MOLECULAR CHARACTERIZATION AND FEED INTAKE INCREASE OF GHRELIN IN STARRY FLOUNDER *Platichthys stellatus*

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Introduction

Classification

- Class : Actinopterygii
- Order : Pleuronectiformes (Flatfishes)
- Family : Pleuromectidae (Righteye flounders)
- Genus : Platichthys
- Species : *P. stellatus*

Starry flounder (*Platichthys stellatus*)

Etymology and Distribution

- Etymology : Platichthys → 'platys = flat(Greek)' + 'ichthys = fish(Greek)'
- Northern Pacific, from the Yellow Sea along coasts of Korean Peninsula, Russian Far East, and Japanese Arch.

Importance

- Alternative species of halibut
- Increased fish farm for starry flounder
- Replaceable without facility cost (Halibut to starry flounder)

Appetite

- Appetite : [noun] a physical desire for food
- increase in feed intake

Feed intake

- Feed intake : act of feeding
- increase the growth rate
- Food intake control is critical for fish growth and survival (Ronnestad et al., 2017)

Growth rate

- shorten the breeding period
- improve productivity

Purpose

- **Identification of factors affecting ghrelin expression.**
- **Determine if ghrelin ultimately causes growth enhancement.**

Methods

Molecular characterization

Experimental fish and conditions

- Size : 121.4 ± 2.91 mm, weight : 25.35 ± 2.45 g
- Rearing conditions : water temp(15℃), DO(8.59 mg/L), Salinity (33.7 psu), pH 7.55, natural photoperiod.
- Anesthesia : 200ppm triciane methanesulfonate
- Samples stored in liquid nitrogen(-196℃) for RNA extraction.

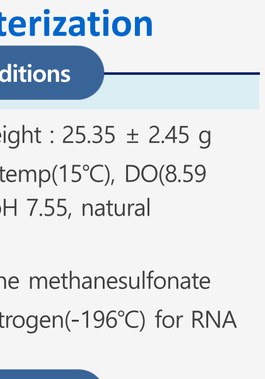
RNA extraction and cDNA synthesis

50mg of tissue → Homogenization → RNA extraction kit → cDNA synthesis

5′ and 3′ RACE PCR and cloning

5′ and 3′ RACE PCR → ligation to pRACE vector → Transformation to competent cell → Amp selection → Plasmid extraction → Sequencing with M13 primer set

Ghrelin Injection exp tank set



Starvation

Experimental fish and conditions

- Size : 56.76 ± 0.3 mm, weight : 2.09 ± 0.03 g
- Rearing conditions : water temp(13.5℃), DO(7-8 mg/L), Salinity (30-32 psu), pH 7.5, natural photoperiod
- Tank size : 83X57X42.5 cm (two tank), 60 individuals/a tank
- Anesthesia : 200ppm triciane methanesulfonate
- Sampling point (0, 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 88 and 24 hour)

Stomach contents dry weight

Sampling stomach contents → Keeping at -20℃ → Dry with dry oven(40℃) → measure

Ghrelin Injection

Experimental fish and conditions

- Size : 68.7 ± 1.09 mm, weight : 4.94 ± 0.25 g
- Rearing conditions : water temp(15℃), DO(8.59 mg/L), Salinity (33.7 psu), pH 7.55, natural photoperiod.
- Tank size : 83X57X42.5 cm (six tanks), 60 individuals/a tank
- Anesthesia : 200ppm triciane methanesulfonate
- Samples stored in liquid nitrogen(-196℃) for RNA extraction.
- Injection concentration : 0.8% saline and ghrelin(100ng/g)
- Intraperitoneal injection once per 2 weeks.

Results & discussion

1. Gene structure

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Ghrelin
1                                                           AC   2
3    ATG GGG AGA TTT AGA ACA GCA GAT TTG ATT TGA AAT CTT TTA ACG  47
48   TTC TAT GTT GCT TCA TCA TTT TGC AGC AGA GAC TCT GGT TCC ACC  92

93   ATG TTT TTG AAA AGA AAC ACC CAG CTG CTG GTG TTT CTA CTC TGT  137
1     M  F  L  K  R  N  T  Q  L  L  V  F  L  L  C               15
138  TCT CTG ACC TTG TGG TGC AAG TCG ACC AGC GCA GGT TCC AGT TTC  182
16    S  L  T  L  W  C  K  S  T  S  A  G  S  S  F               30
183  CTC AGC CCT TCA AAC AAA CCT CGG AAC AAG GGG AAA CCT CCG AGA  227
31    L  S  P  S  N  K  P  R  N  K  G  K  P  P  R               45
228  GCC GGC CGC CAA ATC ACA GAG GAG CAG AGT CAA CCC ACC GAG GAC  272
46    A  G  R  Q  I  T  E  E  Q  S  Q  P  T  E  D               60
273  CAG CAC ATC ACG GTG AGT GCC CCA TTT GAA ATT GGC ATC ACC ATG  317
61    Q  H  I  T  V  S  A  P  F  E  I  G  I  T  M               75
318  ACA CCA GAG GAC TTT GAG GAG TAC GGC GTG TTG CTG CGG GAG ATC  362
76    T  P  E  D  F  E  E  Y  G  V  L  L  R  E  I               90
363  GTT CAG CGC CTG CTG GGA AAC ACG GAG GCA GCA GAG AGA CCC TCT  407
91    V  Q  R  L  L  G  N  T  E  A  A  E  R  P  S              105
408  TAA                                                        410
      *
411       CTT TGA AGA TTA AGG ACA AGA TTT GCA GAT TTA CCT TTT TAT  452
453  TTC TTT AAA TTT CTA CTT CAT TAG AAA GTG ATC ATT AAA ATA TGA  497
498  GTA AAA ATG TCA GTT GCG TTA AAA ACA TTG TTA CTC ACA CTG TTG  542
543  GTA GTT AAT CTG TAC AAT TAA CCG ATG TTC ATC AAG TGA ATC TAT  587
588  GAG TAT GTC TAT ATT CAG AGC TTT CAA CCA CAT ATC AGT GCA ACC  632
633  ATT AGT GAG ATT GTT GTT AAA AAT AAT AAA GTT AAT ATC AAT TCA  677
678  ATA TTG ATA TAT ATA TAA AAC TAG AGT TTT AAT TTA TTT GTC AAA  722
723  CTA TGG TTC TCA TAC ACA AAT TTA GGT CAC TGC TGT ATA TTT TAT  767
768  TTT CAA AAT CAC TTG CTG CAG TAC ATA TAA CAT AAA TTA GTC TTT  812
813  ATT TGT CTT CAG AGA CTT ACA TGT GGT GGT AAA AAT AAA GCA TCA  857
858  ACT GAC AAA AAA AAA AAA AAA AAA AAA AAA AAA AAG              893
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Figure 1. Nucleotide and deduced amino acid sequences of starry flounder ghrelin cDNA. Sequence registered at Genbank data base(accession number: MZ043898).

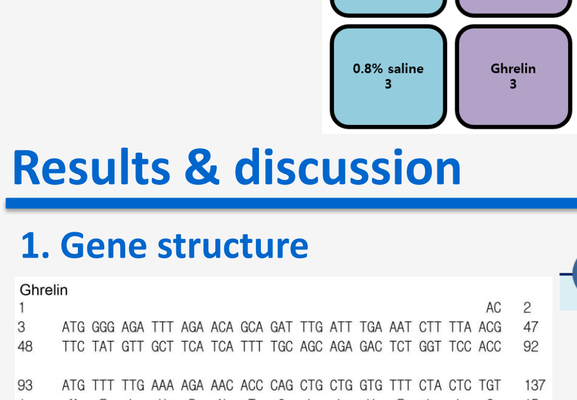

Figure 2. Schematic diagram illustration of the exon/intron organization of starry flounder ghrelin gene.

Ghrelin in other species

- 3 introns and 4 exons in goldfish and human
- 4 introns and 5 exons in rainbow trout, rat, and mice

Starry flounder Ghrelin

- 318 bp and 105 amino acid residues
- Mature peptide : 23 amino acid residues
- Polyadenylation signal (AATAAA) at 3′UTR

Ghrelin in other fishes

- Mature peptide : 19 or 12 amino acids(Goldfish)
- Mature peptide : 24 or 21 amino acids(Rainbow trout)
- Mature peptide : 20 amino acids(Nile tilapia)

Starry flounder Ghrelin

- 3 introns and 4 exons
- 5′ and 3′ UTR, signal peptide, mature peptide, C-terminal peptide
- Des acyl ghrelin has active with acylation

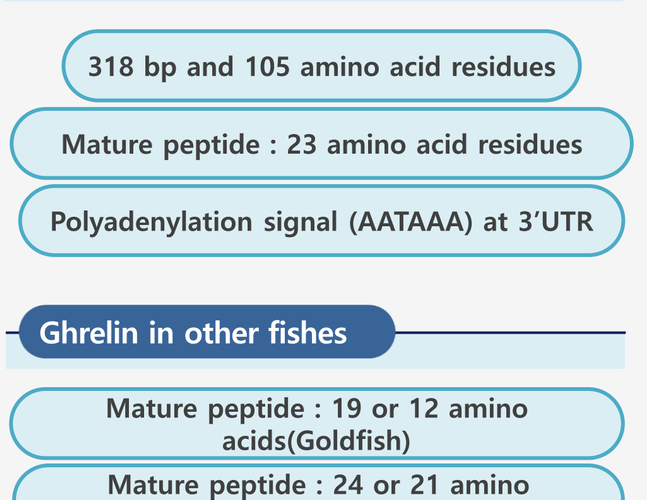

Figure 3. Relative ghrelin expression normalized to GAPDH of starry flounder in various tissues.

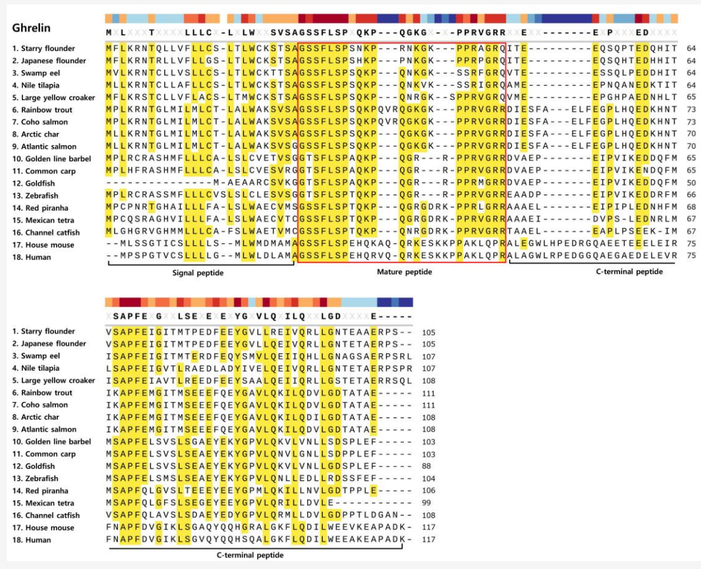

Figure 4. Multiple alignment of starry flounder ghrelin amino acid sequences with other vertebrate species.

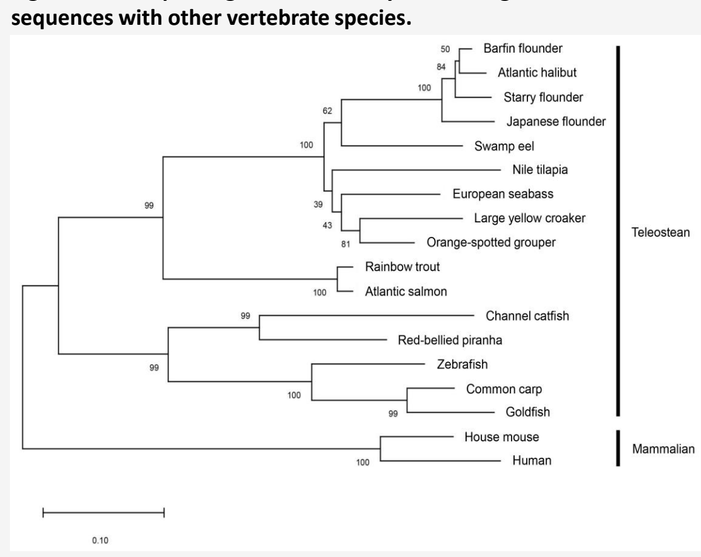

Figure 5. Phylogenetic tree based on complete ghrelin gene with other vertebrate species. The tree was contructed using maximum likelihood method available in MEGA X. The evolutionary distances were computed using the maximum composite likelihood method and 1000 bootstrap replications.

Multiple Alignment

- The GSSFLSP portion of the mature peptide was relatively well conserved in the vertebrate lineage.
- The PPR*GR portion of the mature peptide was relatively well conserved in bony fish.
- In addition, the 12th amino acid of the mature peptide was composed of proline (P) in three flatfishes (Barfin flounder, Atlantic halibut, and Japanese flounder) except for starry flounder, but that of starry flounder was composed of arginine (R)

Phylogenetic tree

- The ghrelin amino acid sequence of the starry founder showed the highest similarity with Barfish flounder and Japanese flounder at 91.43%.
- The genetically similarity with Human was the lowest at 20.95%.

2. Starvation and ghrelin trigger

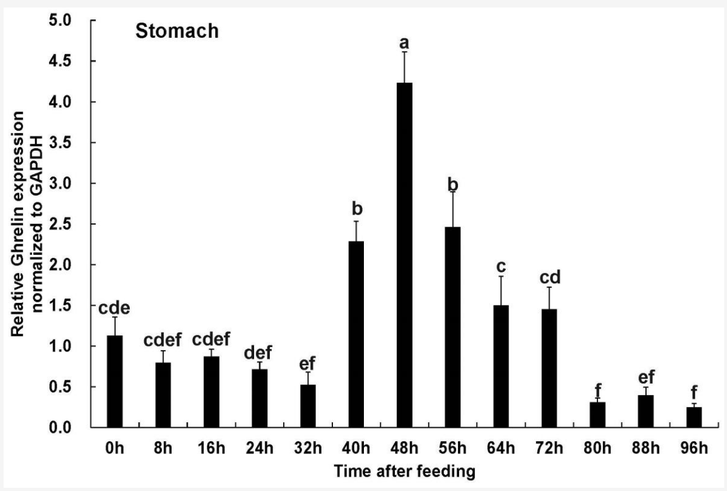

Figure 6. Ghrelin expression level of starry flounder by time after feeding.

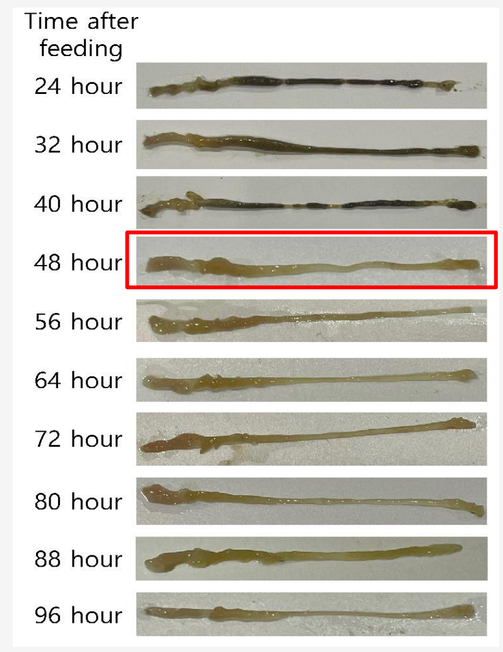

Figure 7. Gastrointestinal tract photograph by time after feeding

Intestinal transit time : 40-48 hour → Match time → Ghrelin expression: 48 hour

Intestinal emptying → Ghrelin expression index

3. Effect of human ghrelin

3.1. Growth hormone

Figure 8. growth hormone level of control and ghrelin group starry flounder brain by time after injection.

- Significantly increase at 48 hour after ghrelin intraperitoneal injection (about 1 pg/mg)
- In human, ghrelin stimulates growth hormone secretion.
- In this study, ghrelin recombinant increased growth hormone.

3.2. Feed intake and efficiency

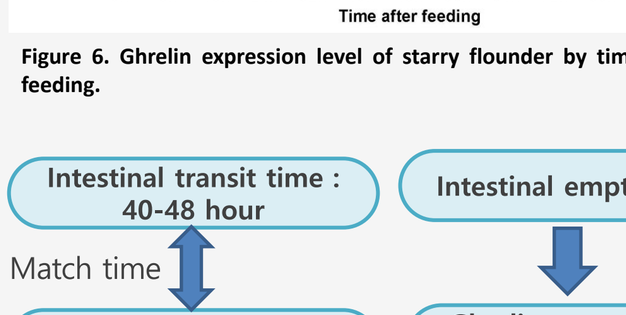

Figure 9. Daily feed intake and feed efficiency of saline and ghrelin injection groups for 64 days rearing.

- Ghrelin recombinant (intraperitoneal(IP) injection, 100ng/g) increased daily feed intake.
- In rainbow trout, IP injection of recombinant ghrelin stimulated food intake(Shepherd et al., 2007)
- Ghrelin recombinant triggered increases of feed efficiency.
- This result suggests that ghrelin induces more growth with less feed.

3.3. Growth

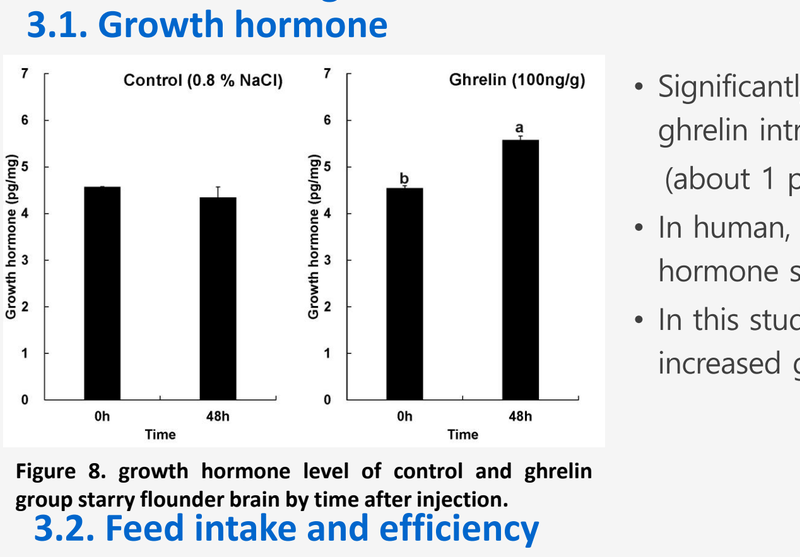

Figure 9. Final body weight(g) and weight gain(%) of saline and ghrelin injection groups for 64 days rearing.

- Ghrelin recombinant increased final body weight.
- Weight gain also increased at ghrelin injection.
- Ghrelin recombinant triggered more increases of body weight and weight gain.
- In rainbow trout, IP injection of recombinant ghrelin stimulated food intake(Shepherd et al., 2007).

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