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COMPARATIVE INSIGHTS OF THE KISSPEPTIN/KISSPEPTIN RECEPTOR SYSTEM: LESSONS FROM NON-MAMMALIAN VERTEBRATES

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ABSTRACT

Kisspeptins, the peptide products of the *Kiss1* gene, were initially identified in mammals as ligands of the G protein-coupled receptor 54 (GPR54; also termed Kiss1R) with ability to suppress tumor metastasis. In late 2003, the indispensable role of kisspeptins in the control of reproductive function was disclosed by the seminal observations that humans and mice carrying inactivating mutations of GPR54 displayed hypogonadotropic hypogonadism. Since then, numerous experimental studies, conducted initially in several mammalian species, have substantiated the roles of kisspeptins as essential players in the physiologic regulation of key aspects of reproductive maturation and function, including the timing of puberty onset, the dynamic control of gonadotropin secretion via stimulation of GnRH neurons, the transmission of the negative and positive feedback effects of sex steroids, the metabolic regulation of fertility and the control of reproductive function by environmental (photoperiodic) cues. Notably, while studies about kisspeptins in non-mammals appeared initially to lag behind, significant efforts have been devoted recently to define the genomic organization and functional characteristics of kiss/ kisspeptins and gpr54 in different non-mammalian species, including fish, reptiles and amphibians. These analyses, which will be comprehensively revised herein, have not only substantiated the conserved, essential roles of kisspeptins in the control of reproduction, but have also disclosed intriguing evolutionary aspects of kisspeptins and their receptors. Such *comparative* approaches will be instrumental to fuel further studies on the molecular regulation and physiological roles of kisspeptins, thus helping to unveil the complex biology of this system as indispensable regulator of the reproductive axis in a wide diversity of animal species.

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1. INTRODUCTION

In spite of the diversity of strategies adopted by different species to ensure reproductive success, a conserved hierarchical element in the neurohormonal axis governing reproduction in vertebrates is the family of gonadotropin-releasing hormones (GnRH). While various GnRH forms have been identified, some of which do not appear to display hypophysiotropic actions, the consensus exists that specific populations of GnRH neurons operate as integrators and major output pathway for a diversity of regulatory signals that modulate reproductive maturation and function in a wide range of species [38, 76]. Our knowledge of the regulatory afferents to GnRH neurons, and their roles in the control of the gonadotropic axis, has enlarged considerably in recent years. A major breakthrough in the field took place in late 2003, when kisspeptins, as the products of the *Kiss1* gene acting via the previously orphan receptor, GPR54, were first suggested to be important players in the central control of reproduction. This assumption stemmed from human and rodent data showing that inactivating mutations of GPR54 induced a state of hypogonadotropic hypogonadism [9, 60]. Indeed, in the relatively short period of time elapsed since these seminal observations, a large body of experimental evidence has accumulated that unambiguously support an essential role of kisspeptins as major stimulators of GnRH secretion and, hence, of gonadotropin release [47, 51, 58].

In mammals, detailed neuroanatomical studies have documented the existence of discrete populations of Kiss1 neurons in key hypothalamic areas, such as the arcuate/infundibular nucleus (ARC) and, in rodents, the anteroventral periventricular region (AVPV) [47, 51, 58]. In addition, functional analyses have demonstrated the involvement and putative relevant roles of kisspeptins in virtually all facets of reproductive physiology. These roles are likely to include: (*a*) the process of sexual differentiation of the brain; (*b*) the proper timing of puberty; (*c*) the dynamic control of gonadotropin secretion, via stimulation of GnRH neurons; (*d*) the transmission of the negative feedback effects of sex steroids; (*e*) the mediation of the positive feedback effects of estrogen and, hence, the generation of the pre-ovulatory surge of gonadotropins; (*f*) the metabolic regulation of

fertility; and (*g*) the control of reproductive function by environmental (photoperiodic) cues [8, 47, 51, 58].

While most of the initial advancements in the field of kisspeptin physiology came from studies in different mammalian species, including laboratory rodents and primates, as extensively reviewed elsewhere recently [8, 47, 51], in the last three years we have witnessed a significant upsurge of studies aiming to define the genomic organization and functional characteristics of the kisspeptin systems in different non-mammalian species, including fish, reptiles and amphibians. These *comparative* analyses that have allowed gaining an in-depth knowledge of essential aspects of the evolution and conserved functions of kisspeptins and their receptors in the control of reproduction will be comprehensively summarized in this work.

2. MOLECULAR CHARACTERIZATION AND EVOLUTIONARY INSIGHTS OF THE KISS/ GPR54 SYSTEM IN NON-MAMMALIAN VERTEBRATES

As indicated above, the identification of the *Kiss*/*Gpr54* pair as a new regulatory system of GnRH, and consequently gonadotropin secretion, has revolutionized our understanding of the mechanisms for the neuroendocrine control of reproduction in mammals, and has fueled also related research fields in other vertebrate species [58, 67, 68, 76]. Contrary to mammals where, with the exception of the platypus (monotreme), only one gene coding for the ligand and one for the receptor are present, compelling evidence has now demonstrated the presence of two distinct genes encoding kisspeptins (*kiss1* and *kiss2*), and up to four different genes encoding their cognate receptors, *gpr54*s, in non-mammalian vertebrates, such as amphibians, reptiles and, preferentially, bony fish [1, 4, 25, 69, 76] (see **Tables 1** and **2**).

In fish, isolation of kisspeptin coding genes has been accomplished in the zebrafish *Danio rerio* [23, 70], medaka *Oryzias latipes* [22, 23], sea bass *Dicentrarchus labrax* [18], goldfish *Carassius auratus* [26], grass puffer *Takifugu niphobles* [63], orange-spotted grouper *Epinephelus coioides* [64], chub mackerel *Scomber japonicus* [59] and striped bass (*Morone saxatilis*) [75]. All of these teleost species possess the *kiss2* gene, while there is also evidence that the *kiss1* gene is

present in the genomes of zebrafish, medaka, sea bass, goldfish and chub mackerel (**Table 1**). It is, however, worth pointing out that searches in different available genome databases have evidenced that several fish species, such as *Takifugu rubripes* (tiger puffer), *Tetraodon nigroviridis* (green puffer) and *Gasterosteus aculeatus* (stickleback), lack the *kiss1* gene and possess only *kiss2* [18, 23, 26, 74]. On the other hand, there is also evidence that both *kiss1* and *kiss2* genes are present in cartilaginous fish such as the elephant shark *Callorhinchus milii* (Chondrichthye) and in the sea lamprey *Petromyzon marinus* (Agnatha) [18, 23, 25, 26, 64, 70, 74]. In amphibians, the identification of three forms of *kiss* genes has been reported in the Western clawed frog *Silurana tropicalis* [25], while the Anole lizard (*Anolis carolinensis*) has only *kiss2* (XM_003220766) [1, 18, 69].

Of note, while the first reports of cloning of *kiss* genes in fish were released only in 2008 [70], a different situation occurred for the kisspeptin receptor. From 2004 onwards, a number of studies reported the isolation and (partial) characterization of *gpr54* genes in non-mammalian vertebrates [1, 25, 69]. Indeed, to date, kisspeptin receptors have been identified and cloned in up to twenty different teleost fish species (**Table 2**). So far, the only fish species that appear to have two *gpr54* genes are medaka [22, 23], zebrafish [2, 70], goldfish [26], sea bass [17] and striped bass (*Morone saxatilis*) [75]. Recently, recommendations to unify nomenclatures for the receptors among vertebrates have been reported [1, 17, 69]. For this review, the two kisspeptin receptors in fish species have been tentatively referred using the terms gpr54-1b and gpr54-2b [17]. Importantly, *gpr54-2b* is the kisspeptin receptor gene commonly found in most of the teleost genomes, including Perciforms, Pleuronectiforms, Mugiliforms, Tetraodontiforms, Beloniforms, Gasterosteiforms and Cypriniforms (**Table 2**).

To the best of our knowledge, searches in Chondrichthye and Agnatha genomes have failed to identify kisspeptin receptors. In amphibians, molecular cloning of *gpr54-2b* has been reported in the bullfrog (*Rana catesbeiana*) [41], whereas characterization of three cDNAs encoding *gpr54* receptors (i.e., *gpr54-1a*, *gpr54-1b*, *gpr54-2b*) has been recently reported in the Western clawed

frog [25]. In the particular case of reptiles, searches in the available genome database of Anole lizard (*Anole carolinensis*) have shown that the lizard genome has a single kisspeptin receptor gene, the *gpr54-2a* form (Chromosome 2 at location 102,914,244-102,924,790; assembly AnoCar 2.0) [17]. Overall, kisspeptin receptor sequences display a greater degree of conservation across species and phyla than the *kiss* genes; a feature that has facilitated their molecular characterization in non-mammalian vertebrates. Indeed, while *kiss1* and *kiss2* share only 7-16% identity in their deduced amino acid sequences in vertebrates, the different forms of gpr54 exhibit a higher percentage of amino acid identity among them (**Table 3**).

Genome and cDNA analyses of the kisspeptin genes have revealed that the structural organization of both *kiss1* and *kiss2* genes is similar, containing two coding exons, with the exon 2 coding for the kisspeptin-10 sequence (i.e., YNWNSFGLRY for kiss1 and FNFNPFGLRF for kiss2) [18, 23, 74, 76]. On the other hand, it has been commonly considered that the *gpr54* genes contain five exons, although, interestingly, medaka *gpr54-1b* and sea bass *gpr54-1b* have six exons [17]. Furthermore, a similar tissue distribution of *gpr54-1b* and *gpr54-2b* has been observed in medaka and sea bass using RT-PCR. The conserved genomic organization of the *kiss* and *gpr54* genes indicates that each of them originated from a common ancestral gene [1, 25, 69]. In support of this fact, mapping of *kiss1* and *kiss2* as well as of the different *gpr54*s in amphibian, reptile and fish genomes reveals the presence of conserved synteny around each gene across species [18, 25, 44, 69, 70]. This conserved genomic organization is observed in the vertebrate lineage, including mammalian and non-mammalian species. In addition, these findings also show that when two genes co-exist in a same species, these are located in different chromosomes. The synteny analysis coupled to phylogenetic analysis suggest that *kiss* and *grp54* genes have diversified through genome duplication and gene modification and deletion in all vertebrates [1, 69] (**Fig. 1**).

Of particular interest, searches in the avian lineage have failed in the identification of *kiss*/*gpr54* pair in this taxonomic group. In addition, little information exists so far regarding the presence of kisspeptin system in invertebrates. Recently, it has been reported that several

predicted kisspeptin receptor-like gene annotations exist in Branchiostoma (Cephalochordate), sea urchin (Echinodermate) and Saccoglossus (Hemichordate), with no records predicted from Ciona (Urochordate) genomic sequences [2, 17, 25]. The phylogenetic analyses of these invertebrate kisspeptin receptor-like gene annotations, together with those in mammalian and non-mammalian vertebrates, suggest a complex evolutionary history for these genes. Regarding the ligands, no kisspeptin-like gene or sequences have been found in invertebrates. The marked divergence found among invertebrate and vertebrate species makes comparisons of molecular co-evolution of the kisspeptin system difficult in animal phyla. Nevertheless, because *kiss1* and *kiss2* have been found in Agnatha (lamprey) and Chondrichthyes (elephant shark) and, on the other hand, kisspeptin receptor-like genes have been predicted in invertebrates, at least in Cephalochordata (lancelets), Echinodermata (sea urchin) and Hemichordata (Saccoglossus), the *kiss*/*gpr54* pair is proposed to be present before vertebrate radiation. Thus, the history of these genes is suggested to date back over more than 600 million years [25, 36, 44]. This striking feature makes it clear that a better knowledge of the evolutionary history of this ligand/receptor pair would be instrumental to improve our understanding of its functional roles in mammalian and non-mammalian vertebrates.

3. TISSUE EXPRESSION AND NEUROANATOMICAL DISTRIBUTION OF KISSPEPTINS AND GPR54 IN AMPHIBIANS AND FISH

In fish, expression of the genes encoding kisspeptins and their receptors have been found in different brain areas, namely in the hypothalamus, telecephalon, thalamus, optic tectum, mid brain tegumentum, olfactory bulbs and tracts, optic tectum, optic nerves, medulla oblongata, cerebellum and pituitary. Nevertheless, the expression of these genes has been also reported in a range of other tissues including testes, ovary, heart, muscle, stomach, intestine, spleen, liver, kidney, adipose tissue, pancreas, gills, eye and skin, to a variable extent depending on the fish species and gene [47]. In the brain, Parhar et al. provided the first evidence of the expression of a non-mamalian *gpr54* in GnRH neurons of a cichlid fish, the tilapia (*Oreochromis niloticus*),

193 suggesting a potential anatomical association of gpr54 with the GnRH system [49]. Thereafter,
194 two distinct kisspeptin receptor transcripts (*gpr54-1b and gpr54-2b*) have been isolated in a
195 variety of fish species and its expression examined by quantitative PCR. The detection of *gpr54-1b*
196 and *gpr54-2b* mRNAs in brain and gonads at different reproductive stages reinforces their
197 potential reproductive role, although their functional relevance may vary among gender and
198 species [1, 47, 76].

199 The neuroanatomical distribution of the pair kiss/kisspetin receptor has been recently
200 reviewed in a variety of fish species [1, 47, 76]. Fish inhabit a wide range of environmental niches
201 and thus they have developed a great variety of reproductive strategies. Moreover fish
202 investigators have used different experimental approaches to study the kiss/kisspetin receptor
203 system(s). This makes it difficult to provide a unified overview of all studies conducted so far. In
204 this section, we will review only the information related to the neuroanatomical organization
205 gained by *in situ* hybridization (*ISH*) and immunocytochemical studies. *ISH* studies in medaka [22]
206 identified for the first time in fish neuronal *kiss1* cell bodies in two hypothalamic nuclei, the
207 nucleus posterioris periventricularis (NPPv) and the nucleus ventralis tuberis (NVT). The number
208 of neurons of NVT was larger in breeding than in non-breeding fish, and more numerous in males
209 than in females. From these results, it was concluded that kiss1 system is pivotal for the regulation
210 of reproduction in that species. Afterwards, Kitahashi et. al. [23] cloned a novel kisspeptin gene
211 (*kiss2*) in the zebrafish and medaka, and by using *ISH* and laser capture microdissection coupled
212 with real-time PCR identified *kiss1* mRNA expression in the ventromedial habenula and the
213 periventricular hypothalamic nucleus, and *kiss2* expressing neurons in the posterior tuberal
214 nucleus and the periventricular hypothalamic nucleus. Almost simultaneously, *kiss1* and *kiss 2*
215 genes were cloned in a marine teleost, the sea bass [18]. Neuroanatomical distribution of their
216 expression has been characterized recently by *ISH* assays, which point out that the mediobasal
217 hypothalamus, and specially the nucleus of the lateral recess, is an important area of expression of

218 both *kiss1* and *kiss2* genes. Furthermore, *kiss1* expressing cells were also found at the level of the
219 habenular region in this fish species [12].

220 Of note, studies conducted in zebrafish on brain expression of *kiss* genes during
221 development, and on the effects of kiss1 and kiss2 decapeptide administration suggested that the
222 habenular kiss1 and the hypothalamic kiss2 are potential regulators of reproduction, and that
223 kiss2 is the predominant regulator of gonatropin synthesis [23]. In the same vein, Felip et al.
224 observed that the kiss2 decapeptide robustly elicited LH release, both in prepubertal and adult
225 male sea bass, therefore reinforcing the important role of the kiss2 system in central regulation of
226 the fish reproductive axis [18].

227 Two kisspeptin receptors genes have been characterized in the sea bass [4], and
228 interestingly *gpr54-1b and gpr54-2b*-expressing cells have been shown to be mostly located in the
229 same regions as their cognate ligands [12]. Additionally, these authors observed that GnRH1-
230 expressing neurons co-express *gpr54-2b* indicating that they are target for kisspeptins via gpr54,
231 in line with the functional observations of potent LH-releasing effects of kiss2 in this species [18].

232 The information on the targets of the axonal projections of kiss1 and kiss2 neurons in fish
233 has been hindered mainly due to the difficulty in obtaining specific antibodies that selectively
234 recognize kiss1 or kiss2. Recently, it has been documented for the first time the organization of
235 kiss1 and kiss2 systems in zebrafish by using highly specific antibodies that allowed to
236 unequivocally distinguish preprokiss1 from preprokiss2 [61]. Immuno-cytochemistry studies
237 performed by these authors confirmed previous *ISH* data, as preprokiss1-immuno-reactive
238 neurons were found in the ventromedial habenula; neurons which send axons only to the
239 interpeduncular and raphe nuclei. Double immunostaining showed that these same neurons
240 expressed *gpr54-1b*. Prokiss2 neurons were mainly located in the dorsal and ventral
241 hypothalamus projecting widely into the subpallium, preoptic area, the thalamus, the ventral and
242 caudal hypothalamus and the mesencephalon. All these regions strongly expressed *kiss2* [61].
243 Double staining with sea bass proGnRH3 (80% identity) antiserum revealed immunoreactive

neurons in the subpallium and the anterior preoptic area, some of which were contacted by prokiss2 fibers. Treatment with estradiol caused an increase of *kiss1*, *kiss2* and *gpr54-2b* but not *gpr54-1b*. Moreover, estrogen caused a significant increase of the number of *kiss2* neurons in the hypothalamus [61] .

Apart from fish, neuroanatomical information regarding the kiss system in non-mamalian vertebrates is very scarce, although consistent, in amphibians. In the Western clawed frog expression of three *kiss* genes, *kiss1a*, *kiss1b* and *kiss2*, and three *gpr54*s has been documented [25]. Localization of *kiss* genes by *ISH* showed that *kiss1* mRNA was expressed in the ventral hypothalamus (VH) whereas neurons expressing *kiss2* were detected in the preoptic area (POA) and VH. Further, it was confirmed by immunocytochemistry using an anti *S. tropicalis* kiss2 peptide antiserum that these neurons were restricted to the POA and VH. Moreover, fibers originating from these POA and VH neurons terminated in the median eminence. In this same species, the three *gpr54* genes were abundantly expressed in the hypothalamus, as shown by RT-PCR; expression that was also observed in the pituitary for some of the forms [25]. In the bullfrog, *gpr54* has been found to be expressed in the forebrain, hypothalamus and pituitary gland [41].

The localization of the kisspeptin/gpr54 system in the brain of fish and the determination of the equivalent mammalian brain regions are, at present, far from being established and general statements can be outlined. Teleostean forebrain (telencephalon/diencephalon) neuroanatomy varies considerably given that this subclass is extremely species-rich and a diversified clade within the class of Actinopterygii. Besides, eversion is the principal morphogenetic event in the formation of actinopterigian cerebral hemispheres while evagination is the common one in other vertebrate groups. These facts have greatly hampered the hodological studies with other vertebrates [72]. However, molecular, hodological and behavioral studies using model species (i.e zebrafish, mouse) have generally demonstrated great similarities of teleostean brain circuitry with other vertebrates supporting the idea that the posterior zone of the area dorsalis telencephali (Dp) is homologous to the lateral pallium of other vertebrates (including the piriform

cortex of mammals) the medial zone of the area dorsalis telencephali (Dm) is homologous to the pallial amygdale (ventral pallium derivative) and the lateral zone of the area dorsalis telencephali (Dl) is homologous to the hippocampus (medial palial derivative). Concerning the dorsal tier nuclei of the area ventralis telencephali or subpallium (Vd, Vc) represent the striatal formation, whereas the ventral tier subpallial nuclei (Vv, Vl) correspond to the septal formation, respectively [42, 45, 73]. In addition to these complexities encountered at the comparative neuroanatomy level, the information about localization of kisspeptins in the brain of teleost has been also hampered by difficulty to raise specific antibodies to kisspeptins only achieved in one fish species, the zebrafish [61]. This explains why the neuroanatomical localization of the kiss-producing neurons in the brain has been mainly studied at the mRNA level [22, 23, 25, 39]. Thus there is an important lack of knowledge in fish compared to mammals in this matter. Notwithstanding, a preliminary comparative approach for brain localization of *kiss* genes that are predominant for regulation of gonadotropin synthesis either in fish or in mammals could be envisaged from the study of Kitahashi [23]. In this paper, the *kiss2* mRNA-containing cells were seen in the posterior tuberal nucleus and the periventricular hypothalamus of the zebrafish and medaka suggesting that those cells could be similar to those expressing *Kiss1* RNA in the arcuate and periventricular nuclei described in mammals [37]. Undoubtedly, more work is still needed to decipher if these homologous areas/nuclei of teleost are functionally equivalent to their mammalian counterparts.

4. KISSPEPTIN SIGNALING IN NON-MAMMALS: LIGAND-RECEPTOR SPECIFICITY AND INTRACELLULAR PATHWAYS

In mammals, kisspeptins are the only known ligands for GPR54, which was initially identified as an orphan receptor in 1999. Thereafter, it was demonstrated that kisspeptins from placental extracts were able to bind and activate this receptor and thus were defined as their natural ligands [24, 48]. Different studies using *Gpr54*-transfected cell lines have shown that mammalian Gpr54s couple to G-proteins of the $G\alpha_q$ type, activating the phospholipase C pathway and finally leading to Ca2+ mobilization [5, 24, 52]. The existence of this pathway in GnRH

296 neurons has been confirmed *ex vivo* using brain explants [6, 27]. Other reported downstream
297 effectors include arachidonic acid, protein kinase C (PKC) or the mitogen activated protein kinases
298 (MAPK) ERK1/2. However, no activation of the cAMP/Protein kinase A (PKA) pathway has been
299 described for mammalian Gpr54s [6, 24, 43].

300 As mentioned above, in some fish species two different *kiss* genes and two *gpr54* co-exist,
301 raising the question of how promiscuous or specific are ligand-receptor interactions. Although the
302 use of transfected heterologous cell lines may not exactly reflect what happens *in vivo*, these *in*
303 *vitro* systems are nonetheless appropriate to perform initial studies aimed to delineate ligand-
304 receptor interactions. In addition, they are useful to elucidate which are the signaling pathways
305 that may be used by these receptors. Therefore, heterologous cell systems have been used so far
306 to analyze fish gpr54-kisspeptin pairs. Binding and activation of the receptors has been indirectly
307 recorded by transactivation of the luciferase gene placed under promoters that signal different
308 intracellular pathways [7]. The two pathways analyzed so far have been PKC-MAPKs activation, by
309 using "serum responsive element" (SRE) motifs to drive luciferase expression, and the cAMP/PKA
310 pathway through "cAMP responsive elements" (CRE) driven luciferase expression.

311 To date, the functionality and ligand specificity of the kiss/gpr54 system has been analyzed
312 in three fish species containing a duplicated kiss system, namely zebrafish [2, 25], goldfish [26]
313 and European sea bass [17]; and in one species, orange spotted grouper, with a single kiss/gpr54
314 pair [64]. Ligand-receptor selectivity has also been studied in the Western clawed frog, which
315 contains a triplicate system [25]. All the fish gpr54 functionally tested were able to activate
316 luciferase expression driven by a SRE promoter, indicating that, as it is the case in mammals,
317 activation of PKC-MAPKs is involved as signaling pathway. However, differences have been
318 observed depending on the receptor-ligand combination, and also on the length of the kisspeptins
319 used. In mammals, a ten amino acid kisspeptin (kiss-10) is the minimum peptide size able to
320 activate Gpr54 with maximum potency [24]. Thus, in fish the deduced sequences for kiss1-10 and
321 kiss2-10 were initially assumed as the minimum functional peptides. However, a natural 12 amino

322 acid long kiss2 peptide was isolated from brain of Western clawed frog [25]. Indeed, a conserved
323 Arg in position 13 exists in all available fish kiss2 sequences, indicating the existence of a putative
324 cleavage site that would produce a mature kiss2-12 peptide. Similarly, a basic motif in fish kiss1
325 sequences would produce a kiss1-15 peptide containing a conserved N-terminal Gln. Moreover,
326 Lee et al. proved that pyroglutamilation of that N-terminal Gln gives rise to a more active
327 kisspeptin [25].

328 The kiss-10 peptides have been tested in the four fish species studied to date (*see above*).
329 When we consider the action of these short peptides on activating the PKC-MAPK route, we
330 observe that gpr54-1b is more efficiently activated by kiss1-10 than by kiss2-10 in zebrafish [25]
331 and sea bass [17], while the opposite occurs for goldfish gpr54-1b [26]. However, gpr54-2b is
332 preferably activated by kiss1-10 in goldfish, but similarly activated by kiss1-10 or kiss2-10 in
333 zebrafish and sea bass. It is worthy to mention that in the orange spotted grouper, which only
334 harbors a kiss2/grp54-2b ligand-receptor pair, human kiss1-10 was as effective as the
335 homologous grouper kiss2-10 in activating the gpr54-2b receptor [64]. Kisspeptins longer than
336 kiss-10 (kiss1-15 and kiss2-12) have only been tested in zebrafish and sea bass. In all cases, they
337 have been more potent activators of gpr54s than their corresponding kiss-10 peptides. However,
338 while kiss1-15 showed the highest potency for the activation of gpr54-1b both in zebrafish and
339 sea bass, gpr54-2b was maximally activated by kiss2-12 in the case of sea bass and with kiss1-15
340 in zebrafish [17, 25].

341 Whereas it has been reported that mammalian Gpr54s do not activate the cAMP/PKA
342 signaling pathway, even when transfected in heterologous cell lines where this pathway is
343 functional [6, 24], the same does not stand for fish receptors. The ability to activate this pathway
344 has been tested in gpr54 receptors of four fish species, as well as in bullfrog, by measuring CRE-
345 driven luciferase activity. In sea bass, goldfish and zebrafish, gpr54-1b elicits stronger activation
346 of the cAMP/PKA pathway than gpr54-2b. Yet, while for sea bass gpr54-1b, kiss1-15 is the most
347 potent ligand [17], maximum activation of gpr54-1b is achieved with kiss2-10 stimulation in the

case of goldfish [26], as described for the PKC-MAPK pathway. In the case of zebrafish, where only kiss1-10 was tested, this ligand was able to activate the cAMP/PKA pathway through gpr54-1b activation, but not via gpr54-2b [2]. In orange spotted grouper [64] or the amphibian bullfrog [41], where only the gpr54-2b receptor has been studied, no signaling through the PKA pathway was observed. These data are thus consistent with the observations in the other fish species where gpr54-2b receptors are less efficient signaling through this pathway.

The neuroanatomical distribution of *kiss*- and *gpr54*-expressing neurons in zebrafish brain [61], as summarized in previous sections, is fully compatible with the preferred activation of zebrafish (and sea bass) gpr54-1b by the kiss1 ligand. The expression sites in the zebrafish brain also agree with the higher potency of kiss2 in activating gpr54-2b *vs.* gpr54-1b. In addition, in those fish species where only one ligand/receptor pair has been described, this corresponds to the kiss2/gpr54-2b pair. All in all, the data summarized in this section show that there are not highly specific ligand/receptor pairs in fish, but rather different levels of activation for the different ligand/receptor combinations; a phenomenon that is also influenced by the fact that native mature kisspeptins in non-mammalian vertebrates are possibly longer than in mammals. In addition, some signaling differences in the gpr54 duplicates of a given species have also been observed. However, more fish (and other non-mammalian) species need to be analyzed in order to specify defined signaling patterns.

5. PHYSIOLOGICAL ACTIONS OF KISSPEPTINS: MAJOR REGULATORS OF GONADOTROPIN SECRETION AND PUBERTY IN NON-MAMMALIAN VERTEBRATES

The biological effects of kiss-10 forms after systemic administration have been studied in some fish species. As relevant end-points, the expression of *gnrh* genes in the brain and of gonadotropin subunits in the pituitary has been measured, and variations in gonadotropin levels in blood monitored. In early-mid pubertal fathead minnow, kiss1-10 injection provoked an increase in the expression of *gpr54-2b* and *gnrh3* in the brain, but not of *gnrh2*. In this species, *gnrh3* is likely to be the hypophysiotropic form [19]. However, systemic injections of kiss1-10 and

kiss2-10 in sexually mature female zebrafish did not elicit any expression variation in *gnrh3* (hypophysiotropic form) or *gnrh2* [23]. On the other hand, in orange spotted grouper, whose genome contains only *kiss2*, the administration of the kiss2-10 peptide in sexually mature females evoked an increase in hypothalamic expression of *gnrh1*, which is probably the hypophysiotropic form in this species, but not of *gnrh3* [64]. Finally, in sea bass, no variation of GnRH1 content was detected in brain or pituitary after injection of kiss1-10 or kiss2-10 in prepubertal and pubertal fish (Felip et al. unpublished data).

At the pituitary level, systemic administration of kisspeptins induced detectable responses in all cases, either in gene expression or hormone secretion. In female zebrafish, kiss2-10 was significantly more potent than kiss1-10 inducing expression of the *fshβ* and *lhβ* subunits [23]. In female orange spotted grouper, kiss2-10 injection also provoked an increase in *fshβ* expression, but had no effect on expression of the *lhβ* gene [64]. In the European sea bass and goldfish, the effects of kiss-10 administration on gonadotropin release from the pituitary were analyzed by measuring gonadotropin levels in blood. In sea bass, kiss2-10 was more potent than kiss1-10 in inducing LH and FSH release in pre-pubertal fish, and LH secretion in pubertal males [18]. On the contrary, kiss1-10 administration in goldfish significantly increased blood LH levels in a dose dependent manner, while kiss2-10 showed no effect [26].

The observed effects of peripheral kisspeptins at the pituitary level opened the question of whether these actions are conducted through activation of GnRH neurons at the brain level or directly through gpr54 activation at the level of gonadotrophs, where this receptor has been shown to be expressed also [74]. In mammals, the presence of kisspeptins in the hypophysial portal circulation and the expression of *Gpr54* and *Kiss1* in gonadotrophs is compatible with a role of kisspeptins in the direct control of gonadotropic function at the pituitary level; however, the physiological relevance of these pituitary actions is still not clear [51, 57]. In non-mammals, direct kisspeptin activation of pituitary cells has been evaluated in goldfish by using an *in vitro* primary culture, but the results obtained are contradictory. In one report neither kiss1-10 nor kiss2-10

400 could elicit LH release at different doses [26], while in another work similar doses of kiss1-10
401 were able to promote LH secretion (as well as prolactin and growth hormone) from pituitary cells
402 [74]. Moreover, long term treatment (24h) with kiss1-10 resulted in up-regulation of *lhβ*, growth
403 hormone and prolactin genes [74]. These data, together with the clear expression of *kiss1* in
404 goldfish somatotrophs, have led to propose that kisspeptin could act as a paracrine/autocrine
405 factor to modulate secretion of pituitary hormones from different neuroendocrine axes [74]. In
406 the same line, kiss2-producing cells were detected by immunohistochemistry in the pars
407 intermedia of the zebrafish pituitary, where MSH and somatolactin expressing cells are located
408 [61]. Similarly, in mammals, some data point to the involvement of kisspeptins in the stimulation
409 of growth hormone and prolactin secretion [28, 66]. On the other hand, a very recent work on
410 European eel has demonstrated for the first time an inhibitory effect of kisspeptins on
411 gonadotropins. In this study, long term treatments (10 days) of eel pituitary cells with kisspeptins
412 from different origins resulted in all cases in an inhibition of *lhβ* expression, while no effect was
413 observed in the expression of other glycoprotein subunits or in the growth hormone gene [50].

414 All together, these data conclusively demonstrate that kisspeptins influence gonadotropin
415 release in fish. In those species with two *kiss* genes, different potencies of kiss1- or kiss2-derived
416 peptides are observed, but their relative potency depends on the species. It should be noted,
417 however, that to date only the kiss-10 forms have been used for *in vivo* studies. Therefore, the
418 results obtained so far might not be conclusive, as there are clear indications that longer
419 kisspeptin forms are more efficient in driving receptor activation. On the other hand, the action of
420 kisspeptins at the pituitary level in fish needs further investigation, and in particular, the
421 expression of *gpr54* in the pituitary and the direct response of cultured pituitary cells to
422 kisspeptin stimulation.

423 In good agreement with their proven ability to activate the gonadotropic axis, it is now
424 recognized that kisspeptins play an important role in the timing of puberty in mammals. This
425 contention stems from the observation of the state of impuberism of rodents and humans with

426 inactivating mutations of GPR54 or Kiss1, and has been documented by a number of expression
427 and functional analyses [51, 68]. These analyses have revealed a complex and multifaceted
428 mechanism for the control of puberty by kisspeptin pathways, which appear to involve not only an
429 enhancement of the hypothalamic kisspeptin tone and GPR54 signaling efficiency during the
430 pubertal transition, but also substantial plastic changes of the populations of Kiss1 neurons, which
431 become more abundant and increase the number of appositions (putative synaptic contacts) with
432 GnRH neurons along pubertal maturation [51, 68]. While characterization of the roles of
433 kisspeptins in the control of puberty in fish is still at its infancy, recent studies performed in the
434 very diversified and evolutionary ancient group of teleosts, such as zebrafish, grey mullet (*Mugil*
435 *cephalus*), fathead minnow (*Pimephales promelas*), tilapia and cobia (*Rachycentron canadum*),
436 have suggested that kisspeptin pathways are also involved in timing the onset of puberty in these
437 non-mammalian species [2, 19, 30, 40, 46]. Yet, it is admitted that most of the evidence linking
438 kisspeptins and puberty in fish is circumstantial, as coming from expression analyses, and
439 characterization of the mechanisms and major sites of action of kisspeptins for such a role in
440 pubertal maturation remains largely incomplete.

441 **6. KISSPEPTINS AS MEDIATORS IN THE PHOTOPERIODIC CONTROL OF REPRODUCTION**

442 There is cumulative evidence that in mammals the photoperiodic control of reproduction
443 involves direct or indirect modulation of the kisspeptin system, which seems to be posed with a
444 central position in the seasonal control of reproduction and would operate as putative mediator of
445 the effects of melatonin (see [55] for review). Among the wide variety of environmental factors
446 that change seasonally and may trigger the BPG axis, photoperiod is the most unvarying
447 geophysical cue from year to year that most vertebrate species use to predict the changing
448 seasons and hence drive gametogenesis and anticipate spawning time. However, how animals
449 obtain information on daily and seasonal changes of day length, including transduction and
450 transmission throughout the BPG axis is still poorly understood despite the latest scientific
451 advances [54]. The pineal organ is responsible for the correct timing of daily and seasonal

452 physiological rhythms on which the circadian production of melatonin by this organ can be
453 modulated by the retinal and biological clock processes. Despite, the neural circuitry that connects
454 the supra chiasmatic nucleus (SCN) to the pineal gland and the signaling mechanisms of melatonin
455 via its membrane receptors are highly complex and vary with the cell type being species specific.
456 In all vertebrates, melatonin is released during the dark phase ensuring high levels of this
457 hormone in plasma and cerebrospinal fluid at night and low during the day. There are convincing
458 evidences that the melatonin mediates the central effects of photoperiod to the neuroendocrine
459 circuitry controlling pituitary functions. Among the pleiotropic functions of this molecule, [54],
460 the seasonal reproduction is an important one because the cyclic production of melatonin not only
461 operates as a “clock” but also as a “calendar” [53], Thus, photoperiod may determine the duration
462 of the melatonin signal while temperature affects its amplitude, providing an accurate definition
463 of both the daily and annual cycles [14]. On the other hand, seasonally changing melatonin
464 message can be used by both, long-day and short-day breeders to adjust their reproductive cycle
465 with the suitable season pointed up that melatonin is neither antigonadotropic nor
466 progonadotropic. More specifically, melatonin can be considered as a factor that mediates
467 photoperiod signals in the regulation of gonadal development since seasonal changes in daylength
468 are likely transduced in the appropriate melatonin rhythms which in turn will regulate the BPG
469 axis. However, many questions still remain incompletely answered in most vertebrate species: At
470 what level of the BPG axis is the melatonin acting? ; What are the systems on which melatonin
471 shows an important interaction?; Could the kiss system be regulated by melatonin?. Some specific
472 information can be found at the reviews of [10, 14, 16, 37, 54]. Collectively, these data indicate the
473 indisputable role of melatonin in synchronizing seasonal reproduction. However, the cellular and
474 molecular sites of melatonin action are so far unknown except that melatonin does not act directly
475 on GnRH neurons and responsiveness to GnRH are not modified by photoperiod treatments [29].
476 Notwithstanding, melatonin binding sites have been recognized in diverse brain structures with
477 important species differences [14, 16, 31]. In seasonal breeders the photoperiodic control of

478 reproduction may involve direct or indirect modulation of the kisspeptin system, which seems to
479 be posed with a central position in the seasonal control of reproduction and would operate as
480 putative mediator of the effects of melatonin. However, data linking photoperiod and the
481 kisspeptin system have been mostly reported in seasonal mammals, such as hamsters and sheep
482 [20, 56, 71]. One of the major advances for the understanding of the molecular mechanisms
483 reinforcing seasonal breeding came from the studies in Syrian hamsters where expression of *kiss1*
484 in the arcuate nucleus was down-regulated by melatonin, whereas kisspeptin administration in
485 photoinhibited animals reactivated reproductive activity [65]. However, it is still not known
486 whether melatonin acts on arcuate *kiss1* expressing neurons or mediates its action via
487 interneurons. Of note, one of the few studies conducted in teleosts addressing this topic
488 demonstrated that long-day photoperiods, which inhibit the onset of puberty, also inhibit
489 kisspeptin receptor expression in tilapia [30]. Likewise, Kanda et al. reported a correlation
490 between photoperiod response and *kiss1* expression in medaka; i.e. long photoperiods
491 (permissive to reproduction) induced higher number of NVT *kiss1* neurons than short
492 photoperiods (inhibitory to reproduction) [22]. Recent studies performed in teleost have shown
493 that the pineal organ exhibits bidirectional connections with the brain through pinealofugal
494 (efferent) and pinealopetal (afferent) projections [11, 15]. Of note, GnRH-2 fibers originating from
495 the synencephalic population of GnRH-2 neurons project to the pineal gland in sea bass [62], thus
496 suggesting that GnRH-2 cells could be an intermediary system mediating the
497 integration/modulation of photoreceptor information perceived by the pineal organ. This is in
498 agreement with the fact that most organisms may modulate their reproductive activity
499 responding to photoperiod by the nocturnal release of melatonin as has been proved recently in
500 zebrafish [3, 62]. In situ hybridization studies identified *kiss1* in the habenula of zebrafish and sea
501 bass suggesting that kiss1 neurons are probably engaged in the perception of environmental and
502 metabolic signals [12, 13, 61]. In addition, studies of the effect of melatonin in zebrafish revealed
503 the ability of melatonin to increase the transcription of *kiss1*, *kiss2*, *gnrh3* genes in the brain, and

504 *lhβ* in the pituitary; expression effects that were associated with an increase of fecundity induced
505 by melatonin in this species.

506 All in all, these results suggest that melatonin may act as a signal mechanism to trigger
507 reproductive capacity in teleosts, by activating a cascade involving kisspeptin pathways which, in
508 turn, stimulate hypothalamic GnRH neurons to switch on the gonadotropic axis, thus supporting
509 the hypothesis that the photoperiod, via melatonin, modulates kiss1 neurons to drive
510 reproductive axis. Very recent studies in zebrafish suggest that while *kiss2* gene is likely involved
511 in the control of the reproductive functions (see previous sections), *kiss1* neurons (that are
512 predominantly located in the habenular nucleus) are probably engaged in the perception of
513 environmental (and possibly) metabolic signals [61]. As a whole, the scarce data gathered to date
514 in fish strongly suggest that, as it has been proposed in seasonal mammals [56], the photoperiod
515 modulates the secretion of melatonin from the pineal gland that, in turn, would directly or
516 indirectly regulate *kiss1* neurons.

517 **7. OTHER REGULATORS OF THE KISSPEPTIN SYSTEM IN FISH: SEX STEROIDS AND**
518 **METABOLIC FACTORS**

519 A large body of evidence has demonstrated that, in mammals, sex steroids are among the
520 most important regulators of Kiss1 expression, with opposite effects in the ARC (inhibitory) and
521 the AVPV (stimulatory) [41, 44, 49]. Although similar studies are scarce in fish, it has been shown
522 that estrogen can play an important regulatory role of the kisspeptin system in non-mammals.
523 Indeed, in medaka ovariectomy significantly reduced the number of *kiss1* neurons in the NVT
524 compared to control but treatment with estradiol (E2) completely reversed this effect, therefore
525 suggesting that these neurons could be involved in the positive feedback control of the brain-
526 pituitary-gonadal axis [21]. In contrast, kiss1 neurons from of NPPv did not show sensitivity to E2,
527 suggesting possible additional roles of *kiss1* in functions not related with reproduction [21]. In
528 good agreement with the above data in medaka, treatment of juvenile zebrafish with E2 caused an
529 increase in the number of kiss2 neurons in the hypothalamus, as shown by ISH. RT-PCR analyses

530 confirmed these results and showed that E2 treatment also caused a significant increase of brain
531 *kiss1* mRNA, although of lower magnitude than *kiss2* responses. Additionally, *gpr54-2b*, but not
532 *gpr54-1b*, expression has been reported to increase following E2 treatment in zebrafish [61].
533 Similarly, in the orange-spotted grouper, changes in the expression of the elements of the
534 kisspeptin system during 17 alpha-methyl-testosterone-induced sex reversal have been reported
535 [64]. Thus, both *kiss2* and *gpr54-2b* expression decreased in the first week after 17 alpha-
536 methyltestosterone implantation, but *kiss2* increased in the fourth week coinciding with a
537 significant increase of *gnrh1* in the hypothalamus. It is interesting to note that hypothalamic
538 estrogen-sensitive kiss2 neurons in the zebrafish exhibit the same location than estrogen-
539 sensitive kiss1 neuron population in medaka so both populations are positioned in the ventral
540 hypothalamus, thus supporting that the kisspeptin system in teleosts may show important
541 variations among species [61], despite the conserved responsiveness of different kiss neurons to
542 sex steroids.

543 In addition to sex steroids, compelling evidence, gathered in various mammalian species,
544 has documented that the hypothalamic Kiss1 system is responsive to changes in the metabolic
545 status of the organism [7, 44, 50]. This has been substantiated in a number of models of metabolic
546 stress, known to inhibit reproductive function, where expression of *Kiss1*/ kisspeptins is also
547 suppressed. In addition, a number of metabolic signals, with key roles in energy homeostasis, such
548 as leptin, ghrelin and NPY, have been shown to influence, either directly or indirectly, the
549 expression of *Kiss1* [7, 44, 50]. Surprisingly, the potential impact of energy status and metabolic
550 cues on the expression of the elements of the kiss/gpr54 system in non-mammalian species
551 remain virtually unexplored until very recently, when Mechaly and co-workers described in the
552 Senegalese sole (*Solea senegalensis*) that fasting results in a significant increase of *kiss2* mRNA
553 levels in the hypothalamus, which is concomitant with an increase of *lhβ* and *fshβ* gene expression
554 in the pituitary [33]. To our knowledge, this is the first evidence in fish suggesting a possible role
555 of kiss2 in the metabolic control of reproduction; yet, it is intriguing that the reported changes in

kiss2 expression following fasting are opposite (increase) to the changes in hypothalamic *Kiss1* mRNA levels observed in rodents under conditions of negative energy balance. The physiological relevance of this phenomenon, as well as the nature and mechanism of action of the signals putatively involved in such a metabolic regulation of the kisspeptin system in fish, are yet to be elucidated.

8. FINAL REMARKS

In the last seven years, we have witnessed an astonishing progress of our understanding of the neuroendocrine and molecular mechanisms responsible for the regulation of the reproductive axis in vertebrates; identification of kisspeptins and their receptor, GPR54, as major players of the reproductive- brain being a significant breakthrough in the area. While mammalian studies have *dominated* the research activities in the field of kisspeptin physiology, in the last few years, an increasing number of reports have been released, which have helped to define the specific characteristics of the elements of the kisspeptin system in non-mammalian vertebrates. These studies have not only substantiated the particular molecular, anatomical and functional features of this system in a variety of fish, amphibians and reptiles, but have underscored also more general aspects, pertaining to the complex molecular evolution and specialization of the genes and peptides of the kiss/gpr54 tandem. Taking the paradigmatic example of GnRH, where specific analyses in non-mammalian vertebrates disclosed the functional diversity of this key neuropeptide family, it is anticipated that further progress in the *comparative physiology* of kisspeptins and their receptors will be crucial to provide an integral knowledge of this essential element of the regulatory circuits governing reproduction in a wide variety of vertebrate species.

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TABLES

Table 1. Identification of the different forms of kisspeptin (*kiss1* and *kiss2*) genes in fish.

Species name	Peptide	Accession Nº or Ensembl	References
Zebrafish	kiss1	EF641126	[70]
	kiss2	AB439561	[23]
Medaka	kiss1	AB272555	[22]
	kiss2	AB439562	[23]
Sea bass	kiss1	FJ008914	[18]
	kiss2	FJ008915	
Goldfish	kiss1	FJ465137	[26]
	kiss2	FJ465138	
Chub mackerel	kiss1	GU731672	[59]
	kiss2	GU731673	
Striped bass	kiss1	GU351864	[75]
	kiss2	GU351865	
Elephant shark	kiss1	AAVX01162971	see [25]
	kiss2	AAVX01172388	
Stickleback	kiss2	assembly BROAD S1[c]	see [18]
Tiger puffer	kiss2	assemblyFUGU4 [a]	see [18]
Green puffer			see [18] [23] [26] [74]
	kiss2	assembly TETRAODON8[b]	
Grass puffer	kiss2	AB548304	[63]
Grouper	kiss2	GQ258777	[64]
Senegalese sole	kiss2	HM116743	[34]
Nile tilapia	kiss2	JN565693	Direct submission

Locations: [a]kiss2 scaffold_171: 390,310-392,041, [b]kiss2, Un_random: 8,262,675-8,266,896; [c]kiss2, scaffold_221: 3,202-3,411

Table 2. Identification of multiple forms of *gpr54* genes in fish.

Species name	Receptor	Accession Nº or Ensembl	References
Nile tilapia	gpr54-2b	AB162143	[49]
Cobia	gpr54-2b	DQ790001	[40]
Grey mullet	gpr54-2b	DQ683737	[46]
Fathead minnow	gpr54-2b	EF672266	[19]
Zebrafish	gpr54-1b	EU047918	[2]
	gpr54-2b	EU047917	
Medaka	gpr54-1b	assembly MEDAKA1[a]	see [25]
	gpr54-2b		
Striped bass	gpr54-1b	-	[75]
	gpr54-2b	GU351869	
Atlantic croaker	gpr54-2b	ABC75101	see [35]
Senegalese sole	gpr54-2b	EU136710	[33]
Goldfish	gpr54-1b	FJ465140	[26]
	gpr54-2b	FJ465139	
Atlantic halibut	gpr54-2b	GQ330487	[32]
Astatotilapia burtoni	gpr54-2b	GQ860302	[21]
Grass puffer	gpr54-2b	AB548356	[63]
Grouper	gpr54-2b	GQ258778	[64]
Sea bass	gpr54-1b	submitted	[17]
	gpr54-2b	submitted	
Tiger puffer	gpr54-2b	assembly FUGU4 [b]	see [17]
Green puffer	gpr54-2b	assembly TETRAODON8 [c]	see [17]
Stickleback	gpr54-2b	assembly BROADS1 [d]	see [17]
Bluefin tuna	gpr54-2b	GQ150542	Direct submission
European eel	gpr54-2b	FR667382	[50]

Locations: [a] gpr54-1b, Ch 9: 4,484,331-4,490,709 and gpr54-2b, Ch 17: 29,839,926-29,854,747; [b]gpr542b, scaffold_80:1,035,934-1,046,929; [c]gpr54-2b, Ch 15_random: 3,123,498-3,129,467; [d]gpr54-2b, groupIII: 13,328,256-13,335,295.

Table 3. Amino acid sequence identity kisspeptins and their cognate receptors in vertebrates.

Forms of kisspeptins			**Forms of kisspeptin receptors**				
	kiss1	*kiss2*		*gpr54-1a*	*gpr54-1b*	*gpr54-2a*	*gpr54-2b*
kiss1	**12-81**		*gpr54-1a*	**40-92**			
kiss2	7-16	**9-82**	*gpr54-1b*	34-66	**57-84**		
			gpr54-2a	36-57	49-61	**100**	
			gpr54-2b	21-59	27-63	43-63	**38-97**

The numbers in bold indicate percent identity in amino acid sequence with the members within each form of *kiss* and *gpr54* genes. The kisspeptin sequences used for amino acid sequence similarity analysis are: for *kiss1*, human (*Homo sapiens*) (AY117143), rat (*Rattus norvegicus*) (AY196983), mouse (*Mus musculus*) (AB162440), opossum (*Monodelphis domestica*) (assembly MonDom 5), platypus (*Ornithorhynchus anatinus*) (assembly Ornithorhynchus_anatinus-5.0), clawed frog (*Silurana tropicalis*) (assembly Version 4.1), lamprey (*Petromyzon marinus*) (assembly 5.9X), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), sea bass (*Dicentrarchus labrax*), goldfish (*Carassius auratus*), chub mackerel (*Scomber japonicus*); for *kiss2*, platypus (assembly Ornithorhynchus_anatinus-5.0), western clawed frog (assembly Version 4.1), lamprey (assembly 5.9X), lizard (*Anolis carolinensis*) (assembly AnoCar1.0), zebrafish, medaka, sea bass, goldfish, chub mackerel, stickleback (*Gasterosteus aculeatus*), tiger puffer (*Takifugu rubripes*), green puffer (*Tetraodon nigroviridis*), grass puffer (*Takifugu niphobles*) and grouper (*Epinephelus coioides*). The kisspeptin receptor sequences used for amino acid sequence similarity analysis are as follow: human *GPR54* (NM_032551) and those orthologs to human GPR54 and tentatively referred as G*pr54-1a* in non-primate mammals including, mouse (NM_053244), rat (NM_023992), pig (*Sus scrofa*) (NM_0011044624), opossum (*Monodelphis domestica*) (XM_001374715) and platypus (assembly Ornithorhynchus_anatinus-5.0; UltraContig Ultra266 at location 390,566-395,434) and *gpr54-1a* for non-mammalian species such as western clawed frog (EU853678) and bullfrog (*Rana catesbeiana*) (EU681171). The remaining sequences from non-mammalian species are as follow: western clawed frog (EU853679 for *gpr54-1b* and EU853680 for *gpr54-2b*), lizard (assembly AnoCar 2.0; Chromosome 2 at location 102,914,244-102,924,790 for *gpr54-2a*), nile tilapia (*Oreochromis niloticus*), cobia (*Racycentron canadum*), grey mullet (*Mugil cephalus*), fathead minnow (*Pimephales promelas*), zebrafish, medaka, atlantic croaker (*Micropogonias undulates*), senegalese sole (*Solea senegalensis*), goldfish, atlantic halibut (*Hippoglossus hipoglossus*), *Astatotilapia burtoni*, grass puffer, grouper, sea bass, tiger puffer, green puffer, stickleback, bluefin tuna (*Thunnus maccoyii*), european eel (*Anguilla anguilla*)

and striped sea bass (*Morone saxatilis*). For accession numbers or Ensembl Genome Browser database see Table 1 and Table 2.

Figure 1. A schematic representation of the known *kiss* and *gpr54* genes during vertebrate evolution. *kiss* and *gpr54* genes probably originated from a common ancestral gene and diversified through gene or genome duplication, and gene modification or deletion. The symbol (●) indicates whole-genome duplications, whereas the asterisk (*) indicates the presence of gene(s). The number (1) indicates the identification of a second kisspeptin receptor-like gene in platypus whose sequence displays a low degree of conservation when it is compared with the available sequences of four different forms of *gpr54*. The number (2) indicates the characterization of a third *kiss* gene has been reported in the Western clawed frog [25]. The letters A-F indicate different orders in Teleostei: (A) Tetraodontiformes, (B) Gasterosteiformes, (C) Pleuronectiformes, (D) Perciformes, (E) Cypriniformes, (F) Anguilliformes.