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Cloning and Characterisation of Gonadotropin-Releasing Hormone (GnRH) Receptors in the Cichlid (*Haplochromis burtoni*) and the Zebrafish (*Danio rerio*)

University of Cape Town

MSc Thesis
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Preface

The experimental work described in this report was carried out in the Department of Molecular and Cell Biology, University of Cape Town, from April 2000 to August 2002, under the supervision of Professor N. Illing.

This study represents the original work of the author and has not otherwise been submitted in any form for any degree or diploma to any other university. Where use has been made of the work of others, it is acknowledged in the text.

Michelle Gaye Morley

March 2003

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Abstract

The identification of multiple forms of gonadotropin-releasing hormone (GnRH) in a single species is becoming a common occurrence. The highly conserved chicken GnRH II is present along with one or two other GnRHs, composing a combination unique to particular species. This multifunctional peptide is widely distributed through the central nervous system and peripheral tissues. Also, endogenous GnRHs demonstrate distinct patterns of spatial expression within the brain, suggesting they may have separate functions. In addition to being the primary regulator of gonadotropin secretion in vertebrates, GnRH is also involved in the release of GH and prolactin and may fulfil a possible neuromodulatory role.

GnRHs exert their actions through the stimulation of distinct GnRH receptors on pituitary gonadotrophs. The presence of multiple GnRH receptor subtypes has been demonstrated in several species and is likely to be a common characteristic of most vertebrates. This thesis describes the cloning and characterisation of GnRH receptors in two species of teleost fish, *Haplochromis burtoni* (cichlid) and *Danio rerio* (zebrafish). A type I GnRH receptor has previously been shown to exist in the cichlid. In the present study degenerate primers designed to extracellular loop three of the mammalian GnRH receptors were used to identify a second putative receptor subtype from cichlid (*Haplochromis burtoni*) genomic DNA. Furthermore, a near full-length cDNA, encompassing transmembrane domain 1 through to transmembrane domain 7 of the GnRH receptor, was cloned from cichlid RNA by reverse transcriptase PCR. This region of the receptor shares approximately 80% amino acid homology with corresponding regions of type III GnRH receptors previously identified in species of perciform fish.

Partial sequences of a type IA and a type IB GnRH receptor have previously been identified in the zebrafish. Two sets of degenerate primers were used to elucidate the possible existence of a third receptor in the zebrafish using both genomic DNA and RNA. However, this strategy failed to result in the amplification of novel receptor subtypes in the zebrafish.

Controversy surrounds the developmental origins of GnRH neurons and their temporal expression in relation to GnRH receptors. The zebrafish is a model organism, widely used

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for the study of reporter gene expression during development. Hence an attempt was made to isolate the zebrafish GnRH receptor genes using a genomic DNA library and identify the promoter regions for use as reporter genes in the study of GnRH and GnRH receptor expression during development. Southern blot analysis revealed six genomic clones with sequences homologous to zebrafish GnRH receptor cDNA. Comparison with genomic and cDNA sequences of other GnRH receptors revealed that those regions of the genomic clones that were sequenced only encoded exons 2 and 3. The presence of large introns in the GnRH receptor gene made it difficult to identify genomic clones containing the entire gene and the promoter region. The cloning of part of the zebrafish GnRH receptor genes will make their complete characterisation somewhat less problematic since an idea of their basic intron/exon structure has been obtained. Exons 2 and 3 of the zebrafish type IA and type IB GnRH receptor genes show a high degree of conservation when compared to the same regions of the goldfish type IA and type IB GnRH receptor cDNAs, demonstrating approximately 90% homology in both cases. In this study sequence information was obtained for the regions between transmembrane domains 4 and 7, and 3 and 7 of the zebrafish type IA and type IB GnRH receptor genes, respectively, and was subsequently used clone zebrafish GnRH receptor full-length cDNAs.

This study describes the discovery of a type III GnRH receptor in the cichlid but suggests its presence may be restricted to only certain orders of teleost since a type III receptor was not identified in the zebrafish on this occasion. The information acquired from this study may help to reveal patterns, which relate the presence of particular GnRHs and GnRH receptors in single species to specific reproductive requirements.

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Abbreviations

bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
cfGnRH	Catfish GnRH
cGnRHI	Chicken GnRH I
cGnRHII	Chicken GnRH II
cpm	Counts per minute
DAG	Diacylglycerol
dgGnRH	Dogfish GnRH
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EC	Extracellular loop
FSH	Follicle stimulating hormone
GAP	GnRH associated peptide
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GnRH-R	Gonadotropin-releasing hormone receptor
GPCR	G-protein coupled receptor
GSP	Gene specific primer
gpGnRH	Guinea pig GnRH
hGnRH	Herring GnRH
IC	Intracellular loop
$InsP_3$	1, 4, 5-triphosphate
kbp	Kilo base pair
lGnRH	Lamprey GnRH
LB	Luria broth
LH	Luteinising hormone
MB	Midbrain
mdGnRH	Medaka GnRH
mGnRH	Mammalian GnRH
mRNA	Messenger RNA
MW	Molecular weight
NGSP	Nested gene specific primer
PCR	Polymerase chain reaction
pfu	Plaque forming units
pGlu	Pyro-glutamate
PKA	Protein kinase A
PKC	Protein kinase C
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PLC	Phospholipase C
POA	Preoptic area
rGnRH	Rana GnRH
RNA	Riboxynucleic acid
RT-PCR	Reverse transcriptase PCR
SbGnRH	Seabream GnRH
sGnRH	Salmon GnRH
tGnRH	Tunicate GnRH
TM	Transmembrane domain
TN	Terminal nerve
UPM	Universal primer mix

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Introduction

GnRH and GnRH receptors

1.1 Introduction

This introduction aims to give a brief overview of the role of gonadotropin releasing hormone (GnRH) and the GnRH receptor. The structure, function, evolution, distribution and regulation of GnRH will be covered in this chapter. The GnRH receptor, with reference to its structure, function, signal transduction and expression will also be described.

1.2 Molecular forms of GnRH

GnRH is the primary regulator of reproduction in vertebrates. It is a decapeptide, synthesised and released from the hypothalamus into the hypophyseal portal circulation. It subsequently binds to specific receptors on the pituitary gonadotrope, resulting in the synthesis and release of the pituitary gonadotropins, luteinising hormone (LH) and follicle stimulating hormone (FSH) (Fink, 1988). GnRH was originally isolated from pig and sheep hypothalamus (Matsuo *et al*., 1971 and Amoss *et al*., 1971, respectively) and was found to be conserved in most mammalian species including the rat (Adelman *et al*., 1986), human (Adelman *et al*., 1986) and mouse (Seeburg *et al*, 1987). Mammalian GnRH (mGnRH), as it was subsequently termed, was thought to be the solitary form of this peptide until the existence of GnRH structural variants was demonstrated in nonmammalian brains (King and Millar, 1979). At present, fifteen structural variants of GnRH have been identified and have been named after the species from which they were first isolated. Though if the GnRH-like mature peptide, identified in the common octopus (*Octopus vulgaris*) is to be included, a total of sixteen GnRH variants have in fact been identified (Iwakoshi *et al*., 2002). Most vertebrate species express at least two, and often three forms of GnRH (see reviews King and Millar, 1997 and Sealfon *et al*., 1997). This, along with other evidence, suggested GnRH may have other functions besides that of regulating gonadotropin release (see reviews King and Millar, 1997 and Millar *et al*., 1997).

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Chicken GnRH II (cGnRHII) is the most conserved, and possibly the most ancient, form of GnRH. It is expressed in all classes of vertebrates studied to date, along with the more species-specific form of GnRH and even, in some cases, a third form. The species-specific form varies greatly, for example chicken GnRH I (cGnRHI) is found in species of birds and reptiles, mammalian GnRH (mGnRH) in mammals and amphibians and dogfish GnRH (dgGnRH) in sharks. A unique form has been identified in the guinea pig (gpGnRH), in place of mGnRH (Grove-Strawser *et al.*, 2002). So far, the presence of a third form of GnRH has been only demonstrated in a few species, mainly species of bony fish, for example cGnRHII, seabream GnRH (sbGnRH) and salmon GnRH (sGnRH) coexist in the cichlid, *Haplochromis burtoni* (White *et al.*, 1998). The most recently discovered form of GnRH, rana GnRH (rGnRH), was isolated from the species of frog, *Rana dybowskii*, along with cGnRHII and mGnRH (Yoo *et al.*, 2000). A mammalian species, the capybara (*Hydrochaeris hydrochaeris*), is reported to also express three forms of GnRH: mGnRH, sGnRH and cGnRHII (Montaner *et al.*, 1998 and 1999). It is feasible that in the future many more species will be found to synthesise multiple forms of GnRH.

GnRH	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Chicken II	**pGlu**	**His**	**Trp**	**Ser**	**His**	**Gly**	**Trp**	**Tyr**	**Pro**	**Gly-NH_2**
Mammalian	pGlu	His	Trp	Ser	**Tyr**	Gly	**Leu**	**Arg**	Pro	Gly-NH_2
Rana	pGlu	His	Trp	Ser	**Tyr**	Gly	**Leu**	**Trp**	Pro	Gly-NH_2
Catfish	pGlu	His	Trp	Ser	His	Gly	**Leu**	**Asn**	Pro	Gly-NH_2
Salmon	pGlu	His	Trp	Ser	**Tyr**	Gly	Trp	**Leu**	Pro	Gly-NH_2
Herring	pGlu	His	Trp	Ser	His	Gly	**Leu**	**Ser**	Pro	Gly-NH_2
Medaka	pGlu	His	Trp	Ser	**Phe**	Gly	**Leu**	**Ser**	Pro	Gly-NH_2
Seabream	pGlu	His	Trp	Ser	**Tyr**	Gly	**Leu**	**Ser**	Pro	Gly-NH_2
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	**Leu**	Pro	Gly-NH_2
Chicken I	pGlu	His	Trp	Ser	**Tyr**	Gly	**Leu**	**Gln**	Pro	Gly-NH_2
Guinea Pig	pGlu	**Tyr**	Trp	Ser	**Tyr**	Gly	**Val**	**Arg**	Pro	Gly-NH_2
Lamprey I	pGlu	His	**Tyr**	Ser	**Leu**	**Glu**	Trp	**Lys**	Pro	Gly-NH_2
Lamprey III	pGlu	His	Trp	Ser	His	**Asp**	Trp	**Lys**	Pro	Gly-NH_2
Tunicate I	pGlu	His	Trp	Ser	**Asp**	**Tyr**	**Phe**	**Lys**	Pro	Gly-NH_2
Tunicate II	pGlu	His	Trp	Ser	**Leu**	**Cys**	**His**	**Ala**	Pro	Gly-NH_2

Figure 1A - Comparison of the amino acid sequence of the fifteen naturally occurring GnRH forms. The amino acids printed in red are variable regions, with respect to chicken GnRHII.

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GnRH controls reproductive function in molluscs (Zhang *et al.*, 2000) and protochordates (King and Millar, 1992) and has been referred to as a functionally old peptide (Dubois *et al.*, 2002). All fifteen forms of GnRH are decapeptides in which the NH_2- and COOH-terminal sequences are highly conserved along with amino acids in positions 4 and 9. Position 8 is most variable followed by positions 5 and 7 with less variation seen in position 6. Two forms of GnRH have unique substitutions relative to all other forms of GnRH. These are position 2 of gpGnRH and position 3 of Lamprey GnRH I (see Fig 1A). Further structural diversity of GnRH is found in the form of hydroxyproline mGnRH (Gautron *et al.*, 1991). This post-translational modification of the GnRH peptide has been demonstrated in frog and rat brains (Gautron *et al.*, 1991). It is expressed in relatively high levels in mammalian fetal brain where the modification may be necessary to maintain the peptide in an inactive state (Gautron *et al.*, 1991).

1.3 The GnRH gene

The GnRH gene consists of three introns and four exons. This basic modular structure is common in all genes encoding the different forms of GnRH. The nucleotide sequence of the gene encoding the GnRH preprohormone has been determined for several different forms of GnRH including mGnRH (Seeburg and Adelman 1984 ; Adelman *et al.*, 1986), cGnRHII (Bogerd *et al.*, 1994, Gothilf *et al.*, 1995 and White *et al.*, 1995), sGnRH (Bond *et al.*, 199, Klungland *et al.*, 1992 and Gothilf *et al.*, 1995), cGnRHI (Dunn *et al.*, 1993), catfish GnRH (Bogerd *et al.*, 1994) and seabream GnRH (Gothilf *et al.*, 1995 and White *et al.*, 1995). The second, third and part of the fourth exon encode the GnRH preprohormone. This preprohormone consists of a signal peptide (21 to 23 peptides), the GnRH peptide, a cleavage site (Gly-Lys-Arg) and the GnRH-associated peptide or GAP (40-60 amino acids) (King and Millar, 1992). The second exon is relatively conserved compared to the first, third and fourth exons. The GnRH hormone and Gly-Lys-Arg processing site are highly conserved but the GnRH-associated peptide shows less homology among species (Dubois *et al.*, 2002). The exact function of the GnRH-associated peptide is unclear. It has been suggested that it plays a role in the inhibition of prolactin (Nikolics *et al.*, 1985) or as gonadotropin releasing factor (Millar *et al.*, 1986).

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1.4 Evolutionary aspects of GnRH

GnRH has been identified in vertebrates that evolved over 500 million years ago. The structure of GnRH may have diverged but the peptide has been highly conserved throughout evolution indicating the importance of GnRH in the control of reproduction in all vertebrates. The presence of more than one form of GnRH in a single species and the fact that they are transcribed from separate genes, suggests that the ancestral GnRH gene underwent duplication early in vertebrate evolution (King and Millar, 1994 and Sherwood *et al.*, 1997). A model was proposed based on there being two main lineages in the evolution of GnRH; the cGnRHII lineage and the mammalian lineage giving rise to the other forms of GnRH (King and Millar, 1992). More recently, the model has been revised to accommodate three evolutionary distinct lineages based on their distribution in the brain of a single vertebrate species (White *et al.*, 1998 and Fernald and White, 1999). They are referred to as the hypothalamic form or GnRH1, the form localised in the midbrain or GnRH2 and GnRH3 which is present in the terminal nerve. The species-specific form or GnRHI includes mGnRH, sbGnRH, gpGnRH and cGnRHI. They have a predominantly hypophysiotropic function, regulating LH release from the pituitary. The conserved cGnRHII (GnRH2) is reported to function as a neurotransmitter. Salmon GnRH is classed as GnRH3. A separate lineage has been proposed for sGnRH, which originated during teleost evolution, and may be the original member of a new evolutionary GnRH lineage (Dubois *et al.*, 2002) (refer to figure 1B). The tunicate and lamprey GnRH genes are still to be cloned but it is proposed that they precede cGnRHII in evolution (Dubois *et al.*, 2002). Dogfish GnRH differs from cGnRHII by a single amino acid and probably diverged directly from the cGnRHII lineage, whereas rana, guinea pig, chicken I, medaka and seabream GnRH are more closely related to mammalian GnRH. Based on their localisation in the brain (ventral forebrain) and predominantly hypophysiotropc function, herring, catfish, seabream and medaka GnRH are more likely to be derived from the mammalian GnRH lineage. Analysis of the sGnRH precursor gene and its unique location in the terminal nerve suggests a new evolutionary line may be more applicable to this form (Okubo *et al.*, 2000; Wang *et al.*, 2001 and Dubois *et al.*, 2002).

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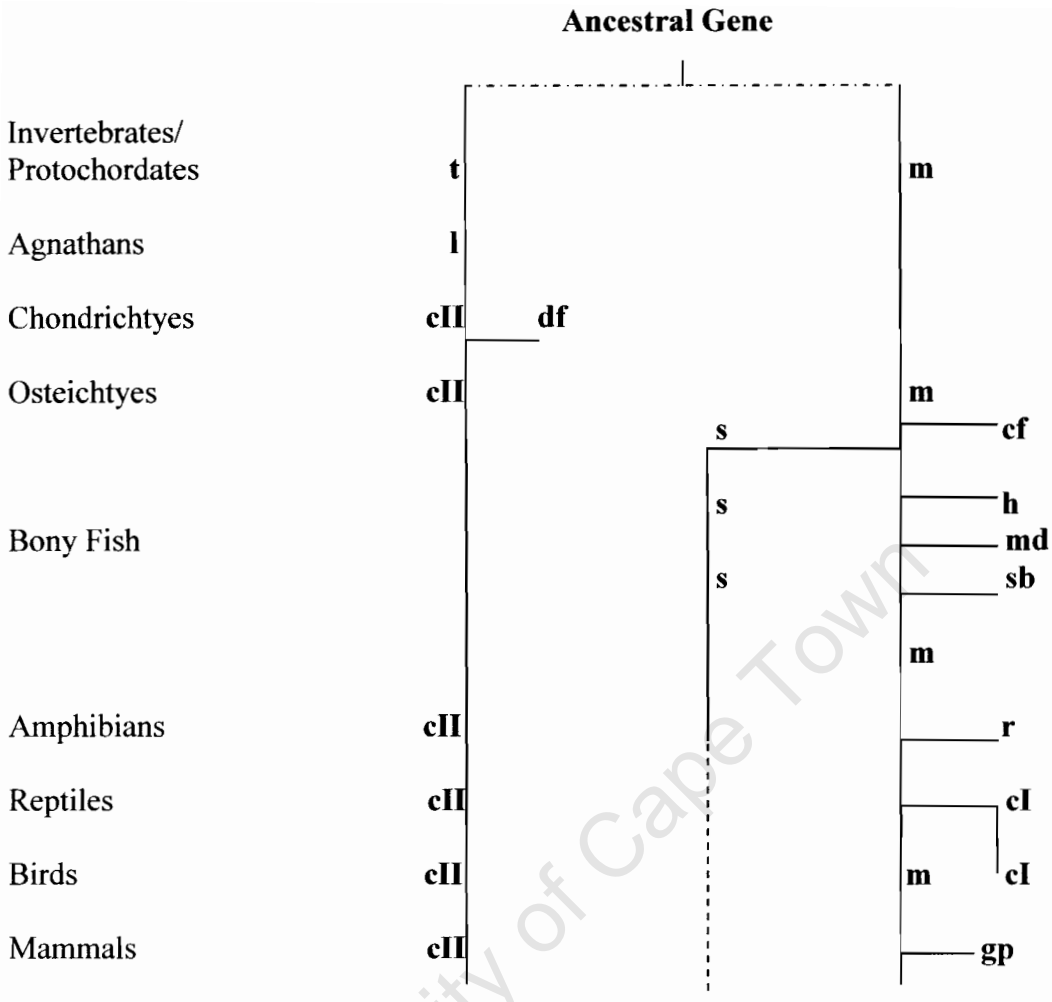

Fig 1B - Dubois *et al.* (2002) proposed a hypothetical tree for the evolution of GnRH incorporating the three lineages of GnRH, the conserved cGnRHII lineage, the species specific mammalian lineage and the salmon lineage. Broken lines represent uncertain origins. t, tunicate GnRH; l, lamprey GnRH; cII, chicken GnRHII; dg, dogfish GnRH; m, mammalian GnRH; c, catfish GnRH; h, herring GnRH; md, medaka GnRH; sb, seabream GnRH; r, rana GnRH; cI, Chicken GnRHI; gp, guinea pig GnRH.

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1.5 Distribution and function of GnRH

The two or three forms of GnRH are differentially localised in the brain. The more species-specific form of GnRH (GnRH1), which predominates in the hypothalamus, is thought to be the primary regulator of gonadotropin release from the pituitary. The conserved cGnRHII (GnRH2) is localised in the midbrain. If a third form is present in the

terminal nerve (TN), usually sGnRH, it is referred to as GnRH3 (White and Fernald, 1999).

It has been proposed that the more species-specific form (GnRH1) is the primary form involved in regulating gonadotropin release due to its presence in the pituitary at higher concentrations, however, when present, all three native GnRHs are powerful LH secretogogues (Powell *et al.*, 1995). In the African cichlid fish, *H. burtoni*, reproductive maturation is suppressed in non-territorial males. These non-territorial males show elevated cortisol levels resulting from an increase in stress due to aggressive interactions with dominant males. In territorial males, decreased cortisol levels are related to the size of sbGnRH neurons, which are reportedly 8 times larger in territorial males (Fox *et al.*, 1997).

The role of GnRH2 is not fully understood. *In vitro* GnRH2 is an extremely efficient stimulator of gonadotropin release (Bosma *et al.*, 2000) but *in vivo*, the pituitary levels are low, which may be necessary due to its high potency. Originally, the presence of GnRH2 was only demonstrated in primitive placental mammals, for example cGnRHII and mGnRH were identified in the brains of the musk shrew and tree shrew (Dellovade *et al.*, 1993 and Kasten *et al.*, 1996, respectively). Subsequently, GnRH2 has been identified in the brains of primates (Lescheid *et al.*, 1997) and humans (White *et al.*, 1998). The high degree of selective pressure on GnRH2 suggests a broad range of actions and target cells. GnRH2 is known to be expressed in the gonads (Yu *et al.*, 1998) and GnRH2 cells are responsive to feedback of sex steroids (Montero *et al.*, 1994). It is likely that GnRH2 is also involved in reproductive behaviour and has been shown to be more effective than GnRH1 at promoting reproductive behaviour in several species (King and Millar, 1997). In the European sea bass, GnRH2 producing neurons are present as early as four days post hatching, before differentiation of the gonads, and remain functional whilst migrating from their origin in the synencephalon to the midbrain, thus indicating an essential role in early development (González-Martínez *et al.*, 2002). The number of GnRH2 producing neurons is seen to decrease in adulthood which has also been observed in tadpoles which tend to have increased GnRH2 levels compared to adult frogs (Muske and Moore, 1990).

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The selective pressure on GnRH2 alone strongly supports evidence that besides reproduction, GnRH2 also functions as a neurotransmitter (King and Millar, 1994). Neuromodulation by cGnRHII has been demonstrated in the sympathetic ganglion neurons of amphibians (Troskie *et al.*, 1997) and has been implicated in the modification of sensory-motor activity (White *et al.*, 1995). GnRH has also been shown to increase the excitability of olfactory receptor neurons and possibly intensify odorant sensitivity during the mating season (Eisthen *et al.*, 2000). The expression of GnRH2 in the immune system, particularly in mast cells has been demonstrated (Silverman *et al.*, 1994, Rissman *et al.*, 1995 and Marchetti *et al.*, 1996). On the basis of these results, it was proposed that GnRH2 originated in the immune system and evolved its role as a neuromodulator in the brain (White *et al.*, 1998).

The exact function of GnRH3 is unclear. However, its location, at the junction of the olfactory nerve and telencephalon, suggests involvement in the relation of sensory information to reproductive behaviour (Yamamoto *et al.*, 1997).

Many other possible functions have been proposed for this versatile peptide. In the goldfish, *Carassius auratus*, GnRH has been shown to stimulate growth hormone (GH) release in addition to gonadotropin release demonstrating co-ordination between reproduction and growth (Marchant *et al.*, 1989 and Klausen *et al.*, 2001). An increase in GnRH levels has been demonstrated in the brain of sea lampreys undergoing spontaneous metamorphosis and is thought to be essential for its success (Youson and Sower, 2001). In the perciform teleost, *Oreochromis mossambicus*, sGnRH functions as a prolactin-releasing factor (Weber *et al.*, 1997) and in striped bass, prolactin producing cells were identified in the pituitary immediately after the emergence of the first sGnRH neurons (González-Martínez *et al.*, 2002). There is evidence that GnRH plays a paracrine role in the regulation of steroidogenesis (White *et al.*, 1998). In goldfish testis, GnRH has been shown to increase DNA fragmentation which is indicative of apoptosis and may help regulate testicular maturation and regression (Andreu-Vieyra and Habibi, 2001). The autocrine role of GnRH and the presence of GnRH binding sites has been demonstrated in endometrial, ovarian, breast and prostate tumours (Gründker *et al.*, 2002).

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When three forms of GnRH are present in a single species, for example, in perciforms, which expresses, sGnRH, sbGnRH and cGnRHII, a distinct expression pattern has been observed. Salmon GnRH predominates in the cells of the olfactory bulb, sbGnRH in the pre-optic area, whose neurons project into the pituitary and cGnRHII in the dorsal synencephalon (Gothilf *et al.*, 1996; Okuzawa *et al.*, 1997; White *et al.*, 1998). The origin of GnRH neurons is unclear. Original evidence from studies in amphibians, birds and mammals suggested that all GnRH neurons develop in the olfactory placode and migrate to the hypothalamus (Wray *et al.*, 1989 and Muske *et al.*, 1994). In the European sea bass, sGnRH and sbGnRH producing neurons have been shown to have a common origin in an olfactory primordium whereas cGnRH producing neurons develop from a synencephalic primordium (González-Martínez *et al.*, 2002). However, the consensus seems to suggest that although sGnRH neurons originate in the olfactory placode, sbGnRH neurons differentiate from the preoptic area (Gothilf *et al.*, 1996 and White and Fernald, 1998). The functional significance of a species having three forms of GnRH is unclear but it may result in a more refined control of reproduction offering an evolutionary advantage.

1.6 Regulatory aspects of GnRH

Regulation of GnRH synthesis is controlled at the transcriptional and translational level, in addition to being under the influence of various feedback mechanisms. The peptide is primarily synthesised in the hypothalamus but also in a number of peripheral tissues. Characterisation of the 5' flanking region of the both rat and human GnRH genes has provided information regarding the regulation of GnRH gene expression. Enhancer regions have been identified which bind transcription factors necessary for neuron-specific expression of the GnRH gene and confer tissue specificity (Nelson *et al.*, 1998). The human 5' flanking region has been shown to contain 2 major regulatory elements, one which is active in the hypothalamus and a distal regulatory element which is active in reproductive tissues (Dong *et al.*, 1997).

The pulsatile manner by which GnRH is released from hypothalamic stores is necessary to maintain gonadotropin levels required at various reproductive states. The phasic release of

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GnRH results in the pulsatile release of gonadotropins important for activation of the gonads. The frequency and amplitude of the GnRH pulse has been shown to affect GnRH receptor mRNA expression, where continuous GnRH secretion results in down regulation of receptor expression (Plosker and Brogden, 1994). Although not fully understood, the ability to release GnRH in a pulsatile manner seems to be an intrinsic property of the GnRH neurons themselves and not under hypothalamic control. This pulsatile release of GnRH seems to be under the control of various inhibitory and excitatory amino acids, the release of which is regulated by steroid hormones (see review by Brann and Mahesh, 1997). Glutamate is thought to be the major stimulatory force behind the GnRH surge along with nitric oxide, neuropeptide Y and galanin. This excitatory effect is negated by opioid peptides such as neuropeptide K, effective during nonsurge conditions. When steroid hormone levels in the brain increase, the inhibitory effect of opioids is lifted and the excitatory effect of glutamate is exerted (see review by Brann and Mahesh, 1997).

The regulation of GnRH secretion appears to be dependent on many factors. For example, it has been demonstrated that in a single species, the stimulation of LH release by one form of GnRH can be inhibited by another from of GnRH (Bosma *et al.*, 2000). There is also evidence of GnRH and LH being secreted simultaneously indicating the presence of an autoregulatory feedback mechanism involving GnRH. Testosterone has been shown to effect up-regulation of mGnRH mRNA at different rates depending on the mRNA species concerned and reproductive status (Okubo *et al.*, 2002 and Dubois *et al.*, 1998). In the pituitary, locally produced GnRH may be involved in maintaining sensitivity of the pituitary gonadotrope to hypothalamic GnRHs, creating a baseline stimulation between pulses (Krsmanovic *et al.*, 2000). This priming effect has been shown to enhance the effects of subsequent stimulation by exogenous GnRH (Fink *et al.*, 1976).

1.7 Structure-activity relations of GnRH

The binding of a ligand to its receptor is influenced by the affinity of the ligand for its receptor and by its ability, once bound, to activate the receptor. Where GnRH is concerned, amino acid composition and conformation play a critical role in receptor binding. As a highly flexible peptide, GnRH is capable of existing in multiple

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conformational states, few of which retain their biological activity. It has been proposed that a bend in the middle of the GnRH peptide is essential for maintaining a biologically active state (see reviews Flanagan *et al.*, 1997 and Sealfon *et al.*, 1997). The highly conserved residue, Gly^6, promotes this flexibility and hence the 'bend' by being relatively small. The substitution of the Gly^6 residue with a D-amino acid (Monahan *et al.*, 1973), or incorporating a γ-lactam ring at the Gly-Leu peptide bond, has been shown to confine the peptide to its active state thus enhancing binding and increasing its potency (Freidinger *et al.*, 1980). However, the effectiveness of this constraint is more pronounced with the mammalian GnRH receptor when compared to the chicken receptor emphasising conformational preference with respect to species (Millar *et al.*, 1986).

Although all fifteen natural GnRHs are capable of stimulating gonadotropin release there are significant differences in their potencies. The mammalian GnRH receptor shows extreme preference for mammalian GnRH and demonstrates low specificity for all other natural GnRHs except cGnRHII (King and Millar, 1994). In comparison, non-mammalian GnRH receptors show comparable binding affinity with most vertebrate GnRHs.

All naturally occurring GnRH peptides have been extremely well conserved with respect to their length, NH_2- and COOH- terminal domains, signifying the functional relevance of these particular features. The variation seen within the central domain is obviously related to receptor/ligand selectivity. Several thousand synthetic GnRH variants have been studied, having both agonistic and antagonistic properties. They have provided valuable information regarding the importance of individual residues (see review Sealfon et al., 1997).

The consequence of substituting pGlu in the NH_2-terminal sequence is an almost total loss of activity. If it is replaced with either a residue with a similar cyclic structure or D-pGlu, some restoration of activity is observed (for review see Sealfon *et al.* 1997).

The aromatic amino acid histidine, in position 2 of the peptide, is thought to be essential for proper receptor binding/activation especially the mammalian receptor. Reduced

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activity is seen as a result of residue substitution. The unique replacement with Tyr^2 in gpGnRH lends support to the theory that an aromatic residue is required in this position. It has been proposed that the electropositive Lys^{121} of the GnRH receptor, necessary for receptor function, requires an electron dense aromatic residue for the formation of H-bonds (Flanagan *et al.*, 1997).

Trp^3 is conserved in all but one of the natural GnRH peptides. In Lamprey I GnRH, Tyr replaces Trp i.e. the substitution of one aromatic amino acid for another. The formation of π-π complexes between aromatic molecules are critical for receptor-ligand interactions. Substitutions in position 3 with non-aromatic residues result in an almost total loss of activity (Sealfon *et al.*, 1997).

There is no variation in position 4, Ser being invariant among the fifteen natural GnRH peptides. A relatively small residue seems to be required but generally, replacements are well tolerated. Substitution with large amino acids, primarily those with large side chains, does result in some loss of activity possibly due to spatial constraints within the receptor-binding pocket (Sealfon *et al.*, 1997).

Position 5 exhibits high amounts of variation between the natural GnRHs. Substitutions here are tolerated with little or no loss of activity. Along with residues in positions 7 and 8, the variable domain is responsible for receptor/ligand selectivity (Sealfon *et al.*, 1997).

As previously mentioned, Gly^6 lends flexibility to the GnRH peptide and is only substituted in lamprey and tunicate GnRHs. This may be due to the tunicate and lamprey receptors having different conformational requirements (Sealfon *et al.*, 1997).

Substitutions in position 7 are well tolerated as would be expected due to the high degree of variation seen among natural GnRHs in this position. Amino acids with large bulky side chains are preferred in this position and have been shown to result in increased LH secretion in sheep (Millar *et al.*, 1989).

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Position 8 demonstrates the most variation, although Arg^8 in mammalian GnRH is vital for high binding affinity with the mammalian receptor (Millar and King, 1983). Glu^{301} in extracellular loop III of the mammalian receptor is important in the recognition of Arg^8 and this interaction appears to be responsible for stabilising the ligand in a high-affinity conformation (Flanagan *et al.*, 1997).

Substitutions of Pro^9 result in a considerable loss of activity and it has been proposed that Pro^9 imposes necessary conformational restraint upon the GnRH peptide (see review Sealfon *et al.*, 1997).

Although highly conserved, Gly-NH_2 can be substituted without consequential loss of activity. Replacement with ethylamide for example can result in a substantial increase in the activity (Arimura *et al.*, 1974).

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1.8 The GnRH receptor

The isolation of the αT3 mouse gonadotrope cell line was the turning point that resulted in the cloning of the mouse GnRH receptor cDNA (Tsutsumi *et al.*, 1992). The GnRH receptor was characterised and consequently identified as being a member of the G-protein coupled receptor (GPCR) superfamily. Presently, other mammalian GnRH receptors have been cloned including rat (Eidne *et al.*, 1992), human (Kakar *et al.*, 1992), cow (Kakar et al., 1993), sheep (Brooks *et al.*, 1993 and Illing *et al.*, 1993), pig (Wesner and Matheri, 1994) and primate (Millar et al., 2001, Neill et al., 2001). More recently, full-length GnRH receptor sequences have been obtained for non-mammalian species including the African catfish (Tensen *et al.*, 1997), Striped bass (Alok *et al.*, 2000), *Xenopus laevis* (Troskie *et al.*, 2000) Rainbow trout (Madigou et al., 2000) and cDNAs encoding two receptor subtypes have been characterised in the goldfish (Illing *et al.*, 1999) and the Medaka (Okubo et al., 2001). Also, Wang *et al.* (2001) identified three distinct GnRH receptor types in the bullfrog.

1.9 Classifying the GnRH receptor

The evolution of the various GnRH analogues occurred concomitantly with the evolution of diverse forms of the GnRH receptor (see review Sealfon *et al.*, 1997). The known GnRH receptors can be separated into two groups; Type I GnRH receptors which have a greater affinity for GnRHI than GnRHII and include all identified mammalian receptors, and Type II GnRH receptors which demonstrate preference for the conserved GnRH2 and include the non-mammalian receptors. Conversely, the recently cloned GnRH receptor from the marmoset is an example of a mammalian GnRH receptor highly selective for GnRHII (Millar *et al.*, 2001), signifying the generalisation of this terminology. An alternative method of classification was proposed based primarily upon differences in the amino acid composition of extracellular loop 3 (EC3) of the GnRH receptor (Troskie *et al.*, 1998). Extracellular loop III is known to be important for ligand selectivity and similarities in this region may relate to receptor type. Using this model, the GnRH receptors can be one of three subtypes, type IA, type IB or type II. However, the cloning of the Type II receptor in species of primate (Millar *et al.*, 2001 and Neill *et al.*, 2001), the presence of three receptor subtypes in the bullfrog (Wang *et al.*, 2001) and a unique receptor subtype being isolated in the medaka (Okubo *et al.*, 2001) and striped bass, (Alok *et al.*, 2000) has impelled the inclusion of an additional GnRH receptor type. This alternative categorisation is based on whether or not the particular GnRH receptor has a C-terminal tail and the presence or absence of a third intron in the GnRH receptor gene (Okubo *et al.*, 2001). The first subtype includes GnRH receptors possessing a C-terminal tail but lacking intron A for example the medaka GnRH-R1, bullfrog receptors 1 and 3, the striped bass GnRH receptor and the type II primate GnRH receptors. The second GnRH receptor subtype encompasses GnRH receptors having both a C-terminal tail and intron A, for example, the medaka GnRH-R2, goldfish type IA and IB, bullfrog 2, catfish, *X. laevis* and chicken GnRH receptors. The remaining mammalian receptors are included in the third subtype, deficient in both a C-terminal tail and intron A. This system of classification is appropriate to this particular study and places the known GnRH receptors in to three distinct groups. However, it is confusing because the numbering of the receptors, according to the original literature, varies between members of a particular group, for example, Medaka 1 appears in the same lineage as Bullfrog 3, African green

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monkey II and cichlid III. These problems will only be overcome when a method of GnRH receptor classification is agreed upon and used exclusively. For the purpose of this thesis, GnRH receptors will be referred to as either mammalian type I receptors for example the human type I receptor, non-mammalian type I receptors i.e. goldfish IA and IB, bullfrog 2 and cichlid type I GnRH receptors, type II receptors which include the primate GnRH receptors and type III non-mammalian GnRH receptors for example striped bass, bullfrog 1 and amberjack receptors. Alternatively, GnRH receptors will be termed according to how they are cited in the relavant reference.

1.10 G-protein coupled receptors

GPCRs are known under this name due to their ability to activate heterotrimeric G-proteins resulting in the activation of multiple signal transduction pathways and a diverse range of cellular responses. Molecular cloning of GPCRs has categorised them as belonging to three separate groups; the metabotropic glutamate receptors, the secretin-calcitonin-PTH receptors and the rhodopsin-like GPCR family, of which the GnRH receptor is a member. Collectively, GPCRs are responsible for the transduction of many important neural and endocrine signals.

Members of the three GPCR families do not share a huge amount of sequence homology. Their common characteristic feature is having a single amino acid chain comprising seven hydrophobic domains. The seven hydrophobic membrane-spanning domains are arranged in a α-helical conformation around a hydrophilic core and need to be at least 18 amino acids in length to cross the membrane. The amino acid homology is greatest within the transmembrane domains where conserved motifs can be used to assign a GPCR to a particular family. The transmembrane helices are unified through intracellular and extracellular loops. Both transmembrane domains and extracellular loops of GPCRs contain residues necessary for ligand binding whilst intracellular loops are important for interactions with specific G-proteins.

Consensus post-translational modifications of GPCRs include N-linked glycosylation, phosphorylation of intracellular serine and threonine residues and the formation of

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disulphide bridges between conserved cysteines in first and second extracellular loops (see review Sealfon *et al.*, 1997). Receptor expression, responsiveness and stability are influenced by these modifications.

1.11 Mechanisms of GnRH induced signal transduction

GnRH exerts its effect as a gonadotropin releasing factor via GnRH receptors on the gonadotropes in the anterior pituitary. Upon binding of GnRH, various intracellular signalling events are mediated via the GnRH receptor and its associated $G_{q/11}$ family of G-proteins, resulting in the synthesis and release of the pituitary gonadotropins. Ligand binding to the GnRH receptor initiates a conformational change in the receptor, resulting in the dissociation of the heterotrimeric G-protein into α-subunits and $\beta\gamma$-subunits and subsequent activation of phospholipase C (PLC) (see review Stojikovic *et al.*, 1994). The increase in PLC activity results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and the formation of inositol phosphate ($InsP_3$) and diacylglycerol (DAG). GnRH stimulation of $InsP_3$ accumulation is necessary for the generation of an intracellular calcium spike-plateau (see review McArdle *et al.*, 2002). The spike phase is generated from $InsP_3$-mediated mobilisation of calcium from intracellular stores, whereas the plateau phase is a result of an $InsP_3$ independent influx of calcium through voltage sensitive calcium channels. A feedback mechanism seems to be in place where low GnRH concentrations produce calcium oscillations which, although capable of up-regulating GnRH receptor and LH subunit gene expression, do not result in gonadotropin release (Krsmanovic *et al.*, 2000). When GnRH concentrations increase, calcium spike-plateau conditions come into play which result in the release of gonadotropins but decrease gene expression. The combination of calcium release and DAG production results in the activation of protein kinase C (PKC) which exerts positive feedback, maintaining levels of DAG. PKC is also involved in the integration of various phospholipases into the signalling cascade and is essential for gonadotropin release (see review Stojikovic *et al.*, 1994).

1.12 Characteristic features of the GnRH receptor

Although retaining many traits of the GPCRs, which have aided its characterisation, the GnRH receptor has unique attributes. Dissimilar to any other member of the rhodopsin

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GPCR family, the mammalian Type I GnRH receptors lack a carboxy-terminal tail. This characteristic feature was thought to have been conserved throughout the mammalian GnRH receptors, however, the recently identified Type II primate receptors posses a C-terminal tail (Millar *et al.*, 2001 and Neill *et al.*, 2001). Sustained stimulation of GPCRs results in rapid desensitisation, mediated by phosphorylation of the carboxy-terminal tail (McArdle *et al.*, 1992). Desensitisation is a consequence of the receptor binding β-arrestin, preventing G-protein coupling and leading to subsequent internalisation of the receptor. Following internalisation, the receptor is either recycled or degraded (Hislop *et al.*, 2001). The mammalian GnRH receptor does not exhibit rapid desensitisation and whilst being subject to agonist-induced internalisation, the process occurs at a reduced rate. In contrast, the type II primate and non-mammalian GnRH receptors, which both possess a C-terminal tails, experience rapid desensitisation and internalisation (see review McArdle *et al.*, 2002). Threonine and serine residues within the C-terminal tail act as possible phosphorylation sites, implicated in receptor desensitisation and internalisation. Lack of receptor desensitisation may be beneficial regarding the generation of the preovulatory LH surge following an increase in exposure of the gonadotrophs to GnRH. It has been proposed that the lack of a C-terminal tail is a recent evolutionary adaptation resulting in the ability of the receptor to respond effectively to GnRH pulses necessary for mammalian physiological requirements. The increased length of the first intracellular loop of the mammalian receptor is thought to compensate somewhat for the absence of the C-terminal tail (Tsutsumi *et al.*, 1992). Alignment of all nine fully sequenced mammalian GnRH receptors shows complete conservation of the last fifteen residues of the carboxy terminus. Whether these amino acids replace the C-terminal tail with regard to desensitisation and internalisation is not fully understood. However, the presence of these conserved residues are essential with respect to conformation of the ligand binding site (Brothers *et al.*, 2002). Addition of the catfish GnRH receptor C-terminal tail to the rat receptor had no effect on binding but did increase levels of cell surface receptor expression (Lin *et al.*, 1998). Receptor regulation was also affected, probably as a result of an increase in the rate of internalisation. A monophasic pattern of regulation was observed as opposed to the usual biphasic pattern of regulation resulting from cyclical changes in GnRH concentration (Lin *et al.*, 1998).

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1.13 Post-translational modifications

The human GnRH receptor has two potential N-linked glycosylation sites, one in the amino terminal tail and the other in the first extracellular loop (EC1). The amino terminus of the rodent GnRH receptor has an additional N-linked glycosylation site. Site directed mutagenesis of potential glycosylation sites of the mouse GnRH receptor resulted in a decline in the level of expression and was primarily due to the loss of the amino terminal sites (Davidson *et al.*, 1995). An increase in the level of expression of the human GnRH receptor was observed when an extra glycosylation site was introduced into the amino terminus using the same technique (Davidson *et al.*, 1996).

The occurrence of Cysteine bridges is a conserved feature of most GPCRs. The presence of a disulphide bridge between cysteine residues in the first and second extracellular loops is believed to be important for stabilising the receptor in its functional state (see review Sealfon *et al.* 1997). The consequence of mutating these cysteine residues is a loss of function. A disulphide bridge is present between Cys^{114} and Cys^{196} of the mouse GnRH receptor and is conserved in other GnRH receptors although the positions vary slightly. There is evidence of a second bridge existing between Cys^{14} and Cys^{200} of the human receptor (Davidson *et al.*, 1997).

The positioning of interacting residues, Asp in transmembrane helix II and Asn in transmembrane helix VII, is known to be crucial for G-protein coupling and is conserved among GPCRs. In the GnRH receptor these residues are interchanged resulting in Asn^{87} in helix II and Asp^{318} in helix VII. Mutation of Asn^{87} to Asp^{87} results in a loss of receptor function which is restored when a second mutation is applied, Asp^{318} to Asn^{318}. By re-establishing the proximity of these residues, their interaction and consequent binding of G-protein is restored (Flanagan *et al.*, 1997).

1.14 The GnRH receptor binding site

Mutagenesis studies on GPCRs have helped expose some of the individual residues central to interactions that occur within the ligand-binding pocket. In many GPCRs, there

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appears to be a concentration of binding sites in the transmembrane domains (Strader *et al.*, 1995). Alignment of the Type I mammalian GnRH receptors reveals highest homology within the transmembrane helices supporting evidence of a role in ligand binding and conserved preference for GnRH1. In transmembrane helix III, Asp^{113} of other GPCRs, such as monoamine receptors, has the ability to form salt bridges with amine groups of the ligand. In the GnRH receptor this function appears to be performed by Lys^{121}. Substitution of Lys^{121} results in a loss of binding affinity for agonists but not antagonists (Zhou *et al.*, 1995). As GnRH agonists and antagonists differ primarily in their amino terminal region, this is the proposed site for the interaction between Lys^{121} and the GnRH peptide.

Arg^{8} of mammalian GnRH restricts the ligand in a conformation favoured by the mammalian GnRH receptor. In the mouse GnRH receptor, Glu^{301} appears to be significant in the recognition of this high affinity confirmation. Mutation of Glu^{301} to Gln results in a loss of affinity for GnRH analogues containing Arg^{8}. However, affinity is restored when the conformation of the GnRH peptide is constrained (Flanagan *et al.*, 1994). In the human and other nonrodent receptors the corresponding residue is Asp^{302} and is similarly responsible for determining selectivity for Arg^{8}-containing GnRH by induction of a high-affinity ligand conformation (Fromme *et al.*, 2001).

Asn^{102}, situated at the extracellular end of transmembrane domain II interacts with the glycine amide group at the C-terminal of the GnRH peptide sustaining high potency ligand-induced signal transduction (Davidson *et al.*, 1996). Mutations to Ala have resulted in a loss of potency whereas replacement with Gln increases the potency of ligand-induced signal transduction (Davidson *et al.*, 1996).

$Lys^{121}$of helix III, Asn^{102} of helix II and Glu^{301} of helix VII may be adjacent within the three dimensional structure of the GnRH receptor, thus forming a high affinity binding site for GnRH.

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1.15 Intracellular domains

The interaction between the GnRH receptor and associated G-proteins is mediated through the intracellular domains, particularly intracellular loops two and three. Located in the second intracellular loop of GPCRs is the highly conserved 'DRY' (Asp-Arg-Tyr) motif. In the mammalian Type I GnRH receptor, Tyr is substituted forming a 'DRS' (Asp-Arg-Ser) motif. In these positions, Asp^{138} and Arg^{139} are important for receptor expression, activation and internalisation (Arora *et al.*, 1997) and also binding. The Tyr/Ser residue may be a potential phosphorylation site, making it important for desensitisation and internalisation by acting as a substrate for G-protein receptor kinase. This is supported by evidence that mutating Ser^{140} to Tyr results in an increase in the rate of internalisation (Arora *et al.*, 1995). Replacement with Ser in the mammalian GnRH receptor may be an adaption to mammalian reproductive strategy, decreasing the rate of internalisation during surge conditions. In the marmoset GnRH receptor Ser^{140} is substituted with Phe and site directed mutagenesis of Ser to Phe in the rat resulted in an increase in the rate of internalisation (Byrne *et al.*, 1999). Tyr and Phe are aromatic amino acids whereas Tyr and Ser are potential phosphorylation sites. Possibly the aromatic nature of the residue is more essential than being a phosphorylation site where receptor internalisation is concerned. In the non-mammalian catfish GnRH receptor the Asp-Arg-Tyr motif becomes Asp-Arg-His, reiterating the theory that phosphorylation is not critical at this site (Tensen *et al.* 1997). However, the presence of a C-terminal tail in the catfish receptor may compensate a lack of phosphorylation sites.

1.16 GnRH receptor distribution

The distribution of GnRH receptors is relatively widespread. The pituitary and brain GnRH receptors have been well characterised and distinct patterns of expression are apparent. In the gonads, GnRH receptors are expressed in the Leydig cells, which are primarily responsible for the synthesis and release of testosterone. GnRH receptors have also been identified in the granulosa and luteal cells of the ovary and are present in placental tissue although they differ significantly from the pituitary receptor (see review Stojilkovic *et al.*, 1994). Expression of GnRH receptors has also been seen in certain hormone-dependent tumours including those of the testis, breast, endometrium, prostate

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and ovary (see review McArdle *et al.*, 2002). GnRH receptors isolated from these tumours appear to be identical to the pituitary receptor but may be functionally diverse. This is an intensive area of research as the use of various GnRH analogues has already been shown to inhibit the rate of proliferation of some tumour cells (Gründker *et al.*, 2002). GnRH receptor mRNA has been identified in a diverse range of rat tissue including heart, brain, lung, duodenum, small intestine, spleen, kidney, epididymus and skeletal muscle indicating a multifunctional role for GnRH and its receptor (Matsubara *et al.*, 1995).

1.17 GnRH receptor gene structure and regulation

The gene structure of the human and mouse GnRH receptor have been well defined, both being composed of three exons and two introns spanning 18.9kbp and 22kbp respectively (see review Stojilkovic *et al.*, 1994). The human GnRH receptor is encoded by 328 amino acids and the mouse receptor is 327 amino acids long, the additional residue of the human receptor is Lys^{191} in extracellular loop II. The organisation of various GnRH receptor genes is shown in figure 1C. Type I non-mammalian GnRH receptor gene identified in the medaka consists of four exons separated by three introns, termed introns A, B and C (Okubo *et al.*, 2001). The same basic structure is observed in the *X. laev*is Type I receptor gene however the size of the introns differ (Troskie *et al.*, 2000). Intron A is lost in the Type III non-mammalian and the mammalian GnRH receptor genes characterised to date. Introns B and C are retained but differ in size between species. Interestingly, the positioning of introns B and C are completely conserved in all the known GnRH receptor genes.

GnRH is actively involved in the regulation of its own receptor. Continuous GnRH administration results in decreased levels of receptor mRNA and subsequent reduction in receptor number, while pulsatile GnRH stimulation increases receptor expression. Therefore, as GnRH levels fluctuate during the estrous cycle, pregnancy or lactation, pituitary GnRH receptor levels and mRNA expression echo the effect (Bauer-Dantoin *et al.*, 1995). Glucocorticoids, thyroid hormones and sex steroids are also implicated in the regulation of the GnRH receptor (Nelson *et al.*, 1998).

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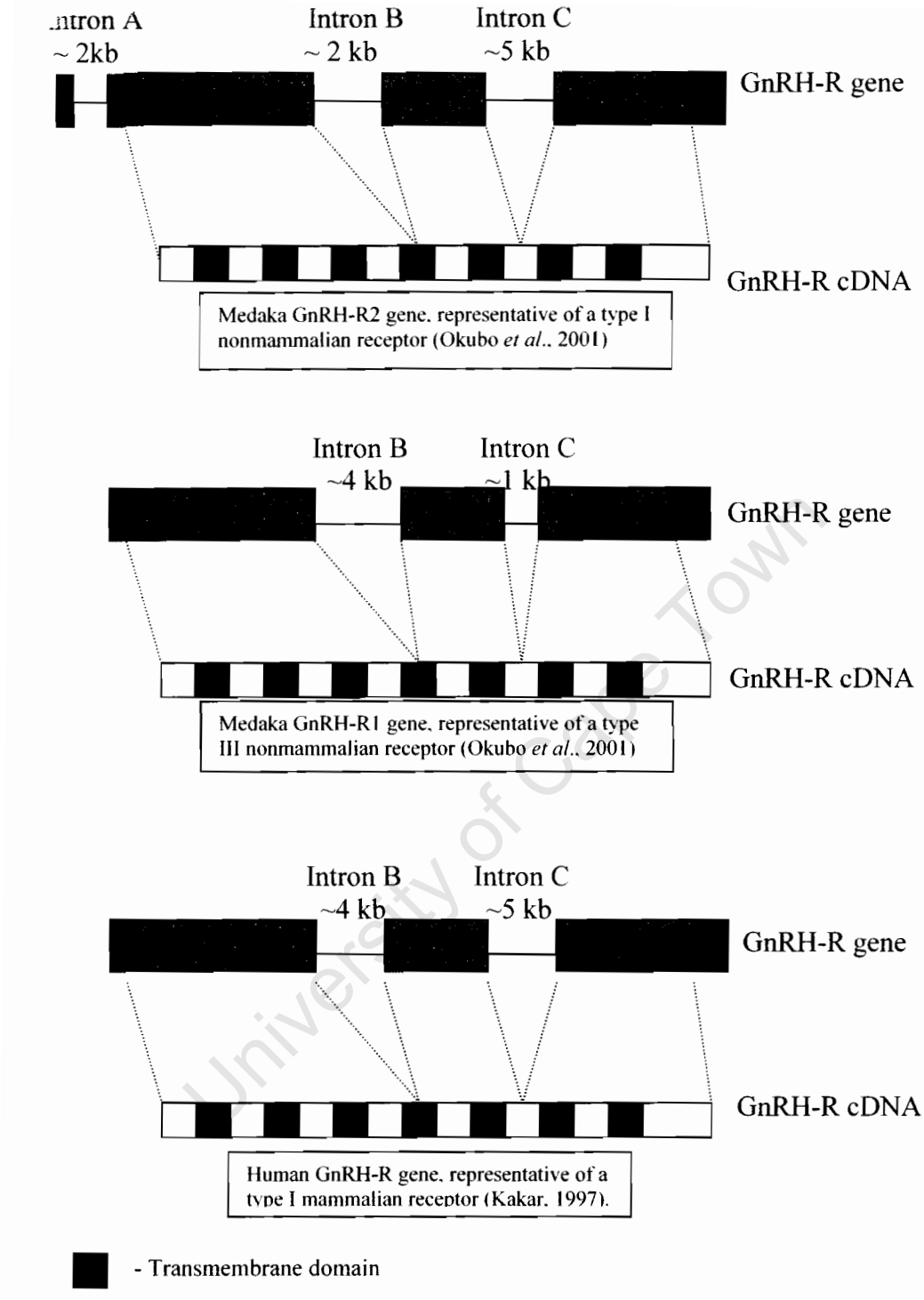

Fig.1C Schematic diagram illustrating the organisation of the medaka GnRH-R1, GnRH-R2 and human Type I GnRH receptors as a representation of non-mammalian and mammalian receptor genes, respectively.

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The promoter regions of the mouse (Albarracin *et al.*, 1994), rat (Pincas *et al.*, 1998) and human (Fan *et al.*, 1995) GnRH receptor genes have been characterised providing information concerning their transcriptional regulation. Key regulatory elements have been recognised within the promoter region such as SF-1 (steroidogenic factor), GRAS (GnRH receptor activity sequence), AP-1 and AP-2 (activating protein binding sites), GRE (glucocorticoid response element) and specific hormone response elements. GRAS has been identified as a activin response element and activin A in particular is known to enhance GnRH-mediated transcriptional activation of the mouse GnRH receptor gene (Norwitz *et al.*, 2002). GRAS, AP-1 and SF-1 motifs have also been implicated in mediating cell-specific expression (Duval *et al.*, 1997). SBE (SMAD binding element) SURG-1 (Sequence underlying responsiveness to GnRH-1) and Pit-1 sites have also been identified, the latter being critical for GnRH receptor gene expression (Kakar, 1997). Parts of the mouse, rat and human 5' flanking regions, which show high sequence homology, may be important for gonadotrope-specific expression (Pincas *et al.*, 1998).

1.18 Summary and Aims

There is still much information to be acquired on the subject of GnRH and GnRH receptors. Theories regarding the purpose of multiple GnRHs occurring in single species and the developmental origins of the distinct GnRH-producing neurons are conflicting. It is unclear whether certain GnRH receptors preferably bind certain endogenous GnRHs or are required to be present at specific stages of development for particular functions. Phylogenetic relationships seem to be emerging between groups of organisms and the types of GnRH receptors they possess. However, more information is required about ancestral GnRH receptor genes before the evolutionary significance can be realised. Bony fish demonstrate the greatest diversity with respect to the expression of GnRHs and GnRH receptor subtypes. Based on this information, this study aims to examine the presence GnRH receptor subtypes in species of teleost fish by:

1 Cloning and characterising putative GnRH receptor subtypes in the cichlid fish (*H. burtoni*).

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2 Isolating the GnRH receptor genes in the zebrafish (*Danio rerio*) to be used as reporter genes in developmental studies.

3 Identifing putative GnRH receptor subtypes in the zebrafish.

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Chapter 2

Molecular cloning of a putative Type III GnRH receptor in the cichlid (*H. burtoni)*

2.1 Introduction

In the African cichlid fish, *H. burtoni,* reproductive status is socially regulated. Territorial males are physiologically distinct having brightly coloured and aggressive markings. In contrast, non-territorial males have no distinctive colouring. Reproductive maturation of non-territorial males is suppressed, thus allowing dominant males to account for all reproductive successes. Fluctuations in environmental conditions can produce changes in social status i.e. territorial males becoming non-territorial and vice-versa. Consequent alteration of physiological characteristics and behaviour rapidly transpire. In addition to colouring, territorial males have larger testes and greater amounts of mature sperm (Francis *et al.*, 1993). In addition, the population of GnRH neurons found primarily in the preoptic area (POA) of the brain can be up to eight times larger in territorial males than in non-territorial males. Therefore, it is likely that GnRH and its receptor have a direct involvement in socially regulated reproduction in the cichlid.

The greatest diversity in the number of forms of GnRH present in the brain can be seen in fish. In teleosts, the conserved GnRH2 is present along with either catfish GnRH (cfGnRH), salmon GnRH (sGnRH) or mGnRH. Where identified, herring GnRH (hrGnRH), medaka GnRH (mdGnRH) or seabream GnRH (sbGnRH) occur as a third form. Three forms of GnRH have been identified in the brain of the cichlid and have been shown to have a distinct pattern of expression (White *et al.*, 1995 and Powell *et al.*, 1995). GnRH2 predominates in the anterior midbrain and synencephalon, sGnRH or GnRH3 in the terminal nerve ganglion including the olfactory nerve and telencephalon and sbGnRH (GnRH1) is found in the ventral forebrain encompassing the preoptic area and the pituitary. All three endogenous GnRH forms are capable of inducing gonadotropin release. However, sbGnRH predominates in the pituitary and is thought to be the main gonadotropin releasing GnRH in the cichlid (White *et al.*, 1995 and Powell *et al.*, 1995). It is possible that sbGnRH stimulates the release of gonadotropin I (FSH-like), gonadotropin II (LH-like) and growth hormone (GH). However, using the goldfish assay sbGnRH was shown to be ineffective at stimulating

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gonadotropin II release and only slightly more efficient at stimulating GH release. This may be due to differences in GnRH receptor types in the goldfish compared to cichlid or seabream, though *in vivo* injection of sbGnRH in seabream showed low potency gonadotropin II release compared to GnRH3 and GnRH2. In the ancient teleost herring, hrGnRH is the primary form found in pituitary and effectively stimulates the release of gonadotropin II. Herring GnRH is also capable of releasing GH, although with less potency than the other endogenous GnRHs, sGnRH and GnRH2 (Carolsfeld *et al.*, 2000). The presence of multiple GnRH forms in a single organism and the distinct differences in the potencies of endogenous GnRHs with respect to gonadotropin and GH release suggests that an individual species may possess more than one associated receptor type. Only recently has the existence of multiple GnRH receptor types been demonstrated in a single species, for example in zebrafish, goldfish, *X. laevis* (Illing *et al.*, 1999 and Troskie *et al.*, 1998) and the bullfrog (Wang *et al.*, 2000). In both goldfish and bullfrog, GnRH receptor types exhibited distinct differences in their ligand selectivity. In the teleost fish, medaka *Oryzias latipes*, three endogenous GnRHs, mdGnRH (GnRH1), GnRH2 and sGnRH (GnRH3) have been identified along with two distinct GnRH receptor subtypes with clear ligand preferences (Okubo *et al.*, 2001). Unlike the goldfish receptors, which are variant forms within the same subtype, the medaka receptors fall into two separate lineages, namely the Type I and Type III (Okubo *et al.*, 2001). The Type III receptor shows greatest homology to the GnRH receptor identified in striped bass (Alok *et al.*, 2000).

A functional GnRH receptor has been characterised in the African cichlid fish, *H. burtoni* (Robison *et al.*, 2001). Its widespread distribution and binding properties suggest that the cichlid GnRH receptor is capable of responding to all three endogenous GnRHs. However, it is highly unlikely that this is the only GnRH receptor expressed in cichlid since RT-PCR showed the receptor to be expressed at relatively low levels in the pituitary (Robison *et al.*, 2001). This suggests the presence of a more abundant pituitary GnRH receptor subtype yet to be identified in the cichlid. It would be plausible to assume the cichlid GnRH receptor would be similar to GnRH receptors isolated from other species of perciforms. Type III GnRH receptors have been identified in amberjack, sea bass and striped bass, all of which belong to the same order of fish as the cichlid. If the gene duplication giving rise to

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multiple receptor subtypes did, as proposed (Okubo *et al.*, 2001), predate the divergence of teleosts and tetrapods, the Type III receptor should have been retained in the cichlid. However, the cDNA of the cichlid receptor shows the highest homology to GnRH receptors identified in tetraploid fish such as goldfish and catfish. Hence, the cichlid receptor has been designated a Type I GnRH receptor (Robison *et al.*, 2001). As the cichlid was not shown to possess a second Type I receptor it may be possible that the additional GnRH receptor in the cichlid is similar to the Type III receptor identified in other perciform species.

The possibility of an additional GnRH receptor subtype in the African cichlid fish (*H. burtoni*) was, therefore, investigated. Partial cDNA encoding a unique receptor subtype in the cichlid was identified. This receptor subtype shows greatest homology to the GnRH receptor identified in the perciform species, *Seriola dumerili* (amberjack). Low homology is seen with the cichlid and other Type I GnRH receptors identified in fish and also with the mammalian GnRH receptors.

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2. 2 Materials and methods

2.2.1 Genomic DNA and total RNA

Cichlid genomic DNA and RNA were obtained via personal communication with Prof. Russel Fernald (Stanford University, USA). DNA, stored under 100% ethanol, was centrifuged at 13,000 rpm for 10 minutes. DNA was washed in 70% ethanol, air-dried and resuspended overnight at 4°C in Tris-EDTA buffer (10mM Tris-HCl, pH 7.5; 1mM EDTA, pH 8.0). A 1% TAE (40mM tris-acetate; 2mM $Na_2EDTA{\bullet}2H_2O$, pH 8.5) agarose gel containing ethidium bromide (0.1μg/ml) was used to check the quality of the DNA and was visualised using UV light.

RNA, stored under 100% ethanol, was centrifuged at 13,000 rpm for 10 minutes and washed in 70% ethanol made up with 0.1% diethylpyrocarbonate (DEPC) treated water. RNA was air-dried, resuspended in DEPC water and stored at -70°C. All microfuge tubes and pipette tips were newly opened and autoclaved to minimise RNA degradation by

ribonucleases. The RNA was visualised on a 1% formaldehyde agarose gel to check quality.

2.2.2 Degenerate primer PCR

Degenerate PCR primers, designed to conserved regions that straddle the transmembrane (TM) domains TM6 and TM7 of the cloned mammalian GnRH receptors, were used to amplify cichlid genomic DNA (Illing *et al.*, 1999).

The degenerate primers were as follows: (where N = G, A, T OR C; R = G OR A; H = A, C OR T; D = A, G OR T):

JH5s 5' CTCGAATTCGGNATHTGGTADTGGTT

JH6a$_2$ 5' ACACTCGAGCCRTADATNTRNGGRTC

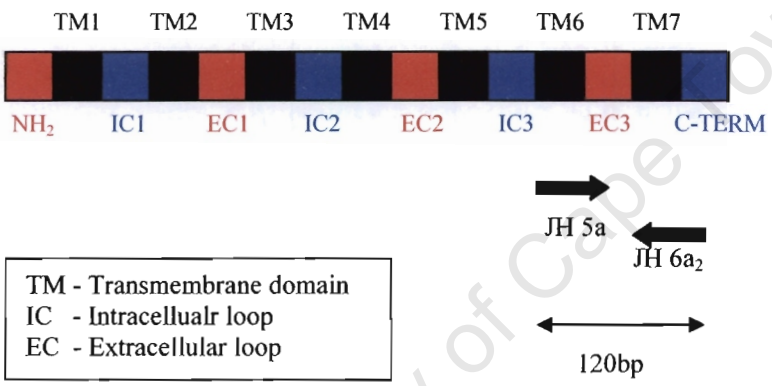

Fig. 2A. Location of the degenerate primers with respect to the GnRH receptor mRNA (not to scale)

PCR reactions were set up in a designated DNA free area to avoid contamination. 1µg of cichlid genomic DNA was amplified in a Perkin-Elmer GeneAmp hot-lid thermal cycler in a 50µl volume containing 10mM Tris-HCl (pH 9), 50mM KCl, 0.1% Triton, 2mM $MgCl_2$, 200µM dNTPs, 200ng of each degenerate primer (JH5s and JH6a$_2$) and 5 units of Taq DNA polymerase (Promega). PCR conditions were as follows: 93°C for 2.5 minutes, and 35 cycles of 93°C for 1 minute, 55°C for 2 minutes and 72°C for 3 minutes followed by a final extension step at 72°C for 5 minutes. 10µl of PCR products were electrophoresed through a 2% TAE agarose gel containing ethidium bromide (0.1µg/ml).

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2.2.3 PCR product subcloning

PCR products of interest were excised from a low melting point agarose gel (FMC Bioproducts), purified using Gene Clean II (Bio101) and subcloned using Promega pGEM-T Easy vector kit, according to the manufacturer's instructions (see below for map of pGEM-T Easy vector). Recombinant colonies were screened for inserts using restriction enzyme analysis. DNA was prepared from a 5ml overnight culture of LB (10g/l bacto-tryptone, 5g/l yeast extract, 5g/l NaCl, pH 7) containing ampicillin (100µg/ml). Plasmid DNA was extracted using the alkaline lysis method (Ish-Horowicz and Burke, 1981) and DNA was analysed by appropriate restriction endonuclease digestion. DNA of interest was sequenced using the T7 Sequenase system (Amersham).

2.2.4 Degenerate primer design

The internet-based BLAST facility (http://www.ncbi.nlm.nih.govt) was used to search for GnRH receptors with sequence homology to clones identified in the section above. Identified sequences were aligned in the DNAMan program and primers were designed to conserved regions.

2.2.5 Reverse Transcriptase PCR (RT-PCR)

2.5µg of total RNA in a volume of 12.5µl DEPC treated water was used for the first strand synthesis. After denaturing at 70°C for 10 minutes, reverse transcription was performed using the MMLV system (Promega), in a volume of 20µl containing 200ng of random primer, 1X MMLV buffer (250mM Tris-HCl (pH 8.3 at 25°C); 250mM KCl; 50mM $MgCl_2$; 2.5mM spermidine; 50mM DTT), 500µM dNTPs, 20 units of RNAsin and 200 units of MMLV enzyme. The above was incubated at room temperature for 10 minutes followed by 37°C for 50 minutes and 70°C for 10 minutes. An aliquot of reverse transcribed reaction was amplified in a Perkin-Elmer GeneAmp hot-lid thermal cycler in a 50µl volume containing 10mM Tris-HCl (pH 9), 50mM KCl, 0.1% Triton, 2mM $MgCl_2$, 200µM dNTPs, 200ng of each degenerate primer (S2, S4, as1 and as2) and 5 units of *Taq* DNA polymerase (Promega). PCR conditions were as follows: 94°C for 5 minutes, and 30 cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes followed by a final

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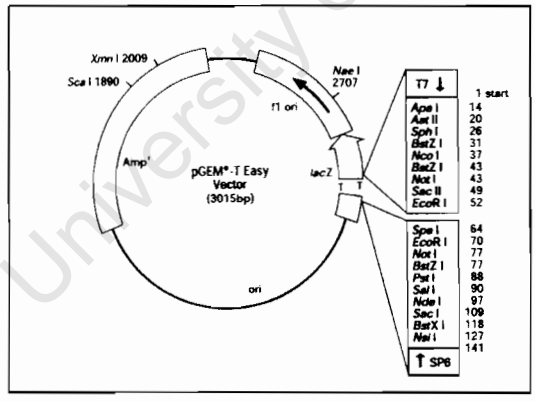
extension step at 72°C for 5minutes. 8µl of PCR products were electrophoresed through a 1% TAE agarose gel containing ethidium bromide (0.1µg/ml).

2.2.6 RT-PCR product subcloning and screening

PCR products of interest were excised from a 1% low melting point agarose gel (FMC Bioproducts). The agarose was melted at 70°C for 10 minutes and equilibrated at 37°C for a further 10 minutes. 3µl of the sample was used for cloning of the fragment into the pGEM-T Easy vector (Promega). Ligations were incubated overnight at room temperature and transformed according to the manufacturer's instructions (see below for map of pGEM-T Easy vector). Recombinant colonies were screened for inserts using restriction enzyme analysis. DNA was prepared from a 5ml overnight culture of LB (10g/l bacto-tryptone, 5g/l yeast extract, 5g/l NaCl, pH 7) containing ampicillin (100µg/ml). Plasmid DNA was extracted using the alkaline lysis method (Ish-Horowicz and Burke, 1981) and DNA was analysed by appropriate restriction endonuclease digestion. The nucleotide sequence of a putative positive clone was determined using automated sequencing on a MegaBACE 500 sequencer (Molecular Dynamics, USA) using both forward and reverse primers.



pGEM-T Easy Vector

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2.2.7 5'RACE (Rapid Amplification of cDNA Ends)

Sequence information from section 2.2.6 was used to design gene specific primers to amplify the 5' end of the cichlid GnRH receptor. The gene specific primers were as follows:

CH3pa (GSP) 5' TGCAGGCAAAGTCTCCGGCAAGC

CH3nc (nested GSP) 5' AATCAGCACCCTCACGTGGGATTTTCG

The 5' end of cichlid cDNA was amplified using rapid amplification of cDNA ends or RACE. The SMART RACE cDNA Amplification kit (Clontech) was used in this method. 1μg of cichlid total RNA was used for cDNA synthesis, which was performed according to the kit's specifications. The cDNA was subsequently amplified in a Perkin-Elmer GeneAmp hot-lid thermal cycler in 50μl containing 10mM dNTPs, 1X universal primer mix (Clontech) and gene specific primers (CH3pa or CH3nc) both at 10μM and 1X Advantage 2 Polymerase Mix with 1X Advantage 2 PCR buffer (Clontech). PCR conditions were as follows: 5 cycles of 94°C for 5 seconds and 72°C for 3 minutes followed by 5 cycles of 94°C for 5 seconds, 70°C for 10 seconds and 72°C for 3 minutes and finally 25 cycles of 94°C for 5 seconds, 68°C for 10 seconds and 72°C for 3 minutes. Negative controls were included in the PCR reactions, which were universal primer only and gene specific primer only. These are included to determine whether any PCR products are the result of non-specific amplification. A positive control cDNA and applicable gene specific primers are included in the SMART RACE kit. 10μl of PCR products were electrophoresed through a 1% TAE agarose gel containing ethidium bromide (0.1μg/ml). PCR product, resulting from amplification with gene specific primer CH3pa, was diluted 1 in 100 and further amplified as before but using the nested gene specific primer, CH3nc, to verify specificity. A single product of the expected size was subcloned into pGEM-T vector (Promega) and sequenced using automated sequencing.

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2.2.8 3'RACE

5'RACE product sequence analysis was used to design gene specific primers to be used in a 3'RACE reaction. The primers are as follows:

CH5'a 5' CGCACATTAACGCACTAACACACGAAGC

CH5'n 5' TCATGGTGGATGGGCACAACGTTCCTTC

The SMART RACE cDNA Amplification kit (Clontech) was used for 3' cDNA synthesis, which was performed according to the kit specifications. The cDNA was amplified as in section 2.2.7 but using gene specific primers CH5'a or CH5'n. PCR conditions were as folllows: 5 cycles of 94°C for 5 seconds and 72°C for 3 minutes followed by 5 cycles of 94°C for 5 seconds, 70°C for 10 seconds and 72°C for 3 minutes and finally 25 cycles of 94°C for 5 seconds, 67°C for 10 seconds and 72°C for 3 minutes. A PCR product of approximately 1.4kbp was subcloned into pGEM-T vector (Promega) and sequenced using automated sequencing.

2.3 Results

2.3.1 Identification of a putative Type III GnRH receptor from cichlid genomic DNA

Degenerate PCR primers, designed to conserved regions that straddle the transmembrane (TM) domains of TM6 and TM7 of the cloned mammalian GnRH receptors were used to amplify cichlid genomic DNA (Illing *et al.*, 1999). A positive band of approximately 120bp was identified after amplification of genomic DNA using the degenerate primer pair, JH5s and JH6a$_2$. The 120bp fragment was subcloned and sequenced. From seventeen clones analysed, two different subtypes were identified. Thirteen clones were homologous to the Type I cichlid GnRH receptor (Robison *et al.*, 2001). Four clones were identified which demonstrated novel sequences.

2.3.2 Primer design

Type III GnRH receptor sequences were obtained from the Genebank database and aligned in order to identify conserved domains. The alignments are shown in Figures 2B and 2C, as well as the location of the degenerate primers designed against amino acids that

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were specifically conserved in the other Type III receptors (Illing, personal communication).

The degenerate primers were as follows:

S2 5' GTGGGCIGCICA(T/C)(A/T)(G/C)IGA(C/T)GGIAA

S4 5' GGAT(G/C)TT(C/T)(C/T)TIAA(G/A)(C/T)TICA(A/G)GCIATGTA

as1 5' GTTICC(C/T)TCIA(A/G)(G/A)TC(G/A)TCIGG(G/A)AA

as2 5' AC(T/C)TTICC(T/C)TCIA(A/C)(G/A)TC(G/A)TCIGG

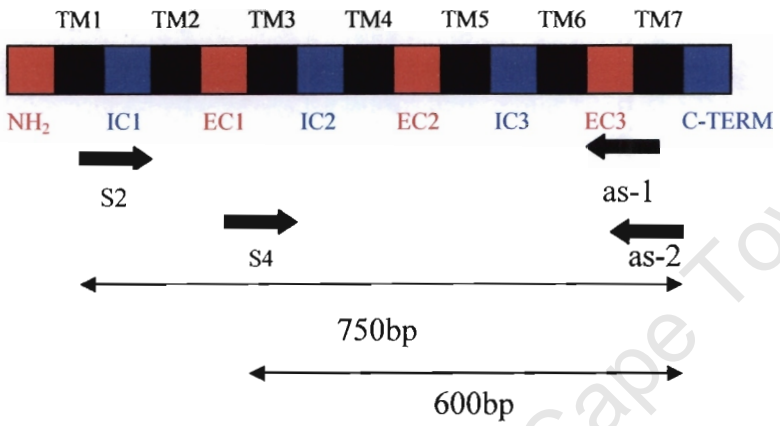

Fig. 2B - Location of the degenerate primers with respect to the Type III GnRH receptor mRNA. (Not to scale)

TM – transmembrane domain
EC – extracellular loop
IC – intracellular loop

```
Sea bass          1     MNTTLCDSAVALYHLTTDHQLNASCNYSSPTSNWTS------GGGALQLPTFT 47
Striped bass      1     MNTTLCDSAVAMYHLTTDHQLNASCNYSSPTSNWTS------GGGSLQLPTFT 47
Amberjack         1     MDTTLCDAAVTMYHLTTDHQLNASCNCSSPACNWTA------GGDAPQLPTFT 47
bullfrog1         1        MNISKEVSIKGCNNAQWLSSSCDLDVNMTSTNG------THTHFQLPTFS 44
bullfrog3         1  MNASDQPMGDGEAAPPGLCAFKGFNFSCVHANGFEKPHGPNITFLNEDHFVLPTFS 56
                                                 ——S2—>
sea bass         48  TAAKVRVIITCILCGISAFCNLAVLWAAHSDGKRK-SHVRVLIINLTVADLLVTFI 102
striped bass     48  TAAKVRVIITCILCGISAFCNLAVLWAAHSDGKRK-SHVRVLIINLTVADLLVTFI 102
amberjack        48  TAAKVRVIITFILCAISAFCNLAVLWAAHSDGKRK-SHVRVLIINLTVADLLVTFI 102
bullfrog1        45  PAAKARVIITFVIFTLSATCNLAALWSAARTSRKKRSHVRILILNLTTADLLVTFI 100
bullfrog3        57  TAAKIRVAITCVLFISSACFNMATLWTITYKYRKK-SHIRILIINLVAADLLITFV 111
                                                 ——S4—>
sea bass        103  VMPVDAVWNITVQWLAGDLACRLLMFLKLQAMYSCAFVTVVISLDRQSAILNPLAI 158
striped bass    103  VMPVDAVWNITVQWLAGDLACRLLMFLKLQAMYSCAFVTVVISLDRQSAILNPLAI 158
amberjack       103  VMPVDAVWNITVQWLAGDFACRLLMFLKLQAMYSCAFVTVVISLDRQSAILNPLAI 158
bullfrog1       101  VMPLDAIWNITVQWHAGDIACRILMFLKLLSMYSCAFVTVVISVDRQSAILNPLAI 156
bullfrog3       112  VMPLDAVWNVTIQWYAGDVACRILMFLKLVAMYSSAFVTVVISLDRHAAILNPLGI 167

sea bass        159  NKARKRNRVMLTVAWGMSVVLSVPQLFLFHNVTIIHPEDFTQCTTRGSFVTHWHET 214
striped bass    159  NKARKRNRVMLTVAWGMSVVLSVPQLFLFHNVTIIYPEDFTQCTTRGSFVTHWHET 214
amberjack       159  NKARKRNRVMLSVAWGMSTVLSVPQIFLFRNVTINHPEDFTQCTTRGSFVTHWHET 214
bullfrog1       157  NDAKKKNKIMLSVAWLMSAVLSLPQLFLFHTVTITEPHNFTQCTTRGSFQQHWQET 212
bullfrog3       168  GDAKKKNKAMLSVAWTLSLLLATPQLFVFHTVSRSQPVHFVQCATVGSFKAHWLET 223

sea bass        215  AYNMFTFSCLFLLPLVIMITCYTRIFCEISKRMKKDNLPSNEVHLRRSKNNIPRAR 270
striped bass    215  AYNMFTFSCLFLLPLIIMITCYTRIFCEISKRLKKDNLPSNEVHLRRSKNNIPRAR 270
amberjack       215  AYNMFTFSCLFLLPLVIMITCYTRIFCEISKRLKKDNLPSNEVHLRCSKNNIPRAR 270
bullfrog1       213  VYNMVSFVCLFLLPLLIMICCYSRILLEISKRMSKGTLSSKEVYLRCSKNNIPKAR 268
bullfrog3       224  LYNMFTFCCLFLLPLLIMVFCYGRILVEISRKMKKAEVSSREVNLRRSYNNIPRAR 279
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```
                                                <-<-as1/as2
sea bass          271 MRTLKMGIVIVSSFIVCWTPYYLLGLWYWFFPDDLEG-KVSHSLTHILFIFGLVNA 325
striped bass      271 MRTLKMSIVIVSSFIVCWTPYYLLGLWYWFFPDDLEG-KVSHSLTHILFIFGLVNA 325
amberjack         271 MRTLKMSIVIVLSFIICWTPYYLLGLWYWFFPDDLEG-KVSHSLTHILFIFGLVNA 325
bullfrog1         269 MRTLKMSVVIVSSFIICWTPYFLLGLWYWFYPEIMEE-KVSQSTTHILFIFGLVNA 323
bullfrog3         280 MRTFKMSLVIVLTFIVCWTPYYLLGIWYWFSPEMLTSRKVPPSLSHILFLFGLFNT 335

sea bass          326 CLDPVIYGLFTIHFRKGLRRYYCNATKAADLDNNTVITGSFICAANSLPLKREAS- 380
striped bass      326 CLDPVIYGLFTIHFRKGLRRYYCNATKASDLDNNTVITGSFICAANSLPLKREVSP 381
amberjack         326 CLDPVIYGLFTIHFRKGLRRYYCNAATASDLDTNTVITGSFTCATNSLPLKRQVSP 381
bullfrog1         324 CLDPITYGLFTIHFRKSLQR-YCGGRRTSDADTSSSVTGSFRCSMSSFRAKKMIVL 378
bullfrog3         336 CLDPIIYGLFTIHFRREIRRVCRCATQGKDADATSLGTGSFRISTAAVPLKRSAGA 391

sea bass          381 ---QERFMLYSDNHSRAESTSPRSSFL-------RDPNQSSSESNL 416
striped bass      382 AS-QERFVLYSDNHSRAELTSPRSSFL-------RDPNQSSSESNL 419
amberjack         382 SS-QERFMLCSDNHSKAESASPGSSFLTADNDAERDLNQSSPESVI 426
bullfrog1         379 N--QELQVLQSCNGNFNNPELRLNGLG-------------T--SCL 407
bullfrog3         392 SGGSCKFDLEVTGVGLHSGKCEHCKRQ-------------IVESFM 424
```

Fig 2C - Alignment of Type III GnRH receptors identified in species of perciform fish and bullfrog GnRH-R1 and GnRH-R3. Degenerate primers designed to areas of high sequence homology.

2.3.3 Cloning of a putative Type III receptor using RT-PCR

Due to the presence of large introns in the genomic clones of the GnRH receptor, RT-PCR was used to clone the rest of the putative cichlid Type III GnRH receptor. RT-PCR using the primer pair S2 and as1 gave a product of the expected size (see Fig 2D). The translated sequence of the 750bp partial cDNA fragment amplified from cichlid total RNA encoded for 250 amino acid residues. Blast results and multiple sequence alignment revealed homology to other Type III GnRH receptors.

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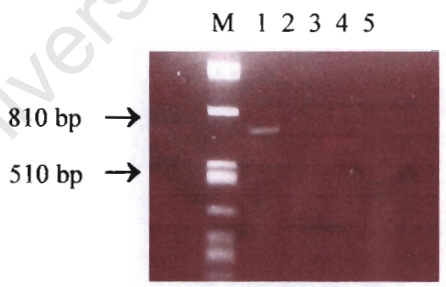

Fig 2D – UV gel of the products after RT-PCR on *H. burtoni* total RNA with degenerate primers designed to conserved regions of Type III GnRH receptors. M represents the MW marker, 1 - 4 are the combinations of degenerate primers used. 1) S2/as1; 2) S2/as2; 3) S4/as1; 4) S4/as2; 5) negative control. A 750bp product of the expected size could be seen with the primer pair S2 and as1.

2.3.4 Cloning of a full-length Type III receptor using 5' and 3'RACE

The nucleotide sequence information from the clones that were derived from the RT-PCR with degenerate primers to Type III receptors, was used to design gene specific primers that could be used in a 5'RACE PCR reaction. There was insufficient time to do Southern blot analysis and confirm the 5'RACE result so two rounds of amplification were performed. PCR product, obtained following amplification with universal primer and the gene specific primer, was reamplified using the universal primer and the nested gene specific primer. A slightly smaller band of about 380bp was observed, confirming that the product of the first round of amplification was specific. The positive band obtained after one round of amplification was subcloned and sequenced (see Fig 2E). The 5' 450bp partial cDNA encoded for approximately 80bp of 5' untranslated region and spanned the 5' region up until the area to which the gene specific primer was designed.

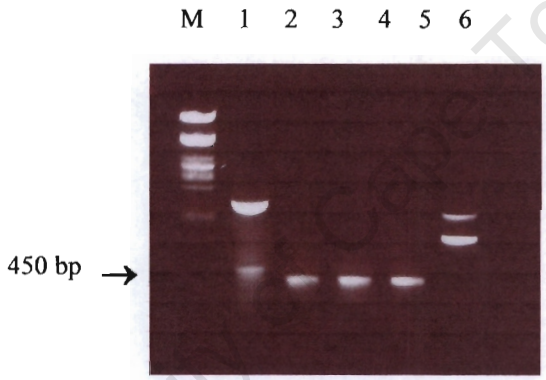

Fig 2E – UV gel of the products after 5'RACE on *H.burtoni* total RNA with gene specific primers. M represents the MW marker, 1-6 are the combinations of gene specific (GSP) or nested gene primers (NGSP) and universal primer (UPM) used. 1) GSP/UPM; 2) NGSP/UPM; 3) GSP/UPM→NGSP/UPM; 4) NGSP/UPM→NGSP/UPM; 5) UPM blank; 6) negative control. Products of approximately 450bp with GSP and 380bp with NGSP could be seen.

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The nucleotide sequence information obtained from 5' amplification of the Type III cichlid GnRH receptor was used to design gene specific primers that could be used in 3'RACE PCR reaction and possibly clone the full-length Type III receptor cDNA. A 1.4kbp band was subcloned and sequenced but appeared to be a truncated version of the Type III cichlid GnRH receptor. Due to time constraints no further attempts could be made to obtain a full-length clone for the cichlid Type III receptor.

2.3.5 Sequence analysis

Fig 2F shows the nucleotide and deduced amino acid sequence of 5'RACE and RT-PCR products combined. Blast results and multiple sequence alignments of the cichlid Type III receptor sequence to the corresponding regions of other Type III receptors revealed the cichlid Type III GnRH receptor has the highest similarity (87%) with the amberjack (*Seriola dumerili*) followed by 85% with the European sea bass (*Dicentrarchus labrax*) and striped bass (*Morone saxatilis*) GnRH receptors (see Fig. 2H). The identity of the cichlid Type III receptor to the human GnRH receptor is 53% (see Fig. 2I). A phylogenetic tree was generated by the MEGA software package (Nei and Kumar, 2000) following sequence alignment using CLUSTALW (Thompson *et al.*, 1994) of the corresponding regions of various cloned GnRH receptors. The cichlid receptor, identified in this study, is clearly grouped with other Type III GnRH receptors (see Fig. 2J). Sheep, cow, pig, mouse, rat and possum GnRH receptors fall into the same lineage as the human type I receptor. However, these sequences were not included in the construction of this phylogenetic tree as the focus was mainly on those lineages containing the fish GnRH receptors.

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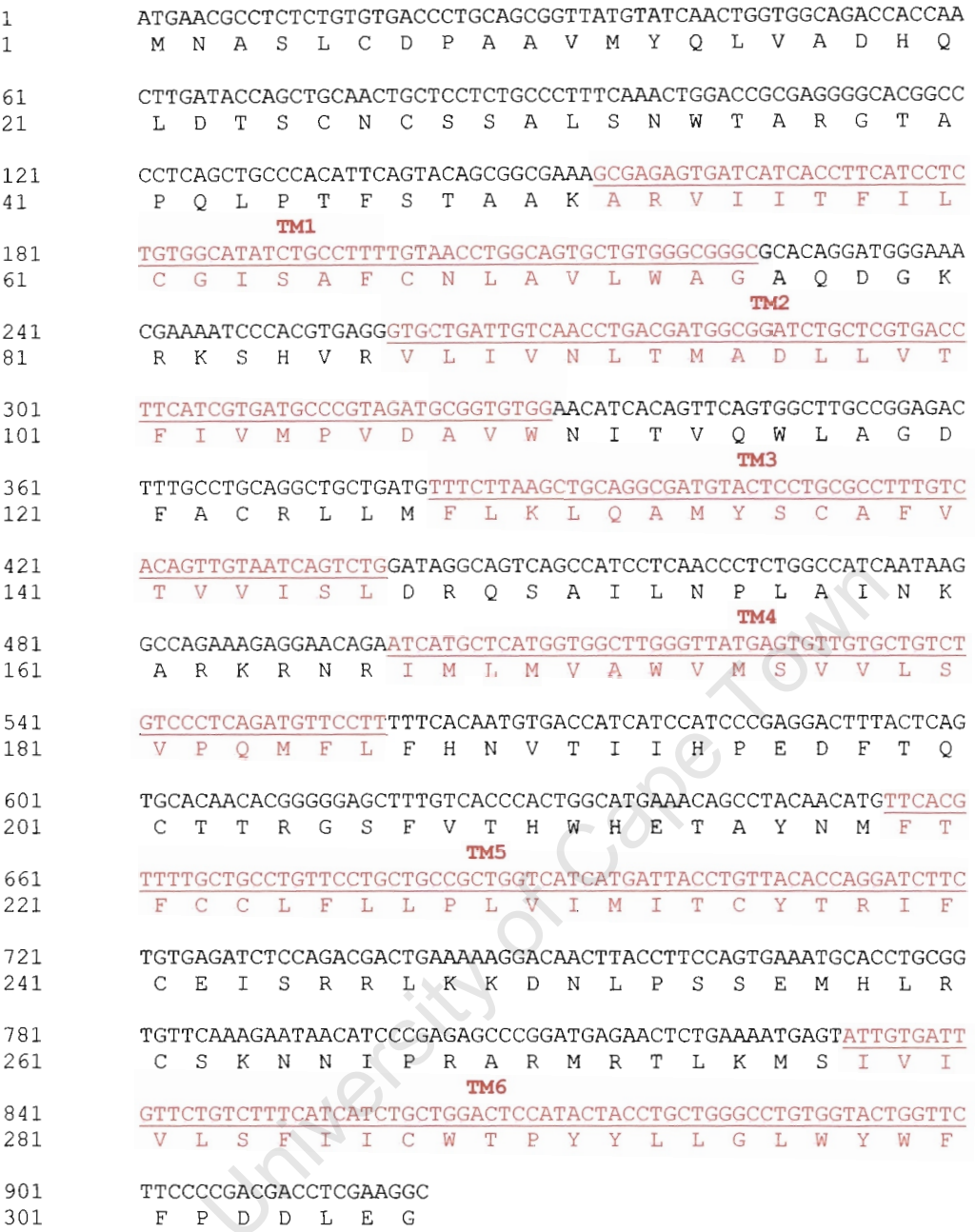

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Fig 2F – Nucleotide and deduced amino acid sequence following 5'RACE and RT-PCR of part of the putative Type III cichlid GnRH receptor cloned from *H. burtoni* total RNA.

```
cichlid Type I     1   MAGNWS---------IIRLSLPPTSSTAITLHNTSQYPPLPDWEAPSFTRAAQFRV  47
cichlid Type III   1   MNASLCDPAAVMYQLVADHQLDTSCNCSSALSNWTARGTAP--QLPTFSTAAKARV  54

cichlid Type I     48  GATFVLFLFAACSNLALLVSVWRGQRLASHLRPLMLSLASADLMMTFVVMPLDAVW  103
cichlid Type III   55  IITFILCGISAFCNLAVLWAGAQDGKRKSHVRVLIVNLTMADLLVTFIVNPVDAVW  110

cichlid Type I     104 NMTVQWYGGDALCKLLCFLKLFAMHASAFILVVISLDRQHAILHPLDALSAHSRNR  159
cichlid Type III   111 NITVQWLAGDFACRLLMFLKLQAMYSCAFVTVVISLDRQSAILNPLAINKARKRNR  166

cichlid Type I     160 RMLLLAWTLSILLASPQLFIFRTIR-YDSVDFTQCASHGSFSRHWQETVYNMFHFT  214
cichlid Type III   167 IMLMVAWVMSVVLSVPQMFLFHNVTIIHPEDFTQCTTRGSFVTHWHETAYNMFTFC  222

cichlid Type I     215 TLYVVPLLVMSCCYSRILLHIHQQHLRDKAGESPLR-RSGTDIIPKARMKTLKMTV  269
cichlid Type III   223 CLFLLPLVIMITCYTRIFCEISRRLKKDNLPSSEMHLRCSKNNIPRARMRTLKMSI  278

cichlid Type I     270 VIVLSFVVCWTPYYLLGIWYWFQPDMLRTTPEYVHHALFVFGNLNTCCDPIIYGFY  325
cichlid Type III   279 VIVLSFIICWTPYYLLGLWYWFFPDDLEG                          307

cichlid Type I     326 TPSFRADLAACCRWVRCDADTSSQYIDRMSTREGPHSREHEPQPSTNNQTAADQPM  381
cichlid Type III   308                                                       307

cichlid Type I     382 KARDFSM 388
cichlid Type III   308         307
```

Fig 2G – Alignment of deduced amino acid sequences of GnRH receptors in the cichlid. Homologous regions are highlighted in purple.

```
Sea bass           1   MNTTLCDSAVALYHLTTDHQLNASCNYSSFTSNWTSGGGALQLPTFTTAAKVRVII  56
Striped bass       1   MNTTLCDSAVAMYHLTTDHQLNASCNYSSFTSNWTSGGGSLQLPTFTTAAKVRVII  56
Amberjack          1   MDTTLCDAAVTMYHLTTDHQLNASCNCSSPACNWTAGGDAPQLPTFTTAAKVRVII  56
Cichlid Type III   1   MNASLCDPAAVMYQLVADHQLDTSCNCSSALSNWTARGTAPQLPTFSTAAKARVII  56

Sea bass           57  TCILCGISAFCNLAVLWAAHSDGKRKSHVRVLIINLTVADLLVTFIVMPVDAVWNI  112
Striped bass       57  TCILCGISAFCNLAVLWAAHSDGKRKSHVRVLIINLTVADLLVTFIVMPVDAVWNI  112
Amberjack          57  TFILCAISAFCNLAVLWAAHSDGKRKSHVRVLIINLTVADLLVTFIVMPVDAVWNI  112
Cichlid Type III   57  TFILCGISAFCNLAVLWAGAQDGKRKSHVRVLIVNLTMADLLVTFIVNPVDAVWNI  112

Sea bass           113 TVQWLAGDLACRLLMFLKLQAMYSCAFVTVVISLDRQSAILNPLAINKARKRNRVM  168
Striped bass       113 TVQWLAGDLACRLLMFLKLQAMYSCAFVTVVISLDRQSAILNPLAINKARKRNRVM  168
Amberjack          113 TVQWLAGDFACRLLMFLKLQAMYSCAFVTVVISLDRQSAILNPLAINKARKRNRVM  168
Cichlid Type III   113 TVQWLAGDFACRLLMFLKLQAMYSCAFVTVVISLDRQSAILNPLAINKARKRNRIM  168

Sea bass           169 LTVAWGMSVVLSVPQLFLFHNVTIIHPEDFTQCTTRGSFVTHWHETAYNMFTFSCL  224
Striped bass       169 LTVAWGMSVVLSVPQLFLFHNVTIIYPEDFTQCTTRGSFVTHWHETAYNMFTFSCL  224
Amberjack          169 LSVAWGMSTVLSVPQIFLFRNVTINHPEDFTQCTTRGSFVTHWHETAYNMFTFSCL  224
Cichlid Type III   169 LMVAWVMSVVLSVPQMFLFHNVTIIHPEDFTQCTTRGSFVTHWHETAYNMFTFCCL  224

Sea bass           225 FLLPLVIMITCYTRIFCEISKRMKKDNLPSNEVHLRRSKNNIPRARMRTLKMGIVI  280
Striped bass       225 FLLPLIIMITCYTRIFCEISKRLKKDNLPSNEVHLRRSKNNIPRARMRTLKMSIVI  280
Amberjack          225 FLLPLVIMITCYTRIFCEISKRLKKDNLPSNEVHLRCSKNNIPRARMRTLKMSIVI  280
Cichlid Type III   225 FLLPLVIMITCYTRIFCEISRRLKKDNLPSSEMHLRCSKNNIPRARMRTLKMSIVI  280

Sea bass           281 VSSFIVCWTPYYLLGLWYWFFPDDLEGKVSHSLTHILFIFGLVNACLDPVIYGLFT  336
Striped bass       281 VSSFIVCWTPYYLLGLWYWFFPDDLEGKVSHSLTHILFIFGLVNACLDPVIYGLFT  336
Amberjack          281 VLSFIICWTPYYLLGLWYWFFPDDLEGKVSHSLTHILFIFGLVNACLDPVIYGLFT  336
Cichlid Type III   281 VLSFIICWTPYYLLGLWYWFFPDDLEG                            307

Sea bass           337 IHFRKGLRRYYCNATKAADLDNNTVITGSFICAANSLPLKREAS---QERFMLYSD  389
Striped bass       337 IHFRKGLRRYYCNATKASDLDNNTVITGSFICAANSLPLKREVSPASQERFVLYSD  392
Amberjack          337 IHFRKGLRRYYCNAATASDLDTNTVITGSFTCATNSLPLKRQVSPSSQERFMLCSD  392
Cichlid Type III   308                                                       307

Sea bass           390 NHSRAESTSPRSSFL-------RDPNQSSSESNL 416
Striped bass       393 NHSRAELTSPRSSFL-------RDPNQSSSESNL 419
Amberjack          393 NHSKAESASPGSSFLTADNDAERDLNQSSPESVI 426
Cichlid Type III   308                                    307
```

Fig 2H – Alignment of deduced amino acid sequences of type III GnRH receptors in species of perciform.

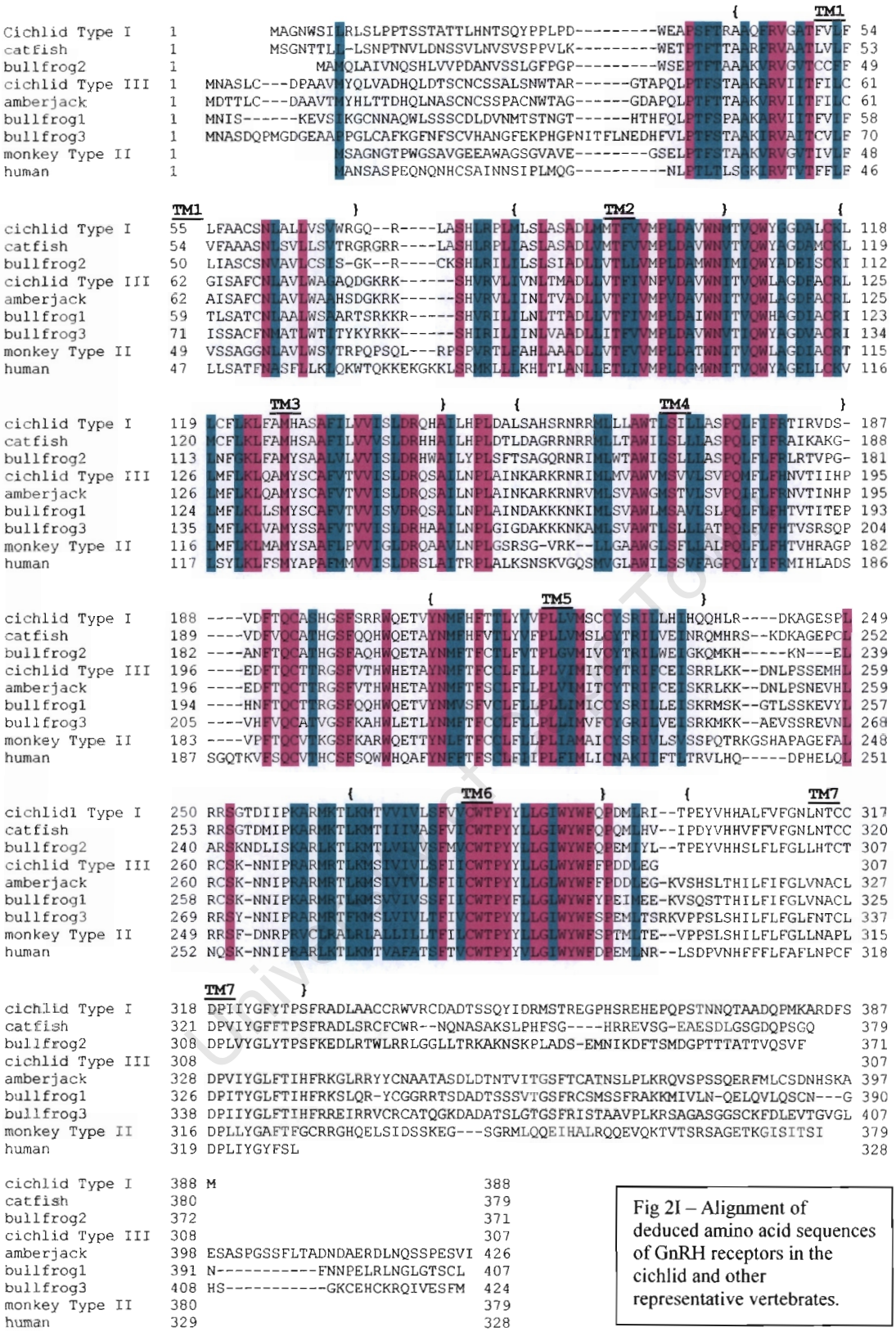

Fig 2I – Alignment of deduced amino acid sequences of GnRH receptors in the cichlid and other representative vertebrates.

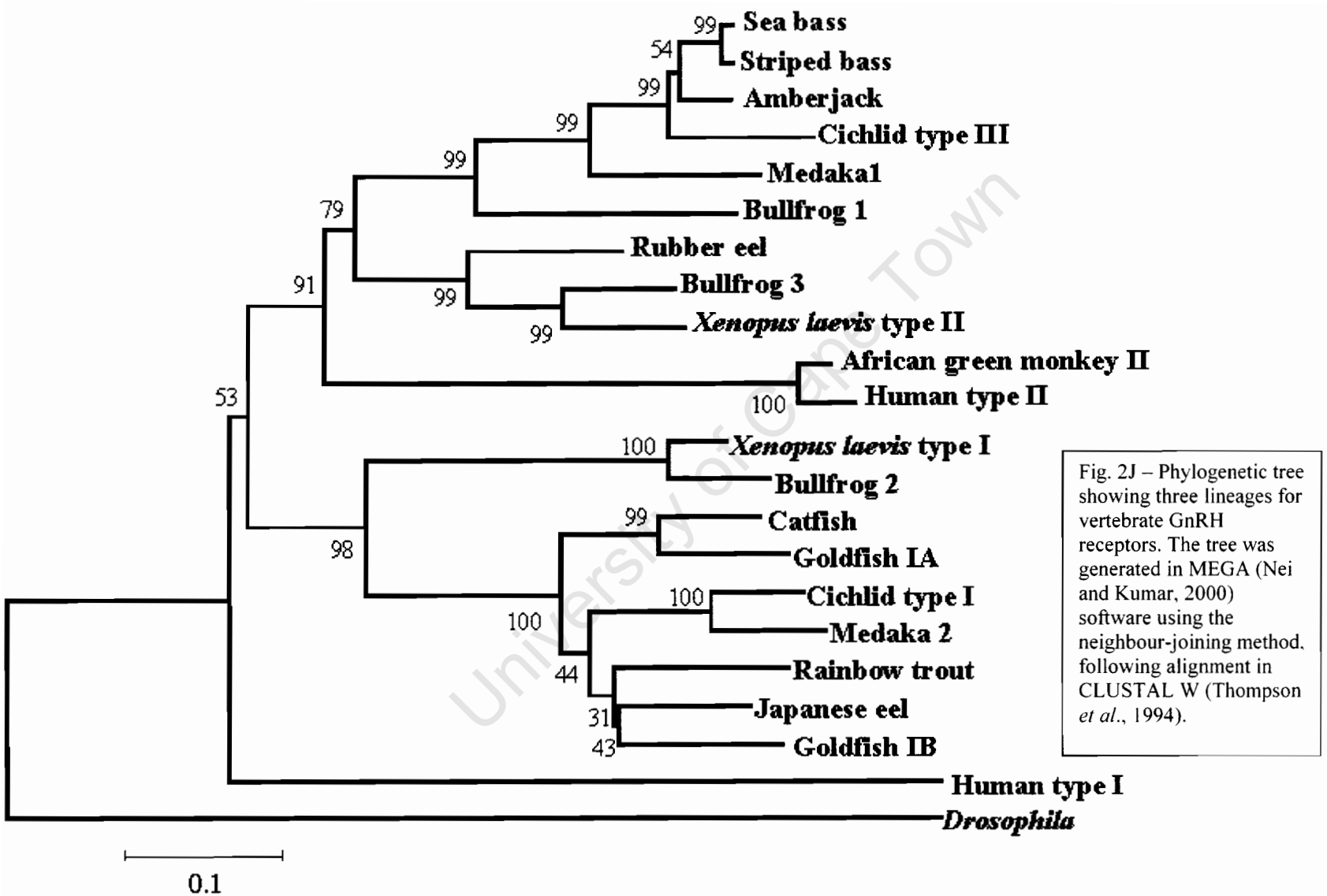

Fig. 2J – Phylogenetic tree showing three lineages for vertebrate GnRH receptors. The tree was generated in MEGA (Nei and Kumar, 2000) software using the neighbour-joining method, following alignment in CLUSTAL W (Thompson *et al*., 1994).

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2.4 Discussion

Three separate genes encoding GnRHs have been identified in *H. burtoni* with distinct patterns of expression. Seabream GnRH (GnRH1), expressed primarily in the preoptic area, has been implicated in the regulation of gonadotropin release. It appears that social influences directly affect levels of sbGnRH mRNA expression (White *et al.*, 2002). The occurrence of multiple GnRHs in a single species makes it reasonable to assume that more than one associated receptor will be present. Previously, only one GnRH receptor was identified in the cichlid and was designated a Type I GnRH receptor (Robison *et al.*, 2001). The Type I receptor shows extensive distribution in the brain but is not excessively expressed in the pituitary. It also demonstrates a higher affinity for cGnRHII as opposed to the other endogenous GnRHs. With the identification of multiple GnRH receptor types in other teleost species and the complexity of reproductive regulation in *H. burtoni*, it is highly probable that additional GnRH receptor subtypes exist in this species.

In this chapter, the occurrence of additional receptor subtypes was investigated. PCR amplification of genomic DNA revealed a short fragment of a putative Type III GnRH receptor. Partial cDNAs encoding this receptor were cloned from total RNA. A 450bp 5' cDNA fragment was identified. This spanned approximately 80bp of UTR up to the middle of TM2. In addition, a 750bp cDNA fragment spanning the intracellular end of TM1 to the center of EC3, was cloned using RT-PCR. There was significant overlap between the two sequences. Amalgamation of partial sequences revealed 307 amino acids of coding region. The sequences encoding EC3, TM7 and probable C-terminal tail are yet to be elucidated.

Attempts to obtain a full-length cDNA clone were unsuccessful yielding only a 1.4kbp truncated transcript. A polyA tail was present in the mRNA transcript and appears to contain only the first exon. The relevance of multiple mRNA transcripts for the GnRH receptor is not fully understood but may result from multiple transcription start sites, alternative splicing of the gene or alternative polyadenylation signals (Albarracin *et al.*, 1994).

As expected, the Type III cichlid GnRH receptor shows a common phylogenetic origin with other non-mammalian receptors, particularly Type III receptors identified in other species of

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perciforms. The highest similarity (87%) is seen with the amberjack (*S. dumerili*) GnRH receptor, followed by 85% with the sea bass (*D. labrax*) and striped bass (*M. saxitilis*) receptors. Low homology is observed with the Type I GnRH receptors, 53% with the catfish and only 50% with the previously identified cichlid Type I receptor. Like other non-mammalian GnRH receptors, the Type III cichlid receptor shows low amino acid identity with the mammalian receptors, for example 53% with the human GnRH receptor.

The cichlid Type I and Type III GnRH receptors are highly divergent as seen from their low amino acid homology. The phylogenetic tree, shown in Fig. 2J, clearly places them in two distinct lineages. Interestingly, the cichlid Type III GnRH receptor is more closely related to the Type II primate receptors than the cichlid or other fish Type I receptors. A similar pattern is observed in the phylogenetic comparison performed by Okubo *et al*, (2001). It has been suggested that the amphibian and mammalian Type II receptors are selective for GnRH2 (see review Millar, 2003). Based on their phylogenetic relationship to the Type II receptors, it may be that the Type III receptors identified in species of bony fish, are also specific for GnRH2.

The duplication of genes, observed in the teleost lineage, was thought to be the result of a genome duplication, which occurred after the divergence of teleosts and tetrapods. However, these results indicate that the Type I and Type III receptors evolved early in teleost evolution, predating the divergence of tetrapods. Also, although the Type IA and Type IB GnRH receptors identified in the goldfish, a tetraploid species, share high amino acid identity of 71%, they demonstrate sufficient divergence to suggest they arose before tetraploidisation. It is therefore likely that the presence of a Type III receptor in addition to both Type IA and IB GnRH receptors may be a common feature of teleosts and tetrapods, if it is indeed true that these receptor subtypes arose prior to divergence. One might also expect to see evidence of the duplication of type III GnRH receptors, i.e. the presence of two type III GnRH receptor subtypes in a single species. The possibility of a second type III receptor in the cichlid can not be ruled out, although further studies would be required to prove or disprove this theory. Although the cDNA or the gene encoding a second Type I receptor has not yet been identified in *H. burtoni*, antisera raised to EC3 has been used to demonstrate differential distribution of Type IA, IB and Type III GnRH receptors in the pituitary of a cichlid fish, the tilapia (Parhar *et*

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al., 2002). Interestingly, the Type IA GnRH receptor was shown to colocalise in the pituitary with cells containing LHβ-subunit, the Type IB receptor was identified in cells expressing prolactin and those expressing LH and the Type III GnRH receptor was expressed in growth hormone expressing cells. Three GnRH receptor subtypes have been identified in the bullfrog where bfGnRH-R1 and bfGnRH3 fall into the same lineage and bfGnRH-R2 falls into a separate distinct lineage (Wang *et al.*, 2000). It seems likely that most, if not all, vertebrates possess two, if not more, distinct GnRH receptors.

Amino acid alignment indicates that the cichlid Type III GnRH receptor is a typical G-protein coupled receptor with characteristic transmembrane domains and an extracellular N-terminus. Although a full-length cDNA of the Type III receptor was not identified in this study, the existence of a C-terminal tail is highly likely since it is conserved in all non-mammalian GnRH receptors cloned to date.

Several features distinguish the cichlid Type III receptor from the mammalian GnRH receptor. The mammalian GnRH receptors are characterised by the mutual exchange of Asp in TM2 and Asn in TM7 to give Asn^{87} and Asp^{318}, a feature unique among GPCRs. The interaction of these residues seems to be conformationally essential as mutation of Asn^{87} to Asp results in loss of receptor function. In the catfish GnRH receptor, aspartic acid in TM2 is restored (Asp^{90}) thus leaving aspartic acid residues in both TM2 and TM7. Asp^{90} is known to be essential for proper receptor functioning in the catfish, thus it is likely that it interacts with residues in other transmembrane domains. This feature is common among non-mammalian GnRH receptors and appears as Asp^{96} in the Type III cichlid receptor.

Another typical characteristic of the GnRH receptor is the modification of the 'DRY' motif, conserved among other GPCRs but which becomes 'DRS' in the human GnRH receptor. In non-mammalian GnRH receptors this signature sequence differs again. This motif is changed to Asp^{147}, Arg^{148}, Gln^{149} (DRQ) in the Type III cichlid receptor and is homologous with the amberjack, striped bass, sea bass, medaka and bullfrog GnRH-R1. However, catfish, GfA, GfB and bullfrog GnRH-R2 and GnRH-R3 contain the sequence DRH in this region. Interestingly,

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the DRH motif seen in other Type I receptors is not maintained in the cichlid Type I GnRH receptor. Instead DRQ is once again observed.

Two predicted phosphorylation sites, Protein Kinase A (PKA) specific Ser^{83} and calmodulin II specific Thr^{273}, are conserved in the cichlid Type III GnRH receptor. The majority of the serine sites, which are also potential phosphorylation sites in amberjack, striped bass and sea bass GnRH receptors are also present in the Type III cichlid receptor.

Prospective glycosylation sites, the Asn residues in the N-terminal domain are important for expression in the mammalian GnRH receptor and have been conserved in the catfish and goldfish receptors. The Type III cichlid receptor also contains such sites at positions 2, 26 and 33, which are conserved in other Type III receptors identified in perciforms and similar to bfGnRH-R1. Likewise, the putative ligand binding site in the human GnRH receptor (Asp^{98}, Asn^{102} and Lys^{121}), also present in the catfish and goldfish receptors, is conserved as Asp^{107}, Asn^{111} and Lys^{130} in the Type III cichlid receptor. This suggests a similar binding site is present in both mammalian and non-mammalian GnRH receptors.

The conserved Cys^{25} in the N-terminal domain and Cys^{123} in EC loop II, which are necessary for proper receptor folding in the mammalian GnRH receptor, are also present in the Type III cichlid receptor.

A greater degree of conservation is seen within the transmembrane domains, as opposed to the extracellular and intracellular loops, when comparing the Type III cichlid receptor with cichlid Type I and mammalian GnRH receptors. The significance of these domains in maintaining the conformational structure of the receptor is unclear. The intracellular and extracellular loops of the cichlid Type I and Type III receptors do not show significantly high levels of homology. Intracellular loop I is more similar among teleosts than compared to mammalian GnRH receptors, with the only residue completely conserved being a single serine. Intracellular loop III, that is thought to be important for G-protein coupling, shows a greater degree of homology between the Type III receptor and the human GnRH receptor. Extracellular loop II appears to be the most highly conserved between Type I and Type III cichlid receptors and with the

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human GnRH receptor. Extracellular loop III plays a critical role in ligand specificity and can be used to classify GnRH receptors into their prospective subgroups. The role of extracellular loop III in the non-mammalian GnRH receptor has not yet been elucidated but significant variation is apparent in this domain between receptor subtypes. This region is completely conserved in the Type III receptors identified in species of perciforms i.e. sea bass, striped bass, medaka and amberjack.

To conclude, in addition to the Type I receptor, a putative Type III GnRH receptor has been identified in a perciform species known to have three forms of native ligands, the cichlid fish, *H. burtoni*. This novel cichlid receptor shows a common phylogenetic origin with other non-mammalian GnRH receptors but is more similar to Type III receptors characterised in other species of perciform, primarily the amberjack GnRH receptor. It is possible that the two cichlid GnRH receptors may correspond to a GH and a gonadotropin releasing receptor. A recent study has demonstrated expression of the Type III receptor primarily in the somatotropes with the Type I cichlid GnRH receptor being localised in the pituitary gonadotropes (Parhar *et al.*, 2002). The presence of a second Type I receptor was also suggested, however, this was not confirmed in this study. However, it is becoming increasingly likely that three GnRH receptors will be identified in a single species, in this case type IA, type IB and type III, and that endogenous GnRH variants have cognate receptors involved in the regulation of discrete cell populations. GnRH1 mRNA levels have been shown to be directly influenced by social status and it is highly likely that fluctuations in cognate receptor levels will be concurrent. In a species where reproduction is socially regulated, elucidation of native Type I and Type III GnRH receptor sequences will help provide information on how GnRH receptors are regulated under social conditions.

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Chapter 3

Cloning and characterisation of zebrafish (*D. rerio*) GnRH Receptor genes

3.1 Introduction

The presence of multiple GnRH forms in a single species along with more than one cognate GnRH receptor poses questions concerning their developmental origin. Initially, it was suggested that GnRH producing cells originate embryonically in the olfactory placode and migrate to their adult destination early in embryogenesis (Muske, 1993). More recent studies imply that GnRH neurons may have distinct origins. The cranial neural crest and anterior pituitary are stipulated to be possible sources of GnRH producing cells as an alternative to the olfactory placode (Amraoui and Dubois, 1993 and Tobet *et al.*, 2001). A recent study in the European sea bass (*D. labrax),* a teleost species, suggests that cells expressing three native GnRHs have varying origins. Salmon GnRH and seabream GnRH-producing cells appear to have a common origin in the olfactory placode, whilst chicken GnRHII neurons are derived from the synencephalon (González-Martínez *et al.*, 2002). In the sea bass, cells showing expression of cGnRHII mRNA were observed just four days after hatching, whilst still migrating to their final destination in the midbrain. Since differentiation of the gonads only occurs later in development, the possibility of cGnRHII having a neuromodulatory as opposed to a reproductive role is reinforced. Expression of cGnRHII at an earlier time point compared to other native GnRHs also indicates a critical role for this conserved GnRH in development. Salmon GnRH expressing cells, first seen on day seven after hatching, are also suggested to be of developmental importance although a possible role in the processing of reproductive-related visual and odorant information is likely. Seabream GnRH expressing cells are located predominantly in the preoptic area with fibres innovating the pituitary. Expression is first seen on day 30, shortly before the proliferation of gonads and the appearance of the first germ cells, reiterating the belief that sbGnRH is the primary gonadotropin releasing GnRH in perciforms (Powell *et al.*, 1994). However, more information is required before the developmental origins and final location of GnRH neurons, in relation to function, is clarified.

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Clear patterns of expression have been observed for GnRH-producing neurons. However, little is known about the developmental distribution of cognate GnRH receptors in a single species. The relevance of possessing two distinct receptor subtypes, expressed in the pituitary, is unclear. For example, in the goldfish, GfA and GfB receptors demonstrate varying affinities for endogenous GnRHs and differential release of gonadotropins and GH (Illing *et al.*, 1999). These differences may have developmental significance in promoting or silencing certain effects relevant only to particular developmental or reproductive stages.

A recent study established a differential distribution pattern of GnRH receptors in the pituitary of tilapia (Parhar *et al.*, 2002). As early as day eight post fertilisation expression of a Type III GnRH receptor is seen in GH producing cells. By day fifteen, expression of Type IA and IB receptors is observed in LH and prolactin cells respectively. Intense Type IA receptor expression is identified in the pituitary by day 25, which is tightly coupled to the onset of gonadal differentiation. Relationships were observed between expression of GnRH receptor subtypes and the appearance of native GnRHs helping to elucidate possible developmental roles.

The zebrafish (*D. rerio*) is a model organism for studying developmental biology in vertebrates. Zebrafish transparency has allowed expression of specific genes to be visualised and neurons to be tracked during development from origin in transparent embryos to their final destination. Developmental programs are comparative in all vertebrates hence, the zebrafish can be used as a model organism for many species. The zebrafish is a tetraploid species that is thought to have retained a significant number of duplicated genes, which arose from the genome duplication early in teleost evolution. It has been proposed that rather than being silent, duplicated genes subdivide functions of ancestral genes (Fishman, 2001).

Two endogenous GnRHs have been identified in the zebrafish, sGnRH and cGnRHII (Powell *et al.*, 1996), along with partial sequences, 120 bp of extracellular loop 3, encoding two distinct GnRH receptor Types, IA and IB (Troskie *et al.*, 1998). We therefore aimed to identify and isolate the genes encoding the two receptor subtypes in zebrafish, and possibly identify their promoter regions. This information could subsequently be used to produce promoter-GFP

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constructs of zebrafish GnRH receptors and visualise their expression during development in zebrafish. The genes encoding two GnRH receptor subtypes were identified by screening a zebrafish genomic library. However, the clones extended from intron B to the 3' untranslated region and none of the clones included exon 1. Consequently, cloning the promoter region of the zebrafish GnRH receptors was not possible.

3.2 Materials and methods

3.2.1 Preparation of probe DNA

The plasmids, S12 and Mp14, were used as a source of EC3 of the zebrafish Type IA and IB GnRH receptors, respectively (obtained via personal communications with Elaine Rumback). The degenerate primers JH5s and JH6a$_2$ (see section 2.2.2) were used to amplify both inserts from plasmid DNA.

PCR reactions were made up in molecular grade water in a 50µl volume containing 50ng of plasmid DNA, 10mM Tris-HCl (pH 9), 50mM KCl, 0.1% Triton, 2mM $MgCl_2$, 200µM dNTPs, 200ng of each degenerate primer (JH5s and JH6a$_2$) and 5 units of *Taq* DNA polymerase (Promega). The PCR cycles were: 93°C for 2.5 minutes, and 35 cycles of 93°C for 1 minute, 55°C for 2 minutes and 72°C for 3 minutes followed by a final extension step at 72°C for 5 minutes. 30µl of the reactions were electrophoresed on a 2% low melting point agarose gel (FMC) containing ethidium bromide (0.1µg/ml). The selected band was cut from the gel under long wavelength UV light and purified using a PCR Purification Kit (Promega).

3.2.2 Screening of a zebrafish genomic DNA library

A zebrafish Lambda EMBL3 SP6/T7 (see below for map) genomic library was purchased from Clontech. The library was plated using *Escherichia coli* K820 (Clontech) and the titre was established to be 1 x 10^{10}pfu/ml. One million plaques were screened by filter hybridisation with Hybond N+ (Amersham) according to the manufacturer's instructions (Clontech). Approximately 50ng of zebrafish GnRH receptor partial cDNA, generated as in section 3.2.1, was random labelled using the Amersham Megaprime labelling kit. Probe was labelled to contain at least 10^7cpm using [α^{32}P]dCTP (3000Ci/mmol), also obtained from Amersham.

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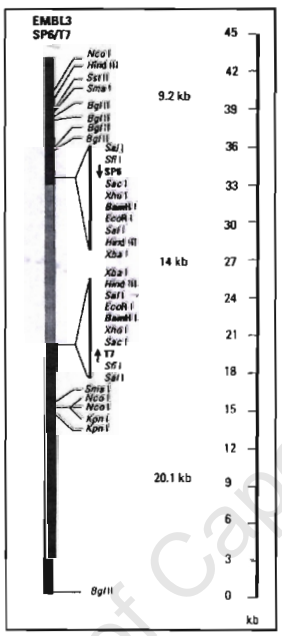
Labelled probes were purified from unincorporated deoxynucleotides using Sigma Spin Columns. Positive plaques were subjected to secondary screening. Six recombinant phage clones, which contained sequences homologous to the zebrafish GnRH receptor partial cDNA, were plaque purified and phage DNA was prepared using the Qiagen Lambda DNA Midi-Preparation kit.



Map of Lambda Phage vector

3.2.3 Southern blot analysis

Phage DNA was further characterised by appropriate restriction endonuclease digestion followed by Southern blot analysis. *SacI* digested DNA was electrophoresed through a 0.8% TAE agarose gel. The DNA containing gel was depurinated for 10 minutes in depurination solution (0.125M HCl). This was necessary due to the size of the DNA involved i.e. DNA fragments larger than 10kbp. The DNA gel was subsequently denatured for 30 minutes in denaturing solution (1.5M NaCl, 0.5M NaOH), followed by neutralisation for 30 minutes in neutralising solution (1.5M NaCl, 0.5M Trizma Base, pH 7.5). DNA was transferred to a nitrocellulose membrane, Hybond N+ (Amersham) in 20x SSC (0.3M tri-sodium citrate, 3M NaCl, pH 7-8) for a minimum of 16 hours. The DNA was fixed to the membrane using an optimised UV crosslinker (70,000 microjoules/cm^2). Zebrafish Type IA and IB GnRH receptor partial cDNA were used as probes and were labelled using the Megaprime Labelling Kit

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(Amersham). Blots were prehybridised for at least one hour in hybridisation solution (50% formamide, 100µg/ml denatured salmon sperm DNA, 0.1% SDS, 5x SSPE (20x stock; 3MNaCl, 0.2M $NaH_2PO_4.H_2O$, 0.5M EDTA, pH 7.4) and 5x Denhardt's solution (0.5g Ficoll, 0.5g polyvinylpyrrolidone, 0.5g BSA)). Labelled probe was denatured at 95°C for 5 minutes before being added to the hybridisation solution. The blots were incubated in hybridisation solution containing probe at 42°C overnight. Membranes were washed for 20 minutes at room temperature in 2x SSC, 0.5% SDS followed by a 60 minute wash at 65°C in 1x SSC, 0.1% SDS. Membranes were subjected to autoradiography with an overnight exposure at -70°C.

Further restriction mapping and Southern blotting were used to fully characterise the six recombinant phage clones. Phage DNA was digested with combinations of *SacI, SalI, XhoI, EcoRI* and *NotI* and analysed by Southern blot analysis. Four separate probes were used to attempt to identify the smallest bands, which contained sequences homologous to 5' regions of the zebrafish GnRH receptors. Probes used are specified in the section 3.3.

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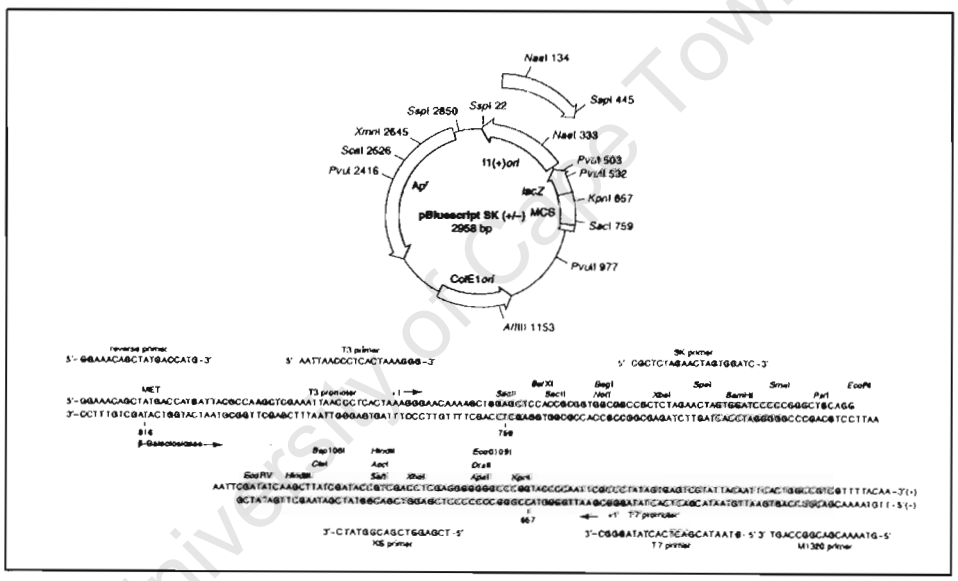
3.2.4 Subcloning and analysis of zebrafish GnRH receptor genomic clones

Following restriction mapping of zebrafish GnRH receptor genomic clones, bands of approximately 6kbp and less, that hybridised positively to the GnRH receptor probes, were excised from a 0.8% low melting agarose gel and subcloned into pBluescript II SK (+/-), see below for map. pBluescript II SK was linearised by digestion with *SacI* and *EcoRI* for directional cloning or with *SacI* alone for non-directional cloning. Vector, digested with *SacI* alone, was dephosphorylated to prevent re-annealing following linearisation. 500ng of *SacI* digested pBluescript II SK was incubated at 37°C for 15 minutes with 0.5 units of Shrimp alkaline phosphatase (Promega) and 1x reaction buffer (Promega). The amount of insert required to give an insert to vector ratio of 1:1, using 50ng of vector, was calculated using the following equation:

$$\text{ng of insert required} = \frac{\text{ng of vector (50)} \times \text{kbp size of insert}}{\text{kbp size of vector (2.9)}}$$

Approximately 83ng of insert was ligated into 50ng of pBluescript II SK in 10µl containing 1x ligation buffer (Promega) and 1 unit of T4 DNA ligase (Promega). Ligations were incubated at 4°C overnight before being transformed into XL1 Blue competent cells. 200µl of competent cells were added to 5µl of ligation and incubated on ice for 20 minutes. Transformations were heat shocked at 42°C for 45 seconds before being incubated on ice for a further 2 minutes. SOB (2% tryptone; 0.5% yeast extract; 10mM NaCl; 2.5mM KCl, pH 8.6 and 10mM MgCl) was added to a final volume of 1ml and transformations were incubated at 37°C for 1 hour before being plated onto LB plates containing ampicillin (100µg/ml), X-Gal (20µg/ml) and IPTG (40µg/ml). Recombinant colonies were screened for inserts using appropriate restriction enzyme digestion and DNA of interest was sequenced using automated sequencing and primer walking.



pBluescript SK vector

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3.3 Results

3.3.1 Isolation of six genomic clones for zebrafish GnRH receptor genes

Partial cDNA, encoding zebrafish GnRH receptors Type IA and Type IB, was used to screen a zebrafish genomic DNA library. Type IA and type IB receptor probes were used independently initially. As very little difference in intensity was observed, due to the high degree of homology the probe sequences share, they were combined in further screens. Approximately 1 x 10^6 plaques were screened and following secondary screening six positive recombinant clones, numbers 1-6, were selected (see Fig. 3A). Phage clones were purified and phage plate lysates were used to prepare Lambda DNA.

3.3.2 Identification of genomic fragments containing the GnRH receptor genes

The phage DNAs were digested with *SacI* (see Fig. 3B), *XhoI or SalI* (see Fig. 3D) and subjected to Southern blot analysis to identify genomic fragments of the zebrafish GnRH receptors (see Fig. 3C and 3D). Inserts of zebrafish genomic DNA in phage clones ranged from approximately 14 – 18kbp. Following restriction enzyme digestion with *SacI,* it was clear that phage clones 1, 5 and 6 shared the same *SacI* digest pattern and were likely to contain the same genomic DNA inserts. Subsequent restriction analysis and Southern blot analysis were performed on phage clones 2, 3, 4 and 5.

The Type IA and Type IB zebrafish receptor cDNAs have relatively high homology to each other, so as expected, there was considerable binding of both probes to all six clones. However, Southern blot analysis using zebrafish GnRH receptor Type IA and Type IB cDNA probes, respectively, under stringent conditions, revealed that clones 1, 4, 5 and 6 contained fragments hybridising strongly to Type IA receptor cDNA and clones 2 and 3 contained fragments which hybridised stronger to Type IB receptor cDNA (see Fig. 3C and 3D). Clones 1, 5 and 6 each contained a 5kbp fragment hybridising to zebrafish GnRH receptor cDNA confirming that they probably contained the same genomic DNA insert.

In order to map whether any of the genomic clones had inserts which included exon 1 of the GnRH receptors, the Southern blots were stripped and reprobed with either the full-length

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cDNA clone for the Type IA and IB goldfish GnRH receptors, or a 500bp cDNA fragment which included the start codon, stretching to the extracellular end of TM4. There was no difference between bands that hybridised to the full-length or 500bp cDNA probes (see Fig 3C and 3D), although binding was weaker using the 500bp probe.

Due to the large size of the hybridising fragment of genomic clone 2, further characterisation, using restriction enzyme mapping was necessary prior to subcloning. Digestion of clone 2 with *SacI* and *EcoRI* restriction endonucleases followed by Southern blot analysis revealed a fragment of approximately 6kbp, which hybridised to 5' goldfish GnRH receptor cDNA (see Fig. 3E). The 6kbp *EcoRI/SacI* fragment of the genomic clone was subcloned and sequenced. A 5kbp fragment, hybridising to 5' goldfish GnRH receptor cDNA, was identified in clones 1 and 4 which were also subcloned and sequenced. Clone 3 contained hybridising fragments which were very similar in size to the right arm of the phage vector and would therefore have been difficult to isolate in the time available.

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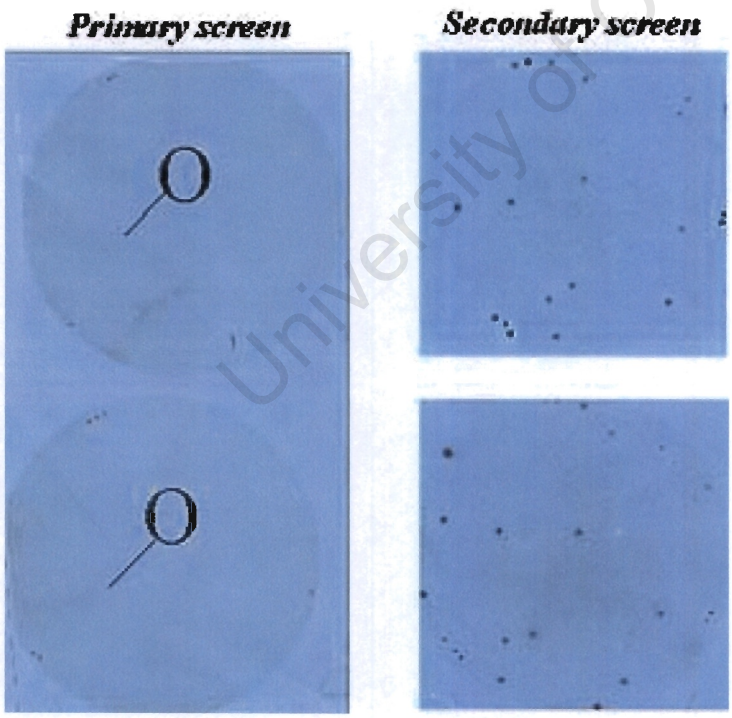

Fig 3A - Autoradiographs showing primary and secondary screen positive plaques identified after screening a lambda phage zebrafish genomic library

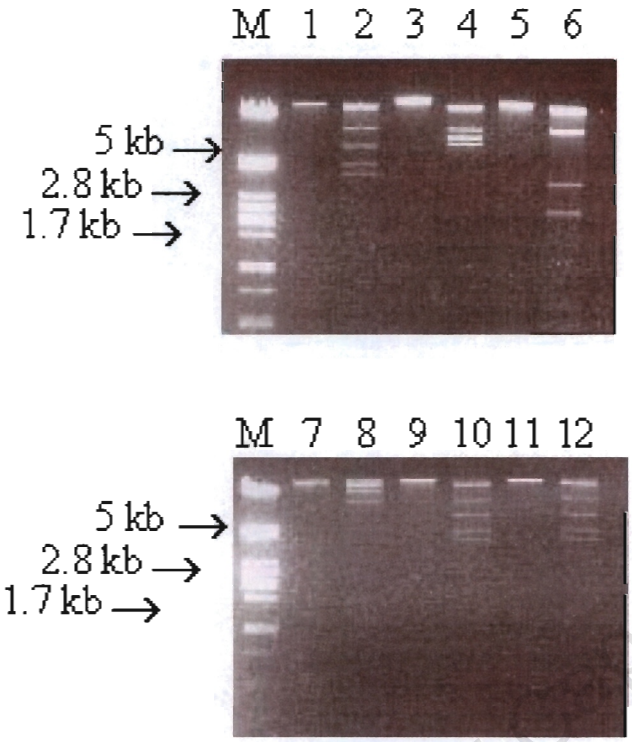

Fig. 3B – Ethidium bromide stained agarose gel showing the genomic clones isolated after secondary screening of the zebrafish genomic DNA library. The clones are digested with *SacI* to excise the inserts from EMBL3 SP6/T7. M represents the MW marker; 1: Clone 1 undigested; 2: Clone 1 digested with *SacI*; 3: Clone 2 undigested; 4: Clone 2 digested with *SacI*; 5: Clone 3 undigested; 6: Clone 3 digested with *SacI*; 7: Clone 4 undigested; 8: Clone 4 digested with *SacI*; 9: Clone 5 undigested; 10: Clone 5 digested with *SacI*; 11: Clone 6 undigested; 12: Clone 6 digested with *SacI*.

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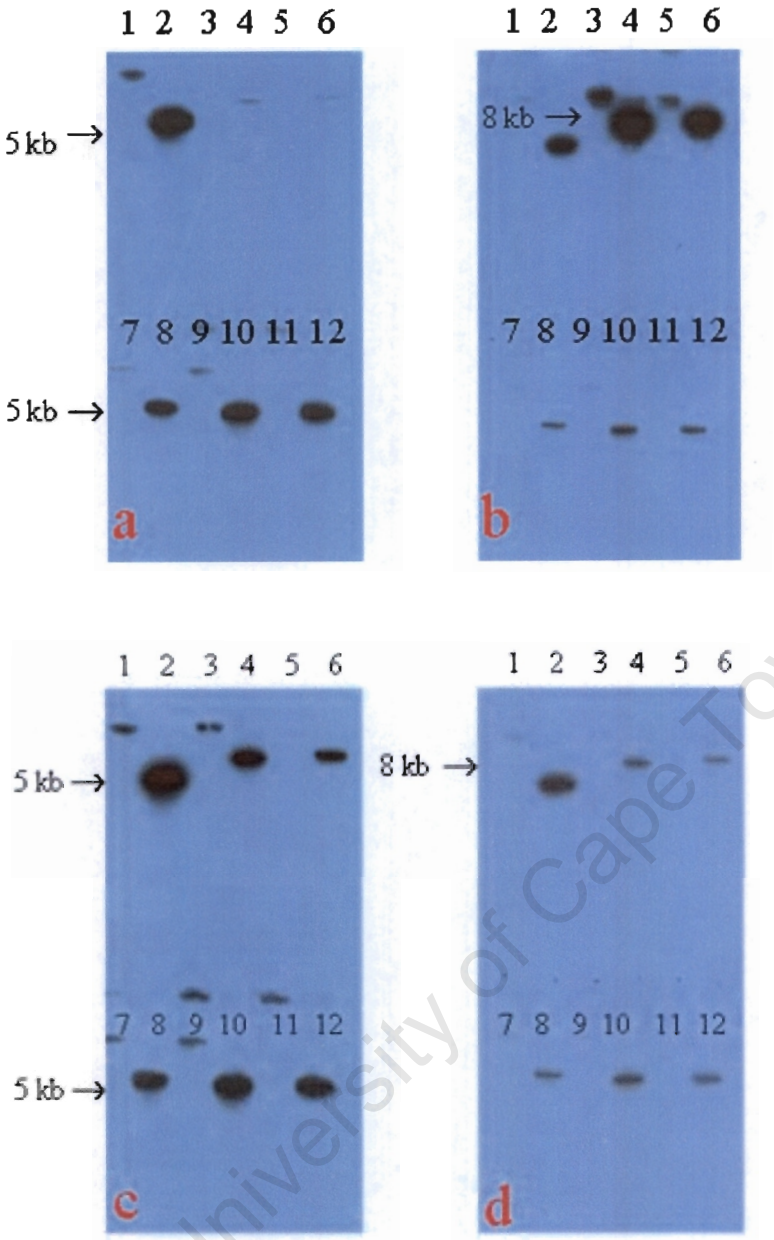

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Fig 3C - Southern blot of *SacI* digested genomic clones shown in Figure 3B. 1: Clone 1 undigested; 2: Clone 1 digested with *SacI*; 3: Clone 2 undigested; 4: Clone 2 digested with *SacI*; 5: Clone 3 undigested; 6: Clone 3 digested with *SacI*; 7: Clone 4 undigested; 8: Clone 4 digested with *SacI*; 9: Clone 5 undigested; 10: Clone 5 digested with *SacI*; 11: Clone 6 undigested; 12: Clone 6 digested with *SacI*.

DNA probes as follows; **a**: EC3 of Type IA zebrafish GnRH receptor; **b**: EC3 of Type IB zebrafish GnRH receptor; **c**: full-length goldfish GnRH receptor cDNA; **d**: 500bp fragment encoding the 5' region of the goldfish GnRH receptor.

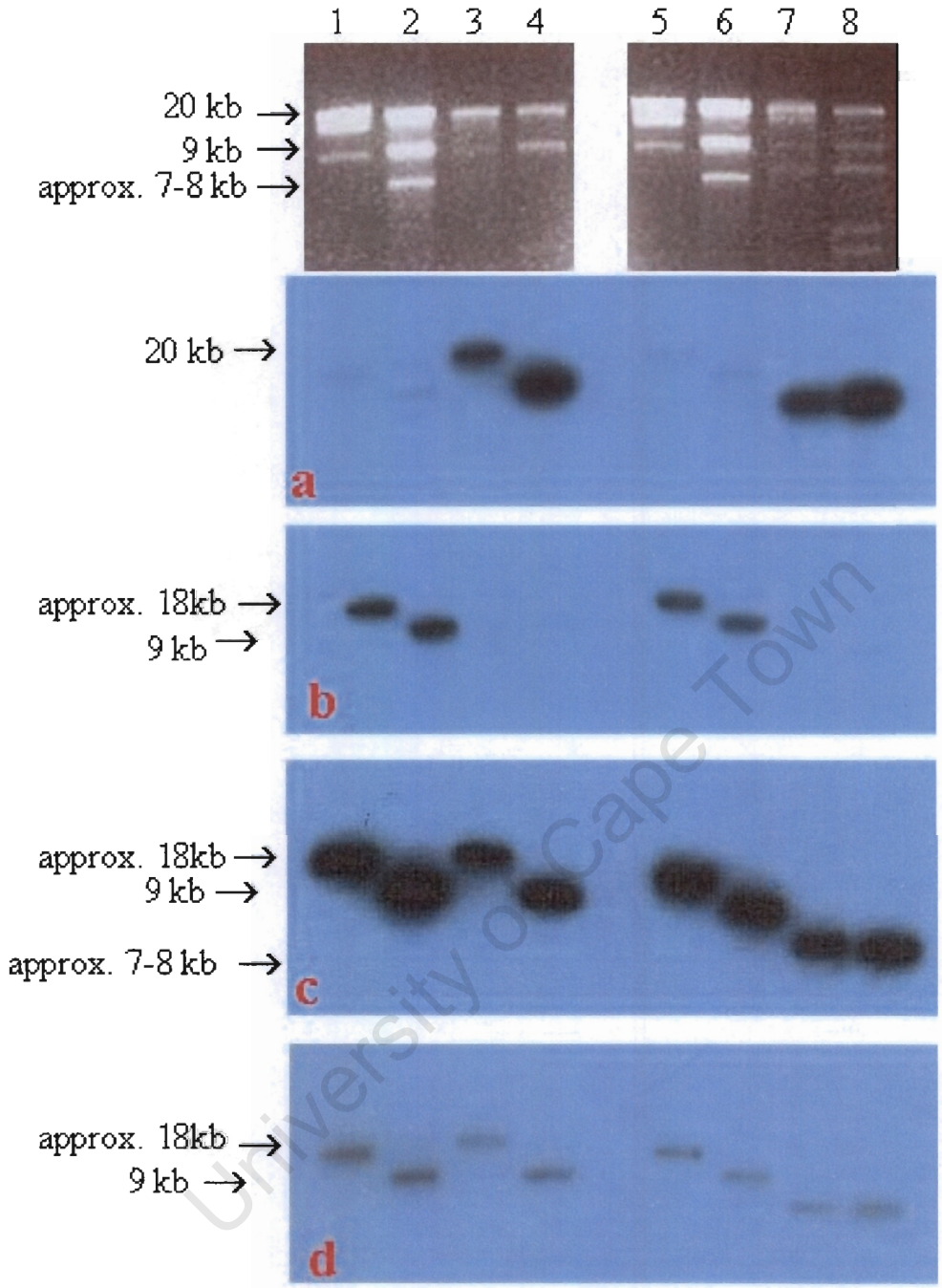

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Fig 3D – Ethidium bromide stained agarose gel and Southern blot of *XhoI and SalI* digested genomic clones. 1: Clone 2 digested with *SalI*; 2: Clone 3 digested with *SalI*; 3: Clone 4 digested with *SalI*; 4: Clone 5 digested with *SalI*; 5: Clone 2 digested with *XhoI*; 6: Clone 3 digested with Xho*I*; 7: Clone 4 digested with *XhoI*; 8: Clone 5 digested with *XhoI*. DNA probes as follows; **a**: EC3 of Type IA zebrafish GnRH receptor; **b**: EC3 of Type IB zebrafish GnRH receptor; **c**: full-length goldfish GnRH receptor cDNA; **d**: 500bp fragment encoding the 5' region of the goldfish GnRH receptor.

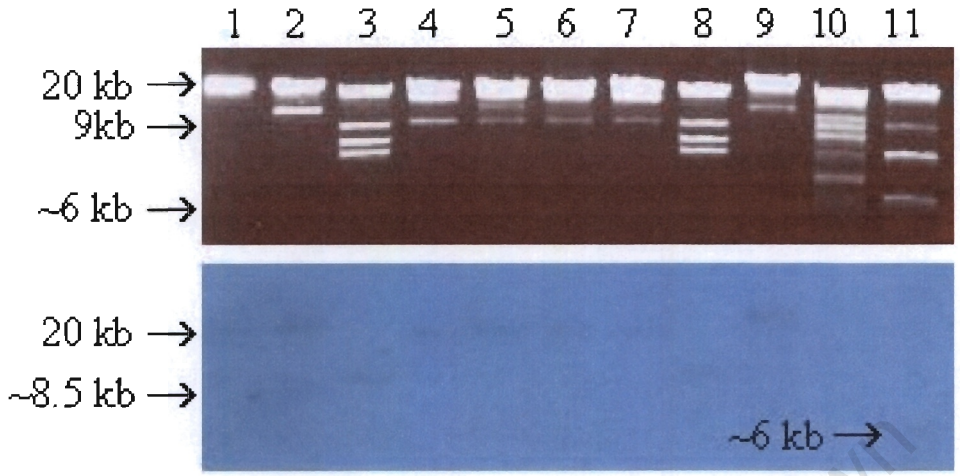

Fig 3E - Ethidium bromide stained agarose gel and corresponding Southern blot of clone 2 multiple digests. 1: *NotI*; 2: *EcoRI*; 3: *SacI*; 4: *XhoI*; 5: *SalI*; 6: *NotI/SalI*; 7: *NotI/XhoI*; 8: *NotI/SacI*; 9: *NotI/ EcoRI*; 10: *XhoI/EcoRI*; 11: *SacI/EcoRI*. The 500bp fragment encoding the 5' region of the goldfish Type IA GnRH receptor was used as a probe.

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3.3.3 Characterisation of the zebrafish GnRH receptor genes

BLAST search revealed that the nucleotide sequence of genomic clones 1 and 2 were in agreement with that of other Type I non-mammalian GnRH receptors previously identified. Part of the sequence obtained from genomic clone 1 showed high homology (90%) to the corresponding region of the goldfish Type IA GnRH receptor cDNA (see Figure 3G). Alignment with other non-mammalian Type I GnRH receptor genes revealed that clone 1 lacked the 5' end of the gene, only encoding part of exon 3. Due to time constraints, the remainder of clone 1 was not sequenced. The rest of the 5kb insert that was sequenced was intronic. The sequence of the coding region obtained from genomic clone 2 showed the greatest sequence homology to the goldfish Type IB GnRH receptor, confirming provisional Southern blot results (see Figure 3G). The gene sequence encoded by genomic clone 2 encompasses exons 2 and 3 with 90% and 91% homology to GfB, respectively. The remainder of the 6kb genomic clone encoded the intron located between exons 2 and 3. The intron/exon structure of both genomic clones, align with the positions of the introns and exons of other non-mammalian Type I receptor genes apart from 3 amino acids at the beginning of exon 3. Further experiments would need to be undertaken to confirm sequences as only one clone was used at this stage. A schematic representation of genomic clones with respect to the medaka Type I GnRH receptor gene (GnRH-R2) can be seen in Figure 3F. The three introns of the Type I non-mammalian receptor gene are termed introns A, B and C and are located in the extracellular N-terminal domain, TMIV and the third extrcellular loop, respectively. These positions are conserved in zebrafish genomic clones 1 and 2. Intron C of clone 2 was approximately 5kb in length similar to that of the medaka Type I receptor gene. Clone 4 was partially sequenced, but again, was found to be primarily intronic sequence and was not pursued.

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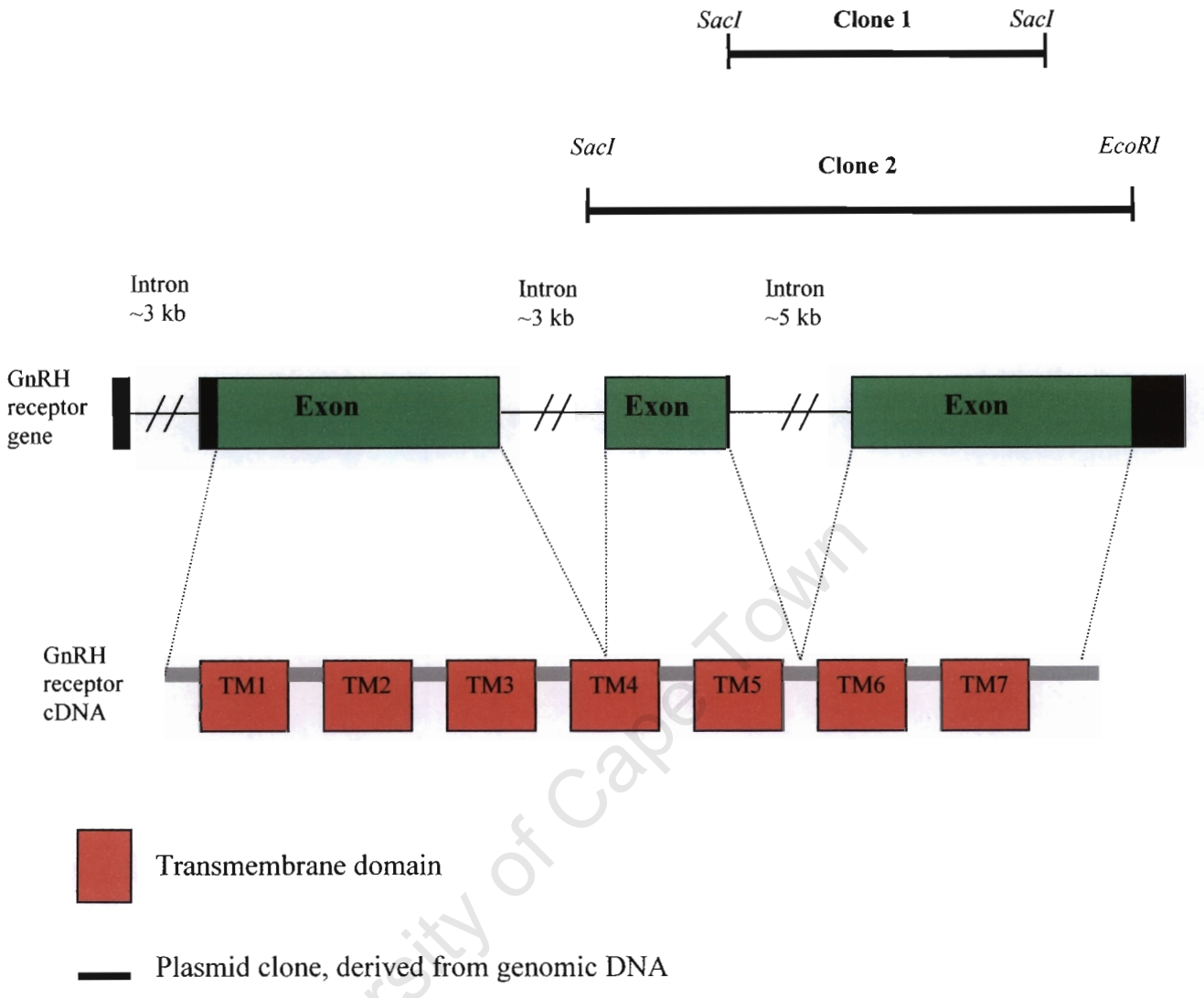

Fig 3F - Schematic diagram showing the Type I GnRH receptor gene identified in the medaka (Okubo *et al.*, 2001). Regions identified in zebrafish plasmid clones 1 and 2 are indicated at the top of the diagram. Intron sizes are based on the medaka GnRH receptor gene.

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// - Intron boundries
* - Not sequenced beyond this point

Figure 3G - Alignment of zebrafish genomic clones 1 and 2 with goldfish Type IA and Type IB GnRH receptor cDNA sequences, respectively. Conserved regions are highlighted. The positions of the introns are indicated with //.

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3.4 Discussion

In this study, parts of the genes encoding the zebrafish Type IA and Type IB GnRH receptors have been identified. Previously, only the sequence information encompassing EC3 was known. However, in this study exon 2 and exons 2 and 3 have been sequenced for zebrafish type IA and type IB GnRH receptors, respectively. These receptors share several common features with other non-mammalian GnRH receptors. The presence of a C-terminal tail in the zebrafish Type IB receptor is evident from exon 3 sequence alignment, similar to other non-mammalian receptors such as GfB and the catfish receptor (see Figure 3G). The intracellular carboxy-terminal tail is necessary for ligand-induced receptor desensitisation and internalisation (Pawson *et al.*, 1998). Low homology is observed in the C-terminal tails of non-mammalian receptors of different subtypes in a single species i.e. between bullfrog 1, 2 and 3 and medaka GnRH-R1 and GnRH-R2. This suggests distinct receptor types in a single species may exhibit diverse patterns of desensitisation and internalisation (Okubo *et al.*, 2001 and Wang *et al.*, 2001). The Type IA goldfish GnRH receptor also possesses a carboxy-terminal tail, although it is fifteen residues shorter than that of GfB. The presence of a C-terminal tail in the zebrafish Type IA receptor is evident from the sequence obtained from genomic clone 1, though the complete C-terminal sequence was not elucidated.

The gene organisation, for the regions of the receptor sequenced in this study, of both zebrafish receptor genes is similar to other GnRH receptor genes with respect to intron/exon structure. Intron B is positioned within TMIV and intron C is located between TMV and TMVI. The presence of a third intron had been demonstrated in the extracellular domain of the medaka GnRH-R2 gene (Okubo *et al.*, 2001) and the Type I GnRH receptor gene isolated in *X. laevis* (Troskie *et al.*, 2000). All mammalian GnRH receptor genes lack intron A but the positions of introns B and C are conserved in the corresponding positions in TMIV and intracellular loop 3 (Kakar *et al.*, 1997 and Neill *et al.*, 2001). It appears that the Type III GnRH receptors identified in species of bony fish also lack intron A. It is not yet known whether the zebrafish GnRH receptor genes possess a third intron, intron A, as the genes were not sequenced preceding exon 2. The conservation of the locations of introns B and C in all GnRH receptor subtypes, irrelevant of the species, reinforces the theory that a gene duplication event occurred early in vertebrate evolution. In the medaka, the GnRH-R1 and GnRH-R2 genes have been

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assigned to distinct chromosomes making it unlikely they arose from gene duplication within the teleost lineage.

It is unclear if intron A was lost in the mammalian GnRH gene or gained in Type I non-mammalian GnRH receptor gene. Whether the ancestral gene had a 3 intron/4 exon or 2 intron/3 exon structure remains to be seen. However, Type III non-mammalian and the mammalian GnRH receptor genes share the 2 intron/3 exon structure suggesting this may be the ancestral gene which arose early in vertebrate evolution and the 3 intron/4 exon GnRH gene evolved later in non-mammalian species.

The presence of such large introns in the GnRH receptor genes makes obtaining an entire gene from a single genomic clone problematic. It may be that the entire 15kbp of genomic clones 1 and 2 do contain the 5' regions of the Type IA and IB zebrafish GnRH receptor genes, respectively. However it would be more practical to repeat the primary screen of the genomic library with a short 5' goldfish receptor probe, only encoding sequence within exon 1, as opposed to the zebrafish EC3 probe used in the initial screening. Although genomic clones were Southern blotted and probed with a 500bp 5' goldfish GnRH receptor cDNA, it overlapped significantly enough with exon 2 to produce faint and misleading binding. Useful information was obtained regarding the zebrafish GnRH receptor genes but more time is required to elucidate the complete gene structure. However, information from the genomic clones was used to design primers for 5'RACE reaction, which has subsequently lead to the cloning of the full-length Type IA and Type IB zebrafish receptors by others in this laboratory (Illing, personal communication).

The zebrafish GnRH3 promoter has been characterised and used by other members of the laboratory in reporter gene studies in conjunction with GnRH1 to demonstrate cell-specific expression in transgenic zebrafish (Torgersen *et al.*, 2002). Co-localisation of GnRH1 and GnRH3 was observed, reaffirming the suggestion that teleost GnRH3 genes are orthologues of GnRH1 i.e. the ancestral gene can be traced prior to the divergence of the species in question. The development of multi-transgenic zebrafish expressing both GnRH and GnRH receptor reporter genes can be used in conjunction with confocal microscopy to help divulge

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information regarding developmental origins and tissue specificity surrounding GnRH and its receptor. Unlike *in-situ* and immunostaining techniques, this method should be highly specific and results clearly visible.

The zebrafish genome project is close to completion and may accelerate the elucidation of the 5' region of the zebrafish GnRH receptor genes. Thus, discrepancies concerning developmental origins of GnRH neurons, ligand selectivity of native GnRH receptors and the regulation of gonadotropin and GH release in teleosts (Illing *et al.*, 1999 and Klausen *et al.*, 2001) may soon be clarified using reporter studies in this model fish.

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Chapter 4

Gonadotropin-releasing hormone receptor subtypes in the zebrafish (*D. rerio*)

4.1 Introduction

The zebrafish (*D. rerio*) has been established as a model organism for vertebrate developmental biology (Wang *et al.*, 2001). Transparent zebrafish embryos have been used in the study of the expression of various reporter genes during development. If more information can be gathered regarding GnRH and GnRH receptors in the zebrafish, the controversy surrounding developmental origins of GnRH neurons and their spatio-temporal expression in relation to their cognate GnRH receptors could soon be resolved.

Two endogenous GnRHs have been identified in the zebrafish. These are cGnRHII and sGnRH. Some species of teleost express three native GnRHs, which can be termed GnRH1, GnRH2 and GnRH3 on the basis of their locations within the brain (Dubois *et al.*, 2001). This can be confusing when used to describe species with two GnRHs where the same form is expressed in the preoptic area (POA) and the terminal nerve (TN). When considering the zebrafish, it may be simpler to refer to them as midbrain GnRH (MB GnRH) which is predominantly GnRH2, and TN GnRH or POA GnRH when referring to sGnRH.

The evolutionary explanation for the multiplicity of native GnRHs in the teleost lineage is confusing. Ancient teleost species have been shown to possess two endogenous GnRHs, suggesting the occurrence of three GnRHs in a single species may be a more recently evolved characteristic of modern teleosts. However, three endogenous GnRHs have been identified in the herring, an ancient teleost. Of the teleosts which are supposed to have evolved after the herring, some express three GnRHs, for example, species of perciform, whilst others have only two, including tetraploid species of fish such as zebrafish and goldfish. It may be that the herring did not give rise to all species of modern teleost or that a third form is present in tetraploids but has not yet been identified. Another suggestion is that the 'new' GnRH gene

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was lost during tetraploidisation (Carolsfeld *et al.*, 2000). Whether two or three forms of GnRH are present in a single species, their spatial distribution suggests they have distinct functions.

GnRH has long been known to regulate the release of GtHII (LH-like gonadotropin II) and GtHI (FSH-like gonadotropin I) in fish but has also been shown to function as a prolactin-releasing factor (Weber *et al.*, 1997) and to stimulate the release of GH (see review Melamed *et al.*, 1998 and Klausen *et al.*, 2001). There must be reasoning behind the occurrence of multiple GnRHs in a single species. The refined control of reproduction gained by having multiple GnRHs may offer an evolutionary advantage.

In a species expressing three native GnRHs, each GnRH is capable of stimulating the release of gonadotropins *in vitro,* though only the neurons of the POA GnRH actually infiltrate the pituitary. It seems likely that distinct native GnRHs perform separate functions and the spatial distribution of cognate receptors may help preserve the distinct functions of endogenous GnRHs. The identification of multiple receptor types in a single species is becoming a common occurrence since the discovery of two receptor subtypes in the goldfish, a tetraploid species (Illing *et al.*, 1999). Two receptor subtypes have also been identified in other species of teleost fish such as the medaka (Okubo *et al.*, 2001) and cichlid (Robison *et al.*, 2001 and see chapter 2 of thesis). The goldfish receptors have been designated as a Type IA and a Type IB GnRH receptor on the basis of their amino acid sequence homology. However, the receptors identified in the cichlid and medaka have been shown to represent a Type I but also a Type III GnRH receptor. The presence of a second Type I receptor has not yet been demonstrated in either species although it has been suggested that a third GnRH receptor may exist in the medaka as neither of the two identified receptors shows preference for GnRH1. In other species of teleost, either a Type I or a Type III receptor has been identified (Tensen *et al.*, 1997, Alok *et al.*, 2000).

Extracellular loop three (EC3) of the mammalian GnRH receptor is known to be important in determining ligand selectivity (Flanagan *et al.*, 1994). Hence, differences in this region may be equated to receptor type (Troskie *et al.*, 1998). Degenerate primers designed to conserved regions flanking EC3 were used to identify partial sequences encoding a Type IA and a Type

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IB receptor in the zebrafish (Troskie *et al.*, 1998). Therefore, the possibility of the existence of a third GnRH receptor type in the zebrafish was investigated using the above mentioned EC3 degenerate primers and primers specific for the Type III GnRH receptor.

4.2 Materials and methods

4.2.1 Genomic DNA and total RNA

Zebrafish genomic DNA and RNA were obtained via personal communication with Dr. Kate Whitlock (Cornell University, USA). The quality was assessed and they were stored as described in section 2.2.1.

4.2.2 Degenerate primer PCR

The degenerate PCR primers, JH5s and JH6a$_2$ (see section 2.2.2) were used to amplify zebrafish genomic DNA. Amplification was carried out as in section 2.2.2 using 1µg of zebrafish genomic DNA. 10µl of PCR products were electrophoresed through a 2% TAE agarose gel containing ethidium bromide (0.1µg/ml).

4.2.3 PCR product subcloning and colony PCR

PCR products of interest were excised from a low melting point agarose gel (FMC Bioproducts), purified using Gene Clean II (Bio101) and subcloned using Promega pGEM-T Easy vector kit, according to the manufacturer's instructions (see map of pGEM-T Easy vector below). Recombinant colonies were screened using blue/white selection. Colonies were picked using a sterile pipette tip and placed directly into the 20µl reactions containing 10mM Tris-HCl (pH 9), 50mM KCl, 0.1% Triton, 2mM $MgCl_2$, 200µM dNTPs, 200ng of SP6 and T7 primers, and 5 units of *Taq* DNA polymerase (Promega). PCR conditions were as follows: 94°C for 3 minutes, and 10 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 3 minutes followed by 20 cycles of 94°C for 30 seconds, 51°C for 30 seconds and 72°C for 3 minutes with a final extension step at 72°C for 5minutes. 4µl of PCR products were electrophoresed through a 1.2% TAE agarose gel containing ethidium bromide (0.1µg/ml).

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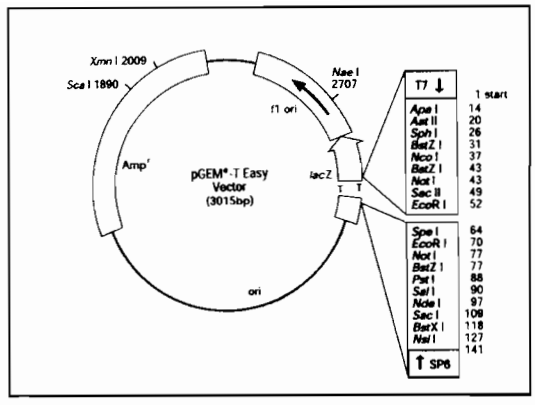

pGEM-T Easy Vector

4.2.4 Restriction enzyme screening

The restriction enzymes, *RsaI* and *BstEII,* were used to eliminate clones having Type IA and Type IB GnRH receptor inserts, respectively. Clones identified as having a unique restriction pattern or neither *RsaI* nor *BstEII* restriction sites were sequenced using an automated sequencer.

4.2.5 Reverse transcriptase PCR (RT-PCR)

The degenerate primers described in section 2.3.2, which are specific for the Type III non-mammalian GnRH receptor, were used to amplify zebrafish total RNA. Reverse transcription was performed by heating 2.5µg of total RNA, in a volume of 12.5µl DEPC treated water, to 70°C for 10 minutes. Subsequent reactions were carried out in a final volume of 20µl containing 200ng of random primer, 1X MMLV buffer (250mM Tris-HCl (pH 8.3); 250mM KCl; 2.5mM spermidine; 50mM DTT), 500µM dNTPs, 20 units of RNAsin and 200 units of MMLV enzyme. The above was incubated at room temperature for 10 minutes followed by 37°C for 50 minutes and 70°C for 10 minutes. An aliquot of reverse transcribed reaction was amplified in a Perkin-Elmer GeneAmp hot-lid thermal cycler in a 50µl volume containing 10mM Tris-HCl (pH 9), 50mM KCl, 0.1% Triton, 2mM $MgCl_2$, 200µM dNTPs, 200ng of each degenerate primer (S2, S4, as1 and as2) and 5 units of *Taq* DNA polymerase (Promega). PCR conditions were as follows: 94°C for 5 minutes, and 30 cycles of 94°C for 1 minute, 50°C for 1

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minute and 72°C for 2 minutes followed by a final extension step at 72°C for 5minutes. 8µl of PCR products were electrophoresed through a 1% TAE agarose gel containing ethidium bromide (0.1µg/ml).

4.3 Results

4.3.1 Amplification of extracellular loop 3 of the zebrafish GnRH receptor genes

A product of the expected size, approximately 120bp, was visible following PCR amplification with the degenerate primer pair, JH5s and JH6a$_2$ (see fig. 4A). The product was subcloned and recombinant colonies were screened for inserts using colony PCR followed by restriction enzyme digest.

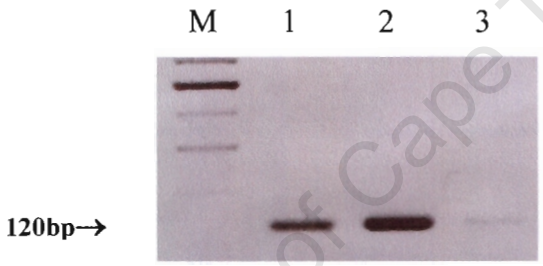

Fig. 4A - Amplification of EC3 of zebrafish GnRH receptors. M – 100bp molecular weight marker; 1) Using 0.5 µg of genomic DNA; 2) Using 1µg of zebrafish genomic DNA; 3) Negative control.

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4.3.2 Restriction enzyme screening

Inserts were non-directionally cloned into the multiple cloning site of pGEM-T. Therefore, restriction digests produced two distinct patterns depending on the orientation with regard to SP6 and T7 primers (see Fig. 4B). *RsaI* restriction enzyme was specific for the Type IA GnRH receptor and produced two bands of sizes 137bp and 173bp or 157bp and 153bp. The restriction enzyme *BstEII* was used to identify inserts of receptor Type IB producing two possible restriction patterns of 164bp and 146bp or 184bp and 126bp. Forty-six recombinant clones were subjected to colony PCR and subsequent restriction enzyme digest with both *RsaI* and *BstEII* (see Fig. 4C). Sixteen clones had a *RsaI* restriction site and produced a restriction

digest pattern indicative of a Type IA GnRH receptor. Also, sixteen clones were designated Type IB receptors after inserts were shown to cut with *BstEII* having the correct restriction pattern. Thirteen clones which did not cut with either restriction enzyme or showed dubious restriction patterns, such as clone 20 in Fig. 4C, were sequenced. Sequencing results only revealed inserts with homology to Type IA (six clones) and Type IB (seven clones) GnRH receptor sequences. These were in complete agreement with the zebrafish Type IA and Type IB GnRH receptor EC3 sequences obtained by Troskie *et al*, (1998). One colony did not amplify as no bands were observed using agarose gel electrophoresis. No other receptor types were identified in this experiment.

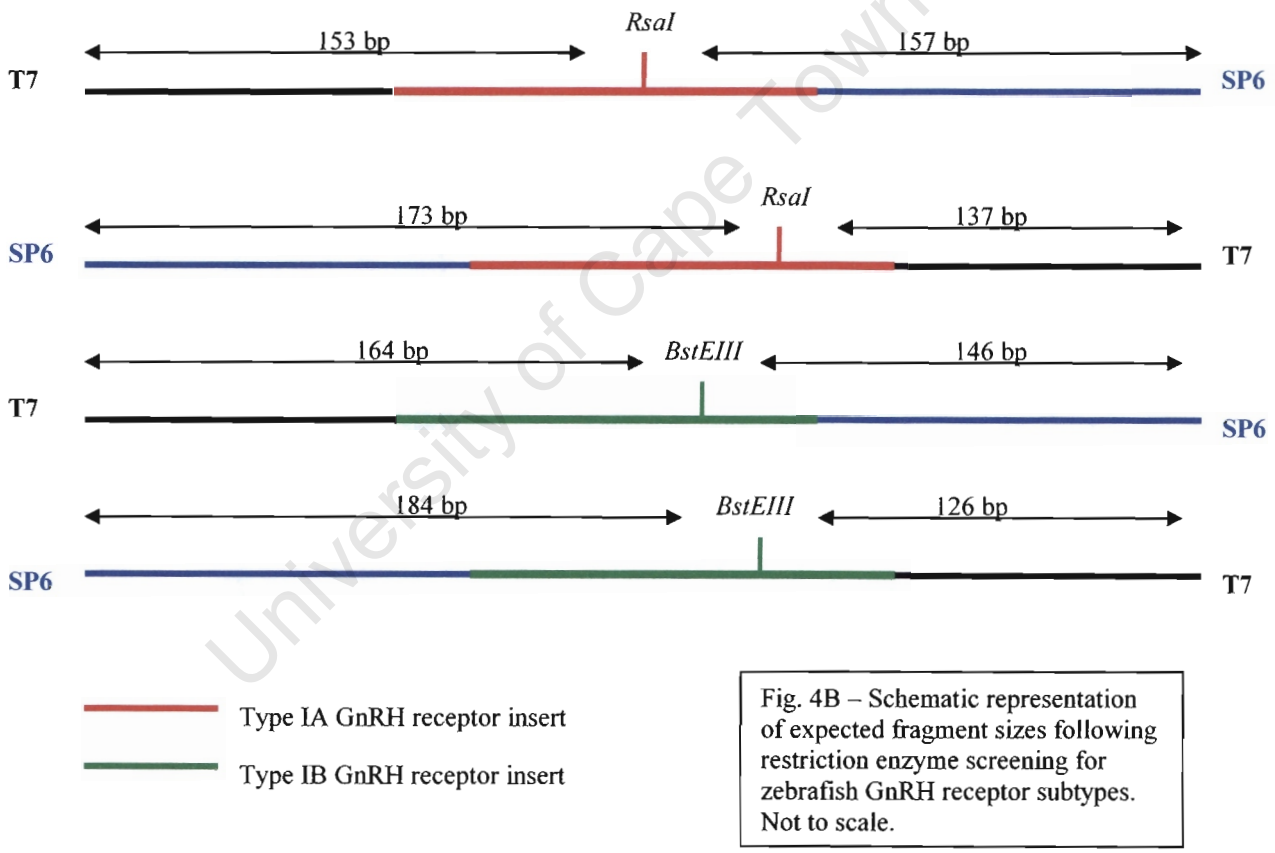

Fig. 4B – Schematic representation of expected fragment sizes following restriction enzyme screening for zebrafish GnRH receptor subtypes. Not to scale.

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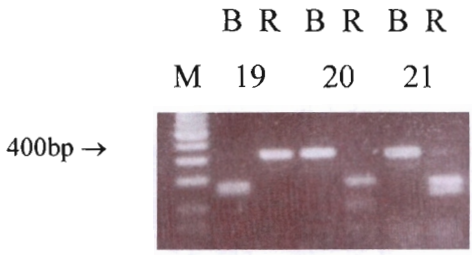

Fig. 4C - Example of three colony PCR clones subjected to restriction enzyme digest. M is the molecular weight marker. B – *BstEII,* R – *RsaI.* Clone 19 cuts with *BstEII* (Type IB), Clone cuts with *RsaI* (Type IA), Clone 21 cuts with *RsaI* (Type IA).

4.3.3 RT-PCR specific for the Type III GnRH receptor

Degenerate primers designed to conserved regions unique to the known Type III GnRH receptors, were used in an attempt to identify a putative Type III receptor in the zebrafish. Amplification was performed in tandem with both zebrafish and cichlid total RNA. The cichlid sample was included as a positive control and produced a band of approximately 750bp using the degenerate primer pair S2 and as1 (see Fig 4D). No bands were observed following amplification in the zebrafish.

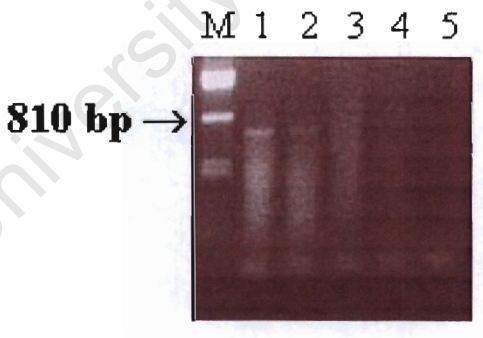

Fig. 4D – RT-PCR of cichlid and zebrafish RNA using primers specific for the Type III GnRH receptor. M represents the MW marker, 1) and 2) are duplicate cichlid samples amplified with S2 and as1 primers; 3) and 4) are duplicate zebrafish samples amplified with S2 and as1 primers; 5) negative control.

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4.4 Discussion

These results provide an interesting insight to the multiplicity of GnRH receptors seen in a single species. Previously, Troskie *et al* (1998) identified two distinct receptor types in the zebrafish (*D. rerio*). Using degenerate primers encoding conserved sequences flanking EC3 of the mammalian GnRH receptor, partial cDNAs encoding Type IA and IB receptors were identified. More recently, the presence of Type III GnRH receptors has been demonstrated in species of bony fish, alone and in addition to a Type I GnRH receptor. This study therefore aimed to establish whether or not a Type III receptor is present in the teraploid species, the zebrafish. Neither amplification of genomic DNA and RNA with EC3 nor Type III specific primers, respectively, resulted in the identification of an additional receptor subtype in this species. Full-length cDNAs encoding a Type IA and IB GnRH receptor have been identified in another tetraploid teleost, the goldfish (Illing *et al.*, 1999). It is suggested they may represent separate receptors which have distinct functions in relation to the regulation of gonadotropin and growth hormone release via different endogenous GnRHs. The phylogenetic relationship of these two receptors indicates they might be the result of fairly recent gene duplication although they do not demonstrate sufficient homology to suggest they arose from tetraploidisation. In addition, the catfish is an example of a tetraploid species in which only a single Type I receptor has been detected but it may be that the second is yet to be identified.

The Type I and Type III receptors identified in the cichlid share only 51% homology, similar to the degree of homology observed between receptors isolated in the medaka. In both cases these receptors fall into two distinct phylogenetic lineages unlike Type IA and IB receptors which are variants within the same lineage (see Fig. 2J). It has been suggested that the Type I and Type III receptors arose from a gene duplication that took place early in vertebrate evolution, predating the divergence of teleosts and tetrapods. It also shows that the multiplicity of the GnRH receptor is not simply the result of ploidy, as the cichlid and medaka are both diploid species. This is supported by the fact that a Type I and two Type III receptors have been identified in the bullfrog (Wang *et al.*, 2000). It seems logical that the presence of a Type III receptor should be preserved throughout the teleost line. It may however have been lost during tetraploidisation or was simply not retained during their evolution.

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It is interesting that many non-mammalian characteristics have been conserved between bony fish and amphibian GnRH receptors. The divergence of amphibians and bony fish predates that of amphibians and mammals by approximately 150 million years. It may not be that the Type III receptor was lost in humans but that selective pressure has resulted in the accelerated divergence of mammalian GnRH receptors. In the mammalian Type I receptor the C-terminal tail is absent, a unique feature thought to promote an absence of rapid ligand-induced desensitisation and subsequent internalisation. This may be an adaptation required during LH-surge conditions observed in mammals. The presence of a second human GnRH receptor has been demonstrated. However, a functional transcript could not be obtained and it is still unclear whether this receptor is actually functional (see review Millar, 2003).

GnRH has been shown to effectively stimulate gonadotropin, GH, prolactin and somatolactin cells in bony fish (Weber *et al.*, 1997). It may be that the presence of three native GnRHs, observed in certain species of bony fish, has encouraged the paralogous evolution of three cognate GnRH receptors necessary for the undertaking of their diverse functions. In the cichlid fish (tilapia; *O. niloticus*), immunological methods and *in-situ* hybridisation have been used to demonstrate the presence of Type IA and IB receptors in addition to a Type III receptor. Each receptor type demonstrates a distinct pattern of spatio-temporal expression indicative of having separate functions. Expression of Type IA and IB receptors was seen in LH and prolactin producing cells, respectively, where the Type III receptor was identified in GH cells (Parhar *et al.*, 2002). It is interesting that in the bullfrog it is the Type III receptor that is present as two variant forms of the same subtype as opposed to the Type I receptor in the zebrafish and goldfish. If it is true that the Type III receptor is primarily involved in the regulation of GH release, the occurrence of two Type III GnRH receptors may be indicative of the need for a more refined co-ordination between growth and reproduction in the bullfrog. Possibly, in teleost species such as zebrafish and goldfish, which so far have been shown to possess only two native GnRHs, there may be no requirement for a third GnRH receptor. Functional necessity may be met adequately with two GnRHs and their cognate receptors. Following gene duplications, it is suggested that resulting genes share the functions of the ancestral gene rather than simply mimicking each other, which may be the case here.

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It may be that a third GnRH receptor type is present in the zebrafish but was not identified in this study. Regions of a putative additional zebrafish receptor may not exhibit high homology with EC3 or Type III receptor sequences and therefore was not amplified using the PCR primers selected for this study. This may have been clarified by attempting to amplify zebrafish genomic DNA with type III gene specific primers. However, this was not undertaken in this study but should be addressed in future studies. Maybe the receptor is present as a rare transcript expressed in discrete tissues that were not represented sufficiently in the total RNA sample. Another possibility is that of a temporally expressed receptor type, which can only be identified, using samples from varying developmental stages. If the Type III receptor is primarily involved in regulating GH release it may only be expressed at detectable levels early on in development up until maturity.

It is evident from the above data that further information is required before a clear picture of the reasoning behind the multiplicity of GnRH and cognate receptors can be understood. The possible cloning and characterisation of GnRH receptors in more ancient vertebrate species, and in particular species of teleost, may help the understanding of how and why these receptor subtypes diverged from their ancestral genes and the impact this has had on their reproductive mechanisms and subsequent evolutionary success.

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5 Conclusion

Understanding the significance of multiple GnRHs and GnRH receptors is of both scientific and therapeutic value. Information regarding its control of reproduction in vertebrates may aid the development of more effective contraceptives and answer questions regarding infertility. In addition, GnRH is reported to influence tumour cell growth and possibly GnRH agonists and antagonists can be exploited for use as therapeutic agents.

A complex interplay between ligand and receptor must exist to manage the multiplicity of GnRH in a single species. Multiple cognate receptors may have evolved in parallel to accommodate specific requirements and it is likely that at least two GnRH receptor subtypes are present in most, if not all, vertebrate species. Exactly which GnRH receptor corresponds to which native GnRH, and exactly what effect the affiliation results in, is unclear. It is unlikely to be as simple. The temporal and spatial pattern of GnRH and GnRH receptor expression may also have significant influence on their effectiveness.

The present study on gonadotropin-releasing hormone receptors aimed to generate information that could possibly elucidate some of the questions surrounding the diversity of GnRH and GnRH receptors and their developmental origins. Two species of teleost fish were used as model systems in which to investigate this trend. Although previous studies demonstrated that mammalian and non-mammalian GnRH receptors possess some unique features, they are significantly comparable.

In the present study, a Type III GnRH receptor was identified in the cichlid fish, *H. burtoni*. This is in addition to a Type I receptor that was previously described by Robision *et al.* (2001). The significance of this finding is unclear, with respect to the social control of reproduction, which is observed in this species. However, it will be interesting to see how and if these receptors are differentially controlled under social conditions. In addition, only Type IA and IB GnRH receptors were identified in the zebrafish, consistent with previous findings. Whether this is a characteristic feature of tetraploid species of fish remains to be seen.

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Little is known about the origins and expression of GnRH neurons and GnRH receptors during development. The screening of a zebrafish genomic library was employed to help elucidate the sequence of the 5' and promoter regions of the zebrafish GnRH receptors and use this information to construct reporter genes for developmental studies. The presence of large introns made this objective difficult to achieve in the time available. However, the information that was obtained regarding these genes has lead to the cloning of full-length zebrafish GnRH receptor cDNAs (Illing, personal communication).

This work provides a basis for understanding the multiplicity observed with respect to GnRH and GnRH receptors and may hopefully contribute to the elucidation of their diverse functional roles.

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