BMC Veterinary Research

RESEARCH

Open Access

Assessing salivary cortisol and testosterone as non-invasive biomarkers for GnRH-immunocastration efficiency in heavy pigs

E. Atallah[1], G. Pesenti Rossi[1*], J. F. Soares Filipe[1], E. Dalla Costa[1], S. M. Mazzola[1], M. Minero[1], A. Pecile[1], A. Motta[2] and S. Barbieri[1]

Abstract

Background Efforts to enhance animal welfare have led to the investigation of alternative methods of performing surgical castration on young pigs that induce stress and anxiety. This study was conducted to establish the efficacy of immunocastration in heavy pigs, with non-invasive diagnostic indicators being salivary cortisol and testosterone levels.

Results At 225 days, a significant difference was noted in the cortisol levels of pigs in the treatment groups, with the immune-castrated (IC) recording higher levels than the surgically castrated (SC) animals. Furthermore, 196-day and 225-day-old IC pigs had remarkably elevated testosterone amounts. The changes that were seen to be significant after immunocastration indicate that the use of salivary tests can be a good way of evaluating how effective this alternative method is. Among SC pigs, a significant positive relationship was found between salivary cortisol and salivary testosterone concentrations at 225, 240, and 268 days. Moreover, no association was observed in IC animals. These findings signal that the hormonal levels and their interactions are related to different physiological reactions of pigs to immunological and surgical castration.

Conclusions The results underline the significance of salivary cortisol and testosterone as stress and hormonal balance indicators in heavy pigs with immunocastration. This study provides insights into the complex hormonal reactions to stress post-castration and emphasizes the need for further research to enhance animal welfare practices. Overall, salivary testing offers a practical approach to evaluating immunocastration efficacy and monitoring pig health and well-being.

Keywords Animal welfare, Immunocastration, Heavy pig, Salivary testosterone, Salivary cortisol, Enzyme-linked immunosorbent assay (ELISA)

*Correspondence:
G. Pesenti Rossi
gaia.pesenti@unimi.it
[1]Dipartimento di Medicina Veterinaria e Scienze Animali, Università degli Studi di Milano, Lodi, Italy
[2]Centro Ricerche Produzioni Animali, Reggio Emilia, Italy

Background

Animal welfare has become one of the main aspects considered for farm animal products as a result of an increasing public perception towards ethical aspects and implications around farming practices and consuming animals. Castration of male piglets, performed to avoid boar taint (i.e., an undesired off-odour and off-flavour present in the fat of entire males), has been pointed out as a welfare issue, considering it is mostly performed without analgesia or anaesthesia [1], and therefore causing both acute and long-term signs of pain and stress in piglets [2]. For this reason, in 2010, some European stakeholders agreed on a voluntary declaration to end surgical castration of piglets and to adopt alternative practices by 2018: although up to date surgical castration has not been eliminated yet, the interest toward alternatives has also driven research, aiming at finding welfare-friendly solutions, while still avoiding the presence of boar taint [3].

Apart from finding effective pain control protocols during and after surgical castration, the main alternatives resulted in raising entire males (slaughtered before puberty onset) and immunocastration, which is a vaccination against gonadotropin-releasing hormone (GnRH). This hypothalamic hormone regulates reproductive functions through the hypothalamic-pituitary-gonadal axis [4]. The production of antibodies against GnRH causes the suppression of this endocrine cascade, inhibiting testicular steroid synthesis with subsequent testicular atrophy [5]. This process requires at least two injections, with the first given at about 12 weeks of age and priming the immune system, and the second after at least 4 weeks from the first and 4–6 weeks before slaughter [6]. According to Kress and colleagues [7], after the second vaccine, the production of testicular hormones stops, and behavioral issues unique to boars decrease within two weeks.

Additionally, Reiter et al. [8] found that the number of injuries, such as penile injuries, also decreases. The European Medicines Agency (EMA) licensed Improvac® for use in the European Union in 2009 [9]. Currently, just one vaccine (Improvac®) is available for commercial use in Europe [7] and immunocastrates still only account for 2.8% of the European market [10]. The lack of commercial acceptability of this technology may be attributed to numerous issues, mostly stemming from insufficient understanding of the best application of immunocastration to meet different market requirements [11].

Surely, alternatives must be considered in relation to the pig farming contexts: in heavy pig production, which is fundamental in the Italian pig sector, where pigs are raised for 9 months (largely after puberty onset) and reach more than 180 kg body weight, certainly entire male pig production is not feasible. On the other hand, some studies have considered the use of immunocastration for Italian heavy pigs, the first of which Pinna et al. ([12] have pointed out the necessity of 3 doses at 11, 26–27 and 36–37 weeks of age, resulting in meat with comparable characteristics to pigs surgically castrated [12].

As immunocastration is a reversible intervention, the timeframe for injections must be relatively accurate. The effectiveness of immunocastration has been frequently based on the comparison with surgical castration in terms of products' quality, behaviour of pigs (i.e., comparing the level of total activity of animals, but also focusing on sexual and aggressive behaviours), and in some works on blood hormonal patterns [13–15]. However, when considering the process in real-time, finding a method to assess the situation simultaneously might help improve the latter's efficiency. Saliva has gained increasing interest in research as a matrix, providing helpful information with a low impact on animal welfare and behaviour. Although largely used to evaluate hormones like cortisol, few studies assessed salivary testosterone in pigs [16, 17].

The aim of this study was firstly to evaluate the efficacy of immunocastration by monitoring the salivary testosterone levels in a group of heavy pigs compared to surgically castrated pigs. Secondly, to assess the presence of a possible correlation with cortisol levels for the whole duration of the experimental period.

Results

Salivary cortisol levels

Cortisol levels showed a significant difference between the treatment groups only at the age of 225 days ($P = 0.022$), with cortisol concentration being higher in the immune-castrated (IC) group compared to the surgically castrated (SC) pigs (Table 1; Fig. 1A). Within the SC group, cortisol levels significantly decreased at 196 days compared to 150 days ($P < 0.001$) and at 240 days compared to 225 days ($P = 0.044$). In the IC group, cortisol levels significantly increased at 225 days compared to 196 days ($P = 0.041$), while they significantly decreased at 196 days compared to 150 days ($P < 0.001$) and at 240 days compared to 225 days ($P = 0.001$) (Table 2).

Although a significant difference was observed between groups at 225 days, the overall time × group interaction for salivary cortisol was not statistically significant ($P = 0.134$), indicating that the trajectory of cortisol levels over time did not differ significantly between the IC and SC groups.

Salivary testosterone levels

Testosterone levels in the IC group were significantly higher than in the SC groups at 225 days ($P < 0.001$) (Table 1; Fig. 1B). Within the IC treatment group, the

Table 1 Comparison of salivary biomarkers levels between surgically castrated and immunocastrated pigs at different time points

Time point (Days)	Salivary cortisol (pg/mL)			Salivary testosterone (pg/mL)		
	SC	IC	*P*-value	SC	IC	*P*-value
100	4171.3 (1870–4831)	2154.7 (1631–6791)	0.569	531.4 (178–571)	558.3 (294–1035)	0.997
150	2531.7 (1444–2630)	2139 (1588–3745)	0.880	674.5 (149–1368)	804.8 (462–2774)	0.571
196	895.6 (702–955)	706.2 (635–867)	0.139	445 (442–539)	982.1 (484–1360)	0.586
225	1072.1 (838–1300)	1561.7 (1186–2482)	0.022*	305.4 (268–563)	3980.4 (2549–5717)	< 0.001**
240	720.9 (667–926)	815.5 (390–833)	0.591	178.2 (171–230)	341.4 (158–434)	0.998
268	798.2 (773–1037)	751.8 (712–920)	0.743	299.3 (251–333)	411.6 (282–507)	0.999

n = 10 animals/group. Values are presented as median with interquartile ranges (IQR, 25th -75th percentiles). *(*P* < 0.05) and ** (*P* < 0.001) indicate statistically significant differences.

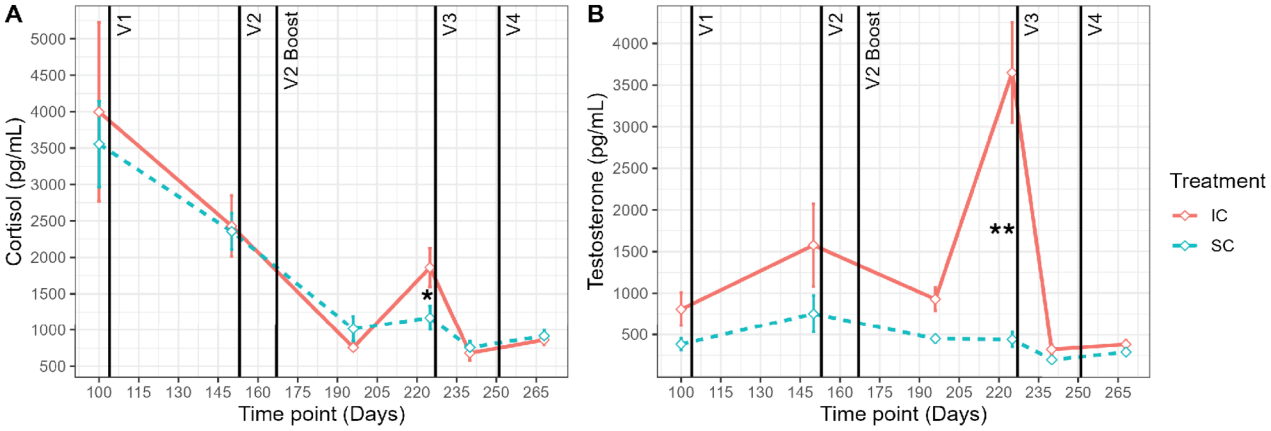

Fig. 1 Graphical representation of changes in salivary testosterone (**A**) and cortisol (**B**) levels in pigs at the ages of 100, 150, 196, 225, 240, and 268 days between surgically castrated (SC) vs. immuno-castrated (IC) pigs. Values are expressed as means ± SEM (*n* = 10 animals/group). *(*P* < 0.05) and ** (*P* < 0.001) indicate a significant difference between the two types of castration at each time point

Table 2 Comparison of salivary biomarkers levels within surgically castrated and immunocastrated groups between different time points

Time points comparison (Days)	SC *P*-value		IC *P*-value	
	Cortisol	Testosterone	Cortisol	Testosterone
100 vs. 150	0.055	0.838	0.204	0.226
150 vs. 196	< 0.001**	0.995	< 0.001**	0.982
196 vs. 225	0.495	0.937	0.002*	0.008*
225 vs. 240	0.044*	0.537	0.001*	< 0.001**
240 vs. 268	0.326	0.924	0.705	0.991

n = 10 animals/group. *(*P* < 0.05) and ** (*P* < 0.001) indicate statistically significant differences.

testosterone levels were significantly higher at 225 days compared to 196 days ($P = 0.008$) and significantly lower at 240 days compared to 225 days ($P < 0.001$). In contrast, no statistically significant variations in testosterone levels were observed within the SC treatment group across the different time points ($P > 0.05$) (Table 2).

The overall time × group interaction for salivary testosterone concentrations was statistically significant ($P < 0.001$), suggesting that the testosterone changes over time differed significantly between the IC and SC groups.

Table 3 Correlations between the salivary testosterone and salivary cortisol concentrations of the groups at each time point

Time point (Days)	SC		IC	
	Correlation	*P*-value	Correlation	*P*-value
150	0.702	0.121	0.466	0.175
196	-0.514	0.157	0.045	0.903
225	0.824	0.012*	0.427	0.218
240	0.701	0.035*	0.567	0.112
268	0.754	0.019*	-0.513	0.194

n = 10 animals/group. * Indicates significant difference (*P* < 0.05).

Interaction between salivary biomarkers

Cortisol and testosterone correlated significantly only for the surgically castrated pigs; as for the immunocastrated groups, no significant correlation was found ($P > 0.05$). Spearman's correlation results between salivary testosterone and cortisol concentrations within each group at the different time points are shown in Table 3. For the SC pigs, a positive correlation ($P < 0.05$) was found between both salivary hormones at 225, 240, and 268 days of age with P-values of 0.012, 0.035, and 0.019, respectively.

Discussion

The determination of salivary biomarkers in heavy pigs following castration appears as a promising non-invasive solution to understand their physiological condition and welfare status. Since saliva collection is less invasive and stressful than blood sampling, it benefits animal welfare and enables frequent non-invasive monitoring compared to conventional matrixes like blood [18, 19]. According to available literature, the efficacy of immunocastration in pigs has been assessed by measuring testosterone levels in blood samples [20]. However, salivary testosterone levels have been found to be strongly correlated with free serum testosterone levels in different mammal species, including humans [21, 22], primates [23] and goats [24].

To determine the levels of salivary testosterone in each animal in both groups at each time point, one sample was collected shortly before the next dose was administered. Saliva samples were collected without causing distress to the animals, and a volume of 50–100 µl was then used for the analysis. This confirms that this technique is a simple, feasible, and non-invasive approach to assess testicular function. Compared to the SC group, immunocastrated pigs had higher testosterone concentrations at all time points except for the last 2, at 240 and 268 days of age, where the testosterone levels of both groups were nearly the same. Starting at 100 days of age, the concentration in IC pigs gradually increased and reached its highest point at 225 days of age, before the third vaccine dose (V3). However, there was a significant decrease after the second vaccine boost (V2) for approximately 7 weeks. The concentration then dropped to levels similar to that of SC pigs at 240 days of age. The elevated testosterone levels seen 7 weeks after the V2 boost dosage contradict the findings of Zamaratskaia et al. [25], who reported a decrease in testosterone that persisted for up to 22 weeks following the second treatment. We hypothesize that the peak observed at 225 days of age might be associated with a stressful event such as handling, management interventions, or environmental changes, rather than a recovery of testicular function. Although the last immunization occurred at 167 days, these events could have triggered an acute stress response, leading to increased adrenal testosterone production. This hypothesis is supported by the significant increase in cortisol levels observed at this time point, suggesting an acute stress response. This hypothesis is supported by the absence of increased activity levels and body lesions in the immunocastrated animals observed in our previous study [26]. Additionally, testosterone has been suggested as a marker of acute stress [27], rising alongside cortisol following stressful events. This idea aligns with our findings, which show significantly higher cortisol concentrations in immunocastrated pigs compared to those surgically castrated at 225 days of age.

Several studies have demonstrated that the activity of vaccinated pigs was comparable to that of intact male pigs prior to the second injection. However, after the second injection, the activity level decreased to a level like that of surgically castrated pigs [13, 28, 29]. Additionally, testicular functions such as hormone secretion and production of androsterone were effectively suppressed starting from day eight after injecting an appropriate dose, with this inhibition lasting for 10–24 weeks, depending on the individual [30].

Regarding the measurement of cortisol levels, both groups exhibited nearly the same levels at different recorded time points, starting with high levels prior to the first dose administration (100 days of age) and gradually decreasing until 196 days of age. An increase was recorded at 225 days in IC treatment group (Supplementary material 1), with significantly higher levels prior to the V3 dose. Salivary cortisol is a well-established indicator of stress and is tightly linked to physiology [17] varying according to the circadian rhythm. In pig saliva, basal cortisol concentration is higher in the morning and lower in the evening [31]. In addition, measuring salivary cortisol levels enables the assessment of stress reaction to immunocastration, hence facilitating a comprehensive evaluation of pig welfare [32]. It has been shown that different types of stressors elicit diverse reactions in salivary biomarkers, as reported by Ott et al. [33]. Evaluating salivary cortisol concentration is frequently employed to determine stress levels in animals during activities such as handling, transportation, or regrouping [34, 35]. When evaluating stress caused by castration, tooth excision, or tail docking, serum or plasma samples are more commonly used [36–38]. According to our research, only one study has investigated salivary cortisol concentrations in castrated piglets [19], making this the first study comparing salivary cortisol levels between castrated and immunocastrated heavy pigs.

It is desirable to determine how these two altered biological risk factors, salivary cortisol and testosterone, in castrated pigs correlate to ensure better animal welfare and other aspects of management. The results of the present investigation indicate that a strong positive connection between the two salivary biomarkers was seen exclusively in the group of surgically castrated subjects during the final three time points (225, 240, and 268 days of age). The study by Escribano et al. [16] also showed a positive correlation, but this was in non-castrated pigs. Both hormones collaborate to sustain a suitable physiological and psychological equilibrium [39]. Salivary cortisol and testosterone levels rise in reaction to immediate psychosocial stress [40, 41]. The complex interaction between high levels of testosterone and low levels of cortisol during challenging social situations leads to effective decision-making [42]. Thus, all these results

underline the complexities of hormonal responses to stress following castration. Understanding the strong positive correlation between these hormones, particularly in surgically castrated pigs, suggests that managing stressors in pig husbandry can significantly improve animal welfare. Elevated cortisol indicates stress, which can compromise welfare, while high testosterone levels may suggest aggressive behaviors in response to stressors, influencing social dynamics within groups. Monitoring these hormonal levels can guide management practices aimed at reducing stress, such as improving housing conditions and minimizing handling stress. This highlights the importance of considering both physiological and psychological aspects of animal welfare, emphasizing the need for strategies that reduce psychosocial stressors.

Continuous research and improvements in sensitive detection methods have highlighted the value of salivary biomarkers despite challenges arising from differences in saliva composition and lower concentrations for most biomarkers [43, 44]. These improvements make it easier to use salivary biomarkers in different situations to monitor the pigs' health. Consequently, such results emphasize how salivary testing can be a practical means of evaluating immunocastration while supporting animal well-being.

Conclusion

To the best of our knowledge, this is the first study that explored the relationship between the levels of cortisol and testosterone in the saliva of castrated and immunocastrated heavy pigs. According to the findings, pig saliva could be used for confirming that castration was performed successfully. However, the peak observed between the second and the third dose suggest a potential confounding effect associated with a stressful event, which should be further investigated. Valuable insights into welfare of pigs and hormonal dynamics could be provided by salivary biomarkers, promoting the effectiveness of immunocastration as a welfare-friendly alternative to surgical castration. Future studies should concentrate on improving the use of salivary biomarkers for monitoring the welfare in pigs, as well as in other animal species and humans, to provide more reliable and practical information on these biomarkers.

Methods

Animals and housing

The study was approved by the Animal Welfare Committee of the University of Milan (OPBA_26_2020) according to Directive 2010/63/EU and was carried out in accordance with the ARRIVE guidelines. Animals were housed and managed in compliance with Council Directive 120/2008/EC.

The experimental research involved twenty commercial-hybrid male piglets (Topigs Norsvin, Helvoirt, The Netherlands) housed in two different intensive private farms specialized in heavy pig production in Northern Italy. Buildings were naturally ventilated and had at least 8 h of artificial light. The National Research Council Nutrient Requirements of Swine [45] were used to create diets for the growing finishing phase that would meet the nutritional needs of the animals. Throughout both the grower and fattening stages, pigs remained in uniform housing and feeding conditions without any mixing. The farm veterinarian did regular checks on how the pigs were growing as well as on their overall health status. This was meant to ensure that all animals from experimental groups were in perfect health during vaccination times hence, any pig identified as sickly or having insufficient body weight throughout raising thereafter was isolated and handled differently according to its requirements.

The pigs, with an initial weight of 35 kg and a final weight of 75 kg, were housed singly in two straw-bedded pens. The floor space per pig when body weight reached 70 kg was 1 m^2. The arrangement of the housing was treatment-wise. Each pen had an automatic feeder. Animals were given unrestricted amounts of dry commercial feed. The fattening pens for animals weighing 75 kg for slaughter were individually assigned based on their treatment group. All these pens were made up of fully slatted concrete floors where each pig had 1.06 m^2 area at body weight of 160 kg. Pigs were fed three times daily through a liquid commercial meal using a trough. Water was supplied *ad libitum* from nipples (1 nipple/8 pigs). In each pen within the fattening area, enrichment material, consisting of metallic chains containing wooden bars, was installed on the walls of the cages. Pigs were slaughtered in a commercial abattoir following standard slaughterhouse procedures (according to Reg. 1099/2009/EC) by the end of the raising phase at about forty and forty-one weeks old.

Treatment groups

At birth, the male pigs were divided equally into two treatment groups: surgical castration and immunocastration. Surgical castration was performed on a total of 10 animals from the SC group following guidelines stipulated in Council Directive 2008/120/EC and regular husbandry procedures when they were four days old. The group of individuals who had chemical castration ($n = 10$) received immunization with Improvac® (Zoetis Italia Srl, Roma, Italy), following the methodology outlined in the research by Pesenti Rossi et al. [26]. Improvac® was recommended to be given at the ages of 104, 167, and 227 days old and then also at 35 days before slaughter at the age of 251 days old.

However, due to the elevated degree of aggressivity within the IC group, the second vaccination was anticipated and then administered again after two weeks, following the supplier's recommendations. Additionally, a fourth intervention was introduced. Animals were watched closely for any side effects after each dose of Improvac®. Injection site swellings are common, according to the leaflet that accompanies it. Anaphylactoid-type reactions, which are thought to be very rare, are also mentioned.

Data recordings

Data were collected on days 100, 150 and 225, 2–4 days prior to each Improvac® injection, when the male pigs were most likely to exhibit their behaviour, as it is anticipated that minimum vaccination impact occurs during this period. Moreover, 2–4 weeks after V2 administration (the second dose) at 196, 240 and 268 days, the effectiveness of applied immunization on observed salivary biomarkers was tested in immunocastrated male pigs. The data sets collected these days were used to assess any possible effects of therapy. Additionally, levels of cortisol and testosterone were determined at 100, 150, and 196 age days in intact boars to establish normal ranges for these particular analytic modalities.

Data were gathered at days 100, 150, and 225, 2–4 days before each Improvac® injection, when animals were most likely to exhibit their behavior, as this is when the least amount of vaccination impact is anticipated. Also, after 2–4 weeks following the administration of the "V2 boost", "V3", and "V4" doses, respectively, at days 196, 240 and 268, the efficacy of vaccination on the studied salivary biomarkers was evaluated. The data obtained from each time point was utilized to examine any potential therapy effects.

Sample collection and salivary biochemical measurements

The testosterone levels of the two test groups were measured through the collection of saliva samples at each time point. To accommodate the different group sizes in the two types of housing and minimize the number of pigs sampled, the number of animals selected for testosterone analysis varied throughout the study. At the ages of 100, 150, 196, 225, 240, and 268 days, 10 pigs from each treatment group were randomly chosen for saliva collection. In order to reduce any stress associated with the process, saliva was obtained in the late morning, between 11:00 and 12:00, from pigs that willingly approached the operator.

After being freed, the pigs were given long tongs and told to chew on a cotton swab (Salivette, Aktiengesellschaft & Co., Sarstedt, Nümbrecht, Germany) until it was completely wet. This was done for at least one minute. The Salivette® rolls were thereafter frozen and stored at a temperature of -20 °C. During the ELISA examination, the samples were subjected to centrifugation with a force of 2000 times the acceleration due to gravity for 15 min. The resulting liquid above the sediment, known as the supernatant, was collected. All of the samples were then examined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Salimetrics LLC, located in State College, PA, USA) as previously reported [27]. The analysis was performed in accordance with the instructions provided by the manufacturer. In short, the testosterone found in the samples competes with testosterone that is bound to horseradish peroxidase for the locations on an antibody where they may attach on a microtiter plate. The optical density is measured using a conventional plate reader at a wavelength of 450 nm. The level of testosterone enzyme conjugate found is negatively correlated with the level of testosterone in the sample. The assays have a sensitivity and detection range of 0.1–20 ng/mL. The coefficients of variation for intra-assay and inter-assay were 4.21% and 5.38%, respectively.

Salivary cortisol levels were quantified using a commercially available ELISA kit (LDN Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany), with ELISA-based salivary cortisol measurement in pigs previously validated by Thomsson et al. [46]. The samples were analyzed twice according to the manufacturer's instructions. The measurable range of cortisol concentration was 0.1–30 ng/mL, and the within-assay and between-assay coefficients of variation were 5.01% and 5.76%, respectively. In order to reduce the variability across assays, samples from all groups and time points were analyzed using the same test.

Statistical analysis

The data were analyzed using SPSS 29 (SPSS Inc., Chicago, IL, USA). Normality was assessed using the Shapiro–Wilk test, and homogeneity of variance was tested using Levene's test. As the data were not normally distributed, a Generalized Linear Model (GLM) was conducted using a gamma distribution and a log link function to appropriately handle the non-normality of the data. The GLM analysis was performed with treatment group and time points as fixed factors, including their interaction (time × group). When significant effects were found, post-hoc pairwise comparisons were conducted to evaluate between-group differences at each time point and within-group changes across time points, with Sidak correction applied for multiple comparisons. Data visualization was carried out using the ggplot2, Rmisc, geomtextpath, and cowplot packages in RStudio (version 4.2.1), with mean ± SEM for better visualization of trends over time and group comparisons. Relationships between hormone concentrations at each time point were examined using Spearman's correlation test. Statistical significance

was set at $P < 0.05$, and data are presented as median with interquartile ranges (IQR, 25th–75th percentiles) since data are not normally distributed.

Abbreviations

BW	Body weight
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
GnRH	Gonadotropin hormone-releasing hormone
IC	Immunocastrated
SEM	Standard error of the mean
SC	Surgically castrated

Acknowledgements
The authors would like to thank Mattias Azzolari and Marzia Borciani for their support in data collection. The authors are also grateful to all the staff of pig farms who participated in data collection.

Author contributions
E.D.C., S.M.M., M.M. and S.B.: conceptualization; M.M., J.F.S.F., E.D.C. and S.B: methodology; E.A.: formal analysis; M.M., E.D.C. and S.B.: resources; E.A. and G.P.R: data curation, E.A., G.P.R, J.F.S.F. and E.D.C.: writing—original draft preparation; E.A., G.P.R., J.F.S.F., E.D.C., S.M.M., A.M., A.P., A.M. and S.B.: writing—review and editing; E.D.C., and S.B.: supervision; E.D.C., M.M. and S.B.: funding acquisition. All authors read and approved the final manuscript.

Funding
This research was funded by Regione Lombardia, Bando 2018, d.d.s. n. 19441 of 21/12/2018, grant number 27 (PorCastro Project).

Data availability
The data presented in this study are available upon request from the corresponding author. The data are not publicly available due to privacy restrictions.

Declarations

Ethics approval and consent to participate
The protocol was approved by the Animal Welfare Committee of the University of Milan (OPBA_26_2020), according to the Directive 2010/63/EU and was carried out in compliance with the ARRIVE guidelines (https://arriveguidelines.org). Animals were housed and managed in compliance with Council Directive 120/2008/EC. Informed consent was obtained from the owner of the pig farms involved in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Authors' affiliations
1- Università degli Studi di Milano, Departimento di Medicina Veterinaria e Scienze Animali, Lodi, Italy.
E. Atallah, G. Pesenti Rossi, J.F. Soares Filipe, E. Dalla Costa, S.M. Mazzola, M. Minero, A. Pecile & S. Barbieri.
2- Centro Ricerche Produzioni Animali, Reggio Emilia, Italy.
A. Motta.

Received: 19 July 2024 / Accepted: 24 March 2025
Published online: 09 April 2025

References

1. De Briyne N, Berg C, Blaha T, Temple D. Pig castration: will the EU manage to ban pig castration by 2018? Porcine Health Manag. 2016;2:1–11.
2. Prunier A, Bonneau M, Von Borell EH, Cinotti S, Gunn M, Fredriksen B, et al. A review of the welfare consequences of surgical castration in piglets and the evaluation of non-surgical methods. Anim Welf. 2006;15(3):277–89.
3. Bonneau M, Weiler U. Pros and cons of alternatives to piglet castration: welfare, Boar taint, and other meat quality traits. Animals. 2019;9(11):884.
4. Thompson DL Jr. Immunization against GnRH in male species (comparative aspects). Anim Reprod Sci. 2000;60:459–69.
5. Aponte PM, Gutierrez-Reinoso MA, Sanchez-Cepeda EG, Garcia-Herreros M. Active immunization against GnRH in pre-pubertal domestic mammals: testicular morphometry, histopathology and endocrine responses in rabbits, Guinea pigs and Ram lambs. Animal. 2018;12(4):784–93.
6. Čandek-Potokar M, Škrlep M, Zamaratskaia G. Immunocastration as alternative to surgical castration in pigs. Theriogenology. 2017;6:109–26.
7. Kress K, Millet S, Labussière É, Weiler U, Stefanski V. Sustainability of pork production with immunocastration in Europe. Sustainability. 2019;11(12):3335.
8. Reiter S, Zöls S, Ritzmann M, Stefanski V, Weiler U. Penile injuries in immunocastrated and entire male pigs of one fattening farm. Animals. 2017;7(9):71.
9. European Commission. Improvac® solution for injection. Annex I Product Characteristics. Retrieved from European Commission Public Health [Internet]. 2009 [cited 2024 May 16]. Available from: https://ec.europa.eu/health/documents/communityregister/2011/20110905107948/anx_107948_en.pdf
10. Backus G, Higuera M, Juul N, Nalon E, De Briyne N. Second progress report 2015–2017 on the European declaration on alternatives to surgical castration of pigs. Brussels, Belgium. 2018.
11. Kress K, Weiler U, Schmucker S, Čandek-Potokar M, Vrecl M, Fazarinc G, et al. Influence of housing conditions on reliability of immunocastration and consequences for growth performance of male pigs. Animals. 2019;10(1):27.
12. Pinna A, Schivazappa C, Virgili R, Parolari G. Effect of vaccination against gonadotropin-releasing hormone (GnRH) in heavy male pigs for Italian typical dry-cured Ham production. Meat Sci. 2015;110:153–9.
13. Rydhmer L, Lundström K, Andersson K. Immunocastration reduces aggressive and sexual behaviour in male pigs. Animal. 2010;4(6):965–72.
14. Santos R, de KS FR, Moi M, Santos LS dos, Nääs IA, Foppa L et al. Behavior of immunocastrated pigs. Revista Brasileira de Zootecnia. 2016;45:540–5.
15. Pérez-Ciria L, Miana-Mena FJ, Álvarez-Rodríguez J, Latorre MA. Effect of castration type and diet on growth performance, serum sex hormones and metabolites, and carcass quality of heavy male pigs. Animals. 2022;12(8):1004.
16. Escribano D, Gutiérrez AM, Tecles F, Cerón JJ. Changes in saliva biomarkers of stress and immunity in domestic pigs exposed to a psychosocial stressor. Res Vet Sci. 2015;102:38–44.
17. Cerón JJ, Contreras-Aguilar MD, Escribano D, Martínez-Miró S, López-Martínez MJ, Ortín-Bustillo A, et al. Basics for the potential use of saliva to evaluate stress, inflammation, immune system, and redox homeostasis in pigs. BMC Vet Res. 2022;18(1):81.
18. Martínez-Miró S, Tecles F, Ramón M, Escribano D, Hernández F, Madrid J, et al. Causes, consequences and biomarkers of stress in swine: an update. BMC Vet Res. 2016;12:1–9.
19. Němečková M, Popelková T, Chloupek P. Sampling methods for determination of cortisol in pig saliva and their use in the assessment of pig welfare. Acta Vet Brno. 2022;91(3):261–6.
20. Batorek-Lukač N, Kress K, Čandek-Potokar M, Fazarinc G, Škrlep M, Poklukar K, et al. Immunocastration in adult boars as a model for late-onset hypogonadism. Andrology. 2022;10(6):1217–32.
21. Arregger AL, Contreras LN, Tumilasci OR, Aquilano DR, Cardoso EML. Salivary testosterone: a reliable approach to the diagnosis of male hypogonadism. Clin Endocrinol (Oxf). 2007;67(5):656–62.
22. Cardoso EML, Contreras LN, Tumilasci EG, Elbert A, Aguirre EC, Aquilano DR, et al. Salivary testosterone for the diagnosis of androgen deficiency in end-stage renal disease. Nephrol Dialysis Transplantation. 2011;26(2):677–83.
23. Arslan M, Bint Akhtar F, Nieschlag E. Salivary testosterone levels in normal and testosterone treated monkeys. J Steroid Biochem. 1984;21(4):461–4.
24. Samir H, Nyametease P, Elbadawy M, Fathi M, Mandour AS, Radwan F, et al. Assessment of correlations and concentrations of salivary and plasma steroids, testicular morphometry, and semen quality in different Climatic conditions in goats. Theriogenology. 2020;157:238–44.
25. Zamaratskaia G, Andersson HK, Chen G, Andersson K, Madej A, Lundström K. Effect of a Gonadotropin-releasing hormone vaccine (ImprovacTM) on steroid hormones, Boar taint compounds and performance in entire male pigs. Reprod Domest Anim. 2008;43(3):351–9.
26. Pesenti Rossi G, Dalla Costa E, Filipe JFS, Mazzola SM, Motta A, Borciani M, et al. Does immunocastration affect behaviour and body lesions in heavy pigs?? Vet Sci. 2022;9(8):410.
27. Escribano D, Fuentes-Rubio M, Cerón JJ. Salivary testosterone measurements in growing pigs: validation of an automated chemiluminescent immunoassay and its possible use as an acute stress marker. Res Vet Sci. 2014;97(1):20–5.

28. Fàbrega E, Velarde A, Cros J, Gispert M, Suárez P, Tibau J, et al. Effect of vaccination against gonadotrophin-releasing hormone, using Improvac®, on growth performance, body composition, behaviour and acute phase proteins. Livest Sci. 2010;132(1–3):53–9.
29. Andersson K, Brunius C, Zamaratskaia G, Lundström K. Early vaccination with Improvac®: effects on performance and behaviour of male pigs. Animal. 2012;6(1):87–95.
30. Claus R, Rottner S, Rueckert C. Individual return to Leydig cell function after GnRH-immunization of boars. Vaccine. 2008;26(35):4571–8.
31. Griffith MK, Minton JE. Free-running rhythms of adrenocorticotropic hormone (ACTH), cortisol and melatonin in pigs. Domest Anim Endocrinol. 1991;8(2):201–8.
32. Einarsson S, Andersson K, Wallgren M, Lundström K, Rodriguez-Martinez H. 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. 2009;71(2):302–10.
33. Ott S, Soler L, Moons CPH, Kashiha MA, Bahr C, Vandermeulen J, et al. Different stressors elicit different responses in the salivary biomarkers cortisol, haptoglobin, and chromogranin A in pigs. Res Vet Sci. 2014;97(1):124–8.
34. Coutellier L, Arnould C, Boissy A, Orgeur P, Prunier A, Veissier I, et al. Pig's responses to repeated social regrouping and relocation during the growing-finishing period. Appl Anim Behav Sci. 2007;105(1–3):102–14.
35. Merlot E, Meunier-Salaün MC, Prunier A. Behavioural, endocrine and immune consequences of mixing in weaned piglets. Appl Anim Behav Sci. 2004;85(3–4):247–57.
36. Backus BL, McGlone JJ. Evaluating environmental enrichment as a method to alleviate pain after castration and tail Docking in pigs. Appl Anim Behav Sci. 2018;204:37–42.
37. Prunier A, Mounier AM, Hay M. Effects of castration, tooth resection, or tail Docking on plasma metabolites and stress hormones in young pigs. J Anim Sci. 2005;83(1):216–22.
38. Sutherland MA, Davis BL, Brooks TA, Coetzee JF. The physiological and behavioral response of pigs castrated with and without anesthesia or analgesia. J Anim Sci. 2012;90(7):2211–21.
39. Willemsen G, Ring C, McKeever S, Carroll D. Secretory Immunoglobulin A and cardiovascular activity during mental arithmetic: effects of task difficulty and task order. Biol Psychol. 2000;52(2):127–41.
40. Fan Y, Cui Y, Tang R, Sarkar A, Mehta P, Tang YY. Salivary testosterone and cortisol response in acute stress modulated by seven sessions of mindfulness meditation in young males. Stress. 2024;27(1):2316041.
41. Mehta PH, Prasad S. The dual-hormone hypothesis: a brief review and future research agenda. Curr Opin Behav Sci. 2015;3:163–8.
42. Wang J, Rao H, Wetmore GS, Furlan PM, Korczykowski M, Dinges DF et al. Perfusion functional MRI reveals cerebral blood flow pattern under psychological stress. Proceedings of the National Academy of Sciences. 2005;102(49):17804–9.
43. Contreras-Aguilar MD, Escribano D, Martínez-Subiela S, Martínez-Miró S, Cerón JJ, Tecles F. Changes in alpha-amylase activity, concentration and isoforms in pigs after an experimental acute stress model: an exploratory study. BMC Vet Res. 2018;14:1–8.
44. Escribano D, Fuentes-Rubio M, Cerón JJ. Validation of an automated chemiluminescent immunoassay for salivary cortisol measurements in pigs. J Vet Diagn Invest. 2012;24(5):918–23.
45. Council NR, Earth D, on, Studies L, Swine C. on NR of. Nutrient requirements of swine. 2012.
46. Thomsson O, Ström-Holst B, Sjunnesson Y, Bergqvist AS. Validation of an enzyme-linked immunosorbent assay developed for measuring cortisol concentration in human saliva and serum for its applicability to analyze cortisol in pig saliva. Acta Vet Scand. 2014;56:1–5.

Publisher's note