Development of real-time reverse transcription polymerase chain reaction assays to quantify insulin-like growth factor-1 receptor and insulin receptor expression in equine tissue

by

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Submitted in partial fulfillment of the requirements for the degree

MMedVet: Theriogenology

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Declaration

I, Stephen Bernard Hughes, do hereby declare that the research presented in this dissertation, was conceived and executed by myself, and apart from the normal guidance from my supervisor and co-supervisors, I have received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted for degree at this University or any other University.

This dissertation is presented in partial fulfillment of the requirements for the degree in MMedVet in Theriogenology.

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Abstract

There has been significant progress in the development of new technologies and methodologies to characterise gene expression. The fluorescent-based real-time reverse transcription (RT) polymerase chain reaction (PCR) is an important tool used for clinical and molecular research, biotechnology and as a diagnostic test. Insulin-like growth factors (IGF-1 and IGF-2) and insulin are ubiquitously expressed and play important roles in the regulation of cell growth, differentiation and the maintenance of cell differentiation in mammals. The IGF system (IGF-1, IGF-2, IGF -1 receptor, IGF-2 receptor and six IGF-binding proteins) and insulin are consequently essential to most aspects of male and female reproduction. IGF-1 is produced in multiple tissues but predominately in the liver, from where it enters the circulation. Insulin is secreted by β-cells of the pancreas' islets of Langerhans. Both IGF-1 and insulin polypeptides bind to specific cell surface receptors. These receptors are members of the superfamily known as tyrosine protein kinases, and are composed of two α and two β subunits linked by disulfide bonds to form an αβ–αβ heterotetramer. The α subunits include ligand binding sites, whereas the β subunits contain tyrosine kinase activity. The aim of this project was to develop real-time RT-PCR assays for quantification of equine insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor (INS-R) mRNA.

The assays were developed using stallion testicular tissue samples, obtained by excisional biopsy, from three horse breeds (Friesan, Thoroughbred and Warmblood). The assays developed were efficient, sensitive and had a broad linear range of detection (seven logs for IGF-1R and six logs for INS-R). The assays worked well in our hands and were both sensitive and specific for the detection of equine IGF-1R and INS-R mRNA in a variety of equine tissues.

Chapter 1 Literature review

1.1. Introduction to insulin-like growth factor and insulin

The polypeptide hormones insulin-like growth factor-1 (IGF-1) and insulin are similar structurally (Rinderknecht and Humbel 1978; Torres *et al.* 1995). They elicit similar biological responses, regulating cell proliferation (examples include promote foetal and post-natal growth and differentiation) and mediate metabolic signals (an example being glucose transport), but with differing potencies (Morgan *et al.* 1986). The biological effects of both are mediated by interactions with cell surface, trans-membrane receptors (Naz and Padman 1999). These ligands competitively cross-react with insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor (INS-R) (Torres *et al.* 1995).

The insulin-like growth factor-1 receptor (IGF-1R) and the insulin receptor (INS-R) are closely related members of the tyrosine kinase receptor super-family (Garrett *et al.* 1998). These receptors are expressed in almost all mammalian cell types and are large trans-membrane proteins consisting of several structural domains (Garrett *et al.* 1998). The IGF-1R and the INS-R are tetrameric glycoproteins composed of two alpha- and two beta-subunits ($\alpha_2\beta_2$) linked by disulfide bonds. Each alpha-subunit contains the extra-cellular ligand-binding site, and is ~125-140 kilo-Daltons (kDa), whereas each beta-subunit has a trans-membrane domain and the catalytic intra-cellular tyrosine kinase domain, and is ~95-97 kDa. Tyrosine kinases are enzymes that phosphorylate the tyrosine portion of proteins (Abbott *et al.* 1992). These receptors, like their ligands, are encoded by distinct genetic loci believed to

have evolved from a common ancestral gene (Kim and Accili 2002). They share >50% overall amino acid sequence identity and 84% identity with tyrosine kinase domains (Jones and Clemmons 1995; Pandini *et al.* 2002).

The aim of this project was to develop real-time, reverse transcription polymerase chain reaction (RT-PCR) assays to evaluate IGF-1R and INS-R gene expression in equine tissues.

The benefits arising from this project are:

- It forms part of requirements for the completion of the MMedVet in Theriogenology by Dr S.B. Hughes.
- The assays may be applied to gene expression studies in the fields of equine reproduction and medicine.
- It promotes cooperative research between departments within the Faculty of Veterinary Science, University of Pretoria.

1.2. General protein structure

Proteins are associated with almost every physiological process in living cells which include: transport and storage of molecules, immune support, provision of structure to tissues and membranes, ordering of biological reactions as enzymes, receptors located either on the plasma membrane or within the cytosol of the target cells and the control of metabolic processes as hormones (Widmaier *et al.* 1998).

The subunits of proteins are amino acids and all proteins are constructed from the same set of twenty amino acids. The amino acids are linked together by a polar covalent bond (peptide bond), which form between the amino group of one amino acid and the carboxyl group of another amino acid (Widmaier *et al.* 1998). A sequence or chain of amino acids linked by peptide bonds is known as a polypeptide. If one or more mono-saccharides bond covalently to the side chains of specific amino acids, glycoproteins are formed (Widmaier *et al.* 1998).

Protein structure may be classified as primary, secondary, tertiary, and quaternary (Widmaier *et al.* 1998). The structure of an individual protein determines the functioning thereof. The primary protein structure is determined by both the number of amino acids in the sequence and the specific type of amino acid at each position along the sequence. Secondary protein structure is formed by the folding of the amino acid chain and is considered to be the conformation of the protein. The folding of the amino acid chain is determined by hydrogen bonds between side chains or with surrounding water molecules, ionic bonds between polar and ionized side chains, and weak van der Waals' forces between non-polar side chains. Tertiary protein structure is derived from the strong disulfide bonds that form between sulfhydryl groups. A protein can fold over itself in a variety of different ways giving it a unique shape, allowing it to interact with other molecules. Quaternary protein structure is when proteins are composed of more than one polypeptide chain (multimeric proteins). A change in the primary structure of a protein may alter the secondary, tertiary, and quaternary structure (Widmaier *et al.* 1998).

Receptors are specific proteins commonly located either on the plasma membrane or within the cytosol of target cells. The binding of the ligand to the receptor induces a conformational change in the receptor which in turn has a biological effect on the cell. Plasma-membrane receptors may be distributed over the entire surface of the cell or are confined to a particular region. The latter case permits localisation of the receptor's actions to that region of the cell (Widmaier *et al.* 1998).

1.3. General nucleic acid chemistry

Deoxyribonucleic acid (DNA) is contained within the cell nucleus. DNA directs the synthesis of all proteins in the body, specifies the amino acid sequence of the proteins and, with exception of mutational and recombinational events, remains constant from generation to generation (Unger *et al.* 2009). DNA is a polymer of nucleotides, and a particular sequence of nucleotides will specify the amino acid sequence of a single polypeptide chain. This sequence of nucleotides is known as a gene. A gene is a unit of hereditary information and

a single molecule of DNA contains many genes. The total genetic information coded in the DNA of a typical cell in an organism is known as its genome (Unger *et al.* 2009).

1.4. Molecular structure and chemical properties of deoxyribonucleic acid and ribonucleic acid

DNA has two chains of nucleotides arranged around each other in a double helical conformation. A nucleotide is composed of a sugar deoxyribose with a phosphate residue at the 5' carbon and either a purine or pyrimidine base at the 1' carbon. The purines are adenine (A) and guanine (G), and the pyrimidines are cytosine (C) and thymine (T). The nucleotides are linked by phosphodiester bonds that link the 5'-phosphate group of one sugar to the 3'-hydroxyl group of the adjacent sugar. The two nucleotide chains of a DNA polymer contain a specifically ordered sequence of base side chains, one chain being complementary to the other. The sequence of bases imparts the unique identity to each DNA molecule and forms the basis of the transfer of information from DNA to ribonucleic acid (RNA) and the duplication of DNA during cell division. The double-stranded helical structure of DNA is formed due to the two nucleotide chains running in opposite directions (3'-5' versus 5'-3') and specific base paring. Adenine (A) forms two hydrogen bonds with thymine (T) and the guanine (G) forms three hydrogen bonds with cytosine (C). The sequences of bases in each strand must be such that A:T and G:C hydrogen bonds always form (Unger *et al.* 2009).

The phosphate esters of the nucleic acid backbone are strong acids that exist as anions at neutral pH. DNA is soluble in water up to 1% weight of solute per volume of solution and is precipitated by the addition of alcohol. The bases are only weakly basic and uncharged over a pH range of approximately 4 to 9, outside these limits the base pair hydrogen bond are disrupted and unwinds. Nucleic acid molecules absorb ultraviolet light maximally at 260 nm almost entirely because of the constituent bases. This property is used to quantify the nucleic acid content of a solution (Unger *et al.* 2009).

RNA is chemically similar to DNA, except that the sugar unit in RNA is ribose (containing an additional hydroxyl group at the 2' position) and thymine (T) is replaced by the demethylated pyrimidine uracil (U). RNA is a single-stranded polymer and is much shorter than DNA. RNA is less stable than DNA because it lacks the double stranded helical conformation and because the 2'-hydroxyl group of ribose makes it subject to alkaline hydrolysis.

Production of proteins is mediated by messenger RNA (mRNA) molecules that carry information for the specific proteins from DNA in the nucleus to the cytoplasm, where the proteins are synthesized. An mRNA molecule is synthesized directly from that part of the DNA that specifies the protein to be produced in a process termed transcription. Transcription requires the separation of the duplex DNA strands and the use of the appropriate DNA strand as a template. An RNA polymerase recognizes and binds specific DNA sequences, termed the initiating site, and starts joining the ribonucleotide units that pair with the template. The linkage of the nucleotides continues as the polymerase moves across the gene template. Specific DNA sequences result in chain termination, and the newly synthesized mRNA quickly detaches from the template DNA.

When mRNA is first synthesized, it is a complimentary copy of the DNA gene sequence. Before mRNA molecules are exported to the cytoplasm, non-coding regions known as introns are excised from the mRNA by a molecular complex that enzymatically cleaves and ligates RNA at specific recognition sequences (splicing). After this step the mRNA contains only exons which are the sequences of codons of a gene that are transcribed and retained during the mRNA processing within the nucleus. Mature mRNA then exits the nucleus and directs protein synthesis in the cytoplasm via a process known as translation. Translation takes place on the ribosomes, which are ribonulcleotide protein complexes that synthesizes proteins (Unger *et al.* 2009).

1.5. Insulin-like growth factor-1 and insulin

IGF-1 actions are best described as stimulatory effects on target cell proliferation and differentiation, and the ability to inhibit apoptosis (Jones and Clemmons 1995). The biological actions of IGF ligands (IGF-1 and IGF-2) are mediated through the cell surface receptors type 1 and type 2 by a family of high-affinity IGF binding proteins (IGFBP's 1 to 6) and by IGFBP proteases, which regulate ligand bioavailability (Henricks *et al.* 1998). The IGF-1 receptor (IGF-1R) mediates most actions of both IGF-1 and IGF-2. The affinity of this receptor for the ligands varies; slightly higher for IGF-1 than for IGF-2, and much higher than for insulin. The effects of IGF-2 are likely mediated by IGF-1R. IGF-2R binds IGF-2 and IGF-1 with a low affinity, but not insulin (Silva *et al.* 2009).

Insulin exerts a large array of intracellular effects that are triggered by its binding to specific membrane receptors on target cells. The receptor itself contains the information required to initiate the biochemical effects of insulin (Zick 1989). These effects range from stimulating glucose, amino acid and ion uptake, to stimulation of anabolic pathways through the modulation and induction of specific enzymes, and the enhancement of cell growth by triggering RNA and DNA synthesis (Zick 1989). The effects of insulin and INS-R binding are mainly mediated through phosphorylation and dephosphorylation reactions. Phosphorylation is a common type of reversible modification used to regulate biological functions. At a molecular level, insulin action appears to be mediated through translocation, phosphorylation, dephosphorylation of several hormone receptors, transporters and enzymes located at key regulatory positions of fat and carbohydrate metabolic pathways (Desoye *et al.* 1997).

In domestic animals, IGF-1 and insulin play essential roles in reproduction (Velazquez *et al.* 2008). In an effort to explain how local factors regulate the formation and competence of mammalian oocytes and spermatozoa, many more studies are being performed using molecular technology, such as quantification of gene expression.

1.6. Insulin-like growth factor-1 receptor and insulin receptor

IGF-1R and INS-R proteins are ubiquitous in mammalian tissues, and the target cell plasma membrane appears to be the predominant site of receptor location (Desoye *et al.* 1997; LeRoith *et al.* 1995).

The human IGF-1 receptor (hIGF-1R) cDNA was cloned and sequenced in 1986 (Ullrich *et al.* 1986). The hIGF-1R is a product of a single-copy gene located at bands q25-26, at the distal end of chromosome 14 (Abbott *et al.* 1992). It consists of 4,989 nucleotides and codes for a 1,367 amino-acid precursor. The α-chain and 195 residues of the β-chain comprise the extra-cellular portion of the hIGF-1R. There is a single trans-membrane sequence and a 408-residue cytoplasmic domain containing the tyrosine kinase (Adams *et al.* 2000). The hIGF-1R gene is > 110 kb in size and contains 21 exons, 10 in the alpha and 11 in the beta chain (Abbott *et al.* 1992).

Human insulin receptor (hINS-R) is a 350, 000-kDa transmembrane, tetrameric glycoprotein (Zick 1989). The hINS-R gene is located on the short arm of chromosome 19 and spans a region > 120,000 base pairs (bp). It consists of 22 exons and 21 introns. The exons vary in size from 36 bp (exon 11) to > 2, 500 bp (exon 22) and 21 introns vary in size from 500 to > 2,500 bp and divide the gene into segments that encode structural and functional elements to the protein (Seino *et al.* 1990). Tyrosine kinase is an enzyme that add a phosphate group onto the amino acid tyrosine portion of proteins. This is believed to play an important role in the internal regulation of the cell and the transmission of information to the cell nucleus.

The ligands, IGF-1 and IGF-2 and insulin all share a common architecture and competitively cross-react with IGF-1R and INS-R (Ward *et al.* 2001). IGF-1R has considerable identity with the INS-R gene in overall size (100kb) and in the number and size of individual exons. The exon that could not be detected in IGF-1R is the exon analogous to the alternatively spliced exon 11 of the INS-R (Abbott *et al.* 1992). Twelve exons out of the 21 exons of IGF-1R are identical

to the homologous exon of INS-R. Overall amino acid identity between INS-R and IGF-1R is ~50-60 %, with the region of highest amino acid identity (80-95%) being the tyrosine kinase domain (consist of five exons, 16-20) in the beta-subunit. Despite these similarities, the two receptors differ in ligand specificities and tissue distribution (Abbott *et al.* 1992). Ligand binding specificity is conferred by the cysteine-rich regions of the α–subunit extra-cellular domain. There is only 48% identity between the cysteine-rich domains of IGF-1R and INS-R, but there is complete conservation of the cysteine residue spacing between these two receptors. The IGF-1R and INS-R have a high affinity for their cognate ligand, and 100 to 1000 fold lower affinity for the converse ligand (Jones and Clemmons 1995).

1.7. Determining mRNA expression

Gene expression analysis is of fundamental importance in biomedical research (Bustin 2002). Determining mRNA expression is of particular interest, and the various methods used include: Northern blotting, *in situ* hybridization, RNAse protection assays, cDNA arrays and RT-PCR (Giulietti *et al.* 2001).

The PCR method of DNA amplification is a powerful and sensitive technique, with broad applications in the fields of molecular biology and epidemiology, diagnostics, and forensic analysis (Holland *et al.* 1991). Genetic material can be extracted and detected with great sensitivity and specificity in a biological sample. It was developed in the 1980s by Kary Mullis and colleagues (Mullis *et al.* 1992). When designing a PCR assay, the sequence of the target gene or template must be known. The template may then be amplified between two points, producing an amplicon (a short piece of DNA of known size and sequence). PCR is performed by temperature cycling and generally relies on the following three phases: a) denaturation of double-helical DNA into single strands at a high temperature (e.g. 94 ˚C); b) the temperature is then lowered (e.g. 55 ˚C) to allow short oligonucleotides (primers), complementary to the DNA strands flanking the sequence of interest, to anneal to these single strands of DNA; and c) elongation phase, where the temperature is increased to 72 ˚C, which is optimal for DNA polymerase (enzyme that catalyses the

synthesis of DNA) to manufacture a copy of the template extending from the primer site. These three phases are completed within a single cycle, which may take 60 to 90s, and the instrument (thermocycler) that takes the samples through the phases of changing temperature may be set to run for 20 to 45 cycles (Willoughby 2003). The exact conditions for the PCR and the times for each phase of the cycle are determined by the sample, length of the region to be amplified, the sequence of the template and primers, and also on the instrument and reaction containers used.

PCR-based assays are the most commonly used method for characterizing gene expression and comparing mRNA levels in different samples (Bustin 2002). RNA cannot serve as a template for PCR, hence the mRNA in the sample must first be transcribed to cDNA by reverse transcription (RT). Numerous techniques have been used to quantify the results of RT-PCR, such as semi-quantitative and quantitative competitive RT-PCR and quantitative real-time RT-PCR (Giulietti *et al.* 2001). Regardless of the method used, accurate quantification is highly dependent on the RT step, as the amount of cDNAs produced must correctly reflect the input amounts of mRNAs (Kubista *et al.* 2004). Quantifying PCR product in real-time is based on fluorescence-kinetic RT-PCR and requires the combination of fluorescent techniques and suitable instrumentation (Giulietti *et al.* 2001). Real-time RT-PCR is a technique used for the analysis of mRNA in extremely low abundance, in material such as cells and tissues, made possible by including in the reaction a fluorescent marker (probe or dye) that binds to the product formed and reports the amount of amplified product after every cycle by fluorescence. The use of specialised thermal cyclers equipped with fluorescence detection modules, allows for the detection of the amplicon as it accumulates by measuring fluorescence emission. It is a fast and reproducible method that allows for the amplified product to be detected and measured as the reaction progresses (Pfaffl *et al.* 2002). Additional advantages include: high sensitivity, high throughput, use of a closed tube system, reduced variation, the ability to multiplex and the lack of post-PCR manipulations (Schmittgen 2001). There are various fluorescent chemistries available that can detect amplified product, and the two main categories are: double-stranded DNA-binding dyes (SYBR

Green); and dye-labeled, sequence specific oligonucleotide probes (TaqMan probes, molecular beacons, scorpions) (Bustin 2000; Bustin 2002). All these chemistries allow detection of PCR products via the generation of a fluorescent signal. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to a dsDNA (Giulietti *et al.* 2001) TaqMan probes, molecular beacons and scorpions depend on the principle of Förster or fluorescence resonance energy transfer (FRET) and a fluorescence signal is emitted only on cleavage of a dual-labeled oligonucleotide probe. Using any of the developed chemistries, the increase in fluorescence signal can be read during the course of the reaction, and is a direct consequence of target sequence amplification during PCR (Giulietti *et al.* 2001).

The real-time PCR progresses through a number of amplification cycles. Fluorescent values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. There is an initial weak signal that cannot be distinguished from the background. This is followed by an exponential growth phase as the amount of product accumulates and finally a plateau phase as the signal levels off because some component (e.g. primer, polymerase molecules) of the reaction is limiting (Kubista *et al.* 2004). The level of the plateau tells us nothing about the initial amount of template molecules in the sample tested, as the curve for each sample tested will reach the same plateau level. It is only the exponential growth phase of the curves that differ, and this reflects the difference in the initial amount of template within each sample. A threshold fluorescent signal level is chosen and is called the cycle threshold value (C_t). The C_t is defined as the cycle number of the PCR at which the sample fluorescence signal exceeds a fixed baseline fluorescence level during the exponential phase of amplification (Johnson *et al.* 2000). The difference between each sample is quantified by comparing the C_t values. The C_t is directly proportional to the starting copy number of template molecules in the sample. The higher the starting copy number, the sooner a significant increase in fluorescence is observed, resulting in a low C_t number (Johnson *et al.* 2000). This relationship forms the basis for the quantitative aspect of real-time PCR, as the C_t value can be translated into a quantitative

result by constructing a standard curve (Bustin 2000). The standard curve gives a good estimation of the efficiency of the PCR reaction (Kubista *et al.* 2004).

1.8. Potential application of mRNA expression assays to male factor infertility

Male gonadal function is regulated by endocrine and local factors. There are individual males with observed poor fertility that lack demonstrable endocrine abnormalities. Hence, more research is now focused on the role of local factors regulating spermatogenesis and the events of sperm capacitation. Local factors that have been investigated include; differentiation factors, cytokines, growth factors, and sperm surface receptors (Champion *et al.* 2002, Henricks *et al.* 1998, Hess and Roser 2001, Hoeflich *et al.* 1999, Macpherson *et al.* 2002, Naz and Padman 1999, Ostermeier *et al.* 2005, Skinner 1991).

It has been proposed that spermatozoal mRNA transcription profiles may be used as a non-invasive tool to investigate poor fertility in the male (Ostermeier *et al.* 2005). In human spermatozoa, many mRNA's have been discovered that reflect gene expression during spermatogenesis. These may serve as markers for male fertility (Ostermeier *et al.* 2002, Ostermeier *et al.* 2005). However, spermatozoal RNA isolation protocols require species-specific optimization. Spermatozoal mRNA isolation protocols have been described for the human (Goodrich *et al.* 2007, Lambard *et al.* 2004), bovine (Gilbert *et al.* 2007), boar (Yang *et al.* 2009) and recently the stallion (Das *et al.* 2010).

1.9. Involvement of IGF and insulin in some aspects of male reproduction

Ejaculated mammalian sperm must undergo a set of biochemical and physiological changes termed capacitation before they can undergo the acrosome reaction and are capable of fertilising an oocyte (Yanagimachi 1994). Some of the physiological changes constituting capacitation may be regulated by IGF-1R and INS-R located in the plasma membrane of human

spermatozoa (Aquila *et al.* 2005, Naz and Padman 1999, Sanchez-Luengo *et al.* 2005).

IGF-1 is recognised as an important regulator of several male reproductive functions, including modulation of gonadal steroidogenesis in the rat, and spermatogenesis and sperm function in man (Lin *et al.* 1986, Naz and Padman 1999, Ovesen *et al.* 1995, Spiteri-Grech and Nieschlag 1992). The identification of receptors for both IGF-1 and IGF-2 on Leydig and Sertoli cells, spermatogonia, spermatocytes, and spermatids indicate that these growth factors are local regulators of testicular function (Funk *et al.* 1992, Hansson *et al.* 1989, Henricks *et al.* 1998, Zhou and Bondy 1993). In the bovine it has been demonstrated that IGF-1 is present in seminal plasma and can interact with a specific IGF-1R on the acrosomal region of ejaculated spermatozoa, increasing sperm motility and straight line velocity (Henricks *et al.* 1998). The location of IGF-1R primarily on the apical ridge of the acrosome in bovine spermatozoa, suggests that growth factor signaling may be important in capacitation and the acrosome reaction (Henricks *et al.* 1998).

Macpherson and coworkers identified IGF-1, IGFBP-2, and IGFBP-5 in equine seminal plasma. They examined the relationship between seminal plasma IGF-1 concentration and fertility parameters in stallions, and demonstrated a positive but variable association. Stallions with high seminal plasma IGF-1 levels had better sperm motility and sperm morphology, and resultant pregnancy rates in bred mares were higher (Macpherson *et al.* 2002). The source of equine seminal plasma IGF-1 has not been determined, but is probably similar to men, which has been shown to be primarily of testicular or epididymal origin (Ovesen *et al.* 1995).

The onset of puberty in the stallion appears to be closely associated with plasma and testicular IGF-1 levels, and thus its primary reproductive role may be to promote cell division and regulate spermatogenesis (Hess and Roser 2001). Hess and Roser (2001) demonstrated that plasma and testicular concentrations of IGF-1 are significantly higher in colts younger than 2 years of age than in adult stallions between 5-23 years of age. They also compared

stallions with differing fertility status and the results showed that there was no significant difference in IGF-1 levels between these groups of stallions. They therefore suggest that neither plasma nor testicular IGF-1 levels are reliable markers for diagnosing sub-fertility and infertility in stallions (Hess and Roser 2001).

Champion *et al.* (2002), showed that GH and IGF-1 are effective in promoting the in vitro longevity of equine sperm. Motility was maintained longer in spermatozoa treated with either recombinant bovine growth hormone (rbGH) or recombinant human IGF-1 (rhIGF-1) during a 24 hour period at room temperature.

Mice with an IGF-1 null mutation had significantly poorer reproductive parameters, both sexes were affected, female mice failed to ovulate even after supplemental gonadotropin administration, and the male mice had dramatically reduced serum testosterone, concentrations of spermatozoa and libido (Baker *et al.* 1996). In the male growth-hormone (GH)-deficient dwarf (*dw/dw*) rat, fertility is reduced and appears to be associated with compromised spermatogenesis as well as impaired sperm motility (Gravence *et al.*1997). Sub-fertile *dw/dw* rats treated with recombinant bovine growth hormone (rbGH) demonstrated an increase in percentage of motile spermatozoa (Breier *et al.* 1996). IGF-1 treatment showed similar effects on improving motility, and additionally improved morphological parameters of spermatozoa in GH-deficient rats (Vickers *et al.* 1999). These findings together with the data published by Henricks *et al.* (1998) describing IGF-1 receptors on spermatozoa, suggests that the ability of IGF-1 to improve motility is the direct effect of elevated IGF-1 levels in seminal plasma acting through the IGF-1R in the sperm plasma membrane.

Insulin has been shown to play a central role in male gonadal function. In human spermatozoa, receptor-mediated binding of insulin to the acrosome has been demonstrated, and both the sperm plasma membrane and the acrosome represent cytological binding targets for insulin (Silvestroni *et al.* 1992). Aquila and coworkers showed that insulin mRNA and insulin protein are present in

ejaculated human spermatozoa. They also detected insulin secretion from ejaculated human spermatozoa and suggested that this sperm-derived insulin may be involved in the induction of capacitation (Aquila *et al.* 2005)

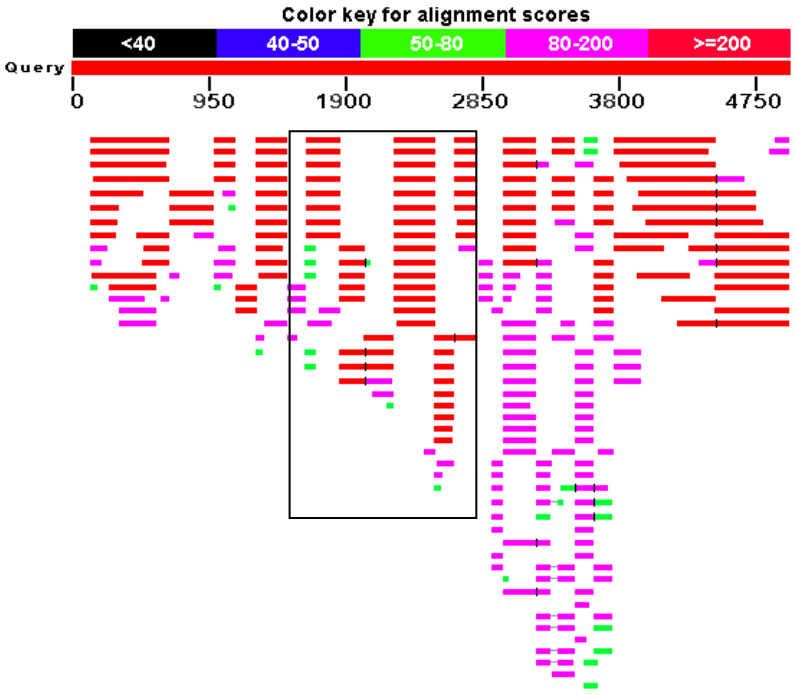
CHAPTER 2 Materials and Methods

2.1. Sequencing of equine IGF-1R and INS-R mRNA

2.1.1. Design of sequencing primers

The complete *Equus caballus* genome had not been published at the time of the study. Only a trace-file archive of the genome was available publically on GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed). The human mRNA sequences for IGF-1R (accession number NM 000875.2) and INS-R (accession number NM 000208.1) were assumed to be closely related to the equine equivalents and were used to perform a discontinuous megablast (Altschul *et al.* 1990) with the equine trace-file archive to identify equine IGF-1R and INS-R trace file sequences (Figure 1). A region of the human mRNA of approximately 500 to 1500 base pairs (bp) in length was randomly selected and trace files within this region assembled using the Staden package (Staden 1996; Staden *et al.* 2000). Four contigs (a length of contiguous sequence assembled from partial, overlapping sequences) were obtained for IGF-1R and five for INS-R. A local alignment between each contig (genomic DNA) and the respective human mRNA sequence was performed using Water EMBOSS (Rice *et al.* 2000).

a) Human IGF-1R mRNA



b) Human INS-R mRNA

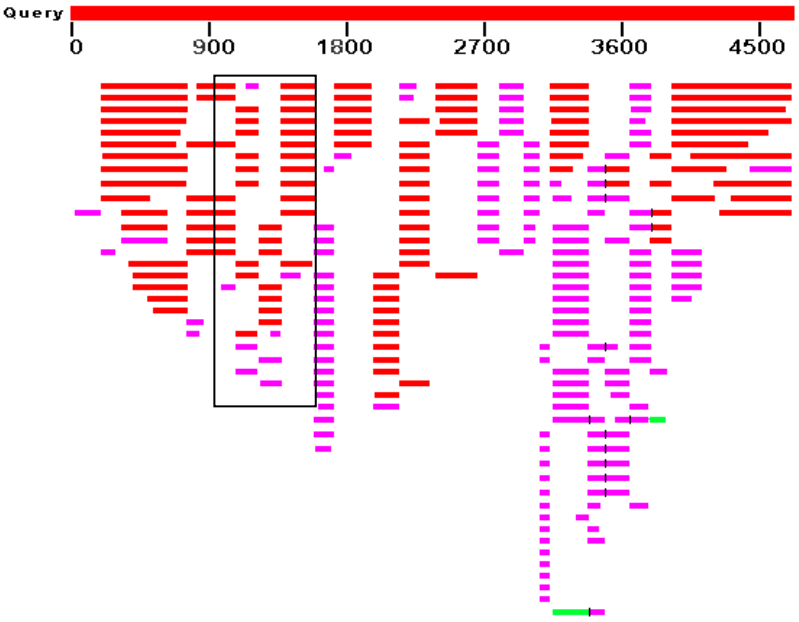


Figure 1. An overview of the *Equus caballus* trace file archive aligned to human insulin-like growth factor 1 receptor (IGF-1R) (a) and insulin receptor (INS-R) (b) mRNA (Acc. No. NM_000875.2 and NM_000208.1). The alignment score is indicated by one of five different colours. Blocked regions refer to the trace files selected for alignment and assembly. IGF1R (1650 – 2850 bp) and INSR (1000 – 1550 bp).

An exon was identified as a region with a high degree of alignment. The non-exon sequences were deleted from the trace file and exons were aligned with the human mRNA (Figure 2 and Figure 3). Exon-exon junctions were identified which corresponded with the human exon-exon junctions. The putative equine mRNA sequences were used to design sequencing primers with the aid of FastPCR® software v3.6.89 (Kalender 2007). Two pairs of primers were designed and used for IGF-1R due to the large sequence size (>1 000 bp) and one pair for INS-R (Table 1, Figure 2 and Figure 3).

Table 1. Primer names and sequence for sequencing of equine IGF-1R and INS-R.

IGF-1R	
SH_IGF1R_EQSEQ_F1878_1900	5'-gct aat tgt gaa gtg gaa ccc ac-3'
SH_IGF1R_EQSEQ_R2378_2398	5'-gta caa agt gaa tgg ccg gag-3'
SH_IGF1R_EQSEQ_F2289_2311	5'-aca atg tca cag acc cag agg ag-3'
SH_IGF1R_EQSEQ_R2742_2764	5'-aga aca cag gat cag tcc acg ac-3'
INS-R	
SH_INSR_EQSEQ_F1037_1057	5'-tga tgt gta ccc cgt gcc tgg-3'
SH_INSR_EQSEQ_R1505_1525	5’-tgg tct tca ggg cga tgt cgt-3'

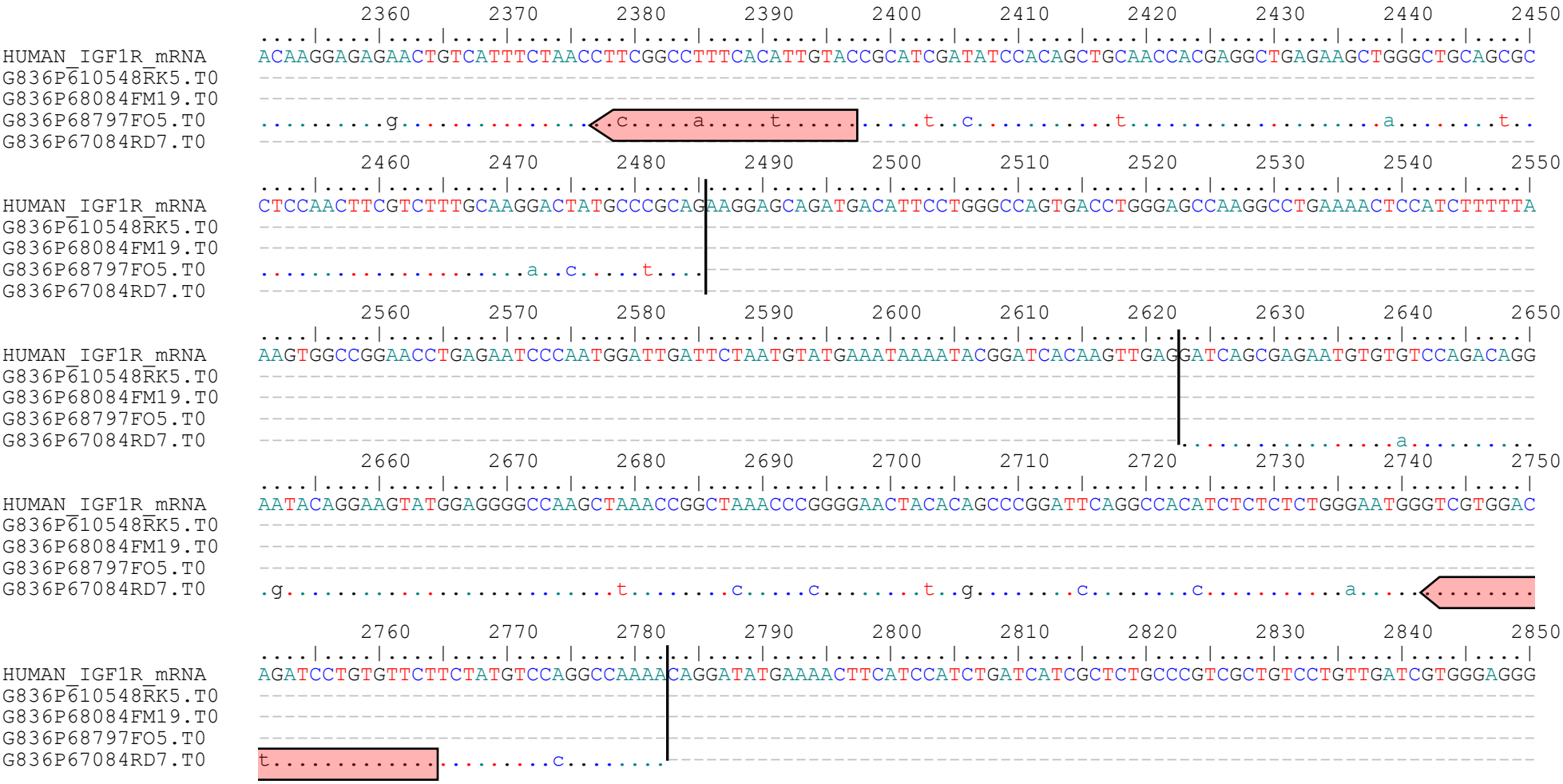


Figure 2. Alignment of four equine trace-file contigs (non-exons deleted) with the human IGF-1R mRNA. Two pairs of sequencing primers, forward primer (⇨) and reverse primer (⇦). Exon-exon junctions (|).

Figure 3. Alignment of four equine trace-file contigs (non-exons deleted) with the human INS-R mRNA. One pair of sequencing primers, forward primer ( ) and reverse primer ( ). Exon-exon junctions (|).

2.1.2. RNA extraction from equine testes

Testes were harvested from each of the three healthy Friesian, Thoroughbred, and Warmblood stallions admitted for routine orchidectomies at the Onderstepoort Veterinary Academic Hospital (OVAH). Samples of parenchyma from these testes were collected using sterile forceps and a surgical blade. To avoid DNA contamination between stallion samples, a new set of instruments were used per stallion. The samples were approximately 5.0 x 5.0 x 5.0 mm in size, and were placed into plastic 2.0 ml cryotubes (Nunc) containing approximately 10 volumes of RNA*later*® (Ambion). The samples were kept at 4 °C for 24 h and then stored at -80°C until analysed.

To extract RNA, a sample of testicular tissue from each stallion (n=3) was thawed at room temperature and 30 mg of this tissue was placed into a MagNA Lyser® Green Beads tube (Roche) containing 1.4 mm ceramic beads, 900 µl RLT buffer (Qiagen) and 9 µl 14.3 M β-mercaptoethanol (β-ME) (Sigma), The samples were homogenized with a MagNA Lyser® Instrument (Roche) and two runs of 7 000 rpm for 45 s were performed, with cooling on ice for 2 min between runs. Samples were kept at room temperature for 30 min, then centrifuged at 14 000 rpm for 3 min. The supernatant was removed (600 µl) and transferred to 1.5 ml microcentrifuge tubes.

RNA was extracted from the samples using the RNeasy® Mini kit (Qiagen) according to the manufacturer's instructions and eluted in 50 µl nuclease-free water. RNA concentration and purity was determined from the A_{260}/A_{280} ratio, as measured by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

2.1.3. Reverse transcription polymerase chain reaction (RT-PCR)

A one-step reverse transcription polymerase chain reaction (RT-PCR) was performed with the GeneAmp® Gold RNA PCR Core Kit (Applied Biosystems)

according to the manufacturer's instructions. Two 25 µl reactions were performed per RNA sample for each primer pair (Table 1, Table 2).

Table 2. RT-PCR mix for sequencing equine IGF-1R and INS-R mRNA.

	Volume (µl)
5 X RT-PCR Buffer	5.00
25 nM MgCl2	1.75
10 nM dNTP blend	2.00
100 nM DTT	1.25
RNase inhibitor	0.25
Amplitaq Gold	0.25
Multiscribe RT	0.15
Forward primer (20 nM)	0.50
Reverse primer (20 nM)	0.50
RNA	2.00
RNase-free H2O	11.35
Total Volume	**25.00**

Cycling conditions for the RT-PCR were 42 °C for 12 min, 95 °C for 10 min and 40 cycles at 94 °C for 20 s, 57 °C for 30 s and 72 °C for 60 s, followed by a final extension step at 72 °C for 7 min and a hold at 4 °C on a GeneAmp® PCR System 9700 (Applied Biosystems).

Five µl of PCR product was mixed with 1 µl loading dye (Fermentas), loaded onto an ethidium bromide stained 1.5 % agarose gel, electrophoresed at 110 volts and visualized by UV trans-illumination**.**

The two RT-PCR reactions per sample were combined and purified using a QIAquick® PCR purification kit (Qiagen), according to the manufacturer's instructions. The purified DNA was eluted in 50 ul EB buffer (Qiagen).

2.1.4. Sequencing

A BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used for sequencing. A minimum of two forward and two reverse ¼ reactions (4 – 7 µl DNA, total volume per reaction = 20 ul) were performed per sample according to the manufacturer's instructions. Sequence reactions were performed in 96-well plates using a GeneAmp® PCR System 9 700 (Applied

Biosystems). Cycling conditions were as described in the Applied Biosystems protocol.

After cycling, 50 µl 100% ethanol (Merck), 2 µl 125 mM EDTA (Ambion) and 2 µl 3M sodium acetate pH 5.5 (Ambion) was added to each sample and incubated for 15 min at room temperature. Samples were centrifuged at 4 230 rpm (2 500 g) for 30 min in a Sorvall® RC6 centrifuge (Kendro Laboratory Products). The plate was inverted immediately after centrifugation and spun at 1 000 rpm (acceleration 7) for 1 min to remove the supernatant. Seventy µl 70% ethanol was added to each reaction and the plate centrifuged at 3 140 rpm (1 650 g) for 15 min. The supernatant was removed (as described above) and the pellet air dried.

Ten µl Hi-Di™ formamide (Applied Biosystems) was added to each sample and the mixture heated to 94 °C for 2 min, followed by cooling at 4 °C. Samples were analyzed with an ABI 3130xl Genetic Analyzer using POP-7 polymer and a 36 cm capillary (Applied Biosystems).

2.1.5. Sequence analysis

Phred (Ewing and Green 1998; Ewing *et al.* 1998) and the Staden package (Staden 1996; Staden *et al.* 2000) were used for base calling and sequence assembly. The assembled IGF-1R and INS-R sequence for each of the three breeds of stallion was aligned with the equivalent human mRNA sequence (GenBank accession numbers NM 000875.2 and NM 000208.1) using ClustalW (www.ebi.ac.uk/Tools/clustalw).

The identities of the sequence results were compared to the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990).

2.2. Development of real-time RT-PCR assays for equine IGF-1R and INS-R mRNA

2.2.1. Primer and probe design

The sequences obtained were analyzed with Primer Express® software (Applied Biosystems) in order to design equine IGF1-R and INS-R mRNA primers and TaqMan® TAMRA™ probes. The probes were designed to span exon-exon junctions to ensure detection mRNA amplification over genomic DNA (

Table 3). The probes were also and labeled with different fluorescent dyes (FAM and VIC) to allow differentiation between IGF1-R and INS-R.

Table 3. Primer and TaqMan® TAMRA™ probe names and sequences for a real-time RT-PCR assay of equine IGF-1R and INS-R.

IGF1R	
SH_IGF1R_F2445_2465	5'-cag tgc ctc caa ctt cgt ctt-3'
SH_IGF1R_R2496_2512	5'-ccg gcc cag gaa tgt ca-3'
SH_IGF1R_P2468_2493	5'VIC-caa gaa cca tgc ctg cag aag gag ca-3'
INSR	
SH_INSR_F1286_1306	5'-tcc gga agt tac gcc taa ttc-3'
SH_INSR_R1340_1359	5'-ctg gtt gtc caa ggc gta ga-3'
SH_INSR_P1311_1338	5'FAM-tga gta gtt ccc gat ttc caa ggt ctc t-3'

The additional criteria used to select the TaqMan probes were: a guanine-cytosine content in the range of 30-80%; no runs of an identical nucleotide; no guanine on the 5’ end; a strand with more cytosine’s than guanine’s, and for the primer selection: primers chosen after the probe; the primers designed as close as possible to the probe without overlapping the probe; a guanine-cytosine content in the range of 30-80%; no runs of an identical nucleotide; the five nucleotides at the 3’ end should have no more than two guanine and/or cytosine bases.

a)

```
TGAGATGGCA GCGACAGCCT CAGGACAGCT ACCTTTACCG GCACAATTAC        50
TGCTCCAAAG ACAAAATCCC CATCAGGAAG TATGCCGATG GCACCATTGA       100
TGTTGAAGAG GTGACGGAGA ATCCCAAGAC CGAAGTGTGT GGTGGAGAGA       150
AAGGGCCTTG CTGTGCTTGC CCCAAAACCG AAGCCGAGAA GCAGGCCGAG       200
AAGGAGGAAG CCGAGTACCG CAAAGTCTTT GAGAATTTCC TGCACAACTC       250
CATCTTTGTG CCCAGACCTG AAAGGAAGCG GAGAGATGTC ATGCAGATTG       300
CCAACACTAC CATGTCCAGC CGAAGCAGGA ACACCACGAT GGCAGACACC       350
TACAATGTCA CAGACCCAGA GGAGCTGGAG ACAGAATACC CTTTCTTTGA       400
GAGCAGAGTG GACAACAAGG AGAGGACTGT CATTTCTAAC CTCCGGCCAT       450
TCACTTTGTA CCGCATTGAC ATCCACAGCT GTAACCACGA GGCTGAGAAG       500
CTAGGCTGCA GTGCCTCCAA CTTCGTCTTT GCAAGAACCA TGCCTGCAGA       550
AGGAGCAGAT GACATTCCTG GGCCGGTGAC CTGGGAGCCA AGGCCTGAAA       600
ATTCCATCTT TTTAAAGTGG CCAGAACCTG AGAATCCCAA TGGATTGATT       650
CTAATGTATG AAATAAAATA TGGATCACAA ATCGAGGATC AGCGAGAATG       700
TGTATCCAGA CAGGAGTACA GGAAGTATGG AGGGGCCAAG CTTAACCGGC       750
TCAACCCCGG                                                  760
```

b)

```
GTCCCTGTCC CAAGGTGTGC CACCTCCTGG AAGGCGAGAA GACCATCGAC        50
TCGGTGACGT CCGCCCAGGA GCTCCGAGGC TGCACGGTCA TCAACGGGAG       100
CCTGATCATC AACATCCGAG GGGGCAACAA CCTGGCAGCT GAGCTAGAGG       150
CCAACCTTGG ACTCATTGAA GAAATTTCAG GGTATCTGAA AATCCGCCGG       200
TCCTACGCTC TCGTGTCGCT TTCCTTCTTC CGGAAGTTAC GCCTAATTCG       250
GGCAGAGACC TTGGAAATCG GGAACTACTC ATTCTACGCC TTGGACAACC       300
AGAACCTGAG GCAGCTATGG GACTGGAGCA AGCACAACCT CACCATCACT       350
CAGGGGAAAC TCTTCTTCCA CTATAATCCC AAACTCTGCT TGTCGGAAAT       400
TCACAAGATG GAGGAAGTTT CAGGAACCAA AGGGCGCCAG GAGAGGA          447
```

Figure 4. Primers (arrows) and TaqMan® TAMRA™ probes (blocks) for a) IGF-1R and b) INS-R. real-time RT-PCR assays (▬ = exon-exon junction).

2.2.2. Cloning and characterization of PCR amplicons

The real-time RT-PCR products were purified with the QIAquick PCR purification kit (Qiagen) and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The amplicons were cloned using the pGEM® - T Easy Vector System (Promega) (Figure 5) according to the manufacturer's instructions. An insert:vector molar ratio of 3:1 was used for ligation. Transformation reactions were set up using JM109 High Efficiency Competent Cells (Promega). Transformation cultures were plated onto LB/ampicillin/IPTG/X-Gal plates (Invitrogen) and incubated overnight at 37 °C. White colonies which contained the inserts were selected, inoculated into 4 ml of LB medium (Invitrogen) and incubated overnight at 37 °C. An aliquot of each sample was mixed with an equal volume of glycerol, snap frozen in liquid nitrogen and stored at -80 °C. The remaining sample was purified using the

High Pure Plasmid Isolation Kit (Roche) according to the manufacturer's instructions.

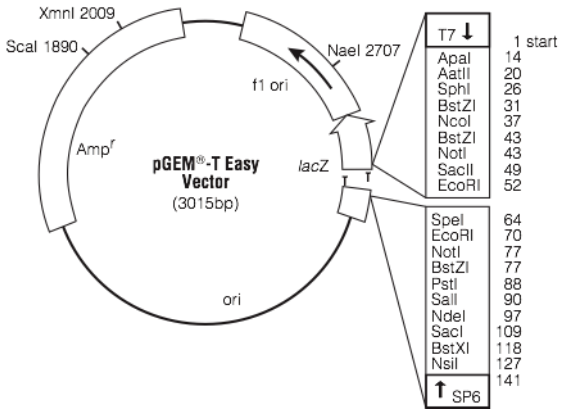


Figure 5. pGEM®-T Easy Vector circle map and sequence reference points.

Plasmids were sequenced using T7 and SP6 primers and those with mutations were discarded. The identities of the sequence results were compared to the GenBank nucleotide database using the megablast option (http://www.ncbi.nlm.nih.gov/sites/entrez).

2.2.3. Generation of IGF-1R and INS-R standard curves

Sal I (Promega) was selected to linearize the plasmid, as: i) the enzyme's restriction site is downstream from both the plasmid insert and T7 promoter, ii) it does not digest the plasmid insert, as checked using the Staden package and iii) it does not leave 3' overhangs, as a low level of transcription from such templates has been reported (Schenborn and Mierendort 1985). The linearised template DNA was run on a 1.5% agarose gel to confirm that cleavage was complete.

The linearized plasmid was transcribed using the MEGAshortscript™ Kit (Ambion), according to the manufacturer's instructions. The DNA template was removed by treatment with 3 µl TURBO DNase/sample and an additional purification with QIAzol (Qiagen) was done according to the manufacturer's instructions. RNA was resuspended in 20 µl TE buffer (Ambion) and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific),

using an extinction coefficient of 33 for small RNA < 200 nucleotides (MEGAshortscript™ protocol). The RNA concentration and the molecular weight of the transcript, as indicated in the Staden package, were used to calculate the RNA copy numbers. A 10-fold dilution series (1×10^0 to 1×10^9 copies/µl) was aliquoted and stored at -80 °C until needed. The control step for the presence of plasmid DNA in the serial RNA dilutions was checked by running real-time PCR on the RNA, without first performing a reverse transcription reaction (no-RT control).

Real-time RT-PCR of the standard dilution series was repeated in triplicate on two separate days. All the data were used to calculate a linear regression equation in Microsoft Excel® of cycle threshold (C_T) against log copy number. The regression equation was used to calculate mRNA copy numbers per reaction from the C_T. Efficiency of the assay was calculated using the following equation: efficiency = $10^{-1/slope}$ - 1. The limit of detection was defined as the copy number concentration where 95 % of the RT-PCR's would yield a positive result ($C_T \leq 40$) and was calculated using the Karber equation (Karber 1931).

2.2.4. Quantification of IGF-1R and INS-R mRNA in equine tissues

2.2.4.1. Collection and RNA extraction from equine tissues

Tissue samples from four mixed-breed adult horse mares were collected immediately after slaughter at a horse abattoir. The samples collected were obtained from the liver, spleen, kidney, lung, heart, skeletal muscle and endometrium and RNA was extracted (as described in 2.1.2.), with the one exception that RNA was eluted in 30 µl volumes, .

2.2.4.2. Quantitative two-step real-time RT-PCR

TaqMan® reverse transcription reagents (Applied Biosystems) were used as described in the supplied protocol. Reverse transcription was performed with random hexamers. A volume of 7.7 µl RNA was added to the reagents up to a total volume of 20 µl/reaction. Samples were placed in an Applied Biosystems

9 700 thermal cycler and the following conditions were used: 25 °C for 10 min, 42 °C for 30 min, 95 °C for 5 min and a hold at 4 °C.

A LightCycler® TaqMan® Master kit (Roche) was used as described in the supplied protocol for the real-time PCR. Five µl of cDNA, a final concentration of 0.5 µM primers and a final concentration of 0.1 µM probe were used per reaction. Cycling conditions were as described in the protocol, with an annealing temperature of 60˚C. Simultaneously with each sample tested, a no-RT control was run as a control to screen for genomic DNA contamination. Copies per gram of tissue were calculated using the following formulae:

$$\text{copies/mg tissue} = \frac{\text{copies/reaction}}{\text{RNA/reaction}^{b}} \times \frac{\text{RNA}^{a}}{\text{mg tissue extracted}^{c}}$$

$$\text{copies/mg tissue} = \frac{\text{copies/reaction}}{7.7 \div 20 \times 5} \times \frac{30}{\text{mg tissue sample} \times 600 \div 900}$$

$$\text{copies/mg tissue} = \frac{\text{copies/reaction}}{1.925} \times \frac{45}{\text{mg tissue sample}}$$

a The volume (µl) of RNA extracted using the RNeasy spin column (Qiagen).
b 7.7 µl of RNA were converted into 20 µl of cDNA and 5 µl of cDNA used/reaction.
c Tissue was homogenized in 900 µl RLT buffer (Qiagen) and 600 µl used for RNA extraction.

CHAPTER 3 Results

3.1. Sequencing of equine IGF-1R and INS-R mRNA

After total RNA extraction from the equine testes and amplification thereof, the RT-PCR products were visualised on a gel and were of the expected size (Figure 6). For IGF-1R the expected sizes were 520 and 453 base pairs and for INS-R 488 base pairs, respectively. The sequences obtained for IGF-1R and INS-R mRNA were identical for each breed of horse (Friesian, Warmblood and Thoroughbred). Use of the BLAST showed a 92 % and 93 % identity of the equine IGF-1R and INS-R mRNA sequences with the human homologs (Figure 7).

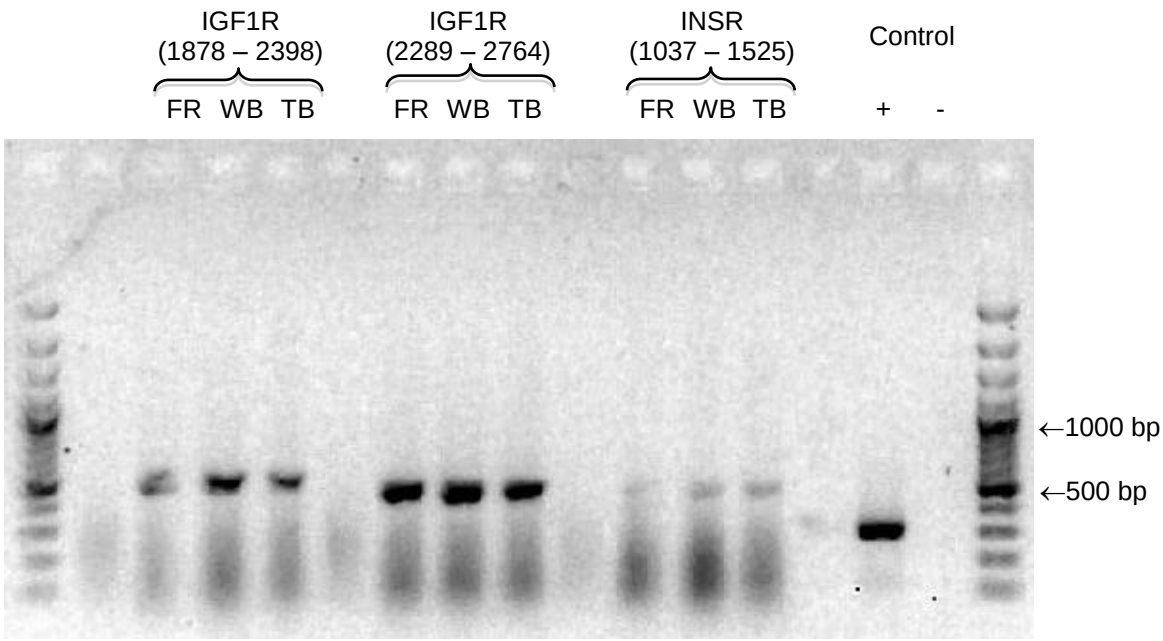
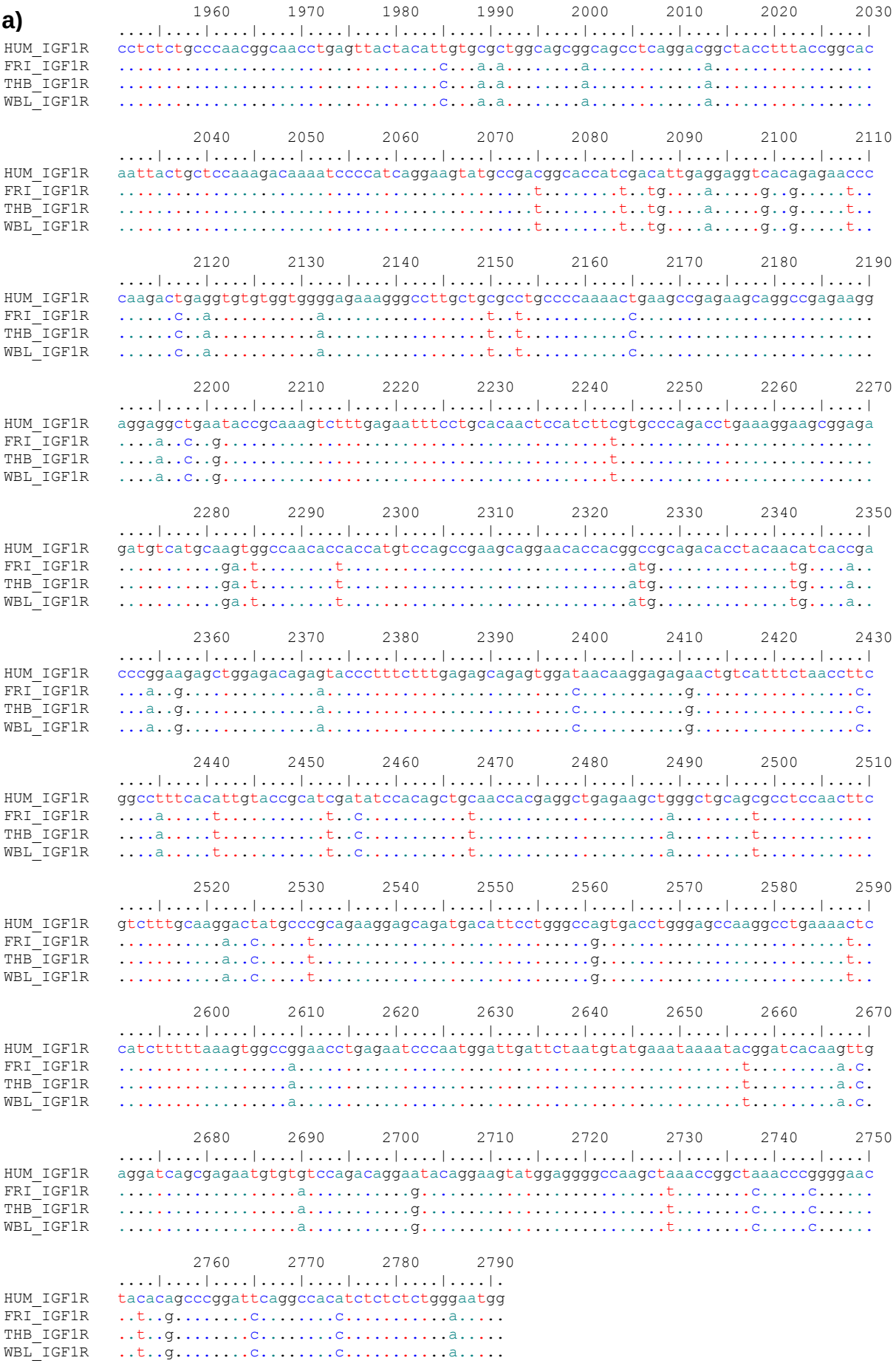


Figure 6. Agarose gel of IGF-1R (520 and 453 bp) and INS-R (488 bp) PCR products, with positive (300 bp) and negative RT-PCR controls and DNA ladders in first and last wells. Amplified regions of IGF-1R and INS-R mRNA indicated in legends. FR – Friesan, WB – Warmblood, TB – Thoroughbred.

a)



b)

```
             1110      1120      1130      1140      1150      1160      1170      1180
            ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
HUM_INSR    gtccctgtcccaaggtgtgccacctcctagaaggcgagaagaccatcgactcggtgacgtctgcccaggagctccgagga
FRI_INSR    ........................g.............................c.................c
THB_INSR    ........................g.............................c.................c
WBL_INSR    ........................g.............................c.................c

             1190      1200      1210      1220      1230      1240      1250      1260
            ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
HUM_INSR    tgcaccgtcatcaacgggagtctgatcatcaacattcgaggaggcaacaatctggcagctgagctagaagccaacctcgg
FRI_INSR    .....g.............c.............c.....g........c.................g........t..
THB_INSR    .....g.............c.............c.....g........c.................g........t..
WBL_INSR    .....g.............c.............c.....g........c.................g........t..

             1270      1280      1290      1300      1310      1320      1330      1340
            ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
HUM_INSR    cctcattgaagaaatttcagggtatctaaaaatccgccgatcctacgctctggtgtcactttccttcttccggaagttac
FRI_INSR    a.........................g...........g...........c.....g......................
THB_INSR    a.........................g...........g...........c.....g......................
WBL_INSR    a.........................g...........g...........c.....g......................

             1350      1360      1370      1380      1390      1400      1410      1420
            ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
HUM_INSR    gtctgattcgaggagagaccttggaaattgggaactactccttctatgccttggacaaccagaacctaaggcagctctgg
FRI_INSR    .c..a.....g................c...........a.....c..................g........a...
THB_INSR    .c..a.....g................c...........a.....c..................g........a...
WBL_INSR    .c..a.....g................c...........a.....c..................g........a...

             1430      1440      1450      1460      1470      1480      1490      1500
            ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
HUM_INSR    gactggagcaaacacaacctcaccatcactcaggggaaactcttcttccactataaccccaaactctgcttgtcagaaat
FRI_INSR    ..........g.....................................t................g.....
THB_INSR    ..........g.....................................t................g.....
WBL_INSR    ..........g.....................................t................g.....

             1510      1520      1530      1540      1550
            ...|....|....|....|....|....|....|....|...
HUM_INSR    ccacaagatggaagaagtttcaggaaccaaggggcgccaggagagaa
FRI_INSR    t..........g...............a.............g.
THB_INSR    t..........g...............a.............g.
WBL_INSR    t..........g...............a.............g.
```

Figure 7. Similarity between human and equine a) IGF-1R and b) INS-R mRNA**.**

3.2. Development of quantitative real-time RT-PCR assays for equine IGF-1R and INS-R mRNA

Clones with the real-time RT-PCR product insert were sequenced to confirm the presence and sequence of the insert. BLAST results were used to confirm the specificity of the assay - only IGF-1R and INS-R mRNA sequences from various species (Table 4) were identified.

Table 4. Discontiguous megablast of the real-time RT-PCR product sequence showing the five most similar results.

	Query coverage (%)	Maximum identity (%)
IGF-1R mRNA		
Equus caballus	100	100
Sus scrofa	97	98
Bos taurus	97	96
Canis familiaris	97	96
Homo sapiens	97	93
INS-R mRNA		
Equus caballus	100	100
Pan troglodytes	100	93
Homo sapiens	100	91
Macaca mulatta	100	91
Ovis aries	100	91

The plasmid was linearised (circular DNA linearised) completely with *Sal* 1 restriction enzyme. Transcription of the plasmid yielded 2.40 and 4.51 µg RNA/µl for IGF-1R and INS-R respectively.

Plasmid DNA contamination was evaluated by running a no-RT control and a no-template control in the real-time PCR. Plasmid DNA was detected, but for IGF-1R, the DNA concentration was 9 800, and for INS-R 76 000, times less, respectively than the RNA, and was therefore considered to be not significant (Table 5).

Table 5. Comparison between RT and no-RT controls, using cycle threshold (C_T) values of an IGF-1R and INS-R dilution series.

INS-R			IGF-1R		
log copies/µl	C_T (RT)	C_T (no-RT)	log copies/µl	C_T (RT)	C_T (no-RT)
9	18.55	31.98	9	16.49	30.32
8	22.46	35.33	8	19.78	34.04
7	27.61		7	23.89	35.89
6	30.99		6	26.81	44.71
5	35.05		5	29.65	
4	38.39		4	33.61	
3	40.39		3	36.10	
2			2	38.50	

The calculated regression equation for the IGF-1R dilution series is: y = -3.301x + 45.59, and for the INS-R dilution series: y = -3.444x + 47.42 (Figure 8). The efficiency and sensitivity of the IGF-1R assay are 1.01 and 192 copies/µl and for the INS-R assay 0.95 and 891 copies/µl respectively.

3.3. Application of quantitative real-time RT-PCR assays for equine IGF-1R and INS-R mRNA

The log transformed mean IGF-1R mRNA concentration of equine (n = 4) tissue samples (liver, spleen, heart, lung, kidney, muscle and endometrium) is $10^{4.91} \pm 10^{1.04}$ standard deviations (SD) copies per mg tissue with a range of $10^{3.66}$ (spleen) to $10^{6.19}$ (heart) (Figure 9a). Using RNA as the unit of measurement the mean IGF-1R mRNA concentration is $10^{4.78} \pm 10^{1.63}$ SD per µg RNA with a range of $10^{2.92}$ (spleen) to $10^{6.83}$ (muscle). In all cases, with the exception of heart and muscle, the IGF-1R mRNA concentrations per mg tissue are higher than the concentration per µg RNA.

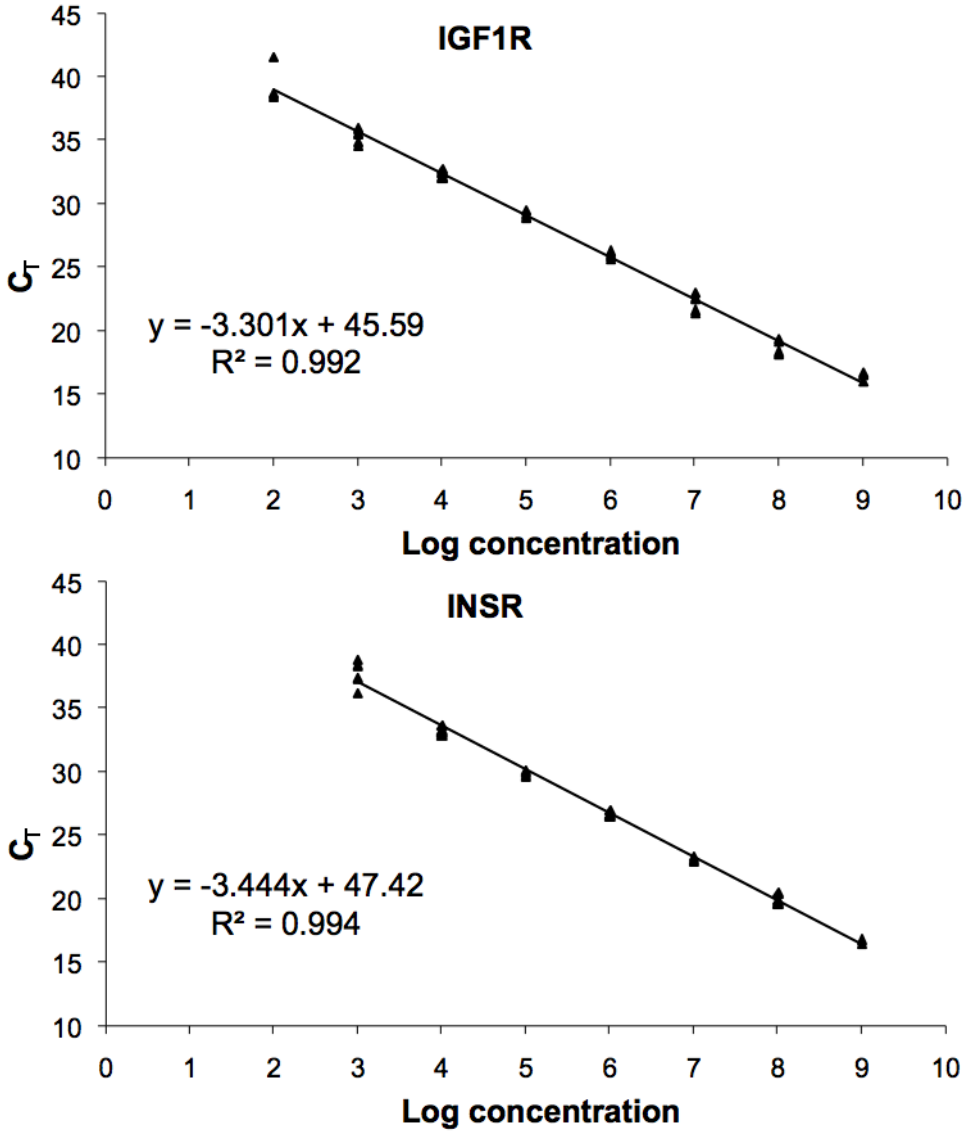


Figure 8. Linear regression of 6 replicates of IGF-1R and INS-R standard curves (from CT real-time PCR results).

The log transformed mean INS-R mRNA concentration of the same tissue samples is $10^{5.32} \pm 10^{1.35}$ SD copies per mg tissue with a range of $10^{3.75}$ (spleen) to $10^{6.54}$ (kidney), and $10^{5.19} \pm 10^{1.89}$ SD per µg RNA with a range of $10^{2.97}$ (endometrium) to $10^{7.48}$ (muscle) (Figure 9b). In all cases, with the exception of heart and muscle, the INS-R mRNA concentrations per mg tissue are higher than the concentration per µg RNA.

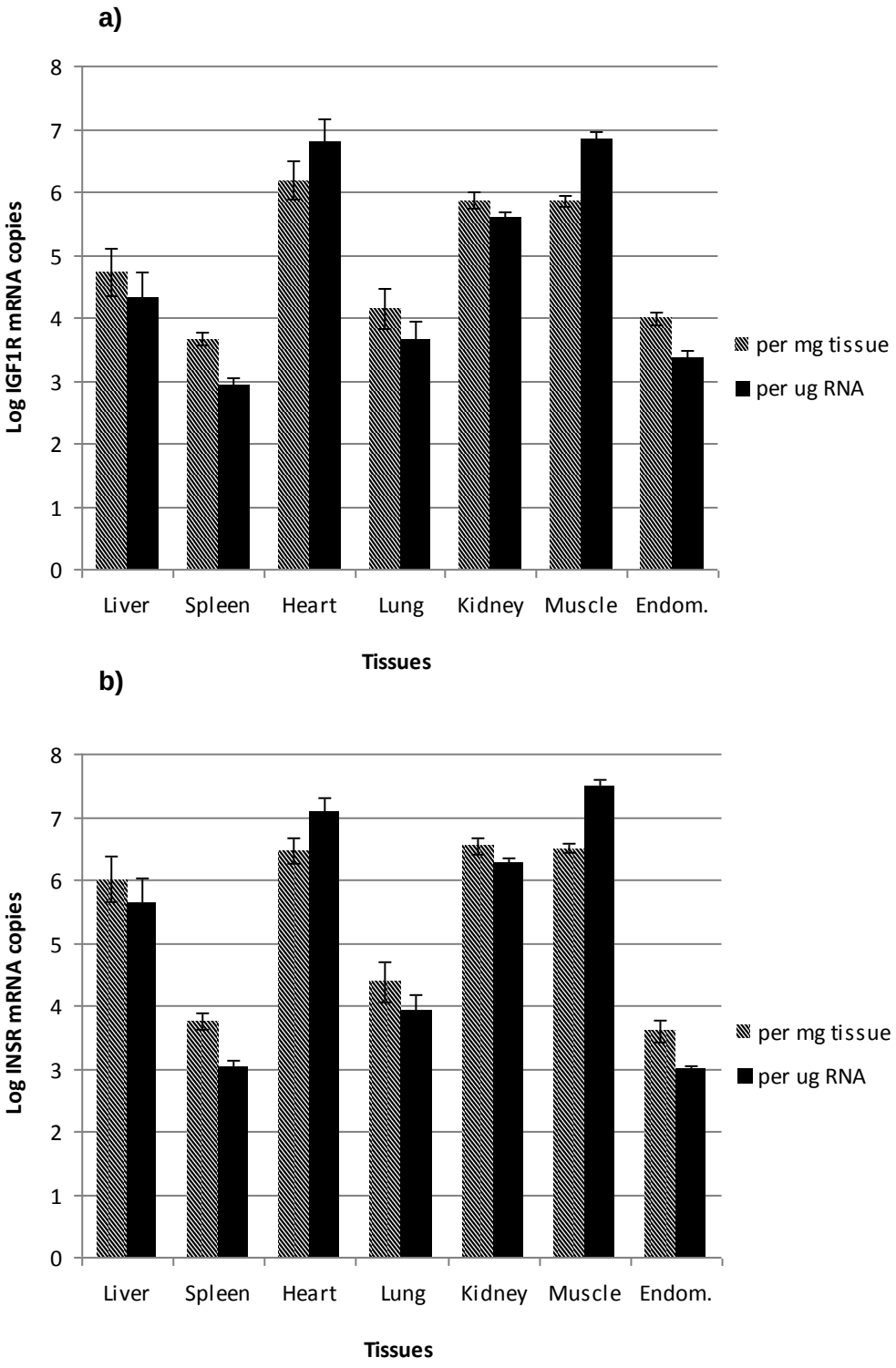


Figure 9. Mean log a) IGF-1R and b) INS-R mRNA copies per mg tissue (downward diagonal) and µg RNA (black) ± standard error (n = 4) (Endom. – endometrium).

The IGF-1R and INS-R profiles in various tissues are similar. Low concentrations of both IGF-1R and INS-R mRNA were detected in endometrium, lung and spleen samples, while high concentrations were detected in heart, muscle and kidney. For all tissues, the INS-R mRNA concentration is higher than the IGF-1R mRNA concentration (Table 6). The mean INS-R:IGF-1R mRNA ratio for equine liver, spleen, heart, lung, kidney, muscle and endometrium tissue samples is 1.08 ± 0.02 (95% confidence interval). Liver has the highest (22 times), and endometrium the lowest (0.5 times) concentration of INS-R to IGF-1R mRNA.

Table 6. Log INS-R:IGF-1R mRNA ratios in various tissues from four mares (Horses 1 – 4), C.I. – 95% confidence interval.

	Liver	Spleen	Heart	Lung	Kidney	Muscle	Endometrium
Horse 1	17.70	0.98	1.40	2.85	2.74	3.32	0.26
Horse 2	37.39	1.00	1.22	1.38	9.01	5.08	0.36
Horse 3	11.16	0.89	1.89	1.51	4.74	5.49	0.26
Horse 4	21.14	2.71	4.23	1.74	4.31	4.49	1.16
Mean	**21.85**	**1.39**	**2.19**	**1.87**	**5.20**	**4.60**	**0.51**
C.I.	**10.93**	**0.86**	**1.36**	**0.66**	**2.63**	**0.93**	**0.43**

Chapter 4 Discussion

This study describes the development of real-time RT-PCR assays for the preliminary characterisation of IGF-1R and INS-R mRNA expression in selected equine tissues. In order to develop these real-time RT-PCR assays, equine IGF-1R and INS-R trace files were identified from a megablast with human IGF-1R and INS-R mRNA. This initial step was necessary as the only public horse genome data that were available at the time of our study, was a DNA trace-file archive, accessible through GenBank. There is more equine genome data now available, but much of the sequence data are predicted, whereas the sequence data used in this study were generated from equine mRNA. This step in the experimental design increased the complexity of the study.

The assays were designed to span exon-exon junctions to preferentially amplify mRNA over genomic DNA. Exons were identified as areas with a high degree of identity between the equine DNA trace-files and the human mRNA sequences. The exons in the equine trace files were separated by large regions of non-similar sequences. The exon-exon junctions of the equine mRNA were identical to that of the human, suggesting a common homology.

A region of both equine IGF-1R and INS-R mRNA that spanned exon-exon junctions was amplified by PCR and we obtained products of the expected size when compared to a DNA ladder on agarose gel. The PCR products were sequenced and were shown to be highly homologous to the human IGF-1R and INS-R mRNA.

Both assays are efficient, sensitive and have a broad linear range of detection (7 logs for IGF-1R and 6 logs for INS-R). The assays worked well in our hands and were sensitive and specific for the detection of equine IGF-1R and INS-R mRNA.

The IGF-1R and INS-R's are present on the surface of almost all cell types (Desoye *et al.* 1997; LeRoith *et al.* 1995; Seino *et al.* 1990). However, the expression of these receptors in different tissue types may vary depending on the metabolic and mitogenic characteristics of the cells within these tissues and there may be variation between individual animals due to age (Georgieva *et al.* 2003; Hess and Roser 2001; Lackey *et al.* 2000), nutritional state (Balage *et al.* 1990; LeRoith *et al.* 1995) and stage of reproductive cycle (Desoye *et al.* 1997; Ginther *et al.* 2003; Lackey *et al.* 2000; Shimizu *et al.* 2008; Silva *et al.* 2009). In our study the IGF-1R and INS-R profiles in various equine tissues were similar. Low concentrations of both IGF-1R and INS-R mRNA were detected in endometrium, lung and spleen samples, while high concentrations were detected in heart, muscle and kidney samples. The high level of INS-R expression in heart, muscle and kidney is probably associated with the high level of glucose metabolism and utilization by these tissues. For all tissues, except endometrium, the INS-R mRNA concentrations were higher than the IGF-1R mRNA concentrations. The mean INS-R:IGF-1R mRNA ratio was highest in liver (22:1) and lowest in endometrium tissue (1:2). The liver manufactures most of the circulating IGF-1 (Jones and Clemmons 1995; Navarro *et al.* 1999) and has the highest levels of IGF-1 mRNA and peptide expression, but the lowest expression of IGF-1R mRNA (Jones and Clemmons 1995). It is highly probable that an organ manufacturing a hormone with an endocrine function would have a low concentration of receptors for that hormone. This would ensure that the hormone enters the general circulation and exerts its endocrine effect elsewhere in the body.

The sensitivities of the assays at 95% using the Karber equation are 192 copies/ µl and 891 copies /µl for IGF-1R mRNA and INS-R respectively. This allows analysis of very small amounts of mRNA. However, for these assays to be reliable they need extensive and accurate optimization. Hence, a study describing the steps used to optimize and validate the assays for real-time RT-PCR of equine IGF-1R and INS-R mRNA is needed, and should attempt to avoid co-amplification of genomic DNA, reduce inter-assay variability, and be normalized to a relevant housekeeping gene.

Measuring gene expression at the mRNA level requires the inclusion of a reliable housekeeper gene for accurate data interpretation (housekeeper genes are evenly expressed among different tissues of an organism, at different stages of development, between normal and diseased states and should not be affected by experimental treatment itself e.g. beta-actin, glyceraldehyde-3-phosphate dehydrogenase, 18S ribosomal RNA). The assays may then be used routinely and incorporated into gene expression research in the field of equine medicine, such as joint cartilage injury and repair, as well as in the field equine reproduction, such as stallion fertility. Quantifying the amount of mRNA indirectly using real-time PCR enables the measurement of differential gene expression, but the level of mRNA transcription may not be necessarily proportional to the level of mRNA translation due to RNA-binding translational activators and repressors. Hence, real-time PCR should be coupled to techniques for measuring or quantifying protein levels (TaqMan® Protein Assays, Western blotting, ELISA or immuno-precipitation immuno-fluorescence).

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