THE UNIVERSITY *of* EDINBURGH

IDENTIFICATION OF NOVEL CANDIDATE GENES FOR REGULATION OF FOLLICLE SELECTION IN THE AVIAN OVARY

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This thesis is presented for the degree of

Doctor of Philosophy

The University of Edinburgh

2012

Dedication

I would like to dedicate this thesis to my parents, without whose unwavering support it could not have been completed.

Declaration

I declare that this thesis, and the work presented herein is entirely my own except where explicitly stated. All work presented here was undertaken for the degree of Doctor of Philosophy at the University of Edinburgh and has not been submitted for any other degree or academic award.

Neil A. McDerment

Acknowledgements

Special Thanks

I would firstly like to thank my primary supervisor Paul Hocking for his continuous guidance over the last 4 years, in particular, his patience when faced with my early attempts at more complex statistical analysis. I would also like to thank my secondary supervisor Ian Dunn for his guidance and input both in the lab and with what occasionally seemed like endless drafts of abstracts and the paper that arose from the first chapters of this thesis. Special thanks should also be accorded to Peter Wilson for his general assistance with all things lab-based, particularly for the training in new techniques and familiarisation with local procedures, and David Waddington for his assistance with the early analysis of the microarray data. Recognition should also be given to Athanasios Theocharidis, the Lead Developer of Biolayout Express3D, for implementation of many features of BioLayout as their need arose in my analysis of the microarray data.

I would also like to express my gratitude towards all my friends and colleagues at The Roslin Institute who have contributed towards making my time at the Institute such a wonderful experience, particularly those that shared in the ever more eclectic range of conversations over lunch and coffee over the years.

Funding

I would also like to acknowledge the sources of funding for this work. Core funding was provided by the BBSRC. Additional CASE funding was provided by the Biosciences KTN (Genesis Faraday) and Aviagen Ltd.

Data & Resources

This thesis has drawn from several experiments carried out prior to commencement of my PhD. All experimental populations, save the White Leghorns, were provided by Paul Hocking. All data collection for these was carried out by Paul Hocking, Ian Dunn, Peter Wilson and Graeme Robertson. The microarray was printed by ARK Genomics with sample preparation carried out by Peter Wilson. David Waddington carried out the first analysis of the microarray data. Natasha Whenham provided purified RNA for the AIL Tissue panel.

Publications arising from this Thesis

Papers:

McDerment, N., Wilson, P., Waddington, D., Dunn, I. and Hocking, P. (2012). *Identification of novel candidate genes for follicle selection in the broiler breeder ovary.* BMC Genomics, 13:494.

Abstracts:

McDerment, N., Dunn, I. and Hocking, P. (2012) *PDGFRL: a novel candidate for regulation of follicle development in the broiler breeder ovary.* British Poultry Abstracts, 8:1.

McDerment, N., Wilson, P., Waddington, D., Dunn, I. and Hocking, P. (2010). *Using gene expression profiling to identify gene candidates in ovarian follicular development of broiler breeders*. British Poultry Abstracts, 6: 28.

Proceedings:

McDerment, N., Wilson, P., Waddington, D., Dunn, I. and Hocking, P.(2011) *Identification of novel candidates for follicle selection in the broiler breeder ovary.* Proceedings of the 4th International Symposium on Animal Functional Genomics, Dublin, Ireland.

McDerment, N., Wilson, P., Waddington, D., Dunn, I. and Hocking, P.(2011) *Identification of novel candidates for follicle selection in the broiler breeder ovary.* Proceedings of the 7th European Poultry Genetics Symposium, Peebles, UK

McDerment, N., Wilson, P., Waddington, D., Dunn, I. and Hocking, P.(2010) *Identification of novel candidates for follicle selection in the broiler breeder ovary.* Proceedings of the 13th European Poultry Conference, Tours, France

McDerment, N., Wilson, P., Waddington, D., Dunn, I. and Hocking, P.(2009) *Visualisation of gene expression patterns in ovarian follicular development* Proceedings of the 6th European Poultry genetics Symposium, Poznan, Poland

Abstract

Selective breeding of chickens for high growth rate and other production traits has led to the modern commercial broiler, a bird that has the genetic potential for reaching an average body weight of 2.7kg within 6 weeks of hatch. However, the breeding stock for modern broilers has to be feed controlled in order to lay large numbers of viable hatching eggs. Broiler breeders, when fed ad libitum, have a propensity to produce internal ovulations, double-yolked, misshapen or shell-less eggs. This is due to the release of multiple ova at ovulation, which results in a significant loss of production. Feed control has been shown to mitigate this effect but welfare concerns have been raised as to the side-effects for the birds. The main objective of this research was to determine the genetic basis for the regulation of ovarian follicle selection and its dysfunction in ad libitum-fed broiler breeders, and how this might be addressed by genetic selection to limit the impact on the management and welfare of future broiler breeders.

A multi-layered statistical, expression profiling and cluster analysis of ovarian gene expression data from a microarray study was carried out to identify candidate genes for further study.Key stages of development were investigated for feed restricted and ad libitum-fed broiler breeders. Several gene candidate genes were validated by qPCR in a comparison of different ovarian tissues in layer type hens for subsequent analysis in broiler breeders. Sequencing of the founders of an Advanced Intercross Line (AIL) of commercial broiler breeders and White Leghorn layers was performed covering 3 regions of each of the primary candidate genes in order to identify genetic variation that could account for differences in follicle number between broilers and layers.

Expression data from a microarray study highlighted a number of potential candidate genes for regulation of follicle development. One of these genes, Platelet Derived Growth Factor Receptor Like (PDGFRL), shares significant sequence homology with the active domains of Platelet Derived Growth Factor Receptor β. Expression profiling in layers showed peak PDGFRL expression in 5-6 mm follicles and the F2 follicle ($P < 0.001$). PDGFRL was also up-regulated in response to ad libitum feeding in broiler breeders in 6-8 mm follicles ($P < 0.016$), the point at which follicle selection and recruitment is considered to occur. In addition to this, while PDGFRL expression remains relatively constant between tissues under ad libitum conditions, it shows a clear

reduction in expression (P <0.001) in prehierarchical follicles relative to the stroma and the F1 follicle under feed restriction. This observation is consistent with results from the original microarray study. Sequencing of the AIL Founders highlighted several SNPs in the broiler that have the potential to be used as markers for incorporation into commercial selection programs. EST alignment in preparation for targeted sequencing of PDGFRL also highlighted three potential forms of the protein, each with a different 5' starting sequence. Initial investigation has shown all three to be expressed in ovarian follicles. QPCR in a panel of 13 tissues shows marked differences between the 3 variants, implying different and perhaps specialised roles for each. The PDGFR family has a potential role in steroidogenesis, and the expression profiling, combined with the clear effect on expression from ad libitum feeding in broiler breeders, suggest that PDGFRL is a strong candidate for involvement in the regulation of follicle development GDF9, shown to be associated with multiple ovulation in sheep, and FSH receptor, a mediator of neuroendocrine signalling to the ovary, were also investigated. They behaved as expected in layer type birds but both showed significant differential expression (P = 0.005 and 0.018 respectively) as a result of ad libitum feeding in broiler breeders. Though these two genes have been extensively investigated, these are previously unobserved effects. SNPs have also been identified in these genes which have the potential to be used as markers for incorporation into commercial selection programs. To fully exploit these results, additional investigation is recommended to confirm these results in commercial populations and to determine how they can be employed to best effect.

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List of Abbreviations

ABLIM3	Actin binding lim protein family, member 3
ACAT2	Acetyl-coa acetyltransferase 2
ADRA1B	Adrenergic receptor, alpha 1
AIL	Advanced intercross line
BMP	Bone morphogenetic protein
BTN1A1	Butyrophilin, subfamily 1, member a1
CAMK2A	Calcium/calmodulin-dependent protein kinase ii alpha
CAPRIN2	Caprin family member 2
EGF	Epidermal growth factor
F(1)	Hierarchical follicle (n-1 stages prior to ovulation)
FGF	Fibroblast growth factor
FLNB	Filamin b, beta
FOXI1	Forkhead box i1
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GDF9	Growth differentiation factor 9
GHR	Growth hormone receptor
GnIH	Gonadotrophin-inhibiting hormone
GnRH	Gonadotrophin-releasing hormone
GnRHR	Gonadotrophin-releasing hormone receptor
GRP	Gastrin-releasing peptide
GULP1	Gulp, engulfment adaptor ptb domain containing 1
HPGA	Hypothalamo-pituitary-gonadal axis
ID	Inhibitor of differentiation
IGF	Insulin-like growth factor
KRT75	Keratin 75
LH	Leutinising hormone
LHR	Leutinising hormone receptor
LSP1	Lymphocyte-specific protein 1
MAMDC2	Mam domain containing 2
MFGE8	Milk fat globule-egf factor 8
MFHAS1	Malignant fibrous histiocytoma amplified sequence 1
MOSPD1	Motile sperm domain-containing 1
MYO1C	Myosin ic
NPY	Neuropeptide y
PAK3	P21 protein (cdc42/rac)-activated kinase 3
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PDGFRL	Platelet-derived growth factor receptor-like

List of Abbreviations (cont'd)

POSTN	Periostin, osteoblast specific factor
PPARGC1B	Peroxisome proliferator-activated receptor gamma, coactivator 1 beta
RXRG	Retinoid x receptor gamma
SH3BGRL3	Sh3 domain binding glutamic acid-rich protein like 3
SLIT3	Slit homolog 3
SMAD2	Smad family member 2
SMAD3	Smad family member 3
SPTY2D1	Spt2, suppressor of ty, domain containing 1 (s. Cerevisiae)
SRP68	Signal recognition particle 68kda
STaR	Steroidogenic acute regulatory protein
TBC1D13	Tbc1 domain family, member 13
TGF	Transforming growth factor
TGFB	Transforming growth factor beta
TGFBR1	Transforming growth factor-beta receptor 1
TGM2	Transglutaminase 2 (c polypeptide, protein-glutamine-gamma-glutamyltransferas
TUBD1	Tubulin delta 1
TXN2	Thioredoxin 2
VDAC1	Voltage-dependent anion channel 1
YAP1	Yes-associated protein 1
ZNF593	Zinc finger protein 593

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Review of Reproduction in the Chicken & its Impact on Commercial Production

1.1. Overview

The sexually mature hen will, given the appropriate endocrine stimuli, ovulate approximately once daily during their laying periods [1]. Follicles of the ovarian hierarchy contain the oocyte and yolk which is deposited over the latter stages of follicular maturation [1]. At ovulation, the yolk and oocyte are released into the oviduct where fertilisation will occur and where the albumen, or egg white, and the shell will be laid down prior to laying of the complete egg [1]. If, on the other hand, multiple simultaneous ovulations occur, the resulting eggs are unviable due to the constraints of external incubation [2, 3].

Reproductive development and behaviour are regulated by a number of physiological and environmental factors to ensure that resources are redistributed from growth and maintenance towards reproduction at the appropriate times, i.e. when the likelihood of survival of offspring is at its highest [4]. Although the rearing environments for commercial poultry in the developed world are tightly regulated [5], the biological imperatives that govern reproduction in wild birds still manifest themselves [6-8]. Understanding of these mechanisms has allowed adjustment of the rearing environments of commercial poultry to maximise production [5]. However, there are still gaps in our understanding of these regulatory mechanisms, particularly in local regulation of ovarian development and activity. Lack of appreciation for the complex associations between growth and reproduction at the molecular level has led to unforeseen side-effects of early selection programs such as multiple ovulation [9]. Further elucidation of these mechanisms would not merely expand our knowledge, but would also allow for improved genetic selection. This would provide opportunities for more precise adjustments of commercial environments in order to develop more efficient production systems with reduced negative impact on the birds as a result. Given the implications of an increasing global population, sustainable and cost-effective food production will become increasingly important.

1.2. Ovarian & Follicle Development

1.2.1. The Ovarian Follicle

Despite obvious differences in post-ovulatory mechanisms, the principles underlying the ovulatory cycle in chickens are generally comparable with that of mammalian species save for deposition of yolk [10]. The ovarian follicle houses the ovum until the bird is ready to ovulate. As such, it provides protection for the ovum as well as acting as a conduit for nutrient flow to sustain the ovum and deposit the yolk. As well as these supporting functions, the follicle also has a role in regulation of reproductive development in the form of steroidogenesis and production of other regulatory signals. The follicle is comprised of a series of membranes and highly vascularised cell layers that surround, support, and protect the oocyte [11]. These are described in *Table 1.1*.

Table 1.1. The layers of the ovarian follicle, progressing outwards from the centre, and their primary function [11, 12].

Follicle Layer	Primary Function
Vitelline Membrane	Surrounds the Yolk and Oocyte
Perivitelline Layer	Sperm binding & Fertilization
Granulosa	Gonadotrophin detection, Steroidogenesis & Oocyte development
Basement Membrane	Surrounds outer Granulosa cells
Theca Interna	Gonadotropin detection & Steroidogenesis
Theca Externa	Structural support & Ovulation
Follicle Stalk	Structural support & nutrient delivery

1.2.2. The Stages of Development

Follicles within the chicken ovary are ordered progressively by size from many follicles <1 mm in diameter to a single pre-ovulatory or F1 follicle at around 40 mm [12]. The growth represented by this progression takes approximately 3 weeks, with the proportion of atretic follicles rapidly increasing in the later stages of pre-hierarchical development. Follicles can be divided into 2 basic groups, white follicles, which range from 1-6 mm, and yellow follicles, ranging from approximately 8-40 mm, which are distinguishable not only by their size but by the deposition of yolk and increased vascularisation that gives them their colour [12].

The majority of follicles will not grow sufficiently to undergo ovulation. Most follicles <8 mm in diameter will ultimately become atretic and be reabsorbed. However, under the correct endocrine conditions, following ovulation a single follicle from the 6-8 mm pool is recruited into the pre-ovulatory hierarchy [12-14]. The current understanding is that selection, or the process that determines whether follicles will be recruited, happens prior to rather than during recruitment itself. The key decision is believed to occur between 5-7 mm follicles [13, 15]. Once recruited, follicles are highly likely to mature and, unless gonadotrophin support is removed, proceed to ovulation in about 10 days [12].

1.2.3. At the Molecular Level

In pre-hierarchical development, Follicle Stimulating Hormone receptor (FSHR) is the predominant gonadotrophin receptor mediating signals from the Hypothalamo-Pituitary-Gonadal Axis (HPGA). However, once follicles are drafted into the hierarchy, this predominance shifts towards Luteinising Hormone receptor (LHR) [16]. Receptors for the gonadotrophins FSH and LH are expressed in both the granulosa and theca layers. While FSHR is expressed relatively uniformly in theca cells [16, 17], expression in the granulosa differs between developmental stages [17]. Unlike FSHR, LH signalling induces increased expression of its own receptor [18].

There are many changes in molecular signalling pathways at the different stages of follicle development. Expression of many members of the Transforming Growth Factor Beta (TGFB) superfamily is altered between stages. For example, activin and BMP2 through 7 are up-regulated in Granulosa and Theca cell layers in early stages of development [12]. activin A production results in up-regulation of gonadotrophin receptors [19]. Presence of activin A also enhances gonadotrophin-induced secretion of progesterone and inhibin A [12].

A considerable amount of research has gone into elucidating various TGFB superfamily signal cascades. Besides the effects of activins and inhibins, the BMP sub-group of signalling molecules are considered to be important to follicle development, as they are in the development of many tissues. The research on BMPs has been recently reviewed by Knight [12]. BMPs 3 through 7 and 15 have been implicated in paracrine signalling in Theca cells from prehierarchical follicles, and

BMP2 through 7 in pre-ovulatory follicles [20]. However, functional significance has yet to be ascribed to these observations in the chicken. It has been shown, however, that BMP6 does stimulate secretion of progesterone from Granulosa cells as well as increased expression of FSHR and LHR in the F1 or pre-ovulatory follicle [20]. Several other growth factor families have been independently implicated in follicle development, including EGF [21], FGF [22], IGF [23], and TGF [22]. However, these are all important growth factors in many tissues and there is, as yet, no comprehensive model that relates each to the other and their combined effects on follicle development.

1.2.4. Factors Affecting Follicle Number

Reproduction in wild birds is seasonal and is usually associated with availability of their preferred food source, nesting material and other environmental cues. As well as influencing behaviour, the changing seasons result in physiological changes. Although the specific mechanism governing follicle number has yet to be determined, the association between availability of food and follicle number has been well established [24]. This association should not come as a surprise as reproduction is an energy-intensive process, particularly in avian species with their redistribution of resources for deposition of yolk, albumen and shell to form the complete egg. Getting the timing or the balance wrong can be devastating in the wild, possibly resulting in the loss of an entire breeding season. Lay too few eggs and the chances of offspring surviving to maturity are vastly diminished. Too many follicles developing over too short a time may lead to multiple ovulation [9], which in turn, leads to unviable eggs.

1.2.5. Multiple Ovulation

As introduced previously, multiple ovulation, or the release of 2 or more follicles simultaneously, results in the production of unviable eggs. Multiple ovulation arises when 2 or more follicles develop in parallel beyond the 8 mm stage. This has been established by the work [9] from which this study derives. In broiler breeders fed *ad libitum*, the mechanism that regulates follicle selection and recruitment fails to do so and they develop abnormally large ovarian follicular hierarchies (Figure 1.1). This

will ultimately lead to their experiencing multiple ovulation, despite their pre-hierarchical (<8 mm) follicle numbers not being significantly affected. This demonstrates that the cause of this problem lies in the control mechanism underpinning follicle recruitment to the hierarchy, and not in folliculogenesis. Excess feed intake has often been cited as the cause of multiple ovulation as feed restriction stabilises ovarian function in commercial broiler breeders. However, commercial turkeys, particularly male-line breeders, also develop parallel follicular hierarchies, with minimal beneficial effect of feed restriction [24]. Follicle number is perhaps therefore more appropriately correlated with body weight than with feed intake [25, 26].

1.2.6. Follicle Development in Mammals

Ovarian follicle development is directly comparable between many mammalian species [27, 28]. In humans, as in other mammals, follicle development follows the same basic process within the ovary as in chickens; only the duration of the ovulatory cycle and the size to which follicles grow differs [28]. Cyclic increases in FSH levels are believed to 'rescue' a cohort of antral (pre-hierarchical) follicles from atresia [27, 28]. Unlike chickens, in humans, cohorts of ~10 antral follicles are taken together into the equivalent of the hierarchical phase of follicle development [28]. Of these, one becomes dominant and grows more rapidly, excreting oestrogens and inhibins that slow the growth of the others in the cohort [28]. It is understood that this is achieved through a combination of suppression of FSH and LH secretion by the pituitary and potentially interfering with the activity of local growth factors that enhance downstream signalling of FSH and LH [28]. It is unclear why one follicle emerges as dominant but it is hypothesised that this is due to heightened sensitivity to FSH [28]. It has been demonstrated in monkeys that inhibition or neutralisation of oestrogens produced during the mid-follicular phase, resulting in continuous FSH release, leads to development of multiple pre-ovulatory follicles [28], supporting this theory.

While atresia occurs at earlier stages, it occurs predominantly immediately prior to the stage at which recruitment can occur [27, 29-31]. Data from some species indicate that this is still the case even in the absence of the pituitary – suggesting that

regulation of pre-recruitment development is intra-ovarian [27-29] once it has been initiated. Pre-recruitment development would therefore constitute the normal lifespan of follicles, with only progression beyond recruitment and subsequent ovulation being triggered externally [27, 29, 32]. This being true, it would suggest that conditions have been altered for follicles selected for recruitment to make them receptive to an extra-ovarian signal to which younger follicles will not respond, or that allows them to supersede their neighbours.

An increase in the blood-plasma levels of FSH has been shown to occur in temporal synchronisation with selection for recruitment [27, 33-36] but while this is a plausible extra-ovarian stimulus for recruitment, it is unclear whether this would be the direct cause or whether it is the trigger for further downstream effects that cumulatively result in selection.

Figure 1.1. Ovaries showing a normal layer/restricted broiler breeder follicular hierarchy (left) **and a large ad libitum fed broiler breeder hierarchy** (right)**.** Image courtesy of P. M. Hocking.

1.3. The HPG Axis in the Chicken

1.3.1. The Hypothalamus

Endocrine regulation of the ovary is managed through the hypothalamo-pituitary-gonadal axis, with input signals being generated in the hypothalamus and transferred to the pituitary where hormone secretion is initiated [17]. The hypothalamus co-ordinates reproductive development and activity in response both to physiological and environmental signals such as ovarian steroids [17] and photoperiod [6].

Gonadotrophin-Releasing Hormone (GnRH), of which there are two sub-types [37], is the primary signal from the hypothalamus to the pituitary. GnRH-containing neurones can be detected from day 4 of embryonic development [38], the cell bodies of which are localised to the pre-optic hypothalamus with axonal termini in the median eminence [17]. GnRH plays an important regulatory role throughout sexual development and activity. Puberty in cockerels is also characterised by increased GnRH in the hypothalamus [17, 39] as well as increased steroid-based feedback mechanisms [6, 40-42].

"Photoperiod is the most important environmental manipulator of sexual development and reproductive activity...for all species of poultry" [7]. As with vernalisation in many plants prior to reproduction, at hatch birds that undertake seasonal breeding are photorefractory and require a period of short days followed by an extended period of longer days before reproduction can take place [7]. Increased photoperiod, or day length, is not dependent on ocular detection [43] and is registered in the hypothalamus [7, 8] and stimulates GnRH activity while a week or more of exposure to long days will induce initiation of the ovulatory cycle in sexually mature birds [6, 44].

A surge in GnRH is responsible for the pre-ovulatory surge of LH that triggers ovulation [45]. This surge has been shown to be caused by feedback mechanisms stemming from the ovary [46] and will be discussed in a later section. There are many neurochemical factors believed to be involved in regulating GnRH production and release, however much of the data derive from temporal correlation and/or physical proximity rather than demonstrated functional relationships. This having been said, some factors are better understood. Reduction of Neuropeptide Y (NPY) for example has been shown to stimulate release of GnRH in the median eminence

during the LH surge associated with ovulation [47] while expression of NPY is increased in feed restricted birds [48]. GnRH's major counterpart, however, is Gonadotropin Inhibitory Hormone (GnIH). Like GnRH, GnIH is produced in neurones with cell bodies in the hypothalamus and axonal termini in the median eminence [49, 50]. GnIH has been shown to reduce the expression of FSHβ and the common α-subunit in the pituitary [51], and is regulated by melatonin [52].

1.3.2. The Pituitary

GnRH is released in pulses into the pituitary and the frequency and strength of these pulses modulate reproductive development [17] by inducing the production and release of LH and FSH from gonadotrophs in the anterior pituitary [12, 16, 53-57] (Figure 1.2). In chickens, LH and FSH are produced in separate cells [58], distinguishable from day 8 of embryonic development [59]. As in mammals, increases in pituitary and blood-plasma FSH have been shown in chickens to temporally correlate with increased follicular growth [60, 61].

As avian FSH has proven challenging to study due to difficulty in its isolation from LH, it is not as well understood as LH, the mechanisms of which are quite well characterised [17]. This being said, research suggests that follicular responsiveness to FSH is required for follicles to be recruited successfully to the hierarchy [21]. It has been shown in mammals that other factors besides GnRH are involved in the regulation of FSH production and release in the pituitary. While these factors are present in the chicken [9], less experimental evidence exists to confirm what has been shown in mammals [17]. GnRH release into the pituitary triggers production of activin and follistatin [62]. activin in mammals stimulates production of FSH while follistatin inhibits this process [63]. These interactions are also the likely point of action of several feedback mechanisms initiated in the ovary to regulate FSH production and release [64, 65].

LH secretion pulses at a frequency of approximately once an hour upon stimulation [66] and is known to generate a positive feedback effect by increasing expression of its own receptor within ovarian tissue [18]. Increased photoperiod, through GnRH signalling, has been clearly shown to stimulate LH [67] as has ovarian feedback from increased progesterone [68].

1.3.3. The Ovary & Steroidogenesis

While gonadotrophin signalling leads to cell growth and proliferation, follicular steroidogenesis, and ultimately ovulation, [12, 56, 69-72], mapping of the downstream pathways is less complete. Their effects, however, have been studied. It has been shown that several positive and negative feedback mechanisms exist within the HPGA to regulate reproductive development. Many of these incorporate steroid hormones produced in the ovary. This association can be detected in early embryonic development by the presence of oestrogen receptors within gonadotrophs in the pituitary [73] and by the fact that removal of the embryonic pituitary prevents development of steroid-producing cells in the ovary [74, 75], indicating that the interaction works in both directions**.** Oestrogen has a clear inhibitory effect on FSH produced by the pituitary; however it is unclear how much of this effect is due to inhibition of hypothalamic GnRH [6, 42], or through direct action on the pituitary through GnRH receptor inhibition [41]. Inhibins, secreted from the ovary following FSH stimulation, also have a negative impact on circulating levels of FSH [64, 65].

progesterone, on the other hand, is involved in positive feedback signalling [17]. Released from the F1 pre-ovulatory follicle [46], Progesterone acts on the hypothalamus to increase release of GnRH, which in turn triggers the LH surge responsible for ovulation [46, 76]. Testosterone is also required for the pre-ovulatory surge of LH [77] as blocking it's receptor appears to inhibit production of the pre-ovulatory LH surge, as well as preventing release of progesterone [17]. Increases in testosterone have also been shown to increase expression of LHR in the F1 [77], increasing receptiveness to the subsequent surge in LH.

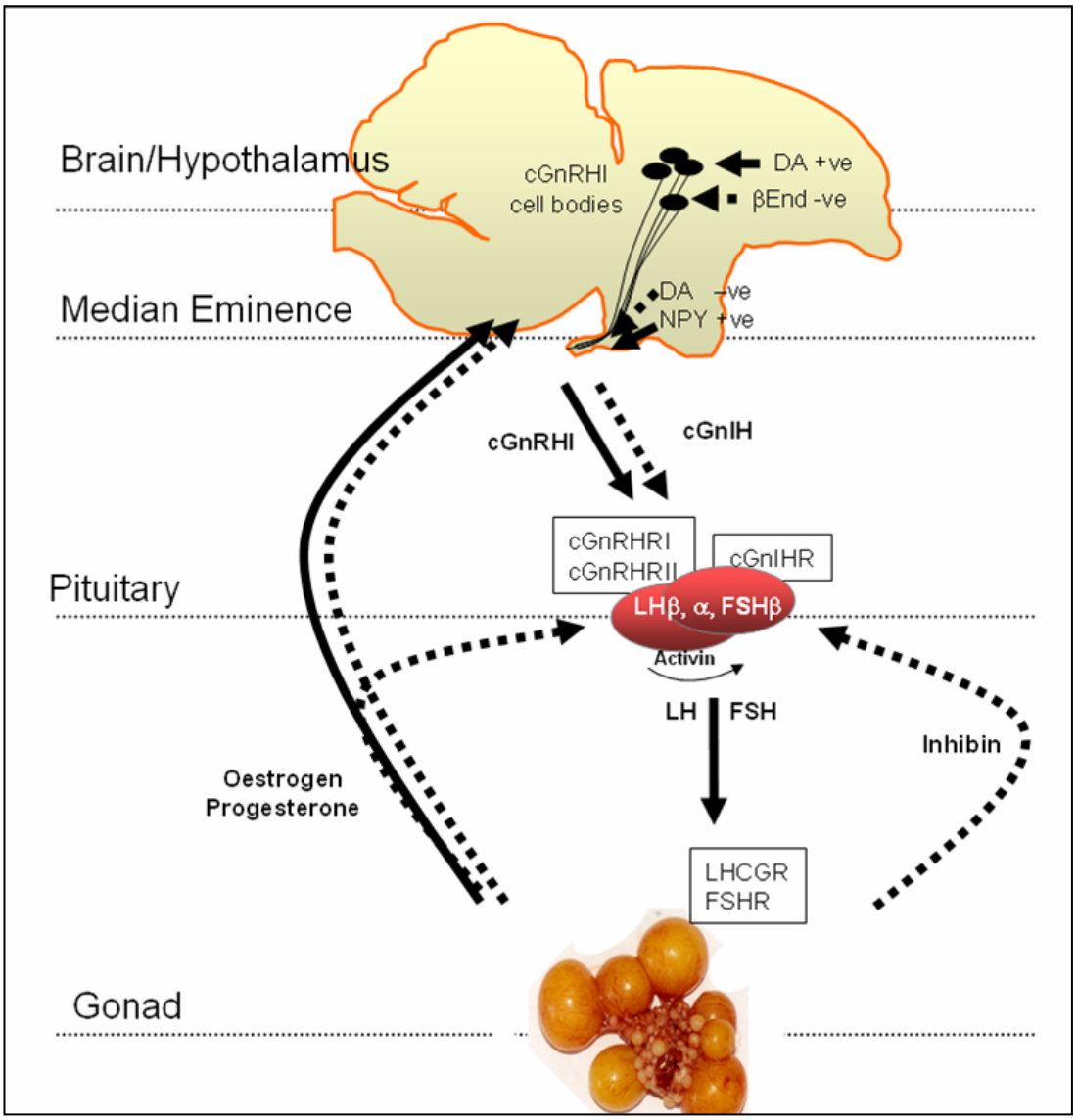

Figure 1.2. The Hypothalamo-Pituitary-Gonadal Axis showing key regulators and their general function within the axis. Solid arrows (+ve) indicate positive effect, hashed arrows (-ve) indicate negative effect. Image courtesy of I. C. Dunn

1.4. The Modern Poultry Industry

1.4.1. Development of the Modern Poultry Industry

At last estimate, the global poultry industry produces at least one third of all animal-derived food for human consumption [3]. Over the 45 years from 1961 to 2006, global poultry meat production rose from less than < $1x10^7$ tonnes to $8.1x10^7$ tonnes per annum [3]. Approximately 86% of total poultry meat production is accounted for by broiler chickens, $7x10^{10}$ tonnes at last estimate. Other poultry (turkeys, ducks, geese etc.) produce a further $1.15x10^{10}$ tonnes of meat for human consumption [3]. The demand for egg production is also high, with $6x10^9$ layers producing over $1x10^{12}$ eggs globally per year [3]. All in all, the global poultry industry is a massive entity. The market is now controlled by 2 main competitors, the EW Group (incorporating Aviagen, Hy-Line, and Lohmann Tierzucht), and Cobb-Vantress (incorporating Hybro and Hendrix Genetics). Both groups employ extensive selective breeding programs [78] that have resulted in the modern broiler breeder and layer (Figure 1.3). However, while many of the primary traits selected for, growth rate, Feed Conversion Rate (FCR), and reproduction traits for example [78], have significant and direct economic impact, the market drives selection for many 'secondary' traits, such as health and welfare-related traits [78]. Some companies may focus on one group of 'secondary' traits while competitors focus more on others. Different lines of commercial birds can thus be very different (See Figure 6.7).

1.4.2. The Cost of Multiple Ovulation

Although modern breeding programmes consider many more traits [3], in early breeding programmes, commercial broiler breeders, selected primarily for rapid growth and high meat yield, produced fewer eggs [2, 79]. The result of that selection pressure is that broiler breeders fed *ad libitum* produce multiple ovulations through development of a numerically large ovarian hierarchy [80]. While many ova from multiple ovulations are lost into the body cavity, some result in double- or multiple-yolked eggs, or eggs with defective shells [2]. Such eggs either cannot be incubated or have poor hatchability. If left uncontrolled, this would constitute a significant loss of production in a commercial flock. It has been shown that under commercial conditions, wastage due to defective or damaged eggs is approximately 4.5%, under *ad libitum* conditions, this rises to over 14% [2].

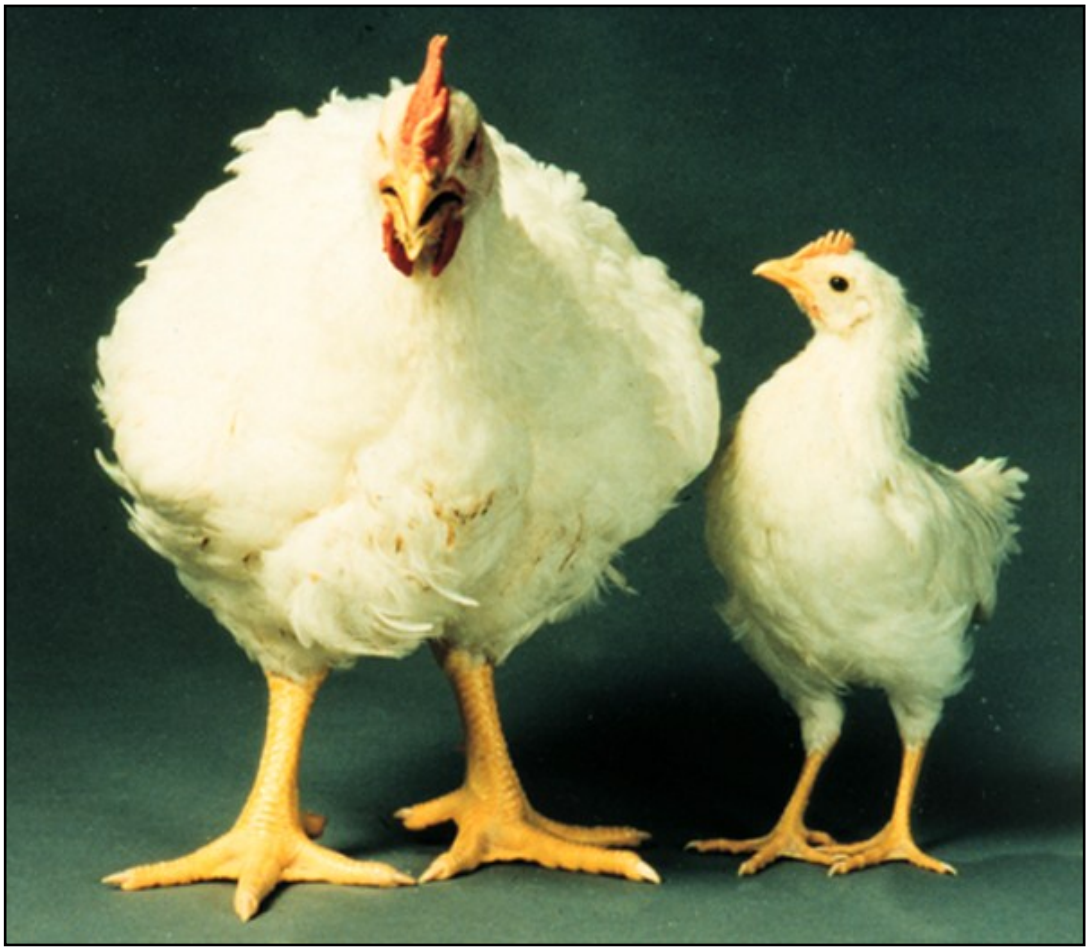

Figure 1.3. Image of a commercial broiler (left) and egg layer (right), both at 10 weeks of age, showing the vast physical differences between the two types of commercial production chicken. Image courtesy of P. M. Hocking.

1.4.3. Food Restriction in Broiler Breeders

The mechanism by which food restriction regulates follicle number is not completely elucidated, but it does act through hormonal regulation at the hypothalamic and pituitary level [9, 81]. It is clear, however, that there is a natural mechanism in place that this measure exploits. As might be expected, in wild birds clutch size is affected by availability of food and the energy required to obtain it [4]. Thus food intake and related growth influences follicular selection in the ovary of birds and the result of selection for these traits has affected the native regulatory mechanisms that govern follicle selection within the ovary [82, 83].

Ad-libitum fed broiler breeders will become very heavy and develop various weight-related problems. Consequently, although juvenile broiler breeders are fed *ad libitum* to reach target body weights, they are required to be limited to at least 40% of their natural nutritional intake as they reach sexual maturity [24]. However, this leaves the birds in a permanently hungry state. Several studies, recently reviewed in Hocking (2009) [24], have shown increased foraging and other behaviour in response to restricted diets [84, 85] that support this conclusion. It cannot be denied however, that broiler breeders are, on balance, in a state of greater physical health when feed restricted as opposed to when fed *ad libitum*. Feed restriction also reduces follicle numbers within the ovarian hierarchy [9, 24], though not the number of pre-hierarchical follicles [9], and consequently decreases the chance of multiple ovulation. It is interesting to note that, while commercial turkeys also produce multiple parallel hierarchies, restriction does not have the same effect as it does in broiler breeders, often disrupting laying patterns altogether [24]. Despite this added benefit, the degree of feed restriction has become a welfare issue [2, 84, 86], which is of concern to both producers and consumers, indeed over 30% of the traits selected for by Aviagen in their broiler breeder programme are associated with welfare indicators.

The effects of *ad libitum* vs. restricted feeding on HPGA signalling have been well studied in recent years. While GnIH expression in the hypothalamus is unaffected, GnRH expression is reduced under restricted feeding [9]. In the pituitary, expression of LHβ and FSHβ is unaffected by restricted feeding, though circulating levels of LH and FSH are reduced [9], as would be expected if GnRH levels are reduced.

1.5. Hypotheses, Aims, and Approach

1.5.1. Hypotheses

From the literature discussed above, control of follicle number is clearly a highly complex multi-faceted mechanism, incorporating many interconnected processes within the neuroendocrine signalling axis and reproductive tissue. However, examination of changes in pre-hierarchical and hierarchical follicle numbers in response to feed restriction in broiler breeders, as well as the work done in mammalian species confirms that the problem that results in multiple ovulation lies at recruitment to the ovulatory hierarchy of excessive numbers of pre-hierarchical follicles, as their development prior to recruitment is not visibly affected by standard feed restriction and they are capable of developing to the point of recruitment without neuroendocrine input once development has begun.

There are several possible explanations for why follicle recruitment is increased under *ad libitum* conditions. 1: Follicles' responses to neuroendocrine signalling are purely dose-dependent and they are responding to the increased levels of circulating gonadotropins under *ad libitum* feeding. 2: Neuroendocrine signalling results in inhibitory signals being sent by the receiving follicle to neighbouring follicles to reduce their receptiveness to neuroendocrine signalling and this mechanism is either failing in broiler breeders and being balanced by the reduction in circulating gonadotropins in restricted birds; or the rise in circulating gonadotropins in *ad libitum* fed birds overshadows the follicle's ability to respond to inhibitory signalling. 3: Follicle selection for recruitment is determined prior to neuroendocrine signalling and signalling simply puts into action the decision already made. This is also likely to be dose-dependent in some way as it must respond to increased circulating gonadotropins in *ad libitum* fed birds.

It is also possible that selective breeding in broiler breeders has resulted in undesirable mutations becoming fixed in the population that affect part of the mechanism, whatever that may be, which would otherwise behave normally. The fact that laying birds only rarely develop this problem while without feed restriction the broiler industry would be unsustainable supports the idea that selective breeding may have played a part in creation of this problem in broiler lines. Selective breeding therefore may also be the best solution to the problem.

1.5.2. Aims

The aim of this study was to investigate intraovarian regulation of follicle number in chickens by identifying gene candidate genes which have a potential role in regulating follicle number in the ovary, either through intrafollicular signalling, or feedback mechanisms that affect the HPGA. Ultimately this information will increase our understanding of the mechanisms by which dysfunction in broiler breeder hen ovaries occurs and may lead to genetic or alternative strategies to reduce dependence on food restriction.

1.5.3. Approach

The first phase of the project focused primarily on identification of novel candidate genes (experiment 1). Available literature was used to prioritise candidate genes only after the microarray analysis was complete so as to reduce its effect as a potential source of bias. Phase 2 involved characterisation of selected candidate genes' activity in different conditions (experiment 2 & 3). Phase 3 then investigated the genetic characteristics of the primary candidate genes and possible association with follicle number.

The research described in chapters 3, 4 and 5 utilised comparisons of ovarian function between 3 sets of animals; 1) a broiler breeder line that was feed restricted (FR) or 2) fed *ad libitum* (AL) and 3) a line of layer hens fed *ad libitum*. The feed restricted broiler breeder and *ad libitum* layer share a comparable ovarian hierarchy, though the former is artificially maintained through the restricted diet. The 3 groups were used to examine changes in gene expression between key stages in follicle development in 3 experiments:

Experiment 1, gene expression in FR vs. AL broiler breeders was compared using microarray analysis of key stages of follicle development. Subsequent analysis of changes in gene expression between these stages was carried out. Two analytical approaches (in R [87] and BioLayout Express [88]) were used to identify significant differences within these two comparisons;

Experiment 2, laying hens, having normal follicle hierarchies, were used to screen candidate genes from experiment 1 for changes in expression in a more detailed set of follicular stages. It was reasoned that genes showing large changes around the

stage associated with follicle selection would be the most likely to be involved in recruitment;

Experiment 3, candidate genes that were identified after screening in experiment 2 were validated by QPCR within and between key stages of follicle development in FR vs. AL broiler breeders to confirm their changes and to estimate the effect of dietary treatment on their expression. It was hypothesised that genes showing differences associated with level of feeding were likely to be involved in follicle recruitment;

Experiment 4, candidate genes confirmed as showing differential expression between feed regimes were taken forward for sequencing in the founder birds of a broiler x layer Advanced Intercross Line (AIL) to identify SNPs for genotyping in subsequent generations.

1.6.References

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2

Experimental & Statistical Methods

This chapter describes the standard methods for data collection and analysis. Where the standard method required optimisation, this is indicated in the appropriate data chapter. Where non-standard or case-specific methods were employed, this is also discussed in the relevant data chapters. For all standard methods, data chapters will reference the relevant sections laid out here.

2.1. Birds and Sampling

2.1.1. Broiler Breeders Chapters 3, 5 & 6)

Female Ross 308 broiler breeder chicks (n=16) were reared on a standard restricted diet, as per the management manual guidelines [1], to 29 weeks. A photoperiod of 8L:14D was maintained during rearing from 8 days to 16 weeks, rising to 16L:8D by 25 weeks of age using standard incremental increases [1]. At 29 weeks of age half the birds were allowed *ad libitum* access to feed and all were killed 2 weeks later by an overdose of sodium pentabarbitone. Birds were killed 11 to 16 hours after dusk. Initially a larger broiler breeder population was reared for use in multiple studies. Sixteen birds were selected for the microarray from this population at post-mortem to represent extreme ovarian phenotypes as regards numbers of hierarchical follicles. All birds had eggs present in the oviduct at sampling. Body weight and the numbers of follicles >8 mm and 5-8 mm diameter were recorded at post mortem. Tissues taken for probing the microarray were the F1 follicle wall, 5-6 and 6-8 mm whole follicles and the ovarian anterior stroma, containing primordial follicles. All yolk was removed from F1 follicles and the follicle walls washed prior to snap-freezing in LN_2. All other follicles were sorted using a follicle measuring gauge; a device which contained a channel along which follicles could be rolled and within which were holes of fixed diameter, increasing incrementally by 1 mm, at intervals. This allowed fast sorting of follicles to ensure minimum time between collection and snap-freezing in LN_2. All conditions were repeated for birds to be used in experiment 3 (n=23, 12 AL, 11 FR), with the additional inclusion of the smallest hierarchical follicle.

2.1.2. Layers (Chapter 4)

Mature *ad libitum* fed White Leghorn layers (n=8) were kept on a 28 hour photoperiodic cycle (14L:14D) for 3 weeks to synchronise ovulatory cycles. Birds were killed by an overdose of sodium pentabarbitone over 3 consecutive days, based on their laying record, to ensure minimal variation between individuals as a result of time of kill relative to the ovulatory cycle. Birds were killed 20 hours after dusk [2]. All birds used had eggs present in the oviduct at sampling. Body weight and the numbers of follicles of each sample category were recorded at post mortem (Table 2). Sampled tissues were the anterior stroma, pre-hierarchical follicles of diameter 1-

4 mm, 4-5 mm increasing in 1 mm increments to 8 mm and the F6-F1 hierarchical follicles. All yolk was removed from hierarchical follicles and the follicle walls washed prior to snap-freezing in LN_2. All other follicles were sorted using a follicle measuring gauge as above.

2.1.3. Advanced Intercross Line (Chapter 6

Offspring from mating a WL male to a Ross 308 parent female were used to establish a full-sib advanced intercross (AIL) flock. The 14 male and 14 female F1 were divided into 12 families that formed the parents of the F2. It was subsequently discovered that duplicate wing bands were used for offspring of a second mating and an F1 offspring of a broiler male x layer female cross had contributed to the F2. For the F2, and all subsequent generations, 5 males and 5 females were selected from each of the 12 families. Subsequent matings were conducted in a scheme described by Falconer [3] to minimise inbreeding. Mating was by artificial insemination (single male-female pairs from the F2 onwards).

Chicks used for data collection were reared in floor pens 1.45 m wide x 2.45 m deep, littered with wood shavings and supplied with a suspended feeder and drinker. They were fed *ad libitum* on standard layer rations. The photoperiod was 8L:18D from hatch and 14L:10D after caging at 20 weeks. Body weights were recorded at 7, 21, 42, 63, 84 and 140 d. Age at first egg was recorded and the birds were killed by an overdose of sodium pentabarbitone after they had laid at least 2 eggs. At post mortem, numbers and weights of white follicles 5-8 mm diameter and yellow follicles were recorded. The weights of the ovarian stroma, oviduct, liver, abdominal fat and pituitary were recorded.

2.1.4. Multistrain Population Chapter 6)

The multistrain populations comprised i) 13 traditional or pure breeds with a range of growth phenotypes (Auracana, Barnevelder, Brown Leghorn, Buff Orpington, Cornish Game, Friesian Fowl, Ixworth, Jersey Giant, J-line, Light Sussex, Maran, White Dorking, White Sussex), ii) 12 modern meat type lines, similar to the female used in the AIL, which were originally derived from heavy breeds and iii) 12 modern layer lines which included lines of a White Leghorn background and from brown egg

layers which were from a number of breeds including some of a heavier type. The populations have been described previously by Sandercock *et al.* [4]. Twelve individuals of each line were available at 6, 8 & 10 weeks of age (4 per age group), except where otherwise stated. Birds were fed ad-libitum. Birds from the 10 week group were selected for use in restriction digests.

2.2. RNA Processing

2.2.1. RNA Purification Chapters 4-6)

Total RNA was isolated using the Ultraspec II RNA kit (AMS Bioscience, Abingdon, UK). Yolks were removed from hierarchical follicles prior to their being snap-frozen in LN_2. Whole pre-hierarchical follicles were pooled for each bird according to sample groups described in section 2.1 and snap-frozen LN_2. On thawing, all samples were maintained on ice during homogenisation in a solution of Ultraspec II at a ratio of 1ml per 100mg tissue.

The Ultraspec II kit uses a phenol/chloroform solution to isolate RNA in the aqueous phase of samples separated by centrifugation. RNA quality was checked using an Agilent Bioanalyzer (Agilent Technologies UK, Stockport, UK) for the RNA required for the microarray analysis, and on a nanodrop (Thermo Scientific, Wilmington, DE, USA) for RNA to be used for QPCR.

2.2.2. Reverse Transcription (Chapters 4-6)

For confirmatory real-time quantitative PCR (QPCR) in layer tissue 1 µg of total RNA was reverse transcribed using a First Strand Synthesis Kit (GE Healthcare, Little Chalfont, UK). The First Strand Synthesis Kit uses a murine Reverse Transcriptase (FPLC*pure*™) and provides the NOT *I*-d$(T)_{18}$ bifunctional primer to facilitate the reaction. Reverse transcription of the broiler breeder tissue for QPCR used the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems (Life Technologies), Paisley, UK) using the NOT *I*-d$(T)_{18}$ primer from the First Strand Synthesis Kit and MultiScribe™ Reverse Transcriptase.

2.3. QPCR

2.3.1. Primer Design (Chapters 4-6)

Primers for RT-PCR were designed for all candidate genes. A reference sequence for each gene was identified from the Chicken Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway?org=chicken) using the 3rd (2006) build of the genome. The primary search key used was the HGNC gene name; however, if this returned multiple alternately spliced variants or was unknown then the ChEST ID or Ensembl ID from the microarray annotation file was used to identify the variant specific to the microarray probe. Primer3 (http://frodo.wi.mit.edu/) [5] was used for designing the primers. Default settings were used to retrieve products of length 100-250bp which spanned intronic regions in order to clearly distinguish product from possible contamination by gDNA in samples for PCR. The primer sequences produced by Primer3 were then tested in the *in silico* PCR tool on the Chicken Genome Browser Gateway to confirm their suitability. While primers for sequencing do not require it, primers for expression analysis should ideally sit within separate exons and the product should span the intronic region. This makes it possible to distinguish between cDNA and gDNA, should any remain. If primers did not span an intron, that region was excluded from the sequence provided to Primer3 and the search repeated until primers that conformed to the desired parameters were identified. As several genes would be analysed on the same 96-well plate for both assay development and high-throughput screening, it was also important that primers were effective within both FastStart and SybrGreen PCR reactions at similar annealing temperatures so that reactions could be run simultaneously.

2.3.2. Production of Standard Curves (Chapter 4)

PCR was carried out using FastStart Taq (Roche, Burgess Hill, UK) and the product run out on a 2% agarose gel to ensure a single band was present. The band was then excised and the cDNA extracted using a Qiaex II Gel Extraction Kit (Qiagen, Crawley, UK). This kit solubilises agarose and uses silica gel particles to bind DNA in the presence of high salt concentrations. Subsequent washing of the particles in a solution of low salt concentration allows dissociation of the DNA from the silica gel.

A Top Standard was produced by diluting the purified cDNA 1/500. A series of 10-fold serial dilutions were carried out to produce the Standard Curve.

Table 2.1 FastStart PCR Mastermix components and standard reaction conditions

FastStart PCR Mix per sample:

Reagent	Volume
10x Buffer	3µl
10x NTP	3µl
20µm primer F	3µl
20µm primer R	3µl
FastStart Taq	0.15µl
cDNA	2µl
H_2O	15.85µl

FastStart PCR Conditions:

Cycles	Time	Temperature
1x	4 mins	95˚c
|	30sec	95˚c
40x|	30sec	60˚c
|	30sec	72˚c
1x	7 mins	72˚c

Table 2.2 SybrGreen QPCR Mastermix components and standard reaction conditions

SybrGreen PCR Mix per sample:

Reagent	Volume
SybrGreen	12.5µl
Rox	0.5µl
20µm primer F	0.5µl
20µm primer R	0.5µl
H_2O	1µl
cDNA	10µl

SybrGreen PCR Conditions:

Cycles	Time	Temperature
1x	2 mins	50˚c
1x	2 mins	95˚c
40x |	15 sec	95˚c
|	30 sec	60˚c
1x	1 mins	95˚c
1x	30 sec	60˚c

2.3.3. Expression Profiling in Layers (Chapter 4)

Candidate genes were initially screened using two 4-bird pools of anterior stroma, 5-6 mm, 6-8 mm, and F4 material taken from layers. QPCR was carried out on cDNA according to a Platinum SybrGreen (Life Technologies Ltd, Paisley, UK) protocol with duplicates using a standard curve on an MX3000 Sequence Detection System (Stratagene). Controls (no template) were run for all primer pairs. For the first stage - high-throughput screening of pooled material - fold change between consecutive tissues was estimated using the ΔCt method [6], where Ct is the cycle number at which the detection threshold is crossed. PAK3, a gene associated with cytoskeleton assembly, was selected from the gene list as a normalising control for this stage as it

showed only minimal variation between samples. This was done to prioritise candidate genes for comprehensive profiling by QPCR as described above. All sampled tissues were used for comprehensive profiling. In the comprehensive profiling, QPCR for each candidate was run across 2 plates (4 birds per plate) with each plate replicated: see Figure 2.1 for plate schematic. Lamin B Receptor (LBr) values were used for normalisation during this second stage. LBr, a protein located in the nuclear envelope [7] was used as traditional controls such as GAPDH showed increased variation across samples in rapidly growing tissues such as ovarian follicles. LBR was recommended as an alternative by members of Dr Mike Clinton's research group at The Roslin institute. ANOVA confirmed no significant differences between stages of follicle development.

2.3.4. Validation of Dietary Effect in Broiler Breeders (Chapter 5)

Twelve bird pairs of 1 AL and 1 FR were randomised with replication over 4 plates. QPCR was carried out on cDNA as above with a positive control sample of pooled layer follicle cDNA run in triplicate across plates to normalise between plates. See Figure 2.2 for plate schematic. LBr values were used for normalisation.

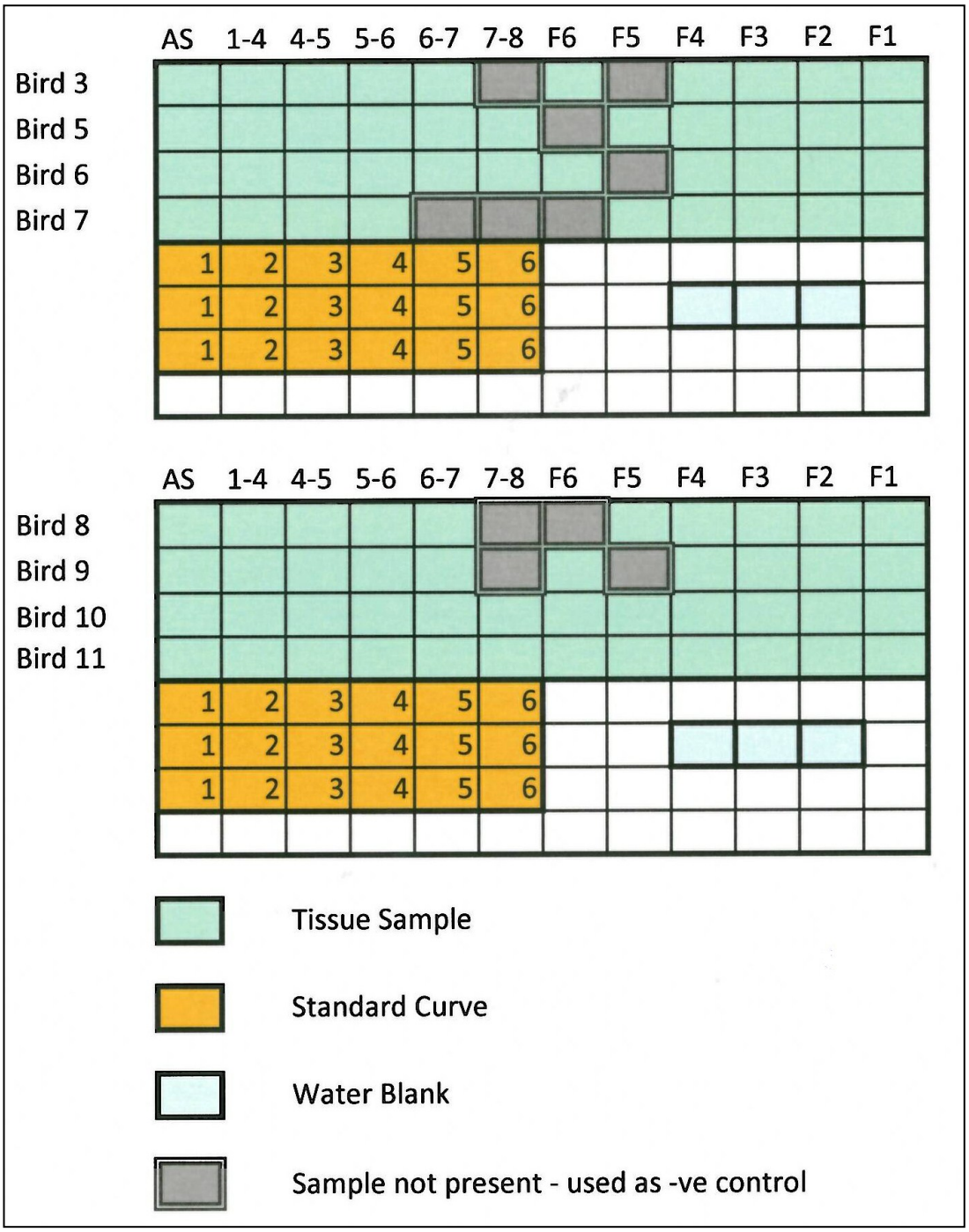

Figure 2.1: 96-well Plate layout for qPCR in White Leghorns. AS - Anterior Stroma, numbering (e.g. 1-4) refers to follicle diameter or hierarchical position.

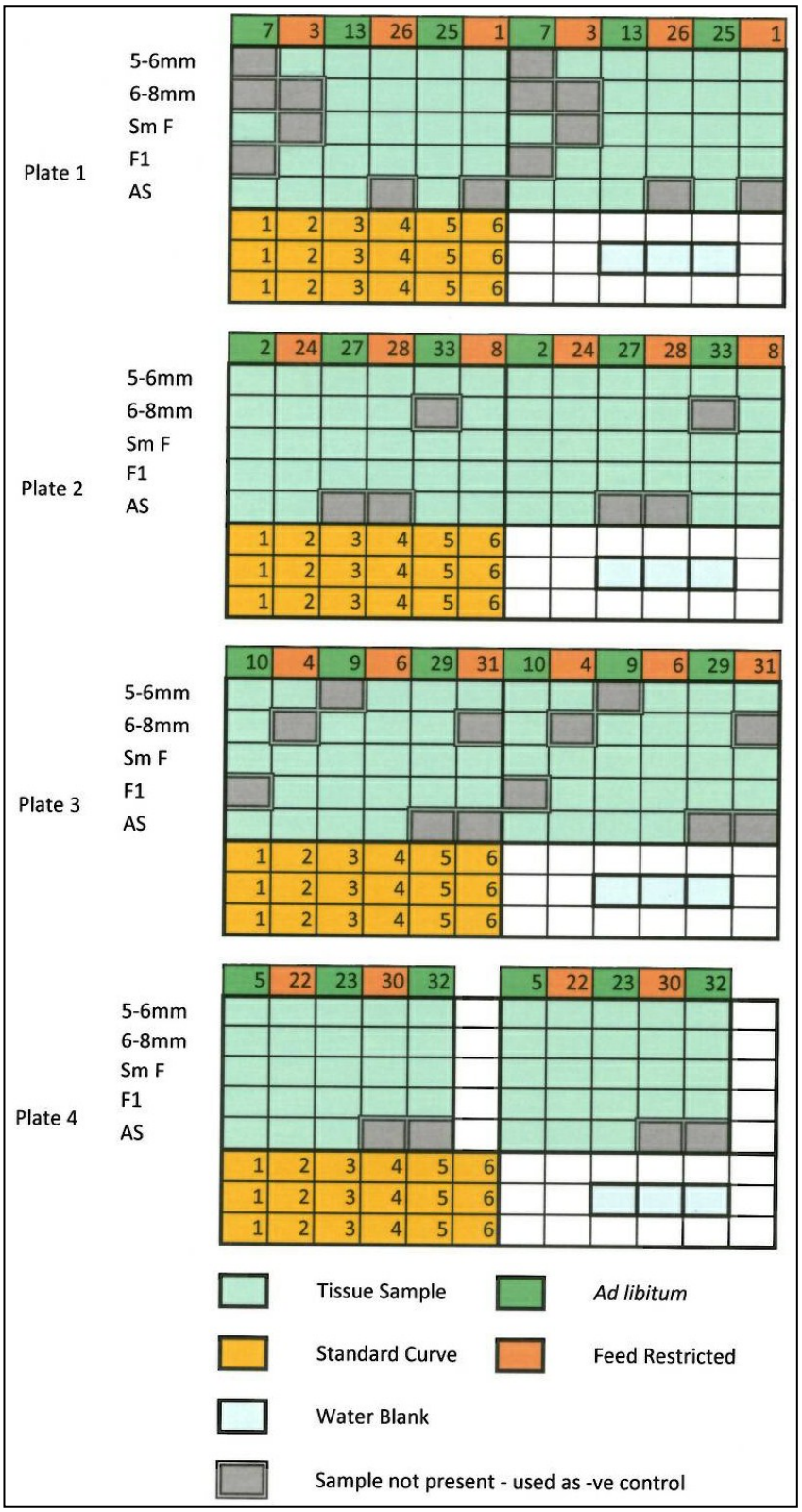

Figure 2.2: 96-well Plate layout for qPCR in *ad libitum* vs. restricted broiler breeders. AS - Anterior Stroma, Sm F- Smallest Hierarchical follicle, F1 - pre-ovulatory follicle5-6mm/6-8mm indicates pre-hierarchical follicle diameter. Other numbering indicates Bird ID.

2.4. Statistical Analyses

2.4.1. Between-Tissue Statistical Analysis of Microarray Data (Chapter 3)

For each probe, mean values were calculated for each bird-pair within the microarray to remove the dietary variable prior to between-tissue comparison. The datasets for the individual ovarian tissues were combined and then quantile-normalised within R prior to performing a Kruskal-Wallis one-way ANOVA to identify probes that showed significant differences between tissues. The Kruskal-Wallis test allows comparison between 3 or more sample populations and allowed the analysis of all 4 ovarian tissues without having to conduct multiple tests. A threshold of $P < 0.01$ was used for statistical significance.

2.4.2. Between-Tissue Cluster Analysis of Microarray Data (Chapter 3)

An expression file was created using normalised bird-pair mean intensity values from R. This consisted of annotation columns and 32 data columns representing the 4 ovarian tissues from the 8 bird pairs. BioLayout Express3D (www.biolayout.org/) was used to analyse this data file. File construction and data analysis were carried out according to the protocol available from the website [8], and the various stages thereof will be discussed further in the next chapter. A Pearson correlation threshold of 0.9 was used in the initial analysis and the embedded clustering algorithm (MCL) was used to cluster genes by expression profile. Clusters were limited to $n \geq 3$ where n = number of probes to ensure clusters were not formed merely by probes clustering with their own replicates.

2.4.3. Between-Tissue Statistical Analysis of QPCR Data (Chapter 4)

QPCR datasets for each candidate gene from the expression profiling in layers were log-transformed using natural logarithms. An Analysis of Variance was run in GenStat [9], using the model containing fixed effects for tissue within bird and Plate as the Block effect.

2.4.4. Treatment by Tissue Statistical Analysis of QPCR Data (Chapter 5-6)

QPCR datasets for each candidate gene from the validation of dietary effect in broiler breeders were log-transformed using natural logarithms. Following correction for

plate effect and Total RNA, replicate means were calculated and log-transformed. A Linear Mixed Model (REML) was run in GenStat [9] using a model with fixed effects for Tissue x Treatment with Bird as the random effect.

For the 3 PDGFRL variants discussed in chapter 6, the same approach was followed using a Linear Mixed Model (REML) with fixed effects for Tissue x Treatment x Gene_Variant with Bird x Tissue as the random effect.

2.5. Sequencing and Genotyping (Chapter 6)

2.5.1. EST Alignment and In Silico SNP Detection

For each gene, Expressed Sequence Tags (ESTs) were identified from the UCSC Chicken Genome Browser website (http://genome.ucsc.edu/) by blast search with the reference sequence and downloaded in FASTA format with the reference sequence from the NCBI database. The PreGAP and GAP4 tools in the Staden package [10] were used to align the ESTs to the Reference sequence. GAP4 was then used to identify discrepancies between aligned sequences. Where the same discrepancy was repeated across multiple ESTs, a possible SNP was marked.

2.5.2. AIL Founder Sequencing

To confirm the presence of SNPs predicted as above and to identify further SNPs in the region, primers were designed against the Reference sequence using Primer3 [5]. FastStart PCR was carried out using gDNA from the AIL founders and the product purified using a PureLink PCR Purification Kit (Life Technologies Ltd, Paisley, UK). The kit uses a silica membrane to selectively bind dsDNA using the same principles as the Qiaex II Gel Extraction kit described above. Sequencing of the purified product was carried out by GATC (GATC Biotech Ltd. London, UK).

2.5.3. AIL F8 Genotyping

DNA for 360 AIL F8s was shipped to KBioscience for Genotyping. Association between follicle number and genotype was tested using a general Analysis of Variance.

2.5.4. Restriction Digests in the Multistrain Population

Restriction digest assays were designed against the 2 multi-base inserts identified in PDGFRL and GDF9 using NEBCutter 2.0 [11], and were tested using DNA from the AIL founders. Assays were then carried out on DNA from the multistrain population described in section *2.1.4* above. The multistrain population was divided into broiler, layer, and traditional lines and assays carried out on the 3 groups separately for ease of data handling and experimental implementation. For PDGFRL, a sequence spanning 385bp was amplified by PCR. The presence of the insert was detected

using the restriction enzyme MnlI, which cut between base 105 and 106 if the extra sequence was not present and between bases 171 and 172 where it was present. For GDF9, a region spanning 628 bases was amplified and the enzyme BlpI used to determine presence of the insert. BlpI cut the sequence between bases 98 and 99 and between bases 419 and 420 if the insert was present. Enzymes and other reagents were supplied by New England BioLabs, Ipswich, MA, USA. A chi-square test was used to analyse the data.

2.6. References

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3

The Broiler Breeder Microarray Study

This chapter introduces the initial dataset provided for this study and describes identification and selection of initial candidate genes for investigation in subsequent chapters. Selected results are published in McDerment et. al. (2012).

3.1. Introduction

3.1.1. Background

As discussed in chapter 1, feed restriction is a standard practice employed in the poultry industry to mitigate the adverse effects that mature broiler breeders would otherwise experience, of which multiple ovulation is the major problem preventing an effective improvement to their welfare [1]. Lower follicle number in the ovary is, perhaps, a predictable effect of reduced food intake. However, our understanding of the signalling and regulatory mechanisms behind the genetic and nutritional effects is far from complete [1, 2].

The main gap in our collective knowledge as regards follicle development concerns the transition of follicles from the 6-8 mm pre-hierarchical pool to the rapidly growing hierarchy [2]. Since the number of pre-hierarchical follicles remains relatively unchanged in response to food restriction or *ad libitum* feeding [3], it is likely that, as in various mammalian species [4], regulation of follicle growth after initial stimulation is internal to the ovary during pre-hierarchical development, though this has yet to be conclusively proven in birds. What is known about food restriction shows the major effects occurring at the level of the hypothalamus and pituitary [1, 3, 5]. However, whereas this will affect the rate and quantity of gonadotrophin signalling to the ovary, it does not explain why, under ideal conditions, one follicle is selected from the 6-8 mm pool to survive and the others become atretic. This suggests that there are factors that set the selected follicle apart from the remainder of the pool.

3.1.2. Strategy

To study this process, a Defra-funded microarray study was undertaken in 2006 to identify genes involved in regulating follicle number by comparing the response to food restriction (FR) with that of *ad libitum* feeding (AL) in tissues of the Hypothalamo-Pituitary-Gonadal Axis (HPGA) and follicles of different classes. Although results from the different follicles proved inconclusive from this standpoint, an overview of that study will be given here in order to provide the context for the data received from it that formed the starting point of this thesis.

Although the stroma and each class of follicle in the study had been examined individually with respect to treatment in the initial analysis, they had not been examined collectively to identify the changes as follicles develop. This was an obvious avenue to pursue in the first instance.

3.1.3. Microarray Technology

Microarrays utilise a combination of the technologies of DNA hybridisation that underpins Southern Blotting with the principles behind high-throughput methods such as the yeast-2-hybrid system. This allows for examination of large numbers of features in parallel [6]. The technology has enabled genome-wide gene expression profiling [7, 8] and large scale SNP genotyping [9], as well as alternate splice variant [10] and phylogenetic analysis [11]. As this suggests, many different types of microarray exist; cDNA and oligo arrays were the first to be developed [8, 12], partly as a method for elucidation of expression patterns for groups of known genes in specific cell types, tissues etc. but also for identifying novel genes through ESTs (Expressed Sequence Tags) [12] co-expressed with known genes of a given pathway. Using microarrays is a tried and tested method for gene candidate identification between defined physiological states, genotypes or during temporal changes or development, and although microarrays have been used as much for diagnostic as research purposes in recent years [7, 8]; it was in their primary capacity that they were used for this study.

3.1.4. Aims & Objectives

The primary aim of this chapter was to determine if any further information could be gained from the 2006 microarray study, in the context of regulation of follicle development in the ovary. The first objective was to conduct a second analysis of the microarray dataset, removing the dietary variable and focussing on differences between tissues representing the different stages of development, in order to compile a list of candidate genes for further investigation. The second objective was to incorporate other sources of information into a composite analysis to refine this candidate list prior to examination of individual candidate genes.

3.2. Experimental Design

3.2.1. The 2006 Microarray Study

Female Ross broiler breeder chicks were reared as described in section *2.1.1.* Birds were organised into 8 randomised pairs (FR v AL) for the hypothalamus, pituitary, anterior stroma, 5-6 mm follicles, 6-8 mm follicles and the F1 pre-ovulatory follicle. The anterior stroma was separated from the posterior stroma because the vast majority of primordial follicles are restricted to this region. The posterior stroma contains very few follicles so is unlikely to yield many insights into genes involved in their regulation. Paired samples were then hybridised to a 2-channel spotted microarray in a dye-swap design using Cy3 and Cy5 dye (GE Healthcare, Little Chalfont, UK) labelled RNA. Cy3 and Cy5 have similar but not identical binding efficiencies. To ensure this did not bias results, each chip on the microarray was duplicated, reversing the labelling dyes for the two treatment groups, so that all birds in both treatment groups were measured using both dyes. The chicken oligo microarray used was produced by ARK Genomics (http://www.ark-genomics.org) and contained 17,000 unique features and multiple controls. Labelling was performed using a Stratagene Fairplay kit (Agilent Technologies Ltd, Stockport, UK) and hybridised using an automated GeneTAC hybridisation station (Genomic Solutions (Digilab), Huntingdon, UK).

3.2.2. Between-Treatment Statistical Analysis

The original statistical analysis of the microarray data was carried out in an R environment [13] using the Bioconductor Limma package [14] and the protocol outlined by IC Dunn*, et al.* [15]. The data was quantile-normalised to account for the different binding efficiencies of the Cy3 and Cy5 dyes used in the microarray and means were calculated for replicate spots. A split-plot ANOVA was used to estimate the treatment effect. A Mann-Whitney non-parametric *t*-test was used to validate the normalisation process.

3.2.3. Results and Conclusions

The study clearly demonstrated that, while pre-hierarchical follicle numbers were not significantly altered by feed restriction (Table 3.1.), under *ad libitum* feeding there

were twice as many hierarchical follicles compared with feed restricted birds. A number of candidate genes were identified in the pituitary and were followed up. However nothing from the ovarian tissues surpassed the threshold of statistical significance set for the study. As a result, the ovarian data from the microarray was set aside while the candidate genes in the pituitary were investigated. The ovarian portion of the microarray data was subsequently provided for use in this study.

Table 3.1: Follicle numbers, body, pituitary and ovarian stroma weight in broiler breeders kept on restricted feeding or released for two weeks to simulate ad-libitum feeding.

Variable	Restricted		*Ad-libitum*		P-value
	Mean	SEM	Mean	SEM	
Body weight (Kg)	2.89	±0.11	3.79	±0.07	<0.001
Number of follicles >8mm	5.62	±0.18	10.25	±0.37	<0.001
Number of follicles 5-8mm	10.6	±1.5	12.2	±1.4	NS
Pituitary weight (mg)	8.03	±0.55	11.18	±0.42	<0.001
Ovarian stroma Weight (g)	4.55	±0.31	6.91	±0.38	<0.001

3.3. Revisiting the Ovarian Data

3.3.1. Data Handling

As the data for the original experiment had been analysed in R, it was decided to continue to use R for subsequent analyses. This required familiarisation with R from first principles.

It was decided, in discussion with David Waddington, to mean the raw values for each bird-pair for each probe from the 2 channels of the microarray as a method for removing the dietary variable at minimal cost to variation within the dataset. The datasets for the individual ovarian tissues were consolidated and then quantile-normalised within R prior to performing a Kruskal-Wallis one-way ANOVA to identify probes that showed significant differences between tissues. A threshold of $P < 0.01$ was used.

3.3.2. Results from Basic Analysis in R

Statistical analysis of the data on a between-tissue basis, after removing the treatment effect, produced 5571 probes with significant differential expression between one or more tissues at $P < 0.01$ and an additional 1149 at $P < 0.05$. Plotting the distribution of the *P*-value frequency provides a visual indication of where the greatest significant differences exist between groups. Figure 3.1a shows the *P*-value distribution for the whole ovarian dataset while figure 3.1b shows the distributions for the comparisons of what amount to consecutive tissues in the context of this study. As would be expected, the fewest significant differences occur between 5-6 mm and 6-8 mm follicles, as these are genuinely adjacent stages in development. There are, however, still a large number of probes that show significantly altered expression between these stages. Conversely, the stroma to 5-6 mm transition and the 6-8 mm to F1 transition each show just over 10,000 significantly differentially expressed probes at $P < 0.05$ when compared in isolation.

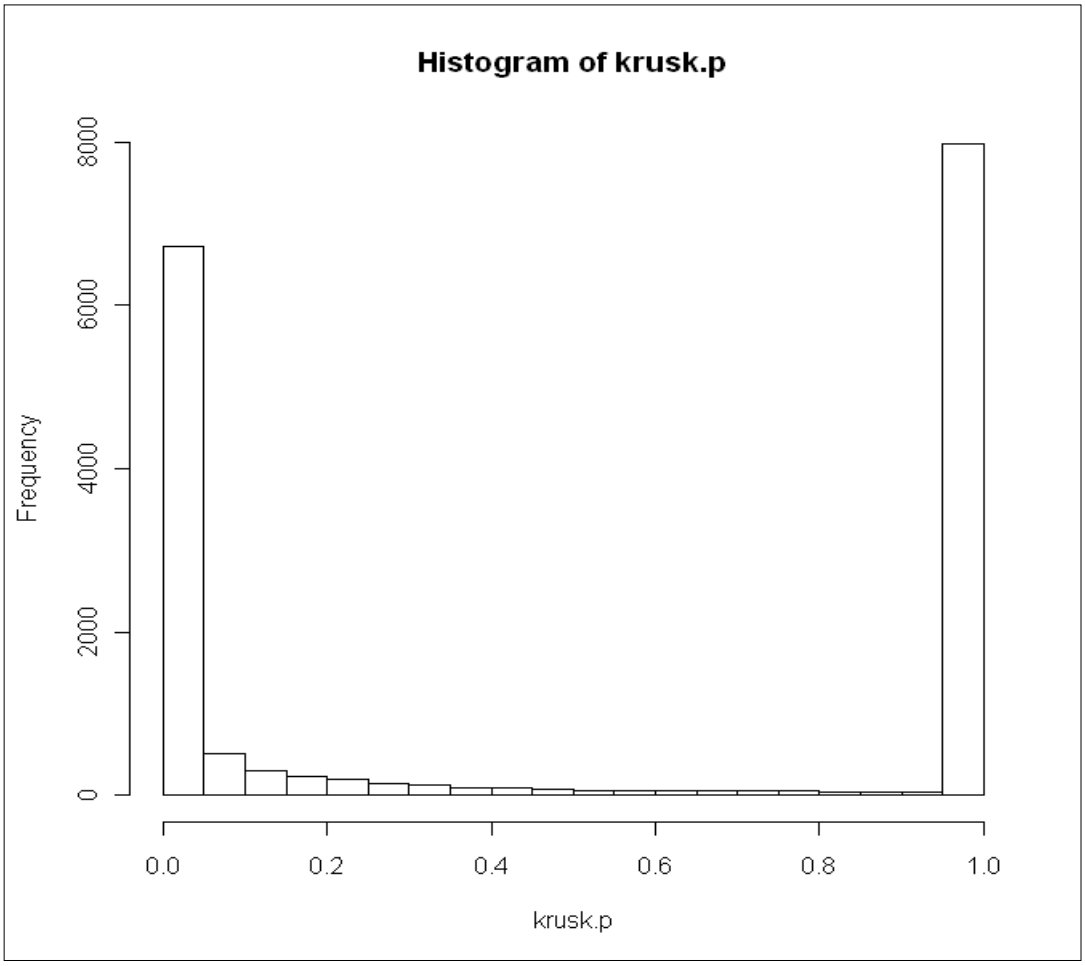
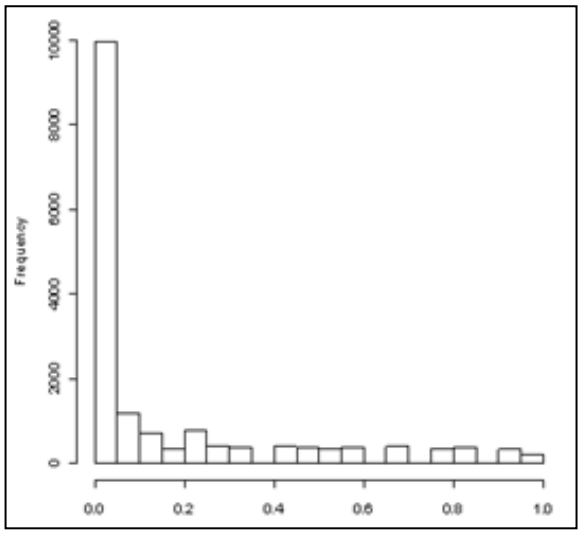
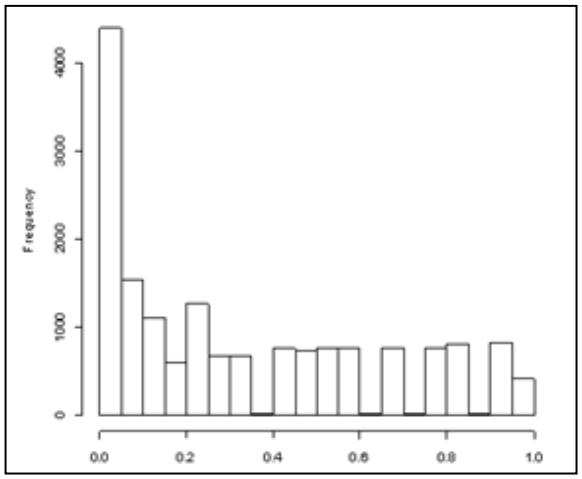


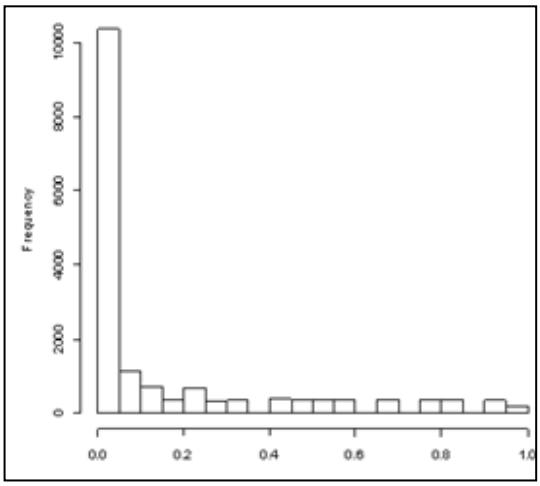
Figure 3.1a Histogram showing the frequency distribution of *P*-values calculated by the Kruskal-Wallis test (See section 2.4.1) for the microarray probe-set in all ovarian tissues from the FR vs. AL broiler breeder microarray to identify probes showing significant differential expression between 2 or more stages of follicle development.



Progression from anterior stroma to 5-6 mm



Progression from 5-6 mm to 6-8 mm follicles



Progression from 6-8 mm to the F1 follicle

Figure 3.1b Histogram showing the frequency distributions of *P*-values calculated by the Kruskal-Wallis test (See section 2.4.1) for the microarray probe-set in comparisons of consecutive stages of development from the FR vs. AL broiler breeder microarray.

3.4. Cluster Analysis in BioLayout Express3D

3.4.1. Expansion of Annotation

The original annotation for the microarray, received with the data, was lacking a considerable amount of information as the latest build of the chicken genome was not available when the annotation was compiled. Before proceeding with the cluster analysis, it was clear that the annotation required updating to reflect the current level of genome annotation available. This task was initially carried out by manual curation. This initially proved to be a time-consuming process due to the distributed nature of the information, though during the later stages more comprehensive resources were made available. The process of updating the annotation is shown in figure 3.2.

3.4.2. Results from Cluster Analysis

An initial network of 5189 probes was produced from the BioLayout Express analysis (Figure 3.3) using a Pearson Correlation threshold of 0.9. The Markov Clustering Algorithm (MCL) produced 260 clusters of probes, of which 101 exhibited expression profiles indicative of a possible role in follicle development based on changes in expression between tissues. Inspection of the profiles identified 4 distinct profile types that are presented in Figure 3.4. No probe in the profile lists from BioLayout Express had a Kruskal-Wallis (K-W) *P* value of >0.002 which suggests that BioLayout Express successfully filters out the vast majority of non-significant data at point of entry. With this double-filtering by the Kruskal-Wallis *P*-value and the BioLayout Express Pearson correlation, the number of probes under consideration was reduced to 1,227. The Kruskal-Wallis/BioLayout-filtered probe lists were compared with the Top-50 probes (by *P* value) from the feed restriction vs. *ad libitum* feeding comparison within tissues from the original analysis in order to identify any genes common to both analyses. This process identified 13 common genes (Table 3.2). No apparently related function for these genes could be identified through literature mining, although a number have functions of potential relevance to different aspects of follicular development.

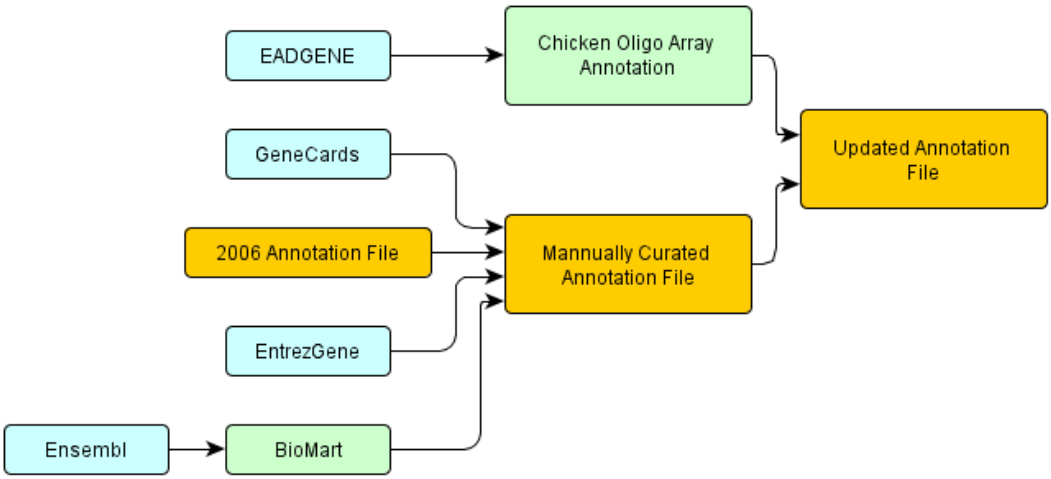


Figure 3.2 Schematic representation of workflow for updating and consolidating available annotation of the microarray data. Nodes in orange: Primary data file, nodes in blue: Data sources, nodes in green: processes where annotation consolidation was all or partly automated.

Table 3.2a. Layout of gene list comparisons of the between-treatment and between-tissue analyses of the microarray data showing the number of common genes.

BioLayout Profile	F5-6 Top 50	F6-7 Top 50	F1 Top 50	Stroma Top 50
F5-8 Up	2	4	3	2
F5-8 Upward	-	-	-	-
F5-8 Downward	-	-	-	-
F5-8 Down	-	-	-	3

Table 3.2b. Genes identified by comparison of the BioLayout profile lists with the top 50 genes by *P*-value from the initial microarray analysis.

Comparison	5-6 mm Top 50 vs. F5-8 Up	6-8 mm Top 50 vs. F5-8 Up	F1 Top 50 vs. F5-8 Up	Stroma Top 50 vs. F5-8 UP	Stroma Top 50 vs. F5-8 Down
Common Genes	MYO1C YAP1	MYO1C GULP1 RIGG03908 RIGG05331	GRP ZNF593 MAMDC2	RIGG01740 SPTY2D1	POSTN PDGFRL TBC1D13

Note: F5-8 Up refers to the profile seen in Figure 3.4 top left, F5-8 Upward refers to the profile seen in Figure 3.4 top right, F5-8 Downward refers to the profile seen in Figure 3.4 top right, F5-8 Down refers to *the profile seen in Figure 3.4 bottom left.*

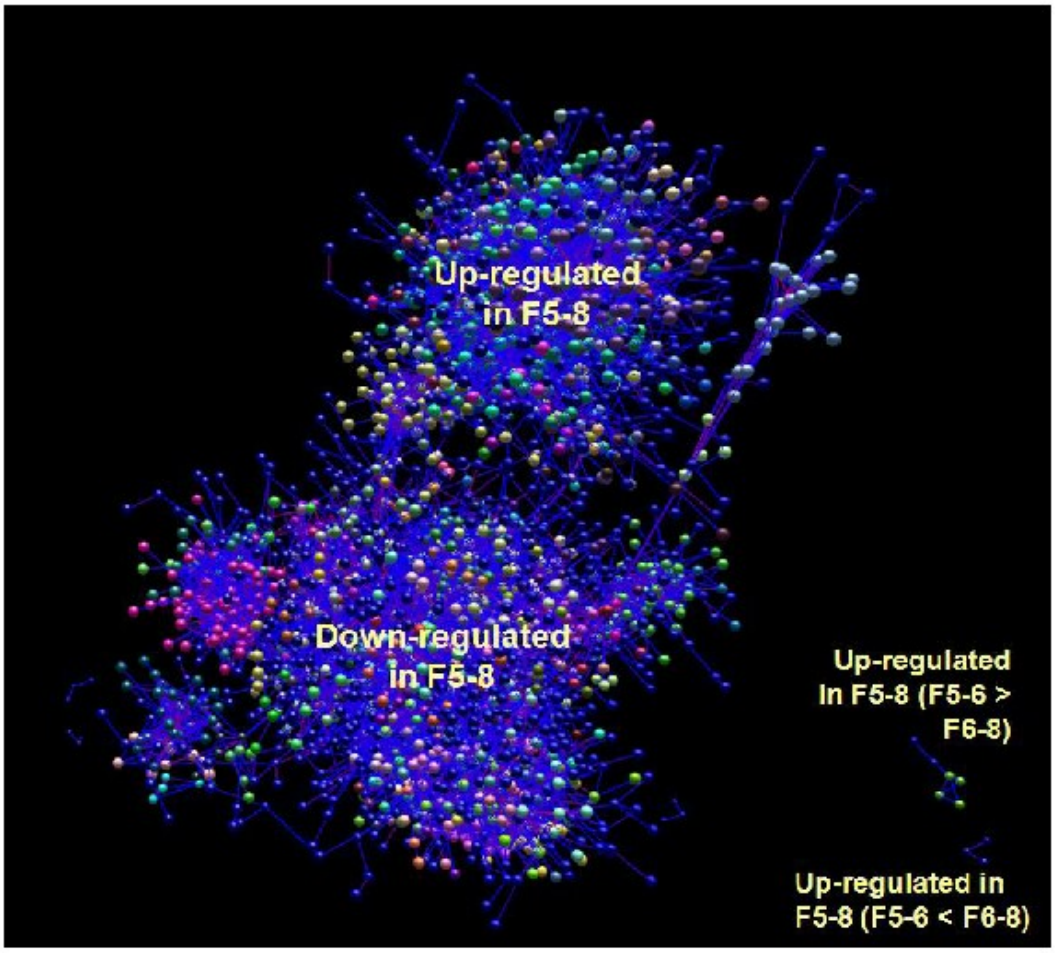


Figure 3.3. The 3D network of nodes generated in BioLayout Express3D from the quantile-normalised microarray data excluding the dietary variable. Labels indicate localisation of key expression profile types. Individual nodes represent probes from the microarray. Colours indicate nodes' cluster membership. Edges connecting nodes indicate relatedness based on the Pearson correlation. A Pearson Correlation threshold of 0.9 was used in the primary analysis (Section 2.4.2).

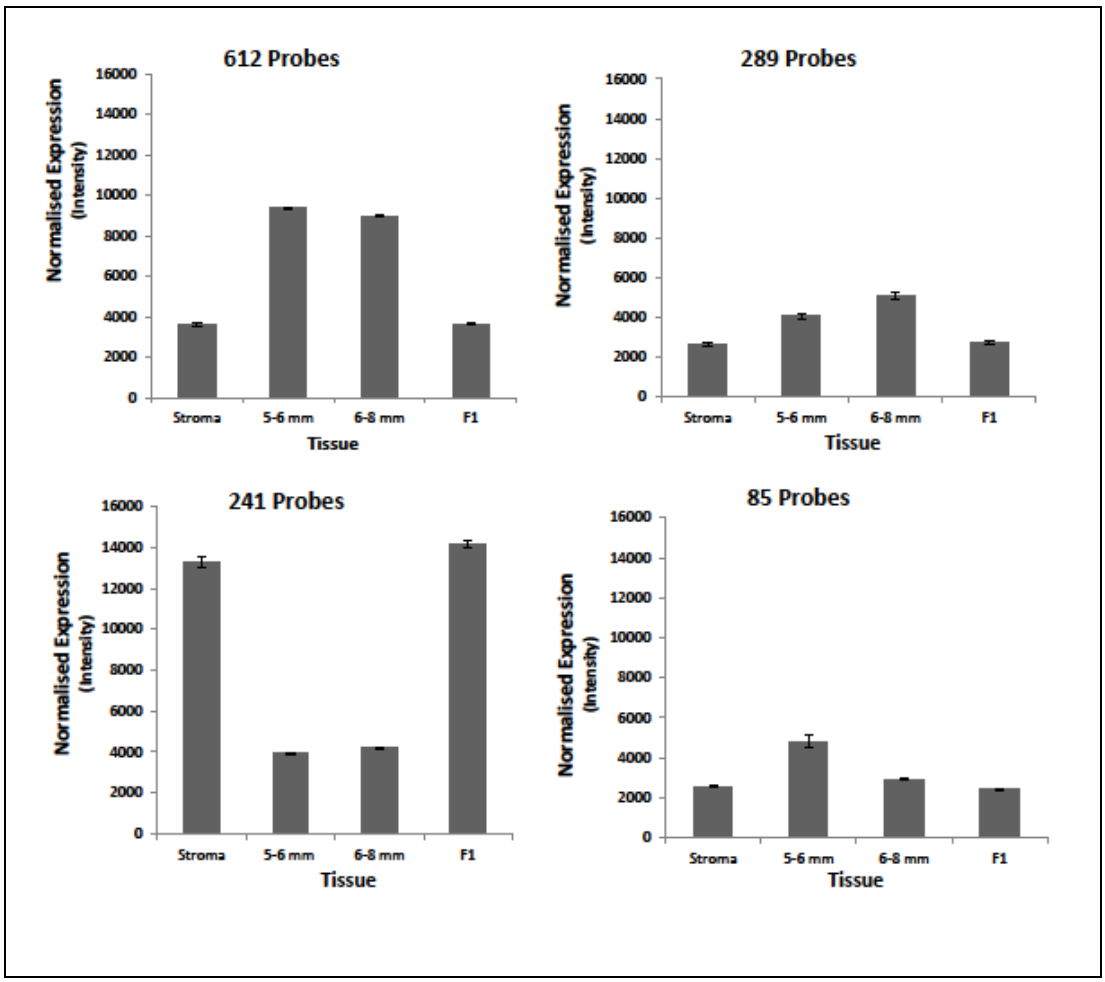


Figure 3.4 Examples of general expression profile types identified from the BioLayout Express3D cluster analysis (For method see section 2.4.2). Top left: 5-8 Up top right: 5-8 Upward, bottom left: 5-8 Down, bottom right: 5-8 Downward. Probe numbers indicate total probes from the microarray that exhibit the profile type. Data represents mean normalised intensity values with Std Error (n = 8).

3.4.3. Other Data Patterns

3.4.3.1. Enrichment Analysis

The BioLayout profile gene lists were put through GeneCoDis (http://genecodis.dacya.ucm.es/) an online tool for grouping genes with related annotations such as molecular function or sub-cellular location. A number of common processes, functions, and pathways were identified for the different profile groups by GeneCoDis. These are summarised in Tables 3.3, 3.4, and 3.5 respectively.

In terms of prominent biological processes represented in these profile gene lists, in the 5-8 mm Up profile there is a strong trend towards cell growth and proliferation, whereas in the 5-8 mm upward profile, the emphasis is towards intracellular transport. Certain other specific transport processes are represented in the 5-8mm Down and Downward profiles but the strongest signals are associated with cell adhesion, which one might be expected to be reduced in tissues where cells are proliferating. Annotations for KEGG pathways could only be found for the profiles showing increased expression in 5-8 mm follicles. The most prominent pathways identified, Parkinson's disease and Oxidative Phosphorylation, can, in this instance, be interpreted as leading to apoptosis, again, something to be expected in a rapidly growing tissue with high cell turnover.

The profiles also show a wide range of molecular functions, though in each case, the most common is protein binding, suggesting that protein-protein interaction networks and therefore signalling pathways are being detected.

While these can be taken as indicators only, the results provide an additional level of consistency and strengthen the case for using these profiles as the basis for candidate selection. It should be noted that, as with many online resources, databases on this site were not as comprehensive for the chicken as for mammalian species so the searches shown were run against human and mouse databases. Where annotations were available for the chicken, these were consistent with the results in tables 3.3, 3.4 and 3.5.

Table 3.3. Biological Processes associated with the genes from the 4 BioLayout profiles identified from the between-tissue analysis of the broiler breeder microarray.

	BioLayout Profiles as seen in Figure 3.4			
Biological Process	5-7 Up	5-7 Upward	5-7 Downward	5-7 Down
Carbohydrate Metabolism	-	5	-	-
Cell Adhesion	-	-	2	11
Cell Cycle	19	-	-	-
Cell Differentiation	-	-	4	-
Cell Division	11	-	-	-
DNA Damage	-	5	-	-
DNA Replication	8	-	-	-
Electron transport Chain	-	5	-	-
Endosome organisation	-	-	-	3
innate immune response	-	-	2	-
Intracellular protein transport	8	-	-	5
Mitosis	15	-	-	-
Nuclear mRNA Splicing	10	-	-	-
Oxidation-Reduction	-	8	-	5
Potassium ion Transport	-	-	2	-
Protein Complex Assembly	-	-	2	-
Protein Folding	-	-	-	6
Protein Modification	-	6	-	-
Protein transport	-	7	-	-
Regulation Catalysis	-	-	-	3
RNA Splicing	11	6	-	-
Signal transduction	-	-	7	-
Translation	-	-	-	5
Translational Elongation	-	-	-	4
Transport	-	9	-	-
Ubiquitin-dependent Catabolism	13	-	-	-
Other	62	72	64	10

Table 3.4. Molecular Functions associated with the genes from the 4 BioLayout profiles identified from the between-tissue analysis of the broiler breeder microarray.

	BioLayout Profiles as seen in Figure 3.4			
Molecular Function	5-7 Up	5-7 Upward	5-7 Downward	5-7 Down
Actin Binding	-	-	-	8
ATP Binding	28	18	-	17
DNA Binding	27	-	-	-
Electron Carrier Activity	-	7	-	-
FAD Binding	-	4	-	-
Helicase Activity	-	-	2	-
Hydrolase Activity	-	12	-	11
Ligase Activity	-	-	3	-
Metal ion Binding	45	-	-	19
Nucleotide Binding	40	-	8	22
Oxidation-Reduction	-	9	-	-
Protein Binding	131	58	19	55
RNA Binding	23	12	4	11
Transferase Activity	28	-	-	-
Zinc Ion Binding	43	23	-	18
Other	101	37	20	115

Table 3.5. KEGG Pathways associated with the genes from the 4 BioLayout profiles identified from the between-tissue analysis of the broiler breeder microarray.

	BioLayout Profiles as seen in Figure 3.4			
Pathway	5-7 Up	5-7 Upward	5-7 Downward	5-7 Down
Citrate cycle	-	3	-	-
Mismatch repair	3	-	-	-
Parkinson's Disease	6	5	-	-
Oxidative Phosphorylation	6	5	-	-
Ribosome	5	-	-	-
Styrene Degradation	2	-	-	-

3.4.3.2. Profiles of Annotated Signalling Pathways

A useful feature of BioLayout is that it creates a different class–set for each column of annotation e.g. Molecular Function or KEGG Pathway. This makes it possible to plot collectively expression profiles for all probes sharing a given annotation. Where the annotation was available, the profiles for different signalling pathways were identified within BioLayout in order to determine if the data was comparable with what was already known about intrafollicular signalling. For example, as can be seen in Figure 3.8, the probes associated with the Progesterone-mediated Oocyte maturation pathway clearly show, with the exception of one outlier, relatively constant levels of expression in the stroma and pre-hierarchical follicles followed by a dramatic increase in the F1 follicle. The expression profile for probes in the TGFB signalling pathway also show the expected pattern of expression.

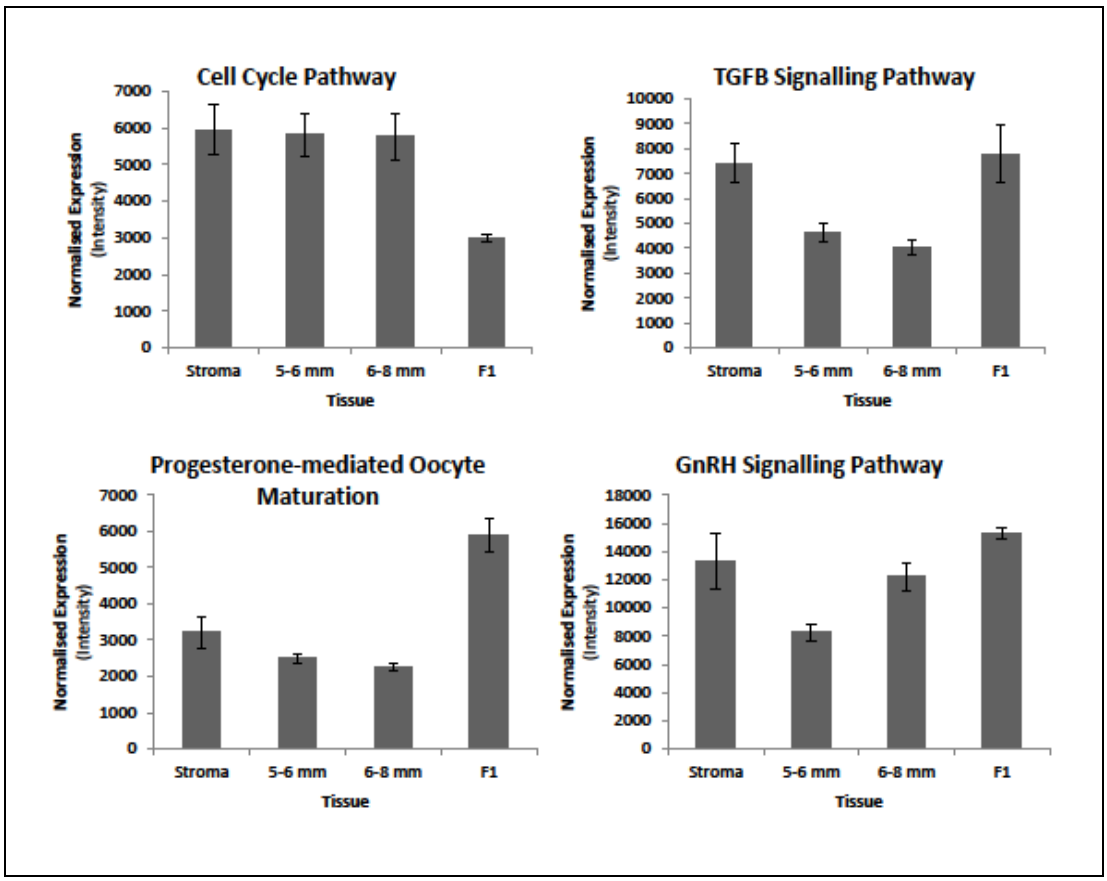


Figure 3.5. Expression Profiles from BioLayout for genes annotated as being members of the Cell Cycle (Top Left), TGFB (Top Right), Progesterone-mediated Oocyte Maturation (Bottom Left) and GnRH (Bottom Right) signalling pathways from the FR vs. AL broiler breeder microarray. Data represents mean normalised intensity values with Std Error (n = 8). For method, see section 2.4.2.

3.4.4. Refinement of the Candidate Lists

3.4.4.1. Reintroduction of Dietary Effect

In order to refine candidate selection, it was decided to reintroduce the dietary effect into the BioLayout analysis. Initially, LOG2 ratio data from the original microarray output files was used. This involved creating a new expression file incorporating all 6 tissues (the anterior stroma, 5-6 mm and 6-8 mm follicles, the F1 follicle, the pituitary and the hypothalamus) from the 2006 experiment. Therefore, 16 data columns for each tissue were brought together forming a file of 96 data columns to which annotation was then added.

As the original experiment was a 2-channel dye-swap, this had to be taken into account when selecting columns for inclusion in the BioLayout expression file. For chips 1-8, channel 1 (CY3) represented *ad libitum* samples and channel 2 (CY5) represented feed restricted. This was reversed for chips 9-16. Therefore, to ensure that the same comparison was being made (feed restricted vs. *ad lib*), for chips 1-8, the Channel 2 : Channel 1 LOG2 ratio, and for chips 9-16, the Channel 1 : Channel 2 LOG2 ratio were used.

When this file was viewed in BioLayout, an inverse pattern was observed between chips 1-8 and 9-16 across tissues. Where genes were up-regulated in 1-8, they appeared to be down-regulated to the same degree in 9-16. This could not be explained as it was confirmed that the correct columns had been used.

To attempt to rectify this, it was decided to use the channel intensity data to create an expression file thus incorporating dietary effect, but also dye effect into the overall analysis. This produced consistent patterns of behaviour across replicates and dyes, albeit at varying levels of intensity. As it was appreciated that CY3 and CY5 are not identical in their binding efficiency, the observed differences in intensity between dyes was not wholly unexpected. Consequently, care had to be taken in drawing conclusions from the data. It was decided to use this analysis for prediction only. The original gene candidate list, excluding literature-sourced markers, was screened in the intensity values datasets for the ovarian tissues. Table 3.6 lists those members of the original list that were predicted to be differentially expressed based on their profiles in BioLayout.

Table 3.6. Genes predicted to be up-regulated in response to *ad libitum* feeding from the BioLayout analysis of the FR vs. AL broiler breeder microarray. Quoted *p*-values are from the Kruskal-Wallis test performed in R for differential expression between developmental stages. A Pearson correlation of 0.9 was used in BioLayout Express.

Symbol	Chromosome	BioLayout Profile	K-W *P*-value	Known Function
ACAT2	Gga 3	UP F5-7	<0.001	Cellular Metabolism
BTN1A1	Gga 28	DOWN F5-7	<0.001	Lipid Transport
FLNB	Gga 12	UP F5-7	<0.001	Regulation of Cytoskeleton
GHR	Gga Z	Downward F5-7	<0.001	Growth
LSP1	Gga 5	DOWN F5-7	<0.001	Signal transduction & Chemotaxis
MFGE8	Gga 10	DOWN F5-7	<0.001	Cell Proliferation
RXRG	Gga 8	DOWN F5-7	<0.001	Inhibition of Cell Proliferation
SH3BGRL3	Gga 23	DOWN F5-7	<0.001	Regulation of Retinoic Acid Signalling
SRP68	Gga 18	DOWN F5-7	<0.001	Protein Transport
TGM2	Gga 20	DOWN F5-7	<0.001	Regulation of Apoptosis

3.4.4.2. Candidate Genes from QTL Regions

The locations of two recently identified follicle number QTL regions on chromosomes 4 and 13, from a parallel study (unpublished data) were provided and a list of known genes from these regions was downloaded from the NCBI Chicken Genome map viewer. This information was incorporated where possible into the BioLayout annotation. Once in BioLayout, the expression profiles of all QTL-associated genes could be viewed. Table 3.7 lists those QTL-associated genes for which both replicate spots indicated a change in *ad libitum* and had a potentially relevant documented function.

Table 3.7. Genes within follicle number QTL on chromosomes 4 and 13 where BioLayout analysis predicted up-regulation in *ad libitum*-fed birds. *P*-values are from the Kruskal-Wallis test performed in R for differential expression between developmental stages. A Pearson correlation of 0.9 was used in BioLayout Express.

Symbol	Chromosome	*K-W P*-Value	Known Function
ADRA1B	Gga 13	0.002	Cell Growth
CAMK2A	Gga 13	NS	Ca2+ Signalling & Cell Cycle
FGF13	Gga 4	0.003	Embryonic Development & Cell Growth
FOXI1	Gga 13	NS	Cell Growth & Proliferation
GDF9	Gga 13	<0.001	Folliculogenesis
PAK3	Gga 4	0.012	Multicellular Organism Development
PPARGC1B	Gga 13	NS	Oestrogen Receptor Binding
SLIT3	Gga 13	NS	Axon guidance & Pro-Apoptotic Signalling
VDAC1	Gga 13	<0.001	Ca2+ Signalling & Regulation of Apoptosis

3.4.4.3. The Hypothalamus, Pituitary, and Co-expression Analysis

Distinctive expression profiles such as were observed in the ovarian data would not be evident in the hypothalamus and pituitary as it represented a 'snapshot' rather than a time-course. Using resources such as KEGG Pathway and knowledge accumulated from the literature, key members of the GnRH signalling pathway were identified in the microarray data in order to determine their cluster membership after MCL had been implemented on the dataset. Few key components of the pathway successfully clustered within BioLayout as far as could be determined with the available annotation so it was decided to focus on the ovarian data.

Apolipoprotein O (APOO) and motile sperm domain containing 1 (MOSPD1) were shown to be co-expressed with FSHR in follicles according to BioLayout clustering. While there were several other genes that also seemingly co-expressed with FSHR, these two were the only ones to have potentially relevant functions. APOO is involved in lipid transport and thus there is the potential for its involvement in yolk formation. While the specific function of MOSPD1 is as yet unknown, it has been implicated in regulation of transcription.

3.5. Candidate Selection

Candidate genes were prioritised for follow-up based on the accumulated results of the above analyses. Of an initial 60 genes being considered from their expression pattern, 36 were confirmed as being of considerable potential interest. The list can be broken down to: 10 genes predicted to be up-regulated in *ad libitum* in BioLayout (Table 3.8), 9 genes in proximity to putative Follicle Number QTL also predicted to be up-regulated in *ad libitum* (Table 3.7.), and 10 of the 13 differentially expressed genes identified from the comparison with the AL vs. FR analysis (Table 3.2.). The remaining 6 (*) candidate genes were added to the list based on their BioLayout expression profile and functions derived from the available literature (also Table 3.8)

Table 3.8. Remaining candidate genes selected from the microarray for investigation. *P*-values are from the Kruskal-Wallis test performed in R for differential expression between developmental stages. The change in *ad libitum* was estimated from the original microarray analysis, and is predictive only

Symbol	Chromosome	BioLayout Profile	K-W *P*-value	Change in *ad lib*	Known Function
ABLIM3*	Gga 13	DOWN F5-7	<0.001	-	Embryonic Development
CAPRIN2*	Gga 1	UP F5-7	<0.001	-	Cell Growth and Differentiation
GRP	Gga Z	UP F5-7	<0.001	Down	Cell Proliferation
GULP1	Gga 7	UP F5-7	<0.001	Up	Phagocytosis
KRT75*	Gga 15	DOWN F5-7	<0.001	-	Possible Lipid transport
MAMDC2	Gga Z	UP F5-7	<0.001	Up	Signal Transduction Activity
MFHAS1*	Gga 4	DOWN F5-7	<0.001	-	Potentially Cell Cycle related
PDGFRL	Gga 4	DOWN F5-7	<0.001	Up	Implied Tumour Suppressor Activity
RIGG01740	Gga 1	UP F5-7	<0.001	Down	Unknown
RIGG03908	Gga 5	UP F5-7	<0.001	Up	Unknown
SPTY2D1	Gga 5	UP F5-7	<0.001	Down	Unknown
TBC1D13	Gga 17	DOWN F5-7	<0.001	Up	Cell Growth and Differentiation
TUBD1*	Gga 19	DOWN F5-7	<0.001	-	Cell Differentiation and
TXN2*	Gga 1	DOWN F5-7	<0.001	-	Anti-Apoptosis
YAP1	Gga 1	UP F5-7	<0.001	Up	Signal Transduction Activity
ZNF593	Gga 23	UP F5-7	<0.001	Up	Negative Transcriptional Regulation
MOSPD1	Gga 4	Expressed with FSHR	<0.001	-	Unknown

3.6. Discussion

In summary, a total of 36 prospective candidate genes were identified through the combination of the different analyses of the microarray data and the QTL study. These results have subsequently been published [16]. With the exception of 4 QTL-associated genes, all showed significant differential expression between at least two stages of follicle development ($P \leq 0.012$) from the R analysis and exhibited patterns of expression consistent with a role in follicle development (Figure 3.4), or were located in or around putative QTL for follicle number (Table 3.7). Though validation will be required, the expression of several candidate genes appears to be sensitive to changes in food intake.

Of course each method has its merits and drawbacks, which is why, in order to narrow down the list of prospective candidate genes, as well as to mitigate any inbuilt bias in the individual methods, several approaches were taken and the results combined. Examination of the *P*-value distributions from the complete and sub-divided dataset (Figure 3.1) highlights the importance of considering all the angles in approaching a problem. Comparing consecutive tissues in isolation indicated many more significant probes than analysis of the whole dataset. Analysis of the whole dataset also produced a large number of highly non-significant probes ($P > 0.9$) in addition to the general right-skewed distribution seen in all the comparisons. This would suggest a large number of false positives from the individual comparisons between consecutive stages are filtered out in the combined comparison of all stages.

The poor annotation of the microarray probes is a source of lost opportunities in large-scale analyses such as was carried out here: it is always easier to justify selecting one probe over another if one has supporting annotation and the other does not. While the annotation for this microarray was expanded considerably in preparation for the BioLayout phase of analysis, easily interpreted annotation was not available for the complete array. As a result, there may yet be useful information contained within the microarray that we have not identified. Improved annotation may be more accessible with the latest build of the chicken genome, which has just been released (WGS Project: AADN03).

BioLayout Express3D was used for the 2nd phase of microarray analysis because it utilised different principles for the analysis of the data [17]. The inbuilt clustering

algorithm and the data visualisation tools (Figures 3.3, 3.4 and 3.5) make it easier to identify patterns in the data and how they relate to each other than analysis in programs like R. BioLayout also permits fast and efficient QC on the data.

As discussed earlier, whereas many different expression profiles were observed in the data with BioLayout using the standard MCL clustering algorithm [18] (Figure 3.4), as opposed to clustering by annotation (Figure 3.5), only a subset of these profiles were used for candidate selection. However, there were other profiles within the data, such as probes only up-regulated in the F1 follicle. This means that there is scope for exploiting this data even further to answer other questions about follicle development.

The 4 expression profile types selected (Figure 3.4) represent either up- or down-regulation of probes in pre-hierarchical follicles, relative to the stroma and F1, or a progressive increase or decrease in expression across all stages of development. In this regard, it would have been advantageous to have had a greater representation of different stages of follicle development. However, the experiment was not originally designed with this in mind, and it would have added significantly to the overall cost of the microarray experiment.

There are several factors used in the various analyses that are predictive rather than quantitative and this needs to be acknowledged when drawing conclusions utilising these aspects. As was discussed above, reintroduction of the dietary effect proved problematic, and the successful solution, namely using the intensity values rather than ratios, is likely to have introduced greater variation than intended. However the results can still provide hypotheses to test in a dedicated experiment, where more of the variation can be accounted for. As such, the results of this endeavour were used to give weight to candidate genes already supported by other evidence, but no candidate was selected on this alone.

Likewise, the use of a candidate in proximity to a putative Follicle Number QTL was considered with care. This data was received from a parallel study that is, as yet, incomplete and so additional evidence was required for any candidate highlighted through this approach.

Though the list of candidate genes has been refined there are still a comparatively large number to investigate. As outlined in section *1.5.3.*, the next phase will focus

on characterisation of the gene candidate genes in different ovarian phenotypes. This characterisation will take the form of determining how they are expressed in the 'ideal' phenotype and validation of predicted effects of *ad libitum* feeding. This will facilitate further refinement of candidate selection, allowing deeper investigation of primary candidate genes, as well as providing data for improving current models of follicle development.

3.7. References

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4

Expression Profiling of Candidate Genes

This chapter focusses on qPCR expression profiling of selected gene candidates from the previous chapter in layer-type chickens in order to observe their behavior in the 'ideal' reproductive phenotype. Selected results are published in McDerment et. al. (2012).

4.1. Introduction

4.1.1. Overview

As discussed in the opening chapter, a marked difference is observed in the reproductive phenotypes of commercial unrestricted broiler breeders and commercial egg-laying birds [1-3]. With respect to the follicular hierarchy, egg-laying birds represent the 'ideal' phenotype. Laying birds do not need to be feed restricted, or otherwise controlled in order to develop a normal and healthy follicular hierarchy [2].

In the previous chapter, 4 different expression profile types were observed across different ovarian follicle classes from broiler breeders, and 36 potential candidate genes were selected for investigation. The next step was to examine these genes in the context of the ideal phenotype and determine whether their behaviour was consistent with a role in follicle development. Consequently, White Leghorn layers were used for this phase.

4.1.2. Aims & Objectives

The aims of this chapter were firstly to determine whether expression of the prospective candidate genes changed between different follicle classes in layer type birds, and by this, prioritise candidate genes to be taken forward for further investigation. It was important that a further experimental mandate be established rather than continue to depend on literature mining alone. The ultimate objective, however, was to develop quantitative assays for the selected candidate genes and comprehensively profile their expression across the full range of pre-hierarchical and hierarchical follicle classes.

4.2. Experimental Design

4.2.1. The White Leghorn Study

Given the large number of candidate genes under consideration, and to make it manageable, a two-tier process was adopted. Prospective candidate genes were first screened by qPCR in two pools of four samples of selected stages of follicle development, comparable with those of the microarray discussed in the previous chapter, as a crude method for determining which genes were changing most dramatically. Fully quantitative assays were then developed for those genes considered of greatest interest prior to expression profiling across all stages of follicle development. The White Leghorn layers used for this study were reared as described in section 2.1.2. Other than a lengthening of the day to 28 hours in order to synchronise ovulations [4], the birds were kept in standard, *ad libitum* conditions.

4.2.2. Data Collection

Seven of the final 8 birds were culled over a period of 2 days after 3 weeks in the climate chamber to ensure that their ovulatory cycles were as synchronised as possible at time of death. Birds were selected on the basis of their egg-laying record. Birds known to have laid on the morning of the cull, and with a good laying history, were selected for sampling. All follicle classes were taken, with prehierarchical classes divided into 1 mm increments. Birds 3, 5, 6 and 9 were culled on the 1st day and tissues collected 20 hours after dusk, as described in section 2.1.2. Only birds 7, 10 and 11 had laid on the following day and were culled 4 hours later than the previous day to account for the 28 hour photoperiodic cycle. A final bird was culled for sampling on the third day, along with the remainder of the population which had gone out of lay and provided training samples. Trait means for the White Leghorns used (as compared to broiler breeders in Table 3.1) are displayed in Table 4.1.

Table 4.1. Trait means for White Leghorn layers (n = 8) used for expression profiling of candidate genes.

Variable	Mean	SEM
Body Weight (Kg)	2.05	0.10
Number of Follicles >8 mm	6.00	0.38
Number of Follicles 5-8 mm	12.13	2.22
Ovarian Stroma Weight (g)	5.68	0.10

4.2.3. Introduction of Literature-sourced Marker Genes

Much work has been done to elucidate several signalling pathways known to be active during follicle development, as was discussed in the opening chapter. It was decided to include a number of genes from these pathways and assess them as potential positive controls for the novel candidate genes selected from the microarray. For example, it is known that FSH receptor is up-regulated in early pre-hierarchical development but is subsequently down-regulated as follicles mature. TGFβ signalling should also exhibit this general pattern of expression. A total of 17 genes were selected to represent HPGA, TGFβ superfamily and steroidogenic signalling pathways. Table 4.2 lists the literature-sourced genes that were assessed.

Table 4.2. Literature-sourced genes selected for investigation for use as possible positive controls.

Symbol	Chromosome	Associated Pathway	Literature
FSHR	Gga 3	HPGA Signalling	[5-13]
FST	Gga Z	HPGA Signalling	[5, 7]
INHA	Gga 7	HPGA Signalling	[5, 7, 11]
INHBA	Gga 2	HPGA Signalling	[5, 7, 11]
ACVR1	Gga 7	TGFB/Activin Signalling	[14]
ACVR1B	LGE22C19W28	TGFB/Activin Signalling	[14]
ACVR2A	Gga 7	TGFB/Activin Signalling	[14]
ACVR2B	Gga 2	TGFB/Activin Signalling	[14]
SMAD2	Gga Z	TGFB/Activin/BMP Signalling	[15]
SMAD3	Gga 10	TGFB/Activin/BMP Signalling	[12, 15]
TGFBR1	Gga 2	TGFB Signalling	[10, 11, 14-16]
ID1	Gga 20	TGFB Signalling	[10, 15]
ID2	Gga 3	TGFB Signalling	[10, 15]
ID4	Gga 2	TGFB Signalling	[10, 15]
BMPR1B	Gga 4	BMP Signalling	[14]
BMPR2	Gga 7	BMP Signalling	[14]
STaR	Gga 22	Steroidogenesis	[9, 15]

4.3. Results - The Initial Screen & its Implications

4.3.1. Literature-sourced Controls

Of those genes that changed at the crucial stage of transition from 5-6 mm to 6-7 mm in the qPCR screen, TGFBR1 and follistatin were shown to change most dramatically (Figure 4.1.). TGFBR1 mediates incoming signals to the follicle, whereas follistatin is primarily an outgoing signal that regulates gonadotropin release from the pituitary [17], though there is some evidence for paracrine activity [18], particularly in the regulation of FSH-induced activin A signalling [14]. Therefore, while still important, it is more likely to affect subordinate follicles than the source follicle. TGFBR1 also has a greater body of literature characterising its activity and therefore was deemed to be more useful as a positive control for verification. SMAD3, as a mediator of TGFB superfamily signalling, was also selected as recent literature had implicated it in several different pathways (Table 4.2) within ovarian follicles in the chicken [12, 15]. FSHR, with its clear role in follicle development was also selected.

4.3.2. Candidate Genes

QPCR was carried out along with the literature-sourced controls on the pooled material. Fold change was calculated using the ΔCt method [19], using PAK3 (P21 protein (cdc42/rac)-activated kinase 3), a gene associated with cytoskeleton assembly, as a normalising control as this gene showed minimal variation across samples.

The results, as can be seen in figure 4.2., allowed visual grouping of 31 of the candidate genes into 3 basic groups; RIGG03908 through to PDGFRL, which showed increases in expression of >30-fold, SPTY2D1 through to GULP1, which showed increases in expression between 5- and 10-fold, SH3BGRL3through to MFHAS1, showing increases of <5-fold, and GDF9, FOXI1 and PPARGC1B, which showed a fold change of <1. Four potential candidate genes were set aside due to difficulties encountered during primer design. It was decided, given the number of candidate genes under consideration, to revisit these at a later date, should the opportunity arise.

The third group was removed from consideration as the estimated changes in expression were comparatively small relative to the other groups that showed increases in expression. A subset of candidate genes from the remaining groups was then selected based on the gene's ranking within its group and a more stringent assessment of supporting literature. Table 4.3 lists the candidate genes selected for full expression profiling in White Leghorn layers.

Table 4.3. Summary of supporting evidence for candidate genes taken forward for QPCR profiling in White Leghorn Layer ovarian tissues

Gene	BioLayout Profile	QTL	AL vs. FR (Predicted)	Literature
FSHR	-	-	-	Key mediator of reproductive signalling
TGFBR1	-	-	-	Key mediator of cell growth + survival
SMAD3	-	-	-	Promotes Cell Survival
SLIT3	-	Gga13	Up	Promotes Apoptosis
PDGFRL	Down 5-8 mm	-	Up	Homologs involved in steroidogenesis
VDAC1	Up in 5-8 mm	Gga13	Up	Central to pro-apoptotic signalling
YAP1	Up in 5-8 mm	-	Up	Possible pro-cell survival signalling
MOSPD1	Clustered with FSHR	-	-	Up-regulated in ovarian cancer
KRT75	Down 5-8 mm	-	-	Possible Lipid Transport
SPTY2D1	Up in 5-8 mm	-	Down	Unknown
RIGG1740	Up in 5-8 mm	-	Down	Unknown
GULP1	Up in 5-8 mm	-	Up	Phagocytosis of apoptotic cells

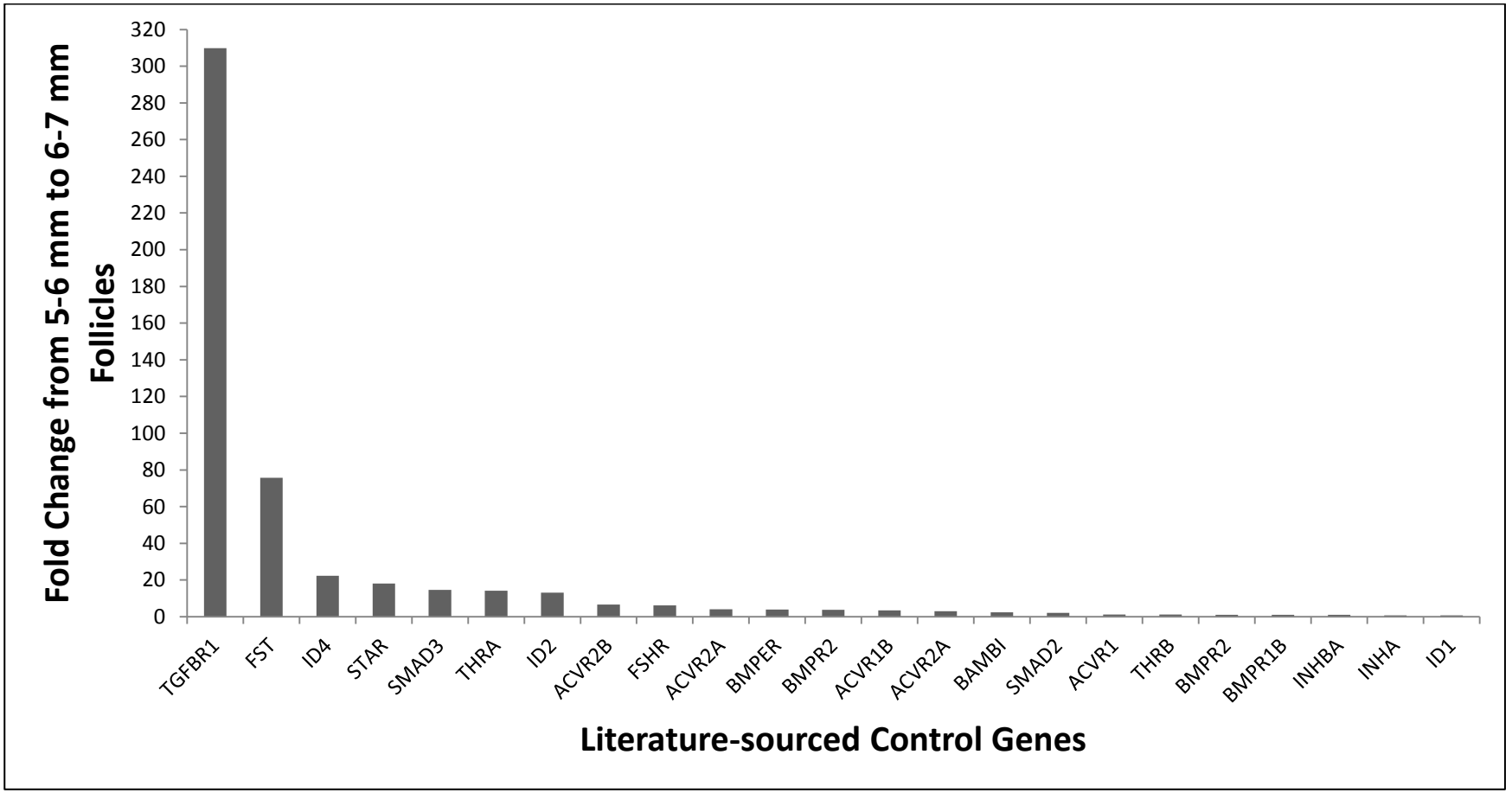

Figure 4.1. Graph showing the fold change of selected literature-sourced genes over the 5-6 mm to 6-7 mm transition in 2 pools of follicles from *ad libitum* fed mature White Leghorns (2x n=4) examined in the qPCR screen (Section 2.3.3). PAK3 was used for normalisation.

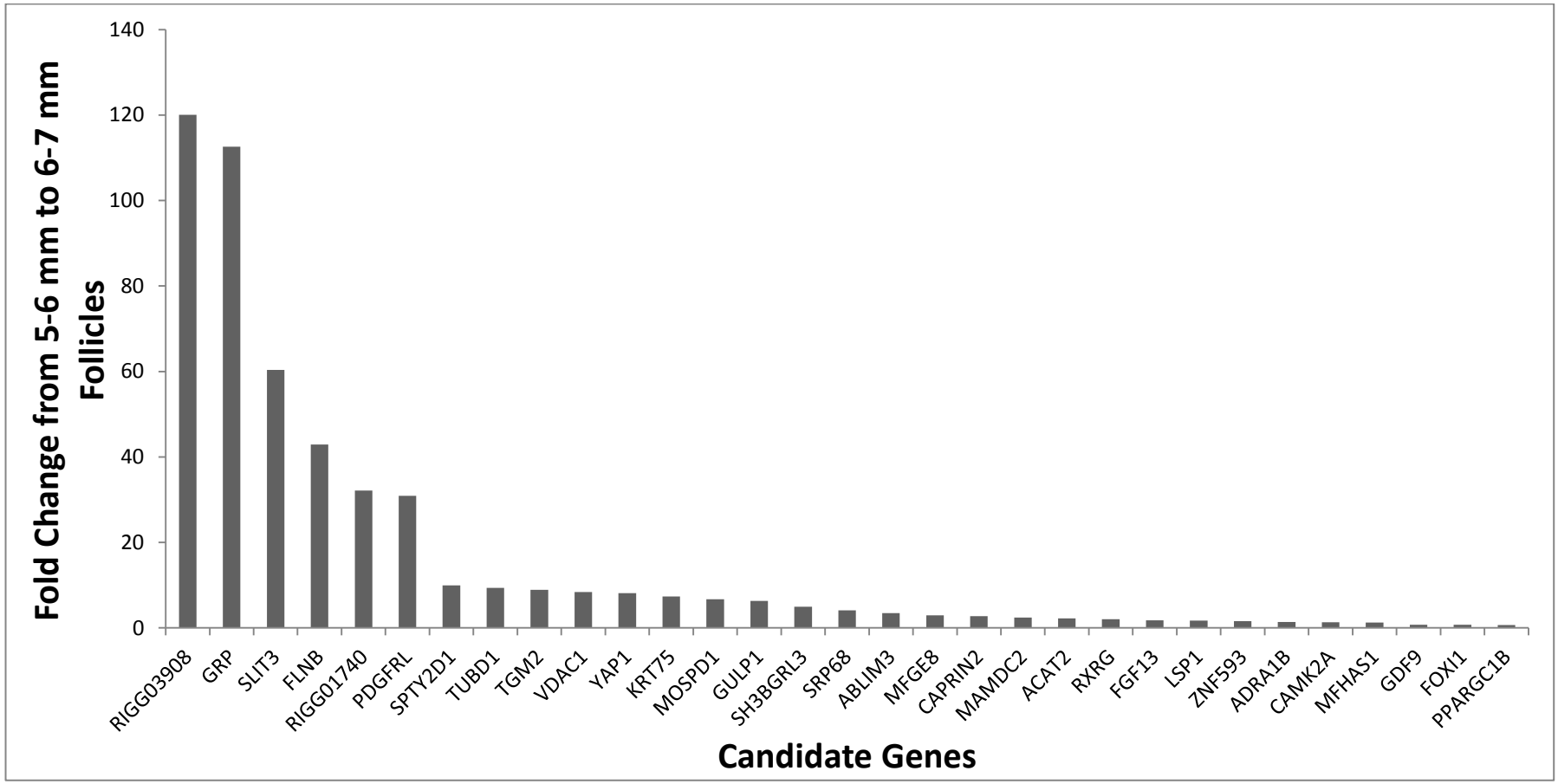

Figure 4.2. Graph showing the fold change of selected candidate genes during the 5-6 mm to 6-7 mm transition in 2 pools of follicles from *ad libitum* fed mature White Leghorns (2x n=4) examined in the qPCR screen (Section 2.3.3). PAK3 was used for normalisation.

4.4. Results - Full Profiling of Top Candidate Genes

Quantitative assays for all selected candidate genes were developed with minimal requirement for optimisation. Standard Curves (section 2.3.2) were used in place of the ΔCt method [19] for quantification of all qPCR assays described in this and all subsequent sections. Examples of plots for Standard and Dissociation curves for these assays can be seen in Figures 4.6 and 4.8. Laminβ Receptor (LBR) was used as a control for normalisation of data. The plots for this assay can be seen in Figure 4.3.

Having established these assays, full expression profiling was carried out using all follicle classes available. It was decided to pool follicles between 1 and 4 mm in diameter partly as early follicular development has been well studied [20-25].

Figures 4.4 and 4.6 show the expression profiles for the prospective candidate genes, while Figures 4.5 and 4.7 show the plots for the Standard and Dissociation curves for each assay. All primer pairs produced single products, as observed from gel electrophoresis. For all assays except SPTY2D1 the dissociation curves confirmed this. SPTY2D1 produced 2 peaks in the dissociation curve. As this was not confirmed by gel electrophoresis, the smaller peak is likely primer-dimer. All standard curves had an R^2 value >0.97, indicating a good correlation between concentration and Ct and, therefore, accurate quantification.

FSHR (P = 0.001) and TGFBR1 (P <0.001) showed bell-shaped curves of expression during early stages of development, each peaking at the 4-5 mm stage. They also exhibited peaks at the F6 stage immediately post-recruitment. SMAD3 (P <0.001) mimics this pattern to a certain extent, though the later peak occurs at the F1 rather than the F6. PDGFRL (P <0.001) has a far less distinct pattern of expression. However the observed expression levels are considerably higher than any of the other candidate genes. There are also 2 distinct peaks, at the 5-6 mm and F2 stages, the stages preceding recruitment and ovulation respectively.

SLIT3 (P <0.001) showed an oscillating pattern of expression, which can be divided into a series of ramped increases in expression followed by a prominent peak in the F1. VDAC1 (P <0.001), on the other hand, exhibited a pattern of expression that might be compared to an exponential curve leading up to early pre-hierarchical development. YAP1 and MOSPD1 show similar oscillating patterns to SLIT3.

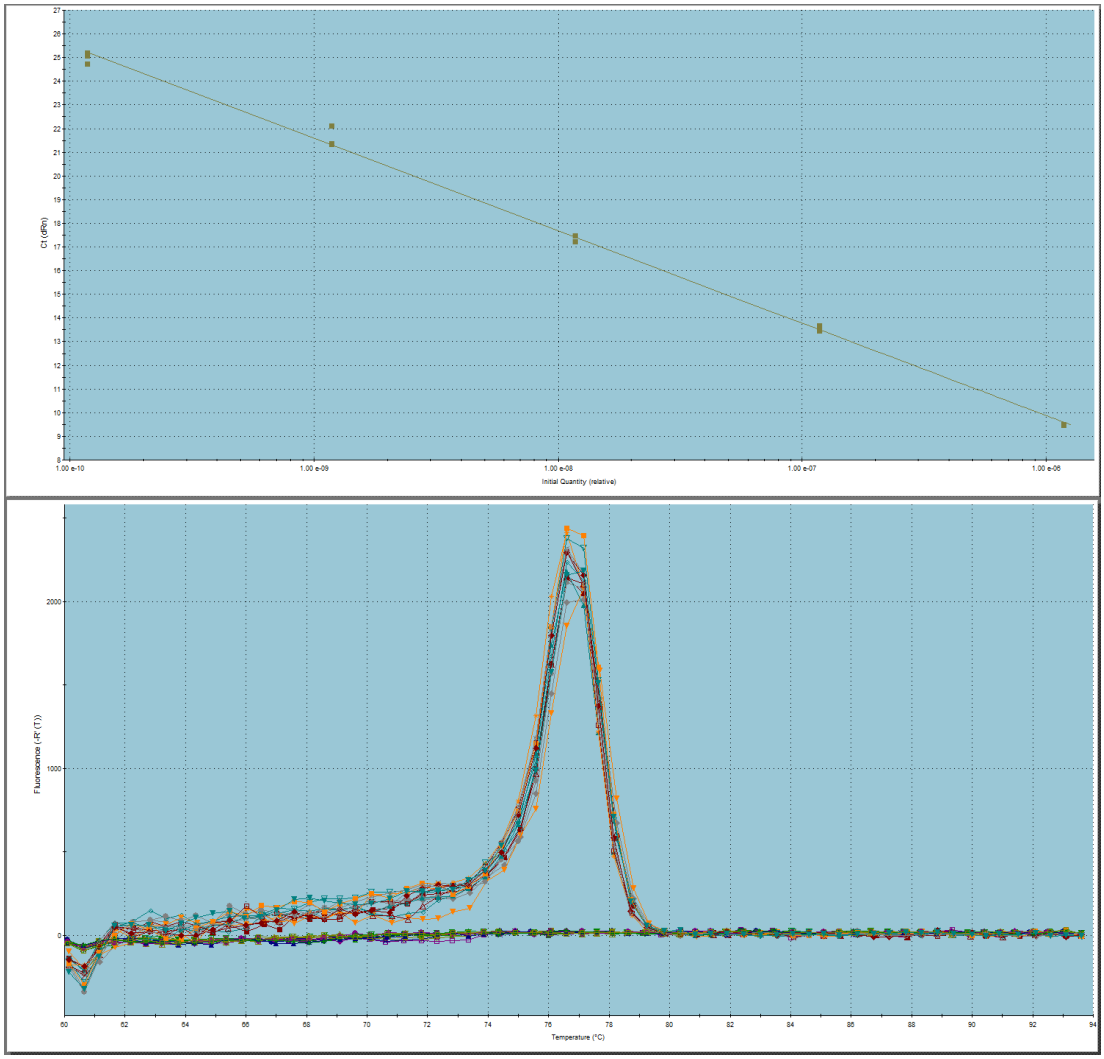

Figure 4.3. Standard Curve (Top), showing Ct plotted against sample concentration (ng/µl), and Dissociation Curve (Bottom), showing fluorescence against temperature (ºC) for the Laminβ receptor (LBR) qPCR assay developed from the basal hypothalamus of White Leghorns, R^2=0.998.

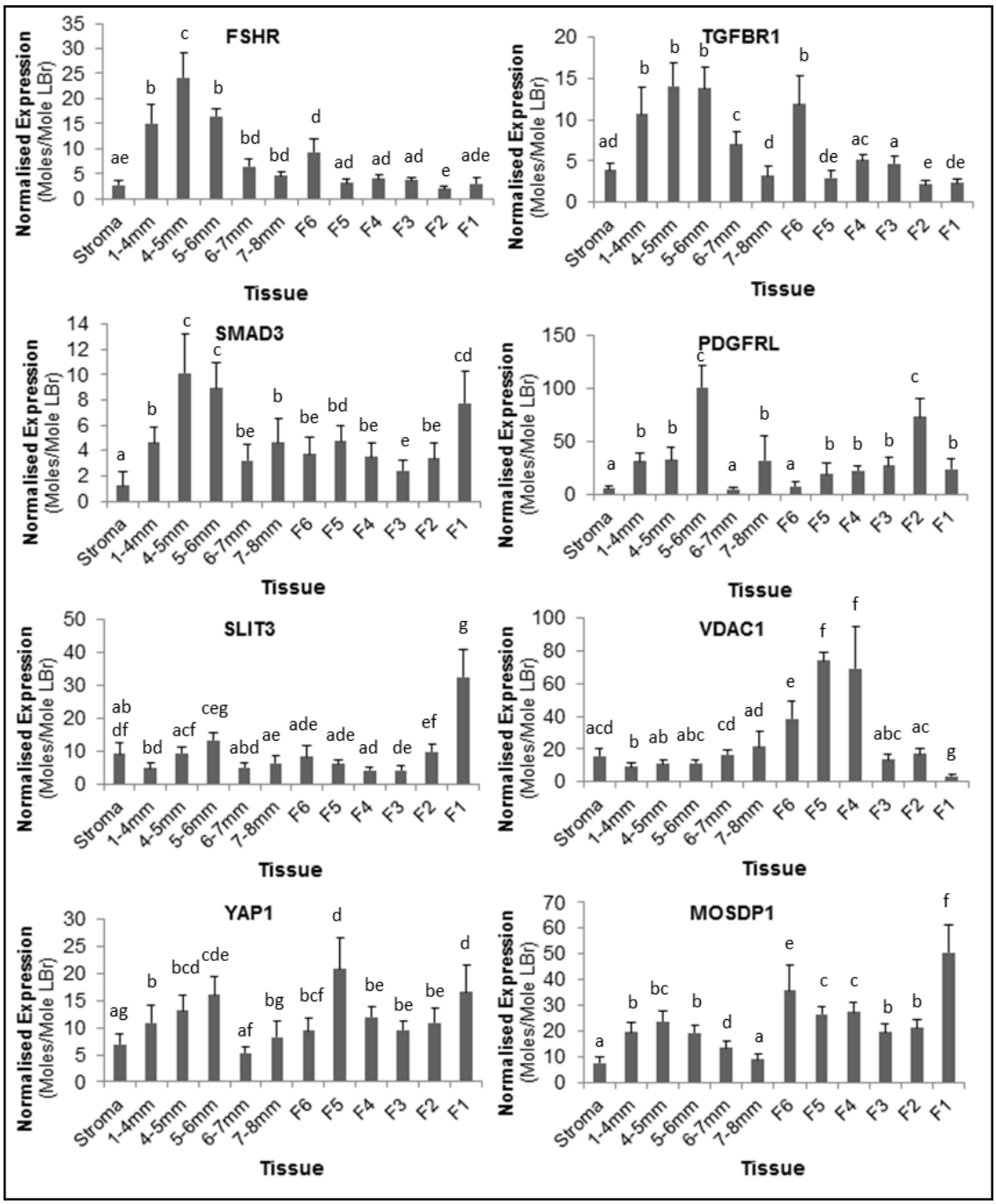

Figure 4.4. QPCR-derived expression profiles (section 2.3.3) for top candidate genes across all stages of follicle development in White Leghorn layers (n=8) (Section 2.1.2). Different letters above data points indicate significant differences between tissues within each gene as determined by ANOVA (Section 2.4.3) of log-transformed data (LOG_e) Calculation tables for these assays can be found in Appendix 2. Least Significant Difference (LSD) was used for comparison of means. LBR was used for normalisation.

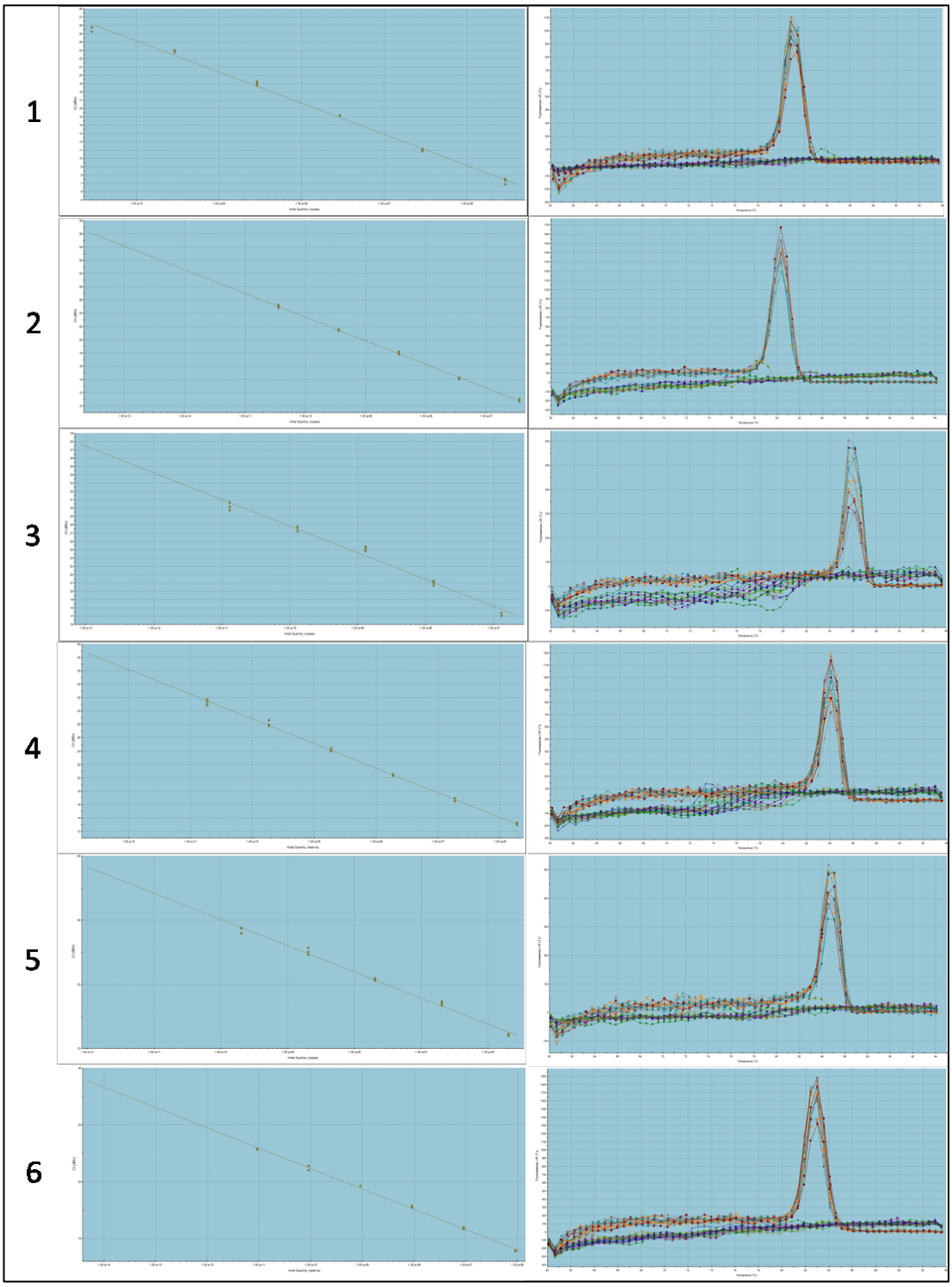

Figure 4.5. Standard Curves (Left), showing Ct plotted against sample concentration (ng/µl), and Dissociation Curves (Right), showing fluorescence against temperature (°C) for the top candidate gene assays developed from White Leghorn Ovarian Stroma: FSHR (1), R^2=0.996; TGFBR1 (2), R^2=0.999; SMAD3 (3), R^2=0.982; PDGFRL (4), R^2=0.998; SLIT3 (5), R^2=0.994; VDAC1 (6), R^2=0.997.

The genes whose profiles can be seen in Figure 4.6, namely GULP1 (P <0.001), RIGG1740 (P 0.017), SPTY2D1 (P <0.001) and KRT75 (P <0.001), are candidate genes of secondary interest. Expression of GULP1 is relatively low and unchanging over the period of follicle recruitment, while RIGG1740 and SPTY2D1 have somewhat indistinct profiles. RIGG1740 does peak in early prehierarchical development but this peak is not large. While SPTY2D1 expression is generally lower in hierarchical follicles, beyond this there is no distinct trend. KRT75 expression does oscillate, however expression levels are relatively low in comparison with many of the other candidate genes.

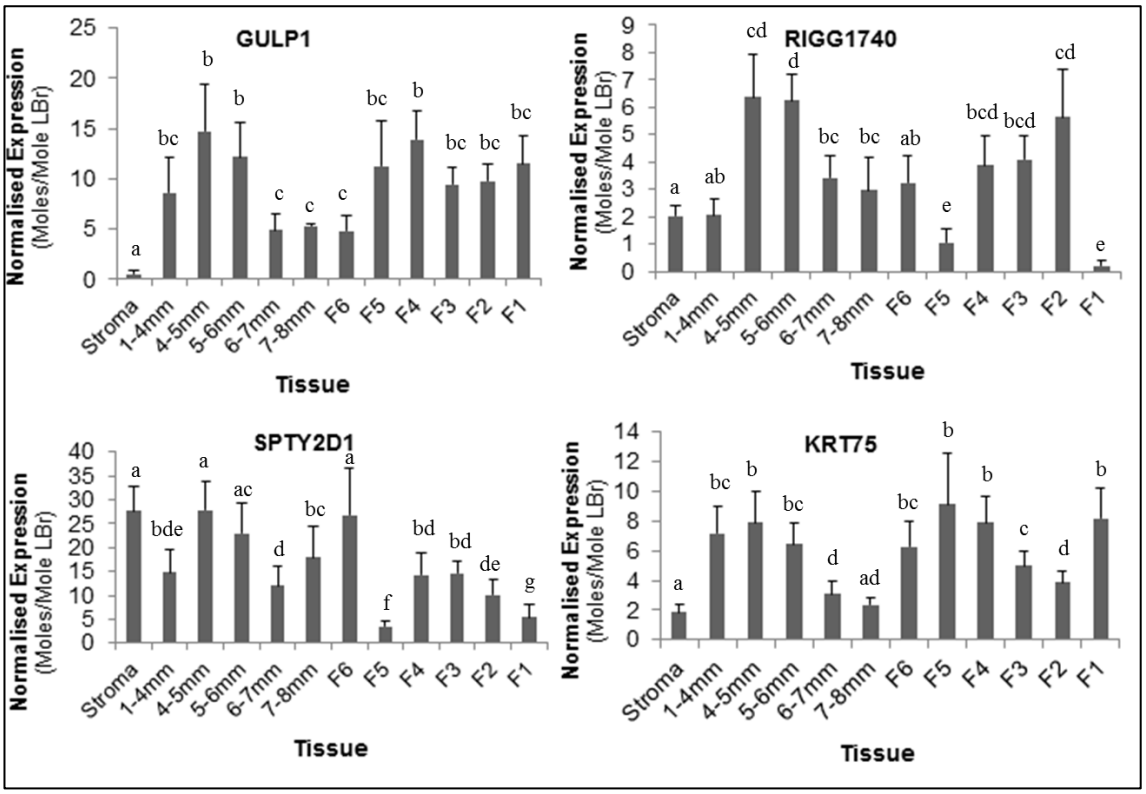

Figure 4.6. QPCR-derived expression profiles (section 2.3.3) for remaining candidate genes across all stages of follicle development in White Leghorn layers (n=8) (Section 2.1.2). Different letters above data points indicate significant differences between tissues within each gene as determined by ANOVA (Section 2.4.3) of log-transformed data (LOG_e) Calculation tables for these assays can be found in Appendix 2. Least Significant Difference (LSD) was used for comparison of means. LBR was used for normalisation.

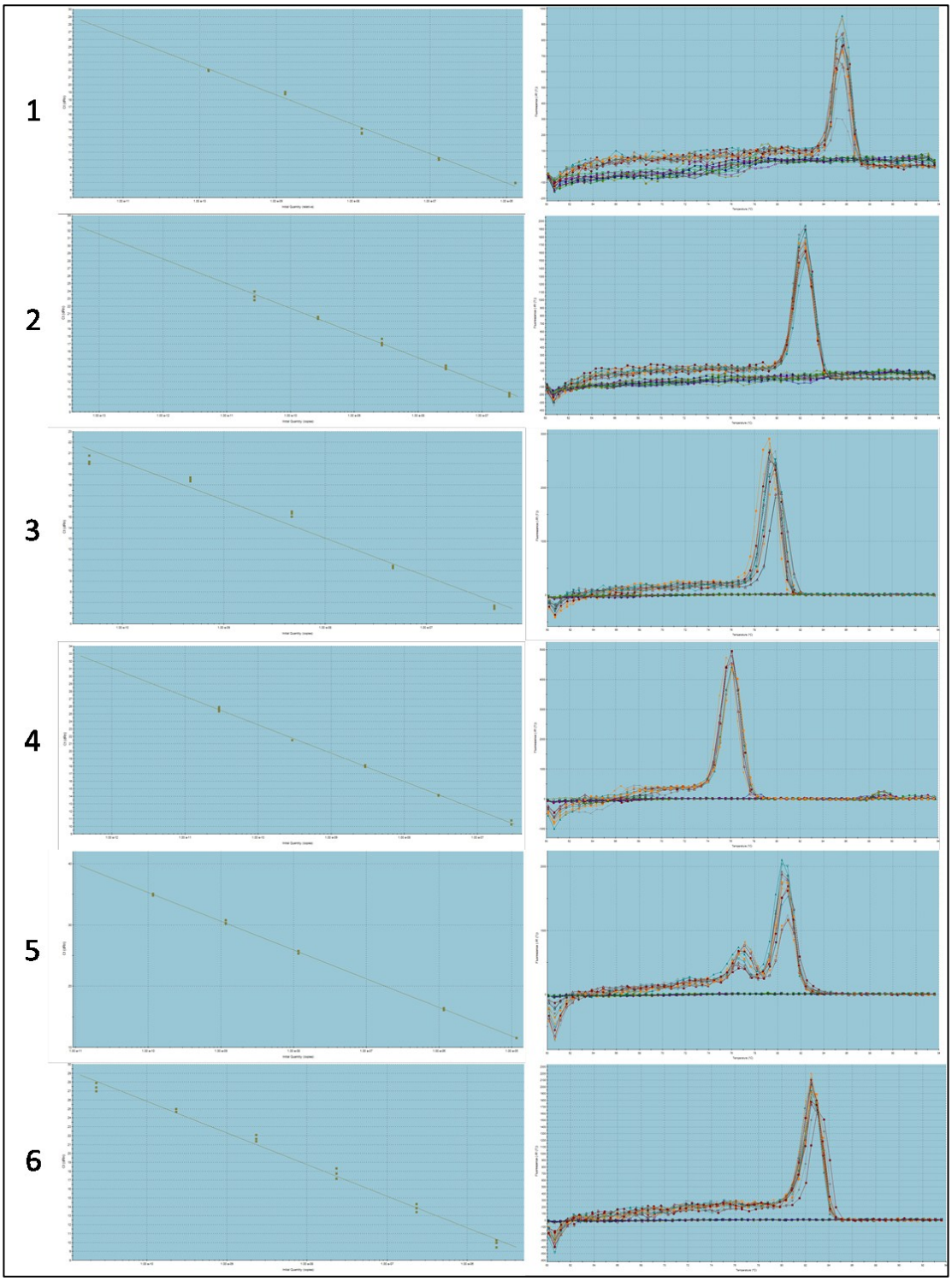

Figure 4.7. Standard Curves (Left), showing Ct plotted against sample concentration (ng/µl), and Dissociation Curves (Right), showing fluorescence against temperature (ºC) for the top candidate gene assays developed from White Leghorn Ovarian Stroma: YAP1 (1), R^2=0.992; MOSPD1 (2), R^2=0.994; GULP1 (3), R^2=0.974; RIGG1740 (4), R^2=0.999; SPTY2D1 (5), R^2=1.000; KRT75 (6), R^2=0.991.

4.5. Discussion

While it would have been quite easy to round up the usual suspects in terms of genes involved in follicle development, the aim of this project was to identify novel candidate genes as the genes already known to be involved have already been intensively studied.

From the 36 prioritised candidate genes considered for multi-level QPCR profiling, 10 candidate genes, with the addition of FSHR and TGFBR1 for validation of the approach, had sufficient experimental and/or literature-sourced evidence for basic hypothesis generation as to their role in follicle selection and recruitment. All of these candidate genes are associated with regulation of apoptosis [26-31], cell growth [8, 32, 33] and survival [34] or steroidogenesis [35]. While these processes are all prerequisite to follicle survival, there is insufficient information at present to create a single model system incorporating all of our candidate genes, although FSHR, TGFBR1 and SMAD3, with the added possibility of MOSPD1, are known to interact with common signalling pathways [8, 32, 33].

The QPCR results from the layer experiment show that FSHR and TGFBR1, genes known to be involved in follicle growth, peak in their expression during early prehierarchical development. This agrees with previous results [8, 32]. Interestingly, both FSHR and TGFBR1 show a prominent peak of expression in the F6 follicle, indicating that follicles immediately post-recruitment may have a heightened sensitivity to the ligands of these receptors. As far as FSHR is concerned, this result agrees with previous studies [8]. As both receptors activate pathways leading to cell growth, proliferation and differentiation, higher expression at those stages would be consistent with patterns of follicular growth. SMAD3, a known downstream signal mediator of TGFB family receptors demonstrates a very similar pattern of expression to TGFBR1. MOSPD1 has been implicated in mesenchymal cell differentiation [33] and is up-regulated in ovarian cancer [36]. BioLayout Express analysis showed it clustered with FSHR in broiler breeders and QPCR profiling in layers corroborated this. MOSPD1 is a membrane-associated protein [37] and may be involved in supporting or mediating signal transduction from the FSH receptor but further work will be required to determine this.

PDGFRL produces a product which is homologous with the functional domains of Platelet-derived Growth Factor Receptors that are involved in intrafollicular cell

signalling associated with steroidogenesis in mice [35]. QPCR profiling in layers clearly shows significant (P <0.001) and substantial peaks in expression at the 5-6 mm and F2 stages, i.e. immediately prior to selection and ovulation respectively. This evidence would support a function in regulatory feedback mechanisms within the HPGA. The BioLayout Express profile from the broiler breeder microarray data suggests that the gene is down-regulated in 5-7 mm follicles relative to the stroma and F1. The observed down-regulation in 5-7 mm follicles from broiler breeders is in marked contrast to the layers, which would support the hypothesis of potential dysregulation of part of the steroid-based feedback mechanism, given what is already known of the PDGFR family in other species.

SLIT3 and VDAC1 have both been shown to be involved in pro-apoptotic signalling [26-29] and are located in the putative QTL for follicle number on chromosome 13. SLIT3 is also involved in ovary and follicle development in sheep [24] and its expression profile in layers is consistent with phases of increased apoptosis. Expression profiling of SLIT3 and VDAC1 in BioLayout Express for broiler breeders is consistent with the layer QPCR profiling. The major peak in SLIT3, however, is in the F1 follicle. As well as its role in pro-apoptotic signalling, SLIT3 is known to act as a chemorepellent in axonal growth [38, 39], but also exhibit chemoatractant capabilities in other systems [40]. It has been observed that the infundibulum, the mouth of the oviduct moves towards and engulfs the F1 follicle [41]. It would be interesting to verify this observation, and determine whether SLIT3 plays a part in this process.

YAP1 is believed to be involved in cell survival signalling through regulation of the p53 signalling pathway [34]. The BioLayout Express profile of the broiler breeders suggests that it is up-regulated in late prehierarchical follicles. However, profiling in layers shows higher expression in early prehierarchical follicles and the F5 follicle, where pro-survival signals would be expected to occur more prominently.

GULP1 is expressed in macrophages and is involved in engulfment of apoptotic cells [30, 31]. Profiling in layers is consistent with this activity. While this is not likely to be a candidate for follicle selection, it does highlight the transitional stages of the follicle as it progresses through development, and therefore may be useful as a temporal marker of selection, though further work would be required to verify this.

Very little is known about the remaining genes. RIGG1740 is the identifier given to the oligo from the microarray. Whereas the sequence is similar to that of a human ATPase, ATAD2B, this gene is yet to be formally annotated in the chicken. KRT75 is a member of the keratin family of structural proteins and is localised to epithelial keratinocytes. In the chicken these cells are involved in lipid synthesis [42]. However, it is a structural protein and is most likely produced as a response during phases of rapid growth rather than acting as a regulator thereof. The QPCR profiling does not preclude either option; however logic would indicate a supporting rather than a regulatory role for this gene. Very little is also known about SPTY2D1. From the QPCR profile, it is clearly more highly active in prehierarchical follicles but there is no clear pattern of expression that would indicate a regulatory role in layers. In the BioLayout analysis of broiler breeders it was shown to be up-regulated in 5-7 mm follicles and predicted to be down-regulated in response to *ad libitum* feeding. Of course, the production of what is believed to be primer-dimer in the qPCR assay for SPTY2D1 reduces confidence in the results of the assay.

When examining the data, one must take into account a number of factors. A certain part of the variation in the stages of follicle development prior to recruitment can be explained by follicles in the pool that were in the initial stages of atresia but showed no outward sign at the time of sampling, despite every effort being made to identify and remove atretic follicles. In addition, follicular growth is not a series of discrete stages but a continuous process. Thus, we cannot expect every follicle to adhere strictly to the artificial delimiters imposed by sampling techniques. Nor can we eliminate variation between birds entirely, but only minimise it through tight regulation of the environment.

In conclusion, the aims of this chapter were to develop quantitative PCR assays for candidate genes to be investigated, profile the expression of candidate genes, and by this prioritise candidate genes for further study. Quantitative assays were unsuccessfully developed for all but 1 candidate selected from the original screen, namely SPTY2D1. All the candidate genes were profiled in White Leghorns and prioritised as discussed above.

In the previous chapter, BioLayout Express3D predicted that expression of some of these candidate genes would be affected by changes in feed intake. The next phase of investigation, therefore, is to verify these predictions by returning to the broiler breeder

model and examining expression of the genes selected here. Following validation of dietary effect, potential sources of variation will need to be investigated, particularly at the sequence level.

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5

Validation of Dietary Effect in Broiler Breeders

This chapter examines the predictions from chapter 3 that some candidate genes' expression in broiler breeders is altered by different dietary regimes. Selected results are published in McDerment et. al. (2012).

5.1. Introduction

5.1.1. Overview

In the opening chapter, we discussed commercial broiler production and the need for broiler breeders to be kept on a restricted diet to maintain proper ovarian function. In chapter 3, analysis in BioLayout Express3D of the broiler breeder microarray data predicted that the expression of several genes would be up-regulated in the ovary in response to *ad libitum* feeding. Several of these genes were selected as primary candidate genes from the expression profiling in White Leghorns in the previous chapter. In this chapter the genes identified in this process will be examined in mature broiler breeders that have been kept on restricted or *ad libitum* diets to determine whether expression is altered as a result of the change in diet.

5.1.2. Aims & Objectives

The aims of this experiment were firstly to verify predicted effects of changes in dietary regime on expression of candidate genes, and secondly, to determine the presence or absence of any dietary effects that were not anticipated.

5.2. Experimental Design

5.2.1. The Second Broiler Breeder Study

Broiler breeders (n=23, FR=11, AL=12) were reared as described in section 2.1.1. The population was originally reared to provide samples for a repeat of the microarray experiment described in chapter 3. Tissues were available for the stroma, 5-6 and 6-8 mm follicles, the smallest hierarchical follicle, and the F1 pre-ovulatory follicle that represent the key stages of follicle development. Table 5.1 summarises the genes taken forward from the previous chapter with the conclusions and predictions, where available, from chapter 3. On further review of the literature [1, 2], it was decided to reconsider GDF9 as a candidate, despite the results in the previous chapter, in addition to those genes listed below.

Table 5.1a. Selected candidate genes with responses to changes in feed strategy predicted by BioLayout Express3D.

Gene	BioLayout Profile	QTL Chromosome	AL vs. FR (Predicted)	Literature
PDGFRL	Down 5-8 mm	-	Up	Steroidogenesis [3]
YAP1	Up in 5-8 mm	-	Up	Possible pro-cell survival signalling [4]
SLIT3	-	Gga13	Up	Promotes Apoptosis [5-7]
VDAC1	Up in 5-8 mm	Gga13	Up	Central to pro-apoptotic signalling [8, 9]

Table 5.1b. Remaining candidate genes where BioLayout Express3D did not predict responses to changes in feed strategy

Gene	BioLayout Profile	Literature
FSHR	-	Key mediator of reproductive signalling [10-18]
TGFBR1	-	Key mediator of cell growth + survival [15, 16, 19-21]
SMAD3	-	Promotes Cell Survival [17, 20]
MOSPD1	Clustered with FSHR	Up-regulated in ovarian cancer [22, 23]

5.3. Results

5.3.1. Effects of Ad Libitum vs. Restricted Feeding on Gene Expression

Of the genes that were predicted to alter their expression in response to a change in feed strategy, only PDGFRL demonstrated such an effect (Figure 5.1). It proved to be up-regulated in response to *ad libitum* feeding, relative to restriction, in 6-8 mm follicles. Though not predicted to do so, FSHR and GDF9 were also affected by *ad libitum* feeding (Figure 5.1). FSHR was down-regulated in the F1 follicle while GDF9 was down-regulated in the stroma and in 6-8 mm follicles. All the significant effects are outlined in Table 5.2. (It should be noted that statistical analysis was carried out on log-transformed (LOGe) data, while the graphs present the untransformed data.)

5.3.2. Changes in Gene Expression Throughout Development

In terms of changes in expression between developmental stages, 7 of the 9 genes examined showed significant differential expression (figures 5.2 & 5.3). In contrast to the results of the previous chapter, VDAC1 and MOSPD1 showed no significant difference in expression between tissues.

Table 5.2. Summary of effects observed in broiler breeders (n=23) following qPCR investigation of dietary effect on gene expression

Gene	Candidate Source	Tissue *P* value	Treatment *P* value	Interaction *P* value	Interaction Effect	Location of Interaction Effect
GDF9	Literature/QTL	<0.001	NS	0.005	Down in *ad lib*	6-8 mm
FSHR	Literature	<0.001	NS	0.018	Down in *ad lib*	F1
PDGFRL	Microarray	<0.001	NS	0.016	Up in *ad lib*	6-8 mm
					Down in *ad lib*	F1
SLIT3	QTL/Microarray	<0.001	NS	NS	-	-
SMAD3	Literature	<0.001	NS	NS	-	-
TGFBR1	Literature	0.005	NS	NS	-	-
YAP1	Microarray	0.027	NS	NS	-	-
VDAC1	QTL/Microarray	NS	NS	NS	-	-
MOSPD1	Microarray	NS	NS	NS	-	-

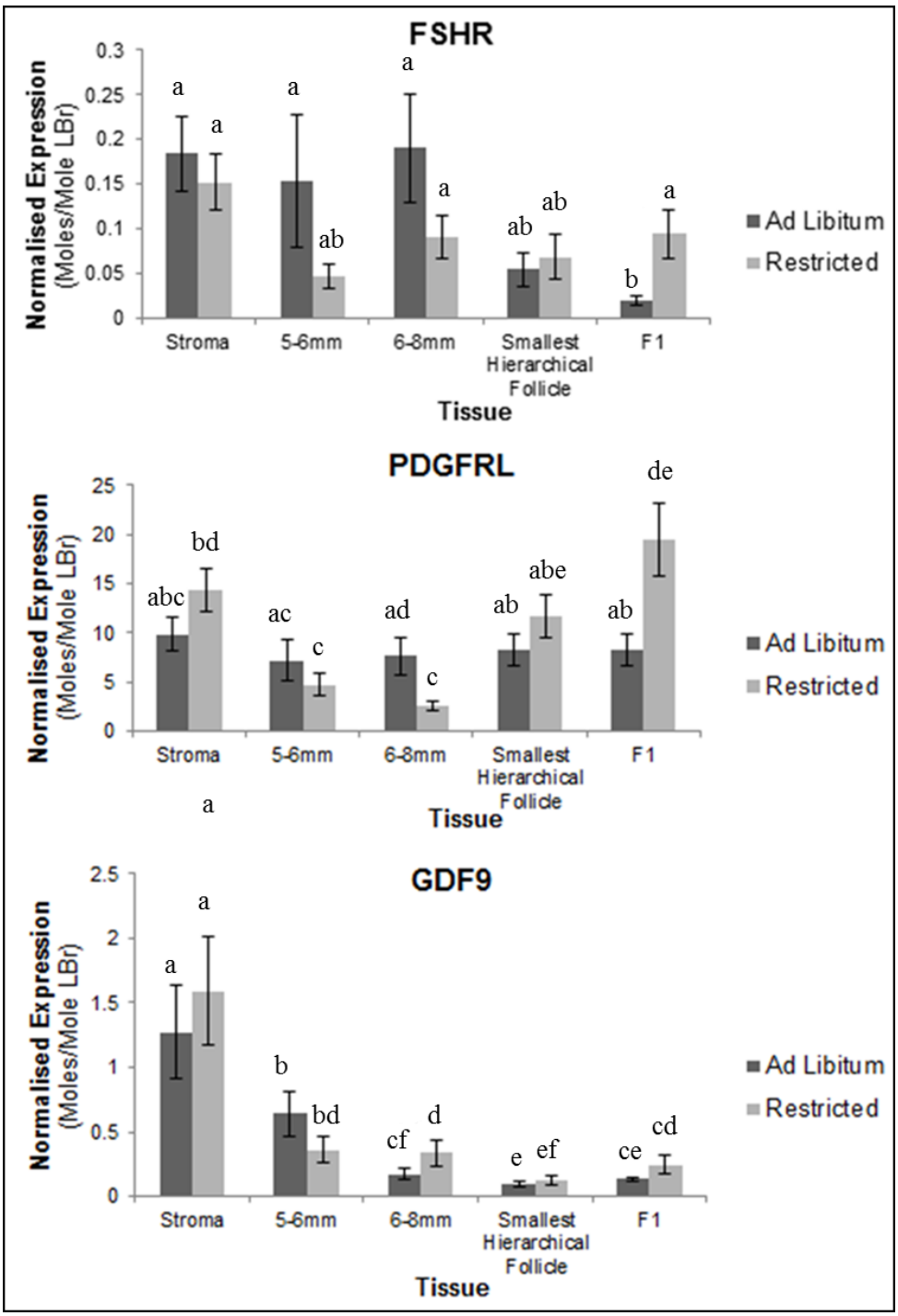

Figure 5.1. QPCR expression profiles for genes shown to be significantly altered in their expression in response to *ad libitum* feeding (section 2.3.4) in broiler breeders (n=23) (Section 2.1.1). Note different axes. Graphs represent untransformed tissue means for each feeding strategy. Statistical analysis was carried out on Log-transformed data (LOG_e). Error bars indicate standard error. Different letters above data points indicate significant differences between samples within each gene (tissue/treatment interaction) as determined by GLM. Calculation tables for these assays can be found in Appendix 2. Least Significant Difference (LSD) was used for comparison of means. LBR was used for normalisation.

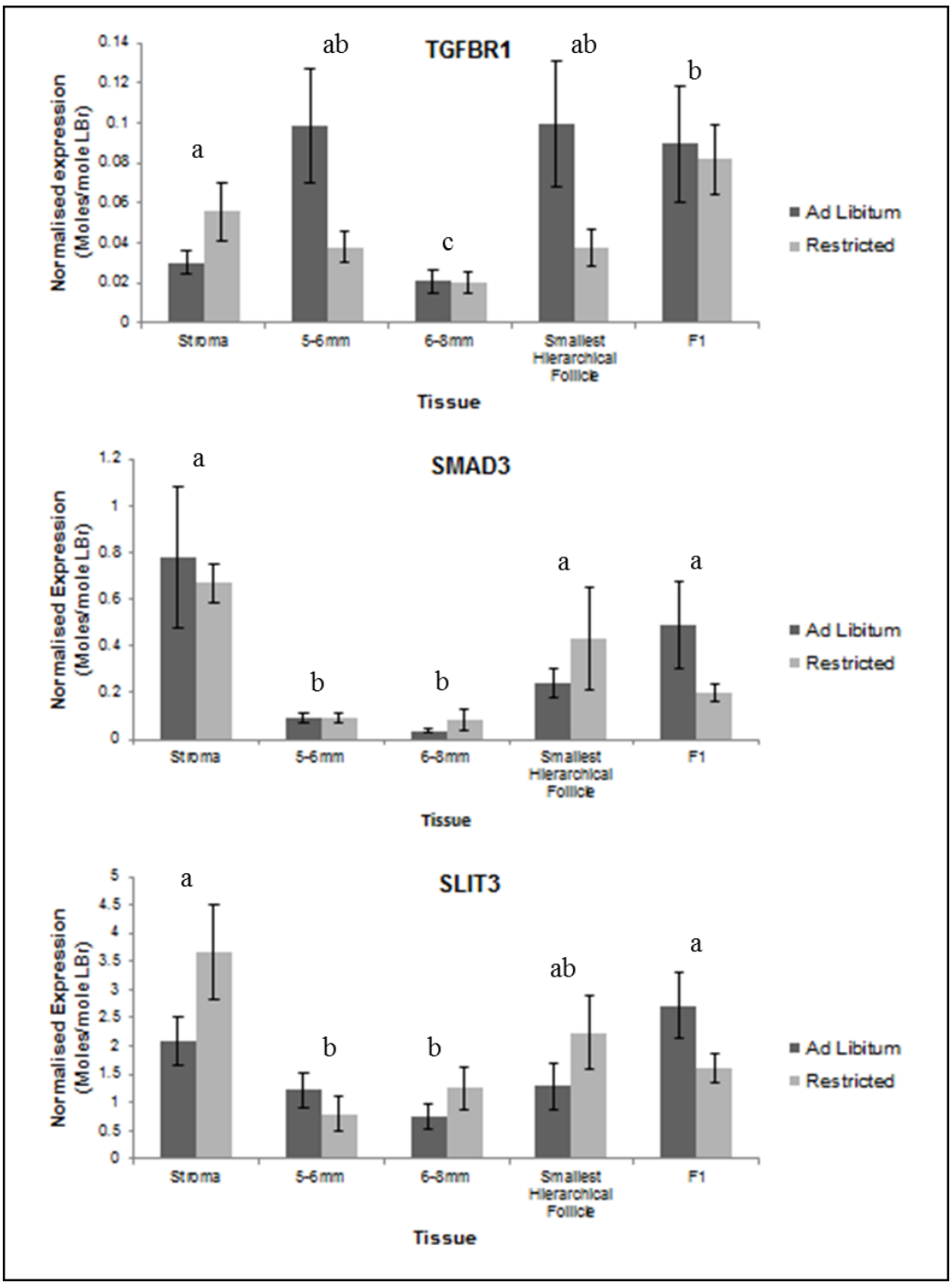

Figure 5.2. QPCR expression profiles for genes that showed altered expression across developmental stages (section 2.3.4) in broiler breeders (n=23) (Section 2.1.1). Note different axes. Graphs represent untransformed tissue means for each feeding strategy. Statistical analysis was carried out on Log-transformed data (LOG_e). Error bars indicate standard error. Different letters above data points indicate significant differences between tissue means within each gene as there was no effect or interaction with treatment for these genes determined by GLM. Calculation tables for these assays can be found in Appendix 2. Least Significant Difference (LSD) was used for comparison of means. LBR was used for normalisation.

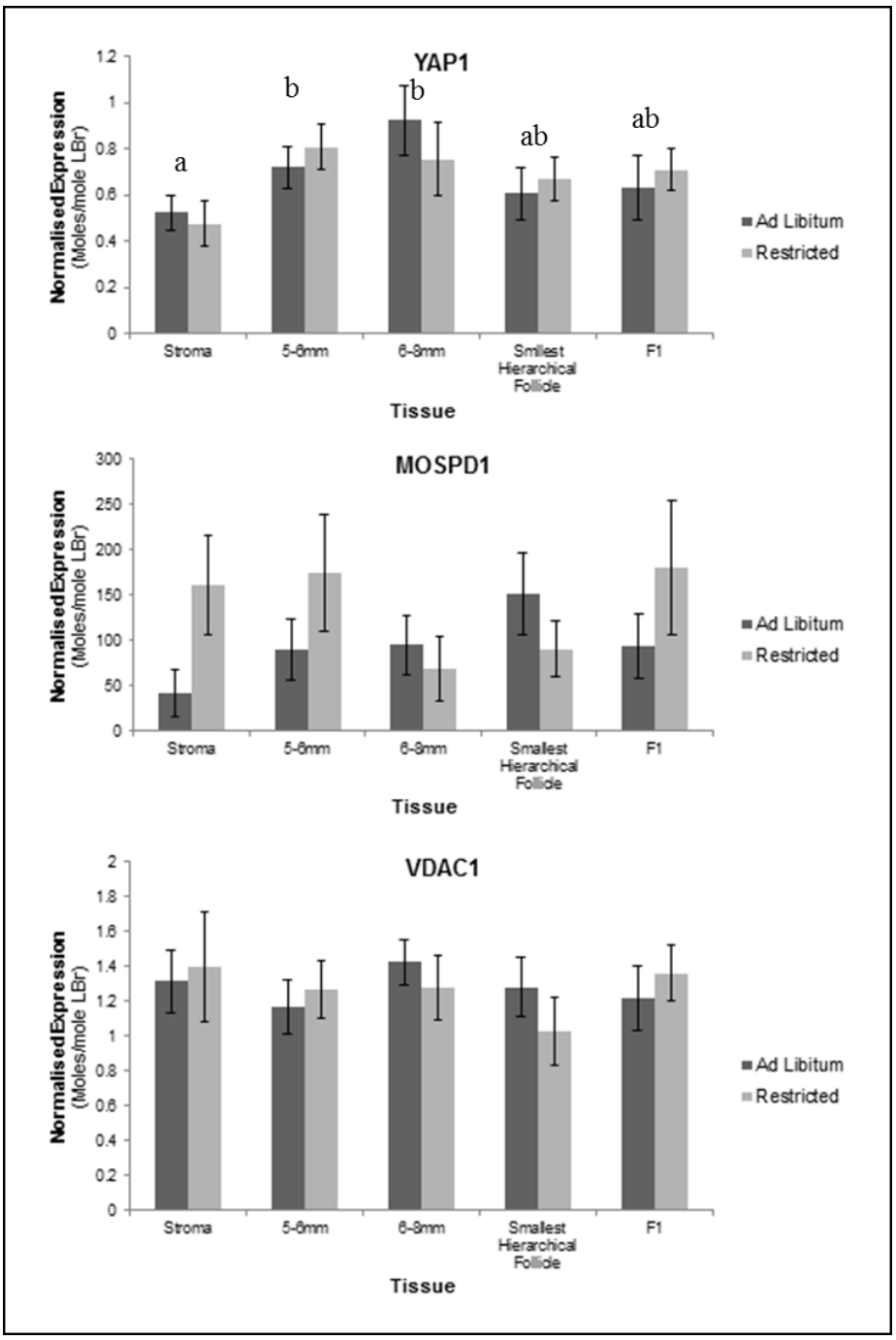

Figure 5.3. QPCR expression profiles for remaining genes (section 2.3.4) in broiler breeders (n=23) (Section 2.1.1). Note different axes. Graphs represent untransformed tissue means for each feeding strategy. Statistical analysis was carried out on Log-transformed data (LOG_e). Error bars indicate standard error. Different letters above data points for YAP1 indicate significant differences between tissue means. MOSPD1 and VDAC1 have not been annotated as there was no significant tissue or treatment effect for MOSPD1 or VDAC1 as determined by GLM. The calculation table for YAP1 can be found in Appendix 2. Least Significant DIFFERENCE (LSD) was used for comparison of means. LBR was used for normalisation.

5.4. Discussion

Of the literature-sourced genes, TGFBR1 and SMAD3 did not show a significant effect in response to *ad libitum* feeding, whereas FSHR was significantly down-regulated in the F1 follicle under *ad libitum* feeding. It is most likely, given that the lower FSHR expression shown in broiler breeders fed *ad libitum* is more comparable with profiles observed in other studies [18], that the increased expression in restricted birds leads to negative feedback resulting from steroidogenic factors. Little functional significance has been placed on FSH control in the F1, despite observations by Johnson *et al*. that FSHR mRNA is up-regulated by Activin signalling in the F1 [24]. Further investigation is warranted to explore the potential roles for FSHR in this follicle in light of these results. Interestingly, MOSPD1, which in the previous chapter was proposed to be a potential mediator of FSHR signalling, due to its clustering in BioLayout, and because of its cellular localisation and role in mesenchymal differentiation [22, 23], showed no significant change in expression, either between feed strategies or, indeed, between stages of development. This is in marked contrast to the experiment in layers, which clearly showed significant differential expression between stages of development. Further investigation of this candidate to determine the nature of its relationship, if any, with FSHR would be required before the implications of this result can become clear.

Based on its homology with members of the PDGFR family, it was proposed that PDGFRL is involved in regulation of steroidogenesis [3]. The results from the previous chapter would also support a role in regulatory feedback mechanisms. As discussed in the previous chapter, the BioLayout Express profile from the microarray indicated down-regulation in in 5-7 mm follicles relative to the stroma and F1. This is consistent with the broiler breeder QPCR expression pattern for feed restricted birds. In contrast, the expression levels across tissues in *ad libitum* fed birds show no significant difference in expression. In *ad libitum* fed birds, where hierarchical follicle number is increased, the increased expression of PDGFRL expression, relative to feed restricted birds, suggests that, if it were involved in recruitment, directly or otherwise, it is likely to be in activation or up-regulation of positive feedback signalling to the HPGA. This observed increase in expression in *ad libitum* fed birds adds additional support to the hypothesis that PDGFRL may be responsible

for increased recruitment through dysregulation of part of the steroid-based feedback mechanisms governing the HPGA. However, the fact that, in those same *ad libitum* fed birds, it shows a drop in expression in the F1, a major source of steroid-based regulation, clearly demonstrates that, whatever its precise role, it is likely to be more complex than first anticipated.

GDF9 was not identified through the original microarray analysis but was included due to its location near the putative QTL for follicle number in the chicken and because studies in sheep report an association between mutations in GDF9 and increased ovarian follicle number and ovulation rate [1, 2]. Work published by other groups on GDF9 in the chicken has retrospectively supported this decision [25, 26].

Despite showing little apparent change in expression between tissues in the initial screen in layers, GDF9 does show significant down-regulation in response to *ad libitum* feeding in broiler breeders in 6-8 mm follicles ($P = 0.005$). In conjunction with results from mammalian studies this result would imply an inhibitory effect on follicle number. It is interesting to note that the expression profile for GDF9 in broiler breeders, regardless of diet, is comparable with other studies, and across species [25-28], indicating a high level of inter-species conservation for this gene. Reported inter-species sequence conservation from the UCSC Chicken Genome Browser supports this, with sheep being most comparable in terms of exon coverage. Further investigation is underway to determine if there are mutations in the chicken, as there are in sheep, which might be associated with multiple ovulation.

The remaining QTL-associated candidate genes, SLIT3 and VDAC1, were considered good candidate genes given their known functions as discussed in the previous chapter [5-9], which the layer results corroborated. However, there was no significant differential expression between dietary regimes in the broiler breeder QPCR validation for either candidate. VDAC1 also showed no significant difference in expression between stages of development. This is interesting in itself as VDAC1 demonstrated a distinctive expression pattern in the layers. If VDAC1 is not being expressed to the extent it should be at certain stages of development, this could contribute to increased follicle number, being a possible indicator of reduced levels of atresia.

YAP1, most likely involved in cell survival through regulation of p53 [4], was considered a secondary candidate. Though predicted to be up-regulated in *ad libitum* fed birds, this could not be validated by QPCR. This does not negate a role for YAP1 in follicular development; however it is unlikely to be responsible for multiple ovulation in broiler breeders.

Whereas comparisons have been made in this chapter with the White Leghorn results from chapter 4, it should be noted that these two experiments were independent. While trends in the data can be compared, one should be cautious in making too close a comparison of, for example, magnitude of effects. After all, each run of a PCR will introduce variation, whether it be fluctuations in heating between or during cycles, the mixing of the reaction volume, time between preparation and initiation of the reaction, or a number of other factors. Most importantly, however, the experiments were designed independently of each other and the birds used were of different ages, aside from the obvious breed differences.

In summary, the prime candidate genes, PDGFRL, GDF9 and FSHR all have strong cases for further investigation. GDF9 and FSHR are not novel candidate genes, indeed, FSHR was initially included in this study as a form of positive control and the identification of a previously unreported dietary effect on its expression was unexpected. PDGFRL however, is a novel candidate, and its implicated role in regulation of steroidogenesis, along with its response to *ad libitum* feeding makes it of primary importance.

In order to attempt to fulfil the ultimate objectives of the project, i.e. develop strategies for selection for reduced follicle recruitment in broiler breeders, the next chapter will focus on identifying SNPs and other genetic variation that may be causative or merely indicative of increased follicle recruitment.

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6

Genetic Variability in Candidate Genes

This chapter looks at the genetic variation within the primary candidate genes in various populations in order to identify possible features that could be associated with follicle number and used to select against multiple ovulation

6.1. Introduction

6.1.1. Genetic Variation

Within all populations there is a certain amount of inherent genetic variation. This is required to maintain a viable population free from the build-up of harmful mutations. Indeed, recombination during meiosis occurs precisely to maintain this diversity, providing a mechanism by which homologous chromosomes can 'swap' whole sections of DNA, linkage groups, with each other.

Even within controlled or selectively bred populations such as commercial livestock, unintended genetic variation will arise through errors resulting from DNA replication [1]. Of course, this variation will not be inherited if the mutations occur in somatic cells, or if the mutation is lethal. Mutations will only persist if they occur within the ancestral population of the germ cell lineage, and do not result in the knock-out or deactivation of important genes.

Through genetic linkage, mutations arising in this manner can become fixed in association with commercially desirable traits [2, 3] in commercial livestock populations. The 3 basic types of mutation to be discussed here are point mutations, where single nucleotides are incorrectly replaced with an alternative nucleotide, insertion mutations, where an additional nucleotide or nucleotides are inserted into the genomic sequence, or deletion mutations, where the reverse occurs.

6.1.2. Potential Effects of Mutations

If the mutation is a point mutation, a single nucleotide polymorphism (SNP), different alleles can have either a direct impact on the resulting trait phenotype, as long as they occur within the coding or promoter region of a key gene, or can simply be used as a marker for a desired phenotype through statistical association where there is no direct causal link but there is genetic linkage [2, 3].

SNPs will often have no measurable effect on genome function or the resulting phenotype of the organism [1]. Even SNPs occurring within the coding region of genes may still have little effect on the resulting protein sequence [1], unless in the 1st, and occasionally 2nd position of an in-frame codon, or alters the codon to a STOP codon (TAA, TAG or TGA) [1]. This is due to there being 64 possible codon combinations, 61 of which code for amino acids, with only 20 amino acids to code for. However, a

change to the 1st position in the codon can have implications for protein structure or activity, as it will often result in alteration of the amino acid sequence [4, 5]. In most cases even such a change will not significantly impact on the protein, due to the inbuilt redundancy in the system [1] i.e. the alternative amino acid is often of the same type as the original (polar/nonpolar, positively/negatively charged etc.). However changes can and do occur, with varying results.

Insertion and deletion mutations can be much more damaging in their effect, if they occur in the coding region of a gene as they force the sequence out of frame, i.e. the codons will all shift one base. This will often effectively knock-out the gene. Contiguous insertions or deletions of multiples of 3 in the coding region however, will simply result in the addition or deletion of amino acids from the protein. This can impact on protein folding or function of the active sites of domains in which they occur, but are unlikely to result in the loss of the protein, unless they remove the transcriptional start site or promoter region, or introduce a premature STOP codon as described above.

6.1.3. Selection

The concept of using markers associated with commercially important traits is not a new one. Selective breeding using phenotype as an indicator has been employed for thousands of years in farming of both crops and livestock species. As our knowledge of genetics has increased, methods of selection have improved to incorporate various forms of phenotypic markers, often associated with individual genes, associated with desirable traits [6-8]. This form of indirect selection, whilst being beneficial on the whole, has had unforeseen correlated effects, such as multiple ovulation in poultry [9, 10] or any number of disorders in various dog breeds [11-13]. However, in recent decades, developing technology has allowed detection of markers at the DNA level, making selection methods considerably more precise, and with fewer unforeseen negative side-effects [14]. Genomic selection, using results from QTL studies and SNP genotyping, has great potential in poultry [15] and is heavily used in the poultry industry.

6.1.4. Aims & Objectives

The aim of this set of experiments was initially to identify any SNPs associated with the primary candidate genes, PDGFRL, GDF9 and FSHR, that might have an effect on their activity, or, at the least, could be used as markers for increased follicle number. However, it became apparent in the early stages that there were several types of genetic variation besides SNPs present in the genes and the remit of this phase of the study was expanded to include these.

6.2. Experimental Design

6.2.1. Experimental Populations

This set of experiments utilised 3 different populations of birds: i) the Advanced Intercross Line (AIL) – a repeatedly crossed line deriving from a mating of a White Leghorn and a commercial Ross 308 broiler breeder (as described in section 2.1.3), ii) A Multistrain population – comprising of several lines of broiler, layer, and traditional breeds (as described in section 2.1.4), iii) the population of broiler breeders used for qPCR in the previous chapter (described in section 2.1.1).

6.2.2. Data Collection

There are various methods for approaching SNP detection. In a species such as the chicken, where there have been several builds of the genome, the initial phase can be carried out *in silico* using alignment of Expressed Sequence Tags (ESTs). These are short stretches of sequence that, when aligned *in silico*, overlap to allow compilation of a consensus sequence for the genome. Using ESTs sourced from multiple labs (often from different lines of birds), assuming there is sufficient coverage of the desired region, allows for observation of repeated sequence discrepancies. Where these discrepancies are consistent, a putative SNP can be called. Once a list of putative SNPs was produced, the SNPs were tested by sequencing of the founder birds of the AIL population to determine allele origin. DNA from the F8 generation of the AIL, where data on follicle number was available, was then genotyped to determine whether an association between the SNP(s) and follicle number could be made.

During the *in silico* and founder sequencing stages of this process, other genetic anomalies were identified. These were investigated using the broiler breeder and Multistrain populations described above.

6.3. Results

6.3.1. SNP Detection

6.3.1.1. In Silico Alignment

From *in silico* alignment of ESTs downloaded from the NCBI database, as described in section 2.5.1, putative SNPs could only be confidently called for PDGFRL based on the Trace plots. These were located within the 3' UTR of the gene and were in close proximity to each other. The EST alignment is shown in Figure 6.2. The putative SNPs can be seen in Table 6.1. As can be seen in Figure 6.1, if allele 1, as labelled in Table 6.1, is present for the first SNP, then Allele 1 for the second SNP is also present. This is consistent across all ESTs used for the alignment.

Table 6.1. Putative SNPs identified through *in silico* EST Alignment.

SNP ID	Allele 1	Allele 2
PDGFRL_UTR_1	T	C
PDGFRL_UTR_2	A	G

While conducting the EST alignment for PDGFRL, it was observed that there were 3 proposed transcriptional start sites for the gene (Figure 6.1). Two different start sites were suggested by ESTs, while a 3rd was predicted by Ensembl based on homology with other species, such as *Homo sapiens, Bos taurus, Rattus norvegicus, and Xenopus laevis*. Each version had an apparently unique 1st exon. This will be discussed in more detail in section 6.3.3.

6.3.1.2. AIL Founder Sequencing

Since *in silico* EST alignment only proved successful for PDGFRL, and there, only in the 3' UTR, it was decided to select 3 regions from each of the primary candidate genes for sequencing in the AIL founders. For sequencing by GATC Biotech (London, UK), any region had to be no more than 1kb in length. Three regions were selected for each gene, focussing on exons, with the possibility of additional regions where needed, depending on results and gene length. The regions selected can be seen in Figures 6.2, 6.3, and 6.4 for PDGFRL, GDF9, and FSHR respectively. The results from the sequencing of these regions is summarised in table 6.2.

Figure 6.1. Schematic representation of the EST alignment (section 2.5.1) and gene prediction evidence for the 3 potential alternate transcriptional start site variants of PDGFRL: The Ensembl predicted site (EnsPred), and the 2 sites supported by ESTs (ESTCons and ESTAlt) as described in Table 6.4. The gene is present on the negative strand and runs right to left as viewed in this figure. Further details and additional alignments can be observed in figure 6.3.

Figure 6.2. Alignment of available ESTs for PDGFRL, showing a portion of the 3' UTR. Alternative alleles of the 2 putative SNPs are highlighted in blue. Accession Numbers for ESTs are displayed in the left-hand panel. * indicate gaps in the alignment or possible sequencing errors in individual ESTs

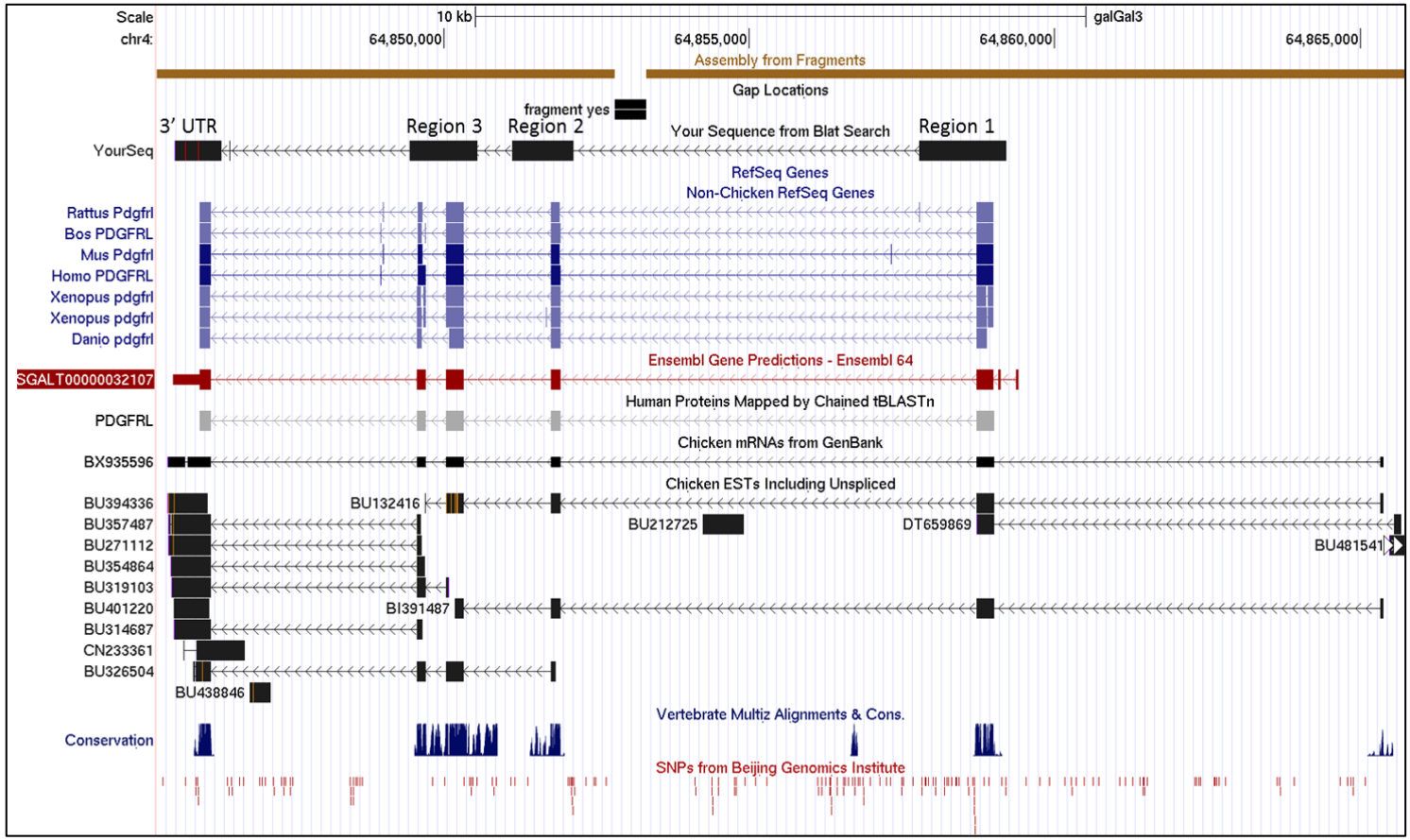

Figure 6.3. Sequence alignment against build 3 of the chicken genome for PDGFRL. From top to bottom, features include alignment with homologs from other species (blue), the Ensembl predicted gene (red), chicken mRNAs and ESTs (black), and the positions of BGI SNPs. The topmost feature (BLAT Search) indicates the regions selected for sequencing in the AIL founders (R1, R2, R3 and 3' UTR from right to left)

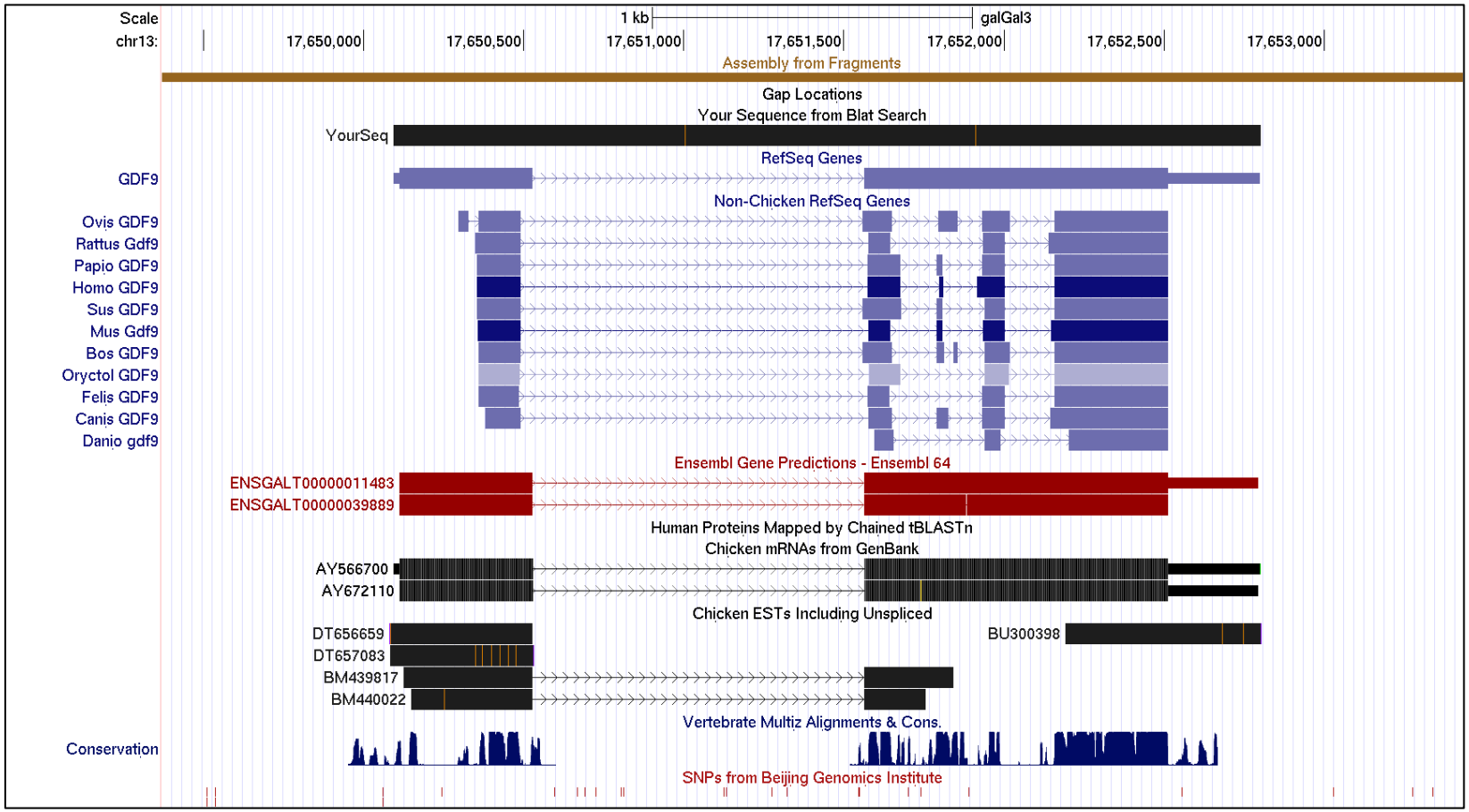

Figure 6.4. Sequence alignment against build 3 of the chicken genome for GDF9. From top to bottom, features include alignment with Chicken RefSeq genes & homologs from other species (blue), the Ensembl predicted gene (red), chicken mRNAs and ESTs (black), and the positions of BGI SNPs. The topmost feature (BLAT Search) indicates the regions selected for sequencing in the AIL founders. The length of the gene allowed the 3 regions to overlap, providing complete coverage.

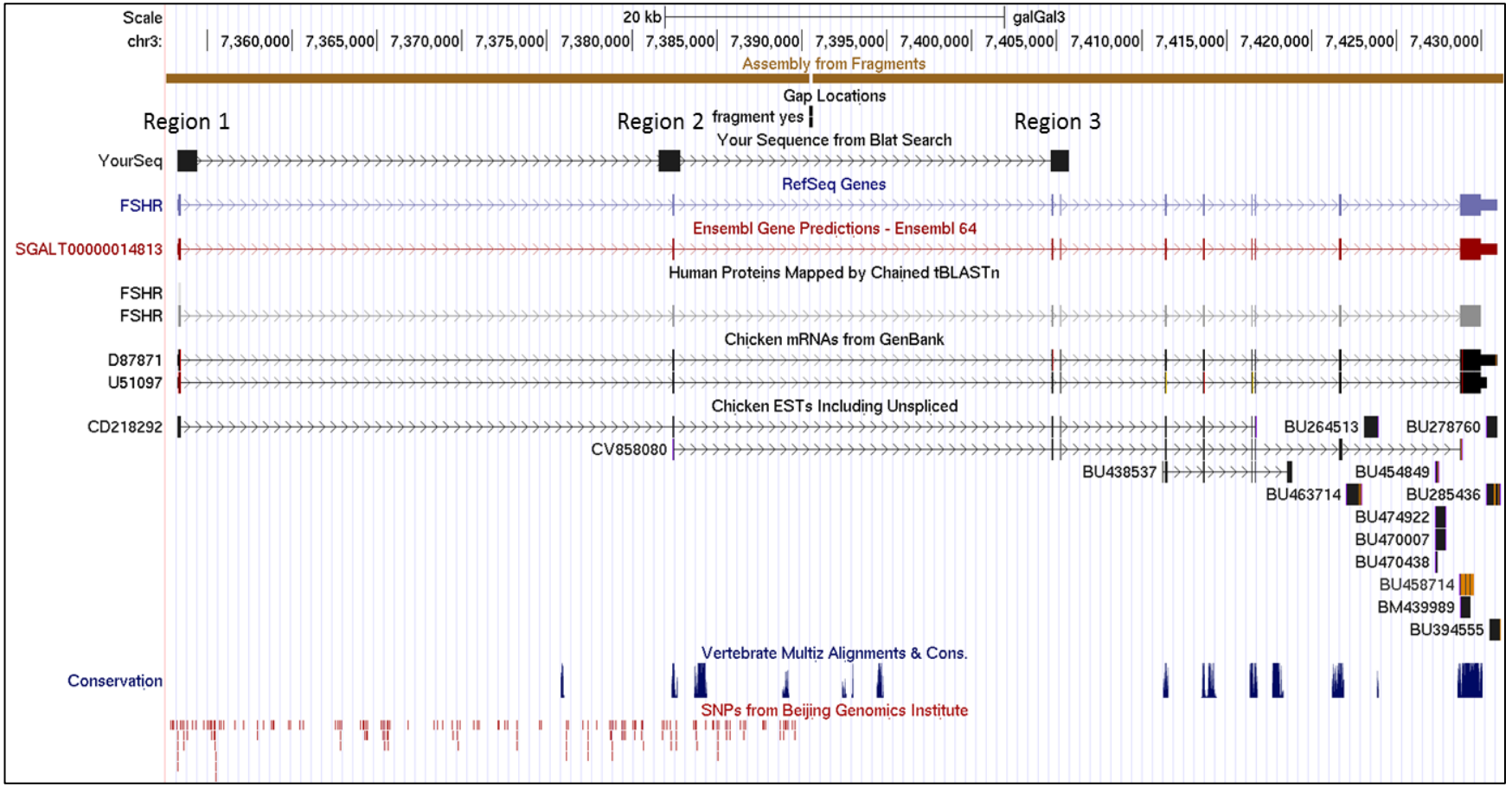

Figure 6.5. Sequence alignment against build 3 of the chicken genome for FSHR. From top to bottom, features include alignment with Chicken RefSeq genes (blue) Ensembl predicted genes (red), chicken mRNAs and ESTs (black), and the positions of BGI SNPs (red). The topmost feature (BLAT Search) indicates the regions selected for sequencing in the AIL founders.

Table 6.2. Summary of the results from the sequencing of the AIL founders listing SNPs and other features for each sequenced region of each gene. Region locations can be seen in Figures 6.3, 6.4, and 6.5.

Gene	Region	Result
PDGFRL	Region 1	Sequencing failed
	Region 2	Sequencing failed
	Region 3	5bp Deletion in Layer in 3rd intronic region
	3' UTR	SNP at positions: 226, 356, 396, 435, 533, 719, 722, 749
GDF9	Region 1	9bp Insertion in Broiler in coding region
	Region 2	No significant variation
	Region 3	No significant variation
FSHR	Region 1	SNP at positions: 76, 146
	Region 2	Sequencing failed
	Region 3	No significant variation

In all, 10 regions were sequenced in the 3 AIL founder birds. The 3'UTR of PDGFRL was sequenced first to test for the presence of the putative SNPs identified *in silico*. While those SNPs were monomorphic in the founder birds, 8 other SNPs were identified. All the SNPs were homozygous for 1 allele in the broiler and homozygous for the other allele in the layer and F1 from the other mating. The genotypes are listed in Table 6.3.

Table 6.3. SNPs identified by sequencing of AIL founders for the PDGFRL 3'UTR region. Quoted positions refer to locations relative to the start of the sequencing region. Note: the F1 originated from a different mating and is not the offspring of the other two birds.

SNP Position	Broiler	Layer	F1
226	A/A	C/C	C/C
356	G/G	A/A	A/A
396	T/T	C/C	C/C
435	T/T	C/C	C/C
533	C/C	T/T	T/T
719	C/C	G/G	G/G
722	C/C	T/T	T/T
749	A/A	C/C	C/C

6.3.1.3. F8 Genotyping

As all the identified SNPs in the 3' UTR sequenced region of PDGFRL presented 1 allele in the broiler breeder and the second consistently in the layer and F1 from the other mating, the SNP at position 396 was selected to represent all, as the trace file plot (Figure 6.6) showed strong and unambiguous peaks. However, no significant association could be observed between genotype and large yellow follicle number for this SNP by ANOVA. The SNP at position 76 in Region 1 of FSHR was selected for genotyping by KBioscience under the same criteria as in PDGFRL. However, despite 360 birds of the AIL F8 being used, the genotyping returned monomorphic results. The assay was repeated but returned the same results.

6.3.2. Multi-Nucleotide Insertions & Deletions

Sequencing of PDGFRL and GDF9 highlighted 2 multi-base anomalies (Table 6.2). Region 3 of PDGFRL contained a 5bp deletion in the layer, compared with the broiler breeder and the Reference sequence (Figure 6.7), top panel). A restriction digest was designed as described in section 2.5.4 for use in the Multistrain population (section 2.1.4.) to determine whether this feature was conserved in broiler-type birds, and absent in layer types. The results can be seen in Table 6.4. The chi-square test produced a value of 17.84 with 4 degrees of freedom, equating to a *P* value of 0.001. This effect most likely arose from the small number of broiler type birds with no copies of the sequence, though an apparent correlation can be observed in the Layers and Traditional breeds in Figure 6.8 between mean body weight and percentage of birds within each line (as opposed to type) possessing 2 copies of the sequence .

Table 6.4. Frequency table showing occurrence of the homozygous +, -, and heterozygous genotypes for the deleted sequence within PDGFRL in the 3 bird types

Type	2 Copies	No Copies	1 Copy	Total
Broiler	33	2	9	44
Layer	17	13	16	46
Traditional	28	12	10	50
Total	78	27	35	140

Figure 6.6. Putative SNP at position 396 of the 3' UTR sequenced region of PDGFRL, showing the sequence (top panel) of the F1, broiler breeder and layer respectively. The lower panel shows the raw trace file plots for each sequence, indicating a 'T' for the broiler breeder where the other birds show a 'C'.

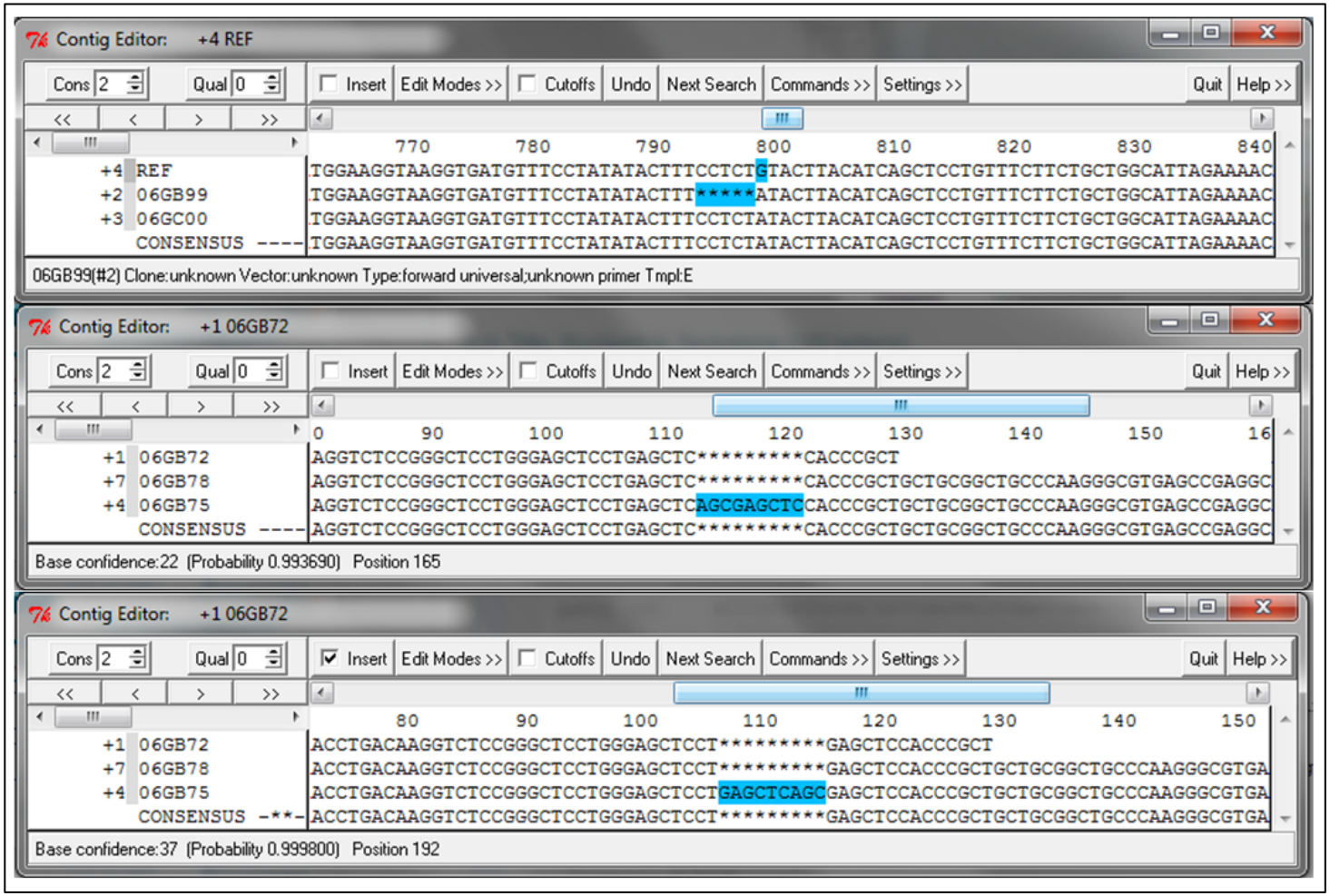

Figure 6.7. Sequencing results showing the 5bp deletion in the 3rd intron of PDGFRL in the layer (top) and the 9bp insertion in GDF9 in the broiler (galGal3 = middle, galGal4 = bottom).

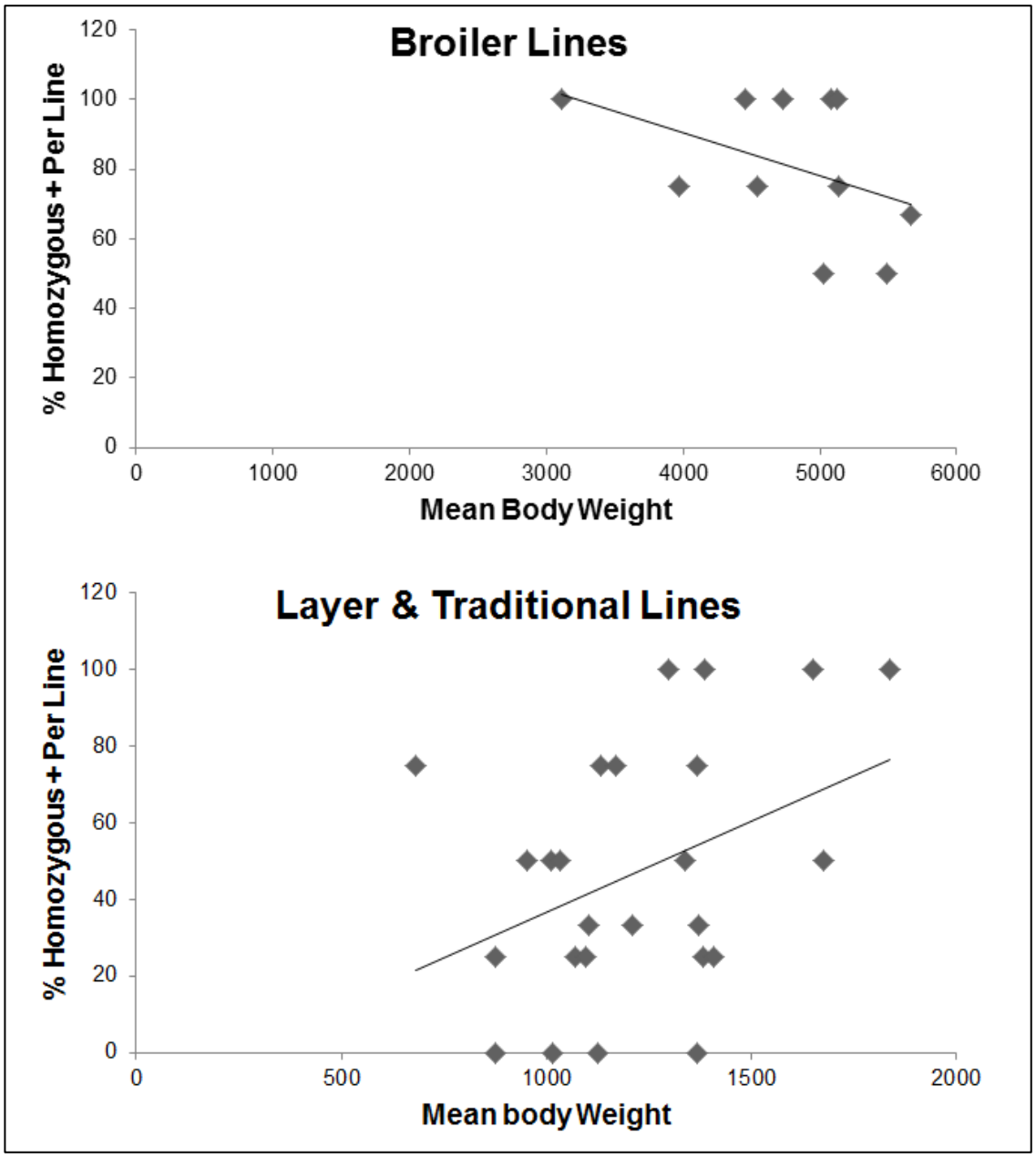

Figure 6.8. Graphs showing % of birds per line of the multistrain population possessing 2 copies of the anomalous sequence in PDGFRL plotted against mean body weight for each line. Each line contained 4 birds, with 12 broiler lines, 12 layer lines, and 13 traditional breeds.

6.3.3. PDGFRL Alternate Start Variants

6.3.3.1. Expression

As has been stated, during the process of selecting regions for sequencing of PDGFRL, it was observed that there were 3 different proposed transcriptional start sites for this gene. The evidence is summarised in Table 6.4 and the sequence alignments can be seen in Figure 6.3. There is also a small coding sequence (BU481541) that overlaps the start of the ESTAlt variant, though not the consensus start variant, and runs in the opposite direction, as can be seen in the lower right of Figure 6.13.

Table 6.4 Sources for sequences supporting alternate start sites for PDGFRL

Sequence	Start Site	Development Stage	Tissue	Source
BU132416	ESTCons	Stage 36 Embryo	Not stated	[16]
BI391487	ESTCons	Embryo	Not stated	[17]
DT659869	ESTAlt	Not stated	Testis, Ovary, Oviduct	[18]
ENSGALT00000032107	ENSPred	n/a	n/a	Ensembl Prediction
BU481541	ESTAlt Antisense	Adult	Growth Plate Cartilage	[16]

Designing primers for the 3 variants proved challenging due to the unique regions being in the range of 150-200bp, however it was possible to show that all 3 variants were expressed in the ovary (Figure 6.9) through amplification by PCR from pooled cDNA from the White Leghorns used in chapter 4. Having established this, it was decided to investigate these 3 variants of PDGFRL in the broiler breeder material remaining from the validation of dietary effect in broiler breeders (chapter 5). Though this population was reduced to 17 from the original 23, due to the need for experimental re-runs in the previous chapter, it was still possible to determine that there was significant differential expression between the 3 variants (P <0.001), as can be seen in Figure 6.10.

A cDNA tissue panel from a single AIL F16, comprised of 13 samples from various organ systems in duplicate, provided the opportunity to get an impression of the behaviour of PDGFRL, i.e. the original assay established in chapter 4, and the 3 alternate start variants of it across these tissues. The results of this can be seen in Figure 6.11. While ‘generic’ PDGFRL expression is greatest in the skin in the individual

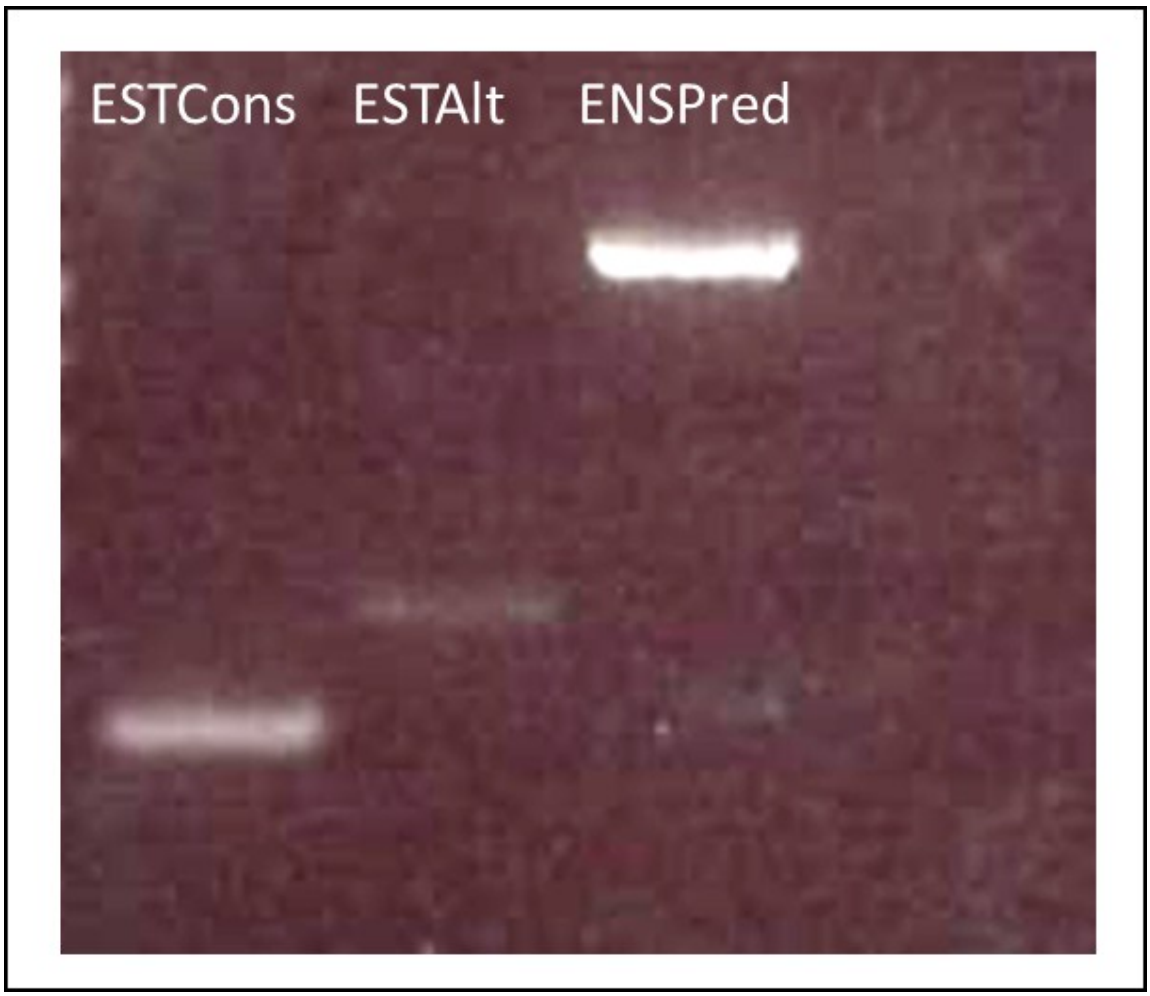

Figure 6.9. 2%Agarose gel showing amplification of single PCR products for primers designed against the three alternate start variants of PDGFRL in pooled ovarian cDNA from White Leghorn layers.

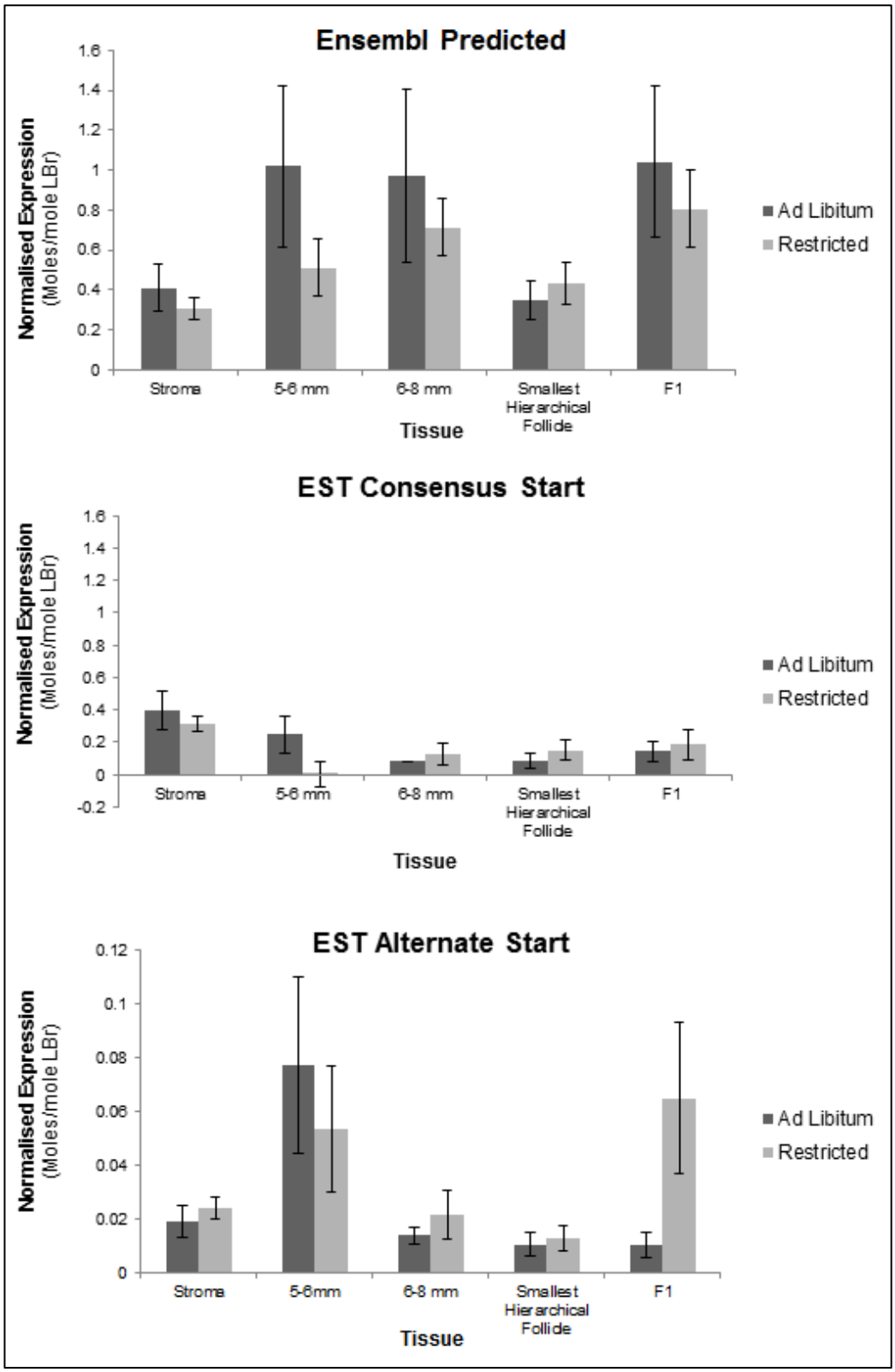

Figure 6.10. Expression of PDGFRL alternate start variants in broiler breeders (n=17) fed *ad libitum* (n=9) or restricted (n=8) diets. Note different scale for the bottom graph.

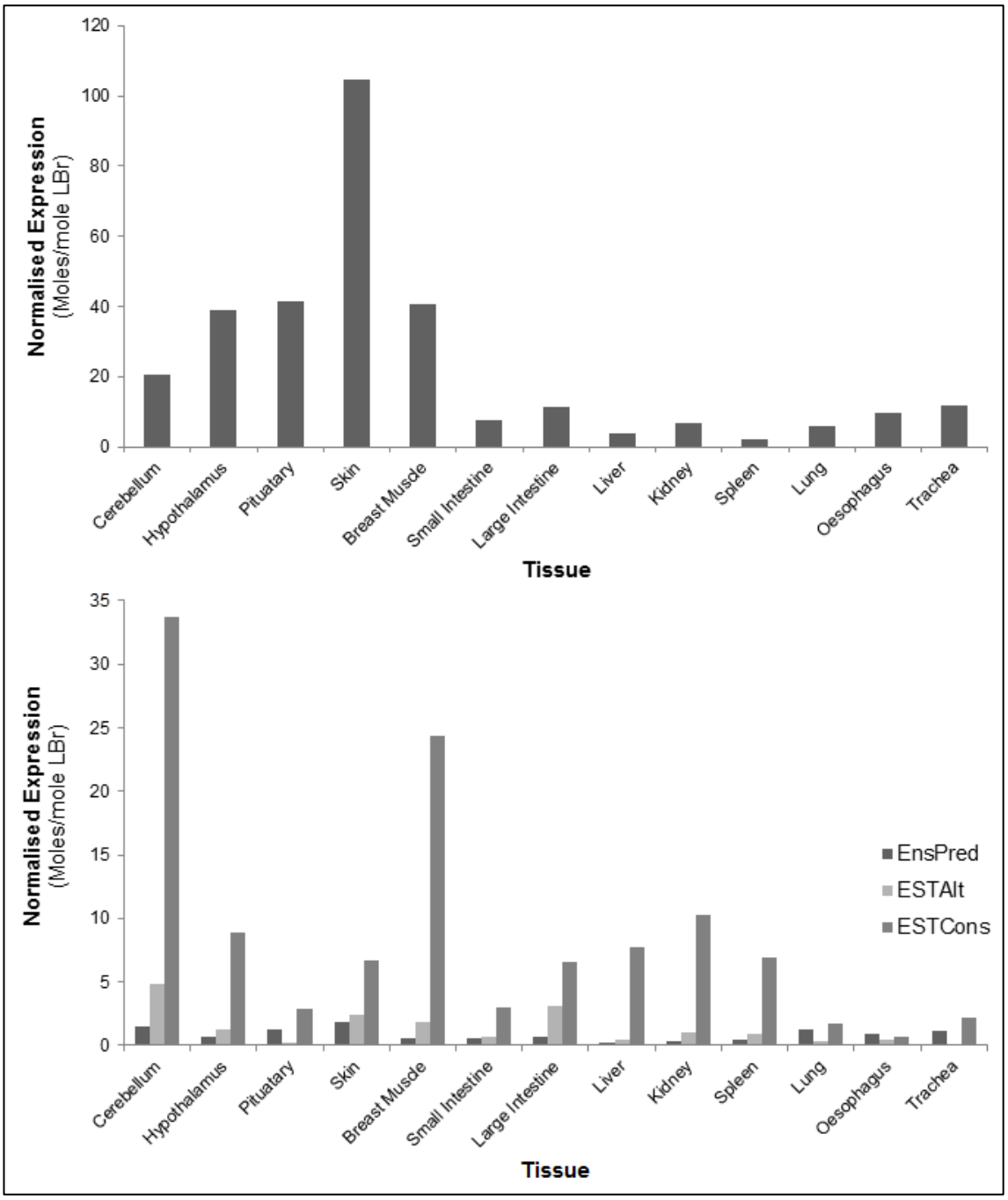

Figure 6.11. Expression of 'generic' PDGFRL (Top) and Alternate start variants (Bottom) in the White Leghorn tissue panel.

analysed; with the exception of the breast muscle, it is most commonly found in various regions of the brain, most noticeably, in the hypothalamus and pituitary, both components of the HPGA. Interestingly, the same pattern is not observed in the 3 variants. While the consensus start variant was not particularly strongly expressed in the ovary compared with the Ensembl predicted start variant, it is clearly the dominant variant expressed elsewhere. Except for the pituitary, lung, oesophagus, and trachea, the alternate start variant is dominant over the Ensembl predicted start variant.

6.3.3.2. Protein Variants

The protein translation from the ESTCons variant (Figure 6.12) aligns closely to the human protein for PDGFRL, albeit marginally truncated at the N terminus (Figure 6.13). The ESTCons variant protein does not incorporate coding from the upstream 1st exon from the ESTs. The other 2 variants conform to the ESTCons protein, with the addition of the sequences displayed in Figure 6.12. The EnsPred protein sequence, the alignment of which can be seen in Figure 6.14, does utilise elements of the unique exons predicted by Ensembl, and aligns well with the human protein. The proposed protein sequence for the ESTAlt variant, however, does utilise the extreme 5' sequence predicted as coding in the EST (Figure 6.15). Figure 6.16, output from InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/), designed to identify protein domains, clearly shows that the predicted translated protein is consistent with being a member of the PDGFR family.

Sequence analysis of the likely signal peptide region (Figure 6.17), however, shows little indication of a functional signal peptide for either ESTCons or ESTAlt. EnsPred shows the highest score, though even this does not surpass the threshold recommended by SignalP (http://www.cbs.dtu.dk/services/SignalP/), the program used for the analysis. In contrast, the human PDGFRL shows a a high quality signal peptide (Figure 6.18).

Consensus Protein Sequence (ESTCons):

M K E R E P V D S S S K S Q S I L **M** Q V **M** D K G R F Q K L T A T L S L S A G Q S I E L R C K G S N V T W S Y P S Y L D T F K D
S R L S I K Q L D R Y G Q L I L T N S T A A D T G E Y S C W L Q L C N G N K C R K D E T K T G S T Y I F F T D K E E L F V P T P
S Y F E I V Y L N P D K P A V I P C R V T T P L A K V T L H R E F P A E E I E T D E T N I I Y D A K K G F V Y Q H P T S D H K G I
V Y C K A E S Q G A P Q I S I K Y H L L Y V E V P R G P P S A T I V A S P S R A K V S D G I H V A C T V L G E P D V D V N F S W
Q Y P G Q E Q L E R P V I I Q N F W R L I N R G T G H T T R I S K S V L I I E D F E A R D A G N Y I C I A Q N L Q G T T T V A T R
V E L N **Stop**

5' Additions in other Variants:

Variant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
ESTAlt	**M**	T	L	Q	A	E	D	N	L	Q	S	V	R	V	V	R	G	R	V	I	G	Q	*	S	A	K
EnsPred	**M**	K	I	W	H	L	C	N	A	A	Q	K	N	R	I	L	V	I	L	C	G	N	K	S	A	K

Variant	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
ESTAlt	S	K	R	P	K	E	P	G	E	N	K	I	K	P	I	N	K	K	V	K	P	K	G	L	K
EnsPred	S	K	R	P	K	E	P	G	E	N	K	I	K	P	I	N	K	K	V	K	P	K	G	L	K

Figure 6.12. Predicted protein sequence for the ESTCons PDGFRL Variant and the additional 5' sequences preceding it in the other 2 variants.

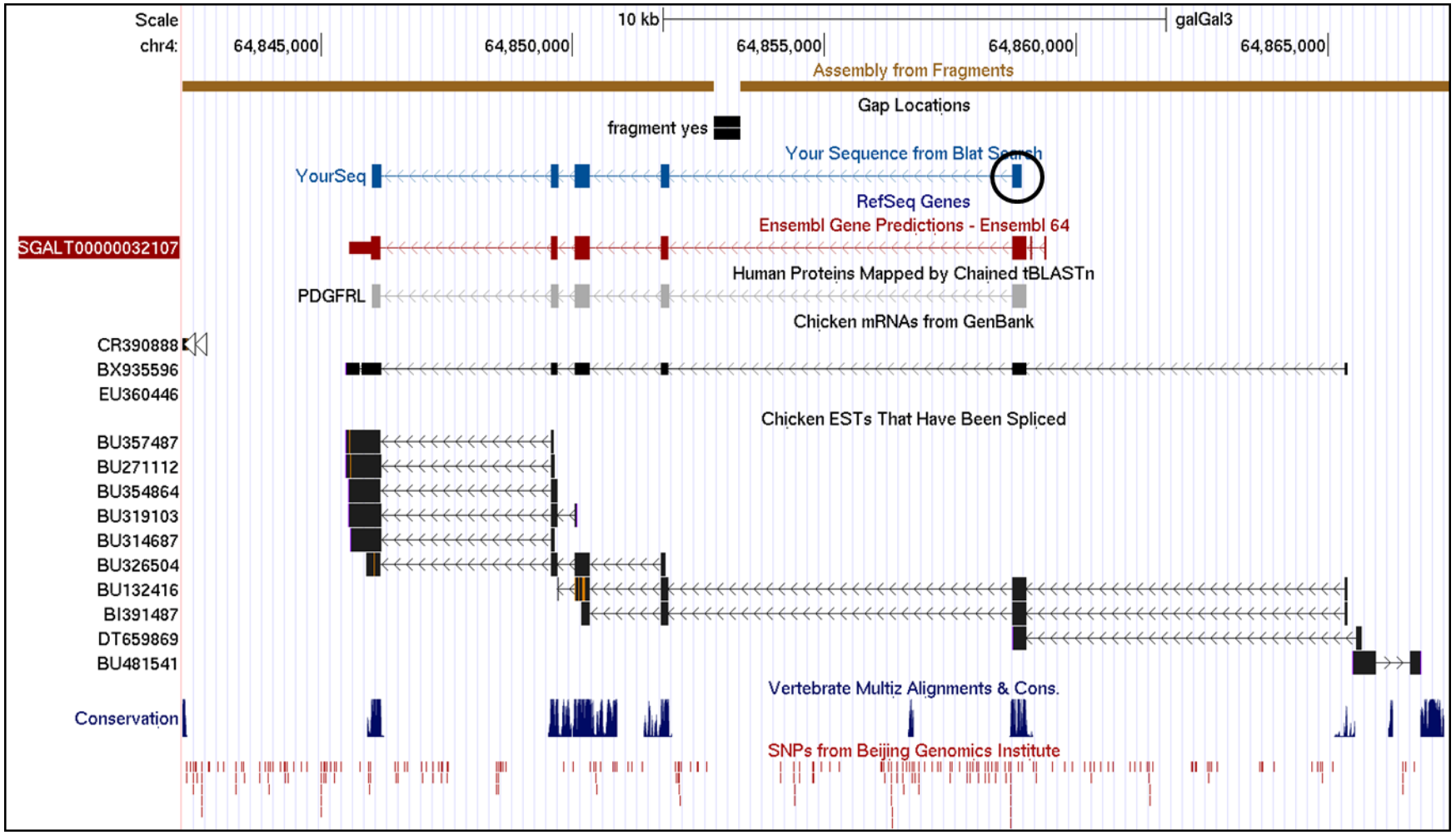

Figure 6.13. Protein alignment for the ESTCons variant of PDGFRL (BLAT Search) against the Ensembl predicted gene sequence, the human protein, and Chicken ESTs. Circled area shows truncated region.

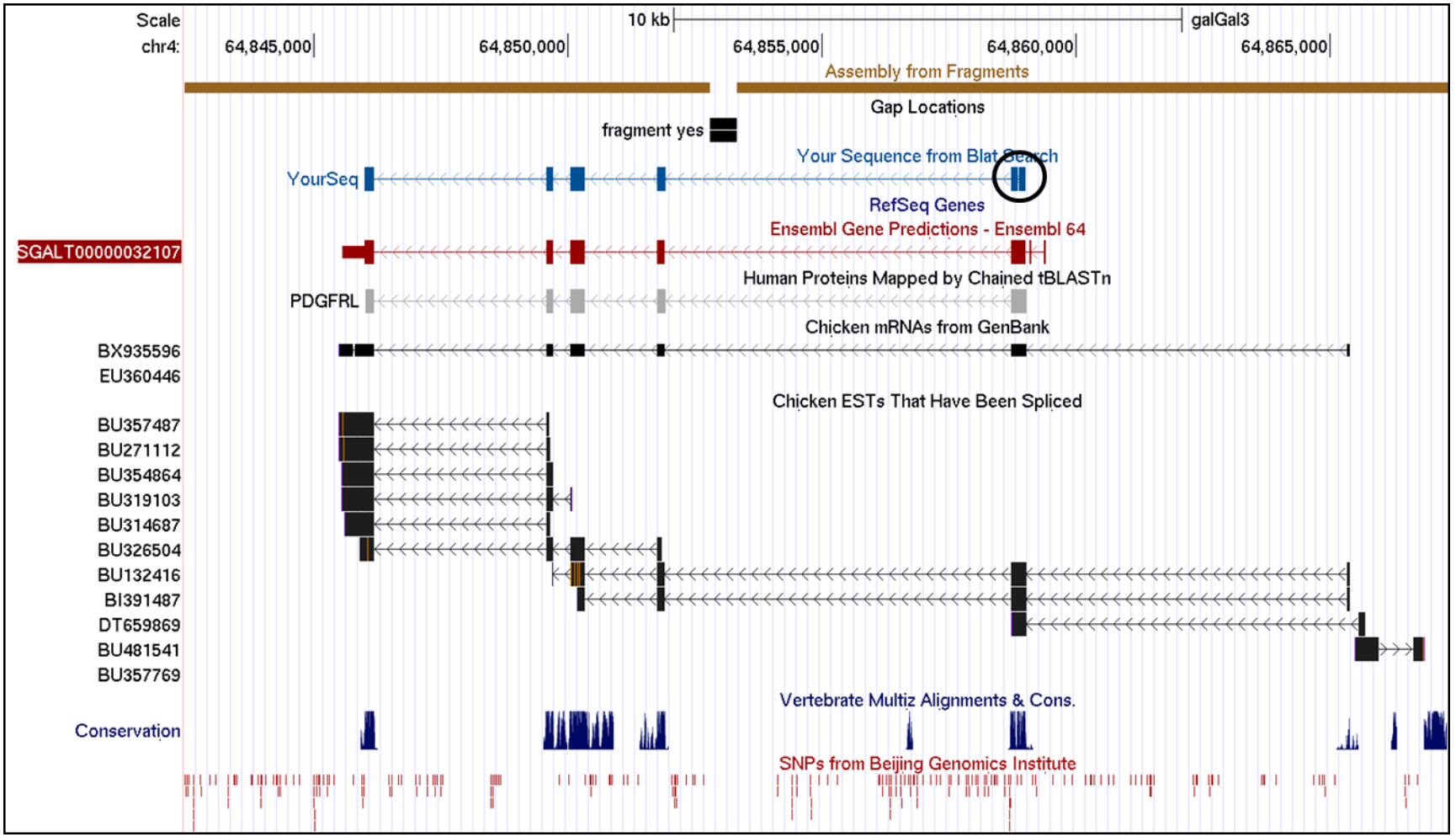

Figure 6.14. Protein alignment for the EnsPred variant of PDGFRL (BLAT Search) against the Ensembl predicted gene sequence, the human protein, and Chicken ESTs. Circled area shows truncated region.

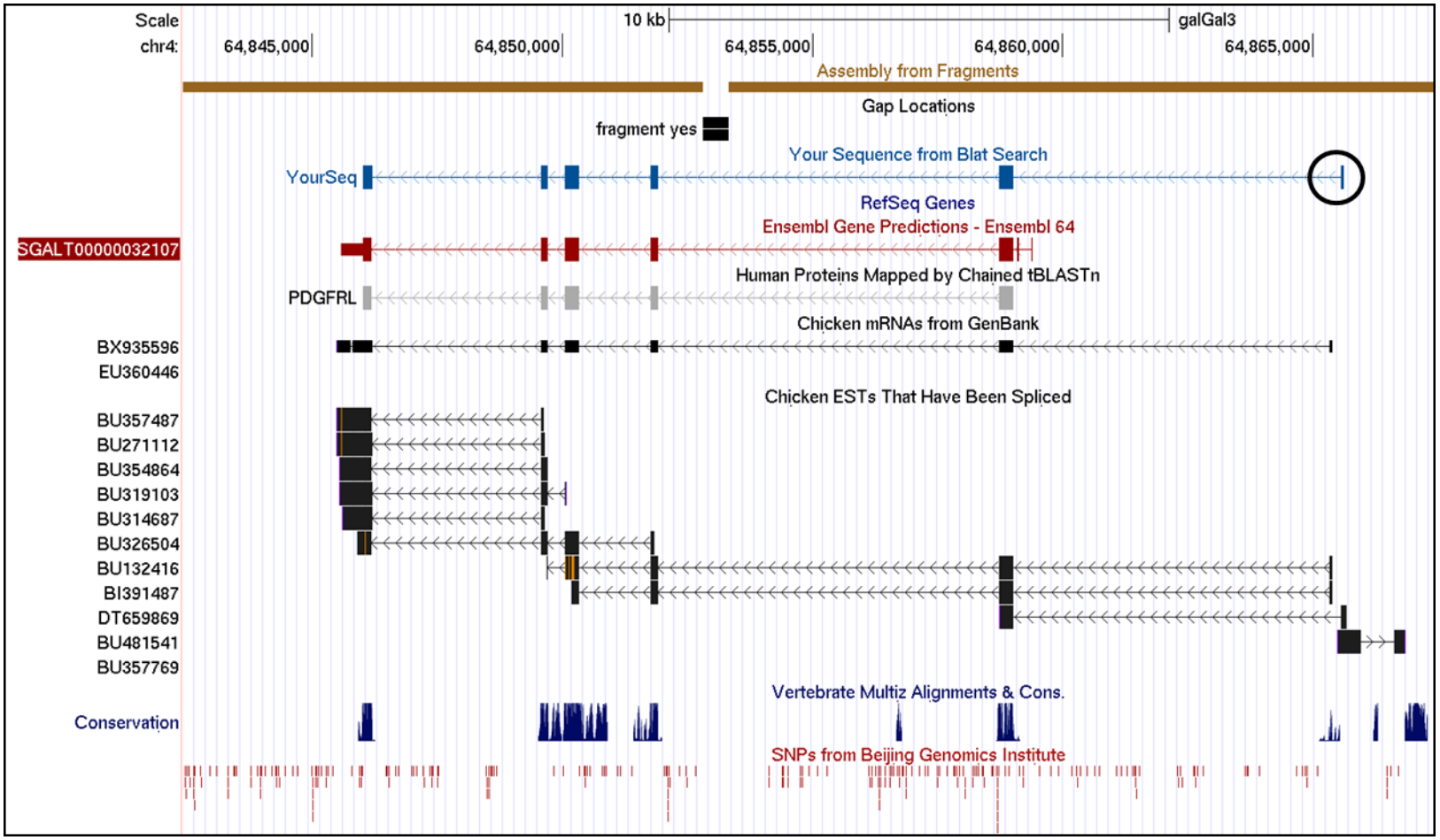

Figure 6.15. Protein alignment for the ESTAlt variant of PDGFRL (BLAT Search) against the Ensembl predicted gene sequence, the human protein, and Chicken ESTs. Circled area shows novel region.

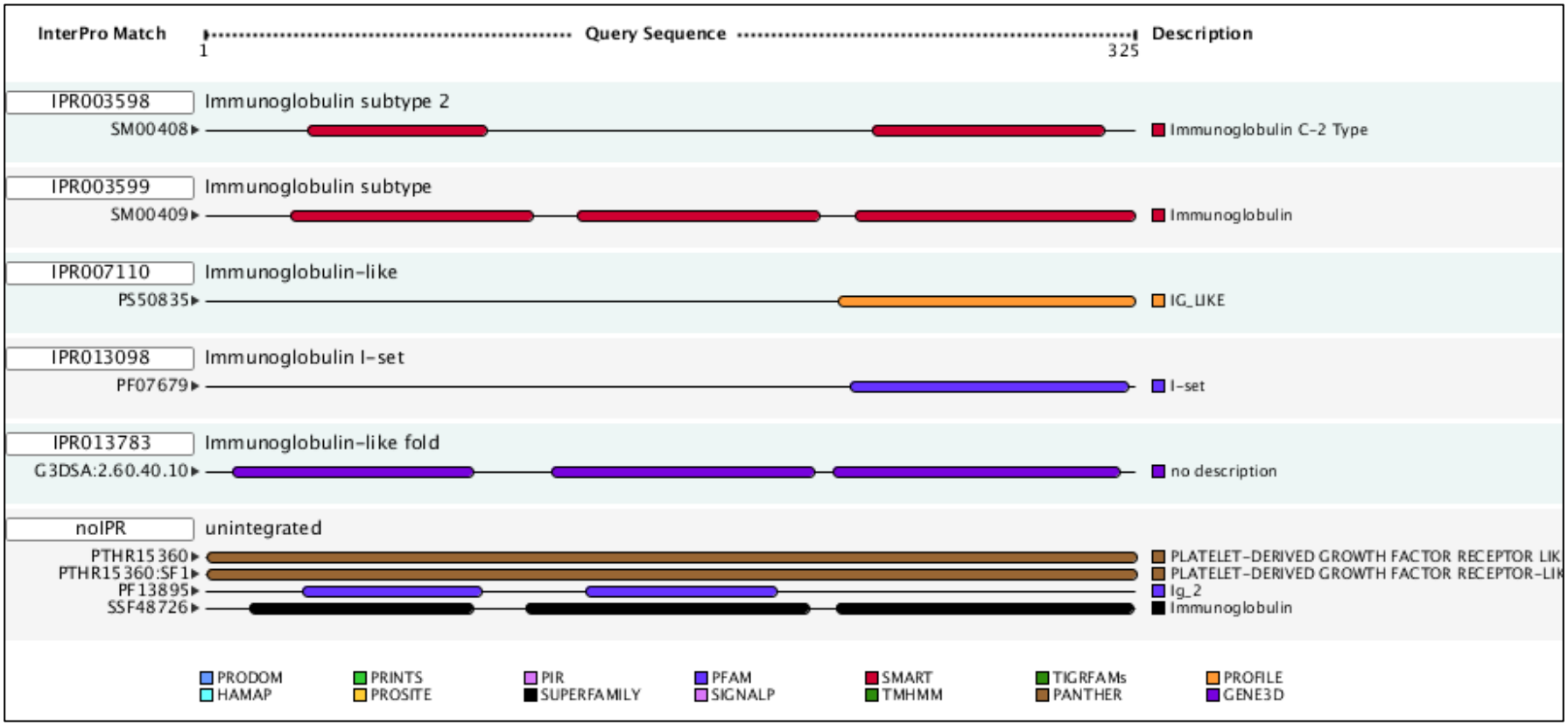

Figure 6.16. Putative domains located in the ESTCons protein translation variant of PDGFRL as predicted by InterProScan.

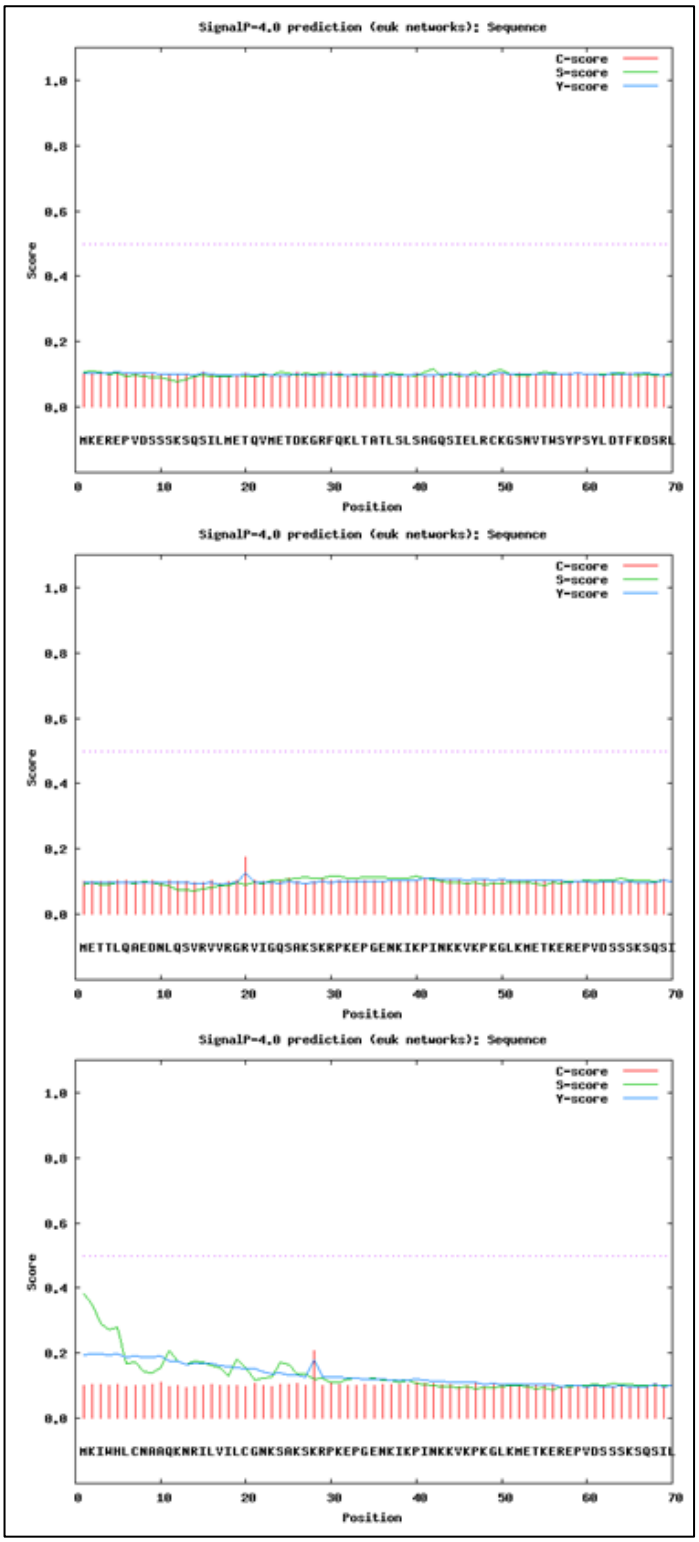

Figure 6.17. Predicted signal peptide quality for ESTCons (top) ESTAlt (middle) and EnsPred (bottom). The C-score indicates likelihood of a cleavage site; the Y-score is a further estimate of the cleavage site, while the S-score indicates suitability of the amino acid sequence as regards functionality as a signal peptide.

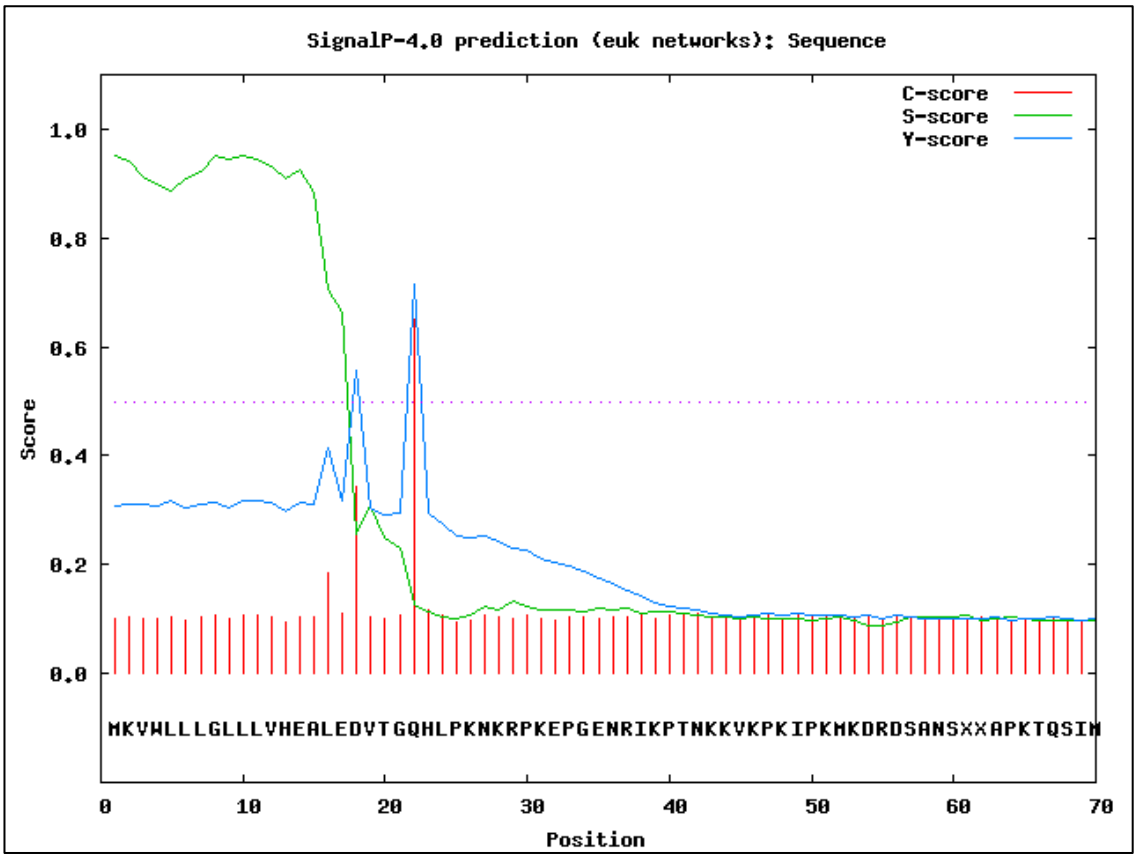

Figure 6.18. Predicted signal peptide quality for the human PDGFRL. The C-score indicates likelihood of a cleavage site; the Y-score is a further estimate of the cleavage site, while the S-score indicates suitability of the amino acid sequence as regards functionality as a signal peptide.

6.4. Discussion

This chapter has presented a large amount of results. However, these can be divided into 3 sections; i) SNP genotyping, ii) the discovery of short inserted/deleted nucleotide sequences, and iii) discovery of 3 alternate start variants of PDGFRL.

The original aim of this chapter was to identify SNPs in any or all of the primary candidate genes and investigate the possibility of their use as markers for increased follicle number. While many SNPs were identified, both through *in silico* EST alignment, and through direct sequencing, the majority of these were in the same region, and in linkage with one another. While no association could be made between the SNPs selected for genotyping and follicle number in the AIL, for reasons as yet unexplained, sequencing of some of the regions by GATC returned poor results. As some were resubmitted using the same stock material and returned good quality sequence, the error is thought to lie after dispatch of samples to GATC. Regardless of the nature or source of the error, this does mean that there are regions of some of the candidate genes that were not fully sequenced in the AIL founders and may yet yield results were they to be repeated. As things stand, the original aim of the chapter cannot be said to have been met.

However the sequencing did yield other and unexpected results. The 5bp deletion in the layer ancestor of the AIL within PDGFRL, and the 9bp insertion in the broiler within GDF9 are both worthy of further investigation. The deletion in PDGFRL is clearly not randomly distributed between different types of commercial bird. While only 2 out of 44 broiler type birds had no copies of the deleted sequence, 29 out of 46 layer type birds in the multistrain population either had only one or no copies of the sequence. Only 20% of the traditional types had no copy of the deleted sequence, and among the layer and traditional lines, there was indication of a correlation between the number of copies of the sequence and increased body weight. This having been said, it should be made clear that this is a deletion from the official reference sequence for the chicken, no doubt why so many of the traditional breeds have two copies. As such, this deletion is, by its very nature, unlikely to be responsible, in whole or in part, for the problem of multiple ovulation in broiler breeders. It is an interesting observation but it does not further the aims of this project.

The sequence that broiler breeders seem to have gained as part of GDF9, however, is far more interesting. Found in the coding region of the gene, it codes for 3 additional amino acids. Interestingly, this inserted sequence is held in common with the turkey, and various primate species, as was observed through blast search on the NCBI database. While, due to technical problems, time did not allow for follow-up of this result, it certainly needs to be investigated as its presence in the more heavily muscled turkey could suggest a correlation with body weight, especially as commercial turkeys also develop multiple follicle hierarchies [10].

The most interesting feature, however, is the discovery of the 3 alternate transcriptional start sites for PDGFRL. The results show that the 3 variants are all expressed in the ovary, but at significantly different levels. No alternate splice variants have been reported for this gene in other species, though 3 RNA transcripts are reported in humans on the Ensembl database. Only single products were observed when testing the original assay described in chapter 4, both by agarose gel electrophoresis and by dissociation curve during qPCR. It is interesting to note that the greatest expression in the ovary is exhibited by the EnsPred variant, which had no prior experimental evidence to support it. In contrast, the ESTCons variant, which is very highly expressed in tissues of the single AIL F16 compared with the other variants, shows very low levels of expression in the ovary.

Expression of the ESTAlt variant, despite its prior evidence coming from reproductive organs (Table 6.4), is extremely low. This variant may have been affected by expression of the product of the anti-sense sequence BU481541 (bottom right, Figures 6.13-15), which was not investigated here. Looking at the expression of this sequence may provide context for activity of the ESTAlt variant, particularly if it was possible to block expression of BU481541.

In addition, the sum totals of the variants in any of the tissues examined here by qPCR only occasionally equate to a value close to the value for the generic assay in the same tissue. While there will be variation between runs of any given assay, some tissues demonstrate quite large discrepancies, leading to the question of whether there are other variants, as yet unaccounted for, which would theoretically make up the difference. This would, however, require an extensive investigation to determine.

Of course, the standard errors for all the variants are quite high and it would be necessary to repeat this experiment, preferably with a larger sample population, before proper conclusions can be drawn.

While it has been shown that the common element of the predicted protein sequence from the 3 variants aligns to the human PDGFRL protein, as the functional protein is localised to the cell membrane, a signal peptide is required for intracellular transport. Based on the sequence analysis by SignalP, there is no strong signal peptide present in any of the variants. The EnsPred variant shows the highest score in the method used but even this does not surpass the recommended threshold. This could be a reflection of the quality of the tool; however it does provide a good score for the human protein and other groups have successfully used SignalP in comparing chicken and human signal peptides [19]. It is more likely that the nucleotide sequences used for prediction of the protein variants are lacking a 5' region, or that the methionine codons selected as the start sites are not the start sites used *in vivo*. It is possible, though unlikely, that PDGFRL is actually inactive in the chicken, through loss of its signal peptide. The 3 alternate start variants could be evidence of genetic restructuring that has disrupted the 5' end of the gene and prevents the gene from functioning appropriately. In humans PDGFRL has been identified as having potential tumour suppressor activity [20] and it is not impossible that the signalling mechanisms are related. However, to determine this, considerable work will have to be undertaken to isolate the proteins, examine their primary structure, and determine their subcellular localisation.

In conclusion, this set of experiments has clearly demonstrated that there is a considerable amount of genetic variability within PDGFRL, including SNPs, a multi-nucleotide deletion and at least 3 alternate transcriptional start sites. With the 9bp insertion in GDF9, there is much to follow up. Although the ultimate aim here was to identify possible features to be used for selection against multiple ovulation, the results do not allow for such conclusions to be drawn at this time. With further work however, it may well be possible to exploit one or more of the features described here to that end.

6.5. References

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7

General Discussion

This chapter reviews the results presented in the previous data chapters and lays out a proposed model for several of the candidate genes. It also discusses possible modes of action for those where there is insufficient data for them to be included in the proposed model, and provides recommendations for further avenues of investigation.

7.1. Overview

This work began with the aim of identifying novel candidate genes for regulation of hierarchical follicle number in the avian ovary, with a view to providing tools for selection in broiler breeders prone to multiple ovulation. The study has utilised several models of ovarian development to approach this problem and has reported a range of results supporting several strong candidate genes for a role in follicle selection and recruitment [1, 2], namely FSHR, GDF9 and PDGFRL.

In chapter 3, concerning the initial broiler breeder microarray study, the cases with supporting evidence for over 30 proposed candidate genes were laid out prior to full gene expression profiling in egg layer type birds in chapter 4. Chapter 5 then explored the effects of *ad libitum* vs. restricted feeding, a treatment known to radically affect follicle number [3], on the expression of the top candidate genes in broiler breeders. This analysis highlighted a number of significant effects at key stages of follicle development for 3 of the candidate genes, further supporting their predicted roles in determining follicle number.

The final data chapter explored genetic variability within the 3 top candidate genes shown to be affected by *ad libitum* feeding, and identified a number of features within PDGFRL and GDF9 which had the potential to contribute to their functionality. This chapter will discuss these results, their implications for current understanding of regulation of follicle number, and how the greatest benefit may be realised, both for commercial breeders and the birds themselves.

7.2. Approach & Methodology

As has been described, this study used a range of models for follicle selection and recruitment from which to collect its data. These different models were used through the candidate prioritisation process to discount successive layers of non-significant results, in the context of follicle recruitment, to identify key genes involved in that process. The path of this study has been linear, with one phase leading directly from the previous phase, with minimal need for parallel avenues of investigation. The initiation of that path was not so simple however.

The initial project from which this study arose focussed on the effect of feed restriction, compared with *ad libitum* feeding, on genes active in the ovary as feed restriction had long been established as a limiting factor on follicle number in broiler breeders [3]. The experimental design was simple; subject 2 groups of birds to 2 conditions known to result in different ovarian phenotypes and identify differences in gene expression between these two groups. This reflected a standard scientific approach of direct testing of evidence-based hypotheses. However, as discussed in chapter 3, in this instance the direct approach failed [2]. Were the experiment to be repeated, it would no doubt benefit from increased sample size and/or commercially produced microarray chips. However this scale of sample size, 16 individuals in this instance, had been shown to be sufficient in the past [4, 5] and cost-effective commercial microarrays for the chicken were unavailable at the time.

Consequently, it was decided to take a somewhat more circuitous approach to the problem using the available data by focussing on changes between stages of development. If key genes responsible for growth and development of follicles could be identified, they could subsequently be tested individually to determine whether they could be affected by level of feed intake. Given the high rate of false positives this approach was likely to generate, two different statistical approaches were used to analyse the microarray data and what effectively amounted to a methodological Venn diagram was used to filter the results such that only genes significant to both analyses were taken forward. As discussed above, the remainder of the study observed a linear structure and the results have been presented in that fashion. As with any scientific study, there is always a ‘wish list’ in terms of experimental approaches. As it is known that different cell types in the follicle wall have different roles in development and

function of the follicle, the question has been raised as to whether distinction of these cell types would have been beneficial in furtherance of the aims of this project. As acknowledged in the relevant chapters, this study has greatly benefited from the availability of pre-existing sample stocks so that only the layer experiment described in chapter 4 was conducted from conception within the timeframe of this study. As this experiment was intended to examine gene expression across all stages of follicle development, it was felt that to introduce cell type as an additional factor in the experiment would create a prohibitively large sample size, both in terms of qPCR setup and potentially reagent cost for the number of genes being investigated at the time. Having considerably reduced the number of candidate genes being investigated, the study of the genes in different cell types becomes very important and is a clear avenue to pursue in the future, particularly in light of the alternate start variants of PDGFRL that have been identified. There are several methods by which this may be achieved, immunohistochemistry for example. Though this may be effective for most of the candidate genes, for the different forms of PDGFRL this method could prove extremely challenging. Separation of the granulosa and theca layers at sampling, followed by the approach used hitherto for gene quantification would probably be the best method for approaching accurate identification of the PDGFRL variants. However, *in vitro* culture of granulosa and theca cell lines would also be quite useful in studying the candidate genes further. Cell lines could be exposed to individual stimuli, or different combinations thereof, sourced from the HPGA, thus providing information as to how the observed effects were triggered. Additional, more gene-specific routes of investigation will be proposed in section 7.5 in association with discussion of the appropriate gene.

7.3. Primary Candidate Genes

FSHR and GDF9, with the inclusion of SLIT3, can feasibly be fitted into a loose model for follicle development. Figure 7.1 shows a schematic representation of the major interactions involved in HPGA signalling and follicle development, as well as the key observations from chapter 5 as regards gene expression [2].

FSHR shows reduced expression under *ad libitum* conditions, or increased expression under feed restriction, in the F1 follicle (Figure 5.1). FSHR expression is supposed to be reduced in hierarchical follicles relative to earlier stages, as shown in the profiling in White Leghorns (Figure 4.4) and other studies [6], in favour of increasing LHR expression in preparation for ovulation. This correlates with what is already known about FSH and LH signalling in mammals [7], particularly the fact that FSH in mammals has been shown to 'rescue' or trigger recruitment of follicles [7, 8]. However in mammals follicles are recruited in waves or cohorts of up to 10 at a time [7], suggesting that while FSH indeed triggers recruitment, it does not necessarily determine the number of follicles recruited. Regulation of the receptor by intraovarian factors, however, could well play a part in determining follicular fate.

In chickens, ovulation is immediately followed by recruitment of a single additional follicle to the hierarchy, though, as described in the introduction, under certain conditions multiple follicles are recruited. As discussed in earlier chapters, FSH has proven challenging to study in the chicken due to difficulty in isolating it from LH. However it has been shown that, as in mammals, receptiveness to FSH is required for recruitment [9]. While the downstream pathways leading from FSHR activation are also not fully characterised in the chicken, increased steroidogenesis is a known outcome and the F1 follicle is a major source of steroid-based feedback. Again, this is borne out in mammals, however there are important differences. Progesterone in humans, for example, is a post-ovulatory hormone that is important for preparing the uterus for implantation of the fertilised ovum [10]. In chickens, on the other hand, it is released from the F1 and is responsible for triggering the pre-ovulatory LH surge [11-13], taking the role that oestrogen plays in humans [14].

It is conceivable therefore, that a consequence of increased FSHR expression in the F1 follicle is subsequent regulation of steroid-based feedback to the HPGA such that the mechanism of recruitment is dampened, perhaps through release of oestrogen, which is

known to have a negative effect on gonadotropin release in the chicken [15-17]. This is supported by the fact that the lower FSHR expression shown in the F1 follicles of *ad libitum* fed broilers relative to the other tissues (Figure 5.1), is more comparable with previous studies [18]. This model (Figure 7.2.) would support the hypothesis of recruitment being a dose-dependent response with respect to circulating gonadotropins, as outlined in chapter 1.

However, increased expression of FSHR in the F1 follicle was not the only observed effect of feed restriction. GDF9 expression is also increased under feed restriction in 6-8 mm follicles in broiler breeders (Figure 5.1), and mutations in GDF9 have been associated with increased ovarian follicle number and ovulation rate in sheep [19, 20]. The expression patterns of both *ad libitum* fed and restricted broiler breeders for GDF9 is comparable with other species (Figure 5.1) [21, 22] and therefore the role is also likely to be conserved. However, the observation in this study, showing that increased GDF9 expression is associated with reduced follicle recruitment, conflicts with a previous study that shows GDF9 positively influencing granulosa cell proliferation [23]. This having been said, GDF9 is most active in small white follicles [24], and its expression is significantly reduced in later stages of development [2, 23]. A possible explanation for this apparent contradiction, in terms of the effects of GDF9, would be that a dose-dependent conditional switch operates for GDF9 signalling, with a pro-development signal being activated below a certain threshold and a pro-atresia signal above that threshold. This sort of mechanism is not unprecedented in the TGFB superfamily [25], of which GDF9 and the BMPs are part, though no observations have been reported specifically for GDF9. Of course this threshold would have to be lowered or receptor levels proportionally reduced during later stages of follicle development, compared with the initial stages, since the expression at early stages of development surpasses expression levels under both *ad libitum* and restricted feeding in later stages.

While it is possible that the effects on FSHR and GDF9 under restricted feeding are unrelated, it is equally if not more likely that they are part of a common mechanism. A proposed structure for this mechanism is outlined in Figure 7.2, also incorporating a secondary candidate gene, SLIT3. SLIT3 is known to trigger pro-apoptotic signalling under certain conditions [26, 27] and has been shown to be regulated by steroid hormones in the ovary [26]. Increased SLIT activity has also been associated with

reduced oocyte proliferation in the foetal ovary in mammals [28]. The White Leghorn study also showed it to be most highly expressed in the F1 follicle (Figure 4.4) [2], and it is also located within a putative QTL for follicle number. There was, however, no effect on SLIT3 expression in response to *ad libitum* feeding (Figure 5.2) [2], suggesting that, if it is involved, regulation would need to be at the level of extracellular release of SLIT3 rather than regulation of its expression (Figure 7.2).

While this is speculation, FSHR activation could lead to extrafollicular release of SLIT3, which is, in turn, detected by receptors in 6-8 mm follicles. These receptors would, then trigger up-regulation of GDF9, increasing levels above the hypothetical threshold discussed earlier, causing the follicle to not be recruited to the hierarchy. There are many gaps in this model, and again it relies on dosage-dependence. As GDF9 is in close proximity to a putative QTL for follicle number in the chicken, this and other evidence [23, 29] adds additional weight to the need for its inclusion in the model.

Extrapolating the role for PDGFRL in follicle recruitment is far more complex. Three potentially different forms of the gene transcript have been identified (Figure 6.9), with the implication that there may be others (Figure 6.11). Chapter 6 showed that the form that is most highly expressed in the ovary of broiler breeders (Figure 6.10) is not the same as the dominant form in most other tissues from the AIL tissue panel (Figure 6.11). PDGFRL has been implicated in regulation of steroid-producing cells in the follicle [30] and it has been observed to be considerably up-regulated in 5-6 mm and the F2 follicle, relative to other follicle classes, in White Leghorns (Figure 4.4), and up-regulated in 6-8 mm follicles in *ad libitum* fed broiler breeders (Figure 5.1) [2]. The up-regulation correlates with increased follicle recruitment from this follicle class to the hierarchy. This evidence would support a function in follicle recruitment. However, until the number and nature of the different variants of PDGFRL and how they are regulated are determined, it is difficult to speculate as to how PDGFRL would regulate follicle number. However, if it does play a part, then the increased expression observed in 6-8 mm follicles of *ad libitum* fed broiler breeders would suggest that it supports positive feedback to the HPGA, causes up-regulation of gonadotropin receptor mechanisms in 6-8 mm follicles necessary for recruitment, or down-regulation of mechanisms that would otherwise limit recruitment.

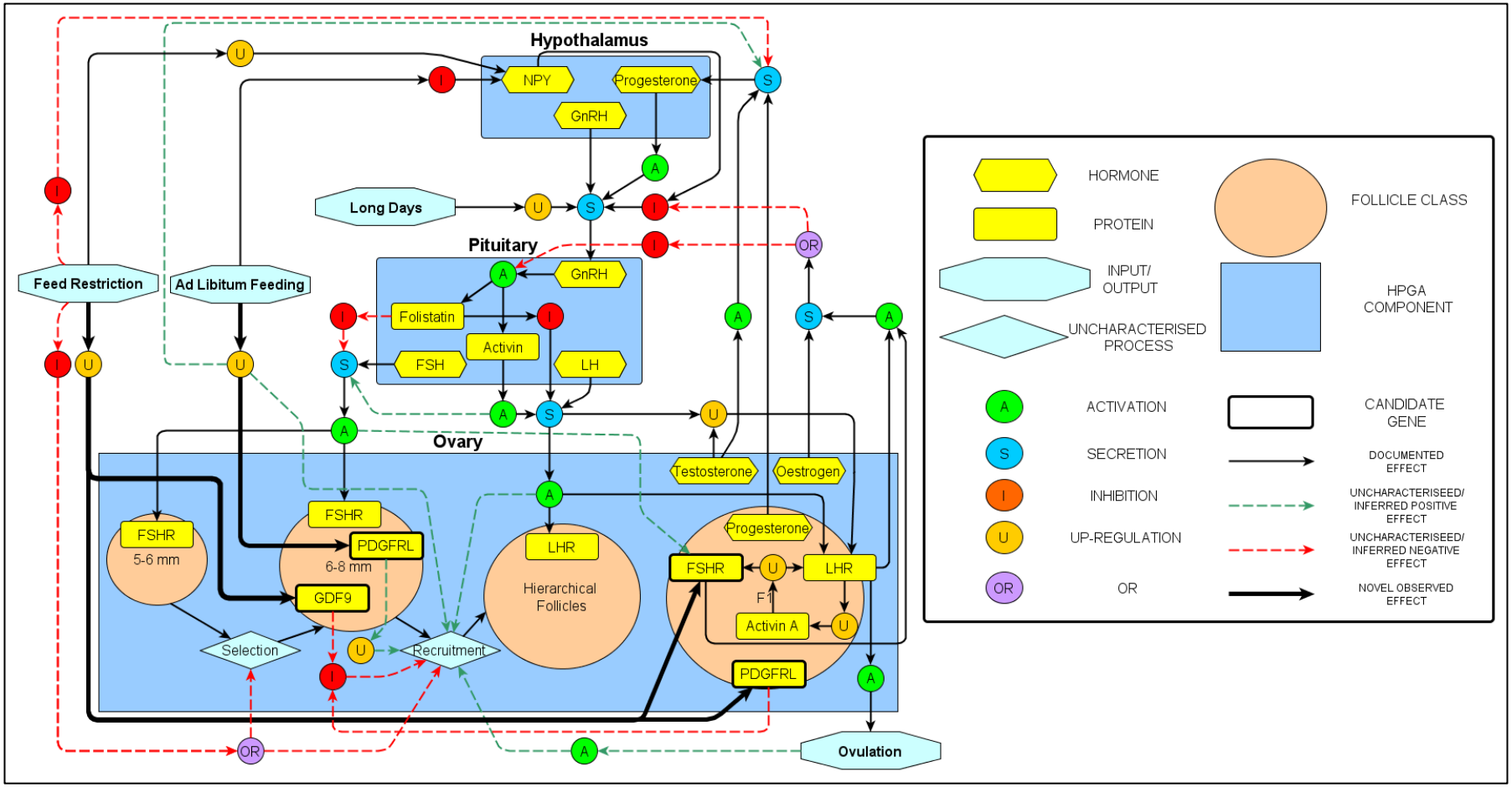

Figure 7.1. Pathway diagram based on the Edinburgh Pathway Notation (EPN) system [31], showing an overview of the major HPGA signalling and feedback mechanisms as discussed in chapter 1 with the addition of the observed effects (bold arrows) reported by McDerment *et al.* [2].

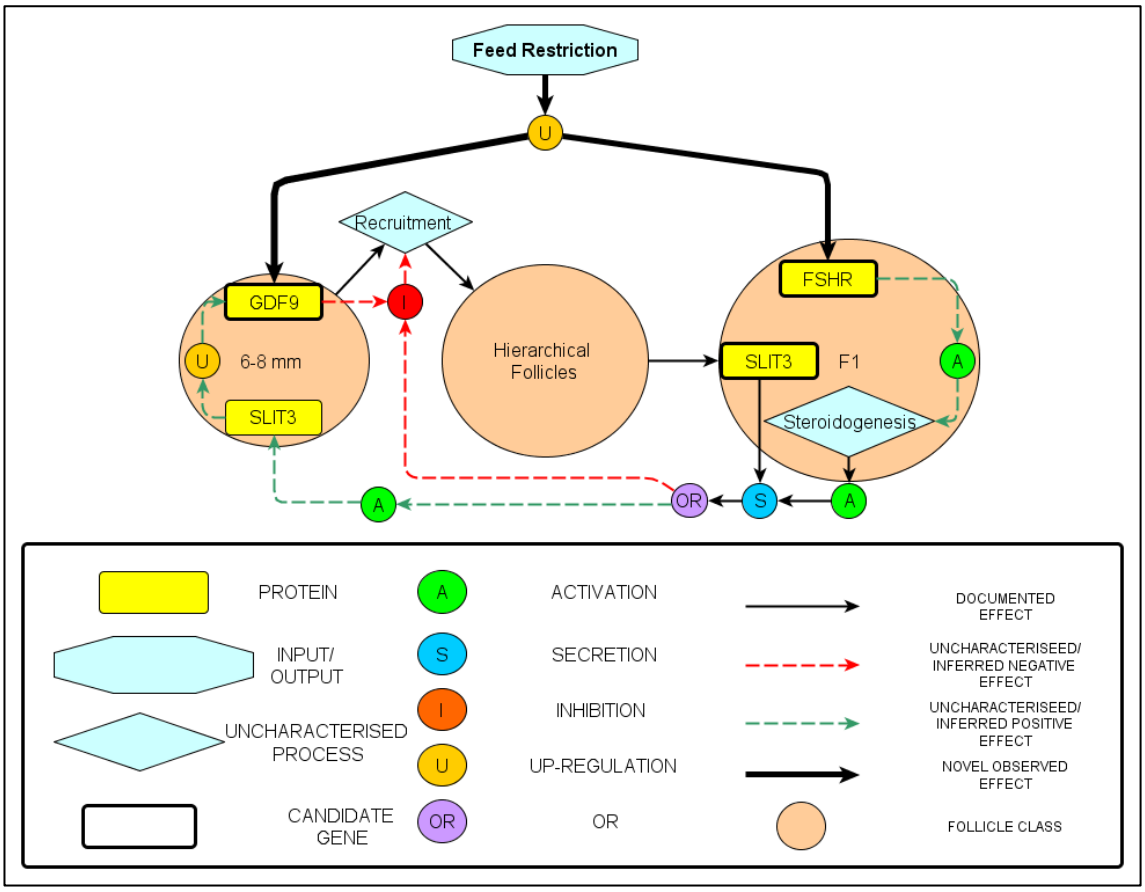

Figure 7.2. Pathway diagram based on the Edinburgh Pathway Notation (EPN) system [31], illustrating the hypothetical signalling mechanism of the candidate genes FSHR, SLIT3 and GDF9 and how they might affect follicle recruitment based on the observed effects reported by McDerment *et al.* [2].

7.4. Other Candidate Genes

In terms of secondary candidate genes, some features of SLIT3 have already been discussed. SLIT3 has several different reported functions, though much of the research was carried out in mammals. As well as pro-apoptotic signalling [27], it can also function as a chemo-repellent in axon guidance [32, 33], and as a chemo-attractant in monocyte migration [34]. As discussed in chapter 4, in light of this, SLIT3 may have one or more of several functions in the reproductive system. The infundibulum is known to move towards and engulf the F1 follicle during ovulation [35]. It is possible that, since SLIT3 has chemo-attractant abilities, and is most highly expressed in the F1 in White Leghorns (Figure 4.4), it mediates this process. It could also be involved in triggering cell death in the follicle wall prior to rupture of the follicle to release the yolk and ovum during ovulation. However, this is theoretical and would need further investigation. Both these hypotheses could be tested, at least in a preliminary fashion by culturing the appropriate cells and exposing them to a concentration gradient of SLIT3. This would show whether cells of the infundibulum migrated towards higher concentrations of SLIT3, and whether follicular cells underwent apoptosis in response to exposure to SLIT3.

VDAC1, the other gene located within the same QTL for follicle number, was not pursued beyond the *ad libitum*/feed restriction experiment as no significant effect was observed. It has been shown to be involved in Ca^{2+}-dependent apoptotic signalling [36, 37] and exhibited a pattern of expression in White Leghorns that is reminiscent of an exponential curve, beginning in early prehierarchical development and peaking after recruitment in the F5 follicle (Figure 4.4). However, in broiler breeders, there was no significant change whatsoever either between feed strategies or developmental stages (Figure 5.3). Not much is known about VDAC1 in the chicken, though in other species it has been associated with cancer [38]. The fact that such a marked difference in the expression of this gene exists between broiler breeders and layer-type birds is intriguing and is worth investigating further. Although less is known about MOSPD1, the same observation can be made as again there was significant differential expression in layer-type birds (Figure 4.4) between developmental stages but no significant difference in broiler breeders (Figure 5.3).

This having been said, the relative levels of expression for all of the candidate genes between the White Leghorn experiment (Chapter 4) and the broiler breeder experiment (Chapter 5) are quite different. The broiler breeder experiment consistently shows considerably lower normalised expression than the White Leghorn experiment, despite using the same gene for normalisation, LBR. There are a couple of potential reasons for this. Given the consistency of the variation across so many genes, while it is possible that this is representative of a real effect, it is more likely that it is an artefact resulting from methodological differences. The experiments were not designed to be directly compared, though results from both have been used in hypothesis generation. Different reverse transcription kits were used for the different experiments (see section 2.2.2.) and this may have had an impact on relative cDNA yield. This is likely to have had the largest effect but was unavoidable. The kit was changed for the broiler breeder experiment due to repeated supply problems with GE Healthcare. Alternatively, the White Leghorn experiment was using 'fresh' RNA, reverse transcribed a matter of days after collection while RNA for the broiler breeder experiment had been isolated from tissue in -80 storage for 4-5 years prior to reverse transcription for this experiment. While there are opposing colloquial views on the effect of long term storage on RNA quality, quantitative work to establish the effects of long term storage on human tissue have noted only small effects on quality [39, 40] as a result of long term storage. It is not impossible that the reduced levels of expression, if they are a result of storage or some other technical artefact, are masking what would otherwise be significant effects. However, a further experiment, looking at expression of these genes in both broiler breeders and layer type birds together would be the only way to resolve the nature of this effect and its potential implications.

7.5. General Conclusions & Further Work

As is often the case, this research would benefit greatly from further investigation. The data presented in this thesis has demonstrated that several novel candidate genes show expression patterns consistent with a possible role in follicle development in the 'ideal' ovarian phenotype (Figures 4.4 & 4.6). The 3 primary candidate genes, PDGFRL, GDF9, and FSHR were also shown to be affected in their expression by alteration to the feeding strategy in broiler breeders (Figure 5.1). Many of the candidate genes have not been well characterised in the chicken but, using these observations and available literature on the candidate genes in other model organisms, including primates, rodents, and ruminants, a basic model for regulation of follicle recruitment has been proposed for further testing, incorporating 3 of the candidate genes (Figure 7.2). Theoretical modes of action for other candidate genes have also been proposed separately where they cannot be easily incorporated into the main model.

Variation in the primary structure of both PDGFRL and GDF9 has also been identified between different types of commercial chickens which provides a potential source of selection tools for industry should they be proven to be associated with follicle number. Allele frequency for the 5bp sequence shown to be absent in the layer founder of the AIL population was indicated as potentially being correlated with body weight in the layers and traditional breeds of the multistrain (Figure 6.8), while only 2 birds, labelled as broiler type, (out of 44) had no copy of the insert (Table 6.4). Verification of this apparent correlation in the AIL population, where follicle number data is available, would be greatly beneficial. The assay for the 9bp inserted sequence identified in the coding region of GDF9 in the broiler type founder of the AIL also needs to be revisited in light of the discrepancy observed between the 2 builds of the genome, especially as this insert would affect the amino acid sequence of the GDF9 protein, and is also present in the turkey reference genome.

Only one set of the multistrain population was used to assess these features. There are two other sets, birds killed at 6 and 8 weeks of age, which could be used to improve the statistical power for identifying correlations in the multistrain. If a correlation can be observed for the GDF9 insert then the same course can be followed as with PDGFRL. Of course, the fact that this insert appears in the coding region highlights the necessity for the effect on the resulting protein to be investigated, perhaps through isolation of the

2 isoforms and subsequent treatment of cultured cells from the follicle wall. This would give an indication of the relative effects on follicular growth of the 2 isoforms.

The 3 alternate transcriptional start sites identified for PDGFRL (Figure 6.9) pose quite a different problem. While the 3 variants have been confirmed in both broiler breeders and the AIL, and they have been shown to exhibit markedly different profiles (Figures 6.10 & 6.11), sample size was a limiting factor on statistical significance as material remained for only 17 of the original 24 broiler breeders and tissues were only collected from 1 bird from the F16 AIL population. Sample size needs to be increased both for the broiler breeders and for other bird types in order to improve the expression results.

For the implications of this variation to be realised, functional studies will have to be carried out. *In-situ* hybridisation is a possible method for confirming the presence of functional signal peptides, given that they were predicted to be of poor quality in *in silico* analysis (Figure 6.17). Determining whether all 3 variants are localised to the plasma membrane is an important step in confirming their functionality. However, as discussed previously, this is not the only line of investigation indicated for PDGFRL (see section 7.2). Investigation of the anti-sense EST that, based on its alignment, has the potential to regulate translation of the ESTAlt transcript would also be beneficial as it would potentially allow for an *in vivo* study into the activity of the ESTAlt variant. It may also explain why the expression of ESTAlt is so comparatively low in some tissues. However, as the EnsPred variant appears to be the most abundant in the ovary, this is, perhaps, where the focus of an investigation should reside initially.

All in all, though this thesis reports a considerable array of results, the work is by no means over and much remains to be done before a complete picture of the regulatory mechanisms governing follicle recruitment to the hierarchy can be developed. If the results reported here can be followed up then it is likely to significantly improve the chances of being able to develop successful tools for selection against multiple ovulation and through this mitigate the concerns raised over welfare as regards the current levels of feed restriction of commercial broiler breeders. The results, based on what has been demonstrated here, which could be of greatest commercial benefit would likely be the multi-base insertion and deletion in GDF9 and PDGFRL respectively. Certainly for PDGFRL there are a range of genotypes present that could be used for selection, if it could be confirmed that selecting against this sequence had no

detrimental effects on other traits. However this would require an extended study looking at the effects of selection on successive generations of commercial lines. However, after further investigation along the lines proposed above of the other results reported here, other avenues may prove as beneficial in the context commercial selection.

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8

Appendices

8.1. Appendix 1 – Published Manuscript

The following section contains the final PDF version of the manuscript "Identification of novel candidate genes for follicle selection in the broiler breeder ovary" submitted to BMC Genomics.

Title	Identification of novel candidate genes for follicle selection in the broiler breeder ovary
Authors	N. A. McDerment, P. W. Wilson, D. Waddington, I. C. Dunn & P. M. Hocking
Journal	BMC Genomics 2012, 13:494
D.O.I	10.1186/1471-2164-13-49422992265
Submitted	13/04/12
Accepted	14/09/12
Published	19/09/12

Note: There are editorial errors in the formatted PDF and online version of this publication. These errors were introduced during post-production and are the responsibility of the BMC production team. Efforts have been made to have the publication amended.

8.2. Appendix 2 – QPCR Formulae & Calculations

The following section contains the Standard Curve formulae and calculation tables for determining significance for each gene examined by qPCR in chapters 4 and 5. Calculation tables show the differences between means of logged data (LOG_e). Bold values indicate differences greater than twice the s.e.d. In the layer experiment, the s.e.d. for tissue is quoted, in the broiler breeder experiment; the tissue/treatment interaction s.e.d. is quoted where a significant interaction was detected. In all other cases, tissue means (AL + FR) are quoted with the tissue s.e.d. Assays showing no significant effects have been omitted.

FSHR

Standard Curve: $y = -3.746 \times LOG(X) - 13.31$

Layer experiment:
s.e.d. = 0.87

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		0.38	2.12	3.00	2.75	1.66	1.48	1.25	0.94	1.06	1.16	0.00	0.42
Stroma	0.38	0.00	**-1.75**	**-2.62**	**-2.37**	**-1.29**	**-1.10**	**-0.87**	-0.57	-0.69	-0.78	0.38	-0.05
1-4	2.12		0.00	**-0.87**	-0.63	0.46	0.64	**0.88**	**1.18**	**1.06**	**0.97**	**2.13**	**1.70**
4-5	3.00			0.00	0.25	**1.33**	**1.52**	**1.75**	**2.06**	**1.94**	**1.84**	**3.00**	**2.58**
5-6	2.75				0.00	**1.09**	**1.27**	**1.51**	**1.81**	**1.69**	1.60	**2.75**	**2.33**
6-7	1.66					0.00	0.18	0.42	0.72	0.60	0.51	**1.67**	**1.24**
7-8	1.48						0.00	0.24	0.54	0.42	0.33	**1.48**	**1.06**
F6	1.25							0.00	0.30	0.18	0.09	**1.25**	0.82
F5	0.94								0.00	-0.12	-0.21	**0.95**	0.52
F4	1.06									0.00	-0.09	**1.07**	0.64
F3	1.16										0.00	**1.16**	0.73
F2	0.00											0.00	-0.43
F1	0.42												0.00

Broiler breeder experiment:
s.e.d. = 0.71

		Stroma AL	Stroma FR	5-6 AL	5-6 FR	6-8 AL	6-8 FR	Sm F AL	Sm F FR	F1 AL	F1 FR
		-1.69	-1.89	-1.88	-3.07	-1.66	-2.40	-2.92	-2.67	-3.94	-2.48
Stroma AL	-1.69	0.00	0.20	0.19	1.38	-0.03	0.71	1.23	0.98	**2.25**	0.79
Stroma FR	-1.89		0.00	-0.01	1.18	-0.23	0.51	1.03	0.78	**2.05**	0.59
5-6 AL	-1.88			0.00	1.19	-0.22	0.52	1.04	0.79	**2.06**	0.60
5-6 FR	-3.07				0.00	-1.41	-0.67	-0.15	-0.40	0.87	-0.59
6-8 AL	-1.66					0.00	0.74	1.26	1.01	**2.28**	0.82
6-8 FR	-2.40						0.00	0.52	0.27	**1.54**	0.08
Sm F AL	-2.92							0.00	-0.25	1.02	-0.44
Sm F FR	-2.67								0.00	1.27	-0.19
F1 AL	-3.94									0.00	**-1.46**
F1 FR	-2.48										0.00

GDF9

Standard Curve: $y = -3.444 \times LOG(X) - 14.02$ $y = -3.444 \times LOG(X) - 14.02$

Broiler experiment:
s.e.d. = 0.29

		Stroma AL	Stroma FR	5-6 AL	5-6 FR	6-8 AL	6-8 FR	Sm F AL	Sm F FR	F1 AL	F1 FR
		0.24	0.46	-0.45	-1.02	-1.76	-1.09	-2.36	-2.05	-2.02	-1.40
Stroma AL	0.24	0.00	-0.22	**0.69**	**1.26**	**2.00**	**1.33**	**2.60**	**2.29**	**2.26**	**1.64**
Stroma FR	0.46		0.00	**0.91**	**1.48**	**2.22**	**1.55**	**2.82**	**2.51**	**2.48**	**1.86**
5-6 AL	-0.45			0.00	0.57	**1.31**	**0.64**	**1.91**	**1.60**	**1.57**	**0.95**
5-6 FR	-1.02				0.00	**0.74**	0.07	**1.34**	**1.03**	**1.00**	0.38
6-8 AL	-1.76					0.00	**-0.67**	**0.60**	0.29	0.26	-0.36
6-8 FR	-1.09						0.00	**1.27**	**0.96**	**0.93**	0.31
Sm F AL	-2.36							0.00	-0.31	-0.34	**-0.96**
Sm F FR	-2.05								0.00	-0.03	**-0.65**
F1 AL	-2.02									0.00	**-0.62**
F1 FR	-1.40										0.00

GULP1

Standard Curve: $y = -3.565 \times LOG(X) - 15.50$

Layer experiment:
s.e.d. = 0.44

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		-0.67	2.15	2.69	2.50	1.59	1.67	1.57	2.42	2.63	2.25	2.28	2.44
Stroma	-0.67	0.00	**-2.82**	**-3.36**	**-3.17**	**-2.26**	**-2.34**	**-2.24**	**-3.09**	**-3.30**	**-2.92**	**-2.95**	**-3.11**
1-4	2.15		0.00	-0.54	-0.35	0.56	0.48	0.58	-0.27	-0.48	-0.10	-0.13	-0.29
4-5	2.69			0.00	0.19	**1.10**	**1.02**	**1.12**	0.27	0.06	0.44	0.41	0.25
5-6	2.50				0.00	**0.91**	0.83	**0.93**	0.08	-0.13	0.25	0.22	0.06
6-7	1.59					0.00	-0.08	0.02	-0.83	**-1.04**	-0.66	-0.69	-0.85
7-8	1.67						0.00	0.10	-0.75	**-0.96**	-0.58	-0.61	-0.77
F6	1.57							0.00	-0.85	**-1.06**	-0.68	-0.71	-0.87
F5	2.42								0.00	-0.21	0.17	0.14	-0.02
F4	2.63									0.00	0.38	0.35	0.19
F3	2.25										0.00	-0.03	-0.19
F2	2.28											0.00	-0.16
F1	2.44												0.00

KRT75

Standard Curve: $y = -3.552 \times LOG(X) - 9.67$

Layer experiment:
s.e.d. = 0.19

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		0.61	1.97	2.07	1.87	1.13	0.86	1.83	2.21	2.07	1.60	1.35	2.10
Stroma	0.61	0.00	**-1.36**	**-1.46**	**-1.26**	**-0.52**	-0.25	**-1.22**	**-1.60**	**-1.46**	**-0.99**	**-0.74**	**-1.49**
1-4	1.97		0.00	-0.10	0.10	**0.84**	**1.11**	0.14	-0.24	-0.10	0.37	**0.62**	-0.13
4-5	2.07			0.00	0.20	**0.94**	**1.21**	0.24	-0.14	0.00	**0.47**	**0.72**	-0.03
5-6	1.87				0.00	**0.74**	**1.01**	0.04	-0.34	-0.20	0.27	**0.52**	-0.23
6-7	1.13					0.00	0.27	**-0.70**	**-1.08**	**-0.94**	**-0.47**	-0.22	**-0.97**
7-8	0.86						0.00	**-0.97**	**-1.35**	**-1.21**	**-0.74**	**-0.49**	**-1.24**
F6	1.83							0.00	-0.38	-0.24	0.23	**0.48**	-0.27
F5	2.21								0.00	0.14	**0.61**	**0.86**	0.11
F4	2.07									0.00	**0.47**	**0.72**	-0.03
F3	1.60										0.00	0.25	**-0.50**
F2	1.35											0.00	**-0.75**
F1	2.10												0.00

MOSPD1

Standard Curve: $y = -3.885 \times LOG(X) - 12.63$

Layer experiment:
s.e.d. = 0.10

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		2.04	2.98	3.17	2.97	2.59	2.20	3.58	3.28	3.31	2.98	3.06	3.92
Stroma	2.04	0.00	**-0.94**	**-1.13**	**-0.93**	**-0.55**	-0.16	**-1.54**	**-1.24**	**-1.27**	**-0.94**	**-1.02**	**-1.88**
1-4	2.98		0.00	-0.19	0.01	**0.39**	**0.78**	**-0.60**	**-0.30**	**-0.33**	0.00	-0.08	**-0.94**
4-5	3.17			0.00	0.20	**0.58**	**0.97**	**-0.41**	-0.11	-0.14	0.19	0.11	**-0.75**
5-6	2.97				0.00	**0.38**	**0.77**	**-0.61**	**-0.31**	**-0.34**	-0.01	-0.09	**-0.95**
6-7	2.59					0.00	**0.39**	**-0.99**	**-0.69**	**-0.72**	**-0.39**	**-0.47**	**-1.33**
7-8	2.20						0.00	**-1.38**	**-1.08**	**-1.11**	**-0.78**	**-0.86**	**-1.72**
F6	3.58							0.00	**0.30**	**0.27**	**0.60**	**0.52**	**-0.34**
F5	3.28								0.00	-0.03	**0.30**	**0.22**	**-0.64**
F4	3.31									0.00	**0.33**	**0.25**	**-0.61**
F3	2.98										0.00	-0.08	**-0.94**
F2	3.06											0.00	**-0.86**
F1	3.92												0.00

PDGFRL

Standard Curve: $y = -3.419 \times LOG(X) - 7.32$

Layer experiment:
s.e.d. = 0.30

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		1.76	3.45	3.49	4.61	1.60	3.46	1.99	2.96	3.10	3.30	4.30	3.17
Stroma	1.76	0.00	**-1.69**	**-1.73**	**-2.85**	0.16	**-1.69**	-0.23	**-1.20**	**-1.34**	**-1.53**	**-2.53**	**-1.41**
1-4	3.45		0.00	-0.04	**-1.16**	**1.85**	-0.01	**1.46**	0.49	0.35	0.16	**-0.84**	0.28
4-5	3.49			0.00	**-1.12**	**1.89**	0.03	**1.49**	0.53	0.38	0.19	**-0.81**	0.32
5-6	4.61				0.00	**3.01**	**1.16**	**2.62**	**1.65**	**1.51**	**1.32**	0.32	**1.44**
6-7	1.60					0.00	**-1.86**	-0.39	**-1.36**	**-1.50**	**-1.70**	**-2.70**	**-1.57**
7-8	3.46						0.00	**1.46**	0.50	0.35	0.16	**-0.84**	0.28
F6	1.99							0.00	**-0.96**	**-1.11**	**-1.30**	**-2.30**	**-1.18**
F5	2.96								0.00	-0.15	-0.34	**-1.34**	-0.21
F4	3.10									0.00	-0.19	**-1.19**	-0.07
F3	3.30										0.00	**-1.00**	0.12
F2	4.30											0.00	**1.12**
F1	3.17												0.00

Broiler breeder experiment:
s.e.d. = 0.29

		Stroma AL	Stroma FR	5-6 AL	5-6 FR	6-8 AL	6-8 FR	Sm F AL	Sm F FR	F1 AL	F1 FR
		2.29	2.66	1.98	1.50	2.10	0.94	2.12	2.46	2.10	2.97
Stroma AL	2.29	0.00	-0.37	0.31	0.79	0.19	**1.35**	0.17	-0.17	0.19	**-0.68**
Stroma FR	2.66		0.00	**0.68**	**1.16**	0.56	**1.72**	0.54	0.20	0.56	-0.31
5-6 AL	1.98			0.00	0.48	-0.12	**1.04**	-0.14	-0.48	-0.12	**-0.99**
5-6 FR	1.50				0.00	**-0.60**	0.56	**-0.62**	**-0.96**	**-0.60**	**-1.47**
6-8 AL	2.10					0.00	**1.16**	-0.02	-0.36	0.00	**-0.87**
6-8 FR	0.94						0.00	**-1.18**	**-1.52**	**-1.16**	**-2.03**
Sm F AL	2.12							0.00	-0.34	0.02	**-0.85**
Sm F FR	2.46								0.00	0.36	-0.51
F1 AL	2.10									0.00	**-0.87**
F1 FR	2.97										0.00

RIGG01740

Standard Curve: $y = -3.785 \times LOG(X) - 13.39$

Layer experiment:
s.e.d. = 0.25

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		2.04	2.98	3.17	2.97	2.59	2.20	3.58	3.28	3.31	2.98	3.06	3.92
Stroma	2.04	0.00	**-0.94**	**-1.13**	**-0.93**	**-0.55**	-0.16	**-1.54**	**-1.24**	**-1.27**	**-0.94**	**-1.02**	**-1.88**
1-4	2.98		0.00	-0.19	0.01	**0.39**	**0.78**	**-0.60**	**-0.30**	**-0.33**	0.00	-0.08	**-0.94**
4-5	3.17			0.00	0.20	**0.58**	**0.97**	**-0.41**	-0.11	-0.14	0.19	0.11	**-0.75**
5-6	2.97				0.00	**0.38**	**0.77**	**-0.61**	**-0.31**	**-0.34**	-0.01	-0.09	**-0.95**
6-7	2.59					0.00	**0.39**	**-0.99**	**-0.69**	**-0.72**	**-0.39**	**-0.47**	**-1.33**
7-8	2.20						0.00	**-1.38**	**-1.08**	**-1.11**	**-0.78**	**-0.86**	**-1.72**
F6	3.58							0.00	**0.30**	**0.27**	**0.60**	**0.52**	**-0.34**
F5	3.28								0.00	-0.03	**0.30**	**0.22**	**-0.64**
F4	3.31									0.00	**0.33**	**0.25**	**-0.61**
F3	2.98										0.00	-0.08	**-0.94**
F2	3.06											0.00	**-0.86**
F1	3.92												0.00

SLIT3

Standard Curve: $y = -3.573 \times LOG(X) - 9.12$

Layer experiment:
s.e.d. = 0.25

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		0.37	0.20	0.74	0.90	0.25	0.75	0.71	0.60	0.34	0.22	0.85	1.19
Stroma	0.37	0.00	-0.17	0.37	**0.53**	-0.12	0.38	0.34	0.23	-0.03	-0.15	0.48	**0.82**
1-4	0.20		0.00	**0.54**	**0.70**	0.05	**0.55**	0.50	0.40	0.13	0.01	**0.64**	**0.98**
4-5	0.74			0.00	0.16	-0.49	0.01	-0.04	-0.14	-0.41	**-0.53**	0.10	0.44
5-6	0.90				0.00	**-0.65**	-0.15	-0.19	-0.30	**-0.56**	-0.68	-0.05	0.29
6-7	0.25					0.00	0.50	0.45	0.35	0.08	-0.03	**0.59**	**0.93**
7-8	0.75						0.00	-0.04	-0.15	-0.41	**-0.53**	0.10	0.44
F6	0.71							0.00	-0.11	-0.37	-0.49	0.14	**0.48**
F5	0.60								0.00	-0.27	-0.38	0.25	**0.59**
F4	0.34									0.00	-0.12	**0.51**	**0.85**
F3	0.22										0.00	**0.63**	**0.97**
F2	0.85											0.00	0.34
F1	1.19												0.00

Broiler breeder experiment:
s.e.d. = 0.57

		Stroma	5-6	6-8	Sm F	F1
		1.04	-0.81	-1.00	0.08	0.53
Stroma	1.04	0.00	**1.85**	**2.04**	0.96	0.51
5-6	-0.81		0.00	0.19	-0.89	**-1.34**
6-8	-1.00			0.00	-1.08	**-1.53**
Sm F	0.08				0.00	-0.45
F1	0.53					0.00

SMAD3

Standard Curve: $y = -3.908 \times LOG(X) - 9.54$

Layer experiment:
s.e.d. = 0.25

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		0.22	1.53	2.31	2.19	1.16	1.53	1.31	1.56	1.25	0.89	1.21	2.05
Stroma	0.22	0.00	**-1.31**	**-2.09**	**-1.97**	**-0.94**	**-1.31**	**-1.09**	**-1.34**	**-1.03**	**-0.67**	**-0.99**	**-1.83**
1-4	1.53		0.00	**-0.79**	**-0.66**	0.36	0.00	0.21	-0.03	0.28	**0.63**	0.32	**-0.52**
4-5	2.31			0.00	0.12	**1.15**	**0.78**	**1.00**	**0.75**	**1.07**	**1.42**	**1.10**	0.26
5-6	2.19				0.00	**1.03**	**0.66**	**0.88**	**0.63**	**0.94**	**1.30**	**0.98**	0.14
6-7	1.16					0.00	-0.37	-0.15	-0.40	-0.08	0.27	-0.05	**-0.89**
7-8	1.53						0.00	0.22	-0.03	0.28	**0.64**	0.32	**-0.52**
F6	1.31							0.00	-0.25	0.07	0.42	0.10	**-0.74**
F5	1.56								0.00	0.31	**0.67**	0.35	-0.49
F4	1.25									0.00	0.35	0.04	**-0.80**
F3	0.89										0.00	-0.32	**-1.16**
F2	1.21											0.00	**-0.84**
F1	2.05												0.00

Broiler breeder experiment:
s.e.d. = 0.55

		Stroma	5-6	6-8	Sm F	F1
		-0.33	-2.38	-2.86	-1.13	-1.16
Stroma	-0.33	0.00	**2.05**	**2.53**	0.80	0.83
5-6	-2.38		0.00	0.48	**-1.25**	**-1.22**
6-8	-2.86			0.00	**-1.73**	**-1.70**
Sm F	-1.13				0.00	0.03
F1	-1.16					0.00

SPTY2D1

Standard Curve: $y = -4.693 \times LOG(X) - 11.62$

Layer experiment:
s.e.d. = 0.19

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		3.32	2.69	3.33	3.13	2.49	2.89	3.29	1.24	2.66	2.68	2.32	1.70
Stroma	3.32	0.00	**0.63**	-0.01	0.19	**0.83**	**0.43**	0.03	**2.08**	**0.66**	**0.64**	**1.00**	**1.62**
1-4	2.69		0.00	**-0.64**	**-0.44**	0.20	-0.20	**-0.60**	**1.45**	0.03	0.01	0.37	**0.99**
4-5	3.33			0.00	0.20	**0.84**	**0.44**	0.04	**2.09**	**0.67**	**0.65**	**1.01**	**1.63**
5-6	3.13				0.00	**0.64**	0.24	-0.16	**1.89**	**0.47**	**0.45**	**0.81**	**1.43**
6-7	2.49					0.00	**-0.40**	**-0.80**	**1.25**	-0.17	-0.19	0.17	**0.79**
7-8	2.89						0.00	**-0.40**	**1.65**	0.23	0.21	**0.57**	**1.19**
F6	3.29							0.00	**2.05**	**0.63**	**0.61**	**0.97**	**1.59**
F5	1.24								0.00	**-1.42**	**-1.44**	**-1.08**	**-0.46**
F4	2.66									0.00	-0.02	0.34	**0.96**
F3	2.68										0.00	0.36	**0.98**
F2	2.32											0.00	**0.62**
F1	1.70												0.00

TGFBR1

Standard Curve: $y = -3.568 \times LOG(X) - 12.26$

Layer experiment:
s.e.d. = 0.17

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		1.37	2.37	2.65	2.63	1.96	1.17	2.48	1.10	1.63	1.55	0.77	0.87
Stroma	1.37	0.00	**-1.00**	**-1.28**	**-1.26**	**-0.59**	0.20	**-1.11**	0.27	-0.26	-0.18	**0.60**	**0.50**
1-4	2.37		0.00	-0.28	-0.26	**0.41**	**1.20**	-0.11	**1.27**	**0.74**	**0.82**	**1.60**	**1.50**
4-5	2.65			0.00	0.02	**0.69**	**1.48**	0.17	**1.55**	**1.02**	**1.10**	**1.88**	**1.78**
5-6	2.63				0.00	**0.67**	**1.46**	0.15	**1.53**	**1.00**	**1.08**	**1.86**	**1.76**
6-7	1.96					0.00	**0.79**	**-0.52**	**0.86**	0.33	**0.41**	**1.19**	**1.09**
7-8	1.17						0.00	**-1.31**	0.07	**-0.46**	**-0.38**	**0.40**	0.30
F6	2.48							0.00	**1.38**	**0.85**	**0.93**	**1.71**	**1.61**
F5	1.10								0.00	**-0.53**	**-0.45**	0.33	0.23
F4	1.63									0.00	0.08	**0.86**	**0.76**
F3	1.55										0.00	**0.78**	**0.68**
F2	0.77											0.00	-0.10
F1	0.87												0.00

Broiler breeder experiment:
s.e.d. = 0.33

		Stroma	5-6	6-8	Sm F	F1
		-3.20	-2.80	-3.89	-2.79	-2.46
Stroma	-3.20	0.00	-0.40	**0.69**	-0.41	**-0.74**
5-6	-2.80		0.00	**1.09**	-0.01	-0.34
6-8	-3.89			0.00	**-1.10**	**-1.43**
Sm F	-2.79				0.00	-0.33
F1	-2.46					0.00

VDAC1

Standard Curve: $y = -4.181 \times LOG(X) - 13.67$

Layer experiment:
s.e.d. = 0.19

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		2.76	2.23	2.39	2.43	2.80	3.06	3.65	4.31	4.24	2.61	2.85	1.21
Stroma	2.76	0.00	**0.53**	0.37	0.33	-0.04	-0.30	**-0.89**	**-1.55**	**-1.48**	0.15	-0.09	**1.55**
1-4	2.23		0.00	-0.16	-0.20	**-0.57**	**-0.83**	**-1.41**	**-2.07**	**-2.00**	-0.38	**-0.61**	**1.02**
4-5	2.39			0.00	-0.04	**-0.41**	**-0.67**	**-1.26**	**-1.92**	**-1.85**	-0.22	**-0.46**	**1.18**
5-6	2.43				0.00	-0.37	**-0.63**	**-1.22**	**-1.88**	**-1.80**	-0.18	**-0.42**	**1.22**
6-7	2.80					0.00	-0.26	**-0.85**	**-1.51**	**-1.44**	0.19	-0.05	**1.59**
7-8	3.06						0.00	**-0.59**	**-1.25**	**-1.17**	**0.45**	0.21	**1.85**
F6	3.65							0.00	**-0.66**	**-0.59**	**1.04**	**0.80**	**2.44**
F5	4.31								0.00	0.07	**1.70**	**1.46**	**3.10**
F4	4.24									0.00	**1.63**	**1.39**	**3.03**
F3	2.61										0.00	-0.24	**1.40**
F2	2.85											0.00	**1.64**
F1	1.21												0.00

YAP1

Standard Curve: $y = -4.214 \times LOG(X) - 15.48$

Layer experiment:
s.e.d. = 0.14

		Stroma	1-4	4-5	5-6	6-7	7-8	F6	F5	F4	F3	F2	F1
		1.93	2.38	2.58	2.78	1.68	2.11	2.24	3.03	2.48	2.25	2.39	2.81
Stroma	1.93	0.00	**-0.45**	**-0.65**	**-0.85**	0.25	-0.18	**-0.31**	**-1.10**	**-0.55**	**-0.32**	**-0.46**	**-0.88**
1-4	2.38		0.00	-0.20	**-0.40**	**0.70**	0.27	0.14	**-0.65**	-0.10	0.13	-0.01	**-0.43**
4-5	2.58			0.00	-0.20	**0.90**	**0.47**	0.34	**-0.45**	0.10	0.33	0.19	-0.23
5-6	2.78				0.00	**1.10**	**0.67**	0.54	-0.25	0.30	0.53	0.39	-0.03
6-7	1.68					0.00	**-0.43**	-0.56	**-1.35**	-0.80	-0.57	-0.71	**-1.13**
7-8	2.11						0.00	-0.13	**-0.92**	-0.37	-0.14	-0.28	**-0.70**
F6	2.24							0.00	**-0.79**	-0.24	-0.01	-0.15	**-0.57**
F5	3.03								0.00	0.55	0.78	0.64	0.22
F4	2.48									0.00	0.23	0.09	**-0.33**
F3	2.25										0.00	-0.14	**-0.56**
F2	2.39											0.00	**-0.42**
F1	2.81												0.00