



# The gastrointestinal tract as target of steroid hormone action: Quantification of steroid receptor mRNA expression (AR, ER $\alpha$ , ER $\beta$ and PR) in 10 bovine gastrointestinal tract compartments by kinetic RT-PCR<sup>☆</sup>

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## Abstract

We have examined the tissue-specific mRNA expression pattern of androgen receptor (AR), both estrogen receptor (ER) subtypes ER $\alpha$  and ER $\beta$  and progesterin receptor (PR) in 10 bovine gastrointestinal compartments. Goal of this study was to evaluate the deviating tissue sensitivities and the influence of the estrogenic active preparation Ralgro<sup>®</sup> on the compartment-specific expression regulation. Ralgro<sup>®</sup> contains Zeranol which shows strong estrogenic and anabolic effects. Eight heifers were treated for 8 weeks with Ralgro<sup>®</sup> at different dosages (0, 1, 3, and 10 times). To quantify the very low abundant steroid receptor mRNA transcripts sensitive and reliable real-time (kinetic) reverse transcription (RT)-PCR quantification methods were validated on the LightCycler. Expression results indicate the existence of AR and both ER subtypes in all 10 gastrointestinal compartments. PR receptor was expressed at very low abundance. Gastrointestinal tissues exhibit a specific ER $\alpha$  and ER $\beta$  expression pattern with high expression levels for both subtypes in rectum, colon and ileum. With increasing Zeranol concentrations a significant down-regulation for ER $\alpha$  and ER $\beta$  was observed in jejunum ( $P < 0.001$  and  $< 0.05$ , respectively). Significant up-regulations under estrogen treatment could be shown in abomasum for ER $\alpha$  ( $P < 0.05$ ) and in rectum for ER $\beta$  ( $P < 0.001$ ). The authors conclude, that especially estrogens and the expression of their corresponding receptor subtypes may play an important role in the modulation and regulation in gastric as well as gut functions, cell proliferation and possibly in the pathophysiology of cell cancer. The different expression patterns of ER $\alpha$  and ER $\beta$  can be regarded as support of the hypothesis that the subtype proteins may have different biological functions in the gastrointestinal tract. AR and PR seem to be not estrogen dependent.

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## 1. Introduction

Steroid hormones regulate cell growth, cell differentiation, protein accumulation and carbohydrate utilization in numerous tissues, primarily in uterus, mammary gland, ovary, testis, prostate and muscularity. The effects of steroid hormones are mediated through interaction with specific intracellular receptors which are members of the nuclear receptor family [1,2]. Numerous tissues were shown to express the mRNA transcript for both estrogen receptor (ER) subtypes ER $\alpha$  and ER $\beta$  [3–6], androgen receptor (AR) [7–9] and progesterin receptor (PR) [10,11]. Sex steroids do

not only exert effects in organs related to reproductive function [12,13] or muscularity [10], as mentioned previously, but also in tissues that were not regarded as classical targets so far. The effects of steroid hormones on gastrointestinal function, related to intermediary metabolism and absorption of nutrients and minerals [14,15], have been reported. For estrogens [16–18] and androgens [19] a number of intestinal functions seem to be affected by alterations in hormone concentration [20]. For progesterone no such investigations were made in the gastrointestinal tract. Beside this sex steroids and steroid hormone receptors play a critical role in the pathophysiology of carcinogenesis [21,22].

Cloning and sequencing of ER $\beta$  in various species [23,24] has provided the first example, that the estrogen receptors exist in two isoforms, each of which is encoded by a separate gene. The ER $\beta$  protein is smaller than the previously identified ER $\alpha$  and the DNA-binding domains of both subtypes are highly conserved (~60%) over several species [25,26]. ER $\alpha$  and ER $\beta$  tissue distribution and relative level

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of expression are quite different since various tissues express either one or both receptors. Most tissues in cattle [6,10] or reproductive organs in rat express both subtypes [12]. The classical tissues for estrogen action like uterus, mammary gland and liver showed high ER $\alpha$  abundance. Also high ER $\alpha$  mRNA expression could be observed in muscularity, whereas high ER $\beta$  expression was observed in uterus, kidney and spleen [6,10]. The relative expression levels of these receptors (ER $\alpha$ /ER $\beta$  expression ratio) may play a major role in mediating estrogen actions in a particular tissue. Both ER $\alpha$  and ER $\beta$  activate transcription, they can work in opposite directions and activate protein-1 response elements. In general, ER $\alpha$  is an activator, whereas ER $\beta$  is known to be an inhibitor or without effects, at activating protein-1 sites [13].

The AR as well is a member of the family of cytoplasmic steroid hormone receptors. Proper functioning of this receptor protein is a prerequisite for normal male sexual differentiation and development [9]. It is widely expressed in non-reproductive tissues [7–10], but outside the urogenital tract, it generally does not exhibit a sexually dimorphic expression pattern [27]. It is reported, that the AR mRNA expression can correlate positively [28] or negatively [29] with the functional activity of the mature protein. Only little is known about the AR expression in gut compartments [20].

PR is composed of two protein isoforms, termed A and B, both expressed by a single gene in rodents and humans [30]. The selective physiologic roles of the two isoforms are still unknown, although isoform A seems to be the dominant form [31]. In vitro studies have shown that A and B form can have different functions in the same cell and that the activity of the individual form of the receptor can vary among different cell types [32]. Comparable isoforms are not yet described for large domestic animals. In reproductive organs PR expression is under the control of estrogen, estrogen increases PR expression, whereas progesterone decreases PR expression [33,34]. No literature about PR expression in different compartments of the gastrointestinal tract is available up to date.

First aim of the presented paper was to study expression of AR, ER $\alpha$ , ER $\beta$  and PR by real-time reverse transcription (RT)-PCR in various bovine gut departments. To detect and quantify these rare mRNA transcripts real-time RT-PCR was performed on the LightCycler system (Roche Diagnostics, Mannheim, Germany). Real-time RT-PCR with an external calibration curve is a fully quantitative methodology and therefore an absolute comparison of all transcripts within the investigated tissue RNA preparation is possible [35]. Second aim was to evaluate the deviating tissue sensitivities in different gut departments of cattle, as well as the influence of the estrogen active preparation Ralgro<sup>®</sup> on the tissue-specific expression pattern and regulation of steroid receptor expression levels. The results may help to improve our understanding of the possible role of steroid receptors' regulating function especially in single gastrointestinal tract compartments.

## 2. Materials and methods

### 2.1. Animal experiment and sampling

Six Holstein Friesian heifers were treated with the implant preparation Ralgro<sup>®</sup> (Mallinckrodt Veterinary, Inc., Mundelein, IL, USA) 8 weeks before slaughter. Two animals served as control, two got single (1 $\times$ ), two three-fold (3 $\times$ ) and two 10-fold dose (10 $\times$ ) of the preparation. One Ralgro<sup>®</sup> implant contains 36 mg  $\alpha$ -Zearalanol (Zeranol), a derivative of the mycotoxin Zearalenon. Zeranol shows strong estrogenic and anabolic effects in farm animals. Besides this, it exhibits all symptoms of hyper-estrogenism in particular reproductive and developmental disorders. Subsequently after slaughtering tissue samples from all four stomach (rumen, reticulum, omasum, abomasum) and six different gut regions (duodenum, jejunum, ileum, caecum, colon, rectum) were taken and frozen in liquid nitrogen.

### 2.2. Total RNA extraction

Five hundred milligrams of frozen tissue were homogenized in 4 M guanidinium thiocyanate buffer to destroy RNase activity [36]. In the following steps, the RNA clean protocol (AGS RNA-Clean; AGS, Heidelberg, Germany) with phenol/chloroform extraction for total RNA was used, as described earlier [6,10]. In order to quantify the amount of total RNA extracted, the optical density (OD) was determined (Ultraspec 3000 photometer, Pharmacia). The total RNA integrity was electrophoretically verified by ethidium bromide staining and by optical density measurement of OD<sub>260</sub>/OD<sub>280</sub> nm absorption ratio (>1.92).

### 2.3. Primer design

AR, ER $\alpha$  and PR primers were derived from earlier publications [6–8,10]. ER $\beta$  primers were designed newly, to achieve better sensitivity and assay repeatability. Steroid receptor RT-PCR products were designed in the region of the receptor ligand-binding domain to produce amplification products of 172, 234, 262, and 227 bp for AR, ER $\alpha$ , ER $\beta$  and PR, respectively. PR primer are located near the 5'-end of mRNA and therefore cover all two receptor isoforms A and B. Primer sequences are summarized in Table 1.

### 2.4. Reverse transcription

One microgram total RNA from the sample preparation was reverse transcribed in 40  $\mu$ l as follows: M-MLV RT buffer (Promega, Mannheim, Germany), and 300  $\mu$ M dNTPs (Roche Diagnostics) were denaturated for 5 min at 65 °C in a Mastercycler Gradient (Eppendorf, Hamburg, Germany). The subsequent RT was done at 37 °C for 60 min by adding 2.5 mM random hexamer primers (Roche Diagnostics), 200 U of M-MLV H<sup>-</sup> reverse transcriptase

Table 1

Primer sequences derived from earlier publications: AR [8], ER $\alpha$  [6], ER $\beta$  and PR [10]

AR forward primer	5'-CCT GGT TTT CAA TGA GTA CCG CAT G-3'
AR reverse primer	5'-TTG ATT TTT CAG CCC ATC CAC TGG A-3'
ER $\alpha$ forward primer	5'-AGG GAA GCT CCT ATT TGC TCC-3'
ER $\alpha$ reverse primer	5'-CGG TGG ATG TGG TCC TTC TCT-3'
ER $\beta$ forward primer	5'-CTT CGT GGA GCT CAG CCT GT-3'
ER $\beta$ reverse primer	5'-GAG ATA TTC TTT GTG TTG GAG TTT-3'
PR forward primer	5'-GAG AGC TCA TCA AGG CAA TTG G-3'
PR reverse primer	5'-CAC CAT CCC TGC CAA TAT CTT G-3'

(Promega), 12.5 U of RNAs in RNase inhibitor (Roche Diagnostics). The samples were finally heated for 1 min at 99 °C to terminate RT.

### 2.5. Optimization of real-time RT-PCR master mix and temperature profiles

Conditions for RT-PCRs were optimized in a gradient cycler with regard to Taq DNA Polymerase (Roche Diagnostics), primers (MWG Biotech, Ebersberg, Germany), MgCl<sub>2</sub> concentrations and various annealing temperatures. Optimized settings were transferred to real-time RT-PCR protocols on the LightCycler platform (Roche Diagnostics). A master mix of the following reaction components was prepared to the indicated end-concentration (given in parenthesis): 6.4  $\mu$ l water, 1.2  $\mu$ l MgCl<sub>2</sub> (4 mM), 0.2  $\mu$ l forward primer (0.2  $\mu$ M), 0.2  $\mu$ l reverse primer (0.2  $\mu$ M) and 1.0  $\mu$ l LightCycler DNA Master SYBR Green I (Roche Diagnostics). Nine microliters of master mix was filled in the glass capillaries and 25 ng reverse transcribed total RNA (=cDNA) in 1  $\mu$ l was added as PCR template. The capillaries were closed, centrifuged in a micro-centrifuge and placed into the cycling rotor. To improve SYBR Green I quantification a high temperature target-specific fluorescence measurement point was included in the amplification protocol performed (Table 2) [37]. Unspecific PCR products, e.g. primer dimers, melt at the elevated temperature, the non-specific fluorescence signal is eliminated and accurate quantification of the desired product is ensured. The following cycling protocols were used: *denaturation program* (95 °C for 10 min), a four-segment amplification and

*quantification program* repeated 40 times (factor-specific amplification condition with a single fluorescence measurement at an elevated temperature level), *melting curve program* (60–99 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurements) and finally a *cooling program* down to 40 °C.

### 2.6. Calibration curves

For all quantitative assays an external calibration curve was used, based on a single stranded DNA (ssDNA) molecule calculation. AR, ER $\alpha$ , ER $\beta$  and PR real-time RT-PCR products from *Bos taurus* were cloned separately in pCR-4.0 (Invitrogen, Leek, The Netherlands) and linearized by a unique restriction digest. Dilutions of each linear plasmid preparation from single ssDNA up to 10<sup>10</sup> ssDNA molecules (Table 3) were amplified in the individual calibration curves, as described previously [6,10].

### 2.7. Confirmation of primer specificity and sequence analysis

For exact length verification RT-PCR products were separated on 4% high resolution gel electrophoresis. Each of the amplified gradient MasterCycler (Eppendorf) and LightCycler (Roche Diagnostics) PCR products showed a single band of the expected length [6,10]. Specificity of the desired products was additionally documented with single peaks in melting curve analysis of LightCycler Software (Roche Diagnostics). Sequence analysis (MWG Biotech) of cloned real-time RT-PCR products from *B. taurus* showed 100% homology to the GenBank and EMBL published sequences.

### 2.8. Real-time RT-PCR assay validation

All applied real-time RT-PCR assays were product-specific, and effective PCR amplification kinetic was shown by high PCR efficiency per cycle (Table 3). Assay sensitivities were confirmed by detection limits lower than 14 ssDNA molecules and linear quantification ranges between 10<sup>2</sup> and 10<sup>9</sup> molecules. Intra- and inter-assay variation of maximal 30% were determined over the entire quantification range from 10<sup>2</sup> to 10<sup>9</sup> molecules. The advantage of a high temperature fluorescence acquisition in the fourth segment during the amplification program results in reliable

Table 2

Cycling conditions of AR, ER $\alpha$ , ER $\beta$  and PR in four-segment LightCycler real-time RT-PCR

Segment number	Name of segment	Duration (s)	Temperature profile (°C)			
			AR	ER $\alpha$	ER $\beta$	PR
I	Denaturation	15	95	95	95	95
II	Product-specific annealing	10	60	64	65	65
III	Elongation	20	72	72	72	72
IV	Fluorescence acquisition	5	83	82	86	81

Amplification and quantification programs are repeated 40 times with a single fluorescence acquisition point at elevated temperature (segment IV).

Table 3

Characteristics and validation parameters of real-time RT-PCR assays in bovine gastrointestinal compartments

	AR	ER $\alpha$	ER $\beta$	PR
Product length (bp)	172	234	262	227
Detection limit (mc)	<12	<2	<4	<14
Quantification limit (mc)	120	165	100	760
Quantification range (test linearity) (mc)	120–1.20 $\times 10^{10}$ ( $r = 0.996$ )	165–1.65 $\times 10^9$ ( $r = 0.995$ )	100–1.0 $\times 10^{10}$ ( $r = 0.997$ )	760–7.60 $\times 10^9$ ( $r = 0.998$ )
PCR efficiency	1.91	1.81	1.83	1.94
Intra-assay variation (%)	28.2 ( $n = 3$ )	18.7 ( $n = 4$ )	13.8 ( $n = 4$ )	5.7 ( $n = 4$ )
Inter-assay variation (%)	19.7 ( $n = 7$ )	28.6 ( $n = 4$ )	19.7 ( $n = 4$ )	25.7 ( $n = 4$ )

Here, mc: molecules,  $r$ : Pearson correlation coefficient. Intra-assay (test precision) and inter-assay variation (test variability) of assays were determined over the complete quantification range over several orders of magnitude. Detection limit, quantification limit and variations were based on molecule basis.

and sensitive steroid receptor mRNA-specific quantification with high linearity (Pearson correlation coefficient:  $r > 0.995$ ) over seven orders of magnitude. By high temperature fluorescence acquisition in the fourth segment the unspecific RT-PCR products melt between 81 and 86 °C (specific for each steroid receptor type) and the non-specific fluorescence signal derived from primer dimers disappear.

### 3. Results

#### 3.1. Tissue-specific ER $\alpha$ and ER $\beta$ mRNA expression

ER $\alpha$  and ER $\beta$  mRNA expression could be observed in all 10 gastrointestinal compartments. Table 4 summarizes the mean mRNA expression rates of the ER subtypes and the ER $\alpha$ /ER $\beta$  ratio of all eight investigated animals with the corresponding variation coefficients (CV = S.D./mean on molecule basis). Highest expression levels of both receptor subtypes were shown in rectum, colon and ileum (Fig. 1). The following expression abundance series could be quantified for ER $\alpha$ : rectum > colon > jejunum > caecum = duodenum > abomasum > omasum > ileum > rumen > reticulum. ER $\beta$  showed high abundance in rectum > ileum > colon, and very low abundant ex-

pression under 100 molecules (determined quantification limit for ER $\beta$ ) in the remaining ones. High ER $\alpha$ /ER $\beta$  ratios were examined in rumen > duodenum > reticulum. Medium ratios were shown in abomasum > jejunum  $\approx$  caecum > colon > omasum. An inverse relation, i.e. a higher ER $\beta$  than ER $\alpha$  expression, was shown only in ileum and rectum. To make the tissue-specific expression pattern evident for both ER subtypes, expression rates were plotted (Fig. 1) with bi-directional error bars (mean  $\pm$  S.E.M.). To determine the influence of estrogen treatment on mRNA expression levels, linear or exponential fit and corresponding coefficient of correlation ( $r$ ) between expression levels and increasing Ralgro<sup>®</sup> concentrations were calculated (Fig. 2a–d). Significant relationships of increasing estrogen concentrations and ER $\alpha$  expression were observed in abomasum (linear regression,  $r = 0.72$ ;  $P < 0.05$ ) and a decreasing relation in jejunum (exponential decay,  $r = 0.98$ ;  $P < 0.001$ ). ER $\beta$  showed increasing expression levels in rectum (exponential regression,  $r = 0.99$ ;  $P < 0.001$ ), decreasing expression abundance in jejunum (exponential decay,  $r = 0.87$ ;  $P < 0.05$ ) and a linear trend of regulation in reticulum ( $r = 0.68$ ;  $P = 0.06$ ; graph not shown). In the remaining gastrointestinal compartments no trends of change (linear or exponential) in neither ER $\alpha$  nor ER $\beta$  expression levels could be detected.

Table 4

Mean expression data shown in mRNA molecules per 25 ng total RNA, CV (in %) of AR, ER $\alpha$ , ER $\beta$ , PR and ER $\alpha$ /ER $\beta$  mRNA expression ratio of eight heifers

Tissue	Estrogen receptors				ER $\alpha$ /ER $\beta$	Androgen receptor		Progesterone receptor	
	Mean ER $\alpha$	CV ER $\alpha$ (%)	Mean ER $\beta$	CV ER $\beta$ (%)		Mean AR	CV AR (%)	Mean PR	CV PR (%)
Rumen	316	85	11 <sup>a</sup>	78	29.3	6131	73	n.d.	82
Reticulum	254	78	11 <sup>a</sup>	69	23.4	3941	51	n.d.	130
Omasum	517	40	79 <sup>a</sup>	52	6.5	8465	25	23 <sup>a</sup>	93
Abomasum	578	127	31 <sup>a</sup>	53	18.6	420	33	211 <sup>a</sup>	106
Duodenum	641	75	26 <sup>a</sup>	54	24.3	969	49	117 <sup>a</sup>	40
Jejunum	790	85	74 <sup>a</sup>	90	10.7	226	28	12 <sup>b</sup>	86
Ileum	412	83	1053	85	0.4	648	36	6 <sup>b</sup>	43
Caecum	642	135	61 <sup>a</sup>	148	10.5	395	45	2 <sup>b</sup>	80
Colon	1753	69	227	56	7.7	242	67	8 <sup>b</sup>	108
Rectum	2328	111	2418	169	0.96	577	74	12 <sup>b</sup>	87

Here, n.d.: mRNA not detected.

<sup>a</sup> Most mRNA samples under quantification limit.

<sup>b</sup> Some mRNA samples under detection limit.

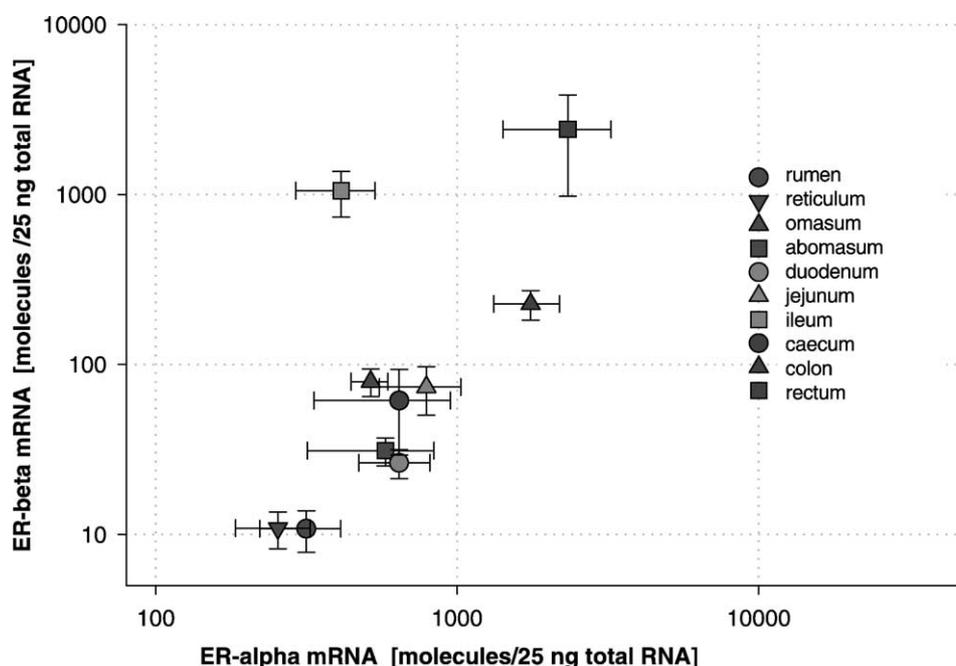


Fig. 1. Tissue-specific ER $\alpha$  and ER $\beta$  mRNA expression cluster in 10 different gastrointestinal compartments. Transcripts were measured on molecule number basis with real-time RT-PCR in 25 ng total RNA ( $n = 8$ ) and quantified results are shown as mean with bi-directional error bars (S.E.M.).

### 3.2. Tissue-specific AR and PR mRNA expression

AR mRNA expression could be detected in all investigated tissues. Table 4 summarizes the mean mRNA expression levels and CV. Highest AR mRNA expression was found in all three fore-stomachs with the abundance series: omasum > rumen > reticulum. Medium expression levels were quantified for: duodenum > ileum > rectum > abomasum > caecum > colon > jejunum. No trend of regulation of the AR expression under Ralgro<sup>®</sup> treatment was given in all investigated gastrointestinal compartments.

PR mRNA was very low abundant in bovine stomach and guts, and not detectable in rumen and reticulum with expression abundances under 14 molecules per capillary and 25 ng total RNA. Expression levels in omasum, abomasum and duodenum are within quantification range, but most of the gut compartments were under the quantification limit of the assay with 760 PR mRNA molecules per capillary, and therefore only a semi-quantitative measurement could be done. In the remaining gut compartments only a theoretic expression mean could be calculated, which is under the real detection limit. No trends of change in the mRNA expression levels under estrogen influence could be found for the PR.

## 4. Discussion

Treatment with Zeranol, known as the estrogen active component in Ralgro<sup>®</sup>, was chosen as a good model in heifers to mimic an estrogenic activity. Plasma Zeranol levels were measured by enzyme-immuno-assay and concen-

trations in treated animals resulted in measurable and elevated Zeranol levels in comparison to the control group [6,38]. Zeranol ( $\alpha$ -Zearalanol) possesses 50–60% of the estrogenic potencies of the natural ER ligand estradiol-17 $\beta$  [39,40]. Therefore, a 1-fold dosage results approximately to the physiological estrogen concentration in comparison to estradiol-17 $\beta$  occurring at cattle estrus. After application of a simple Ralgro<sup>®</sup> dose the estrus cycle obviously is arrested at pre-ovulatory stage. Multiple dosage results in a possible blockage of the estrus cycle, accompanied by distinctively small uterus sizes [6,41]. Estradiol-17 $\beta$  directly regulates the gonadotropin-releasing hormone (GnRH) expression at the level of GnRH neurons and may exert its neuroendocrine control through direct interaction with specific receptors expressed in these cells [42]. This clearly indicates the negative feedback mechanism on the gonadotropic axis, caused by high estrogen concentrations.

This paper focused on the tissue distribution and expression pattern of steroid receptors in the gastrointestinal tract in *B. taurus* under physiological conditions and under estrogen treatment. The steroid receptor AR, ER $\alpha$  and PR mRNA expression was measured with established quantitative kinetic RT-PCR assays [6,10]. RT followed by real-time PCR is a sensitive method to quantify low amounts of mRNA molecules and offers important insights into the local expression and para- and autocrine regulation. With regard to a better sensitivity and reliability herein new primers were designed for ER $\beta$ . The ER $\beta$  assay was validated on the Light-Cycler platform in 25 ng reverse transcribed total RNA and resulted in a more sensitive and reliable quantification, compared to earlier studies [6–8,10]. The sensitivity, linearity

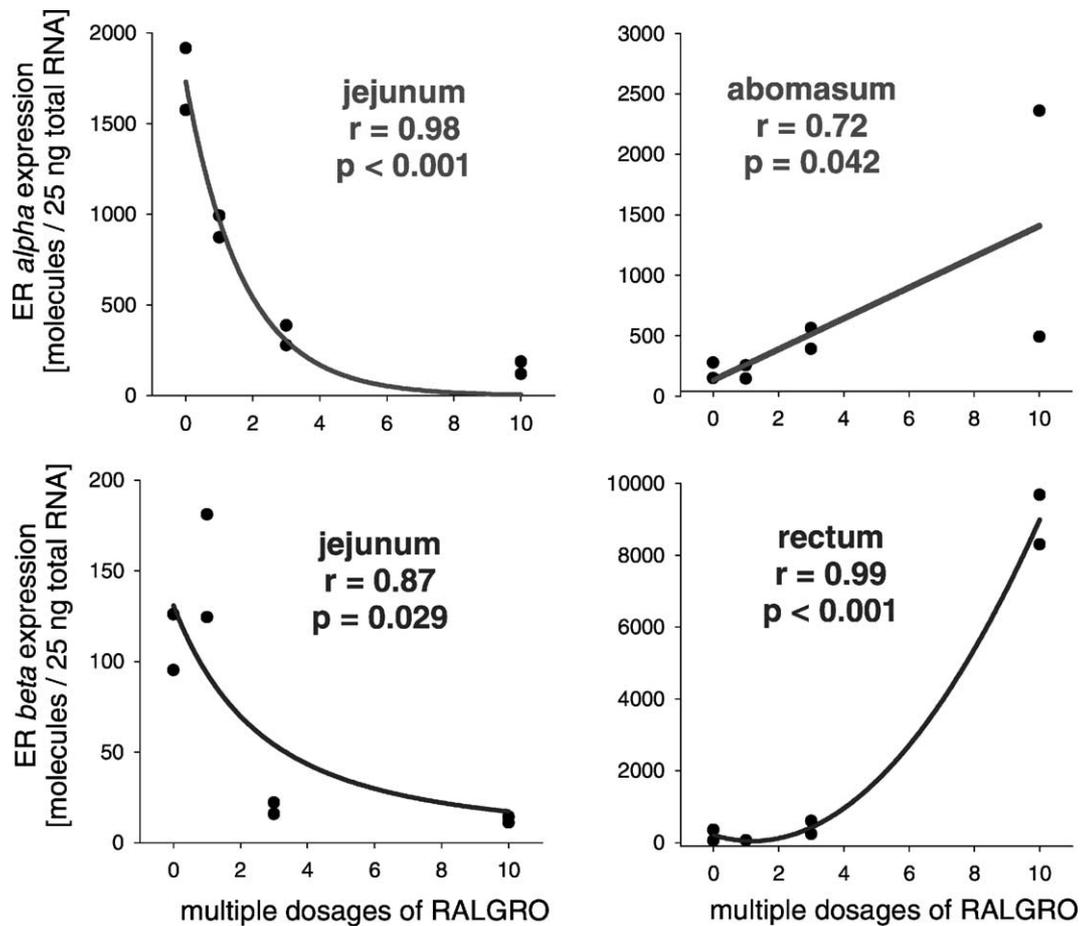


Fig. 2. Comparison between multiple Ralgro<sup>®</sup> treatment ( $1 \times 36$  mg Zeranol) and ER $\alpha$  mRNA expression in (a) jejunum and (b) abomasum and ER $\beta$  expression in (c) jejunum and (d) rectum. Relations are expressed in coefficient of correlation ( $r$ ) and level of significance ( $P$ -value).

and reproducibility of the applied assays allow for absolute and accurate quantification even in gastrointestinal tissues with very low mRNA levels down to a few molecules. We have used these assays to compare the expression rates in a multiple dosage Zeranol treatment in heifers. The presence and the amount of the steroid receptor mRNA in the tissue is the first determination of specificity and magnitude of response to active estrogens present in this tissue [1,43].

Except PR, all investigated receptor transcripts could be quantified with high accuracy. The PR mRNA quantities were mostly under the detection limit of the applied quantitative kinetic RT-PCR of 14 molecules per reaction. A successful quantification could only be performed in omasum, abomasum and duodenum. Integrity and reproducibility of the real-time PR assay were confirmed previously in uterus total RNA [10]. In mice the PR protein was quantified in several tissues via immuno-histochemistry under the influence of estrogen treatment. Neither stomach, nor duodenum, jejunum, ileum, colon, nor rectum showed a successful PR staining. Estrogen treatment had no effect on PR expression in the gastrointestinal tract [11]. Also in human and rabbit no PR expression could be found in small intestine and colon of healthy individuals [44]. But, PR and ER mRNA

were expressed at low levels in seven established gastrointestinal tumor lines and colon [45] and concentrations were strongly correlated in both cancers and normal tissues [46]. However, Ralgro<sup>®</sup> has no effects on PR expression level, but might have more influence on other members of the steroid receptor family.

Herein AR could be detected and quantified mainly in the three fore-stomachs. As shown in other studies, AR was present in liver [47], muscularity [7,8,10], and gastrointestinal tract [20,48], and therefore may mediate the action of sex hormones or androgenic steroids. In our study a significant coherence between increasing estrogen concentrations and AR mRNA expression could not be shown in all 10 gastrointestinal compartments. This suggests, that estrogens have no effect on the AR expression in the gastrointestinal tract.

Cloning of ER $\beta$  has introduced a new level of complexity of estrogen action. Since it was discovered, no definite statement regarding its expression pattern in the gastrointestinal tract and its unique function could be made. RT-PCR real-time assays with an external calibration curve are directly quantitative and therefore an absolute comparison of ER $\alpha$  and ER $\beta$  mRNA molecules is possible [35]. As demonstrated herein, ER $\alpha$  and ER $\beta$  kinetic RT-PCR,

meets the assay parameter requirements described in the tables with excellent performance. As shown previously [6], the detection and dominant expression of ER $\beta$  in kidney medulla, kidney cortex and in the jejunum leads to the hypothesis that ER $\beta$  plays a dominant role in kidney and the gastrointestinal tract. Other tissues were not influenced by estrogens [2,6,46,48]. However, notations on physiological direct effects of estrogens on gastrointestinal tissues remain speculative, but there are some indications, that estrogens may influence calcium transport and cell proliferation [14,15]. Herein a more detailed study of all 10 gastrointestinal compartments was performed with an optimized ER $\beta$  assay to increase sensitivity and reliability. Abomasum, rectum and particularly jejunum were shown to be very estrogen sensitive tissues with regard to the expression results of ER $\alpha$  and ER $\beta$ . All tissues showed a significant regulation of the receptor expression under multiple Ralgro<sup>®</sup> treatment. Due to the given results of a mainly non-significant relationship between estrogen and expression levels within one tissue all data were pooled ( $n = 8$ ). The derived mean expression concentrations and variations are characteristic for all investigated tissues and the relation of both ER subtypes results in a tissue-specific expression cluster. Each of the gut compartments possesses a characteristic ER $\alpha$  and ER $\beta$  expression pattern which stays relatively stable even under Zeranol treatment and results in a gastrointestinal ER $\alpha$ /ER $\beta$  expression cluster. Highest ER $\alpha$  and ER $\beta$  mRNA expression levels were derived in ileum, colon and rectum. ER protein concentrations and localization were investigated earlier [20] and showed high concentrations in colon, duodenum and ileum as well as a clear localization in bovine rumen. High mRNA and protein concentrations imply an important role in the cell proliferation in these tissues [22,49]. Only little is known about ER expression in the stomach. In rats both ER protein subtypes could be localized in the stomach [50]. ER mRNA is expressed in gastric mucosa of human and rats [48,50,51]. But in these references it is not properly distinguished between ER $\alpha$  and ER $\beta$  mRNA expression, and therefore the expression data can only provide an idea, if ER is expressed in general or not.

The authors conclude, that especially estrogens and the expression of their corresponding receptor subtypes may play an important role in the modulation and regulation in gastric as well as gut functions, cell proliferation and possibly in the pathophysiology of cell cancer. The functionalities of the ERs have to be demonstrated in further studies. The different expression patterns of ER $\alpha$  and ER $\beta$  can be regarded as support of the hypothesis that the subtype proteins may have different biological functions in the gastrointestinal tract. AR and PR seem to be not estrogen dependent.

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## References

- [1] M. Beato, P. Herrlich, G. Schutz, Steroid hormone receptors: many actors in search of a plot, *Cell* 83 (6) (1995) 851–857.
- [2] O.M. Conneely, Perspective: female steroid hormone action, *Endocrinology* 142 (6) (2001) 2194–2199.
- [3] E. Enmark, M. Peltö-Huikko, K. Grandien, S. Lagercrantz, J. Lagercrantz, G. Fried, M. Nordenskjöld, J.A. Gustafsson, Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern, *J. Clin. Endocrinol. Metab.* 82 (1997) 4258–4265.
- [4] J.A. Gustafsson, Estrogen receptor beta: a new dimension in estrogen mechanism of action, *J. Endocrinol.* 163 (1999) 379–383.
- [5] A.W. Brandenberger, M.K. Tee, J.Y. Lee, V. Chao, R.B. Jaffe, Tissue distribution of estrogen receptors alpha (ER-alpha) and beta (ER-beta) mRNA in the midgestational human fetus, *J. Clin. Endocrinol. Metab.* 82 (10) (1997) 3509–3512.
- [6] M.W. Pfaffl, I.G. Lange, A. Daxenberger, H.H.D. Meyer, Tissue-specific expression pattern of estrogen receptors (ER): quantification of ER alpha and ER beta mRNA with real-time RT-PCR, *APMIS* 109 (5) (2001) 345–355.
- [7] A.M. Brandstetter, M.W. Pfaffl, J.F. Hocquette, D.E. Gerrard, B. Picard, Y. Geay, H. Sauerwein, Effects of muscle type, castration, age, and compensatory growth rate on androgen receptor mRNA expression in bovine skeletal muscle, *J. Anim. Sci.* 78 (3) (2000) 629–637.
- [8] A. Malucelli, H. Sauerwein, M.W. Pfaffl, H.H.D. Meyer, Quantification of androgen receptor mRNA in tissues by competitive co-amplification of a template in reverse transcription-polymerase chain reaction, *J. Steroid Biochem. Mol. Biol.* 58 (5–6) (1996) 563–568.
- [9] A.O. Brinkmann, G.G. Kuiper, C. Ris-Stalpers, H.C. van Rooij, G. Romalo, M. Trifiro, E. Mulder, L. Pinsky, H.U. Schweikert, J. Trapman, Androgen receptor abnormalities, *J. Steroid Biochem. Mol. Biol.* 40 (1–3) (1991) 349–352.
- [10] M.W. Pfaffl, A. Daxenberger, M. Hageleit, H.H.D. Meyer, Effects of synthetic progestagens on the mRNA expression of androgen receptor, progesterone receptor, estrogen receptor  $\alpha$  and  $\beta$ , insulin-like growth factor (IGF)-1 and IGF-1 receptor in heifer tissues, *J. Vet. Med. A* 49 (2002) 57–64.
- [11] N. Uotinen, R. Puustinen, S. Pasanen, T. Manninen, M. Kivineva, H. Syvala, P. Tuohimaa, T. Ylikomi, Distribution of progesterone receptor in female mouse tissues, *Gen. Comp. Endocrinol.* 115 (3) (1999) 429–441.
- [12] P.J. Shughrue, M.V. Lane, P.J. Scrimo, I. Merchenthaler, Comparative distribution of estrogen receptor-alpha (ER-alpha) and beta (ER-beta) mRNA in the rat pituitary, gonad, and reproductive tract, *Steroids* 63 (10) (1998) 498–504.
- [13] S. Saji, H. Sakaguchi, S. Andersson, M. Warner, J.A. Gustafsson, Quantitative analysis of estrogen receptor proteins in rat mammary gland, *Endocrinology* 142 (2001) 3177–3186.
- [14] B.H. Arjmandi, M.A. Salih, D.C. Herbert, S.H. Sims, D.N. Kalu, Evidence for estrogen receptor-linked calcium transport in the intestine, *Bone Miner.* 21 (1993) 63–74.
- [15] G. Picotto, V. Massheimer, R. Boland, Acute stimulation of intestinal cell calcium influx induced by 17-beta-estradiol via the cAMP messenger system, *Mol. Cell Endocrinol.* 119 (2) (1996) 129–134.
- [16] M.L. Thomas, M.J. Ibarra, Effects of ovariectomy on duodenal calcium transport in the rat: altered ability to adapt to low-calcium diet, *Proc. Soc. Exp. Biol. Med.* 185 (1) (1987) 84–88.

- [17] M.L. Thomas, W.G. Hope, M.J. Ibarra, The relationship between long bone growth rate and duodenal calcium transport in female rats, *J. Bone Miner. Res.* 3 (5) (1988) 503–507.
- [18] R. Dahiya, T.A. Brasitus, Estrogen-induced alterations of hematoside of rat small intestinal mucosa, *Biochim. Biophys. Acta* 918 (3) (1987) 230–235.
- [19] H. Cheng, M. Bjerknes, Variation of mouse intestinal epithelial whole population cell kinetics during the estrous cycle, *Anat. Rec.* 220 (4) (1988) 397–400.
- [20] H. Sauerwein, M.W. Pfaffl, K. Hagen-Mann, A. Malucelli, H.H.D. Meyer, Expression of estrogen and androgen receptor in the bovine gastrointestinal tract, *Dtsch. Tierarztl. Wochenschr.* 102 (4) (1995) 164–168.
- [21] M.G. Catalano, U. Pfeffer, M. Raineri, P. Ferro, A. Curto, P. Capuzzi, F. Corno, L. Berta, N. Fortunati, Altered expression of androgen-receptor isoforms in human colon-cancer tissues, *Int. J. Cancer* 86 (3) (2000) 325–330.
- [22] M. Campbell-Thompson, J.J. Lynch, B. Bhardwaj, Expression of estrogen receptor (ER) subtypes and ERbeta isoforms in colon cancer, *Cancer Res.* 61 (2) (2001) 632–640.
- [23] S. Mosselman, J. Polman, R. Dijkema, ER beta: identification and characterization of a novel human estrogen receptor, *FEBS Lett.* 392 (1996) 49–53.
- [24] G.G. Kuiper, E. Enmark, M. Peltö-Huikko, S. Nilsson, J.A. Gustafsson, Cloning of a novel receptor expressed in rat prostate and ovary, *Proc. Natl. Acad. Sci. U.S.A.* 93 (1996) 5925–5930.
- [25] P. Walter, S. Green, G. Greene, A. Krust, J.M. Bornert, J.M. Jeltsch, A. Staub, E. Jensen, G. Scrace, M. Waterfield, Cloning of the human estrogen receptor cDNA, *Proc. Natl. Acad. Sci. U.S.A.* 82 (1985) 7889–7893.
- [26] S. Green, P. Walter, G. Greene, A. Krust, C. Goffin, E. Jensen, G. Scrace, M. Waterfield, P. Chambon, Cloning of the human oestrogen receptor cDNA, *J. Steroid Biochem.* 24 (1986) 77–83.
- [27] H. Takeda, G. Chodak, S. Mutchnik, T. Nakamoto, C. Chang, Immunohistochemical localization of androgen receptors with mono- and polyclonal antibodies to androgen receptor, *J. Endocrinol.* 126 (1) (1990) 17–25.
- [28] H. Takeda, T. Nakamoto, J. Kokontis, G.W. Chodak, C. Chang, Autoregulation of androgen receptor expression in rodent prostate: immunohistochemical and in situ hybridization analysis, *Biochem. Biophys. Res. Commun.* 177 (1) (1991) 488–496.
- [29] V.E. Quarby, W.G. Yarbrough, D.B. Lubahn, F.S. French, E.M. Wilson, Autologous down-regulation of androgen receptor messenger ribonucleic acid, *Mol. Endocrinol.* 4 (1) (1990) 22–28.
- [30] O.M. Conneely, B.L. Maxwell, D.O. Toft, W.T. Schrader, B.W. O'Malley, The A and B forms of the chicken progesterone receptor arise by alternate initiation of translation of a unique mRNA, *Biochem. Biophys. Res. Commun.* 149 (2) (1987) 493–501.
- [31] W. Schneider, C. Ramachandran, P.G. Satyaswaroop, G. Shyamala, Murine progesterone receptor exists predominantly as the 83-kilodalton 'A' form, *J. Steroid Biochem. Mol. Biol.* 38 (1991) 285–291.
- [32] E. Vegeto, M.M. Shahbaz, D.X. Wen, M.E. Goldman, B.W. O'Malley, D.P. McDonnell, Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function, *Mol. Endocrinol.* 7 (1993) 1244–1255.
- [33] O.M. Conneely, J.P. Lydon, F. de Mayo, B.W. O'Malley, Reproductive functions of the progesterone receptor, *J. Soc. Gynecol. Invest.* 7 (1) (2000) S25–S32.
- [34] C.M. Peterson, Estrogen and progesterone receptors: an overview from the year 2000, *J. Soc. Gynecol. Invest.* 7 (1) (2000) S3–S7.
- [35] M.W. Pfaffl, M. Hageleit, Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR, *Biotechnol. Lett.* 23 (2001) 275–282.
- [36] J.M. Chirgwin, E.A. Przybyla, J. MacDonald, W.J. Rutter, Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases, *J. Biochem.* 18 (1979) 5291–5299.
- [37] M.W. Pfaffl, Development and validation of an externally standardised quantitative insulin like growth factor-1 (IGF-1) RT-PCR using LightCycler SYBR<sup>®</sup> Green I technology, in: S. Meuer, C. Wittwer, K. Nakagawara (Eds.), *Rapid Cycle Real-time PCR, Methods and Applications*, Springer, Heidelberg, 2001, pp. 281–291.
- [38] I.G. Lange, A. Daxenberger, H.H.D. Meyer, Hormone contents in peripheral tissues after correct and off-label use of growth promoting hormones in cattle: effect of the implant preparations Finaplix-H<sup>®</sup>, Ralgro<sup>®</sup>, Synovex-H<sup>®</sup> and Synovex Plus<sup>®</sup>, *APMIS* 109 (2001) 53–65.
- [39] A. Arukwe, T. Grotmol, T.B. Haugen, F.R. Knudsen, A. Goksoyr, Fish model for assessing the in vivo estrogenic potency of the mycotoxin Zearalenone and its metabolites, *Sci. Total Environ.* 236 (1999) 153–161.
- [40] M. Seifert, S. Haindl, B. Hock, In vitro analysis of xenoestrogens by enzyme linked receptor assays (ELRA), *Adv. Exp. Med. Biol.* 444 (1998) 113–117.
- [41] C. Moran, D.J. Prendiville, J.F. Quirke, J.F. Roche, Effects of oestradiol, zeranol or trenbolone acetate implants on puberty, reproduction and fertility in heifers, *J. Reprod. Fertil.* 89 (1990) 527–536.
- [42] D. Roy, N.L. Angelini, D.D. Belsham, Estrogen directly represses gonadotropin-releasing hormone (GnRH) gene expression in estrogen receptor-alpha (ERalpha)- and ERbeta-expressing GT1-7 GnRH neurons, *Endocrinology* 140 (1999) 5045–5053.
- [43] R.J. King, Effects of steroid hormones and related compounds on gene transcription, *Clin. Endocrinol.* 36 (1992) 1–14.
- [44] M.F. Press, G.L. Greene, Localization of progesterone receptor with monoclonal antibodies to the human progestin receptor, *Endocrinology* 122 (3) (1988) 1165–1175.
- [45] E. Jacobs, S.A. Watson, J.D. Hardcastle, J.F. Robertson, Oestrogen and progesterone receptors in gastrointestinal cancer cell lines, *Eur. J. Cancer* 32A (13) (1996) 2348–2353.
- [46] S. Singh, M.C. Sheppard, M.J. Langman, Sex differences in the incidence of colorectal cancer: an exploration of oestrogen and progesterone receptors, *Gut* 34 (5) (1993) 611–615.
- [47] C. Cohen, D. Lawson, P.B. DeRose, Sex and androgenic steroid receptor expression in hepatic adenomas, *Hum. Pathol.* 29 (1998) 1428–1432.
- [48] P. Waliszewski, M. Blaszczyk, E. Wolinska-Witort, M. Drews, M. Snochowski, R.E. Hurst, Molecular study of sex steroid receptor gene expression in human colon and in colorectal carcinomas, *J. Surg. Oncol.* 64 (1) (1997) 3–11.
- [49] N. Arai, A. Strom, J.J. Rafters, J.A. Gustafsson, Estrogen receptor beta mRNA in colon cancer cells: growth effects of estrogen and genistein, *Biochem. Biophys. Res. Commun.* 270 (2) (2000) 425–431.
- [50] M. Campbell-Thompson, K.K. Reyher, L.B. Wilkinson, Immunolocalization of estrogen receptor alpha and beta in gastric epithelium and enteric neurons, *J. Endocrinol.* 171 (1) (2001) 65–73.
- [51] S. Singh, R. Poulosom, N.A. Wright, M.C. Sheppard, M.J. Langman, Differential expression of oestrogen receptor and oestrogen inducible genes in gastric mucosa and cancer, *Gut* 40 (4) (1997) 516–520.