Thyroid hormone deficiency suppresses fetal pituitary-adrenal function near term: implications for the control of fetal maturation and parturition

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Key words: fetus, adrenal gland, pituitary gland, corticotrophs, thyroid hormones, ACTH, cortisol

Abstract

Background

The fetal hypothalamic-pituitary-adrenal (HPA) axis plays a key role in the control of parturition and maturation of organ systems in preparation for birth. In hypothyroid fetuses, gestational length may be prolonged and maturational processes delayed. The extent to which the effects of thyroid hormone deficiency *in utero* on the timing of fetal maturation and parturition are mediated by changes to the structure and function of the fetal HPA axis is unknown.

Methods

In twin sheep pregnancies where one fetus was thyroidectomized and the other sham-operated, this study investigated the effect of hypothyroidism on circulating concentrations of adrenocorticotrophic hormone (ACTH) and cortisol, and the structure and secretory capacity of the anterior pituitary and adrenal glands. The relative population of pituitary corticotrophs, and the masses of the adrenal zones, were assessed by immunohistochemical and stereological techniques. Adrenal mRNA abundances of key steroidogenic enzymes and growth factors were examined by qPCR.

Results

Hypothyroidism *in utero* reduced plasma concentrations of ACTH and cortisol. In thyroid-deficient fetuses, the mass of corticotrophs in the anterior pituitary gland was unexpectedly increased, while the mass of the zona fasciculata and its proportion of the adrenal gland were decreased. These structural changes were associated with lower adrenocortical mRNA abundances of insulin-like growth factor-I (IGFI) and its receptor, and key steroidogenic

43 enzymes responsible for glucocorticoid synthesis. The relative mass of the adrenal medulla
44 and its proportion of the adrenal gland were increased by thyroid hormone deficiency *in*
45 *utero*, without any change in expression of phenylethanolamine N-methyltransferase or the
46 IGF system.

47 **Conclusions**

48 Thyroid hormones are important regulators of the structure and secretory capacity of the
49 pituitary-adrenal axis before birth. In hypothyroid fetuses, low plasma cortisol may be due
50 to impaired adrenocortical growth and steroidogenic enzyme expression, secondary to low
51 circulating ACTH concentration. Greater corticotroph population in the anterior pituitary
52 gland of the hypothyroid fetus indicates compensatory cell proliferation and that there may
53 be abnormal corticotroph capacity for ACTH synthesis and/or impaired hypothalamic input.
54 Suppression of the development of the fetal HPA axis by thyroid hormone deficiency may
55 contribute to the delay in fetal maturation and delivery observed in hypothyroid offspring.

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Introduction

Birth, and the successful transition from the intra- to extrauterine environment, is arguably the most important physiological event in life. Survival of the offspring depends upon the maturation of a wide range of fetal tissues towards term, including the lungs, gastrointestinal tract and brown adipose tissue (1). Developmental changes in these fetal organs are essential to activate pulmonary gas exchange, enteral nutrient uptake and thermoregulation, all physiological processes that are required for the first time at birth. The mechanisms that induce fetal maturation are closely linked to the mechanisms of parturition to ensure the delivery of a viable offspring.

The fetal hypothalamic-pituitary-adrenal (HPA) axis is a key regulator of fetal maturation and the onset of parturition (2). Hypothalamic neurons release corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) which stimulate corticotrophs in the anterior pituitary gland to secrete adrenocorticotrophic hormone (ACTH). In the sheep fetus, there are changes in corticotroph ultrastructure, the molecular capacity to synthesize ACTH, and responsiveness to CRH and AVP with increasing gestational age (3, 4). Concentrations of ACTH rise in the fetal circulation towards term and promote the growth and secretory capacity of the adrenal gland. In the zona fasciculata of the adrenal cortex, ACTH binds its receptor (MC2R) to promote the expression of proteins responsible for the synthesis of adrenal hormones from cholesterol. The steroidogenic acute regulatory protein (StAR) transports cholesterol from the outer to the inner mitochondrial membrane and a series of steroidogenic enzymes convert cholesterol into glucocorticoids (cholesterol side chain cleavage, CYP11A1; 17α-hydroxylase, CYP17; 3β-hydroxysteroid dehydrogenase, 3βHSD; 21-hydroxylase, CYP21; 11β-hydroxylase, CYP11B1). Increasing plasma

concentrations of adrenal hormones stimulate the process of parturition (glucocorticoids in sheep and androgens in humans) and maturational events in key tissues which are essential for neonatal survival (glucocorticoids in both species; 5, 6).

A range of experimental and clinical studies have shown that thyroid hormones also have an important role in the control of fetal maturation and the timing of birth. In the sheep fetus, hypothyroidism prolongs gestation and causes abnormal growth and development of a range of fetal organs (7, 8). These effects may be a direct consequence of thyroid hormone deficiency and/or may occur secondary to changes in other hormones in the hypothyroid fetus, including insulin, leptin and insulin-like growth factors (IGF; 8-10). Prolonged gestational length is also observed in human pregnancy where the fetus has congenital hypothyroidism (CH) which affects 1:2000 human births worldwide. Pregnancies complicated by CH are more likely to extend past 40 weeks of gestation (35-48% CH versus 15-19% control) and to require induction (11, 12). Furthermore, even in studies where infants with CH, prematurity and low birth weight are excluded, high neonatal thyroid-stimulating hormone (TSH) concentration, indicative of low systemic thyroid hormone levels, is associated with a longer pregnancy (13). Cases of CH and dysmaturity syndrome have also been reported in horses in Canada and Europe; gestational length is increased and the hypothyroid foals show skeletomuscular abnormalities, poor temperature control and reduced survival (14). The extent to which thyroid hormones influence fetal maturation and gestational length via development of the HPA axis before birth, however, is unknown.

In neonatal and adult rats, hypothyroidism induced by surgical and pharmacological methods decreases the expression of components of the HPA axis involved in the production of ACTH and glucocorticoids (15, 16). Little is known, however, about the

consequences of thyroid hormone deficiency before birth for the structure and function of the developing pituitary and adrenal gland, especially in a species like the sheep that has a similar pattern of maturation of thyroid hormone activity to human infants (8). The aims of the present study were, therefore, to determine the effect of fetal hypothyroidism on (i) circulating concentrations of ACTH and cortisol, (ii) the corticotroph population in the anterior pituitary gland, (iii) the zonal structure of the adrenal gland, and (iv) the adrenal mRNA abundance of key genes responsible for glucocorticoid synthesis. It was hypothesised that thyroid hormone deficiency in the sheep fetus would lead to lower circulating ACTH due to a reduction in the number of corticotrophs in the anterior pituitary gland and, as a consequence, lower plasma cortisol due to impaired growth and steroidogenic capacity of the adrenal gland.

Materials and Methods

Animals

All surgical and experimental procedures were carried out in accordance with UK Home Office legislation and the Animals (Scientific Procedures) Act 1986, after approval by the Animal Welfare and Ethical Review Body, University of Cambridge, UK. Sixteen Welsh Mountain pregnant ewes of known gestational age and carrying twin fetuses were used in this study. The ewes were housed in individual pens and were maintained on 200 g/day concentrates (14% crude protein, energy content 12 MJ/kg dry matter; H & C Beart Ltd, Stowbridge, UK) with hay and water ad libitum and access to a salt block. Food, but not water, was withheld from the ewes for 18-24 hours before surgery.

126

127 **Experimental procedures**

128 Under general anaesthesia (2% isoflurane in O_2-N_2O) and at 102-110 days of gestation (dGA;
129 term ~ 145 ± 2 days), the twin fetuses of each ewe underwent either surgical removal of the
130 thyroid gland (thyroidectomy, TX) or a sham operation in which the thyroid gland was
131 exposed but not removed (sham), as described previously (7). At surgery, antibiotics were
132 administered to each fetus intravenously and into the amniotic cavity of each fetus (total
133 600 mg benzylpenicillin in 5 ml of 0.9% saline: Crystapen, Schering-Plough, Welwyn Garden
134 City, UK). The ewes were treated with antibiotics (30 mg/kg procaine benzylpenicillin I.M.;
135 Depocillin, Intervet UK Ltd, Milton Keynes, UK) immediately before the start of surgery and
136 daily for 3 days thereafter. The animals were monitored over the recovery period and
137 resumed normal feeding within 24 h of surgery.

138 Between 140-145 dGA, the fetuses were delivered by Caesarean section under
139 general anaesthesia (20 mg/kg maternal body weight sodium pentobarbitone I.V.). Blood
140 samples were collected by venipuncture of the umbilical artery into EDTA-containing tubes.
141 Each fetus was weighed and a variety of fetal organs, including the pituitary and adrenal
142 glands, were dissected and weighed after administration of a lethal dose of barbiturate (200
143 mg/kg sodium pentobarbitone I.V.). One adrenal gland was snap frozen in liquid nitrogen
144 and stored at -80°C for molecular analysis, and the other adrenal gland and the pituitary
145 gland were immersion fixed whole in 4% paraformaldehyde (with 0.2% glutaraldehyde in 0.1
146 M phosphate buffer, pH 7.4) for 2 days for histological analysis. After washing in phosphate-
147 buffered saline, the fixed adrenal and pituitary glands were processed and embedded in
148 paraffin wax.

149

150 **Plasma hormone measurements**

151 Umbilical plasma triiodothyronine (T3) and thyroxine (T4) concentrations were determined
152 by radioimmunoassay (RIA) kits (MP Biomedicals, Loughborough, UK); the intra-assay
153 coefficients of variation were 3% and 5%, and the minimum levels of detection were 0.14
154 and 7.0 ng/ml, respectively. Plasma concentrations of ACTH, cortisol and insulin were
155 determined using ELISA kits (ACTH 1-39: Demeditec Diagnostics GmbH, Kiel, Germany;
156 cortisol: IBL International, Hamburg, Germany; insulin: Mercodia, Uppsala, Sweden); the
157 intra-assay coefficients of variation were all <10%, and the minimum levels of detection
158 were 0.22 pg/ml, 2.5 ng/ml and 0.025 ng/ml, respectively. Plasma leptin concentration was
159 measured by RIA as previously described (17). The intra-assay coefficient of variation was
160 5%, and the minimum level of detection was 0.09 ng/ml.

161

162 **Histology and immunohistochemistry**

163 The fixed pituitary and adrenal glands were exhaustively sectioned at 5µm. For the pituitary
164 gland, ACTH-positive cells were identified in 10 equally spaced sections by
165 immunohistochemistry using a rabbit polyclonal antibody against human ACTH (10 µg/ml;
166 Bio-Rad Laboratories, Watford, UK). Detection was achieved using the Vectastain Elite ABC
167 kit (Vector Laboratories, Peterborough, UK) and diaminobenzidine, and haematoxylin was
168 used to counter-stain the sections. All sections were scanned using a NanoZoomer digital
169 slide scanner (Hamamatsu Photonics, Welwyn Garden City, UK) to create digital images for
170 analysis. Section images were analyzed blinded to the treatment group using NewCAST

stereological software (Visiopharm, Hoersholm, Denmark). In each section of the pituitary gland, approximately 5-10% of the tissue was sampled to estimate the fractional volume of anterior pituitary and, specifically, ACTH-positive pituitary cell types by point-counting and application of Cavalieri's principle (18). At least 150 points were counted for each variable. Absolute masses of the anterior pituitary gland and the corticotroph population were calculated by expressing the estimated fractional volume as a proportion of the total pituitary weight, and relative mass was calculated by dividing the absolute mass by fetal body weight at delivery.

For the adrenal gland, 8-10 equally spaced sections were stained with haematoxylin and eosin in order to distinguish the medulla and zones of the cortex. In each section of the adrenal gland, approximately 10% of the tissue was sampled to determine the fractional volumes of the adrenal compartments. Using the point-counting method as detailed for the pituitary gland, the regions of the adrenal gland were classified into four different compartments: capsule, zona glomerulosa, zona fasciculata and adrenal medulla. In sheep, the zona reticularis is not present in the adrenal gland until postnatal life. Absolute masses of the adrenal compartments were calculated by expressing the estimated fractional volumes as a proportion of the total adrenal weight, and relative mass was calculated by dividing the absolute mass by fetal body weight at delivery.

Quantification of adrenal mRNA abundance

Frozen adrenal glands were separated into cortex and medulla regions on the basis of appearance using a dissecting microscope. Dissected tissue was homogenised in Lysing

193 Matrix-D tubes (MP Biomedicals, Loughborough, UK) using a MagNA Lyser (Roche
194 Diagnostics, Almere, The Netherlands). The RNA was extracted following the protocol of the
195 RNeasy Plus Mini Kit (Qiagen, Manchester, UK) and RNA extraction yields were assessed
196 using a Nanodrop (Thermo Fisher Scientific, Loughborough, UK).

197 Reverse transcription of the extracted mRNA was performed using the High-Capacity
198 cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Negative controls were prepared
199 where the multiscribe reverse transcriptase and RNase inhibitor were replaced with equal
200 volumes of RNase free water. Reverse transcription was carried out in the Gene Pro
201 thermocycler (Bioer Ltd, Hangzhou, China) at 25°C for 10 minutes, 37°C for 120 minutes, and
202 85°C for 5 minutes.

203 Quantitative polymerase chain reaction (qPCR) was performed on cortex samples for
204 the genes MC2R, StAR, CYP11A1, CYP17, 3βHSD, CYP21, CYP11B1, and on medulla samples
205 for the gene phenylethanolamine N-methyltransferase (PNMT: the enzyme that converts
206 noradrenaline into adrenaline). Expression of IGFI, IGFII, IGF type 1 and type 2 receptors
207 (IGF-1R, IGF-2R), and the housekeeping gene YWHAZ, were examined in both cortical and
208 medullary regions. The sequences of the primers used are presented in Supplementary
209 Table 1. The primer efficiencies were 83-115% and all melting curves showed a single
210 product with melting temperatures between 58-62°C.

211 Each well of a 96-well PCR plate (STARLAB Ltd, Blakelands, UK) was loaded with
212 sample cDNA, forward and reverse primers, RNase free water and MESA BLUE qPCR
213 MasterMix Plus for SYBR® Assay No ROX (Eurogentec, Seraing, Belgium). Separate plates
214 were used to measure mRNA levels for each gene and each sample or standard was
215 measured in duplicate. Samples were amplified for qPCR using a DNA Engine Opticon 2

Sequence Detection System (Bio-Rad Laboratories) at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by a melting curve stage of 60°C to 90°C and held for 10 seconds to read at every 1°C increment. Data were processed using Opticon Monitor Version 3.1 (Bio-Rad Laboratories). The negative control samples did not generate any detectable amplicons, confirming the absence of genomic contamination. In order to compare mRNA abundance of target genes between the treatment groups, cycle thresholds (Ct) were expressed relative to YWHAZ and analysed using the delta-delta-Ct (ΔΔCt) method as all standard curves were linear and parallel.

Statistical analyses

Data from the two treatment groups are presented as mean ± SEM. Following assessment for normality using the D'Agostino-Pearson test, data from the treatment groups were compared by Student's unpaired t-test or Mann-Whitney test, as appropriate. Relationships between variables were determined by Pearson correlation using $\log_{10}$-transformed data where necessary. $P<0.05$ was regarded as significant. Statistical tests were not performed on data that included values below the lower limit of assay detection (plasma T4 and T3 concentrations).

Results

Hypothyroidism *in utero* suppressed circulating ACTH and cortisol concentrations

Umbilical plasma T4 and T3 concentrations decreased to below the limits of assay detection in the TX fetuses (Table 1). Compared to the sham fetuses, the TX fetuses had significantly lower plasma ACTH and cortisol, and higher plasma insulin and leptin concentrations ($P<0.05$; Table 1). When data available from all fetuses were combined, regardless of treatment group, negative correlations were observed between $\log_{10}$ plasma ACTH and both insulin ($R=-0.64$, $P<0.01$, $N=16$) and leptin concentrations ($R=-0.81$, $P<0.0005$, $N=16$).

Low plasma ACTH in hypothyroid fetuses was associated with an increase in the pituitary corticotroph population

In the TX compared to sham fetuses, body weight was reduced ($P<0.05$; Table 1) and both absolute and relative weights of the total pituitary gland were increased ($P<0.005$; Table 1). The fractional volume of corticotrophs in the anterior pituitary gland was reduced by hypothyroidism (sham 31.4 ± 1.2%, TX 27.1 ± 1.7%, $P<0.05$; Figure 1A), however, when expressed as absolute and relative masses, the populations of both corticotrophs (absolute mass: sham 25.0 ± 1.7 mg, TX 33.6 ± 1.9 mg; relative mass: sham 6.8 ± 0.4 mg/kg, TX 10.6 ± 0.5 mg/kg) and other pituitary cell types were greater in TX compared to sham fetuses ($P<0.005$; Figure 1B and C). The estimated absolute and relative weights of the anterior pituitary gland were also increased in the TX fetuses ($P<0.05$; Figure 1B). The relative total pituitary weight correlated negatively with $\log_{10}$ plasma ACTH ($R=-0.58$, $P<0.01$, $N=21$) and positively with plasma concentrations of insulin ($R=0.62$, $P<0.05$, $N=16$) and leptin ($R=0.59$, $P<0.05$, $N=16$). Plasma concentrations of insulin and leptin also correlated with the relative masses of the anterior pituitary (both $R=0.59$, $P<0.05$, $N=16$), corticotrophs (insulin: $R=0.53$,

leptin: R=0.56, P<0.05, N=16) and other pituitary cell types (insulin: R=0.57, leptin: R=0.55, P<0.05, N=16).

Hypothyroidism *in utero* decreased zona fasciculata and increased medulla sizes in the fetal adrenal gland

There was no difference in total adrenal gland weight, expressed in absolute or relative terms, between the groups of TX and sham fetuses (Table 1). Hypothyroidism caused a decrease in zona fasciculata percentage volume and an increase in medulla percentage volume of the total adrenal gland (P<0.05; Figure 2A). When expressed as a percentage volume of the adrenal cortex (zona glomerulosa and zona fasciculata combined), the zona fasciculata in the TX fetuses was a smaller fraction of the adrenal cortex than in the sham fetuses (sham 86.4 ± 0.7%, TX 83.2 ± 0.8%, P<0.01). The absolute mass of the zona fasciculata was lower, and the relative mass of the medulla was greater in the TX compared to sham fetuses (P<0.05; Figures 2B and C). There were no significant differences in the absolute (P=0.07) or relative mass of the adrenal cortex between the TX and sham fetuses (Figure 2B and C). Overall, $\log_{10}$ plasma cortisol concentration correlated positively with the absolute and relative masses of both the adrenal gland (R=0.54 and R=0.40, respectively, P<0.05, N=27) and the zona fasciculata (R=0.67 and R=0.64, respectively, P<0.0005, N=27), and negatively with the relative mass of the medulla (R=-0.46, P<0.05, N=27)

Hypothyroidism *in utero* reduced mRNA abundance of steroidogenic and growth factor genes in the adrenal cortex

In the TX relative to the sham control fetuses, mRNA abundance in the adrenal cortex was reduced for StAR, steroidogenic enzymes CYP11A1, CYP17, 3βHSD, CYP21 and CYP11B1 ($P<0.05$; Figures 3A-F), and for IGFI and IGF-1R ($P<0.05$; Figures 3G and H). Overall, $\log_{10}$ plasma cortisol concentration correlated with the mRNA abundance for StAR and all of the steroidogenic enzymes ($R=0.52$-0.58, $P<0.005$, $N=26$), except CYP21 and CYP11B1. A significant relationship was also observed between $\log_{10}$ plasma cortisol and adrenocortical IGF-1R mRNA abundance ($R=0.44$, $P<0.05$, $N=26$). Fetal hypothyroidism had no effect on MC2R, IGFII or IGF-2R mRNA abundance in the adrenal cortex, or PNMT, IGFI, IGFII or IGF receptor mRNA in the adrenal medulla (Table 2).

Discussion

Thyroid hormone deficiency in the ovine fetus suppressed the activity of the HPA axis near term with actions on the structure and secretory capacity of both the anterior pituitary and adrenal glands. Hypothyroidism *in utero* caused reductions in circulating ACTH concentration, the size of the zona fasciculata in the adrenal cortex, mRNA levels of steroidogenic enzymes and plasma cortisol concentration. This study has, therefore, elucidated a potential mechanism by which thyroid hormone deficiency before birth affects the timing of fetal maturation and parturition.

Contrary to the study hypothesis, the reduction in plasma ACTH concentration observed in the hypothyroid fetuses was not due to a deficit in corticotrophs in the anterior pituitary gland. Indeed, although the corticotroph population formed a smaller percentage of the anterior pituitary gland, both absolute and relative corticotroph masses were

increased in the thyroid-deficient fetuses near term. Using the estimates based on the fractional volume of total tissue mass, however, it was not possible to determine whether the increase in corticotroph and anterior pituitary mass was due to hyperplasia and/or hypertrophy of pituitary cell types. Overgrowth of the pituitary gland in the hypothyroid sheep fetus was likely to be due to expansion of the thyrotroph and corticotroph populations. Previous studies in hypothyroid adult rats have reported increased numbers of thyrotrophs in the anterior pituitary gland, in response to the lack of negative feedback from thyroid hormones, and a more moderate increase in corticotroph cell number (19). The low circulating concentration of ACTH in the thyroid-deficient sheep fetus may be due, therefore, to impaired corticotroph function. Analysis of the ultrastructure of pituitary corticotrophs in hypothyroid adult rats has shown abnormalities in the formation of endocrine vesicles which may impair the capacity for ACTH secretion (20). Little is known, however, about the control of corticotroph number and function by thyroid hormones before birth.

Hypothyroidism may delay structural and functional maturation of pituitary corticotroph types with consequences for the activity of the fetal HPA axis. This may occur via direct actions on the anterior pituitary gland and/or via indirect actions on the neural control of hypothalamic development and neuroendocrine hypothalamic control of pituitary corticotroph function. In the sheep fetus, sub-populations of corticotrophs have been described previously, based on morphology or the expression of genes important for ACTH synthesis, which change in relative proportions as the pituitary gland matures towards term and in response to glucocorticoid treatment (3, 4, 21, 22). In the present study, however, it was not possible to identify morphologically-distinct corticotroph subtypes in the

histological images. Moreover, the morphology of corticotrophs in the fetal pituitary gland does not appear to relate to functional characteristics identified *in vitro*, such as the amount of ACTH stored in CRH-responsive corticotrophs (21). Further studies are required to assess the structural and functional properties of the corticotrophs present in the hypothyroid fetus, including cell ultrastructure and responsiveness to CRH and AVP.

It will also be important to determine the effects of thyroid hormone deficiency *in utero* on the development of the hypothalamus and its control of corticotroph structure and function. Surgical disconnection of the pituitary gland from the hypothalamus in the ovine fetus increases both thyrotroph and corticotroph numbers and delays the maturation of corticotroph sub-types, adrenal growth and expression of steroidogenic enzymes normally seen near term (23-26). Hypothyroidism is likely to influence neural control of hypothalamic function in the fetus, especially over the latter stages of gestation when the fetal HPA axis is activated. Thyroid hormones are well known to play an important role in the development of the central nervous system, including the hippocampus and hypothalamus (27, 28), although little is known about the effects of thyroid hormone deficiency on the maturation of the neural networks that regulate the fetal HPA axis in late gestation. In neonatal rats, hypothyroidism blunts ACTH and corticosterone responses to stress and suppresses the developmental increments in plasma ACTH and corticosterone concentrations that normally occur over the early postnatal period in rodents (15, 31). The changes in neonatal pituitary-adrenal function induced by thyroid hormone deficiency were associated with lower CRH mRNA abundance and numbers of CRH-positive neurones in the paraventricular nucleus of the hypothalamus (15). Previous studies in the thyroidectomized sheep fetus have shown that neurotransmitter contents of noradrenaline and serotonin are altered in the

hypothalamus and other brain regions, in a manner dependent on the timing of thyroid hormone deficiency (29, 30). The consequences of hypothyroidism for the development and activity of hypothalamic CRH and AVP neurone networks towards term, however, and the extent to which changes in these pathways are responsible for suppression of ACTH and glucocorticoid production in the present study, remain to be established.

The effects of thyroid hormone deficiency before birth on the developing adrenal gland appear to be mediated, at least in part, by suppression of plasma ACTH. Towards term, the rising plasma concentration of ACTH promotes the growth and secretory function of the fetal adrenal gland (32). In the present study, the mRNA abundance of the ACTH receptor was unchanged by hypothyroidism *in utero*, but the expression of the enzymes responsible for the conversion of cholesterol to glucocorticoids was reduced. Thyroid hormone deficiency also decreased the size of the zona fasciculata in association with lower expression of IGFI and the IGF type 1 receptor in the adrenal cortex. IGFI infusion in the ovine fetus stimulates adrenal growth without affecting plasma cortisol concentration or the expression of steroidogenic or catecholamine-synthesizing enzymes (33) and IGFI may, therefore, mediate the growth-promoting effects of ACTH during late gestation. Thyroid hormones may influence adrenocortical IGF mRNA levels in a manner similar to that reported in other fetal tissues such as the liver and skeletal muscle, and indeed, interactions with the IGF system may be responsible, in part, for the reduction in fetal body weight seen in response to hypothyroidism in this and previous studies (8, 34, 35).

Although thyroid hormone deficiency impaired the growth of the zona fasciculata in the adrenal cortex, the relative mass of adrenal medulla was increased in the hypothyroid fetuses. Overgrowth of the adrenal medulla occurred without any changes in the

371 expression of the IGFs or their receptors, or in the mRNA level of PNMT, the enzyme that
372 converts noradrenaline to adrenaline. While adrenomedullary and circulating
373 concentrations of the catecholamines were not measured in the present study, it has been
374 shown previously that thyroid hormone deficiency in fetal sheep impairs the plasma
375 catecholamine response to hypoxemia (29). Thyroid hormone deficiency may influence the
376 structural and functional innervation of the adrenal medulla that occurs during late
377 gestation with consequences for adrenomedullary growth (36, 37). It is also possible that
378 the lower levels of glucocorticoids synthesised within the adrenal cortex of the hypothyroid
379 fetus may impact growth of the adrenal medulla. Indeed, glucocorticoids have been shown
380 to suppress proliferation of chromaffin cells in fetal and neonatal rats both *in vivo* and *in*
381 *vitro* (38, 39). Overgrowth of the adrenal medulla may, therefore, be a consequence of
382 reduced levels of cortisol perfusing the adrenal medulla from the cortex.

383 The effects of thyroid hormone deficiency *in utero* on the developing HPA axis may
384 be direct and/or secondary to other endocrine changes. Hypothyroidism in the sheep fetus
385 is associated with increased circulating concentrations of insulin and leptin, which originate
386 from changes in the structure and function of the fetal pancreas and adipose tissue,
387 respectively (9, 10). In fetal sheep, intravenous infusion of leptin to supraphysiological
388 concentration has been shown to prevent the normal increments in plasma ACTH and
389 cortisol seen towards term (40), and to suppress adrenal responsiveness to ACTH challenge
390 and decrease the adrenal mRNA and protein content of the ACTH receptor, StAR and CYP21
391 in mildly hypoxic fetuses (41). Furthermore, intracerebroventricular infusion of leptin
392 suppresses the amplitude of ACTH and cortisol pulses in the fetal circulation without any
393 change in responsiveness to CRH and AVP administration (42). This suggests that leptin may

394 inhibit HPA activity *in utero* via central mechanisms, although the extent to which increased
395 systemic levels contribute to the consequences of thyroid hormone deficiency in the
396 present study remains to be determined. A moderate increase in circulating leptin has no
397 effect on basal plasma cortisol concentration in the thyroid-intact sheep fetus (43).

398 The findings of the present study contribute to the understanding of the endocrine
399 control of fetal maturation and parturition. Although not assessed in the present study,
400 prolonged gestational length and delayed fetal maturation have been reported in previous
401 research using the same sheep model of fetal hypothyroidism (7, 8). Impaired development
402 of the anterior pituitary and adrenal glands, and suppression of steroidogenic enzymes,
403 observed in the hypothyroid fetus will have consequences for the initiation of labour and
404 delivery. While the structure of the adrenal gland differs between ovine and human
405 species, adrenal hormones induced by ACTH are key regulators in the timing of parturition
406 in both species (2, 5). Indeed, gestation is prolonged in both human and other animal
407 models of congenital hypothyroidism (7, 11, 12, 14). Impaired adrenal steroidogenesis will
408 also impact development of fetal organs in preparation for birth since many of the
409 maturational processes that take place over the perinatal period are known to be
410 glucocorticoid-dependent (6). Dysmaturity observed in hypothyroid fetuses and neonates,
411 therefore, may be due to suppression of the HPA axis and a delay in the prepartum cortisol
412 surge (8). Through interactions with the developing HPA axis, thyroid hormones produced
413 by the fetus appear to have a role in the coordinated delivery and survival of the neonate.

414

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549 carbohydrate metabolism in the ovine fetus during late gestation. J Physiol 586:2393-2403.

550

551 **Table 1.** Mean (± SEM) plasma hormone concentrations, and body and organ weights, in
552 sham (n = 15) and thyroidectomized (TX, n=12) fetuses at 143 days of gestation. *,
553 significantly different from sham fetuses, P<0.05; ND, not detectable (limit of assay
554 detection: T4 7.0 ng/ml, T3 0.14 ng/ml). Thyroxine (T4), triiodothyronine (T3),
555 adrenocorticotrophic hormone (ACTH).

	Sham (n=12-15)	**TX (n=10-12)**
Plasma hormone concentrations		
T4 (ng/ml)	92.6 ± 9.7	ND
T3 (ng/ml)	0.68 ± 0.09	ND
ACTH (pg/ml)	149.4 ± 37.4	21.6 ± 7.0*
Cortisol (ng/ml)	54.9 ± 10.1	23.7 ± 2.8*
Insulin (ng/ml)	0.56 ± 0.13	1.52 ± 0.18*
Leptin (ng/ml)	0.68 ± 0.05	1.03 ± 0.10*
Body and organ weights		
Body (kg)	3.70 ± 0.17	3.18 ± 0.11*
Pituitary gland (mg)	93 ± 8	141 ± 13*
Relative pituitary gland (mg/kg)	25 ± 2	44 ± 3*
Adrenal glands (mg, total)	434 ± 33	373 ± 19
Relative adrenal glands (mg/kg, total)	118 ± 8	118 ± 5

556

557

558 **Table 2.** Mean (± SEM) mRNA abundance, expressed as -ΔΔCt values, in the adrenal cortex
559 and medulla of sham and thyroidectomized (TX) fetuses. No significant differences were
560 observed between the treatment groups. Delta-delta cycle threshold (ΔΔCt),
561 adrenocorticotrophic hormone receptor (MC2R), insulin-like growth factors I and II (IGFI,
562 IGFII), IGF type 1 and 2 receptors (IGF-1R, IGF-2R), phenylethanolamine-N-
563 methyltransferase (PNMT).

	Sham (n=15)	**TX (n=11-12)**
Adrenal cortex		
MC2R	0.00 ± 0.28	-0.37 ± 0.25
IGFII	0.00 ± 0.25	-0.41 ± 0.40
IGF-2R	0.00 ± 0.20	-0.56 ± 0.34
Adrenal medulla		
PNMT	0.00 ± 0.55	0.26 ± 0.41
IGFI	0.00 ± 0.21	-0.56 ± 0.34
IGFII	0.00 ± 0.23	-0.40 ± 0.28
IGF-1R	0.00 ± 0.21	-0.29 ± 0.22
IGF-2R	0.00 ± 0.17	-0.25 ± 0.24

564

565

566

567 **Figure Legends**

568 1. Mean (± SEM) populations of corticotrophs and other cell types in the anterior pituitary
569 gland, expressed as (A) fractional volume percentage, (B) absolute mass and (C) relative
570 mass, in sham (n=15) and thyroidectomized (TX, n=12) fetuses at 143 days of gestation.
571 Example of immunohistochemical localisation of corticotrophs in the anterior pituitary gland
572 of a sham fetus (D). *, significantly different from sham fetuses, $P<0.05$.

573 2. Mean (± SEM) adrenal compartments, expressed as (A) fractional volume percentage, (B)
574 absolute mass and (C) relative mass, in sham (n=15) and thyroidectomized (TX, n=12)
575 fetuses at 143 days of gestation. Measurements made in one adrenal gland from each
576 fetus. *, significantly different from sham fetuses, $P<0.05$.

577 3. Mean (± SEM) mRNA abundance, expressed as -ΔΔCt values, of adrenocortical StAR and
578 steroidogenic enzymes (A-F), and IGFI and IGF type 1 receptor (G and H), in sham (n=15) and
579 thyroidectomized (TX, n=12) fetuses at 143 days of gestation. *, significantly different from
580 sham fetuses, $P<0.05$. Delta-delta cycle threshold (ΔΔCt), steroidogenic acute regulatory
581 protein (StAR), cholesterol side chain cleavage (CYP11A1), 17α-hydroxylase (CYP17), 3β-
582 hydroxysteroid dehydrogenase (3βHSD), 21-hydroxylase (CYP21), 11β-hydroxylase
583 (CYP11B1), insulin-like growth factor I (IGFI), IGF type 1 receptor (IGF-1R).

584

585

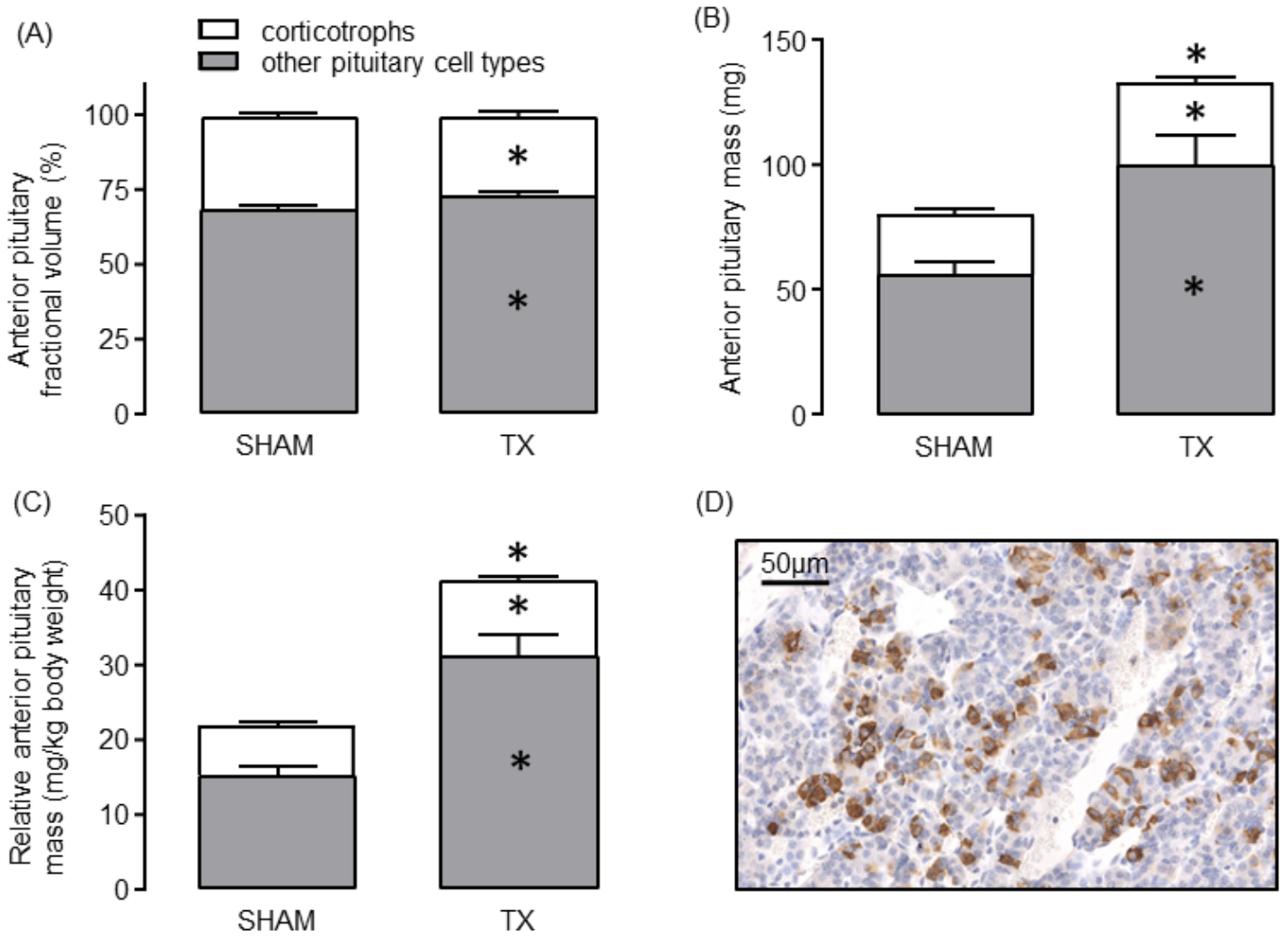
(A)
corticotrophs
other pituitary cell types
Anterior pituitary fractional volume (%)
100
75
50
25
0
SHAM
TX
(B)
Anterior pituitary mass (mg)
150
100
50
0
SHAM
TX
(C)
Relative anterior pituitary mass (mg/kg body weight)
50
40
30
20
10
0
SHAM
TX
(D)
50µm

Figure 1.

586

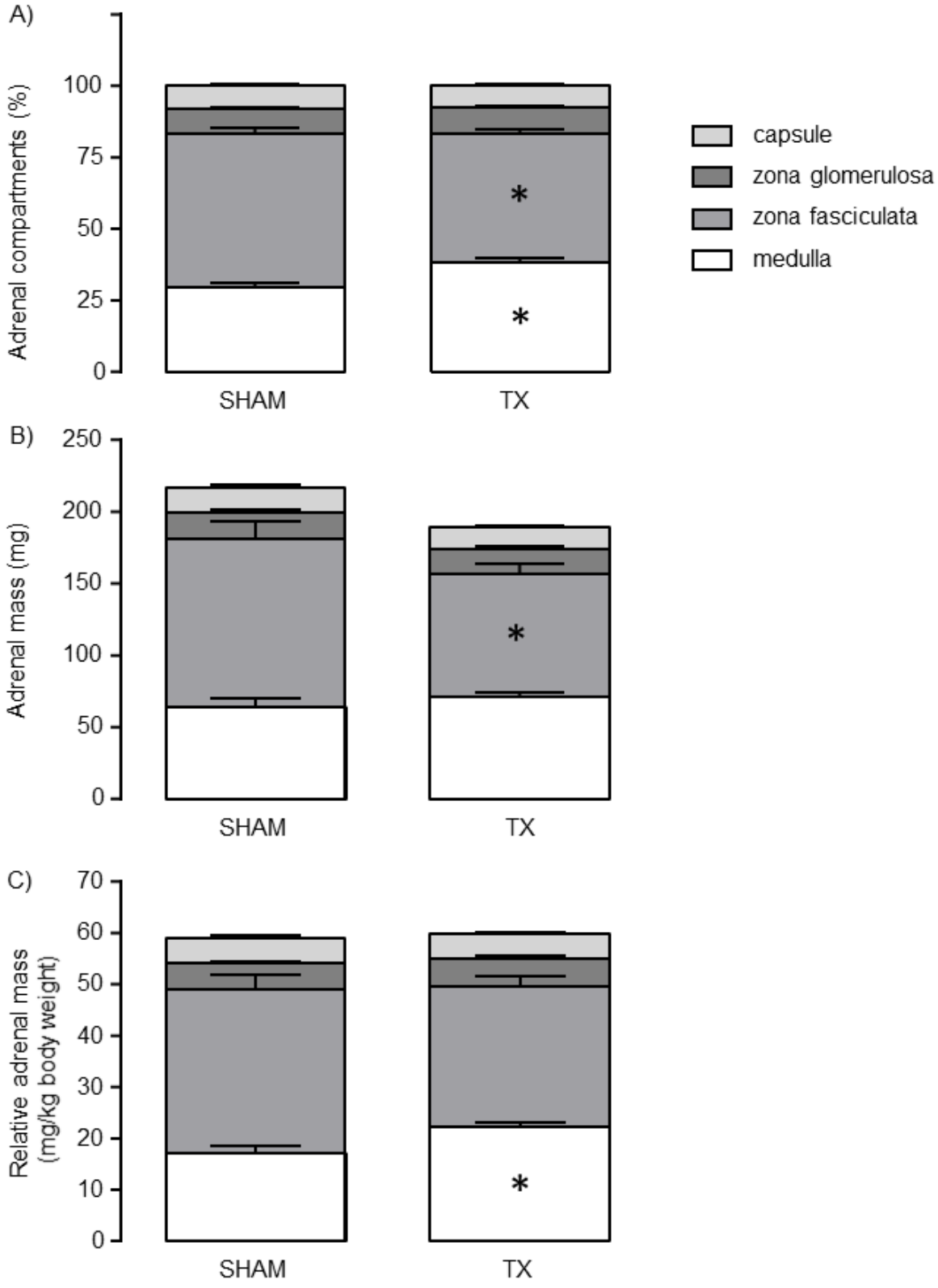
A)
Adrenal compartments (%)
100
75
50
25
0
SHAM
TX
*
*
capsule
zona glomerulosa
zona fasciculata
medulla
B)
Adrenal mass (mg)
250
200
150
100
50
0
SHAM
TX
*
C)
Relative adrenal mass (mg/kg body weight)
70
60
50
40
30
20
10
0
SHAM
TX
*

Figure 2.

587

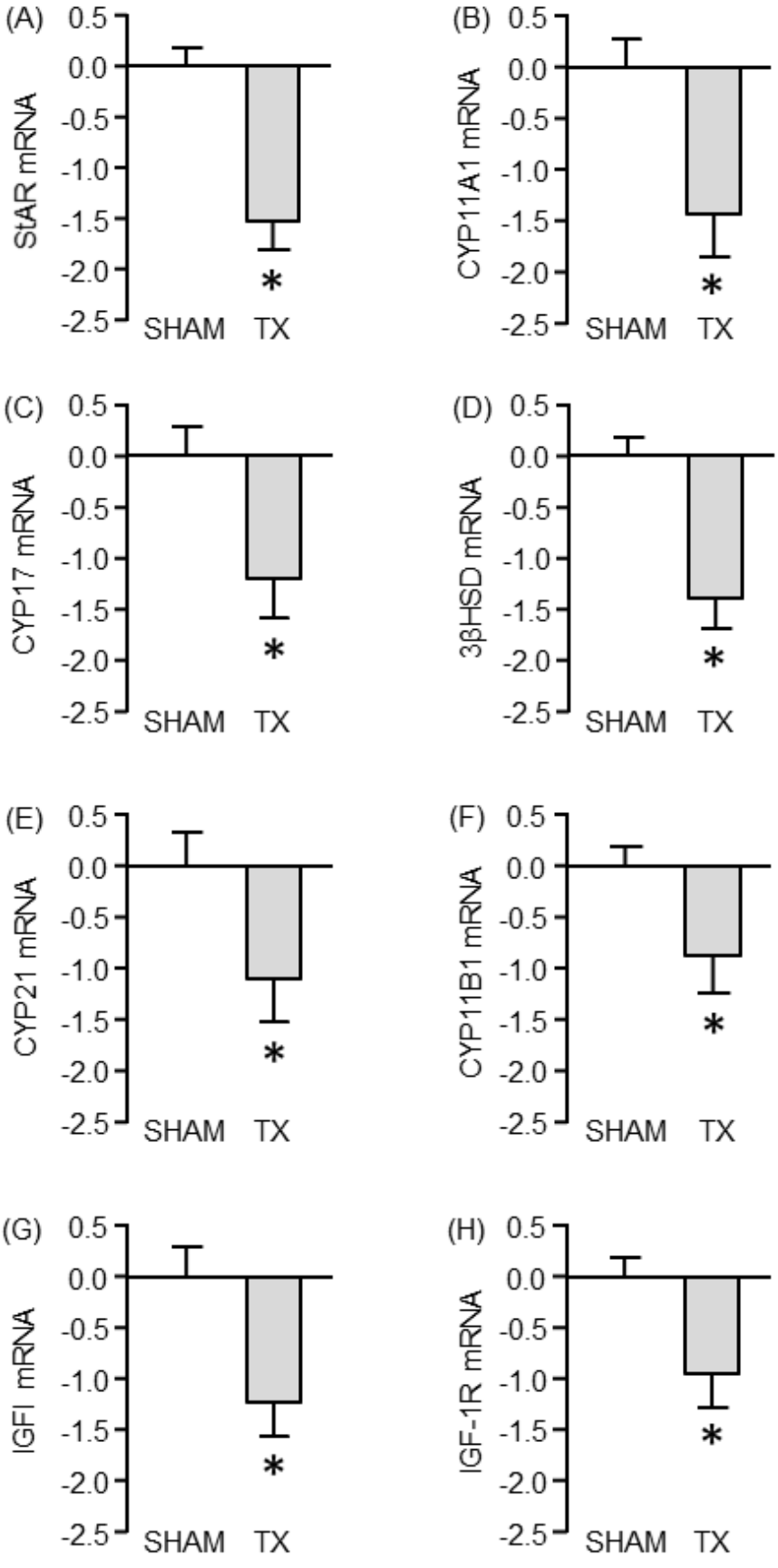
(A)
StAR mRNA
(B)
CYP11A1 mRNA
(C)
CYP17 mRNA
(D)
3βHSD mRNA
(E)
CYP21 mRNA
(F)
CYP11B1 mRNA
(G)
IGFI mRNA
(H)
IGF-1R mRNA
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
SHAM
TX
*

Figure 3.

589 **Supplementary Table 1.** Sequences and accession numbers of the primers used in qPCR to determine adrenal mRNA abundance.

Gene	Forward Primer	Reverse Primer	Accession number
MC2R	GTATGAAAACATCAACAGTACAGCAAGAA	AAAACTCCGACAATGGATACTGTGA	AF116874
StAR	GCATCCTCAAAGACCAGGAG	CTTGACACTGGGGTTCCACT	NM_001009243
CYP11A1	GGCTCACAGAGAATCCACTTTCG	TGATGTCCCCTACAAACTTTCCG	D50057
CYP17	CATCAGAGAAGTGCTCCGAATCC	TCCTGCTCCAAAGGGCAAGTAG	AF251388
3β-HSD	CCTGCTGGAAGGAGACATTCTG	GTGCTGGTGTGGATAAAGACCG	NM_174343
CYP21	TGCCTCGGTGTCTCCTTTTATTG	GGTGCCCCTTCACGGAAATG	M11267
CYP11B1	GGAGACACATGGTGTTCGTG	CACCAAGGGCGTGTACTTCT	NM_174638.3
IGF-I	GAATCGTGGATGAGTGCTGCT	AGCAGCACTCATCCACGATTC	NM_001009774.3
IGF-II	GCTTCTTGCCTTCTTGGCCTT	TCGGTTTATGCGGCTGGAT	NM_001009311.1
IGF-IR	AAGAACCATGCCTGCAGAAGG	GGATTCTCAGGTTCTGGCCATT	XM_012098367.2
IGF-IIR	GATGAAGGAGGCTGCAAGGAT	CCTGATGCCTGTAGTCCAGCTT	XM_004011550.1
PNMT	CCCTCATTGACATCGGTTCAGG	CGGTTCACCTCCAGGAAATCTG	M14318
YWHAZ	TGTAGGAGCCCGTAGGTCATCT	TTCTCTCTGTATTCTCGAGCCATCT	AY970970

590

591