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Investigation of equine testis contribution to vitamin D bioactivation

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1. Introduction

Vitamin D is a key regulator of cell differentiation and proliferation, calcium homeostasis and bone metabolism (Bouillon et al., 1995; Fleet et al., 2008; Holick and Chen, 2008). Indeed, vitamin D regulates the intestinal absorption of calcium, renal reabsorption and bone metabolism thus allowing the maintenance of the calcium concentration for the calcium-dependent physiological functions (Bouillon et al., 2008; Haussler et al., 2013). In humans, the main source of vitamin D is synthesized in the skin where UV-B radiations emitted by the sun's rays convert cutaneous 7-dehydrocholesterol into cholecalciferol, namely the inactive form of vitamin D. Then, the biological form of vitamin D results from two successive hydroxylation reactions (Prosser and Jones, 2004; Gray et al., 1971). To this end, cholecalciferol is transported to the liver where the first hydroxylation reaction occurs, mainly mediated by CYP2R1 and supported by CYP27A1. The newly formed 25-hydroxyvitamin D₃ will be transported to the kidneys where the second hydroxylation reaction mediated by CYP27B1 occurs (Morris and Anderson, 2010). A gene mutation of CYP2R1 or CYP27B1 results in low levels of 1 α ,25-Dihydroxyvitamin D₃ (1,25(OH)₂ vitamin D₃) as well as defective calcium homeostasis leading to bone damages (Kato, 2001; Kitanaka et al., 1998). The biologically active form of vitamin D, 1 α ,25(OH)₂ vitamin D₃ generates its transcriptomic effects by binding to its receptor VDR (Vitamin D Receptor) in target tissues until its inactivation by CYP24A1 enzyme (Prosser and Jones, 2004).

The expression of vitamin D metabolizing enzymes (CYP2R1, CYP27A1, CYP27B1 and CYP24A1), was previously described as being limited exclusively to the liver and kidneys. However, the expression of these enzymes, as well as VDR, have been evidenced in a variety of tissues including, male reproductive organs (Choudhary et al., 2005; Tirabassi et al., 2016; De Angelis et al., 2017). Indeed, in humans, these enzymes and VDR are expressed in the testis, epididymis, seminal vesicles and prostate (Aquila et al., 2009; Blomberg Jensen et al., 2010; Choudhary et al., 2005; Corbett et al., 2006). In the human testis, all enzymes and the receptor are co-located in Leydig cells (Blomberg Jensen et al., 2010; Foresta et al., 2011), in spermatocytes, round and elongated spermatids and spermatozoa. Only, CYP27B1, CYP24A1 and VDR are expressed in spermatogonia (Blomberg Jensen et al., 2011, 2010). Moreover, the expression of vitamin D metabolizing enzymes in the male reproductive system has been reported in many species. In addition to being expressed in the human testis, the

enzymatic expression has also been demonstrated in rodent testis (Johnson et al., 1996; Mahmoudi et al., 2013), sheep (Yao et al., 2018) and roosters (Oliveira et al., 2008). In these species, enzymes have also been demonstrated in male reproductive tissues, in particular in the efferent ductules (Oliveira et al., 2008), the epididymis (caput, corpus and cauda) (Dornas et al., 2007; Johnson et al., 1996; Mahmoudi et al., 2013; Oliveira et al., 2008; Yao et al., 2018), ductus deferens, epithelial cells of the seminal vesicles (Johnson et al., 1996; Mahmoudi et al., 2013) and prostate (Johnson et al., 1996).

The existence of a link between the testis and cartilage or bone tissues has been demonstrated in humans (Kato et al., 2001) and rodents (Dardenne et al., 2001). In fact, men with hypogonadism had significantly lower vitamin D levels than healthy individuals (Lee et al., 2012; Wang et al., 2015). Avitaminosis associated with hypogonadism resulted in an altered bone architecture and a low bone mineral density (BMD) which increased the risk of fractures (Misra, 2012). Elevated vitamin D levels have also been shown to be associated with hypogonadism (Lerchbaum et al., 2014). In addition, a decrease in vitamin D levels has been demonstrated in orchiectomized patients after a diagnosis of testicular cancer (Foresta et al., 2013; Foresta et al., 2010; Schepisi et al., 2016). Low levels of vitamin D resulted in altered BMD (Foresta et al., 2013). These various dysfunctions have demonstrated a testicular contribution to the vitamin D bioactivation in humans and the impact of vitamin D on osteoarticular metabolism (Kitanaka et al., 1998). To help reverse this testicular deficiency, vitamin D supplementation is necessary (Bischoff-Ferrari et al., 2009; Trivedi, 2003).

Unlike other species, the horse is considered as an exception with regard to its calcium homeostasis, which is characterized by high plasma calcium level, high intestinal calcium absorption capacity, weaker renal reabsorption of calcium with a high urinary calcium excretion and a low plasma concentration of vitamin D metabolites (Breidenbach et al., 1998; Mäenpää et al., 1988; Schryver et al., 1970; Toribio, 2007; Toribio et al., 2001). In equine species, the kidney plays a more significant key role in calcium homeostasis than vitamin D (Toribio, 2007). The low renal 1α -hydroxylase CYP27B1 activity leads to a low vitamin D level in this species (Breidenbach et al., 1998; Toribio, 2007).

The presence of enzymes metabolizing vitamin D has not been documented in the testis of horses, but the castration of male horses is a regular surgical breeding practice aimed at removing the primary source of sex steroid hormones, especially testosterone which has an impact on behavior through its action on the brain (Mhaouty-Kodja et al., 2018). This practice is carried out in a veterinary context without age recommendation to

avoid side effects beyond the desired effects. In other words, this surgery is performed at any age: in foals, during the growth period or in adulthood. However, the consequences of castration on vitamin D metabolism have not been studied in equine species. Through this study, we sought to assess the contribution of the equine testis to the vitamin D bioactivation as it has been described in humans and some herbivores. To this end, the gene expression of vitamin D metabolizing enzymes in equine testis was studied. Subsequently, plasma levels of $1\alpha,25(\text{OH})_2$ vitamin D_3 were quantified before and after castration in order to investigate the impact of castration on vitamin D levels in horses. For this purpose, three groups of castrated animals at different ages (1 week, 2.2 and 6.6 years old) and a non-castrated control group were studied.

2. Materials and methods

2.1 Materials and reagents

Dulbecco's Phosphate Buffered Saline (PBS) was purchased from Pan Biotech (D. Dutscher, Aidenbach, Germany). A RT-PCR kit was purchased from Promega (Madison, USA): deoxyribose nucleotide triphosphate (dNTP), Random hexamers, Ribonuclease Inhibitor (RNasin), Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT), GoTaq PCR Master Mix were purchased from Promega (Madison, USA). Primers were purchased from Eurogentec (Seraing, Belgium). A testosterone ELISA Kit was purchased from Enzo Life Sciences (Farmingdale, USA) and $1,25(\text{OH})_2$ vitamin D_3 ELISA Kit from MyBioSource (California, USA).

2.2 Animals and samples

The experiment was carried out with four horse groups (*Equus Caballus*) which were classified according to the age at which they were castrated: castrated neonates at birth ($n=8$; 1week), uncastrated neonates ($n=8$), castrated horses at post-pubertal stage ($n=8$, mean age: 2.2 years old) and castrated at adult stage ($n=6$; mean age: 6.6 years old) (Figure 1). For each group, a blood sample was collected before castration (P0). The blood samples from the male neonates' group with castration following birth and control group neonates without castration (Welsh ponies), were collected monthly over a period of 12 months. The foals from these two groups were born in the same period (June) and come from the "Animal Physiology Experimental Facility (PAO; INRAE

Val de Loire, France). Regarding the group comprising animals castrated after puberty and the group comprising animals castrated at the adult stage, blood samples were collected at 3 and 6 months after castration. These animals come from the Saint-Michel de Livet veterinary clinic (Calvados, France).

Blood samples were collected into a tube with anticoagulant (EDTA), the plasma was separated by centrifugation at 3500 r.p.m. for 10 min at 4°C and stored at -80°C for subsequent hormone measurement.

Testes from 4 neonates (1 week), 5 post-pubertal (2.2 years old), 5 adults (6.6 years old) stallions were collected during routine castrations at the INRAE of Nouzilly (Indre et Loire, France) and the Centre Hospitalier Vétérinaire Équin de Livet (Calvados, France). One piece (1 cm³), localized near the middle of the testis, was snap-frozen on dry ice and stored at -80°C for mRNA extraction.

2.3 RNA extraction and RT-PCR analysis

The total RNA was extracted from the testis of experimental herds with Nucleozol[®] reagent according to the manufacturer's instructions. Their concentration was quantified by absorbance at 260nm with NanoDrop[®] (Thermo Scientific). RNA quality was checked by measuring optical density at 260nm and 280nm. 1µg of total RNA was reverse transcribed into cDNA for 90min at 37°C with 100 IU of Moloney Murine Leukemia Virus Reverse Transcriptase, 25UI of RNasin, 0.5µg of random primers and 400µM of dNTP in a final volume of 40µL. In the absence of reverse transcriptase, a reaction was also included to check for genomic contamination. Negative control was used by adding RNase-free water instead of RNA. The resulting cDNA was used in PCRs with primer (Eurogentec, Belgium) designed to amplify specific regions of enzymes metabolizing vitamin D (Table 1). The reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified. The PCR mixture consisted of 8µL of cDNA, 1.5mM of MgCl₂, 0.2mM of each dNTP, 1.25UI of Go Taq G2 Polymerase (Promega) and 16.6µM of each primer. The samples were amplified in Mastercycler (Eppendorf) as follows: one cycle of 2min at 95°C, followed by 30 cycles of 30s at 60°C, followed by an inactivation phase of 5min at 72°C. The PCR products were resolved onto agarose gels (1,5%) containing ethidium bromide (5%) and visualized under UV transillumination.

The reaction specificity was confirmed by PCR products sequencing (GENEWIZ, Germany).

2.4 Immunohistochemistry procedure

Tissue sections were deparaffinised in xylene and rehydrated through a series of graded ethanol (70%, 96%, 100%). To inhibit endogenous peroxidase activity, all sections were incubated in H₂O₂ 3%/PBS for 10 minutes, following a sodium citrate buffer incubation (pH 6) in microwave oven at 90W for 10 minutes. The glass slides were incubated in Triton 0.2%/PBS for 20 minutes in order to permeabilize testicular sections. Then, all sections were blocked in 3% BSA / PBS solution for 20 minutes. The sections were exposed to the mouse anti-CYP27A1 antibody (Santa Cruz, USA) at 4°C overnight in a humid chamber followed by goat anti-mouse-HRP antibody (Cell signaling, USA) for 1h30 at room temperature. Then, the sections were visualized using the diaminobenzidine detection system (Sigma-Aldrich, USA). After counterstaining with Mayer's hematoxylin, testicular sections were dehydrated and covered with Eukitt. Negative control was processed using identical protocols except for the primary antibody was omitted.

2.5 ELISA

Testosterone and 1 α ,25(OH)₂ vitamin D₃ levels were measured using ELISA kits following the manufacturer's instructions (Enzo Life Science; detection limit: 3.9 – 1000 pg/mL (Human reactivity) for testosterone and MyBioSource; detection limit: 31.2 – 1000 pg/mL for 1,25(OH)₂ vitamin D₃). The samples were assayed in duplicate.

2.6 Statistical analysis

All data were presented as the mean \pm SEM. A one-way ANOVA followed by a Tukey test was performed to compare the blood samples obtained before and after the castration of the experimental groups (adult, foals, castrated neonates and non-castrated neonates). A statistical analysis based on a t-test was carried out to compare the samples of both groups. Each sample was performed in duplicates. The differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Testicular expression of CYP2R1, CYP27A1 and CYP27B1

To investigate the ability of testes to play a role in the vitamin D metabolism, the gene expression of CYP2R1, CYP27A1 and CYP27B1 was analyzed in the testis of neonates, post-pubertal and adult horses (Figure 2). A weak signal for CYP27A1 mRNA was detected in all three groups. **The reaction specificity was confirmed by PCR products sequencing. Moreover, the expression of CYP27A1 was demonstrated in the interstitial tissue by immunohistochemistry (Figure 3).** No signal was detected for mRNAs of CYP2R1 and CYP27B1 regardless of the developmental stages studied. The absence of gene expression was confirmed by different pairs of primers, some of them were validated by other studies (Azarpeykan et al., 2016).

3.2. Age-dependent circulating levels of $1\alpha,25(\text{OH})_2$ vitamin D_3

The circulating levels of $1\alpha,25(\text{OH})_2$ vitamin D_3 were quantified in plasma samples obtained from different groups formed for the study, before castration: neonates (1 week) ($142,28 \pm 5,71$ pg/mL), post-pubertal horses (2.2 years old) ($232,76 \pm 11,82$ pg/mL) and adults (6.6 years old) ($194,01 \pm 11,82$ pg/mL) (Figure 4). A significant age-depending difference was observed. Indeed, in neonates, the $1\alpha,25(\text{OH})_2$ vitamin D_3 levels were significantly lower than in the post-pubertal and adult groups. On the other hand, no significant difference was observed between foals and adults.

3.3. $1\alpha,25(\text{OH})_2$ vitamin D_3 levels in neonates castrated or not castrated before puberty

The circulating $1\alpha,25(\text{OH})_2$ vitamin D_3 levels were measured in plasma samples obtained from foals from the neonate stage to the puberty stage (1 year). Two groups were compared monthly for one year: the first included foals castrated during the first week after birth and the second included non-castrated foals (Figure 5). Puberty is characterized by a significant level of testosterone (425 ± 59.2 pg/mL) as observed for the control group at 12 months compared to castrated horses at the neonate stage (Figure 6). A low testosterone level

confirmed the castration ($11,93 \pm 0,97$ pg/mL). A monitoring of vitamin D levels was performed from blood samples collected monthly from birth to 12 months (Figure 5). For geldings, the $1\alpha,25$ (OH)₂ vitamin D₃ level did not present any significant variations during the first year of life (mean level: $215,05 \pm 30,35$ pg/mL). Non-castrated horses exhibited the same pattern with a constant level during the first year of life (mean level: $170,21 \pm 17,34$ pg/mL). For each sample, the two groups were compared, but the geldings did not show significantly different levels in non-castrated horses. Both groups had similar $1\alpha,25$ (OH)₂ vitamin D₃ levels regardless of gonadal status.

3.4. $1\alpha,25$ (OH)₂ vitamin D₃ levels in stallions and horses castrated after puberty

The same analysis was carried out on horses castrated after puberty or in adulthood. A monitoring of vitamin D levels was performed from blood samples collected before castration and 3- and 6-months after castration.

A group of animals was studied at the post-pubertal stage in which castration was carried out after puberty between 18 months and 2 years (mean age: 2.2 years old). No significant difference in the vitamin D levels was observed before castration and 3 and 6 months after castration (mean level: $285,91 \pm 16,03$ pg/mL) (Figure 7A).

Finally, the same analysis was carried out on the last group of the study in which horses were castrated in adulthood (mean age: 6.6 years old). Likewise, the level of $1\alpha,25$ (OH)₂ vitamin D₃ showed no variation between stallions and geldings (mean level: $213,73 \pm 15,66$ pg/mL) (Figure 7B). In the absence of testis, the same circulating levels of vitamin D were observed, whether the animals were castrated or not.

4. Discussion

The aim of the study was to determine the putative testicular contribution to vitamin D bioactivation in equine species, as demonstrated in different species. Subsequently, the consequences of castration on circulating vitamin D levels were studied. A surprising finding was that castration did not affect circulating $1\alpha,25$ (OH)₂ vitamin D₃ levels. This has been observed at any stage of testicular development. An age-dependent

difference has been observed in plasma vitamin D levels. To our knowledge, few studies have described the effect of age on vitamin D levels in equine species. Pozza and colleagues also described age-related differences with higher levels in adults than in yearlings, and showed no significant difference between yearlings and adults as we observed for post-pubertal and adult horses in our study (Pozza et al., 2014). However, vitamin D levels in adult horses were lower than those observed in humans. All these results suggested an absence of equine testis's contribution to the vitamin D bioactivation whatever the stage of testicular development. The gene expression study of enzymes metabolizing vitamin D confirmed this hypothesis with an absence of the expression of CYP2R1 and CYP27B1 in opposite to situation described in other species and low levels of CYP27A1 in horses's testis.

Given the studies conducted in other species, it might have been feared that castration would have an osteoarticular impact as has been demonstrated in humans or rodents. Indeed, in human, gonadal deficiency was often correlated to low level of vitamin D in plasma (Foresta et al., 2013). Vitamin D deficiency is implicated in a wide range of skeletal diseases such as altered bone mineral density (BMD), bone disorders, bone mineral deficiency and increased bone fractures. However, this study did not show any avitaminosis in equine species after castration.

The specificity of equine species can be explained by its evolutionary difference compared to other species (Uhl, 2018). Indeed, as mentioned previously, horses have an atypical vitamin D metabolism. Vitamin D may play a less important physiological role in this species and therefore fewer organs will contribute to its bioactivation. Rickets are rare in equine species because they have developed calcium homeostasis differently in other animals. Compared to humans and rodents, calcium homeostasis is a specific characteristic of equine species with a high capacity of intestinal calcium absorption and high renal excretion (Breidenbach et al., 1998; Mäenpää et al., 1988; Schryver et al., 1970; Toribio et al., 2004, 2001). The renal activity of 1α -hydroxylase CYP27B1 is low, and thus results in low vitamin D levels in this species (Toribio et al., 2004). Therefore, CYP27B1 activity and consequently the active vitamin D metabolism depend on the calcium status. In horses, 70% of the absorbed calcium comes from their diet (Schryver et al., 1970). To balance the high intestinal absorption of calcium, kidneys have become accustomed to removing large amounts of calcium. The urinary excretion of calcium in horses is 5 to 10-fold higher than in other animals or in humans (Toribio et al., 2001). In fact, 98 to 99% of the filtered calcium is reabsorbed by the renal tubules in domestic animals (Blaine et al., 2015). Vitamin D exerts its endocrine activity on calcium transporters to maintain calcium homeostasis and the low vitamin D

amounts in horses suggest that paracellular calcium transport may be more important than transcellular transport and therefore less dependent on vitamin D (Alexander et al., 2014). Vitamin D may not play a central role in calcium homeostasis regulation in horses as has been observed in other species. Thus, in case there should be a vitamin D independence, it is likely that less tissues are involved in vitamin D metabolism. This was shown in this study with an absence of contribution from the equine testis to this bioactivation. The lack of effect may be due to the timing of the analysis. It would be interesting to study the acute effect, with analyses performed on the days following castration.

Moreover, this particularity can be linked to its herbivore status. Indeed, in evolutionary terms, calcium metabolism is more or less dependent on vitamin D. In carnivorous animals such as cats and dogs, 7-dehydrocholesterol levels are low. Indeed, during evolution, these animals became more dependent on their carnivorous diet which is a good source of vitamin D and have lost the capacity to produce sufficient levels of vitamin D by skin synthesis (How et al., 1994). On the contrary, herbivores are more dependent on endogenous synthesis. Besides, there is a vegetable form of vitamin D: Vitamin D₂ (ergocalciferol). Ergocalciferol is found in fungi, yeast, grass and hay and can be a second source of vitamin, especially in equine species. However, in a grazing ruminant such as ram, almost all vitamin D comes from dermal photosynthesis and the expression of vitamin D metabolizing enzymes has been demonstrated in the male reproductive tract (Yao et al., 2018), which makes the equine species a special model.

5. Conclusion

This study has highlighted the non-contribution of the equine testis in vitamin D bioactivation. Consequently, from a practical point of view, castration did not impact long-time vitamin D levels. There is currently no specific recommendation on castration, *i.e.*, it can be performed at any age. Regarding the vitamin D metabolism, this study did not show any contraindication to horses' castration regardless of the age at which it is performed. As equine testis did not contribute to vitamin D bioactivation, horses are an exception when compared to the other species.

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Conflict of interests

Conflict of interests: none

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Table 1: Primers used for RT-PCR analysis

Gene	Full Gene name	GeneBank (Accession code)	Primer (5'-3')	Amplicon Length (bp)
GAPDH	Gluceraldehyde-3-phosphate deshydrogenase	NM_001163856.1	F: CCAGAACATCATCCCTGCTT R: CGTATTTGGCAGCTTTCTCC	159
CYP2R1	Cytochrome P450 family 2 subfamily R member 1	XM_023646084.1	F: ATAACCCTTTTGGCGTGCT R: CGAGGCATTTTTGCGTGTGA	355
			F: GTTGCAAGGAACTGCTCAACA R: CGCCAGACGTGTCAGTACAA	539
			F: GCATTTTCCGCATGAACTGGT R: AGGCGCTTATACAGCCTTG	136
CYP27A1	Cytochrome P450 family 27 subfamily A member 1	XM_023642348.1	F: GTTAGGGCAAGGCTACGTTT R: AACTGGCTCAGTCGAACCAT	360
			F: ACACGGATGCTCTGAACGAG R: AGCTTCCAAGGCAAAGTGGT	114
CYP27B1	Cytochrome P450 family 27 subfamily B polypeptide 1	NM_001163957	F: GGAATGTGACGGAGCTGCTA R: TCAGCACTTCCTTGACCACG	210
			F: GGAAATTCCCGTGTCCCAGA	306

			R: GATTGGAGTAGCACCTGGCT	
			F: CAGAGACATTCATGTGGGTGA R: GCTGGACGAAAAGAATTGG	117 * Azarpeykan et al., 2016

Figure 1: experimental design of collected blood samples from experimental herds. Blood samples were collected monthly until puberty (17 months of age) for the two experimental herds from INRAE: castrated foals following birth (n=8) and uncastrated foals (n=8). Blood samples from Saint-Michel de Livet veterinary clinic were obtained at 3 and 6 months after castration: adult (n=6; 6.6 years old) and post-pubertal horses (n=8; 2.2 years old). A blood sample was collected for all groups before castration (P0).

Figure 2: Testicular expression of CYP27A1, CYP27B1 and CYP2R1 relative to ages. Identification of CYP27A1, CYP27B1 and CYP2R1 transcripts in testis from adults (lane 1 to 5), post-pubertal horses (lane 6 to 10) and neonates (lane 11 to 14) by RT-PCR. The last lane represents negative control. The PCR products were resolved on 1,5% agarose gel electrophoresis visualized by ethidium bromide staining. Molecular standard size (M) is a 100 pb DNA ladder (Biolabs). * indicates 200pb. GAPDH was used as an internal control.

Figure 3: Immunodetection of CYP27A1 protein in adult equine testis. The positive CYP27A1 signal is visible in interstitial tissue. Insert: no positive staining was observed when the primary antibody was omitted (100µm scale bar).

Figure 4: Circulating levels of 1α,25-dihydroxyvitamin D₃ relative to the age. 1α,25-dihydroxyvitamin D₃ levels were measured from neonates (n=8; 1week), post-pubertal (n=8; 2.2 years old) and adult (n=6; 6.6 years old) groups before castration. Data are shown as mean ± SEM. Statistical analyses were conducted using one-Way ANOVA followed by Tukey test (a ≠ b; p ≤ 0.001).

Figure 5: Circulating rates of 1α,25-dihydroxyvitamin D₃ in plasma samples of foals. 1α,25-dihydroxyvitamin D₃ levels were measured before and after castration of foals castrated following birth and of non-castrated foals for 12 months. Data are shown as mean ± SEM (n=8). The statistical analyses were performed over a period of 12 months in each group using one-Way ANOVA followed by a Tukey test. The samples of both groups were compared using a t-test.

Figure 6: Puberty in the NC group. The testosterone levels were measured in castrated and non-castrated groups at puberty stage (12 months). Data are shown as mean ± SEM. Statistical analyses were conducted using an unpaired T-test. *** p ≤ 0.001 (non-castrated n=8; castrated n=8).

Figure 7: Circulating rates of 1α,25-dihydroxyvitamin D₃ in plasma samples of post-pubertal (A) and adult horses (B). 1α,25-dihydroxyvitamin D₃ levels were measured in adults (n=6; 6.6 years old) and post-pubertal horses (n=8; 2.2 years) at 3 and 6 months after castration. Data are shown as mean ± SEM. The statistical analyses were conducted using one-Way ANOVA followed by a Tukey test.

Figure 1

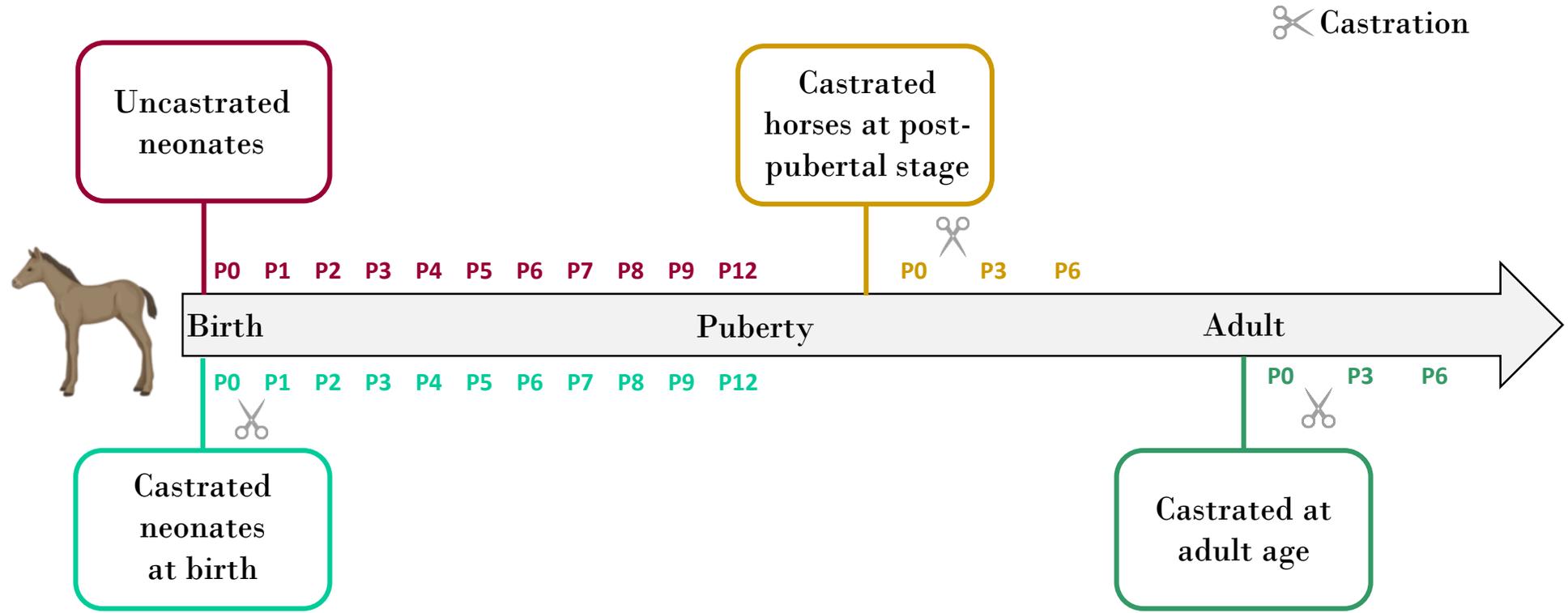


Figure 2

* : 200pb

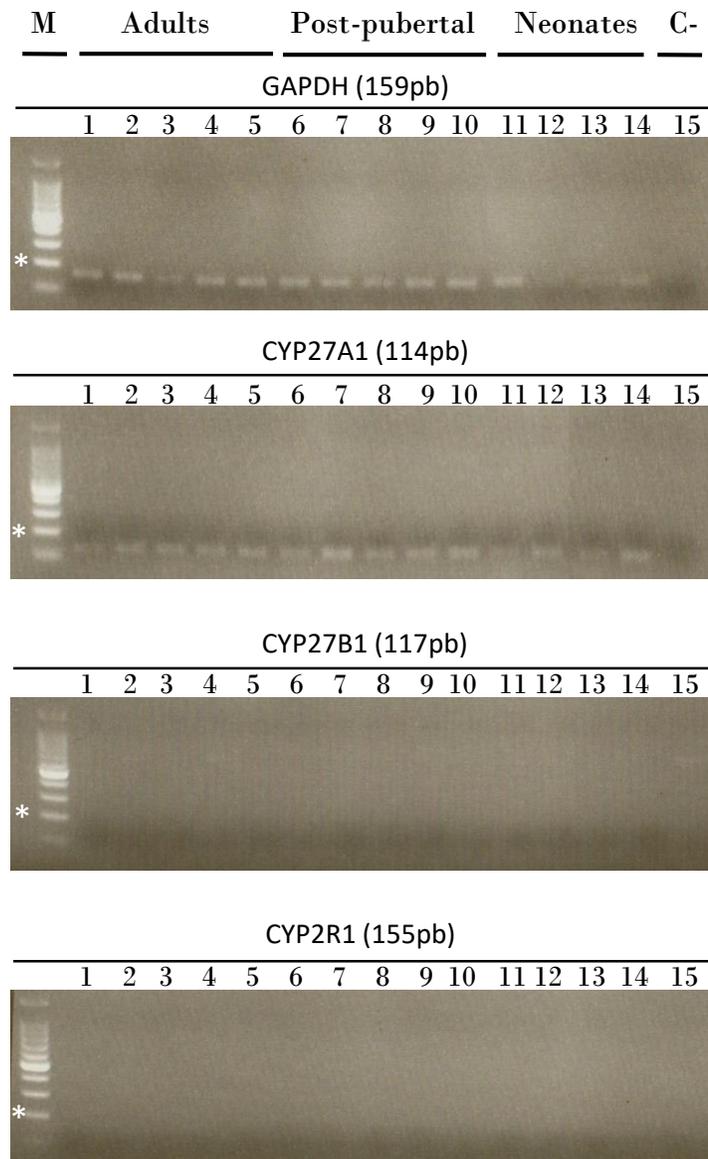


Figure 3

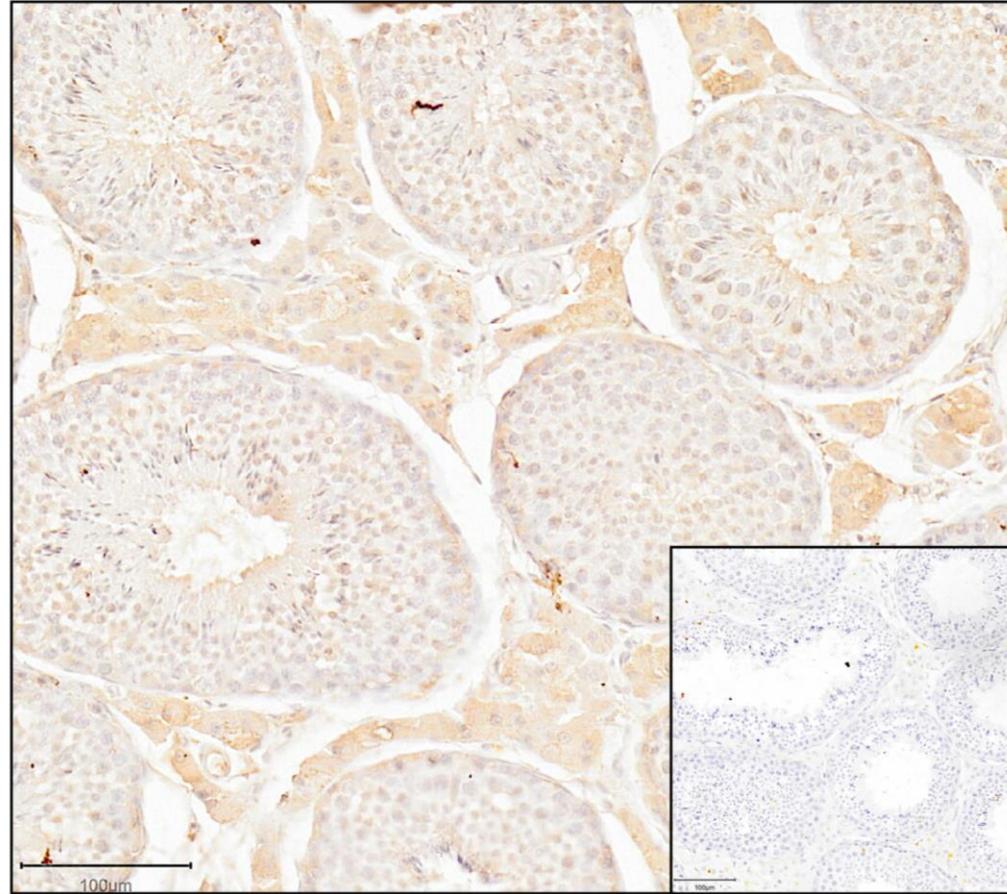


Figure 4

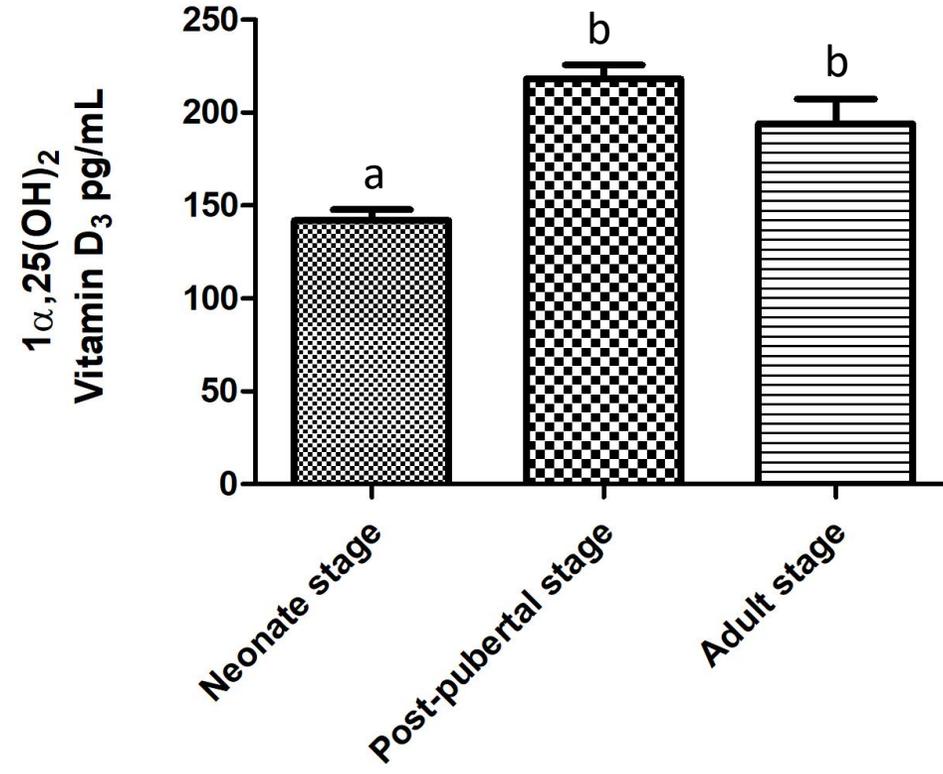


Figure 5

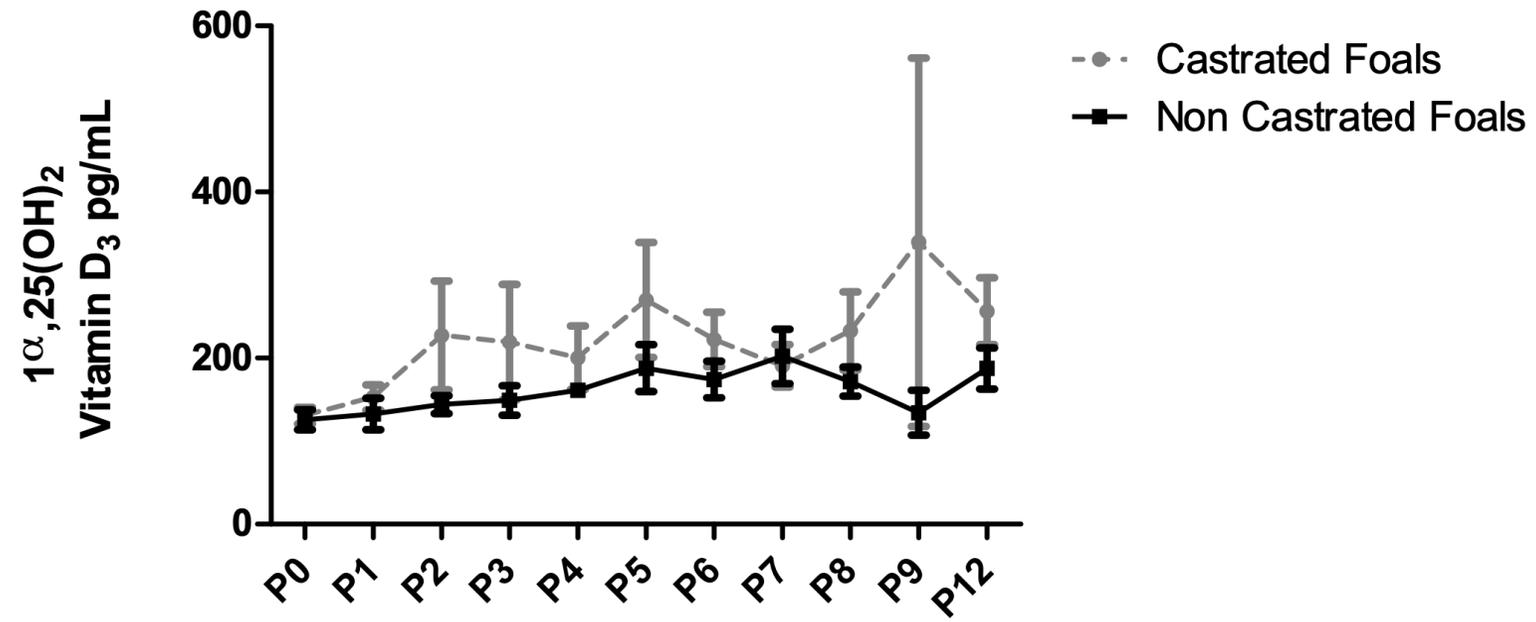


Figure 6

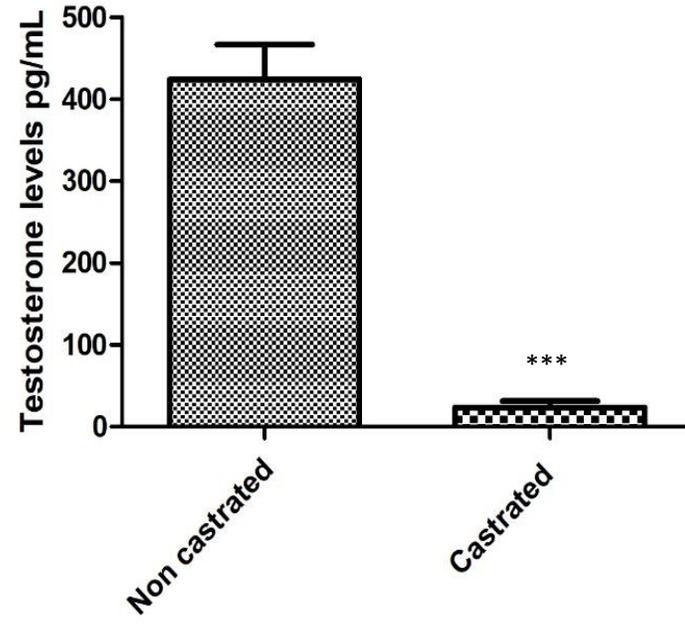


Figure 7

