THE UNIVERSITY *of* EDINBURGH

Role of glucocorticoids in development and growth of the cardiovascular system in the zebrafish

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Table of Contents

Declaration

I have read and understood The University of Edinburgh guidelines on Plagiarism and declare that this written thesis "Role of glucocorticoids on development and growth of the cardiovascular system in the zebrafish" is all my own work except where I indicate otherwise by proper use of quotes and references. I hereby declare that this thesis was composed by me under the supervision of Dr Martin Denvir and Dr Patrick Hadoke. All the work which is described in this thesis was performed entirely by myself and has not been submitted for any other degree or professional qualification.

Kathryn S. Wilson

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Thesis abstract

Introduction

Glucocorticoids (GCs) are synthesised endogenously in mammals by the hypothalamic pituitary adrenal (HPA) axis in response to stress. These hormones can elicit a number of physiological roles by binding to and activating specific receptors (glucocorticoid or mineralocorticoid receptors- GR or MR). GCs are important in tissue development and maturation and commonly used therapeutically. Mammalian animal studies have suggested that over-exposure to GCs, whether pharmacologically or through induction of maternal stress, is associated with increased cardiovascular disease risk in adult life. The underlying mechanisms underpinning this early life programming are poorly understood, however GC exposure during development may have direct and indirect effects on the structure and function of developing tissues and organs which may predispose to disease in later life. Current mammalian models of programming do not lend themselves well to studying organ development during embryogenesis. The zebrafish provides an ideal model to study this phenomenon due to the transparent nature of developing larvae and the availability of transgenic lines expressing fluorescent markers.

Methods

GC pathways were comprehensively characterised during zebrafish embryo development using qRT-PCR and steroid ELISAs. The physiological roles of GCs were assessed during early zebrafish development (first 120 hours post fertilisation (hpf)) assessing stress response, swim activity and global development following various genetic and pharmacological manipulations of the GC system. The impact that GC manipulation had on the cardiovascular system was also investigated. Embryos which had been exposed to GC manipulation during early development were then allowed to develop to adulthood in order to assess the long term impact. The same parameters were investigated in the adult as in the embryo.

Results

The key components of the GC system are present and functional in the developing embryo with *de novo* cortisol biosynthesis evident from 48hpf. A functioning hypothalamic pituitary inter-renal (HPI) axis is demonstrable from 72hpf. Manipulation of specific components of the GC pathway during early embryonic development influences growth-rate, head-trunk angle, chorion hatch-rate and swim behaviour. Manipulation of GCs during embryogenesis resulted in altered body weight, length and girth in adulthood, with altered stress response and swim behaviour also detected. Embryonic heart development was also affected with a reduction in ventricle cardiomyocyte number, cardiac gene abundance (*vhmc*) and cardiac function during embryogenesis resulting in structural abnormalities such as fewer trabeculae and increased intra-ventricular space. Embryonic GC manipulation also alters the formation and patterning of intersegmental blood vessels by 120hpf. In adulthood this manifests as a reduced angiogenic capacity.

Conclusion

The zebrafish embryo represents a valid and physiologically relevant model for GC research. Manipulation of GCs during early development results in altered growth, gene abundance and cardiovascular structure. These findings have significant implications for on-going research addressing GC mediated programming and suggest that the zebrafish is a highly suitable model for GC research

Publications arising from this work

Wilson, K.S., Matrone, G., Livingstone, D.E.W., Al-Dujaili, E.A.S., Mullins, J.J., Tucker, C.S., Hadoke, P.W.F., Kenyon, C.J., Denvir, M.A. (2013) Physiological roles of glucocorticoids during early embryonic development of the zebrafish (Danio rerio) *Journal of Physiology* **15**: 6209-20

Matrone, G., Taylor, J. M., **Wilson, K. S.,** Baily, J., Love, G. D., Girkin, J. M., Mullins, J. J., Tucker, C. S., Denvir, M.A. (2013) Laser-targeted ablation of the zebrafish embryonic ventricle: A novel model of cardiac injury and repair. *International Journal of Cardiology* **168:** 3913-3919

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Glucocorticoids play a key role in maturation and structural organisation of the developing zebrafish cardiovascular system. **Wilson, K.S**., Matrone, G., Tucker, C.S., Mullins, J.J., Kenyon, C.J., Hadoke, P.W.F., Denvir, M.A. European Heart Journal (2013) 34 (suppl 1)

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Effects of pharmacological and genetic manipulation of glucocorticoids during early development of the zebrafish embryo. Wilson, K.S., Matrone, G., Tucker, C.S., Mullins, J.J., Kenyon, C.J., Hadoke, P.W.F., Denvir, M.A. Endocrine Abstracts (2013) 32 P336

Presentations

7th European Zebrafish Meeting, Edinburgh 2011

Corticosteroids and cardiovascular development in the zebrafish embryo **KS Wilson**, G Matrone, CS Tucker, CJ Kenyon, JJ Mullins, PWF Hadoke, MA Denvir

Scottish Society of Experimental Medicine 2011-2013

2013 University of Edinburgh -Can the zebrafish be used as a model of glucocorticoid induced programming? **KS Wilson**, G Matrone, CS Tucker, CJ Kenyon, JJ Mullins, PWF Hadoke, MA Denvir

2012 University of Dundee- Modelling Glucocorticoid mediated programming in the Zebrafish (*Dario rerio*) **KS Wilson**, G Matrone, CS Tucker, CJ Kenyon, JJ Mullins, PWF Hadoke, and MA Denvir

2011 University of Edinburgh- Zebrafish (Danio rerio) as a model for investigation of corticosteroid manipulation during early vertebrate development. **KS Wilson**, G Matrone, CS Tucker, CJ Kenyon, JJ Mullins, PWF Hadoke, MA Denvir.

Scottish Cardiovascular Forum 2012-2013

2013 University of Strathclyde- Glucocorticoids are important for maturation and development of the zebrafish embryo cardiovascular system. **KS Wilson**, G Matrone, CS Tucker, CJ Kenyon, JJ Mullins, PWF Hadoke, MA Denvir (**1st place poster prize**).

2012 University of Dundee-The role of glucocorticoids in zebrafish cardiovascular structure and function **KS Wilson**, G Matrone, CS Tucker, CJ Kenyon, JJ Mullins, PWF Hadoke, MA Denvir

BHF student symposium, Kings College, London 2011

Zebrafish (*Danio rerio*) as a model for investigation of corticosteroid manipulation during early vertebrate development. **KS Wilson**, G Matrone, CS Tucker, CJ Kenyon, JJ Mullins, PWF Hadoke, MA Denvir.

BHF student symposium, Oxford University 2013

Glucocorticoids are important for maturation and development of the zebrafish embryo cardiovascular system. **KS Wilson**, G Matrone, CS Tucker, CJ Kenyon, JJ Mullins, PWF Hadoke, MA Denvir

UoE/BHF Centre for Cardiovascular Science symposium 2010-2013

2010 11β-hydroxylase activity in the zebrafish embryo **KS Wilson**, CS Tucker, CJ Kenyon, PWF Hadoke, MA Denvir, 3rd **place poster prize**

2011 The zebrafish glucocorticoid system is important for normal embryo development **KS Wilson**, CS Tucker, CJ Kenyon, PWF Hadoke, MA Denvir, 2nd **place poster prize**

2012 The role of glucocorticoids in zebrafish cardiovascular structure and function **KS Wilson**, G Matrone, CS Tucker, CJ Kenyon, JJ Mullins, PWF Hadoke, MA Denvir, **2nd place poster prize**

List of figures

List of tables

List of equations

Abbreviations

(-/-)	global knockout
α	alpha
β	beta
γ	gamma
µ	micro
11βHSD1	11 beta hydroxysteroid dehydrogenase type 1
11βHSD2	11 beta hydroxysteroid dehydrogenase type 2
11β-hydroxylase	11 beta hydroxylase
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
atg-Mo	translational blocking mo
av	atrio-ventricular
BA	bulbus arteriosus
bpm	beats per minute
BSA	bovine serum albumin
CAH	congenital adrenal hyperplasia
CCTV	close circuit television
CMLC2	cardiac myosin light chain two
CRH	corticotrophin-releasing hormone
DAPI	4',6-diamidino-2-phenylindole
DCP	direct electrical current pulse
Dex	dexamethasone (glucocorticoid receptor agonist)
DLAV	dorsal longitundal anastomotic vessel
DNA	deoxyribonucleic acid
Doc	11-deoxycorticosterone
Doxy	11-deoxycortisol
dpa	days post amputation
dpf	days post fertilisation
EC	endothelial cells
ECL	enhanced chemiluminescence
EDA	end diastolic area
EDTA	ethylenediaminetetraacetic acid
EF	ejection fraction
ef1α	elongation factor 1 alpha
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ERK	extracellular-regulated kinase
ESA	end systolic area

EtOH	ethanol
expts	experiments
FLi1	friend leukaemia integration 1 transcription factor
GAPDH	glyceraldehyde 2-phosphate dehydrogenase
GC(s)	glucocorticoid(s)
GFP	green fluorescent protein
GR	glucocorticoid receptor
h	hour
H&E	haematoxylin and eosin
H_2O	water
HPA	hypothalamic-pituitary-adrenal
hpf	hours post fertilisation
HPI	hypothalamic-pituitary-interrenal
HR	heart rate
HRP	horse radish peroxidase
hsp	heat shock protein
in utero	in the womb
in vitro	studies out with organism
in vivo	studies in whole living organism
IP	intraperitoneal
ISV	inter segmental vessels
L	litre
LBD	ligand binding domain
m	milli
M	moles
MAPK	mitogen-activated protein kinase
MC(s)	mineralocorticoid(s)
MC2R	melanocortin 2 receptor
Met	metyrapone
min	minute
mm-Mo	5-mispair mo
mmp	matrix metalloprotease
Mo	antisense morpholino
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NF-kB	nuclear factor kappa-light-chain-enhancer of activated b cells
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween-20
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase

PFA	paraformaldehyde
PG_2	prostacyclin
PR	progesterone receptor
qRT-PCR	quantitative real time polymerase chain reaction
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	rotations per minute
RT-PCR	real-time polymerase chain reaction
RU486	mifepristone (gr and pr antagonist)
SEM	standard error of mean
ss-Mo	splice site specific mo
star	steriodogenic acute regulatory protein
SV	sinus venosus
TALENs	transcriptor activator-like effector nucleases
tg	transgenic
TNFα	tumour necrosis factor alpha
UPL	universal probe library
VEGF	vascular endothelial growth factor
VI	vascularisation index
VSMC	vascular smooth muscle cells
WIK	wild-type
Zfns	zinc finger nucleases

Chapter 1 Introduction

1 Introduction

1.1 Introduction

Mothers at risk of pre-term delivery are often prescribed glucocorticoids (GCs) in order to promote maturation of the lung and reduce infant morbidity and mortality. In their role as mediators of the stress response, GCs may rise as a direct result of maternal stress or indirectly through alterations in maternal diet or environment. Concerns remain that this early exposure of the foetus to high GCs could result in reprogramming of cellular and molecular mechanisms which could in turn lead to increased susceptibility to disease later in life. Current animal models are not ideally suited to assess the developmental impact of both excess and low levels of GC during critical developmental time points. In contrast, the zebrafish embryo allows ready access to this developmental window. This thesis will investigate the suitability of the zebrafish as a model of embryonic GC manipulation and its impact on subsequent structure and function in the adult cardiovascular system.

1.2 Steroid hormones

Steroids are a family of endocrine hormones derived from the same common precursor, cholesterol and thus have closely related structures. There are two main sub-categories; sex hormones (androgens, oestrogens and progestins) and corticosteroids. Steroid biosynthesis or steroidogenesis occurs throughout the body but the main sites are the adrenal cortex, placenta, the testes and ovaries. Although steroidogenesis is often thought of as separate processes with distinct gland dependent roles, e.g. oestrogen and the ovaries, steroid generation is often through common pathways. There are numerous mutual features amongst the different steroid sub-categories with enzymes, reactions and regulatory processes often common (Miller & Auchus, 2011) with termination reactions being the obvious difference (Davies & MacKenzie, 2003). Thus alteration in the expression of these features may ultimately alter the levels of many different types of steroid hormone. The work presented here will focus on the corticosteroid sub-family GCs.

1.2.1 Corticosteroids

Corticosteroid hormones are a family comprising two subcategories, GCs and mineralocorticoids (MCs) (Payne & Hales, 2004). In mammals, corticosteroids play important roles in response to stressful stimuli such as trauma, starvation, sepsis (Nakano *et al*, 1987) and tissue ischemia (Sapolsky *et al*, 2000; Sellevold & Jynge, 1988) and are also involved in a range of physiological activities. MCs (for example aldosterone) regulate sodium and potassium levels in extracellular fluids (Hu *et al*, 2001), thus making an important contribution to regulation of electrolyte balance and blood pressure (Davies & MacKenzie, 2003; Sanderson, 2006). GCs, such as cortisol, are important for neural processes, carbohydrate, protein and fat metabolism as well as inflammation regulation and other immune responses (Moritz *et al*, 2005). Although both corticosteroid families play important roles, the focus for this work will be GCs as GC exposure during development has been shown to influence later adult health (Seckl & Meaney, 2004).

1.3 Glucocorticoid hormones

1.3.1 Glucocorticoid function

Normal mammalian GC physiology can be broadly categorised into three main roles, (1) maintenance, with respect to metabolic, autonomic, psychological, haemostatic and cardiovascular functions (2) suppressive, by keeping in check pathways involved in cellular proliferation, differentiation, tissue maturation and death, and infection or inflammation mechanisms; and (3) partitioning of body components to maximise efficiency, through processes such as proteolysis and insulin resistance in muscle and increased insulin sensitivity and lipidogenesis in fat depots (Chapman & Seckl, 2008; Fowden & Forhead, 2004; Girod & Brotman, 2004; Moritz *et al*, 2005).

GCs are administered therapeutically to regain/replace physiological concentrations in deficiencies and, at higher doses, are frequently prescribed for their anti-inflammatory or immunosuppressive roles in the treatment of such conditions as asthma, rheumatoid arthritis (Bello & Garett, 1999), transplant rejection and lympho-proliferative disease (Almawi *et al*, 2002). Therapeutic GCs are one of the most commonly prescribed drugs worldwide (Bello & Garett, 1999).

1.3.2 Glucocorticoid biosynthesis

GCs are produced endogenously under the control of the neuroendocrine system, the hypothalamic pituitary adrenal (HPA) axis (Walker, 2007). Briefly, upon stimulation by stressor (immune, physical, metabolic, emotional) or circadian (a diurnal pattern with evening troughs and early morning peaks) cues, the hypothalamus releases corticotrophin-releasing hormone (CRH) stimulating the pituitary to produce and release adrenocorticotrophic hormone (ACTH). ACTH binds to the melanocortin 2 receptor (MC2R) on the surface of the adrenal cortex activating signal transduction pathways and resulting in GC biosynthesis in the zona fasciculata region of the adrenal cortex (Sanderson, 2006). Steroidogenesis from cholesterol is through a series of mostly hydroxylation reactions catalysed by various members of the cytochrome P450 enzyme family. The main features will briefly be highlighted. Cholesterol is transported into the mitochondria, via steroidogenic acute regulatory protein (StAR), where side-chain cleavage of cholesterol is catalysed by monooxygenase P450scc (Cyp11a1). Afterwards, 17α-hydroxylation (Cyp17), 3β-hydroxysteroid dehydrogenation (Hsd3b), and 21-hydroxylation (Cyp21a1) occur prior to the last step in cortisol biosynthesis, which is 11-hydroxylation mediated by 11β-hydroxylase (Cyp11b1) (Tokarz *et al*, 2013b). The structural organisation and some key components of the HPA axis are highlighted in Figure 1.1.

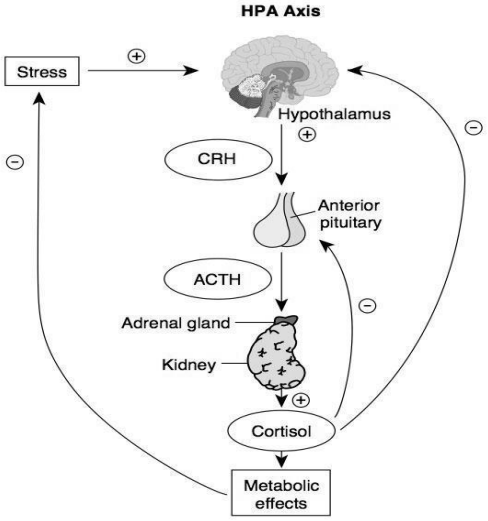

Figure 1.1 Hypothalamic-pituitary-adrenal (HPA) axis.
A schematic highlighting the key structures of the mammalian hypothalamic pituitary adrenal (HPA) axis along with the main signal transduction features in the production of the glucocorticoid hormone cortisol in response to stress or stimulus. Figure reproduced with consent from Hiller-Sturmhöfel S, Bartke A (1998) The endocrine system: an overview. *Alcohol health and research world* **22**: 153-164.

1.3.3 Glucocorticoid action

GCs predominately produce their central and peripheral effects through activation of the relative low affinity but ubiquitously expressed GC receptors (GR). In some instances however actions are mediated through the higher affinity MC receptors (MR) which are found less ubiquitously expressed in a tissue specific manner (Chapman & Seckl, 2008; Walker, 2007). While this may imply that GC action is regulated by the receptor expression in particular tissue types it should be noted, that GC action in a particular tissue is not governed solely by the level of receptor expression but is also governed by ligand availability. As the circulating level of the main GC cortisol (corticosterone in rodents) generally outweighs that of the main mammalian MC, aldosterone, it may be perceived that cortisol always occupies MR, if this ligand is present in MR rich tissue. To prevent this unnecessary activation of MR in most tissues these receptors are co-expressed with 11β hydroxysteroid dehydrogenase type 2 (11βHSD2) which acts as a barrier to MR activation by GCs by converting them into their inactive form (this will be discussed in more detail later).

1.3.4 Glucocorticoid receptor

As mentioned above, many of the effects mediated by GCs are via the GR. The human form of this receptor is a protein member of the superfamily of ligand regulated nuclear receptors, known as the nuclear receptor 3 subfamily (Hollenberg *et al*, 1985). Cloning of human GR in the 1980s led to the discovery of two functional isoforms referred to as alpha (α) and beta (β). These isoforms have identical N-terminal regions (encoded by exons 2-8) and differing C-terminal regions (encoded by exons 9α and 9β respectively). The α-isoform is 777 amino acids in length, is expressed in virtually all cell types, and is responsible for mediating most GC effects. The shorter β-isoform is 742 amino acid residues in length and only accounts for less than 1% of GR found in the body. While the β-isoform is present in the cytoplasm like the α-isoform it is incapable of hormone binding due to alterations in the ligand binding domain (LBD); a proposed role for GR-β is in inhibitory action on GR-α but this is un-confirmed. Further sub-types of GR (mainly GR-α subtypes) have been discovered more recently, the purpose of which is hypothesised to confer cellular heterogeneity. As the physiological and pharmacological actions of GC are

primarily mediated through the activation of the GR-α LBD, GR herein will refer to all functional α-isoforms.

GR are expressed in almost every cell in the body, where activation by GCs can have pleiotropic effects. The unbound form of the receptor is present within the cytoplasm in an inactive oligomeric complex with some regulatory proteins such as the Heat Shock Protein (HSP) 90 and 70 KD forms, the p59 immunophilin, FKBP52 and the small p23 phosphoprotein (Davies *et al*, 2005). The presence of these compounds is thought to maintain the C-terminal domain in a favourable conformation for ligand binding. As GCs are lipid soluble they are able to diffuse easily through the plasma membrane and bind to the specific, high-affinity GR within the cytoplasm. The resulting complex, the GR-GC complex, releases the GR from its interactions with the inhibitory complex, resulting in a conformational change, unmasking of the receptor nuclear localization signal (Figure 1.2). Upon activation, GR-GC translocate to the nucleus and binds as a dimer to DNA through its central domain. GR can interact either with DNA by targeting specific nucleotide palindromic sequences termed Glucocorticoid Response Elements (GRE) or Negative GRE (nGRE) (Ruegg *et al*, 2004). Depending on the nature of the GRE, the overall process of GR binding can result in activation or repression of genes which contain these GR-binding sites (Davies *et al*, 2005).

Receptor activation can, therefore, alter gene expression in target tissues through the processes of transactivation and transrepression (Spokoini *et al*, 2010). GCs have also been shown to exert a number of rapid actions which are independent of gene transcription, such as the production of endothelial nitric oxide (eNO) by eNO synthase (eNOS) (Hafezi-Moghadam *et al*, 2002; Lowenberg *et al*, 2006)**.**

As for many ligand-regulated nuclear receptors, transgenic mice have allowed the specific roles of GR to be investigated. Homozygous disruption of GR (GR (-/-) highlights the multiple roles of this receptor as knockdown results in reduction of gluconeogenic enzymes and amino acid catabolising gene expression and infant mortality shortly after birth (Cole *et al*, 1995). Furthermore dysregulation of the HPA axis components, such as raised ACTH, has also been observed (Newton, 2000) suggesting GR-mediated effects on the HPA axis.

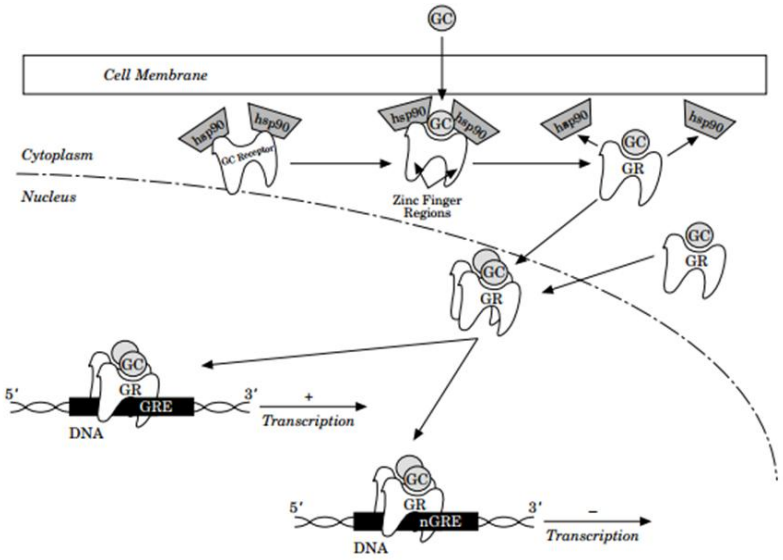

Figure 1.2 Glucocorticoid receptor (GR) interactions with DNA.
The inactive GR is found in the cellular cytoplasm and is bound to heat shock proteins (hsps) such as the hsp90 shown here. Association with hsps prevents the movement of the GR into the cell nucleus. Once glucocorticoid (GC) crosses the cell membrane and binds to the GR, the hsps then disassociate allowing the GR-GC complex to translocate from the cytoplasm into the nucleus where it undergoes dimerization. The zinc finger domains of the GR are then free to bind specific DNA sequences, the Glucocorticoid Response Elements (GRE), found within the 5′ promoter region of the target gene. GR are able to activate gene transcription, or repress transcription if the region contains a negative GRE (nGRE). This figure was reproduced with consent from "Umland SP, Schleimer RP, Johnston SL (2002) Review of the molecular and cellular mechanisms of action of glucocorticoids for use in asthma. *Pulmonary Pharmacology and Therapeutics* **15**: 35-50.

1.3.5 Glucocorticoid regulation

As in the case of many other endocrine hormones, GC biosynthesis is controlled by a negative feedback inhibition loop (Figure 1.1). This is a GR-mediated method of regulation is where the final product of the cascade, cortisol in humans, corticosterone in rodents, feeds back on both the hypothalamus and pituitary gland suppressing ACTH stimulation of the adrenal cortex and, thus, inhibiting the release of hormones in the signalling cascade.

Another way of keeping in check GR activity is through local pre-receptor metabolism by the 11βHSD isoenzymes (Nagalski & Kiersztan, 2010) which regulate tissue GC exposure through alterations in enzyme activity. This family catalyses the inter-conversion of cortisol and corticosterone (in rodents) with their inert 11-keto forms (cortisone and 11-dehydrocorticosterone respectively). There are two isozymes of 11βHSD. Type 2 (11βHSD2) is a dehydrogenase which is

responsible for the conversion of corticosterone or cortisol to their biologically inactive 11-keto derivatives. This has a key role in preventing GC from binding to the non-selective MR in MC target tissues. In contrast, the type 1 isozyme (11βHSD1) functions as an 11β-reductase. It is responsible for local regeneration of active GCs from circulating inert 11-keto forms in specific tissues (eg liver, lung and adipose tissue). These tissues have been shown to express GR but not MR, suggesting that 11β-HSD1 is important for amplifying GC-dependent activation of GR in target tissues (Klusonova *et al*, 2009; Kotelevtsev *et al*, 1997).

The importance of these enzymes in GC regulation has been described elsewhere (Ferrari, 2010; Morton, 2010; Seckl *et al*, 2004). However, some observations will be summarised here. Human 11βHSD2 gene mutations result in apparent MC excess, which is characterized by activation of renal MR by GC resulting in sodium retention, severe hypertension, and hypokalaemia (Wilson *et al*, 1995). Furthermore deregulated HPA axis activity has been observed in 11βHSD2 -/- mice (Kotelevtsev *et al*, 1997). Exposure of mice to raised levels of 11-dehydrocorticosterone, the substrate for 11βHSD1, has been shown to result in increased circulating GCs, and down-regulation of HPA activity axis. As the 11βHSD1 enzyme is highly expressed in metabolically active tissues (such as liver and adipose tissue), impairment of local GC regeneration in 11βHSD1 -/- mice results in fat accumulation and hepatic insulin resistance (Harno *et al*, 2013).

1.3.6 Glucocorticoid dysregulation

Although short-term elevations in GC levels can be beneficial (for survival in severely stressful conditions or when administered as therapy for inflammatory or malignant disease) chronic elevation of GCs is detrimental (Chapman & Seckl, 2008; Stumpf, 1990). Constant activation of the HPA axis, whether this is as a result of defective control mechanisms/biosynthesis pathways or pharmacotherapy, can threaten the dynamic equilibrium and unless allostasis (adaptive changes) occurs, system defects such as adrenal hyperplasia can result and if left untreated survival can be impaired (O'Connor *et al*, 2000).

Cushing's disease is a well-documented condition of prolonged high circulating cortisol levels, commonly as a result of a pituitary adenoma (pituitary tumour) and/or uncontrolled ACTH production (Stewart & Petersenn, 2009). This condition is characterised by increased cardiovascular disease risk factors, including elevated systolic blood pressure, central obesity (Girod & Brotman, 2004; Ng & Celermajer, 2004), dyslipidaemia, insulin resistance (Trayhurn & Beattie, 2001) and hyperglycaemia (Stewart & Petersenn, 2009). Prior to advanced medical treatment it was not uncommon for those suffering from Cushing's disease to die prematurely of myocardial infarction or stroke (Colao *et al*, 1999). GR genetic variants, such as the common haplotype 3, have been associated with Cushing-like symptoms such as hypertension, visceral obesity and hyperinsulinaemia. This genetically determined cortisol sensitivity is involved in the pathogenesis of cardiovascular disease (van den Akker *et al*, 2008). Many of the cardiovascular risk factors associated with raised GC levels are summarised in Figure 1.3.

In contrast people with chronic GC insufficiency in a group of autosomal recessive disorders characterised by defective steroidogenesis enzymes, referred to as congenital adrenal hyperplasia (CAH) (Mullins *et al*, 2009), or with Addison's disease, (an insufficient production of adrenal steroids) exhibit chronically low blood pressure, hypoglycaemia, fatigue and weight loss.

It is not uncommon for those receiving GC pharmacologically to experience a number of side-effects, including osteoporosis, delayed wound healing, glaucoma, diabetes, vasculitis, ulceration, pancreatitis and increased risk of infection (Schacke *et al*, 2002) and increased cardiovascular risk (Souverein *et al*, 2004). Many side-effects of GC therapy can be attributed to the non-selective nature of these compounds with the treatment of particular conditions leading to disruption of healthy anabolic processes. In some cases at extreme doses, MC side effects, such as water retention, may also be observed; however, generally this would only occur along with impaired 11βHSD2 activity (see glucocorticoid regulation 1.3.5).

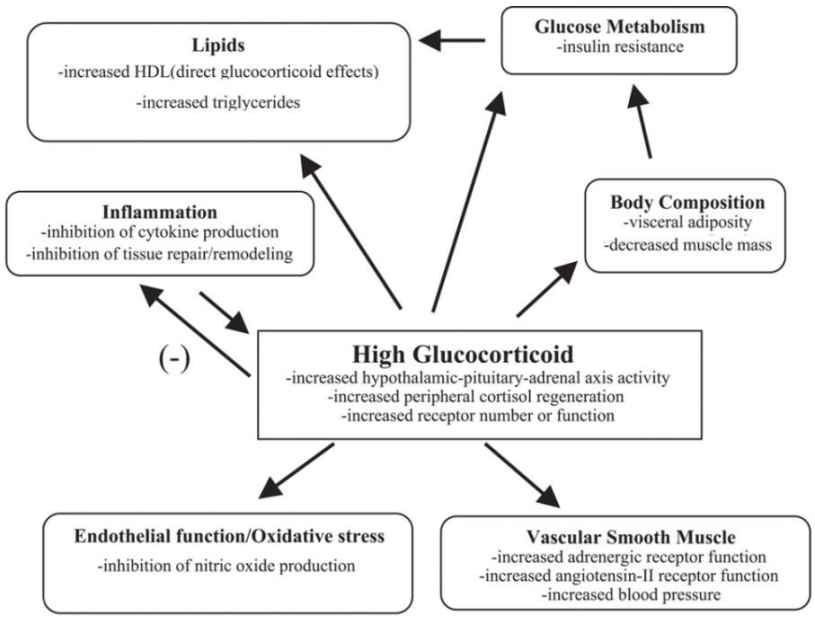

Figure 1.3 Impact of raised glucocorticoid levels on cardiovascular risk factors.
The above schematic summarises some of the risk factors associated with chronically raised glucocorticoid (GC) levels. Features highlighted are those which are known cardiovascular risk factors. This Figure has been adapted from Girod JP, Brotman DJ (2004) Does altered glucocorticoid homeostasis increase cardiovascular risk? *Cardiovascular Research* **64**: 217–226.

1.4 Glucocorticoids and the stress response

Stress is an evolutionary conserved mechanism which enables fast carefully orchestrated physiological responses to cope with life-threatening dangers such as predation, trauma, noxious stimuli or environmental stressors. The perception of stress in vertebrates is through the hypothalamus and subsequent activation of the sympathetic nervous system. GCs play an important role in this stress response along with catecholamine production. Catecholamines such as epinephrine initiate the rapid fight or flight response to stress, while GC produce a more moderate slower physiological response to sustained or prolonged stressor. The roles of GCs in the stress response include an increase in the mobilisation of energy from storage locations throughout the body into the bloodstream (for mitochondrial energy requirements), an increase of cardiovascular tone (increasing cardiovascular efficiency) and a delay in the non-essential processes within the body such as feeding, digestion, growth, and reproduction. Stress induced GC production also enhances the body's immune system to allow better protection from harmful

pathogens, thus reducing the risk of infection (Sapolsky *et al*, 2000). When the stressor has been removed the negative action of GC feeds back on the HPA axis to prevent chronic pathological activation (Sapolsky *et al*, 2000) (Figure 1.1). Further post-stressor roles of GC include returning energy homeostasis through replenishment of energy stores. As mentioned previously chronic unregulated stress response can have serious pathophysiological effects.

1.5 Glucocorticoids and the cardiovascular system

During the stress response short-term alterations in GC levels act to maintain vascular tone and modify cardiovascular inflammatory, proliferative and remodelling responses to injury (Walker, 2007). As highlighted in section 1.3.5 instances of GC dysregulation have been associated with numerous direct and indirect actions on the cardiovascular system (Stumpf, 1990) with prolonged effects maladaptive, resulting in severe metabolic and cardiovascular consequences (summarised in Figure 1.3).

The pathology of conditions such as Cushing's disease, Addison's disease and CAH further highlight the role of GC the cardiovascular system. Complete cardiovascular collapse and death can occur with the absence or very low levels of adrenal steroid biosynthesis (Grunfeld & Eloy, 1987; Hammer & Stewart, 2006) and in instances of chronic steroid biosynthesis (Cushing's disease) premature atherosclerosis, hypertension, cardiac myopathy, reduced ejection fraction, congestive heart failure and ischaemic heart disease have been documented (De Leo *et al*, 2010). Chronic therapeutic use of GCs is also known to result in several adverse cardiovascular complications including hypertension, atherosclerosis, and heart failure (Grunfeld & Eloy, 1987; Saruta, 1996). Although there is strong evidence to allow us to describe an association between GCs and the cardiovascular system, little is known on specific GC (exogenous and/or endogenous)-mediated cardiovascular responses and their mechanisms (Sainte-Marie *et al*, 2007) and whether the observations are a direct GC mediated effect on the cardiovascular tissues or indirect effect, such as alteration of systemic risk factors.

1.5.1 Glucocorticoid signalling on the cardiovascular system

The possibility of direct actions on the cardiovascular system (blood vessels and the heart itself) has been suggested through the finding of widespread expression of both MR and GR in on the myocardium (Katz *et al*, 1988), blood vessel walls (Christy *et al*, 2003), cultured vascular smooth muscle cells (VSMC) (Scott *et al*, 1987) and isolated endothelial cells (EC) (Yang & Zhang, 2004). Further support is provided by evidence indicating that manipulation of GCs results in altered cardiac and vascular cell structure and function. Altered cardiac morphology and function (in the form of decreased heart rate and cardiac output (Roy *et al*, 2009)) has been observed in rats following exposure to GC, with vascular collapse observed in the complete absence of adrenal GC steroidogenesis (Hammer & Stewart, 2006).

Indirect actions of GCs on the cardiovascular system may be due to interaction with other organ systems such as the liver, kidney, central nervous system and adipose tissue (Ferrari, 2003). It is thought that cardiovascular dysfunction then results as a consequence of increased cardiovascular risk factors such as insulin resistance, hyperglycaemia, hypertension (in a dose-dependent fashion), dyslipidaemia, cholesterolaemia, and central obesity (Girod & Brotman, 2004; Ng & Celermajer, 2004; Trayhurn & Beattie, 2001). These risk factors are often associated with Cushing's disease supporting an indirect role of GC (Figure 1.3 highlights these risk factors).

1.5.2 Glucocorticoids and the vasculature- human and animal studies

GC have been shown to be essential in the modulation of peripheral vascular tone, with raised blood pressure observed in dogs and rats treated with non-physiological concentrations of the GC deoxycorticosterone (Ullian, 1999). Clinical observations of Cushing's and Addison's sufferers or those who have side-effects from therapeutic application also highlight a link between vascular tone and GC exposure, with hypertension observed in instances of raised GC. It remains unclear as to whether alterations observed in the vasculature are as a result of direct effects on the vascular wall or indirectly such as alteration of systemic risk factors.

1.5.2.1 Glucocorticoids and vascular endothelial function

GR has been detected in both VSMC and EC, and GC have been shown regulate vascular reactivity on both cell types (Yang & Zhang, 2004). The importance of GR in endothelial function was demonstrated in vascular-endothelium-specific GR knockout mice where animals were found to be resistant to dexamethasone (Dex-a synthetic GC/GR selective agonist)-induced hypertension and showed a decreased contractile response to Dex (Goodwin *et al*, 2011).

In humans, endothelial cell dysfunction has been noted following GC administration and is thought to contribute to the increased incidence of stroke and heart attack observed in this setting (Ullian, 1999). Endothelial cell dysfunction is associated with impaired nitric oxide (NO) production, perturbed interactions between platelets, leukocytes and the vessel wall, and alterations in thrombosis and thrombolysis (Girod & Brotman, 2004). While it has been shown that endothelial cell dysfunction can be caused by hyperglycaemia, hypertension and dyslipidaemia, all of which are well-known effects of chronic GC exposure (Jensen-Urstad *et al*, 1997), direct actions on the vasculature have been shown when cortisol administered to healthy normotensive individuals was found to impair cholinergic vasodilation (Mangos *et al*, 2000). Furthermore organ bath assessments have also shown to GC suppress the synthesis of potent vasodilators such as prostaglandin 2 (PG_2) and NO in healthy tissue.

NO is a potent endothelium derived vasodilator; imbalance of this compound has been associated with many cardiovascular disorders. GC overexposure has been shown to reduce NO levels, which may play a key role in GC mediated hypertension observed in Cushing's for example. A number of possible mechanisms by which GC inhibit NO synthesis (mainly by interactions with endothelial nitric oxide synthase (eNOS)) and have been reviewed comprehensively elsewhere (Ullian, 1999) these include 1) reduction in transcription of the gene encoding eNOS, 2) increased rate of eNOS messenger ribonucleic acid (mRNA) degradation, 3) reduced stability of the eNOS protein stability, 4) impaired agonist induced calcium mobilisation from intracellular stores (eNOS requires the binding of a calmodulin/calcium complex)

and finally 5) a reduction in the availability of eNOS cofactor (GC are thought to reduce the synthesis of tetrahydrobiopterin an important cofactor of eNOS).

Rodent studies have documented a decrease in the levels of the inflammatory mediator PG_2 following Dex induced hypertension. Further studies using isolated endothelial cells confirm the role of GR in GC mediated inhibition of PG_2 by demonstrating the ability of GR antagonists to prevent the GC induced effects (Ullian, 1999). While it unclear how GC inhibit the synthesis of PG_2, GC have been shown to suppress the transcription of 2 cyclooxygenase (COX-2) in bovine arterial endothelial cells suggesting a possible mechanism for the suppression of PG_2 by GC (Inoue *et al*, 1999). It has also been suggested that a decrease in PG_2 is as a result of inhibition of phospholipase A2 and arachidonic acid (Lewis *et al*, 1986). The regulation of peripheral vascular tone is balance between the effect of vasoconstrictors and vasodilators, as highlighted above vasodilation is regulated by GC. It has also been shown that GC can influence vascular resistance by regulation of vasoconstriction too.

1.5.2.2 Non-endothelial effects of glucocorticoids

GC administration to healthy adults increases the vessel total peripheral resistance (Pirpiris *et al*, 1992). It may be that GC modulate vascular tone by endothelium-independent mechanisms such as the enhancement of response to vasoconstrictor hormones such as norepinephrine, angiotensin II, arginine vasopressin, endothelin I and thromboxanes (Girod & Brotman, 2004; Ullian, 1999) an example of this using *ex vivo* rabbit aortic strips showed increased contractile sensitivity to norepinephrine vasoconstriction when co-treated with corticosterone (Fowler & Chou, 1961). Further support for a non-endothelial effect was observed in rat VSMC where GC exposure was found to up-regulate the expression of alpha-1 adrenergic and angiotensin II receptors and display a potentiation of the vasoconstrictive actions of these factors (Ullian, 1999).

It has also been documented that GC increase the expression of ACE and the production of angiotensinogen (Aubert *et al*, 1997) and that GC alter vascular tone by activation of the renin-angiotensin system (Suzuki *et al*, 1982). The influence of

GC on VSMC hypertrophy has been investigated; however the data thus far suggest that this is not the causative mechanism of altered vascular tone.

1.5.2.3 Glucocorticoids and angiogenesis

Although high blood pressure is a common feature of Cushing's disease, vascular remodelling may develop independently of raised blood pressure. As alluded to earlier GC are important for proliferative and remodelling responses to injury (Walker, 2007) with sufferers of Cushing's disease often displaying vascular contusion (bruising to artery wall), impaired wound healing atherosclerosis and vascular remodelling (Rizzoni *et al*, 2009). GC, through the action of GR, have been shown to alter vascular remodelling by inhibiting endothelial cell tube formation (Logie *et al*, 2010) and the migration and proliferation of human (Goncharova *et al*, 2003) and animal (Pross *et al*, 2002) VSMC in culture.

The ability of GCs to inhibit the process of angiogenesis is well-documented, with cortisol often used as a positive control substance (inhibitor) in many investigations of angiogenesis. GC suppression of angiogenesis is thought to contribute to impaired wound healing in Cushing's disease, furthermore cutaneous wound healing and myocardial infarction recovery have been shown to be inhibited by local generation of GC by 11βHSD1 (Small *et al*, 2005). There is also an increasing body of evidence which suggests that endogenous GC contribute to regulation of new vessel formation (Hadoke *et al* 2009).

A great deal of interest has focused on the anti-angiogenic properties of GC, with the suggestion of possible therapeutic applications. GC are used therapeutically in the inhibition of angiogenesis in capillary haemangioma and certain cancers (Yano *et al*, 2006a; Yano *et al*, 2006b) and also in the inhibition of neointimal proliferation following intra-vascular injury (Hadoke *et al*, 2006).

While it is clear that GC have a role in angiogenesis it remains unclear as to the mechanisms by which GCs inhibit blood vessel formation. It has been suggested that GC-mediated anti-angiogenesis activity is linked to their anti-inflammatory roles but in cultured human cells, endothelial cell tube formation is inhibited even in the absence of an immune or anti-inflammatory response (Logie *et al*, 2010). Exposure

of GC to cultured human cells was found to impair cell to cell contacts and morphology rather than impairing the proliferative, migratory or viability of the cells (Logie *et al*, 2010). It, therefore, may be that GCs provide their anti-angiogenic action through alterations in the cytoskeletal structure. While this suggests a possible mechanism for GC reduction in angiogenesis it is unlikely that this is the only mechanism involved as GC administration has also been shown to decrease the expression of crucial regulatory component of angiogenesis (Logie *et al*, 2010).

As mentioned previously GC administration impairs the activity of PG_2 which is also important in angiogenesis. It has been suggested that GC inhibit angiogenesis by inhibiting production of PG_2, as GC have been shown to decrease endothelial cell PG_2 this may be possible. Vascular endothelial growth factor (VEGF), another key mediator of angiogenesis, has been reduced in several cell types (Koedam *et al*, 2002) including VSMC following exposure to GCs. VEGF, has a number of specialised effects on vascular EC such as the increase of proliferation, migration and vascular permeability (Iwai *et al*, 2004). VEGF production and activity increases during hypoxia (Tintu *et al*, 2009) or instances where increased blood flow is desirable (e.g. the placenta), and has been shown to be prevented by GC exposure in those instances (Hewitt *et al*, 2006).

GC have been shown to play an important role in the vascular response to injury *in vivo* (Fishel *et al*, 1995). The mechanical removal of vascular occlusion can result in fibroproliferative vascular response (restenosis). The use of GC in the prevention of neointimal lesion formation or restenosis was suggested in part due to their antiproliferative and anti-inflammatory properties. GC administration has been shown to successfully reduce post angioplasty neointimal proliferation in rats, rabbits, and dogs, and with mixed success in humans (Hadoke *et al* 2009: Hadoke *et al* 2013). It is unclear mechanistically how GCs prevent restenosis. However, it may be due to suppression of pro-inflammatory pathways such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway, the reduced production of inflammatory cytokines, or tumour necrosis factor alpha (TNF-α) release (Pesarini *et al*, 2010). GCs have also been shown to reduce matrix metalloprotease (MMP) activity and secretion suggesting that prevention of

neointimal lesion formation may be due to the inhibition of smooth muscle cell migration (Fishel *et al*, 1995).

While the data summarised here may suggest that GCs act as potent anti-inflammatory agents or by the prevention of tissue remodelling inhibit atherogenesis and thus offer therapeutic application it should also be considered that GC excess, such as Cushing's disease, is associated with increased atherosclerosis (Hadoke *et al*, 2013). While it may be that atherosclerosis in these instances is due to increased cardiovascular risk factors such as dyslipidaemia it should also be considered that GC have complex, and often contradictory, influences on cardiovascular disease and cardiovascular risk (Walker, 2007).

1.5.3 Glucocorticoids and the heart

As highlighted above excess levels of GC have been associated with numerous cardiovascular disease risk factors and adverse cardiac events such as left ventricular hypertrophy and cardiac failure. The elucidation of tissue-specific effects of GR action can often be difficult, since the GR overlaps functionally MR and most metabolically active organs, including the heart, express substantial levels of both GR and MR (Tokudome *et al*, 2009).

Much of the role of GC modulation on the heart has been gathered using genetic manipulation techniques of these receptors in rodent models. GR knockdown mice die shortly after birth with multiple organ defects and widespread somatic oedema. This suggest the importance of these receptors in early development but makes investigation of specific roles problematic, often investigation occurs in late gestation however notably the heart is smaller, with abnormal cellular and ultra-structural architecture (Rog-Zielinska *et al*, 2012). Over-expression of GR in mice has been shown to induce atrio-ventricular (AV) block (Sainte-Marie *et al*, 2007). Conditional knock down of the other GC receptor MR has been shown to produce severe heart failure and cardiac fibrosis, which is fully reversible with the addition of MR antisense mRNA (Beggah *et al*, 2002). Conditional over-expression of MR in mice triggers cardiac arrhythmias resulting in excess mortality which can be reduced with the addition of MR antagonist (Ouvrard-Pascaud *et al*, 2005). These GR and MR

manipulation studies highlight novel effects of GR and MR activation in the heart, suggesting that both receptors have direct and specific cardiac effects in rodent models (Wintour, 2006), with important roles in development of this systems (Rog-Zielinska *et al*, 2012). While both GR and MR have been found to be expressed in the heart (Brown *et al* 2005), the influence which these receptors play in cardiac development are poorly characterised, with much of published data thus far focusing on the role of endogenous and exogenous GC on the developed heart.

As with the vasculature, GCs have also been shown to have a direct and short term effect on contractile function in the heart. Dex treatment enhances the development of contractile tension and increases contraction and relaxation velocities in cardiac muscles (Katz *et al*, 1988; Penefsky & Kahn, 1971; Sainte-Marie *et al*, 2007), similar observations were noted following exposure to cortisol (Rossier *et al*, 2008). Further support for a more chronic or longer term GC-mediated role on the myocardium was observed in adrenalectomised rats, thus unable to produce corticosterone, demonstrating decreased cardiac contractile force, which is was rescued upon Dex treatment (Rao & Narayanan, 2000). A number of rodent and cell culture studies have also demonstrated GC-induced cardiac hypertrophy (Ohtani *et al*, 2009). However, cardiac hypertrophy, which develops with MR over-expression, has not been documented in mouse hearts with over-expression of GR, suggesting that cardiac hypertrophy is predominantly MR-mediated. Cardiac MR have been shown to play a key role in cardiac remodelling, with the MR antagonists spironolactone and eplerenone shown to reduce mortality in patients with heart failure and either impaired systolic function (Stevenson, 2006) or diabetes following acute myocardial infarction.

1.5.3.1 Glucocorticoids in heart protection

GCs have also been shown to elicit a cardio-protective effect in the acute setting of myocardial ischemia reperfusion in both animals (Sapolsky & Meaney, 1986; Sapolsky *et al*, 2000; Tokudome *et al*, 2009) and humans (Giugliano *et al*, 2003). In accordance, blockade of the GR has been shown to increase infarct size (Hafezi-Moghadam *et al*, 2002). However, as highlighted previously prolonged exposure to

high systemic levels of GC can be detrimental following myocardial infarction, due to suppression inflammatory processes critical for infarct healing.

1.5.4 Problems with glucocorticoid cardiovascular research

There are substantial weaknesses in this field, since very few experiments have dissected systemic (e.g. actions in liver or kidney) from local (intravascular) effects of either GC or MC. It has also been difficult to recapitulate *in vivo* findings in experiments in isolated vessels or cardiomyocytes *in vitro*. The potential for occupancy of MR as well as GR by cortisol has not always been taken into account in interpretation of results, with the potential effects of aldosterone often ignored. Furthermore the concentrations and time-course of GC exposure has often not reflected normal physiological relevance suggesting that while indicative of the role that GC may play in cardiovascular pathophysiology it is unclear what their activity is in normal physiology.

1.6 Glucocorticoids during development

GCs have also been shown to be critical during development, where they are thought to regulate tissue and organ development, maturation and morphology by promoting cellular differentiation (Fowden & Forhead, 2004). Towards the end of gestation GC levels rise rapidly, preparing the foetus for the extra-uterine environment, resulting in maturational changes in organ systems such as the heart, gut, kidneys and, importantly, stimulating surfactant production by the lung (Cottrell & Seckl, 2009). It is thought these effects result from a shift from cellular proliferation to differentiation events. Furthermore, it has been shown that maturation can be induced prematurely through exogenous GC administration (Ballard, 1979; Ballard *et al*, 1975; Fowden & Forhead, 2004) and it is due to this well-known effect that GCs are often used in the treatment of mothers at risk of preterm delivery to promote infant lung maturation (Bolt *et al* 2001: Seckl & Meaney 2004). The importance of these hormones in organ maturation is highlighted in GR -/- mice which die within min of birth as a result of retarded lung development (Cole *et al*, 1995), and kidney and heart immaturity (Rog-Zielinska *et al*, 2012).

1.6.1 Glucocorticoids in cardiac maturation

Numerous mechanical and hormonal factors, such as GCs, are thought to play important roles in the maturation of the mammalian heart. While the expression of GR and MR in the developing heart is indicative of a role it is difficult to investigate the actions specifically in organ development. However it has been shown in global GR -/- mice that disruption of GR signalling results in immature late gestation hearts with tissue specific cardiomyocyte GR -/- mice displaying impaired contractile function and altered compact myocardium structure (Rog-Zielinska *et al*, 2012). Infants which are born prematurely have an increased risk of cardiovascular disorder, it has been hypothesised by Rog-Zielinska and colleges (Rog-Zielinska *et al*, 2012) that since premature births occur before the surge in GC in late gestation, that organs are immature for the extra-uterine environment and thus disorder risk increases. While this hypothesis may be possible, in humans it is particularly difficult to dissect out the long-term effects of premature birth from the long-term effects of therapeutic GC administration in maturation of pre-term foetus.

1.6.2 Glucocorticoid treatment of pre-term infants

Although GC treatment results in an overall reduction in neonatal death and cerebro-ventricular haemorrhage (Roberts & Dalziel, 2006) in certain circumstances the benefit of accelerated lung maturity may be associated with detrimental consequences of GC excess since GR are found in most tissues (Harris & Seckl, 2011). Short-term side effects include hypertension, hyperglycaemia, infections, intestinal perforations, gastrointestinal bleeding, inhibition of somatic growth and hypertrophic cardiomyopathy (Bolt *et al*, 2001). The putative long-term effect on the foetus of antenatal GC administration remains a point of much debate with uncertainty about the optimal number and timing of doses of GC administered to mothers (Newnham & Moss, 2001). Concerns remain with regard to the longer term consequences which may include inhibition of lung growth and neurodevelopmental abnormalities in later life (Bolt *et al*, 2001; Phillips *et al*, 2005).

Numerous studies both in humans and in animal models have shown that prenatal exposure to GCs can influence environment significantly enough to alter growth and increase the likelihood of cardio-metabolic disease, HPA axis perturbations and

affective disorders in later life (Cottrell & Seckl, 2009; Phillips *et al*, 2005; Seckl & Meaney, 2004). Furthermore other factors of pre-natal programming such as maternal diet, drug use or stress are thought to be mediated by alterations in GC levels. As a result GC administration is the second most common model of *in utero* early-life programming of adult disease after diet restriction (Moritz *et al*, 2005).

1.7 Early life programming

During intrauterine development, every organism has a critical window of organ and system developmental plasticity. This key window provides the animal or organism with the ability to phenotypically alter or adapt in response to environmental conditions to maximise the chances of survival (Gluckman *et al*, 2005; Hochberg *et al*, 2011; Moczek *et al*, 2011). When this change or adaptation is permanent, it is termed a "early-life programming" (referred to herein as programming) change and is associated with long-lasting effects on tissue structure and/or function (Brenseke *et al*, 2013). A number of epidemiological studies have suggested that intrauterine environment influences development and risk of disease in later life (Barker & Osmond, 1988; Barker, 1998; Barker *et al*, 1993; De Blasio *et al*, 2007). This suggests that an adverse environment (De Blasio *et al*, 2007), during sensitive periods or windows of tissue morphological/physiological development and organisation, can result in compensatory or adaptive cellular processes (morphological remodelling by disruption or activation of cellular proliferative or differentiation events (Langley-Evans, 1997) and alteration in gene abundance to name a few), resulting in altered growth and long-term disturbances in the structure and function of tissues, (De Blasio *et al*, 2007; Stocker *et al*, 2005; Wintour *et al*, 2003). This concept of early-life-programming is displayed well in the so called "thrifty phenotype" (Barker *et al*, 1989; Hales & Barker, 1992) where maternal starvation and thus reduction in nutrient and oxygen supply, correlates with adaptive changes in the foetus to maximise metabolic efficiency and reduce growth.

While this adaption ordinarily is beneficial, at least in the short term, for the health and survival of the organism, a "mismatch" has been described where adapted individuals are subsequently exposed to a persistent environmental condition unlike those originally adapted to (Bateson *et al*, 2004; Byrne & Phillips, 2000; Godfrey *et*

al, 2007). The degree of disparity between the plasticity-induced environment and the adult environment can have a profound effect on the risk of developing disorder in later life. Barker *et al* (Barker *et al* 1993) found in the "thrifty phenotype" group that the offspring of starved mothers had an increased incidence of metabolic disorders in later life when the nutritional status was markedly different to that experienced during early development.

A plethora of processes and systems have been highlighted as programmable through alteration of uterine environment with a number of diseases or disorders being attributed to early-life programming such as cardiovascular disorders, neuropsychiatric disorders (Bale *et al*, 2010)**,** obesity (Cottrell & Ozanne, 2008) metabolic syndrome (Wang, 2013) and chronic kidney disease (Kett & Denton, 2011; Vehaskari, 2010) to name a few**.**

1.7.1 Possible mechanisms of cardiovascular early-life programming

A number of mechanisms have been proposed to explain the effects of early-life alterations in adult cardiovascular health, such as epigenetic, morphological, physiological and metabolic changes.

1.7.1.1 Epigenetic modifications

Epigenetic modifications are alterations in gene expression or cellular phenotypes which are as a result of mechanisms other than changes in the underlying DNA sequence (no alterations in nucleotide sequence); occasionally these alterations are found to be heritable. There are three main forms of epigenetic modifications *I)* Histone modifications *ii)* MicroRNAs and *iii)* DNA methylation (Santos & Joles, 2012). Several studies have shown that programming effects lead to alterations in the epigenetic pattern of crucial genes involved in growth, metabolism and cardiovascular development, with experimental rodent models demonstrating that DNA methylation has a role in atherosclerotic and cardiovascular disease progression (Baccarelli *et al*, 2010).

1.7.1.2 Structural and physiological changes

Numerous animal studies have suggested that alterations in morphology and physiology of the cardiovascular and renal systems have been associated with the adverse long-term early-life programming effects (Santos & Joles, 2012).

Direct cardiovascular programming has been associated with altered vasoconstrictor responses with VSMCs displaying increased superoxide production and some arteries (e.g. carotid, mesenteric) showing impaired endothelium-dependent vasodilation. Rat offspring of food-restricted (protein and/or calorific) mothers have aortic and mesenteric artery remodelling and also display reduced levels of elastin in their aorta accompanied with persistent abnormalities in arterial stiffness. Although unclear in these rat models, abnormalities in arterial stiffness in adult humans increases systolic and pulse pressure. This is particularly interesting as hypertension is a common phenotype associated with programming (Alexander, 2006; Ojeda *et al*, 2008). Alterations in composition and structure of extracellular matrix of the vessels has also been observed in rats which has been found to impair angiogenesis and reduce capillary density (Nuyt & Alexander, 2009; Struijker Boudier, 1999).

It has been proposed that adaptations associated with early-life programming can alter mechanisms of cardiac growth, resulting in permanent changes to cardiac structure and function (Wang *et al*, 2012). However, this hypothesis is based on known features of heart growth but little work has been carried out to ascertain whether it is correct. In many mammals, heart growth in early gestation is predominantly due to the proliferation of mono-nucleated cardiomyocytes. As gestation progresses there is a shift from hyperplastic to hypertrophic growth and these mono-nucleated cardiomyocytes differentiate into a bi-nucleated form. Post natal heart growth, therefore, is thought to be a result of hypertrophy of these bi-nucleated cardiomyocytes rather than the process of proliferation (Mollova *et al*, 2013). During development mechanisms are in place to regulate cardiomyocyte proliferation, apoptosis and the timing of terminal differentiation. However, numerous models of early-life programming have suggested a reduction in cellular proliferation and an increase in apoptosis as central adaptive changes in other organ types (Botting *et al*, 2012). If this was to occur in the heart it may result in reduced

cardiomyocyte number, increasing the vulnerability of the heart in situations of increased demand, of particular significance given the importance of cardiac hypertrophy as a cardiovascular mortality and morbidity indicator (Porrello *et al*, 2008)**.** Although the number of studies investigating the cellular basis of programming of cardiovascular disease is limited there is some indication that a reduction in cardiomyocyte number may be a central feature (Porrello *et al*, 2011; Porrello *et al*, 2008).

Although physical/structural alterations in the cardiovascular system itself may be causative in the development of cardiovascular disorder in later life, the physiological changes in other tissues should also be taken into consideration after all the likelihood of developing cardiovascular disease or disorder is increased by the presence of risk factors. With structural and functional alterations in the kidney, pancreas and liver observed in programming models, an increase of cardiovascular risk factors such as hypertension, glucose intolerance and insulin resistance (Santos & Joles, 2012) may occur indirectly.

1.7.2 Contributors to early-life programming

A number of possible *in utero* insults to the foetus have been associated with the concept of programming, including maternal nutrition (both starvation and more recently increased food consumption as a result of the "obesity epidemic"), malnutrition (leading to iron or vitamin deficiency)(Wu *et al*, 2004), narcotic drug use, alcohol consumption (Minnes *et al*, 2011), intrauterine exposure to therapeutic drugs (reviewed by Fowden & Forhead, 2004) to environmental factors such as pollution (Hong *et al*, 2013) and tobacco smoke (Suter *et al*, 2013) or physical and psychological stressors (Davis & Sandman, 2010). A commonly studied and well-documented model of altered environment and disease programming is early-life overexposure to GCs. This concept has been reviewed extensively (Dotsch *et al*, 2012; Khulan & Drake, 2012; Raikkonen *et al*, 2009; Raikkonen *et al*, 2011; Szostak-Wegierek & Szamotulska, 2011) and some of the key features will be described here.

1.8 Glucocorticoid-induced early-life programming

1.8.1 Animal studies

Numerous animal (mostly rodent) models have been used to study GC modulation. There is considerable evidence to suggest that foetuses exposed to increased levels of GCs (Dex or cortisol) in the womb have intrauterine growth retardation (IUGR) as observed both in sheep (Ikegami *et al*, 1997; Jobe *et al*, 1998; Nyirenda *et al*, 1998) and in rats (Langdown & Sugden, 2001; Nyirenda *et al*, 2006; Nyirenda *et al*, 1998). In rats, an increased heart weight/body weight ratio has also been noted; upon closer inspection of these hearts a number of features were observed. These included altered actin and total protein levels, disrupted protein/DNA ratio and larger cardiomyocyte volume, length and width 5 and 7 days after birth. These data are indicative of myocardial hypertrophy (Rademaker & de Vries, 2009). Furthermore these rat offspring also show increased premature mortality compared to control littermates in adulthood which was attributed to end stage cardiac and renal failure. Cardiac failure could be explained as a result of reduced cardiomyocyte proliferation, and thus reduced cardiomyocytes in hearts which could in turn impair normal contractile and functional activity of the heart (Rademaker & de Vries, 2009).

Further studies in rats and sheep have shown increased neonatal systolic blood pressure (Dodic *et al*, 2002; Langley-Evans *et al*, 1996; Oritz *et al*, 2003) following excess intrauterine Dex or cortisol exposure. The alteration in blood pressure has been shown to persist into adulthood in sheep (Dodic *et al*, 2002). However comparable findings were not observed in a similar model in the guinea pig suggesting this may be a species-dependant observation (Rademaker & de Vries, 2009). Findings in animal models have also shown that the time-point of GC treatment may be crucial, with hypertension noted in sheep treated in the first month of gestation but not in those treated in the third (Dodic *et al*, 2002). In contrast, IUGR is most notable when GCs are administered towards the end of gestation, specifically in the third trimester, in rats (Nyirenda *et al*, 1998; Seckl, 2004) although effects are still evident if exposure is continued throughout pregnancy. The mechanisms by which GC can programme adult hypertension are unknown but a variety of explanations have been suggested. These include reduction in nephron number (Oritz *et al*, 2003; Wintour *et al*, 2003), altered response to vasoactive

substances (Derks *et al*, 1997; Nuyt & Alexander, 2009), influence on altered function of the renin-angiotensin system (Dodic *et al*, 2002) and altered post natal HPA axis activity (Marciniak *et al*, 2011). In instances of IUGR not only is vascular dysfunction observed, but altered vascular structure is also noted, such as mesenteric artery and aorta remodelling in rats (Khorram *et al*, 2007a; Khorram *et al*, 2007b)

It is not only cardio-metabolic features which can be programmed by GC exposure. GCs are essential for many aspects of normal brain development; exposure of the brain to raised levels of GC whether through maternal stress or therapeutically has been shown to result in life-long alterations in neuroendocrine function and behaviour. Male rat models of GC overexposure display anxiety-related behaviours and by non-human primate maternal stress studies, where there is an association with attention deficits in offspring (Cottrell *et al* 2009). Animal studies have also shown that GC exposure during development does not only affect behaviour but can also reprogram the function of the HPA axis in the offspring as evident by long-term effects on basal GC levels in the offspring (Seckl *et al* 2004).

1.8.2 Human studies

The effects of GC treatment in humans have been studied with findings suggesting that children of mothers treated antenatally, even with short term Dex, suffer from IUGR (Bloom *et al*, 2001; French *et al*, 1999). A number of human studies looking at IUGR have shown an increased risk of cardiovascular death in adult life, it is unclear whether this is due to programming or confounding health feature of being born with IUGR.

The longer-term, life-time consequences of excess GC exposure during development in the human remain poorly documented. This can be in part attributed to the fact that preterm administration of GC only emerged as a recognised and widely used treatment in the 1980s and so the oldest of these offspring are now in their third or fourth decade. Hypertension, hypertrophic cardiomyopathy and left ventricular outflow tract obstruction have been described in association with Dex treatment in some young neonatal humans (Rademaker & de Vries, 2009). These observations may be due to a direct effect of GCs on the heart, as noradrenergic and autonomic

processes have been found to be altered following GC overexposure in human (Bian *et al*, 1993).

Epidemiological evidence suggests that an adverse environment also programs increased risk of neuroendocrine and cognitive disorders in adulthood. Very little is known about the mechanisms by which GC influence neurodevelopment in the human, however a common behavioural outcome of pregnancies affected by prenatal stress is an increased incidence of attention deficit hyperactivity disorder (O'Connor *et al* 2002). Further features which have been thought to be influenced by GC exposure include cognitive development (impaired language development) and psychoactive disorders such as schizophrenia; however supporting data are limited. Not only is there limited evidence of long-term effects published findings are often contradictory which may be due to difficulties in separating the detrimental effects of GC treatment with the detrimental effects of pre-term birth.

1.9 Developmental programming models

Various models have been used to study the developing foetus and the influence of which subtle alterations during early vertebrate developmental stages may produce on organogenesis and function. Despite this the precise roles of GCs in the embryo and the impact that manipulation during early development remains unclear. A particular gap in our knowledge lies in the actions of GCs in the developing foetus and the importance of GC regulation in normal organ development. It is also unclear as to what the underlying molecular mechanisms linking impaired development and adult disease or disorder are (Alsop & Vijayan, 2008) and whether there are particular windows in embryogenesis where alterations have a more profound and specific effect. In understanding these better it may be possible to intervene early in life of those susceptible to reduce the incidence and severity of the disease or disorder in later life.

1.9.1 Human studies

Whilst developmental programming is a well-established observational theory, there remains a deficit in our knowledge with regard to the underlying cellular and molecular mechanisms occurring in the embryo that lead to this phenomenon.

Human epidemiological studies have provided much of the support for Barker *et al*'s work but numerous caveats and confounding features should be considered.

At a maternal/ level, for example, maternal age, parity, multiple foetuses (twins are generally smaller at birth - Multiple Births in England and Wales for 2011 obtained from the Office of National Statistics: twin average weight 2.5kg vs singletons 3.5kg- and according to Barker's hypothesis should have greater morbidity and mortality. However further complexity in data analysis is shorter gestational length- 37 weeks for twin vs 40 weeks for singletons (thus prematurity may be a factor)) and /placental relationship to name but a few. At a juvenile level, catch-up growth and discrepancies in displaying body weight/mass and body composition can skew data.

At adult level socio-economic factors, lifestyle choices, and environment should all be taken into consideration when interpreting results. Human studies have exclusively used retrospective data which rely on health professionals recording the appropriate information and for the health of the children to be accurately monitored throughout life, in many instances for 80+ years (the mean life expectancy in the UK is 80yrs). Often the initial study cohort decreases with time and cases lost to follow up for various reasons may further confound key findings. Furthermore, there have been marked improvements in sanitation, housing, education and health-care (particularly pre, post and neonatal) in the last 50 years alone, resulting in improved infant survival (especially in those with additional -environmental complications such as preeclampsia for which historically mortality was high) resulting in a trend for increasing life expectancy. A further confounding factor is the potential time-frame for a person to experience adverse health; when does the disease or disorder manifest, and does the increasing life-span of humans alter the risk of "mis-match" and disease? With an aging population it may be that we are not seeing the true extent of programming yet.

In summary, human studies are complicated by a number of variables coupled with challenges of accurate and comprehensive follow up which means that very large cohorts are required to see both cross sectional and longitudinal effects over time. Despite modern data systems and information technology, studies of this scale are increasingly difficult and expensive.

1.9.2 Animal studies

Animal models have been proposed as a means to address the gap in understanding of the cellular and molecular mechanisms underlying programming by allowing accurate measurements, follow-up and documentation of phenotype in a more controlled way. A further advantage of animal models is the relative homogeneity of animal cohorts which reduces the confounding effects of genetic variation, typical of human studies (Drake and Walker 2004). Numerous animal models have been used to explore the cellular and molecular mechanisms of programming and have revealed an association with growth, metabolic status and cardiovascular risk. However, none of these models achieve the gold standard as a clinically-relevant model.

One difficulty is variations between species; for example, mouse models of maternal hyperglycaemia result in normal or small-sized pups whereas human offspring exposed to hyperglycaemia develop macrosomia (larger than normal birth weight) (Brenseke *et al*, 2013). Furthermore, particularly relevant to tissue plasticity programming is that rodent species are altricial with organ maturation largely occurring after birth, thus the influence of postnatal factors may be highly relevant in these rodent models. This may explain why pre- and peri-natal conditions have a more profound effect in human, primate or ovine models of programming (Brenseke *et al*, 2013).

A number of approaches used to monitor development in vertebrate model systems are invasive for both mother and foetus and can be associated with high morbidity, furthermore confounding factors such as activation and induction of the maternal HPA axis, make monitoring GC activity problematic. A further feature of current models which makes investigation and subsequent findings difficult to interpret is the influence of the placental barrier. It is known that the placenta offers a controlling mechanism for perturbed GC levels as it contains both 11βHSD1 and 11βHSD2 regulating the trans-placental transfer of cortisol and cortisone in many mammals (Burton & Waddell, 1999; Waddell *et al*, 2005). The placenta is also susceptible to excess levels of GC, with a reduction in placental weight, accompanied by reduced vascularity and increased apoptosis (Hewitt *et al*, 2006; Waddell *et al*, 2005). Thus, it is hard to determine whether the effects induced by prenatal exposure to GCs

occurred secondary to placental changes, direct effects on the foetus or a combination of both. Although work carried out by Holmes and colleagues does suggest a key role of feto-placental 11βHSD2 in prenatal GC programming (Holmes *et al*, 2006).

1.9.3 The placental influence

The mammalian placenta possesses a number of protective mechanisms which help to prevent potentially harmful compounds from entering the environment. Placental 11βHSD2 expression regulates over-exposure to cortisol by converting large amounts into the inactive metabolite cortisone (Yang, 1997). Suppression of placental 11βHSD2 activity either genetically (heredity disorder) or by maternal liquorice consumption allows greater levels of maternal GCs to cross the placenta, resulting in a physiologically-relevant model replicating Dex treatment (Holmes *et al*, 2006). In humans, a reduction in placental 11βHSD2 correlates with lower birth weight and higher blood pressure in later life (Cottrell & Seckl, 2009). Mice displaying global 11βHSD2 knockout also exhibit reduced birth weight along with heightened anxiety in adulthood (Holmes *et al*, 2006). Furthermore placental 11βHSD2 expression is altered by maternal environment, with maternal starvation or stress resulting in reduced 11βHSD2 expression and activity (Langley-Evans, 1997; Langley-Evans *et al*, 1996; Mairesse *et al*, 2007). In addition, mammalian growth is directly related to placental growth and development, with a number of placental abnormalities (such as decreased placental blood flow, placental abruption or previa, and infection) associated with small-for-gestational-age offspring. Growth is also dependent on adequate placental nutrient transfer. As embryos exposed to GCs *in utero* display IUGR, it is possible that the effects of GCs on development are due to direct effects on the placenta with reduction in placental weight (Korgun *et al*, 2012), altered vascularisation (dilation of the -placental circulation (Clifton *et al*, 2002)), suppression of extracellular matrix (e.g. fibronectin and laminin expression (Yoon *et al*, 1998)) and up-regulation of placental glucose transporters (Korgun *et al*, 2012) all reported.

While it is apparent that the models currently in use have allowed progress in understanding programming, this section highlights the requirement for alternative

and complementary models in which we can further improve our understanding and knowledge by diminishing the effects of confounding factors such as the placenta and *in utero* development; one such model is the zebrafish (Figure 1.4).

1.10 The zebrafish as a research model

There are a number of benefits of the zebrafish as a vertebrate model system for the study of development and organ system dysfunction (Ackermann & Paw, 2003; Dodd *et al*, 2000; Hu *et al*, 2001). These benefits include external fertilisation, embryonic transparency (Glickman & Yelon, 2002) (Figure 1.4) and rapid embryogenesis (Lieschke & Currie, 2007). Contrary to their appearance, zebrafish have many similarities to humans at a molecular and cellular level, with greater genetic similarities than many invertebrates (Veldman & Lin, 2008). Like humans, zebrafish possess immune, digestive, nervous and cardiovascular systems with many of these organs systems analogous to their human counterparts at anatomical and physiological levels.

A number of distinct phenotypes, many which are consistent with human disorders, have been produced in zebrafish (Barrett *et al*, 2006; Veldman & Lin, 2008). In addition, Zebrafish are amenable to generating transgenic lines (Udvadia & Linney, 2003), many with tissue specific expression resulting in fluorescence in various organs and tissue systems, thus allowing enhancement of *in vivo* imaging capabilities (Veldman & Lin, 2008). Antisense morpholino (Mo) (Ackermann & Paw, 2003; Veldman & Lin, 2008) zinc finger nucleases (Zfns) (Meng *et al*, 2008) and transcriptor activator-like effector nucleases (TALENs)(Dahem *et al*, 2013) offer the opportunity to knockdown or modify gene expression.

Another important feature of zebrafish is the ability to regenerate a number of organ systems and structures. For example the complete regeneration of the caudal fin (Mathew *et al*, 2007), heart (Poss *et al*, 2002) and spinal cord have been reported. Ordinarily following injury or damage scar tissue forms in mammalian hearts. In zebrafish, however, up to 20% of the heart ventricle can be removed with complete regeneration within 60 days (Poss *et al*, 2002). Caudal tail fin regeneration has been shown to be inhibited by incubation with a number of pharmacological agents

(Mathew *et al*, 2007). The zebrafish (Figure 1.4) therefore provides a unique model to test the impact of interventions on tissue regeneration pathways.

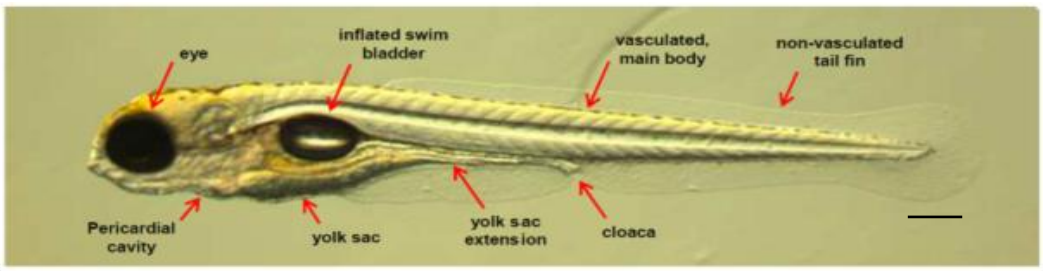

Figure 1.4 Image of a 120 hour post fertilisation zebrafish embryo.
Image is of a wild-type embryo, embedded in agar. At this time point all major organ systems are developed, swim bladder is inflated and embryos are free swimming. Important features are highlighted.

1.11 Zebrafish glucocorticoid system

1.11.1 Teleost steroidogenesis

In teleost fish such as the zebrafish the process of steroidogenesis is very similar to mammalian steroid biosynthesis, with many of the steroids and enzymes present in humans being found in zebrafish (Hsu *et al*, 2009). Like humans the predominant circulating corticosteroid is cortisol. This is produced from the piscine stress axis analogous to the HPA axis, the hypothalamus-pituitary-interrenal (HPI) axis in which cortisol is produced by the inter-renal gland, a homologue to the mammalian adrenal gland (Schoonheim *et al*, 2010; Ziv *et al*, 2013). The inter-renal gland is a cluster of specialised cells embedded within the head kidney, the formation of this gland has been comprehensively reviewed elsewhere (Liu, 2007). The proposed pathway of zebrafish steroidogenesis is highlighted in Figure 1.5. To date most of the highlighted enzymes have been cloned in the adult zebrafish and their expression has been confirmed using polymerase chain reaction (PCR) or *in situ* hybridisation (Tokarz *et al*, 2013a; Tokarz *et al*, 2013b). Although the numerous studies carried out would suggest a well-characterised steroidogenic system, the putative physiological significance of these findings is unclear. Consequently, more work is required to determine functionality.

1.11.2 Zebrafish glucocorticoid receptors

There has been great debate in recent years as to whether teleost fish produce aldosterone, with the current consensus being that they lack the capacity to synthesise aldosterone (Alsop & Vijayan, 2008; Colombe *et al*, 2000; Jiang *et al*, 1998; Strum *et al*, 2005). Despite the absence of aldosterone, zebrafish possess both GR and MR and cortisol has been shown to have both MC and GC activity, controlling both metabolic and electrolyte processes (McGonnell & Fowkes, 2006). This has important roles in seawater and freshwater adaption (Strum *et al*, 2005); however in freshwater fish such as zebrafish the specific roles are unclear. 11-Deoxycorticosterone has also been shown to exhibit strong MR affinity, but there is a lack in evidence to suggest that this is of physiological significance (Lohr & Hammerschmidt, 2011).

The role of steroids in zebrafish is similar to that observed in terrestrial vertebrates with most of the cellular responses associated with cortisol in the zebrafish thought to be facilitated by GR activation. Cortisol has been shown to be involved in the zebrafish for the regulation of metabolism, immune response, osmoregulation, along with reproductive influences and circadian cell-cycle rhythmicity (Tokarz *et al* 2013a). Similarities between the zebrafish and mammalian systems are highlighted in the increase of phosphoenolpyruvate carboxykinase (PEPCK) activity following cortisol administration with inhibition observed in response to the GR (and PR) antagonist RU486 (Aluru & Vijayan, 2009).

Despite the teleost genome wide duplication event (an evolutionary multiplication of some genes), unlike most teleost fish, zebrafish only have one GR type which has a high level of similarity with the human GRα form (Schoonheim *et al*, 2010), with splice variant isoforms being almost identical between the two (Schaff *et al*, 2008). This again supports the zebrafish as an appropriate and relevant model for comparative functional and genomic studies of the GC system (Aluru & Vijayan, 2009).

While the role of steroids in the adult zebrafish is reasonably well-characterised comparatively little is known about the role of steroids such as GC during development. A limited number of studies have been carried out in zebrafish and

other teleost species concerning corticosteroid biosynthesis (Alsop & Vijayan, 2008; Alsop & Vijayan, 2009), and steroid activity during development (Pikulkaew *et al*, 2010). The expression of some steroid biosynthesis genes, such as *cyp11a1* and *hsd3b* has been detected during early stages in zebrafish embryogenesis (Hsu *et al*, 2009). However, the physiological role of steroids in the developing zebrafish are poorly characterised (Pikulkaew *et al*, 2011). Experimental alterations in steroids during development in other species of teleost have been shown to cause distinct changes in offspring, such as growth delay following cortisol incubation (Brooks *et al*, 1997). This suggests that these compounds are biologically active throughout the early embryonic stages (Pikulkaew *et al*, 2010). These data support the role of GCs in embryonic development in the fish.

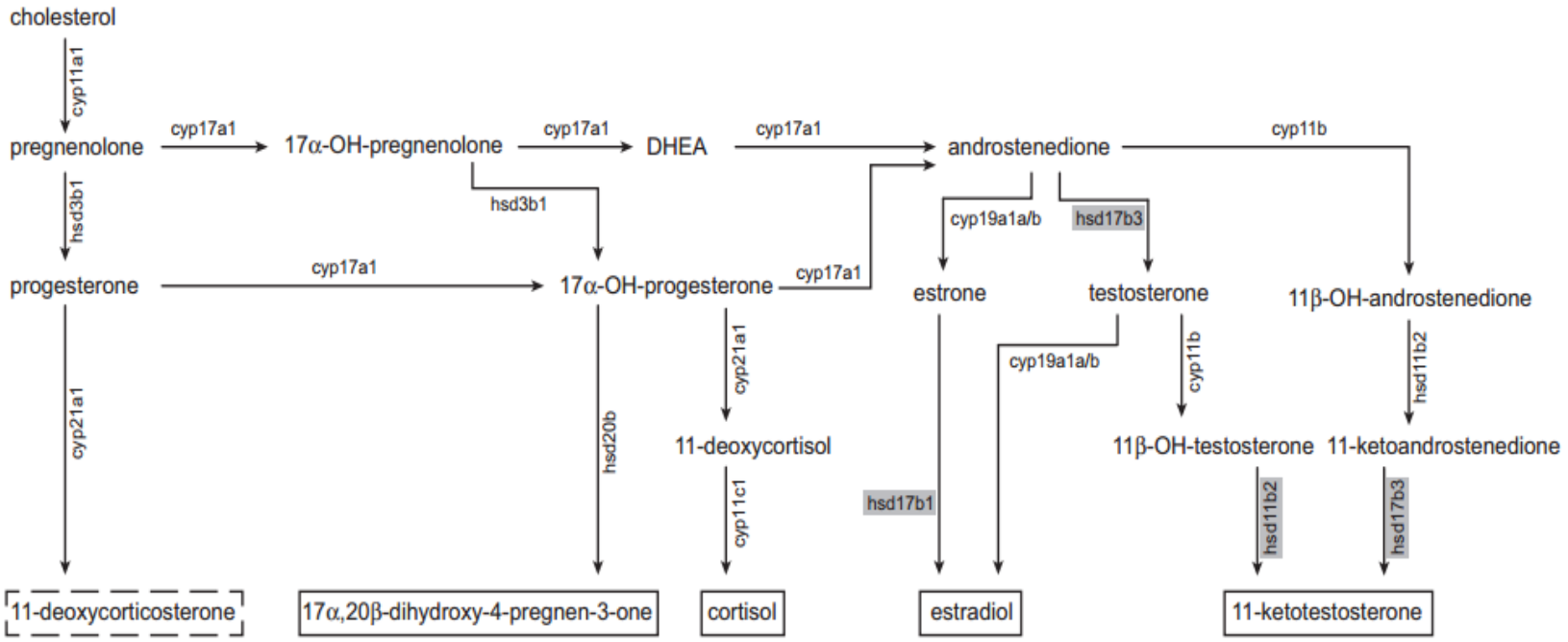

Figure 1.5 The proposed steriodogenic pathway in the zebrafish
Features highlighted are genes encoding suggested enzymes, those marked in grey have confirmed catalytic activity. Major circulating steroids are highlighted by boxes, with the dashed box highlighting a proposed mineralocorticoid receptor (MR) ligand. This figure has been reproduced with consent from Tokarz J, Möller G, Hrabe de Angelis M, Adamski J (2013a) Zebrafish and steroids: What do we know and what do we need to know? *Journal of Steroid Biochemistry and Molecular Biology* **137**: 165-173.

1.12 The zebrafish as a model of cardiovascular development

Zebrafish are now widely used for the study of cardiovascular disorders. Zebrafish do not spontaneously develop cardiovascular disorders analogous to humans (Chico *et al*, 2010) but a number of conditions can be readily modelled and many gene specific transgenic strains have been generated. There are numerous features in the zebrafish which make them a desirable cardiovascular model.

The heart is the first functioning organ to be formed during vertebrate embryogenesis (Stainier, 2001). The morphological processes of heart formation and development have been shown to occur in a similar way in many vertebrate species (Schwerte & Fritsche, 2003; Schwerte & Pelster, 2000). The embryonic heart is best described as a tube like structure, contracting and moving blood in a peristaltic fashion which undergoes morphogenetic movements leading to the formation of a looped, multi-chambered organ (Glickman & Yelon, 2002) consisting of 2 chambers, an atrium where the blood flows into the heart and a ventricle where the blood flows out. A 2 day old zebrafish heart is comparable to a 12 day mouse or 35 day human embryonic heart (Stainier, 2001). The genetic processes which regulate cardiac development in these early vertebrate stages thought to be highly conserved between vertebrates, and so the zebrafish offers an ideal model for early mammalian cardiovascular development (Glickman & Yelon, 2002). Furthermore the adult zebrafish heart is anatomically similar to the embryonic mammalian heart prior to septation (the formation of the septum); unlike in mammalian hearts, however, adult zebrafish cardiomyocytes are mono-nucleated, small in size and retain proliferative capacity (Porrello *et al*, 2011; Poss *et al*, 2002). It has been shown that these mature zebrafish cardiomyocytes can proliferate to help regenerate following injury (Jopling *et al*, 2010) a feature which has also been shown in the early postnatal mammalian ventricle (Poss *et al*, 2002).

In the zebrafish embryonic heart, cardiomyocyte contractions are first detected as uncoordinated irregular contractions in the linear heart tube. However, as formation of the heart progresses cardiac contractility become more coordinated (Bakkers, 2011). In addition to the cellular composition and the similarities in contractile

machinery, the processes which regulate cardiac rhythmicity such as ion flux and thus cardiac contraction being remarkably similar in fish to those observed in mammals (Nguyen *et al*, 2008).

Various cardiovascular parameters can be assessed easily in the developing zebrafish, due to embryonic transparency, allowing *in vivo* observation using bright field microscopy without invasive techniques; it is even possible to track individual erythrocytes using image analysis software (Chico *et al*, 2010). A number of other technologies also allow determination of heart rate, cardiac wall motion amplitude, and systolic/diastolic wall velocity (Denvir *et al*, 2008).

Through the use of endothelial transgenic fish and other technologies it has been possible to increase our knowledge of the zebrafish vascular system, allowing investigation of the mechanisms underlying early embryonic blood vessels formation and the signals and factors which are responsible for arterial and venous differentiation (Glickman & Yelon, 2002). The complex circulatory system found in zebrafish is, in most respects, very similar to that of other vertebrates, including humans–however an added advantage of the zebrafish embryo for cardiovascular research is the non-reliance of a functional cardiovascular system over the first few days of development (Pelster & Burggren, 1996). The zebrafish embryo can survive without circulating blood and only by oxygen passive diffusion for several days allowing the study of dysfunctional hearts (Glickman & Yelon, 2002; Pelster & Burggren, 1996), in contrast to rodent and avian models in where similar mutations would result in mortality.

1.13 Experimental rationale

The data presented here display a clear correlation between environmental alterations and disease progression in later adult life, with cardiovascular disease and alteration in HPA axis function often displayed. It is believed that this alteration in adult phenotype is as a result of adaptive changes at an organ level occurring in the developing foetus. These adaptive changes may not manifest as disease in adulthood unless further confounding features are also displayed, these may be in the form of environmental alterations or further disease risk factors.

Current models of programming do not allow us to understand better whether environmental alterations can have a direct and subtle effect on the system of interest, for example the cardiovascular system. The zebrafish has many desirable features which allow assessment of the organ development, providing a possible model of programming. As GC modulation is one of the most well characterised models of programming, this approach will be used to test the zebrafish as a programming model

1.14 PhD hypothesis

Modulation of embryonic GC function will result in direct effects on the structure and function of the developing cardiovascular system leading to long-lasting structural, functional and molecular changes in the adult.

1.15 PhD aims

- To characterise the GC system in the embryonic and adult zebrafish to confirm the suitability of this species as a model of GC modulation.
- To investigate classical mammalian programmable features (development, stress response and behaviour) in the zebrafish embryo (up to 120 hpf) and adult (up to 120 days post fertilisation (dpf)) after GC manipulation.
- To investigate programmable features of the cardiovascular system in the zebrafish embryo and adult after GC manipulation.

1.16 Experimental plan

The experimental work described in this thesis was designed to determine the suitability of the zebrafish as a programming model, by manipulating the embryonic environment (either by pharmacological or molecular means) during early stages of development (from fertilisation up to 120 hpf) and then assessing the long-term impact of this early-life environmental change in adulthood (120 dpf). At both embryonic and adult phases a number of parameters were assessed such as growth and development, HPI axis function, gene expression, behaviour and cardiovascular structure and function. More detail about specific assays will be given in the relevant experimental chapters.

Chapter 2 Materials and methods

2 Materials and methods

2.1 Materials

Unless stated otherwise all solvents were purchased from BDH (VWR, Leicestershire, UK) and were of analytical reagent grade. All pharmacological agents were purchased from Sigma Aldrich Company Ltd (Dorset, UK).

2.2 Zebrafish maintenance

Zebrafish of the wild type (WIK) (obtained from University College London, UK), transgenic cardiac myosin light chain 2: green fluorescent protein tg(CMLC2:GFP)(Burns *et al* 2005)(obtained from Harvard University, Boston, USA) and the transgenic friend leukaemia integration 1 transcription factor (FLi1):enhanced green fluorescent protein tg(FLi1:EGFP) (Lawson & Weinstein, 2002) (obtained from University of Sheffield, UK) lines were housed in the QMRI (BRR) zebrafish facility and maintained under standard culture conditions, with a 14h light and 10h dark cycle, at 28.5^{o}C, as recommended by (Lawrence, 2007).

2.2.1 Embryo maintenance

All procedures for embryos were carried out on eggs and larvae up to a maximum of 120hpf, in accordance with Home Office regulations. Age of embryos was determined according to the commonly accepted staging protocol of Kimmel *et al.* (Kimmel *et al*, 1995). Embryos were stored in 30 mL Petri dishes at a density of <50 embryos (~1.5 embryos/ mL) and housed in an incubator at 28.5^{o}C. All embryos used in this current work were on a no feeding regimen as nutrient supply before this age is reliant on yolk sac reserve. For "grow-up" experiments and for general breeding of lines, the feeding regimen began at 5 dpf (according to Home Office guidelines) when embryos were transferred to 1 L larval raising tanks.

2.2.2 Adult maintenance

All procedures on adult zebrafish were carried out in accordance to Home Office regulations, following approval by the local Ethical Review Committee. Adult fish were staged according to Parichy *et al* (Parichy *et al*, 2009). Health screens were carried out according to BRR guidelines and animals which were deemed unwell or

of detrimental health (impaired swimming, emaciated, egg associated oedema) were disposed of humanely. Adult zebrafish were housed in 10 L tanks at a density of 2-3 fish per L (maximum of 30 fish per tank).

2.3 Light microscopy

All visual observations were carried out using a Leica Stereomicroscope MZ16F dissecting microscope. All light microscopy images were obtained using standard white light, whilst fluorescent (GFP, EGFP, DAPI, Texas red and mCherry) images were produced by using UV mercury bulb with an appropriate filter. Photographic images were collected using an attached Leica Camera (DFC320FX).

2.4 Zebrafish embryo studies

2.4.1 Embryo collection

Following onset of light cycle, adult (sexually mature) zebrafish initiate spawning and fertilisation of eggs in desirable environmental conditions (Westerfield, 2000).

Containers with marbles were placed into the bottom of tanks prior to onset of the light cycle to encourage spawning and prevent predation/cannibalism by adult fish (Lawrence, 2007), whilst also allowing ease of egg removal. Collected eggs were stored in systems water (6g salt, Tropic Marin® Wartenberg, Germany) in 20 L of in-house deionised H_2O) with 0.5 mg/L of the antiseptic methylthioninium chloride (methylene blue). All unfertilised eggs were discarded and the remaining healthy fertilised eggs were scored according to Kimmel *et al* (Kimmel *et al*, 1995). Embryos > 8 cell stage (~2 hpf) were also discarded.

2.4.2 De-chorionation of embryos

Although spontaneous hatch-rate was monitored for developmental assessment in a number of cases manual de-chorionation was required. This is a simple procedure consisting of gently teasing apart the chorion, using fine Dumont 5# forceps, and releasing the larvae.

2.4.3 Scoring of embryonic phenotypic characteristics

To assess the influence of environmental, pharmacological or molecular manipulation on the developing zebrafish embryos, gross morphology was observed under a dissecting light microscope (x10 (Section 2.3)). For each manipulation used in this study, determination of suitable non-teratogenic concentrations was achieved by embryonic exposure from 2 hpf to a dilution series of the drug, the vehicle and a negative control, or in the case of molecular manipulation; Mo and their relevant controls.

Mortality and the presence of malformations were assessed for each concentration and time point of development, in incidences where high drug or Mo concentrations were used this has been determined as a concentration for which no malformations or lethality were observed in the first 24 hpf in at least 90% of the individuals in three consecutive experiments.

A scoring system was devised where a number of abnormal physical characteristics were assessed: pulmonary and pericardial swelling/ballooning, tail deformities such as truncated coiled or bending; blood pooling, altered heart rate and small /deformed heads. Normal embryos were determined subjectively compared to controls, head size and body axis were assessed according to the Kimmel paper (Kimmel *et al*, 1995) with normal heart rate of around 150bpm and no detectable blood pooling (Figure 2.1). The number of normal factors was determined for embryos; a score was assigned with regard to severity of phenotype, with 6 representing normal and 1 a severe phenotype as detailed in table 2.1 (examples are shown in Figure 2.1).

Table 2.1 Classification of zebrafish embryo morphological characteristics
Embryos were scored according to a number of normal factors. Normal factors were as follows, normal head size, straight body axis, normal heart rate (HR), blood present within the heart, no pooling of red blood cells and normal tail circulation.

Number of normal characteristics	Phenotype
6	Normal
4-5	Mild
2-3	Moderate
1	Severe
0	Dead

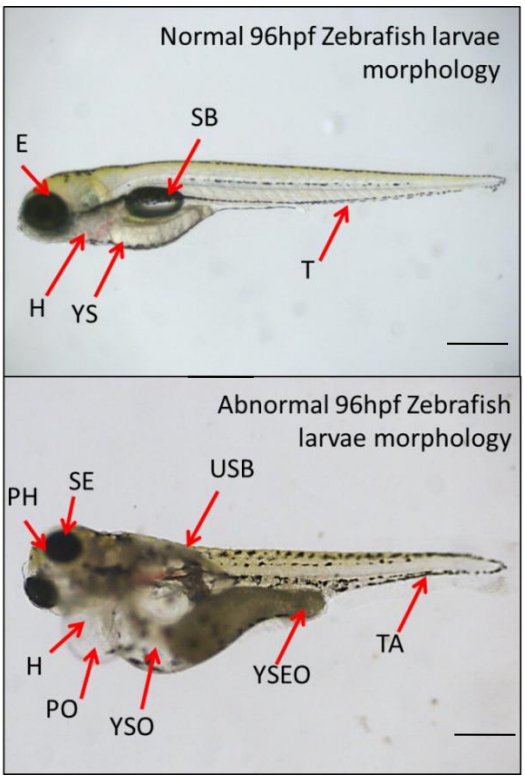

Figure 2.1 Normal and abnormal zebrafish larvae appearance
Normal appearance 96 hours post fertilisation (hpf) embryo shown in the upper panel with some of the abnormal features shown in the bottom panel. Normal features highlighted include eye size (E), location of heart (H), typical appearance of yolk sac (YS) at 96hpf, inflated and detectable swim bladder (SB) and the straight tail (T). Features highlighted as abnormal are pin head (PH), small eyes (SE), pericardial oedema (PO), yolk sac oedema (YSO), yolk sac extension oedema (YSEO), tail axis abnormalities (TA). Scale bars represent 0.5mm.

2.4.4 Embryonic manipulation

2.4.4.1 Embryonic pharmacological manipulation

Embryos from the 2 cell stage to 120 hpf were exposed to a number of pharmacological agents at varying concentrations. Drugs of interest which were investigated were as follows: the GR agonist Dex, the GR (and progesterone receptor (PR)) antagonist RU486 (also known as mifepristone), the 11β-hydroxylase inhibitor metyrapone (Met) the 11β-hydroxylase substrates 11-deoxycorticosterone (Doc) and 11-deoxycortisol (Doxy), and the zebrafish MR agonist spironolactone (Scott *et al*, 2005). All drug optimisation is described in Appendix 1. Unless stated otherwise a standard drug exposure protocol was used for each drug of interest. Groups of 30 embryos were placed in Petri dishes containing 30 mL of systems water. Embryos

were exposed to the drug solution continuously up to 120 hpf and drugs were replaced daily.

2.4.4.2 Embryonic genetic manipulation

Genetic manipulation of embryos was carried out by Mo antisense gene knock-down (Heasman, 2002; Summerton, 1999) targeted towards the genes encoding *cyp11b1* and *gr*. Mo were purchased from GeneTools (Philomath, Oregon, USA) (http://www.gene-tools.com) designed specifically to the zebrafish *cyp11b1* and *gr* gene sequence. Mo design and region targeting is explained in more detail elsewhere (Moulton & Yan, 2008). For each gene three 25mer Mo sequences were produced:

1) Translational blocking Mo, which is targeted to the ATG start codon, preventing ribosomal assembly and mRNA production (referred herein as atg-Mo).
2) Splice site specific Mo which is targeted to pre-mRNA across or near exon-intron boundaries inhibiting splicing, resulting in exon excision and shortened or truncated mRNA (referred to herein as ss-Mo).
3) 5-Mispair Mo with an almost identical sequence to the ss-Mo but with a number of mismatched base pairs throughout the course of the sequence. This oligo sequence controls against off-target effects which may be introduced by high concentrations of antisense molecules (referred to herein as mm-Mo).

The sequences of all Mo are shown (Appendix 1). For *cyp11b1* Mo all sequences designed have 3' modifications of carboxyfluorescein, a green emitting fluorescent tag; for GR Mo all sequences designed have a 3' modification of lissamine, a red emitting fluorescent tag. These 3' modifications allow visualisation of morpholino uptake following microinjection

2.4.4.2.1 Morpholino microinjection

Microinjection is a fast, high throughput technique for injection of synthetic oligonucleotide sequences into newly fertilised eggs at the 2-8 cell stage. Microinjection was carried out as described by Nusslein-Volhard *et al.* (Nusslein-Volhard & Dahm, 2002). To summarise, injection needles with an inner diameter of

0.1mm were pulled from glass capillary tubes (borosilicate glass 1.0mm O.D x 0.78 mm I.D from Harvard Apparatus, Kent, UK) with care using a micropipette puller (P-97 Flaming/Brown Micropipette Puller from Sutter Instruments (Novato, CA, USA)). For needles used here the following ramp features were deemed optimum, heat 450, pull 85, velocity 55 and time 150. Due to the buoyant nature of the embryos, 2% agar (melted in H_2O) microinjection plates were used to line up and hold embryos in an appropriate injection position with sufficient water surrounding the embryos to prevent dehydration and to provide surface tension.

The Narishige IM-300 programmable micro-injector was set up and the needle was loaded with approximately 3 µL of morpholino (diluted to appropriate concentration as shown in Appendix 1). A calibration injection onto mineral oil over a graticule slide was carried out, which allowed the measurement of bolus sphere diameter. From the radius (r) of this bolus, the volume of injection was calculated as below so that the amount of morpholino to be injected could be accurately determined (normal injection volume was 5 nL). When injector pressure and bolus size were optimal the needle was inserted through the chorion into the yolk sac of the embryo below the cell mass, where, if accurately injected, the morpholino was taken up into the cytoplasm of the newly forming cells through a process known as cytoplasmic streaming. Optimisation of morpholino dosage is shown in Appendix 1.

Equation 2.1 Morpholino bolus

$$\boldsymbol{bolus\ volume = 4/3\pi r^3}$$

2.4.5 Embryonic developmental assessments

A number of assessments were carried out through the course of 120 hpf to determine whether manipulation had any impact on development.

2.4.5.1 Hatch rate, embryogenesis, body length and growth rate

The normal window of spontaneous chorion hatching occurs between 48 and 72 hpf, the rate of which embryos hatched in embryos manipulated pharmacologically or genetically was monitored at 24h intervals over 120 hpf to determine whether treatment increased or decreased the rate at which hatching occurs. The total number

of embryos hatched was expressed as percentage of the surviving population at a given time point.

Body length, growth rate and embryogenesis at a given time were determined on embryos which had been manually de-chorionated (as described in section 2.4.2) and imaged. All images were captured using a Leica Stereomicroscope MZ16F dissecting microscope with a Leica camera (DFC320FX), as described in section 2.3, then quantitatively analysed using ImageJ software.

2.4.5.1.1 Head-trunk angle

Head-trunk angle was used as a marker of global developmental during normal zebrafish embryogenesis from 20 to 70 hpf. The head-trunk angle (in degrees) increases uniformly as a result of the body straightening shown in technique optimisation (Figure 2.2) and as previously described (Kimmel *et al*, 1995). Using still images obtained at 10X magnification the head trunk angle is the angle obtained by drawing a line through the middle of the head connecting the eye and ear, a parallel line is then drawn through the notochord; the angle is obtained at the point which these lines cross (as shown in Figure 2.2).

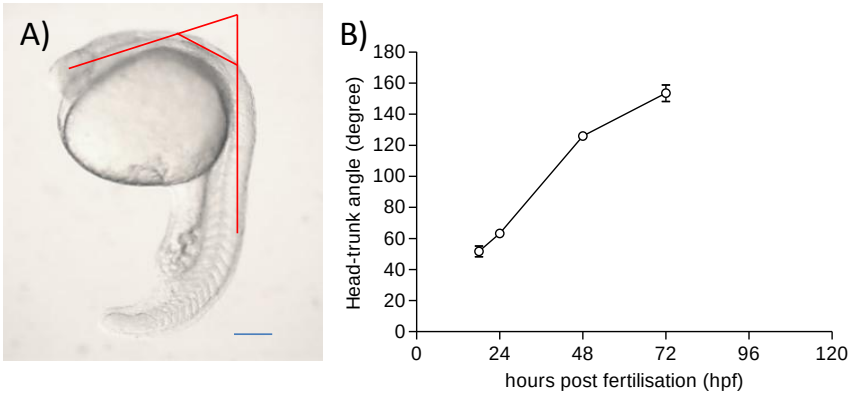

Figure 2.2 Assessment of embryonic head-trunk angle
A) A) Head trunk angle measurement example. B) Head trunk-angle throughout the first 72 hours post fertilisation (hpf) of zebrafish development; data are mean ± SEM for control embryos throughout development (n=4; (10 embryos per experiment)).

2.4.5.1.2 Total body length

Total body length was also determined from still images at x20 magnification. Images were analysed using a method previously described (Kimmel *et al*, 1995)(Figure 2.3). For each length measurement time was also recorded allowing growth-rate to be determined as the change in length per hour (μm/h).

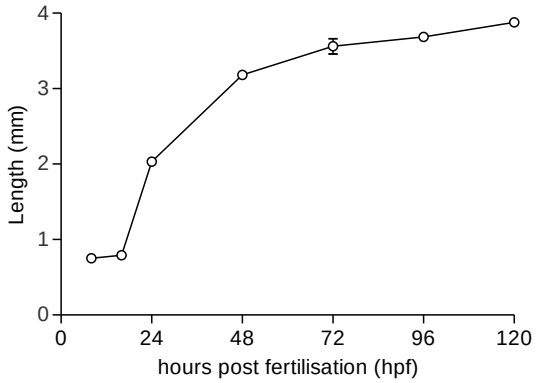

Figure 2.3 Assessment of embryonic length with time
Total body length (mm) throughout the first 120 hours post fertilisation (hpf). Data are mean ± SEM for control embryos throughout development (n=4; 10 embryos per experiment)).

2.4.5.2 Eye length and area

Further developmental assessment and determination of craniofacial abnormalities following manipulation was carried out by determining eye length relative to body length. For this 30x magnification images were captured as in section 2.3, and the length (b) and width (a) of the eye was determined using ImageJ software. From this, eye area was determined using the following formula.

Equation 2.2 Eye area

$$\boldsymbol{area\ of\ an\ elipse = \pi\ \frac{1}{2}a\ \times\ \frac{1}{2}b}$$

2.4.5.3 Embryo swim activity

Embryonic swim activity was observed at 72 and 120 hpf through open-field observations as previously described (Levin & Cerutti, 2009). Briefly, a single embryo was placed in a 5cm Petri dish delineated into three evenly spaced concentric rings, described as inner, mid and outer regions. Each embryo was introduced into

the mid region of a marked dish containing 3 mL of fresh systems water. Embryo movements were recorded from above using a digital CCTV camera (Baxall, AD group, UK). Data were analysed by commercially available software (Limelight Ltd, UK) to determine total distance travelled, percentage of time spent in each region of the Petri dish and average speed of movement for each embryo (n=12 embryos per group).

2.4.5.4 Embryo movement

At 48 hpf all movements produced by the embryo are in the form of random twitching rather than coordinated locomotor activity. The influence of pharmacological or genetic manipulation on this phenomenon was determined by placing 20 embryos into a 5cm Petri dish. The embryos were then filmed at 10x magnification for 5 min using a digital CCTV camera (Baxall, AD group, UK). The mean number of twitches recorded for the group in the footage was counted using slow speed play back.

At 96 hpf coordinated muscular movement is usually present in response to touch stimulus. Here embryos were touched gently with Dumont #5 forceps; if embryos swim to remove themselves from the stimulus this was scored as a swim=1, if no movement was detected this was recorded as a no swim=0. A group cumulative score was produced. N=5 experiments (10 embryos per experiment).

2.4.6 Vascular formation analysis

tg(FLi1: EGFP) embryos were used to determine the influence of GC on vascular formation. In this transgenic line enhanced green fluorescent protein (EGFP) is expressed by the *fli1* transcription factor, this is expressed in cells of presumptive haemangioblast lineage and allows vascular visualisation (Lawson & Weinstein, 2002b)(Figure 2.4).

2.4.6.1 Intersegmental vessel number

Normal intersegmental vessels (ISV) were considered to be vessels which expressed GFP, joined the aorta to the dorsal longitudinal anastomotic vessel (DLAV) and were not branched in appearance (Figure 2.4). Under a fluorescent microscope (40 x) normal ISV were counted dorsally from the yolk sac throughout the length of the tail,

within a similar focal plane. The number of complete ISV crossing completely between the two primary vessels un-branched was determined at 48, 72, 96 and 120hpf. Embryos were maintained in 24 well plates with 2 mL of incubation solution, to allow observation throughout development.

2.4.6.2 Vascularisation index

On images obtained at 40 x magnification a region of 5 somites was selected in the tail dorsally from the yolk sac. Here 3 parallel lines were drawn using ImageJ software. The number of vessel intersections were then counted and the sum of these counts was then plotted to produce the vascularisation index of the embryos as described elsewhere (Pelster *et al*, 2003)(Figure 2.4).

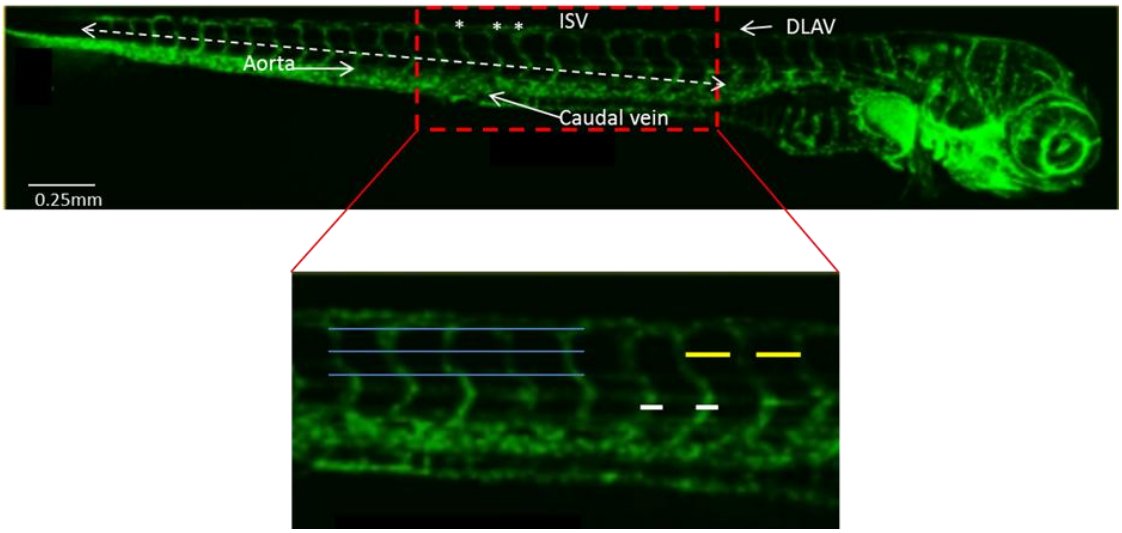

Figure 2.4 Representative image of tg (FLi1: EGFP) zebrafish embryo.
Vasculature in a 96 hour post fertilisation (hpf) zebrafish embryo, visible as a result of enhanced green fluorescent protein (EGFP) expressed under the control of the *fli*1 transcription factor. Intersegmental vessels (ISV) can be observed between dorsal longitudinal anastomotic vessels (DLAV) and aorta. High magnification of the region highlighted with a red dashed square shows the distance between vessels (yellow lines), vessel diameter (white lines) and the region for calculation of vascularisation index (blue horizontal lines.

2.4.7 Cardiac assessment

tg(CMLC2: GFP) embryos were used to determine the influence of GC on cardiac formation. In this transgenic line green fluorescent protein (GFP) is expressed under the control of the CMLC2 promoter resulting in green hearts when observed under fluorescent microscope (Figure 2.5)

2.4.7.1 Heart removal from tg (cmcl2: GFP) embryos

tg(CMLC2:GFP) (Figure 2.5), embryos at 120 hpf were euthanized by anaesthetic overdose (neat MS-222) and then fixed in 4% paraformaldehyde (PFA) overnight at 4^{o}C, washed 3 times with 1x Phosphate Buffered Saline Tween-20 (PBST) and then stored in absolute methanol at -80^{o}C until required. Following storage, methanol was removed from embryos which were then washed 3 times with Phosphate Buffered Saline (PBS) in preparation for heart removal.

Hearts were removed either manually by pinching the heart (under a fluorescence microscope) above the yolk sac using fine forceps (Dumont #5) and gently teasing it out, or using a mechanical disruption procedure. The mechanical procedure was adapted from a previously published protocol (Burns & MacRae, 2006). Briefly, embryos were pooled in a 1.5 mL Eppendorf tube and a 19 gauge needle with regular bevel attached to a 5 mL syringe was inserted and repeatedly plunged (30-40 times) to homogenise the embryos. The resulting homogenate was filtered through a 105µm nylon mesh, the perfusate was collected and GFP positive hearts were selected under fluorescent microscope (Figure 2.5). Isolated hearts were stored in RNAlater (Life Technologies, Paisley, UK) for PCR (see gene analysis section 2.6), fixed again in 4% PFA for histology (see histology section 2.8) or quickly frozen by placing on dry ice.

2.4.7.2 Assessment of cardiomyocyte number

PFA-fixed isolated hearts were incubated in 200 µL DAPI (1/1000 with PBS) for 1h at room temperature then washed in PBS for 1h at room temperature under gentle agitation. The hearts were then mounted on microscope slides in Flouromount-G (Southern Biotech, Birmingham, USA) and imaged using confocal microscopy

(Leica SP5 confocal). Z stacks were produced at intervals of 2-5µM, and collected using the supplied Leica software.

Tiff images were analysed using ImageJ software and the number of DAPI positive cells (those DAPI marked cells within the GFP area) were counted using a cell counter plugin. This software allows analysis of Z-stack images while tracking previously-counted cells, thus preventing duplication. To eliminate the possibility of erythrocyte, atrial or bulbus arteriosus cell staining only cells which were double stained (green and blue) were considered. To accurately count cells, intra and inter operator counts were analysed (see statistical section 2.10). Each heart was counted 3 times on separate days and a mean was taken.

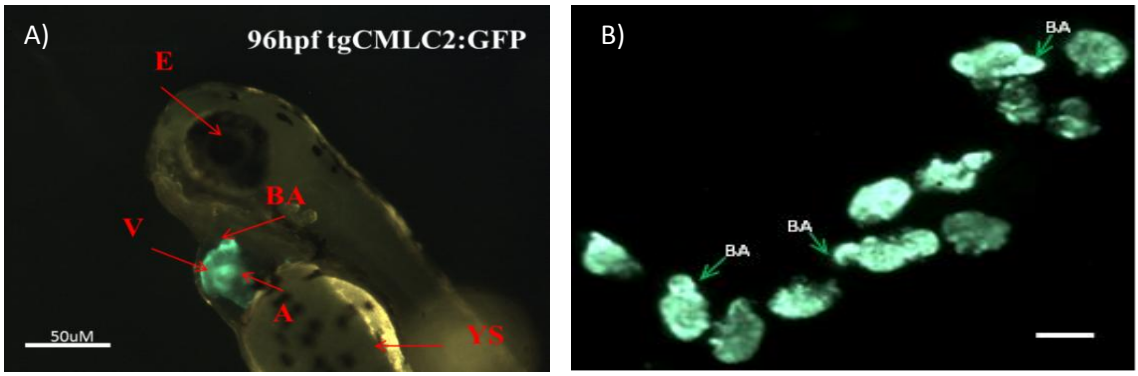

Figure 2.5 Representative image of tg (CMLC2:GFP) zebrafish embryo
A) 96 hours post fertilisation (hpf) zebrafish embryo under UV light; fluorescence is due to GFP expression under the cardiomyocyte light chain 2 (CMLC2) promoter which is highly expressed in the heart of the zebrafish. Some key structures have been highlighted: eye (E), bulbus arteriosus (BA), ventricle (V), atrium (A) and the yolk sack (YS). A) image of ventricle in zebrafish embryo. B) isolated 120 hpf zebrafish embryonic hearts with fluorescent ventricles the bulbus arteriosus (BA) is highlighted for orientation. (Scale bar is 50µm).

2.4.7.3 Cardiac functional assessment

tg(CMLC2:GFP) embryos (Figure 2.5) were used to determine the influence of manipulation on heart structure and function. Hearts were visualised under fluorescence using an Axioskop II MOT compound microscope with a (X40) dipper objective lens. Images were captured throughout the course of the cardiac cycle using a black and white camera connected to a computer. Videos were processed using VirtualDub software (www.virtualdub.org) which converted the compressed video

files into a number of frames. These still images were opened in ImageJ software for further assessment.

The ventricle was assessed for diastole and systole throughout three cardiac cycles. The outer side of the ventricle was outlined using the draw ellipse feature at the maximum during the cardiac cycle -the end diastolic area (EDA) and the minimum- end systolic area (ESA). These data were used to calculate the ejection fraction using an area subtraction method.

Equation 2.3 Ejection fraction

$$\boldsymbol{Ejection\ fraction}\ \% = \frac{\boldsymbol{end\ diastolic\ area\ \ EDA\ - end\ systolic\ area\ \ ESA}}{\boldsymbol{EDA}} \times \mathbf{100}$$

Further measurements were obtained using the images. The ventricular width (a) and length (b) were determined using the line utility. These data were then input into the formula for prolate spheroid to calculate ventricle volume (μl^3) during systole and diastole.

Equation 2.4 Ventricle volume

$$\boldsymbol{Ventricle\ volume}(\boldsymbol{\mu l^3}) = \mathbf{4/3} \times \boldsymbol{\pi} \times (\frac{\mathbf{1}}{\mathbf{2}}\ \boldsymbol{b}) \times (\frac{\mathbf{1}}{\mathbf{2}}\boldsymbol{a^2})$$

The difference between diastolic and systolic volume enables calculation of stroke volume (the volume of blood pumped by the ventricle per cardiac cycle).

Equation 2.5 Stroke volume

$$\boldsymbol{Stroke\ volume}\ (\boldsymbol{\mu l^3})\ = \boldsymbol{EDV - ESV}$$

Heart rate was measured by timing 15 heart beats and then calculating the number of beats per minute (bpm). Bpm were then combined with the stroke volume to calculate cardiac output.

Equation 2.6 Cardiac output

$$\boldsymbol{Cardiac\ output}\ (\boldsymbol{\mu l^3})/\ \mathbf{min} = \boldsymbol{stroke\ volume}\ \times \boldsymbol{heart\ beats\ per\ min}$$

2.4.7.4 Assessment of cardiac morphology

Alterations in cardiac morphology as a consequence of manipulation were measured by determining the distance between two regions of the heart -the sinus venosus (SV), the region of blood inflow to the heart, and the bulbus arteriosus (BA) the outflow from the ventricle by using the straight line function in ImageJ software. The measured length (µm) was then normalised by body length (mm)(Antkiewicz *et al*, 2005).

2.4.7.5 Assessment of pericardial oedema

As oedema is often a feature of cardiac morphological or functional defects, the area of oedema was also determined. Here the area between the yolk sac and the jaw and the outer pericardial tissue was traced using lateral images and ImageJ software, and the freehand trace tool. The area contained within this freehand trace was calculated. For each fish three traces were determined on separate occasions, a mean of the three measurements was then produced for each embryo.

2.5 Adult zebrafish studies

2.5.1 Adult fish anaesthesia

Fish were moved from 10 L holding tanks to small 1 L tanks containing 4.2% v/v MS-222 and left until equilibrium was lost (fish float on side), operculum movement slowed and fish did not respond to touch stimuli. When satisfactory anaesthesia was achieved fish were removed from water and placed in a moistened sponge for either visual observation under dissecting microscope, tail resection or morphometric analysis. Period and frequency of anaesthesia was kept to a minimum in accordance with Home Office guidelines. After procedure fish were placed in a recovery tank of systems water at 28.5ºC. Fish were monitored during recovery and when satisfactory operculum movement and swim activity were observed fish were returned to holding tank.

2.5.2 Adult fish culling

Adult fish were culled according to Home Office guidelines for Schedule 1B. Adult fish were added to an appropriated volume of neat MS-222 to give anaesthetic overdose. Fish were left until equilibrium was lost, operculum movement had

completely ceased and no response to touch was noted. From here Home Office schedule 1 culling procedures were followed to destroy brain function: decapitation anterior to the gills using a sharp scalpel blade.

2.5.3 Adult morphometric assessments

Adult fish were weighed as follows. A 500 mL beaker of systems water was placed on a balance, the balance was then set to zero. Fish were individually collected from 10 L holding tank using a net, excess water was removed by briefly dabbing the net on a paper towel. The fish was then gently transferred to the weighing balance beaker. Measurements for all fish were taken in triplicate, and results displayed as a mean per fish.

Adult fish length was determined from anaesthetised fish (to prevent undue stress). When suitable anaesthesia was achieved, fish were placed laterally on a moistened sponge. Digital callipers (Digital Electronic Vernier Calliper (0-150 mm) Sealey) were then used to measure the total body length anterior to posterior (not including tail fin rays). After satisfactory measurements fish were transferred to the recovery tank. The fish length and weight data were then used to calculate the condition factor (Siccardi *et al*, 2009).

Equation 2.7 Condition factor

$$K = \frac{weight \times 100}{length^3}$$

2.5.4 Adult fish dissection and tissue harvesting

Adult fish were culled as in section 2.5.2 and placed, ventral side up, on a moistened sponge, adult tissue was removed in an adapted protocol (Gupta & Mullins, 2010) .

2.5.4.1 Heart removal

As head was removed by decapitation, gills were pulled aside to expose the heart, using fine forceps; the heart (ventricle, atrium and the BA) was removed by gently pinching above the BA. For RNA extraction beating hearts were transferred to ice cold PBS to remove some of the blood which is known to be protease rich. For histology hearts were fixed in neutral buffered formalin overnight at 4^{o}C then rinsed 3 times in PBS. After this they were stored in methanol until required. Hearts were

weighed on a microbalance (analytical semi-micro balance, Denver) by adding the tissue to an Eppendorf of known weight. Heart lengths were determined by placing them on an agar lined Petri dish and capturing images using a Leica Stereomicroscope MZ16F dissecting microscope with a Leica camera (DFC320FX). Heart length was determined using ImageJ software.

2.5.4.2 Liver and gut removal

After heart removal, a cut was made ventrally from cloaca to gills. The skin of the peritoneal cavity was then removed to expose liver and gut. Due to the lobular nature of the liver, some liver tissue may be entwined with the gut; when this occurred the gut tissue was teased out to clearly display the tan coloured vascularised tissue of the liver. Great care was taken to not puncture the gut which contains numerous RNases. Liver tissues were collected and stored in 1 mL of RNAlater to stabilise tissue, this was left at room temperature overnight and then stored at -20^{o}C until RNA extraction.

2.5.4.3 Kidney removal

After gut removal, ovaries or testes were then removed from female and male fish, respectively. The swim bladder was then removed, by pinching the inflated region just posterior to head with forceps and gently pulling towards the tail. Removal of the swim bladder exposes the kidney (including head kidney and interrenal gland) which is located along the dorsal body wall and can be removed. The translucent, faintly pink coloured kidney structure is teased away gently from the vertebrate of the spine.

2.5.4.4 Smooth muscle removal

Smooth muscle was dissected by de-scaling a region of the tail and removing bones to expose pink coloured muscle tissue.

2.5.4.5 Brain removal

The brain was removed by breaking open the skull and simply teasing out the lobed milky white tissue within. Due to the site of decapitation, the cerebellum and medulla were often found in the body section and were teased out using fine forceps (Dumont #5). Isolated brain tissue was stored in RNAlater for PCR investigation.

2.5.5 Adult blood glucose measurements

Blood glucose was determined using a commercial blood glucose meter (Freestyle freedom lite blood glucose monitoring system) with Freestyle lite blood glucose test strips, as directed by manufacturer's instructions. To acquire sufficient blood, this was a terminal procedure and blood was removed from fish which had been culled, blood collection was by exsanguination and blood placed directly on the blood glucose strips.

2.5.6 Adult behavioural assessments

Of the numerous zebrafish behavioural assays described (Levin & Cerutti, 2009) three were used in the current work (schematics shown in Figure 2.6). These were open-field, forced swim and novel object assays. For each assay fish were transferred from the 10 L holding tank into the observation tank by netting. The test tank contained fresh, temperature-controlled water which was replaced for each fish monitored. After the assay the fish were netted from the observation tank and transferred to a new 10 L tank (post observation tank). Room temperature was monitored throughout the assay, with light and ambient noise controlled.

2.5.6.1 Forced dive swim assay

Zebrafish were placed individually in a 1.5 L trapezoidal tank (15.2 height × 27.9 top × 22.5 bottom × 7.1 width (cm).) The tank was filled ¾ with fresh 28.5ºC systems water. This tank was illuminated from above using a standard angle poise desk lamp and from below by placing the tank on a light-box (X-Ray MG-7 Standard 17" X 14" Illuminater Light Box, Wolf). The CCTV camera was mounted laterally to capture a side view of this tank (as indicated in Figure 2.6). Limelight software was used to divide the tank into two equal horizontal portions. The fish was then placed in the tank and the behaviour was recorded over a 5 min period. Total distance, zone crossing, time spent in each region and the average velocity were recorded to determine the boldness of fish. Generally fish will spend longer at the bottom of a novel tank and bold fish will spend more time at top.

2.5.6.2 Open-field assay

The open field test consisted of transferring a single fish from the holding tank to the centre of a square tank (15x15cm) the tank contained 28.5ºC systems water to a depth of 3 cm ~ 1.5 L. The tank was lit from below using a light box, light from above was provided by a desk lamp. To minimize interference or disturbance a 20cm box surrounded the edges of the tank. A CCTV camera was suspended above the tank for recording movement within the test area. Limelight software was used to record and analyse behaviour. The software delineates a 3x3 (21 cm^2) square grid arena and over the course of 5 min records the movement within the tank. For each fish introduced to the observation tank the total distance travelled, number of grid crossings, time spent in each area and the average velocity were calculated.

2.5.6.3 Novel object assay

The same tank and camera set up as for the open-field assay was used for novel object assay. However, in this assay a novel object (aquarium enrichment ornament, Pets at home, UK wide) was used to represent a predator. The novel object was placed in the centre of the tank and Limelight software was then used to delineate the tank into two regions, the central region (predation area) containing the novel object (plus a 2 cm border) and the rest of the tank (avoidance area). The time spent in either the predation or the avoidance area was recorded over a 5 min period. This test is another method for determination of the boldness of a fish, with the longer spent in the predation region indicating the bolder the fish.

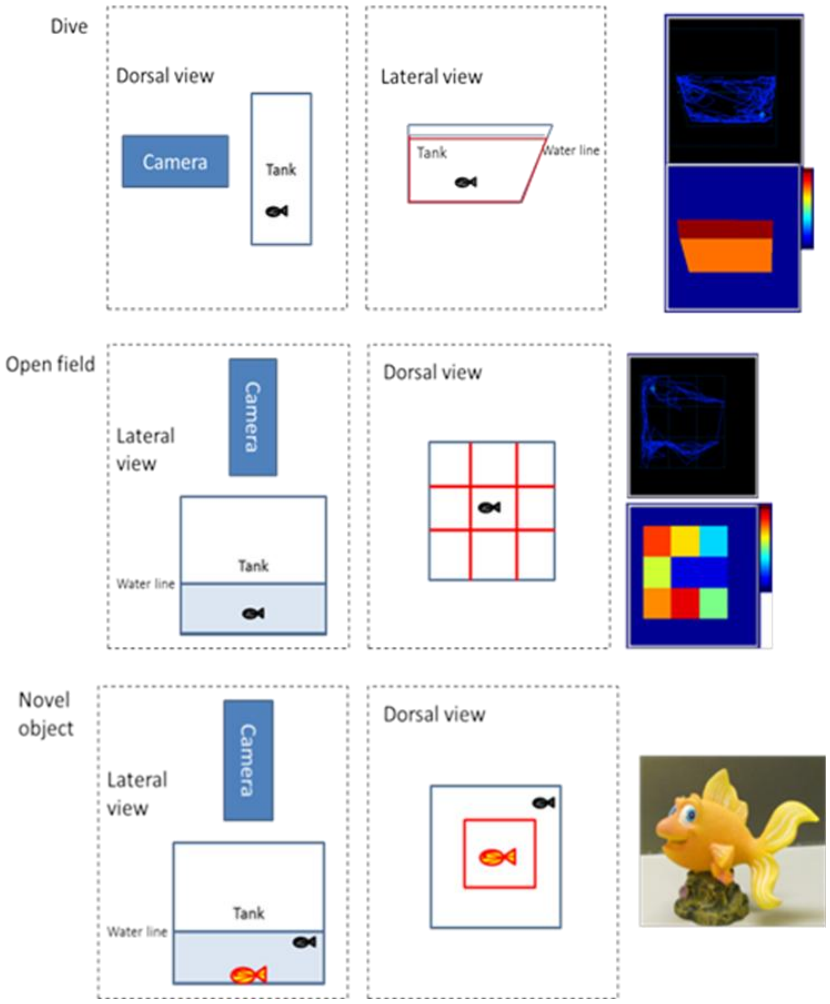

Figure 2.6 Schematic representation of experimental set up for behavioural assays
Shown are dive, open-field and novel object behavioural assays. For each assay, dorsal and lateral views are shown to illustrate the experimental set-up. For dive and open-field assays typical zebrafish software traces are shown. For the novel object assay, the predator/novel object (aquarium enrichment ornament) is shown.

2.5.7 Tail fin regeneration

To determine whether there was any difference in regenerative capabilities between embryonically-manipulated adult groups, a tail fin regeneration assay was carried out. To visualise and image blood vessels, adults of the tg(FLi1: EGFP) line were used and anaesthetised as described (section 2.5.1). Fish were placed, lateral side up, on a Petri dish, tails fin rays were gently spread out and images of tails pre amputation were recorded using light microscopy. Using dissection scissors and fine forceps, tails were lifted from the Petri dish surface and a single vertical cut was

made perpendicular to the fin rays. Dissected tail sections were retained for further analysis. Images of the tail were then recorded post-amputation. Fish were placed in a recovery beaker post amputation and, following recovery, were housed singularly and tail regrowth monitored over 30 days. Regrowth was calculated relative to total body length.

2.5.7.1 Assessment of tail-fin vascular patterning

Determination of tail vascularisation in adult zebrafish was carried out as previously described (Pugach *et al*, 2009) and the fish were euthanized (section 2.5.2) at the end of the procedure. Briefly, following administration of anaesthetic overdose fish were placed dorsal side upwards and facing to the right on a slotted sponge. A Hamilton syringe was filled with 4 µL of 10 mg/mL 70kDa dextran, Texas Red ® dissolved in PBS. With the bevel facing upwards the needle was positioned such that if the fish eye were a clock the needle would be pointing at the 7 o'clock position at a 45-65 degree angle to the fish. The needle was gently inserted and the plunger was gradually depressed. After around 5 min, the dye can be discerned in the tail vasculature of the fish (Figure 2.7).

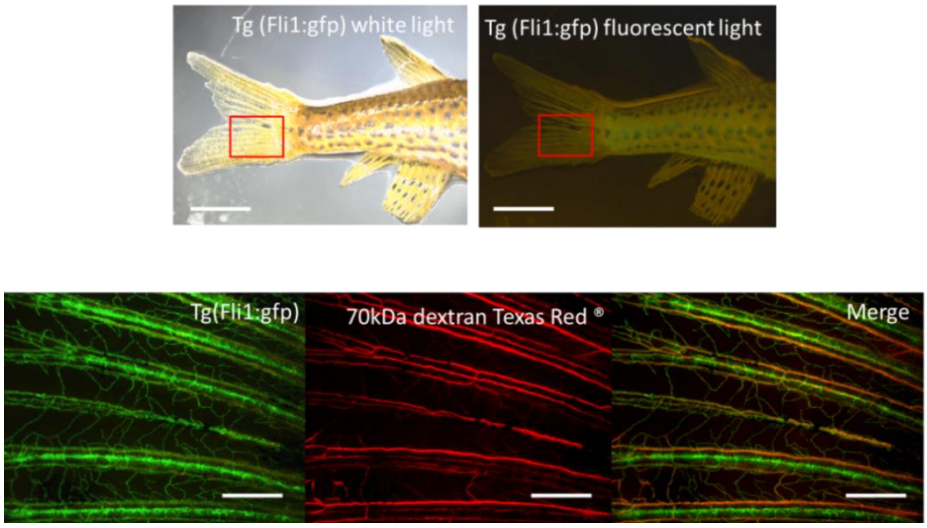

Figure 2.7 Assessment of tail-fin vascular patterning.
Shown are white light and fluorescent 10X magnification images of the tail fin region of adult tg (FLi1: GFP) fish. Higher magnification images are shown of the GFP expression in tail ray fins, with 70kDa dextran injections and a merge of the two images to show blood flow within ray fins of the tail.

2.6 Gene abundance study

Gene nomenclature used throughout will be as follows (unless highlighted in text), Example, glucocorticoid receptor

GR, human (Homo sapiens) GR gene isoform, protein designation GR

Gr, rodent (Mus musculus, Rattus norvegicus) GR isoform; protein designation GR

gr zebrafish (Danio rerio) GR gene isoform, protein designation Gr

2.6.1 Ribonucleic acid (RNA) extraction

Multiple methods for total RNA extraction were used as some methods were found to yield higher mRNA concentrations in a tissue specific manner.

2.6.1.1 Whole embryo ribonucleic acid (RNA) extraction

In whole embryos the RNeasy Mini Kit (Qiagen, Crawley, West Sussex, UK) was used to extract RNA from pooled embryos (<48hpf, 20 embryos; >48hpf, 10 embryos). Tissue was homogenised in Buffer RLT (provided with kit) using a bead mixer mill (3 min at 15Hz) immediately prior to RNA extraction according to the provided manufacturer's protocol for RNA extraction from animal cells.

2.6.1.2 Embryonic heart ribonucleic acid (RNA) extraction

Total RNA was extracted from isolated embryo hearts (see heart isolation protocol section 2.4.9.1). 100 hearts from 120 hpf embryos were pooled. Here an RNeasy Micro Kit (Qiagen, Crawley, West Sussex, UK) was used as per manufacturer's protocol for RNA extraction for cultured cells.

2.6.1.3 Adult tissue ribonucleic acid (RNA) extraction

Total RNA was isolated from adult organ tissue (see section on adult organ dissection section 2.5.4) by addition of TRIzol ® reagent (Invitrogen Ltd, Paisley UK) and subsequent homogenisation in a bead mixer mill. RNA was then extracted from the resultant homogenate according to manufacturer's directions.

2.6.2 Ribonucleic acid (RNA) quantity

RNA quantity was determined using a Nano-drop ® spectrophotometer ND-1000 (Fisher Scientific, Loughborough, UK) to determine extracted RNA concentration at

260nm (molar extinction coefficient 40ng-cm/µL). RNA integrity was verified by a 260/280 nm absorbance ratio of ~2.

2.6.3 Ribonucleic acid (RNA) quality

To further determine the quality of RNA a 1 % agarose gel was produced using 0.5X Tris/Borate/ethylenediaminetetraacetic acid (EDTA) (TBE) solution (5 x solution 54g Tris base, 27.5 g boric acid, 20 mL of 0.5 M (pH 8) u to 1 L with distilled water (ddH_20)) and GelRED reagent (Invitrogen, Paisley, UK). The gel was then run in a standard horizontal gel electrophoresis tank at 80 volts for 1 h using molecular weight ladders (18S and 28S Ribosomal RNA from calf liver, Sigma Aldrich, Poole, UK) for identification of 18S and 28S RNA Bands under a UV light transilluminator in the GelDoc gel imaging system (BioRad, Hertfordshire) using associated software (Image Lab™ Software, BioRad, Hertfordshire). These bands correspond to the 28S and 18S ribosomal subunits and are found in a ratio of 2:1 (28S:18S) in eukaryote samples, such as zebrafish (Peterson & Freeman, 2009).

2.6.4 Ribonucleic acid (RNA) clean-up

Samples which were found to have gDNA contamination (appearing as a smear on the RNA gel) were treated with DNA-free ™ kit (Applied Biosystems, Warrington, UK) to eliminate possible DNA presence. The protocol was followed as per manufacturer's instructions using 40 µL RNA, 4µL 10x DNase buffer-1 and 4µL DNase inactivation Reagent. RNA was re-analysed as before (sections 2.6.2 and 2.6.3) to determine successful DNA removal.

2.6.5 Ribonucleic acid (RNA) reverse transcription

RNA (0.5µg) was reverse transcribed into cDNA using the High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK) according to manufacturer's guidelines using 10 µL 2x RT Master Mix (provided with the kit) and 10 µL RNA (0.5 µg total RNA). A no enzyme control was also included to assess reverse transcription activity and demonstrate that there is no genomic DNA (gDNA) contamination within the sample. The following reverse transcription conditions were used, 25^{o}C for 10 min, 37 ^{o}C for 120min, 85 ^{o}C for 5 min and a final hold at 4^{o}C.

2.6.6 Polymerase chain reaction (PCR) primer design

Gene of interest nucleotide sequences were obtained by searching Ensembl Genome Browser, zebrafish database. Here transcript sequence and accession number were obtained. This gene information was then used to design appropriate oligonucleotide primer pairs. Primer design is a key determinant of PCR success, without a successful primer set PCR products are not produced. Real-time PCR primers were designed using free online Primer3 software through insertion of gene of interest nucleotide sequence or accession number. This software takes into consideration three main criteria 1) primers should contain between 40-60% of the nucleotide bases guanine (G) or cytosine (C), with minimal clusters of the two bases together 2) primers should be 20-40 base pairs in length and not self-complimentary 3) primer melting temperatures (Tm) should be no more than 5^{o}C different and around 65-70 ^{o}C. For qRT-PCR, primers were designed using the Universal Probe Library Assay design online software (Roche Diagnostics) by insertion of the gene name or accession number into the zebrafish database. Here primer pair sequences were produced along with the complimentary UPL probe, with multiple assays described ranked in order of criteria success. After primers (either RT-PCR or qRT-PCR) were designed the sequences were then checked using BLAST software, which checks the alignment of a nucleotide sequence against all sequenced zebrafish genes. All primer pairs were purchased as lyophilised powders from Sigma-Aldrich and were reconstituted in TE Buffer (10 mM Tris at pH 8.0 with HCl 1 mM EDTA). Assay success was determined by using high quality cDNA pooled from either a range of aged embryos or from various adult tissues.

2.6.7 Real time polymerase chain reaction (RT-PCR)

Real time PCR (RT-PCR) reactions were carried out using PCR Master Mix (Promega (www.promega.com)) according to manufacturer's instructions. For a 20µl reaction volume, 10 µL 2x PCR Master Mix, 1 µL of each primer (forward and reverse) 6 µL dH_2O and 2 µL cDNA were used. The following PCR parameters were used for all genes initially: an initial 5min denaturation at 94^{o}C followed by 30 annealing cycles of 94 ^{o}C for 15 sec, 55 ^{o}C for 1 min and 72 ^{o}C for 10 sec and then a final extension at 72 ^{o}C for 5 min. PCR products were run out on a 1.5% TBE agarose gel containing 1/10000 (v/v) Gelred ™ Nucleic acid stain (Cambridge

Bioscience, Cambridge, UK). The gel was then run in a standard horizontal gel electrophoresis tank at 100 volts for 1.5 h using molecular weight ladders (QuickLoad © PCR Marker, New England Biolabs) for transcript identification. The gel was then observed under a UV light transilluminator in the GelDoc gel imaging system (BioRad, Hertfordshire) using associated software Image Lab™ Software, BioRad, Hertfordshire, UK). If the assay was unsuccessful (no detectable bands), the annealing and denaturing parameters were altered or a small amount of $MgCl_2$ was added to the reaction mixture. If these alterations still did not produce bands or bands were detected faintly or at the wrong band size, further PCR troubleshooting was carried out using the online resource https://www.neb.com/tools-and-resources/troubleshooting-guides/pcr-troubleshooting-guide

2.6.8 Quantitative real time polymerase chain reaction (qRT-PCR)

The following procedure was used to quantify numerous genes of interest using the LightCycler 480 system (Roche, Hertfordshire, UK). To determine the concentration of a given gene in a sample a standard curve was produced. Initial investigation of primer success and determination of sample dilution factor was carried out, this allows sample amplification to occur within the standard curve, and a dilution factor of 1:40 was selected for all tissue samples. As genes are expressed in varying levels in early development, a pooled time-course was used to determine the suitability of an assay (primer pairs and probe) for detection of the gene of interest. Those deemed successful were linear and had an efficiency of between 1.7 and 2.1. The error should be as close to 0 as possible, with slight increases allowed, an error greater than 0 is likely to be as a result of poor pipetting or problems mixing cDNA and master mix.

All standards and samples (including controls) were run in triplicate for each gene of interest. Probes used were from the Universal Probe Library (UPL) (Roche Diagnostics Ltd, UK). To each well 8µl of master mix containing probe and primer combination for each gene of interest (recipe for master mix is shown in table 2) was added. The plates were thoroughly sealed and centrifuged at 4^{o}C for 3 min at 8,000g. The LightCycler system was used at the following operating conditions 95^{o}C for 5 min, 50 cycles (95^{o}C 10 sec, 60^{o}C 30 sec, 72^{o}C 1 sec), 40^{o}C 30 sec. All quantification analysis was carried out using the LightCycler software provided by

manufacturer, data produced were through the maximum second derivative method, whereby the entire amplification curve is considered rather than just the threshold point. For each experiment an appropriate housekeeping gene was run concurrently. For each assay, samples were quantified relative to the standard curve to produce a concentration in arbitrary units (AU). From here the mRNA concentration for the gene of interest was then corrected by the mean of two appropriate housekeeping genes.

A number of housekeeping genes were assessed for suitability and it was found that, particularly in developmental studies, the abundance of genes can alter with time. For all developmental studies, the housekeeping genes of choice were elongation factor 1α (*ef1α*) and *18s (*Figure 2.8). For all studies which included only samples of the same developmental stage, the house keeping genes used were *ef1α* and, on occasion, *gapdh.* For adult tissue *ef1α, gapdh* and beta actin *βactin* were used.

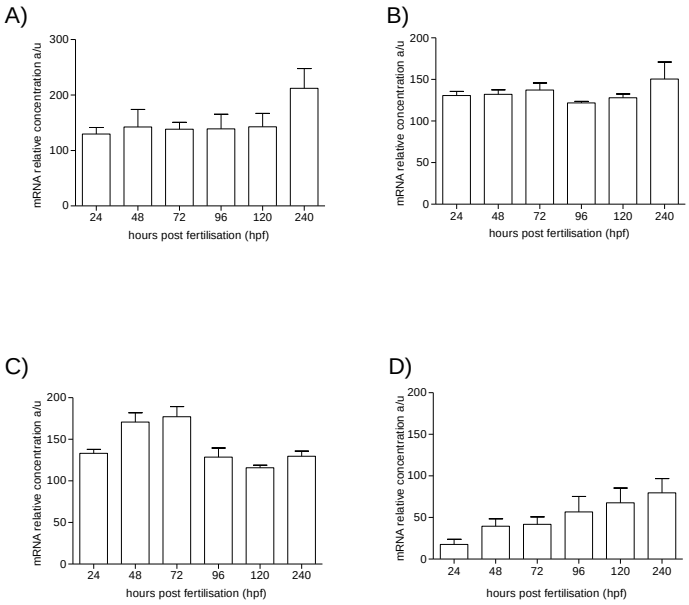

Figure 2.8 Relative abundance of housekeeping genes
Determination of suitability of housekeeping genes for developmental studies. Concentration is determined through the standard curve of pooled samples and thus expressed as a relative concentration (AU). Data shown are for determination of suitable housekeeping gene for the developmental study (24-240hpf). A) Elongation factor 1 alpha (e*f1α*) B) *18s* and C) *β actin* and D) *gapdh*. Data shown are mean ± SEM (n=4; 10 embryos per group).

2.7 Protein study

2.7.1 Radioimmunoprecipitation assay (RIPA) extraction

Tissue was incubated for 3 min in radioimmunoprecipitation assay (RIPA) buffer on ice (6 embryos in 50 µL) containing phenylmethylsulfonyl fluoride (1 mM) and protease inhibitors [1:100] (Sigma-Aldrich protease inhibitor cocktail P2714: AEBSF 2 mM, E-64 14 µM, Bestatin 130 µM, Leupeptin 0.9 µM, Aprotinin 0.3 µM, EDTA 1 mM). Tissue was then homogenised in a Soniprep Ultrasonic Homogenizer (Sonciator - Model 3000MP Ultrasonic Homogenizer, BioLogics). Following sonication, tissues were centrifuged at 12,000 g for 5 min at 4^{o}C. The subsequent protein supernatant was collected to a fresh Eppendorf and then stored at -20^{o}C.

2.7.2 Protein quantification

Protein concentration was determined using a Bradford Assay. Standards of bovine serum albumin (BSA) in RIPA buffer were produced in the range of 0.05 - 1.0 mg/mL. 5 µL of standards, samples or blank (RIPA alone) were added in triplicate to a 96 well plate followed by 25 µL of Reagent A (BioRad, Hertfordshire, UK) and 200 µL of Reagent B (BioRad), and incubated for 15 min at room temperature (approximately 20^{o}C). The absorbance was measured at 750 nm and the concentrations of the samples were calculated using MRX microplate plate reader (Dynex Laboratories Ltd, West Sussex, UK) software and interpolation from the standard curve.

2.7.3 Western blotting

2.7.3.1 Sample preparation and gel loading

Protein samples were diluted to give the desired amount of protein (10µg), using Laemmli 2x buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue and 0.125 M Tris HCl (pH 6.8).

Prior to loading onto the gel, protein samples were incubated at 95^{o}C for 5 min, 20µl of sample was then loaded per well of a 2% Bis-Tris gel (Fisher Scientific UK Ltd, Loughborough, UK). Samples were run as per manufacturer's instructions, with SDS Bis-Tris buffer at 100V for 45 min. The gel was then transferred to nitrocellulose

membrane by a semi-dry transfer method (200mA for 120 min) using transfer buffer (Tris base, pH 8.3, 25 mM, glycine 192mM, methanol 20%).

2.7.3.2 Membrane blocking

The nitrocellulose membrane was blocked using 5% dried milk powder (Tesco, UK) in TBST (NaCl 137 mM, Tris base pH 7.5 20 mM, 0.1% Tween 20) for 1 h at room temperature under gentle agitation.

2.7.3.3 Primary and secondary antibody incubation

Following blocking, the membrane underwent an overnight incubation at 4^{o}C with gentle agitation with the primary antibody (goat anti-trout GR a GR antibody which was a kind gift from Dr M Vijayan, University of Waterloo, Canada) diluted in TBST with 5% dried milk powder (1:250). After the overnight incubation the membrane was washed with TBST 3 x 5min then incubated with TBST containing secondary antibody, a rabbit anti goat antibody conjugated to horse radish peroxidase (HRP) (1:1000) (Santa Cruz Biotechnology, Heidelberg, Germany, Cat no sc-2768) for 2 h at room temperature with gentle agitation. After this a final 3 x 5 min washes were performed with TBST to remove any unbound secondary antibody.

2.7.3.4 Protein detection

Enhanced chemiluminescence (ECL) reagent (GE Healthcare, Buckinghamshire, UK) was applied to the washed membrane as per manufacturer's instructions. In a dark room, the ECL loaded membrane was exposed to photographic film for 5 min to visualise the protein bands. The film was then developed.

2.7.3.5 Membrane stripping and re-probing

To quantify the band and thus the protein content the membrane was stripped of the ECL reagent and the bound GR primary and secondary antibodies and re-probed with the house keeping protein α-tubulin. The membrane was placed in stripping buffer (20 mL 10% sodium dodecyl sulphate (SDS), 12.5 mL Tris HCl (pH 6.8), 67.5 mL H_2O and 0.8 mL β-mercaptoethanol) for 30 min at 50^{o}C. After incubation the membrane was rinsed 3x 5 min with TBST at room temperature. After washing, the membrane was then re-blocked. The membrane was then incubated in primary and secondary antibodies as previously carried out, however for α-tubulin a concentration

of 1:500 (in TBST) of the primary antibody (rabbit host catalogue number AB4074, Abcam, Cambridge, UK) was used with 1:1000 anti-rabbit secondary antibody (Goat anti rabbit, Santa Cruz Biotechnology, Heidelberg, Germany, Cat no sc-2030). After this detection and film exposure were carried out as in section 2.7.3.4. However, in this instance membrane film exposure was for 30 sec and not 5 min as for the GR antibody.

2.7.3.6 Protein density quantification

Protein bands were quantified to determine changes throughout development. To do this densitometry of Western blots was carried out. The films from GR antibody and α tubulin exposure were scanned into a computer using a standard office scanner. The images were then saved as Tiff files; this format of image can then be opened using ImageJ software where the band densitometry plug-in tool was used to subtract the bands obtained from the GR antibody from the α-tubulin band. Each membrane was measured 3 times and the Western blot protocol was carried out 3 times to accurately quantify GR protein abundance.

2.7.3.7 Zebrafish antibody problems

Unlike rodent species, a limited number of antibodies have been produced specifically for the zebrafish. Although the number of proteins which have complementary antibodies in the zebrafish is increasing rapidly there is still a short-fall. A number of papers have reported success in using different antibodies specific for other species. For this work a number of GR-related antibodies were used. However, even with optimisation few were successful. Antibodies which did not work were anti-GR antibody catalogue number Ab3578 (Abcam, Cambridge, UK) and anti-GR antibody catalogue number SC-1004 (Santa Cruz Biotechnology, Heidelberg, Germany). There are many possible factors why these antibodies did not work, including isotope or epitope location.

2.8 Histology

Whole embryos or isolated adult hearts were used for histological examination using a protocol previously published (Sabaliauskas *et al,* 2006) with mounting in a precast agarose mould. Agarose blocks were paraffin embedded by the SuRF@ QMRI

facility (http://www.surf.ed.ac.uk/histology) and paraffin blocks were sectioned, using a microtome, to give 5µm thin sections which were mounted onto microscope slides. After slides were dried overnight, haematoxylin and eosin (H&E) staining was performed using a standard protocol. Masson's trichrome collagen staining was performed by SuRF@QMRI facility staff using a standard protocol. Embryos were sectioned laterally to give full body sections and hearts were sectioned longitudinally through the ventricle.

2.9 Steroid quantification

2.9.1 Steroid extraction-embryonic whole body cortisol

The cortisol content of fish at different stages of development and in response to various GR and pathway manipulations was determined. Groups of 15 embryos from each time point (8-120hpf) or treatment group (pharmacologically or genetically manipulated) of interest were homogenised in 1 mL of systems water using a mixer mill (MM301, Retsch® Leeds, UK) and metal beads. This homogenate was added to 7 volumes of 100% methanol and the mixture was left at 4^{o}C for 24h to remove insoluble matter. 1 mL of this supernatant was dried down under nitrogen then reconstituted with 500 µL of cortisol ELISA assay buffer (0.05 mol/L PBS (pH 7.4) containing 0.1% BSA).

2.9.2 Steroid extraction-adult swim water

An advantage of using adult zebrafish was the possibility to detect cortisol in the swim water- the water surrounding the fish in the tank. Here static stand-alone (those not on the system) tanks were used. Pilot studies were carried out to optimise the experimental set-up.

2.9.2.1 Water volume and stocking density

The volume of water which was found to yield the greatest cortisol levels without compromising animal welfare was found to be 1L. Unsurprisingly it was determined that cortisol expression increased linearly with fish number in tank (Figure 2.9), with 10 fish displaying the greatest level. 5 fish per L was found to be optimum for this work.

2.9.2.2 Sample time

Although levels of cortisol were detectable after only 1 h in the tank, to eliminate for circadian influence, fish were held within test tanks for a 24 h period (to control for diurnal cortisol flux). All stressor experiments, tank transfers, feeding were also carried out at the same time of day (2pm).

2.9.2.3 Experimental considerations

Detectable levels of cortisol were observed in the flow systems water (data not shown). This water flows through all tanks which are present in a system so detectable levels of cortisol would not be surprising. To control for this, enough fresh systems water was made for all tanks investigated at a given time (Section 2.2). The feeding regimen was also found to interfere with cortisol levels, as the SDS feeds (Section 2.2) show minor but detectable levels of cortisol (data not shown). However, as control fish and treatment fish were treated identically this was found not to affect the data, but may influence investigation of food restriction as a stressor; for the latter a fed-water control would be used. To eliminate any food-related influence all tanks received exact rations of feed once in the 24 h test period.

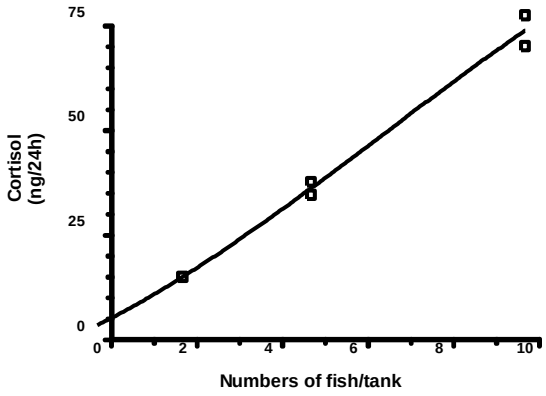

Figure 2.9 Optimisation of quantification of cortisol release by adult zebrafish.
An increasing linear relationship can be seen between the number of adult fish (2, 5, 10 fish in duplicate) per tank (litre volume) and cortisol detected in swim water after 24 hours. Every open square correspond to a sample; n=2.

2.9.2.4 Swim water cortisol extraction

To concentrate the steroids within 500 mL of the holding water, samples were filtered through Sep-Pak®Classic C18 Cartridges-360 mg Sorbent per Cartridge, 55-

105 µm Particle Size (Waters, Hertfordshire, UK) connected to a vacuum system (Waters, Hertfordshire, UK). Prior to sample filtration, cartridges were primed as per manufacturer's instructions with 5 mL methanol, followed by 5 mL of ddH_2O. Samples were fed through the cartridges by a syphon approach, whereby glass bottles containing fish swim water were placed on an elevated platform above the Sep-Pak cartridges with a narrow hose with one end submerged in the water. The other end of the hose was attached to the Sep-Pak cartridge and, when the vacuum system was started, the swim water was syphoned through the cartridge. The flow rate from the hose was regulated by the presence of small taps prior to the cartridge.

When sufficient sample had been filtered through the cartridge the steroids were eluted, using 2 mL of methanol, into glass tubes and stored. Pilot studies with radioactive steroid showed that approximately 100% of steroid was recovered from 1 L of water in this way. The methanol eluent was evaporated to dryness under a stream of nitrogen and was reconstituted in 0.5 mL ELISA assay buffer.

2.9.3 Cortisol enzyme-linked immunosorbent assay (ELISA)

Total cortisol was measured using a sensitive and specific in-house ELISA modified from methods previously described (Al-Dujaili *et al*, 2009).

2.9.3.1 Assay sensitivity

Assay sensitivity is 0.025 ng/mL (0.06 nmol) with extraction recoveries ranging from 94.8-106.7 % for cortisol levels in the range of 2.6-140.8 ng/mL. Cross reactivity of the anti-sheep cortisol antibody were 0.68% for cortisone, 1.12% for deoxycortisol, 0.4% for testosterone,0.01% for progesterone and other interfering steroids <0.01%. Intra- and inter- assay precision values were 3.2% and 5.7% respectively.

2.9.3.2 Enzyme-linked immunosorbent assay (ELISA) methodology

Briefly a 96-well plate (Griener Bio-One, Germany) was coated overnight at 4^{o}C with coating buffer (0.25 mol/L PBS (pH 7.4)) containing 200 ng/mL Cortisol-CMO-BSA conjugate: plates were then (0.02 mol/L PBS (pH 7.4) containing 0.05% Tween 20) and blocked (0.025 mol/L PBS (pH 7.4) containing 0.1% BSA) at 37^{o}C for 2 h. Cortisol standards (0-1000 ng/mL) and samples were added to wells, along with antibody solution (1:20,000 sheep anti-cortisol antibody (Micropharm, London, UK))

and incubated at room temperature for 1 h. Samples were then washed and treated with enzyme solution (1:20,000 horseradish peroxidase-donkey-anti-sheep IgG Sigma Chemical Company, Poole, UK) for 1h. After washing, peroxidase substrate solution (0.33 mg/mL Tetramethylbenzidine (TMB) and 30% H_2O_2 in 0.2M acetate citrate buffer) was added. The reaction was stopped after 15 min incubation at room temperature by the addition of 5 µL 1M H_2SO_4 to each well. Absorbance at 450 nm was measured in an MRX Microplate reader (Dynatech Laboratories).

2.9.4 Deoxycortisol enzyme-linked immunosorbent assay (ELISA)

Total Doxy was measured with a specific in-house ELISA adapted from the above cortisol ELISA protocol. For Doxy ELISA, however, standards were in the range of (0-50 ng/mL). The primary antibody was used at the concentration of 1:10,000 (rabbit anti-deoxycortisol antibody, Micropharm, London, UK). The enzyme solution was used at 1:10,000 (horseradish peroxidase-goat anti rabbit IgG, Sigma Chemical Company, Poole, UK).

2.9.5 Enzyme-linked immunosorbent assay (ELISA) quantification

The readings obtained from the microplate reader were analysed using AssayZap software (Biosoft ® Cambridge) where the standard solutions were used to produce a standard curve and the sample concentrations were interpolated. The concentrations produced by software were given in nmol/L from this a concentration of ng/fish could then be calculated.

2.10 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) unless stated otherwise. Statistical analysis was carried using GraphPad Prism 5.0, as follows, unless stated otherwise in the relevant text. Comparison of two matched groups was carried out as follows, parametric un-paired data by (Welch) unpaired *t*-test, parametric paired data by paired *t*-test, non-parametric and unpaired data by The Mann-Whitney test and non-parametric and paired data by the Wilcoxon matched pairs test. Comparison of three or more groups at one sample point was carried out by one-way analysis of variance (ANOVA) followed by appropriate post-hoc tests (e.g. comparison of every mean to a control mean, by Dunnett's post hoc test or

comparison of every mean with every other mean by Tukey's post hoc test). For studies where groups were affected by two factors (for example time and drug treatment) a two-way (not repeated measures) ANOVA was carried out followed by Tukey or Bonferroni post-hoc tests to compare every column mean with every other column mean or to control mean. Hatch-rate was analysed by Chi Squared analysis of proportions by calculating the degree of difference between the observed data and the null hypothesis (based on the control data). A Bland-Altman plot was produced for ejection fraction to determine the assay accuracy. This analysis was carried out on one sample group where observer 1 determined the ejection fraction, multiple times on separate occasions (intra-observer variation), the same population sample was analysed by observer 2 multiple times on separate occasions (inter-observer variation). The data are plotted as the difference between the two measurements on the Y axis, and the average of the two measurements on the X axis (Figure 2.10). Statistical significance throughout was accepted as $p<0.05$ (*$p=0.05$, **$p\leq0.001$, *** $p\leq0.0001$).

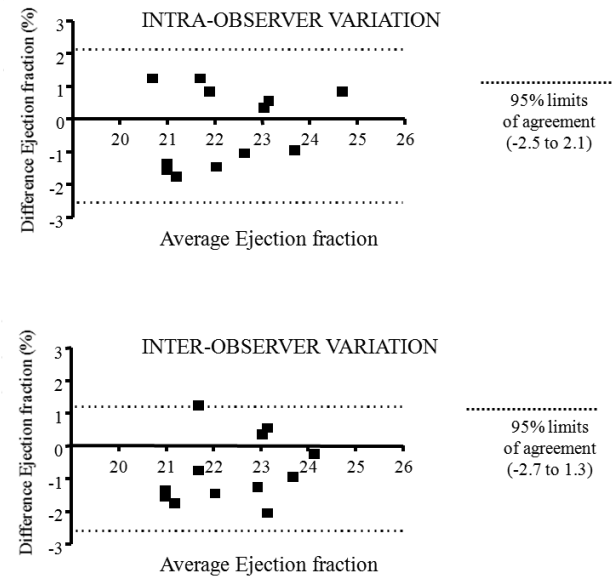

Figure 2.10 Bland-Altman analysis for embryonic ejection fraction.
This statistical analysis was applied to assess intra-observer (A) and inter-observer (B) variation in the measurement of area with ImageJ. The differences between the measures are plotted against the mean for the two tests. The dotted line represents the 95% limits of agreement. Ejection Fraction was assessed twice in the same embryos (number of embryos=12).

Chapter 3: Characterisation of the zebrafish glucocorticoid system

3 Characterisation of the zebrafish glucocorticoid system

3.1 Introduction

As described in chapter 1, GC biosynthesis is a tonically active process (with a circadian diurnal pattern). However, a well-documented increase in GC biosynthesis occurs in response to stressful stimuli where, along with catecholamine signalling pathways, GCs enable the body to deal with and respond to the stressor by increasing essential and decreasing non-essential bodily functions. This increase in GC levels is an evolutionary conserved, physiological homeostatic mechanism common amongst most species (Steenbergen *et al*, 2011). GC are also used therapeutically for conditions such as asthma, rheumatoid arthritis and inflammatory bowel disease (Wei *et al*, 2004) and have often been shown to produce similar therapeutic effects in animals as observed in humans (Miller, 1999).

GC levels are regulated by negative feedback of the HPA axis and tissue specific local enzyme inactivation. Short-term increases in circulating GC are advantageous but chronic increases, due to impaired regulatory mechanisms, can be detrimental (Chapman & Seckl, 2008) and lead to side-effects such as elevated blood pressure (Dixon & Bansback, 2012; Schacke *et al*, 2002)). As highlighted in chapter 1, understanding better the cellular and molecular actions of GC will allow greater knowledge of the physiological and pathophysiological roles of these hormones. Few animal models have been widely accepted for investigation of GC physiology; while the mouse model is the most commonly used it is not without problems.

The teleost fish has been suggested as a complementary model for GC research (Hsu *et al*, 2006; Pikulkaew *et al*, 2010; Steenbergen *et al*, 2011). Previous studies in adult zebrafish have shown that their corticosteroid system has many similarities with the human system at a molecular and cellular levels (Steenbergen *et al*, 2011; Veldman & Lin, 2008). The GC synthesis pathway in the adult zebrafish is believed to be virtually identical to that found in mammals (Leatherland *et al*, 2010; Lohr & Hammerschmidt, 2011; Schaaf *et al*, 2009; Schoonheim *et al*, 2010; Steenbergen *et al*, 2011; Tokarz *et al*, 2013). Furthermore the zebrafish embryo allows relatively easy observation of cellular and organ specific development which is particularly

important for determining physiological function. In addition, these developmental processes can be readily manipulated by altered pharmacological and environmental conditions (Zon & Peterson, 2005).

While these features support the utility of the zebrafish as a model for GC research, a limited number of published studies have fully assessed the functional roles of the GC system during embryonic development. For example, a number of issues remain unanswered in relation to the biochemical, anatomical, organisational and mechanistic control of GC biosynthesis in zebrafish embryos. A comprehensive review of the importance of this pathway in zebrafish development is particularly lacking. While cortisol is a known indicator of stress in the adult zebrafish it is unclear whether the machinery is in place to mediate such a response in the embryo and, if so, when. While it has previously been shown that cortisol is present in the zebrafish embryo (Alsop & Vijayan, 2008) the physiological relevance during development has never been investigated.

3.2 Hypothesis and aims

The zebrafish embryo has a physiologically functional GC system which is responsive to physiological or environmental stimulus.

Data presented here aimed to address the following questions:

1) Are the key components for GC biosynthesis present in the zebrafish?
2) When does *de novo* GC biosynthesis occur in the zebrafish embryo?
3) Is there endogenous production of GC in response to stress?
4) Does maternal stress impact on embryonic cortisol levels?

3.3 Methods

3.3.1 Embryonic glucocorticoid modulation

Modulation of GC activity in zebrafish embryos was carried out as described (Section 2.4.4). Briefly all modulations were carried out at the 2 cell stage (approx. 1h after egg collection) until 120 hpf in accordance with Home Office regulations. Embryos were housed in standard husbandry conditions as described in a 10cm Petri dish at a density of 1 embryo/mL.

3.3.1.1 Pharmacological manipulation

Pharmacological manipulation was performed by bathing embryos in various concentrations of the following drugs: Doxy, Met and Dex. All drugs were dissolved in EtOH (0.1%) (optimisation in Appendix 1) and diluted to final concentration in systems water. Embryonic survival, phenotype scoring, and drug replacement were performed daily over the 120h study.

3.3.1.2 Genetic manipulation

Genetic manipulation was by Mo targeted to the *cyp11b1* gene, which is responsible for encoding the catalytic enzyme 11β-hydroxylase responsible for the conversion of 11-deoxycortisol to cortisol (Figure 3.1). Mo design, sequence and dosage optimisation are described in Appendix 1. Successful genetic manipulation was determined by a significant reduction in *cyp11b1* mRNA gene abundance in ss-Mo (ss-Cyp Mo) injected embryos compared to mm-Mo (mm-Cyp Mo (Figure 3.2)). A wide dose ranging study demonstrated that the concentration of ss-Cyp Mo that produced a significant (30%) knockdown of *cyp11b1* mRNA, without altering survival or phenotype, was 6 ng/nL. This concentration was found to also produce comparable survival and phenotype data for the translational blocking (atg-Mo) targeted for *cyp11b1* knockdown (atg-Cyp Mo) and mm-Cyp Mo. As a result a concentration of 6 ng/nL was used for all. As previously highlighted, unless stated otherwise, data shown from here on in will be for translational blocking atg-Mo, and will be referred to as Cyp Mo throughout (Chapter 2).

As for pharmacological manipulations embryos were stored in systems water which was replenished daily over the 120 h study whilst scoring survival and phenotype

occurred daily. Confirmation of embryonic Mo uptake was achieved by the cytoplasmic presence of the fluorescent fluorescein 5' Mo modification (Section 2.4.4).

3.3.1.3 Embryonic experimental controls

For all pharmacological manipulations carried out in this chapter controls are vehicle only controls (0.1% EtOH as determined in appendix 1). For molecular manipulation using morpholino the controls shown here are injected with mm-Cyp Mo.

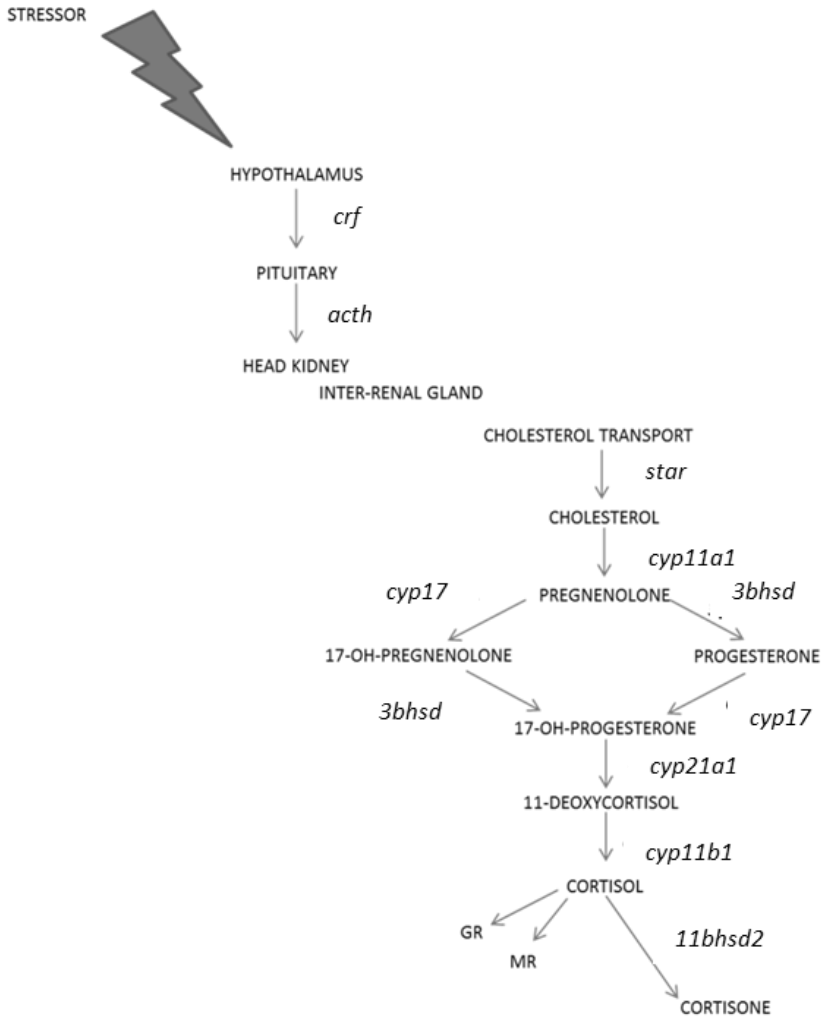

Figure 3.1 Schematic of the mature zebrafish glucocorticoid system.
Summary of key reactions, enzymes and components of the mature zebrafish GC systems. This schematic shows corticotrophin releasing factor (*crf*), adrenocorticotropic hormone (*acth*), steroidogenic acute regulatory protein (*star*), cholesterol side-chain cleavage enzyme (*cyp11a1*), 17α-hydroxylase (*cyp 17*), 3β-hydroxysteroid dehydrogenase (*3βhsd*), 11β-hydroxylase (*cyp11b1*), 21α-hydroxylase (*cyp 21a2*).

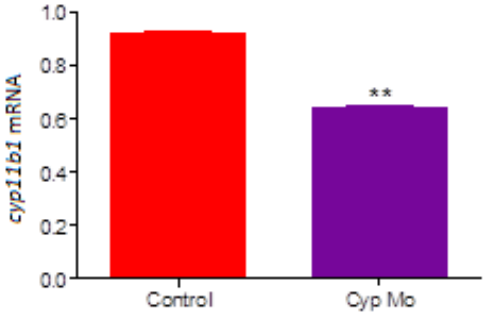

Figure 3.2 Confirmation of *cyp11b1* knockdown by Cyp Mo
Gene abundance analyses for *cyp11b1* mRNA at 72 hours post fertilisation (hpf) in control (mism-Mo-injected) whole embryo homogenate compared to those which had been injected with 6 ng/nL *cyp11b1* splice site specific morpholino (Cyp Mo). N=3 expts (10 embryos per group). Gene abundance is given as an arbitrary concentration (AU) and normalised to house-keeping gene. Columns represent mean ± SEM. Comparisons were made using Student's *t*-test, ** $p<0.001$.

3.3.2 Maternal glucocorticoid manipulation

To determine whether the early detectable GC in the embryo was maternally-derived a maternal GC modulation experiment was carried out using Dex. Two forms of modulation were investigated, maternal bathing and injection.

For bathing modulation adult female WIK zebrafish were placed in a stand-alone 1 L tank containing distilled water at a density of 6 fish per L. To this Dex was added to give a final tank concentration of 10 µM Dex. The drug was replaced every second day (after pH, chlorine and salt levels were assessed and found to be comparable to un-altered distilled water).

For the injection experiment, intraperitoneal (IP) injection of Dex was carried out using an adapted protocol of Kinkel and colleagues, (Kinkel *et al*, 2010). Following anaesthesia fish were placed ventral side upwards in a slotted, moistened sponge and a 5 µL bolus of 1 nM Dex was injected.

3.3.2.1 Maternal experimental controls

Controls for mothers bathed in Dex were mothers bathed in vehicle only (0.1% EtOH). Vehicle was replaced every second day. Controls for maternal Dex injections

were injected with a 5 µL vehicle (0.1% EtOH) only bolus. Injections of vehicle occurred twice weekly. Experiment was repeated in triplicate (6 adult fish per tank)

3.3.2.2 Maternal manipulation offspring

After 35 days of treatment the manipulated mothers were placed in pair-mating tanks with un-manipulated WIK male fish (1:1 ratio). Embryo production was assessed daily with fecundity and survival recorded. Embryos were collected for assessment of cortisol concentration at 8, 24 and 120 hpf.

3.3.3 Gene abundance study

Gene abundance analysis was performed for a range of GC genes of interest in whole embryo homogenates over the course of 120 h. Embryonic genes investigated were *cyp11b1*, *cyp11a1*, *gr*, *mr*, *11βhsd2*, *20βhsd*, *star* and *crf* as these have been highlighted in mammalian models as key indicators of active GC production (the primer sequences for these genes are shown in Table 3.1). Gene abundance was analysed throughout the course of development over a range of time points - 8, 24, 48, 72, 96 and 120 hpf (n=5 samples per time point (10 embryos per sample)). All embryonic time-course studies were normalised to *ef1α* and *18s.* Adult gene abundance was measured in isolated kidney, heart, brain and liver for *gr*, *mr*, *11βhsd2* and *cyp11b1*.

Table 3.1 mRNA gene abundance primer sequences.
Glucocorticoid genes of interest with forward and reverse primer sequence used for qRT-PCR

Gene	Forward Primer	Reverse Primer
cyp11b	gcagacacagcaaaggagtct	gacagacgaggacaccatca
cyp11a1	ggatataaaggccagtgtcacc	tcataaagcgtccacagcag
gr	ttctcaagcagcccctattc	tctttccaccagctgacgat
mr	cctgcagagtacgcaagtgt	cccaacttcttcgacttcc
11βhsd2	gggggtcaaagtttccactat	gtactctgcgttactgctctgc
star	cctggagctagcacttggat	agcactggtcagcttcactg
crf	tccaaggatttaccaattacgc	tcatacggcggtggaaag
20βhsd2	aatggttgaaaggggggaaag	ttatgggtcatgttcgtgga

3.3.4 Cortisol enzyme-linked immunosorbent assay (ELISA)

Whole embryo cortisol was extracted and analysed from homogenates as described (section 2.9). Adult cortisol excretion was determined by analysing swim water.

Cortisol levels either in tissue homogenates or in the swim water were calculated by interpolation from cortisol standard curve in the range of 1000-10 ng/µL, using AssayZap, assay calculating software (BioSoft).

3.3.4.1 Embryonic cortisol: time-course

To determine the expression profile of cortisol in the embryo a time-course study over 120 hpf was performed using whole embryo homogenates. The time points investigated here were 8, 18, 24, 36, 48, 72, 96 and 120 hpf (n=5 samples, (n=10 embryos per sample)). Embryos were collected at the time-points of interest and processed as described (section 2.9).

3.3.4.2 Embryonic cortisol: stress

For embryonic stress experiments, unless stated otherwise, all embryos were sampled at the same time of day (between 14.00-15.00h) under standard light: dark cycle conditions to control for the possible influence of diurnal circadian cortisol production (Dekens *et al*, 2003; Dickmeis, 2009; Lohr & Hammerschmidt, 2011). Stressed embryos were stored in an incubator for 1 h after stressor (at the time-point of interest) prior to euthanizing and homogenisation. This allows for induction of the stress response as the release of cortisol in teleostean and other bony fishes is delayed relative to catecholamine release (Barton, 2002) with cortisol synthesis and release from interrenal cells being shown to have a lag time of several min after stressor (Iguchi *et al*, 2003). Cortisol was extracted from whole embryo homogenates as described (section 2.9). Controls are described in section 3.3.6. Experiment was repeated in triplicate (10 embryos pooled).

3.3.4.3 Embryonic cortisol: drug treatment

To determine whether drug incubation had any influence on cortisol levels, embryos which had been continuously incubated in drug (from the 2-cell stage) were thoroughly rinsed through a series of system water prior to cortisol extraction as described in section 2.9.1. For drug data ELISA, the concentrations determined were normalised to an embryo free drug solution (containing the equivalent dose of drug) to determine whether background levels of cortisol are measured as a result of drug presence. Experiment was repeated in triplicate (10 embryos pooled).

3.3.4.4 Adult cortisol: drug treatment

For determination of the maternal cortisol influence on embryonic cortisol levels, adult fish were treated with Dex (section 3.3.2). Water was collected at two time points during Dex treatment (24h after initial exposure and 24h after final drug exposure). All samples were collected at 2pm on sampling day then cortisol extraction and ELISA were carried out as per adult swim water cortisol (described in Section 2.9.2). Experiment was repeated in triplicate (6 adult fish per tank).

3.3.5 Cortisol radioimmunoassay (RIA)

The cortisol was further determined over the time-course study using an appropriate radioimmunoassay (RIA). The RIA was only used here to confirm the ELISA data and was not used for any other study as the ELISA was felt to be faster, cheaper, more reproducible and safer (no radio conjugate is required for ELISA). The same homogenisation/ cortisol extraction method was used as for the ELISA (Section 2.9.1). However after the samples were dried down under N_2, they were reconstituted with 100µl of borate buffer (0.133 M boric acid, 67.5 mM NaOH and adujusted to pH7.4 with 1M HCl containing 0.5% bovine serum albumin, 1% methanol and 0.01% ethylene glycol). For the RIA 25 µL of either sample extract or standards (0.31-320 nM cortisol) were incubated with 25 µL of cortisol sheep antibody (MicroPharm, 1:3000), 25 µL of anti-sheep SPA scintillation beads (GE healthcare) and 25 µL of ^{125}I labelled cortisol (MP, Biomedicals), in 96-well plates. Plates were covered with transparent film, shaken and left to incubate at room temperature for 6 h for the reaction to reach equilibrium. The γ-emission of bound ^{125}I was measured by counting each well for 5 min in a Gamma scintillation counter (Wallac, PerkinElmer, Cambridge, UK). Sample concentrations were determined by interpolation from the standard curve using AssayZap assay quantification software (BioSoft). Experiment was repeated in triplicate (10 embryos pooled).

3.3.6 Embryo stressor

A number of "stressors" were assessed for activation of HPI activity. HPI activity was determined by a rise in cortisol concentrations (determined by ELISA; section 2.9) following stress, compared with un-stressed controls. Experiment was repeated in triplicate (10 embryos pooled).

3.3.6.1 Embryonic hypoxia

To assess hypoxia as an environmental stressor, embryos (24, 72 and 120hpf) in 30mL Petri dishes containing systems water (10 embryos/group; 3 h) were placed in a hypoxic chamber (Coy cabinet) in the following conditions: 1% oxygen, 5% carbon dioxide, 94% nitrogen at 28.5°C and a relative humidity of 45%.

3.3.6.2 Embryonic kinetic stress

Two forms of kinetic stressors were investigated for their impact on cortisol production. 10 embryos were placed in 30 mL Petri dishes containing systems water at 28.5°C. For constant movement stressor, the Petri dish was placed on a horizontal shaker at 30rpm for 1h. For brief kinetic stressors embryos were placed on a horizontal shaker for 100 rpm for 1min.

3.3.6.3 Embryonic electrical stress

Direct electrical current pulse (DCP)-induced stress was induced by placing 10 embryos in a 30 mL Petri dish and passing a 2 mA electrical current (10 m/s duration, 3 times per h) through the bathing water over a 2h period.

3.3.6.4 Determination of stress cortisol

For all methods of stress induction embryos were left in an incubator (28.5°C) for 1 h after exposure to the stressor to allow for induction of stress-induced cortisol production. Embryonic cortisol was then measured by extraction followed by ELISA, as described (section 2.9). Controls were treated the same as controls, i.e. were taken out of incubator for the duration of experiment and kept on a bench near stressor protocol thus received all same handling, lighting, temperature apart from stressor.

3.3.7 Experimental controls

In the following experiments data were obtained primarily in the un-manipulated zebrafish (embryo and adult). These results are displayed in the open (colourless) columns. Where a manipulation (pharmacological or molecular) was carried out controls (red column) were either vehicle only (0.1% EtOH for drug studies) or mm-Mo (for Mo studies). Adult drug studies were carried out by treating adults with Dex

(black columns) the controls were treated with vehicle only (0.1% EtOH - red columns). For embryonic stress experiments temporal controls were used.

3.4 Results

3.4.1 Aim 1

Are the key components for GC biosynthesis present in the zebrafish?

3.4.1.1 Important GC genes are found in isolated adult tissue

To confirm previously reported adult zebrafish data the abundance of *gr*, *mr*, *cyp11b1* and *11βhsd2* was detected in isolated adult tissues. *gr* and *mr* mRNA were both detected at varying levels in all tissues investigated (brain, kidney, heart and liver), with *gr* mRNA abundance highest in the brain. *11βhsd2* was found in the brain and at lower levels in the heart and kidney, there was virtually no *11βhsd2* in the liver. *cyp11b1* was found in the kidney with levels in other tissues negligible (Figure 3.3).

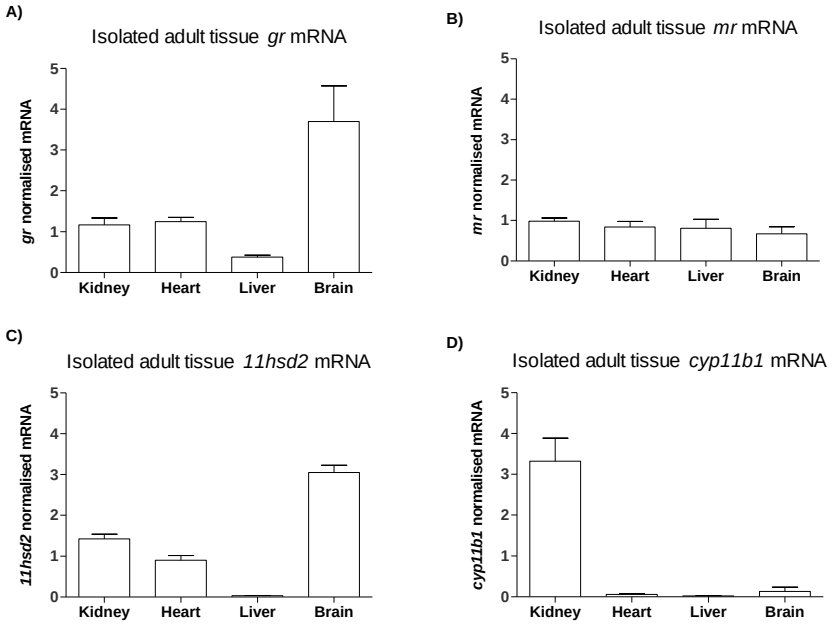

Figure 3.3 Relative abundance of glucocorticoid genes in adult zebrafish
Relative mRNA abundance of key glucocorticoid genes in isolated adult zebrafish tissue. A) *gr* mRNA, B) *mr* mRNA, C) *11βhsd2* mRNA and D) *cyp11b1*mRNA. All gene abundance is n=3 (5-10 pooled organs) mean ± SEM. Gene abundance is quantified through standard curve production and normalisation to the housekeeping genes *ef1a* and *βactin* Data are presented as arbitrary units (AU).

3.4.1.2 Important GC genes are found throughout development in embryos

A number of key genes important for GC biosynthesis, metabolism and action were investigated in the developing embryo (8-120hpf). Changing patterns of relative abundance were shown for mRNA of all of the factors associated with GC system over the 120 hpf time-course of the study (Figure 3.5).

gr mRNA (Figure 3.5A) abundance increased significantly ($p \leq 0.0001$) by 48 hpf (0.96 ± 0.09AU) compared with 24 hpf (0.38± 0.04AU). This higher level was sustained until 120 hpf ($p \leq 0.0001$ vs. 24 hpf). Similar to *gr* mRNA relative abundance, Gr protein abundance in whole embryo homogenates (n=3, 5 embryos per sample) was found to increase significantly at 48 hpf compared to 24 hpf, with levels thereafter sustained until 120 hpf at which point a rise in protein levels was observed (Figure 3.4).

Unlike *gr*, the mRNA levels of *mr* remained constant over the first 48 hpf (0.33 ± 0.07 and 0.39 ± 0.06 AU for 24 and 48 hpf, respectively) then increased significantly by 72 hpf ($p \leq 0.0001$). This level of relative abundance was maintained for 96 and 120 hpf (Figure 3.5B). A similar temporal pattern of abundance was observed for the Mr regulatory enzyme *11βhsd2*, with no difference in levels between 24 and 48 hpf but with a significant increase noted at 72 hpf compared to 24 hpf ($p<0.001$(Figure 3.5C)). Another regulatory enzyme investigated was the novel 20βhsd2 enzyme which is found in teleost fish and is proposed to be an important enzymes in cortisol catabolism in zebrafish where it is responsible for converting cortisone into 20β-hydroxycortisone, providing a route for cortisol catabolism (Tokarz *et al*, 2012). When the abundance profile was investigated it was found to be a similar to *11βhsd2*, that is, expressed at low levels in the first 48 hpf but significantly increased significantly at 72hpf; a feature which was sustained over the remaining 24h until 120 hpf (Figure 3.5H).

The temporal abundance pattern of *crf* mRNA, the main mediator of stress-induced GC biosynthesis showed low levels initially with a significant increase from 72 hpf, with the levels detected at 96 ($p<0.001$) and 120 hpf ($p<0.05$) significantly higher than those detected at 24 hpf (Figure 3.5D)).

star, the gene responsible for cholesterol transportation in steroidogenesis, displayed higher levels at 72 compared to 24 hpf ($p<0.05$). However the levels detected at all other time points were comparable to the 24 hpf level (Figure 3.5E). The levels of *cyp11a1* mRNA remained constant during the course of development with no significant alteration with time (Figure 3.5F). In contrast *cyp11b1* mRNA relative abundance showed a biphasic pattern, initially lowest at 24 hpf, rising significantly at 48 hpf compared to 24 hpf ($p\leq0.0001$), and then falling again at 72 hpf. Higher levels were then detected at 96 and 120 hpf ($p\leq0.0001$).

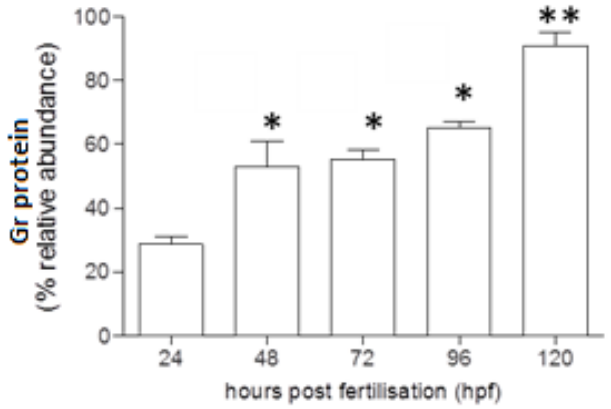

Figure 3.4 Glucocorticoid receptor protein levels in whole zebrafish embryos
Embryonic zebrafish glucocorticoid receptor protein (Gr) abundance studied over 120 hours post fertilisation (hpf). Gr is presented as percentage (%) relative abundance as determined by band density normalised to the house-keeping protein α-tubulin, determined using ImageJ image analysis software with band density plugin. Data are mean ± SEM (n=3 blots (n=8 embryos <72hpf; n=5 embryos >72hpf)) analysed by 1-way ANOVA and Dunnett's post hoc test. *$p \leq 0.05$, **$p \leq 0.001$ compared to 24hpf.

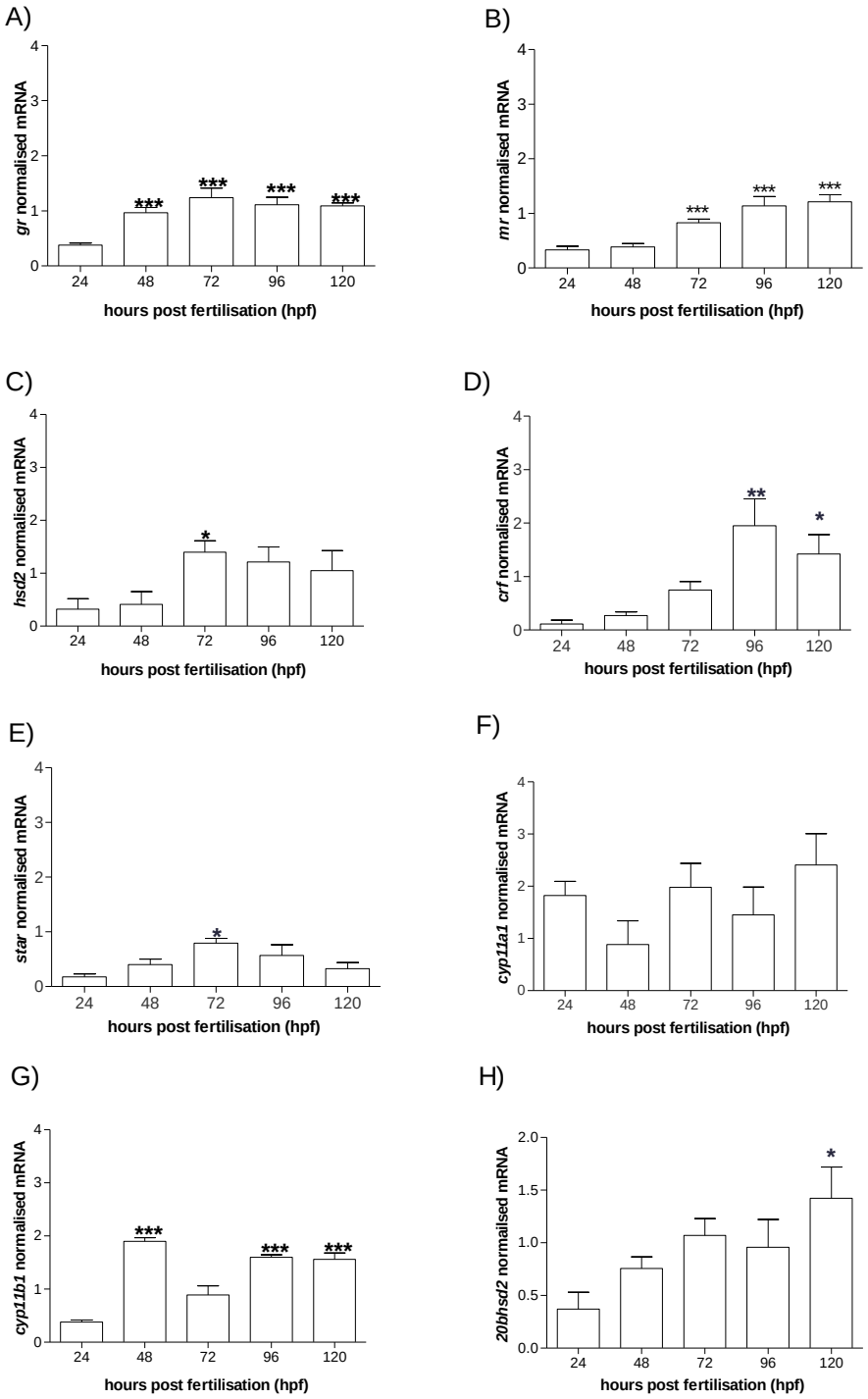

Figure 3.5 Relative abundance of genes regulating glucocorticoid biosynthesis
Relative mRNA abundance of glucocorticoid related genes over the course of the first 120 hours post fertilisation (hpf). A) *gr*, B) *mr*, C) *11βhsd2*, D) *crf*, E) *star*, F) *cyp11a1*, G) *cyp11b1* and H) *20βhsd2*. In all cases columns represent mean ± SEM (n=6 (10 embryos per time pooled)). Gene abundance is presented as arbitrary units (AU) after standard curve production and normalisation to *ef1a* and *18s*. Data were analysed by 1-way ANOVA and Dunnett's post hoc test. *$p \leq 0.05$, **$p \leq 0.001$ and ***$p \leq 0.0001$ compared to 24hpf.

3.4.1.3 Embryonic zebrafish cortisol levels show a biphasic pattern

Following demonstration of components of the GC system in the zebrafish embryo, investigations were performed to determine whether cortisol levels were actively regulated in the embryo. Cortisol was detected at all time-points investigated. Levels were found to decrease from initial levels at 8hpf (2.2 ± 0.29 ng/embryo) until 36 hpf (0.4 ± 0.42 ng/embryo; p=0.01). Thereafter, the levels increased steadily from 48 hpf onwards reaching 3.39 ± 0.44 ng/embryo at 120 hpf; more than 1 ng/embryo greater than the initial detectable levels at 8 hpf (Figure 3.6A). To confirm these data and ELISA specificity further a RIA method was used for cortisol detection. A similar cortisol profile was observed as for the cortisol ELISA, supporting ELISA specificity (Figure 3.6 B).

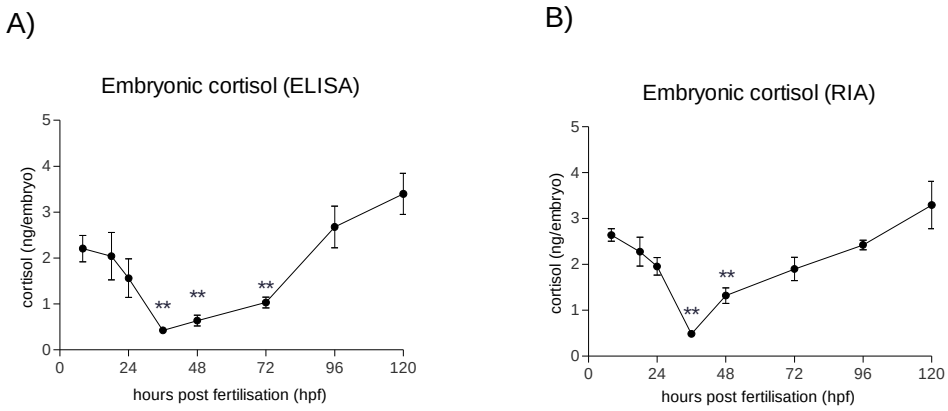

Figure 3.6 Zebrafish whole embryo cortisol levels
Cortisol concentration over the course of 120 hours post fertilisation (hpf) in whole embryo homogenate. Cortisol levels were determined by two methods. A) Cortisol ELISA (n=6 (10 embryos per sample)). B) Cortisol radioimmunoassay (n=3 (10 embryos per sample)). Data are mean ± SEM and were analysed by 1-way ANOVA and Dunnett's post hoc test. ** $p \leq 0.001$ vs 8 hpf levels..

3.4.2 Aim 2

When does* de novo *GC biosynthesis begin in the zebrafish embryo?

3.4.2.1 Embryonic cortisol pharmacological manipulation

Incubation of embryos over 120 hpf with the 11β-hydroxylase substrate Doxy [1μM] resulted in a significant increase in embryo cortisol levels compared to vehicle only controls (5.43 ± 0.01 vs. 3.68 ± 0.02 ng/embryo (p=0.05(Figure 3.7A)). In embryos incubated with increasing concentrations of Met there was a concentration-dependent

decrease in cortisol levels compared to controls at 120hpf, with a 15% reduction noted for 0.1μM Met, 47% reduction for 1μM Met and a 66% reduction at the highest concentration (10μM Met) (Figure 3.7C). Conversely an ELISA specific for Doxy demonstrated a concentration-dependent increase in the levels of substrate, Doxy, with the highest detectable level of Doxy observed for the highest concentration of Met investigated (10 μM (Figure 3.7 D)).

Co-incubation of Met with 0.5μM Doxy impaired the increase in cortisol production observed with Doxy alone, a concentration-dependent reduction in cortisol levels was observed with increasing concentrations of Met. Incubation in a further 11β-hydroxylase substrate Doc [0.5μM], as expected had no influence on the forward reaction and produced comparable data to controls with increasing concentrations of Met (Figure 3.7 E).

A further pharmacological manipulation which was carried out to determine HPI axis activity was Dex treatment, following continuous exposure from the 2-cell stage. Whole embryonic cortisol levels were reduced by ~25% at 120 hpf ($p<0.05$) (Figure 3.7 B).

Continuous incubation in 1μM Met resulted in a significant reduction in whole embryo cortisol by 120 hpf, at time points prior to this (24 and 72 hpf) however, the effects of Met on cortisol were not significantly different from controls (Figure 3.8).

Furthermore, the Cyp Mo treated embryos (30% reduction in *cyp11b1* mRNA at 72 hpf) displayed a significant (~45%) reduction in cortisol levels at 120 hpf ($p<0.01$). Similar to the observations for Met there was no difference between this group and the controls at 24 or at 72 hpf ($p>0.05$ (Figure 3.8A)).

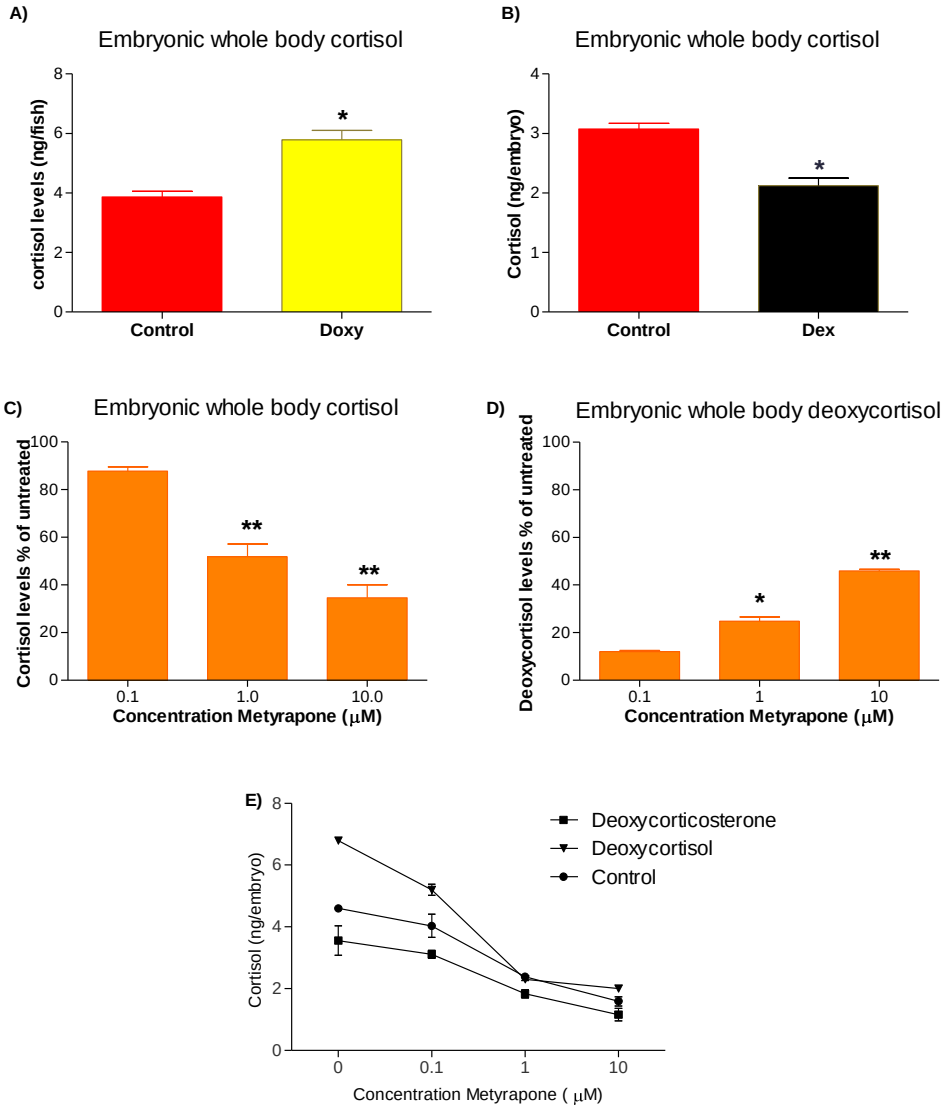

Figure 3.7 Embryonic cortisol levels following pharmacological manipulation
Whole embryo steroid levels in embryos 120 hours post fertilisation (hpf) as determined by ELISA following various forms of pharmacological manipulation. A) Whole embryo cortisol data for embryos incubated in 1μM deoxycortisol (Doxy) for 4 hours prior to cortisol sampling vs. controls (vehicle only). B) Cortisol levels in embryos treated continuously with dexamethasone (Dex) from the 2 cell stage vs. controls. C) Cortisol levels following continuous incubation in 0.1, 1 and 10μM metyrapone (Met) from 2 cell stage vs. control. D) whole embryo Doxy levels following continuous incubation in 0.1, 1 and 10μM Met from 2 cell stage vs. controls. E) cortisol data for embryos continuously incubated with Met at 0.1, 1 and 10μM, co-incubated with 0.5 μM Doxy or 0.5 μM deoxycorticosterone for 4 h prior to sample preparation. All data are mean ± SEM (n=3 (10 embryos per group)), A and B were analysed by Student's *t*-test, C-E by 1-way ANOVA and Dunnett's post hoc analysis); *p≤0.05, ** p≤0.01.

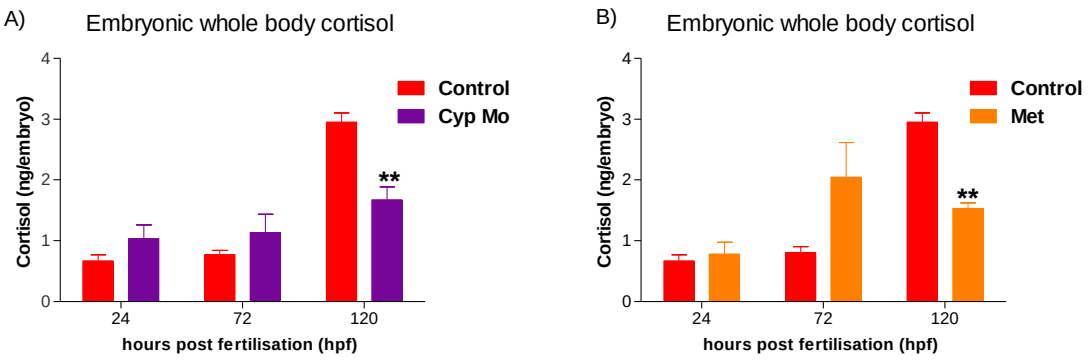

Figure 3.8 Embryonic cortisol levels following 11β-hydroxylase manipulation
Whole embryo cortisol levels at 24, 72 and 120 hours post fertilisation (hpf) following manipulation of 11β-hydroxylase activity by A) *cyp11b1* targeted morpholino (Cyp Mo) injected at the two cell stage, or B) 10µM metyrapone (Met) compared in both cases to control data (mismatch morpholino for Cyp Mo and vehicle only for Met). Data are mean ± SEM (n=3 (10 embryos per group)) and were analysed by 2-way ANOVA and Bonferroni post hoc analysis.**$p \leq 0.01$ compared with control.

3.4.2.2 Pharmacological manipulation of cortisol in adults

As part of the experiment to manipulate maternally-derived cortisol in zebrafish embryos, adult female zebrafish, were treated with the synthetic GR agonist Dex for 35 days. This duration of treatment was chosen to reflect the time period of gametogenesis in the fish (Carnevali *et al*, 2010).

Two routes of administration were investigated, 1) Dex in the bathing water (Dex-B) and 2) IP injection (Dex-Inj). Each Dex administration group had an appropriate vehicle control group (C-B or C-Inj). As part of the experiment, cortisol was measured in the swim water (the water surrounding the fish) over the course of the 35 day study. On occasion cortisol was measured in an adaption of a human Dex suppression test in which Dex is used to assess the functionality of the patient's HPA axis. Dex should produce a negative feedback on the pituitary gland to supress the production of Acth and Crh, and thus reduce cortisol production. In adult fish cortisol water was collected 24h after the first treatment (either injection or bathing) to assess the negative feedback regulation of cortisol production (Figure 3.9). It was observed that Dex Inj produced a reduction in cortisol levels.

When cortisol levels were measured at a later stage (35 days) of the experiment -24h after final Dex treatment- a more pronounced alteration was observed between the groups. Dex-Inj fish had significantly lower cortisol than C-Inj similarly Dex-B fish also displayed a reduction in cortisol levels compared to the C-B. Overall, the trend for Dex–Inj and Dex-B adults was similar, with both displaying reduced cortisol level in the swim water suggesting Dex suppression of endogenous cortisol production in the adult female zebrafish.

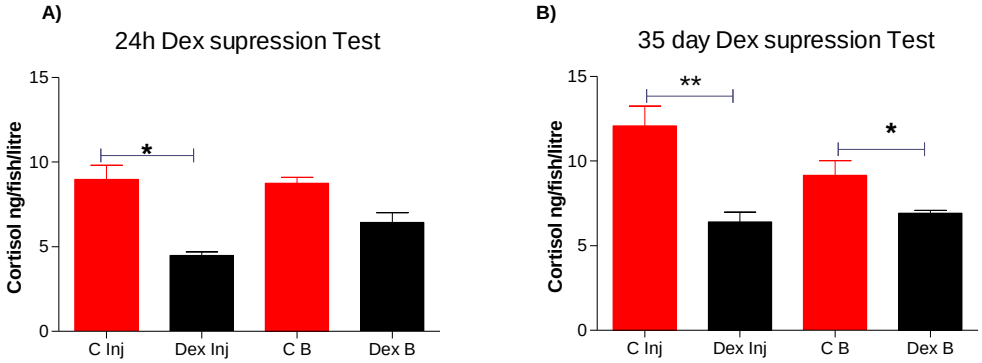

Figure 3.9 Adult zebrafish swim water cortisol after dexamethasone treatment
Measurement of cortisol in swim water from tanks containing adult zebrafish for 24 h. Zebrafish were pre-treated with with dexamethasone (Dex) or vehicle only controls (C). Exposure to Dex was achieved either by injection (Dex-Inj or C-inj) or by administration to the swim water (Dex-B or C-B). Data displayed are of sample collections at A) 24 hour after initial treatment or B) 24h after final treatment (35 days after the first treatment). Cortisol levels were quantified by ELISA and concentrations determined through standard curve intrapolation. Data are mean ± SEM (n=3 (6 adult fish per tank) analysed by Student's *t*-test. *p≤0.05, ** p≤0.001 vs respective controls.

3.4.3 Aim 3

Is there endogenous production of GC in response to stress?

3.4.3.1 Cortisol is produced in response to stressful stimuli

A number of stressors were assessed including kinetic agitation (both brief and prolonged exposure), 3 h of hypoxia (1% O_2) and Direct Current electrical impulse passed through the bathing water. Changes in cortisol were assessed using these three stressors at 3 developmental time points 24, 72 and 120 hpf (Figure 3.10). At 24 hpf none of the stressors caused an increase in cortisol compared to controls (Figure 3.10A). A stress response (as determined by a significant rise in cortisol levels) was detected at 72 hpf. At this time-point, hypoxia, prolonged kinetic agitation and DCP

all significantly increased cortisol ($p<0.001$). No significant increase in cortisol was observed for brief kinetic agitation compared to controls (0.77 ± 0.06 ng/embryo, $p=0.57$ (Figure 3.10B)). At 120 hpf brief kinetic agitation and hypoxia did not alter the levels of cortisol compared to controls ($p>0.05$). A significant increase in cortisol was noted however for prolonged kinetic agitation and for DCP ($p<0.001$; Figure 3.10C).

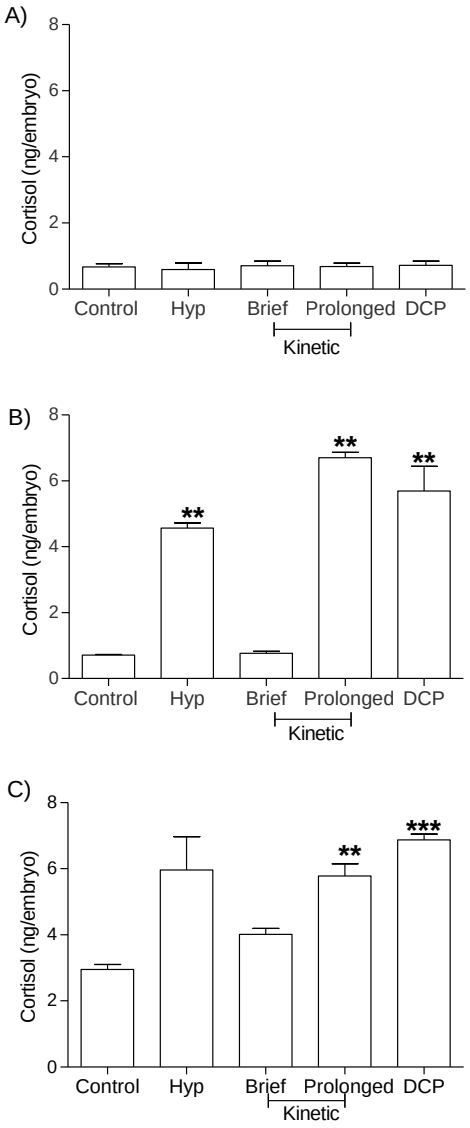

Figure 3.10 Embryonic cortisol levels after stress
The impact of hypoxia (Hyp), brief or prolonged kinetic stressor, or direct electrical current pulses (DCP) were investigated on embryos aged 24, 72 or 120 hours post fertilisation (hpf). Stimuli were investigated for activation of stress response by measuring increases in whole embryo cortisol levels by ELISA. Data shown are following stressor at A) 24 hpf B) 72 hpf and C) 120hpf. Data are mean ± SEM (n=3 (10 embryos per experiment)) and were analysed by 1-way ANOVA and Tukey's post hoc test. ** $p\leq0.01$, ***$p\leq0.001$ compared to age- matched controls.

3.4.3.2 Embryonic stress induced cortisol production is controlled by HPI axis

While a marked increase in cortisol levels was associated with prolonged kinetic agitation at 120 hpf in Figure 3.10 this was found to be abolished in embryos that had been treated with either Met or Cyp Mo from the 2 cell stage (Figure 3.11) (Met + prolonged kinetic stress 0.89 ± 0.08 vs. prolonged kinetic stress 5.80 ± 0.80 ng/embryo ($p<0.001$) and (Cyp Mo + prolonged kinetic stress 1.10 ± 0.15 v prolonged kinetic stress 5.80 ± 0.80 ng/embryo, ($p<0.001$).

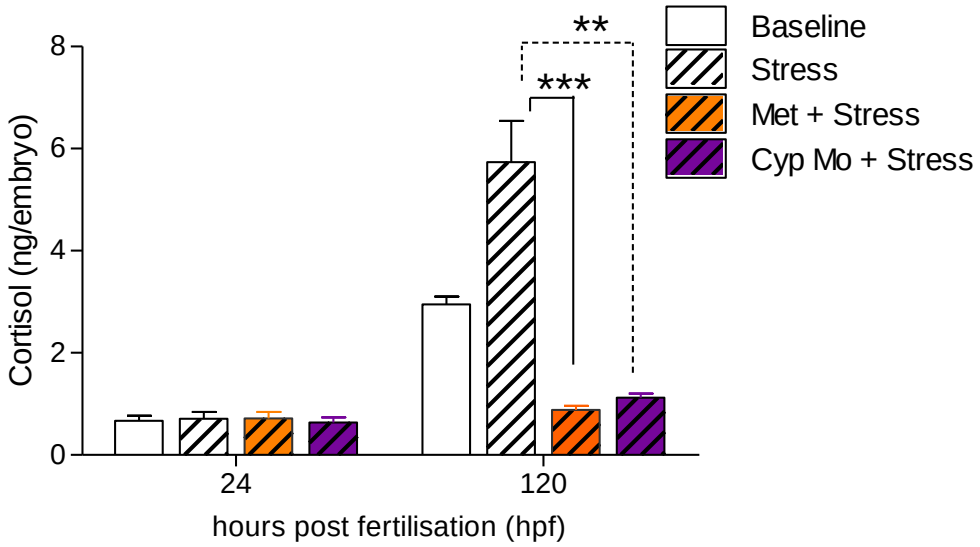

Figure 3.11 Embryonic cortisol levels after stress and 11β-hydroxylase manipulation
A stressor stimulus (kinetic agitation) was investigated 24 and 120 hours post fertilisation (hpf) for activation of the stress response with or without 11β-hydroxylase inhibition using metyrapone (Met) [10μM] or by targeting the *cyp11b1* gene using Cyp Mo. Data are mean ± SEM (n=3 (10 embryos per group)) and were analysed by 1-way ANOVA and Tukey's post hoc test. ** $p\leq0.01$, ** $p\leq0.001$.

3.4.4 Aim 4

Does maternal stress impact on embryonic cortisol levels?

3.4.4.1 Adult cortisol manipulation alters whole embryo cortisol levels

As can be noted from the embryonic time-course data, whole embryo cortisol appears to be high in the early stages of embryogenesis (8hpf), reducing over the next few h reaching a minimum around 48hpf. From this point a steady and progressive rise in cortisol develops until 120hpf. The initial decrease may be attributed to a maternal component within the developing embryo yolk sac which is depleted over time as the embryo requires the particular gene or protein. If this is the case for cortisol it may be that maternal cortisol levels have a direct effect on the levels in the embryo prior to the inflection point. As described (section 3.4.2) adult females exposed to Dex both by bathing and by IP injection display altered swim water cortisol.

When the offspring of these mothers were investigated for cortisol production it was found that only those embryos from the mothers injected with Dex had an altered whole embryo cortisol compared to the control basal data (Figure 3.12A). Whole embryo cortisol in this group was found to be greater at the initial time point of 8 hpf compared to controls ($p<0.05$). Cortisol levels in this group then fell at 24 hpf following the trend of the controls (0.72 ± 0.16 v 1.50 ± 0.30 ng/embryo $p=0.059$). At 120 hpf there was no difference between the Dex-inj offspring and the controls (5.7 ± 0.98 v 3.285 ± 0.3 $p= 0.06$). All other groups investigated showed no significant alteration in cortisol (Figure 3.12B, C and D).

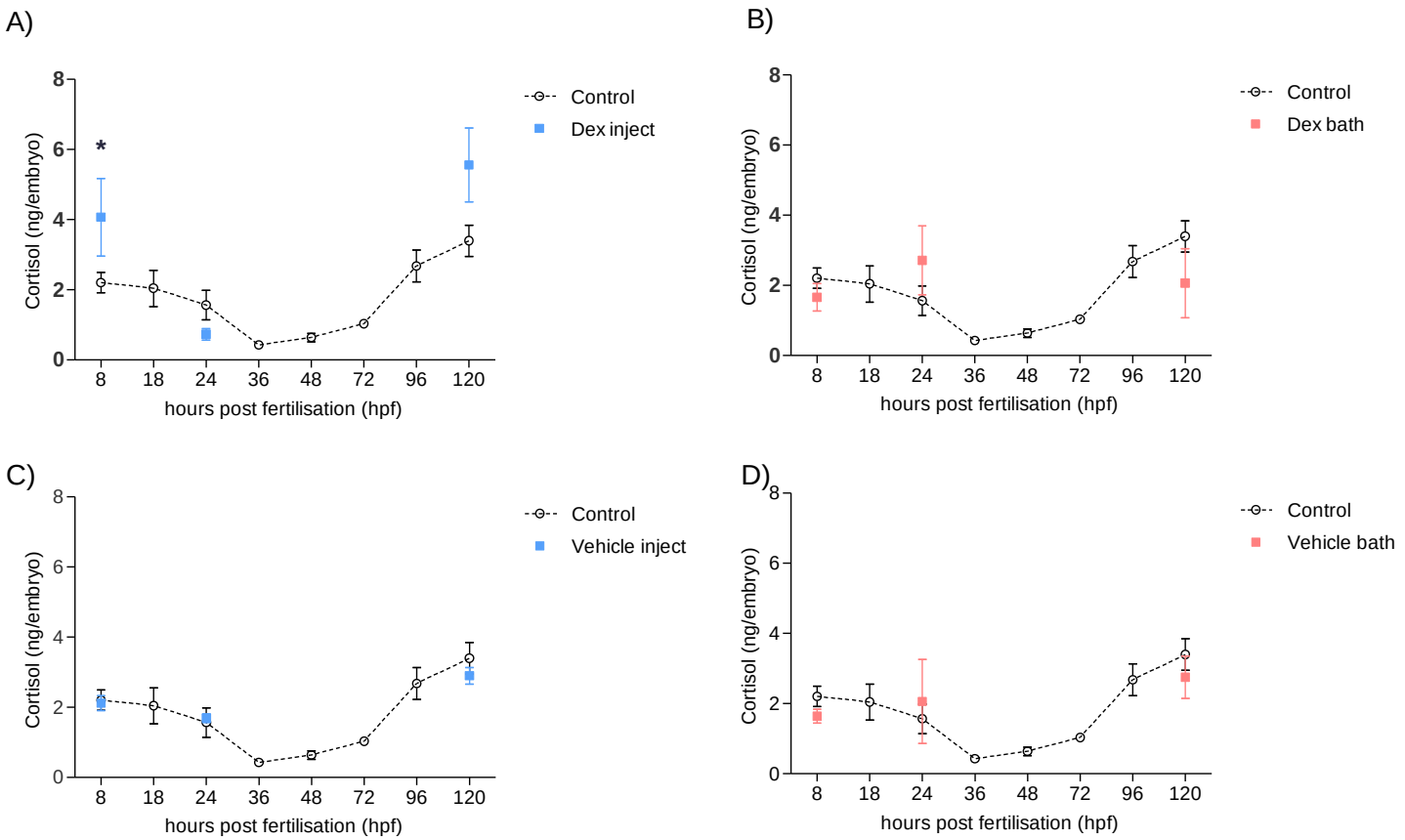

Figure 3.12 Influence of maternal dexamethasone treatment on offspring cortisol
Whole embryo homogenate cortisol levels (time-course over 8, 18, 24, 36, 48, 72, 96 and 120 hours post fertilisation (hpf)). Each graph shows data of offspring from maternal dexamethasone (Dex) or vehicle overlaid at 8, 24 and 120 hpf to compare with controls (untreated embryos). A) Dex injected (Dex inject) B) dex bathed (Dex bath) C) vehicle injected, and D) vehicle bathed (Vehicle Bath). Data are mean ± SEM (n=4 experiments (10 embryos per experiment)) analysed by Student's *t*-test *p ≤ 0.05, vs control at each time point.

3.5 Discussion

This chapter set out to investigate the hypothesis that the zebrafish GC system is functional. On the basis of the limited evidence available on the zebrafish GC system and current understanding of the mammalian system the following hypothesis was produced. The zebrafish embryo has a physiologically functional GC system, responsive to physiological or environmental stimulus.

Data presented here aimed to answer the following questions

1) Are the key components for GC biosynthesis present in the zebrafish?
2) When does *de novo* GC biosynthesis occur in the zebrafish embryo?
3) Is there endogenous production of GC in response to stress?
4) Does maternal stress impact on embryonic cortisol levels?

Data presented here clearly shows that the zebrafish embryo has many of the key components critical for a functional GC system from 24 to 48 hpf. Using pharmacological and genetic manipulations it has been shown that endogenous GC biosynthesis is active from 48 hpf onwards. It has also been shown for the first time, using stress experiments, that the HPI axis becomes functional and responsive in the zebrafish embryo at 72 hpf. With the stress induced rise in cortisol at 120 hpf abolished by pharmacological and genetic inhibition of 11β-hydroxylase. Furthermore it has been shown for the first time that maternally derived embryonic cortisol can be influenced by maternal exposure to the synthetic GC Dex.

3.5.1 Aim 1

Are the key components for GC biosynthesis present in the zebrafish?

It has been shown previously that there is maternal deposition of a number of endocrine components, such as oestrogen, in the zebrafish embryonic yolk sac which progressively deplete during embryogenesis (Alsop & Vijayan, 2008); in accordance with this data presented here shows a biphasic pattern of whole embryo cortisol levels during early development. This pattern suggests that cortisol detected from 48 hpf onwards is newly synthesised by the embryo itself. Endogenous embryonic biosynthesis of GC is further supported by my data showing an increase in mRNA of

key genes required for cortisol biosynthesis *crf*, *star*, *cyp11a1* and *cyp11b1* from 48-72hpf. These genes each play a critical role in HPI axis activity as highlighted in Figure 3.1. Star and Cyp11a1 proteins are important in the conversion of cholesterol into steroid hormones. Their pattern of gene relative abundance during development, in conjunction with observed changes in the gene responsible for the final rate limiting phase of cortisol production *cyp11b1* (Hagen *et al*, 2006), would strongly support *de novo* embryonic cortisol biosynthesis as primarily responsible for the secondary rise in cortisol levels between 72 and 120 hpf. CRF in mammals plays a central role in the response to stressors through regulation of the activity of the HPA axis (Smith & Vale, 2006). In the experiments described here a rise in the *crf* mRNA relative abundance was detected from 72 hpf which strongly supports the acquisition of a functional HPI axis at this developmental time-point.

The data presented clearly display a rise in *gr* mRNA and Gr protein relative abundance increase significantly at 48 hpf. This increase continues until 120 hpf confirming a key functional role during this period of rapid growth and development of the embryo, consistent with other vertebrate models (Cole *et al*, 1995; Liggins, 1994; Murphy *et al*, 2006; Smith & Shearman, 1974) and other fish species (Hillegass *et al*, 2007; LaLone *et al*, 2012; Nesan *et al*, 2012). The *mr* is another receptor for GCs, particularly in the fish in which the classical mammalian MR ligand aldosterone is not present. *mr* relative abundance also increased 4 fold between 24 and 120hpf. However the increase in abundance appears to double at 72 hpf suggesting that the action of GCs on Mr may not be important until later phases of development while their actions via Gr may be more important during earlier stages of development. This has been previously highlighted in rodent and human studies where a role for MR has been implicated in lung maturation, electrolyte and fluid dynamics in the developing foetus (Hirasawa *et al*, 1999; Keller-Wood *et al*, 2005).

Although the role of Mr in fish remains uncertain, since there appears to be no aldosterone-like molecule, studies have suggested that Mr plays a similar role in fluid homeostasis in fish as it does in mammals (Colombe *et al*, 2000; Gilmour, 2005; Pippal *et al*, 2011). It also appears to play a further fish specific role in salt water

adaptation (Gilmour, 2005; Strum *et al*, 2005). The lack of aldosterone in fish suggests that these roles are primarily activated by cortisol but recent work has suggested that 11-deoxycorticosterone (a *cyp11b1* substrate) may act as a ligand for MR in fish (Kiilerich *et al*, 2011; Strum *et al*, 2005). These findings do not exclude the possibility that Mr plays an important role in the developing embryo. Indeed, the late rise in levels of abundance of *11βhsd2* further support a possible role of Mr in development since this enzyme confers specificity on the Mr through the conversion of biologically active GCs to inactive 11-keto metabolites (Hirasawa *et al*, 1999; Mommsen *et al*, 1999; Tokarz *et al*, 2013b). Other work has suggested that, through increased exposure to cortisol, the abundance level of the *11βhsd2* is increased as a compensation mechanism in order to reduce circulating levels of GCs (Tokarz *et al*, 2012). Another GC catabolic enzyme 20βhsd2 has also been implicated in GC regulation in the zebrafish (Tokarz *et al*, 2013b). A rise in *20βhsd2* mRNA abundance was also detected from 72hpf. These data support the hypothesis that the increase in *11βhsd2* and *20βhsd2* abundance coincides with the cortisol surge at around 72 hpf in order to regulate GC activity and to prevent over activation of both zebrafish Gr and Mr.

Numerous studies to date have described gene abundance patterns for steriodogenic enzymes and pathways in the adult zebrafish but these abundance profiles have largely focused on the steriodogenic tissues such as the interrenal cells, the gonads, ovaries and brain (Diotel *et al*, 2011; Goldstone *et al*, 2010; Sakamoto *et al*, 2001) or have assumed that expression will be in accordance to mammalian systems due to the striking conservation of the systems (Tokarz *et al*, 2013a). Work here was carried out to confirm the expression of the two main receptors for GC function, Gr and Mr, a key gene required for local synthesis (*cyp11b1),* and *11βhsd2* which is required for local GC catabolism. In accordance with animal studies, *gr* and *mr* were both found in all of the tissues investigated, suggesting that these receptors may have similar functions in adult zebrafish as in other mammals. The abundance of *mr* but lack of classic ligand (aldosterone) are intriguing and may offer a mean of characterizing the cortisol mediated Mr effects. Growing evidence in mammals suggests that the roles of GCs in target tissues are critically determined by the abundance of not only the receptors (GR and MR) but also by the presence of 11βHSDs. The data here also

suggest a varying expression profile for *11βhsd2* in different tissues. *cyp11b1* mRNA abundance was only found at quantifiable levels in the kidney, suggesting that local GC generation does not occur in the other tissues investigated here, although this does not eliminate the possibility in other tissue types. The interrenal tissue of teleostian fish is found in close proximity to, or embedded in, the head-kidney; therefore it is likely that the *cyp11b1* mRNA abundance found in the kidney tissue here is as a result of interrenal tissue presence.

3.5.2 Aim 2

When does de novo GC biosynthesis begin in the zebrafish embryo?

Taking the *cyp11b1* gene abundance data and the developmental cortisol profile into consideration, steps were taken to confirm the hypothesis that cortisol detected after 36 hpf is not derived from the mother but synthesised by the embryo. To do this Mo technology was used to target the enzyme 11β-hydroxylase, which catalyses the final rate-limiting step in cortisol production. In keeping with the presence of a functional enzyme at this developmental time-point an increase in cortisol following incubation in 11-deoxycorticosteroid substrates was observed. Doxy incubation increased cortisol levels by around 35% consistent with Doxy acting as the immediate precursor for cortisol biosynthesis confirming the likelihood of *de novo* GC synthesis. To further explore the action of this enzyme in the zebrafish, embryos were incubated in the known inhibitor of 11β-hydroxylase activity, Met. With increasing concentrations of Met a dose-dependent reduction in whole embryo cortisol was observed at 120hpf. Similar observations have been made in adult fish of other teleost species (Doyon *et al*, 2006; McConnachie *et al*, 2012; Milligan, 2003). Further to this when Doxy was co-incubated with Met a reduction in cortisol was observed consistent with inhibition of the forward reaction of doxy to cortisol. Additionally Met-induced inhibition of 11β-hydroxylase activity was confirmed by an increase in Doxy levels determined by ELISA for this steroid. The possibility of molecular suppression of cortisol by Mo knockdown of the *cyp11b1* gene was also investigated and showed that with a modest mRNA reduction of ~30% there was a significant decrease in cortisol at 120hpf; however this genetic manipulation was found to not significantly alter cortisol at earlier time points investigated (24 and

72hpf). These data clearly indicate that the zebrafish embryo has the machinery in place to control its own production of cortisol at least by 120hpf.

3.5.3 Aim 3

Is there endogenous production of GC in response to stress?

This work confirms that at 120hpf, zebrafish respond to a variety of stressors with an increase in cortisol (Alsop & Vijayan, 2008; Pikulkaew *et al*, 2010; Steenbergen *et al*, 2011). Hypoxia and prolonged stirring both increased whole embryo cortisol more than twofold. Varying the intensity of stress altered the level of response: a shorter period of stirring caused a 1.5 fold increase whereas electrical stimulation caused a 2.5 fold increase. Lesser degrees of stress and assay sensitivity may explain why other studies failed to show significant cortisol changes in larval fish (Barry *et al*, 1995; Long *et al*, 2012). The ontogeny of the stress response was investigated by comparing stress-induced cortisol changes at different stages of development. No response to stress at 24 hpf was determined indicating that detectable cortisol at this stage is most likely due to residual maternal cortisol in the yolk-sac since at this early stage the embryo lacks the elements required for *de novo* cortisol biosynthesis.

A marked response to stress at 72 hpf was noted however, at a time when *crf, star and cyp11b1* are clearly active. Taken together these data suggest that once all of the elements of the embryonic HPI axis are in place then endogenous cortisol production becomes stress-responsive. Indeed, it is significant that inhibition of 11β-hydroxylase or Mo knockdown of *cyp11b1* both abrogated the stress response at 72 and 120 hpf. Interestingly, at 72 hpf, 11β-hydroxylase blockade lowered stressed cortisol levels to unstressed control levels but at 120 hpf, levels were reduced even further, below unstressed controls. This implies that some unstressed cortisol at 72 hpf may still be maternally-derived and not susceptible to 11β-hydroxylase inhibition.

Data presented also shows that a main cortisol catabolism gene, *11βhsd2*, is present at a significant level from 48 hpf onwards. The enzyme encoded by this genes catalyses the local inactivation of cortisol to cortisone, the activity of this gene along with 11β-hydroxylase activity is therefore important for regulating cortisol levels (Tokarz *et al*, 2013b). This is an apparent conserved homeostatic regulatory response

found in mammals and other species. However, unlike mammals the opposing reaction (cortisol production from cortisone by *11βhsd1*)- does not occur (Tokarz *et al*, 2013a). The presence of key genes for homeostatic regulation of cortisol in the developing embryo from 48 hpf onwards lends further support for a functional HPI axis and an active stress response.

3.5.4 Aim 4

Does maternal stress impact on embryonic cortisol levels?

It has previously been shown that progeny can be influenced by non-genetic factors derived from the mothers environmental/physiological status during gametogenesis, possibly by affecting the mother-to-embryo transfer of proteins or other factors (McCormick, 1998). In this way, maternal environmental conditions may influence oogenesis and, thus, influence the next generation (Li & Leatherland, 2012). On the basis of this the ability of the mother to influence the embryonic cortisol levels was investigated by altering the environmental conditions experienced by the mother during gametogenesis.

Maternal exposure to the synthetic GC Dex clearly influences maternal cortisol production. Chronic exposure to Dex suppresses basal cortisol production in treated adult females. This finding mirrors the phenomenon seen in the diagnosis of Cushing's disease (al-Saadi *et al*, 1998), the Dex suppression test. Where Dex is acting as a GR agonist on the cortisol biosynthesis pathway; activating a negative feedback loop resulting in suppression of endogenous cortisol production. Subsequently the offspring of mothers injected with Dex displayed a significant increase in whole embryo cortisol levels at the earliest time point investigated (8hpf). Previous studies have shown that prenatal Dex treatment mimics the effect of maternal stress and in rat studies offspring of mothers treated in late gestation with Dex have increased plasma corticosterone (the primary GC produced by rodents) (Levitt *et al*, 1996). Reduced birth weight (an indicator of maternal stress, GC exposure) has also been associated with increased plasma cortisol levels in humans (Phillips *et al*, 2005; Phillips *et al*, 2000).

Importantly no significant effect on cortisol levels was observed on the offspring of maternal Dex treatment at later time points confirming that by these later stages of development the endogenous production of cortisol by the embryo is the main contributor to cortisol. These data clearly suggest that cortisol detected in the embryo in the first 36 hpf is maternally-derived and that by altering the environmental/ physiological status in the mother, using injected Dex, we can influence the cortisol composition of the egg.

3.6 Conclusion

This chapter has detailed a comprehensive investigation of the temporal abundance of a number of key components of the GC system, highlighting their activity during early development in the zebrafish embryo. The corticosteroid biosynthesis pathway becomes physiologically active and relevant from 72hpf, with positive and negative HPI axis control from 72hpf. It also supports the suitability of the zebrafish embryo as a model system to study physiological and pathophysiological consequences of corticosteroid excess and deficiency. While this chapter has confirmed the presence of the GC system in the zebrafish embryo it does not determine the role that this system plays during embryogenesis and whether manipulating this system will result in long term consequences in the adult. Chapter 4 will address the short and long-term effects on development, HPI axis function and behaviour while chapter 5 will address the role in the cardiovascular system.

Work arising from this chapter has been published. Physiological roles of glucocorticoids during early embryonic development of the zebrafish (Danio rerio) Wilson, K.S., Matrone, G., Livingstone, D.E.W., Al-Dujaili, E.A.S., Mullins, J.J., Tucker, C.S., Hadoke, P.W.F., Kenyon, C.J., Denvir, M.A. 2013 Journal of Physiology 15;591(Pt 24):6209-20

Chapter 4: Embryonic developmental programming by glucocorticoids in the zebrafish

4 Embryonic developmental programming

4.1 Introduction

Maternal-foetal signalling and the *in utero* environment are known to influence foetal development. Exposure to adverse *in utero* environmental factors can result in altered foetal cellular and molecular development (Cottrell & Ozanne, 2008) which can have long-lasting, and possibly permanent, physiological and pathophysiological effects; this is called foetal programming.

Numerous studies, observational in humans and experimental in other mammals, have highlighted a number of abnormalities as a consequence of foetal environmental alterations such as reduced birth weight, raised blood pressure, increased adult body mass index, glucose intolerance, insulin resistance and dyslipidaemia (Seckl & Meaney, 2004). Foetal exposure to GCs can lead to permanent modification of HPA function (Kapoor *et al*, 2006; Kapoor *et al*, 2008) with evidence of an association with increased anxiety and depressive behavioural traits in adult life (Hack, 2006) including increased incidence of disruptive behavioural disorders (Latimer *et al*, 2012).

As highlighted in chapter 1, GCs have been suggested as a primary candidate for programming. It is clear that GCs ordinarily play a role in growth and tissue development, by acting as nutritional and maturational signals (Fowden & Forhead, 2004), as a rise in GC exposure coincides with a reduction in growth rate, in preparation for birth (Fowden & Forhead, 2004). GCs are also given to mothers at risk of preterm delivery to assist development and maturation (Stocker *et al*, 2005).

Animal models of programming have been developed in order to further evaluate and characterise features and mechanisms involved following GC exposure. Pregnant rats treated with excess GCs in the mid-trimester, produce fewer, lighter pups per litter. These pups show alterations in glucose handling and in liver glycolytic pathways in adulthood (Nyirenda *et al*, 2006; Nyirenda *et al*, 1998).

Current models of GC-induced programming have, however, limitations which can affect the interpretation of experimental studies. For example, environmental

manipulation and follow-up investigation can introduce confounding maternal stress (Steenbergen *et al*, 2011). In mammalian models the placenta adds further complexity, firstly due to the high level of abundance of the GC catabolising enzyme 11βHSD2 (Burton & Waddell, 1999) protecting the foetus from GC over-exposure. Secondly, there are conflicting and controversial views as to whether changes in the foetus are directly caused by GCs or whether are secondary to maternal or placental effects (Barker & Thornburg, 2013; Braun *et al*, 2013). A further limitation of mammalian *in utero* embryogenesis is that real-time observation of organogenesis is challenging (Hsu *et al*, 2006) and often invasive.

Zebrafish could provide a solution as they develop externally, removing a direct maternal influence and also allowing ease of environmental and non-invasive observation of growth and development. The concept of maternal programming has previously been looked at in a number of salmonoids, including zebrafish (Aluru & Vijayan, 2009; Cai *et al*, 2012; Cole *et al*, 2012; Mommer & Bell, 2013; Zhang *et al*, 2006). The concept of GC modulation in the zebrafish has generated considerable interest recently (De Marco *et al*, 2013; Nesan *et al*, 2012; Pikulkaew *et al*, 2011). However, investigation has largely focused on the profound effects of global abolition of GCs on morphology rather than subtle adaptive effects on tissues which may underpin reprogramming events.

4.2 Experimental hypothesis and aims

Manipulation of GC activity in the embryonic zebrafish during the first 5 days post fertilisation will result in developmental abnormalities in the embryo leading to longer term changes in the adult.

Data presented here aimed to answer the following questions:

1) Does embryonic GC manipulation impact on embryonic and adult growth and development? (Aim 1)
2) Does embryonic GC manipulation impact on embryonic and adult HPI axis function? (Aim 2)
3) Does embryonic GC manipulation alter embryonic and adult swim behaviour? (Aim 3)

4.3 Methods

4.3.1 Embryonic glucocorticoid modulation

Modulation of GC activity was performed in zebrafish embryos at the 2 cell stage (approx. 1h after egg collection), as described (Appendix 1). Embryos were housed under standard conditions (section 2.2) in a 10cm Petri dish at a density of 1 embryo/mL.

4.3.1.1 Pharmacological manipulation

Pharmacological manipulation (Appendix 1) was achieved by bathing embryos in: Dex [100 µM], RU486 [10 µM], Met [10 µM] or spironolactone [0.1 µM]. Controls for all drug bathing studies in this chapter were embryonic bathing in vehicle only (0.1% EtOH). Experiment was repeated in triplicate (10 embryos pooled).

Pharmacological manipulation in a small cohort was achieved by injection (section 2.4) of a small bolus (2 ng/mL) of drug dissolved EtOH then diluted in systems water.

4.3.1.2 Genetic manipulation

Genetic manipulation was achieved by injection of targeted Mo for knockdown of two key zebrafish genes (*gr* and *cyp11b1*). Design, dosage and injection is summarised in Appendix 1. Unless stated otherwise, data shown subsequently will be for atg-Mo and will be referred throughout as either GR Mo or Cyp Mo. Controls for each of the Mo is injection of respective mm-Mo for each of the genes of interest. Experiment was repeated in triplicate (10 embryos pooled).

4.3.1.3 Embryonic developmental assessments

Embryonic development was undertaken by daily measurement of total body length, head-trunk angle, eye size/length, self-hatch rate and swim bladder inflation throughout 120 hpf (section 2.4.5). All experiments were n=3 (20 embryos per group) apart from self-hatch which was n=3 (20-60embryos per group).

4.3.2 Embryo to adult programming study

To assess the long term effects of embryonic GC manipulation, three groups were allowed to grow to adulthood after manipulation had ceased at 120 hpf (un-

manipulated from 120 hpf -120 dpf). The adult fish are referred to as embryonic modification (Emmod). The experimental plan is summarised in Figure 4.1. Two of these groups were pharmacological; exposure to Dex [100 µM] (EmmodDex), or exposure to RU486 [10 µM] (EmmodRU486) for the first 120 hpf and then left un-manipulated under normal fish husbandry conditions (section 2.2). The third manipulation group investigated was the genetic knockdown of *gr* (EmmodGR Mo) at the 2 cell stage, for the first 120 hpf and then left un-manipulated under normal fish husbandry (section 2.2). In each of these manipulations treated embryos and adults were compared with age-matched controls. For pharmacological manipulation the control was a "vehicle only" group, whilst for the GR Mo it was injection of mm-Mo. All control groups are referred to as "Control" in figures. However, where two-way ANOVA was used they are referred to as treatment group and control (e.g. EmmodDexControl).

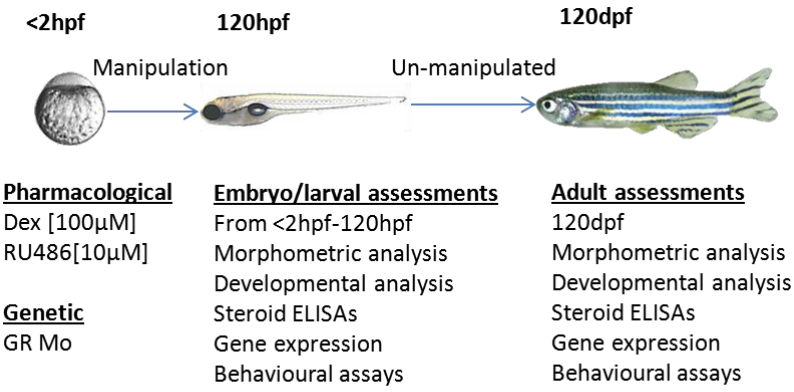

Figure 4.1 Embryo to adult programming experimental plan
Schematic (images not to scale) highlighting the protocol for investigating longitudinal effects of embryonic cortisol manipulation on adult phenotype. Embryonic manipulations were exposure to dexamethasone (Dex), or RU486, or targeted glucocorticoid receptor morpholino (GR Mo) from the 2-cell stage. Manipulation lasted from ~2 hours post fertilisation (hpf) until 120 hpf during the course of which embryonic assessments were performed. From 120 hpf until 120 days post fertilisation (dpf) embryos/larvae were allowed to develop under normal husbandry conditions. At 120 dpf a number of phenotypic characteristics were assessed in adults.

4.3.2.1 Adult developmental assessments

Adult phenotype and growth were assessed throughout the course of development as described (in section 2.5). Length and weight were measured in all fish at 10, 20, 40,

60, 90 and 120 dpf. A condition factor (K) was derived for each fish from length (mm) and weight (mg) measurements. The formula is given in section 2.5.3. Adult zebrafish male/female ratio confirmed by dissection *post mortem*.

4.3.3 Gene abundance analysis

Abundance analysis was performed for several genes, during embryogenesis (whole embryo homogenate) or in adulthood (isolated tissue). Whole embryo homogenate or adult isolated tissue were processed, RNA extracted, cDNA synthesised and qRT-PCR performed as described (section 2.6).

Abundance analysis was performed on a gene previously associated with zebrafish development, insulin-like growth factor-1 (*igf1*) (White *et al*, 2009) and two genes which have been previously suggested to be mediated by GC activity, FK506 binding protein 5 (*fkbp5)* (Mathew *et al*, 2007) and matrix metallopeptidase 13 (*mmp13*) (Hillegass *et al*, 2007; Hillegass *et al*, 2008)). mRNA relative abundance analysis was carried out on three genes key genes associated with downstream GC activity *gr*, *mr* and *11bhsd2*. Gene selection is summarised in Appendix 2.

4.3.4 Measurement of cortisol levels

Whole embryo homogenate and adult swim water cortisol were measured using a sensitive and specific in-house ELISA (section 2.9). This method has previously been validated and applied extensively within our laboratory for buffer-reconstituted urine, saliva and cell cultures (Al-Dujaili *et al*, 2011; Al-Dujaili *et al*, 2009; Moore *et al*, 2011).

4.3.4.1 Basal whole embryo cortisol

Whole embryo cortisol was determined from pooled homogenates of 20 embryos (section 2.9) following pharmacological or genetic manipulation.

4.3.4.2 Optimisation of measurement of cortisol in stressed adult fish.

A series of techniques were assessed to determine a suitable protocol by which to adequately and humanely stress adult zebrafish. To determine whether the stress response was altered in Emmod adults the following protocol was employed. Briefly, five fish were placed in a 1 L tank. For each set of fish (EmmodDex, EmmodRU486

or EmmodGR Mo, and their respective controls) 3 groups of 5 fish were assigned as basal (unstressed) controls and 3 groups of 5 adult fish were assigned as stressed (n=3 (5 fish per tank)). Stress was induced using netting protocol. Protocol began with a 24 h baseline familiarisation period, followed by a 24 h stressed period (consisting of 5h of repeated stressor - netting every 30 min for 5 h) then a 24 h recovery period.

4.3.5 Behavioural assessments

Behavioural characteristics (swim/movements) were determined in embryos and adults which had undergone pharmacological or genetic manipulation of the GC system at the 2-cell stage.

4.3.5.1 Embryonic behaviour

Briefly, a single embryo was placed in a 5cm Petri dish containing 3 mL fresh systems water. Embryo movement was recorded from above using a digital CCTV camera (Baxall, AD group, UK). Twitch response was counted manually and swim/movement data were analysed by commercially available software (Limelight Ltd, UK).

4.3.5.2 Adult swim behaviour

Adult fish swim behaviour was assessed as described (section 2.5.6). These studies included an open field assay, a novel object assay and a forced swim/dive assay. Movement of the fish over a given period was recorded using a digital CCTV camera (Baxall, AD group, UK) connected to a PC. Swim behaviour were then analysed by commercially available software (Limelight Ltd, UK).

4.3.6 Experimental controls

In the following experiments data were obtained both in embryos and in adult zebrafish. For embryonic pharmacological manipulations controls are vehicle only (0.1% EtOH). For embryonic Mo manipulation control data are for injected control Mo. For data where embryonic pharmacological and molecular manipulation are represented together (e.g. gene abundance) control data are a mean of vehicle only and control Mo together (Student's *t*-test shows no significance between groups) to

ease graphical representation and interpretation. For adult investigations, control data for EmmodDex and EmmodRU486 are adult fish which were treated with vehicle only during embryogenesis. EmmodGR Mo controls are adults which were injected with control Mo during embryogenesis.

4.4 Results

4.4.1 Aim 1

Does embryonic GC manipulation alter embryonic and adult growth and development?

4.4.1.1 Embryonic growth and development

4.4.1.1.1 Total body length

None of the embryos which were treated with Dex, Met or Cyp Mo had any difference in total body length when compared to controls at any of the time-points investigated (Figure 4.2). However targeting the GR receptor (RU486 and GR Mo) delayed growth/total body length such that by 48 hpf embryos treated with RU486 were shorter than controls ($p<0.001$). This significant difference was lost by 120 hpf in embryos incubated with RU486 (Figure. 4.2A). Similarly GR Mo embryos were also shorter than controls at 48 hpf ($p <0.05$), this significant reduction in length was maintained at 72 hpf (<0.05), however there was no significant difference between GR Mo and controls at 96 and 120 hpf (Figure 4.2 B).

4.4.1.1.2 Embryonic Growth rate

RU486 and GR Mo displayed significantly reduced growth rate (the length (µm) grown in 1 h) in the early stages of the study with comparable growth rate to controls by 120 hpf (Figure 4.4 (A and B)). There was no reduction or enhancement in growth rate for Met, Cyp Mo or Dex (Figure 4.3 (C-E)).

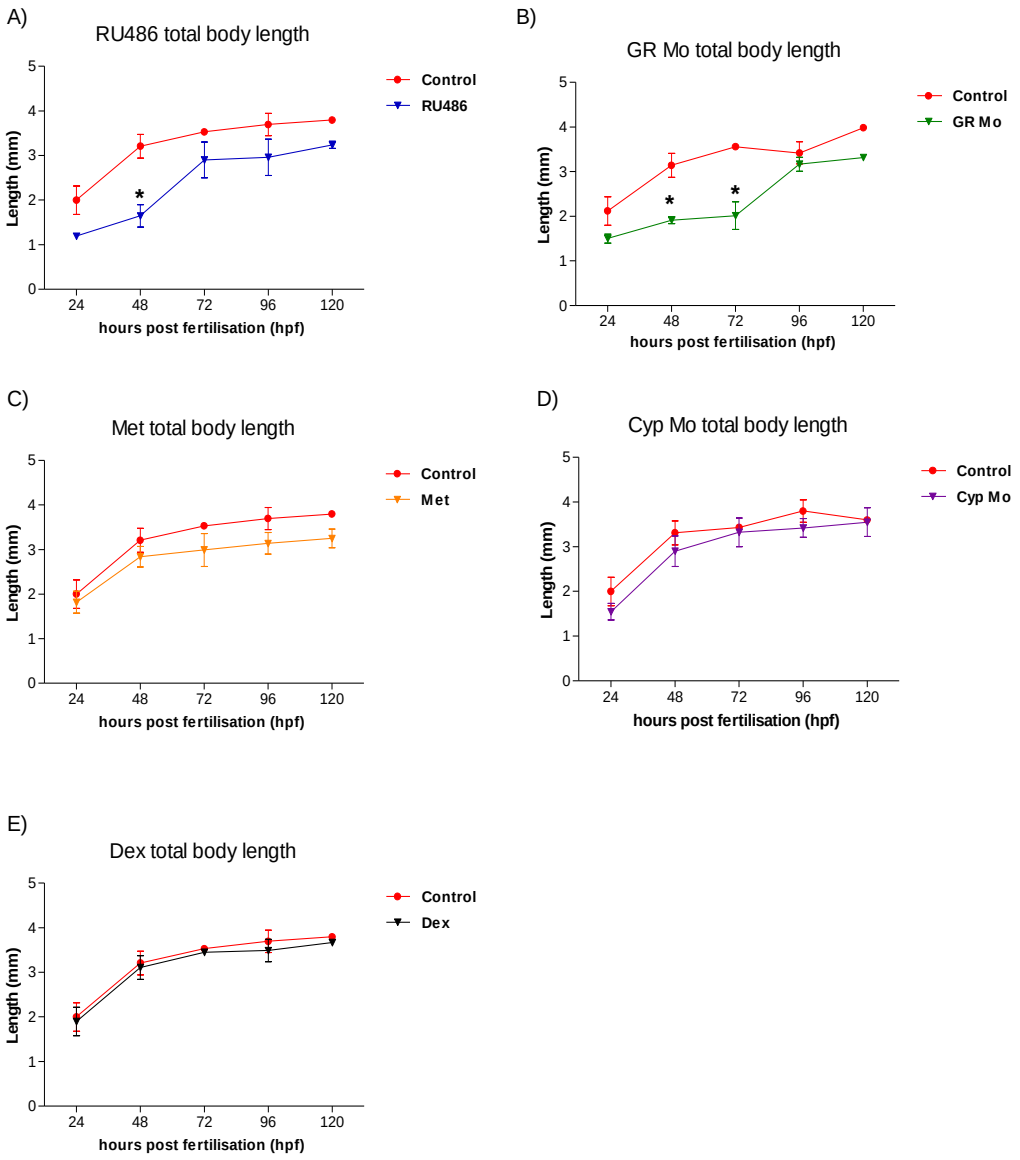

Figure 4.2 Embryonic body length following glucocorticoid modulation
Total body length (mm) throughout development up to 120 hours post fertilisation (hpf) in zebrafish embryos A) treated with 10µM RU486, B) injected with *gr*-targeted morpholino (GR Mo), C) treated with 10µM metyrapone (Met), D) injected with *cyp11b1*-targeted morpholino (Cyp Mo), or E) treated with 100µM dexamethasone (Dex). All data from treated fish were compared with appropriate controls (vehicle only for drugs and mismatch morpholino injection for morpholino treated- red line on each graph). Data are mean ± SEM for n=3 (20 embryos per group). Data were analysed by 2-way ANOVA and Bonferroni post hoc test: *$p \leq 0.05$.

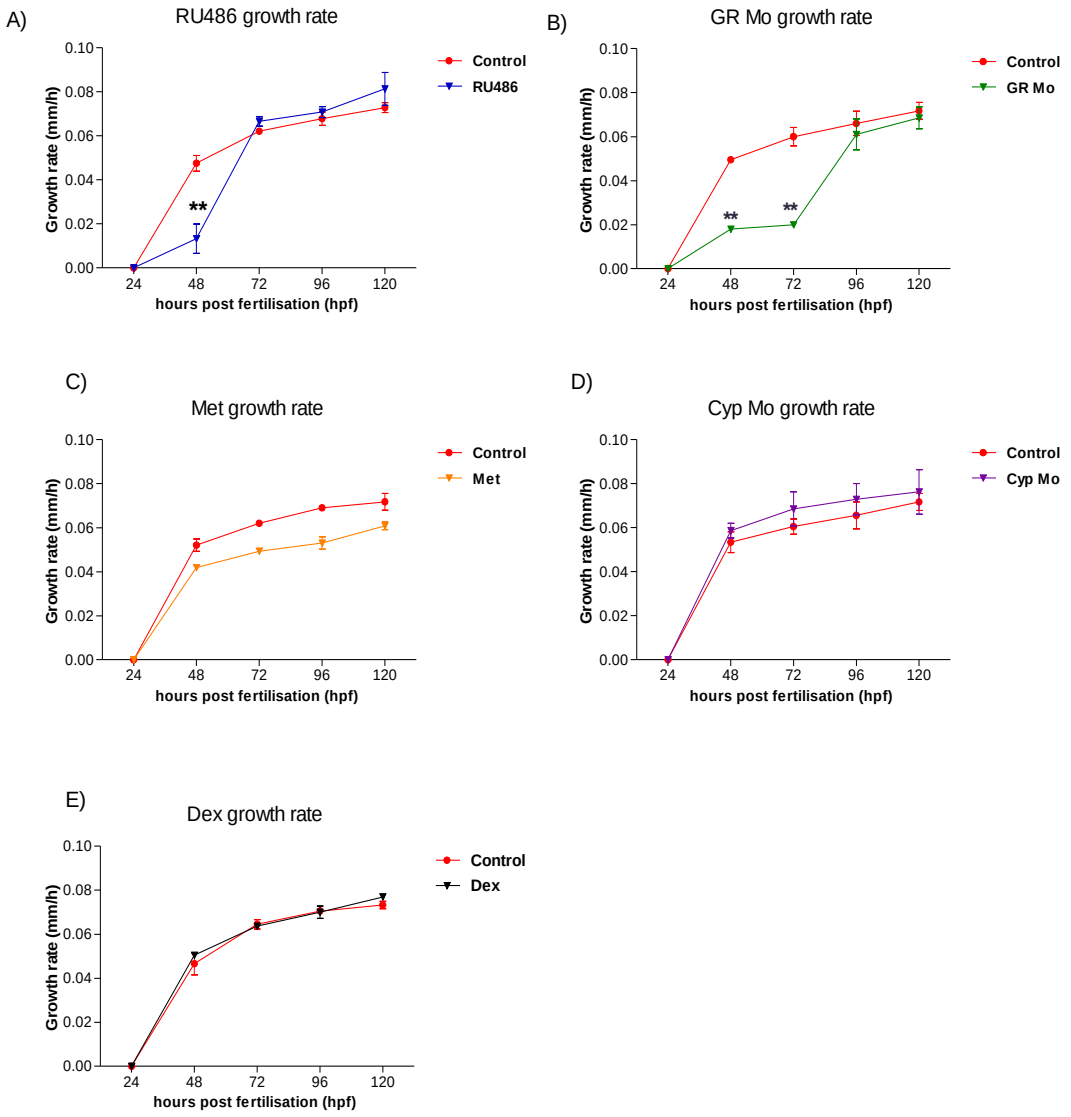

Figure 4.3 Embryonic growth rate following glucocorticoid modulation
Growth rate (mm grown/ h) over the course of 120 hours post fertilisation (hpf) for embryos: A) treated with 10µM RU486, B) injected with *gr*-targeted morpholino (GR Mo), C) treated with 10µM metyrapone (Met), D) injected with *cyp11b1*-targeted morpholino (Cyp Mo), or E) treated with 100µM dexamethasone (Dex). All data from treated fish were compared with appropriate controls (vehicle only for drugs and mismatch morpholino injection for morpholinos - red line on each graph). Data are mean ± SEM for n=3 (20 embryos per group). Data were analysed by 2-way ANOVA and Bonferroni post hoc test; *$p \leq 0.05$, *$p < 0.05$, **$p<0.001$.

4.4.1.1.3 Head-trunk angle

When visual observation of embryos was carried out it was noted that embryos treated with RU486 and GR Mo appeared to have a delay in reaching straight body axis compared to controls, this is an indicator of embryogenesis in developmental biology and is quantified by head-trunk angle. The reduced head trunk angle in the GR Mo group was equivalent to a 6h developmental delay (in accordance with Kimmel (Kimmel *et al*, 1995) delay shown in Figure. 4.4). Embryos treated with RU486 had a significantly reduced head-trunk angle compared to controls at all time-points. Likewise embryos treated with GR Mo showed changes in head-trunk angle at all time-points ($p<0.001$; Figure 4.5 B). Embryos treated with Met and Cyp Mo did not show significantly impaired head-trunk angle at any of the time-points (Figure 4.5 C and D). Dex treatment accelerated development with more advanced changes in head trunk angle over the time-points studied compared to controls, $p<0.001$; Figure 4.5 E).

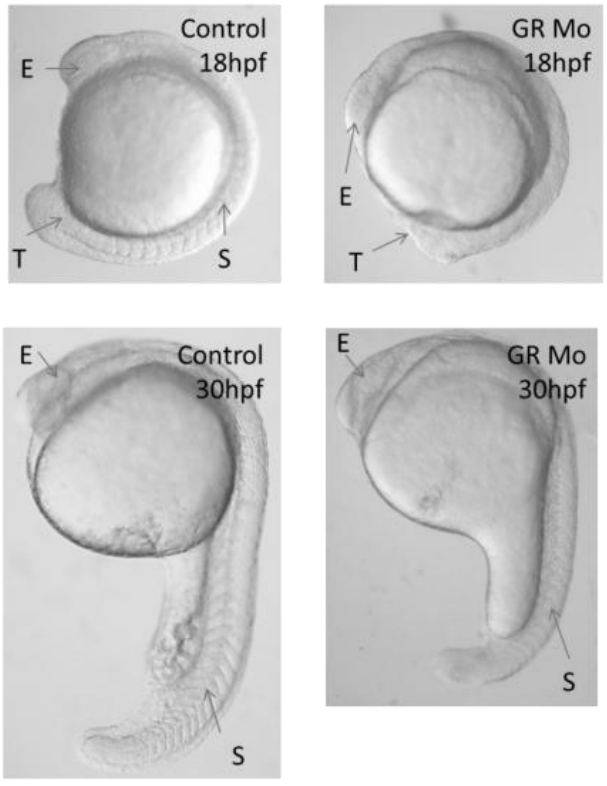

Figure 4.4 Body axis in glucocorticoid receptor morpholino embryos
Body development at 18 and 30 hours post fertilisation (hpf) in control embryos and those which had been injected with an oligonucleotide morpholino sequence specific for the glucocorticoid receptor (GR Mo) producing a transient 40% knockdown in *gr* mRNA (Figure 4.2). Images show developmental delay of 8h in those embryos treated with morpholino compared to the controls. Regions of interest have been highlighted E= Eye, T=Tail, S= Somite patterning.

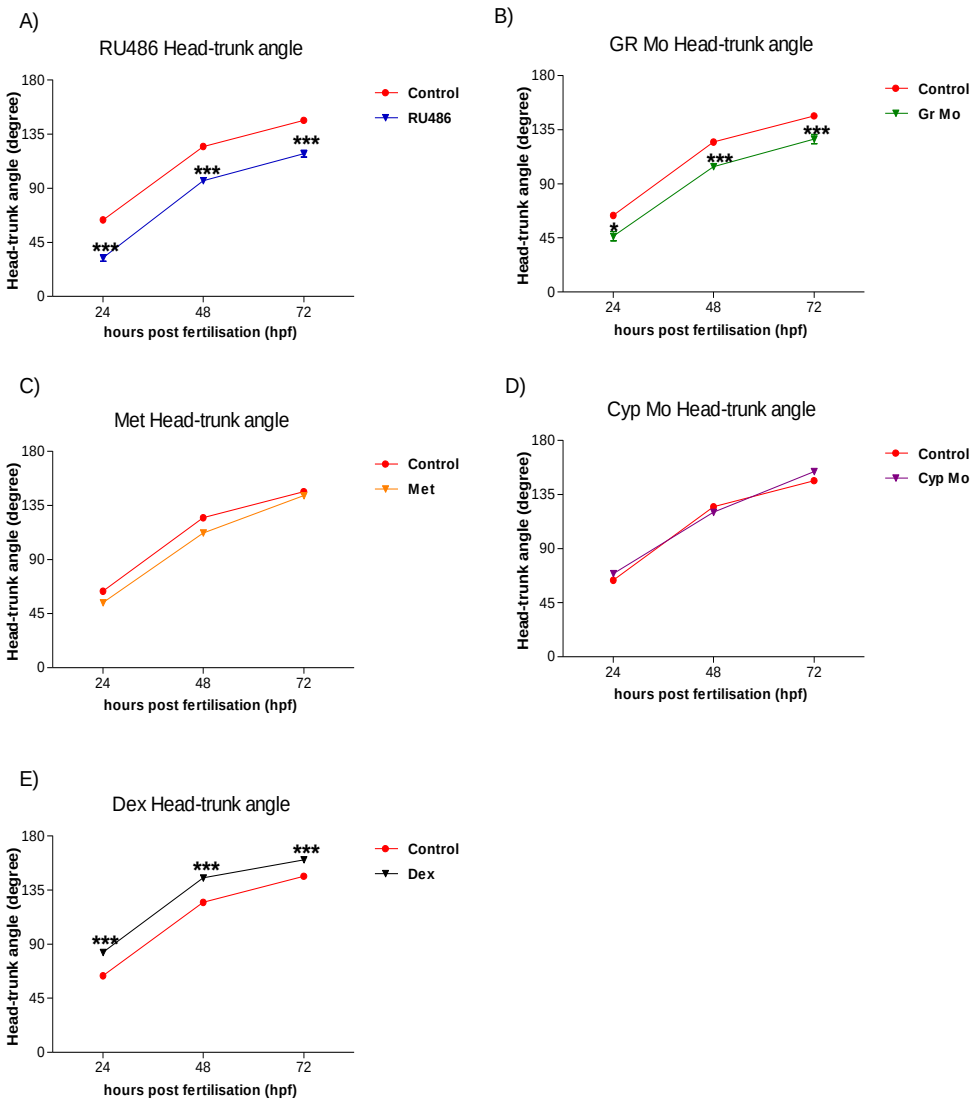

Figure 4.5 Embryonic head-trunk angle following glucocorticoid modulation
Head trunk angle over the course of 120 hours post fertilisation(hpf) for embryos: A) treated with 10µM RU486, B) injected with *gr* morpholino (GR Mo), C) treated with 10µM metyrapone (Met), D) injected with *cyp11b1* morpholino (Cyp11b1 Mo), or E) treated with 100µM dexamethasone (Dex). All data from treated fish were compared with appropriate controls (vehicle only for drugs, for morpholino these were mismatch morpholino - red line on each graph). Data are mean ± SEM for n=3 (20 embryos per group). Data were analysed by 2-way ANOVA and Bonferroni post hoc test; ***p<0.001.

4.4.1.1.4 Abundance of key growth, maturation and regeneration genes

mRNA relative abundance was measured at 72 and 120 hpf in GC modulated embryos for three genes previously highlighted as having a role in growth (*igf2)* or GC activity (*fkbp5* and *mmp13*)(Figure 4.6(A-C)).

Embryos treated with GR Mo had lower *igf2* mRNA levels than control injected embryos at 72 and 120 hpf ($p<0.001$). Lower *igf2* mRNA abundance was also observed at 120 hpf for embryos treated with RU486 compared to vehicle exposed embryos ($p<0.001$) but not at 72 hpf. No other treatments altered *igf2* mRNA abundance at either time point (Figure 4.6A).

There was an increase in *fkbp5* mRNA abundance in Dex treated embryos at 72 and 120 hpf (both $p<0.05$). In contrast, a decrease in *fkbp5* mRNA was observed at 120 hpf in embryos treated with the GR Mo ($p<0.05$). No other treatment altered *fkbp5* abundance (Figure 4.6 B).

Abundance of *mmp13* mRNA has previously been shown to be altered following GC treatment (Matthews *et al*, 2004) however was found here to be similar at 72 and 120 hpf in all of the treatment groups investigated (Figure 4.6 C).

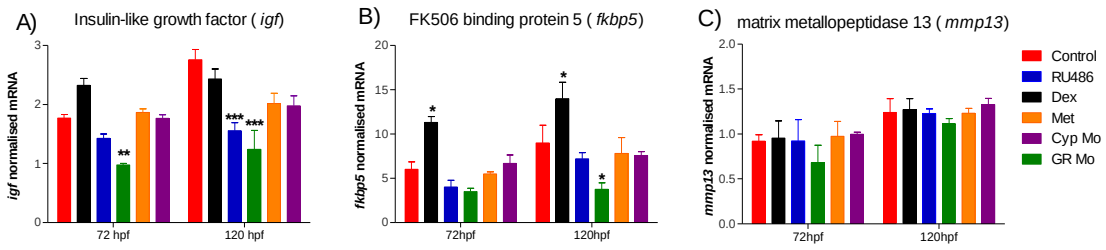

Figure 4.6 Gene relative abundance following glucocorticoid modulation
Gene mRNA relative abundance in whole zebrafish embryos at 72 and 120 hours post fertilisation (hpf). Embryos had previously been treated from the 2-cell stage with 100µM dexamethasone (Dex-black bars), 10 µM RU486 (blue bars), glucocorticoid receptor targeted morpholino (GR Mo-green bars), metyrapone (Met-orange bars) or targeted *cyp11b1* morpholino (Cyp Mo-purple bars) compared to controls (red bars) as detailed in section 4.3.6. Abundance data are mean ±SEM for n=3 (10 pooled embryos each time) for A) *igf2* mRNA B) *fkbp5* mRNA, and C) *mmp13* mRNA. Gene abundance is presented as arbitrary units (AU) after standard curve production and normalisation to *ef1a* and *18s* as described (section 2.5). Data are mean ± SEM and were analysed by 2-way ANOVA and Bonferroni post hoc test; *$p < 0.05$, **$p<0.001$.

4.4.1.1.5 Eye length and area

Embryos treated with Met and the Cyp Mo had similar eye length and similar eye area to their controls ($p>0.05$). Embryos treated with Dex, RU486, or GR Mo however all showed reduced eye length compared to controls (Dex 0.79 ± 0.02mm, RU486 0.78 ± 0.01mm, GR Mo 0.78 ± 0.02mm vs controls 0.91 ± 0.01 mm, $p\leq 0.05$) resulting in a smaller eye area ($p\leq 0.001$ (Table 4.1)).

4.4.1.1.6 Chorion hatch-rate

The number and proportion (%) of embryos spontaneously hatching from the chorion (hatch-rate) was carefully documented over 120 hpf in each of the treatment groups (Figure 4.7). Dex incubation increased the percentage hatched by 48 hpf (43 ± 3% v controls 16.5 ± 3.5 % $p<0.05$). Injecting the embryos with a bolus of 2 ng/nL Dex increased the proportion of embryos hatched by 48hpf, (97.5 ± 0.5 % vs controls 16.5 ± 3.5 % $p=<0.0001$) (Figure 4.7B).

In embryos treated with RU486 there was a reduction in the proportion of embryos hatched by 48 hpf ($p<0.05$), this difference was sustained at 72 hpf (67.5 ±2.5 v 99 ±1 % $p<0.05$) but lost by 96 hpf (Figure 4.7C). A more profound delay in self-hatching was observed in embryos injected with RU486 where hatch rate remained lower at all the time-points studied up to 120 hpf at which time a substantial proportion of embryos (~34% of those injected) remained un-hatched (Figure 4.7D).

Similar findings were observed for embryos injected with GR Mo. There was a reduction in the number/proportion of self-hatched embryos compared to controls at all time-points 48 hpf ($p<0.001$). As with RU486, a many (~85%) of the GR-Mo injected embryos remained un-hatched by 120 hpf. No significant difference in hatch-rate was noted in embryos treated with Met or Cyp Mo.

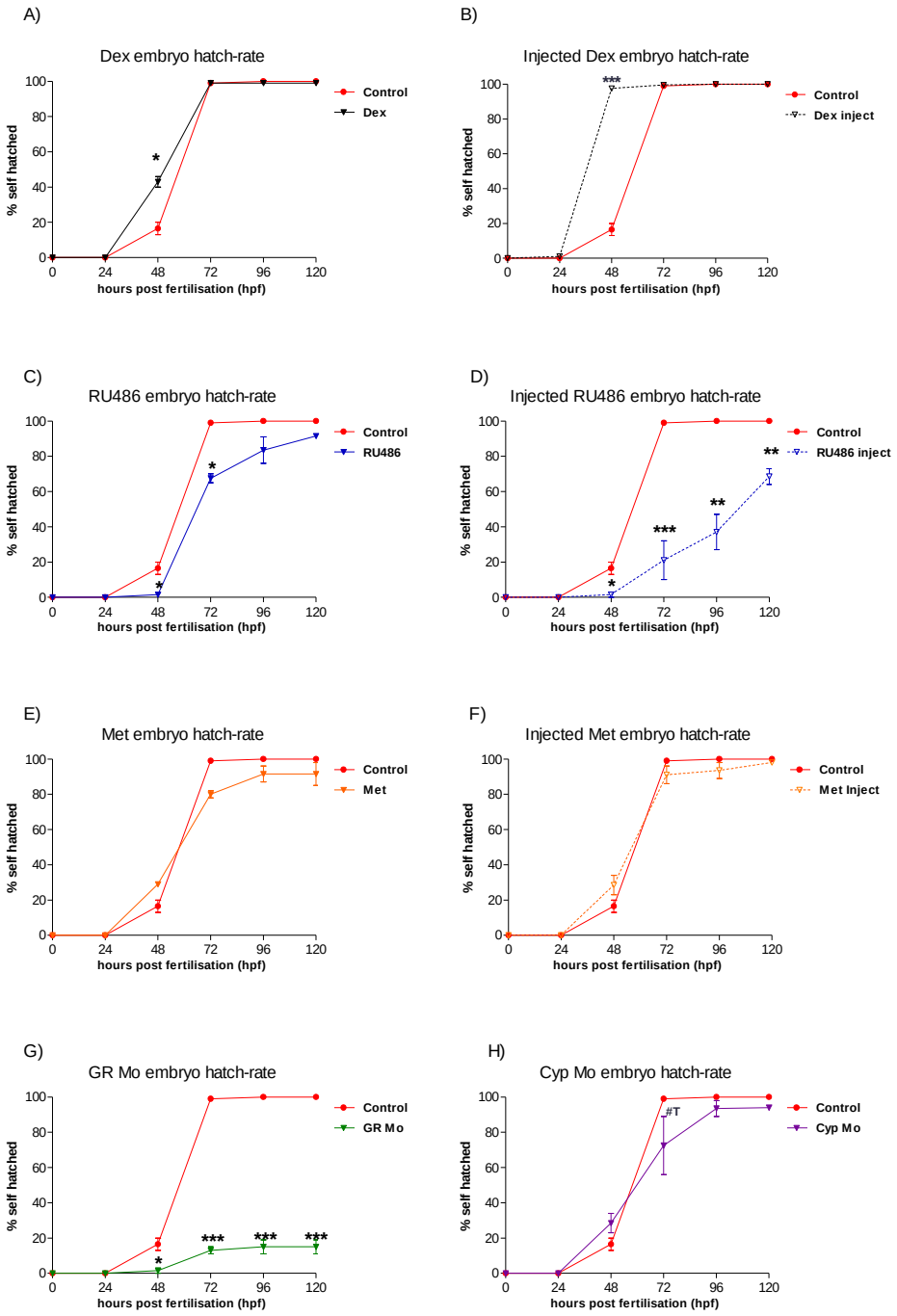

Figure 4.7 Embryonic hatch rate following glucocorticoid modulation
Proportion of embryos with spontaneous chorion hatching during development vs controls (red lines). A) Effects of 100µM dexamethasone (Dex -black line), B) Dex injection (black dashed line), C) 10µM RU486 (blue line), D) RU486 injection (blue dashed line), E) 10µM metyrapone (Met -orange line), F) Met injection (orange dashed line), G) glucocorticoid receptor targeted morpholino (GR Mo-green line), F) *cyp11b1* targeted morpholino (Cyp Mo-purple line). Values are mean ± SEM for n=3 experiments (20-60 embryos per experiment). Effects were compared to vehicle/injected control using Chi squared probability test (*p≤0.05, **p≤0.001, ***p≤0.0001).

4.4.1.1.7 Gross developmental phenotype and morphology

Careful phenotype observations were undertaken for dose optimisation (Section 2.4) of both the pharmacological and genetic manipulations. The final selection of doses for Dex, RU486 and Cyp Mo used in this study did not significantly alter the gross phenotype characteristics assessed using a 6 point scoring system (section 2.4.3). GR Mo embryos were found to be significantly different at 96 hpf compared to age matched controls ($p<0.05$), the difference in phenotype score at this point is due to reduced head trunk angle, a feature which was not apparent at 120 hpf when the phenotype score was comparable to controls. Embryos treated with Met displayed different phenotype scores are 96 and 120 hpf, (both $p\leq 0.01$) which was mainly due to an excess of pericardial oedema in Met treated embryos. No GC manipulations in this work resulted in a mean phenotype score of less than 4, indicating that they caused only mild to moderate alterations in gross developmental phenotype (Figure 4.8).

Histological examination of treated embryos showed no significant differences in the cellular architecture and the structure of non-cardiovascular organs at 120 hpf (Figure 4.10), with many of the key anatomical features present (in consultation from a departmental vet pathologist). However a common observation noted by 120 hpf in embryos where GR activity was suppressed (RU486 and GR Mo) was an alteration in swim bladder inflation (recorded as the percentage of the total treatment group which had an inflated swim bladder at 120 hpf).

Embryos incubated in RU486 or treated with GR Mo were less likely to have an inflated swim bladder at 120 hpf compared to controls (RU486 7.3 ± 1.3 % vs 99.4 ±0.2 %, respectively, $p<0.001$) with similar findings in the GR Mo embryos (25.4 ± 3.4 % vs 99.4 ± 0.2 % $p<0.001$). Examples of a lack of swim bladder inflation in the RU486 group are shown in Figure 4.9. Treatment of embryos with Dex, Met or Cyp Mo had no significant impact on swim bladder inflation (Table 4.1).

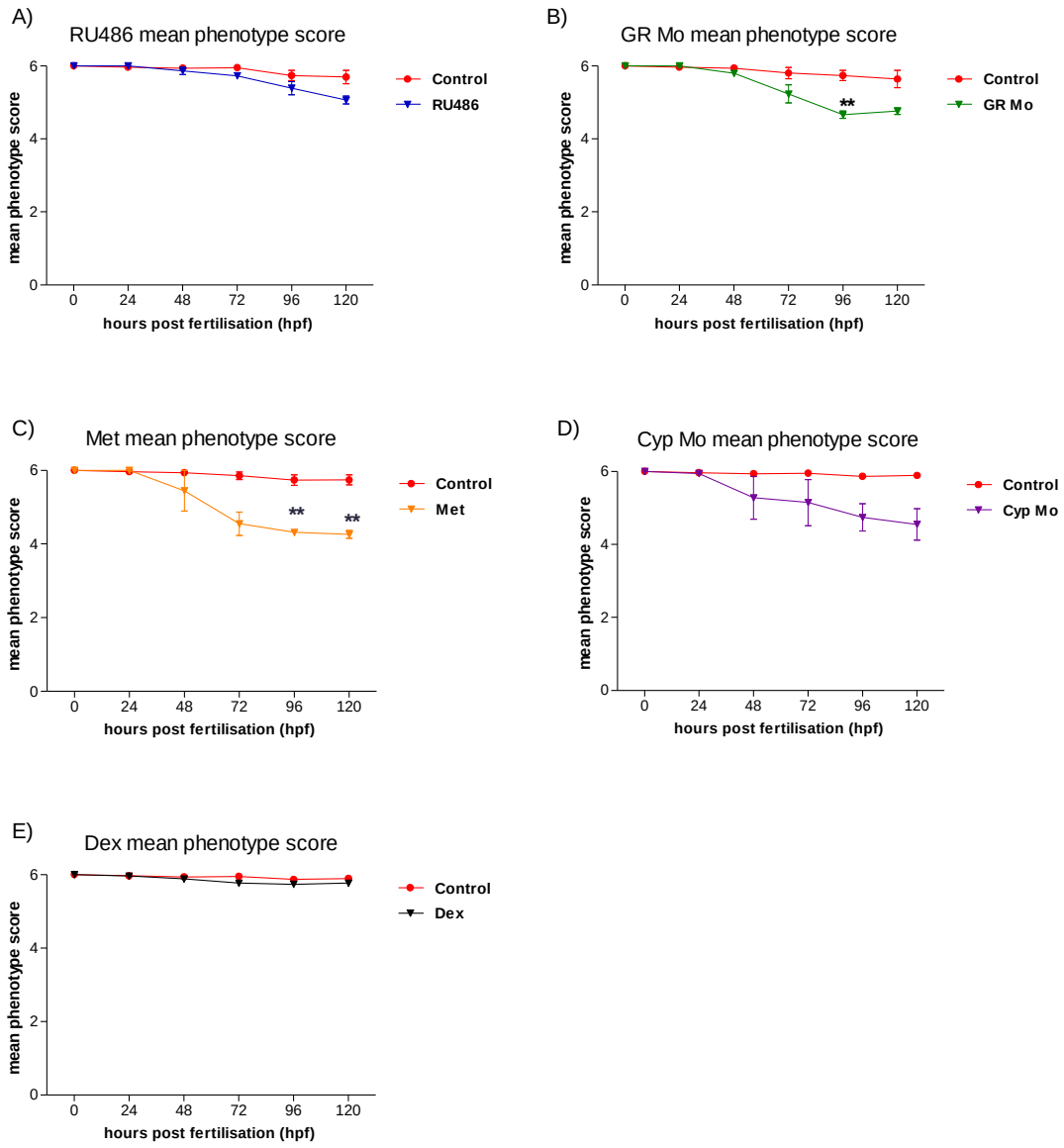

Figure 4.8 Embryonic phenotype score following glucocorticoid modulation
Mean phenotype score for groups of embryos treated either pharmacologically or genetically to manipulate the effect of glucocorticoids. A) 10µM RU486 B) glucocorticoid receptor-targeted morpholino (GR Mo), C) 10 µM metyrapone (Met), D) *cyp11b1*-targeted morpholino (Cyp Mo), and E) 100 µM dexamethasone (Dex) all vs control (vehicle alone 0.1 % EtOH or mismatched morpholino) over 120 hours post fertilisation (hpf). Scoring system is as follows 6 (Normal), 4-5 (Mild), 2-3 (Moderate), 1 (Severe) and 0 (dead). Data are mean ± SEM (n=3, duplicated results of 35-40 embryos per experiment) and analysed by 2-way ANOVA and Bonferroni post hoc test; **p≤0.01.

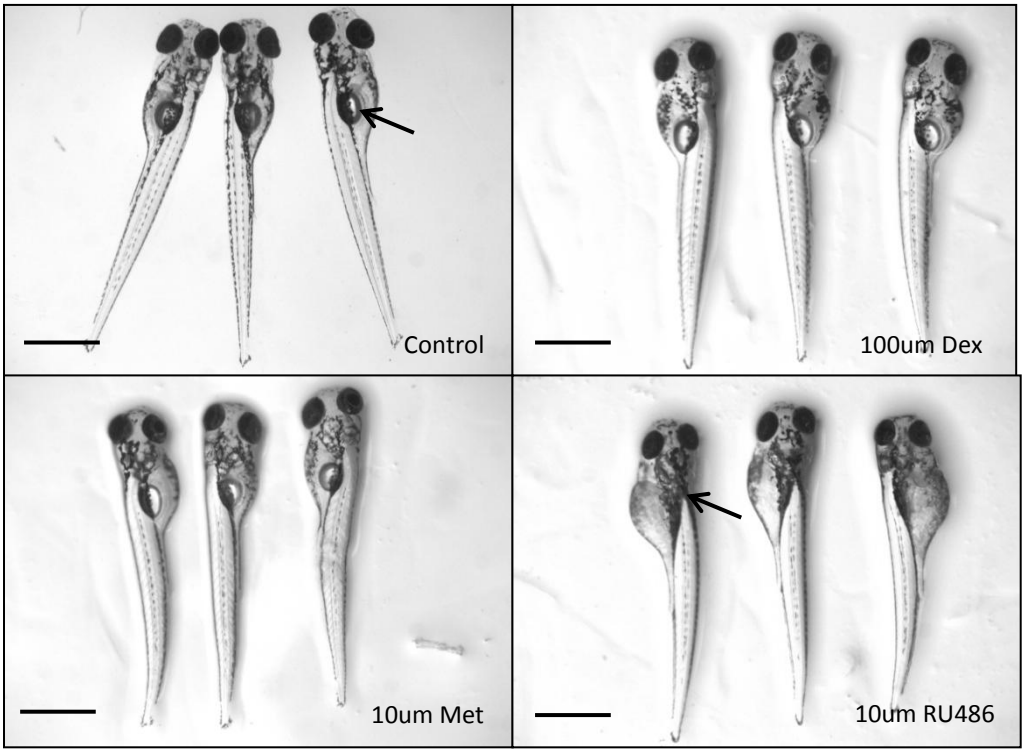

Figure 4.9 Effects of glucocorticoid modulation on swim bladder inflation
Images depicting swim bladder inflation in embryos at 120 hour post fertilisation (hpf) in controls and in those which had been treated with 100µM dexamethasone (Dex); 10µM metyrapone (Met) or 10µM RU486. Arrow in control image depicts location of inflated swim bladder. Arrow in RU486 image shows absence of inflated swim bladder. Calibration bar is 1mm.

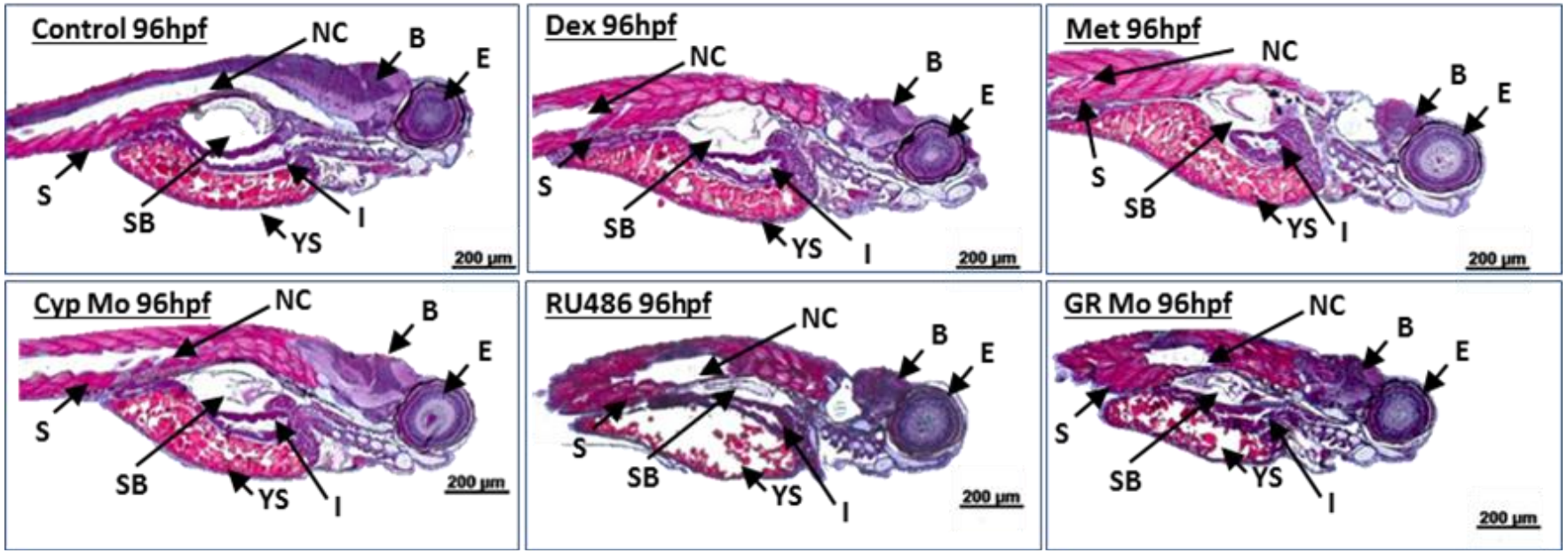

Figure 4.10 Embryonic histology following glucocorticoid modulation
Typical sagittal-sectioned embryos at 96 hours post fertilisation (hpf) in untreated controls or those which had been continuously pharmacologically or molecularly manipulated to modulate the activity of the GC system using the drugs dexamethasone (Dex), RU486 (a GR antagonist), metyrapone (Met) or morpholinos targeted towards *gr* (GR Mo) or *cyp11b1* (Cyp Mo). Sections were stained with haematoxylin (H) and eosin (E). H stains nuclei blue/indigo while E stains eosinophilic structures (proteins etc.) shades of red, pink and orange. Images shown may have planar differences and are only used to highlight anatomical features; histological images have not been used for quantitative analyses. Features highlighted are: somite (S), swim bladder (SB), yolk sac (YS), intestine (I), notochord (NC), brain (B) and eye (E).

Table 4.1 Embryonic phenotype observations

Summary of eye length (72 hours post fertilisation (hpf)), eye area (72hpf) and swim bladder inflation (120 hpf) in zebrafish embryos which had undergone glucocorticoid (GC) manipulation from the 2-cell stage by using dexamethasone (Dex), RU486, glucocorticoid receptor morpholino (GR Mo), metyrapone (Met), or *cyp11b1* targeted morpholino (Cyp Mo). Data are mean ± SEM for n=3 (12 per group for eye data/50 per group for swim bladder inflation). Eye data were compared with controls using 1-way ANOVA, swim bladder inflation was analysed by chi-square analysis.

	Control	**Dex**	**P value**	**RU486**	**P valued**	**GR Mo**	**P value**	**Met**	**P value**	**Cyp Mo**	P value
Eye length @72 hpf (mm)	0.91± 0.01	0.79±0.02	p<0.0001	0.78±0.01	p<0.05	0.78±0.02	p<0.05	0.89±0.02	NS	0.87± 0.02	NS
Eye area @72 hpf (mm^2)	0.51±0.01	0.39±0.02	p<0.0001	0.38±0.02	p<0.0001	0.42±0.02	p<0.001	0.49±0.02	NS	0.46±0.03	NS
Swim bladder inflation @120 hpf	99.4±0.2 %	99.5±0.3	NS	7.3 ±1.3	p<0.001	26.3± 3.4	p<0.001	94.2 ±5.6	NS	97.4±1.0	NS

4.4.1.2 Adult growth and development

4.4.1.2.1 Embryo to adult Survival

No differences were observed in terms of group survival over the course of the study with comparable numbers surviving until 120 dpf. These data include fish that died spontaneously and those that were humanely disposed of due to major structural abnormalities in accordance with Home Office regulations. The majority of deaths in all groups occurred between 10 dpf and 20 dpf (EmmodDex, 24% dead vs control, 14% dead, $p<0.05$; EmmodRU486, 17% dead vs control, 21% dead; and EmmodGR, Mo 28% dead vs control. 31% dead (Figure 4.11)).

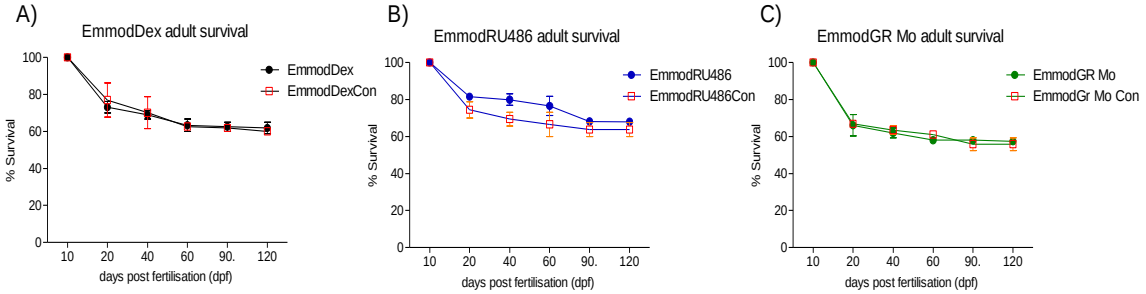

Figure 4.11 Adult survival following embryonic glucocorticoid modulation
Adult survival from 10-120 days post fertilisation (dpf) following embryonic glucocorticoid system manipulation with either A) 100µM dexamethasone (EmmodDex), B) 10µM RU486 (EmmodRU486) or C) glucocorticoid receptor morpholino (EmmodGR Mo). In each treatment data mean± SEM (60-80 fish per group) and compared with a control (red symbol); for A) and B) the control is embryonic exposure to vehicle (0.1% EtOH) and C) is a mismatched glucocorticoid receptor morpholino.

4.4.1.2.2 Juvenile swimming behaviour

Beyond 120 hpf it was noted that a high percentage of juveniles treated with RU486 (EmmodRU486) were not free swimming at 10 dpf compared to the controls ($p<0.04$). By 20 dpf, however, most fish in this group were able to swim freely ($p>0.05$). Similarly, fewer EmmodGR Mo fish were able to swim freely at 10 dpf compared to controls ($p<0.05$); again this was not observed at 20 dpf when all surviving fish in these groups were able to swim freely (Table 4.2).

Table 4.2 Adult development
Data presented in this table are a summary of male: female ratio, total body length at 90 days post fertilisation (dpf) and 120 dpf, weight at 90 dpf and 120 dpf in adult zebrafish which had been treated with glucocorticoid manipulating compounds during embryogenesis. Data are for those treated during embryogenesis with dexamethasone (EmmodDex), RU486 (EmmodRU486) or with glucocorticoid receptor morpholino (EmmodGR Mo). Data are mean ± SEM (n=35-45 adult fish per group). Statistical analysis was achieved using Student's *t*-test vs. relevant controls.

Measurement	EmmodDex	EmmodDex Con	P value	EmmodRU486	EmmodRU486 Con	P value	EmmodGR Mo	EmmodGR MoCon	P value
Male: Female ratio (%)	57:43	61:39	0.66	43:57	50:50	0.36	53:47	60:40	0.39
Total body length @90 dpf (mm)	21.5± 0.9	18.9± 0.9	0.05	22.5 ± 0.9	19.4 ± 0.8	0.02	19.4 ± 0.9	17.1 ± 0.8	0.154
Total body length @ 120 dpf (mm)	26.1 ± 0.3	23.8 ± 0.3	<0.001	27.5 ± 0.3	25.0 ± 0.3	<0.0001	23.1 ± 0.7	22.1 ± 0.6	0.105
Total body weight @ 90 dpf (g)	198.0±9.0	225.3±15.0	0.13	189.2±11.0	195.4±10.1	0.68	196.2±6	192.4±8.8	0.71
Total body weight @ 120 dpf (g)	532.0 ± 22.1	340.6 ± 15.6	<0.001	495.0 ± 20.0	387.1 ± 19.0	0.002	257.6 ± 26.1	345.5 ± 36.0	0.055
Condition factor	1.42 ± 0.06	0.84 ± 0.04	<0.001	1.33 ± 0.07	1.02 ± 0.06	0.002	0.58 ± 0.07	0.82± 0.09	0.008

4.4.1.2.3 Total body length

Body length was recorded at 20-30 day intervals over the course of 120 dpf. EmmodDex adults showed no difference in length up to 60 dpf. However, by 90 dpf they were longer than their controls ($p<0.05$. This difference was maintained until 120 dpf ($p<0.001$ Figure 4.12A)). EmmodRU486 adults were of similar length to controls until 60 dpf after which they were longer (EmmodRU486 22.5 ±0.9mm vs Controls 19.4± 0.8mm, $p=0.02$). This difference was maintained until 120 dpf ($p<0.05$ (Figure 4.12B)). EmmodGR Mo adults showed no significant difference in body length at any point throughout the course of the experiment ($p>0.05$ (Figure 4.12C)).

4.4.1.2.4 Body weight

EmmodDex showed a weight-gain trajectory similar to controls up to 90 dpf. However, by 120 dpf these fish were significantly heavier than controls ($p<0.01$ (Figure 4.12D)). No sex difference was observed (Table 4.2)). EmmodRU486 adults showed no difference in body weight compared with controls until 120 dpf by which point they were heavier (495 ± 20 mg vs. 387 ± 19 mg, $p=0.002$ (Figure 4.12E)). There was also no detectable difference between the sexes within each group (Table 4.2)). EmmodGR Mo adults had a similar weight gain trajectory as controls throughout the 120 day time-course of the study. However, a trend towards significance was observed at the end of the experiment at 120 dpf, when the GR Mo were slightly smaller than the controls (258 ± 26 mg vs. 346 ±36 g, $p=0.056$ (Figure 4.12F)) increasing the group number here may determine the importance of this result.

4.4.1.2.5 Condition factor (fish girth)

EmmodDex adults had a greater condition factor than controls at 120 dpf (1.42 ± 0.06 k vs. 0.84 ± 0.04 k, $p<0.0001$). EmmodRU486 also displayed a greater condition factor compared to controls (1.327 ± 0.07k vs 1.023 ± 0.06 k, $p<0.05$, whereas EmmodGR Mo displayed a significantly lower condition factor compared with their controls (0.58 ± 0.07 vs. 0.82 ± 0.09, $p<0.001$ (Table 4.2).

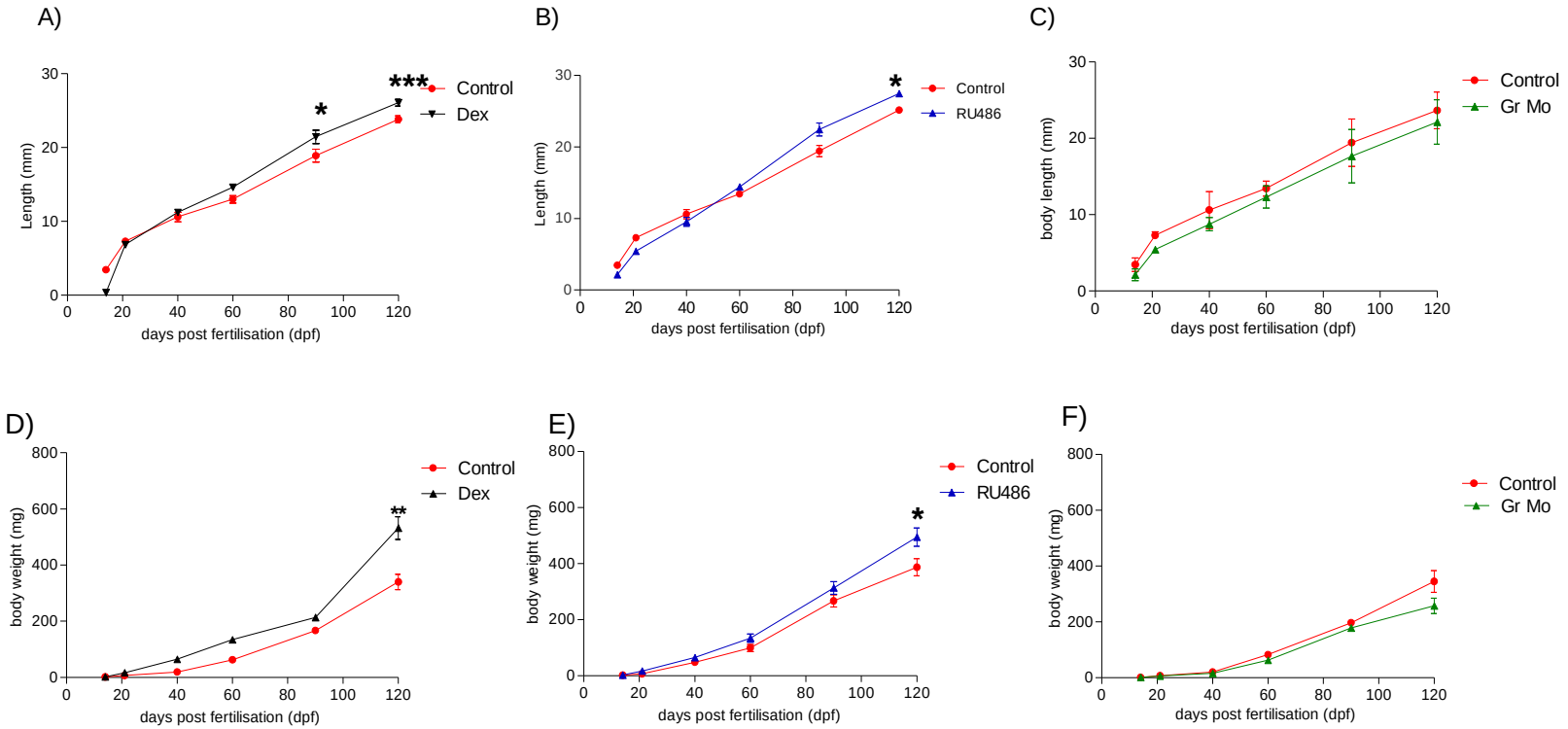

Figure 4.12 Adult growth following embryonic glucocorticoid modulation
Growth (length and weight trajectories) of adults derived from embryos which underwent glucocorticoid manipulation during embryogenesis. Data presented here are from 10 days post fertilisation (dpf) until 120 dpf. Adults are from the groups treated during embryogenesis with: A) and D) dexamethasone (EmmodDex-black line), B) and E) RU486 (EmmodRU486-blue line), or C) and F) glucocorticoid receptor-targeted morpholino (EmmodGR Mo-green line). All data are compared with their own specific controls (for drugs this was vehicle only (0.1% EtOH) for morpholino this was mismatched morpholino - red line). A-C) is for total body length and D-F) is for total body weight. Data shown are mean length per group ±SEM (n=28-40 adult per group). Data were analysed by 2-way ANOVA and Bonferroni post hoc test; *p≤0.05, **p≤0.01***p<0.001.

4.4.2 Aim 2:

Does embryonic GC manipulation alter embryonic and adult HPI axis function?

To investigate whether GC manipulation altered HPI axis activity cortisol levels were monitored using cortisol ELISA, and the abundance of key GC genes was determined using qRT-PCR.

4.4.2.1 Embryonic HPI axis activity

4.4.2.1.1 Embryonic Basal cortisol

Changes in cortisol levels were determined by ELISA of whole embryo homogenates at 72 and 120 hpf following pharmacological or genetic modulation of the GC system. There were no differences in cortisol for any of these modulations at 72 hpf compared to controls ($p>0.05$ (Figure 4.13(A)). However, by 120 hpf, all embryos treated with drugs or Mo displayed reduced cortisol levels compared to controls Dex, GR Mo, Cyp MO ($P<0.05$), RU486 ($P<0.01$) and Met ($P<0.001$)(Figure 4.13 (B)).

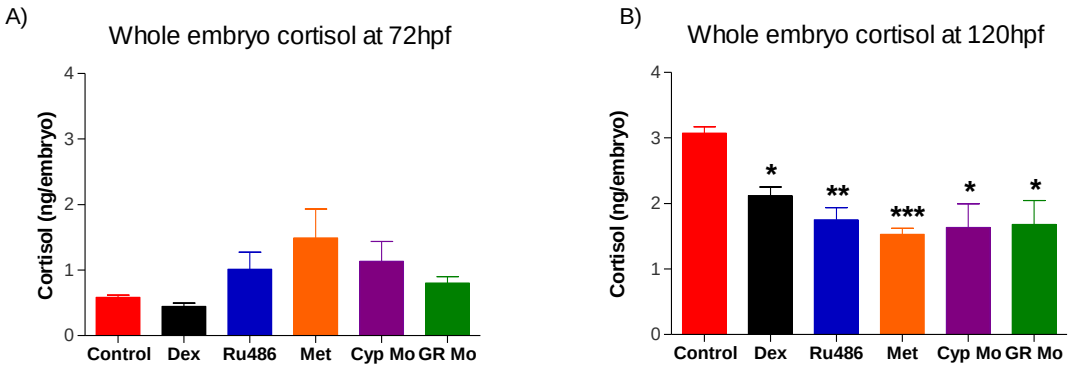

Figure 4.13 Embryonic cortisol after glucocorticoid modulation
Whole embryo cortisol in embryos exposed to dexamethasone (Dex) [100µm], RU486 [10µm], metyrapone (Met) [10µm] or morpholino targeted towards *cyp11b1* (Cyp Mo) or glucocorticoid receptor (GR Mo) from 1 hour post fertilisation (hpf) until 72 hpf (A) and 120 hpf (B) Data shown are mean ± SEM for n=3 (10 embryos). Data were analysed by 1-way ANOVA and Dunnett's post hoc analysis versus control (*$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$).

4.4.2.1.2 Embryonic gene relative abundance after glucocorticoid manipulation

Incubation in Dex or Met had no effect on abundance of *gr* mRNA relative abundance at 72 or at 120 hpf. Incubation in RU486 had no effect on detectable *gr* levels at 72 hpf ($p>0.05$) but increased abundance of *gr* mRNA at 120 hpf ($p<0.001$). Embryos treated with Cyp Mo showed a reduction in *gr* mRNA at 72 hpf ($p<0.05$) but not at 120 hpf (0.88 ± 0.10 vs. controls 0.97 ± 0.01 AU, $p>0.05$). Meanwhile for GR Mo embryos a reduction in *gr* mRNA was noted at 72 hpf ($P<0.0001$) the detectable levels at 120 hpf were found to be significantly lower than controls but the difference here was less extreme ($p<0.05$ (Figure 4.14 A)).

mr mRNA abundance was also assessed at 72 and 120 hpf following continuous incubation with drug (from 1 hpf) or treatment with Mo (Figure 4.14 B). Neither of the Mo gene knockdown treatments altered *mr* mRNA levels significantly compared to controls at 72 hpf (both $p>0.05$) or at 120 hpf (both $p>0.05$). Likewise no difference in *mr* mRNA was detected in embryos which had been constantly incubated in RU486 at 72 hpf or at 120 hpf ($p>0.05$). Met incubation did, however, result in increased levels of *mr* mRNA at 120 hpf (1.23 ± 0.02 vs. control 0.75 ± 0.02, $p<0.0001$), but not at 72 hpf ($p>0.05$). Following Dex incubation there was an obvious increase in *mr* mRNA at 72and at 120 hpf ($p<0.001$ (Figure 4.14B)).

hsd11b2 mRNA relative abundance was also assessed (Figure 4.14C). RU486 treatment did not alter abundance of this gene at 72 or 120 hpf ($p>0.05$). Met incubation and treatment with the Cyp Mo showed similar findings at 120 hpf where both of these groups displayed and increase in *hsd11b2* mRNA relative abundance compared to the controls ($p<0.01$). Embryos treated with GR Mo showed higher mRNA levels of *hsd11b2* at 120 hpf ($p>0.001$), however, there was no difference at 72 hpf (0.99 ± 0.1 vs. control 0.91 ± 0.03AU.). Constant incubation in Dex resulted in an increased in *hsd11b2* mRNA levels at 72 and at 120 hpf ($p<0.0001$ Figure 4.14C)).

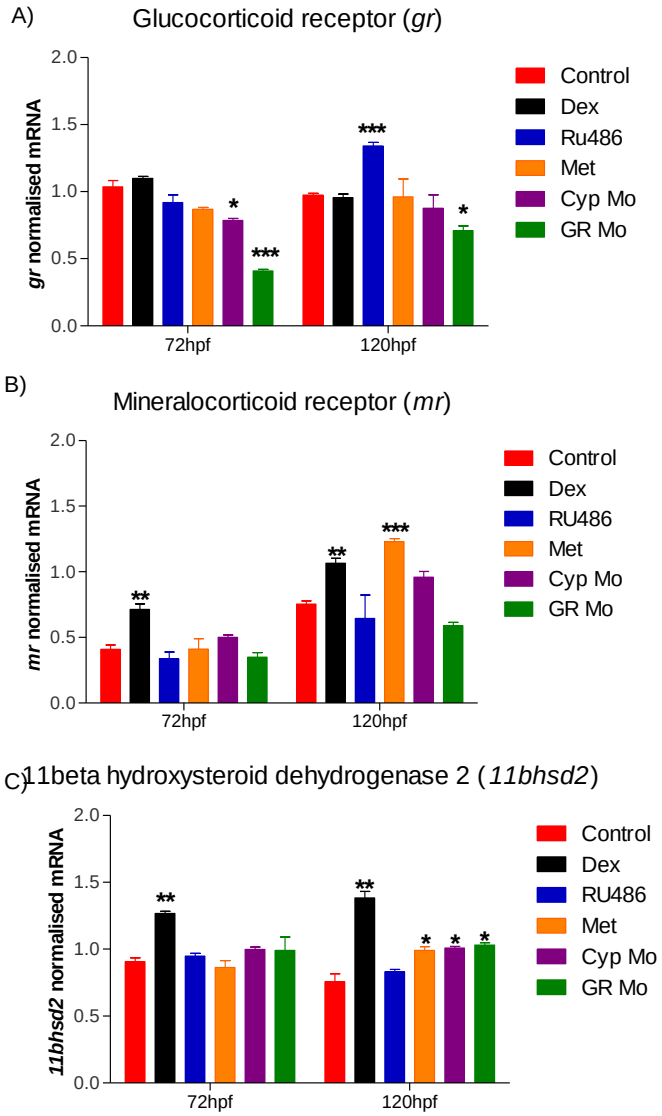

Figure 4.14 Embryonic gene abundance after glucocorticoid modulation
Gene mRNA relative abundance in whole zebrafish embryos at 72 and 120 hours post fertilisation (hpf). Embryos had previously been treated from the 2-cell stage with dexamethasone (Dex-black bars), RU486 (blue bars), morpholino targeted towards glucocorticoid receptor (GR Mo-green bars)), metyrapone (Met-orange bars) or a morpholino targeted towards *cyp11b1* (Cyp Mo-purple bars) compared to control embryos (red bars) as detailed in section 4.3.6. Relative mRNA abundance is shown for A) *gr*, B) *mr*, and C) *11βhsd2*. Data are mean ± SEM for n=3 (10 embryos per time pooled), presented as arbitrary units (AU) after standard curve production and normalisation to *ef1a* and *18s*. Data were analysed by 1-way ANOVA and Dunnett's post hoc test, compared to controls at each time-point; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

4.4.2.2 Adult HPI axis activity

4.4.2.2.1 Adult basal and stressed cortisol

To determine the long-term effects of embryonic GC manipulation on adult HPI axis function 24h cortisol was measured in the bathing water of Emmod-adults. Basal cortisol levels were determined as the cumulative concentration achieved in the bathing water of a 5L tank containing 5 adult fish over a 24 h period prior to stress, followed by the same measurement in the 24h following the stressful stimulus.

EmmodDex adults had a similar basal 24h cortisol level to controls (EmmodDex 8.15 ± 0.35 ng/fish/ L vs. control 6.87 ± 0.50 ng/fish/ L, p=0.09 (Figure 4.15A). Following netting stress, however, EmmodDex showed a higher 24h tank cortisol level compared to controls (EmmodDex 19.80 ± 0.60 ng/fish/ L vs. control 15.10 ± 0.46 ng/fish/ L, p=0.008 (Figure 4.15 B).

EmmodRU486 adults showed no difference in 24h tank cortisol levels compared to controls both basally (10.37 ± 1.89 vs. 9.45 ± 0.97 ng/fish/ L, p >0.05) and following stress, (p>0.05(Figure 4.16A and B)). EmmodGR MO showed no detectable difference in basal tank cortisol levels compared to controls (p>0.05), however did display lower stress tank cortisol level following stress compared to controls (p<0.01 (Figure 4.17B))

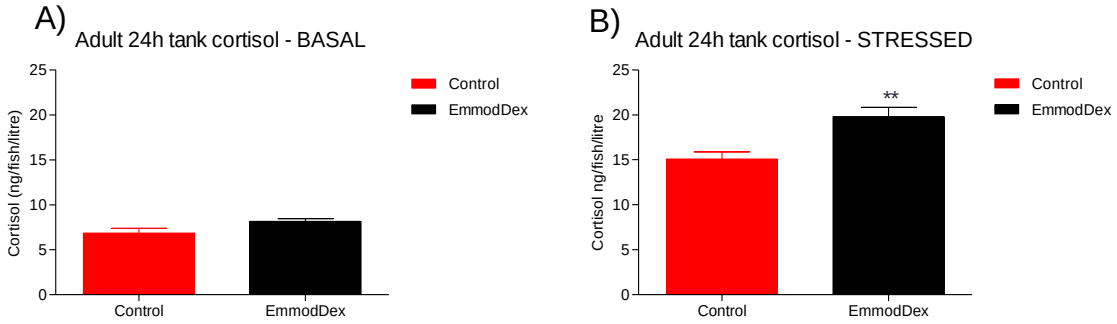

Figure 4.15 Embryonic dexamethasone exposure and adult cortisol
Tank Cortisol levels in 24 h collection pre (basal) and post (stressed) net/restraint stressor protocol for adult fish treated with dexamethasone during embryogenesis (EmmodDex) vs. vehicle only controls. Data are mean ± SEM for n=3 (5 fish per L) expressed as mean cortisol levels in ng/fish/ L and analysed using Student's *t*-test (**p≤0.01).

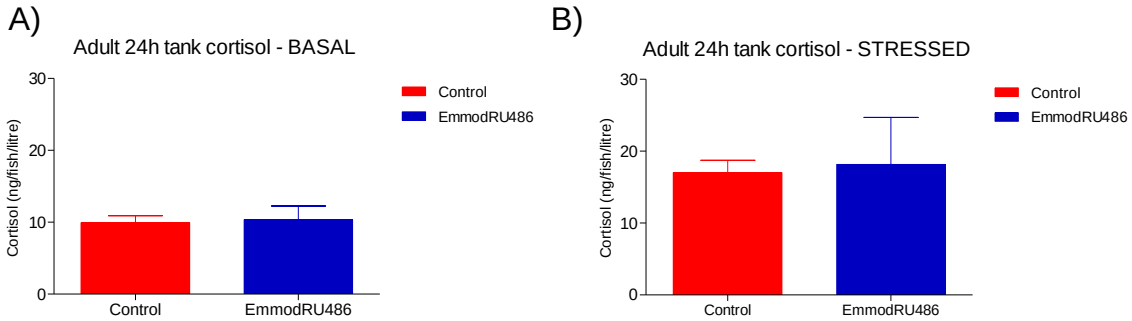

Figure 4.16 Embryonic RU486 exposure and adult cortisol

Tank Cortisol levels in 24 h collection pre (basal) and post (stressed) net/restraint stressor protocol for adults which had been treated with RU486 during embryogenesis (EmmodRU486) vs vehicle only controls. Data are mean ± SEM for n=3 (5 fish per L) expressed as the mean cortisol levels in ng/fish/ L, analysed using Student's *t*-test.

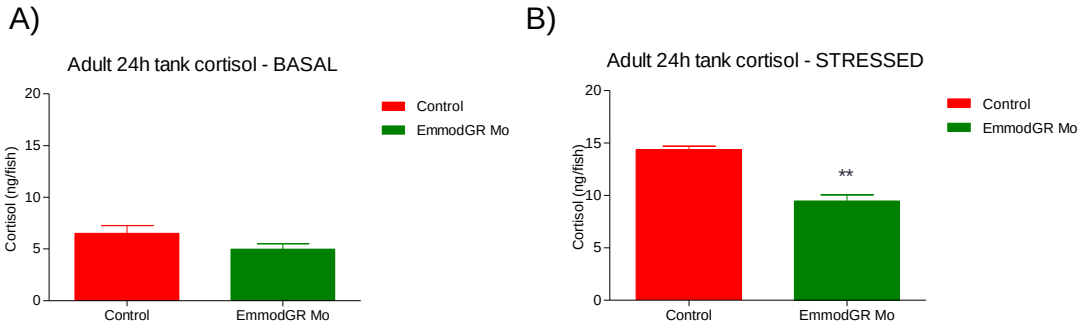

Figure 4.17 Embryonic glucocorticoid receptor knock down and adult cortisol

Tank Cortisol levels in 24 h collection pre (basal) and post (stressed) net/restraint stressor protocol for adults which had been treated with glucocorticoid receptor targeted morpholino during embryogenesis (EmmodGR Mo) vs. injected mismatch controls. Data are mean ± SEM for n=3 (5 fish) expressed as the mean cortisol levels in ng/fish/ L, analysed using Student's *t*-test (** $p \leq 0.001$).

4.4.2.3 Adult mRNA abundance of key Glucocorticoid genes

Abundance of GC function genes was determined in various isolated adult tissues following embryonic manipulation (Tables 4.3 and 4.4). EmmodDex increased *gr* mRNA relative abundance in the kidney ($P<0.001$) and in the liver ($p<0.05$). No other tissues investigated (including brain and smooth muscle) showed altered *gr* mRNA levels. In EmmodDex *mr* mRNA abundance was unaltered in all of tissue-types studied.

EmmodRU486 adults, showed lower *gr* mRNA gene abundance in isolated liver compared to controls ($p<0.05$) with no differences in other isolated tissues (kidney, brain, smooth muscle). *mr* mRNA levels were higher in isolated brain tissue compared to controls (11.21 ± 0.52 vs. 7.55 ± 0.31 AU, $p<0.001$), with abundance of *mr* mRNA comparable to controls all other tissues investigated.

EmmodGR Mo adults, showed lower *gr* mRNA in isolated liver preparations compared to controls ($p<0.01$) with no differences in other isolated tissues (kidney, brain, smooth muscle). *mr* mRNA abundance levels in EmmodGR Mo were higher in brain than for controls ($p<0.01$) but unaltered in all other tissues investigated.

Table 4.3 Glucocorticoid receptor (*gr*) mRNA abundance

gr gene relative abundance as determined using qRT-PCR following embryonic treatment with dexamethasone (EmmodDex), RU486 (EmmodRU486) or morpholino targeted towards glucocorticoid receptor (EmmodGR Mo), versus their respective controls. Gene abundance is quantified as relative concentration in arbitrary units derived from a standard concentration curve and normalised to the house keeping genes e*f1α* and *gapdh.* Data are mean ± SEM of n=3 (5 organs pooled) analysed by Student's *t*-test.

gr mRNA abundance	EmmodDex	Control	P value	EmmodRu486	control	P value	EmmodGR Mo	Control	P value
Liver	0.47±0.03	0.34±0.05	0.056	0.29±0.02	0.35±0.02	0.03	0.21±0.02	0.37±0.03	0.01
Kidney	2.03±0.07	1.17±0.17	0.0007	1.22±0.21	1.15±0.16	0.80	1.45±0.09	1.20±0.26	0.41
Brain	7.23±0.85	6.35±0.59	0.44	7.35±0.21	6.89±0.15	0.15	6.74±0.14	7.01±0.65	0.71
Muscle	2.34±0.04	2.48±0.09	0.22	1.85±0.04	1.98±0.17	0.09	2.10±0.12	2.01±0.23	0.75

Table 4.4 Mineralocorticoid receptor (*mr)* mRNA abundance

mr gene relative abundance as determined using qRT-PCR following embryonic treatment with dexamethasone (EmmodDex), RU486 (EmmodRU486) or morpholino targeted towards glucocorticoid receptor (EmmodGR Mo), versus their respective controls. Gene abundance is quantified as relative concentration in arbitrary units derived from a standard concentration curve and normalised to the house keeping genes e*f1α* and *gapdh.* Data are mean ± SEM. of n=3 (5 organs pooled) analysed by Student's *t*-test.

mr mRNA abundance	EmmodDex	Control	P value	EmmodRu486	control	P value	EmmodGR Mo	Control	P value
Liver	0.68±0.03	0.62±0.02	0.17	0.56±0.03	0.68±0.08	0.24	0.62±0.08	0.71±0.07	0.44
Kidney	1.12±0.05	1.20±0.09	0.48	1.32±0.08	1.29±0.05	0.76	1.28±0.05	1.32±0.06	0.71
Brain	11.7±0.06	10.05±0.63	0.10	11.21±0.57	7.55±0.31	0.001	10.33±0.42	9.35±0.31	0.01
Muscle	0.89±0.23	0.85±0.65	0.95	0.91±0.05	0.92±0.12	0.94	0.87±0.03	0.91±0.02	0.31

4.4.3 Aim 3:

Does embryonic glucocorticoid manipulation alter embryonic and adult swim behaviour?

4.4.3.1 Embryonic swim/movement behaviour

4.4.3.1.1 Spontaneous twitch behaviour

The first locomotor behaviour observed in zebrafish embryos is in the form of spontaneous movement, whereby the embryos repeatedly perform slow, alternating twitch like movements (Selderslaghs *et al*, 2013). The influence of GC manipulation on locomotor development was observed in all treatment groups at 18hpf. Embryos which were continuously incubated in Dex and Met or injected with Cyp Mo displayed no difference in spontaneous twitches compared to controls. However, embryos which had been treated with RU486 or GR Mo showed fewer twitches compared to controls ($p<0.001$ Figure 4.18A).

4.4.3.1.2 Response to touch stimulus

Coordinated muscular movements in response to touch stimulus were observed in embryos which were continuously incubated in drugs or following treatment with morpholino. Dex, Met or Cyp Mo embryos showed no alteration in the response to the gentle touch of a forcep tip. RU486 and GR Mo embryos did, however, display muted responses to this stimulus, with a lower percentage responding than in controls ($p<0.001$, Figure 4.18B).

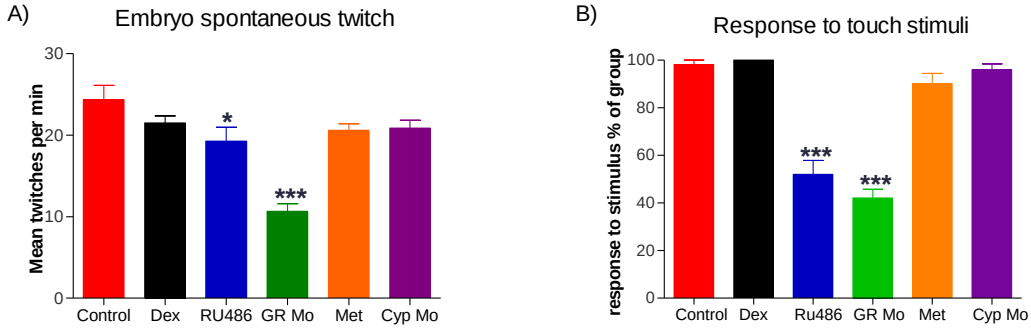

Figure 4.18 Embryonic movement after glucocorticoid modulation
Embryonic movement following either pharmacological or molecular glucocorticoid modulation. Pharmacological manipulations include: dexamethasone (Dex), RU486 and metyrapone (Met). Molecular manipulations include: morpholino targeted towards glucocorticoid receptor (GR Mo) or cyp11b1 (Cyp Mo). Shown are A) mean twitches per min, the spontaneous uncoordinated movements observed in embryos at 18 hours post fertilisation (hpf)(n=20 embryos per group). B) The response to gentle touch stimulus given as a percentage of the total study group (n=5, 10 embryos per test). Data are mean ± SEM, analysed by 1-way ANOVA and Dunnett's post hoc test; *p≤0.05, ***p≤0.001.

4.4.3.1.3 Distance travelled

Velocity of movement was observed at 72 and 120 hpf in embryos continuously treated with drug, or after Mo treatment. Treatments did not impair velocity of movement at 72hpf, when embryos are not actively exploring their environment (Figure 4.19A). However, by 120hpf, some treatments altered embryonic velocity. All treatments which suppress the activity of GC resulted in a reduced velocity compared to controls ($p<0.001$). Embryos which were treated with Dex had comparable velocity to controls (Figure 4.19 B). Locomotor activity of embryos was compared between treatment groups at 120 hpf over a standardised 5 min time period. Embryos treated with Dex showed no difference from controls in distance travelled ($p>0.05$) while treatment with either RU486 or GR Mo resulted in less distance travelled compared to controls ($p<0.05$). Embryos treated with Met or Cyp Mo also showed lower locomotor activity compared to controls ($p<0.001$ ((Figure 4.19 C)).

At 72 hpf control embryos spend a large proportion of their time in the mid region of the Petri dish with typically 73.25 ± 1.93% of their time spent in this region compared to only 12.75 ± 1.37 % of the time in the outer region. Met, RU486, Cyp Mo and GR Mo-treated embryos mirrored this pattern of swim behaviour (Table 4.5). In contrast, Dex treated embryos spent less time in the central region ($p<0.0001$) with more time spent in the outer region ($p<0.0001$ (Table 4.5)). At 120 hpf control embryos spent 20.05 ± 3.20% of their time in the outer region, this was similar to the time spent by, Met and Cyp Mo ($p>0.05$). Less time was spent in this zone by RU486 and GR Mo embryos compared to controls ($p<0.05$). These embryos spent longer in the mid region (entry region) of the Petri dish than any other group, significantly more than the un-manipulated controls ($p<0.001$). Conversely, embryos which were treated with Dex spent much longer in the outer region of the dish ($p<0.001$), with very little of their time spent in the mid or inner regions ($p<0.001$) and ($p<0.001$) of the time, respectively (Table 4.3)). These findings are clear in the heat maps (Figure 4.19).

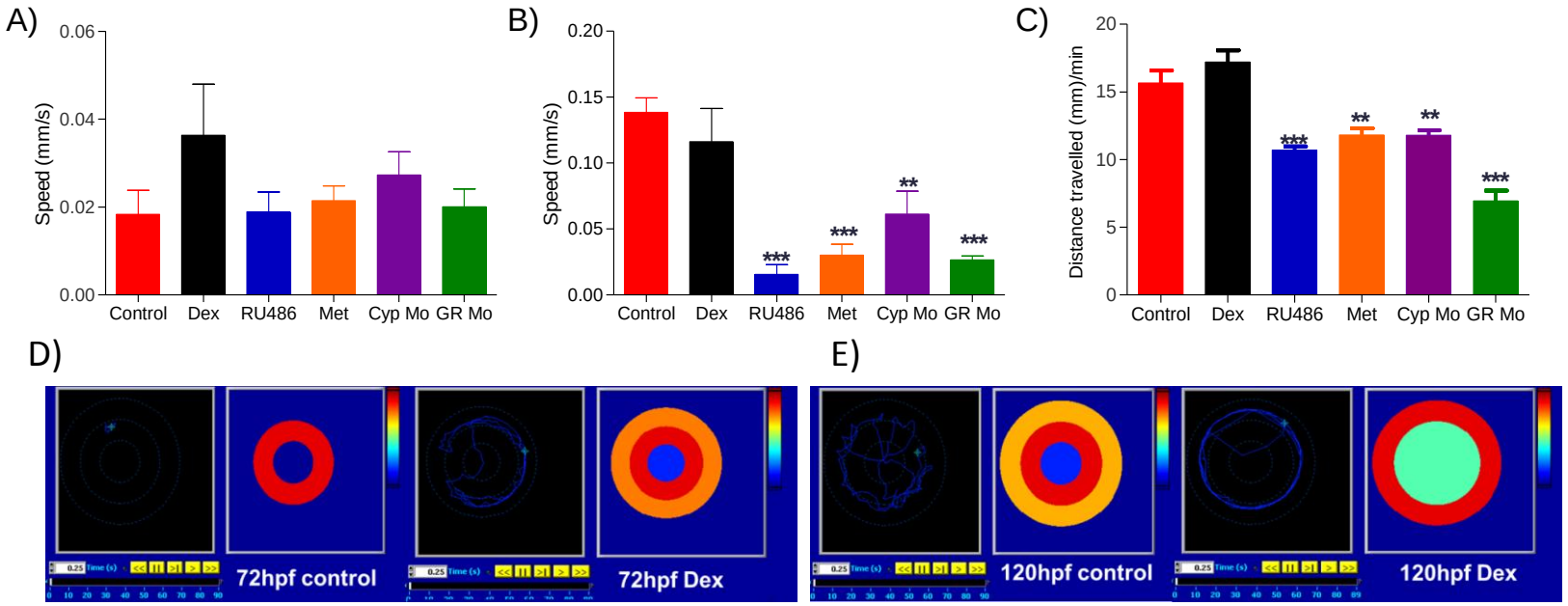

Figure 4.19 Embryonic swim activity after glucocorticoid modulation
Locomotive/swim activities in embryos following various treatments with drugs and morpholinos. Drugs investigated include: dexamethasone (Dex-black bars), RU486 (blue bars) and metyrapone (Met-orange bars). Morpholinos were targeted towards the glucocorticoid receptor (GR Mo) or *cyp11b1* (Cyp Mo). Data shown are A) Speed of 72 hours post fertilisation (hpf) embryos (n=3 (8 embryos per expt.)), B) Speed of 120 hpf embryos, after constant incubation in drug or treatment with drug n=3 (8 embryos per expt), C) Distance travelled (mm/min) of 120 hpf embryos (n=3 (8 embryos per group)). Data are mean ± SEM, analysed by 1-ANOVA compared to controls and analysed with Dunnett's post hoc test (*p≤0.05, **p≤0.001, ***p≤0.0001). D-E) Typical heat maps and traces of embryo movement at 72 and 120hpf. LimeLight video analysis software was used to digitally delineate the Petri dish into 3 concentric regions. Shown are traces of a single embryo's movement over a 5 min period (blue line on black background) with corresponding graded heat map with areas of high activity indicated by the warmer colours (red) in a downward gradient towards cooler colours (blue) for less activity.

Table 4.5 Embryonic zone preferences after glucocorticoid manipulation
Embryonic zone preference at 72 and 120 hours post fertilisation. Zone preference refers to the time spent in a particular delineated region of a Petri dish. Three concentric regions were marked, the inner, mid and outer. The time spent in each zone was given as a percentage of the total study time. Data are mean ± SEM (n=12 embryos). Statistical analysis was carried out by 1-way ANOVA of variance, p values displayed with those highlighted deemed statistically significant; $p \leq 0.05$.

72hpf	Inner	P value	Mid	P value	Outer	P value
Control	1.20 ±2.15	/	73.25±1.93	/	12.75± 1.37	/
Dex	0.75±1.85	0.88	20.25± 0.95	0.0001	68.25±1.54	0.0001
RU486	1.75±1.63	0.84	79.00 ± 1.08	0.22	9.25±1.10	0.06
GR Mo	0.75±2.86	0.90	67.75± 2.98	0.14	12.50±0.65	0.87
Cyp Mo	3.75±2.16	0.42	76.25± 3.09	0.42	16.00±0.82	0.06
Met	9.25±3.19	0.05	75.50 ± 2.84	0.52	15.25±1.03	0.15
120hpf	**Inner**	**P value**	**Mid**	**P value**	**Outer**	**P value**
Control	18.80±2.15	/	40.32±1.56	/	20.05± 3.20	/
Dex	4.75±1.06	0.0001	10.25± 1.25	0.0001	59.53±3.02	0.0001
RU486	2.75±0.52	0.0001	59.00 ± 3.06	0.0001	6.20±5.01	0.03
GR Mo	5.75±2.86	0.001	72.75± 1.68	0.0001	8.50±1.05	0.002
Cyp Mo	11.75±2.86	0.06	36.25± 2.07	0.13	17.0±0.82	0.37
Met	16.25±5.19	0.08	42.50 ± 1.64	0.35	18.50±1.23	0.66

4.4.3.2 Adult swim behaviour

4.4.3.2.1 Forced swim/dive assay

This assay exploits the zebrafish natural tendency to initially dive to the bottom of a novel or unfamiliar tank after which vertical activity should gradually increase. The time used for this assay was four min during which it was apparent that control fish spent longer in the lower region of the tank (on average 86% of time) with an average of only 14% of total time spent in the upper region of the tank.

There was no difference between EmmodRU486 and controls in the time spent in the upper region of the tank or, consequently, in the time spent in the lower region of the tank ($p>0.05$; Figure 4.21). Likewise the EmmodGR Mo adults spent a similar proportion of their time in the upper region and the lower regions as controls ($p>0.05$ Figure 4.22. A difference however was noted in the EmmodDex adults which spent longer in the upper region of the tank compared to controls (36.5 ±8.9 vs. 14.2 ± 6.4 %, $p=0.05$), and, consequently, less time in the lower region of the tank (53.3 ± 8.9 vs. controls 89.2 ± 4.2%, $p=0.05$; Figure 4.20).

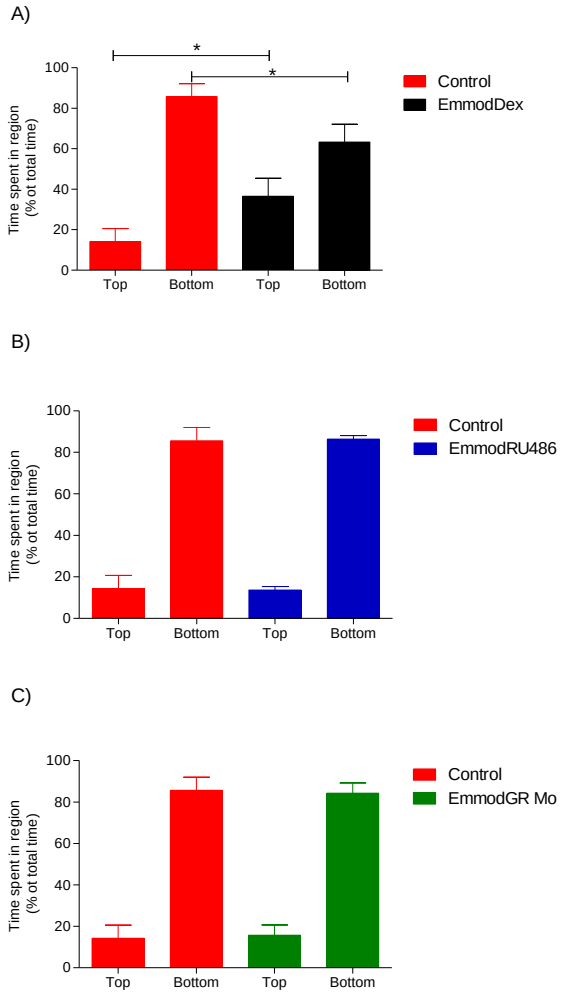

Figure 4.20 Adult dive tank/forced swim assay after embryonic modulation
Forced swim/dive tank region preference (either upper or lower region of tank) in adult fish embryonically manipulated by: A) dexamethasone (EmmodDex-black bars), B) RU486 (EmmodRU486-blue bars), or C) glucocorticoid receptor targeted-morpholino (EmmodGR Mo –green bars). Data are mean ± SEM (n=12 fish) and were compared with respective controls (red bars) by Student's *t*-test (*$p\leq0.05$).

4.4.3.2.2 Open field assay

An open field test was used to assess adult swim behaviour. This assessment measures the number of zones that the fish crosses in a 3 min period using a 9-square grid template to track fish movements using LimeLight software. The percentage of time spent in the central zone (a feature of "boldness") and the speed at which the fish travels whilst exploring the tank are documented and analysed by the tracking software. EmmodRU486 and EmmodGR Mo displayed no difference in zone crossing or the time spent in the central zone compared to their controls. There was, however a difference in the time spent in the central region by the EmmodDex groups compared to controls ($p<0.05$, Figure 4.21A). The speed at which adults explored the open field area was also calculated. No group showed any difference in swim velocity compared to their controls ($p>0.05$).

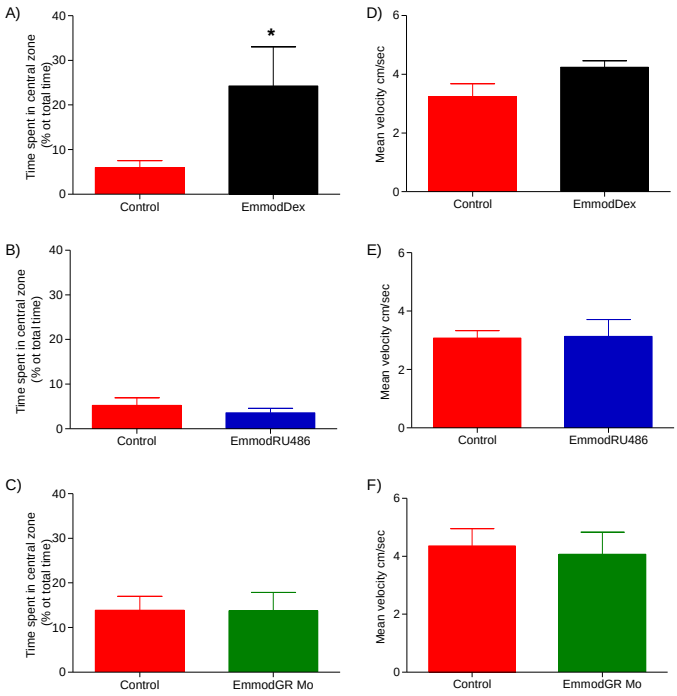

Figure 4.21 Adult open-field assay after embryonic manipulation
Open field quantitative analysis of adults which had glucocorticoid modulation during embryogenesis. Manipulation was achieved using: dexamethasone (EmmodDex-black), RU486 (EmmodRU486-blue), or targeted glucocorticoid receptor morpholino (EmmodGR Mo-green). All group data are versus respective control (red). A-C) Percentage of total time in the open field arena which was spent in the central square of a nine square grid open field arena, and D-F) Mean velocity (cm/s) of adults in an open field arena. Data are mean ± SEM for n=12 fish. Data were analysed by Student's *t*-test vs controls (* $p\leq0.05$).

4.4.3.2.3 Novel object assay

The aim of this assay is to measure "boldness" by recording the amount of time a single fish spends close to a foreign object, representing a predator, in the testing arena. The average number of times the fish crossed into the novel object zone was recorded over a 2 min period. EmmodRU486 adults showed very similar behaviour to controls ($p>0.05$, Figure 4.22B)). EmmodGR Mo displayed an apparent reduction in the number of crosses into the novel region but this did not reach statistical significant (EmmodGR Mo 2.2 ± 0.6 vs. 4.0 ± 0.9 crosses/2min, $p=0.09$. In contrast, EmmodDex showed increased time spent in the novel object zone compared to controls ($p<0.001$ (Figure 4.22).

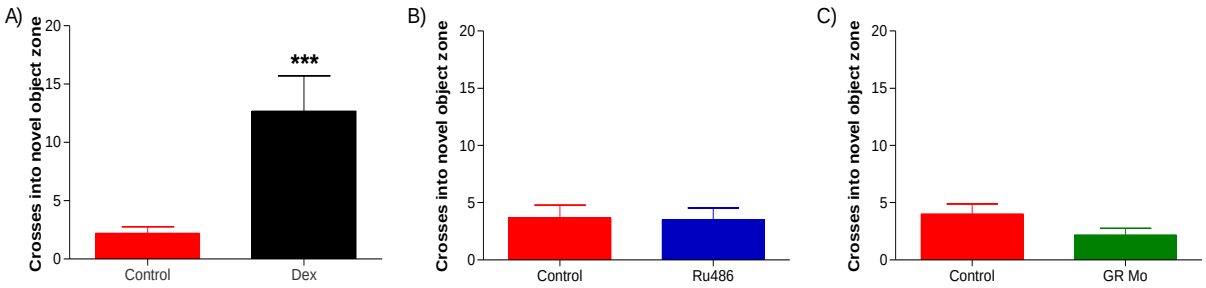

Figure 4.22 Adult object avoidance after embryonic glucocorticoid manipulation
Total number of crosses into the novel object region by adult zebrafish which were manipulated during embryogenesis. Data shown are for adults treated during embryogenesis with: A) dexamethasone (EmmodDex-black), B) RU486 (EmmodRU486-blue), or C) injected with a targeted glucocorticoid receptor morpholino (EmmodGR Mo). Data are mean ± SEM for n=12 fish and were analysed by Student's *t*-test vs. control (*** $p\leq0.001$).

4.5 Discussion

This chapter has carefully explored and documented whether pharmacological or genetic manipulation of the GC system during early embryonic development alter development and growth, HPI axis function and swim behaviour in the developing zebrafish embryo, and whether long-term disruption of these processes is "programmed" in the adult.

4.5.1 Aim 1:

Does embryonic GC manipulation alter embryonic and adult growth and development?

GCs play a crucial role in mammalian foetal maturation during the late phases of gestation which is why they are used clinically to accelerate lung maturation in preterm infants (Hewitt *et al*, 2006). However, this pre-natal GC exposure reduces birth-weight, possibly as a result of decreasing cell proliferation and increasing tissue differentiation (Cottrell & Seckl, 2009; Seckl, 2004). The underlying cellular and molecular events are poorly characterised. To assess the suitability of the zebrafish as a model of GC programming model, the effects of manipulating the GC system on growth and development were assessed by both inhibiting and activating GC-related pathways.

Despite differences in growth observed in the early stages of development, by 120 hpf none of the pharmacological treatments investigated altered the length of the embryos. There was, however, a clear lag in development/embryogenesis with a reduced growth rate and head-trunk angle during the first 72 hpf for embryos in which the GC activity pathway was impaired. This lag was not sustained, however, as a return to normal developmental rate and head-trunk angle was observed by 120hpf. Exposure to GC, using Dex incubation, did not cause an increase in growth by 120hpf, in contrast to the low birth weight observations in GC over-exposure mammals (Reynolds, 2013). Similar investigations using Damselfish (McCormick & Nechaev, 2002), however, were consistent with the findings shown in the current work. McCormick and colleagues suggested that, as growth is pulsatile in teleost fish, there are periods of faster growth followed by longer periods of no growth. By

introduction of cortisol global effects on growth may appear more dilute, resulting in a highly variable cortisol effect as observed here. However, by exploring growth through numerous time-points and assessing numerous features (i.e. head-trunk angle) it is evident that GC exposure does have a direct effect on specific periods of growth and development, particularly in the early phases of development (less than 72hpf).

While an impairment of growth is observed here the effects are less profound than in animal models where the foetus and offspring are known to be smaller, a possible explanation for this is the likely direct placental effects of GC administration. Whilst it is always difficult to extrapolate directly from fish to mammals, the unexpected growth shown here could suggest that the placenta plays a key role in the Dex influence on growth, possibly potentiating the effects of GC on growth, i.e. a subtle growth inhibition is noted in the developing tissue however the gross effect on size is as a result of the placenta. Support for this is given from rodent studies where the impact of Dex on placental growth has been found to be greater than on growth (Hewitt *et al*, 2006). In the zebrafish, therefore, we may be seeing the true direct impact of GCs on growth rather than a combination of direct and indirect effects on the foetus via the placenta.

The most profound effects of GR inhibition on growth were observed in the first 24-72hpf. As previous data confirmed that GR is present (Chapter 3), this would suggest a GR role in early cell growth, even before the endogenous GC system has become fully active. Indeed the very early inhibition of GR expression by RU486 suggests that the maternal cortisol (identified in Chapter 3) signalling through GR must be contributing to some extent to somatic growth. This is in accordance with previous research which suggests that GR signalling is essential for mesoderm formation and is required for growth and patterning of the neural tube and somites (Reshef *et al*, 1998). The delay observed with GR inhibition in the current studies represents a 6-8h developmental delay (staged according to Kimmel *et al*, 1995), since defined somite patterning was not observed at 18hpf. The reduction in spontaneous twitching, an early marker of somatic muscle innervation, is consistent with these findings. Eye size may also be indicative of development as the eye is one of the first detectable

features in the embryo. The reduction in eye length and area in the RU486 and GR Mo embryos, suggests a global developmental delay. However, interestingly, embryos which were incubated in Dex also had a smaller eye. These embryos had no other detectable impairment in growth and, thus, it might be that Dex treatment at this stage interrupts critical proliferative steps in the formation of the eye. While retinal formation data in GC over-exposure studies is limited previous studies in GR over-expressing mice have indicated that GR activity may be important for normal retinal formation and gene expression analysis of GR morphant zebrafish embryos suggests altered expression of critical genes involved in eye formation (Nesan *et al*, 2012).

Chorion hatch-rate, which normally occurs between 48 and 72 hpf (Kimmel *et al*, 1995), was delayed in embryos incubated in RU486 and in embryos treated with GR Mo. These data also suggest global developmental delay, possibly related to changes in the chorion itself or changes in the developing skeletal muscle of the embryo (rendering it less capable of mechanically breaking through the chorion). In contrast, Dex incubation increased the rate of spontaneous-hatching. This could be due to softening of the chorion (Kim *et al*, 2006). To try to address this, Dex was injected directly into the embryo at the 2-cell stage. This produced an even greater increase in early hatching, suggesting that this process is initiated predominantly from within the embryo itself, as a GR-mediated process. There are at least two possible explanations for these findings. Firstly, it is possible that GR promotes hatching-gland maturation and subsequent production of proteolytic enzymes that induce softening of the chorion (Hagenmaier, 1974). Secondly, Dex has been shown to reduce embryonic energy stores within the yolk sac resulting in the need for embryos to hatch earlier to seek alternative energy sources (McCormick & Nechaev, 2002). Met incubation and Cyp Mo, both influencing endogenous GC biosynthesis pathways, had no significant effect on hatch-rate in early development suggesting that endogenous synthesis of GC has limited functional role in hatching before 72 hpf and, therefore, hatching is a feature of maternal cortisol and functional embryonic GR.

As mentioned, mammals prenatally exposed to excess GC show reduced birth weight. This, in turn, is associated with metabolic alterations and abnormal feeding

behaviour (Cottrell *et al*, 2012). These models typically result in delayed growth with subsequent catch-up in growth later in development (Coupe *et al*, 2012). Catch-up growth is known to occur following removal of the mediator of growth restriction which results in a steeper trajectory of growth (Wit & Boersma, 2002).

Developmental regulation, and the underlying cellular mechanisms responsible for catch-up, growth are poorly understood with two suggested mechanisms 1) a neuroendocrine mechanism, as a result of mis-match in growth between the organism's target size and its actual size; resulting in alteration in the production of growth factors, neuroendocrine factors, hormones, and other signal molecules to adjust the growth rate accordingly to the degree of the mismatch. 2) the senescence model, in which cell senescence is delayed following GC exposure (Coupe *et al*, 2012). The findings shown here clearly show that EmmodDex are longer and heavier than controls from around 90 dpf. Using length and weight data to calculate the condition factor (the fish girth) confirmed that these fish were indeed larger than controls. In contrast for EmmodGR Mo adults there was a trend towards smaller and lighter adult fish with a clearly reduced condition factor (girth) than their controls.

In the "senescence hypothesis" exposure to GCs delays cell senescence (in the form of cell division), thus delaying proliferation and preserving the proliferative capacity of the cells (Coupe *et al*, 2012). When cessation of treatment occurs, the proliferative capacity is reinstated and the cells are capable of proliferating like younger cells. This results in an increased rate of proliferation and, as such, an increase in growth velocity (Chagin *et al*, 2010). This senescence hypothesis is very complex to investigate *in vitro* with numerous cell types thought to be contributing to the phenomenon; the role of GC in growth plate stem cell proliferation, longitudal bone growth, and catch-up growth has been described in detail (Baron *et al*, 1994). While investigation of cellular proliferation may confirm the senescence model, the favourable regenerative capacity held by the fish may make investigation of this problematic.

To date there has been little convincing data to suggest that an increase in growth hormone signalling is present in catch-up growth. However, there are numerous cell culture studies which show a relationship between GCs, IGF expression and cellular

growth (Fowden, 2003; Fowden & Forhead, 2004). The data presented here further suggest a neuroendocrine mechanism may be causative in altered adult growth trajectory as there is a reduction in the expression of *igf* mRNA in the zebrafish embryo when GC activity is reduced. The adults derived from these embryos are smaller than controls. These findings are contradictory to previously published data generated using GR Mo knockdown in the zebrafish embryo which showed no detectable reduction in *igf* mRNA (Nesan *et al*, 2012). However, Nesan *et al.* investigated gene expression earlier in development (<36hpf) than the data shown here. It may be that GR-mediated *igf* signalling pathways are more critical in later stages of zebrafish development and earlier unchanged levels of *igf* detected by Nesan and colleagues are due to maternally-derived transcript.

Somewhat contradictory to the data of EmmodDex and EmmodGR Mo, is an increase in length and weight in EmmodRU486. It has been previously been shown that RU486 treatment of rats increases their growth rate, possibly by antagonising the anti-proliferative actions of GC (Soro *et al*, 1995). However, as embryos were only exposed to GR antagonism for 5 days and the detectable difference between treatment groups and controls only became apparent at 90+ dpf, this may be unlikely. Another possible explanation is in RU486's other role as a progesterone receptor (PR) antagonist (Cadepond *et al*, 1997). PR have been found in osteoclasts and osteoblasts, and the activity of these has been shown to have a direct action on bone mass, with PR knock-out mice showing greater bone mass than wild type littermates (Yao *et al*, 2010). It might be possible that in exposing embryos to RU486 during critical stages of linear bone growth the bone mass of these adults is greater. However, as no investigation of bone mass was carried out this is purely speculative.

4.5.2 Aim 2:

Does embryonic GC manipulation alter embryonic and adult HPI axis function?

GCs are important for normal brain development, promoting myelination and terminal maturation (Huang, 2011; Lupien *et al*, 2009). The developing HPA axis has been shown, in many species, to exhibit a number of differences to the HPA axis at various postnatal life stages, with the hypothalamus, pituitary and adrenal glands all undergoing significant morphological and/or functional changes during

maturation (Tegethoff *et al*, 2009). Numerous animal studies have highlighted that maternal over-exposure to GCs can result in higher than normal levels crossing the placenta and exposing the foetal brain to elevated GC levels. In mammalian models this can affect development of the HPA axis (Kapoor *et al*, 2008) resulting in long-term changes in neuroendocrine function that can persist throughout life (Kapoor *et al*, 2006; Kapoor *et al*, 2008). Epidemiological studies have shown that, in humans, low-birth weight babies have higher (basal and stress-induced) plasma cortisol levels throughout adult life, indicating life-long HPA axis programming (Seckl, 2004; Seckl & Meaney, 2004).

As shown (Chapter 3), zebrafish GC biosynthesis, including an intact and functional HPI axis, is present in the embryo from 72hpf. The impact of embryonic GC manipulation on embryonic HPI axis activity was assessed by measuring cortisol at 72 and 120hpf. In keeping with a functional HPI axis, it was shown that Met treatment and the Cyp Mo reduced cortisol levels at 72 and 120hpf, confirming that GC biosynthesis is an evolutionarily conserved process (Steenbergen *et al*, 2011) ultimately regulated by 11β-hydroxylase activity (as found in numerous species; chapter 3), this also abolished the stress-induced cortisol production. Interestingly reduced HPI axis activity (in the form of reduced whole embryo cortisol) was demonstrated in embryos treated with Dex. It is well known that the mature HPI axis is subject to negative feedback by GCs via corticosteroid receptors (Alderman *et al*, 2012). Similar effects on basal GC following Dex-exposure have been made in guinea pigs (McCabe *et al*, 2001), human foetal cord blood (Ballard *et al*, 1975) and neonates 1 day after birth (Karlsson *et al*, 2000). At 72 hpf however, cortisol levels were unaffected by Dex incubation suggesting that residual maternal cortisol within the yolk sac remains important at this point.

Interestingly, there was also an unexpected reduction in cortisol levels in RU486-treated embryos which, as predicted by mammalian systems, would be expected to antagonise the negative pituitary feedback of either endogenous or exogenous GC, resulting in enhanced GC production. However, these data (combined with reduced head-trunk uncoiling, delayed hatching and impaired swim activity) are consistent with developmental delay, and it may be that in these embryos the HPA axis itself is

immature. Steps were taken to assess the functionality of the HPI axis in the RU486 embryos by co-incubation with Met [5µM RU486 and 0.5µM Met]. However, the survival of these embryos was severely impaired with an almost 100% mortality noted by 72 hpf (data not shown) and severe developmental delay. While neither Met or RU486 treatment alone resulted in such high mortality, the effects observed may be GR independent or it may be highlighting the importance of not only GR but of *de novo* cortisol biosynthesis.

During normal development, there was an increase in *mr* and *11βhsd2* mRNA abundance at both 72 and 120hpf. In mammals 11BHSD2 is responsible for the inactivation of cortisol to its 11-keto metabolites, conferring selectivity for aldosterone at MR (Chapman & Seckl, 2008). It has also been suggested that in the developing foetus 11BHSD2 is important for foetal growth and development by playing a protective role in regulation GR-mediated responses. However, since aldosterone, the classical MR agonist in many vertebrate species is not synthesised by teleosts it is possible that cortisol serves as a MR agonist in this setting. This possibility is supported by the demonstration of increased *mr* mRNA abundance at 120 hpf in embryos treated with Met (reduced basal cortisol). Therefore, the *11βhsd2* up-regulation seen in Dex-treated embryos may enable selective-prevention of cortisol access to MR binding sites, further controlling the levels of circulating cortisol by inactivation. *11βhsd2* is also minimally capable of metabolizing Dex (Tang *et al*, 2011), further suggesting that the up-regulation of this gene will reduce availability of GC and, hence, reduce activation of GR.

Mammalian studies of chronic maternal Dex exposure suggest a trend for reduction in MR mRNA abundance (Vellucci *et al*, 2002) but this was not seem in the current investigation. As mentioned, this may be due to cortisol activity at fish MR. Studies of MR function in teleost fish are compromised by the lack of specific pharmacological antagonists. Recent studies suggest that both spironolactone and eplerenone (MR antagonists in mammalian species) are MR agonists in zebrafish (Fuller *et al*, 2012; Pippal *et al*, 2011). The use of spironolactone here supports that the effects of GC modulation on growth and development are GR-mediated. While it is unclear what role MR plays in zebrafish embryogenesis, the expression of this

receptor throughout embryonic development would suggest a functional role. Future work should be carried out on the physiological role of MR in zebrafish development including determination of whether effects are cortisol-mediated or whether other hitherto unrecognised ligands are responsible.

It is well documented in mammals that GC over-exposure via maternal stress reprograms the developing HPA axis and stress-related behaviours, resulting in an altered ability to deal with a stressful environment (Jellyman *et al*, 2012; Kapoor *et al*, 2006; Kapoor *et al*, 2008; Seckl & Meaney, 2004; Tegethoff *et al*, 2009). The long term impact of embryonic GC manipulation (up to 120hpf) was examined by measuring the basal and stress-induced cortisol levels in the bath water of EmmodDex, EmmodRU486 or EmmodGR Mo. In accordance with data generated in other species (e.g. rhesus monkey; (Slobada *et al*, 2006; Uno *et al*, 1994)), cortisol levels were elevated in bathing water from EmmodDex adults, suggesting enhanced HPI axis responsiveness to an acute stressor with no change in basal cortisol levels (as has been shown by others). There were no alterations in either basal or stress-induced cortisol in EmmodRU486 adults. However, there was a reduction in stress-induced cortisol in EmmodGR Mo adults. This is not the first report showing that suppression of GC action during embryogenesis results in a markedly reduced stress response in the subsequent adult animals. Work by Ridder *et al* (Ridder *et al*, 2005) suggests that mice heterozygote for GR knockdown (GR-/+) have no alteration in basal corticosterone but have a reduced corticosterone stress response. Furthermore zebrafish with abnormal negative feedback due to abolished transcriptional activity of GR display higher than normal cortisol levels (Ziv *et al*, 2013).

4.5.3 Aim 3:

Does embryonic glucocorticoid manipulation alter embryonic and adult swim behaviour?

The stress response is important in a number of physiological roles. Of particular interest here is the role of arousal and emotional salience in allowing the organism to adapt and respond to this detrimental stressor and, thus, increasing the chance of survival. However, when the HPA axis is chronically activated or is in a state of deregulation this can increase the susceptibility to behavioural abnormalities (Kolber

et al, 2008). Early life stress alters the neuroendocrine stress response, particularly the HPA axis. This has been associated with psychiatric, cognitive and behavioural disorder (Bolten *et al*, 2013; Paschetta *et al*, 2013; Starkman, 2013). To determine whether modulation in GC activity had any impact on zebrafish behaviour, swimming and locomotor activity were observed in the embryo and in the adult following embryonic GC manipulation.

RU486 and Met treatment reduced swim speed and swim distances indicating a role for embryonic rather than maternal GC signalling through GR. It has previously been shown that swim movements increase as a fish grows and changes shape during early development, particularly between 72 and 120 hpf (Muller & van Leeuwen, 2004). This allows a more active exploration of their environment - mainly to forage for food (Brustein *et al*, 2003; Thirumalai & Cline, 2008). Our findings confirm these reports and show that inhibiting GC synthesis and its actions alters normal swim activity. This could be a consequence of delayed development of the skeletomotor system, although detailed phenotype scoring of all treated and control embryos failed to detect any significant structural differences (other than a lack of swim bladder inflation) in the majority of RU486 treated embryos. Swim bladder inflation usually occurs at 72 hpf and assists buoyancy. It has previously been shown that "swim-up" to the surface behaviour is critical in the larval zebrafish for swim bladder inflation (Lindsey *et al*, 2010) and it has been suggested that swim bladder development is a corollary of early mammalian lung development (Winata *et al*, 2009) which is well known to be modified by GC administration. It is likely therefore that the lack of swim bladder inflation simply reflects the delayed development of normal swim behaviour. Increased time spent in the centre of the Petri dish was observed in embryos treated with Met or RU486 and is once again likely to be related to the impact of GCs on development and maturation.

Swimming activity in fish is linked to defined behavioural characteristics such as escape and avoidance. Zebrafish larvae have been shown to be thigmotactic from 96hpf, i.e. they spend longer in the periphery of a tank or open-field area, than in the centre; an evolutionary response to avoid predation (Colwill & Creton, 2011). As Dex embryos spend longer in the periphery of the test area, this again suggests a

more mature phenotype compared to the other groups investigated, further supporting an important developmental role for GCs in adaptive responses linked to survival and feeding.

The adult zebrafish is gaining more support as a model of complex behaviours such as learning and memory, anxiety, reward and aggression (as reviewed by Norton & Bally-Cuif, 2010). Much of the zebrafish behavioural assessments determine fish "boldness". Boldness is mostly assessed by monitoring individual zebrafish behaviour in: (1) an unfamiliar open-field environment, (2) the same environment with and unfamiliar/novel object, (3) forced swim in a narrow novel tank. The alterations observed in embryonic swim behaviour following GC manipulation, plus the altered adult cortisol profile following embryonic GC manipulation, suggested there would be a similar effect on swim behaviour in the adult.

The open field test was used to assess novelty exploration, a feature which has been shown to be evolutionarily conserved (Stewart *et al*, 2012) with similar observations noted in rodent and zebrafish. Generally, due to the averse environment, adult zebrafish show limited exploration and increased thigmotaxis (wall hugging (Steenbergen *et al*, 2011)). In this investigation, EmmodDex showed a reduced thigmotaxis compared to controls, with significantly more time spent in the central region of the open field arena. This behaviour is referred to as "exploratory boldness" (Wisenden *et al*, 2011). No other treatment groups showed changes in thigmotaxis or boldness. Introduction of a novel object into the tank demonstrated a further boldness characteristic in the EmmodDex-approaching the possible predator. Again, no other group showed this characteristic. The dive tank test was used to exploit the zebrafish's natural tendency to dive to the bottom of a novel tank followed, with time, by a gradual increase its vertical activity with increasing familiarity with the tank. The proportion of time that the fish spends at the bottom of the tank has been interpreted as an index of anxiety (Subbiah & Kar, 2013). A clear difference was seen in EmmodDex adults, which showed a bolder swim pattern with significantly less time spent at the lower region of the tank. These findings are interesting since these "bold" fish also showed similar basal cortisol levels but raised cortisol levels following stress. This suggests that there is no direct relationship

between boldness and stress-responsiveness, i.e. the bold behaviour is not a result of increased basal cortisol. While these findings differ slightly than in mammalian models it may be that in fish behavioural programming differs from mammalian models.

4.6 Conclusion

GCs play important maturational effects at early stages of development in the zebrafish embryo, with suppression of GC activity (RU486 or GR Mo) resulting in delayed growth rate, reduced head-trunk angle and decreased hatch-rate. Phenotypically these embryos are normal with a low mortality; the only observable feature is an absent, or poorly-inflated, swim bladder which may be a consequence of decreased swim activity in these embryos. Increased GR activation, in the form of Dex treatment, results in enhanced growth rate with more mature swim behaviour. The enhanced growth rate here appears to alter growth trajectory of these fish post 10 dpf, as by adulthood (120 dpf) they are heavier and more robust than their control counterparts. Not only are these fish heavier but they display a bolder behavioural phenotype than un-manipulated fish. Alteration in behaviour may be a consequence of the altered HPI axis activity observed in both the embryonic Dex group and the EmmodDex group.

Chapter 5: Early-life glucocorticoid manipulations and cardiovascular developmental programming

5 *Early-life manipulations and cardiovascular developmental programming*

5.1 Introduction

In many animal species, including humans, GCs become crucially important towards the end of gestation where a dramatic peak in circulating levels is obvious (Fowden & Forhead, 2004). GR is also detected in most tissues; in the mouse from as early as E9.5, with gene abundance rising throughout gestation (Harris & Seckl, 2011). The peak in circulating GC and GR expression corresponds to a period of accelerated maturation of tissues and organs in preparation for birth (Roberts & Dalziel, 2006; Smith & Shearman, 1974), suggesting a developmental role for GCs.

The key role of GCs in organ development was first highlighted in the global GR knockdown mouse ($GR^{-/-}$) which has a 100% perinatal mortality rate as a result of multi-organ failure (Cole *et al*, 1995; Opherk *et al*, 2004). Furthermore, organ specific GR manipulation has suggested that GR are important for maturation of many systems (including the gastrointestinal tract (Babyatsky, 2004), skin, liver and kidney (Liggins, 1994)) and in the process of erythropoiesis (Tang *et al*, 2011). More recently convincing evidence has emerged to indicate a possible role for GR in early heart development and maturation (Rog-Zielinska *et al*, 2012).

Although there is fairly strong evidence to allow us to describe an association between GC exposure and alterations in the mature cardiovascular system (such as cardiac and vessel remodelling, structural alterations (Christy *et al*, 2003; Katz *et al*, 1988) and increased disease risk factors (Girod & Brotman, 2004; Ng & Celermajer, 2004; Stewart & Petersenn, 2009; Trayhurn & Beattie, 2001)) there is less evidence to support a direct and specific action of GCs (whether exogenous and/or endogenous) on the developing cardiovascular system (Sainte-Marie *et al*, 2007). It is also unclear whether abnormalities in GC signalling during critical stages of development can alter cardiovascular structure and function.

As highlighted in chapter 4, exposure to raised GC levels during gestation can have life-long detrimental effects on health (Seckl & Meaney, 2004), such as increased

risk of cardio-metabolic disease in later life (Seckl, 2004). Synthetic GC administration in rodent models causes myocardial hypertrophy and increased neonatal systolic blood pressure (Dodic *et al*, 2002; Langley-Evans, 1997; Oritz *et al*, 2003). While these cardiovascular abnormalities may be a result of the systemic effects of GC, the work here will investigate whether the long-term effects of GC on cardiovascular programming may be due to direct cardiovascular actions during development.

While there are clear anatomical differences between fish and mammalian cardiovascular systems there are sufficient similarities to allow relevant experimental observations of heart and blood vessel growth to be made. The vertebrate embryo heart, for example, is the first organ to form and function in all species (Glickman & Yelon, 2002). In its earliest incarnation the mammalian heart is a tube shaped structure with two basic chambers -atrium and ventricle, thus the zebrafish heart is a relevant model of early mammalian heart development (Glickman & Yelon, 2002; Yelon, 2001). These near identical heart developmental processes coupled with embryonic transparency and the ability to survive many days without functional cardiac or circulatory systems (Glickman & Yelon, 2002), allow investigation of manipulations, modifications and mutations which would otherwise result in mortality in rodent models (e.g. the mouse $GR^{-/-}$ model).

5.2 Experimental hypothesis and aims

The work described in this chapter directly assesses the effects of early life GC manipulations, constituting both excess and diminished GC levels, on development of the heart and vasculature in the zebrafish, from embryonic stages through to adulthood. The hypothesis which will be investigated is that modulation of GC activity during embryogenesis will impair normal embryonic heart and blood vessel development and function resulting in life-long alterations in the adult cardiovascular system.

Data presented here aimed to answer the following questions:

1) Does manipulation of the GC system alter early embryonic vascular development?
2) What are the long term effects of early life GC manipulations on the adult vascular system?
3) Does manipulation of the GC system alter embryonic heart development?
4) What are the long term effects of early life GC manipulations on the adult heart?
5) Is the embryonic phenotype caused by GR Mo rescued by capped *gr* mRNA

The above chapter aims will be addressed in three parts:

Part 1: The influence of embryonic GC manipulation on the embryonic and adult vasculature (aims 1 and 2).

Part 2: The influence of embryonic GC manipulation on the embryonic and adult heart (aims 3 and 4).

Part 3: Genetic manipulation of GC activity, can GR Mo be rescued? (aim 5).

5.3 Materials and methods

5.3.1 Embryonic glucocorticoid modulation

A number of zebrafish embryonic GC modulations were performed as described (Chapters 2 and 4). Briefly, all embryonic modulations were performed at the 2 cell stage (approx. 1h after egg collection). Embryos were housed under standard husbandry conditions (section 2.3.1) in a 10cm Petri dish at a density of 1 embryo/mL.

5.3.1.1 Pharmacological manipulation

Pharmacological manipulation was performed as described (section 2.3.4) by bathing embryos in the following: Dex [100 µM], RU486 [10 µM], Met [10 µM], or spironolactone [0.1 µM]. All drugs were dissolved in EtOH (0.1%) and diluted to final concentration in systems water. Embryonic survival, phenotype scoring and drug replacement occurred daily over the 120h of the study.

5.3.1.2 Genetic manipulation

Genetic manipulation was achieved as described (Chapters 2 and 3) by the injection of targeted Mo knockdown of two key genes, *gr* and *cyp11b1* (referred to as GR Mo and Cyp Mo, respectively). Appropriate mm-Mo was used as controls. The design, sequence and dosage optimisation are described in section 2.3.5. Embryos were stored in systems water which was replenished daily over the 120h study whilst scoring survival and phenotype and drug replacement occurred daily.

5.3.2 Embryo to adult longitudinal study

In keeping with the experimental hypothesis underpinning this work, embryos undergoing pharmacological and genetic GC manipulations during the first 120 hpf were then maintained under standard fish husbandry conditions (un-manipulated) from 120 hpf until adulthood (120 dpf) to determine programming. The embryo to adult programming study was carried out as described (section 4.3.2). Briefly, three groups were assessed until adulthood, EmmodDex, EmmodRU486 and EmmodGR Mo. For each of these adult groups a contemporaneous age-matched group acted as the control. For pharmacological manipulation this was a vehicle only (0.1% EtOH) group and for the GR Mo this was mm-Mo. All control groups are referred to in

graphs as “Control” except in instances where confusion may arise (e.g. two-way ANOVA) when they are referred to as treatment group and control, for example EmmodDex Control.

5.3.3 Gene abundance analysis

Gene abundance analysis was carried out on a number of key cardiovascular, structural, angiogenic and maturational genes in the embryo and the adult using qRT-PCR.

vegfaa, il-8 and *flk* mRNA abundance were determined in whole embryos and in adult caudal tail fins to elucidate signalling in angiogenesis. In both embryonic and adult isolated myocardial tissue *mef2c, vmhc, gata4, igf, gr, mr* and *11βhsd2* mRNA abundance were assessed. Gene selection is summarised in Appendix 2.

All adult tissue or whole embryo homogenate samples were processed, RNA extracted, cDNA synthesised and qRT-PCR carried out as described (section 2.5).

5.3.4 Protein abundance analysis

Protein abundance of Gr was carried out by Western blot analysis as described (section 2.7). Samples were prepared from pooled tissue: 100 embryonic hearts or 5 adult hearts.

5.3.5 Embryonic vascular assessment

Embryonic vascular assessments performed as described (section 2.4.6) using tg(FLi1: EGFP) zebrafish embryos. Briefly, after manipulation the number of ISV were determined in a given region of the embryonic tail. Vascularisation index was also calculated (as in section 2.4.6.2).

5.3.6 Adult vascular assessment

Adult zebrafish of the tg(FLi1: EGFP) strain were used to observe tail vasculature, as described (section 2.5.7). The pattern of tail blood vessels was observed under fluorescent microscopy and still-frame images recorded using Leica software. Images were analysed using ImageJ software to determine caudal ray vessel

patterning including the number of vessel-crosses between rays and the number of visible angiogenic sprouts present post-tail-fin resection (Section 2.5.7).

5.3.7 Adult tail fin regeneration

To determine whether embryonic GC manipulation altered the angiogenic properties of the adult zebrafish a tail-fin regeneration model was adapted (Azevedo *et al*, 2011) as described fully in section 2.5.7.

5.3.8 Embryonic cardiac assessments

A number of structural and functional embryonic cardiac assessments were carried out as described (section 2.4.7) using tg(CMLC2:GFP) embryos. Structural assessments included ventricle length, inflow/outflow distance, pericardial area and heart area. Functional assessments included heart rate, ventricular ejection fraction, stroke volume and cardiac output (as in section 2.4.7.3).

5.3.9 Adult cardiac assessment

Adult hearts were isolated as described (section 2.5.4). After isolation, hearts were weighed and bright-field microscope images taken to determine ventricle length. Heart weight was normalised to body weight, and heart length was normalised to body length. Further cardiac assessments involved histology which was performed as in section 2.8 with H&E or Masson's trichrome staining. Inter-trabecular space was measured from theses stained sections. For this hearts were imaged as previously described (Section 2.3), these images were then analysed using ImageJ software, ventricle size was calculated by tracing around the outer ventricular wall using the free-hand tool, inter-trabecular space was then determined by tracing around areas of free-space (unstained with no apparent tissue) within this ventricle. The regions of empty space were then totalled to give the free area within the section. This inter-trabecular space was then subtracted from the total ventricular area and the total percentage of inter-trabecular was thus given as a percent.

5.3.10 Messenger ribonucleic acid (mRNA) morpholino rescue

To determine proof of specificity of GR Mo an mRNA rescue experiment was performed by co-administration of Mo with mRNA encoding the gene of interest

(GR). The rescue mRNA was constructed with an altered 5' UTR (capped) and thus no GR Mo target (the five prime un-translated region).

Rescue of GR Mo was deemed successful if the associated GR Mo phenotype observed after administration was not detected or was significantly attenuated in the rescue mRNA embryos, indicating that the observed Mo phenotype is due to knocking down the target gene and not due to an unexpected interaction with an off-target RNA.

5.3.10.1 DNA linearization

Template DNA was purchased in the form of IMAGE clone (Source Bioscience, Cambridge), in plasmid Miniprep form, meaning it is a purified plasmid. The GR gene was cloned into the pDNR-LIB clone (Figure 5.1) with the insert being found between Sfil (77) and (298) sites. The plasmid was then linearized with XhoI restriction enzyme (Applied Biosystems) with cleavage sites downstream of the insert. Confirmation that this restriction enzyme cuts the vector at one site only and does not cleave the DNA sequence of interest was carried out using SerialCloner software mapping the pDNR-LIB donor vector (vector shown in Figure 5.1).

A typical linearization reaction was as follows: to a 2 mL Eppendorf tube on ice were added 500 ng DNA, 10X NEB Buffer, BSA, ddH_20 and the XhoI restriction enzyme. This was then warmed to 37^oC for 1 h to activate the enzyme, and the reaction then stopped by the addition of 1/20th volume 0.5 M EDTA, 1/10th volume 5 M sodium acetate and 2 volumes ethanol. The sample was then chilled at -20^oC for 30 min. The tube was then centrifuged at 10,000 g at 4^oC for 15 min to pellet the DNA, supernatant was removed and discarded and the pellet was reconstituted in TE buffer at a concentration of 500 ng/µL. Confirmation of completed cleavage was carried out on a DNA gel.

Figure 5.1 Restriction map and multiple cloning site of pDNR-LIB vector.
Multiple cloning sites (MCS) A and B are highlighted to the left and right of the stuffer sequence. MCS A is shown in frame with the loxP site. T7 promoter location is also highlighted. The "stuffer" fragment shown here is replaced by the cDNA insert of interest, in our case with the zebrafish GR sequence. This image was produced using SnapGene software (from GSL Biotech; available at snapgene.com).

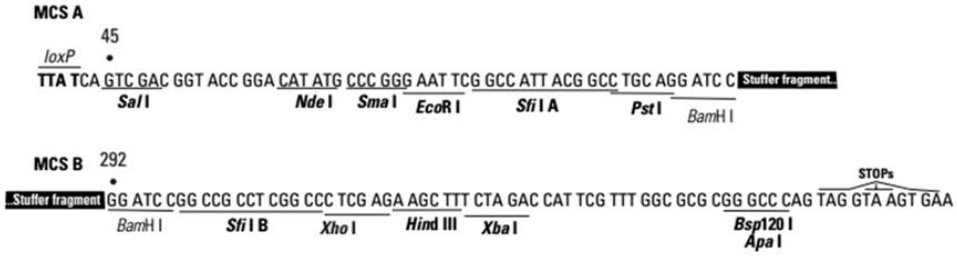

Figure 5.2 Multiple cloning site of pDNR-LIB vector.
Unique restriction sites are shown in bold. MCS A is shown in frame with the loxP site with the last four nucleotide bases of the loxP highlighted at the left hand side of MCS A in bold. The "stuffer" fragment shown here would be replaced by the cDNA insert of interest, in our case with the zebrafish GR sequence. This image was provided with IMAGE clone (Source Bioscience, Cambridge).

5.3.10.2 Proteinase K treatment

After linearization, the template DNA was treated with proteinase K (100 µg/mL) and 0.5% SDS for 30 min at 50^{o}C to eliminate for RNase or other transcription inhibitors to prevent suboptimal transcription.

5.3.10.3 Phenol/chloroform extraction and ethanol precipitation

Proteinase K treatment was followed by phenol/chloroform extraction using a well-documented protocol. Briefly, 1 volume of phenol was added to the DNA, this was mixed and then centrifuged for 2 min at 15, 000 g at 4^{o}C. The resulting supernatant was removed into a clean tube and an equal volume of chloroform was added followed by vortexing. The resulting solution was then centrifuged for 2 min at 15,000 g at 4^{o}C. The supernatant was transferred to a fresh tube where 0.1 volume of sodium acetate and 2.5 volumes of 100 % ethanol were added. This was then allowed to precipitate at -20^{o}C overnight. Following precipitation, the tube was spun at 15,000 g for 20 min at 4^{o}C. The resulting supernatant was removed and 1 mL of 70% ethanol was added to wash out any salts, following a final 10 min centrifugation at 15,000 g at 4^{o}C. Liquid was removed and the pellet was allowed to air dry after which it was reconstituted in TE buffer.

5.3.10.4 Capped transcription reaction

Capped transcription reaction was carried out using the mMessage mMachine Kit (Ambion) according to manufacturer's instruction. Briefly, 500 ng/µL linearized DNA was added to a chilled Eppendorf tube. To this 2x NTP/CAP solution was added along with 10X reaction buffer and T7 enzyme mix. The volume of the tube was then made up to 20 µL by addition of ddH_2O. The tube was then gently mixed and incubated at 37^{o}C for 1.5 h. After the incubation, 1 µL TURBO DNase (Ambion) was then added to the tube and incubated at 37^{o}C for 15 min. This step removes any residual template DNA.

5.3.10.5 RNA recovery and quantification

Lithium chloride was used as an effective way to remove unincorporated nucleotides and proteins. This step follows directly from the previous protocol (Capped transcription reaction). The reaction was stopped by the addition of 30 µL nuclease

free ddH_2O and 30µL lithium chloride. The tube content was then mixed gently and chilled for 1 h at -20^oC. After chilling, samples were centrifuged at 4^oC for 15 min at 15,000 g. The supernatant was then carefully removed and the remaining pellet was washed once with 1 mL of 70% ethanol and then centrifuged again as before. The supernatant was then removed and the pellet was reconstituted in 20 µL DEPC treated water. The final concentration of RNA was determined using a nanodrop spectrophotometer (as described for RNA extraction methods chapter 2) and stored at -20^oC.

5.3.10.6 Injection titration

It has been documented that incorporation of rescue RNA may result in teratogenic effects. It was, therefore, important to carry out titration reactions, similar to those carried out for Mo dosage determination. Here, 4 nL boli of various diluted concentrations of rescue RNA were injected into developing zebrafish embryos, at the 2-cell stage. The embryos were then assessed and scored using the 6 point phenotype scoring system as described (section 2.4.3). The final concentration of rescue RNA which was found to not significantly alter gross morphology was 3 ng/nL. The effects of rescue success were determined by assessing global and cardiovascular development and will be described further part 3.

5.3.11 Experimental controls

In the following experiments data were obtained both in embryos and in adult zebrafish. For embryonic pharmacological manipulations controls are vehicle only (0.1% EtOH). For embryonic Mo manipulation control data are for injected control Mo. For data where embryonic pharmacological and molecular manipulation are represented together (e.g. gene abundance) control data are a mean of vehicle only and control Mo together (Student's *t*-test shows no significance between groups) to ease graphical representation and interpretation. For adult investigation control data for EmmodDex and EmmodRU486 were obtained in adult fish which were treated with vehicle only during embryogenesis. EmmodGR Mo controls are adults which were injected with control Mo during embryogenesis.

5.4 Part 1

The influence of embryonic glucocorticoid manipulation on the embryonic and adult vasculature.

The following section will address the impact which GC manipulation has on vascular development in the zebrafish embryo, and the long-term impact that this embryonic GC manipulation has on adult vasculature.

5.4.1 Part 1: Results

5.4.1.1 Aim 1:

Does manipulation of the glucocorticoid system alter embryonic vascular development?

tg(FLi1: EGFP) zebrafish were used to determine whether manipulation of the GC system altered the formation of the embryonic vasculature. As mentioned previously (section 2.4.6) the *fli1* promoter is a known endothelial marker in mouse models and known to be expressed during vascular formation, haematopoietic cell types and the jaw mesenchyme (Lawson 2002) making it an ideal gene for vasculature. This EGFP expression enables visualisation of the vasculature for qualitative and quantitative assessment of blood vessel formation and function (Figure 5.3).

5.4.1.1.1 Intersegmental vessel formation

In the first instance the number of ISV expressing EGFP which crossed completely between the aorta and the DLAV following the tail somite patterning were counted throughout the length of the tail, referred to here as "normal ISV".

Pharmacological and genetic manipulations that reduced production of active cortisol (Chapter 3), namely incubation of embryos in the drug Met or injection with the Cyp Mo, had no effect on the number or patterning of these normal ISV expressing EGFP at any of the time-points investigated (Figure 5.4 C and D).

Incubation of embryos in (the GR agonist) Dex (100µM), reduced the number of ISV expressing GFP compared to controls at all time-points investigated ($p<0.0001$ (Figure 5.4 E)). Embryos incubated in Dex typically showed an incomplete EGFP signal throughout the length of the somite, making the ISV appear truncated, dense or in some instance absent along the length of the tail (Figure 5.3).

Incubation of embryos with (the GR antagonist) RU486 altered ISV EGFP expression reduced the number of "normal" ISV at all time-points investigated ($p<0.0001$(Figure 5.4A)). Embryos incubated with RU486 showed increased expression of GFP in branching appenditures from the ISV with many forming a "Y" shape which typically did not follow the normal somatic patterning arrangement observed in controls (Figure 5.3).

Similarly, embryos treated with GR Mo showed a change in ISV patterning compared to mm-Mo (controls) at several time points during early development ($p<0.0001$). No quantifiable ISVs expressing EGFP were detected in this group at 48 hpf (data for GR Mo begins at 72 hpf on Figure 5.4B) possibly as a consequence of severe retardation in vessel sprouting (Figure 5.5). EGFP expressing ISV detected in the later developmental stages were largely found towards the yolk sac of the embryo with very few "normal" ISV observed further along the length of the tail.

In order to try to ascertain whether these observations were mediated by GR or MR (GCs may have MR agonist activity in fish), ISV patterning was also examined in embryos which had been incubated in a known teleost MR agonist* spironolactone (Pippal *et al*, 2011) [1uM]. Spironolactone was found to have no effect on the number of complete EGFP expressing ISVs compared to the controls at any of the time-points investigated (Figure 5.4 F).

* Spironolactone has classical MR antagonistic properties in most mammalian systems and although it has been used as an antagonist previously in zebrafish studies it has more recently been shown to have agonist properties (Pippal *et al* 2011).

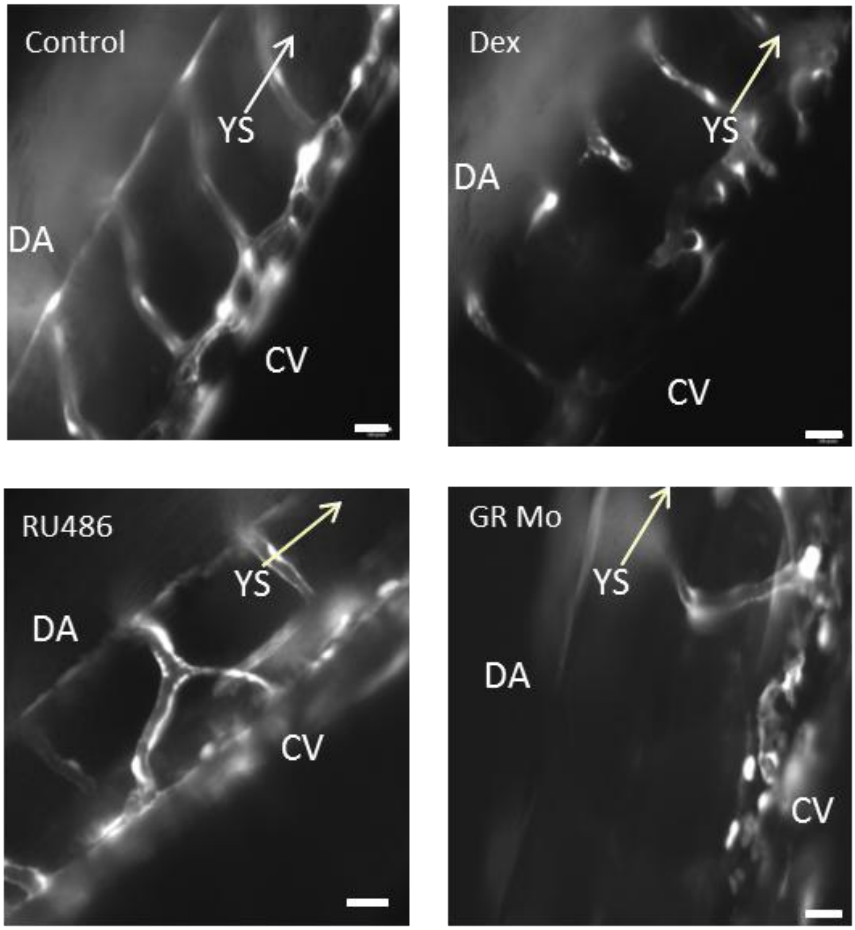

Figure 5.3 Typical intersegmental vessel (ISV) appearance
ISV appearance in 96 hours post fertilisation (hpf) tg(FLi1:GFP) zebrafish tail mid-sections which were either (untreated) controls or had been treated with Dex [100uM], RU486 [10uM] or targeted morpholino knockdown of the GR (GR Mo). The dorsal longitudinal anastomotic vessel (DA), aorta (A) are marked with the location of the yolk sac (YS) highlighted for orientation. Normal ISV are observed in the control fish, GFP expression is present in vessels following the normal somite patterning of the tail connecting the A and the DLAV. Low expression of GFP is detected in ISV of Dex and GR Mo embryos, with ISV appearing missing or incomplete. RU486 embryos display increased GFP expression with expression observed in many branched appenditures. Scale bars represent 100µm.

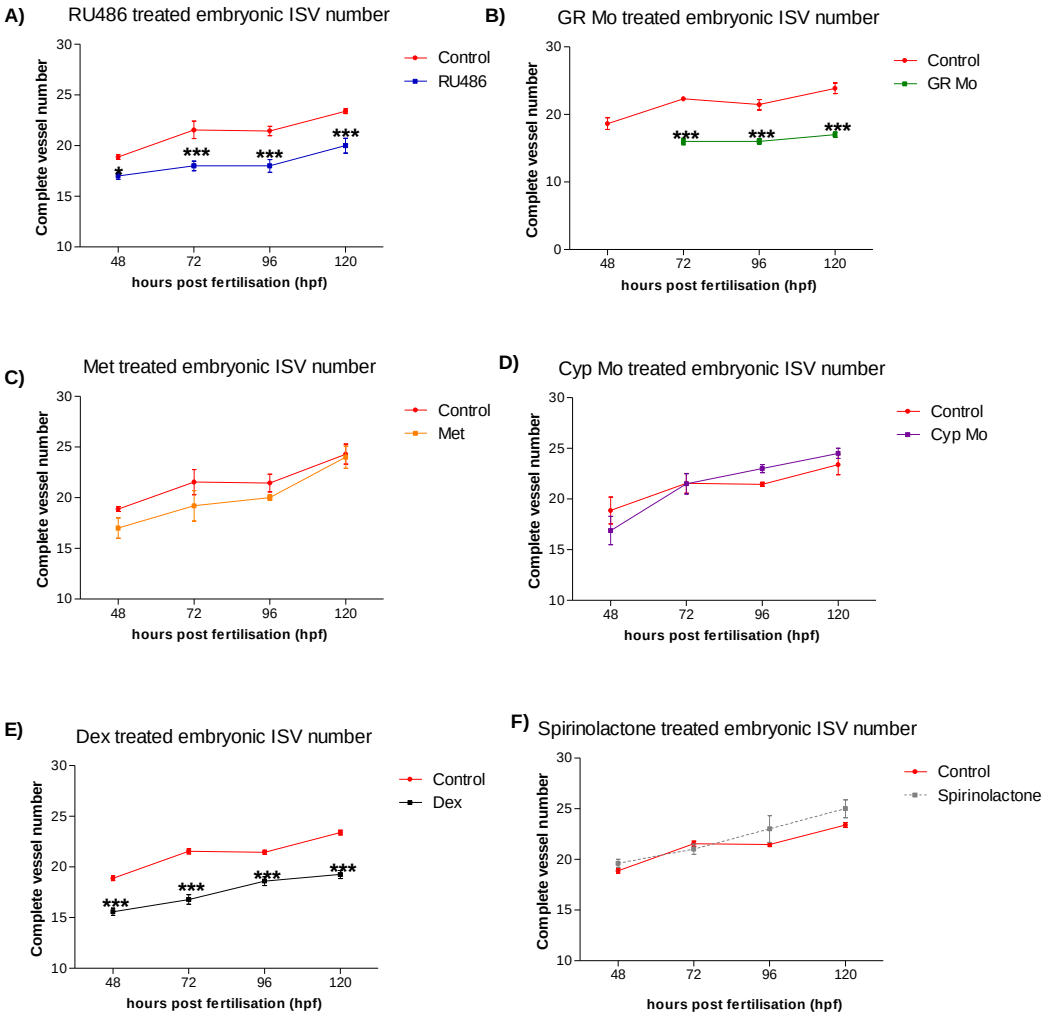

Figure 5.4 Number of complete inter-segmental vessels (ISV)
ISV within a given range of the tail, between yolk sac extension and last detectable ISV on the tail tip. The number of complete ISV is shown after: A) RU486 (blue) [10µM], B) targeted glucocorticoid receptor morpholino (GR Mo-green), C) metyrapone (Met-orange) [10µM], D) targeted *cyp11b1* morpholino (Cyp Mo-purple), E) dexamethasone (Dex-blue) [100µM], or F) spironolactone (grey), treatment from the 2-cell stage. At 48hpf; there were no quantifiable ISV for GR Mo at this point. Data are mean ± SEM for n=5 (5 fish per group) and were analysed by 2-way ANOVA and Bonferroni post hoc test. ***$p \leq 0.001$.

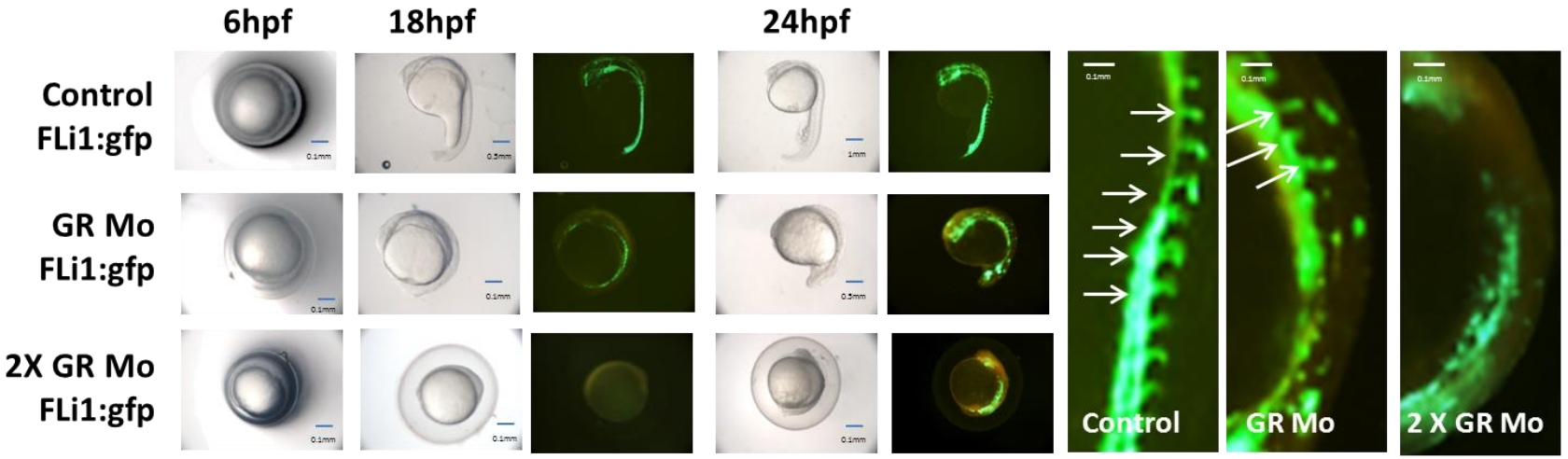

Figure 5.5 Typical vessel appearance in glucocorticoid receptor morpholino embryos
Shown are embryos which were either (untreated) controls or had been treated with targeted knockdown of the glucocorticoid receptor (*gr)*. Images shown are for two concentrations of Mo (5ng/nL (GR Mo) and 10ng/nL (2x GR Mo)) to show concentration-dependent effects. Each row shows images of the same fish throughout the first 24 h of development (6-24 hours post fertilisation (hpf)) from embryo collection under white light and also under UV fluorescence. The green vasculature is imaged by fluorescent imaging of the EGFP expressed in the tg(FLi1: EGFP) line. High magnification images are shown of 24 hpf embryos on the right hand side. Sprouting ISV in control and GR Mo are highlighted by arrows.

5.4.1.1.2 Intersegmental vascularisation index

While it was apparent that the GC manipulation was altering vessel EGFP expression it was also clear that the manipulations were have differing effects. To better quantify the level of variation in vascular patterning, a scoring system was devised (referred to here as the Vascularisation Index (VI)) for a given region of the tail at 96hpf. This was determined using a scheme which assessed the number of EGFP expressing vessel intersections by three perpendicular lines in a defined region of the tail (Figure 5.6).

Embryos treated continuously with Met or Cyp Mo from fertilisation showed no difference in VI compared to control (un-manipulated). Those treated with RU486 however showed a higher VI ($p<0.05$) at 96 hpf as a result of a greater number of line intersections by ISV (Figure 5.6 A). In contrast, embryos treated with GR Mo showed an opposite effect at 96 hpf with a reduction in the number of grid-line intersections ($p<0.001$ (Figure 5.6 B)).

A similar reduction in the number of vascular intersections was observed in embryos treated with the GR agonist Dex, ($p<0.001$ (Figure 5.6 E)). The typical abnormal appearance of ISV in Dex-treated embryos is shown in Figure 5.7, with the mid-region (the area used to determine VI), marked to show absent or truncated vessels. To determine whether this was a GR or MR mediated effect, again embryos were incubated with spironolactone. This caused no alteration in the VI (Figure 5.6 F)).

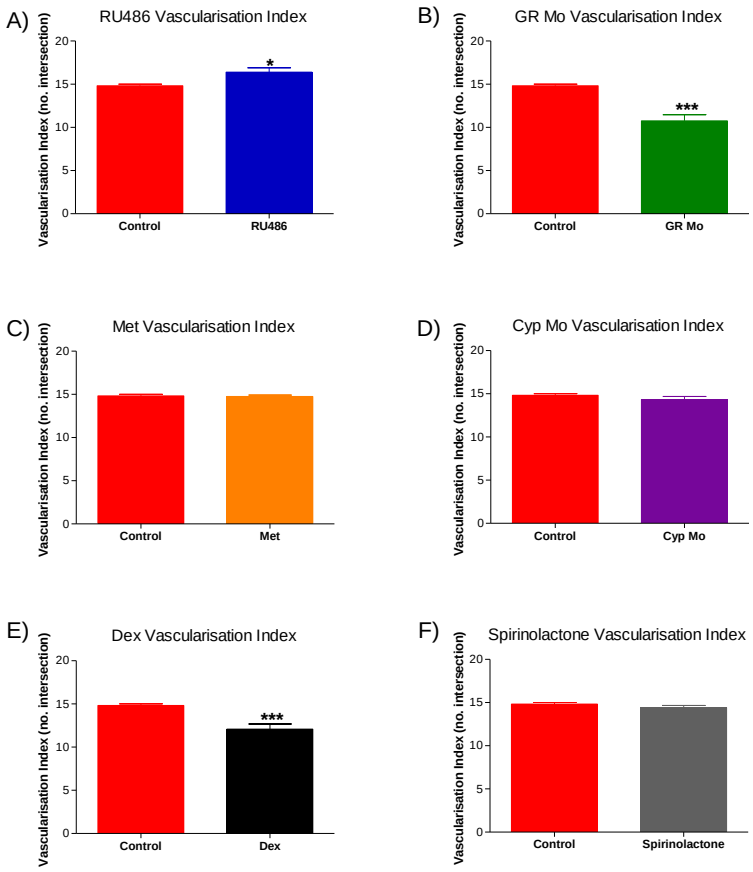

Figure 5.6 Embryonic vascularisation index (VI)
VI of 5 somite mid-tail section from cloaca towards the head in 96 hours post fertilisation (hpf) Fli1: GFP zebrafish embryos which had been continuously incubated in drug or treated with morpholino from 2-cell stage (~1hpf). All data show control (red) versus A) RU486 (blue) B) targeted glucocorticoid receptor morpholino (GR Mo-green), C) metyrapone (Met-orange), D) morpholino targeted towards *cyp11b1* (Cyp Mo-purple) E) dexamethasone (Dex-black) and F) spironolactone. Data are mean ± SEM n=3 (5 embryos per n), analysed by Student's *t*-test, * P≤0.05, *** p≤0.001.

5.4.1.1.3 Embryonic vasculature specific gene abundance

flk1/kdr is a prominent receptor in *vegf* signalling in zebrafish (Bussmann *et al*, 2008). To determine whether alteration in abundance of this gene may be associated with abnormal ISV appearance, its abundance was measured at 120 hpf in whole embryo homogenates for each treatment group and respective controls. Continuous incubation in GC modifying drugs or GR Mo had no detectable effect on gene abundance (Figure 5.7A).

VEGF A is a key determinant of normal vascular formation in mammals, and is recognised as a key angiogenic factor (Bussmann *et al*, 2008). Previous studies have highlighted alterations in abundance pattern of the zebrafish form (*vegfaa*) associated with altered vascular developmental in the zebrafish (Gore *et al*, 2012). Here, embryos treated with Dex showed a lower abundance of *vegfaa* mRNA than controls at 120 hpf ($p<0.05$). Conversely embryos treated with RU486 showed increased abundance of *vegfaa* mRNA ($p<0.05$ (Figure 5.7B). No other treatment altered *vegfaa* mRNA abundance.

IL-8, a member of the chemokine signalling family, is a key pro-angiogenic and pro-metastatic factor (Li *et al*, 2003; Stoll *et al*, 2011) The expression profile of the gene encoding this protein was examined at 120 hpf in GC manipulated embryo-homogenates. There was a lower level of abundance in embryos treated with Dex compared to controls ($p<0.001$), while RU486 incubation increased the abundance of *il-8* mRNA when compared to controls ($p<0.05$) at this time point however GR Mo treatment did not alter the abundance of this gene at all.

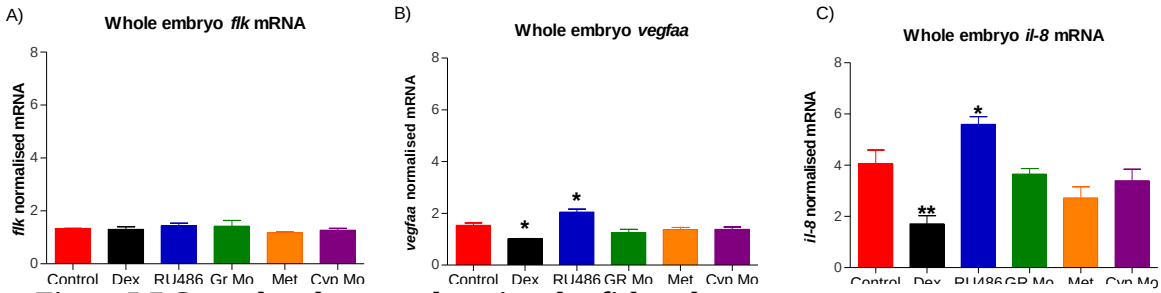

Figure 5.7 Gene abundance analyses in zebrafish embryos
Relative mRNA abundance in embryos which had been treated with the glucocorticoid modulating pharmacological agents dexamethasone (Dex-black bars), RU486 (blue bars) or metyrapone (Met-orange bars), or genetically using morpholinos directed against glucocorticoid receptor (GR Mo-green bars) or *cyp11b1* (Cyp Mo-purple bars). Data shown are mRNA abundance of key vascular angiogenic genes *flk* (A), *vegfaa* (B) and *il-8* (C) quantified by standard curve production and normalised to the housekeeping gene *ef1α*. All data are mean± SEM presented as AU, n=4 (10 embryos per group). Data were analysed by 1-way ANOVA and Dunnett's post hoc test; * $p\leq0.05$, ** $p\leq0.01$ compared with control.

5.4.1.2 Aim 2:

What are the long term effects of embryonic GC manipulation on the adult vascular system?

To determine the long term impact of embryonic GC manipulation on adult vascular phenotype, the caudal fins of anaesthetised adult tg(FLi1:GFP) zebrafish were assessed under fluorescent microscopy at 120 dpf. While it appeared that GC manipulated embryos had varying levels of branching, as indicated by altered GFP expression, steps were taken to investigate whether there was a similar effect on the fine capillary like appenditures between caudal ray vessels (red arrows Figure. 5.8) in the adult. However, measuring the interconnections between these vessels which expressed GFP proved challenging due to: the wide branching/meshing (blue arrows Figure 5.8) both in treated and in control adult fish; the plane of observation; and also the length and curvature of the caudal fin.

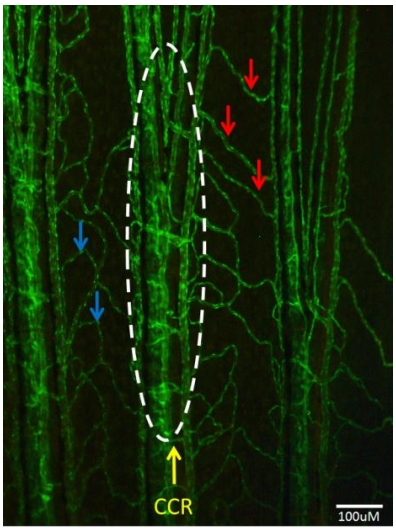

Figure 5.8 High magnification image of adult zebrafish caudal fin vasculature.
Highlighted features include the central caudal ray (CCR) in yellow, ray to ray vessel crossing as highlighted by red arrows, complex meshing of vessels between rays (blue arrows) and around the main ray vessels (white ellipse).

5.4.1.2.1 Ray fin vessel crosses

In an adaptation of the ISV quantification the number of complete vessels connecting rays in a given region of the caudal fin was determined. The defined region was two caudal rays on the left hand side (LHS) of the central caudal ray (CCR) and two

caudal rays on the right hand side (RHS) of the CCR (so 4 inter-ray spaces). The total number of connections between the rays was then counted, as for ISV. Only those which completely joined the rays and were un-branched were counted throughout the length of each of the inter-ray spaces. With this method no differences in fin ray vascular patterning was observed in any of the treatment groups ($p>0.05$ (Figure 5.9)). In determining the number of ray fin vessel crosses there appeared to be a greater number of fine vessels connecting rays towards the tip of the homocercal tail in EmmodDex adults compared to the other groups investigated (Figure 5.9). Measuring these subtle differences was problematic with varying caudal fin length and non-uniform tail appearance not allowing an accurate method of quantification. Caudal fin patterning is, however, highlighted in Figure 5.9 for qualitative assessments. To investigate whether this apparent increase in fine vessels in the EmmodDex group conferred an increase in blood vessel perfusion, Dextran injections were carried out. Many of the fine vessels (which were thought to be in greater abundance in the EmmodDex group) did not have active blood flow as indicated by lack of red fluorescence following injection of 70kDa dextran tetramethylrhodamine (Dextran-rhodamine). This was also noted in control fish so was not as a result of treatment. This dye was chosen as it is comparable in particulate size to the mononucleated blood cells found in the circulation (Bayliss *et al*, 2006). Typical images 5 min after intra-ocular dextran are shown in Figure. 5.11 These data suggest that these fine vessels towards the end of the tail in the EmmodDex adults are not functional.

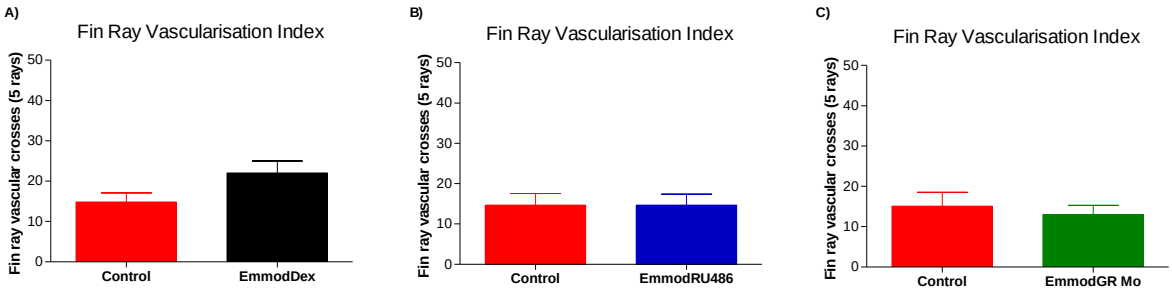

Figure 5.9 Adult fin ray vascularisation index (VI)
Total number of vessel crosses between the central caudal fin rays of the adult zebrafish tail in a 5 ray section. Data show adults treated during embryogenesis with A) dexamethasone (EmmodDex-Black bars) B) RU486 (EmmodRU486-Blue) or C) glucocorticoid receptor targeted morpholino (EmmodGR Mo-green bars). All data are versus respective controls (red). Data are mean n=10 adult tails ± SEM. Statistical analysis was carried out by Student's *t*-test.

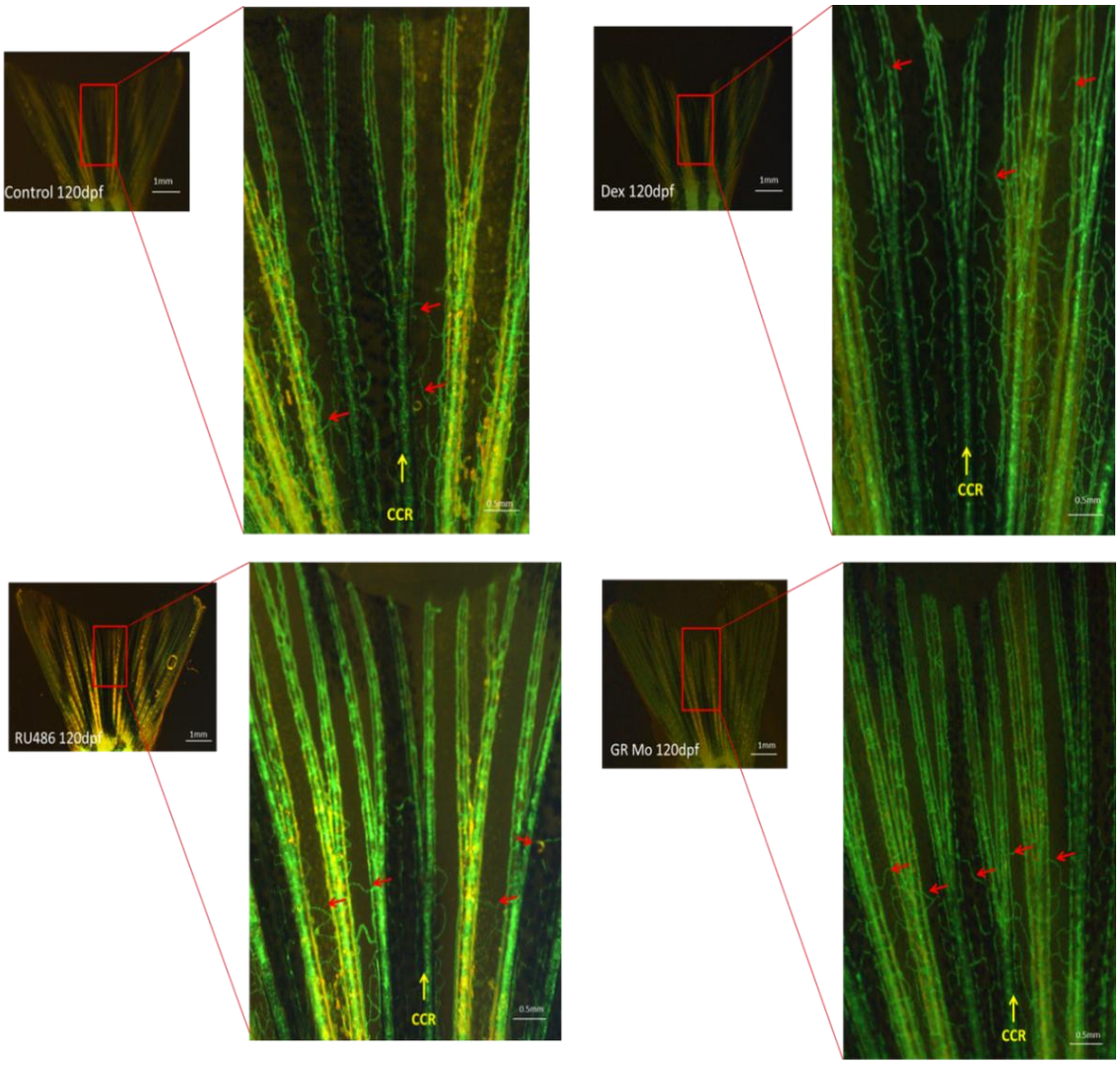

Figure 5.10 Typical caudal fin vascular patterning in adult tg(FLi1:GFP) zebrafish
Typical caudal fin appearance in adults which had been treated with dexamethasone (Dex), RU486, targeted glucocorticoid receptor morpholino (GR Mo) or vehicle control for the first 120 hours post fertilisation (hpf) and then left to develop un-manipulated until 120 days post fertilisation (dpf). Low magnification images are shown to highlight the area of interest. High magnification images show the central caudal ray (CCR), denoted by a yellow cross, and the most dorsal vessel crossing between rays, marked by red arrow heads.

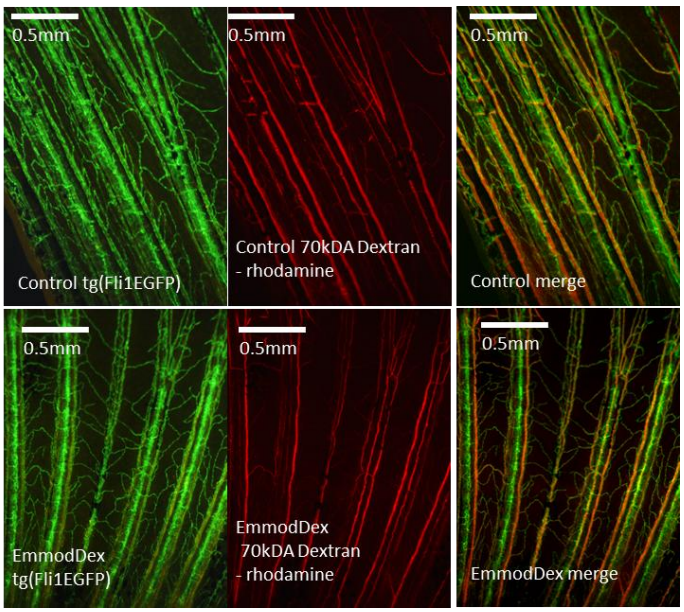

Figure 5.11 Adult caudal fin patency
Images of a caudal fin of an adult zebrafish treated with dexamethasone (Dex) as an embryo (EmmodDex). Upper panel images show control caudal fin vasculature under fluorescent microscopy, lower panels show EmmodDex adult caudal fin vasculature. Images on the left hand side show GFP expression in the tg(FLi1:GFP) line highlighting the vasculature, central panels show 70kDA Dextran-rhodamine expression in circulation following 10 min of cardiac cycles. The right hand side panels show the green and red channels merged to show the level of blood flow within the vasculature.

5.4.1.2.2 Caudal fin angiogenesis model

To determine whether the intrinsic angiogenic capacity of the adult zebrafish vessels had been reprogrammed by embryonic environmental GC manipulation, a classical zebrafish regenerative model was adapted to examine angiogenic blood vessel formation and patterning following caudal fin resection. Before the angiogenic capacity was investigated basic assessments of tail regenerative capacity were carried out.

There was no gross difference in the appearance of regenerated fins in adults that had undergone GC manipulations during embryonic development compared to respective controls. Adults from all treatment groups showed a classical homocercal tail regeneration pattern (Figure 5.12).

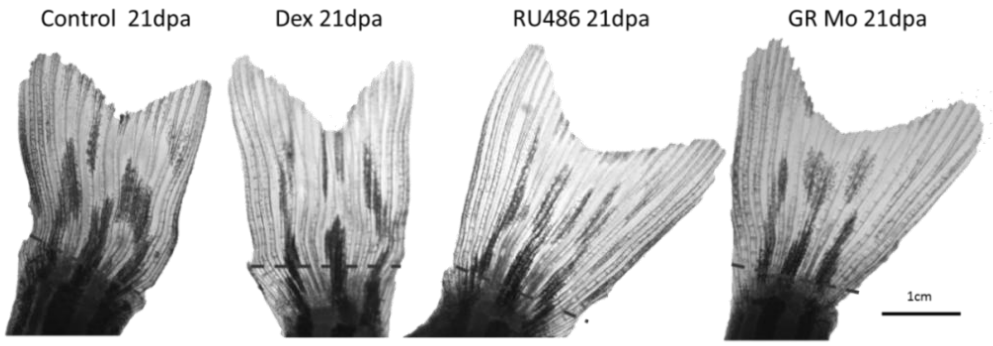

Figure 5.12 Typical caudal fin regrowth 21days post amputation (dpa).
Caudal fin regrowth in adult tg(FLi1:GFP) zebrafish which had been exposed to dexamethasone (Dex), RU486 or a morpholino targeted towards the glucocorticoid receptor (GR Mo) during embryogenesis. Images show tail appearance and site of initial amputation (dashed line) and regrowth.

5.4.1.2.3 Tail regenerative capacity

The regenerative capacity of EmmodDex, quantified as rate of fin regrowth, was found to be similar to controls ($p>0.05$). EmmodGR Mo showed similar fin regrowth as controls, likewise there was no difference in fin regrowth in the EmmodRU486 group either (Figure 5.13 A-C).

5.4.1.2.4 Angiogenic gene abundance in tail fin regenerates.

After tail re-growth was investigated, the total area of fin regrowth was assessed for the abundance of pro-angiogenic genes, *vegfaa* and *il-8*, mRNA abundance, were assessed in the adult by qRT-PCR. *il-8* mRNA abundance in tail fin regenerates was found to be unaffected by embryonic GC manipulation with none of the adult groups displaying any differences in *il-8* mRNA abundance. Similar observations were noted for *vegfaa* mRNA abundance in the EmmodRU486 and EmmodGR Mo groups, with neither group showing a detectable difference in *vegfaa* mRNA abundance. However, a reduction in *vegfaa* mRNA abundance was detected for the EmmodDex group ($p<0.05$).

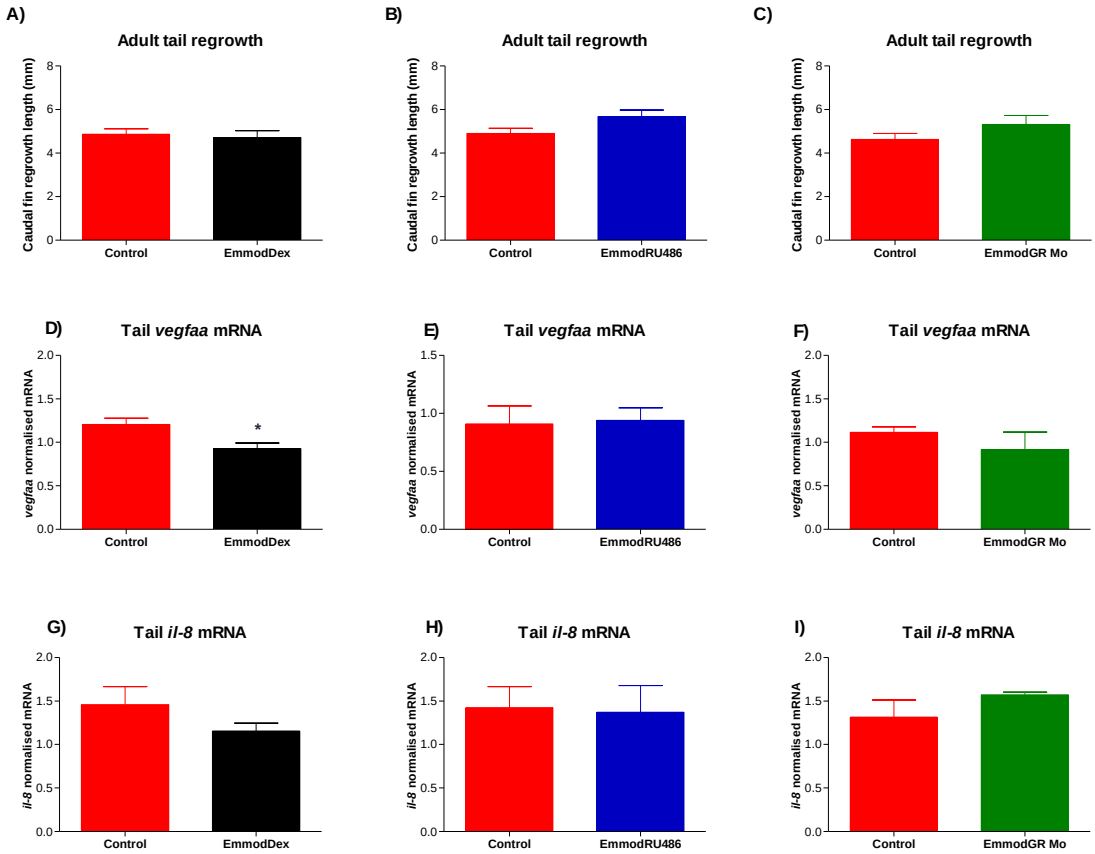

Figure 5.13 Caudal fin regrowth assessments
Data shown are from adults which had been manipulated during embryogenesis with dexamethasone (EmmodDex-black bars), RU486 (EmmodRU486-blue bars), or a morpholino targeted towards the glucocorticoid receptor (EmmodGR Mo-green bars); all versus their respective controls (red bars). (A-C) data shown are the mean length (mm) of three regions of the homocercal tail (central ray, left hand side lateral ray, and right hand side lateral ray) per fish displayed as the mean ± SEM (n=10 adult fish per group). Data were analysed by Student's *t*-test. (D-F) Data shown for *vegfaa* mRNA abundance in tail regrowth. (G-I) Data shown for *il-8* mRNA abundance in tail regrowth. mRNA abundance data were quantified through standard curve production and normalisation to house-keeping gene *ef1α*. Data are given as mean ±SEM (AU), n=5 tails. Data analysed by Student's *t*-test, * $p<0.05$.

5.4.1.2.5 Angiogenic vessel sprouting in tail regenerates

While there was no clear difference in vascular patterning between treatment groups and controls 21dpa (data not shown) a difference was observed at 3dpa in EmmodDex vs. their controls (Figure. 5.15). In 64% of control fish vessel sprouting from the regenerate wound site occurred earlier than in EmmodDex fish, with vessel sprouts being detected by 3dpa (Figure. 5.15). There was no observable difference detected for EmmodRU486 and EmmodGR Mo fish when compared to their controls.

Quantification of these sprouting vessels in the caudal fin rays indicated that EmmodDex-treated fish did indeed have fewer sprouts at 3dpa compared to the controls ($p < 0.01$) while this difference was not observed in EmmodRU486 or the EmmodGR Mo.

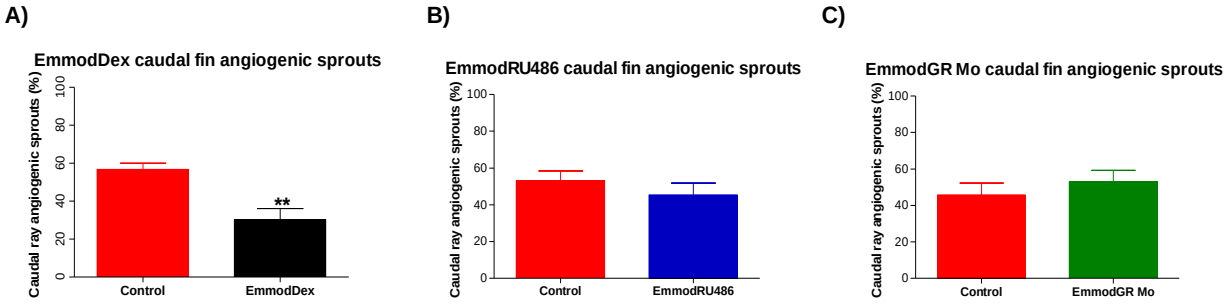

Figure 5.14 Quantification of angiogenic sprouts at 3 days post amputation (dpa)
Data are of caudal tail fin angiogenic sprouts in adult zebrafish 3 dpa which had been subjected to glucocorticoid manipulation during embryogenesis. Data are shown for adults treated as embryos with (A) dexamethasone (EmmodDex-black bars), (B) RU486 (EmmodRU486-blue bars), or (C) morpholino targeted towards glucocorticoid receptor (EmmodGR Mo) and their respective controls (red bars). Angiogenic sprouts were recorded as the number of caudal rays which display an angiogenic sprout in relation to the total number of caudal rays (percentage). Data are mean± SEM (n=6) and were analysed using Student's *t*-test **p<0.01.

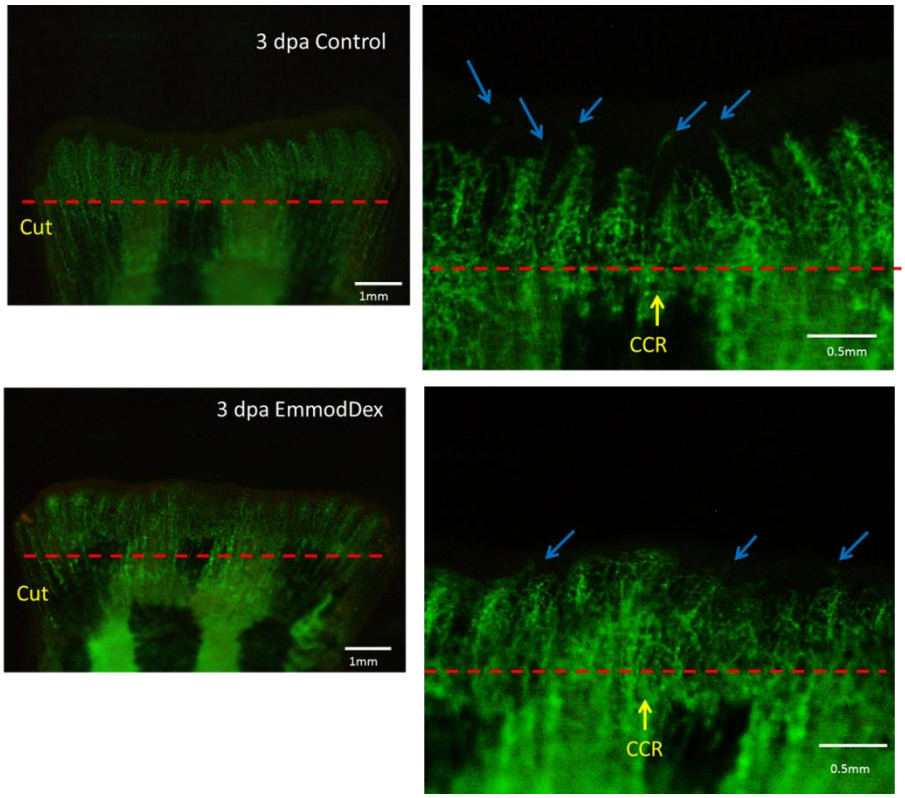

Figure 5.15 Adult zebrafish angiogenic sprouts 3 days post amputation (dpa)
Vessel sprouting in the caudal fin of adult tg (Fl1: GFP) zebrafish tails 3 days post amputation (3dpa), in adults which had been treated during embryogenesis with dexamethasone (EmmodDex) compared to controls. Low magnification images show location of tail amputation (red dashed line). A higher magnification image is shown highlighting vessel-sprouting (blue arrows) from the injury site (red dashed line). For orientation, the high magnification image has the central caudal ray (CCR) highlighted (yellow arrow).

5.4.2 Part 1: Discussion

This section of work has focused on the direct and specific actions of GC modulation on the developing embryonic zebrafish vasculature and the long-term effects this has in the adult.

5.4.2.1 Short-term effects of embryonic GC manipulation on the vasculature

Hypertension is widely believed to be a programmable cardiovascular risk factor but the mechanism underpinning this remains uncertain (Alexander, 2006). Many years of hypertension research have suggested that changes in peripheral vascular resistance may be linked to endothelial dysfunction, structural changes in the vessel wall, and micro vascular rarefaction (Pladys *et al*, 2005). However, the association with hypertension and a direct causative role has been difficult to establish (Nuyt, 2008). While this work has not looked at blood pressure, we have been able to assess whether specific manipulations of the GC system result in short- and longer-term changes in vascular structure and associated molecular signalling by virtue of the transparency of the embryo and the ability to readily observe angiogenesis as part of the regeneration of the caudal fin in the tg (FLi1: EGFP) zebrafish line.

It is well established that the molecular and cellular pathways controlling vascular anatomy are conserved amongst vertebrate models (Kamei *et al*, 2006) which makes it possible to assign homologies between distinct blood vessels and to directly compare the formation of these vessels in different vertebrate species. During the processes of vertebrate embryogenesis the first blood vessels to form are the large midline artery and vein. The formation of these vessels is through the process of vasculogenesis - the migration, differentiation and coalescence of angioblasts (Ellertsdottir *et al*, 2010). When these vessels are established the trunk vessels then form, by the migration of endothelial cells from those midline vessels, through a process referred to as sprouting angiogenesis. It is apparent that the formation of vessels by angiogenesis is as a result of highly-regulated signalling events to coordinate cell migration, rearrangement and proliferation. However, the underlying cellular and molecular events are not yet fully characterised. A number of important signalling molecules and pathways have, however, been identified in the regulation

vasculogenesis and angiogenesis: for example, VEGFs, ephrins, angiopoietins, netrins, chemokines, and their receptors, (Stoll *et al*, 2011).

The zebrafish has been previously used as a model of angiogenesis, with many of the major molecular pathways regulating angiogenesis in mammalian systems found to be conserved in this species (Hasso & Chan, 2011). Indeed, treatment of zebrafish embryos with known mammalian anti-angiogenic compounds has been shown to inhibit angiogenesis (Evensen *et al*, 2010). The success of this model is in part due to the availability of transgenic strains which allow easy visualisation of blood vessels, such as the transgenic line used in this chapter tg(FLi1:EGFP).

In the work presented in this chapter manipulation of GCs in the embryo resulted in marked changes in the formation of the embryonic zebrafish vasculature, with a strong association with ISV formation in the first 120hpf. This was clear from simple counts of complete ISV or calculation of VI. Often the vessels were completely missing (as observed by somite patterning but no EGFP expression) or were truncated and denser in appearance, suggesting abnormalities in the migration and/or differentiation of endothelial cells.

The primary axial vessels were formed similarly in all groups investigated, suggesting that the formation of these vessels by vasculogenesis is unaffected by GC manipulation. However, it may be that subtle effects did occur which were not detected by the methodology used and future work could focus on the origin and specificity of endothelial cells, the formation of the dorsal aorta and caudal vein, the anterior and posterior differences in artery formation and the processes of lumen formation for these primary vessels (Ellertsdottir *et al*, 2010) which may be affected.

To enhance our understanding of angiogenesis numerous vessel networks have been assessed in the zebrafish; for example the sub-intestinal vessels, a vascular network ventral to the gut (Serbedzija *et al*, 1999). However, one well documented zebrafish model of angiogenesis is the formation of the ISV network in the tail of the developing embryo (Cannon *et al*, 2010; Childs *et al*, 2002; Gore *et al*, 2012). The formation of the ISV in the trunk of the zebrafish embryo has previously been used as a model of endothelial tube formation *in vivo* (Blum *et al*, 2008).

ISVs sprout from the dorsal aorta at around the 24 somite stage (Kimmel *et al*, 1995) and extend and connect to the dorsal longitudinal anastomotic vessel (DLAV). Cell lineage labelling experiments indicate that ISVs form when a tip-cell embedded in the dorsal aorta carrying a dorsally sprouting filopodia-like appendage this grows dorsally along vertical somite boundaries, leaving elongated cells behind (Gore *et al*, 2012). The migration of these cells is a very fast and dynamic process. As the tip-cell reaches the neural tube the cells branch rostrally and caudally to form a T-shaped cell in the DLAV (Childs *et al*, 2002). Cellular rearrangement and division occurs, allowing the interconnected cells to become lumenised. These tube structures have been shown, through time-lapse microscopy, to develop as a result of the formation of endothelial vacuoles which then undergo intracellular and intercellular fusion (Kamei *et al*, 2006).

In the current work, it is clearly demonstrated that alteration in GC activity impairs the formation of the ISV, with both Dex and RU486 treatment resulting in abnormal ISV EGFP expression. Manipulation of cortisol biosynthesis, using Met or Cyp Mo, did not affect ISV number suggesting that the effects of GC on ISV formation are receptor mediated rather than reliant on embryonic cortisol biosynthesis which, as shown in chapter 3, is present from 48hpf. GR and MR are both present very early in development (8hpf) (Chapter 3) suggesting a possible physiological role for GC in early formation of the vascular tree.

Both GR and MR have been associated with mammalian angiogenic effects. Decreased MR receptor expression is associated with angiogenesis and increased microvasculature density, with the converse observed with increased MR activity (Tiberio *et al*, 2013). Reduced GR activation has been associated with increased angiogenesis (Small *et al*, 2005). Spironolactone (used here as an MR agonist (Pippal *et al*, 2011)) incubation during embryogenesis had no effect on ISV number or appearance while GR Mo treated embryos (and the selective GR agonist Dex) had reduced numbers of ISVs, suggesting that these effects are mediated by GR rather than MR. However, it is possible that, by altering GR expression and/or activity, we are also indirectly altering MR activation. Genetic MR knockdown, through targeted

MR Mo, may provide a more specific way to assess and define MR mediated effects in the zebrafish model.

vegfaa is important in the formation of ISV, and is known to be produced and secreted by the somites between which the sprouting ISV cells migrate (Cleaver & Krieg, 2001; Liang *et al*, 2001a). Furthermore it has been documented that suppression of activity of its receptor, inhibits the formation of ISV (Habeck *et al*, 2002). In addition, drugs which promote angiogenesis have been shown to increase expression of *vegfaa* in zebrafish (Tian *et al*, 2012). Interestingly the Dex-treated embryos displayed both reduced angiogenic phenotype and a reduced level of *vegfaa* mRNA, suggesting a possible interaction between increased GC levels and suppression of angiogenesis through reduction in *vegfaa* signalling. These findings are in keeping with similar work in mammalian placenta (Hewitt *et al*, 2006), human chrondocytes (Koedam *et al*, 2002) and in angiogenesis linked to prostate cancer (Yano *et al*, 2006a).

Unlike previous studies, however, no alteration in the expression of *vegf (flk/kdr*) receptor mRNA was observed, suggesting that the reduction in ISV formation is as a result of reduced ligand-dependent (*vegfaa*) receptor activation rather than the unavailability of receptor. This may explain the sporadic appearance of vessels observed here rather than a complete absence of ISV as previously observed with *(flk/kdr*) receptor antagonism (Zhang *et al*, 2011).

While the findings presented here may also suggest that the vascular abnormalities following GC manipulation may be independent of *vegfaa* signalling. Further support for a role of GR in Vegfaa signalling is that it has previously been shown that Vegfaa is not required for the establishment of the axial vasculature patterning i.e. midline vessel development. In contrast, the development of ISV by angiogenesis is strongly dependent on *vegfaa* signalling (Nasevicius *et al*, 2000). The data presented here is in accordance with this, as reduced ISV was accompanied with apparently normal vasculogenesis.

A reduction in *il-8* abundance in embryos treated with Dex was also observed. The most widely recognized role of IL-8 in mammals is in the inflammatory system

where it promotes release of neutrophil granular enzymes (de Oliveira *et al*, 2013; Rahman *et al*, 1999). Similar observations have been noted in fish (Renshaw *et al*, 2006). However, IL-8 is also recognized as an important regulator of angiogenesis in mammals with suppression of IL-8 activity known to have anti-angiogenic effects (Yano *et al*, 2006a). Not only has IL-8 been associated with angiogenesis but has also been linked with the GC system; impaired local regeneration of GC in the $11\beta HSD1^{-/-}$ mouse increased IL-8 activity, which was suggested as the mechanism for increased angiogenesis *in vitro* and *in vivo* within sponges, wounds, and infarcted myocardium (McSweeney *et al*, 2010; Small *et al*, 2005) in these mice.

In contrast to these data, embryos which were treated with the GR antagonist RU486 had a higher level of *il-8* associated with an increased VI (consistent with increased branching or sprouting). The observed sprouting and branching out-with the somite boundaries may also be due to altered Vegfaa signalling of the tip cells as the data presented here shows in contrast to embryos incubated in Dex, those which were treated with RU486 displayed raised *vegfaa* mRNA levels. However, as for Dex treatment there was no alteration in the levels of the *vegfa* receptor mRNA.

The data shown here for Dex and RU486 suggest that GR modulation could influence zebrafish angiogenesis by altering the expression of key angiogenic factors *vegfaa* and *il-8* while this may suggest a mechanism for the alteration in the embryonic vasculature there are numerous other angiogenic factors which could be investigated further. The data here is particular interesting as mammalian studies have highlighted a signalling pathway common to VEGF and IL-8 (amongst other chemokines), the mitogen activated protein kinase (MAPK) pathway, in which mRNA expression of these pro-inflammatory factors is negatively regulated by the activity of GR through the p38MAPK subgroup (Holehouse *et al*, 2012; Imasato *et al*, 2002), again suggesting a possible route for further investigation.

GR Mo embryos also displayed an altered vascular phenotype with missing ISV throughout the length of the tail during the course of the first 120 hpf. It has been established that ISV form dorsally as the embryo grows and this treatment caused impaired sprouting of vessels with many found to be truncated in appearance. While no alteration in the mRNA abundance profile of the angiogenic factors was observed

in this group a reduction in the VI was noted. This suggests that the developmental rate of angiogenesis is likely to be impaired. The initial sprouting delay observed at 24 hpf, may result from an overall developmental delay, (as highlighted in chapter 4 there is an 8-12h delay in formation of somites accompanied by delayed vessel sprouting in the GR Mo embryos). Interestingly, a clear return of global developmental milestones was observed in these embryos by 120 hpf (chapter 4) meanwhile ISV number and VI remained reduced compared to controls. As these embryos maintain a reduced *gr* mRNA at this time point, data presented here suggests that GR signalling is important for ISV angiogenesis in the developing zebrafish.

While the data presented here indicates that Dex treatment reduced the number of ISV with detectable EGFP expression, it is also apparent that the patency of ISV does not appear to be affected by Dex treatment during embryogenesis, suggesting that while the endothelial EGFP expressing component of the vasculature appears to be reduced the lumenisation of these vessels was not impaired. GCs have previously been shown to directly inhibit tube formation in cultured endothelial cells in human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAoECs) treated with cortisol (Logie *et al*, 2010). This inhibition of tube structure formation was proposed to be the result of a GR-mediated effect on cellular morphology rather than impaired migration, differentiation and cellular viability (Logie *et al*, 2010). It should, however, be noted that Logie *et al.*, used cultured endothelial cells, whereas the current observations in the zebrafish may offer new opportunities to investigate the mechanism of GC mediated inhibition of tube structure formation which currently remain unclear. While there is detectable Dextran within the somite boundaries this is however not a direct indication of blood cell flow, and although care was taken to select a Dextran of molecular weight as close to red blood cells as possible there are obvious differences between this and the properties of blood such as adherence and movement.

5.4.2.2 Long-term effects of embryonic GC manipulation on vasculature

Hypertension is characterized by an increase in peripheral vascular resistance and micro-vascular abnormalities (Noon *et al*, 1997; Wong *et al*, 2001). It is well

established that the appearance of these abnormalities often precedes alterations in blood pressure. The association with later-life hypertension and /embryonic reprogramming is well established from numerous observational studies in humans and in a number of animal models, including rodents (Barker, 1997). While endothelial dysfunction and artery remodelling are common observations in both animal and human studies of programming (Nuyt, 2008; Nuyt & Alexander, 2009) it is only more recently that negative changes in small vessel form and function have been investigated as an early indicator of cardiovascular risk in adult life (Ciuffetti *et al*, 2003; Noon *et al*, 1997).

Early pathogenesis of hypertension is thought to result from direct structural effects on the artery and on the capillary bed (Ligi *et al*, 2010) with reduction in capillary density (rarefaction) observed in the offspring of rats following maternal nutrient restriction (Nuyt & Alexander, 2009). These offspring also have reduced angiogenic capacity accompanied by reduced in Vegf expression (Goligorsky, 2010; Pladys *et al*, 2005). In humans vascular rarefaction, which may elevate peripheral vascular resistance, has been detected in capillary beds of young men with hypertension risk and those who had low birth weight (Irving *et al*, 2004). Furthermore it has also been documented that suboptimal maternal nutrition and growth can result in reduced microvascular perfusion and functional dilator capacity, reduced expression of VEGF proteins, decreased angiogenic potential, shorter cultured neo-vessel formation and increased levels of cellular apoptosis (Khorram *et al*, 2007a; Pladys *et al*, 2005). These changes in small vessel molecular composition, structure and function can often be observed long before the onset of cardiovascular and metabolic abnormalities (Goligorsky, 2010; Pladys *et al*, 2005). It is therefore highly likely that altered vascular pathophysiology is as a result of some direct alteration in vascular phenotype.

Small vessels (those less than 150μm in diameter) are particularly important physiologically in the regulation of solute transfer, a process which is largely controlled by the number of vessels and by the local flow of blood (Clough & Norman, 2011). Therefore, alterations in microvasculature structure and flow can have a profound impact on functional integrity, with reduced surface area resulting in

suboptimal tissue perfusion and failure to meet the metabolic demand (Clough & Norman, 2011). These vessels are also important in the maintenance of overall peripheral resistance, with reduced capillary density found to increase peripheral resistance (Gregório *et al*, 2007).

Given this apparent association of microvasculature structure with programming, the primary aim here was to investigate whether the alterations in embryonic blood vessel appearance can impair adult vasculature. To do this a caudal fin ray vasculature assessment was performed in adult fish that had undergone embryonic GC manipulation. The adult caudal fin vasculature is an interconnected tubular multi-tissue structure which allows the transportation of blood throughout the caudal fin, similar to fine capillary networks. An increase in tail fin vascularisation has been observed in some fish models of exercise training (Sanger & Stoiber, 2001) suggesting that capillarisation, a feature which allows increased oxygen supply to working muscle, may be an adaptable feature for increased energy demand. This fin is commonly used as a relatively accessible model for studying regenerative processes in the adult zebrafish following amputation (Bayliss *et al*, 2006) and was adapted here to assess whether capillary density and angiogenesis could be altered in the adult fish of GC-manipulated embryos.

In the work presented no significant alteration in the number of vessels joining caudal rays was observed, suggesting that the features which alter embryonic ISV formation do not result in long-term alteration in these fine adult vessels, at least in terms of inter-ray connections. This is in contrast to mammalian studies in which developmental programming altered neonatal capillary number or length/volume density in key organs (such as skeletal muscle, pancreas, brain, and kidney (Clough & Norman, 2011)). Vascularisation in the heart has also been effected with reduced intra-myocardial vascularisation in male rat offspring of low protein diet mothers (Gregório *et al*, 2007). However, whether these adaptations for short-term survival are sustained through the life course in these models remains unclear.

While the data presented here do not highlight any differences in microvasculature structure, it does not mean that differences in capillary density do not occur in other tissue capillary beds, such as skeletal muscle. It may be that decreased capillary

density allows redistribution of blood and nutrients to central tissues at the expense of peripheral tissues, therefore to fully understand vessel effects other capillary networks should be investigated in the adult fish. In keeping with the energy demands, the highly vascularised gills may offer a more suitable model.

Altered angiogenesis in embryos treated with Dex also resulted in long term alteration in angiogenic capacity of EmmodDex adults which manifests as a reduction in angiogenic sprouting in the amputated tailfin at 3dpa. These data are consistent with data published by Pladys and colleagues, (Pladys *et al*, 2005) where a decrease in cultured aortic ring angiogenesis was observed from offspring of maternal low protein diet rats. Interestingly, the EmmodDex impaired angiogenesis was not accompanied by reduced tail tissue regrowth suggesting that the tissue regeneration pathways are not altered by embryonic GC manipulation and also showing that impaired angiogenesis is not a result of impaired tail regrowth. By 21dpa there were no obvious differences in tail morphology or in blood vessel appearance, in any of the groups investigated, suggesting that the angiogenesis processes are only temporally delayed and not completely impaired.

5.5 Part 2

The influence of embryonic glucocorticoid manipulation on the embryonic and adult heart

5.5.1 Part 2: Results

5.5.1.1 Aim 3:

Does manipulation of the glucocorticoid system alter embryonic cardiac development?

5.5.1.1.1 Cardiac functional assessments following embryonic GC manipulation

tg (CMLC2: GFP) zebrafish were used to investigate the structure and function of the developing heart in the zebrafish embryo following pharmacological and genetic manipulation of the GC system (Figure 5.16(A-D)).

Reduced heart rate was observed at 120 hpf in embryos where treatment resulted in reduced GR activity i.e. the RU486 or GR Mo groups ($p<0.001$). Ejection fraction

was also lower in these groups (RU486 $p<0.01$ and GR Mo $p<0.001$) when compared to controls. None of the other treatment groups investigated (Met, Cyp Mo or Dex) altered these parameters at 120 hpf (Figure 5.16(A and B)).

Dex incubation increased stroke volume by 120 hpf (0.14± 0.03 μl^3 vs 0.08± 0.03 μl^3 Figure 5.16 (C)). This resulted in an increase in cardiac output ($p<0.05$(Figure 5.16 (D)). Conversely a reduction in cardiac output was observed in embryos treated with GR Mo by 120 hpf (15.7± 3.0 μl^3/min vs controls 11.3 ± 0.7 μl^3/min, $p<0.05$) although no difference in stroke volume was noted. No other treatment altered stroke volume or cardiac output (Figure 5.16 (C and D)).

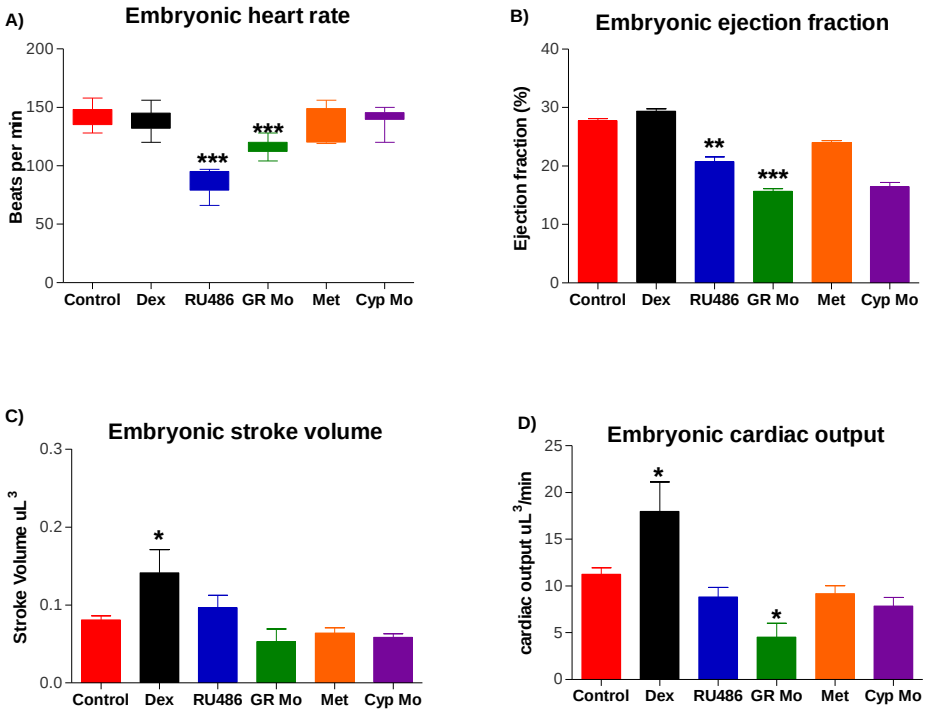

Figure 5.16 Embryonic cardiac assessments
Cardiac assessments at 120 hours post fertilisation (hpf) in zebrafish embryos continuously incubated in 100µM dexamethasone (Dex - black bars), 10µM RU486 (blue), morpholino targeted towards glucocorticoid receptor (GR Mo-green), 10µM metyrapone (Met-orange) or morpholino targeted towards *cyp11b1* (Cyp Mo-purple). Data shown are A) heart rate, B) embryonic ejection fraction, C) embryonic stroke volume and D) embryonic cardiac output. Data are mean± SEM (n=21 hearts/group; maximum to minimum for (A)) and analysed by 1-way ANOVA and Dunnett's post hoc test; p* <0.05, p**<0.01, *** p<0.001.

5.5.1.1.2 Cardiac structural assessments following embryonic GC manipulation

In order to quantify the effect of GC manipulation on embryonic zebrafish heart morphology, the distance between the junction of the heart with the inflow tract at the sinus venosus (SV) and the junction with the outflow tract at the region of the bulbus arteriosus (BA) (an indicator of heart looping) was determined. This parameter was unaffected by Dex or Met treatment but increased in RU486 (0.83 ± and GR Mo ($P<0.0001$).

While the gross phenotype of embryos was unaffected by drug treatments at the selected concentrations, mild pericardial oedema was detected in some embryos (Figure 5.17). This can often be associated with cardiac dysfunction/enlargement as a result of impaired circulation and pooling of erythrocytes. As the cardiac function and heart looping had already been assessed, the pericardial area was calculated to determine whether there was any correlation with abnormal cardiac function and the observed oedema. Unlike the heart looping investigation and ejection fraction data, RU486 or GR Mo treatment did not alter pericardial area (neither did Dex treatment). However, embryos treated with Met or Cyp Mo ($P\leq0.001$) showed increases in area compared to controls.

Reduction in cardiac function (for example in GR Mo and RU486 embryos) is often associated with a fall in blood flow which would be expected to reduce filtration at the developing pronephros (Rider *et al*, 2012). As a result of the alteration in filtration activity an increase in fluid volume would be expected which would ultimately lead to oedema. To determine whether alteration in pericardial area was a result of increased fluid accumulation, the distance between the heart wall and the pericardial wall was investigated. This distance was greater in embryos treated with Met or Cyp Mo ($p\leq 0.001$) than in controls but no other groups were found to be different.

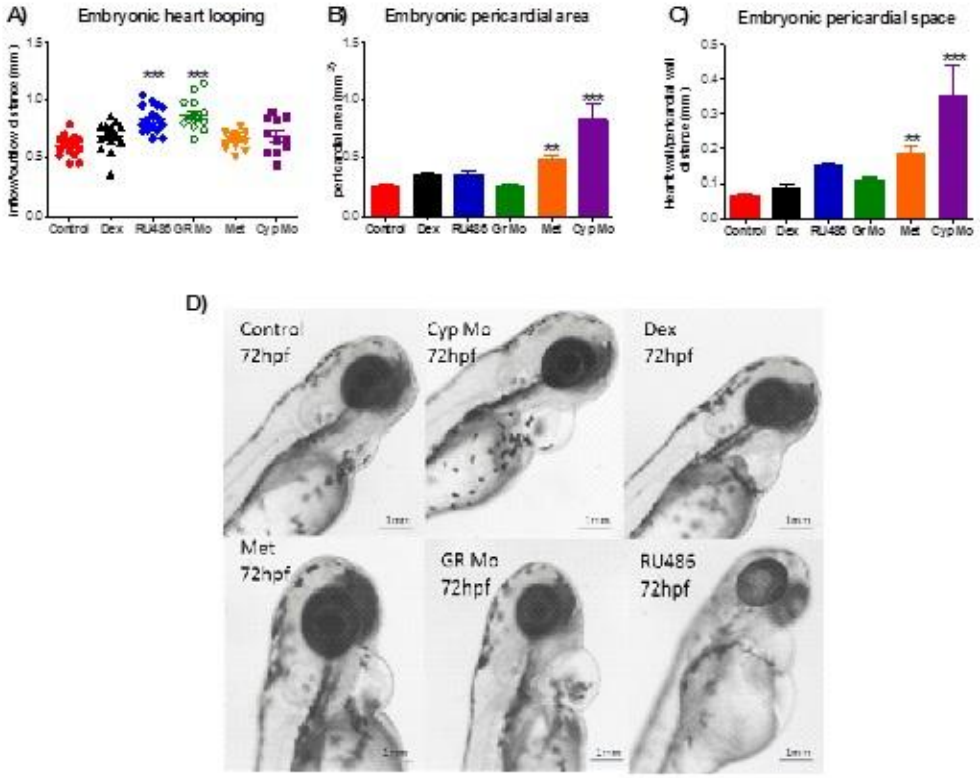

Figure 5.17 Heart morphology after glucocorticoid modulation
Quantitative assessments of embryonic heart morphology in zebrafish embryos at 72 hours post fertilisation (hpf) in controls or in those which had been treated continuously with dexamethasone (Dex-black bars), RU486 (blue), morpholino targeted towards glucocorticoid receptor (GR Mo-green), metyrapone (Met-orange) or morpholino targeted towards *cyp11b1* (Cyp Mo-purple). Data shown are for A) blood inflow/outflow distance of the heart, B) the pericardial area (area between head and yolk sack), and C) the heart wall/pericardial wall distance. Data are mean ± SEM (n=21 embryos/group), analysed by 1-way ANOVA and Dunnett's post hoc test; **p≤0.01, *** p≤ 0.001 D) Typical images of 72 hpf zebrafish embryos treated continuously from the 2-cell stage with the glucocorticoid-manipulating drugs, listed above. Images show pericardial appearance following continuous incubation.

Embryonic heart size was assessed *in situ* throughout the course of development (Figure.5.18). At 72 hpf ventricle length was unchanged following Dex; Met or Cyp Mo treatment compared to controls (controls 82.9 ± 2.3 µm; Dex 85.6 ± 2.6 µm; Met 89.4 ± 1.0 and Cyp Mo 88.7 ± 2.9 µm).

Embryos with reduced GR activity, resulting from treatment with RU486 or the GR Mo, showed a reduction in ventricle length compared to controls at 72 hpf and at 120 hpf. In contrast, hearts from embryos continuously incubated in Dex displayed an increase in ventricle length at 72 hpf ($p<0.001$). Embryos which had been incubated with Met or treated with Cyp Mo were of similar length to controls. The differing heart sizes at 120 dpf are evident in the isolated hearts shown in Figure 5.19.

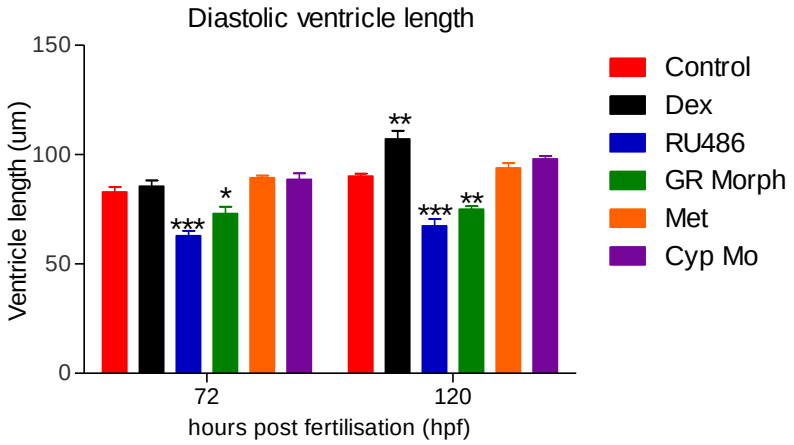

Figure 5.18 Embryonic ventricular length after glucocorticoid modulation
Ventricle long axis length during maximum diastole. Data shown are for 72 and 120 hours post fertilisation (hpf) for control untreated embryos and embryos which had been continuously incubated with dexamethasone (Dex), Ru486 or metyrapone (Met), or treated with morpholino targeted towards glucocorticoid receptor (GR Mo) or *cyp11b1* (Cyp Mo) from the 2 cell stage. Data are mean ± SEM (n= 10 embryos per group where,. each n represents the mean of three cardiac cycles). Data were analysed by 2- way ANOVA and Bonferroni post hoc analysis; *$p\leq0.05$, **$p\leq0.01$, *** $p\leq 0.001$, compared with control.

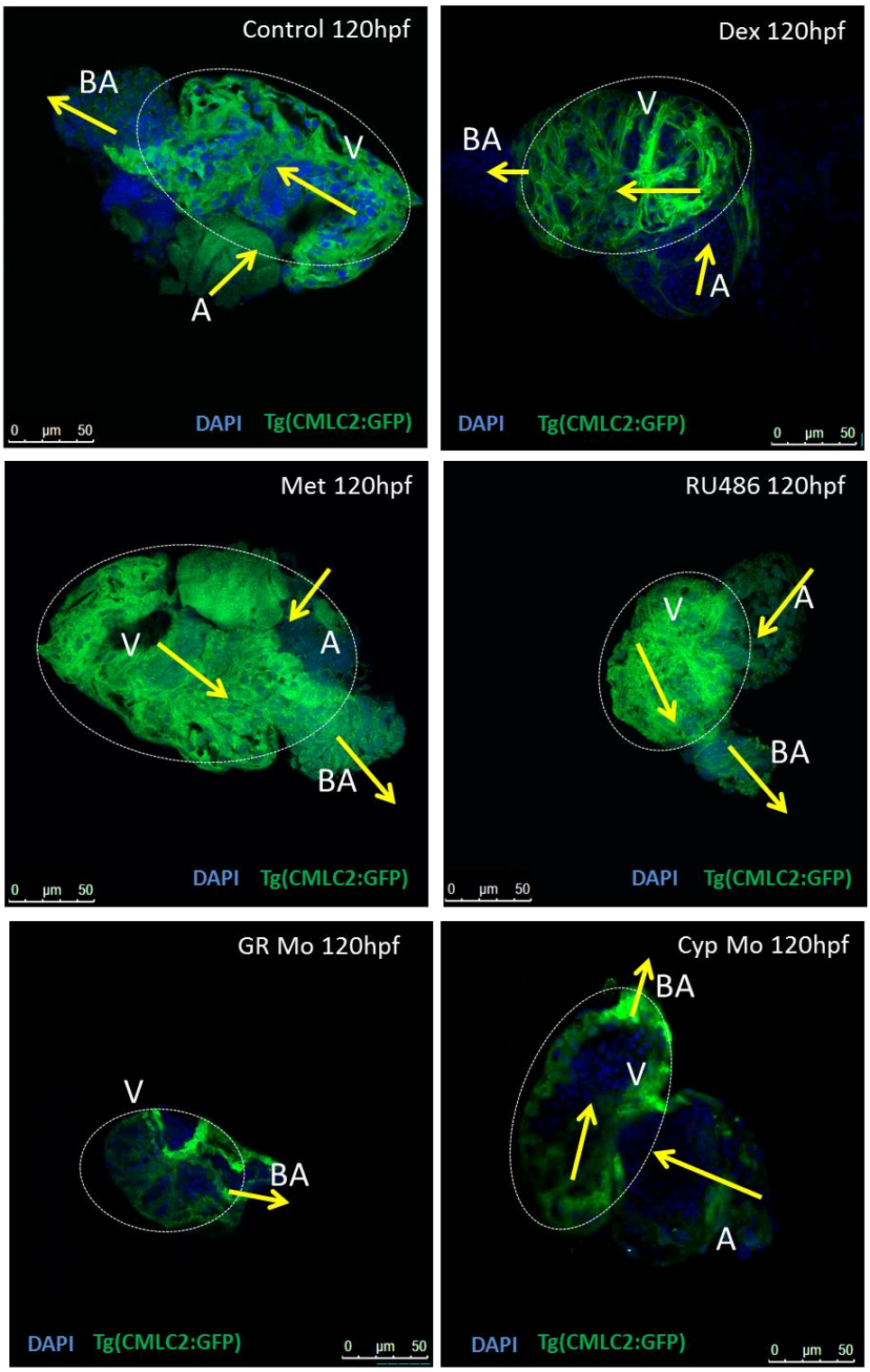

Figure 5.19 Isolated embryonic hearts following glucocorticoid modulation
Representative images of isolated tg(cmlc2:GFP) hearts 120 hours post fertilisation (hpf) co-stained with nuclear stain following exposure to dexamethasone (Dex), metyrapone (Met), or RU486, or to morpholinos targeted towards the glucocorticoid receptor (GR Mo) or *cy11b1* (Cyp Mo). Features annotated are ventricle (V, and dotted ellipse), atrium (A), and bulbous arteriosus (BA). Yellow arrows show direction of blood flow.

5.5.1.1.3 Isolated embryonic heart cardiomyocyte number

Total ventricular cardiomyocyte number was assessed in 120 hpf embryonic hearts. Following treatment, hearts from tg(CMLC2:GFP) fish were surgically isolated and stained with DAPI, ventricular cardiomyocyte nuclei were distinguished from other cell types by the close association of the DAPI-stained nuclei to the GFP expression and counted (Figure 5.20 (B)). Treatments which reduced cortisol production, namely incubation with the drug Met or treatment with Cyp Mo (2.04 ± 0.15 cells per heart) caused no change in cardiomyocyte number compared with controls ($p>0.05$). However, treatments that reduced GC activity, RU486 or GR Mo ($p\leq0.001$) reduced the mean ventricular cardiomyocyte number compared with controls. When this was normalised to total ventricle length, the difference was maintained (both $p<0.001$). Although no reduction in mean cardiomyocyte number was detected for hearts of embryos treated with Dex (187.5± 11.35 cells per heart vs controls 194.6± 11.8 cells per heart), when this number was normalised by the ventricular length it revealed a reduction in total ventricle cardiomyocyte number ($p<0.05$).

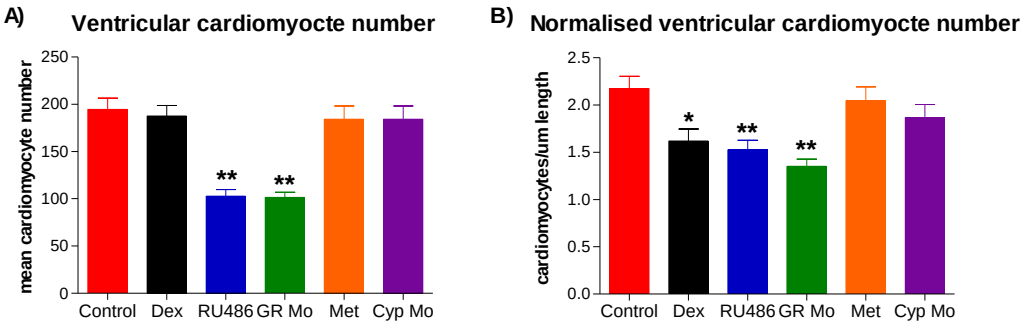

Figure 5.20 Cardiomyocyte number in isolated embryonic zebrafish hearts
Cardiomyocyte number in hearts isolated 120 hours post fertilisation (hpf) following continuous treatment with glucocorticoid modulators dexamethasone (Dex-black), RU486 (blue), targeted glucocorticoid receptor morpholino (GR Mo-green), metyrapone (Met-orange) or targeted *cyp11b1* morpholino (Cyp Mo (purple). A) Shown is the mean ventricular cardiomyocyte number of isolated hearts and B) following normalisation to the ventricular length (µm). Data are mean± SEM (n=12 hearts). Data were analysed by 1-way ANOVA and Dunnett's post hoc analysis; *$p \leq 0.05$, ** $p \leq 0.01$ compared with controls.

5.5.1.1.4 Glucocorticoid gene abundance in isolated embryonic hearts

Three key genes of GC activity were determined in the isolated 120hpf embryonic heart. Constant incubation in either Dex or RU486 had no effect on relative abundance of *gr, 11hsd2* and *mr* genes. No alteration in *mr* or *11hsd2* mRNA abundance was observed in isolated hearts from GR Mo embryos, however in accordance with gene knock down data (chapter 3) a 39% reduction in *gr* mRNA was observed in isolated hearts ($p \leq 0.05$) (Figure 5.21 (C)).

5.5.1.1.5 Cardiac gene abundance in isolated embryonic heart

The abundance of four key cardiac genes was determined in isolated embryonic hearts at 120 hpf following continuous incubation in drug (Figure 5.22). For gene abundance studies, due to challenges in isolation and extraction of RNA from these very small hearts only 3 treatment groups were chosen for investigation. These were: Dex, RU486 and GR Mo which were found to have the greatest impact on cardiovascular structure and function in the embryo (Sections 5.5.1.1.1 to 5.5.1.1.4).

Myocyte enhancer factor-2c (Mef2c) through its role as a transcription factor can regulate skeletal and cardiac muscle-specific genes during development, influencing cellular differentiation and consequently playing a critical role in cardiac development (Xin *et al*, 2013). The abundance profile of this gene in isolated embryonic hearts was unaffected ($p>0.05$) by treatment with Dex or RU486. Isolated hearts from embryos treated with GR Mo however showed a reduction ($P<0.001$) in *mef2c* abundance compared with controls (Figure. 5.22(A)).

Gata 4 is involved in the development of numerous zebrafish organs (Holtzinger & Evans, 2005). It has an important influence on cardiac development and cardiomyocyte proliferation (Singh *et al*, 2010), a role which appears to be conserved in zebrafish and rodent models (Holtzinger & Evans, 2005). In isolated embryonic hearts there was no difference in the abundance levels *gata 4* compared with controls for any of the treatments investigated (all $p<0.05$) (Figure 5.22(B)).

Ventricular myosin heavy chain (*vhmc*) is a gene homologue to the human MYH7 gene which is responsible for the production of myosin heavy chain beta (Miyata *et al*, 2000). Vmhc therefore, required for the normal contractile activity of the heart

and is particularly important in the later stage of cardiac development. There was a rise in *vmhc* abundance in hearts treated with Dex ($p<0.05$). Conversely, embryos treated with RU486 or GR Mo showed a reduction ($p<0.0001$) in abundance compared with controls (Figure 5.22(C)).

Insulin-like growth factor 1 (IGF1) is important for cardiomyocyte proliferation during cardiac development (Li *et al*, 2011). Following incubation of embryos in Dex or RU486 for 120 hpf there were no differences in the abundance of *igf1* levels $p>0.05$). A significant reduction in *igf1* abundance was however detected in isolated hearts from embryos treated with GR Mo, ($p<0.05$) (Figure 5.22(D)).

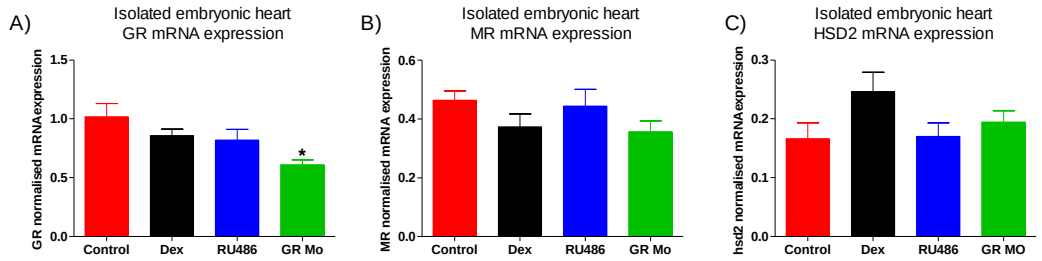

Figure 5.21 Glucocorticoid gene abundance analyses in isolated embryonic heart
Relative mRNA abundance in isolated embryonic hearts at 120 hours post fertilisation (hpf) Data shown are for hearts isolated from embryos constantly incubated in the drugs dexamethasone (Dex-black) or RU486 (blue), or treated with targeted glucocorticoid receptor morpholino (GR Mo-green). Abundance data are for A) *gr* mRNA B) *mr* mRNA and C) *hsd2* mRNA. Data shown are quantified by normalisation to the house-keeping genes *ef1α* and *gapdh*. Abundance is presented as arbitrary units (AU). Data are mean ± SEM (n=3 (100 hearts per group)) and are analysed by 1-way ANOVA and Dunnett's post hoc test. * $p\leq0.05$.

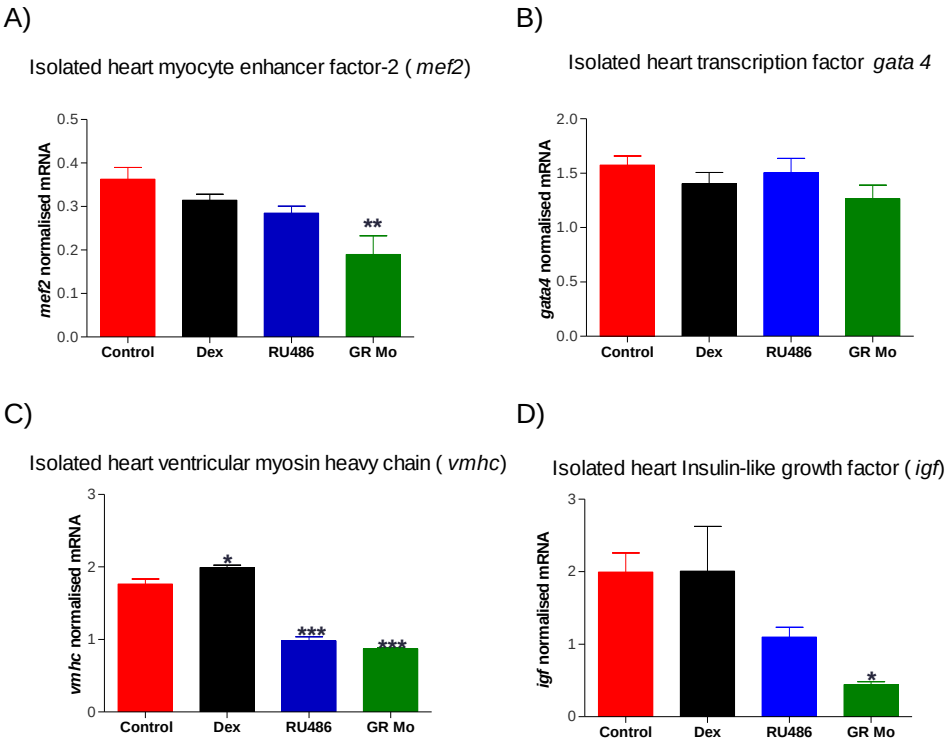

Figure 5.22 Cardiac gene abundance in isolated embryonic heart.
Cardiac mRNA abundance in embryonic zebrafish hearts isolated 120 hours post fertilisation (hpf). Data shown are for hearts constantly incubated in the drugs dexamethasone (Dex-black bars) or RU486 (blue) or treated with morpholino targeted towards glucocorticoid receptor (GR Mo-green), versus un-manipulated controls (red). Abundance data are for: A) *mef2*, B) *gata4*, C) *vmhc* and D) *igf* mRNA. Data shown are quantified by normalisation to the house-keeping genes *ef1α* and *gapdh*. Abundance is presented as arbitrary units (AU). Data are mean ± SEM (n=3 (100 hearts per group)) and were analysed by 1-way ANOVA and Dunnett's post hoc test. *p≤0.05, **p≤0.01 and ***p ≤0.001, compared with controls.
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Aim 4:

What are the long term effects of embryonic manipulation of the GC system on the adult heart?

5.5.1.1.6 Isolated adult hearts

Hearts were isolated from adult zebrafish that had undergone GC manipulation during the first 120 hpf (embryogenesis) and been allowed to develop un-manipulated until 120 dpf (as described in section 5.3.2). A number of parameters and features were investigated in the isolated hearts from each of the 3 treatment groups (EmmodDex, EmmodRU486 and EmmodGR Mo) and their controls (Figure 5.23).

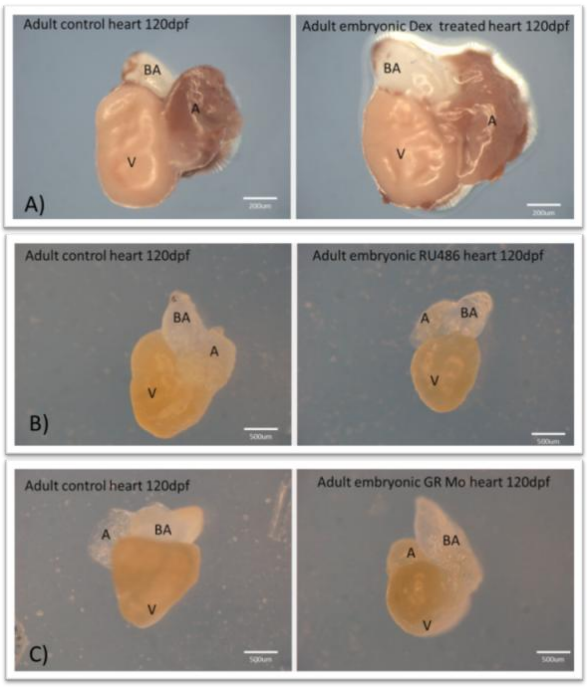

Figure 5.23 Isolated adult hearts after embryonic glucocorticoid modulation
Light microscopy images of typical hearts isolated from adult (120 days post fertilisation (dpf)) zebrafish. Images shown are excised hearts from those treated during embryogenesis with: A) dexamethasone (EmmodDex) – shown with controls, B) RU486 (EmmodRU486) – and controls, and C) injected with a targeted *gr* morpholino (EmmodGR Mo) – shown with controls. Highlighted on images are: bulbus arteriosus (BA) - blood outflow from heart, atrium (A) – in some instances only partial atrium is shown (B and C), and ventricle (V). Images in A) were taken using a microscope to which access was no longer available at the time required for images B and C. Images shown are purely representative and have not been used for quantification of any feature.

5.5.1.1.7 Cardiac size and weight

The wet weight of isolated hearts (the weight of the hearts directly after removal) was normalised to each individual adult fish body weight. Hearts from EmmodDex adults were heavier than controls ($p<0.001$) Figure 5.24 (A)). Hearts derived from the EmmodRU486 group were found to be no different in weight from their controls $p > 0.05$(Figure 5.24 (B)). EmmodGR Mo group were found to be similar in weight to those of the control fish ($p=0.05$) (Figure 5.24 (C)). Normalised ventricular length was no different for EmmodDex or EmmodRU486 treatments compared to their controls ($p>0.05$). A difference was however noted for the EmmodGR Mo adult group, which displayed a smaller normalised ventricular length ($p<0.001$(Figure 5.24(F)).

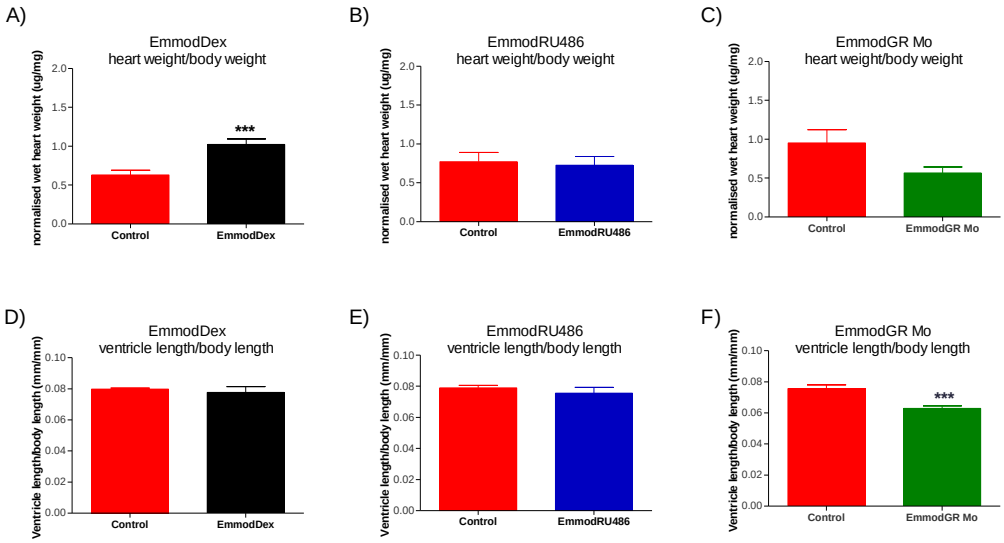

Figure 5.24 Isolated adult heart weight and length
Isolated hearts from adult zebrafish which were embryonically glucocorticoid manipulated with dexamethasone (EmmodDex - black bars), RU486 (EmmodRU486 - blue bars) or targeted glucocorticoid receptor morpholino (EmmodGR Mo - green bars), compared with their corresponding controls (red bars). Data shown A-C) are normalised heart weight/body weight for isolated wet hearts; D-F) are ventricular length normalised to total body length. Data are mean± SEM (n=12 hearts/fish per group) and were analysed by Student's *t*-test; *** $p \leq 0.001$.

5.5.1.2 Heart histology

Detailed histological assessment was carried out in conjunction with a trained Veterinary pathologist. In EmmodDex fish the cardiac anatomy was grossly normal although some of these hearts did display features of cardiac enlargement with mild thickening of the compact myocardium but otherwise normal trabecular of the spongy myocardium (Figure 5.25). Staining with Masson's trichrome, a stain used to differentiate between collagen and smooth muscle cells, failed to show interstitial or sub-endocardial fibrosis which often suggests cardiac hypertrophy (Data not shown). There was no apparent difference in inter-trabecular space between the EmmodDex and control hearts (13.86 ± 0.68 % ventricular area vs controls 12.06 ± 1.04 % ventricular area).

EmmodRU486 also showed normal cardiac architecture including shape and size of bulbous, ventricle and atria; valve morphology; cardiomyocyte size and arrangement; sarcoplasmic integrity (cross striation, fragmentation, vacuolation); endocardial morphology and epicardial morphology (Figure 5.26). As for EmmodDex fish, there were no obvious differences in the interstitial collagen component of these hearts (Data not shown) and there was no obvious difference in the inter-trabecular space (12.42± 1.07 % ventricular area vs 12.24 ±1.07 % ventricular area).

In contrast, at least 70% of the hearts removed from the EmmodGR Mo adults displayed changes in the ventricle affecting both compact and spongy muscle layers. In these hearts spongy trabecular were reduced in number and thickness. An increase in the inter-trabecular space was also apparent (20.32 ± 1.24 % ventricular area vs 11.89 ± 1.22 % ventricular area) reducing the thickness of the compact myocardium along the full circumference in these hearts when compared to the control hearts which displayed a thick compact myocardial layer (Figure 5.27).

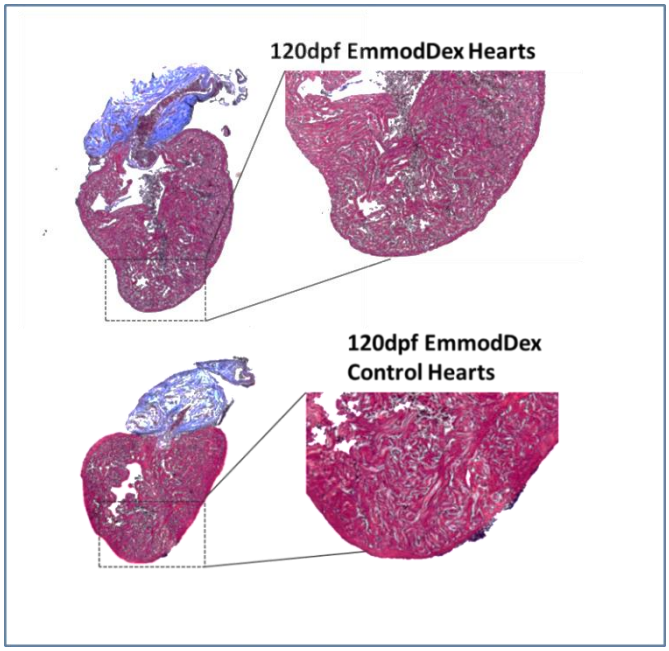

Figure 5.26 Embryonic dexamethasone exposure: adult heart morphology
Heart histology from adult fish at 120 days post fertilisation (dpf) following manipulation with dexamethasone [100µM] during embryogenesis (EmmodDex), and the respective control (EmmodDex Controls). Images shown are low power(4x) and high power (20x) magnification images. The magnified region is highlighted. Images show near identical trabeculae with mild cardiac enlargement in EmmodDex hearts.

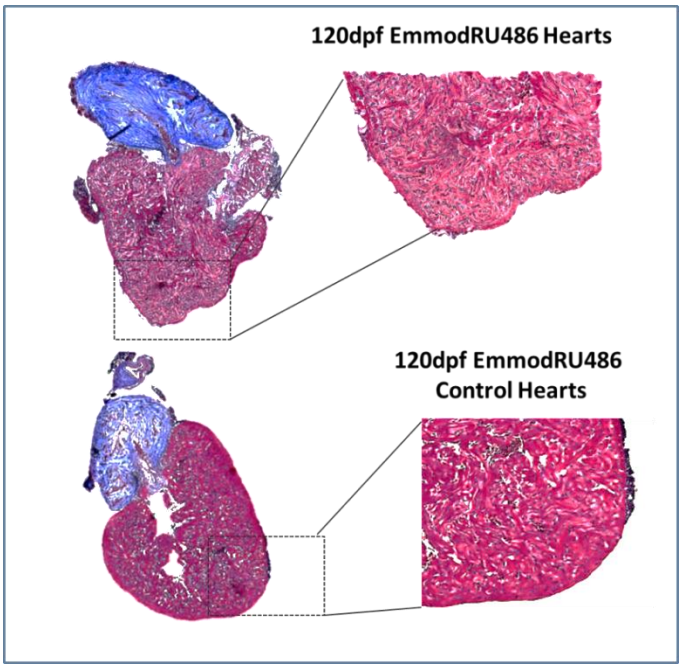

Figure 5.25 Embryonic RU486 exposure: adult heart morphology
Heart histology from adult fish (at 120 days post fertilisation (dpf)) manipulated with RU486 [10µM] during embryogenesis (EmmodRU486), and the respective control (EmmodRU486 Control). Images shown are low power (4x) and high power (20x) magnification images. The magnified region is highlighted. Images are of the near identical cardiac structures of the EmmodRU486 and EmmodRU486 controls.

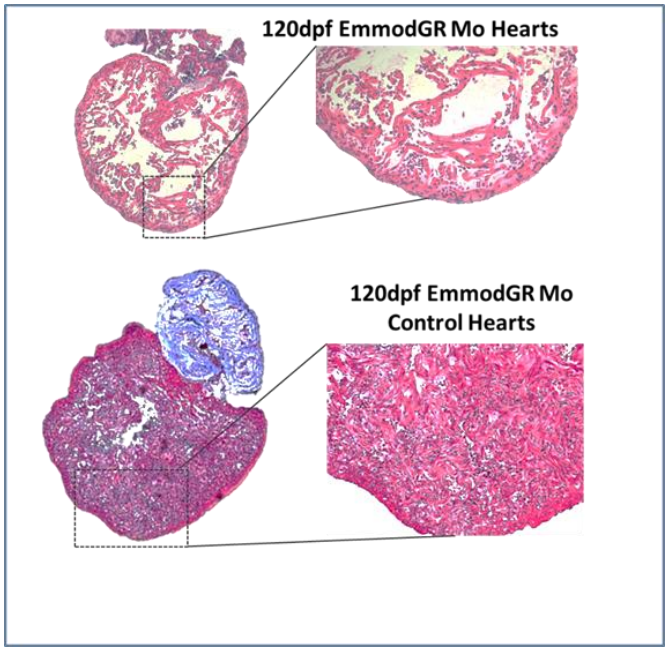

Figure 5.27 Embryonic glucocorticoid receptor knockdown: adult heart morphology
Heart histology from adult fish (at 120 days post fertilisation (dpf)) manipulated with GR Mo during embryogenesis (EmmodGR Mo), and the respective control (EmmodGR Mo Controls). Images shown are low power magnification (4x) and high power (20x) magnification images. The magnified region is highlighted. Images show the substantially reduced compact myocardium along with the increase in the inter-trabecular space noted in 70% of the GR Mo hearts. The control hearts for this treatment did not display such features.

5.5.1.2.1 Adult heart GC gene abundance analysis

In EmmodDex adults *gr* mRNA abundance was higher than in controls ($p<0.05$). In EmmodRU486 adult hearts there was no difference in *gr* mRNA levels when compared to controls. Similarly no difference was detected in EmmodGR Mo (Figure 5.28 (A-C)). *mr* mRNA relative abundance was found to be unaffected in all of the groups investigated (Figure 5.28 (E-G)). *11βhsd2* mRNA abundance was significantly raised in EmmodDex ($p<0.05$). A reduction in levels in *11βhsd2* was observed in EmmodGR Mo isolated hearts ($p<0.04$) but no difference was detected in hearts from EmmodRU486 (1.82 ± 0.26 AU vs controls 1.74 ± 0.16AU).

5.5.1.2.2 Adult heart cardiac gene abundance analysis

Isolated EmmodDex hearts had lower ($p=0.003$) *mef2c* mRNA abundance than controls (Figure 5.29 (A)). In contrast, hearts obtained from EmmodGR Mo adults

displayed raised *mef2c* mRNA levels compared to controls ($p<0.05$). No difference was detected in *mef2c* mRNA relative abundance in EmmodRU486 hearts ($p>0.05$(Figure 5.29(B))). *gata4* mRNA was decreased in EmmodGR Mo adult hearts compared to their controls ($p<0.05$) but was unaffected in both EmmodDex and EmmodRU486 groups. Similarly, EmmodDex and EmmodRU486 groups showed no alteration in *vmhc* relative abundance either. However, in EmmodGR Mo hearts there was a notable decrease in *vmhc* mRNA levels ($p<0.01$). None of the treatments affected abundance of *nkx2.5* in any of the Emmod adult hearts ($p>0.05$).

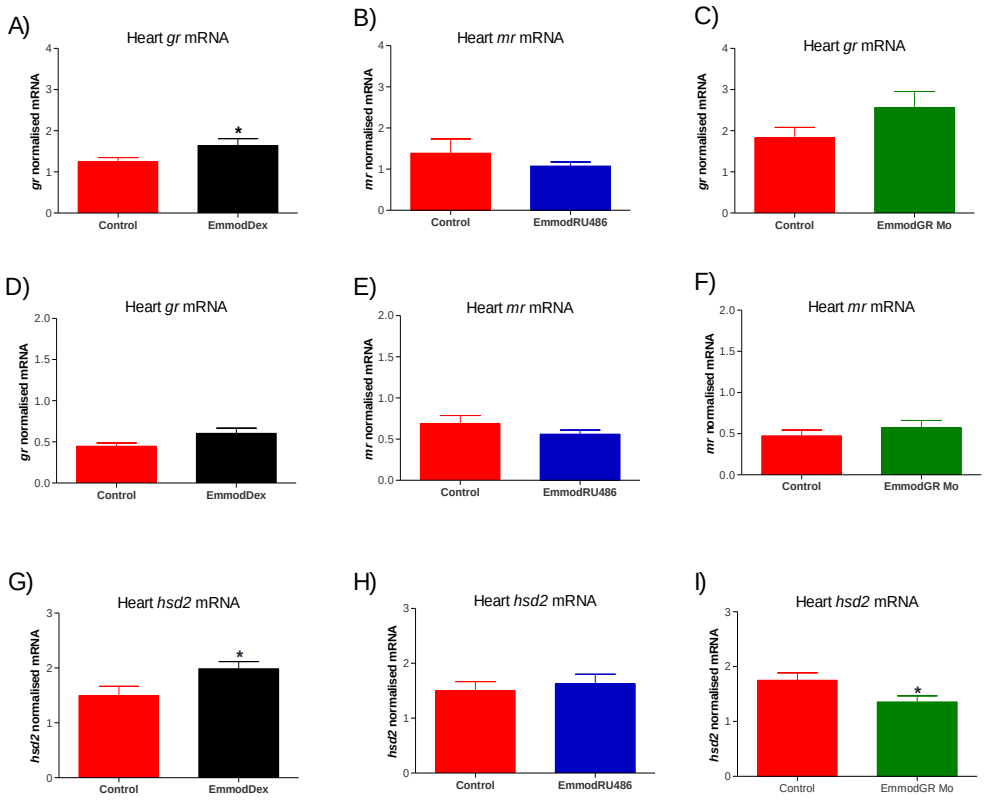

Figure 5.28 Glucocorticoid gene abundance in adult hearts
Glucocorticoid gene mRNA abundance analyses in adult zebrafish hearts isolated 120 days post fertilisation (dpf). Data shown are for hearts constantly incubated in the drugs during embryogenesis and allowed to develop normally until adulthood. Embryonic treatments were with dexamethasone (EmmodDex - black bars) or RU486 (EmmodRU486 - blue bars) or targeted morpholino towards the glucocorticoid receptor (EmmodGR Mo - green bars), versus their respective un-manipulated controls (red bars). mRNA abundance data for A-C) *gr*, D-F) *mr* and G-I) *11βhsd2*. Gene abundance data shown were quantified by normalisation to the house-keeping gene *ef1α*, and expression is presented as AU. Data are mean ± SEM (n=5 (5 hearts per group)) and were analysed by Student's *t*-test; *$p\leq0.05$.

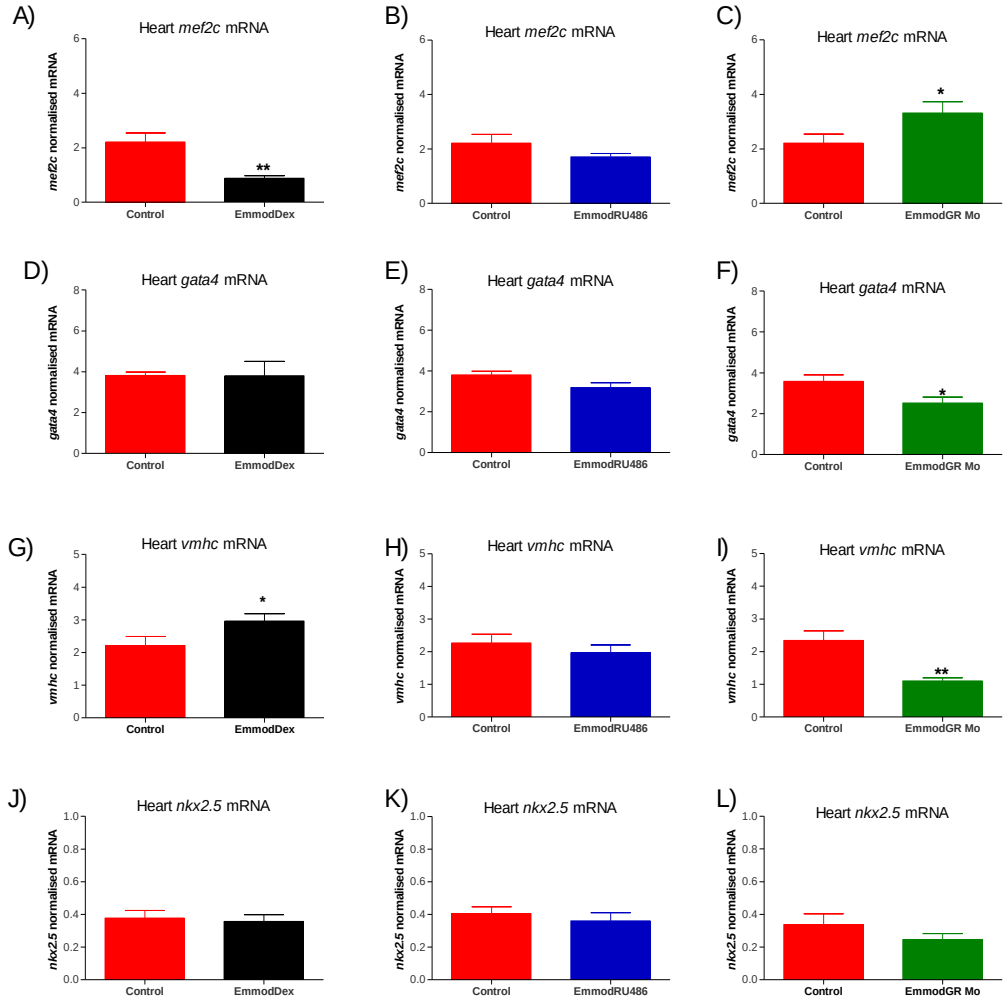

Figure 5.29 Cardiac gene abundance in adult heart
Cardiac gene abundance mRNA analyses in adult zebrafish hearts isolated 120 days post fertilisation (dpf). Data shown are for hearts constantly incubated in the drugs during embryogenesis and allowed to develop normally until adulthood. Embryonic treatments were with dexamethasone (EmmodDex - black bars) or RU486 (EmmodRU486 - blue bars) or treated with a targeted glucocorticoid receptor morpholino (EmmodGR Mo - green bars) versus their respective un-manipulated controls (red bars). Abundance data are for A-C) *mef2c* mRNA abundance, D-F) *gata 4* mRNA abundance, G-I) *vmhc* abundance and J-L*) nkx2.5* mRNA abundance. Gene abundance data shown are quantified by standard curve construction and normalisation to house-keeping gene data and are presented as arbitrary units. Data are mean ± SEM (n=5 (5 hearts per group)) and analysed by Students *t*-test; *p≤0.05, **p≤0.001.

5.5.2 Part 2: Discussion

This section of work has focused on the direct and specific action of GC modulation on the developing embryonic zebrafish heart and the long-term effects this has in the adult.

5.5.2.1 Short-term effects of embryonic GC manipulation on the heart

Corticosteroids such as GCs produce both protective and adverse effects on the heart (Hadoke *et al*, 2009). In addition to their effects on cardiovascular risk factors, the relative abundance of GC receptors in adult cardiac tissue suggests that they can directly influence both physiological and pathophysiological processes in the heart (Greenwood *et al*, 2003). The role of GCs on the developing heart however is less clear. Conditional knockdown of MR in the mouse produces severe heart failure and cardiac fibrosis, which can be fully reversed with the addition of MR antisense mRNA (Beggah *et al*, 2002). Furthermore in mice conditional overexpression of MR triggers cardiac arrhythmias with a high death rate. Again this phenotype can be mitigated using MR antagonist (Ouvrard-Pascaud *et al*, 2005).

In MC target tissues, MR specificity for aldosterone is conferred by the activity of the enzyme 11βHSD2 which, by inactivating GCs (converting cortisol and corticosterone to cortisone and 11-dehydrocorticosterone, respectively), protects MR from inappropriate activation by GCs (Chapman *et al*, 2013). Human and rodent studies have highlighted that MR expression is largely restricted to MC target tissues, but it is also expressed in the heart without 11β-HSD2 co-localisation. Thus, in the heart, MR can bind endogenous cortisol/corticosterone and some actions of MR antagonist drugs may be derived from their ability to prevent action of GCs at cardiac MR. Overexpression of GR induces atrio-ventricular (AV) block (Sainte-Marie *et al*, 2007). Global GR knockout ($GR^{-/-}$) mice die shortly after birth with multiple organ defects and widespread somatic oedema. Notably the heart is smaller with abnormal cardiomyocyte architecture and impaired contractile function (Rog-Zielinska *et al*, 2012). Taken together these data confirm a key role for GCs acting directly on the heart via GR, and possibly MR, in rodents. Similar published data from lower vertebrates, including the zebrafish, is sparse.

The heart is one of the earliest functioning organs in the zebrafish and data generated in this investigation indicate that supressed GC action via inhibition of GR activity, either by Mo knockdown of GR or pharmacological antagonism at GR using RU486, results in early impairment of cardiac function (reduced heart rate, ejection fraction and cardiac output). This is consistent with findings in $GR^{-/-}$ mouse embryos which display severely impaired Doppler-derived indices of myocardial performance by ultrasound assessment (Rog-Zielinska *et al*, 2012). In addition to impaired function of the zebrafish embryonic heart, the ventricle (corrected for total body size) is also smaller. As shown (Chapter 4), these embryos have impaired growth rate and reduced head-trunk angle in early stages of development, suggesting an initial developmental delay. However by 120hpf, there is no apparent difference in development rate, suggesting that GC have a less critical role in later stages of development, and that developmental catch-up is occurring. Interestingly, although there is a somatic catch-up, cardiac development remains impaired, as indicated by small ventricular size, in embryos where GR activity was reduced either pharmacologically or with Mo. It is well documented that GCs have a profound effect on cell proliferation and differentiation and a smaller ventricle indicates that GCs play a key developmental role in the heart. Indeed in the mouse GR knockouts, hearts are smaller. In addition to smaller sized ventricles, these hearts also displayed an increased inflow/outflow distance at 72 hpf suggesting a delay in cardiac looping, consistent with delayed cardiac development.

Embryos exposed to GR supressing treatments also displayed reduced expression of key cardiac maturational genes (including *igf* and *vmhc*) which also indicates that the hearts are developmentally immature and may at least partly explain some of observed functional abnormalities. Reduced cardiac function, in these embryos may also be the result of a loss of the direct ionotropic effects of GCs which has been described in rodent models (Rao & Narayanan, 2000; Rossier *et al*, 2008; Sainte-Marie *et al*, 2007) although until now such observations have not been detected in the zebrafish. The findings reported here provide the first evidence for a direct effect of GCs on contractile function in the developing zebrafish heart.

There is some evidence that corticosteroid treatment of preterm infants is associated with post-natal adverse cardiovascular effects (Katz *et al*, 1988; Kelly *et al*, 2012) although some studies have suggested that this is not the case (Mildenhall *et al*, 2009). When the rat foetus is exposed to a high cortisol preterm, increases in ventricular cardiomyocyte size are observed (Bal *et al*, 2009). In the work presented here, zebrafish embryos treated with Dex showed contrasting effects to those treated with GR Mo at 120hpf, with an increase in ventricular length and an increased cardiac output associated with normal heart rate. These hearts also displayed greater levels of *vmhc* mRNA, suggesting greater maturity and which could at least partly explain the observed increase in ejection fraction /stroke volume.

When embryonic hearts treated with Dex were isolated at 120 hpf it was found that they contained the same total cardiomyocyte numbers as control hearts. However, after normalising for the larger ventricle these hearts appear to have fewer cardiomyocytes than controls. While this may need confirmation using volumetric analysis it suggests that the cardiomyocytes of the Dex treated embryos were larger than controls, pointing towards possible cardiac hypertrophy. A number of rodent and cell culture studies have demonstrated GC-induced cardiac hypertrophy (Ohtani *et al*, 2009), similarly early postnatal treatment with Dex in rats has been linked to a hypertrophic heart and increased MHC mRNA abundance (Muangmingsuk *et al*, 2000) supporting the observations documented in the work presented here. Microscopic examination of the isolated embryonic Dex hearts showed them to be structurally more mature than those treated with GR Mo showing a well-defined muscle striation pattern. These hearts also displayed a normal cardiac morphology, with a normal folding pattern and normal trabeculation of the ventricle. Histologically there was no significant alteration in cellular morphology of these hearts in although hearts were significantly larger than controls. Further work is needed to establish whether these hearts had altered gene abundance consistent with cardiac hypertrophy, however, decreased cardiomyocyte number, increased *vmhc* mRNA abundance and increased heart size following Dex treatment would support a direct and dynamic role for GCs and GR signaling in the developing zebrafish heart. Further supporting this is the striking structural and functional abnormalities

resulting from a 39% reduction in cardiac GR mRNA during early development (GR Mo).

Two further groups of GC manipulation which were investigated here were those treated with Met and Cyp Mo. As previously described, these treatments caused a significant reduction in cortisol levels in the embryo (chapter 3). This reduction in cortisol, while detrimental in stress-induced HPI axis activity appears to have had little or no effect on heart formation in the developing zebrafish embryo. This suggests that the role of GCs in early zebrafish cardiac development is largely attributable to maternally-derived cortisol rather than *de novo* synthesis within the embryo. While no morphological alterations were observed in the developing heart in these groups, an increase in pericardial edema was observed, which could be indicative of cardiac dysfunction. This does not appear to be the case here, as ventricular ejection fraction in these embryos was similar to controls. An alternative explanation for the pericardial edema is activation of MR via 11-deoxycortisol (a proposed Cyp11b1 substrate) ligand activity (Strum *et al*, 2005) resulting in excess sodium and water retention (Flynn *et al*, 1994).

5.5.2.2 Long-term effects of embryonic GC manipulation on the heart

Before the long-term cardiac effects of GC manipulation are discussed, it should be emphasised that all embryos, larvae and adults which were used in the longitudinal studies had stringent phenotypic/morphological assessments and were monitored for health and welfare throughout. The long-term survival of all embryos into adulthood was similar amongst all treatment groups with no increase in mortality observed following any of the embryonic manipulations.

When embryonic fish that had undergone 5 days of GC manipulation were then allowed to mature in a normal environment, adult hearts displayed a number of structural, molecular and histological abnormalities. These long-term effects resulting from embryonic GC manipulation are consistent with the phenomenon of GC-induced early life programming (Barker, 1995). The most convincing evidence of a programming-like long-term alteration in the heart was in EmmodDex adults in which activity of GR was up-regulated in the embryo for the first 120h of

development (enhanced GR), and in EmmodGR Mo in which GR activity was down-regulated for at least 120h (reduced GR).

It has been fairly well established in humans that prolonged GC exposure in the adult can result in cardiac remodelling and cardiac dysfunction (Scheuer & Mifflin, 1998). Cardiac remodelling is characterised by myocyte hypertrophy and apoptosis, accompanied by hyperplasia and hypertrophy of non-muscular cells, with alterations to the extracellular matrix, inflammation and fibroblast function (Swynghedauw, 1999). The teleost heart has a high level of plasticity, both anatomically and physiologically, in response to environmental changes, and ventricular growth can occur by hypertrophic (growth of cardiomyocytes) or hyperplasic (proliferation of cardiomyocytes) processes (Gamperl and Farrell, 2004). The association between stress, environment and cardiac remodelling has recently been investigated in salmonoid fish, with cardiac enlargement observed in high stress groups (Johansen *et al*, 2011): a feature seen here in the hearts of the enhanced GR adults. These hearts were larger and heavier than controls, in accordance with embryonic data (ventricles were larger than controls), and suggestive of cardiac hypertrophy. The classical histological markers of hypertrophy such as sarcomere reorganisation, increased vascularisation (to satisfy increased metabolic demand of hypertrophic cells) and increased fibrosis were absent from the EmmodDex zebrafish hearts.

Cardiac hypertrophy in adult mammals is typically associated with alterations in genetic and molecular signals in the cardiomyocytes. This results in a switch to a pattern of gene expression (or re-expression) normally observed during early cardiac development. Alterations in the pattern of gene abundance associated with cardiac hypertrophy is less well defined in teleost fish with only a limited number of genes affected. This is mainly due to the lack of fish models which mimic mammalian models such as aortic banding or models of hypertension for example. However, some of the changes seen in mammals have been reported in fish (Chen *et al*, 2013). One such gene is *vmhc* (homologous to mammalian β-MHC). In the current investigation a similar increase in *vmhc* abundance was not observed in the EmmodDex group. Furthermore, a reduction in *mef2* levels was observed. This is interesting as, Mef2c and GR act cooperatively in controlling gene transcription, with

GR influencing Mef2 activity (Speksnijder *et al*, 2012). The Mef 2 transcription factor family is important in early cardiac development and hypertrophy, and contributes to regulation of cardiac energy metabolism (such as cardiac fatty acid oxidation regulation and maintenance of mitochondrial function (Czubryt & Olson, 2004)). This raises the question as to why *mef2* abundance is low in this setting. It may be that manipulation of GC results in perturbations in various regulatory mechanisms in the heart via modification of Mef2c.

Further investigation of additional molecular markers of hypertrophy, such as slow myosin light chain (*smlc2*) and muscle LIM protein (*mlp*) is clearly required. For example Mef2 synergistically regulates the transcription of several cardiac genes along with transcription factor Gata4.

Numerous rodent studies have highlighted the importance of GATA4 in cardiac morphogenesis, with *Gata4* -/- mice displaying embryonic lethality and a poorly formed heart tube (Kuo *et al*, 1997). The role of GATA4 in cardiac morphogenesis isn't surprising given its role as a transcription factor regulating a number of cardiac specific genes (such as αMHC and atrial natriuretic peptide (anp)) in rodents. Furthermore, GATA4 is important in instances of hypertrophy and subsequent inducible expression of hypertrophic genes, with overexpression of GATA4 resulting in hypertrophy of cultured cardiomyocytes (Liang *et al*, 2001a; Liang *et al*, 2001b). The transcriptional activity of GATA4 is thought to be regulated by interaction with a number of cofactors, including nkx2.5. GATA4 is also activated through direct serine phosphorylation by the p38 MAPK pathway may be mechanism by which GCs can influence the vasculature. While EmmodDex adults did not show an up-regulation of *gata4* mRNA relative abundance as may have been expected, EmmodGR Mo hearts display a reduction in *gata4* relative abundance associated with quite marked changes in in cardiac structure. These data suggest that GC modulation may alter cardiac and vascular morphogenesis via common pathways. While this is not particularly ground-breaking considering numerous compounds produce signal transduction through this GATA4 signalling pathway, it does offer a means of explaining the multiple actions of GCs on target tissues.

As the classical cellular and molecular features of cardiac hypertrophy were not observed in the EmmodDex hearts, future work should focus on exploring further the underlying molecular and cellular characteristics causing cardiac enlargement in this model. An increase in size could be as a result of cardiomyocyte hyperplasia. Since fish hearts have been shown to regenerate using this process, cell proliferation may contribute to cardiac alterations observed. However, no apparent increase in the number of nuclei was observed in the histological sections of the adult hearts. Further investigation of cellular proliferation could be assessed using carried out using proliferating cell nuclear antigen (PCNA). While histological examination did not identify structural abnormalities, some EmmodDex hearts displayed thickening of the compact myocardium. It is unlikely that this has much influence on overall cardiac size but further investigation of cardiac cellular composition could be completed to determine whether there is any alteration in the expression of cardiac fibroblasts, myocytes, endothelial cells, or vascular smooth muscle cells.

Not only did the EmmodGR Mo alter abundance of some key cardiac genes (reduction of Gata4), there was clear evidence of altered cardiac morphology. The heart of salmonoid fish is composed of an inner spongy myocardium, which is supplied with oxygen from venous blood returning to the heart, and an outer more compacted myocardium (Pieperhoff *et al*., 2009). The ventricular wall in the adult zebrafish ordinarily has a thin compact layer with extensive elongated and thickened trabeculae. In EmmodGR Mo adult hearts there were long-lasting effects of GC manipulation on the gross anatomical structure of the heart muscle, with a reduction in spongy trabeculated myocardium muscle accompanied by an increase in inter-trabecular space and an apparent reduction in compact myocardium. Normally the long thin appenditures of the trabeculae form a mesh like structure with intra-trabecular spaces. Cardiac trabeculae are thought to enhance cardiac contractility and intra-ventricular conduction. The ventricular trabeculae of the adult zebrafish heart resemble those of the chick, where the arrangement of the trabeculae, ventricular shape and size are critical for ventricular contraction (Pieperhoff *et al*., 2009). The organization of the adult zebrafish myocardium is, in part, determined by the haemodynamic forces which occur during ventricular wall development. As embryos the EmmodGR Mo group displayed reduced ejection fraction and decreased heart

rate so this may explain why these hearts appear immature and not formed as normal. While it is unclear whether the hearts of these adult fish are functionally alike the controls or not it has been shown previously that the trabecular folds are vital for ventricular stability during contraction, suggesting that the EmmodGR Mo hearts may have impaired function.

While it is clear that future work needs to be carried out on the adult heart of GC modulated embryos to understand the underlying cellular and molecular mechanisms involved, it is apparent that altered embryonic cardiac development can result in long-term alterations in the structure and genetic composition of the heart, i.e. programming. While it is unclear as to the functional impact of these abnormal hearts in this model, normal mortality rate, gross phenotype and normal motility would suggest that cardiac performance is not altered significantly enough to impair survival under normal conditions. However, these alterations may impair the ability of the cardiovascular system of these individuals to react to altered environmental, behavioural or mechanical stress.

5.6 Part 3:

Is the embryonic phenotype caused GR Mo be rescued by capped gr mRNA

While the data presented in this chapter suggest that manipulation of GC activity through the GR can impact on cardiovascular development, phenotypic rescue would provide further support that the observations are the direct result of manipulation of Gr actions and not a secondary or non-specific effect. While pharmacological rescue is possible and has been investigated previously, rescue of the GR Mo phenotype was performed to avoid possible drug toxicity.

5.6.1 Part 3: Results

5.6.1.1 Aim 5

5.6.1.1.1 Glucocorticoid receptor mRNA and protein abundance: GR Mo rescue

To assess whether cardiovascular phenotypes resulting from GC manipulation were associated with altered *gr* mRNA abundance, *gr* mRNA abundance was confirmed in both the zebrafish embryonic and adult heart (Figure 5.30 A). Furthermore, Gr protein was also found both in embryonic and adult zebrafish hearts, using a custom-

designed zebrafish Gr antibody (a kind gift from Professor M. Vijayan, University of Waterloo, Ontario, Canada) (Figure 5.30 B). Indeed, immunohistochemistry suggests that Gr is located in the DAPI: GFP positive mono-nucleated cardiomyocytes (Figure 5.30.D). Further optimisation of this technique and enhanced imaging would be required, however, to quantify the abundance and determine whether localisation is cytoplasmic or nuclear.

As previously shown (Chapter 4 and 5 previously), *gr* mRNA and protein signals were reduced in whole embryos following GR Mo treatment, with a clear reduction in both *gr* mRNA and Gr protein 72hpf. Embryos treated with GR Mo (splice) had a 41.5 ± 6.3 % reduction in GR mRNA compared to age matched controls (representing 100%), p=0.004 (Figure. 5.30 E) and a 28.7 ± 5.4 % reduction in GR protein compared to controls, (p=0.006, Figure. 5.30 F).

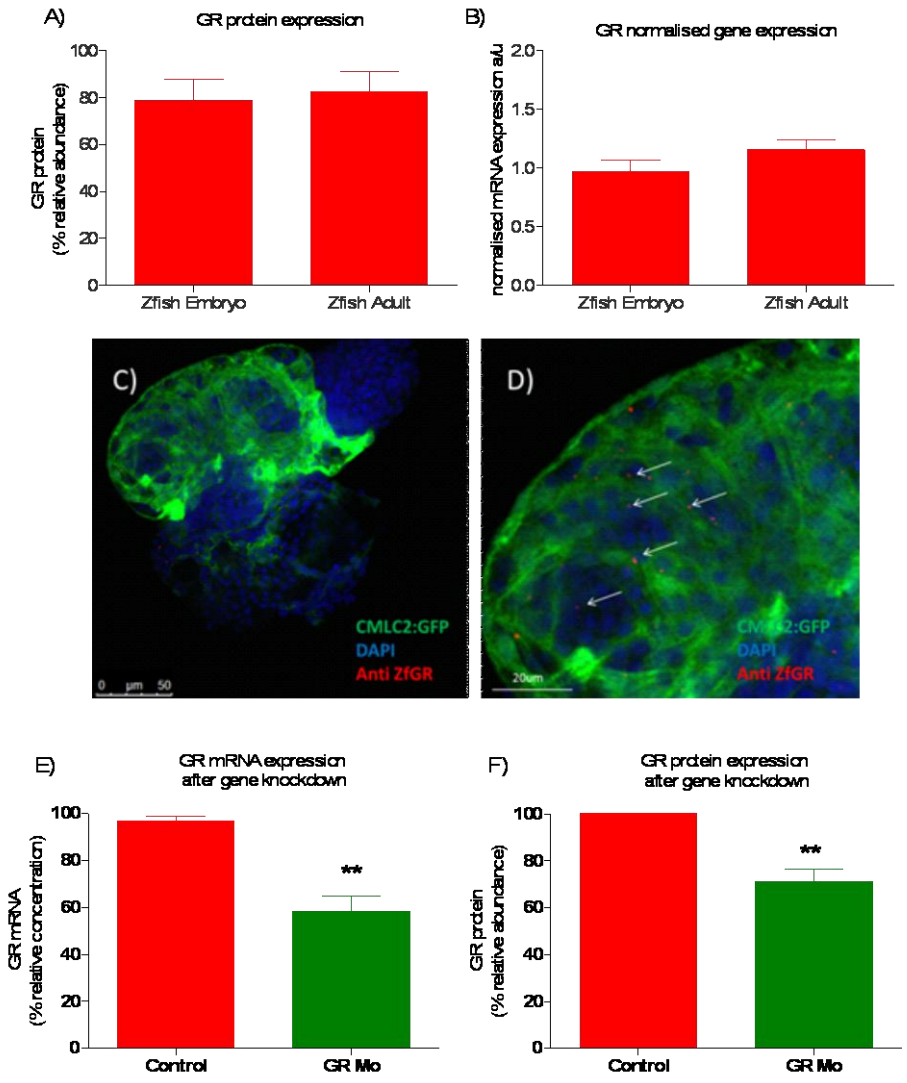

Figure 5.30 Confirmation of glucocorticoid receptor presence in zebrafish heart
A) *gr* mRNA abundance in isolated embryonic and adult zebrafish heart tissue as determined by qRT-PCR normalised to house-keeping gene *βactin*. B) Gr protein abundance in isolated embryonic and adult hearts as determined by Western blot analysis and densitometry with α-tubulin house-keeping protein. C) Representative immunohistochemistry of isolated embryonic zebrafish heart expressing GFP under the *cmlc2* promoter (green) stained with an anti-Gr antibody (Alexa 555 conjugated secondary-red) and co-stained with the nuclear stain DAPI (blue). D) Higher magnification image to allow observation of weakly-expressed anti-GR antibody. Arrows indicate possible abundance and cytoplasmic localisation within the mononucleated cardiomyocytes. Image capture was with Leica SP5 confocal microscope. E) qRT-PCR. GR Mo gene knockdown as a percentage of control *gr* mRNA abundance at 72 hours post fertilisation (hpf). N=3 (10 embryos per group). F) Western blot densitometry data of GR Mo protein relative knockdown as a percentage of control Gr protein abundance at 72hpf. N=3 (5 embryos per group). Data are mean ± SEM, statistical analysis by Student's *t*-test; ** P<0.001.

5.6.1.1.2 Rescue experiment for GR Morpholino on cardiac phenotype

To determine the specificity of the GR Mo, an mRNA rescue experiment was carried out as described (Section 5.3.10). Capped mRNA encoding GR was co-injected into embryos treated with the GR Mo. To determine whether successful rescue was achieved a number of parameters were investigated (Figure 5.31) based on features which could be assessed easily in the embryo and were observed to be altered by GR Mo; for example, ventricle length, cardiomyocyte number, head-trunk angle and blood vessel patterning.

While ventricle length was reduced in GR Mo (<0.001) compared to controls, an increase was detected after rescue ($p<0.05$ vs GR Mo). However, the rescue group still had smaller hearts than controls ($p<0.05$) (Figure. 5.31 (A) and Figure. 5.32).

Cardiomyocyte number remained lower in rescued, compared to control untreated, hearts (1.76 ± 0.09 cardiomyocytes/µm vs 2.18 ±0.13 cardiomyocytes/µm, respectively; $p<0.05$), although cardiomyocyte number was higher in rescued hearts than in those from embryos treated with GR Mo alone (($p<0.05$) (Figure 5.31 (B)).

It previously was shown (chapter 4) that GR Mo treatment resulted in developmental delay which manifested as impaired head-trunk angle. Co-injection of rescue significantly attenuated the effect of GR Mo on head-trunk angle at all time-points investigated (24hpf, rescue 58.87 ± 1.22 ^{O} vs GR Mo 46.30 ± 3.87 ^{O}, $p<0.05$; 48 hpf rescue 116.43 ± 2.86 ^{O} vs GR Mo 104.15 ± 2.47 ^{O}, $p<0.001$; 72 hpf rescue 140.35 ± 1.02 ^{O} vs GR Mo 127.09 ± 3.57 ^{O}, $p< 0.001$). No difference was observed in head trunk angle for rescue embryos vs controls apart from at 48 hpf ($p<0.05$) (Figure 5.31(C)).

Blood vessel patterning was also assessed in the rescue embryos. As reported previously (Section 5.4.1.1), a reduction in ISV number was observed in GR Mo ($P<0.001$), while rescued embryos had fewer ISV than mismatch controls. The mean number of ISV in rescued embryos was greater than in GR Mo-treated embryos ($p<0.01$) indicating a partial rescue of the blood vessel phenotype (Figure 5.31(D)).

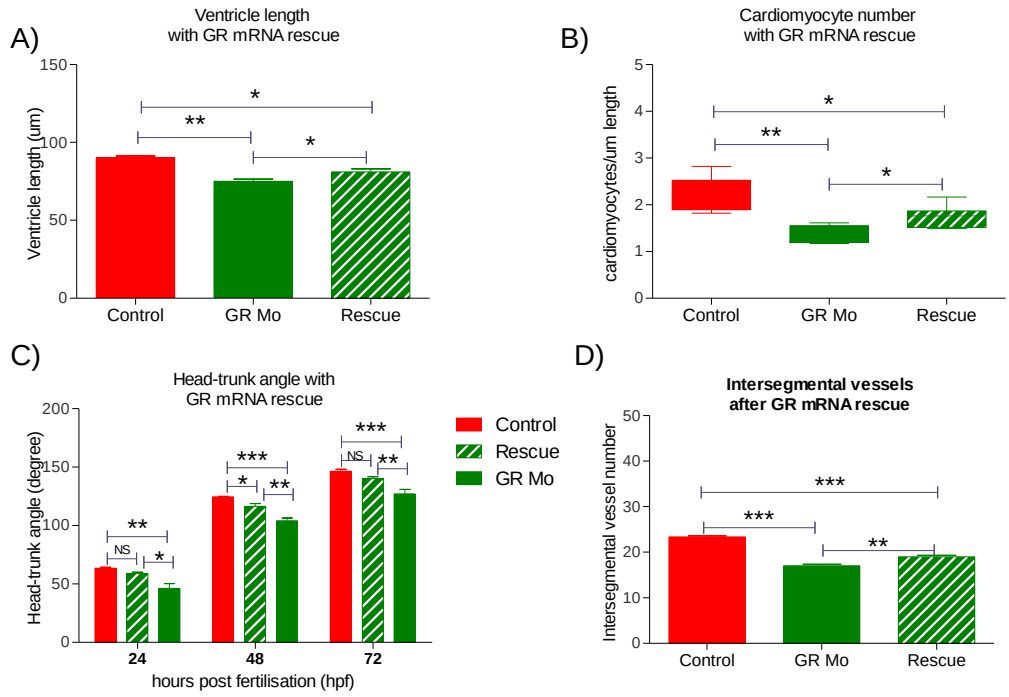

Figure 5.31 Glucocorticoid receptor morpholino phenotype rescue analysis
Features known to be altered in zebrafish embryos by *gr* morpholino (GR Mo) treatment were assessed in embryos following co-injection of rescue *gr* mRNA with GR Mo. Data shown are for embryos treated with GR Mo (green bars), GR Mo co-injected with *gr* rescue (dashed green bars), and controls (mm-Mo – red bars). Features investigated were A) ventricle length at 120hpf, B) cardiomyocyte number at 120 hours post fertilisation (hpf), C) head-trunk angle 24-72 hpf and, D) intersegmental vessel number at 120hpf. Data are mean ± SEM (n=3 (6 embryos per group)). A, B and D) were analysed by 1-way ANOVA and Dunnett's post hoc test; C) was analysed by 2-way ANOVA and Bonferroni post hoc test; *p≤0.05, **p≤0.01 and ***p≤0.001.

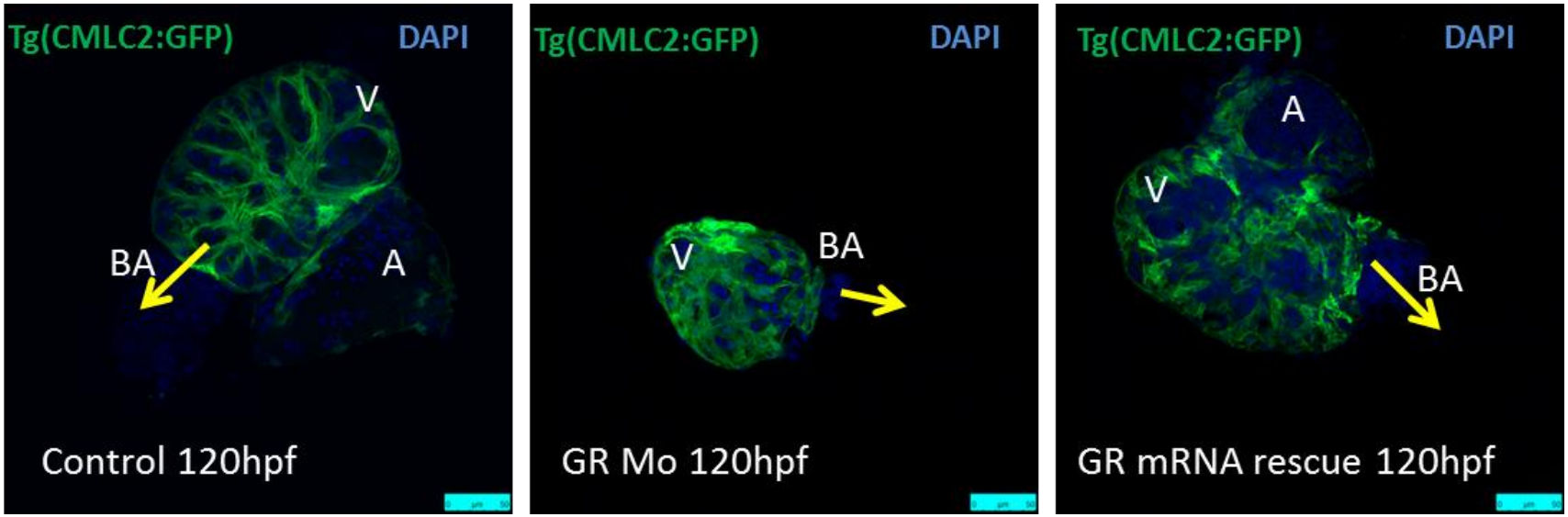

Figure 5.32 Isolated embryonic hearts from zebrafish embryos
Representative images of hearts excised from embryos 120 hours post fertilisation (hpf). Examples shown are of a control (untreated) heart, one which was treated with targeted glucocorticoid receptor morpholino (GR Mo) at the 2-cell stage, and one which was co-injected with capped *gr* mRNA with GR Mo. Features annotated on images include ventricle (V), atrium (A - where applicable) and bulbus arteriosus (BA). Yellow arrowhead depicts blood outflow from ventricle through bulbus arteriosus.

5.6.2 Part 3: Discussion

The mRNA rescue experiment of the GR Mo demonstrated a partial rescue of the structural phenotype in the heart. Specific notable features, compared with GR Mo embryos, included increased heart ventricle length, increased ventricular cardiomyocyte number and accelerated development (as shown by head-trunk angle). These findings provide support for a direct role for GR on the cardiovascular phenotype observed with GR Mo. While the effects of GR Mo may be partly related to developmental delay a direct impact on maturation and development of the cardiovascular system is also likely.

Evidence of altered cardiomyocyte architecture, was also observed in GR Mo which was partly rescued by *gr* mRNA. While these findings may be attributable to altered cardiac gene abundance, they may also partly explain the finding of impaired cardiac function. In addition to being structurally immature the ventricles of GR-suppressed hearts also contained fewer cardiomyocytes than controls. Again, this lends support for a key role played by GC in cardiomyocyte proliferation. Indeed, cortisol triggers isolated cardiomyocytes to enter the cell cycle (Giraud *et al*, 2006). Further examination of the cellular microscopic structure and gene/protein abundance may allow a more complete understanding of the processes underlying the reduced cardiac size and function resulting from GR suppression and its rescue by capped GR mRNA.

5.7 Conclusion

GCs have both direct and indirect effects on the adult mammalian cardiovascular system as a consequence of the abundant expression of GR and MR in the vasculature and in the heart (Walker, 2007). Direct effects of GC include alterations to vascular function and cardiac and vascular remodelling (Hadoke *et al*, 2009). It is also fairly well established that foetal exposure to excess GC can alter embryonic development and increase the risk of developing cardiovascular disorders in later adult life (Seckl & Meaney, 2004). In this chapter, the effects of up- and down-regulation of GC activity on the embryonic cardiovascular system were assessed during embryogenesis and the effects of this modulation on the longer term structure and molecular signalling in the adult cardiovascular system were investigated. The

zebrafish has provided a unique opportunity to achieve the main aims of this chapter within a relatively short period of time, highlighting its utility for this type of experiment. The use of organ-specific GFP-expressing transgenic fish and the ability to readily manipulate GC activity in parallel experiments and then allow the embryos to develop into adults represents a unique and highly efficient research approach.

The main findings of this chapter are: 1) GC signalling through the GR is important for embryonic angiogenesis, with enhanced embryonic GR activity appearing to have long-term effects on regenerative angiogenesis in the adult caudal fin. 2) Enhanced embryonic GC activity through GR increases cardiac maturational events during embryogenesis; this enhanced early cardiac growth manifests in adulthood as a larger heart. 3) Genetic suppression of the embryonic zebrafish GR results in impaired cardiac maturation with resulting structural and functional abnormalities; leading on to similar abnormalities in adulthood.

While these findings alone are interesting it is unclear what the wider implications may be. When the activity of GR is enhanced (exposure to excess GC in the form of Dex) in the embryo a reduced number of ISV was observed, however this did not prevent or impede the normal growth and development of these embryos by 120 hpf or into adulthood (120 dpf). In the adults there was evidence of reduced angiogenic response to injury in the caudal fin. Furthermore a reduction in key molecular contributors to pro-angiogenic signalling was also observed. These data could represent a diminished capacity to repair injured tissue and could be the first evidence of altered regenerative capacity in the zebrafish resulting from an early-life programming intervention with GC. This finding, along with published data on GC inhibition of angiogenesis (Khorram *et al*, 2013) suggest that documented programmed vascular dysfunction in adulthood (such as hypertension) may be as a result of reduced angiogenic capacity. Enhanced GR activity in the embryo also appeared to accelerate heart maturation resulting in enhanced cardiac performance at 120hpf. Strikingly, in the adults this resulted in larger hearts with altered molecular signalling (e.g. increased *vmhc* mRNA abundance) this clearly highlighted a programmed effect of the early life GC exposure. While no functional data was observed in the adult hearts, survival data would suggest that normal functional

activity was maintained. However it is unclear whether this would affect long-term health. It may be that the plasticity of the heart which normally allows the heart to adapt in instances of increased workload may be finite and thus the observed rapid cardiac maturation following enhanced GR activity during embryogenesis may impair cardiac plasticity in later life.

Reduced GR activity namely in the GR Mo group reduced cardiovascular development in the first 120 hpf by which point normal somatic development was observed clearly highlighting a developmental role of GC in the zebrafish cardiovascular system. Notably in the Gr Mo embryos hearts were smaller, structurally immature and subsequently had impaired cardiac function. By adulthood, long after the intervention was removed cardiac abnormalities persisted, as evident by the smaller hearts with increased intra trabecular space. Furthermore, molecular signalling in these hearts is also altered, with reduced *vmhc* abundance. While it is unclear as to the functional consequence of this in the adult it is likely that normal contractile action will be impaired.

Chapter 6: General discussion

6 General Discussion

6.1 Introduction

Previous research has highlighted a clear association between environmental alterations and disease progression in later adult life, with cardiovascular disease and alteration in HPA axis function often displayed. This association, termed programming, is thought to be as a result of adaptive changes at an organ level occurring during critical stages of embryonic development. While the exact mechanism through which these adaptive changes result in adult onset disease is unclear, it is likely that alterations in tissue cellular and molecular composition may increase disease susceptibility. This disease risk can be further exacerbated by confounding features such as food abundance, environmental alterations or additional disease risk factors.

Current models of programming do not allow the level of investigation required to determine the direct and subtle effects of programming on the system of interest. The zebrafish has many desirable features which make it suitable for this form of study. The work presented in this thesis investigates the impact of GC manipulation on zebrafish development.

6.1.1 PhD hypothesis

The overlying hypothesis which was investigated in this thesis was that modulation of embryonic GC system will result in direct effects on the structure and function of the developing cardiovascular system.

6.1.2 PhD aims

- To characterise the GC system in the embryonic and adult zebrafish to confirm the suitability of this species as a model of GC modulation.
- To investigate programming in the zebrafish by assessing classical mammalian programmable features 1) development, 2) stress response, and 3) behaviour in the embryo (up to 120 hpf) and the adult (up to 120 days post fertilisation (dpf)).

- To investigate the long-term impact of early-life GC manipulations on cardiovascular developmental.

6.2 Relevance of findings and future direction

6.2.1 Physiological roles of glucocorticoids in embryonic development

A number of studies have described the HPI axis in adult salmonoid fish, including the adult zebrafish, and have suggested that this system may be present in the embryo (Alderman *et al*, 2012; Alsop & Vijayan, 2008; Alsop & Vijayan, 2009; Mommsen *et al*, 1999; Nesan *et al*, 2012; Nesan & Vijayan, 2013). Few, however, have characterised whether the system is physiologically active in the zebrafish embryo (Tokarz *et al*, 2013a).

This work clearly demonstrates a functional HPI axis in zebrafish embryos as indicated by the detection of key functional components of the system and receptors for cortisol from early in development (8hpf-fertilised oocyte). These findings are consistent with a number of factors: **1)** GCs have well-documented developmental roles in the mammalian foetus (Brown & Seckl, 2005), with a correlation between increased basal circulating GC levels and maturational events in numerous organ systems (e.g. lung maturation, although the underlying cellular and molecular mechanisms are complex (Harris & Seckl, 2011); **2)** the teleosteon HPI axis shares a high degree of homogeneity with the mammalian HPA axis in terms of composition, structural organisation and function (Steenbergen *et al*, 2011), and **3)** the widespread expression pattern of GR and its ligand cortisol during very early zebrafish embryogenesis (Alderman *et al*, 2012; Nesan & Vijayan, 2013) would strongly support a developmental role.

The data reported in this thesis support a key role for GCs in embryogenesis and development. Increased GR activation, resulting from Dex incubation, increased the rate of development (growth, hatching and swim bladder inflation) over the first 72hpf. Conversely, diminished GR activity, using pharmacological (RU486) and genetic (GR Mo) manipulation, impaired these important developmental features. Two recent publications have also identified a role for GCs in zebrafish development (Nesan *et al*, 2012; Pikulkaew *et al*, 2011). These studies defined a role for GR in the formation of mesoderm structures, as GR Mo knockdown caused tail kinking and

malformed somites. While this supports a role for GC, it should be acknowledged that a relatively high concentration of Mo was used (10.3-20.6ng/embryo) and some of the features described can occur as off-target non-specific effects of Mo treatment. However, work by Pikulkaew (Pikulkaew *et al*, 2011) describes an mRNA rescue experiment similar to that described here, indicating that the effects seen are indeed due to a direct effect of greater GR knock-down.

Capped GR mRNA rescue (Chapter 5) partially rescued the developmental delay by normalising the head trunk angle. A complete rescue was not observed, however, as higher concentrations of the capped mRNA alone caused phenotype abnormalities (pericardial oedema and craniofacial abnormalities). A possible explanation for this is over-expression of GR since exogenous RNA is not metabolised in the usual way and this over-expression could result in structural abnormalities (Summerton, 1999). The gold standard of mRNA rescue experiments, which was not carried out here but could be addressed on future work, would be the introduction of an mRNA from another species which has sufficient 5'-UTR sequence divergence but which can complement the missing protein's activity- thus rescuing the phenotype while minimising Mo RNA interactions (Summerton, 1999).

The work presented here also demonstrates that maternally-derived cortisol within the embryo can be influenced, with the offspring of mothers injected with Dex displaying raised cortisol levels in the initial phase of development. This is the first (to date) documented alteration of maternally-derived cortisol in zebrafish. Consistent with a role of GC in hatching, a larger proportion of these offspring hatched earlier than controls (Data not shown). The significance of these findings would require further investigation but it may represent a form of natural selection, where individual embryos within this population have reproductive modifications or alterations conferring greater survival advantage in their environment.

6.2.2 Embryonic glucocorticoid manipulation and adult development

It is now widely recognised that small-for-gestational age, IUGR or pre-term infants have a greater risk of later onset cardio-metabolic disease (Boubred *et al*, 2013; Hales & Ozanne, 2003) and some neurological disorders (Phillips *et al*, 2000; Rugolo, 2005). There is emerging evidence that this risk is further amplified by rapid catch-up growth (Stein *et al*, 2013). The mechanisms involved are unclear and it is widely suspected that diseases in later life are more likely to result from this association of rapid catch-up growth postnatally rather than intrauterine growth restriction *per se* (Tedner *et al*, 2012).

This phenomenon of catch-up or compensatory growth shows fairly wide variation around an optimal level in large populations (Stein *et al*, 2013). The trajectory of growth rate can increase, as seen in the EmmodDex fish reported here which, although not smaller at 120hpf, displayed increased growth rate between the juvenile and adulthood stages. Previous studies have suggested, however, that compensatory growth has a finite capacity (Tedner *et al*, 2012). This means that despite catch up growth, once there is cessation of cell proliferation, the final cell number may be lower than would have occurred under normal circumstances. This can result in irreversible alterations in organ cell number and size. Although cell number could not be determined in the isolated adult heart, cardiomyocyte number was reduced at embryonic stages of development when GR activity was inhibited by drugs or genetic manipulations.

Abnormalities in organ size and or cellular structure have detrimental consequences on cellular and physiological activity with alterations in fat deposition, telomere abrasion, glucose tolerance, insulin resistance, obesity, risk of cardiovascular disorder and reduced lifespan shown in humans and other vertebrates (Metcalfe & Monaghan, 2001). It is, therefore, reasonable to suggest that the cellular abnormalities reported here could lead to alterations in function in later life in the zebrafish. However, the physiological consequences of such changes for the zebrafish, either in captivity or in their natural environment, remain to be determined.

6.2.3 Embryonic glucocorticoid manipulation and embryonic stress response

The data generated also suggest that, although the critical signalling and steriodogenic features of the stress response are present and functional from early in zebrafish development, the earliest detectable stress response does not occur until 72hpf. The lag between functional activity of the system and stressor-induced cortisol biosynthesis has been reported in other teleost species, including the rainbow trout (Barry *et al*., 1995) and, more recently, the tilapia (Pepels and Balm, 2004). Although a delay in stress-induced activity is observed in other teleost species (Leatherland *et al*., 2010), its functional relevance remains unclear. It may suggest a further role of GCs as developmental regulators; the lag period being protective of key growth phases without which sudden surges in GCs may result in disruption of GC-sensitive developmental events. Indeed, this feature is observed following embryonic Dex exposure where there is an increase in growth rate and chorion hatching when the HPI axis stress response is absent. This is also referred to as the stress hyporesponsive (SHR) period in rodent models (Sapolsky & Meaney, 1986).

Further support for a developmental role for GCs in the zebrafish embryo is the detection of significant levels of cortisol in the early oocyte. In zebrafish, as for other fish species, a number of developmental components are transferred from the mother into the yolk sac during gametogenesis. These hormones are thought to control developmental rate and are depleted as embryogenesis progresses prior to *de novo* synthesis (Gagliano & McCormick, 2009; McCormick & Nechaev, 2002). Furthermore, evidence suggests that there may be a direct link between maternal plasma hormonal levels and the eggs which are produced. Numerous maternal hormones are known to be transferred, such as those involved in growth (e.g. thyroxin (Brown *et al*, 1988)), reproduction (testosterone) and metabolism (cortisol). Maternally-derived cortisol is not thought to be important for stress response during this phase but, rather, it may play key functional interactive roles with a number of developmental hormones. These may include the suppression of reproductive activity and involvement in several aspects of gametogenesis and embryogenesis in fish (Schreck, 2010). As a consequence of this, raised oocyte cortisol levels can have a major impact on embryogenesis. In this work, embryonic GC incubation (Dex treatment) increased the abundance of a number of growth-related hormones,

including *igf*. This suggests that, increasing activity of GR at key developmental phases where GC levels are normally low (the SHR period), can produce a downstream effect on growth hormones and, subsequently, on tissue growth.

The fall and rise in cortisol and in GC-related transcripts in this work showed a clear inflection point at, or near, 48hpf. This suggests that cortisol detected before this time-point was of maternal origin and subsequent increases represent *de novo* synthesis by the embryo itself. In mammals, maternal stress results in raised circulating cortisol levels and this subsequently influences mortality in the offspring. Similar observations have been detected in salmonoid fish, in which raised maternal plasma cortisol levels during the period of oocyte vitellogenesis can alter oocyte cortisol content (Leatherland *et al*, 2010; Veillette *et al*, 2007). Here to an increase in cortisol was also observed in the offspring of Dex injected mothers. The alteration of offspring cortisol in fish is said to represent evolution of reproductive strategies for generational life history and ecology progression (Burton *et al*, 2013; McCormick, 1998).

From published data on the environmental influences on fish reproductive function it is apparent that teasing apart the effects of cortisol on reproduction *per se*, versus the intrinsic survival mechanisms of the species under stressful conditions, is extremely difficult (Aluru & Vijayan, 2009; Segner, 2009). An important paper by Carl Schreck (Schreck, 2010), is one of a limited number which describe the impact of stress on the number and quality of surviving offspring, describing a reduction in egg production and viability following maternal stress. One other study reported that administration of cortisol to female tilapia (fish) by injection resulted in impaired oocyte growth and reduced adult offspring condition factor (Foo and Lam, 1993). These findings are particularly interesting as it may be an initial indication of adaptive programming of the embryo in fish species.

6.2.4 Embryonic glucocorticoid manipulation and adult stress response

As discussed, the function of the HPA or HPI axis is to provide an animal or organism with an adaptive response to environmental or physical stress to ensure homeostasis via production and release of GCs (cortisol in humans and cortisone in

rodents). This neuroendocrine system is present after the formation of the hypothalamus, pituitary brain structures and adrenal gland (found on the kidney), along with the presence of various catalytic cytochrome p450 enzymes and steroidal substrates. GCs released via this pathway act upon numerous target tissues which express either GR or, to a lesser extent, MR; the HPA/HPI axis itself also expresses these receptors creating auto-regulation or negative feedback of GC production (Chapman & Seckl, 2008).

The ontogeny of this system, like many developing organ systems, is influenced by maternal GCs, with GC over-exposure during the perinatal period associated with programming of HPA axis function (Huang, 2011; Jellyman *et al*, 2012; Kapoor *et al*, 2006; Seckl & Holmes, 2007). GC over-exposure towards the end of gestation permanently increases basal and stress-induced plasma GC levels in rodents (Cottrell & Seckl, 2009). Consistent with these rodent studies, Dex treatment during zebrafish embryogenesis increased stress-induced cortisol in the adults. Furthermore, alterations in basal and stress-induced cortisol alters behaviour, resulting in increased incidence of anxiety-related behaviours (Welberg *et al*, 2000). This was demonstrated in EmmodDex adults who displayed differing behaviour in a number of assays; open-field, dive and response to a novel object in the tank. These data are of particular significance since, not only do they confirm a physiological role of the HPI axis in swim behaviour in zebrafish, but they also suggest that altered behaviour can be programmed in the zebrafish following embryonic GC exposure.

Published data suggests that environmental modulation (such as GC over-exposure) can influence anxiety, learning, memory, and cognition in rodents (Cottrell *et al*, 2012) and humans (Reynolds, 2013). The data presented here confirms behavioural alterations in the zebrafish model too following GC manipulation, not only confirming the suitability of the zebrafish as a behavioural model but also suggests that features such as learning and memory could be readily investigated using in zebrafish too. Indeed, Pavlovian learning (e.g. electric shock avoidance (Pradel *et al*, 2000)) and operant conditioning (e.g. choosing between one arm of a maze and another (Colwill *et al*, 2005)) have been carried out successfully in zebrafish (Levin & Cerutti, 2009).

6.2.5 Embryonic glucocorticoid manipulation and vascular development

Previous studies suggest an association between foetal GC exposure and increased cardiovascular abnormalities in adult animals (Wintour *et al*, 2003). GC inhibit the formation of vascular tubes by endothelial cells (Logie *et al*, 2010; Nicosia & Ottinetti, 1990) and prevent migration and proliferation of vascular smooth muscle cells (Longenecker *et al*, 1982), resulting in vascular remodelling and structural alterations (Christy *et al*, 2003; Katz *et al*, 1988) which is associated with an increased cardiovascular risk (Girod & Brotman, 2004; Ng & Celermajer, 2004; Stewart & Petersenn, 2009; Trayhurn & Beattie, 2001). However, less is known about the direct actions of GCs (whether exogenous and/or endogenous) on the growth and structure of the developing cardiovascular system (Sainte-Marie *et al*, 2007).

The work presented here not only confirms a role for GCs in global maturation and development of the zebrafish embryo but also suggests an important role in the development of the heart and blood vessels (Chapter 5). GCs have anti-angiogenic properties (Folkman *et al*, 1983) and here GC manipulation (either suppression or enhancement of receptor activity and not endogenous production) resulted in alteration of the process of angiogenesis but not vasculogenesis (the primary axial vessels are formed similarly in all groups investigated). This suggests that GC signalling plays no role in aggregation of angioblasts into blood vessels but does play an important role in the sprouting of vessels from existing vasculature. GC-induced abnormalities in the formation of embryonic ISV suggest that the migration and/or differentiation of endothelial cells are influenced by GC signalling. Increased GR activity (Dex treatment) results in reduced vessel/sprout formation with the converse observed for decreased activity (especially apparent in the increased branching in RU486).

vegfaa mRNA abundance correlated with the observed alteration in ISV vessel formation. Reduced GR activity (Dex) reduced *vegfaa* abundance and also reduced *il8* abundance. These data are consistent with cell culture data where GC overexposure resulted in reduced *vegfa*a and *il8* abundance (Yano *et al*, 2006a). GCs are thought to produce a potent anti-angiogenic effect partly via their influence on

other growth and transcription factors, such as NFκB, resulting in the trans-repression of inflammatory gene transcription (King *et al*, 2009). Activated GRs are also known to negatively regulate the MAPK subgroups JNK, ERK1, ERK2, and p38 by inhibition of the phosphorylation step required for their activation (Figure 6.1). The defined molecular mechanisms of these GC-mediated effects have not been fully characterized and may be cell-type and stimulus specific (Stellato, 2004). However, similar observations in the zebrafish provide further support for future studies of this nature. This would permit greater understanding of the anti-angiogenic roles of GCs and could allow investigation of reduced vessel formation as a therapeutic strategy for cancer treatment.

Hypertension is arguably the greatest risk factor for cardiovascular disease, a number of studies have highlighted that elevated blood pressure (basal and stress-induced) is programmed (O'Regan *et al*, 2008). Despite this, little is known about the underlying mechanism(s) that lead(s) from *in-utero* programming to hypertension later in life. Much of the work carried out to address this has focused on endothelial dysfunction and microvascular rarefaction when hypertension has already developed (Nuyt & Alexander, 2009) and it is unclear whether the observed changes are a cause or effect of the elevated blood pressure (Khorram *et al*, 2007a).

Work carried out by Khorram *et al* (Khorram *et al*, 2013; Khorram *et al*, 2007a; Khorram *et al*, 2007b) characterised the vascular phenotype of IUGR offspring early in development, well before the hypertensive phenotype is apparent. The published work suggests that, in a rat model of maternal under-nutrition, there are marked vascular structural abnormalities such as extracellular matrix (ECM) remodelling and collagen deposition (Khorram *et al*, 2007b). These offspring were also found to have decreased vascular VEGF expression and a markedly reduced angiogenic capacity in microvascular endothelial cells (Khorram *et al*, 2007a). These data, taken together with what is known about GC ant-angiogenic actions both in complex systems (Hadoke *et al*, 2009) and in cell culture (Logie *et al*, 2010), leads to the hypothesis that increased foetal exposure to GCs results in adult onset hypertension by direct inhibition of embryonic *vegfaa* activity.

In the zebrafish, not only did enhanced GR activity (Dex) reduce blood vessel number, and abundance of key angiogenic factors in the embryo, but reduced angiogenic capacity in the adult as determined by reduced angiogenic sprouts 3 days following tailfin amputation. These data support a role for GC during embryogenesis on developing vasculature and also raises the possibility of a subtle life-long impairment of blood vessel angiogenesis. It may be that the observed alterations in vascular phenotype are subtle enough to permit normal growth and maturation and only result in pathophysiology when combined with further genomic, lifestyle or environmental risk factors such as diet, pollution or oxidative stress during the lifetime of the organism/animal.

To conclude, enhanced GR activity (Dex) results in reduced ISV formation in the embryo and diminished capacity to repair blood vessels in the adult. To date this is the first documented study in the zebrafish to record programmed vascular dysfunction in the adult following embryonic GC exposure.

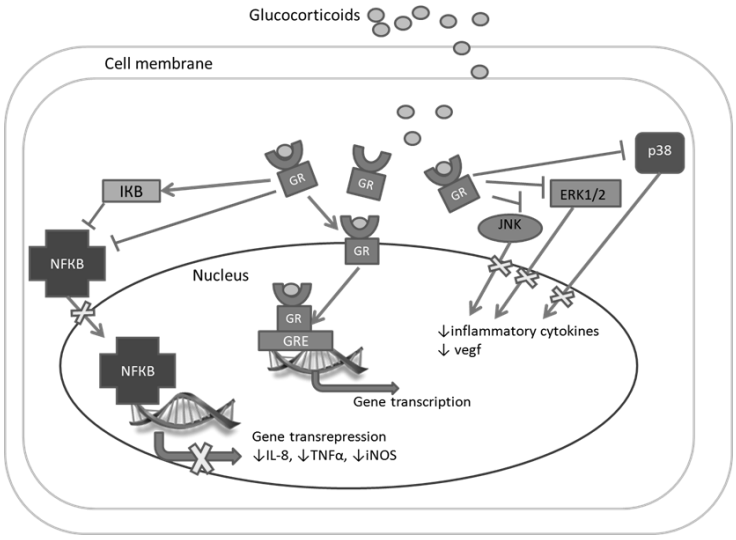

Figure 6.1 Schematic summarising possible glucocorticoid vascular interactions
Features shown are glucocorticoid receptor (GR), glucocorticoid responsive element (GRE), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), Interleukin 8 (IL-8), Tumour necrosis factor alpha (TNFα), inducible nitric oxide synthase (iNOS), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, (IκB), c-Jun N-terminal kinases (JNK), Extracellular signal-regulated kinases 1 or 2 (ERK1/2) and P38 mitogen-activated protein kinases (p38). Figure shows three possible methods of anti-angiogenic function: trans-repression (for example of inflammatory cytokines), gene transcription of negative regulators of angiogenesis, and direct inhibition of the activity of kinase signalling pathways.

6.2.5.1 Future studies of vasculature

Further investigation of the vascular phenotypes is clearly required using these programming models. Extracellular matrix composition and collagen deposition could not be investigated fully during the course of this study although no significant alteration in *mmp13* mRNA abundance was determined at 120 hpf in whole embryos (Chapter 4). However, it may be that alterations occur later in development or may occur in the composition of other ECM components. Initial histological assessment of collagen using Masson's trichrome staining on microtome sections (Figure 6.2) did not show significant changes in collagen deposition in the Dex treated embryo. However, the GR Mo embryos did show a generalised increase in collagen staining.

GCs have previously been shown to decrease Type I collagen synthesis *in vivo* and in fibroblast cell culture (Meisler *et al*, 1995). Since there was an increase in collagen staining in the GR Mo group but not in the Dex group, the effects observed may be mediated via MR. Furthermore, a developmental delay (which is apparent in the images shown in Figure 6.2) may also influence collagen deposition. The Masson's trichrome staining used here was a qualitative assessment and more sophisticated quantitative assessment possibly using an alkaline phosphatase assay may provide further useful data.

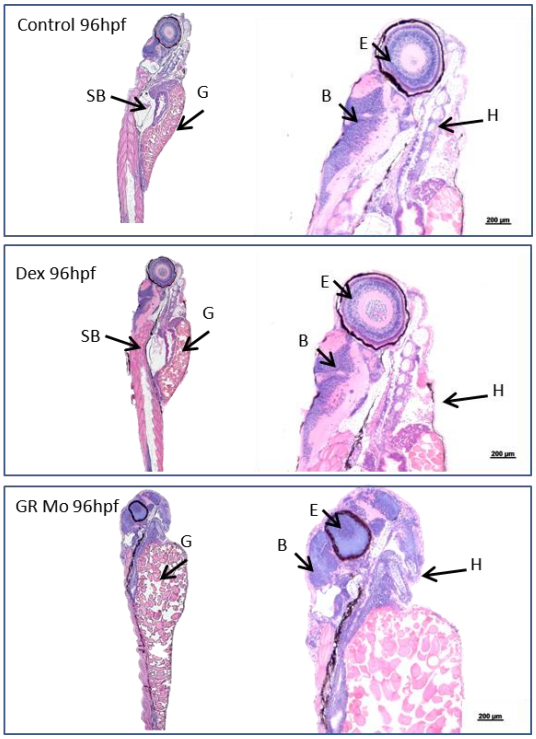

Figure 6.2 Embryonic Masson's trichrome staining
Typical sagittal sectioned embryos at 96 hours post fertilisation (hpf) in untreated controls or those which had been continuously treated pharmacologically with dexamethasone (Dex) or molecularly-manipulated using targeted *gr* morpholino (GR Mo) to modulate the activity of the GC system. Sections are stained with Masson's Trichrome histological stain where connective tissue is stained blue, nuclei are stained dark red/purple, and cytoplasm is stained light red/pink. Images shown may have planar differences and are only used here to highlight anatomical features. Features shown are eye (E), brain (B), heart (H), gut (G) and swim bladder (SB).

6.2.6 Embryonic glucocorticoid manipulation and cardiac programming

Exposure to GC during development may influence the maturation and development of a number of different organ systems. The effects of GC on cardiac development have not been fully investigated, although previous evidence has suggested that foetal exposure to excess GC can compromise cardiac growth (Torres *et al*, 1997). The work presented here indicates a clear impairment in cardiac growth and development during embryogenesis with reduced GR activity (GR Mo). These hearts were both structurally and functionally impaired compared to controls. These findings are similar to data published on $GR^{-/-}$ and cardiac specific GR knockdown mice (Rog-Zielinska *et al*, 2012) in which GR suppression caused poor alignment of

cardiomyocytes and abnormal myocardial architecture. In adulthood GR Mo fish displayed smaller hearts, increased intra-trabecular area with abnormal myocardial architecture despite similar survival and gross phenotype to controls. This suggests that alterations in cardiac structure may be subtle enough to not influence baseline cardiac function under normal environmental circumstances. However, it may be that as in human programming counterparts (Barker *et al*, 1989), further confounding features such as adverse environment occur the stress, load and exposure to risk factors placed on the heart will have consequences that lead to disease or disorder.

GR Mo treated embryos showed a significant reduction in GR mRNA this may suggest the suitability of the zebrafish GR Mo model for assessing the heart specific roles of GC during development. This would certainly overcome the poor survival observed in $GR^{-/-}$ mice (Rog-Zielinska *et al*, 2013). While knockdown of GR mRNA produced an abnormal maturational phenotype in the heart, Dex exposure during embryogenesis also altered cardiac development with GR Mo impairing cardiac maturation and Dex enhancing it. These findings are consistent with the maturational role of Dex on other organ types such as the lungs. Indeed, the work of Torres and colleagues (Torres *et al*, 1997) also suggests that GCs can have a direct role in cardiac development, with prenatal treatment of rats altering development of the heart by restricting growth. These authors also describe an increase in cardiomyocyte cell volume, consistent with results reported in Chapter 5. This probably reflects a GC-mediated decline in the rate of cell division (withdrawal from the cell cycle) or influence on the actions of other growth factors.

As GC treatment appears to have an influence on IGF (see chapter 4) further studies on the relationship between GCs and this growth factor and other maturational markers of cardiac development should be performed. GC may also exert cardiac effects on myocardial ECM, in a similar way to its effects on vascular ECM. Once again, maturational delay may be associated with a reduction in ECM as tissue which is actively proliferating would have less ECM (Gospodarowicz & Ill, 1980). While no changes in collagen content were detected in adult hearts (chapter 5) other workers have shown that chick hearts exposed to excess GCs have reduced collagen levels (Oikarinen *et al*, 1988): although this may be a transient effect during drug

exposure rather than a programming related alteration. Furthermore the alteration in chick heart ECM may be a result of delayed maturation rather than a true alteration in ECM composition. The changes in *vmhc* mRNA observed in the current investigations is interesting and has been seen in previous studies using animal models of excess GC exposure. It is linked to increased αMHC in rat models (Sheer & Morkin, 1984; Torres *et al*, 1997). Again opposing effects were noted in the adults, with *vmhc* abundance with enhanced Gr activity resulting in increased abundance and reduced Gr activity (GR Mo) reducing abundance.

To conclude the cardiac data, supressing or enhancing GR activity in the developing zebrafish heart can have long-lasting effects on the adult heart structure and molecular composition long after the stimulus (Dex treatment or GR Mo) has been removed. Once again, while the effects of this change appear minimal under normal physiological conditions further work is needed to determine whether structural alterations and small change in *vhmc* abundance could have on cardiac function. The extent that these subtle cardiac alterations may have might not become apparent until the heart is placed under increased haemodynamic load or suffered acute injury.

6.2.6.1 Future studies of the heart

To truly understand the extent of these cardiac abnormalities, investigation into cardiac function should be carried out. While it was relatively easy to investigate various cardiac functional parameters in the embryo, investigation in the adult was more problematic. Initial pilot studies using ultrasound presented a number of problems, particularly the poor signal produced by the adult heart; the intra-ventricular space is small and diffuse and, thus, accurate functional assessment is not possible. Further cardiac functional investigation could be carried out through respiratory assessments and exercise fatigue.

6.3 Future work

Strong evidence was generated in this thesis to show that early-life GC action in the zebrafish embryo has a direct influence on growth in later life. While this is an interesting observation, these data open up many further avenues of study to

investigate life- trajectory and potential long-term effects on the cardiovascular and other organ systems.

6.3.1 Role of glucocorticoids in programming adult metabolic pathways

Published data on GC exposure suggests a relationship between GC manipulation and altered cardio- metabolic profile in adulthood such as altered lipogenic capacity, insulin resistance and raised blood glucose (Fowden, 2003). A small associated study was carried out, during the course of this thesis, on the blood glucose profile of Emmod adults, data generated could be the basis for future investigation

Blood glucose levels were determined using a commercially available blood glucose meter (Section 2.5.5) in a small cohort (n=8) of adult fish that had undergone GC manipulation as embryos (Figure 6.3). Higher blood glucose was observed in EmmodDex adults ($p<0.01$). Blood glucose in EmmodRU486 or EmmodGR Mo adults were the same as controls. This is similar to published work in rodents and humans showing a relationship between embryonic GC exposure, birth size, catch-up growth and disadvantageous insulin and glucose profiles in adulthood (De Blasio *et al*, 2007; Stocker *et al*, 2005). Further support for an altered metabolic status is provided by increased *pepck* mRNA abundance in the livers of EmmodDex adults ($p<0.05$ (Figure. 6.3 D). Pepck catalyses the rate-limiting step in gluconeogenesis and, therefore, is essential in glucose metabolism. In this pilot study transcription of *pepck* was enhanced by GC activity. These data are intriguing as in rats, maternal exposure to Dex in late pregnancy can programme permanent induction of hepatic PEPCK abundance, hyperglycaemia and insulin resistance in the adult (Nyirenda *et al*, 2001). The data generated support the hypothesis that GC elevation during development has a programming role in glucose metabolism leading to permanent hyperglycaemia in adulthood.

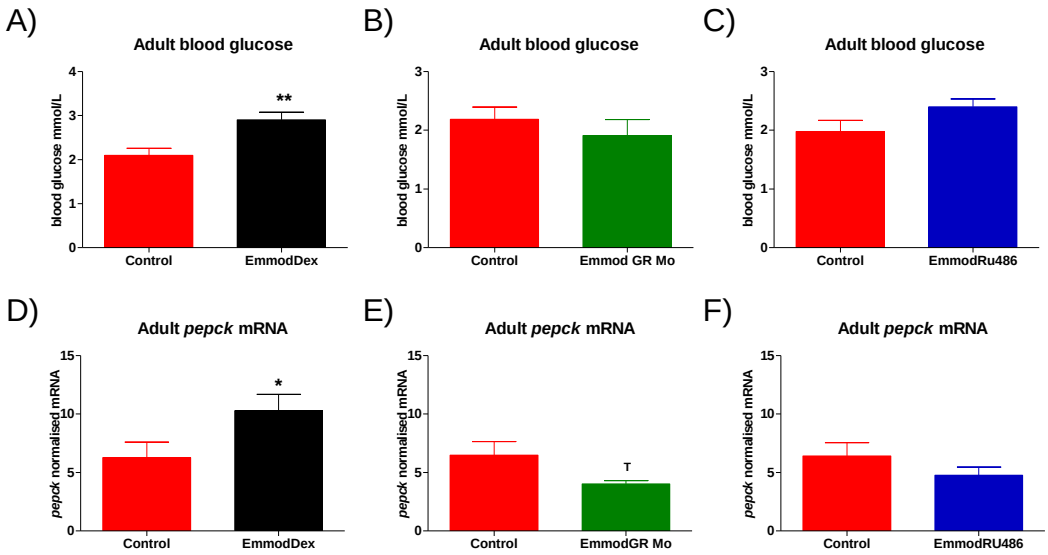

Figure 6.3 Impact of embryonic glucocorticoid modulation on adult blood glucose
Blood glucose (mmol/L) and liver *pepck* mRNA expression. Data shown are blood glucose levels (mmol/L) for, A) EmmodDex, B) EmmodGR Mo, and C) EmmodRu486 fish compared to their respective controls (n=12 per treatment). D-F data shown is for isolated liver pepck mRNA expression n=4 (4 pooled livers) from D) EmmodDex, E) EmmodGR Mo, and F) EmmodRu486 fish compared to their respective controls. All data are mean ± SEM and were analysed by Student's *t*-test (*p≤0.05, **p≤0.001, T=0.06).

6.3.2 Role of glucocorticoids in programming aging

A further feature that could be explored in this GC-programming model using the zebrafish is the aging process and cell senescence. This is thought to be an important contributor to chronic disease progression and may be a link between catch-up growth and adult onset disease.

Rat pups with IUGR display increased rate of kidney and heart senescence (Luyckx *et al*, 2009) and this is proposed as a mechanism to explain later life hypertension, chronic kidney disease, and cardiovascular disease. In the work presented here adult fish assessments were carried out at 120 dpf as this is when zebrafish are generally accepted to be sexually mature (Gerhard *et al*, 2002). However it may be important to look at later time-points to assess the influence of GC manipulation on the aging process. The zebrafish is thought to have a median lifespan of around 36 months (~1080 dpf) and can live for up to 66 months (~1980 dpf) (Gerhard *et al*, 2002). Given how the zebrafish is used for research in neurodegenerative and age-related

disorders, it is surprising that aging is a relatively unexplored process in this species (Gerhard & Cheng, 2002). This may be as a result of the complexity of the aging process and its multifactorial nature in all species. However, in the quest to understand better the similarities between aging in zebrafish and mammals, work published by Kishi and colleagues over the last 10 years (Kishi, 2004; Kishi *et al*, 2009) describes the gradual senescence process in the zebrafish, highlighting its suitability for the study of age-related alterations in musculoskeletal and eye morphology, endocrine factors, gene expression, circadian clock, sleep and cognitive functions (Kishi, 2004; Kishi *et al*, 2009). This group has further highlighted various pathological processes in aging zebrafish, coupled with age-dependent declines in their reproductive and regenerative capacity (Kishi *et al*, 2003). Kishi and colleagues also highlight the suitability of zebrafish for longevity studies, and their work supports the possibility of using it as a model of long-term programming.

While it is not suggested that aging itself is a programmable feature, the long-term impact of programming remains poorly understood; and, as the zebrafish is well suited for the investigation of the basic processes implicated in aging (such as insulin signalling and oxidative stress) it may be used as a comparative model to species with widely divergent longevities (Gerhard, 2007).

6.3.3 Role of glucocorticoids in multi-generational programming

The experimental work and discussion in this thesis up until now has focused on the concept of embryonic exposure to an adverse environment resulting in altered developmental signals in that offspring (F1). However, it has also been suggested that these physiologically programmed changes, with or without a continuing environmental stressor, can result in a developmental signal being passed-on to the next generation (F2). This perpetuation of the alteration has been referred to as the cycle of inter-generational programming (Drake & Seckl, 2011; Drake & Walker, 2004; Godfrey *et al*, 2010; Khulan & Drake, 2012; Roseboom & Watson, 2012).

Work carried out in the rat suggests that alterations in birth-weight are sustained in the F2 generation of F1 offspring whose mothers were either calorie restricted or had altered exercise (both increased and sedentary life-style) (Drake and Walker, 2004). Abnormal glucose tolerance has also been demonstrated in F2 rats (Roseboom &

Watson, 2012) whilst studies in rabbits with surgically-induced hypertension displayed increased blood pressure in the F2 generation although this study suggested a sex-specific phenotype in the female F2 offspring (Drake and Walker, 2004). Alterations in the HPA axis activity across generations has also been reported in these rodent models (Roseboom & Watson, 2012). From these data it would appear that programming of maternal physiology can persist through multiple generations and thus could form the basis of future work.

6.4 Study limitations

Whilst the work presented in this thesis strongly supports the suitability of the zebrafish as a programming model it is important to consider possible limitations.

Much of the work presented in this thesis has focused on GR-mediated actions of GCs. Since GR modulation was the aim of this study one may assume that alterations in cardiovascular structure and physiology are as a direct effect of GR-mediated actions. However, as the ligand for MR has not been identified in the zebrafish and as there are limited *11βhsd2* mRNA found in the heart (Tokarz *et al* 2013); it is likely that GC stimulates both MR and GR in the zebrafish heart. Therefore, some of the observations may result from activation of MR. To understand the specific roles of the receptors, targeted Mr manipulation could be carried out concurrently with the Gr modulation as presented in mouse studies by Ren and colleagues (Ren *et al* 2012).

Many investigations into the physiological influence of GC manipulation have focused on the genomic effects resulting from GC-GR interactions in the form of alterations in the relative abundance of mRNA for relevant genes. The genes included in this study were carefully selected to investigate the potential underlying mechanisms of programming. While a genome wide microarray analysis could have provided a greater insight into the potential positive and negative mediators of the observed alterations, the hypothesis-driven approach described in this thesis has provided important quantitative data for the genes concerned and highlighted potential downstream targets for future investigation.

In various studies it has been demonstrated that classic steroid hormone receptors may be involved not only in genomic steroid action, but also in rapid non-genomic

steroid effects (Falkenstein *et al* 2000). As highlighted in drug optimisation studies (Appendix 1) the interpretation of the data presented here is based on the assumption that changes are mediated through activation of GR-dependent genomic effects rather than non-genomic effects. This interpretation is supported in part by the results obtained using GR Mo knockdown but, given that some of the non-genomic effects of GCs are mediated by GR in a non-nuclear location a more robust investigation of the genomic and non-genomic effects of GC would be required. Work could involve investigating the sensitivity of response in the presence of transcription and protein synthesis inhibitors. Genomic actions of GCs have been investigated by the treatment with actinomycin D (an inhibitor of transcription) or cycloheximide (an inhibitor of translation) (Lee *et al* 2012).

The work presented in this thesis uses the zebrafish as a model for developmental programming of growth, behaviour, stress handling and cardiovascular function. While this model was chosen because of features which aid analysis (external fertilisation, embryonic transparency) there are clearly a number of limitations with regard to translation into human disease. Of particular importance in the cardiovascular programming chapter (Chapter 5, Part 2) are the anatomical differences between the zebrafish and the human heart. The zebrafish heart has many features that are similar to the foetal human heart, but the hearts of adult fish and adult humans have considerable differences; for example, zebrafish hearts lack septation and have fewer chambers (Glickman *et al* 2002). This should be taken into consideration when changes observed in zebrafish are translated to humans.

While the current work (Chapter 5, Part 2) describes adult zebrafish cardiac structure and molecular composition it does not describe adult cardiac function. This was due to limited success with *in vivo* cardiac imaging in adult zebrafish. Despite the benefits of the zebrafish (compared with mammalian) embryos for *in vivo* imaging, real-time assessment of cardiac function remains challenging in the adult zebrafish. Pilot studies using cardiac Doppler ultrasound have identified a distinct pattern of filling and emptying of the ventricle, similar to mammals, although the actual images of the beating heart remain of relatively poor quality and would not have allowed

adequate assessment of cardiac structure and function. Further optimisation may produce better quality images..

Because the analysis compared groups of equal sample size and because data were often found to meet classic homogeneity of variance assumptions, the likelihood of producing a type 1 error was considered to be low. However, increasing group size would have helped prevent the rejection of a true null hypothesis, particularly where data showed a trend towards significance.

6.5 Concluding remarks

The data presented here represent a comprehensive assessment of the use of the zebrafish as a model of foetal origins of adult disease and programming by modulating GCs during early development. The physiological roles of the GC system were assessed in the developing zebrafish embryo, through pharmacological and genetic manipulation. This highlighted various functions of the GC system in the zebrafish which have not previously been documented. Furthermore, it demonstrated that although these transient manipulations result in subtle alterations in development, organ structure and function, they are significant enough to alter the adult phenotype long after the manipulation has ceased. It is proposed that these alterations have indeed been "programmed" as a result of cellular and molecular alterations, in keeping with mammalian models of foetal programming.

The data generated here, coupled with the desirable experimental features of the zebrafish (such as short generation time, ease of manipulation, *ex utero* fertilisation and low cost), could result in the zebrafish becoming a prominent model of foetal programming in the future. Indeed the possible embryonic manipulations that could be used with this model are extensive. The obvious anatomical (gills, fins, scales etc.) and physiological (osmoregulation) differences from mammals do, however, mean that the zebrafish may not provide evidence directly translatable to humans and the patient suffering from cardio-metabolic disorders. However, the data derived from this model, coupled with data from rodent (or other mammalian models) and cell culture studies, may provide complementary assessments of what appears to be a very complex relationship.

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8 Appendix 1: Pharmacological and molecular optimisation

In the work presented in this thesis a number of pharmacological and molecular manipulations were carried out. To determine suitable concentrations for use in the relevant experimental chapters, comprehensive dose-ranging studies were performed and these are summarised here.

8.1 Pharmacological optimisation

The zebrafish embryo is widely recognised as a useful tool for pharmacological and novel-compound screening using a high-throughput approach (Brannen *et al* 2013). In the work described here the features which make the zebrafish embryo a suitable drug screening model (small size, high fecundity, rapid development and embryonic transparency) are utilised to determine viability, growth and other functional endpoints resulting from pharmacological manipulation of the glucocorticoid (GC) system. Before these functional endpoints could be investigated a series of model optimisation steps were carried out.

When investigating any pharmacological manipulation assay it is important to consider whether the correct drug has been selected and whether the optimal dosage regimen has been established. This is particularly relevant when considering the translation of dose, exposure and subsequent response into human models (Gabrielssona *et al* 2010).

8.1.1 Drug selection

The primary aim of this work was to assess the developmental effects of GC receptor (GR) manipulation during embryogenesis in the zebrafish. A small group of drugs was selected which primarily alter GR activity and alter ligand availability by targeting the enzyme 11β-hydroxylase (which is responsible for the final step in cortisol biosynthesis). The following drugs were selected based on published pharmacological data from mammalian (and, where possible, teleostean fish) studies: the GR agonist dexamethasone (Dex), the GR (and progesterone receptor (PR)) antagonist RU486 (also known as mifepristone) and the 11β-hydroxylase inhibitor metyrapone (Met). Some experiments also included: the 11β-hydroxylase substrates

11-deoxycorticosterone (Doc) and 11-deoxycortisol (Doxy), and the zebrafish mineralocorticoid (MR) agonist spironolactone (Scott *et al*, 2005).

8.1.2 Vehicle selection

Work by Hillegass and colleagues (Hillegass *et al*., (2007); Hillegass *et al*., (2008)) highlighted the suitability of the zebrafish model for continuous drug exposure by dissolving drug in the bathing water in either dimethyl sulfoxide (DMSO) or an aqueous vehicle. Ethanol (EtOH) readily dissolves steroids but has detrimental effects of zebrafish development and survival at concentrations of more than 3% (Billotta *et al* 2004). It was, therefore, important to determine a concentration of EtOH which readily dissolved the drugs of interest but did not have a significant impact on development or survival (Figure 8.1).

8.1.2.1 Vehicle concentration optimisation protocol

It was found that concentrations of less than 1% EtOH had minimal impact on both survival and phenotype of embryos. Consequently, a concentration of 0.1% EtOH, which was sufficient for dissolving all the relevant compounds, was selected for all further drug solutions. For all experimentation where drug exposure occurred, a vehicle only (0.1% EtOH) control was included.

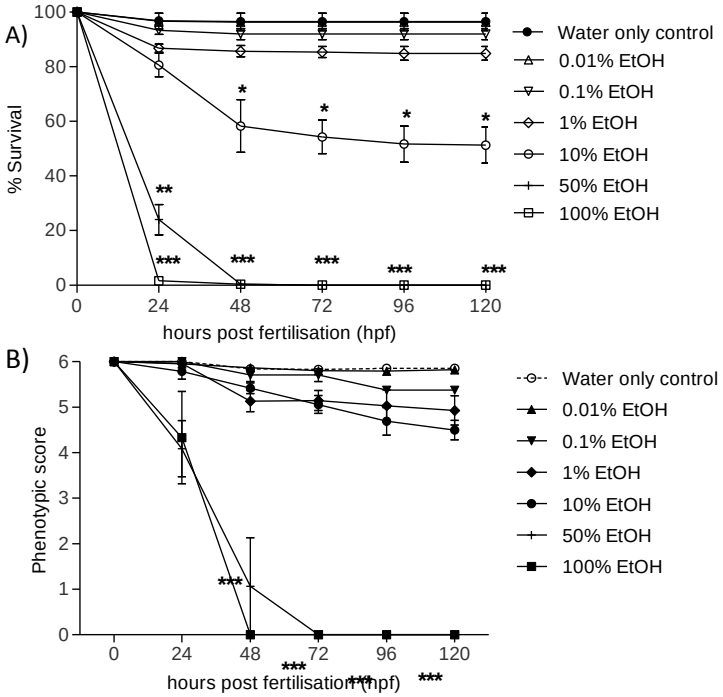

Figure 8.1 Determination of concentration of ethanol used as a vehicle.
A) influence of varying concentrations of ethanol (EtOH (%)) on survival over 120hours post fertilisation (hpf) B) influence of varying concentrations of EtOH (%) on phenotype score n=3 experiments 30 embryos per group. Mean ±SEM, data analysed by 2-way ANOVA with Bonferroni post hoc test vs water only control *P<0.05, **P<0.01 and ***P<0.001.

8.1.3 Drug concentration range selection

As there were no previously published data on the effective doses (ED) and ligand/receptor activity of these drugs on zebrafish embryos, a typical drug optimisation screen was carried out. This consisted of treatment over a range of concentrations of the test compound and assessment of survival and phenotype throughout the course of exposure (120 h). The concentration ranges of drugs used were, where possible, based on previously published data.

Dex was investigated over a range of 25-200 µM; while the upper limit may appear relatively high in comparison to mammalian studies, these concentrations were selected based on previous publications (Hillegass *et al* 2008; Schaaf *et al* 2009). These studies also use RU486 as a potential GR antagonist but used a lower concentration range (up to 1 µM) than described here. Park and colleagues (Park et *al*. 2012) used 12 µM RU486 without toxic effects. Since a number of concentrations

have been used in published work, a concentration range up to 100µM was investigated in pilot studies.

At the start of the current research no publications were available describing exposure of zebrafish embryos to Met; in the absence of data, a concentration range up to 100 µM was chosen. Subsequently, published work has shown that zebrafish can tolerate doses up to 45 µM Met without adverse effect (Liu *et al* 2013). Concentration optimisation for Doc, Doxy and Spironolactone was produced during work towards a Master of Science (MSc) dissertation (K Wilson 2009) and cannot be included here. However, the doses used for this study were Doc [1 µM], Doxy [0.1 and 1 µM], and spironolactone [0.1 µM]. All drugs were dissolved completely in vehicle and diluted to their final concentrations in a 30 mL Petri dish.

8.1.3.1 Drug exposure protocol

A standard drug exposure protocol was used for optimisation of each drug of interest. Groups of 30 embryos were placed in Petri dishes containing 30 mL of drug solution at the desired concentration. Embryos were exposed continuously up to 120 hpf, with fresh drug added daily. Drug toxicity was monitored daily by assessing survival (as a percentage of the initial group size) and morphology (using a 6 point mean phenotype score-methods section 2.4.3). Optimisation for the drugs Dex, RU486 and Met are shown in Figures 8.2 and 8.3 and summarised in Table 8.1.

8.1.3.2 Drug exposure concentration selection

For Dex it was found that concentrations of 25, 50, and 100 µM did not significantly alter the survival or phenotype score of the embryos. 100 µM Dex was used for further studies as this was a concentration which was found to enhance a number of developmental features (Chapter 5) but did not significantly alter global morphology. Furthermore this concentration has been used previously in studies of GR activity in zebrafish (Schaaff *et al* 2009). For RU486, 10 µM was selected for further investigation as it did not impair survival or gross phenotype. In contrast, 50 and 100 µM RU486 reduced survival and phenotype significantly. For Met concentrations of 100 and 150 µM reduced survival and altered the phenotype the embryos whereas

concentrations of 0.1, 1 and 10 µM did not alter any of the features investigated. A summary of the drug concentrations used throughout the thesis is given in Table 8.1.

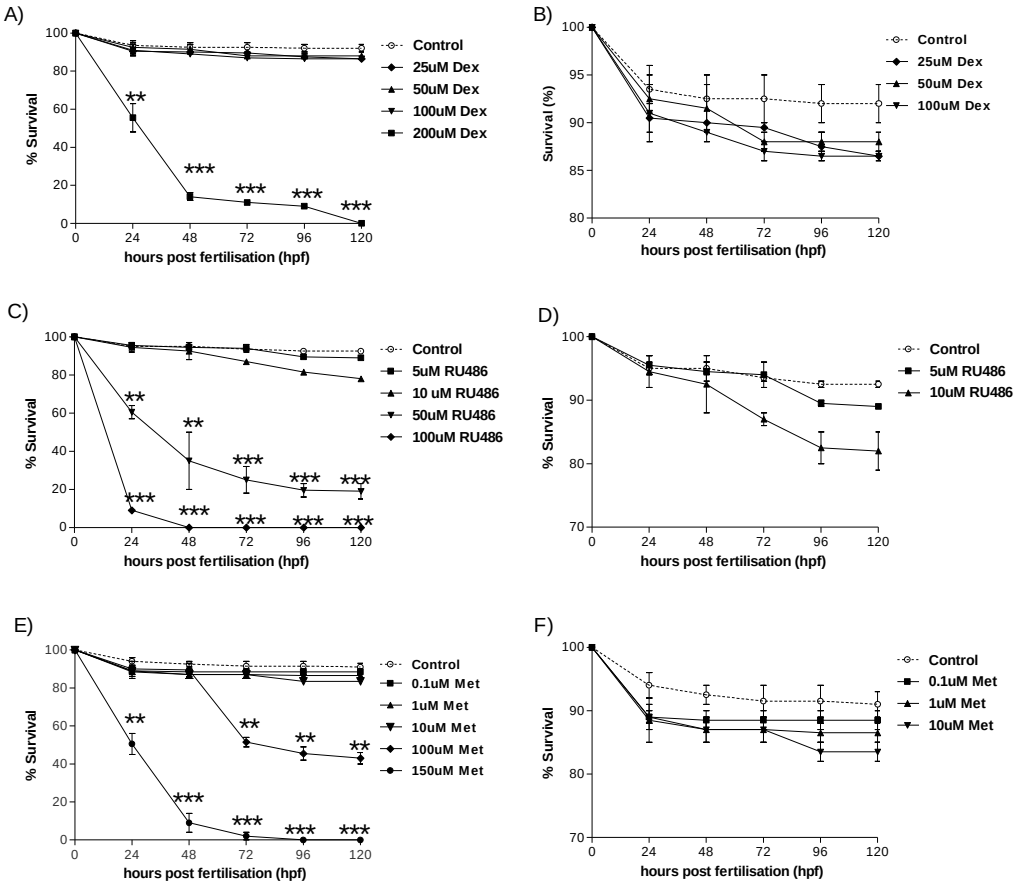

Figure 8.2 Embryonic pharmacological survival curves
Varying drug concentrations (µM) for optimisation of exposure to A) dexamethasone (Dex) B) Dex (reduced y axis for clearer interpretation). C) RU486 D) RU486 (reduced y axis for clearer interpretation) E) Metyrapone (Met) F) Met (reduced y axis for clearer interpretation). All data were for embryos compared to age matched vehicle 0.1% EtOH treated controls. Survival is shown as percentage of original embryo clutch; n=3 experiments, 35-40 embryos per experiment. Mean ± SEM data analysed by 2-way ANOVA and Bonferroni post hoc analysis vs vehicle only control *p≤0.05, **p≤0.01 and ***p≤0.001

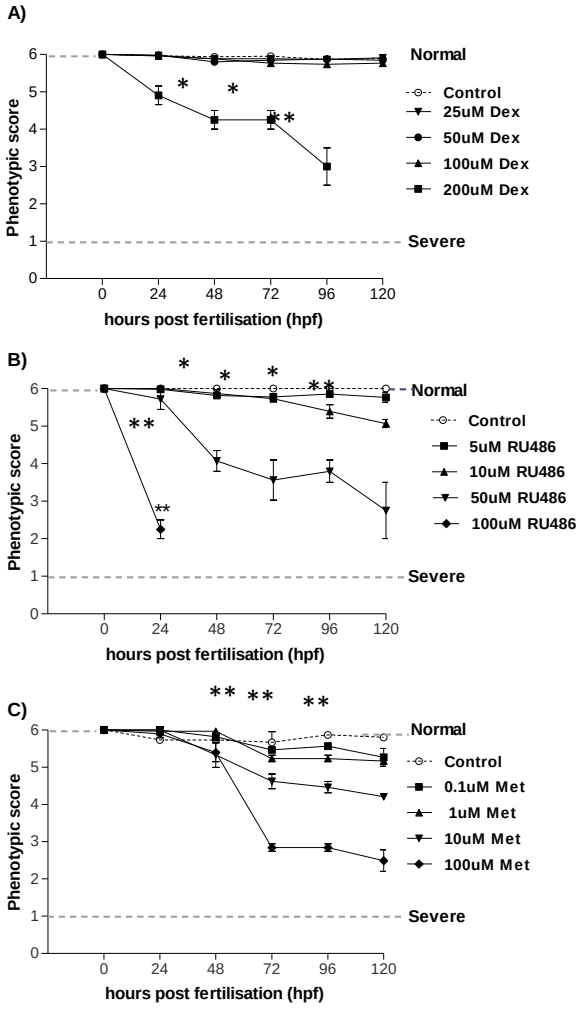

Figure 8.3 Mean phenotypic score for pharmacological manipulation
Concentration (μM) optimisation. A) dexamethasone (Dex) B) RU486 and C) metyrapone (Met) concentration determination vs. control (vehicle alone 0.1 % EtOH) over 120hpf. Scoring system is as follows 6 (Normal), 4-5 (Mild), 2-3 (Moderate), 1(Severe) and 0 (dead). n=3, duplicated results of 35-40 embryos per experiment, mean ± SEM Data are analysed by 2-way ANOVA and Bonferroni post hoc test *p≤0.05, **p≤0.01 and ***p≤0.001

Table 8.1 Concentrations (µM) of pharmacological agents-embryos
Shown are drug concentrations which were selected for further investigation. Agents investigated were the glucocorticoid receptor (GR) agonist dexamethasone (Dex), the GR (and progesterone receptor -PR) antagonist RU486, the 11β-hydroxylase inhibitor metyrapone (Met), the 11β-hydroxylase substrates deoxycortisol (Doxy) and deoxycorticosterone (Doc) and the zebrafish mineralocorticoid (MR) agonist spironolactone. Concentrations shown are those used to bathe the embryos for the experimental chapters. The potential cross-reactivity is the amount of drug which would be present within the tissue homogenate assuming all drug is taken up into the embryo, the cross reactivity of cortisol ELISA is <0.01 % for interfering steroids so a greater potential cross-reactivity would suggest a greater likelihood of the drug interfering with cortisol concentration.

Pharmacological agent	Concentration	Potential assay read-out (pg/fish)
Dex	100 µM	<897
RU486	10 µM	<128.70
Met	0.1, 1 and 10 µM	<0.68-67.80
Doxy	0.1 or 1 µM	<1.05
Doc	0.1 µM	<0.99
spironolactone	0.1 µM	<1.25

8.1.4 Maternal glucocorticoid manipulation

To determine whether the early detectable GC in the embryo was maternally-derived a maternal GC modulation experiment was carried out using Dex. Two forms of modulation were investigated, maternal bathing and injection. No previous investigation using maternal Dex treatment had been carried out so a maternal Dex exposure protocol was produced (Section 3.3.2). The potential cortisol ELISA cross-reactivity of the doses used has been calculated and highlighted in Table 8.2.

Table 8.2 Concentrations (µM) of pharmacological agents-adults
Shown are drug concentrations which were selected for further investigation for maternal modulation of embryonic cortisol. The agents which was used for further investigation was the glucocorticoid receptor (GR) agonist dexamethasone Concentrations shown are those used to bathe the adult in or inject intraperitoneally. The potential assay read-out is the amount of drug which would be present within the swim water if all the injected bolus is present or the maximum amount of drug has been taken up by the adult fish.

Pharmacological agent	Concentration	Potential assay read-out (ng/fish/litre)
Dex Injected	5 nL of 10 uM stock	3.92
Dex Bathing	10 µM	1.96

8.1.5 Pharmacological manipulation-points to consider

While the Dex concentration used here (100µM or 39 mg/30ml) may appear to be high in comparison to human therapeutic applications (orally 4-20 mg daily) (Bello *et al* 1999) or in rodent models (50 mg/kg) absorption and distribution should be taken into consideration. By bathing the embryo in the drug one would assume that absorption occurs through epithelial surface diffusion (skin) throughout the course of the study. However, pilot studies with a fluorescently-tagged Dex (Dexamethasone Fluorescein, Life Technologies, Paisley, UK) suggest that from 48 hpf onwards tagged Dex is detectable within the gut of the developing embryo. This suggests that Dex may be taken up orally as well as through skin, particularly in later stages of development. While both of these routes of administration are commonly used for GC therapy, it is unclear what the absorption and relative bioavailability of the drug is in this setting. Injection of a bolus of Dex (100 µM) directly into the developing embryo potentiated the physical effect observed following bathing in the drug, suggesting that a lower effective dose is experienced by embryos bathed in the drug. While tagged Dex did offer some information with regard to the uptake route, it did not fulfil its purpose in allowing quantification of Dex within the embryo. Steps were taken to determine the levels of Dex and its metabolite 11-dehydrodexamethasone using liquid chromatography–mass spectrometry (LC-MS) but there was insufficient time to develop this technique. Nevertheless this does offer a possible approach for future work.

While absolute levels of Dex within the embryo remain uncertain it does appear clear that the concentration used throughout the thesis is having a functional effect in the embryo. Data in chapter 3 (Figure 3.8) suggest that this concentration of Dex suppresses endogenous cortisol in accordance to mammalian models; suggesting that enough Dex is being taken up by the embryo to suppress cortisol biosynthesis through negative feedback. Endogenous cortisol levels were determined using cortisol ELISA. Numerous steps were taken to optimise the ELISA in terms of calculating the cross reactivity of other steroids (endogenous and synthetic e.g. Dex) but it should be taken into consideration that (although the ELISA cross reactivity for Dex is low; <0.01 %) the relatively high concentration of drug used may interfere with the assay, providing an over-estimation of the cortisol concentration (a false

positive result). The maximum possible interference of each drug has been calculated for all drug doses used (Table 8.1). These calculations are based on the assumption that all drug present within the Petri dish is taken up by the embryos and thus any cross-reactivity would likely be lower than levels calculated here. Calculations are based on a zebrafish embryonic volume of 0.3 cm^3 as determine by length, width and height measurements (3 mm x 1 mm x 1mm). For Dex the maximum interference for the assay was calculated as <897 pg/embryo. The ELISA data for this group of embryo embryos suggested cortisol levels were 2.18 ± 0.40 ng/embryo; therefore it is likely that the dose of Dex used is interfering with the ELISA. While this does suggest a degree of cross-reactivity it should be highlighted that Dex treatment resulted in a decrease the detected levels of cortisol, the risk of falsely showing an increase due to cross reactivity were therefore low, and Dex treatment may in fact have a more profound effect on cortisol than is shown here. The cross –reactivity is also highlighted for adults treated with Dex (Table 8.2). The data calculated for Dex injection is based on the injection of a bolus of 5 nLand the data for bathing is based on the assumption that all drug which the adult is exposed to is taken up (as for embryo this is calculated volumetrically (50 cm^3)). Maximum cross-reactivity calculated are likely to interfere with the ELISA, as detected levels were found to be ~5 ng/fish/litre for injection and ~6ng/fish/litre for bathing.

As described in greater detail in chapter 1, GCs exert genomic effects by binding to, and activating, the transcription factors MR and GR. However, non-genomic effects have also been observed in mammals (Stellato *et al* 2004) and more recently in zebrafish (De Marco 2013). The mechanisms underlying the non-genomic effects are poorly understood and it is unclear whether non-genomic and genomic effects interact (De Marco 2013). It is likely that Dex, particularly at the higher concentration used here, is producing genomic and non-genomic effects as increased ligand concentration increases the likelihood of receptor saturation and thus more compound is available for non-receptor mediated non-genomic effects. The steroid-binding characteristics of the receptors may also determine whether genomic or non-genomic effects are responsible, with the calculated Dex dissociation constant (Kd) in trout gr being 5.5 ± 0.41nM (Strum *et al* 2005) (high degree of homogeneity to zebrafish gr). It is likely that some non-genomic effects are observed at the

concentration used throughout this study. While it is unclear whether the physiological effects observed following Dex exposure are as a result of genomic or non-genomic effects, steps were taken to address this, mainly through a non-pharmacological approach (morpholino (MO) knocking down the zebrafish GR (*gr*) - GR Mo), however, as non-genomic effects can also occur through GR this does not allow confirmation of genomic effects. To determine whether the observations described in this thesis are as a result of genomic or non-genomic GC-mediated effects transcription and protein synthesis inhibitors could be investigated (Lee *et al* 2012).

8.2 Molecular optimisation

As a control for the pharmacological investigation, a molecular manipulation was carried out to reduce the abundance of gr mRNA in the zebrafish embryo using MO oligonucleotides. While this form of manipulation may appear to circumvent the issues associated with pharmacological manipulation, as with all other forms of anti-sense gene knock-down, off-target effects may occur following Mo treatment. This can make it difficult to interpret whether the effects observed result from knock-down of the gene of interest or of other genes (Eisen and Smith, 2008). To control for off-target effects and to confirm gene of interest function, a comprehensive scoring system was used for assessing the influence of Mo, this was the same as the pharmacological 6 point scoring system and allowed the grading of severity of Mo effects.

8.2.1 Morpholino concentration optimisation protocol

Molecular manipulation of embryos was carried out by Mo antisense gene knock-down (Heasman, 2002; Summerton, 1999) targeted towards the genes encoding *cyp11b1* and *gr*. Mo were purchased from GeneTools (Philomath, Oregon, USA) (http://www.gene-tools.com) designed specifically to the zebrafish *cyp11b1* and *gr* gene sequence. MOs were injected as per section 2.4.4. This entailed injecting approximately a 5nl bolus of various concentration stock solutions 3-7 ng/nL as calculated in section 2.4.4. Data for the ATG Mo for each gene are shown for illustrative purposes (Figure 8.4).

8.2.1.1 Morpholino concentration selection

An appropriate concentration was selected for both Cyp Mo and GR Mo by scoring for survival and gross morphology over the course of 120 h with concentrations of 5 and 7 ng/nL GR Mo significantly reducing survival and morphology. After carrying out the dose ranging studies it was felt that a concentrations of 3 ng/nL would be used for the GR Mo groups (Mo, splice and mism) and a concentration of 6 ng/nL would be used for the Cyp11b1 Mo groups (Mo, splice and mism). For all data represented in this thesis the controls for the Mo and splice groups is the mism injection. Concentrations used throughout are summarised in Table 8.3.

8.2.1.2 Molecular manipulation-points to consider

The use of both atg-Mo and ss-Mo allows greater assessment of effects and confirmation of gene specificity. Titration studies where carried out for both ss-Mo and atg-Mo to determine suitable concentrations for this study (only atg-Mo are shown in Figure 8.4). Although atg-Mo offer a wider translational knockdown, this form of Mo does not confirm knock-down of gene of interest, ss-Mo therefore are used to quantify the efficiency of the Mo. Injection of Mo itself can be problematic with mechanical effects often resulting in phenotype alterations. Therefore a control base mispair mm-Mo was used here as this is the most similar to the Mo being injected, this is the control for all Mo investigations shown in the thesis.

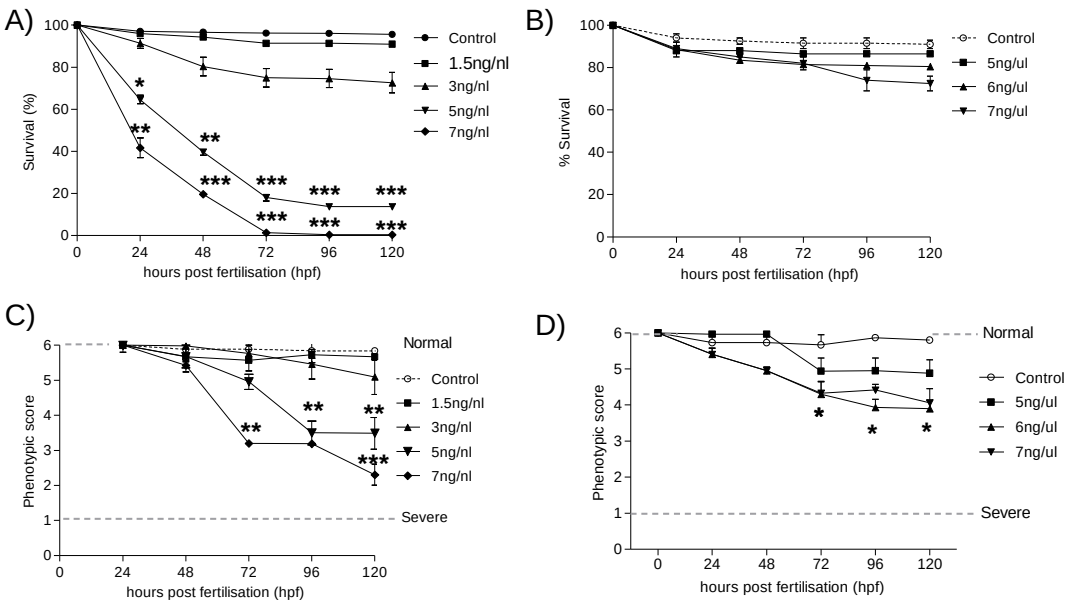

Figure 8.4 Optimisation of morpholino injection dose
Embryonic survival curves of varying concentrations (ng/nL) of either A) glucocorticoid receptor (GR) targeted ATG morpholino (MO) knockdown and B) cyp11b1 targeted ATG MO knockdown. Mean phenotypic score for molecular manipulation concentrations by C) GR targeted knockdown and D) cyp11b1 targeted knockdown, scoring system is as follows 6 (Normal), 4-5 (Mild), 2-3 (Moderate), 1(Severe) and 0 (dead). N=3 experiments, 30 embryos per experiment, data are mean + SEM*p≤0.05, **p≤0.01 and ***p≤0.001

Table 8.3 Morpholino sequences and concentrations
Summary of morpholino (MO) concentrations used throughout this work unless stated otherwise in specific experimental chapters.

Morpholino	Sequence	Concentration
GR atg (GR Mo in text)	CATTCTCCAGTCCTCCTTGATCCAT	3 ng/nL
GR splice site	GCCAGAGATATATGGAATACCTTCA	3 ng/nL
GR mism	CATTGTCCACTCCTGCTTCATCGAT	3 ng/nL
Cyp11b1 atg (Cyp Mo in text)	ATTTCCTCCTGAAATGAGCCCTCAT	6 ng/nL
Cyp11b1 splice site	CTCTCTTTGTGAGACTTTACATCAC	6 ng/nL
Cyp11b1 mism	CTCTCTTTGTGAGCTTTACATCAC	6 ng/nL

8.3 Pharmacological and molecular optimisation conclusion

The work described in this appendix details the steps which were taken in the concentration optimisation during GC pharmacological and molecular manipulation model set up. Concentrations used for further investigation are detailed along with concentrations which were found to detrimentally impact on survival or phenotype.

Appendix 2: Gene selection

Table 0.1 Gene of interest selection
Summary of the reasons for selection of a number of genes investigated during the course of the work presented in this thesis. More detail is given within chapters 4, 5 and 6. Genes highlighted are the zebrafish transcript encoding insulin-like growth factor-1 (*igf),* FK506 binding protein 5 *(fkbp5),* vascular growth factor *(vegfaa),* interleukin 8*(il-8,)* myocyte enhancer factor 2*(mef2c),* ventricular myosin heavy chain*(vmhc)* and GATA transcription factor 4 (*gata4).*

Gene	Reason for investigation
igf1	*igf* signalling is important for zebrafish development (White *et al* 2009). Glucocorticoid treatment has been shown to reduce *igf* mRNA in many models and is important for cardiomyocyte proliferation during cardiac development (Li *et al*, 2011).
fkbp5	FK506 binding protein is a co-chaperone for heat shock protein (hsp) 51. *fkbp5* mRNA has been found to increase after chronic stress or glucocorticoid exposure. Fkbp5 has been shown to be altered in zebrafish after glucocorticoid treatment (Mathew *et al*, 2007)
vegfaa	Vegfaa signalling has been shown to be involved in the formation of zebrafish intersegmental vessels (Nasevicius *et al*, 2000). Vegf concentration has been found to decreased following glucocorticoid exposure in tumours (Koedam *et al*, 2002).
il-8	Glucocorticoids have been shown to reduce the expression of inflammatory cytokines (Yano *et al*, 2006a) Il8 has been associated with the formation of intersegmental vessels in the zebrafish
flk	*flk1/kdr* is a prominent receptor in *vegf* signalling in zebrafish (Bussmann *et al*, 2008).
mef2c	The Mef 2 transcription factor family is important in early cardiac development and hypertrophy (Czubryt & Olson, 2004). Mef2c and GR act cooperatively in controlling gene transcription, with GR influencing Mef2 activity (Speksnijder *et al*, 2012)
vmhc	Vmhc is required for the normal contractile activity of the heart and is particularly important in the later stage of cardiac development (Miyata *et al*, 2000)
gata4	Gata 4 is involved in the development of numerous zebrafish organs (Holtzinger & Evans, 2005) and is an important influence on cardiac development and cardiomyocyte proliferation (Singh *et al*, 2010)