JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2018, 69, 5, 747-753
www.jpp.krakow.pl | DOI: 10.26402/jpp.2018.5.09

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LOCAL REGULATORS OF SEASONAL REPRODUCTION PROCESSES IN UTERUS MASCULINUS OF AN ADULT MALE EUROPEAN BISON (*BISON BONASUS*, LINNAEUS 1758)

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Growth factors, hypoxia-inducible factor 1-alpha (HIF-1α) and klotho protein all have very important functions in the male reproduction; however their role in the regulation of seasonal reproductive processes in the male European bison remains unclear. Similarly, although the *uterus masculinus* is very frequently found in the bison, its importance and functions remain unknown. It is likely that, this organ may have secretory functions and thus be a target for various regulatory factors. Therefore, the aim of this study was to investigate expression and activity of several factors: vascular endothelial growth factor (VEGF-A), fibroblast growth factor (FGF-2), transforming growth factor β1 (TGF-β1), nerve growth factor (NGF), insulin-like growth factor receptor (IGF-IR β), hypoxia-inducible factor 1 alpha (HIF-1α), and klotho protein in the *uterus masculinus*, immediately after the season of the reproductive activity (November and December). Our study reveals that the growth factor expression levels are significantly higher in November, when compared to December, while expression of HIF-1α and klotho was higher in December. These results provide novel data on differences in the expression levels of several factors in the *uterus maculinus* of European bison bulls after the breeding season. The described factors may, therefore, be potent regulators of the seasonal reproduction.

Key words: *growth factors, hypoxia-inducible factor 1-alpha, klotho, uterus masculinus, seasonal reproduction, European bison, vascular endothelial growth factor, nerve growth factor*

INTRODUCTION

European bison is the largest wild land mammal in Europe, still at a risk of extinction and included in the Red List of Threatened Species (1966). Despite the upward trend in the bison population size, they still face numerous threats, with problems related to their reproduction belonging to the most important issues. Observed pathological changes in the reproductive system gave rise to concerns for the further fate of these animals. The most frequently registered problems include hypoplasia, asymmetry, and testicular atrophy (1). The European bison are the seasonal breeders, with a short rutting season at the turn of August and September (2), but spermatogenesis needs to begin earlier to reach the higher level, and then the body begins to prepare for the 'reproductive silence' (3). Normal testis function requires endocrine regulation based on the hypothalamic-pituitary-gonadal axis, and a local control by autocrine/paracrine factors (4). Normal mechanisms of proliferation and spermatogenesis are crucial for production and development of fertile male reproductive cells. These mechanisms are regulated by androgens, of which testosterone is the most important. Furthermore, various growth factors are involved in paracrine/autocrine regulation (5). The observed steroid-influenced changes in growth factors expression suggest that steroid hormones strictly determine the way in which the growth factor influences both genomic and non-genomic pathways (6, 7). Growth factors are secreted by a number of cells and are crucial for control of cellular proliferation (8). Transforming growth factor β (TGF-β1) (9), nerve growth factor (NGF) (10), insulin-like growth factor (IGF-1) (11), fibroblast growth factor (FGF-2) (12), and vascular endothelial growth factor (VEGF-A) (4) may have an effect on reproductive organs, affecting quality of germ cells in testes and epididymides. Additionally, hypoxia-inducible factor 1-alpha (HIF-1α) is a very important transcription factor involved in cell adaptation to hypoxia conditions and which also may contribute to cell survival (13). Klotho protein, on the other hand, is an important factor in control of spermatogenesis, so it may be critical for normal functioning of male reproductive cells and, indirectly, also for their development, possibly in the bison, too (14). Correct cooperation of all elements of the reproductive system results in the production of fertile sperm. In European bison bulls, the *uterus masculinus* frequently forms a part of the reproductive system. This organ was found in various animal species, such as hamster, horse and donkey, Canadian beaver, cat and dog, but as well as in humans (15).

Uterus masculinus functions remain unknown, but its presence in European bison males suggests a significant regulatory importance in the reproductive system. This organ can be a site of production, secretion, and, eventually, action of various regulatory factors. The aim of our study was to verify

presence and expression of the above-mentioned growth factors, HIF-1α and klotho protein, in *uterus masculinus* tissues of Polish European bison bulls immediately after the breeding season.

MATERIAL AND METHODS

Animals and tissues

Uterus masculinus tissues of European bison (*Bison bonasus*, Linnaeus 1758) bulls were collected from mature adult males (500 – 620 kg body mass) during November (n = 6) and December (n = 6) (time after the breeding season, falling in September and October). Although both months belong to the period post the intense reproductive activity, processes occurring in reproductive tissues may change intensively due to gradual inhibition of spermatogenesis. As the bison population is covered by increased protection, elimination of bison and thus access to *uterus masculinus* tissues are strictly limited. However, it will be a priority in further seasonal analyzes. Animals were culled 'on the spine' (a shot from a firearm in the area of 2 – 3 cervical vertebrae causes defragmentation of the spinal cord, the animal completely loses consciousness and does not feel any pain, next, circulation is stopped and death occurs) without the use of anesthetics, during selective eliminations in the Bialowieza National Park and Bialowieza Forest (northeaster Poland), according to the approved guidelines for ethical treatment of animals in accordance with Polish legal requirements. The individuals for the analyses were carefully selected, and mechanical injury to the limbs was adopted as the enrolment criterion. For mRNA and protein analyzes, tissues were collected from the *uterus masculines* (20 mg) containing all tissue layers: endometrium, myometrium and adventitia. The obtained tissues were shock-frozen in liquid nitrogen (–196ºC) and stored at –80ºC, until further analyzes.

Real time polymerase chain reaction

RNA was isolated from *uterus masculinus* tissues using a column-based kit according to the manufacturer protocol (11-100, A&A Biotechnology, Poland). One µg of the total RNA was reverse-transcribed into cDNA using the Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer protocol. Bovine TaqMan gene expression assays for VEGF-A (Bt03213282; Entrez Gene ID 281572), FGF-2 (Bt03259205; Entrez Gene ID 281161) and β-actin (Bt03279174; Entrez Gene ID 280979) were performed using the StepOne Plus system (Applied Biosystems, USA) and protocols provided by the supplier. β-actin was used as the reference gene for normalization, and mRNA abundance was quantified using the $2^{-\Delta\Delta CT}$ method (16).

Polymerase chain reaction

cDNA samples used for RT-PCR analyzes were the same as used for the real-time polymerase chain reaction (PCR). Received cDNAs were amplified using primers specific to *TGF-β1*, *NGF* and *β-actin* (*Table 1*). The reactions were conducted as follows: 5 µl of 2 × PCR TaqNova-RED Master Mix (DNA Gdansk; Gdansk, Poland), 4 µl of primers (2 µl of 1 µM forward and reverse primer each) (Genomed; Warsaw, Poland) and 1 µl of cDNA (10 ng). Amplification of PCR included 35 cycles of denaturing (95ºC for 45 s), annealing (51ºC for *TGF-β1*, 49ºC for *NGF*, 55ºC for *β-actin*) and extension (72ºC for 45 s), and finally extension (72ºC for 10 min). In 2% agarose gel with ethidium bromide, PCR reaction products were visualized by electrophoresis. Using the GelQuantNET software, the relative density was calculated and the results were normalized to *β-actin*.

Western blot

The *uterus masculinus* tissues homogenized in 2% SDS were centrifuged at 15,000 g at 4ºC for 15 min. Protein concentrations were determined using the BCA protein assay according to the manufacturer protocol (Thermo Scientific, USA). Bovine serum albumin (BSA) was used as a calibration standard. 30 µg of proteins were separated by SDS-PAGE and electroblotted onto the PVDF membrane (Thermo Scientific, USA). Membranes were blocked at RT for 1 hour in 1% BSA for VEGF-A, FGF-2, IGF-IRβ, HIF-1α and 3% BSA for klotho protein in TBST (20 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1% Tween 20). Then they were incubated overnight in one of the primary antibodies: mouse monoclonal anti-VEGF-A, 1:1000 (#MA1-16626, Thermo Scientific, USA), mouse monoclonal anti-FGF-2, 1:1000 (#MA1-24682, Thermo Scientific, USA), rabbit polyclonal anti-IGF-IRβ, 1:500 (#sc-9038, Santa Cruz, USA), rabbit polyclonal anti-klotho, 1:1000 (#PA5-21078, Thermo Scientific, USA), goat polyclonal anti-HIF-1α,1:200 (sc-12542, Santa Cruz, USA), or rabbit polyclonal anti-ACTB, 1:10,000 (#PA1-16889, Thermo Scientific, USA) prepared in 1% BSA in TBST. Then membranes were carefully rinsed (4×) with TBST for 5 min and next incubated in RT with secondary anti-rabbit or anti-mouse antibody 1:80,000 in 1% BSA in TBST (#A0545 and #A9044, Sigma, respectively) for 1 hour. The ECL Western Blotting Kit (BioRad) and the Fusion Fx7 system (Viber Lourant) were used to visualize the immunocomplexes. The protein levels were calculated from the measured band densities. The obtained results were normalized to ACTB (GelQuantNET Software).

Statistical analysis

Data was presented as a mean ± SD. A statistical analysis was conducted using GraphPad Prism 6.0 with the unpaired t-test for

Table 1. Primer sequences for *TGF-β1*, *NGF* and *β-actin* (ACTB) genes used in PCR.

Gene	**Primer sequence**	**Product length (bp)**	**T** (ºC)
TGF-β1	Forward: 5'-GGACACCAACTACTGCTTCA-3' Reverse: 5'-ACCTTCACCTAAGTGCTTGG-3'	99	51
NGF	Forward: 5'-CTGGGAGAGGTGAACATCAAC-3' Reverse: 5'-TGTGGAAGCAGTTCCGC-3'	115	49
ACTB	Forward: 5'-CATCGGCAATGAGCGGTTCC -3' Reverse: 5'-CCGTGTTGGCGTAGAGGTCC -3'	147	55

comparisons between two independent groups (n = 6) in November and in December. P value of < 0.05 was considered statistically significant, and statistical significances are shown as *P < 0.05, **P < 0.01, and ***P < 0.001.

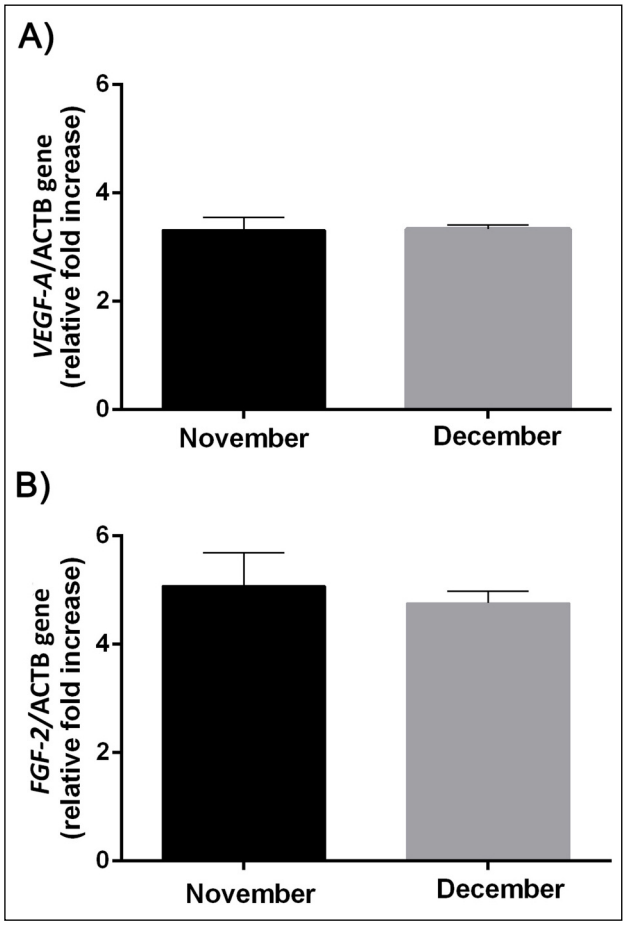

Fig. 1. Real time PCR analysis of: *A*) *VEGF-A*, *B*) *FGF-2* mRNA expression in the *uterus masculinus* of European bison. Bars indicate SD; n = 6; ***P < 0.001, **P < 0.01, *P < 0.05; no indication - no statistical significance (P > 0.05). The results were normalized to *β-actin*.

RESULTS

Growth factor mRNA expression in uterus masculinus of the European bison male

The analysis of VEGF-A mRNA expression in *uterus masculinus* showed no significant differences between November and December (P > 0.05) (*Fig. 1A*). Similarly, no differences were found in *FGF-2* mRNA expression for the analyzed months (P > 0.05) (*Fig. 1B*).

The *TGF-β1* mRNA expression level was significantly higher in November than in December, by 2.032 times (P < 0.05) (*Fig. 2A*). Furthermore, there was no statistically significant difference in *NGF* gene expression between November and December (P > 0.05), although its value in December was clearly higher than in November (*Fig. 2B*).

Growth factors, hypoxia-inducible factor 1-alpha and klotho expression in uterus masculinus of the European bison

Similarly as in the case of mRNA VEGF-A expression, measurements of the protein levels revealed no variations between the analyzed months (P > 0.05) (*Fig. 3A*). Similarly, the relative optical density of FGF-2 protein synthesis in *uterus masculinus* of the European bison did not show any differences between November and December (P > 0.05) (*Fig. 3B*). However, the expression of IGF-IRβ was 3.599 times higher in November when compared to December (P < 0.01) (*Fig. 3C*). As shown in *Fig. 3D*, the quantitative analysis of HIF-1α protein revealed its significant increase in December (3.168-fold increase) (P < 0.05).

The relative optical density of klotho revealed that its level was higher in December versus November, for both membrane and secreted forms (P < 0.01) (2.318- and 1.685-fold increases, respectively) (*Fig. 4*).

DISCUSSION

In the European bison duration of its reproductive season is clearly marked and limited (2). According to our knowledge, this

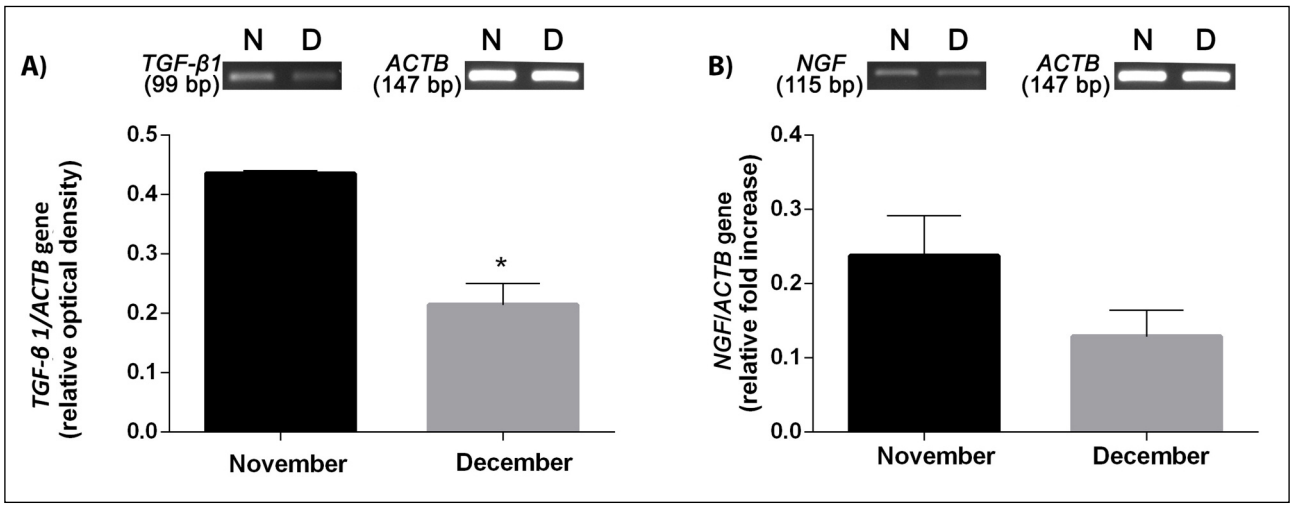

Fig. 2. PCR technique analysis of: *A*) *TGF-β1* and *B*) *NGF* mRNA expression in the *uterus masculinus* of European bison. Bars indicate SD; n = 6; ***P < 0.001, **P < 0.01, *P < 0.05; no indication - no statistical significance (P > 0.05); N, November; D, December. The results were normalized to *β-actin*.

is the first study showing the expression of mRNA and protein levels for different factors: TGF-β1, NGF, IGF-IRβ, FGF-2, VEGF-A, HIF-1α and klotho in *uterus masculinus* of the European bison after the breeding season. In this paper, we demonstrated the stable levels of FGF-2 and VEGF-A in November and December. As it has been previously proven, VEGF is an important factor responsible for both neovascularization and vascular densities, phenomena directly affecting the seasonality of the reproduction (2, 17). The seasonal changes in *VEGF* in roe deer testes have been already linked to testicular involution and recrudescence (4). However, authors do not report any differences in *VEGF* mRNA expression at other seasons, and this could explain the lack of detectable differences in European bison *uterus masculinus* between November and December as observed in our study (4). Furthermore, another theory suggests that the increase in angiogenesis is caused by a decrease in production and secretion of anti-angiogenic factors, including angiostatin, endostatin, thrombospondins, and the platelet factor (18). Additionally, increased expression of VEGF stimulating testosterone secretion from Leydig cells may indirectly regulate the male reproductive function (19). Perhaps similar mechanisms may be found in the European bison. Supposedly, a higher level of VEGF in *uterus masculinus* during and after the reproductive season may stimulate proliferation of reproductive cells in a paracrine manner. Initiation of testosterone production and secretion by Leydig cells in the bison testes may also be a result of paracrine regulation. For *NGF*, our results show that there were no differences in the level of its expression between analyzed months. According to our assumptions, NGF may be not needed in the post-reproductive season. November and December are months when sperm cells are not produced. However, it is possible that NGF affects the quality of bison semen during the breeding season. Because this neurotrophin is produced in the reproductive tract specifically by the accessory glands, it may indirectly affect quality of the produced semen in the

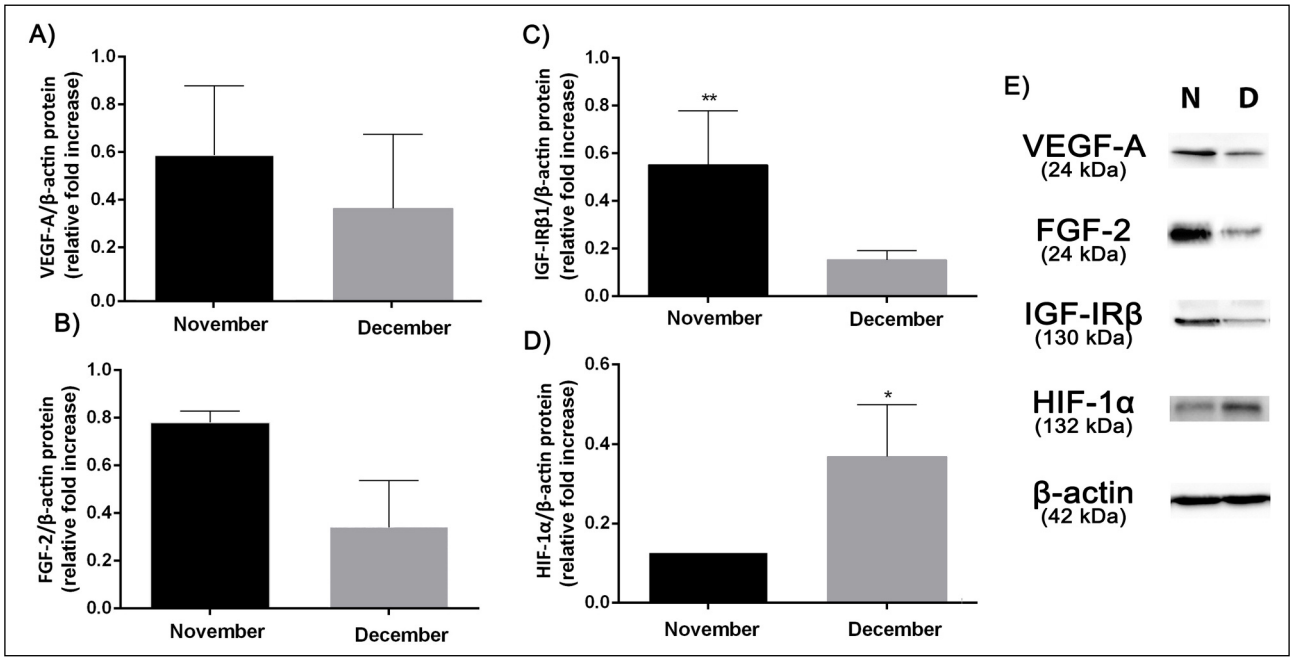

Fig. 3. Western blot analysis of: *A*) VEGF-A, *B*) FGF-2, *C*) IGF-IRβ1, *D*) HIF-1α, *E*) representative immunoblots in *uterus masculinus* of European bison. Bars indicate SD; n = 6; ***P < 0.001, **P < 0.01, *P < 0.05; no indication - no statistical significance (P > 0.05); N, November; D, December.

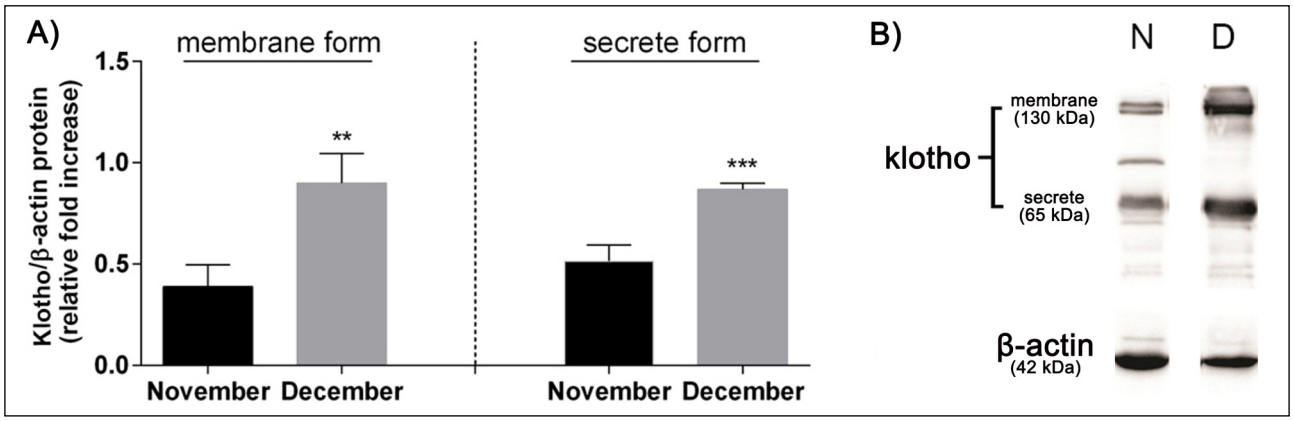

Fig. 4. Western blot analysis of klotho protein and representative immunoblots in *uterus masculinus* of European bison.; n = 6; ***P < 0.001, **P < 0.01, *P < 0.05; no indication - no statistical significance (P > 0.05); N, November; D, December.

paracrine/autocrine pathway, controlling motility, acrosome reaction, necrosis and apoptosis, and thus indirectly affecting male infertility (20-23). In this study, upregulated expression of mRNA *TGFβ1* in the bison *uterus masculinus* was observed in November, in the period after the intensive production of reproductive cells. The decline in its expression in December suggests that *uterus masculinus* may be a source of *TGFβ1* for reproductive cells and its unknown functions in the breeding season. Additionally, the demand for this growth factor during this season may be higher in general. The high levels of testicular TGFβ1 molecules in male roe deer throughout the year suggest that they may influence overall regulation of reproductive processes throughout the year, and additionally have an effect on spermatogenesis during the breeding season. In male deer, an increased expression of TGFβ1 was observed in the period when semen production was at its highest levels (June/August) (24). The changes in growth factor expression may result from the fact that development of germ cells is variable, but their unknown functions in such specific sites as the *uterus masculinus* should also be considered.

On the other hand, the insulin family of growth factors and IGF-IRβ, also being of interest to us, consists of small single-chain mitogenic polypeptides transmitting signals crucial for growth control and metabolism but, also for the reproductive functions. IGF 1 is a potent regulator of reproductive functions and its action is mainly affected by IGF-IR (25). This growth factor is responsible for regulation of spermatogenesis (26). Both testosterone and estradiol are necessary to maintain the process of spermatogenesis, and a positive effect on estradiol and testosterone production and secretion was observed after IGF 1 administration (26, 27). In our study, the protein expression of IGF-IRβ in *uterus masculinus* of the bison was significantly higher in November than in December. This downregulated trend suggests its reduced demand after the reproductive season. Similarly, in males of white-tailed deer, the level of IGF 1 falls after the breeding season (27), while in captive male reindeer, the IGF 1 levels do not decrease, and this is probably associated with supplemental feed (28). Continuing, all types of cells are sensitive to reactive oxygen species (ROS), and the reproductive cells in particular (29). HIF-1α is the transcription factor enabling adaptation to hypoxic conditions (30). In contrast, the presence of HIF-1α was also demonstrated in testes and epididymides of various animal species in normoxic conditions (31). In our analyzes, the level of HIF-1α protein expression in *uterus masculinus* was much higher in December than in November. In roe deer testes and epididymides the stable low levels of *HIF-1α* mRNA expression were found during the pre-rut, rut and post-rut seasons, whereas during the reproductive season, the level of HIF-1α protein expression was high (31). Our results indicating the increase in the levels of HIF-1α protein expression after the reproductive season suggest a protective effect against free radicals, which may appear after the intense biochemical processes. The high ROS levels produced during the intense biochemical processes, associated with the reproductive activity, may require activity of the HIF-1α protein in *uterus masculinus* to maintain the proper oxygen environment after the reproductive season.

Klotho protein occurs in two forms: membrane and secrete, and each of them has a different function. The membrane form is a co-receptor for fibroblast growth factor (FGF-23) responsible for phosphate homeostasis and regulation of vitamin D metabolism (32, 33). The secrete form is a humoral factor and determines activity of ion channels, membrane transporters and receptors for growth factors. Klotho also contributes to maintenance of calcium (Ca^{2+}) homeostasis and inhibition of the insulin/insulin-like growth factor (IGF 1) pathway (34-36). Our results suggest that expression of klotho protein in *uterus masculinus* of the European bisons differs significantly between November and December for membrane and secrete forms alike. In both cases, much higher protein expression was found in December. Current literature reports that klotho can regulate IGF 1 levels (36, 37). Our results also show such dependence. It was observed that expression of klotho protein in *uterus masculinus* of adult bison, especially secrete form, is significantly higher in December, when compared to November, while the trends for the IGF-IRβ protein expression were opposite. The obtained protein levels confirm the theory of an inhibitive klotho effect on the insulin/insulin-like growth factor (IGF 1) pathway (35). A relationship between klotho and the reproductive system found may imply that it is highly probable that klotho regulates the normal function of this system (14). The above assumption was confirmed by the results obtained by Miranda *et al.*, where klotho-deficient mice were characterized by disturbed gonadotropin regulation (38). Additionally, it has been proven that klotho protein can act as an enzyme in the steroid hormone synthesis pathway (39). Therefore, klotho protein can act as humoral or as hormonal factor, as required by target organs (40).

Very different experimental observations *in vivo* and *in vitro*, shed more insight into understanding of the role played by various physiological, pathophysiological factors and mechanisms in control and regulation of gonadal activity, the seasonal reproduction, pregnancy and lactation. The results obtained in this study seem to be of great importance as the regulators of the seasonal activity of the male reproductive system, similarly to other factors *e.g.* Rho and Rho-associated kinases in pregnant uterus (16), adiponectin in steroidogenesis (41) or the impact of specific maternal high-fat diet on offspring (42).

Taken together, presented findings of this study demonstrate differences suggesting seasonal variation in growth factors, HIF-1α and klotho protein in *uterus masculinus* of the European bison. Nevertheless, further analyses of these tissues at different times of a year are required to fully understand the influence of a season. Unfortunately, the importance of *uterus masculinus* is poorly understood, but the seasonal changes in various factors found in this organ imply its important regulatory function in the reproductive processes. However, it would be very desirable to determine in further studies the exact regulatory mechanisms underlying the seasonal reproduction.

Authors contribution: A. Tabecka-Lonczynska performed the experiments, carried out data interpretation, wrote the paper, conceived and designed the experiments; J. Mytych performed the experiments, carried out data interpretation; P. Solek analyzed the data; A. Abrachamowicz performed the experiments; M. Welz carried out data interpretation; M. Koziorowski contributed materials, carried out data interpretation. All authors read and approved the final version of this manuscript.

Acknowledgments: The authors are grateful to the Management and Personnel of the Bialowieza National Park for their assistance in collecting the materials.

Conflict of interest: None declared.

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Received: August 10, 2018
Accepted: October 30, 2018

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