UNIVERSTY OF SOUTHAMPTON

Environment of the early embryo and its effect on development and postnatal life

by

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A thesis submitted for the degree of PhD

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July 2004

UNIVERSTY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

BIOLOGICAL SCIENCES

Doctor of Philosophy

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Past studies have demonstrated that early embryo environment can alter the relative size of the blastocyst trophectoderm (TE) and inner cell mass (ICM) populations, and can lead to alterations in the viability, growth and physiology of the fetus, conceptus and during postnatal life. For example, rats fed a maternal low protein diet exclusively during the preimplantation period (0-4.25 days post mating), produced blastocysts with significantly reduced numbers of ICM and TE cells (Kwong *et al.*, 2000). Offspring developing from these embryos showed gender specific altered birth weights (female offspring), development of adult hypertension (male offspring), and disproportionate growth of specific organs compared to body weight (male offspring).

My hypothesis is that the environment of the preimplantation embryo can affect aspects of pre and post-implantation development, which can then subsequently impinge upon postnatal growth, development and physiology. I have investigated the impact of mouse preimplantation environment on embryo lineage allocation and overall size, and have related this to possible long term effects after embryo transfer. Blastocysts developed *in vitro* from the 2-cell stage had reduced total cell numbers compared to blastocysts developed *in vivo* at 96 hours post hCG; however, the ICM:TE ratio was equivalent between these two groups. *In vitro* culture in either IGF-I (1.7 nM and 8.5 nM) or insulin (170 nM and 850 nM) significantly increased ICM cell numbers and the ICM:TE ratio ($p < 0.05$). Altering the size of the pre-compact embryo so as to half or double the normal number of cells resulted in significantly altered total blastocyst cell numbers ($p < 0.05$), with the ICM:TE ratio increasing as the cell number increased ($p < 0.05$). Aggregated ICMs isolated from early blastocysts produced blastocyst-like structures with significanly increased ICM:TE ratios ($p < 0.05$).

Using embryo transfer techniques, I have compared the postnatal development of embryos derived from an *in vitro* environment (termed '*in vitro*' mice) with embryos derived from an *in vivo* environment (termed '*in vivo*' mice). In turn, these two groups have been compared with mice derived from naturally mated and superovulated mothers, which are acting as non-embryo transfer controls. One group of offspring have had their litter size corrected at birth to a mean of 6 ('naturally mated (6)' mice), whilst another to 4 ('nautrally mated (4)' mice) so as to be comparable with experimental groups involving embryo transfer.

No significant differences in birth weight were observed between *in vitro* and *in vivo* offspring. However, *in vitro* offspring were significantly lighter that *in vivo* offspring in a gender dependent manner at 2 weeks of age (males, $p = 0.009$) and at 6 and 11 weeks of age (females, $p = 0.037$ and 0.035 respectively). In addition, at 4 weeks of age, the *in vivo* males became significantly lighter when compared to the naturally mated males ($p = 0.034$). At 10, 12 and 13 weeks of age, male offspring derived from embryos cultured in the presence of 1.7 nM IGF-I became significantly lighter than naturally mated (4) males ($p < 0.05$). Male mice derived from embryos disaggregated at the 8-cell stage became significantly heavier from 12 weeks of age, and remained heavier for the remainder of the 27 week study.

At 8, 15 and 21 weeks of age, mice derived from embryos developed *in vivo* or cultured *in vitro* had significantly elevated systolic blood pressure when compared to non-embryo transfer controls ($p < 0.05$). At 15 and 21 weeks of age, female offspring from embryos cultured in T6 PVP or 1.7 nM IGF-I, and male and female offspring derived from disaggregated and reaggregated embryos all had significantly elevated systolic blood pressure when compared to naturally mated (4) offspring ($p < 0.05$). At 27 weeks of age, significant differences were observed in specific organ:body weight ratios between embryo transfer and the naturally mated (6) and (4) groups.

One gene that has been shown to influence the rate of mouse preimplantation development is the *Ped* (preimplantation embryo development) gene. Linkage analysis has shown the *Ped* gene maps to the Qa-2 sub-region of the mouse major histocompatibility complex (MHC) and is expressed on the surface of *Ped* plus embryos from the oocyte stage. Embryos expressing the *Ped* gene (B6 K2, *Ped* plus) develop at a faster rate and containing more cells than those which do not express the gene (B6 K1, *Ped* minus).

I have investigated the postnatal development of B6 K1 and B6 K2 mice to determine the influence of *Ped* gene expression on growth and physiology. I have observed that B6 K2 mice were heavier at birth than the B6 K1 mice. There are significant differences in the post natal growth rates between the two strains ($p < 0.05$), with the B6 K1 mice becoming significantly heavier during specific early periods of postnatal life. B6 K1 mice had significantly elevated systolic blood pressure at 21 weeks of age when compared to B6 K2 mice ($p = 0.006$). Altered organ:body weight ratios were observed, with the B6 K1 females having a significantly higher ratio for their lungs, when compared to B6 K2 female mice.

B6 K1 mice were observed to have significantly elevated serum angiotensin converting enzyme activity, possibly contributing to the elevated blood pressure observed.

Collectively, the data from my thesis support the hypothesis that the environment of the early embryo may influence its long term postnatal development and physiology.

This study has been supported by the Medical Research Council of the UK, and the University of Southampton DOHaD Centre.

List of contents

List of Figures

List of Tables

Acknowledgements

Firstly, I would like to acknowledge my two supervisors, Professors Mark Hanson and Tom Fleming who, through their constant encouragement, enthusiasm and belief, have guided me thought the past four years of my Ph.D. In particular, I would like to thank Mark for giving me the opportunity to work upon a Ph.D that was virtually tailor made for me and my interests, and to Tom for his endless commitment, support and for an office door that was always open. Along with my supervisors, I would like to acknowledge Tom Fleming's group at Boldrewood. In particular I would like to thank Bhav, Judith and Lydia for answering my constant stream of questions and for their invaluable input and suggestions regarding my experiments. A special thanks also goes to Dr Clive Osmond, whos' help and suggestions regarding the statistical analysis of my data has been invaluable.

Intellectual input out side of the lab has been provided by Lee Brawley, Rohan Lewis and Chris Torrens, usually of an evening and over a pint or two. I must thank these individuals for not only being good friends over then past four years, but also (Chris) being a great house mate. These, and a cohort of others, have also provided much needed social relief, particularly when experiments were not going as well as I would have hoped. Cheers guys.

I particularly wish to thank my parents. Without their ceaseless support, encouragement and love, I would never have got to where I am now, and I would never have had the opportunities that I have had. Thank you both!

Finally I would like to thank Jane, who over the past two years has had to put with my general complaining and moaning, but has always been there for me. I feel very privileged to be with you. Thank you!

I would therefore, like to dedicate this thesis to
my parents, my niece Lily and to Jane

List of abbreviations

anti-DNP	Anti-Dinitrophenyl-BSA antibody solution
ART	Assisted reproductive techniques
BSA	Bovine serum albumin
cdk	Cyclin dependent kinase
Cdx2	Caudal-related homeodomain protein 2
DMEM	Dulbecco's modified Eagles medium
DMR	Differentially methylated region
DnmtI	DNA methyltransferase 1
ESC	Embryonic stem cells
GLUT	Energy-independent facilitative glucose transporters
FCS	Fetal calf serum
FGF-4	Fibroblast growth factor-4
FST	Fine Scientific Tools
DOHaD	Developmental Origins of Health and Disease
ICM	Inner cell mass
IGF-I	Insulin like growth factor-1
IGF-IR	Insulin like growth factor-1 receptor
IP_3	Inositol 1,4,5-trisphosphate
GFP	Green Fluorescent Protein
GPI	Glycosylphosphatidylinositol
hCG	Human chorionic gonadotrophin
LH	Luteinizing hormone
LIF	Leukaemia inhibitory factor
LOS	Large offspring syndrome
MHC	Major Histocompatability Complex
MPF	Maturation promoting factor
NIDDM	Non insulin dependent diabetes mellitus
NLS	Nuclear localising sequence
PBS	Phosphate buffered saline

PE	Primitive endoderm
Ped	Preimplantation Embryo Development gene
PIP_2	Phosphtidylinositol 1,2 bisphosphate
PLC	Phospholipase C
PMS	Pregnant mare serum gonadotrophin
POU	Pit, Oct, Unc
PVP	Polyvinylpyrrolidone
SEP	Sperm entry point
Sox2	SRY-box containing gene
TE	Trophectoderm
TNBS	Trinitrobenzenesulfonic acid
ZGA	Zygotic genome activation

Chapter 1
Introduction

The initial stages of mammalian preimplantation embryo development are influenced by both genetic determinants and environmental factors. During the preimplantation stages, the embryo forms the first two cell lineages, the trophectoderm (TE) which will predominantly form the placental tissues, and the inner cell mass (ICM) from which will form the fetal tissues. Numerous studies have shown that alterations to the surrounding environment of the preimplantation embryo can have both short and long term effects on development (Bowman and McLaren, 1970; Lane and Gardner, 1997a, 1997b; Kaye and Gardner, 1999; Kwong *et* al., 2000; Khosla *et al*, 2001; Young *et al.*, 2001). It has previously been shown that *in vitro* culture of preimplantation mammalian embryos not only slows developmental rates (Bowman and McLaren, 1970), but can result in altered patterns of gene expression (Khosla *et al.*, 2001) and altered patterns of postnatal growth and organ allometery (Young *et al.*, 2001). It is also possible to increase the rate of preimplantation embryo development via the addition of growth factors such as insulin and insulin like growth factor 1(IGF-I) to the culture media, specifically enhancing development of the ICM (Harvey and Kaye, 1990; Kaye and Gardner, 1999).

It would therefore be of interest to investigate the effect of manipulating the normal patterns of mouse preimplantation embryo development *in vitro* on both short term (preimplantation) and long term (postnatal) development. Such an analysis would enable the relationship between blastocyst cell numbers and cell ratios and postnatal growth patterns, physiology and organ allometery to be evaluated.

1.1 Development of the mouse preimplantation embryo

1.1.1.1 Oocyte maturation and ovulation

In the perinatal mouse, oocytes that have arrested at the diplotene stage of meiosis I become surrounded by a single layer of granulosa cells which in turn, are surrounded by a basal lamina to form primordial follicles (reviewed in Amleh and

Dean, 2002). The granulosa cells and oocyte are connected to each other via gap junctions which enable the cells to co-ordinate development (Boland and Gosden, 1994). In the mouse, after birth, groups of primordial follicles are recruited to undergo a 3 week growth period in which the granulosa cells proliferate into multi-cellular layers leading to the formation of the preantral follicle (reviewed in Amleh and Dean, 2002). After puberty, the primordial follicles undergo further growth, accelerated by the presence of gonadotrophins, developing into antral follicles (Richards *et al.*, 2002). A surge in luteinizing hormone (LH) then initiates ovulation of the oocyte (Lee *et al.*, 1996)

1.1.1.2 Fertilization

Upon fertilization, the developmental program is initiated by a series of Ca^{2+} oscillations, the first of which occurs 1-3 minutes after the sperm cytoplasm becomes incorporated into that of the egg's (Cuthbertson *et al.*, 1981; Cuthbertson and Cobbold, 1985). The first oscillation is observed as a wave that sweeps across the entire egg, originating from the sperm entry point (SEP). These low frequency oscillations then occur repeatedly over the next several hours (Cuthbertson *et al.*, 1981; Cuthbertson and Cobbold, 1985). The exact mechanism of how the sperm initiates these oscillations is still unclear. One hypothesis is that the sperm introduces a factor into the cytoplasm of the egg that leads to inositol 1,4,5-trisphosphate (IP_3) formation, and thus ultimately Ca^{2+} release (Figure 1.1; reviewed in Stricker, 1999). Saunders *et al.*, (2002) identified and cloned a novel sperm-specific Phospholipase C (PLC), PLCξ, capable of triggering Ca^{2+} oscillations exactly like those seen at fertilization. It was also demonstrated that sufficient PLCξ was present within a single sperm to trigger Ca^{2+} release, and that the specific removal of PLCξ from the sperm abolished Ca^{2+} oscillations completely. A nuclear localisation signal sequence (NLS) within mouse PLCξ has recently been identified (Larman *et al.*, 2004), suggesting a possible mechanism for the cell cycle dependency of the Ca^{2+} oscillations observed in fertilised mouse embryos. Larman *et al.*, (2004) hypothesise that at fertilisation, Ca^{2+} oscillations are triggered by sperm PLCξ diffusing through the eggs cytoplasm. As the pronuclei form, PLCξ is sequestered by virtue of its NLS thus resulting in the

cessation of Ca^{2+} oscillations. Upon pronuclear envelope breakdown, PLCξ is released allowing for mitotic cell division, but is subsequently removed from the cytoplasm as the 2-cell nuclei form.

Prior to fertilization, the egg is arrested in metaphase of meiosis II. Upon fertilization, the egg completes meiosis II with the extrusion of the second polar body. Meiotic cell division is under the control of a complex signal transduction pathway comprised of different kinases and maturation promoting factor (MPF). MPF is comprised of two proteins, cyclin B and cyclin dependent kinase (cdk).The Ca^{2+} oscillations initiated at fertilization result in the degradation of cyclin B, enabling the fertilized egg to exit meiosis and subsequently enter into the first mitotic cycle (reviewed in Carroll, 2001). However, Ca^{2+} oscillations are seen to continue until the formation of the pronuclei, approximately two hours later. Therefore, one hypothesis suggests that cessation of Ca^{2+} oscillations may be brought about by the sequestering of either IP_3 or Ca^{2+} releasing factors by the newly formed pronuclei. This hypothesis has been supported by the observation that inhibition of pronuclear formation results in persistent Ca^{2+} oscillations (Marangos *et al.*, 2003).

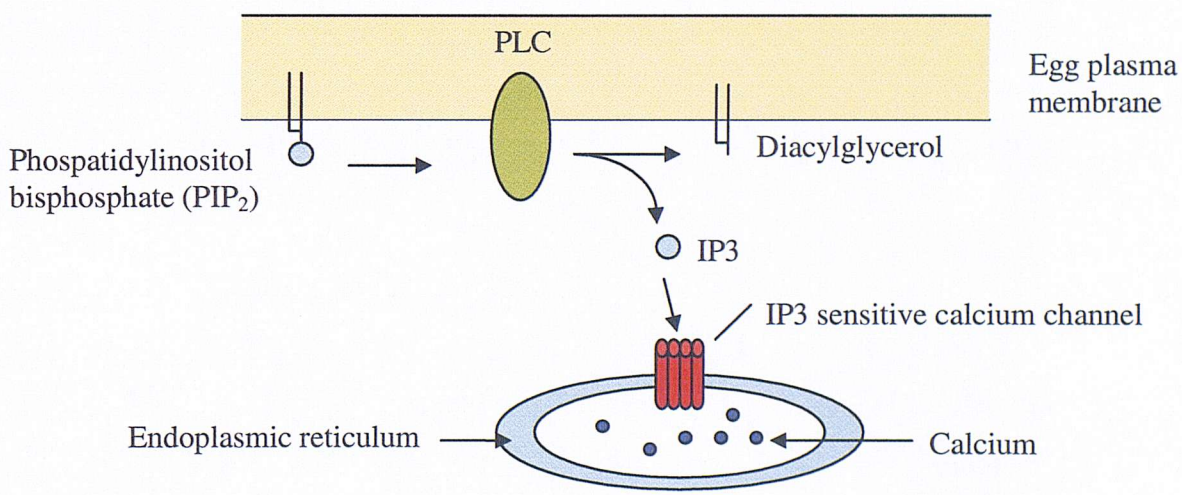


Figure 1.1 Diagram showing the actions of PLC on PIP2 to form IP3. IP3 diffuses through the cytoplasm to the endoplasmic reticulum where it binds to an IP3 sensitive calcium channel causing a release of stored Ca^{2+}.

Studies have demonstrated that different processes in the egg-to-embryo transition are driven by differential responses to Ca^{2+} oscillation number, with the termination of these processes requiring a greater number of oscillations than for their initiation (Ducibella *et al.*, 2002). Manipulation of the number, frequency and amplitude of the Ca^{2+} oscillations can also affect the number of ICM and TE cells in the blastocyst (Bos-Mikich *et al.*, 1997) as well as influencing fetal development (Ozil and Huneau, 2001).

1.1.1.3 Formation of pronuclei

Upon entry into the egg cytoplasm, the sperm nucleus undergoes several dramatic changes. Whilst the egg progresses from metaphase II into telophase II of meiosis, the nuclear envelope of the sperm is broken down, and the disulphide bonds within the protamines associated with the sperm DNA are reduced (reviewed in Collas and Poccia, 1998). Protamines are a group of proteins that bind the DNA and package it to reduce the amount of space the DNA occupies within the nucleus. As the male pronucleus decondenses, the DNA becomes more accessible to the environment of the egg's cytoplasm. The protamines packaging the sperm DNA are replaced by histones derived from the egg. The male and the female pronuclei begin to migrate towards each other, replicating their DNA as they do so. As they meet, their chromatin condenses into chromosomes and align on a common mitotic spindle so that after the first mitotic division a diploid genome becomes established (Gilbert, 1997).

1.1.1.4 Zygotic genome activation (ZGA)

The fertilized egg is able to control the first embryonic cell division via the maternal mRNAs, proteins and macromolecules stored within the oocyte at ovulation. However, maternal and paternal genes must be transcribed for normal development to be maintained. The activation of the zygotic genome takes place in a series of steps with some genes being transcribed earlier and later than the main activation event (reviewed in Latham and Schultz, 2001). In the mouse, the majority of activation occurs at the 2-cell stage (Figure 1.2 **a**), but some gene activation also takes place at

the late 1-cell and 8-cell stages (Wang and Latham, 1997, Wang *et al.*, 2001). A similar stepwise activation process is believed to occur in the human embryo, but with the majority of zygotic genome activation taking place at the 4 to 8-cell stage (Braude *et al.*, 1979).

In order for zygotic genome transcription to take place there needs to be a reorganisation of the chromatin structure. The tight association of histones with the DNA can effectively silence genes by making them inaccessible to the transcription proteins. Alternatively, by changing the composition of the core histones and/or the linker histones, the DNA can become more open to the gene transcription machinery. The binding affinity of histones can be altered by their patterns of phosphorylation and acetylation (Dimitrov *et al.*, 1993; Wiekowski *et al.*, 1997). In the Xenopus egg, histone H4 is initially acetylated on two lysine residues, but these are removed as the histone becomes incorporated into the chromatin after fertilization (Dimitrov *et al.*, 1993). At the 2-cell stage in the mouse, acetylated forms of histone *H2A, H3* and *H4* are seen to accumulate at the nuclear periphery, possibly to be incorporated into the newly formed genomic DNA (Stein *et al.*, 1997). These observations suggest that the degree of acetylation of the histones may reflect the relative transcriptional states of the DNA into which they are to be incorporated.

In the mouse, the maternal mRNA transcripts laid down within the oocyte are degraded, so that by the 2-cell stage the majority of these transcripts are no longer present (Bachvarova *et al.*, 1985; Paynton *et al.*, 1988). The activation of the zygotic genome therefore allows for the replacement of the maternal mRNAs with embryonic transcripts. The 1-cell stage mouse embryo has been shown to alter its levels of gene transcription as it develops. At the early 1-cell stage the embryo is transcriptionally silent, but by the late 1-cell stage there is a step-wise increase in the activation of embryonic genes (Wang *et al.*, 2001). This would imply that the late 1-cell embryo either possesses the necessary transcription factors for embryonic gene transcription, or that it has increased its levels of proteins such as RNA polymerase II. For both of these situations, *de novo* protein synthesis is necessary. Both entry into the

transcriptionally-permissive state at the late 1-cell stage, and ZGA have been shown to be dependent upon protein synthesis, possibly via the recruitment of specific transcription factors controlling the expression of key house-keeping genes necessary for development (Wang and Latham, 1997; Wang *et al.*, 2001).

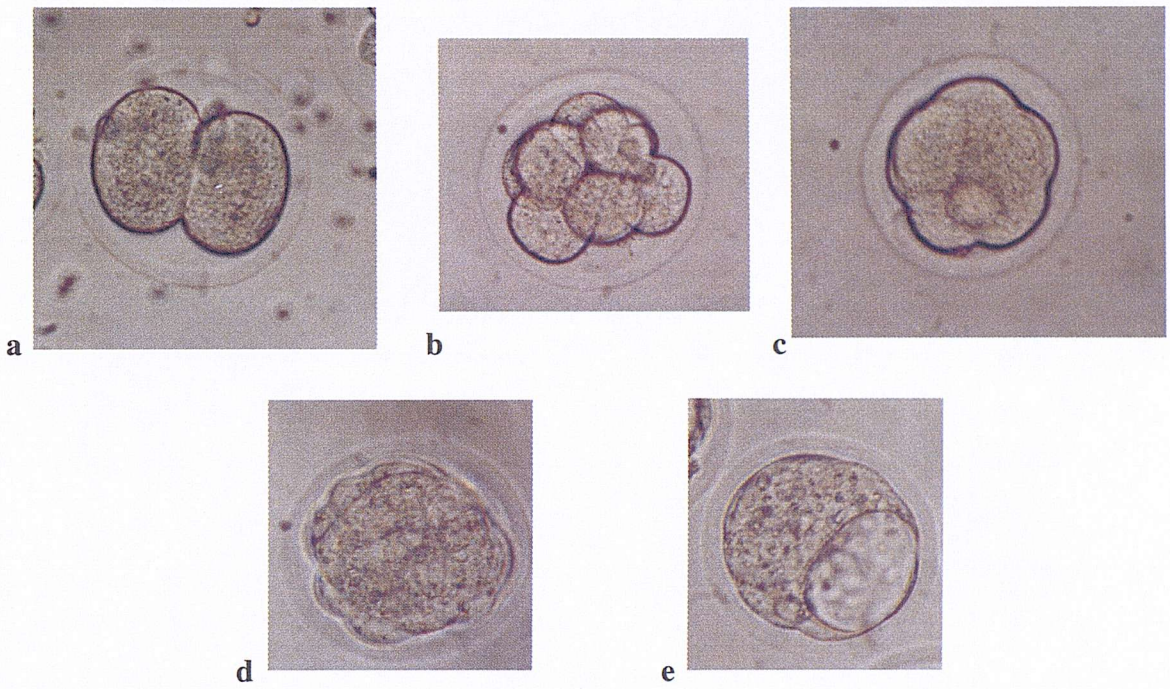


Figure 1.2. Phase-contrast micrograpghs showing stages of mouse preimplantation embryo development, (**a**) the 2-cell stage, the major time of zygotic genome activation. (**b**) The 8-cell stage, at this point all the blastomeres are nonpolar and totipotent. (**c**) Compact 8-cell stage, here the cells are polarising and cell-cell adhesion has taken place. (**d**) The morula (16-32 cells), the formation of the first two distinct cell populations, an outer polarised population surrounding an inner core of nonpolar cells. (**e**) Blastocyst formation (32-64 cells) characterised by the formation of the blastocoel cavity and the establishment of the TE and ICM lineages.

1.1.1.5 Embryo compaction

After the 2-cell stage, the embryo undergoes two reduction cleavage divisions resulting in the generation of eight non-polar, loosely associated, totipotent blastomeres all having a face on the outside of the embryo (Figure 1.2**b**). At this time in the mouse, each of the cells has the capacity to develop into any tissue type found within the fetus or the placenta (reviewed in Fleming and Johnson, 1988). At the 8-cell stage the blastomeres undergo a process of polarisation, characterised by accumulation of actin, microtubules, endosomes and clathrin in the apical (outward

facing) cytoplasm, and microvilli at the apical membrane, whilst the nucleus locates towards the basal cytoplasm (Reeve and Kelly, 1983; Johnson and Maro, 1985; Maro *et al.*, 1985). At compaction, the transmembrane protein E-cadherin redistributes from all membrane surfaces to areas of cell-cell contact (Vestweber *et al.*, 1987), whilst at the basolateral cell surface gap junctions form (reviewed Fleming *et al.*, 2001). The localisation of E-cadherin defines an axis along which the blastomeres can polarise (Ziomek and Johnson, 1980). These structural and cytoplasmic changes in the blastomeres enable the generation of a proto-epithelial phenotype that will ultimately lead to the development of the first transporting epithelium within the embryo.

The process of compaction does not appear to be solely regulated by the expression of the proteins involved in cellular adhesion, since if protein synthesis is inhibited via the use of anisomycin, the timing of compaction is advanced (Levy *et al.*, 1986). As neither incipient protein transcription or translation are required for compaction, then the timing of compaction would appear to be controlled by post-translational modification of existing proteins (Kidder and McLachlin, 1985; Levy *et al.*, 1986; Fleming *et al.*, 2001).

1.1.1.6 Morula formation

The establishment of cell polarity marks the beginning of the ability for the blastomeres to form the first two distinct cell types of the early embryo. After compaction, the plane of cleavage of dividing polarised 8-cell blastomeres generates either polar:polar (conservative division) daughters, or polar:non polar daughters (differentiative division; Figure 1.3). In the 16-cell mouse morula, polar and non-polar cell types occupy outer and inner positions respectively, and tend to give rise to the TE and ICM lineages (Johnson and Ziomek., 1983; Fleming and Johnson, 1988).

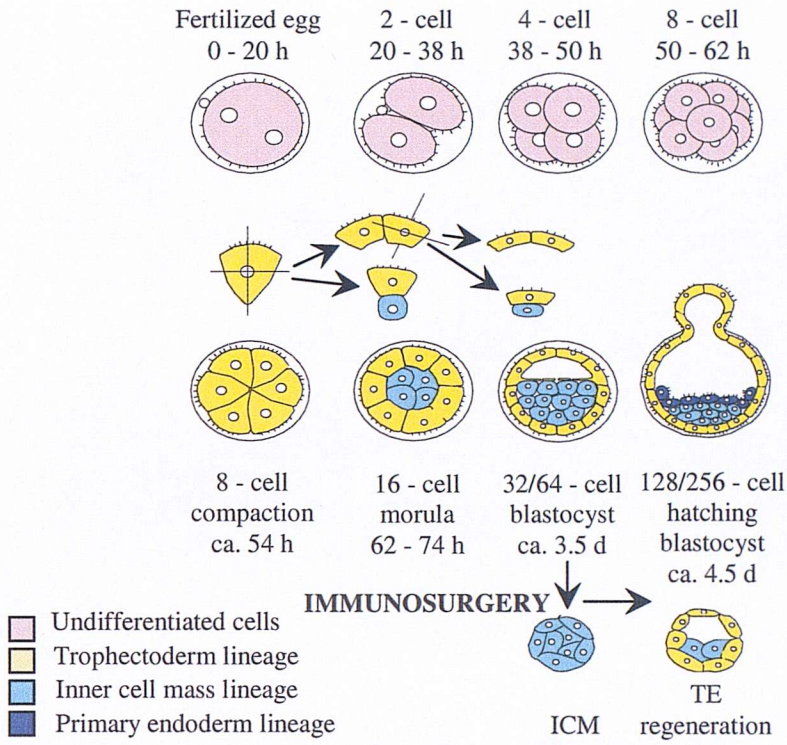


Figure 1.3. Diagram showing the development of the preimplantation mouse embryo. Two rounds of differentiative and conservative cell divisions result in the production of the inner cell mass and trophectoderm lineages respectively (taken from Collins and Fleming, 1995).

At the 16-cell stage (Figure 1.2**d**), the proportions of inner and outer cells can vary from embryo to embryo. Differentiative divisions at the 8-cell stage generate two distinct populations of cells occupying different regions of the embryo (Figure 1.3). A second round of differentiative and conservative cell division of outer polar cells occurs at the 16-32-cell stage, and appears to be regulated by cell contact patterns (reviewed in Fleming *et al.*, 2001). An inverse relationship exists between the number of inner non-polar cells generated from the first and second rounds of differentiative divisions (Figure 1.4). Therefore, the embryo is able to regulate the proportion of cells allocated to the two cell lineages. This regulative capacity appears to be controlled mainly by cell shape and number (Fleming and Johnson, 1988).

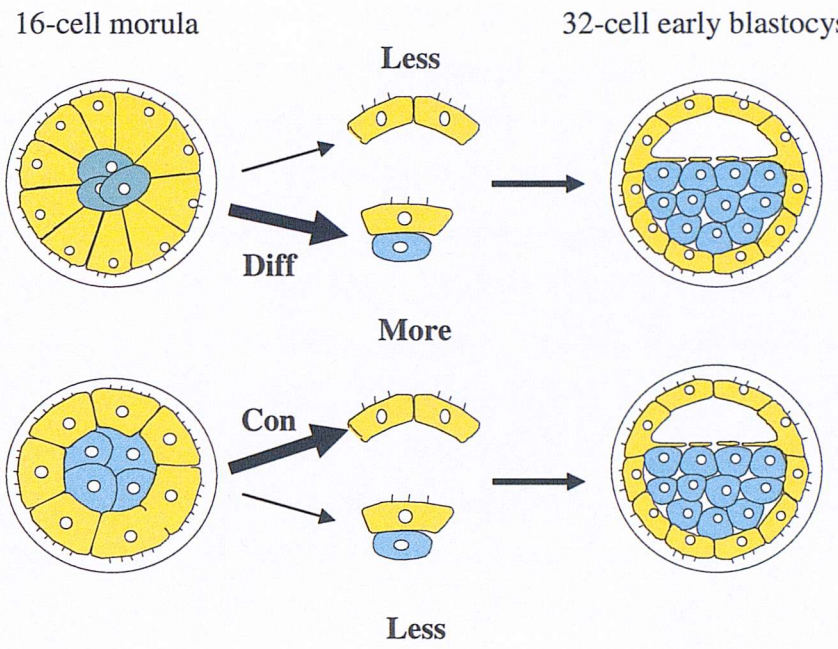


Figure 1.4 Diagram of two 16-cell morula which vary in their outer polar cell (yellow) and inner non-polar (blue) cell populations. Polar cells from morulae with a small inner cell population (top) will tend to divide differentiatively (Diff) more frequently and conservatively (Con) less frequently than polar cells from morula containing a larger non-polar inner cell population (bottom) (adapted from Collins and Fleming, 1995).

1.1.1.7 Blastocyst formation

Formation of the blastocyst (32-cell stage in the mouse) is characterised by the accumulation of the fluid filled blastocoel cavity (Fig 1.2**e**), the development of the TE into a distinct epithelial layer and the generation of the non-polar pluripotent ICM. Two criteria central to the formation of the blastocoel are the formation of tight junctions between the TE cells and presence of appropriate transporter systems within the apical and basolateral membranes of the TE. Tight junctions are composed of several transmembrane and cytoplasmic proteins that bring the two opposing membranes into contact with each other to form a belt-like permeability seal (Fleming *et al.*, 2001; Watson and Barcroft, 2001). The membranes are in such close proximity to each other that molecules can no longer pass between the cells of the TE. This allows the TE to create a sealed environment around the developing ICM into which it can transport proteins, growth factors, amino acids and metabolites, all of which are likely to facilitate the development of the ICM. The fluid within the blastocoel cavity

is formed by the selective transport of specific ions across the TE into extracellular spaces which leads to the production of a concentration gradient driving the movement of water into the extracellular spaces. Na^+ is believed to be one of the main ions transported in the formation of the blastocoel via apically-located Na^+ channels, or via a Na^+ co-transporter (Manejwala *et al.*, 1989). It is then believed that Na^+ is transported across the basolateral membrane of the TE via a Na/K-ATPase which is expressed predominantly from the late morula stage onwards (Watson and Kidder, 1988; Zhao *et al.*, 1997). The Na/K-ATPase is composed of three subunit types, α, β and γ. The α subunit is involved in the transportation of ions, whilst the β subunit is involved in the insertion of the Na/K-ATPase into the plasma membrane (Herrera *et al.*, 1987; Greering, 1991). The exact role of the γ subunit has not yet been characterised (Mercer *et al.*, 1993; Jones *et al.*, 1997). Both the α and β subunits have many isoforms, however, it appears that the α_1 β_1 isoform is the major type involved in blastocyst formation, as it is the only isoform found within the basolateral plasma membrane of mouse TE cells (MacPhee *et al.*, 2000).

Blastocoel formation is also regulated by Cl^- transport into cells via apical membranbe Cl^- channels and then efflux into the blastocoel via a basolateral membrane HCO_3^-/ Cl^- exchanger (Zhao *et al.*, 1997). After selective transport of these ions, water would then relocate into the blastocoel cavity by moving down the concentration gradient, and via aquaporin channels within the TE membranes (reviewed in Watson and Barcroft, 2001).

1.1.1.8 Formation of the primitive endoderm

After four and a half days of development, three cell lineages are apparent within the mouse blastocyst; the epiblast, formed from the ICM and which will give rise to the entire fetus, and the TE and primitive endoderm (PE) which both form extraembryonic tissues including the chorioallantoic placenta and visceral yolk sac respectively. Like the TE, the PE is an epithelial structure, and lies over the free face of the ICM. At this time the TE becomes regionally specialised with respect to its morphology and developmental potential. Those TE cells which lie over the

blastocoel cavity, known as mural TE cells, cease cell division, endoreplicate their DNA and become enlarged trophoblast giant cells. Those TE cells that are in direct contact with the ICM remain proliferative, are known as the polar TE, and go on to form the extraembryonic ectoderm and ectoplacental cone.

1.1.2 Allocation of cells to the blastocyst lineages

Early experiments, which involved the isolation and culture of individual ICMs from blastocysts of different developmental ages, demonstrated that by the expanded blastocyst stage, the ICM was no longer capable of contributing cells to the TE, but did contain cells capable to contributing to the PE (Rossant, 1975a, 1975b; Gardner, 1982). However, ICMs isolated from earlier blastocysts (32-64 cells) could form TE as well as PE (Rossant and Lis, 1979; Nichols and Gardner, 1984). Lineage tracing studies, involving the use of Green Fluorescent Protein (GFP), within individual cells of the ICM, have shown that the daughters of these cells contribute to either the epiblast or the PE, but never both (Weber *et al.*, 1999; Novak *et al.*, 2000). It would therefore appear that by the mid blastocyst stage, ICM cells have become predisposed to either the PE or epiblast lineages.

As the mouse blastocyst develops, differential expression of regulatory transcription factors underlie lineage diversifications. Oct-4 is a member of the POU (Pit, Oct, Unc) family of transcription factors, and in the mouse is present in the nuclei of all cleavage stage cells, but become restricted to the ICM and subsequently the epiblast, thus suggesting that it is a marker of pluripotency (Palmieri *et al.*, 1994). Similar expression patterns are seen in human preimplantation embryos (Hansis *et al.*, 2000), suggesting that Oct-4 may have similar roles in humans and mice (reviewed in Pesce and Scholer, 2001). Oct 4 -/- preimplantation embryos are able to form blastocyst-like structures before arresting development, but the cells of the ICM are not pluripotent, and are only able to differentiate along the extraembryonic trophoblast lineage (Nichols *et al.*, 1998). Oct-4 expression is therefore an essential factor during the segregation of the TE and the ICM lineages.

Oct-4 has been shown to be co-expressed with Sox2 (SRY-box containing gene) throughout cleavage stages and within the ICM and epiblast (Collignon *et al.*, 1996). These together regulate FGF-4, a key ICM-specific growth factor (Yuan *et al.*,1995) which promotes proliferation within the polar TE. Knockout of Sox2 leads to preimplantation embryo lethality, suggesting that for proper development of the epiblast an Oct-4/Sox2 pathway is necessary (Avilion *et al.*, 2003). PE specific gene transcription has also been shown to take place. Transcription factors such as HNF4, GATA-4 and GATA-6 are all expressed within the 4.5 day old PE (reviewed in Rossant *et al.*, 2003).

Development of the trophoblast lineage is not solely the default pathway in the absence of Oct-4 expression. In the early embryo, the caudal-related homeodomain protein, Cdx2, is expressed in a reciprocal pattern to that of Oct-4, becoming restricted to the TE lineage by the blastocyst stage (Beck *et al.*,1995). Knock-out of Cdx2 results in homozygous embryos that fail to implant, indicative of a major trophoblast defect (Chawengsaksophak *et al.*, 1997). Cdx2-/- blastocysts are unable to attach and outgrow in culture, and differentiated trophoblast cells are not detected. All cells within the mutant blastocysts continue to express Oct-4 (reviewed in Rossant *et al.*, 2003). Therefore, loss of Oct-4 and gain of Cdx2 expression is required for TE formation. It is unclear however, whether a direct regulatory network exists between Oct-4, Sox2 and Cdx2 in the early embryo (Figure 1.5). To date, these three transcription factors appear to be the earliest regulators of cell fate in the embryo.

By day 4.5, Oct-4 and GATA-6 have become restricted to the epiblast and PE respectively, but at day 3.5 there appears to be co-expression of these two factors within the ICM (Rossant *et al.*, 2003). It would appear that as cells move to the developing PE then Oct-4 expression is down regulated. Therefore, the level of GATA-6 expression could be a factor determining whether cells of the ICM become PE or epiblast.

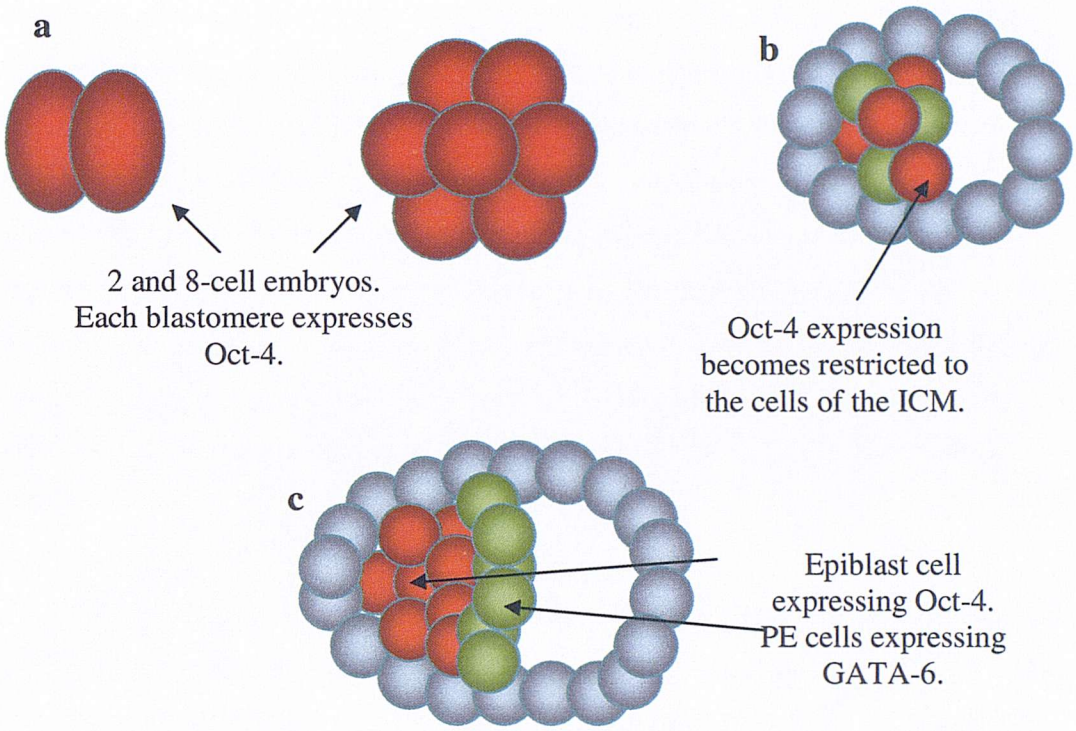


Figure 1.5 Diagram showing the development and expression profiles of key transcripton factors during mouse early development. a, all the blastomeres within cleavage stage embryos express Oct-4 (orange). **b**, Oct-4 becomes restricted to the developing ICM. Some cells of the ICM express GATA-6 (green). **c**, those cells expressing GATA-6 move to the face of the ICM to form the PE (adapted from Rossant *et al.*, 2003)

1.1.3 Patterning of the embryo and the generation of body axes

Establishment of body axes is essential for development and the mapping of the body plan. Until recently it was believed that in the mouse, certain axes were only established after implantation (a quarter of the way through gestation). This, coupled with the extensive developmental plasticity evident especially during the preimplantation stages, led to the belief that establishment of body axes was imposed upon the early embryo, rather than being intrinsic to it (reviewed in Zernicka-Goetz, 2002).

In the mouse, the first axis is evident upon fertilisation and completion of meiosis II with the extrusion of the second polar body (Figure 1.6**a**). This remains as a marker of the animal pole for as long as the polar body exists, and subsequently marks the animal-vegetal axis (Gardner, 1997). However, it has been observed that *in*

vitro the polar body can lie some distance from the furrow between the developing two-cell blastomeres, and that it then moves into the furrow as cytokinesis progresses (reviewed in Gardner and Davies, 2003). The development of the embryonic-abembryonic axis, which is perpendicular to the animal-vegetal pole, is then established at the 3-cell stage by the division plane of the earlier dividing 2-cell blastomere (Figure 1.6**b**). This plane marks the regions of the embryo in which the ICM and blastocoel cavity will preferentially develop, as it has been observed that the earlier dividing 2-cell blastomere contributes disproportionately more cells to the ICM than to the TE (Graham and Deussen, 1978; Garbutt *et al.*, 1987; Gardner, 2001).

Certain studies have suggested that the sperm entry point (SEP) into the egg has a patterning role for the embryo (Piotrowska and Zernicka-Goetz, 2001; Plusa *et al.*, 2002). Piotrowska and Zernicka-Goetz (2001) marked the SEP on the surface of the egg with fluorescent beads and found that the first cleavage division was aligned with respect to the animal pole and SEP. As in previous studies (Graham and Deussen, 1978; Garbutt *et al.*, 1987), Piotrowska and Zernicka-Goetz (2001) also found that the 2-cell blastomere which inherited the SEP went on to divide first, and that the daughter cells of this blastomere contributed disproportionately more to the polar trophectoderm and developing ICM, whereas the later dividing 2-cell blastomere contributed more to the mural trophectoderm and the surface ICM cells that go on to form the primitive endoderm. Thus, Piotrowska and Zernicka-Goetz (2001) believe that as the SEP appears to predict the plane of first cleavage, then it will also establish the embryonic-abembyonic axis via the cleavage of the earlier 2-cell blastomere (Figure 1.6**c**). This observation has recently been disputed; Davies and Gardner (2002) claim that the method employed by Piotrowska and Zernicka-Goetz (2001) to mark the SEP is flawed and, from their own studies, found that there was no relationship between the SEP and the first cleavage plane.

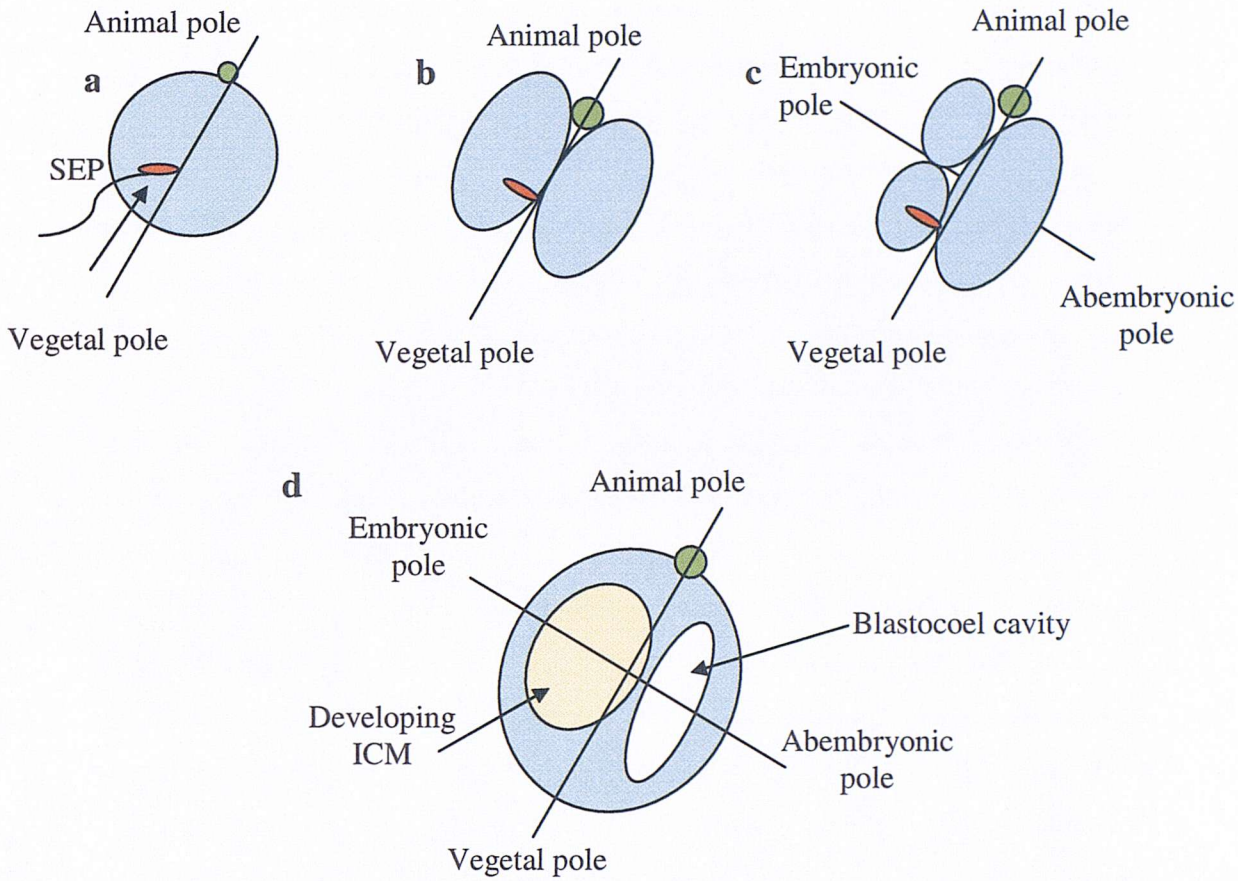


Figure 1.6 The generation of the first two axes within the mouse embryo as suggested by Piotrowska and Zernicka-Goetz (2001). **(a)** the animal-vegetal axis identified via the presence of the second polar body after fertilisation. **(b)** the SEP predicts the plane of the first cleavage. **(c)** the SEP also leads to the establishment of the embryonic-abembryonic axis due to the 2-cell blastomere which inherits the SEP dividing first and subsequently contributing more to the embryonic region of the embryo. **(d)** the early blastocyst with the ICM (embryonic region) and the blastocoel cavity (abembryonic region) (adapted from Johnson, 2001).

Another recent study in which both the SEP and the sperm were labelled, did find a correlation between the SEP and the plane of first cleavage (Plusa *et al.*, 2002). Despite this, there was a population of embryos (22%) that did not show such a correlation, implying that whilst the SEP may be a factor influencing the position of the first cleavage plane, it may not be the only factor.

1.2 Regulation of embryo size and cell number

In vivo, the allocation of cells to the TE and the ICM is a tightly regulated process, resulting in the generation of a blastocyst containing specific proportions of TE and ICM cells. However, *in vitro* this process can be manipulated so that the normal size, cell number and proportions of ICM to TE can become disturbed. This then allows for the study of how the embryo compensates for changes in its overall cell number and size. From initial experiments, it was believed that within the preimplantation embryo there was an inherent control mechanism enabling the embryo to regulate its size, cell number and developmental rate, so that normal development could still occur (Tarkowski, 1959; Mintz, 1965).

Half sized embryos, created via the removal of cells from 8-cell embryos or via the destruction of a single blastomere at the 2-cell stage, are seen to have significantly increased numbers of TE cells, whilst the ICM cell proportion remains similar to that of the controls (Rands, 1986b; Hardy and Handyside, 1993; Hishinuma *et al.*, 1995). Compensatory development has been observed to take place within half sized embryos between days 7.5 to 10.5 *post coitum* (Rands, 1986b; Hishinuma *et al.*, 1995). Somers *et al.*, (1990) observed that in three quarter sized embryos, the ICM to TE ratio was significantly lower at 84, 96 and 102 hours *post coitum.*

Embryos generated to be double the normal size and cell number remain double the normal size for up to 5 days *post coitum* (Lewis and Rossant 1982). Over the next 24 hours these embryos undergo significant size remodelling, so that by 6 days 16 hours *post coitum*, there is no significant difference between the double sized embryos and control embryos in their total cell numbers. Quadruple sized embryos are observed to undergo a period of size regulation lasting several days (Rands, 1986a). By 5.5 days *post coitum*, the quadruple embryos are just over double the size of control embryos and by 6.5 days *post coitum* size regulation is complete. Lewis and Rossant (1982) drew the conclusion that there is a "window" during preimplantation development during which size remodelling of the embryo occurs.

Lewis and Rossant (1982) believed that the pre-implantation embryo is able to alter the length of its cell cycle, in order to compensate for the increase in cell number.

In fetuses derived from half sized embryos there are significantly smaller amounts of ICM derived tissues and more TE derived tissues, implying that the effects of altering the ICM:TE cell ratio are still present during fetal development (Rands, 1986b). Somers *et al.*, (1990) observed that the process of gastrulation was halted by up to 12 hours in three quarter sized embryos. This delay accompanied an increased level of cell proliferation, allowed the embryo to develop to a point where it has the correct number of cells, or was at the right size, to progress through development (Power and Tam, 1993). Hardy and Handyside (1993) suggested that as the volume of the reduced sized embryos is decreased disproportionately to the surface area, then more of the cells would become positioned on the outer face and so the proportion of cells developing as TE would therefore increase. In a quadruple sized embryo, the ICM:TE cell ratio is already akin to that of the controls by day 5.5 *post coitum* despite size regulation still being incomplete (Rands, 1986a).

Biggers and Papaioannou (1991) found that there was no significant difference in the growth rates between mice from the transferred half embryos and transferred controls. It was observed that the process of embryo transfer itself resulted in the production of smaller litters of relatively large animals at birth. These animals went on to remodel their growth rates to the effect that by 4 weeks of age, there were no significant differences between transferred and control mice. Three quarter sized embryos are observed to undergo significant increases in cell proliferation rates between days 6.5 and 11.5 *post coitum* so that by term, offspring are of normal birth weight and had normal body proportions when compared to controls (Somers *et al.*, 1990; Power and Tam, 1993).

Recent studies have focused on the extent to which individual embryos contribute to the various cell lineages present in aggregation chimaeras. Tang and West (2000) examined whether a 4-cell embryo would contribute equally to the ICM

and the TE in a 4-cell diploid-8-cell diploid mouse aggregate. The 4-cell embryo contribution to the aggregate was lower than that of the 8-cell embryo. The 4-cell embryo did not appear to contribute preferentially to any tissue type in the 12.5 day old embryo. The general trend was that the 4-cell embryo with its larger cells, generally contributed to the primitive endoderm rather than other lineages. In a follow up study, Tang and West (2001) investigated the effects of aggregating two 8-cell mouse embryos from different species. In line with observations from previous studies, size regulation was complete by day 12.5 of gestation.

1.3 Preimplantation embryo metabolism

The mouse preimplantation embryo travels through the maternal reproductive track towards the uterus over a period of approximately 3.5 days (Figure 1.7). During this time, the embryo depends heavily on the maternal reproductive tract to provide it with necessary nutrients, metabolites and developmental cues required for cell division and development. During pre- and post-compaction development, the embryo displays different metabolic requirements. Up to the 8-cell stage, the mammalian embryo mainly metabolises pyruvate (Wales *et al.*, 1987; Conaghan *et al.*, 1993; Martin and Leese, 1999). As the embryo proceeds through compaction and towards the blastocyst stage it begins to take up and utilize more glucose and less pyruvate (Wales *et al.*, 1987; Conaghan *et al.*, 1993; Martin and Leese, 1999; Gardner *et al.*, 2001). *In vitro* culture experiments have shown that high glucose levels can inhibit development of the zygote and pre-compact embryo (Conaghan *et al.*, 1993, reviewed in Gardner, 1998). Gardner *et al.*, (2002) proposed that as the pre-compact embryo displays relatively low levels of biosynthesis, an increased ATP:ADP ratio would accumulate within the blastomeres. This would have the effect of inhibiting the influx of glucose via the glycolytic pathway. As the embryo proceeds into compaction, its energy demands increase and the ATP:ADP ratio would fall allowing the increased influx of glucose. Other explanations come from studies into the expression patterns of the energy-independent facilitative glucose transporters (GLUT). In the mouse, GLUT 3 has been shown to be expressed only from the compaction stage onwards (Pantaleon *et al.*, 1997). GLUT3 localises in the apical

membranes of polarised cells within the morula, and then to the apical membranes of the TE where it can then transport maternal glucose (Pantaleon *et al.*, 1997). However, GLUT1 is expressed throughout preimplantation development, and so glucose would be available to the pre-compact embryo. It has therefore been suggested that GLUT1 may act as a detector of the surrounding glucose concentration so that the embryo can coordinate the switch to glucose metabolism with the expression of GLUT3 (reviewed in Pantaleon and Kaye, 1998). Along with the expression of the GLUTs, the activity of the enzymes involved in glucose metabolism have also been show to coincide with the timing of glucose utilization. Hexokinase is the first enzyme in the glycolytic pathway and has some rate limiting control over the entry of glucose into glycolysis. During preimplantation development, hexokinase activity is low but then increases as the embryo proceeds towards cavitation (Houghton *et al.*, 1996), thus matching the profile of glucose utilization in the mouse embryo.

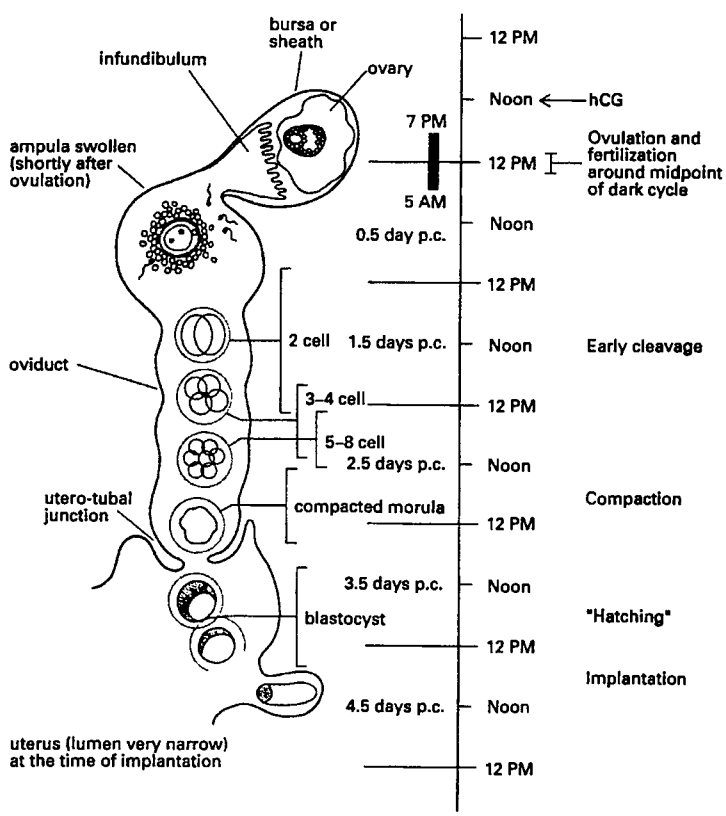


Figure 1.7 Diagram showing the stages of *in vivo* preimplantation development in the mouse (taken from Hogan *et al.*, 1994).

After the activation of the zygotic genome, the embryo increases its levels of protein synthesis (Wang and Latham, 1997; Wang *et al.*, 2001). The maternal reproductive tract is an important source of exogenous proteins and amino acids that the embryo can utilize in the synthesis of its own proteins (Gardner and Leese, 1990). From *in vitro* studies it has been shown that different amino acids enhance different developmental stages. Non-essential amino acids and glutamine significantly increase the rate of cleavage in mouse (Lane and Gardner, 1997a, 1997b), human (Devreker *et al.*, 1998), bovine (Steeves and Gardner, 1999) and sheep (Garnder *et al.*, 1994) pre-compaction embryos. After compaction, essential amino acids have been shown to increase the rate of embryo development, in particular that of the ICM (Gardner *et al.*, 1994, Lane and Gardner, 1997a, 1997b).

Amino acids have also been shown to play an important role in regulating the pH within preimplantation embryos. Whilst mouse and human pre-compact embryos have the necessary transporters to regulate against an increasing alkaline environment, they are not able to regulate against acidosis (Dale *et al.*, 1998; Edwards *et al.*, 1998). One view is that amino acids present within the female reproductive tract may act as zwitterions, being able to chelate or release H^+ ions, thus controlling intracellular pH. In the preimplantation mouse embryo, those amino acids with the greatest buffering capacities, taurine, glycine, alanine, glutamine and aspartate, have all been detected at high levels (Schultz *et al.*, 1981; Van Winkle and Dickinson 1995, Edwards *et al.*, 1998).

Cellular proliferation and differentiation of preimplantation embryos can be stimulated by various growth factors found within the maternal reproductive tract. The preimplantation embryo expresses the receptors for certain growth factors, and even expresses some growth factors itself. Insulin and insulin like growth factor-1(IGF-I) treatment increases the number of cells within mouse, bovine and human preimplantation embryos, as well as increasing the rate of blastocyst formation (Harvey and Kaye, 1990, 1992; Palma *et al.*, 1997; Lighten *et al.*, 1998; Mihalik *et al.*, 2000). Insulin and IGF-I have also been shown to increase the amount of protein

endocytosed by the TE of mouse embryos (Dunglinson and Kaye, 1993). Insulin and IGF-I may therefore improve embryo development by increasing the amount of growth promoting factors available to the embryo and the developing ICM. Spanos *et al.*, (2000) demonstrated that IGF-I also reduced the number of apoptotic cells within human preimplantation embryos, and increased the proportion of embryos reaching the blastocyst stage.

Within the blastocyst there are differences in the levels of metabolism between the TE and the ICM. Hewitson and Leese (1993) showed that the ICM had a higher uptake of glucose, and formation of lactate, compared with the TE. Whilst the ICM converted all of the glucose into lactate, the TE only converted 55%. Analysis of the glucose concentration within the blastocoel cavity (0.59mM) showed it to be lower than that of the surrounding culture media (1mM). This, coupled with the detection of high levels of lactate, suggested an important role for the TE in maintaining a supply of glucose, amongst other molecules, to the ICM. These studies again demonstrate that the TE acts as a protective homeostatic barrier for the developing ICM.

1.3.1 The uterine environment

As discussed above, the preimplantation embryo has different metabolic and nutritional requirements at different stages of its development. It is therefore necessary that the maternal reproductive tract fluid reflects these changing requirements. After ovulation, the human embryo is exposed to an oviductal environment high in pyruvate (0.32mM) and lactate (10.50mM), but low in glucose (0.5mM; Gardner *et al.*, 1996). This mirrors the requirements of the pre-compaction embryo to metabolise pyruvate rather than glucose up until the 8-cell stage. The low levels of glucose found in this region of the female reproductive tract are beneficial to embryo development. *In vitro* studies have shown glucose to be inhibitory to early human embryo development (Conaghan *et al.*, 1993). In the uterus the situation is reversed, there is a fall in the concentrations of pyruvate (0.10mM) and lactate (5.87mM), but an increase in the glucose concentration (3.15mM), again reflecting

the metabolic needs of the embryo at that particular stage of development (Gardner *et al.*, 1996). Human Fallopian tubule fluid contains high concentrations of Cl^- and Na^+ ions compared to serum levels (Borland *et al.*, 1980). Cl^- and Na^+ are important for the production of the blastocoel cavity as they are used to drive the movement of water into the intracellular spaces of the embryo.

The mouse maternal reproductive tract contains a high concentration of protein (10.9 mg/ml; Gardner and Leese, 1990), which can be endocytosed by the embryo and broken down to be used in the embryo's own biosynthesis of proteins. Certain amino acids such as glycine, taurine, glutamine and glutamate are all found in high concentrations within the female reproductive tract (Casslen *et al.*, 1987). Not only are these amino acids important in promoting embryo development, but they can also act to regulate the embryo's internal pH.

The pH of the reproductive tract changes between the oviduct and the uterus, with the uterus being more acidic. The alkaline oviductal environment is beneficial as, before compaction, the embryo is incapable of regulating for acidosis (Dale *et al.*, 1998; Edwards *et al.*, 1998). As the embryo compacts it develops the necessary H^+ transporters to be able to cope with the acidic environment of the uterus (Dale *et al.*, 1998; Edwards *et al.*, 1998).

1.4 The effects of embryo culture

The ability to culture mammalian embryos *in vitro* has led to many advances within biomedical science. These include the production of transgenic and cloned animals, the possibility of using embryonic stem cells (ESC) to regenerate tissues and even body parts, and assisted reproductive techniques (ART) to combat infertility. However, over the past few years there has been a growing body of evidence that although the ability of the preimplantation embryo to develop to the blastocyst stage may not be compromised by culture conditions, postimplantation and fetal development may be.

1.4.1 Preimplantation embryo culture systems

The analysis of female reproductive tract fluid, and studies into *in vitro* culture methods have led to the development of sequential embryo culture procedures for human embryos. The aim is to provide for the embryo's changing needs by using two separate media. The first is used for the first three days of culture, then a separate medium is employed to promote development to the blastocyst stage (day 5 in humans). Not all sequential media are the same in composition (reviewed in Smith, 2002). In general, most sequential media are based around the composition of human tubal fluid (Quinn *et al.*, 1985). Most media reduce the level of glucose, or remove it altogether, from the first medium, instead using amino acids as a potential energy source. Glucose is then used as the main energy source in the second culture medium. Different media differ in their osmolarity, pH and amount of glucose but all are designed to improve blastocyst formation so as to give a better chance of implantation (reviewed in Smith, 2002).

Along with changes in media, the duration of culture has also been analysed. The use of sequential media means that human embryos can be cultured to the blastocyst stage, and so, for ART purposes, allows the selection of better quality embryos. This can encourage a reduction in the number of embryos transferred, thereby reducing the risk of multiple births. Several studies have investigated the implantation and pregnancy rates between human embryos transferred at day three *versus* day five of culture. 8-cell embryos at day three have a higher rate of blastocyst formation compared to 6-cell embryos (76% and 54% respectively), implying that it may be possible to identify viable embryos within a group (Langley *et al.*, 2001). Implantation rates are seen to be higher for embryos transferred at day five compared to day three, regardless of the age of the recipient (Marek *et al.*, 1999). Gardner *et al.*, (2000) used a three point scoring system (based on blastocyst expansion and ICM and TE development) to grade human embryos. Upon transfer of two high scoring blastocysts, an implantation rate of 70% was achieved. This was higher than the implantation rates achieved for those embryos assessed at the pronucleate (28%), or cleavage stage (48%). Studies examining the depletion/appearance of amino acids

from singly cultured human embryos observed that embryos which subsequently arrested had a higher amino acid turnover that those embryos capable of developing to the blastocyst stage (Houghton *et al.*, 2002). These finding were in line with other studies which observed that mouse embryos which had elevated glycolytic rates were less likely to implant after embryo transfer, whilst human embryos which had a lower pyruvate consumption were more likely to result in a pregnancy post transfer (reviewed in Leese, 2002).

The use of a sequential media system is also beneficial to embryo development as it prevents the build up of toxic substances within the culture medium. One such example is the break down of amino acids into ammonium (reviewed in Gardner, 1994). As amino acids have been seen to improve preimplantation embryo development, their inclusion in culture media has increased. However, one of the byproducts of amino acid metabolism is ammonium (Figure 1.8). Under periods of prolonged culture, the ammonium levels can build up to embryo-toxic levels. Along with this, amino acids spontaneously break down at 37°C. These two factors mean that the ammonium concentration can reach embryo toxic-levels within 48 hours of culture (reviewed in Gardner, 1994). The use of sequential media means that the embryo is placed into fresh medium at day three, thus preventing the embryo from being exposed to toxic levels of waste compounds.

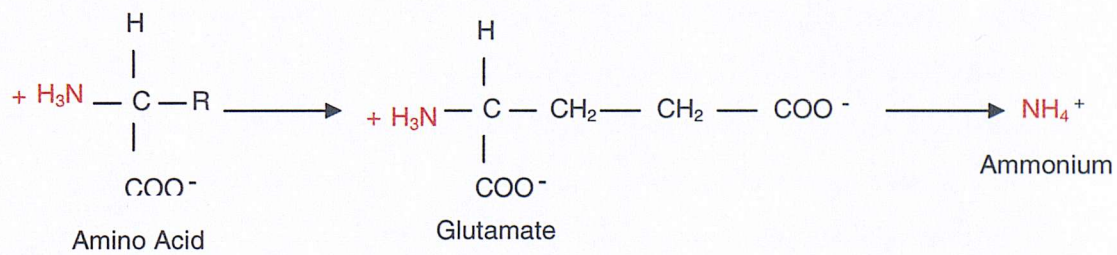


Figure 1.8 Production of ammonium from amino acid breakdown. The α-amino group of many amino acids is transferred to α-ketoglutarate to form glutamine, which is then oxidatively deaminated to yield ammonium (Stryer, 4th Ed, 1995).

In vivo, the build up of embryo-toxic factors during development is believed to be prevented by the reproductive tract and the dynamic environment that it

provides. This has led to the development of mouse co-culture systems to try to improve the rates of blastocyst formation for *in vitro* culture. Co-culture involves the use of helper somatic cells to try to provide an environment more akin to that *in vivo*. Firstly, if the co-culture cells are oviductal then these can release a range of growth factors, cytokines and other embryo-trophic factors similar to those found within the maternal reproductive tract (Leppens *et al.*, 1996; Lee *et al.*, 2001; Xu *et al.*, 2000, 2001). Secondly, these cells can remove deleterious substances from the culture medium via enzymes such as superoxide dismutase which scavenge reactive oxygen species (Joo *et al.*, 2001). The co-culturing of mouse embryos on human oviductal cells significantly increases the rates of blastocyst formation and hatching (Lee *et al.*, 2001). With this, there is a significant increase in the number of ICM and TE cells with an increase in the ICM:TE ratio (Leppens *et al.*, 1996; Xu *et al.*, 2000, 2001).

1.4.2 The use of exogenous growth factors

During the preimplantation period, the embryo develops despite lacking a blood supply or any direct contact with the reproductive tract wall (Figure 1.7). For development and implantation to occur normally there must be effective maternal-embryonic communication. *In vitro* studies have shown that exogenous growth factors are not necessary for mouse preimplantation development to occur; in fact the mouse embryo can survive to the blastocyst stage in a salt solution supplemented only with pyruvate and albumin (Devreker *et al.*, 1998). However, comparisons between *in vitro* and *in vivo* cultured embryos suggest that maternally-and embryonically-derived factors play an important role during development (Bowman and McLaren, 1970; Harlow and Quinn, 1982). Culturing mouse embryos in groups not only increases developmental rates when compared to single embryo culture, but also reduces the levels of apoptosis, suggesting the production of autocrine growth factors by the embryos themselves (Brison and Schultz, 1997; O'Neill *et al.*, 1998).

Various growth factors and their receptors have been shown to be expressed by the embryo or within the female reproductive tract. Two such growth factors are insulin and the insulin like growth factors (IGFs). Receptors for both insulin and

IGF-1 are expressed from the 8-cell stage in the mouse, and are located on the apical surface of the TE, and on the ICM (Heyner *et al.*, 1989). The mouse embryo is able to produce its own IGF-1 but relies on the maternal tract for insulin. Both insulin and IGF-1 have been shown to increase the number of cells within the mouse, bovine and human preimplantation embryo (Harvey and Kaye 1990, 1992; Palma *et al.*, 1997; Lighten *et al.*, 1998; Mihalik *et al.*, 2000; Spanos *et al.*, 2000), primarily by increasing the number of ICM cells (Harvey and Kaye, 1990, 1992; Palma *et al.*, 1997; Lighten *et al.*, 1998). IGF-1 has been shown to reduce the levels of apoptosis within cultured preimplantation embryos (O'Neill *et al.*, 1998; Spanos *et al.*, 2000). Spanos *et al.*, (2000) reported that *in vitro*, 50% of human embryos arrest after six days in culture. Spanos *et al.*, (2000) reported that IGF-1 reduced the number of apoptotic nuclei within human embryos, and that subsequently the rate of blastocyst formation increased. Although IGF-1 and insulin have been shown to have similar effects, the timing of their effects differs. IGF-1 is responsible for promoting pre-compaction development of embryos, whereas insulin increases the proportion of embryos reaching the blastocyst stage and so exerts its effects post-compaction (Harvey and Kaye, 1992).

Insulin and IGF-1 also influence embryo metabolism, increasing the rate and amount of protein endocytosed (Dunglinson *et al.*, 1995; Dunglinson and Kaye, 2000), and reducing the rate at which the incorporated protein is catabolised (Dunglinson and Kaye, 2000). As uterine fluid contains approximately 10 mg/ml protein, it is an important source of exogenous proteins and amino acids for the embryo (Gardner and Leese, 1990). The embryo can break these proteins down to add to its own amino acid pool or use them for metabolism. Dunglinson *et al.*, (1995) concluded that insulin stimulates the endocytic apparatus at the apical face of the TE, in order to enhance the internalization of the surrounding fluid. As the mouse embryo begins to increase the levels of protein synthesis around the time of compaction, the increased amount of protein available to the ICM may promote an accelerated rate of embryo development. Mouse embryos cultured in the presence of insulin and/or albumin showed an increase in ICM proliferation, but not TE or placental

proliferation (Kaye and Gardner 1999). Upon transfer and development of embryos cultured in the presence of insulin and albumin, offspring developing from these embryos have increased fetal weight by 4-6%. Kaye and Gardner (1999) suggested that a larger pool of ICM cells could produce an increased stem cell population, thereby leading to increased fetal mass.

1.4.3 Growth factor receptor signalling

The insulin and IGF-I receptors (IRs and IGFRs) are both members of the receptor tyrosine kinase (TKs) family. They are distinguished by the proteolytic cleavage and disulphide linkage of pro-receptor monomers that generate mature receptors with a β-α-α-β subunit structure. Both receptors are widely expressed in mammalian tissues. In cells expressing both IR and IGFR, pro-receptors undergo hetero- as well as homo-dimerization, creating IR–IGFR hybrids alongside classical IRs and IGFRs (Moxham *et al.*, 1989; Soos and Siddle, 1989). Sequence analysis and molecular modelling has predicted six structural domains of approximately 150 amino acids in total.The intracellular region of the IR and IGFR contains the juxtamembrane (JM), TK and C-terminal (CT) domains, with the TK domain being the most conserved region of the receptor (reviewed in Siddle *et al.*, 2001). The JM domains contain an autophosphorylation motif which aids in substrate binding.

Upon ligand binding, the b-subunits undergo intramolecular *trans*-autophosphorylation. Both IR and IGFR phosphorylate IR substrates (IRS-1–IRS-4) and Shc (Src homology/collagen) proteins (p46/p52/p66), triggering two major signalling pathways (reviewed in Siddle *et al.*, 2001). The phosphorylated IRSs recruit and activate class 1a phosphoinositide 3-kinase (PI 3-kinase), leading to synthesis of membrane-associated phosphatidyl inositol 3,4,5 trisphosphate. This in turn recruits and activates phosphoinositide-dependent kinases (PDKs), which then phosphorylate and activate other protein kinases, including Akt/protein kinase B (PKB), p70rsk and protein kinase Cξ (PKCξ) (Vanhaesebroeck and Alessi, 2000). The small G-proteins Ras and Rac have also been identified as down stream targets of IR and IGFR autophosphorylation. There is substantial evidence that PI 3-kinase

activity mediates a wide range of insulin/IGF effects, including stimulation of glucose transport, glycogen synthesis, protein synthesis and mitogenesis, inhibition of apoptosis and regulation of gene transcription (Shepherd *et al.*, 1998).

1.5 The 'Developmental Origins of Health and Disease' (DOHaD) hypothesis

Embryonic and fetal development represent a time of rapid cell division, cell differentiation and growth. It could therefore be assumed that perturbations which affect the normal patterns of embryonic cell number and rate of development could then impinge upon fetal development, and possibly upon aspects of potnatal development. In humans, there is a wealth of evidence from numerous epidemiological studies which suggest that low birth weight, thinness at birth or a low ponderal index, correlate with an increased susceptibility of developing adult diseases such as hypertension, non insulin dependent diabetes mellitus (NIDDM) and obesity (Barker *et al.*, 1989; Forsen *et al.*, 1997; Rich-Edwards *et al.*, 1997; Stein *et al.*, 1997; Leon *et al.*, 1998). These conditions have been collectively termed the "metabolic syndrome" or "syndrome X". Early epidemiological studies in Hertfordshire (Barker *et* al., 1989) and in Preston (Baker *et al.*, 1990) found that low birth weight babies (<3.4 kg) born with large placentas were three times more likely to develop hypertension, and more likely to develop ischaemic heart disease than larger babies with normal sized placentas. Later studies from America (Rich-Edwards *et al.*, 1996), India (Stein *et al.*, 1997), Finland (Forsen *et al.*, 1997), Hong Kong (Cheung *et al.,* 2000) and Sweden (Leon *et al.*, 1998) also revealed correlations between smallness at birth and a higher incidence of adult onset diseases. They also demonstrated that factors such as parental social class and smoking appeared to have little or no effect on predisposition to the metabolic syndrome. It has also been observed that boys who were small at birth and at 1 year of age, but then subsequently had accelerated gain in weight and body mass index during infant growth, showed a higher prevalence of coronary heart disease in adult life (Eriksson *et al.*, 1999, 2001). Despite the increased weight gain, the heights of these children remained significantly lower between 1 and 5 years of age when compared to children of average birth weight (Figure 1.9)

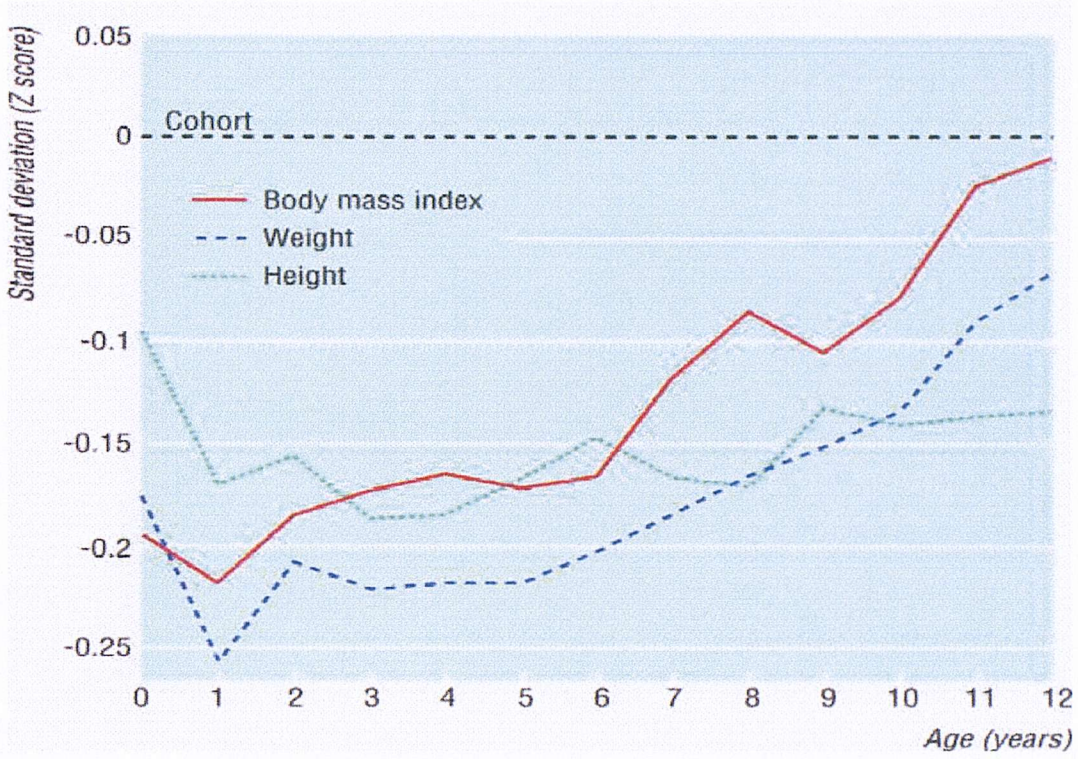


Figure 1.9. Growth of 357 boys who later developed coronary heart disease in a cohort of 4630 boys in Helsinki (taken from Eriksson *et al.*, 2001).

From these initial observations, attention was drawn towards potential mechanisms through which reduced fetal growth could affect adult physiology. During periods of under-nutrition, the fetus makes compensatory adaptations in order to maintain the development of vital organs such as the brain. However, these compensations can have negative impacts on the growth of other organs developing at the same time. Slower rates of cell proliferation during periods of intrauterine growth restriction could produce permanent changes in the structure and function of certain organs within the body. Several animal models of early growth restriction have been developed in an attempt to understand the mechanisms linking growth restriction to adult disease.

In the rat, the feeding of a low protein diet to pregnant mothers results in reduced numbers of ICM and TE cells within blastocysts, disproportionate fetal growth, disproportionate growth of specific organs compared to body weight, hypertension and reduced nephron number in the offspring (Langley-Evans *et al.*,

1996a, 1996b, 1999; Kwong *et al.*, 2000). Neonates from dams fed a low protein diet during pregnancy show significantly reduced β-cell proliferation and islet size in the pancreas (reviewed in Ozanne, 2001). As well as this, islet vascularisation is decreased and apoptosis in β-cells is increased. It has been hypothesised that this reduction in β-cell numbers produces insulin sensitivity and metabolic overload resulting from the existing β cells having to produce higher amounts of insulin (Hales *et al.*, 1991). In sheep, offspring of nutrient restricted pregnant mothers have increased kidney weights and significantly increased angiotensin II type one receptor (AT1) expression in kidney, liver and lung tissue (Whorwood *et al.*, 2001).

In sheep, prenatal exposure to the synthetic glucocorticoid dexamethasone, produces offspring with hypertension, increased cardiac output, left ventricular hypertrophy and reduced numbers of glomeruli within the kidney (reviewed in Mortiz *et al.*, 2003). In the rat, offspring exposed to excess prenatal glucocorticoids undergo postnatal catch-up growth so that body weights have normalised by weaning (3 weeks of age; reviewed in Ozanne, 2001). Fetal glucocorticoid overexposure in rats has also been shown to be associated with elevated blood pressure and raised blood glucose levels in adulthood (reviewed in Ozanne, 2001).

It is hypothesised that a poorly nourished mother will give the fetus a forecast of the nutritional environment into which it will be born. The fetus then sets in motion a series of compensatory mechanisms that will give the offspring a metabolism adapted to survival under conditions of poor postnatal nutrition. These adaptations only become detrimental when the postnatal environment differs from the mother's forecast. Under conditions of increased postnatal nutrient availability the offspring undergo rapid postnatal catch-up growth. These factors combined with the possible altered organ physiology will increase the chances of the offspring developing the "metabolic syndrome" and possibly having a shorter life span (Figure 1.10; Barker and Clark, 1997).

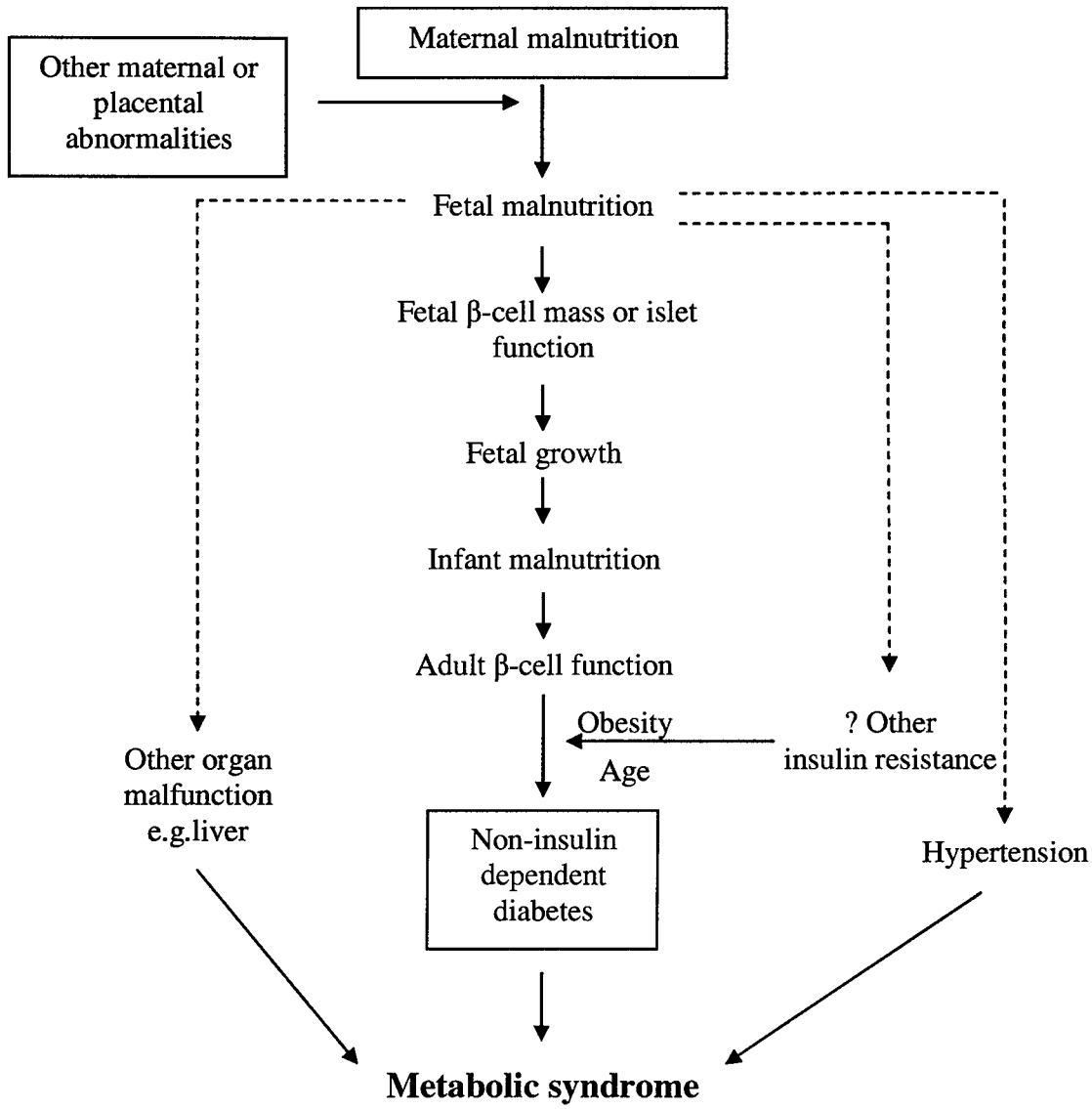


Figure 1.10 Diagram showing the mechanisms leading to the metabolic syndrome (taken from Hales and Barker, 2001).

1.6 Long term consequences of embryo culture, 'Large Offspring Syndrome'

Even though *in vitro* culture is now providing an environment similar to that of the female reproductive tract, there is growing evidence that *in vitro* culture may alter pre and postnatal development, producing specific phenotypic abnormalities (Thompson *et al.*, 1995; Holm *et al.*, 1996; Sinclair *et al.*, 1999; Khosla *et al.*, 2001). One of the most striking examples of this is the phenomenon of large offspring syndrome (LOS). LOS is predominantly seen in sheep and cattle, and is primarily

associated with a significant increase in fetal birth weight. Other physiological abnormalities reported in conjunction with LOS include increased muscle mass, cerebellar dysplasia, skeletal and facial malformations, changes in the normal size and weight of internal organs such as the liver, heart, kidney and plantaris muscle and sudden perinatal death (Thompson *et al.*, 1995; Holm *et al.*, 1996; Young *et al.*, 1998; Sinclair *et al.*, 1999; Wells *et al.*, 1999).

Since the first report by Willadsen *et al.*, (1991), it has become apparent that LOS is correlated with the introduction of the embryo into unusual *in vitro* and/or *in vivo* environments. These include the addition of serum (of various sources) in the culture medium, cloning by nuclear transfer, the co-culture of embryos on granulosa cells, the transfer of embryos to an asynchronous uterine environment, and the exposure of the embryo to a high progesterone uterine environment (Farin and Farin, 1995, Thompson *et al.*, 1995, Holm *et al.*, 1996, Young *et al.*, 1998, Sinclair *et al.*, 1999). In the majority of cases, embryos have been exposed to these environments between the time of fertilization and the hatching of the blastocyst. The belief is that the embryo may become exposed to developmental cues different from those normally received *in vivo*. These could then alter the patterns of gene expression, increase future growth rates or alter embryo metabolism, all of which could change the normal pattern of fetal development.

Serum is used for *in vitro* culture as it contains growth factors, proteins and other beneficial compounds required for embryo development. However, serum also contains many factors that the embryo would not normally be exposed to *in vivo* (Gardner, 1994a). Although embryos cultured in the presence of serum have a higher developmental rate, they also have altered metabolism, morphology and postimplantation development (Gardner *et al.*, 1994a; Thompson *et al.*, 1995; Holm *et al.*, 1996; Sinclair *et al.*, 1999). Embryos cultured in the presence of serum are seen to accumulate large amounts of lipid droplets within their cells, particularly those of the TE (Figure 1.11; Thompson *et al.*, 1995). Gestation length and mean birth weights of fetuses from embryos cultured in the presence of serum are significantly increased when compared to control groups (Thompson *et al.*, 1995; Holm *et al.*, 1996).

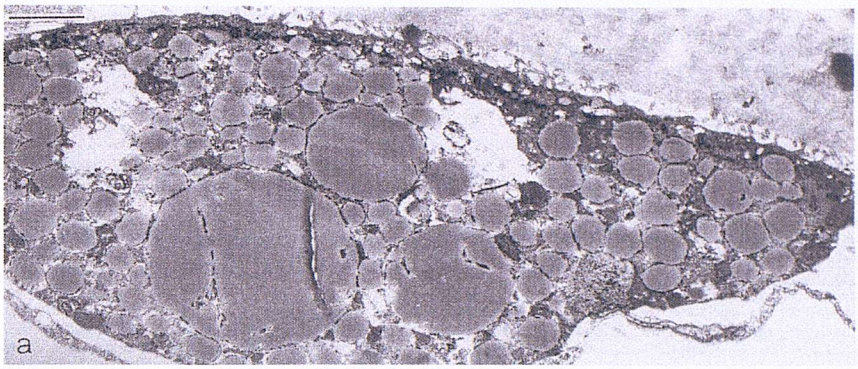


Figure 1.11 Accumulation of lipid droplets in the TE cells of sheep embryos cultured in medium supplemented with human serum (taken from Thompson *et al.*, 1995)

It has been reported that culture in the presence of fetal calf serum reduces the number of cells within bovine blastocysts, and increased the level of apoptosis (Byrne *et al.*, 1999). Subsequent studies have reported that the inclusion of serum in the culture media results in reduced numbers of cells within the TE and ICM when compared to blastocysts produced either *in vivo* or blastocysts cultured in the absence of serum (reviewed in McEvoy *et al.*, 2000). In particular, there was a reduction in the allocation of cells to the ICM.

It is believed that the addition of serum to the culture medium could accelerate embryo development, possibly altering the ICM:TE ratio. This could subsequently impart on the embryo a property which promotes increased gestation length and promotes increased fetal growth. The accumulation of lipid within the TE cells could also alter the embryo's metabolism, leading to an increased fetal growth rate (Thompson *et al.*, 1995). Usually, the embryos are manipulated before the activation of the zygotic genome.

Studies have shown that serum can be replaced by the use of amino acids, bovine serum albumin (BSA) or inert polymers (reviewed in Gardner, 1994; Gardner *et al.*, 1994; Thompson *et al.*, 1995). It was found that under these conditions premature blastulation was alleviated, blastocyst cell numbers were increased and

birth weight and organ size were comparable to those of the controls. However, these studies did report lower blastocyst yields, and lower rates of pregnancy upon transfer of these embryos.

1.7 DNA methylation and imprinted genes

One molecular explanation for the altered patterns of fetal growth observed in LOS could be the transcription patterns of imprinted genes that influence fetal growth (Young *et al.*, 1998). Imprinted genes are genes that are only expressed from one of the parental chromosomes. DNA methylation is central to the process of imprinting, and occurs at certain cytosine residues adjacent to guanidine, so termed a CpG group. During the early cleavage stages of embryo development both the maternal and paternal genomes are demethylated, allowing for the establishment of a zygotic methylation pattern (Santos *et al.*, 2002). The reprogramming process is conserved across several mammalian species, in that there is active demethylation of the paternal genome shortly after fertilization but before DNA replication (Dean *et al.*, 2001). It has been speculated that the paternal genome may be targeted for demethylation during the histone-protamine exchange when the DNA is effectively bare (Santos *et al.*, 2001). The exact process by which the active demethylation takes place it currently unknown. After completion of the first cell cycle, passive demethylation is carried out by the DNA methyltransferase 1 (DnmtI) enzyme (reviewed in Reik *et al.*, 2001). At the blastocyst stage there are differences in the levels of methylation within the ICM and TE, with the ICM displaying high levels of methylation whilst the TE has relatively no methylation (Santos *et al.*, 2001). DNA methylation in the ICM is essential of early postimplantation development as embryos devoid of Dnmt3a and b show abnormalities within all embryonic lineages whilst the extraembryonic tissues appear unaffected (Okano *et al.*, 1999).

Several imprinted genes have been shown to influence fetal growth. These include the genes for the potent fetal growth factor, insulin like growth factor II (IGF-2) and its receptor (IGF-2r). Mice with null mutations for the *Igf-2* exhibit reduced fetal growth (reviewed in Hastie, 1997). Loss of imprinting within the *Igf-2* gene

results in biallelic expression of *Igf-2* and increased expression of Igf-2 protein leading to increased fetal growth (reviewed in Hastie, 1997). In LOS sheep fetuses, born after *in vitro* culture of fertilised eggs, Igf-2r expression was reduced by 30-60% relative to the control group. Levels of DNA methylation within fetal liver sections of the IGF-2r gene were 70% of the control group (Young *et al.*, 2001)

In vitro culture conditions have been shown to affect other imprinted genes in other mammals. In the mouse, H19 is a maternally expressed gene, the paternal gene being highly methylated over a 7kb region and subsequently transcriptionally silent (Doherty *et al.*, 2000). In the mouse, the H19 and IGF-2 genes are located 90kb apart and share an upstream differentially methylated region (DMR) necessary for the imprinting of both genes. Culture of mouse embryos in Whitten's medium (without serum) resulted in biallelic expression of H19, with a loss of methylation within the paternal DMR. Embryos cultured in KSOM medium in the presence of amino acids showed no such effects (Doherty *et al.*, 2000). Khosla *et al.*, (2001) observed that only one third of embryos cultured in the presence of FCS went on to develop to day 14 of gestation, and that these fetuses were on average 12% lighter than the control fetuses. In those fetuses that did develop, levels of H19 and IGF-2 expression were decreased by 31% and 15% respectively. Altered patterns of imprinting may therefore be dependent upon the culture medium used.

Altered patterns of development have also been observed in animals derived from nuclear cloning. This process involves the transfer of nuclei from somatic cells into enucleated oocytes. The factors present in the oocyte's cytoplasm are then able to reprogramme and remodel the somatic cell nucleus. As with normal development, the DNA methylation patterns of the donor nucleus must be removed, and then replaced with a new zygotic methylation pattern. Studies have shown that the growth anomalies associated with nuclear cloned animals may be due to incomplete reprogramming of the donor nucleus. Studies have shown that in cloned bovine embryos the donor nucleus was not totally demethylated, and the patterns of *de novo* methylation that followed were consistent with the patterns seen in fully

differentiated nuclei (Dean *et al.*, 2001). In mice, embryos cloned using cumulus cell nuclei, display reduced levels of Oct-4 expression (Boiani *et al.*, 2002). In a further study, the expression of ten genes related to Oct-4 were examined in cloned embryos derived from donor cumulus nuclei. The target genes were related to Oct-4 in that they shared similar expression profiles and are expressed in pluripotent cells but repressed in differentiated cells. With the exception of one gene (*Dppa 5*), all the genes showed failures to be expressed in the cloned embryos (Bortvin *et al.*, 2003).

Wells *et al.*, (1999) produced cloned calves following nuclear transfer from cultured adult mural granulosa cells. The nuclei were exposed to the oocyte's cytoplasmic environment for prolonged periods of time before activation. Only 10% of the cloned calves survived to term, and none lived for more than 100 days of age. It was proposed that the high degree of loss during gestation might have been due to placental dysfunction.

1.8 Assisted Reproductive Techniques (ART)

It is estimated that since 1978 when the birth of the first 'test tube baby' from an oocyte fertilized *in vitro* took place, there have been approximately 1,000,000 children conceived from ART procedures (Schultz and Williams, 2002). Infertility within the human population has increased over the past few decades to the point that approximately 35 to 70 million couples worldwide have required ART in order to conceive (Schultz and Williams, 2002). This has led to the development of several systems that enable the collection, culture, storage and manipulation of both male and female gametes and embryos. Despite the increased ability to culture and manipulate gametes and embryos, the pregnancy rate from ART still remains low with only approximately 23% of women undergoing treatment becoming pregnant (Figure 1.12; reviewed in Hardy *et al.*, 2002).

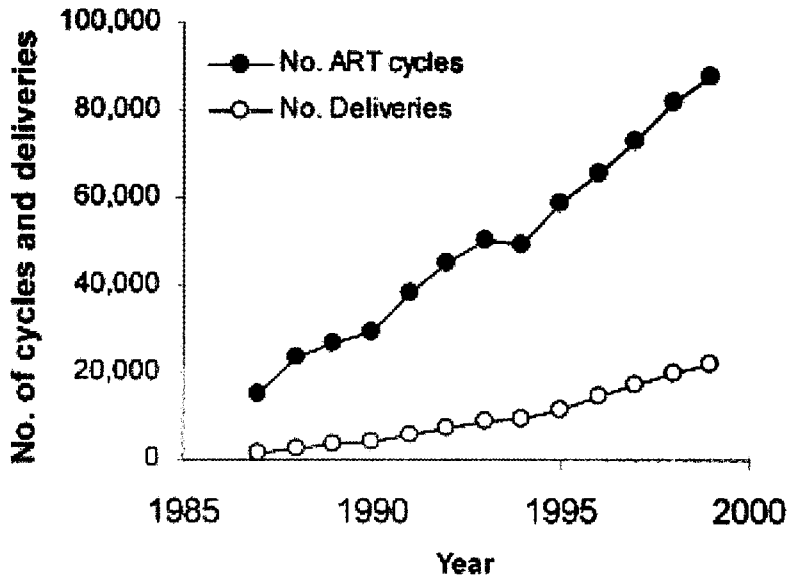


Figure 1.12 The number of total ART cycles and deliveries reported to the national registry in the United States of Americain up to 1999 (taken from Toner, 2002).

With the increase in the number of ART cycles taking place, concern has arisen about the possible long term effects of gamete and embryo manipulation on the children produced. As discussed above, several studies have shown that in other mammalian models, culture of preimplantation embryos can not only alter embryo development, but can also affect post-implantation and postnatal life. As yet, there has been no such reporting of similar effects with human children born from ART.

As male infertility accounts for approximately 40% of infertility cases (reviewed in Schultz and Williams, 2002), the use of IntraCytoplasmic Sperm Injection (ICSI) has increased. This procedure involves the injection of the head of the sperm, which contains the nucleus, into the cytoplasm of the oocyte. However, there are several concerns regarding the procedure of ICSI. One consideration is that ICSI bypasses all the processes of natural selection mechanisms that sperm would normally encompass in a natural fertilization. There could also be considerable damage caused by the injection needle to the oocyte and cytoplasmic structures such as the spindle. These could then lead to developmental abnormalities such as aneuploidy. Other problems include the introduction of foreign material into the oocyte upon sperm injection such as the culture medium or extra sperm structures. In

some studies, it has been observed that ICSI leads to increased rates of chromosomal aberrations (reviewed in Hardy *et al.*, 2002).

Questions regarding the long term effects of ART on aspects of postnatal development are starting to be addressed. Studies have observed that incidences of multiple pregnancies are increased in patients receiving ART. As a consequence, children conceived through ART were more likely to be born premature, were more likely to be born with a low birth weight (<2500g), and were observed to have higher incidences of some congenital abnormalities (D'Souza *et al.*, 1997; Bergh *et al.*, 1999; Tarlatiz and Grimbizis, 1999). However, at the current point in time, it is not possible to assess the long term effects of ART, as the majority of children conceived via ART are still under the age of twenty.

1.9 The Preimplantation Embryo Development (*Ped*) gene as a possible genetic modulator of postnatal development

As discussed above, alterations to the normal pattern of embryonic development can have a range of consequences on both fetal and postnatal development, ranging from a slowing in the rate of preimplantation development to altered patterns of fetal gene expression to increased perinatal mortality (Bowman and Mclaren, 1970; Young, 1998; Khosla *et al.*, 2001). *In vitro*, the slowing of embryonic developmental rates has been associated with poor fetal development. Within a population of embryos, cleavage rates may vary, with some embryos developing at a faster rate than others. One gene that has been shown to influence the rate of mouse preimplantation embryo development is the *Ped* (preimplantation embryo development) gene. Studies have linked this gene to the H-2 region of the mouse Major Histocompatability Complex (MHC; Warner *et al.*,1987b), in particular the Q7 and Q9 genes within the Q region (Cai *et al.*, 1996; Wu *et al.*, 1999). The product of the *Ped* gene, the Qa-2 protein, is a Class 1b molecule and is expressed on the mouse embryo from the 2-cell stage (Warner *et al.*, 1987a; McElhinny and Warner, 1997) and throughout preimplantation embryo development being expressed on both ICM and TE cells (McElhinny *et* al., 1998). The Qa-2 protein is linked to the

surface of the cell by a glycosylphosphatidylinositol (GPI) anchor. Two alleles of the *Ped* gene, *fast* and *slow*, have been defined, based upon the number of cells contained within embryos at specific times (Goldbard *et al.,* 1982), with *Ped fast* embryos having an increased rate of embryo cleavage, and containing more cells when compared to *Ped slow* embryos (Goldbard *et al.,* 1982; Brownell and Warner, 1988). Microinjection of the Q7 and/or Q9 genes into Qa-2 negative zygotes increased the rate of embryo cleavage, leading to the expression of Qa-2 upon the surface of injected embryos (Wu *et al.,* 1999). Removal of the Qa-2 antigen from the embryonic cell surface, through enzymatic cleavage by phosphotidylinositol phospholipase C, resulted in a slowing of embryo development (Tian *et al.*, 1992). Addition of exogenous GPI linked Qa-2 protein into the membrane of *Ped* negative embryos also increased the rate of cleavage divisions (McElhinny *et al.,* 2000).

The Qa-2 protein also correlates with patterns of postimplantation development. Offspring developing from *Ped fast* embryos have a higher chance of intrauterine survival, are heavier at birth, come from larger litters and are heavier at weaning (Warner *et al.,* 1991, 1993; Exley and Warner, 1999). Both *Ped* fast and *Ped* slow embryos have equivalent chances of developing to mid-gestation, it is only after this time that a higher degree of fetal loss occurs within the *Ped* slow mice (reviewed Warner and Brenner, 2001). As *Ped* fast embryos have a higher number of cells within the TE and ICM, these embryos may have a larger pool of stem cells within the ICM, have a higher rate of placentation, and so have a higher rate of fetal developtment.

Warner and Brenner, (2001) suggest that the Qa-2 antigen might have a role in protecting the developing fetus from maternal natural killer cells or macrophages, which could account for the higher levels of fetal loss in the embryos lacking the Qa-2 antigen. They also suggest that the Qa-2 antigen may have some influences on postnatal growth and health, however, what these influences are were not specified.

1.9.1 A human homologue of the mouse *Ped* gene

The *Ped* gene phenotype, a population of faster cleaving preimplantation embryos that appear to have a preferential survival rate, has also been observed in human embryos (reviewed Warner and Brenner, 2001). In humans, 8-cell embryos at day three have a higher rate of blastocyst formation compared to 6-cell embryos (Langley *et al.*, 2001). Thus, it is believed that a similar molecule could be influencing human preimplantation embryo development.

Currently, the human HLA-G is believed to be the functional homologue to the mouse Qa-2 antigen (Allcock *et al.*, 2000). Although they do not share exactly the same DNA sequence, they are believed to be funtionally the same as a result of convergent evolution (Allcock *et al.*, 2000). Although HLA-G is also a class 1b molecule, it differs from the Qa-2 in that it is inserted into the cell membrane with a 6 amino acid cytoplasmic tail and not via a GPI anchor (Tian *et al.*, 1992; Warner and Brenner, 2001). However, it is believed that this difference does not lead to a difference in function between HLA-G and Qa-2 (reviewed in Warner and Brenner, 2001). Jurisicova *et al.*, (1996) observed a correlation between HLA-G expression and the rate of pre-implantation development in human embryos, thus supporting the proposal that HLA-G is a functional homologue of Qa-2.

The proposed role for HLA-G in embryo development is similar to that for Qa-2, in that it may protect the embryo from the maternal immune system, thus enhancing the probability of fetal survival (Table 1.1; reviewed in Warner and Brenner, 2001). HLA-G has been proposed to attenuate unwanted immune responses through its interactions with T-cell receptors. This would be beneficial to implanting embryos in protection against maternal immune responses to paternal antigens at the maternal/fetal interface (Loke and King, 1991). It has also been proposed that HLA-G protects from natural killer cell mediated cell lysis via the transmission of an inhibitory signal through NK receptors (Chumbley *et al.*, 1994). HLA-G has been proposed to protect the fetus from transmission of maternal pathogens across the placenta (Lee *et al.*, 1995).

Table 1.1 Comparisons of the HLA-G and the Qa-2 gene products

Feature	Qa-2	HLA-G
MHC class 1b molecule	Yes	Yes
DNA sequence	Similar to HLA-G	Similar to Qa-2
Expression in preimplantation embryos	Yes	Yes
Increases preimplantation growth rate	Yes	Yes
Enhances fetal survival	Yes	Yes
Limited tissue expression	Yes	Yes
Interacts with T-cell receptor	Yes	Yes
Interacts with CD8 (on T cells)	Yes	Yes

Recreated from Warner and Brenner, (2001).

Rationale

Mammalian preimplantation embryo development requires the co-ordinated actions of cellular differentiation, metabolism changes and an appropriate synchronised uterine environment. At the time of implantation the embryo contains two distinct cell lineages, the TE and the ICM. In the blastocyst, the TE acts as a transporting epithelial barrier for the developing ICM, effectively sealing it off from the surrounding environment and protecting it from perturbations in the environment. During preimplantation development, the embryo has different metabolic and environmental needs at different stages of development. The female reproductive tract is able to cater for these needs by providing an environment that is suitable in its pH, metabolite levels and developmental cues. However, changes to any of these factors can affect embryo development in a variety of ways, resulting in impaired development, altered gene expression and, potentially, increased susceptibility to abnormal postnatal development.

Therefore, the hypothesis of this thesis is that the environment in which the preimplantation embryo develops can not only affect blastocyst development, but that it may also impinge upon aspects of postimplantation growth and development. These may then manifest in altered pattern of postnatal growth, development and physiology. The aims of this thesis are therefore, to investigate the impact of mouse early embryo *in vitro* culture environment on (a) short-term blastocyst development and (b) long-term postnatal growth and physiology after embryo transfer. In order to ascertain the effect of each manipulation upon blastocyst cell numbers and ICM:TE ratios, three series of *in vitro* experiments will be performed. The first series of experiments will compare the effect of *in vivo* development to a series of *in vitro* culture environments and durations on blastocyst formation. Embryos will be cultured from the 2-cell to blastocyst or expanded blastocyst stages in media supplemented with, or devoid of, an exogenous protein source. These embryos will then be compared against embryos allowed to develop to the blastocyst stage *in vivo*. A second series of experiments will investigate the effect of different concentrations of exogenous insulin and IGF-1 on preimplantation developmental rates, and the ratios

of ICM:TE cells in embryos cultured from the 2-cell stage to the blastocyst and expanded blastocyst stages. Insulin and IGF-I have previously been shown to accelerate the rate of blastocyst formation, specifically increasing ICM cell numbers (Harvey and Kaye 1990, 1992; Palma *et al.*, 1997; Lighten *et al.*, 1998). Significant increases in fetal mass have also been observed from embryos that have been cultured in the presence of insulin and albumin (Kaye and Gardner 1999). The final set of experiments will examine the effect of physical manipulation on the ICM:TE ratio within preimplantation embryos. This will be investigated using two separate approaches; (1) altering the size of the pre-compact embryo so as to half or double the normal number of cells; (2) by aggregating isolated ICMs to produce a blastocyst-like structure that comprises the normal number of cells, but where the TE has been derived from the ICM which does not occur during normal development.

Following manipulation and subsequent characterization at the blastocyst stage, embryos from each of the above treatments will be transferred into suitable recipient mothers and allowed to develop to term. Increased or decreased rates of preimplantation embryo development could influence aspects of fetal development. Slower rates of cell proliferation during periods of in-uterine growth could produce permanent changes in the structure and function of certain organs within the body. The feeding of a low protein diet to pregnant rats results in reduced numbers of ICM and TE cells within blastocysts, disproportionate fetal growth, disproportionate growth of specific organs compared to body weight, hypertension and reduced nephron number in the offspring (Langley-Evans *et al.*, 1996a, 1996b; Kwong *et al.*, 2000). It is hypothesised that a mother gives the fetus a forecast of the nutritional environment into which it will be born. The fetus then sets in motion a series of compensatory mechanisms that will give the offspring a metabolism adapted to survival within a specific postnatal nutrition condition. The adaptations only become detrimental when the postnatal environment differs from the mother's forecast. Under conditions of increased postnatal nutrient availability the offspring undergo rapid postnatal catch-up growth. These factors combined with the possible altered organ

physiology could increase the chances of the offspring developing aspects of the "metabolic syndrome".

Along with these studies, experiments investigating the influence of *Ped* gene expression upon postnatal development and physiology will also be conducted. These studies are of interest as the *Ped* gene has been shown to correlate to increased embryo cell numbers, increased birth weight and increased weight at weaning (Warner *et* al., 1987; Brownell and Warner, 1988). Increased embryonic developmental rates and higher ICM cell number have been associated with an increase in fetal survival (Kaye and Gardner, 1999).

Therefore, postnatal factors such as birth weight, growth rates, blood pressures and organ allometry will be monitored to assess the impact of *in vitro* culture, manipulation and *Ped* gene expression upon postnatal growth, development and physiology (see Figure 1.13 for a time line of proposed experiments).

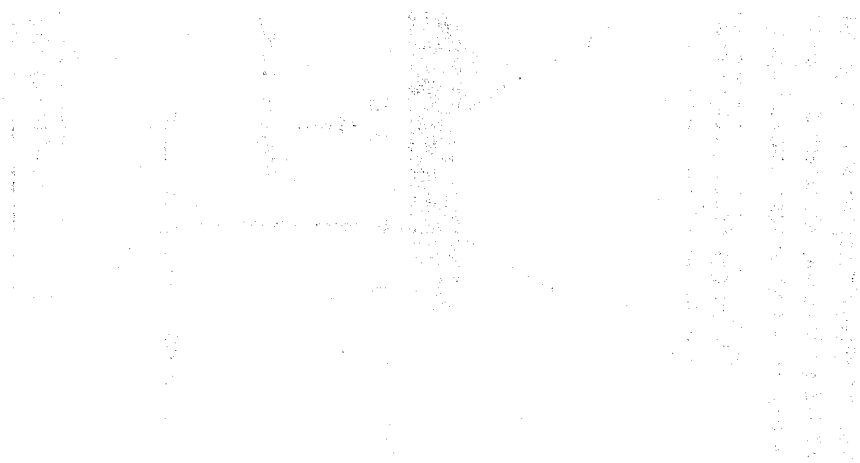
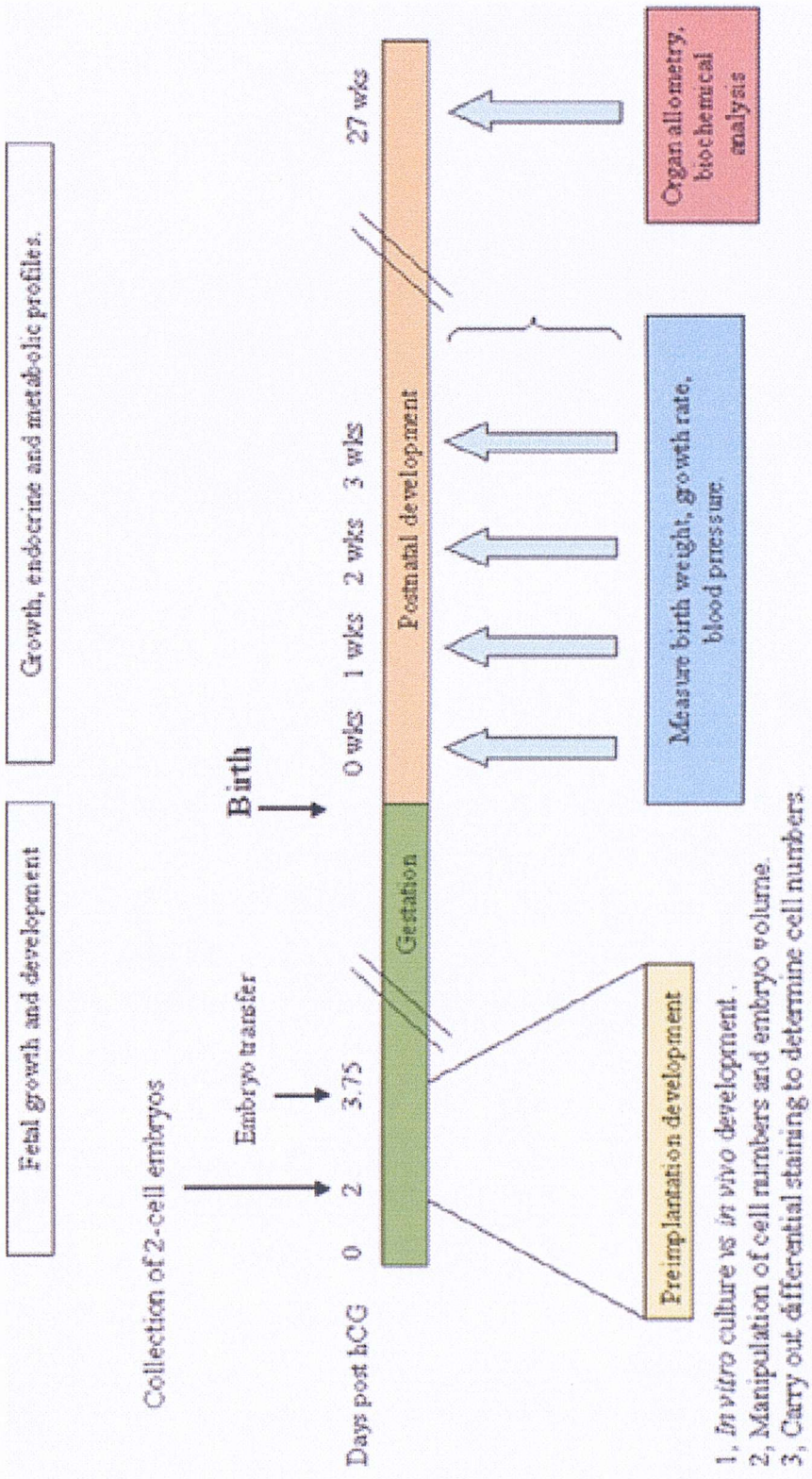


Figure 1.13 Time line of proposed experiments.



Chapter 2

Materials and methods

2.1 Embryo culture and manipulations

2.1.1 Superovulation

5-6 week old virgin female F1 CBAx C57/BL6 mice (University of Southampton, Biomedical Research Facility) were superovulated by intraperitoneal injection of 5-10 IU pregnant mare serum gonadotrophin (PMS, Intervet), then approximately 46 hours later with 5-10 IU human chorionic gonadotrophin (hCG, Intervet) and caged overnight with MF1 males. The presence of a vaginal plug the following morning was taken as a sign of mating.

2.1.2 Embryo collection and culture

Mice were killed by cervical dislocation and embryos were flushed from the dissected oviduct or the uterus depending on the stage of development required. Embryos were flushed using pre-warmed H6 medium supplemented with either 4 mg/ml BSA (Sigma; embryo culture tested; H6 BSA; appendix 1) or 6 mg/ml polyvinylpyrrolidone (Sigma; embryo culture tested; H6 PVP, appendix I). Embryos were then cultured in either T6 medium supplemented with either 4 mg/ml BSA (T6 BSA, appendix I) or 6 mg/ml polyvinylpyrrolidone (T6 PVP, appendix I) under mineral oil (Sigma; embryo culture tested). Embryos were cultured at 37°C, 5% CO_2 in air for up to 116 hours post hCG.

2.1.3 Incubation with insulin and IGF-I

850 nM, 170 nM and 34 nM concentrations of insulin (Sigma, sodium salt, human recombinant), or 8.5 nM, 1.7 nM and 0.34 nM IGF-I (Sigma, human recombinant) were prepared in either T6 BSA or T6 PVP and pipetted as 20 µl droplets in culture dishes (Sterilin), covered in mineral oil and allowed to equilibrate at 37°C, 5% CO_2 in air for at least one hour. Embryos were flushed from oviducts at the 2-cell stage (48 hours post hCG) using pre-warmed H6 BSA or H6 PVP, pooled and temporarily maintained in equilibrated T6 BSA or T6 PVP under oil at 37°C, 5%

CO_2 in air, before assigning ten embryos per 20 µl drop for each growth factor concentration. Embryos were cultured for up to 116 hours post hCG with the media being changed every 24 hours. Embryo developmental rates were scored twice daily (9 am and 5 pm). Embryos were allowed to develop to the blastocyst and expanded blastocyst stage at which point they were removed from culture for differential nuclear staining to determine the number of cells present in the TE and ICM cell lineage (section **2.1.6**).

2.1.4 Production of half, whole and double sized embryos

Embryos were flushed from oviducts at the 2-cell stage and cultured for approximately 20 hours in T6 BSA at 37°C, 5% CO_2 in air, to the early 8-cell stage (70 hours post hCG). The zona pellucidae were removed by brief incubation (15-30 seconds) in 37°C acid Tyrode's solution (pH 2.2, appendix I) before extensive washing and recovery in pre-warmed T6 BSA for a minimum of 20 minutes at 37°C. Embryos were placed into drops of Ca^{2+}-free H6 medium containing 6 mg/ml BSA (appendix I) for 15 minutes which caused loss of intercellular adhesion. Embryos were disaggregated by gentle sucking and blowing into and out of a fine flame-polished glass micropipette (attached to a mouth pipette) of smaller internal bore than the embryos. The micropipettes were made by pulling glass capillaries (internal diameter 0.58 mm, external diameter 1.0 mm, Harvard Apparatus) over an alcohol flame and broken in the centre to form two micropipettes. These were then placed onto a microforge (De Fonbrune) with the pulled region in contact with a glass bead on the filament. The filament was heated so as to fuse the micropipette with the filament and then cooled. The pipette was retracted so that a clean break was formed at the tip. The tip was then flame polished by placing it close to the heated filament. Disaggregated blastomeres were transferred from Ca^{2+}-free H6 BSA into micro-drops (~5 µl) of equilibrated T6 BSA and aggregated in groups of either 4, 8 or 16 cells to produce half, whole and double sized embryos, respectively. The aggregates were cultured in T6 BSA at 37°C, 5% CO_2 in air until they had reached the blastocyst stage with a cavity, at which point they were removed from culture for differential nuclear staining.

2.1.5 Immunosurgery of the trophectoderm

Embryos were flushed from uteri at the morula stage (~74 hours post hCG) using pre-warmed H6 BSA. Embryos were cultured for approximately 20 hours in T6 BSA at 37°C, 5% CO_2 in air, to the early blastocyst stage. The zonae pellucidae were removed by brief incubation in acid Tyrode's solution (section **2.1.4**). Embryos were allowed to recover in pre-warmed T6 BSA for a minimum of 20 minutes. Embryos were incubated at room temperature in trinitrobenzenesulfonic acid (TNBS, Sigma; 1:10 dilution in phosphate buffered saline, PBS, with 0.1% PVP, adjusted to pH 7.4 with NaOH; stored in the dark) for 10 minutes then washed in pre-warmed H6 PVP. The embryos were then incubated in a 24 µl drop comprising 7.5 µl goat Anti-Dinitrophenyl-BSA antibody solution (anti-DNP, ICN Biochemicals; 3 mg/ml in PBS with 0.1% PVP) in 16.5 µl H6 BSA for 10 minutes at room temperature. Embryos were again washed in H6 PVP and then incubated in 23 µl guinea pig complement protein (Cedarlane; Diluted 1:10 in H6 BSA,) for 10 minutes at 37°C, 5% CO_2 in air, and then washed in H6 PVP. The embryos were cultured in H6 BSA for at least 30 minutes to allow for lysis of the outer TE cells, before the ICMs were isolated using a flame polished glass micropipette (section **2.1.4**).

Isolated ICMs were aggregated in groups of three or left as single ICMs and cultured in Dulbecco's modifled Eagles's medium (DMEM, Sigma) supplemented with 10% heat inactivated fetal calf serum (Labtech), until they had re-cavitated at which point they were taken out of culture for differential nuclear staining.

2.1.6 Differential nuclear staining

The method of Handyside and Hunter (1984) was used with modifications. If necessary, the zona pellucidae were removed by brief incubation in acid Tyrode's solution (section **2.1.4**). Embryos were allowed to recover in pre-warmed H6 BSA for a minimum of 20 minutes. Embryos were then incubated at room temperature in TNBS, anti DNP, washed in PVP and incubated in guinea pig complement as in the procedure for the immunosurgery of the TE (section **2.1.5**). For the current procedure, 2 µl of propidium iodide (Sigma ; 1 mg/ml in distilled water) was added to the

23 µl of guinea pig complement protein (section **2.1.5**). Again, the embryos were incubated for 10 minutes at 37°C, 5% CO_2 in air and then washed in H6 PVP. Embryos were fixed in 990 µl of absolute ethanol to which 10 µl of BisBenzimide H 33258 stain (Hoechst stain, Sigma; 2.5 mg/ml in distilled water) was added. Embryos were left at 4°C for a minimum of 90 minutes, and a maximum of 1 week.

Embryos were placed into absolute ethanol for 5-8 minutes, and then into ultra pure glycerol (Amersham). A tiny drop of ultra pure glycerol was placed onto a glass slide and an embryo was transferred into the middle of the drop. A cover slip was placed over the drop, and firmly pressed. The embryos were viewed under a fluorescence microscope (Leitz Diaplan), and the number of nuclei counted. The nuclei of the TE were stained red (propidium iodide) and the nuclei of the ICM were stained blue (Hoechst).

2.2 Procedures for embryo transfer

2.2.1 Production of vasectomized males for the generation of pseudopregnant females

4-6 week old male MF1 mice (University of Southampton, Biomedical Research Facility) were anaesthetized by an intraperitoneal injection of a ketamine (Fort Dodge) acepromazine (C-Vet) anaesthetic (appendix II). The mice received approximately 7 µl/gm of body weight (i.e. a 40 g mouse received ~280 µl of anaesthetic). The abdomen of the mice was shaved, and the area cleaned using a surgical scrub (7.5% Providone-Iodine, USP). A 1.5 cm transverse incision was made into the skin and body wall, using fine dissection scissors (Fine Scientific Tools, F.S.T), at a point level with the top of the legs. The hole in the body wall was marked using a small piece of suture (vicryl absorbable suture, Johnson and Johnson). Using blunt forceps, the left testicle, vas deferens and epididymis were excised from the body cavity. A loop of approximately 1 cm of the vas deferens was held in the forceps and removed. The two exposed ends of the vas deferens were then cauterized using a red hot spatula. The testicle, vas deferens and epididymis were carefully replaced back inside the body wall, and the procedure was repeated on the right vas

deferens. The hole in the body wall was sutured using an absorbable suture (vicryl absorbable suture, Johnson and Johnson). The cut in the skin was sutured (ethilon non-absorbable suture, Johnson and Johnson), and covered with a tissue adhesive (Vetbond, 3M Animal Care Products, USA). After surgery, each mouse received 150 µl of analgesic (Temgesic 300 µg/ml; Reckitt & Colman Products, appendix II) by intraperitoneal injection and allowed to recover before being returned to their cages.

The males were allowed to recover for 2 weeks before their sterility was assessed. 5-12 week old virgin female F1 CBAxC57/BL6 mice (University of Southampton, Biomedical Research Facility) were housed overnight with the vasectomized males. Each morning, the females were checked for a vaginal plug (taken as a sign of copulation). Plug positive females were individually housed and weighed at regular intervals for the next 20 days. Plug negative females were placed back in their original cages during the day, but then returned to the same male in the afternoon. Each male was mated with at least two females to confirm sterility.

2.2.2 Embryo transfer

5-6 week old virgin female F1 CBAxC57/BL6 mice were superovulated (section **2.1.1**) and mated with MF1 males. At the time of hCG injection and mating, 8-12 week old female F1 CBAxC57/BL6 mice were mated (without superovulation) with vasectomised MF1 males. Females which had copulated with the vasectomised males (determined by the presence of a vaginal plug the next morning) were individually housed for the next 3 days. To provide embryos derived from an *in vivo* environment, some of the mice that had been superovulated and mated with a fertile male were not flushed until an hour before the time of transfer (~ 93 hours post hCG). These embryos were flushed from the uterus (section **2.1.2**) and kept in pre-warmed H6 BSA under mineral oil until transferred, approximately 2 to 4 hours after flushing.

Immediately prior to their transfer, embryos were removed from their *in vitro* culture conditions and placed into pre-warmed H6 BSA or H6 PVP (depending on the

type of manipulation being performed on the embryos) under mineral oil for up to three hours, and kept at 37°C. This was to buffer against any pH changes until the embryos were transferred.

Day 3.5 pseudo-pregnant female F1 CBAxC57/BL6 mice were anaesthetized by an intraperitoneal injection of a ketamine (Fort Dodge) acepromazine (C-Vet) anaesthetic (section **2.2.1**). The lower back of the anaesthetized mice was then shaved, and the area cleaned using a surgical scrub (7.5% Providone-Iodine, USP). A small longitudinal incision (1 cm) was made in the skin at the level of the first rib. Connective tissue holding the skin to the body wall was carefully cut. The skin was manipulated so that both ovaries could be seen through the body wall. Using fine forceps and dissection scissors (Fine Scientific Tools, F.S.T), a small cut was made in the body wall just over the left ovary. Using a small piece of suture (vicryl absorbable suture, Johnson and Johnson) the cut in the body cavity was marked. The ovary was excised from the body via the fat pad taking great care not to handle or damage the ovary, oviduct or uterus. The ovary was held outside the body via the use of a serrated bulldog type serrefine (Fine Scientific Tools). Using a polished transfer pipette (section **2.1.4**. bore of pipette is made large enough to fit an expanded blastocyst) attached to a mouth pipette, 6 blastocysts were taken up. The transfer pipette was kept in place until required using plasticine, whilst the culture dish was replaced in the 37°C incubator. Under a stereomicroscope (Zeiss), a hole was made in the top of the uterus using a 25 gauge needle, a few millimetres down from the uteruo-tubal junction. Approximately 5 mm of the transfer pipette was inserted into the hole, and the blastocysts were transferred. The mouse was removed from the microscope and the hole in the body cavity was sutured (vicryl absorbable suture, Johnson and Johnson). This procedure was repeated on the right hand side of the mouse.

The cut in the skin was sutured (ethilon non-absorbable suture, Johnson and Johnson), and covered with a tissue adhesive (Vetbond, 3M Animal Care Products USA). After surgery, each mouse received 100 µl of analgesic (Temgesic, 300 µg/ml.

Reckitt & Colman Products, section **2.2.1**) by intraperitoneal injection and allowed to recover before being returned to their cages.

2.3 Analysis of postnatal growth and development

2.3.1 Postnatal maintenance of offspring

On day of birth, mice were weighed on an electronic balance, sexed and if necessary, litter size was adjusted so as to contain specific numbers of males and females (see Chapter 4, section **4.2.1**, and Chapter 5, section **5.1**). Care was taken not to transmit the smell of other mice onto the new born pups via the wearing of gloves and washing of hands, as this may have resulted in the rejection of the pups by their mother. At three weeks of age, offspring were weaned from their mothers, and the sexes caged separately. Mice had access to standard chow and water *ad libitum*.

2.3.2 Measurement of postnatal growth

Mice were weighed on day of birth and then subsequently on the same day every week for the next 27 weeks.

2.3.3 Systolic blood pressure

Systolic blood pressure was determined at 8, 15 and 21 weeks of age by tail-cuff plethysmography using an ITC model 229 blood pressure monitor (Linton Instruments, Norfolk, UK). Mice were allowed to acclimatize to a room temperature of 27-30°C for at least 90 minutes before readings were taken. Mice were restrained within a Perspex tube whilst the tail was placed into the tail cuff. The mice were allowed to acclimatize to the tube and tail cuff for several minutes. Four systolic blood pressure recordings were taken per mouse for each of the ages studied. If after 20 minutes all four recordings had not been taken, the mouse was released and allowed to recover for 30 minutes before obtaining the remaining blood pressure values. The mouse's heart rate was monitored as an indicator of stress. If the animal's heart rate rose above 500 beats per minute then readings were stopped until the heart rate had reduced. See appendix III for an example of a blood pressure trace.

2.3.4 Measurement of organ weight

27 week old mice were weighed before being killed by cervical dislocation ensuring not to rupture any blood vessels. Blood samples were taken by puncturing the heart with a pulled glass pipette (internal diameter ~ 2 mm) attached to a mouth pipette, and kept on ice. Liver, left and right kidneys, abdominal and retro kidney fat, heart and lungs were dissected out, weighed and snap frozen in liquid nitrogen, and stored at -80°C. Blood samples were centrifuged at 10,000 g, 4°C for 10 minutes, after which the serum was aliquoted into 10 µl samples and frozen at -80°C.

2.3.5 Measurement of ACE activity in mouse serum

5 µl of serum was mixed with 70 µl buffer (300 mM KH_2PO_4, 150 mM NaCl, pH 8.3, see appendix IV). To each sample, 50 µl Hippuryl-$_L$-histidine-$_L$-leucine acetate salt (5 mM, Sigma) in buffer was added. Immediately after mixing, the samples were transferred to a shaking water bath and incubated for 45 minutes at 37°C. The reaction was terminated by the addition of 875 µl ice cold, chloride-free buffer (100 mM KH_2PO_4, pH 8.3, appendix IV) and by keeping the samples on ice for 10 minutes. As a negative control, duplicates containing only serum and chloride buffer were prepared and treated exactly as the incubates. After 10 minutes, 500 µl of 0.16 M cyanuric chloride (2-4-6 trichloro 1-3-5 triazine; Sigma) in 1,4 dioxane (Sigma) was added to each sample, mixed and left at room temperature for 10 minutes for a yellow colour to develop. After centrifugation at 3,000 g for 10 minutes at room temperature, the clear yellow suppernatant was removed. From each sample, four 200 µl aliquots were pipetted into a 96 well plate, and were analysed on a Dynatech MR5000 plate reader at 380 nm against a blank containing 1ml of 100 mM buffer (pH 8.3) and 0.5 ml cyanuric chloride in 1,4 dioxane. For each analysis, a standard curve was prepared from a 1mM hippuric acid (Sigma) solution in 100 mM chloride buffer pH 8.3 (appendix IV). Solutions of 1 mM, 750 µM, 500 µM, 250 µM, 100 µM and 50 µM hippuric acid were treated exactly as the incubates. Each sample was analysed in duplicate, and an average activity was taken.

Serum ACE activity was expressed as nanomoles of hippurate formed per millilitre of serum per minute.

2.3.6 Measurement of ACE activity in lung tissue

Samples of lung (approximately 50 mg) were homogenized in 300 µl ice cold buffer (0.2 M H_3BO_3, 2 M NaCl, pH 8.3, appendix IV) using a PowerGen 125 homogeniser. Samples were centrifuged at 16,400 rpm for 10 minutes at 4°C. The supernatant was removed and stored at -80°C until analysis. The pellet was homogenised in a further 300 µl of buffer and the supernatant was removed after centrifugation as before. 10 µl samples of lung extracts were mixed with 50 µl of buffer (0.2 M H_3BO_3, 2 M NaCl, pH 8.3, appendix V) and 20 µl of water before being incubated at 37°C in a water bath for 5 minutes. 20 µl of Hippuryl-$_L$-histidine-$_L$-leucine acetate salt (20 mM, Sigma) in buffer was added to each tube. Controls were set up where 1 M HCl was added prior to the addition of the Hippuryl-$_L$-histidine-$_L$-leucine. Samples and blanks were then incubated in a water bath at 37°C for 30 minutes. The reaction was terminated by the addition of 100 µl of 1 M HCl, 100 µl of 1 M NaOH, 400 µl 0.2 M KH_2PO_4 (pH 8.3) and 400 µl 0.16 M cyanuric chloride (2-4-6 trichloro 1-3-5 triazine; Sigma) in 1,4 dioxane (Sigma). Samples were mixed and left at room temperature for 10 minutes for a yellow colour to develop. Samples were centrifuged and analysed as described in section **2.3.4** against a blank containing buffer (0.2 M H_3BO_3, 2M NaCl, pH 8.3, appendix IV) and water, which had been treated exactly as the samples. For each analysis, a standard curve was prepared from a 4 mM hippuric acid (Sigma) solution in 1M NaOH. Solutions of 4 mM, 3 mM , 2 mM, 1 mM, 400 µM and 20 µM hippuric acid were treated exactly as the incubates. Each sample was analysed in duplicate, and an average activity was taken. Tissue protein content was measured using a commercially available protein assay kit (Bio-Rad).

Tissue ACE activity was expressed as nanomoles of hippurate formed per milligram of protein per minute.

2.4 Statistical analysis of data

For all statistical analyses, significance was taken as $p \leq 0.05$. A trend was assumed to exist if a p value of between 0.1 and 0.051 was observed.

All data regarding blastocyst cell numbers and rates of blastocyst development were analysed using a one way ANOVA (SigmaStat statistical software, version 2.0). A one way ANOVA followed by a pairwise t-test with Bonferroni correction analysis was used to analyse the litter size from the different treatment groups (SigmaStat statistical software, version 2.0).

Data regarding correlations of litter size to birth weight were assessed for normality using the SigmaStat statistical software package (version 2.0). If the distribution passed the normality test, the correlation was analysed using a Pearson correlation. If the distribution failed the normality test, the correlation was analysed using a Spearman correlation.

All postnatal data regarding birth weights, growth weights, blood pressures, organ weights and ratios, serum and lung ACE levels and ACE correlations were analysed using a multilevel random effects regression model which takes into account between-mother and within-mother variation in litter size and parameters measured from individual animals, thereby preventing differences that are due to abnormal litters. The model used was the following:

$$y(lp) = a + b.treatment + u(l) + e(lp) + c$$

Where l is the litter
p is the pup
y(lp) is the response (=outcome) variable as measured on pup p in litter l
a is the intercept
treatment is either the mouse group from the different embryo origins e.g. *in vitro*, or in the case of the *Ped* mice the strain (0 for *Ped* minus, 1 for *Ped* plus)

b is the regression coefficient which measures the amount by which y is increased due to the treatment as opposed to the control treatment

u(l) is a random effect applying to all pups in litter l, if u(l) is large then all pups in that litter tend to have large values of y. The programme estimates the variance of all the u's that apply to the various litters

e(lp) is a random effect applying to all pups, it represents measurement errors within-pup variability and other uncaptured determinants of outcome.

c is the number of animals wthin the litter, either before and/or after correction

The program estimates the variance of all the e(lp) terms. The random effects model analysis was conducted in consultation with Dr Clive Osmond, senior Medical Statitician at the Medical Research Council Epidemiology uint at Southampton General Hospital.

Chapter 3

Effect of *in vitro* culture and manipulation on blastocyst development

3.1. Introduction

In vitro cultured mammalian embryos have been shown to develop at slower rates when compared to embryos developing *in vivo* (Harlow and Quinn, 1982; Sakkas *et al.*, 1989; Gardner and Sakkas, 1993). Specific *in vitro* culture conditions, such as the presence of exogenous protein, have been shown to alter developmental rates, embryo morphology, metabolism and embryonic gene expression patterns (Thompson *et al.*, 1995, Lane and Gardner 1997a, 1997b; Young *et al.*, 1998; Khosla *et al.*, 2001a). Whilst *in vitro* culture may slow preimplantation development, the addition of growth factors such as insulin and IGF-1 to the culture medium has been shown to stimulate embryo development *in vitro*, in particular development of the ICM (Harvey and Kaye 1990, 1992; Palma *et al.*, 1997; Lighten *et al.*, 1998; Mihalik *et al.*, 2000). Embryos cultured in the presence of these growth factors have been shown to develop at a faster rate, have increased levels of endocytosis and protein synthesis, and have a significantly increased total blastocyst cell number.

Studies have shown that the mouse preimplantation embryo is able to compensate for changes in its total volume and cell number, whilst still being able to develop into a viable offspring (Rands, 1986a, 1986b; Somers *et al.*, 1992; Biggers and Papaioannou 1992; Hardy and Handyside, 1993). These studies have shown that embryos containing altered total numbers of cells remodel their development so as to compensate for the changes imposed upon them. It has been observed that embryos manipulated to contain increased or decreased number of cells reschedule events of morphogenesis and gastrulation so as to keep the cell number and body size appropriate for each developmental stage (Tarkowski, 1959; Mintz, 1965; Tarkowski and Wroblewska, 1967; Power and Tam, 1993).

Under the correct *in vitro* conditions, isolated ICMs are able to generate a

TE-like structure via the differentiation of those cells located on the outer face of the ICM. In an intact embryo, the cells of the ICM display total cell-cell contact. Upon isolation, those cells located on the outer face of the ICM now display contact asymmetry. The loss of total cell-cell contact leads to the up-regulation of an epithelial phenotype within these cells, allowing for the reformation of a blastocoel cavity (reviewed in Fleming *et al.*, 2001).

The aim of the experiments within this Chapter was to determine the effect of specific *in vitro* culture conditions or manipulations on blastocyst development, cell number and ICM:TE cell ratios. Such an analysis to characterise the proliferation and morphogenesis of blastocysts was a pre-requisite for subsequent studies of their developmental potential after embryo transfer. The first series of experiments compare the effect of *in vivo* development with a series of *in vitro* culture environments and durations on blastocyst formation. Embryos were either cultured from the 2-cell to the blastocyst and expanded blastocyst stages in T6 BSA or T6PVP, or were flushed at the blastocyst stage. A second series of experiments investigated the effect of exogenous insulin and IGF-1 on preimplantation developmental rates, and the ratios of ICM:TE cells, in embryos cultured from the 2-cell stage to the blastocyst and expanded blastocyst stages. As previous studies have observed significant effects on blastocyst and ICM cell number at insulin and IGF-1 concentrations of 170 nM and 1.70 nM respectively, it was decided to base this study around these concentrations (Harvey and Kaye, 1990, 1992; Palma *et al.*, 1997; Lighten *et al.*, 1998). It was also decided to examine the effects of concentrations five fold higher and lower than 170 nM insulin and 1.70 nM IGF-I in order to observe any dose dependent effects. The final set of experiments examined the effect of physical manipulation on the ICM:TE ratio within the preimplantation embryo. This was studied using two separate approaches; (1) altering the size of the pre-compact embryo so as to half or double the normal number of cells; (2) aggregating ICMs isolated from early blastocysts to produce a blastocyst-like structure that comprises the normal number of cells, but where the TE has been derived from the ICM which does not occur during normal development.

For all three series of experiments the numbers of cells within the TE and ICM were then determined so as to monitor effects on allocation and proliferation within these two lineages.

3.2 Methodology

3.2.1 Superovulation and embryo collection

Female F1 CBAxC57/BL6 mice were superovulated and mated with MF1 males (section **2.1.1**). Embryos were either flushed at the 2-cell stage (~50 hours post hCG) and cultured in T6 BSA or in T6 PVP to the 8-cell stage (~70 hours post hCG), blastocyst stage (~96 hours post hCG), expanded blastocyst stage (~116 hours post hCG), or flushed at the blastocyst stage (~96 hours post hCG; section **2.1.2**).

3.2.2 Embryo culture in the presence of growth factors

2-cell stage (~50 hours post hCG) embryos were cultured in different concentrations of insulin or IGF-1 for up to 116 hours post hCG (section **2.1.3**). Control embryos were also flushed at the 2-cell stage and cultured in the absence of growth factors. Stages and rates of embryo development were assessed at two time points for each day of culture (10 am and 5 pm). Morula stage embryos were recorded when no individual cell outline was discernable within the embryo. Expanded blastocysts were determined by eye as having a blastocoel cavity occupying 75% or more of the embryos' volume. Data on blastocyst cell numbers and rates of embryo development were combined from a minimum of four repeated experiments.

3.2.3 Production of half, whole and double sized embryos

8-cell embryos (cultured from the 2-cell stage in T6 BSA) were disaggregated to single blastomeres and then reaggregated so as to contain 4, 8 or 16 cells, thus creating half, whole and double sized embryos, respectively (section **2.1.4**). These embryos were then cultured until they developed to the blastocyst stage (~114 hours post hCG).

3.2.4 Immunosurgery of the trophectoderm

The TE of early blastocysts was removed by immunosurgery, and the ICMs isolated (section **2.1.5**). Isolated ICMs were then aggregated (groups of three) or cultured individually in DMEM plus 10% fetal calf serum for up to 114 hours post hCG.

3.2.5 Differential nuclear staining

All embryos were differentially stained at either the blastocyst or expanded blastocyst stage (section **2.1.6**) and the number of TE and ICM nuclei counted. Alongside each data set presented here is an *in vitro* reference group taken from Figures 3.1 and 3.2. Although the experiments within Figures 3.1 and 3.2 were carried out at a different time to those of the other experiments within this section, they do provide an outside reference of the developmental competence of the manipulated embryos.

3.2.6 Statistical analysis

All data were analysed using a one way analysis of variance (one way ANOVA test; Sigmastat statistical software package, version 2.0). Statistical significance was assumed at $p \leq 0.05$.

For these experiments, the control group have been designated the letter **a**; any significant difference between control and experimental groups is shown by the use of a different letter, as indicated in the Figures in this chapter. The * above the bars indicates the level of significance observed.

3.3 Results

3.3.1 Comparison of *in vitro* and *in vivo* environments on blastocyst development

The effect of *in vitro* culture on the development of embryos to the early blastocyst (96 hours post hCG) and the expanded blastocyst (116 hours post hCG) stage, when compared to embryos derived from an *in vivo* environment at 96 hours post hCG, is shown in Figures 3.1 and 3.2. Embryos analysed from an *in vivo*

environment have significantly increased numbers of TE and ICM cells compared to *in vitro* derived embryos for the same time post hCG. If the *in vitro* derived embryos at 96 hours post hCG are cultured for a further 20 hours (116 hours post hCG) then they achieve cell numbers comparable to those of the *in vivo* derived embryos at 96 hours post hCG. However, there is no significant difference in the ratio of ICM:TE cells for any of the *in vitro* culture conditions when compared to the *in vivo* derived blastocysts (Figure 3.2).

The data within Figures 3.1 and 3.2 also compare the effects of two different culture media (T6 BSA and T6 PVP) on embryo development. No significant differences were observed in the total cell number or the ICM:TE ratio between embryos cultured in the two media at either 96 or 116 hours post hCG.

Figure 3.1

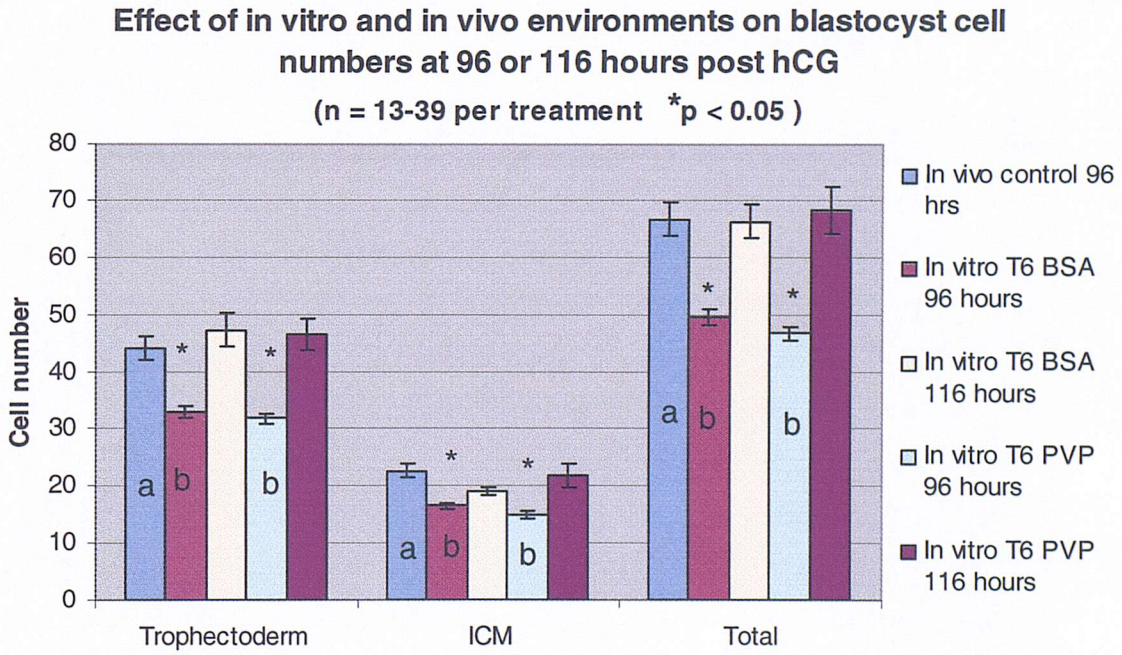
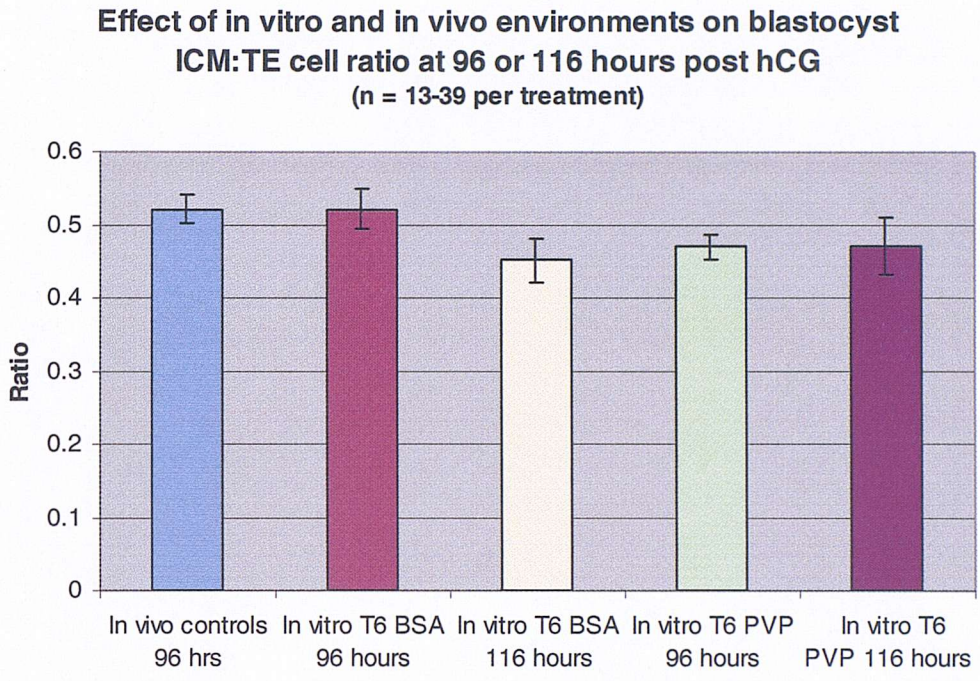


Figure 3.2



3.3.2 Embryo culture in T6 BSA medium and the presence of different concentrations of insulin

The developmental rate of embryos cultured from the 2-cell to the expanded blastocyst stage in T6 BSA medium supplemented with different concentrations of insulin (see section **2.1.3**) are shown in Figures 3.3 to 3.7. Figure 3.3 and 3.4 show that between 70 and 77 hours post hCG, those embryos cultured in the absence of insulin have the lowest percentage of 4 and 8-cell embryos, whilst those embryos in the presence of 850nM insulin have the highest percentage of 8-cell embryos.

Figure 3.5 shows that between 70 and 92 hours post hCG, those embryos cultured in the presence of 850 nM insulin have the highest percentage of compacting embryos. At 114 and 120 hours after hCG, those embryos cultured in the presence of 850 nM insulin have a significantly higher percentage of blastocysts and expanded blastocysts respectively, when compared to those embryos cultured in the absence of insulin (Figures 3.6 and 3.7).

Figure 3.3

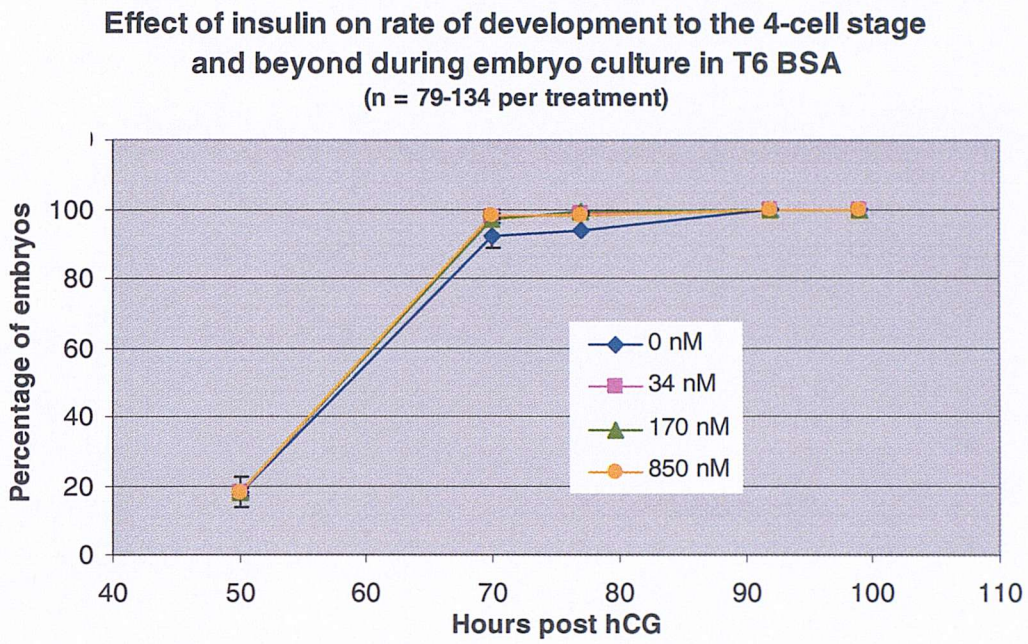


Figure 3.4

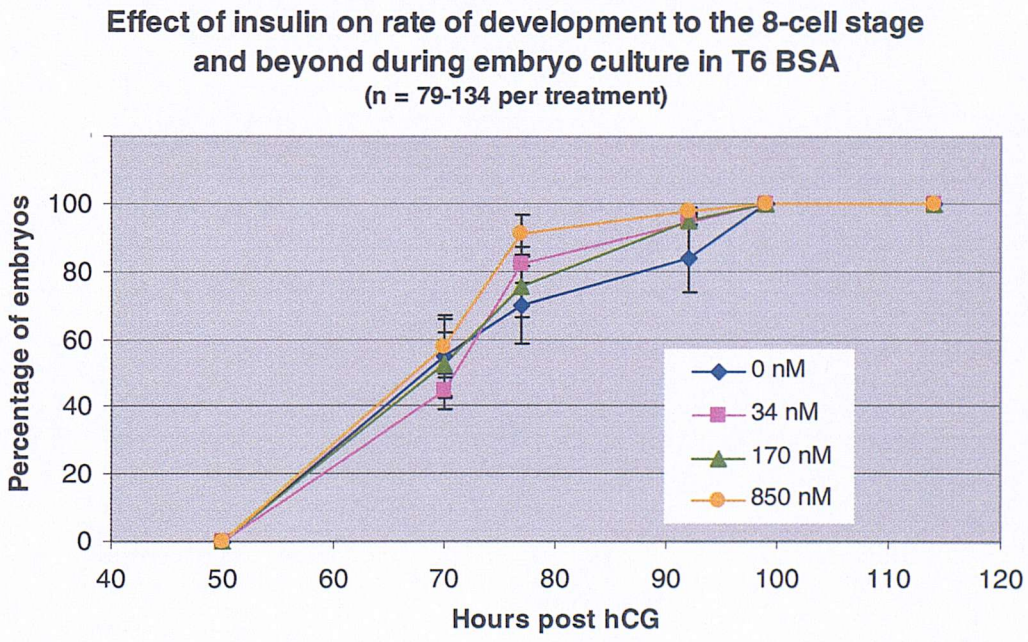


Figure 3.5

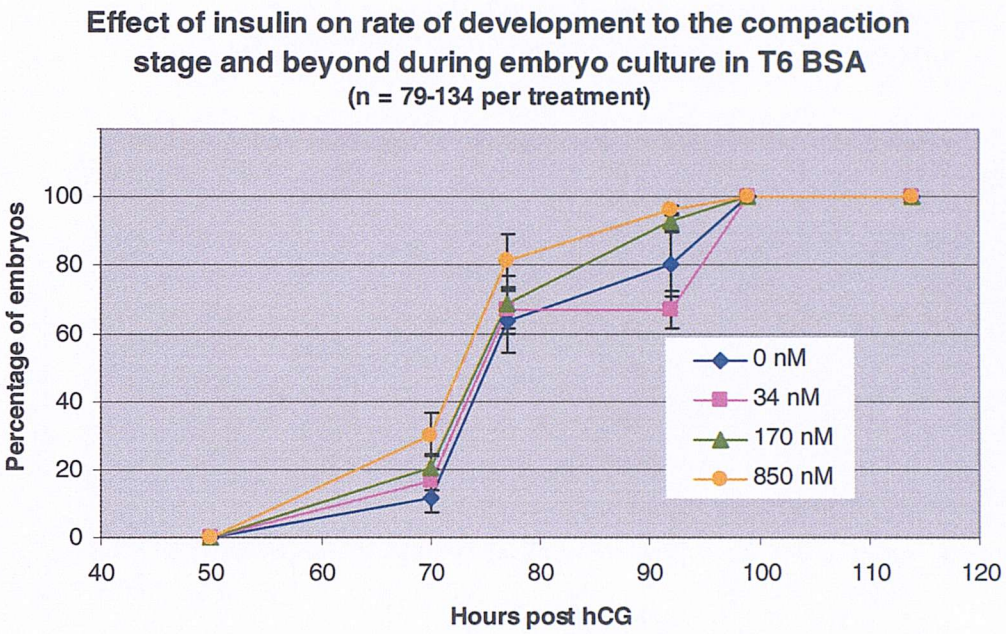


Figure 3.6

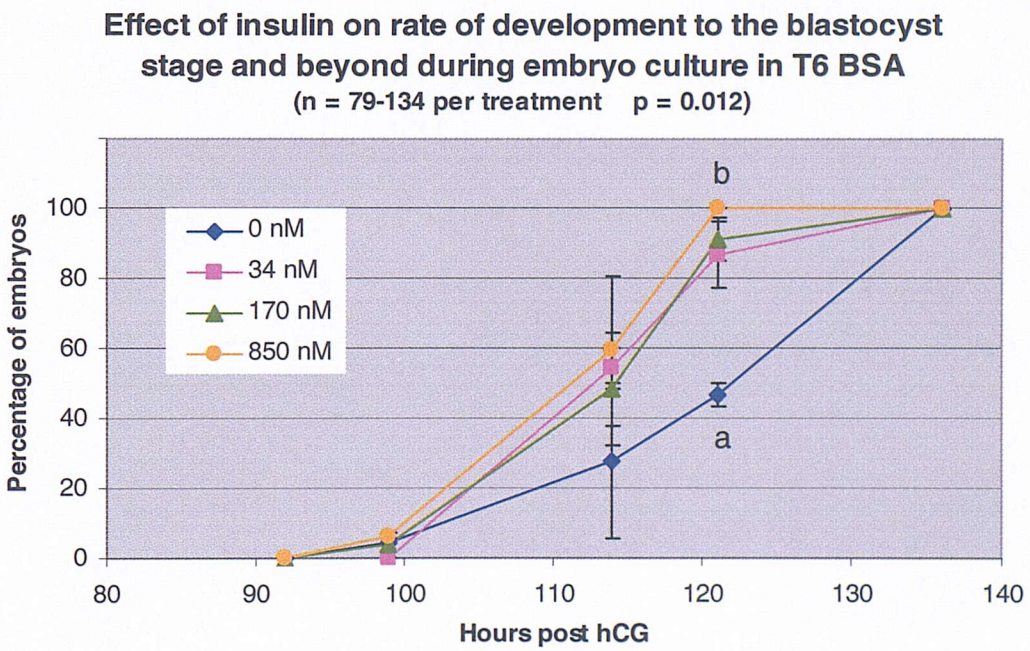
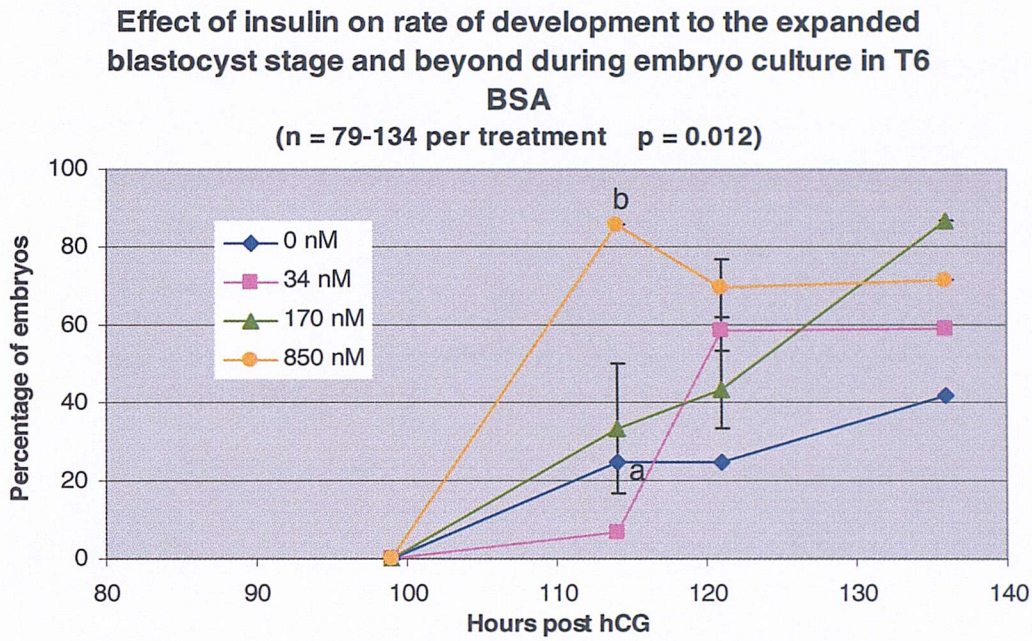


Figure 3.7


Effect of insulin on rate of development to the expanded blastocyst stage and beyond during embryo culture in T6 BSA
(n = 79-134 per treatment p = 0.012)
Percentage of embryos
100
80
60
40
20
0
80
90
100
110
120
130
140
Hours post hCG
0 nM
34 nM
170 nM
850 nM
b
a

The effects of culturing embryos from the 2-cell stage in different concentrations of insulin in T6 BSA medium for up to 96 hours post hCG on blastocyst cell numbers are shown in Figures 3.8 and 3.9. Figure 3.8 shows that there is a significant increase in the total number of ICM cells within those blastocysts cultured in the highest concentration of insulin (850 nM) when compared to the control group (0 nM insulin). This increase, however, has no significant effect on the ICM:TE ratio, although these blastocysts do have the highest overall ratio.

Figure 3.8

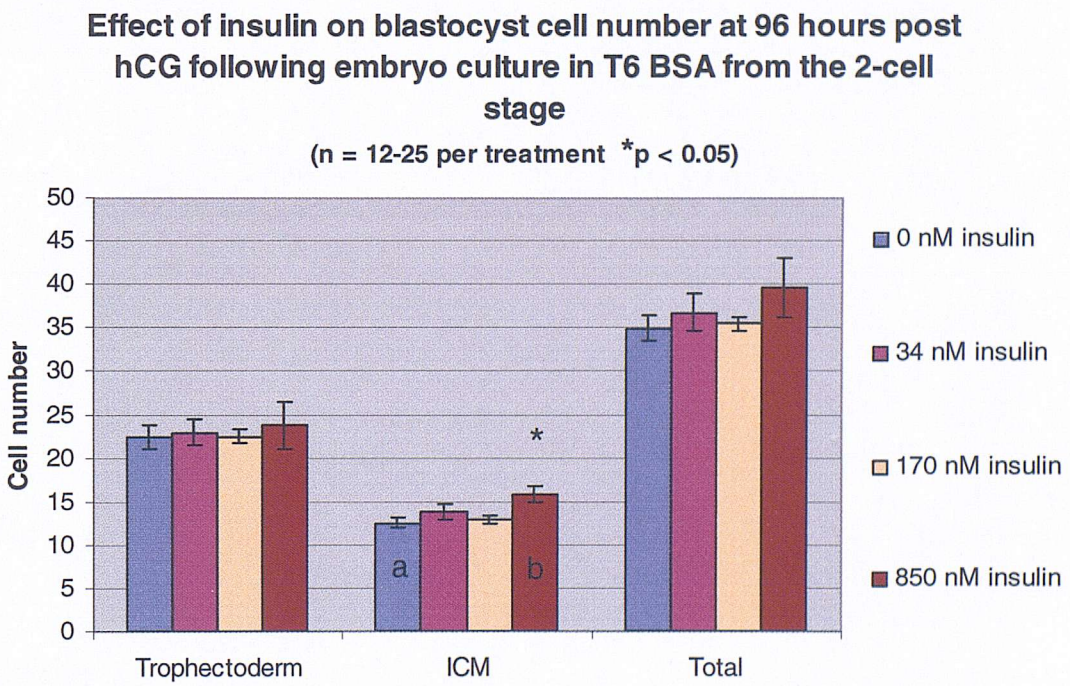
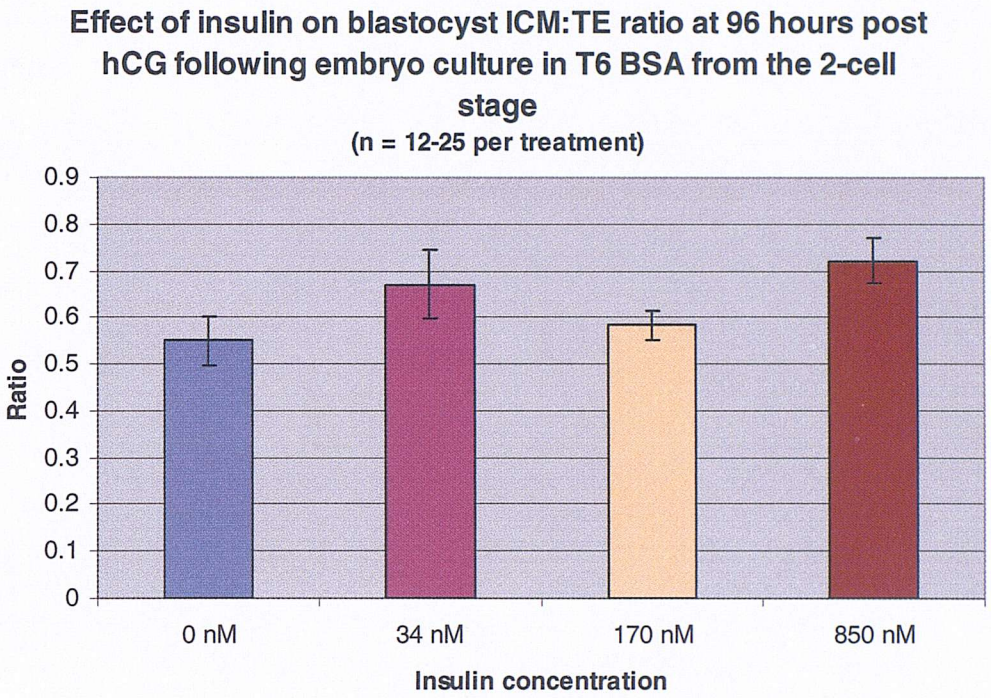


Figure 3.9


Effect of insulin on blastocyst ICM:TE ratio at 96 hours post hCG following embryo culture in T6 BSA from the 2-cell stage
(n = 12-25 per treatment)
Ratio
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 nM
34 nM
170 nM
850 nM
Insulin concentration

The mean blastocyst cell number of embryos cultured from the 2-cell stage in different concentrations of insulin in T6 BSA for up to 116 hours post hCG are shown in Figures 3.10 and 3.11. There is no significant difference between any of the groups. However, Figure 3.10 shows that there is a slight but non-significant increase in the total cell number within the 850 nM insulin group, caused by an increased number of ICM cells. This increased ICM cell number does have the effect of giving the 850 nM insulin group the highest ICM:TE ratio for all the concentrations studied.

Figure 3.10

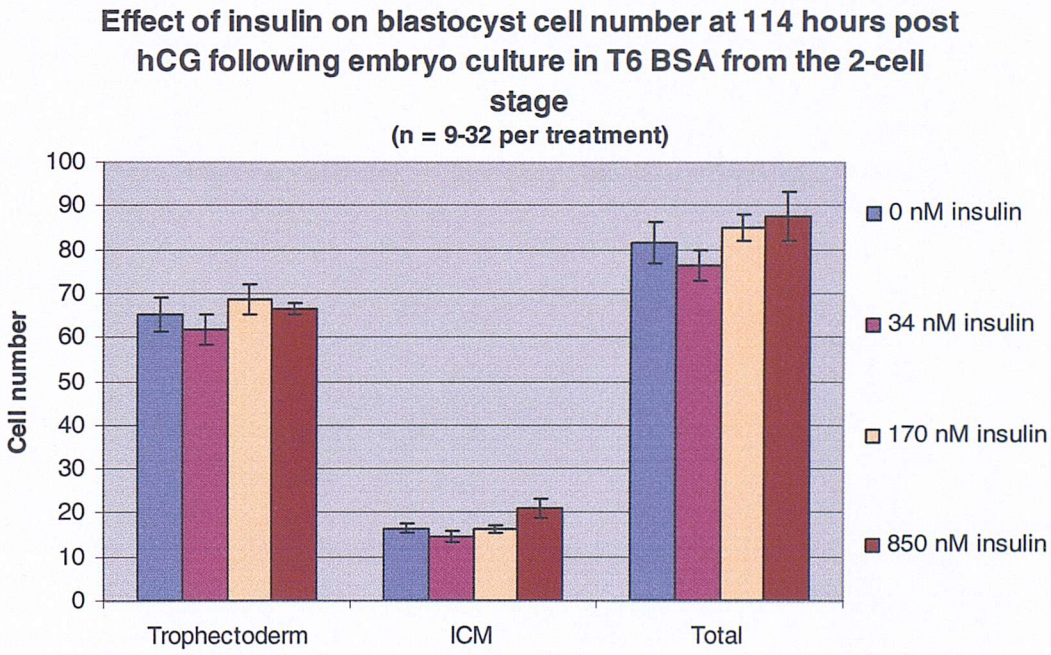
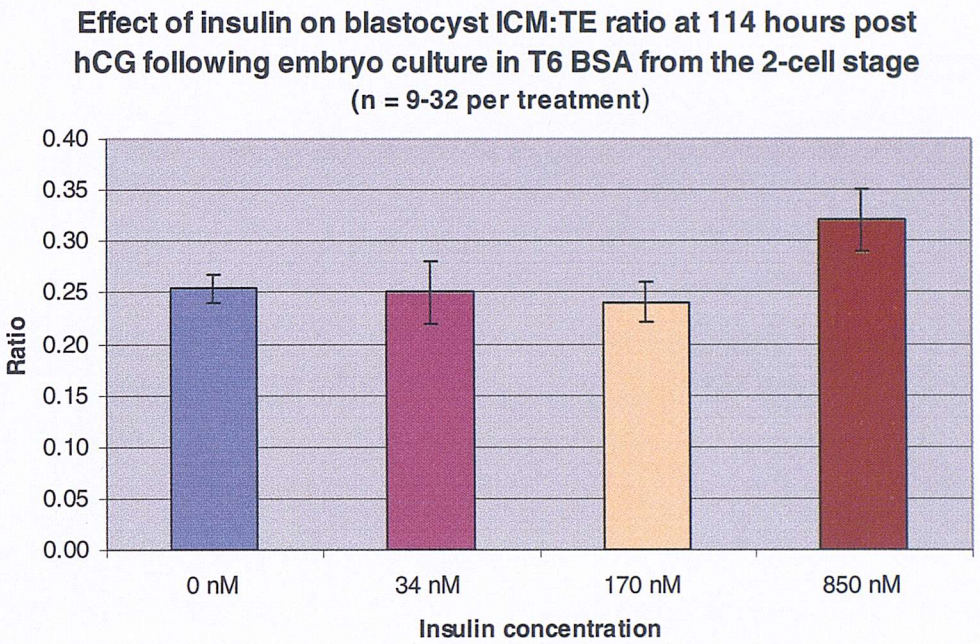


Figure 3.11


Effect of insulin on blastocyst ICM:TE ratio at 114 hours post hCG following embryo culture in T6 BSA from the 2-cell stage
(n = 9-32 per treatment)
Ratio
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 nM
34 nM
170 nM
850 nM
Insulin concentration

3.3.3 Embryo culture in T6 PVP medium and the presence of different concentrations of insulin

The developmental rate of embryos cultured from the 2-cell to the blastocyst stage in T6 PVP medium supplemented with different concentrations of insulin (section **2.1.3**) are shown in Figures 3.12 to 3.16. No significant difference was observed between any of the treatment groups for any of the stages analysed.

Figures 3.14 and 3.15 show that at 70 and 90 hours post hCG, those embryos cultured in the presence of 850 nM insulin have the highest percentage of compacting embryos and blastocysts respectively, when compared to the other groups. Figure 3.15 shows that at 86 hours after flushing, the 850 nM and 170 nM insulin groups have the highest percentage of expanded blastocysts, whilst the 0 nM and 34 nM insulin groups have the lowest. Figure 3.16 shows that those embryos cultured in the highest concentration of insulin have the highest percentage of expanded blastocysts, whilst those embryos cultured in the absence of insulin have the lowest percentage.

Figure 3.12

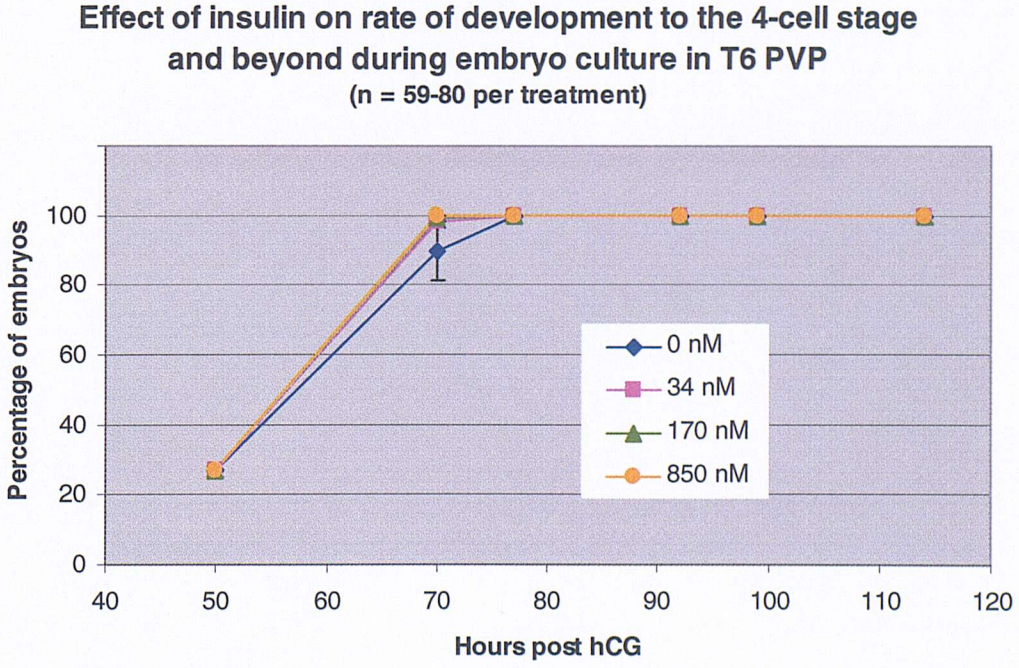


Figure 3.13

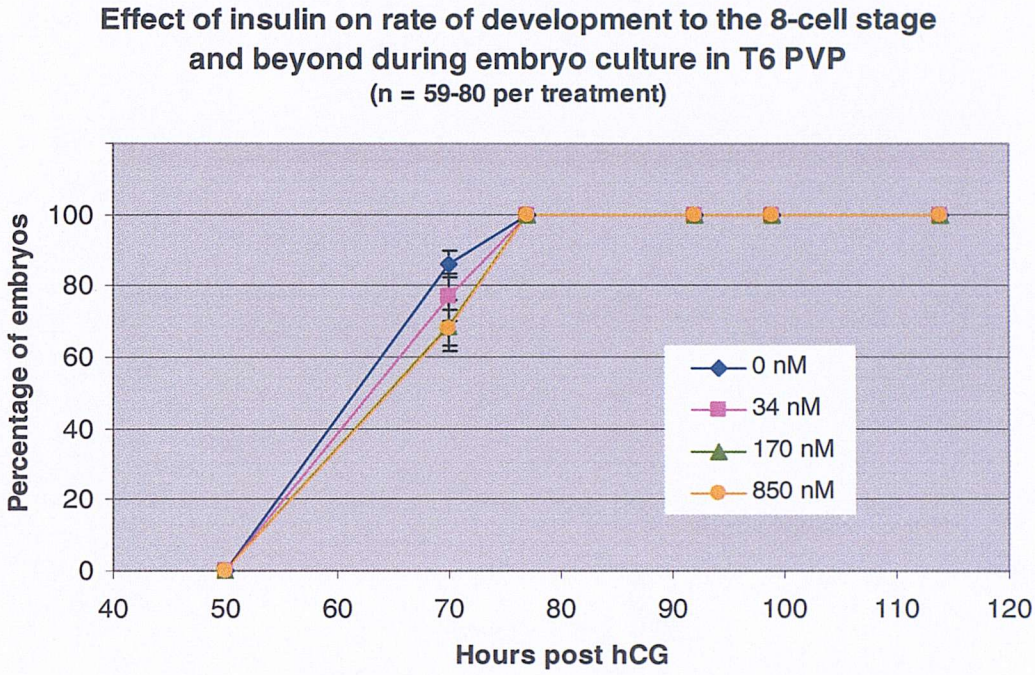


Figure 3.14

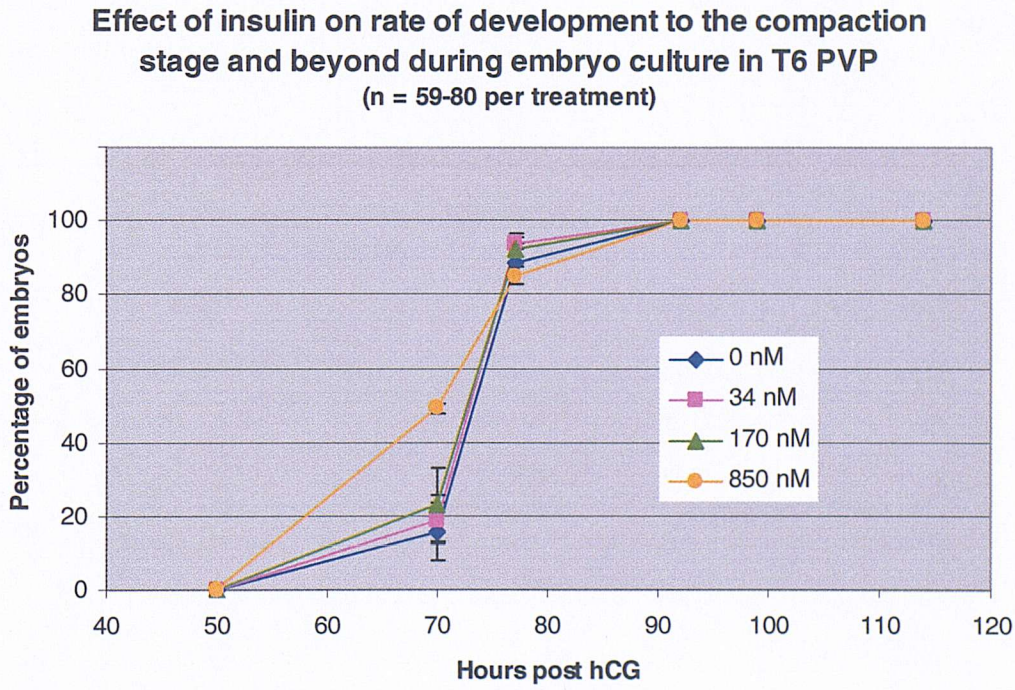


Figure 3.15

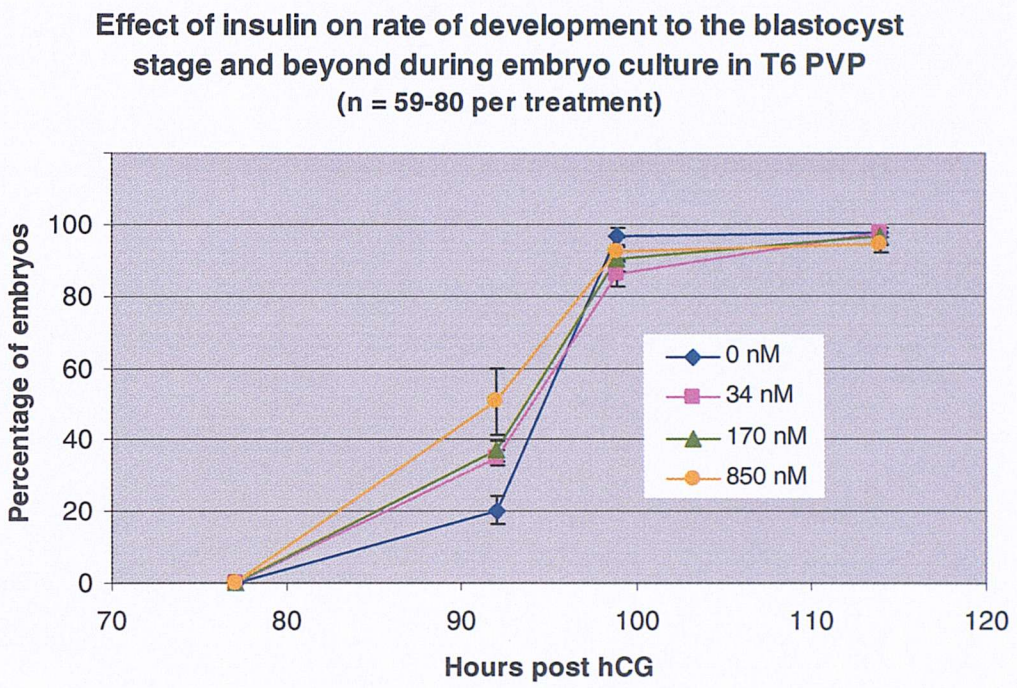
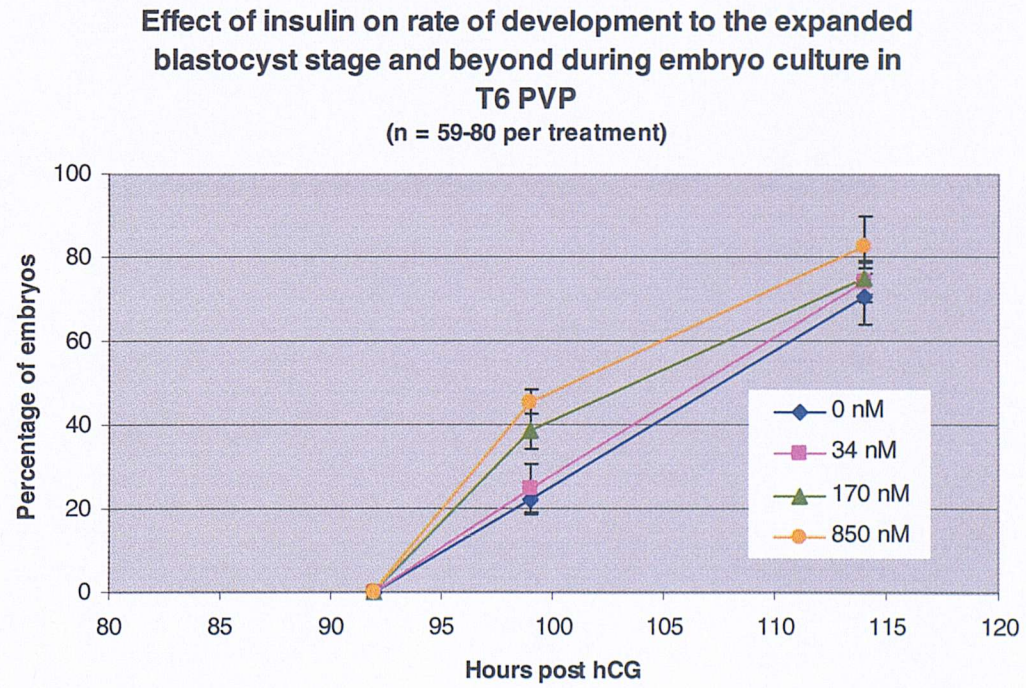


Figure 3.16



The mean blastocyst cell number of embryos cultured from the 2-cell stage in different concentrations of insulin in T6 PVP for up to 96 hours post hCG are shown in Figures 3.17 and 3.18. There is a slight reduction in the number of TE cells, whilst there is a gradual increase in the number of ICM cells as the insulin concentration increases. At 850 nM insulin, there is a significant increase in the number of ICM cells when compared to the control (0 nM insulin). There is no overall significant increase or decrease in the total number of cells within any of the blastocyst groups, however, it can be seen that as the insulin concentration increases there is a gradual increase in the numbers of ICM cells. There is a significant increase in ICM:TE ratio at 170 nM insulin and above.

Figure 3.17

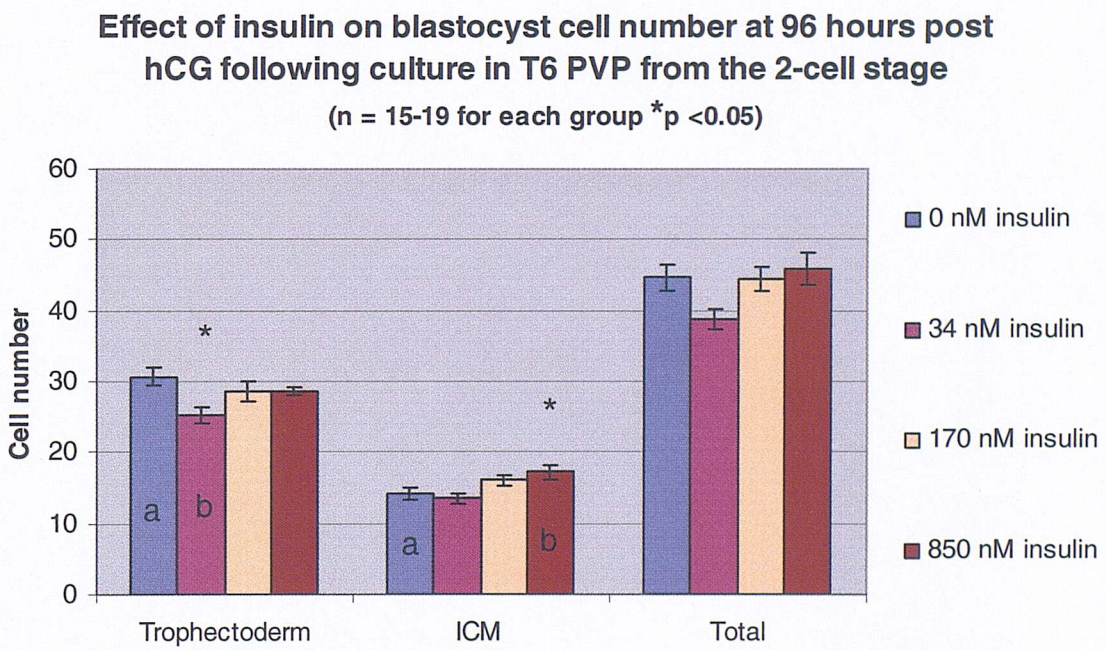
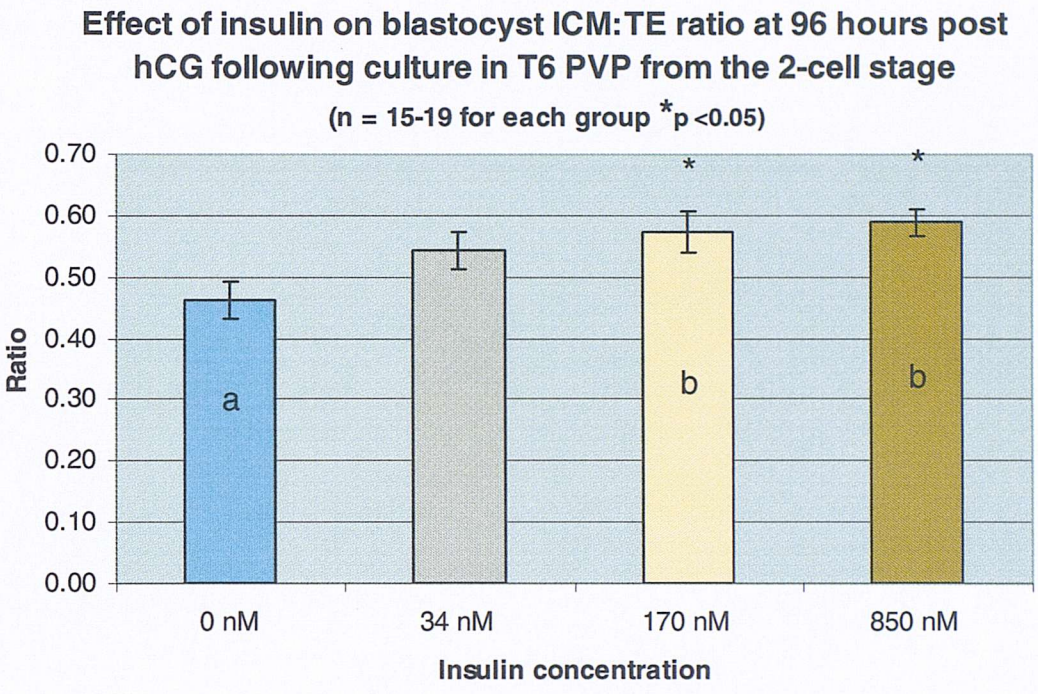


Figure 3.18


Effect of insulin on blastocyst ICM:TE ratio at 96 hours post hCG following culture in T6 PVP from the 2-cell stage
(n = 15-19 for each group *p <0.05)
Ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
a
b
b
*
*
0 nM
34 nM
170 nM
850 nM
Insulin concentration

3.3.4 Embryo culture in T6 PVP medium in the presence of different concentrations of IGF-1

The developmental rate of embryos cultured from the 2-cell to the expanded blastocyst stage in T6 PVP medium supplemented with different concentrations of IGF-1 is shown in Figures 3.19 to 3.22. Figure 3.19 shows that there is no difference in the rate of embryos reaching the 8-cell stage between any of the groups. Figure 3.20 shows that at 70 hours post hCG, there are significantly more compacted embryos within the 8.50 nM IGF-I group when compared to those embryos cultured in the absence of IGF-I.

At 92 and 99 hours post hCG, there are significantly more blastocysts and expanded bloastocysts respectively in the 8.50 nM IGF-I group when compared to those embryos cultured in the absence of IGF-I (Figures 3.21and 3.23).

Figure 3.19

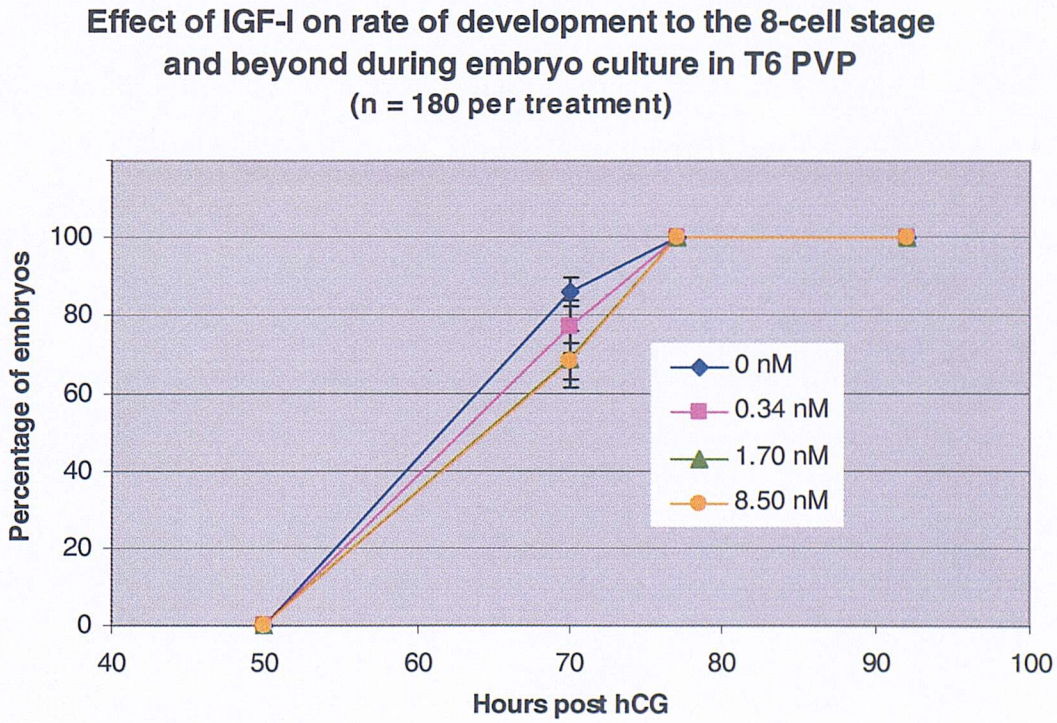


Figure 3.20

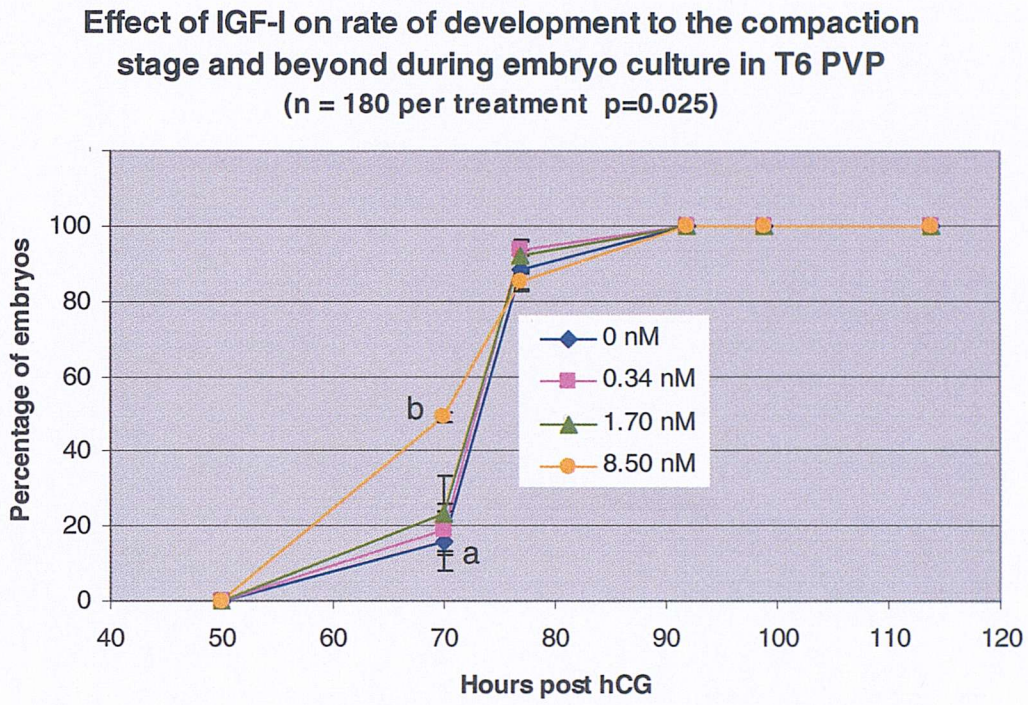


Figure 3.21

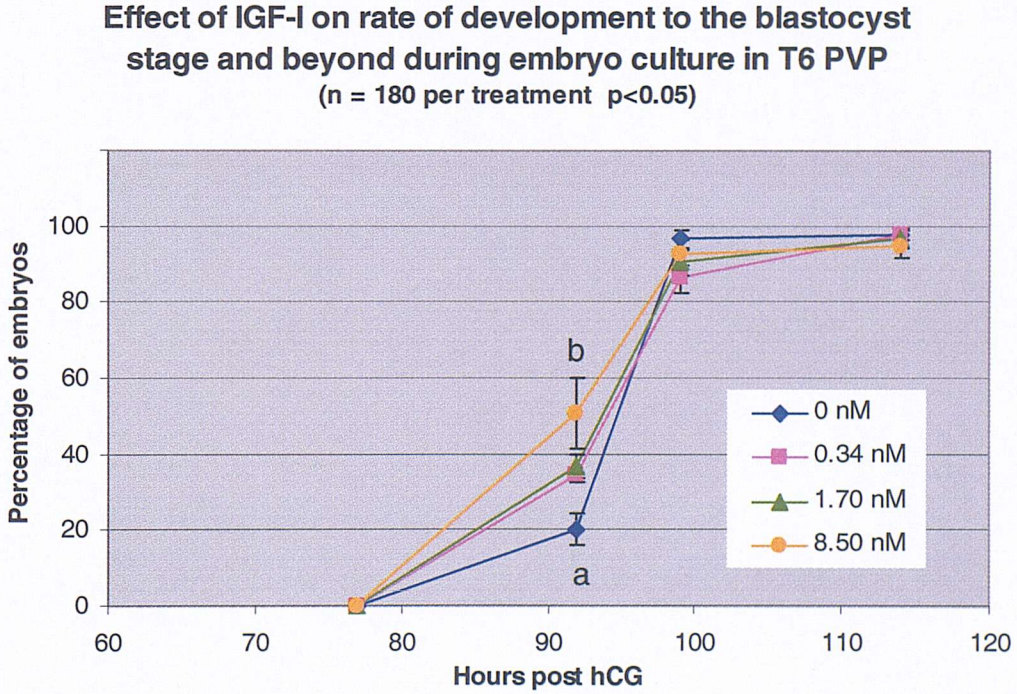
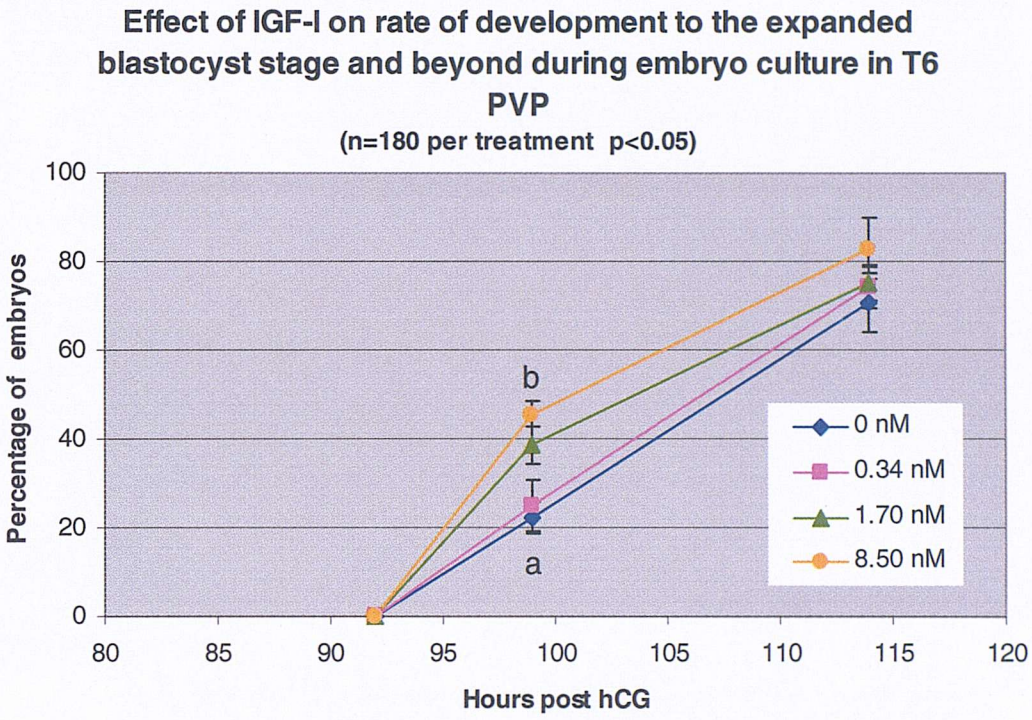


Figure 3.22


Effect of IGF-I on rate of development to the expanded blastocyst stage and beyond during embryo culture in T6 PVP
(n=180 per treatment p<0.05)
Percentage of embryos
100
80
60
40
20
0
80
85
90
95
100
105
110
115
120
Hours post hCG
b
a
0 nM
0.34 nM
1.70 nM
8.50 nM

From Figure 3.23 it can be seen that the overall trend in blastocyst cell number and ICM:TE ratio after culture with IGF-1 is similar to that seen in response to insulin (Figures 3.17 and 3.18). There is a general decline in the number of TE cells, whilst there is a general increase in the number of ICM cells and total blastocyst cell numbers. 1.70 nM and 8.50 nM IGF-I produce significant increases in the number of ICM cells ($p < 0.05$) when compared to the control group (0 nM). There is also a significant increase in the ICM:TE ratio at the highest IGF-I concentrations (1.70 nM and 8.50 nM, $p < 0.05$, Figure 3.24). From the Figures it would appear that the reduction in the TE cell number, matched by a significant increase in the ICM cell number contributes to the increase in the ICM:TE ratio.

Figure 3.23

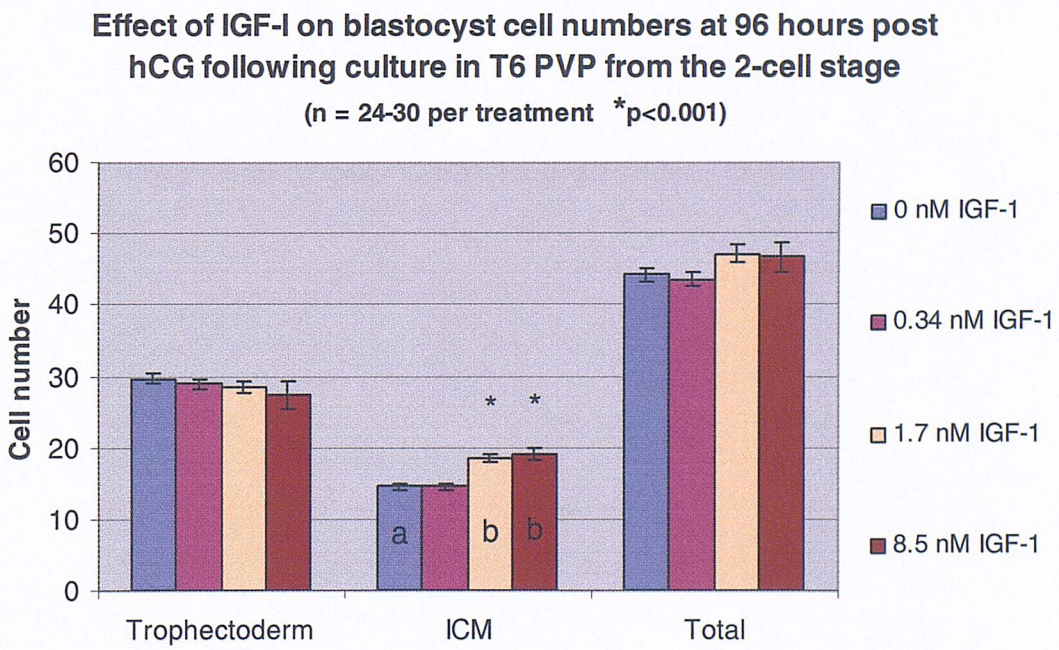
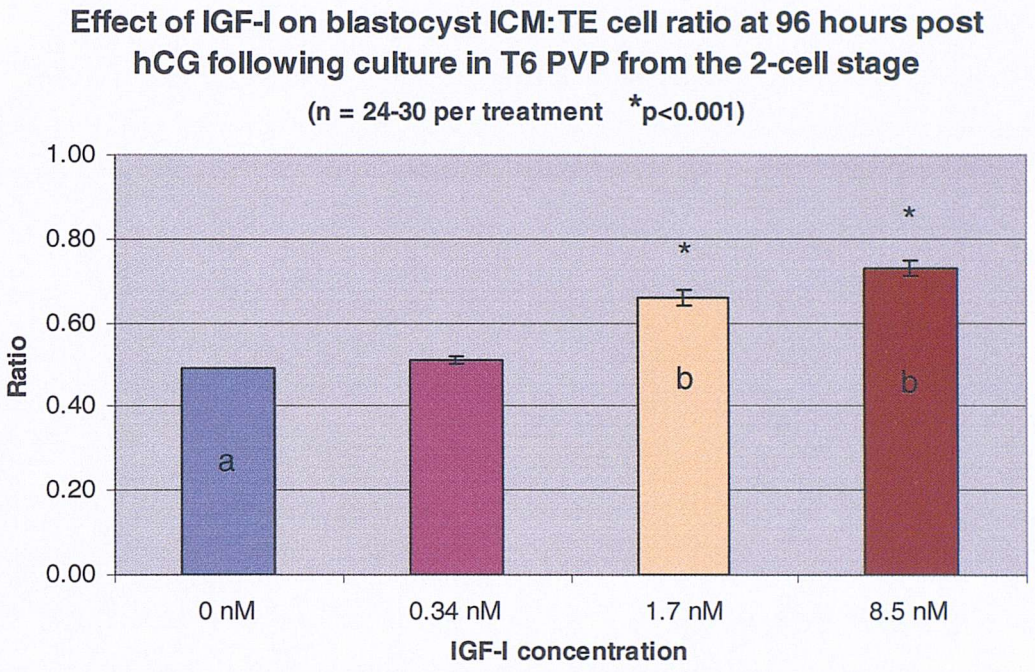


Figure 3.24


Effect of IGF-I on blastocyst ICM:TE cell ratio at 96 hours post hCG following culture in T6 PVP from the 2-cell stage
(n = 24-30 per treatment *p<0.001)
Ratio
1.00
0.80
0.60
0.40
0.20
0.00
a
b
b
*
*
0 nM
0.34 nM
1.7 nM
8.5 nM
IGF-I concentration

3.3.5 Preimplantation development of manipulated embryos

By the use of embryo disaggregation and reaggregation, half, whole (normal, taken as controls) and double 8-cell embryos were generated. Half sized embryos had significantly reduced numbers of TE and ICM cells, whilst the double sized embryos contain significantly more TE and ICM cells when compared to the control embryos ($p < 0.05$, Figure 3.25). The re-aggregated control embryos have approximately the same number and proportion of cells as the *in vitro* reference blastocysts (taken from Figures 3.1 and 3.2), showing that the process of disaggregation and then reaggregation does not appear to have an impact on cell division, or on the process of allocation to the ICM and TE lineages. At 116 hours post hCG, there has been no noticeable regulation for the increase or decrease in the total cell number. The half sized embryos had approximately half the total number of cells compared to the controls (38 and 79 cells respectively). The double sized embryos had approximately double the number of cells compared to the controls (79 cells and 164 cells respectively).

Figure 3.26 shows that the half sized embryos have a significantly reduced ICM:TE ratio when compared to the controls, with the double sized embryos having a significantly increased ICM:TE ratio ($p < 0.05$) again showing that as yet no noticeable regulation has taken place to redress the altered ratios of ICM to TE. For the controls there is no difference in their ratios when compared to the *in vitro* reference embryos.

Figure 3.25

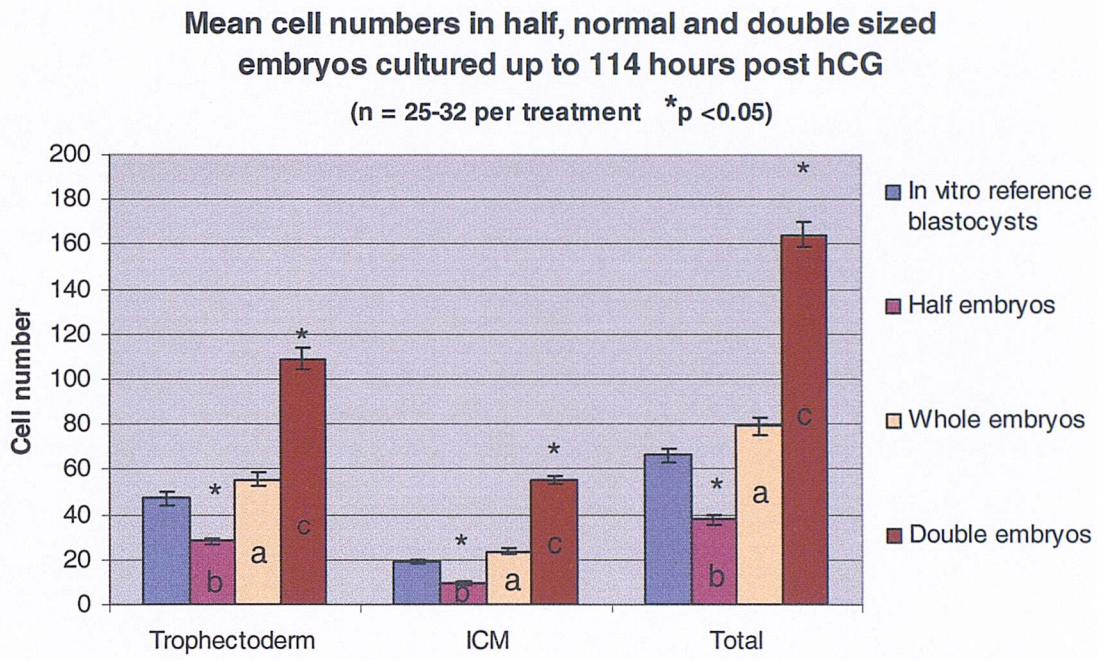
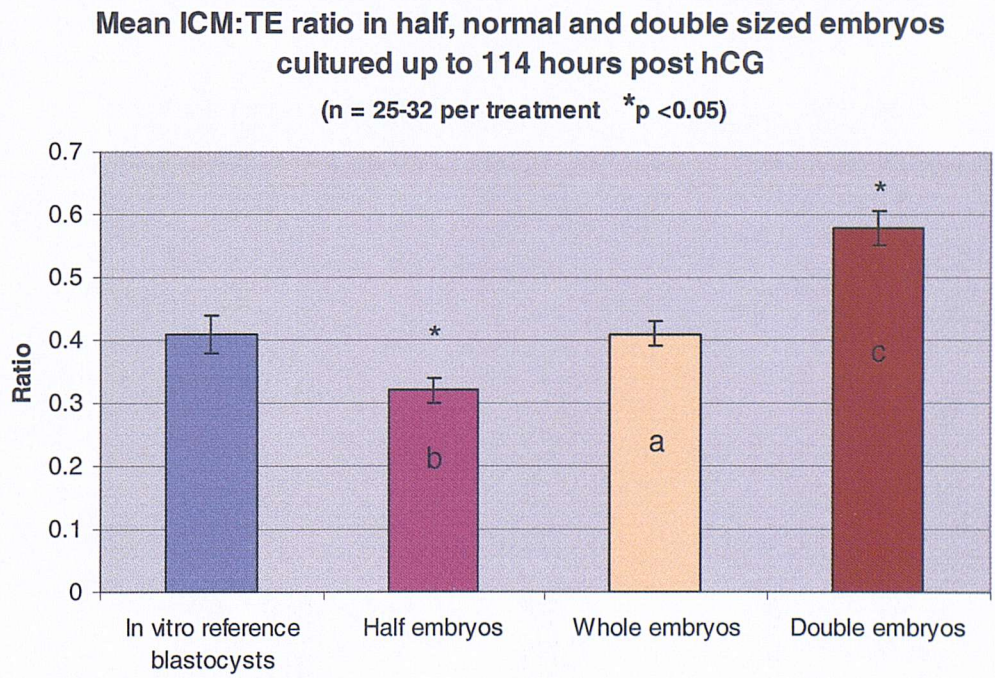


Figure 3.26



Blastocyst-like structures generated from a single, or an aggregate of three, isolated ICMs had significantly reduced numbers of ICM, TE and total cells when compared to the *in vitro* reference blastocysts (Figure 3.27). Blastocyst like structures generated from three isolated aggregated ICMs had significantly more TE, ICM and total cell numbers when compared to the blastocyst like structures generated from a single isolated ICM.

Figure 3.28 shows that for blastocyst-like structures generated from both single and aggregated ICMs, the ICM:TE ratio is significantly increased when compared to the *in vitro* reference ($p < 0.05$). The blastocyst-like structures generated from aggregated ICMs have a significantly higher ICM:TE ratio when compared to the single ICM group.

Figure 3.27

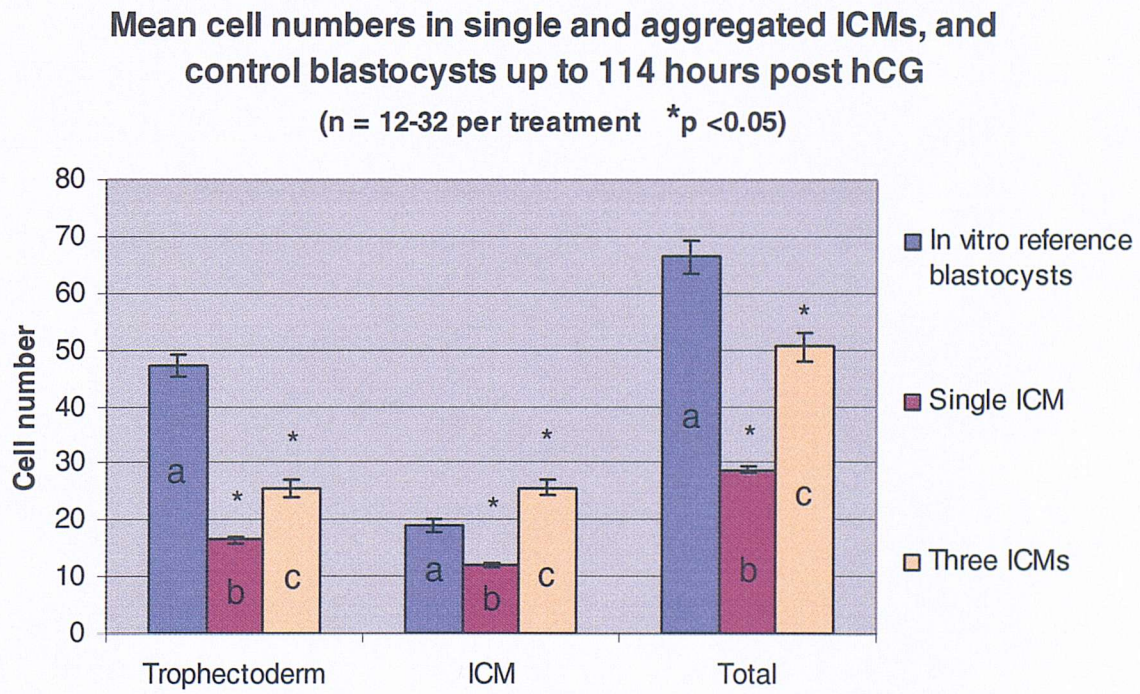
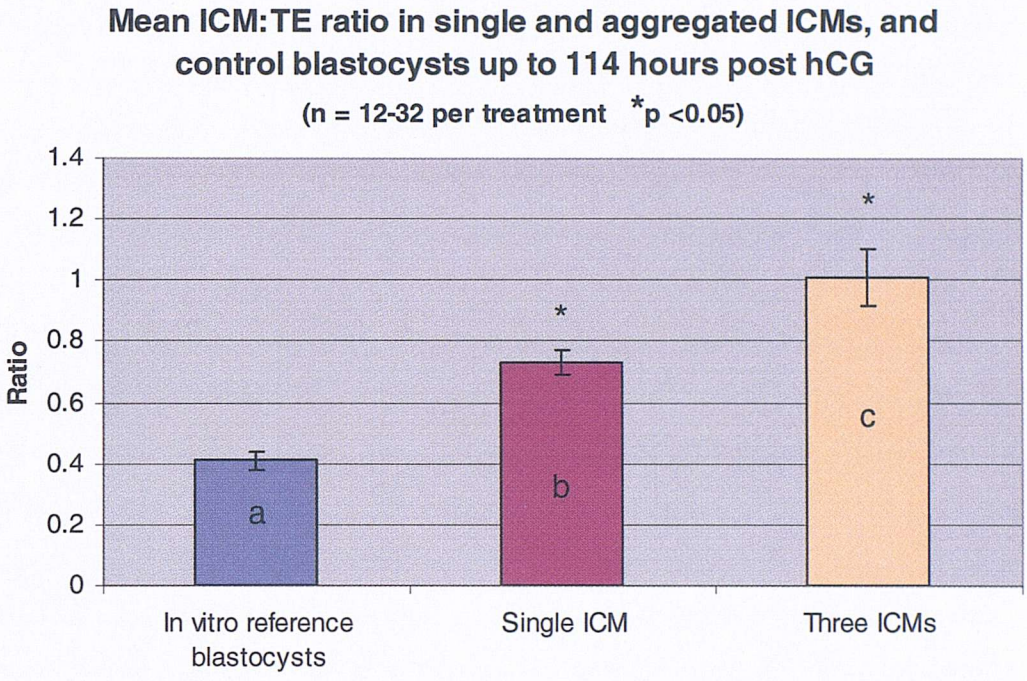


Figure 3.28


Mean ICM:TE ratio in single and aggregated ICMs, and control blastocysts up to 114 hours post hCG
(n = 12-32 per treatment *p <0.05)
Ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
c
*
*
*
In vitro reference blastocysts
Single ICM
Three ICMs

3.4 Discussion

3.4.1 Comparison of *in vitro* and *in vivo* environments on blastocyst development

The differences in cell numbers between *in vivo* derived and *in vitro* cultured embryos are shown in Figures 3.1 and 3.2. These results are in agreement with previous studies which showed that *in vitro* cultured embryos develop at a slower rate and with a lower total cell number when compared to *in vivo* derived embryos at the same time post hCG (Harlow and Quinn, 1982, Sakkas *et al.*, 1989, Gardner and Leese, 1990). *In vivo* derived blastocysts have significantly more TE and ICM cells when compared to the *in vitro* embryos at 96 hours post hCG. After a further 20 hours in culture, the *in* vitro embryos acquire comparable cell numbers to the 96 hour *in vivo* derived embryos. Figure 3.2 shows that despite the slower developmental rate of the *in vitro* derived embryos, their ICM:TE ratio is unaffected. This would imply that although the rate of cell division might be slower, the process involved in ICM and TE allocation and the mechanisms employed to maintain the correct ICM:TE ratio are unaffected. Figures 3.1 and 3.2 also show the comparison of development between two different culture media (T6 BSA and T6 PVP). It can be seen that there is no significant difference in the ICM, TE or total cell numbers between embryos from the two media at either of the times analysed (96 and 116 hours post hCG). As well as this, there is no significant difference in the ICM:TE ratio at either culture time.

Those embryos developing *in vitro* may have slower rates of cleavage due to the culture medium not providing optimal conditions for preimplantation embryo development. Mammalian embryos have changing metabolic and environmental requirements during different stages of preimplantation development (Lane and Gardner 1997a, 1997b; Martin and Leese, 1999; Gardner *et al.*, 2001). *In vivo*, the female reproductive tract caters for these changing needs by providing different environmental conditions (concentrations of metabolites, growth factors and developmental cues) as the embryo travels towards the uterus (Gardner and Leese, 1990; Gardner *et al.*, 1996). In the experiments reported here, all the embryos were cultured in a single medium during development from the 2-cell to blastocyst stage. Therefore, the metabolite concentrations would effectively be the same throughout

the culture period even though the mouse embryo appears to prefer different concentrations of metabolites during pre and post compaction development (Martin and Leese, 1999; Gardner *et al.*, 2001. Therefore, whilst the culture conditions would likely be optimal for certain stages during development, at others they may be less beneficial, thereby affecting the rate of cleavage.

BSA is a component of some culture media as a source of fixed nitrogen, a provider of free amino acids (either through the degradation of BSA itself, or via amino acids being bound to the BSA molecule), and trace levels of growth factors and other beneficial compounds such as chelators of heavy metals (Gardner, 1994; Biggers e*t al.*, 1997). In the mammalian preimplantation embryo, rates of protein synthesis are relatively low until the blastocyst stage (Brinster, 1967; Epstein and Smith, 1973; Sellens *et al.*, 1981). Coupled with this, endogenous pools of amino acids are also seen to increase during the transition to the blastocyst stage (Schultz *et al.*, 1981; Sellens *et al.*, 1981). At the same time, the TE endocytic apparatus matures and becomes organized so that the majority of endocytosis occurs from the outer (apical) membrane (Fleming and Pickering, 1985). Radioiodinated BSA has been shown to be endocytosed by 16-cell morulae and broken down by secondary lysosomes (Pemble and Kaye, 1986). This increased rate of endocytosis and degradation would provide the embryo with a valuable source of amino acids. Therefore, by the blastocyst stage the mouse embryo has the ability to endocytose maternally-derived nutrients (from the uterine fluid) in order to support and sustain its increasing requirements for protein synthesis and metabolism (Brinster *et a*l., 1979; Merz *et a*l., 1981; Pemble and Kaye, 1986).

Given the potential contribution of endocytosed protein to embryo development, it might therefore be assumed that embryos developing in the absence of BSA (those embryos cultured in T6 PVP) would be at a developmental disadvantage and have a slower rate of cleavage when compared to embryos cultured in the presence of BSA. Figures 3.1 and 3.2 show that there is no significant difference in cell numbers between embryos cultured in T6 BSA and T6 PVP. One

possible explanation is that the removal of BSA might only be detrimental to the embryo after the mid blastocyst stage. Sellens *et al.*, (1981) observed that the levels of amino acids within blastocysts cultured in the absence of any protein or amino acids fell to undetectable levels. Despite this, these embryos were able to develop normally to the blastocyst stage. It was therefore proposed that embryos cultured in the absence of protein would use up their endogenous amino acids pools during preimplantation development. However, these embryos would then be prevented from developing through to the expanded blastocyst stage as they would be unable to maintain the high levels of *de novo* protein synthesis required at this time. As all the embryos from Figures 3.1 and 3.2 were analysed at the early to mid/late blastocyst stage, this may explain why embryos developing in T6 PVP are seen to have comparable cell numbers, and to develop at the same rate as embryos developing in T6 BSA medium.

Studies have shown that whilst serum can be replaced by inert polymers such as PVP (Brinster *et al.*, 1967; Biggers *et al.*, 1997; Eckert *et al.*, 1998), alternative nitrogen sources, such as exogenous amino acids or glutathione, are necessary for development of the mammalian embryo to the blastocyst stage. On the other hand, the removal of BSA from the culture medium has been shown to have some benefits for mammalian embryo culture. In the case of cattle and sheep preimplantation embryo culture, the use of serum is associated with altered embryo morphology and disproportionate postnatal growth (Thompson *et al.*, 1995, Sinclair *et al.*, 1999). However, all of these studies replaced BSA with an exogenous nitrogen source such as amino acids, so enabling the embryo to carry out *de novo* protein synthesis. However, in the experiments reported here, an exogenous nitrogen source was not added.

3.4.2 Embryo culture in the presence of different concentrations of insulin or IGF-1

Previous studies have shown that insulin and IGF-I concentrations of 170 nM and 1.7 nM respectively, or above, produce significant increases in embryo developmental rates with a significant increase in total blastocyst cell number particularly within the ICM (Harvey and Kaye, 1990, 1992; Dunglinson and Kaye, 1993; Smith *et al.*, 1993; Pantaleon and Kaye, 1996; Lighten *et al.*, 1998; Chi *et al.*, 2000; Markham and Kaye, 2003). As well as this, IGF-I has been observed to reduce the levels of apoptosis in cultured embryos (Spanos *et al.*, 2000). In the experiments reported in this thesis, rates of embryo development appear to be influenced by different insulin or IGF-I concentrations. Despite increased rates of embryo development, total blastocyst cell numbers were not affected by culture in medium containing BSA and insulin. However, if BSA was replaced with the inert polymer PVP, then ICM cell numbers were significantly increased at insulin and IGF-I concentrations of 170 nM and 1.7nM respectively, and above.

Insulin and IGF-I may be expected to increase embryo development through various pathways. Previous studies have shown that IGF-I increases the rate of pre-compaction embryo development, whilst insulin increases the rate of post-compaction development in the mouse (Harvey and Kaye, 1992). In the results reported within this Chapter, both insulin and IGF-I had their maximal effect on developmental rates post-compaction (Figures 3.6, 3.7, 3.20, 3.21 and 3.22), where those embryos cultured in the highest concentrations of insulin or IGF-I had significantly more compacting embryos, blastocysts and expanded blastocysts when compared to the control embryos. These accelerated rates of development could have been caused by increases in levels of embryo metabolism. As the mouse embryo approaches the blastocyst stage, there is a dramatic increase in the level of protein synthesis taking place (Brinster, 1967; Epstein and Smith, 1973; Sellens *et al.*, 1981). At the same time the embryos' endocytic apparatus increases in activity, enabling the embryo to take up nutrients from the surrounding environment (Fleming and Pickering, 1985). Insulin and IGF-I have both been shown to increase the level of endocytosis (Heyner

et al., 1989; Dunglinson *et al.*, 1995), glucose uptake (Pantaleon and Kaye, 1996) and protein uptake and synthesis (Harvey and Kaye, 1988; Dunglinson and Kaye, 2000) within mouse preimplantation embryos. It may therefore be expected that insulin and IGF-I would increase the amount of metabolites (glucose and amino acids) and exogenous protein being endocytosed by the post-compaction embryo. The protein may then be degraded, so increasing the embryos amino acid pool and potential for protein synthesis. At the same developmental time, the mouse embryo is starting to switch from pyruvate to glucose as its main metabolite. Insulin has been shown to regulate glucose transport in mouse blastocysts (Pantaleon and Kaye, 1996) and may therefore be accelerating embryo development by increasing the levels of glucose available for metabolism.

In the mouse embryo, receptors for insulin and IGF-I are both expressed throughout the cleavage stages with increased expression during blastocyst formation. In the blastocyst, insulin and IGF-I receptors are present on the apical and basolateral surfaces of the TE, and on the cells of the ICM (Heyner *et al*; 1989, 1993). Once bound to their receptors, insulin and IGF-I are internalized via receptor-mediated endocytosis and delivered into spaces between the TE and ICM where they have been shown to bind to receptors expressed on the surface of the ICM (Heyner *et al.*, 1989, 1993). Insulin and IGF-I may therefore be stimulating the TE in its role as a transporting epithelium, to increase the delivery of growth factors, metabolites and other developmental factors from the external environment to the developing ICM. This would enable an increased rate of cell division, particularly within the ICM, thus leading to the increase in ICM:TE ratio observed.

IGF-1 has also been shown to reduce the levels of apoptosis taking place within mouse (Chi *et al.*, 2000) and human embryos (Spanos *et al.*, 2000) specifically within the ICM. Removal of IGF-1 and thus IGF1 receptor (IGF-IR) signalling by either removing IGF-1 from the culture medium, or by the use of an antibody against IGF1R, results in blastocysts with reduced ICM cell numbers (Markham and Kaye,

2003). Thus, an IGF-1-IGF1R circuit would act to increase ICM cell numbers by reducing the levels of apoptosis.

Although insulin increased the rate of embryo development in the presence of BSA, no differences were observed in blastocyst cell numbers or in the ICM:TE ratio, as had been previously reported. One possible reason for this could be that the strain of mouse used in the experiments reported within this thesis was different to those used in previous studies (Quakenbush strain; Harvey and Kaye, 1990; Dunglinson and Kaye, 1993; Smith *et al.*, 1993; Pantaleon and Kaye, 1996). The different embryo genotype used in the previous studies may have subtle differences in their culture specifications or responsiveness to insulin (e.g. receptor number and/or timing of expression).

Alternatively, it may be the case that the presence of BSA within the medium may be masking the stimulatory effects of insulin by producing its own stimulatory effect on all the embryos. BSA is believed to be beneficial for preimplantation embryo development. However, as well as being an exogenous protein source, it also contains undefined levels of growth factors and beneficial compounds such as chelators of heavy metals (Gardner, 1994; Biggers e*t al.*, 1997). This notion is supported by the experiments involving the inert polymer PVP as a replacement for BSA (Figures 3.12 to 3.24), where it is observed that specific concentrations of both insulin and IGF-I significantly increase the number of ICM cells when compared to the controls.

3.4.3 Preimplantation development of manipulated embryos

3.4.3.1 Preimplantation development of half, whole and double sized embryos

Previous studies have shown that increasing or decreasing the number of cells within early mammalian embryos produces blastocysts with altered total cell numbers and ICM:TE ratios (Buehr and McLaren, 1974; Rands, 1986a, 1986b; Lewis and Rossant, 1982; Biggers and Papaioannou 1992; Hardy and Handyside, 1993). These studies have also shown that manipulated embryos undergo periods of size and cell

number remodelling between days 5.5 and 10.5 *post coitum*, so that by day 10.5 *post coitum* no significant difference in cell number are observed between manipulated and control embryos.

For the current study, it is necessary for the embryo manipulation to take place at the pre-compact 8-cell stage, whilst all the cells are still totipotent, as after compaction, cells are allocated to the ICM and TE lineages and their potential gradually becomes restricted (section **1.1.1.5**). Even though all the blastomeres are still totipotent, developmental fates of the cells are already becoming ascribed. Studies in mice have shown that the daughter cells of the first 2-cell blastomere to divide will contribute disproportionately more to the developing ICM, whilst the daughter cells of the later dividing 2-cell blastomere will contribute disproportionately more to the TE (Gardner, 2001; Piotrowska *et al.*, 2001). The division of the earlier 2-cell blastomere influences the orientation of the embryonic-abembryonic axis from which the ICM and mural TE will predominantly develop respectively. Therefore, within an intact 8-cell embryo, there will be cells located within certain regions of the embryo that will preferentially allocate daughter cells to the ICM or TE lineages. In the experiments reported within this thesis, pre-compact 8-cell stage embryos are disaggregated, the blastomeres pooled, and then reaggregated. This would mean that the cells predisposed to ICM lineage allocation may become re-positioned into regions of the embryo that would preferentially allocate cells to the TE lineage. Whilst it has been shown that disaggregated and reaggregated embryos are able to proceed through embryonic and fetal development, giving viable offspring (Biggers and Papaioannou, 1992), the altered positioning of predisposed blastomeres within the embryo, and/or that disruption to already established axes of the preimplantation embryo could cause subtle changes in cell allocation patterns, possibly affecting normal patterns of postimplantation and fetal development.

In the undisturbed embryo, those cells allocated to the embryo interior will predominantly form the ICM, whilst those cells located on the outer surface will

predominantly form the TE (Johnson *et al.*, 1981; Johnson and Ziomek., 1983; Fleming, 1987). If the size of the embryo is increased then the volume will increase disproportionately to the surface area. In double-sized embryos, this will result in proportionately more cells located inside the embryo relative to its surface, and therefore a larger than normal population of cells from which to form the ICM (Figure 3.29). In half sized embryos it would be expected that the majority of the cells would be located on the outer face of the embryo, and so should develop with a significantly reduced ICM:TE ratio.

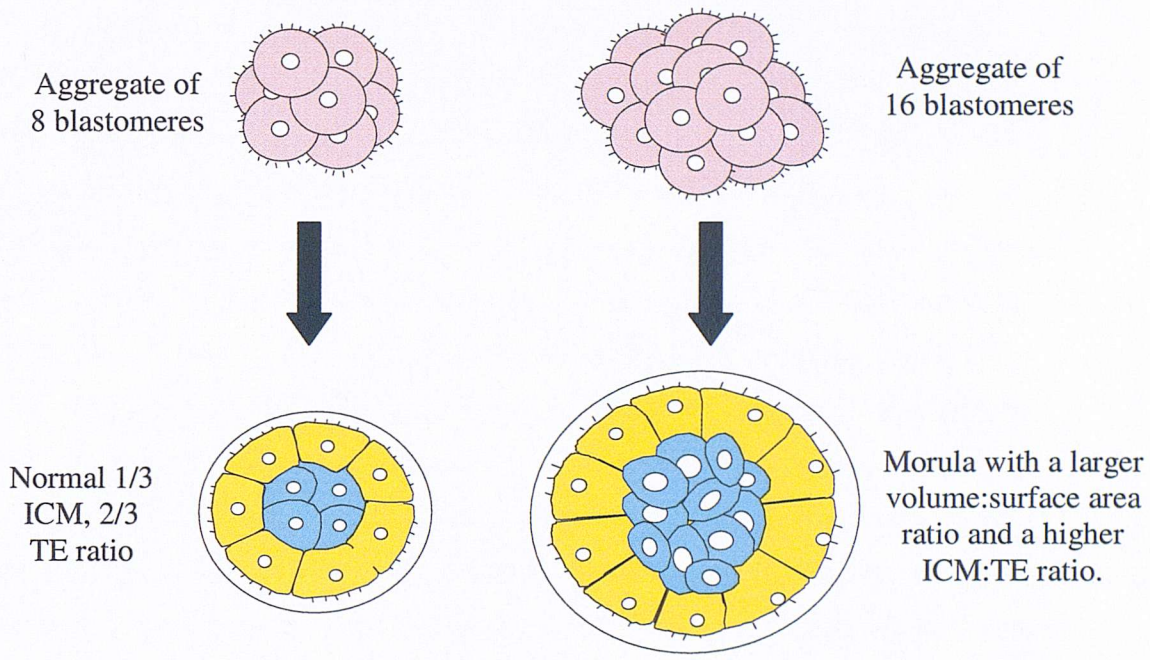


Figure 3.29. Diagram showing the development of a control and a double sized reaggregated embryo. In the control embryo, those cells located on the outer face will go on to form the TE lineage (yellow) whilst those cell located within the embryo will go on to form the ICM lineage (blue). In the double sized embryo, because the volume has increased disproportionately to the surface area, more cells will be located within the embryo thus increasing the proportion of cells allocated to the ICM lineage. The case is the opposite for half sized embryos.

In the experiments reported within this thesis, doubled sized embryos have a significantly increased total cell number (164.13 ± 5.53 vs 79.15 ± 3.89, $p < 0.05$, an increase of 207.37%), and ICM:TE ratio (0.578 ± 0.027 vs 0.41± 0.02, $p < 0.05$) when compared to the controls at 114 hours post hCG (Figures 3.25 and 3.26). The half sized embryos have a significantly reduced total cell number (37.62 ± 2.032 vs 79.15

± 3.89, $p < 0.05$, a reduction of over 47%) and ICM:TE ratio (0.32 ± 0.0185 vs 0.41± 0.02, $p < 0.05$) when compared to the controls at 144 hours post hCG (Figures 3.25 and 3.26).

The results reported within this thesis indicate that the double and half sized embryos have not yet begun to compensate for their altered total cell numbers as they still contain approximately double and half the total cell numbers of the controls. Studies have shown that mouse embryos will compensate for alteration in their total cell numbers between day 5.5-10.5 *post coitum*, so that by 10.5 days *post coitum* initial differences in total cell numbers have disappeared (Lewis and Rossant 1982; Rands 1986a, 1986b; Somers *et al.*, 1990; Hardy and Handyside, 1993; Powers and Tam, 1993). As the embryos from Figures 3.25 and 3.26 were analysed at approximately 4.3 days *post coitum* (114 hours post hCG), then it may be expected that these embryos may not yet have begun to compensate for their altered size and cell number. Table 3.1 shows cell number results from previous studies where embryo size has been manipulated. Although in these studies embryos were analysed at different times post hCG, these times are similar to those reported here. As Table 3.1 shows, previous studies found double and half sized embryos also still contain approximately double and half the total cell number respectively when compared to the controls for between 90 and 120 hours post hCG. Lewis and Rossant (1982) drew the conclusion that there is a "window" during development at which size remodelling of the embryo occurs. It is believed that the preimplantation embryo is able to make compensations for increased or decreased cell numbers by either lengthening or shortening the duration of the cell cycle respectively (Lewis and Rossant 1982). Therefore, a half sized embryo will effectively reduce the cell cycle length so enabling an increased rate of cell division.

Table 3.1 Results from previous studies examining the preimplantation development of embryos with altered total cell numbers.

Size of embryos	Time analysed (post hCG)	Mean number of TE cells	Mean number of ICM cells	Mean total cell number	Cell % of controls	Reference
½ sized	114			53.7 ± 1.7	51%	1
½ sized	90-93	10.18 ± 0.3	5.42 ± 0.3	15.61 ± 0.3	53%	2
½ sized	92-94	19.5 ± 3.3	4.4 ± 1.9	23..9 ± 4.8	56.2 %	3
½ sized	92-94			25.5 ± 2.9	49.42 %	4
Double	~114			248 ± 31.4	215.7 %	5
Double	~120			243.7	219%	6

References, (1) Hishinuma et al., (1995), (2) Hardy and Handyside, (1993), (3) Power and Tam, (1993), (4) Rands, (1986), (5) Lewis and Rossant, (1982), (6) Bueher and McLaren (1974).

Manipulation of the embryo to contain half and double the normal number of cells results in an altered cell allocation pattern to the ICM and TE when compared to the controls (Figure 3.26). Previous studies have shown that within half sized embryos there is a significant increase in the number of TE cells (Hardy and Handyside, 1993), whilst in fetuses derived from half sized embryos there is significantly smaller amounts of ICM derived tissues and more TE derived tissues (Rands, 1986b). Chimaeras derived from the aggregation of two separate embryos develop with significantly increased total cell numbers and with a significant increase in the number of ICM cells (Bueher and McLaren, 1974; Lewis and Rossant, 1982; Rands, 1986). It would therefore appear that altering the volume of the preimplantation embryo not only affects the numbers of cells within the embryo, but also affects the allocation of cells to the ICM and TE. However, it would then be expected that the embryo would go through a period of compensatory growth so that by day 10.5 *post coitum* there would be no significant difference when compared to the controls.

3.4.3.2 Preimplantation development of aggregated and single ICMs

If an ICM is isolated from an early blastocyst and cultured under the appropriate *in vitro* conditions, the cells located on the outside will differentiate into a polarised epithelium, thus forming a TE like structure (Spindle, 1978; Rossant and

Lis, 1979; Nichols and Gardner, 1984). Consequently, as the ICM comprises approximately one third the total number of cells within an early blastocyst, it would be expected that if three ICMs were aggregated then a cellular mass would be created comprising approximately the same number of cells as an intact blastocyst. In the intact blastocyst, the cells within the ICM have complete cell-cell contact, whether from other cells of the ICM, the cells of the polar TE or from the TE cell projections that overlie the face of the ICM exposed to the blastocoel. Upon isolation, those cells located on the outer face of the ICM display incomplete cell-cell contacts, resulting in the up-regulation of an epithelial phenotype within these cells (reviewed in Fleming *et al.*, 2001). This allows for the re-formation of a blastocyst like-structure; however, TE-like cells will have subsequently been derived from a population of cells (ICM) which they would not normally derive from (Figure 3.30).

Blastocyst-like structures generated from the aggregation of three isolated ICMs had significantly reduced total numbers of cells, but a significantly increased ICM:TE ratio caused by a significant reduction in the number of regenerated TE cells and a significant increase in the number of ICM cells (Figures 3.27 and 3.28). The high ICM:TE ratio could be attributed to the way in which the outer cells on the isolated ICM differentiate and encompass the ICM. It may be the case that upon isolation, a small proportion of the cells located upon the outerface of the ICM take on a TE-like phenotype and subsequently surround the remaining cells of the ICM. In doing so they re-establish a pattern of total cell-cell contact within the other cells of the ICM, thus inhibiting them from expressing a TE phenotype. This would effectively create a blastocyst-like structure containing relatively few of outer cells surrounding a proportionately larger non-polar inner cell population.

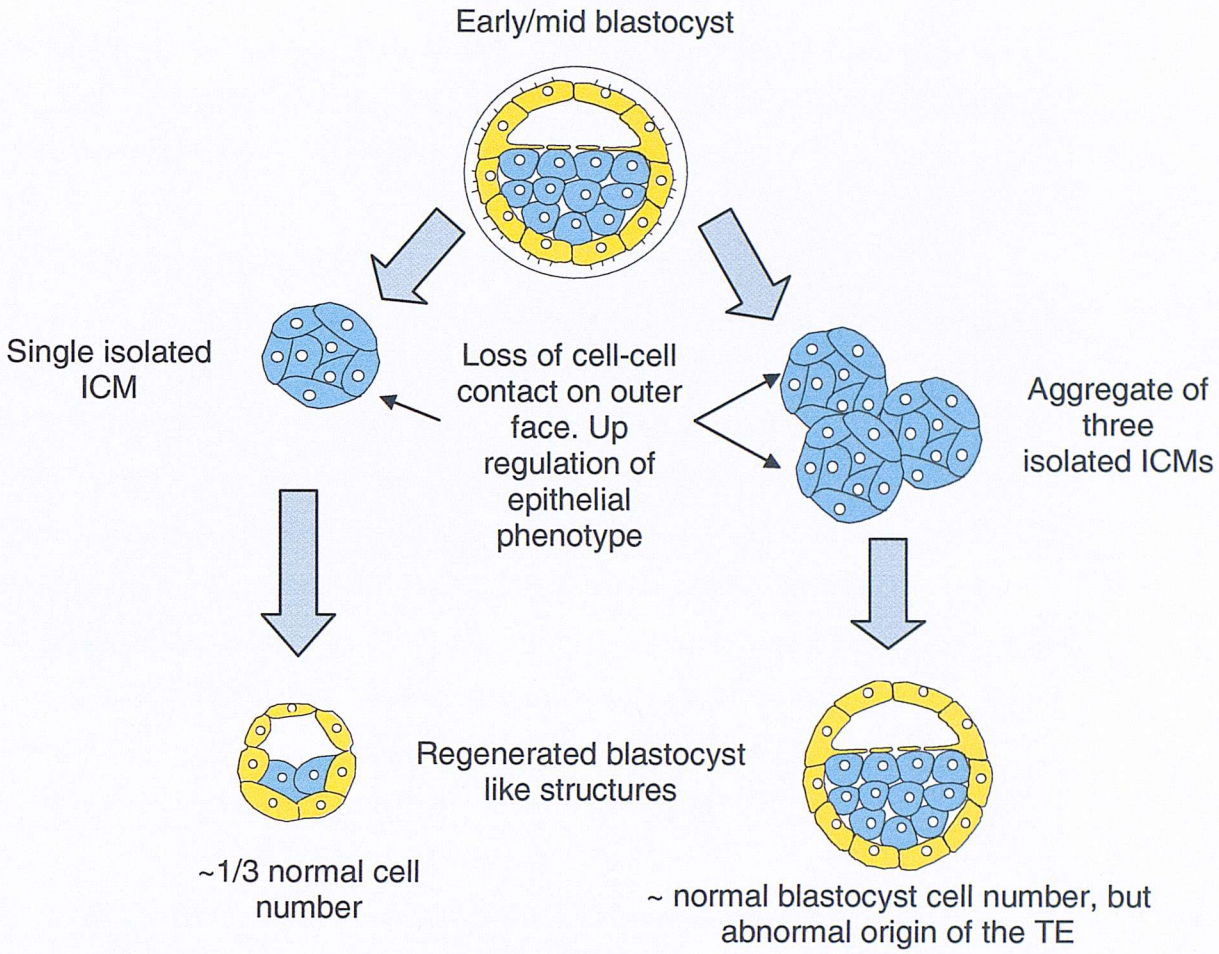


Figure 3.30. Diagram showing the regeneration of a TE-like cell layer from isolated single and aggregated ICMs. Upon isolation, the cells on the outer surface of the ICM no longer have total cell-cell contact. These cells then undergo polarisation and reform an epithelial-like structure enabling the regeneration of a blastocoel cavity. If three ICMs are aggregated then a blastocyst-like structure will form comprising the normal number of cells, but with an abnormal origin of the TE.

A second possible explanation could be the rate of cell proliferation within the isolated and aggregated ICMs. Several studies have shown varying rates of development of individual and aggregated ICMs *in vitro*. Spindle, (1978) observed no proliferation within either isolated ICMs or in aggregates of five ICMs, for up to 24 hours of culture, whereas other studies have reported an approximate doubling in the number of cells within isolated ICMs between 12 and 24 hours post isolation (Nichols and Gardner, 1984; Chisholm *et al.*, 1985). As the blastocyst-like structures reported within this thesis were analysed for their cell numbers only 20 hours after isolation and aggregation, the level of cell proliferation within single and aggregated ICMs

may still have been low, as reported by Spindle, (1978). This low level of cell proliferation, coupled with the low level of TE regeneration, could result in the reduced total blastocyst cell number, whilst producing the increased ICM:TE ratios observed within Figures 3.27 and 2.28.

3.5 Conclusions from preimplantation embryo manipulation.

The findings reported within this Chapter, and those of previous studies, show that *in vitro* culture leads to a slower rate of preimplantation embryo development when compared to embryos from an *in vivo* environment for the same developmental time post hCG. This slower rate of development results in significantly reduced numbers of ICM and TE cells, although the ratio of the cells within these two lineages is not altered. It is possible to increase the rate of preimplantation embryo development *in vitro* via the addition of specific concentrations of exogenous growth factors to the culture medium. It can be seen that at certain growth factor concentrations and above, cells of the ICM are stimulated to proliferate, thus significantly increasing the ICM:TE ratio within these embryos.

Ratios of ICM to TE cells are also affected by embryo volume and total number of cells present within the embryo. Alterations in cell numbers will affect the volume of the embryo, and thus the proportions of cells located within and on the outer face of the embryo. Increases or decreases in embryo volume result in significantly altered total cell numbers and altered ICM:TE ratios. Blastocyst like structures created from isolated and aggregated ICMs display significantly altered ICM:TE ratios and significantly decreased total numbers of cells.

These findings have shown that specific *in vitro* conditions or manipulations to preimplantation mouse embryos can alter the size, cell number and ICM:TE ratio, whilst allowing development to the blastocyst stage. It would therefore be of interest to ascertain the effects of these manipulations upon subsequent fetal and postnatal development. Previous studies have demonstrated that specific *in vitro* environments or manipulations can affect aspects of fetal and postnatal development (Biggers and

Papaioannou, 1992; Kaye and Gardner, 1999; Khosla *et al.*, 2001). *In vivo*, the embryo develops in synchrony with the female reproductive tract. It has previously been shown that embryos cultured *in vitro* develop at a slower rate and have a reduced ability to develop after uterine transfer when compared to embryo developing *in vivo* (Bowman and McLaren, 1970). Slower rates of preimplantation embryo development may result in periods of accelerated postimplantation growth or development to compensate for their initial slower rates of preimplantation development. These periods of compensatory catch up development may affect aspects of fetal or postnatal development and physiology. *In vitro* embryo culture has also been demonstrated to alter patterns of fetal gene expression, resulting in disproportionate fetal and postnatal growth (Khosla *et al.*, 2001, Young, 2001).

Those embryos cultured in the presence of exogenous growth factors, or manipulated to contain different numbers of cells, contain disproportionate ratios of TE and ICM cells. As these populations form the placenta and fetus respectively, then the offspring developing from these embryos may have disproportionate amounts of fetal and placental tissues. Offspring developing from embryos cultured in the presence of insulin and albumin have been shown to have an increased birth weight by 4-6% (Kaye and Gardner, 1999).

Subsequent chapters of this thesis report the effects of the above embryo culture conditions and manipulations on aspects of postnatal development and physiology, studying parameters including growth profiles, blood pressure and organ allometry.

Chapter 4

Influence of preimplantation embryo environment on postnatal development and physiology

4.1 Introduction

Studies have shown that *in vitro* culture and subsequent transfer of mammalian embryos can have numerous effects on preimplantation, fetal and postnatal development. *In vitro* culture has been shown to slow preimplantation embryo developmental rates and reduce total blastocyst cell numbers (Chapter 3 of this thesis; Bowman and McLaren, 1970; Harlow and Quin, 1982; Conaghan *et al.*, 1993). In mice, day 14 fetuses derived from embryos cultured in the presence of fetal calf serum had reduced weight, and decreased expression of the imprinted H19 and IGF-2 genes when compared to control fetuses (Koshla *et al.*, 2001). The culture and transfer of sheep and cattle embryos (specifically when cultured in the presence of serum) has been associated with significantly increased fetal growth, increased birth weight, increased muscle mass, skeletal and facial malformations and higher incidences of postnatal mortality (Thompson *et al.*, 1995, Holm *et al.*, 1996, Young *et al.*, 1998, Sinclair *et al.*, 1999; Jacobsen *et al.*, 2000).

Therefore, the aim of this study was to examine the effect of an *in vitro* culture environment upon aspects of postnatal development and physiology. Five treatment groups were generated, compared, and name as (a) '*in vitro* mice', (b) '*in vivo* mice', (c) 'superovulated mic'e, (d) 'naturally mated mic'e and (e) 'naturally mated (6) mice' (see Table 4.1 for treatment details). These five treatment groups enabled the comparison of postnatal effects between embryos dependent upon whether derived from *in vitro* preimplantation embryo culture, *in vivo* preimpantation embryo development, embryo transfer procedures or a superovutaled maternal environment. Within this study, mice derived from embryos developed *in vivo* are acting as controls for mice derived from embryos cultured *in vitro*, as both groups underwent embryo transfer, the only distinction between groups is *in vitro* culture. As both of these groups were derived from embryos obtained from mothers who had

been superovulated, a third treatment group comprising superovulation, but no *in vitro* culture or embryo transfer, was generated so as to assess the impact of embryo transfer. Finally, two groups originating from natural mating (with no superovulation or embryo transfer) were generated in order to study the effect of superovulation and embryo culture and transfer. So as to minimise the postnatal effects derived from altered litter size, one naturally mated group had their litter size corrected at birth to a size comparable with the *in vitro* and *in vivo* treatment groups (mean litter size corrected to 6, termed naturally mated (6) mice).

Table 4.1 Outline of the five groups of mice studied within this experiment, their embryonic treatments and whether they had their litter size corrected at birth.

Group name	Embryo treatment	Purpose of treatment/group	Embryos derived from superovulated mothers	Embryo transfer	Litter size corrected at birth
'*In vitro* mice'	Derived from embryos cultured *in vitro* (2-cell to blastocyst stages)	Examine the postnatal effect of *in vitro* culture	Yes	Yes	No
'*In vivo* mice'	Derived from embryos developed *in vivo*	Control for *in vitro* culture	Yes	Yes	No
'Superovulated mice'	Maternal superovulation	Control for *in vitro* culture and embryo transfer	Yes	No	No
'Naturally mated mice'	None	Control for superovulation, *in vitro* culture and embryo transfer	No	No	No
'Naturally mated (6) mice'	None	Control for superovulation, *in vitro* culture, embryo transfer and postnatal litter size	No	No	Yes (mean litter size of 6)

As one of the parameters being investigated is blood pressure, factors involved in directly regulating blood pressure will also be examined. It was decided to assay the activity of angiotensin converting enzyme (ACE) within the serum at 27 weeks of age. ACE is a dipeptidyl carboxypeptidase that is a component of the renin angiotensin system (RAS). It has a critical role in the cleavage of angiotensin I to

angiotensin II, a potent vasoconstrictor leading to an elevation of blood pressure (Skeggs *et al.*, 1956). ACE also acts to stimulate the release of aldosterone from the adrenal cortex, thus leading to the re-absorption of sodium, and inactivates bradykinin which is a potent vasodilator, again facilitating in the raising and overall regulation of blood pressure (Yang and Erdos, 1967; Yang *et al.*, 1970). ACE is located within the renal tubular epithelium, gut epithelium, areas of the brain, the testes and within the plasma and serum (Friedland *et al.*,1978; Strittmatter and Snyder, 1978; Corvol *et al.*, 1995). However, due to the high levels of vascularisation and endothelial tissue, it is viewed that the ACE within the lungs contributes the main effect to the control of blood pressure. The N and C terminal domains can be cleaved to yield a soluble active form of the enzyme that circulates within the blood stream (Serum ACE, Figure 4.1). This is brought about by the actions of a group of secretases that cleave the juxtamembrane stalk (Beldent *et al.*, 1995; Sadhukhan *et al* 1998; Woodman *et al.*, 2000; Eyries *et al.*, 2001).

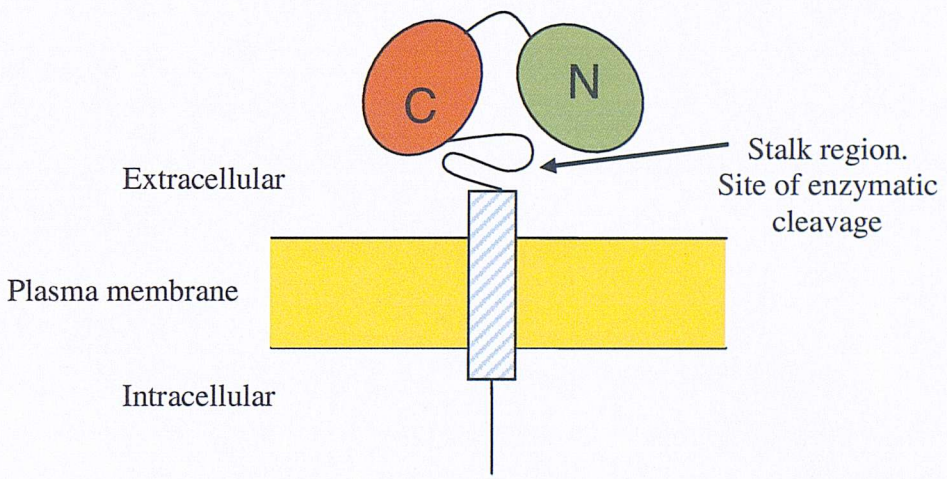


Figure 4.1. Diagram of somatic ACE showing the transmembrane region (blue striped box) and the two extracellular domains (C and N). The arrow indicates the region of enzymatic cleavage yielding a free floating catalytic unit that becomes serum ACE.

Knockout studies have shown ACE -/- mice to be hypotensive, having a systolic blood pressure ~ 30 mm Hg below wild type mice (Cole *et al.*, 2003). Mice manipulated to express one, two or three copies of the ACE gene show no difference in their blood pressure (Krege *et al.*, 1997). Therefore, it would be of great interest to

assay the activity of ACE within the serum and examine its correlation to blood pressure.

4.2 Methodology

4.2.1 Production of embryos for embryo transfer

5-6 week old female F1 CBAxC57/B16 mice were superovulated and mated with male MF1 mice (section **2.1.1**). For *in vitro* derived embryos, 2-cell embryos were flushed and cultured in T6 BSA for approximately 45 hours, until the blastocyst stage (section **2.1.2**). *In vivo* derived embryos were flushed from the uteri at the blastocyst stage (~93 hours post hCG). At the time of transfer both *in vivo* and *in vitro* derived embryos were placed in H6 BSA for up to three hours, to buffer against pH changes until they were transferred to recipient uteri (section **2.2.3**).

For the production of non-embryo transfer groups, six superovulated females were allowed to develop to term with their pregnancies. Along with these, two groups of female mice were naturally mated and allowed to develop to term with their pregnancies (minimum of six females each). One subset of offspring from the natural mating had their litter size adjusted at birth to a size comparable to that of the *in vitro* and *in vivo* treatment groups (termed naturally mated (6) mice).

4.2.2 Embryo transfer

The ovaries and top 1 cm of the uterus of anaesthetised 3.5 day pseudo pregnant female CBAxC57 mice were carefully exposed from the body cavity. Six blastocysts were then transferred to each uterine horn (section **2.2.2**). Six to ten litters of each embryo treatment group were generated.

4.2.3 Measurement of postnatal growth rates

Mice were weighed on day of birth and then subsequently on the same day for the next 27 weeks (section **2.3.1**).

4.2.4 Measurement of systolic blood pressure

Systolic blood pressure was determined at 8, 15 and 21 weeks of age by tail-cuff plethysmography (section **2.3.2**).

4.2.5 Measurement of organ weights

Organs were dissected out of the mice at approximately 27 weeks of age. Organs were weighed before being snap frozen in liquid nitrogen, and stored at -80°C (section **2.3.3**). Blood samples were collected by vacuum puncture of the heart using a pulled glass pipette (internal diameter ~1 mm) attached to a mouth pipette, and then centrifuged at 10,000 g, 4°C for 10 minutes, after which the serum was aliquoted into 10 µl samples and frozen at -80°C.

4.2.6 Measurement of serum ACE activity

Activity was determined as described in section **2.3.4**. Serum ACE activity was expressed as nanomoles of hippurate formed per millilitre of serum per minute.

4.2.7 Statistical analysis of data

A Bonferroni one way analysis of variance t-test was used to analyse the litter size from the different treatment groups (SigmaStat statistical software, version 2.0).

The correlation between birth weight and litter size was assessed for normality using the SigmaStat statistical software package (version 2.0). As the distribution failed the normality test, the correlation was analysed using a Spearmans correlation.

Data for animal weights, blood pressure, organ ratios and weights and serum ACE activity were analysed using a multilevel 'random effects' regression model which took into account between-mother and within-mother variation in litter size and parameters measured from individual animals, thereby preventing any differences derived from abnormal litter sizes influencing the data (section **2.4**). Statistical significance was assumed at $p \leq 0.05$. A trend was assumed to occur if a p value

between 0.1 and 0.051 was observed. Differences between the naturally mated 2 (control) and other treatment groups were assessed using this method.

4.3 Results

In order to minimise the effect of between-mother variation in litter size influencing the data for any one group, six to ten litters for each embryo treatment were generated. The mean litter size at birth for the five treatment groups is shown in Figure 4.2. The *in vitro* and *in vivo* groups have a significantly reduced number of offspring per litter when compared to the three non-embryo transfer groups (before they had their litter size was adjusted). There is no significant difference between the mean litter size of the *in vitro* and *in vivo* treatment groups (5.32±0.42 *vs* 6.00±0.42 respectively, $p = 1.00$), nor is there any difference between any of the non-embryo transfer groups. On the day of birth the naturally mated (6) group had their litter sizes adjusted to a mean size similar to that of the *in vitro* and *in vivo* groups (mean litter size of 6 animals, orange bar on Figure 4.1). Whilst the mean litter size for the superovulated group tended to be larger than the two naturally mated groups, this did not reach statistical significance ($p = 0.359$).

The mean birth weights of male and female offspring from the five treatment groups studied are shown in Figures 4.3 and 4.4. There is no significant difference between the mean birth weights of the *in vitro* and *in vivo* derived mice ($p = 0.746$), or between these two groups and the naturally mated (6) group. Males derived from mothers who were superovulated but did not undergo embryo transfer, are significantly lighter when compared to the naturally mated (6) males ($p = 0.015$, Figure 4.3). There are no significant differences between any of the female groups.

A negative correlation exists between birth weight and litter size for males and females from all treatment groups (Figure 4.5, $r = -0.645$). It can be seen that the mice from the largest litter (mice derived from a superovulated mother, litter size of 19) have the lowest mean birth weight, whilst those mice from the smallest litter (*in vitro* mice, litter size of 3) have the largest mean birth weight. The mice developing from

transferred embryo groups all came from significantly smaller litters (3-8 pups) when compared to the the naturally mated (6) group (9-14 pups, $p < 0.05$), and so subsequently had larger mean birth weights (Figure 4.3 and 4.4).

Figure 4.2

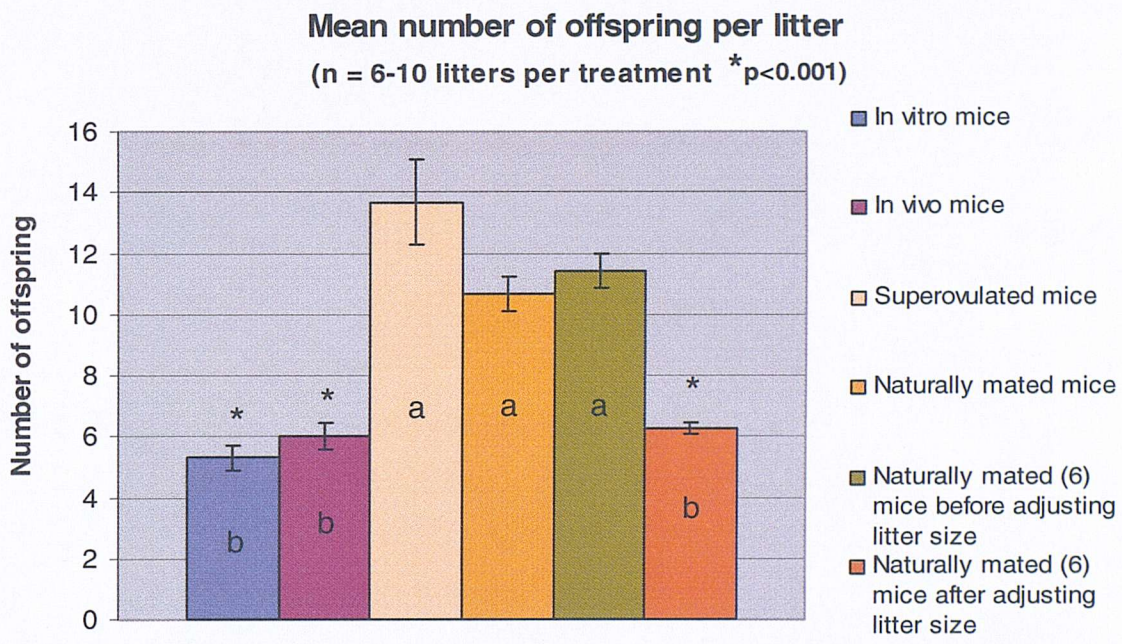


Figure 4.3

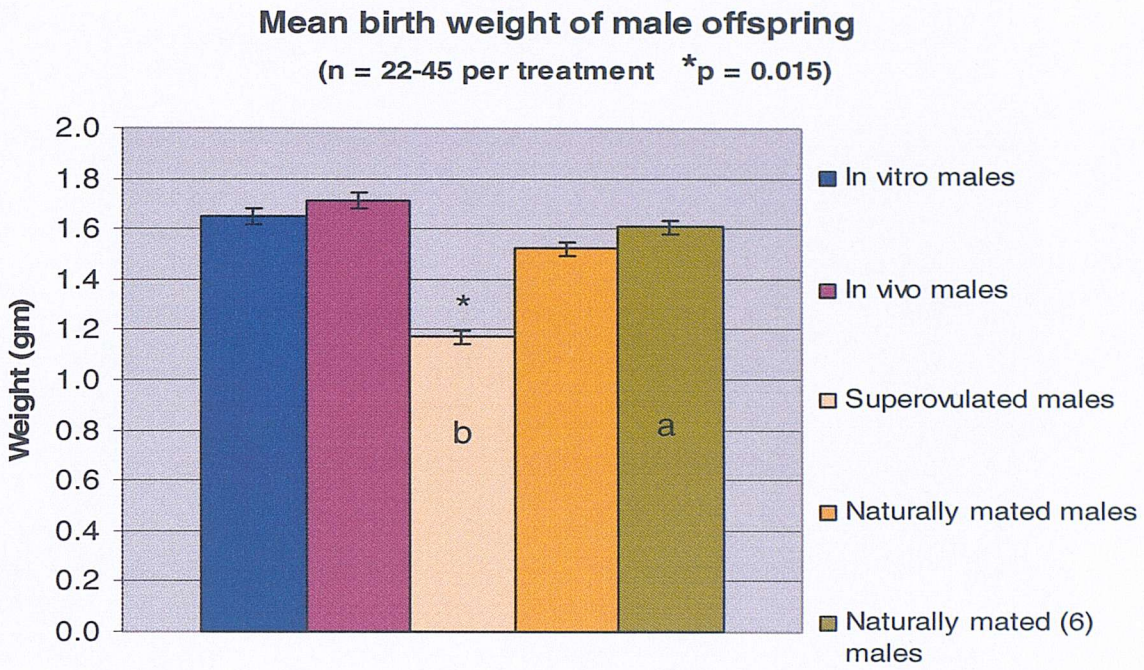


Figure 4.4

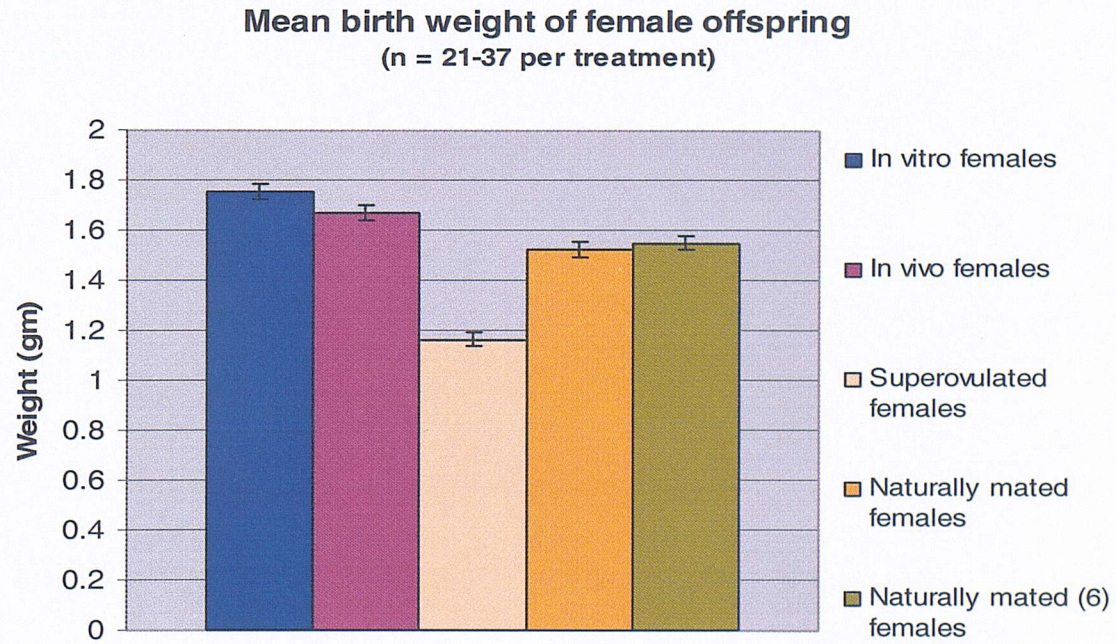
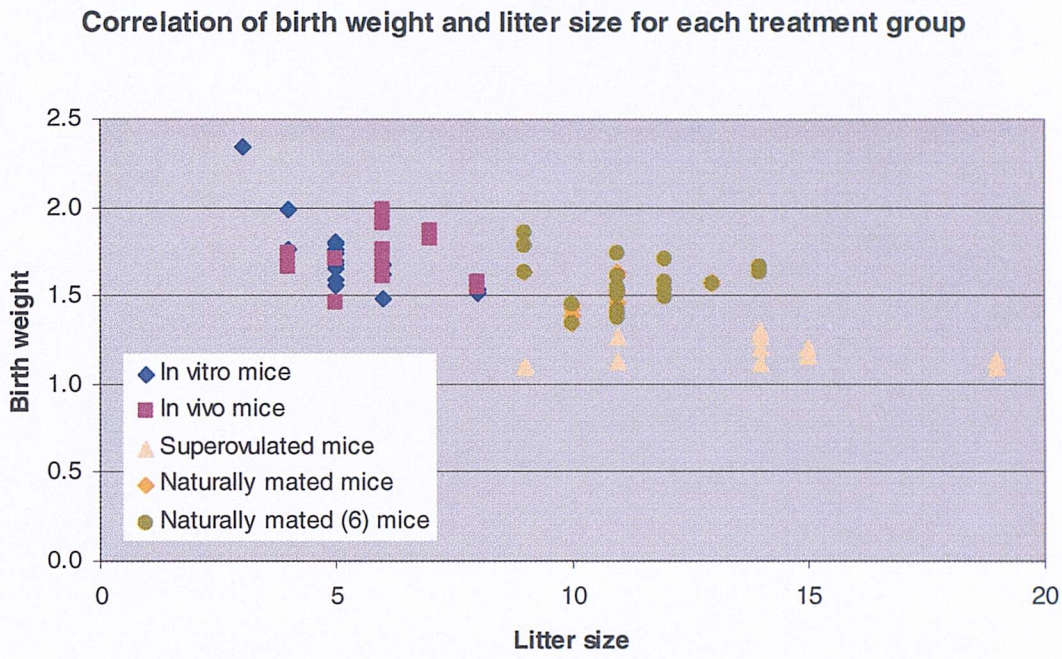


Figure 4.5



The mean weekly weights for all five treatment groups from birth to 27 weeks of age are shown in Figures 4.6 and 4.7 (mean ± standar error). It can be seen that all the mice undergo a dramatic growth spurt between 3 and 5 weeks of age. During this time the average increase in weight is approximately 2- 2.5 times that occurring in previous and in subsequent weeks. After approximately 7 weeks, the increase in weight begins to plateau, so that by approximately 11 weeks of age they are only increasing their weight by approximately 1 gram a week.

At 2 weeks of age the *in vivo* males are significantly heavier than the *in vitro* males ($p = 0.009$). Table 4.2 shows that at 3 weeks of age, the naturally mated and superovulated males are significantly lighter than the naturally mated (6) males. At 4 weeks of age, the *in vivo* males also become significantly lighter when compared to the naturally mated (6) males. However, at 5 weeks of age, the superovulated and *in vivo* males only show a trend towards being lighter than the naturally mated (6) males. For the remainder of the 27 weeks, there are no further significant differences or trends between any of the male groups.

Table 4.3 shows a similar pattern for the female mice. At 3 weeks of age, the superovulated females are significantly lighter than the naturally mated (6) females. At 4 weeks of age, the naturally mated, superovulated and *in vitro* females are all significantly lighter than the naturally mated (6) females. The *in vivo* females display a trend towards being lighter than the naturally mated (6) females ($p = 0.056$). At 5 weeks of age, only the superovulated and the naturally mated females are significantly lighter than the naturally mated (6) females. At 8, 10, 11 and 12 weeks of age, the superovulated females remain significantly lighter than the naturally mated (6) females. From 14 weeks of age and for the remainder of the 27 weeks, there are no further significant differences or trends between any of the female groups. At 6 and 11 weeks of age, the *in vivo* females are significantly heavier than the *in vitro* females ($p = 0.037$ and 0.035 respectively).

For both male and the female treatment groups, the litter size at birth or after adjustment, had no significant influence on the growth patterns observed within Figures 4.6 and 4.7.

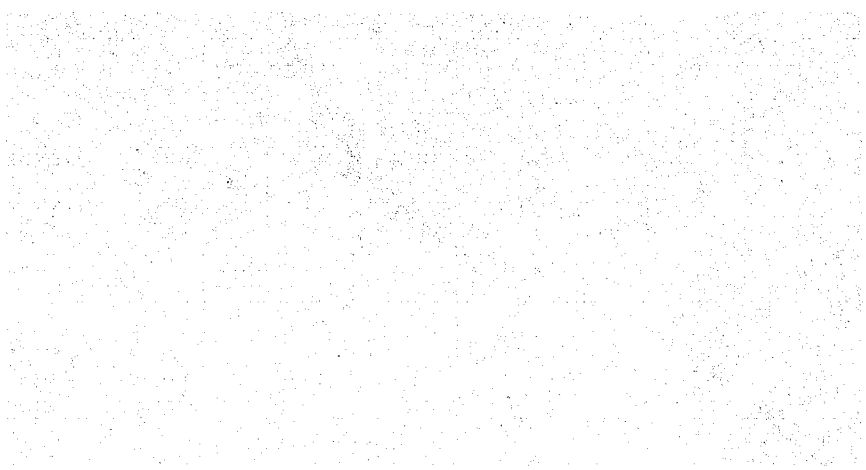


Figure 4.6

Mean weekly weight of male offspring
(n = 11-45 per treatment)

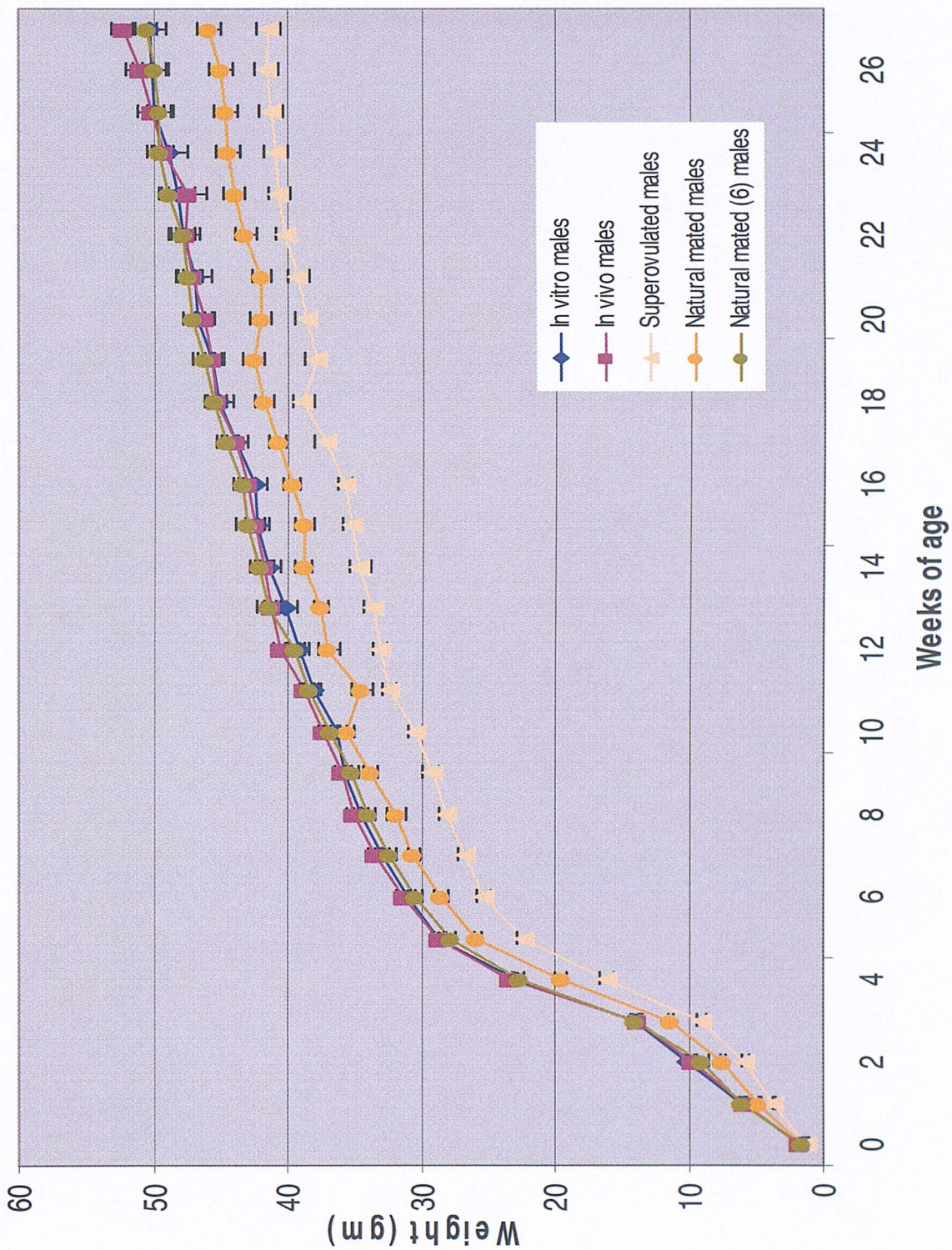
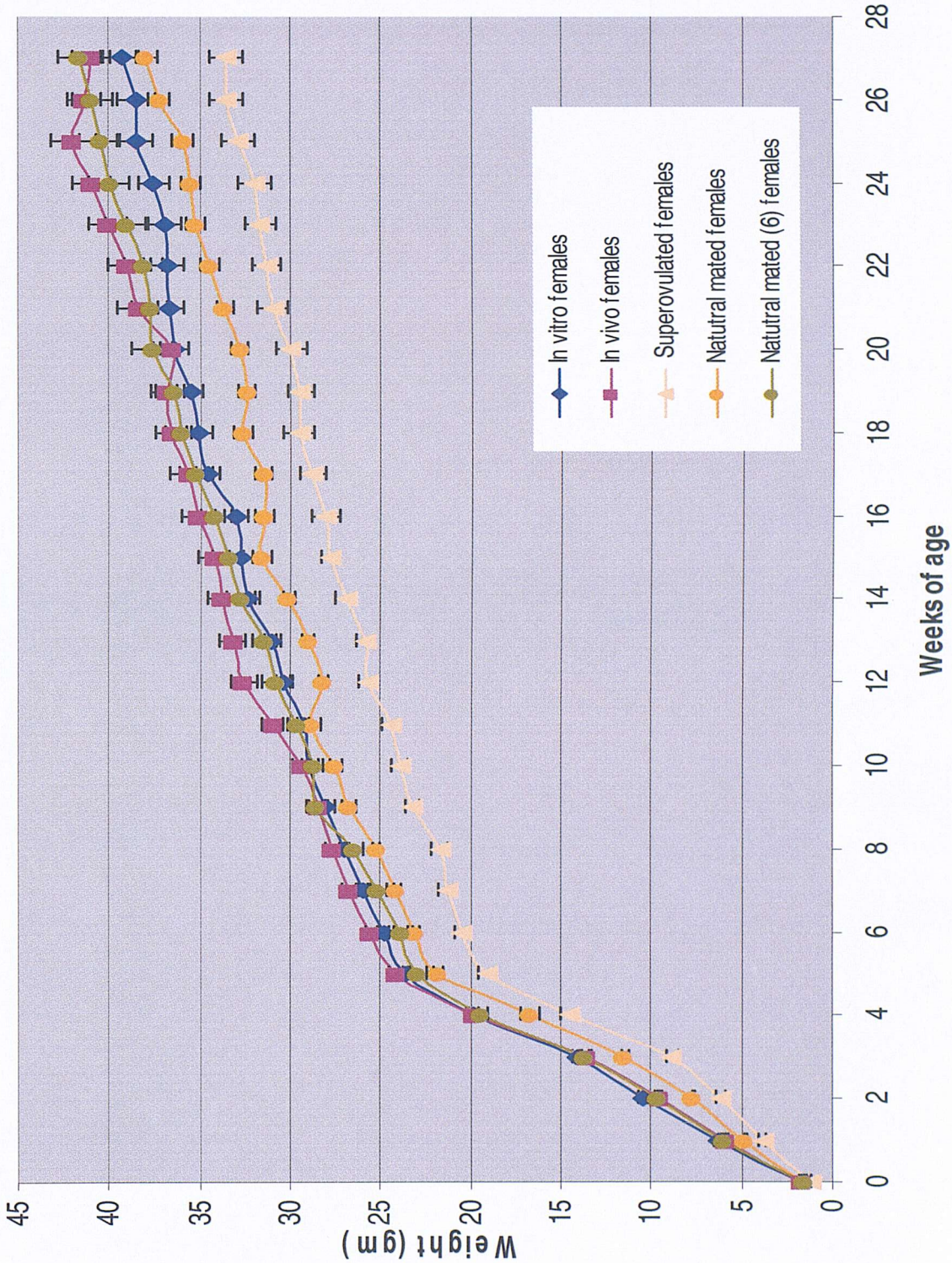


Figure 4.7

Mean weekly weight of female offspring
(n = 9-37 per treatment)



Each mouse was weighed on its day of birth and then subsequently every week for the next 27 weeks. All the weights for the mice from each treatment at each week have been averaged and plotted along with the standard error (Figures 4.6 and 4.7). Due to the complex nature and high number of data points within Figures 4.6 and 4.7, the results from the statistical analysis have been displayed in Tables 4.2 and 4.3 along side the raw data. All groups have been analysed against the naturally mated (6) group. Statistical differences ($p \leq 0.05$) have been highlighted in blue, whilst trends (a p value between 0.051 and 0.1) have been highlighted in orange.

Table 4.2 Results from statistical analysis of male offspring weekly weights

	Weight	Error	P value
	Day of birth		
In vitro males	1.648	0.0315	
In vivo males	1.713	0.0324	
Superovulated males	1.168	0.0256	0.015
Natural mated males	1.52	0.0284	
Natural mated (6) males	1.607	0.027	

	Weight	Error	P value
	Week 1		
In vitro males	5.995	0.113	
In vivo males	5.889	0.164	
Superovulated males	3.633	0.121	0.028
Natural mated males	4.743	0.0743	
Natural mated (6) males	6.01	0.124	

	Week 2		
In vitro males	10.032	0.207	
In vivo males	9.786	0.177	
Superovulated males	5.753	0.206	
Natural mated males	7.388	0.155	
Natural mated (6) males	9.005	0.461	

	Week 3		
In vitro males	14.03	0.331	
In vivo males	13.796	0.335	
Superovulated males	9.016	0.328	0.014
Natural mated males	11.348	0.226	0.035
Natural mated (6) males	14.042	0.28	

	Week 4		
In vitro males	22.779	0.405	0.034
In vivo males	23.427	0.454	
Superovulated males	16.051	0.535	0..033
Natural mated males	19.492	0.433	< 0.001
Natural mated (6) males	22.716	0.404	

	Week 5		
In vitro males	28.738	0.442	0.062
In vivo males	28.597	0.428	
Superovulated males	22.229	0.564	0.075
Natural mated males	25.801	0.289	
Natural mated (6) males	27.848	0.416	

	Week 6		
In vitro males	30.943	0.442	
In vivo males	31.404	0.404	
Superovulated males	25.305	0.501	
Natural mated males	28.468	0.492	
Natural mated (6) males	30.409	0.464	

	Week 7		
In vitro males	32.96		
In vivo males	33.458		
Superovulated males	26.658		
Natural mated males	30.563		
Natural mated (6) males	32.468		

	Week 8		
In vitro males	34.437	0.526	
In vivo males	35.099	0.467	
Superovulated males	28.124	0.62	
Natural mated males	31.829	0.722	
Natural mated (6) males	34.054	0.517	

Week 9		
35.834		
35.872		
29.283		
33.769		
35.302		

	Week 10		
In vitro males	36.361	0.744	
In vivo males	37.332	0.435	
Superovulated males	30.445	0.565	
Natural mated males	35.647	0.571	
Natural mated (6) males	36.805	0.616	

Week 11		
38.101	0.633	
38.718	0.456	
32.406	0.524	
34.448	0.779	
38.358	0.654	

	Week 12		
In vitro males	39.189	0.746	
In vivo males	40.474	0.569	
Superovulated males	33.083	0.564	
Natural mated males	36.941	0.807	
Natural mated (6) males	39.447	0.662	

Week 13		
40.094	0.775	
41.206	0.607	
33.623	0.648	
37.524	0.549	
41.411	0.88	

	Week 14		
In vitro males	41.340	0.83	
In vivo males	41.764	0.636	
Superovulated males	34.607	0.736	
Natural mated males	38.795	0.612	
Natural mated (6) males	42.046	0.779	

Week 15		
42.28	0.889	
42.515	0.677	
35.18	0.697	
38.816	0.686	
43.027	0.787	

	Week 16		
In vitro males	42.415	0.907	
In vivo males	43.095	0.655	
Superovulated males	35.77	0.591	
Natural mated males	39.723	0.57	
Natural mated (6) males	43.286	0.812	

Week 17		
43.938	1.009	
43.84	0.804	
37.25	0.758	
40.796	0.625	
44.54	0.807	

	Week 18		
In vitro males	45.15	1.047	
In vivo males	45.276	0.74	
Superovulated males	38.862	0.75	
Natural mated males	41.782	0.64	
Natural mated (6) males	45.417	0.836	

Week 19		
45.727	1.414	
45.586	0.689	
37.951	0.754	
42.518	0.826	
46.258	0.809	

	Week 20		
In vitro males	46.765	1.502	
In vivo males	46.117	0.702	
Superovulated males	38.644	0.762	
Natural mated males	42.004	0.755	
Natural mated (6) males	47.019	0.823	

Week 21		
46.881	1.667	
47.093	0.706	
39.221	0.846	
42.004	0.736	
47.712	0.889	

	Week 22		
In vitro males	47.957	1.622	
In vivo males	47.643	0.715	
Superovulated males	40.13	0.732	
Natural mated males	43.164	0.812	
Natural mated (6) males	47.957	1.031	

Week 23		
48.566	1.824	
47.446	1.44	
40.656	0.831	
43.977	0.839	
48.783	0.975	

	Week 24		
In vitro males	48.931	1.852	
In vivo males	49.163	0.754	
Superovulated males	40.88	0.849	
Natural mated males	44.461	0.87	
Natural mated (6) males	49.455	1.075	

Week 25		
49.938	1.868	
50.114	0.996	
41.244	0.88	
44.65	0.861	
49.593	1.045	

	Week 26		
In vitro males	49.919	1.81	
In vivo males	51.05	0.987	
Superovulated males	41.572	0.88	
Natural mated males	45.001	0.887	
Natural mated (6) males	49.256	0.993	

Week 27		
49.905	1.824	
52.205	0.843	
41.409	0.93	
45.92	0.886	
50.487	1.029	

Table 4.3 Results from statistical analysis of female offspring weekly weights

	Weight	Error	P value
	Day of birth		
In vitro females	1.754	0.0476	
In vivo females	1.669	0.04	
Superovulated females	1.164	0.0328	
Natural mated females	1.522	0.0234	
Natural mated (6) females	1.548	0.0314	

Weight	Error	P value
Week 1		
6.314	0.174	
5.818	0.14	
3.836	0.146	
4.82	0.0656	
5.984	0.116	

	Week 2		
In vitro females	10.413	0.259	
In vivo females	9.551	0.203	
Superovulated females	6.147	0.228	
Natural mated females	7.644	0.0855	
Natural mated (6) females	9.608	0.0829	

Week 3		
14.119	0.259	
13.625	0.341	
8.809	0.383	0.009
11.433	0.196	
13.674	0.238	

	Week 4		
In vitro females	19.83	0.306	0.026
In vivo females	19.828	0.325	0.056
Superovulated females	14.478	0.552	< 0.001
Natural mated females	16.745	0.536	0.002
Natural mated (6) females	19.427	0.4	

Week 5		
23.546	0.234	
24.123	0.369	
19.032	0.469	< 0.001
21.769	0.224	0.0014
22.979	0.487	

	Week 6		
In vitro females	24.765	0.243	
In vivo females	25.546	0.326	
Superovulated females	20.435	0.461	0.004
Natural mated females	23.039	0.222	
Natural mated (6) females	23.857	0.421	

Week 7		
25.894	0.273	
26.746	0.366	
21.32	0.517	0.033
24.07	0.222	
25.187	0.527	

	Week 8		
In vitro females	27.018	0.286	
In vivo females	27.65	0.38	
Superovulated females	21.7	0.496	0.001
Natural mated females	25.234	0.231	
Natural mated (6) females	26.461	0.452	

Week 9		
28.044	0.534	
28.472	0.497	
23.171	0.406	0.0057
26.743	0.377	
28.628	0.476	

	Week 10		
In vitro females	28.911	0.468	
In vivo females	29.341	0.559	
Superovulated females	23.934	0.519	0.014
Natural mated females	27.500	0.342	
Natural mated (6) females	28.748	0.633	

Week 11		
29.173	0.372	
30.932	0.572	
24.387	0.529	0.003
28.804	0.504	
29.553	0.573	

	Week 12		
In vitro females	30.327	0.407	
In vivo females	32.578	0.744	
Superovulated females	25.664	0.545	0.037
Natural mated females	28.217	0.272	
Natural mated (6) females	30.808	0.78	

Week 13		
30.986	0.416	
33.152	0.733	
25.839	0.52	0.072
28.98	0.315	
31.437	0.665	

	Week 14		
In vitro females	32.305	0.581	
In vivo females	33.724	0.82	
Superovulated females	26.903	0.582	
Natural mated females	30.096	0.35	
Natural mated (6) females	32.687	0.773	

Week 15		
32.751	0.603	
34.221	0.828	
27.781	0.519	
31.545	0.484	
33.401	0.843	

	Week 16		
In vitro females	32.985	0.637	
In vivo females	35.047	0.929	
Superovulated females	27.99	0.773	
Natural mated females	31.433	0.474	
Natural mated (6) females	34.14	0.846	

Week 17		
34.533	0.647	
35.646	1.019	
28.787	0.735	
31.497	0.48	
35.166	0.914	

	Week 18		
In vitro females	35.034	0.756	
In vivo females	36.47	0.941	
Superovulated females	29.5	0.832	
Natural mated females	32.593	0.503	
Natural mated (6) females	35.989	0.945	

Week 19		
35.531	1.108	
36.81	0.943	
29.445	0.726	
32.386	0.422	
36.421	0.969	

	Week 20		
In vitro females	36.878	1.080	
In vivo females	36.560	0.994	
Superovulated females	29.949	0.810	
Natural mated females	32.770	0.472	
Natural mated (6) females	37.622	1.052	

Week 21		
36.648	0.966	
38.408	1.076	
30.972	0.906	
33.620	0.432	
38.073	0.989	

	Week 22		
In vitro females	37.159	1.319	
In vivo females	39.022	0.958	
Superovulated females	31.253	0.776	
Natural mated females	34.448	0.485	
Natural mated (6) females	38.427	1.121	

Week 23		
37.299	1.408	
40.069	1.004	
31.654	0.869	
35.202	0.537	
39.218	1.211	

	Week 24		
In vitro females	37.699	1.295	
In vivo females	40.924	1.059	
Superovulated females	31.984	0.903	
Natural mated females	35.505	0.521	
Natural mated (6) females	40.225	1.224	

Week 25		
38.283	1.380	
41.948	1.193	
32.887	0.946	
35.887	0.572	
40.719	1.178	

	Week 26		
In vitro females	37.791	1.426	
In vivo females	41.399	0.921	
Superovulated females	33.543	0.953	
Natural mated females	37.238	0.614	
Natural mated (6) females	40.820	1.164	

Week 27		
39.086	1.289	
41.000	1.063	
33.530	0.947	
37.896	0.575	
41.819	1.211	

The mean weight gained per week for the different treatment groups for the first 10 weeks of age are shown in Figure 4.8 and 4.9. The first and most prominent feature of these graphs is the dramatic increase in weight gain seen over weeks 3, 4 and 5. After 4 weeks of age, the increase in weight diminishes, and then appears to fluctuate from week to week. Figure 4.8 shows that at 1 and 3 weeks of age, the naturally mated (6) males have the highest weight gain for any of the groups. For the first four weeks, and subsequently at week 7, the superovulated males have the lowest weight gain.

A similar pattern for the female mice is seen in Figure 4.9, in that there is dramatic weight gain for all groups during weeks 3, 4 and 5. Again, the superovulated females have the lowest weight gain, particularly during weeks 1, 2, and 3. Unlike the naturally mated (6) males, the naturally mated (6) females do not appear to have the highest rates of weight gain during the first few weeks after birth. Instead they have the largest weight gain during weeks 7, 8 and 9. It can also be seen that the naturally mated females appear to have a two week period of maximal weight gain, from weeks 4 to 5. No other group appear to have such a pattern.

Figure 4.8

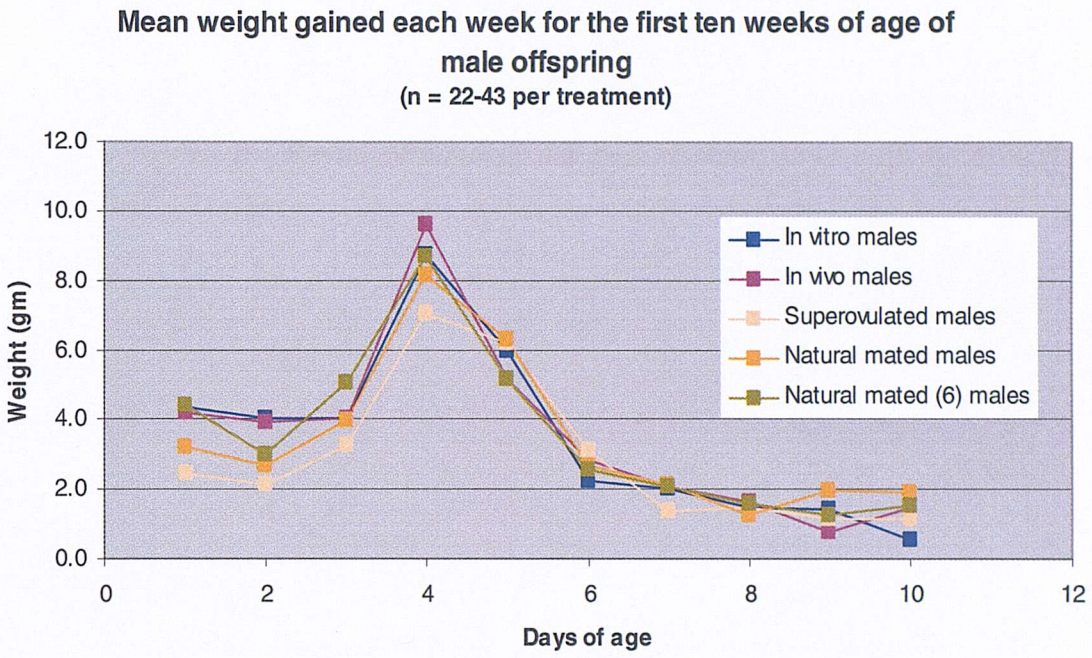
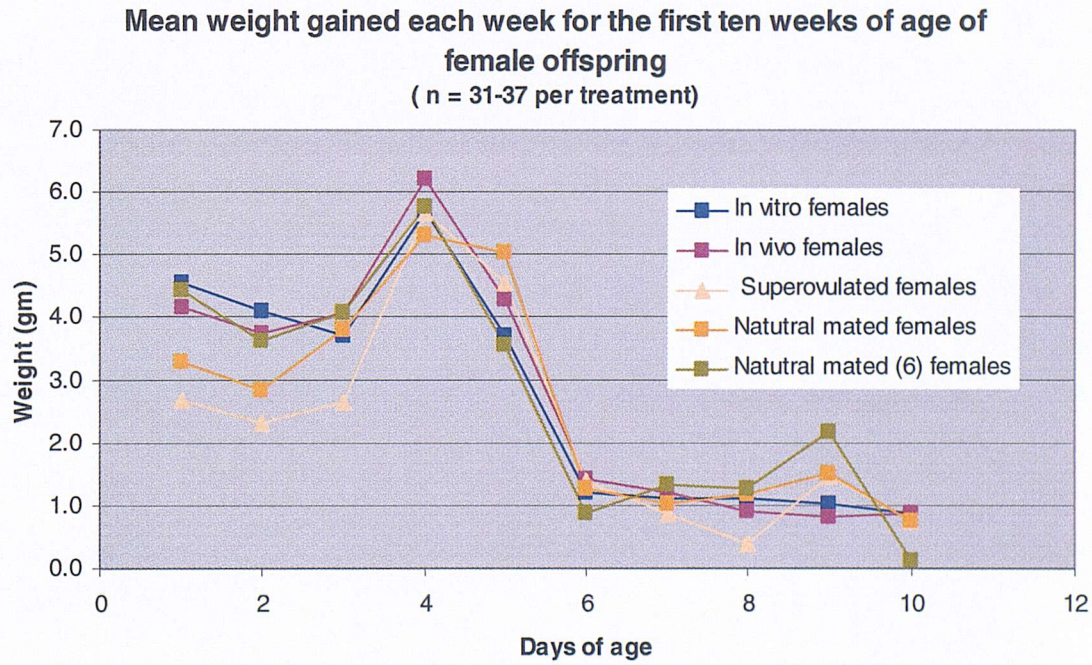


Figure 4.9



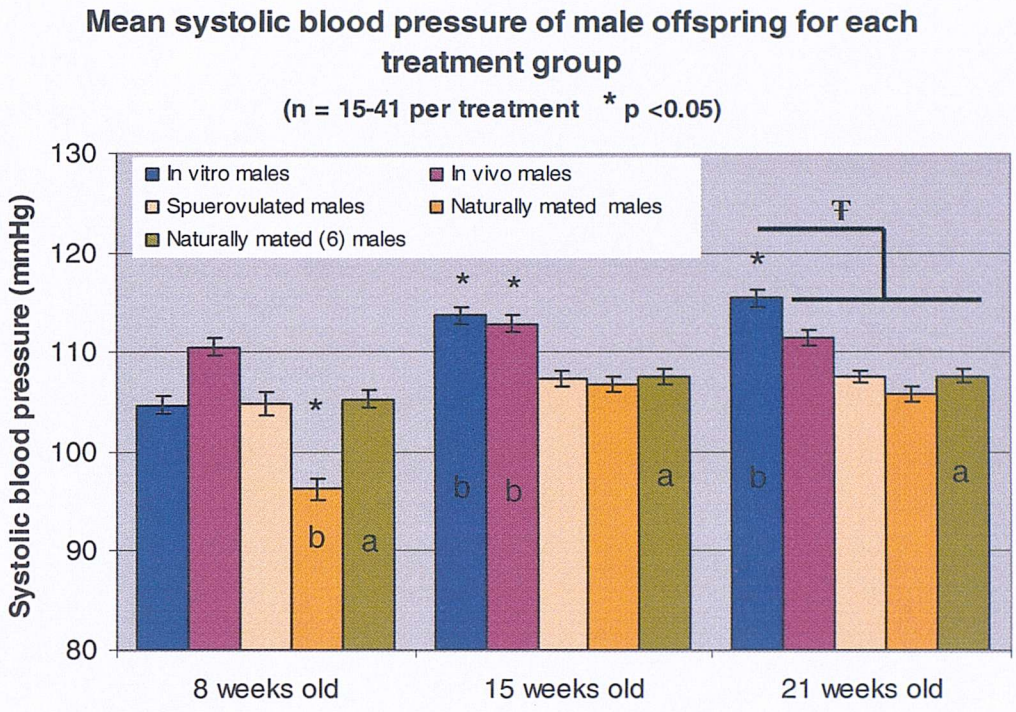
The mean systolic blood pressures for the different treatment groups at three ages (8, 15 and 21 weeks of age) are shown in Figures 4.10 and 4.11.

For the males at 8 weeks of age, mice from the naturally mated group have significantly lower systolic blood pressure when compared to the naturally mated (6) males. There is no significant difference between the *in vitro*, *in vivo* and the superovulated males when compared to the naturally mated (6) males. There is also no significant difference between the *in vivo* and *in vitro* males, although there does appear to be a trend towards the *in vivo* mice having a higher blood pressure ($p = 0.059$). At 15 weeks of age, the *in vitro* and *in vivo* males have a significantly elevated blood pressure when compared to all the non-embryo transfer groups. There is no significant difference between the naturally mated and superovulated males when compared to the naturally mated (6) males. At 21 weeks of age, the *in vitro* males have a significantly elevated blood pressure when compared to all the *in vivo* males ($p = 0.003$), and when compared to the non-embryo transfer groups (indicated by the ₸ symbol on Figure 4.10, $p < 0.01$).

There is a similar pattern observed for the females. At 8 weeks of age the *in vivo* females have a significantly elevated blood pressure when compared to the *in vitro* females (as indicated by the ₸ symbol on Figure 4.11, $p = 0.003$). At 15 weeks of age, there is no significant difference between any of the females groups, although it can be seen that the *in vivo* and *in vitro* females have the highest overall blood pressure. At 21 weeks of age, the *in vitro* females have a significantly elevated blood pressure when compared to the *in vivo* females ($p < 0.001$), and when compared to the non-embryos transfer female groups (indicated by the ₸ on the graph, $p < 0.025$).

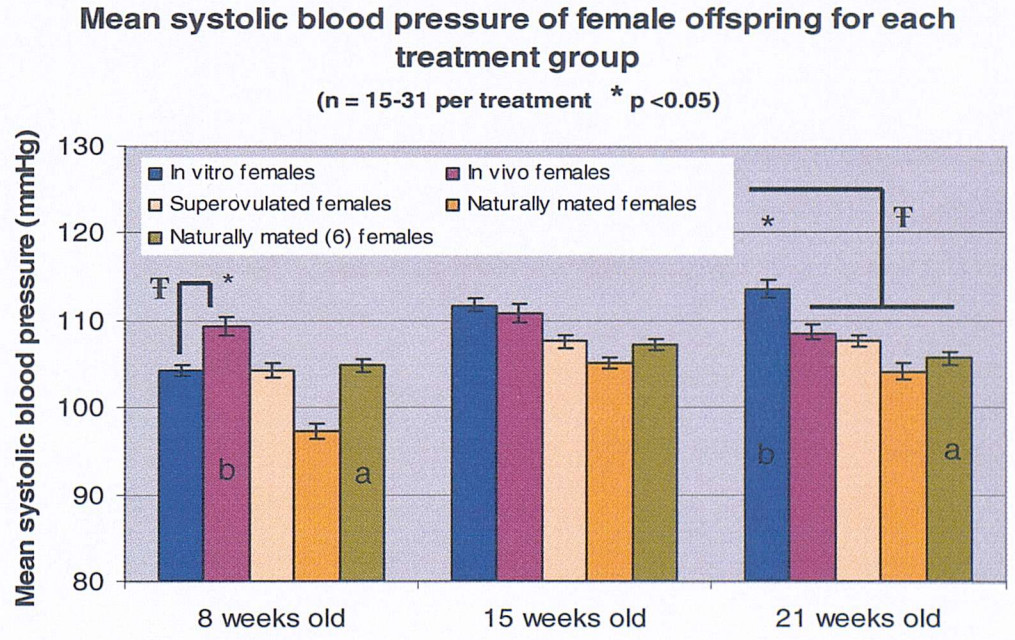
For both male and the female treatment groups, litter size at birth or after adjustment, has no significant influence on the blood pressure results observed within Figures 4.10 and 4.11.

Figure 4.10



The symbol Ŧ used on Figures 4.8 and 4.9 indicates that there is significant difference between the *in vitro* group and other groups within the study.

Figure 4.11



The mean organ:body weight ratios, and organ weights alone, for the five treatment groups studied at 27 weeks of age are shown in Figures 4.12 to 4.15.

Figure 4.12 shows that for the male mice, there is no significant difference between any of the groups for any of the organ:body weight ratios when compared to the non-embryo transfer groups. The *in vitro* males have a significantly elevated liver:body weight ratio when compared to the *in vivo* males ($p = 0.023$). It can also be seen that *in vitro* males do have the highest overall ratio for the liver and both kidneys. The naturally mated (6) males have the lowest organ:body weight ratio for both of the kidneys and for the heart.

Figure 4.13 shows that the *in vitro* females have a significantly lower liver:body weight ratio when compared to the naturally mated (6) females ($p = 0.038$). There are no other significant differences between the naturally mated (6) females and any of the other groups, however, for all the other organs, the *in vitro* and *in vivo* females are seen to have the largest ratios.

When organ weights are analysed independently of body size, there are no significant difference between any of the treatment groups for either the males or the females (Figures 4.14 and 4.15).

For both male and the female treatment groups, litter size at birth or after adjustment, has no significant influence on either organ: body weight ratios, or when the organ data is analysed independently of body weight.

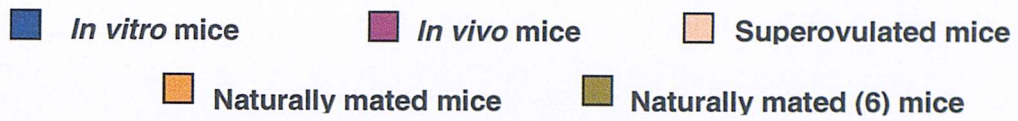


Figure 4.12

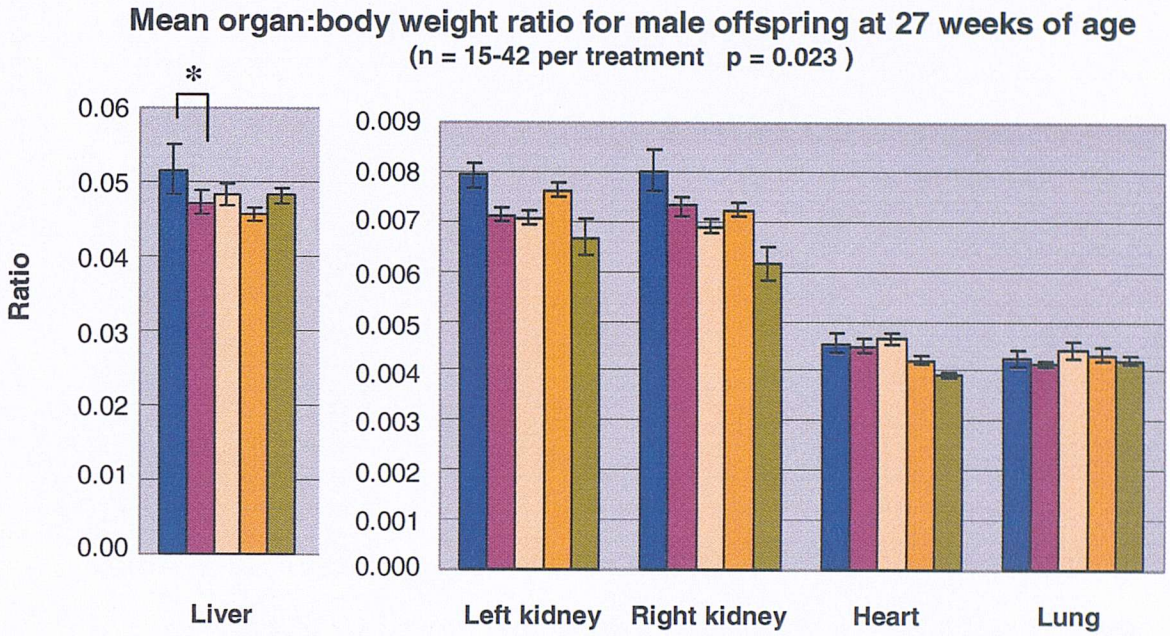
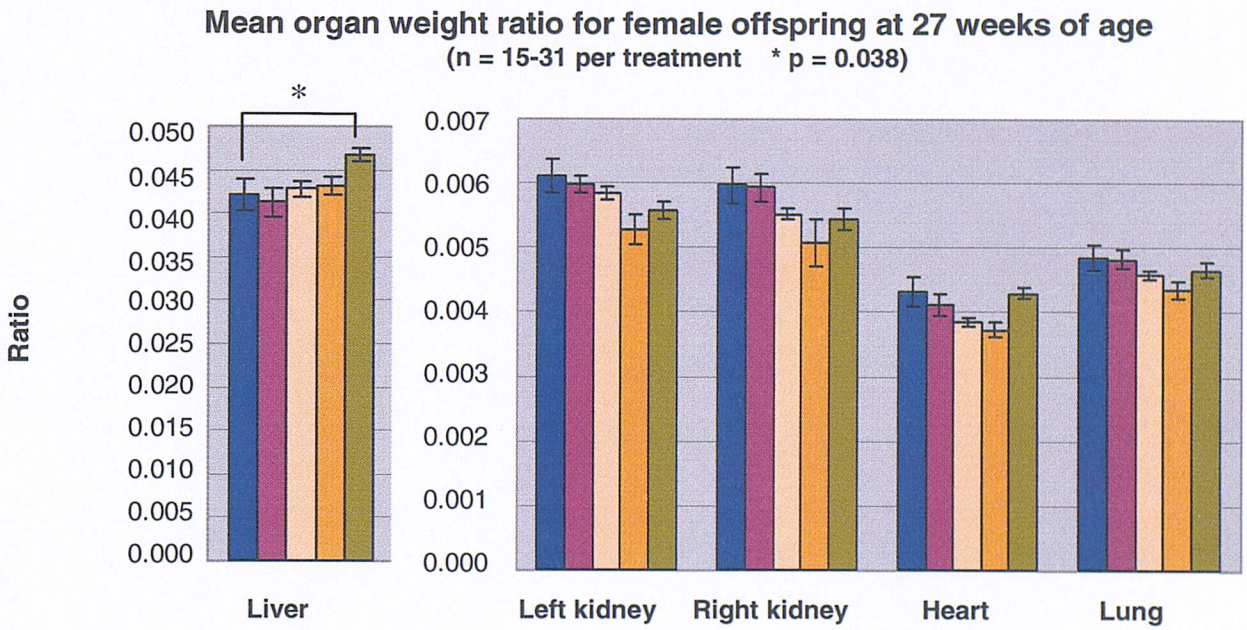


Figure 4.13



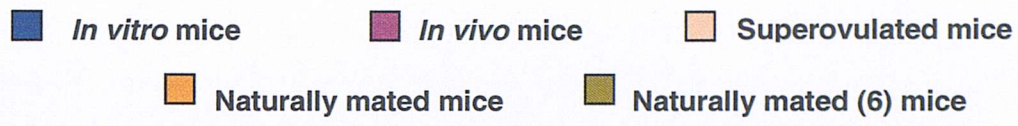


Figure 4.14

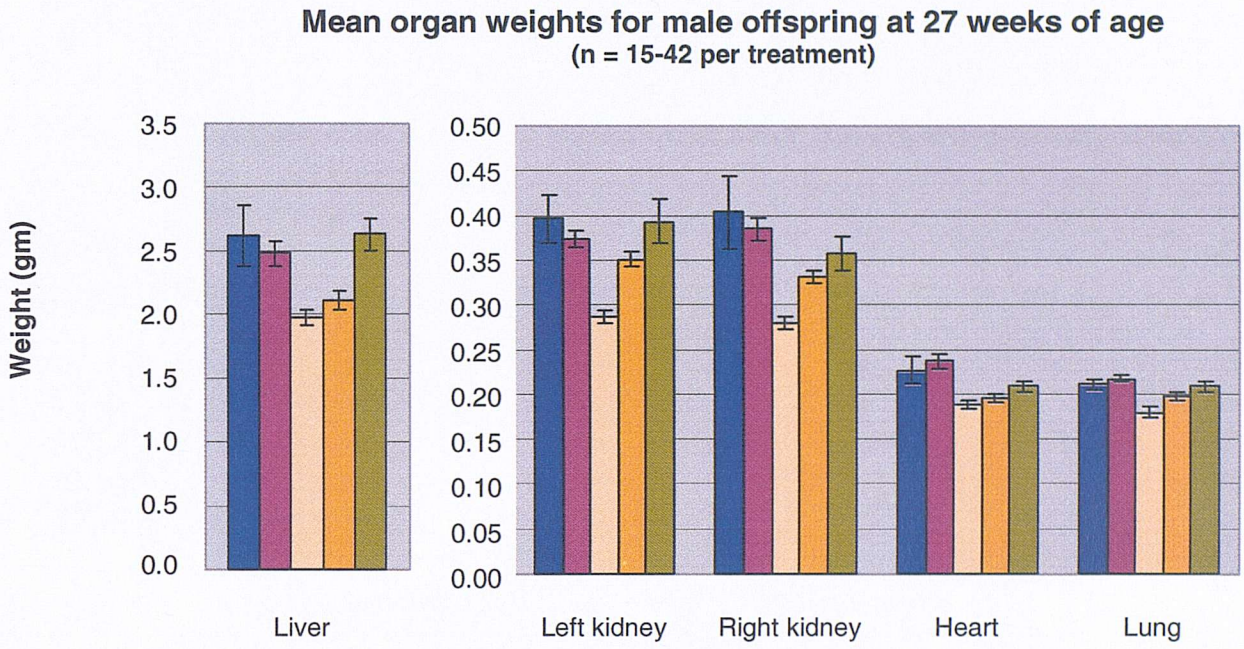
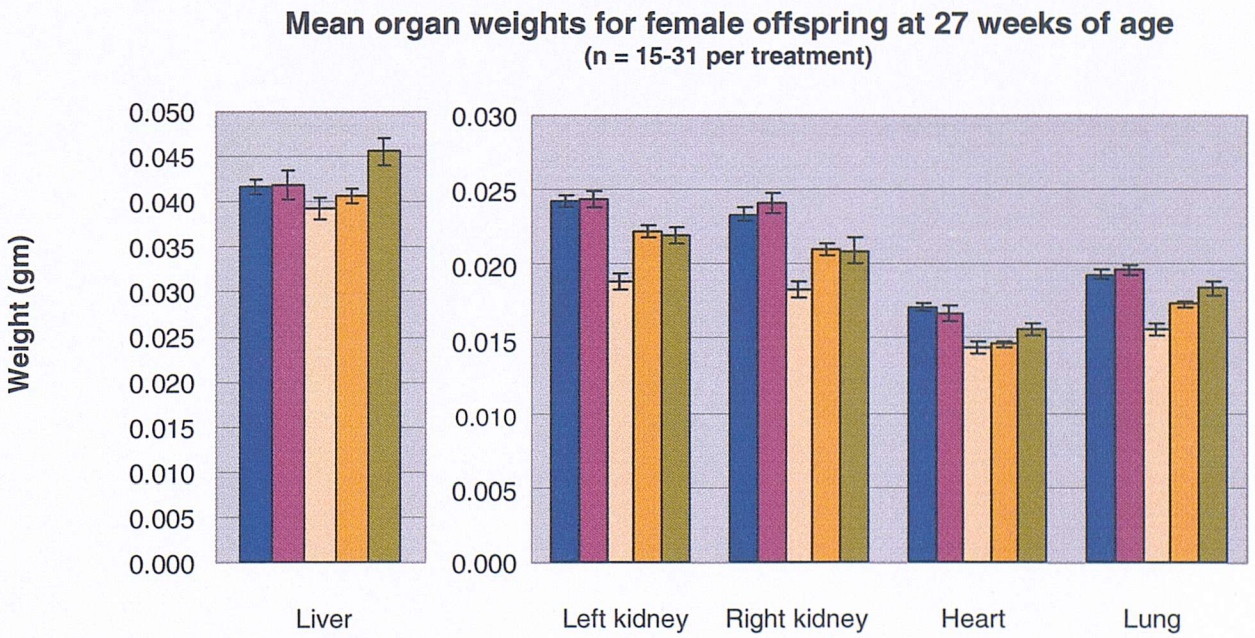


Figure 4.15



The mean activity of ACE within the serum of the treatment groups at 27 weeks of age are shown in Figures 4.16 and 4.17. Due to the fact that this study is currently ongoing, serum samples from the naturally mated (6) mice were unavailable to analyse. Figures 4.15 and 4.16 show that there is no significant difference between any of the groups, for either males or females, in terms of serum ACE activity. However, it can be seen that for both the male and female groups, the naturally mated mice have the lowest levels of ACE activity. There is no significant difference between the *in vitro* and *in vivo* groups ($p = 0.12$ for the male groups, $p = 0.824$ for the female groups). There is no significant influence from litter size at birth, or after correction, for either the male or female treatment groups.

Figure 4.16

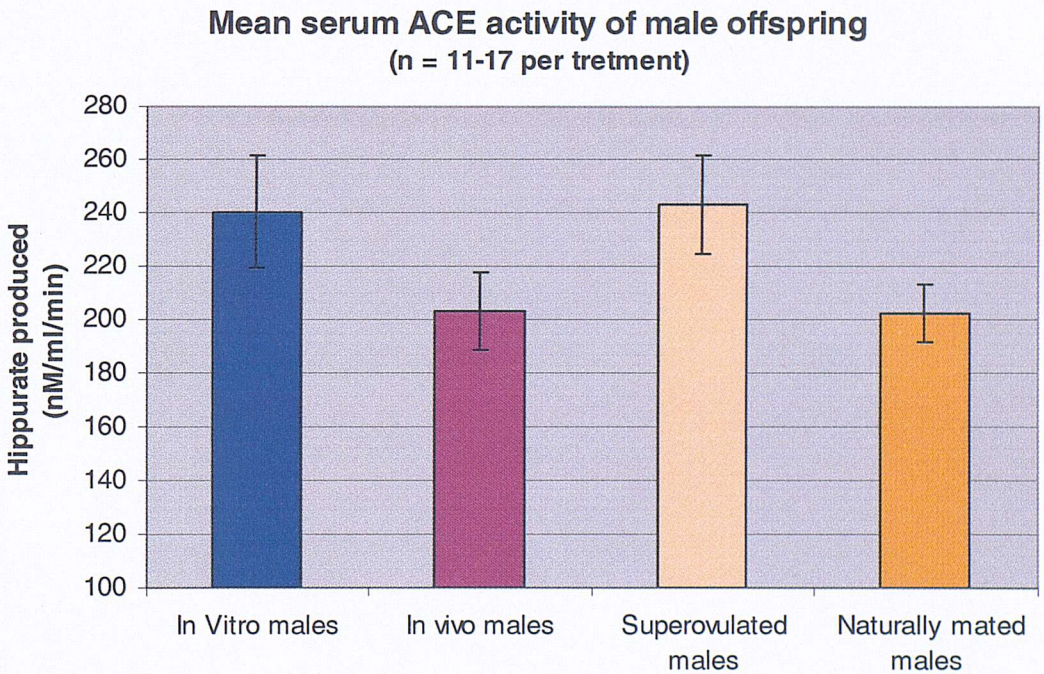
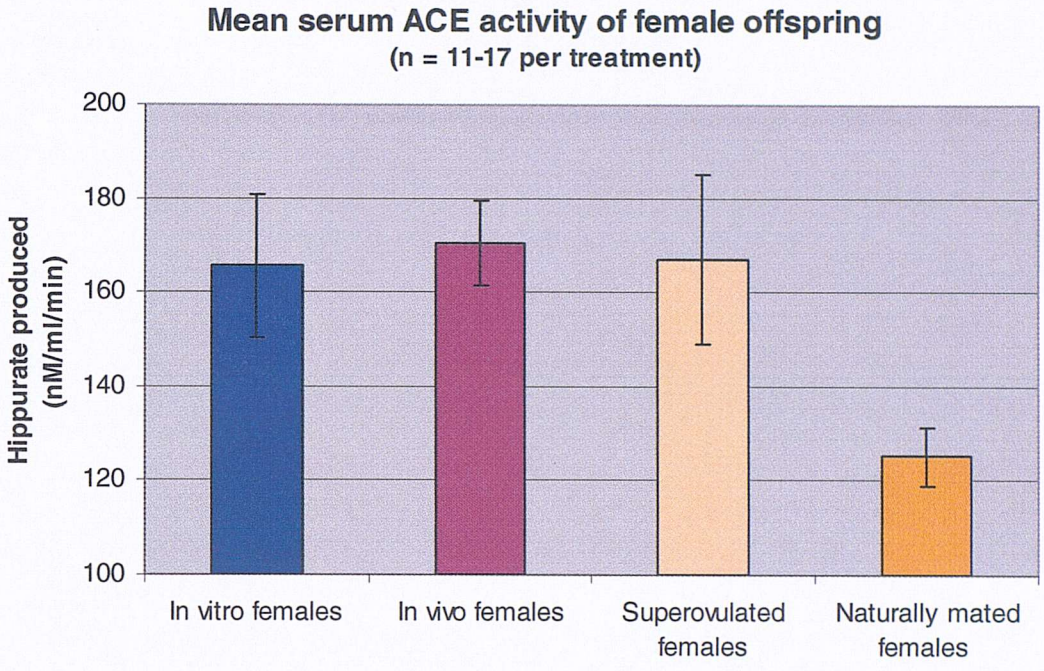


Figure 4.17


Mean serum ACE activity of female offspring
(n = 11-17 per treatment)
Hippurate produced (nM/ml/min)
200
180
160
140
120
100
In vitro females
In vivo females
Superovulated females
Naturally mated females

4.4 Discussion

4.4.1 Environment of the preimplantation embryo and its effects on birth weight and litter size

Previous studies have shown that *in vitro* culture not only affects preimplantation embryo development, but can also impact upon aspects of fetal and postnatal development. Altered patterns of preimplantation embryo development can result in reduced numbers of cells within the embryo, increased or decreased fetal growth, altered patterns of gene expression and increased incidences of postnatal mortality (Bowman and McLaren, 1970; Harlow and Quin, 1982; Thompson *et al.*, 1995, Holm *et al.*, 1996, Young *et al.*, 1998, Sinclair *et al.*, 1999; Koshla *et al.*, 2001).

In the experiments reported within this Chapter, it was observed that the superovulated males had significantly reduced birth weights when compared to the naturally mated (6) males. It was also observed that there was no significant difference between the *in vitro* and *in vivo* animals for either males or females. Within this experiment, predominantly the largest factor affecting weight at birth was litter size. A negative correlation was found between litter size and birth weight, such that as litter size increased then the mean birth weight within that litter decreased (Figure 4.4, R =-0.645). Restricted growth in larger litters can be attributed to over-crowding within the uterus preventing the fetus reaching maximal size. There would also be competition between siblings for the available maternal nutrients and blood supply.

As anticipated, litters born to mothers who were superovulated had the lowest mean birth weight, as superovulation would result in the production of significantly more fertilized eggs and so an increased litter size (n = 11-19 pups, significantly increased when compared to the *in vitro* and *in vivo* groups n = 3-8, $p < 0.05$). Those mothers who were naturally mated gave birth to litters of a size that could be considered normal (9-14 pups).

Although there is no difference in litter size or birth weight between the *in vivo* and *in vitro* derived mice ($p = 0.116$ and $p = 0.656$ respectively), it might be

anticipated that embryos derived from an *in vitro* environment would be at a developmental disadvantage when compared to embryos derived from an *in vivo* environment. Studies have shown that *in vitro* culture can slow developmental rates, reduce total blastocyst cell numbers and even alter the patterns of gene expression (Bowman and McLaren, 1970; Harlow and Quinn, 1982; Conaghan *et al.*, 1993; Khosla *et al.*, 2001). As preimplantation development is slower for *in vitro* cultured embryos (Figure 3.1), then the rate of postimplantation development may also be affected when compared to *in vivo* derived embryos. If this is the case, birth size could be reduced for mice generated from *in vitro* derived embryos. Alternatively, reduced rates of development and lower total cell numbers within *in vitro* derived blastocycsts (Figure 3.1) may compromise postimplantation development, so producing a smaller population of embryos that are able to implant and produce viable offspring. In this situation fetal growth might be increased due to increased uterine space and availability of maternal nutrients. It may also be the case that fetuses derived from *in vitro* derived embryos might have increased their rate of postimplantation development, compensating for the initial slower rate of preimplantation development (Rands, 1986; Hardy and Handyside, 1993; Power and Tam, 1993; Hishinuma *et al.*, 1995).

Twelve embryos were transferred into each pseudo pregnant mouse, however, a maximal implantation and gestation rate of only 66% was observed for both *in vitro* and *in vivo* derived embryos (maximum litter size observed of 8). This finding has two possible conclusions; (1) that although *in vitro* derived embryos have a slower rate of development, this does not affect their ability to implant and develop into viable offspring when compared to *in vivo* derived embyos; (2) exposure to an *in vitro* environment, regardless of the duration (60-120 minutes for the *in vivo* developed embryos just prior to their transfer compared with culture for up to 96 hours post hCG for the *in vitro* group) alters the ability on an embryo to implant and develop into a viable offspring. However, as implantation rates were not directly observed, increased loss of embryos at the implantation stage can only speculated.

4.4.2 Environment of the preimplantation embryo and its effect on postnatal growth rates

Altered patterns of postnatal growth and development occurring from periods of *in vitro* culture have been well documented. The culture and transfer of sheep and cattle embryos (specifically in the presence of serum) has been associated with significantly increased birth weight, increased muscle mass and skeletal and facial malformations (Thompson *et al.*, 1995, Holm *et al.*, 1996, Young *et al.*, 1998, Sinclair *et al.*, 1999; Jacobsen *et al.*, 2000). In mice, it has been seen that day 14 fetuses developing from embryos cultured in the presence of fetal calf serum were lighter when compared to controls (Koshla *et al.*, 2001). However, offspring developing from mouse embryos manipulated to contain increased or decreased numbers of cells, have similar birth weights to control animals (Tsunoda and McLaren, 1983: Biggers and Papaioannou, 1989, 1991).

During the period of 3-5 weeks of age, the superovulated, naturally mated and *in vitro* groups are all observed to become significantly lighter than the naturally mated (6) males and females (Figure 4.6 and 4.7, Table 4.2 and 4.3). After these periods, the superovulated animals remain significantly lighter for up to 12 weeks of age. Therefore, whilst at birth there is no significant difference between the naturally mated (6), naturally mated and *in vitro* groups, during specific periods of early postnatal growth and development, growth patterns become significantly altered when compared to the naturally mated (6) animals. However, these animals go on to increase their weight to a mean value comparable to that of the naturally mated (6) animals. At 2 weeks of age the *in vivo* males were observed to be significantly heavier than the *in vitro* males ($p = 0.009$) whilst at 11 and 16 weeks of age, the *in vivo* females became significantly heavier than the *in vitro* females ($p = 0.037$ and 0.035 respectively). At no other times were significant differences observed between the *in vivo* and *in vitro* animals. During the statistical analysis of the data, it was possible to assess the influence of litter size at birth and subsequent litter size after correction (naturally mated (6) animals, litter size corrected to a mean of 6). Litter size was observed to significantly influence birth weight only. For no other measurement did litter size have a significant influence on the results observed. For a

more detailed discussion on the effect of litter size on postnatal development see Chapter 5.

The overall patterns of weight gain appeared to be similar for all groups. Figures 4.8 and 4.9 show that all the groups go through a dramatic increase in weight over weeks 3, 4 and 5. One possible explanation for this increase in mean weight gain could be due to weaning (taken place at 3 weeks of age) and the subsequent altering of diet from maternal nutrient supply to pellet chow. After this period, the increase in weight diminishes and then appears to fluctuate from week to week. However, it can be seen that whilst the superovulated group shows the same trend, their average weight gain is dramatically less than that of all other groups, so that at no time do they achieve a weight comparable to that of the controls. Naturally mated (6) males are observed to have the highest weight gain for any of the groups during the first and third weeks of age. This could account for the significantly lower mean body weight of the superovulated, naturally mated and *in vitro* treatment groups during the second to fifth week of age. The naturally mated (6) males also have the largest weight gain during weeks 7, 8 and 9. The naturally mated (6) females do not appear to show such a similar pattern, although they do have the highest weight gain at 7, 8 and 9 weeks of age. Therefore, *in vitro*, *in vivo*, superovulated and naturally mated groups, particularly for the males, appear to have a reduce rate at which they increase their growth during these early postnatal periods.

Environmental stresses occurring during early development can have long lasting effects on physiology and behaviour. Long lasting changes in stress-induced corticosterone levels and anxiety-like behaviours have been observed in Long-Evans rats as a consequence of neonatal maternal separation and neonatal handling (Durand *et al.*, 1998; Kalinichev *et al.*, 2002). As the reduced levels of postnatal weight gain are observed to occur from 3 weeks of age, and persist through the period of weaning, it may be the case that the experimental groups are more prone to the stresses of handling, weighing and short term maternal separation. This increased stress response may manifest itself as a reduction in weight gain during this time.

4.4.3 Origin of the embryo and its effect on systolic blood pressure

Previous studies have shown that the environment in which the preimplantation embryo develops can affect postnatal systolic blood pressure. Offspring from female rats fed a low protein diet during the preimplantation period have been shown to have raised systolic blood pressure in later life (Kwong *et al.*, 2000).

Previous studies examining the blood pressure of adult mice have shown similar mean systolic blood pressure to those values reported for the naturally mated (6) mice (see Table 4.4). In these earlier studies, mean systolic blood pressure ranges for similar strains of mice to those studied within this thesis, were found to be within 90-100 mm Hg, depending upon the method used to measure the blood pressure. In the experiments reported within this Chapter, the average systolic blood pressure was approximately 108 mm Hg (Figures 4.11 and 4.12).

Table 4.4 Results from previous studies examining the blood pressure in mice

Strain of mice	Age of mice	Monitoring method	Mean systolic pressure (mmHg)	Reference
C57/BL6	19-30gm	Radiotelemetry	98.4 (abdominal)	1
C57BL10J		Tail cuff	80	2
C57BL/6J		Tail cuff	93	2
CBA/J		Tail cuff	97	2
C57BL6J	8-13 weeks	Catheritization	120	3

References (1) Carlson and Wyss (2001), (2) Schlager, (1966), (3) Mattson (2001).

At 8 weeks of age, significant differences are observed between the naturally mated (6) and the naturally mated mice. It might be anticipated that this finding is due to the altered litter size of the naturally mated (6) mice, however, statistical analysis showed that the litter size at birth, or after correction was not having a significant influence on the blood pressure results. Therefore, other factors such as body weight may be contributing to the significant difference. As the progress of individual animals was not traced from week to week, this factor within these studies can-not be

assessed. Significant differences were also observed between the naturally mated (6) and the *in vivo* female mice. In addition, the *in vivo* females have a significantly elevated blood pressure when compared to the *in vitro* females. At 15 weeks of age, the male *in vitro* and *in vivo* mice have a significantly elevated blood pressure when compared to the naturally mated (6) mice. At 21 weeks of age it is seen that the *in vitro* mice have significantly elevated blood pressure when compared to all the other groups. From these results it would appear that at all times analysed, those offspring developing from embryos exposed to an *in vitro* environment have an elevated systolic blood pressure when compared to the non-embryo transfer groups. Therefore, even though the *in vivo* group were only exposed to an *in vitro* environment for a relatively short period of time, 90-180 minutes, this appears to be sufficient to programme an increased blood pressure during periods of postnatal growth. It may also be the case that the duration of exposure to an *in vitro* environment may influence blood pressure. This is demonstrated by the observation that at 21 weeks of age, the *in vitro* mice have a significantly elevated blood pressure when compared to all the other treatment groups, whilst the *in vivo* mice (derived from embryos exposed to an *in vitro* environment for only a few hours) only show a significantly elevated blood pressure at 8 and 15 weeks of age.

One factor important in the regulation of blood pressure is ACE. Figures 4.16 and 4.17 show that there is no significant difference between any of the treatment groups in the activity of ACE within the serum. However, it was observed that the *in vitro* mice (which had the highest blood pressure at 21 weeks of age) did have the highest serum ACE activity at 27 weeks of age. Some studies have shown that serum ACE levels in humans are significantly higher in hypertensives when compared to normotensives, that there is a positive correlation to BMI in males and females and that serum ACE activity correlates positively with plasma angiotensin II levels (Forrester *et al.*, 1997; Nystrom *et al.*, 1997). As the *in vivo* and *in vitro* mice were observed to be the heaviest groups at 27 weeks of age, this increased weight could account for the increased ACE activity observed within these groups.

Previous experiments have found a correlation between low birth weight and a higher adult postnatal systolic blood pressure (Barker *et al.*, 1990: Barker 1995; Forsen *et al.*, 1997; Rich-Edwards *et al.*, 1997). In the data reported within this thesis, the reverse is observed, in that those animals with the largest birth weights have the highest blood pressures. However, recent re-evaluation of the data from previous studies has suggested that the inverse relationship between birth weight and blood pressure may not be as reliable as previously thought (Huxley *et al.*, 2002). Studies have found there to be no difference or even a reduced mean arterial blood pressure in intrauterine growth restricted (IUGR) sheep when compared to controls (Edwards *et al,*. 1999; Louey *et al,*. 2000). Therefore, in the context of the experiments within this thesis, it appears that the environment of the early embryo may have more of an effect on postnatal development than factors such as birth weight.

4.4.4 Origin of the embryo and its effect on organ weight

Embryo culture in the presence of serum (sheep and cattle) has been shown to lead to changes in the normal size of internal organs (Thompson *et al.*, 1995, Holm *et al.*, 1996, Young *et al.*, 1998, Sinclair *et al.*, 1999; Jacobsen *et al.*, 2000). McEvoy et al., (1998) found that hearts from bulls produced from *in vitro* derived embryos were heavier than hearts from bulls produced from *in vivo* derived embryos. In rodents, offspring from rats fed a low protein diet exclusively during the preimplantation period of development have been shown to have disproportionate growth of specific organs compared to body weight (Kwong *et al.*, 2000). *In vitro* males were observed to have an elevated liver:body weight ratio when compared to the *in vivo* males, whilst the naturally mated (6) females were observed to have an elevated liver:body weight ratio when compared to the *in vitro* females. There were no other significant differences between any of the treatment groups for either the males or the females. However, it was observed that the naturally mated (6) males had the lowest organ:body weight ratio for both of the kidneys and for the heart, whilst the *in vitro* males had the highest ratio for the liver and kidneys. It may therefore be the case that within this study, the environment of the early embryo has minimal impact on organ allometry at 27 weeks of age.

4.5 Conclusions

From the results reported within this chapter, the environment in which the embryo develops within the first 3.5 days can affect aspects of postnatal physiology and development of the offspring produced. It would appear that whilst birth weight is influenced by litter size, subsequent growth rates, blood pressure, organ ratios and weights, and serum ACE activity are not. It would, therefore, seem that exposure to an *in vitro* environment during preimplantation development can affect parameters of postnatal development in particular growth patterns and cardiovascular physiology. Exposure to an *in vitro* environment for a relatively short period can have some of the same long term effects as those observed from prolonged culture. This would imply that the preimplantation mouse embryo is particularly sensitive to its surroundings, and that perturbations to the normal patterns of development can have long term consequences.

From the results reported within this chapter, it would be of interest to investigate the differences in blood pressure further. It would be of interest to examine the activity of ACE within the lung tissue and compare it with that within the serum. Alternatively, the expression profiles of proteins involved in blood pressure regulation such as type 1 angiotensin II receptor (AT1), mineralocorticoid receptor and the glucocorticoid receptor using western blotting techniques.

Studies examining the growth and development of an F2 generation derived from the mice studied within this chapter would reveal whether exposure to an *in vitro* environment results in transmission of the observed phenotypic effects into future generations. This could have major implications for numerous areas of mammalian embryos culture and manipulation.

Chapter 5

Effects of growth factors and other treatments during preimplantation development on postnatal life

5.1 Introduction

Embryos cultured in the presence of specific concentrations of insulin and IGF-1 are seen to develop at a faster rate, increase their levels of protein synthesis, and have a significantly increased total blastocyst cell number (Harvey and Kaye 1990, 1992; Palma *et al.*, 1997; Lighten *et al.*, 1998; Mihalik *et al.*, 2000). Upon the transfer and development of embryos cultured in the presence of insulin and albumin, fetal weight is seen to increase by 4-6% (Kaye and Gardner, 1999).

Studies have shown that the mouse preimplantation embryo is able to compensate for changes in its size and cell number, whilst still being able to develop into viable offspring (Rands, 1986a, 1986b; Somers *et al.*, 1992; Biggers and Papaioannou 1992; Hardy and Handyside, 1993). These studies have shown that embryos containing altered total numbers of cells remodel their development so as to compensate for the changes imposed upon them, so that at birth there is little difference between manipulated and control offspring. Any significant differences observed at birth were no longer present at 5 weeks of age (Biggers and Papaioannou 1992).

It is hypothesised that as insulin and IGF-I both caused a significant increase in ICM cell numbers within cultured blastocysts (section **3.2**), then these embryos may have an increased pool of cells from which the fetus will form. This may then produce an increased rate of fetal growth and subsequently impact upon aspects of postnatal growth and development. Those embryos developing after being disaggregated and then reaggregated, whilst containing the correct number and ratios of cells, would have endured significant physical manipulation just prior to the formation of the ICM and TE lineages. Along with the effects of *in vitro* culture, the impact of manipulation may alter the patterns of postimplantation development within

the offspring developing from these embryos. As discussed in section **3.4.3.1**, the process of disaggregation and reaggregation at the pre-compact 8-cell stage could resulted the altered positioning of predisposed blastomeres within the embryo. Previous studies have demonstrated that the parental origin of the cells within the pre-compact 8-cell embryo influences future allocation to the ICM or TE lineages (Gardner, 2001; Piotrowska *et al.*, 2001). Disruption of this pattern by disaggregation and reaggregation may result in cells predisposed to ICM lineage allocation becoming re-positioned into regions of the embryo that would preferentially allocate cells to the TE lineage. As well as this, disruption to the already established embryonic-abembryonic axis could also have subtle effects on post-implantation or postnatal development.

Within the experiments reported within this chapter, five treatment groups of mice have been generated (See Table 5.1 for group terminology and a description of the embryo treatments). The first group of mice are derived from embryos cultured *in vitro* in T6 PVP media (referred to as 'PVP mice'). These will act as an *in vitro* embryo transfer control group to which the development of mice derived from embryos cultured in the presence of insulin or IGF-I in T6 PVP media (referred to as 'insulin' or 'IGF-I mice' respectively) can be assessed. These three groups of mice in tern will be analysed along-side a group of mice derived from natural mating who have their mean litter adjusted to a size of 4 (these mice will be termed 'naturally mated (4) mice' within this Chapter).

It is also of interest to compare the three groups of mice produced from natural mating. These are the two naturally mated groups from Chapter 4, and the group generated within this Chapter. This will enable the effect of litter size (the only variable between the three groups) on postnatal growth and development to be determined. As well as this, they have not been subjected to any other manipulation or treatment which could lead to any differences observed. For the purpose of this study, those mice which had no adjustment of their litter size (taken from chapter 4) are termed 'naturally mated' mice.

In a third study, the postnatal development of mice derived from embryos that were disaggregated and then reaggregated at the 8-cell stage (referred to as 'disaggregated embryo mice'; see Table 5.1) have also been studied. It was always the intention of this study to examine the postnatal development of mice derived form embryos disaggregated and reagggregated to contain half, double and the normal number of cells. Within this proposed study, those embryos containing the normal number of cells would have been the control group. However, due to the ongoing nature of this study, at this point in time only mice derived from embryos that were reaggregated to contain the normal number of cells have been generated. Therefore, in order to compare their postnatal development, they have been analysed along side the '*in vitro*', '*in vivo*' and 'naturally mated (6)' mice from Chapter 4. This is because the *in vitro* and *in vivo* mice were exposed to periods of *in vitro* culture, underwent embryo transfer and had a similar mean litter size (approximately 6). The naturally mated (6) mice were included as a non-embryo transfer control group, again with an appropriate mean postnatal litter size.

Therefore, the aim of the following experiments was to observe the postnatal growth, development and physiology of mice derived from the experimental and control groups described above. Due to the ongoing nature of these experiments, the number of animals within some of the treatment groups is low. This has meant that statistical analysis between certain groups has not been possible.

Table 5.1 Outline of the five groups of mice studied within these experiments, their embryonic treatments and whether they had their litter size corrected at birth

Group name	Treatment	Purpose of treatment/group	Derived from superovulated mothers	Embryo transfer	Litter size corrected at birth
'PVP mice'	Derived from embryos cultured in T6 PVP	Postnatal control for *in vitro* culture and embryo transfer	Yes	Yes	If necessary
'Insulin mice'	Derived from embryos cultured *in vitro* in T6 PVP and insulin	Examine the postnatal effects of *in vitro* culture in insulin	Yes	Yes	If necessary
'IGF-I mice'	Derived from embryos cultured *in vitro* in T6 PVP and IGF-I	Examine the postnatal effects of *in vitro* culture in IGF-I	No	Yes	If necessary
'Disaggregated embryo mice'	Derived from reaggregated 8-cell embryos	Examine the postnatal effects of embryo dissaggregation	Yes	Yes	If necessary
'Naturally mated (4) mice'	None	Control for superovulation, *in vitro* culture, embryo transfer and postnatal litter size	No	No	Yes (mean litter size of 4)

5.2 Methodology

5.2.1 Embryo culture and manipulation

Female F1 CBAxC57/BL6 mice were superovulated and mated with male MF1 mice (section **2.1.1**). 2-cell embryos were flushed and cultured in either T6 PVP (section **2.1.2**) or in T6 PVP containing 170 nM insulin or 1.7 nM IGF-I (section **2.1.3**), for approximately 45 hours, until the blastocyst stage. The insulin and IGF-I concentrations were chosen as these significantly increased ICM cell numbers and ICM:TE ratios (section **3.3.3** and **3.3.4**). It was also decided to culture using 170 nM insulin or 1.7 nM IGF-I to minimize any possible side effects that higher concentrations may produce.

2-cell embryos were flushed and cultured in T6 BSA until the 8-cell stage (section **2.1.2**). Pre-compact 8-cell embryos were disaggregated to single blastomeres and pooled. Groups of 8 blastomeres were reaggregated so as to create embryos of

normal size and cell number (section **2.1.4**). These embryos were then cultured in T6 BSA until the blastocyst stage (section **2.1.2**).

5.2.2 Embryo transfer

Immediately prior to their transfer, embryos were removed from their *in vitro* culture conditions and placed into pre-warmed H6 BSA or H6 PVP (depending on the type of manipulation performed on the embryos) under mineral oil, and kept at 37°C. This was to buffer against any pH changes until the embryos were transferred (section **2.2.2**).

The ovaries and top 1cm of the uterus of anaesthetized 3.5 day pseudo pregnant female F1 CBAxC57/BL6 were carefully exposed from the body cavity. Six blastocysts were then transferred to each uterine horn (section **2.2.2**).

For the production of the naturally mated (4) group, female F1 CBAx C57/BL6 mice were naturally mated with MF1 studs and allowed to develop to term. The offspring produced would not have been exposed to the embryo transfer procedure, or to a superovulated uterine environment. It was also decided to adjust the litter size at birth to a size comparable with that of the experimental groups (adjusted to a litter size of 4, 2 males and 2 females). This was so that these mice would have the same availability to postnatal maternal nutrient as those of the experimental groups, and to remove any possible effects caused by the size of the litter. Any litters derived from transferred embryos comprising of more than 6 animals at birth were corrected to a mean litter size of 6 (3 males and 3 females where possible).

5.2.3 Measurement of postnatal growth rates

Mice were weighed on day of birth and then subsequently on the same day for the next 27 weeks (section **2.3.1**).

5.2.4 Measurement of systolic blood pressure

Systolic blood pressure was determined at 8, 15 and 21 weeks of age by tail-cuff plethysmography (section **2.3.2**).

5.2.5 Measurement of organ weights

Organs were dissected out of the mice at approximately 27 weeks of age. Organs were weighed before being snap frozen in liquid nitrogen, and stored at -80^{0}C (section **2.3.3**). Blood samples were collected by vacuum puncture of the heart using a pulled glass pipette (internal diameter ~1 mm) attached to a mouth pipette, and then centrifuged at 10,000 g, 4°C for 10 minutes, after which the serum was aliquoted into 10µl samples and frozen at -80°C.

5.2.6 Statistical analysis of data

A Bonferroni one way analysis of variance t-test was used to analyse the litter size from the different treatment groups (SigmaStat statistical software, version 2.0).

The correlation between birth weight and litter size was assessed for normality using the SigmaStat statistical software package (version 2.0). As the distribution failed the normality test, the correlation was analysed using a Spearmans correlation.

Data for animal weights, blood pressure, organ ratios and weights and serum ACE activity were analysed using a multilevel 'random effects' regression model which took into account between-mother and within-mother variation in litter size and parameters measured from individual animals (section **2.4**). Statistical significance was assumed at $p \leq 0.05$. A trend was assumed to occur if a p value between 0.1 and 0.051 was observed.

5.3 Results

5.3.1 Effect of litter size on the postnatal growth and development of offspring from natural mating

The mean litter size at birth of the three of naturally mated groups are shown in Figure 5.1. There are no significant differences between any of the three groups. The mean birth weight of offspring from the three naturally mated groups, before litter size correction, is shown in Figures 5.2 and 5.3. The naturally mated (6) males have an elevated birth weight when compared to the naturally mated (4) males ($p < 0.001$, Figure 5.2). Figure 5.3 shows that both the naturally mated and naturally mated (6) females are significantly heavier at birth than the naturally mated (4) females ($p < 0.05$).

Figure 5.1

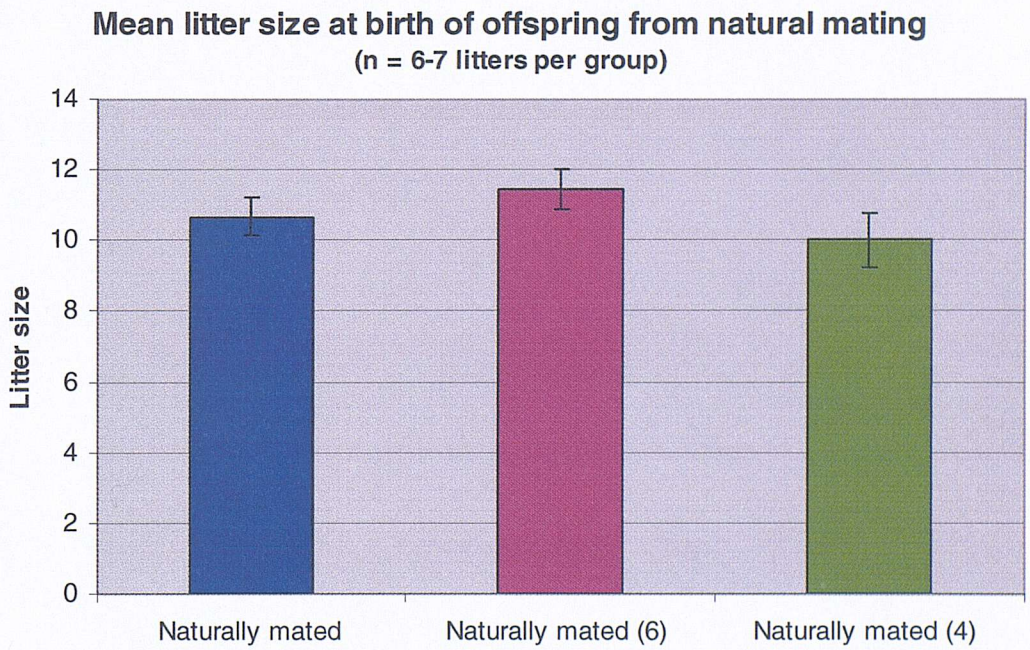


Figure 5.2

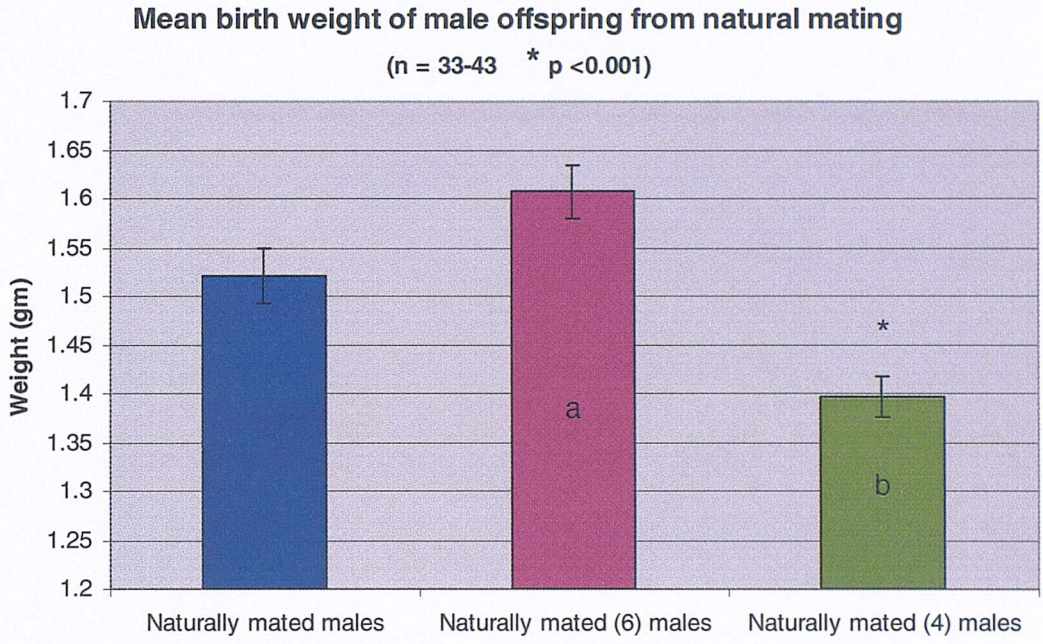
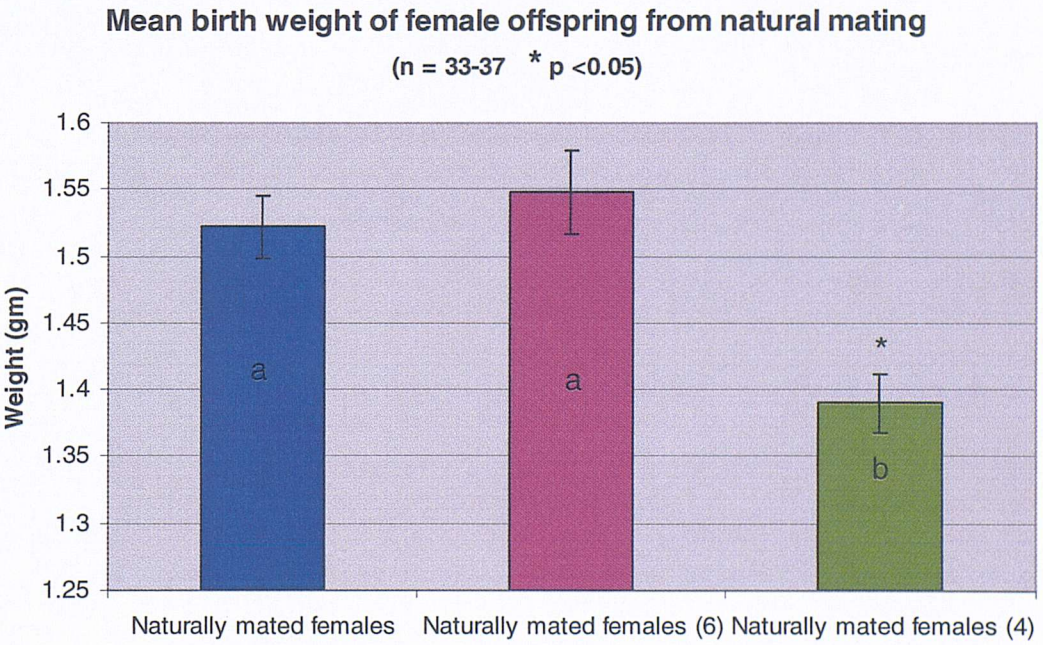


Figure 5.3



The mean weekly weights of the three naturally mated groups from birth to 27 weeks of age are shown in Figures 5.4 to 5.7(mean ± standar error). Each mouse was weighed on its day of birth and then subsequently every week for the next 27 weeks. All the weights for the mice from each treatment at each week have been averaged and plotted along with the standard error. The data at zero weeks of age represents data presented within Figures 5.2 and 5.3, therefore, incorporating weights of mice that were subsequently removed from the study during the standardising of litter sizes. The same pattern of weight gain and growth is observed as within Figures 4.5 and 4.6. All mice undergo a dramatic growth spurt between 2 and 5 weeks of age, during which weight gain per week is approximately 2- 3 times that occurring during previous and subsequent weeks. After 7 weeks of age, the increase in weight plateaus, such that by approximately 11 weeks of age they are only increasing their weight by on average 1 gram per week.

At 2, 3 and 4 weeks of age, the naturally mated (6) and (4) males and females both become significantly heavier than the naturally mated males and females ($p < 0.015$). Despite the naturally mated males and females remaining lighter for the remainder of the study, at no other time are the three groups significantly different in their mean weight.

Figure 5.4

Mean weekly weight of male offspring from natural mating
(n = 14-43 per treatment)

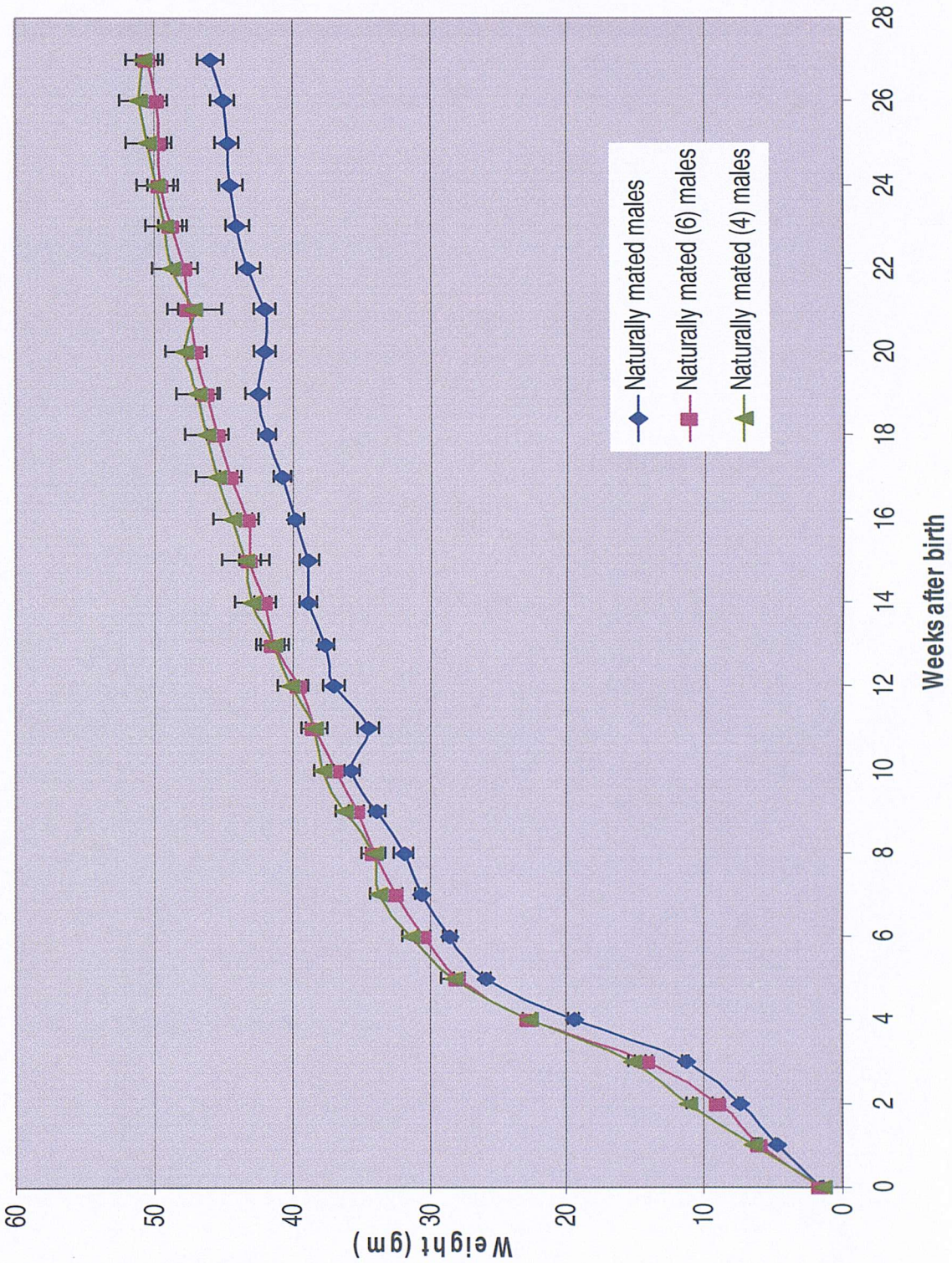


Figure 5.5

Mean weekly weight of female offspring from natural mating
(n = 14-37 per treatment)

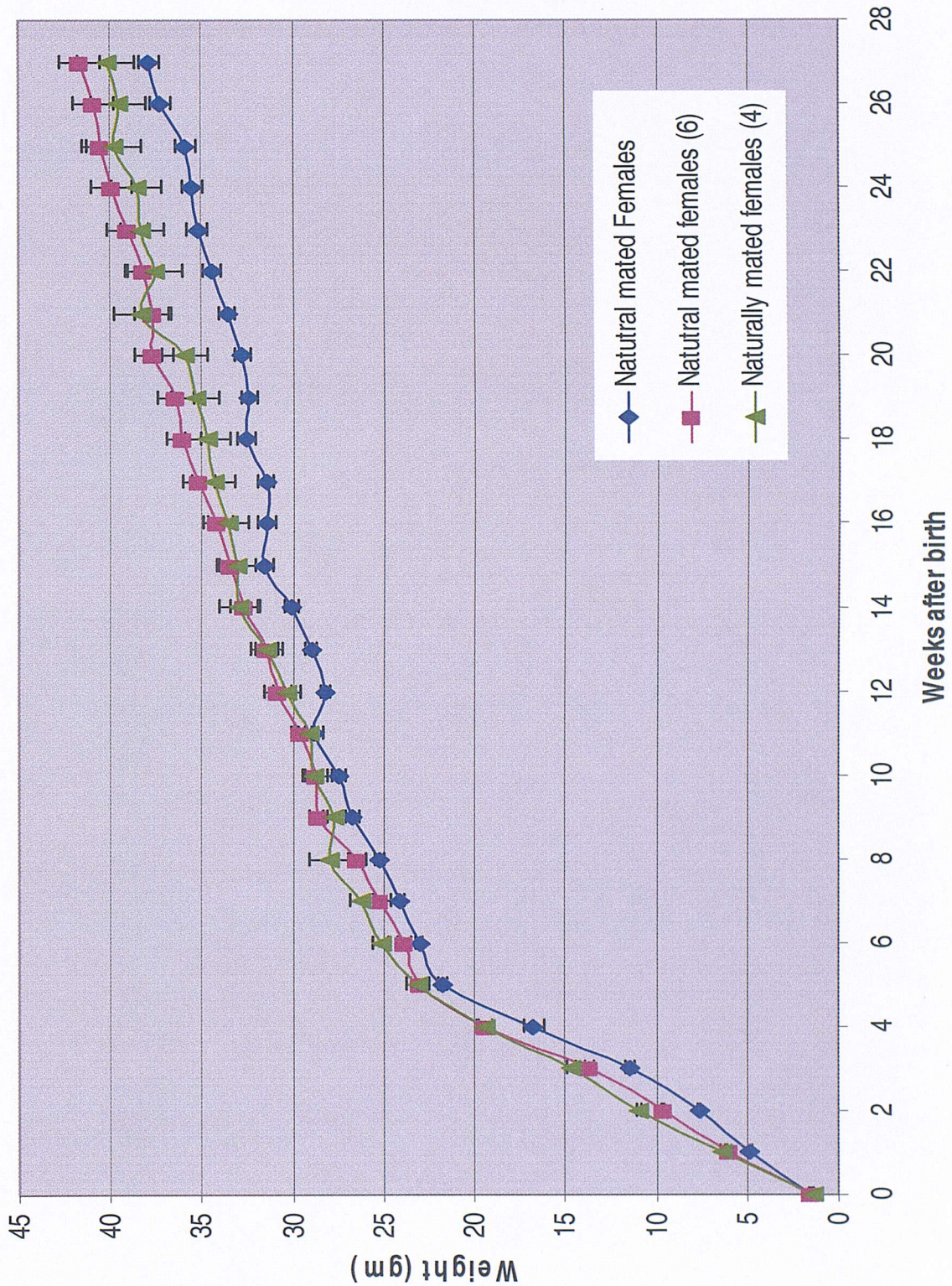


Figure 5.6

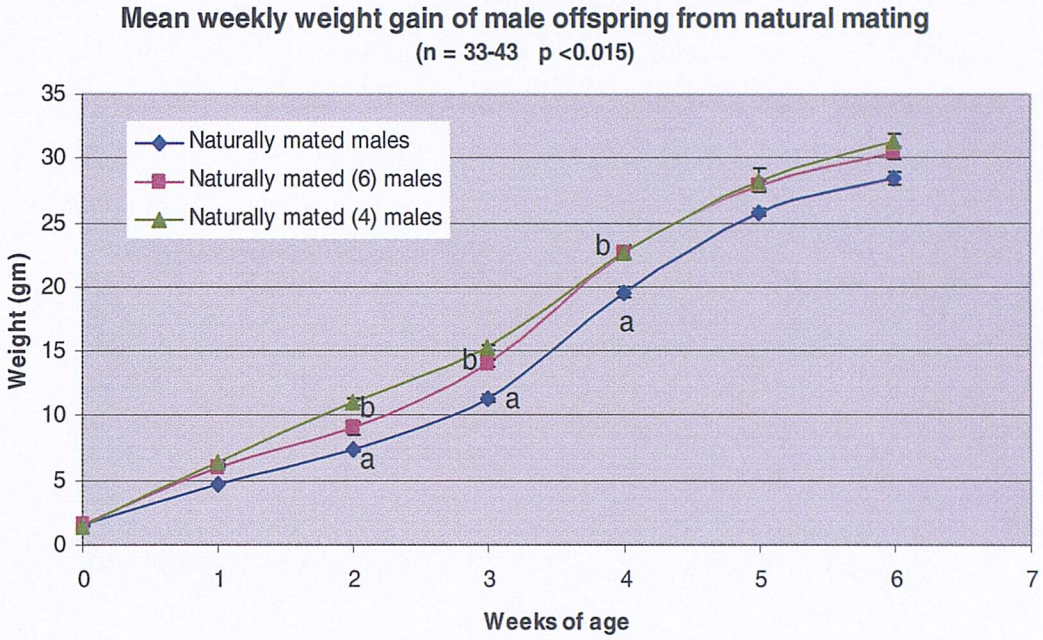
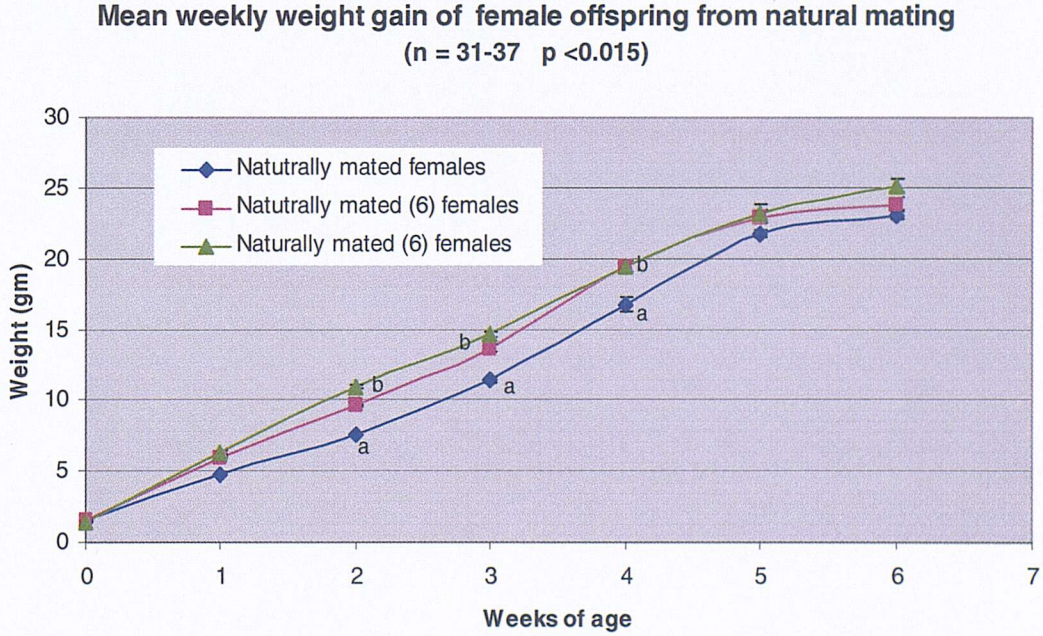


Figure 5.7



The mean systolic blood pressure of the three naturally mated groups at three ages (8, 15 and 21 weeeks of age) are shown in Figures 5.8 and 5.9. At 8 weeks of age, both naturally mated (6) and (4) males and females have significantly elevated systolic blood pressure when compared to the naturally mated males and females. At 15 and 21 weeks of age, there are no significant differences between any of the naturally mated groups for either the males or the females.

Figure 5.8

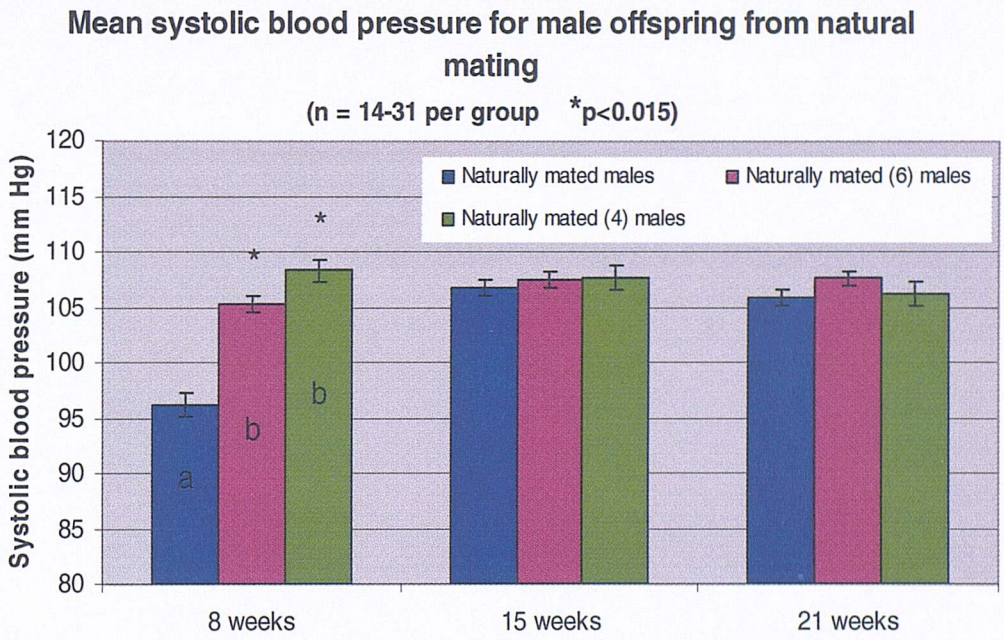
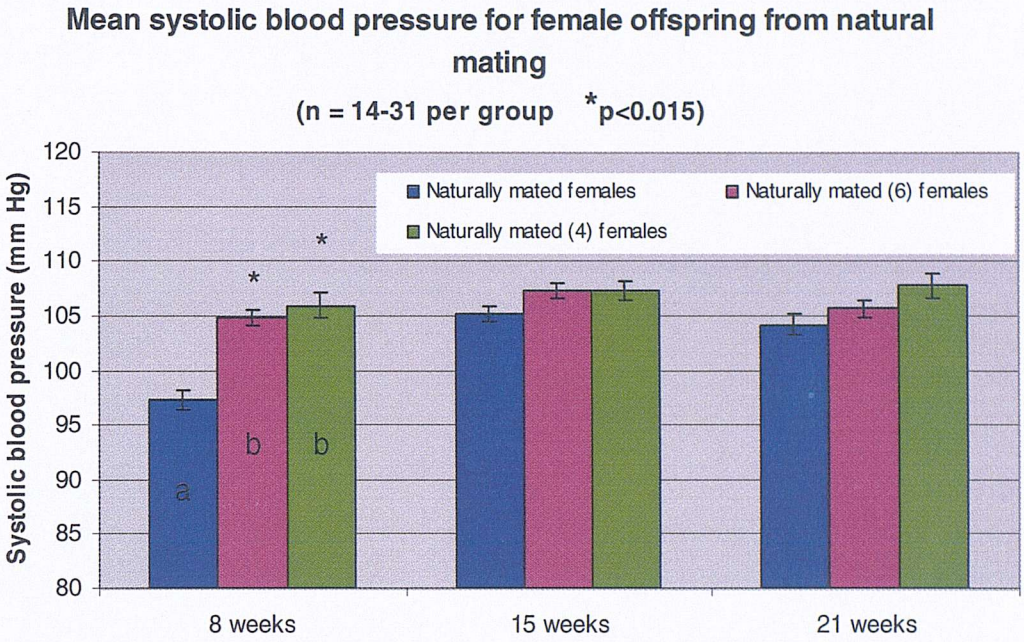


Figure 5.9


Mean systolic blood pressure for female offspring from natural mating
(n = 14-31 per group *p<0.015)
Systolic blood pressure (mm Hg)
120
115
110
105
100
95
90
85
80
Naturally mated females
Naturally mated (6) females
Naturally mated (4) females
*
*
a
b
b
8 weeks
15 weeks
21 weeks

The mean organ:body weight ratio and mean organ weights from the three naturally mated groups are shown in Figures 5.10 to 5.13. Figure 5.10 shows that the naturally mated (4) males have a significantly lower lung:body weight ratio when compared to the naturally mated (6) males. There are no other significant differences between the three naturally mated male groups.

Figure 5.11 shows that the naturally mated (4) females have a significantly lower liver:body weight ratio when compared to the naturally mated (6) females. There are no other significant differences between the three naturally mated female groups.

When organ weight is analysed independently of body weight, both the naturally mated (6) and (4) males have a significantly heavier liver when compared to the naturally mated males ($p = 0.003$, Figure 5.12). The naturally mated (6) females have a significantly heavier liver when compared to the naturally mated females, whilst the naturally mated (4) females have a significantly heavier heart weight when compared to the naturally mated females ($p < 0.03$, Figure 5.13). It is observed that body weight has a significant influence upon organ weight ($p < 0.001$).

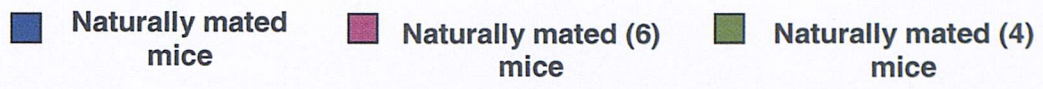


Figure 5.10

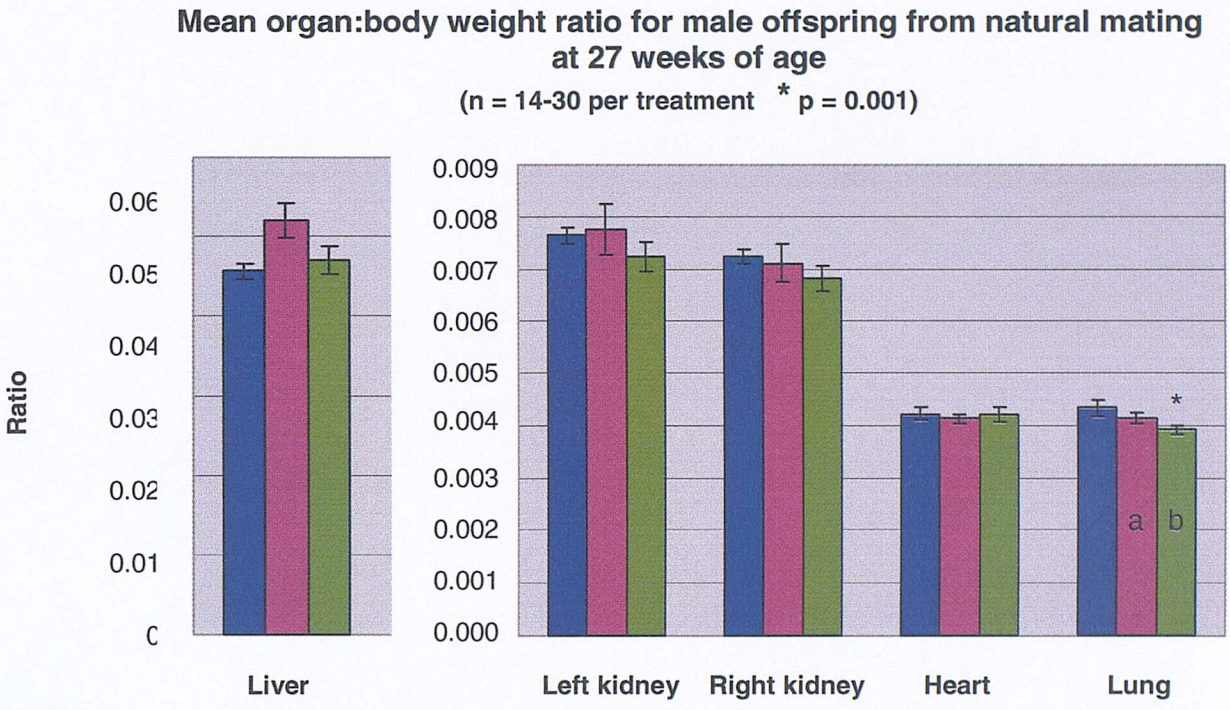


Figure 5.11

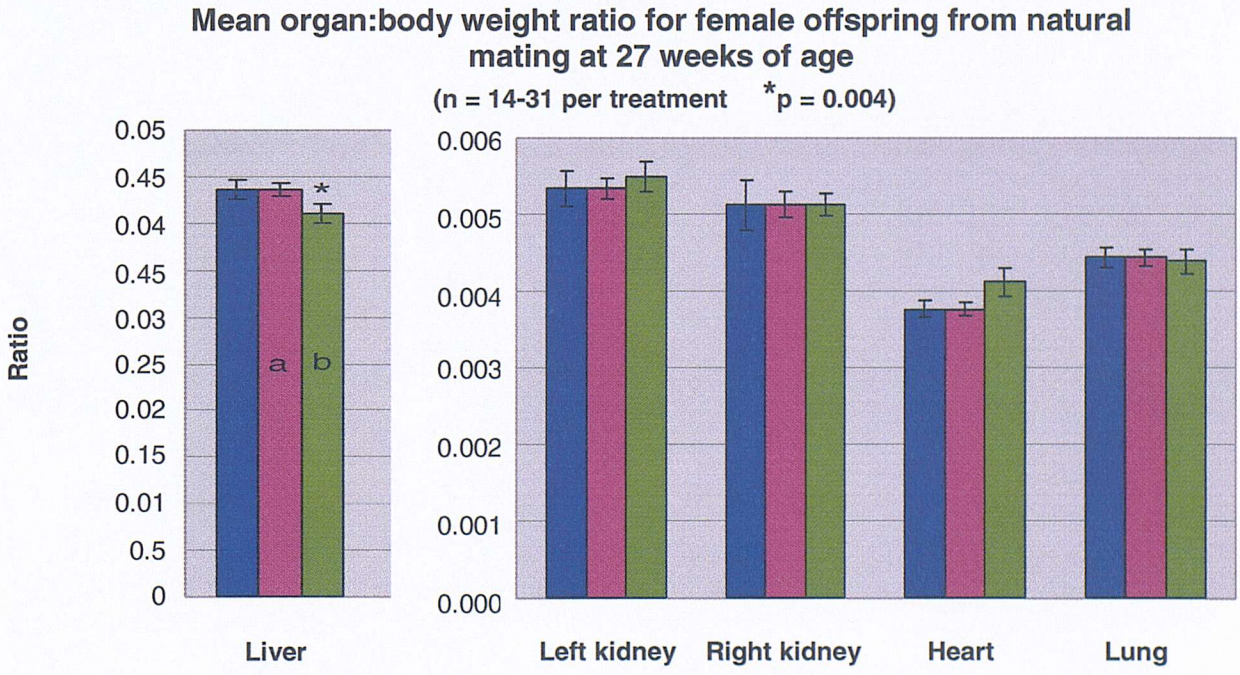


Figure 5.12

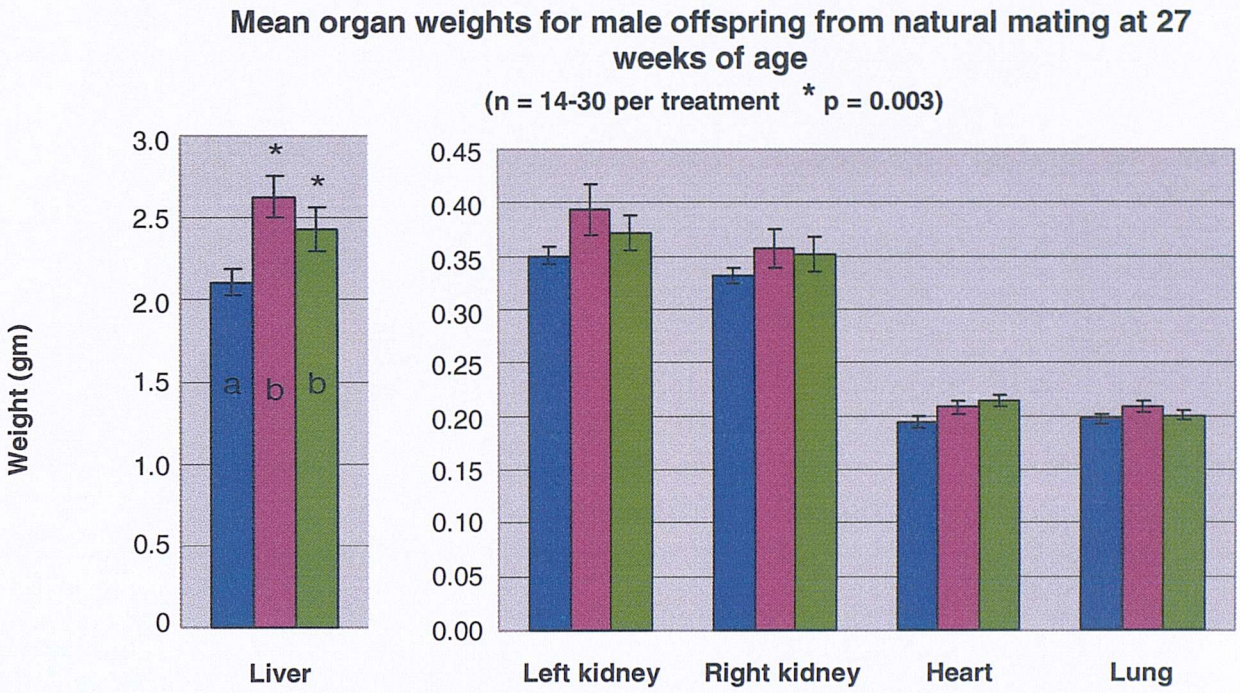
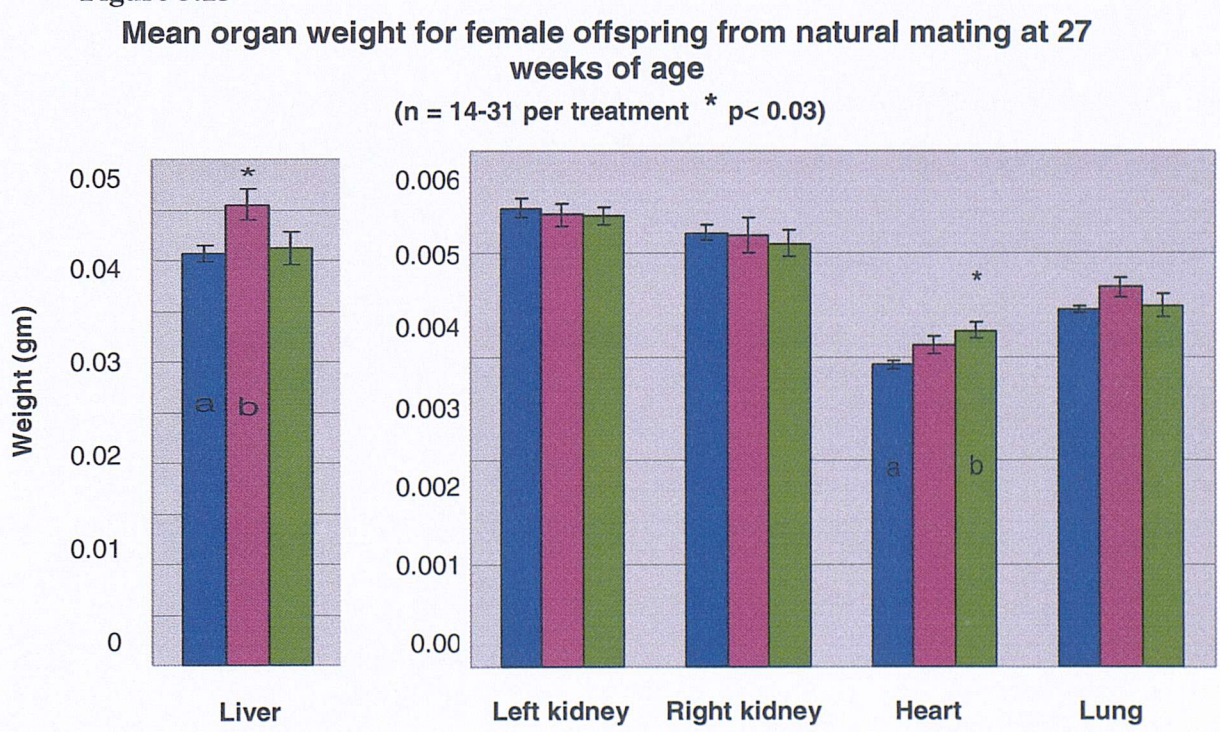


Figure 5.13



5.3.2 Effect of embryo culture in the presence of growth factors on postnatal growth and development

The mean litter size of offspring developing from embryos cultured in exogenous growth factors, T6 PVP medium or from natural mating are shown in Figure 5.14. For offspring derived from embryo transfer procedures, the mean litter size ranged from two to six (number of litter shown in bars of the Figure). Although the mean litter size for the naturally mated group was ten, after birth the mean litter size was adjusted to 4 offspring (values shown in Figure 5.1), so as to be similar to the mean litter size of the embryo transfer treatment groups. There are no significant differences between the mean litter size of offspring from the embryo transferred treatment groups and those of the naturally mated (4) group after adjustment.

Figures 5.15 and 5.16 show that there are no significant differences in birth weight, for either the males or the females, for any of the treatment groups before correction (number of offspring per treatment group is shown in the bars of each Figure). However, the insulin and IGF-I males and females have the highest birth weights out of all the groups, although these values did not reach statistical significance.

As observed in Figure 4.4, a negative correlation exists between birth weight and litter size (Spearman correlation r = -0.892, Figure 5.17, number of animals per treatment group at birth is shown in brackets). It can be seen that the mice from the largest litter (mice derived from naturally mated mothers, litter size of 12) have the lowest mean birth weight, whilst those from the smallest litter (PVP mice, litter size of 1) have the largest mean birth weight.

Figure 5.14

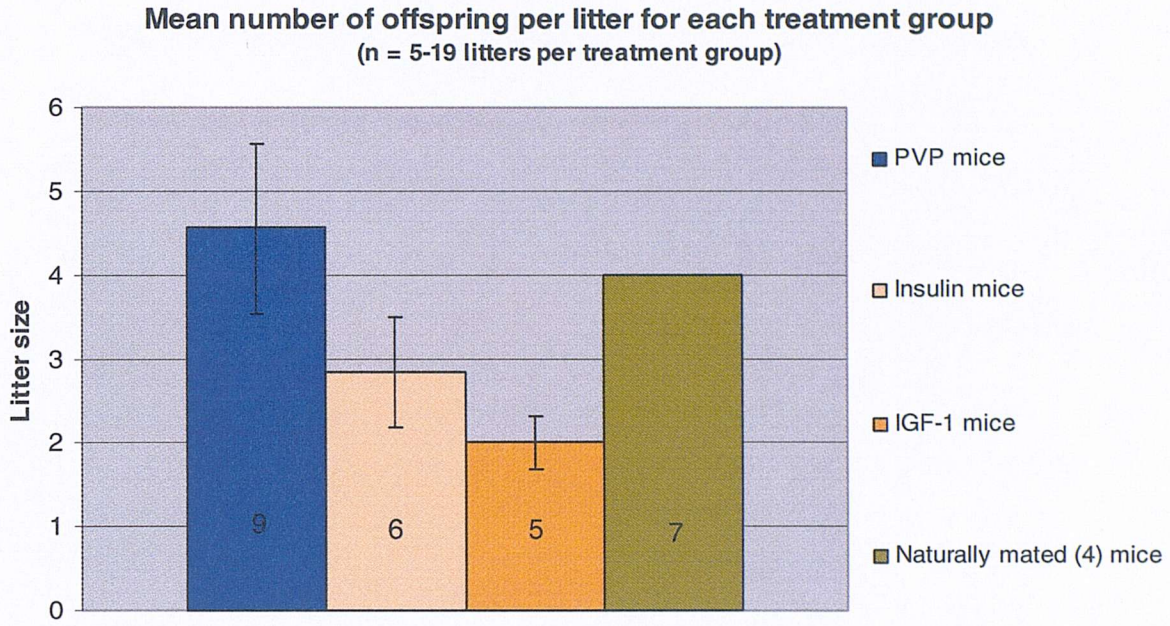


Figure 5.15

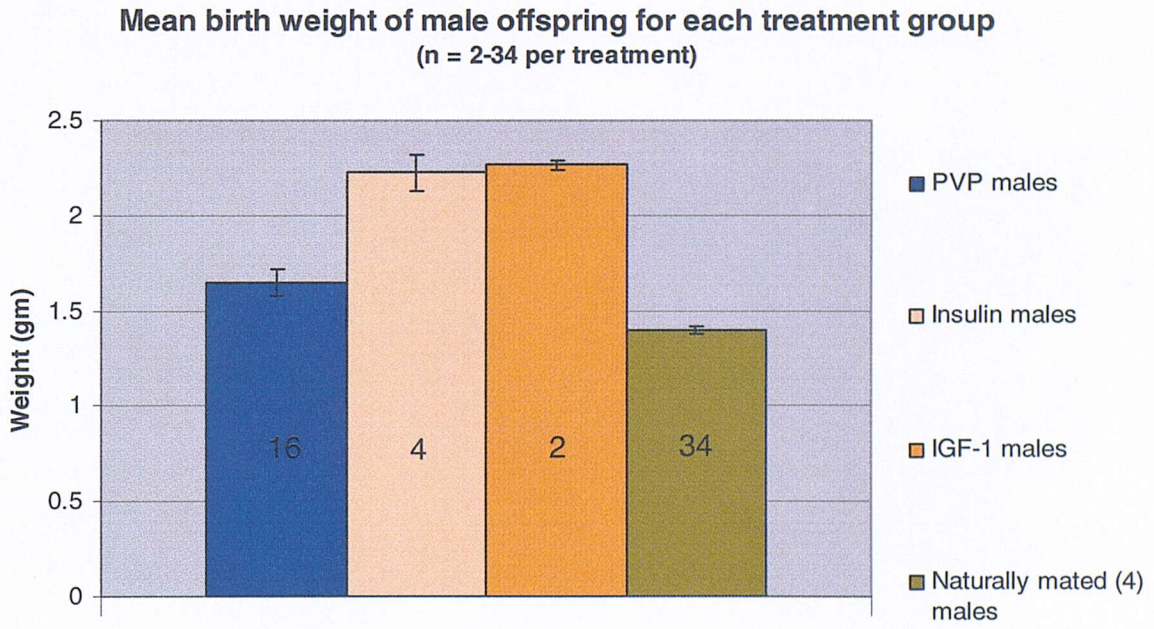
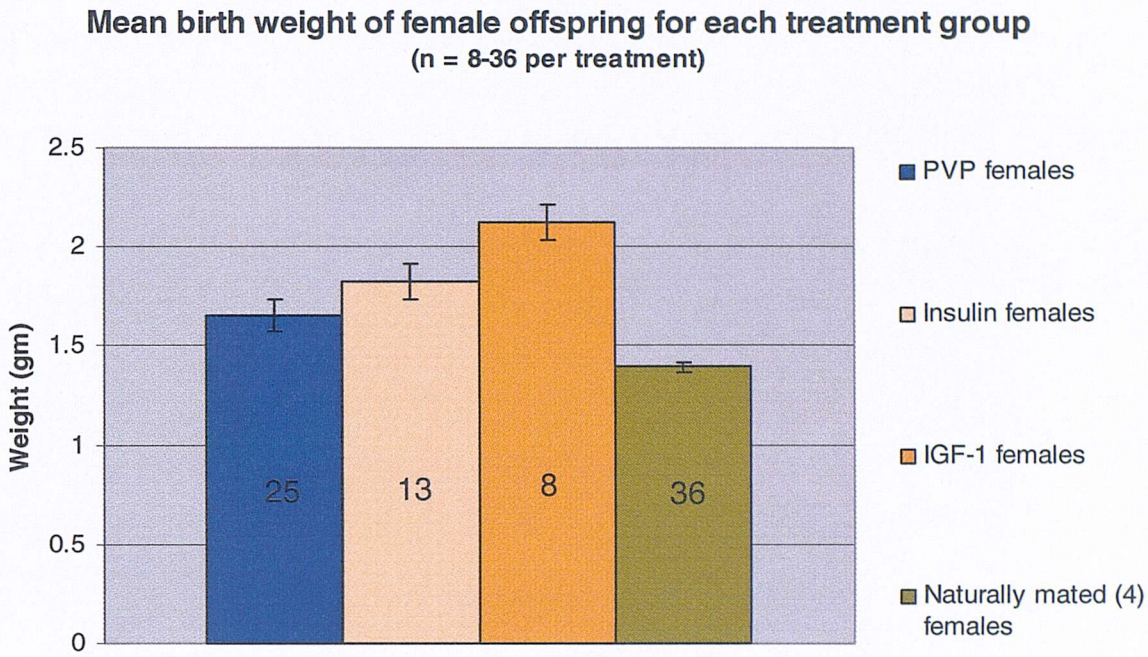
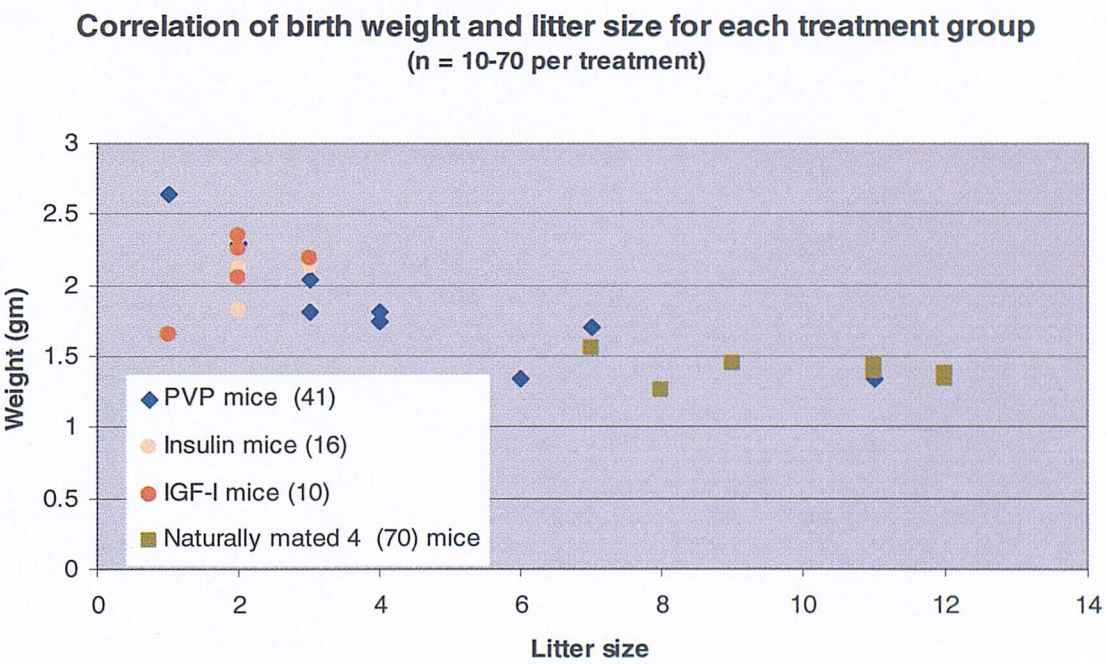


Figure 5.16



Figures 5.17



The mean weekly weights of the five treatment groups studied within this study from birth to 27 weeks of age are shown in Figures 5.18 and 5.19 (mean ± standar error). Each mouse was weighed on its day of birth and then subsequently every week for the next 27 weeks. All the weights for the mice from each treatment at each week have been averaged and plotted along with the standard error. The data at zero weeks of age represents data presented within Figures 5.15 and 5.16, therefore, incorporating weights of mice that were subsequently removed from the study during the standardising of litter sizes As in Figures 4.5 and 4.6, it can be seen that all the mice undergo a dramatic growth spurt between 2 and 5 weeks of age. During this time, weight gain per week is approximately 2-3 times that occurring during previous and subsequent weeks. After 7 weeks of age, the increase in weight begins to plateau, such that by approximately 11 weeks of age they are only increasing their weight by on average 1 gram per week.

At 2 weeks of age, the PVP males are significantly lighter than the naturally mated (4) males. At 10, 12 and 13 weeks of age, the IGF-I males are significantly lighter than the naturally mated (4) males ($n = 2$, $p < 0.05$). At 1, 2, 3 and 13 weeks of age, the PVP females are significantly lighter than the naturally mated (4) females ($p < 0.05$).

Figure 5.18

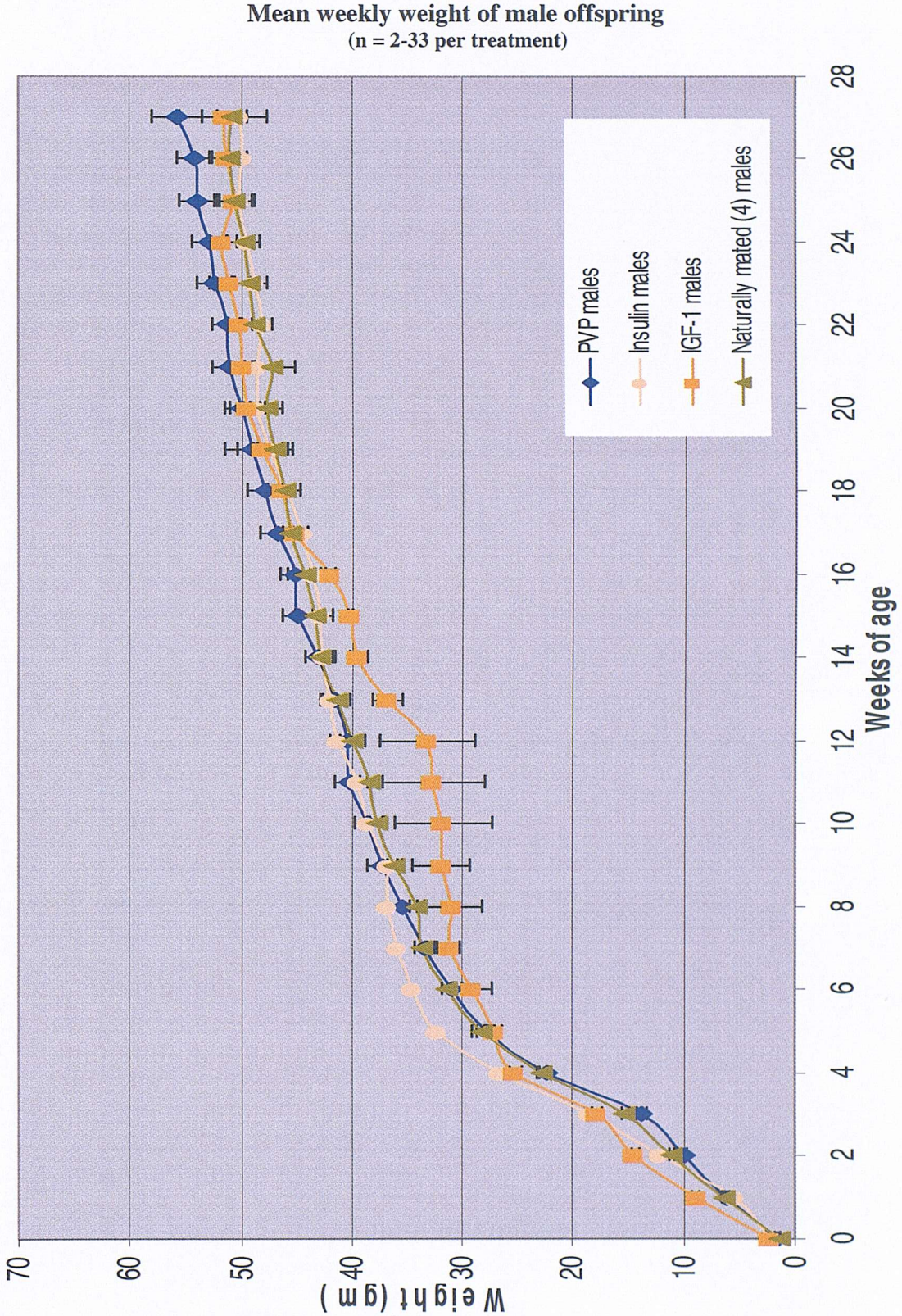
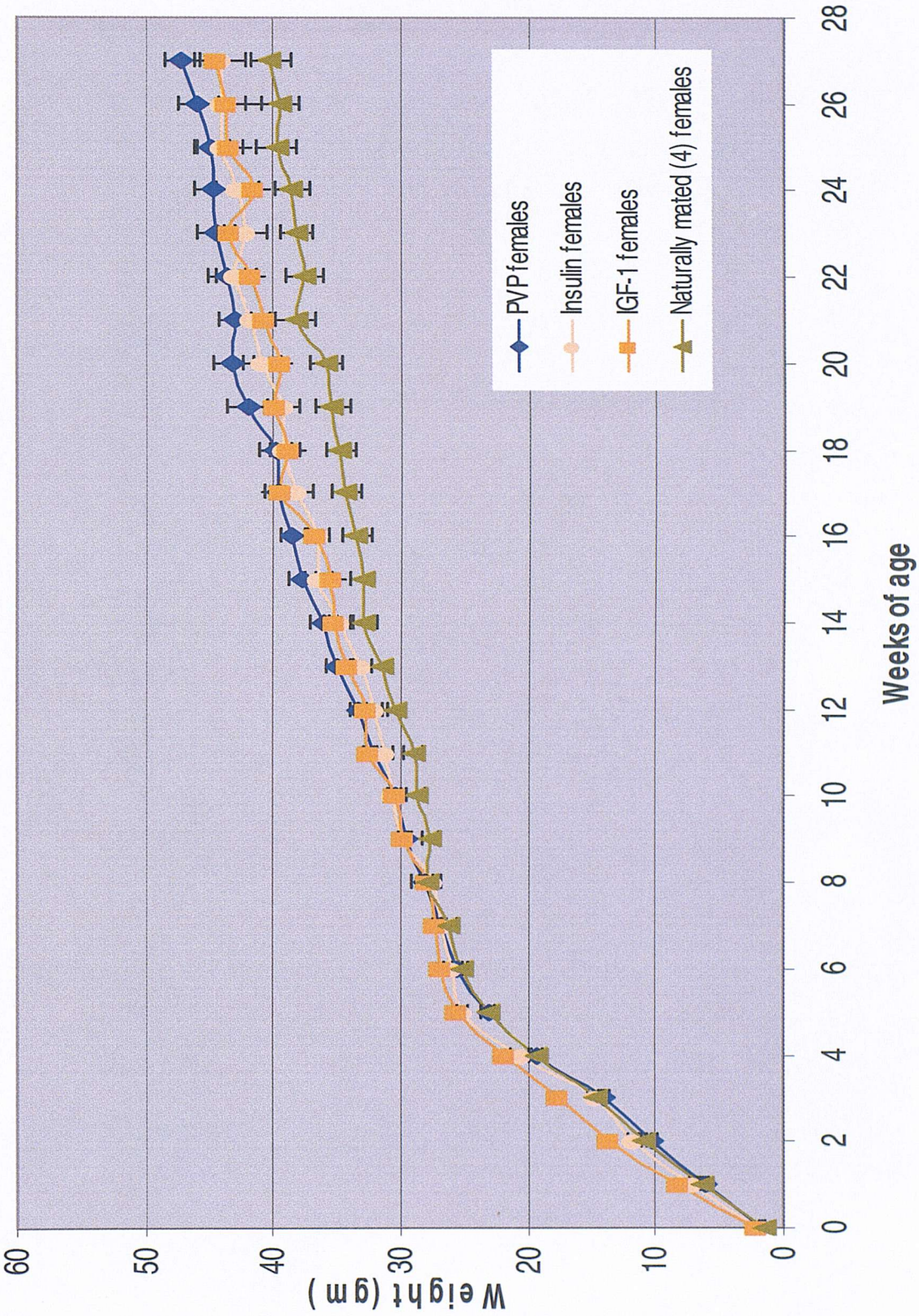

Mean weekly weight of male offspring
(n = 2-33 per treatment)
Weight (gm)
0
10
20
30
40
50
60
70
Weeks of age
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
PVP males
Insulin males
IGF-1 males
Naturally mated (4) males

Figure 5.19

Mean weekly weight of female offspring
(n = 7-31 per treatment)



The mean weight gain per week of the five different treatment groups during the first ten weeks of age are shown in Figures 5.21 and 5.22. Figure 5.21 shows that whilst the IGF-I males have the highest weight gain at 1 week of age, they subsequently have the lowest weight gain during weeks 5 and 6. It can also be seen that at 2 and 3 weeks of age, the insulin males have the highest weight gain for all of the male groups. Therefore, during the first three weeks of age, those mice derived from embryos cultured in the presence of growth factors have the highest mean weight gains.

Figure 5.22 shows a similar pattern for the female mice, in that at 1 and 2 weeks of age, the IGF-I females have the highest weight gain with the insulin females having the second highest weight gain. At 4 weeks of age it can be seen that the IGF-I females have the lowest weight gain for any of the groups, whilst at 5 weeks of age, the insulin females have the highest weight gain.

Figure 5.20

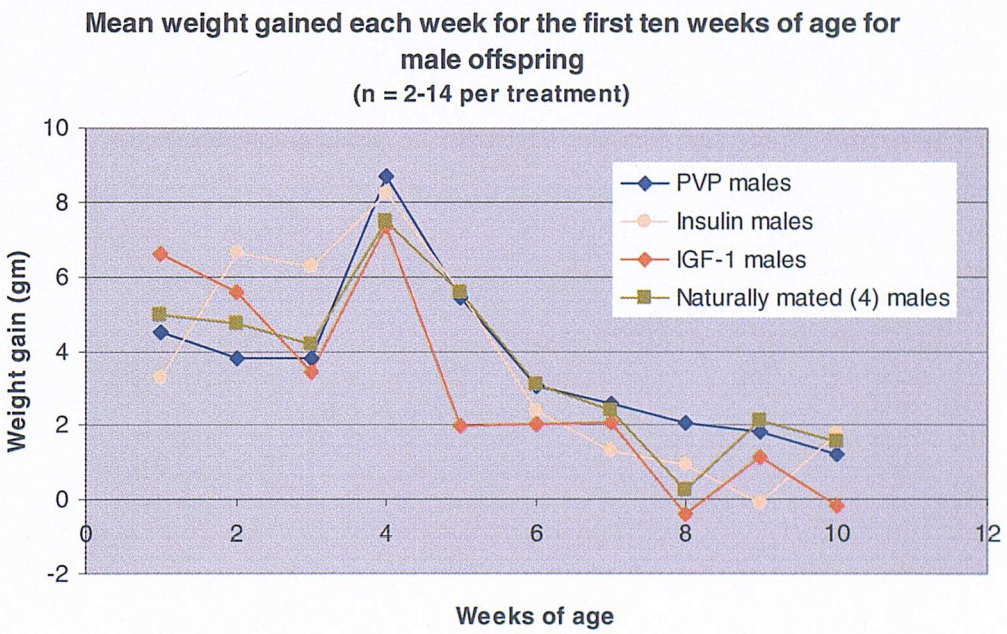
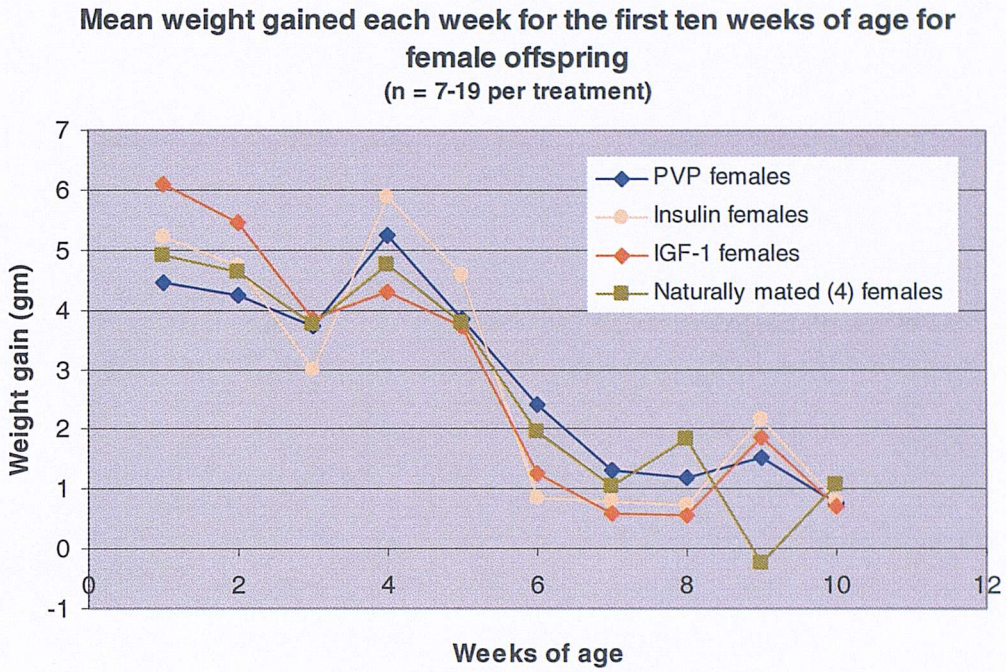


Figure 5.21


Mean weight gained each week for the first ten weeks of age for female offspring
(n = 7-19 per treatment)
Weight gain (gm)
7
6
5
4
3
2
1
0
-1
0
2
4
6
8
10
12
Weeks of age
PVP females
Insulin females
IGF-1 females
Naturally mated (4) females

The mean systolic blood pressure of the four different treatment groups studied are shown in Figures 5.23 and 5.24. At 8, 15 and 21weeks of age, there are no significant differences between any of the male treatment groups (Figure 5.23).

Figure 5.24 shows that for the female groups, there is no difference in blood pressure at 8 or 15 weeks of age. At 21 weeks of age, the IGF-I and the PVP females have a significantly elevated systolic blood pressure when compared to the naturally mated (4) females.

Figure 5.22

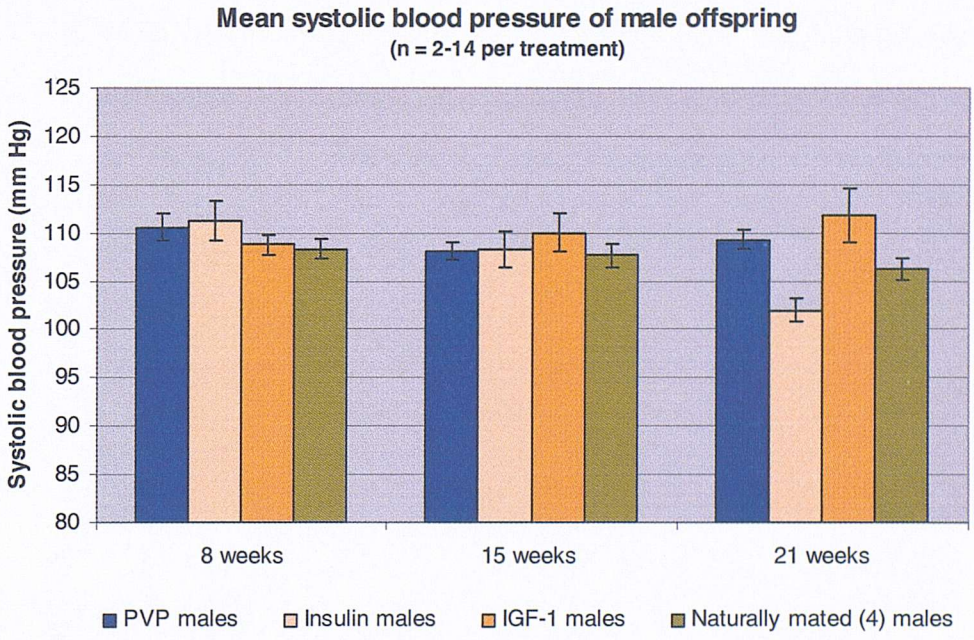
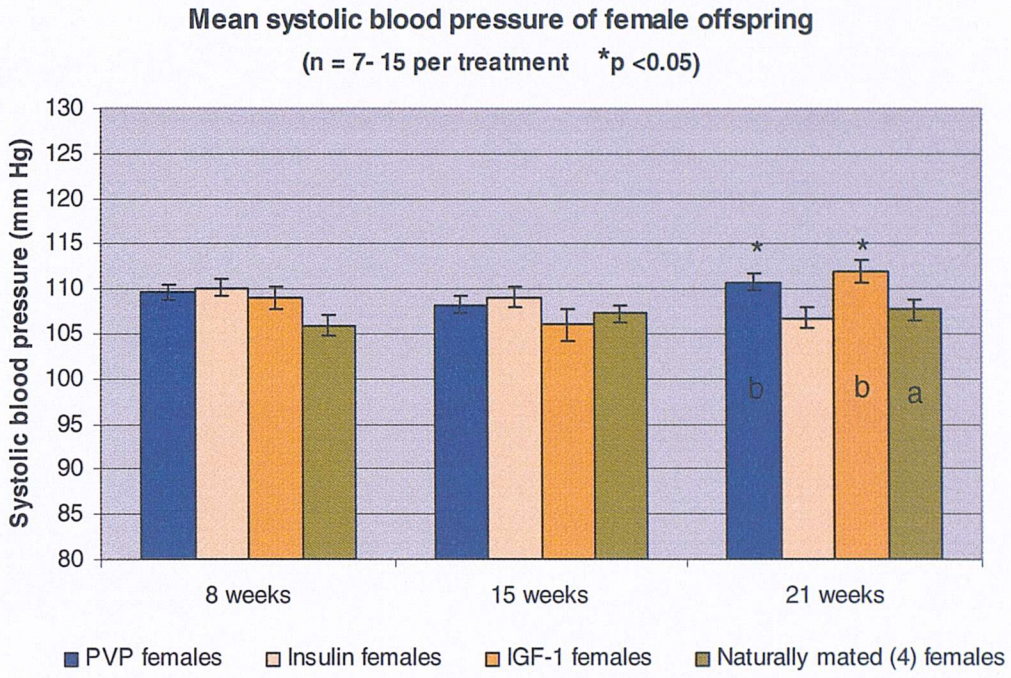


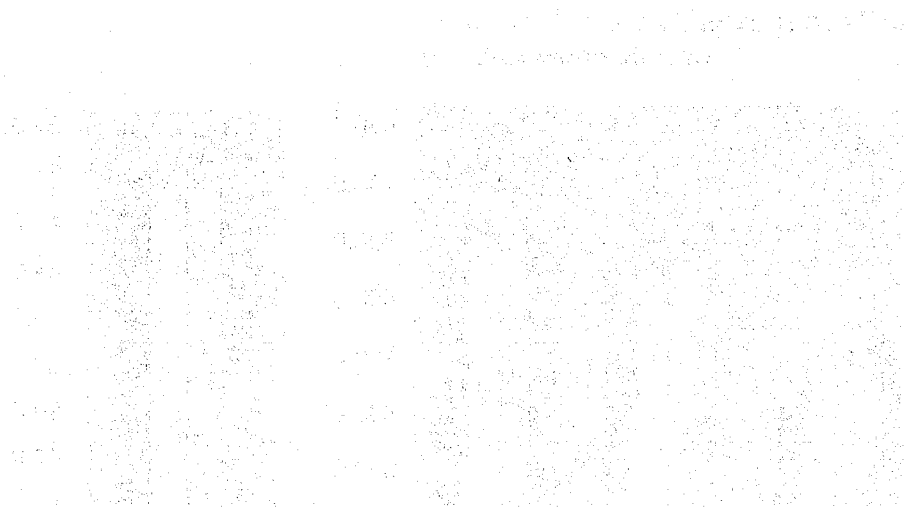
Figure 5.23


Mean systolic blood pressure of female offspring
(n = 7- 15 per treatment *p <0.05)
Systolic blood pressure (mm Hg)
130
125
120
115
110
105
100
95
90
85
80
*
*
b
b
a
8 weeks
15 weeks
21 weeks
PVP females
Insulin females
IGF-1 females
Naturally mated (4) females

The mean organ:body weight ratio and mean organ weight from the different treatment groups studied are shown in Figures 5.24 to 5.27. Figure 5.24 shows althought not significant, the insulin males have the highest ratio for the liver, both of the kidneys, the heart and the lungs.

As observed in Figure 5.25, the IGF-I females have elevated ratios for all the organs studied, although, as with the male mice, these do not reach statistical significance.

There are no significant differences between any of the groups studied when organ weight is analysed independently of body weight (Figures 5.26 and 5.27) for either the males or the females. Figure 5.27 shows that the IGF-I females have the heaviest liver, left and right kidney and lungs.



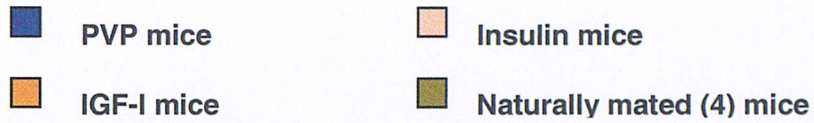


Figure 5.24

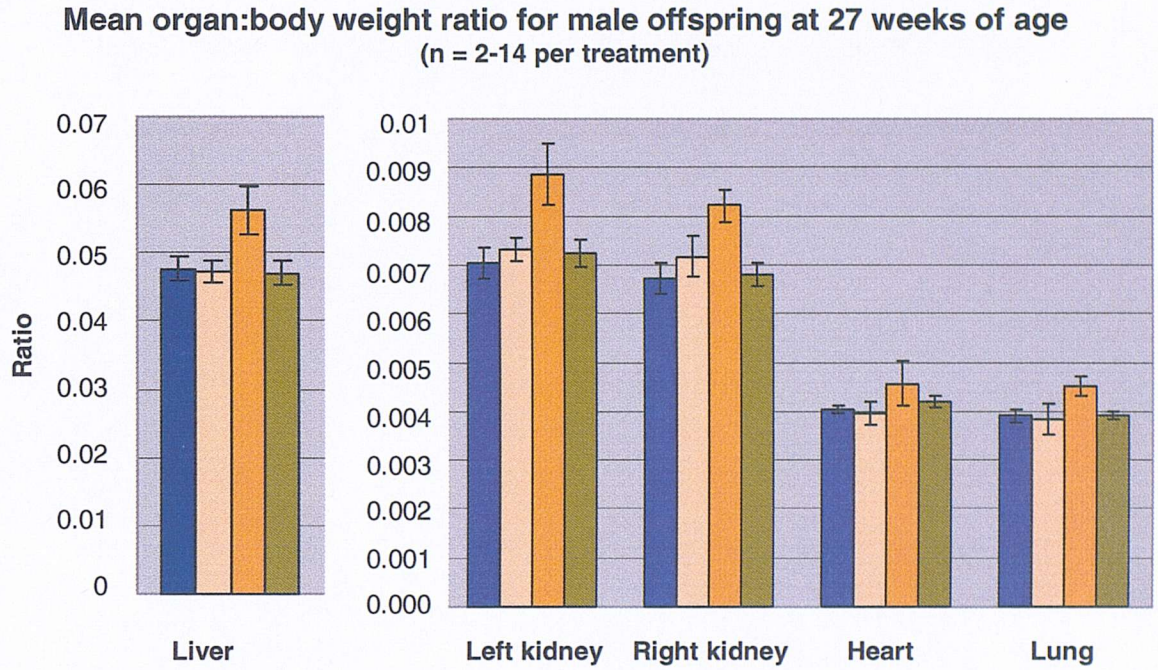
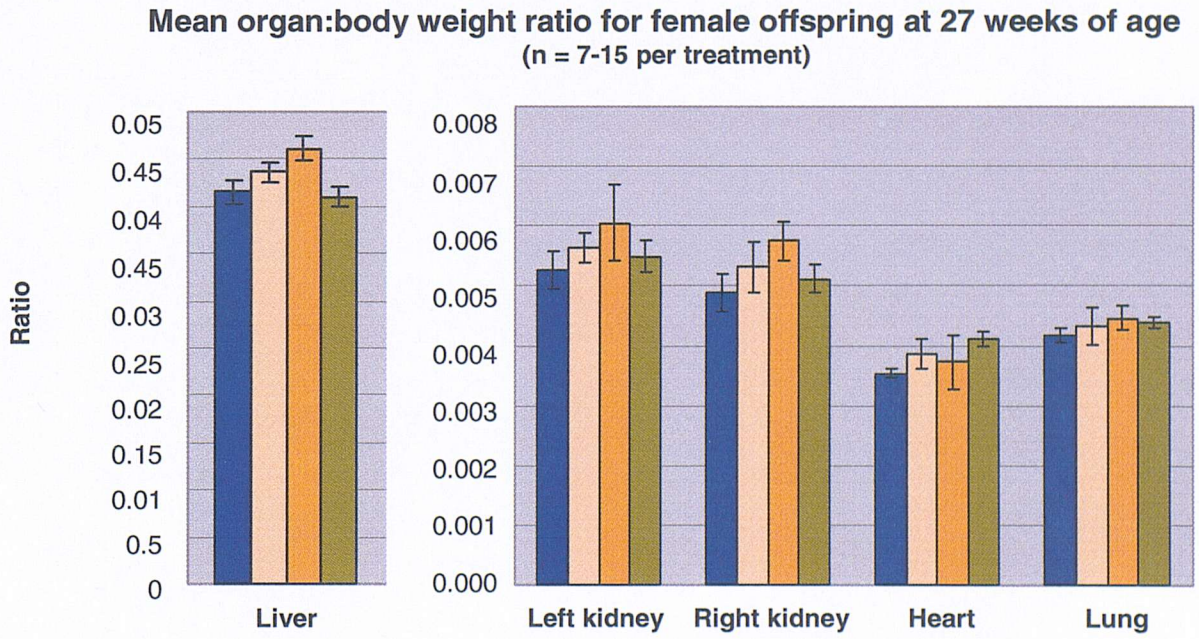


Figure 5.25



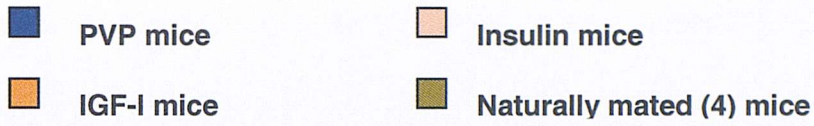


Figure 5.26

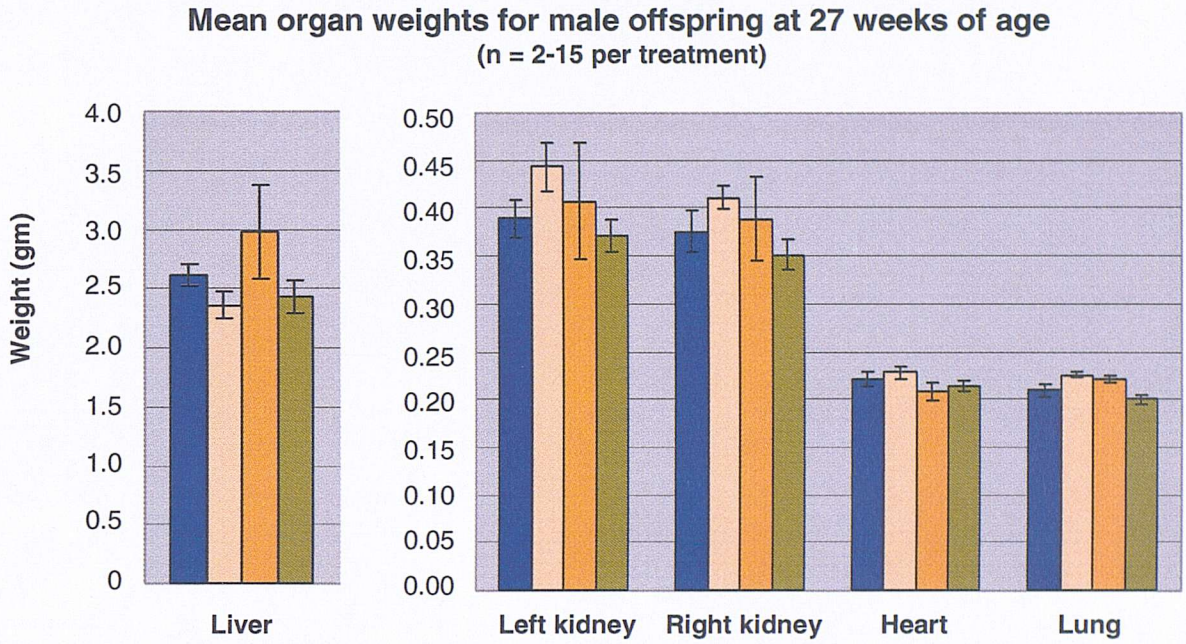
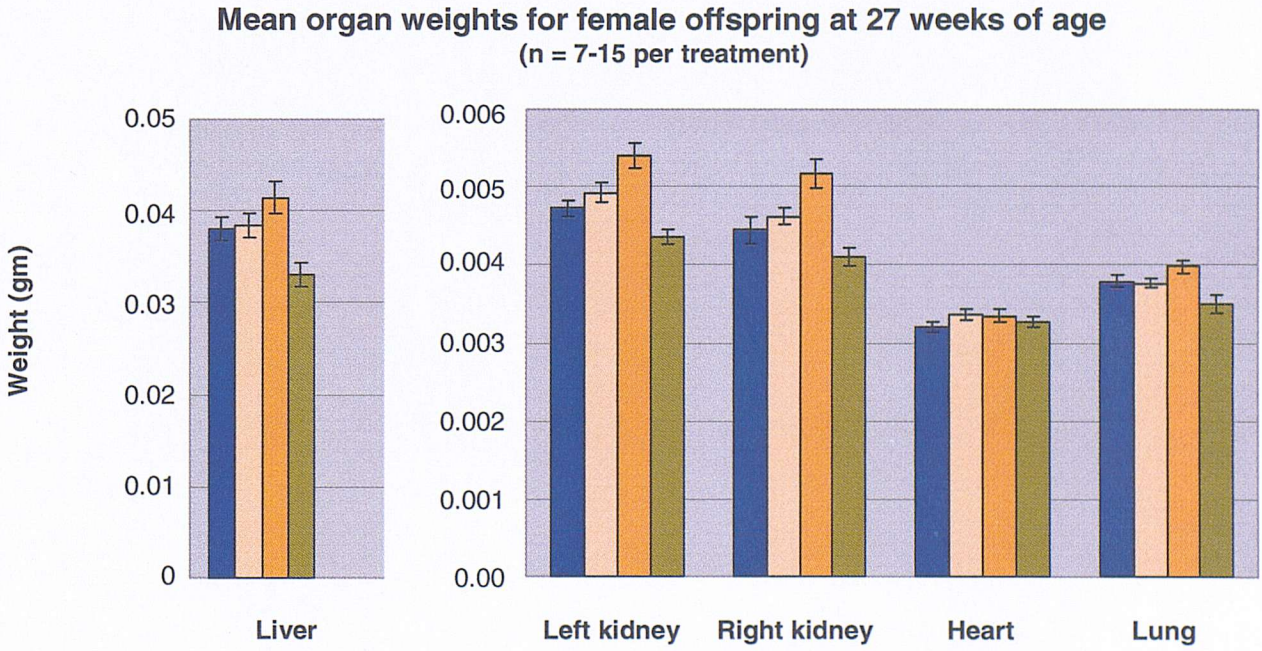


Figure 5.27



5.3.3 Effect of embryo disaggregation and reaggregation upon postnatal growth and development.

The mean litter size of offspring from the disaggregated embryo, *in vitro*, *in vivo* and naturally mated (6) groups (taken from Chapter 4), are shown in Figure 5.28. It can be seen that after adjustment (naturally mated (6) group), there is no significant difference between any of the groups in their mean litter size.

The mean birth weight of mice derived from disaggregated embryo, *in vitro*, *in vivo* and naturally mated (6) groups are shown in Figures 5.29 and 5.30. Figure 5.29 and 5.30 show that there is no significant difference between any of the treatment groups, for either the males or the females.

As statistical analysis between the *in vitro* and *in vivo* treatment groups has already been performed in Chapter 4, it will not, therefore, be referred to again within this chapter.

Figure 5.28

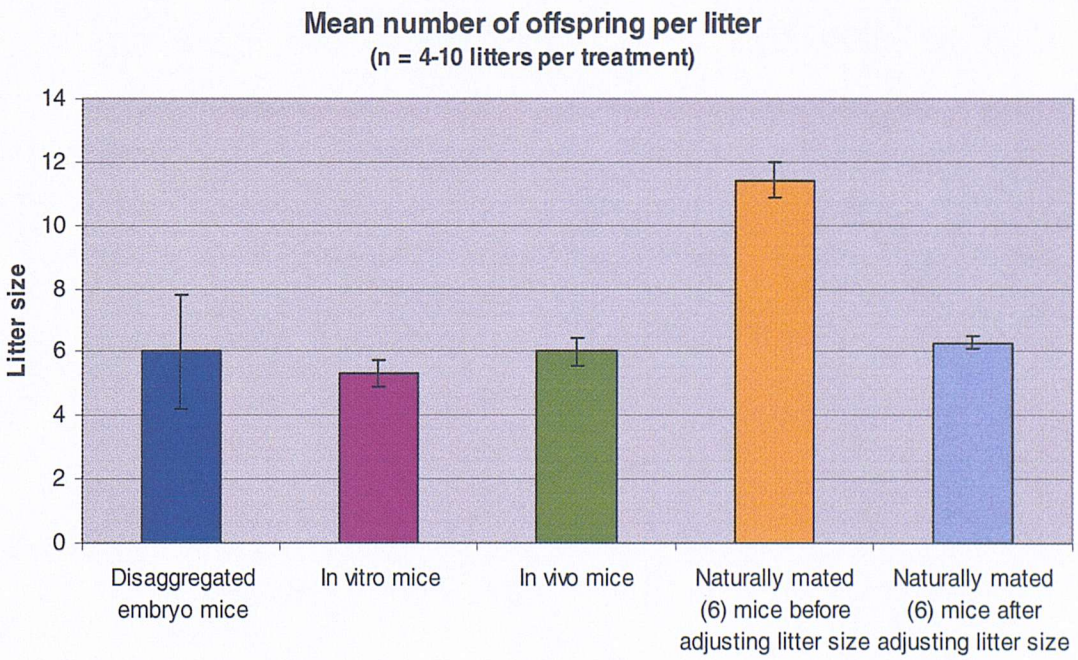


Figure 5.29

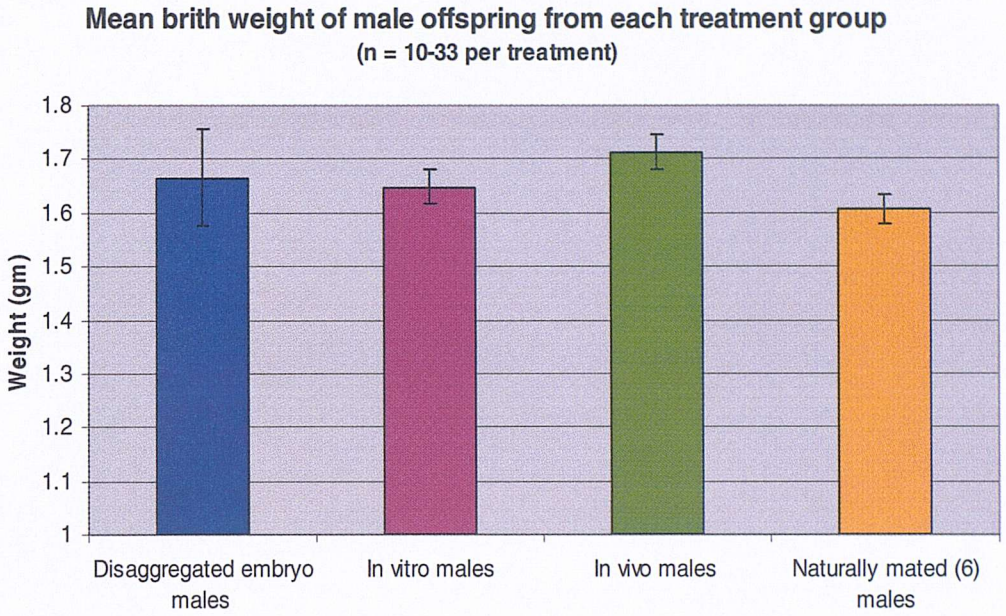
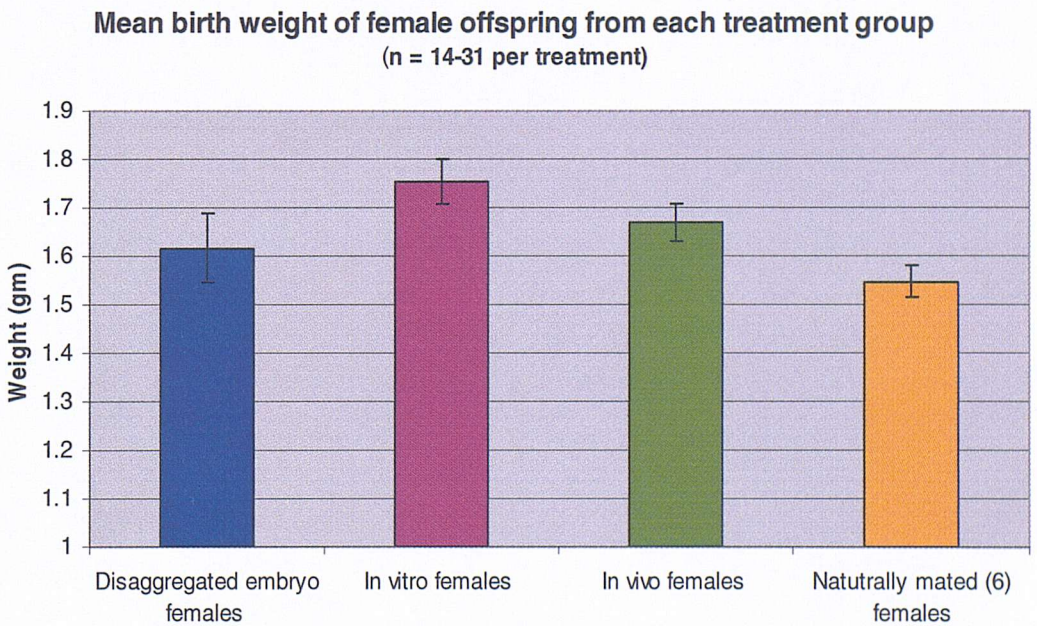


Figure 5.30



The mean weekly weights of the four treatment groups analysed from birth to 27 weeks of age are shown in Figures 5.31 and 5.32 (mean ± standar error). Each mouse was weighed on its day of birth and then subsequently every week for the next 27 weeks. All the weights for the mice from each treatment at each week have been averaged and plotted along with the standard error.

At 12 and 13 weeks of age, the *in vitro* males have a significantly lower weight when compared to the disaggregated embryo males (Figure 5.31, p =0028 and 0.02 respectively). Between 14 to 17 weeks of age both the *in vivo* and *in vitro* males are significantly lighter than the disaggregated embryo males. Between 18 and 21 weeks of age, the *in vitro*, *in vivo* and naturally mated (6) males all become significantly lighter when compared to the disaggregated embryo males. Between 22 and 27 weeks of age only the naturally mated (6) males are significantly lighter when compared to the disaggregated embryo males.

There are no significant differences between the disaggregated embryo females and any of the other female groups (Figure 5.32).

Figure 5.31

Mean weekly weight of male offspring
(n = 9-33 per treatment)

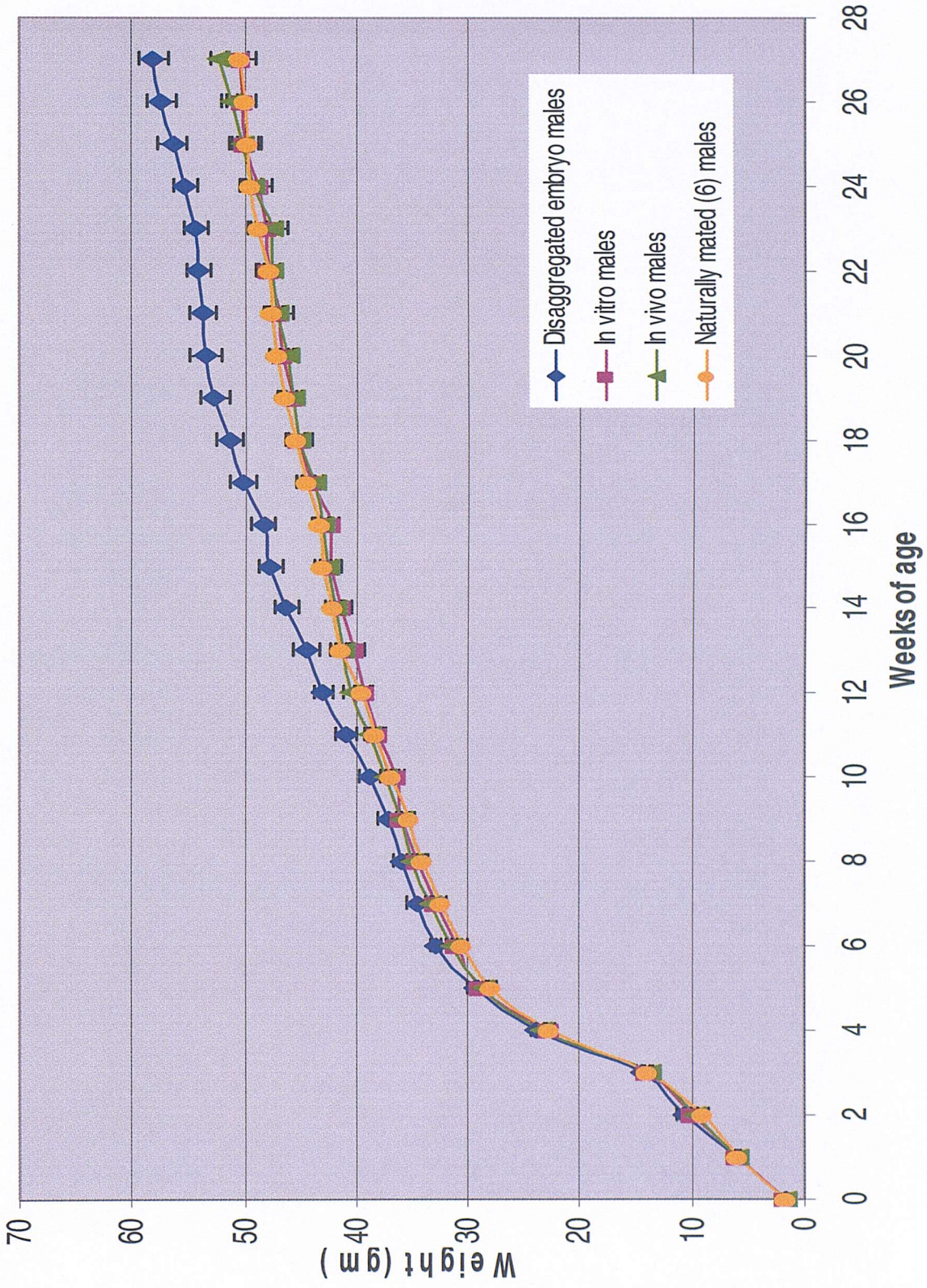
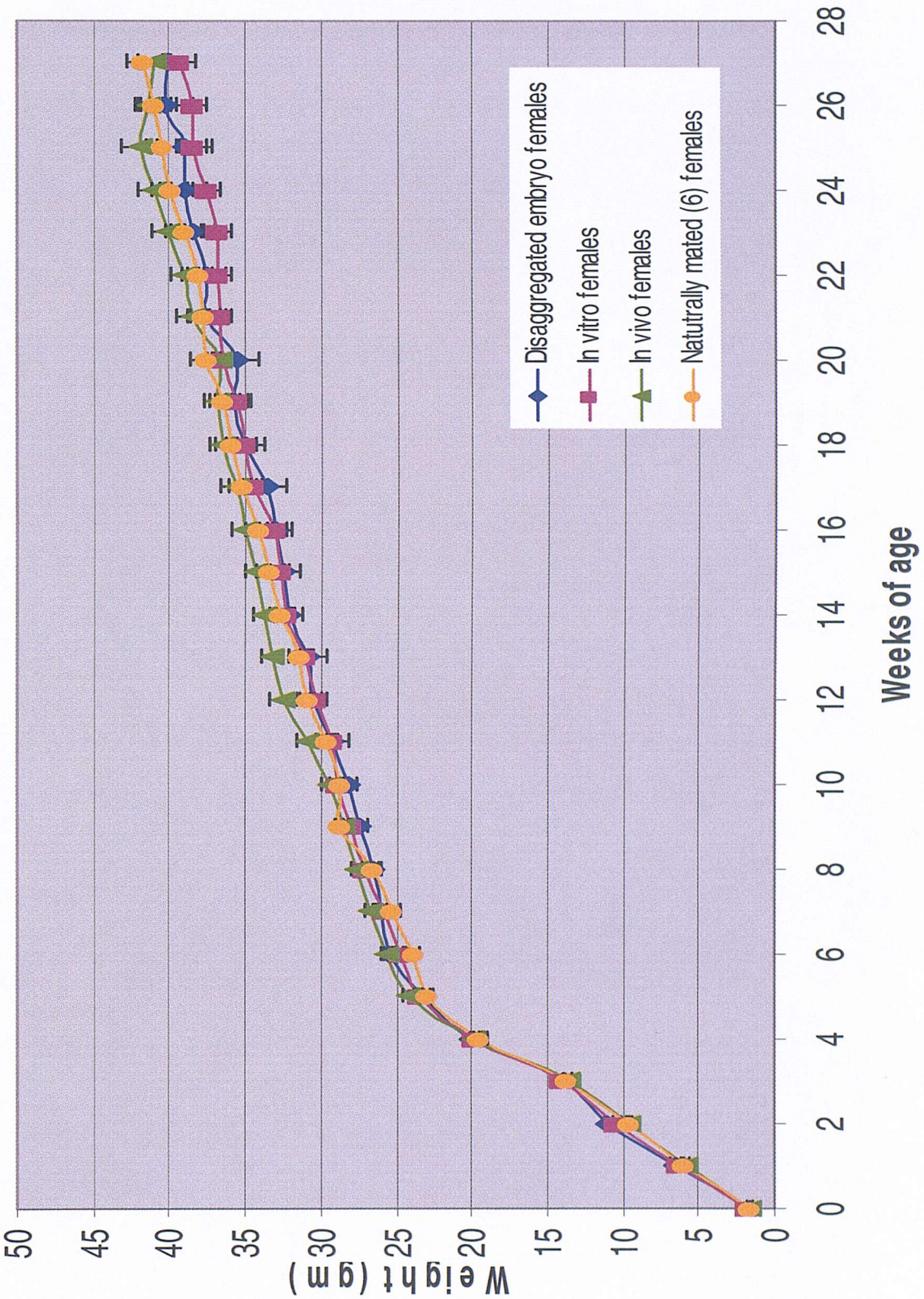


Figure 5.32

Mean weekly weight of female offspring
(n = 8-31 per treatment)



The mean systolic blood pressure of the four treatment groups analysed at three ages (8, 15 and 21 weeks of age) are shown in Figures 5.33 and 5.34. There are no significant differences between any of the males groups at 8 weeks of age. At 15 weeks of age, the disaggregated, *in vitro* and *in vivo* males all have a significantly elevated systolic blood pressure when compared to the naturally mated (6) males. At 21 weeks of age, the disaggregated and *in vitro* males have a significantly elevated systolic blood pressure when compared to the naturally mated (6) males. At no time is there any significant difference between the disaggregated males and the *in vitro* or *in vivo* males.

At 15 weeks of age, the disaggregated females have a significantly elevated blood pressure when compared to the naturally mated (6) females. At 21 weeks of age, only the *in vitro* females are significantly different when compared to the naturally mated (6) females. As observed with the male groups, there are no significant differences between the disaggregated, *in vitro* and *in vivo* female groups.

Figure 5.33

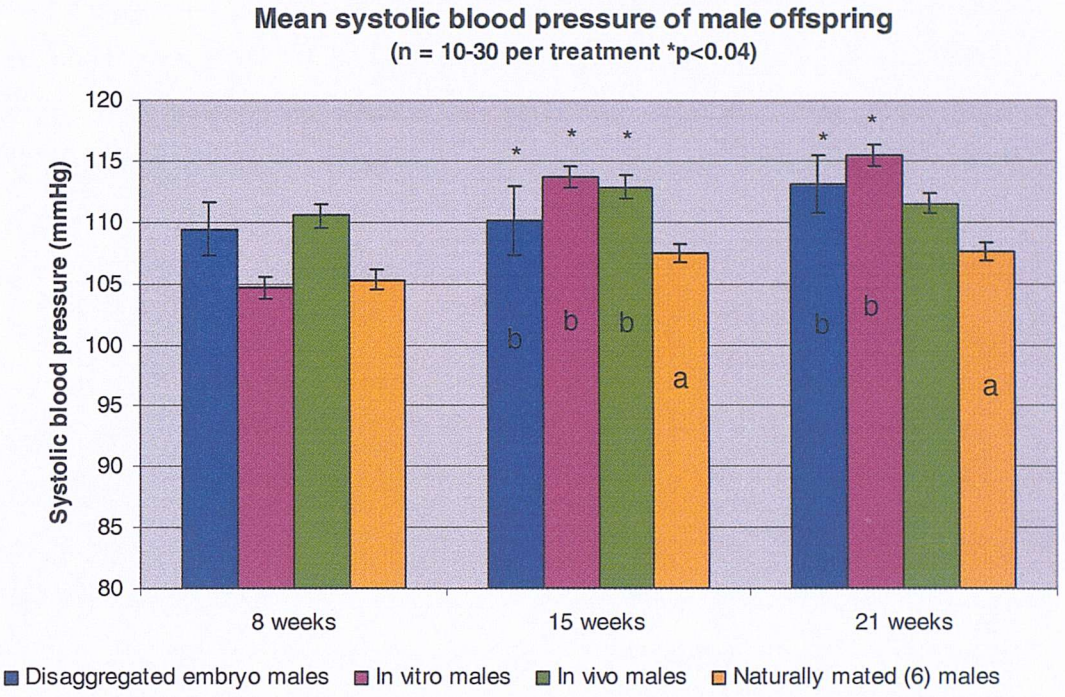
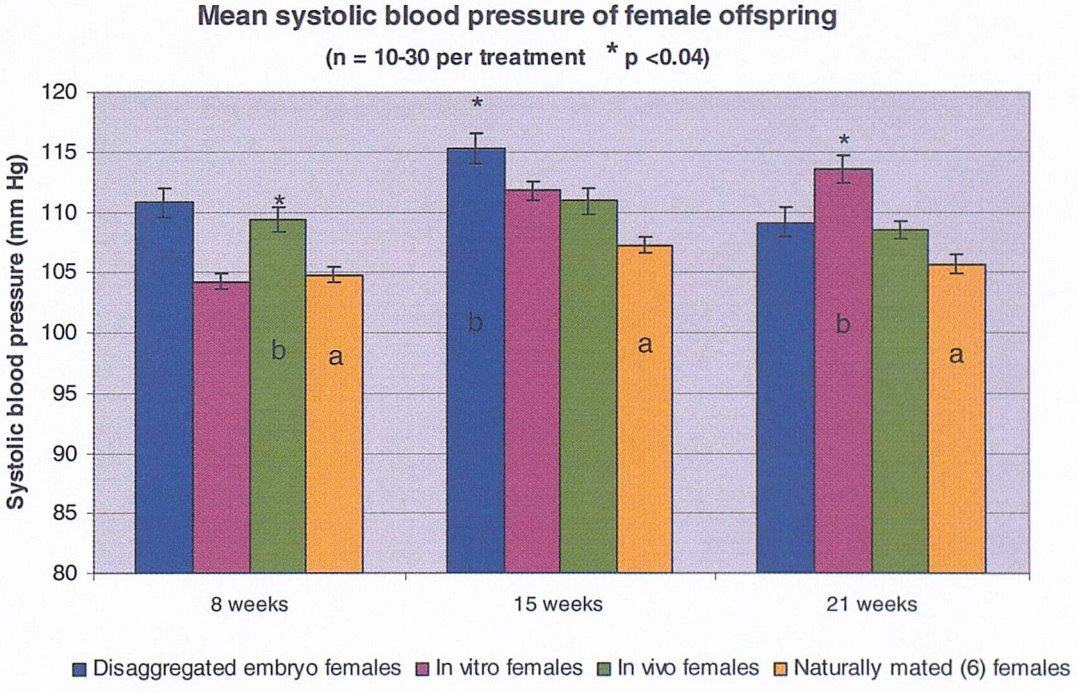


Figure 5.34



The mean organ:body weight ratio and mean organ weights from the five treatment groups studied are shown in Figures 5.35 to 5.38. There are no significant differences between any of the male or female groups for any of the organ:body weight ratios studied (Figure 5.35 and 5.36). The disaggregated males do have the smallest ratio for their liver, hearts and lungs despite being the heaviest groups at 27 weeks of age. The disaggregated female groups have the largest ratio for liver and heart when compared to the other female groups (Figure 5.36).

When organ weight is analysed independently of body weight, the disaggregated males have a significantly heavier liver when compared to the *in vivo* males (Figure 5.37). The disaggregated males also have the heaviest left and right kidney and lung weights, although these values did not reach statistical significance. These findings are likely to be due to the increased mean weight of the disaggregated males at 27 weeks of age when compared to the other males groups. There are no significant differences between any of the female groups when organ weight is analysed independently of body weight (Figure 5.38).

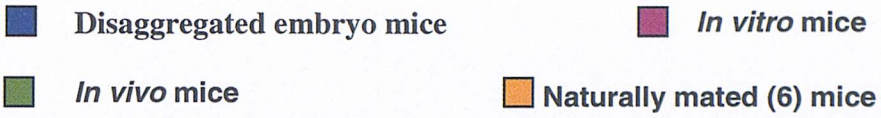


Figure 5.35

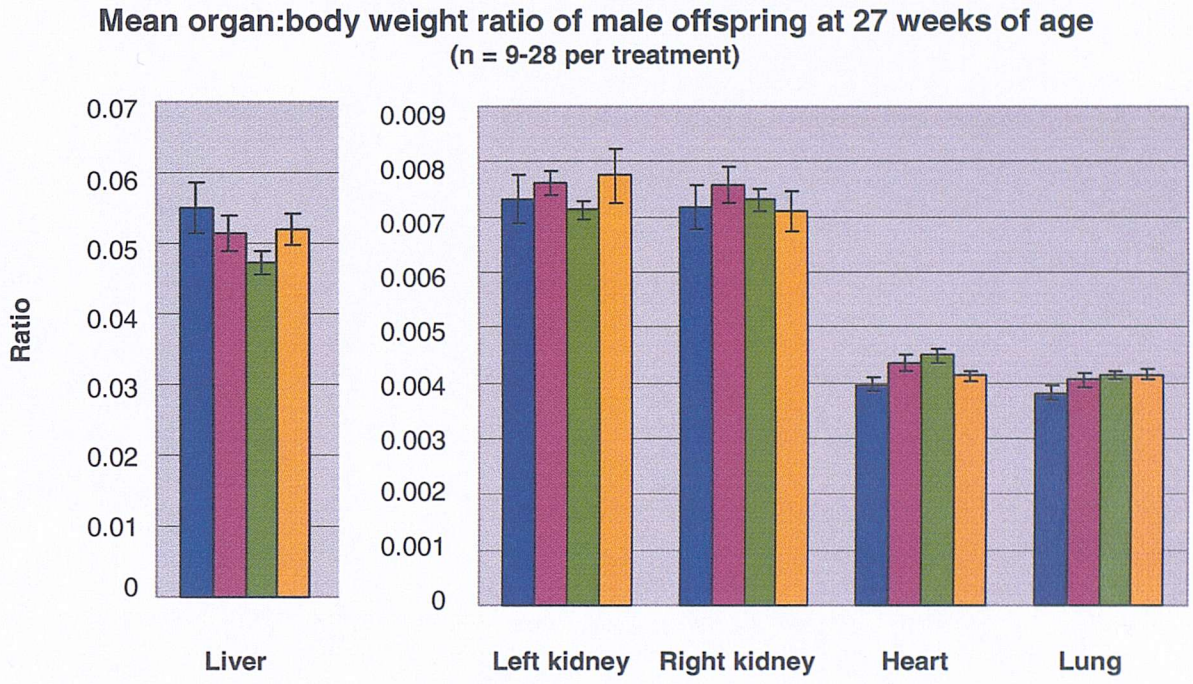
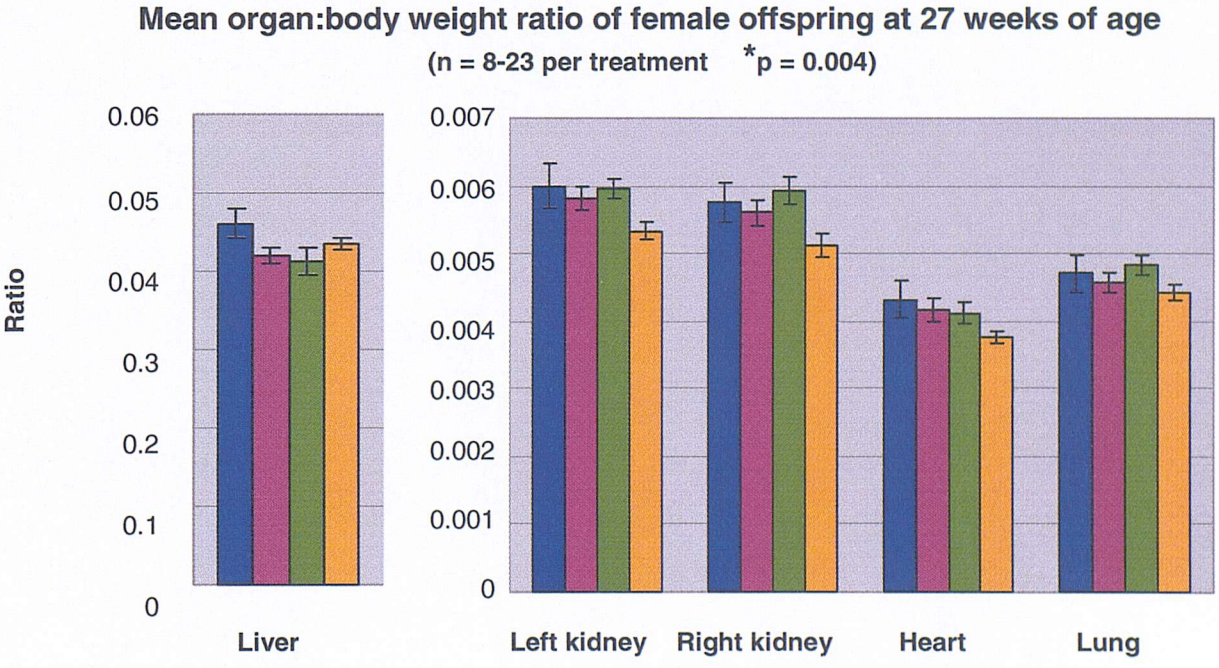


Figure 5.36



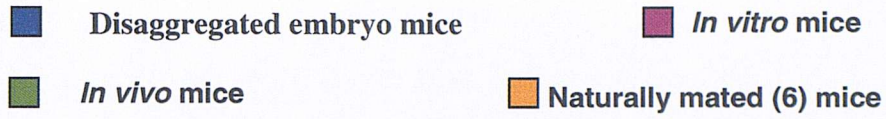


Figure 5.37

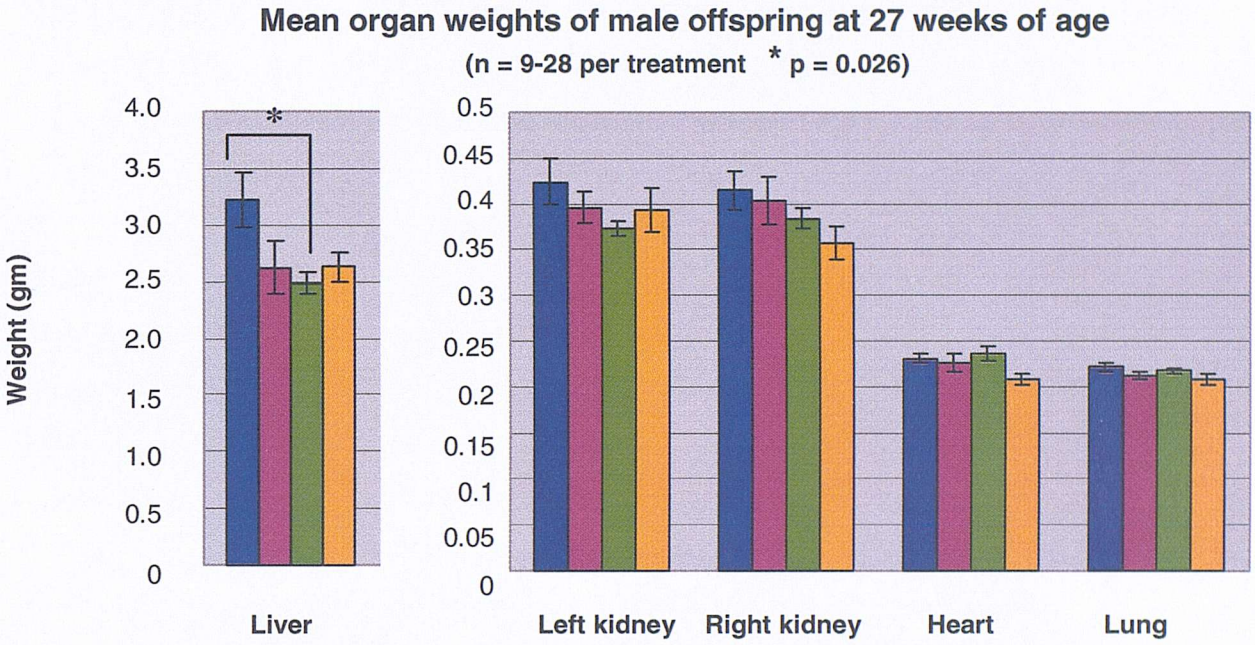
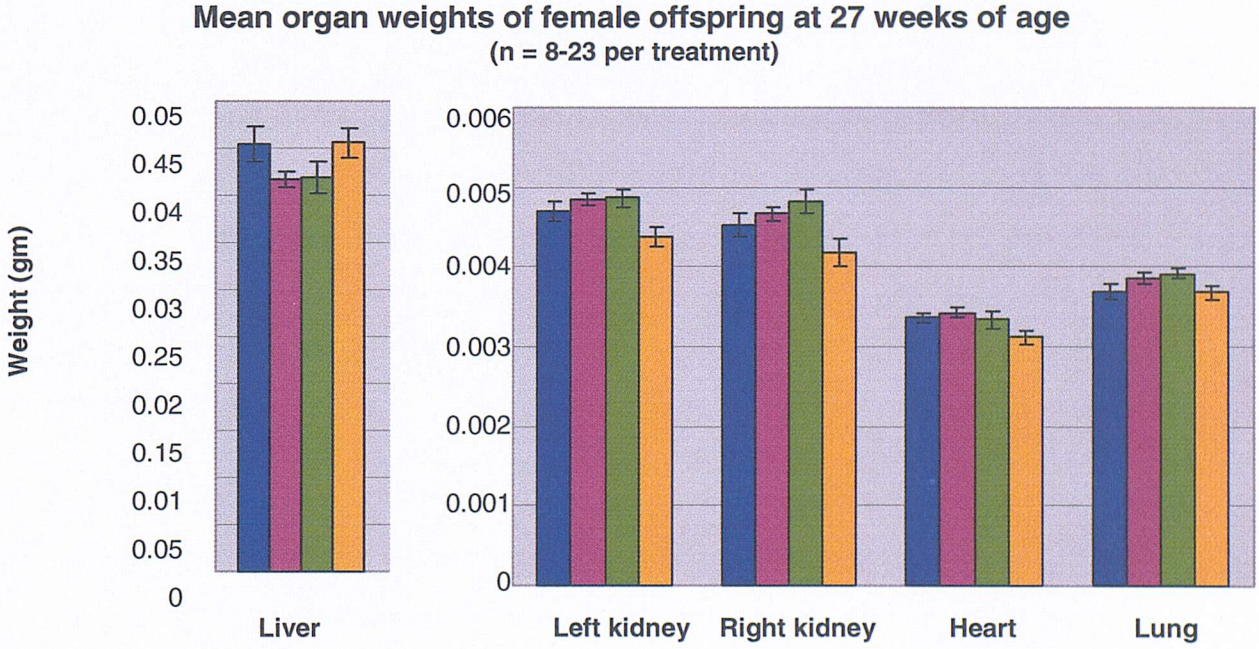


Figure 5.38



5.4 Discussion

5.4.1 Effect of embryo manipulation on birth weight and litter size

The discussion and conclusions for the experiments reported within this chapter are based upon the data available at this point in time. As some of these experiments are currently ongoing, the number of animals within some of the treatment groups is currently low. Clearly, this may have consequences for some of the results observed for postnatal growth and physiology.

Insulin and IGF-1 have both been shown to increase the number of ICM cells within blastocysts of several mammalian species (Harvey and Kaye 1990, 1992; Palma *et al.*, 1997; Lighten *et al.*, 1998, Sections **3.3.3** and **3.3.4**). These effects are believed to be brought about by increased levels of metabolism and protein synthesis, by a reduction in the number of apoptotic cells within the embryo, or by a combination of the two (see section **3.4.2** for discussion; Dunglinson and Kaye 1993; Dunglinson *et al.*, 1995 Lighten *et al.*, 1998; Spanos *et al.*, 2000). Insulin has also been shown to influence fetal development. Embryos cultured in the presence of insulin and albumin result in offspring of increased fetal weight by 4-6% (Kaye and Gardner, 1999).

The results reported within this Chapter showed that mice developing from embryos cultured in the presence of insulin or IGF-1 had increased birth weights when compared to other treatment groups, althought these results did not reach statistical significance. These findings are in line with previous studies (Kaye and Gardner, 1999). Whilst the naturally mated males and females have the lowest birth weight, this can be attributed to the size of the litters from which they came. Although it is possible to control for litter size after birth, it is not possible to have an undisturbed control group for which litter size in utero is altered.

Within this Chapter, the mean birth weight of offspring derived from disaggregated embryos was not significantly different from that of offspring derived from *in vitro* cultured embryos or those of the naturally mated (6) mice (Figures 5.29

and 5.30). This may suggest that physical manipulation may have less of an affect on fetal growth and subsequent birth weight than the process of *in vitro* culture alone.

As observed in Chapter 4, the process of *in vitro* culture and/or manipulation appears to affect the ability of the embryo to implant and develop into a viable offspring. Within this Chapter, the average litter size recorded for offspring derived from embryo transfer is just 4 (Figure 5.4). Therefore, on average, only one third of the embryos transferred were able to implant and develop to term. Again, these results would suggest that some aspect of *in vitro* culture and/or embryo transfer is resulting in a reduction in viability of the embryo to develop into offspring as discussed in section **4.4.1**. Of particular interest is the observation that the insulin and IGF-I treatment groups had the lowest mean litter size out of all the treatment groups. It may have been assumed that the increased rate of embryonic development observed within sections **3.3.3** and **3.3.4** may have given these embryos a post-implantation developmental advantage. However, this appears not to have been the case. It may be the case that as these embryos were cultured in the absence of protein (T6 PVP medium) that this may have negated any of beneficial effects of the insulin or IGF-I. However, those offspring derived from embryos cultured in T6 PVP alone, had a higher mean litter size. One possible explanation may be that, for the strain of embryos used within these experiments, the concentrations of insulin and IGF-I used may not be optimal for postimplantation development. As fetal development was not assessed within this study, it is not possible to ascertain whether a reduced implantation rate, an impairment in fetal development or some other factor was responsible for the small litter sizes observed within these treatment groups.

5.4.2 Effect of embryo manipulation on postnatal growth rates

Upon analysis of the naturally mated groups, those animals which had their litter size adjusted at birth become significantly heavier than those who did not have their litter size adjusted (Figures 5.4 to 5.7). As there was no significant difference in orifinal litter size, all of the naturally mated groups should have received similar *in utero* levels of nutrition, and should therefore have made the same predictions about

the extent of postnatal nutrient availability. However, those mice from litters adjusted at birth would subsequently experience an altered level of postnatal nutrient availability to that which they had predicted *in utero*. This increase in nutrient availability could have resulted in over compensatory growth, leading to the increased weights observed within these groups at 2, 3 and 4 weeks of age. At no other time were there any significant difference between the three naturally mated groups. This would imply that for non-embryo trasfered mice, adjusting litter size may only have significant effect during early periods of postnatal growth rates, possibly attributed to differences in postnatal nutrient availability.

At 10, 12 and 13 weeks of age, the IGF-I males become significantly lighter than the naturally mated (4) males ($p < 0.05$). It may therefore be the case that whilst culture in the presence of IGF-I can stimulate an increase in birth weight, this increased growth rate is finite, or that the IGF-I mice can no longer sustain the increase rate of weight gain. Previous studies reporting increased postnatal weight of offspring from embryos cultured in the presence of insulin also supplemented the embryos with albumin (Kaye and Gardner, 1999). This would have provided the embryos with an exogenous protein source, thus aiding embryo development further. In the studies reported within this thesis, the insulin and IGF-I culture medium was not supplemented with albumin. This could have lead to an altered rate of development compared to that observed in previous studies.

During fetal and postnatal development, insulin and IGF-I predominantly have differing roles. Knockout studies have revealed that *IGF-I* null mice are approximately 60% lighter at birth than control mice, whilst null mice for IGF-I receptor (*IGFIR*) are approximately 45% lighter (Baker *et al.*, 1993). Postnatal development of the brain and long bones are particularly retarded in mice lacking *IgfI* (reviewed in Nakae *et al.*, 2001). Whilst insulin stimulates fetal growth, its effect in comparison to IGF-I is meagre, demonstrated by the fact that insulin null mice are only growth retarded by approximately 15-20% when compared to controls (reviewed in Nakae *et al.*, 2001). Insulin has its predominant effect upon postnatal metabolism.

Mice lacking either insulin genes develop diabetic ketoacidosis and die within days of birth (reviewed in Nakae *et al.*, 2001).

At 1, 2, 3 and 13 weeks of age, the PVP females became significantly lighter than the naturally mated (4) females ($p < 0.05$). This result may be attributed to the *in vitro* environment experienced by the PVP group. The lack of protein could have slowed the rate of preimplantation development resulting in a slower rate of fetal growth, and significantly lighter weights during postnatal growth. This coupled with the fact that female embryos develop at a slower rate when compared to male embryos (Xu *et al.*, 1992; Sato *et al.*, 1995; Krackow and Burgoyne 1998), could explain why a reduction in weight is only observed in the PVP females and not the males.

Male mice derived from disaggregated embryos became significantly heavier than the other male groups from 12 weeks of age, and remained heavier for up to 27 weeks of age. In particular, between 14 and 21 weeks of age, the disaggregated males were significantly heavier than the *in vitro*, *in vivo* and the naturally mated (6) males. As discussed within Chapter 3, the process of disaggregation and reaggregation could disturb already established axies of the preimplantation embryo. As the embryonic-abembryonic axis is generated through the division patterns of the earlier and later dividing 2-cell blastomeres, by the 8-cell stage there will already be cells predisposed to allocating to the ICM or TE lineages. Therefore, disaggregation and reaggregation would not only disturb the axes of the embryo, but also reposition predisposed cells into alternative regions of the embryo. For the males, it would appear that this process results in significantly increased postnatal growth as they were significantly heavier than the *in vitro* males. The disaggregation and reaggregation process did not appear to affect the female offspring in the same manner as there were no significant differences between any of the female groups. One possible explanation for this observation again could be due to the different rates at which male and female embryos develop (Xu *et al.*, 1992; Sato *et al.*, 1995; Krackow and Burgoyne 1998). The increased rate of male embryo development coupled with the stress of

disaggregation could have induced the increased rates of postnatal development observed for male offspring. However, the relatively small number of animals and litters within this treatment group must be kept in mind until the sample size can be increased.

5.4.3 Effect of embryo manipulation on systolic blood pressure

At 8 weeks of age, both the naturally mated (4) and (6) groups show an elevated systolic blood pressure when compared to the naturally mated group (Figures 5.8 and 5.9). The significant differences observed in the early growth rates between the naturally mated and naturally mated (6) and (4) mice (possible caused by differences between *uterine* nutrient prediction and postnatal nutrient availability) may be a factor in the raised systolic blood pressure observed at 8 week of age. As the growth profiles no longer significantly differ after 6 weeks of age, this could explain why it is only at 8 weeks of age that significant differences in systolic blood pressure are observed between the naturally mated groups.

There were no significant differences between the PVP, insulin, IGF-I or naturally mated (4) male groups at 8, 15 or 21weeks of age (Figure 5.22). For the female groups, there were no differences in blood pressure at 8 or 15 weeks of age. However, the naturally mated (4) females did have the lowest blood pressure of any of the groups at 8 amd 21 weeks of age. At 21 weeks of age, the IGF-I and the PVP females had significantly elevated systolic blood pressures when compared to the naturally mated (4) females. For the PVP females, the significantly reduced rates of postnatal development during 1, 2 and 3 weeks of age may have lead to the increased systolic observed at 21 weeks of age. The lack of numbers of animals within some of the groups (particularly for the male groups) is an issue that must be considered.

The process of embryo disaggregation and reaggregation resulted in male offspring having an elevated systolic blood pressure at 15 and 21 weeks of age when compared to the naturally mated (6) males. Despite this, there were no significant differences in blood pressure between *in vitro*, *in vivo* and the disaggregated embryo

males. The female disaggregated embryo group had a significantly elevated systolic blood pressure at 15 weeks of age when compared to the naturally mated (6) females, despite there being no significant difference in weight at this time. It was also observed that the female disaggregated embryo group had the highest blood pressure at 8 and 15 weeks of age when compared to all the other groups. However, as with the males, there were no significant differences in blood pressure between *in vitro*, *in vivo* and the disaggregated embryo females. Again, this may suggest that the disaggregation and reaggregation procedure affects postnatal blood pressure to the same extent as *in vitro* culture alone which in turn is consistent with the previous data.

5.4.4 Effect of embryo manipulation and its effect on organ weight

The naturally mated (4) mice had a significantly lower lung:body weight ratio when compared to the naturally mated (6) mice. There are no other significant differences between the three naturally mated male groups for any of the other organ:body weight ratios. When organ weight was analysed independently of body weight, both the naturally mated (6) and (4) males had a significantly heavier liver when compared to the naturally mated males ($p = 0.003$, Figure 5.12). The naturally mated (6) females had a significantly heavier liver when compared to the naturally mated females, whilst the naturally mated (4) females have a significantly heavier lung weight when compared to the naturally mated females ($p < 0.03$, Figure 5.13). However, body weight was observed to have a significant influence upon organ weight ($p < 0.001$).

In vitro mammalian embryo culture has been shown to affect aspects of organ growth and allometry. Culture of sheep and cattle embryos in the presence of serum has been shown to lead to changes in the normal size of internal organs (Thompson *et al.*, 1995, Holm *et al.*, 1996, Young *et al.*, 1998, Sinclair *et al.*, 1999; Jacobsen *et al.*, 2000). There were no significant differences between any of the groups in section **5.3.2** for either the males or the females. Although not significant, the insulin males

and IGF-I females had the highest ratio for the liver, both of the kidneys, the heart and the lungs.

The disaggregated males had a significantly heavier liver (independent of body weight) when compared to the *in vivo* males (Figure 5.37). The disaggregated males also have the heaviest left and right kidney and lung weights, although these values did not reach statistical significance. These findings would most likely be due to the increased mean weight of the disaggregated males at 27 weeks of age when compared to the other males groups. There are no significant differences between any of the female groups when organ weight was analysed independently of body weight (Figure 5.38).

It would therefore appear that all of the above manipulations have minimal effect on the organ:body weight ratios. The main influence upon actual organ weight was observed to be body weight. However, as stated during this Chapter, these results are based on small numbers of animals. This means that the observed results could possibly differ once more data is added.

5.5 Conclusions

In conclusion manipulation of litter size resulted in significant changes in weight and blood pressure during early periods of development, such that weight gain and systolic blood pressure were observed to increase as postnatal litter size was decreased however. After 8 weeks of age no further differences were observed (apart from organ weight, though this was not significantly influenced by postnatal litter size). The differences in early development could have been attributed to inuterine predictions of postnatal nutrient availability being different to that which was available.

The addition of growth factors to the culture medium not only affected blastocyst cell numbers, but also altered patterns of postnatal growth and cardiovascular physiology. These changes may have caused as a result of increased

fetal development (stemming from increased blastocyst ICM cell numbers), subsequently increasing birth weight.

Embryos that underwent disaggregation gave rise to offspring of similar birth weights to offspring from *in vitro* and *in vivo* treatment group embryos. This may imply that the process of physical manipulation may affect postimplantation development to the same extent as *in vitro* culture alone. However, disruption to the embryonic-abembryonic axis though embryo disaggregation resulted in significantly increased weight gain during postnatal life (in male offspring only), and elevated systolic blood pressure when compared to naturally mated groups.

In all of the groups studied there was minimal difference in the organ:body weight ratios, or organ weight independent of body weight, between treatment groups. It would therefore appear that whilst certain manipulations can affect aspects of postnatal growth and development, the act of *in vitro* culture itself may be having the larges influence on long term development and physiology.

The main future aim arising from these studies is to increase the numbers of animals within each of the treatment groups. It would also be necessary to create and transfer embryos disaggregated and reaggregated to contain half and double the normal cell number. As well as these, it would be of interest to conduct studies into the postnatal development of embryos created from the aggregation of the isolated ICMs from Chapter 3.

Chapter 6

Ped gene expression and its influence on postnatal development

6.1 Introduction

Expression of the *Ped* gene product, the Qa-2 protein, has been shown to correlate with increased rates of mouse preimplantation embryo development, such that those embryos expressing the *Ped* gene (*Ped* plus) are seen to have an increased rate of embryo cleavage, and contain more cells when compared to embryos not expressing the Qa-2 protein (*Ped* minus) (Goldbard *et al.,* 1982; Warner *et* al., 1987; Brownell and Warner, 1988). The Qa-2 protein is expressed on the surface of *Ped* plus embryos from the oocyte stage and throughout preimplantation development (Warner *et al.*, 1987). Previous studies have shown that offspring developing from *Ped* plus embryos have a higher incidence of intrauterine survival, are heavier at birth, come from larger litters and are heavier at weaning when compared to *Ped* minus offspring (Warner *et al.,* 1991, 1993; Exley and Warner, 1999).

The relationship between preimplantation embryo development and postnatal growth and physiology is a central theme within this thesis. As previous studies have shown that *Ped* gene expression influences preimplantation embryo cell numbers, with subsequent correlations to birth weight, it was decided to involve the *Ped* model in a study examining the effects of *Ped* gene expression on aspects of postnatal growth and physiology. Using two congenic strains of mice, B6 K1 (*Ped* minus) and B6 K2 (*Ped* plus), the effects of *Ped* gene expression on postnatal parameters including growth profiles, systolic blood pressure and adult organ allometery have been investigated.

6.2 Methodology

6.2.1 Matings

6-8 week old B6 K1 and B6 K2 females were housed overnight with males of the same strain and age (6-8 weeks old). Each morning the females were checked for a vaginal plug (taken as a sign of copulation). Plug positive females were individually

housed and allowed to develop to term with their pregnancies. Plug negative females were placed back in their original cages during the day, but then returned to the same male in the afternoon. Eight females of each strain were allowed to develop to term with their pregnancies. No postnatal manipulation of litter size took place.

6.2.2 Measurement of postnatal growth rates

Offspring were weighed on day of birth and then subsequently on the same day for the next 27 weeks (section **2.3.1**).

6.2.3 Measurement of systolic blood pressure

Systolic blood pressure was determined at 8, 15 and 21 weeks of age by tail-cuff plethysmography (section **2.3.2**).

6.2.4 Measurement of organ weights

Organs were dissected from mice at approximately 27 weeks of age. Organs were weighed before being snap frozen in liquid nitrogen, and stored at -80°C (section **2.3.3**). Blood samples were collected by vacuum puncture of the heart using a pulled glass pipette (internal diameter ~1mm) connected to a mouth pipette, and then centrifuged at 10,000g, 4°C for 10 minutes, after which the serum was aliquoted into 10 µl samples and stored at -80°C.

6.2.5 Measurement of serum ACE activity

Serum ACE activity was determined as described in section **2.3.4**. Serum ACE activity was expressed as nanomoles of hippurate formed per millilitre of serum per minute.

6.2.6 Measurement of ACE activity in lung tissue

Lung ACE activity was determined as described in section **2.3.5**. Tissue protein content was measured using a commercially available protein assay kit (Bio-Rad). Tissue ACE activity was expressed as nanomoles of hippurate formed per milligram of protein per minute.

6.2.7 Statistical analysis of data

Data was analysed was using a multilevel 'random effects' regression model which took into account between-mother and within-mother variation in litter size for parameters measured from individual animals, thereby preventing any differences derived from abnormal litter sizes influencing the data (section **2.4**). Statistical significance was assumed at $p \leq 0.05$.

6.3 Results

No significant difference in birth weight between the two strains was observed, or if the males and females were analysed separately (Figure 6.1, Table 6.1). However, both male and the female B6 K2 mice are heavier that B6 K1 males and females. There are no significant differences in the number of pups per litter between the two strains (Table 6.1).

Figure 6.1

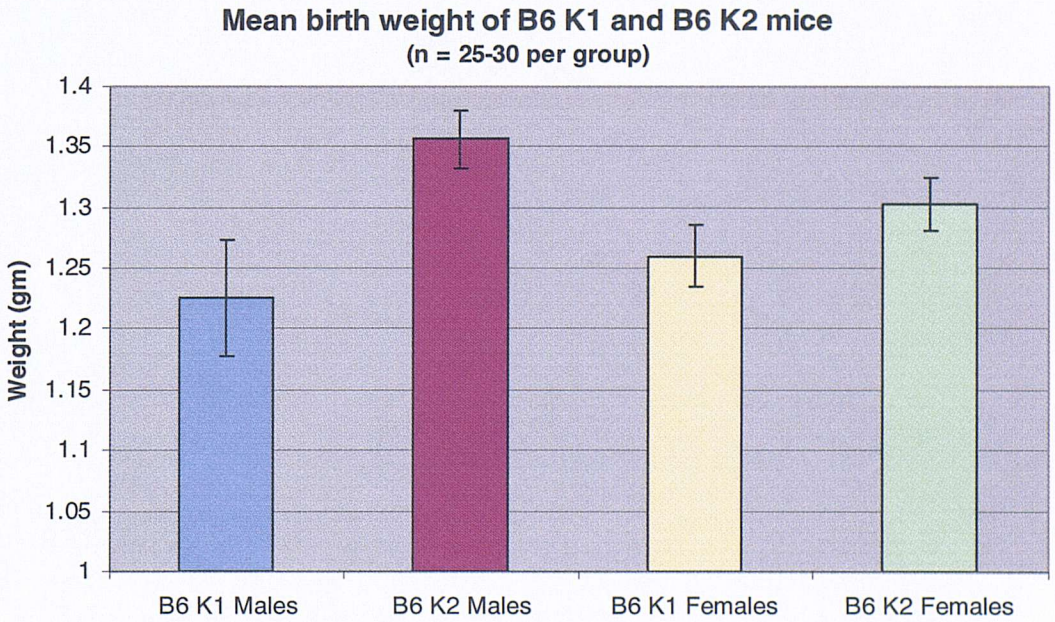


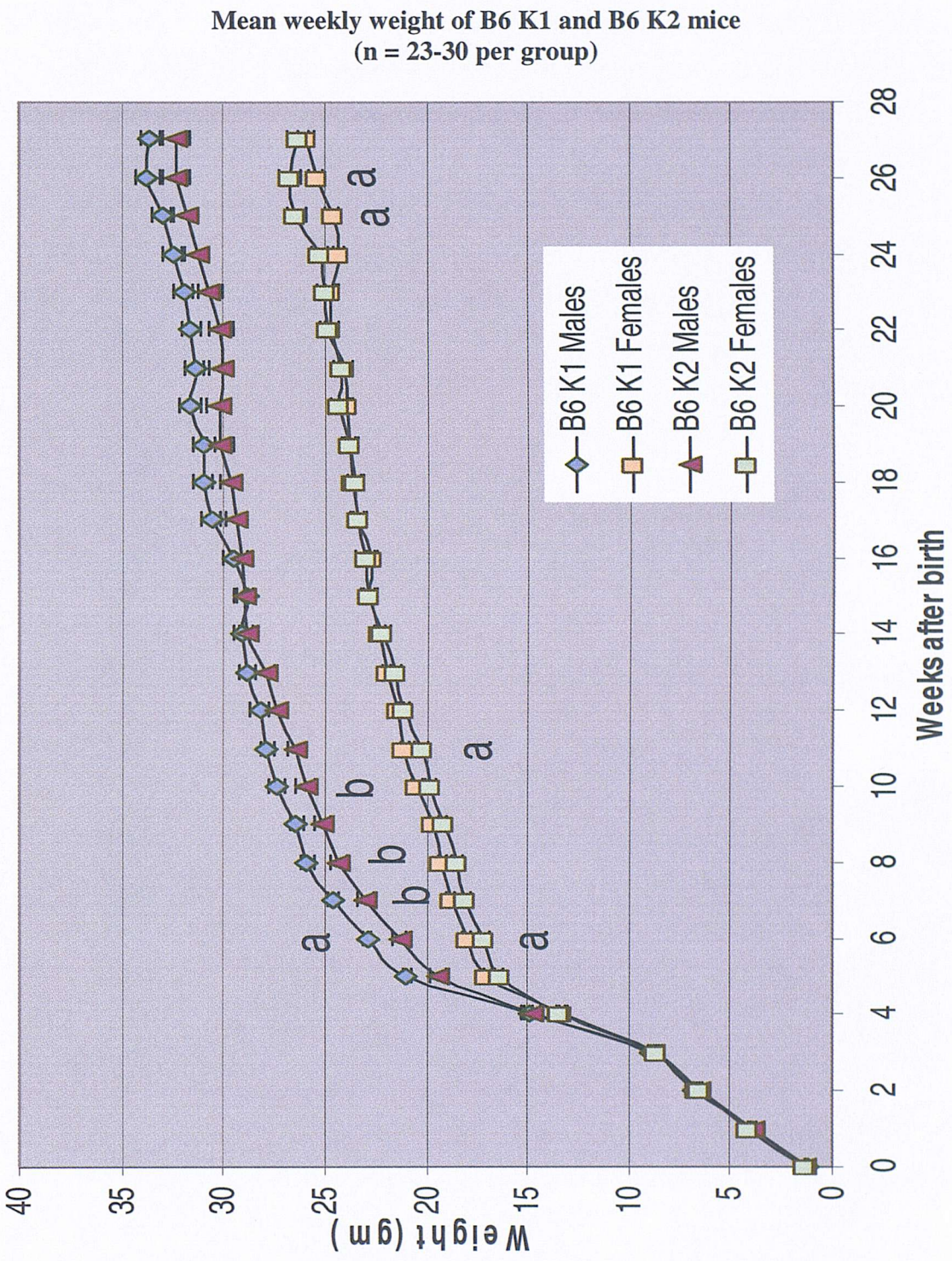
Table 6.1

	B6 KI mice	B6 K2 mice	p value
Male birth weight (gm)	1.225	1.336	0.186
Female birth weight (gm)	1.260	1.304	0.240
Male and female combined birth weights (gm)	1.243	1.329	0.194
Mean litter size	7.25	6.375	0.997

The mean weights of both B6 K1 and B6 K2 mice for up to 27 weeks of age are shown in Figure 6.2 (mean ± standard error). From five weeks of age, male and female weights differed, with the males becoming significantly heavier than the females. As seen in Figures 4.5 and 4.6, male and female mice go through a period of accelerated growth during four and five weeks of age. After this time, the mean weight gain begins to decrease. However, it can be seen that the mice are still growing as the mean weight has not yet reached a plateau by 27 weeks of age.

From five weeks of age, and up to the end of the study, the B6 K1 males have an elevated mean weight when compared to the B6 K2 males. After eleven weeks of age, there appears to be no such separation in the females. At six weeks of age, both the male and female B6 K1 mice are significantly heavier (denoted by the letter **a** above and below the male and female data points respectively, $p < 0.05$) than the B6 K2 mice. During the following six weeks, there are further significant differences between the two strains, but at specific weeks there is only a significant difference when the data for males and females are combined (denoted by the letter **b** in between the male and female data points, $p < 0.05$). This is the case at seven, eight and ten weeks of age where the B6 K1 mice are significantly heavier than the B6 K2 mice. At twenty five and twenty six weeks of age, the B6 K1 females become significantly lighter than the B6 K2 females.

Figure 6.2



Mean weight for B6 KI and B6 K2 male and females from 1-27 weeks of age (± SEM). Letter **a** denotes a significant difference between the B6 K1 and B6 K2 strains for either the males or the females. Letter **b** denotes that there is an overall significant difference between the two strains but only when the male and female data are combined.

The mean systolic blood pressures for B6 K1 and B6 K2 mice at three ages (8, 15 and 21 weeks of age) are shown in Figures 6.3 and 6.4. At 8 weeks of age there is no significant difference in the mean systolic blood pressure between the two strains. At 15 weeks of age the B6 K1 females have a significantly reduced blood pressure, but this is reversed at 21 weeks of age, where the B6 K1 females have a significantly elevated blood pressure. Although the same trend is observed in the males, this did not reach statistical significance (15 weeks $p = 0.055$, 21 weeks $p = 0.256$). At 15 weeks of age, the B6 K1 mice have a significantly lower systolic blood pressure when compared to the B6 K2 mice (105.76 ± 1.27 vs 111.45 ± 0.64, $p = 0.002$, Figure 6.5). However at 21 weeks of age the B6 K1 mice have a significantly elevated systolic blood pressure (110.31 ± 0.58 vs 107.38 ± 0.54, $p = 0.006$, Figure 6.5).

Figure 6.3

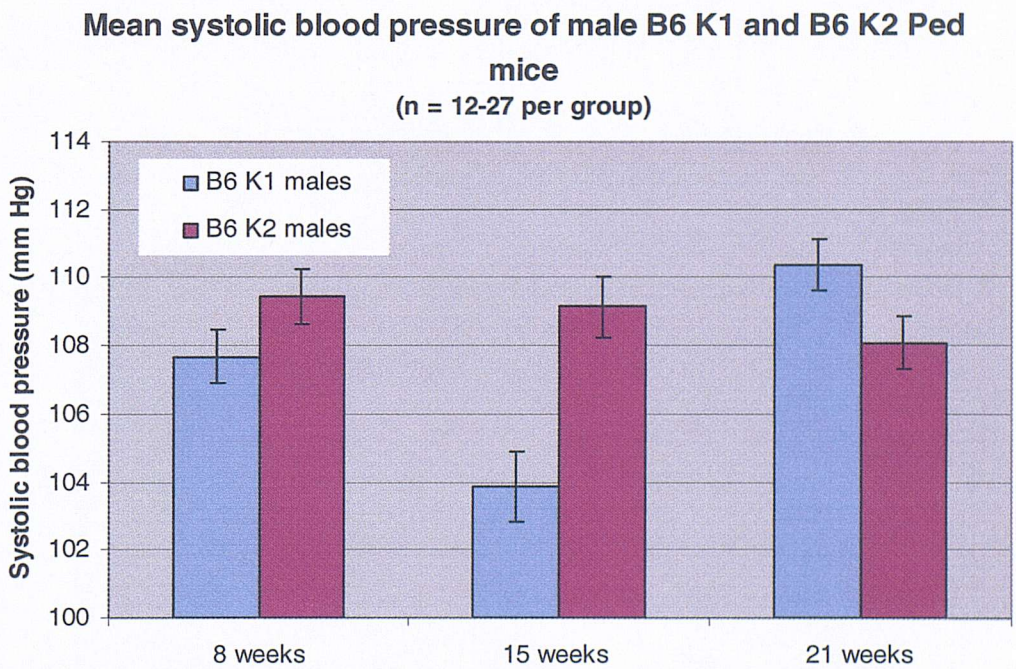


Figure 6.4

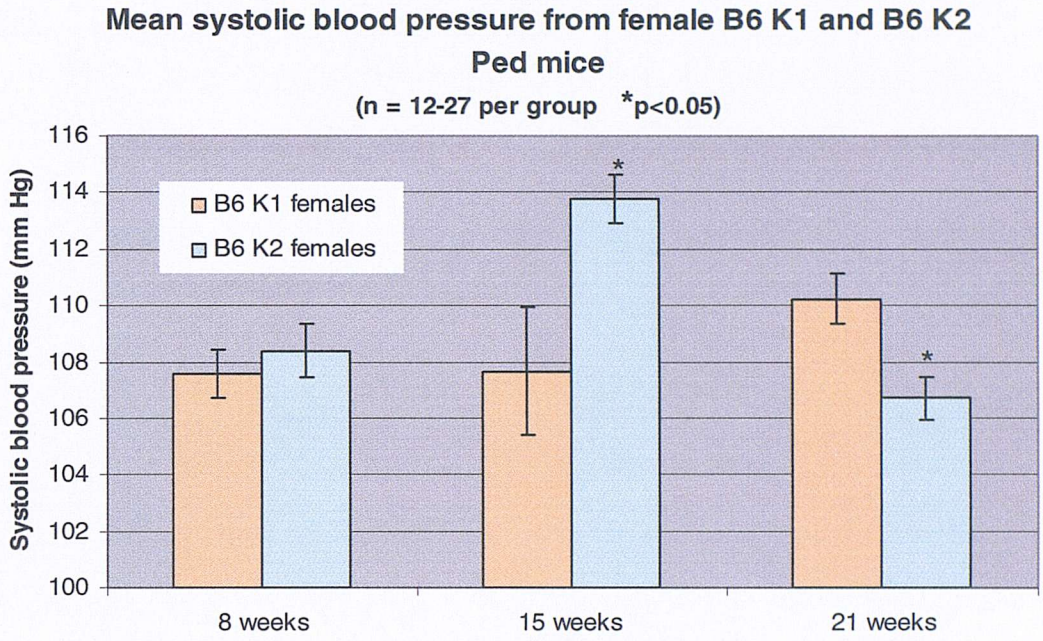
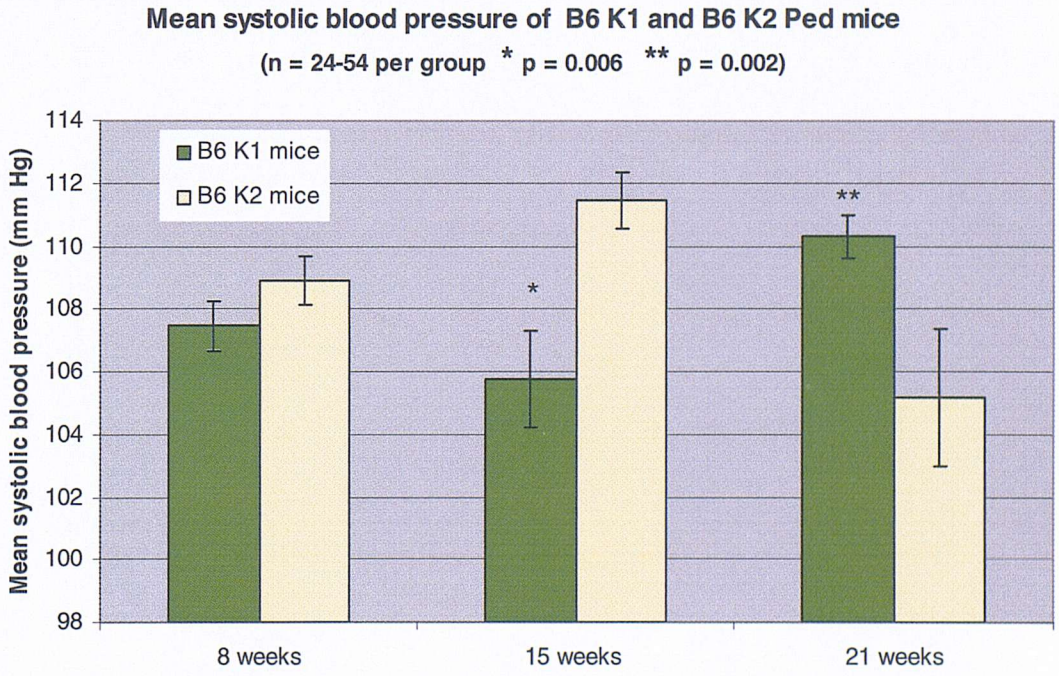


Figure 6.5



The mean organ:body weight ratios in B6 K1 and B6 K2 mice at 27 weeks of age are shown in Figures 6.6 and 6.7. Figure 6.6 show that there is no significant difference between male B6 K1 and B6 K2 mice for any of the organs analysed. However, the B6 K1 males have a lower ratio for all of the organs when compared to the B6 K2 males.

Figure 6.7 shows that the B6 K1 females have a significantly elevated ratio for the lungs when compared to the B6 K2 females. The B6 K1 females also have an elevated ratio for their left and right kidney and their hearts, although these ratios are not significantly different. If the data for the male and female lung:body weight ratios are combined then the B6 K1 mice have a significantly elevated ratio when compared to the B6 K2 mice ($p = 0.013$).

The mean organ weights for liver, left and right kidney, heart and lungs for B6 K1 and B6 K2 mice are shown in Figures 6.8.and 6.9. Figure 6.8 shows that there are no significant difference between male B6 K1 and B6 K2 mice for any of the organs studied. Figure 6.9 shows that the B6 K1 females have a significantly elevated lung weight when compared to the B6 K2 females.

Figure 6.6

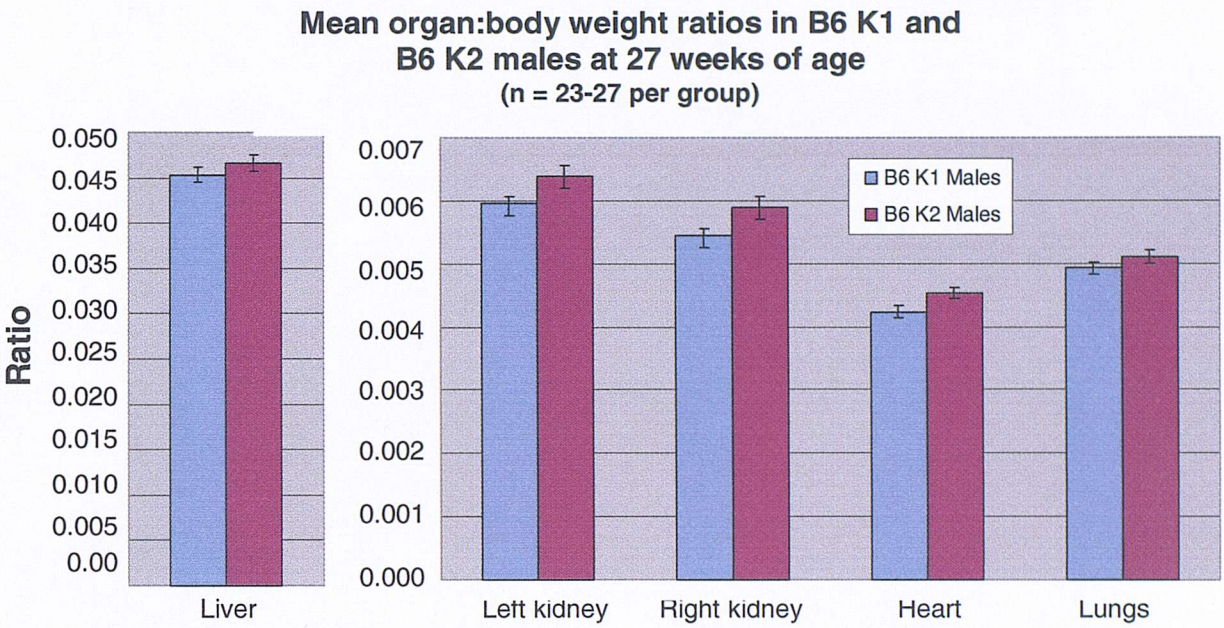


Figure 6.7

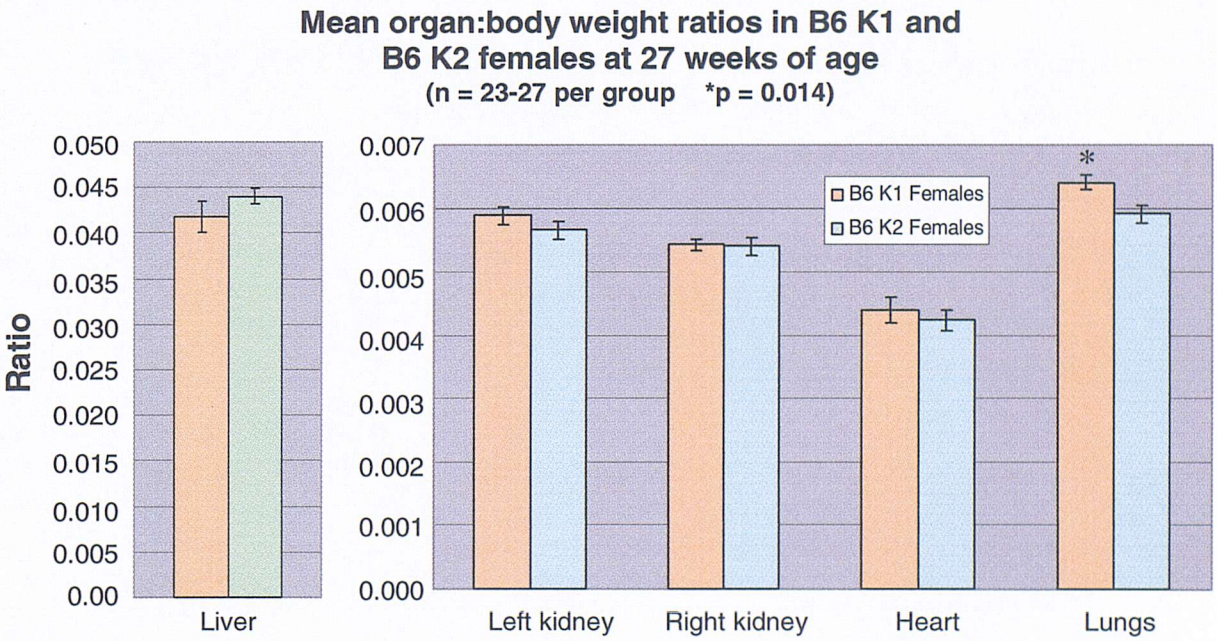


Figure 6.8

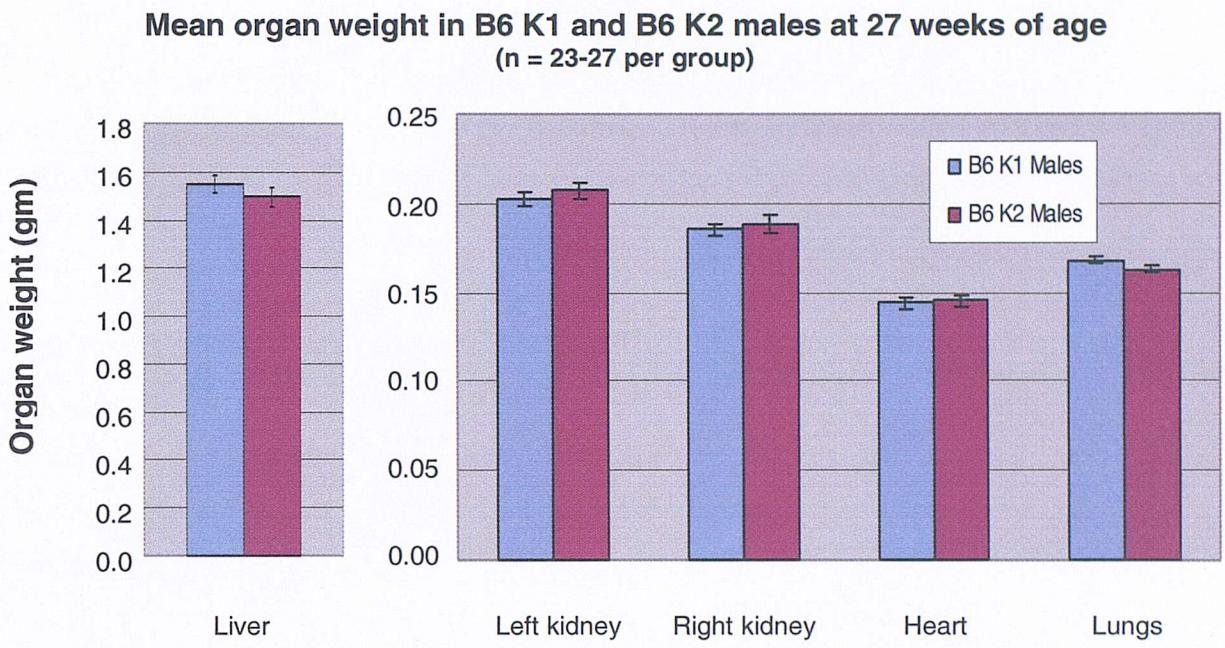
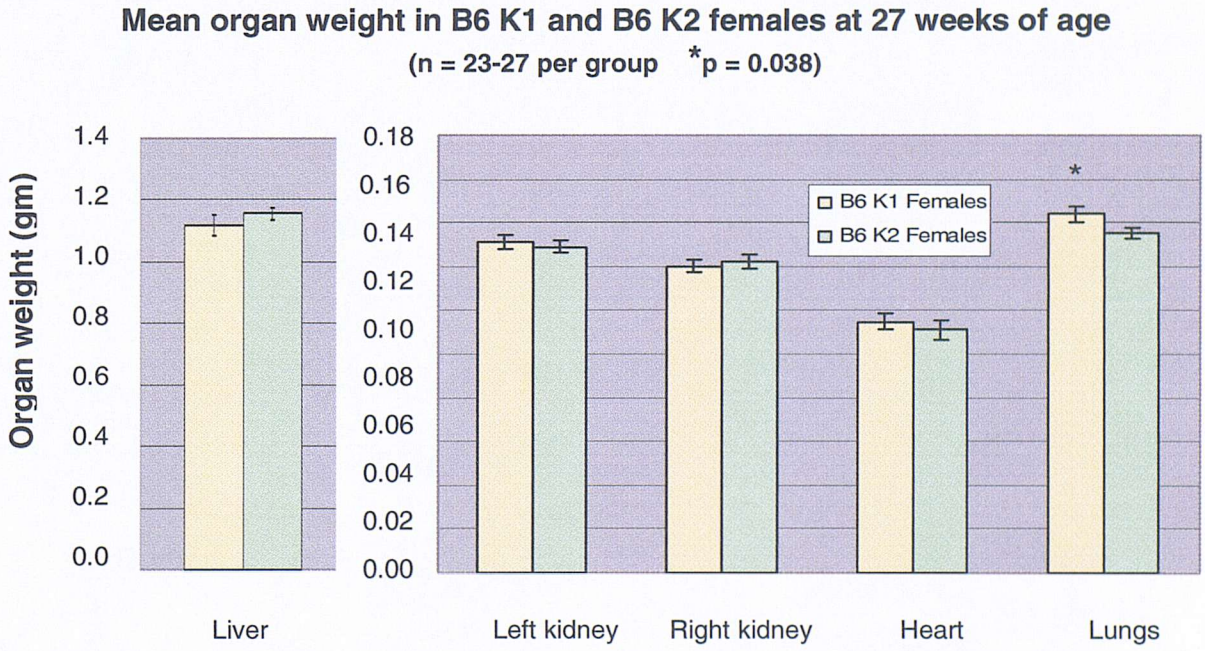


Figure 6.9



Correlations between organ weight and body weight for B6 K1 and B6 K2 mice at 27 weeks of age are shown in Table 6.2. Each organ has been individually correlated with each of the other organs and with body weight. Significant correlations ($p \leq 0.05$) have been highlighted in blue, whilst trends ($p = 0.051 - 0.1$) have been highlighted in orange. Overall, there are relatively strong correlations for both strains between the left and the right kidney, and between the left and right kidney and the liver. This analysis shows that the two strains vary in the level of correlation that exists between specific organs and/or body weight. Taking for example, the correlation of the right kidney weight with lung weight correlation (highlighted by the blue dots●), it can be seen that the B6 K1 mice do not have a significant correlation ($p = 0.172$), whilst the B6 K2 mice do have a significant correlation ($p = 0.001$).

Table 6.2 Data from statistical analysis of organ:organ and organ:body weight correlations

	Left kid-liver	Left kid-weight
B6 K1 males	<0.001	0.341
B6 K1 females	0.833	0.262
B6 K2 males	0.654	0.178
B6 K2 females	0.006	0.622
B6 K1 mice	<0.001	0.203
B6K2 mice	0.183	0.064
All males	0.006	0.479
All females	0.216	0.329
All mice	<0.001	0.991

	Right kid-liver	Right kid-weight
B6 K1 males	0.003	0.717
B6 K1 females	0.665	0.034
B6 K2 males	0.116	0.259
B6 K2 females	0.048	0.806
B6 K1 mice	0.041	0.469
B6K2 mice	0.003	0.430
All males	0.018	0.642
All females	0.453	0.082
All mice	0.001	0.380

	Heart-liver	Heart-weight
B6 K1 males	0.092	0.094
B6 K1 females	0.699	0.791
B6 K2 males	0.378	0.023
B6 K2 females	0.618	0.298
B6 K1 mice	0.545	0.539
B6K2 mice	0.906	0.008
All males	0.345	0.004
All females	0.801	0.166
All mice	0.860	0.037

	Lung-liver	Lung-weight
B6 K1 males	<0.001	0.220
B6 K1 females	0.813	<0.001
B6 K2 males	0.229	0.022
B6 K2 females	0.007	0.703
B6 K1 mice	0.018	0.330
B6K2 mice	0.582	0.031
All males	0.061	0.513
All females	0.101	0.013
All mice	0.013	0.147

	Right kid-left kid	Right kid-weight
B6 K1 males	<0.001	0.901
B6 K1 females	<0.001	0.009
B6 K2 males	0.418	0.126
B6 K2 females	0.235	0.221
B6 K1 mice	<0.001	0.035
B6K2 mice	<0.001	0.628
All males	<0.001	0.384
All females	0.001	0.025
All mice	<0.001	0.083

	Right kid-heart	Right kid-weight
B6 K1 males	0.184	0.280
B6 K1 females	0.536	0.010
B6 K2 males	0.038	0.500
B6 K2 females	0.412	0.134
B6 K1 mice	0.364	0.015
B6K2 mice	0.403	0.030
All males	0.016	0.072
All females	0.573	0.040
All mice	0.072	0.003

	Right kid-lung	Right kid-weight
B6 K1 males	0.334	0.297
B6 K1 females	0.919	0.062
B6 K2 males	<0.001	0.862
B6 K2 females	0.053	0.536
B6 K1 mice	0.172	0.098
B6K2 mice	0.001	0.409
All males	0.015	0.114
All females	0.140	0.093
All mice	0.001	0.048

	Left kid-heart	Left kid-weight
B6 K1 males	0.398	0.057
B6 K1 females	0.067	0.125
B6 K2 males	0.034	0.582
B6 K2 females	0.469	0.037
B6 K1 mice	0.048	0.168
B6K2 mice	0.562	0.001
All males	0.370	0.009
All females	0.770	0.017
All mice	0.041	0.015

	Left kid-lung	Left kid-weight
B6 K1 males	0.190	0.114
B6 K1 females	0.443	0.703
B6 K2 males	<0.001	0.334
B6 K2 females	0.621	0.230
B6 K1 mice	0.012	0.961
B6K2 mice	0.011	0.083
All males	<0.001	0.049
All females	0.464	0.087
All mice	<0.001	0.167

	Heart-lung	Heart-weight
B6 K1 males	0.571	0.350
B6 K1 females	0.218	0.371
B6 K2 males	0.005	0.004
B6 K2 females	0.252	0.270
B6 K1 mice	0.708	0.852
B6K2 mice	0.050	0.024
All males	0.381	0.012
All females	0.528	0.299
All mice	0.410	0.068

Differences, such as the example above, between *Ped* plus and *Ped* minus in their correlation between specific organs and/or body weight are also presented graphically in Figures 6.10 and 6.11. In many cases, as seen in Figure 6.11, it was observed that the two data sets overlapped and the differences between the B6 K1 and B6 K2 correlations were not readily distinguishable. This reflects the fact that the p values given within Table 6.2 have been derived from data corrected during statistical analysis for the effect of individual animal weights and litter sizes, whereas the data presented within Figures 6.10 and 6.11 have not been. Thus they may not accurately display the significant differences present between the two strains.

It was also decided to examine whether organ:organ weight correlations for the B6 K1 mice were significantly different to those for the B6 K2 mice (Figures 6.12 to 6.15). There are significant differences between the B6 K1 and B6 K2 mice in the correlation between lung and liver weight (Figures 6.12 and 6.13). Figure 6.14 shows that there is a trend towards a significant difference between the correlation of the right kidney to lung weight between the B6 K1 and B6 K2 males ($p = 0.063$). Figure 6.15 shows that there is a significant difference between the correlation of the left kidney and lung weight between the B6 K1 and B6 K2 males.

Within Table 6.2, certain organs have been correlated with body weight on more than one occasion e.g. the left kidney. However, it was observed that the results for each analysis were not always the same. The explanation for these differences is due to the level of correlation that each organ had with the organ to which it was being correlated. Taking the example of the B6 K1 males and their correlation of the left kidney to liver or weight, it can be seen that there is a strong significant correlation between the left kidney and the liver. This means that body weight becomes a poorer predictor of kidney weight and so its correlation to kidney weight is weak. However, when the left kidney is then correlated to the heart, there is a weak organ to organ correlation. This then means that body weight has a larger influence on left kidney weight. Therefore, in predicting left kidney weight the liver weight is a better predictor than body weight, which in turn is a better predictor than the heart weight.

Figure 6.10

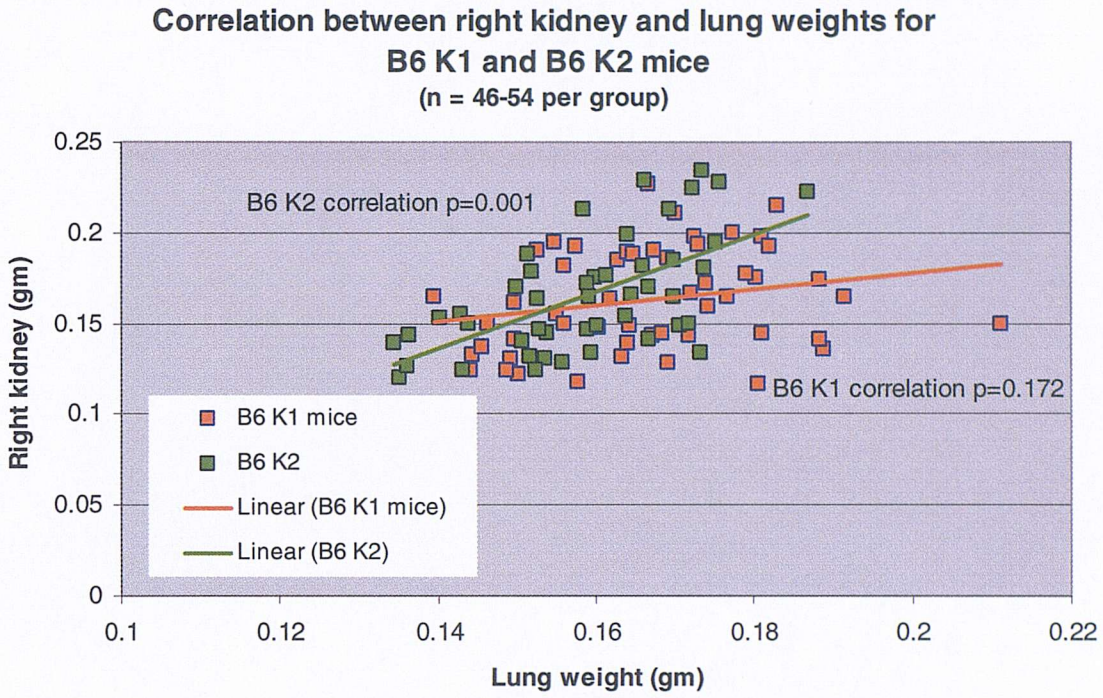


Figure 6.11

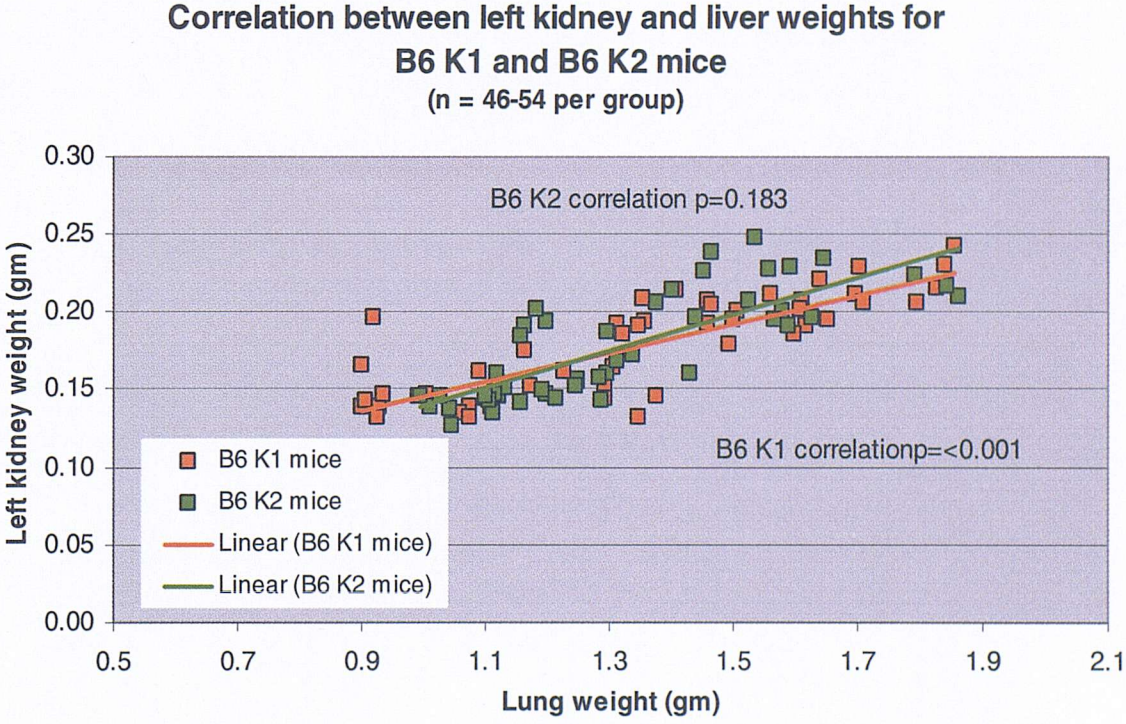


Figure 6.12

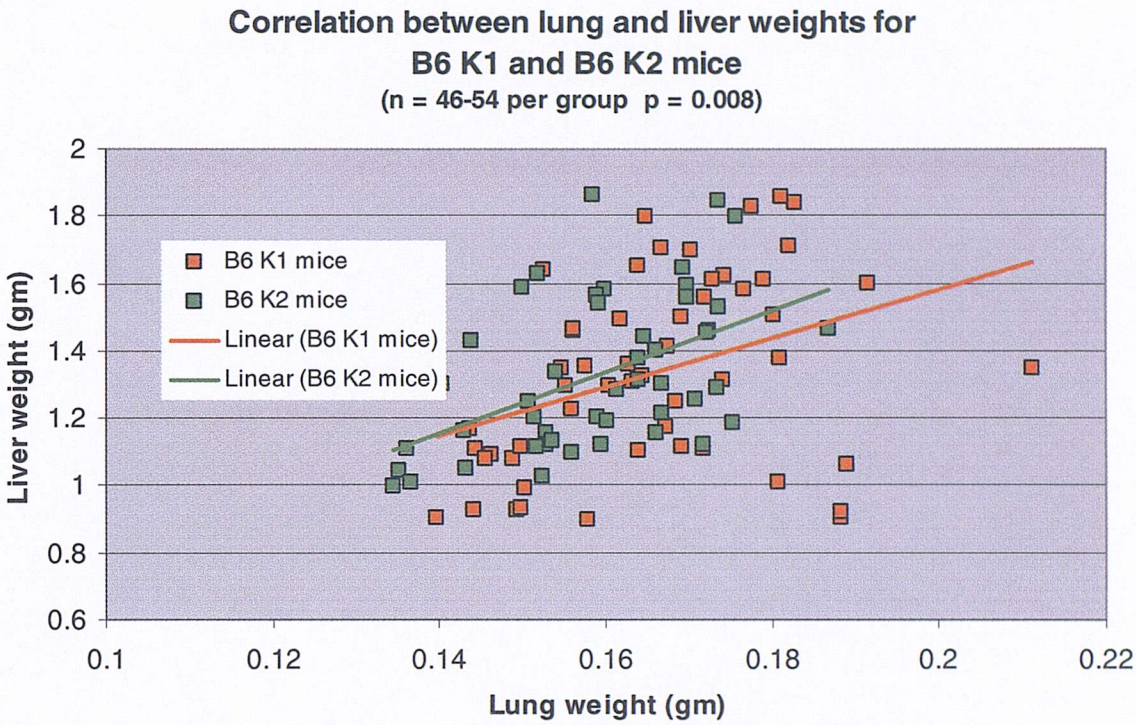


Figure 6.13

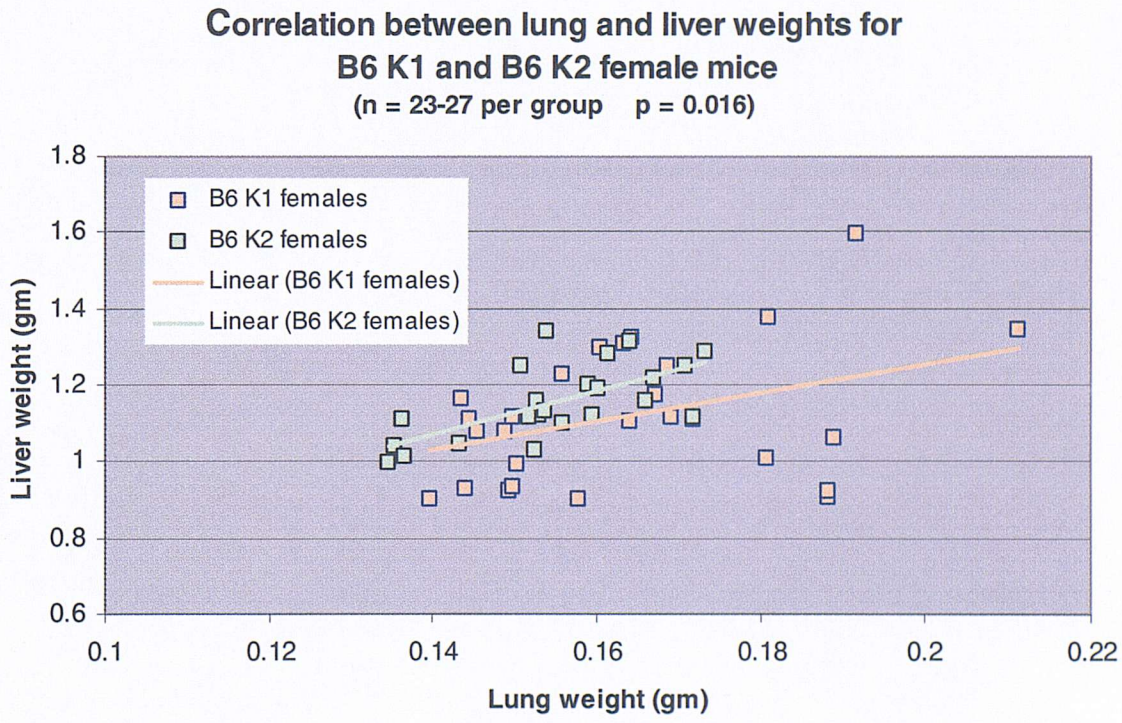


Figure 6.14

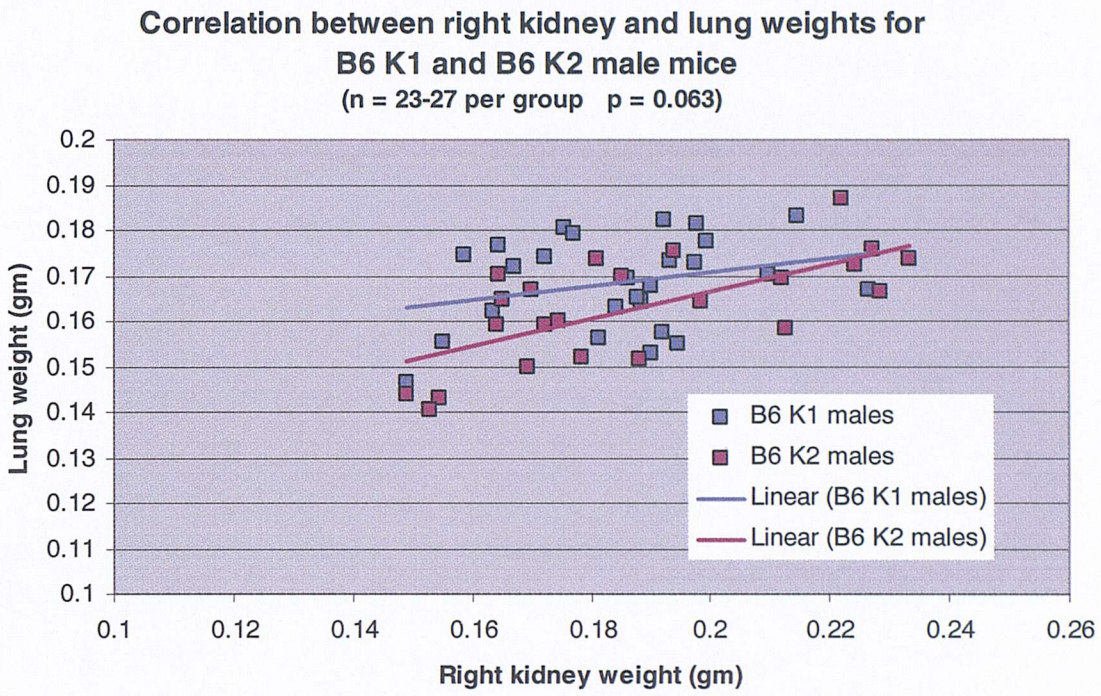
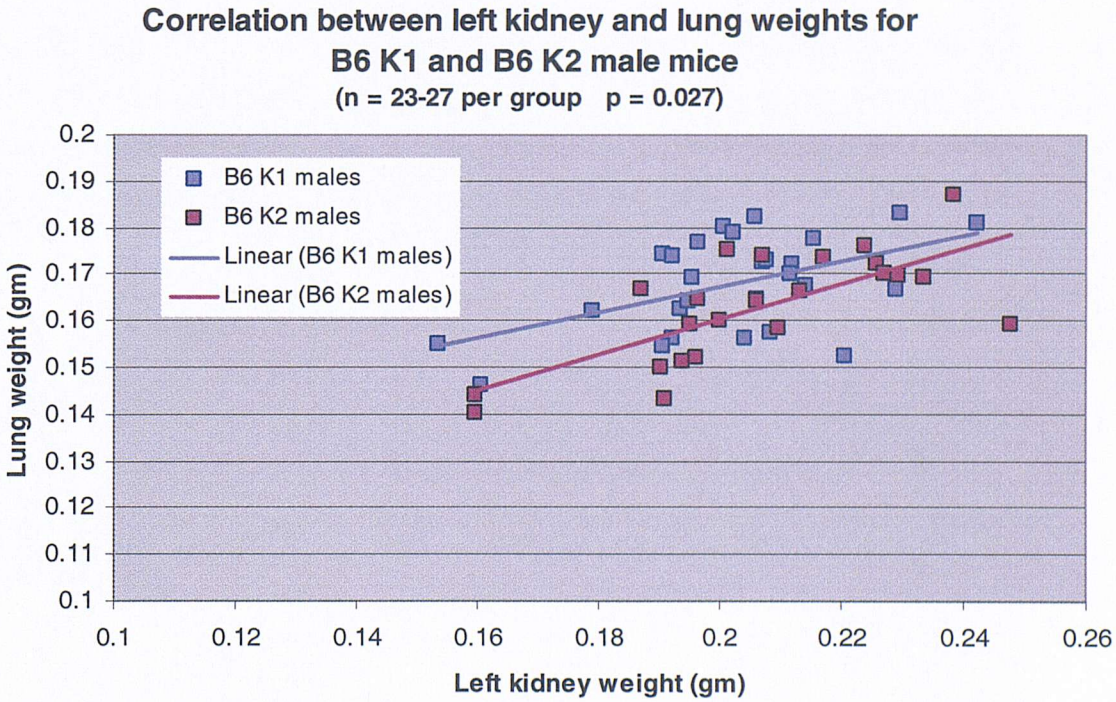


Figure 6.15



The mean ACE activity of B6 K1 and B6 K2 mice serum and lung tissue are shown in Figures 6.16 and 6.17. B6 K1 males have a significantly elevated serum ACE activity when compared to the B6 K2 males. No significant difference is observed between the females. If the data for the males and the females are combined then the B6 K1 mice have a significantly elevated serum ACE activity when compared to the B6 K1 mice ($p = 0.037$). Figure 6.17 shows the mean ACE activity from B6 K1 and B6 K2 mice lung extracts. As in Figure 6.16, the B6 K1 mice have an elevated activity, but these results do not reach statistical significance. If data for the B6 K1 males and the females are combined then a trend is observed ($p = 0.055$).

The correlation between serum ACE activity, lung ACE activity and body weight are shown in Figures 6.18 to 6.26. There is a slight negative correlation for the B6 K1 mice, so that as the serum ACE activity increases, then the lung activity decreases, the B6 K2 mice display a significant positive correlation. This positive correlation is primarily attributed to the correlation for the B6 K2 males ($p = 0.004$), as the B6 K2 females show no significant correlation between serum ACE and lung ACE activity ($p = 0.491$, Figures 6.19 and 6.20).

Figures 6.21 to 6.23 show that there is no significant correlation between serum ACE activity and body weight for the B6 K1 and B6 K2 mice. There are no significant correlations when the data for the B6 K1 and B6 K2 males and females are analysed independently of each other (Figure 6.22 and 6.23).

Figure 6.24 shows that there is no significant correlation between lung ACE activity and body weight for the B6 K1 and B6 K2 mice. However, when the data for the males and females are analysed independently, the B6 K2 males have a significant negative correlation, so that as body weight increases then lung ACE activity decreases ($p < 0.001$). There are no such correlations for the B6 K1 males or females, or for the B6 K2 females.

Figure 6.16

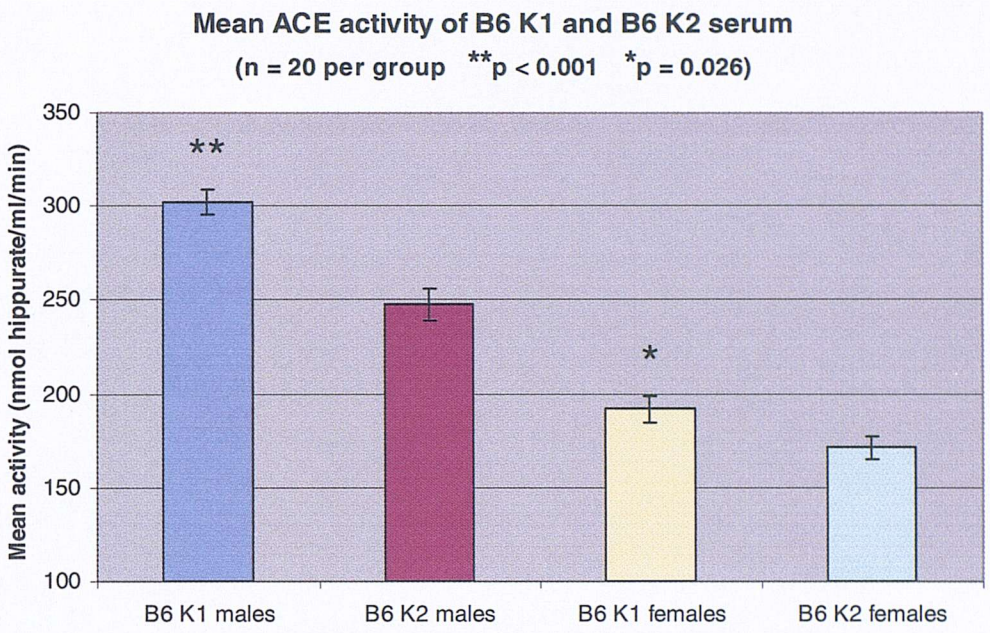


Figure 6.17

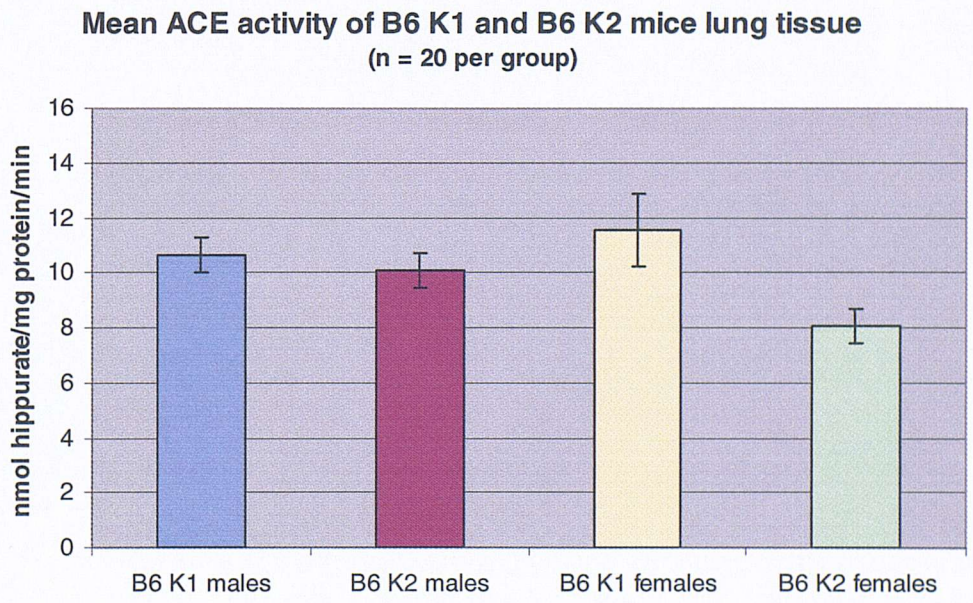
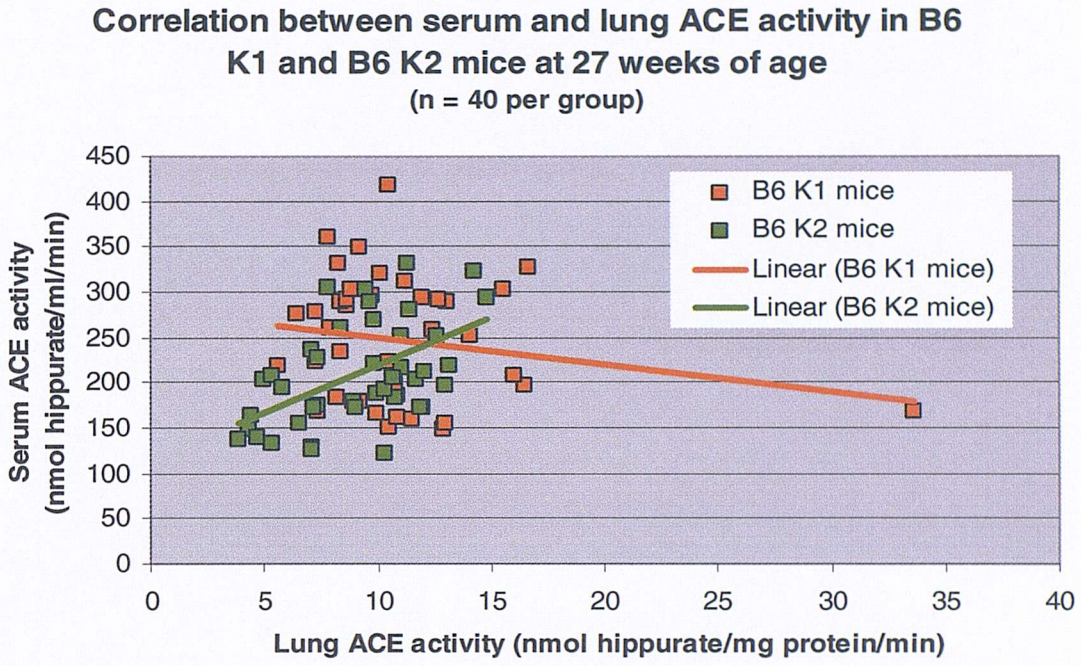
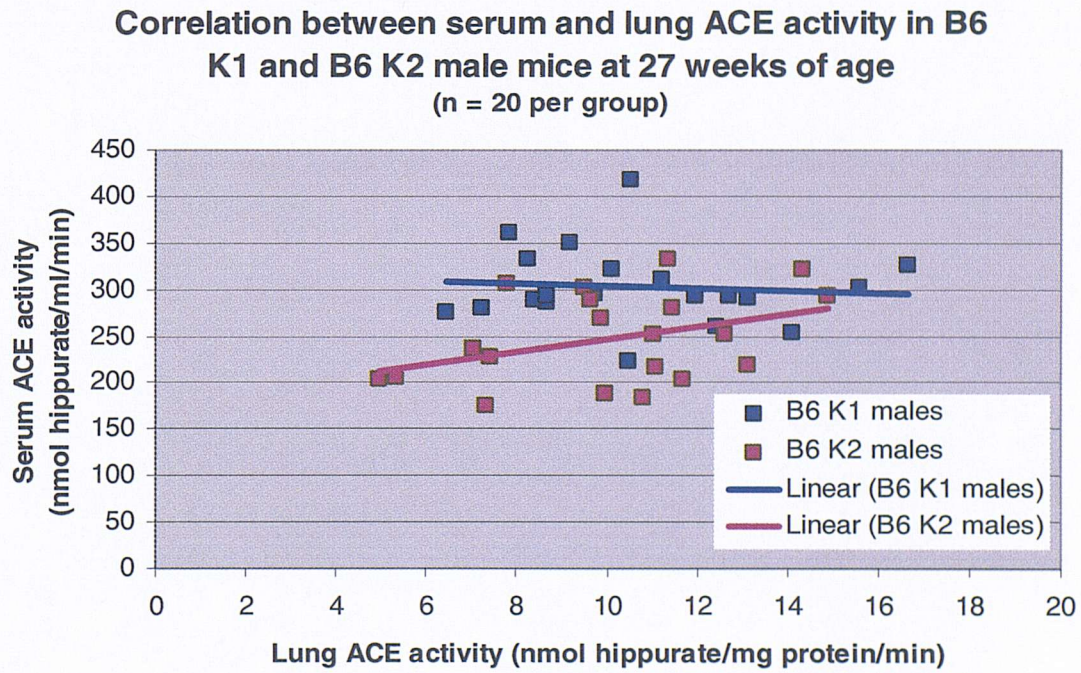


Figure 6.18



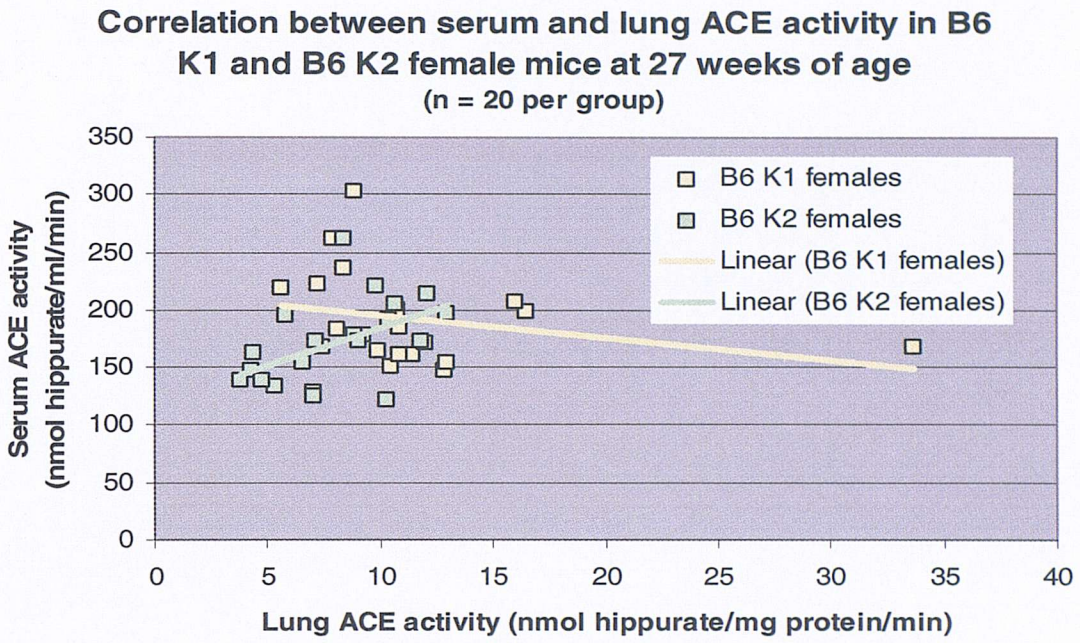
B6 K1 correlation p = 0.523 B6 K2 correlation p =0.018

Figure 6.19



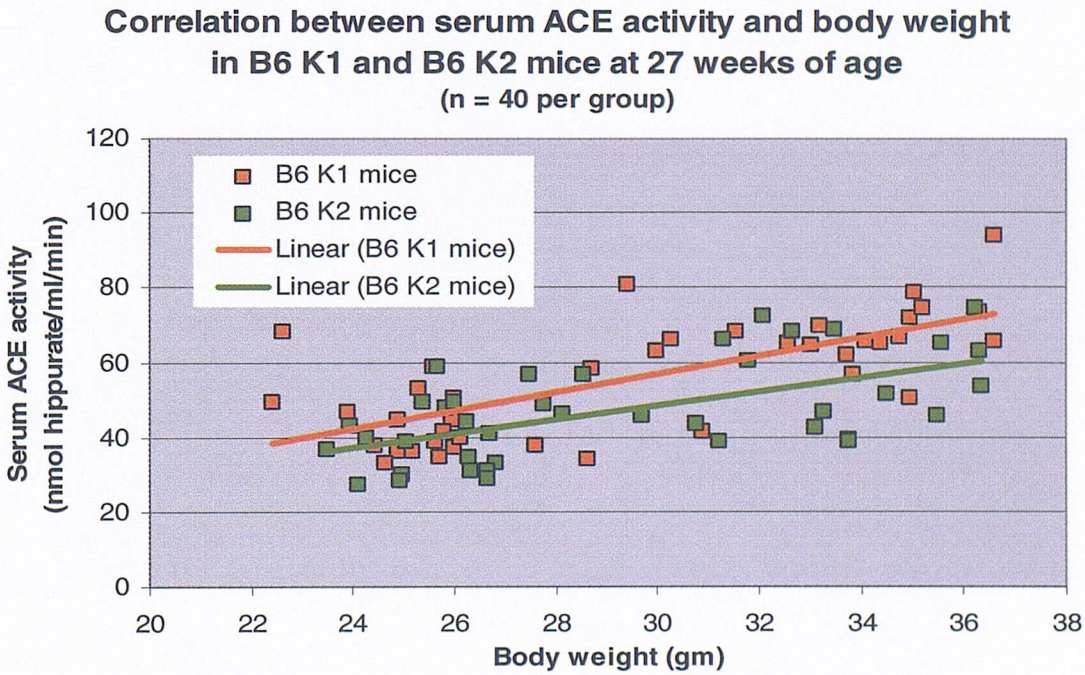
B6 K1 correlation p = 0.519 B6 K2 correlation p =0.004

Figure 6.20



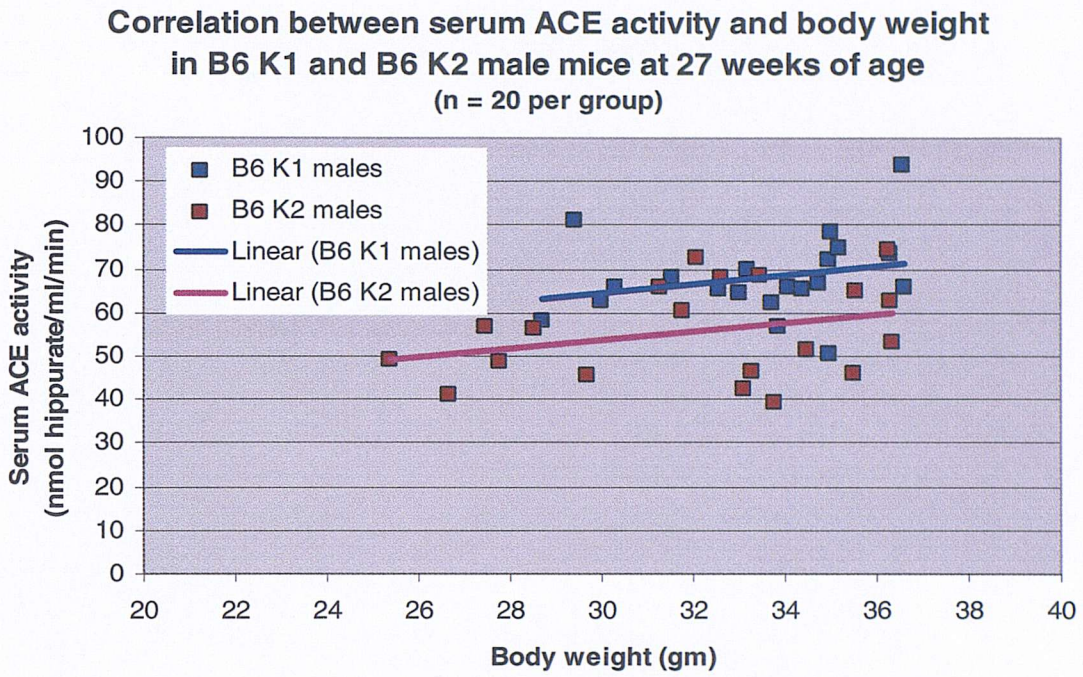
B6 K1 correlation p = 0.275 B6 K2 correlation p =0.491

Figure 6.21



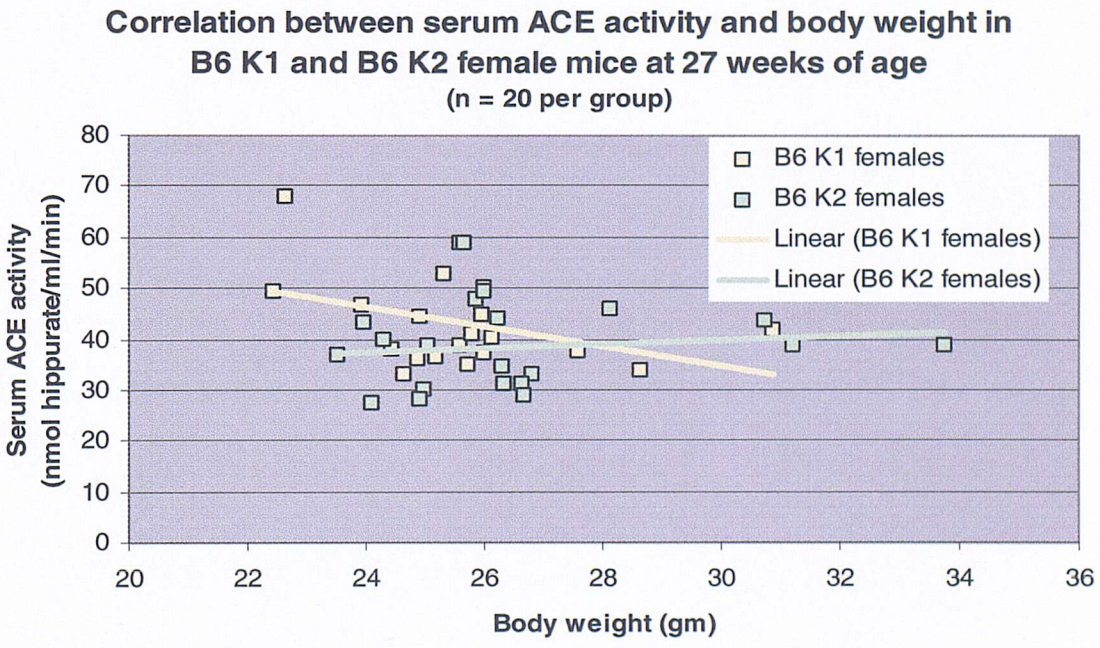
B6 K1 correlation p = 0.425 B6 K2 correlation p =0.204

Figure 6.22



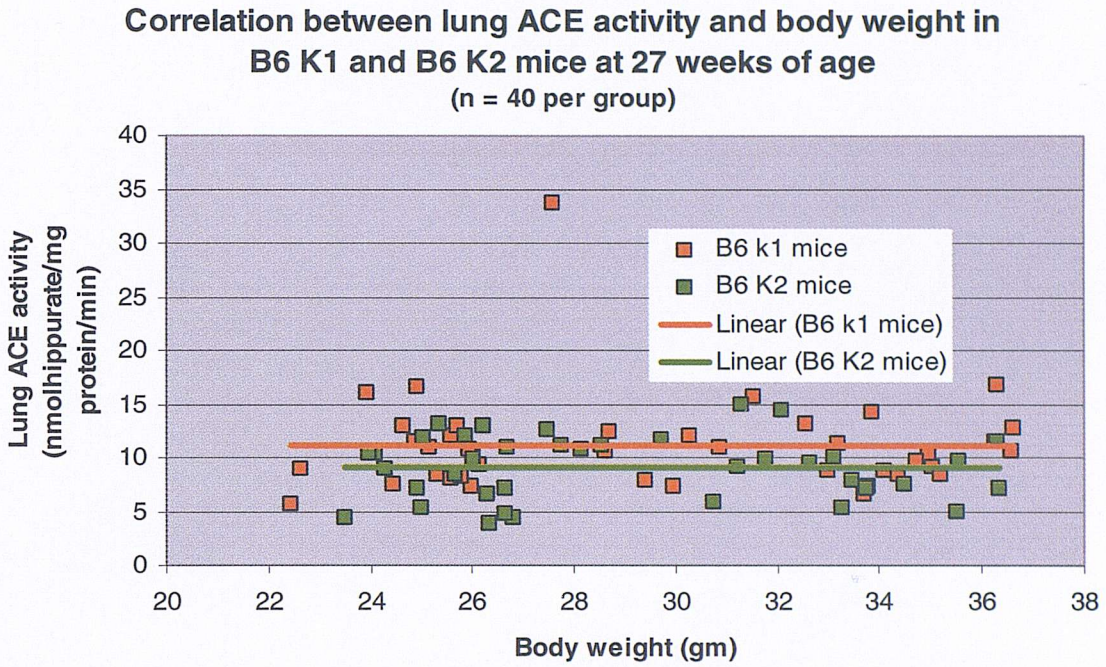
B6 K1 correlation p = 0.163 B6 K2 correlation p =0.120

Figure 6.23



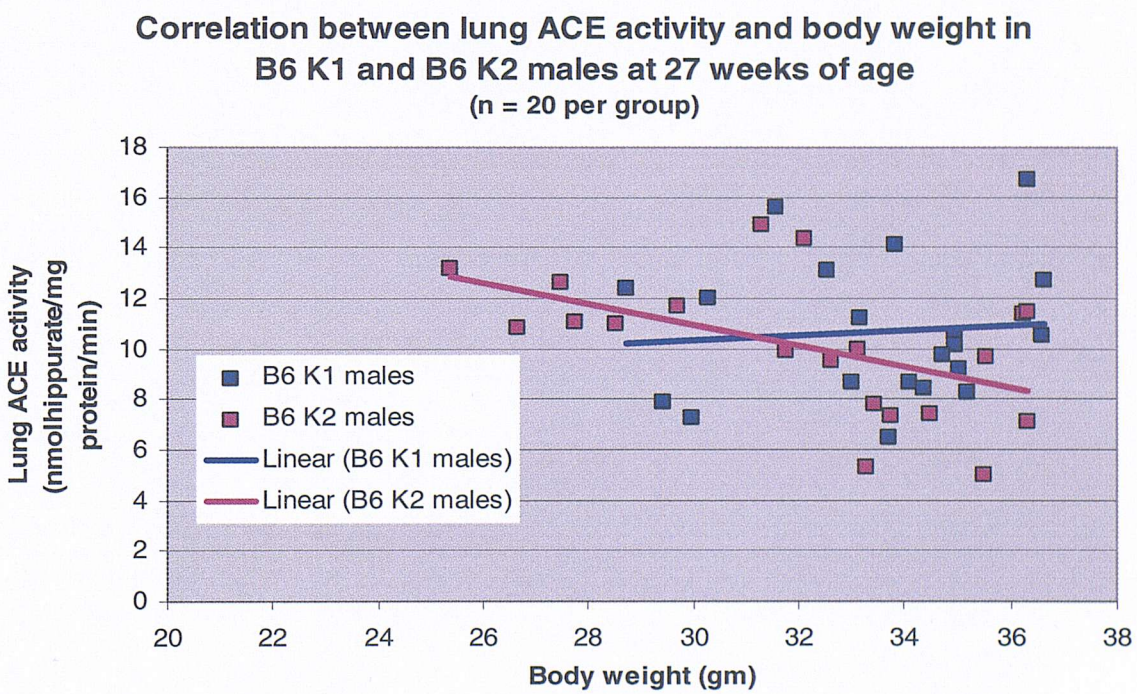
B6 K1 correlation p = 0.468 B6 K2 correlation p =0.508

Figure 6.24



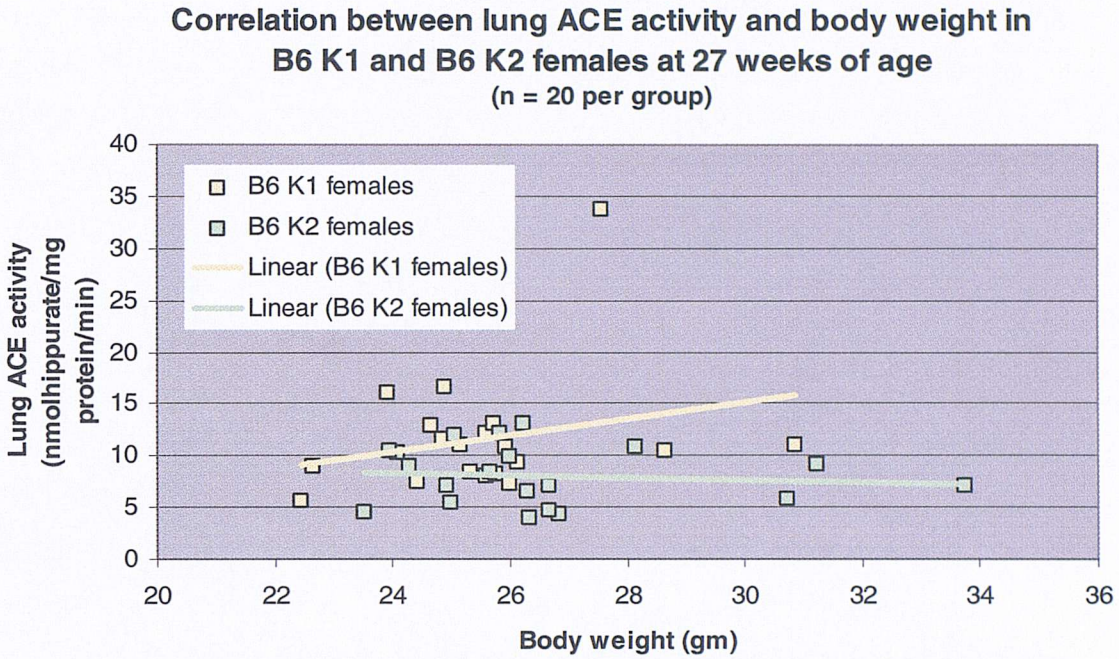
B6 K1 correlation p = 0.126 B6 K2 correlation p =0.126

Figure 6.25



B6 K1 correlation p = 0.823 B6 K2 correlation p < 0.001

Figure 6.26



B6 K1 correlation p = 0.464. B6 K2 correlation p =0.738

6.4 Discussion

Numerous animal and human studies have demonstrated that alterations to the normal pattern of embryonic or fetal development can have long term effects on postnatal health and physiology (Langley-Evans *et* al., 1996a, 1996b; Kwong *et al.*, 2000; Hales and Barer, 2001). In particular, a reduced rate of fetal development is associated with juvenile over compensatory catch-up growth resulting in adult obesity, increased risk of developing cardiovascular diseases and increased prevalence of NIDDM (Hales and Barker, 2001; Ozzane, 2001). It was therefore of interest to investigate the postnatal development and physiology of two congenic strains of mice, B6 K1 (*Ped* plus) and B6 K2 (*Ped* minus), which differ in the rate of their preimplantation embryo development influenced by the presence or absence of *Ped* gene expression.

As reported in previous studies, B6 K2 mice were heavier than B6 K1 mice at birth (Figure 6.1, Warner *et al.,* 1991, 1993). However, there were no significant differences in birth weight, litter size or weight at weaning, as had been previous been observed (Warner *et al.,* 1991, 1993; Exley and Warner, 1999). The data measured from individual animals reported within this thesis have been statistically analysed to take into account the between-mother and within-mother variation in litter size, thereby preventing any differences derived from abnormal litter sizes influencing the data. Previous studies reporting birth weights, litter sizes and weaning weights of B6 K1 and B6 K2 mice did not analyse their data in such a manner (Warner *et al.,* 1991, 1993; Exley and Warner, 1999). This is one possible explanation for the differences observed between the results reported within this thesis and those of previous studies.

It has been proposed that as *Ped* plus embryos appear more advanced and contain higher number of cells within the TE and ICM lineages when compared to *Ped* slow embryos, they may reach the uterus ahead of *Ped* minus embryos, have improved vascularised implantation sites leading to better placentation and so increased fetal development (Goldbard *et al.,* 1982; Warner *et al.,* 1987; Brownell

and Warner, 1988; McElhinny *et al*; 1998; Warner and Brenner, 2001). Studies in mice involving the asynchronous transfer of embryos have shown that more advanced embryos can implant earlier, and can give rise to offspring of increased birth weight (Doyle *et al.,* 1963; Aitken *et al.,* 1977; Marsk, 1977; Ueda *et al.*, 2003). As the *Ped* gene product displays many functional and structural similarities with the human HLA-G protein (Sharrow *et al.*, 1994; Lee *et al.*, 1995; Jurisicova *et al.*, 1996), embryos expressing the Qa-2 protein may be protected from unwanted maternal immune responses (Loake and King, 1991; Chumbley *et al.*, 1994). This may be an extra mechanism through which *Ped* gene expression results in increased levels of fetal development.

In contrast to increased rates of development, reduced rates of preimplantation embryo development have been linked with impaired fetal growth and altered patterns of postnatal physiology (Kwong *et al.*, 2000). It is hypothesised that if development in utero is restricted, the fetus will adapt by implementing a series of compensatory mechanisms for survival in a poor nutritional postnatal environment. If however, the postnatal environment then has an over abundance of nutrients, the offspring will undergo rapid postnatal catch-up growth, subsequently increasing the chances of developing cardiovascular diseases, obesity and NIDDM (Barker and Clark, 1997; reviewed in Ozanne, 2001). This hypothesis may explain the results observed from Figures 6.2, 6.3, 6.4 and 6.5, where between six and eleven weeks of age, both B6 K1 males and females became significantly heavier than B6 K2 mice, despite being initially lighter at birth (Figure 6.2). It was also observed that the B6 K1 males remained heavier for the remainder of the study. At 15 weeks of age, B6 K1 mice had a significantly reduced systolic blood pressure when compared to the B6 K2 mice. However, at this time it was not possible to obtain blood pressure measurements from a cohort of B6 K1 mice, and the results may have been affected by the reduced number of observations. In contrast to the data at 15 weeks of age, at 21 weeks of age the B6 K1 males and females have a significantly elevated systolic blood pressure when compared to the B6 K2 males and females. It would therefore appear that the reduced birth weight and subsequent postnatal catch up growth have

resulted in the B6 K1 mice developing with an elevated systolic blood pressure at specific times during adult life.

As hypertension is a multifactorial disease its origin may lie in more than one cause, one being altered organ allometry. It was observed that the B6 K1 females had a significantly elevated lung:body weight ratio compared to the B6 K2 females (Figure 6.7). The B6 K1 females also had an elevated ratio for their left and right kidney and their hearts, although these ratios did not reach statistical significance. B6 K1 males had a lower ratio for all of their organs when compared to the B6 K2 males (Figure 6.6). During periods of impaired fetal development, the fetus makes compensatory adaptations in order to maintain the development of vital organs such as the brain. These compensations have been shown to have negative impacts on the growth and development of other organ structures (Hales *et al.*, 1991; Kwong *et al.*, 2000; Whorwood *et al.*, 2001).

Another factor that influences postnatal blood pressure is ACE activity. ACE cleaves angiotensin I to yield angiotensin II, a potent vasoconstrictor, thus resulting in the raising of blood pressure. ACE -/- mice are hypotensive, having a systolic blood pressure ~ 30 mm Hg below wild type mice (Esther *et* al., 1997; Klein *et al.*, 2002). It may therefore be hypothesised that an increased level of ACE enzyme, or an increased activity of existing ACE, may result in an elevated blood pressure. Figure 6.12 show that the B6 K1 males have a significantly elevated serum ACE activity when compared to the B6 K2 males. It can also be seen that the B6 K1 females have an elevated serum ACE activity, although this did not reach statistical significance. However, when the data for the males and females were combined, the B6 K1 mice have a significantly elevated serum ACE activity when compared to the B6 K2 mice. Figure 6.13 shows that whilst the B6 K1 mice have elevated levels of lung ACE activity, there is no significant difference between the two strains. Previous studies have shown that the expression profile of ACE appears to be important in the regulation of blood pressure. In humans, studies have shown that serum ACE levels are significantly higher in hypertensives as compared to normotensives. There is also

a positive correlation to body mass index (BMI) in males and females, and serum ACE activity correlates positively with plasma angiotensin II levels (Forrester *et al.*, 1997; Nystrom *et al.*, 1997).

As lung ACE activity is negatively correlated to body weight for the B6 K2 mice, and as serum ACE activity is positively correlated to lung ACE, then, it may be assumed that as body weight increases, overall ACE activity decreases. This reduced activity could be one factor in the lower systolic blood pressure observed for the B6 K2 mice when compared to the B6 K1 mice. *In vivo*, serum ACE is derived from the cleavage of endothelial bound somatic ACE, predominantly that of the lung (Belent *et* al., 1995; Sadhukhan *et al* 1998; Woodman *et al.*, 2000; Eyries *et al.*, 2001). It may therefore be hypothesised that the more ACE that is expressed on the surface of the epithelia, then the more ACE will be available for cleavage, so raising serum ACE protein and activity levels. However, for the B6 K1 mice as lung ACE activity increases, the serum ACE activity falls slightly. There could be several possible reasons for this observation. One possibility could be a decrease in the amount of enzymatic activity of the secreatases. This would mean that cleavage of ACE from the epithelial surface would take place at a slower rate than the expression of ACE. Alternatively, upon cleavage the activity of the serum ACE could become modified so reducing its activity. However, B6 K1 mice had elevated serum ACE activity when compared to the B6 K2 mice. It may therefore be the case that the serum ACE may derive from another source such as the vasculature or the kidneys.

6.5 Conclusions

In conclusion, it has been observed that the different rates of preimplantation development between the *Ped* plus and *Ped* minus embryos produces differences in postnatal development and physiology. One explanation could be that the *Ped* minus mice have developed some aspects of the "metabolic syndrome", in that they are lighter at birth, go through a period of postnatal catch-up growth and become heavier in adult life. At 21 weeks of age, the *Ped* minus mice have elevated systolic blood pressure possibly attributed to altered patterns of serum and lung ACE activity, or due

to the differences in organ:body weight ratios between the two strains. It would therefore appear that an altered rate of preimplantation embryo development can have long term consequences on postnatal health and physiology.

From the observations reported within this Chapter, several possibilities for future work have emerged. It would be of great interest to perform embryo transfer studies between the B6 K1 and B6 K2 mice. This would help address the influence of the *Ped* plus or minus maternal uterine environment on postnatal development, and allow for the manipulation of litter sizes. It would then be possible to increase the litter size of the B6 K2 mice to examine whether an increased birth weight is still observed. It would also be of interest to examine possible mechanisms for the differences in blood pressure further. Techniques such as Western blotting would allow for the quantification of expressed proteins involved in blood pressure regulation such as the type 1 angiotensin II receptor (AT1), mineralocorticoid receptor and the glucocorticoid receptor. Further examination of the actions of ACE and its interactions using specific inhibitors such as Captopril would also be of interest. Other aspects of the renin-angiotensin system could also be studied in similar way to those outlined above.

Chapter 7
General discussion

7.1 Preimplantation development of *in vitro* cultured and manipulated embryos.

In vitro cultured embryos were observed to develop at a slower rate and with significantly lower total cell numbers when compared to *in vivo* derived embryos, for the same time post hCG. *In vitro* cultured embryos were retarded in their development by approximately 20 hours when compared to embryos developing *in vivo* (Figure 3.1). These findings are line with those of previous studies (Harlow and Quinn, 1982, Sakkas *et al.*, 1989, Gardner and Leese, 1990). Despite the slower developmental rate, the ICM:TE ratio was equivalent between these two groups (Figure 3.2). Therefore, although rates of cell division were slower, mechanisms allocating cells to the ICM and TE lineages were unaffected.

Slower rates of *in vitro* preimplantation development could be attributed to the culture medium providing a less than optimal environment for the different stages of preimplantation embryo development. Mammalian embryos have different metabolic and environmental requirements during preimplantation development (Gardner, 1998). In the experiments reported within this thesis, all embryos were cultured in a single medium. Therefore, whilst at specific developmental stages the embryo's needs would be catered for, at other stages the medium would be of a less optimal composition.

Comparison of preimplantation development in media supplemented with, or devoid of, exogenous protein, revealed no significant difference in blastocyst cell numbers or ICM:TE ratios. As BSA is believed to provide a source of fixed nitrogen, free amino acids and trace levels of growth factors, it might have been anticipated that embryos developing in the absence of BSA (those embryos cultured in T6 PVP) would be at a developmental disadvantage when compared to embryos cultured in the presence of BSA. Previous studies have demonstrated that the preimplantation embryo is capable of developing to the blastocyst stage in a medium devoid of protein

(Sellens *et al.*, 1981). It was hypothesised that these embryos would exhaust their own endogenous stores of amino acids and protein in order to sustain development. As those embryos cultured in T6 PVP were analysed at the blastocyst stage, any impairment in their development may not yet have been manifested at the blastocsyt stage. Analysis of the postimplantation developmental capacity would therefore be a necessary subsequent study to those already performed.

Embryo culture in media supplemented with insulin and IGF-I concentrations of 170 nM and 1.7 nM respectively, or above, resulted in significant increases in ICM cell numbers and ICM:TE ratios (Section 3.3.2 to 3.3.4). These findings are in line with those of previous studies (Harvey and Kaye, 1990, 1992; Dunglinson and Kaye, 1993; Smith *et al.*, 1993; Pantaleon and Kaye, 1996). However, ICM:TE ratios were not altered by culture in medium containing BSA. Insulin and IGF-I both had their maximal effect on developmental rates post compaction, possibly by increasing levels of embryo metabolism. After compaction, the mouse embryo predominantly metabolises glucose, as well as dramatically increases protein synthesis levels (Brinster, 1967; Epstein and Smith, 1973; Sellens *et al.*, 1981). The binding of both insulin and IGF-I to their receptors increases the level of endocytosis within the mouse preimplantation embryo (Heyner *et al*; 1989, 1993; Dunglinson *et al.*, 1995). As the receptors are located upon the apical and basolateral surfaces of the TE, and on the cells of the ICM (Heyner *et al*; 1989, 1993; Dunglinson *et al.*, 1995), insulin and IGF-I could stimulate an increased delivery of exogenous protein, growth factors and metabolites via the TE to the developing ICM, thus inducing the increased rate of cell proliferation observed within the ICM.

Embryos manipulated to contain double or half the normal numbers of cells at the 8-cell stage, had significantly altered total blastocyst cell numbers and ICM:TE ratios when compared to control blastocysts (Figures 3.25 and 3.26). If the size of the embryo is increased, then it would be expected the volume will increase disproportionately to the surface area. In double-sized embryos, this will result in proportionately more cells located inside the embryo relative to its surface, and

therefore a larger than normal population of cells from which to form the ICM. In half sized embryos it would be expected that the majority of the cells would be located on the outer face of the embryo, and so should develop with a significantly reduced ICM:TE ratio. At the time of analysis, the manipulated embryos had not yet begun to compensate for the altered total cell numbers as they still contained approximately double and half the total cell numbers of the controls. It would therefore be of future interest to examine the disaggregated embryos at later time points than those within this thesis, to examine at what time embryo size and cell number remodelling takes place.

An additional point for consideration with the reaggregated embryos is the disruption to the spatial organisation of the blastomeres within the embryo. The different daughter cells from the 2-cell stage blastomeres differentially contribute to the ICM and TE lineages. Therefore, dissaggregation and reaggregation at the 8-cell stage could result in the altered positioning of predisposed blastomeres within the embryo. Due to the different allocation patterns of the daughter blastomeres, the embryonic-abembryonic axis is already established by the time of disaggregation. Whilst disaggregated and reaggregated embryos are able to proceed through embryonic and fetal development, giving rise to viable offspring (Biggers and Papaioannou, 1992), the altered spatial organisation of predisposed lineage blastomeres to alternative regions of the embryo, and /or the destruction of established embryonic axes could cause subtle changes in cell allocation patterns, possibly affecting patterns of postimplantation, fetal or postnatal development.

Blastocyst-like structures generated from the aggregation of three isolated ICMs had significantly reduced total numbers of cells, but a significantly increased ICM:TE ratio caused by a significant reduction in the number of regenerated TE cells and a significant increase in the number of ICM cells (Figures 3.27 and 3.28). The high ICM:TE ratio could be attributed to the way in which the outer cells on the isolated ICM differentiate and encompass the ICM. Upon isolation, those cells located upon the outer face of the aggregate would have asymmetrical cell-cell

contact patterns. This will result in the up-regulation of a TE-like phenotype within these cells. However, if a small proportion of the cells located upon the outer face of the ICM take on a TE-like phenotype and subsequently encapsulate the remaining cells of the ICM, re-establishing total cell-cell contact within the other cells of the ICM, thus inhibiting them from expressing a TE-like phenotype. This would result in the generation of a blastocyst-like structure containing relatively fewer numbers of outer cells surrounding a proportionately larger non-polar inner cell population.

A second explanation could be a relatively low rate of cell proliferation within the isolated and aggregated ICMs. Previous studied have reported low rates of cell division within aggregated ICMs for up to 24 hours after isolation (Spindle, 1978). As the blastocyst-like structures reported within this thesis were only 20 hours after isolation and aggregation, the level of cell proliferation within single and aggregated ICMs may still have been low, resulting in the reduced total blastocyst cell number.

As the results from Chapter 3 revealed, the development of the preimplantation embryo could be altered through various manipulations. The predominant question arising from these findings was how would the subsequent postnatal growth profiles and physiology of offspring arising from these embryos be affected. Although not performed within this thesis, it would have been of great interest to have studied the fetal development of embryos manipulated with the above treatments. This would have given insight into implantation rates, fetal development and growth, and through studies of placental tissues it would have been possible to examine fetal-maternal exchange and communication.

7.2 Environment of the preimplantation embryo and its effects on birth weight and litter size

Within the experiments of Chapters 4 and 5, predominantly the largest factor affecting weight at birth was litter size. A negative correlation was shown to exist between litter size and birth weight, such that as litter size increased the mean birth weight within that litter decreased. These findings are in agreement with previous

studies (Epstein, 1977). No significant difference in litter size or birth weight were observed between *in vivo* and *in vitro* derived mice (Figures 4.2 and 4.3, $p = 0.116$ and $p = 0.656$ respectively). It may have been anticipated that the slower rates of preimplantation embryo development observed for *in vitro* cultured embryos might disadvantage their postimplantation and fetal development, resulting in altered birth weights. However, previous studies have shown that the mouse preimplantation embryo is capable of compensating for altered cell numbers and developmental rates, such that birth weight is not altered (Rands, 1986a, 1986b; Somers *et al.*, 1992; Biggers and Papaioannou 1992; Hardy and Handyside, 1993).

Previous studies have shown that *in vitro* culture not only affects preimplantation embryo development, but can also impact upon aspects of fetal and postnatal development. Altered patterns of preimplantation embryo development have been observed to alter the rate of fetal growth, alter patterns of gene expression, increase birth weights and incidences of postnatal mortality (Bowman and McLaren, 1970; Harlow and Quinn, 1982; Thompson *et al.*, 1995, Holm *et al.*, 1996, Young *et al.*, 1998, Sinclair *et al.*, 1999; Koshla *et al.*, 2001).

Mice developing from embryos cultured in the presence of insulin or IGF-1 had elevated birth weights when compared to control animals (Figures 5.2 and 5.3), which are in line with previous studies (Kaye and Gardner, 1999). However, the growth factor manipulated mice within this thesis came from dramatically smaller litters than those of other treatment groups within the same study. Therefore, the litter size would be having a substantial effect on birth weight. The low mean birth weight of the naturally mated and superovulated groups could also be attributed to the size of the litters from which they came. For this reason, the naturally mated groups were analysed together so that the effect of adjusting the mean litter size after birth could be assessed. Despite there being no significant difference in litter size between the three naturally mated groups (prior to postnatal adjustment), those mice who later had their litter size adjusted to 4 had a significantly lower mean birth weight when compared to the other naturally mated groups.

One striking factor that emerged from the embryo transfer experiments was that a maximal implantation and gestation rate of only 66% was observed for both *in vitro* and *in vivo* derived embryos (maximum litter size observed of 8). For offspring developing from physically manipulated or growth factor cultured embryos the average litter size was approximately 4 (Figure 5.4). Two possible conclusions were drawn from these findings; (1) that although the *in vitro* derived embryos had a slower rate of development, this did not affect their ability to implant and develop into viable offspring; (2) exposure to an *in vitro* environment, regardless of the duration affects the ability on an embryo to implant and develop into a viable offspring. From these conclusions it may appear that the mouse preimplantation embryo is extremely sensitive to its environment, and that some aspect of *in vitro* culture is affecting the ability of the embryo to implant and develop. This low rate of postimplantation development is still apparent despite the increased rates of preimplantation development caused by the addition of growth factors to the culture medium. The suggestion that the embryo might be more susceptible to its environment is drawn from the fact that at this point in time, disaggregating and reaggregating embryos resulted in the production of larger mean litter sizes when compared to those derived from *in vitro* culture in the presence of growth factors. As well as this, there was no significant difference in birth weight between the diaggregated embryo group and the *in vivo* group (Figures 5.27 and 5.28). It may therefore be concluded that environmental manipulation may have a greater influence on postimplantation survival and development than physical manipulation.

7.3 Environment of the preimplantation embryo and its effect on postnatal growth

In Chapter 4, it was observed that during specific periods of early postnatal growth and development, the superovulated, naturally mated and *in vitro* groups all became significantly lighter than the naturally mated (6) males and females (Figure 4.5, Tables 4.1 and 4.2). At 2, 11 and 16 weeks of age, the *in vivo* mice were significantly heavier than the *in vitro* mice. At no other times were significant differences observed between the *in vivo* and *in vitro* animals. During the statistical

analysis of the data it was possible to assess the influence of litter size at birth and the subsequent litter size after adjustment. Litter size was only a significant influence on birth weight. At no other time did litter size have any significant influence on the growth profile results for any of the treatment groups analysed.

Altered patterns of postnatal growth and development occurring from periods of *in vitro* culture have been previously observed. The culture and transfer of sheep and cattle embryos has been associated with the phenomenon of LOS (Thompson *et al.*, 1995, Holm *et al.*, 1996, Young *et al.*, 1998, Sinclair *et al.*, 1999; Jacobsen *et al.*, 2000). However, offspring developing from mouse embryos manipulated to contain increased or decreased numbers of cells, have similar birth weights to those of controls (Tsunoda and McLaren, 1983: Biggers and Papaioannou, 1989, 1991).

Upon analysis of the three naturally mated groups, those mice whom had their litter size adjusted at birth became significantly heavier at 2, 3 and 4 weeks of age when compared to those mice who had no litter size adjustment. As there was no significant difference in mean birth litter size, it would be anticipated that all of the naturally mated groups would have received similar *in utero* levels of nutrition. Therefore, each group would have anticipated to the same extent the level of postnatal nutrient availability. However, those mice from litters adjusted at birth would have altered levels of postnatal nutrient availability to that which they had predicted *in utero*. This increase in nutrient availability could have caused over compensatory growth, resulting in the increased weight observed within these groups at 2, 3 and 4 weeks of age. At no other time were any significant differences between any of the naturally mated groups observed. This would imply that the adjusting of litter size does not have any significant long term effect on postnatal growth rates.

At 2 weeks of age, the PVP males were significantly lighter than the naturally mated (6) males. At 10, 12 and 13 weeks of age, the IGF-I males were significantly lighter than the naturally mated (4) males ($p < 0.05$). At 1, 2, 3 and 13 weeks of age, the PVP females were significantly lighter than the naturally mated (4) females

($p < 0.05$). Due to the low numbers of animals within the insulin and IGF-1treatment groups, it was not possible to analyse the growth patterns of these animals in relation to each other, or in relation to the PVP males and females.

As the majority of the differences on birth weight were observed to occur during the earlier periods of development, environmental stresses endured during this time could have affected development. Changes in stress-induced corticosterone levels have been observed in Long-Evans rats as a consequence of neonatal maternal separation and neonatal handling (Durand *et al.*, 1998; Kalinichev *et al.*, 2002). It may be the case that certain experimental groups are affected by the weekly weighing and short term maternal separation. This increase in stress levels and responses could affect weight gain. After a period of repeated exposure to the weighing procedure, the mice become accustomed and so the differences in weight are no longer observed.

Male offspring derived from disaggregated embryos became significantly heavier than other male groups between 12 and 17 weeks of age (Figure 5.29). In particular, they became significantly heavier than the *in vitro* males. This finding would suggest that disruption to the normal spatial organisation and/or the destruction of the already established embryonic-abembryonic axis results in significantly increased postnatal weight gain.

7.4 Environment of the preimplantation embryo and its effect on postnatal systolic blood pressure

Previous studies have shown that the environment in which the preimplantation embryo develops can affect postnatal systolic blood pressure. Offspring from female rats fed a low protein diet exclusively during the preimplantation period have been shown to have raised systolic blood pressure in later life (Kwong *et al.*, 2000).

From the results displayed within Chapter 4, it would appear that at all times analysed, those offspring developing from embryos exposed to an *in vitro*

environment had an elevated systolic blood pressure when compared to non-embryo transferred offspring. At 8 and 15 weeks of age, the *in vivo* females had a significantly elevated blood pressure when compared to the both naturally mated groups, whilst the *in vivo* males had a significantly elevated blood pressure at 15 weeks of age. At 21 weeks of age, the *in vitro* mice have significantly elevated blood pressure when compared to all the other treatment groups, whilst the *in vivo* males and females are not significantly different from the two naturally mated groups. Again, it would appear that even though the *in vivo* group were only exposed to an *in vitro* environment for a relatively short period of time, 90-180 minutes, this appears to be a sufficient enough insult to programme an increased blood pressure during early periods of postnatal growth. However, prolonged exposure to an *in vitro* environment results in elevated systolic blood pressure during periods of later postnatal life. These data could have possible implications for the field of assisted reproductive techniques.

The possibility that exposure to an *in vitro* environment for either short or prolonged periods of time could predispose the offspring to elevated systolic blood pressure is of potential concern. Although there are limited data regarding the postnatal development of children conceived via ART, especially with regard to their postnatal systolic blood pressure, there are data to suggest that children conceived through ART are more likely to be born premature, to have a lower birth weight (<2500g) and to have a higher incidence of some congenital abnormalities (D'Souza *et al.*, 1997; Bergh *et al.*, 1999; Tarlatiz and Grimbizis, 1999). The present data might suggest that systolic blood pressure may also be prone to alteration as a consequence of embryo manipulation and/or culture. It will therefore be of great interest to follow data collected from children conceived via ART as they develop into middle age, to see whether these people are more prone to developing aspects of the metabolic syndrome, in particular elevated systolic blood pressure.

Previous experiments have suggested a correlation between low birth weight and a higher adult postnatal systolic blood pressure (Barker *et al.*, 1990: Barker 1995;

Forsen *et al.*, 1997; Rich-Edwards *et al.*, 1997). In the data reported within Chapter 4, the reverse is observed, in that those animals with the largest birth weights had the highest blood pressures. However, recent re-evaluation of the data from previous studies has suggested that the inverse relationship between birth weight and blood pressure may not be as reliable as previously thought (Huxley *et al.*, 2002). Therefore, in the context of the experiments within this thesis, it appears that the environment of the early embryo may have more of an effect on postnatal development than factors such as birth weight.

There were no significant differences between any of the treatment groups in the activity of ACE within the serum. However, the *in vitro* mice (which had the highest blood pressure at 21 weeks of age) did have the highest serum ACE activity. Some studies have shown that serum ACE levels in humans are significantly higher in hypertensives as compared to normotensives, and that serum ACE levels positively correlate to BMI scores in males and females (Forrester *et al.*, 1997; Nystrom *et al.*, 1997).

At 8 weeks of age, both the naturally mated (4) and (6) groups have an elevated systolic blood pressure when compared to the naturally mated group (Figures 5.15 and 5.16). These results could have been caused by the increased growth rates of these two groups observed during early postnatal development (Figures 5.7 to 5.10) leading to the increased systolic blood pressure observed at 8 weeks of age. Postnatal over-compensatory growth has been demonstrated to increase the chances of developing cardiovascular disease and hypertension in later life (Barker and Clark, 1997; reviewed in Ozanne, 2001).

At 21 weeks of age, the IGF-I and the PVP females had a significantly elevated systolic blood pressure when compared to the naturally mated (4) females. The increased systolic blood pressure for the PVP females could be linked to the first three postnatal weeks where they were significantly lighter than the naturally mated (4) females. For the female groups, there was no difference in blood pressure at 8 or

15 weeks of age. However, the naturally mated (6) females do have the lowest blood pressure of any of the groups at all three ages studied. There were no significant differences between any of the male groups studied for any of the times. It should again be stated that the numbers of offspring within some of the treatment groups is low at this point in time. Therefore, as more numbers of animals are added to this long-term study, significant differences that are present now may no be present later, and significant differences that are not present at this point in time may well become evident.

Male offspring derived from disaggregated embryos had a significantly elevated systolic blood pressure at 15 and 21 weeks of age when compared to the naturally mated (6) males (Figure 5.31), whilst the female disaggregated embryo group had a significantly elevated blood pressure at 8 weeks if age (Figure 5.32). For the male offspring in particular, the increased systolic blood pressure occurs at the same time as the observed significant increase in weight.

7.5 Environment of the preimplantation embryo and its effect on postnatal organ weight

Embryo culture in the presence of serum (sheep and cattle) has been shown to result in changes tothe normal size of internal organs (Thompson *et al.*, 1995, Holm *et al.*, 1996, Young *et al.*, 1998, Sinclair *et al.*, 1999; Jacobsen *et al.*, 2000). Offspring from rats fed a low protein diet exclusively during the preimplantation period of development have been shown to have disproportionate growth of specific organs compared to body weight (Kwong *et al.*, 2000).

In vitro males were observed to have an elevated liver:body weight ratio when compared to the *in vivo* males, whilst the naturally mated (6) females were observed to have an elevated liver:body weight ratio when compared to the *in vitro* females. There were no other significant differences between any of the treatment groups for either the males or the females. However, it was observed that the naturally mated (6)

males had the lowest organ:body weight ratio for both of the kidneys and for the heart, whilst the *in vitro* males had the highest ratio for the liver and kidneys.

The PVP and naturally mated (4) males had a significantly lower ratio for their lung when compared to the naturally mated (6) males ($p < 0.005$). The naturally mated (4) females had a significantly lower lung:body weight ratio when compared to the naturally mated (6) females ($p = 0.004$). The insulin males and the IGF-I females had the highest ratios for all organs studied, although these values do not reach statistical significance. There were no significant differences between any of the groups studied when organ weight was analysed independently of body weight (Figures 5.13 and 5.14) for either the males or the females. Male offspring derived from disaggregated embryos had significantly heavier livers when compared to the naturally mated (6) and *in vitro* males (Figure 5.33). However, this finding is likely to be attributed to the increased weight observed for these animals at 27 weeks of age.

7.6 Effect of *Ped* gene expression on postnatal development and physiology

The results reported within Chapters 3, 4 and 5 indicated that changes to the developmental rates and cell ratios within preimplantation embryos could result in altered patterns of postnatal development, growth and physiology. In light of these findings, it was decided to study the postnatal development of two congenic strains of mice, differing only in the expression of one gene, the *Ped* gene. These mice were of interest to our studies in that expression of the *Ped* gene has been correlated to increased rates of preimplantation embryo development and increased cell numbers when compared to embryos not expression the *Ped* gene. This study therefore examined the effect of altered rates of preimplantation embryo development *in vivo* on postnatal development, growth and physiology.

As previous reported, B6 K2 (Ped plus) mice were heavier than B6 K1 (*Ped* minus) mice at birth (Figure 6.1, Warner *et al.,* 1991, 1993). However, there were no significant differences in birth weight, litter size or weight at weaning as had been previous been observed (Warner *et al.,* 1991, 1993; Exley and Warner, 1999).

Between six and eleven weeks of age, both B6 K1 males and females became significantly heavier than B6 K2 mice, with the B6 K1 males remaining heavier for the remainder of the study.

At 15 weeks of age, B6 K1 mice had a significantly reduced systolic blood pressure when compared to the B6 K2 mice, however, at 21 weeks of age the B6 K1 males and females had a significantly elevated systolic blood pressure when compared to the B6 K2 males and females. B6 K1 mice were observed to have significantly elevated serum ACE activity when compared to the B6 K2 mice. Lung ACE was also elevated in B6 K1 mice, though this did not reach statistical significance. As lung ACE activity was negatively correlated to body weight for the B6 K2 mice, and as serum ACE activity was positively correlated to lung ACE, then, it may be assumed that as body weight increased, overall ACE activity decreased, thus resulting in the lower systolic blood pressure observed for the B6 K2 mice. As the B6 K1 mice had a significantly elevated lung:body weight ratio, this could have resulted an increased level of endothelial ACE, and subsequently increased level of serum ACE, giving rise to the increased blood pressure seen at 21 weeks of age. However, it was observed that for the B6 K1 mice as lung ACE activity increased, the serum ACE activity fell. One explanation could be a decrease in the amount of enzymatic cleavage of ACE from the epithelial surface. Alternatively, upon cleavage the activity of the serum ACE could become modified so reducing its activity. However, B6 K1 mice had elevated serum ACE activity when compared to the B6 K2 mice. It may therefore be the case that the serum ACE may derive from another source such as the vasculature or the kidneys.

Numerous animal and human studies have demonstrated that alterations to the normal pattern of embryonic or fetal development can have long term effects on postnatal health and physiology (Langley-Evans *et* al., 1996a, 1996b; Kwong *et al.*, 2000; Hales and Barer, 2001). It is hypothesised that if development in utero is restricted, the fetus will adapt by implementing a series of compensatory mechanisms for survival in a poor nutritional postnatal environment. If however, the postnatal

environment then has an over abundance of nutrients, the offspring will undergo rapid postnatal catch-up growth, subsequently increasing the chances of developing cardiovascular diseases, obesity, NIDDM and altered organ allometry (Barker and Clark, 1997; reviewed in Ozanne, 2001). Lack of *Ped* gene expression results in a slower rate of preimplantation development, and lighter birth weights. This reduced rate of fetal development may programme the fetus into adapting its nutritional requirements towards a poor postnatal environment. However, after birth, both B6 K1 and B6 K2 mice were fed *ad libitum*. This difference in pre and postnatal nutrient availability could have been instrumental in the postnatal over compensatory catch-up growth, resulting in the increased systolic blood pressure at 21 weeks and the altered organ allometery and ACE activities.

In conclusion, this study has demonstrated that specific *in vitro* culture environments or manipulations to the preimplantation mouse embryo result in significantly altered rates, cell numbers and ICM:TE ratios within the blastocyst. Upon transfer and development to term, *in vitro* culture and manipulation had little effect on birth weight, although offspring derived from embryos cultured in the presence of exogenous insulin and IGF-1 were observed to have the highest birth weight. Growth profiles were modestly affected by *in vitro* culture or manipulation, with the majority of differences observed during the first 10 week of age. However, disaggregation and reaggregation of embryos resulted in significant increases in weight gain from 12 to 27 weeks of age when compared to *in vitro*, *in vivo* and naturally mated (6) males. *In vitro* culture and manipulation resulted in significantly elevated systolic blood pressures when compared to control animals, and altered patterns of organ allometry.

Reduced rates of embryo development *in vivo*, influenced by the absence of *Ped* gene expression, resulted in altered patterns of early postnatal growth, increased systolic blood pressure at 21 weeks of age, altered organ allometery and raised serum ACE activity.

Although answering some question, this thesis has raised many others. It would be of interest to examine the underlying causes of the altered blood pressure further. As suggested within Chapter 4, other key regulators of blood pressure such as the AT-1 receptor, glucocorticoid and mineralocorticoid receptors and other components of the renin-angiotensin system would all be key candidates for further studies. Examination of trans-generational effects within an F2 progeny would give insight into whether the effects observed within this thesis are limited to the offspring directly derived from the manipulated embryos, or whether the manipulations have affected the germ line. The findings of such studies would have particular relevance to the fields of ART and livestock biotechnologies, which regularly manipulated and culture embryos *in vitro*. From the study involving the *Ped* mice, similar questions have been raised. However, with this model it would be of particular interest to perform embryo transfer studies. These would allow for the examination of the degree of influence that the maternal uterine environment has on postnatal growth and physiology. Also, embryo transfer would allow for the manipulation and possible control of litter size. Therefore, it may be possible to reduce the mean litter size of the B6 K1 mice and subsequently analyse whether the over-compensatory growth sill occurs.

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Apendix 1

Components of H6 BSA, H6 PVP, T6 BSA and T6 PVP medium.

Solution F (per100 ml, osmolarity, 2555 +/. 20 mOsm)

Sodium chloride	4.720g
Potassium chloride	0.11g
Sodium dihydrogen orthophosphate	0.06g
Magnesium chloride	0.1g
D- glucose	1.0g
DL- lactic acid (60%)	3.4 ml

Solution G (per 10 ml, osmolarity 60 +/. 10 mOsm)

Pyruvic acid	0.03g
Penicillin	0.06g
Streptomycin	0.05g

Solution B (per 10 ml, osmolarity 444 +/. 20 mOsm)

Sodium hydrogen carbonate	0.2106g

Solution H (per 10 ml, osmolartiy 415 +/. 20 mOsm)

Calcium chloride 2-hydrate	0.26g

Solution E (per 50 ml, osmolarity 354 +/. 20 mOsm)

Hepes	2.9785g

Need to pH to 7.4 with 5 M NaOH before checking mOsm.

Phenol red	6 mg/ml
Sodium chloride	20%

Sodium chloride 15mg/ml

Embryo culture media

Stock	T6 BSA	T6 PVP	H6 BSA	H6 PVP	H6 BSA Calcium free
F	1.00 ml	1.00 ml	1.00 ml	1.00 ml	1.00 ml
B	1.00 ml	1.00 ml	0.16 ml	0.16 ml	0.16 ml
G	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
H	0.1 ml	0.1 ml	0.1 ml	0.1 ml	N/A
E	N/A	N/A	0.84 ml	0.84 ml	0.84 ml
H_20	7.8 ml	7.8 ml	7.8 ml	7.8 ml	7.8 ml
BSA	40 mg	N/A	40 mg	N/A	60 mg
PVP	N/A	60 mg	N/A	60 mg	N/A
20% NaCl	40 ul	40 ul	60 ul	60 ul	60 ul
NaCl 15mg/ml	N/A	N/A	N/A	N/A	0.1 ml
Phenol red	25 ul	25 ul	N/A	N/A	N/A

Acid Tyrode's solution (per 100ml, pH 2.3)

NaCl	0.8g
KCl	0.02g
$CaCl_2.2H_2O$	0.0265g
$MgCl_2.2H_2O$	0.01g
D-glucose	0.1g
PVP	0.4g
Or	
$CaCl_2$	0.02g

Pregnant mare's serum

1 vial (1000 i.u.) diluted in 20 ml 0.9% saline solution. Sterile filtered and aliquoted in 4 ml samples. Stored at -20^{0}C.

Human chorionic gonadotrophin

1 vial (1500 i.u.) diluted in 30 ml 0.9% saline solution. Sterile filtered and aliquoted in 4 ml samples. Stored at -20^{0}C.

Appendix II

Anaesthetic for embryo transfer surgery

375 µl Saline (0.9%)

100 µl Ketaset (Fort Dodge)

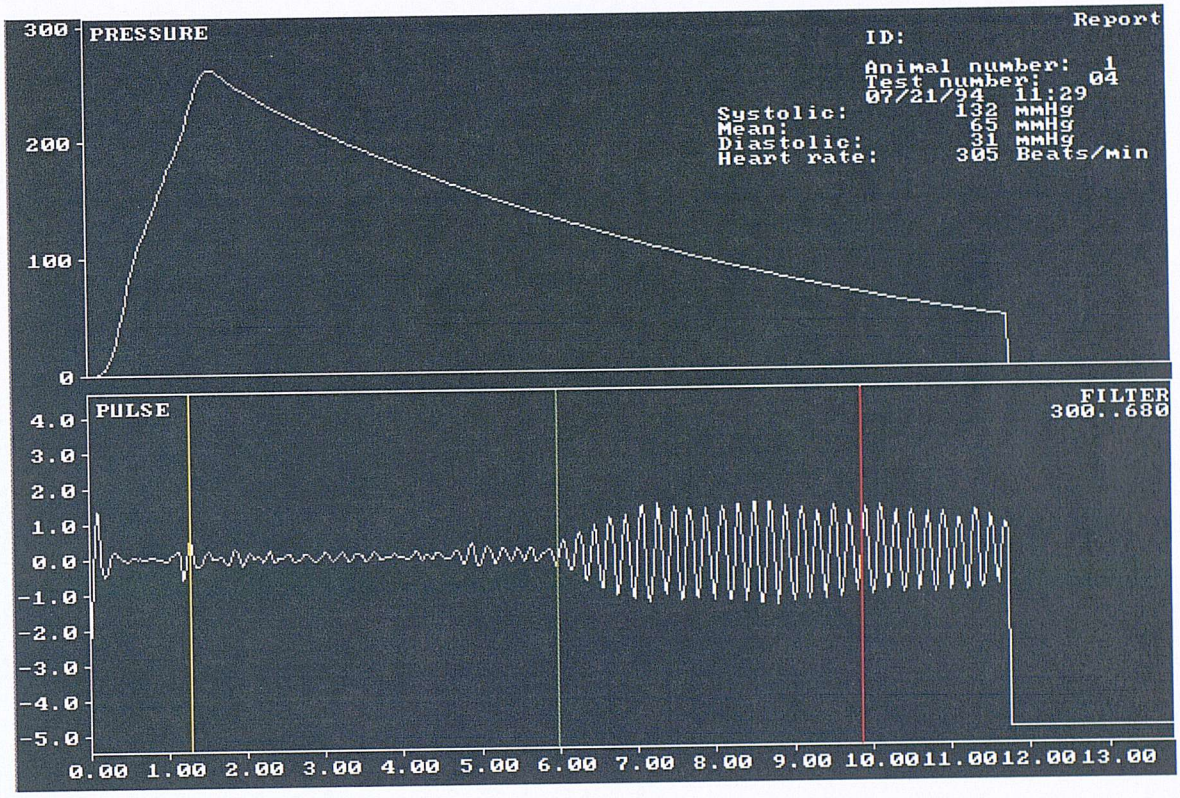
25 µl Acepromazine (C-Vet)

Analgesic used after embryo transfer surgery

Temgesic (Reckitt & Colman Products Ltd.). One capsule (300 µg/ml) diluted 1:10 in 0.9% saline solution to give a working concentration of 30 µg/ml.

Appendix III

Example of a blood pressure trace by tail-cuff plethysmography using an ITC model 229 blood pressure monitor (Linton Instruments, Norfolk, UK).



Appendix IV

Solutions used for the serum ACE assay

300 mM phosphate buffer containing 150mM sodium chloride
4.106g KH_2PO_4
0.876g NaCl

p.H to 8.3 using NaOH

100 mM phosphate buffer
1.3609g KH_2PO_4

p.H to 8.3 using NaOH

5 mM Hippuryl-His-Lue acetate salt (per 5 ml of 300 mM phosphate buffer containing 150 mM NaCl)
10.737mg

1 mM Hippuric acid (per 100 ml of 100 mM chloride buffer)

100 mM chloride buffer
1.6264g $MgCl_2$
0.294g $CaCl_2$

p.H to 8.3 using NaOH

Solutions used for the lung ACE assays (per 100ml)

200 mM Boric acid buffer
1.236g H_3BO_3
11.688g NaCl

p.H to 8.3 using NaOH

20 mM Hippuryl-His-Lue acetate salt (per 1 ml of Boric acid buffer)
10.1mg/ml

200 mM phosphate buffer
2.722g KH_2PO_4

p.H to 8.3 using NaOH

0.16M cyanuric chloride

2.96g	cyanuric chloride
100 ml	1,4-dioxane

4 mM Hippuric acid (per 100 ml 1 M NaOH)

71.68 mg	Hippuric acid