Effects of temperature and melatonin on day-night expression patterns of arginine vasotocin and isotocin mRNA in the diencephalon of a temperate wrasse *Halichoeres tenuispinis*

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Abstract

Most wrasses are protogynous species that swim to feed, reproduce during the daytime, and bury themselves under the sandy bottom at night. In temperate and subtropical wrasses, low temperature influences emergence from the sandy bottom in the morning, and induces a hibernation-like state in winter. We cloned and characterized the prohormone complementary DNAs (cDNAs) of arginine vasotocin (AVT) and isotocin (IT) in a temperate wrasse (*Halichoeres tenuispinis*) and examined the effects of day/night and temperature on their expression in the diencephalon, because these neurohypophysial peptides are related to the sex behavior of wrasses. The full-length cDNAs of pro-AVT and pro-IT were 938 base pairs (154 amino acids) and 759 base pairs (154 amino acids) in length, respectively. Both pro-peptides contained a signal sequence followed by the respective hormones and neurophysin connected by a Gly–Lys–Arg bridge. Reverse-transcription polymerase chain reaction (RT-PCR) revealed that pro-AVT mRNA expression was specifically observed in the diencephalon, whereas pro-IT mRNA expression was seen in the whole brain. Quantitative RT-PCR revealed that the mRNA abundance of pro-AVT and pro-IT was higher at midday (zeitgeber time 6; ZT6) than at midnight (ZT18) under 12 h light and 12 h darkness (LD 12:12) conditions, but not under constant light. Intraperitoneal injection of melatonin decreased the mRNA abundance of pro-AVT, but not of pro-IT. When fish were reared under LD 12:12 conditions at 25°C, 20°C, and 15°C, day high and night low mRNA expressions of pro-AVT and pro-IT were maintained. A field survey revealed seasonal variation in the number of swimming fish at observatory sites; many fish emerged from the sandy bottom in summer, but not in winter, suggesting a hibernation-like state under the sandy bottom under low-temperature conditions. We conclude that the day-night fluctuation of pro-AVT and pro-IT mRNA abundance in the brain is not affected by temperature and repeated under the sandy bottom in winter.

Keywords: Arginine vasotocin, circadian, hibernation, isotocin, melatonin, qPCR, temperature

Introduction

Arginine vasotocin (AVT) and isotocin (IT) are neurohypophysial nonapeptides in teleosts and homologous forms to arginine vasopressin (AVP) and oxytocin (OXT) in mammals, respectively (Urano and Ando 2011). They are synthesized in the neurosecretory neurons within the preoptic area, stored in the neurohypophysis as part of a large precursor molecule with a neurophysin carrier protein, and released in the circulatory system and to the extra-hypothalamic regions in response to appropriate stimuli or stresses (Clements and Funder 1986). These peptides in the central neural system play crucial roles in responses related to reproductive and social behaviors in many teleost species (Goodson and Bass 2001); AVT has effects on courtship exertion (Bastian et al. 2001; Semsar et al. 2001; Salek et al. 2002; Carneiro et al. 2003) and aggression (Semsar et al. 2001; Lema and Nevitt 2004; Santangelo and Bass 2010). IT appears to be related to social approach interactions in goldfish *Carassius auratus* (Thompson and Walton 2004) and parental care in the monogamous cichlid *Amatitlania nigrofasciata* (O'Connell et al. 2012).

Daily patterns of AVT and IT have been examined in certain teleost fishes. The levels of AVT in the blood circulation increase during daytime in the European flounder *Platichthys flesus* (Kulczykowska et al. 2001) and during sunset in the rainbow trout *Oncorhynchus mykiss* (Kulczykowska and Stolarski 1996; Kulczykowska 1999). High transcript levels of AVT and AVT/IT have been detected during the daytime in the brain of the rainbow trout (Gilchriest et al. 1998) and the threespot wrasse *Halichoeres trimaculatus* (Hur et al. 2011), respectively. Under constant conditions, transcript levels of AVT/IT oscillated in the threespot wrasse (Hur et al. 2011), but not in the Atlantic salmon (Gozdowska et al. 2006). These results suggest that despite species or age variation, reproductive and social behaviors in relation to AVT and/or IT are regulated by the circadian system, and their action is restricted to the daily active phase. In addition, it is likely that melatonin plays a role in controlling these neurohypophysial

75 nonapeptides, because melatonin exhibits a daily and circadian pattern with an increase during
76 scotophase (Kulczykowska 2001). To date, few studies have been conducted on the effects of
77 temperature on their expression in the brains of poikilothermic animals, although the circadian
78 system exhibits temperature compensation (Ruoff and Rensing 2004).

79 Most wrasses are protogynous hermaphroditic species and exhibit daily rhythm in their
80 activity; they bury themselves into the sandy bottom before the sunset and appear from it around
81 the sunrise. This daily behavior guarantees foraging and reproduction during the daytime, while
82 it attains a sleep-like state and avoids predation risk at night (Nishi 1989; Nishi 1990; Nishi
83 1991). In the bluehead wrasse *Thalassoma bifasciatum*, a coral reef fish, numbers of AVT
84 mRNA-producing cells in the hypothalamus were greater in dominant males than in females,
85 and similar increases in numbers occur in accordance with sexual and aggressive behavior
86 during sex change (Godwin et al. 2000). It is hypothesized that AVT and IT expressions change
87 daily with temperature compensation in temperate wrasses, because these species undergo
88 reproductive and social behavior under large temperature fluctuations. The goal of this study
89 was to clone and characterize complimentary DNAs (cDNAs) of pro-AVT and pro-IT from the
90 brains of the honbera wrasse *H. tenuispinis*, a temperate wrasse inhabiting northwest Pacific
91 waters (Randall 1999). Expressions of pro-AVT and pro-IT mRNA were studied mainly in the
92 diencephalon of the honbera, because the previous study revealed that they expressed mainly
93 in the hypothalamus including preoptic area of the threespot wrasse (Hur et al. 2011). We
94 evaluated their expression patterns under day/night and constant light conditions at different
95 temperatures as well as under administration of melatonin. The relationship between water
96 temperature and emerging behavior from the sandy bottom was observed in the honbera wrasse
97 to determine the physiological roles of AVT and IT in different seasons.
98
99 **Materials and Methods**

Fish and experimental design

Mature honbera wrasse with a body mass ranging from 15.0 g to 23.5 g were collected in June from a pier in the reefs off the northern Jeju Island, South Korea, by fishing with a hook and line. They were transferred to the Marine Science Institute at Jeju National University, South Korea, and were acclimatized in concrete tanks (7 metric ton capacity) with running seawater and aeration under ambient water temperature and photoperiod. Plastic boxes with sand were set at the bottom of each tank. The fish were fed daily at 09:00 h and 17:00 h with commercial pellets (EP3; Daehan Co., Pusan, South Korea). For pro-AVT and pro-IT cDNA cloning, fish (n = 10) were sampled from the tanks and anesthetized in seawater containing 0.01% 2-phenoxyethanol (Kanto Kagaku, Tokyo, Japan) at 12:00 h, and then were immediately euthanized by decapitation. The whole brain was taken from each fish. Fish (n = 7) were also sampled from the tanks for pro-AVT and pro-IT mRNA tissue distribution. Following anesthetization with 2-phenoxyethanol and decapitation, the whole brain, gonads, gills, intestines, skin, liver, heart, spleen, and kidneys were dissected. The whole brain was further divided into the diencephalon (including hypothalamic area) and the remaining part of the brain (brain except diencephalon). Collected samples were immediately frozen in liquid nitrogen and stored at -80°C until subsequent analyses.

To assess day-night change in pro-AVT and pro-IT mRNA abundance, fish were housed in fiber-reinforced plastic aquaria (500 L capacity; n = 20 each) with running seawater at ambient temperature and acclimated under a light-dark cycle (LD = 12:12; light on at 06:00 h, light off at 18:00 h) and constant light (LL). The bottom of each aquarium was covered with sand at a depth of 5 cm. Fluorescent bulbs (20 W) were set on the aquaria, and illuminance at the water surface was 1500 lx. After anesthetization and decapitation, the brain was taken from fish at 12:00 h (ZT6) and 00:00 h (ZT18) for LD = 12:12 and at 12:00 h (78 hours under LL) and 00:00 h (90 hours under LL) for LL, and the diencephalon was separated. Sample collection

at 00:00 h under LD condition was performed under conditions of dim LED red light (KR3, SSLight Co., Seoul, Korea) approximately 1.5 lx, 0.0 $\mu molm^{-2}s^{-1}$ at 670 nm. Collected samples were immediately frozen in liquid nitrogen and stored at -80°C until analyses were performed. To assess the effects of temperature on pro-AVT and pro-IT mRNA abundance in the diencephalon, fish were transferred into three aquaria (500 L capacity; n = 20 each) with filtration equipment and acclimated under LD = 12:12 (light on = 06:00 h; ZT0) conditions. The three aquaria were maintained at 15°C, 20°C, and 25°C using a programmable temperature controller. The bottom of each aquarium was covered with sand at a depth of 5 cm. Fluorescent bulbs (20 W) were set on the aquaria, and illuminance at the water surface was 1500 lx. After anesthetization and decapitation, the brain was taken from fish at 12:00 h (ZT6) and 00:00 h (ZT18), and the diencephalon was separated. Sample collection at 00:00 h was performed under dim light conditions. Collected samples were immediately frozen in liquid nitrogen and stored at -80°C until analyses were performed. The effects of melatonin on pro-AVT and pro-IT mRNA abundance in the diencephalon were evaluated by intraperitoneal injection. Melatonin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ethanol and diluted with saline to a final concentration of 1 mg/mL. Melatonin (1 μg/g body weight) was given to fish (n = 12) at 11:00 h. Saline without melatonin was injected into the control fish (n = 12). Three hours after injection, fish (n = 6) were taken from the aquarium and anesthetized to collect diencephalon samples. Collected samples were immediately frozen in liquid nitrogen and stored at -80°C until analyses were performed.

All of the experiments were conducted in compliance with the Animal Care and Use Committee guidelines of the University of the Ryukyus and regulations for the care and use of laboratory animals in Japan.

Field survey

Seasonal change in emergence of the honbera wrasse from the sandy bottom was observed by scuba diving. Observatory sites (stations 1–5) were selected off the northern shore of Jeju Island, South Korea (Fig. 1). The fish swimming around divers at each site were counted three times between 11:00 h and 12:00 h and averaged among sites. A field survey was conducted every 2–4 months. The water temperature was monitored simultaneously.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from the frozen samples using the RNAiso Plus (TaKaRa Bio, Otsu, Japan) according to the manufacturer's protocol. The quantity of total RNA was assayed spectrophotometrically at 260 and 280 nm, and samples with an A260/A280 ratio of 1.7–2.0 were used for cDNA synthesis. We synthesized cDNA from 1 μg total RNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa Bio) for cDNA cloning and quantitative polymerase chain reaction (qPCR) according to the manufacturer's instructions.

Pro-AVT and pro-IT cDNA cloning

The pro-AVT and pro-IT cDNA fragments of honbera were amplified by RT-PCR using degenerate primers (AVT-Forward, AVT-Reverse for AVT and IT-Forward, IT-Reverse for IT; Table 1) designed from several fish species (GenBank Accession Numbers: *P. flesus*, AB036517; *Takifugu niphobles*, AB297919; *T. bifasciatum*, AY167033 for AVT; *T. rubripes*, AB297920; *T. niphobles*, U90880; and *O. keta*, D10941 for IT). PCR was performed as follows: denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 1 min. PCR products were sub-cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and then sequenced using a PRISM 3730XL Analyzer (Applied Biosystems, Foster City, CA, USA). After identity of the amplified cDNA fragments had been confirmed by BLAST analysis, full-length cDNA was obtained by rapid amplification of cDNA

ends (RACE) using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. AVT- and IT-specific and nested primers for RACE were designed based on 277 base pairs (bp) and 217 bp partial cDNA fragment sequences, respectively (Table 1). The initial PCR was performed using 5 cycles at 94°C for 5 s, 72°C for 3 min; 5 cycles at 94°C for 5 s, 70°C for 10 s, and 72°C for 3 min; and 25 cycles at 94°C for 5 s, 68°C for 10 s, and 72°C for 3 min. Nested PCR was performed using 28 cycles and the following conditions: 94°C for 5 s, 68°C for 10 s, and 72°C for 2 min. To determine the nucleotide sequence of full-length pro-AVT/pro-IT cDNA, sub-cloning and sequencing of these cDNA fragments were performed as described above.

RT-PCR and qPCR

Primer sets used in RT-PCR and qPCR are shown in Table 1. RT-PCR was performed using Go Taq Green master mix (Promega) according to the manufacturer's protocol. PCR reactions were performed as follows: 28 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 58°C, and extension for 1 min at 72°C. Then the reaction mixture was electrophoresed on 2% agarose gels with ethidium bromide. We performed qPCR using the SYBR Green premix PCR kit (TaKaRa Bio). Each PCR reaction mix contained 50% SYBR Premix Ex Taq, 10 μM of each primer, and 20 ng cDNA template. The qPCR reactions were run on the CFX96^{TM} Real Time System (Bio-Rad, Hercules, CA, USA). The cycling conditions comprised initial denaturation at 95°C at 1 min, followed by 40 cycles of denaturation for 5 s at 95°C, and annealing and extension for 1 min at 60°C. To ensure specificity, melting curve analyses were performed by slowly raising the temperature of the sample from 60°C to 95°C. Temperature curves showed a single amplified product with complete absence of primer–dimer formation. The Gene study tools software (Bio-Rad) was used to determine a normalizing factor from β–actin as the internal reference gene, which was used to calculate normalized expression for the target genes.

200

201 Statistical analyses

202 Data are expressed as the mean ± standard error of the mean (SEM). Daily and circadian
203 variation and the effects of melatonin injection on pro-AVT and pro-IT mRNA abundance in
204 the diencephalon were compared using the Student's *t*-test. Two-way analysis of variance
205 (ANOVA) was performed to compare the effects of temperature on pro-AVT and pro-IT mRNA
206 abundance in the diencephalon, followed by the Newman–Keuls test. Statistical differences
207 were significant at a probability of $P < 0.05$.

208

209 **Results**

210 The full-length cDNAs of pro-AVT and pro-IT were 938 base pairs (GenBank Accession No.
211 GU212654) and 759 base pairs (GenBank Accession No. GU212655) in length, respectively,
212 encoding proteins of 154 and 156 amino acids, respectively. The putative honbera wrasse pro-
213 AVT and pro-IT had structural features including signal peptides, hormones, a Gly–Lys–Arg
214 bridge, and neurophysin. Neurophysin included a leucine-rich core segment that resembled
215 mammalian AVP copeptin (Fig. 2) and OXT (Fig. 3). Pro-AVT mRNA expression was
216 specifically observed in the diencephalon, whereas pro-IT mRNA in the whole brain including
217 the diencephalon. No expression of either gene was observed in peripheral tissues (data not
218 shown).

219 Day–night differences in pro-AVT and pro-IT mRNA expression in the diencephalon were
220 determined at 12:00 h (ZT6) and 00:00 h (ZT18) by qPCR (Fig. 4). The abundance of pro-AVT
221 mRNA in the diencephalon at 12:00 h was significantly higher ($P < 0.05$) than that at 00:00 h
222 (Fig. 4A). Similarly, significantly high expression of pro-IT mRNA was observed at 12:00 h
223 (Fig. 4A). When fish were reared under LL conditions, there were no differences in the mRNA
224 abundance of either gene in the diencephalon between 12:00 h and 00:00 h, which could be

assumed as subjective day and subjective night, respectively (Fig. 4B). An intraperitoneal injection of melatonin lowered the mRNA expression of pro-AVT (Fig. 5A), but not that of pro-IT (Fig. 5B), in the diencephalon within 3 h.

Thermal responses to day–night differences in pro-AVT and pro-IT mRNA abundance in the diencephalon were examined in fish reared at three water temperature conditions (15°C, 20°C, and 25°C), which simulated temperatures in winter, spring/fall, and summer in the sampling sites, respectively. As previously shown, a day high and night low pattern of mRNA abundance of both genes was observed in the diencephalon at 20°C. The same day–night patterns of mRNA abundance were maintained in high (25°C) and low (15°C) temperatures (Fig. 6A and B).

Figure 7 shows seasonal changes in the number of fish emerging from the sandy bottom off the northern shore of Jeju Island, South Korea. Many individuals were observed in summer (August and September), when water temperature increased, whereas few individuals were observed in winter (February), when water temperature decreased.

Discussion

The cDNAs cloned from the brain of the honbera wrasse had a prohormone-like sequence, which comprised a signal peptide followed by a nonapeptide and neurophysin. Nonapeptide was linked by a Gly–Lys–Arg bridge. These structures were similar to those of pro-AVT and pro-IT in the threespot wrasse (Hur et al. 2011), the white sucker *Catostomus commersoni* (Heierhorst et al. 1989), the European flounder *P. flesus* (Warne et al. 2000), the grass puffer *T. rubripes* (Motohashi et al. 2008), and salmonid species (Hyodo and Urano 1991; Hiraoka et al. 1997). Because nonapeptide structures in this study were identical to those of AVT and IT, we infer that we successfully cloned the neurohypophysial hormone sequence of the honbera wrasse.

Quantitative RT-PCR analysis revealed that pro-AVT mRNA expression was observed in the diencephalon of the honbera wrasse. Pro-AVT mRNA seems to specifically express in this region of the wrasse brain, because it was previously reported that the distribution of pro-AVT mRNA was detected in the hypothalamus including the preoptic area (Hur et al. 2011). *In situ* hybridization analysis revealed that pro-AVT mRNA expression was observed in the preoptic area in the brain of the bluehead wrasse *T. bifasciatum*; its abundance varies among sexual phenotypes of this species. Numbers of AVT mRNA-producing cells in the magnocellular preoptic area are greater in terminal phase (TP) males than in females, and stronger signals in the cells were observed in initial phase (IP) and TP males than in females (Godwin et al. 2000). An intraperitoneal injection of AVT to non-territorial bluehead wrasse males induced territorial TP male-like behaviors including courtship, chases toward IP individuals, and territorial behavior (Semsar et al. 2001). It was also found that giving a AVT-V_{1a} receptor antagonist, Manning compound [(β-mercapto- β, β-cyclopentamethyl-enepriopionyl^{1}, *O*-Me-Tyr^{2},Arg^{8})-vasopressin] to territorial TP males had the opposite effect (Semsar et al. 2001). These data strongly support the idea that variation in AVT in the hypothalamic areas of different sexual phenotypes plays a crucial role in maintaining and ranking the social system in certain fishes, including wrasses (Godwin and Thompson 2012). The expression of pro-IT mRNA appeared to be widespread in the brain of the honbera wrasse. When the brain of threespot wrasse was divided into several parts to measure pro-IT mRNA, its expression was observed in the telencephalon, hypothalamus (including the preoptic area), optic tectum, cerebellum, and medulla oblongata of the threespot wrasse (Hur et al. 2011). Widespread innervation of IT-like immunoreactivity was also observed in the brains of the rainbow trout (van den Dungen et al. 1982) and the plainfin midshipman fish *Porichthys notatus* (Goodson et al. 2003). The detection of IT in the broad area of the vocal-acoustic circuitry is associated with the entire vocal-acoustic

behavior process in a sonic fish (Goodson et al. 2003). Therefore, it may be that IT is involved in various physiological processes related to teleost behavior (Hur et al. 2011).

Our results confirmed a day high and night low fluctuation of pro-AVT and pro-IT mRNA abundance in the diencephalon. Because higher transcript levels of pro-AVT and pro-IT mRNA during photophase than during scotophase were also notified in the hypothalamus of the threespot wrasse (Hur et al. 2011), it appears that the day–night variation in these neuropeptides plays a role in diurnal-based physiological and behavioral processes in wrasses. Similar day–night variation in AVT have been reported in the blood circulation and pituitary of certain fishes (Gilchriest et al. 1998; Kulczykowska 1999; Kulczykowska et al. 2001; Rodríguez-Illamola et al. 2011), although this may be attenuated with the progress of sexual maturation in the rainbow trout (Saito et al. 2004). In contrast to these consistent results with AVT among species, little or high night fluctuation in IT has been observed in the blood circulation and brain of salmonids (Kulczykowska and Stolarski 1996; Saito et al. 2004). Therefore, it is likely that AVT and IT synthesis are separately regulated in individual neurons, because the rhythmicity of these neuropeptides differs among neurons (Gozdowska et al. 2006).

Similar to the pituitary and plasma AVT contents, a diurnal rhythmic pattern of pro-AVT mRNA abundance in the hypothalamus persisted after transferring immature rainbow trout from 14 h light and 10 h darkness (LD 14:10) to constant darkness (DD) conditions for two consecutive days, although the amplitude and duration of its peak was attenuated and increased gradually (Rodríguez-Illamola et al. 2011). This result suggests that AVT synthesis in the hypothalamus is regulated in a circadian manner. Experiments with cannulated European flounder reared under LD 12:12 showed a reciprocal day–night pattern in plasma concentrations of AVT (increasing during the light phase) and melatonin (increasing during the dark phase) (Kulczykowska et al. 2001). An intraperitoneal injection of melatonin lowered the abundance of pro-AVT mRNA in the diencephalon of the honbera wrasse in this study and in the whole

299 brain of the threespot wrasse (Hur et al. 2011), suggesting that melatonin may be a regulator of
300 AVT synthesis in fish including the honbera wrasse. Melatonin production from the cultured
301 pineal organ also exhibited daily variation, with an increase during the night and a decrease
302 during daytime under LD, which persisted under DD, but not LL in the threespot wrasse (Hur
303 et al. 2012). The results of this study suggest no circadian variation in pro-AVT and pro-IT
304 mRNA expression in the diencephalon of the honbera wrasse under LL, when melatonin
305 fluctuation is likely to be damped. Taken together, the results imply that these neuropeptides in
306 the hypothalamus change in a circadian manner under the influence of melatonin, although we
307 did not test for daily fluctuations of pro-AVT and pro-IT mRNAs under constant conditions.
308 The abundance of pro-AVT/IT mRNAs in the diencephalon under LD 12:12 conditions
309 maintained day–night fluctuation at three different temperatures, which were set within the
310 natural temperature range of honbera wrasse habitats (15°C in February, 20°C in May, and 25°C
311 in July). It was clear that the day-night fluctuation of pro-AVT and pro-IT mRNA abundance in
312 the brain is not affected by physiological temperatures. Although their contents were not
313 evaluated, seasonal changes in AVT and IT contents at high temperatures have been reported in
314 the brain of the three-spined stickleback *Gasterosteus aculeatus*: AVT decreased in December
315 in both sexes, whereas IT in males exhibited little difference, and that in females increased in
316 July (Gozdowska et al. 2006). *In situ* hybridization and immunohistochemistry using immature
317 female masu salmon (*O. masou*) revealed that AVT mRNA signals in neurosecretory cells in
318 the magnocellular part of the preoptic nucleus increased in November, when plasma levels of
319 testosterone and estradiol-17β peaked (Ota et al. 1999). It has been proposed that the seasonality
320 of AVT and IT abundance in the brains of these two species is closely related to changes in sex
321 hormone levels. Because this study was performed using mature females, the effects of sex
322 hormones on mRNA abundance of pro-AVT and pro-IT in the brain of the honbera wrasse

remains unknown. However, the involvement of sex hormones in day–night variation in AVT and IT can be assumed in the brain of the wrasse.

In conclusion, a day high and night low fluctuation of pro-AVT and pro-IT mRNA abundance persists annually in the diencephalon of this temperate wrasse. Field observation in this study revealed that most individuals of this wrasse species enter a hibernation-like state during low temperature periods and repeatedly bury themselves in the sandy bottom (Fig. 8). The present findings imply that transcript levels of pro-AVT and pro-IT mRNA in the diencephalon are repeated under the sandy bottom even in winter. However, daytime behaviors in relation to these peptides may not be undertaken in this species in winter because of a hibernation-like state. Additional studies are needed to clarify the interplay between the annual and daily rhythms of reproductive behavior and of AVT/IT in the nerve tissues of wrasses.

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447 **Figure legends**

448 Fig. 1. Field survey points in northern shore of Jeju Island, South Korea. Five observatory sites
449 (ST1 to ST5) were selected in the shallow waters near Marine Science Institute, Jeju
450 National University.

451 Fig. 2. Comparison of the deduced amino acid sequence of arginine vasotocin (AVT) of honbera
452 wrasse (upper) with that of threespot wrasse (GenBank accession no. GU212657),
453 cichlid fish (AF517936), grass puffer (AB297919) and chicken (X55130), and arginine
454 vasopressin (AVP) of human (BC126224) and rat (NM016992). The alignment was
455 generated by ClustalW. Amino acid residues conserved in all vertebrates are marked
456 with an asterisk. Signal peptide, hormone, and neurophysin are indicated by solid lines,
457 while copeptin by a dot line. Bold characters in copeptin suggests leucine-rich core
458 segment.

459 Fig. 3. Comparison of the deduced amino acid sequence of isoticin (IT) of honbera wrasse
460 (upper) with that of threespot wrasse (GenBank accession no. GU212658), European
461 flounder (AB036518), and grass puffer (AB297919), mesotocin (MT) of chicken
462 (AB194408) and toad (M126232), and oxytocin (OXT) of human (NM000915) and rat
463 (NM0011025). The alignment was generated by ClustalW. Amino acid residues
464 conserved in all vertebrates are marked with an asterisk. Signal peptide, hormone, and
465 neurophysin are indicated by solid lines, while copeptin by a dot line. Bold characters
466 in copeptin suggests leucine-rich core segment.

467 Fig. 4. Variations of mRNA abundance of pro-AVT and pro-IT in the diencephalon of the
468 honbera wrasse under light-dark cycle (A) and constant light (B). After acclimation
469 under conditions of light-dark cycle (LD 12:12) and constant light (LD), the brain of
470 fish was sampled at 12:00 (ZT6, n = 10) and 00:00 (ZT18, n= 10) for LD and 12:00 (n
471 = 10) and 00:00 (n = 10) for LL. Following total RNA isolation and reverse-transcription,

mRNA abundance of pro-AVT and pro-IT in each sample was measured real-time qPCR, normalized against mRNA abundance of β-actin, and then averaged. Values are expressed as means ± standard error of the mean (SEM). Horizontal bar with white and block colors in each figure indicates light and dark phases, respectively. Asterisk represents statistical difference at $P < 0.05$.

Fig. 5. Effects of a single melatonin injection on mRNA abundance of pro-AVT (A) and pro-IT (B) in the diencephalon of the honbera wrasse. Melatonin was intraperitoneally injected to the experimental fish group (n = 6) at a concentration of 1 mg/kg body weight. The vehicle was injected to the control fish group (n = 6). Three hours after injection, the diencephalon was sampled and used for total RNA isolation and reverse-transcription. The mRNA abundance of pro-AVT and pro-IT in each sample was measured real-time qPCR, normalized against mRNA abundance of β-actin, and then averaged. Values are expressed as means ± standard error of the mean (SEM). Asterisk represents statistical difference at $P < 0.05$.

Fig. 6. Day-night variations of mRNA abundance of pro-AVT (A) and pro-IT (B) in the diencephalon of the honbera wrasse at different water temperatures. Under programmable light-dark cycle (LD 12:12), fish were acclimated at 15, 20, and 25 ^{o}C, and then sampled at 12:00 h (ZT6, n = 6) and 00:00 h (ZT18, n= 6). Following total RNA isolation and reverse-transcription, mRNA abundance of pro-AVT and pro-IT in each sample was measured real-time qPCR, normalized against mRNA abundance of β-actin, and then averaged. Values are expressed as means ± standard error of the mean (SEM). White and black columns indicate 12:00 h and 00:00 h, respectively. Different alphabet represents statistical difference at $P < 0.05$.

Fig. 7. Seasonal changes in water temperature and numbers of individuals emerging from the sandy bottom of the northern shore of Jeju Island, Korea, during daytime. Fish around

497 divers were counted three times between 11:00 h and 12:00 h and their number at
498 observatory points was averaged. Values are expressed as means ± standard error of the
499 mean (SEM).
500

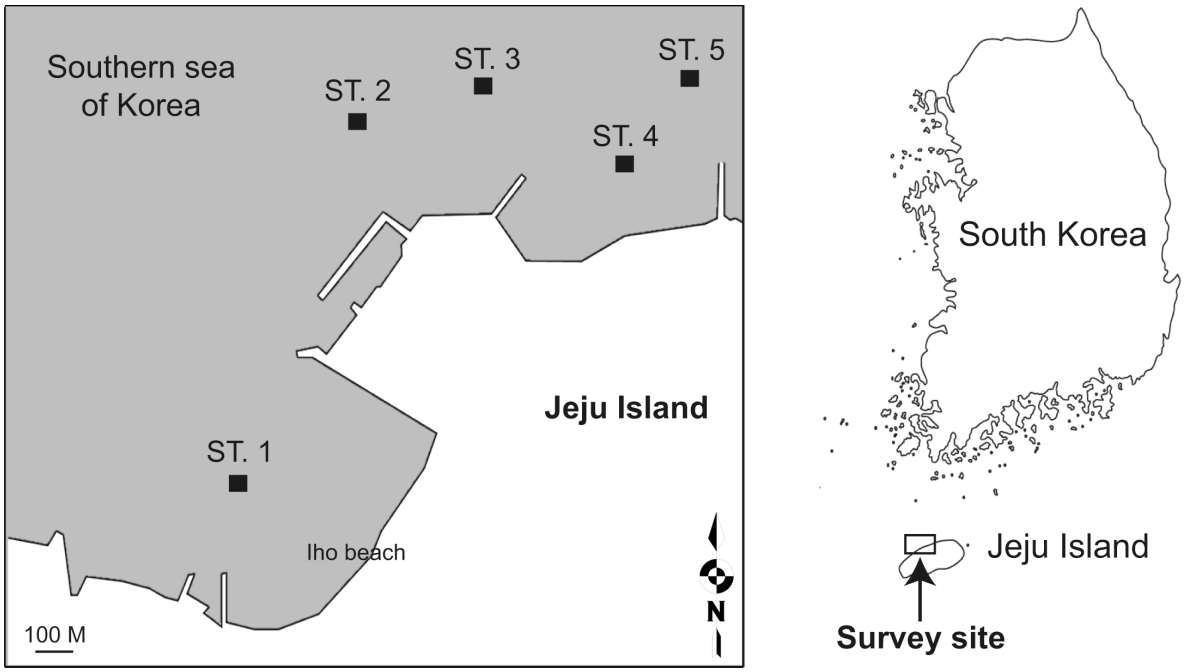

Southern sea
of Korea
ST. 2
ST. 3
ST. 5
ST. 4
Jeju Island
ST. 1
Iho beach
100 M
N
South Korea
Jeju Island
Survey site

Figure 1

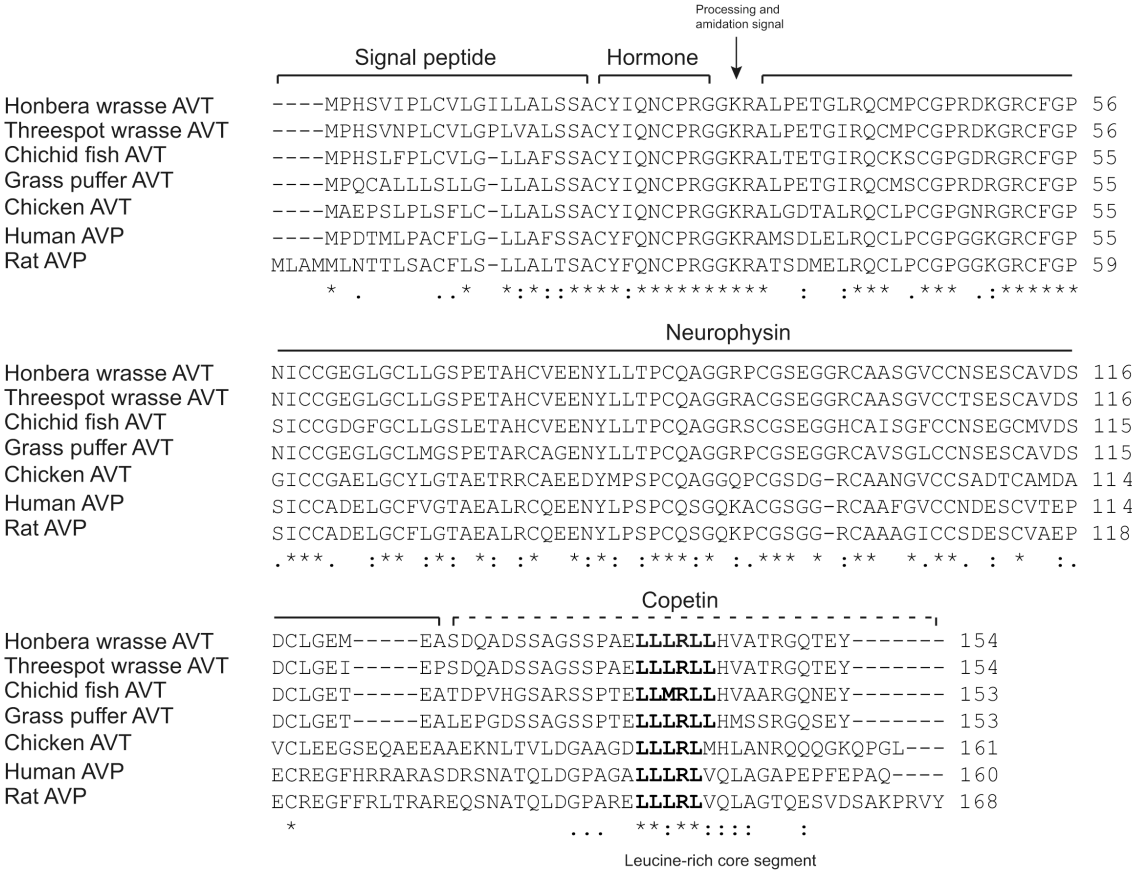

Processing and amidation signal
Signal peptide
Hormone
Honbera wrasse AVT ----MPHSVIPLCVLGILLALSSACYIQNCPRGGKRALPETGLRQCMPCGPRDKGRCFGP 56
Threespot wrasse AVT ----MPHSVNPLCVLGPLVALSSACYIQNCPRGGKRALPETGIRQCMPCGPRDKGRCFGP 56
Chichid fish AVT ----MPHSLFPLCVLG-LLAFSSACYIQNCPRGGKRALTETGIRQCKSCGPGDRGRCFGP 55
Grass puffer AVT ----MPQCALLLSLLG-LLALSSACYIQNCPRGGKRALPETGIRQCMSCGPRDRGRCFGP 55
Chicken AVT ----MAEPSLPLSFLC-LLALSSACYIQNCPRGGKRALGDTALRQCLPCGPGNRGRCFGP 55
Human AVP ----MPDTMLPACFLG-LLAFSSACYFQNCPRGGKRAMSDLELRQCLPCGPGGKGRCFGP 55
Rat AVP MLAMMLNTTLSACFLS-LLALTSACYFQNCPRGGKRATSDMELRQCLPCGPGGKGRCFGP 59
Neurophysin
Honbera wrasse AVT NICCGEGLGCLLGSPETAHCVEENYLLTPCQAGGRPCGSEGGRCAASGVCCNSESCAVDS 116
Threespot wrasse AVT NICCGEGLGCLLGSPETAHCVEENYLLTPCQAGGRACGSEGGRCAASGVCCTSESCAVDS 116
Chichid fish AVT SICCGDGFGCLLGSLETAHCVEENYLLTPCQAGGRSCGSEGGHCAISGFCCNSEGCMVDS 115
Grass puffer AVT NICCGEGLGCLMGSPETARCAGENYLLTPCQAGGRPCGSEGGRCAVSGLCCNSESCAVDS 115
Chicken AVT GICCGAELGCYLGTAETRRCAEEDYMPSPCQAGGQPCGSDG-RCAANGVCCSADTCAMDA 114
Human AVP SICCADELGCFVGTAEALRCQEENYLPSPCQSGQKACGSGG-RCAAFGVCCNDESCVTEP 114
Rat AVP SICCADELGCFLGTAEALRCQEENYLPSPCQSGQKPCGSGG-RCAAAGICCSDESCVAEP 118
Copetin
Honbera wrasse AVT DCLGEM-----EASDQADSSAGSSPAELLLRLLHVATRGQTEY------- 154
Threespot wrasse AVT DCLGEI-----EPSDQADSSAGSSPAELLLRLLHVATRGQTEY------- 154
Chichid fish AVT DCLGET-----EATDPVHGSARSSPTELLMRLLHVAARGQNEY------- 153
Grass puffer AVT DCLGET-----EALEPGDSSAGSSPTELLLRLLHMSSRGQSEY------- 153
Chicken AVT VCLEEGSEQAEEAAEKNLTVLDGAAGDLLLRLMHLANRQQQGKQPGL--- 161
Human AVP ECREGFHRRARASDRSNATQLDGPAGALLLRLVQLAGAPEPFEPAQ---- 160
Rat AVP ECREGFFRLTRAREQSNATQLDGPARELLLRLVQLAGTQESVDSAKPRVY 168
Leucine-rich core segment

Figure 2

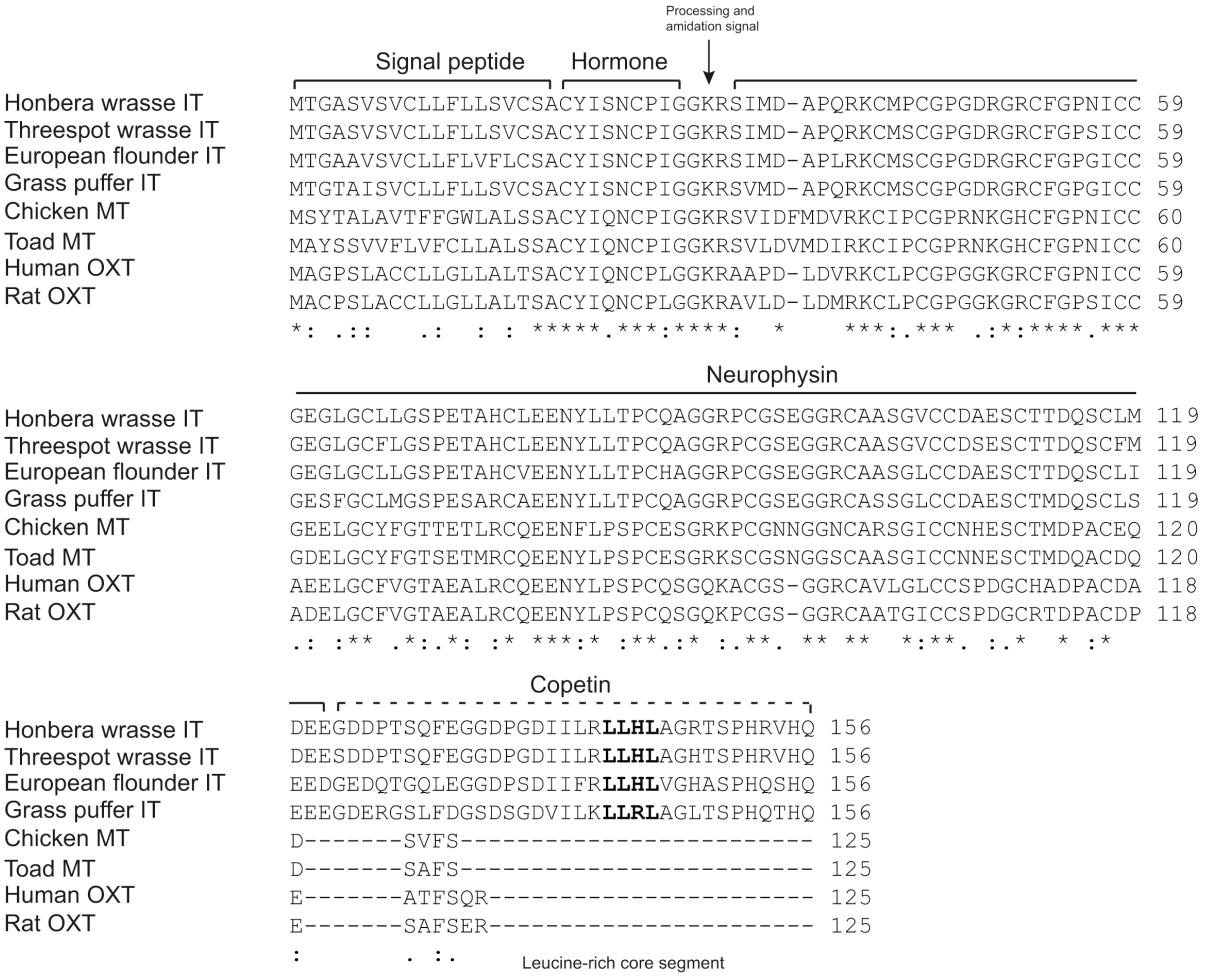

Processing and amidation signal
Signal peptide
Hormone
Honbera wrasse IT MTGASVSVCLLFLLSVCSACYISNCPIGGKRSIMD-APQRKCMPCGPGDRGRCFGPNICC 59
Threespot wrasse IT MTGASVSVCLLFLLSVCSACYISNCPIGGKRSIMD-APQRKCMSCGPGDRGRCFGPSICC 59
European flounder IT MTGAAVSVCLLFLVFLCSACYISNCPIGGKRSIMD-APLRKCMSCGPGDRGRCFGPGICC 59
Grass puffer IT MTGTAISVCLLFLLSVCSACYISNCPIGGKRSVMD-APQRKCMSCGPGDRGRCFGPGICC 59
Chicken MT MSYTALAVTFFGWLALSSACYIQNCPIGGKRSVIDFMDVRKCIPCGPRNKGHCFGPNICC 60
Toad MT MAYSSVVFLVFCLLALSSACYIQNCPIGGKRSVLDVMDIRKCIPCGPRNKGHCFGPNICC 60
Human OXT MAGPSLACCLLGLLALTSACYIQNCPLGGKRAAPD-LDVRKCLPCGPGGKGRCFGPNICC 59
Rat OXT MACPSLACCLLGLLALTSACYIQNCPLGGKRAVLD-LDMRKCLPCGPGGKGRCFGPSICC 59
Neurophysin
Honbera wrasse IT GEGLGCLLGSPETAHCLEENYLLTPCQAGGRPCGSEGGRCAASGVCCDAESCTTDQSCLM 119
Threespot wrasse IT GEGLGCFLGSPETAHCLEENYLLTPCQAGGRPCGSEGGRCAASGVCCDSESCTTDQSCFM 119
European flounder IT GEGLGCLLGSPETAHCVEENYLLTPCHAGGRPCGSEGGRCAASGLCCDAESCTTDQSCLI 119
Grass puffer IT GESFGCLMGSPESARCAEENYLLTPCQAGGRPCGSEGGRCASSGLCCDAESCTMDQSCLS 119
Chicken MT GEELGCYFGTTETLRCQEENFLPSPCESGRKPCGNNGGNCARSGICCNHESCTMDPACEQ 120
Toad MT GDELGCYFGTSETMRCQEENYLPSPCESGRKSCGSNGGSCAASGICCNNESCTMDQACDQ 120
Human OXT AEELGCFVGTAEALRCQEENYLPSPCQSGQKACGS-GGRCAVLGLCCSPDGCHADPACDA 118
Rat OXT ADELGCFVGTAEALRCQEENYLPSPCQSGQKPCGS-GGRCAATGICCSPDGCRTDPACDP 118
Copetin
Honbera wrasse IT DEEGDDPTSQFEGGDPGDIILRLLHLAGRTSPHRVHQ 156
Threespot wrasse IT DEESDDPTSQFEGGDPGDIILRLLHLAGHTSPHRVHQ 156
European flounder IT EEDGEDQTGQLEGGDPSDIIFRLLHLVGHASPHQSHQ 156
Grass puffer IT EEEGDERGSLFDGSDSGDVILKLLRLAGLTSPHQTHQ 156
Chicken MT D-------SVFS---------- 125
Toad MT D-------SAFS---------- 125
Human OXT E-------ATFSQR---------- 125
Rat OXT E-------SAFSER---------- 125
Leucine-rich core segment

Figure 3

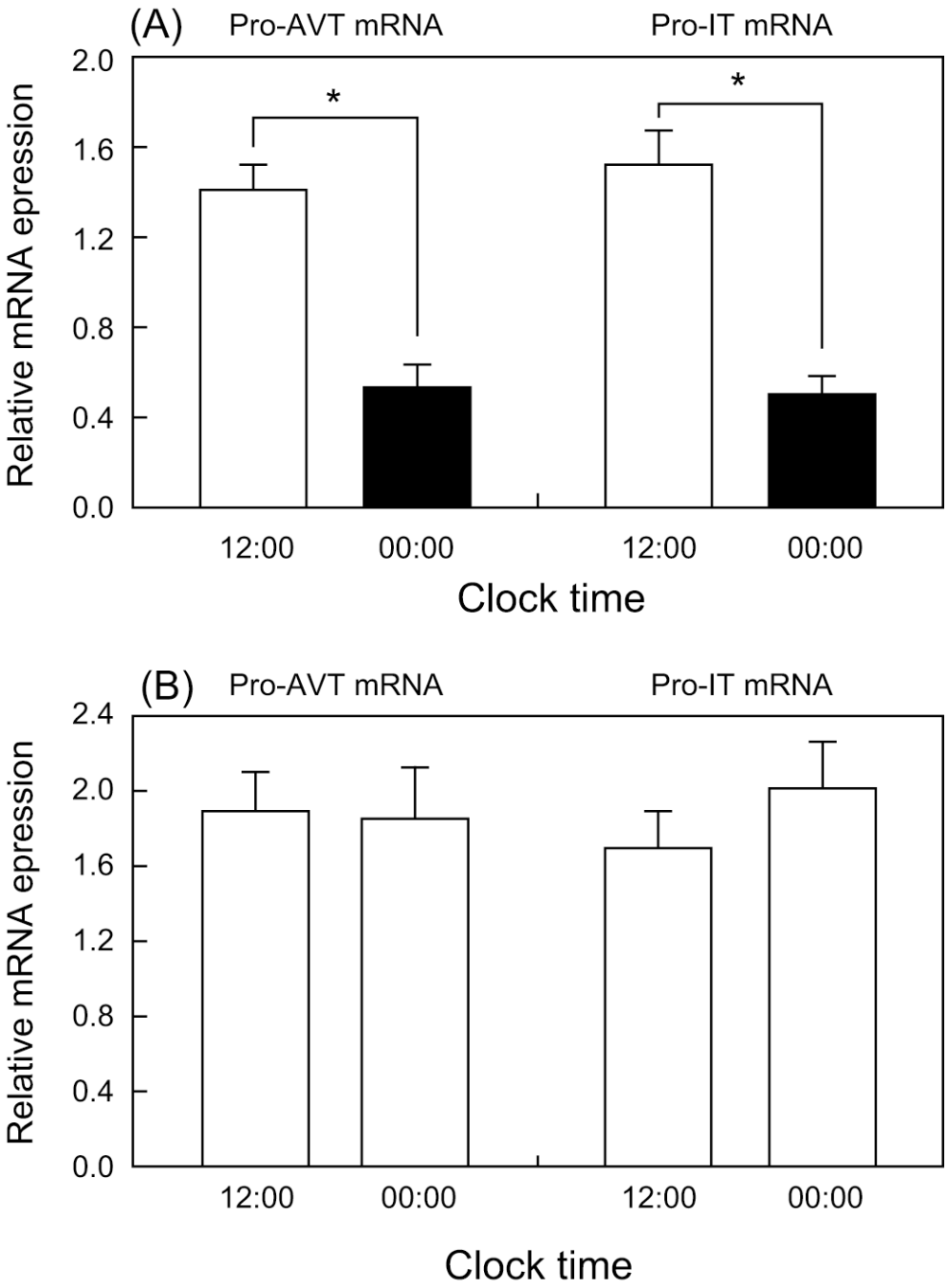

(A)
Pro-AVT mRNA
Pro-IT mRNA
Relative mRNA epression
2.0
1.6
1.2
0.8
0.4
0.0
*
*
12:00
00:00
12:00
00:00
Clock time
(B)
Pro-AVT mRNA
Pro-IT mRNA
Relative mRNA epression
2.4
2.0
1.6
1.2
0.8
0.4
0.0
12:00
00:00
12:00
00:00
Clock time

Figure 4

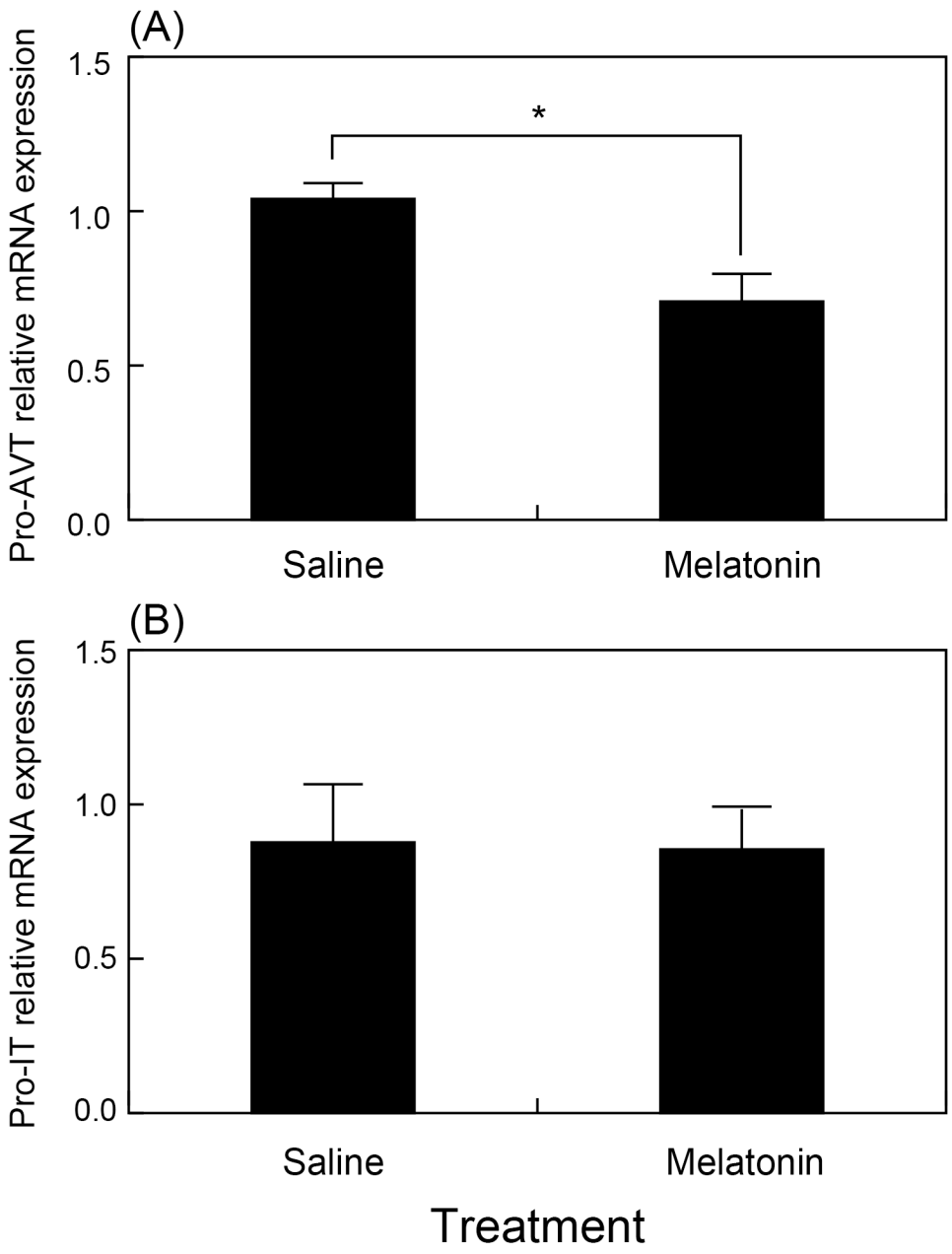

(A)
Pro-AVT relative mRNA expression
1.5
1.0
0.5
0.0
*
Saline
Melatonin
(B)
Pro-IT relative mRNA expression
1.5
1.0
0.5
0.0
Saline
Melatonin
Treatment

Figure 5

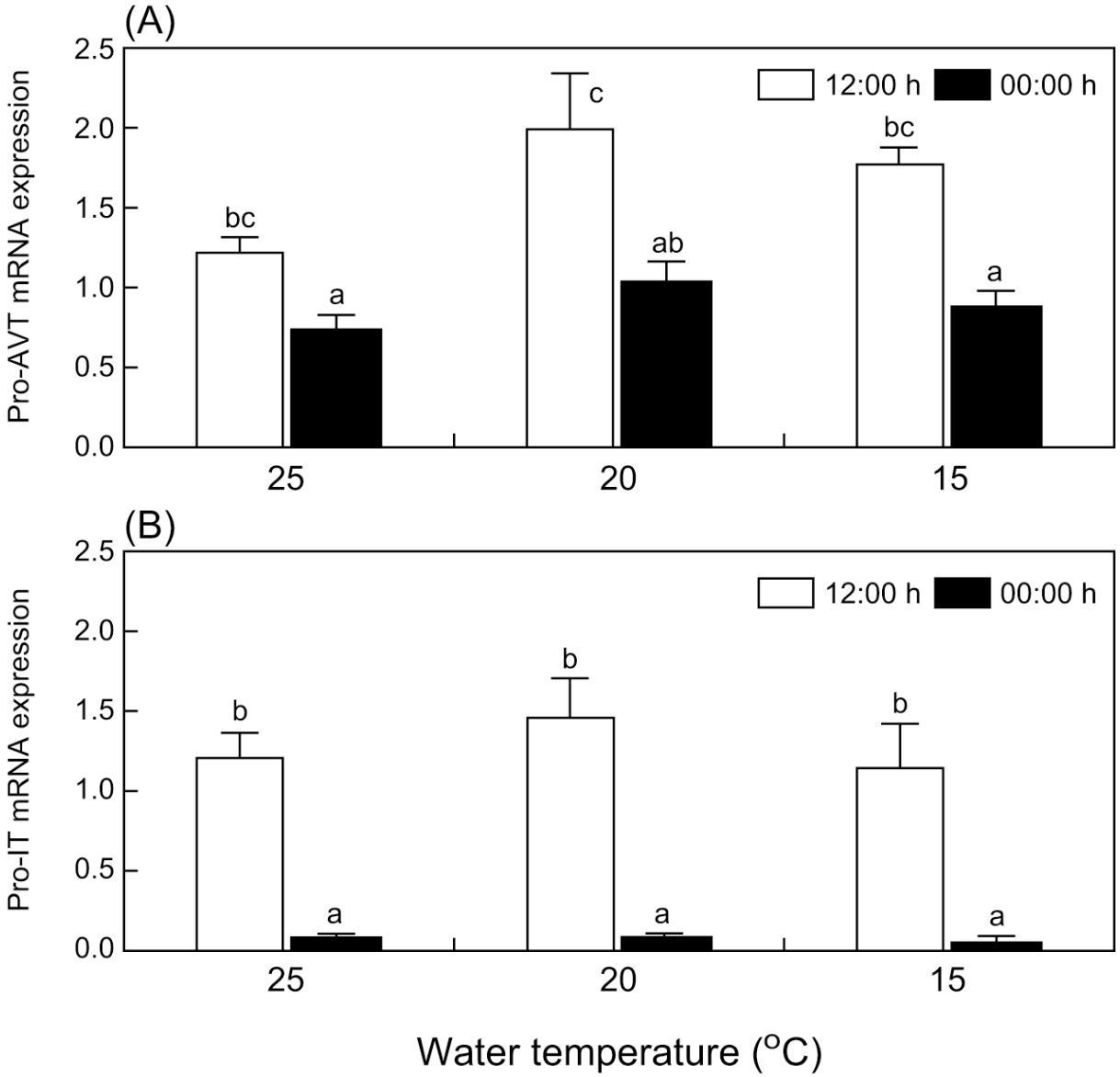
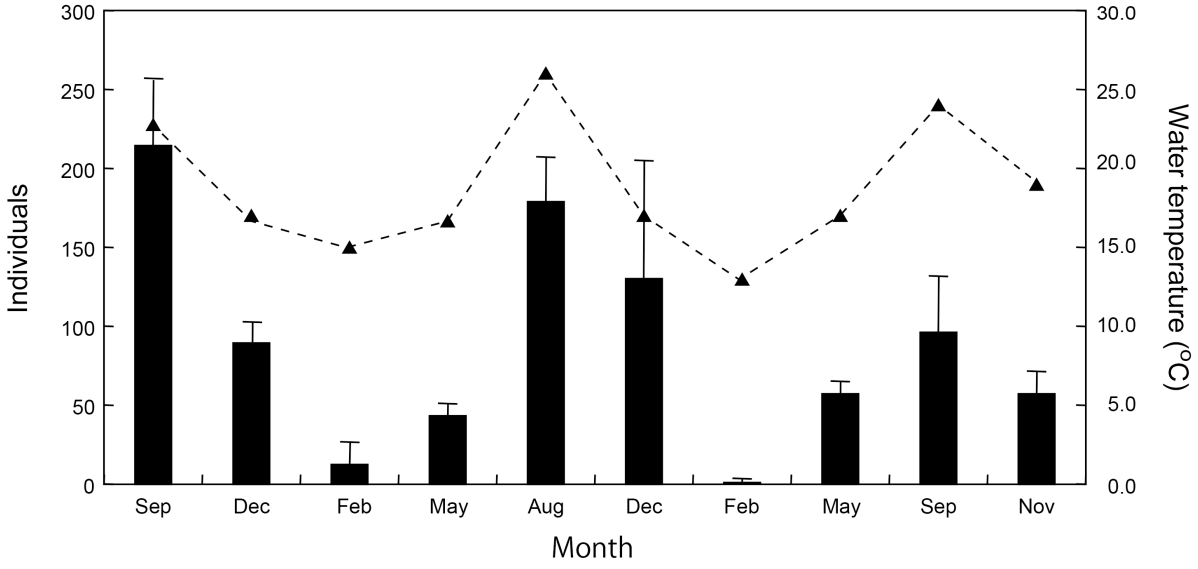

(A)
Pro-AVT mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
12:00 h
00:00 h
bc
a
c
ab
bc
a
25
20
15
(B)
Pro-IT mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
12:00 h
00:00 h
b
a
b
a
b
a
25
20
15
Water temperature (°C)

Figure 6


Individuals
Water temperature (°C)
Month
300
250
200
150
100
50
0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Sep
Dec
Feb
May
Aug
Dec
Feb
May
Sep
Nov

]

Figure 7

Table 1. Primer sets used in the present study.

Primer	Sequence
Cloning primer for partial	
AVT-Forward	5'-GAC AGT GCA TGT CGT GYG-3'
AVT-Reverse	5'-ARC TCT CTG TGT TAC AGC AG-3'
IT-Forward	5'-ACA TCT CCA ACT GTC CCA TC-3'
IT-Reverse	5'-GCA GGA GCT TCA GGA TGA C-3'
Cloning primer for RACE PCR	
AVT-GSP1	5'-TCC CTC AGA TCC ACA GGC TCT ACC TC-3'
AVT-GSP2	5'-AGT GCA TGT CGT GTG GTC CCA GAG-3'
AVT-NGSP1	5'-GGG GTG AGC AGG TAG TTC TCC TCC A-3'
AVT-NGSP2	5'-CCC AAT ATC TGC TGT GGG GAA GGT C-3'
IT-GSP1	5'-TGG CAG GGG GTG AGC AGG TAG TTC T-3'
IT-GSP2	5'-GAG GTC AAT CAT GGA CGC ACC TCA G-3'
IT-NGSP1	5'-CAG CAG ATA CTG GGG CCA AAG CAG-3
IT-NGSP2	5'-GCT GCT TTG GCC CCA GTA TCT GCT-3'
RT-PCR primer	
AVT-PCR-Forward	5'-CAC TCC GTG AAC CCT CTG TG-3'
AVT-PCR-Reverse	5'-GTG GAA CAG GGA TGG TCT TC-3'
IT-PCR-Forward	5'-GTG TCC GTG TGC CTT CTT TT-3'
IT-PCR-Reverse	5'-TCA GCA TCA CAG CAG ACT CC-3'
β-actin-PCR-Forward	5'-ACT ACC TCA TGA AGA TCC TG-3'
β-actin-PCR-Reverse	5'-TTG CTG ATC CAC ATC TGC TG-3'
Real-time qPCR primer	
AVT-qPCR-Forward	5'-GAC AGG GAT CAG ACA GTG CAT-3'
AVT-qPCR-Reverse	5'-CTC CTC CAC ACA GTG AGC TG-3'
IT-qPCR-Forward	5'-CAC AGC GCA AGT GCA TGT-3'
IT-qPCR-Reverse	5'-CCA AAC AGT GGG CTG TCT CT-3'
β-actin-qPCR-Forward	5'-GAG ATT GTG CGT GAC ATC AAG GAG-3'
β-actin-qPCR-Reverse	5'-CAT CAG GCA GCT CGT AGC TCT TC-3'