

**TITLE:** Some endocrine traits of transgenic rabbits. I. Changes in plasma and milk hormones

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**SHORT TITLE:** blood and milk hormones in transgenic rabbits

**KEY WORDS:** transgenesis, factor VIII, progesterone, testosterone, estradiol, IGF-I, corticosterone

## SUMMARY

The aim of these studies was to compare some endocrine and non-endocrine characteristics of transgenic (carrying mammary gland-specific mWAP-hFVIII gene construct) and non-transgenic rabbits. The concentrations of corticosterone (C), progesterone (P<sub>4</sub>), testosterone (T), estradiol (E<sub>2</sub>), IGF-I and human factor VIII (hFVIII) in the blood plasma of adult females (9 months of age; 3rd generation transgenic animals), adult males, and young females (1-2 months of age; 4th generation of transgenic animals), as well as in the milk of lactating adult females, were analysed by using RIA. In addition, litter size and body mass of pups born by transgenic and non-transgenic females from the 3rd generation were compared. Transgenic animals were compared with their non-transgenic siblings (the same genetic and epigenetic background).

Transgenesis did not influence plasma hFVIII, but significantly increased C (in all animals), reduced IGF-I (in adult males and females), T and E<sub>2</sub> (in young females) and altered P<sub>4</sub> (increase in adult males and decrease in adult females) concentrations in blood plasma. In addition, transgenic females had higher milk concentrations of T, but not P<sub>4</sub> or IGF-I, than their non-transgenic sisters. These endocrine changes were not associated with changes in litter size. **Transgenic male (but not female) pups have lesser body mass, than control animals.** These observations demonstrate the influence of transgenesis *per se* on the animal endocrine system (secretion of reproductive and stress steroid hormones and growth factor) **and growth** over four generations.

## INTRODUCTION

Production of transgenic organisms plays a key role in modern biotechnology and in the pharmaceutical industry. Transgenesis is used mainly for the production of specific biologically active substances, which are accumulated in the cells or released into blood, milk or incubation media. Most publications on transgenic organisms deal with the quantity and quality of produced recombinant proteins, and not the effects of transgenesis *per se* on the physiological state of recipients. Both specific and non-specific action of transgenesis on target organs and physiological processes is reported (Lo et al., 1999; Davey and MacLean, 2006; Palmer et al., 2006). Nevertheless, despite their great importance, the consequences of introduction of foreign gene constructs on the physiological state, especially over several generations, have been insufficiently examined. Transgenic rabbits could be used not only as a source of animal products or recombinant proteins, but also as models for the study of the influence of foreign genes and transgenesis itself on living processes (Bősze et al., 2003). One such models could be transgenic rabbits in which the mammary gland produces human factor VIII (hFVIII), the anti-haemophilic A factor (Tuddenham et al., 1991). Rabbits with the mWAP-hFVIII gene, which is shown to be integrated into the majority of cells but which induces production of recombinant hFVIII only in mammary gland (Chrenek et al., 2005a, 2005b), have been produced and analysed in our Institute over four generations. It was observed that the founders transmitted mWAP-hFVIII to their offspring in a Mendelian fashion (Chrenek et al., 2005a). The presence of mWAP hFVIII increased the incidence of pathological changes in organs, blood leucocytes concentration and aneuploidy (Suvegova et al., 2004; Parkanyi et al., 2005), the appearance of new type bone tissue and increase in bone marrow cell aneuploidy (Martiniakova et al., 2005), and altered the mobility, concentration, osmolarity and viability of sperm, but did not influences its fertility (Chrenek et al., 2006). The effect of this gene on rabbit female reproductive traits has not been reported.

The endocrine system is responsible for the control of reproduction, adaptation and a number of other biological processes. In rabbits, as in other mammals, gonadal hormones could be both regulators and markers of the state of the reproductive system (Spies et al., 1997; Hillier, 2001). Adrenal corticosteroids play an important role in adaptation and stress. In rabbits, a large corticosterone surge is associated with stress and inhibition of reproduction (Viau, 2002; Brecchia et al, 2005) and growth (Aghajafari et al., 2002). Growth factors, especially insulin-like growth factor I (IGF-I), have not yet been described in rabbit blood. Nevertheless, in mammals including rabbits IGF-I is a known stimulator of growth and meat production (Oksbjerg et al, 2004), gonadal development, ovarian folliculogenesis, oocyte maturation (Fair, 2003; Mazerbourg et al., 2003), and ovarian cell proliferation and steroidogenesis (Makarevich et al, 2000). The association of mWAP-hFVIII with changes in the secretion of hormones and growth factors regulating reproduction and growth has not yet been studied.

The aim of the present work was to compare the secretion of hormones and a growth factor in transgenic (mWAP-hFVII) and non-transgenic rabbits in relation to their fertility and growth *in vivo*.

## MATERIAL AND METHODS

In our experiments we have try to examine:

- (1) the concentrations of gonadal steroid hormones (progestagen progesterone, P<sub>4</sub>, the androgen testosterone, T, the estrogen estradiol 17beta, E<sub>2</sub>), the 'stressor' steroid hormone corticosterone (C) and the growth factor IGF-I in the plasma of transgenic and non-transgenic adult rabbits,
- (2) the concentration of these molecules in the plasma of young, sexually immature transgenic and non-transgenic females,
- (3) the concentrations of P<sub>4</sub>, T and IGF-I in the milk of adult lactating transgenic and non-transgenic animals,
- (4) the litter size (number of pups) of transgenic and non-transgenic females,
- (5) the body mass of these pups (at 1 month of age).

### *Animals*

New Zealand White broiler line rabbits from the Slovak Agricultural Research Centre were used. The animals were housed in individual flat-deck wire cages, under a constant photoperiod of 14 h light per day. The temperature and humidity of the building were recorded continually by means of a thermograph positioned at the same level as the cages. The rabbits were fed *ad libitum* with a commercial diet and water was provided *ad libitum* from nipple drinkers. Rabbits were bred in the standard industrial conditions for intensive breeding of broiler rabbits.

Transgenic rabbit founders carrying a mammary gland specific construct consisting of a 2.5 kb murine whey acidic protein promoter (mWAP), 7.2 kb cDNA of the human clotting factor VIII (hFVIII), and 4.6 kb of 3' flanking sequences of mWAP gene provided by Dr. H. Lubon (American Red Cross, Maryland, USA) were used (Chrenek et al 2005a). To obtain the F<sub>3</sub> generation, transgenic females from the F<sub>2</sub> generation were bred with non-transgenic males. The F<sub>4</sub> generation was produced by breeding transgenic females no.1-3-5 with non-transgenic male (line I) and transgenic females no. 1-9-7 with transgenic male (line II). In our experiment we analysed both transgenic and non-transgenic offspring, each from the same litters from generation F<sub>3</sub> (adult sexually mature males and females 9 months of age) and F<sub>4</sub> (young, immature females, 1-2 months of age).

### *Detection of transgene*

Total DNA was isolated from the ear tissue of newborn rabbits and subjected to PCR analysis for the amplification of the hFVIII transgene as reported by Chrenek et al. (2005a), using primers hFVIII-F: 5'-GTA GAC AGC TGT CCA GAG GAA-3' and hFVIII-R: 5'-GAT CTG ATT TAG TTG GCC CAT C-3' which define a 578 bp region of human FVIII cDNA.

### *Animal treatment and sample collection*

Blood from young (2 month of age) transgenic or non-transgenic females and adult (9 month of age) transgenic or non-transgenic males from the same litter was collected 3 times at intervals of 2 days, always between 10.00 and 14.00. Blood from adult transgenic or non-

transgenic females from the same litter was collected 1 h after stimulation of superovulation by i.m. injection of pregnant mare serum gonadotropin (PMSG, Werfaser, Alvetra und WERFFT, Vienna, Austria, 20 IU/kg body mass). Blood was aspirated by syringe from the central ear vein, collected into heparinized tubes, centrifuged by  $\times 2000$  g, and the resultant plasma frozen at  $-80^{\circ}\text{C}$  to await RIA.

Milk samples (2-4 ml per sample) were taken from lactating transgenic and non-transgenic female rabbits on days 10, 20 and 30 of lactation and processed as reported previously (Chrenek et al., 2005a). In order to stimulate milk down, intramuscular injection of 5IU of oxytocin (Veyx Pharma, Germany) was applied 10 min before milk collection. The milk was immediately centrifuged at 5000 g for 10min, and the upper lipid layer was removed. The samples were stored at  $-80^{\circ}\text{C}$  until RIA.

### *Hormone analysis*

Concentrations of progesterone (P4), testosterone (T), estradiol (E2), corticosterone (C) and insulin-like growth factor I (IGF-I) in 20-50  $\mu\text{l}$  of blood plasma and milk was measured using RIA/IRMA kits from DSL (Webster, TX, USA) according to the manufacturer's instructions. The characteristics of the assay were described previously (Sirotkin et al., 1998; Makarevich et al., 2004). The concentration of rhFVIII in 200  $\mu\text{l}$  of plasma was determined by using an ELISA kit (Asserachrom FVIII:Ag) from Diagnostica Stago (Asniers-sur-Seine, France) according to the manufacturer's instructions. No cross-reactivity of mouse monoclonal antiserum against rhFVIII used with any infectious agents, rheumatoid and growth factors and hormones was detected.

### *Statistics*

Hormones were analysed in the blood of 18 adult animals (transgenic: 5 males and 3 females, non-transgenic: 6 males and 4 females) and of 18 young rabbits (transgenic: 4 males and 6 females, non-transgenic: 4 males and 4 females). Milk was collected from 3 transgenic and 3 non-transgenic females. Quantitative data were processed by standard statistical methods. Differences between the groups (transgenic and non-transgenic animals of the same age and sex) were determined by Duncan test by using the computer programmes SigmaStat and SigmaPlot 9.0. (Systat Software, Inc., Erkrath, Germany). Differences at  $P < 0.05$  were considered as significant.

## **RESULTS**

### *Concentration of hormones in plasma of adult transgenic and non-transgenic males and females*

RIA revealed the presence of substantial amounts of C, P<sub>4</sub>, T, E<sub>2</sub> and of low amounts of rhFVIII immunoreactivity in plasma of both transgenic and non-transgenic rabbits. Relatively high concentrations of IGF-I were detected in plasma of control, but not of transgenic animals.

Males had **lower** concentration of C than females. Transgenic rabbits, both males and females, had increased concentrations of plasma C compared with non-transgenic animals (Fig. 1a).

Analysis of IGF-I showed that transgenic males and females had extremely low plasma concentrations of this growth factor (Fig. 1b)

Analysis of gonadal steroid hormones revealed substantial sexual differences: females had more progestagen P<sub>4</sub> and estrogen E<sub>2</sub> and less androgen T in comparison to males. Transgenic males had enhanced plasma concentrations of P<sub>4</sub>, than non-transgenic animals of the same sex. Transgenic females however had reduced plasma P<sub>4</sub> concentration (Fig. 1c). No significant differences between transgenic and non-transgenic animals (either males or females) in plasma T (Fig. 1d) or E<sub>2</sub> (Fig. 1e) were observed.

No differences in plasma rhFVIII immunoreactivity were found between sexes and groups (transgenic and non-transgenic animals; Fig. 1f).

#### *Concentration of hormones in plasma of young transgenic and non-transgenic females*

The blood of young animals contained substantially less C, P<sub>4</sub> and E<sub>2</sub> and more IGF-I and T, than that of adult females (Fig. 2). Transgenic young females, like adult animals, had higher plasma concentrations of C than non-transgenic (Fig. 2a). The mean concentration of IGF-I in young transgenic animals was lower than in non-transgenic rabbits, but in the controls these differences were statistically insignificant due to large S.E.M. (Fig. 2b). Transgenic young females had significantly less T (Fig. 2d) and E<sub>2</sub> (Fig. 2e) in plasma than non-transgenic animals. No differences in plasma P<sub>4</sub> concentration between the groups were observed (Fig. 2c).

#### *Concentration of hormones in milk of transgenic and non-transgenic females*

Substantial amounts of IGF-I, P<sub>4</sub> and T were detected in rabbit milk (Fig. 3). No influence of transgenesis on milk concentration of IGF-I (Fig. 3a) and P<sub>4</sub> (Fig. 3 b) was detected, but the concentration of T in milk of transgenic animals was significantly higher than in controls (Fig. 3c).

#### *Some indices of fertility and growth in transgenic and non-transgenic animals*

Comparison of number of offspring born by transgenic and non-transgenic females from the 3rd generation of transgenes showed no differences in litter size between the groups (Fig. 4). Measurement of body mass of these offspring **showed reduction in this index in male, but not female transgenic pups** (Fig. 5).

## **DISCUSSION**

Our observations confirm previous reports of presence of C (Aghajafari et al., 2002; Viau, 2002; Brecchia et al., 2005) and of gonadal steroids and their sexual differences (Spies et al., 1997; Hillier, 2001) in rabbit plasma. This is the first report of the presence of IGF-I and hFVIII-like immunoreactivity in rabbit blood.

Increased amounts of C in the blood of both adult and young transgenic animals, suggest the higher expression of non-specific stress in transgenic rabbits. Increased C in these transgenic rabbits was shown to be associated with the occurrence of pathological changes in organs, bones and blood leucocytes, urea and aspartat-aminotransferase (Parkanyi et al., 2004; Suvegova et al., 2004; Martiniakova et al., 2005). All these observations suggest the existence

of some pathological processes or decreased adaptability of transgenic animals in comparison with their non-transgenic relatives. On the other hand, lack of differences in viability of transgenic and non-transgenic rabbits from the same litter described previously (Chrenek et al., 2005a) suggest that the observed changes in physiological and endocrine parameters did not alter the efficacy of adaptive mechanisms or the viability of transgenic animals.

In rabbits, high C concentration can suppress reproduction (Viau, 2002; Brecchia et al, 2005) and growth (Aghajafari et al., 2002). It is possible that the increased C observed in our studies could be responsible for altered mobility, concentration, osmolarity and viability of sperm described in these transgenic animals previously (Chrenek et al., 2006). Nevertheless, the observed changes in C seem not to affect fertility (litter size) or growth (body mass of pups).

Transgenesis in our experiments was associated with a dramatic decline in plasma IGF-I concentration. Since IGF-I may be a stimulator of growth, reproduction, ovarian secretory activity, adaptogene and anti-apoptotic factors (Makarevich et al, 2000; Fair, 2003; Mazerbourg et al., 2003; Oksbjerg et al, 2004), the suppression of some these processes in transgenic rabbits might be expected. On the other hand, transfection-induced overproduction of IGF-I by rabbit mammary gland did not influence their fertility (Zinovieva et al., 1998). In our experiments, reduced IGF-I in transgenic animals was associated with an increased C concentration in blood. These changes in hormone secretion, together with some signs of pathological changes reported previously (Suvegova et al, 2004), might indicate transgenesis-induced suppression of mechanisms controlling growth, adaptation or repair. **This hypothesis was confirmed by lower body mass in transgenic young males, which could be due to reduced plasma IGF-I level, but not to IGF-I received from maternal milk (where IGF-I, in contrast to plasma, was not reduced).** These observations confirm the previous report (Zinovieva et al., 1998) of lack of influence of milk IGF-I level on rabbit physiological and reproductive performance.

The differences in  $P_4$ , T and  $E_2$  in blood and milk between transgenic and control animals could indicate some transgenesis-associated changes in secretory activity of both male and female gonads. These differences could be responsible for changes in some gonadal steroid-regulated tissues: the bones (Martiniakova et al., 2005) and sperm (Chrenek et al., 2006) as described previously in these transgenic rabbits. Some of the observed changes in gonadal steroids could be due to changes in their regulators, IGF-I and C. Nevertheless, the observed transgenesis-induced alterations in plasma progesterone, androgen and estrogen concentration were not associated with significant changes in female fecundity (litter size).

Our observations confirm previous reports (Lo et al., 1999; Davey and MacLean, 2006; Palmer et al., 2006) of non-specific changes in transgenic animals seemingly not related to introduced gene. It provides however the first data on non-specific inherited effect of transgenesis on endocrine system and growth in rabbits. The present observations suggest that transgenesis in rabbits could be associated with substantial changes in endocrine system (plasma and milk concentration of adrenal and gonadal steroid hormones and growth factor), but not in fertility or growth.

The mechanisms whereby transgenesis affects endocrine traits remain unknown. The introduced WAP-hFVIII gene construct did not contain genes regulating substances other than hFVIII. Moreover, it promoted production of hFVIII only in mammary gland, but not in other tissues (Chrenek et al., 2005b). The fact that hFVIII is produced only in mammary gland and secretes only to milk but not to other tissues, was confirmed by absence of WAP-hFVIII gene expression in non-mammary tissues, accumulation of hFVIII in milk (Chrenek et al., 2005b) and by the low concentrations of hFVIII-like immunoreactivity in blood of both transgenic and non-transgenic animals observed in our experiments. Therefore, the observed differences

between transgenic and non-transgenic animals were not due to hFVIII introduced into somatic cells because hFVIII is absent in males and non-lactating females. Furthermore, these differences could not be an effect of hFVIII taken up from maternal milk because both transgenic and non-transgenic pups from the same litter, differences between which were described here, were fed with the same milk. Therefore, the observed differences appear to be due to changes induced by transgenesis itself and inherited over several generations. These unknown factors require further study due to the growing importance of transgenesis, the consequences of which on present and future generations should be known and taken into account. Furthermore, the differences in some endocrine and growth parameters observed in our experiments, should be taken into account by production of transgenic animals in future due to potential effect of these hormonal alterations on physiology, adaption, viability, growth and reproduction of transgenic animals in further generations..

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## LEGENDS TO FIGURES

Fig. 1. Concentration of hormones in blood plasma of adult male and female non-transgenic and transgenic rabbits. A-corticosterone, b – IGF-I, c – progesterone, d – testosterone, e – estradiol, f – hFVIII. Values are means  $\pm$  S.E.M. \* - significant ( $p < 0.05$ ) differences between control and transgenic animals of the same sex.

Fig. 2. Concentration of hormones in blood plasma of young female non-transgenic and transgenic rabbits. A-corticosterone, b – IGF-I, c – progesterone, d – testosterone, e – estradiol. Values are means  $\pm$  S.E.M. \* - significant ( $p < 0.05$ ) differences between control and transgenic animals.

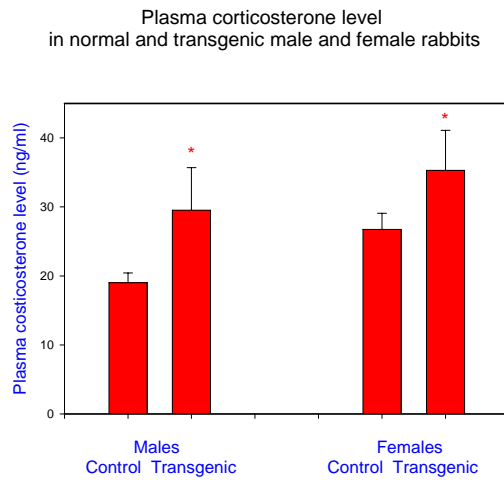
Fig. 3. Concentration of hormones in milk of non-transgenic and transgenic rabbits. A-IGF-I, b – progesterone, c – testosterone. Values are means  $\pm$  S.E.M. \* - significant ( $p < 0.05$ ) differences between control and transgenic animals.

Fig. 4. Litter size in non-transgenic and transgenic rabbits. Values are means  $\pm$  S.E.M.

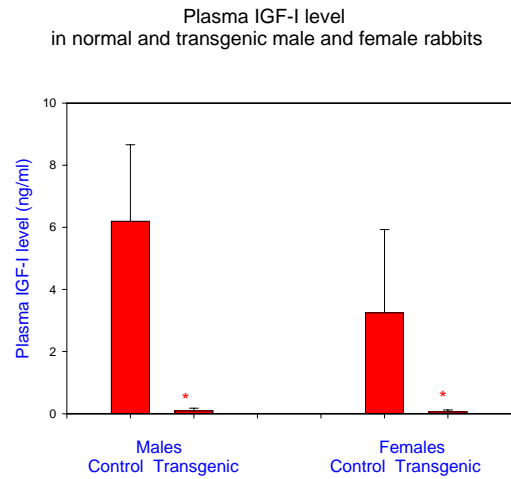
Fig. 5. Body mass of pups (1 month of age) of non-transgenic and transgenic male and female rabbits. Values are means  $\pm$  S.E.M.

Fig. 1. Hormones in plasma of adult males and females

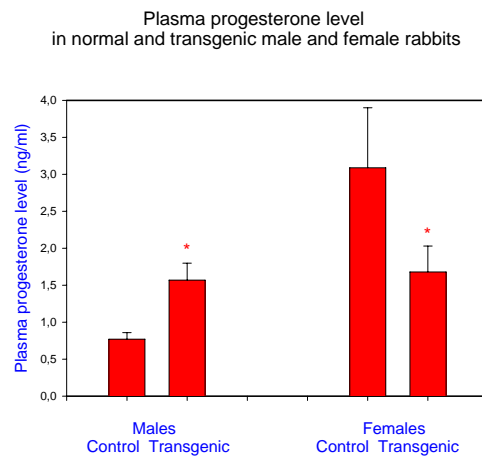
A.



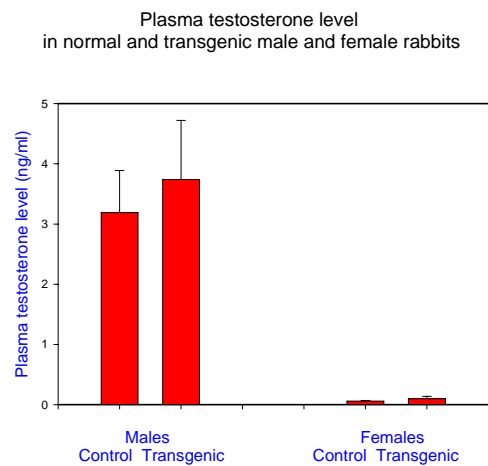
B.



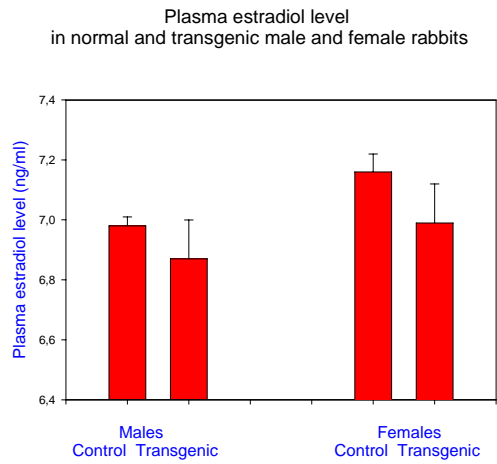
C.



D.



E.



F.

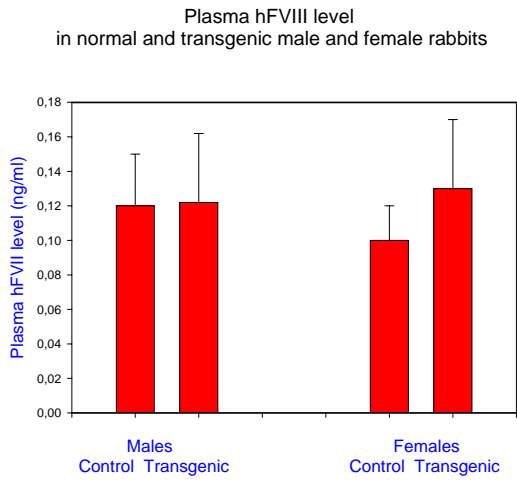
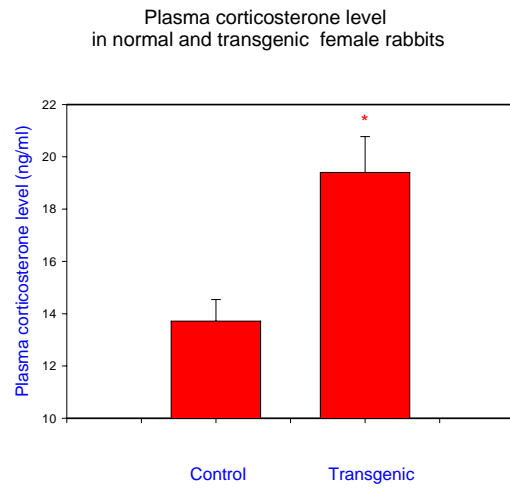
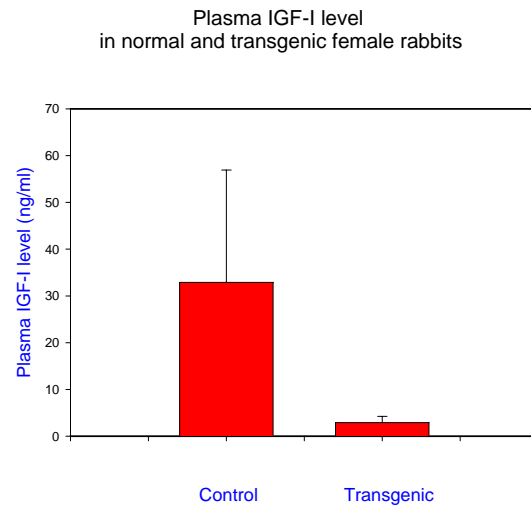


Fig. 2. Hormones in plasma of young females

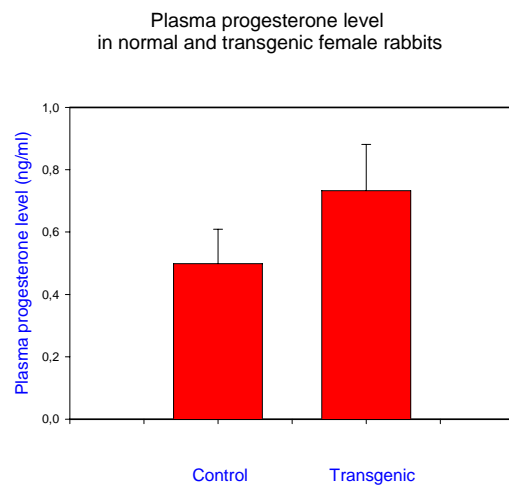
A.



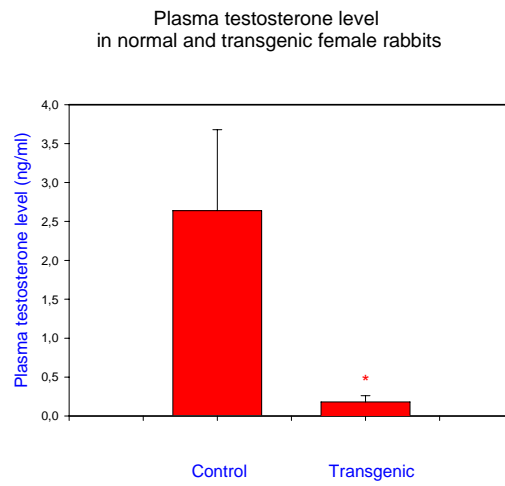
B.



C.



D.



E.

Plasma estradiol level  
in normal and transgenic female rabbits

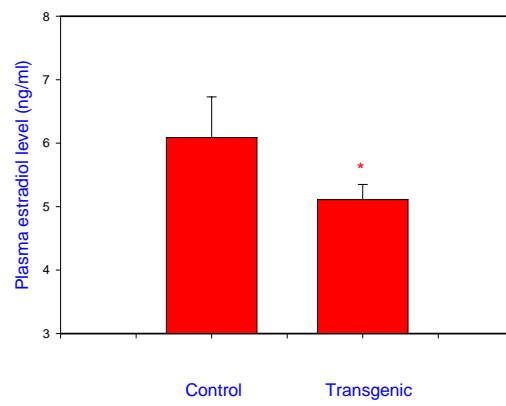
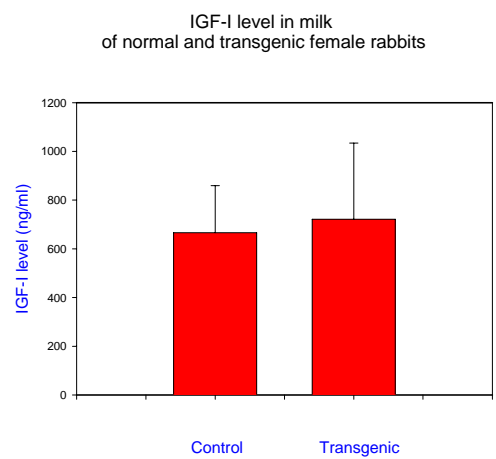
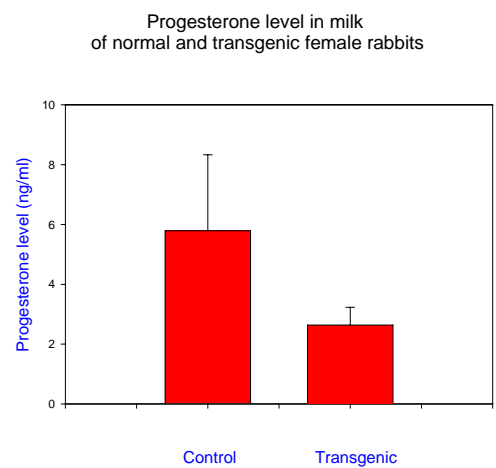


Fig. 3. Hormones in milk of adult females:

A.



B.



C.

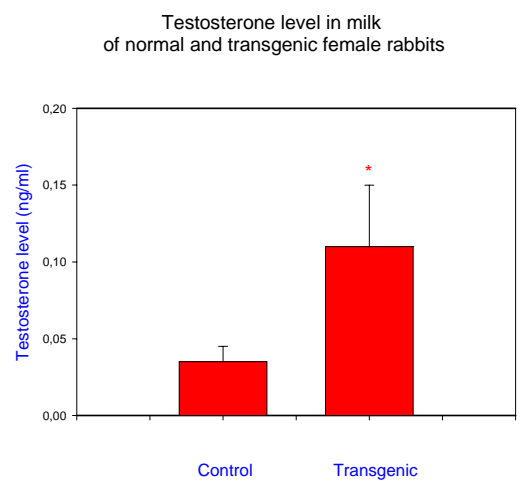


Fig. 4. Litter size

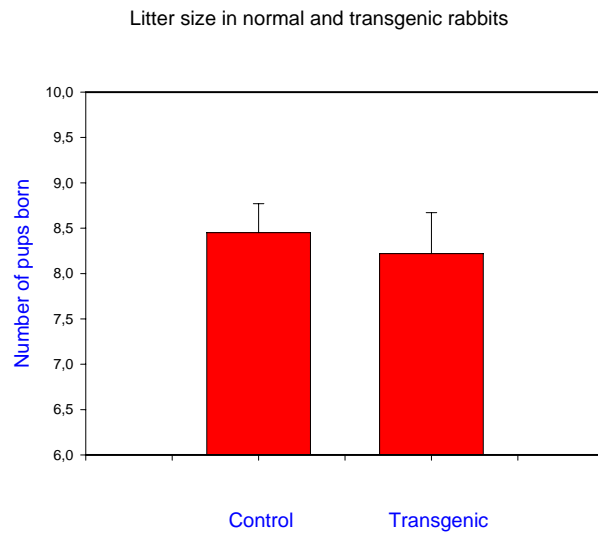


Fig. 5. Body mass

