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Distribution of ghrelin immunoreactivity in artificially reared Japanese eel, *Anguilla japonica*, leptocephalus and metamorphosed glass eel

Nam-Sil Lee, Dae-Jung Kim*, Bae-Ik Lee, Shin Kwon Kim and Kyung-Kil Kim

New Strategy Research Center, National Fisheries Research and Development Institute, Busan 619-705, South Korea

*Corresponding Author's Email : djkim4128@korea.kr

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Abstract

Ghrelin plays a role in regulation of appetite and satiety, and the orexigenic effects of peripheral or central ghrelin administration in fish have been reported. Ghrelin distribution in artificially reared eel leptocephalus and metamorphosed glass eels were examined in the present study using immunohistochemical methods. Immunoreactivity was detected in spinal cord, mucinous pouch, vascular tubules, and skin of eel leptocephalus on various days-after-hatching. Moreover, predominant immunopositive reactions were detected in the vascular system, including heart, along with spinal cord, notochord, head kidney and skin of the metamorphosed glass eel. Ghrelin immunopositivity was elevated after development of hematopoietic and vascular systems of the metamorphosed eel. Ghrelin thus, has more marked effects on physiology after the metamorphosis stage in Japanese eel.

Key words

Anguilla japonica, Eel leptocephalus, Ghrelin, Glass eel, Immunohistochemistry, Immunoreactivity

Introduction

Ghrelin is a 28-amino-acid peptide isolated from rat and human stomach (Kojima *et al*., 1999). The peptide is predominantly produced by stomach and lesser amounts are produced by small and large intestines, pancreas, pituitary gland, kidney, placenta, immune system organs and cells, testis, ovary and the arcuate nucleus of hypothalamus (Kojima *et al*., 1999). "Ghre-" is a Proto-Indo-European word meaning 'to grow,' and ghrelin is involved in regulation of many physiological functions, including regulation of growth hormone secretion and food intake in mammals (Kojima *et al*., 1999; Date *et al*., 2000).

As in mammals, ghrelin mRNA is expressed primarily in the gut of fish. Ghrelin in fish is involved in regulation of a number of physiological functions, including secretion of growth hormone (GH) and prolactin (Kaiya *et al*., 2003a; Unniappan and Peter, 2005). Among teleosts, ghrelin has been identified in goldfish, *Crassius auratus* (Unniappan *et al*., 2002), Japanese eel, *Anguilla japonica* (Kaiya *et al*., 2003a), Mozambique tilapia, *Oreochromis mossambicus* (Kaiya *et al*., 2003b), Nile tilapia, *Oreochromis niloticus* (Parhar *et al*., 2003), and rainbow trout, *Oncorhynchus mykiss* (Kaiya *et al*., 2003c).

Both octanoulated and decanoylated ghrelins are present in some fish species, but the form of ghrelin that play major role in fish is unknown (Schwndt *et al*., 2010), and neither quantitative data nor distribution ranges in various tissues have been reported. Eel ghrelin is a 21-amino-acid peptide (GenBank accession no. AB062427), unlike its counterpart mammalian ghrelin that consist of 28 amino acids. As in other fish and mammals, two molecular forms of eel ghrelin have been reported, 'eel ghrelin-21' and 'eel ghrelin-21-C10,' and the N-terminal seven amino acids exhibit 100% sequence homology to mammalian ghrelins (Kaiya *et al*., 2003a; Schwndt *et al*., 2010). Although eel ghrelin was identified previously (Kojima *et al*., 1999), distribution was detected only in Japanese eel (over 200 g body weight)

stomach (Kaiya *et al*., 2003a; Kurokawa *et al*., 2011).

This study was performed to determine ghrelin distribution in eel leptocephalus and metamorphosed glass eel developmental stages by immunohistochemistry (IHC). Which of the two kinds of ghrelin reacted could not be determined in the present study. However, it is likely that both ghrelins reacted with the polyclonal rabbit antibody against human ghrelin. Based on these results, the pattern of ghrelin immunopositivity and functions of ghrelin in eel leptocephalus were discussed at several stages after hatching, and at the metamorphosed glass eel stage.

Materials and Methods

Rearing of leptocephali : Artificially fertilized eggs were obtained by hand stripping mature adults induced by hormonal treatments according to the previous reports of Kim *et al*. (2006) and Kim *et al*. (2007a, b). Eel leptocephalus, *Anguilla japonica*, experiments were conducted by using the facilities available at National Fisheries Research and Development Institute. A feeding trial was carried out in 20 l acrylic resin tanks, continuously supplied (1 l min^{-1}) with filtered seawater at 23°C, except during feeding. The composition of larval diet was modified slightly from that described previously (Tanaka, 2003; Kim *et al*., 2014). The slurry diet (10 ml) was pipetted onto the bottom of each tank five times per day. Then, all larvae were transferred to a separate tank by siphoning. Another feeding operation was carried out as described by Tanaka (2001).

Sample preparation : Growing leptocephali and metamorphosed glass eels were randomly sampled at 10, 60, 100, 200 and 250 days-after-hatching (dah). Samples at each time point were fixed in 10% buffered neutral formalin (BNF; Sigma). The fixed tissues were washed with tap water for 3-5 hr and then dehydrated, impregnated and embedded in paraffin wax using an automated system (Leica TP 1020 & EG1150M, Germany). Paraffin-block samples were cut at 4-µm thickness serially using a microtome (Leica RM2165, Germany). Sections were stained with hematoxylin and eosin (H&E) using a Leica Autostainer XL (Germany). Selected paraffin sections were then subjected to IHC staining after microscopic examination of H&E-stained samples.

Immunohistochemistry (IHC) : Ghrelin H-40 is a rabbit polyclonal antibody raised against amino acids 21-60, mapping at the N-terminus of ghrelin of human origin (Santa Cruz Biotechnology, Inc.). The 28 amino acid sequence of human ghrelin (AB29433) and 21 amino acid sequence of eel ghrelin (ab062427) were available from DDBJ/EMBL/GenBank databases. The Vectastain Elite ABC kit (VECTOR Labs., USA), which includes normal goat serum and a biotinylated anti-rabbit polyclonal antibody, and ImmPACT DAB as peroxidase substrate, were used (VECTOR Labs.). The immunohisto chemical staining procedure followed kit protocol. Sections were deparaffinized and hydrated in xylene and a graded alcohol series, and then rinsed for 5 min in distilled water. Then, proteinase K solution (IHC World, USA) was added, followed by incubation at 37°C for 10 min. After rinsing with distilled water, 0.3% hydrogen peroxide solution was added for 30 min to quench endogenous peroxide. Phosphate buffer, pH 7.5, was used with wash buffer, and normal goat serum was used for blocking according to kit protocol. The diluted primary antibody (1:500) was then added, followed by an overnight incubation at 4°C. The secondary antibody (biotinylated antibody in the kit) was then added, followed by incubation for 1 hr at room temperature (RT). The Vectastain ABC reagent was added for 30 min at RT and the peroxidase substrate was added for about 5 min. A 1% toluidine blue solution was used for counterstaining.

Slides were viewed under microscope (Zeiss Axio Imager A1, Germany) and images were captured using camera (Carl Zeiss Axio Cam MRC5) and the AxioVision software (Axiovs40, v. 4.6.3.0). Ghrelin in tissue sections was quantified by determining the density and extent of brown reaction product.

Results and Discussion

Immunoreactivity in leptocephali : 10-dah leptocephalus had simple structure. The skin and intestine were composed of monolayers of epidermis; the primitive muscular layer had not yet developed. Immunopositive reactions, colored light brown or brown, were detected primarily in the anterior portion of leptocephali. Immunoreactivity was also evident in the dorsal mucinous pouch region (Fig. 1A), central region, immediately beneath notochord, renal system region, and at the inter-space between epidermis and primitive muscle layer (Fig. 1B). In the middle and posterior portions of 10-dah leptocephali, the major immunopositive reactions were seen at the central region, immediately beneath the notochord, and around intestine (Fig. 1C, 1D).

Sixty-dah leptocephali showed immunopositive reactions immediately beneath the notochord; *i.e*., the central aorta region as in 10-dah leptocephali (Fig. 2A). Weak immunopositive reactions were detected at the spinal cord and in skin epithelial cells, and between the epithelium and muscle layer (Fig. 2A, 2B). The majority of immunopositive reactions were detected around the digestive tubules and endothelial cells of intestine (Fig. 2C, 2D). Immunopositive reactions in the anterior portion of 60-dah leptocephali were evident immediately above the oesophagus, in skin epithelial cells, and at canaliculi in the osophageal musculature (Fig. 2B). Immunoreactivity was also detected around the renal tubules and endothelial cells of intestine (Fig. 2C-1). In rectum, immunopositive reactions were detected at the apical area in intestinal endothelial cells and in mucinous pouch-like region, immediately beneath the rectum (Fig. 2D-1). The immunopositive reactions in 100-dah leptocephali were similar to those at 60-dah (data not shown).

Table 1 : Summary about various degrees of ghrelin immunopositive reactions were detected in tissue sections of different stages of the artificially produced eel leptocephalus and glass eel in the present study

Classification of reactions	Type of reactions (color)	Mainly reacted portions of tissue sections
Weak positive	Thin line scattered fine spots (Light brown)	Mucinouse pouch/Spinal cord/Canaliculi around intestine/Between epithelium and musculature of skin (in leptocephalus)
Mild positive	Thin or thick line some size of spot (Brown)	In vasculature under notochord/Around renal tubules / Epithelium of skin Endothelium of intestine / Canaliculi around intesitine (in leptocephalus)
Heavy positive	Thick line full space mass of spots (Dark brown)	Head kidney/Gill filamentIn vasculature / Around renal tubules / Lamina propria of intestine / Epithelium of skin (in glass eel)
negative	no-reactive (Blue)	Most of no-reacted portions (including nuclei of cells)

In 200-dah and 250-dah leptocephali, immunopositive reactions were detected in canaliculi and in various regions of vasculature. Immunopositive reactions in about 200-dah leptocephali were seen in canaliculi in the lateral body stroma and weakly in spinal cord (Fig. 3A, 3A-1). Immunopositive reactions were also detected immediately beneath the notochord (Fig. 3A-2). Numerous tubule structures had developed around the intestine, and immunopositive reactions were evident in these regions (Fig. 3B-1). Widespread weak immunopositive reactions were evident in the primitive reticular tissue region, coinciding with the mucinous pouch region (Fig. 3A, 3C). Weak or mild immunopositive reactions were detected around intestine and in skin epithelium (Fig. 3C). The results for 250-dah leptocephali were not shown because they were largely similar to those of 200-dah leptocephali.

Strong immunopositive reactions were detected in metamorphosed glass eels. Definite tissue and organ formation had progressed in various body regions in the metamorphosed individual, and development of the vascular system was vigorous. In the anterior portion of animal, immunopositive reactions were detected in heart and notochord (Fig. 4A), and strong immunopositive reactions were detected in head kidney and endothelium of oesophagus (Fig. 4A-1). Additionally, immunopositive reactions were detected in gill filament tissue (Fig. 4B). In vasculature and in the lamina propria region of intestine, strong immunopositive reactions were seen (Fig. 4C-1), a predominant immunopositive reaction around the renal tubules were evident (Fig. 4C). A cross-section of the posterior region showed weak immunopositive reactions in the endothelium of intestine and renal tubules, and lymphoid tissue around renal tubules and lamina propria in intestine showed strong immunopositive reactions (Fig. 4D). The vasculature and canaliculi around the inner abdominal wall showed strongly immunopositive reactions (Fig. 4D). The immunohistochemistry results are shown in Table 1.

Immunopositive reactions were detected in the digestive tissues, spinal cord, skin, primitive reticular tissue (mucinous pouch region) and around or in the vascular system of leptocephali. Moreover, immunopositive reactions in the central vasculature of gill filaments and head kidney were detected in glass eel. Distribution of ghrelin-producing cells in digestive tract of Japanese eels during metamorphosing leptocephali, glass eel and adult stage (326~350 g body weight) was reported by Kurokawa *et al.* (2011). Expression of eel ghrelin gene in various tissues has been determined by RT-PCR in brain, heart, stomach, intestine, and kidney to various degrees, but not gills (Kaiya *et al*., 2003a). However, presence of ghrelin was confirmed in gill tissue at glass eel stage in this study.

The interesting finding was predominant reaction in the notochord of glass eel. According to Fukushima *et al.* (2005), ghrelin plays a role in osteoblastic differentiation and regulation of bone mineral density in rats. The positive reaction in notochord suggests that ghrelin is related to bone growth, because notochord is the most important skeletal tissue for growth of eels, and active bone hardening and growth have progressed in glass eel. Strong ghrelin immunopositivity was detected in the vascular system and digestive system of glass eel. Also, mild or weak signals were detected in spinal cord, mucinous pouch region, vasculature and skin epithelium during the leptocephalus stage (Table 1). The density and extent of immunopositive reactions suggest that ghrelin expression increased markedly in glass eel after metamorphosis. These results suggest that ghrelin is involved in formation of vascular and hematopoietic systems.

In mammals, the primary physiological function of ghrelin is stimulation of growth hormone release and increase food intake. The importance of ghrelinergic system in regulation of appetite and satiety is well established and the orexigenic effects of peripheral or central ghrelin administration in rodents and humans have been documented (Tschop *et al*., 2001; Asakawa *et al*., 2003; Rolland *et al*., 2011). However, some reports indicate that ghrelin does not play a primary role in initiating feeding or as a regulator of feeding patterns (Sato *et al*., 2008). Moreover, ghrelin has been shown to exhibit a range of effects on cardiovascular, gastrointestinal and pancreatic function, as well as lipogenic and glucogenic activities (Ghigo *et al*., 2005; Sato *et al*., 2012). Additionally, ghrelin is secreted by other organs, such as hypothalamus and pancreas, in rat and mRNA is expressed in various organs (Date *et al*., 2002; Sato *et al*., 2005).

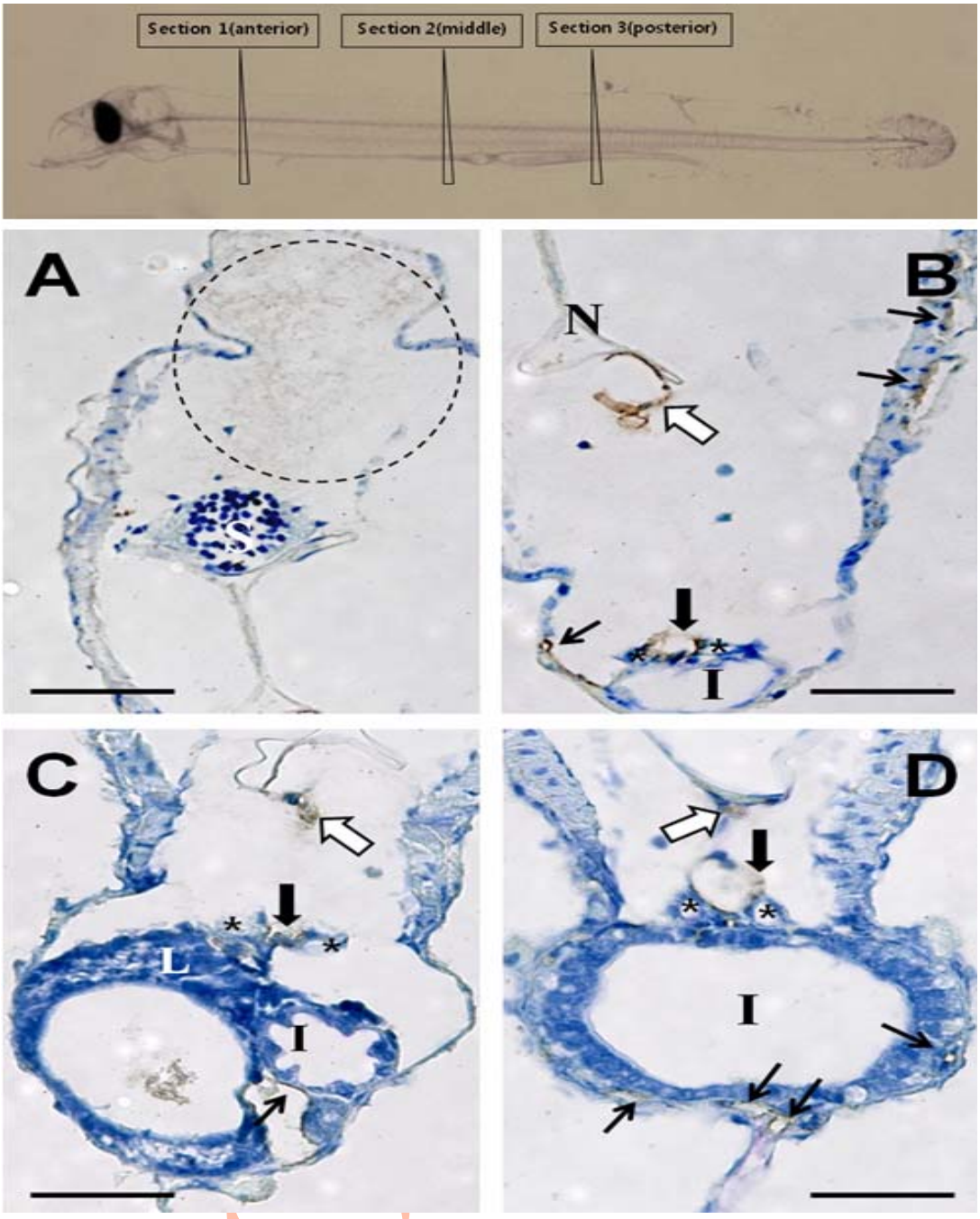

Fig. 1 : Ten-dah leptocephalus (body length: 6.7 mm). Figures A-D show examples of ghrelin immunoreactivities in three cross-sections. **A**: Anterior portion of 10-dah eel leptocephalus (section 1). S; Spinal cord. Immunopositive reaction, colored light brown, is seen at the dorsal mucinous pouch (circle). **B**: Ventral region of section 1. Immunopositive reaction, colored brown, is seen between the skin epithelium and primitive musculature (small arrows), immediately above the anterior intestine (*I*, large arrow), and immediately beneath the notochord (*N*, white arrow). *Asterisks,* renal tubules. **C**: Middle portion of 10-dah eel leptocephali (section 2). A mild immunopositive reaction was seen immediately beneath the notochord (white arrow), between the intestine (I) and the internal organs (arrow), and between the renal tubules (*asterisks*, large arrow). *L*, liver. **D**: Posterior portion of 10-dah eel leptocephali (section 3). Immunopositive reactions were detected between the renal tubules (large), underneath the notochord (white arrow), and at the canaliculi around the intestine (*I*, arrows). Bar: 20 µm.

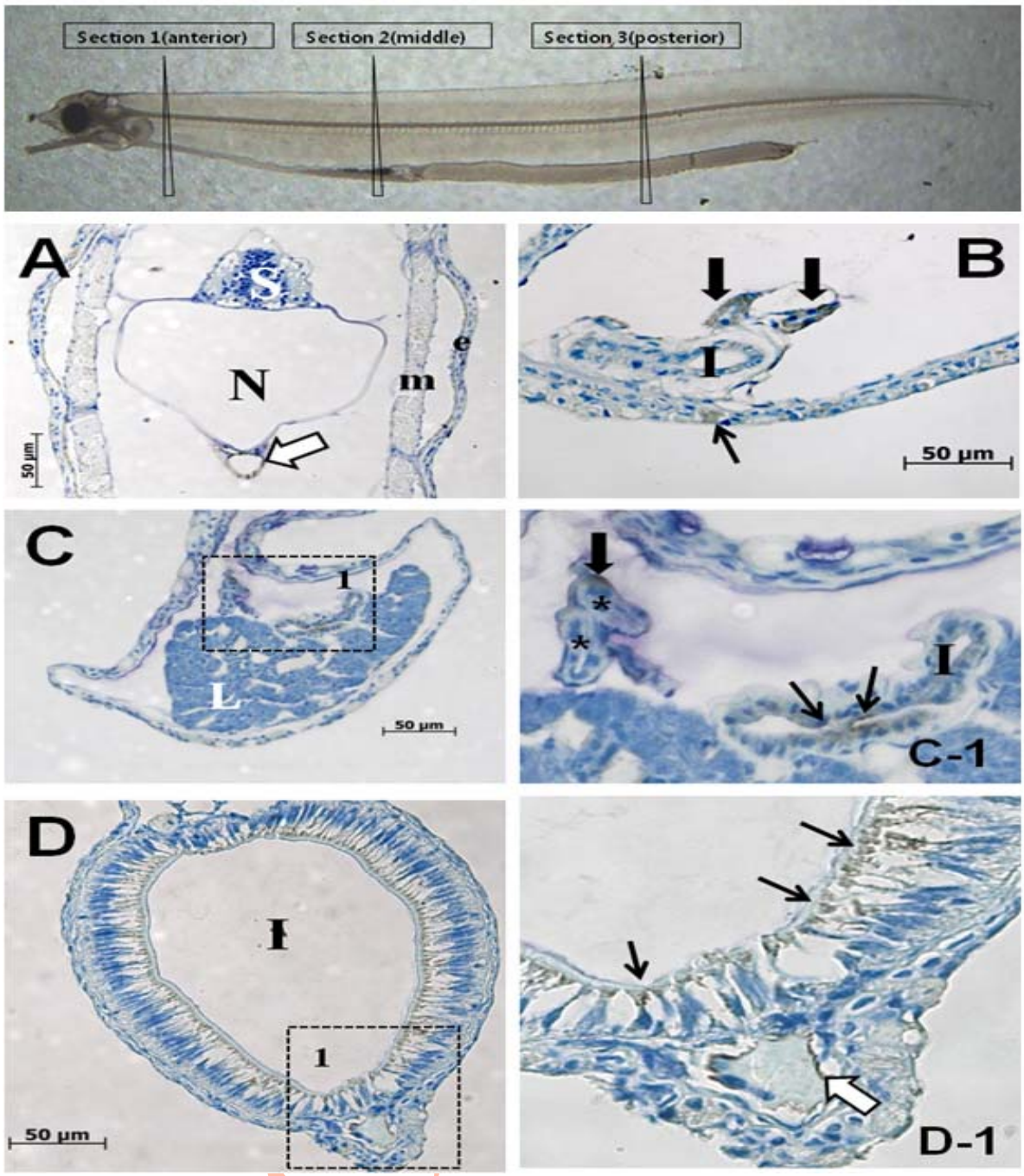

Fig. 2 : Sixty-dah eel leptocephalus (body length: 22.0 mm). Figures A-D show ghrelin immunoreactivity in three cross-sections. **A**: Section 1. Immunopositive reactions were seen immediately beneath the notochord (*N*, white arrow) and weak reactions, colored light brown, were seen at the spinal cord (*S*) and between the skin epithelium (*e*) and the musculature (*m*). **B**: Ventral region of section 1. An immunopositive reaction was seen around canaliculi and renal tubules immediately above the intestine (*I*) (large arrows) and scattered cells in the skin epithelium (arrow). **C**: Ventral region of section 2. Immunopositive reactions were seen between the renal tubules (*asterisk*s, large arrow) and the endothelium of the intestine (I). C-1 is a large-scale view of square 1 in C. *L*, liver. **D**: Ventral region of section 3. *I*, intestine. D-1: Large scale view of square 1 in D. Apical area of endothelial cells (arrows) and fluidal substance (white arrow) under the posterior intestine showed a mildly immunopositive reaction

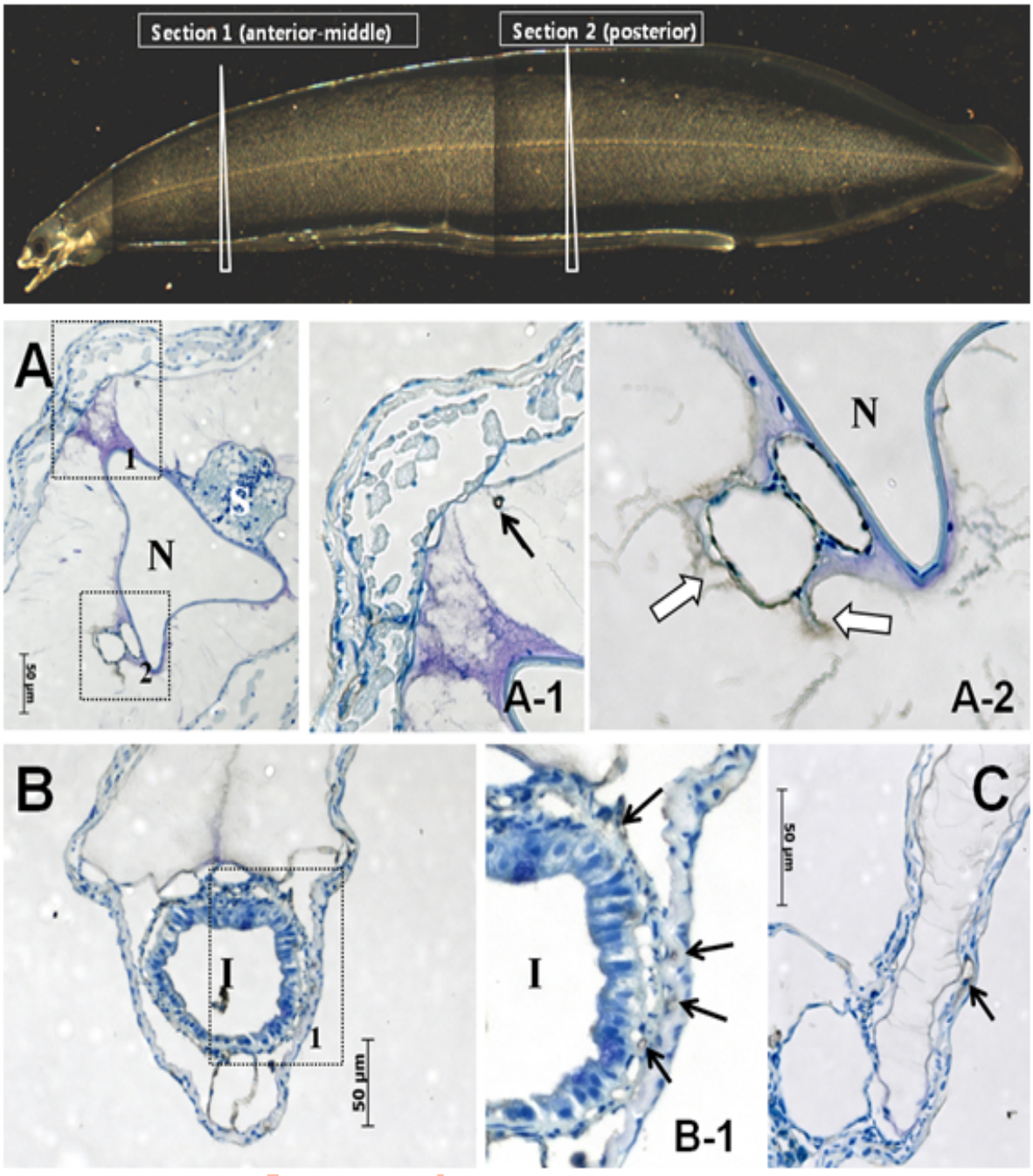

Fig. 3 : Two-hundred-dah eel leptocephalus (body length: 50.3 mm). Figures A- C show examples of ghrelin immunoreactivities in two cross-sections. **A**: Section 1. Predominant immunopositive reactivity was seen in various tubule structures and a weak reaction was seen in the spinal cord. A-1: Large-scale view of square 1 in A. Arrow: immunoreactivity indicating a microtubule structure in the body stroma adjacent to the lateral line. A-2: Large-scale view of square 2 in A. There are two tubule structures underneath the notochord (*N*), around which immunopositive reactions were detected (white arrows). **B**: Ventral region of section 2. Weak and widespread immunopositive reactions appeared at the mucinous pouch and mild reactions were seen in various tubule structures. B-1: Large-scale view of square 1 in B. Canaliculi around the intestine showed immunoreactivity (arrows). **C**: Body region of section 2. Immunopositive reactions appeared in the skin epithelium (arrow) and immunopositivity was widespread at the mucinous pouch in the body stroma

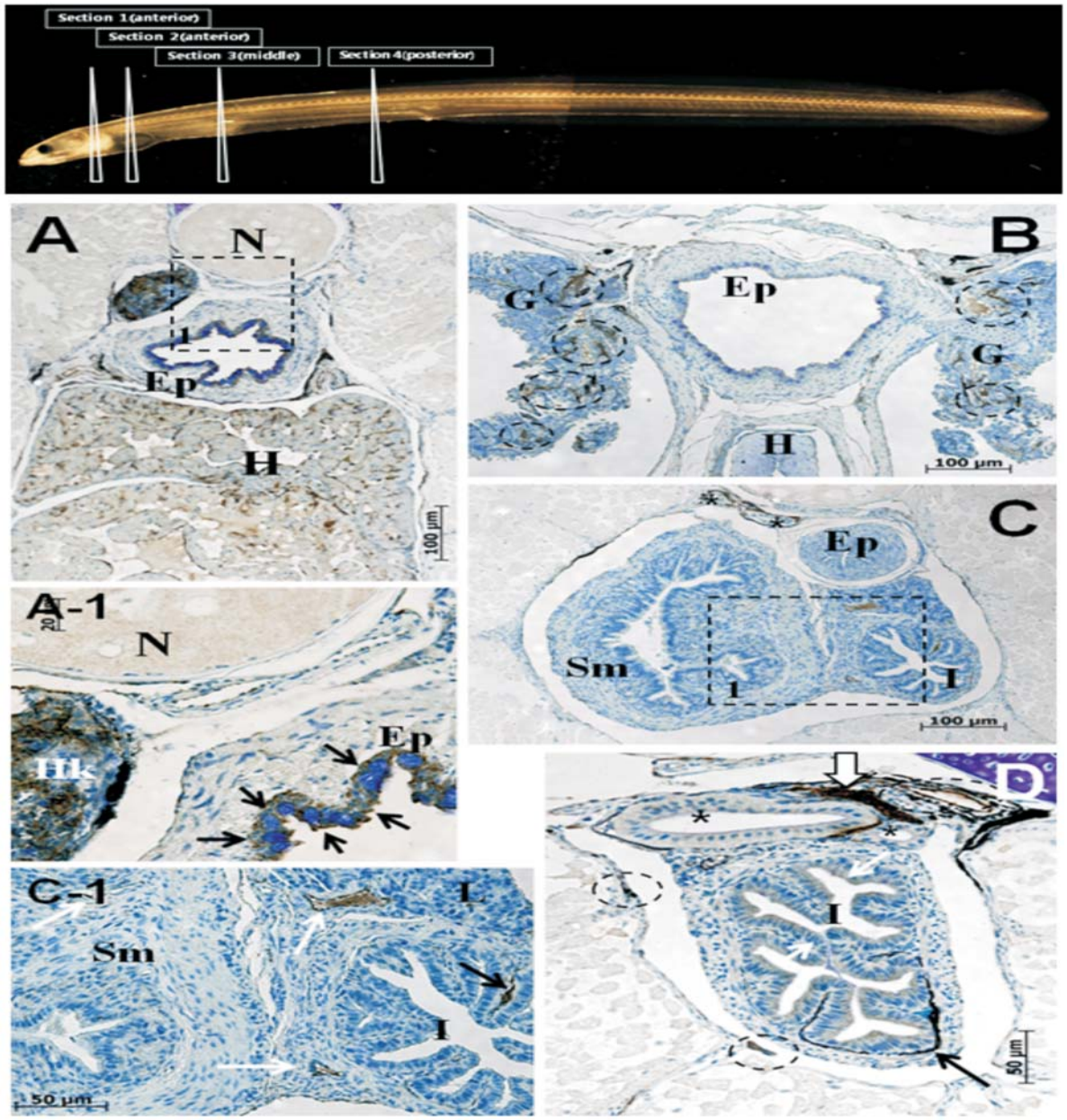

Fig. 4 : Metamorphosed glass eel (body length: 58.2 mm). Figures A-D show examples of ghrelin immunoreactivities in four cross-sections. **A**: Cross-section of the anterior portion of a glass eel (section 2). Immunopositive reactions were detected at the notochord (*N*), heart (*H*), head kidney, and endothelial cells of the esophagus (*Ep*). A-1: Large-scale view of square 1 in A. Scattered immunopositive reactions appeared in the notochord (*N*), and strong immunopositive reactions, colored dark brown, appeared in the head kidney (*Hk*) and endothelial cells of the esophagus (*Ep*, arrows). **B**: Cross-section of the head region (section 1). Endothelial cells of the esophagus (*E*) and the central vascular region of gill filaments (circles) showed immunopositive reactions. *H*, bulbus arteriosus of the heart. *Ep*, esophagus, *G*, gill. **C**: Cross-section of the middle position (section 3). The posterior region of the esophagus (*Ep*), stomach (*Sm*), and anterior intestine (*I*) appear in this section. Immunopositive reactions were seen around the digestive tubules, and a strong reaction was detected around the renal tubules (asterisks). C-1: Large-scale view of square 1 in C. Heavy and dominant immunopositive reactions appeared in the vascular tubules around the intestine (white arrows) and at the lamina propria in the intestine (*I*, black arrow). *Sm*, stomach; *L*, liver. **D**: Cross-section of the posterior region (section 4). Weak immunopositive reactions were evident in the endothelium (white arrows) of the intestine and renal tubules (asterisks). Lymphoid tissue around the renal tubules (large white arrow) and the lamina propria in the intestine (*I*, arrow) exhibited strongly immunopositive reactions, as did the vasculature and canaliculi around the abdominal inner wall (circles).

Two acylated forms of ghrelin have been identified in fish. However, which of these play the major role in fish is not known (Schwndt *et al*., 2010). Several physiological functions of fish ghrelin, such as regulation of growth hormone, luteinizing hormone, prolactin, somatolactin secretion, food intake and water intake were discussed by Unniappan and Peter (2005), and the experimental study about physiological function of eel ghrelin with prolactin release was coducted only in Japanese eel related (Riley *et al*., 2002; Kaiya *et al*., 2003a). Prolactin plays an important role in fresh water osmoregulation by preventing both the loss of ions and the uptake of water (Manzon, 2002), and Kozaka *et al*. (2003) suggested that ghrelin might involved in fluid homeostasis in eels by regulating water intake.

The results of this study explained ghrelin distribution in various regions of leptocephalus and glass eel, and that immunopositivity was more marked at the glass eel stage. There was no variation in immunoreactivity that could explain the relation of ghrelin with growth or metamorphosis during leptocephalus. Therefore, it was considered that ghrelin likely played more important physiological roles in plasma or other body fluids after metamorphosis stage in Japanese eel.

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