

RESEARCH ARTICLE



Testosterone levels and the genetic variation of sex hormone-binding globulin gene of *Bubalus bubalis*, bulls in Egypt

SAMY NAEEM¹, AHMED GHONEIM^{2*} , GAMAL ABD-ALLAH² and OMAIMA HASSAN¹

¹Physiology and Hormones Department, Animal Health Research Institute, Agricultural Research Center, Cairo 12618, Egypt

²Faculty of Science, Department of Zoology, Damietta University, New Damietta P.O. 34517, Egypt

*For correspondence. E-mail: am_ghoneim@du.edu.eg.

Received 11 March 2017; revised 20 August 2017; accepted 24 August 2017; published online 15 March 2018

Abstract. Testosterone is the main plasma androgen produced and secreted by male testis. To be bioactive, it binds to sex hormone-binding globulin (SHBG), i.e. the major transporter protein of sex steroids in the blood of mammals and other vertebrate species. Firstly, this study aimed to determine the levels of testosterone in different-age groups of buffalo bulls (*Bubalus bubalis*) in Egypt, and secondly to screen the genetic polymorphisms in their SHBG gene and to investigate whether these polymorphisms are associated with the level of the circulating serum testosterone. Blood samples were collected from 67 Egyptian buffalo bulls representing four different age-groups and testosterone concentration was measured by radioimmunoassay. Selected DNA samples from different age-groups were sequenced for the full length of their SHBG gene. A significant increase in the concentration of serum testosterone in buffalo bulls was recorded with the advancement of age from 12/18 to 24/36 months. Based on the number of polymorphisms in SHBG gene, the current study reveals the presence of three bull genotypes, of which one is likely to be associated with low testosterone concentration, while other is likely to be associated with an increased testosterone concentration. Such genetic associations can provide a good tool for the selection of bull genotypes with higher testosterone concentrations, which are indispensable for breeding purposes.

Keywords. testosterone; radioimmunoassay; sex hormone-binding globulin; gene; mutations; *Bubalus bubalis*.

Introduction

Human male testosterone is the major circulating androgen and more than 95% of it is secreted by the testis, which produces ~ 6–7 mg per day (Coffey 1988). Testosterone is secreted in most vertebrates and it circulates in the blood, bound mainly to plasma proteins with only 1–2% being free, 40–50% being loosely bound to albumin and 50–60% being specifically and strongly bound to sex hormone-binding globulin (SHBG) (Dunn *et al.* 1981). SHBG is a plasma glycoprotein synthesized and secreted by the liver (Siiteri *et al.* 1982; Westphal 1986). Plasma SHBG is expressed in the testes of most mammals and is known as the testicular androgen-binding protein (ABP), which is synthesized and excreted by Sertoli cells (Joseph 1994). Plasma SHBG is a dimer of two essentially identical monomers and it binds only to one steroid molecule (Turner *et al.* 1984; Petra *et al.* 1986). SHBG is considered as the primary plasma transport

protein for sex hormones (Westphal 1986) and it binds to its ligands 5 α -dihydrotestosterone and testosterone with high affinity (Becchis *et al.* 1996). Plasma SHBG not only transports the steroids but also regulates the access of sex steroids to their target tissues (Siiteri *et al.* 1982; Westphal 1986).

A number of studies were directed to the sequence and cloning of ABP/SHBG gene (*abp/shbg*) of many different species (Joseph *et al.* 1988; Hammond *et al.* 1989; Ger-shagen *et al.* 1989; Ip *et al.* 2000). Sequence analysis of *shbg* showed that rabbit *shbg* spans 2.5 kb and the coding region is distributed over eight exons (Ip *et al.* 2000), whereas the same eight exons in the human gene span 3.2 kb (Hammond *et al.* 1989). The transcription unit encoding zebrafish *shbg* spans 12,945 bp and it contains eight exons of 81–189 bp intervened by intron sequences of 78–5108 bp in length (Miguel-Queralt *et al.* 2004). In rainbow trout, there are two SHBG genes, *shbga* and *shbgb*, the main significant distinction between them is the length

of introns, which are bigger in *shbgb* compared to other SHBG genes (Marivin et al. 2014). Like rainbow trout *shbga* and rainbow trout *shbgb* comprises eight exons, but is almost twice the length of *shbga* (Marivin et al. 2014).

Some studies have shown that the serum level of human testosterone is controlled by genetic factors (Ahn et al. 2009; Ohlsson et al. 2011; Sung and Song 2016). As per our knowledge, no studies were directed to the genetic variation in SHBG gene in *Bubalus bubalis*.

This study aimed first to determine the concentration of testosterone in different-age groups of buffalo bulls (*B. bubalis*) in Egypt, and second to sequence the SHBG gene of these buffalo bulls and determine whether the mutations, if any, had an impact on the testosterone levels in the buffalo's blood.

Materials and methods

Animals and blood sampling

This study was carried on 67 adult buffalo bulls of the species *B. bubalis* from different farms in Damietta and Kafr El-Sheikh governorates. Animals were subdivided into four age groups: 12, 18, 24 and 36 months.

Peripheral blood samples were collected in both EDTA-rinsed vacutainer tubes and tubes without anticoagulant by venipuncture from the jugular vein during the period, from October 2013 to March 2014. Blood samples were collected twice at 2 weeks interval between 08:00 and 11:00 am from all the animals. After 1-h incubation at room temperature, samples were centrifuged at 2500× g for 10 min, and the sera were collected, immediately frozen and kept at -20°C until testosterone assay by radioimmunoassay.

Radioimmunoassay

The concentration of plasma testosterone in each blood sample was determined by radioimmunoassay kit (Direct REF IM 1119, Immunotech, Czech Republic) according to the manufacturer's instructions. Samples or calibrators

were incubated with ¹²⁵I-labelled testosterone in antibody-coated tubes. The liquid content of tubes was then aspirated and the bound radioactivity was determined in a gamma counter. A standard curve was prepared, and the unknown values were obtained from the curve by interpolation.

PCR and DNA sequencing

Ten samples representing the four buffalo groups were selected based on the concentration of plasma SHBG and their DNA was extracted using Gene JET Genomic DNA Purification kit (#K0722, Thermo Scientific) according to the manufacturer's instructions. Extracted DNA was subjected to PCR to amplify SHBG gene using the oligonucleotides listed in table 1. Oligonucleotides were designed based on *B. bubalis* breed Mediterranean unplaced genomic scaffold, UMD_CASPUR_WB_2.0 scf7180021617181, whole genome shotgun sequence (NCBI ref. NW_005785061). All polymerase chain reactions (PCR) contained 20 µL of Maxima Hot Start PCR master mix kit (#K1051, Thermo Scientific), 3.2 µL of the forward and reverse primers and 3.84 µL of each DNA sample. Reactions were brought to a final volume of 40 µL with nuclease-free water and cycled for 4 min at 95°C for initial enzyme activation followed by 45 cycles (30 s at 95°C for denaturation, 30 s at the optimal annealing temperature and 1–2 min at 72°C for extension) and 10 min at 72°C for final extension. After visualization, PCR products were purified and sequenced using the oligonucleotides listed in table 1 in MacroGen sequencing services (MacroGen, Seoul, South Korea).

Statistical analysis

All data were processed by STATISTICA software ver. 10. The differences between testosterone concentration levels in the different age groups were analysed by one way ANOVA test and the mean values were considered significantly different when $P \leq 0.05$. P values were adjusted by Bonferroni correction. χ^2 test was used to assess the Hardy–Weinberg equilibrium.

Table 1. List of oligonucleotides used in PCR and DNA sequencing.

Name	Sequence (5' – 3')	Use
SH3_F	ACTTAAGCACACTGGTGGGG	Amplification, sequence
SH4_F	AAGACTCTCCTGCTCTGCAC	Sequence
SH4_R	AGCCCCAGCATAAACCAGTC	Amplification, sequence
SH5_F	GTCTATGCTCAAAGCGCACG	Amplification
SH8_F	GGGGAATAAACAGGGTGGGG	Amplification, sequence
SH9_F	AGCCTAGGAGCAATGGGAGA	Sequence
SH10_F	ACCATGGGAAACGGCTAAGG	Sequence
SH10_R	AGGCCACCATCTCTCTGTGA	Amplification
SH11_R	CATTCCCACCACCTCAGTCC	Amplification, sequence

Results

Serum testosterone concentration

Measurements of serum testosterone in the Egyptian buffalo bulls (figure 1) showed no statistically significant differences ($P = 1.0$) in testosterone concentration between the 12 and 18 months age buffalo bulls. Although the 24 months age buffalo bulls showed slightly higher serum testosterone concentration, there was no statistically significant difference ($P = 1.0$) between the 12 and 24 months age buffalo bulls. Nevertheless, the concentration of serum

testosterone differed significantly ($P = 0.000$) between the 12 and 36 months age buffalo bulls (figure 1).

Although the 24 months age buffalo bulls showed slightly higher serum testosterone concentration, there was no statistically significant difference ($P = 0.857$) between the 18 and 24 months age buffalo bulls. The concentration of testosterone, however, was significantly high in 36 months age buffalo bulls ($P = 0.001$) compared to 18 months age group. The concentration of serum testosterone was significantly higher ($P = 0.007$) in the 36 months age bulls compared to the 24 months age bulls (figure 1). Overall, there was a significant increase in the concentration of serum testosterone in buffalo bulls with the increase of age from 12/18 to 24/36 months of age.

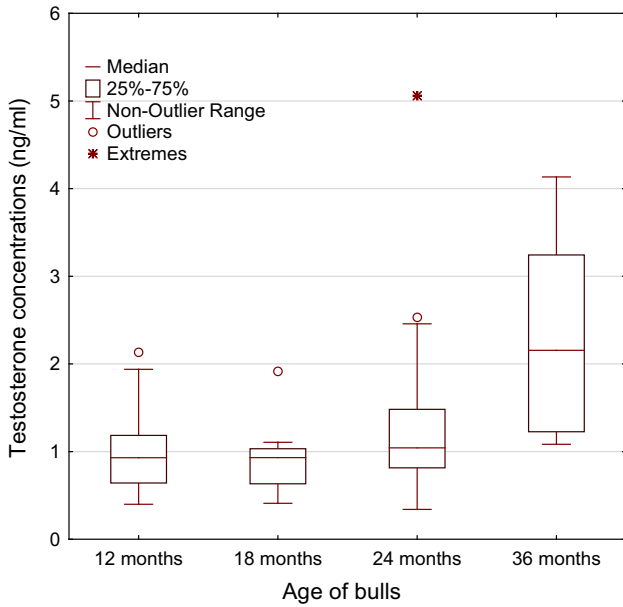


Figure 1. Plot box representing testosterone concentration (ng/mL) in buffalo bulls at 12, 18, 24 and 36 months of age.

Genetic variation of SHBG gene

DNA samples from 10 Egyptian buffalo bulls were sequenced for the full length of their SHBG gene and compared to the predicted *B. bubalis* SHBG gene sequences within the genomic scaffold NW_005785061. As shown in the nucleotide variance report (table 2), from 4 to 11 polymorphic sites were recorded in these 10 DNA samples (table 2). Based on variance of these samples, they can be categorized into three genotypes. One genotype contains four polymorphic sites; represented by bulls K4, K11, K32, K37 and K47. The second genotype contains nine polymorphic sites; represented by bulls D18 and D27. The third genotype contains 11 polymorphic sites; represented by bulls K3, K6 and K43. The sequences of these genotypes are deposited in the GenBank under the accession numbers KY653957 (genotype 1), KY653958 (genotype 2) and KY653959 (genotype 3). Based on the predicted transcript variant X1 (NSCBI ref. XM_006062863) of SHBG gene, the recorded polymorphisms result in two amino acid substitutions (S315L and R337G) in genotype

Table 2. Nucleotide variance report of *shbg*.

Reference	Buffalo bulls									
	K11	K3	K32	K37	K4	K43	K47	K6	D18	D27
36	G	R			R		R	R	R	R
2648	C	Y			Y		Y	Y		
2720	G								R	R
2787	A	R			R		R	R	R	R
2844	A	R			R		R	R	R	R
2850	A	R			R		R	R		
3076	C	Y			Y		Y	Y	Y	Y
3089	C	T	Y	T	T	Y	T	Y	Y	Y
3093	G	A	R	A	A	R	A	R	R	R
3154	A	G	G	G	G	G	G	G	R	R
3401	A		W			W		W		
3472	A	G	G	G	G	G	G	G	R	R
Total	4	11	4	4	4	11	4	11	9	9

R, A or G; Y, C or T; W, A or T; M, A or C.

Table 3. The relation between testosterone concentration and different genotypes of buffalo bulls.

Bull Age	K3 12 Mon.	K4 12 Mon.	K6 12 Mon.	K11 12 Mon.	D18 12 Mon.	K32 24 Mon.	K37 24 Mon.	D27 24 Mon.	K43 36 Mon.	K47 36 Mon.
Mean of testosterone concentrations (ng/mL)*	0.996	1.012	0.947	1.017	1.027	1.252	1.334	1.343	2.092	2.431
Testosterone concentrations (ng/mL)	1.001	0.702	1.938	0.609	0.415	2.532	0.555	0.341	4.134	1.083
Genotype	3	1	3	1	2	1	1	2	3	1

*Mean of testosterone concentration does not include the sample under comparison, e.g. when the sample K3 is compared, the mean of testosterone concentration refers to the mean of all samples except K3.

1, two amino acid substitutions (S315L/S and R337G/R) in genotype 2 and three amino acid substitutions (P297S/P, S315L and R337G) in genotype 3.

Compared to the mean of testosterone concentration in the other buffalo bulls within the same age group (table 3), the first genotype (bulls K4, K11, K32, K37 and K47) does not seem to be associated with a distinctive testosterone level. The second genotype (bulls D18 and D27) is likely to be associated with a decreased concentration of testosterone; however, the third genotype is likely to be associated with an increased testosterone concentration. An attempt to assess whether any of the 12 polymorphisms within the second genotype or the third genotype deviates from Hardy–Weinberg equilibrium revealed that each individual polymorphism is still with equilibrium ($P = 0.514–0.659$).

Discussion

In this study, comparatively a low concentration of serum testosterone was recorded in 12 and 18 months age buffalo bulls. A significant increase in testosterone concentration was recorded with the advancement of age to 24–36 months of age. These results are in accordance with some previous reports. According to Hemeida *et al.* (1985), serum testosterone of Egyptian buffalo bulls started to increase in 8–9 months of age and at 13–15 months of age and sharply increased in 17–19 months of age. In Pakistani Nili-Ravi buffalo bulls, testosterone levels were moderately low in 5–21 months of age then, a significant rise was seen from 21 to 26 months (Ahmad *et al.* 1984). Testosterone levels at 25 and 38 months of age were significantly different in relation to each other and were significantly different from the testosterone levels at 5–21 months of age (Ahmad *et al.* 1984). According to the same research group, there were no differences in mean plasma testosterone values measured in Nili-Ravi buffalo bulls at 5, 15,

17 and 21 months of age. According to Ahmad *et al.* (1989), plasma testosterone concentrations of Nili-Ravi buffalo bulls remained low, but variable between neonates to 12 months of age and markedly rose at 14 months of age, followed by a second peak at 18 months of age. Levels of plasma testosterone of Indian buffalo males were low and in the same range as in the 15 days–6 months of age, but could be estimated in almost all blood samples up to 15 months of age (Sharma *et al.* 1984). Testosterone concentration started rising at 15–18 months of age, followed by a remarkable increase at 24–30 months of age and the elevated concentration of testosterone was kept up to 42 months followed by sharp increments up to 48 months of age (Sharma *et al.* 1984). Testosterone concentrations in Murrah buffalo males in India showed low levels in ages from 1 to 24 months, followed by an increase in testosterone levels at 3.5–4 years of age (Gulia *et al.* 2010).

The above studies support the correlation between testosterone concentration and the advancement of age as a result of the development and functional differentiation of the testis (Sharma *et al.* 1984; Gulia *et al.* 2010; He *et al.* 2014). The slight variations among these reports could be related either to the differences in the species under study or some environmental and behavioural factors.

Based on the nucleotide variance in the full length of SHBG gene reported here, the current study suggests the presence of three genotypes in the studied bulls. NCBI database released five predicted transcript variants (X1–X5), the largest of which is the variant X1 (NSCBI ref. XM_006062863). Based on this transcript variant, the first genotype produces two amino acid substitutions (S315L and R337G) and also, the second genotype produces two amino acid substitutions (S315L/S and R337G/R), while the third genotype produces three amino acid substitutions (P297S/P, S315L and R337G). The second genotype is likely to be associated with a decreased concentration of testosterone, while the third genotype is likely to be associated with an increased testosterone concentration. None

of the polymorphisms within the second or third genotype deviated from Hardy–Weinberg equilibrium. Keeping in mind the small sample size in this study, it is worthy to quote that agreement with Hardy–Weinberg proportions is not a guarantee that observed variation has a genetic basis, nor does it guarantee that other factors such as selection and drift are not influencing genotypic frequencies (Waple 2015). The Hardy–Weinberg equilibrium test generally has low power to detect actual departures unless sample sizes are large (Haiman *et al.* 2005; Waple 2015) and allele frequencies in small sample sizes could have no meaning (B-Rao 2001).

So far, there are no reports about the genetic variation in SHBG gene in *B. bubalis* bulls; however, several studies on SHBG in human and other animals correlated circulating testosterone levels to some polymorphisms in SHBG gene. The circulating human SHBG and sex-steroid hormone levels were generally thought to be affected by genetic factors (Ahn *et al.* 2009; Ohlsson *et al.* 2011; Sung and Song 2016).

The SHBG polymorphism rs1799941, located 67-bp upstream from the transcription start site, predicted circulating testosterone levels and was associated with increasing testosterone levels in human males (Ahn *et al.* 2009). Ohlsson *et al.* (2011) have demonstrated that men with GG genotype for the polymorphism rs12150660, located 11.5-kb upstream of the transcription start site of the human SHBG gene, have lower serum testosterone concentrations than those with TT genotype. Serum human SHBG levels decreased significantly with increasing copies of the minor (T) allele for the polymorphism rs727428, located 1.1 kb beyond the 3' end of the SHBG gene (Wickham *et al.* 2011).

Testosterone concentration was not only associated with polymorphisms in the 5' and 3' UTR, but also with polymorphisms in the coding sequence. Men with the CT genotype for the polymorphism rs6258, located in exon 4 of human SHBG, had lower serum testosterone concentrations than those with CC genotype (Ohlsson *et al.* 2011). This single-nucleotide polymorphism was reported to result in the production of SHBG variant with reduced affinity for testosterone thus explaining the low serum testosterone concentrations and the reduced action at the target tissue cells (Ohlsson *et al.* 2011).

The polymorphism rs6259, located within exon 8 of the human SHBG gene, results in the substitution of D327N in the SHBG polypeptide sequence (Power *et al.* 1992). Pang *et al.* (2014) reported that the rs6259 variant allele (AG or AA) carriers had increased serum levels of human SHBG and total testosterone than the wild-type allele (GG). An association between both the N327 allele and the (TAAAA)₆ allele and human SHBG and sex-steroid serum concentrations was reported (Vanbillemont *et al.* 2009). Middle-aged men, carriers of the N327 allele were found to have higher SHBG and testosterone levels (Vanbillemont *et al.* 2009). Carriers of the six-repeat-allele were found to have higher SHBG, testosterone and free testosterone

levels (Vanbillemont *et al.* 2009), however, genotypes with long alleles ((TAAAA)_n repeat) were thought to be associated with lower SHBG levels and higher free testosterone and 17 β -estradiol levels than those with shorter alleles and the variant N allele Safarinejad *et al.* (2011). The increased production of human SHBG caused by genetic variations in the SHBG gene was thought to result in an increase in the levels of inactive, bound sex-steroids such as testosterone and a decrease in the concentration of active, unbound, or free sex-steroids, such as free testosterone Safarinejad *et al.* (2011). Hogeveen *et al.* (2001) found that a 46-kD nuclear factor preferentially binds this six-repeat allele and can alter transcriptional activity together with downstream elements of the SHBG promoter region.

SHBG levels increased with increasing copies of the minor (A) allele for the polymorphism rs1799941, located in exon 1L within the 5' untranslated region of the gene (Wickham *et al.* 2011). The relationship between rs1799941 genotype and serum SHBG suggested a dominant role for this splice variant, in the control of circulating SHBG levels (Wickham *et al.* 2011).

Tsung-Sheng and Hammond (2014) found that the G195E substitution in human SHBG caused a general reduction in steroid-binding affinity. This mutant was secreted at very low levels and was thought to disturb the normal spatial configuration of the 2 LG domains with respect to each other and there by influencing both the steroid-binding activity and the processing of N-linked oligosaccharides attached to sites within the C-terminal LG domain (Tsong-Sheng and Hammond 2014). Another human SHBG mutant (T48I) was associated with a 2-fold reduction in steroid-binding affinity due to impairment in its dimerization, and a consequent more extensive glycosylation than the wild-type SHBG (Tsong-Sheng and Hammond 2014).

Tsung-Sheng and Hammond (2014) found that the rate of dihydrotestosterone association with the SHBGR123H mutant is normal, whereas its dissociation rate is abnormally fast, and therefore they concluded that amino acid substitutions at the dimer interface exert long-range allosteric effects that influence how specific ligands are accommodated within the steroid-binding pockets of each subunit.

Based on the above reports, the fluctuation in testosterone concentrations in Egyptian buffalo bulls in the current study may be due to a possible alteration in the binding affinity of SHBG for testosterone, or testosterone bioavailability or a change in SHBG transcription level. The present study provides an initial screening for the SHBG variants in the Egyptian buffalo bulls. Further studies on a larger number of samples are required to confirm the association between these mutations and the concentration of serum testosterone, which will provide a good tool for the selection of bull genotypes with higher testosterone concentrations of great importance for breeding purposes.

Acknowledgements

We thank Dr Laila N. Eid, Buffalo Breeding Research Department, Animal Production Research Institute, Agriculture Research Center, Egypt, for her sincere help during sample collection.

References

- Ahmad M., Latif M., Ahmad M., Qazi M. H., Sahir N. and Arslan M. 1984 Age-related changes in body weight, scrotal size and plasma testosterone levels buffalo bulls (*Bubalus bubalis*). *Theriogenology* **22**, 651–656.
- Ahmad N., Shahab M., Khurshid S. and Arslan M. 1989 Pubertal development in the male buffalo: longitudinal analysis of body growth, testicular size and serum profiles of testosterone and oestradiol. *Anim. Reprod. Sci.* **19**, 161–170.
- Ahn J., Schumacher F. R., Berndt S. I., Pfeiffer R., Albanes D., Andriole G. L. et al. 2009 Quantitative trait loci predicting circulating sex steroid hormones in men from the NCI-Breast and Prostate Cancer Cohort Consortium (BPC3). *Hum. Mol. Genet.* **18**, 3749–3757.
- Becchis M., Sullivan P. M., Ordronneau P., Petrusz P. and Joseph D. R. 1996 Distribution of immunoreactive androgen-binding protein/sex hormone-binding globulin in tissues of the fetal rat. *Steroids* **61**, 392–400.
- B-Rao C. 2001 Sample size considerations in genetic polymorphism studies. *Hum. Hered.* **52**, 191–200.
- Coffey D. S. 1988 Androgen action and the sex accessory tissues. In *The physiology of reproduction* (ed. E. Knobil and J. Neill), pp. 1081–1119. Raven Press, New York.
- Dunn J. F., Nisula B. C. and Rodbard D. 1981 Transport of steroid hormones-binding of 21 endogenous steroids to both testosterone binding globulin and corticosteroid-binding globulin in human plasma. *J. Clin. Endocrinol. Metabol.* **53**, 58–68.
- Gershagen S., Lundwall A. and Ferlund P. 1989 Characterization of the human sex hormone binding globulin (SHBG) gene and demonstration of two transcripts in both liver and testis. *Nucleic Acids Res.* **17**, 9245–9258.
- Gulia S., Sarkar M., Kumar V., Meyer H. H. D. and Prakash B. S. 2010 Divergent development of testosterone secretion in male zebu (*Bos indicus*) and crossbred cattle (*Bos indicus* x *Bos taurus*) and buffaloes (*Bubalus bubalis*) during growth. *Trop. Anim. Health Prod.* **42**, 1143–1148.
- Haiman C. A., Riley S. E., Freedman M. L., Setiawan V. W., Conti D. V. and Le Marchand L. 2005 Common genetic variation in the sex steroid hormone-binding globulin (SHBG) gene and circulating SHBG levels among postmenopausal women: the multiethnic cohort. *J. Clin. Endocrinol. Metab.* **90**, 2198–2204.
- Hammond G. L., Underhill D. A., Rykx H. M. and Smith C. L. 1989 The human sex hormone-binding globulin gene contains exons for androgen-binding protein and two other testicular messenger RNAs. *Mol. Endocrinol.* **3**, 1869–1876.
- He Z. X., Huang M. F., Wang A. K., Zhang J. C., Zhao G., Yuan X. et al. 2014 Patterns of plasma hormone concentrations in mithun bulls under a semi-management. *J. Anim. Vet. Adv.* **13**, 732–739.
- Hemeida N. A., El-Baghdady Y. R. and El-Fadaly M. A. 1985 Serum profiles of androstenedione, testosterone and LH from birth through puberty in buffalo bull calves. *J. Reprod. Fret.* **74**, 311–346.
- Hogveen K. N., Talikka M. and Hammond G. L. 2001 Human sex hormone-binding globulin promoter activity is influenced by a (TAAAA)n repeat element within an *Alu* sequence. *J. Biol. Chem.* **276**, 36383–36390.
- Ip Y. C., Lee W. M. and Hammond G. L. 2000 The rabbit sex hormone-binding globulin gene: structural organization and characterization of its 5' flanking region. *Endocrinology* **141**, 1356–1365.
- Joseph D. R. 1994 Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. *Vitam. Horm.* **49**, 197–280.
- Joseph D. R., Hall S. H., Conti M. and French F. S. 1988 The gene structure of rat androgen-binding protein: identification of potential regulatory deoxyribonucleic acid elements of a follicle-stimulating hormone-regulated protein. *Mol. Endocrinol.* **2**, 3–13.
- Marivin E., Yano A., Guerin A., Nguyen T. V., Fostier A., Bobe J. and Guiguen Y. 2014 Sex hormone-binding globulins characterization and gonadal gene expression during sex differentiation in the rainbow trout, *Oncorhynchus mykiss*. *Mol. Reprod. Dev.* **81**, 757–765.
- Miguel-Queralt S., Knowlton M., Avvacumov G. V., Al-Nouno R., Kelly G. M. and Hammond G. L. 2004 Molecular and functional characterization of sex hormone binding globulin in zebrafish. *Endocrinology* **145**, 5221–5230.
- Ohlsson C., Wallaschofski H., Lunetta K. L., Stolk L., Perry J. R., Koster A. et al. 2011 Genetic determinants of serum testosterone concentrations in men. *PLoS Genet.* **7**, e1002313.
- Pang X. N., Yuan Y., Sun Y., Shen J. P., Zha X. Y. and Hu Y. 2014 The relationship of sex hormone-binding globulin (SHBG) gene polymorphisms with serum SHBG level and metabolic syndrome in Chinese Han males. *Aging Clin. Exp. Res.* **26**, 583–589.
- Petra P. H., Namkung P. C., Titani K. and Walsh K. A. 1986 Characterization of the plasma sex steroid-binding protein. In *Binding proteins of steroid hormones* (ed. M. G. Forest and M. Pugeat). John Libbey, London.
- Power S. G., Bocchinfuso W. P., Pallesen M., Warmels-Rodenhiser S., Van Baelen H. and Hammond G. L. 1992 Molecular analyses of a human sex hormone-binding globulin variant: evidence for an additional carbohydrate chain. *J. Clin. Endocrinol. Metab.* **75**, 1066–1070.
- Safarinejad M. R., Shafiei N. and Safarinejad S. 2011 Association of the (TAAAA)n repeat and Asp327Asn polymorphisms in the sex hormone-binding globulin (SHBG) gene with idiopathic male infertility and relation to serum SHBG concentrations. *J. Steroid Biochem. Mol. Biol.* **123**, 37–45.
- Sharma I. J., Agarwal S. P. and Dwaraknath P. K. 1984 Changes in profiles of serum sex steroids of male buffaloes from birth to maturity. *Theriogenology* **22**, 175–186.
- Siiteri P. K., Murai J. T., Hammond G. L., Nisker J. A., Raymoure W. J. and Kuhn R. W. 1982 The serum transport of steroid hormones. *Recent Prog. Horm. Res.* **38**, 457–510.
- Sung J. and Song M. I. 2016 Genetic effects on serum testosterone and sex hormone-binding globulin in men: a Korean twin and family study. *Asian J. Androl.* **18**, 786–790.
- Tsung-Sheng W. U. and Hammond G. L. 2014 Naturally occurring mutants inform SHBG structure and function. *Mol. Endocrinol.* **28**, 1026–1038.
- Turner E. E., Ross J. B. A., Namkung P. C. and Petra P. H. 1984 Purification and characterization of the sex steroid binding protein from macaque serum. Comparison with the human protein. *Biochemistry* **23**, 492–497.
- Vanbillemont G., Bogaert V., Bacquer D., Lapauw B., Goemaere S., Toye K. et al. 2009 Polymorphisms of the SHBG gene contribute to the interindividual variation of sex steroid hormone blood levels in young, middle-aged and elderly men. *Clin. Endocrinol.* **70**, 303–310.

- Waple R. S. 2015 Testing for Hardy–Weinberg proportions: have we lost the plot? *J. Hered.* **106**, 1–19.
- Westphal U. 1986 Steroid–protein interactions II. *Monogr Endocrinol.* **27**, 1–603.
- Wickham E. P. III., Ewens K. G., Legro R. S., Dunaif A., Nestler J. E. and Strauss, J. F. III. 2011 Polymorphisms in the SHBG gene influence serum SHBG levels in women with polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.* **96**, E719–E727.

Corresponding editor: INDRAJIT NANDA