UNIVERSITY OF ALBERTA

INSULIN-LIKE GROWTH FACTOR I AS A MEDIATOR OF PORCINE OVARIAN REPONSES TO NUTRITIONAL REGIMEN

BY

SUSAN T. CHARLTON

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **INSULIN-LIKE GROWTH FACTOR I AS A MEDIATOR OF PORCINE OVARIAN RESPONSES TO NUTRITIONAL REGIMEN** submitted by **SUSAN T. CHARLTON** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **ANIMAL REPRODUCTION.**

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ABSTRACT

The objective of this thesis was to develop and apply methodology to test the hypothesis that IGF-1 is a locally produced hormone that may mediate ovarian responses to changes in metabolic state.

Northern analysis and RNase protection procedures were used to detect IGF-1 gene expression in porcine ovarian and hepatic tissues. The RNase protection procedure was found to be more senstive and, therefore, more suitable for detecting IGF-1 mRNA in individual animal samples.

A preliminary study established a seven day restrict-fed gilt model for studies in nutrition-reproduction interactions in our laboratory. A second study developed this model to determine whether nutritional manipulation of the metabolic state of a prepubertal gilt alters ovarian and hepatic IGF-1 gene expression and subsequent production of IGF-1. Eleven pairs of littermate gilts (75kg) were placed on a maintenance level of feeding for seven days. On d8, littermates were split into two groups and either maintenance-fed or fed to-appetite for a further six days. Blood samples were taken, via indwelling jugular cannula, on d13 (07.00-16.00h) to determine plasma insulin and IGF-1, and on d14 (02.00-06.00h) to determine plasma GH. Following slaughter on d14, one ovary from each animal was retained to measure follicular fluid IGF-1 and estradiol. The remaining ovary and a sample of liver were retained for mRNA analysis. Analysis indicated significant increases in plasma IGF-1 ($P<0.005$) and basal insulin ($P<0.05$), in response to six days of re-feeding, with no effect on plasma GH. Ovarian follicular volume and diameter were significantly larger after re-feeding ($P<0.05$) with no changes in follicular fluid estradiol. Mean follicular fluid IGF-1 concentration was unaffected by treatment, however regression analysis revealed significant

effects on individual follicular fluid IGF-1 content ($P<0.05$). Analysis of IGF-1 mRNA indicated increases in hepatic IGF-1 gene expression but decreases in ovarian IGF-1 gene expression in response to re-feeding.

The results from this study suggest that IGF-1 may act as a paracrine or autocrine indicator of metabolic status at the level of the ovary, and that IGF-1 gene expression is differentially regulated by metabolic status in hepatic and ovarian tissues.

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CHAPTER 1

Insulin-Like Growth Factor I as a Mediator of Porcine Ovarian Responses to Nutritional Regimen

1.1. INTRODUCTION

The metabolic state of an animal is defined as whether the animal is in a state of anabolism or catabolism although the metabolism of an animal is in fact dynamic with continuous tissue gain (anabolism) and loss (catabolism). If an animal is in an anabolic state, there is a surplus of energy available above that required to maintain the animal at its present body condition and weight. As a consequence, the balance between anabolism and catabolism shifts in favour of anabolism and there is a net gain in tissue mass. In a growing animal, surplus energy, which may be carbohydrate or protein in nature, is used initially to fuel tissue proliferation and differentiation. Excess energy to the requirement for growth are either stored (ie. lipids and carbohydrates) or metabolised and excreted (ie proteins and amino acids).

If an animal is in a catabolic state, insufficient energy is available from dietary sources to maintain the animal's condition and body weight. In this case, the balance between anabolism and catabolism shifts in favour of catabolism as the animal metabolises the most accessible stored energy (for example glycogen and lipids) to make up the deficit in energy required. These stores are rapidly depleted and the animal then begins to breakdown tissue protein in order to produce the necessary energy to maintain essential functions.

When an animal becomes catabolic, reproductive function is deprioritized in favour of more essential bodily functions. In essence, reproductive activity is suspended. It therefore follows that nutrition has important effects on reproductive status. In the following review, I discuss the effects of feeding level on the reproductive development of

the prepubertal gilt, and particularly the effects on ovarian development. I propose that insulin-like growth factor 1 (IGF-1) is a growth factor that might be a metabolic signal mediating the effects of nutrition at the level of the ovary. Therefore, I investigate the effects of feeding level on IGF-1 gene expression and circulating levels, in addition to the characteristics of the IGFs and the proposed autocrine/paracrine functions of IGF-1 in ovarian tissue.

1.2. NUTRITION-REPRODUCTION INTERACTIONS

The effects of nutrition on the reproductive status of the female are well documented in both humans and animals. For comprehensive reviews on this subject the reader is referred to I'Anson *et al.* (1991), Bronson & Manning (1989) and Cameron (1989). In general, undernutrition of immature animals has been found to delay or completely inhibit attainment of puberty, while in mature animals, reproductive cycles become erratic or stop completely. If an undernourished animal does succeed in becoming pregnant, conception rates are low and the return to a fertile cyclic state following parturition will be delayed.

The exact mechanisms mediating the interactions between the nutritional status of an animal and its reproductive system are obscure, but several theories have emerged to explain this complex aspect of animal physiology (reviewed by I'Anson *et al.*, 1991). Initially it was hypothesised that the interaction between nutrition and reproduction was related to body composition (Frisch & Revelle, 1971). Now evidence suggests that the interaction is metabolic in nature, with dynamic changes in metabolism regarded as possible signals for reproduction rather than

changes in body condition. Gut peptides, released from the gastrointestinal tract as a result of stimulation by specific constituents of food, have also been implicated as possible signals. These gut peptides may act as neuropeptides, and as such, influence the reproductive system via the central nervous system (Snyder, 1980).

Undernutrition of females of many species results in hypogonadism. For example, using prepubertal gilts that had been restrict-fed for seven days, Booth (1990) demonstrated that short term feed restriction reduced luteinizing hormone (LH) pulsatility in the absence of changes in body composition. Ovarian and uterine development were also impaired. Re-feeding of the same gilts with either a single feed to appetite, or a parenteral infusion of glucose, resulted in restored LH pulsatility within six hours. These results also suggest that the reproductive status of the prepubertal gilt is not primarily affected by body composition or, changes in levels of gut peptides, but is instead under the influence of changes in metabolic state.

1.3. MEDIATORS OF NUTRITION-REPRODUCTION INTERACTIONS

If reproduction is dependent on changes in the metabolic state of an animal, the next question asked must be what aspects of this change in metabolic state mediate the interaction between nutrition and reproduction?. Further more, at what level do these interactions occur? Are such the interactions systemic or local? Experimental manipulation of metabolic state by feeding level, or by other means, has been used in studies designed to identify possible links between metabolism and reproduction. Several ideas are emerging as to what these links are.

One theory suggests that blood glucose level is a possible metabolic cue for reproduction. McClure (1967) demonstrated that hypoglycaemia inhibited reproduction in mice. Fasting, or treatment with both insulin or 2-deoxyglucose (a drug that decreases intracellular glucose utilization), reduced fertility. Administration of human chorionic gonadotropin (HCG) prevented the observed infertility, suggesting that induced hypoglycaemia affected reproduction indirectly at the level of the hypothalamus-pituitary axis rather than directly at the level of the gonads. Similar observations were made using 2-deoxyglucose in cyclic heifers (McClure *et al.*, 1978) and in anoestrus ewes (Crump & Rodway, 1986).

The mechanism by which hypoglycaemia inhibits reproductive activity may be simply a consequence of limited fuels available for reproductive processes. However, as low blood glucose levels affect metabolism on a whole body scale, including the reproductive system, this is unlikely to be the case unless the reproductive system is more sensitive to hypoglycaemia. It seems more likely that other more specific metabolic cues, such as insulin, are also involved in the deprioritization of the reproductive system in favour of other more essential bodily functions.

Specific insulin binding sites have been located within the hypothalamus and particularly in the region of the arcuate nucleus (Van Houten *et al.*, 1980). Therefore, although glucose uptake by the brain is independent of both insulin and normal blood glucose levels, binding of insulin by these sites may regulate the utilization of glucose by the brain, particularly as cerebral spinal fluid levels of insulin have been found to be correlated to basal levels of plasma insulin (Woods & Porte, 1977). In this way insulin levels may be able to act as a metabolic

signals for reproduction at a central level, and in particular at the level of the hypothalamus.

In direct relation to reproduction, however, insulin has been found to have both inhibitory and stimulatory effects. McClure (1967), as described above, demonstrated that insulin treatment has an inhibitory effect on the reproductive status of mice. Insulin treatment of young cyclic gilts, however, has been shown to enhance reproductive status (Jones *et al.*, 1983): insulin had positive effects on reproductive activity at the level of the ovary without affecting gonadotropin, secretion as insulin-treated gilts that exhibited estrus had higher ovulation rates, while neither LH peak amplitude or basal levels were altered. These data suggest that, in swine, there may be differential control of reproduction by metabolic state at the level of the ovary compared to the hypothalamus-pituitary axis.

These results were confirmed in a second study by the same group that determined the effects of short or long term acting insulin treatment on the ovulation rates and pattern of LH secretion in cyclic gilts fed a high energy diet (Cox *et al.*, 1987). Both forms of insulin treatment were found to significantly increase ovulation rates in the absence of significant increases in LH peak amplitude or basal levels. Similar results were found using a modification of the seven day restrict-fed gilt model developed by Booth (1990). The LH secretion of prepubertal gilts was partially, but chronically, suppressed using the oral progestagen allyl trenbolone ("Regumate"), and the gilts were placed on a maintenance level of feeding. After seven days on this feeding regimen, animals were either re-fed to appetite or continued at a maintenance level of feeding. The

results from this study indicated that, even in the absence of increasing LH secretion, re-feeding of short-term restrict-fed gilts can of stimulate ovarian development and function (Cosgrove *et al.*, 1991), again suggesting that there is regulation of ovarian development and function by changing metabolic state independent of effects at the level of the hypothalamic-pituitary axis.

To summarize, there must be one or more metabolic signals that mediate the effects of metabolic state on reproduction, and these signals do not necessarily have the same effects at the level of the ovary as they do at the level of the hypothalamus. Blood glucose level and insulin have both been implicated as having such roles. Supporting evidence, however, is conflicting and as a result other metabolic factors are being considered. In particular, attention has switched to finding possible candidates for the role of a metabolic signal at the level of the ovary. As mentioned earlier, the hypothesis for this thesis was that the peptide hormone Insulin-Like Growth Factor 1 (IGF-1) fulfils the role of such a metabolic signal. The remainder of this review will therefore provide the theoretical basis for this hypothesis.

1.4. INSULIN-LIKE GROWTH FACTORS - THE EFFECTS OF NUTRITION

It is well established that growth hormone stimulates the production of IGF-1, and that many of the actions of growth hormone are mediated by this growth factor (for a comprehensive review on this subject the reader is referred to Sara and Hall, 1990). However, studies of the effects of nutrition on circulating IGF-1 levels and IGF-1 gene expression, provide

evidence suggesting that IGF-1 production is also dependent on the nutritional status of an animal. In 1976, Phillips and Young, using a cartilage growth bioassay, showed that IGF-1 activity in cartilage tissue was reduced in young male rats after a 72 hour period of fasting. Following six hours of re-feeding, IGF-1 activity increased and was restored to control levels after 24 hours. The observed decrease in IGF-1 activity appeared to be a result of a nutritionally-induced uncoupling of the growth hormone-IGF axis, rather than as consequence of a growth hormone deficiency, as treatment of the fasted rats with bovine growth hormone did not prevent the drop in IGF activity. Therefore another stimulant for IGF-1 production, acting either directly or indirectly, was absent in the fasted animal.

Similar studies have been carried out in humans and pigs on the effects of nutrition on plasma levels of IGF-1 rather than on tissue IGF-1 content. Obese men were found to have a 75% reduction in plasma immunoreactive IGF-1 levels following ten days of fasting, which were rapidly restored to pre-fasting levels upon re-feeding (Clemmons *et al.*, 1981). Restrict-feeding of pigs also resulted in a decrease in plasma IGF-1 (Buonomo & Baile, 1991). In this study the effects of short-term fasting and subsequent re-feeding on circulating IGF-1 and other hormone levels were examined in barrows. Plasma IGF-1 levels were found to be reduced by 53% after 48 hours, even though there was an elevation of growth hormone. Re-feeding resulted in a significant decrease in growth hormone within two hours, concomitant with increases in insulin and triiodothyronine (T_3); however, IGF-1 levels did not return to pre-feeding levels until 24 hours after re-feeding. It was therefore suggested that insulin or T_3 may be

mediating growth hormone-stimulated production of IGF-1.

Molecular evidence demonstrating the effects of nutrition on IGF-1 production was provided by Elmer and Schalch (1987). They studied the effects of fasting on hepatic gene expression for IGF-1 in rats. They identified three hepatic IGF-1 mRNA transcripts with sizes of 8.0, 1.8 and 1.1 kb. Upon fasting, the levels of the 8.0 kb transcript decreased progressively after six hours, to reach only 39% of control levels after 30 hours. When rats were re-fed after 24 hours of fasting, by six hours the amount of 8.0 kb transcript had exceeded normal levels and was 18 times higher than in the fasted rats after 30 hours of re-feeding.

The site of the nutrition-sensitive control point in the growth hormone-IGF-1 axis is unclear, but a number of studies have been carried out in an attempt to identify such a site. Using a streptozotocin-induced diabetes, the effects of reduced insulin levels may be studied without alterations of feed intake. Maes *et al.* (1986) used this model to look at the responses of diabetic and control rats to bovine growth hormone treatment. Their hypothesis was that reduced insulin levels, incurred in a state of nutritional deprivation, altered either (or both) growth hormone receptor affinity or numbers. However, their results actually refuted their hypothesis as reduced insulin levels had no effect on either hepatic growth hormone binding affinity constants or binding capacity. They therefore concluded that the growth hormone-resistant state observed is due to a post-receptor defect rather than an inability of growth hormone to interact with its receptor.

The results obtained by Maes *et al.* (1986) are consistent with those of Bornfeldt *et al.* (1989). In a study comparing diabetic (streptozotocin-

induced), fasted (three days) and protein-deficient rats, Bornfeldt *et al.* (1989) demonstrated that hepatic gene expression for IGF-1 was reduced in all three states, while expression of growth hormone receptors was reduced in only the fasted and protein-deficient states. It appears therefore, that although insulin levels and IGF-1 gene expression are reduced in all three metabolic states, the site of the uncoupling of the growth hormone-IGF-1 axis depends on whether the reduction in circulating insulin is as a consequence of nutritional deprivation or due to insulin deprivation. In a diabetic state, the uncoupling site appears to be distal to receptor binding, while in nutritional deprivation the uncoupling site is a reduction in receptor binding capacity. These conclusions are supported by an earlier study demonstrating a 60% decrease in the number of hepatic binding sites, but no change in binding affinities, in rats that were starved for three days (Baxter *et al.*, 1981).

The difference between diabetes and the nutritional deprivation may be that in diabetes the treatment focuses on insulin and effects on other endocrine systems are secondary, while in nutritional deprivation model treatment affects are less focused and other endocrine systems in addition to insulin production are primarily affected. An example of such an alternative endocrine system is the thyroid system. Thyroid function has also been shown to play a role in insulin-mediated growth hormone stimulation of IGF-1 production. IGF-1 and 2 are both moderately reduced in hypothyroid patients (Furlanetto, 1983). Thyroid function interacts with the growth hormone-IGF-1 axis at two points: thyroxine (T_4) plays a regulatory role in the secretion of growth hormone and also potentiates the action of growth hormone on hepatic IGF-1 synthesis. In relation to

nutrition, undernutrition (as may result in high, normal or low T_4 levels, depending on the ability of the patient to adapt to nutritional deprivation. In general though, starvation results in a hypothyroid state with a deficiency of T_3 (Mosier & Knauer, 1983).

From the evidence presented above, it is possible to conclude that IGF-1 production is sensitive to nutritional status. In states of undernutrition, growth hormone binding capacity is diminished due to reduced production of growth hormone receptors. Evidence strongly supports the hypothesis that insulin plays an important role in the coupling of the growth hormone-IGF-1 axis. However, evidence suggests that other endocrine systems, such as that of the thyroid hormones, are also likely to be involved and therefore must not be discounted.

Evidence provided so far introduces the concept that insulin-like growth factor 1 may be an important mediator of the effects of nutrition on reproductive function. The following will be a discussion of the characteristics and the functions of IGF-1, particularly with respect to local effects in the ovary.

1.5. THE INSULIN-LIKE GROWTH FACTOR SYSTEM - CHARACTERISTICS AND FUNCTIONS

1.5.1 Historical Background

The insulin-like growth factors (IGFs) were first regarded as three separate biological factors present in serum. They exerted quite distinct biological activities: 1. Sulphation activity, 2. Non-suppressible insulin-like activity, and 3. Multiplication-stimulating activity. In

1957, Salmon and Daughaday observed that rat serum stimulated a two-fold increase in the uptake of ^{35}S-sulphate into *in vitro* incubations of cartilage. They also demonstrated that the serum of hypophysectomised rats was unable to exhibit such sulphation activity. In their incubation system, the sulphation activity could not be reinstated by the addition of growth hormone (GH) to the medium but was induced by adding the serum of growth hormone-treated hypophysectomised rats. From these findings, the authors concluded that GH may induce the formation of serum factors that mediate the action of GH. They referred to these factors as "Sulphation Factors".

Meanwhile, several other properties of serum were being investigated. Following the advent of the insulin radioimmunoassay, it became evident that the effects of serum on insulin target tissues, such as muscle and adipose tissues, were greater than expected on the basis of the serum insulin content. Froesch *et al.* (1963) demonstrated that 90% of the insulin-like effect of serum on rat adipose tissue was not suppressed by anti-insulin serum. They therefore concluded that the "non-suppressible insulin-like activity" (NSILA) was due to an insulin-immunologically distinct substance.

In addition to exerting sulphation and insulin-like activity, serum was also found to have growth promoting effects. In 1972, Pierson and Temin extracted factors from serum that were found to have multiplication-stimulating activity (MSA). These polypeptide substances were found to have a molecular weight of approximately 10,000 Da and when added to a culture medium, they stimulated cell proliferation. In the following year, Dulak and Temin (1973) found that cultured rat liver cells would secrete

multiplication-stimulating activity into a culture medium. This was the first demonstration that MSA is produced by the liver.

When the three isolated factors were compared, it soon became apparent that these substances were members of one large family of polypeptide hormones. In 1972, the term "Somatomedin" was introduced by the consensus of the workers in the field (Daughaday *et al.*, 1972) to classify the uncharacterised factor(s) that exhibited both somatic growth stimulating activity and insulin-like effects. Now the term "insulin-like growth factor" has been adopted to describe this group of growth factors.

1.5.2. IGF Structure

Rinderknecht and Humbel (1978a, b) determined that IGF-1 and IGF-2 are homologous peptides that are structurally related to pro-insulin. Both growth factors are single-chain molecules with three intra-chain disulphide bridges located in the same positions as in insulin (Froesch, 1983). IGF-1 consists of 70 amino acids while IGF-2 consists of 67. Their molecular weights are 7,646 and 7,471, respectively.

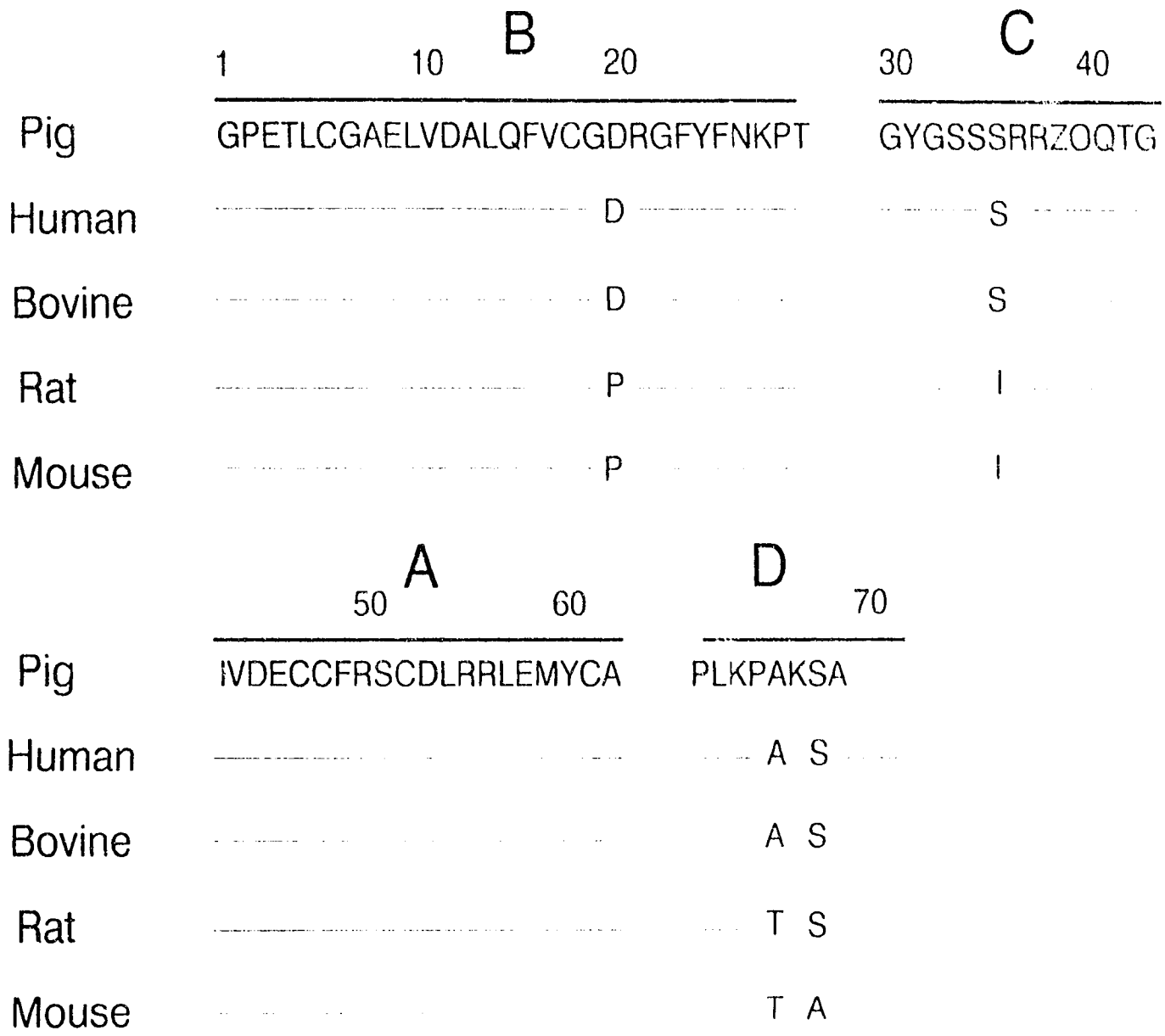
The mature IGF peptides consist of four different regions: A, B, C and D. The A and B domains are joined by a short connecting C region that consists of twelve amino acids in the IGF-1 molecule and eight amino acids in the IGF-2 molecule. The D region is an eight amino acid (IGF-1) or a six amino acid (IGF-2) extension of the A chain. The amino acid sequences of the two IGFs are identical in 45 positions, resulting in a sequence homology of 62%. In addition to the A, B, C and D regions found in the mature IGF peptides, the precursor IGF-1 peptide contains an E region. The role of the E peptide is unclear, however it has been suggested that the

E region is an extension peptide that plays an important role in the efficient transport of the newly synthesized IGF molecules across the endoplasmic reticulum (Tavakkol *et al.*, 1988). Pro-insulin contains similar A and B domains to the IGF peptides, resulting in an approximate 43% sequence homology between pro-insulin and the two growth factors. However, unlike insulin, there is no loss of the C region in the production of the IGFs. Insulin does not contain a D region.

Due to the similarities in the structures of the IGFs and insulin and their receptors, and their biological actions, it is thought that all three molecules and other related hormones such as relaxin and nerve growth factor (NGF) probably evolved from the same ancestral gene (Bradshaw & Niall, 1978). Blundell *et al.* (1978) estimated that the diversion of the ancestral gene to insulin on one hand and IGF on the other occurred approximately 600 million years ago, when vertebrates appeared on Earth. The diversion of the common IGF gene to either IGF-1 or IGF-2 is estimated to have occurred at about the time that mammals first appeared on Earth ie. 300 million years ago. This diversion of the ancestral gene resulted in a family of genes that code for insulin and insulin-like growth factors and their related growth factors.

The structure of the IGFs appears to be highly conserved between species as determined by comparing the amino acid sequences characterised from either the protein or the cDNA sequence. Bovine, porcine and human IGF-1 have been found to be identical (Sara & Hall, 1990) while rat and mouse IGF-1 differ from bovine by three and four amino acids, respectively (illustrated in Figure 1.1.). Differences in IGF-2 structure are found mainly within the B and C domains. Human and bovine IGF-2 differ only by

Figure 1.1. Schematic comparison of amino acid sequence in human, bovine and rodent mature IGF-1 peptides compared to porcine IGF-1, using an alphabet code to differentiate amino acid residues. Amino acids, in single-letter code, are numbered above the sequence; the peptide is divided into domains B, C, A and D. Sequences are identical unless a different amino acid residue is denoted.


B
1
10
20
C
30
40
Pig
GPETLCGAELVDALQFVCGDRGFYFNKPT
GYGSSSRRZOQTG
Human
D
S
Bovine
D
S
Rat
P
I
Mouse
P
I
A
50
60
D
70
Pig
IVDECCFRSCDLRRLEMYCA
PLKPAKSA
Human
A S
Bovine
A S
Rat
T S
Mouse
T A

three amino acids while human and rat IGF-2 differ by four amino acids.

Computer-aided modelling of the IGFs suggests that they have a tertiary structure that is similar to that of insulin (Blundell *et al.*, 1983). This structural similarity between the two IGFs and insulin helps to explain the cross reactivity of these hormones at the level of their membrane receptors and thus their similar biological activity. By studying differences in three-dimensional models of the IGFs and insulin, and then comparing these differences with the biological activities of the molecules, it has been possible to tentatively define the various functional sites within the IGF structures. It has been suggested that a region toward the end of the B domain may be important in binding to the IGF-1 receptor. Binding to the IGF-2 receptor, however, must involve a region that is not shared by insulin as there is negligible cross-reactivity between insulin and the IGF-2 receptor (Dafgard *et al.*, 1985).

1.5.3. Gene Structure

The gene for human IGF-1 is located on chromosome 12, while the gene for IGF-2 is located on chromosome 11 in close proximity to the insulin gene (Brissenden *et al.*, 1984). Both of the IGF genes span a considerable length of their respective chromosomes and are also similar in that they have a discontinuous structure containing several exons.

The length of the human IGF-1 gene is not known exactly but it does contain 5 exons with an undetermined gap between exons 2 and 3 (Jansen *et al*, 1983). Exon 1 contains 5' untranslated sequences, while exons 2 and 3 encode the signal peptide as well as the intact IGF-1 A, B, C and D regions and the first 16 amino acids of the E region. Exons 4 and 5

consist of sequences that encode the remainder of the E region as well as the 3' untranslated sequences that encode alternative polyadenylation sequences.

Differential splicing of the primary hIGF-1 mRNA transcript results in two different mRNA transcripts that differ in their E regions (Rotwein *et al*, 1986). The two alternative mRNA molecules are known as IGF-1a and IGF-1b: IGF-1a mRNA contains exons 1, 2, 3 and 5; while IGF-1b mRNA contains exons 1, 2, 3 and 4. (illustrated in Figure 1.2.). The IGF-1 gene therefore encodes two alternative precursor proteins for the IGF-1 protein: one, a protein containing 153 amino acids, 35 of which are the E peptide (IGF-1a) and the second, a protein containing 195 amino acids, 77 of which are the E peptide (IGF-1b).

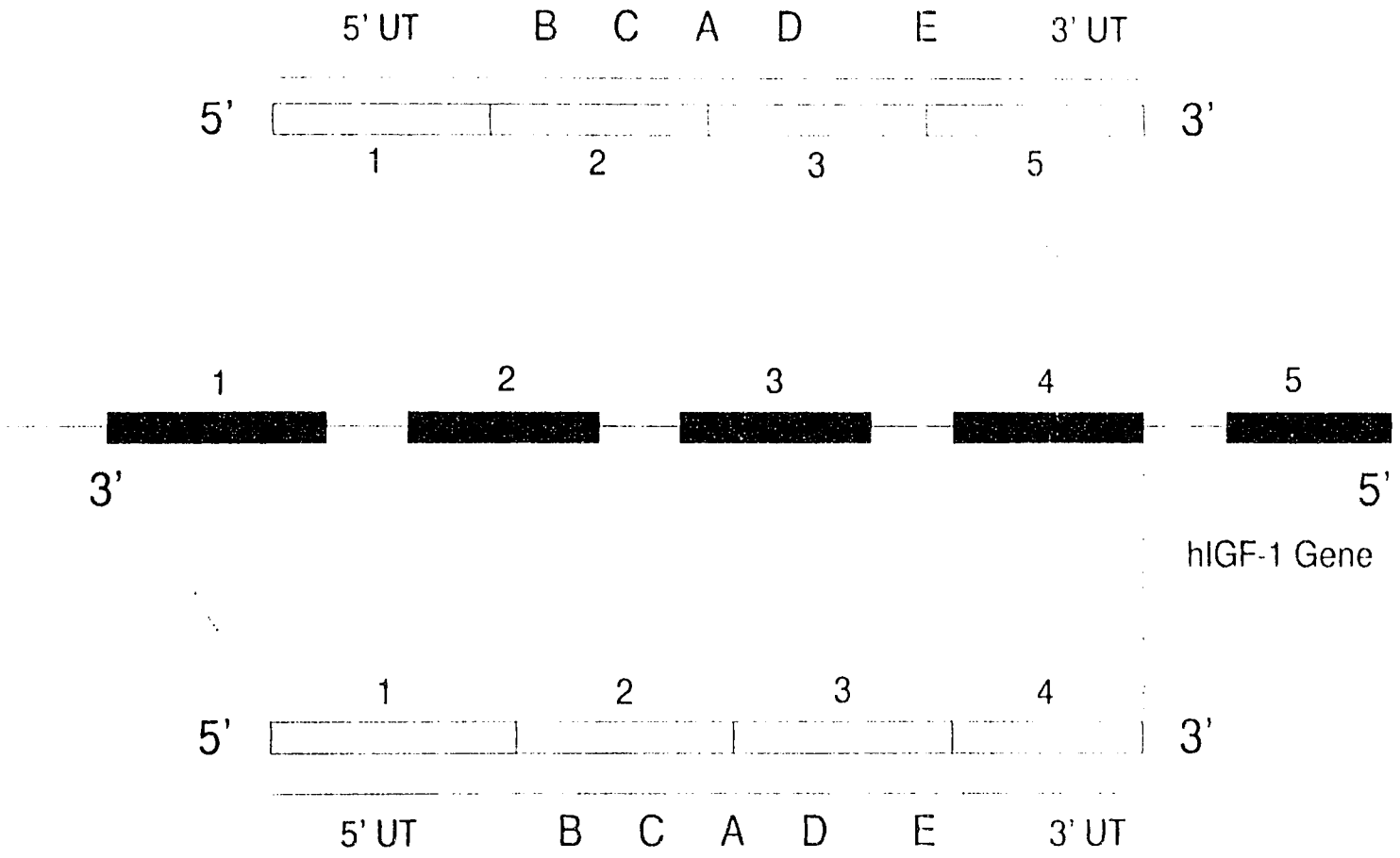
In addition, there appears to be differential expression of other sequences of the IGF-1 gene. Lowe *et al.* (1987) demonstrated that there is differential expression of alternative 5' untranslated regions in mRNAs encoding rat IGF-1. They demonstrated that three distinct IGF-1 mRNA species exist that are dissimilar in the 5' untranslated regions. They classed the three species as Class A (323 bases), Class B (297 bases) and Class C (242 bases).

Multiple IGF-1 mRNA transcripts have been identified in different species that may reflect the use of alternative 5' untranslated regions as well as variable polyadenylation sites at the 3' untranslated region. In rat (Murphy *et al.*, 1987), human (Shimatsu & Rotwein, 1987), bovine (Glimm *et al.*, 1991) and porcine tissues (Tavakkol *et al.*, 1988; Simmen *et al.*, 1990), two major transcripts have been identified: 8.0 Kb and 0.8-1.1 Kb. In the porcine endometrial tissue, a 2.3 Kb transcript has also been

Figure 1.2. A schematic representation of the human IGF-1 gene and its two alternative IGF-1a and IGF-1b mRNA transcripts.

The IGF-1 gene consists of five exons (solid regions) separated by introns of undetermined lengths. Both mRNA transcripts contain exons 1, 2 and 3, encoding the 5' untranslated region and the B, C, A and D regions of the mature IGF-1 peptide. The transcripts differ in their E and 3' untranslated regions, encoded by either exon 4 or 5.

IGF-1a


5' UT
B
C
A
D
E
3' UT
5'
3'
1
2
3
5
1
2
3
4
5
3'
5'
hIGF-1 Gene
1
2
3
4
5'
3'
5' UT
B
C
A
D
E
3' UT

IGF-1b

identified (Tavakkol *et al.*, 1988; Simmen *et al.*, 1990).

As yet no differences in the biological activities of the heterogeneous transcripts from the IGF-1 gene have been found. However, Sara and Hall (1990) suggest that the observed alternative mRNA processing may provide a mechanism by which IGF-1 synthesis and subsequent functions are regulated.

The human IGF-2 gene consists of eight exons that extend over 30 kilobases of the DNA (Sara & Hall, 1990). Exons 1-4, 4b and 5-7 contain the sequences that encode the precursor protein as well as the 3' untranslated region. Like IGF-1 expression, there is differential regulation of the expression of the IGF-2 gene. The various promoters present result in the production of different transcripts.

1.5.4. IGF Receptors

1.5.4a. *Receptor Structure and Properties*

The following will be a brief account of the structure and properties of the IGF receptors. For more extensive reviews, the reader is referred to Rechler and Nissley (1985) and Nissley *et al.* (1985).

As the IGFs are polypeptide hormones, in order to initiate a biological response in the appropriate target tissue, they must bind to specific and saturable sites on the plasma membranes known as receptors. Two different types of IGF receptor have been characterised (Rechler & Nissley, 1983). The two subtypes of IGF receptors were identified by the binding of tracer amounts of iodinated IGF-1 or IGF-2. IGF-1 was found to bind preferentially to the Type 1 receptor while IGF-2 bound preferentially to the Type 2 receptor. However, although preferential

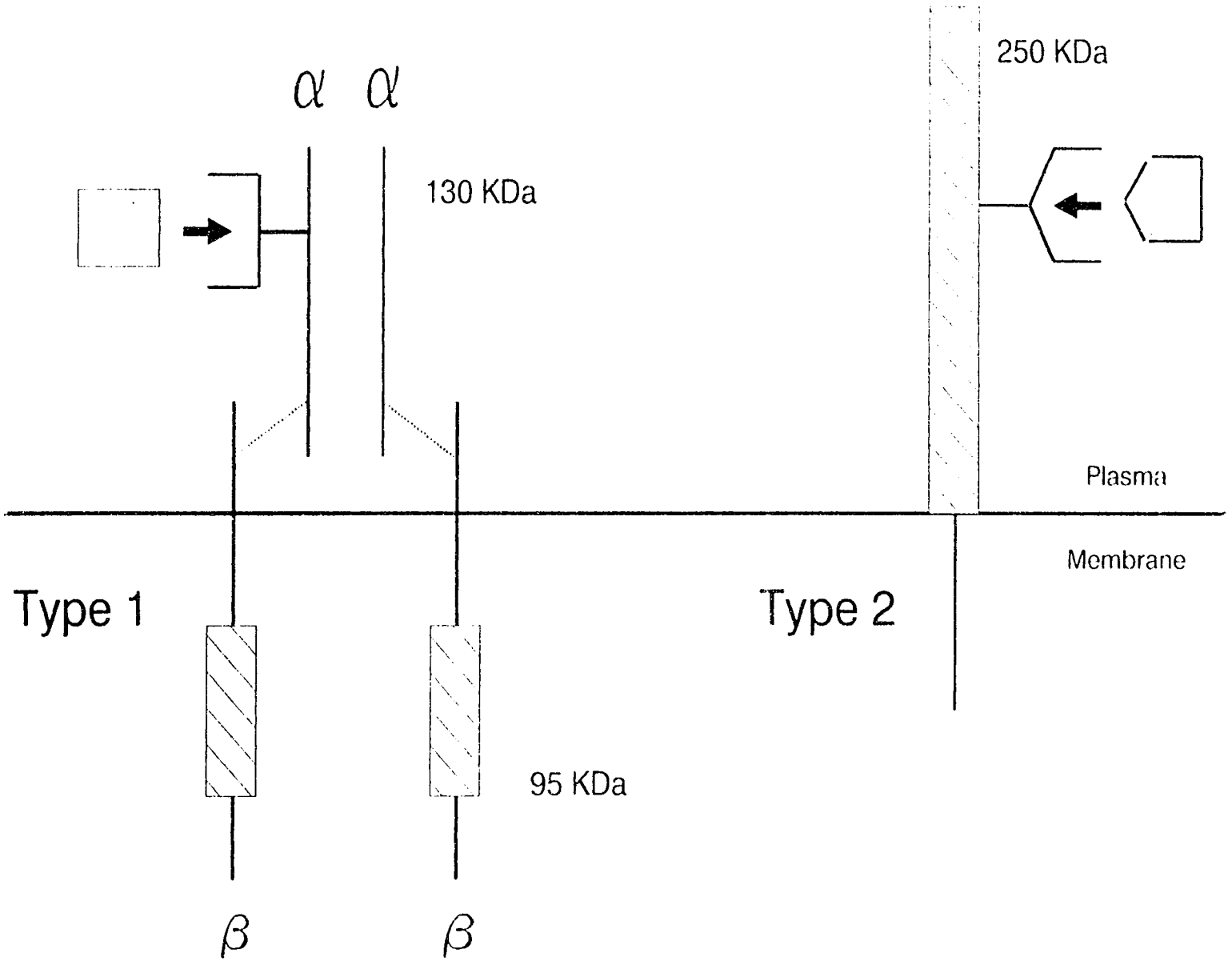
binding was observed, studies showed that there was also potential for extensive cross-reactivity (10-100%) (Rechler & Nissley, 1985). For this reason, the two receptors were designated as Type 1 and Type 2, respectively, rather than the IGF-1 receptor and the IGF-2 receptor.

Both receptors are thought to be located within the plasma membrane, as illustrated in Figure 1.3. Using immuno-precipitation techniques, the Type 1 receptor has been found to consist of two extracellular alpha subunits (approximately 130 KDa) and two transmembranal beta subunits (approximately 95 KDa) (Kull *et al.*, 1983). The structure of the human Type 1 receptor has also been identified using placental cDNA (Ullrich *et al.*, 1986). The Type 1 receptor is encoded in a precursor protein (approximately 152KDa) consisting of one alpha subunit and one beta subunit. This protein is then glycosylated, dimerized and then proteolytically processed to produce the mature transmembranal complex.

The primary structure of the Type 1 receptor is very similar to that of the insulin receptor. This explains why there is cross-reactivity between IGF-1 and insulin and their associated receptors. At high concentrations, insulin is able to cross-react weakly with the Type 1 receptor. Both receptor types contain alpha and beta subunits although there is evidence for small differences in the sizes of the respective subunits (Stuart *et al.*, 1984). However despite discrepancies in subunit sizes, the insulin and Type 1 receptor structures are remarkably similar, leading to the conclusion that they may have evolved from the same ancestral gene in the same way that IGF-1 and insulin may have done.

The Type 2 receptor is structurally unrelated to both the Type 1 and insulin receptors. As already mentioned, the Type 2 receptor

Figure 1.3. A schematic representation of the IGF type 1 and type 2 receptors.


α α
130 KDa
250 KDa
Plasma
Membrane
Type 1
Type 2
95 KDa
β β

preferentially binds to IGF-2. There is some cross-reactivity with IGF-1, but the Type 2 receptor is insensitive to insulin. Morgan *et al.* (1987) characterised the primary structure of the human Type 2 receptor from the cDNA sequence obtained from the human hepatoma cell line Hep G2. The Type 2 receptor consists of a large extracellular domain attached to a single transmembranal region with a small intracellular domain and has a total molecular weight of 250 KDa. As a consequence, approximately 93% of the receptor protein is extracellular.

The binding of the IGFs or insulin to the Type 1, Type 2 and insulin receptors appears to result in the autophosphorylation of the receptor proteins. The phosphorylation sites of the insulin and Type 1 receptors appear to be on the beta subunit of the transmembranal receptor complex (Jacobs *et al.*, 1983). Phospho-amino acid analysis indicated that the phosphorylation occurred on the tyrosine residues as a result of protein kinase activity. Haskell *et al.* (1984) demonstrated that the Type 2 receptor is also phosphorylated in intact rat cells. Autophosphorylation of the Type 2 receptor and the presence of protein kinase activity have yet to be confirmed.

1.5.4b. *Regulation of IGF Receptors*

There is extensive down-regulation of the Type 1 receptors. IGF-1 and insulin have been found to down regulate the Type 1 receptors on monolayer cultures of human skin fibroblasts (Rosenfeld & Dollar, 1983). Scatchard analysis of the binding data indicates that down-regulation is accounted for by a reduction in receptor number rather than a decrease in binding affinity.

Type 2 receptors do not appear to be down-regulated. However there is evidence for extensive up-regulation of this receptor type. Following a brief incubation with insulin (10^{-10}M), IGF-2 binding to rat adipose Type 2 receptors is increased (King *et al.*, 1982). IGF-2 binding was also increased by pro-insulin and antibodies antagonistic to insulin receptors. It appears that the pro-insulin and insulin receptor antibodies are acting via the insulin receptor. Using Scatchard analysis, it appears that this indirect up-regulation of the Type 2 receptor by insulin is via an increase in the Type 2 receptor affinity rather than by an increase in receptor numbers.

1.5.5. Circulating Levels of IGFs

Unlike many other hormones, production of the IGFs is not associated with any one particular tissue or cell type, although the liver is thought to produce the majority of circulating IGF-1. It is not synthesised by a specialised cell, retained within vacuoles and then released upon an appropriate stimulus. The IGFs are released by a wide range of tissues directly into the blood circulation (as will be elaborated on later in this review) and in fact it is the blood that acts as the storage depot for these growth factors.

Studies have been carried out to characterise circulating levels of both the IGF-1 and IGF-2 with respect to age in pigs, humans and rats. In growing swine, IGF-1 levels increase from approximately 10 to 140 days of age, just prior to puberty (Owens *et al.*, 1991; Louveau *et al.*, 1991). In humans, Daughaday and Rotwein (1989) describe a progressive rise in IGF-1 during childhood, reaching mean adult levels just prior to puberty. During

puberty there is a two to three fold rise in serum IGF-1 which is presumably associated with the sudden increase in sex steroids at this time. At any particular time, serum IGF-1 levels were higher in girls when compared to boys of the same age which is consistent with the more rapid maturation observed in girls. After puberty, serum IGF-1 continues to increase as growth velocity subsides until growth is negligible. IGF-1 levels then begin to decrease until average adult IGF-1 levels are reached in the early twenties. These levels are then maintained until about sixty years of age when IGF-1 levels once again begin to decline. A similar pattern for IGF-1 secretion has been observed in rats where circulating IGF-1 levels begin to rise when growth becomes growth hormone dependent.

When the rat, human and pig are all compared, one thing is apparent in all three species: IGF-2 plays a minor role in postnatal growth and development when compared to IGF-1. In the human, IGF-2 levels increase rapidly during the first year and are then maintained at a relatively constant level throughout life, suggesting that the growth factor may play an important role in the first twelve months of life but not in later maturational events, as no significant changes in IGF-2 levels have been found at puberty (Sara & Hall, 1990). In the rat IGF-2 levels are high prenatally but decrease at birth, again suggesting a minimal role in the attainment of puberty (Sara *et al.*, 1980). In the pig, IGF-2 levels do increase in the first few months of life, but the rate of increase is lower than for IGF-1 (Owens *et al.*, 1991).

1.5.6. Binding Proteins

Because of the high degree of insulin-like activity of the growth

factors, it is important that there is a mechanism by which the body is protected from this insulin-like activity and prevented from becoming hypoglycaemic. The production of high affinity binding proteins to which the IGFs immediately bind upon their secretion by tissues fulfils this role. These binding proteins play an integral yet unclear role in the regulation of the IGFs. In recent years, those working in the area of IGF research have turned their attention to elucidating the complex roles played by these high molecular weight proteins in the regulation of IGF activity. For an extensive review on this aspect of the IGF physiology, the reader is referred to Baxter and Martin (1989).

There appear to be two classes of the serum binding proteins: the larger 150 KDa protein binds 80% of the circulating IGFs, while the remaining IGFs are bound to the smaller 25 KDa binding protein. The exact physiological roles of these two major forms of binding protein, and their many variants, remain unclear but it is thought that the major role of the 150 KDa protein is to act as a storage depot for the large amounts of circulating growth factors.

The smaller binding protein possibly act as an intermediatory binding protein which binds to the IGF when it first enters the circulation. Kaufmann and co-workers (1977) found that when iodinated IGF was injected into rats, although the radioactive IGF was initially bound to the smaller binding protein, binding activity was rapidly associated with the larger binding protein. Overall, both forms of binding protein serve to extend the half life of the circulating IGFs: free IGF-1 has a half life of about 20 min, whereas that bound to the smaller binding complex has a half life of three hours and that bound to the larger

complex, 13-20 hours (Holly & [illegible]s, 1989).

1.5.7. Autocrine-Paracrine Roles of IGF-1

Early classic studies on the production of the IGFs, including those by Dulak and Temin (1973), led to the conclusion that IGF was produced by the liver. It is now generally accepted that circulating IGF-1 is primarily of hepatic origin. However, although it was originally thought that the only source of IGF-1 was the liver, it is now known that the majority of body tissues are capable of producing this growth factor.

IGF-1 production by other tissues was initially demonstrated with *in vitro* cell culture studies, and more recently from the detection of IGF-1 gene expression in tissues other than hepatic tissue using molecular biology techniques. Murphy and co-workers (1987) demonstrated the presence of IGF-1 gene expression in rat fore brain, lung, ovary, testis, uterus, kidney, heart, skeletal muscle, mammary gland and liver, with ovarian tissue demonstrating the third highest level of gene expression after hepatic and uterine tissue. In growing and mature swine, IGF-1 gene expression has been detected in cardiac and skeletal muscle (Leaman *et al.*, 1990), the uterus (Tavakkol *et al.*, 1988), ovary (Cameron *et al.*, 1990) and adipose tissue (Duffy, *personnel communication*), in addition to the liver (Leaman *et al.*, 1990).

Evidence for the local production of IGF-1 in a wide range of tissues suggests that as well as having an endocrine role, IGF-1 could have very important local effects in its tissue of origin through paracrine or autocrine actions. The exact nature of the paracrine/autocrine effects of IGF-1 depends on the tissue involved, but

both mitogenic and metabolic effects have been demonstrated in a number of tissues using *in vitro* cultures involving both exogenous treatment with IGF-1 and growth hormone-induced production of endogenous IGF-1. The remainder of this review will focus on the apparent paracrine/autocrine effects of IGF-1 within ovarian tissue.

1.6. THE OVARIAN INSULIN-LIKE GROWTH FACTOR SYSTEM

1.6.1. Ovarian Production of IGF-1

There is an abundance of convincing evidence for the local production of IGF-1 in the ovary. For extensive reviews on this subject the reader is referred to those written by Adashi *et al.* (1985a) and Hammond *et al.* (1991). In 1985, Hammond and co-workers established that porcine granulosa cells were capable of producing IGFs and IGF-binding proteins under serum-free conditions *in vitro*. They suggested that there was also evidence for *in vivo* ovarian production of IGFs as follicular fluid from preovulatory follicles contained significantly more IGF-1 than serum or immature follicles. However the value of this evidence for the local production of IGF-1 is debatable, as these authors did not determine whether the higher follicular IGF-1 was due to local production of IGF-1 or due to the transport of circulating IGF-1 into the follicle antral space.

Recently, more direct evidence for the ovarian production of IGF-1 has been presented. Oliver *et al.* (1989) detected transcripts of mRNA in rat ovarian tissue with sizes of 7.0, 1.6 and 0.4 - 0.9 Kb, which are similar to those found in rat liver. They also used *in situ* hybridization to determine the distribution of IGF-1 gene expression in the rat ovary.

The major site of IGF-1 gene expression appears to be the granulosa cell and particularly those cells in the antral cell layers and cumulus oophorus of the preantral and antral follicles. IGF-1 gene expression in atretic follicles or in the corpus luteum was undetectable.

The regulation of the expression of the IGF-1 gene in the ovary is complex and not as yet fully understood. Growth hormone administration to hypophysectomised rats has been found to increase follicular fluid levels of IGF-1 . There was a rapid rise in tissue IGF-1 within eight hours of growth hormone treatment of GH-deficient rats (Davoren & Hsueh, 1986). However, the *in vivo* nature of this study raises the question of what tissue was the source of the increase in IGF-1? Growth hormone is known to increase hepatic production of IGF-1, so it may well be that the increase in follicular IGF-1 is actually due to increased endocrine levels of IGF-1 rather than increased local production.

A more recent study provides some more convincing evidence regarding growth hormone-stimulated ovarian IGF-1 production. Hernandez *et al.* (1989) demonstrated using Dot Blot and RNase Protection techniques that ovine growth hormone on its own actually *inhibited* rat ovarian IGF-1 gene expression. This was in contrast to the five-fold increase in hepatic IGF-1 gene expression observed following growth hormone treatment. However this study was carried out using hypophysectomised animals, and given that other important gonadotrophic hormone stimuli, and also subsequent steroidal stimuli, were absent, the effects of exogenous growth hormone treatment were probably not typical of the effects of endogenous growth hormone in the intact animal. Indeed, when hypophysectomised rats were given an estrogenic implant containing DES, the subsequent steroid-induced

increase in IGF-1 gene expression changed from a two-fold increase to a three-fold increase upon treatment with growth hormone.

Other hormones have also been shown to increase the production of IGF-1 in ovarian tissue. *In vitro* studies using porcine granulosa cells have demonstrated an increase in the secretion of immunoreactive IGF-1 as a result of treatment with gonadotropin (pregnant mare serum gonadotropin (PMSG) and hCG) and estradiol (Hsu & Hammond, 1987). Cyclic AMP, the presumed mediator of gonadotropic action, was also found to enhance IGF-1 secretion. In 1988, the same group provided evidence for *in vivo* effects of gonadotropin on ovarian IGF-1 production. Their results indicated that gonadotropin treatment increased intrafollicular levels of immunoreactive IGF-1 in prepubertal gilts (Hammond *et al.*, 1988).

1.6.2. Ovarian IGF-1 Receptors

Work has also been carried out to characterize the receptors mediating actions of IGF-1 in granulosa cells. Adashi *et al.* (1990a) demonstrated that treatment of cultured rat granulosa cells with antibodies raised against the Type 2 receptor failed to inhibit the cytodifferentiative action of either IGF-1 or 2. The receptors mediating both the actions of IGF-1 and IGF-2 were therefore considered to be Type 1 and not Type 2 receptors. The number of IGF-1 receptors appears to be regulated by several different factors, including LH, FSH and beta adrenergic agonists (Adashi *et al.*, 1988). FSH has also been found to enhance IGF-1 binding to these receptors (Adashi *et al.*, 1986).

1.6.3. Effects of IGF-1 in the Ovary

As discussed earlier, the effects of locally produced IGF-1 are potentially both paracrine and autocrine. The exact nature of the action of locally produced IGF-1 is difficult to elucidate *in vivo* as any IGF-1 stimulated effects may be due to the action of endocrine or ovarian IGF-1. Techniques have yet to be developed that would allow a distinction between the two. As a result of this, much of the work carried out to determine IGF-1 action in the ovary has been carried out using *in vitro* cell culturing techniques.

Many of the effects of IGF-1 are by the amplification of the actions of the gonadotropins on the ovary. For example, IGF-1 has been shown to enhance FSH-induced acquisition of LH receptors in cultured rat granulosa cells in a dose and time dependent fashion (Adashi *et al.*, 1985b). Growth hormone has also been shown to increase FSH-induction of LH receptors in rat granulosa cells (Jia *et al.*, 1986). However, it is tempting to speculate that these effects were probably not as a result of the direct action of GH, but instead due to action of GH-induced production of IGF-1.

As well as playing a role in the acquisition of granulosa LH receptors, IGF-1 has also been shown to have a key regulatory role in the steroidogenic pathways of the granulosa cells, and as a consequence of this, may have an important role in the development, selection and subsequent luteinization of a follicle. Studies carried out by Veldhuis and co-workers (1985a) demonstrated that swine granulosa cells, cultured under serum-free conditions, responded in a dose and time dependent fashion to IGF-1 in that they showed increases in progesterone, pregnenolene and estradiol production. They provided evidence that IGF-1

was affecting actual steroidogenesis, and not just release, by showing that there was a decrease in the levels of the metabolite, 20 alpha hydroxy-pregn-4-en-3-one. Further proof was obtained using aminoglutethimide, an inhibitor of the mitochondrial cholesterol side-chain cleavage system. This agent blocked the stimulatory effects of IGF-1 on progesterone production, therefore supporting the hypothesis that IGF-1 increases the activity of the side-chain cleavage system (Veldhuis *et al.*, 1985b). Other groups have demonstrated similar effects of IGF-1 in other species: IGF-1 has been found to augment FSH-stimulation of estradiol and progestin production in the rat (Davoren *et al.*, 1985; Dorrington *et al.*, 1987; Adashi *et al.*, 1985c) and human (Erickson *et al.*, 1989).

IGF-1 also has mitogenic effects within the ovary. In 1984, Baranao and Hammond carried out a study to compare the effects of both IGF-1 and IGF-2 and insulin on the stimulation of thymidine incorporation into DNA (an indication of mitogenesis) and the facilitation of FSH-induced progesterone secretion in porcine granulosa cells. IGF-1 exhibited both a greater mitogenic effect, as well as a greater trophic effect, than IGF-2. Both growth factors were more potent than insulin. It is suggested that a possible explanation for why IGF-1 is the more potent factor is due to differing binding affinities for the receptor mediating the hormonal effects. In fact the same study showed that IGF-1 was most efficient in the displacement of iodinated IGF-1 from specific membrane receptor sites, with IGF-2 the next most efficient and insulin the least efficient.

Further evidence for a mitogenic paracrine/autocrine action of IGF-1 was published in 1989 by Mondschein *et al.* Porcine granulosa cells were cultured in serum-free medium, thus rendering them dependent on

endogenously produced factors. The cells were then treated with growth hormone, FSH, estradiol and combinations thereof. Upon treatment with a neutralizing monoclonal antibody raised against IGF-1, the stimulatory effects on progesterone production by growth hormone, FSH and estradiol were inhibited by approximately 50%. The effects on cell growth were less dramatic: FSH and estradiol induced an 18% increase in cell numbers which was abolished by the IGF-1 antibody. The apparent differences in the level of inhibition of the stimulatory effects of Growth Hormone, FSH and Estradiol may suggest that ovarian IGF-1 is actually more important to granulosa cell cytodifferentiation than it is to cell replication.

As well as having steroidogenic and mitogenic effects in the ovary, IGF-1 has also been shown to have metabolic effects. IGF-1 has been found to increase the metabolism of low density lipoproteins in swine granulosa cells (Veldhuis *et al.*, 1987) as well as increase glucose oxidation (Weber & LaBarbara, 1988). In the latter case, IGF-1 enhanced glucose metabolism at a much lower concentration than insulin, therefore suggesting that *in vitro* the effects of insulin on glucose metabolism may be mediated via the Type 1 receptor. This suggests that IGF-1 plays a more important role in the modulation of the metabolic status of the ovary than the classic metabolic hormone, insulin.

1.6.4. Ovarian IGF-Binding Proteins

In recent years, studies have shown that IGF-binding proteins, as well as IGF-1 itself, may be produced locally by many tissues, including the ovary. There is now much speculation as to the possible roles of these locally produced binding proteins, and what their effects may be on

ovarian function. In 1985, Hammond *et al.* demonstrated that porcine granulosa cells were capable of producing both IGFs and IGF-binding protein *in vitro*. Since then, work has been carried out in an effort to characterize these binding proteins, to establish that they are indeed produced locally, and to determine their role in the ovary.

From work carried out by Koistinen *et al.* (1990) and Shimasaki *et al.* (1990), there is evidence for ovarian production of several different species of IGF-binding proteins. Human granulosa cells have been found to express the gene for a binding protein designated as IGF-BP1 which is similar to that produced in human decidua (Koistinen *et al.*, 1990). Meanwhile, using a cDNA probe encoding the porcine homologue of human IGF-BP3, a mRNA transcript of 2.6 Kb has also been detected in porcine ovaries (Shimasaki, *et al.*, 1990).

The regulation of the release of ovarian IGF-BPs seems to depend on the species of IGF-BP. Bicsak *et al.* (1990) reported that IGF-BP3 is GH-dependent, while another binding protein, IGF-BP2, is GH-independent. IGF-BP1 is also thought to be GH-dependent. There may also be another binding protein present in porcine follicular fluid that is actually regulated by the production of IGF-1 itself (Grimes *et al.*, 1991), therefore suggesting that there may be complex positive or negative feedback systems in operation. This binding protein has only just recently been identified and designated as IGF-BP5. It is characterised as an IGF-BP that has a similar molecular weight to IGF-BP1 but is not recognised by antibodies raised against IGF-BP1. Porcine cultures of granulosa cells have been found to produce more of this binding protein upon IGF-1 treatment as determined by gel electrophoresis. This discovery of IGF-1-mediated regulation of IGF-

BP5 production may suggest that the GH-regulation of some of the other binding proteins may also be IGF-1 mediated.

The production of IGF-BPs also seems to be related to the gonadotropin status of the animal, as FSH has been found to modulate their production. Adashi and co-workers (1990b) demonstrated that FSH treatment of rat granulosa cell cultures resulted in a dramatic inhibition of binding activity of iodinated IGF-1 (an 89% decrease at 100 ng/ml). Similar results were also shown *in vivo*; when rats were given 10 μg FSH/rat/day for two days, they also observed a dramatic decrease in granulosa cell binding activity. Further work by the same group indicated that there may actually be a biphasic response to FSH levels. FSH treatment at low levels (1-3 ng/ml for 72h) increased IGF-1 binding activity while higher levels of FSH (>10 ng/ml) decreased binding activity as in their previous study (Adashi *et al.*, 1991). In the same study, the effects of hypophysectomy on ovarian IGF-1 binding activity were studied. Hypophysectomy was found to inhibit any subsequent *in vitro* release of IGF-1 binding activity therefore suggesting that pituitary factors other than FSH are involved in the regulation of granulosa cell production of IGF-1 binding proteins. However, although FSH may not be the sole regulator of the production of IGF-BP, the apparent biphasic FSH regulation of IGF-BP activity may play a role in follicle development and subsequent selection.

It appears that the binding proteins (IGF-BPs) have an important regulatory function with respect to the actions of IGF-1, although the exact nature of this regulation is unclear at present. IGF-BPs have been shown to have both stimulatory and inhibitory effects. In 1987, Elgin and

co-workers found that an IGF-BP enhanced the biological response to IGF-1 in a number of different species. They demonstrated marked increases in DNA synthesis in chick embryo, human and mouse fibroblasts, and porcine smooth muscle cells in the presence of a 150 KDa binding protein purified from human amniotic fluid. Similar findings were published by Blum *et al.* in 1989: they found that optimum DNA synthesis by baby hamster and human skin fibroblasts occurred when cultures were exposed to equimolar quantities of IGF-1 and IGF-BP, whereas free IGF-1 had no effect. On the basis that hourly treatments of small amounts of free IGF-1 *did* increase DNA synthesis, the authors suggest that the binding protein is acting as a reservoir that continually releases low amounts of the growth factor thus creating a steady state of receptor occupancy. It appears that IGF-1 is a better mitogenic stimulator at constant low concentrations, than at a temporary high concentration.

In the ovary, the majority of the effects of binding proteins found to date have been inhibitory to the actions of IGF-1. Using cultured granulosa cells from DES-treated immature rats that were exposed to porcine follicular fluid, both IGF-BP2 and IGF-BP3 were found to inhibit estradiol and progesterone production in a dose and time dependent fashion (Bicsak *et al.*, 1990). In the same study both binding proteins inhibited tritiated thymidine uptake by granulosa cells, therefore suggesting that the binding proteins were inhibiting the effects of IGF-1 on both steroidogenesis and mitogenesis. In a later study, Bicsak *et al.* (1991) demonstrated inhibitory actions of IGF-BPs *in vivo*. Immature female rats were primed with equine chorionic gonadotropin (eCG) and then given a dose of human chorionic gonadotropin (hCG). When the rats were given an

intrabursal infusion of IGF-BP3, there was a significant decrease (55%) in the number of hCG-stimulated ovulations. These results not only suggest that IGF-BP3 may inhibit the synthesis of LH receptors on the granulosa cells, but also provides evidence that endocrine-derived binding proteins are able to perfuse into the ovarian interstitial space.

It has been tentatively suggested that IGF-BPs may have their own direct actions on ovarian function irrespective of IGF-1. Ui *et al.* (1989) demonstrated that inhibition of FSH-stimulated estradiol production could be removed by the addition of equimolar quantities of IGF-1 and IGF-2. They suggest that the inhibitory effect of the binding proteins could be via the sequestering of IGFs produced by the culture, so that any additional IGFs added to the system would be in a non-sequestered state and as such would be able to exhibit their positive effects on FSH activity. However the authors suspect that the action of the IGF binding proteins is not as simple as this theory suggests and therefore they hint at a possible IGF-independent role of the binding proteins. Before this proposal can be generally accepted though, more convincing data must be produced to either support or refute their statement.

The roles of the IGF binding proteins in the ovary have so far all been inhibitory in nature. However one can not discount the idea that as more and more species of the binding proteins are discovered, they may also have some positive effects such as those found in fibroblasts and other tissue types. On one hand the IGF-BPs may inhibit IGF-1 action by sequestration and thus preventing an interaction with the membrane-bound Type 1 receptors, but on the other hand they may actually serve to enhance the actions of IGF-1 by perhaps facilitating binding to their receptors

(as has been suggested in fibroblasts), or even indirectly by increasing the half life of IGF-1 within the ovary. Whatever their exact roles, and what part endocrine versus locally produced binding proteins play, more work needs to be carried out to determine the exact physiological roles of the IGF-binding proteins in the ovary.

1.7. SUMMARY

To summarize, the literature reviewed indicates that undernutrition of the female of all species studied impairs reproduction both at the level of the hypothalamic-pituitary axis and at the level of the reproductive tract. It is understood that there must be one or more cues that mediate between nutrition status and reproduction. Evidence favours that these cues are metabolic in nature. Nutrition may actually have a two tiered affect on the reproductive system, as studies have demonstrated ovarian responses to nutrition in the absence of changes in gonadotropin release. This suggests that the ovary is able to respond independently to a change in metabolic state.

I propose that Insulin-Like Growth Factor 1 may be a metabolic signal that may act as a mediator of changing metabolic status at the level of the ovary. The basis for this hypothesis was that: 1. Hepatic IGF-1 gene expression and plasma IGF-1 levels are dependent on nutritional status, 2. IGF-1 gene expression has been detected in the ovarian tissue of several species, and 3. IGF-1 has been demonstrated to have steroidogenic, mitogenic and metabolic affects within the ovary.

The following experimental work aims to: 1. Provide evidence that the IGF-1 gene is expressed in the follicular and hepatic tissue of

prepubertal gilts, and 2. To determine whether ovarian gene expression is independently regulated by feeding level, with respect to hepatic IGF-1 gene expression.

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CHAPTER 2

Detection of Insulin-Like Growth Factor I Gene Expression in Porcine Ovarian and Hepatic Tissues

INTRODUCTION

The insulin-like growth factor 1 (IGF-1) gene is expressed in a wide range of tissues in several species. In the rat, IGF-1 gene expression has been detected in the liver, uterus, ovary, lung, kidney, heart, skeletal muscle, testes, brain and mammary gland (Murphy *et al.*, 1987). In growing and adult swine, the IGF-1 gene has been found to be expressed in liver, skeletal and cardiac muscle (Leaman *et al.* 1990), uterus (Tavakkol *et al.*, 1988) and adipose tissue (Duffy, *personnel communication*). The discovery of IGF-1 gene expression in such a wide range of tissues has revealed the possibility of autocrine and paracrine actions of IGF-1 at the level of specific cells and tissues.

In the ovary, many studies have suggested that locally-produced IGF-1 might play an important role in the regulation of ovarian function and development in an autocrine/paracrine manner (Adashi *et al.*, 1985a & 1985b; Davoren *et al.*, 1985; Veldhuis *et al.*, 1987; Weber & LaBarbera, 1988; Hammond *et al.*, 1991). However, the majority of this evidence was derived from the use of *in vitro* procedures. The *in vivo* studies conducted so far have not provided definitive evidence to support the existence of autocrine or paracrine IGF-1 actions in the ovary. The reason for this short-coming is that the methods used have not allowed the differentiation between IGF-1 of endocrine and autocrine/paracrine origin. We have applied molecular biology techniques to resolve this issue and developed an experimental approach that allowed the measurement of physiological and treatment effects on ovarian IGF-1 production *in vivo*.

Preliminary work by Cameron *et al.* (1990) revealed that the IGF-1 gene is expressed by porcine granulosa cells *in vivo*. However, this work

was not extended to the study of treatment effects on individual animals. The objectives of this study were therefore two-fold: 1. To confirm that the IGF-1 gene is expressed in both porcine ovarian and hepatic tissue using two different procedures, northern analysis and an RNase protection assay. 2. To determine the suitability of these procedures for analysing tissue from individual animals subjected to different treatments *in vivo*.

MATERIALS AND METHODS

Total RNA Isolation

Prepubertal gilt ovarian and hepatic tissues were collected from a local packing plant and frozen in liquid nitrogen. The samples were stored at -70°C. Total RNA was isolated from approximately 0.5 g of tissue using a procedure described by Chirgwin *et al.* (1979) with some modifications. Briefly, samples were pulverised in liquid nitrogen using a pestle and mortar, added to 9 ml of 4 M guanidine isothiocyanate (GIT) and then homogenized for 1 min at high speed using a polytron tissue homogenizer (Brinkman Instr., Westbury, NY, U.S.A.). The homogenate was centrifuged at 5000*g* at 4°C for 10 min to pellet cell debris and the supernatant removed and layered onto 3.3 ml of caesium chloride (5.7 M CsCl, 0.1 M EDTA, pH 7.0) in a polyallomer ultracentrifuge tube (Beckman Canada, Burnaby, BC). Total RNA was then pelleted by centrifugation at 30,000 rpm in a Sw 41-Ti rotor (Beckman Instruments Inc., Mississaugua, ON) for 23 hours at 20°C. After ultracentrifugation, the tissue homogenate and CsCl were aspirated and the RNA pellet was resuspended in 300 μl SET buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8) and 0.1% Sodium dodecyl sulphate (SDS) (v/v). The quantity and purity of the RNA was determined by ultraviolet

spectrophotometry at 260 and 280 nm.

Northern Analysis

Polyadenylated RNA (poly A^+ RNA) was isolated from pooled ovarian or hepatic total RNA using one round of oligodeoxythymidylate (oligo d(t)) cellulose Type 3; Collaborative Research Inc., Bedford, Mass., U.S.A.) chromatography. The poly A^+ RNA yields from 0.5 g each of ovarian and hepatic tissues were 5.3 and 100 μg, respectively. This represents a recovery of poly A^+ RNA from total RNA of 1.7 % and 10% for ovarian and hepatic tissue, respectively. Aliquots of total and poly A^+ RNA were added to 25 μl of loading buffer (50% deionized formamide (v/v), 2 M formaldehyde, glycerol (v/v), 0.02 M MOPS, 5 mM sodium acetate, 1 mM EDTA and 0.1% of bromophenol blue (w/v)), heated at 65°C for 5 min to separate the RNA strands and then placed on ice for 5 min. After adding 1 μl of Ethidium bromide (EtBr; 1 mg/ml) to each sample, mixing and briefly centrifuging to consolidate the samples at the bottom of the tubes, the samples were loaded onto 1% agarose (w/v) gels containing 0.66 M formaldehyde. RNA was then fractionated according to size by electrophoresis in the presence of recirculating 1 X MOPS running buffer (10 h at 40 V). A 0.24-9.49 kb RNA ladder (Bethesda Research Laboratories, Gaithersberg, MD) was used as a size marker and treated exactly as RNA samples. After electrophoresis, the gels were soaked in two changes of 10 X standard saline citrate (SSC) (1.5 M NaCl, 0.15 M trisodium citrate, pH 7.0) and then blotted onto a quaternary amine-derived nylon membrane (Zeta-probe, Bio-rad Laboratories, Richmond, California, U.S.A.) employing the capillary method described by Southern (1975), but using a sponge

configuration. The RNA was then fixed onto membranes by baking *in vacuo* at 80°C for 2 hours.

Prior to hybridization with the [^{32}P] CTP-labelled riboprobe, membranes were prehybridized for two hours at 50°C in hybridization buffer (60% deionized formamide (v/v), 10% dextran sulphate (w/v), 1 X SSPE (0.18 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA, pH 7.4), 1% SDS (w/v), 0.5% blotto (low fat milk powder) (w/v) and 0.5 mg/ml sheared salmon testes DNA (ssDNA)). Following prehybridization, membranes were incubated for 16 hours at 50°C in an identical volume (0.15 ml/cm^2 membrane) of fresh hybridization buffer, but with the addition of 2 X 10^6 dpm/ml of riboprobe. The riboprobe used in this procedure was a single-stranded antisense riboprobe (710 bases) synthesized from a Pvu II linearized pGEM-1/hIGF-1b plasmid (Rotwein, 1986) using SP6 RNA polymerase, [α-^{32}P] CTP (NEN Superconcentrated, NEG-008C) and a Gemini riboprobe core system kit (Promega, Madison, Wisconsin, U.S.A.). Unincorporated [P^{32}] CTP was removed from the reaction solution using Sephadex G-50 (Pharmacia Canada, Dorval, PQ) spun column chromatography.

Following hybridization, membranes were rinsed briefly at room temperature in 2 X SSC and then washed in 2 X SSC/0.1% SDS for 15 min each. They were then washed for 15 min at 70°C in 0.2 X SSC/1% SDS. After a final brief rinse in 0.2 X SSC at room temperature, membranes were blotted on 3MM Whatman filter paper, heat sealed in plastic bags and then exposed at -70°C to Kodak XAR 5 film (Eastman Kodak, Rochester, NY, U.S.A.) using an intensifying screen (Dupont Canada, Missaugua, ON).

Controls

Several controls were used to test the specificity of the

hybridization reaction. Samples of both porcine ovarian and hepatic non-polyadenylated (Poly A^-) RNA were included on gels to test for non-specific binding of the riboprobe. Poly A^- RNA is obtained from the eluate collected during the final step of the poly A^+ RNA purification by oligo d(t) cellulose chromatography. In theory, all of the poly A^+ RNA should be removed from the total RNA preparation during oligo d(t) cellulose chromatography. However, in practice it is likely this chromatography procedure does not remove all poly A^+ RNA. Nonetheless, the poly A^- RNA preparation still serves as an important control, to detect possible non-specific riboprobe interactions, because it provides an RNA preparation that is depleted of poly A^+ RNA molecules.

Rat liver RNA (either total or poly A^+ RNA) was also included as a control in the northern hybridization to assist in the identification of authentic IGF mRNA species. IGF-1 mRNA has been previously characterised in this tissue (Murphy *et al.*, 1987; Daughaday & Rotwein, 1989). Furthermore, it has been demonstrated that rat liver contains a high abundance of IGF-1 mRNA.

RNase Protection Assay

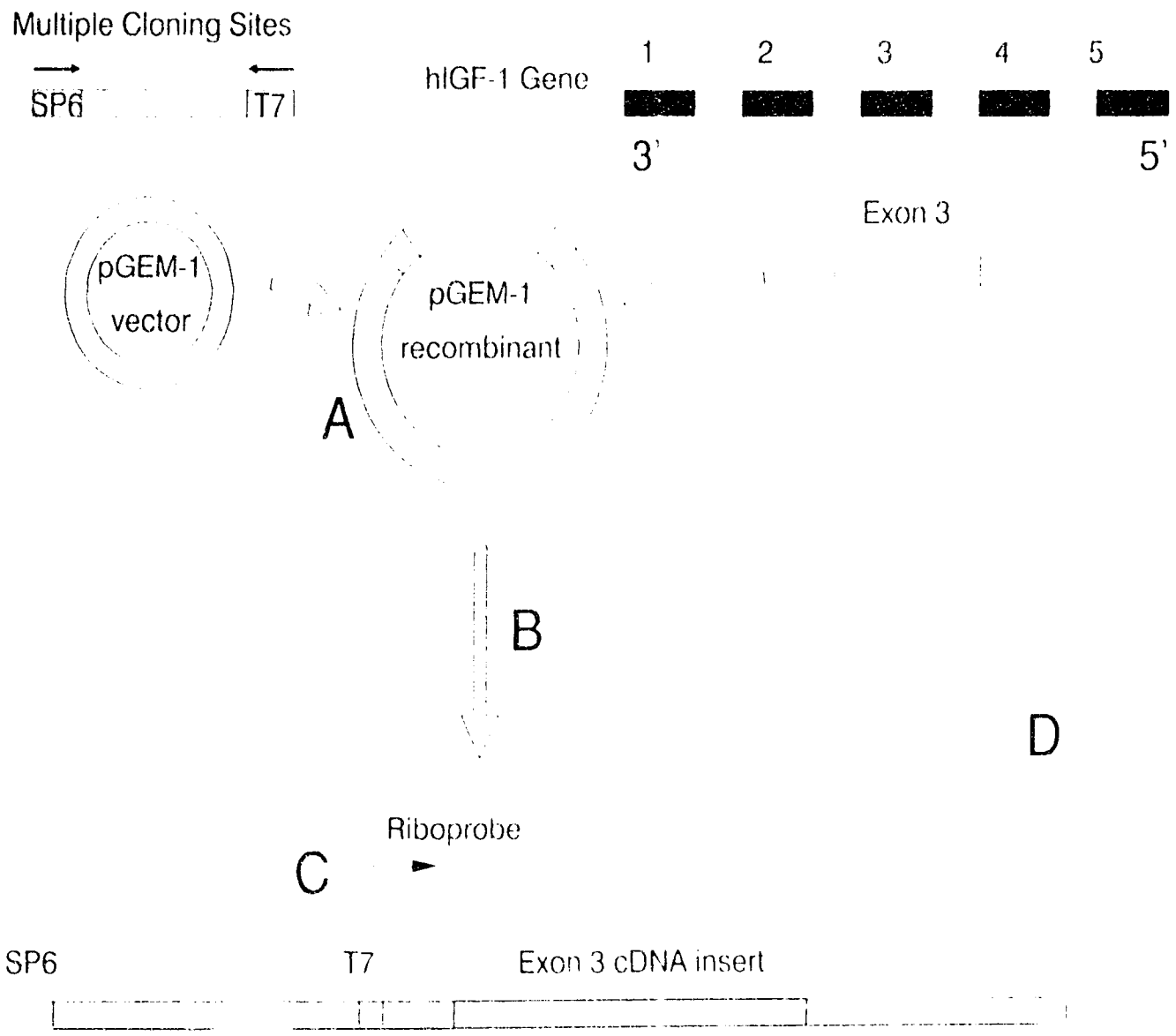
An RNase protection procedure was developed based on a procedure described by Ausubel *et al.* (1987). Samples of pooled ovarian or hepatic total RNA were precipitated overnight at -20°C in 0.1 volumes of 3 M sodium acetate (pH 5.4) and 2.5 volumes of cold (-20°C) 95% ethanol. RNA was pelleted by centrifugation (12 000g) in a microcentrifuge at 4°C for 30 min, the ethanol aspirated and the RNA pellet was dissolved in 30 μl of a hybridization buffer consisting of 80% deionized formamide (v/v), 20% 5 X

PIPES solution (220 mM PIPES, 2 M NaCl, 5 mM EDTA) (v/v) and 5 X 10^5 c.p.m. of the radiolabelled exon 3 hIGF-1 riboprobe (Rotwein, 1986). The single stranded antisense riboprobe (1038 bases) was synthesized and purified as described for northern analysis except for using a linearized pGEM-1 (hIGF-1 Exon 3 plasmid and T7 RNA polymerase. Figure 2.1. depicts schematically how the antisense riboprobe is produced. RNA samples in hybridization buffer were then heated at 85°C for 5 min to eliminate secondary structure and then transferred to a 45°C water bath to incubate for 16 hours.

Following hybridization, 300 μl of digestion buffer (10 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, 40 μg/ml RNase A and 2 μg/ml RNase T1) was added to each sample and the mixture incubated at 30°C for 1 hour. After digestion, 10 μl of 20% SDS and 2.5 μl of 10 mg/ml proteinase K were added to each sample. The samples were incubated at 37°C for 15 min and then extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1). The aqueous phase was transferred to a clean microfuge tube containing 1 μl of 10 mg/ml yeast tRNA, and then precipitated for 1 hour at -20°C with 1 ml of cold (-20°C) 95% ethanol. Following precipitation, all samples were centrifuged (12 000g) in a microfuge at 4°C for 30 min. After aspirating the ethanol, the RNA pellets were dissolved in 3 μl of loading buffer (80% deionized formamide (v/v), 1 mM EDTA, 0.1% xylene cyanol (w/v) and 0.1% bromophenol blue (w/v)). The samples were then heated at 100°C for 5 min and loaded onto a denaturing polyacrylamide/urea sequencing gel (Little and Jackson, 1987). The protected RNA was then size fractionated by electrophoresis in the presence of TBE running buffer (1.8 M tris-base, 1.8 M boric acid, 5.5 mM EDTA) using 40 W constant power and approximately 1200 V.

Figure 2.1. A schematic representation of the synthesis of the IGF-1 exon 3 riboprobe utilized in the RNase protection assay.

A. The plasmid vector (pGEM-1) is prepared for ligation with the IGF-1 exon 3 cDNA insert by restriction digestion within the multiple cloning site using the restriction enzyme Bam H1. **B**. The recombinant plasmid is linearized with the restriction enzyme XBal. **C**. RNA synthesis reaction components are added, including the T7 RNA polymerase, and then incubated at 40°C for 60 min. **D**. Synthesized antisense riboprobe transcripts


Multiple Cloning Sites
SP6
T7
hIGF-1 Gene
1
2
3
4
5
3'
5'
Exon 3
pGEM-1
vector
pGEM-1
recombinant
A
B
D
C
Riboprobe
SP6
T7
Exon 3 cDNA insert

Electrophoresis was stopped when the bromophenol stain had reached the bottom of the gel. The gel plates were then separated and the gel rinsed in 10% glacial acetic acid (v/v) and 10% methanol (v/v) for 15 min. After draining for 15 min, the gel was transferred to 3MM Whatman filter paper and then heat-sealed into a plastic bag and exposed to Kodak XAR 5 film at room temperature using an intensifying screen (Dupont Canada, Missaugua, ON).

Controls

Several controls were used to confirm the authenticity of protected fragments in the protection assay. Samples containing only tRNA, in amount equal to that used in all other RNA samples, were included in each assay and treated exactly as all other RNA samples. Because there is no mRNA present in the yeast tRNA, any protected bands detected would reflect non-specific binding of the probe.

Because the probe is transcribed from a human cDNA sequence, samples of human placenta RNA were included to allow comparison between protection of IGF-1 mRNA in human and pig RNA samples. Samples of "synthetic" IGF-1 were included in each assay. To produce "synthetic" IGF-1 mRNA, T7 polymerase was used to generate sense strands of IGF-1 mRNA from a cDNA template in which the IGF-1 exon 3 nucleotide sequence was inserted in the opposite orientation to that of the riboprobe template; these molecules were therefore identical to a portion of IGF-1 mRNA transcribed *in vivo* in humans. In theory, the use of this "synthetic" material in the hybridization reaction should not generate any non-specific binding and should produce only a single, authentic, protected IGF-1 exon 3 fragment.

Another control consisted of adding yeast tRNA to hybridization

mixture, containing no test RNA, after the RNase digestion. This control was used to determine the efficiency of digestion of the antisense riboprobe by the RNases in the digestion reaction. Samples of porcine ovarian and hepatic poly A^+ RNA were also included to test for non-specific riboprobe binding, and consequently protection of non-IGF-1 mRNA sequences. Unhybridized, undigested antisense riboprobe was also loaded onto the sequencing gel to confirm the size of the probe.

RESULTS

Northern Analysis

Two IGF-1 mRNAs of 8.0 kb and 0.8 - 1.1 kb were detected in both rat liver total and poly A^+ RNA, (Figure 2.2.a and b). The abundance of IGF-1 mRNAs in porcine ovarian tissue was low compared to rat liver. In ovarian total RNA, the 0.8 - 1.1 kb mRNA transcripts were detected in 30 μg and 10 μg aliquots but not in the 5 μg aliquot (Figure 2.2.a). The 8.0 kb transcript was not detected in any preparation of ovarian total RNA. Northern analysis of porcine ovarian poly A^+ RNA revealed the presence of four IGF-1 mRNAs (Figure 2.2b). In addition to the 8.0 and 0.8 - 1.1 kb transcripts, two other IGF-1 transcripts of 3.6 and 2.3 kb were detected. The 2.3 kb transcript appeared as a diffuse band, indicating that there may be range of mRNA transcripts with an average size of 2.3 kb. All four transcripts were detected in 25 μg and 5 μg of poly A^+ RNA, but only the 0.8 - 1.1 kb mRNA transcripts were detected in an 2.5 μg aliquot of poly A^+ RNA. The authenticity of the IGF-1 mRNA detected in the ovarian tissue is supported by the absence of equivalent-sized bands in the control poly A^+ RNA (Figures 2.2.a and b and Figures 2.3.a and b).

Figure 2.2. Northern hybridization analysis of total (2.2a), poly A^+ and poly A^- RNA (2.2b) from porcine ovary (PO) and rat liver (RL), hybridized with a Pvu II linearized [^{32}P] hIGF-1b riboprobe.

The estimated molecular weight (in kilobases (Kb)) of the major transcripts, relative to the migration of an RNA ladder, are indicated on the left of each blot. The position of 18S and 28S ribosomal RNA is indicated on the right of each blot. RNA quantity (in μg) is indicated below each lane; suprascript ($^+$) denotes poly A^+ RNA, suprascript ($^-$) denotes poly A^- while the absence of a suprascript denotes total RNA. The autoradiograms are both seven day exposures at -70°C.

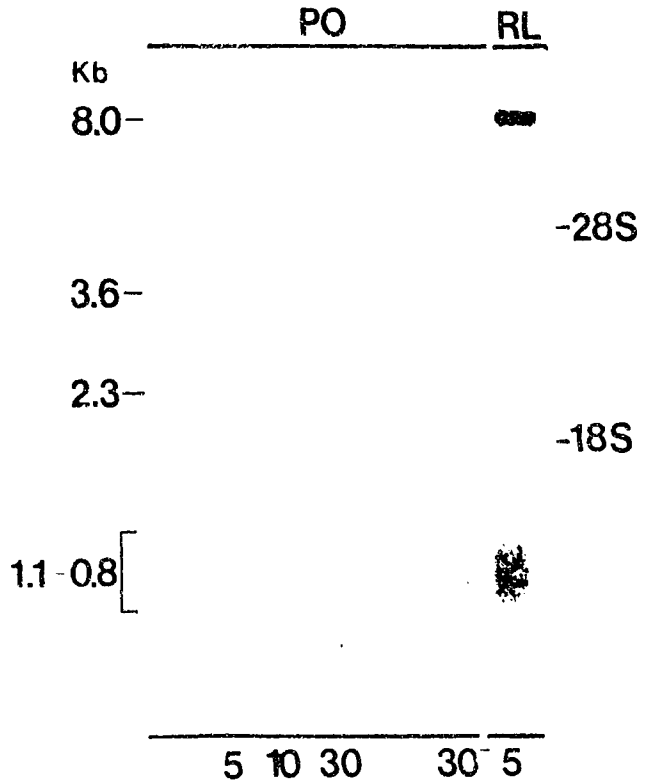


Figure 2.2a.

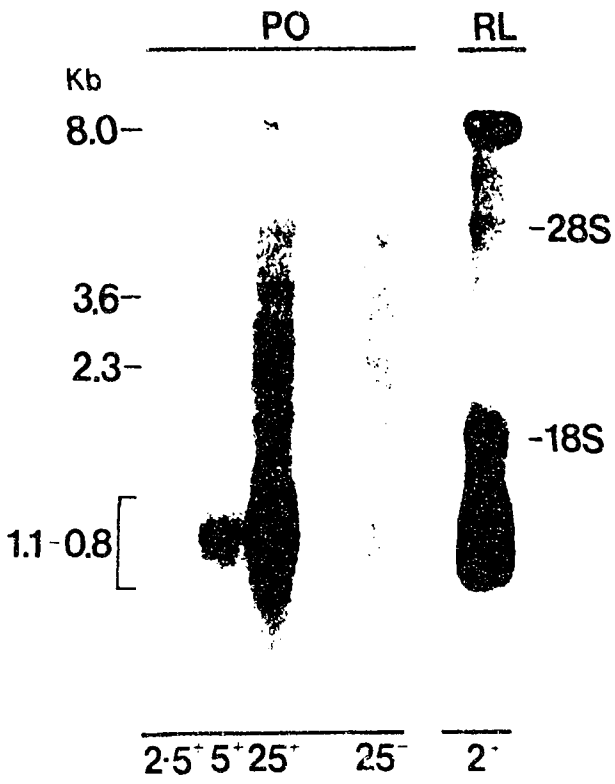


Figure 2.2b.

Figure 2.3. Northern hybridization analyses of total, poly A^+ and poly A^- RNA from porcine ovary (PO) and liver (PL) and rat liver (RL), hybridized with a Pvu II linearized [^{32}P] hIGF-1b riboprobe.

The estimated molecular weight (in kilobases (Kb)) of the major transcripts, relative to the migration of an RNA ladder, are indicated on the left of each blot. The position of 18S and 28S ribosomal RNA is indicated on the right of each blot. RNA quantity (in μg) is indicated below each lane; suprascript ($^+$) denotes poly A^+ RNA, suprascript ($^-$) denotes poly A^- while the absence of a suprascript denotes total RNA. The autoradiograms are both seven day exposures at -70°C.

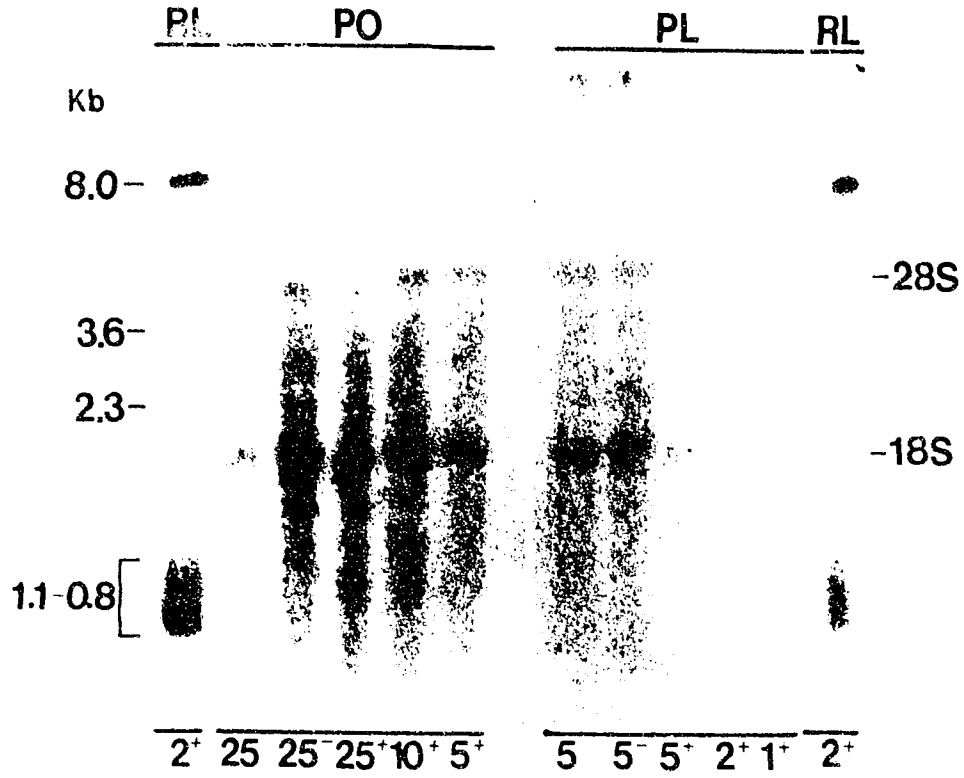


Figure 2.3a.

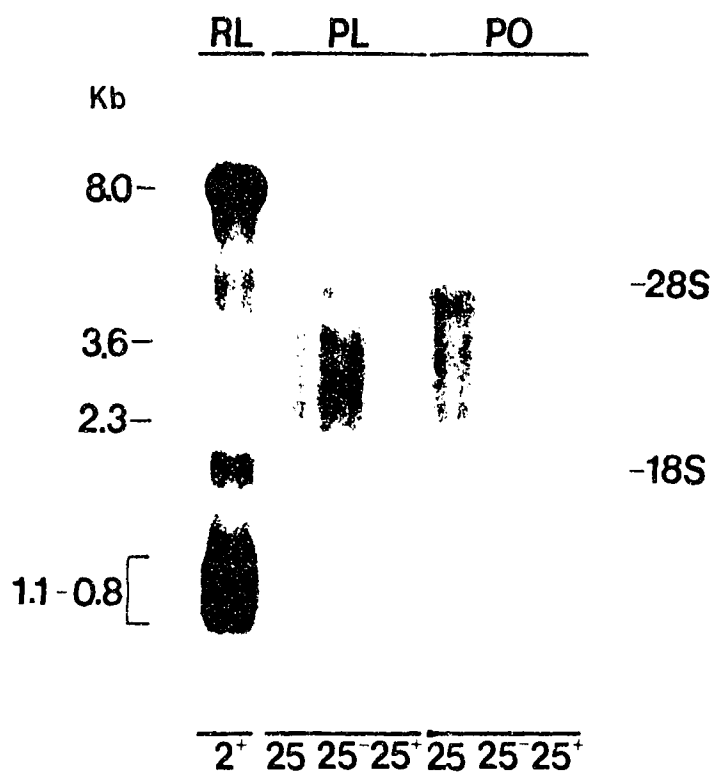


Figure 2.3b.

The 0.8 - 1.1 and 8.0 kb mRNA transcripts detected in rat liver and porcine ovary are similar to those reported previously in porcine (Tavakkol *et al.*, 1988 and Simmen *et al.*, 1990), rat (Murphy *et al.*, 1987) and human tissues (Shimatsu & Rotwein, 1987). The ovarian 2.3 kb IGF-1 mRNA species has been reported previously in porcine endometrial tissue (Tavakkol *et al.*, 1988 and Simmen *et al.*, 1990); however, there are no previous reports of a 3.6 kb mRNA species in any porcine tissue.

The data obtained from the northern analysis of porcine hepatic RNA in the present study does not convincingly establish that the IGF-1 gene is expressed in porcine liver (Figure 2.3.a and 2.3.b). Low abundance of hepatic IGF-1 mRNA in swine has been reported previously by Leaman *et al.* (1990). Tavakkol *et al.* (1988) and Duffy (*personnel communication*). Indeed Tavakkol *et al.* (1988) failed to identify distinct IGF-1 mRNAs in porcine hepatic tissue using northern analysis of poly A^+ RNA. There did not appear to be any hybridization of the probe at all in the 25 μg sample of poly A^+ hepatic RNA - to mRNA or to ribosomal RNA - suggesting that there may have been some RNase contamination within this sample. However, there was non-specific binding of the riboprobe to 18S and 28S ribosomal RNA in all other preparations indicating that these preparations were intact. The results presented in Figures 2.3a and b indicate the presence of the 2.3 kb and 3.6 kb transcripts which appear similar to those detected in porcine ovarian tissue. However, the 2.3 and 3.6 kb transcripts were also detected in the poly A^- RNA control, indicating that these two mRNAs may not be authentic and are instead artifacts due to non-specific binding of the riboprobe. In addition, the abundance of 2.3 and 3.6 kb hybridization in the poly A^+ RNA aliquot is lower than that in

either total RNA or the poly A$^-$ control, revealing that the signals detected does not represent hybridization to IGF-1 mRNA. In theory, the abundance of IGF-1 mRNA in the poly A$^+$ RNA should be higher, due to its enrichment by oligo d(t) cellulose chromatography.

It therefore appears that, like Tavakkol and co-workers (1988), this study failed to detect the presence of authentic IGF-1 mRNA in porcine hepatic tissue. However the apparent authenticity of the 2.3 kb and 3.6 kb mRNAs in porcine ovarian tissue throws suspicion on the validity of the poly A$^-$ controls used. Aliquots of porcine ovarian and hepatic poly A$^-$ were also subjected to the RNase protection procedure to act as controls for the detection of IGF-1 exon 3 fragments. If the oligo d(t) cellulose had been 100% efficient in separating the poly A$^+$ RNA from the total RNA, there should not have been any mRNA within these samples, therefore no 182 base protected fragment should have been detected. However Figure 2.4. indicates that there was a 182 base protected fragment present in these samples, suggesting that the oligo d(t) poly A$^+$ purification procedure was less than 100% efficient in separating the poly A$^+$ from the rest of the total RNA and that there was mRNA within the control. As the poly A$^-$ used for the RNase protection assay was from the same stock as that used for the northern analysis (Figures 2.3.a and b), the 2.3 kb and 3.6 kb mRNA species within the poly A$^-$ aliquot may actually be authentic mRNA rather than non-specific binding.

RNase Protection Assay

A a protected RNA fragment of 182 bases corresponding to the IGF-1 exon 3 RNA fragment was detected (Figure 2.4). This fragment was detected in porcine ovarian and hepatic total RNA, as well as in human placenta,

Figure 2.4. Autoradiogram of RNase protection of porcine liver (PL) and ovary total RNA (PO), and yeast tRNA, with controls **a - f**: **a.** 30 μg human placenta total RNA, **b.** 250 pg synthetic IGF-1 mRNA preparation, **c.** 50 μg porcine hepatic poly A^- RNA, **d.** 50 μg porcine ovarian poly A^- RNA, **e.** 7,500 dpm unhybridized, undigested IGF-1 exon 3 riboprobe, and **f.** 5 x 10^6 dpm unhybridized, digested IGF-1 exon 3 riboprobe.

Control markers (M) (in bases) are indicated on left of the autoradiogram, estimated weights (in bases) of the protected fragment and the riboprobe are depicted to the right of the autoradiogram. RNA quantity (in μg) is indicated below each lane. The autoradiogram was exposed for 24 hours.

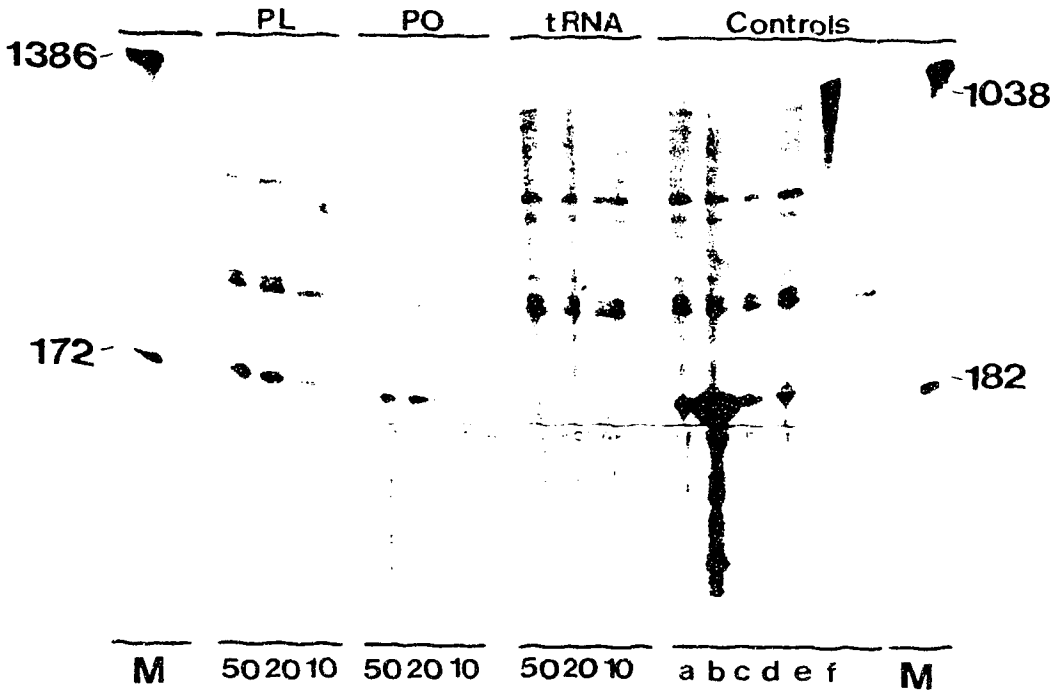

PL
PO
tRNA
Controls
1386
1038
172
182
M
50 20 10
50 20 10
50 20 10
a b c d e f
M

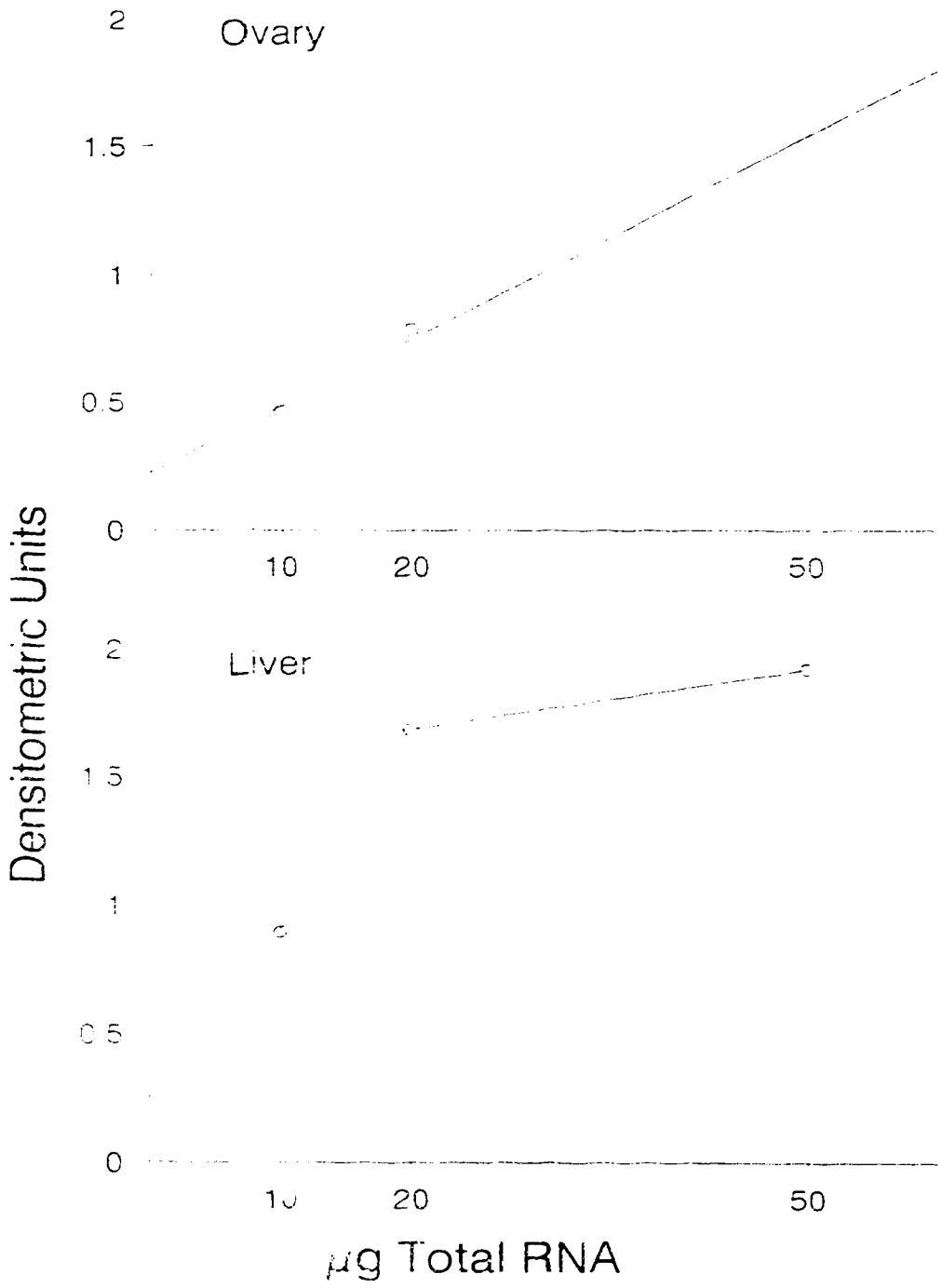
Figure 2.4.

synthetic IGF-1 RNA and porcine ovarian and hepatic poly A$^+$ RNA preparations. The undigested probe was detected as a single band of approximately 1038 bases. This is in agreement with the size of the exon 3 cDNA insert as well as the predicted size of the riboprobe. The presence of a single band suggests that the transcription reaction conditions are such that only full length transcripts are being synthesized.

No 182 base fragment was protected in the control samples in which yeast tRNA was added either before or after RNase digestion of the riboprobe. These two controls therefore confirm the identity of the 182 base exon 3 fragment observed in the test samples. Several larger fragments were also detected in all test samples and in these two controls. The presence of these bands in the yeast tRNA control containing no test samples, indicates that the non-specific binding is the result of hybridization of undigested probe to yeast tRNA.

Various amounts of porcine ovarian and hepatic total RNA were analysed using the RNase protection procedure to test whether this procedure had the potential to quantitate the level of IGF-1 mRNA abundance in different tissues. Aliquots of 10, 20 and 50 μg of total RNA, from each tissue, were hybridized with 5 x 10^5 dpm of the exon 3 riboprobe (Figure 2.4.). The optical densities of protected bands were then measured using a densitometer to produce a semi-quantitative value for each aliquot. The densitometry readings (illustrated in Figure 2.5.) indicate that the abundance of IGF-1 exon 3 mRNA increases with an increasing amount of total RNA analysed. This procedure therefore has the potential to provide a semi-quantitative representation of the level of IGF-1 mRNA abundance.

Figure 2.5. IGF-1 mRNA abundance detected by RNase protection in varying levels of ovarian and hepatic total RNA.


Ovary
2
1.5
1
0.5
0
10
20
50
Liver
2
1.5
1
0.5
0
10
20
50
Densitometric Units
µg Total RNA

The relationship between total RNA analysed and the abundance of IGF-1 mRNA revealed by densitometry was nonlinear. The nonlinearity was primarily due to the nature of the autoradiographic film as the relationship between the sensitivity of the x-ray film and radiation exposure is only linear over a very narrow range of radiation intensities. Therefore, the film itself is unable to to represent a linear relationship between the amount of total RNA analyzed and the yield of protected RNA produced when large ranges of total RNA are analyzed. Nonlinearity may also be due to the following: Firstly, the ratio of riboprobe to sample RNA in each aliquot was not constant; this likely resulted in more efficient hybridization of the riboprobe to smaller amounts of total RNA and therefore in an apparently higher level of IGF-1 mRNA abundance per unit of RNA than observed in samples containing a larger amount of total RNA. In order to avoid this effect the total amount of RNA in each sample should be normalized by adding an appropriate amount of tRNA. Secondly, it is also likely that some of the nonlinearity was due to methodological error which would be reduced through the use of replicates.

DISCUSSION

Northern analysis succeeded in detecting four IGF-1 mRNA transcripts in porcine ovarian tissue (8.0, 3.6, 2.3 and 0.8 -1.1 Kb). The 0.8 - 1.1, 2.3 and 8.0 kb mRNA species have been reported previously in porcine tissues (Tavakkol *et al.*, 1988; Simmen *et al.*, 1990); however, this is the first time that a 3.6 kb IGF-1 mRNA species has been reported. Application of the northern procedure for analysing tissue from individual animals revealed that the success of this procedure is dependent on both the

abundance of the RNA of interest and the amount of tissue available for analysis. For example, although hepatic IGF-1 gene expression is low, the yields of poly A^+ RNA from 0.5 g of liver tissue are high (approximately 100 μg), therefore there is possibly enough poly A^+ available from each animal to allow detection of hepatic IGF-1 levels in individual animals. However, this procedure may still not be suitable, as the greater the amount of RNA analysed as a single aliquot, the greater the amount of non-specific hybridization which may obscure the authentic signal.

Although IGF-1 gene expression was detectable in relatively small amounts of ovarian poly A^+ RNA, the ovaries of pigs are very small. In addition, evidence from *in vivo* work indicates that the expression of the IGF-1 gene in rat ovaries is confined to granulosa cells (Oliver *et al.*, 1989). Therefore, follicular rather than stromal tissue was collected for analysis in the present study. Little more than 0.5 g of total follicular material could be harvested from the ovaries of prepubertal gilts. In addition to the small amount of follicular tissue available, a large proportion of the follicular mass harvested was made up of fluid, particularly in larger follicles. Therefore, the amount of tissue mass per 0.5 g ovarian sample was low compared to a tissue such as liver. Although the 0.8 - 1.1 kb IGF-1 mRNA transcript was detected in total RNA, the 8.0, 3.6 and 2.3 kb IGF-1 transcripts could only be detected in poly A^+ RNA. The approximate yield of poly A^+ RNA from 0.5 g of follicular material was 5 μg, which is barely enough to allow the detection of IGF-1 mRNA, certainly not enough to allow replication of the procedure.

The sensitivity of RNase protection assays have been reported to be twenty times higher than that of northern analyses (Maniatis *et al.*,

1982). In support of this contention, the present study demonstrated that IGF-1 mRNA could be detected in as little as 10 μg of both porcine ovarian and hepatic total RNA.

The difference in sensitivity between the two procedures is due to differences in the number of potential hybridization sites available for binding to the riboprobe in each procedure. In northern analysis a proportion of the potential hybridization are unavailable for binding as they are used to covalently link the RNA to the solid support. In the RNase protection assay procedure, hybridization occurs in solution and therefore all binding sites are available for hybridization with the riboprobe. The disadvantage of the RNase protection assay is that only a single protected fragment is quantified. Although this may give a more sensitive estimate of the absolute amount of IGF-1 expression it does not identify which species of mRNA are present.

In this study a human - derived riboprobe was successfully used to detect a porcine mRNA. Usually the use of heterologous probes in an RNase protection assay is not advised, as the presence of miss-matched pairs in the riboprobe-sample RNA hybrid produces sites that may be targeted by the RNase enzymes used to digest non-hybridized single-stranded sample RNA and riboprobe. The degree of damage caused by RNase digestion at miss-matched pair sites depends on how much the gene sequence for the target gene of the two species differ. If there is little homology between the two species, then the hybrid may be totally digested. On the other hand, if there are only a few different codons present, then the protected hybrid may only be broken into a few smaller fragments. The results of the RNase protection assay demonstrate that the nucleotide sequences of the IGF-1

exon 3 has a high degree of homology in humans and pigs. Further support for a high degree of homology between the IGF-1 exon 3 sequences in these two species is provided by the finding that the protected fragment detected in human placenta total RNA was identical to that detected in porcine hepatic and ovarian total RNA. This is not surprising, considering that the amino acid sequences of human and porcine IGF-1 are identical (Sara & Hall, 1990).

This procedure not only succeeded in detecting IGF-1 exon 3 mRNA in porcine ovarian and hepatic tissue, but also has the potential to quantitate changes in the expression of the IGF-1 gene. As discussed earlier, this ability for quantification of gene expression may be limited as the relationship between the amount of RNA analysed and the level of IGF-1 gene expression was not linear. Nonetheless, as long as the samples are processed in the same manner, and the absolute amount of RNA in each sample is the same, the RNase protection assay has the potential do detect physiological changes in gene expression.

The observation that the level of gene expression in porcine hepatic tissue is lower than that in ovarian tissue was unexpected. In all other species examined, the highest IGF-1 mRNA abundance has been found in hepatic tissue (Murphy *et al.* 1987; Han *et al.*, 1988). While the primary source of circulating IGF-1 in rats and humans is believed to be the liver, the results of the present study suggest that in the pig other tissues may contribute significant amounts of this growth factor to the circulating pool. There are reports that in this species, cardiac and skeletal muscle (Leaman *et al.*, 1990), uterine (Tavakkol *et al.*, 1988) and adipose tissue (Duffy, *personnel communication*) all contain higher levels

of IGF-1 mRNA than the liver.

Considering IGF-1 gene expression on a tissue basis, it is possible that the majority of circulatory IGF-1 may still be of hepatic origin in the pig. Because the mass of liver is considerably larger compared to the ovary, heart and other tissues it is still likely to produce the greater total amount of IGF-1. However, the mass of skeletal muscle exceeds that of the liver and total adipose tissue exceeds that of liver (Schaefer, *personnel communication*), so it is possible that these tissues still contribute a significant amount of IGF-1 to the circulating pool. However, the yield of total RNA from hepatic tissue are approximately twice that of cardiac or skeletal muscle (Leaman *et al.*, 1990) and likely to be greater than that from adipose tissue based on the number of cells per unit mass. Therefore, although relative IGF-1 gene expression is lower in hepatic tissue, this may be due in part to the dilution effect of greater amounts of total RNA rather than due to a simple lack of IGF-1 gene expression. Thus, the level of total IGF-1 gene expression per unit mass of tissue may actually be similar in porcine hepatic tissue, if not higher.

Another possible explanation for the apparent lack of IGF-1 gene expression in the liver may be that clearance rate of the IGF-1 mRNA may be higher in hepatic tissue than in other tissues. Hepatic tissue is considered to be highly metabolically active compared to other tissues, therefore the turnover rate of IGF-1 mRNA may be higher.

In conclusion, both the northern analysis and the RNase protection procedures have the ability to detect the expression of the IGF-1 gene in porcine ovarian tissue. The detection of IGF-1 gene expression in this tissue supports the hypothesis that IGF-1 is produced locally by the ovary

and therefore has the potential for paracrine and (or) autocrine actions within the ovary *in vivo*. Hepatic IGF-1 gene expression was detected using the RNase protection procedure, whereas northern analysis did not convincingly detect IGF-1 gene expression in extracts of comparable sizes. The level of IGF-1 gene expression per unit of total RNA appears to be lower in the porcine liver than in the ovary. In terms of the suitability of these procedures for the detection of IGF-1 gene expression in the tissues of individual animals, the higher sensitivity of the RNase protection assay suggests that this would be the preferred procedure to use in *in vivo* animal studies.

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CHAPTER 3

Ovarian and LH Responses to Restrict-Feeding/Re-Feeding in the Prepubertal Gilt

INTRODUCTION

It is well established that growth hormone stimulates the production of insulin-like growth factor 1 (IGF-1) and that many of the actions of growth hormone are mediated by this growth factor. Originally it was thought that growth hormone could only induce the production of IGF-1 in hepatic tissue and that any immunoreactive IGF-1 found in other tissues originated from the liver. However, recently, using *in vitro* incubation and molecular biological techniques that are capable of detecting the messenger ribonucleic acid (mRNA) for IGF-1, it has been demonstrated that IGF-1 is produced in other tissues. Using cDNA probes for rat IGF-1, Murphy *et al.* (1987) investigated the tissue distribution of mRNA for this growth factor in adult rats. They provided evidence for the expression of the IGF-1 gene in the liver, brain, lung, heart, uterus and ovary and, to a lesser extent, in the testes and mammary gland. In the growing and adult pig, IGF-1 gene expression has been detected in cardiac and skeletal muscle (Leaman *et al.*, 1990), the uterus (Tavakkol *et al.*, 1988), ovary (Cameron *et al.*, 1990) and adipose tissue, in addition to the liver (Leaman *et al.*, 1990). This widespread distribution of message for IGF-1 therefore suggests a potential paracrine/autocrine role for IGF-1.

The growth hormone-IGF-1 axis has an important effect on the development of the ovarian follicle. Growth hormone has been shown to increase ovarian levels of immunoreactive IGF-1 *in vitro* (Davoren and Hsueh, 1986). It appears that IGF-1 then acts in a paracrine or autocrine manner as it regulates granulosa cell growth and function (for review see Hammond *et al.*, 1991). IGF-1 has been shown to potentiate the action of follicle-stimulating hormone (FSH) in the FSH-induced differentiation and

the progestin biosynthetic capacity of granulosa cells (Davoren *et al.*, 1985; Adashi *et al.*, 1985a), as to augment the FSH-stimulated induction of luteinizing hormone receptors on granulosa cells (Adashi *et al.*, 1985b). In addition to its mitogenic and indirect steroidogenic effects, this growth factor has also been shown to have classic metabolic effects on granulosa cells. IGF-1 has stimulatory effects on protein synthesis and glucose oxidation (Weber and LaBarbera, 1988) and low density lipoprotein metabolism during steroidogenesis (Veldhuis *et al.*, 1987).

As IGF-1 is considered to be a metabolic hormone, its complex role in the ovary makes it a good candidate for a possible link between the metabolic and reproductive state of an animal. Booth (1990a) investigated the effects of restrict-feeding followed by re-feeding on the reproductive status of the prepubertal gilt. This study showed that restrict-feeding of prepubertal gilts reduced LH pulsatility, and impaired ovarian and uterine development, in the absence of any changes in body weight or composition. Re-feeding of the restrict-fed gilt, with a single feed to-appetite or a parenteral infusion of glucose, restored LH pulsatility within six hours, corresponding to an increase in plasma levels of insulin and IGF-1. No changes in GH secretion were observed. These findings indicate a rapid restoration of the reproductive status of the animal as a result of the increased plane of nutrition.

In humans, Clemmons *et al.* (1981) reported that fasting for ten days resulted in IGF-1 levels as low as those found in GH-deficient patients. Merimee *et al.* (1982) later concluded that as GH administration to starved humans failed to increase IGF-1 levels, an adequate level of nutrition is

a prerequisite for GH-induced IGF-1 production. It appears that an adequate level of insulin is necessary for GH-induced generation of IGF-1 and that fasting or restrict-feeding uncouples the GH - IGF-1 axis. In the rat, fasting has been demonstrated to reduce circulating IGF-1 levels and the expression of the IGF-1 gene in hepatic tissue (Elmer and Schalch, 1987). However it is unknown whether fasting or restrict-feeding has the same effect in the ovary.

There are indications that insulin levels influence IGF-1 levels within the ovaries of gilts and that this effect may be independent of gonadotropins. Cox *et al.* (1987) investigated the effects of insulin treatment on ovulation rates and pattern of LH secretion in cyclic gilts fed a high energy diet. Insulin treatment was found to significantly increase ovulation rates in the absence of significant increases in LH peak amplitude or basal levels. Hammond *et al.* (1988) demonstrated that follicular fluid levels of IGF-1 increased in response to PMSG treatment, suggesting that intrafollicular IGF-1 levels may be regulated by gonadotropins. However, in a more recent study, Meurer *et al.* (1991) demonstrated that IGF-1 levels in the large follicles of diabetic gilts were lower compared to those of normoglycaemic gilts, even when treated with PMSG. They concluded that the absence of insulin, and subsequent lower follicular fluid levels of IGF-1, may have compromised steroidogenesis, as positive correlations between follicular fluid estradiol and IGF-1 in normoglycaemic animals were not observed in diabetic animals. This suggests that decreased insulin levels may have detrimental effects on ovarian function via the production of IGF-1.

In study that used feeding level to manipulate insulin levels,

Cosgrove *et al.* (1991) demonstrated that in the chronically gonadotropin-suppressed and restrict-fed gilt, re-feeding was capable of stimulating ovarian development in the absence of increasing gonadotropin. After five days of re-feeding for example, both follicular size and aromatase activity were significantly higher in re-fed compared to the restrict-fed gilts.

The data from the studies carried out by Cox *et al.* (1987), Meurer *et al.* (1991) and Cosgrove *et al.* (1991) suggest that ovarian development may be to some extent influenced directly by changing metabolic status, rather than indirectly by metabolic influences on the hypothalamic-pituitary axis. If nutrition does affect the production of IGF-1 in the ovary, the proposed paracrine/autocrine role of this growth factor would help to explain such gonadotropin-independent changes in the ovary. The objective of the following experiment was to use the same prepubertal gilt model as used by Booth (1990a) and Cosgrove (1991), to confirm the effects of restrict-feeding/re-feeding on the reproductive status of the prepubertal gilt under University of Alberta conditions. The effects of changing metabolic state on ovarian development were also further investigated, focusing on the hypothesis that IGF-1 may be a growth factor that acts as a metabolic signal at the level of the ovary.

MATERIALS AND METHODS

Animals

Prepubertal gilts (Camborough x Canabrid; PIC Canada, Ltd.) were obtained from the University of Alberta swine unit. Five groups of four littermates were selected from a pool of *ad libitum* fed animals on the

basis of comparable growth rates. The average weight of the gilts was 80.6 ± 6.5 Kg. One day prior to the start of the experimental study, groups of gilts were moved into individual animal crates in which they were within close visual and aural contact with each other and received water *ad libitum*. The following day (d1), they were placed on a 7 day restrict-feeding regimen whereby they were fed a maintenance level of feeding twice daily at 09.00h and 17.00h (approximately 30% of individual *ad libitum* feed intake). The level of feeding was calculated on the basis of NRC (1988) maintenance requirements.

On d8, the animals were split into four groups of five, with one litter mate in each treatment (ie. employing a 2 x 2 factorial experimental design with five replicates per treatment). Two groups of gilts remained on a maintenance level of feeding, either from d8 to d9 (R8) or d8 to d13 (R12). The remaining two groups were fed to appetite, either from d8 to d9 (A8) or d8 to d13 (A12).

Blood Sampling

An indwelling jugular cannula was surgically placed under general anaesthesia and aseptic conditions on day 6 of the study to facilitate stress-free withdrawal of blood. Blood samples were taken at 10 minute intervals from 08.00h until 17.00h on day 8. Groups R12 and A12 were also sampled from 08.00h until 17.00h on day 12. Blood was collected into heparinized tubes and plasma obtained by centrifugation at 2000*g* for 20 min immediately after collection. The plasma was then stored at -20 C until analysis for LH, insulin, IGF-1 and GH.

Collection Of Ovarian Tissue

Groups R8 and A8, and R12 and A12 were slaughtered at approximately 09.00h on d9 or d13 respectively in order to facilitate the collection of the ovaries. One ovary (chosen at random) was examined to determine follicular dimensions. The diameters of the ten largest follicles were measured directly on the surface of the ovary, while their follicle volumes were determined by aspiration of the follicular fluid using either a 50 μl or a 200 μl Hamilton syringe. The follicular fluid was retained for estradiol analysis by adding it to 1 ml of PBS buffer.

The remaining ovary was snap frozen in liquid nitrogen and stored at -70°C until analysis for IGF-1 mRNA.

Radioimmunoassays

Luteinizing Hormone

Plasma LH concentrations were measured using the homologous double-antibody radioimmunoassay previously described by Cosgrove (1991). The detection limit of the assay was approximately 0.01 ng/tube, at a binding of 87.9% (defined as mean total bound minus two standard deviations from the mean total bound). Intra- and inter-assay coefficients of variation were 10.6% and 2.2% respectively.

Insulin

Plasma insulin concentrations were measured in a single radioimmunoassay using the homologous double-antibody radioimmunoassay previously described by de Boer and Kennelly (1989), with modifications as described by Cosgrove (1991). The detection limit of the assay was

approximately 0.008 ng/tube, at a binding of 90.9% (defined as mean total bound minus two standard deviations from the mean total bound). The intra-assay coefficient of variance for this assay was 10.2% respectively.

Growth Hormone

Growth hormone plasma concentrations were determined in a single assay using the double-antibody radioimmunoassay procedure as described by Marple and Aberle (1972) with modifications as described by de Passille *et al.* (*in press*). The detection limit of the assay was approximately 0.06 ng/tube, at a binding of 93.9% (defined as total bound minus two standard deviations from the mean total bound). The intra-assay coefficient of variance was 5.8%.

Insulin-Like Growth Factor 1

IGF-1 plasma concentrations were quantified in a single radioimmunoassay following acid-ethanol extraction to remove binding proteins. The acid-ethanol extraction procedure used was that described by Booth (1990b), with the following modifications; A ratio of 3000 μl acid-ethanol:100 μl of sample was used, followed by a 20 hour incubation at room temperature. The extracted protein was then precipitated by centrifugation at 3000g at 4°C, thus leaving the unbound IGF-1 in the supernatant. The extraction efficiency of this procedure was estimated to be 100% , based on estimates of cold recoveries of hIGF-1 added to a standard pool of plasma. The radioimmunoassay employed was that described by Glimm *et al.* (1990), with modifications as described by Cosgrove (1991). The detection limit was 0.002 ng/tube, at a binding of 93.7%

(defined as mean total bound minus two standard deviations from mean total bound). The intra-assay coefficient of variance for this assay was 4.74%.

Estradiol

Follicular fluid estradiol-17ß concentrations were estimated in a single tritiated radioimmunoassay using an antiserum (NCR Rabbit All, obtained from Dr. N.C. Rawlings, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada) raised in rabbit against 1,3,5,(10)-estratrien-3,17ß-diol-6-one-6-CMO:BSA (STERALOIDS E1361). Cross-reactivity for this antiserum reported by the supplier were: estrone 8%, estriol 0.4%, 17 α-estradiol 0.4%, testosterone 0.08%, dihydrotestosterone 0.1%, 5 α-androstan-3ß,17ß-diol 0.08% and cortisone 0.03%. Androstenedione, 5 α-androstan-3α,17ß-diol, progesterone, pregnenolene, cholesterol, corticosterone and cortisol did not cross-react with this antiserum. 100 μl of sample diluted in PBS buffer, 150 μl of assay buffer (2.77 mM sodium phosphate, 7.22 mM disodium phosphate, 139.66 mM NaCl, 15.38 mM NaN_3 and 0.1% (w/v) gelatin) and 200 μl antiserum (final dilution 1:440,000) were added to a 12 x 75 mm glass tube. After vortexing, the mixture was cultured at room temperature (20°C) for one hour, and followed by the addition of 100 μl (approx. 20,000 dpm) ^{3}H-labelled estradiol-17ß (Du Pont NEN Products) to each assay tube. After incubation at 4°C for approximately 20 hours, 200 μl of well mixed dextran coated charcoal (Dextran 0.125 g, Carbon Decolouring Alkaline Norit-A 1.25 g, mixed thoroughly in 250 ml assay buffer and cooled to 4°C) was added to each assay tube, incubated for 10 min at 4°C and then centrifuged at 1450g for 10 min. The supernatant from each tube was poured off into a 6 ml vial

containing 5.6 ml liquid scintillation fluid (8824757), mixed thoroughly, and then counted in a beta-counter for 2 min. Duplicate tubes were assayed for each sample. A series of standards was prepared by diluting estradiol-17B (1,3,5,(10)-Estratrien-3,17ß-diol; 3,17ß-dihydroxy-1,3,5,(10)-estratriene, Sigma) in assay buffer. Specificity was confirmed by showing parallelism between a series of diluted quality control follicular fluid pools (1/50, 1/100, 1/200, 1/400, 1/800 and 1/1600) and standard inhibition curves. The detection limit of the assay was 4.1 pg/tube, at a binding of 84.9% (defined as mean total bound minus two standard deviations from mean total bound). The intra-assay coefficient of variance for this assay was 11.3%.

RNA Analysis

From each ovary, 0.5 g of frozen follicular tissue was harvested by dissection with a sterile scalpel blade. The follicles were harvested in descending order of size until 0.5 g of tissue was collected. Total RNA was isolated from each sample using the guanidine isothiocyanate/caesium chloride procedure as described in Chapter 2 and then analysed in a single RNase protection assay, using the same procedure as described in Chapter 2. As in Chapter 2, sample total RNA was hybridized against a single stranded antisense hIGF-1 Exon 3 riboprobe. The level of gene expression for each animal was quantified using densitometry, expressing the readings as a percentage of the reading for a standard aliquot of human placenta (kindly supplied by a co-worker in our laboratory, using identical isolation techniques). This method of quantifying gene expression was adopted to allow comparisons of data generated in this study with that

generated in future studies. The controls utilized to determine the authenticity of the protected fragment are listed in Figure 3.4.

Statistical Analysis

Although a total of 20 gilts were originally selected for this study, data was only collected from 12 animals. At slaughter one animal from a litter of 4 was found to be a hermaphrodite and, as another sibling (not selected for the study due to a poor growth rate) was also found to be a hermaphrodite, this litter of four gilts was eliminated from the trial. Two further gilts were eliminated from the trial prior to surgery as one gilt appeared to be in estrus while the other showed inappetance. Data on hormone changes were unavailable for another gilt, as the patency of her cannula failed during the d8 sampling period; however her ovaries were still collected and included in analysis of follicular parameters. Similarly, the cannula of one gilt from the R12 group remained patent throughout the d8 sampling period but failed on during the d12 sampling period. Enough samples were collected to make fair comparisons for insulin, IGF-1 and GH secretion but not to give a fair estimate of LH pulsatility. Once again her ovaries were still collected and included in analysis of follicular parameters. Finally, a fifth gilt was withdrawn from the experiment at slaughter as examination of her ovaries revealed the presence of corpora lutea (ie she was no longer prepubertal!).

Luteinizing hormone (LH) profiles were subjected to analysis using a sliding window technique, as described by Shaw and Foxcroft (1985), to generate mean, maximum and minimum secretion levels. The procedure was also modified to generate the area of the secretion profile and as such,

give information regarding the total amount of LH secreted within the sampling period, taking into account the baseline sensitivity of the assay. LH pulses in each profile were also quantified to give an indication of LH pulsatility. Pulses were quantified by using a visual appraisal procedure as described by Foxcroft *et al.* (1988) in which a pulse was defined as an increase in secretion that occurs within two sampling intervals, followed by a decline that takes at least three sampling intervals to reach the baseline level of secretion and represents a logarithmic decline in LH concentrations.

The effect of treatment on hormone secretion, on d8, was analysed using a split-plot analysis of variance, fitting treatment as the main effect and animal weight at the onset of the study as a covariate, using the general linear model procedure (PROC GLM) within the SAS statistical analysis computing software package. For d8 plasma hormone analysis, data for treatments R8 and R12 were pooled, as until d9 these two groups were subjected to an identical treatment regimen. Using the same rational, data from groups A8 and A12 were also pooled. Treatment effects on insulin secretion were analysed during three different time periods: 08.00-09.00h (Pre-prandial secretion), 09.00-11.00h (Prandial secretion) and 11.00-17.00h (Post-Prandial secretion). On d12, secretion of insulin (pre-prandial, prandial and post-prandial), IGF-1 and GH were again analysed using a split-plot analysis of variance. However, due to lack of animal replicates with sufficient samples to justify using the sliding window procedure, LH secretion on d12 was analysed using the Student's *t*-test testing LH characteristics against treatment.

Comparisons were made between d8 and d12 hormone secretions.

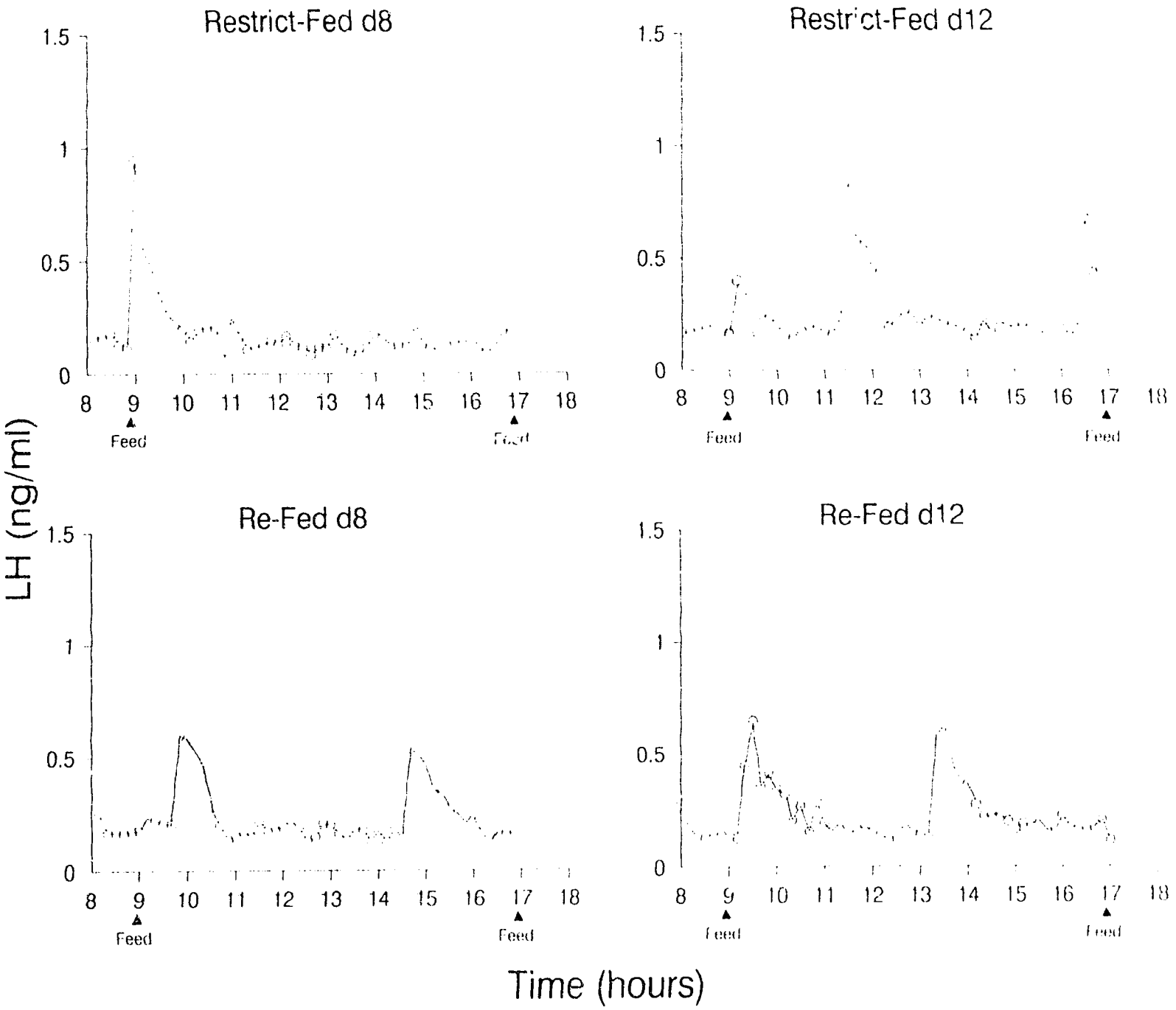
Repeated measures procedures were carried out within the SAS statistical package to compare d8 insulin (pre-prandial, prandial and post-prandial), IGF-1 and GH secretion with that of d12. Again due to a lack of animal replicates, this comparison could not be made for LH.

Follicular parameters were also analysed using a split-plot analysis of variance. As described above for hormone secretion, treatment was fitted as the main effect with gilt weight as a covariate. Individual follicle volume, for each gilt, was regressed against diameter, for each treatment, utilizing the regression procedure (PROC REG) within the SAS statistical package. The resulting coefficients and intercepts for each gilt were then analysed using the split-plot analysis of variance as before. Similar comparisons were made for individual follicular estradiol content (pg/follicle) and concentration (ng/ml) against follicle volume. Follicle IGF-1 gene expression was regressed against mean follicle volume and then mean follicular estradiol concentration (ng/ml) utilizing a general linear model procedure for analysis of variance (PROC GLM) within the SAS statistical package, testing follicular volume or estradiol as covariates and their interactions with treatment as main effects.

RESULTS

LH secretion was estimated at 10 min intervals to allow the characterization of pulsatility. The LH profiles from two littermates from groups R12 and A12, on d8 and d12, are illustrated in Figure 3.1 and are representative of the effects of re-feeding and duration of re-feeding on LH secretion in all gilts. On d8, re-feeding of restrict-fed gilts had no effect on mean, maximum and minimum LH secretion, or on LH profile area.

Figure 3.1. Plasma LH profiles of two littermates from groups RLP and ALP, on d8 and d12. Arrows denote time of feed.


Restrict-Fed d8
Restrict-Fed d12
Re-Fed d8
Re-Fed d12
LH (ng/ml)
Time (hours)
1.5
1
0.5
0
8 9 10 11 12 13 14 15 16 17 18
Feed
Feed

On d12, however, minimum LH secretion was significantly increased by re-feeding ($P<0.05$) with similar trends for increases in mean and maximum secretion, and profile area. LH episodes were also quantified: no treatment effects on LH pulsatility were observed on d8, however on d12 there were distinct increases in LH pulsatility although gilt variation was responsible for this increase not being significant. All LH data is presented in Table 3.1.

On d8 there was no significant effect of treatment on preprandial insulin secretion. Prandial insulin secretion was greater in re-fed animals but this difference was insignificant. Post-prandial secretion was significantly increased by re-feeding on d8 ($P<0.001$). By d12, re-feeding of previously restrict-fed gilts significantly decreased preprandial insulin ($P<0.05$) but did not change prandial or post-prandial insulin secretion. When d8 insulin secretion was compared with that on d12, no effects of time or time x treatment interactions were observed. Least square means data for insulin secretion is presented in Table 3.2.

There were no significant effects of re-feeding on plasma GH secretion on either d8 or d12. When d8 GH secretion was compared with d12, there was no effect of time or any time x treatment interaction on GH secretion. Weight was found to have an effect on GH secretion ($P<0.05$). There were no effects of treatment on plasma IGF-1 secretion on either d8 or d12 and no significant differences between d8 and d12 secretion. Least square mean data for GH and IGF-1 are presented in Table 3.2.

There appeared to be no significant effects of re-feeding restrict-fed gilts on ovarian development. No changes in follicular diameter or volume were observed, although weight was found to have an effect as a

Table 3.1 The Effect of Re-feeding Restrict-Fed Gilts on Plasma LH Secretion on D8 and D12

	Restrict-Fed	Re-Fed
Day 8		
Mean LH (ng/ml)	0.235 ± 0.037	0.225 ± 0.030
Maximum LH (ng/ml)	0.319 ± 0.056	0.314 ± 0.046
Minimum LH (ng/ml)	0.170 ± 0.026	0.156 ± 0.021
LH Profile Area (ng/ml)	2.041 ± 0.327	1.878 ± 0.269
LH Pulses	1.511 ± 0.638	2.063 ± 0.526
Day 12		
Mean LH (ng/ml)	0.264 ± 0.007	0.399 ± 0.080
Maximum LH (ng/ml)	0.391 ± 0.007	0.558 ± 0.111
Minimum LH (ng/ml)	0.176 ± 0.001	0.273 ± 0.059*
LH Profile Area (ng/ml)	2.279 ± 0.049	3.495 ± 0.757
LH Pulses	2.500 ± 0.500	4.250 ± 1.031

Values are least square means ± SEM for D8 and means ± SE for D12

* $P<0.05$ compared with restrict-fed group

Table 3.2 The Effect of Re-Feeding Restrict-Fed Gilts on Plasma Insulin, GH and IGF-1 Secretion on D8 and D12

	Restrict-Fed	Re-Fed
Day 8		
Pre-prandial Insulin (ng/ml)	0.488 ± 0.048	0.526 ± 0.042
Prandial Insulin (ng/ml)	1.100 ± 0.442	2.376 ± 0.386
Post-prandial Insulin (ng/ml)	0.344 ± 0.068	1.076 ± 0.059**
Growth Hormone (ng/ml)	0.789 ± 0.166	1.126 ± 0.145
IGF-1 (ng/ml)	86.129 ± 7.224	85.712 ± 6.312
Day 12		
Pre-prandial Insulin (ng/ml)	1.974 ± 0.115	0.166 ± 0.088
Prandial Insulin (ng/ml)	1.120 ± 0.801	0.896 ± 0.611
Post-prandial Insulin (ng/ml)	1.059 ± 0.321	0.469 ± 0.245*
Growth Hormone (ng/ml)	1.693 ± 0.405	0.725 ± 0.309
IGF-1 (ng/ml)	110.326 ± 8.305	85.445 ± 6.331

Values are least square means ± SEM

* $P<0.05$, ** $P<0.001$ compared with restrict-fed group.

covariate ($P<0.05$). In addition, there were no treatment effects on mean follicular fluid estradiol content (pg/follicle) or concentration (ng/ml), although there are trends for an increase in follicular estradiol with re-feeding on d13. Follicular volume and diameter are depicted in Figure 3.2, and follicular estradiol is illustrated in Figure 3.3.

Individual follicle volumes were regressed against individual follicle diameter to give an indication whether re-feeding affected the ratio of follicle cell content to antral fluid. A similar comparison was made for the relationship between individual follicle volume and individual follicular fluid estradiol concentration and content. Analysis of the intercepts and slopes for each animal did not indicate any effects of treatment on the relationship between follicle volume and follicle diameter, follicular estradiol concentration or follicular estradiol content.

Authentic IGF-1 exon 3 protected fragments were detected in ovarian total RNA from all gilts in all treatments, suggesting that the IGF-1 gene was expressed in ovarian tissue from both restrict-fed and re-fed gilts (Figure 3.4.). Re-feeding was found to have a highly significant effect on ovarian IGF-1 gene expression, with re-feeding resulting in increases in mRNA detected on d9 and d13 compared with their respective restrict-fed treatment groups ($P<0.001$). No significant difference between two days of re-feeding (d9) and five days of re-feeding (d13) were observed, although five further days of restrict-feeding resulted in higher gene expression than two further days of restrict-feeding. Mean densitometric readings for IGF-1 mRNA expression in ovarian tissue from each group are illustrated in Figure 3.5. Litter was also found to have an effect on IGF-1 gene

Figure 3.2. Mean follicle diameters and volumes for four treatment groups. Treatment abbreviations are defined in the text.

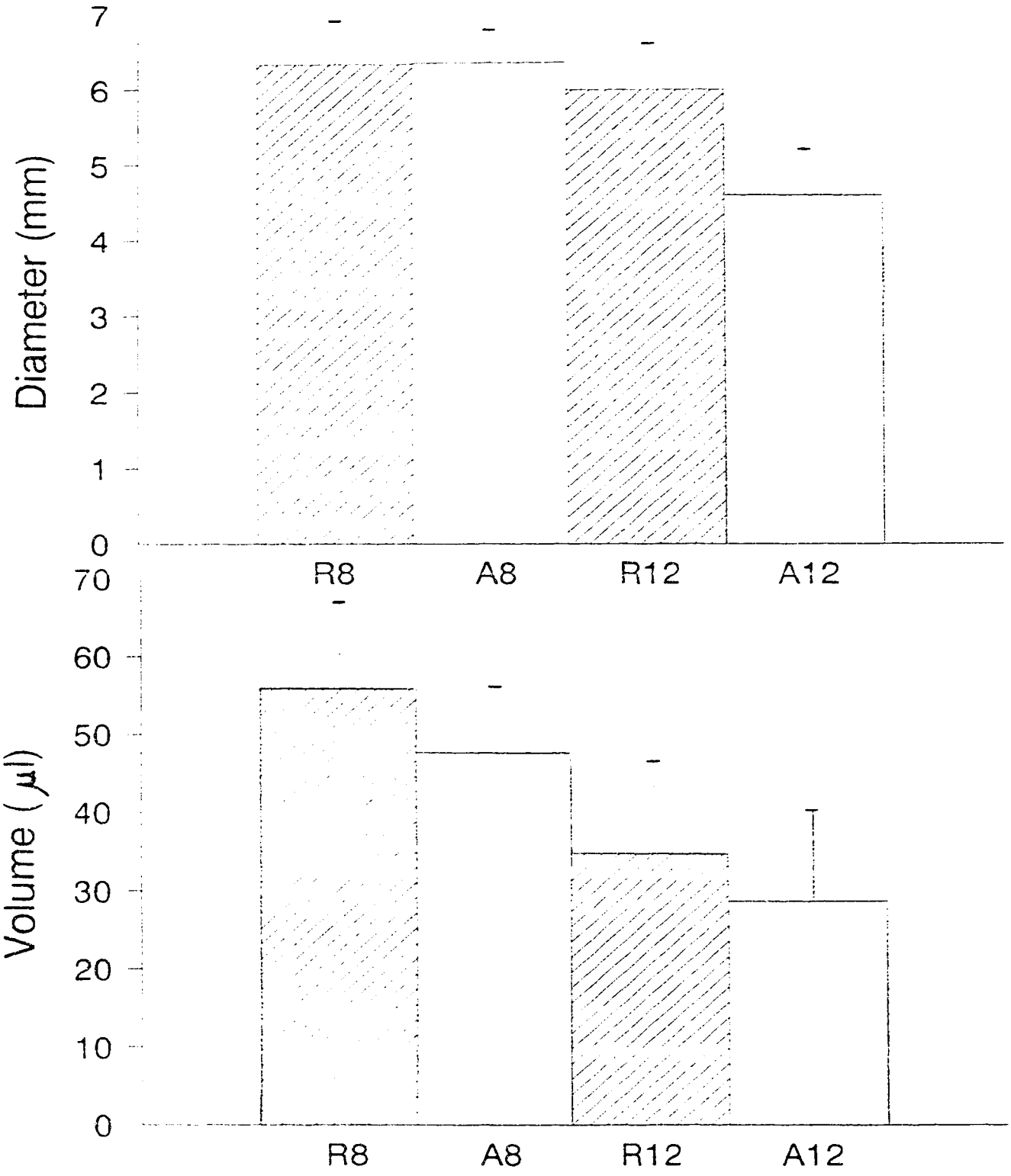

Diameter (mm)
7
6
5
4
3
2
1
0
R8
A8
R12
A12
Volume (μl)
70
60
50
40
30
20
10
0
R8
A8
R12
A12

Figure 3.3. Mean follicle estradiol concentrations and contents for four treatment groups. Treatment abbreviations are defined in the text.

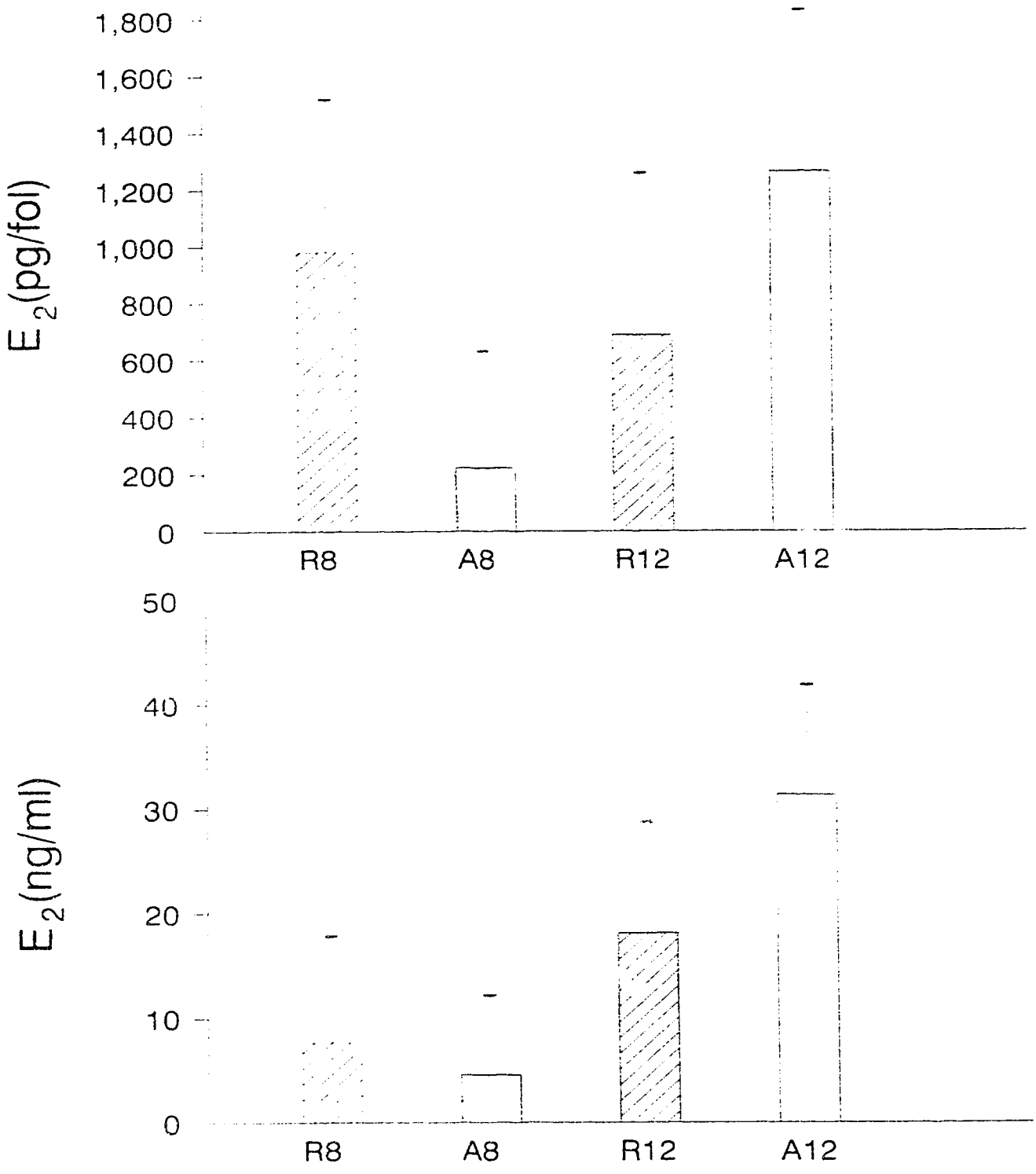

1,800
1,600
1,400
1,200
1,000
800
600
400
200
0
E_2(pg/fol)
R8
A8
R12
A12
50
40
30
20
10
0
E_2(ng/ml)
R8
A8
R12
A12

Figure 3.4. Autoradiograms of RNase protection of 50 μg of porcine ovarian total RNA from re-fed and restrict-fed gilts on d13 and d9 (3.4b), with controls **a - f** and **A - C** (3.4a and 3.4b): **a & C.** 30 μg human placenta total RNA, **b & B.** 250 pg synthetic IGF-1 mRNA preparation, **c.** 50 μg porcine hepatic poly A^- RNA, **d.** 50 μg porcine ovarian poly A^- RNA, **e.** 7,500 dpm unhybridized, undigested IGF-1 exon 3 riboprobe, **f.** 5 x 10^6 dpm unhybridized, digested IGF-1 exon 3 riboprobe, and **A.** 5 μg ovarian total RNA, undigested.

Control markers (M) (in bases) are indicated on right of the autoradiogram, estimated weights (in bases) of the protected fragment and the riboprobe are depicted to the left of the autoradiogram. Each lane represents individual gilt samples. The autoradiogram was exposed for 40 hours.

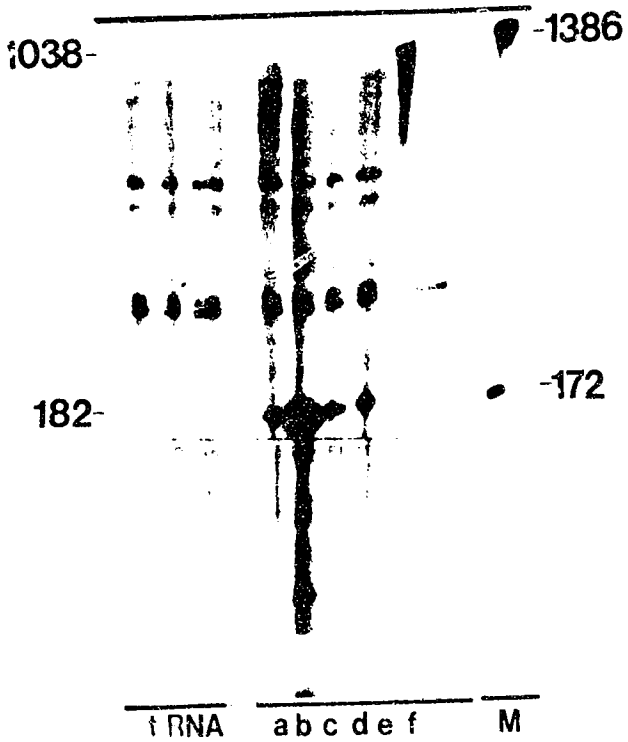


Figure 3.4 a.

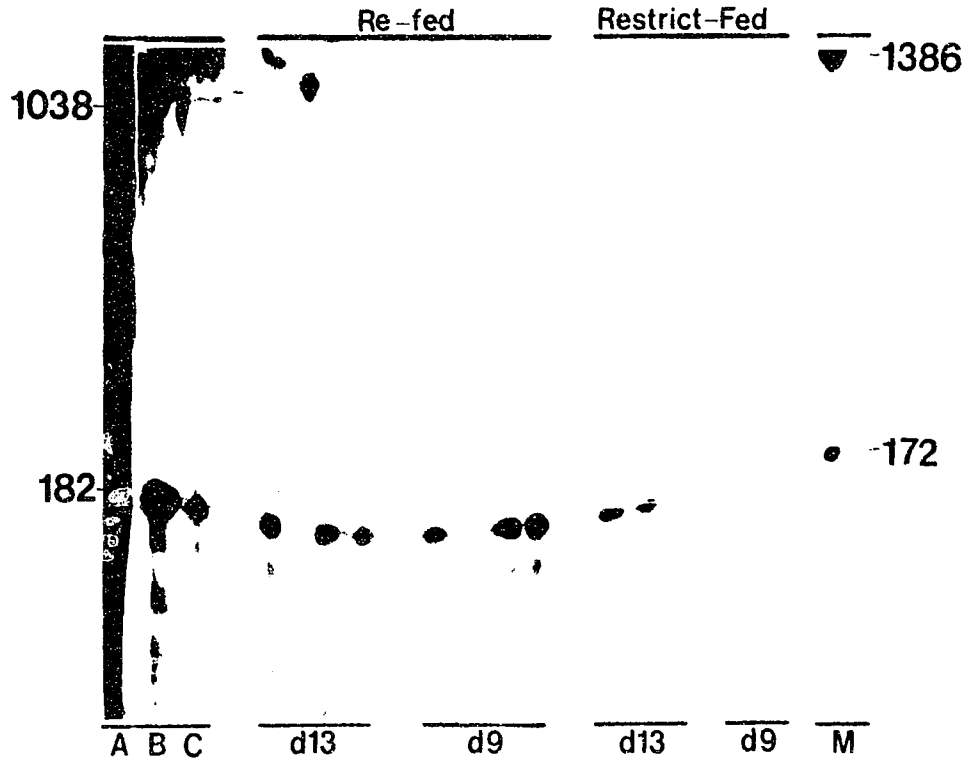
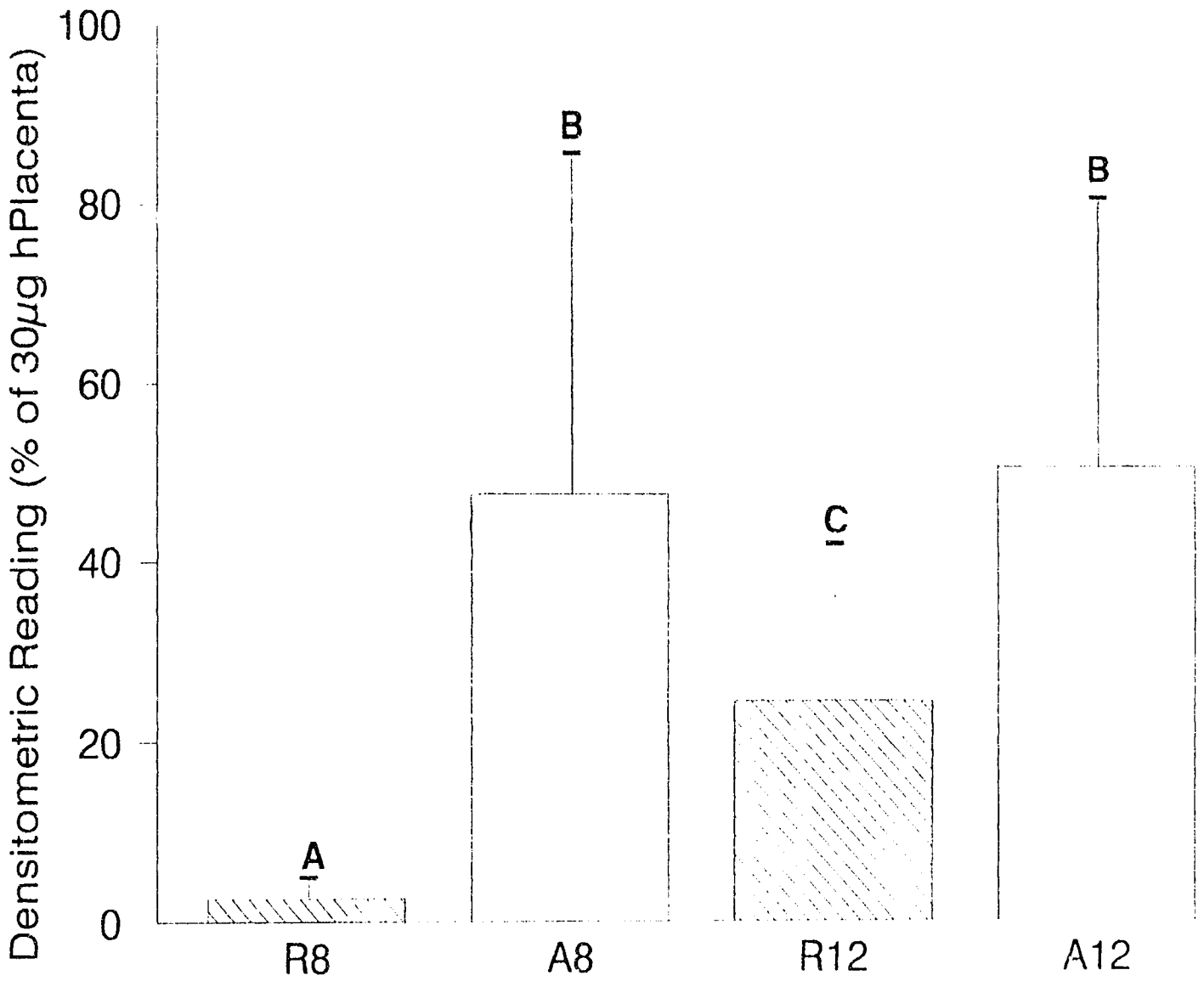


Figure 3.4b.

Figure 3.5. Mean abundance of IGF-1 mRNA detected in 50 μg of ovarian total RNA from four treatments. Treatment abbreviations are defined in the text. Bars with different superscripts were significantly different ($P<0.001$).


Densitometric Reading (% of 30μg hPlacenta)
100
80
60
40
20
0
A
B
C
B
R8
A8
R12
A12

expression ($P<0.005$), as was the covariate weight ($P<0.001$). No correlations be made between ovarian IGF-1 gene expression and follicle volume estradiol concentrations.

DISCUSSION

Despite the loss of 40% of the animals from this study, we did succeed in confirming the effects of re-feeding restrict-fed gilts on LH secretion as re-feeding gilts significantly elevated minimum levels of LH by d12 and trends indicated that all other characteristics of LH secretion were also elevated. The lack of differences between the two treatment groups on d8 were not surprising. Booth (1990a) failed to detect any increases in LH pulsatility until 6 hours after re-feeding. In the present experiment this would be equivalent to 15.00h, with only two hours of frequent sampling following this time. Considering the low numbers of animals successfully sampled and the short duration of this period, any differences would have to be dramatic in order to be statistically significant. In conclusion we can be confident that we have succeeded in establishing the restrict-fed/re-fed prepubertal gilt model under the University of Alberta conditions. This has since been confirmed more extensively by Cosgrove (1991).

On d8 there were no treatment effects on pre-prandial insulin, which was to be expected, as until the 09.00h feed following the pre-prandial period of sampling, all four groups of animals had been treated exactly the same. Prandial levels were not significantly elevated by re-feeding, however trends did indicate a slight increase in insulin in response to the feed-to-appetite. Post-prandial insulin was significantly elevated,

therefore suggesting that a single feed-to-appetite was able to elevate basal levels of insulin secretion.

There were very little effects of treatment on insulin secretion by d12 of the study. Preprandial levels of insulin were lower in the re-fed compared to the restrict-fed, which is perhaps surprising. Booth (1990a) and Cosgrove (1991) failed to detect differences in the pre-feeding period which was probably due to the fact that the re-fed animals were only full-fed at 16.00h for a one hour period following which they were subjected to an overnight fast. The overnight fast may have succeeded in promoting a marginally catabolic metabolic state similar to that of the restrict-fed gilts. Why pre-prandial insulin should be significantly lower in the re-fed animals is unclear. The answer may actually lie in the number of gilts replicates in each treatment.

The present study failed to reveal any effects of re-feeding restrict-fed gilts on circulating levels of growth hormone on either d8 or d12. This is in contrast to a study carried out by Buonomo and Baile (1991) to study the effects of 48 hours of fasting, followed by re-feeding on the metabolic status of barrows. They demonstrated that there was a decrease in growth hormone within two hours of re-feeding concomitant with increases in insulin and triiodothyronine (T_3). The results from the present study were more similar to those found by Booth (1990b) who failed to detect any changes in growth hormone in a subset of two gilts between d7 and d14. The results from Booth's study and the present study may be different from that of Buonomo and Baile due the differences in the treatment regimen: 48 hours of fasting may have a more severe effect on growth hormone release than seven days of maintenance feeding. The level

of nutrition may alter pituitary responses to hypothalamic regulatory factors such as growth hormone releasing factor (GRF), or 48 hour of fasting may reduce circulating IGF-1 concentrations thus partially removing the negative feedback effects of IGF-1 on growth hormone secretion (Guyda *et al.*, 1983).

On d8, there was no effect of re-feeding on plasma IGF-1 levels. This result agrees with the findings of Buonomo and Baile (1991), who failed to detect changes in IGF-1 until 24 hours after the return to *ad libitum* feeding following the 48 hour fast. Similarly there were no changes in IGF-1 levels on d12, which is not surprising considering the lack of treatment effects on plasma insulin or growth hormone.

The failure to achieve different levels of insulin or IGF-1 by d12 suggests that physiologically this experiment is not very representative of the effects of re-feeding restrict-fed gilts when compared to other studies. This was probably due to poor replication within treatments. The ovarian data supports this conclusion, as there appeared to be no indication of ovarian responses to re-feeding in either mean follicle diameter, volume or follicular estradiol on d9 or d13.

Evidence for expression of the IGF-1 gene was detected in all ovarian samples. The RNase protection assay succeeded in detecting a protected fragment corresponding to the exon 3 of the transcribed IGF-1 mRNA. Re-feeding of restrict-fed gilts was found to significantly increase the amount of IGF-1 mRNA detected on both d8 and d12. This finding suggests that ovarian IGF-1 gene expression is sensitive to feeding level, and that the response of this gene in the ovary is similar to that of hepatic IGF-1 gene expression in the rat (Elmer and Schalch, 1987).

It is not surprising that IGF-1 gene expression may be affected on d8 while circulating levels were not, as this suggests that there may be a lag period between gene transcription and production of the gene product. However, the lack of changes in circulating IGF-1 levels observed on d12 might suggest that hepatic IGF-1 gene expression was not effected by re-feeding in the same way as ovarian IGF-1 gene expression, assuming that the majority of circulating IGF-1 is of hepatic origin. Due to the lack of a suitable number of replicates, this conclusion must be very tentative, as the observed differences may be due to inter-animal variation. In addition, the methodology for detecting the IGF-1 mRNA is still in its preliminary stages of development.

To summarize, it is difficult to make any firm conclusions from the results of this study due to the lack of replicates. However, we can be confident that the restrict-fed/re-fed prepubertal gilt model used with locally available animals produces similar affects on gonadotropin release as observed in previous studies (Booth, 1990a and Cosgrove, 1991).

Evidence for the expression of the IGF-1 gene was detected in the ovarian tissue of individual gilts, therefore demonstrating the enormous potential for applying RNase Protection assay methodology to answering physiological questions at a molecular level in individual animals. This procedure is particularly useful when only small amounts of tissue are available and when the level of gene expression is low. In addition, ovarian gene expression was found to be regulated by feeding level, suggesting that local production of IGF-1 in the ovary may be regulated directly by feeding level and as a consequence of this, supports the hypothesis that IGF-1 might be a link between metabolic status and ovarian

development.

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CHAPTER 4

Effects of Restrict-Feeding/Re-Feeding on Metabolic Status, Ovarian Follicular Development and on Ovarian and Hepatic insulin-Like Growth Factor 1 (IGF-1) Gene Expression and Production in the Prepubertal Gilt.

INTRODUCTION

The study carried out in Chapter 2 established the validity of using the seven day restrict-fed prepubertal gilt model in our laboratory for further studies of nutrition-reproduction interactions. However, due to low numbers of replicates, that experiment was not representative of the effects of restrict-feeding followed by re-feeding on the metabolic and reproductive status of the gilts. For this reason the following more extensive study was designed. As there is unequivocal evidence demonstrating the central effect of restrict-feeding/re-feeding on LH secretion (Booth, 1990a; Cosgrove, 1991; results presented in Chapter 3), no estimation of LH secretion was carried out.

Data from the previous study and that of Cosgrove *et al.* (1991) and Booth (1990) indicated that there were few effects of re-feeding on ovarian function during the first 24 hours, but that differences were observed on days 12 and 13. The present study was therefore simplified from that in Chapter 3, to only examine the metabolic and reproductive status of the animals following six days of re-feeding.

Another development from the previous study was the strategy adopted to evaluate circulating growth hormone levels. Kirkwood *et al.* (1987) demonstrated that there may be a night time elevation in growth hormone secretion in growing swine. Therefore if there were to be any effects of re-feeding restrict-fed gilts on growth hormone secretion, they may be more easily detected during the night. In addition, studies by Thomas *et al.* (1990), using the ovariectomized ewe, indicated that restrict-feeding may induce pulsatile secretion of growth hormone. Growth hormone secretion was therefore evaluated using a night time sampling window (02.00-06.00 on

d14) and samples were taken at 10 min intervals in order to allow the characterization of pulsatile secretion should it exist.

In addition to obtaining tissue for detecting the level of IGF-1 gene expression in ovarian tissue, as in the previous study, samples of hepatic tissue were also analysed. Hepatic IGF-1 gene expression has been shown to be sensitive to feeding level in the rat: fasting reduces circulating levels of IGF-1 and the expression of the IGF-1 gene in hepatic tissue (Elmer and Schalch, 1987). Analysis of the IGF-1 gene expression in both ovarian and hepatic tissue collected from restrict-fed and re-fed gilts would therefore allow a comparison of the changes in IGF-1 gene expression to feeding level in the different tissues and may provide evidence for dependent or independent responses.

The main objectives of the following experiment were therefore two-fold: Firstly to confirm the effects of restrict-feeding followed by re-feeding on the metabolic status of the gilt and therefore determine whether decreased insulin levels do indeed uncouple the GH - IGF-1 axis; secondly to determine whether ovarian IGF-1 gene expression responds to feeding level in the same manner as the hepatic IGF-1 gene expression.

MATERIALS AND METHODS

Animals

Prepubertal gilts (Camborough x Canabrid; PIC Canada Ltd.) were obtained from the University of Alberta swine unit. The animals were selected from a group of gilts fed a commercial grower ration *ad libitum*. The average weight of the gilts was 36.2 $\pm$ 5.1 Kg at the time of selection. All gilts were then weighed at weekly intervals to determine

growth rates until a target weight of 70 Kg was achieved. Due to housing restrictions, two groups of gilts were selected two weeks apart, resulting in a replicated experimental design. Overall, twenty-two gilts from seven litters were selected for the study on the basis of comparable growth rates, with four litters in the first replicate (n=10) and three in the second (n=12). One week prior to the start of the study, all animals were fed to appetite twice daily at 09.00h and 16.00h. One day prior to the start of the experimental study, the gilts were moved into individual holding crates within visual and aural contact of each other and received water *ad libitum*. An additional feature of the replicated design was that the sampling protocols were carried out in two different barns: the first group was housed in a newly constructed experimental animal housing facility (designated as Barn 1), while the second group was housed in an older barn used previously for similar intensive studies (designated as Barn 2).

On d1 of the study, all gilts were placed on a 7 day restrict-feeding regimen, whereby they were fed a maintenance level of feed twice daily at 09.00h and 17.00h (approximately 30% of individual *ad libitum* feed intake). The level of feeding was calculated on the basis of NRC (1988) maintenance requirements. On d8 the animals were allocated within litter to one of two treatments, balanced for at least one litter mate in each treatment (ie. employing a split plot experimental design) and to achieve a mean body weight across treatments of 70.7 $\pm$ 4.7 Kg. Restrict-fed gilts remained on a maintenance level of feeding from d8 to d14, while re-fed gilts were fed to-appetite from d8 to d14 for two one hour periods starting at 09.00 and 16.00h. Weekly live weight measurements are

illustrated in Figure 4.1.

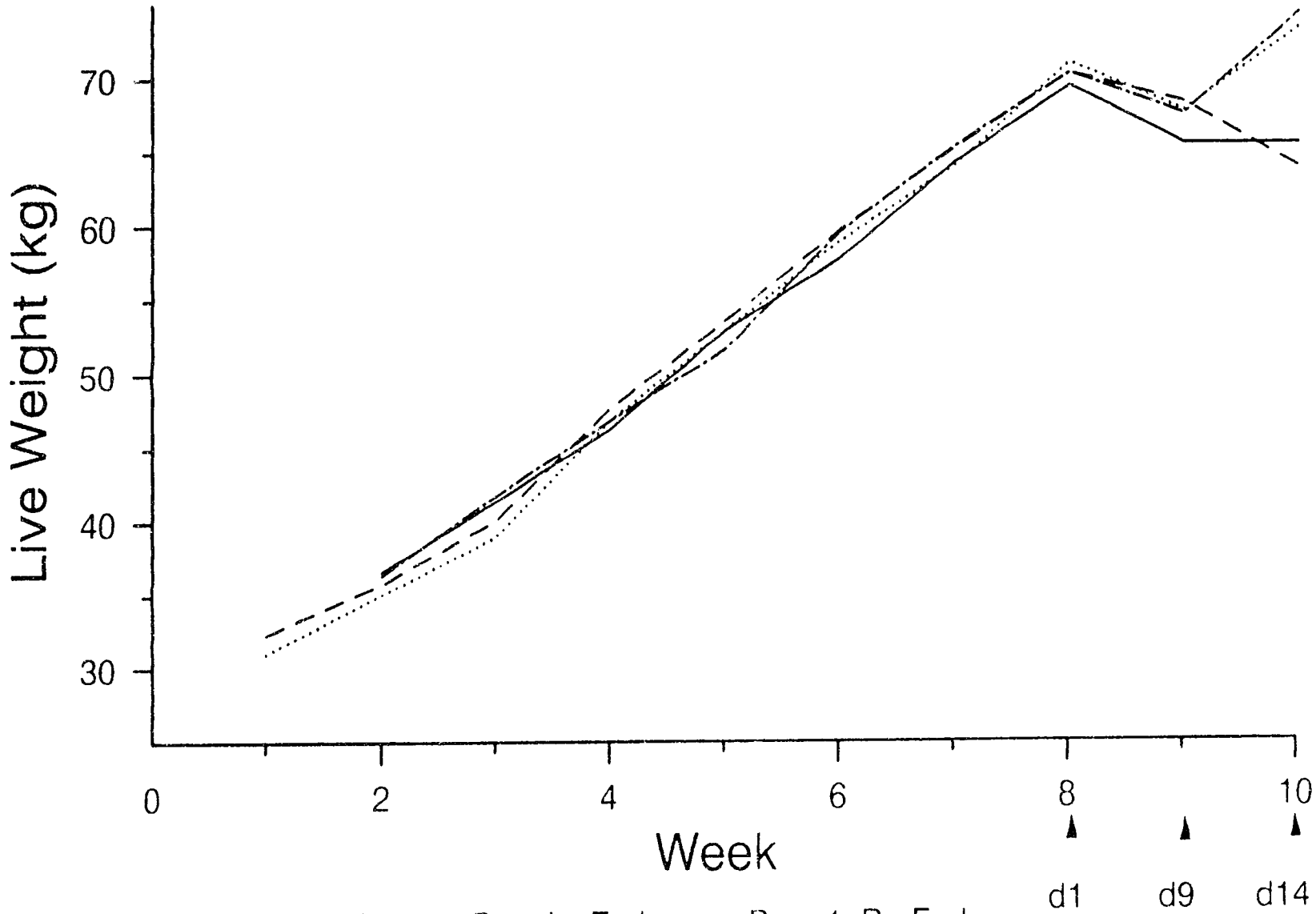
Blood Sampling

An indwelling jugular cannula was surgically placed under general anaesthesia via the superficial cephalic vein, under aseptic conditions on day 9 of the study to facilitate stress-free withdrawal of blood. On d13, 10 ml blood samples were taken at half hourly intervals from 07.00h until 11.00h and then hourly until 16.00h. The blood samples were collected into heparinized tubes and plasma obtained by centrifugation at 2000 *g* for 20 min immediately after collection. The plasma was then stored in two aliquots per sample at -20°C until analysis for plasma insulin and IGF-1. On d14, 3 ml blood samples were taken at 10 min intervals from 02.00h to 06.00h for analysis of GH secretion. At 06.00h a 10 ml blood sample was taken to determine insulin and IGF-1 status prior to shipping and slaughter.

Collection Of Ovarian Tissue

All animals were slaughtered at a local packing plant at approximately 09.00h on d14 to facilitate the collection of the ovaries and a 10 g liver sample. One ovary (chosen at random) was examined to determine follicular dimensions. The diameters of the ten largest follicles were measured directly on the surface of the ovary, while their follicle volumes were determined by aspiration of the follicular fluid using either a 50 μl or a 200 μl Hamilton syringe. The follicular fluid was retained for estradiol and IGF-1 analysis by adding it to 250 μl of phosphate buffered saline (PBS).

Figure 4.1. Weekly average live weights of gilts in each treatment and barn.


Live Weight (kg)
70
60
50
40
30
0
2
4
6
8
10
Week
d1
d9
d14
Barn 1, Restrict Fed
Barn 1, Re-Fed
Barn 2, Restrict Fed
Barn 2, Re-fed

The remaining ovary and liver sample were snap frozen in liquid nitrogen and stored at -70°C until analysis for IGF-1 mRNA.

Radioimmunoassays

Insulin

Plasma insulin concentrations were measured in a single assay using the homologous double-antibody radioimmunoassay previously described by de Boer and Kennelly (1989), with modifications as described by Cosgrove (1991). The detection limit of the assay was approximately 0.005 ng/tube, at a binding of 94.8% (defined as mean total bound minus two standard deviations from mean total bound). The intra-assay coefficient of variance for this assay was 10.0%.

Growth Hormone

Growth hormone plasma concentrations were determined using the double-antibody radioimmunoassay procedure as described by Marple and Aberle (1972), with the following modifications as described by de Passillé *et al.* (*in press*). The detection limit of the assay was 0.150 ng/tube, at a binding of 86.7% (defined as mean total bound minus two standard deviations from the mean total bound). The intra- and inter-assay coefficients of variance were 5.79% and 11.19% respectively.

Insulin-Like Growth Factor 1

Plasma and follicular levels of IGF-1 were quantified in a single radioimmunoassay following acid-ethanol extraction to remove binding proteins. The acid-ethanol extraction procedure used was that described by

Booth (1990b), with the following modifications; A ratio of 3000 μl acid-ethanol:100μl of sample was used, followed by a 20 hour incubation at room temperature. The extracted protein was then precipitated by centrifugation at 3000g at 4°C, thus leaving the unbound IGF-1 in the supernatant. The extraction efficiency of this procedure was estimated to be 100%, based on estimates of cold recoveries of hIGF-1 added to a standard pool of plasma. The radioimmunoassay employed was that described by Glimm *et al.* (1990), with modifications as described by Cosgrove (1991). The detection limit of the assay was 0.002 ng/tube, at a binding of 93.7% (defined as mean total bound minus two standard deviations from the mean total bound). The intra-assay coefficient of variance for this assay was 4.74%.

Estradiol

Follicular fluid estradiol concentrations were estimated in a single radioimmunoassay using the same procedure as that described in Chapter 3. The detection limit of the assay was 1.56 pg/tube, at a binding of 89.2% (defined as mean total bound minus two standard deviations from the mean total bound). The intra-assay coefficient of variance for this assay was 6.83%.

RNA Analysis

Frozen follicular tissue was harvested from the ovaries of all 22 gilts by dissection with a sterile scalpel blade. The follicles were harvested from each ovary in descending order of size until 0.5 g of tissue was collected. Total RNA was isolated from each ovarian sample, as well as a 0.5 g sample of liver from the same animal, using the guanidine

isothiocyanate/caesium chloride procedure as described in Chapter 2. All samples from each animal were then subjected to the RNase protection assay procedure described in the same chapter. Aliquots of 50 μg of sample total RNA were hybridized against 5 X 10^6 dpm of single-stranded antisense hIGF-1 Exon 3 riboprobe. As all 44 RNA samples (22 ovarian and 22 hepatic) could not be analysed in the same RNase protection assay, a 30 μg aliquot of human placenta total RNA (kindly provided by D.R.Glimm, using identical total RNA isolation techniques) was included on each gel to act as a standard control across assays. The controls utilized to determine the authenticity of the protected fragment are listed in Figure 4.9.

Statistical Analysis

Complete blood samples were only collected from 18 animals as cannula patency failed in 2 gilts in Barn 1 (both re-fed) and 2 gilts in Barn 2 (1 restrict-fed and 1 re-fed). Their ovaries and liver sample were collected, however, and included in the appropriate analyses. Follicular estradiol was estimated in all follicles, however, due to insufficient follicular fluid remaining after analysis for estradiol, IGF-1 levels could not be measured in all follicles. Therefore, in order to allow a fair comparison of follicular IGF-1 concentration in all gilts, only the mean of the five largest follicles was used.

Due to the differing environments within the two barns, the effect of treatment on hormone secretion, ovarian development and IGF-1 gene expression was analysed using a 2-way split-plot analysis of variance, fitting feeding level and barn as the main effects and animal live weight on d0 as a covariate. An error term for barn of barn(litter) was used to

test the effect of barn as, although treatments were equally distributed across the two barns, litters were not due to the two week difference in age between the two groups For statistical analysis, litters with more than one littermate pair were considered as representing separate litters with pairings based on similarity of weights at the onset of the study. This increased the of degrees of freedom for litter to increase from 6 to 10. The general linear model procedure (PROC GLM) within the SAS statistical analysis computing software package was used for all statistical analyses.

Plasma insulin was estimated at half hourly intervals from 07.00-11.00h to give a detailed profile of the prandial response of insulin secretion to feeding at 09.00h. Insulin was then measured at hourly intervals until 16.00h to give an indication of basal post-prandial levels of secretion. Treatment effects on insulin secretion were analysed during three different time periods: 07.00-09.00h (pre-prandial secretion), 09.00-11.00h (prandial secretion) and 11.00-16.00h (post-prandial secretion). Night time secretion of GH was characterized using a sliding window procedure as used by Shaw and Foxcroft (1985). A window width of two hours was used, as examination of night time profiles for GH revealed surges of GH secretion with durations of approximately two hours. This procedure provided mean, maximum and minimum characteristics of GH secretion. A modification of this procedure estimated the area under the profile (profile area), therefore giving an indication of the total amount of GH circulating in the four hour period.

Mean follicular parameters were analysed using the 2-way split-plot analysis of variance, as described above. In addition, individual follicle

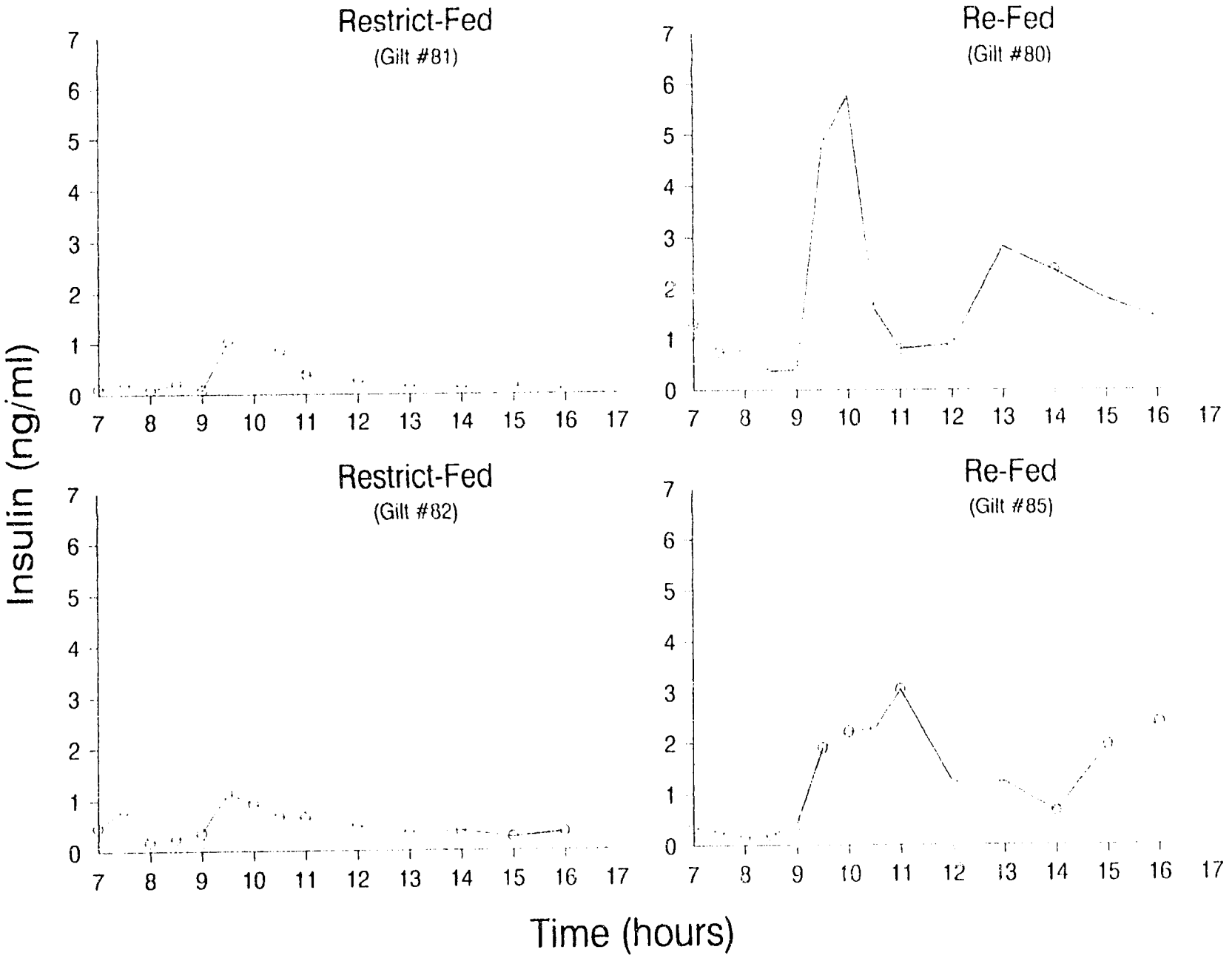
volumes, for each gilt, were regressed against diameter, utilizing the regression procedure (PROC REG) within the SAS statistical package. The resulting coefficients and intercepts for each gilt were then analysed using the 2-way split-plot analysis of variance as before. Similar comparisons were made for individual follicular estradiol content (pg/follicle) and concentration (ng/ml) against follicle volume, and for individual follicular IGF-1 content (ng/follicle) and concentration (ng/ml). The regression analysis for follicular IGF-1 concentrations and contents used all available data and not only the data from the five largest follicles.

Follicle IGF-1 gene expression was regressed against mean follicle volume, mean follicular fluid IGF-1 concentration (ng/ml), mean plasma IGF-1, preprandial, prandial and post-prandial plasma insulin, and plasma GH. The general linear model for analysis of variance was used for this analysis, with follicle volume, follicular IGF-1, plasma IGF-1, plasma insulin and plasma GH as covariates. The interactions between each covariate and treatment were also analysed.

RESULTS

The insulin profiles from four littermates (two restrict-fed and two re-fed) are illustrated in Figure 4.2. Six days of re-feeding restrict-fed gilts significantly increased pre-prandial ($P<0.05$) and post-prandial ($P<0.01$) insulin secretion. No significant increases in prandial insulin levels were detected, although least square means of prandial secretion suggest that there are distinct trends for re-feeding to increase the insulin response to feeding. There were no barn effects for pre- and post-

Figure 4.2. Plasma insulin profiles from four littermates, two restricted and two re-fed. Profiles represent insulin secretion during three time periods: pre-prandial (07.00 - 09.00h), prandial (09.00 - 11.00h) and post-prandial (11.00 - 16.00h).


Restrict-Fed
(Gilt #81)
Re-Fed
(Gilt #80)
Restrict-Fed
(Gilt #82)
Re-Fed
(Gilt #85)
Insulin (ng/ml)
Time (hours)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

prandial insulin secretion, however there were significant effects of barn on prandial secretion ($P<0.05$), which may offer an explanation why treatment was not significant despite apparent changes in mean prandial insulin. All insulin data is presented in Table 4.1.

The GH profiles from four littermates (two restrict-fed and two re-fed) are illustrated in Figure 4.3. Night time secretion of GH was not affected by treatment or barn. There were no changes in mean, maximum or minimum plasma GH or in profile area as illustrated in Table 4.2. There were highly significant effects on plasma IGF-1 levels ($P<0.005$): realimentation induced a 60% increase in circulating levels. Housing also had an effect on circulating IGF-1 ($P<0.05$), however there were no barn x treatment interactions, thus indicating a uniform effect of re-feeding across both barns (illustrated in Figure 4.4.).

Plasma levels of insulin and IGF-1 were estimated in a single sample at 06.00h on d14 to determine their status prior to slaughter. Their values were found to be comparable to mean pre-prandial insulin levels and mean IGF-1 on d13, respectively.

In this study there were associated effects of re-feeding gilts on ovarian development. Mean follicular diameter was increased by re-feeding ($P<0.05$), as was volume ($P<0.01$). Mean follicular fluid estradiol and IGF-1 levels were not however affected by treatment. Follicular parameter data is presented in Table 4.3.

Individual follicle volumes were regressed against individual follicle diameters for each treatment to give an indication whether re-feeding had effects on the ratio of follicle cell content to antral fluid. Regression analysis of these particular data revealed significant linear

Table 4.1 The Effect of Re-feeding Restrict-Fed Gilts on Insulin Levels.

	Restrict-Fed	Re-Fed
Pre-prandial Insulin (ng/ml)	0.363 ± 0.168	1.075 ± 0.174*
Prandial Insulin (ng/ml)	1.601 ± 0.362	2.641 ± 0.374
Post-prandial Insulin (ng/ml)	0.454 ± 0.175	1.536 ± 0.181**

Values are Least-square Means ± S.E.M.
* $P<0.05$, ** $P<0.01$
compared with restrict-fed group

Figure 4.3. Plasma GH profiles from four littermates, two restrict-fed and two re-fed. Samples were taken at 10 min intervals from 02.00 until 06.00 on d14.

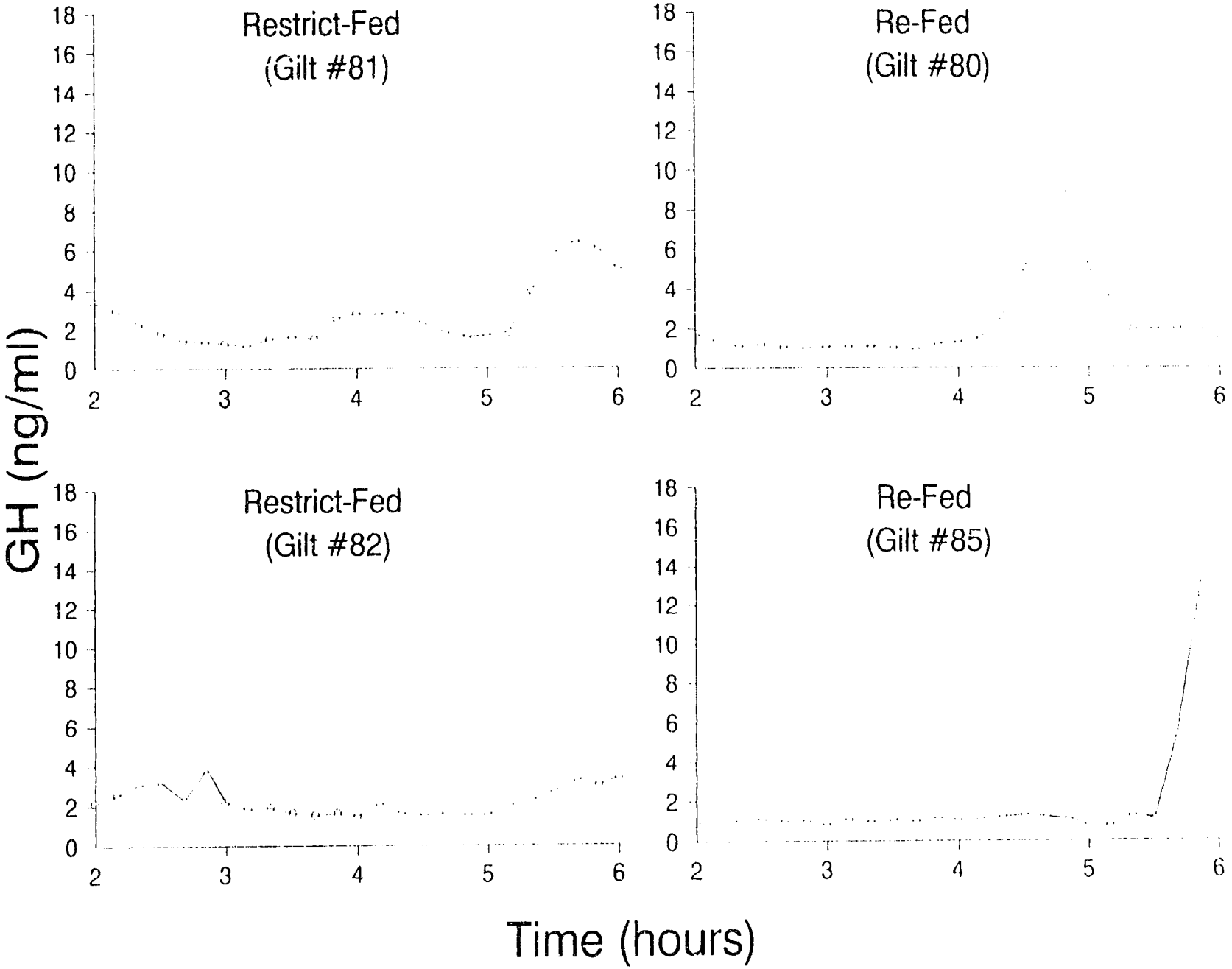

Restrict-Fed
(Gilt #81)
Re-Fed
(Gilt #80)
Restrict-Fed
(Gilt #82)
Re-Fed
(Gilt #85)
GH (ng/ml)
Time (hours)
0
2
4
6
8
10
12
14
16
18
2
3
4
5
6

Table 4.2 The Effect of Re-Feeding Restrict-Fed Gilts on Plasma GH and IGF-1 Levels.

	Restrict-Fed	Re-Fed
Mean GH (ng/ml)	2.016 ± 0.373	1.806 ± 0.386
Maximum GH (ng/ml)	3.322 ± 0.636	3.546 ± 0.657
Minimum GH (ng/ml)	1.087 ± 0.187	0.972 ± 0.193
GH Profile Area (ng/ml)	4.091 ± 1.138	4.091 ± 1.176
Mean IGF-1 (ng/ml)	75.912 ± 6.213	120.483 ± 6.42*

Values are Least-square Means ± S.E.M.
* $P<0.005$
compared with restrict-fed group

Figure 4.4. Mean plasma IGF-1 concentrations in restrict-fed and re-fed gilts in each barn.

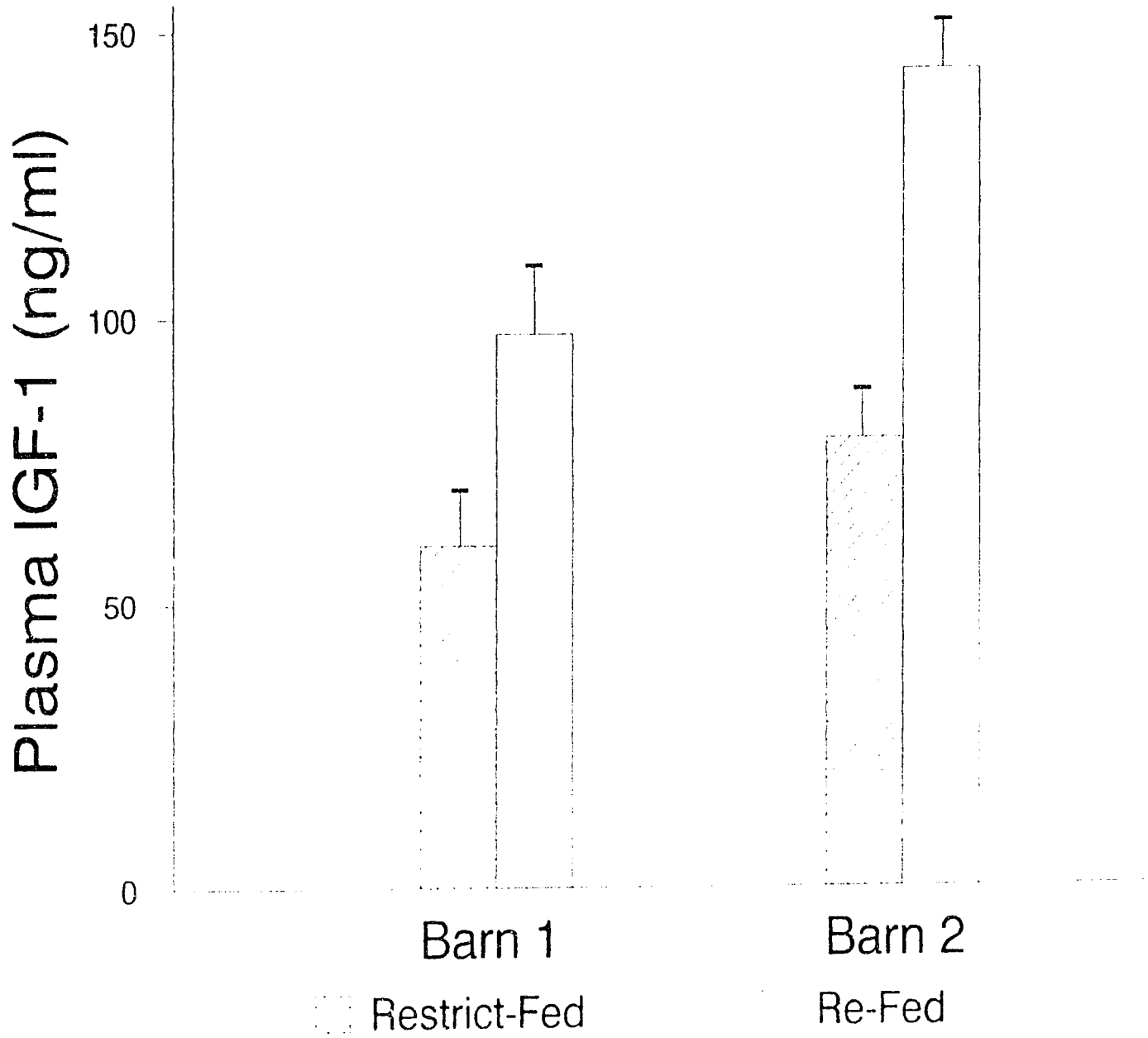

Plasma IGF-1 (ng/ml)
150
100
50
0
Barn 1
Barn 2
Restrict-Fed
Re-Fed

Table 4.3 The Effect of Re-Feeding Restrict-Fed Gilts on Follicular Diameter and Volume, and Estradiol and IGF-1 Concentration.

	Restrict-Fed	Re-Fed
Diameter (mm)	3.634 ± 0.333	5.122 ± 0.333*
Volume (μl)	16.916 ± 3.493	34.383 ± 3.487**
Estradiol (ng/ml)	1.956 ± 0.592	2.266 ± 0.591
IGF-1 (ng/ml)	192.490 ± 14.736	199.565 ± 14.816

Values are Least-square Means ± S.E.M.
* $P<0.05$, ** $P<0.01$, compared with restrict-fed group.

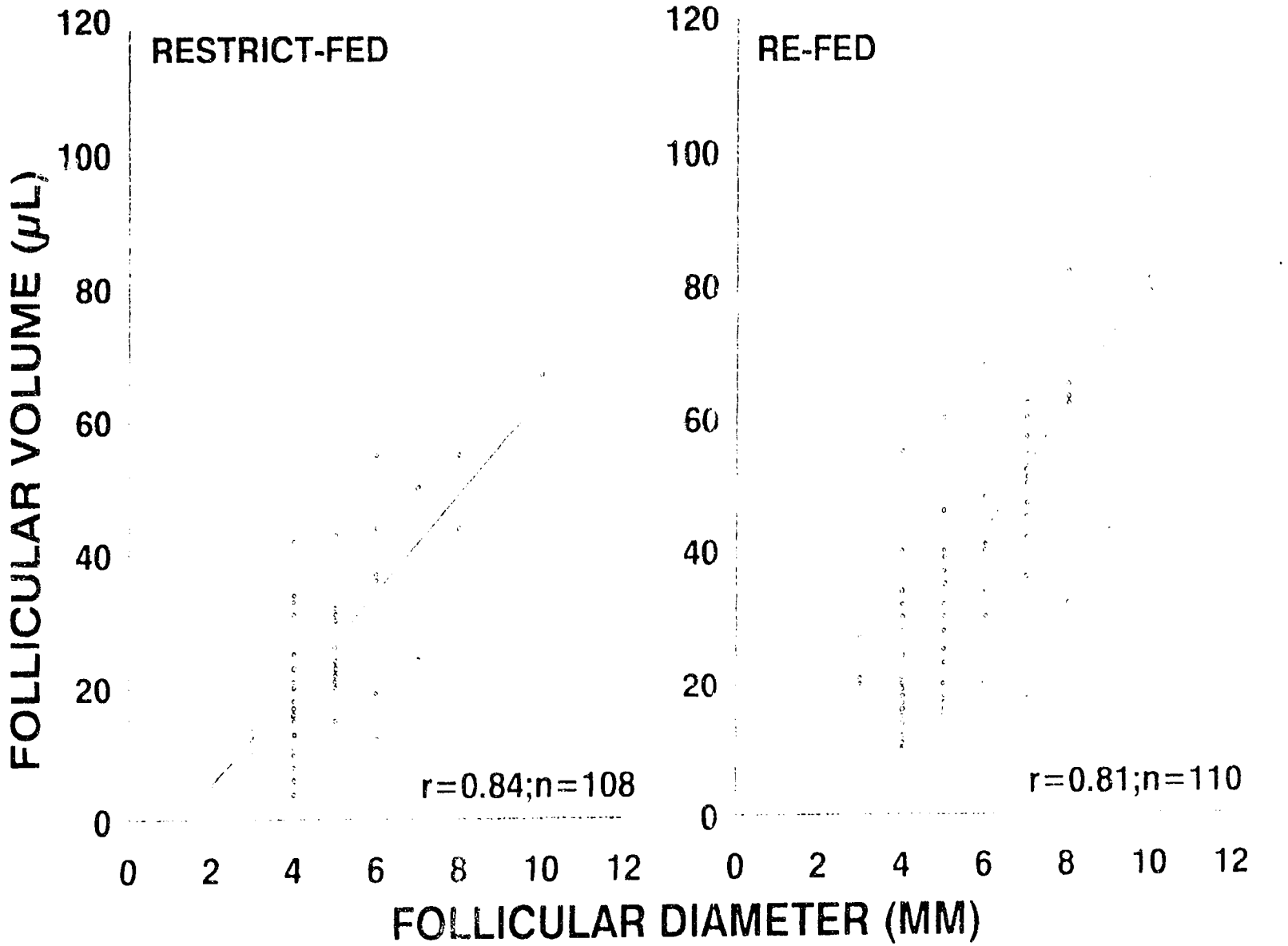
relationships between follicle volume and diameter in both restrict-fed ($P<0.001$) and re-fed gilts ($P<0.001$) (Figure 4.5.). Analysis of the intercepts and slopes for each animal did not reveal any effect of treatment (or barn) on the relationship between follicle diameter and volume.

Individual follicular estradiol concentrations and content were also regressed against individual follicle volume. Regression analysis revealed significant linear relationships between follicular estradiol (concentration and content) and volume in both restrict-fed ($P<0.001$) and re-fed gilts ($P<0.001$), as depicted in Figures 4.6. and 4.7. Analysis of the intercepts and slopes for each animal did not indicate any effects of treatment or barn on this relationship.

Follicular IGF-1 concentrations and contents for individual follicles were regressed against individual follicle volumes. Regression analysis revealed a significant inverse relationship between follicle IGF-1 concentration and volume for both restrict-fed ($P<0.001$) and re-fed gilts ($P<0.001$), as illustrated in Figure 4.8. However, the relationship between follicular IGF-1 content and volume was found to be linear (depicted in Figure 4.9.) for both restrict-fed ($P<0.001$) and re-fed gilts ($P<0.001$). Analysis of the intercepts and slopes for both characteristics of follicular IGF-1 revealed significant effects of treatment in that re-feeding significantly increased the intercepts for both follicle IGF-1 concentration and content. There were, however, no effects of re-feeding on the gradients for either concentration or content, and there was no effect of barn on any characteristic of follicular IGF-1.

Gene expression for IGF-1 was detected in follicular tissue from all

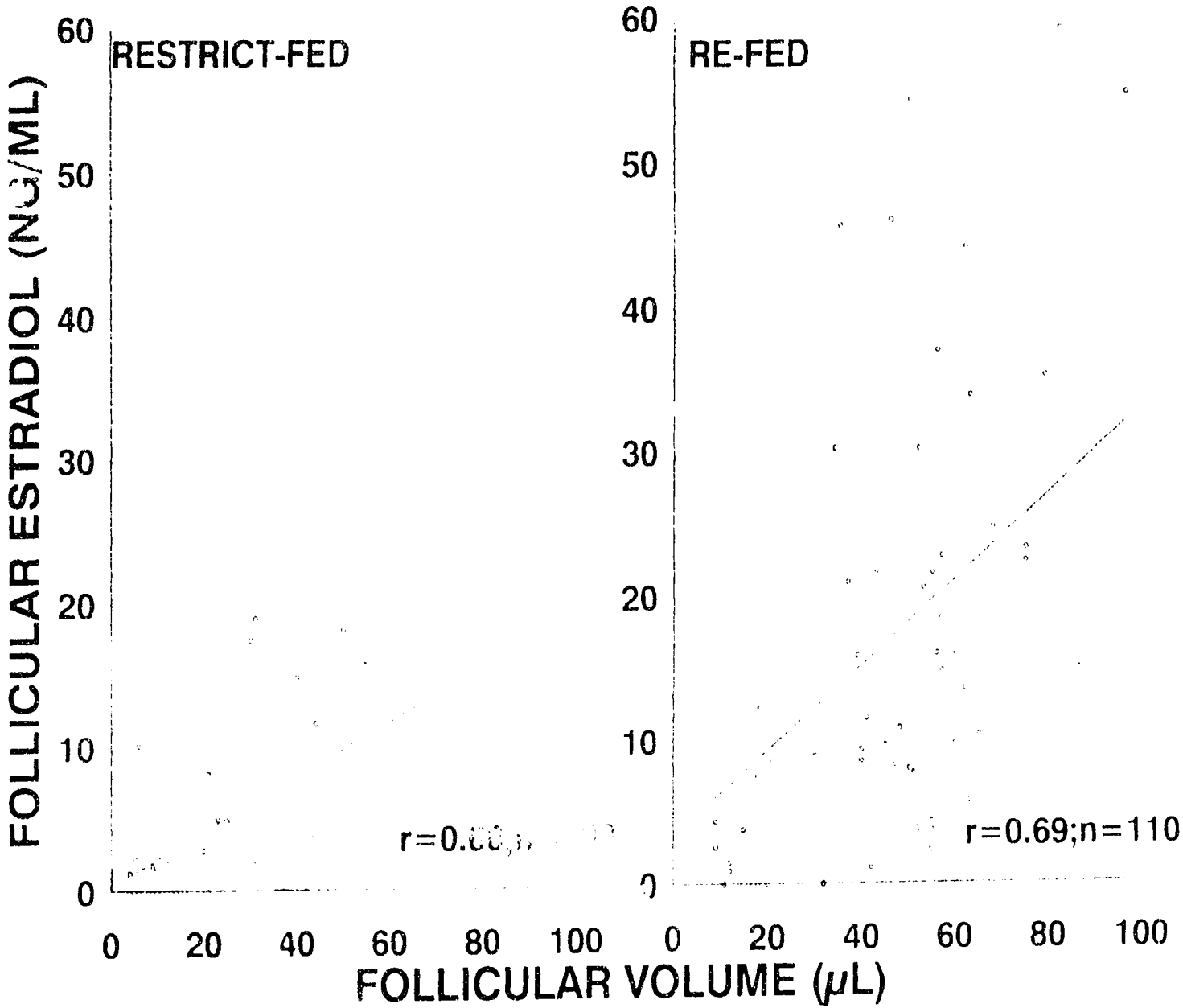
Figure 4.5. Linear regression of individual follicle volume against follicle diameter in restrict-fed ($P<0.001$) and re-fed gilts ($P<0.001$). Circles denote individual data, while the solid lines denote fitted line. Fitted lines were not significantly different.



RESTRICT-FED FOLLICULAR VOLUME = 7.312(FOLLICULAR DIAMETER) - 9.474

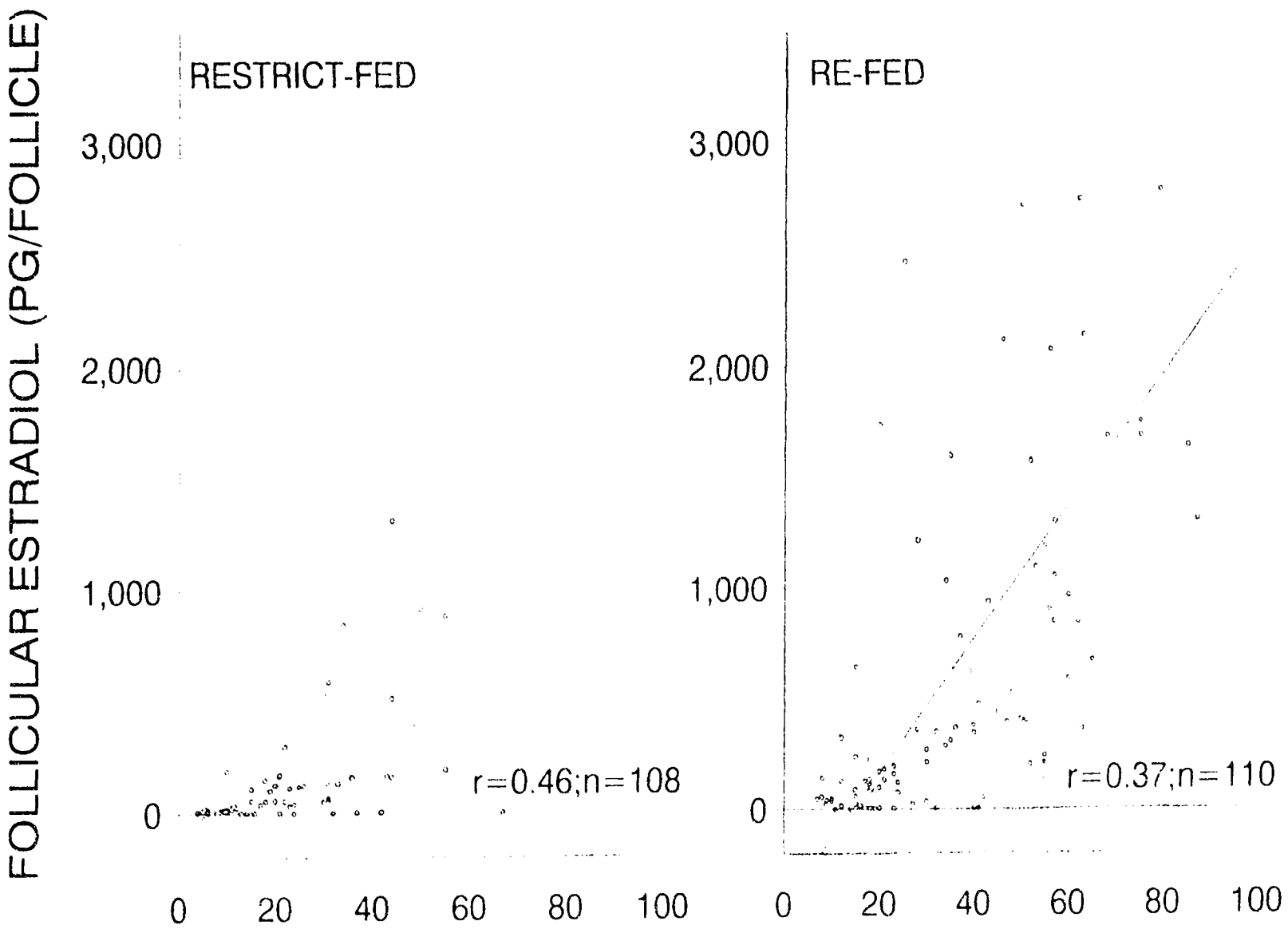
RE-FED FOLLICULAR VOLUME = 9.683(FOLLICULAR DIAMETER) - 15.076

Figure 4.6. Linear regression of individual follicle estradiol concentration against follicle volume in restrict-fed ($P<0.001$) and re-fed gilts ($P<0.001$). Circles denote individual data, while the solid lines denote fitted line. Fitted lines were not significantly different.



RESTRICT-FED FOLLICULAR E2 = 0.190(FOLLICULAR VOLUME) + 0.295
RE-FED FOLLICULAR E2 = 0.299(FOLLICULAR VOLUME) + 3.308

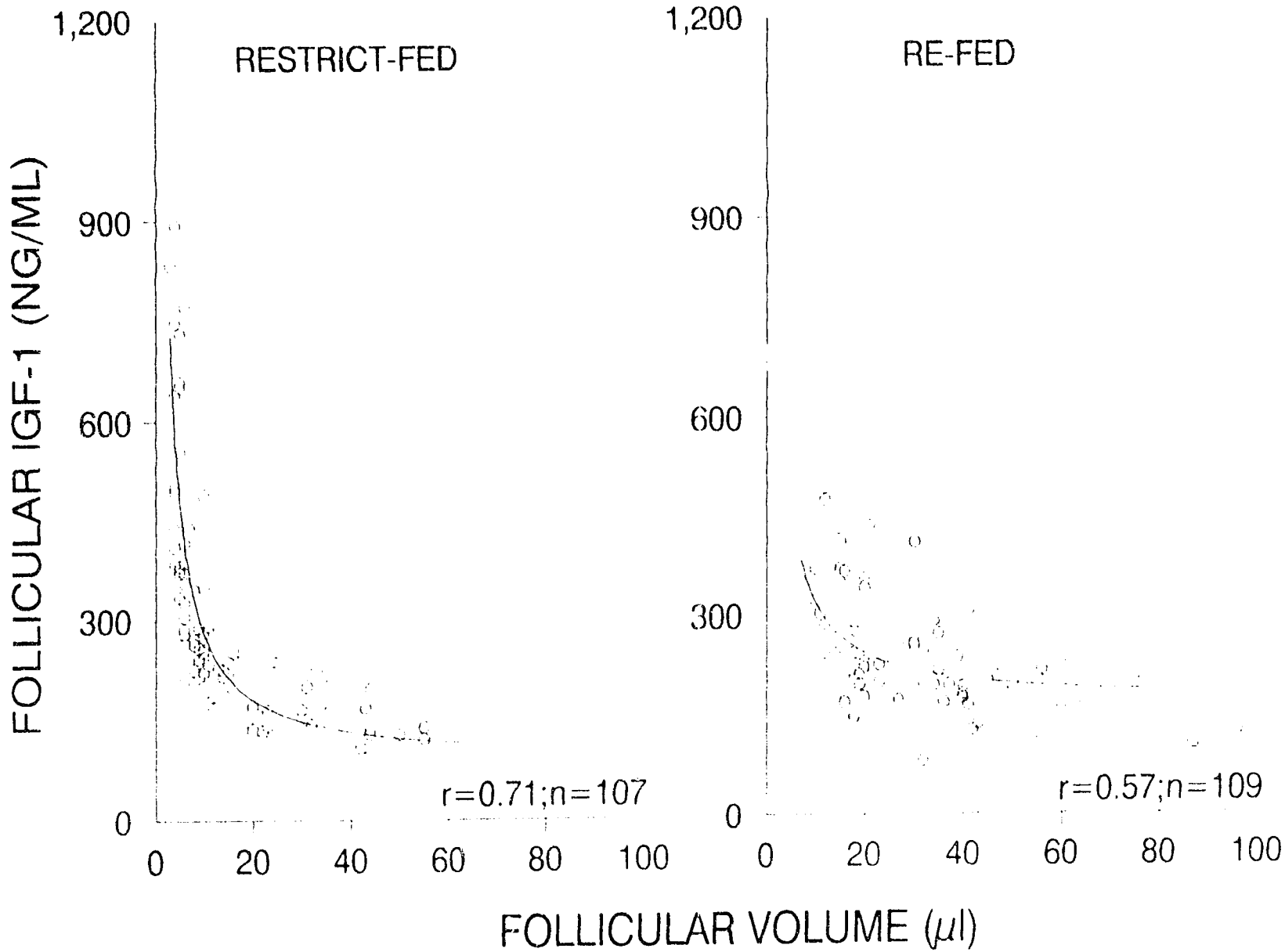
Figure 4.7. Linear regression of individual follicle estradiol content against follicle volume in restrict-fed ($P<0.001$) and re-fed gilts ($P<0.001$). Circles denote individual data, while the solid lines denote fitted line. Fitted lines were not significantly different.



RESTRICT-FED FOLLICULAR E2 = 9.476(FOLLICULAR VOLUME) - 66.267

RE-FED FOLLICULAR E2 = 30.085(FOLLICULAR VOLUME) - 434.653

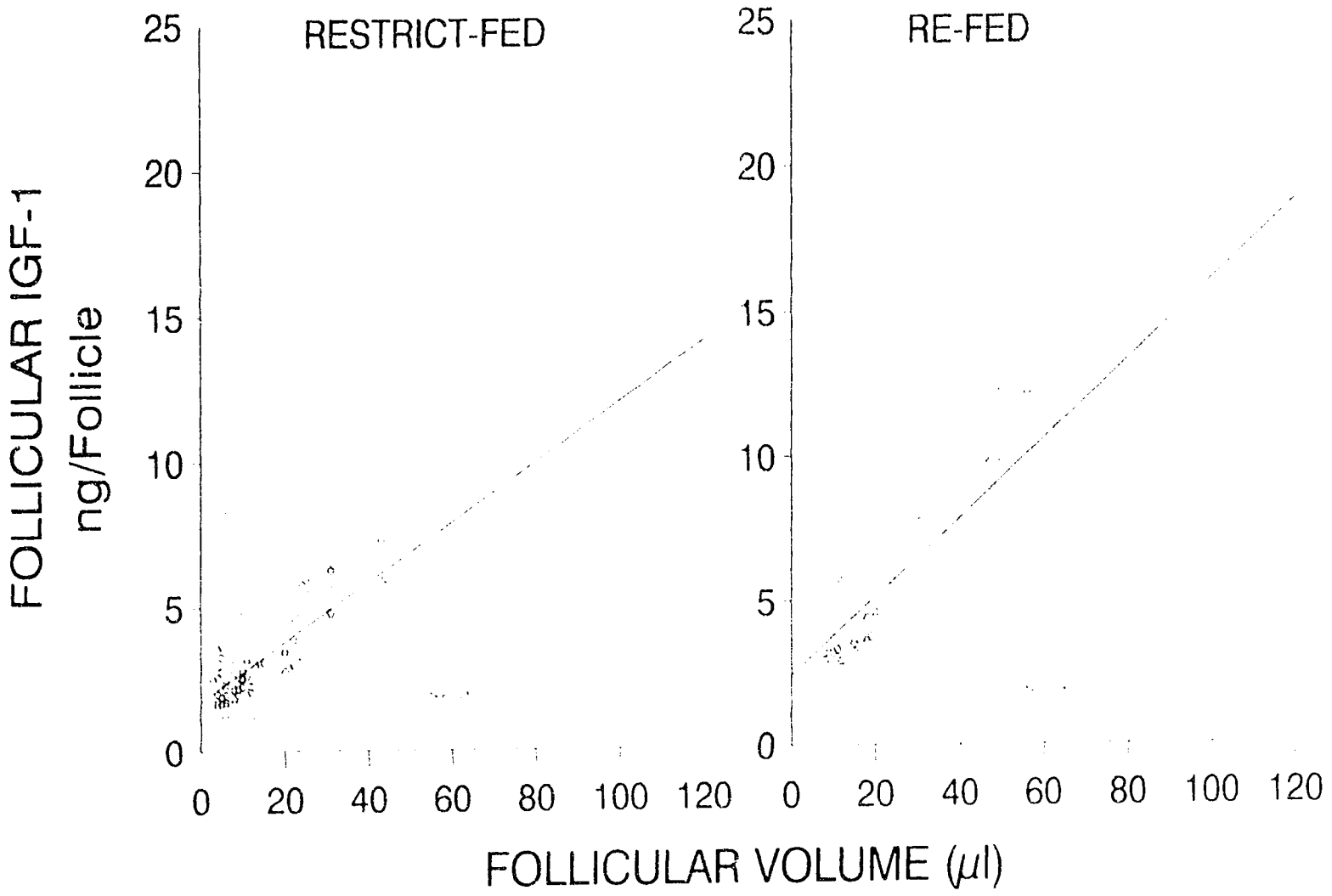
Figure 4.8. Reciprocal regression of individual follicle IGF-1 concentration against follicle volume in restrict-fed ($P<0.001$) and re-fed gilts ($P<0.001$). Circles denote individual data, while the solid lines denote fitted line. The intercepts of the fitted lines were significantly different ($P<0.05$).



RESTRICT-FED FOLLICULAR IGF-1 = 1923.958/(FOLLICULAR VOLUME) + 85.510

RE-FED FOLLICULAR IGF-1 = 1508.764/(FOLLICULAR VOLUME) + 167.818

Figure 4.9. Linear regression of individual follicle IGF-1 content against follicle volume in restrict-fed ($P<0.001$) and re-fed gilts ($P<0.001$). Circles denote individual data, while the solid lines denote fitted line. The intercepts of the fitted lines were significantly different ($P<0.05$).



RESTRICT-FED FOLLICULAR IGF-1 = 0.103 (FOLLICLE VOLUME) + 1.712

RE-FED FOLLICULAR IGF-1 = 0.137 (FOLLICLE VOLUME) + 2.414

22 gilts, as illustrated in Figure 4.10. IGF-1 gene expression was detected in hepatic tissue from 17 gilts (8 restrict-fed and 9 re-fed gilts). As the absence of expression liver mRNA could have been due to methodological problems, data from these gilts were treated as missing values, rather than attributing them with a 0% level of expression with respect to the level of mRNA expression in a standard aliquot of total human placenta RNA. No effect of re-feeding gilts was found on either ovarian or hepatic IGF-1 gene expression. However, the lack of significance at the 5% level may well be due to the enormous variations in gene expression between replicates, as the mean levels of expression suggest that there are marked decreases in ovarian IGF-1 gene expression and increases in hepatic IGF-1 gene expression in response to re-feeding (illustrated in Figure 4.11.).

Regression analyses were carried out to determine whether there were correlations between ovarian and hepatic IGF-1 gene expression, and follicular volume and IGF-1 concentration, and plasma levels of IGF-1, insulin and GH. No correlations could be made between hepatic or ovarian IGF-1 gene expression and any of the parameters chosen, although there were changes in the abundance of IGF-1 abundance coincident with changes in the parameters examined. This was presumably due to the variation observed between replicates.

DISCUSSION

The effects of re-feeding on plasma insulin, growth hormone and IGF-1 support the hypothesis that restrict-feeding uncouples the growth hormone-IGF-1 axis. Re-feeding gilts increased both basal insulin levels and

Figure 4.10. Autoradiograms of RNase protection of 50 μg of porcine ovarian (O) and hepatic (L) total RNA from four littermates (two re-fed and two restrict-fed) (4.10a), with controls **a** - **f** and **A** - **C** (4.10a and 4.10b): **a** & **C**. 30 μg human placenta total RNA, **b** & **B**. 250 pg synthetic IGF-1 mRNA preparation, **c**. 50 μg porcine hepatic poly A^+ RNA, **d**. 50 μg porcine ovarian poly A^+ RNA, **e**. 7,500 dpm unhybridized, undigested IGF-1 exon 3 riboprobe, **f**. 5 x 10^6 dpm unhybridized, digested IGF-1 exon 3 riboprobe, and **A**. 5 μg ovarian total RNA, undigested.

Control markers (M) (in bases) are indicated on right of the autoradiogram, estimated weights (in bases) of the protected fragment and the riboprobe are depicted to the left of the autoradiogram. Each lane represents either the ovarian or hepatic sample from one gilt. The autoradiogram was exposed for 16 hours.

Figure 4·10a.

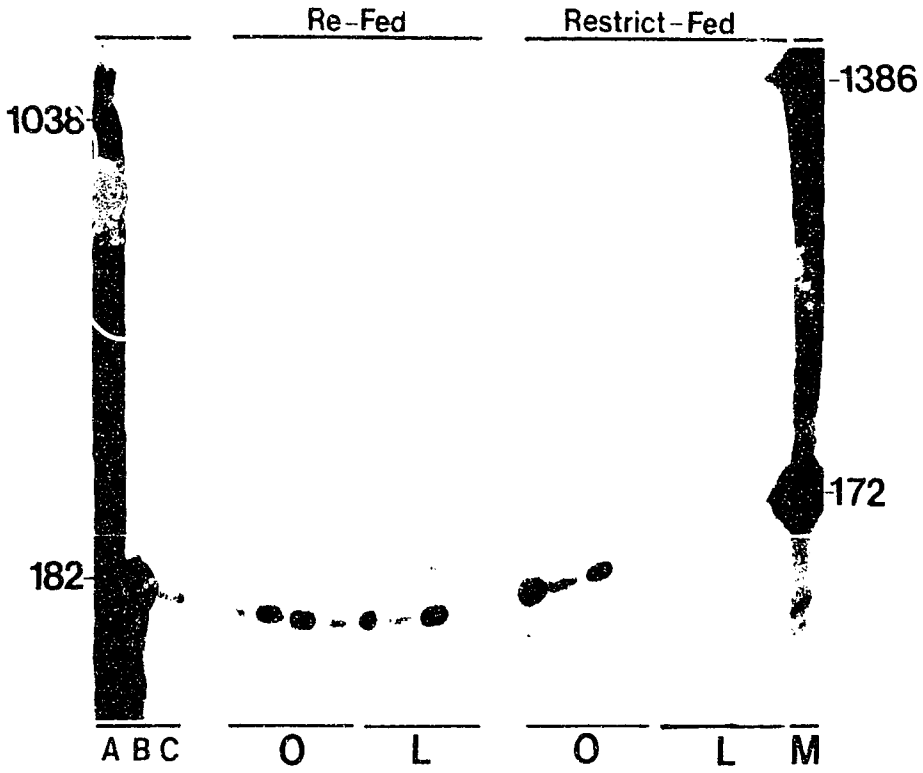
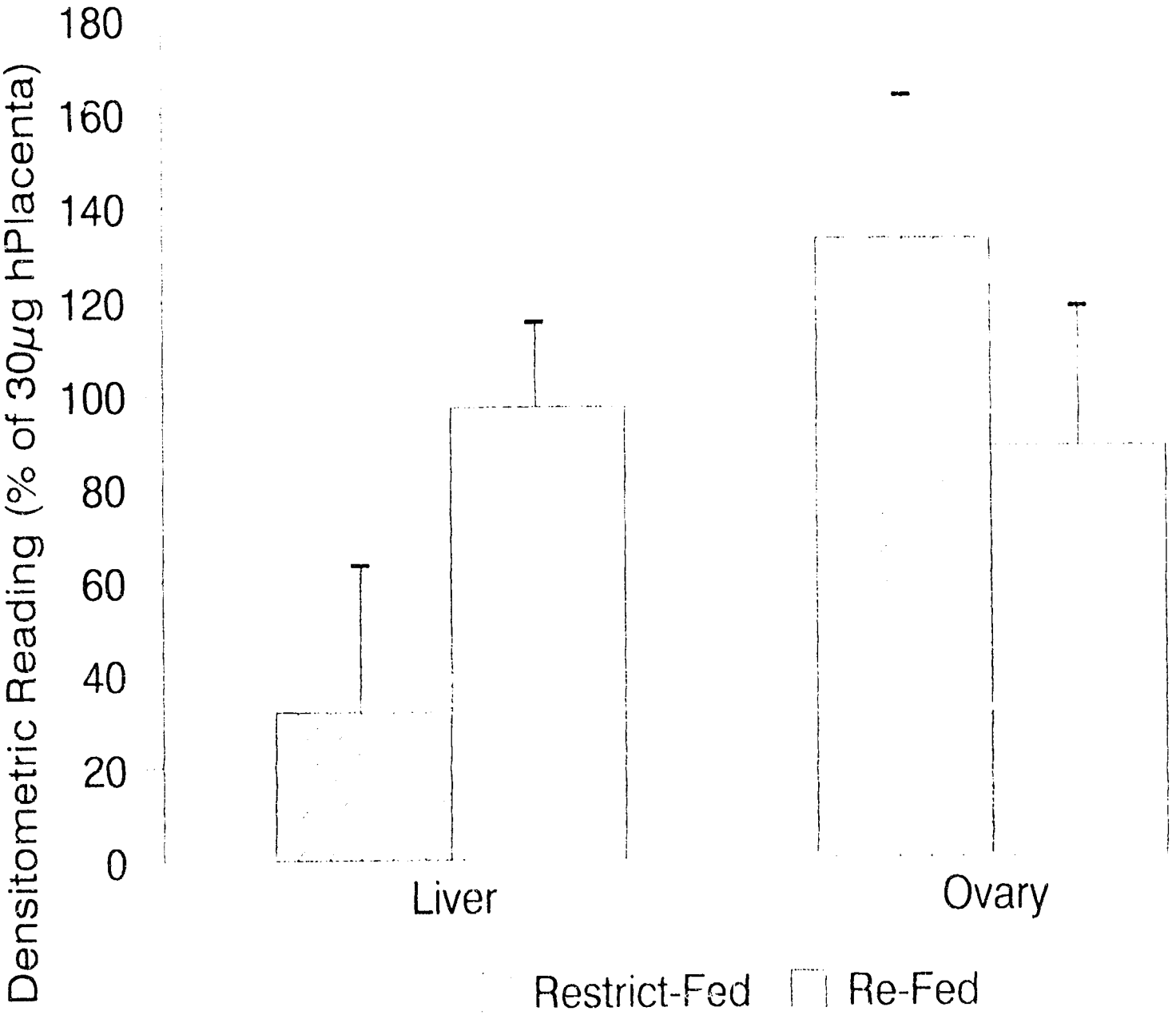


Figure 4·10b.

Figure 4.11. Mean abundance of IGF-1 mRNA detected in 50 μg of restrict-fed and re-fed hepatic and ovarian total RNA.


Densitometric Reading (% of 30μg hPlacenta)
180
160
140
120
100
80
60
40
20
0
Liver
Ovary
Restrict-Fed
Re-Fed

plasma IGF-1, in the absence of any changes in circulating growth hormone. These effects of re-feeding are similar to those reported by Booth (1990b) and Cosgrove (1991), who both found that five days of re-feeding in restrict-fed gilts increased plasma IGF-1 levels, coincident with increases in insulin. Cosgrove did not measure plasma growth hormone, however Booth demonstrated that growth hormone levels were not affected by re-feeding in the limited number of gilts in which GH was analysed.

Prandial insulin was not significantly elevated by re-feeding, however prandial insulin levels were significantly affected by barn: mean insulin levels were higher in Barn 1 than in Barn 2. Although re-feeding did elevate mean levels of insulin in both barns, the elevation measured in barn 1 was greater than that measured in Barn 2. Barn-induced variation across the two treatments therefore confounded the effect of re-feeding on the insulin response to eating a single meal.

It appears that either the different environments within the barns or differences between the two groups of animals or a combination of the two, were responsible for affecting the gilts' response to re-feeding. As growth rates for the two groups of animals were similar (Figure 4.1), the cause of barn effect is not clear.

Frequent night time measurements of growth hormone of both re-fed and restrict-fed gilts, revealed what might be described as a surge of growth hormone secretion that lasted approximately two hours and occurred at random within the sampling period. The duration of the surges of growth hormone were similar to those observed by Klindt and Stone (1984) in *ad libitum* fed barrows weighing 70 kg, sampled at 15 min intervals for a 24 hour period. However profile of the rise in GH secretion did not have the

classic pulse profile as observed by Klindt and Stone (1984) in barrows or in ovariectomized ewes (Thomas *et al.*,1990), where there was a rapid increase in GH within two sampling intervals, followed by a logarithmic decline towards basal levels. Instead the surges observed in both the restrict-fed and re-fed gilts demonstrated a gradual increase in GH followed by a gradual decrease returning to basal levels. The observed differences between the observation in the barrow study and the present study may be related to the different feeding regimens employed. The barrows were fed to *ad libitum*, while the gilts in both treatments in the present study were subjected to an overnight fast. Why an overnight fast should alter the secretory profile of GH is unclear.

Ovarian development was stimulated by six days of re-feeding. As in previous studies (Booth, 1990b and Cosgrove, 1991), realimentation significantly increased follicular diameter and volume. There were, however, no effects of treatment on follicular fluid estradiol concentration. In fact the estradiol concentrations in the follicles collected in the present study were so low that they appear to be non-estrogenic, suggesting that the ovaries collected from the gilts in this study were not sufficiently developed to possess estrogenic follicles.

Although plasma levels of IGF-1 were significantly increased by re-feeding, there was a marked lack of response in the mean level of IGF-1 in follicles of the same gilts. In addition, follicular IGF-1 concentrations were higher than both restrict-fed and re-fed gilts. A similar difference has also been observed in the dominant follicles of human ovaries. Eden *et al.* (1989) reported that follicular fluid IGF-1 levels of cohort follicles are comparable to circulating IGF-1 levels, but IGF-1 levels in dominant

follicles are significantly higher than circulating levels. As it is likely that the ten largest follicles from a porcine ovary would be among those destined for ovulation at puberty (unless they became atretic), the higher concentrations measured within these follicles are consistent with the conclusion of Eden *et al.* (1989) that the intrafollicular levels of "dominant" follicles are higher than those of subordinate follicles.

The higher levels of IGF-1 within follicles may indicate either that there is local production of IGF-1 by the granulosa cells, as suggested by *in vitro* studies, or that IGF-1 clearance rates within follicular fluid are slower than in plasma - perhaps by sequestration by locally-produced IGF Binding Proteins (Holly & Wass, 1989). The lack of effect of re-feeding restrict-fed gilts on mean follicular IGF-1, in contrast to the dramatic effects observed on circulating IGF-1, may suggest that in some way the ovary maintains its internal concentrations of IGF-1 by either, or both, of the methods suggested above. The analysis of total RNA for mRNA for the IGF-1 gene suggests that the intra-follicular levels of this growth factor are maintained by local production by follicular tissue, as restrict-fed gilts were actually found to have a higher level of follicular expression of the IGF-1 gene. It appears that ovarian and hepatic IGF-1 gene expression are regulated differentially and this allows ovarian production of IGF-1 to make up a deficit in any flux of circulating IGF-1 into the follicular compartment when hepatic gene expression and circulating levels are low.

These conclusions are all circumspect, however. They assume that a proportion of IGF-1 measured in follicular fluid is of circulating origin. To date there is little evidence to indicate how IGF-1 is transported

across capillary membranes into tissues and to the author's knowledge, no work has been carried out to determine how circulating IGF-1 is transported into the follicular antrum.

Individual concentrations of follicular fluid IGF-1 were found to have an inverse relationship with follicle volume, as illustrated in Figure 4.9. This may suggest that absolute levels of follicular IGF-1 remain constant and that as the follicles grow and fill with more fluid, the IGF-1 held within is diluted with the increasing amounts of fluid. However, regression of absolute follicular IGF-1 content against follicle volume indicates that this may not be the case: absolute IGF-1 content was found to linearly increase with increasing follicle volume. These findings may allow us to conclude that IGF-1 production is greater in smaller follicles compared to larger follicles, and that as follicles increase in size, the metabolic clearance rates of this growth factor are decreased , perhaps by sequestration by locally-produced specific binding proteins.

Regression analysis of individual follicle volume against follicular IGF-1 concentrations and content suggest that there are effects of re-feeding restrict-fed gilts on ovarian IGF-1. The intercepts of both follicle IGF-1 concentration and content with volume were found to be significantly higher in the re-fed animals compared to the restrict-fed animals. Plots of individual follicular IGF-1 concentrations and contents against follicular volume reveal that the effects of re-feeding are related to follicle volume rather than a change in the relationship between follicle volume and IGF-1. This conclusion is based on the observation that re-feeding effectively "pushes" the population of follicles along the fitted line in the direction of increasing follicle

volume.

Examination of the distribution of the individual data upon the regression line for follicular IGF-1 concentration and volume (Figure 4.9.) may reveal why there were no observed changes in mean follicular IGF-1 concentrations of the five largest follicles, although there were differences in ovarian IGF-1 gene expression. The fitted regression line, illustrated in Figure 4.9., indicates that once a follicle reaches a volume of approximately 15 μl, the concentrations of IGF-1 do not change markedly thereafter. As the volumes of the five largest follicles from each animal were all greater than 15 μl, it is unlikely that any differences in follicle volume would result in significantly different IGF-1 concentrations.

The discovery that the granulosa cells of smaller follicles are exposed to an intra-follicular environment containing a higher concentration of IGF-1 than larger follicles poses several questions: Why would the granulosa cells of a smaller follicle require a greater exposure to this growth factor than those of a larger follicle? What are the implications of this finding on the supposed roles of this growth factor within the growing follicle? How does this relate to an ovarian response to metabolic state? This experiment alone can not answer all of these questions, however one may philosophise possible answers based on published literature.

As discussed earlier, IGF-1 has mitogenic, metabolic and steroidogenic functions within follicles. Before a follicle can become steroidogenic and then proceed towards ovulation, there must be sufficient cells that possess the necessary biochemical pathways to be classed as

"steroidogenic". Perhaps the initial role of IGF-1 in smaller follicles is that of a "priming" factor that initially has a mitogenic role, stimulating the granulosa cells to undergo mitosis. Once the follicle has grown to a volume of approximately 15 μl, the primary role of IGF-1 may then switch from that of a mitogenic factor to that of a factor that potentiates the role of the gonadotropins within the granulosa cells.

In terms of the metabolism of granulosa cells, and possible regulation by IGF-1, high concentrations of this growth factor may again be acting as a priming mechanism. High levels of IGF-1 may be important in smaller follicles to maximize the potential to use substrates for cell metabolism, particularly important in cells that should be undergoing rapid proliferation as the follicle grows from a subordinate follicle into a dominant follicle. In terms of follicular response to metabolic status, as manipulated by feeding level, short term restrict-feeding as used in the present study, results in a larger population of smaller follicles with higher concentrations of IGF-1. This may suggest that the follicles are in a state of stasis, with the high concentration of IGF-1 present to maintain the follicles in a primed state - primed to respond to an improved supply of substrates for granulosa cell metabolism once the animal is realimented. If the animal was subjected to a long term period of feed restriction, follicular levels of IGF-1 may be different but perhaps the ovaries do maintain the potential for follicular growth and development, although actual development is ceased, by maintaining high intra-follicular concentrations of IGF-1.

This experiment did not provide for the possible effects of feeding level on other growth factors within the ovary. IGF-II, EGF, TGF, FGF and

PDGF have also been shown to play a role in follicular development (Hammond *et al.*, 1988; Schomberg *et al.*, 1983; Gospodarowicz & Bialecki, 1979). This study also did not investigate the roles of the IGF binding proteins with respect to feeding level. Additional studies need to be carried out to determine their exact roles within this model. However, this study did succeed in demonstrating that IGF-1 is a locally produced growth factor that may be sensitive to feeding level, and as such may be one several factors that acts as a mediator between metabolic state and ovarian function.

To summarize, several conclusions can be made from this study. Changes in circulating IGF-1 and insulin and hepatic IGF-1 gene expression, in the absence of changes of plasma growth hormone, support the hypothesis that restrict-feeding uncouples the growth hormone-IGF-1 axis. Ovarian development was stimulated by the re-feeding of restrict-fed prepubertal gilts, as demonstrated by the increases in follicular diameter and volume. Follicular IGF-1 concentrations decrease as follicle volume increases until a volume of approximately 15 μl is achieved, after which follicular fluid IGF-1 concentrations appear to be maintained, irrespective of metabolic state. Data on follicular IGF-1 gene expression, as determined by the level of mRNA for IGF-1, supports this hypothesis, as IGF-1 expression was higher in follicular tissue harvested from a restrict-fed gilt ovaries than from re-fed gilt ovaries. Higher intra-follicular IGF-1 concentration may be important in "priming" granulosa cells for future steroidogenic activity. Finally ovarian IGF-1 gene expression is differentially regulated by feeding level compared to expression in hepatic tissue, therefore suggesting that local IGF-1

production may indeed act as a metabolic regulator at the level of the ovary.

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CHAPTER 5

General Discussion

A number of studies have demonstrated that ovarian development is dependent on metabolic status. Futhermore, there is evidence to suggest that the ovary is able to respond to changes in metabolic status independently of the response of the hypothalamic-pituitary axis. Ovarian development can be restored by re-feeding of restrict-fed gilts, in the absence of changes in increasing LH (Cosgrove *et al.*, 1991). Also insulin treatment of gilts, fed a high energy diet, can increase ovulation rates in the absence of changes in LH secretion (Cox *et al.*, 1987).

The hypothesis for this thesis was that IGF-1 is a locally produced hormone that may mediate ovarian responses to changes in metabolic state. The basis for this hypothesis was that IGF-1 has been shown to be secreted by granulosa cells *in vitro* (Adashi *et al.*, 1985; Hammond *et al.*, 1985), and that circulating levels of IGF-1 are dependent on the metabolic status of an animal (Clemmons *et al.*, 1981; Buonomo & Baile, 1991). It is postulated that local production of this growth factor *in vivo* may be sensitive to feeding level and subsequent metabolic state. Indeed, Meurer *et al.* (1991) demonstrated that the IGF-1 content of large follicles in diabetic gilts was lower than in those of normoglycaemic gilts, even when treated with PMSG. This study suggested that insulin levels may in turn influence follicular levels of IGF-1, and that these lower follicular fluid levels of IGF-1 may influence ovarian development.

A criticism of many studies in this area is that there is little evidence to indicate whether follicular fluid concentrations of IGF-1 are of ovarian or circulating origin. The objective of this thesis was to develop and apply methodology that would allow investigation of the

effects of changing metabolic state on ovarian levels of IGF-1 mRNA in individual animals and, therefore, to determine these effects on ovarian production of IGF-1 *in vivo*.

Before one can investigate effects of changing metabolic state on ovarian gene expression, as determined by IGF-1 mRNA abundance, it must be established that the IGF-1 gene is expressed in porcine ovarian tissue. Although IGF-1 was originally believed to be produced by the liver only, many studies have now demonstrated that IGF-1 is produced by tissues other than liver *in vitro*. In addition, studies by Murphy *et al.* (1987) have shown that the IGF-1 gene is expressed in a wide range of tissues in the rat, as well as hepatic tissue. Other studies have provided similar evidence in the pig (Tavakkol *et al.*, 1988; Lowman *et al.*, 1990; Simmen *et al.*, 1990; Duffy, *personnel communication*). Preliminary work by Cameron *et al.* (1990) demonstrated that the IGF-1 gene is expressed in porcine follicular tissue, however, this work was not extended to the study of treatment effects on individual animals. Therefore, the objectives of the first study, described in Chapter 2., were to confirm the that the IGF-1 gene is expressed in porcine ovarian tissue, and to determine the potential of current methodology to detect IGF-1 mRNA in samples of RNA on an individual animal basis.

Using northern analysis, it was possible to detect IGF-1 mRNA in porcine ovarian total and poly A^+ enriched RNA. However, as in studies by other workers (Tavakkol *et al.*, 1988), the northern analysis procedure did not convincingly detect the presence of IGF-1 mRNA in either porcine hepatic total or poly A^+ enriched RNA. The sensitivity of the RNase protection assay was considerably higher than that of the northern

procedure. IGF-1 mRNA was detected in as little as 10 μg of total RNA from both porcine ovarian and hepatic tissue. The RNase protection assay, therefore, appeared to be the more suitable procedure for analyzing the expression of the IGF-1 gene in samples from individual animals.

The objectives of the studies described in Chapters 3 and 4, were to apply the RNase methodology to determine the effects of changing feeding level on ovarian IGF-1 expression. In Chapter 4, application of RNase protection methodology allowed a comparison of the effects of feeding level on ovarian and hepatic IGF-1 mRNA abundance in the pig.

Due to lack of replicates in a relatively complicated experimental design, the usefulness of the results from the experiment described in Chapter 2 is limited. However, this study did establish the seven day restrict-fed model in our laboratory, and also indicated how the experimental design could be modified to answer questions more concisely and successfully.

The study described in Chapter 4 succeeded in demonstrating the effects of changing nutrition regimen on gilt metabolic status and reproductive status. In addition, this study provided evidence suggesting that IGF-1 may be behaving as an indicator of metabolic status at the level of the ovary. Restrict-feeding was found to uncouple the growth hormone - IGF-1 axis in terms of circulating IGF-1 and hepatic IGF-1 mRNA abundance. Plasma IGF-1 concentration and hepatic IGF-1 mRNA were related to plasma insulin levels rather than plasma GH.

However, at the level of the ovary, the relationships between follicular IGF-1 concentrations, IGF-1 mRNA abundance with plasma insulin and GH appear to be different. Decreased basal insulin was associated with

higher levels of IGF-1 mRNA abundance and no change in mean IGF-1 concentration in the five largest follicles from each animal. In addition, individual follicular fluid IGF-1 concentrations were found to be inversely related to follicle volume, therefore the smaller follicles associated with the restrict-fed state had higher concentrations of IGF-1 in their antral fluid compared to larger follicles.

Unfortunately, variability in mRNA replicates did not allow correlations of IGF-1 gene expression with either plasma insulin or any other parameter. However, these results do indicate the possibility that there is differential control of IGF-1 gene expression by feeding level. Alternatively, the higher levels of IGF-1 mRNA transcripts within the follicular tissue of restrict-fed gilts may reflect changes in mRNA turnover. The turnover rate of IGF-1 mRNA may be reduced by restrict-feeding, possibly reflecting reduced translation of the mRNA transcripts and subsequent production of the IGF-1 peptide. However, the higher observed levels of IGF-1 within the smaller follicles suggest that the higher levels of follicular fluid IGF-1 are due to increased IGF-1 gene expression, rather than lower turn over rates, unless of course the metabolic clearance rate of the IGF-1 peptide itself is reduced by restrict-feeding. This study alone can not answer these questions, indeed the RNase protection work should be repeated on the same samples to confirm that the effects of restrict-feeding/re-feeding are repeatable using this methodology.

Nonetheless, this study does pave the way for more detailed studies to investigate why porcine small follicles should have higher concentrations of IGF-1 than larger follicles. For example, detection of

IGF-1 gene expression in individual follicles may be a good starting point. The RNase protection methodology could be further developed to allow the detection of the abundance of IGF-1 mRNA at the level of individual follicles. Since the present RNase protection procedure has determined the authenticity of the protected fragment, this method may be adaptated into a simple in-solution hybridization assay. In this procedure the radioactivity of digested samples and suitable controls are counted directly, rather than indirectly via electrophoretic separation and subsequent exposure to x-ray film. This modification of the RNase protection assay should increase the sensitivity of the assay further. In fact, the limitation of this methodology is more likely to be associated with the ability to isolate sufficient total RNA from small tissue samples, rather than the ability to detect the mRNA for specific genes within the RNA.

A criticism of the present RNase protection assay is that this procedure only gives an indication of total IGF-1 gene expression; the gene is either being expressed or it is not. There is no indication of what species of mRNA are present as the IGF-1 exon 3 nucleotide sequence is one that is conserved in all IGF-1 mRNA transcripts identified to date. Relative abundancies of different IGF-1 mRNA transcripts have been shown to be regulated by feeding level (Elmer & Schalch, 1987). Although there is no evidence to date to suggest that there is any physiological significance in the expression of different transcripts, in the future subtle changes in the processing of the IGF-1 mRNA transcripts may prove to be important. Therefore, the results of the present studies suggest that ovarian samples should either be pooled to provide enough total RNA

to allow enrichment of the samples for poly A+ RNA and subsequent northern analysis, or the RNase protection assay should be modified to allow detection of different mRNA transcripts within samples from individual animals.

Suitable modifications of the RNase protection procedure are possible. In addition to the hIGF-1 exon 3 probe, there are also hIGF-1 exon 4 and 5 probes available. Exons 4 and 5 are differentially spliced into the two primary IGF-1 mRNA transcripts, IGF-1b and IGF-1a respectively (Illustrated in Figure 1.2.). Therefore, hybridization of sample total RNA with a combination of the exon 3, 4 and 5 riboprobes would produce a range of protected fragments that would give an indication of qualitative changes in IGF-1 gene expression.

In conclusion, in these studies a procedure that was capable of detecting IGF-1 mRNA in small samples of tissues with low levels of expression of the IGF-1 gene was established. In addition, this procedure was applied to test the hypothesis that IGF-1 is a locally produced growth factor that may mediate ovarian responses to changing metabolic state. The results indicate that in the pig IGF-1 may be acting as an indicator of metabolic status in a paracrine or autocrine manner at the level of the ovary, and that expression of the IGF-1 is differentially regulated by metabolic status in hepatic and ovarian tissues.

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APPENDICES

APPENDIX I

RNASE PROTECTION PROCEDURE

DAY 1 Preparation of Probe

1. Mix in an autoclaved microcentrifuge tube:

1 μl	1 mg/ml DNA Template
3.5 μl	RNase-free water
3 μl	RNTP mix (10 mM ATP, GTP & UTP)
2 μl	100mM Dithiothretiol (DTT)
0.5 μl	RNasin (25-40 units/μl)
4 μl	5 * polymerase enzyme buffer (0.2 M Tris-HCl, pH [illegible], 30 mM $MgCl_2$, 10 mM spermidine $(HCL)_3$
5 μl	^{32}P-CTP
1 μl	Polymerase enzyme (15 units/μl)

2. Incubate 30 to 60 min at 37°C.
3. Add 0.5 μl RNasin and 1 μl RQDNase (Promega).
4. Add 28.5 μl of TE (10 mM Tris-HCl, 1 mM EDTA) to produce a final volume of 50 μl.
5. Extract with phenol/choroform/isoamylalcohol.
6. Add all 50 μl of probe mixture onto prepared spun sephadex column and spin at 6000 rpm to remove unincorporated label. The column should pretreated by running 2 volumes of 1 x PIPES stock solution.
7. Count 1 μl of eluate to determine incorporation and to determine volume required to add to 30 μl hybridization solution (preferably require $\sim$ x 10^6 for each sample).

Hybrization

8. Precipitate sample RNAs with 1/10 vol 3M sodium acetate (pH5.4) and 2.5 vols of cold (-20°C) 95% ethanol. *NB* This step may be done the previous day to optimise precipitation and to reduce preparation on Day 1. Following precipitation centrifuge samples for 30 min at 4°C.
9. Redissolve in 30 μl hybridization buffer containing probe RNA.
10. Incubate 5 min at 85°C to denature RNA.
11. Rapidly transfer to 45°C water bath and incubate overnight (~8 hr).

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RNASE PROTECTION PROCEDURE

DAY 1 Preparation of Probe

1. Mix in an autoclaved microcentrifuge tube:

1 μl	1 mg/ml DNA Template
3.5 μl	RNase-free water
3 μl	RNTP mix (10 mM ATP, GTP & UTP)
2 μl	100mM Dithiothretiol (DTT)
0.5 μl	RNasin (25-40 units/μl)
4 μl	5 * polymerase enzyme buffer (0.2 M Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine $(HCl)_3$
5 μl	^{32}P-CTP
1 μl	Polymerase enzyme (15 units/μl)

2. Incubate 30 to 60 min at 37°C
3. Add 0.5 μl RNasin and 1 μl RQDNase (Promega).
4. Add 28.5 μl of TE (10 mM Tris-HCl, 1 mM EDTA) to produce a final volume of 50 μl.
5. Extract with phenol/choroform/isoamylalcohol.
6. Load all 50 μl of probe mixture onto prepared spun sephadex column and spin at 6000 rpm to remove unincorporated label. The column should pretreated by running 2 volumes of 1 x PIPES stock solution.
7. Count 1 μl of eluate to determine incorporation and to determine volume required to add to 30 μl hybridization solution (preferably require 5 x 10^6 for each sample).

Hybrization

8. Precipitate sample RNAs with 1/10 vol 3M sodium acetate (pH5.4) and 2.5 vols of cold (-20°C) 95% ethanol. *NB* This step may be done the previous day to optimise precipitation and to reduce preparation on Day 1. Following precipitation microfuge samples for 30 min at 4°C.
9. Redissolve in 30 μl hybridization buffer containing probe RNA.
10. Incubate 5 min at 85°C to denature RNA.
11. Rapidly transfer to 45°C water bath and incubate overnight (>8 hr).

DAY 2 Ribonuclease Digestion

12. To each hybridization reaction add 350 µl ribonuclease digestion buffer containing 40 µg/ml ribonuclease A and 2 µg/ml ribonuclease T1. Incubate 30 to 60 min at 30°C.
13. Add 10 µl of 20% SDS and 2.5 µl of 20 mg/ml proteinase K. Incubate 15 min at 37°C.
14. Extract once with 400 µl phenol/choroform/isoamylalcohol, removing the aqueous phase to a clean microfuge tube containing 1 µg/ml yeast tRNA.
15. Add 1 ml cold 95% ethanol and precipitate for 30 min at -70°C. Following precipitation, microfuge samples for 30 min at 4°C.
16. Air dry pellet *thoroughly* and redissolve in 3 to 5 µl RNA loading buffer. Denature samples by heating at 100°C for 5 min.
17. Analyse on a denaturing polyacrylamide/urea (sequencing) gel.

Preparation of Polyacrylamide/Urea Gel

1. Prepare stock gel solution:

   For 1 litre: 57 g acrylamide
   3 g bisacrylamide
   460 g urea

   Dissolve above in about 900 ml water, heat may be required. Once dissolved, add about 20 g Biorad mixed bed resin and stir for about 30 min to deionize. Filter through a 0.45 µm nitrocellulose filter. Add 50 ml 20 x TBE (1.8 M Tris-HCl, 1.8 M boric acid, 5.5 mM EDTA) and make up to 1 litre.
2. De-gas mix by evacuating with a vacuum apparatus for a few minutes. About 50 ml will provide excess for a large 30 cm wide "sequencing" gel. The gel mix should be kept on ice to slow down polymerization.
3. Add 650 µl freshly made 10% ammonium persulphate and 100 µl TEMED. Pour the gel, insert well former. Leave to set for approx. 1 hour.
4. Prerun gel for 30 min or longer under running conditions.
5. Add 5 µl of loading buffer to each sample. Vortex briefly to dissolve RNA. Place in boiling water bath for 5 min prior to loading onto gel.

6. Run gel at 40 watts for a 30 cm wide gel or 25 - 30 watts for a 20 cm gel. The voltage will increase during electrophoresis and run from 950 V to 1200 V.

7. The length of run depends on the predicted size of the protected fragments. As a rough guide, on 6% polyacrylamide gels, bromophenol blue dye runs at about 40 bases while xylene cyanol runs at about 120 bases. For our purposes a suitable running time appears to be 45 mins or until the bromophenol blue marker is 3 cm from the base of the gel.

8. Following electrophoresis, separate plates carefully. Fix gel on the plate by soaking it in 10% methanol, 10% acetic acid for 15 min, then drain dry for 15 min. The gel and plate can then be wrapped in cling film and autoradiographed or the gel transferred to filte paper by firmly pressing a dry sheet of Whatman 3MM onto the gel and peeling it away from the plate. After covering the gel with Clingfilm, the gel can then be dried onto the paper on a heated vacuum drier and autradiographed in a standard cassette. Dried gels provide a much sharper and more sensitive signal.

Reagents and Solutions

1. Hybridization Buffer

5 x Stock Solution:	To make 100ml
220 mM PIPES	6.05 g PIPES
2 M NaCl	11.69 g NaCl
5 mM EDTA	0.186 g EDTA

Working Solution:

4 parts formamide
1 part 5 x stock buffer

2. Ribonuclease Digestion Buffer

10 mM Tris-HCL	Ribonuclease Mix:
300 mM NaCl	2 mg/ml ribonuclease A
5 mM EDTA	0.1 mg/ml ribonuclease T1

1/50 vol of 50 x ribonuclease mix (see below)

To make 100 ml

0.158 g Tris-HCL
1.75 g NaCl
0.186 g EDTA

Make 5 aliquots of 19.6 ml and store at -20°C. Add 400 µl of RNAse mix prior to use.

3. RNA Loading Buffer (Make on day of use)

80% Formamide
1 mM EDTA, pH 8.0
0.1% Bromophenol Blue
0.1% Xylene Cyanol
Water

To Make 10 ml:

8 ml formamide
20 µl 0.5M EDTA
10 µg Bromophenol Blue
10 µg Xylene Cyanol
1.6 ml Water

4. RNase Solution

Require 1/50 vol of 50 x RNase mix:
2 mg/ml RNase A
0.1 mg/ml T1
RNase A 20 ml x 40 µg/ml = 800 µg
@10 000 µg/ml = 10 ug/µl
Therefore require 80 µl of RNase A in 20 ml.
RNase T1 20 ml x 2 µg/ml = 40 µg
@ 1.1 µg/µl
Therefore require 37 µl T1 in 20 ml.

APPENDIX II

RNASE PROTECTION PROCEDURE CONTROLS

Controls

A. IGF-1 mRNA (ie. synthesised from Bam H1/T7 template)

1 µl	IGF-1 Bam H1 (1µg)
7.5 µl	sterile water
4 µl	RNTP mix (500 µM each of U,G,A,C)
2 µl	100 mM DTT
0.5 µl	RNasin
4 µl	5 x Transcription Buffer
1 µl	T7
20 µl	

1. Incubate the above for 1.5 hours at 40cC.
2. Remove DNA template: i) Add 0.5 µl RNasin.
   ii) Add 1 µl RQ1 DNase.
   iii) Incubate for 15 min at 37°C.
3. Make up reaction volume to 100 µl with TE (ie. add 78.5 µl TE).
4. Extract proteins using phenol/chloroform/iso amylalcohol.
5. Remove phenol by extracting with chloroform/iso amylalcohol.
6. Purify reaction using spun-column chromatography.
7. Record yield (µl).
8. Add 1/10 recovered volume of 3M sodium acetate, mix.
9. Add 2.5 volumes of 95% cold ethanol (-20oC).
10. Incubate overnight at -20°C.
11. Microfuge for 30 min at 4oC.
12. Aspirate ethanol and allow the pellet to air dry.
13. Resuspend pellet in 10 µl H_2O.

Preparation For RNase Protection Assay

Yield eg. 4.6 µg in 10 µl, concentration = 460 ng/µl

	1 µl RNA solution (460,000pg)
+	9.199 ml sterile H_2O
	9.2 ml @ 50 pg/µl

1. Take 5 µl (250 pg) for RNase Protection Assay, freeze remainder as 1 ml aliquots at -70°

2. Add 15 µl 95% cold ethanol, mix well.
3. Incubate at -20oC for 30 min.
4. Microfuge for 15 min at 4°C.
5. Remove ethanol, dry pellet.
6. Resuspend pellet in 30 µl hybridization buffer and proceed as routine for remainder assay.

B. Control Template Marker

1. Carry out transcription reaction as routine, but use 1 µl ^{32}P-CTP (ie. 50 µCi) rather than 5 µl. Also add 0.5 µl of cold CTP to achieve a final concentration of approximately 250 µM.
2. Place 500 dpm in an eppendorf and add 1 µl of 10 mg/ml tRNA.
3. Add 2.5 volumes of 95% cold ethanol.
4. Incubate at -20°C for 30 min.
5. Microfuge for 15 min at 4oC.
6. Aspirate ethanol and allow pellet to air dry.
7. Resuspend pellet in 5 µl of RNA loading buffer.
8. Heat for 3 min at 85oC and then load onto the gel.

Transcription Reaction:

1 µl	Template DNA (ie Control Marker)
7 µl	RNase-free water
3 µl	RNTP mix
0.5 µl	cold CTP
2 µl	100mM DTT
4 µl	SP6 5 x Transcription buffer
0.5 µl	RNasin
1 µl	^{32}P-CTP
1 µl	SP6 polymerase enzyme

Suggestions:

1. Load 7500 dpm of unhybridised, undigested probe.
2. Load 7500 dpm of marker synthesised from control template.