APPETITE REGULATION IN WINTER FLOUNDER (Pseudopleuronectes americanus): CHARACTERIZATION OF MELANIN-CONCENTRATING HORMONE (MCH) AND GONADOTROPIN-RELEASING HORMONE (GnRH) TRANSCRIPT FAMILIES AND THEIR ROLE IN FEEDING BEHAVIOUR

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Appetite regulation in winter flounder (*Pseudopleuronectes americanus*): characterization of melanin-concentrating hormone (MCH) and gonadotropin-releasing hormone (GnRH) transcript families and their role in feeding behaviour

by

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Abstract

In vertebrates, appetite regulation is a complex process involving both nervous and endocrine systems. Melanin-concentrating hormone (MCH) and gonadotropin-releasing hormone (GnRH) are two endocrine factors that have been found to regulate food intake in fish and other vertebrates. These hormones were characterized in winter flounder, *Pseudopleuronectes americanus*, a flatfish common in Newfoundland bottom waters. mRNAs encoding several forms of both hormones were identified and shown to be expressed in tissues previously found to be involved in appetite regulation, including the brain (telencephalon, optic tectum, and hypothalamus) and midgut. Adult fish submitted to fasting displayed higher brain expression levels of transcripts encoding MCH and its receptor, MCH-R1, suggesting that the MCH system might stimulate feeding in flounder. Conversely, both chicken- and salmon-GnRH mRNA levels were lower in fasted fish suggesting an appetite-inhibiting effect. Our results suggest that MCH and GnRH, along with their receptors, might play an important role in regulating feeding in winter flounder.

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List of Abbreviations

aa: amino acid

AgRP: agouti gene-related protein

ARC: arcuate nucleus

B: brain

BBMS: Bonne Bay Marine Station

BLAST: basic local alignment search tool

bp: base pairs

C: cerebellum

CART: cocaine-and amphetamine regulated transcript

cDNAs: complementary deoxyribonucleic acid

cfGnRH: catfish gonadotropin-releasing hormone

cGnRH: chicken gonadotropin-releasing hormone

Con: control

CRF: corticotrophin-releasing factor

Ct: cycle threshold

DNA: deoxyribonucleic acid

dNTP: deoxynucleotide triphosphate

EF-1α: elongation factor 1α

ES: eyed-side

FG: foregut/stomach

FSH: follicle-stimulating hormone

G: gill

GAP: gonadotropin-releasing hormone associated peptide

GB: gall bladder

GH: growth hormone

GI: gastrointestinal tract

GnRH: gonadotropin-releasing hormone

GnRH-R: gonadotropin-releasing hormone receptor

GSI: gonadosomatic index

H: hypothalamus

He: heart

HEK293T: human embryonic kidney-293T

HPG: hypothalamic-pituitary-gonadal

HIS: hepatosomatic index

ICV: intracerebroventricular

ir: immunoreactive

L: ladder

LH: luteinizing hormone

LHRH: luteinizing hormone-releasing hormone

Li: liver

LVR: lateral ventricular recess

M: muscle

MC3-R: melanocortin-3 receptor

MC4-R: melanocortin-4 receptor

MCH: melanin-concentrating hormone

MCH-R: melanin-concentrating hormone receptor

mdGnRH: medaka gonadotropin-releasing hormone

MEGA: Molecular evolutionary genetics analysis

MG: midgut/intestine

mGnRH-1: mammalian gonadotropin-releasing hormone 1

MO: medulla oblongata

mRNA: messenger ribonucleic acid

MSDB: medial septum/diagonal band of Broca

MSH: ∝-melanocyte stimulating hormone

MUSCLE: multiple accurate and fast sequence comparison by log-expectation

NCBI: National Centre for Biotechnology Information

NEI: neuropeptide E-I

nAP: nucleus anterioris periventricularis

nLT: nucleus lateralis tuberis

nPPv: nucleus preopticus paraventricularis

nPPv: nucleus posterioris periventricularis

nPT: nucleus posterior tuberis

NPY: neuropeptide Y

NS: non-eyed side

NSERC: Natural Sciences and Engineering Council

NSV: nucleus saccus vasculosa

O: ovaries

Oct-GnRH: octopus gonadotropin-releasing hormone

OSC: Ocean Sciences Centre

OT: optic tectum/thalamus

OVLT: organum vasculosum of the lamina terminalis

OX: orexins

P: pituitary gland

PCR: polymerase chain reaction

POA: preoptic area

PVO: paraventricular organ

PVH: paraventricular nucleus of the hypothalamus

qPCR: quantitative real-time polymerase chain reaction

RACE: rapid amplification of complementary deoxyribonucleic acid ends

RNA: ribonucleic acid

RT-PCR: reverse transcription-polymerase chain reaction

sbGnRH: seabream gonadotropin-releasing hormone

SC: spinal cord

SEM: standard error of means

sGnRH: salmon gonadotropin-releasing hormone

T: telencephalon/preoptic area

T_3: triiodothyronine

TCAG: The Centre for Applied Genomics

Te: testes

UTR: untranslated region

VMN: ventromedial nucleus

WGD: whole genome duplication

Chapter 1: Introduction and Overview

1.1. Background information

1.1.1. Appetite regulation

Fish feeding behaviour is a complex neuroendocrine process involving multiple levels of organization. Within the brain, the primary control centres consist of various nuclei of the hypothalamus, which receive positive and negative feedback signals from the brain and the periphery. Other brain regions (telencephalon/preoptic area and optic tectum/thalamus) have also been found to regulate food intake (Volkoff *et al.* 2005). Peripheral tissues, including adipose tissue and the gastrointestinal (GI) tract, have also been shown to be intricately involved in appetite regulation. Appetite regulation involves both orexigenic (appetite-stimulating) and anorexigenic (appetite-inhibiting) factors, which work together to maintain energy homeostasis. Orexins (OX) (Volkoff *et al.* 1999) and neuropeptide Y (NPY) (Lopez-Patino *et al.* 1999) are examples of central nervous system orexigenic peptides, whereas cocaine- and amphetamine-regulated transcript (CART) (Volkoff and Peter 2000) and corticotrophin-releasing factor (CRF) (de Pedro *et al.* 1997) are central nervous system anorexigenic hormones. Leptin (Johnson *et al.* 2000) and adiponectin (Nishio *et al.* 2008) are anorexigenic peptides produced by liver and adipose tissue, and ghrelin (Unniappan *et al.* 2002) is an orexigenic factor secreted within the GI tract.

The decision to eat (hunger) or not to eat (satiety) is achieved by the brain

following the integration of a variety of internal and external cues. Internal cues include peptides from other endocrine systems, such as the reproductive and stress axes, and biological rhythms. For example, behavioural studies have shown that both Atlantic cod (*Gadus morhua*) and winter flounder (*Pseudopleuronectes americanus*) reduce and even cease their food intake during the winter when they are preparing to spawn, suggesting that reproductive hormones might affect feeding behaviour (Fordham and Trippel 1999; Stoner *et al.* 1999). Indeed, in teleosts, interactions between gonadotropin-releasing hormone (GnRH), a major reproductive hormone, and appetite-related hormones, such as OX (Hoskins *et al.* 2008), growth hormone (GH) (Marchant *et al.* 1989), and NPY (Chiba *et al.* 1996) have been demonstrated. Appositions between GnRH and OX-immunoreactive (ir) cell bodies and fibres have been identified in pigs (Su *et al.* 2008) and OX brain implants in rats (*Rattus norvegicus*) induce GnRH release (Russell *et al.* 2001). Hormone intracerebroventricular (ICV) injections in goldfish (*Carassius auratus*) have shown that GnRH inhibits food intake (Hoskins *et al.* 2008), while appetite regulators, such as OX, have been shown to be associated with reproductive behaviours and associated changes in reproduction-related transcripts, including spawning behaviour in goldfish (Hoskins *et al.* 2008) and differential mRNA expression during various stages of the estrous cycle in rats (Porkka-Heiskanen *et al.* 2004), linking the two endocrine systems.

The stress axis is another physiological system that influences food intake in fish. CRF, a major hypothalamic hormone of the stress axis, decreases food intake in goldfish (de Pedro *et al.* 1993) and tench (*Tinca tinca*) (de Pedro *et al.* 1995) following ICV injections. CRF has been found to interact with appetite-related peptides, such as α-

melanocyte-stimulating hormone (MSH) (Tran *et al.* 1990) and galanin (Batten *et al.* 1990) indicating a possible neuromodulation of food intake-related factors by stress axis hormones.

The internal clock of an organism has also been associated with food intake in vertebrates (Challet 2010). However, external factors, including photoperiod, have been found to strongly affect internal mechanisms regulated by the biological clock, such as the feeding cycle and food anticipatory behaviour. Regimented feeding allows the animal to predict feeding time and display behaviours [e.g. increased locomotion, body temperature and corticosterone release (Mistlberger 1994; Stephan 2002; Feillet *et al.* 2006)] in response to internal cues from the circadian clock. Manipulating photoperiod has been found to change food anticipatory behaviours and feeding cycles in sea bass (*Dicentrarchus labrax*) (Sanchez-Vazquez *et al.* 1995), golden shiners (*Notemigonus crysoleucas*) (Lague and Reebs 2000), and rainbow trout (*Oncorhynchus mykiss*) (Bolliet *et al.* 2001). Disruption of circadian rhythms by changes in photoperiod can alter food intake and the expression of appetite-related hormones. In goldfish, circadian (*Clock* and *Per1* genes) and appetite-related (OX) rhythms appeared to be abolished during constant light conditions (Hoskins and Volkoff 2010).

1.1.2. Melanin-concentrating hormone (MCH)

Melanin-concentrating hormone (MCH) is a 15 amino acid (aa) peptide, which was first recognized as a hormone that induced colour changes in basal vertebrates, including fish, which utilize colour change as a mechanism of protection against

predation. MCH and its antagonist α-melanocyte-stimulating hormone (MSH) induce changes in the aggregation of melanosomes (melanin granules) within the dermal melanophores (or melanocytes) (Jain and Patil 1990). The colour change occurs when the melanosomes move up and down the dendritic processes of the melanophore to and from the cell centre. The dispersion (MSH) or aggregation (MCH) of these granules results in the darkening or lightening, respectively, of the fish due to changes in the refractive index (Oshima *et al.* 1985). To date, there is no evidence for a role of MCH in mammalian skin colouration. It has been suggested that MCH could interact with MSH to regulate melanocyte dispersal and conglomeration in mammals (Hoogduijn *et al.* 2002).

The role of MCH in food intake in vertebrates is not fully understood. MCH has been shown to be an appetite-stimulator in mammals, such as rat (Rossi *et al.* 1997), mouse (Qu *et al.* 1996) and sheep (Whitlock *et al.* 2005) using hormone ICV implants and mRNA expression analyses following fasting experiments. However, in fish the function of MCH in appetite regulation is not as clearly defined. Like mammals, barfin flounder (*Verasper moseri*) exhibit a concomitant increase in brain MCH mRNA expression and MCH-ir cell numbers in the nucleus lateralis tuberis (NLT) and lateral ventricular recess (LVR) of the hypothalamus during fasting, suggesting that in this species, as in mammals, MCH might act as a hunger factor (Takahashi *et al.* 2004). Similarly, fasted zebrafish (*Danio rerio*) exhibit an increase in MCH2-ir producing cells, a second form of MCH found only in fish(Berman *et al.* 2009). However, in goldfish, a significant decline in feeding was observed following ICV MCH injections (Matsuda *et al.* 2009), and a decrease in MCH-ir cell bodies in the LVR was seen in fasted fish (Matsuda et al. 2007), suggesting that MCH has anorexigenic effects in this species.

It has been proposed that MCH acts more as a neuromodulator or neurotransmitter than a hormone since its concentrations are very low in mammalian plasma. As MCH nerve fibres co-localize with other neurons producing appetite-related hormones, including OX (Broberger *et al.* 1998; Amiya *et al.* 2007), NPY (Broberger *et al.* 1998), agouti gene-related protein (AgRP) (Broberger *et al.* 1998) and CART (Broberger 1999), it is likely that MCH acts as a neuromodulator for appetite regulation in the brain.

MCH cell bodies are primarily found in the hypothalamus of all vertebrates. The location of MCH cell bodies within specific nuclei of the hypothalamus differs between vertebrates and, more specifically, within fish. In rats, MCH-ir cells bodies were identified in the posterior hypothalamic area, medial forebrain bundle-lateral hypothalamic area, subzona incerta, perifornical area (*i.e.* periventricular nucleus) (Skofitsch *et al.* 1985), nucleus of the diagonal band of Broca (Skofitsch et al. 1985; Zamir *et al.* 1986a), and medial mammillary nucleus (Zamir *et al.* 1986b).

In fish, MCH cell bodies tend to be confined to the hypothalamus, specifically the NLT and paraventricular organ (PVO) of the LVR. Early studies of the most ancestral fish, lamprey (*Lampetra fluviatilis*), showed that in this species, MCH cell bodies are localized within the hypothalamus close to the third ventricle (Al-Yousuf and Mizuno 1991), later identified as the periventricular dorsal hypothalamic nucleus, part of the PVO (Bird *et al.* 2001). Within the NLT, MCH-ir cell bodies were identified in the sailfin molly *(Poecilia latipinna*) (Batten and Baker 1988), Chinese grass carp (*Ctenopharyngodon idella*) and trout (Bird *et al.* 1989), barfin flounder (Amano *et al.* 2003), medaka (*Oryzias latipes*) (Amiya *et al.* 2007) and goldfish (Matsuda *et al.* 2007). MCH-ir neurons were discovered in the PVO of the LVR of trout (Baker *et al.* 1995),

barfin flounder (Amano *et al.* 2003), medaka (Amiya et al. 2007) and goldfish (Matsuda *et al.* 2007). Other hypothalamic nuclei where MCH-ir cells have been identified in teleosts include the pars lateralis in sailfin molly (Batten and Baker 1988), nucleus posterioris periventricularis (NPPv) in medaka (Amiya et al. 2007) and nucleus posterior tuberis (NPT) in goldfish (Matsuda et al. 2007). Unlike mammals, in fish there is an abundance of MCH neuronal projections into the pituitary (Rance and Baker 1979), suggesting that MCH could play a role in modulating secretions from the hypophysis, including appetite-related factors and skin colour-related peptides, through MCH-ir fibres projections either into the median eminence in early evolved ray-finned fish or into the anterior pituitary in teleosts (Kawauchi and Baker 2004).

In order to completely understand how hormones influence feeding, it is necessary to determine how their receptors react to various physiological states, such as fasting. In most vertebrates, including humans (Rodriguez *et al.* 2001) and teleosts (Mizusawa *et al.* 2009), two MCH receptors (MCH-R) have been identified, while in rodents only one has been reported (Hervieu *et al.* 2000). In the rat brain, MCH-R1 mRNA is found in regions related to food intake, including the nucleus accumbens, as well as nuclei of the hypothalamus [ventromedial nucleus (VMN), arcuate nucleus (ARC) and zona incerta)] (Saito *et al.* 2001). In goldfish, MCH-Rs are present in the brain and intestine, amongst other tissues, indicating a possible role in food intake regulation (Mizusawa *et al.* 2009). MCH-R1 mRNA expression in barfin flounder is restricted to the brain, while MCH-R2 mRNA is expressed almost ubiquitously around the body, although higher levels are present in the pituitary gland, brain and intestine (Takahashi *et al.* 2007).

MCH-Rs have been key players in studies on reducing obesity in rodent models.

ICV injected MCH-R1 antagonists reduce obesity and feeding in mice (Ito *et al.* 2010) and rats (Audinot *et al.* 2009), while MCH-R1 agonists caused rats to maintain an obese phenotype (Shearman *et al.* 2003). Fasting in rats induces an increase in MCH-R1 neurons in the vagal nerve demonstrating the receptor's appetite-stimulating effects (Burdyga *et al.* 2006). Obese rats have higher levels of MCH-R1 mRNA in the hypothalamus compared to fasted rats (Elliott *et al.* 2004). In humans, MCH-R2 does not appear to be involved in food intake, since there is no correlation between MCH-R2 single nucleotide polymorphisms in normal and obese children (Ghoussaini *et al.* 2007). In barfin flounder, MCH-R2 has been implicated as the receptor responsive to environmental cues and control of colour change since barfin flounder in tanks with white backgrounds have higher brain MCH-R2 mRNA expression, while mRNA expression of MCH-R1 in brain of barfin flounder could suggest a role for this form as a neuromodulator (Takahashi *et al.* 2007).

1.1.3. Gonadotropin-releasing hormone (GnRH)

GnRH is the primary hypothalamic hormone that regulates reproduction in vertebrates. It is known for its neuromodulation of other hormones, such as orexins (Hoskins *et al.* 2008) and growth hormones (Melamed *et al.* 1996), and neurotransmitter activities in the brain (Williamson-Hughes *et al.* 2005). The underlying endocrine mechanisms of reproduction have been conserved throughout vertebrates. The control of reproductive events occurs via the hypothalamic-pituitary-gonadal (HPG) axis of the organism and begins with the synthesis and release of GnRH [*i.e.* luteinizing hormone-

releasing hormone (LHRH)] from the hypothalamus. GnRH is released in a pulsatile manner and regulates the secretion of the gonadotropin hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary. The exact mechanism behind the pulsatility is unknown, but several factors have been suggested to alter the natural rhythmicity of the release. Some of these factors include feedback by the sex steroids, as well as other hormones and proteins (Chhabra *et al.* 2005; Matsuyama *et al.* 2005; Rispoli and Nett 2005; Kowase *et al.* 2007; Suter and O'Farrell 2008), a paracrine feedback response of GnRH onto its own receptors in the hypothalamus (Khadra and Li 2006; Xu *et al.* 2008), or an electrophysiological response in the mediobasal hypothalamus correlated with pulses of LH (Knobil 1989). Binding of GnRH to the receptors on the gonadotrope cells initializes the release of LH and FSH. Previous studies have shown that LH release is synchronized with GnRH release. This finding was observed when using GnRH agonists to induce LH production (Brussow *et al.* 2007) and by measuring serum LH levels (Clarke and Cummins 1982). In contrast to LH, FSH secretion is not in synchrony with the GnRH pulse generator. In a study where ovariectomized dogs were given GnRH intravenously, LH levels rose, whilst FSH levels remained unchanged, indicating that GnRH may not be the sole regulator of FSH regulation (Beijerink *et al.* 2007).

In vertebrates, 16 GnRH isoforms have been reported with the majority of variants coming from the teleost lineage. The most prominent forms found are the mammalian form (first identified in rodents; mGnRH), chicken GnRH (cGnRH), and salmon GnRH (sGnRH). Isoforms are named according to the vertebrate from which they were first isolated. Most vertebrates have at least two of these structural variants

expressed in their brain; cGnRH is almost always one of them and is evolutionary conserved from teleosts to humans (Kah *et al.* 2007).

Seabream GnRH (sbGnRH) neurons are typically seen within the hypothalamus of vertebrates. In the lamprey, sbGnRH-ir cells have been segregated to the POA, hypothalamus and pituitary gland (Youson *et al.* 2006). Siberian sturgeon (*Acipenser baeri*) contains sbGnRH-ir positive cells within the hypothalamus, as well as the POA, telencephalon and olfactory nerves and bulbs (Lepretre *et al.* 1993). In the frog (*Xenopus laevis*), mGnRH-ir cells are present in the forebrain, more specifically the ventral telencephalon, preoptic recess, boundary of the telencephalon and diencephalon, thalamus and hypothalamus (Hayes *et al.* 1994).

The predominant distribution of cGnRH cells in the midbrain has been conserved throughout vertebrate history. In the primitive lamprey, cGnRH-ir cells have been localized to the preoptic nucleus of the hypothalamus adjacent to the third ventricle (Kavanaugh *et al.* 2008). cGnRH-ir positive cells are solely found in the nucleus of the medial longitudinal fasciculus of the midbrain tegmentum in Siberian sturgeon (Lepretre *et al.* 1993). cGnRH mRNA is expressed in the optic tectum, as well as the telencephalon and hypothalamus in goldfish (Hoskins *et al.* 2008). Musk shrew (*Suncus murinus*) cGnRH-ir cells are found in the midhabenula and adjacent to the periaqueductal grey zone, both regions of the midbrain, as well as the VMN (Dellovado *et al.* 1993).

sGnRH has only been found in fish species, and cells are principally located in the forebrain, more specifically the boundary between the olfactory bulbs and the telencephalon, in goldfish (Yu *et al.* 1988), Atlantic salmon (*Salmo salar*) and rainbow trout (Bailhache *et al.* 1994), dwarf gourami (*Colisa lalia*) (Yamamoto *et al.* 1995) and

seabream (*Sparus aurata*) (Gothilf *et al.* 1996). sGnRH-3-ir cells are also present in the pituitary gland and hypothalamus in goldfish (Yu *et al.* 1988).

The coupling of GnRH with energy status and metabolism was first identified in rodent puberty studies (Bronson 1986). Food restriction inhibits prepubescent female rats from ovulating, while GnRH injections and reintroduction to feeding both reversed the effect (Bronson 1986; Bronson 1988). In ram lambs, immunization against GnRH caused decreases in both reproductive (mounts and ejaculations) and appetite-related (food efficiency and feeding times) behaviours (Kiyma *et al.* 2000). Alterations of reproductive behaviours and physiology, such as reduced numbers of corpora lutea (Temple and Rissman 2000), induction of maturation (Pati and Habibi 2000) and degeneration of gonads (Soverchia *et al.* 2007), as well as stimulation of gonadotropin release (Bowen *et al.* 2006; Proudman *et al.* 2006), have been reported following GnRH ICV injections and implants, indicating that cGnRH-2 could have a dual function in regulation of energy status and reproduction.

The dual-functional role of GnRH in reproduction and energy status (food intake) is further demonstrated by implants and ICV injections of appetite-related hormones, such as leptin and NPY, and NPY agonists, which all decrease the pulse interval of GnRH in prepubescent rats (Lebrethon *et al.* 2000) and goats (Ichimaru *et al.* 2001). NPY receptor antagonists and CART anti-serum increase the GnRH pulse interval, countering the effects of NPY and leptin, respectively (Lebrethon *et al.* 2000). Dense matrices of OX-ir terminals have been found in close proximity to GnRH-ir cells in the organum vasculosum of the lamina terminalis (OVLT), the lateral and medial parts of the POA and some cells in the diagonal band of Broca [medial septum (MS); MSDB] in ovine

hypothalami (Iqbal *et al.* 2001). In rat POA, OX fibres are also found in the vicinity of GnRH-ir cells, and OX-receptor-ir terminals co-localize with GnRH-ir neurons (Campbell *et al.* 2003). The interaction between OX and GnRH in food intake is observed in ICV-injected goldfish; OX treatment causes a decrease in cGnRH mRNA expression and concomitant increases in food intake whereas cGnRH-2 ICV injections inhibits both food intake and OX mRNA expression in the optic tectum/thalamus and hypothalamus compared with the saline-injected controls (Hoskins *et al.* 2008).

Only a few direct studies on the role of GnRH in feeding have been completed. In goats, fasting increased staining of GnRH-ir cells in areas adjacent to the third ventricle, ARC, dorsomedial nucleus of the hypothalamus (DMN) and periventricular regions, with a few GnRH-ir cells in the paraventricular nucleus of the hypothalamus (PVH) that are not observed in fed animals (Ichimaru *et al.* 2001). Food restriction decreases cGnRH-1 levels in the median eminence of immature hens (*Gallus gallus*) (Bruggeman *et al.* 1998), whereas *ad libitum* food intake increased GnRH-1 hypothalamic mRNA levels in broiler chickens (Ciccone *et al.* 2007). In goldfish, ICV injections of cGnRH reduce feeding and ICV injections of orexin induce increases in cGnRH-2 mRNA brain levels, suggesting that there are interactions between appetite- and reproductive-related processes and peptides (Hoskins *et al.* 2008).

GnRH and GnRH-R have been isolated from an invertebrate, the octopus (*Octopus vulgaris*), demonstrating the conservation of GnRH throughout the evolutionary history of metazoans. In octopus, GnRH- producing cells are localized in the supraesophageal and subesophageal parts of the octopus central nervous system, as well as the subpedunculate lobe and posterior olfactory lobe-optic gland (analogous to

hypothalmo-hypophysial axis) possibly regulating reproduction and appetite (Iwakoshi-Ukena *et al.* 2004). Octopus-GnRH (oct-GnRH) activates a GnRH-R in clawed toad oocytes and GnRH-R mRNA is expressed ubiquitously around the central nervous system (Kanda *et al.* 2006). The presence of oct-GnRH-R mRNA in the superior buccal lobe as well as functional assays linking the role of GnRH-R to movement of muscles surrounding the buccal mass suggests GnRH as having a role in food intake regulation in invertebrates. These results suggest that the GnRH peptide system, with regards to both form and function, might be highly conserved throughout the animal kingdom.

1.1.7. MCH and GnRH

Within the last few years, studies have been conducted to examine the interactions between MCH and GnRH in fish. It has been demonstrated that background colour affects the expression of both cGnRH and MCH in barfin flounder (*Verasper moseri*) (Amiya *et al.* 2008). When fish were subjected to a white background, the expression of MCH in their brain and pituitary was higher than that of animals held in a black background. In contrast, cGnRH mRNA expression was higher in the brain of fish in the black tank (Amiya *et al.* 2008). There were no significant differences with respect to the sbGnRH and sGnRH between animals held in different background, suggesting that these peptides are unaffected by background colour (e.g. environmental cues) and do not play a role in colour adaptation. The interactions between MCH and GnRH were further demonstrated by immunohistochemistry studies showing that cGnRH-ir fibres were in close proximity to MCH-ir fibres in the hypothalamus, suggesting that cGnRH could be

regulating MCH neural function (Amiya *et al.* 2008). However, regardless of the mechanisms involved, it appears that GnRH and MCH both affect appetite in barfin flounder and could be applied to other teleost species.

Since MCH acts as a neuromodulator in the brain of vertebrates it is likely that MCH alters the release of other hormones, specifically GnRH (Williamson-Hughes *et al.* 2005). In Sprague-Dawley rats, treatment by low doses of MCH either by *in vivo* MCH injections into the median eminence or by *in vitro* treatment of pituitary cells stimulates GnRH release, indicating that MCH might indeed act as a neuromodulator of GnRH secretion at the level of the hypothalamus as well as the pituitary (Chiocchio *et al.* 2001). It is noteworthy that the regulation of the gonadotropins and GnRH by MCH appears to be dependent on the reproductive stage as differences in MCH-induced protein levels were more prevalent in rats at the proestrous stage. This indicates that MCH could indirectly be regulating reproduction in vertebrates. In addition, gender might affect the interactions between these hormones. Higher levels of MCH and GnRH proteins were observed in the median eminence of female rats compared with males at the start of estrous, but similar MCH- and GnRH-ir cell and fibre patterns were observed (Gallardo *et al.* 2004).

MCH-ir and GnRH-ir fibres are in fact present within the same vicinity in the preoptic area and anterior hypothalamus in rats (Williamson-Hughes *et al.* 2005). Furthermore, using an antibody generated against synaptophysin (a protein found in the synaptic cleft), MCH fibre terminal buttons are shown to be in close contact with the GnRH neurons and that there was extensive overlap between the GnRH nerve endings and MCH-ir fibres in the median eminence. In addition, MCH-R expression in the rat

brain was found to be colocalized with that of GnRH-ir neurons in areas such as the cortex, nucleus accumbens, bed nucleus of stria terminalis, anterior hypothalamic area and the POA (Williamson-Hughes *et al.* 2005).

Similarly, in mice, MCH and GnRH axons and cell bodies are in close proximity in the MSDB (Wu *et al.* 2009). Patch and voltage clamps studies in brain slices of the MSDB show that MCH inhibits GnRH neurons via the MCH-R1, as the MCH-R1 antagonist, PMC-3881-PI, blocks the MCH-induced hyperpolarisation on these neurons (Wu *et al.* 2009). MCH can also indirectly inhibit GnRH neuron activation through other pathways, such as kisspeptin neurons (Wu *et al.* 2009).

1.1.8. Study species: Winter flounder

Winter flounder are bottom-dwelling fish found in the cold waters off the coast of Newfoundland. During the winter, flounder undergo a natural state of fasting, which, surprisingly, coincides with the development of their gonads and an increasing gonadosomatic index (GSI), a process that normally requires large amounts of energy (Stoner *et al.* 1999). The fish thus likely switch from the use of internal body reserves in the pre-spawning season to consumption of external foodstuffs following spawning. The mechanisms by which fasting is initiated and terminated are not known, but probably involve both appetite-related hormones such as MCH and reproductive hormones such as GnRH.

1.2. Research objectives

The objectives of this study are to identify the MCH and GnRH cDNAs families, and their receptors, in winter flounder. The identification and sequence analyses of the cDNAs will be used to determine evolutionary relationships among winter flounder MCH and GnRH and their homologues in other vertebrates including other fish. Central nervous system and peripheral tissue distributions of mRNA allow for the localization of the sites of synthesis (peptides) and action (receptors) of these peptides and might give some insight into their possible physiological functions. Quantification of MCH and GnRH mRNAs and their receptors in fed and fasted fish will help to determine whether these peptides play a role in appetite and energy homeostasis regulation in winter flounder.

The results of this study will contribute to understanding the roles of MCH and GnRH isoforms and receptors in food intake regulation of winter flounder. These results can be applied and compared with other vertebrate models, such as flounders, model fish and othe higher vertebrates, to resolve the role of MCH and GnRH in the overall model of appetite regulation. If MCH and GnRH appear to be involved in food intake, then the outcomes can be implicated in the selection of fish that eat more and grow larger for aquaculture.

1.3. Coauthorship Statement

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Chapter 2: The role of melanin-concentrating hormone (MCH) and its receptors in appetite regulation of winter flounder (*Pseudopleuronectes americanus*)

Abstract

Melanin-concentrating hormone (MCH) has been implicated in teleost appetite regulation, but its role is not yet completely understood. In this study, the mRNAs of two forms of MCH, prepro-MCH and MCH2, and two forms of MCH receptors, MCH-R1 and MCH-R2, were isolated from winter flounder (*Pseudopleuronectes americanus)*. MCH1 and MCH2 mRNAs were both expressed in the forebrain and midbrain, as well as pituitary and most peripheral tissues examined, including gut and gonads. MCH-R1 and MCH-R2 were ubiquitously expressed in the brain and periphery. To better understand the role of this peptide family in the regulation of feeding, the mRNA expression of these peptides and their receptors was determined under fed and fasted conditions. Fasting induced higher expressions of MCH and MCH-R1 mRNAs in optic tectum/thalamus and hypothalamus but had no effect on either MCH2 or MCH-R2 mRNA expressions. My results suggest that MCH and MCH-R1, but not MCH2 and MCH-R2 might have a role in the regulation of appetite in flounder.

2.1. Introduction

The endocrine mechanisms of appetite regulation in fish consist in a complex system. Numerous intrinsic (*i.e.* energy and reproductive status) and extrinsic (*i.e.* photoperiod and environmental colour) factors influence how food intake is synchronized with the energy requirements of an individual (Volkoff *et al.* 2009). The regulation of feeding in vertebrates has been shown to be under the control of peptides that are produced either within the brain or in the periphery. Central feeding-stimulating (orexigenic) hormones include orexins (OX) (Volkoff *et al.* 1999) and neuropeptide Y (NPY) (Lopez-Patino *et al.* 1999), whereas cocaine-amphetamine regulated transcript (CART) (Volkoff and Peter 2001) and gonadotropin-releasing hormone (GnRH) (Matsuda *et al.* 2008) are central appetite-inhibiting (anorexigenic) peptides. Ghrelin is an example of an orexigenic hormone found in the periphery (stomach) (Terova *et al.* 2008), while the anorexigenic peptide leptin is synthesized in liver (Johnson *et al.* 2000) and adipose tissue (Ronnestad *et al.* 2010).

Melanin-concentrating hormone (MCH) is a 17-amino acid peptide that was first identified in fishes as a hormone that regulated colour change (Kawauchi *et al.* 1983). Although in most teleosts, only one form of MCH has been identified, two or more variants of MCH have been isolated in Japanese flounder (*Paralichthys olivaceus*), zebrafish (*Danio rerio*) (Berman *et al.* 2009), green-spotted and Japanese pufferfish, (*Tetraodon nigroviridis* and *Takifugu rubripes*, respectively) and salmonids (Ono *et al.* 1988; Baker *et al.* 1995). In zebrafish, one of the forms, MCH2, is most similar to mammalian MCH (Berman et al. 2009), while the other, MCH1, appears to have closer

resemblance to other teleost MCH amino acid sequences.

Early hypothalamic lesion studies in mammals showed that MCH release is regulated by the ventromedial hypothalamus (VMH), a brain area implicated in the control of feeding (Deray *et al.* 1994; Griffond *et al.* 1995). Only recently, studies have demonstrated that, in addition to its role in regulating skin colour, MCH can indeed act as a neuromodulator of food intake directly or via other appetite-related peptides (Santollo and Eckel 2008; Shimakura *et al.* 2008). In rodents, MCH treatments increased food intake (Rossi *et al.* 1997; Gomori *et al.* 2003), MCH-deficient animals display decreases in feeding and body weight (Shimada *et al.* 1998) and fasted animals display greater brain MCH mRNA expression than fed animals (Presse *et al.* 1996; Qu *et al.* 1996).

In fish, the exact role of MCH in the regulation of feeding remains unclear as few studies have been conducted. In barfin flounder (*Verasper moseri*), MCH mRNA levels and numbers of brain immunoreactive (ir) cell bodies are higher when fish are fasted, suggesting an orexigenic effect of MCH in this species (Takahashi *et al.* 2004). On the other hand, goldfish (*Carassius auratus*) injected with MCH display decreased food intake, suggesting an anorexigenic effect of MCH (Matsuda *et al.* 2006). MCH regulation has been linked to background colour, as MCH brain mRNA expression and plasma levels increase in several fish species when animals are placed in white tanks (Green *et al.* 1991; Groneveld *et al.* 1995; Amiya *et al.* 2008). Correlations between background colour, somatic growth and MCH mRNA expression have been determined in barfin flounder (Takahashi *et al.* 2004), where both greater growth rates and high MCH are seen in white-adapted animals and could indicate an increase in food intake. In zebrafish, MCH appears to be the regulator of skin colour, while MCH2 is thought to be involved in

food intake (Berman *et al.* 2009).

In humans (*Homo sapiens*) and lower vertebrates, there are at least two G-protein-coupled MCH receptors (MCH-R), whereas only one receptor, MCH-R1, has been isolated in rodents. Post-mortem analyses of cachectic (with loss of weight and muscle mass) humans demonstrate 1.6 times higher MCH-R1 mRNA expression in the infundibular nucleus, which suggests a role of this receptor in energy homeostasis (Unmehopa *et al.* 2005). Furthermore, MCH-R1-deficient mice tend to be hypophagic and resistant to obesity with high-fat diets (Chen *et al.* 2002) and do not increase their food intake when injected intracerebroventricularly (ICV) with MCH (Marsh *et al.* 2002). MCH-R1 antagonists (Takekawa *et al.* 2002; Shearman *et al.* 2003) and agonists (Shearman et al. 2003) have been shown to mimic and inhibit the orexigenic stimulation of MCH on feeding, respectively.

Few studies have looked at the functional importance of MCH-R2 in animals. In mammals, MCH-R2 has only been characterized in dogs (*Canis familiaris*) and ferrets (*Mustela putorius*) (Tan *et al.* 2002), rhesus monkeys (*Macaca mulatta*) (Fried *et al.* 2002) and humans (Sailer *et al.* 2001). The only evidence that MCH-R2 is involved in energy homeostasis is that MCH-R2 is present is human adipose tissue (Hill *et al.* 2001) and mediates the differentiation of preadipocytes into mature cells when exposed to MCH (Yang *et al.* 2009).

Fish appear to have at least two MCH-Rs. In goldfish, MCH-Rs (MCH-R1 and MCH-R2) present in the brain are postulated to mediate the central effects of MCH (Mizusawa *et al.* 2009). Both goldfish MCH-Rs are also present in several peripheral tissues, including skin, where MCH-Rs might mediate colour changes, as well as

intestine and fat tissue, where MCH-Rs might regulate appetite and energy homeostasis (Mizusawa *et al.* 2009). Three MCH-Rs (MCH-R1a, MCH-R1b and MCH-2R) have been identified in zebrafish (Logan *et al.* 2003). MCH-R1a and MCH-R1b are thought to be a product of the teleost-derived whole genome duplication (WGD) event as they are both orthologues of human and mouse MCH-R1.When the MCH-R1 gene is knocked down, melanosome dispersal is impaired (Richardson *et al.* 2008), indicating a role for MCH-R1 in mediating colour change. In barfin flounder, MCH-R2 is present throughout the organism and appears to have widespread effects, including the induction of melanosome dispersal (Takahashi *et al.* 2007; Mizusawa et al. 2009). No studies have looked at the function of fish MCH receptors with respect to energy homeostasis.

Winter flounder (*Pseudopleuronectes americanus*) is a bottom-dwelling flatfish inhabiting the shores of Newfoundland. These fish are readily available year round and represent an interesting model to study seasonal feeding behaviour as they undergo a period of fasting during the winter months. Surprisingly, this fasting occurs before spawning, at a time when animals increase their gonadal weight [gonadosomatic index (GSI)] (Stoner *et al.* 1999).

In this study, I isolated transcripts of the MCH-peptide family in winter flounder. cDNAs for two variants of MCH (MCH and MCH2) and MCH-Rs (MCH-R1 and MCH-R2) were isolated. To further typify the transcripts encoding these peptides and their receptors, I examined their central nervous system including pituitary and peripheral tissue distributions. In order to assess a possible role of MCH peptides in winter flounder, I examined the effects of fasting on the brain mRNA expression of both peptides and receptors.

2.2. Materials and Methods

2.2.1. Animals

Winter flounder brain tissue used for cloning was sampled from 3-4 wild fish collected by scuba divers off the shore of St. John's (Logy Bay, Newfoundland and Labrador, Canada). After collection, fish were kept in 2 m × 2 m flow through tanks at the Ocean Sciences Centre (OSC of Memorial University of Newfoundland; St. John's, Newfoundland and Labrador, Canada). Fish were kept under natural photoperiod and temperature conditions (11.9 °C). The sex ratio was approximately 50:50 in all tanks. Fish were fed frozen herring to satiety two or three times a week at the same time of the day (10:00).

Fish for the food deprivation experiment were obtained by seining and held at the Bonne Bay Marine Station (BBMS; Norris Point, Newfoundland, Canada). Fish (five per tank) were maintained in four white 0.5 m x 0.5 m flow through tanks with a sandy substrate to imitate their natural environment, at ambient water temperatures and lighting (see below). Males and females were used with an approximate 50:50 ratio in each treatment. Fish were fed cut up frozen squid every 2-3 days at the same time of the day (21:00). On the sampling day, fish were fed 1 h prior to sampling. Weights were obtained before the experiment began and during sampling. Gonad and liver weights were determined following sampling for calculation of GSI and hepatosomatic index (HSI). All experiments were conducted in accordance with the principles found in the Canadian Council on Animal Care guidelines.

2.2.2. Food deprivation experimental design

Winter flounder ($n = 20$; average weight of 115.59 ± 22.67 g) were acclimated for two weeks in four tanks (five fish per tank) under natural photoperiod and an average water temperature of 10 °C (July 6th to July 30th 2009). The same feeding regime was used as previously described (section 2.2.1). Following acclimation, two tanks were selected as controls (fed as described above), while the remaining tanks were starved for 10 days. Duplicate tanks were used to account for any tank effect. Following experimentation, fish were sacrificed with an overdose (100mg/L) of tricaine methanesulfonate (Syndel Laboratories, Vancouver, British Columbia, Canada), and brains were dissected and stored in RNAlater® stored at -20°C (Qiagen Inc., Mississauga, Ontario, Canada) until further processing.

2.2.3. RNA extraction and cDNA synthesis

For cloning and tissue distribution, tissues from the brain (telencephalon/pre-optic area, optic tectum/thalamus, hypothalamus, cerebellum, medulla oblongata and spinal cord) including the pituitary gland and from the periphery (gill, eyed-side skin, blind-side skin, muscle, heart, liver, gall bladder, foregut, midgut, male gonad and female gonad) were removed from two adult flounder. An annotated anatomy of the flatfish brain was utilized for brain regional dissections (Evans 1937).

RNA was isolated using the Tri-reagent/chloroform (BioShop, Burlington, Ontario, Canada) extraction technique using the manufacturer's protocol. Final RNA

concentrations, 260/280 and 260/230 data were determined using NanoDrop (NanoDrop, Wilmington, North Carolina, USA) spectrophotometry at a wavelength of 260-nm. RNA was then reverse-transcribed (RT) into cDNA via the Quantitect Reverse-Transcriptase Kit (Qiagen, Mississauga, Ontario, Canada) using 20 µl samples consisting of 2 ng RNA, 6X Quantitect buffer, 7X genomic DNA wipeout buffer, 0.5 nM of each dNTP, 0.5 µg each random hexamer and oligo dT primers and 200 U Quantitect Reverse Transcriptase.

2.2.4. Isolation of prepro-MCH, prepro-MCH2 and MCH-receptors cDNAs from flounder brain

Regions of mRNA sequences in various teleost fishes, including the Japanese flounder (GenBank EU232720/AF236090), barfin flounder (GenBank AB117947), goldfish (GenBank AM403731), zebrafish (GenBank FJ392644/ACO35934), the perciform cichlid fish (*Cichlasoma dimerus*) (GenBank GQ253057), rainbow trout (*Oncorhynchus mykiss*) (GenBank X73837) and pufferfish (*Tetraodon nigroviridis*) (ENSEMBL ENSTNIT00000003019/ENSTNIT00000008042) (Table 2.1) were used to design degenerate primers for MCH and MCH2. In order to obtain the initial amplicon, a 25 µl polymerase chain reaction (PCR) was set up using 6X Go Taq Flexi Buffer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 0.2 µM of each primer 1 U Go Flexi Taq Polymerase (Promega, Madison, Wisconsin, USA) and 2.5 µl of cDNA corresponding to 1 µg of initial RNA. An initial 5 min denaturation at 95°C, followed by 30 cycles of: 30 s denaturation at 95°C, 30 s annealing with temperatures ranging from 48 to 62°C and a 1 to 2 min extension at 72°C, with a final extension of 5 min at 95°C using the eppendorf

Table 2.1. Sequences of primers used in the melanin-concentrating hormone (MCH) study.

Gene	Forward primer / RACE primer 1	Reverse primer/ RACE primer 2
Degenerate primers		
MCH2	5′-ACSCTVGTGCTGTTCTCTGAGC-3′	5′-TCCBAYCATRCACCGCAGCA-3′
MCH-R1	5′-TGTCGGABTTCAAGCTHGGG-3′	5′-CCGGAGCCTCCTCTCDATGA-3′
MCH-R2	5′-TTCTTCCAAGCTCACAGTCG-3′	5′-CGGTATCCATTTCCAGTTGTG-3′
3′ RACE primers		
MCH	5′-CCACCTGGAAGATCTTCACC-3′	5′-GATCTTCACCATGAGGCAGTCG-3′
MCH2	5′-TCCGAGAGGATGAAGATCTTAGG-3′	5′-ATCTTAGGATTCCCCGCTTCATCC-3′
MCH-R1	5′-GCATGGGYTACGCYAACAGTTGC-3′	5′-AACAGCTGCATTAACCCNTTYCTC-3′
MCH-R2	5′-CATCTGTCTCAGCTACTCKCACA-3′	5′-AGCTGCATCAACCCRCTYATG-3′
5′ RACE primers		
MCH – specific	5′-GCGCGAGTTGTCGAGTGTCC-3′	
MCH2 – specific	5′-CCATTTTGAAACTGTACTCGGCCA-3′	
MCH-R1 – specific	5′-CCTGCCCAGCGGCCTTTACC-3′	
MCH	5′-CGTCGCTGAAGTCGTTTTCC-3′	5′-CGTTTTCCGTGGCCTTGTCGC-3′
MCH2	5′-CATCCCTGATGCTGTTGTCC-3′	5′-TGTTGTCCAGCAGGAGGCTTCC-3′
MCH-R1	5′-CAAAGATCTTAGCTCCGTTGC-3′	5′-CTCCGTTGCCATCTGCATCG-3′
Specific primers for RT-PCR		
MCH	5′-CGTGTTTGTCCATCGTCTTCG-3′	5′-TCCTCGGCGGCCAGGTCG-3′
MCH2	5′-ATCATGCACCGCAGCAAGT -3′	5′-TGGTGCTGTTCTCTGAGCTG -3′
MCH-R1	5′-GACAAAATACACACTAATTGACGAGC-3′	5′-GAGACTGCTCCCTTTGTTTGCTGGA-3′
MCH-R2	5′-TTCTTCCAAGCTCACAGTCG-3′	5′-CGGTATCCATTTCCAGTTGTG-3′
Primers for internal control of RT-PCR		
EF-1α	5′-CCTGGACACAGGGACTTCAT-3′	5′-CGGTGTTGTCCATCTTGTTG-3′
Specific primers for qPCR		
MCH	5′-CGTGTTTGTCCATCGTCTTCG-3′	5′-TCCTCGGCGGCCAGGTCG-3′

MCH2	5′-GCTTCACCTACGACGAAAGG-3′	5′-CCCTGATGCTGTTGTCCAG-3′
MCH-R1	5′-GACAAAATACACACTAATTGACGAGC-3′	5′-GAGACTGCTCCCTTTGTTTGCTGGA-3′
MCH-R2	5′-TCACCGCAAAAGGAAGTAGC-3′	5′-CACTCAGGGCTGAAGTTGC-3′
EF-1α	5′-CCTGGACACAGGGACTTCAT-3′	5′-CGGTGTTGTCCATCTTGTTG-3′
Adapter primers		
dT-AP	5′-GGCCACGCGTCGACTAGTAC(T17)-3′	
AP	5′- GGCCACGCGTCGACTAGTAC-3′	

* Degenerate bases: W (A or T), S (C or G), M (A or C), K (G or T), R (A or G), Y (C or T), B (not A), D (not C), H (not G), V (not T) and N (any base)

Mastercycler® 5333 and Mastercycler® personal 5332 for all PCR reactions. In all PCRs, a negative control was used for each primer set by excluding cDNA from the reaction. Products were electrophoresed in a 1.25% ethidium bromide stained agarose gel using 1X TAE buffer and bands were excised and purified with the GeneJET™ Gel Extraction Kit (Fermentas, Burlington, Ontario, Canada) according to manufacturer's instructions, ligated in the pGEM easy vector (Promega; Madison, Wisconsin, USA) transformed into JM109 competent cells, according to manufacturer's instructions, grown on ampicillin and X-gal treated agar plates, white colonies were grown further followed by minipreps of selected colonies using GeneJET™ Plasmid Miniprep Kit using the manufacturer's protocol (Fermentas, Burlington, Ontario, Canada). A 10 µl EcoR1 (Promega; Madison, Wisconsin, USA) digestion was set-up using 1 µl 1X buffer H, 0.5 µl EcoRI restriction enzyme and 3 µl DNA and incubated at 37°C for 1h for each miniprep to determine if the plasmid contained an insert. Plasmid DNA containing inserts were then sequenced by The Centre for Applied Genomics lab (TCAG; The Hospital for Sick Children, Toronto, Ontario, Canada).

Once the initial fragment was isolated, a 3′ Rapid Amplification of cDNA Ends (RACE) was completed with two rounds of nested PCR using gene- specific primers and adaptor primers, where the first round of amplification included the RACE primer 1 and dtAP and the second used RACE primer 2 and AP (Table 2.1). A 25 µl PCR was set up using 6X Go Taq Flexi Buffer, 0.2 mM of each dNTP, 2-3 mM $MgCl_2$, 0.2 µM of each primer 1 U Go Flexi Taq Polymerase (Promega, Madison, Wisconsin, USA) and 2.5 µl of cDNA corresponding to 1 µg of initial RNA. An initial 5 min denaturation at 95°C,

followed by 30 cycles of: 30 s denaturation at 95°C, 30 s annealing with temperatures ranging from 48 to 62°C and a 1 to 2 min extension at 72°C, with a final extension of 5 min at 95°C using the eppendorf Mastercycler 5333 and Mastercycler personal 5332 for all PCR reactions. A 5´RACE was further completed with a reverse transcriptase (RT) - PCR using gene-specific primers (Table 2.1). cDNA was then purified using Montage PCR Millipore™ kit (Bedford, Massachusetts, USA) according to manufacturer's instructions and polyA-tailed using 6 µl cDNA, 2.5 µl 10X tailing buffer, 1 µl 5mM dATP which was incubated for 3 min at 94°C and 1 µl of Terminal Deoxynucleotidyl Transferase was added (Invitrogen, Burlington, Ontario, Canada). A final incubation of 37°C for 10 min and 65°C for 10 min was completed. The tailed cDNA was then amplified with two rounds of nested PCR with gene-specific primers and adaptor primers, where the first round of amplification included the RACE primer 1 and dtAP and the second used RACE primer 2 and AP (Table 2.1). A 25 µl PCR was set up using 6X Go Taq Flexi Buffer, 0.2 mM of each dNTP, 2-3 mM $MgCl_2$, 0.2 µM of each primer 1 U Go Flexi Taq Polymerase (Promega, Madison, Wisconsin, USA) and 2.5 µl of cDNA corresponding to 1 µg of initial RNA. An initial 5 min denaturation at 95°C, followed by 30 cycles of: 30 s denaturation at 95°C, 30 s annealing with temperatures ranging from 48 to 62°C and a 1 to 2 min extension at 72°C, with a final extension of 5 min at 95°C using the eppendorf Mastercycler 5333 and Mastercycler personal 5332 for all PCR reactions.. For both the 3´ and 5´RACE, PCR products were run on 1.25% stained with ethidium bromide agarose gels and run with 1X TAE buffer, visualized and bands were excised, as previously described. These bands were cloned and sequenced as described previously.

2.2.5. Distribution of prepro-MCH, prepro-MCH2 and MCH-receptors cDNA expression in flounder brain regions and peripheral tissues

Gene-specific primers were designed based on the sequence information previously obtained. cDNAs were synthesized from the peripheral and central tissues based on the procedures described above. A 25 µl PCR reaction was used containing 10X GoTaq Master Mix (Promega; Madison, Wisconsin, USA), 0.2 µM of each primer (Table 2.1) and 2.5 µl cDNA. Negative no-template controls were included in all PCRs, where an initial 5 min denaturation at 95°C, followed by 30 cycles of: 30 s denaturation at 95°C, 30 s annealing with temperatures ranging from 48 to 62°C and a 1 to 2 min extension at 72°C, with a final extension of 5 min at 95°C using the eppendorf Mastercycler 5333 and Mastercycler personal 5332 for all reactions. Products were electrophoresed in a 1.25% agarose gel stained with ethidium bromide and run using 1X TAE buffer and visualized using the Epichemi Darkroom Bioimaging System (UVP, Upland, California, USA) equipped with a 12-bit cooled camera. Image processing and analysis were performed using LabWorks 4.0 software (UVP, Upland, California, USA). The control gene used was elongation factor-1α (EF-1α) (GenBank AW013637).

2.2.6. Changes in prepro-MCH, prepro-MCH2 and MCH-receptors cDNA expression in flounder brain during varied nutritional states

Following reverse-transcription of mRNA into cDNA (section 2.2.3), products were diluted 1:2 in nuclease-free water (Qiagen, Mississauga, Ontario, Canada) and

quantitative RT-PCR (qPCR) was completed using primers specific to the winter flounder genes of interest (Table 2.1). Although the RT protocol included a DNase step, at least one primer was designed across an exon-exon junction to further avoid the risk of amplifying genomic DNA. Primers were designed to have melting temperatures that were approximately the same and result in amplicon sizes between 75-130 base pairs (bp). An automated pipetting system (epMotion® 5070, Eppendorf) was used to set up all PCRs. The final volume was 10 µl containing 2 µl of cDNA from 1 µg of RNA, 1 µM of each sense and antisense primer and 5 µl of QuantiFast SYBR Green PCR kit master mix (Qiagen; Mississauga, Ontario, Canada). Samples were run in duplicates on 96-well plates using the Mastercycler® ep realplex 2S system (Eppendorf; Hamburg, Germany, Europe). A "no DNA" negative control where cDNAs were replaced by water was included on each plate. Primer optimization PCRs were conducted to ensure primer specificity ($0.9 > R^2 > 1.0$) using a 5 point standard curve for each primer pair to make certain that amplifications with each primer pair had similar efficiencies (EF-1α = 1.07; MCH = 0.93; MCH2 = 0.98; MCH-R1 = 0.99; MCH-R2 = 1.09). The Realplex1.5 software (Eppendorf; Hamburg, Germany, Europe) was used for amplification, dissociation curves and mRNA expression analyses. The relative cycle threshold (Ct; ΔΔCt) method was used to quantify expression. Fold-change in expression of the target gene was normalized to the housekeeping gene (EF-1α) and were compared with the calibrator sample from the control group (a single fed fish). The average fold mRNA expression from the control group was set to 100% and expression of genes in the fasted groups was relative to EF-1α using the following formula: (relative quantification of fed or fasted fish*100)/average relative quantifications of fed fish. Verification that the

housekeeping gene was not affected by feeding regimen in the brain was demonstrated by similar Ct values between fed and starved fish.

2.2.7. Sequence analysis

DNA sequences and deduced amino acid sequences were analyzed using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/blast) from the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). The signal peptide was predicted with the program SignalP 3.0 (www.cbs.dtu.dk/services/SignalP/). Multiple alignments were performed using Multiple Accurate and Fast Sequence Comparison by Log-Expectation (MUSCLE) tool with CLUSTAL2 (strict) output (Edgar 2004). Neighbour-joining phylogenetic analyses (1000 bootstrap iterations) were completed using Molecular Evolutionary Genetic Analysis 4.0.2 (MEGA; Tamura *et al.* 2007) based on a Poisson distance matrix of the MUSCLE alignments.

2.2.8. Data analysis and statistics

Expression levels were described as a percentage relative to the control (fed) group (100%). One-tailed (MCH) and two-tailed (MCH2, MCH-R1, MCH-R2) Student's t-tests were used to determine the significance of differences between fed and fasted groups in GraphPad Prism 5 (GraphPad Software, San Diego, California, USA). The threshold for significance was set at $p < 0.05$.

2.3. Results

2.3.1. Sequence identitiesof winter flounder MCH, MCH2 and MCH-receptor isoforms

A complete MCH (GenBank HQ406773) fragment is 597 base pairs (bp) long encoding a putative 137 amino acid (aa) protein. The transcript contains a 78 bp 5′ untranslated region (UTR), a 107 bp 3′ UTR, a putative 24 aa signalling peptide at the N-terminus end of prepro-MCH and a 15 aa mature peptide at the C-terminus (Figure 2.1). BLAST results indicate 35-87% amino acid sequence identities with other teleost MCHs (Figure 2.2).

The complete open reading frame sequence for winter flounder MCH2 (GenBank HQ406771) is 453 bp long, with a partial 12 bp 5′ UTR and a 128 bp 3′ UTR (Figure 2.3). The 151 aa prepro-MCH2 protein encodes a 23 aa signal peptide and the 19 aa MCH2. MCH2 is most similar (77% aa identity) to Japanese flounder MCH-like peptide (GenBank AF236090). The active MCH2 peptide is quite conserved with other MCH and MCH2 peptides (Figure 2.2), however more variation occurs in the prepro-MCH2 region. MCH-R1 (GenBank HQ406772) is 228 aa and contains a 197 bp 5′ UTR and a 177 bp 3′ UTR (Figure 2.4).

The flounder MCH-R1 protein is 99% conserved with zebrafish (GenBank CAM15133) at the amino acid level and retains a high degree of similarity with mammals and birds, such as 82% with opossum (*Monodelphis domestica*) (GenBank XP_001367230.1), zebra finch (*Taeniopygia guttata*) (GenBank ACH44230), mouse (*Mus musculus*) (GenBank NP_080297), and chicken (GenBank NP_989774) using the

Figure 2.1. Annotated winter flounder melanin-concentrating hormone (MCH) complete cDNA sequence with nucleotides and deduced amino acids. Bolded underline indicates signal peptide, dotted underline is neuropeptide AL, double underline indicates mature peptide and lower case letters represent the 5′ and 3′ untranslated regions.

Figure 2.2. Protein alignment for MCH and MCH2 used for detecting phylogenetic relationships, including frog (*Xenopus tropicalis,* XP002936876), chicken (*Gallus gallus,* ADL61813), rat (*Rattus norvegicus*, AAA41581), human (*Homo sapiens,* AAA63214), green-spotted pufferfish (*Tetraodon nigroviridis* ENSTNIP00000007881/ENSTNIP00000001084), Perciform cichlid (*Cichlasoma dimerus,* ACT33940), winter flounder (*Pseudopleuronectes americanus*, HQ406773/HQ406771), barfin flounder (*Verasper moseri*, BAC82350), Japanese flounder (*Paralichthys olivaceus*, ABY73341/AAF67166), goldfish (*Carassius auratus,* CAL48576), zebrafish (*Danio rerio* ACO35933 /ACO35934) and rainbow trout (*Oncorhynchus mykiss,* AA49420/P56943). Winter flounder sequences are bolded. The signal peptide is indicated by the bolded underline, neuropeptide EI by the single underline, dotted underline is neuropeptide AL, dashed underline is neuropeptide EV, the mature MCH by the double underline. * indicates that all amino acids are identical in the column, : demonstrates that conserved substitutions have been observed and . specifies that semi-conserved substitutions have been observed

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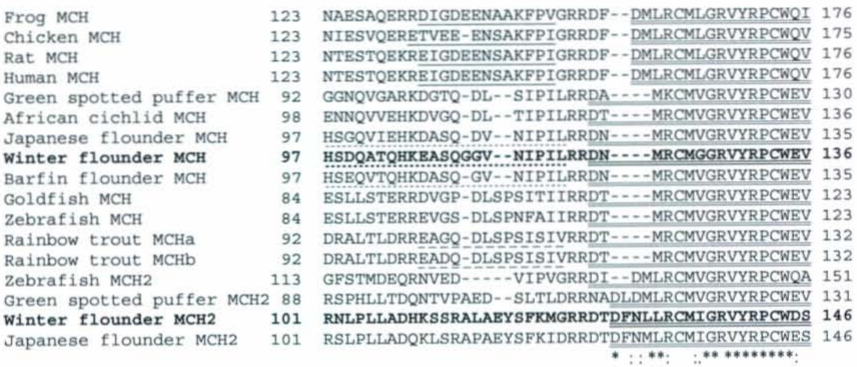
Frog MCH                      1  MVRMISLGYPLLISLSILSQ--GFLIAVSKSRKAENDDLLLDTI  42
Chicken MCH                   1  M---YISSYMLILS--LFSQ--GFLISVSKSLQKAEDEDMLLTA  39
Rat MCH                       1  MAKMSLSSYMLMLAFSLFSH--GILLSASKSIRNVED-DIVFNT  42
Human MCH                     1  MAKMNLSSYILILTFSLFSQ--GILLSASKSIRNLDD-DMVFNT  42
Green spotted puffer MCH      1  M---RQSIMVVVLAAALLCE--CYSLSVALPMSKTEDNSLEQDS  39
African cichlid MCH           1  M---RQSLISIIFAAALFFK--CYALAAALPMGKAEDGSLEQDV  39
Japanese flounder MCH         1  M---RQSFMSIIFAAALLFK--CYVLSGALPMARTEDGSLEQET  39
Winter flounder MCH           1  M---RQSCLSIVFAAALIFK--CCDLSGALPMGKAEDGSLEQET  39
Barfin flounder MCH           1  M---RQSFVSIVFAAALIFK--GYILSGALPMAKTEDGSLEQET  39
Goldfish MCH                  1  M---KLSVGTVLISVALLSE--CYFKTAAIPMTKAEDTEPDLQG  39
Zebrafish MCH                 1  M---KLSAGTVLITVALLSE--CYFKTAAIPMTKSEEIEPDLQS  39
Rainbow trout MCHa            1  M---RHSVLSISFAVALFLE--CYTPSTAIPIGKMDDVALEQDT  39
Rainbow trout MCHb            1  M---RDSVLSVIFALALFLE--CYTPSMAIPMGKMEDTALEQDT  39
Zebrafish MCH2                1  M----ASSYIIIFALALFVE--LTTRSMALPKFKMDEEHTDQER  38
Green spotted puffer MCH2     1  M----ISACSFLYTLVLFSELSSHLVAVAMPPAKVEDEAL----  36
Winter flounder MCH2          1  M----ISFSSIVFTLVLFSELNSPLVTVASPTTKGEDGVIDQDG  40
Japanese flounder MCH2        1  M----VSAYSILFTLVLFSELNSHLLTVASPATKVEDGEIEQDG  40
                                 *         . : :: *     : : .  . ::

Frog MCH                     43  TLRKAFRNGDTLEKPIGDPNN-QYKTDGGNILNEEEERNMKTID  85
Chicken MCH                  40  FNLGKTLRNGDGTEKRGAMPLLKRYKIEE-SFLDEDDDRKLKFF  82
Rat MCH                      43  FRMGKAFQKEDTAERSVVAPSLEGYKNDESGFMKDDDDKTTKNT  86
Human MCH                    43  FRLGKGFQKEDTAEKSVIAPSLEQYKNDESSFMNEEENKVSKNT  86
Green spotted puffer MCH     40  F--TSLLSEEGTDN---------GLEEADLAGPSKAGGRGVIVV  72
African cichlid MCH          40  F--TSLLNDEATEN---------SQGGADLSVVTKARAPRVIVI  72
Japanese flounder MCH        40  L--ASRLSDKATEN---------SLSDADLGTEEKLSGPRIIVV  72
Winter flounder MCH          40  F--ASLLSDKATEN---------DFSDADLAAEEKLSGPRVIVV  72
Barfin flounder MCH          40  F--ASLMSDKATEN---------SFSDADLGAEEKLSGPRVIVV  72
Goldfish MCH                 40  L----------------------SDSLEENALRSAPGNSRIIVV  61
Zebrafish MCH                40  F----------------------SESLEENSLRSTPGGSRIIVV  61
Rainbow trout MCHa           40  L--DSLLRVEVSEN---------------SPDSVRGRSSKIVLL  66
Rainbow trout MCHb           40  L--DSLLNEEVADK---------------NPDSVRSGSSKIIVL  66
Zebrafish MCH2               39  M--VSVTGEDDISELGPGQLPFRRHPIIEGRLVDEDGTKRIFIL  80
Green spotted puffer MCH2    37  ---GLALGDELAAVPH-------VYRPMDATDSGPRDGRSKVIV  70
Winter flounder MCH2         41  L--SSFLGDEPMMEQAMVPPVYRGSLLLDNSIRDEAGNPKIFIL  82
Japanese flounder MCH2       41  L--SSLLGDDPINEHAMVPPAYRGRLMLDNSIRDEDGNRKIIII  82
                                                 :.           *

Frog MCH                     86  SQQNY------LSHDGLPLNIGIKQMPYLAFRNSLHH-SENEDQ  122
Chicken MCH                  83  DTDSRH----DFSNHGVPISVGRKQLPYLALKGAIAFPADTEIQ  122
Rat MCH                      87  GSKQNL------VTHGLPLSLAVK--PYLALKGPAVFPAENGVQ  122
Human MCH                    87  GSKHNF------LNHGLPLNLAIK--GYQALKGSVDFPAENGVQ  122
Green spotted puffer MCH     73  -ASPSLW---RMLHNG--LY-------------------QKRSG  91
African cichlid MCH          73  AADANLWRDLRVLHNGLPLY-------------------KRRAD  97
Japanese flounder MCH        73  -ADPSMWRDLRVLHSGLSLY-------------------KRRAD  96
Winter flounder MCH          73  -ADPSVWRDLRVLHNGLSLY-------------------KRRAD  96
Barfin flounder MCH          73  -ADPGLWRDLRVLHNGLSLY-------------------KRRAD  96
Goldfish MCH                 62  -ADSNLLRTLTSLNRGVPHL---------------------SLT  83
Zebrafish MCH                62  -ADSNLLRTLTSLNRGVPHL---------------------NAP  83
Rainbow trout MCHa           67  -ADSGLWMN---LNRGLPLYKLIA----------------AAAGP  91
Rainbow trout MCHb           67  -ADSGMWKN---LNRGLPLYKLKA----------------AAAGL  91
Zebrafish MCH2               81  -ADTGI-------KGGREGNLAFS----RTFSELLPHGLEHALD  112
Green spotted puffer MCH2    71  VSDLRL--------KGFHGL-------------------NRAFF  87
Winter flounder MCH2         83  -SDMRQ------KGHGTQGL-------------------NSGLT  100
Japanese flounder MCH2       83  -SDMRQ------RGRGTRGL-------------------NSVLT  100
```


Frog MCH 123 NAESAQERRDIGDEENAAKFPVGRRDF--DMLRCMLGRVYRPCWQI 176
Chicken MCH 123 NIESVQERETVEE-ENSAKFPIGRRDF--DMLRCMLGRVYRPCWQV 175
Rat MCH 123 NTESTQEKREIGDEENSAKFPIGRRDF--DMLRCMLGRVYRPCWQV 176
Human MCH 123 NTESTQEKREIGDEENSAKFPIGRRDF--DMLRCMLGRVYRPCWQV 176
Green spotted puffer MCH 92 GGNQVGARKDGTQ-DL--SIPILRRDA----MKCMVGRVYRPCWEV 130
African cichlid MCH 98 ENNQVVEHKDVGQ-DL--TIPILRRDT----MRCMVGRVYRPCWEV 136
Japanese flounder MCH 97 HSGQVIEHKDASQ-DV--NIPILRRDN----MRCMVGRVYRPCWEV 135
Winter flounder MCH 97 HSDQATQHKEASQGGV--NIPILRRDN----MRCMGGRVYRPCWEV 136
Barfin flounder MCH 97 HSEQVTQHKDASQ-GV--NIPILRRDN----MRCMVGRVYRPCWEV 135
Goldfish MCH 84 ESLLSTERRDVGP-DLSPSITIIRRDT----MRCMVGRVYRPCWEV 123
Zebrafish MCH 84 ESLLSTERREVGS-DLSPNFAIIRRDT----MRCMVGRVYRPCWEV 123
Rainbow trout MCHa 92 DRALTLDRREAGQ-DLSPSISIVRRDT----MRCMVGRVYRPCWEV 132
Rainbow trout MCHb 92 DRALTLDRREADQ-DLSPSISIVRRDT----MRCMVGRVYRPCWEV 132
Zebrafish MCH2 113 GFSTMDEQRNVED-----VIPVGRRDI--DMLRCMVGRVYRPCWQA 151
Green spotted puffer MCH2 88 RSPHLLTDQNTVPAED--SLTLDRRNADLDMLRCMVGRVYRPCWEV 131
Winter flounder MCH2 101 RNLPLLADHKSSRALAEYSFKMGRRDTDFNLLRCMIGRVYRPCWDS 146
Japanese flounder MCH2 101 RSLPLLADQKLSRAPAEYSFKIDRRDTDFNMLRCMIGRVYRPCWES 146

```
-12                                                        attgtagatacc    0

1    ATGATCTCATTCTCCTCCATCGTTTTTACTCTGGTGCTGTTCTCTGAGCTGAACAGCCCCTTGGTA   66
1     M  I  S  F  S  S  I  V  F  T  L  V  L  F  S  E  L  N  S  P  L  V    22

67   ACTGTAGCTTCACCTACGACGAAAGGAGAAGATGGTGTAATAGACCAAGATGGCCTGAGTTCCTTT  132
23    T  V  A  S  P  T  T  K  G  E  D  G  V  I  D  Q  D  G  L  S  S  F    44

133  CTGGGAGACGAACCCATGATGGAGCAGGCCATGGTCCCGCCTGTGTACAGAGGAAGCCTCCTGCTG  198
45    L  G  D  E  P  M  M  E  Q  A  M  V  P  P  V  Y  R  G  S  L  L  L    66

199  GACAACAGCATCAGGGATGAAGCGGGGAATCCTAAGATCTTCATCCTCTCGGACATGAGGCAGAAG  264
67    D  N  S  I  R  D  E  A  G  N  P  K  I  F  I  L  S  D  M  R  Q  K    88

265  GGACACGGGACTCAGGGGCTGAACTCGGGCCTCACCAGGAACCTTCCTCTGCTCGCTGACCACAAG  330
89    G  H  G  T  Q  G  L  N  S  G  L  T  R  N  L  P  L  L  A  D  H  K   110

331  TCGAGCCGTGCTCTGGCCGAGTACAGTTTCAAAATGGGCCGAAGAGACACAGACTTCAACTTGCTG  396
111   S  S  R  A  L  A  E  Y  S  F  K  M  G  R  R  D  T  D  F  N  L  L   132

397  CGGTGTATGATAGGACGAGTGTACCGACCCTGCTGGGATTCTTCCGACCCCAACTGAacgtgcagc  462
133   R  C  M  I  G  R  V  Y  R  P  C  W  D  S  S  D  P  N  *            150

463  ggagagttcatcctcgtgtcttacaccgaaatgcatttctatgtcgaaaagaagtgtgaagcaaat  528
529  gtgtacaac                                                           549
```

Figure 2.3. Annotated winter flounder MCH2 partial cDNA sequence with nucleotides and deduced amino acids. Bolded underline indicates signal peptide, double underline is mature peptide, * signifies stop codon and letters in lower case represent the partial 5′ and complete 3′ untranslated regions

```
-197    ctcctgcgccagcagcggtgtgtgtgtcaccgaggctgggctgctgctgccggacagacccggag   -133
-132    tcagcgcgaacacccgaccggtccactcaaactggcatcggggccgaggggctgccccggagaggc    -67
-66     ttagacccgagcgacgttcaggtcggaccccgcgcctctctctgtcaccgccgcccgtccctcccg      0

1       ATGTCGGACTTCAAGCTGGGGATCGTGCGGCTCGGCCGTGTGGCTGGGAAGACAAAATACACACTA     66
1        M  S  D  F  K  L  G  I  V  R  L  G  R  V  A  G  K  T  K  Y  T  L     22

67      ATTGACGAGCAGGACATCCCGCTTGTGGAAAACTATGCCTTTGAGGCGCGCATGGAGGTCGATGCA    132
23       I  D  E  Q  D  I  P  L  V  E  N  Y  A  F  E  A  R  M  E  V  D  A     44

133     GATGGCAACGGAGCTAAGATCTTTGCCTACGCCTTTGACATCGGTAAAGGCCGCTGGGCAGGGAGG    198
45       D  G  N  G  A  K  I  F  A  Y  A  F  D  I  G  K  G  R  W  A  G  R     66

199     CCGCTGCACGAGCTGCTGTGGGAGAAGCACAGAGGAGGCATCGCACCCAGTTTCCAAGTCATTCAC    264
67       P  L  H  E  L  L  W  E  K  H  R  G  G  I  A  P  S  F  Q  V  I  H     88

265     ATCAACTCTGTGACCGTGGACAACCGGTTAGACAACCTGCGACTGGTGCCAGTGGGCTGGAGCCCC    330
89       I  N  S  V  T  V  D  N  R  L  D  N  L  R  L  V  P  V  G  W  S  P    110

331     AAACCGGAGGAGATCTCCAGCAAACAAAGGGAGCAGTCTCTTTACTGGCTGGCCATCCAGCAGGTG    396
111      K  P  E  E  I  S  S  K  Q  R  E  Q  S  L  Y  W  L  A  I  Q  Q  V    132

397     CCGGCCGACCCTGTGGAGGAGCAGTATCTGGAGCTAAGCCGCACACGCTACTACAACGCTAACGGG    462
133      P  A  D  P  V  E  E  Q  Y  L  E  L  S  R  T  R  Y  Y  N  A  N  G    154

463     GAGCTGGTGGAGGAAGAGGAGTGCTCCTGCACCTACTATGAGTGTCATTATCCTCCCTGTTCACTC    528
155      E  L  V  E  E  E  E  C  S  C  T  Y  Y  E  C  H  Y  P  P  C  S  L    176

529     ATTGAGAGGAGGCTCCGGGAGTTCAACATCTGTGGTCGCTGCCAGGTGGCCCGCTACTGCGGCTCC    594
177      I  E  R  R  L  R  E  F  N  I  C  G  R  C  Q  V  A  R  Y  C  G  S    198

595     CAGTGCCAGCAGAGGGACTGGCCCGCCCACAAGAAGCAGTGTCGCGAGCGCAAGCGAGTGTTGGCC    660
199      Q  C  Q  Q  R  D  W  P  A  H  K  K  Q  C  R  E  R  K  R  V  L  A    220

661     CTGGAGTCAGAGCCCGAACGATGAtctgccttcatcctcgcctgggagaggatgggggaggaattg    726
221      L  E  S  E  P  E  R  *                                              242

727     ggctgggggggtctgggtttgacttgtggcagggaagccggtatggagggctaaacggggacattt    792
793     tctggcgagagactaacgacagaggacaatgttggttgtttggattcgggtggtaaagaagactga    858
859     gga                                                                   861
```

Figure 2.4. Annotated winter flounder MCH-receptor 1 (MCH-R1) cDNA sequence with nucleotides and deduced amino acids. * denotes stop codon and lower case letters indicate 5′ and 3′ untranslated regions

BLAST algorithm (Figure 2.5).

A partial fragment of MCH-R2 (GenBank HQ406774) encoding a 270 bp 3′UTR was sequenced (Figure 2.6). The 3′ coding region of MCH-R2 shows 59-93% aa similarity to other fish species such as goldfish (GenBank BAH70339) and barfin flounder (GenBank BAF49518), 59 and 93% respectively (Figure 2.5). No significant similarities are observed with higher vertebrates.

2.3.2. Phylogenetic analyses

Most bootstrap values for the MCH/MCH2 tree were above 95% (Figure 2.7). MCH2 clustered with other vertebrate MCH sequences and appears to have evolved from the MCH gene in their last common ancestor of teleosts and tetrapods. In fish, MCH could be a result of the teleost WGD, since it is more recently derived.

More than half of the MCH-R phylogeny bootstrap values were above 95% (Figure 2.8). MCH-R1 and MCH-R2 appear to be both derived from the mammalian MCH-Rs, where MCH-R1 is ancestral to MCH-R2.

2.3.3. Tissue distribution

A qualitative RT-PCR approach was used to determine the localization and differential expression of mRNAs for MCH and receptor variants in the central nervous system and peripheral tissues of winter flounder. cDNA fragments of 256 bp, 375 bp, 294 bp, and 188 bp were amplified for MCH, MCH2, MCH-R1 and MCH-R2, respectively.

Figure 2.5. Protein alignment for MCH-R1 and MCH-R2 used for detecting phylogenetic relationships, including frog (*Xenopus laevis,* CAD32375), rat (*Rattus norvegicus*, NP_942065), human (*Homo sapiens,* CAC16691/AAK32193), winter flounder (*Pseudopleuronectes americanus*, HQ406772/HQ406774), barfin flounder (*Verasper moseri,* BAF49518), Japanese flounder (*Paralichthys olivaceus,* ACJ45804), goldfish (*Carassius auratus,* BAH70339), and zebrafish (*Danio rerio,* NP_898892). Winter flounder sequences are bolded, * indicates that all amino acids are identical in the column, : demonstrates that conserved substitutions have been observed and . specifies that semi-conserved substitutions have been observed

```
Winter flounder MCH-R1     1   M---------SDFKLGIVRLGRVAGKTKYTLIDEQDIPLVENYAF  36
Zebrafish MCH-R1           1   M---------SDFKLGIVRLGRVAGKTKYTLIDEQDIPLVGNYAF  36
Rat MCH-R1                 1   M---------TDFKLGIVRLGRVAGKTKYTLIDEQDIPLVESYSF  36
Human MCH-R1               1   M---------TDFKLGIVRLGRVAGKTKYTLIDEQDIPLVESYSF  36
Frog MCH-R1                1   M---------TDFKLGIVRLGRVAGKIKYTLIDEQDIPLVDSYAF  36
Human MCH-R2               1   MNPFHASCWNTSAELLNKSWNKEFAYQTASVVDT----VILPSMI  41
Goldfish MCH-R2            1   MNTSDILC-ASEFANSSNFSCVNRTTPSYSIIDIASFMHIFPTIY  44
Winter flounder MCH-R2     0   ----------------------------------------------  0
Japanese flounder MCH-R2   1   MGDTGTFC--NQTANLTDPACLNSTRPSYSHIDITTFMHIFPTIY  43
Barfin flounder MCH-R2     1   MNDTGIFCRNNQTANLTDPACLNSTRPSYNHIDITSFMHIFPTIY  45

Winter flounder MCH-R1    37   EARMEVDADGNGAKIFAYAFDIGKGRWA--------------GR  56
Zebrafish MCH-R1          37   EARMEVDADGNGAKIFAYAFDIGKGRWA--------------GR  56
Rat MCH-R1                37   EARMEVDADGNGAKIFAYAFDKNRGRGS--------------GR  56
Human MCH-R1              37   EARMEVDADGNGAKIFAYAFDKNRGRGS--------------GR  56
Frog MCH-R1               37   EARMEVDADGNGAKIFAYAFDKYKGRGS--------------GR  56
Human MCH-R2              42   GIICSTGLVGNILIVFTIIRSRKKTVPDIYICNLAVADLVHIVGM  86
Goldfish MCH-R2           45   GILCSVGVLANGLVIYAVASCKKKMVSDIYVLNLAIADLLFLLVM  89
Winter flounder MCH-R2     0   ----------------------------------------------  0
Japanese flounder MCH-R2  44   GILCSVGVIANGLVIYAVAACKKKMVSDIYVLNLAIADMLFLLVM  88
Barfin flounder MCH-R2    46   GILCSVGVIANGLVIYAVAACKKKMVSDIYVLNLAIADMLFLLVM  90

Winter flounder MCH-R1    57   PLHELLWEKHRGGIAPSFQVIHINSVTVDNRLDNLRLVPV-----  96
Zebrafish MCH-R1          57   PLHELLWEKHRGGIAPSFQVVHINSVTVDNRLDNLRLVPV-----  96
Rat MCH-R1                57   LLHELLWERHRGGVAPGFQVVHLNAVTVDNRLDNLQLVPW-----  96
Human MCH-R1              57   LLHELLWERHRGGVAPGFQVVHLNAVTVDNRLDNLQLVPW-----  96
Frog MCH-R1               57   LLHELLWQRHKGAIAPGFHVVHLNAITVDNRLDNLHIVPR-----  96
Human MCH-R2              87   PFLIHQWARGGEWVFGGPLCTIITSLDTCNQFACSAIMTVMSVDR 131
Goldfish MCH-R2           90   PFNIHQLVRDRQWVFGHFMCKAVVVVDVSNQFTTVGIVTVLCIDR 133
Winter flounder MCH-R2     0   ----------------------------------------------  0
Japanese flounder MCH-R2  89   PFNIHQLVRDRQWVFGNFMCKA-VVVDVSNQFTTVGIVTVLCIDR 132
Barfin flounder MCH-R2    91   PFNIHQLVRDRQWVFGNFMCKAVVVVDVSNQFTTVGIVTVLCIDR 135

Winter flounder MCH-R1    97   -----------GWSPKPEEIS----------------------- 106
Zebrafish MCH-R1          97   -----------GWSPKPEDLS----------------------- 106
Rat MCH-R1                97   -----------GWRPKAEETS----------------------- 106
Human MCH-R1              97   -----------GWRPKAEETS----------------------- 106
Frog MCH-R1               97   -----------GWRQPVEEVS----------------------- 106
Human MCH-R2             132 YFALVQPFRLTRWRTRYKTIRINLGLWAASFILALPVWVYSKVIK 176
Goldfish MCH-R2          134 YVAIVHP--TSEKRTIQWTIIINIMVWVGSFLLSIPVMIYTKVIS 176
Winter flounder MCH-R2     0   ----------------------------------------------  0
Japanese flounder MCH-R2 133 YIAIVHP--TSEKRTIHWTIMINILVWLGSFLLTVPVMMYAKVER 175
Barfin flounder MCH-R2   136 YIAIVHP--TSEKRTIHWTIMINILVWLGSFLLTVPVMMYAKVER 178
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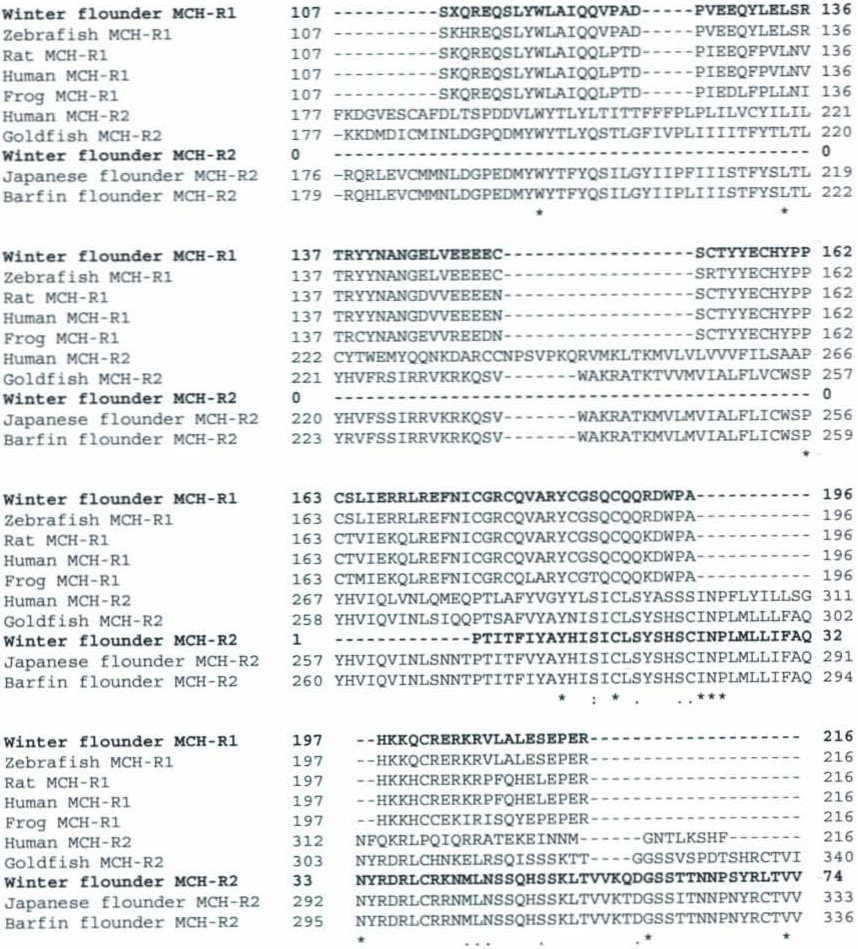
Winter flounder MCH-R1 107 ----------SXQREQSLYWLAIQQVPAD-----PVEEQYLELSR 136
Zebrafish MCH-R1 107 ----------SKHREQSLYWLAIQQVPAD-----PVEEQYLELSR 136
Rat MCH-R1 107 ----------SKQREQSLYWLAIQQLPTD-----PIEEQFPVLNV 136
Human MCH-R1 107 ----------SKQREQSLYWLAIQQLPTD-----PIEEQFPVLNV 136
Frog MCH-R1 107 ----------SKQREQSLYWLAIQQLPTD-----PIEDLFPLLNI 136
Human MCH-R2 177 FKDGVESCAFDLTSPDDVLWYTLYLTITTFFFPLPLILVCYILIL 221
Goldfish MCH-R2 177 -KKDMDICMINLDGPQDMYWYTLYQSTLGFIVPLIIIITFYTLTL 220
Winter flounder MCH-R2 0 ---------- 0
Japanese flounder MCH-R2 176 -RQRLEVCMMNLDGPEDMYWYTFYQSILGYIIPFIIISTFYSLTL 219
Barfin flounder MCH-R2 179 -RQHLEVCMMNLDGPEDMYWYTFYQSILGYIIPLIIISTFYSLTL 222
Winter flounder MCH-R1 137 TRYYNANGELVEEEEC----------SCTYYECHYPP 162
Zebrafish MCH-R1 137 TRYYNANGELVEEEEC----------SRTYYECHYPP 162
Rat MCH-R1 137 TRYYNANGDVVEEEEN----------SCTYYECHYPP 162
Human MCH-R1 137 TRYYNANGDVVEEEEN----------SCTYYECHYPP 162
Frog MCH-R1 137 TRCYNANGEVVREEDN----------SCTYYECHYPP 162
Human MCH-R2 222 CYTWEMYQQNKDARCCNPSVPKQRVMKLTKMVLVLVVVFILSAAP 266
Goldfish MCH-R2 221 YHVFRSIRRVKRKQSV-------WAKRATKTVVMVIALFLVCWSP 257
Winter flounder MCH-R2 0 ---------- 0
Japanese flounder MCH-R2 220 YHVFSSIRRVKRKQSV-------WAKRATKMVLMVIALFLICWSP 256
Barfin flounder MCH-R2 223 YRVFSSIRRVKRKQSV-------WAKRATKMVLMVIALFLICWSP 259
Winter flounder MCH-R1 163 CSLIERRLREFNICGRCQVARYCGSQCQQRDWPA---------- 196
Zebrafish MCH-R1 163 CSLIERRLREFNICGRCQVARYCGSQCQQRDWPA---------- 196
Rat MCH-R1 163 CTVIEKQLREFNICGRCQVARYCGSQCQQKDWPA---------- 196
Human MCH-R1 163 CTVIEKQLREFNICGRCQVARYCGSQCQQKDWPA---------- 196
Frog MCH-R1 163 CTMIEKQLREFNICGRCQLARYCGTQCQQKDWPA---------- 196
Human MCH-R2 267 YHVIQLVNLQMEQPTLAFYVGYYLSICLSYASSSINPFLYILLSG 311
Goldfish MCH-R2 258 YHVIQVINLSIQQPTSAFVYAYNISICLSYSHSCINPLMLLLFAQ 302
Winter flounder MCH-R2 1 ----------PTITFIYAYHISICLSYSHSCINPLMLLIFAQ 32
Japanese flounder MCH-R2 257 YHVIQVINLSNNTPTITFVYAYHISICLSYSHSCINPLMLLIFAQ 291
Barfin flounder MCH-R2 260 YHVIQVINLSNNTPTITFIYAYHISICLSYSHSCINPLMLLIFAQ 294
Winter flounder MCH-R1 197 --HKKQCRERKRVLALESEPER---------- 216
Zebrafish MCH-R1 197 --HKKQCRERKRVLALESEPER---------- 216
Rat MCH-R1 197 --HKKHCRERKRPFQHELEPER---------- 216
Human MCH-R1 197 --HKKHCRERKRPFQHELEPER---------- 216
Frog MCH-R1 197 --HKKHCCEKIRISQYEPEPER---------- 216
Human MCH-R2 312 NFQKRLPQIQRRATEKEINNM------GNTLKSHF------- 216
Goldfish MCH-R2 303 NYRDRLCHNKELRSQISSSKTT----GGSSVSPDTSHRCTVI 340
Winter flounder MCH-R2 33 NYRDRLCRKNMLNSSQHSSKLTVVKQDGSSTTNNPSYRLTVV 74
Japanese flounder MCH-R2 292 NYRDRLCRRNMLNSSQHSSKLTVVKTDGSSITNNPNYRCTVV 333
Barfin flounder MCH-R2 295 NYRDRLCRRNMLNSSQHSSKLTVVKTDGSSTTNNPNYRCTVV 336

```
1      GCCGACAATCACCTTCATCTACGCCTACCACATCAGCATCTGCCTCAGCTACTCTCACAGCTGCATC    67
1        P  T  I  T  F  I  Y  A  Y  H  I  S  I  C  L  S  Y  S  H  S  C  I     22

68     AACCCACTCATGCTGCTCATCTTCGCCCAGAACTACCGTGACCGTCTTTGCCGCAAAAACATGCTA    133
23      N  P  L  M  L  L  I  F  A  Q  N  Y  R  D  R  L  C  R  K  N  M  L     44

134    AACAGCTCCCAGCATTCTTCCAAGCTCACAGTCGTCAAACAAGATGGTTCCAGTACGACCAATAAC    199
45      N  S  S  Q  H  S  S  K  L  T  V  V  K  Q  D  G  S  S  T  T  N  N     66

200    CCCAGCTACCGCTTAACGGTCGTATAAtcccaaagtgtgtgtcctttagtagatacacaatgttct    265
67      P  S  Y  R  L  T  V  V  *                                            74

266    gctgctcaccgcaaaaggaagtagctcgatgaatggtgttgtccgtgaaataacattacccagcat    331
332    tttcacaactggaaatggataccgtagaagtgagcctgggtataaagcatgagcagcaacttcagc    397
398    cctgagtgaaagaagtgacatacaggtcctatcaaatgttttcaacagtgagtgcaagagaaaaaa    463
464    aacacaaatctgcagaggagagcaagcccgcta                                     496
```

Figure 2.6. Annotated winter flounder MCH-R2 partial cDNA sequence with nucleotides and deduced amino acids. * indicates a stop codon and letters in lower case represent the 3′ untranslated region

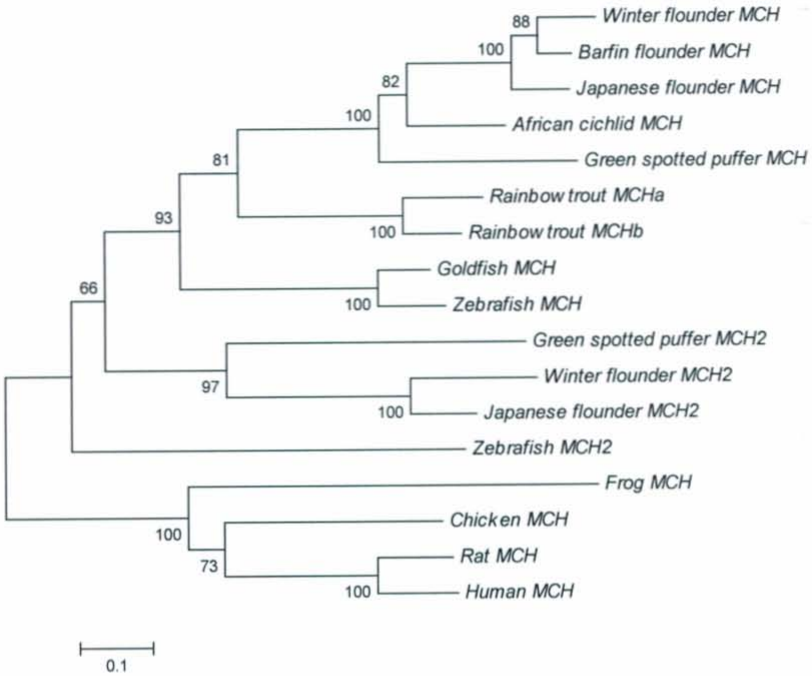

Figure 2.7. Neighbour-joining phylogenetic analyses for melanin-concentrating hormone (MCH) isoforms in vertebrates. Distance matrix is 1.067 bootstrap support (1000 replicates) indicated above nodes for MCH and MCH2, respectively. Accession numbers are the same as in Figure 2.2

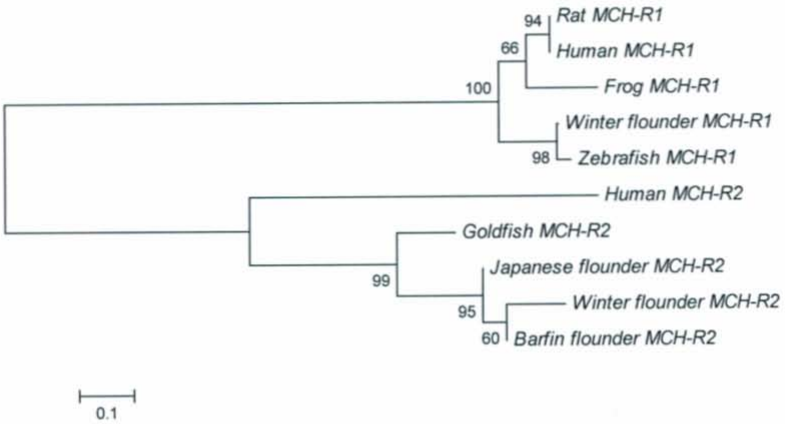

Figure 2.8. Neighbour-joining phylogenetic analysis for melanin-concentrating hormone receptors (MCH-Rs) in vertebrates. Distance matrix is 1.255 bootstrap support (1000 replicates) indicated above nodes for MCH-R1and MCH-R2, respectively. Accession numbers are the same as Figure 2.5

There was no amplification in the no-template negative control and all tissue samples produced a band of 201 bp for the housekeeping gene, EF-1α.

MCH mRNA is present throughout the brain, with apparent highest concentration in the forebrain (telencephalon/preoptic area, hypothalamus and pituitary) and midbrain (optic tectum/thalamus) and extremely low or no mRNA expression in the cerebellum and medulla oblongata (Figure 2.9). MCH was present in brain and all peripheral tissues examined, except heart and liver (Figure 2.10).

MCH2 mRNA is present in the forebrain and midbrain and pituitary gland, but not in hindbrain (cerebellum, medulla and spinal cord), as well as the pituitary of winter flounder (Figure 2.9). MCH2 mRNA appears to be ubiquitously expressed in peripheral tissues, with apparent highest expression in the gut and gonads (Figure 2.10).

MCH-R1 and MCH-R2 transcripts are ubiquitously expressed throughout the brain (Figure 2.9) and peripheral tissues of the winter flounder (Figure 2.10). MCH-R2 appears to be expressed at lower levels than MCH-R1 mRNA. Regarding MCH-R1, transcript expression levels appear to be lowest in the brain and liver. Within the brain, levels of MCH-R2 appear to be highest in the cerebellum and lowest in the hypothalamus. In the periphery, differential mRNA expression is observed, whereby highest expression is observed in the skin, foregut and gonads.

2.3.4. Effects of food deprivation on gene expression

Tissues chosen for qPCR (telencephalon, optic tectum, hypothalamus and gut) were based upon results from tissue distribution analyses and previous knowledge of

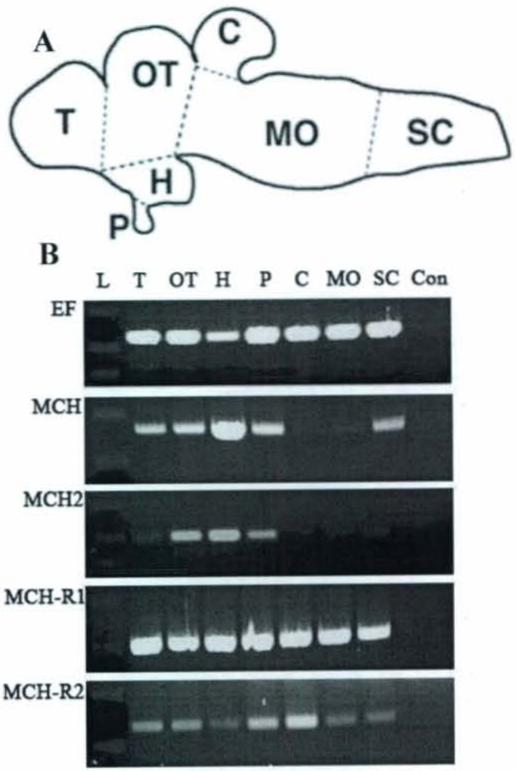

Figure 2.9. A) Schematic diagram of the winter flounder brain dissection. B) Qualitative RT-PCR tissue distribution of elongation factor-1α (EF; control), melanin-concentrating hormone (MCH), melanin-concentrating hormone 2 (MCH2), MCH-receptor 1 (MCH-R1) and MCH-R2 genes in winter flounder, *Pseudopleuronectes americanus*, for various central nervous system tissues including the pituitary (T, telencephalon/preoptic area; OT, optic tectum/thalamus; H, hypothalamus; P, pituitary gland; C, cerebellum; MO, medulla oblongata; SC, spinal cord) as detected by RT-PCR. L, ladder; Con, PCR no-template control. Ladder includes band sizes of 50 bp, 150 bp and 300 bp

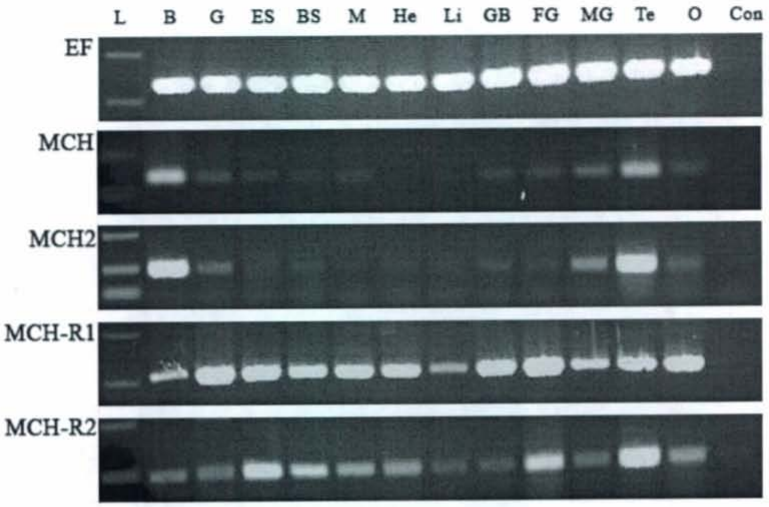

Figure 2.10. Qualitative RT-PCR tissue distribution of elongation factor 1α (EF; control), melanin-concentrating hormone (MCH), melanin-concentrating hormone 2 (MCH2), MCH-receptor 1 (MCH-R1) and receptor 2 (MCH-R2) genes in winter flounder, *Pseudopleuronectes americanus*, for various peripheral tissues. L, ladder; B, brain; ES, eyed side skin; BS, non-eyed side skin; M, muscle; G, gill; He, heart; Li, liver; GB, gall bladder; FG, stomach; MG, intestine; Te, testes ; O, ovaries; Con, no-template control. Ladder includes bands of 50 bp, 150 bp and 300 bp

putative brain regions regulating food intake (Demski and Knigge 1971; Peter 1979).

MCH mRNA expression showed a significant increase in the optic tectum/thalamus in fasted animals compared to fed animals (Figure 2.11). However, no significant differences were observed in either the telencephalon/preoptic area, hypothalamus or midgut. There were no significant differences in any tissue for the MCH2 transcript during food deprivation (Figure 2.11).

Fasting induced an increase in MCH-R1 mRNA expression in the hypothalamus, but no significant difference between fed and fasted fish were seen in the telencephalon, optic tectum and gut (Figure 2.12). There were no significant differences in the mRNA expression of MCH-R2 between fed and fasted fish in any of the tissues examined.

2.4. Discussion

The primary objective of this study was to determine how transcripts encoding MCH isoforms (MCH and MCH2) and their receptors (MCH-R1 and MCH-R2) may play a role in appetite regulation of winter flounder. Transcripts encoding the peptides were cloned and sequenced to determine sequence identities and homology with other teleosts and vertebrates.

I identified a cDNA sequence encoding a complete MCH2 propeptide of 151 aa, and a partial proMCH fragment for MCH. Amino acid sequence similarities, with other teleost MCHs ranged from 35-87% and 35-77%, for MCH and MCH2, respectively. Winter flounder MCH appears to be most similar to barfin flounder MCH (87%) and generally more divergent from zebrafish and goldfish (36 and 38%, respectively).

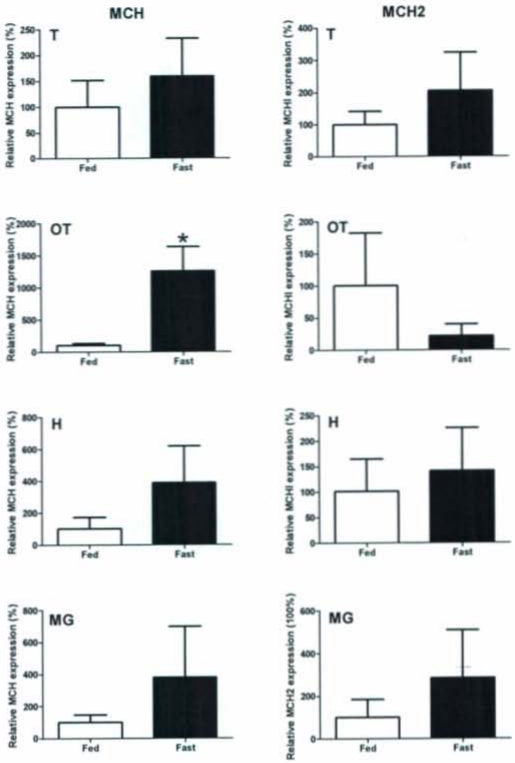

Figure 2.11. Effects of fasting on melanin-concentrating hormone (MCH) and its paralogue (MCH2) in the telencephalon/preoptic area (T), optic tectum/thalamus (OT), hypothalamus (H) and midgut (MG) during the winter flounder, *Pseudopleuronectes americanus*, food deprivation experiment. Fish (n = 5 to 9) were sampled from each the fed and fasted groups. Expression levels are a percentage that is normalized to the control group (fed fish) set at 100%. Data is shown as mean ± SEM. * indicate significant differences between the groups ($p < 0.05$).

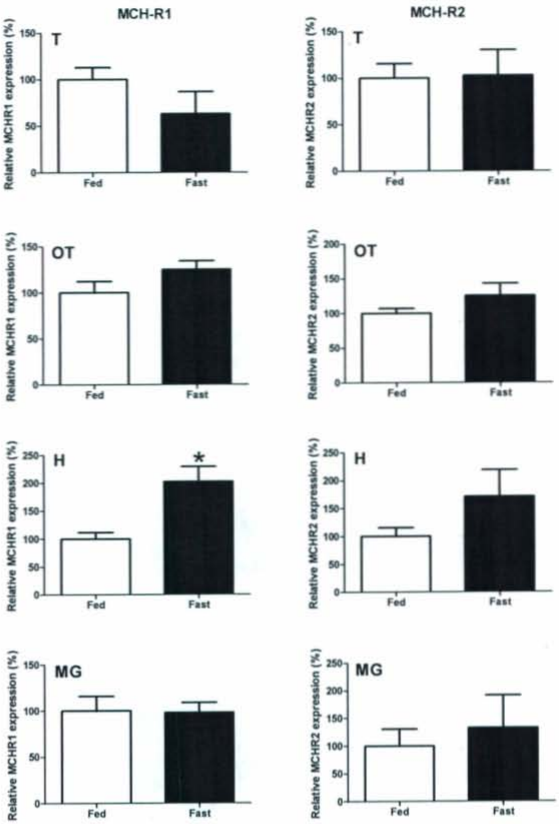

Figure 2.12. Effects of fasting on melanin-concentrating hormone receptor 1 (MCH-R1) and receptor 2 (MCH-R2) in the telencephalon/preoptic area (T), optic tectum/thalamus (OT), hypothalamus (H) and gut (MG) during the winter flounder, *Pseudopleuronectes americanus*, food deprivation experiment. Fish (n = 5 to 9) were sampled from each of the fed and fasted groups. Expression levels are a percentage that is normalized to the control group (fed fish) set at 100%. Data is shown as mean ± SEM. * indicates significant differences between the groups ($p < 0.05$)

Few MCH2 sequences have been identified in teleosts, however winter flounder MCH2 is most conserved with Japanese flounder MCH2 (77%) and least with Chinook salmon (*Oncorhynchus keta*; 35%) As shown by the alignments, the signalling and mature peptides appear to be most conserved, while the intervening amino acids are more divergent. Barring 1 or 2 amino acid substitutions, the mature MCH peptide is relatively well conserved amongst teleosts and appears to have diverged from the higher vertebrate MCH (Figure 2.7). In other vertebrates, a relatively well conserved peptide sequence called neuropeptide E-I (NEI) is cleaved just prior to MCH (Berman *et al.* 2009; Cardinaud *et al.* 2004). It appears that NEI is not present in either of the teleost MCH or MCH2 forms (Figure 2.3). However, a 25 aa neuropeptide AL has been identified in flounder and a 14 aa neuropeptide EV is cleaved just prior to MCH in rainbow trout. The positioning of neuropeptide AL and neuropeptide EV are consistent with the site of NEI in mammals and could indicate that NEI and neuropeptides EV and AL are homologous hormones.

The MCH2 mature peptide is less conserved among vertebrates than MCH, but its length (19 aa) appears to be consistent with that of other higher vertebrates providing evidence of its mammalian-derived evolution. Interestingly, the zebrafish mature MCH2 peptide is nearly identical to the mammalian forms and is less similar to other fish. However, the zebrafish signal peptide is more conserved with other fish compared to mammals and other vertebrates.

I identified a complete 228 aa sequence for MCH-R1, while only a partial fragment of MCH-R2 was isolated. The MCH-R1 amino acid sequence is 82-99% conserved with all vertebrates, including mammals, while the MCH-R2 amino acid

fragment is 59-93% conserved with other teleosts. The presence of two MCH-R forms in fish likely dates to the first whole genome duplication event in vertebrates, since humans contain two MCH-Rs. Based on the phylogenetic analyses (Figure 2.8), MCH-R1 is ancestral to MCH-R2. With regards to the MCH-R1 phylogeny and alignment, one can note the short branch lengths and high sequence conservation between higher vertebrates and fish. On the other hand, MCH-R2 is more recently derived and the human MCH-R2 amino acid sequence is quite divergent from that of fish.

Central and peripheral tissue mRNA distributions for each of the genes were examined. MCH was ubiquitously expressed throughout the brain and most peripheral tissues, although it was not present in heart and liver. Highest expression levels appeared to be present in the forebrain (telencephalon/preoptic area, hypothalamus, pituitary) and midbrain (optic tectum/thalamus), with particularly high expression levels in the hypothalamus, a major MCH production site in all orders of vertebrates that have been examined (Hervieu and Nahon 1995; Qu et al. 1996; Vallarino *et al.* 2009).

In fish, the main hypothalamic nuclei in which MCH-ir cell bodies are localized include the NLT, nucleus posterioralis paraventricularis (NPPv), and the LVR, but species-specific differences in distribution occur. In the agnathid lamprey, MCH-ir perikarya are localized in the periventricular dorsal hypothalamic nucleus surrounding the paraventricular organ (PVO) (Bird *et al.* 2001). In sexually mature lamprey, MCH-ir cell bodies are found in the telencephalon, which is not seen in other teleosts (Bird *et al.* 2001). Dogfish shark (*Scyliorhinus canicula)* appear to have MCH-ir cells in regions of the NLT, as well as the nucleus succus vasculosa (NSV) and the pars distalis and intermedia of the pituitary (Vallarino *et al.* 1989). MCH-ir cell bodies are seen in nuclei

of the dorsal hypothalamus and telencephalon in lungfish (*Protopterus annectens*) (Vallarino *et al.* 1998). In goldfish, zebrafish, barfin flounder and white seabream (*Diplodus sargus*) MCH-ir bodies were found in various regions of the NLT, as well as the LVR and other posterior and inferior parts of the hypothalamus (Duarte *et al.* 2001; Amano *et al.* 2003; Berman et al. 2009; Matsuda *et al.* 2009). Findings in the medaka (*Oryzias latipes*) are consistent with that of the goldfish, zebrafish and barfin flounder with the addition of MCH-ir cell bodies within the pituitary (Amiya *et al.* 2007; Suehiro *et al.* 2009). The presence of MCH mRNA in the telencephalon in our study could be a result of our dissection pattern, which might have included cell bodies from the NLT - a region that is dorsal to the hypothalamic lobes - in our telencephalic/preoptic region. However, it is possible that MCH-producing cells do exist within the actual telencephalon, as is the case in lamprey and lungfish (Vallarino *et al.* 1998; Bird *et al.* 2001). Similarly, the expression of MCH transcript in the optic tectum of winter flounder could be explained by its close proximity to the periventricular organ (PVO) and the LVR or by the presence of MCH cells in the true optic tectum. Future studies using *in situ* hybridization/immunohistochemistry are necessary to confirm the exact localization of MCH-producing cells.

MCH mRNA was seen in most peripheral tissues examined, with the exception of the heart and liver. The presence of MCH transcript in skin has previously been shown (Castrucci *et al.* 1987) and linked to the physiological role of this peptide in colouration. No studies have ever examined the distribution of MCH in other peripheral tissues. The presence of MCH mRNA in the gut and gonads could indicate a peripheral role of MCH in energy balance and/or reproduction.

Little is known about the distribution and function of MCH2 transcript in teleosts. In this study, MCH2 was expressed centrally in the forebrain and midbrain, but not hindbrain, of winter flounder and ubiquitously in the peripheral tissues with highest expression seen in the midgut and gonads. In zebrafish, MCH2-ir cell bodies were localized in the lateral NLT of the hypothalamus, however the peripheral tissue distribution of MCH2 has never been examined (Berman *et al.* 2009). As for MCH, it could be postulated that MCH2 plays a role in reproduction and energy homeostasis, since it was expressed in both the gonads and gut.

MCH2 appears to be more closely related to mammals and is likely a result of the most recent duplication event giving rise to teleosts and tetrapods from their common ancestor making it the more ancestral form compared with the teleost derived MCH. In mammals, MCH is known for its appetite-related functions and the central nervous system and peripheral distributions of MCH2 mRNAs in flounder are consistent with these results. Highest expression is in the telencephalon/preoptic area, optic tectum/thalamus, hypothalamus, and pituitary gland, as well as the midgut and foregut of winter flounder indicate possible roles of MCH2 in appetite regulation. The teleost derived MCH in winter flounder has also evolved mRNA synthesis in the hindbrain and spinal cord, as well as higher expression in the hypothalamus compared with MCH2 and could represent teleost derived functions, such as skin colour change. Similar expression profiles between MCH and MCH2 are observed in the periphery, with the exception of MCH2 mRNA being expressed in very low levels in heart tissue.

Both MCH-R1 and MCH-R2 transcripts were ubiquitously expressed in central and peripheral tissues in winter flounder. Few studies have looked at the functional roles

of the MCH receptors in fish. In goldfish, MCHR1 is found in the brain and pituitary as well as in several peripheral tissues including eyeball and fat (Mizusawa et al. 2009). Interestingly, MCH-R1 is found only in the brain and not in peripheral tissues of barfin flounder (Takahashi *et al.* 2007). In zebrafish, MCH-R1a and MCH-R1b are not expressed in skin tissue, however no other peripheral tissues were examined. MCH-R1a-ir cells are found in the telencephalon, optic tectum, hindbrain, as well as numerous hypothalamic nuclei including the periventricular nucleus, posterior tuberal nucleus and lateral hypothalamus (Berman *et al.* 2009). MCH-R1b displays a similar mRNA expression profile to MCH-R1a, with additional expression in the preoptic area of the hypothalamus. This is the first report of the presence MCH-R1 transcript in the stomach and the intestine of a fish and could indicate a peripheral role for MCH in regulating appetite and digestive processes.

The ubiquitous localization of MCH-R2 mRNA in winter flounder is consistent with results in goldfish and barfin flounder. In goldfish, MCH-R2 transcripts are expressed in pituitary, brain, and in several peripheral tissues - except liver - such as spleen and intestine, eyeball and skin tissues (Mizusawa et al. 2009). In barfin flounder, MCH-R2 is expressed in all tissues examined, with the exception of liver and stomach, including brain, pituitary, intestine, testes, ovaries, eyed- and non-eyed side skin (Takahashi et al. 2007). However, in zebrafish, expression of MCH-R2 is restricted to the skin and not present in the brain (Berman et al. 2009).

Phylogenetically, MCH-R1 is ancestral to the more recently derived MCH-R2 and this relationship can be seen in their respective tissue distributions and physiological functions. Winter flounder MCH-R1 and MCH-R2 transcripts have ubiquitous expression

profiles across all tissues, however MCH-R2 appears to be differentially regulated transcripts in brain and peripheral tissues. The derived form, MCH-R2, might have taken on a fish-specific function in skin colouration in flounder (Takahashi et al. 2007) which is suggested by high expression levels in the skin compared to brain. Lower MCH-R2 mRNA expression than MCH-R1 in brain regions (telencephalon/preoptic area, optic tectum/thalamus and hypothalamus) containing putative appetite control centres could be a result of a functional divergence between the two receptor forms, with MCH-R1 being the primary mediator of appetite regulation in the brain.

qPCR analyses were completed to assess the role of MCH and their receptors in the regulation of feeding. Our results show that MCH mRNA expression is significantly higher in the optic tectum/thalamus of fasted winter flounder, which is consistent with previous mRNA expression results in rodents and barfin flounder (Presse *et al.* 1996; Qu *et al.* 1996; Takahashi *et al.* 2004). Similar trends for an increase in mRNA expression were seen in the telencephalon/preoptic area, hypothalamus and midgut, but were not significant. It is surprising that significant differences were observed in the optic tectum/thalamus and not in the hypothalamus, since MCH is thought to be a strictly hypophysial derived peptide. As our dissections of the optic tectum/thalamus likely included part of the anterior hypothalamus (including the PVO and LVR), it could be hypothesized that the changes in expression seen in the optic tectum result from changes in expression within the PVO and LVR. These regions appear to be key modulators of MCH secretion and feeding in fish. Indeed, ICV injections of MCH antiserum in goldfish causes a concomitant increase in food intake and ir-cells in the LVR (Matsuda *et al.* 2007), contradictory to what has been observed in flounder immunohistochemical studies

(Takahashi *et al.* 2004). Localization of MCH-ir cells in the LVR of goldfish could explain the elevated expression in the optic tectum/thalamus, and not the hypothalamus, during fasting in winter flounder.

Consistent with our results in winter flounder, barfin flounder exhibited higher MCH mRNA expression levels in the hypothalamus and an increase in the number of MCH-ir cells in the NLT and LVR during prolonged fasting (Takahashi *et al.* 2004). As mentioned previously, the locations of the LVR and NLT in the anterior hypothalamus in close proximity to optic tectum/thalamus, might explain the high MCH mRNA expression levels seen in fasted winter flounder.

No significant differences in MCH2 mRNA expression were observed in flounder between fed and fasted fish in any of the tissues examined (brain regions and midgut), which contrasts with zebrafish, for which fasting causes a significant increase in MCH2-ir cell bodies in the brain (Berman *et al.* 2009). This discrepancy could be explained by the low sample sizes in our study and/or individual differences in response to fasting. It is also possible that, since MCH2 mRNA is present at high levels in the gonads of winter flounder, MCH2 could play a larger role in reproduction than feeding or that sex-related differences in feeding behaviour exist in flounder. In fasted rats, it has been shown that female MCH-ir neurons decrease more rapidly following glucose injections compared to males (Mogi *et al.* 2005). Other appetite-related hormones, including ghrelin (Parhar *et al.* 2003) and thyroid hormone, triiodothyronine (T_3) (Toguyeni *et al.* 1996), display sex-related differences in food-restricted tilapia. Future studies with larger samples and fish of the same sex could result in similar mRNA expression of MCH2 as observed in zebrafish.

Until this study, there was no information on a possible role for any MCH receptor in the regulation of feeding in fish. Our results show that fasting has no effects on the expression of MCH-R2 mRNA in either brain or gut, but up-regulates hypothalamic MCH-R1 mRNA expression. These results strongly suggest that MCH-R1, but not MCH-R2, might mediate the effects of MCH on feeding. Such a role in the regulation of feeding for MCH-R1 has been demonstrated in rodents. Rodents ICV-injected with MCH-R1 antagonists display a reduction in food intake, body weight and lipid accumulation (Suply *et al.* 2001; Oh *et al.* 2009) whereas ICV and inter-nucleus accumbens shell injections of MCH-R1 agonists increase feeding (Guesdon *et al.* 2009). In addition, MCH-R1 knock-out mice are resistant to obesity and hyperphagy (Chen *et al.* 2002). In mice, MCH-R1-ir cells and mRNA expression in vagal afferent neurons increase in number and are higher in fasted compared to fed animals (Burdyga *et al.* 2006).

Differential MCH-R2 mRNA expression was not observed between fed and fasted flounder in the brain and gut. In barfin flounder, MCH-R2 is thought to be the receptor involved in colour change (Takahashi *et al.* 2007) and, as previously mentioned, this could be the case in winter flounder, where MCH-R1 is the receptor primarily involved in food intake, while MCH-R2 has evolved other functions (skin colour change). Such a divergence in function for two receptor forms has also been reported for melanocortin receptors [melanocortin receptor -3 (MC3-R) and 4 (MC4-R)] in rodents: a differential expression was observed during food restriction assays between the two forms (Harrold *et al.* 1999). MC4-R appears to be the receptor primarily involved in food intake, while MC3-R is unaffected by food restriction and might be involved in fat metabolism. Rather,

MC3-R has been found to decrease leanness and enhance fat mass in mice (Chen et al. 2000). More studies on the MCH-R2 receptor in other fish are needed to clearly establish its physiological roles.

Transcripts for the MCH peptides and receptors are detectable in the midgut of winter flounder and could implicate a minute role in peripheral appetite regulation. However, since the transcript expression levels are quite low ($\Delta\Delta$CT values > 30), we cannot say with confidence that MCH transcripts do not have a peripheral role in food intake regulation. Future *in situ* hybridization studies and analysis of different gut regions could associate a peripheral role of MCH and MCH2 transcripts to appetite regulation.

MCH-R1 and MCH-R2 mRNA expression in the intestine of winter flounder was not different between fed and fasted fish, suggesting that MCH might not have a crucial role in regulating appetite peripherally. It is also possible that prolonged MCH secretion might have caused an internalization of the receptors in fasted fish. Indeed, in human embryonic kidney-293T (HEK293T) cells transfected with rat MCH-R1, internalization is induced by MCH treatment (Saito *et al.* 2004).

In conclusion, cDNAs for MCH, MCH2, MCH-R1 and MCH-R2 were cloned and sequenced, tissue distribution studies were completed, and expression differences between fed and fasted fish were quantified. All transcripts were present in the forebrain and midbrain, while transcripts encoding MCH and its receptors showed some prevalence in the hindbrain. Genes were ubiquitously expressed in peripheral tissues of winter flounder, with the exception that MCH mRNA was absent in heart and liver tissues using RT-PCR. Furthermore, qPCR analyses indicated that fasting induced higher mRNA expression levels of MCH in the optic tectum/thalamus and of MCH-R1 in the

hypothalamus. This study supports the orexigenic effects of MCH in winter flounder and suggests that these actions might be mediated by the MCHR1 receptor. The presence of high MCH mRNA expression levels in the gonads could indicate a potential role in reproduction due to its neuromodulatory function of other hormones.

2.5. Acknowledgements

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Chapter 3: Characterization of the gonadotropin-releasing hormone (GnRH) transcript family in winter flounder (*Pseudopleuronectes americanus)* and its role in feeding

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Abstract

Gonadotropin-releasing hormone (GnRH) is primarily related to reproductive processes in vertebrates. The ten amino acid active peptide is relatively conserved throughout chordates, more specifically in fish species. Teleosts generally have at least two variants of GnRH present in their genomes. Chicken-GnRH-2 (cGnRH-2) is common to all fish, whereas other prevalent forms include salmon-GnRH-3 (sGnRH-3) and seabream-GnRH-1 (sbGnRH-1). The mRNAs of all three forms were identified in winter flounder (*Pseudopleuronectes americanus)*. In this fish, cGnRH-2 mRNA is highly expressed in the optic tectum/thalamus. Winter flounder sGnRH-3 mRNA is ubiquitously expressed throughout the brain, with apparent highest expression in the telencephalon/preoptic area. The effect of fasting on the expression of each of the three isoforms was assessed. Fasting reduces cGnRH-2 and sGnRH-3 mRNA expression in the optic tectum/thalamus and hypothalamus, and telencephalon/preoptic area, respectively, compared to fed fish. Our results suggest that the GnRH system could be a major regulator of food intake in winter flounder.

3.1. Introduction

Gonadotropin-releasing hormone (GnRH) is a decapeptide classically known in vertebrates for its key regulatory role in reproduction. GnRH was first identified in mammals (Adelman *et al.* 1986), termed mammalian-GnRH (mGnRH), and has subsequently been identified in most other vertebrates, including amphibians (Conlon *et al.* 1993) and teleost fish (King *et al.* 1995). In fish, 16 variants of GnRH have been isolated with up to three forms present in a single species. A homolog of mGnRH is present in several fish, including gilthead seabream (*Sparus aurata*) (Gothilf *et al.* 1996), lungfish (*Protopterus annectens*) (King *et al.* 1995) and eel (*Anguilla anguilla*) (Dufour *et al.* 1982). It has been suggested that mGnRH is the most ancestral form throughout vertebrates (Chen and Fernald 2008; Okubo and Nagahama 2008) from which other teleost-specific variants, including seabream-GnRH (sbGnRH) (Gothilf *et al.* 1996), catfish-GnRH (cfGnRH) (Sherwood *et al.* 1989) and medaka-GnRH (mdGnRH) (Okubo *et al.* 2000), have evolved. In fish such as medaka (*Oryzias latipes*) (Okubo *et al.* 2000), gilthead seabream (Gothilf *et al.* 1996), barfin flounder (*Verasper moseri*) (Amano *et al.* 2002), and European seabass (*Dicentrarchus labrax*) (Gonzalez-Martinez *et al.* 2002), GnRH-1-immunoreactive (ir) neurons have been shown to cluster predominantly in the preoptic area (POA) with some cells in the pituitary gland, olfactory bulbs, ventral thalamus and hypothalamus. The presence of GnRH-1-ir neurons in the POA and of fibre tracts extending into the hypophysis (Gothilf et al. 1996; Amano et al. 2002; Gonzalez-Martinez et al. 2002) suggests that these GnRH forms function as regulators of gonadotropin release [follicle-stimulating hormone (FSH) and luteinizing hormone (LH)].

Another form common in most fish is chicken-GnRH (cGnRH). cGnRH was first isolated from the chicken (King and Millar 1982a; King and Millar 1982b; Miyamoto *et al.* 1983) along with another variant, cGnRH-1. Together, cGnRH-1 and cGnRH are the most common forms in birds and reptiles and appear to have evolved from the diapsid lineage (King and Millar 1995). The mature cGnRH decapeptide structure is conserved in fish (King and Millar 1985), birds (Miyamoto *et al.* 1984), amphibians (Conlon *et al.* 1993), reptiles (Ikemoto and Park 2003), marsupials (King *et al.* 1990) and mammals (Dellovade *et al.* 1993). cGnRH expressing cells are nearly always only found within the midbrain tegmentum as seen in Siberian sturgeon (*Acipenser baeri*) (Lepretre *et al.* 1993), clawed toad (*Xenopus laevis*) (King *et al.* 1994) and musk shrew (*Suncus murinus*) (Dellovade *et al.* 1993). Other regions of the brain where cGnRH has been identified are hypothalamic nuclei such as the infundibular nucleus [ostrich (*Struthio camelus*), (Powell *et al.* 1987); frog (*Rana ridibunda*), Conlon *et al.* 1993], telencephalic areas, such as the preoptic nucleus (frog, Conlon *et al.* 1993), and the pituitary gland [catfish (*Clarias gariepinus*) (Schulz *et al.* 1993; Bogerd *et al.* 1994), frog, Conlon *et al.* 1993].

Early studies in the musk shrew identified cGnRH as the variant linking reproduction and energy status. In female musk shrews, fasting induces an increase in GnRH-ir cells in both the POA and median eminence compared with *ad libitum* fed females (Temple and Rissman 2000). The GnRH protein appears to be produced but not released from the median eminence during food restriction, with secretion occurring only after reinstatement of feeding (Temple and Rissman 2000). Intercerebroventricular (ICV) injections of cGnRH and mGnRH both cause a decrease in food intake in *ad libitum* fed musk shrews (Temple and Rissman 2000; Kauffman and Rissman 2004) and cGnRH

decreases food intake following ICV injections in goldfish (*Carassius auratus*) (Hoskins *et al.* 2008; Matsuda *et al.* 2008).

Salmon-GnRH (sGnRH) is a third variant present in fish and is believed to have major roles in reproduction and in the regulation of gonadotroph secretions and sex steroids synthesis (Zohar *et al.* 2010). Like cGnRH, the structure (amino acid sequence) and neuronal organization of sGnRH is highly conserved amongst fish and amphibians. sGnRH is primarily localized to the olfactory bulbs, telencephalon and hypothalamic nuclei such as the nucleus preopticus periventricularis (nPP), preoptic nucleus and nucleus anterioris periventricularis (nAP), of numerous fish, including Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Bailhache *et al.* 1994), sole (*Solea solea*) (Rodriguez *et al.* 1985), and sea bass (Kah *et al.* 1991).

Although sGnRH has been implicated in the regulation of several reproductive processes such as such as ovulation and oogenesis (Yu *et al.* 1987; Weil and Marcuzzi 1990a), spermatogenesis (Weil and Marcuzzi 1990b; Amano *et al.* 1997), spawning behaviour (Marte *et al.* 1987; Alok *et al.* 1993) and gonadotrophs and steroid hormones secretion (Weil and Marcuzzi 1990a; Weil and Marcuzzi 1990b), very little information is available on its potential role as a regulator of other physiological systems. Since sGnRH-3 has been isolated in the olfactory bulbs in most fish species, including chub mackerel (*Scomber japonicus*) (Selvaraj *et al.* 2009), Nile tilapia (*Oreochromis niloticus*) (Swapna *et al.* 2008) and silver seabream (*Sparus sarba*) (Hu *et al.* 2008), it could be hypothesized that sGnRH plays a role in food sensing and perhaps food intake. However, a recent study in goldfish shows that ICV injections of sGnRH have no significant effect on appetite in this species (Matsuda *et al.* 2008).

GnRH-receptors (GnRH-R) are G-protein-coupled receptors containing seven hydrophobic alpha helix transmembrane domains linked with hydrophobic extra- and intra-cellular loops. It is within the third extracellular loop that a three amino acid motif is used to identify the variant. Four receptor isoforms have been identified in vertebrates, GnRH-Ra1, GnRH-Ra2, GnRH-Rb1 and GnRH-Rb2, with corresponding extracellular loop motifs of PEY, SDP, PPS and SHS. GnRH-Ra1 is considered to be mammalian-specific, while GnRH-Ra2 is non-mammalian-specific. The GnRH-Rb group is much more divergent, where GnRH-Rb1 is restricted to tetrapods and GnRH-Rb2 to lower vertebrates. Two variants, have been identified in humans, however only one has been added to this classification system (GnRH-Ra2), and a single form GnRH-Ra2 has been identified in rodents (Troskie *et al.* 2000; Flanagan *et al.* 2007). GnRH-Ra1 and GnRH-Rb2 have been recognized in fish (Chen and Fernald 2008). From here onward, receptors will be identified based on their nomenclature in studies, unless referring specifically to their phylogeny.

Few studies have looked at the functions of the GnRH-Rs in relation to feeding. In musk shrews implanted with ICV cannulae, treatments with a GnRH-R1 antagonist (Antide) does not affect feeding (Kauffman *et al.* 2005), whereas a GnRH-R2 agonist (135-18) inhibited food intake, suggesting that GnRH-R2 might be the primary receptor involved in appetite regulation (Kauffman *et al.* 2005). Although GnRH-Rs have been implicated in the regulation of reproduction in several fish species such as the African cichlid (*Astatotilapia burtoni*) (Maruska and Fernald 2010), black porgy (*Acanthopagrus schlegeli*) (Lin *et al.* 2010) and goldfish (Khakoo *et al.* 1994), their roles in mediating energy status and appetite regulation remain unknown.

Winter flounder (*Pseudopleuronectes americanus*) is a commercially important fish found along the seabed off Newfoundland shores. Little information is known about how these fish regulate feeding behaviours. Interestingly, winter flounder undergo a natural period of fasting during the winter when energy demands and gonadal development are at their peaks (Stoner *et al.* 1999). Post-spawning, flounder resume their natural feeding habits to prepare for their next fast.

In this study, we isolated and characterized transcripts of the GnRH-peptide family in winter flounder. cDNAs for three forms of GnRH (sbGnRH, cGnRH and sbGnRH) and two forms of GnRH-Rs (GnRH-R1 and GnRH-R2) were isolated. To further characterize cGnRH and sGnRH mRNAs, we examined their brain distributions. Due to time constraints, peripheral tissue distributions were not completed for any transcripts, nor were central nervous system tissue transcript expression profiles for sbGnRH, GnRH-R1 and GnRH-R2 was not studied. In order to assess a possible role of GnRH peptides and receptors in winter flounder food intake regulation, we examined the effects of fasting on their brain mRNA expression.

3.2. Materials and Methods

3.2.1. Animals

Winter flounder brain tissue used for cloning was sampled from 3-4 wild fish collected by scuba divers off the shore of St. John's (Logy Bay, Newfoundland and Labrador, Canada). After collection, fish were kept in 2 m × 2 m flow through tanks at

the Ocean Sciences Centre (OSC of Memorial University of Newfoundland; St. John's, Newfoundland and Labrador, Canada). Fish were kept under natural photoperiod and temperature conditions (11.9 °C). The sex ratio was approximately 50:50 in all tanks. Fish were fed frozen herring to satiety two or three times a week at the same time of the day (10:00).

Fish for the food deprivation experiment were obtained by seining and held at the Bonne Bay Marine Station (BBMS; Norris Point, Newfoundland, Canada). Fish (five per tank) were maintained in four white 0.5 m x 0.5 m flow through tanks with a sandy substrate to imitate their natural environment, at ambient water temperatures and lighting (see below). Males and females were used with an approximate 50:50 ratio in each treatment. Fish were fed cut up frozen squid every 2-3 days at the same time of the day (21:00). On the sampling day, fish were fed 1 h prior to sampling. Weights were obtained before the experiment began and during sampling. Gonad and liver weights were determined following sampling for calculation of GSI and hepatosomatic index (HSI). GSIs ranged from 0 (immature; $n = 2$) to 1.49 in the controls and 0 ($n = 1$) to 1.33 in the fasted group. Fewer males were sampled than females (Fed: 1 male and 8 females; fasted: 1 male and 7 females), however all sampling was done randomly. All experiments were conducted in accordance with the principles found in the Canadian Council on Animal Care guide.

3.2.2. Food deprivation experimental design

Winter flounder ($n = 20$; average weight of 115.59 ± 22.67 g) were acclimated for

two weeks in four tanks (five fish per tank) under natural photoperiod and an average water temperature of 10 °C (July 6th to July 30th 2009). The same feeding regime was used as previously described (section 3.2.1). Following acclimation, two tanks were selected as controls (fed as described above), while the remaining tanks were starved for 10 days. Duplicate tanks were used to account for any tank effect. Following experimentation, fish were sacrificed with an overdose (100mg/L) of tricaine methanesulfonate (Syndel Laboratories, Vancouver, British Columbia, Canada), and brains were dissected and stored in RNAlater stored at -20°C (Qiagen Inc., Mississauga, Ontario, Canada) until further processing.

3.2.3. RNA extraction and cDNA synthesis

For cloning and tissue distribution, tissues from the brain (telencephalon/pre-optic area, optic tectum/thalamus, hypothalamus, cerebellum, medulla oblongata and spinal cord) including the pituitary gland and from the periphery (gill, eyed-side skin, blind-side skin, muscle, heart, liver, gall bladder, foregut, midgut, male gonad and female gonad) were removed from two adult flounder. An annotated anatomy of the flatfish brain was utilized for brain regional dissections (Evans 1937).

RNA was isolated using the Tri-reagent/chloroform (BioShop, Burlington, Ontario, Canada) extraction technique using the manufacturer's protocol. Final RNA concentrations, 260/280 and 260/230 data were determined using NanoDrop (NanoDrop, Wilmington, North Carolina, USA) spectrophotometry at a wavelength of 260-nm. RNA was then reverse-transcribed (RT) into cDNA via the Quantitect Reverse-Transcriptase

Kit (Qiagen, Mississauga, Ontario, Canada) using 20 µl samples consisting of 2 ng RNA, 6X Quantitect buffer, 7X genomic DNA wipeout buffer, 0.5 nM of each dNTP, 0.5 µg each random hexamer and oligo dT primers and 200 U Quantitect Reverse Transcriptase.

3.2.4. Isolation of prepro-GnRH and GnRH-receptors from flounder brain

Regions of mRNA sequences in various teleost fishes, including the Japanese flounder (GnRHs: GenBank AAY28981/ACS88343; GnRH-Rs: AAY28982), zebrafish (*Danio rerio*, GenBank AAM43951/AAL99294; GnRH-Rs: ABU92656/ABU62657/ABU62658/ABU62659), goldfish (GnRHs: GenBank AAB86989/BAB18904; GnRH-Rs: AAD20001/AAD20002), the perciform Burton's mouthbreeder (*Haplochromis burtoni*) (GnRHs: GenBank AAC27716/AAC27717/AAC27718), rainbow trout (GnRHs: GenBank AAB82559/AAD43452; GnRH-Rs: CAB93351), medaka (GnRHs: GenBank BAC06421/BAC06417/BAC06425; GnRH-Rs: BAB70504/BAB70503/BAC97833) and grass pufferfish (*Takifugu niphobles*) (GnRHs: GenBank AAA63214/BAJ07189/BAJ07190) (Table 3.1) were used to design degenerate primers for amplifying cDNA fragments of sbGnRH, cGnRH and sGnRH, as well as the receptors, GnRH-R1 and GnRH-R2. In order to obtain the initial amplicon, a 25 µl polymerase chain reaction (PCR) was set up using 6X Go Taq Flexi Buffer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 0.2 µM of each primer 1 U Go Flexi Taq Polymerase (Promega, Madison, Wisconsin, USA) and 2.5 µl of cDNA corresponding to 1 µg of

Table 3.1. Sequences of primers used in the gonadotropin-releasing hormone (GnRH) study.

Gene	Forward primer / RACE primer 1	Reverse primer/ RACE primer 2
Degenerate primers		
sbGnRH	5′- GCACTGGTCVTATGGACTGA -3′	5′- CTGYCAAKRAATCCTTTCATTCT -3′
cGnRH	5′- CACTGGTCCCAYGGBTGGTA -3′	5′- CTCTGGGGTCTCDAGTAGCTG -3′
sGnRH	5′- AGGTGKTGWTGTTGGCGTTG -3′	5′- CTCTCTKGGRTHTGGGCACT -3′
GnRH-R1	5′- TGCTGGTGACTTTCATCGTG -3′	5′- CYTTVGGGATGTTGYTGT -3′
GnRH-R2	5′- TGCTGGTGACTTTCATCGTG -3′	5′- CYTTVGGGATGTTGYTGT -3′
3′ RACE primers		
cGnRH	5′- AAGAGGGAGCTGGACTCGTTTG -3′	5′- TTTGGCGCATCAGAGATTTCAGAGG -3′
sGnRH	5′- TGGGAGCCATCCATAGGA -3′	5′- GGACCAGTGCTGGGACAG G -3′
GnRH-R1	5′- CTTGTGTGAGATCTCCAAACG -3′	5′- GAGATCTCCAAACGACTGTACACGG -3′
5′ RACE primers		
sbGnRH – specific	5′- GTTTGAGAAAGACTGTCCAG -3′	
cGnRH – specific	5′- TCTTGCCTCCCGGGTACCAA -3′	
GnRH-R1– specific	5′- AACTTCCACGGGTCGTGCACTG -3′	
GnRH-R2 – specific	5′- GGTCCTGAAGATGAAGAGTTGTG G -3′	
sbGnRH	5′- GGAAATCCTCAACTACATTGCCC -3′	5′- GGTGAGTCCACATGAGGGAAAT -3′
cGnRH	5′- CCAAACGAGTCCCAGCTCCCTC -3′	5′- AAATTCTGATGCGCCAAACG -3′
GnRH-R1	5′- ATGACTCTGTTCCTCTTTCTGG -3′	5′- CCTCTTTCTGGCCTCGTTGATAGC -3′
GnRH-R2	5′- CTACGATGAGCACTCAGAGC -3′	5′- ACTCAGAGCATCCAGCGGGTGC -3′
Specific primers for RT-PCR		
cGnRH	5′- GTACCCGGGAGGCAAGAG -3′	5′- TCAGAGGATCAACCTAAAGTTTCAC -3′
sGnRH	5′- CCCAAGCCCAAGAGAGACC -3′	5′- CCCAACACTGATGAAAATGC -3′

Primers for internal control of RT-PCR		
EF-1α	5′-CCTGGACACAGGGACTTCAT-3′	5′-CGGTGTTGTCCATCTTGTTG-3′
Specific primers for qPCR		
sbGnRH	5′- CATGTGGACTCACCTTGCAG -3′	5′- GATTCATCCAAAACCGAACAC -3′
cGnRH	5′-CAGAGGGAGATAAAGCTGTGTG -3′	5′- GTGCATTGTGGGAAACTGTC -3′
sGnRH	5′- CTCAGGAAGAGACACCACTCC -3′	5′- CCTGTCCCAGCACTGGTC -3′
EF-1α	5′- CCTGGACACAGGGACTTCAT -3′	5′- CGGTGTTGTCCATCTTGTTG -3′
Adapter primers		
dT-AP	5′- GGCCACGCGTCGACTAGTAC(T17) -3′	
AP	5′- GGCCACGCGTCGACTAGTAC -3′	

* Degenerate bases: W (A or T), S (C or G), M (A or C), K (G or T), R (A or G), Y (C or T), B (not A), D (not C), H (not G), V (not T) and N (any base)

initial RNA. An initial 5 min denaturation at 95°C, followed by 30 cycles of: 30 s denaturation at 95°C, 30 s annealing with temperatures ranging from 48 to 62°C and a 1 to 2 min extension at 72°C, with a final extension of 5 min at 95°C using the Eppendorf Mastercycler 5333 and Mastercycler personal 5332 for all PCR reactions. In all PCRs, a no-template control was used for each primer set by excluding cDNA from the reaction. Products were electrophoresed in a 1.25% ethidium bromide stained agarose gel using 1X TAE buffer and bands were excised and purified with the GeneJET™ Gel Extraction Kit (Fermentas, Burlington, Ontario, Canada) according to manufacturer's instructions, ligated in the pGEM easy vector (Promega; Madison, Wisconsin, USA) transformed into JM109 competent cells, according to manufacturer's instructions, grown on ampicillin and X-gal treated agar plates, white colonies were grown further followed by minipreps of selected colonies using GeneJET™ Plasmid Miniprep Kit using the manufacturer's protocol (Fermentas, Burlington, Ontario, Canada). A 10 µl EcoR1 (Promega; Madison, Wisconsin, USA) digestion was set-up using 1 µl 1X buffer H, 0.5 µl EcoRI restriction enzyme and 3 µl DNA and incubated at 37°C for 1h for each miniprep to determine if the plasmid contained an insert. Plasmid DNA containing inserts were then sequenced by The Centre for Applied Genomics lab (TCAG; The Hospital for Sick Children, Toronto, Ontario, Canada).

Once the initial cDNA fragments were isolated and sequenced, a 3′ Rapid Amplification of cDNA Ends (RACE) was completed with two rounds of nested PCR using gene- specific primers and adaptor primers, where the first round of amplification included the RACE primer 1 and dtAP and the second used RACE primer 2 and AP

(Table 3.1). A 25 µl PCR was set up using 6X Go Taq Flexi Buffer, 0.2 mM of each dNTP, 2-3 mM $MgCl_2$, 0.2 µM of each primer 1 U Go Flexi Taq Polymerase (Promega, Madison, Wisconsin, USA) and 2.5 µl of cDNA corresponding to 1 µg of initial RNA. An initial 5 min denaturation at 95°C, followed by 30 cycles of: 30 s denaturation at 95°C, 30 s annealing with temperatures ranging from 48 to 62°C and a 1 to 2 min extension at 72°C, with a final extension of 5 min at 95°C using the eppendorf Mastercycler 5333 and Mastercycler personal 5332 for all PCR reactions. A 5′RACE was further completed with a reverse transcriptase (RT) -PCR using gene-specific primers (Table 3.1). cDNA was then purified using Montage PCR Millipore kit (Bedford, Massachusetts, USA) according to manufacturer's instructions and polyA-tailed using 6 µl cDNA, 2.5 µl 10X tailing buffer, 1 µl 5mM dATP which was incubated for 3 min at 94°C and 1 µl of Terminal Deoxynucleotidyl Transferase was added (Invitrogen, Burlington, Ontario, Canada). A final incubation of 37°C for 10 min and 65°C for 10 min was completed. The tailed cDNA was then amplified with two rounds of nested PCR with gene-specific primers and adaptor primers, where the first round of amplification included the RACE primer 1 and dtAP and the second used RACE primer 2 and AP (Table 3.1). A 25 µl PCR was set up using 6X Go Taq Flexi Buffer, 0.2 mM of each dNTP, 2-3 mM $MgCl_2$, 0.2 µM of each primer 1 U Go Flexi Taq Polymerase (Promega, Madison, Wisconsin, USA) and 2.5 µl of cDNA corresponding to 1 µg of initial RNA. An initial 5 min denaturation at 95°C, followed by 30 cycles of: 30 s denaturation at 95°C, 30 s annealing with temperatures ranging from 48 to 62°C and a 1 to 2 min extension at 72°C, with a final extension of 5 min at 95°C using the eppendorf Mastercycler 5333 and Mastercycler personal 5332 for all PCR reactions.. For both the 3′

and 5′RACE, PCR products were run on 1.25% stained with ethidium bromide agarose gels and run with 1X TAE buffer, visualized and bands were excised, as previously described. These bands were cloned and sequenced as described previously.

3.2.5. Distribution of prepro-GnRHs and GnRH-receptors cDNA expression in flounder brain regions and peripheral tissues

Gene-specific primers were designed based on the sequence information previously obtained. cDNAs were synthesized from the peripheral and central nervous system tissues based on the procedures described above. A 25 µl PCR reaction was used containing 10X GoTaq Master Mix (Promega; Madison, Wisconsin, USA), 0.2 µM of each primer and 2.5 µl cDNA (Table 3.1). Negative no-template controls were included in all PCRs, where an initial 5 min denaturation at 95°C, followed by 30 cycles of: 30 s denaturation at 95°C, 30 s annealing with temperatures ranging from 48 to 62°C and a 1 to 2 min extension at 72°C, with a final extension of 5 min at 95°C using the eppendorf Mastercycler 5333 and Mastercycler personal 5332 for all reactions. Products were electrophoresed in a 1.25% agarose gel stained with ethidium bromide and run using 1X TAE buffer and visualized using the Epichemi Darkroom Bioimaging System (UVP, Upland, California, USA) equipped with a 12-bit cooled camera. Image processing and analysis were performed using LabWorks 4.0 software (UVP, Upland, California, USA). The control gene used was winter flounder elongation factor-1α (EF-1α) (GenBank AW013637).

3.2.6. Changes in prepro-GnRHs and GnRH-receptors cDNA expression in flounder brain during varied nutritional states

Following reverse-transcription of mRNA into cDNA (section 3.2.3), products were diluted 1:2 in nuclease-free water (Qiagen, Mississauga, Ontario, Canada) and quantitative RT-PCR (qPCR) was completed using primers specific to the winter flounder genes of interest (Table 3.1). Although the RT protocol included a DNase step, at least one primer was designed across an exon-exon junction to further avoid the risk of amplifying genomic DNA. Primers were designed to have melting temperatures that were approximately the same and result in amplicon sizes between 75-130 base pairs (bp). An automated pipetting system (epMotion® 5070, Eppendorf) was used to set up all PCRs. The final volume was 10 µl containing 2 µl of cDNA from 1 µg of RNA, 1 µM of each sense and antisense primer and 5 µl of QuantiFast SYBR Green PCR kit master mix (Qiagen; Mississauga, Ontario, Canada). Samples were run in duplicates on 96-well plates using the Mastercycler® ep realplex 2S system (Eppendorf; Hamburg, Germany, Europe). A "no template" negative control where cDNAs were replaced by water was included on each plate. Primer optimization PCRs were conducted to ensure primer specificity ($0.9 > R^2 > 1.0$) using a 5 point standard curve for each primer pair to make certain that amplifications with each primer pair had similar efficiencies (EF = 1.07; sbGnRH = 1.02; cGnRH = 0.96; sGnRH = 1.07). The Realplex1.5 software (Eppendorf; Hamburg, Germany, Europe) was used for amplification, dissociation curves and mRNA expression analyses. The relative cycle threshold (Ct; ΔΔCt) method was used to quantify expression. Fold-change in expression of the target gene was normalized to the

housekeeping gene (EF1-α) and were compared with the calibrator sample from the control group (a single fed fish). The average fold mRNA expression from the control group was set to 100% and expression of genes in the fasted groups was relative to EF1-α using the following formula: (relative quantification of fed or fasted fish*100)/average relative quantifications of fed fish. Verification that the housekeeping gene was not affected by feeding regimen in the brain was demonstrated by similar Ct values between fed and starved fish.

2.2.7. Sequence analysis

DNA sequences and deduced amino acid sequences were analyzed using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/blast) from the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). The signal peptide was predicted with the program SignalP 3.0 (www.cbs.dtu.dk/services/SignalP/). Multiple alignments were performed using Multiple Accurate and Fast Sequence Comparison by Log-Expectation (MUSCLE) tool with CLUSTAL2 (strict) output (Edgar 2004). Neighbour-joining phylogenetic analyses (1000 bootstrap iterations) were completed using Molecular Evolutionary Genetic Analysis 4.0.2 (MEGA; Tamura *et al.* 2007) based on a Poisson distance matrix of the MUSCLE alignments.

2.2.8. Data analysis and statistics

Expression levels were described as a percentage relative to the control (fed) group (100%). Two-tailed Students t-tests were used to determine significance between fed and fasted groups in GraphPad Prism 5 (GraphPad Software, San Diego, California, USA). Significance was set at $p < 0.05$.

3.3. Results

3.3.1. Sequence analysis of winter flounder GnRHs and GnRH-receptor isoforms

A 168 base pair (bp) partial cDNA sequence for sbGnRH contains a 17 bp 5′ untranslated region (UTR) (Figure 3.1). The nucleotide sequence encodes an 85 amino acid (aa) peptide including a 22 aa signal peptide, the sbGnRH mature decapeptide and a partial 49 aa GnRH-associated peptide (GAP). Compared with cGnRH and sGnRH, the mature peptide is much less conserved, with multiple substitutions in positions 5 (histidine, tyrosine and phenylalanine), 7 (leucine and methionine), and 8 (asparginine, serine and arginine) (Table 3.2). The amino acid identity ranges from 40-91% in fishes (Figure 3.2).

The full winter flounder cGnRH nucleotide sequence is 610 bp with 135 bp 5' and 216 bp 3′ UTRs, respectively (Figure 3.3). The protein sequence is 87 aa and codes for a 23 aa signalling peptide, a 10 aa mature hormone, and a 52 aa GAP. Overall, the

Figure 3.1. Annotated winter flounder sbGnRH partial cDNA sequence with nucleotides and deduced amino acids. Bolded underline indicates signal peptide, double underline is mature peptide, single underline is GnRH associated peptide and letters in lower case represent the 5′ untranslated region.

Table 3.2. Common amino acid substitutions for each of the gonadotropin-releasing hormone (GnRH) mature peptides compared with winter flounder, *Pseudopleuronectes americanus*. Degenerate bases: W (A or T), S (C or G), M (A or C), K (G or T), R (A or G), Y (C or T), B (not A), D (not C), H (not G), V (not T) and N (any base)

Winter flounder:										
Seabream-GnRH	Q	H	W	S	**Y**	G	**L**	**S**	P	G
Chicken-GnRH	Q	H	W	S	**H**	G	**W**	**Y**	P	G
Salmon-GnRH	Q	H	W	S	**Y**	G	**W**	**L**	P	G
Other teleosts:										
GnRH	Q	H	W	S	**H/Y/ F**	G	**L/ M**	**N/ R/S**	P	G
Chicken-GnRH	Q	H	W	S	**H**	G	**W**	**Y**	P	G
Salmon-GnRH	Q	H	W	S	**Y**	G	**W**	**L**	P	G
Birds and Mammals:										
Human GnRH	Q	H	W	S	**Y**	G	**L**	**R**	P	G
Rat GnRH	Q	H	W	S	**Y**	G	**L**	**R**	P	G
Frog GnRH	Q	H	W	S	**Y**	G	**L**	**W**	P	G
Chicken GnRH-I	Q	H	W	S	**Y**	G	**L**	**Q**	P	G
Chicken GnRH	Q	H	W	S	**H**	G	**W**	**Y**	P	G

Figure 3.2. Protein alignment for sbGnRH, cGnRH and sGnRH used for detecting phylogenetic relationships, including catfish, (*Clarias gariepinus,* GenBank CAA54971/CAA54969), European eel (*Anguilla anguilla,* GenBank ADD92012/ADD92005), whitefish (*Coregonus clupeaformis*, GenBank AAP57221/AAP57219/AAP57220), grass puffer (*Takifugu niphobles,* GenBank AAA63214/BAJ07189/BAJ07190), medaka (*Oryzias latipes,* GenBank BAC06421/BAC06417/BAC06425), rock gunnel (*Fundulus heteroclitus,* GenBank BAF57234/BAF96396/BAF95685), gilthead seabream (*Sparus aurata,* GenBank AAA75469/AAA75447), goldlined seabream (*Rhabdosargus sarba*, GenBank ABS50339/ABS50340/ABS50341), red seabream (*Pagrus major*, GenBank BAA13129 /BAA05104), black porgy (*Acanthopagrus schlegelii,* GenBank ABU92553/ABU92552/ABV03808), Burton's mouthbreeder (*Haplochromis burtoni,* GenBank AAC27716/AAC27717/AAC27718), Nile tilapia (*Oreochromis niloticus,* GenBank BAC56849/BAC56850/BAC56851), European seabass (*Dicentrarchus labrax,* GenBank AAF62898/AAF62900/AAF62899), Asian swamp eel (*Monopterus albus*, GenBank AAW51121/AAV41875/AAW51120), winter flounder (*Pseudopleuronectes americanus,* GenBank HQ623431/HQ623429/HQ623432), zebrafish (*Danio rerio*, GenBank AAM43951/AAL99294), goldfish (*Carassius auratus*, GenBank AAB86989/BAB18904), Atlantic cod (*Gadus morhua,* GenBank ADD92006/ADD92007) common carp (*Cyprinus carpio,* GenBank AAO39753/AAO39975), rainbow trout (*Oncorhynchus mykiss*, GenBank AAB82559/AAD43452), Atlantic salmon (*Salmo salar*, GenBank ACI68188/CAA52912), Japanese flounder (*Paralichthys olivaceus*, GenBank

AAY28981/ACS88343). Winter flounder sequences are bolded. The signal peptide is indicated by the bolded underline, the mature GnRH protein by the double underline. * indicates that all amino acids are identical in the column, : demonstrates that conserved substitutions have been observed and . specifies that semi-conserved substitutions have been observed.

```
Zebrafish sGnRH                 1  M----EWKGRLLVQLLL---LVCVLEVS--LCQHWSYGWL  30
Goldfish sGnRH                  1  M----EWNGRLLVQLLM---LVCVLEVS--LCQHWSYGWL  30
Common carp sGnRH               1  M----EGKGRVLVQLLM---LVCVLEVS--LCQHWSYGWL  30
Atlantic cod sGnRH              1  M----ESSSKVTVQVFF---LLLVVQVT--LTQHWSYGWL  30
Rainbow trout sGnRH             1  M----DLSSKTFVQVVM---LALIAQVT--FSQHWSYGWL  30
Whitefish sGnRH                 1  M----DLSSRTVVQVVV---LVLVAQVT--LSQHWSYGWL  30
Atlantic salmon sGnRH           1  M----DLSNRTVVQVVV---LALVAQVT--LSQHWSYGWL  30
Grass pufferfish sGnRH          1  M----EANSRVMVQVLL---LALALQVT--LSQHWSYGWL  30
Medaka sGnRH                    1  M----DVSSKVVVQVLL---LALVVQVT--LCQHWSYGWL  30
Winter flounder sGnRH           1  ------------------LALVVRVA--LSQHWSYGWL  18
Rock gunnel sGnRH               1  M----KASNRVMVQVLL---LALLAQVT--LCQHWSYGWL  30
Asian swamp eel sGnRH           1  M----EASSRVTVQVLL---LALVVQVT--LSQHWSYGWL  30
Burton's mouthfeeder sGnRH      1  M----EAGSRVIMQVLL---LALVVQVT--LSQHWSYGWL  30
Nile tilapia sGnRH              1  M----EAGSRVIMQVLL---LALVVQVT--LSQHWSYGWL  30
European seabass sGnRH          1  M----EANSRVMVRVLL---LALVVQVT--LSQHWSYGWL  30
Black porgy sGnRH               1  M----EASSRVTVQVLL---LALVVQVT--LSQHWSYGWL  30
Goldlined seabream sGnRH        1  M----EASSRVTVQVLL---LALVVQVT--LSQHWSYGWL  30
Red seabream sGnRH              1  M----EASSRVTVQVLL---LALVVQVT--LSQHWSYGWL  30

European eel cGnRH              1  M---VNTGRLVLILGVL---LCLGAQLS--LCQHWSHGWY  32
Zebrafish cGnRH                 1  M---VLVCRLLLVMGLM---LCLSAQLS--SAQHWSHGWY  32
Goldfish cGnRH                  1  M---VHICRLFVVMGML---MFLSVQFA--SSQHWSHGWY  32
Common carp cGnRH               1  M---VHICRLFVVMGML---LCLSAQFA--SSQHWSHGWY  32
European catfish cGnRH          1  M---VSVCRLLLVAALL---LCLQAQLS--FSQHWSHGWY  32
Atlantic cod cGnRH              1  M---MYVSRLVLLLGLL---LCLGVQLS--TAQHWSHGWY  32
Rainbow trout cGnRH             1  M---VSVARLVFMLGPL---LCLGAQLS--SSQHWSHGWY  32
Whitefish cGnRH                 1  M---VSVARLVFMLGLL---LCLGAQLS--SSQHWSHGWY  32
Atlantic salmon cGnRH           1  M---VSVARLVLMLGLL---LCLGAQLS--SSQHWSHGWY  32
Rock gunnel cGnRH               1  M------SRLVLLLVLF---VCVGAQLC--YAQHWSHGWY  29
Grass pufferfish cGnRH          1  M----RVPGLLLLGLL---LCAGAHLS--NGQHWSHGWY  31
Oryzias latipes cGnRH           1  M------SRLVLLLGVL---LYVGAQLS--QAQHWSHGWY  29
Asian swamp eel cGnRH           1  M------IRLVLLLGLL---LCVGVQLS--GAQHWSHGWY  29
Winter flounder cGnRH           1  M----CASRLVLLLGLL---LCVGAQLS--SGQHWSHGWY  31
Japanese flounder cGnRH         1  M----CASRLVLLLGLL---LCVGAQLS--SGQHWSHGWY  31
Burton's mouthfeeder cGnRH      1  M----CVSRLALLLGLL---LCVGAQLS--FAQHWSHGWY  31
Nile tilapia cGnRH              1  M----CVSRLALLLGLL---LCVGAQLS--FAQHWSHGWY  31
European seabass cGnRH          1  M----CVSRLVLLFGLL---LCVGAQLS--NAQHWSHGWY  31
Gilthead seabream cGnRH         1  M----CVSRLVLLLGLL---LCVGAQLS--NGQHWSHGWY  31
Goldlined seabream cGnRH        1  M----CVSRLVLLLGLL---LCVGAQLS--NGQHWSHGWY  31
Black porgy cGnRH               1  M----CVSRLVLLLGLL---LCVGAQLS--NGQHWSHGWY  31

European catfish cfGnRH         1  M----GIKRALWWMV-----VCVVVLQV--SAQHWSHGLN  29
European eel sbGnRH             1  M----ADKSALLWLGLA---VALVCQ-G--CCQHWSYGLR  30
Whitefish wfGnRH                1  M----EEKKVLLLLLLL---VVALVSQG--CCQHWSYGMN  31
Grass pufferfish sbGnRH         1  M----ATASFSLWLLLL---GSLMSQL---CCQHWSYGLS  30
Medaka mfGnRH                   1  M----VVKTWMPWLL-----VSSVLSQG--CCQHWSYGLS  29
Rock gunnel sbGnRH              1  M----AVKTLSLWLLLA--WTVGLLSLG--SCQHWSYGLS  32
Gilthead seabream sbGnRH        1  M----APQTSNLWILLL---LVVVMMMSQGCCQHWSYGLS  33
Goldlined seabream sbGnRH       1  M----APQTSNLWMLLLVVVVVMMMSQG--CCQHWSYGLS  34
Red seabream sbGnRH             1  M----APQTSNLWLLLV---VMMVMSQG--CCQHWSYGLS  31
Black porgy sbGnRH              1  M----APQTSNLWMLLLLVVMMMMMSRG--CCQHWSYGLS  34
Burton's mouthfeeder sbGnRH     1  M----AAKILALWLLL----AGTVFPQG--CCQHWSYGLS  30
Nile tilapia sbGnRH             1  M----AAKILALWLLL----AGTVFPQG--CCQHWSYGLS  30
European seabass sbGnRH         1  M----AAQTFALRLLLVGTLLGTLLGQG--CCQHWSYGLS  34
Asian swamp eel pjGnRH          1  MHRRMTVKTMALWLLLV---GTLVPQL---YCQHWSFGLS  35
Japanese flounder sbGnRH        1  MHREMAVKTLAVWLLL----VGMLVPQH--SCQHWSYGLS  35
Winter flounder sbGnRH          1  M----AVRTLSVWLLLV----GTLVPQH--CCQHWSYGLS  30
                                   *                        **** .*
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Zebrafish sGnRH                31  PGGKRSVGEMEATFR-----MLDPGDTVLSIPADSPME--  63
Goldfish sGnRH                 31  PGGKRSVGEVEATFK-----MMDAGDAVLSIPADSPME--  63
Common carp sGnRH              31  PGGKRSVGEVEATFR-----MMDAGDAVLSIPADSPME--  63
Atlantic cod sGnRH             31  PGGKRSVGELEATIR-----MMGTGG-EVPLMEDPRDPAL  63
Rainbow trout sGnRH            31  PGGKRSVGELEATIR-----MMDTGG-VMALPEETGAHIP  63
Whitefish sGnRH                31  PGGKRSVGELEATIR-----MMDTGG-EVALPEETSAHVS  63
Atlantic salmon sGnRH          31  PGGKRSVGELEATIK-----MMDTGG-VVALPEETSAHVS  63
Grass pufferfish sGnRH         31  PGGKRSVGELEATIR-----MMGTGG-VVSLPDEESAQTQ  63
Medaka sGnRH                   31  PGGKRSVGELEATIR-----MMGTGR-VVSLPEDASAQTQ  63
Winter flounder sGnRH          19  PGGKRSVGELEATIR-----MMGTGG-VVSLPEEASAQTQ  51
Rock gunnel sGnRH              31  PGGKRSVGELEATIR-----MMGTGG-VVSLPEEASAQTQ  63
Asian swamp eel sGnRH          31  PGGKRSVGELEATIR-----MIGTGG-VVTLPDEASAPIQ  63
Burton's mouthfeeder sGnRH     31  PGGKRSVGELEATIR-----MMGTGG-VVSLPDEANAQIQ  63
Nile tilapia sGnRH             31  PGGKRSVGELEATIR-----MMGTGG-VVSLPDEANAQTQ  63
European seabass sGnRH         31  PGGKRSVGELEATIR-----MMGTGE-VVSLPEEASAQTQ  63
Black porgy sGnRH              31  PGGKRSVGELEATIR-----MMGTGG-VVSLPEEASAQTQ  63
Goldlined seabream sGnRH       31  PGGKRSVGELEATIR-----MMGTGG-VVSLPEEASAQTQ  63
Red seabream sGnRH             31  PGGKRSVGELEATIR-----MMGTGG-VVSLPEEASAQTQ  63

European eel cGnRH             33  PGGKRELDSLTTAE------VLDEIK-----LCDGGECS-  60
Zebrafish cGnRH                33  PGGKREIDLYDTSE------VSEEVK-----LCEAGKCS-  60
Goldfish cGnRH                 33  PGGKREIDVYDPSE------VSEEIK-----LCNAGKCS-  60
Common carp cGnRH              33  PGGKREIDVYDTSE------VSEEIK-----LCEAGKCS-  60
European catfish cGnRH         33  PGGKREIDSYSSPE------ISGEIK-----LCEAGECS-  60
Atlantic cod cGnRH             33  PGGKRELEAFTTSE------VSDEIK-----LCEAGECS-  60
Rainbow trout cGnRH            33  PGGKRELDSFTTSE------ISEGIK-----LCEAEGCS-  60
Whitefish cGnRH                33  PGGKRELDSFTTSE------ISEGIK-----LCEAEGCS-  60
Atlantic salmon cGnRH          33  PGGKRELDSFTTSE------ISEEIK-----LCEAGECS-  60
Rock gunnel cGnRH              30  PGGKRELDSFGASE------MSEEVK-----LCEAGECS-  57
Grass pufferfish cGnRH         32  PGGKRELDPFNPSE------ISEEIK-----LCEAGECS-  59
Medaka cGnRH                   30  PGGKRELDSFE---------VSEEMK-----LCETGECS-  54
Asian swamp eel cGnRH          30  PGGKRELDSFPTSE------ISGEIN-----LCEAGECS-  57
Winter flounder cGnRH          32  PGGKRELDSFGASE------ISEEIK-----LCEAGECS-  57
Japanese flounder cGnRH        32  PGGKRELDSFGTSE------ISEEIK-----LCDAGECS-  59
Burton's mouthfeeder cGnRH     32  PGGKRELDSFGTSE------ISEEIK-----LCEAGECS-  59
Nile tilapia cGnRH             32  PGGKRELDSFGTSE------ISEEIK-----LCEAGECS-  59
European seabass cGnRH         32  PGGKRELDSFGTSE------ISEEIK-----LCEAGECS-  59
Gilthead seabream cGnRH        32  PGGKRELDSFGTSE------ISEEIK-----LCEAGECS-  59
Goldlined seabream cGnRH       32  PGGKRELDSFGTSE------ISEEIK-----LCEAGECS-  59
Black porgy cGnRH              32  PGGKRELDSFGTSE------ISEEIK-----LCEAGECS-  59

European catfish cfGnRH        30  PGGKRAVMQESAEE------IPRRSG----YLCDYVAVSP  59
European eel sbGnRH            31  PGGKRGADSLQDTLQD----IIEELQ-KLD-TSSLPSCND  64
Whitefish wfGnRH               32  PGGKRATGSLSDTQDN----MAEDLL-KIDPSCSLFGCAD  66
Grass pufferfish sbGnRH        31  PGGKRDVDHLSDSVDT----VVVSVP-HAETSSALLGCAE  65
Medaka mfGnRH                  30  PGGKRELKYFPNTLENQIR-LLNSNT-----PCSDLSHLE  63
Rock gunnel sbGnRH             33  PGGKRELDVSPDRLDS----IFEGLA-HVGAPCSVPGCAE  67
Gilthead seabream sbGnRH       34  PGGKRDLDSLSDTLGN----IIERFP-HVDSPCSVLGCVE  68
Goldlined seabream sbGnRH      35  PGGKRDLDGLSDTLGN----IIERFP-HVDSPSSVLSCAE  69
Red seabream                   32  PGGKRDLDSLSDTLGD----IIERFP-HADSPCSVLGCAE  66
Black porgy sbGnRH             35  PGGKRDLDSLSDTLGN----IIERFP-HVDSPCSVLGCAE  69
Burton's mouthfeeder sbGnRH    31  PGGKRDLDNFSDTLGN----MVEEFP-RVEAPCSVFGCAE  65
Nile tilapia sbGnRH            31  PGGKRDLDNFSDTLGN----MVEEFP-RVEAPCSVFGCAE  65
European seabass sbGnRH        35  PGGKRELDGLSETLGNQ---IVGSFP-HVATPCRVLGCAE  70
Asian swamp eel pjGnRH         36  PGGKRELNSLSDTLGN----IVEGFP-HVDAPCSILRCAE  69
Japanese flounder sbGnRH       36  PGGKRELDSLSQSLGN----VVEEFP-PVDPPCSVLGGAE  69
Winter flounder sbGnRH         31  PGGKRELDSLSQTLGNVLQQVVEDFP-HVDSPCSVQGGAE  69
                                   *****                :
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Zebrafish sGnRH                64  ----QLSPIHIVNEVDAEGLPLKGQRYSDRRGRV  94
Goldfish sGnRH                 64  ----QLLPIHIVNEVDADGLPLKEQRFPKRRGRV  94
Common carp sGnRH              64  ----QLSPIHIVNEVDAEGLPLKELRFPKRRGRV  94
Atlantic cod sGnRH             64  E---RLRPYSLVNDEAVRFQ--KKKRLLHD----  88
Rainbow trout sGnRH            64  E---RLRPYDVMS----------KKRMPHK----  80
Whitefish sGnRH                64  E---RLRPYDVIS----------KKWMPHK----  80
Atlantic salmon sGnRH          64  E---RLRPYDVIL----------KKWMPHK----  80
Grass pufferfish sGnRH         64  E---RLRPYNVISEELNRFD--PKRRIQNN----  88
Medaka sGnRH                   64  E---RLRQYNLINDGSTYFD--RKKRFMSQ----  88
Winter flounder sGnRH          52  E---RPRPYNVIDDGPRHFH--RKKRLPDN----  76
Rock gunnel sGnRH              64  E---RLRPYHIND-GSSHFD--RNKRFLNE----  87
Asian swamp eel sGnRH          64  Q---RLRPYNVINDDSSHFD--QRKRFSNK----  88
Burton's mouthfeeder sGnRH     64  E---RLRPYNIINDDSSHFD--RKKRFPNN----  88
Nile tilapia sGnRH             64  E---RLRPYNIINDDSSHFD--RKKRFPNN----  88
European seabass sGnRH         64  E---RLRPYNVINDDSSHFD--RKKRSPNK----  88
Black porgy sGnRH              64  E---RLRPYNVIKDDSSPFD--RKKRFPNK----  88
Goldlined seabream sGnRH       64  E---RLRPYNVIKDDSSPFD--RKKRFPNK----  88
Red seabream sGnRH             64  E---RLRPYNVIKDDSSHFD--RKKRFPNK----  88

European eel cGnRH             61  ----YLRPQRKSLLKNILLDALAREFQRKRK---  87
Zebrafish cGnRH                61  ----YLRPQGRNILKTILLDALIRDFQKRK----  86
Goldfish cGnRH                 61  ----FLIPQGRNILKTILLDALTRDFQKRK----  86
Common carp cGnRH              61  ----YLRPQGRNILKTILLDALIRDFQKRK----  86
European catfish cGnRH         61  ----YLRPLRTNILKSILIDTLARKFQKRK----  86
Atlantic cod cGnRH             61  ----YLRPQHRGTLRNIFMDVLARELQKRK----  86
Rainbow trout cGnRH            61  ----YLRPQRRNILKNVILDALAREFQKRK----  86
Whitefish cGnRH                61  ----YLRPQRRNILKNILLDALAREFEKRK----  86
Atlantic salmon cGnRH          61  ----YLRPQRRNILRNILLDALAREFEKIK----  86
Rock gunnel cGnRH              58  ----YLRPQRRSTLRNIVLDALARELQKRK----  83
Grass pufferfish cGnRH         60  ----YLRPQRRNVLRNFLLDALTRELQKRK----  85
Medaka cGnRH                   55  ----YMRPQRRSFLRNIVLDALARELQKRK----  80
Asian swamp eel cGnRH          58  ----YLRPQRRSILRSILLNVLARELQKRK----  83
Winter flounder cGnRH          58  ----YLRPQRRNILRNILLDALARELQKRK----  83
Japanese flounder cGnRH        60  ----YLRPQRRNILRNILLDALARELQKRK----  85
Burton's mouthfeeder cGnRH     60  ----YLRPQRRSILRNILLDALARELQKRK----  85
Nile tilapia cGnRH             60  ----YLRPQRRSILRNILLDALARELQKRK----  85
European seabass cGnRH         60  ----YLRPQRRSVLRNIILDALARELQKRK----  85
Gilthead seabream cGnRH        60  ----YLTPQRRSVLRNI-----------------  73
Goldlined seabream cGnRH       60  ----YLRPQRRSVLRNIFLDALARELQKRK----  85
Black porgy cGnRH              60  ----YLRPQRRSVLRNILLDALARELQKRK----  85

European catfish cfGnRH        60  ----RNKPFRLKDLLTPVAGREIEE---------  80
European eel sbGnRH            65  L-SPHITLSSLKEILANLAD--RETGRKNI----  91
Whitefish wfGnRH               67  V-SPHAKMYRLRALLASLAD--RQSGLNNI----  93
Grass pufferfish sbGnRH        66  NVAAFSRIFGIKEILNSRTDRRAKRFQTSNK---  96
Medaka mfGnRH                  64  E-SSLAKIYRIKGLLGSVTEAKNGYRTYK-----  91
Rock gunnel sbGnRH             68  E-SPFAKIHRLKGLLVRVHEREHGHQALKQ----  96
Gilthead seabream sbGnRH       69  E-PHVPRMYRMKGFIGSERD--IGHRMYKK----  95
Goldlined seabream sbGnRH      70  E-PHVPKMYRMKGFIGGERD--NGHRTNKK----  96
Red seabream sbGnRH            67  E-PPFPKMYRMKGFIGSGTDRDNGHRTYKK----  95
Black porgy sbGnRH             70  E-PHFPKMYRMKGFIGSDRD--NGHRTYKK----  96
Burton's mouthfeeder sbGnRH    66  E-SPFAKMYRVKGLLASVAERENGHRTFKK----  94
Nile tilapia sbGnRH            66  E-SPFAKMYRVKGLLASLAEGKTDTGHSRNERFL  98
European seabass sbGnRH        71  E-SPFPKIYRMKGFLDAVTDRENGNRTYKK----  99
Asian swamp eel pjGnRH         70  E-LPFASIYRMKGFLDGVTDTENGHRTYKK----  98
Japanese flounder sbGnRH       70  E-SPLAGIYRMKGFLGSIPDRGNGRRTYKK----  98
Winter flounder sbGnRH         70  E-SPFAGIYRMKGFL------------------  83
```

Figure 3.3. Annotated winter flounder cGnRH cDNA sequence with nucleotides and deduced amino acids. Bolded underline indicates signal peptide, double underline is mature peptide, single underline is GnRH associated peptide and letters in lower case represents the 5′ and 3′ untranslated regions.

mature peptide is highly conserved across teleosts with histidine (H), tryptophan (W) and tyrosine (Y) substitutions occurring in the 5, 7, and 8 positions, respectively (Table 3.2). Conservation between winter flounder cGnRH and other fishes range from 70-98%, where the highest amino acid sequence similarities are with other flatfish, including the Japanese (98%), Brazilian (*Paralichthys orbignyanus*) (98%) and barfin (98%) flounder (Figure 3.2).

A partial cDNA sequence for sGnRH is 394 bp, and contains a 160 bp 3′ UTR (Figure 3.4). The deduced partial aa sequence is 78 aa with a partial sequence for the signalling factor (10 aa) and full sequences for the mature decapeptide and GAP protein (58 aa). Like cGnRH, very high conservation of the mature winter flounder sGnRH decapeptide is seen amongst teleosts with changes occurring in the 5 (aa: Y), 7 (aa: W) and 8 [leucine (L)] positions (Table 3.2). Amino acid sequence identities range from 64-96% compared with other teleosts (Figure 3.2). No similarities are observed with other vertebrates, since sGnRH is a fish-specific form.

GnRH-R1 is 1721 bp with a 195 bp 5' and a 264 bp 3′ UTR, respectively (Figure 3.5). The mRNA encodes a 410 aa peptide sequence and the extracellular loop 3 motif is SQS, consistent with the GnRH-R2b classification. Determining conservation of amino acid sequence identities is controversial in that the receptor nomenclature is not well categorized. However, winter flounder GnRH-R1 is most highly similar to Japanese flounder GnRH-R1 (89%) (Figure 3.6).

The partial GnRH-R2 nucleotide sequence is 466 bp and encodes a 233 aa protein (Figure 3.7). It is expected that this receptor belongs to the GnRH-Ra1 category, however more sequence needs to be obtained in order to confirm this hypothesis. The vertebrate

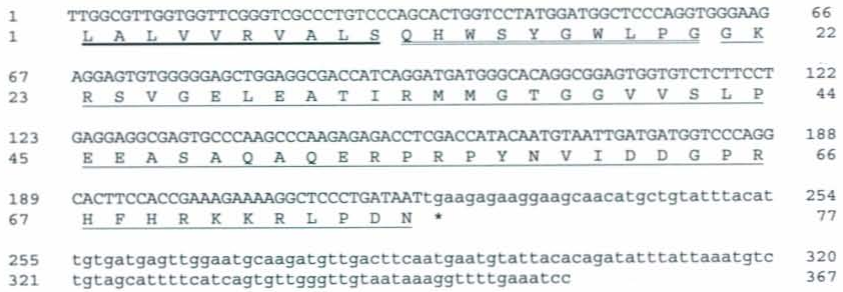

Figure 3.4. Annotated winter flounder sGnRH-3 partial cDNA sequence with nucleotides and deduced amino acids. Bolded underline indicates signal peptide, double underline is mature peptide, single underline is GnRH associated peptide, letters in lower case represent the 3′ untranslated region and * indicates a stop codon.

Figure 3.5. Annotated winter flounder GnRH-R1/b2 sequence with nucleotides and deduced amino acids. Bold underline indicates the conserved transmembrane 3 motif. Letters in lower case represent the 5′ and 3′ untranslated regions and * indicates a stop codon.

```
-194      aaggtggagcggccacacagcggacacacagagcggacacaggacagcggaggagctgcaca  -133
-132  gatgtgggtcttgaacctgacgccttcctgatcgtacagctgctgggatcttctctttctttttt   -67
-66   aataatcggggttccaaacctgcgcatgaggctcaacccgcagcctccacaacacatgcactgacc     0

1     ATGCATCACCTGCCAGCAGACCATCAACTCAACGCCAGCTGCAACTGCTCCTCTCCCCTTTCCAAC    66
1      M  H  H  L  P  A  D  H  Q  L  N  A  S  C  N  C  S  S  P  L  S  N     22

67    TGGACAGCAGGGGGCGACACCCTGCAGCTGCCCACATTCACCACAGCAGCCAAAGTCAGGGTGACC   132
23     W  T  A  G  G  D  T  L  Q  L  P  T  F  T  T  A  A  K  V  R  V  T     44

133   ATTACCTTCATCCTCTGCGCCACGTCGGCCTTCTGCAACCTGGCCGTGCTGTGGGCAGCCCACAGC   198
45     I  T  F  I  L  C  A  T  S  A  F  C  N  L  A  V  L  W  A  A  H  S     66

199   GATGGGAAGCGAAAGTCGCACGTCAGGGTGCTGATCATCAACCTGACTGTGGCTGATCTGCTGATG   264
56     D  G  K  R  K  S  H  V  R  V  L  I  I  N  L  T  V  A  D  L  L  M     88

265   ACCTTCATCGTGATGCCAGTTGACGCAGTGTGGAACATCACAGTCCAGTGGCTTGCTGGGGACCTT   330
89     T  F  I  V  M  P  V  D  A  V  W  N  I  T  V  Q  W  L  A  G  D  L     110

331   GCCTGCAGGCTACTGATGTTTCTCAAGCTGCAGGCGATGTACTCCTGCGCCTTTGTCACTGTGGTG   396
111     A  C  R  L  L  M  F  L  K  L  Q  A  M  Y  S  C  A  F  V  T  V  V   132

397   ATTAGCCTGGACAGGCAGTCAGCTATTCTCAACCCGCTGGCTATCAACGAGGCCAGAAAGAGGAAC   462
133    I  S  L  D  R  Q  S  A  I  L  N  P  L  A  I  N  E  A  R  K  R  N     154

463   AGAGTCATGCTGTCTGTGGCGTGGGCCATGAGTGCTGTGCTGTCAGTCCCTCAGATATTCCTTTTT   528
155    R  V  M  L  S  V  A  W  A  M  S  A  V  L  S  V  P  Q  I  F  L  F     176

529   CACAACGTGACCATCATTCATCCCGAGGAGTTCACTCAGTGCACGACCCGTGGAAGTTTCGTGAGT   594
177    H  N  V  T  I  I  H  P  E  E  F  T  Q  C  T  T  R  G  S  F  V  S     198

595   CACTGGCATGAAACTGCCTACAACATGTTCACCTTCTCCTGCCTGTTCCTGCTGCCGCTGGTCATC   660
199    H  W  H  E  T  A  Y  N  M  F  T  F  S  C  L  F  L  L  P  L  V  I     220

661   ATGATCACCTGTTACACCAGGATCTTGTGTGAGATCTCCAAACGACTGTACACGGACAACTTGTCG   726
221    M  I  T  C  Y  T  R  I  L  C  E  I  S  K  R  L  Y  T  D  N  L  S     242

727   TCCAATGAGGTGCGTTTGCGGTGTTCAAAGAACAACATCCCCAGAGCCCGAATGAGAACTCTTAAA   792
243    S  N  E  V  R  L  R  C  S  K  N  N  I  P  R  A  R  M  R  T  L  K     264

914   ATGAGTATAGTTATTGTCTTGTCTTTTATTATCTGCTGGACTCCGTACTACCTGCTGGGCCTGTGG   858
265    M  S  I  V  I  V  L  S  F  I  I  C  W  T  P  Y  Y  L  L  G  L  W     286

980   TACTGGTTCTTCCCTGATGACCTTGAGGGGAAAGTCTCCCAGTCGCTAACCCACATCCTGTTCATC   924
287    Y  W  F  F  P  D  D  L  E  G  K  V  S  Q  S  L  T  H  I  L  F  I     308

925   TTTGGTCTCCTCAGCGCCTGCCTGGACCCGGTCATCTACGGCCTGTTCACCATTCACTTTCGAAAG   990
309    F  G  L  L  S  A  C  L  D  P  V  I  Y  G  L  F  T  I  H  F  R  K     330

991   GGGCTCCGGAGGTATTTTTTCAAAGCTCCCCCAGCGTCTGACCTGGATAACCACACGGTTATAACT  1056
331    G  L  R  R  Y  F  F  K  A  P  P  A  S  D  L  D  N  H  T  V  I  T     352

1057  GGATCTTTAACTTGTGCTGCCAGTATTTCACCACTGAAAAGAGAGCTGAGCCTTGCTGTCAGCCAG  1122
353    G  S  L  T  C  A  A  S  I  S  P  L  K  R  E  L  S  L  A  V  S  Q     374
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1123   GAGAAGTTCATAACGTACAGAAGCAATCACAGTAAAGAAGAGTCAACGTCACCAAGTGGCAGCTTT   1188
375     E  K  F  I  T  Y  R  S  N  H  S  K  E  E  S  T  S  P  S  G  S  F     396

1310   TTAACAGCAGACAATAATACAGCTAGAGATGTGAACCAGTTCAGCTCCGACAGCACCGTGtgatga   1254
397     L  T  A  D  N  N  T  A  R  D  V  N  Q  F  S  S  D  S  T  V  *       416

1255   caggagaagagacctgacagttttttataatgttgtataactctattatttattcatctttattca   1320
1321   ttgtctgactgtctaatgtgcggggtcctgtattccttgaacactgtcattttacctaaaaaatta   1386
1387   tgcttgtgtataataaaatgaaagaaatttatatttcctattggtaaatgaatgtgtatgtgctct   1452
1453   gtatcttgtgatttgacatcttgaattaaattaaatgtatac                           1494
```

Figure 3.6. Protein alignment for GnRH-Rs used for detecting phylogenetic relationships, including chicken (*Gallus* gallus, GenBank CAC18674/ABK27710), Dybowski's frog (*Rana dybowskii,* GenBank AAO50198/AAO50196/AAO50197), rat (*Rattus norvegicus*, GenBank EDL89848), human (*Homo sapiens,* GenBank EAX05558/EAX05557), common octopus (*Octopus vulgaris*, GenBank BAE66648), medaka (*Oryzias latipes*, GenBank BAB70504/BAB70503/BAC97833), Atlantic cod (*Gadus morhua*, GenBank ADD92008/ADD92009/ADD92010/ADD92011), winter flounder (*Pseudopleuronectes americanus,* GenBank HQ623430/HQ623433), Japanese flounder (*Paralichthys olivaceus,* GenBank AAY28982), European flounder (*Platichthys flesus*, GenBank CAL39150), Coho salmon (*Oncorhynchus kisutch,* GenBank ADH03414/ADH03416/CAB93351), rainbow trout (*Oncorhynchus mykiss*, GenBank CAB93351), blackhead seabream (*Acanthopagrus schlegelii,* GenBank AAV71128/AAV71129), rock gunnel (*Fundulus heteroclitus,* GenBank BAG12379/ABI75337), African cichlid (*Astatotilapia burtoni*, GenBank AAU89433/AAK29745), European seabass (*Dicentrarchus labrax,* GenBank CAD11992/AAS49921), pejerrey (*Odontesthes bonariensis,* GenBank ABI75337), orange-spotted grouper (*Epinephelus coioides*, GenBank ABF93210), catfish (*Clarias gariepinus,* GenBank CAA66128/AAM95605), goldfish (*Carassius auratus,* GenBank AAD20001/AAD20002), and zebrafish (*Danio rerio,* GenBank ABU92656/ABU62657/ABU62658/ABU62659). Winter flounder sequences are bolded, bold underline indicates the conserved transmembrane 3 motif, * indicates that all amino acids are identical in the column, : demonstrates that conserved substitutions have been observed and . specifies that semi-conserved substitutions have been observed.

```
Common octopus GnRH-R              1  M----------------------------------DYL   4
Human GnRH-R2                      1  M----------------------------------ANSAS   6
Rat GnRH-R1                        1  MAGKAPAKQQRFAFSYDKNISSNRDSTLERPWRNMANNAS  40
Human GnRH-R1                      1  M----------------------------------ANSAS   6
Dybowski's frog GnRH-R3            1  M--------------------NASDQPMDGEAALPGLCA  19
Chicken GnRH-R2                    1  M---------------ARLGGGTGQDAAAGGGWLDPGPTV  25
Zebrafish GnRH-R2                  1  M--------------------------------NTTQLIE   8
Dybowski's frog GnRH-R1            1  M--------------------------NSKEVSIKGCSN  13
Atlantic cod GnRH-R2c              0  ----------------------------------------   0
Medaka GnRH-R1                     1  M----------------------NESSCHPPAITYQQSS  17
Atlantic cod GnRH-R2a              0  ----------------------------------------   0
Rock gunnel GnRH-R1                1  M----------------------NTCLETAVTMRDLVT  16
African cichlid GnRH-R1            1  M----------------------NASLDPAAVMYQLVA  16
Black porgy GnRHR1                 1  M----------------------DTTLDSAAAMYHLTT  16
European seabass GnRH-R1           1  M----------------------NTTLDSAVALYHLTT  16
Japanese flounder GnRH-R           1  M----------------------------------HHLPR   6
Winter flounder GnRH-R1            1  M---------------------------------HHLPA   6
European flounder GnRH-R           0  ----------------------------------------   0
Zebrafish GnRH-R4                  1  M----------------------NDSSTSENIMFHQLT  16
Coho salmon GnRH-R1                0  ----------------------------------------   0
Atlantic cod GnRH-R2b              0  ----------------------------------------   0
Medaka GnRH-R3                     1  M----------------------------------FHHLT   6
Black porgy GnRH-R2                0  ----------------------------------------   0
Chicken GnRH-R1                    1  M----------------------------VPAALIEAEPP  12
Dybowski's frog GnRH-R2            1  M--------------------------------AMQLAIVN   9
Medaka GnRH-R2                     1  M----------------------TKADTS-TGNRSSLAPP  17
Rock gunnel GnRH-R2                0  ----------------------------------------   0
Pejerrey GnRH-R1b                  1  M----------------------------SGNVSSLAPS  11
African cichlid GnRH-R             1  M--------------------------ANWSILRLSLPP  13
Winter flounder GnRH-R2            0  ----------------------------------------   0
European seabass GnRH-R2           1  M------------------------SGNSVVRLSSPASP  15
Orange spotted grouper GnRH-R1     1  M----------------------------------------   0
Zebrafish GnRH-R3                  1  M----------------------------------------   0
Goldfish GnRH-R1                   1  M-----------------------------------S   2
European catfish GnRH-R2           1  M----------------------------PRNDSLFSPLE  12
Atlantic cod GnRH-R1b              0  ----------------------------------------   0
Coho salmon GnRH-R2a               0  ----------------------------------------   0
Goldfish GnRH-R2                   1  M----------------------------GKMPLLSVNPT  12
Zebrafish GnRH-R1                  1  M--------------------------------SGNVSLSL   9
European catfish GnRH-R1           1  M----------------------------GNTTLLLSNPT  12
Coho salmon GnRH-R2b               0  ----------------------------------------   0
```

```
Common octopus GnRH-R          5   NDSMFNNMTYNITSTPLPDA------------PRFDNVY  31
Human GnRH-R2                  7   PEQNQNHCSAINNSIPLMQGNL-----------PTLTLSG  35
Rat GnRH-R1                   41   LEQDQNHCSAINNSIPLTQGKL-----------PTLTLSG  69
Human GnRH-R1                  7   PEQNQNHCSAINNSIPLMQGNL-----------PTLTLSG  35
Dybowski's frog GnRH-R3       20   FKGFNLSCVHTNGFERPHGPNIT-FLNEDHFVLPTFSTAA  58
Chicken GnRH-R2               26   GNVSTEPSSSTSHPKRGCAWSPRLESAEEPLLLPTFSPAA  65
Zebrafish GnRH-R2              9   DLLQNSSCKHEA---KHLNSNIA-GDLPHPLHLPRFTAAS  44
Dybowski's frog GnRH-R1       14   AQWLSSSCDLDV---NMTSTNET-LT---RFQLPTFSSAA  46
Atlantic cod GnRH-R2c          0   ----------------------------------------   0
Medaka GnRH-R1                18   RWDLNASCDWSA---PRCNWTSG-DG---PLQLPTFSTAA  42
Atlantic cod GnRH-R2a          0   ----------------------------------------   0
Rock gunnel GnRH-R1           17   DLQANVTCNCSL---ASSNWTEE--E---VPTLPTFTTAA  58
African cichlid GnRH-R1       17   DHQLDTSCNCSS---ALSNWTAR-GT---APQLPTFSTAA  59
Black porgy GnRHR1            17   DHQLNASCNYST---PPSNWTAG-GG---GQQLPTFTTAA  59
European seabass GnRH-R1      17   DHQLNASCNYSS---PTSNWTSG-GG---ALQLPTFTTAA  59
Japanese flounder GnRH-R       7   DRQLNSSWNGSS---PHSNWTAG-GD---TLQLPTFTTAA  49
Winter flounder GnRH-R1        7   DHQLNASCNCSS---PLSNWTAG-GD---TLQLPTFTTAA  49
European flounder GnRH-R       0   ----------------------------------------   0
Zebrafish GnRH-R4             17   ADTLNGSCDL-----PTCNNNTG-EA---ALQLPTFSMAA  57
Coho salmon GnRH-R1            0   ----------------------------------------   0
Atlantic cod GnRH-R2b          0   ----------------------------------------   0
Medaka GnRH-R3                 7   DQTVNGSCLGAS---TDCNKSAD-GD---ALQLPTFSTAA  49
Black porgy GnRH-R2            0   ----------------------------------------   0
Chicken GnRH-R1               13   HHPTTEGDTNTSATHCLEHWVE-----------PRFTKAA  41
Dybowski's frog GnRH-R2       10   RSHLVVPDANISALGFPGPWRE-----------PTFTAAA  38
Medaka GnRH-R2                18   TSSVVPAHLNSSLYPPIPDWEP-----------PSFSQAA  46
Rock gunnel GnRH-R2            0   ----------------------------------------   0
Pejerrey GnRH-R1b             12   TSSVAPTPSNVSHYPPLSNWEP-----------PSFTRAA  40
African cichlid GnRH-R        14   TSSTATTLHNTSQYPPLPDWEA-----------PSFTRAA  42
Winter flounder GnRH-R2        0   ----------------------------------------   0
European seabass GnRH-R2      16   TSSVAPPPPNTSQFPPLTDWEA-----------PSFTCAA  44
Orange spotted grouper GnRH-R1 1   -SGNWSPSPNTSQFPPLTDWEA-----------PTFTRAA  28
Zebrafish GnRH-R3              1   -SGNWSQYNASL----LPVWTA-----------PSFTPAA  24
Goldfish GnRH-R1               3   DNTSLPSVSNASLLPPLTDWRA-----------PSFTPAA  31
European catfish GnRH-R2      13   LDTSLQDVSNSSLSPPLADWVA-----------PTFTRAA  41
Atlantic cod GnRH-R1b          0   ----------------------------------------   0
Coho salmon GnRH-R2a           0   ----------------------------------------   0
Goldfish GnRH-R2              13   SIWENSSVLNATPHFP-SDWET-----------PTFTVAA  40
Zebrafish GnRH-R1             10   ISLLENSSLASSSPQSPQWET-----------PTFTTAA  38
European catfish GnRH-R1      13   NVLDNSSVLNVSVSPPVLKWET-----------PTFTTAA  41
Coho salmon GnRH-R2b           0   ----------------------------------------   0
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Common octopus GnRH-R          32  VSKLCVLGTVFVISFFGNTLVI-----IQIFRIRGSR--S  64
Human GnRH-R2                  36  KIRVTVTFFLFLLSATFNASFLLKLQKWTQKKEKGKK-LS  74
Rat GnRH-R1                    70  KIRVTVTFFLFLLSTAFNASFLVKLQRWTQKRKKGKK-LS 109
Human GnRH-R1                  36  KIRVTVTFFLFLLSATFNASFLLKLQKWTQKKEKGKK-LS  74
Dybowski's frog GnRH-R3        59  KIRVAITCVLFISSACFNMATL-----WTITYKY-RK-KS  68
Chicken GnRH-R2                66  QARVAATFVLFVLSAGCNVAVL----RAAGGRRGGGR--S  99
Zebrafish GnRH-R2              45  QVRVTLTLVLCALSACCNLAVL-----YSANNNQ-RK-RS  77
Dybowski's frog GnRH-R1        47  KARVIITFVIFTLSATCNLAAL-----WAASRTSRKK-RS  80
Atlantic cod GnRH-R2c          0   ----------------------------------------   0
Medaka GnRH-R1                 43  KVRVITTFILCGVSTLCNSAVL-----WAA-IGH-KR-KS  74
Atlantic cod GnRH-R2a          0   ----------------------------------------   0
Rock gunnel GnRH-R1            59  KIRVIITFILCGTSAFCNLAVL-----WAAHRDG-KR-KS  91
African cichlid GnRH-R1        60  KARVIITFILCGISAFCNLAVL-----WAGAQDG-KR-KS  92
Black porgy GnRHR1             60  KVRVVITCILCGVSAFCNLAVL-----WAAHSDG-KR-KS  92
European seabass GnRH-R1       60  KVRVIITCILCGISAFCNLAVL-----WAAHSDG-KR-KS  92
Japanese flounder GnRH-R       50  KVRVTITFILCATSAFCNLAVL-----WAAHSDG-KR-KS  82
Winter flounder GnRH-R1        50  KVRVTITFILCATSAFCNLAVL-----WAAHSDG-KR-KS  82
European flounder GnRH-R       0   ----------------------------------------   0
Zebrafish GnRH-R4              58  KVRVIITFTLCAVSAVCNLGVL-----WAASTNNKRK---  89
Coho salmon GnRH-R1            0   ----------------------------------------   0
Atlantic cod GnRH-R2b          0   ----------------------------------------   0
Medaka GnRH-R3                 50  KVRVIITFTLCAVSAVCNLLVL-----WAAGKGG-KR-KS  82
Black porgy GnRH-R2            0   ----------------------------------------   0
Chicken GnRH-R1                42  KVRVAITAVFFLLAACSNTAVL-----GSLLR---KRRKC  73
Dybowski's frog GnRH-R2        39  KVRVGVTCCFFLMASCSNVAVL-----CSIS---GKRCKS  70
Medaka GnRH-R2                 47  QFRVGAIFILFLFAACSNTALL-----TSVWCGRGRRLAS  81
Rock gunnel GnRH-R2            0   ----------------------------------------   0
Pejerrey GnRH-R1b              41  QFRVCATFILFLFAACSNLALL-----ASVWCGRGRRLAS  75
African cichlid GnRH-R         43  QFRVGATFVLFLFAACSNLALL-----VSVWRG--QRLAS  75
Winter flounder GnRH-R2        0   ----------------------------------------   0
European seabass GnRH-R2       45  QFRVGATFALFLFAASSNLALL-----ASVWCGRGRRLAS  79
Orange spotted grouper GnRH-R1 29  QFRVGATFILFLFTACSNLALF-----ASVWCGRGRRLAS  63
Zebrafish GnRH-R3              25  QARVAATALLFVFAAGSNLALL-------VSVCRSRRLAS  57
Goldfish GnRH-R1               32  QARVAATMVLFLFAAVSNLALL-----ISVSRGRGRRLAS  66
European catfish GnRH-R2       42  QFRVGATLVLFLFAAVSNLALL-----ISVCRGRGRRLAS  76
Atlantic cod GnRH-R1b          0   ----------------------------------------   0
Coho salmon GnRH-R2a           0   ----------------------------------------   0
Goldfish GnRH-R2               41  HFRVVATLVLFVFAAISNLSVL-----ISVTRGRGRHLAS  75
Zebrafish GnRH-R1              39  RYRVVATLVLFVFAAVSNLSVL-----ISVTRGRGRHLAS  73
European catfish GnRH-R1       42  RFRVAATLVLFVFAAASNLSVL-----LSVTRGRGRRLAS  76
Coho salmon GnRH-R2b           0   ----------------------------------------   0
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Common octopus GnRH-R           65  TIQSLILNLAIADLMVSFFNILMDIIWSASTIQSLILNLA 104
Human GnRH-R2                   75  RMKLLLKHLTLANLLETLIVMPLDGMWNISRMKLLLKHLT 114
Rat GnRH-R1                    110  RMKVLLKHLTLANLLETLIVMPLDGMWNISRMKVLLKHLT 149
Human GnRH-R1                   75  RMKLLLKHLTLANLLETLIVMPLDGMWNISRMKLLLKHLT 114
Dybowski's frog GnRH-R3         69  HIRILIINLVAADLLITFVVMPLDAVWNVSHIRILIINLV 108
Chicken GnRH-R2                100  HIRVLLRHLAAADLLVTVVVMPLDAIWNISHIRVLLRHLA 139
Zebrafish GnRH-R2               78  HVRLIITNLAAADLLVTFVVMPTDAVWNISHVRLIITNLA 117
Dybowski's frog GnRH-R1         81  HVRILILNLTTADLLVTFIVMPLDAVWNISHVRILILNLT 120
Atlantic cod GnRH-R2c           0   ----------------------------------------   0
Medaka GnRH-R1                  75  HVRVLIINLTAADLLVTFIVMPVDAAWNISHVRVLIINLT 114
Atlantic cod GnRH-R2a           1   ---VLIVNLTVADLLVTFIVMPVDAAWNI----VLIVNLT  33
Rock gunnel GnRH-R1             92  HVKVLIVNLTVADLLVTFIVMPVDAAWNISHVKVLIVNLT 131
African cichlid GnRH-R1         93  HVRVLIVNLTMADLLVTFI----------SHVRVLIVNLT 122
Black porgy GnRHR1              93  HVRVLIINLTVADLLVTFIVMPVDAVWNISHVRVLIINLT 132
European seabass GnRH-R1        93  HVRVLIINLTVADLLVTFIVMPVDAVWNISHVRVLIINLT 132
Japanese flounder GnRH-R        83  HVRVLIVNLTVADLLVTFIVMPVDAVWNISHVRVLIVNLT 122
Winter flounder GnRH-R1         83  HVRVLIINLTVADLLMTFIVMPVDAVWNISHVRVLIINLT 122
European flounder GnRH-R        1   ---------------VTFVVMPVDAVWNI-----------  14
Zebrafish GnRH-R4               90  HVRILIMNLTVADLLVTFIVMPVDAAWNISHVRILIMNLT 129
Coho salmon GnRH-R1             0   ----------------------------------------   0
Atlantic cod GnRH-R2b           0   ----------------------------------------   0
Medaka GnRH-R3                  83  HVRILIMNLTVADLLVTFIVMPVDAAWNISHVRILIMNLT 142
Black porgy GnRH-R2             1   -------------------------IWNV-----------   4
Chicken GnRH-R1                 74  HVRPLILSLALADLLVTVAVMPLDAAWNVCHVRPLILSLA 113
Dybowski's frog GnRH-R2         71  HLRILILSLSIADLLATLLVMPLDAMWNISHLRILILSLS 110
Medaka GnRH-R2                  82  HLRPLMLSLASADLMMTFVVMPLDAAWNVSHLRPLMLSLA 121
Rock gunnel GnRH-R2             1   -----MLSLASADLMMTFVVMPLDAVWNI------MLSLA  29
Pejerrey GnRH-R1b               76  HLRPLMLSLASADLMMTFVVMPLDAVWNVSHLRPLMLSLA 115
African cichlid GnRH-R          76  HLRPLMLSLASADLMMTFVVMPLDAVWNMSHLRPLMLSLA 115
Winter flounder GnRH-R2         1   -LRPLMLSLASADLMMTFVVMPLDAVWNR--LRPLMLSLA  37
European seabass GnRH-R2        80  HLRPLMLSLVSADLMMTFVVMPLDAVWNVSHLRPLMLSLV 119
Orange spotted grouper GnRH-R1  64  HLRPLMLSLASADLMMTFVVMPLDAVWNVSHLRPLMLSLA 103
Zebrafish GnRH-R3               58  HLRPLILSLAAADLLMTFVVMPLDMVWNVSHLRPLILSLA  97
Goldfish GnRH-R1                67  HLRPLIISLVSADLMMTFIVMPLDMVWNVSHLRPLIISLV 106
European catfish GnRH-R2        77  HLRPLIRSLAVADLMMTFIVMPLDMVWNVSHLRPLIRSLA 116
Atlantic cod GnRH-R1b           0   ----------------------------------------   0
Coho salmon GnRH-R2a            0   ----------------------------------------   0
Goldfish GnRH-R2                76  HLRPLIGSLASADLVMTFVVMPLDAIWNISHLRPLIGSLA 115
Zebrafish GnRH-R1               74  HLRLLIASLASADLVMTFVVMPLDAIWNISHLRLLIASLA 113
European catfish GnRH-R1        77  HLRPLIASLASADLVMTFVVMPLDAVWNVSHLRPLIASLA 116
Coho salmon GnRH-R2b            0   ----------------------------------------   0
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Common octopus GnRH-R           105 IADLMVSFFNILMDIIWSATVEWLAGNTMCKIMKYLTVFG 144
Human GnRH-R2                   115 LANLLETLIVMPLDGMWNITVQWYAGELLCKVLSYLKLFS 154
Rat GnRH-R1                     150 LANLLETLIVMPLDGMWNITVQWYAGEFLCKVLSYLKLFS 189
Human GnRH-R1                   115 LANLLETLIVMPLDGMWNITVQWYAGELLCKVLSYLKLFS 154
Dybowski's frog GnRH-R3         109 AADLLITFVVMPLDAVWNVTIQWYAGDVACRILMFLKLVA 148
Chicken GnRH-R2                 140 AADLLVTVVVMPLDAIWNITLQWRAGDLACRLLMYLRLLA 179
Zebrafish GnRH-R2               118 AADLLVTFVVMPTDAVWNITLQWRAGDLACRTLMFLKLVA 157
Dybowski's frog GnRH-R1         121 TADLLVTFIVMPLDAVWNITVQWHAGDIACRILMFLKLLS 160
Atlantic cod GnRH-R2c           0   ----------------------------------------  0
Medaka GnRH-R1                  115 AADLLVTFIVMPVDAAWNITVQWLAGDAACRFLMFLKLQA 154
Atlantic cod GnRH-R2a           34  VADLLVTFIVMPVDAAWNITVQWLAGDAACRLLMFLKLQA  73
Rock gunnel GnRH-R1             132 VADLLVTFIVMPVDAAWNITVQWLAGDFACRLLMFLKLLA 171
African cichlid GnRH-R1         123 MADLLVTFI-------------------------------- 131
Black porgy GnRHR1              133 VADLLVTFIVMPVDAVWNITVQWLAGDFACRLLMFLKLQA 172
European seabass GnRH-R1        133 VADLLVTFIVMPVDAVWNITVQWLAGDLACRLLMFLKLQA 172
Japanese flounder GnRH-R        123 VADLLVTFIVMPVDAVWNITVQWLAGDLACRLLMFLKLQA 162
Winter flounder GnRH-R1         123 VADLLMTFIVMPVDAVWNITVQWLAGDLACRLLMFLKLQA 162
European flounder GnRH-R        15  -----VTFVVMPVDAVWNITVQWLAGDLACRLLMFLKLQA  49
Zebrafish GnRH-R4               130 VADLLVTFIVMPVDAAWNITVQWLAGDLACRLLMFLKLVA 169
Coho salmon GnRH-R1             0   ----------------------------------------  0
Atlantic cod GnRH-R2b           0   ----------------------------------------  0
Medaka GnRH-R3                  143 VADLLVTFIVMPVDAAWNITVQWQAGDLACRLLMFMKLVA 162
Black porgy GnRH-R2             5   ---------------IWNVTVQWQAGDVACRLLMFMKLVA  25
Chicken GnRH-R1                 114 LADLLVTVAVMPLDAAWNVTVQWYGGDLSCKLLNFLKLFA 153
Dybowski's frog GnRH-R2         111 IADLLATLLVMPLDAMWNIMIQWYADELSCKILNFGKLFA 150
Medaka GnRH-R2                  122 SADLMMTFVVMPLDAAWNVTVQWYGGDALCKLLCFLKLFA 161
Rock gunnel GnRH-R2             30  SADLMMTFVVMPLDAVWNITVQWYGGDALCKLLSFLKLFA  69
Pejerrey GnRH-R1b               116 SADLMMTFVVMPLDAVWNVTVQWYGGDALCKLLCFLKLFA 155
African cichlid GnRH-R          116 SADLMMTFVVMPLDAVWNMTVQWYGGDALCKLLCFLKLFA 155
Winter flounder GnRH-R2         38  SADLMMTFVVMPLDAVWNRTVQWYGGDVLCKLLSFLKLFA  77
European seabass GnRH-R2        120 SADLMMTFVVMPLDAVWNVTVQWYGGDALCKLLCFLKLFA 159
Orange spotted grouper GnRH-R1  104 SADLMMTFVVMPLDAVWNVTVQWYGGDVLCKLLCFLKLFA 143
Zebrafish GnRH-R3               98  AADLLMTFVVMPLDMVWNVTVQWYAGDVVCKLLCFLKLFA 137
Goldfish GnRH-R1                107 SADLMMTFIVMPLDMVWNVTVQWYAGDGLCKLLCFLKLFA 146
European catfish GnRH-R2        117 VADLMMTFIVMPLDMVWNVTVQWHGGDGLCKLLSFLKLFA 156
Atlantic cod GnRH-R1b           0   ----------------------------------------  0
Coho salmon GnRH-R2a            1   ------------------------GDAMCKMLCFLKLFA  15
Goldfish GnRH-R2                116 SADLVMTFVVMPLDAIWNITVQWYAGNAMCKNLCFLKLFA 155
Zebrafish GnRH-R1               114 SADLVMTFVVMPLDAIWNITVQWYAGDAMCKLLCFLKLFA 153
European catfish GnRH-R1        117 SADLVMTFVVMPLDAVWNVTVQWYAGDAMCKLMCFLKLFA 156
Coho salmon GnRH-R2b            1   ------------------------GDTMCKLLCFLKLFA  15
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Common octopus GnRH-R          145 LHLSTYITVSIALDRCFAILSPMSRSKAPLRVRIMITMAW 184
Human GnRH-R2                  155 MYAPAFMMVVISLDRSLAITRPLALKSNSKVGQSMVGLAW 194
Rat GnRH-R1                    190 MYAPAFMMVVISLDRSLAVTQPLAVQSKSKLERSMTSLAW 229
Human GnRH-R1                  155 MYAPAFMMVVISLDRSLAITRPLALKSNSKVGQSMVGLAW 194
Dybowski's frog GnRH-R3        149 MYSSAFVTVVISLDRHAAILNPLGIGDAKKKNRTMLSIAW 188
Chicken GnRH-R2                180 MYASAFVTVVISLDRQAAILRPLAIARARCRNRAMLRAAW 219
Zebrafish GnRH-R2              158 MYSCAFVTVVISLDRQAAILNPLSINKAMRRNKVLLGVAW 197
Dybowski's frog GnRH-R1        161 MYSCAFVTVVISVDRQSAILNPLAINDAKKKNKIMLSVAW 200
Atlantic cod GnRH-R2c          1   ----------------AILNPLGFSEARRRNRAMLSVAW  23
Medaka GnRH-R1                 155 MYSCAFVTVVISLDRQSAILRPLSISAAPRRNRSMLTVAW 194
Atlantic cod GnRH-R2a          74  MYSCAFVTMVISLDRQSAILNPLAINEARRRNRGMLTVAW 113
Rock gunnel GnRH-R1            172 MYSCAFVTVVISLDRQSAILNPLAINKARRRNRIMLAVAW 211
African cichlid GnRH-R1        132 --------------------------ARKRNRIMLMVAW 144
Black porgy GnRHR1             173 MYSCAFVTVVISLDRQSAILNPLAINKARKRNRVMLTVAW 212
European seabass GnRH-R1       173 MYSCAFVTVVISLDRQSAILNPLAINKARKRNRVMLTVAW 212
Japanese flounder GnRH-R       163 MYSCAFVTVVISLDRQSAILNPLAINEARKRNRVMLSVAW 202
Winter flounder GnRH-R1        163 MYSCAFVTVVISLDRQSAILNPLAINEARKRNRVMLSVAW 202
European flounder GnRH-R       50  MYSCAFVTVVISLDRQSAILNPLAINEARKRNRVMLSVAW  89
Zebrafish GnRH-R4              170 MYSCAFVTVVISLDRQSAILNPLAINKAKKKNKIMLSVAW 209
Coho salmon GnRH-R1            1   -----------SLDRQSAILNPLAINEAKKRSKIMLSVAW  29
Atlantic cod GnRH-R2b          1   ---------------HSAILNPLGISEAKRRSQIMLSVAW  25
Medaka GnRH-R3                 163 MYSCAFVTVVISLDRQSAILNPLGISEAKRKSKIMLTVAW 202
Black porgy GnRH-R2            26  MYSCAFVTVVISLDRQSAILNPLGISEAKRKSKIMLTVAW  65
Chicken GnRH-R1                154 MYAAALVLVVISLDRHAAVLQPFA--RARRRNGLLLRAAW 191
Dybowski's frog GnRH-R2        151 MYSAALVLVVISLDRHWAILYPLSFTSAGQRNRIMLWTAW 190
Medaka GnRH-R2                 162 MHASAFILVVITLDRQHAILHPLDALSAHRRNRFMLLLAW 201
Rock gunnel GnRH-R2            70  MHASAFILVVISLDRHHAILHPLDALSAHHRNRCMLLLAW 109
Pejerrey GnRH-R1b              156 MHASAFILVVISLDRQHAIMHPLDALSAHRRNRLMLLLAW 195
African cichlid GnRH-R         156 MHASAFILVVISLDRQHAILHPLDALSAHSRNRRMLLLAW 195
Winter flounder GnRH-R2        78  MHASAFILVVISLDRQHAILHPLDALSAHRRNRRMVLMAW 117
European seabass GnRH-R2       160 MHASAFILVVISLDRQHAILHPLDALSAQCRNRRMLLLAW 199
Orange spotted grouper GnRH-R1 144 MHASAFILVVISLDRQHAILHPLDALNAHRRNRRMLLLAW 183
Zebrafish GnRH-R3              138 MQTSAFILVGISLDRHQAILRPLDTLTAPQRNRRRMLTAW 177
Goldfish GnRH-R1               147 MQTSAFILVVISLDRHHAILHPLDSLNAHQRNRRMLLLAW 186
European catfish GnRH-R2       157 MQASAFILVVISLDRQHAILHPLDTLNAHRRNKRMLVLAW 196
Atlantic cod GnRH-R1b          1   ------------------ILHPLDSLSAHHRNRRMLGLAW  22
Coho salmon GnRH-R2a           16  MHSSAFILVVVSLDRHHAILHPLDSLNSHHRNKRMLGLAW  55
Goldfish GnRH-R2               156 MHSAAFILVVVSLDRHHAILHPLEALDAGRRNRRMLLAAW 195
Zebrafish GnRH-R1              154 MHSAAFILVVVSLDRHHAILHPLEALDAGRRNRRVLLAAW 193
European catfish GnRH-R1       157 MHSAAFILVVVSLDRHHAILHPLDTLDAGRRNRRMLLTAW 196
Coho salmon GnRH-R2b           16  MHSSAFILVVVSLDRHHAILHPLDTLDAGRRNKRMLLVAW  55
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Common octopus GnRH-R          185 VLSAIFSIPQAVIFQEQRK---MFRQGMFHQC--RDSYNA 219
Human GnRH-R2                  195 ILSSVFAGPQLPLHHP-----------SFHH--------- 214
Rat GnRH-R1                    230 ILSIVFAGPQLYIFRMIYLADGSGPA-VFSQCVTHCSFPQ 268
Human GnRH-R1                  195 ILSSVFAGPQLYIFRMIHLADSSGQTKVFSQCVTHCSFSQ 234
Dybowski's frog GnRH-R3        189 TLSLLLATPQLFVFHTVSR---SHPV-HLVQCATLGSFKA 224
Chicken GnRH-R2                220 MLSAALAVPQLFLFHTVTL---HAPH-NFTQCTTHGSFPQ 255
Zebrafish GnRH-R2              198 TMSVVLSIPQVFVFHVVEI---DSPK-QFVQCTTYGSFSS 233
Dybowski's frog GnRH-R1        201 LMSAVLSLPQLFLFHTVTI---TEPH-NFTQCTARGSFQQ 236
Atlantic cod GnRH-R2c          24  SMSLLLSAPQIFIFHSVTI---THPQ-NFTQCTTRGSFQT  59
Medaka GnRH-R1                 195 TMSAVLSVPQMFIFHNVTI---THPA-NFTQCTTRGSFVT 231
Atlantic cod GnRH-R2a          114 AMSALLSVPQVFLFHNVTI---TFPE-RFTQCTTRGSFDT 149
Rock gunnel GnRH-R1            212 GMSVVLSVPQVFLFHNVTI---IHPE-DFTQCTTWGSFAT 247
African cichlid GnRH-R1        145 VMSVVLSVPQMFLFHNVTI---IHPE-DFTQCTTRGSFVT 180
Black porgy GnRHR1             213 GMSVLLSVPQMFLFHNVTI---VHPE-DFTQCTTRGSFVR 248
European seabass GnRH-R1       213 GMSVVLSVPQLFLFHNVTI---IHPE-DFTQCTTRGSFVT 248
Japanese flounder GnRH-R       203 TMSVVLSVPQIFLFHNVTI---VHPE-DFTQCTTRENFVS 238
Winter flounder GnRH-R1        203 AMSAVLSVPQIFLFHNVTI---IHPE-EFTQCTTRGSFVS 238
European flounder GnRH-R       90  AMSAVLSVPQIFLFHNVTI---IHPE-EFTQCTTRGSFVS 125
Zebrafish GnRH-R4              210 AMSVVLSVPQMFLFHNVTI---TVPA-NFTQCTTRGSFVK 245
Coho salmon GnRH-R1            30  AMSVILSVPQMLIFHSVTI---TVPE-KFTQCTTRGSFVQ  65
Atlantic cod GnRH-R2b          26  TMSVLLSLPQIFIFHNVTI---TVPE-RFTQCTTRGSFVQ  61
Medaka GnRH-R3                 203 TTSVILSLPQMFIFHNVTI---SVPE-NFTQCTTHGSFVQ 238
Black porgy GnRH-R2            66  TMSVVLSLPQMFIFHNVTI---TVPE-NFTQCTTHGSFVQ 101
Chicken GnRH-R1                192 LGSVLLASPQLFLFHVHTV---PG-G-NFTQCVTHGSFRA 227
Dybowski's frog GnRH-R2        191 IGSLLLASPQLFLFRLRTV---PG-A-NFTQCATHGSFAQ 226
Medaka GnRH-R2                 202 TLSLLLASPQLFLFRTIKV---DR-A-NFTQCVSHGSFSH 237
Rock gunnel GnRH-R2            110 SLSLLLASPQLFIFRTIRV---DS-V-DFTQCASHGSFSH 145
Pejerrey GnRH-R1b              196 GLSLLLASPQLFIFRTISV---DS-A-DFTQCASHGSFSR 231
African cichlid GnRH-R         196 TLSILLASPQLFIFRTIRV---DS-V-DFTQCASHGSFSR 231
Winter flounder GnRH-R2        118 SLSLLLASPQLFIFRTIMV---DG-V-DFTQCASHGSFRR 153
European seabass GnRH-R2       200 SLSLLLASPQLFIFRAIRV---EA-A-DFTQCVTHGSFHH 235
Orange spotted grouper GnRH-R1 184 SLSLVLASPQLFIFRTIRV---EA-V-DFTQCATHGSFSH 219
Zebrafish GnRH-R3              178 SLSALIASPQLFIFHTVKA---KS-V-DFTQCVTHGSFSE 209
Goldfish GnRH-R1               187 SLSALIASPQLFIFRTVKV---KS-V-DFTQCVTHGSFHE 222
European catfish GnRH-R2       197 SLSLLLASPQLFIFRAIKA---EK-V-DFTQCVTHGSFPE 232
Atlantic cod GnRH-R1b          23  SLSLLLASPQLFIFGALKA---EG-V-DFTQCVTHGSFPR  68
Coho salmon GnRH-R2a           56  GLSVLLALPQLFIFRAIKA---EG-V-DFTQCVTHGSFKE  91
Goldfish GnRH-R2               196 ILSILLASPQLFIFRAIKA---EG-V-DFVQCVTHGSFRQ 231
Zebrafish GnRH-R1              194 ILSVLLASPQLFIYRALKA---EG-V-DFVQCATHGSFRH 229
European catfish GnRH-R1       197 ILSLLLASPQLFIFRAIKA---KG-V-DFVQCATHGSFQQ 232
Coho salmon GnRH-R2b           56  VLSLLLASPQLFIFRAIKA---EG-V-EFTQCVTHGSFHH  91
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Common octopus GnRH-R            220 LWQKQLYSASSLILLFVIPLIIMVTSYLLILKTIVKTSRQ 259
Human GnRH-R2                    215 ---------ADLQC-----------------------KNH 222
Rat GnRH-R1                      269 WWHEAFYNFFTFSCLFIIPLLIMLICNAKII---FALTRV 305
Human GnRH-R1                    235 WWHQAFYNFFTFSCLFIIPLFIMLICNAKII---FTLTRV 271
Dybowski's frog GnRH-R3          225 HWLETLYNMFTFCCLFLLPLLIMVFCYGRIL---LEISRK 221
Chicken GnRH-R2                  256 PWHETLYNMLSFSCLFLLPLLIMVCCYTRIL---LEISRR 192
Zebrafish GnRH-R2                234 RWQETFYNMFTFTFLFLLPLFIMISCYTRIL---FEISKK 270
Dybowski's frog GnRH-R1          237 HWQKTVYNMVSFVCLFLLPLLIMICCYSRIL---LEISKR 273
Atlantic cod GnRH-R2c            60  HGQETAYNMLTFCCLFLLPLVTMITCYSRIL---LQISRR  96
Medaka GnRH-R1                   232 HWQETAYNMFTFTCLFLLPLSIMIICYTRIF---IQISKQ 268
Atlantic cod GnRH-R2a            150 HWQETAYNMFTFCCLFLLPLVIMITCYTRIF---SEISKR 186
Rock gunnel GnRH-R1              248 RWHETAYNMFTFSCLFLLPLVIMITCYARIF---HEISKR 284
African cichlid GnRH-R1          181 HWHETAYNMFTFCCLFLLPLVIMITCYTRIF---CEISRR 217
Black porgy GnRHR1               249 HWHETAYNMFTFFCLFLLPLVIMITCYTRIF---CEISKR 285
European seabass GnRH-R1         249 HWHETAYNMFTFSCLFLLPLVIMITCYTRIF---CEISKR 285
Japanese flounder GnRH-R         239 HWHETAYNMFTFSCLFLLPLAIMIICYTRIF---CEISKR 275
Winter flounder GnRH-R1          239 HWHETAYNMFTFSCLFLLPLVIMITCYTRIL---CEISKR 275
European flounder GnRH-R         126 HWHETAYNMFTFSCLFLLPLVIMITCYTRIL---CEISKR 162
Zebrafish GnRH-R4                246 HWQETIYNMFTFICLFLIPLAIMICCYTRIL---VEISRR 282
Coho salmon GnRH-R1              66  HWQETMYNMFTFACLFLLPLIIMIVCYTRIL---VEISSR 102
Atlantic cod GnRH-R2b            62  HWQETVYNLFTFVCLFLLPLVIMIFCYARIL---VEISSR  98
Medaka GnRH-R3                   239 RWQETLYNMFTFVCLFLLPLVIMIFCYTRIL---VEISSR 275
Black porgy GnRH-R2              102 HWQETLYNMFTFVCLFLLPLVIMIFCYTRIL---IEISSR 138
Chicken GnRH-R1                  228 HWEETVYNMFTFTTLYITPLSIMIVCYVRII---WEISKQ 264
Dybowski's frog GnRH-R2          227 HWQETAYNMFTFCTLFVTPLGVMIVCYTRIL---WEIGKQ 263
Medaka GnRH-R2                   238 RWQETVYNMFHFITLYVVPLLVMSCCYSRIL---LHIHQQ 274
Rock gunnel GnRH-R2              146 RWQETVYNMFHFITLYVIPLLMMSCCYSRIL---LHINQQ 182
Pejerrey GnRH-R1b                232 RWQETVYNMFHFITLYVVPLLVMSCCYSRIL---LHIHLQ 268
African cichlid GnRH-R           232 RWQETVYNMFHFTTLYVVPLLVMSCCYSRIL---LHIHQQ 268
Winter flounder GnRH-R2          154 RWQETLYNMFHFITLYVVPLLVMSCCYSRIL---LHIHQQ 190
European seabass GnRH-R2         236 RWQETVYNMFHFTTLYVFPLLVMSCCYSRIL---LHIHLQ 272
Orange spotted grouper GnRH-R1   220 RWQETVYNMFHFITLYVVPLLVMSCCYSRIL---LHIHLQ 256
Zebrafish GnRH-R3                210 RWHETAYNMFHFVTLYVIPLLVMSCCYTCIL---IEINRQ 246
Goldfish GnRH-R1                 223 RWYETAYNMFHFVTLYVIPLLVMSCCYTCIL---IEINRQ 259
European catfish GnRH-R2         233 RWQETVYNMFHFVTLYVIPLLVMSCCYTRIL---FEINRQ 269
Atlantic cod GnRH-R1b            67  RWQETLYNMFHFVTLYVVPLLVMACCYSRIL---LHINRQ 103
Coho salmon GnRH-R2a             92  RWQETVYNMFYFVTLYVFPLLVMSFCYTHIL---IEINQQ 128
Goldfish GnRH-R2                 232 RWQETAYNMFHFVTLYVFPLLVMSFCYTHIL---VEINRQ 268
Zebrafish GnRH-R1                230 RWQETAYNMFHFVTLYVFPLLVMSFCYTRIL---VEINRQ 266
European catfish GnRH-R1         233 RWQETAYNMFHFVTLYVFPLLVMSLCYTRIL---VEINRQ 269
Coho salmon GnRH-R2b             92  RWQETVYNMFHFVTLYVFPLLVMSFCYTRIL---IEISRQ 128
                                         :      **  *
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Common octopus GnRH-R              260 FHDTPISPTSMSCYSVNHGQIRTHLFERARKRSSRMSAVV 299
Human GnRH-R2                      223 LHPDTGPSSGP---------------PRTTTESVQEQ--- 244
Rat GnRH-R1                        306 LHQDP---RKLQLNQSKNN-I-----PRARLRTLKMTVAA 336
Human GnRH-R1                      272 LHQDP---HELQLNQSKNN-I-----PRARLKTLKMTVAA 302
Dybowski's frog GnRH-R3            222 MKKAEASSREVNLRRSYNN-I-----PRARMRTFKMSLVV 255
Chicken GnRH-R2                    193 MGSSLFSSRDVPLRCSGSN-I-----PRARLRTLKMSLVV 226
Zebrafish GnRH-R2                  271 MTEDRLLSNKVQLRRSKNN-I-----PKARMRTLKMTVVV 304
Dybowski's frog GnRH-R1            274 MSKGTLSSKEVYLRCSKNN-I-----PKARTRTLKMSVVV 307
Atlantic cod GnRH-R2c              97  MRKAS-SCNEAPLRCSRDN-I-----PRARMRTLKMSLVV 129
Medaka GnRH-R1                     269 MTKKNVSSDEPHLRCSKNN-I-----PKARMRTLKMSVVV 302
Atlantic cod GnRH-R2a              187 MRKDNLSSTEVHLRRSKNN-I-----PKARMRTLKMSIVV 220
Rock gunnel GnRH-R1                285 LKKDN-----LDLRCSKNN-I-----PKARMRTLKMSVVV 313
African cichlid GnRH-R1            218 LKKDNLPSSEMHLRCSKNN-I-----PRARMRTLKMSIVV 251
Black porgy GnRHR1                 286 LRKDNLPSNEVHLRRSKNN-I-----PRARMRTLKMSIVV 319
European seabass GnRH-R1           286 MKKDNLPSNEVHLRRSKNN-I-----PRARMRTLKMGIVV 319
Japanese flounder GnRH-R           276 LKSHNLSSNEVHLRCSKNN-I-----PRARMRTLKMSIVV 309
Winter flounder GnRH-R1            276 LYTDNLSSNEVRLRCSKNN-I-----PRARMRTLKMSIVV 309
European flounder GnRH-R           163 LNMDNLSSNEVRLRCSKNN-I-----PRARMRTLKMSIVV 199
Zebrafish GnRH-R4                  283 MTKGNISSKEVHLRRSHSN-I-----PKARMRTLKMSIVV 316
Coho salmon GnRH-R1                103 MTHGNMSSKEIHLRRSHNN-I-----PKARMRTLKMSIVV 136
Atlantic cod GnRH-R2b              99  MGRGSRLSREVHLRRSHNN-I-----PKARMRTLKMSIVV 132
Medaka GnRH-R3                     276 IARTNMVSRDIHLRRSHNN-I-----PKARMRTLKMSIVV 309
Black porgy GnRH-R2                139 MARNNLLSRDVHLRRSHNN-I-----PKARMRTLKMSIVV 172
Chicken GnRH-R1                    265 LKINK------SLVRSQNDHI-----SKARMKTLKMTIVV 293
Dybowski's frog GnRH-R2            264 MKHKN------ELARSKNDLI-----SKARLKTLKMTLVV 292
Medaka GnRH-R2                     275 HLRDK--AGESHLRRSGTDII-----PKARMKTLKMSVVV 307
Rock gunnel GnRH-R2                183 HLQDK--AGESHLRRSGTDII-----PKARMKTLKMTVVV 215
Pejerrey GnRH-R1b                  269 HLRDK--AGESHLRRSGTDII-----PKARMKALKMTVVV 301
African cichlid GnRH-R             269 HLRDK--AGESPLRRSGTDII-----PKARMKTLKMTVVV 301
Winter flounder GnRH-R2            191 HMRDK---GESYLRRSGTDII-----PKARMKT------- 215
European seabass GnRH-R2           273 HLRDK--AGESYLRRSGTDII-----PKARMKTLKMTVVV 305
Orange spotted grouper GnRH-R1     257 HLRDK--AGESYLRRSGTDII-----PKARLKTLKMTVVV 305
Zebrafish GnRH-R3                  247 LHNSN---KGDSLRRSGTDMI-----PKARMKTLKMTLII 294
Goldfish GnRH-R1                   260 LHKST---EGESLRRSGTDMI-----PKARMKTLKMTIII 307
European catfish GnRH-R2           270 EHKNK--AGESCLRRSRTDMI-----PKARMKTLKMTIII 318
Atlantic cod GnRH-R1b              104 HQQNK--AGESYLRRSGTDMI-----PKARMKTLKMTIII 136
Coho salmon GnRH-R2a               129 LHRNK--AGESCLRRSGTDMI-----PKARMKTLKMTIII 161
Goldfish GnRH-R2                   269 MPRGKGKGGEPCLRRSGTNMI-----PKARMKTLKMTIII 303
Zebrafish GnRH-R1                  267 MPRGKGKGGEPCLRRSGADMI-----PKARMKTLKMTIII 301
European catfish GnRH-R1           270 MHRSKDKAGEPCLRRSGTDMI-----PKARMKTLKMTIII 304
Coho salmon GnRH-R2b               129 MHKGK--GGEPCLRRSGADMI-----PKARMKTLKMTIVV 161
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Common octopus GnRH-R           300 IVAAFILCWTPYYIIFLGFAFFQ------WDNSRTVIYFF 333
Human GnRH-R2                   245 --------------------YTKSTAEDSKNDGCICH--F 262
Rat GnRH-R1                     337 FATSFVICWTPYYVLGIWYWFDPEMLN--RVSEPVNHFFF 374
Human GnRH-R1                   303 FATSFTVCWTPYYVLGIWYWFDPEMLN--RLSDPVNHFFF 340
Dybowski's frog GnRH-R3         256 IVLTFIVCWTPYYLLGIWYWFSPEMLTSRRVPPSLSHILF 295
Chicken GnRH-R2                 227 IVSSFILCWTPYYLLGLWYWFCPRAMQQ-KVPPSLSHILF 265
Zebrafish GnRH-R2               305 IVLSFMVCWTPYYMLGLWYWFSPAGLEE-TVSQSLSHILF 343
Dybowski's frog GnRH-R1         308 IVSSFIICWTPYLLLGLWYWFFPEIMEE-KVSQSTTHILF 346
Atlantic cod GnRH-R2c           130 IVLCFIVCWTPYYLLGLWYWFFPDHIEG-KVSHSLTHILF 168
Medaka GnRH-R1                  303 IVVGFIVCWTPYYLLGLWYWFFPDDLEG-KVSHSLTHILF 341
Atlantic cod GnRH-R2a           221 IVLSFIVCWTPYYLLGLWYWFFPDDLEG-KVSHSLTHILF 279
Rock gunnel GnRH-R1             314 IVSSFIICWTPYYLLGLWYWFFPDDLEG-KVSHSLTHILF 352
African cichlid GnRH-R1         252 IVLSFIICWTPYYLLGLWYWFFPDDLEG-KVSHSLTHILF 290
Black porgy GnRHR1              320 IVSSFIICWTPYYLLGLWYWFFPDDLEG-KVSHSLTHILF 358
European seabass GnRH-R1        320 IVSSFIVCWTPYYLLGLWYWFFPDDLEG-KVSHSLTHILF 358
Japanese flounder GnRH-R        310 IVLSFIICWTPYYLLGLWYWFFPDDLEG-KVSHSLTHILF 348
Winter flounder GnRH-R1         310 IVLSFIICWTPYYLLGLWYWFFPDDLEG-KVSQSLTHILF 348
European flounder GnRH-R        200 IVLSFIICWTPYYL---------------------------- 213
Zebrafish GnRH-R4               317 IVTSFIVCWTPYYLLGLWYWFLPEDLEE-TVSHSLTHMLF 355
Coho salmon GnRH-R1             137 IVTSFIVCWTPYYLLGLWYWF------------------- 157
Atlantic cod GnRH-R2b           133 IVTSFIVCWTPYYLLGLWYWPFPEDMEE-TVSHSLTHMLF 171
Medaka GnRH-R3                  310 IVTSFIICWTPYYLLGLWYWLFPEKMEE-TVSHSLTHMLF 348
Black porgy GnRH-R2             173 IVTSFIICWTPYYLITS---------------------- 189
Chicken GnRH-R1                 294 IVASFIICWTPYYLLGLWYWFHPAMIQ--RMPEYINHSFF 331
Dybowski's frog GnRH-R2         293 IVVSFMVCWTPYYLLGIWYWFQPEMIY--LTPEYVHHSLF 330
Medaka GnRH-R2                  308 IVLSFVVCWTPYYLLGIWYWFQPDMLR--VTPEYVHHILF 345
Rock gunnel GnRH-R2             216 IVLSFIVCWTPYYLLGIWYWFQPDMLR--VTPEYVHHALF 253
Pejerrey GnRH-R1b               302 IVLSFVVCWTPYYLLGIWYWFQPGMLR--VTPEYVHHALF 339
African cichlid GnRH-R          302 IVLSFVVCWTPYYLLGIWYWFQPDMLR--ITPEYVHHALF 339
Winter flounder GnRH-R2         215 ------------------------------------------ 215
European seabass GnRH-R2        306 IVLSFVVCWTPYYLLGIWYWFQPDMLH--VTPEYIHH--- 340
Orange spotted grouper GnRH-R1  306 IVLSFVVCWTPYYLLGIWYWFQPDMLR--VTPEYVHHALF 343
Zebrafish GnRH-R3               295 IVLSFVVCWTPYYLLGIWYWFQPEMLT--VTPEYVHHLLF 332
Goldfish GnRH-R1                308 IVLSFVVCWTPYYLLGIWYWFQPEMLK--VTPEYIHHLLF 345
European catfish GnRH-R2        319 IVLSFIICWTPYYLLGIWYWFQPEMLK--VTPEYVHHLLF 354
Atlantic cod GnRH-R1b           137 IVMTFIVCWTPYYLLGIWYWFQPQMLA--VT---------- 165
Coho salmon GnRH-R2a            162 IVMSFVV------------------------------- 168
Goldfish GnRH-R2                304 IVASFVVCWTPYYLLGIWYWFQPRMLQ--SMPEYIHHALF 341
Zebrafish GnRH-R1               302 IVASFVVCWTPYYLLGIWYWFQPRMLQ--VTPVYAHHALF 339
European catfish GnRH-R1        305 IVASFVICWTPYYLLGIWYWFQPQMLH--VIPDYVHHVFF 342
Coho salmon GnRH-R2b            162 IVVSFVI------------------------------- 168
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Common octopus GnRH-R           334 TLGTSNCMLNPLIYGAFT-------IYKVHRGRSGSANSP 366
Human GnRH-R2                   263 IYCLLDSLLCPRNLVLV---------------------- 279
Rat GnRH-R1                     375 LFAFLNPCFDPLIYGYFSL--------------------- 393
Human GnRH-R1                   341 LFAFLNPCFDPLIYGYFSL--------------------- 360
Dybowski's frog GnRH-R3         296 LFGLFNTCLDPIIYGLFTIHFRRE-IRRVCRCAT-QGKDA 334
Chicken GnRH-R2                 266 IFGLFNACLDPITYGLFTIPFRRR-----CGCPCGHSSEP 300
Zebrafish GnRH-R2               344 IFGLLNACLDPIIYGLFTVPLCRG-MRHRQREQNIVAFEL 382
Dybowski's frog GnRH-R1         347 IFGLVNACLDPITYGLFTIHFRKS-LQRYCGGR--RTSDA 383
Atlantic cod GnRH-R2c           169 IFGLFNACLDP---------------------------- 179
Medaka GnRH-R1                  342 IFGLFNTCLDPIIYGLFTTRFHRG-RRKCYGGAT-ATLSL 379
Atlantic cod GnRH-R2a           280 IFGLFNACLDPVIYGLFTVHFRKGLLKRYYRRDTVPASDP 319
Rock gunnel GnRH-R1             353 IFGLVNACLDPVIYGLFTIHFRKG-LRRYYGNAA-STAEV 390
African cichlid GnRH-R1         291 IFGLVNACLDPLIYGLFTIHFRKG-LRRFYTGTT-TSADL 328
Black porgy GnRHR1              359 IFGLVNACLDPVIYGLFTIHFRTG-LRRYYRNAT-TASDL 396
European seabass GnRH-R1        359 IFGLVNACLDPVIYGLFTIHFRKG-LRRYYCNAT-KAADL 396
Japanese flounder GnRH-R        349 IFGLVNACLDPVIYGLFTIHFRKG-LRRYFCRAP-PAPDL 386
Winter flounder GnRH-R1         349 IFGLLSACLDPVIYGLFTIHFRKG-LRRYFFKAP-PASDL 386
European flounder GnRH-R        213 ---------------------------------------- 213
Zebrafish GnRH-R4               356 IFGLFNAILDPITYGLFTIHFRKG-LKRYCRSAV-VLTES 393
Coho salmon GnRH-R1             157 ---------------------------------------- 157
Atlantic cod GnRH-R2b           172 IFGLFNACLDPITYGLFTIHFHHG-LKRHHLNAAAATATA 210
Medaka GnRH-R3                  349 IFGLFNACLDPITYGLFTIHLRQG-AKRRRQISN-AQTEL 386
Black porgy GnRH-R2             189 ---------------------------------------- 189
Chicken GnRH-R1                 332 LFGLLHTCTDPIIYGLYTPSFRED-VQLCLRGIEAAISQH 370
Dybowski's frog GnRH-R2         331 LFGLLHTCTDPLVYGLYTPSFKED-LRTWLRRLGGLLTRK 369
Medaka GnRH-R2                  346 VFGNLNTCCDPVIYGFYTPSFRAD-LAACCR------WSK 378
Rock gunnel GnRH-R2             254 VFGNLNTCCDPLIYGFYTXSFRAD-LAALCR------RTK 286
Pejerrey GnRH-R1b               340 VFGNLNTCCDPVIYGFYTPSFRAD-LAACCR------RTR 372
African cichlid GnRH-R          340 VFGNLNTCCDPIIYGFYTPSFRAD-LAACCR----WVRCD 374
Winter flounder GnRH-R2         215 ---------------------------------------- 215
European seabass GnRH-R2        341 ----------------------KG----------------- 343
Orange spotted grouper GnRH-R1  344 LFGNLNTCCDPIIYGFYTPSFRAD-LAACCR------RTK 376
Zebrafish GnRH-R3               333 VFGNLNSCCDPVIYGLYTPSFRSD-LIRFCC------CRH 365
Goldfish GnRH-R1                346 VFGNLNTCCDPVIYGLYTPSFRAD-LARCWR------CRT 378
European catfish GnRH-R2        355 VFGNLNTCCDPVIYGLYTPSFRAD-LARCWG------CRS 387
Atlantic cod GnRH-R1b           166 ---------------------------------------- 345
Coho salmon GnRH-R2a            169 ---------------------------------------- 354
Goldfish GnRH-R2                342 VFGNLNTCCDPVIYGFFTPSFRAD-IASCFC------RRN 374
Zebrafish GnRH-R1               340 VFGNLNTCCDPVIYGFFTPSFRAD-ITSCFS------RRN 372
European catfish GnRH-R1        343 VFGNLNTCCDPVIYGFFTPSFRAD-LSRCFC------WRN 375
Coho salmon GnRH-R2b            168 ---------------------------------------- 168
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Common octopus GnRH-R            367 SGTRLMIMVNKRGRSTTTTTNRMSGSGRRQLTTGQTITQC 406
Human GnRH-R2                    279 ------------------------------------------ 279
Rat GnRH-R1                      393 ------------------------------------------ 393
Human GnRH-R1                    360 ------------------------------------------ 360
Dybowski's frog GnRH-R3          335 DA-TSLGTGSFRVSTAAVPLKRSAGVS------------- 360
Chicken GnRH-R2                  301 EP-PSPATGSFHCSASSLRGRQGMGGT------------- 326
Zebrafish GnRH-R2                383 ENTNNSLMTSIRTSTSPLTLKRLAVQQ-----------T 410
Dybowski's frog GnRH-R1          384 DT-SSSVTGSFRCSMSSFRAKKMIVLN------------ 409
Atlantic cod GnRH-R2c            180 ------------------------------------------ 180
Medaka GnRH-R1                   380 ES-KVVTAEAVKRSSDASASRGDAGEK------------- 405
Atlantic cod GnRH-R2a            320 EN-STVITGPFRCVSNAFPLKRQPSSA-ARER-------- 349
Rock gunnel GnRH-R1              391 ES-TTVITGSLYSPPNSLSLRREFRRF-SQEKLVVCSDGH 330
African cichlid GnRH-R1          329 EN-NTVITGSFSCAANSLSLKREVSPA-SPERLLLC---- 362
Black porgy GnRHR1               397 DN-NTVITGSFVCAANSLQLKREVSPV-SQERFMLCSDSH 336
European seabass GnRH-R1         397 DN-NTVITGSFICAANSLPLKREASQE----RFMLYSDNH 431
Japanese flounder GnRH-R         387 DN-NTVLTGSFTCTANILPLKREVSPA-SQEKSTMYGNNH 424
Winter flounder GnRH-R1          387 DN-HTVITGSLTCAASISPLKRELSLAVSQEKFITYRSNH 425
European flounder GnRH-R         213 ------------------------------------------ 213
Zebrafish GnRH-R4                394 EN-NSIITGSLKCSPSPFRMKRVTQSG------------- 419
Coho salmon GnRH-R1              157 ------------------------------------------ 157
Atlantic cod GnRH-R2b            211 SATASATASASRRRPATALRAALCPLA------------- 237
Medaka GnRH-R3                   387 EN-NSCLMQM--SCLSAHRQNVSSGLS------------- 410
Black porgy GnRH-R2              189 ------------------------------------------ 189
Chicken GnRH-R1                  371 VRHKPISVSEKTTKDGDVNGQVTSGGS------------- 397
Dybowski's frog GnRH-R2          370 AKNSKPLADSEMNIKDFTSMDGPTTTA------------- 396
Medaka GnRH-R2                   379 ESSSPMSLNRLSTRRGPHSGEHEPQ--------------- 403
Rock gunnel GnRH-R2              287 SDASPRSLDRMSARQGPHSSEDDQQAG------------- 313
Pejerrey GnRH-R1b                373 SDSSPRSLGRLSARQGPHSGEQEPPRP------------- 399
African cichlid GnRH-R           375 ADTSSQYIDRMSTREGPHSREHEPQPS------------- 401
Winter flounder GnRH-R2          215 ------------------------------------------ 215
European seabass GnRH-R2         343 ------------------------------------------ 343
Orange spotted grouper GnRH-R1   377 SDASLRSPDRLSARQDPHSAEHEADPT------------- 403
Zebrafish GnRH-R3                366 HKNTPRP--------QQPTRHT------------------ 379
Goldfish GnRH-R1                 379 PAESPRSLDRIPHENTSPTRPA------------------ 400
European catfish GnRH-R2         388 TSDSPRSLERLSARQGPHSAEQESDPA------------- 414
Atlantic cod GnRH-R1b            345 ------------------------------------------ 345
Coho salmon GnRH-R2a             354 ------------------------------------------ 354
Goldfish GnRH-R2                 375 QNSSLKSLDRLSVRRGGASREAESDLG------------- 401
Zebrafish GnRH-R1                373 QNCSPKSLDRLSARRGGASGEAESDPG------------- 399
European catfish GnRH-R1         376 QNASAKSLPHFSGHRREVSGEAESDLG------------- 402
Coho salmon GnRH-R2b             168 ------------------------------------------ 169
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Common octopus GnRH-R              407 ASLTNPHQPVRPSPGINSTTSPNGKMPTKPPG- 445
Human GnRH-R2                      279 -------------------------------- 279
Rat GnRH-R1                        393 -------------------------------- 393
Human GnRH-R1                      360 -------------------------------- 360
Dybowski's frog GnRH-R3            361 GGSCKFDLEVTGVGLHSGKCEHCKRQIVESFM- 399
Chicken GnRH-R2                    327 EGPHPPIELGLPTGAGSCQSSA-L--------- 349
Zebrafish GnRH-R2                  411 SREKLEWDAEFVLKTGDDGTKNALYSSGVATVE 450
Dybowski's frog GnRH-R1            410 -----QELQVLQSCNGNFNNPEFRLNGLGTSCL 436
Atlantic cod GnRH-R2c              180 -------------------------------- 180
Medaka GnRH-R1                     406 --------------DNNSARTERQSSGGNI-- 420
Atlantic cod GnRH-R2a              350 STPGAKSPRSDRLTVGSDAGGCEARTPSTVSKL 389
Rock gunnel GnRH-R1                331 RRVEPPSLNSSFL-----EDR-DLQQSSPESVL 364
African cichlid GnRH-R1            363 -KAEWTPPRSSFLTADNDTDR-KTHQSSAESIL 400
Black porgy GnRHR1                 337 SREESTPSRSSFLTADNDAEK-DSHQFCSDSII 376
European seabass GnRH-R1           432 SRAESTSPRSSFLRDPNQSSSESNL-------- 456
Japanese flounder GnRH-R           425 GKEESTSQGGSFLTADIYTAR-DRNQSSSDSTM 463
Winter flounder GnRH-R1            426 SKEESTSPSGSFLTADNNTARDVNQFSSDSTV- 464
European flounder GnRH-R           213 -------------------------------- 213
Zebrafish GnRH-R4                  420 -------TGTDPKQNTSTVGEEDKKAADGKTKE 445
Coho salmon GnRH-R1                157 -------------------------------- 157
Atlantic cod GnRH-R2b              238 -------------TEARTTPRSPAPSARCNELE 257
Medaka GnRH-R3                     411 -------KHTEEINDNSSTKNASSPSISVSRI- 435
Black porgy GnRH-R2                189 -------------------------------- 189
Chicken GnRH-R1                    398 ---------------NGTTVNTVC--------- 406
Dybowski's frog GnRH-R2            397 --------------------------TTVQ--- 400
Medaka GnRH-R2                     404 -----------------------------SVF 406
Rock gunnel GnRH-R2                314 ---------------PAHRPPSDGPTREQK--- 328
Pejerrey GnRH-R1b                  400 -------------ADPQAATDCERPIRGQD--- 416
African cichlid GnRH-R             402 ---------------TNNQTAADQPMKARDFSM 419
Winter flounder GnRH-R2            215 -------------------------------- 215
European seabass GnRH-R2           343 -------------------------------- 343
Orange spotted grouper GnRH-R1     404 ---------------TNNQPAGD---------- 411
Zebrafish GnRH-R3                  379 -------------------------------- 379
Goldfish GnRH-R1                   400 -------------------------------- 400
European catfish GnRH-R2           415 ---------------SVKGAEG----------- 421
Atlantic cod GnRH-R1b              345 -------------------------------- 345
Coho salmon GnRH-R2a               354 -------------------------------- 354
Goldfish GnRH-R2                   402 ---------------SGDQPSGQQA-------- 411
Zebrafish GnRH-R1                  400 ---------------SGDQPSGQTA-------- 410
European catfish GnRH-R1           403 ---------------SGDQPSGQ---------- 410
Coho salmon GnRH-R2b               169 -------------------------------- 169
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1     ACTTGCGTCCGCTGATGCTGAGCCTAGCATCGGCTGACCTGATGATGACCTTCGTGGTGATGCCT   65
1       L  R  P  L  M  L  S  L  A  S  A  D  L  M  M  T  F  V  V  M  P     21

66    CTGGACGCAGTGTGGAACCGGACGGTGCAGTGGTATGGAGGAGACGTGCTCTGTAAACTCCTTTCC  121
22     L  D  A  V  W  N  R  T  V  Q  W  Y  G  G  D  V  L  C  K  L  L  S     43

122   TTCCTGAAGCTGTTTGCCATGCACGCCTCGGCCTTCATCCTCGTTGTCATCAGCCTGGACCGCCAG  187
44     F  L  K  L  F  A  M  H  A  S  A  F  I  L  V  V  I  S  L  D  R  Q     65

188   CACGCCATCCTGCACCCGCTGGATGCTCTGAGTGCTCATCGTAGGAATCGGCGAATGGTTCTGATG  253
66     H  A  I  L  H  P  L  D  A  L  S  A  H  R  R  N  R  R  M  V  L  M     87

254   GCCTGGAGCCTCAGCCTGCTGCTCGCATCACCACAACTCTTCATCTTCAGGACCATCATGGTGGAC  319
88     A  W  S  L  S  L  L  L  A  S  P  Q  L  F  I  F  R  T  I  M  V  D    109

320   GACGGTGTGGACTTCACTCAGTGTGCGTCTCACGGCAGCTTCAGGCGCCGTTGGCAGGAGACACTT  385
110    D  G  V  D  F  T  Q  C  A  S  H  G  S  F  R  R  R  W  Q  E  T  L    131

386   TACAACATGTTTCACTTCATCACGCTGTACGTGGTTCCCCTGCTGGTCATGAGCTGCTGCTACAGC  451
132    Y  N  M  F  H  F  I  T  L  Y  V  V  P  L  L  V  M  S  C  C  Y  S    153

452   CGCATCCTGCTGCACATTCACCAGCAGCACATGAGGGACAAAGGGGAATCGTACCTGCGTCGCAGC  517
154    R  I  L  L  H  I  H  Q  Q  H  M  R  D  K  G  E  S  Y  L  R  R  S    175

518   GGCACTGACATCATCCCCAAAGCCCGAATGAAGACC                                553
176    G  T  D  I  I  P  K  A  R  M  K  T                                  187
```

Figure 3.7. Annotated winter flounder GnRH-R2/a1 partial cDNA sequence with nucleotides and deduced amino acids.

classification for GnRH-R2 is more ambiguous than GnRH-R1. GnRH-R2 amino acid similarities are in the range 51-91% with other vertebrate GnRH-Rs (Figure 3.6).

3.3.2. Phylogenetic analyses of winter flounder GnRHs and GnRH-receptor isoforms

The deep nodes clustering all cGnRH sequences and all sGnRH sequences have bootstrap values of 99% and 100%, respectively (Figure 3.8). The phylogenetic distinctiveness of the sbGnRH cluster appears to be less discernable with a bootstrap value of only 41%. The majority of bootstrap values for the shallower nodes within each of these major clusters are typically <85%.

Three main clusters with bootstrap values greater than 95% are observed in the GnRH-R phylogeny (Figure 3.9). Rat and human GnRH-R1s cluster to form the GnRH-Ra2 clade (mammalian-specific) with the SDP aa motif. Human GnRH-R2 and octopus GnRH-R have DGC and NSR aa motifs, respectively, which are not consistent with any previously described extracellular loop 3 amino acid patterns. The GnRH-Ra1 (non-mammalian-specific), GnRH-Rb1 (tetrapod-specific) and GnRH-Rb2 (non-mammalian-specific) clusters conform to the extracellular loop 3 motif observed in the alignments, which are PEY, PPS and SHS, respectively. Winter flounder GnRH-R1 clusters with GnRH-Rb2 clade with an SQS motif, similar to amphibians and not other fish (SHS). Flounder GnRH-R2 belongs to the GnRH-Ra1 clade; therefore, we would expect to find the PEY aa motif in the extracellular loop 3 segment.

Figure 3.8. Neighbour-joining phylogenetic analyses for gonadotropin-releasing hormone (GnRH) isoforms in vertebrates. Box indicates chicken GnRH-2 variant (cGnRH-2), bolded box is seabream GnRH-1 (sbGnRH-1) and dashed box is salmon GnRH-3 (sGnRH-3). Distance matrix is 1.044 bootstrap support (1000 replicates) as indicated above nodes for sbGnRH-1, cGnRH-2 and sGnRH-3, respectively. Accession numbers are the same as Figure 3.2

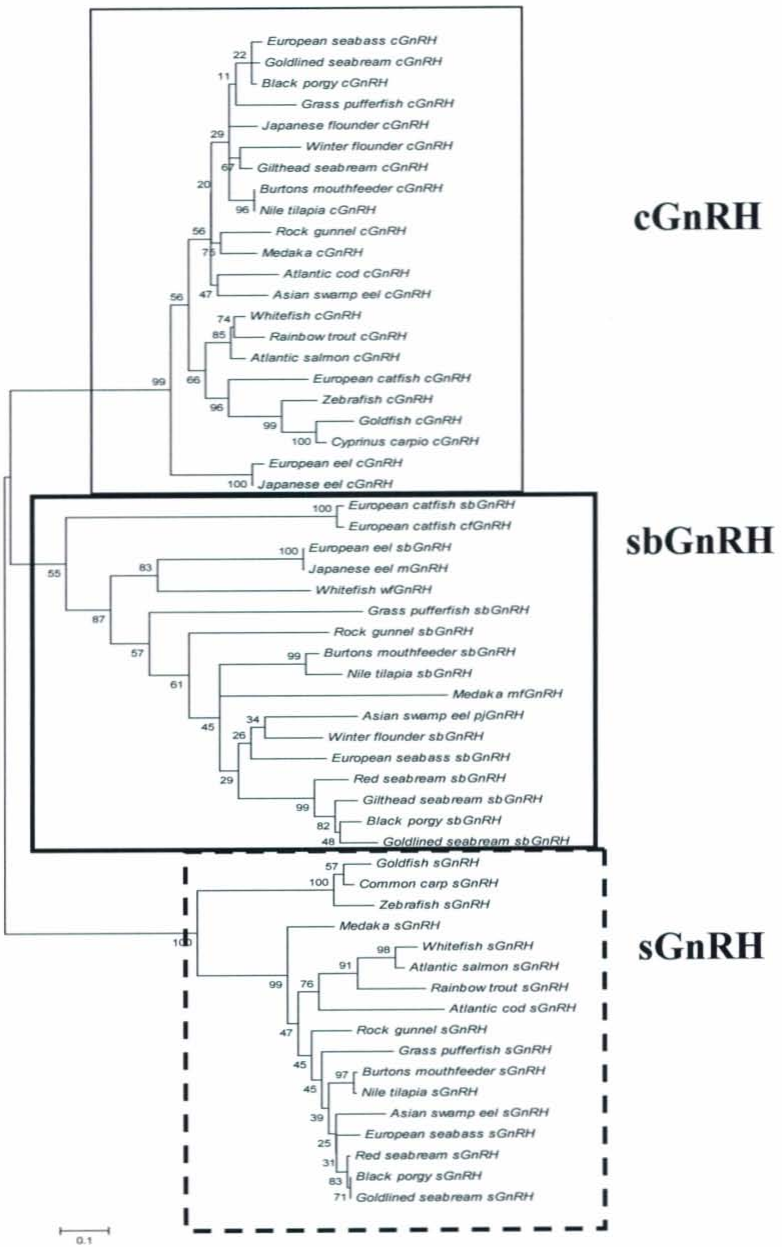
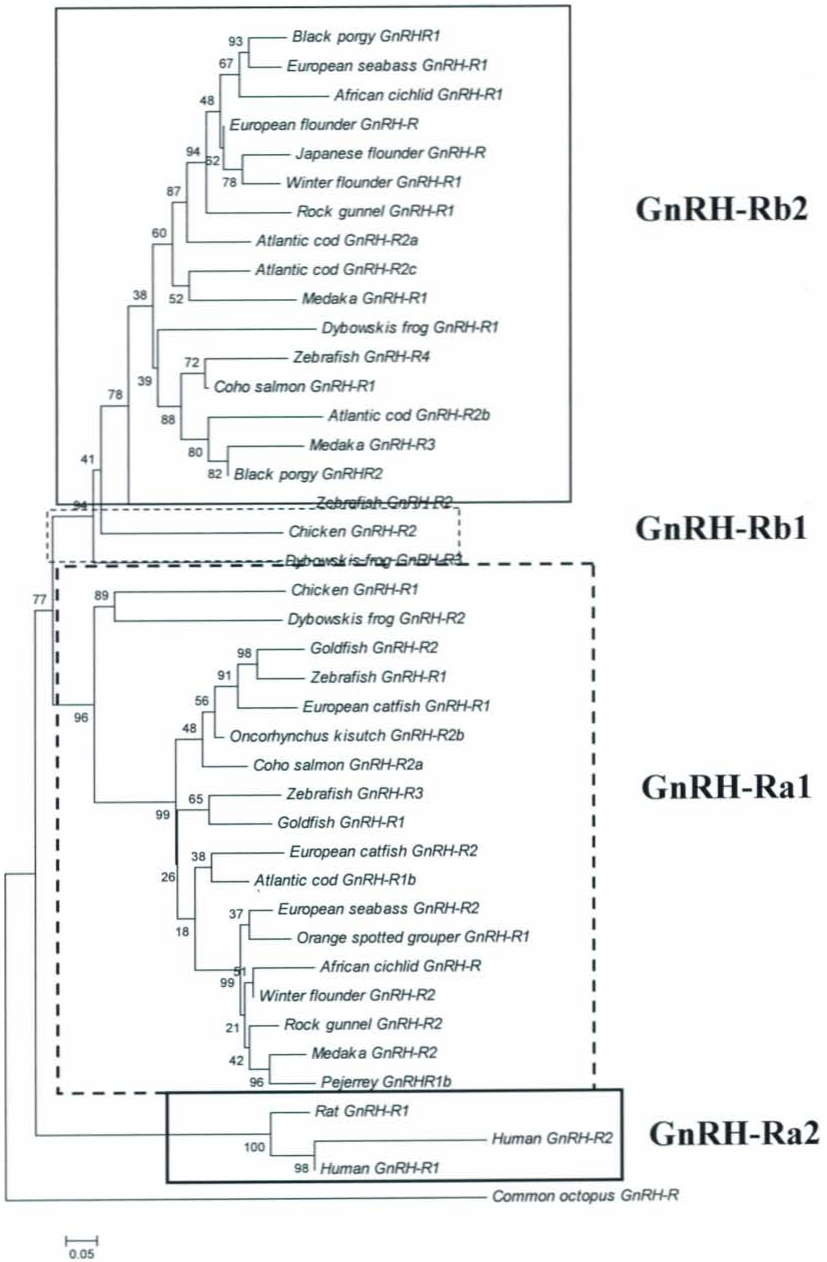

European seabass cGnRH
Goldlined seabream cGnRH
Black porgy cGnRH
Grass pufferfish cGnRH
Japanese flounder cGnRH
Winter flounder cGnRH
Gilthead seabream cGnRH
Burtons mouthfeeder cGnRH
Nile tilapia cGnRH
Rock gunnel cGnRH
Medaka cGnRH
Atlantic cod cGnRH
Asian swamp eel cGnRH
Whitefish cGnRH
Rainbow trout cGnRH
Atlantic salmon cGnRH
European catfish cGnRH
Zebrafish cGnRH
Goldfish cGnRH
Cyprinus carpio cGnRH
European eel cGnRH
Japanese eel cGnRH
cGnRH
European catfish sbGnRH
European catfish cfGnRH
European eel sbGnRH
Japanese eel mGnRH
Whitefish wfGnRH
Grass pufferfish sbGnRH
Rock gunnel sbGnRH
Burtons mouthfeeder sbGnRH
Nile tilapia sbGnRH
Medaka mfGnRH
Asian swamp eel pjGnRH
Winter flounder sbGnRH
European seabass sbGnRH
Red seabream sbGnRH
Gilthead seabream sbGnRH
Black porgy sbGnRH
Goldlined seabream sbGnRH
sbGnRH
Goldfish sGnRH
Common carp sGnRH
Zebrafish sGnRH
Medaka sGnRH
Whitefish sGnRH
Atlantic salmon sGnRH
Rainbow trout sGnRH
Atlantic cod sGnRH
Rock gunnel sGnRH
Grass pufferfish sGnRH
Burtons mouthfeeder sGnRH
Nile tilapia sGnRH
Asian swamp eel sGnRH
European seabass sGnRH
Red seabream sGnRH
Black porgy sGnRH
Goldlined seabream sGnRH
sGnRH
0.1

Figure 3.9. Neighbour-joining phylogenetic analysis for gonadotropin-releasing hormone receptors (GnRH-Rs) in vertebrates. Box indicates GnRH-Rb2 clade, dashed box is GnRH-Rb1 cluster, bolded dashed box is GnRH-Ra1 clade and bolded box indicates GnRH-Ra2 clade. Distance matrix is 0.636 bootstrap support (1000 replicates) as indicated above nodes for GnRH-Ra1, GnRH-Ra2, GnRH-Rb1 and GnRH-Rb2, respectively. Accession numbers are the same as Figure 3.6


Black porgy GnRHR1
European seabass GnRH-R1
African cichlid GnRH-R1
European flounder GnRH-R
Japanese flounder GnRH-R
Winter flounder GnRH-R1
Rock gunnel GnRH-R1
Atlantic cod GnRH-R2a
Atlantic cod GnRH-R2c
Medaka GnRH-R1
Dybowskis frog GnRH-R1
Zebrafish GnRH-R4
Coho salmon GnRH-R1
Atlantic cod GnRH-R2b
Medaka GnRH-R3
Black porgy GnRHR2
Zebrafish GnRH-R2
Chicken GnRH-R2
Dybowskis frog GnRH-R3
Chicken GnRH-R1
Dybowskis frog GnRH-R2
Goldfish GnRH-R2
Zebrafish GnRH-R1
European catfish GnRH-R1
Oncorhynchus kisutch GnRH-R2b
Coho salmon GnRH-R2a
Zebrafish GnRH-R3
Goldfish GnRH-R1
European catfish GnRH-R2
Atlantic cod GnRH-R1b
European seabass GnRH-R2
Orange spotted grouper GnRH-R1
African cichlid GnRH-R
Winter flounder GnRH-R2
Rock gunnel GnRH-R2
Medaka GnRH-R2
Pejerrey GnRHR1b
Rat GnRH-R1
Human GnRH-R2
Human GnRH-R1
Common octopus GnRH-R
0.05
GnRH-Rb2
GnRH-Rb1
GnRH-Ra1
GnRH-Ra2

3.3.2. Tissue distribution for winter flounder cGnRH-2 and sGnRH-3 transcripts

Due to time constraints, all peripheral tissue mRNA distributions and central nervous system tissue distributions for sbGnRH, GnRH-R1 and GnRH-R2 have not been completed. cGnRH mRNA is predominantly expressed in the optic tectum/thalamus, with some expression in the telencephalon/preoptic area and hypothalamus (Figure 3.10 and 3.11) (as seen below in qPCR experiments) sGnRH is found throughout the brain, but not in the pituitary, with apparent highest expression in the telencephalon/preoptic area (Figure 3.10).

3.3.3. Effects of fasting on winter flounder GnRH isoforms mRNA expression

Based on the information from the tissue mRNA distributions as well as previous reports identifying appetite-regulating regions in fish brain (Demski and Knigge 1971; Peter 1979), we chose to quantify GnRH mRNA expression in telencephalon/preoptic area, optic tectum/thalamus and hypothalamus using qPCR (Figure 3.11). No significant differences in sbGnRH mRNA expression levels are observed in any brain region of fasted fish compared with fed fish (Figure 3.11). cGnRH mRNA expression was significantly higher in the optic tectum/thalamus and hypothalamus, but not the telencephalon/preoptic area, of fed fish (Figure 3.11). Significantly higher sGnRH mRNA expression was observed in the telencephalon/preoptic area, but not the optic tectum/thalamus or the hypothalamus of fed fish (Figure 3.11).

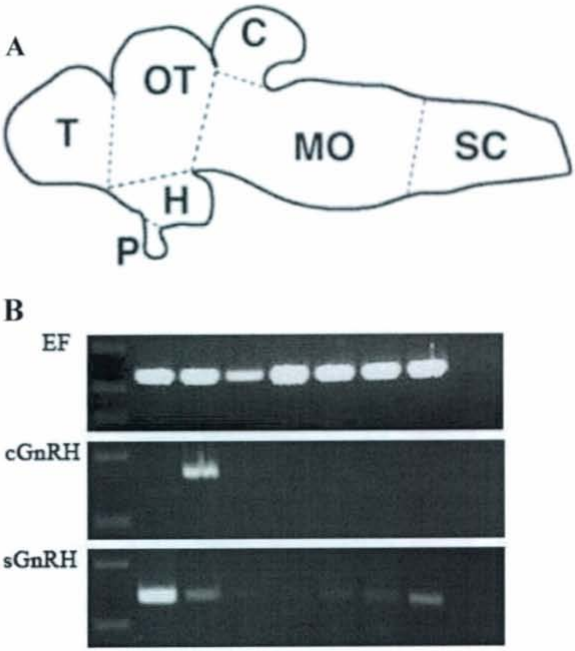


Figure 3.10. A) Schematic diagram of the winter flounder brain dissection. B) Qualitative tissue distribution of elongation factor 1α (EF; control), chicken gonadotropin-releasing hormone (cGnRH), salmon GnRH (sGnRH) transcripts in winter flounder, *Pseudopleuronectes americanus*, for various central nervous system tissues including the pituitary gland as detected by RT-PCR. L, ladder; T, telencephalon/preoptic area; OT, optic tectum/thalamus; H, hypothalamus; P, pituitary gland; C, cerebellum; MO, medulla oblongata; SC, spinal cord; Con, no-template control. Ladder bands included are 50bp, 150 bp and 300 bp.

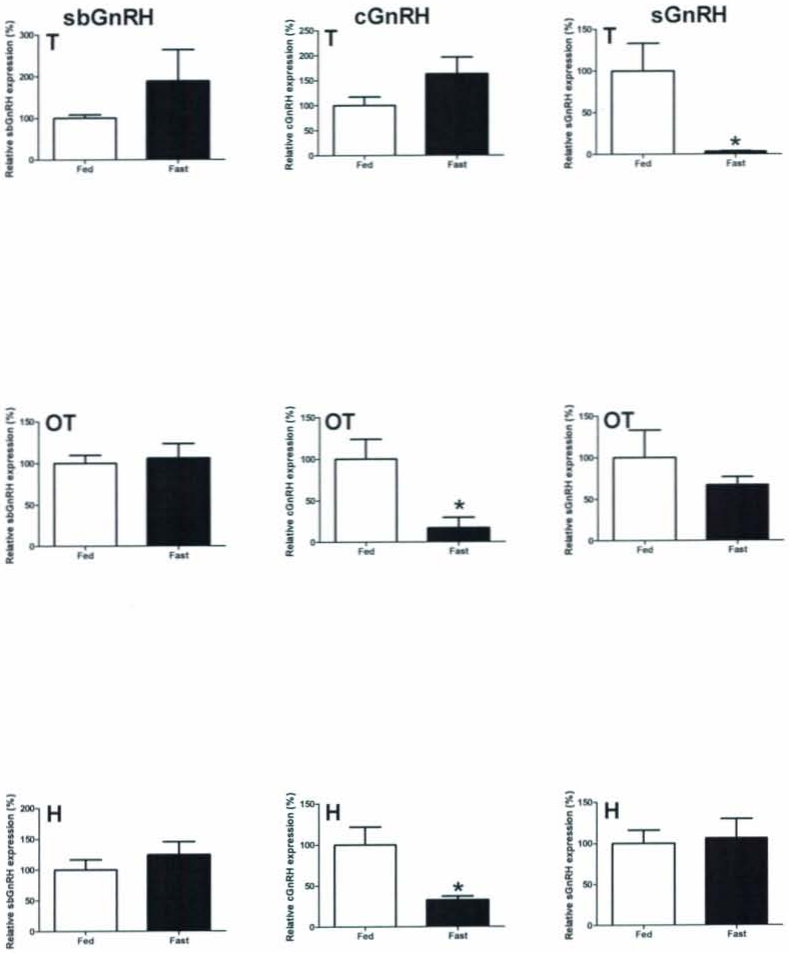


Figure 3.11. Relative mRNA expression levels for seabream GnRH (sbGnRH), chicken GnRH (cGnRH) and salmon GnRH (sGnRH) in fed and fasted winter flounder telencephalon/preoptic area (T), optic tectum/thalamus (OT) and hypothalamus (H). * indicates significance $p < 0.05$.

3.4. Discussion

The primary objective of our study was to determine whether transcripts encoding GnRH isoforms and receptors are influenced at the expression level by nutritional state in winter flounder. We first identified which forms (sbGnRH, cGnRH and sGnRH) and receptors (GnRH-R1 and GnRH-R2) are present in flounder and their phylogenetic relationships with other fish and vertebrates.

Partial nucleotide sequences for sbGnRH- and sGnRH-like forms and a complete cDNA sequence for a cGnRH-like form were identified. The partial winter flounder sbGnRH amino acid sequence is most similar to barfin (91% GenBank BAB83984), Japanese (84%; GenBank AAY83273) and Brazilian (84%; GenBank ACS88343) flounders, whereas a 36% identity is seen with wild turkey (*Meleagris gallopavo*) (GenBank AAT46353) GnRH. According to the phylogenetic analyses in this paper and reviews, sbGnRH appears to be the most recently evolved form of GnRH in teleosts and is the most diverse as it has undergone a higher degree of changes in the mature peptide (substitutions at positions 5, 7 and 8) (Fernald and White 1999; Chen and Fernald 2008; Okubo and Nagahama 2008). sbGnRH is also the teleost GnRH variant that is most similar to mGnRH. Phylogenetically, forms such as mdGnRH and cfGnRH could have evolved from sbGnRH, or vice versa, considering how conserved the mature cGnRH and sGnRH peptides are and based on previous and present phylogenetic analyses (reviewed by Chen *et al.* 2008; reviewed by Sherwood and Adams 2005). In contrast to the mature decapeptide, the signal peptide and GAP amino acid sequence are not well conserved. As the whole propeptide is usually taken into account in phylogenetic studies, these low

similarity levels might be in part the cause of a discordant phylogeny.

The mature peptide for cGnRH appears to be well conserved across vertebrate taxa. The 87 aa winter flounder sequence is most similar to other flounders, including barfin and Japanese flounder (98%; GenBank BAB83983 and AAY28981, respectively), as well as other teleosts such as rainbow trout (84%; GenBank AAF08687), striped bass (*Morone saxatilis*) (92%; GenBank AAD03816), black porgy (*Acanthopagrus schlegelii*; 95%; GenBank ABU92552) and false kelpfish (*Sebastiscus marmoratus*) (93%; GenBank ABS18279). Very low sequence identities are observed in comparisons with other vertebrates, including an ancient teleost, the Indonesian coelacanth (*Latimaria menadoensis*) (59%; GenBank ABZ04537), bullfrog (*Rana catesbeiana*) (63%; GenBank AAL05971), and a caecilian worm, the rubber eel (*Typhlonectes natans*) (63%; GenBank AAD48032). Phylogenetically, the teleost cGnRHs cluster together, however bootstrap values are low. Similar to sbGnRH, lower identities are the result of differences in the aa structure of the signal peptide and GAP, since the mature peptide is similar among vertebrate species.

Like cGnRH, the sGnRH mature decapeptide is extremely conserved in fish, while the signal protein and GAP are more diverged. Relatively high sequence identities are seen in comparisons with other fish, including barfin flounder (96%; GenBank BAB83982), flathead grey mullet (*Mugil cephalus*) (86%; GenBank AAQ83268), Nile tilapia (82%; GenBank BAC65156) and red drum (*Sciaenops ocellatus*) (86%; GenBank AAV74403). The teleost sGnRH cluster has a high bootstrap value (100%), but the shallow branching is less defined with lower statistical significance (bootstraps < 85%) associated with particular groupings. Like the former two GnRHs, this could be a result

of a complicated phylogeny and non-conserved GAP and signal peptides.

A complete nucleotide sequence for GnRH-R1 and a partial cDNA sequence for GnRH-R2 in winter flounder were identified. The amino acid sequence for winter flounder GnRH-R1 is most similar to other teleost GnRH-R1s, including the greater amberjack (*Seriola dumerili*) (86%; GenBank CAB65407), striped bass (84%; GenBank AAF28464), and black porgy (83%; GenBank AAV71128). Some degree of conservation is seen when comparing the flounder receptor to higher vertebrate GnRH-R1s, including mouse (*Mus musculus*) (46%; GenBank EDL37957), guinea pig (*Cavia porcellus*) (48%; GenBank AF426176), and wild boar (*Sus scrofa*) (46%; GenBank AAS68622).

The partial GnRH-R2 amino acid identities appear to be highest with teleost fish [Burton's mouthfeeder GnRH-R (92%; GenBank AAK29745), orange-spotted grouper GnRH-R1 (*Epinephelus coioides*) (91%; GenBank ABF93210), green-spotted pufferfish GnRH-R2 (*Tetraodon nigroviridis*) (85%; GenBank BAE45702), Argentinean silverside GnRH-R1b (*Odontesthes bonariensis*) (91%; GenBank ABI75337), Nile tilapia GnRH-R2 (90%; GenBank BAC77241) and Masu salmon GnRH-R1/R2 (*Oncorhynchus masou*) (75%; GenBank BAC98943/BAC98944), rubber eel (*Typhlonectes natans,* 55%) (GenBank AAD49750] and to show varying degrees with other vertebrates [Dybowski's frog (*Rana dybowskii*) GnRH-R2 and GnRH-R3 (60% and 56%, respectively; GenBank AAO50196/AAO50197), leopard gecko (*Eublepharis macularius*) GnRH-R2 and GnRH-R3 (62% and 57%, respectively; GenBank ABB89901/ABB89900), chicken (*Gallus gallus*) GnRH-R (60%; GenBank ABK27710), common marmoset (*Callithrix jacchus*) GnRH-R2 (54%; GenBank XP_XP_002759859), wild boar GnRH-R2 (54%; GenBank AAS68622) and humans (*Homo sapiens*) GnRH-R2 (51%; GenBank AAL27000)].

Like other flounder, such as barfin (Amano *et al.* 2002), winter flounder have three GnRH forms: sbGnRH, cGnRH and sGnRH. Phylogenetically, the GnRH variants all form three clearly distinct clusters. As previously mentioned, sbGnRH (or mGnRH) appears to be the most recently derived and divergent form of GnRH (Chen and Fernald 2008; Okubo and Nagahama 2008). Two to three amino acid variations are observed at variable sites of the decapeptide (positions 5, 7 and 8), forming different combinations in each of the 16 GnRH isoforms identified in fish.

The GnRH-Rs of all vertebrates form four distinct groups, GnRH-Ra1, GnRH-Ra2, GnRH-Rb1 and GnRH-Rb2 (Troskie *et al.* 2000; Flanagan *et al.* 2007). Winter flounder GnRH-R1 contains the SQS aa motif and falls within the GnRH-Rb2 clade. Our partial GnRH-R2 sequence does not contain the motif but the sequence clusters with other GnRH-Ra1s. Interestingly, the human (GnRH-R2) and octopus (GnRH-R) both contain amino acid motifs, DGC and NSR, respectively, that are quite different from the previously annotated motifs and have not yet been recognized nor have they been incorporated into the most current GnRH-R phylogeny by Chen and Fernald (2008). It has been suggested that the four "modern" GnRH-R subtypes have evolved from an ancestral GnRH-R - lamprey GnRH-R being one of the ancestral types - following two rounds of gene duplication to evolve four subtypes. It could be suggested and that the octopus and/or human GnRH-Rs represent ancestral forms since they do not contain one of the existing motifs present in modern GnRH-R (see review by Chen and Fernald 2008).

A qualitative approach (RT-PCR) was used to determine the expression sites of cGnRH and sGnRH mRNAs within the central nervous system of flounder. cGnRH

mRNA is localized solely within the telencephalon/preoptic area, hypothalamus and optic tectum/thalamus. However, based on previous studies, cGnRH transcripts had been identified in the hypothalamus and telencephalon/preoptic area of other fish (Hoskins *et al.* 2008; Matsuda *et al.* 2008; Selvaraj *et al.* 2009). Although cGnRH mRNA did not appear to be expressed in these regions using RT-PCR, qPCR mRNA expression analyses did confirm that winter flounder express cGnRH in the telencephalon/preoptic area and hypothalamus. cGnRH mRNA and protein (ir-cells) have been shown to be predominantly expressed in the midbrain near the third ventricle of several vertebrates including mammals [musk shrew (Kauffman *et al.* 2006)], birds [house sparrow (*Passer domesticus*) (Stevenson *et al.* 2007)], amphibians [clawed toad (King et al. 1994)], and reptiles [green anole (*Anolis carolinensis*) (Lescheid *et al.* 1997)]), as well as various fish, including chub mackerel (Selvaraj *et al.* 2009), Nile tilapia (Swapna *et al.* 2008), silver seabream (Hu et al. 2008), lamprey (*Petromyzon marinus*) (Kavanaugh *et al.* 2008), African catfish (Goos *et al.* 1985; Bogerd et al. 1994), Siberian sturgeon (Lepretre *et al.* 1993) and goldfish (Hoskins et al. 2008). Aside from the midbrain, cGnRH ir-cells have been isolated in the telencephalon, hypothalamus and hindbrain in vertebrates such as the musk shrew (Kauffman *et al.* 2006), frog (Hayes *et al.* 1994), chimera (*Chimera monstrosa*) (Masini *et al.* 2008), chub mackerel (Selvaraj *et al.* 2009), silver seabream (Hu et al. 2008), lamprey (Kavanaugh *et al.* 2008), goldfish (Hoskins *et al.* 2008), African catfish (Goos *et al.* 1985) and dwarf gourami (*Colisa lalia*) (Yamamoto *et al.* 1995).

In winter flounder, sGnRH mRNA is detected ubiquitously throughout the brain, with no mRNA expression in the pituitary gland. These results are consistent with studies

in other vertebrates, where neurons and mRNA for sGnRH tend to be distributed predominantly in the forebrain, more specifically in the olfactory bulb-telencephalic boundary. In fish, sGnRH is thought to be a product of sbGnRH, derived from the teleost whole genome duplication event, and to be a fish specific form (see review by Chen and Fernald 2008). sGnRH-ir cells have been identified in the olfactory bulb, terminal nerve ganglion, and the transitional area between the olfactory bulb and telencephalon in the chub mackerel (Selvaraj *et al.* 2009), Nile tilapia (Swapna *et al.* 2008), silver seabream (Hu *et al.* 2008), lamprey (Youson *et al.* 2006), goldfish (Yu *et al.* 1988), Atlantic salmon and rainbow trout (Bailhache *et al.* 1994), and dwarf gourami (Yamamato *et al.* 1995). sGnRH-ir neurons have also been detected in the preoptic nucleus in the hypothalamus and pituitary of white carp (*Cirrhinus cirrhosus*) (Sakharkar *et al.* 2008), lamprey (Youson *et al.* 2006), goldfish (Yu *et al.* 1988), Atlantic salmon and rainbow trout (Bailhache *et al.* 1994), and platyfish (*Xiphophorus maculatus*) (Magliulo-Cepriano *et al.* 1994).

Finally, qPCR expression analyses of GnRH transcripts demonstrate the relative abundance of mRNA in fed and fasted flounder for each of the GnRH peptides. sbGnRH mRNA was not affected by fasting in any of the tissues examined, suggesting that sbGnRH, like its mGnRH homolog, might not affect appetite but instead evolved a strict regulatory role in reproduction. However, most studies have focused on the roles of sbGnRH in reproduction, with little attention on its role in appetite regulation (Khalil *et al.* 2007). Further studies are needed to determine whether sbGnRH is involved in food intake behaviour.

Fed fish displayed significantly higher levels of cGnRH and sGnRH mRNA in the

optic tectum/thalamus and hypothalamus and telencephalon, respectively, than fasted fish, suggesting a possible appetite-inhibiting effect for these hormones. Higher mRNA expression of cGnRH in fed fish is consistent with data in other vertebrate models, such as chickens (Bruggeman *et al.* 1998). In musk shrew, food restriction down-regulates both cGnRH mRNA and protein levels in the midbrain and of animals fed *ad libitum* or re-fed after a food restriction period food. Food restricted animals display higher cGnRH mRNA and protein levels than regularly fed animals without food restriction and re-feeding in the midbrain (midhabenula and periaqueductal grey) (Kauffman *et al.* 2006). Evidently, goldfish ICV-injected with cGnRH display decreases in food intake compared with controls (Hoskins *et al.* 2008; Matsuda *et al.* 2008). One study in goldfish found that sGnRH ICV injections did not affect food intake (Matsuda *et al.* 2008). Our study is the first to demonstrate that sGnRH could in fact play a role in food intake in winter flounder. The presence of sGnRH-ir cells and mRNA in the olfactory bulb and telencephalon might suggest that sGnRH plays a role in relaying food-related sensory cues to the brain as well as the actual endocrine process of appetite regulation.

In conclusion, we identified three GnRH forms (sbGnRH, cGnRH and sGnRH) and two forms of GnRH receptors (GnRH-R1 and GnRH-R2) in winter flounder. Phylogenetic analyses of the GnRH isoforms showed clear delineations of subtypes. While the receptor nomenclature is becoming more consistent, outliers (human GnRH-R2 and octopus) still exist. Our results suggest that cGnRH and sGnRH, but not sbGnRH, might be regulators of food intake in winter flounder. cGnRH and sGnRH mRNAs are both expressed in regions of the brain that have been previously implicated as centres involved in appetite regulation, including telencephalon/preoptic area, optic

tectum/thalamus and hypothalamus, as well as the pituitary gland, and their brain expression appears to be affected by nutritional status.

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Summary

I examined the roles of melanin-concentrating hormone (MCH) and gonadotropin-releasing hormone (GnRH) gene families in appetite regulation of winter flounder (*Pseudopleuronectes americanus*). In the MCH study, we cloned two forms of MCH (MCH and MCH2) and two MCH receptors (MCH-R1 and MCH-R2). Sequence and phylogenetic analyses indicate that winter flounder MCH2 is homologous to mammalian-MCH, while flounder MCH might be the result of the teleost whole genome duplication. A high degree of conservation at the amino acid level (82-99%) is observed for MCH-R1 between winter flounder and other teleosts, and between winter flounder and both birds (chicken) and mammals (mouse). The partially cloned winter flounder MCH-R2 cDNA fragment is most similar to fish putative orthologous sequences seems to be more diverged from the human MCH-R2, unlike MCH-R1.

Winter flounder MCH mRNA is expressed in all brain regions, the pituitary, and most peripheral tissues examined, except heart and liver. Winter flounder MCH2 mRNA is present throughout the brain, (except hindbrain), as well as in the pituitary gland and in all peripheral tissues examined. Most notably, the presence of MCH and MCH2 transcripts in the winter flounder forebrain (telencephalon/preoptic area, hypothalamus), pituitary and gastrointestinal tract (foregut and midgut) could be indicative of their role in food intake regulation. Winter flounder MCH-R1 and MCH-R2 transcripts are ubiquitously expressed in central and peripheral tissues, but MCH-R2 appears to be expressed at lower levels than MCH-R1. Like MCH and MCH2, expression of the transcripts encoding receptors in tissues involved in appetite regulation suggests roles in

the control of food intake.

Quantitative real-time polymerase chain reactions (qPCR) in distinct brain regions (telencephalon/preoptic area, optic tectum/thalamus and hypothalamus) and midgut suggests that MCH is an appetite stimulator in winter flounder. Increased expression is observed in fasted fish compared to fed fish for both MCH and MCH-R1 in the optic tectum/thalamus and hypothalamus, respectively. No significant differences in MCH2 and MCH-R2 mRNA expression are seen in the brain or gut of fasted winter flounder.

In the GnRH study, we isolated three forms of GnRH [seabream-GnRH (sbGnRH), chicken-GnRH (cGnRH) and salmon-GnRH (sGnRH)] and two GnRH receptors (GnRH-R1 and GnRH-R2). The mature decapeptide is highly conserved among fish and mammals, with amino acid substitutions solely at positions 5, 7 and 8, whereas the signalling and GnRH-associated (GAP) peptides are not very conserved, even among teleost fish. Phylogenetically, three main clusters are observed for the GnRH isoforms, with sGnRH being the ancestral form and sbGnRH derived from cGnRH and/or sGnRH. Clear delineations of relationships between the fish within each of these clades are not observed as indicated by low bootstrap values.

Relatively high conservation is observed for each of the GnRH-Rs (typically > 80%) between winter flounder sequences and those of other vertebrates, including fish. However, a poorly defined GnRH-R nomenclature renders the sequence and phylogenetic analyses as well as the interpretation of the functional significance of different receptor forms more complicated. Mammalian and invertebrate GnRH-Rs are categorized as outgroups to the fish GnRH-Rs, whereas frog and chicken GnRH-Rs group with teleosts. However the taxonomy is still unclear for GnRH-Rs.

Central tissue distributions were completed only on cGnRH and sGnRH due to time constraints and technical difficulties. cGnRH mRNA is expressed primarily in the optic tectum/thalamus, with low expression in the telencephalon/preoptic area and hypothalamus (as per qPCR results). sGnRH is ubiquitously expressed throughout the brain and pituitary gland with highest apparent expression in the telencephalon/preoptic area.

Expression studies of fed and fasted winter flounder demonstrate that cGnRH and sGnRH could play an inhibitory role in food intake via anorexigenic stimulation. Fed fish have significantly higher cGnRH and sGnRH mRNA expression in the optic tectum/thalamus and hypothalamus, and telencephalon/preoptic area, respectively. No significant differences of sbGnRH, GnRH-R1 and GnRH-R2 mRNA expression are observed between fed and fasted fish.

Future directions for this study include: (1) completion of sequence analyses for MCH and GnRH isoforms and receptors; (2) examining tissue distributions for GnRH, including central distributions for sbGnRH and GnRH-Rs and peripheral distributions for all GnRH variants and receptors; (3) qPCR analyses for MCH and MCH2 in the midgut, as well as GnRH-Rs (centrally and midgut); (4) integration of immunohistochemistry of MCH and GnRH immunoreactive (ir) cells in the brain and pituitary, as well as peripheral tissues, including gut and gonads in winter flounder with previous tissue distribution studies; and (5) examining the direct effects of MCH and GnRH variants on food intake via intracerebroventricular and intraperitoneal injections in winter flounder.

My study sheds light on the endocrine regulation of a complex and relatively new area of research, appetite regulation, in fish. Understanding how these hormones, MCH

and GnRH, are affected by feeding and/or fasting in different fish species could lead to a consensus of how this energy balance is regulated not only in fish but in vertebrates in general. Given the complexity of this regulation and the existence of species-specific differences, examining new fish models, such as Atlantic cod (*Gadus morhua*) and cunners (*Tautogolabrus adspersus*), as well as other putative regulators, might yield important new information. Exploring the integration of feeding regulation with other physiologic systems, such as reproduction and sensory physiology (e.g. the role of visual cues) might also be of great interest.

In addition to the general physiological importance, our study could also provide important information for the aquaculture industry. A better knowledge of how fish regulate food intake could lead to the understanding of their growth mechanisms and consequently be used to achieve better yields. For example, one could use hormones and genes known to control appetite and growth to develop genetic markers that would assist in the selection of larger, fast-growing fish.