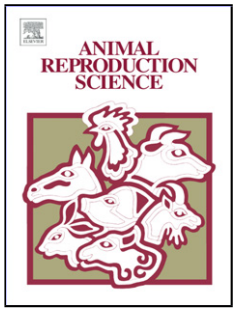

Immuno-castration of female and male pigs with anti-gonadotrophin releasing hormone vaccine: morphometric, histopathological and functional studies of the reproductive system

Olga Mitjana, Cristina Bonastre (Investigation) (Methodology), Mª Teresa Tejedor (Formal analysis) (Data curation) (Writing - review and editing), Laura Garza (Investigation) (Methodology), Mª Ángeles Latorre (Investigation) (Methodology), Bernardino Moreno (Investigation) (Methodology) (Validation) (Writing - review and editing), Mª Victoria Falceto (Investigation) (Methodology) (Validation) (Writing - review and editing)

PII: S0378-4320(20)30471-1

DOI: https://doi.org/10.1016/j.anireprosci.2020.106599

Reference: ANIREP 106599

To appear in: *Animal Reproduction Science*

Received Date: 13 April 2020

Revised Date: 4 September 2020

Accepted Date: 5 September 2020

Please cite this article as: Mitjana O, Bonastre C, Tejedor MT, Garza L, Latorre M, Moreno B, Falceto MV, Immuno-castration of female and male pigs with anti-gonadotrophin releasing hormone vaccine: morphometric, histopathological and functional studies of the reproductive system, *Animal Reproduction Science* (2020), doi: https://doi.org/10.1016/j.anireprosci.2020.106599

Immuno-castration of female and male pigs with anti-gonadotrophin releasing hormone vaccine: morphometric, histopathological and functional studies of the reproductive system

Olga Mitjana[a], Cristina Bonastre[a], Mª Teresa Tejedor[c], Laura Garza[a], Mª Ángeles Latorre[d,e], Bernardino Moreno[b,e,*], Mª Victoria Falceto[a,e]

[a]*Departamento de Patología Animal, Universidad de Zaragoza, Zaragoza, Spain*

[b]*Departamento de Patología Animal, Centro de Encefalopatías y Enfermedades Transmisibles Emergentes, Universidad de Zaragoza, Zaragoza, Spain*

[c]*Departamento de Anatomía, Embriología y Genética Animal, CIBERCV, Universidad de Zaragoza, Zaragoza, Spain*

[d] *Departamento de Producción Animal y Ciencia de los Alimentos, Universidad de Zaragoza, Zaragoza, Spain*

[e]*Instituto Agroalimentario de Aragón - IA2 - (Universidad de Zaragoza-CITA), Zaragoza, Spain*

[*]Corresponding Author: *E-mail address*: bmoreno@unizar.es (B. Moreno); Bernardino Moreno and Mª Victoria Falceto are equal senior contributors to this work

Highlights

- Immuno-castration causes gross and microscopic changes in gilts and boars
- Immuno-castration in female pigs induces significant changes in ovaries and uterus
- Immuno-castration in gilts does not induce significant changes in vagina and vulva
- Immuno-castration in boars induces a reduction of spermatogenesis and Leydig cells
- Immuno-castration in male pigs causes changes in reproductive accessory glands

ABSTRACT

Immuno-castration is increasingly recommended in pigs due to welfare reasons; however, there are few studies in females compared to males. This aim of this study was to investigate the effects of immuno-castration in female and male pigs. The weight, the morphometric and microscopic characteristics of the reproductive organs, and the hormone concentrations were studied in 12 immunocastrated females (IF) and 12 immunocastrated males (IM) and compared with control animals (C). At slaughter, IF tended to have greater body weights than CF ($P = 0.051$), whereas in IM and CM pigs there were not body weight differences ($P = 0.140$). The weight of the reproductive tract and size of all individual organs were less in IF compared with CF. Results from histological assessments indicated IF had more atretic follicles and a thinner endometrial mucosa than control females. Hormone concentrations were not different between CF and IF **(**$P > 0.050$). As a result of immuno-castration, there was impaired spermatogenesis in most males. Results from microscopic evaluations indicated there

was a marked decrease of spermatogonial cells and size of Leydig cells in the testicles. Accessory gland structures were affected in CM and IM with there being differences in gross and microscopic characteristics. Testosterone concentrations, unlike estradiol, were different in IM compared to CM ($P < 0.001$). These results provide evidence that immuno-castration with the anti-gonadotrophin releasing hormone vaccine is effective in female and male pigs and induces morphological and endocrine changes incompatible with fertility.

Keywords: Immuno-castration; Anti-gonadotropin releasing hormone; Gilts; Boars; Morphometry; Histopathology

1. Introduction

Castration of male pigs is a common practice worldwide for production and commercial reasons, and is typically performed by surgical methods; however, female castration occurs less frequently. In Spain, surgical castration of females is a common practice in the farming of Iberian pigs, a free range traditional breed, to avoid unwanted mating with wild boars (Cava et al., 2000). Conversely, it is not performed routinely in white breeds and important losses are derived from carcase rejections at the slaughterhouse due to lack of intramuscular fat (Latorre et al., 2009).

Surgical castration is currently restricted by international regulations due to animal welfare concerns and different alternative castration methods are being applied. Immuno-castration is a promising alternative and has the same advantages as surgical castration. There are rapid growth rates when imposing this procedure indicating there are positive effects on feed conversion. Furthermore, there are positive effects on meat quality when immuno-castration procedures are imposed, with there being greater

intramuscular fat content in the meat and, thus, an eating experience with an enhanced flavour (Bohrer et al., 2014; Gamero-Negrón et al., 2015; Daza et al., 2016; Martínez-Macipe et al., 2016; Van den Broeke et al., 2016). In boars, there is also a reduction in behavioural problems and adverse meat flavour (Rydhmer et al., 2010; Font i Furnols et al., 2012; Brewster and Nevel, 2013; Heid and Hamm, 2013; Daza et al., 2016).

Immuno-castration is typically conducted using vaccination procedures against GnRH (gonadotropin-releasing hormone) and its effects have been studied in several animal species (Brown et al., 1995; Clarke et al., 1998; Baker et al., 2018; Van den Broeke et al., 2016; Zoels et al., 2020). There have been variable results depending on the animal species, the age of the animals, individual responses or the number of immunisations (Heyrman et al., 2019). In pigs, most studies have been with males; however, reports in female pigs are limited. Furthermore, studies describing macroscopic and microscopic findings are very few. The aim of the present study was to evaluate the effects of immuno-castration in female and male pigs. The growth of the animals, macroscopic and microscopic changes of the reproductive organs, and concentrations of sex hormones were determined and compared with control pigs.

2. Materials and methods

2.1. Animals and vaccination protocol

The care and use of animals was conducted in accordance with the Spanish Policy for Animal Protection RD53/2013, which complies with Directive 2010/63 of the European Union on the protection of animals used for scientific purposes. This study was approved by the Ethical Committee for Animal Experiments, University of Zaragoza, Spain (reference number PI29/18). The pigs were managed using procedures

consistent with the applicable regulations in Spain regarding animal welfare and housing.

Pigs (n = 48), 24 females and 24 males, were selected from a farm in Teruel, in eastern Spain. All pigs were of Duroc x (Landrace x Large White) crossbred lines. Animals were individually identified with an ear tag and assigned to groups of three pigs, depending on body weight and sex, and were assigned to 16 pens. Pigs were subsequently allotted into four groups of 12 animals according to the vaccination protocol: intact females (control female, CF), surgically castrated males (control male, CM), immunocastrated females (IF), and immunocastrated males (IM).

The pigs of the groups that were immunocastrated were vaccinated against GnRH, with Improvac® (Zoetis, Madrid, Spain). Following the manufacturer's recommendation, the first injection was administered at 13 weeks of age (45-47 kg) and the second injection was administered 4 weeks later. The females of the control group (CF) were not administered any vaccine and the males of the control group (CM) were surgically castrated in accordance with the EEC Directive (2001). Surgically ovariectomized females were not available. In addition, four entire males of the same weight (120 kg), age (6 months) and genetic line as the IM were assigned to the control group for histology evaluations of testicles and sexual glands and hormone concentrations.

All pigs were slaughtered 8 weeks after the second vaccine administration, at the age of 25 weeks, when commercial pigs are typically slaughtered. The pigs were weighed after the first and second vaccination and 2 days before the time of slaughter.

2.2. Samples

2.2.1. Reproductive organs

The pigs were slaughtered in the "Alto Mijares" slaughterhouse, located in Formiche Alto (Teruel, Eastern Spain). The reproductive organs of male and female pigs were removed immediately after slaughter and stored in individual plastic bags identified by the pig ear tag number. All bags were refrigerated and transported to the laboratory of the Veterinary Faculty of the University of Zaragoza within 2 hours of collection. Samples from female pigs included ovaries, oviducts, uterus, vagina, vulva, and urinary bladder. Samples from male pigs included both testicles within the scrotum and accessory glands (prostate, vesicular and bulbourethral). Because these glands were small and difficult to identify, these tissues were removed with a portion of the rectum and then carefully dissected in the laboratory (Supplementary Figs. 1-6).

2.2.2. Blood samples

Blood samples were collected in 10-mL sterile serum tubes (Biochemistry serum Venoject ® VT-100STK, TERUMO CORPORATION, Tokyo, Japan) at the time of slaughter. Serum was obtained by centrifugation at 1,600 × *g* for 10 min, transferred to Eppendorf test tubes and immediately frozen and stored at -20 ºC for further analyses.

2.3. Morphometric analysis

The weight, length, area, and volume of the reproductive organs (Supplementary Figs. 1-6) were evaluated using a precision electronic balance (Cobos® (XT-920M, Cobos Precision, S.L. Hospitalet de Llobregat, Spain, with a precision of 0.001 g) and a caliper (Ref.: 6555030 710 pocket vernier caliper, Remscheid, Germany).

For the female pigs, the weight, dimensions (length, width, depth), and the volume of the ellipsoid were measured for both ovaries. Follicles of different sizes (< 2 mm, 2-4 mm, 4-6 mm, and > 6 mm) and corpora rubra, lutea and albicantia were identified.

Oviducts, uterine horns, and the uterine body were weighed and measured. The weight and length of the cervix were measured from the caudal area of the uterine body to the cranial area of the vaginal region where the papillary tubercles are present. The length of the vagina was measured from the caudal area of the cervix to the cranial area of the vestibule; the vaginal vestibule from the caudal area of the vagina cranial area of the vulva, and the length of the vulva from the top to the end of the ventral commissure. The extent of flanges and clitoris development were estimated.

The testicles, epididymides, and sexual glands were studied macroscopically in immunocastrated males, whereas only the glands were examined in surgically castrated animals. The weight, dimensions (length, width, depth), and the volume of the ellipsoid were measured for both testicles. The length and weight of the epididymis were determined and the thickness of the head, body, and tail were estimated. The weight, dimensions, and the area of the ellipse of the bulbourethral and vesicular glands were estimated. There was a cross sectional evaluation of the bulbourethral and vesicular glands to determine whether there were seminal contents in these glands and if so the characteristics of the seminal contents. Due to anatomical characteristics, only the diameter of the prostate was measured and, from the diameter, the volume of the sphere was calculated.

2.4. Histopathological study

Histological studies were performed in ten IF and nine IM. In addition, six CF and four EM pigs were used as controls. The samples were routinely processed. Briefly, the samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm, and stained with haematoxylin and eosin.

In females, the thickness of the cortex, presence of atretic follicles, and size of tertiary follicles in the ovary were assessed. In the other organs, the thickness and characteristics of the mucosa and thickness of the muscular layers were estimated.

In males, the diameter of seminiferous tubules and number and type of cells involved in spermatogenesis were estimated in the testicles. In addition, the number of affected seminiferous tubules was studied. According to these characteristics, four groups were considered subjectively. The classifications of Grade 0 occurred when there were testicles with seminiferous tubules similar to normal pigs; Grade I when there was a lesser number of spermatids and mature spermatozoa; Grade II when there was total absence of spermatids and mature spermatozoa and the presence of dispersed spermatogonia, and Grade III when there were seminiferous tubules containing predominantly Sertoli cells. The number and size of Leydig cells were also estimated. Based on the size of the Leydig cells, the pigs were classified into three groups, those with Leydig cells similar to normal pigs (G0), those with Leydig cells of a moderately reduced size (GI) and those with Leydig cells with a markedly lesser size (GII).

In the epididymis, the characteristics of the mucosa and the presence of luminal spermatozoa, immature cells, exudate, and interstitial fibrosis were evaluated. Based on the number of spermatozoa in the lumen, the epididymides were classified into four groups, those similar to what is typically detected in entire pigs (G0), those with a slightly lesser spermatozoa number (GI), those with markedly lesser spermatozoa numbers (GII) and those without spermatozoa (GIII).

For the accessory glands, the number and size of glandular acini, amount of secretions, and amount of fibrous tissue were evaluated. Based on these variables, the glands were classified into four groups: glands similar to entire males (G0), glands with a slightly lesser acini and secretions than entire males and those with slightly more

fibrous tissue than entire males (GI), glands with a moderately less acini and secretions and more fibrous tissue than entire males (GII), and those with markedly lesser acini and secretions and a large amount of fibrosis (GIII).

2.5. Hormonal study

Progesterone concentrations were quantified in all females and estradiol levels in nine CF and ten IF. In males, free testosterone concentrations were determined in all immunocastrated males and in four entire animals. Estradiol was quantified in six CM and 10 IM. All assays were conducted using serum utilising commercially available radioimmunoassay kits following the manufacturer´s instructions. The E2-RIA-CT, KIPP0629 kit was used for 17β-estradiol quantitation, the PROG-RIA-CT, KIP1458 kit for progesterone quantitation, and the Free TESTOSTERONE-RIA-CT, KIPI19000 kit for free testosterone quantitation.

2.6. Statistical analysis

Statistical analyses were conducted using IBM SPSS statistics version 22 software (IBM, Armonk, NY, USA). Means and standard error of the mean (SEM) were calculated for every variable. Fisher´s exact tests were used to compare presence/absence of follicles between CF and IF groups. Variables from paired organs were analyzed for individuals using the paired sample t- test. The General Linear Model (GLM) procedure was used for comparing variables between control and treated groups; weight at slaughterhouse was used as covariable in every case. Weights at previous stages were used for comparing weight data. Neperian logarithm transformation was applied when volume and area data were compared. Hormone serum concentrations were compared using the nonparametric test (U de Mann-Whitney and Kruskall- Wallis

test). Mean values were considered to be different when there was a $P < 0.050$. When differences were detected and multiple comparisons were applicable, pairwise comparisons occurred using the Bonferroni's correction procedures.

3. Results

Two immunocastrated animals (one female and one male) died during the study for reasons not related to the experiment. There, therefore, were only 46 animals slaughtered.

3.1. Weight of animals and Morphometric analysis

There were no differences in female or male weights at any of the three weighing timepoints between control and immuno-castrated animals. The IF, however, tended to have greater ($P = 0.051$) weights than CF at slaughter. Results are included in Tables 1 and 2.

In female pigs, results from morphometric studies indicated there were differences in the weight of the whole reproductive tract and of most individual organs between the immuno-castrated and control animals (Table 1). There were no differences in the lengths of the vagina, vulva, and vulva-anus length or between paired organs.

The number and size of ovarian follicles differed markedly between control and immuno-castrated females. Follicles, however, were detected in all the females of the control group, while there were follicles detected in only 9.1% (1/11) of the immuno-castrated females ($P < 0.001$). These follicles were < 2 mm in diameter and, therefore, were not affected by gonadotrophins considering the small size of these follicles. Only two control females (2/12; 16.7%) had follicles > 6 mm, therefore, having the capacity to produce relatively greater quantities of estrogen and respond to the release of LH that

induces ovulation, indicating there was development of follicles to extent that there could be ovulations from these follicles. Ovarian activity, therefore, was markedly greater in CF females indicating these females were in the peripubertal period of sexual maturation.

The size and weight of all the reproductive glands were markedly greater in IM compared to CM (Table 2). There were only glandular secretions in immuno-castrated pigs (bulbourethral glands: 10/11 (90.9%); vesicular glands: 6/11 (54.5%)). Results from morphometric evaluations of the testicles and epididymides of immuno-castrated males indicated there were minor differences between the right and left testicles.

3.2. Histopathological study

In females, the differences between immuno-castrated and control animals occurred primarily in the ovaries and the uterus. The ovaries of immuno-castrated females had a thinner cortex, more atretic follicles and smaller tertiary follicles (Fig. 1E) compared with control animals (Fig. 1A). There was only one corpus luteum detected and that was in a control female. The uteri of immuno-castrated females had a thinner mucosa with fewer and smaller endometrial glands and a lesser epithelial layer (Figs. 1M and N) compared with control animals (Figs. 1I and J). The oviducts (Figs. 1B, C, D, F, G, and H), cervix (Figs. 1K and O) and vagina (Figs. 1L and P) were similar in structure in both IF and CF.

Eight out of nine immuno-castrated pigs had testicles with an incremental reduction in diameter of the seminiferous tubules due to there being spermatogenesis (Fig. 2; Table 3). In one pig, vaccination was not effective, and the testicles were similar to the testicles of control pigs. Among the eight pigs in which vaccination was effective, one pig had testicles that were similar in morphological structure as that of control pig,

although there was a lesser spermatogenesis in some tubules of this pig (Fig. 2A). In another pig, there was a complete inhibition of the spermatogenic process with seminiferous tubules lined only by Sertoli cells (Fig. 2D). In the other six pigs, there was a moderate to extensive reduction of spermatogenesis in most of the tubules (Fig. 2C). In most pigs, Leydig cell size was smaller compared to entire males (Fig. 2E), due to reduced nucleus/cytoplasm ratio (Fig. 2F). Apparently, the number of Leydig cells was also slightly less in immuno-castrated pigs, although this estimation was difficult to technically assess.

The epididymis of immuno-castrated pigs had few structural differences compared with control pigs, although there were very few luminal sperm cells because of the lesser spermatogenesis (Figs. 2G and 2H; Table 3). An irregular reduction in the density of microvilli, a reduction in apical cytoplasmic vacuolization and the presence of amorphous eosinophilic material consistent with protein secretions were frequently observed (Fig. 2H).

The accessory glands of immuno-castrated males were characterized by varying increments in acinar atrophy and stromal fibrosis that was consistent with the extent of alterations in testicular development as a result of the treatment (Fig. 3; Supplementary Table 1). In the glands of pigs in which the immuno-castration was not effective and of the pig with a slight reduction in spermatogenesis there were similar characteristics as that of the entire pigs (Figs. 3A, E, and I). In surgically castrated males, the accessory glands were characterised by marked underdevelopment of glandular acini and the presence of abundant fibrous tissue surrounding the acini (Figs. 3B, F, and J).

3.3. Hormone concentrations

There were no differences in serum concentrations of progesterone and estradiol between the CF and IF (Table 4). There were lesser concentrations of serum testosterone in IM and CM compared with EM ($P < 0.001$). There were no differences in concentrations of estradiol among pigs of the treatment groups (Table 5).

4. Discussion

Immuno-castration in female pigs has been studied much less than in males. In the present study, immuno-castration was effective in all females with there being a marked reduction in both length and weight of the entire reproductive system. The main gross differences as a result of treatments were in the ovaries and the uterus, similar to what was previously reported in pigs (Zeng et al., 2002; Dalmau et al., 2015; Van den Broeke et al., 2016) and other species (Brown et al., 1995; Clarke et al., 1998; Baker et al., 2018). The ovaries of IF had markedly fewer and smaller follicle sizes, indicating there was a lack of GnRH actions in inducing increases in gonadotrophin secretion (Clarke et al., 1998; Van den Broeke et al., 2016). Immuno-castration of Iberian pigs is an important production practice because it can prevent unwanted mating with wild boars. Vaccination against GnRH has been reported to be effective in inhibiting reproduction of free-ranging horses (Baker et al., 2018) and wildlife (Powers et al., 2011). Immuno-castration in pigs of white breeds also has advantages by increasing the amount of intramuscular fat and, consequently, fewer carcasses are rejected for the production of dry-cured ham (Latorre et al., 2009).

No morphometric studies have been performed where there was evaluation of effects on external genitalia of immuno-castrated female pigs; however, studies of this type may be important for elucidating effects of immuno-castration. Vulvovaginal atrophy has been associated with the hypo-estrogen state (Cagnacci et al., 2019), and anogenital

distance has been used as a marker of effects of sex hormones in human newborn males and females (Domenici et al., 2018). In the present study, morphometric analyses in immuno-castrated females indicated there were no changes in the vagina, vestibule, and vulva. There were also no differences in the distance between the vulva and the anus and, therefore, no external indications of immuno-castration. The different responses of the internal and external genitalia to immuno-castration could be explained by the variable effect of the hormones or by the different effects on embryonic tissues of origin during reproductive development (Sadler, 2019). The lack of differences in estrogen concentrations are the likely reason for the lack of effects on external genitalia structures in the present study.

Microscopic studies have not been performed on the reproductive organs of immuno-castrated female pigs. The reversibility and duration of immuno-castration effects and effects on subsequent fertility and prolificacy are a relevant aspect, and histological studies may be important for evaluation of the effects of immuno-castration of females. In the present study, morphometric effects of immuno-castration were consistent with the histopathological findings with there being marked differences that were only identified in the ovaries and the uterus of immuno-castrated females. Ovaries of IF contained fewer and smaller follicles, and a larger number of atretic follicles indicating there was delayed and aberrant follicular development. In the uterus of immuno-castrated females, the underdevelopment of epithelial and glandular tissues also indicated there was inhibition of functions of the hypothalamic-hypophyseal-ovarian axis as a consequence of immuno-castration (Oberlender et al., 2014). Findings in the present study indicate morphologic changes are not great enough to negate the possibility of reversibility, although a delay in the time of slaughter after the second

vaccination would have been necessary to confirm if reversibility of the treatment occurs.

Hormone concentrations were not different between CF and IF and this is consistent with results from previous studies (Zeng et al., 2002; Dalmau et al., 2015). There was a baseline progesterone concentration due to the absence of corpora lutea and there was not any indication of previous ovulations based on ovarian structure evaluations. An intriguing result was the differences in morphometric findings in the absence of differences in hormone concentrations. This could be explained by the large individual variability in hormone concentrations within each group (see SEM and range values in Table 5) and hormonal effects on the development of the reproductive system during the prepubertal period. Estrogen concentrations increase during estrus in pubertal sows; however, both immuno-castrated and control females in this study were prepubertal at slaughter and, therefore, basal concentrations were expected. In entire females, the development of the reproductive organs after birth is regulated by gonadotrophins and ovarian factors (Colenbrander et al., 1983). In immuno-castrated females, gonadotropin stimulation was not expected and the reproductive organs were expected to remain undeveloped at the time of slaughter.

In males, immuno-castration was effective in most pigs. As previously reported (Brewster and Nevel, 2013; Han et al., 2017; Lugar et al., 2017), spermatogenesis was clearly less in immuno-castrated pigs. One of the most important aspects of immuno-castration should be the absence of negative effects on production variables (Latorre et al., 2003; Morales et al., 2010; Daza et al., 2014), and this was also observed in the present study. Immuno-castration resulted in microscopic changes in all reproductive organs. In the testis, there was less spermatogenesis leading to a reduction in the diameter of the seminiferous tubules in eight out of nine pigs evaluated. Results from

the present study are consistent with those reported by Han et al. (2017); however compared with their results, in the present study there were no fewer Sertoli cells. Apparently, the number of Sertoli cells remains constant when atrophy occurs and only spermatogonial cells are fewer in the seminiferous tubules, and in the final stages of atrophy only Sertoly cells are present.

Leydig cells are also typically affected by immuno-castration and there is reduced fertility due to hormonal effects as a consequence of immuno-castration (Latorre et al., 2003; Einarsson et al., 2009; Morales et al., 2010). Consistent with what was previously reported (Einarsson et al., 2009; Morales et al., 2010), in the present study there was a marked reduction of the cytoplasm of Leydig cells which is consistent with previous findings where there was a reduced hormonal stimulation and a reduced testosterone production as a result of immuno-castration. Assessing the number of Leydig cells was more difficult, but there seemed to be a slight decrease as a result of immuno-castration. During pubertal development in male pigs, changes in Leydig testicular cells occur between 40 and 250 days of age (Lunstra and Christenson, 1986). In the present study, immuno-castration was performed during this period and this would explain the smaller size and the smaller number of Leydig cells in IM pigs.

The macroscopic and microscopic features of the accessory glands also confirmed that there were effects of immuno-castration. There were marked differences in volume and weight compared to entire and surgically castrated males as has been previously reported (Einarsson et al., 2009; Bonneau, 2010). Microscopic findings in the present study were characteristic of unstimulated and atrophic glands (Einarsson et al., 2009). There have been no previous reports on prostate measurements; however, results from the present study indicate the diameter of the prostate may also be a marker of effectiveness of immuno-castration.

There, however, is some individual variability in response to immuno-castration. There was complete failure of immuno-castration in one pig and partial failure in another in the present study. It appears that immuno-castration may not be effective in some male pigs, although the reasons remain unclear (Kubale et al., 2013; Wicks et al., 2013). Different individual responses to vaccination or incorrect application of the vaccine have been suggested as causes of vaccination ineffectiveness. In the present study, application failure was not a cause of this ineffectiveness, therefore, individual variation in response is suspected. The efficacy of immuno-castration appears to depend on vaccination protocols, but effects of this procedure are often greater than that suggested by the manufacturer (Zamaratskaia et al. 2008; Brunius et al., 2011; Kubale et al., 2013; Moore et al., 2017). Re-immunisation has been reported to increase the effectiveness of immuno-castration in free-ranging horses (Baker et al., 2018). Results from other studies indicate anti-GnRH antibodies persisted after 22 weeks from the last injection (Zamaratskaia et al. 2008) or that there were basal testosterone concentrations 15 weeks after immuno-castration (Claus et al., 2008; Lugar et al., 2017). In the present study, although there were not analyses for antibodies, 8 weeks after the second injection, hormone concentrations still remained basal, indicating the efficacy of immuno-castration persisted. In other studies, there was an irreversibility of immuno-castration that was associated with severe histological lesions in the testicles at 4, 16, and 22 weeks after the last vaccination (Einarsson et al., 2009). In the present study, histological lesions remained until 8 weeks after the last injection, when the pigs were slaughtered. Hormone concentrations and histological findings indicate there was likely reversibility of the effects of immuno-castration in most of the pigs of the present study, and this is consistent with the manufacturer-indicated effects of immuno-castration using their procedures. Irreversibility of immuno-castration in the present study was

apparently only an occurrence in one testicle, in which only Sertoli cells remained in the seminiferous tubules. The other seven testicles in the present study had impaired spermatogenesis; therefore, there was integrity of the basement membranes of the seminiferous tubules that were indicative of reversibility. Brunius et al. (2011) reported that testicular dysfunction may be irreversible; especially when vaccination is performed at a young age. These findings indicate the conditions necessary for the full recovery of the morphology and functionality of the testicles, epididymis, and accessory sex glands after the last injection of the immuno-castration treatment regimen remain unclear.

5. Conclusion

Immuno-castration was effective in female pigs and there were marked effects in the ovaries and uterus as a result of imposing this procedure. The presence of only a few and small antral follicles indicated ovarian development was not consistent with developmental characteristics indicative of the onset of puberty. Behavioural estrus or undesirable mating of IF would not be expected to occur. There, however, were no effects on external reproductive organs, and consequently, no external symptoms of immuno-castration in females. Results from histological evaluations indicated underdevelopment of the ovaries and uterus.

Immuno-castration was also effective in males and there was less development of the genital organs and lesser hormone concentrations (testosterone and estrogens), and effects persisted for 8 weeks after the last injection, when pigs were slaughtered. The histological changes observed in the seminiferous tubules of the IM indicated there was impaired spermatogenesis in most pigs. Results from microscopic evaluations also indicated there was the previously reported variability that occurs with immuno-

castration in male pigs and confirmed that vaccination against GnRH may not be effective in a small percentage of the animals.

The individual author contributions are:

Olga Mitjana: Investigation, Methodology, Data Curation, Validation, Writing - Original Draft.

Cristina Bonastre: Investigation, Methodology.

Mª Teresa Tejedor: Formal analysis, Data Curation, Writing - Review & Editing.

Laura Garza: Investigation, Methodology.

Mª Angeles Latorre: Investigation, Methodology.

Bernardino Moreno: Investigation, Methodology, Validation, Writing - Review & Editing.

Mª Victoria Falceto: Investigation, Methodology, Validation, Writing - Review & Editing

Declaration of Competing Interest

The authors declare no conflicts of interest.

Funding

This research received no financial support.

Acknowledgements

The authors would like to thank Dr. Argimiro Daza (Universidad Politécnica de Madrid) for the great help in this research.

Appendix A. Supplementary data

Supplementary data related to this article can be found, in the version online, at http

References

Baker, D.L., Powers, J.E., Ransom, J.I., McCann, B.E., Oehler, M.W., Bruemmer, J.E., Galloway, N.L., Eckery, D.C., Nett, T.M., 2018. Reimmunization increases contraceptive effectiveness of gonadotropin-releasing hormone vaccine (GonaCon-Equine) in free-ranging horses (*Equus caballus*): Limitations and side effects. PLoS ONE 13, e0201570.

Bohrer, B.M., Flowers, W.L., Kyle, J.M., Johnson, S.S., King, V.L., Spruill, J.L., Thompson, D.P., Schroeder, A.L., Boler, D.D., 2014. Effect of gonadotropin releasing factor suppression with an immunological on growth performance, estrus activity, carcass characteristics, and meat quality of market gilts. J. Anim. Sci. 92, 4719-4724.

Bonneau, M., 2010. Accessory sex glands as a tool to measure the efficacy of immunocastration in male pigs. Animal 4, 930-932.

Brewster, V., Nevel, A., 2013. Immunocastration with Improvac TM reduces aggressive and sexual behaviors in male pigs. Appl. Anim. Behav. Sci. 145, 32-36.

Brown, B.W., Mattner, P.E., Carroll, P.A., Hoskinson, R.M., Rigby, R.D.G., 1995. Immunization of sheep against GnRH early in life: effects on reproductive function and hormones in ewes. J. Reprod Fertil. 103, 131-135.

Brunius, C., Zamaratskaia, G., Andersson, K., Chen, G., Norrby, M., Madej, A., Lundström, K., 2011. Early immunocastration of male pigs with Improvac(®) - effect on boar taint, hormones and reproductive organs. Vaccine 29, 9514-9520.

Cagnacci, A., Xholli, A., Sclauzero, M., Vernier, M., Palma, F., Gambacciani, M., & writting group of the ANGEL study. 2019. Vaginal atrophy across the menopausal age: results from the ANGEL study. Climateric 22, 85-89.

Cava, R., Ventanas, J., Ruiz, J., Andres, A.I., Antequera, T., 2000. Sensory characteristics of Iberian ham: Influence of rearing system and muscle location. Food Sci. Technol. Int. 6, 235-242.

Clarke, I.J., Brown, B.W., Tran, V.V., Scott, C.J., Fry, R., Millar, R.P., Rao, A., 1998. Neonatal immunization against Gonadotropin-Releasing hormone (GnRH) results in diminished GnRH secretion in adulthood. Endocrinology 139, 2007-2014.

Claus, R., Rottner, S., Ruecker, C., 2008. Individual return to Leydig cell function after GnRH-immunization of boars. Vaccine 26, 4571-4578.

Colenbrander, B., Van Rossum-Kok, C.M.J.D., Oxender, C.J.G., Wensing, C.J.G., 1983. Ovarian development in control and decapitated pig fetuses. Biol. Reprod. 29, 217-223.

Dalmau, A., Velarde, A., Rodríguez, P., Pedernera, C., Llonch, P., Fàbrega, E., Casal, N., Mainau, E., Gispert, M., King, V., Slootmans, N., Thomas, A., Mombarg, M., 2015. Use of an anti-GnRF vaccine to suppress estrus in crossbred Iberian female pigs. Theriogenology 84, 342-347.

Daza, A., Latorre, M.A., Olivares, A., López-Bote, C.J., 2014. The effect of immunocastration and a diet based on granulated barley on growth performance and carcass, meat and fat quality in heavy gilts. Animal 8, 484-493.

Daza, A., Latorre, M.A., Olivares, A., López Bote, C.J., 2016. The effects of male and female immunocastration on growth performances and carcass and meat quality of pigs intended for dry-cured ham production: A preliminary study. Livestock Sci. 190, 20-26.

Domenici, L., Musella, A., Bracchi, C., Lecce, F., Schiavi, M.C., Colagiovanni, V., Di Donato., V., Marchetti, C., Tomao, F., Palaia, I., Muzii, L., Panici, P.B., 2018. Comparison of anogenital distance and correlation with vulvo-vaginal atrophy: a

pilot study on premenopausal and postmenopausal women. J. Menopausal Med. 24, 108-112.

Einarsson, S., Andersson, K., Wallgren, M., Lundström, K., Rodriguez-Martinez, H., 2009. Short- and long-term effects of immunization against gonadotropin-releasing hormone, using Improvac, on sexual maturity, reproductive organs and sperm morphology in male pigs. Theriogenology 71, 302-310.

Font i Furnols, M., Gispert, M., Soler, J., Diaz, M., Garcia- Regueiro, J.A., Diaz, I., Pearce, M.C., 2012. Effect of vaccination against gonadotrophin-releasing factor on growth performance, carcass, meat and fat quality of male Duroc pigs for dry-cured ham production. Meat Sci. 91, 148-154.

Gamero-Negrón, R., Sánchez del Pulgar, J.S., Cappellin, L., García, C., Gasperi, F., Biasioli, F., 2015. Immune-spaying as an alternative to surgical spaying in Iberian × Duroc females: Effect on the VOC profile of dry-cured shoulders and dry-cured loins as detected by PTR-ToF-MS. Meat Sci. 110, 169-173.

Han, X., Zhou, Y., Zeng, Y., Sui, F., Liu, Y., Tan, Y., Cao, H., Du, X., Meng, F., Zeng, X., 2017. Effects of active immunization against GnRH versus surgical castration on hypothalamic-pituitary function in boars. Theriogenology 97, 89-97.

Heid, A., Hamm, U., 2013. Animal welfare versus food quality: factors influencing organic consumers' preferences for alternatives to piglet castration without anaesthesia. Meat Sci. 95, 203-211.

Heyrman, E., Kowalski, E., Millet, S., Tuyttens, F.A.M., Ampe, B., Janssens, S., Byus, N., Wauters, J., Vanhaecke, L., Alawé, M., 2019. Monitoring of behavior, sex hormones and boar taint compounds during the vaccination program for immunocastration in three sire lines. Res. Vet. Sci. 124, 293-302.

Kubale, V., Batorek, N., Skrlep, M., Prunier, A., Bonneau, M., Fazarinc, G., Candek-Potokar, M., 2013. Steroid hormones, boar taint compounds, and reproductive organs in pigs according to the delay between immunocastration and slaughter. Theriogenology 79, 69-80.

Latorre, M.A., Lázaro, R., Gracia, M.I., Nieto, M., Mateos, G.G., 2003. Effect of sex and terminal sire genotype on performance, carcass characteristics, and meat quality of pigs slaughtered at 117 kg body weight. Meat Sci. 65, 1369-1377.

Latorre, M.A., Ripoll, G., García-Belenguer, E., Ariño, L., 2009. The increase of slaughter weight of gilts as strategy to optimize the production of Spanish high quality dry-cured hams. J. Anim. Sci. 87, 1464-1471.

Lugar, D.W., Rhoads, M.L., Clark-Deener, S.G., Callahan, S.R., Revercomb, A.K., Prusa, K.J., Estienne, M.J., 2017. Immunological castration temporarily reduces testis size and function without long-term effects on libido and sperm quality in boars. Animal 11, 643-649.

Lunstra, D.D., Christenson, R.K., 1986. Changes in Leydig cell ultrastructure and function during pubertal development in the boar. Biol. Reprod. 34, 145-158.

Martínez-Macipe, M., Rodríguez, P., Izquierdo, M., Gispert, M., Manteca, X., Mainau, E., Hernández, F.I., Claret, A., Guerrero, L., Dalmau, A., 2016. Comparison of meat quality parameters in surgical castrated versus vaccinated against gonadotropin releasing factor male and female Iberian pigs reared in free-ranging conditions. Meat Sci. 111, 116-121.

Moore, K.L., Mullan, B.P., Dunshea, F.R., 2017. Boar taint, meat quality and fail rate in entire male pigs and male pigs immunized against gonadotrophin releasing factor as related to body weight and feeding regime. Meat Sci. 125, 95-101.

Morales, J., Gispert, M., Hortos, M., Pérez, J., Suárez, P., Piñeiro, C., 2010. Evaluation of production performance and carcass quality characteristics of boars immunised against gonadotropin-releasing hormone (GnRH) compared with physically castrated male, entire male and female pigs. Span. J. Agric. Res. 8, 599-606.

Oberlender, G., Pontelo, T.P., Miranda, J.R., Miranda, D.R., Zangeronimo, M.G., Silva, A.C., Menezes, T.A., Rocha, L.G.P., 2014. Morphological and morphometric evaluation of prepubertal gilt ovaries, uterine tubes and uterus at different oestrus cycle stages. Pesq. Vet. Bras. 34, 83-90.

Powers, J.G., Baker, D.L., Davis, T.L., Conner, M.M., Lothridge, A.H., Nett, T.M., 2011. Effects of Gonadotropin-Releasing Hormone immunization on reproductive function and behavior in captive female Rocky Mountain Elk (*Cervus elaphus nelsoni*). Biol. Reprod. 85, 1152-1160.

Rydhmer, L., Lundström, K., Andersson, K., 2010. Immunocastration reduces aggressive and sexual behaviour in male pigs. Animal 4, 965-972.

Sadler, T.W., 2019. Langman´s Medical Embryology, fourteenth ed. Philadelphia Wolters Kluwer.

Van den Broeke, A., Leen, F., Aluwé, M., Ampe, B., Van Meensel, J., Millet, S., 2016. The effect of GnRH vaccination on performance, carcass, and meat quality and hormonal regulation in boars, barrows, and gilts. J. Anim. Sci. 94, 2811-2820.

Wicks, N., Crouch, S., Pearl, C.A., 2013. Effects of Improvac and Bopriva on the testicular function of boars ten weeks after immunization. Anim. Reprod. Sci. 142, 149-159.

Zamaratskaia, G., Rydhmer, L., Andersson, H.K., Chen, G., Lowagie, S., Andersson, K., Lundström, K., 2008. Long-term effect of vaccination against gonadotropin-

releasing hormone, using Improvac, on hormonal profile and behaviour of male pigs. Anim. Reprod. Sci. 108, 37-48.

Zeng, X.Y., Turkstra, J.A., Tsigos, A., Meloen, R.H., Liu, X.Y., Chen, F.Q., Schaaper, W.M., Oonk, H.B., Guo, D.Z., van de Wiel, D.F., 2002. Effects of active immunization against GnRH on serum LH, inhibin A, sexual development and growth rate in Chinese female pigs. Theriogenology 58, 1315-1326.

Zoels, S., Reiter, S., Ritzmann, M., Weiß, Ch., Numberger, J., Schütz, A., Lindner, P., Stefanski, V., Weiler, U., 2020. Influences of immunocastration on endocrine parameters, growth performance and carcass quality, as well as on boar taint and penile injuries. Animals 10, 346.

Figure Legends

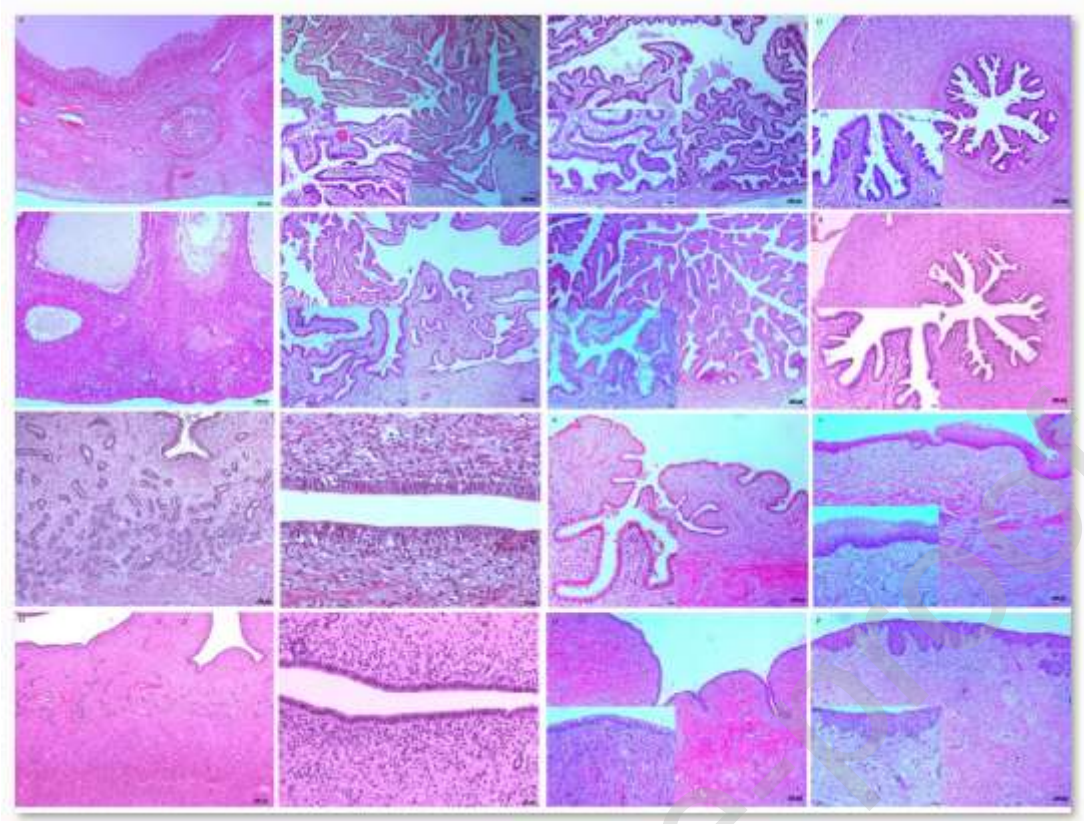

Fig. 1. Histological images of the reproductive tract of control females (A-D and I-L) and immuno-castrated females (E-H and M-P); Inset images depicts details of the epithelial layer of respective parts; Stain used for all images is H&E; A: the ovary of CF contains tertiary follicles of a large size; E: the ovary of IF contains smaller tertiary follicles and more atretic follicles; B, C and D depict different parts of a normal uterine tube (B: infundibulum; C: ampulla; D: isthmus) and F, G and H the same structures in IF, indicating there were no differences; I and J depicts the uterine body of CF with numerous glands (I) and the pseudostratified columnar epithelium (J); M and N depicts the uterine body of IF with a thinner mucosa with scarce glands (M) and simple columnar epithelium (N); K: cervix of CF; O: cervix of IF with similar characteristics to CF; L: vagina of CF; P: vagina of IF with similar features to CF; Cervix and vagina with squamous epithelium and a thick muscular layer.

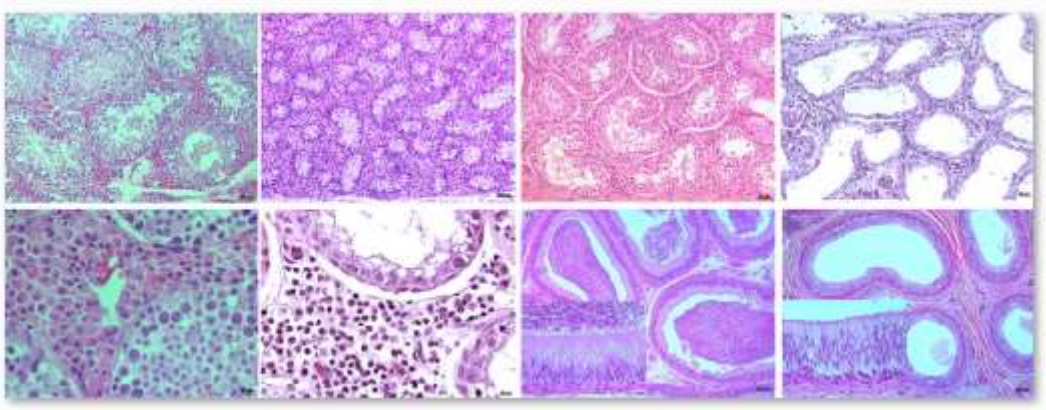

Fig. 2. Histological images of the testis and epididymis of CM (A, E, G), a prepubertal pig (B), IM with moderately lesser spermatogenesis (C), and IM with markedly lesser spermatogenesis (D, F, H); Stain used in all images is H&E; A: testis of CM depicting normal spermatogenic process; B: testis of a prepuberal pig with Sertoli cells and a few spermatogonia; C: testis of IM with moderately less spermatogenesis and a lesser tubule diameter and marked reduction of spermatocytes and spermatids, with some spermatogonial cells remaining; D: testis of IM with marked reduction of spermatogenesis in which Sertoli cells are only observed within tubules; E: testis of CM with a normal population of Leydig cells; F: testis of IM with marked reduction of spermatogenesis; Note the marked reduction of in Leydig cell size; G: epididymis of CM with abundant spermatozoa in the lumen and pseudostratified columnar epithelium with cytoplasmic vacuolation in apical area; Inset image depicts details of the epithelial layer; H: epididymis of IM with absence of spermatozoa in the lumen and pseudostratified columnar epithelium with less vacuolation in apical area; Inset image depicts details of the epithelial layer.

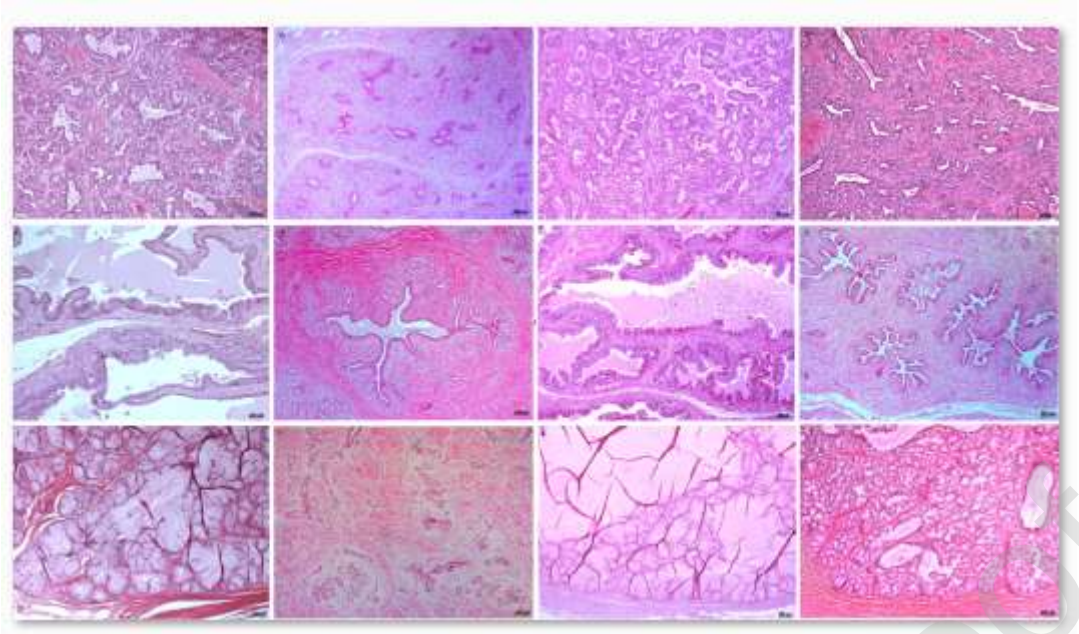

Fig. 3. Histological images of accessory glands of entire males (A, E, I), surgically castrated males (B, F, J), IM with moderate reduction of spermatogenesis (C, G, K) and IM with a marked reduction in spermatogenesis (D, H, L); Stain used in all images is H&E; A: prostate of CM with multiple tubule-alveolar and acinar glands separated by scarce fibrous tissue; B: prostate of surgically castrated male with a few glandular structures separated by abundant fibrous tissue; C: prostate of IM with moderate spermatogenesis depicting a larger abundance of fibrous tissue and less glandular acini; D: prostate of IM with marked reduction of spermatogenesis and larger abundance of fibrous tissue and fewer numbers and sizes of glands; E: vesicular gland of CM with lobules of irregular glandular acini separated by scarce fibrous tissue; F: vesicular gland of surgically castrated male with very few glandular structures separated by abundant fibrous tissue; G: vesicular gland of IM with moderate reduction of spermatogenesis and an abundance of fibrous tissue between glandular acini; H: vesicular gland of IM with marked reduction of spermatogenesis with abundant amounts of fibrous tissue separating glands; I: bulbourethral gland of CM with glands containing an abundant amount of secretory material in the centre separated by a thin fibrous stroma; J:

bulbourethral gland of surgically castrated male with a few glandular structures separated by abundant fibrous tissue; K: bulbourethral gland of IM with moderate spermatogenesis similar to controls; L: bulbourethral gland of IM with markedly less spermatogenesis mainly characterized by moderately lesser glandular secretions.

Table 1

Mean and standard error (SEM) of the weight of control (*n* = 12) and immuno-castrated (*n* = 11) females throughout the experiment, and morphometric measurements and weight of the different parts of the reproductive tract.

Variables	CF	IF	*P* values
1st injection weight (kg)	42.7 ± 0.80	43.9 ± 1.28	0.421
2nd injection weight (kg)	69.4 ± 1.30	67.6 ± 2.04	0.463
Slaughter weight (kg)	126.7 ± 2.42	127.8 ± 3.08	0.770
Whole genital weight (g)	190.0 ± 23.2	49.0 ± 8.26	<0.001
Ovary weight[a] (g)	7.5 ± 0.47	4.5 ± 1.37	<0.001
Ovary volume (cm^3)	4.3 ± 0.54	1.3 ± 0.64	0.002
Oviduct weight[a] (g)	2.5 ± 0.30	1.2 ± 0.16	0.002
Oviduct length (cm)	17.7 ± 1.03	11.5 ± 1.13	0.001
Uterine horn weight[a](g)	88.9 ± 0.14	19.1 ± 4.01	0.001
Uterine horn length (cm)	47.7 ± 4.43	30.6 ± 1.82	0.003
Cervix weight (g)	52.9 ± 6.82	12.3 ± 2.12	<0.001
Cervix length (cm)	16.8 ± 1.02	8.7 ± 1.09	<0.001
Vagina weight (g)	32.2 ± 4.29	10.5 ± 1.63	<0.001
Vagina length (cm)	11.5 ± 1.00	9.4 ± 0.57	0.095
Vulva length (cm)	2.7 ± 0.10	2.6 ± 0.12	0.653
Vagina-anus length (cm)	2.4 ± 0.14	2.3 ± 0.18	0.777
Vestibule length (cm)	9.7 ± 0.63	8.2 ± 0.17	0.034

CF: control females

IF: immunocastrated females

[a]average weight of both organs in paired organs

Table 2

Mean and standard error (SEM) of the weight of surgically castrated (*n* = 12) and immuno-castrated males (*n* = 11) throughout the experiment, and morphometric measurements and weight of the accessory glands.

Variable	IM	CM	*P* Value
1st injection weight (kg)	47.6 ± 1.20	45.2 ± 1.23	0.185
2nd injection weight (kg)	73.3 ± 2.06	71.0 ± 1.66	0.382
Slaughter weight (kg)	130.8 ± 3.42	124.4 ± 2.74	0.156
BG weight (g)	30.6 ± 6.03	3.7 ± 0.81	0.001
BG area (cm^3)	12.2 ± 1.99	3.4 ± 0.69	0.001
VG weight (g)	17.9 ± 7.80	2.2 ± 0.4	0.035
VG area (cm^3)	14.2 ± 4.22	2.3 ± 0.43	<0.001
Prostate diameter (cm)	2.0 ± 0.19	1.5 ± 0.09	0.037
Prostate volume (cm^3)	5.6 ± 1.47	2.2 ± 0.45	0.034

CM: surgically castrated males
IM: immunocastrated males
BG: bulbourethral glands
VG: vesicular glands

Table 3

Categorisation (G0, GI, GII, and GIII) of lesion grades in the testicles and epididymis of immuno-castrated male pigs (described in Material and methods).

	Testis				Leydig	Epididymis			
	G0	GI	GII	GIII	Size	G0	GI	GII	GIII
Pig 1				x	GII	-			
Pig 2			x		GII			x	
Pig 3	x				G0	x			
Pig 4				x	GII			x	
Pig 5			x		GII			x	
Pig 6				x	GII				x
Pig 7			x		GI			x	
Pig 8		X			GI		x		
Pig 9*				x	GII				x

*Male in which all the seminiferous tubules were lined only with Sertoli cells

Table 4

Concentrations of progesterone and estradiol in entire and immuno-castrated female pigs.

Group	*n*	Progesterone (ng/mL) Mean ± SEM (Max-Min)	***n***	Estradiol (pg/mL) Mean ± SEM (Max-Min)
Entire females	12	1.4 ± 0.12 (1.0-2.4)	9	10.3 ± 7.4 (0.0-64.6)
Immuno-castrated females	11	1.2 ± 0.1 (0.5-2.2)	10	12.0 ± 8.7 (0.0-83.9)

Max-Min: refers to the largest and smallest values for each variable

Table 5

Concentrations of testosterone and estradiol in entire, immuno-castrated and surgically castrated male pigs.

Group	*n*	Testosterone (pg/mL) Mean ± SEM (Max-Min)	***n***	Estradiol (pg/mL) Mean ± SEM (Max-Min)
Entire males	4	21.5 ± 4.10^{a} (11.8-31.9)	ND	-
Immuno-castrated males	9	0.3 ± 0.18^{b} (0.06-1.7)	8	0.4 ± 0.15^{a} (0.0-2.4)
Surgically castrated males	12	0.06 ± 0.01^{c} (0.04-0.07)	10	0.4 ± 0.23^{a} (0.0-1.0)

Max-Min: refers to the largest and smallest values for each variable
ND: not done (for entire males, there were no estradiol quantitations)
a,b,c; Different letters in the same column indicate differences ($P < 0.001$)