Title Ontogeny and nutritional programming of the hepatic growth hormone-insulin-like growth factor-prolactin axis in the sheep

Short title: GH-IGF axis and liver development

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Abstract

The liver is an important metabolic and endocrine organ in the fetus but the extent to which its hormone receptor (R) sensitivity is developmentally regulated in early life is not fully established. We, therefore, examined developmental changes in mRNA abundance for the growth hormone (GH) and prolactin (PRL) receptors (R) plus insulin-like growth factor (IGF)-I and –II and their receptors. Fetal and postnatal sheep were sampled at either 80, or 140 days gestation, 1, 30 days or six months of age. The effect of maternal nutrient restriction between early to mid (i.e. 28 to 80 days gestation, the time of early liver growth) gestation on gene expression was also examined in the fetus and juvenile offspring. Gene expression for the GHR, PRLR and IGF-IR increased through gestation peaking at birth, whereas IGF-I was maximal near to term. In contrast, IGF-II mRNA decreased between mid and late gestation to increase after birth whereas IGF-IIR remained unchanged. A substantial decline in mRNA abundance for GHR, PRLR and IGF-IR then occurred up to six months. Maternal nutrient restriction reduced GHR and IGF-IIR mRNA abundance in the fetus, but caused a precocious increase in the PRLR. Gene expression for IGF-I and –II were increased in juvenile offspring born to nutrient restricted mothers. In conclusion, there are marked differences in the developmental ontogeny and nutritional programming of specific hormones and their receptors involved in hepatic growth and development in the fetus. These could contribute to changes in liver function during adult life.

Key words: Growth hormone, insulin-like growth factor, prolactin receptor

Introduction

There are pronounced developmental changes in the expression of many genes that have a critical role in both fetal development as well as ensuring the newborn effectively adapts to the extrauterine environment (1-3). It is now becoming apparent, for some specific genes such as the glucocorticoid receptor (GR), that changes in gene expression continue through to juvenile life and vary greatly between tissues (4, 5). The magnitude of these developmental adaptations is partly determined by organ growth so that, in adipose tissue, which is one of the fastest growing tissues after birth, glucocorticoid action continues to increase between neonatal and juvenile life (5). In contrast, in the lung with its much slower rate of postnatal growth, the reverse adaptation occurs, with glucocorticoid action decreasing after the neonatal period (4). These differences are related, in part, to functions within the inner mitochondria and changes in uncoupling protein abundance. Although the liver does not possess uncoupling proteins (6), it grows as rapidly as some adipose tissue depots after birth (7). The extent to which this period of substantial growth is related to changes in gene expression of the major counter-regulatory hormones, or their receptors, is not known.

It is established that hepatic mRNA abundance for a number of hormones and their receptors, which have a critical role in metabolism and tissue growth, increase near to term in parallel with the pre-partum surge in glucocorticoids (1, 8). These include growth hormone (GH) (9) and prolactin (PRL) receptors (R) (10), together with IGF-I (11) whereas, in contrast, IGF-II is negatively regulated (12). The extent to which such adaptations are confined to the period immediately before birth, or whether they may also change between mid and late gestation, or soon after birth, has yet to be

examined. Determining the fetal and postnatal ontogeny of the aforementioned genes is not only important in understanding the hepatic adaptations involved in the transition form intra- to extra-uterine life but will also enhance our ability to interpret the immediate and long term effects of maternal nutritional manipulation at different stages of gestation (13, 14). In this regard, maternal nutrient restriction between early to mid gestation results in adult offspring in which liver mass is reduced in conjunction with decreased gene expression for a number of important metabolic regulators including the GHR, PRLR and IGF-IIR (15). The extent to which the mRNA abundance for both these and related (i.e. IGF-I, IGF-II and IGF-IR) genes that are unaffected in the adult, may be reset in utero in response to a shorter period of maternal nutrient restriction is currently not known. In contrast, maternal nutrient restriction in late gestation has little impact on hepatic gene expression in the neonate (16).

The aim of our study was, therefore, to examine the ontogenic changes in gene expression of hepatic GHR, PRLR, GR, IGF-I and -II and their receptors between mid gestation through to term, after birth and then up to six months of juvenile life. This was accompanied by an examination of the effects of maternal nutrient restriction between early to mid gestation, coincident with the period of maximal placental growth plus early hepatic growth, on later liver development. In this second part of the study, the fetal liver was examined both at the end of the period of nutrient restriction i.e. 80 days gestation as well as 60 days later i.e. near to term and, subsequently, in the resulting juvenile offspring.

Methods

Ontogeny of liver development

For the ontogeny study, a mixture of Welsh Mountain and Border Leicester cross Swaledale sheep were used. We have previously established that, with respect to the molecular measurements made in the present study, there are no distinguishable differences between breeds at the same developmental age (4, 5, 17). The liver was sampled from fetuses at 80 and 140 days gestation (term ~148 days), and sheep after birth at 1, 30 and 180 days (6 months) of postnatal age (n = 6 at each sampling point, 30 sheep in total), following euthanasia with an overdose of barbiturate (100 mg/kg pentobarbital sodium: Euthatal: RMB Animal Health, UK). All mothers were fed 100% of their total metabolisable energy (ME) requirements (taking into account requirements for both maternal maintenance and growth of the conceptus in order to produce a 4.5 kg newborn at term (18)). Sheep randomised to postnatal sampling were born normally at term. The liver was rapidly dissected, weighed and a representative portion (i.e. 2 g from an 80 day fetus and 20 g at all other ages) from the same position in the right hepatic lobe from each animal placed in liquid nitrogen and stored at -80^{o}C until molecular analysis.

Maternal nutritional manipulation of hepatic gene expression

This study was designed to examine the effects of early to mid gestational nutrient restriction, coinciding with the period of maximal placental growth, on the fetus and offspring and thus utilised singleton bearing sheep. Thirty-six singleton bearing Welsh Mountain sheep of similar age (median 3 years) and weight (36.1 ± 0.9 kg (mean ± SEM)) were entered into the study and individually housed at 28 days gestation, as

described by Bispham et al. (2003). Animals were allocated to one of two nutritional groups using stratified randomisation by body weight. They consumed either 60% (i.e. nutrient restricted, NR) or 150% (i.e. fed to appetite) of their calculated ME requirements (Agricultural Research Council, 1980). Feed intakes were measured daily. Food consumption between 28 and 80 days gestation was 3.2 – 3.8 MJ/day of ME in the NR group (~ 60% of ME requirements) or 8.7 – 9.9 MJ/day of ME in the group fed to appetite (~ 150% of ME requirements) (19). The amount of feed given to each mother was increased at 43 and 61 days gestation to meet the higher energy requirements associated with growth of the conceptus (Agricultural Research Council, 1980). The diet comprised chopped hay that had an estimated ME content of 7.91 MJ/kg dry matter and a crude protein content (nitrogen x 6.25) of 69 g/kg dry matter and barley-based concentrate that had an estimated ME content of 11.6 MJ/kg dry matter and a crude protein content of 162 g/kg dry matter. The proportion of hay to concentrate fed was approximately 3:1, with respect to dry weight. All diets contained adequate minerals and vitamins. These were added separately to the diet with equal amounts provided to all sheep and, thus, were sufficient to fully meet their requirements. After 80 days gestation, all sheep were offered sufficient feed to meet 100% of the ME requirements. They consumed between 6.5 – 7.5 MJ/day of ME with the amount of feed provided being increased at 100 and 120 days gestation to meet the increased ME requirements that accompany the increase in fetal weight with gestation. In those pregnant sheep allowed to go to term, all gave birth normally and the offspring were weaned at 3 months of age. Throughout lactation, all mothers were fed to requirements with hay provided ad libitum together with 1 kg concentrate.

In order to determine the effect of early to mid gestational maternal nutrient restriction on fetal liver development, 6 sheep within each nutrition group were randomised for tissue sampling at either 80 or 140 days gestation. Each animal was humanely euthanised following intravenous administration of 100 mg/kg pentobarbital sodium. The fetus was rapidly dissected, weighed and a representative portion of the liver placed in liquid nitrogen and stored at –80^{o}C until further analysis. The remaining offspring (n = 6 per nutritional group) were sampled at 180 days (6 months) after birth.

All operative procedures and experimental protocols had the required Home Office approval as designated by the Animals (Scientific Procedures) Act (1986) and were approved by the animal experimentation ethics committee at the University of Nottingham.

Laboratory procedures

Messenger RNA detection

Total RNA was isolated from a central region of the right hepatic lobe (see above) using Tri-Reagent (Sigma, Poole, UK). In order to maximise sensitivity, a two-tube approach to reverse transcription (RT) was adopted as previously described for mRNA detection in the ovine liver (15). The conditions used to generate first strand cDNA RT were: 70°C (5 min), 4°C (2 min), 25°C (5 min), 4°C (2 min), 25°C (10 min), 42°C (1 hour), 72°C (10 min), 4°C (5 min). The optimised RT reaction (final volume 20 μl) contained: 1 x reverse transcriptase buffer (250 mM Tris-HCl, 40 mM $MgCl_2$, 150

mM KCl, 5 mM dithioerythritol pH 8.5), 2 mM dNTPs, 1 x hexanucleotide mix, 10 units RNase inhibitor, 10 units M-MLV reverse transcriptase and 1 μg total RNA. All these commercially available products were purchased from Roche Diagnostics Ltd (Lewes, UK).

The expression of each gene was determined by RT-polymerase chain reaction (RT-PCR), as previously described for the liver (15, 16). The analysis used oligonucleotide cDNA primers to each gene of interest by generating specific exon-intron spanning products (see Table 1). Briefly, the PCR programme consisted of an initial denaturation (95 °C (15 min)), amplification (stage I, 94 °C (30 s); stage II, annealing temperature (30 s); stage III, 72 °C (60 s)) and final extension (72 °C (7 min); 8 °C 'hold'). The PCR mixture (final volume 20 ul) contained 7 ul DEPC H_2O, 10 ul Thermo-Start PCR Master Mix® (50 ul contains 1.25 units Thermo-Start® DNA Polymerase, 1 x Thermo-Start® reaction buffer, 1.5 mM $MgCl_2$ and 0.2 mM each of dATP, dCTP, dGTP and dTTP, catalogue number AB-0938-DC-15 ABgene®), 500 nM Forward Primer, 500 nM Reverse Primer and 1 ul RT (cDNA; 2 ng) product. The amount of cDNA used for PCR reactions, annealing temperature and cycle numbers of all primers were optimised so as to be in the same linear range as the internal control, that is, 18S ribosomal RNA (rRNA) as previously described (15, 16). There were no developmental or nutritional effects on the mRNA abundance of hepatic 18S rRNA.

Agarose gel electrophoresis (2.0 – 2.5%) and ethidium bromide staining confirmed the presence of both the product of interest and 18S at the expected sizes. Densitometric analysis was performed on each gel by image detection using a Fujifilm LAS-1000

cooled charge-coupled device camera and mRNA abundance determined for each gene. Consistency of lane loading for each randomly loaded sample was verified from the measurement of 18S rRNA. All results were calculated as a ratio of its own 18S rRNA abundance and expressed as a percentage of a reference sample (hepatic RNA extracted from a 1 day old control sheep) ran on all gels. Each analysis was performed in duplicate with appropriate positive (same as reference sample) and negative (RT: (a) no RNA, (b) no reverse transcriptase; PCR: (a) no cDNA, (b) no taq polymerase) controls and a full range of molecular weight markers. The resultant PCR product was extracted (QIAquick gel extraction kit, catalogue number 28704), sequenced and results cross-referenced against the Genebank database to determine specificity of the target gene. For those genes in which we have previously validated their detection in sheep samples using real-time PCR (20) e.g. the GR, representative samples were reanalysed and in each case confirmed the RT-PCR results with regard to the molecular responses described below.

Protein detection

Plasma membrane, mitochondria and whole cell lysate were prepared from liver tissue sampled from each fetus and sheep (21) and their protein contents was determined using the Lowry protocol (22). The abundance of PRLR protein was determined on 10 ug of plasma membrane protein by Western blot analysis using the R120 polyclonal antibody (23). The intra- and inter-assay coefficient of variation for immunodetection of PRLR was 2.7% and 5.9% respectively. Further Western blots were performed to confirm GHR, IGF-IIR, IGF-IR and GR mRNA findings. Antibodies used included: (a) two GHR antibodies: a mouse anti-rabbit polyclonal

antibody (RDI-GHRabm-5, Research Diagnostics Inc, USA) and a polyclonal anti-mouse GHR (gift from G. Thordarson, University of California, Santa Cruz), (b) an IGF-IIR polyclonal antibody (gift from P. Lobel, Rutgers University, New Jersey as previously described (24)) (c) an IGF-IR polyclonal anti-rabbit antibody: Santa Cruz (catalogue number SC 713) and (d) a GR polyclonal anti-rabbit antibody: Santa Cruz (catalogue number SC 8992). Each antibody was tested at a range of dilutions from 1:250 – 1:1,000 under both mild-reducing and non-reducing conditions on up to 80 μg of relevant protein preparations. Unfortunately, none of these antibodies yielded a signal (in any of the animals) that was in accord with its predicted molecular weight (i.e. signals were non-specific). Non-specificity of detected bands was confirmed through regression analysis of molecular weight markers to determine exact size and through incubation with non-immune rabbit serum. All Western blots were run in duplicate and included a range of molecular weight markers and a reference sample (appropriate protein preparation from a one day old sheep) to allow for comparisons between gels.

Statistical Analyses

Statistical analysis was performed using SPSS software package (version 15.0). Data was firstly subjected to a Kolmogorov-Smirnov normality test to confirm normal distribution. Where appropriate, data were $\log_{10}$ transformed to achieve normality, thereby allowing parametric statistical tests to be performed. Liver weight and mRNA abundances obtained in the ontogeny study were compared by a Restricted Maximum Likelihood (REML) analysis through SPSS univariate General Linear Model test followed by Bonferroni post hoc correction. The terms fitted to the model were

developmental age, genotype and gender. There were no differences in mean values obtained from male and female offspring within each age group. Therefore, data were pooled from these animals. Mean values for organ weight and abundance of target genes between control and nutrient restricted offspring within each age group were compared using a Student's unpaired t test. For consistency, all data are presented as untransformed means and their standard errors. For all statistical comparisons, significance was accepted when $P<0.05$.

Results

Ontogeny of the hepatic GH-IGF axis

GHR, PRLR and GR

Both GHR and GR mRNA abundance within the liver increased from mid gestation to peak one day after birth and then declined up to six months of age (Figure 1). A similar pattern of changes was observed for the PRLR with the modification that mRNA and protein abundance peaked at 140 days gestation rather than after birth. These adaptations were unrelated to changes in liver mass which increased between 80 and 140 days gestation, remained unchanged at birth and then rose up to six months of age (Table 1).

IGF-I, IGF-II and their receptors

The mRNA abundance for hepatic IGF-I exhibited a large increase between 80 and 140 days gestation, but then remained unchanged up to six months of age (Figure 2). In contrast, mRNA abundance for the IGF-IR increased between late gestation and one day after birth to then decline up to six months of age. The ontogeny of gene expression for IGF-II and its receptor was very different to that of IGF-I in that mRNA abundance decreased between 80 and 140 days gestation only to then increase after birth and remain unchanged thereafter. In contrast, there was no effect of developmental age on IGF-IIR mRNA abundance.

Effect of early to mid gestational maternal nutrient restriction on hepatic mRNA abundance for GHR, PRLR, IGF-I, II and their receptors in the fetus and juvenile animal

Liver weight and composition was similar between nutritional groups at each sampling age (data not shown). Gene expression analysis revealed hepatic GHR was consistently lower in offspring sampled from nutrient restricted mothers although this difference was only statistically significant at 140 days gestation (Figure 3). In marked contrast, PRLR mRNA and protein was greater in the livers of nutrient restricted fetuses at 80 days gestation but substantially lower near to term as a consequence of a failure to exhibit any developmental increase with gestational age. By six months of age, however, there was no difference between nutritional groups.

There was no effect of maternal nutrition on gene expression for either IGF-I or its receptor in the fetus. However, by 6 months of age hepatic IGF-I (but not IGF-IR) was significantly raised in offspring born to previously nutrient restricted mothers (Figure 4). A similar pattern of developmental changes, as seen for IGF-I, was also observed for IGF-II. In contrast, hepatic IGF-IIR was significantly reduced in nutrient restricted fetuses, an adaptation that did not persist into juvenile life.

Discussion

We have clearly established the ontogeny of a number of genes that have a critical role in regulating both liver growth and development in utero as well as postnatal growth and endocrine sensitivity (8, 25). The period immediately around the time of birth is a key time point in this process as the liver undergoes a series of maturational processes that ensure adaptation after birth including the onset of hepatic gluocneongenesis (1). With respect to some of the major hormones and their receptors that are implicated in this process, hepatic GHR, PRLR and IGF-IIR were all markedly down regulated near to term following a period of maternal nutrient restriction that ended 60 days earlier. The magnitude of this adaptation is emphasised by our previous finding (incorporating a more prolonged period of nutrient restriction) that the mRNA abundance for each of these genes remains reduced in the resulting adult when its liver mass is decreased (15).

Taken together, a number of aspects of our study are novel. Firstly, gene expression of both GHR and PRLR remained low near to term in previously nutrient restricted fetuses. This is surprising as the developmental increase in both GHR and PRLR are primarily mediated by the prepartum surge in cortisol (9, 10) and there is currently no evidence that plasma cortisol is different between control and previously nutrient restricted offspring (26, 27). Indeed, glucocorticoid action is increased in the livers, as well as adipose tissue, kidneys and lungs of neonates born to mothers nutrient restricted between early to mid gestation (28). Secondly, ontogenic and nutritional manipulation of hepatic PRLR is similar in both its gene and protein expression which lends support to mRNA data for the other genes we examined but are currently unable

to confirm protein expression due to the lack of credible antibodies. Furthermore, we find no differences in GHR, PRLR or GR mRNA abundance in juvenile offspring born to nutrient restricted mothers. This may reflect a transitional period in metabolic adaptation in these animals prior to their attaining mature body weight. Indeed, at this age, only hepatic IGF-I and -II mRNA abundance was significantly higher and may, thus, be the dominant early hepatic growth factors before declining through to adulthood (29), in conjunction with loss of mRNA for the IGF-IR, but maintained IGF-IIR. Taken together, these adaptations would limit liver growth in the adult (15).

It is unlikely that the potential mechanisms by which hepatic gene expression was reset in nutrient restricted fetuses reflects a prolonged change in plasma cortisol, because, as discussed above, there is no evidence to date that this is increased in either nutrient restricted animals either during fetal (27) or later (30) life. Indeed, the fact that hepatic GHR and PRLR were significantly decreased near to term, but not at the time when maternal nutrient intake was reduced, suggests that the endocrine sensitivity to the prevailing plasma cortisol concentration was reset. In this regard, an increase in potential glucocorticoid action in the liver at 80 days of gestation (28) would potentially reduce hepatic sensitivity to the prepartum surge in cortisol, thus explaining why mRNA abundance for potentially glucocorticoid sensitive genes was markedly reduced in late gestation. An adaptation of this type was most apparent for the PRLR with maternal nutrient restriction causing a precocious increase in mRNA and protein abundance at 80 days gestation with no further increase up to 140 days gestation.

A markedly different adaptation was observed for the IGF-IIR in which mRNA abundance was lower at 80 days gestation and remained so up to 140 days gestation. The IGF-IIR gene, however, exhibited a much smaller increase with gestational age compared to the PRLR indicating very different transcriptional regulation and, thereby, potentially explaining differential responses between genes. It may also appear surprising that the reduction in IGF-IIR mRNA was not accompanied by any change in liver growth as, under the artificial conditions of in vitro fertilisation, reduced IGF-IIR increases IGF-II availability and promotes fetal organ growth (24). The same type of adaptation does not appear to be apparent in the nutritionally manipulated fetus or the resulting offspring and suggests very different IGF-II regulation between in vitro fertilised and naturally conceived pregnancies. The lack of any nutritional effect on either liver weight in the fetus or the offspring is in accord with previous studies in which fetal number has been kept constant between nutritional groups (28) but not when a mix of singletons and twins are used (31). It, thus, appears that the reduced liver mass seen in adults born to mothers nutrient restricted between early to mid gestation is an adult adaptation that is mediated by both a reduction in mitogenic stimulation of the liver together with enhanced apoptosis (15).

Of the genes that were not nutritionally responsive in utero (i.e. IGF-I, II and IGF-IR), all appear to show a different ontogeny compared with those that were sensitive to the maternal nutritional environment (i.e. GHR, PRLR, GR and IGF-IIR). Hepatic IGF-II mRNA undergoes a pronounced decrease in gene expression prior to birth which

reflects the negative influence of cortisol (12). The increase in IGF-II gene expression that then occurs between birth and one month of age may be mediated by the pronounced decrease in plasma cortisol over this period (32). In contrast, hepatic IGF-I and IGF-IR mRNA levels were very low at 80 days of gestation and, as such, could be predicted to be nutritionally insensitive in the fetus at this time. These two genes then exhibit differing developmental changes up to one day of postnatal age with hepatic IGF-IR mRNA showing an exponential increase. In contrast, IGF-I mRNA abundance rose up to 140 days gestation but remained unchanged thereafter up to six months of age. Expression of IGF-IR mRNA, however, rapidly decreased after birth and is no longer detectable in the adult liver (15). Taken together, these differences reflect the very different regulation of gene expression during a time when rapid liver growth occurs.

In conclusion, we have shown for the first time the ontogeny of the primary metabolic regulators of liver growth from the mid gestational fetus up to juvenile life. This has enabled us dissection of the differential effects of maternal nutrient restriction over the period of early liver growth in the fetus. As such, the very different responses between the genes examined can be explained by the relative mRNA abundance at the time of this nutritional intervention, or by the subsequent change in gene expression up to the time of birth. At this time, the majority of genes exhibit maximal abundance necessary for ensuring the onset of liver function very soon after birth.

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Figure Legends

Figure 1. Ontogeny of hepatic (a) growth hormone receptor (GHR) (b) prolactin receptor (PRLR) (c) glucocorticoid receptor (GR) and (d) PRLR protein abundance in the sheep. Values are means with their standard errors and n=6 at each sampling point. Representative gel images for each gene at every age are also shown. Different letters denote statistical significant differences ($P < 0.05$) as assessed by REML analysis with Bonferroni *post hoc* comparisons.

Figure 2. Ontogeny of hepatic (a) insulin-like growth factor (IGF)-I (b) IGF-II, (c) IGF-I receptor (R) and (d) IGF-IIR mRNA abundance in the sheep. Values are means with their standard errors and n=6 at each sampling point. Representative gel images for each gene at every age are also shown. Different letters denote statistical significant differences ($P < 0.05$) as assessed by REML analysis with Bonferroni *post hoc* comparisons.

Figure 3. Effect of early to mid gestational maternal nutrient restriction on hepatic (a) GHR mRNA, (b) PRLR mRNA and (c) PRLR protein abundance in the fetal liver at 80 and 140 days gestation (dGA; term 147 dGA) and juvenile sheep at six months of age. Representative gel images for each gene at every age are also shown. Values are mean with their standard errors (n = 6 per group). ***** $P < 0.05$, mean value significantly different from control group (represented by the closed bars) at the same gestational age.

Figure 4. Effect of early to mid gestational maternal nutrient restriction on hepatic (a) IGF-I (b) IGF-II, (c) IGF-IR and (d) IGF-IIR mRNA abundance in the fetal liver at 80

and 140 days gestation (dGA; term 147 dGA) and juvenile sheep at six months of age. Representative gel images for each gene at every age are also shown. Values are mean with their standard errors (n = 6 per group). ***** $P < 0.05$, mean value significantly different from control group (represented by the closed bars) at the same gestational age

Table 1

Total body, absolute and relative liver weight, RNA and mitochondrial protein content of liver tissue from fetuses sampled at 80 and 140 days gestation and sheep at 1 and 30 days and six months of postnatal age (n = 6 per sampling point).

	Fetus		Postnatal Sheep		
	80 days	140 days	1 day	30 days	180 days
Body weight (kg)	0.21 ± 0.008	4.91 ± 0.21	4.60 ± 0.20	16.41 ± 1.35	33.25 ± 1.41*
Liver weight (g)	14.62 ± 2.21	106.55 ± 15.54	101.88 ± 6.45	251.13 ± 12.95	507.20 ± 14.88*
Liver:body weight ratio (g/kg)	68.61 ± 9.14*	21.65 ± 2.87	22.33 ± 1.41	15.58 ± 1.00	15.35 ± 0.60

* denotes maximal ontogenic value significantly different from all other age groups ($P < 0.05$).

Figure 1

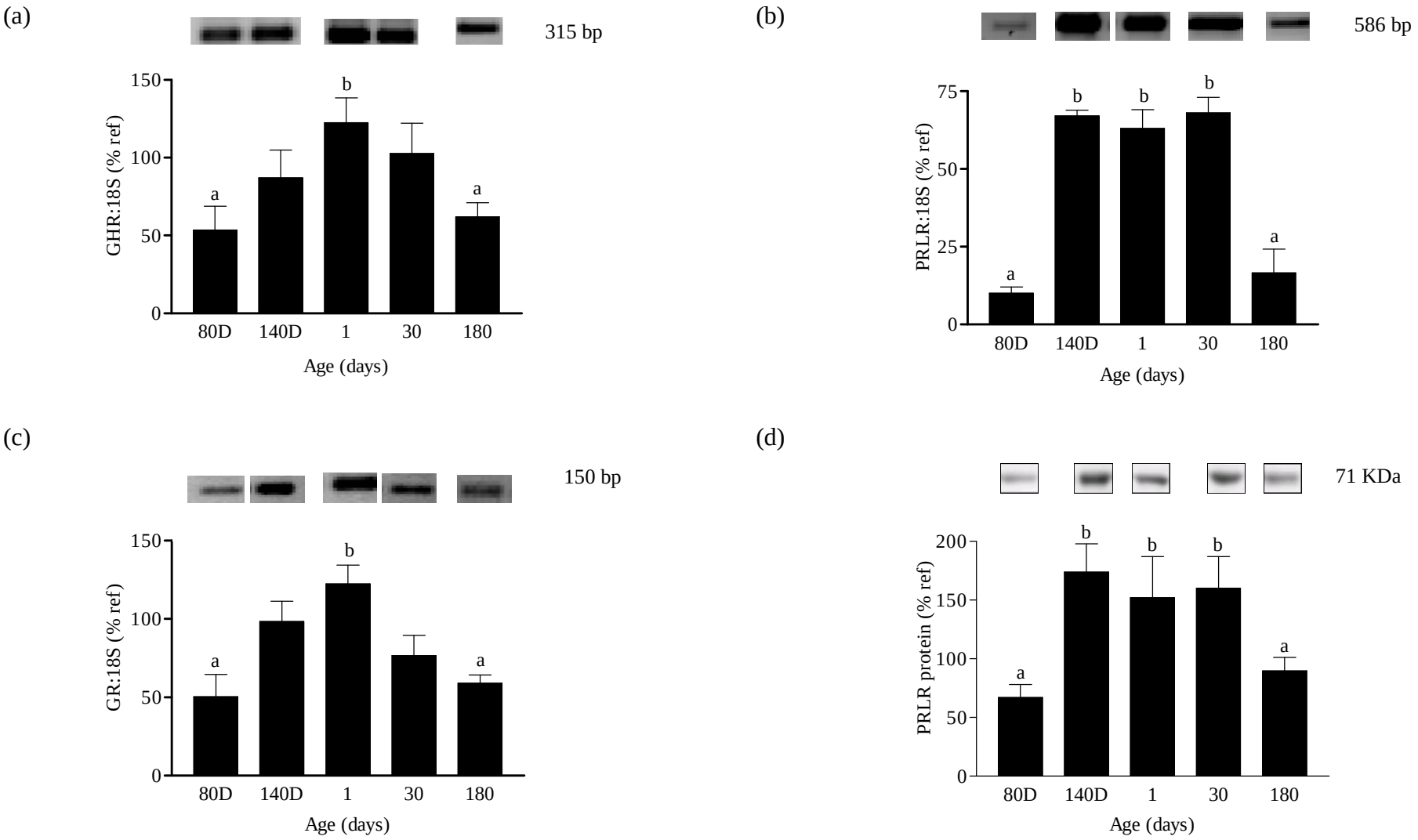

(a)
315 bp
GHR:18S (% ref)
150
100
50
0
a
b
a
80D
140D
1
30
180
Age (days)
(b)
586 bp
PRLR:18S (% ref)
75
50
25
0
a
b
b
b
a
80D
140D
1
30
180
Age (days)
(c)
150 bp
GR:18S (% ref)
150
100
50
0
a
b
a
80D
140D
1
30
180
Age (days)
(d)
71 KDa
PRLR protein (% ref)
200
150
100
50
0
a
b
b
b
a
80D
140D
1
30
180
Age (days)

Figure 2

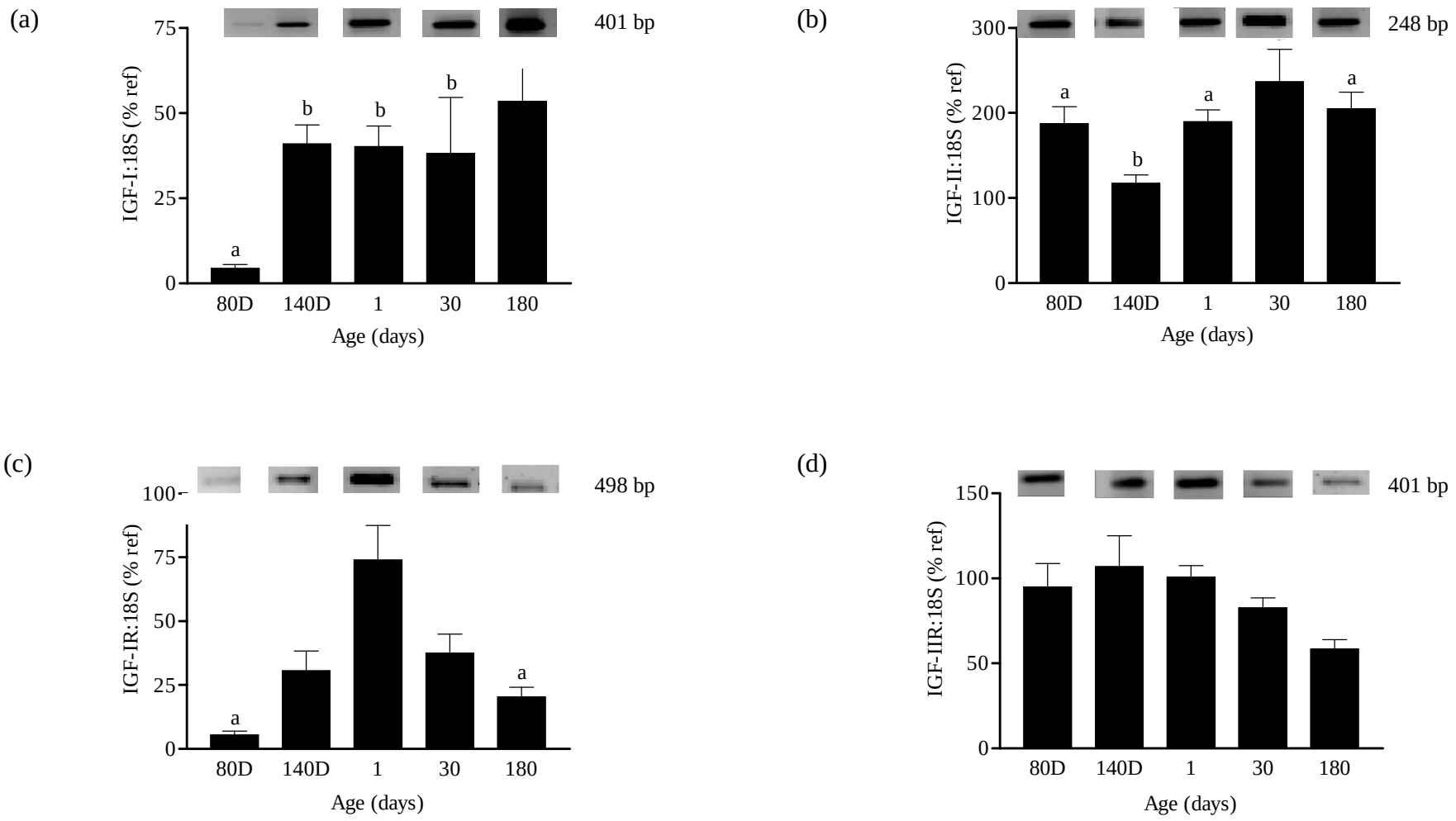

(a)
401 bp
IGF-I:18S (% ref)
75
50
25
0
a
b
b
b
80D
140D
1
30
180
Age (days)
(b)
248 bp
IGF-II:18S (% ref)
300
200
100
0
a
b
a
a
80D
140D
1
30
180
Age (days)
(c)
498 bp
100
IGF-IR:18S (% ref)
75
50
25
0
a
a
80D
140D
1
30
180
Age (days)
(d)
401 bp
IGF-IIR:18S (% ref)
150
100
50
0
80D
140D
1
30
180
Age (days)

Figure 3

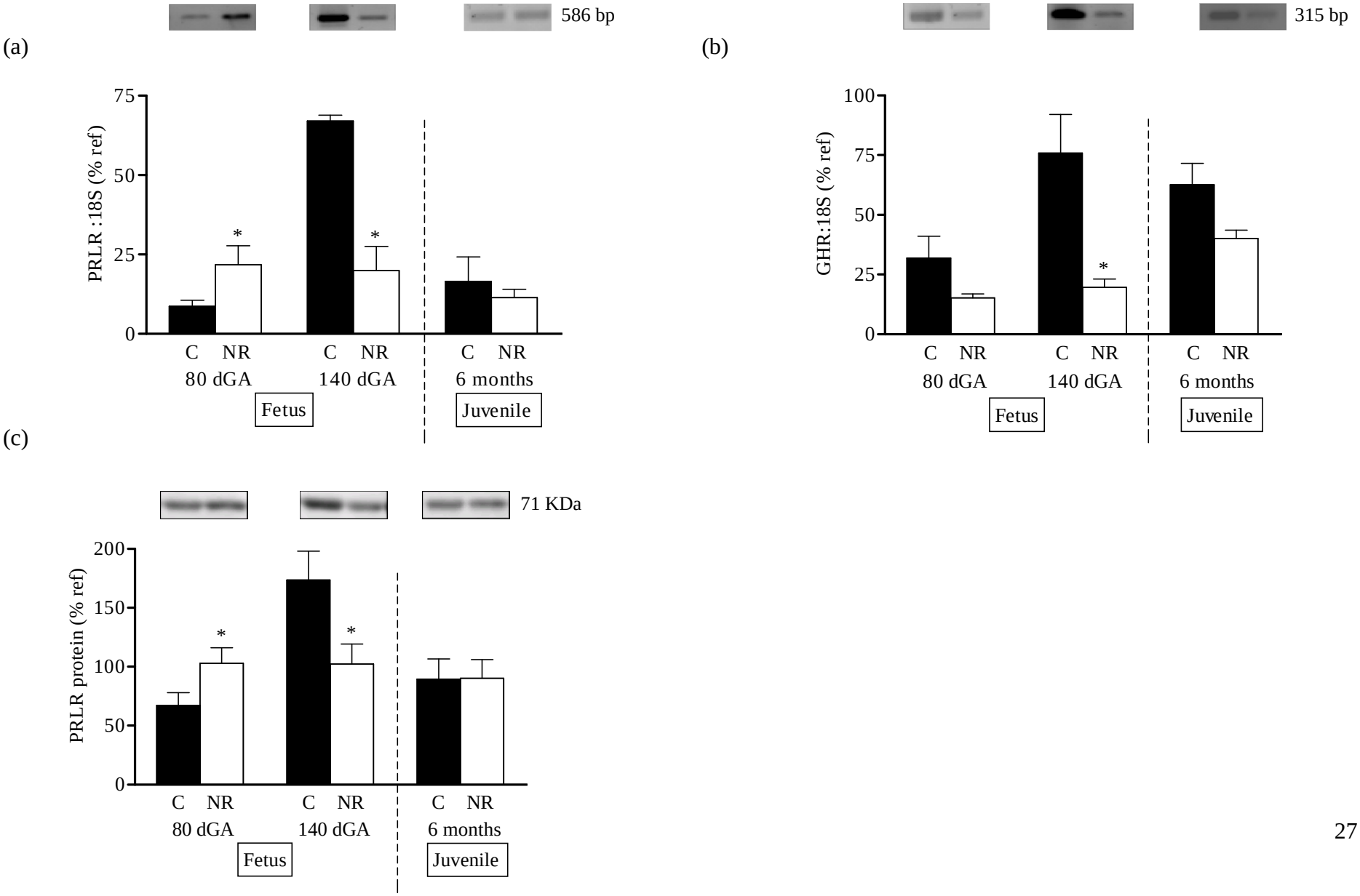
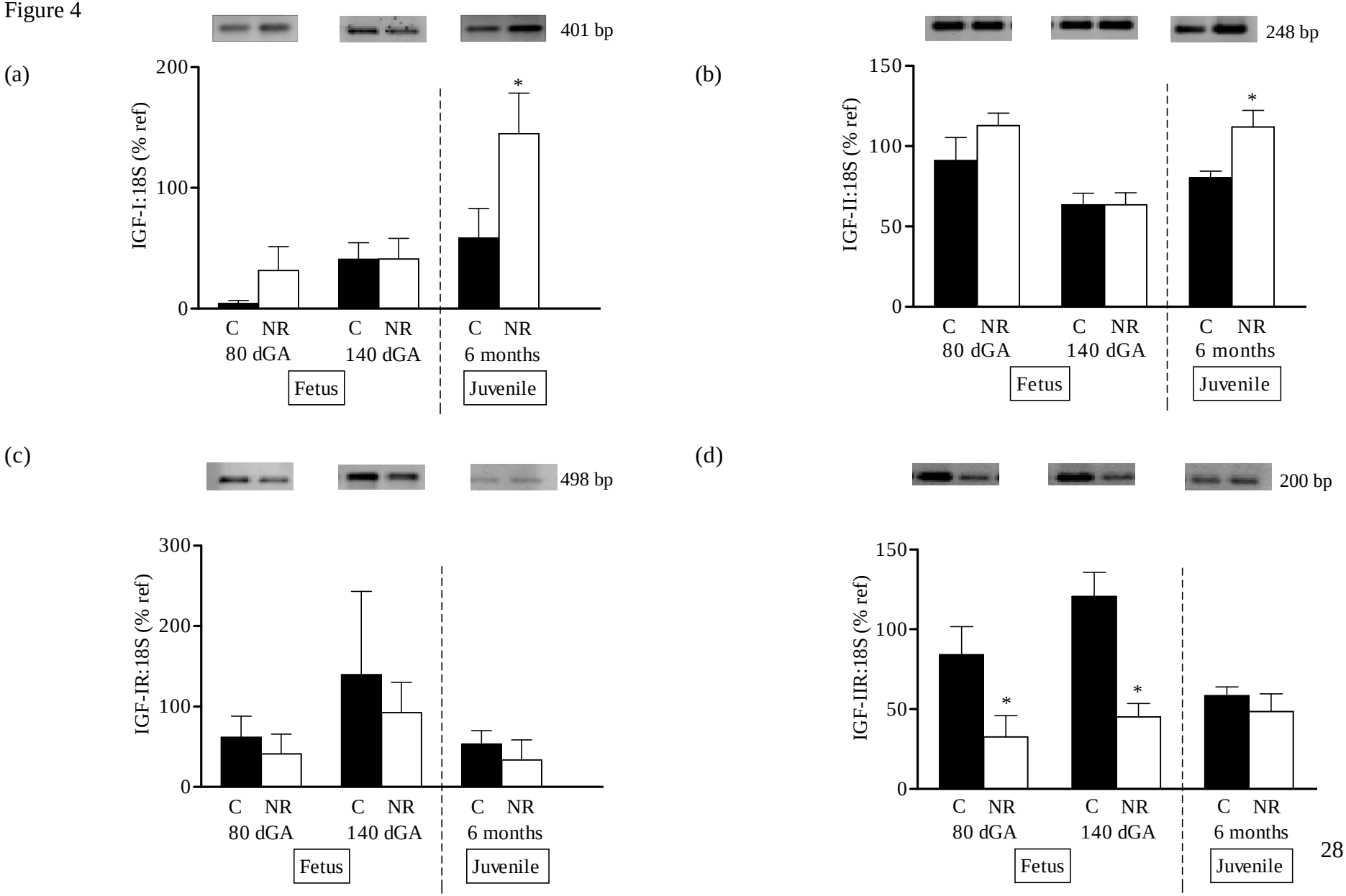

(a)
586 bp
PRLR :18S (% ref)
75
50
25
0
*
*
C NR C NR C NR
80 dGA 140 dGA 6 months
Fetus
Juvenile
(b)
315 bp
GHR:18S (% ref)
100
75
50
25
0
*
C NR C NR C NR
80 dGA 140 dGA 6 months
Fetus
Juvenile
(c)
71 KDa
PRLR protein (% ref)
200
150
100
50
0
*
*
C NR C NR C NR
80 dGA 140 dGA 6 months
Fetus
Juvenile

Figure 4


(a)
401 bp
IGF-I:18S (% ref)
200
100
0
*
C NR
80 dGA
C NR
140 dGA
C NR
6 months
Fetus
Juvenile
(b)
248 bp
IGF-II:18S (% ref)
150
100
50
0
*
C NR
80 dGA
C NR
140 dGA
C NR
6 months
Fetus
Juvenile
(c)
498 bp
IGF-IR:18S (% ref)
300
200
100
0
C NR
80 dGA
C NR
140 dGA
C NR
6 months
Fetus
Juvenile
(d)
200 bp
IGF-IIR:18S (% ref)
150
100
50
0
*
*
C NR
80 dGA
C NR
140 dGA
C NR
6 months
Fetus
Juvenile