In 2001, CNP-deficient mice were reported to show severe dwarfism as a result of impaired endochondral ossification [59], thus indicating that CNP acts locally as a positive regulator of endochondral ossification. In 2004, the phenotype of mice lacking GC-B was reported [60]. The GC-B-null animals exhibited dramatically impaired endochondral ossification and attenuation of longitudinal vertebral or limb bone growth. Therefore, it appears that GC-B is the receptor mediating the CNP action in inducing longitudinal bone growth. Furthermore, homozygous C-receptor-null mice also have skeletal deformities associated with a considerable increase in bone turnover [28], an opposite phenotype to that observed in the mice deficient for CNP. Since CNP is the only natriuretic peptide expressed in bone, it is suggested that one function of the C receptor is to clear locally synthesized CNP from bone and modulate its effects.

Since pharmacological amounts of BNP can stimulate GC-B, these results suggest that activation of the CNP/GC-B pathway in transgenic mice with elevated plasma concentrations of BNP or in mice lacking the C receptor for natriuretic peptides results in skeletal overgrowth. By contrast, inactivation of the CNP/GC-B pathway in mice lacking CNP, GC-B or cGMP-dependent protein kinase II (a downstream mediator of the CNP/GC-B pathway) results in dwarfism caused by defects in endochondral ossification.

Summary

As stated above, studies using genetically engineered animals revealed physiological and pathophysiological roles of the natriuretic peptides/receptor signaling pathways in the regulation of blood pressure/volume, maintenance of the cardiovascular system, and development of the longitudinal bone, acting as not only a circulating hormonal system but also a local regulatory system. Recent evidence also suggests roles for the natriuretic peptide system in renal [61] and neuronal [62] morphology and function. In addition, genetic defects of each component of the system in humans may cause diseases that are also observed in the genetically engineered animals. Furthermore, an interesting hypothesis that needs verification is that these observed phenomena could be the recapitulation of early developmental mechanisms. More studies at tissue, cellular and molecular levels are needed to clarify the mechanisms underlying the intriguing phenotypes observed in transgenic animal models. In addition, more studies at clinical and population levels are needed to elucidate the potential importance of the natriuretic peptide system in humans.

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Review

Recent advances in the phylogenetic study of ghrelin

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ABSTRACT

To understand fully the biology of ghrelin, it is important to know the evolutionary history of ghrelin and its receptor. Phylogenetic and comparative genomic studies of mammalian and non-mammalian vertebrates are a useful approach to that end. Ghrelin is a hormone that has apparently evaded natural selection during a long evolutionary history. Surely ghrelin plays crucial physiological roles in living animals. Phylogenetic studies reveal the nature and evolutionary history of this important signaling system.

Contents

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1. General introduction

More than a decade has passed since ghrelin was discovered [56]. In that time, our knowledge about ghrelin has grown. Ghrelin was originally described as a GH-releasing and appetite-stimulating hormone in mammals, but now it is recognized to be a multifunctional hormone with more features than we originally postulated [57,59,112]. Ghrelin is present not only in mammals, but also in a variety of non-mammalian vertebrates, and it exerts species-specific actions (see review [36,37,43,111]). The growth of genome resources has fueled discovery of ghrelin hormone and receptor sequences (Ensembl Genome Browser, http://www.ensembl.org/index.html). In this review, we will focus on non-mammalian vertebrates and summarize the current literature on the ghrelin system, including both the peptides (by inference from cDNAs) and receptor structures from nineteen species of fish, two species of frog, and four species of birds. Then we will comment on the structural features of ghrelin, its precursor protein, and its receptor.

2. Update on the ghrelin system in fish

Biochemical and physiological studies of ghrelin in fishes are progressing every year. The ghrelin gene or the cDNA have been newly described in ten species, including two species of shark, stingray, Arctic char, Atlantic salmon, carp, cod, goldfish, halibut and zebrafish. In addition, cDNA sequence or the gene for the ghrelin receptor (GHS-R) or the GHS-R-like receptor (GHSR-LR) was also reported in five species including channel catfish, goldfish, orange-spotted grouper, rainbow trout and tilapia. Furthermore, diverse species-specific physiological actions of ghrelin have been reported.

2.1. Cephalochordata – Amphioxus

Weng et al. [117] reported the distribution of ghrelin-like immunoreactivity in a cephalochordate, amphioxus (*Branchiostoma belcheri*), suggesting the existence of ghrelin in the chordate ancestors to vertebrates. Using a polyclonal antiserum against synthetic mammalian ghrelin in this study, ghrelin-like immunoreactivity was observed in the nervous system, Hatchek's pit (the primitive pituitary), wheel organ, digestive tract, testis and ovary. In the nervous system, the immunoreactivity was located in the dorsal and ventral sides of brain vesicle and on the funnel part near Hatchek's pit extending from the right side of the brain vesicle. In Hatchek's pit, ghrelin-like immunoreactivity was detected in each layer of epithelial cells and in wheel organ cells. In the digestive tract, it was found in the hepatic diverticulum and midgut. The distribution patterns of the positive cells are different in each position of digestive tract: most cells in the hepatic diverticulum are spindle-shaped with slightly bulging apex and base, and a narrower middle. These cells are typical open-type gastroendocrine cells. Two types of immunopositive cells are observed in the posterior region of the midgut: one is circular or elliptical shape, and the other is spindle-shaped or coniform in appearance. In gonads, ghrelin-like immunoreactivity was located in the karyotheca, nucleolous membrane, cytoplasmic oogonium, oocyte and follicle cells of the ovary. In the testis, it was found in spermatogonia, primary spermatocytes, and Sertoli cells. The ghrelin immunoreactive cells showed similar distribution patterns to those in other vertebrates, but the chemical characteristics of the ghrelin-immunoreactive substance are still unidentified. The presence of a ghrelin-like substance in amphioxus will be important for elucidating the evolution of ghrelin function in vertebrates and invertebrates.

2.2. Chondrichthyes –Elasmobranchii

2.2.1. Shark

Kawakoshi et al. [51] isolated a ghrelin-like peptide and its cDNA from stomach of two species of shark, the hammerhead shark (*Sphyrna lewini*) and the blacktip reef shark (*Carcharhinus melanopterus*). The cDNAs are 646 bp (GenBank ID: AB254128) and 656 bp (GenBank ID: AB254129) long, respectively, and encode a 108-amino acid protein (Fig. 1). The mature peptides generally consist of 25 amino acids, and only three amino acids differ between the two. In addition, 24-amino acid peptide (des-Lys^{25}), and 22- and 24-amino acid peptides (des-$Asn^{23}Pro^{24}Lys^{25}$ and des-Lys^{25}) have also been identified in the hammerhead and the blacktip reef shark, respectively. Also in these sharks, Ser-3 has been modified by either *n*-octanoic or *n*-decanoic acid, which is characteristic of ghrelins. However, the peptides show only low identity compared to other ghrelin peptides; especially, the N-terminal seven residues (GVSFHPR) are quite different from those of other ghrelins where the sequence is conserved (GSSFLSP) (see Fig. 3, which shows ghrelin precursor for bony fishes). The naming as "ghrelin-like peptide" is due to these different characteristics, and to the fact that overall sequence homology of precursor protein is extremely low compared to others. However, supporting their assignment as "ghrelin"-like peptides are the following: (1) rat GHS-R1a activating property; (2) the presence of acyl modification on Ser-3; (3) predominant mRNA expression in the stomach, and (4) similarity of the gene organization to other ghrelin genes (8541 bp in length, and containing five exons and four introns for the hammerhead shark, GenBank ID: AB254130). This structure might reflect the ancestral form of the ghrelin molecule.

2.2.2. Stingray

The author's group has reported isolation of ghrelin-like peptide from another cartilaginous fish, the red stingray (*Dasyatis akajei*) [38]. The identified peptide consists of only 16 amino acids, and Ser-3 is modified by *n*-octanoic acid. The most interesting feature is that, this peptide has another molecular modification; specifically, glycosylation by mucin-type glycan chains [*N*-acetyl hexosamine $(HexNAc)_3$ hexose $(Hex)_2$] is present on threonine at position 11 (Thr-11) or on both serine at position 10 (Ser-10) and Thr-11, in addition to the acyl modification at Ser-3 (Fig. 2). The

Fig. 1. Comparison of prepro-ghrelin in elasmobranchs. GenBank ID is AB254129 for blacktip reef shark, AB254128 for hammerhead shark and AB4800033 for the red stingray. Amino acid residues that are identical among all species are densely shadowed; those residues conserved in more than half of the listed species are lightly shadowed. Sequences were aligned using the GENETYX-Mac software program version 15.0.1.

stingray ghrelin-like peptide cDNA is 527 bp in length (GenBank ID: AB480033), and the deduced precursor protein consists of 98 amino acids, which is the shortest compared to others (Fig. 1). Comparisons of the amino acid sequence of the precursor proteins revealed that the identity of the stingray peptide to mammals was much lower compared to ghrelin from teleosts (20–27%), but there is relatively high sequence identity for shark ghrelin-like peptide (33% for the hammerhead shark and 35% for the blacktip reef shark) (Fig. 1). The stingray ghrelin-like peptide gene is predominantly expressed in the stomach, and followed by the pituitary, esophagus and duodenum. The unglycosylated synthetic stingray ghrelin-like peptide produced increases in intracellular Ca^{2+} levels in Chinese hamster ovary (CHO) cells stably expressing rat GHS-R1a, although it is less effective than rat ghrelin in this regard. Furthermore, catalysis of the *O*-glycosylation of the native stingray peptide reduced the Ca^{2+}-increasing activity. The functional significance of the glycan-chains of stingray ghrelin-like peptide is still unknown, but the modification seems to be related to the maintenance of biological activity at least in an *in vitro* assay although the glycosylation is not necessary to exhibit ghrelin-like activity. Furthermore, there is the possibility that the modification regulates the physicochemical features of this peptide, and acts as polar molecules that stabilize the peptide in the circulation of the stingray, as observed in an experiment using rat ghrelin fragments [106].

2.3. Actinopterygii – Neopterygii – Teleostei

Alignments of ghrelin precursor protein, ghrelin receptor (GHS-R) and GHS-R-like receptor (GHSR-LR) for bony fishes are shown in Figs. 3 and 4, respectively.

2.3.1. Arctic char

Arctic char (*Salvelinus alpinus*) is both a freshwater and seawater fish in the salmonidae family, native to Arctic, sub-Arctic and alpine lakes and coastal waters. As anadromous (sea-migrating) Arctic char display pronounced seasonal variations in food intake and growth, it becomes an interesting model for studying mechanisms of appetite regulation. Frøiland et al. [26] reported the ghrelin cDNA sequence and changes in ghrelin mRNA expression during a seasonal feeding cycle in this species. The cDNA is 540 bp in length, and

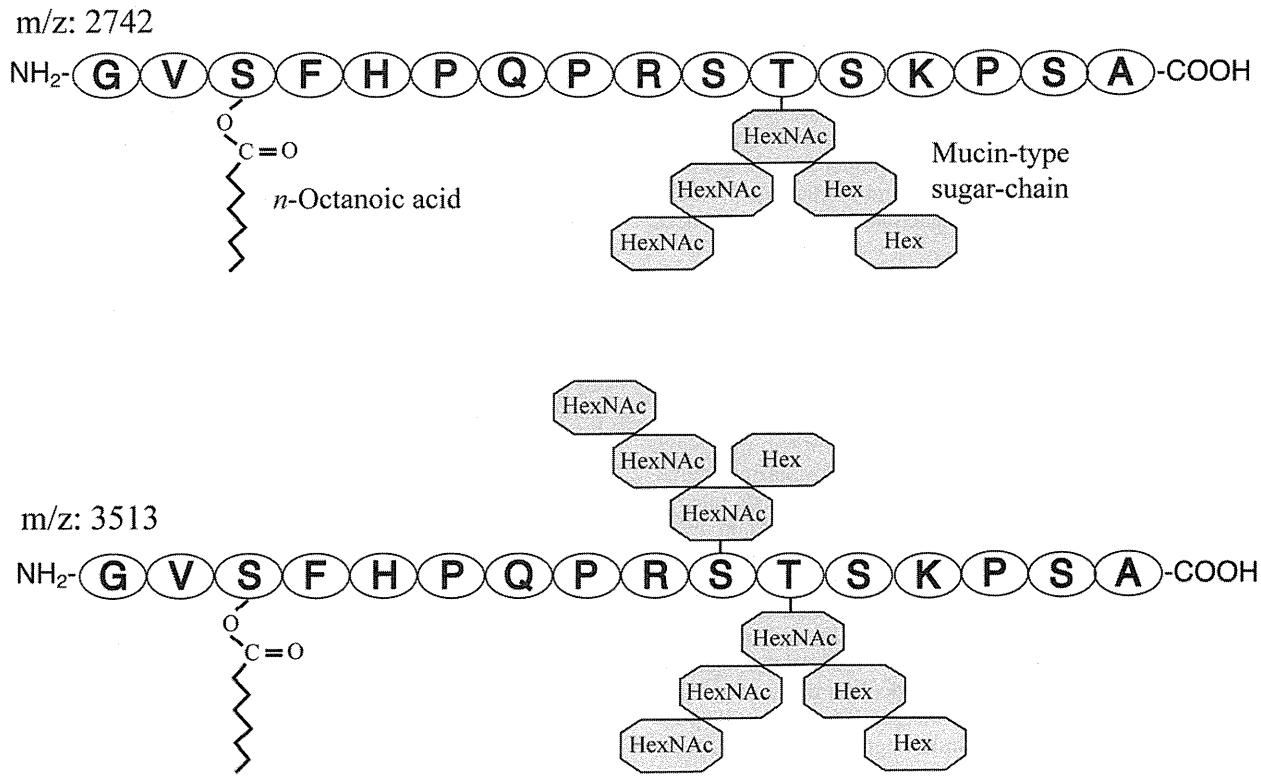

Fig. 2. Predicted primary structure of stingray ghrelin-like peptide. These structures were predicted based on protein sequencing and MALDI-TOF MS/MS of *m/z* 2742.33 and *m/z* 3513.74. Ser-3 is modified by *n*-octanoic acid, and either Ser-10 or Thr-11 is modified by mucin-type glycan chains.

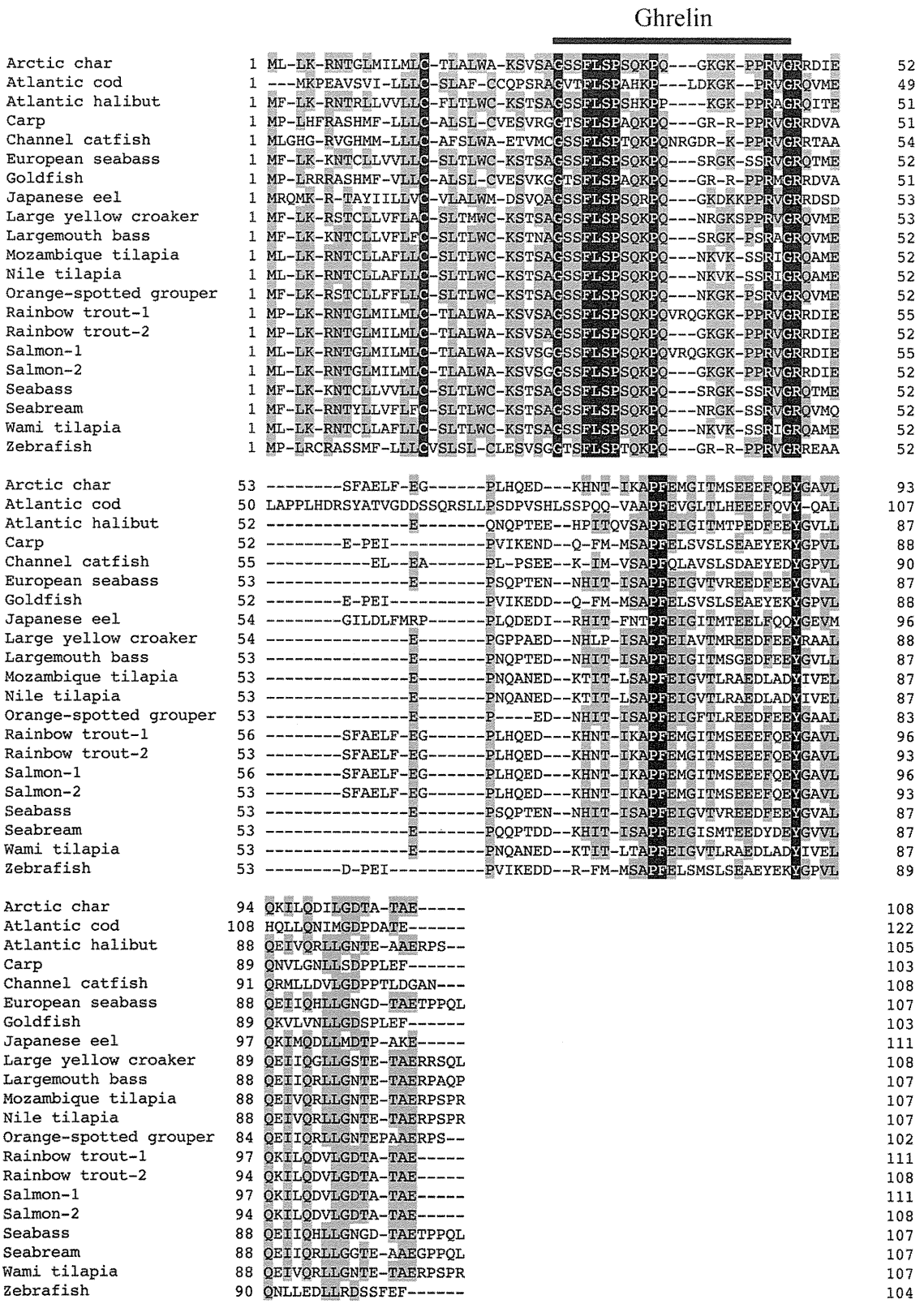

Fig. 3. Comparison of prepro-ghrelin peptides in bony fishes. Peptide sequences were aligned using the GENETYX-Mac software program version 15.0.1. Amino acid residues that are identical among all species are densely shadowed; those residues conserved in more than half of the listed species are lightly shadowed. GenBank accession numbers for sequences are: AB490668 for Arctic char, EU128174 for Atlantic cod, EF493849 for Atlantic halibut, AB332394 for common carp, DQ665912 for European sea bass, FJ560488 for large yellow croaker, EU932862 for largemouth bass, AB443431 for salmon-1 and AB443432 for salmon-2. Other protein sequences have been described in Ref. [43].

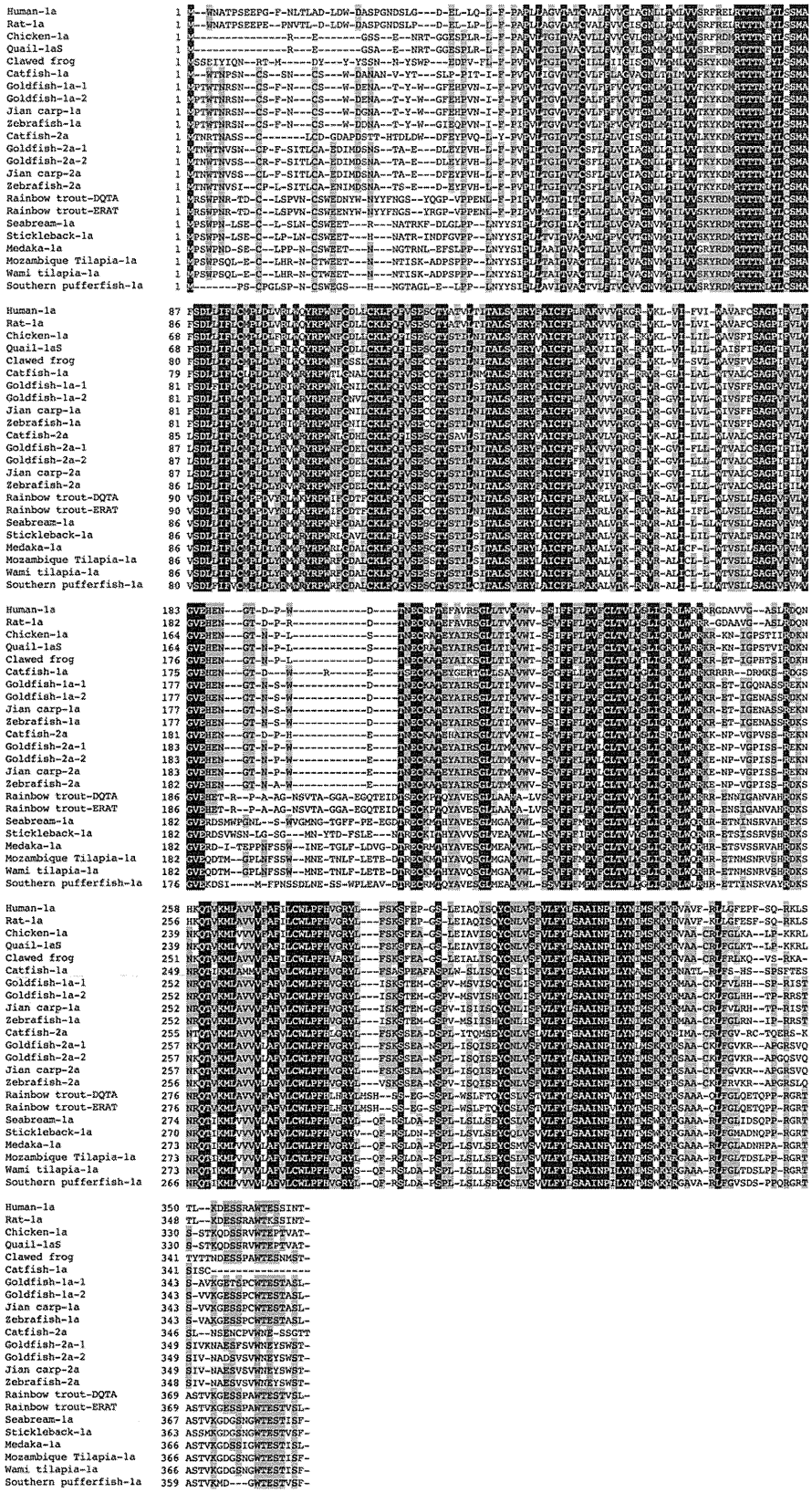

Fig. 4. Multiple sequence alignment of the ghrelin receptor (GHS1a-R) or ghrelin receptor-like receptor (GHSR1a-LR) protein. Peptide sequences were aligned using the GENETYX-Mac software program version 15.0.1. Amino acid residues that are identical among all species are densely shadowed; those residues conserved in more than half of the listed species are lightly shadowed. GenBank accession numbers or Ensembl ID for sequences are: XM_002931572 for clawed frog (*Xenopus tropicalis*), FJ707319 for catfish-1a, AB504275 for goldfish-1a-1, AB504276 for goldfish-1a-2, HM191491 for Jian carp-1a, FJ707321 for catfish-2a, AB504277 for goldfish-2a-1, AB504278 for goldfish-2a-2, HQ162474 for Jian carp-2a, AB362479 for rainbow trout-DQTA, AB362480 for rainbow trout-ERAT, ENSGACP00000014489 in Ensembl for sticleback-1a, ENSORLP00000014678 in Ensembl for medaka-1a, AB361053 for Mozambique tilapia-1a, EU243664 for Wami tilapia-1a. Other protein sequences have been described in Ref. [43].

contains a 327-bp open reading frame (ORF) that encodes a 108-amino acid protein (GenBank ID: AB490668), which was a homolog of ghrelin-2 in Atlantic salmon and rainbow trout (Fig. 3). Specific growth rate and percentage of body total lipids showed positive correlation with the seasonal change in stomach ghrelin mRNA. This suggests that ghrelin may participate in long-term regulation of appetite and energy homeostasis in this fish.

2.3.2. Atlantic cod

Xu and Volkoff [118] reported the cod ghrelin cDNA sequence; the full-length 502 bp ghrelin cDNA contains a 369-bp ORF that encodes a 122-amino acid precursor protein, and deduced 19-amino acid ghrelin peptide. The threonine residue at amino acid position 3 in cod ghrelin differs from the serine present in other teleosts and mammalian ghrelin, but it is the same with bullfrog ghrelin [39]. The ghrelin gene is 904 bp in length, and is comprised of four exons and three introns (GenBank ID: EU128174). They also identified a shorter cDNA encoding a 98-amino acid ghrelin precursor that is missing within the second intron fragment (from Val62 to Glu85).

Ghrelin mRNA is predominantly expressed in the stomach, and also detected in the brain, skin, spleen, kidney, heart, liver and gut. The pituitary gland also expresses ghrelin mRNA, but the level is very low. In terms of ontogenic expression changes, ghrelin mRNA was detected as early as the cleavage stage, and observed throughout development until free-feeding stage 2 (post first-feeding), but the expression level remains unchanged.

Ghrelin mRNA expression in the stomach showed peri-prandial changes in fish fed medium ration, but was not affected by food size ration, indicating that ghrelin plays a physiological role in meal initiation in cod. That is, ghrelin might act as a short-term hunger signal in cod. This is reinforced by the fact that a 1-month starvation followed by 5 days re-feeding did not alter ghrelin expression in cod stomach, suggesting that ghrelin is not involved in regulation of long-term feeding behavior. The effects of fasting on ghrelin expression level appear to vary across species, which may reflect species-specific differences in energy metabolism and resistance to starvation.

2.3.3. Atlantic salmon

Another *Salmonidae*, Atlantic salmon (*Salmo salar*) is also an anadromous fish, and it undergoes its greatest feeding and growth in seawater. Murashita et al. [72] reported the ghrelin cDNA. They cloned two cDNAs, namely ghrelin-1 (GenBank ID: AB443431) and ghrelin-2 (GenBank ID: AB443432). Ghrelin-1 cDNA was 470 bp in length and a 336-bp ORF encodes a 111-amino acid protein, while ghrelin-2 cDNA encoded a 108-amino acid protein, which was identical to prepro-ghrelin-1 except the deletion of GATA-GACAG nucleotides of the cDNA, and this manner is identical to des-VRQ-rainbow trout ghrelin [40]. Only one amino acid of the prepro-ghrelin sequences (Leu2 in trout to Pro2 in salmon) is different between the two species, and the deduced mature ghrelins are identical. Ghrelin mRNA is also predominantly expressed in the stomach, and weakly in the pyloric caeca and adipose tissue. In a 6-day starvation experiment, gastric ghrelin-2 mRNA expression did not change, whereas ghrelin-1 mRNA expression increased after the starvation, suggesting an orexigenic role of ghrelin-1 and a possible different transcriptional regulation between ghrelin-1 and 2 in Atlantic salmon.

Physiological stress generated by normal husbandry practices in aquaculture can have an inhibiting affect on growth of cultured stocks through the suppression of both appetite and feeding behavior [68]. One of the primary endocrine responses to stress in fish is a consistent elevation of plasma cortisol level, and exogenous cortisol inhibits food intake in fish [6]. Pankhurst et al. [79] reported a mechanism for stress suppression of feeding in juvenile cultured Atlantic salmon. Since stress that is accompanied by elevation of plasma cortisol levels results in suppression of feeding, since there is a predicted association of suppressed plasma ghrelin levels with reduced food intake in pre-smolt and smolt juvenile salmon, further study on the direct relationship between cortisol and ghrelin in this fish is indicated.

2.3.4. Common carp

Kono et al. [58] reported isolation of ghrelin cDNA in common carp (*Cyprinus carpio*). The ghrelin cDNA is 461 bp in length, and encodes a 103-amino acid precursor protein (GenBank ID: AB332394), which shows high identity to that of goldfish (82%). The mature peptide was predicted to be 19-amino acids, and only one amino acid (Val19) is different from goldfish ghrelin with its Met19 [69,108]. The carp ghrelin gene consists of four exons and three introns (GenBank ID: AB332394). Carp ghrelin mRNA is predominantly expressed in the gut (a stomach is lacking), followed by spleen and brain. No mRNA expression is detected in the gill, head kidney, liver, heart, skin or muscle. In carp, ghrelin gene expression is detected in the spleen. Ghrelin mRNA expression in the spleen was stimulated by *in vitro* treatment with lipopolysaccharide (LPS) 24 h after incubation exposure, with imiquimod 12 h after exposure, or with phytohemagglutinin (PHA-L_4) 1 h exposure. The results of the former two suggest that ghrelin is involved in down-regulation of cytokine production. Carp ghrelin may control inflammatory or immune responses in carp.

Sokołowska-Mikołajczyk et al. [102] reported effects of ghrelin on spontaneous and salmon GnRH analog (sGnRH)-stimulated luteinizing hormone (LH) release from pituitary cells of common carp. Human ghrelin (10^{-6} M) caused an increase in spontaneous LH release of pituitary cells from females but not from males. Ghrelin in combination with sGnRH increased LH release from pituitary cells of both males and females. These results are in agreement with previous observations in goldfish [110], and indicate ghrelin's involvement in gonadal and reproductive functions in non-mammalian vertebrates [107]. However, this stimulatory action of ghrelin on LH release observed in these two fishes is opposite to that seen in mammals, where ghrelin inhibits LH secretion [27,52,114]. A complex mode of action seems to govern gonadotropin secretion [23].

2.3.5. Channel catfish

Catfish ghrelin was identified in 2005 by the author's group [49]. Peterson et al. [82] has reported endocrine responses of fast- and slow-growing families of channel catfish. In those studies, fast-growing fish consumed 135% more food than slow-growing fish, although the abundances of ghrelin mRNA in the gut and NPY mRNA in the hypothalamus are similar. This result suggests that ghrelin is involved in feeding behavior in channel catfish, but a direct effect has not been proven.

Small et al. [101] reported sequence, and genomic organization of GHS-R-like receptor (GHSR-LR) in channel catfish. They identified two GHSR-LR genes from the pituitary. A GHSR1a-LR cDNA is 1632 bp, with a 1035-bp ORF that encodes a 344-amino acid protein. The GHSR1a-LR gene (GenBank ID: FJ707319) is composed of two exons separated by a 749-bp intron. A GHSR1b-LR sequence with 307 amino acids was also identified in an 1877-bp cDNA (GenBank ID: FJ707320). And another GHSR-LR gene is more similar to zebrafish GHS-R2a. The catfish GHSR2a-LR cDNA is 1490 bp in length, with a 1089-bp ORF encoding a 362-amino acid protein (GenBank ID: FJ707321). The genomic DNA of GHSR2a-LR is comprised of two exons separated by a 1379-bp intron. Catfish GHSR1a-LR and 2a-LR share a 67% sequence identity between them, suggesting that these identified proteins are paralogs that resulted from a duplication during molecular evolution (see [42]). Unfortunately, it has not been proven whether ghrelin can activate those

GHSR1a-LR and 2a-LR. Expressions of GHSR1a-LR and 1b-LR mRNAs were much higher in the pituitary, and lower expression (1/10–1/2 relative to the expression level in the pituitary) was detected in all central and peripheral tissues examined. On the other hand, the highest expression of GHSR2a-LR mRNA was detected in the Brockmann bodies that comprise the pancreatic islet endocrine tissue in teleosts, and the expression level was of similar magnitude to GHSR1b-LR. Expression levels of GHSR2a-LR mRNA were low in central tissues such as the hypothalamus and pituitary.

Small et al. [101] have also examined ontogenetic and ghrelin-induced changes in GHSR1a and 2a-LR mRNA expressions. GHSR1a-LR mRNA expression was high in unfertilized eggs, and gradually declines during embryogenesis, suggesting that GHSR1a-LR mRNA in early embryos is a maternal contribution, and that the expression is regulated by the progress of embryogenesis. On the other hand, GHSR2a-LR mRNA expression was not detected in unfertilized eggs, and was minimally detected at 10, 26 and 34 h post-fertilization (hpf), later increased at 56 hpf, and was highest at the time of hatching.

Intraperitoneal injection of homologous catfish ghrelin-Gly (23-amino acid ghrelin of which Gly extended at the C-terminus), but not ghrelin-amide (22-amino acid ghrelin of which there is the amide structure at the C-terminus), increased pituitary GHSR1a-LR mRNA expression 2 h after injection, whereas no increase was observed in pituitary GHSR2a-LR mRNA expression. In Brockmann bodies, GHSR1a-LR mRNA expressions of both ghrelin-Gly and ghrelin-amide injected fish were relatively higher than that of saline-injected controls, whereas GHSR2a-LR mRNA expression dramatically increased 4 h after injection of ghrelin-amide and 6 h after injection of ghrelin-Gly. It is interesting to note that different actions are caused by these two peptides on GHSR-LR mRNA expression as well as on the regulation of GH release [49].

2.3.6. Flounder

Breves et al. [7] reported localization of ghrelin in the stomach in a juvenile, marine teleost, the summer flounder (*Paralichthys dentatus*). The immuno-reaction was specific for cells of the gastric gland, and the staining was evident in the glandular epithelium. They also looked at the effect of ghrelin on the hypothalamic–pituitary–interrenal (HPI) axis in this fish. There was a trend for human ghrelin (10–1000 ng/g BW) or des-acyl human ghrelin (1000 ng/g BW) to increase plasma cortisol levels, but the changes were not consistent. Further study is needed to clarify the possible effects of ghrelin on the HPI-axis in summer flounder.

2.3.7. Goldfish

Goldfish ghrelin mRNA has two putative processing sites and amidation signals after the 12th and 19th amino acids; consequently, two putative amidated peptides have been proposed in this species [108]. Miura et al. [69] isolated endogenous ghrelin from intestinal extracts of goldfish. In fact, eleven molecular forms of ghrelin were identified, consisting of 14-, 17-, 18- and 19-amino-acid residues, and with the Ser-3 of these peptides expected to be acylated with C8:0, C9:0, C9:1, C10:1 and C10:2 based on mass spectrometric analyses. The predominant form of goldfish ghrelin was the 17-residue peptide without amidation at the C-terminus, being acylated by *n*-octanoic acid (C8:0). It was clear from this study that the number of amino acids in the predominant form of goldfish ghrelin was different from that expected from its cDNA sequence [108]. Actually, it is likely that the peptide is cleaved from pro-ghrelin with a furin-like peptidase recognizing the R-X-X-R sequence. The nineteen-amino-acid ghrelin with C-terminal amidation that had been deduced from cDNA sequence is actually present. On the other hand, a 12-amino-acid ghrelin was not identified by the purification process. The predominant form of 17-residue goldfish ghrelin showed the same potency with the 12-residue form when assessed by *in vitro* Ca^{2+} mobilization assay and *in vivo* feeding activity.

The author's group identified and characterized goldfish GHS-R [42]. Goldfish has two types of GHS-R as seen in zebrafish and catfish, with these receptors named as GHS-R1a and 2a. In addition, subtypes are described for both receptors, namely GHS-R1a-1 (GenBank ID: AB504275 for the cDNA, AB555555 for the gene) and 1a-2 (GenBank ID: AB504276 for the cDNA, GenBank ID: AB555556 for the gene), and GHS-R2a-1 (GenBank ID: AB504277 for the cDNA, GenBank ID: AB555557 for the gene) and 2a-2 (GenBank ID: AB504278 for the cDNA, GenBank ID: AB555558 for the gene). These receptors are generated from four distinct mRNAs that originate from four different genes. The GHS-R genes have a common characteristic composition of two exons separated by an intron. Both GHS-R1a are 360-amino acids protein, while GHS-R2a-1 and 2a-2 are comprised of 366 and 367 amino acids, respectively. GHS-R1a and 2a proteins in goldfish show high sequence identity (96–97%), and there is high sequence identity with zebrafish GHS-R (94–96%). On the other hand, sequence identity between GHS-R1a and 2a is 72–74%. This suggests that the GHS-R1a and 2a resulted from gene duplication (see [42]), and their subtypes by tandem duplication of each gene.

These identified receptor proteins are named GHS-R because their activation by ghrelin is confirmed. Actually, goldfish GHS-R, with the exception of GHS-R2a-2, are activated by goldfish ghrelin and by artificial growth hormone secretagogues (GHSs) with different characters: 1a-1 is activated by goldfish ghrelin 12-C8, 17-C8 and 17-C10 with equal potency and intensity, and rat ghrelin and hexarelin also act with equal potency and intensity, but GHRP-6 does not; 1a-2 is more preferably activated by goldfish 17-C10 than 12-C8 and 17-C8, but not by rat ghrelin and two GHSs (GHRP-6 and hexarelin); 2a-1 is equally activated by all ligands examined, but the intensity is one-third relative to other GHS-Rs. In addition, these three receptors interact with a GHS-R antagonist, [D-Lys^3] GHRP-6, although affinity to GHS-R2a-1 is relatively weak.

The four identified GHS-Rs differentially distributed in body tissues: 1a-1 mRNA is mainly present in the CNS, liver, intestine, ovary and testis; 1a-2 mRNA is in the CNS, liver and testis; 2a-1 mRNA is in the CNS, body kidney, ovary and testis; 2a-2 is in the CNS, testis and gill. Ghrelin can stimulate GH and LH release from goldfish pituitary [31,32,108,110]. GHS-R1a-2 mRNA is the most abundant form in the pituitary, and then low levels of 1a-1 and 2a-2 mRNA are expressed, and 2a-1 mRNA is negligibly level, suggesting that GHS-R1a-2 is involved in the event. Expression of GHS-R1a-1 or 1a-2 mRNA is altered by 7 days of fasting: 1a-1 mRNA declines in the vagal lobe, and 1a-2 mRNA increases in the liver after fasting, though mRNA expressions of both receptors do not change in the olfactory lobe, indicating that negative energy status brings about specific changes in mRNA expression. Further study is needed to clarify the functional relationships among ghrelins and their receptors.

As described above, ghrelin can stimulate GH or LH release from the pituitary of goldfish *in vivo* and *in vitro* [108,110], and Gray et al. [31,32] demonstrated the mechanism of action using homologous goldfish ghrelin-19-amide and dispersed pituitary cells. The entry of extracellular Ca^{2+} through the L-type voltage sensitive Ca^{2+} channels is a key component of the ghrelin signaling pathway leading to GH and LH release from somatotropes and lactotropes, respectively.

De Pedro et al. [19] reported melatonin effects on plasma ghrelin levels in goldfish. IP injection of melatonin (10 μg/g BW) for 10 days 1 h before dark phase reduced body weight gain, and showed a suppressive trend in circulating ghrelin although the effect is not significant level compared with controls. This suggests that ghrelin could be a mediator of feeding and body weight reduction by melatonin in goldfish as seen in mammals [73].

2.3.8. Halibut

Manning et al. [67] have reported cDNA sequence of Atlantic halibut (*Hippoglossus hippoglossus*). The 899-bp cDNA encodes an ORF of a 105-amino acid prepro-ghrelin. The highest identity (69–67%) was shown in the perciform teleosts such as seabream, sea bass and tilapia, intermediate identity (50% and 47%) was seen in rainbow trout ghrelin-1 and -2, and low identity (41–33%) was shown in eel and the ostariophysan teleosts such as goldfish, zebrafish and catfish. The deduced mature ghrelin is 19 amino acids in length with a potential amidation signal of the C-terminus. Ghrelin gene expression in 3-years-old halibut is mainly found in the stomach, with lower expression in pyloric caeca, ovary, intestine and testis. Ontogenic ghrelin expression in halibut is described: low levels of the gene expression are seen over the yolk-sac stage with little change in normalized expression occurring even by pro-metamorphosis; increased expression was associated with metamorphosis; the highest expression level was seen in fish staged as either juveniles or advanced climax metamorphic stage larvae.

2.3.9. Hybrid striped bass

Picha et al. [85] reported the regulation of GH secretion by ghrelin during variable metabolic states in hybrid striped bass. They looked at changes in plasma GH and ghrelin during seasonally based feeding and temperature manipulations. Fasting for 21 days at 24 °C increased plasma GH and ghrelin levels relative to the fed controls. Continued fasting for 90 days at 14 °C resulted in a further 43-fold increase in plasma ghrelin level while plasma GH level remained elevated. A subsequent 19 days of re-feeding at 24 °C elicited hyperphagia and compensatory growth responses, accompanied by declines in plasma GH and ghrelin levels. These observations suggest that ghrelin mediates in part a sustained catabolic-induced rise in plasma GH level during seasonally based period of food deprivation and low temperature in hybrid striped bass. The increase in plasma ghrelin level during negative energy balance is considered to be critical to the dramatic hyperphagic response and accelerated growth after re-feeding.

Furthermore, they examined *in vivo* effects of ghrelin on GH release. Single IP injection of ghrelin in fish reared at 24 °C showed dose-dependent increases in plasma GH level 6 h after injection. In *in vitro* study, ghrelin stimulated GH release from tissue culture of pituitaries during 6 h of incubation. Spontaneous GH release over a 6 h of incubation from pituitaries of 25-day fasted fish was 5.2-fold higher than that of fed fish. In this condition, 10 nM ghrelin increased GH release of fed and 25-day fasted fish by 2.9 and 2.0 times, respectively, than that of their respective controls, and was ineffective in stimulating GH release in the case of fish that were fasted for 21 days followed by 3 days of re-feeding. These results suggest that the metabolic condition of pituitary before incubation influences spontaneous GH release, but that response to ghrelin is not altered by the changes in energy status.

2.3.10. Orange-spotted grouper

The ghrelin sequence in orange-spotted grouper (*Epinephelus coioides*) has been identified and was deposited in GenBank in 2005; since then, there are no further reports on the topic. Before this, Ran et al. [86] had shown *in vitro* effects of rat ghrelin on the release and mRNA expression of GH in the pituitary of this species. Rat ghrelin stimulated GH release and GH mRNA expression 24 h after incubation in a dose-dependent manner over the concentration range 10^{-9} to 10^{-6} M. Ghrelin treatment at 10^{-7} M stimulated GH release and GH mRNA expression from 6 h after incubation. Similar effects on GH release have been observed in tilapia and goldfish [91,110] as well as in rats [56]. A direct effect of ghrelin on GH synthesis has been reported in tilapia [90].

Chen et al. [16] reported the identification of GHS-R1a and 1b in orange-spotted grouper. The gene comprises two exons and one intron, and the cDNA for GHS-R1a is 1512 bp in length with an ORF that encodes a 383-amino acid protein, while the 1703-bp GHS-R1b cDNA contains an ORF that encodes a 303-amino acid protein. Unfortunately, these sequences are not available in the database at present.

The GHS-R1a mRNA is predominantly expressed in the CNS, with the highest expression levels in the pituitary, followed by the hypothalamus. Lower expression is seen in the intestine, thymus, liver and heart. On the contrary, GHS-R1b mRNA shows a different expression pattern compared with GHS-R1a, and is detected to the same extent in all brain regions and peripheral tissues examined.

They have also examined ontogenic changes in GHS-R1a and 1b mRNA. GHS-R1a mRNA is first detected at the neurula stage with little change in the expression level by 2 day post-hatch (dph), and then expression gradually increases during 3 dph to 5 dph. In contrast, GHS-R1b mRNA was found in fertilized eggs but not in unfertilized eggs, and from blastula stage to 5 dph, and its expression remained steady. The physiological significance of this different expression pattern between the two receptors remains unclear yet.

Furthermore, they have reported the detailed localization of GHS-R mRNA in the grouper brain. GHS-R-expressing cells are widely distributed in several brain regions such as olfactory bulbs, telencephalon, diencephalon, mesencephalon, cerebellum and pituitary. Moreover, they looked at the effects of GH or ghrelin on GHS-R mRNA expression in pituitary cells and hypothalamic explants. GH (10^{-7} M) reduced GHS-R1a mRNA only in the primary culture of pituitary cells, and 10^{-5} M rat ghrelin decreases its mRNA expression in both pituitary cells and hypothalamic explants. Expression of GHS-R1b mRNA was decreased by 10^{-7} M GH in both pituitary cells and hypothalamic explants, and was decreased by 10^{-5} M rat ghrelin. No effect from des-acyl ghrelin was seen. These results indicate feedback regulation of GHS-R mRNA expression in the hypothalamus and pituitary.

2.3.11. Rainbow trout

For rainbow trout, changes in plasma ghrelin levels, ghrelin effects on feeding, and identification of GHSR-LR have all been reported.

Pankhurst et al. [78] found that plasma ghrelin levels increase 24 h after a single feeding with concomitantly decreased plasma lactate and glucose levels. A similar peri-prandial increase in serum ghrelin is seen in goldfish [11]. One hour of confinement stress resulted in elevations of plasma cortisol, glucose and lactate levels and depression of plasma ghrelin level, but in another stress experiment there was no depression of plasma ghrelin level. In these two experiments, pre-treatment plasma ghrelin levels differed; the former was high (250 pg/ml) and the latter was low (50 pg/ml).

Regarding the effects of ghrelin on feeding in rainbow trout, Jönsson et al. [34] reported that a single IP injection of rat ghrelin did not influence food intake during the 12 h after injection. On the other hand, Shepherd et al. [97] observed increases in food intake 2 or 5 h after a single IP injection of a GHS, KP-102 or rat ghrelin in juvenile rainbow trout. And Jönsson et al. [35] reported that ICV injection of rainbow trout ghrelin (2 ng/g BW) inhibits food intake in juvenile rainbow trout, and that the inhibitory effect is mediated through the CRF signaling system. In addition, they also observed an inhibitory effect on feeding by long-term (14-day) treatment with rainbow ghrelin delivered via cholesterol pellet. This treatment did not affect swimming activity, growth, lipid content of mesenteric adipose stores, liver and muscle, or plasma GH levels. This inhibitory mechanism of ghrelin on food intake is similar to that demonstrated in the chicken [94]. The controversial findings regarding food intake seen in rainbow trout could be due to the route of injection and to different durations of elevated ghrelin levels in the circulation. It is highly possible that long-term elevation

of ghrelin could produce an inhibitory influence on feeding in rainbow trout. The relevance of ghrelin on homeostatic regulation of energy balance in this fish remains uncertain.

Although the GHS-R in rainbow trout was unknown for a long time, the author's group identified the cDNA sequence and the genomic organisation [44]. Two variants of GHSR1a-LR with 387 amino acid, namely DQTA/LN-type (GenBank ID: AB362479) and ERAT/IS-type (GenBank ID: AB362480) were found, which are designated from specific sequence features of the receptors; amino acid substitutions have occurred at D20E, Q32R, T54A, A62T, L168I and N264S. These receptor proteins showed the highest identity with seabream GHS-R1a (71%), and with pufferfish and zebrafish GHS-R1a (67% identity). In addition, we also identified three GHS-R1b orthologs that are comprised of 297 or 300 amino acid residues with different amino acid sequences at the C-terminus. These two GHSR1a-LRs and three GHSR1b-LRs are generated from three distinct genes. These receptor genes are predominantly expressed in the pituitary, followed by the brain and other peripheral tissues with similar expression levels. Although these receptors share structural and distribution similarities with other GHS-R, functional analysis revealed that these GHSR1a-LRs are not activated by homologous rainbow trout ghrelin or by rat ghrelin when assessed using mammalian cell expression system. The reason was the unexpected splicing of the transfected cDNA. Therefore, it remains unclear whether these identified receptor proteins act as GHS-R1a for rainbow trout. Thus, it has not been designated as GHS-R1a, but instead as GHSR1a-LR.

2.3.12. Sea bass

Ferrando et al. [24] reported the distribution of regulatory molecules in the stomach of juvenile sea bass (*Dicentrarchus labrax*) during compensatory growth by re-feeding for 21 days after fasting for 35 days. Ghrelin-immunopositive (ip) cells were detected in gastric pits and neuronal cell bodies in the myenteric plexus by an antiserum for mammalian ghrelin. The frequencies of ghrelin-ip cells seemed to increase in fasted fish at 15 and 56 days. This increase was associated with reduction in leptin immunoreactivity in the stomach. It is likely that these two peptides could act together in this fish as an emergency mechanism for the regulation of energy balance.

2.3.13. Smallmouth bass

Male smallmouth bass (*Micropterus dolomieu*) exhibit parental care for developing brood, which includes an increase in activity associated with brood defense and a decrease in foraging that results in a decline in endogenous energy reserves. Hanson et al. [33] reported that fluctuations in plasma ghrelin concentrations during the parental care, and that decreases in plasma ghrelin during the egg and egg fry stage may reduce hunger and induce voluntary anorexia. Interestingly, fish injected with ghrelin at the egg stage when plasma ghrelin level is low did not feed despite the higher plasma ghrelin levels for 1.5 weeks after the ghrelin injection. They hypothesized that this resistance to the action of ghrelin could be reinforced through insensitivity of GHS-R1a in the hypothalamus. However, it will be necessary to examine feeding regulation by ghrelin under normal conditions for this species since ghrelin does not necessarily increase feeding in fish [34,35], and there is no available evidence for a feeding effect of ghrelin in this species.

2.3.14. Tilapia

Tilapia (*Oreochromis mossambicus*) is teleost fish species that is often used in ghrelin studies. The tilapia ghrelin peptide was identified in 2003 [41]. Reports in the past few years have linked changes in ghrelin and its receptor to energy status.

GHS-R-like receptor (GHSR-LR) has been reported by the author's group [45]. This GHSR-LR is so-named since the functional activity of the receptor protein to ghrelin has not been confirmed in the mammalian cell expression system as described above. However, it is a putative GHS-R of tilapia because the sequence homology and genomic structure are similar to other GHS-R. The cDNA is 1584 bp in length and encodes a 384-amino acid protein in the ORF of 1155 bp (GenBank ID: AB361053). The GHSR-LR gene is 1815 bp in length and contains two exons separated by an intron (GenBank ID: AB361055), and another ORF was found consisting of the first exon and a part of the intron, which encodes a 298-amino acid GHSR1b-LR (GenBank ID: AB361054). GHSR1a-LR mRNA is mainly detected in the brain, pituitary and liver, with lower expression in the stomach, intestine, and kidney. The amount of GHSR1b-LR mRNA is less than 1/200 of that of GHSR1a-LR mRNA, and the highest expression is seen in the brain, followed by peripheral tissues. As described above, even homologous tilapia ghrelin did not increase intracellular Ca^{2+} of mammalian cells expressing this isolated GHSR1a-LR cDNA. The reason for this is not yet known, but it is commonly noted for the inactive GHS-R1a (GHSR1a-LR) protein that the structure of the receptor's second extracellular loop may affect ligand binding and subsequent receptor signaling. This is also the case with rainbow trout. Furthermore, the test system for receptor activity is not a homologous fish system (fish cell) but instead is a mammalian system, and this may lead to different patterns of transcription and translation of protein [44]. Further studies are needed to confirm that GHSR1a-LR is a true GHS-R1a.

Long-term ghrelin treatment increases feeding, body weight and fat deposition in tilapia [90]. However, Riley et al. [89] reported that fasting for 7 days had no effect on plasma ghrelin levels in spite of increased plasma GH level on day 3, reduction of plasma IGF-I levels on days 3 and 7, and plasma glucose levels on days 3, 5, and 7. In addition, it is likely that mRNA expression of ghrelin, GHSR1a-LR and GHSR1b-LR as well as NPY in the brain do not change during the fasting period. These results indicate that ghrelin does not act as a hunger signal in short-term fasted tilapia, a result that is in contrast to that observed in goldfish [109].

Furthermore, Riley and colleagues looked at pre-and postprandial changes in ghrelin signaling in tilapia [83]. Plasma ghrelin levels were unaltered during the 3-h pre-prandially period, but were increased at 3 h post-feeding. Stomach ghrelin mRNA levels significantly decreased 3 and 2 h before the meal, but increased 1 h before the meal. In fed fish, however, ghrelin mRNA level remained at the elevated levels during 3 h post-feeding. Brain ghrelin mRNA transiently increased 2 h before meal, but changes in the expression level seemed not to be associated with post-prandial metabolic status. Brain GHSR1a-LR and GHSR1b-LR mRNA showed high expression levels 3 h before meal and scheduled feeding time, respectively, but subsequent changes in mRNA expression seemed not to be associated with metabolic status after a meal. Changes in pre-GHSR-LR mRNA (hetero-nuclear RNA) were also measured. The changes were not associated with feeding status, but different patterns of changes in GHSR1a-LR and GHSR1b-LR mRNA were seen. These results suggest that ghrelin is not an acute hunger signal in tilapia, and support the previous hypothesis [89].

Riley et al. [92] further looked at the effect of glucose on plasma ghrelin level or on gastric ghrelin mRNA expression. Glucose load increased plasma ghrelin levels and gastric ghrelin mRNA expression, and reduced GHSR-LR mRNA in the brain 6 h after treatment. This change in plasma ghrelin level is in contrast to human or goldfish, where glucose administration reduces plasma ghrelin levels, in agreement with post-prandial drops in plasma ghrelin levels [98,109]. Similar responses in plasma ghrelin level have been observed in rainbow trout and tilapia, where plasma ghrelin levels are elevated after a meal [79,83]. Together with the suppression of GHSR1a-LR in the brain despite a rise in plasma ghrelin levels,

ghrelin may be playing role in acute adaptation for energy homeostasis that is independent of its orexigenic action.

Fox et al. [25] reported changes in plasma glucose and ghrelin levels after a meal in tilapia; plasma ghrelin levels did not change in spite of a significant increase in plasma glucose at 2, 10 and 24 h after a meal. Peddu et al. [83] observed a significant increase in plasma ghrelin levels 3 h after a meal in the same species. This discrepancy may be due to differences in meal timing: the former was at 0800, and the latter was at 1500. This suggests different ghrelin responses to glucose according to the metabolic status of fish throughout the day.

Fox et al. [25] reported effects of short- and long-term fasting on plasma ghrelin, stomach ghrelin content and stomach ghrelin mRNA expression in tilapia. Plasma ghrelin level increased transiently at 10 h in the pre-prandial group although stomach ghrelin content and mRNA expression did not change. This change did not synchronize the alterations of plasma GH, IGF-I and glucose levels. Intermediate fasting for 4 or 8 days had no effect on plasma ghrelin level, stomach ghrelin content or mRNA expression, although plasma GH level was elevated and plasma IGF-I and glucose levels were decreased. Long-term fasting for 2 or 4 weeks increased plasma ghrelin levels without changes in stomach ghrelin mRNA expression. Elevation of plasma GH level was not observed in this experimental period, but plasma IGF-I and glucose levels were reduced. These results are in agreement with the previous observations [80,89], and similar to the result from bullfrog [48], but different from the results from sea bass and rainbow trout [34,105]. These results support the hypothesis that, in tilapia, ghrelin does not act as a meal-initiated signal but acts as a long-term indicator of negative energy balance. This may be due to different energy metabolism patterns among fish species (e.g., cold-blooded and warm-blooded animals; and herbivorous, omnivorous and carnivorous).

Schwandt et al. [95] published an interesting report on modification of ghrelin action by different fatty acids using tilapia as the model. Ghrelin is modified by *n*-octanoic acid in almost all of the vertebrates examined [43]. However, in tilapia, a major form of ghrelin is modified instead by *n*-decanoic acid [41]. Schwandt et al. [95] focused on the differences in action between octanoylated (C8) and decanoylated (C10) tilapia ghrelin. Brain NPY mRNA expression is stimulated by C10 ghrelin but not by C8 ghrelin when the ghrelin was injected IP. Plasma glucose level increased by C8 ghrelin but not by C10 ghrelin. Both types of ghrelin stimulate glucose release from cultured tilapia hepatocytes, and reduce mRNA expression of the glucose transport protein GLUT4 and insulin receptor in muscle. It will be interesting to learn if the C10 ghrelin found in other vertebrates exhibits different biological action compared with C8 ghrelin.

2.3.15. Zebrafish

Zebrafish is a well-established fish model system to investigate development, reproduction and metabolism. Two different groups have reported a prepro-ghrelin cDNA sequence of zebrafish (GenBank ID: EU908735 [2,62]). The cDNA is 472 bp in length and encodes a 104-amino acids precursor protein in the ORF. The ghrelin gene (GenBank ID: EU908736) is comprised of four exons and three introns. Li et al. [62] described ontogenic changes in ghrelin mRNA expression. There was no maternal ghrelin mRNA detected in eggs, but embryonic mRNA can be detected at 12 h post fertilization (hpf) and that expression gradually increases. At the 32-hpf stage, ghrelin mRNA is clearly detected in the pancreas primordium by *in situ* hybridization. In adult fish, ghrelin mRNA appears in almost all tissues, with the highest level of the expression observed in the intestine, and followed by liver, spleen and kidney, and weak expression detected in the brain, eyes, gills and heart [17]. Amole and Unniappan [2] have also detected ghrelin mRNA expression in the skin, testis and ovary. In the brain, ghrelin mRNA and protein are both located in the hypothalamus and preoptic lobe region (periventricular nucleus [PVN]) and are co-localized with two GHS-R, GHS-R1a and 2a [17].

Piccinetti et al. [84] has reported melatonin effects on ghrelin mRNA expression in zebrafish brain. Melatonin treatments (100 nM or 1 μM for 10 days through exposure of fish to melatonin-containing water) reduced food intake, and this was associated with decreased ghrelin and NPY mRNA expressions, and increased leptin and melanocortin receptor-4 mRNA expressions. These results suggest a link between ghrelin and factors that regulate feeding behavior. Furthermore, ghrelin mRNA expression is stimulated in the brain and gut by fasting for 3, 5 and 7 days, and 4-h re-feeding in the 7-days fasting group reverses the increased expression to the level of fed control, suggesting a possible orexigenic role for ghrelin [2].

Cruz et al. [17] reported *in vivo* effects of exogenous ghrelin (goldfish ghrelin-12 amide at 100 ng/g BW injected IP) on mRNA expression of GHS-R, and on ligand–receptor systems of insulin (INS) and glucagon (GCG) in the brain. It is likely that circulating ghrelin up-regulates the GCG system while down regulating the INS system in the zebrafish brain by mediating its action through GHS-R. This result is in agreement with mammalian studies [8,18], suggesting that ghrelin is involved in a fine-tuning of carbohydrate-glycogen metabolism in the CNS by extra pancreatic system of INS and GCG in zebrafish.

3. Update on the ghrelin system in amphibians

Amphibian ghrelin has been identified in the bullfrog (*Rana catesbeiana*) by the author's group [39], and it is known to stimulate the release of GH and prolactin (PRL) from bullfrog pituitary cells. We have also found that the levels of plasma total ghrelin, stomach ghrelin and stomach ghrelin mRNA increase 10 days after fasting [48].

The author's group identified ghrelin cDNAs of various species of urodele and anuran amphibians (Fig. 5, unpublished data). The aim was to clarify whether threonine is the only acylated amino acid of frog. Seven species of amphibians were examined and it was revealed that ghrelin only of the genus *Rana* has Thr-3, and ghrelins in the other urodele and anuran amphibians have Ser-3. Since the aquatic frog *Xenopus laevis* and the newts *Cynops pyrrhogaster* and *C. ensicauda* have Ser-3, and taking fish ghrelin sequences into consideration, Ser-3 is the residue in the protoform of ghrelin. Two forms could have evolved from the protoform ghrelin, with semi-aquatic frogs such as *Rana* having Thr-3 and terrestrial frogs such as *Hula* and *Bufo* having Ser-3.

Mustonen et al. [74] showed an effect of human ghrelin on lipid metabolism in a common frog, *Rana temporaria*. They injected human ghrelin (10 μg/kg/day) into dorsal lymph sac for 4 days. The ghrelin treatment decreased plasma total cholesterol and LDL-cholesterol levels and increased the HDL-to-LDL ratios. In addition, liver lipase activity decreased and fat body lipase activity increased after the administration. Ghrelin shifts nutrient utilization from lipid to carbohydrate in rodents [71], but this does not seem to occur in the common frog. Interestingly, in contrast to the common frogs, the measured variables did not change in the common toad (*Bufo bufo*). The reason for this difference remains obscure, but may relate to different nutritional or acclimational states of the animals.

4. Update on the ghrelin system in reptiles

Unfortunately, there is no progress in the study of reptilian ghrelins since the ghrelin of a turtle (*Trachemys scripta*)

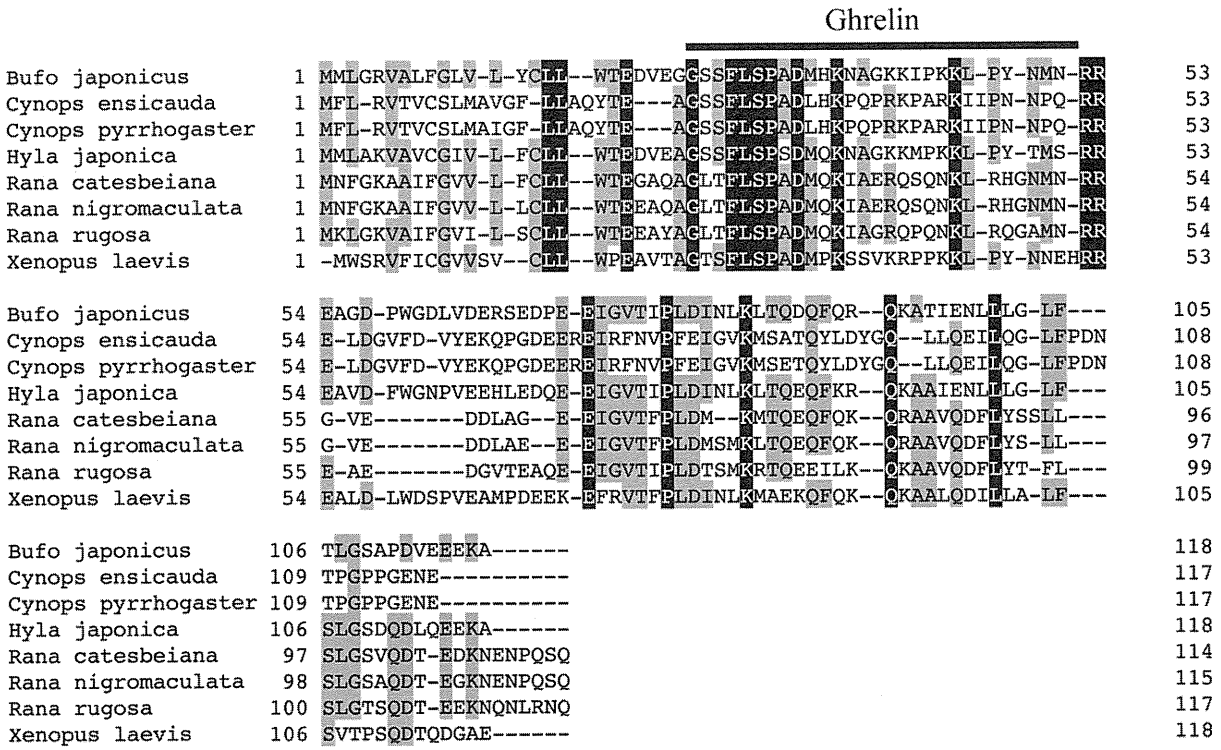

Fig. 5. Comparison of prepro-ghrelin peptides in amphibian. Peptide sequences were aligned using the GENETYX-Mac software program version 15.0.1. Amino acid residues that are identical among all species are densely shadowed; those residues conserved in more than half of the listed species are lightly shadowed.

was identified (Fig. 6 [47]). Alignment of turtle ghrelin precursor with avian ghrelin precursor shows relatively high similarity between them, and the *Trachemys*-1 precursor contains a specific sequence (LNVPFEIGVK) that is similar to that in avian precursors (F(N/H)VPFEIGVK). Revealing a possible evolutionary step in the evolution of vertebrate ghrelins, the *Trachemys* sequence was originated from an intron (H. Kaiya, unpublished data), suggesting that *Trachemys*-2 is produced by alternative splicing of the gene.

5. Update on the ghrelin system in birds

Recent work has expanded our understanding of ghrelin biochemistry in birds. Duck ghrelin cDNA and gene sequence (Fig. 6), and duck GHS-R (data not shown), and quail GHS-R (Fig. 4) are now identified. And there are reports on the identification of new tissue distribution patterns of gene and peptide expression in the body, ontogenic changes in ghrelin gene expression, mechanisms of feeding regulation, novel biological activity, and relationships among GHS-R sequence variations and traits. Since 2008, two excellent reviews have also been published; readers are referred to these [37,87].

5.1. *African ostrich – ghrelin*

Wang et al. [116] reported distribution and developmental changes in ghrelin-ip cells in the gastrointestinal tract of African ostrich (*Struthio camelus*). There is interest in ghrelin function in the ostrich since it is the largest living species of birds. They found ghrelin-ip cells in various region of the gastrointestinal tract such as the proventriculus, gizzard, duodenum, jejunum, ileum, cecum, colon and rectum, and the percentage of open-type ghrelin-ip cell increased gradually from the proventriculus to rectum. This distribution pattern is somewhat different from that of chicken [115,120]. Furthermore, the developmental expression of ghrelin-ip varies according to region: in the proventriculus, the number gradually increases after birth (9.00 ± 2.83 cells/mm^2) until postnatal day 45 (P45) (29.60 ± 9.13 cells/mm^2), and remains steady until P334 (26.60 ± 4.39 cells/mm^2); and in the gizzard and small intestine, the number also gradually increases and reaches a peak at P90 (from 1 to 5 cells/mm^2 for the gizzard; from 3 to 18 cells/mm^2 for the small intestine). This relatively large number of ghrelin-ip cells throughout the gastrointestinal tract may relate to growth performance of ostrich.

5.2. *Chicken – ghrelin*

Chicken ghrelin has been identified by the author's group [50] and we also reported the predominant expression of the mRNA in the proventriculus. In addition, Saito et al. [94] have reported on ghrelin gene expression in various parts of the CNS.

Nie et al. [75] recently published an update on tissue distribution of gene expression. Ghrelin expression was detected not only in the CNS (pituitary, hypothalamus and cerebrum), but also in peripheral tissues such as abdominal fat, subcutaneous fat, small intestine, heart, and breast muscle, but not proventriculus, using quantitative PCR technique. Furthermore, Yoshimura et al. [122] have reported that ghrelin peptide is present in the yolk and albumen of fertilized eggs, and that the ghrelin concentration of fertilized eggs does not change during 5 days of incubation. The origin of ghrelin remains uncertain, but ovary and oviduct may be the source, with the produced ghrelin transmitted to the yolk and albumen [100,121]. The physiological relevance of the ghrelin in the egg remains unknown.

Ghazanfari et al. [29] reported the effects of food restriction and of different energy and protein contents of the diet on ghrelin gene expression in the proventriculus of Ross broiler chickens. The results showed that food restriction increased ghrelin gene expression at 32 days of age but not at 49 days, and that decreasing dietary energy had a trend to increase ghrelin gene expression at 21 days ($P < 0.07$) and 32 days of age ($P < 0.05$). These results are in agreement with previous observations on fasted chickens [46,88]. On the

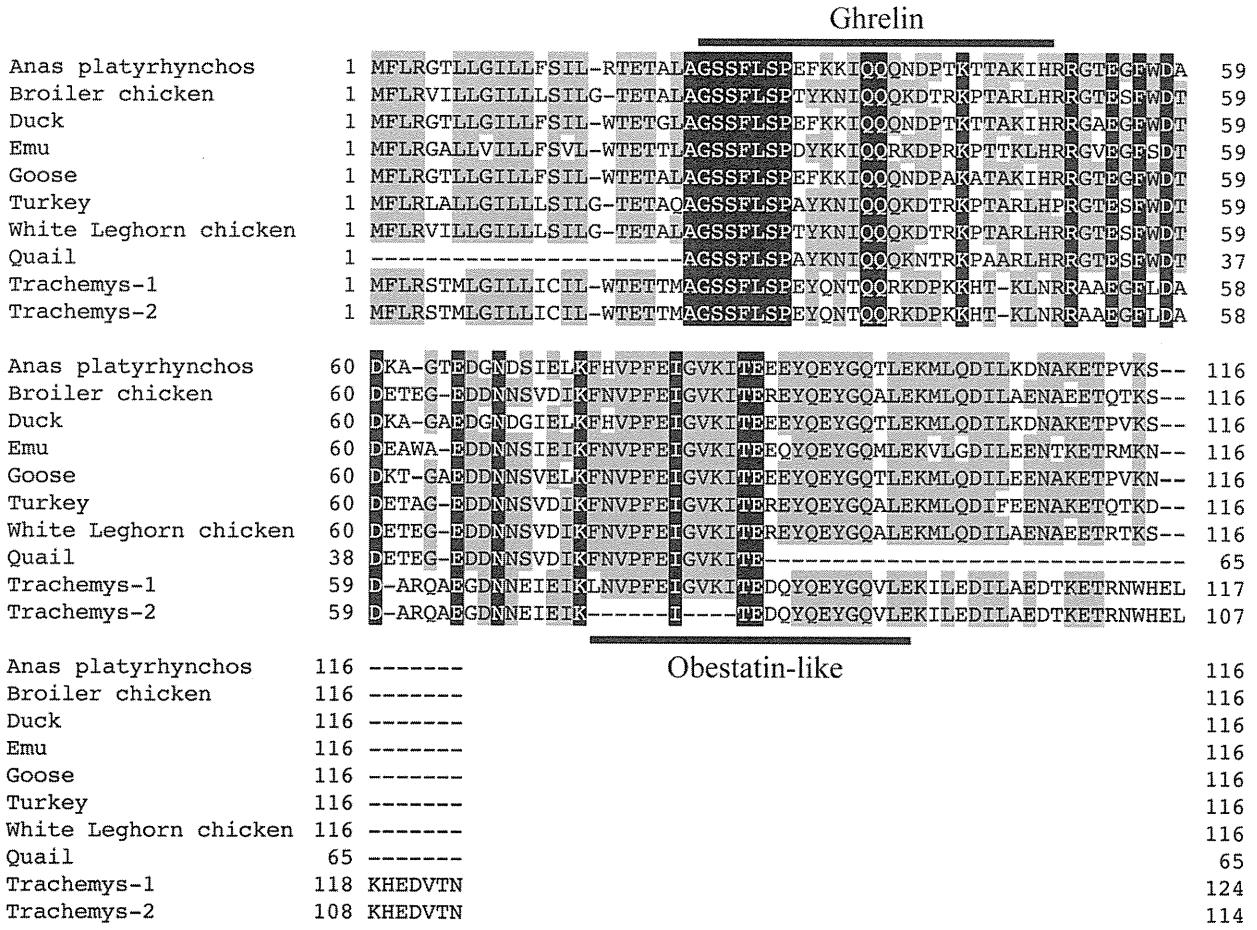

Fig. 6. Comparison of prepro-ghrelin peptides in avian. Peptide sequences were aligned using the GENETYX-Mac software program version 15.0.1. Amino acid residues that are identical among all species are densely shadowed; those residues conserved in more than half of the listed species are lightly shadowed. GenBank accession numbers are: AY338466 for Peking duck (*Anas platyrhynchos*), AB075215 and BAC24980 for broiler chicken, EF613551 for duck, AY338467 for emu, AY338465 for goose, AY333783 for turkey, AY299454 for white leghorn chicken, AB161457 for Trachemys-1, and AB161458 for Trachemys-2.

other hand, decreasing dietary protein had no effect on ghrelin gene expression. Ghazanfari and co-workers [30] re-examined plasma GH levels and ghrelin gene expression in the proventriculus under similar feeding conditions to the previous study. They reported no effects of different dietary energy and protein levels on plasma GH levels or on ghrelin gene expression at 21, 42 and 56 days of age, although feed intake and body weight gain steadily increased in chickens fed on low-energy diet at 21 days of age and on increasing protein levels at all ages examined. Thus, unfortunately, it remains unclear at present whether energy intake affects on the ghrelin gene expression in the proventriculus.

Lu et al. [65] reported changes in ghrelin mRNA expression in relation to the somatotropic, thyrotropic and corticotropic axes of broiler chickens (Langshan (LS) and Arbor Acres (AA)) during embryonic and postnatal development. Hypothalamic ghrelin mRNA levels showed a peak on postnatal day 28 (P28), in agreement with an earlier report [15]. The expression changes were positively correlated with those of GH mRNA during P0 to P42 in both chicken strains, and primary hypothalamic control of GH production was both stimulatory and inhibitory during the corresponding developmental period. Furthermore, hypothalamic ghrelin mRNA was correlated with hepatic IGF-I mRNA (E8 to P28) and CRF mRNA (P0 to P28) in both chicken strains. Moreover, ghrelin may affect cell proliferation and apoptosis by stimulating the release of IGF-I from E8 to P28, and may regulate the hypothalamo-pituitary–adrenal (HPA) axis from P0 to P28.

Rosebrough et al. [93] reported on the influence of dietary protein on growth, and on metabolic plasma hormone levels and regulatory enzymes at representative times in broiler chickens. The results showed that both IGF-I and T_4 are equally positioned in a metabolic priority scheme, and are superior to glucagon and ghrelin, suggesting that ghrelin is involved in growth regulation in chickens. Together with gene expression profiles of ghrelin and its receptor, the ghrelin system may be related to long-term growth performance in chickens although the acute effect on food intake is inhibitory when ghrelin was injected ICV [94].

Richards and McMurtry, in their review [87], reported ontogenic changes in ghrelin mRNA in the proventriculus and plasma total ghrelin levels from hatching to 8 days. They observed a similar pattern of changes in ghrelin mRNA expression to that described in a previous paper [15] in which there was reported a peak at 2 days post-hatch and a delay of feeding that further enhanced the up-regulation. Plasma total ghrelin levels gradually declined by 8 days post-hatch. The divergence in circulating ghrelin and mRNA expression in the proventriculus could depend on events related to the transition from embryo to hatched chick, including a major metabolic shift from the utilization of a high fat nutrient source (yolk) to a high carbohydrate diet (feed). The shift of nutrient source would affect acylation of ghrelin [120].

Concerning the regulation of food intake, the inhibitory mechanism of ghrelin on food intake in chickens is still not fully understood, although Saito et al. [94] have demonstrated the

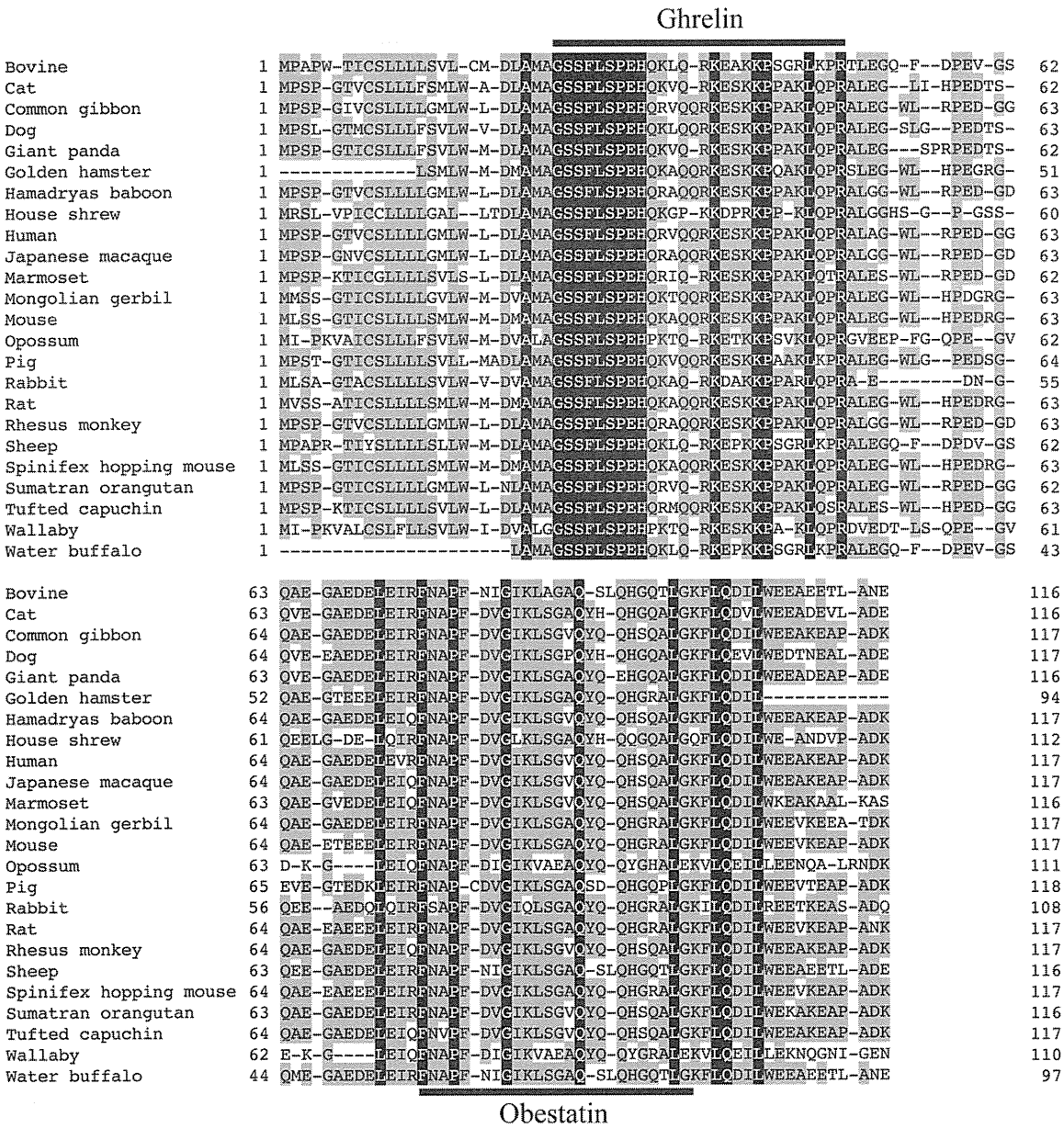

Fig. 7. Comparison of prepro-ghrelin peptides in mammals. Peptide sequences were aligned using the GENETYX-Mac software program version 15.0.1. Amino acid residues that are identical among all species are densely shadowed; those residues conserved in more than half of the listed species are lightly shadowed. GenBank accession numbers are: NM_174067 for bovine, AB089201 for cat, AB365870 for common gibbon, AB060700 for dog, EU375448 for giant panda, EU863658 for golden hamster, DQ987858 for hamadryas baboon, AB364508 for house shrew, NR_024136 for human, AB365871 for Japanese macaque, XM_002758630 for marmoset, AF442491 for Mongolian gerbil, NM_021488 for mouse, XM_001375640 for opossum, AY028942 for pig, XM_002722463 for rabbit, NM_021669 for rat, NM_001032903 for Rhesus monkey, AB060699 for sheep, FJ843089 for spinifex hopping mouse, XM_002813445 for Sumatran orangutan, AB365873 for tufted capuchin, EU677468 for wallaby, and EU604028 for water buffalo.

partial involvement of the CRF system. Khan et al. [53] showed that nitric oxide synthase inhibitor (N^G-nitro-L-arginine methyl ester, L-NAME) completely reversed anorexigenic activity of CRH in neonatal chick although ghrelin-induced anorexigenic activity was attenuated only in part. This suggests that ghrelin-induced anorexigenic activity is not mediated directly through CRF, but through other CRF family peptides, perhaps urocortin or urotensin [124]. Furthermore, Taati et al. [103] indicated that the histaminergic system through the H1 receptor is involved in the inhibitory effect in broiler chickens. These results suggest that feeding regulation by ghrelin links at least two different feeding-inhibition pathways.

ICV injection of ghrelin inhibits food intake, and IV injection of ghrelin reduces respiratory quotient (RQ) [28]. This suggests different physiological roles in energy metabolism in chicken when compared to mammals (e.g., decreased lipogenesis). Buyse et al. [9] reported the *in vivo* effect of chicken ghrelin on the gene expression of fatty acid synthase (FAS), and of important regulators of FAS gene expression, peroxisome proliferatory-activated receptor-γ (PPAR-γ) and sterol regulatory element binding protein-1 (SREBP-1) in the liver and diencephalon. Chicken ghrelin (1 nmol per chick) induced increases in plasma corticosterone level 15 min after injection, and reduced food intake 30 min after injection. In the liver, FAS and PPAR-γ mRNA decreased 30 and 60 min after injection, and SREBP-1 mRNA expression was reduced 15, 30 and 60 min after injection. In diencephalon, FAS and SREBP-1 mRNA levels increased 15 min after injection, and PPAR-γ mRNA showed significantly higher levels than those of time controls. These results suggest that ghrelin reduces *de novo* lipogenic activity in the liver. Also, it is consid-

ered that increased FAS mRNA expression in the diencephalon is associated with inhibition of food intake, in contrast to the effect in mammals, in which ICV or IP injection of ghrelin inhibits hypothalamic FAS mRNA, FAS protein and SREBP protein expression in rats [64].

Erriquez et al. [21] have provided a unique report on calcium signaling after ghrelin stimulation of dorsal root ganglion (DRG) cells from chick embryo. They demonstrated mRNA expression of ghrelin and GHS-R1a in the DRG cells, and that ghrelin triggers calcium signaling in developing DRG cells by regulating two different calcium entry mechanisms, a receptor-activated calcium entry (RACE) and a store-operated calcium entry (SOCE), in a coordinated manner, suggesting a neurotransmitter role for ghrelin. Interestingly, a known GHS-R1a antagonist, [D-Lys^3]-GHRP-6, exerts several activities in the absence of exogenous ghrelin in this cell. For example, it activates calcium release in non-neuronal cells, and it promotes apoptosis of DRG cells by ghrelin-independent activity in a dose-dependent manner. Similar response of a GHS-R1a antagonist has been observed in the contractile activity of chicken crop [54]. This may suggest a possible presence of another type of GHS-R1a as identified in other animals, e.g., fish [42,101], or another conformation of GHS-R1a that is able to bind the GHS-R1a antagonist.

Central ghrelin plays important roles in various physiological functions other than feeding regulation, such as in anxiogenic activity and memory retention [12,13,20]. Carvajal et al. [14] investigated the effects of ICV ghrelin on anxiety and memory retention of neonatal chicks in an Open Field Test and in a one-trial passive avoidance task, respectively. Ghrelin induced anxiogenesis. This is similar to the action in rodents, and is in agreement with a previous paper in which sleep-like behavior was observed in chicks after ICV ghrelin injection [104]. This action would be mediated by the corticotropic axis [60,94]. In addition, ghrelin also decreased memory retention. This effect is opposite to that seen in rodents [12,13,20]. The reason for this response remains unknown, but different mechanisms of feeding behavior may contribute to the impaired effect on memory.

5.3. Chicken – GHS-R

Ghrelin stimulates GH release *in vivo* and *in vitro* [1,5,50]. However, at that time, the distribution of GHS-R1a was still unknown. Yamamoto et al. [119] reported on mRNA and protein expressions of GHS-R1a and on GH protein expression in the cephalic and caudal lobe of pituitary; both expressions are more abundant in the caudal lobe than in the cephalic lobe. Furthermore, they observed that the levels of ghrelin mRNA in the proventriculus and GH mRNA in the pituitary gradually increase in parallel with development, while GHS-R1a mRNA expression remains at a constant level during embryonic and post-hatching periods. It is interesting to note the different expression patterns of mRNA between gastric ghrelin and pituitary GHS-R1a for regulation of GH secretion, suggesting a minor effect of ghrelin on GH release.

Nie et al. [75] reported on tissue distribution and SNP of the chicken GHS-R gene. The highest mRNA expression was detected in the breast muscle, followed by subcutaneous fat, leg muscle, abdominal fat, heat, spleen, liver, uropygial gland, cerebrum, proventriculus, pituitary and testicles. Significant difference in the mRNA expression between two breeds (LY is a faster-growing breed than HB) was found in the hypothalamus and breast muscle. Eight SNPs of the chicken GHS-R gene were found, and it was shown that C-1459T is possibly associated with fat traits.

Fang et al. [22] have reported associations of four SNPs and a "GGTACA" indel of the GHS-R gene with production trails of Chinese native breed chickens (White Recessive Rock and Xinghua) because some polymorphisms of the GHS-R gene are known to affect diseases or abnormal phenotypes or growth performance of humans [4,63,66,70] or cattle [123]. In chickens, some SNPs are associated with fatness and muscle fiber traits [61,76]. Actually, c.739 + 726T > C allel, which locates at an intron of the GHS-R gene, is significantly associated with body weight at 28 and 90 days, dressed weight, eviscerated weight, eviscerated weight with giblet, breast muscle weight and leg muscle weight.

5.4. Duck – ghrelin

Nie et al. [75] reported the prepro-ghrelin sequence in a breed duck (Sanshui (SW), GenBank ID: EF613551), in which it was found six amino acid differences at position W16R, G21A, A53T, T59A, A64T and G71S, compared with the previously reported mallard duck (Peking duck, *Anas platyrhynchos*) (GenBank ID: AY338466) (Fig. 6). The precursor protein showed high identity (70–89%) with those of other birds, but low identity with mouse and human (38% and 37%, respectively). The gene (3670 bp, GenBank ID: EF613552) is comprised of five exons and four introns. In the 5′-flanking region are a typical TATA box and potential transcription factor binding sites for AML-1a, cap-1, GATA-1 and Oct-1. Duck ghrelin gene expression is found predominantly in the proventriculus, followed by the abdominal fat, testicles, subcutaneous fat, uropygial gland and breast muscle.

Interested in what roles ghrelin plays, Shao et al. [96] reported ontogenic changes in ghrelin mRNA expression and ghrelin-ip cells in the gastrointestinal tract of Peking duck (*Anas platyrhynchos*), which has the characteristic of rapid growth. Ghrelin mRNA expression was mainly detected in the proventriculus and proventriculus-gizzard junction. A weak expression was detected on embryonic day 14 (E14), then it increases by E21, and high expression level is maintained from post-hatching days 1 (P1) to P60. A weak expression is also found in the gizzard and duodenum on P60. Ghrelin-ip cells were found in embryos as early as E21 (9.5 ± 1.6 cells/cm^2) and the numbers were similar to those seen at P1 (11.8 ± 1.3 cells/cm^2). This is different from chicken, in which ghrelin-ip cells were not observed on any embryonic days [105,120].

5.5. Duck – GHS-R

Nie et al. [75] reported partial sequence determination, tissue distribution and identification of SNP in the duck GHS-R gene. It is 3717 bp in length (GenBank ID: FJ194548) containing two incomplete exons and one intron, and encodes a 347-amino acid protein. The mRNA is detected in the pituitary, and at lower levels in the subcutaneous fat, hypothalamus, small intestine, testis, cerebellum and cerebrum. Forty-eight SNPs and 5 indels were found in the duck GHS-R gene, indicating that the GHS-R gene is more variable in poultry than in mammals [113]. The A3427G and haplotypes of T404C and A3427G are both possibly associated with fatness traits.

5.6. Quail – ghrelin

In Japanese quail, a partial sequence of prepro-ghrelin (Fig. 6) and the effects of peripheral and central ghrelin on food intake have been reported [99,121], but there are no reports on the receptor or effects on gastrointestinal contraction.

Kitazawa et al. [55] compared the contractile effects of ghrelin between chicken and quail gastrointestinal tract. Chicken and quail ghrelin caused muscle contraction in the crop, proventriculus and colon of both birds, and the contractile efficacy was more potent in the chicken than in the quail. The small intestine was less sensitive to ghrelin, but chicken motilin, a ghrelin family peptide present in the gut, caused marked contraction in both chicken and quail. The different effect of ghrelin on region-specific gastrointestinal contraction between mammals and birds is notable.

5.7. Quail – GHS-R

Kitazawa et al. [55] reported six quail GHS-Rs, namely GHS-R1a-L, 1a-S (GenBank ID: AB469019), 1aV (GenBank ID: AB469020), 1bV(GenBank ID: AB469021), 1b (GenBank ID: AB469022), and 1tv-like receptor (GenBank ID: AB490327, the represented nucleotide number is consecutive from the cDNA [GenBank ID: AB469019]), from quail cerebellum cDNA library [55]. A short-type GHS-R1a (GHS-R1a-S) lacks seven amino acids at the N-terminus of the long-type GHS-R1a (GHS-R1a-L) (Fig. 4). These receptors are composed of 309–354 amino acids. The amino acid sequence of quail GHS-R1a-L is 98% identical to that of chicken GHS-R1a. In these receptors, GHS-R1a-L and 1a-S are functionally active, and they express dose-dependent increases in intracellular Ca^{2+} when the receptor proteins are expressed in HEK293 cells. Interestingly, chicken ghrelin showed higher potency than homologous quail ghrelin and GHSs (GHRP-6 and hexarelin). Regarding GHS-R1a mRNA expression in the gastrointestinal tract, high expression was observed in the colon, moderate levels in the esophagus and crop, and low levels in the proventriculus, gizzard and small intestine. The region specific expression pattern was almost the same as that for chicken GHS-R1a.

6. Comparison of ghrelin precursor protein

An alignment of ghrelin precursor proteins for representative mammals is shown in Fig. 7, and a phylogenetic tree estimate of ghrelin precursors is shown in Fig. 8.

In mammalia, the amino acid sequences are highly homologous, sharing over 90% identity across the group. In opossum and wallaby ghrelins, however, different amino acid sequences are found compared to other mammals, due to the evolutionary position of these two species as primitive mammalia (*Marsupialia*).

The mature ghrelin peptide is composed of 28 amino acids. Another splice variant form, des-Gln^{14} ghrelin, which was firstly found in rats, is seen in many of the reported species such as bovine, cat, giant panda, marmoset, opossum, rabbit, sheep, orangutan, wallaby and buffalo. Nevertheless, the N-terminal-most 9 amino acids are highly conservative across species, and the amino acid residue for acyl modification is Ser-3 in all species. An obestatin sequence is present within the C-terminal segment of the precursor [125], but the C-terminus of the peptide is not likely amidated in primitive mammals such as opossum and wallaby. The amidation is essential for obestatin activity [125].

In aves, amino acid sequences of ghrelin precursor proteins are highly conserved among species (Fig. 6); only one amino acid, Q113R is different between broiler and layer chickens, and six amino acids are different between breed and mallard ducks. Although mature ghrelin is generally comprised of 26 amino acid residues, but in the length of turkey ghrelin is uncertain because the C-terminal Pro^{27}-Arg^{28} sequence of the mature peptide is the same as that in mammalian ghrelin, in contrast to the paired Arg residues that are present in other birds. At the C-peptide, an obestatin-like sequence is present, but it is not known whether this candidate peptide is processed from the precursor protein or whether it is biologically active.

In the reptilia, only turtle ghrelin has been reported [47]. We tried to identify the sequence of a lizard, the green anole, on the Ensembl genome database, but no hits on any ghrelin sequence were obtained. As mentioned earlier, amino acid sequences of the ghrelin precursor protein are very similar between aves and reptilia, suggesting a common evolutionary origin (Fig. 6). This is supported by phylogenetic analysis, where reptiles and birds are sister groups with a clade (Fig. 8). Interestingly, an obestatin-like peptide is present in the *Trachemys*-1 precursor, but a part of this

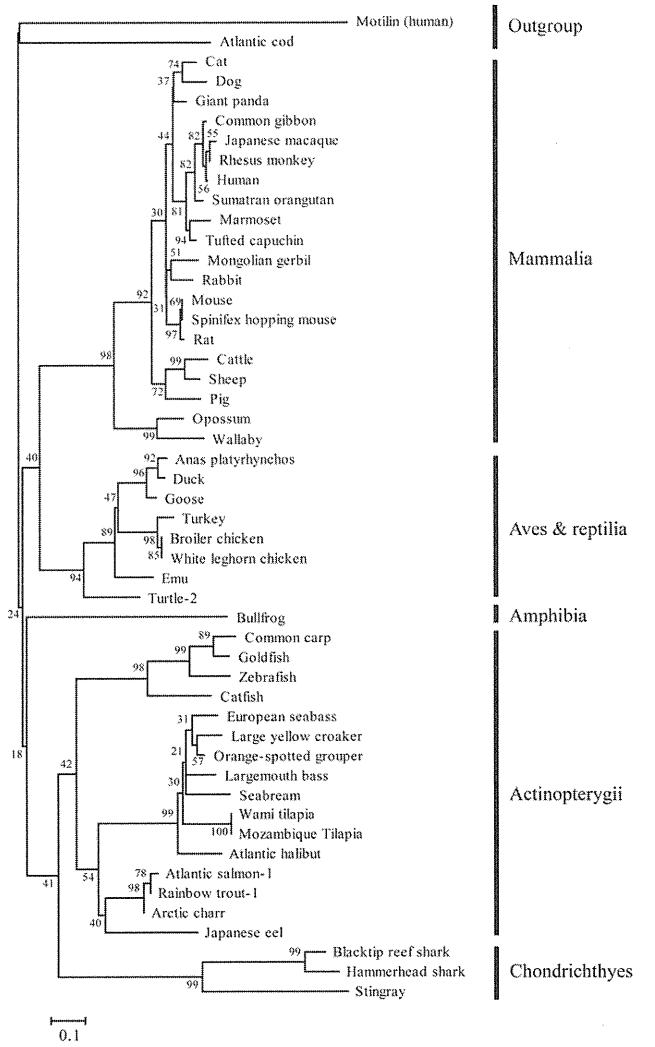

Fig. 8. Phylogenetic analysis of prepro-ghrelin in mammalian and non-mammalian vertebrates using the amino acid sequences. The phylogenetic tree estimate was constructed using the Neighbor-joining method within MEGA4 (http://www.megasoftware.net/). The numbers at branch points represent bootstrap values (as percent, based on 1000 repetitions). Scale bar indicates average substitutions pre position (the relative measure of evolutionary distance).

sequence is deleted in *Trachemys*-2. As mentioned earlier, the segment originated from an intron of the gene (H. Kaiya, unpublished data), and therefore, the existence of obestatin in turtle should be considered with caution.

In amphibia, bullfrog and edible frog (*Rana esculenta*) ghrelin sequences are published to date [39,43]. We recently identified ghrelin in five genera of amphibia including urodele (*Cynops*) and anuran (*Xenopus, Rana, Bufo* and *Hyla*). As shown in Fig. 5, 27- or 28-amino acid ghrelin was identified, and the third amino acid was identified to be threonine only in the genus *Rana*, and was serine in the other frogs and newts examined, similar to the situation in other vertebrates studies to date. Additionally, we noticed that the sequences of amphibian ghrelin are quite variable compared with those of mammals, reflecting species diversity much like that seen in fishes. No obestatin-like sequence has not been found at the C-peptide of amphibian prepro-ghrelins.

In bony fishes (Fig. 3), six species of ghrelin have been newly identified (Arctic char, Atlantic cod, Atlantic halibut, European sea bass, large yellow croaker and Atlantic salmon) since 2008.

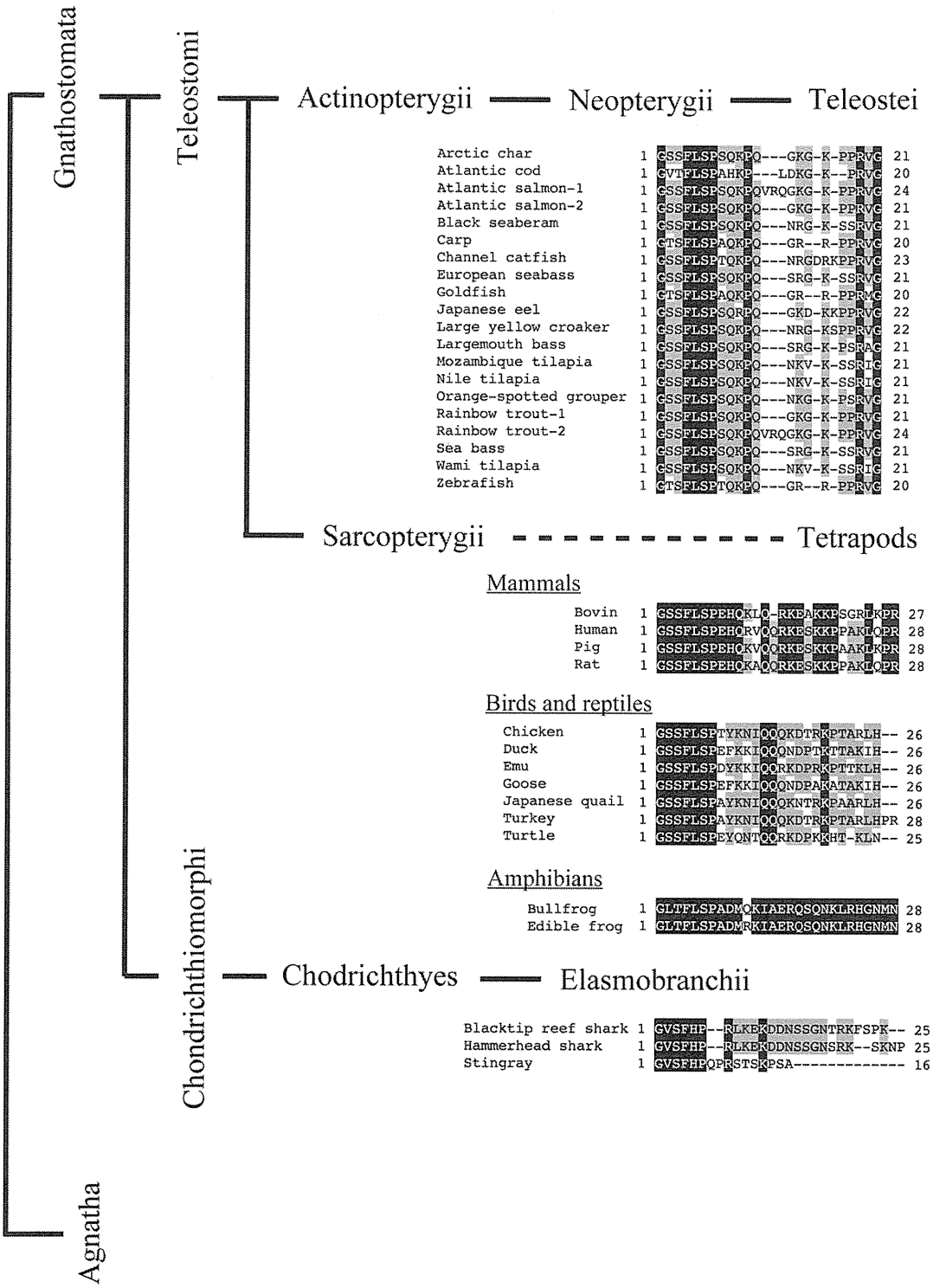

Fig. 9. Schematic drawing of phylogenetic tree of mature ghrelin sequences in mammalian and non-mammalian vertebrates. This figure summarizes the evolutionary changes in mature ghrelins of vertebrates, with consideration of the molecular structure. The details are described in Section 6 of the text.

Phylogenetic analysis revealed that ghrelin of the *Actinopterygii* examined so far are divided into six groups: *Cypriniformes*, *Siluriformes*, *Perciformes*, *Pleuronectiformes*, *Salmoniformes* and *Anguilliformes* (Fig. 8). The basic structures of fish ghrelin, which have Ser-3 and the C-terminal amidation, are conserved across species. In *Salmoniformes*, des-$V^{13}R^{14}Q^{15}$ ghrelin has been found. However, among the described fish ghrelins, cod ghrelin has special features: Thr-3 is present as seen in frog (*Rananidae*) ghrelin (Fig. 5); the second amino acid is valine, as seen in elasmobranches (Fig. 1); and the precursor length is longer than others (122 amino acids for cod, versus 102–111 amino acids in others). As such features are reflected, cod ghrelin appears near the outgroup motilin in our phylogenetic tree estimate of precursor protein (Fig. 8). Further study of other *Gadiform* species is needed.