

# Changes in Testicular Biometry, Steroid Hormones and Receptor Expression in the Peripubertal Period of Indigenous Tenyi-vo Male Pigs of North-eastern Himalayan Region in India

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## Research Article

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# Abstract

Present study was conducted to characterize the testicular changes in the peripubertal period in Tenyi-vo, a miniature size pig of North-eastern Himalayan (NEH) region of India. A total of twenty-four male pigs were randomly selected and categorised for castration at different age groups, G1 (Days 30-45), G2 (Days 60-65), G3 (Days 80-100) and G4 (Days 150-160), n=6 each category. Paired testes and epididymis were used for the assessment of biometry, cauda epididymal spermogram, testicular histology and relative expression of the androgen receptor (AR), estrogen receptors (ER $\alpha$  and ER $\beta$ ), aromatase (CYP19A1), and insulin like growth factor-1 $\beta$  receptor (IGF-1R) in qPCR. Plasma testosterone (T), estradiol (E $_2$ ), tri-iodothyronine (T $_3$ ), thyroxine (T $_4$ ), and cortisol concentrations were estimated on the day of castration in each group of male using commercial ELISA kits. In pigs of G2, a greater testicular weight, volume, epididymis weight was observed relative to G1. The presence of live spermatozoa at  $1240.9 \pm 304.2 \times 10^6$ /mL concentration with 0.65% proximal droplets was recorded as early as day 60. The concentration of T increased steadily over the age of G1 to G4 and a significantly higher concentration was observed in G4 relative to the other categories. Among the transcripts analysed in the testis, the relative fold change of AR was 10.8 fold in G2, which was subsequently reduced in G3 and then down-regulated in G4. CYP19A1 was abundantly expressed in the testis and the fold change ranged from 41-54 fold, although it did not differ significantly from 60-150 days of age. Further, the presence of well-developed seminiferous tubules was evident in the Tenyi-vo male from day 60 onward with a body weight as low as 4.28 kg. The study concluded that the male of Tenyi-vo pig attained puberty at the earliest age of 60 days.

## Introduction

Early onset of puberty is an economic attribute in the swine production system, as it ensures lower spending on extra boar care and improves reproductive life (Kumaresan et al. 2011). A miniature indigenous breed called 'Tenyi-vo' (Accession no. INDIA\_PIG\_1400\_TENYIVO\_09004, National Bureau of Animal Genetic Resource, India) is raised in the backyard by the tribes of Nagaland and Manipur State in the North-East Himalayan (NEH) region of India. With broad pointed heads and relatively short legs, they are black in colour and known for premium meat quality (Patra et al. 2014). In the small holder pig production system prevalent in NEH India, the indigenous pigs are well known for adaptability to harsh environments and thriftiness with negligible inputs (Kumaresan et al. 2007; Kumaresan et al. 2008). The indigenous pig population is decreasing and attempts are made to conserve the germplasm (Bujarbaruah et al. 2007; Vision 2030, 2011; Patra et al. 2015).

It is difficult to ascertain the exact age of attainment of puberty, even though it can be determined on the basis of the presence of functional sperm in the ejaculate or cauda epididymis and the observation of sexual desire (Anderson et al. 1999). Breeds used in the pig industry show initiation of spermatogenesis at around 115 days of age, exhibit synchronised sexual activity and first ejaculation with mature semen characteristics at six months of age (Malmgren 1993; Banaszewska et al. 2011; Chauhan et al. 2016). Early sexual maturity was described previously in Chinese Meishan pigs (Harayama et al. 1991; Lunstra

et al. 1997; Kanematsu et al. 2006). The presence of spermatozoa in the ejaculate and the ability to breed the females at 90 days of age with a mere body weight of 3.0 kg was identified in the Naga local pig (Karunakaran et al. 2009). In another study, early onset of sexual maturity and impregnating ability of local boars in North East India was reported at 108 days based on testicular growth and pregnancy (Kumaresan et al. 2008) compared with the reported age of 5–8 months in commercial breeds (Hurtgen et al. 1986).

Testosterone (T) is necessary for the onset of sexual maturity, and peripheral T concentrations have been well associated with testicular growth, sperm production and onset of puberty (Borg et al. 1993; Perk and Yi 2002). During puberty T concentration increased (Kanematsu et al. 2006) and stayed in the plateau afterwards unless they were sexually stimulated. Apart from T, estradiol ( $E_2$ ) and its  $\alpha$ -receptor (ER $\alpha$ ) are important for regulating normal male fertility (Hess 2003). ER $\alpha$  controls the reabsorption of fluids in efferent ductules and raises the concentration of sperm prior to entry into the epididymis. In adult testis, Leydig cells express CYP19A1 and successfully synthesise  $E_2$  at a far higher rate than that seen in the adult sertoli cells (Levallet et al. 1998; Carreau et al. 1999; Levin 2002; Carreau et al. 2003). The concentration of  $E_2$  in the peripheral blood varied in males, depending on the species (Melnyk et al. 1992; Claus et al. 1992; Bujan et al. 1993). Recently, transcripts of ER and androgen hormone receptor (AR) and CYP19A1 enzymes in the testicular and epididymal tissues have been investigated to explain the capacity for spermatogenesis and steroidogenesis of sexually mature boars (Mutembei et al. 2005; At-Taras et al. 2006; Pearl et al. 2007). However, information on steroid hormones and expression of their receptors in relation to peripubertal changes in Tenyi-vo boar is scanty. The present work documents the gross and microscopical changes of the testis, epididymis, cauda epididymal spermiogram, profile of thyroid and steroid hormones and relative expression of genes related to sex steroid receptors and enzyme associated in the testis of Tenyi-vo pigs in order to characterise the attainment of early puberty.

## Materials And Methods

### Study location

The present study was conducted at the Mega Seed Project on Pig Unit, Indian Council of Agricultural Research (ICAR), Research Complex for North Eastern Hill Region, Nagaland Centre, Medziphema located at 25°45'24" N, 93°50'26" E of Eastern Himalayan subtropical agro-climatic zone. The maximum air temperature ranged from 19.7 °C to 37.5 °C and the minimum air temperature ranged from 6.1 °C to 27 °C, respectively. The area has a humid to rainy climate and receives heavy rainfall of approximately 1800-2500 mm per year.

### Animals and management

The experiment was conducted on Tenyi-vo male pig (n=24) reared under an intensive management system (Supplementary Figure 1). The breeding stock of Tenyi-vo pig were procured from the native tract and maintained in an isomanagerial condition with a commercial concentrate ration. Male piglets were

selected at the age of four weeks and maintained with the dam till weaning at the 45 days of age. At post weaning, group feeding of grower ration was practised with water *ad libitum*. The experiment was performed in compliance with the approved guidelines of the Institute Animal Ethics Committee, ICAR Research Complex for NEH region, Umiam.

### **Collection of testes**

Tenyi-vo male pigs were divided into four groups (n=6/group) viz., G1 (30-45 days), G2 (60-65 days), G3 (80-100 days) and G4 (150-160 days). The body weight of Tenyi-vo boar at birth and weaning was  $0.57 \pm 0.03$  Kg and  $3.81 \pm 0.21$  kg, respectively. Testes were collected by open surgical castration under lignocaine local anesthesia and were transported to laboratory in a sterile thermal flask. After washing with phosphate buffer saline (PBS, pH 7.4), epididymis was dissected. The weight and volume of testis and weight of epididymis were recorded. A piece of testicular tissue was preserved in 10% neutral buffer formalin saline for histology; another piece of tissue weighing about 100 mg was collected in RNAlater (Qiagen, GmbH, Germany) and stored at  $-20^{\circ}\text{C}$  for total RNA extraction. On the day of castration, body weight was recorded and blood was collected in heparinised vials to assay for steroid (T and  $\text{E}_2$ ) and thyroid ( $\text{T}_3$  and  $\text{T}_4$ ) and cortisol hormones.

### **Cauda epididymal spermogram**

The cauda epididymis of each testis was incised and the seminal fluid was aspirated into a micro-centrifuge tube. In the absence of free fluid in the cauda, the lumen was flushed with a few drops of PBS and the fluid was allowed to drain into the micro-centrifuge tube under gravitation. The sperm concentration was estimated by measuring the absorbance of diluted semen sample (2.9% sodium citrate buffer as diluent) at 650 nm in a spectrophotometer (Shimadzu, Japan) using the following the formula [concentration = (Dilution Factor)  $\times$  ( $21.39 \times$  (Absorbance) - 1.09)]. The viability and morphological abnormality were measured by eosin-nigrosin staining methods (Kondracki et al. 2012).

### **Histology of testis**

Preserved tissue samples were trimmed and dehydrated by treatment with increasing alcohol concentration, followed by clearing with acetone and benzene. The tissues were embedded in paraffin and the serial sections of five  $\mu\text{m}$  thickness were cut through a microtome. In the end, tissue sections were stained with haematoxylin and eosin and examined under a light microscope.

### **Hormonal profile**

Plasma T (Cat #AA-E1300),  $\text{E}_2$  (Cat # FR E-2000),  $\text{T}_3$  (Cat # TF E-2100),  $\text{T}_4$  (Cat # TF E-2200) and cortisol (Cat # MS E 5000) concentrations were assayed using commercial ELISA kits (LDN Diagnostica, Germany). The hormone concentration of the test samples was determined using four parameter logistic equations derived from the standard curve in the Multiskan ELISA reader (Thermo scientific Inc., USA).

### **Differential gene expression profile in testicular tissues**

Total RNA was extracted using the RNA isolation kit (Cat # KT151A, Raflex™ Genei, India) and the concentration and purity of RNA was calculated using a micro-cuvet in a UV-VIS spectrophotometer (Shimadzu, Japan). RNA samples having  $A_{260/280}$  between 1.8 to 2.0 were considered for cDNA synthesis. DNase I (1U/μg of RNA, Cat # EN0521, Thermo-Fischer scientific Inc. USA) treatment was performed to eliminate gDNA from the total RNA extracted. cDNA was synthesised using RevertAid M-MuLV reverse transcriptase (Cat # EP0442, Thermo Scientific, USA) and oligo(dT)18 primers (Cat # SO132, Thermo Scientific, USA). A reverse transcriptase control was run in parallel to validate the effectiveness of the DNase I during cDNA synthesis. The primers for AR, CYP19A1, ERα and ERβ, IGF-1R, and endogenous control gene (GAPDH) were designed using the Integrated DNA Technology primer quest tool and custom synthesized (Europhin, India, Table 1). The PCR cyclic condition of the target genes was optimized using Taq PCR Master Mix (Cat # K0171, Thermo-Fischer scientific Inc, USA) in a gradient thermal cycler (Nexus gradient Master cycler, Eppendorf, Germany) and the amplicon size was verified by 1.5 per cent (w/v) agarose gel electrophoresis.

Each target gene was amplified using Maxima SYBR Green qPCR master mix (Cat# K0251, Fermentas, Thermo scientific, USA) in a real-time qPCR system (Pico Real 96, Thermo Scientific, USA). cDNA 10 ng (1 μL) templates were added to 0.2 μL forward and reverse primers (10 pmol each) and 5 μL 2X SYBR Green Master Mix at a final volume of 10 μL. The thermal cyclic conditions included hot start denaturation at 95°C for 15 min, 40 cycles of three segmented amplification and quantification programmes (denaturation at 95°C for 15 s, annealing at a specific temperature for 30 s, an extension at 72°C for 30 s and a melting stage by heating from 55 to 95°C at a rate 1°C/s with the acquisition of fluorescent data. No template control was used as a negative control in the qPCR reaction. GAPDH was used as an endogenous control to generate  $\Delta Ct$  value of the target genes, because its expression was consistent across the age group. The intra-assay coefficient of variation of each target gene at testis was less than 3%. Gr. 1 pigs served as calibrator to generate  $\Delta\Delta Ct$  and the fold change of each gene was determined by  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen 2001).

## Statistical analysis

The variables such as testicular weight, volume, epididymal weight, cauda epididymal spermiogram and hormone were analyzed by One-way ANOVA with Tukey's *post-hoc* test.  $\Delta Ct$  values of each target gene in G1, G2, G3 and G4 was analyzed by non-parametric Kruskal Wallis test with Dunn's *post-hoc* test. Significance was set at 95% and the results are presented as mean  $\pm$  SEM. SPSS 16.0 was used for data analysis and graphpad prism 6.0 was used for constructing the bar diagrams.

## Results

### Body weight and testicular biometry

The age-related biometrical changes of testes and epididymis of Tenyi-vo pig is presented in Table 2. The body weight of G4 male was significantly heavier than those other groups on the day of castration

( $P < 0.001$ ). In contrast, the weight and volume of testis was comparable among G2 to G4; but, significantly heavier than that of G1 (Figure 1). The ratio of testis weight to that of body weight was comparable between G3 and G4; however, it differed significantly from that G1 and G2. Epididymal weight of G4 was significantly heavier than those of other groups; however, it was comparable between G2 and G3.

### **Cauda epididymal spermiogram**

The result of cauda epididymal spermiogram is presented in Table 3. Seminal fluid accumulation was evident in the cauda epididymis of G2 Tenyi-vo pig from 60 days onwards, while only one male in G1 also showed deposition of fluid with the presence of sperm as early as day 45. The amount of fluid in G4 was highest. Despite the fact that the sperm concentration varied from  $1241 \times 10^6$  in G1 to  $2109 \times 10^6$  in G4, the difference was not statistically significant. The proportion of live spermatozoa gradually increased over age and irregular spermatozoa counts, primarily spermatozoa with distal droplet gradually decreased in G2 to G4. The proportion of proximal droplet was upto 2% in various groups.

### **Hormone profile**

Changes in the hormone concentrations during the peripubertal period of Tenyi-vo boars are presented in Figure 2. The concentration of plasma T increased from G1 to G4; however, the T concentration was comparable among G1 to G3. The concentration of T was significantly high in G4 as compared to other groups. The concentration of  $E_2$  ranged from 1.5 to 2.56 ng/mL and was comparable across the age groups. The ratio of T: $E_2$  also remained at similar levels in G1 to G3 (from 7.09 to 8.34); however, G4 showed a significantly high ratio as compared to other groups.

The concentration of  $T_3$  remained at a significant low level in G1, 2 and 4 relative to the G3 while, the concentration of  $T_4$  was higher in G1 to 3 than that of G4. In addition,  $T_4:T_3$  was significantly higher in G1 and 2 than that of other groups. Similar to  $T_3$ , the concentration of cortisol in G3 was significantly higher than those of other groups.

### **Testicular histology**

Presence of differentiated seminiferous tubules with sperm cells was observed in the pre-weaning Tenyi-vo pigs of G1 (Figure 3a). Over age, the compactness of seminiferous tubules and clusters of interstitial cells increased in G2 to 4. In G3 and 4, the seminiferous tubules were more compact and round in shape, consisting of a central lumen with elongated spermatozoa and densely packed interstitial cells between the seminiferous tubules (Figure 3b-d).

### **Expression of steroid hormone receptors in testis**

The relative expression of AR, ER $\alpha$ , ER $\beta$ , CYP19A1 and IGF1R in the testis of Tenyi-vo boar is presented in Figure 4. The AR expression was significantly upregulated with a relative fold change of 10.8 fold in G2;

however, the shift in fold change was reduced in G3 (4.87 fold) and subsequently downregulated in G4 (0.63 fold). The expression of CYP19A1 was most abundant in G2 to G4, ranging from 41 to 54 fold. Similarly, the expression of ER $\alpha$  was up-regulated in the testis, regardless of age group, G2 to G4; however, the fold change was significantly higher in G4. Similarly, the expression of ER $\beta$  appeared to be substantially higher in G4. The expression of IGF-1R transcripts tended to be upregulated in G2 and G3; however, it was moderately downregulated in G4.

## Discussion

The study documents the temporo-spatial changes in the testis, epididymis, hormones and their receptors during the peripubertal period in Tenyi-vo male pigs. The peripubertal changes in the testis and epididymis of Tenyi-vo boar showed that these pig breed had a greater degree of testicular weight and volume, and epididymal weight at day 60. The body weight gain was however, non-significant during the first three months of age. The weight of male piglets of this breed ranged from 300-500 g at birth to 2.5-3.0 kg at 3.0 months of age (Karunakaran et al. 2009) and from 9 to 32 kg at adult age (Borkotoky et al. 2014). The body weight gain remains unchanged during the peripubertal period, attaining a mere 6.28 kg at the age of five months. Higher testicular weight found in the present study in the young age of 60 days old Tenyi-vo male indicates possible early sexual maturity (Kumaresan et al. 2008). The ratio of testis weight to body weight (g/kg) increased steadily from 30 days, reached a significantly higher level at three months of age, and subsequently decreased at around five months of age. Our finding is consistent with the earlier studies describing a significantly higher testis to body weight ratio in local pigs compared to the commercial European breeds (Kumaresan et al. 2008). Early attainment of puberty and sexual maturity of males was also due to faster testicular growth of indigenous pigs compared to Hampshire and Large White Yorkshire (Rohilla et al. 2000). In addition, the change in epididymis weight in respect to the body weight and testicular weight also showed a positive increment; however, a distinct surge was evident from 35 to 62 days. Similarly, at the same time the ratio of epididymis to testis weight was significantly increased, which later remained static up to three months of age, although there was a significant change in the ratio at days 150. Veerabramhaiah et al. (1998) recorded a similar trend in higher growth rates of testis and epididymis over 4 to 6 months of age than 2 to 4 months of age in indigenous pig as compared to the crossbred.

In the present study, the presence of live sperm was evident in the cauda epididymis of a Tenyi-vo boar even at day 45, but it was more distinct at 60 days, where most of the boars had at least 0.5 mL of cauda epididymal fluid consisting of sperm  $>1200 \times 10^6$ /mL with live count  $>60$  per cent and 2% of spermatozoa with proximal droplets. The spermatozoa concentration remained unchanged between days 80 to 150, while a significant rise in live percent and decrease in distal droplets was evident at 150 days of age. This result is consistent with the earlier reports (Kato and Harayama 1990; Lunstra et al. 1992) that the presence of sperm in the lumen of the seminiferous tubules of Chinese Meishan pigs was discernible as early as 60 days and in the cauda epididymis at around 70 days of age. Testicular size and weight are strongly associated with sperm production and total sperm reserves (Lubritz et al. 1991; Rathje et al.

1995) and testicular size is highly heritable in pigs (Johnson et al. 1994). The evolution of the fertilizing ability of spermatozoa has been correlated with changes in progressive motility, alteration of metabolic patterns and structural status of particular tail organelles, changes in nuclear chromatin, changes in the nature of plasma membrane surface and movement of cytoplasmic droplets (Karunakaran et al. 2009). The greater testicular weight and volume, along with the presence of acceptable live count and permissible abnormality in the cauda epididymal spermogram, support the pubertal changes of Tenyi-vo boars by 60 days of age.

Seminiferous tubules and sperm cells existence was observed in Tenyi-vo boars at the early age of day 35 in G1. The more compact seminiferous tubules, consisting of central lumen accumulating spermatozoa and densely packed interstitial cells between the seminiferous tubules, are commonly visible in the boar in days 60. This finding supports the characteristics of histological changes that suggest the attainment of puberty (Avelar et al. 2010). Sexual precocity in male miniature pigs was previously reported (Kakeno 2011), which is characterized by early differentiation of gonocytes as well as active development and proliferation of seminiferous sertoli cells, which shortened the early stage of spermatogenesis. The increase in seminiferous tubule diameter during peripubertal period suggests the ensuing testicular maturation as the diameter of seminiferous tubule, number of seminiferous sertoli cells and tubular fluid volume increases (Creasy 1997; Kangawa et al. 2016; Almunia et al. 2019).

Testicular development, initiation of puberty, sexual maturity, and sperm production are intricately associated with the production of testicular steroid hormones (Borg et al. 1993; Park and Yi 2002). The increased concentration of plasma T found in Tenyi vo boar from 35 to 150 days of age strongly supports the developmental changes in the testis during the process of sexual maturation. The predominant types of circulating androgens differ considerably with age as T becomes more predominant during sexual maturity (Sinclair et al. 2001). T levels recorded in boars showed large variations in age, breed and season, ranging from 0.73 ng/mL to about 50 ng/mL (Borg et al. 1993; Park and Yi 2002). The AR expression was upregulated on day 60, but the fold change decreased on day 90 and subsequently downregulated on day 150. Significantly higher testicular AR gene expression at lower age may be due to early attainment of puberty as seen in spermogram, testicular histology and steroid hormone profile in Tenyi-vo boar. Age related progressive decline in testicular AR gene expression could not be explained convincingly.

The concentration of plasma E<sub>2</sub> in Tenyi-vo males ranged between 1.5 to 2.6 ng/mL across the age groups. The E<sub>2</sub> hormone also plays an important role not only in the control of male sexual activity and sertoli cell function, but essential for normal fertility (Hess 2003). This new paradigm function of E<sub>2</sub> in males started with the discovery that testicular germ cells and epididymal sperm express CYP19A1 and synthesize E<sub>2</sub> (Nita et al. 1993). The expression of CYP19A1 in the Tenyi-vo boar testis was most abundant, ranging from 41 to 54-fold across the age groups from day 60 to 150 in our study. Early studies recorded that the primary source of E<sub>2</sub> in the immature male was Sertoli cell (van der Molen et al. 1981); however, in the adult testis, Leydig cells produce CYP19A1 and actively synthesize E<sub>2</sub> at much higher rate than that seen in the adult Sertoli cell (Carreau et al. 1999; Levin 2002; Carreau et al. 2003).

The expression of ER $\alpha$  was up-regulated in the testis regardless of age group, although the fold change was not significant between days 60 to 150. In comparison, the expression of ER $\beta$  appeared to be significantly higher on day 150. In a recent study using in-situ hybridization and UV-single cell microdissection, ER $\alpha$  mRNA has been shown in the spermatogonia up to mid-pachytene primary spermatocytes of the seminiferous epithelial cycle and ER $\beta$  mRNA in the Sertoli cells. Leydig cells however, did not show either ER $\alpha$  or ER $\beta$  mRNA expression. Thus, a direct effect of E<sub>2</sub> on the Sertoli cell function through ER $\beta$  and germ cell formation via ER $\alpha$  was speculated in the boar testis (Lekhkota et al. 2006). Expression of ER $\alpha$  and  $\beta$  and CYP19A1 in immature and mature boars testis has also been documented (Mutembei et al. 2005).

In spite of the high concentration of steroid hormone and cortisol, the lower body weight during the peripubertal phase suggests the possible involvement of the metabolic hormones in the Tenyi-vo boar. Insulin and insulin-like growth factors (IGF-1) are polypeptides that regulate growth, differentiation and survival in a wide range of cells and tissues. The effect of IGF1-IGF1R on reproductive parameters, specifically release of gonadotropin and interactions between the IGF system and other effectors of gonadotropin release are described (Lackey et al., 1999). In Tenyi-vo boar, the non-significant modulation of IGF-1R expression may be correlated with the poor physical growth of Tenyi-vo breed observed in present study. Thyroid hormones play a significant role in the regulation of the basal metabolic rate. The thyroid gland contributes to the regulation of the growth, development, adaptation and productivity of farm animals (Petkov et al. 2008; Alemneh and Akeberegn 2019). Knowledge on the thyroid profile in the regulation of basal metabolic rate in the Tenyi-vo boar is scanty. The concentration of T<sub>3</sub> remained at a significantly low level between days 35 to 60 as compared to day 90, while the concentration of T<sub>4</sub> was high on day 35 to 90 as compared to day 150. The lower growth potential in Tenyi-vo boar could be associated with reduced concentration of plasma T<sub>3</sub> and higher T<sub>4</sub>:T<sub>3</sub> ratio. The level of thyroid hormone synthesis, which controls the rate and the direction of metabolic events, determines their physiological equilibrium (Breier et al. 2000). In addition, abruptly higher sex steroid levels in males may also be associated with slow growth rate supporting the findings in the Tenyi-vo boar (Duarte-Guterman et al. 2014).

## Conclusion

It is concluded that Tenyi-vo male pigs attained puberty at the earliest by 60 days of age with a body weight around 4 kg. Further researches on the genetic constituents of this particular breed are required for its conservation and utilization in the commercial pig breeding program.

## Declarations

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## **Conflict of interest**

There is no conflict of interest to declare.

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## **Statement of Animal Rights**

The study was conducted following the approved guidelines of the Institutional Animal Ethics Committee (IAEC), ICAR Research Complex for NEH Region, Umiam, Meghalaya.

## **Consent to participate**

We are ready to participate in future program.

## **Consent for publication**

Authors declare their consent for publication of this manuscript in the 'Tropical Animal Health and Production' journal

## **Availability of data and material**

All the research data are available and will be provided on request.

**Code availability** – not applicable

## **Authors' contribution**

M.K.P., G.K. involved in conceptualization; M.K.P., Y.K., E.N., L.N., D.B., involved in methodology; M.K.P., Y.K. and V.S. involved in qPCR and histology, M.K.P., G.K.M. and N.K. wrote, reviewed and edited the manuscript.

## **References**

1. Alemneh, T. and Akebergn, D., 2019. Adaptation strategies of farm animals to water shortage in desert areas. *American Journal of Biomedical Science and Research*, 2(6): 245–250. DOI: 10.34297/AJBSR.2019.02.000617
2. Almunia, J., Nakamura, K., Murakami, M., Takashima, S., Mori, T. and Takasu, M., 2019. Sexual precocity in male microminipigs evaluated immunohistologically using spermatogonial stem cell markers. *Theriogenology*, 130: 120–124.
3. Andersson, H.K., Hullberg, A., Malmgren, L., Lundstrom, K., Rydhmer, L. and Squires, J., 1999. Sexual maturity in entire male pigs: Environmental effects, relations to skatole level and female puberty.

4. At-Taras, E.E., Conley, A.J., Berger, T. and Roser, J.F., 2006. Reducing estrogen synthesis does not affect gonadotropin secretion in the developing boar. *Biology of Reproduction*, 74(1): 58–66.
5. Avelar, G.F., Oliveira, C.F.A., Soares, J.M., Silva, I.J., Dobrinski, I., Hess, R.A. and Franc, L.R., 2010. Postnatal somatic cell proliferation and seminiferous tubule maturation in pigs: A non-random event. *Theriogenology*, 74: 11–23.
6. Banaszewska, D., Kondracki, S. and Wysokińska, A., 2011. Effect of age on the dimensions and shape of spermatozoa of Large White Polish boars. *Archives Animal Breeding*, 54(5): 504–514.
7. Borg, K.E., Lunstra, D.D. and Christenson, R.K., 1993. Semen characteristics, testicular size and reproductive hormone concentrations in mature Duroc, Meishan, Fengjing and Minzhu boars. *Biology of Reproduction*, 49: 515–21.
8. Borkotoky, D., Perumal, P. and Singh, R.K., 2014. Morphometric attributes of Naga local pigs. *Veterinary Research International*, 2(1): 08–11.
9. Breier, L., Oliver, M. and Gallaher, B., 2000. Regulation of growth and metabolism during postnatal development. In: *Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction*, P. Gronje (ed.), New York: CAB I Publishing, pp. 187–200.
10. Bujan, L., Mieusset, R., Audran, F., Lumbroso, S. and Sultan, C., 1993. Increased oestradiol level in seminal plasma in infertile men. *Human Reproduction*, 8: 74–77.
11. Bujarbaruah, K.M., Das, A., Bardoloi, R.K. and Kumaresan, A., 2007. Status and strategies for pig production in the North Eastern India. In: Das, A. et al. (Ed.), *Complementary role of livestock and fisheries towards sustainable farming in North East India*, pp. 79–84.
12. Carreau, S., Genissel, C., Bilinska, B. and Levallet, J., 1999. Sources of oestrogen in the testis and reproductive tract of the male. *International Journal of Andrology*, 22: 211–223. doi: 10.1046/j.1365-2605.1999.00172.x.
13. Carreau, S., Lambard, S., Delalande, C., Denis-Galeraud, I., Bilinska, B., & Bourguiba, S. (2003). Aromatase expression and role of estrogens in male gonad: a review. *Reproductive Biology and Endocrinology*, 1, 35. doi: 10.1186/1477-7827-1-35.
14. Chauhan, A., Patel, B.H.M., Maurya, R., Kumar, S., Shukla, S. and Kumar, S., 2016. Pig production system as a source of livelihood in Indian scenario: An overview. *Interantional Journal of Science Environment and Technology*, 5(4): 2089–96.
15. Claus, R., Dimmick, M.A., Gimenez, T. and Hudson, L.W., 1992. Estrogens and prostaglandin F<sub>2a</sub> in the semen and blood plasma of stallions. *Theriogenology*, 38:687–693.
16. Creasy, D.M.. 1997. Evaluation of testicular toxicity in safety evaluation studies: the appropriate use of spermatogenic staging. *Toxicologic Pathology*, 30:119 e31.
17. Duarte-Guterman, P., Navarro-Martín, L. and Trudeau, V.L., 2014. Mechanisms of crosstalk between endocrine systems: regulation of sex steroid hormone synthesis and action by thyroid hormones. *General and Comparative Endocrinology*, 203: 69–85.

18. Harayama, H., Najo, I., Kanda, S. and Kato, S. 1991. Testicular development in Chinese Meishan boars. *Theriogenology*, 36: 637–43.
19. Hess, R.A., 2003. Estrogen in the adult male reproductive tract: a review. *Reproductive Biology and Endocrinology*, 9(1): 52. doi: 10.1186/1477-7827-1-52.
20. Hurtgen, J.P., 1986. Mating systems and boar management. In: Morrow, DA, editor. *Current Therapy in Theriogenology*. Universidad de Murcia Press; Murcia, Spain, p. 978–80.
21. Jhonson, R.K., Eckardtm, G.R., Rathje, T.A. and Drudik, D.K., 1994. Ten generations of selection for predicted weight of testes in swine: direct response and correlated response in body weight, back fat, age at puberty and ovulation rate. *Journal of Animal Science*, 72: 1978–1981.
22. Kaneko, N., 2011. Microminipig, a non-rodent experimental animal optimized for life science research: preface. *Journal of Pharmacological Science*, 115:112, e4.
23. Kanematsu, N., Jin, W.Z., Watanabe, G. and Taya, K., 2006. Age-related changes of reproductive hormones in young Meishan boars. *Journal of Reproduction and Development*, 52: 651–656.
24. Kangawa, A., Otake, M., Enya, S., Yoshida, T. and Shibata, M., 2016. Histological development of male reproductive organs in microminipigs. *Toxicological Pathology*, 44: 5e22.
25. Karunakaran, M., Mondal, M., Rajarajan, K., Karmakar, H.D., Bhatt, B.P., Das, J., Bora, B., Baruah, K.K. and Rajkhowa, C., 2009. Early puberty in local Naga boar of India: Assessment through epididymal spermogram and in vivo pregnancy. *Animal Reproduction Science*, 111: 112–119. 10.1016/j.anireprosci.2008.02.009.
26. Kato, S. and Harayama, H., 1990. Sexual maturation of Meishan boars. *Proceedings Chinese Pig Symposium*. Toulouse, France, p. 36.
27. Kondracki S., Iwanina M., Wysokińska A. and Huszno M., 2012. Comparative analysis of Duroc and Pietrain boar sperm morphology. *Acta Vet Brno*, 81:141–145.
28. Kumaresan, A., Bujarbaruah, K.M., Kadirvel, G., Khargharia, G., Sarma, G., Goswami, J., Basumatary, R., Palaniappan, K. and Bardoloi, R.K., 2011. Early sexual maturity in local boars of North eastern India: Age-related changes in testicular growth, epididymal sperm characteristics and peripheral testosterone levels. *Theriogenology*, 75: 687–95. 10.1016/j.theriogenology.2010.10.009.
29. Kumaresan, A., Bujarbaruah, K.M., Karunakaran, M., Das, A. and Bardoloi, R.K., 2008. Assessment of early sexual maturity in nondescript local pigs of north east India: testicular development, spermogram and in vivo pregnancy. *Livestock Science*, 116: 342–347.
30. Kumaresan, A., Bujarbaruah, K.M., Pathak, K.A., Chetri B., Das, S.K., Das, A. and Ahmed, S.K., 2007. Performance of pigs reared under traditional tribal low input production system and chemical composition of nonconventional tropical plants used as pig feed. *Livestock Science*, 107: 294 – 328.
31. Lackey, B.R., Gray, S.L. and Henricks, D.M., 1999. The insulin-like growth factor (IGF) system and gonadotropin regulation: actions and interactions. *Cytokine and Growth Factor Reviews*, 10(3–4): 201–217.
32. Lekhkota, O., Brehm, R., Claus, R., Wagner, A., Bohle, R.M. and Bergmann, M., 2006. Cellular localization of estrogen receptor-alpha (ER $\alpha$ ) and beta (ER $\beta$ ) mRNA in the boar testis. *Histochemistry*

- and Cell Biology, 125: 259–264.
33. Levallet, J., Bilinska, B., Mitre, H., Genissel, C., Fresnel, J. and Carreau, S., 1998. Expression and immunolocalization of functional cytochrome P450 aromatase in mature rat testicular cells. *Biology of Reproduction*, 58: 919–926.
  34. Levin, E.R., 2002. Cellular functions of plasma membrane estrogen receptors. *Steroids*, 67: 471–475.
  35. Livak, K. and Schmittgen, T., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta C_t}$  method. *Methods*, 25(4): 402–8.
  36. Lubritz, D., Jhonson, B. and Robison, O.W., 1991. Genetic parameters for testosterone production in boars. *Journal of Animal Science*, 69: 3220–3224.
  37. Lunstra, D.D., Borg, K.E. and Klindt, J., 1992. Characterization of pubertal development in the Meishan Chinese boar. *Journal of Animal Sciences*, 70 (suppl 1): 267.
  38. Lunstra, D.D., Ford, J.J., Klindt, J. and Wise, T.H., 1997. Physiology of Meishan boar. *Journal of Reproduction and Fertility*, 52: 181–93.
  39. Malmgren, L., 1993. Induced testicular alterations in prepubertal and mature boars. Thesis, Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences, Uppsala.
  40. Melnyk, P.M., Sanford, L.M. and Robaire, B., 1992. Moderate increases in peripheral blood estradiol concentration in the adult ram do not directly inhibit testosterone secretion. *Canadian Journal of Physiology and Pharmacology*, 70: 1384–1391.
  41. Mutembei, H.M., Pesch, S., Schuler, G. and Hoffmann, B., 2005. Expression of oestrogen receptors  $\alpha$  and  $\beta$  and of aromatase in the testis of immature and mature boars. *Reproduction in Domestic Animals*, 40(3): 228–236.
  42. Nitta, H., Bunick, D., Hess, R.A., Janulis, L., Newton, S.C., Millette, C.F., Osawa, Y., Shizuta, Y., Toda, K. and Bahr, J.M., 1993. Germ cells of the mouse testis express P450 aromatase. *Endocrinology*, 132: 1396–1401.
  43. Park, C.S. and Yi, Y.J., 2002. Comparison of semen characteristics, sperm freezability and testosterone concentration between Duroc and Yorkshire boars during seasons. *Animal Reproduction Science*, 73: 53–61.
  44. Patra, M.K., Begum, S. and Deka, B.C., 2014. Problems and prospects of traditional pig farming for tribal livelihood in Nagaland. *Indian Research Journal of Extension Education*, 14 (4): 6–11.
  45. Patra, M.K., Deka, B.C., Kent, Y., Ngullie, L., Das, R.K. and Ngachan, S.V., 2015. Promotion of pig breeding in Nagaland through quality piglet production and artificial insemination. Technical bulletin. Published by ICAR Research Complex for NEH Region, Umiam, Meghalaya. pp:1–65.
  46. Pearl, C.A., Berger, T. and Roser, J.F., 2007. Estrogen and androgen receptor expression in relation to steroid concentrations in the adult boar epididymis. *Domestic Animal Endocrinology*, 33(4): 451–459.
  47. Petkov, P., Kanakov, D. and Stoyanchev, K., 2008. Quantitative variations in thyroid hormones -  $T_3$  and  $T_4$  - in pigs of various breeds, gender and age. *Trakia Journal of Sciences*, 6(2): 16–20.

48. Rathje, T.A., Jhonson, R.K. and Lunstra, D.D., 1995. Sperm production in boars after nine generations for increased weight of testes. *Journal of Animal Science*, 69: 2177–2185.
49. Rohilla, P.P., Bujarbaruah, K.M., Kumar, M. and Singh, G., 2000. Carcass traits of Large White Yorkshire, Hampshire and Naga local pigs. *Indian Journal of Animal Sciences*, 70(3): 307–308.
50. Sinclair, P.A., Squires, E.J. and Raeside, J.I., 2001. Early postnatal plasma concentrations of testicular steroid hormones, pubertal development, and carcass leanness as potential indicators of boar taint in market weight intact male pigs. *Journal of Animal Science*, 79: 1868–76.
51. van der Molen, H.J., Brinkmann, A.O., de Jong, F.H. and Rommerts, F.F., 1981. Testicular oestrogens. *Journal of Endocrinology*, 89: 33–46.
52. Veerabramhaiah, K., Naidu, S. and Sreeraman, P.K., 1998. Comparative postnatal development of epididymis in indigenous and crossbred pigs—biometrical studies. *Indian Veterinary Journal*, 75: 226–227.
53. Vision 2030, 2011. National Research Centre on Pig. India: Indian Council of Agricultural Research. Rani, Guwahati. 1–14.

## Tables

**Table 1. Primers used in qPCR for amplification of different target genes**

Target gene	Oligonucleotide sequences (5'>>>>>3') <sup>§</sup>	Amplicon size (bp) <sup>\$\$</sup>	Accession No.
AR	For: AACAGCAGCCTTCACAACAG Rev: TTAAGATCGGTGGAGCAGCT	208	AB052938
IGF-1 $\beta$ R	For: GATCAGCGGGAATGTGTGTC Rev: ACTGGTAGGGCGATGATCAG	203	U58370
Aromatase	For: TTAGCAAGTCCTCAAGTGTG Rev: CCAGGAAGAGGTTGTTAGAG	324	U37311
ER $\alpha$	For: TTGTGTGCCTCAAATCCATC Rev: GACAGGATGAGGAGGAGCTG	202	AF035775
ER $\beta$	For: CATGATGATGTCCCTGACCA Rev: GGTCTGGAGCAAAGATGAGC	200	AF267736
GAPDH	For: CGTCAAGCTCATTTCTGGT Rev: AGTCAGGAGATGCTCGGTGT	201	NM_001206359.1

<sup>§</sup>The length of each primer was 20 bp. <sup>\$\$</sup> Annealing temperature was 54 °C.

**Table 2. Changes in the biometry of testis and epididymis (mean ± SE) of Tenyi-vo pigs during the peripubertal period†**

Attribute	G1 <sup>‡</sup>	G2	G3	G4	P value
Average age at castration (days)	35±3.16 <sup>a</sup> (30-45)	62.67±0.92 <sup>b</sup> (60-65)	87.90±3.09 <sup>c</sup> (80-100)	158.67±2.11 <sup>d</sup> (150-160)	<0.001
Body weight at castration (Kg)	3.37±0.27 <sup>a</sup>	4.28±0.24 <sup>a</sup>	4.35±0.24 <sup>a</sup>	6.28±0.47 <sup>b</sup>	<0.001
Testis weight (g)	7.76±1.01 <sup>a</sup>	14.79±1.13 <sup>b</sup>	16.2±1.55 <sup>b</sup>	17.96±1.34 <sup>b</sup>	0.0002
Testis volume (cm <sup>3</sup> )	15.45±2.03 <sup>a</sup>	32.85±3.31 <sup>b</sup>	41.46±4.82 <sup>b</sup>	39.55±1.67 <sup>b</sup>	0.0005
Epididymis weight (g)	1.33±0.21 <sup>a</sup>	3.65±0.14 <sup>b</sup>	4.10±0.24 <sup>b</sup>	7.15±0.57 <sup>c</sup>	<0.001
Testis weight/body weight (g/kg)	2.28±0.20 <sup>a</sup>	3.57±0.48 <sup>ab</sup>	3.83±0.42 <sup>b</sup>	2.93±0.32 <sup>ab</sup>	0.035
Epididymis wt/body weight (g/kg)	0.385±0.03 <sup>a</sup>	0.869±0.07 <sup>b</sup>	0.958±0.06 <sup>bc</sup>	1.137±0.03 <sup>c</sup>	0.0001
Epididymis/testis weight	0.17±0.01 <sup>a</sup>	0.251±0.01 <sup>b</sup>	0.268±0.02 <sup>b</sup>	0.405±0.03 <sup>c</sup>	<0.001

†Average birth weight 0.57±0.03 Kg and the weaning weight 3.81±0.21 <sup>‡</sup>Sample size n=6/group; Figures with different superscripts across each row differ significantly.

**Table 3. Cauda epididymal spermogram (mean±SE) of Tenyi-vo pig during the peripubertal period†**

Attribute	G2 <sup>‡</sup> (n=6)	G3 (n=6)	G4 (n=6)	P value
Caudaepididymal fluid volume (mL)	0.46±0.02 <sup>a</sup>	0.53±0.06 <sup>a</sup>	0.833±0.06 <sup>b</sup>	0.001
Spermatozoa concentration (×10 <sup>6</sup> )	1240.9±304.2	1653.4±320.8	2109.6±55.8	0.138
Live spermatozoa (%)	63.8±3.87 <sup>a</sup>	67.4±3.72 <sup>a</sup>	86.5±1.92 <sup>b</sup>	0.001
Spermatozoa with proximal droplet (%)	0.65±0.06	2.0±0.95	0.8±0.23	0.303
Distal droplet (%)	17.9±2.49 <sup>a</sup>	14.6±2.04 <sup>ab</sup>	10.8±0.38 <sup>b</sup>	0.041

†In G1, only one boar showed the presence of spermatozoa and cauda epidymal fluid, hence not included in statistical analysis. <sup>‡</sup>Sample size n=6/group; Figures with different superscripts across each row differ significantly

# Figures



A. G1 (day 30)

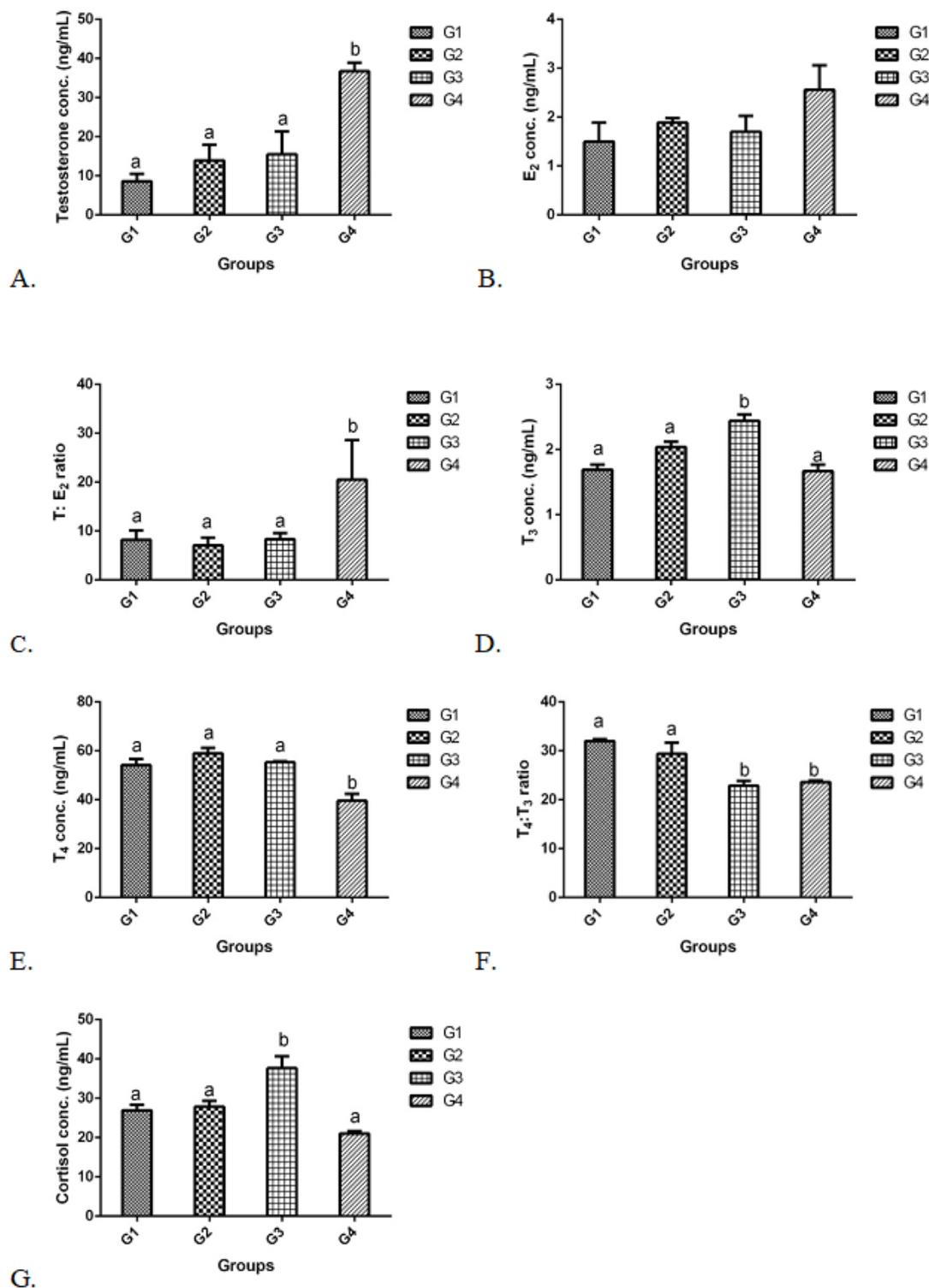
B. G2 (Day- 60)

C. G3 (Day 90)

D. G4 (Day150)

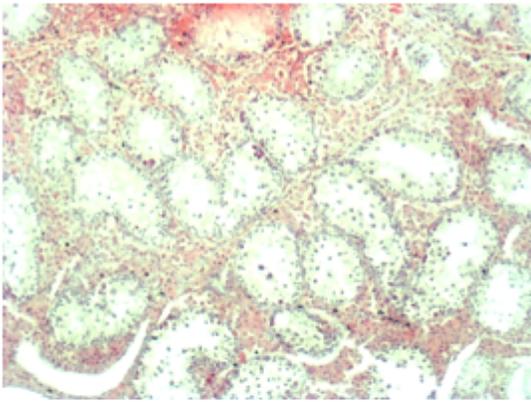
## Figure 1

Representative gross photographs of testis and epididymis of Tenyi-vo pig castrated around the peripubertal period.

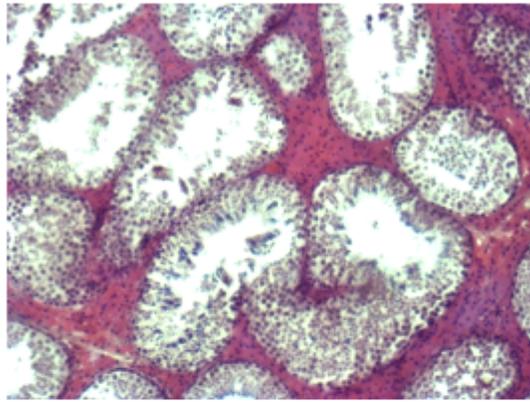


**Figure 2**

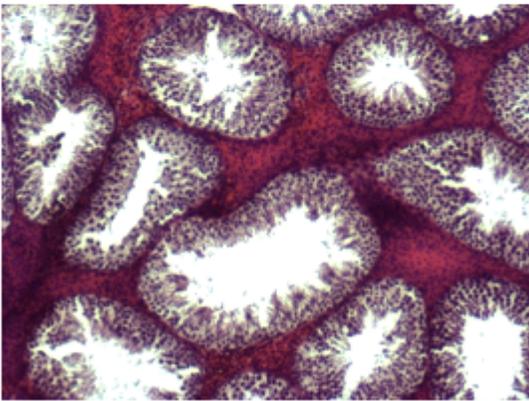
Plasma steroid and thyroid hormone concentration in different age groups of Tenyi-vo pig. A. Testosterone (T), B. Estradiol (E<sub>2</sub>), C. T:E<sub>2</sub> ratio, D. Triiodothyronine (T<sub>3</sub>), E. Thyroxin (T<sub>4</sub>), F. T<sub>4</sub>:T<sub>3</sub> ratio, G. Cortisol. Groups were classified into G1 (30-45 days), G2 (60-65 days), G3 (80-100 days), G4 (150-160 days). Bar with different superscript indicate significant difference at P<0.05.



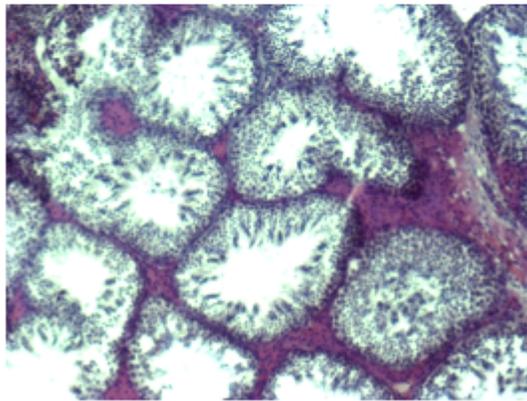
A. G1 (Day 30)



B. G2 (day 60)



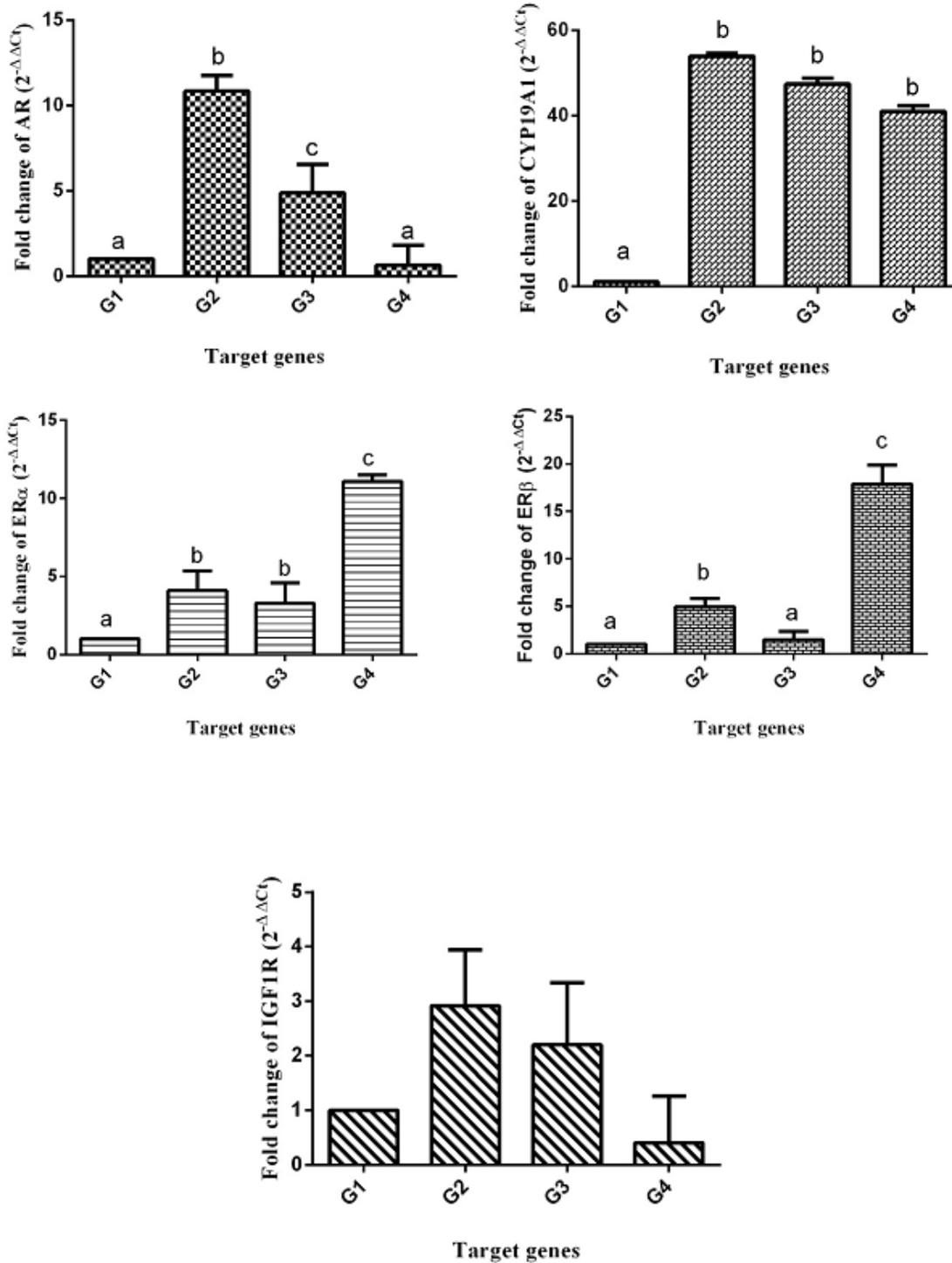
C. G3 (day 90)



D. G4 (day 150)

### Figure 3

Representative photomicrographs of testis of Tenyi-vo pig collected around peripubertal window. Note the apparent increase in the stratification of seminiferous tubules in G2-G4 with decrease in luminal diameter along with well-defined islets of interstitial spaces with Leydig cells between the cross sections of seminiferous tubules. (Magnification: 20X).



**Figure 4**

Relative-fold change ( $2^{-\Delta\Delta C_t}$ ) of transcripts in the testicular tissues of Tenyi-vo male pigs during peripubertal period. A. androgen receptor (AR), B. aromatase (CYP19A1), C. Estradiol receptor  $\alpha$  (ER $\alpha$ ), D. Estradiol receptor  $\beta$  (ER $\beta$ ), E. Insulin like growth factor-receptor (IGF1R). G1 served as control (calibrator group) for calculation of fold change and GAPDH was used as endogenous control. Statistical analysis was done with the  $\Delta C_t$  values. Error bar indicates standard error of difference (SED). SED = square root

$\{(Standard\ deviation\ of\ gene\ of\ interest^2/n1) + (Standard\ deviation\ of\ gene\ of\ reference^2/n2)\}$ , Where  $n1$  and  $n2$  are the number of observations. Bar with different superscript indicate significant difference at  $P<0.05$ .

## Supplementary Files

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- [FigS1.png](#)