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Influence of anabolic combinations of an androgen plus an estrogen on biochemical pathways in bovine uterine endometrium and ovary

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ABSTRACT

The application of anabolic steroids in food producing animals is forbidden in the EU since 1988, but the abuse of such drugs is a potential problem. The existing test systems are based on known compounds and can be eluded by newly emerging substances. The examination of physiological effects of anabolic hormones on different tissues to indirectly detect misuse might overcome this problem.

Two studies were conducted with post-pubertal 24-months old Nguni heifers and pre-pubertal female 2–4 weeks old Holstein Friesian calves, respectively. The animals of the accordant treatment groups were administered combinations of estrogenic and androgenic compounds. The measurement of the gene expression pattern was undertaken with RT-qPCR. Target genes of different functional groups (receptors, angiogenesis, steroid synthesis, proliferation, apoptosis, nutrient metabolism and others) have been quantified.

Several biochemical pathways were shown to be influenced by anabolic treatment. Both studies identified significant regulations in steroid and growth factor receptors (AR, ERβ, LHR, FSHR, Flt-1, PR, IGF-1R, Alk-6), angiogenic and tissue remodeling factors (VEGFs, FGFs, BMPs, ANGPT-2, MMPs, TIMP-2, CTSB), steroid synthesis (S5A1, HSD17, CYP19A1), proliferation (TNFα, IGF-1, IGFBPs, p53, c-fos; CEBPD, c-kit), apoptosis (CASP3, FasL, p53) and others (C7, INHA, STAR). Several genes were regulated to opposite directions in post-pubertal compared to pre-pubertal animals. PCA for Nguni heifers demonstrated a distinct separation between the control and the treatment group.

In conclusion, anabolics modify hormone sensitivity and steroid synthesis, and they induce proliferative effects in the whole reproductive tract (uterus and ovary) as well as anti-angiogenic effects in the ovary. However, the extent will depend on the developmental stage of the animals.

1. Introduction

The use of anabolic agents in animal husbandry is very profitable as treated animals are growing faster and exhibit a higher amount of lean meat due to nitrogen retention and a decrease in total body fat [1]. In some countries, e.g. USA and Canada, the utilisation of these substances is common and widely spread. In Europe, as the health risk for consumers caused by the hormone residues can hardly be estimated, the application of anabolic steroids is not admitted. Nevertheless, their misuse is a potential problem and new and sensitive detection methods have to be established to trace upcoming unknown compounds. One auspicious approach to indirectly uncover the illegal use of growth promoting substances might be by monitoring the different expression levels of genes participating in biochemical pathways, which are known to be influenced by anabolic steroids hormones. The actual study focused on two major reproductive organs (uterine endometrium and ovary) as these organs are primary hormone-dependent tissues. Additionally, few is known about the influences of anabolic steroids on these tissues [2] and the physiological changes induced by growth promoting substances have never been examined in bovine ovary by now. The following target genes have been examined in the actual work due to their involvement in metabolic processes regulated by steroid hormones.

The steroid hormone receptors androgen receptor (AR), estrogen receptors (ERα, ERβ), progestin receptor (PR), glucocorticoid receptor (GR) as well as growth hormone receptor (GHR) and IGF-1 receptor (IGF-1R), which are important for steroid hormone signaling, have been analyzed. Luteinizing hormone receptor (LHR) and follicle stimulating hormone receptor (FSHR) were also investigated, as these are implicated in the negative feedback mechanism of reproductive hormones. The angiogenic vascular endothelial

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growth factors (VEGFs) and their receptors (FLK-1, Flt-1), fibroblast growth factors (FGF-1, -2, -7), thrombospondin (THBS), matrix metalloproteinases (MMP-2, MMP-23B) and their inhibitor (TIMP-2), and angiopoietins (ANGPT-1, ANGPT-2) have been analyzed because angiogenesis is a process naturally occurring in reproductive tissues and is regulated by steroid hormones [3,4]. For the most part, steroid synthesis in the female organism takes place in ovary. This process is physiologically regulated by a negative feedback and though could be influenced by the application of exogenous reproductive hormones. Different key enzymes of steroids synthesis, namely Cytochrome P450 enzymes (CYP11A1, CYP19A1), hydroxysteroid–dehydrogenases (HSD3B1, HSD17B1, HSD17B3, HSD17B8, HSD17B11) and 5α-reductase (S5A1) have been examined [5–7]. The application of anabolic steroids has been correlated to cancer [8]. The development of cancer is associated with the overexpression of so called proto-oncogenes, which affect the regulation of cell growth and differentiation. For this study, the tumour suppressor gene p53 (p53) as well as the transcription factors c-fos, c-jun and c-myc, which are postulated as downstream targets of ER signaling, have been measured [9,10]. Due to the trophic effect of anabolics, apoptosis and proliferation are interesting functional pathways to investigate. Insulin-like growth factors (IGF-1, IGF-2) and their regulators IGFBP-2 and IGFBP-3 [11,12], growth differentiation factor 9 (GDF-9), bone morphogenetic factors (BMP-2, BMP-4, BMP-15) and the receptors mediating their action (Alk-5, -6, BMPRII) [13], transforming growth factor β (TGFβ), tumour necrosis factor α (TNFα), cyclin D2 (CYD2) [8], the negative regulator Prohibitin (PHB) and the transcription factors CCAAT/enhancer binding protein β and δ (CEBPB; CEBPD) represent factors are implicated in proliferative events [10]. The apoptosis group consists of anti-apoptotic members of the Bcl-family (bcl-2, bcl-xl) as well as caspases (CASP3, CASP8), Fas receptor (FAS) and Fas ligand (FASL), which are important mediators of cell death [14]. Glycolysis is an important pathway occurring in every somatic cell. The degradation of glucose conduces to the supply of biological energy for different physiological processes. Hexokinase (HK) and lactatdehydrogenase (LDH), which are key enzymes of glycolysis, have been sorted to the energy metabolism group. Concerning the development of a higher proportion of lean meat in animals treated with anabolic steroids all genes associated with protein turnover are of note. Therefore, cathepsin B (CTSB), cathepsin L (CTSL) and calpastatin (CAST) have been analyzed [15]. Several other factors, which are correlated to anabolic steroid hormone action, like steroidogenic acute regulatory protein (STAR), Lactotransferrin (LTF), complement components (C3, C7), cyclooxygenase 2 (COX-2), Smad2 and Inhibin A (INHA) were investigated [16–20].

Aim of the study was to find differentially regulated metabolic pathways in bovine reproductive tract and to compare the transcriptional response of post- and pre-pubertal animals. Using appropriate biostatistical methods, it should be attempted to find gene expression patterns, which could be used as possible biomarkers for anabolic treatment in cattle [21].

2. Materials and methods

2.1. Experimental design

In the first study ("South Africa study"), 18 healthy, post-pubertal, non-pregnant, 24-months old Nguni heifers were divided into a control and a treatment group of nine animals each. The animal attendance was done by the Onderstepoort Veterinary Institute (Onderstepoort, Pretoria South Africa). The treatment group was implanted with Revalor H (Intervet, Spartan, South Africa), an anabolic preparation licensed in South Africa and containing 140 mg trenbolone acetate (TBA) plus 20 mg estradiol-17β (E2) according to the manufacturer's instructions into the middle third of the pinna of the ear and was treated for 42 days until slaughter.

For the second animal study ("pour on anabolics study"), 20 pre-pubertal, female Holstein Friesian calves were randomly assigned to four groups of five animals each. Group 1 remained untreated and served as control. Animals of group 3 and group 4 were treated once or three times in weekly intervals, respectively, with a hormone mix containing 25 mg estradiol benzoate (Sigma–Aldrich, Zwijndrecht, The Netherlands), 60 mg testosterone decanoate (Sigma–Aldrich) and 60 mg testosterone cypionate (Sigma–Aldrich). The hormone mix was applied in two different ways: per intra muscular injection (one animal per group) or via pour on treatment (four animals per group). For pour on treatment, animals were shaved on the back from neck to tail and 10 mL of the hormone mix were rubbed onto the skin. Four substances served as carrier solvents for the pour on treatment to ensure the transit of the hormone mix from the skin into the organism: Ivomec (Spruyt Hillen, IJsselstein, The Netherlands), dimethyl sulfoxide (DMSO) (Spruyt Hillen), Miglyol 840 (Spruyt Hillen) and diethylen glycol monobutyl ether (DEGMBE) (Spruyt Hillen). For injection, Arachide oil (Spruyt Hillen) was used. Group 2 received only the carrier substances without the hormone mix three times in weekly intervals to serve as a carrier control group. Animals were slaughtered 92 days after the beginning of the experiment. Animal attendance was done according to practice and the treatment protocol has been approved by the ethical committee of the "Regierung von Oberbayern" (Upper Bavaria, Germany).

At slaughter, uterus and ovary samples were collected. Tissues were conserved in RNAlater (Applied Biosystems, Darmstadt, Germany) immediately after the removal and further stored at −80 °C.

2.2. Extraction

Samples were extracted with the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA concentration was measured after extraction using the NanoDrop (peqLab Biotechnologie GmbH, Erlangen, Germany). $OD_{260/230}$ and $OD_{260/280}$ ratios were checked considering sample purity.

2.3. Analysis of RNA integrity

Intactness of sample material is crucial for qPCR experiment [22], so control of RNA integrity was performed with the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) using Eukaryotic Total RNA Nano Assay (Agilent Technologies). The RNA integrity number (RIN) served as RNA integrity parameter. The RIN is calculated based on a numbering system from 1 to 10, with 1 being most degraded and 10 being most intact [23].

2.4. Primer design

Primer pairs (Table 1) were either newly designed using published bovine nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) or previously established primer sequences were used [2,24,25]. Newly designed primers were ordered and synthesized at Eurofins MWG (Ebersberg, Germany).

2.5. Reverse transcription

Per sample, 500 ng (100 ng/μL) of total RNA have been converted to cDNA using M-MLV H^- reverse transcriptase (Promega, Regensburg, Germany). The mastermix for the reverse transcription

Table 1
Primer sequences of target genes measured in uterine endometrium (U) and ovary of Nguni heifers (O_H) and in ovary of Holstein Friesian calves (O_C) with the accordant annealing temperatures (T_M), product length (bp) and accession numbers; primers without accession numbers have been obtained from coworkers.

Gene		Sequence [5′ → 3′]	T_M	Product length [bp]	Accession no.	
UBQ	for	AGA TCC AGG ATA AGG GAA GGC AT	60 °C	198	Z18245	U, O_H, O_C
	rev	GCT CCA CCT CCA GGG TGA T				
H3	for	ACT GCT ACA AAA GCC GCT C	60 °C	233	NM_001014389	U, O_H, O_C
	rev	ACT TGC CTC CTG CAA AGC AC				
AR	for	CCT GGT TTT CAA TGA GTA ACC GCA TG	60 °C	172	AY862875	U, O_H, O_C
	rev	TTG ATT TTT CAG CCC ATC CAC TGG A				
ERα	for	AGG GAA GCT CCT ATT TGC TCC	60 °C	234	AF177936	U, O_H, O_C
	rev	CGG TGG ATG TGG TCC TTC TCT				
ERβ	for	GAG ATA TTC TTT GTG TTG GAG TTT	60 °C	164	NM_174051	U, O_H, O_C
	rev	CTT CGT GGA GCT CAG CCT GT				
PR	for	ACC AGC CCT ATC TCA ACT ACC	60 °C	186	XM_583951.4	U, O_H, O_C
	rev	TAT GCT GTC CTT CCA TTG CCC				
LHR	for	CAG TGT GCT CCT GAA CCA GA	60 °C	192	NM_174381	O_H, O_C
	rev	GTC TGC AAA GGA GAG GTT GC				
FSHR	for	AGTTGCCCTTTTTCCCATCTTTGG	64 °C	150	NM_174061.1	O_H, O_C
	rev	TAGCAGCCACAGATGACCACAA				
GHR	for	CCA GTT TCC ATG GTT CTT AAT TAT	60 °C	136	NM176608	U
	rev	TTC CTT TAA TCT TTG GAA CTG G				
GRα	for	TTC GAA AAA ACT GCC CAG C	62 °C	194	AY238475	U, O_H
	rev	CAG TGT TGG GGT GAG TTG TG				
IGFR	for	CCC AAA ACC GAA GCT GAG AAG	60 °C	314	X54980	U, O_H, O_C
	rev	CAT CCT CCT CGC ATC TCT TC				
EGFR	for	AAC TGT GAG GTG GTC CTT GG	60 °C	169	AY486452	O_H, O_C
	rev	AAA GCA CAT TTC CTC GGA TGT CT				
ALK-5	for	CAG GGA AGA ACG TTC ATG GT	60 °C	128	AF317296	O_H, O_C
	rev	CCA ACC AAA GCT GAG TCC AT				
ALK-6	for	GCC TGT TGT CAC CTC TGG AT	60 °C	106	Z23143	O_H, O_C
	rev	CCT TTC TGT GCA GCA TTC AA				
BMPRII	for	CAA AGA TTG GCC CTT ATC CA	60 °C	109	AJ534390	O_H, O_C
	rev	CTG GAC ATC GAA TGA TCT GA				
Flk-1	for	GCT TCT ACC AGG ACA CTG ACA T	60 °C	144	X94298	U, O_H, O_C
	rev	AAC ACG GAA TCA CCA CCA CAG TT				
Flt-1	for	ATG ACC GAA GGG AAG AAG GTG	60 °C	193	XM_001249768	U, O_H, O_C
	rev	TGA CTG TTG TCT CGC AGG TC				
MMP-2	for	CCC AGA CAG TGG ATG ATG C	60 °C	237	NM_174745	U, O_H, O_C
	rev	TTG TCC TTC TCC CAG GGT C				
TIMP-2	for	GGG TCT CGC TGG ACA TTG	62 °C	255	NM_174472	U, O_H, O_C
	rev	TTG ATG TTC TTC TCC GTG ACC				
MMP-23B	for	CGC GCT ACA GCT GGA AGA AAG GC	62 °C	163	NM_001038556	OC
	rev	ACA GCT CGT CCT GCG ATA GT				
FGF-1	for	TTG TAC GGC TCA CAG ACA CC	60 °C	169	NM_174055	U, O_H, O_C
	rev	CTT TCT GGC CGA TGT GAG TC				
FGF2	for	AGC CTT GCA ACT CTG CTT GT	60 °C	210	NM_174056.3	U, O_H, O_C
	rev	CGA ATT CAG ATC CCT CCT GA				
FGF-7	for	GAC ATG GAT CCT GCC AAG TT	60 °C	129	XM_869016	U, O_H, O_C
	rev	GGG CTG GAA CAG TTC ACA TT				
ANGPT-1	for	TCG GAG ATG GCT CAG ATA CAG	60 °C	229	AF093573	U
	rev	CCA GCA GTT GTA TTT CAA GTC GA				
ANGPT-2	for	AAT TCA GTT CTC CAA AAG CAG C	60 °C	216	NM_001098855	U
	rev	TCC ACC CGT TTC CAT GTC				
ANGPT-2	for	TTA TTC AGC GAC GTG AAG ACG G	62 °C	187	NM_001098855	O_H, O_C
	rev	TAC AGC GAG TAA GCC TGA TT				
VEGFA	for	GGT GGA CAT CCT CCA GGA GTA	60 °C	177	NM_174216.1	U
	rev	CTA TGT GCT GGC TTT GGT GAG				
VEGF 120	for	CCG TCC CAT TGA GAC CCT G	60 °C	296	AB455252	O_H, O_C
	rev	CGG CTT GTC ACA ATT TTT CTT GTC				
VEGF164	for	CCG TCC CAT TGA GAC CCT G	62 °C	278		O_H, O_C
	rev	GCC CAC AGG GAT TTT CTT GC				
LDHA	for	GTG GCT TGG AAG ATA AGT GG	60 °C	155	NM174099	U
	rev	ACT AGA GTC ACC ATG CTC C				
HK1	for	CAA GAC GCA CCC ACA GTA TCC	60 °C	211	NM001012668	U
	rev	TCA CCT CCA GCA GCA TTT CCT T				
CAST	for	GAT CAG AAG TGC TGC TCC A	60 °C	206	NM 174003	U
	rev	GGA CTG TTT CCT CAT CTT ACC				
CTSB	for	GAT CTG CAT CCA CAG CCA	60 °C	192	NM 174031	U
	rev	ATG GAG TAC GGT CTG CAA CC				
CTSL	for	TCC ATA TCT TGC AAC GGA CAC TTT A	60 °C	110	NM 174032	U
	rev	CCT TCA TAA GGG CCT TCT CC				
IGF-1	for	CAT CCT CCT CGC ATC TCT TC	60 °C	239	NM_001077828	U, O_H, O_C
	rev	CTC CAG CCT CCT CAG ATC AC				
IGF-2	for	ACC CTC CAG TTT GTC TGT GG	54 °C	166	BC126514	O_H, O_C
	rev	ACA CAT CCC TCT CGG ACT TG				
IGFBP-2	for	AGC ATG GCC TGT ACA ACC TC	60 °C	157	NM_174555	OH, O_C

Table 1 (*Continued*)

Gene		Sequence [5′ → 3′]	T_M	Product length [bp]	Accession no.	
	rev	CCC TGC TGC TCG TTG TAG AA				
IGFBP-3	for	ACA GAC ACC CAG AAC TTC TCC T	60 °C	202	NM 174556	U, O_H, O_C
	rev	AGA AAC CCC GCT TCC TGC C				
BMP-2	for	CAG TAG GTG GGA GAG CTT CG	60 °C	194	NM_0010099141	U, O_H, O_C
	rev	TGA CAA GCA AGG GCC TTA TCT GT				
BMP-4	for	GAG CTT CCA CCA CGA AGA AC	60 °C	179	NM_001045877	U, O_H, O_C
	rev	TAC GAT GAA GC CCT GT CCC				
BMP-15	for	GCA GGC AGT ATT GCA TCT GAA G	60 °C	250	NM_001031752	O_C
	rev	CAC TCT GAT CCA CCA GCT AC				
CYLD2	for	TGC AGA ACT TGC TGA CCA TCG	57 °C	171	BC120199	O_C
	rev	GGT AAT TGA TGG CGA GAG GAA AG				
c-fos	for	CAG TGC CAA CTT CAT CCC AAC	60 °C	189	NM_182786	U, O_H, O_C
	rev	CTG CCT CCT GTC ATG GTT TTC				
c-Jun	for	CGG CTA TAA CCC CAA GA	60 °C	243	AF_069514	U, O_H, O_C
	rev	CCT GCT CAT CTG TCA CGT TC				
c-myc	for	TCT TGC GCC TAA ATT GAC CTA TTG	54 °C	153	NM_001046074	U, O_H
	rev	GCC AAG GTT GTG AGG TTG TTC				
CEBPB	for	GCA CAG CGA CGA GTA CAA GA	60 °C	152	NM_176788	U
	rev	GTT GCT CCA CCT TCT TCT GG				
CEBPD	for	ATC GAC TTC AGC GCC TAC ATC	62 °C	101	BC133581	U
	rev	GCT TTG TGG TTG CTG TTG AAG AG				
Rb-1	for	CAA ATT CAG AGG CAC AAG CAA	62 °C	179	NM_001076907	O_C
	rev	CTG GAA AAG GGT CCA GAT GAT				
RBBP-1	for	TTT CCA GGT CCA CTG GTC TC	60 °C	226	NM_001034638	U
	rev	CTC AGA CAC CGA GCA AAT GAC				
c-kit	for	AAG TCC ATG CTG TCG AAG AA	60 °C	185	XM_612028	O_C
	rev	TCT GCT GGC TGT TTT CCT TTA				
PHB	for	GTG AGC GAT GAC CTC ACA GA	60 °C	163	NM_001034572.1	U
	rev	CAG CCT TTT CCA CCA CAA ATC T				
THBS	for	ACA CGA CTG CAA CAA GAA CGC	62 °C	199	NM_174196	U
	rev	GGT TGG GGC AAT TAT CCT TTG T				
bcl-2	for	ATG ACT TCT CTC GGC GCT AC	62 °C	245	XM_586976	O_H, O_C
	rev	CCG GTT CAG GTA CTC GGT CA				
bcl-xl	for	GGC ATT CAG CGA CCT GC	60 °C	203	AF245487	U, O_H, O_C
	rev	CC TCC AAG TTG CGA TCC				
FAS	for	TGT TGT CAG CCT TGT CCT CC	60 °C	174	U34794	U
	rev	GTT CCA CTT CTA GCC CAT GTT C				
FasL	for	CAT CTT TGG AGA AGC AAA TAG	60 °C	205	AB035802	U
	rev	GGA ATA CAC AAA ATA CAG CCC				
p53	for	ATT TAC GCG CGG AGT ATT TG GAC	60 °C	174	NMJ74201.2	U, C_H, O_C
	rev	CCAGTGTGATGATGGTGAGGA				
CASP3	for	GAC AGT GGT GCT GAG GAT GA	60 °C	164	NM_001077840	U, O_H
	rev	CTG TGA GCG TGC TTT TTC AG				
CASP8	for	TAG CAT AGC ACG GAA GCA GG	62 °C	294	DQ319070	U
	rev	GCC AGT GAA GTA AGA GGT CAG				
CYP11A1	for	CGG AAA GTT TGT AGG GGA CAT C	62 °C	177	NM_176644	O_C
	rev	ACG TTG AGC AGA GGG ACA CT				
CYP19P1	for	TCA ACA GC GAG AG CTG GAA G	62 °C	181	NM_174305	U, O_H, O_C
	rev	GGG GAT GCT TTG CAA TAA GAA ACA				
HSD3B1	for	TCC ACA CCA GCA CCA TAG AA	57 °C	178	NM_174343	O_C
	rev	AAG GTG CCA CCA TTT TTC AGa g				
HSD17B1	for	CTC ATT ACC GGC TGT TCC TC	57 °C	200	NM_001102365	O_C
	rev	ATG GAA TCT GCA TCC CTC ACg				
HSD17B3	for	CCC AAG CCA TTT CCT TAA CAC G	60 °C	198	BC109700	O_H, O_C
	rev	ACA AAA GCC TTG GAA GCT GAA TAC				
HSD17B8	for	GGG CAT CAC CAG AGA TGA AT	60 °C	228	NM_001046324	O_H, O_C
	rev	CAA TCA CTC CAG CCT TGG AT				
HSD17B11	for	GGT GAA GGC AGA AGT TGG AG	62 °C	228	NM_001046286	O_H, O_C
	rev	AAG AAG GGG ACC CCA GTA TG				
S5A1	for	CCT TCC TAT TGG CGT TCA TCT TC	62 °C	180	NM_001099137	C_H, O_C
	rev	ATT CAA ACA AGC CCC CTC TTG GT				
COX 2	for	GCC AGG GGA GCT ACG ACT A	60 °C	247	NM_174445.2	U
	rev	AAG GAC AAT GGG CAT GAA ACT GTG				
C3	for	ATT GCC AGG TTC TTG TAC GGG	60 °C	258	NM_001040469	U
	rev	GTC ACT GCC TGA TTG CAA GAT G				
C7	for	GGC GGT CAA TTG CTG TTT ATG G	60 °C	232	NM_001045966	U
	rev	GGT CTG CTT TCT GCA TCC TC				
TGFβ	for	ACG TCA CTG GAG TTG TGC GG	60 °C	267	XM5929497	U, O_H, O_C
	rev	TTC ATG CCG TGA ATG GTG GCG				
TNFα	for	CCA CGT TGT AGC CGA CAT C	60 °C	197	NM173966	U, O_H, O_C
	rev	CCC TGA AGA GGA CCT GTG AG				
GDF-9	for	CAT CGG TAT GGC TCT CCA GT	60 °C	122	NM_174681	O_H, O_C
	rev	ATG GCC AAA ACA CTC AAA GGA CT				

Table 1 (*Continued*)

Gene		Sequence [5′ → 3′]	T_M	Product length [bp]	Accession no.	
STAR	for	TGG AAA AGA CAC GGT CAT CA	57 °C	154	NM_174189	O_C
	rev	CTG GGG CAT CTC CTC ATA GA				
Smad 2	for	ATG GTC GTC TTC AGG TGT CC	60 °C	237	NM_001046218	O_H, O_C
	rev	GCA GTT CCG TTA GGA TCT CG				
LTF	for	ACC ATC TCC CAA CCT GAG TG	60 °C	285	NM_180998	U
	rev	AAA GTT GCT GCC CTT CTT CAC G				
INHA	for	TAG TGC ACC CTC CAA GTT TC	60 °C	239	NM_174094	O_H, O_C
	rev	GGT TGG GCA TCT CAT AC				

was prepared as follows: 8 μL RNase free water (5Prime, Hamburg, Germany), 4 μL 5× reaction buffer (Promega), 1 μL Random Primers (Invitrogen, Karlsruhe, Germany), 1 μL dNTPs (Fermentas, St. Leon-Rot, Germany), 1 μL M-MLV H⁻ reverse transcriptase. The reaction mix was inserted in the Eppendorf Gradient Mastercycler (Eppendorf, Hamburg, Germany) and the here stated temperature protocol was started: 21 °C, 10 min; 48 °C, 50 min; 90 °C, 2 min; 4 °C hold. cDNA samples were diluted with 40 μL of RNAse free water (5Prime) to a final volume of 60 μL. Reverse transcription was done in duplicates for every sample.

2.6. Quantitative PCR

Quantitative PCR (qPCR) was performed using MESA Green qPCR MasterMix plus for SYBR Assay w/fluorescein (Eurogentec, Cologne, Germany) by a standard protocol recommended by the manufacturer. The mastermix was prepared as follows: 7.5 μL 2× MESA Green qPCR MasterMix; 1.5 μL forward Primer (10 pmol/μL); 1.5 μL reverse Primer (10 pmol/μL); 3.0 μL RNAse free water (5Prime). For a total volume of 15 μL, 1.5 μL cDNA were mixed with 13.5 μL of the mastermix in a 96-well plate. The plate was heat-sealed with the Eppendorf Heat-Sealer (Eppendorf), placed in the iQ5 Cycler (Bio-Rad, Munich, Germany) and the following PCR protocol was started: denaturation step (95 °C, 5 min), cycling program [95 °C, 3 s; primer specific annealing temperature (see Table 1), 60 s] and melting curve analysis.

2.7. Data evaluation

Expression data were analyzed using relative quantification. Suitable reference genes (RG) were established using GeNorm and Normfinder algorithm of GenEx v.5.0.2.8. software (Multi D Analyses, Gothenburg, Sweden). The geometric mean of three RG [Ubiquitin (UBQ), Histon 3 (H3), βActin (ACTB)] was used as reference index. Data were normalized and relatively compared to the control group according to the ΔΔCq-model with the following formulas [26]:

$$\Delta Cq = Cq_{(\text{target gene})} - Cq_{(\text{reference gene index})}$$
$$\Delta\Delta Cq = \Delta Cq_{(\text{treatment group})} - \text{mean}\ \Delta Cq_{(\text{control group})}$$

The expression ratio of the treatment group compared to the control group is expected as $2^{-\Delta\Delta Cq}$ and represents the *x*-fold regulation with a value of 1.00 indicating no expression change after treatment. Relative expression data were statistically evaluated using Sigma Stat 3.0 (SPSS Inc., Chicago, IL). The determined *p*-values of the statistical significance were examined using Student's *t*-test. Results with $p \leq 0.05$ were considered as statistically significant, results with ≤0.1 were considered to show a statistical tendency. To visualize the multivariate response of the selected classifier genes to the treatment, the method of principle components analysis (PCA) was employed using GenEx v. 5.0.2.8 (multiD Analyses AB). PCA involves a mathematical procedure that transforms a number of variables (here normalized expression values) into a smaller number of uncorrelated variables called principal components. By this, the dimensionality of the data is reduced to a number of dimensions that can be plotted in a scatter plot, here two dimensions. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible [27].

3. Results

3.1. RNA integrity

RNA integrity was controlled to prove the suitability of the sample material for RT-qPCR [22]. In the South Africa study, samples from uterine endometrium horn showed a mean RIN of 8.3 ± 0.49 (n = 12), samples from uterine endometrium corpus showed a RIN of 7.9 ± 0.49 (n = 12) and samples from ovary showed a mean RIN of 7.5 ± 0.98 (n = 11). Ovary samples from the pour on study showed a mean RIN of 7.7 ± 0.70 (n = 19). Therefore, all samples were suitable for RT-qPCR.

3.2. Gene expression results from post-pubertal Nguni heifers

In uterine endometrium corpus, significant regulations could be demonstrated in 7 genes (Table 2). Up-regulations occurred for

Table 2
Regulated genes in uterine endometrium corpus of Nguni heifers after treatment with TBA plus E2.

Functional group	Gene	*x*-Fold regulation		*p*-Value	Significance
		↑	↓		
Receptors	AR	1.71		0.012	**
Angiogenesis	FGF-7	1.62		0.088	*
	VEGFA	1.62		0.044	
Proliferation/apoptosis	IGFBP-3	2.69		0.085	
	CASP3		0.82	0.005	**
	c-fos	2.73		0.028	*
	BMP-4		0.61	0.020	*
	CEBPD	2.28		0.024	*
Others	Complement C7	4.42		0.022	*

* $p < 0.05$.
** $p < 0.01$.

Table 3
Regulated genes in uterine endometrium horn of Nguni heifers after treatment with TBA plus E2.

Functional group	Gene	*x*-Fold regulation		*p*-Value	Significance
		↑	↓		
Receptors	AR	1.65		0.014	**
	IGF-1R		0.73	0.083	
Angiogenesis	ANGPT-1		0.47	0.027	*
	MMP-2		0.65	0.057	
Proliferation/apoptosis	TNFα	5.05		0.018	*
	CASP3		0.72	0.00001	***
	FASL	1.99		0.049	*
	p53		0.72	0.076	
	BMP-4		0.64	0.041	*
Protein metabolism	CTSB		0.63	0.033	*
Others	Complement C7	6.92		0.001	***
	CYP19P1	1.87		0.070	

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

the receptor AR ($p = 0.012$), the angiogenic factor VEGFA ($p = 0.044$), the complement system factor C7 ($p = 0.022$) and the proliferative factors c-fos ($p = 0.028$) and CEBPD ($p = 0.024$). A down-regulation could be shown for CASP3 ($p = 0.005$) and BMP-4 ($p = 0.020$). A trend for up-regulation could be obtained for FGF-7 ($p = 0.088$) and IGFBP-3 ($p = 0.085$), but these differences were not statistically significant.

Significant regulations for 8 genes could be detected in uterine endometrium horn (Table 3). Up-regulations resulted for the receptor AR ($p = 0.014$), the proliferative factors TNFα ($p = 0.018$) and FasL ($p = 0.049$), and the complement system factor C7 ($p = 0.001$). Down-regulations arose for CASP3 ($p = 0.00001$), CTSB ($p = 0.033$) and BMP-4 ($p = 0.041$). IGF-1 R ($p = 0.083$), MMP-2 ($p = 0.057$) and p53 ($p = 0.076$) showed a trend for down-regulation, whereas a trend for up-regulation occurred in CYP19A1 ($p = 0.070$).

20 out of 40 investigated genes showed significant regulations in ovary (Table 4). Regulations occurred for the following receptors: AR ($p = 0.028$), ERβ ($p = 0.010$), FSHR ($p = 0.028$), IGF-1 R ($p = 0.003$) and PR ($p = 0.028$), LHR ($p = 0.004$) and Flt-1 ($p = 0.050$). In the angiogenic group, up-regulations were observed for FGF-2 ($p = 0.038$), ANGPT-2 ($p = 0.015$), and MMP-2 ($p = 0.007$). The proliferative factors IGF-1 ($p = 0.026$) and BMP-2 ($p = 0.005$) and also the anti-cancerogenic protein p53 were up-regulated ($p = 0.021$). Significant down-regulations were demonstrated for VEGF120 ($p = 0.021$), VEGF164 ($p = 0.015$) and TIMP-2 ($p = 0.028$), for the enzymes S5A1 ($p = 0.021$) and HSD17B3 ($p = 0.024$) and for INHA ($p = 0.003$). Trends for up-regulation could be observed in Smad2 ($p = 0.083$) and c-jun ($p = 0.052$), Alk-6 ($p = 0.083$) tended to be down-regulated (Table 4).

Principal components analysis (PCA) for uterine endometrium and ovary was produced as shown in Figs. 1–3 by plotting the normalized Cq values from regulated target genes of all samples by their first and second principal component. In the PCA, close clusters represent experimental groups, with arrange together making it possible to separate these groups due to the response to the

Table 4
Regulated genes in ovary of Nguni heifers after treatment with TBA plus E2.

Functional group	Gene	*x*-Fold regulation		*p*-Value	Significance
		↑	↓		
Receptors	AR	1.67		0.028	*
	ERβ	8.19		0.010	**
	LHR		0.29	0.004	**
	FSHR	3.61	0.56	0.028	*
	ALK-6			0.083	
	FLT-1		0.64	0.050	*
	IGF-1 R	2.03		0.003	**
	PR	2.20		0.028	*
Angiogenesis	VEGF 120		0.34	0.021	*
	VEGF 164		0.21	0.015	*
	FGF-2	2.06		0.038	*
	ANGPT-2	2.32		0.015	*
	MMP-2	2.46		0.007	**
	TIMP-2		0.24	0.028	*
Proliferation/apoptosis	Smad2	1.27		0.083	
	c-jun	2.00		0.052	
	p53	1.68		0.021	*
	IGF-1	2.34		0.026	*
	BMP-2	2.64		0.005	**
Steroid metabolism	S5A1		0.53	0.021	*
	HSD17B3		0.61	0.024	*
Others	INHA	4.67		0.003	**

* $p < 0.05$.
** $p < 0.01$.

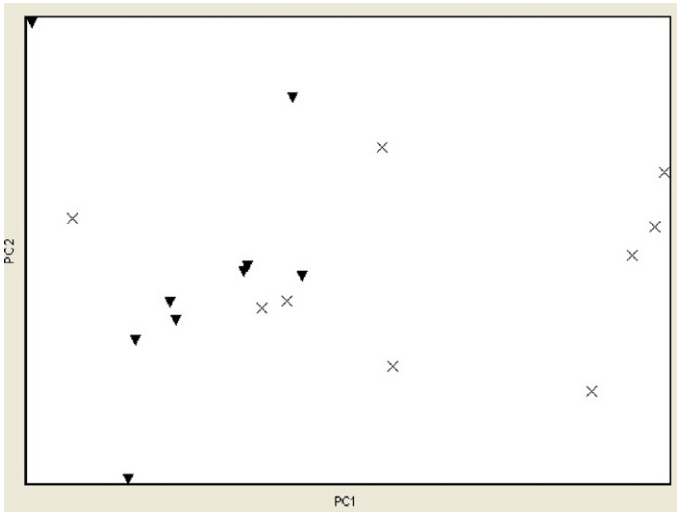

Fig. 1. Slight separation between treated and non-treated heifers after PCA evaluation on the basis of 9 regulated target genes measured in uterine endometrium corpus; (▼) control group, (×) treatment group.

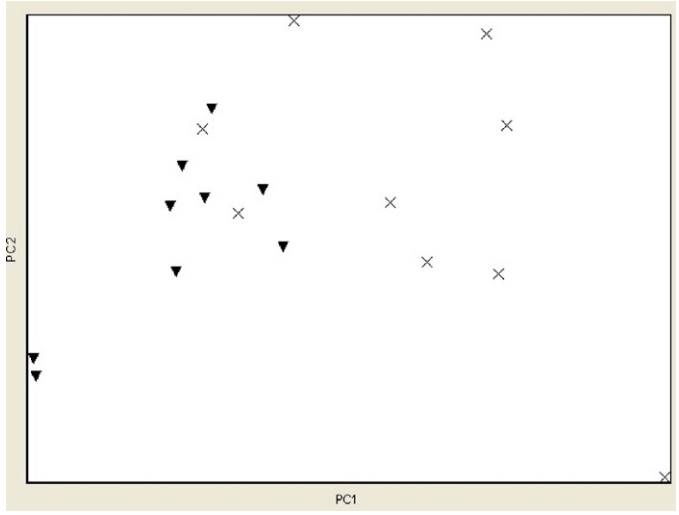

Fig. 2. Slight separation between treated and non-treated heifers after PCA evaluation on the basis of 12 regulated target genes measured in uterine endometrium horn; (▼) control group, (×) treatment group.

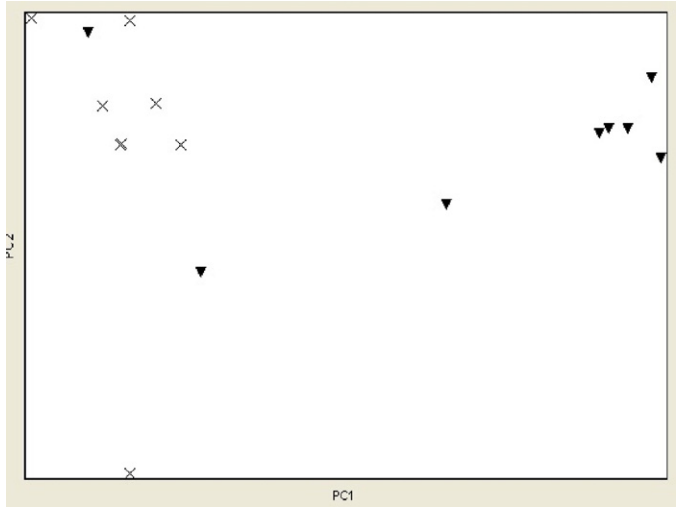

Fig. 3. Distinct separation between treated and non-treated heifers after PCA evaluation on the basis of 23 regulated target genes measured in ovary; (▼) control group, (×) treatment group.

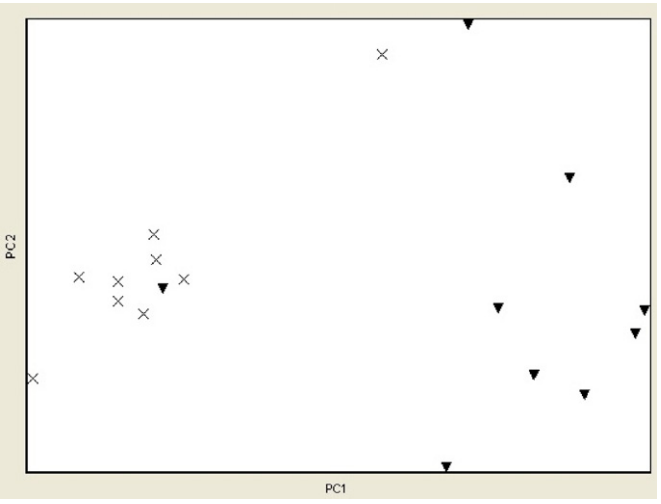

Fig. 4. Distinct separation between treated and non-treated heifers after PCA evaluation on the basis of 44 regulated target genes measured in reproductive tissues; (▼) control group, (×) treatment group.

treatment. Black triangles represent samples of the control group, grey crosses represent the samples of treatment group. In all single tissues, a control group could be seen separating from the animals of the treatment group, showing that there was a multitranscriptional response to the treatment. Additionally, a clear assignment could be observed for the combination of all tissues (Fig. 4). The best separation between the control and the treatment group was achieved for ovary and the composition of all three target organs making these possibilities to the most promising regarding biomarker research.

3.3. Results from pre-pubertal Holstein Friesian calves

In ovary of pre-pubertal Holstein Friesian calves, 23 of 42 measured target genes showed significant regulations (Table 5). Significant regulations of five gens could already be detected in the carrier control group. Herein, an up-regulation of LHR ($p = 0.032$) and down-regulations of VEGF120 ($p = 0.054$) and VEGF164 ($p = 0.030$), PR ($p = 0.028$) and CYP19A1 ($p = 0.025$) could be observed. Most regulations occurred in the one time treated group. Up-regulations could be revealed for the receptors LHR ($p = 0.014$) and Alk-6 ($p = 0.013$), the proliferative factor c-kit ($p = 0.055$), IGFBP-2 ($p = 0.012$) and STAR ($p = 0.053$). In the angiogenic group, FGF-1 ($p = 0.008$), MMP-23B ($p = 0.026$) and the anti-angiogenic inhibitor TIMP-2 ($p = 0.008$) were significantly up-regulated. In the steroid metabolism group, S5A1 ($p = 0.030$), HSD17B3 ($p = 0.034$) and HSD17B8 ($p = 0.022$) were up-regulated. HSD17B11 showed just a trend for up-regulation in the one time treated group ($p = 0.064$), but a statistical significant up-regulation in the three times treated group ($p = 0.031$). Trends for up-regulation could be obtained for Flk-1 ($p = 0.069$), ANGPT-2 ($p = 0.09$), Rb-1 ($p = 0.095$) and HSD3B1 ($p = 0.097$). A trend for down-regulation could be seen for AR ($p = 0.071$). In the three times treated experimental group HSD17B11 ($p = 0.031$), MMP23B ($p = 0.043$) and MMP-2 ($p = 0.016$) were significantly up-regulated. A trend for up-regulation could be observed for ERα ($p = 0.098$), PR ($p = 0.082$) and BMP-2 ($p = 0.063$).

In the PCA of ovarian tissue for pre-pubertal calves, all significantly regulated genes in all four experimental groups were plotted (Fig. 5) by their first and second principal component. Circles represent samples of the control group, crosses display samples of the carrier control group, triangles display the samples of 1× treated group, squares represent samples of the 3× treated group. Obvi-

Table 5
Regulated genes in ovary of Holstein Friesian calves after treatment with hormone mix.

Functional group	Gene	Carrier control		1×		3×	
		x-Fold	*p*-Value	*x*-Fold	*p*-Value	*x*-Fold	*p*-Value
Receptors	AR		n.s.	0.71	0.073		n.s.
	ERα		n.s.		n.s.	1.89	0.098
	PR	0.37	0.028	3.75	n.s.	1.93	0.082
	FLK-1		n.s.		0.069		n.s.
	LHR	1.78	0.032	2.06	0.014		n.s.
	ALK-6		n.s.	3.44	0.013		n.s.
Angiogenesis	VEGF 120	0.78	0.054		n.s.		n.s.
	VEGF 164	0.70	0.030		n.s.		n.s.
	MMP-2		n.s.		n.s.	3.90	0.016
	MMP-23B		n.s.	1.63	0.026	1.67	0.043
	ANGPT-2		n.s.	1.90	0.090		n.s.
	TIMP-2		n.s.	3.78	0.008		n.s.
	FGF-1		n.s.	2.25	0.008		n.s.
Proliferation	BMP-2		n.s.		n.s.	2.87	0.063
	IGFBP-2		n.s.	2.73	0.012		n.s.
	BMP-15		n.s.		n.s.	4.81	0.058
	c-kit		n.s.	1.93	0.055		n.s.
	RB-1		n.s.	1.72	0.095		n.s.
Steroid synthesis	CYP19A1	0.26	0.025		n.s.		n.s.
	S5A1		n.s.	1.96	0.030		n.s.
	HSD3B1		n.s.	4.67	0.097		n.s.
	HSD17B3		n.s.	1.47	0.034		n.s.
	HSD17B8		n.s.	2.01	0.022		n.s.
	HSD17B11		n.s.	2.06	0.064	1.91	0.031
	STAR		n.s.	2.49	0.053		n.s.

ously, no separation could be seen between the two control and the two treatment groups making it debatable, if there was a response to the anabolic treatment on transcriptional level.

4. Discussion

Several biochemical pathways could be shown to be differentially regulated on transcriptional level under the influence of an anabolic combination of an androgen and an estrogen in uterine endometrium and ovary of Nguni heifers. It is generally known that anabolic steroids and especially estrogens cause a trophic response in the reproductive tract [28]. Coincidently, several factors taking place in proliferative and anti-apoptotic events were regulated in the actual study. In ovary and uterus, AR is thought to play a role in physiological proliferation and also in uncontrolled cell growth during tumorgenesis [12]. There are few data concerning AR gene expression in bovine reproductive tract, but estrogens were shown to induce AR expression in rat uterus to mediate the uterotrophic effect [29,30]. Our data suggest that AR is targeted by anabolic steroids in bovine uterus and ovary triggering cell growth. Concordantly, TNFα, CEBPD and c-fos, which were shown to be implicated in cell turnover under the influence of steroid hormones [9,10,12,31,32], were up-regulated in Nguni heifers indicating a higher proliferation rate. While cell growth was induced, a parallel inhibition of apoptotic and tissue degrading factors occurred. BMP-2 and BMP-4 as well as CASP3 are known to cause apoptosis in many

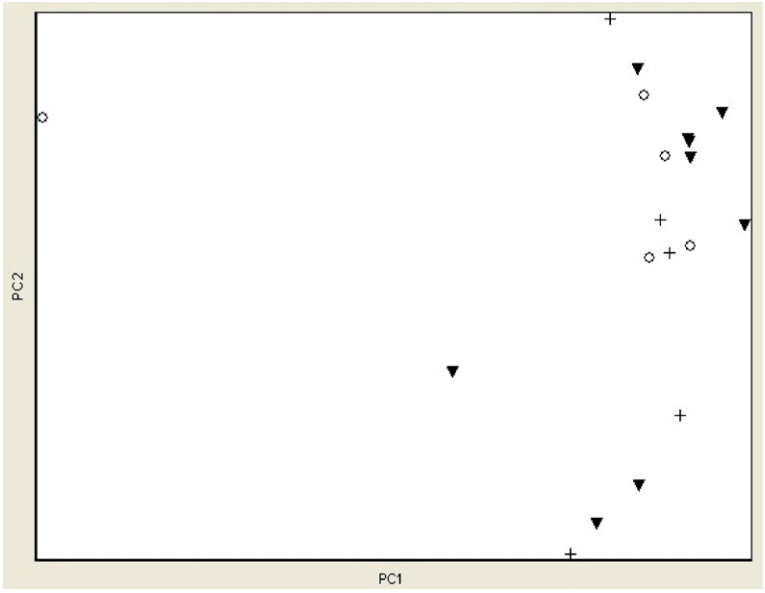

Fig. 5. No separation on the basis of 23 regulations measured in ovary of Holstein Friesian calves after PCA evaluation; (○) control group, (+) carrier control group, (▼) 1× treated group, (■) 3× treated group.

different target cells and have been proven to be implicated in tissue remodeling of the cyclic uterus [33,34]. The protease CTSB plays a role in the degradation of extracellular matrix and the catabolism of intracellular proteins and is therefore physiologically involved in the tissue remodeling of the cyclic uterus [15]. The down-regulation of these factors propose an inhibition of tissue breakdown in the treated animals. In ovary, an up-regulation of the p53 tumour suppressor gene could be detected. p53 is known to be an inhibitor of caspase activity [14,35]. Possibly, caspase-dependent apoptosis in ovary is inhibited by the action of p53. Unlike that, the expression of IGF related factors illustrated another situation. It is generally known that IGF-1 is one of the major growth factors implicated in the proliferation of the uterus [36,37]. The present study showed a down-regulation of IGF-1R and an up-regulation of IGFBP-3 indicating a lower responsiveness and an inhibitory effect towards IGF-1. These regulations might demonstrate a protective adaption mechanism of the organism to prevent the paracrine action of the plentiful IGF-1 originating from liver [42]. Contrarily, up-regulations of IGF-1 and IGF-1R occurred in ovary rather pointing to strong proliferative effects. These results also demonstrate the tissue specific response of the organism to the application anabolic steroids.

A possible adaption mechanism of the organism to the anabolic treatment might also be observed in the regulation of factors implicated in ovarian steroid synthesis in heifers. HSD17B3 and S5A1, whose enzymatic reactions are directed towards the generation of active steroid hormones, are down-regulated. INHA is known to be a suppressor of FSH, whose secretion from the pituitary is crucial for steroid synthesis, and its up-regulation could therefore be part of the negative feedback mechanisms [20]. These data suggest an inhibition of endogenous hormone synthesis and formation of active hormones as a negative feedback response to exogenous application of anabolics. In pre-pubertal calves the up-regulation of HSD17B8, which is responsible for the degradation of E2 into the lower active estrone [5], could be estimated as an induction of decomposition of the applied exogenous hormones. However, the enzymes HSD17B3 and S5A1 are induced not reflecting the possible protective mechanism, which was hypothesized in Nguni heifers. This regulation pattern gives no clear mark on a trend in steroid hormone synthesis in the pre-pubertal ovary.

In ovary, VEGF and the VEGF-receptor Flt-1 are mainly implicated in the formation of new capillary networks during the physiological process of angiogenesis [3]. The expression of VEGF is mainly stimulated by LH secreted from the pituitary [3]. The observed down-regulation of LHR in the ovary of heifers and the following lower responsiveness of this organ towards LH may be responsible for the down-regulation of the VEGF isoforms and their receptor Flt-1. The resulting inhibition of follicular maturation could partly be responsible for known reproductive perturbations like the delayed onset of puberty shown in pre-pubertal animals or the predisposition for non-ovulatory estrus observed in mature cows under the influence of steroidal growth promoters [38]. The changes in gene expression observed in the actual study could also be accounted to mimic the state of regression of the corpus luteum (CL) characterized by a down-regulation of VEGF and an up-regulation of FGF-2 and ANGPT-2 [39]. Thereby, these results could possibly indicate a degrading effect of anabolic steroids on the CL and follicle. In general, there might be an inhibitory effect on angiogenic processes under the influence of anabolic steroid hormones.

In pre-pubertal calves several pro-angiogenic factors like MMPs and FGF-1, as well as the mediating receptors Flt-1 and Alk-6 were up-regulated. This would point to an increase in angiogenic events after the application of exogenous hormones. However, also the anti-angiogenic inhibitor TIMP-2 and the blood vessel degrading factor ANGPT-2 are up-regulated prohibiting an explicit conclusion on angiogenic events. Considering the pour on study, most of the results in ovary are hardly to discuss. Even though the same genes were regulated compared to the ovary of Nguni heifers, the direction of the regulation differed, which was obvious especially in receptors (AR, PR, LHR), but also for steroidogenic enzymes (HSD17B3, S5A1). This could possibly be explained by the different hormonal status of calves and heifers. Accessorily, the reproductive tract of the calves might show no responsivity to the exogenous application of steroid hormones due to the rudimentary developmental stage. A similar hypothesis has been introduced by Caccicatore et al. [40], who observed no effect of hormone administration on different steroid target genes in pre-pubertal animals. Also, different anabolic preparations as well as different application modes (long lasting implants versus pour on) may cause different transcriptional responses in the treated animal. Especially, the route of application may be a reason for the unexpected results as rare valid data exist concerning the pharmacokinetics of steroid esters after pour on treatment. In the course of this study, co-workers investigated the occurrence of the applied steroid hormone esters in hair and plasma and were able to find the applied steroid esters in hair [41]. However, concentrations were under the detection limit after 5–7 weeks (1× treatment) and 9–11 weeks (3× treatment), respectively. Furthermore, no hydrolysates of the applied substances could be detected in plasma. These results indicate that the administered steroid esters compass hair via sweat or sebum excretion at the surface of the skin and reach the blood stream only in negligible amounts. Also, the short duration of the drug effect could be proven. At the time point of slaughter, the applied substances have already been eliminated from the organism, which could possibly be a reason for the absence of specific gene expression changes [41].

Independently of the gene expression results, compared to the control group an anabolic effect of the treatment was visible on the phenotype level by comparing weight gain and carcass weight (unpublished data). Herein, the highest differences could be observed between the control animals and animals from the 3× treatment group on days 28, 63 and 91 after beginning of the treatment. Also, the carcass weight at slaughter was significantly higher in the 3× treated group. No differences could be detected between the two treatment groups. These data demonstrate that an anabolic effect using pour on application becoming manifest in significantly increased weight gain might not be visible at the gene expression level and might therefore remain undetected, when using transcriptomics for surveillance of anabolic misuse.

For biomarker research, results from biostatistical evaluation concerning pattern recognition showed clearly that it would be necessary to establish different biomarkers for specific treatment regimes and different age classes of animals, as the PCA of the ovary results obtained from pre-pubertal calves indicated that the measured genes were not adequate as a certain pattern to divide between treated and untreated animals. Contrarily, the PCA conducted for all tissues from post-pubertal Nguni heifers demonstrated a distinct separation between the control and the treatment group. These results give a first hint that it would be possible to establish a gene expression pattern for the detection of anabolic misuse in adult animals.

5. Conclusion

The observations gained in the actual study indicate a stimulation of cell turnover in the reproductive tract of post-pubertal Nguni heifers characterized by the induction of different transcription and proliferative factors and the inhibition of pro-apoptotic factors. In ovary, anti-angiogenic effects were estimated, which could in part be related to the well known disturbances in fertility following anabolic treatment.

The ambiguous transcriptional regulations in ovary of calves may be due to the non-responsiveness of the immature reproductive tract. The differences in gene expression compared to Nguni heifers could be explained by the different hormonal status of pre- and post-pubertal animals and the differences in the application routes. The application of PCA for pattern recognition demonstrated the possibility to establish a gene expression pattern, which could be used as biomarker to detect the illegal application of exogenous hormones in post-pubertal heifers for uterine endometrium and ovary. Pre-pubertal animals seem to be less suitable for surveillance of anabolic treatment using transcriptomics, as it was not possible to discover a convenient gene expression pattern using PCA in female Holstein Friesian calves.

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