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Growth analysis and blood profile in piglets born by embryo transfer

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ABSTRACT

Assisted reproductive technologies (ART), besides solving several reproductive problems, it has also been used as a tool to improve the animal productivity that is required for feeding the human population. One of these techniques, the embryo transfer (ET), has presented limitations in the porcine species, which could constrain its use in the porcine industry. To clarify the potential of this technique, we aimed to compare the impact of using ET or artificial insemination (AI) on the phenotype of the offspring during its first days of age, in terms of growth and blood parameters. At birth, the body weight was higher for ET-females than AI-females, but this difference was no longer observed at day 15. On day 3, it was observed a higher concentration of red blood cells, haemoglobin, and haematocrit in females-ET and a higher concentration of white blood cells in both ET-derived piglets (males and females) when compared to AI groups. On day 3, the biochemical analysis showed a higher level of albumin for ET-derived males, and a lower level of bilirubin for ET-females than AI controls. However, all values were within the normal ranges. Our results indicate that piglets derived from ET seem to be phenotypically similar to those born by AI, which provides preliminary evidence that the ET procedure is a safe technique, but additional studies beyond 15 days of life are requested to conclude its global impact. Furthermore, the presented reference values of blood parameters in this species are interesting data for the pig industry.

1. Introduction

Compelling evidence about the effects of assisted reproductive technologies (ART) on the short- and long-term health of the offspring (reviewed by (El Hajj and Haaf, 2013; Fernández-Gonzalez et al., 2007)) has led to an increase in research in this field, not only in humans but also in other mammalian species. Nowadays, many studies expose the impact that stressful conditions, derived from gametes and embryo manipulation on the first week of preimplantation development, may have on the epigenetic reprogramming that occurs during this period (Canovas et al., 2017). From ovarian stimulation treatment to embryo transfer (ET), each step in the ART represents a possible alteration in the epigenome with consequences in both transcriptome and physiology of the offspring (reviewed by (Van Montfoort et al., 2012)). Moreover, the phenotype may also be compromised, as it is known that the use of these techniques may affect the birth weight and fetal growth, involving also an increased risk of cardiovascular diseases, diabetes, and obesity in adulthood when compared to naturally conceived offspring (Feuer and Rinaudo, 2017).

While in human ART-derived offspring it is difficult to assess which technology is responsible for the harmful effect(s) (i.e., artificial insemination (AI), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), ET, etc.), or even distinguish between the effect of the global process versus the parental inherited alterations, animal models offer a simpler way to decipher the isolated impact of one specific ART on the phenotype of the offspring. Pig, in particular, represents an excellent model due to its similar anatomical and physiological characteristics with humans (reviewed by (Bellinger et al., 2018)). This creates the opportunity to use the domestic pig as a model of ART, since highly selected healthy male and female breeders are available in commercial farms and AI centres, thus enabling a more restricted/controlled genetic variability under similar handling and feeding conditions.

Aside from being an excellent model to study human reproduction, the use of ART in pigs may also be useful at the commercial level to increase meat production – but apart from AI, these systems are still far from being widely established in the pig industry (Peltoniemi et al., 2019). In fact, the use of ET shows some limitations in porcine species

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since it requires the collection of a large number of viable embryos (ideally cryopreserved) to be transferred to the recipient mothers and, until recent years, it also required the surgical transfer of those embryos (Cameron et al., 2004).

ET enables the exchange and the spread of genetics without animal transport, and it avoids sanitary risks and animal welfare undesired consequences. In addition, ET is mandatory to implement the use of in vitro produced embryos and to benefit swine production from other biotechnologies as cloning by nuclear transfer, sex selection in embryos, or gene editing by CRISPR/Cas, and other technologies. All these factors have revealed highly positive in cattle production and a high impact is also expected in swine. Today, the development of new devices has made it possible to do nonsurgical ET despite the complex anatomy of the sow's genital tract. Nevertheless, to date, in vivo embryo collection by uterine flushing via transrectal palpation is not yet a reality at the industry level due to the length and tortuousness of the gilts' and sows' uterine horns, something that does not occur in species like cattle and horses (reviewed by (Yoshioka et al., 2020)). It is known that total litter size, number of piglets born alive as well as birth weight are very important economic traits in the swine industry. However, even though some studies have focused on obtaining live piglets through the use of ARTs such as ET (Ducro-Steverink et al., 2004; Nakazawa et al., 2008; Suzuki et al., 2004), little is known about its possible consequences on the health of the offspring, in part due to the lack of reference values about the phenotype of piglets, showing, therefore, an incomplete picture at the birth and first days of life.

The analysis of blood profile, including haematological and biochemical parameters, is essential for the physiological study since it provides us with valuable information not only on the general state of health but also on their welfare (Faustini et al., 2003), providing complementary information for a short-cut phenotypic screening.

In new-born piglets, very scarce studies have published reference intervals for consistent comparisons (Casas-Díaz et al., 2015; Perri, 2015). Relatively recent, a very complete report from Ventrella et al. (Ventrella et al., 2017) has supplied a database to be used as a reference, despite showing the high variability occurring in clinical-pathological variables between 5 and 30-day-old piglets.

The present study was conducted, as a preliminary step, to screen the main phenotypic parameters that could be affected by ART, using, in this case, the isolated effect of ET, one of the most used ART. For this purpose, weight, average daily weight gain (ADWG), and the haematological and biochemical profiles of the offspring were analysed, minimizing the confounders from parental origin or another ART.

The results obtained could be useful for future studies by providing an additional source of information and contribute to establishing a protocol for evaluation of the real impact of ARTs, if any, in the short and long-term health of ART-derived piglets or other mammalian species.

2. Material and methods

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Chemical Company (Madrid, Spain).

2.1. Ethics

The experimental work performed in this study was submitted to evaluation by the CEEA (Comité Ético de experimentación Animal) from University of Murcia. After approval, authorization from "Dirección General de Agricultura, Ganadería, Pesca y Acuicultura" – Región de Murcia- nr A13170706 was given to perform the experiments with animals.

2.2. Animals, synchronization and artificial insemination

Crossbred sows (Landrace x Large White) from the same genetic line were used as donors (n = 25, 1–11 parities, 1–5 years old) and as recipients (n = 11, 2–3 parities). All animals were housed and fed under the same conditions and water was provided ad libitum.

Estrus synchronization of donor and recipient sows was carried out naturally after weaning. Sows that showed signs of estrous 4–5 days after weaning were used as donors or recipients. Estrus detection was carried out by exposing a mature boar to stimulate the estrous expression of the sow and applying the back-pressure test (Estill, 1999; Walton, 1986). Those sows that remained immobilized under such pressure were considered in heat.

All donors were artificially inseminated twice, 0 and 24 h after the onset of estrus to optimize the reproductive yield (Belstra et al., 2004), with semen from boars of the same breed and proven fertility. Estrus synchrony of the recipients was between 0 and + 24 h regarding the donors.

2.3. In vivo embryo collection and non-surgical embryo transfer

Considering day 0 as the onset of estrus, donors that were on day 6, 7, or 8 of the cycle were sedated by administration of ketamine (10 mg/kg IM, Imalgene® 1000, Boehringer Ingelheim Animal Health, Merial, France), medetomidine hydrochloride (0.02 mg/kg IM, Domitor®, Orion Pharma, Finland) and midazolam (0.2 mg/kg IM, Dormicum®, Roche, Switzerland). Then, sows were euthanized with an overdose of sodium pentobarbital (0.5 ml/kg IV, Eutanax®, Fatro Iberica, Spain), and immediately after, a midventral laparotomy was performed to excise the uterus.

Embryos were collected by flushing from the uterine bifurcation to the tip of each uterine horn with 60 ml modified TL-Hepes-PVA medium, as described by Funahashi et al. (Funahashi et al., 2000), sterile and tempered at 38.5 °C. Embryo's developmental stage and quality were evaluated under a stereomicroscope and morulae, unhatched, and/or hatched blastocyst were used for further ET. Embryos were washed in the same medium and remained in a culture dish at 38.5 °C until transferred. To get a higher number of embryos, each ET was performed using embryos from three donors to a single recipient. The period between excising the uterus, flushing, selection of embryos, and the non-surgical embryo transfer was between 60 and 90 min.

Before non-surgical ET, the perineal area of the recipients was carefully washed with soap and water and dried gently. A latex glove was placed in the tail to avoid cross contamination of the area.

Nonsurgical ET catheters (DeepBlue® ET catheter, Minitüb, Tiefenbach, Germany) were used to transfer the embryos. The intrauterine insertion method has been previously described (Angel et al., 2014). Briefly, in vivo derived embryos were loaded in a 1 ml syringe using the following sequence of aspiration: 0.1 ml TL-Hepes-PVA medium, 0.1 ml air, 0.1 ml TL-Hepes-PVA medium with embryos, 0.1 ml air, and finally 0.1 ml TL-Hepes-PVA medium. Then, the syringe was attached to the catheter and the content was introduced. An additional 0.3 ml TL-Hepes-PVA medium was used to wash the catheter. Finally, the catheter was removed and re-washed with the same medium on a culture dish verifying under the stereomicroscope that most embryos had been transferred. After the transfer, a dose of amoxicillin (29 mg/kg IM, Clamoxyl LA®; Pfizer, Madrid, Spain) was administrated to each recipient.

The mean number and median of embryos transferred per recipient sow were 50,09 ± 17,80 and 46, respectively. The 551 embryos transferred included 486 (88,20%) hatched blastocysts, 65 (11,80%) blastocysts and 14 (2,54%) morulae (see Supplementary Table S1).

2.4. Pregnancy diagnosis, farrowing data, and collection of piglets' samples

Pregnancy was diagnosed by ultrasound 21–26 days after the onset of estrus. Piglets derived from a total of three sows that were artificially inseminated with the same boars in the same farm under the same conditions were used as control (AI group). All sows were housed in gestation crates located in a parturition unit. Gestation length, farrowing rate, survival rate, and litter size were registered for each sow.

Immediately after birth, piglets from ET and AI groups were identified and weighed using a digital hanging scale and the body weight (BW) was registered. After taking the measurements, the piglets were placed immediately with their mother.

On days 3 and 15 of life, all piglets were weighed, and average daily weight gain (ADWG) was calculated using the formula:

$$\text{ADWG} = (\text{End Weight} - \text{Previous Weight})/(\text{End Date} - \text{Previous Date})$$

Blood samples at days 3 and 15 of life were collected by direct venipuncture of the jugular vein with a 23G x 25 mm needle and lithium heparin tubes (BD Vacutainer®, BD Spain). Blood tubes were transported to the laboratory and haematological analysis was performed using a haematology analyser (Siemens ADVIA® 120, USA). The parameters analysed were concentration of red blood cells (RBC, $x10^6$ cells/μL), haemoglobin (HB, g/dl), haematocrit (HCT, %), mean corpuscular volume (MCV, fL), mean corpuscular haemoglobin (MCH, pg), mean corpuscular haemoglobin concentration (MCHC, g/dl), cell haemoglobin concentration mean (CHCM, g/dl), red blood cell distribution width (RDW, %), haemoglobin concentration distribution width (HDW, g/dl), concentration of white blood cells (WBC, $x10^3$ cells/μL), neutrophils ($x10^3$ cells/μL), lymphocytes ($x10^3$ cells/μL), monocytes ($x10^3$ cells/μL), eosinophils ($x10^3$ cells/μL), basophils ($x10^3$ cells/μL); reticulocytes indices: percentage of reticulocytes (%), mean corpuscular volume of reticulocytes (MCVr, fL) and content of haemoglobin in reticulocytes (CHr, pg), platelets ($x10^3$ cells/μL); and platelets indices: platelecrit (PCT, %), mean platelet volume (MPV, fL), platelet distribution width (PDW, %) platelet component distribution width (PCDW, g/dl), mean platelet mass (MPM, pg), platelet mass distribution width (PMDW, pg), and large platelets ($x10^3$ cells/μL).

Then, blood was centrifuged at 1008*g* for 10 min at room temperature and biochemical analysis was performed using a chemistry analyser for plasma (Olympus AU400, Japan). The biochemical parameters analysed were: creatinine (CREA, mg/dl), urea (mg/dl), amylase (UI/L), creatine kinase (CK, UI/L), cholesterol (mg/dl), alkaline phosphatase (ALP, UI/L), gamma-glutamyl transferase (GGT, UI/L), glucose (mg/dl), aspartate aminotransferase (AST, UI/L), alanine aminotransferase (ALT, UI/L), lipase (UI/L), total protein (TP, g/dl), albumin, (ALB, g/dl), globulin (GLOB, g/dl), triglycerides (TRIGL, mg/dl) and total bilirubin (TBIL, mg/dl).

2.5. Statistical analysis

In vivo embryos were collected from artificially inseminated sows and transferred by a non-surgical approach to recipients. Gestation length, farrowing rate, survival rate, litter size, and phenotypical traits of the offspring at short term (birth weight, average daily weight gain, haematological and biochemical parameters) were analysed.

Data presented in the manuscript were analysed firstly by group (AI vs ET), without considering the sex of the piglets, and secondly by group and sex. All the data (weight, ADWG, and haematological and biochemical parameters) were submitted to D'Agostino-Pearson test to assess normality except for the litter size and the total Kg of piglets per sow, which were evaluated using Shapiro-Wilk normality test due to the too small sample size (n). Both groups were compared by unpaired *t*-test when data were normally distributed, and by non-parametric Mann-Whitney *U* test in the case of non-normal data distribution. The results are presented as mean ± SD (standard deviation). Values of $p < 0.05$ were considered significant. The software used was GraphPad Software, version 7 (La Jolla, California, USA).

3. Results

3.1. Piglets

A total of 83 piglets were obtained from ET and AI groups and phenotypically analysed. There were no significant differences between ET and AI groups ($p = 0.9999$) in the total piglets born nor mean litter size, even it ranged from 6 to 21 and from 11 to 18 in ET and AI groups, respectively (Table 1). A total of 5 piglets from the ET group and 5 from the AI group were excluded from this study, due to being stillbirths or dying of natural causes (i.e., low weight or crushed by the sow). Similarly, when litter size was compared at both days 3 and 15 of age, no significant differences were observed between groups, as shown in Table 1.

Table 2 discriminates the data concerning sex, and birth weight from piglets derived from ET and AI at birth. Fourteen males and 26 females from the ET group, and 27 males and 16 females from the AI group were born, and none of them presented any visible external morpho-anomalies, therefore being anatomically normal and weighing as expected.

D'Agostino and Pearson's normality test showed a normal distribution for males in both groups, while this was only observed in females from the AI group. Moreover, when data were compared by group, the birth weight in ET females was significantly higher than females from the AI group. No differences were observed between males.

Fig. 1 shows the data regarding weight and ADWG of the piglets, separated by sex. D'Agostino and Pearson normality test showed a normal distribution in both males and females from ET and AI groups in terms of weight and ADWG on days 3 and 15 of life. On the other hand, males did not present differences in weight at birth or on days 3 and 15 of life when compared by groups. Similarly, no differences were found when ADWG was measured on days 3 and 15 between groups. However, females showed significant differences in birth weight, being heavier the ET group compared to AI, but no differences were observed on the following days. In the same way, when ADWG was compared between females, no differences were observed. Differences at day 0 between females have also been shown in the figure caption for a better understanding of the growth rate.

3.2. Haematological and biochemical parameters

Data presented in the manuscript are analysed by group and sex of the piglets. An additional analysis, independent of the sex, is presented in more detail (see Supplementary Tables S2–S5). To address the full haematological and biochemical analysis, several additional movie files show this in more detail (see Supplementary Tables S6–S13). When D'Agostino and Pearson normality test was performed, most of the parameters analysed presented a normal distribution with some exceptions. The statistical method used in each one is shown in the title of each figure.

Representative graphs of the principal results are shown below.

Table 1
Piglets born, litter size mean, and litter size range after in vivo embryo transfer or artificial insemination.

Group	Live-born piglets/ Born piglets	Litter size[1]	Litter size range[2]	Piglets alive at 3 days of age (survival percentage[3])	Piglets alive at 15 days of age (survival percentage)
ET	38/40	13.33 ± 7.50	6–21	37 (97,40%)	33 (86,84%)
AI	42/43	14.0 ± 4.0	10–18	38 (90,47%)	33 (78,57%)

[1] No statistical differences were found between groups. Values are mean ± SD.
[2] Litter size and range considered live-born and stillborn piglets.
[3] Survival percentage based on total live-born piglets.

Table 2
Piglets' sex per group and birth weight.

	Group	
	ET	AI
Males (n)	14	27
Females (n)	26	16
Birth weight (g)		
Males[1]	1326.00 ± 374.70	1248.00 ± 374.70
Females[2]	1450.00 ± 496.10[a]	1109.00 ± 299.20[b]

[a-b] Statistically significant differences within rows are annotated with different letter ($p < 0.05$). Values are mean ± SD.
[1] Unpaired t-test
[2] Mann-Witney U test

3.2.1. *Red blood cells*

3.2.1.1. Red blood cell count (RBC). No significant differences were found in male piglets for RBC (Fig. 2A, RBC males). On the contrary, significantly higher values were found in females from the ET group compared with those derived from AI on day 3, but the difference was lost on day 15 (Fig. 2B, RBC females).

3.2.1.2. Haemoglobin (Hb). No significant differences were observed between males of ET and AI in both days (Fig. 2C, Hb males). Female piglets from the ET group had higher values when compared to AI, but only on day 3 (Fig. 2D, Hb females).

3.2.1.3. Haematocrit (HTC). No differences were found in male piglets born by ET or AI on both days 3 and 15 (Fig. 2E, HTC males). Female ET piglets showed significant higher values at day 3 compared to those derived from AI (Fig. 2F, HCT females).

3.2.1.4. Mean corpuscular volume (MCV). No significant differences were found between male piglets born through ET or AI (Fig. 2G, MCV males). Female ET piglets showed lower values on day 15 when compared to AI (Fig. 2H, MCV females).

3.2.1.5. Mean corpuscular haemoglobin (MCH). No significant differences were found in MCH from males at any time (see Supplementary Table S6). However, females from the ET group showed lower levels when compared to the AI group, on day 15 (see Supplementary Table S10).

3.2.1.6. Haemoglobin concentration distribution width (HDW). ET male piglets showed a higher mean value than AI piglets on day 15 (see Supplementary Table S6), but female ET piglets showed lower values at day 3 when compared to AI (see Supplementary Table S10).

3.2.1.7. Reticulocytes. No differences were found in male ET vs. AI piglets (Fig. 3A, Reticulocytes males), but female ET piglets showed significant lower values in comparison to AI female piglets on day 15 (Fig. 3B, reticulocytes females).

3.2.1.8. Mean corpuscular volume of reticulocytes (MCVr). Significant

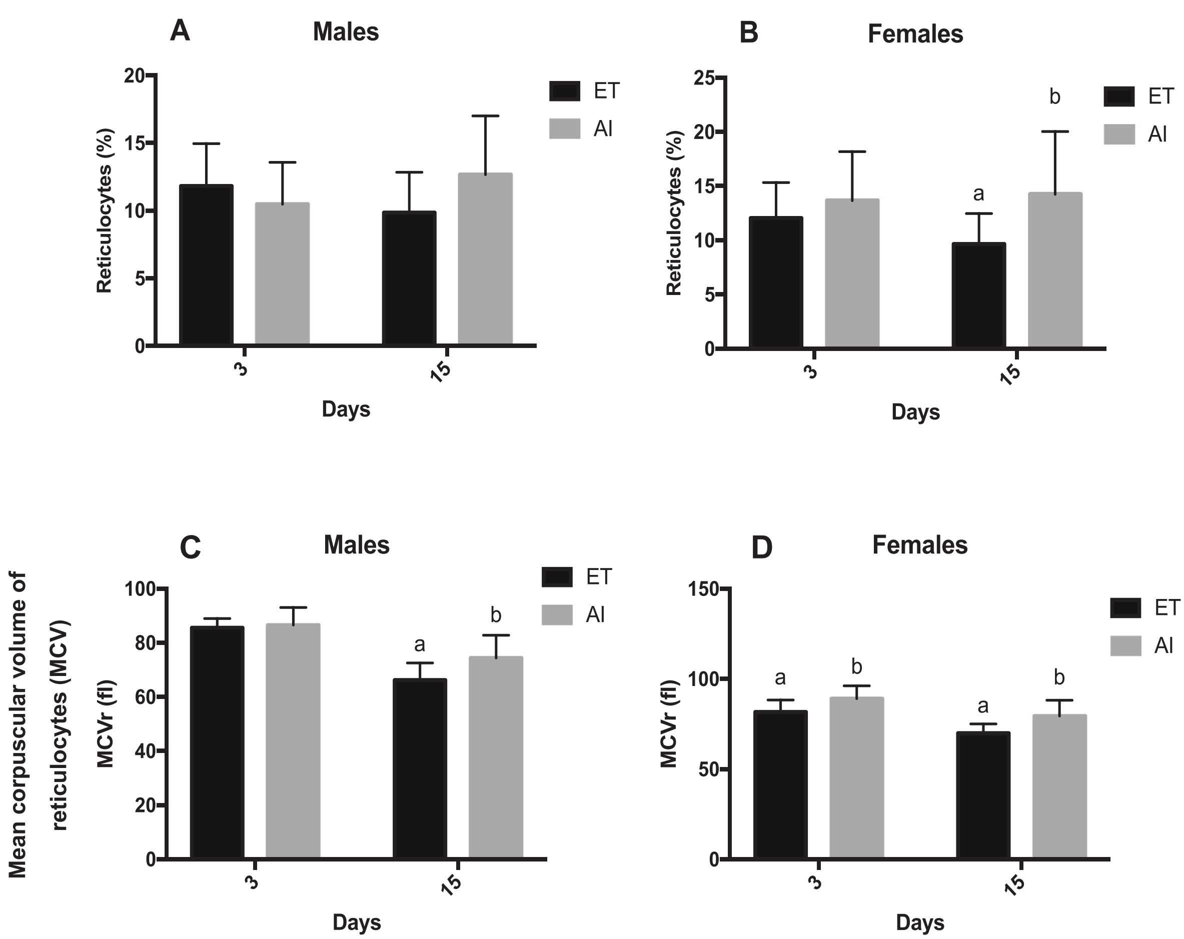

Fig. 1. Weight and average daily weight gain (ADWG) of piglets born by ET or AI. Data are mean ± SD, separated by group (ET or AI) and sex (males or females). (B) The weight at day 0 shows significant differences between females from in vivo embryo transfer (ET, black bar) and females from artificial insemination (AI, grey bar) groups. * Compared with the control $p < 0.05$ (Unpaired *t*-test).

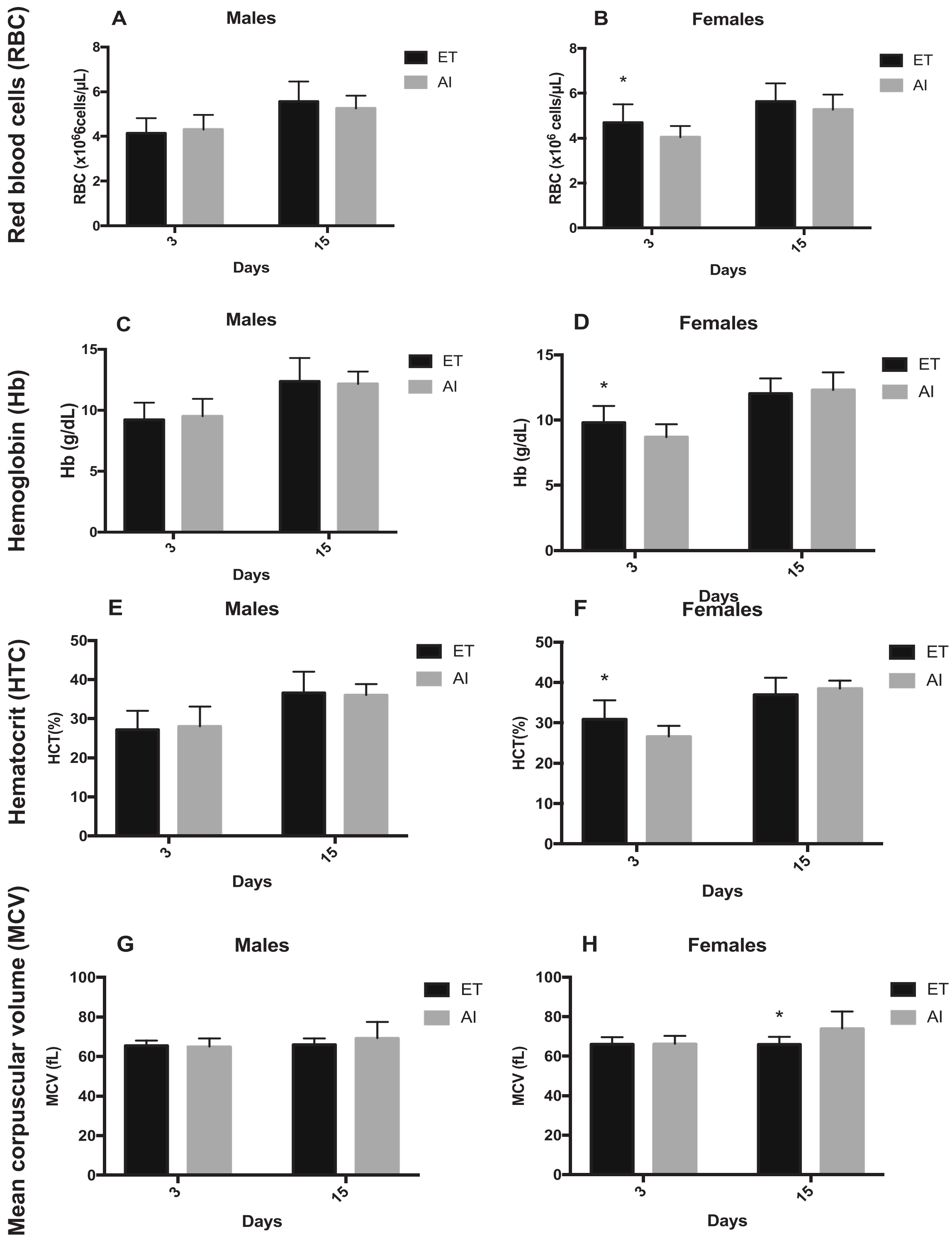

Fig. 2. Haematological analysis of red blood cell parameters of piglets born by ET or AI. Red blood cells count (RBC), haemoglobin (Hb), haematocrit (HCT), and mean corpuscular volume (MCV) in embryo transfer (ET, black bar) or artificial insemination (AI, grey bar) groups. Values are represented by means±SD and separated by age (days) and sex. (B) RBC, (D) Hb, and (F) HCT show significant differences at day 3 between females from ET and AI group whereas (H) MCV shows significant differences at day 15 between females from ET and AI groups. * Compared with the control $p < 0.05$ (Unpaired *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differences were found between both groups on day 15, with ET male piglets showing lower values than their AI counterparts (Fig. 3C, MCVr males) similarly to females (Fig. 3D, MCVr females). However, only females showed the same pattern on day 3, with ET females showing lower values than AI females.

3.2.1.9. Haemoglobin content of reticulocytes (CHr). Both male and female piglets as well as male and female piglets separately from the ET group showed significant lower values than AI counterparts on day 15 (see Supplementary Tables S2, S6 & S10).

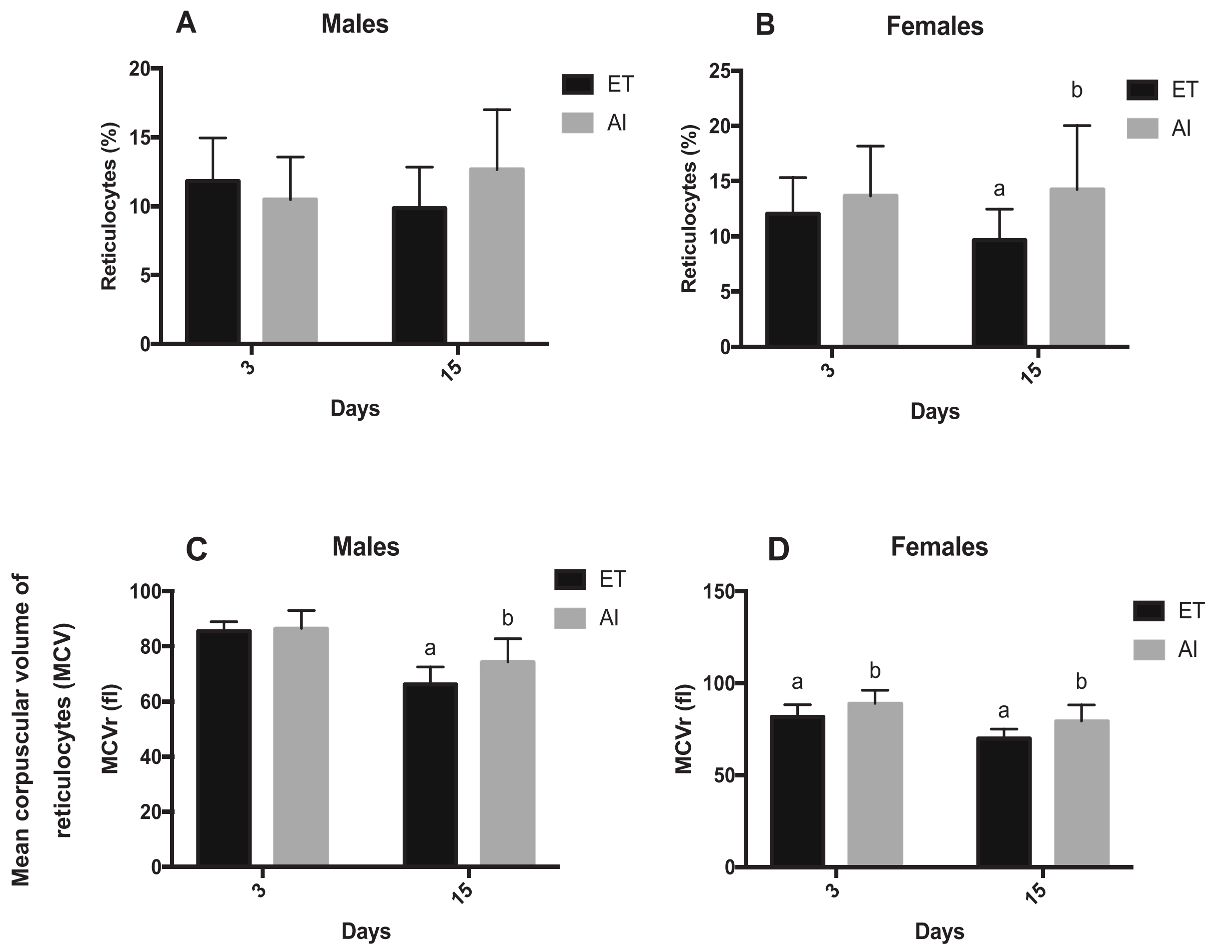

Fig. 3. Haematological analysis of reticulocytes parameters of piglets born by ET or AI. Reticulocytes percentage and mean corpuscular volume of reticulocytes (MCVr) of piglets born by in vivo embryo transfer (ET, black bar) or artificial insemination (AI, grey bar). Values are represented by means±SD and separated by age (days) and sex. The percentage of reticulocytes (B) shows significant differences at day 15 between females from ET and AI groups. * Compared with the control $p < 0.05$ (Mann-Whitney *U* test); (D) MCVr shows significant differences at day 3 between females from ET and AI groups; (C, D) MCVr shows significant differences at day 15 in both males and females from ET and AI groups. * Compared with the control $p < 0.05$ (Unpaired *t*-test).

3.2.2. White blood cells

3.2.2.1. White blood cells count (WBC). The total number of WBC was significantly higher in the ET group for both males and females on day 3 of life (Fig. 4A, WBC males and Fig. 4B, WBC females); while at day 15, these differences were only significant between females.

Neutrophils were significantly higher in both males and females from the ET group vs AI at day 3. Similarly, these differences were also

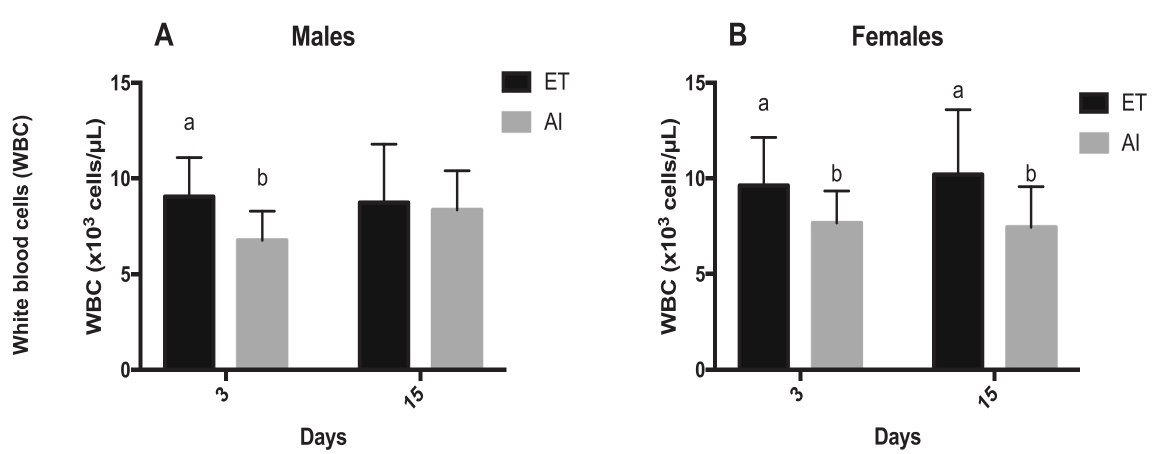

Fig. 4. Haematological analysis of white blood cell count of piglets born by ET or AI. Values are represented by means±SD and separated by age (days) and sex. (A, B) WBC shows significant differences at day 3 in both males and females from in vivo embryo transfer (ET, black bar) and artificial insemination (AI, grey bar) groups; (B) WBC shows significant differences between females from ET and AI groups at day 15. * Compared with the control $p < 0.05$ (Unpaired *t*-test).

observed in males from the ET group vs. AI at day 3, with no further difference (see Supplementary Tables S3 & S7).

Lymphocytes were also significantly higher in both males and females from the ET group, at days 3 and 15. These differences were also found in females from the same group and same days of life (see Supplementary Tables S3 & S11).

3.2.3. Platelets

3.2.3.1. Platelets (PLT). No significant differences in concentration were found between the two groups, although a trend was observed at day 3 in females ($p = 0.0549$), having those derived from the ET group a slightly lower value than those derived from the AI group (see Supplementary Table S12).

3.2.3.2. Platelecrit (PCT). As shown in Fig. 5A and B (PCT males and females respectively), ET-males and females showed significant lower values compared to AI on days 3 and 15 of life, respectively.

Mean platelet volume (MPV): Male ET piglets had higher values at day 15 than AI piglets (Fig. 5C, MPV males). Females did not show any difference between groups (Fig. 5D, MPV females).

Mean platelet mass (MPM) showed only significant differences for males derived from the ET group at day 3 regarding AI (see Supplementary Table S8).

3.2.3.3. Platelet mass distribution width (PMDW). The only difference found was regarding day 15, where ET male piglets had higher values than AI piglets (see Supplementary Table S8).

3.2.3.4. Large PLT. No significant differences were found in males (see Supplementary Table S8) for large PLT but females ET-derived showed lower values in comparison with females AI-derived on day 15 (see Supplementary Table S12).

3.2.4. Biochemical analysis

3.2.4.1. Urea. Males did not present significant differences between groups (Fig. 6A, Urea males). Conversely, females from the ET group showed values below those derived from AI, being significantly different on day 15 of life (Fig. 6B, Urea females).

3.2.4.2. Alkaline phosphatase (ALP). Both males and females derived by ET showed values above AI-piglets on day 3 (Fig. 6C, ALP males and Fig. 6D, ALP females), however, only males from the ET group showed significant differences regarding the AI group. On the other hand, on day 15 of life, no significant differences were found for piglets of any sex.

3.2.4.3. Albumin (ALB). The ALB concentrations were significantly lower on day 3 in males from the ET group compared to those from the AI group (Fig. 6E, ALB males).

3.2.4.4. Total bilirubin (TBIL). Both males and females showed a lower concentration of TBIL on day 3, with only females from the ET group

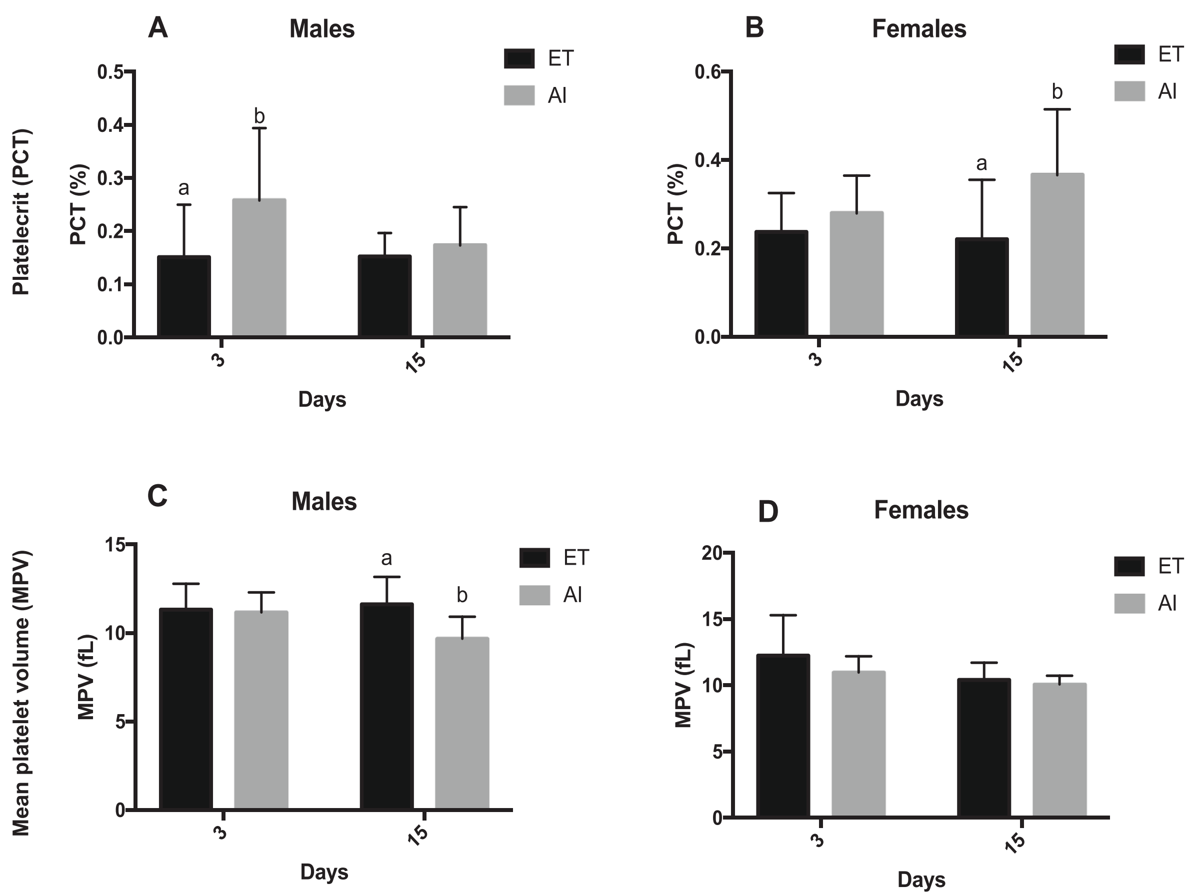

Fig. 5. Haematological analysis of Platelets parameters of piglets born by ET or AI. Platelecrit and mean platelet volume of piglets born by in vivo embryo transfer (ET, black bar) or artificial insemination (AI, grey bar). Values are represented by means±SD and separated by age (days) and sex. (A) PCT shows significant differences at day 3 between males from ET and AI groups. * Compared with the control $p < 0.05$ (Mann-Whitney *U* test); (B) At day 15, PCT shows significant differences between females from ET and AI. * Compared with the control $p < 0.05$ (Unpaired *t*-test); (C) MPV shows significant differences at day 15 between males from ET and AI groups. * Compared with the control $p < 0.05$ (Unpaired t-test).

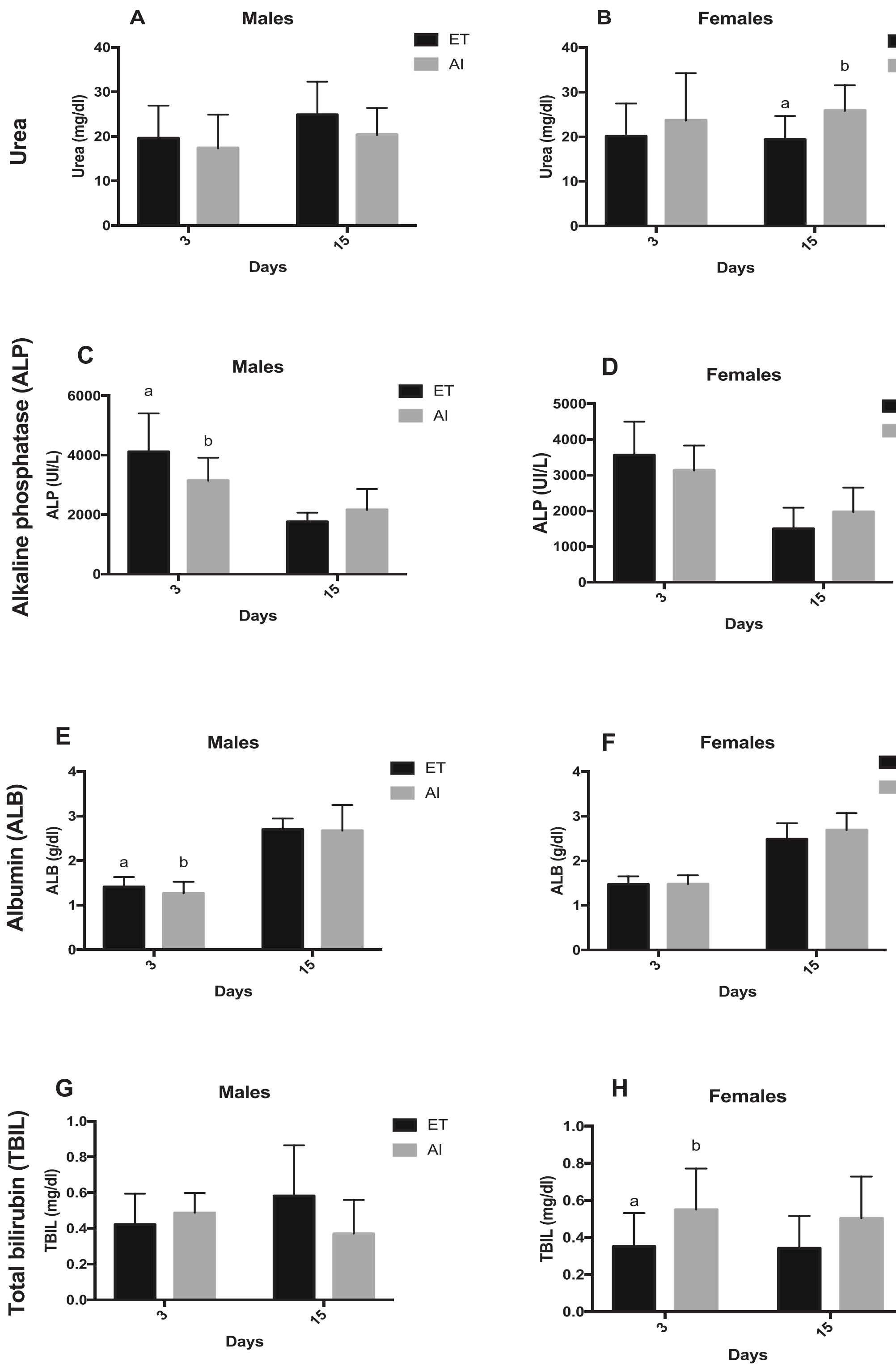

Fig. 6. Biochemical analysis of piglets born by ET or AI. Concentrations of urea, alkaline phosphatase, albumin, and total bilirubin in piglets born by in vivo embryo transfer (ET, black bar) or artificial insemination (AI, grey bar). Values are represented by means±SD and separated by age (days) and sex. (B) Urea shows significant differences at day 15 between females from ET and AI groups. * Compared with the control $p < 0.05$ (Unpaired *t*-test); (C) ALP shows significant differences at day 3 between males from ET and AI groups. * Compared with the control $p < 0.05$ (Unpaired t-test); (E) ALB shows significant differences at day 3 between males from ET and AI groups. * Compared with the control $p < 0.05$ (Mann-Whitney *U* test); (H) TBIL shows significant differences at day 3 between females from ET and AI groups. * Compared with the control $p < 0.05$ (Unpaired t-test).

showing significant differences compared to those from the AI group (Fig. 6G and H, TBIL males and females respectively). However, on day 15 only a trend was observed ($p = 0.0551$) between females.

3.2.4.5. Gamma-glutamyl transferase (GGT). Piglets (males and females) born by ET presented values above those born by AI on days 3 and 15 of life, with only males from ET showing significant differences compared to those born by AI on day 15 (see Supplementary Tables S5 & S9).

3.2.4.6. Aspartate aminotransferase (AST). Higher values were detected in ET-derived male piglets vs. AI on day 15 (see Supplementary Table S9).

3.2.4.7. Lipases. On day 15 of life, piglets from both groups considerably decreased their concentration of lipases compared to day 3. In addition, piglets from the ET group showed lower values compared with the AI group. Similarly, significant differences were found in males from ET regarding those from AI (see Supplementary Tables S5 & S9).

3.2.4.8. Globulins (GLOB). Although no significant differences were found, when the concentration of globulins was analysed, a trend was observed between males from ET having higher values compared to AI on day 15 ($p = 0.0538$) (see Supplementary Table S9).

4. Discussion

The research and development of different ART protocols in the porcine species, with the final objective of improving their efficiency in a holistic sense, represents an area of increasing interest from the economic and strategical point of view since the possible existence of an embryo market would allow farmers the exchange of genetic material without risk of disease transmission and reducing transportation costs (reviewed by (Fowler et al., 2018)).

Besides, the pig has been suggested and largely used as an excellent human-study model (reviewed by (Van Soom et al., 2011)), due to its similar anatomical, physiological and genetic characteristics (Bellinger et al., 2006; Lorenzen et al., 2015), But also, there is compelling evidence that it is a useful model to decipher the long term impact of each aspect of the ART without confounding factors such as those related to the fertility of the parents, a fact that has been recently reinforced with the discovery of the similarities between the pig and the human regarding the DNA methylation reprogramming events during the first week of development (Ivanova et al., 2020).

In this context, this preliminary study focuses on one of the ART, the embryo transfer, using the porcine model to shed light on ART-derived consequences on the phenotype of the offspring from birth to day 15 of life. ET is a biotechnology with very low penetrance in swine production, although it is expected to increase in the next years, according to the advantages reported by its use in cattle production and the latest advances in non-surgical porcine embryo transfer. It could have unprecedented productive and economic implications for the pig sector (Martinez et al., 2019).

Our results represent, to the best of our knowledge, the first approach to describe the impact derived from the use of one isolated assisted reproductive technology, the non-surgical ET in pigs, not only at birth but also at days 3 and 15 of age. Indeed, there have been previous studies that refer to the effect on birth weight, growth parameters, metabolism, etc. in ART offspring (Berntsen et al., 2019; Castillo et al., 2019; Feuer and Rinaudo, 2017), but most of them have been in mice, due to its easy handling, short gestation length, and other beneficial characteristics (Gutierrez et al., 2015). In addition, the few studies performed in pigs have evaluated some productive parameters in IVP-derived piglets such as farrowing rate, litter size, or birth weight, but none of them have described the haematological and biochemical profile of the piglets before weaning, which, in fact, represent an important source of information on the general state of health of the animals. Iron deficiency anemia is one of the most common nutritional disorders in piglets during the perinatal period and it affects regardless of the breed and management system (Godyń et al., 2016). It depends, between other factors, on the capacity of digestive absorption, hepatic iron store, or the exposure to stress conditions which could be derived from the use of ARTs (Starzyński et al., 2013; Venn et al., 1947). Iron requirements are higher for larger litter sizes, higher birth weights, and faster growth which results in a greater blood volume and red blood cell (RBC) count (Starzyński et al., 2013; Ventrella et al., 2017). Considering it, we analysed separately the growth curve and the haematological and biochemical profiles in males and females.

Regarding growth parameters, our results showed that, although ET piglets had a slightly heavier body weight in absolute terms than AI piglets at birth as well as days 3 and 15 of age, no differences were found between groups ($p > 0.05$). This is in disagreement with Ducro-Steverink et al. (Ducro-Steverink et al., 2004), who detected differences in non-surgical ET, showing higher birth weights in comparison with AI piglets. However, these differences could be attributed to the reduced size of non-surgical ET litters in that study, contrary to our results where that difference was not found.

We found that females showed differences when the data were analysed separately, being the weight at birth higher in ET than in AI, as others reported (Ducro-Steverink et al., 2004), but such differences disappeared on days 3 and 15. This fact, however, should be kept in mind for future studies on the long-term phenotypes of ET animals because, as it has been shown in different studies, increased birth weight is one of the most common findings in ART-derived calves and it has been related to the large offspring syndrome (Chen et al., 2013; Li et al., 2019) as well as to the Beckwith–Wiedemann syndrome in humans (Weksberg et al., 2010), although it has not been described in pigs until now. Another reason not to obviate this finding is that the differences were observed only in females, which are the animals kept on the farm as future mothers for the next generations. The likely impact of the increased birth weight of a mother on her further offspring is unknown and deserves future research. Still, weight at birth in all cases was within the normal range and no anatomical abnormalities were detected in any piglet, which is in agreement with other studies (Angel et al., 2014; Yoshioka et al., 2012).

As for ADWG, no differences were found between ET and AI groups. This is consistent with a study in mice, where males and females from IVF had a similar growth phenotype compared to the control group (Donjacour et al., 2014).

Although there are few references to haematological and biochemical parameters in the porcine species (Perri et al., 2017), there are various studies in cloned pigs where different blood parameters have been evaluated (Archer et al., 2003; Gu et al., 2019; Mir et al., 2005). In addition, other studies in piglets (Ventrella et al., 2017) have recently provided some reference intervals that could help us interpret the results obtained. The different parameters analysed to assess the general health status of the piglets showed some differences but also high variability. On day 3 postnatally, females derived from the ET group showed significantly greater values in the concentration of RBC, Hb, and HTC compared to the AI group. Conversely, on the same day a decrease in the percentage of reticulocytes, MCVr, and CHr was observed, although only significant differences were seen in MCVr for the ET group. This is in agreement with Ekert Kabalin et al. (Ekert Kabalin et al., 2008), who previously described that an increase of erythrocytes, due to increased erythropoiesis, is accompanied by a decrease in the reticulocyte count in new-born piglets.

On the other hand, at day 15, lower values were found in the percentage of reticulocytes, MCVr, and CHr for females from the ET group compared to those derived by AI, the latter two parameters also being significantly lower in males derived from ET. Godyn et al. (Godyń et al., 2016) reported that these parameters might be indicators of the existence of iron deficiency; however, none of the values were outside the normal range nor was the Hb concentration altered. Bhattarai et al. (Bhattarai and Nielsen, 2015) reported that due to the differences in weight, most reticulocyte indices vary depending on the size of the piglets.

WBC was significantly greater at day 3 in males and females from ET due to an increase in the levels of neutrophils and lymphocytes, respectively, being elevated in females also on day 15. Although this population of cells is expanding during piglet growth (Cooper et al., 2014) and these values were within the normal range, these significant differences between groups under the same conditions could suggest the existence of moderate exposure to stress (Salak-Johnson and McGlone, 2007) or the possible existence of a subclinical pathology such as bacterial or parasitic infections (Kalai et al., 2012; López-Olvera et al., 2006). However, complementary studies should have depth in these aspects.

Pliszczak-Król et al. (Pliszczak-Król et al., 2016) described that PLT variability in piglets may be due to the rapid growth of these animals and the maturation of their hematopoietic system. Despite no differences being found in the PLT concentration, significantly lower values were found on day 3 in PCT and MPM for males from ET compared to those derived by AI. On the other hand, significantly higher values were seen in males from the ET group on day 15. However, although MPV is an index with some clinical relevance and an increased MPV indicates increased platelet diameter, which can be used as a marker of production rate and platelet activation (Budak et al., 2016), the clinical significance, reference values, and usefulness of most of the platelet indices are still under investigation (Kim et al., 2014) and, in our study, we cannot affirm that any of them could be interpreted as potential markers of any kind of phenotypical difference between our two groups of piglets.

The biochemical analysis showed higher differences on day 3 in ET-derived males compared to males from AI for different parameters, including ALP, GGT, AST, and ALB. Interestingly while on day 15 the concentrations of GGT, AST, and ALB remained higher in the ET group compared to the AI, a decrease was observed for ALP. However, no statistically significant differences were found on this day. According to some authors, an increase in ALP levels is associated with increased production of osteoblasts due to the growth of piglets (Casas-Díaz et al., 2015; Kabalin et al., 2012). In contrast, a decrease in the concentration of this enzyme is related to a decrease in phosphorus levels in diet (Perri, 2015). On the other hand, Stone (Stone, 1984) reported that high ALB levels are associated with the physiological maturation of the liver but it should also be noted that, the ratio of albumin: globulin decreases from day 3 to 15, as expected after colostrum intake during the first days of life (Jerry Kaneko et al., 2008).

In addition, despite an increase in AST concentration being associated with increased physical activity or the existence of muscle damage (Verheyen et al., 2007), all values were within the normal range. According to Yu et al., (Yu et al., 2019), a high concentration in new-born calves is an indication that these enzymes are absorbed from colostrum, at least in the case of GGT and AST. Besides, Dubreuil and Lapierre (Dubreuil and Lapierre, 1997) reported an increase in CK and AST of growing pigs up to week 8 of age, both parameters being related to muscle growth. Despite no differences were found in CK, our results showed high variability in this parameter, also being influenced by the age and sex of the animals (Grindem, 2011; Heffron et al., 1976). On day 15, females from the ET group showed significantly lower values for urea compared with those from the AI group, which has been associated with a decrease in protein intake (Perri et al., 2017).

5. Conclusions

Even though some differences were noted between the ET piglets and their AI counterparts, it does not appear that growth, as well as the haematological or biochemical parameters of the offspring, were clinically affected by the embryo transfer technique and in the absence of other ART at birth nor on days 3 and 15 of age. Nonetheless, additional studies are requested to decipher if the differences could be relevant later in life and to conclude definitively if the embryo transfer technique is a safe procedure in terms of expected development and general health status. Interestingly the results also provide some reference parameters in animals of these ages considering the limited literature that exists on the matter.

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Authorship

PC designed the experiment; all authors conducted the research; EPO, JG and SC analysed the data; EPO, SC and PC wrote the manuscript; all authors reviewed the manuscript and approved the final version.

Declaration of Competing Interest

The authors declare no conflict of interest.

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References

Angel, M.A., Gil, M.A., Cuello, C., Sanchez-osorio, J., Gomis, J., Parrilla, I., Vila, J., Colina, I., Diaz, M., Reixach, J., Vazquez, J.L., Vazquez, J.M., Roca, J., Martinez, E. A., 2014. An earlier uterine environment favors the in vivo development of fresh pig morulae and blastocysts transferred by a nonsurgical deep-uterine method. J. Reprod. Dev. 60 https://doi.org/10.1262/jrd.2014-022.

Archer, G.S., Dindot, S., Friend, T.H., Walker, S., Zaunbrecher, G., Lawhorn, B., Piedrahita, J.A., 2003. Hierarchical phenotypic and epigenetic variation in cloned swine. Biol. Reprod. 69, 430–436 doi:0.1095/biolreprod.103.016147.

Bellinger, D.A., Merricks, E.P., Nichols, T.C., 2006. Swine models of type 2 diabetes mellitus: insulin resistance, glucose tolerance, and cardiovascular complications. ILAR J. 47, 243–258. https://doi.org/10.1093/ilar.47.3.243.

Bellinger, D.A., Merricks, E.P., Nichols, T.C., 2018. Swine models of type 2 diabetes mellitus: insulin resistance. Glucose Tolerance Cardiovas. Comp. 47, 243–258.

Belstra, B.A., Flowers, W.L., See, M.T., 2004. Factors affecting temporal relationships between estrus and ovulation in commercial sow farms. Anim. Reprod. Sci. 84, 377–394. https://doi.org/10.1016/j.anireprosci.2004.02.005.

Berntsen, S., Söderström-Anttila, V., Wennerholm, U.B., Laivuori, H., Loft, A., Oldereid, N.B., Romundstad, L.B., Bergh, C., Pinborg, A., 2019. The health of children conceived by ART: “the chicken or the egg?” Hum. Reprod. Update 25, 137–158. https://doi.org/10.1093/humupd/dmz001.

Bhattarai, S., Nielsen, J., 2015. Early Indicators of Iron Deficiency in Large Piglets at Weaning, 23, pp. 10–17.

Budak, Y.U., Polat, M., Huysal, K., 2016. The use of platelet indices, plateletcrit, mean platelet volume and platelet distribution width in emergency non-traumatic abdominal surgery: a systematic review. Biochem. Med. 26, 178–193. https://doi.org/10.11613/BM.2016.020.

Cameron, R.D.A., Beebe, L.F.S., Blackshaw, A.W., Keates, H.L., 2004. Farrowing rates and litter size following transfer of vitrified porcine embryos into a commercial swine herd. Theriogenology 61, 1533–1543. https://doi.org/10.1016/j.theriogenology.2003.09.003.

Canovas, S., Ivanova, E., Romar, R., García-Martínez, S., Soriano-Úbeda, C., García-Vázquez, F.A., Saadeh, H., Andrews, S., Kelsey, G., Coy, P., 2017. DNA methylation and gene expression changes derived from assisted reproductive technologies can be decreased by reproductive fluids. Elife 6, 1–24. https://doi.org/10.7554/eLife.23670.

Casas-Díaz, E., Closa-Sebastià, F., Marco, I., Lavín, S., Bach-Raich, E., Cuenca, R., 2015. Hematologic and biochemical reference intervals for Wild Boar (Sus scrofa) captured by cage trap. Vet. Clin. Pathol. 44, 215–222. https://doi.org/10.1111/vcp.12250.

Castillo, C.M., Horne, G., Fitzgerald, C.T., Johnstone, E.D., Brison, D.R., Roberts, S.A., 2019. The impact of IVF on birthweight from 1991 to 2015: a cross-sectional study. Hum. Reprod. 34, 920–931. https://doi.org/10.1093/humrep/dez025.

Chen, Z., Robbins, K.M., Wells, K.D., Rivera, R.M., 2013. Large offspring syndrome: a bovine model for the human loss-of-imprinting overgrowth syndrome Beckwith-Wiedemann. Epigenetics 8, 591–601. https://doi.org/10.4161/epi.24655.

Cooper, C.A., Moraes, L.E., Murray, J.D., Owens, S.D., 2014. Hematologic and biochemical reference intervals for specific pathogen free 6-week-old Hampshire-Yorkshire crossbred pigs. J. Anim. Sci. Biotechnol. 5 https://doi.org/10.1186/2049-1891-5-5.

Donjacour, A., Liu, X., Lin, W., Simbulan, R., Rinaudo, P.F., 2014. In vitro fertilization affects growth and glucose metabolism in a sex-specific manner in an outbred mouse Model1. Biol. Reprod. 90, 1–10. https://doi.org/10.1095/biolreprod.113.113134.

Dubreuil, P., Lapierre, H., 1997. Biochemistry reference values for quebec lactating dairy cows, nursing sows, growing pigs and calves. Can. J. Vet. Res. 61, 235–239.

Ducro-Steverink, D.W.B., Peters, C.G.W., Maters, C.C., Hazeleger, W., Merks, J.W.M., 2004. Reproduction results and offspring performance after non-surgical embryo transfer in pigs. Theriogenology 62, 522–531. https://doi.org/10.1016/j.theriogenology.2003.11.010.

Ekert Kabalin, A., Balenović, T., Valpotić, I., Pavičić, Ž., Valpotić, H., 2008. The influence of birth mass and age of suckling piglets on erythrocyte parameters. Vet. Arh. 78, 307–319.

El Hajj, N., Haaf, T., 2013. Epigenetic disturbances in in vitro cultured gametes and embryos: implications for human assisted reproduction. Fertil. Steril. 99, 632–641. https://doi.org/10.1016/j.fertnstert.2012.12.044.

Estill, C.T., 1999. Current concepts in estrus synchronization in swine. J. Anim. Sci. 77, 1–9.

Faustini, M., Bronzo, V., Maffeo, G., Russo, V., Munari, E., Vigo, D., 2003. Reference intervals and age-related changes for platelet count, mean platelet volume and plateletcrit in healthy pre-weaning piglets in Italy. J. Vet. Med. Ser. A Physiol. Pathol. Clin. Med. 50, 466–469. https://doi.org/10.1046/j.1439-0442.2003.00581.x.

Fernández-Gonzalez, R., Ramirez, M., Bilbao, A., Fonseca, F., Gutiérrez-Adán, A., 2007. Suboptimal in vitro culture conditions: an epigenetic origin of long-term health effects. Mol. Reprod. Dev. 74, 1149–1156. https://doi.org/10.1002/mrd.20746.

Feuer, S.K., Rinaudo, P.F., 2017. Physiological, metabolic and transcriptional postnatal phenotypes of in vitro fertilization (IVF) in the mouse. J. Dev. Orig. Health Dis. 8, 403–410. https://doi.org/10.1017/S204017441700023X.

Fowler, K.E., Mandawala, A.A., Griffin, D.K., Walling, G.A., Harvey, S.C., 2018. The production of pig preimplantation embryos in vitro: current progress and future prospects. Reprod. Biol. 18, 203–211. https://doi.org/10.1016/j.repbio.2018.07.001.

Funahashi, H., Ekwall, H., Rodriguez-Martinez, H., 2000. Zona reaction in porcine oocytes fertilized in vivo and in vitro as seen with scanning electron microscopy1. Biol. Reprod. 63, 1437–1442. https://doi.org/10.1095/biolreprod63.5.1437.

Godyń, D., Pieszka, M., Lipiński, P., Starzyński, R.R., 2016. Diagnostics of iron deficiency anaemia in piglets in the early postnatal period – a review. Anim. Sci. Pap. Rep. 34, 307–318.

Grindem, C.B., 2011. Schalm's veterinary hematology. In: Weiss, Douglas J., Wardrop, K. Jane (Eds.), Veterinary Clinical Pathology, 6th edition. https://doi.org/10.1111/j.1939-165X.2011.00324.x.

Gu, T., Shi, J., Luo, L., Li, Z., Yang, J., Cai, G., Zheng, E., Hong, L., Wu, Z., 2019. Study on hematological and biochemical characters of cloned duroc pigs and their progeny. Animals 9, 1–9. https://doi.org/10.3390/ani9110912.

Gutierrez, K., Dicks, N., Glanzner, W.G., Agellon, L.B., Bordignon, V., 2015. Efficacy of the porcine species in biomedical research. Front. Genet. 6, 1–9. https://doi.org/10.3389/fgene.2015.00293.

Heffron, J.J., Bomzon, L., Pattinson, R.A., 1976. Observations on plasma creatine phosphokinase activity in dogs. Vet. Rec. 98 https://doi.org/10.1136/vr.98.17.338, 338 LP – 340.

Ivanova, E., Canovas, S., Garcia-Martínez, S., Romar, R., Lopes, J.S., Rizos, D., Sanchez-Calabuig, M.J., Krueger, F., Andrews, S., Perez-Sanz, F., Kelsey, G., Coy, P., 2020. DNA methylation changes during preimplantation development reveal inter-species differences and reprogramming events at imprinted genes. Clin. Epigenetics 12, 64. https://doi.org/10.1186/s13148-020-00857-x.

Jerry Kaneko, J., Harvey, J.J., Bruss, M.L., 2008. Clinical Biochemistry of Domestic Animals, Clinical Biochemistry of Domestic Animals. Elsevier Inc. https://doi.org/10.1016/B978-0-12-370491-7.X0001-3

Kabalin, A.E., Balenovic, T., Speranda, M., Susic, V., Pavicic, Z., Milinkovic-Tur, S., Stokovic, I., Mencik, S., 2012. Influence of body mass at birth on the activity of enzymes and biocatalysts concentration in the serum of large white piglets until weaning. Vet. Arh. 82, 483–494.

Kalai, K., Nehete, R.S., Ganguly, S., Ganguli, M., Dhanalakshmi, S., Mukhopadhayay, S. K., 2012. Investigation of parasitic and bacterial diseases in pigs with analysis of hematological and serum biochemical profile. J. Parasit. Dis. 36, 129–134. https://doi.org/10.1007/s12639-011-0068-x.

Kim, M.J., Park, P.W., Seo, Y.H., Kim, K.H., Seo, J.Y., Jeong, J.H., Park, M.J., Ahn, J.Y., 2014. Comparison of platelet parameters in thrombocytopenic patients associated with acute myeloid leukemia and primary immune thrombocytopenia. Blood Coagul. Fibrinolysis 25, 221–225. https://doi.org/10.1097/MBC.0000000000000027.

Li, Y., Hagen, D.E., Ji, T., Bakhtiarizadeh, M.R., Frederic, W.M., Traxler, E.M., Kalish, J. M., Rivera, R.M., 2019. Altered microRNA expression profiles in large offspring syndrome and Beckwith-Wiedemann syndrome. Epigenetics 14, 850–876. https://doi.org/10.1080/15592294.2019.1615357.

López-Olvera, J.R., Höfle, U., Vicente, J., Fernández-de-Mera, I.G., Gortázar, C., 2006. Effects of parasitic helminths and ivermectin treatment on clinical parameters in the European wild boar (Sus scrofa). Parasitol. Res. 98, 582–587. https://doi.org/10.1007/s00436-005-0099-2.

Lorenzen, E., Follmann, F., Jungersen, G., Agerholm, J.S., 2015. A review of the human vs. porcine female genital tract and associated immune system in the perspective of using minipigs as a model of human genital Chlamydia infection. Vet. Res. 46, 1–13. https://doi.org/10.1186/s13567-015-0241-9.

Martinez, E.A., Martinez, C.A., Cambra, J.M., Maside, C., Lucas, X., Vazquez, J.L., Vazquez, J.M., Roca, J., Rodriguez-Martinez, H., Gil, M.A., Parrilla, I., Cuello, C., 2019. Achievements and future perspectives of embryo transfer technology in pigs. Reprod. Domest. Anim. 54 (Suppl. 4), 4–13. https://doi.org/10.1111/rda.13465.

Mir, B., Zaunbrecher, G., Archer, G.S., Friend, T.H., Piedrahita, J.A., 2005. Progeny of Somatic Cell Nuclear Transfer (SCNT) Pig Clones Are Phenotypically Similar to Non-Cloned Pigs, p. 7.

Nakazawa, Y., Misawa, H., Fujino, Y., Tajima, S., Misumi, K., Ueda, J., Nakamura, Y., Shibata, T., Hirayama, Y., Kikuchi, K., 2008. Effect of volume of non-surgical embryo transfer medium on ability of porcine embryos to survive to term. J. Reprod. Dev. 54, 30–34. https://doi.org/10.1262/jrd.19132.

Peltoniemi, O., Björkman, S., Oropeza-Moe, M., Oliviero, C., 2019. Developments of reproductive management and biotechnology in the pig. Anim. Reprod. 16, 524–538. https://doi.org/10.21451/1984-3143-AR2019-0055.

Perri, A.M., 2015. An Investigation of Various Hematological and Biochemical Parameters to Asses the Health of Nursery Pigs. Univ. Guelph. https://doi.org/10.1017/CBO9781107415324.004.

Perri, A.M., O'Sullivan, T.L., Harding, J.C.S., Wood, R.D., Friendship, R.M., 2017. Hematology and biochemistry reference intervals for Ontario commercial nursing pigs close to the time of weaning. Can. Vet. J. 58, 371–376.

Pliszczak-Król, A., Rząsa, A., Gemra, M., Król, J., Łuczak, G., Zyzak, A., Zalewski, D., Iwaszko-Simonik, A., Graczyk, S., 2016. Age-related changes of platelet and plasma coagulation parameters in young pigs. J. Vet. Diagn. Investig. 28, 561–567. https://doi.org/10.1177/1040638716658928.

Salak-Johnson, J.L., McGlone, J.J., 2007. Making sense of apparently conflicting data: stress and immunity in swine and cattle. J. Anim. Sci. 85 https://doi.org/10.2527/jas.2006-538.

Starzyński, R.R., Laarakkers, C.M.M., Tjalsma, H., Swinkels, D.W., Pieszka, M., Styś, A., Mickiewicz, M., Lipiński, P., 2013. Iron supplementation in suckling piglets: how to correct iron deficiency anemia without affecting plasma hepcidin levels. PLoS One 8, 1–7. https://doi.org/10.1371/journal.pone.0064022.

Stone, R.T., 1984. Relationship of alpha-fetoprotein and albumin in fetuses and neonates from genetically lean and obese swine. Neonatology 46, 122–130. https://doi.org/10.1159/000242055.

Suzuki, C., Iwamura, S., Yoshioka, K., 2004. Birth of piglets through the non-surgical transfer of blastocysts produced in vitro. J. Reprod. Dev. 50, 487–491. https://doi.org/10.1262/jrd.50.487.

Van Montfoort, A.P.A., Hanssen, L.L.P., De Sutter, P., Viville, S., Geraedts, J.P.M., De Boer, P., 2012. Assisted reproduction treatment and epigenetic inheritance. Hum. Reprod. Update 18, 171–197. https://doi.org/10.1093/humupd/dmr047.

Van Soom, A., Wydooghe, E., Heras, S., Vandaele, L., 2011. Alternative models for the study of embryo maternal cross-talk and signaling molecules from fertilisation to implantation. Reprod. Fertil. Dev. 23, 10–12. https://doi.org/10.1071/RDv23n8_FO.

Venn, J.A.J., Mccance, R.A., Widdowson, E.M., 1947. Iron metabolism in piglet anaemia. J. Comp. Pathol. Ther. 57, 314–325. https://doi.org/10.1016/s0368-1742(47)80037-2.

Ventrella, D., Dondi, F., Barone, F., Serafini, F., Elmi, A., Giunti, M., Romagnoli, N., Forni, M., Bacci, M.L., 2017. The biomedical piglet: establishing reference intervals for haematology and clinical chemistry parameters of two age groups with and without iron supplementation. BMC Vet. Res. 13, 1–8. https://doi.org/10.1186/s12917-017-0946-2.

Verheyen, A.J.M., Maes, D.G.D., Mateusen, B., Deprez, P., Janssens, G.P.J., de Lange, L., Counotte, G., 2007. Serum biochemical reference values for gestating and lactating sows. Vet. J. 174, 92–98. https://doi.org/10.1016/j.tvjl.2006.04.001.

Walton, J.S., 1986. Effect of boar presence before and after weaning on estrus and ovulation in sows. J. Anim. Sci. 62, 9–15. https://doi.org/10.2527/jas1986.6219.

Weksberg, R., Shuman, C., Beckwith, J.B., 2010. Beckwith-Wiedemann syndrome. Eur. J. Hum. Genet. 18, 8–14. https://doi.org/10.1038/ejhg.2009.106.

Yoshioka, K., Noguchi, M., Suzuki, C., 2012. Production of piglets from in vitro-produced embryos following non-surgical transfer. Anim. Reprod. Sci. 131, 23–29. https://doi.org/10.1016/j.anireprosci.2012.01.018.

Yoshioka, K., Uchikura, K., Suda, T., Matoba, S., 2020. Production of piglets from in vitro-produced blastocysts by ultrasound-guided ovum pick-up from live donors. Theriogenology 141, 113–119. https://doi.org/10.1016/j.theriogenology.2019.09.019.

Yu, K., Canalias, F., Solà-Oriol, D., Arroyo, L., Pato, R., Saco, Y., Terré, M., Bassols, A., 2019. Age-related serum biochemical reference intervals established for unweaned calves and piglets in the post-weaning period. Front. Vet. Sci. 6, 1–12. https://doi.org/10.3389/fvets.2019.00123.