# Maternal undernutrition alters triiodothyronine concentrations and pituitary response to GnRH in fetal sheep

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## Abstract

The aims of this study were to determine which hormones may have a role in the expression of maternal undernutrition effects on reproductive function, in both the developing fetus and the adult offspring. This was undertaken by measuring the effects of long-term maternal undernutrition on metabolic hormone profiles and pituitary responses to single doses of GnRH and GH-releasing factor (GRF) in fetal sheep. From mating, groups of ewes were fed rations providing either 100% (HIGH) or 50% (LOW) of estimated metabolisable energy requirements for pregnancy throughout the experiment until slaughter at approximately 119 days of gestation. Fetal and maternal blood samples were collected from 113 until 119 days of gestation, via carotid and jugular catheters respectively, and assayed for insulin, IGF-I, GH, thyroxine and triiodothyronine ($T_3$). Undernutrition had no effects on fetal weight, fetal gonad weight of either sex, fetal insulin or IGF-I concentrations. Male LOW fetuses exhibited a significantly attenuated response ($P<0{\cdot}05$) to a bolus challenge of GnRH compared with HIGH fetuses. Basal fetal GH concentrations and the response to exogenous GRF were similar in both treatment groups, although LOW fetuses exhibited more secretory episodes ($P<0{\cdot}01$). Mean $T_3$ concentrations were significantly lower in both the maternal ($P<0{\cdot}01$) and fetal ($P<0{\cdot}05$) plasma of LOW animals compared with HIGH animals. It is concluded that pituitary function was altered in fetal males and could influence male reproductive development. On the other hand, in female sheep, fetal gonadal abnormalities and reductions in reproductive capacity in adult life which are associated with fetal undernutrition are unlikely to be attributable to altered pituitary function. Additionally, these studies raise the possibility that thyroid hormones may have a role in the expression of maternal undernutrition effects on fetal development.

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## Introduction

Maternal undernutrition during pregnancy is known to cause a permanent reduction in the reproductive capacity of female offspring in sheep (Gunn *et al.* 1995, Rhind *et al.* 1998*a*); equivalent effects in the male offspring have not been investigated. Additionally, maternal undernutrition is thought to be a causal factor in the aetiology of a number of adult diseases in humans (Barker *et al.* 1992). The mechanisms through which these effects are exerted on the fetus and expressed in the adult organism are unknown.

In recent studies of the effects on fetal ovary development of maternal undernutrition, oogonial meiosis and follicular development were found to be delayed (Rae *et al.* 2001). These studies highlighted the importance of the timing of nutrient restriction with respect to the stage of development of the fetus, and indicated that changes in gonadal development occurred in response to periods of undernutrition. To date, there are no such studies which address the possibility of effects of maternal undernutrition on fetal male development.

The present study was designed to identify differences in adult and fetal hormone profiles associated with undernutrition through which the previously reported effects of undernutrition on fetal gonad development (Rae *et al.* 2001) and adult function (Gunn *et al.* 1995, Rhind *et al.* 1998*a*) could be mediated. Effects were assessed by measuring basal hormone concentrations together with responses to single doses of each of two releasing hormones (gonadotrophin-releasing hormone (GnRH) and growth hormone (GH)-releasing factor (GRF)) administered in physiological doses.

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## Materials and Methods

### *Animal management and nutritional treatments*

All experimental procedures involving animals were conducted under the authority of the Animals (Scientific Procedures) Act (1986), after UK Home Office and local ethical committee approval.

Since limited numbers of blood samples could be collected from each fetus, owing to their relatively small blood volume, the experimental protocols were conducted during two consecutive years using comparable groups of ewes from the same flock, maintained under the same daylength and temperature conditions.

Mature Scottish Blackface ewes were fed to achieve similar, moderately high, levels of body condition and were mated at a synchronised oestrus following treatment, for 14 days, with intravaginal progestagen pessaries (Chronolone, 30 mg; Intervet, Cambridge, Cambs, UK). At the time of mating, ewes were allocated randomly, within body condition score (BCS) class (Russel *et al.* 1969), to one of two nutritional treatment groups. They were fed rations designed to meet either the estimated metabolisable energy (ME) requirements of the pregnant ewe, according to stage of pregnancy (designated HIGH) (Robinson *et al.* 1983), or 50% of that amount (designated LOW). At day 70 of pregnancy, ewes were subjected to ultrasound scanning to determine fetal burden. The rations were then adjusted according to stage of pregnancy, litter size and treatment group to maintain the treatment difference while taking account of the increasing fetal burden.

The diet comprised pelleted feed (Green Keil; North Eastern Farmers Ltd, Bannermill, Aberdeen, UK) and hay and was estimated to provide 8·0 MJ ME/day and 4·0 MJ ME/day to HIGH and LOW animals respectively during the first 75 days of pregnancy. From the time of mating, sheep were housed individually under natural daylength conditions with free access to water.

All ewes were weighed and BCSs determined every 21 days throughout the experiments.

### *Surgery and hormone challenges*

Ewes ($n=10$/group per year) with a mean liveweight ($\pm$ S.E.M.) of 59·0 $\pm$ 0·62 kg and a mean BCS of 2·5 $\pm$ 0·02 at mating, were housed in individual pens and fed as follows: HIGH ewes, 100% maintenance from mating to 119 days of gestation; LOW ewes, 50% maintenance from mating to 119 days of gestation.

Following feed withdrawal, for a 12 h period at a time between 108 and 110 days of gestation (term = 147 days), fetal jugular and carotid catheters (polyvinyl, i.d. = 0·72 mm; o.d. = 1·22 mm) were inserted, surgically, under general anaesthesia, in single and twin fetuses, using the method of Brooks & White (1990). Catheters (polyvinyl, i.d. = 1·68; o.d. = 2·39 mm) were also inserted in the amnionic cavity and exteriorised through the maternal flank. Immediately following surgery, and daily during a 3 day recovery period, antibiotics were administered (i.m.) to the ewes (4 ml Streptopen; Pitman and Moore, Crewe, Cheshire, UK) and into the fetal amniotic cavity (1 ml Crystapen, Britannia Pharmaceuticals, Redhill, UK) via the catheter. During the first 2 days of post-operative recovery, all ewes received an analgesic (buprenorphine) (Vetergesic; Alsoe Animal Health, Melton Mowbray, Leicestershire, UK). Catheter patency was maintained by flushing twice daily with sterile heparinised saline (50 IU/ml). In all cases, a 3 day recovery period was allowed between surgery and collection of maternal and fetal blood for hormone analysis.

Fetal arterial blood was collected twice daily, using aseptic techniques, during both recovery and experimental periods, and analysed for pH, $pO_2$ and $pCO_2$; since there were no observed differences in these parameters with respect to nutrition these data were used solely as indices of fetal well-being. These analyses were repeated at the end of each experimental period to confirm fetal health throughout all experiments.

In year 1, maternal jugular vein and fetal arterial blood samples were collected twice daily for 6 days. On day 6, fetuses were sampled at 15 min intervals for 4 h via arterial catheters. Immediately after the end of this sampling period, fetuses were injected (i.v.) with 20 μg GRF (synthetic human peptide, amino acids 1–29-$NH_2$ acetate; BACHEM Feinchemikalien AG, Bubendorf, Switzerland), and blood samples were collected every 15 min for a further 2 h. This dose was based on the studies of Bauer *et al.* (1995), which indicated that this dose of the synthetic human peptide elicited a pulse of GH in sheep fetuses which was within a measurable range.

In year 2, fetal arterial blood samples were collected at 15 min intervals for 45 min (basal samples). Immediately after the third sample, a 150 ng bolus of GnRH (Sigma Chemical Co., Poole, Dorset, UK) in 500 μl sterile saline was administered (i.v.) to each fetus via the jugular venous catheter. Fetal arterial blood samples were then collected at 7·5 and 15 min after injection and thereafter at 15 min intervals for a further 90 min. Samples were centrifuged at 2000 ***g*** for 10 min and the plasma was removed and stored at $-70$ °C until analysis. The dose of GnRH used was based upon a dose of 100 ng used in a previous study of fetuses at day 104 of gestation (Miller *et al.* 1998). This was increased to 150 ng in the current study to take account of the increase in fetal size expected between day 104 and day 115, in accordance with other studies (Miller & Brooks 1999). Dose–response studies were not performed for either of the two releasing hormones to identify an optimal dose because results could have been compromised by serial dosing and the difficulty of surgical preparation precluded the use of one set of animals for each of several doses.



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At the end of the experimental period ewes were killed by barbiturate overdose (Euthatal, 30 ml (i.v.); Rhone Merieux, Harlow, Essex, UK). At this stage, fetuses were weighed, gonads dissected out, weighed, and visually examined to identify macroscopic abnormalities and to determine fetal sex. Discrepancies between numbers of ewes and fetuses at the beginning of each experiment and at analysis at the end of the experiment are attributable to non-pregnant animals in the case of ewes and catheter failure in the case of fetuses.

### *Hormone analysis*

Samples were analysed in single assays for: luteinising hormone (LH) (Adams & Findlay 1998), sensitivity 0·05 ng/ml, coefficient of variation (CV) 5·2%; insulin (MacRae *et al.* 1991), sensitivity 2·6 ng/ml, CV 6%; GH (Rhind *et al.* 1998*b*), sensitivity 0·63 ng/ml, CV 11%; thyroxine ($T_4$) (DPC, Los Angeles, CA, USA), sensitivity 2·5 ng/ml, CV 3·2%; triiodothyronine ($T_3$) (DPC), sensitivity 0·07 ng/ml, CV 5·5%; and insulin-like growth factor-I (IGF-I) (Bruce *et al.* 1991), sensitivity 1·95 ng/ml, CV 5%.

### *Statistical analyses*

The effects of nutritional treatment on fetal weight, gonad weight and hormone concentrations were analysed by one-way ANOVA, using litter size as a covariate. Data from both experiments were combined where appropriate. Sex differences were observed only within treatment groups with respect to LH (but not metabolic hormones) and so male and female fetuses were analysed separately for LH. Where a skewed distribution was evident, data were transformed (log (value + 1)) before analysis.

Basal LH and GH concentrations were defined as the mean value for samples collected before exogenous hormone infusion. GH secretory episodes were defined as a minimum of two consecutive samples which exceeded the mean baseline concentration by at least 1 S.D. The magnitudes of the peaks induced by infusion of exogenous hormones were defined by subtraction of the mean basal values from the mean induced peak values.

## Results

### *Maternal weights and BCSs*

HIGH ewes exhibited no change in mean BCS during the study while LOW ewes exhibited a mean reduction of 0·24 BCS units over the course of the experiment. The mean liveweight of HIGH ewes increased by 1%, while that of LOW ewes decreased by 12% during the experiment (Table 1).

**Table 1** Liveweights and body condition scores (BCSs) (means ± S.E.M.) of HIGH and LOW ewes at mating and before slaughter at 110 days of gestation. No significant differences were recorded between treatment groups for either parameter at the beginning of the experiment; however, at 110 days of gestation liveweight of LOW ewes was significantly different from their mating liveweight and from the liveweight of HIGH ewes at 110 days of gestation ($P<0·05$)

| | **HIGH** ($n=16$) | | **LOW** ($n=20$) | |
|---|---|---|---|---|
| | Mating | Slaughter | Mating | Slaughter |
| Liveweight (kg) | 57·6 ± 1·97 | 59·6 ± 1·27 | 57·3 ± 1·90 | 50·2 ± 1·74 |
| BCS | 2·6 ± 0·09 | 2·6 ± 0·04 | 2·6 ± 0·08 | 2·3 ± 0·03 |

**Table 2** Fetal weights and fetal gonad weights (means ± S.E.M.) from HIGH and LOW pregnancy nutritional treatment groups at 119 days of gestation

| | **HIGH** | **LOW** | **Significance** |
|---|---|---|---|
| Females | | | |
| *n* | 8 | 9 | — |
| Fetal weight (kg) | 1·48 ± 0·06 | 1·28 ± 0·05 | NS |
| Fetal ovary weight (mg) | 22·4 ± 1·35 | 22·9 ± 2·21 | NS |
| Males | | | |
| *n* | 12 | 11 | — |
| Fetal weight (kg) | 1·57 ± 0·09 | 1·59 ± 0·06 | NS |
| Fetal testes weight (mg) | 342·0 ± 13·9 | 358·4 ± 16·2 | NS |

**Table 3** Mean concentrations (± S.E.M.) of hormones in plasma of ewes at 113–119 days of gestation in HIGH and LOW treatment groups

| | **HIGH** ($n=6$) | **LOW** ($n=10$) | **Significance** |
|---|---|---|---|
| IGF-I | 214·2 ± 11·9 | 246·9 ± 18·2 | NS |
| Insulin | 15·7 ± 0·59 | 16·1 ± 0·58 | NS |
| $T_3$ | 1·13 ± 0·09 | 0·70 ± 0·04 | $P<0·01$ |

### *Fetal and gonad weights*

There were no significant effects of nutritional treatment on either the mean fetal weights or mean fetal gonad weights of either male or female fetuses (Table 2). The mean fetal weight of LOW males was significantly higher than that of LOW females ($P<0·01$) but there was no significant difference with sex in the mean weight of HIGH fetuses.

### *Metabolic hormones*

**Maternal** There were no significant differences between treatment groups in maternal plasma insulin or IGF-I concentrations, but $T_3$ concentrations were significantly lower in LOW animals ($P<0·01$) (Table 3).

**Fetal** There were no differences between treatments in mean plasma insulin or IGF-I concentrations. The mean



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**Table 4** Mean concentrations (± S.E.M.) of hormones in fetal plasma from HIGH and LOW nutritional treatment groups at 113–119 days of gestation, the number of GH pulses, and the response to GRF stimulation

| | HIGH | LOW | Significance |
|---|---|---|---|
| IGF-I (ng/ml) | 242·4 ± 14·9 ($n$=8) | 213·2 ± 7·9 ($n$=8) | NS |
| Insulin (μIU/ml) | 18·3 ± 1·15 ($n$=8) | 19·2 ± 1·76 ($n$=8) | NS |
| $T_3$ (ng/ml) | 0·24 ± 0·01 ($n$=8) | 0·19 ± 0·02 ($n$=8) | $P$<0·05 |
| $T_4$ (ng/ml) | 82·9 ± 5·2 ($n$=12) | 72·4 ± 4·6 ($n$=11) | NS |
| Basal GH (ng/ml) | 7·85 ± 1·2 ($n$=8) | 12·2 ± 2·75 ($n$=6) | NS |
| No. of GH episodes | 1·25 ± 0·1 ($n$=8) | 2·0 ± 0·26 ($n$=6) | $P$<0·01 |
| GRF-induced peak (ng/ml) | 16·4 ± 1·53 ($n$=8) | 18·8 ± 5·14 ($n$=6) | NS |

fetal plasma $T_3$ concentration was significantly lower in LOW than HIGH fetuses ($P$<0·05) (Table 4); fetal $T_4$ was also lower in LOW than in HIGH fetuses but the difference was not significant. Mean basal GH concentrations and GRF-induced GH increases were similar in both groups but the number of GH secretory episodes was significantly greater in LOW than HIGH fetuses ($P$<0·01) (Table 4).

**Reproductive hormones** No differences between treatment groups in mean basal LH concentrations were recorded for either sex. However, the response to an exogenous GnRH challenge, as defined by the magnitude of the increase in LH concentration, was significantly lower in male LOW fetuses as compared with male HIGH fetuses ($P$<0·05) (Fig. 1); no such difference in response was recorded in female fetuses (Fig. 1).

## Discussion

Previous studies have shown the sheep fetus to be sensitive, in terms of metabolic hormonal status, to acute changes in maternal nutrition, usually involving a sudden, short-term reduction in the intake of an otherwise well-nourished pregnant ewe (Schreiner *et al.* 1980). However, the current study was designed to determine the effects of chronic undernutrition such as that to which sheep grazing unimproved upland pasture in the UK are exposed in practice. Some human populations may be subject to equivalent nutritional insufficiencies, particularly in the third world.

The similar weights of HIGH and LOW fetuses indicated that intrauterine growth retardation had not occurred and so the effects reported in this study cannot be directly compared with studies involving a placentally mediated reduction in fetal growth (Wallace *et al.* 1997), although the placenta may nevertheless be involved in the mediation of the effects observed in the current study. The current study demonstrates that effects of maternal nutrition on the developing fetus can occur in the absence of any reductions in fetal weight, as reported previously in studies of humans (Lumey *et al.* 1998).

Fetal and maternal $T_3$ concentrations were significantly reduced in response to undernutrition, raising the possibility of thyroid hormones playing a role in mediation of effects of undernutrition on fetal reproductive development. On the other hand, profiles of insulin, GH and IGF-I were not affected, indicating that the previously observed effects on fetal ovarian development, including delayed fetal ovarian folliculogenesis (Rae *et al.* 2001), were unlikely to be directly mediated by changes in profiles of these hormones.

Thyroid hormone concentrations are known to be nutritionally sensitive, and, in the case of fetal and perinatal animals, thyroid status is largely determined by the nutrient supply to the fetus (Symonds 1995). It has been demonstrated that there is no transfer of thyroid hormones across the placenta and so the fetal thyroid is autonomous (Nathanielz 1975). Thyroid hormones are known to regulate components of fetal growth and development, including the final stages of development of fetal brown adipose tissue, liver and lungs (Symonds 1995), and they are thought to interact with other hormones to control the rate of fetal growth (Latimer *et al.* 1993). These effects may be induced through alterations in expression or action of other hormones, such as GH or IGF-I, or their receptors (Richards *et al.* 1993, Forhead *et al.* 2000). Collectively, these observations indicate that thyroid hormones, in particular $T_3$, are a possible mediator of the effects of maternal undernutrition on fetal reproductive development. This suggestion is supported by other studies in both rodents and sheep; hypothyroidism in rats is associated with a reduction in Sertoli cell numbers (Maran *et al.* 1999) while sub-optimal feeding of mature rams leads to reduced Sertoli cell numbers in the testes (Hotzel *et al.* 1998) and hypothyroidism in mature rams results in pituitary insensitivty to GnRH, reduced testicular testosterone secretion and spermatozoal motility (Chandrasekhar *et al.* 1985). All of these observations are indicative of effects of the thyroid hormones on the development and function of the male gonad, and by inference, they may



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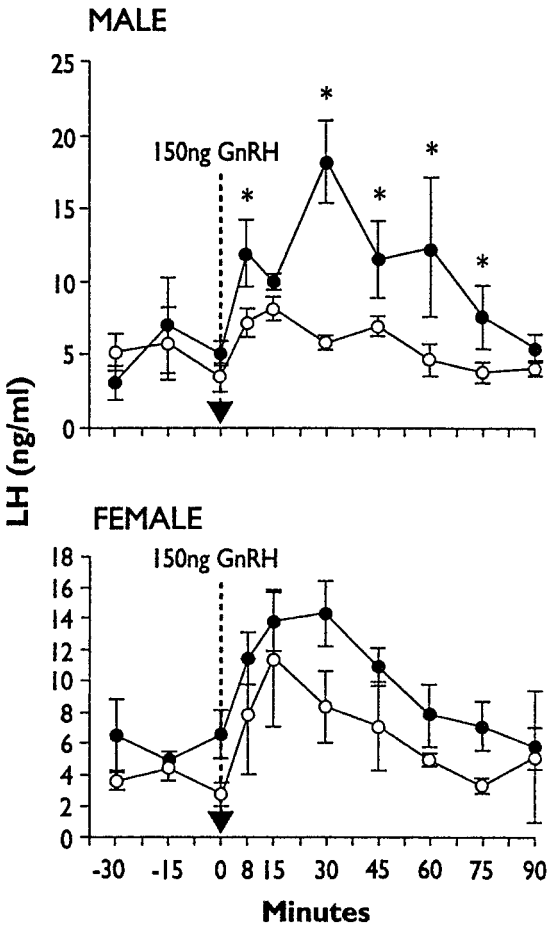


**Figure 1** LH concentrations in response to a 150 ng GnRH injection in HIGH (●) and LOW (○) fetuses. *$P<0{\cdot}05$ for LOW fetuses ($n=4$) compared with HIGH fetuses ($n=5$) for the male fetal response. For the female fetal response, there were no significant differences between HIGH ($n=6$) and LOW ($n=4$).

also play a role in the development and function of the ovary, since the two systems share many common regulatory features.

Previous studies have indicated that at the stage of gestation examined in the current study no responses to thyrotrophin-releasing hormone (TRH) stimulation, measured in terms of $T_4$ secretion, could be detected (Klein & Fisher 1980). These authors reported that injection of TRH raised fetal TSH levels without any downstream effects on $T_4$ levels at 105–115 days of gestation, as observed in pilot studies conducted in our own laboratory (M T Rae, S M Rhind & A N Brooks, unpublished observations). Thus, although the current study showed a reduction in fetal $T_3$ concentrations in response to maternal undernutrition, it appears unlikely that this was under hypothalamic–pituitary control at this stage of development. However, since there was no significant difference in circulating $T_4$ concentrations, an alternative explanation may be that the liver concentrations of tissue deiodinase enzymes, which are responsible for the conversion of $T_4$ to $T_3$, were altered by undernutrition.

The absence of an effect of nutrition on fetal profiles of IGF-I and insulin contrasts with previous reports involving acute undernutrition/starvation during pregnancy (Schreiner *et al.* 1980, Bassett *et al.* 1990). This difference in response between experiments probably reflects the difference in the pattern of undernutrition, and highlights the different effects of acute and chronic undernutrition.

As indicated above, effects of the thyroid hormones could be mediated through changes in profiles of other hormones, including GH and IGF-I. The role of GH in fetal growth and development is of lesser importance in fetal than in postnatal animals, probably as a result of GH receptor immaturity in fetal life (Gluckman *et al.* 1985). Basal GH secretion, and the GRF-stimulated increase were similar in both groups of fetuses in the present study. However, the number of secretory episodes of GH was higher in LOW than in HIGH fetuses, although the overall mean concentrations were not significantly affected by treatment. The increased frequency of pulses would serve to maintain the supply of energy-providing nutrients to the fetal organs, inhibiting deposition in tissue reserves, but its significance with respect to development of the fetal hypothalamic–pituitary–gonadal system, if any, remains unknown. Previous studies have shown that fetal GH concentrations were increased in response to long-term maternal undernutrition but in these studies the reduction in feeding was applied after a period of adequate feeding and the restriction was more severe than in the current study (Bauer *et al.* 1995). These experimental differences may account for the differences in the observed fetal responses.

One mechanism through which changes in the metabolic hormones could affect development and function of fetal gonads is through effects on pituitary response to GnRH. The observation that male LOW fetuses exhibited a lesser response to GnRH injection compared with HIGH fetuses could be attributable to an increased rate of clearance of GnRH from the LOW fetuses but it is more likely that the LOW fetuses had a reduced pituitary sensitivity to GnRH compared with HIGH fetuses since hypothyroidism has been shown to cause pituitary insensitivity to GnRH in adult rams (Chanrasekhar *et al.* 1985). In male sheep fetuses, it has been demonstrated that the growth of the testes, and Sertoli cell number, are dependent on adequate gonadotrophin secretion during fetal life (Thomas *et al.* 1994); thus, a reduction in pituitary responsiveness to GnRH could contribute to reproductive insufficiency in later life. On the other hand, the observation of a similar pituitary response to GnRH in female fetuses of both treatments in the present study is consistent with previous reports which indicated that gonadotrophin secretion suppression has no effects on fetal ovarian weight



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or morphology (Thomas *et al.* 1994). It is possible that the responses observed in the two treatment groups could have been different if the GnRH dose administered had been altered but this is considered unlikely since the dose of GnRH used was considered to be within the physiological range.

It is known that the effects of maternal undernutrition on ovarian development and fetal folliculogenesis can be expressed before the onset of fetal gonadotrophin secretion (Borwick *et al.* 1997, Rae *et al.* 2001). Taking this observation together with the absence of a difference in pituitary response to GnRH in females, it is concluded that it is unlikely that gonadotrophin insufficiency during fetal life is a major factor contributing to the reduced lifetime reproductive performance in ewes.

In summary, we have shown that plasma concentrations of $T_3$ in the sheep fetus are reduced in response to long-term, chronic maternal undernutrition and that in male fetuses this may have induced a reduction in pituitary responsiveness to GnRH which, in turn, raises the possibility of effects on reproductive function in later life.

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