Investigation of the Gene Encoding Isotocin and its Expression in Cinnamon Clownfish, *Amphiprion melanopus*

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Isotocin (IT), a nonapeptide homolog of oxytocin in mammals, has been suggested to be involved in physiological processes including social behaviors, stress responses, and osmoregulation in teleost fish. To study its structure and function, the gene encoding the IT precursor was cloned from the genomic DNA and brain cDNA of the cinnamon clownfish, *Amphiprion melanopus*. The IT precursor gene consists of three exons separated by two introns, and encodes an open reading frame of 156 amino acid (aa) residues, comprising a putative signal peptide of 19 aa, a mature IT protein of 9 aa, a proteolytic processing site of 3 aa, and 125 aa of neurophysin. Tissue-specific analysis of the IT precursor transcript indicated its expression in the brain and gonads of *A. melanopus.* To examine its osmoregulatory effects, the salinity of the seawater (34 ppt) used for rearing *A. melanopus* was lowered to 15 ppt. Histological analysis of the gills indicated the apparent disappearance of an apical crypt on the surface of the gill lamella of *A. melanopus*, as pavement cells covered the surface upon acclimation to the lower salinity. The level of Na^+/K^+ATPase activity in the gills was increased during the initial stage of acclimation, followed by a decrease to its normal level, suggesting its involvement in osmoregulation and homeostasis. The only slight increase in the level of IT precursor transcript in the *A. melanopus* brain upon low-salinity acclimation suggested that IT played a minor role, if any, in the process of osmoregulation.

Key words : Arginine vasotocin, cinnamon clownfish, isotocin, osmoregulation

Introduction

In the marine ornamental industry, the importance of ornamental fish has increased due to their vivid and sometimes exquisite phenotypes, which are attractive to consumers [21]. However, the growth of this industry was limited by inadequate capturing techniques that damaged ecosystems and the overexploitation of marine resources [33]. To maintain growth in the market of marine ornamentals and to meet increasing demand, more efficient production techniques are needed for breeding and culturing marine ornamental fish. It is thus important to understand the physiology of ornamental fish, which might help to improve the efficiency of culture systems as the development and growth of fish are affected by environmental factors [2].

Attempts have been made to produce several marine ornamental species using aquaculture systems. The cinnamon clownfish *Amphiprion melanopus* is a representative species of the marine ornamental fish trade. It is found in lagoons and outer reefs in the Great Barrier Reef of Australia, Indonesia, and the Solomon Islands [3]. Since tropical marine habitats are often inundated with freshwater from seasonal rainfall, clownfish may have evolved the ability to survive low-salinity conditions. Assuming that water salinity influences the development and growth of fish [7, 29], ideal growth of marine fish would occur under optimal salinity. However, the growth of marine fish at lower salinities seems to be advantageous as many marine fish exhibit better growth and feeding efficiency under conditions of intermediate salinity similar to those of brackish water, i.e., 8-20 ppt [9, 10, 14]. This might be due to reduced osmotic stress and disease associated with parasites that prefer higher salinity. It could also be cost-effective to maintain fish in

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a hatchery using artificial saltwater.

The social behaviors and sex determination of clownfish are also of interest for understanding their development and growth. Clownfish are protandric hermaphrodites, changing sex from male to female at a later stage of the life cycle and often living in groups. Social hierarchies of clownfish units consisting of a female, a male, and several subadults and/or juveniles seem to be maintained by a disparity in the sizes of members in the pecking order and their behaviors [20, 35]. Differences in aggressive behavior and sound production observed in the size- and sex-based hierarchic society may even affect the survival rate of the group. Therefore, understanding the social-unit-associated physiology of clownfish is not only important for improving their production yield but also for studying their social behavior.

The development and growth of fish are affected by environmental factors such as temperature, salinity, and photoperiod. Various peptides are involved in signaling pathways important for such responses to the environment [5, 13, 17]. The hormone prolactin (PRL) has been shown to be essential for osmoregulation and growth in fish, but arginine vasotocin and isotocin (IT) were also suggested to be involved in various biological processes ranging from social behavior to osmoregulation [6, 22, 25]. To determine the optimal growth conditions for this marine organism, it is important to understand the mechanisms associated with its osmoregulation and social behaviors affecting growth and survival. In this study, we identified the gene encoding IT, one of the signaling molecules associated with social behavior and osmoregulation in clownfish, and examined its tissue-specific expression pattern as well as its level in the brain upon a change in salinity.

Materials and Methods

Materials

Cinnamon clownfish *A. melanopus* were obtained from a marine ornamental fish breeding company, Corea Cheju Origin Rho's Aquariums (CCORA, Jeju, Korea). Formula-one marine pellet was obtained for feeding from Ocean Nutrition (Newark, CA, USA). Chemicals used for experiments including TRI reagent, Tris.Cl and 2-phenoxyethanol were obtained from Sigma-Aldrich (St Louis, MO, USA). RNase-free DNase I and ImProm-II™ Reverse Transcriptase were obtained from Promega (Madison, WI, USA). AccuPrep® Genomic DNA extraction Kit was obtained from Bioneer

(Daejeon, Korea). Reagents used for plasmid DNA isolation and gel extraction were obtained from NucleoGen (Daejeon, Korea). Topcloner™TA kit and RNase Inhibitor were obtained from Enzynomics (Daejeon, Korea) for cloning. Kit used for 5′-/3′- rapid amplification of cDNA ends (RACE) was obtained from Clontech (Palo Alto, CA, USA).

Fish and sampling

Thirty cinnamon clownfish (67.3±7.9 mm total length, 7.3±2.5 g body weight) divide into six groups were acclimated in 120 l circular tank with a recirculating system (34 ppt, 13L:11D, 26.5±1.0℃) for 5 days [23]. Fish were fed commercial feed Formula-one marine pellet. In order to examine the effect of low salinity, salinity of the seawater (34 ppt) was shifted to 15 ppt by adding freshwater. Five fish were sampled with different intervals for 0, 4, 8, 24, 48, and 144 hr of adaptation to 15 ppt salinity. Fish were collected upon anesthetized with 200 ppm 2-phenoxyethanol and then killed by spinal transection for the collection of the brain.

Cloning and characterization of the IT gene in cinnamon clownfish

Genomic DNA was extracted from the whole blood of cinnamon clownfish using the AccuPrep® Genomic DNA Extraction Kit (Bioneer Inc., Daejeon, South Korea). To obtain the DNA fragments encoding IT in the clownfish, degenerate primers were designed from the conserved regions of the IT gene identified in other teleost fish. The primers used for polymerase chain reaction (PCR) amplification and DNA walking were as follows: ITDegF (5′-tggagcctctgtgtccgtgtgcct), ITDegR (5′-gagaactacctgctcaccccctg), DW-IT F1 (5′-tgcttcggccccagtatctgctgt), DW-IT F2 (5′-gctccccagaaacagctcactgtgt), DW-ITR1 & 5r-IT (5′-tgcatctatgatggacctcttcccacc), DW-ITR2 (5′-ggacagttggagatgtaacaggctgag), CFITF1 (5′-atgaccggagctgctgtgccc), and CFITR1 (5′-tggtggattcggtgaggagaagt), where Deg refers to a degenerate primer, DW refers to a DNA walking primer, and F and R indicate the forward and reverse directions of the IT gene.

PCR was carried out in a 20 μl mixture containing genomic DNA (0.05 μg/μl) or cDNA templates, ITDegF and ITDegR primers (1 μM), and 1× HiQ-PCR Mix. Amplification was carried out with an initial denaturation step at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 5 min. DNA walking was performed according to the manufacturer's in-

structions using DW primers to obtain the regions corresponding to the 5′- and 3′-ends of the IT gene. The PCR products were electrophoresed on an agarose gel, purified using a gel extraction kit, and subcloned into the Topcloner™ TA kit for sequencing analysis. Total RNA was extracted from each tissue using TRI Reagent, followed by treatment with DNase I for 30 min at 37°C. cDNAs encoding fragments of the IT gene were amplified based on the resulting genomic DNA sequence, and then 5′/3′-rapid amplification of cDNA ends was performed in accordance with the manufacturer's instructions.

Expression of IT mRNA by reverse transcription (RT)-PCR

Total RNA was extracted from the brain of *A. melanopus* (n=4) using TRI Reagent and treated with DNase I for 30 min at 37°C. First-strand cDNA was synthesized in a 20 μl reaction containing 0.5 μg total RNA and 0.5 μM dT_{15}, ImProm-II™ Reverse Transcriptase, 6 mM $MgCl_2$, 0.5 mM dNTPs, and 20 units RNase inhibitor. RT-PCR was carried out using cDNA templates and primers CFITF1 and CFITR1 at 94°C for 4 min, followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 50 s, and a final polymerization step at 72°C for 5 min. PCR products were resolved by electrophoresis on a 2% agarose gel, followed by staining with ethidium bromide and quantification using the Gel Doc System/Station (Bio-rad, Hercules, CA, USA). The levels of β-actin mRNA were evaluated as a control. The expression level of IT mRNAs was normalized with respect to the level of β-actin transcript as described [23]. The amplified fragments of IT and β-actin were 471 and 392 bp, respectively.

Histological analysis of the gills using electron microscopy

To examine the effect of salinity changes in fish, the structure of the gills, which are important for absorbing the surrounding water, was analyzed by transmission electron microscopy. The gills were treated with 2.5% glutaraldehyde at 4℃ for 2 hr, washed with 1× phosphate-buffered saline (PBS) for 10 min, and then fixed in 1% osmium tetroxide at 4℃ for 2 hr. Specimens were washed with 1× PBS and then dehydrated using stepwise treatments with ethanol from 50% to 100% for 15 min, followed by treatment with a mixture of propylene oxide and Epon 812. Specimens were cut into 0.5 μm-thick sections, stained with toluidine blue,

and then cut into 70 nm-thick slices. The transmission electron micrograph was analyzed upon double staining with uranyl acetate and lead citrate.

Na$^+$/K$^+$-ATPase (NKA) activity analysis

The activity of NKA was analyzed by a slightly modified version of a previously reported protocol [15]. Four gill filaments separated from the gill arch were stored in 100 μl ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) at -80°C until analysis. Frozen gill filaments were slowly thawed and homogenized in 25 μl SEID (0.5 g deoxycholate/100 ml SEI) for 10 s. Upon centrifugation at 5,000× g for 30 s, the supernatant was subjected to NKA activity analysis by measuring the inorganic phosphate concentration.

Sequencing and phylogenetic analysis

The identified sequence was subjected to homology analysis with other vertebrate orthologs and paralogs from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were generated by ClustalW [30]. Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (ver. 6.06) program and the neighbor-joining method with 1,000 bootstrap replicates.

Statistical analysis

Data were analyzed using SPSS statistical package (ver. 20; SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by a post hoc multiple comparison tests (Tukey's test) was used to compare differences in the groups.

Results and Discussion

Gene structure and amino acid sequence of IT precursor

To explore the structure and functional role of IT, the gene encoding its precursor in cinnamon clownfish was amplified by PCR using oligonucleotide primers corresponding to the conserved regions of this gene [34]. The sequence of the full-length IT gene and its neighboring regions was obtained by DNA walking, as described previously [23], using genomic DNA isolated from clownfish muscle and cDNA prepared from RNA isolated from the brain. A comparison of the sequences obtained from the cDNA and genomic DNA templates indicated that the gene encoding the IT precursor
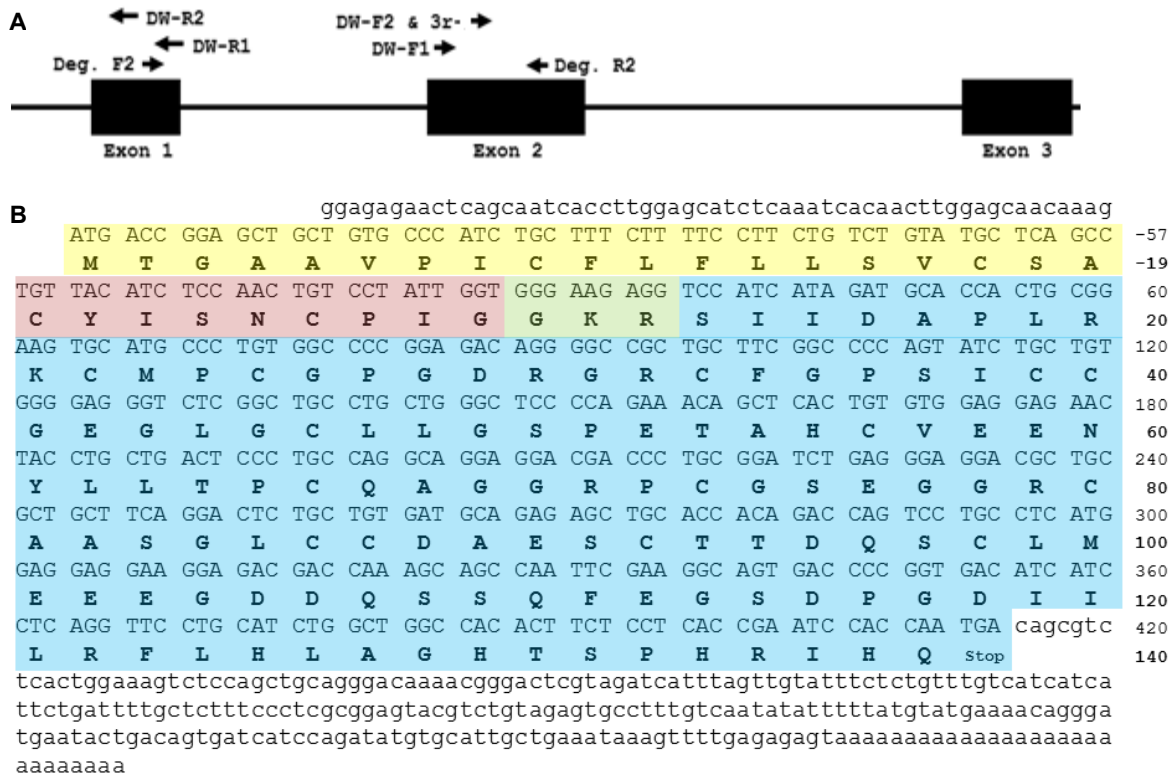
A

B

Fig. 1. (A) Structure of the gene encoding isotocin in *Amphiprion melanopus*. Exons and introns are represented by dark boxes and lines, respectively. The arrow indicates the positions corresponding to the primers used for polymerase chain reaction amplification using degenerate primers and DNA walking. (B) Nucleotide sequence of the isotocin gene. Coding regions are shown as lower-case letters and non-coding regions as upper-case ones. The corresponding amino acid sequences are indicated in bold. The regions corresponding to the signal peptide sequence, mature isotocin, proteolytic processing site, and neurophysin are marked by shaded boxes.

consists of three exons of 120 bp, 205 bp, and 146 bp, separated by two introns of 321 bp and 491 bp (Fig. 1A). The IT cDNA (GenBank accession no. HQ441173.1) contains a single open reading frame (ORF) of 471 bp together with 5′- and 3′-untranslated regions of 57 bp and 266 bp, respectively. The IT precursor encodes an ORF of 156 amino acids (aa), comprising a 19-aa signal peptide, 9 aa of IT, and 125 aa of neurophysin (Fig. 1B). It also contains the glycine-lysine-arginine sequence, which is the signal for proteolytic processing and carboxyl-terminal amidation between the hormone and neurophysin. The latter is believed to act as a carrier protein involved in the transport of the hormone from the hypothalamus. The structure and sequence of the clownfish IT gene were found to be similar to those of IT genes previously identified in other teleosts [1, 32, 34]. The calculated molecular mass of the mature peptide is 16.3 kDa, with a theoretical pI of 4.94. Amino acid sequence comparison of the *A. melanopus* IT precursor with those of other teleosts using ClustalW (Fig. 2) indicated high amino acid

identities (62-88%). The highest (88%) amino acid identity was to that of the Chinese wrasse *Halichoeres tenuispinis* (ADB28875), followed by 85% to that of the European flounder *Platichthys flesus* (BAA98141), 79% to the pufferfish *Takifugu rubripes* (AAC60289), and 73% to both the chum salmon *Oncorhynchus keta* (BAD12146) and the cherry salmon *Oncorhynchus masou* (BAA01738). It also showed 65% similarity to the gene encoding the arginine vasotocin (AVT) precursor [24] of *A. melanopus* (AEB00559). The phylogenetic tree also indicated similarity between cinnamon clownfish and other teleosts, but a distant separation from non-teleost ITs (Fig. 3).

Tissue-specific expression of IT precursor

To examine the tissue-specific expression profiles of the IT precursor, various tissues including the brain, muscle, gills, gonads, heart, intestines, liver, muscle, and stomach were dissected from adult clownfish. RNA was isolated from each tissue and used for cDNA synthesis using oligo-dT

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Ci.clownIT     --MTGAAVP-ICFLFLLSVCSACYISNCPIGGKRSIIDAPLRKCMPCGPGDRGRCFGPSI 57
Se.clownIT     --MTGAAVP-VCFLFLLSVCSACYISNCPIGGKRSIIDAPLRKCMPCGPGDKGRCFGPGI 57
WrasseIT       --MTGASVS-VCLLFLLSVCSACYISNCPIGGKRSIMDAPQRKCMSCGPGDRGRCFGPSI 57
Gi.SeabreamIT  --MTGAAVS-VCLVFLLSVCSACYISNCPIGGKRSIMDAPLRKCMSCGPGDRGRCFGPSI 57
Eu.FlounderIT  --MTGAAVS-VCLLFLVFLCSACYISNCPIGGKRSIMDAPLRKCMSCGPGDRGRCFGPGI 57
RicefishIT     --MTGATVS-VCLLFIMSVCSACYISNCPIGGKRSVMDAPLRKCMSCGPGDRGHCFGPSI 57
PufferfishIT   --MTGTAIS-VCLLFLLSVCSACYISNCPIGGKRSIMDAPQRKCMSCGPGDRGRCFGPGI 57
Ma.SalmonIT    MAMFGTSVSALCLLFLLSVCTACYISNCPIGGKRSALAFPSRKCMSCGPGDRGRCFGPNI 60
At.SalmonIT    MAMFGTPVSALCLLFLLSVCTACYISNCPIGGKRSALAFPSRKCMSCGPGDRGRCFGPNI 60
ZebrafishIT    --MSGGLLSAAALLCLLSVCSACYISNCPIGGKRSVQDWPIRQCMPCGPGDRGRCFGPSI 58
CarpIT         --MSGSLLSSGGLLCLLSICSACYISNCPIGGKRAVQDWQSRQCLSCGPGDHGQCFGPSI 58
RatOT          --MACPSLA-CCLLGLLALTSACYIQNCPLGGKRAALDLMRKCLPCGPGGKGRCFGPSI 57
HumanOT        --MAGPSLA-CCLLGLLALTSACYIQNCPLGGKRAAPDLDVRKCLPCGPGGKGRCFGPNI 57
                *      :.    :: :: : :****.***:****:       *:*:.****.:*:****.*

Ci.clownIT     CCGEGLGCLLGSPETAHCVEENYLLTPCQAGGRPCGSEGGRCAASGLCCDAESCTTDQSC 117
Se.clownIT     CCGEGLGCLLGSPETAHCVEENYLLTPCQAGGRPCGSEGGRCAASGLCCDAESCTTDQSC 117
WrasseIT       CCGEGLGCFLGSPETAHCLEENYLLTPCQAGGRPCGSEGGRCAASGVCCDSESCTTDQSC 117
Gi.SeabreamIT  CCGEGLGCLLGSPETAHCVEENYLLTPCQAGGRPCGSEGGRCAASGLCCDAESCTTDQSC 117
Eu.FlounderIT  CCGEGLGCLLGSPETAHCVEENYLLTPCHAGGRPCGSEGGRCAASGLCCDAESCTTDQSC 117
RicefishIT     CCGDGFGCLLGSPESAACVEENYLLTPCQAGGRPCGSDGHCATSGLCCDAESCTTDQSC 117
PufferfishIT   CCGESFGCLMGSPESARCAEENYLLTPCQAGGRPCGSEGGLCASSGLCCDAESCTMDQSC 117
Ma.SalmonIT    CCGEGMGCYVGSPEAAGCVEENYLPSPCEVGGRVCGSEEGRCAAPGICCDVEGCSIDQSC 120
At.SalmonIT    CCGEGMGCYVGSPEAAGCVDENYLPSPCEAGGRVCGTEEGRCAAPGICCDVEGCSIDQSC 120
ZebrafishIT    CCGEGIGCLVGSPETLRCLEEDFLPSPCEMSGKACG-YEGRCAAPGVCCDSEGCSVDQSC 117
CarpIT         CCGEGIGCLVGSPETLRCLEEDFLPSPCEVSGKACG-YEGHCAAPGVCCDSEGCSMDQSC 117
RatOT          CCADELGCFVGTAEALRCQEENYLPSPCQSGQKPCG-SGGRCATAGICCSPDGCRTDPAC 116
HumanOT        CCAEELGCFVGTAEALRCQEENYLPSPCQSGQKACG-SGGRCAVLGLCCSPDGCHADPAC 116
                **.: :** :*:.*:   * :*::* :**. . : **    * **  *:**. :.*   * :*

Ci.clownIT     LIEEEGDDQSSQFEGSDPGDIILRFLHLAGHTSPHRIHQ 156
Se.clownIT     LMEEEGDDQSSQFEGSDPGDIILRFLHLAGHTSPHRIHQ 156
WrasseIT       LMDEESDDPTSQFEGGDPGDIILRLLHLAGHTSPHRVHQ 156
Gi.SeabreamIT  LIEEEGDDQSSQFEGGDTGDIILRLLHLAGHTSPHRIHQ 156
Eu.FlounderIT  LIEEDGEDQTGQLEGGDPSDIIFRLLHLVGHASPHQSHQ 156
RicefishIT     FMEENGDDQSSPLEVSDPADILLRLLHLAGHTS-HRIHQ 155
PufferfishIT   LSEEEGDERGSLFDGSDSGDVILKLLRLAGLTSPHQTH- 155
Ma.SalmonIT    TEEDEAEYISQSVSSSHGHDLLMKLLNMISHTPPHRVHK 159
At.SalmonIT    TEEDEAEYISQSVSRSHGHDLLMKLLNMISHTPPHRVHQ 159
ZebrafishIT    VDGDADAAVNQPANSP--DLLLKLLHLSSHTHPSRIHQ 154
CarpIT         ADGDADGPADTQPASSQ--DLLLKLLHLSSHAHPLRLHQ 154
RatOT          DPESAFSER--------------------------- 125
HumanOT        DAEATFSQR--------------------------- 125
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Fig. 2. Alignment of the isotocin amino acid sequences in *Amphiprion melanopus* with those in other teleosts. Compared with the other isotocin sequences, the highest identity (88%) was with that from the Chinese wrasse *Halichoeres tenuispinis* (ADB28875), followed by 85% with the European flounder *Platichthys flesus* (BAA98141), 79% with the pufferfish *Takifugu rubripes* (AAC60289), 73% with both the chum salmon *Oncorhynchus keta* (BAD12146) and the cherry salmon *Oncorhynchus masou* (BAA01738), and 65% similarity with the gene encoding the arginine vasotocin precursor of *A. melanopus* (AEB00559), aligned using ClustalW. Identical amino acids among proteins are indicated by asterisks.

primers. RT-PCR analysis indicated that the IT precursor was expressed mainly in the brain, with lower expression in the gonads (Fig. 4). This is consistent with a previous finding of the expression of the nonapeptide in the brain and ovaries of an ostariophysian catfish [1]. Detection of the IT-specific transcript in the gonads reflected its expression in the ovaries, as the largest clownfish in the social group, probably a female, was used for RNA isolation.

Histological observation of gill lamellae using transmission electron microscopy

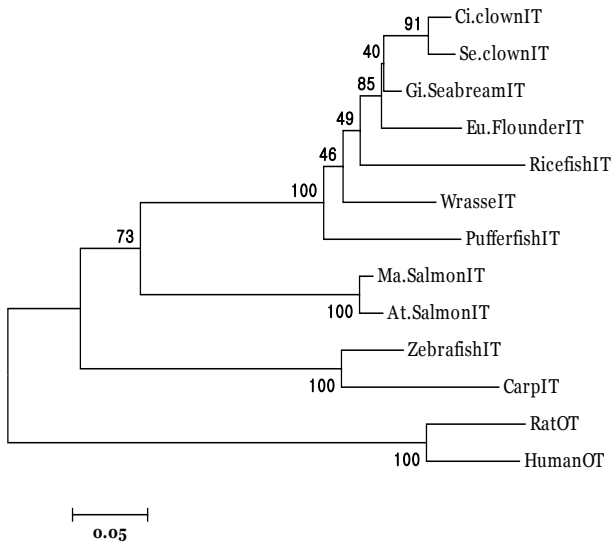The gills are a major organ that absorbs the surrounding

Fig. 3. Construction of the neighbor-joining tree based on the coding regions of the isotocin gene in teleosts and mammals. Bootstrap values are indicated for each node. Taxonomic groups are indicated on the right. Sequences of isotocin in *Amphiprion melanopus* (HQ441173.1) were compared with those of other teleosts including the Chinese wrasse *Halichoeres tenuispinis* (ADB28875), the European flounder *Platichthys flesus,* (BAA98141), the pufferfish *Takifugu rubripes* (AAC60289), the chum salmon *Oncorhynchus keta* (BAD12146), and the cherry salmon *Oncorhynchus masou* (BAA01738). Isotocin sequences from *Papio hamadryas* (ADG56475) and *Homo sapiens* (AAH88370) were included as outgroups, together with a gene encoding the arginine vasotocin precursor of *A. melanopus* (AEB00559).
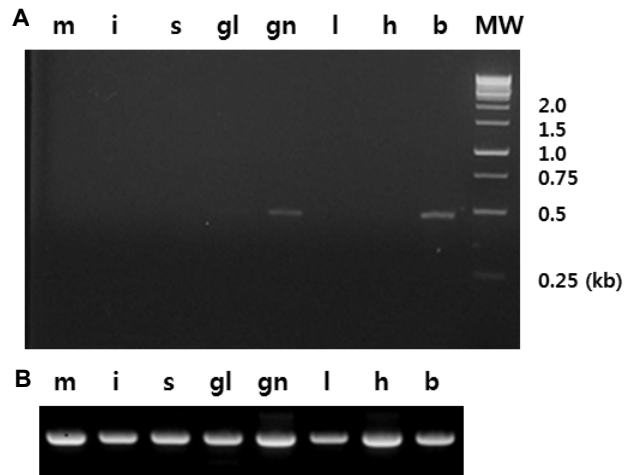

Fig. 4. Tissue-specific distributions of the transcripts encoding the isotocin precursor in the cinnamon clownfish *Amphiprion melanopus*. Reverse transcription polymerase chain reaction (RT-PCR) was carried out using primers specific to the isotocin precursor (CFITF1 and CFITR1 : A) and β-actin (B). Total RNA isolated from muscle (m), intestines (j), stomach (s), gills (gl), gonads (gn), liver (l), heart (h), and brain (b) was used for cDNA synthesis, followed by polymerase chain reaction amplification. Lane MW includes molecular weight markers with the sizes (in kb) indicated on the right.
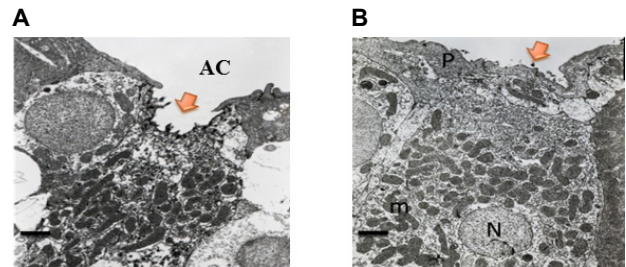

Fig. 5. Transmission electron micrographs of gill lamella of *Amphiprion melanopus* reared in seawater (A) and after transfer to 15-ppt salinity (B). AC: apical crypt, P: pavement cell, L: lamella, M: mitochondrion, N: nucleus. Bar: 2 μm.

water and plays an important role in osmoregulation, together with the kidneys and intestines, by adjusting ionic and osmotic balances between the body fluid and external environment. Fish gill epithelium comprises several types of cells, including pavement cells occupying more than 90% of the gill surface, tubular systems, accessory cells, and mitochondrion-rich cells (MRCs). MRCs, also known as chloride cells, are interspersed along the pavement cells in the epithelium and actively involved in ion transport by NKA in the membrane. The surface structure of the gill lamella seems to be characterized by the presence of apical pores or crypts, depending on the salinity conditions of the surrounding water [4, 11]. To examine the physiological changes associated with osmoregulation, the salinity of the seawater (34 ppt) used for rearing clownfish was shifted to 15 ppt, which is similar to that of brackish water, but has no effect on the consumption of feed or the survival of clownfish [23]. Electron microscopic analysis of the gills

showed a difference in the shape of the apical surface of clownfish depending on the level of salinity to which they were exposed (Fig. 5). However, the concave shape of the apical crypt was transformed to a flattened surface at 15 ppt salinity, reflecting an acute change in salinity resulting in transformation of the apical membrane of the gills during exposure to low-salinity water. This is consistent with the morphological change in the gills observed in euryhaline fish adapting to low salinity [12], with a change from a concave to a convex surface exhibited in the apical region of

MRCs in the killifish *Fundulus heteroclitus*. These morphological transformations of gill epithelium are likely to maintain the ion concentration in the body via NKA [27, 28].

Gill NKA activity of cinnamon clownfish adapting to 15-ppt salinity

Gill NKA plays an important role in osmoregulation by coupling the exchange of two extracellular K^+ ions and three intracellular Na^+ ions to the hydrolysis of ATP. While an increase in gill NKA activity was observed in milkfish, killifish, and striped bass [26, 31], other fish species including tilapia, eel, and salmon showed an alternative NKA response, namely, an increase in hyperosmotic medium [8, 16, 18]. This difference appears to be caused by the dependence of H^+-ATPase or Na^+/H^+-exchanger on the NKA isoforms of fish adapting to the fluctuating salinity. The analysis of NKA activity in the gills of clownfish indicated its increase 4 and 24 hr after the shift to 15 ppt, but a return to its original level as its exposure continued for 48 and 144 hr (Fig. 6). These changes in the initial stage of adaptation, followed by a return to its original level, suggesting homeostasis, are consistent with previous findings regarding the recovery of osmolality in clownfish upon hypo-osmotic shock [23].

Expression of IT in cinnamon clownfish upon acclimation to low salinity

IT has been suggested to play a central role in regulating social behavior and to have peripheral effects on stress responses and osmoregulation [25]. To explore its possible involvement in osmoregulation in clownfish, the levels of the IT precursor transcript were analyzed in cinnamon clown-
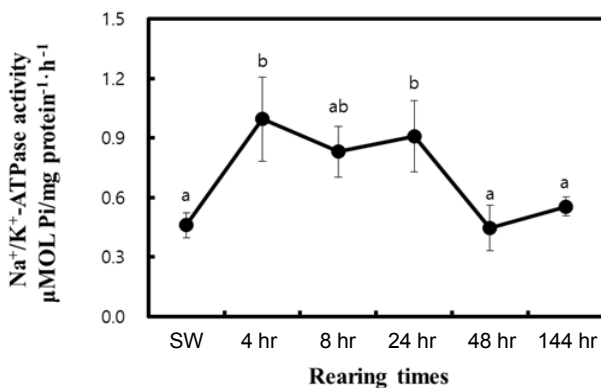

Fig. 6. Analysis of Na^+/K^+-ATPase activity in the gills of *Amphiprion melanopus* after transfer to 15-ppt salinity. Values are mean ± S.E. (n=6) and different letters indicate significant differences ($p<0.05$).

fish reared in seawater after a salinity shift to 15 ppt. Specifically, fish collected after 0, 4, 8, 24, 48, and 144 hr of acclimation were subjected to this analysis. The levels of IT transcripts among RNA isolated from the brain analyzed by RT-PCR (Fig. 7) increased slightly until 144 hr of acclimation. This contrasts with the results showing similar changes in the intracellular levels of PRL and Na^+/K^+-ATPase, which play major roles in the adaptation to lower salinity. This difference in the pattern of changes in IT levels from that of PRL suggested a limited role of IT, if any, in low-salinity-mediated, short-term osmoregulation in clownfish.

Functional implications of IT based on expression analysis

Cinnamon clownfish is a representative species in the marine ornamental fish trade. To develop an efficient culturing system for cinnamon clownfish, more information about the physiological factors affecting its growth is needed. This includes data on sexual characteristics such as protandric hermaphroditism, the social unit and its hierarchy, and clownfish growth under a wide salinity range. To survive in different salinity environments, such as in tropical habitats subjected to a range of salinities, fish need to maintain constant osmolality by releasing and/or absorbing ions from the surrounding water [19]. Among the several hormones involved in osmoregulation, PRL is a well-known example affecting growth rate and osmoregulation. Our previous study also confirmed its role in osmoregulation and homeostasis in *A. melanopus* [23]. Neurophyseal hormones, AVT and IT, were also suggested to have effects on stress responses and osmoregulation, in addition to a central effect on the regulation of social behaviors. While substantial attention has been focused on examining the roles of AVT in regulating social and reproductive behaviors, relatively little was known about the role of IT.

To explore the structure and functional role of IT, the gene encoding its precursor in cinnamon clownfish was evaluated. This gene consists of three exons, separated by two introns, similar to that of other teleosts, as well as to the structure of the gene encoding AVT [24]. Tissue-specific analysis of IT transcripts among RNA isolated from various tissues indicated that the IT precursor is primarily expressed in the brain, with a lower level in the gonads. This is consistent with a previous finding showing expression of the nonapeptide in the brain and ovaries of an ostariophysian

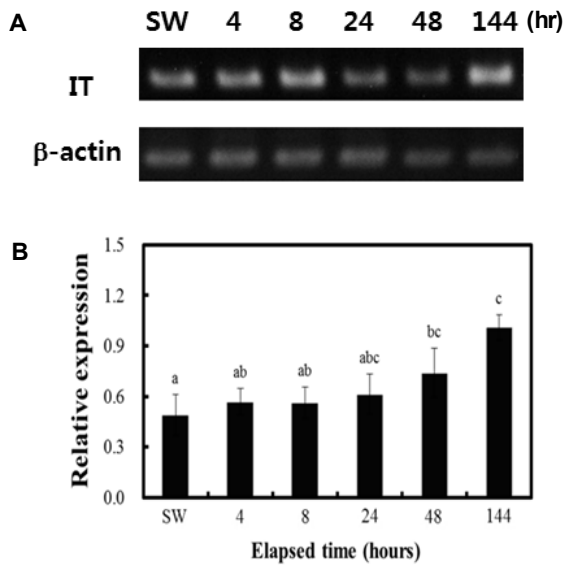**A**   SW   4   8   24   48   144 (hr)

IT

β-actin

B

Fig. 7. Analysis of isotocin precursor mRNA level in *Amphiprion melanopus* after exposure to 15-ppt salinity. (A) Total RNA prepared from the brain tissue of *A. melanopus* acclimated to 15-ppt salinity for the indicated time was used for generating cDNA by reverse transcription. Polymerase chain reaction was carried out using primers specific to isotocin and β-actin genes, respectively. (B) The expression level of isotocin precursor mRNA was normalized using the level of β-actin transcript as a control. Each value represents the mean ± S.E. (n=4), and the same letters indicate no significant difference (p>0.05).

catfish [32].

To explore the possible role of IT in the salinity-associated phenotype of clownfish, the levels of the IT transcript in the brain were analyzed upon exposure of cinnamon clownfish to lower-salinity water. Only a slight difference in its level was noted upon short-term acclimation to 15 ppt (Fig. 7). This contrasts with a previous analysis showing a clear increase in the level of PRL during the early stage, followed by a decrease as acclimation extended to 144 hr, coinciding with an opposite pattern of changes in the plasma levels of Na^+, Cl^-, and osmolality. A correlation between the induction of the PRL transcript and osmolality changes indicates that PRL is strongly involved during the early stage of adaptation to low salinity in cinnamon clownfish. However, a slight increase in the level of the transcript encoding the IT precursor in the brain, together with its inconsistent changes compared with an ion-pumping enzyme, suggests that IT may not play a major role in osmoregulation upon exposure to low salinity. It should also be stressed that the possibility of IT having a functional role in osmor-

egulation at the post-transcriptional and post-translational processing levels should not be ruled out, as the level of IT gene expression was only examined at the transcriptional level.

The results showing the expression of IT in the brain and gonads suggested its functional involvement in these behaviors and sex-related phenotypes. This might be associated with social behaviors linked to the sex-dependent hierarchy in this species. A typical clownfish social unit consists of one mature female, one mature male, and often a few adolescents, with a size-based dominance hierarchy from the largest to the smallest. Behavioral patterns ranging from aggressive behavior by dominant individuals to appeasing behavior by smaller ones were observed in a social unit of clownfish. This suggests that the dominance in a social unit affects the development and growth of the subordinate members. The social unit in clownfish seems to be maintained by a signaling system of molecules such as IT, which mediate or influence the size- and sex-dependent hierarchical system. Further study will be needed to explore the functional role of IT in the social behavior- and sex-related phenotypes of clownfish.

In summary, in this study, the gene encoding the IT precursor in cinnamon clownfish was characterized. It consists of three exons and two introns, encoding an ORF with a putative signal peptide of 19 aa, 9 aa of mature IT, a 3-aa signal cleavage site, and 125 aa of neurophysin. The IT transcript was detected in the brain and gonads of adult clownfish. Cinnamon clownfish is capable of living at salinities as low as 10 ppt and grows more rapidly at 25 ppt. This information will be useful for further studies examining the role of IT in the regulation of social behaviors and sex-dependent phenotypes.

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초록 : Cinnamon clownfish *Amphiprion melnaopus*의 이소토신 유전자 구조와 삼투압 조절이 미치는 영향

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Isotocin (IT)은 포유류의 oxytocin과 유사한 호르몬으로 어류의 행동 조절과 스트레스에 대한 반응, 그리고 삼투압 조절에 이르기까지 다양한 생리반응에 관여한다고 추정된다. 해수 관상어인 cinnamon clownfish의 IT 유전자 구조와 기능을 연구하기 위하여 genomic DNA와 뇌의 cDNA 주형으로부터 유전자를 확보하였다. 세 개의 exon과 156개의 아미노산을 표지하는 ORF로 이루어진 IT 전구물질 유전자는 19개 아미노산으로 이루어진 신호 부위, 9개 아미노산 호르몬 부위, 3개 아미노산의 효소 조절 부위, 그리고 125개 아미노산의 neurophysin 호르몬 부위로 구성되었다. 조직별 RT-PCR 분석결과는 IT 전구물질 유전자가 뇌와 생식소에서 발현됨을 보여준다. 해수 (34 ppt) 적응 개체의 아가미에서는 염류세포 바깥 표면에 apical crypt가 보이는데 비하여, 15 ppt로 낮춘 저염분 적응 개체의 아가미는 표면이 pavement cell로 덮여있는 평평한 구조를 보여준다. 아가미 세포의 Na^+/K^+-ATPase 활성은 저염분 순응 초기에 증가하다 시간이 지날수록 정상 수준을 회복하는 양상을 보여주는데, 이는 prolactin과 같이 저염분 초기순응 및 항상성 유지에 관여함을 의미한다. 하지만 저염분 순응 시 미량 증가하는 IT 전구체 mRNA는 초기 삼투조절에 미치는 역할이 미미함을 보여준다.