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Development of gas exchange and ion regulation in two species of air-breathing fish, *Betta splendens* and *Macropodus opercularis*

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ABSTRACT

Aquatic air-breathing anabantoids, a group of fish species characterized by the presence of a labyrinth organ and some gills, exhibit morphological variations. This study aimed to examine whether unequal gill growth begins during the early stages and described the sequence of the early gill developmental events in *Betta splendens* and *Macropodus opercularis*. To determine when the ion regulatory and gas exchange abilities first appear in the gills, mitochondria-rich cells (MRCs) and neuroepithelial cells (NECs) were examined in young *B. splendens*. To evaluate the relative importance of the gills and the labyrinth organ under different levels of oxygen uptake stress, the levels of carbonic anhydrase II (CAII) and Na^+/K^+–ATPase (NKA) protein expressions in 2 gills and the labyrinth organ were examined in *M. opercularis*. We found that the first 3 gills developed earlier than the 4th gill in both species, an indication that the morphological variation begins early in life. In *B. splendens*, the MRCs and NECs clearly appeared in the first 3 gills at 4 dph and were first found in the 4th gill until 11 dph. The oxygen-sensing ability of the gills was concordant with the ionoregulatory function. In *M. opercularis*, the hypoxic group had a significantly higher air-breathing frequency. CAII protein expression was higher in the labyrinth organ in the hypoxic group. The gills exhibited increased NKA protein expression in the hypoxic and restricted groups, respectively. Functional plasticity in CAII and NKA protein expressions was found between the gills and the labyrinth organ in adult *M. opercularis*.

1. Introduction

Aquatic air-breathing fish are equipped with various accessory air-breathing organs, such as the labyrinth organ, lungs, respiratory gas bladders, digestive tracts, and structures derived from the buccal, pharyngeal, and branchial cavities (Graham, 1997). All species of the suborder Anabantoidei are aquatic air-breathing fishes and have a labyrinth organ protruding from the 1st gill arch on both sides of the branchial cavity to assist in gas exchange (Munshi et al., 1986; Graham, 1997). Previous studies on anabantoid fishes have primarily focused on their vascular organization. With the preparation of cardiac and/or vascular casts, it is evident that anabantoid fishes possess branchial and systemic circuits that are almost analogous to the double circulatory system (Munshi et al., 1986; Olson et al., 1986, 1994, 1995; Olson, 2002). That is, the anterior (1st and 2nd) gills receive blood from the heart and are the site for gas exchange (particularly CO_2 excretion); the blood then flows to the labyrinth organ for further oxygen uptake before returning to the heart. Enlarged vessels in the posterior (3rd and 4th) gills carry the oxygenated blood from the heart to the systemic circulatory system (Munshi et al., 1986, 2001; Olson et al., 1986).

Fish gills are responsible for performing gas exchange and ion regulation (Perry, 1998; Hirose et al., 2003; Evans et al., 2005; Hwang and Lee, 2007). There are 4 major types of cells in the gill epithelia: pavement cells, mitochondria-rich cells (MRCs), mucous cells and undifferentiated cells (Perry, 1997; Evans, 1999). Na^+/K^+–ATPase (NKA), a membrane-spanning enzyme in MRCs, is critical to maintain ion homeostasis (Evans et al., 2005). Under ionic stress, the gills can increase ion uptake and/or excretion in adults (Greco et al., 1996). In addition, the skin MRCs in both tilapia (*Oreochromis niloticus*) and Japanese flounder (*Paralichthys olivaceus*) are also involved in an ion regulatory mechanism during early development (Hiroi et al., 1998; Fridman et al., 2011).

In the present study, the MRCs of the gills and skin were examined during early development. It has recently been proposed that neuroepithelial cells (NECs) behave as an oxygen chemoreceptor and

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are involved in respiratory regulation in the fish gills (Jonz and Nurse, 2003; Regan et al., 2011). NECs are distributed in the efferent filament region between the leading edge and the filament artery (Jonz and Nurse, 2003, 2006). In mammals, this type of cell contains 5-HT, which is the neurotransmitter, serotonin, within cytoplasmic synaptic vesicles that is highly conserved among mammalian O_2 chemoreceptors (carotid body) (Jonz and Nurse, 2005, 2008). Therefore, NECs might play a role in sensing changes in the dissolved oxygen level in fish (Jonz et al., 2004; Jonz and Nurse, 2006; Saltys et al., 2006; Regan et al., 2011). The ontogeny of the NECs in the gills of the hypoxia-tolerant anabantoid fish may be an indication of the initiation of gas exchange at early life stages. By examining the first appearance of the MRCs and NECs in the gills, we may determine the timing of the ion or respiratory regulation functions in aquatic air-breathing fish.

Hypoxia represents a well-known environmental stressor, and fish have a variety of levels of physiological and molecular responses to this pressure. The fish gills can compensate for changes in ambient oxygen levels by undergoing an extensive modification, as observed in the crucian carp (*Carassius carassius*) (Sollid and Nilsson, 2006; Nilsson, 2007). The 1st gill of *Trichogaster lalius* can compensate for pH changes by both morphological and biochemical adjustments (Huang and Lin, 2011a). Several studies used carbonic anhydrase II (CAII), an enzyme that catalyzes the rapid and reversible hydration and dehydration of CO_2, to evaluate the gas exchange ability of the fish gills at different developmental stages in either aerial or aquatic environments (Henry and Swenson, 2000; Gilmour et al., 2009). In the present experiment, we used CAII and NKA to evaluate the function of gas exchange and ion regulation between the gills and the labyrinth organ under oxygen uptake stresses.

In two of our previous studies on air-breathing fishes, we used 12 species from three families and nine genera of Anabantoidei to examine the morphological and biochemical differences among the gills (Huang et al., 2008, 2011b). In the present study, we chose two species to contrast their gill development. *Betta splendens* showed no apparent morphological difference among the gills, whereas *Macropodus opercularis* differed in both filaments and lamellae among the gills, with a smaller length in the 4th gill (Huang et al., 2011b). Our study addresses three experimental objectives. First, we obtained detailed descriptions of the sequence of developmental events in the gills of *B. splendens* and *M. opercularis* and found unequal gill growth in the early stages. Second, we tested whether ion regulation appeared before gas exchange in the gills of *B. splendens*. The quantitative trade-off between the MRCs (NKA-IR cells, NKA-immunoreactive cells) and the NECs (5-HT-IR cells, 5-HT-immunoreactive cells) was examined in the gills and three skin regions in young *B. splendens*. Third, we examined the biochemical responses of CAII and NKA among the 1st gill, 4th gill, and labyrinth organ in adult *M. opercularis* after acclimatization to hypoxic and restricted treatments.

2. Materials and methods

2.1. Animals

B. splendens and *M. opercularis* are aquatic air-breathing fish found primarily in southern Malaysia, Thailand, Singapore, and Vietnam. Both species can colonize stagnant water bodies with very low oxygen contents and can be found in streams, paddy fields and ditches. The experimental water temperature was maintained at 28 ± 1 °C with a 12L:12D photoperiod. Water was changed at least once every week. The dissolved oxygen levels were monitored (Orion model 810, UK) and maintained at 7.48 ± 0.46 mg/L, and the pH was maintained at 7.31 ± 0.24 (Jenco, pH vision 6071, HK). The animals were kept in compliance with the current laws in Taiwan.

2.2. Experimental designs

The first aim of this study was to provide a detailed description of the early development of the gills in *B. splendens* and *M. opercularis*. Gill morphology was examined by histological sections and scanning electron microscopy. The larvae were sampled and examined at 1, 2, 3, 4, 5, 7, 9, 11, 15, and 21 dph (days of post hatching).

The second part of this study examined the timing of the first appearance of the MRCs (NKA-IR) and the NECs (5-HT-IR cells) in the gills and three skin regions (yolk sac, trunk, and tail regions) of *B. splendens*. The gill size of this species was suitable for the examination of the MRCs and the NECs. The larvae were sampled and examined only at 1, 4, 7, 11, 15, and 21 dph. In addition, NKA-IR cells were also quantified in three different skin regions. By simultaneously examining the NKA-IR cells in the gills and the NKA-IR cells in the skin, the transitions of the numbers of the MRCs between the skin and the gills in the early stages of development were confirmed.

The third part of this study examined the response of the 1st and 4th gills and the labyrinth organ to oxygen stresses in adult *M. opercularis*. This species has an apparent morphological variation in the 4th gill, which is large enough to extract protein from the gills and the labyrinth organ. The experimental treatments included the control (aquatic normoxia and air-breathing allowed), hypoxic (aquatic hypoxia and air-breathing allowed), and restricted (aquatic normoxia and air-breathing restricted) groups. The fish in the restricted group were restricted from performing aerial gas exchange by submerging the fish cage. The tissues of the 1st gill, 4th gill, and the labyrinth organ were sampled 7 d after being transferred to the designated treatment conditions. The relative abundances of CAII and NKA serve as indicators of gas exchange and ion regulation performance, respectively. Sample sizes were 8 fish at each sampling time.

2.3. Egg collection and maintenance

To obtain fertilized eggs, one adult male [4.6 to 5.3 mm in standard length (LS) in *B. splendens* and 5.3 to 6.4 mm in standard length (LS) in *M. opercularis*] and one adult female [4.3 to 4.9 mm LS in *B. splendens* and 4.2 to 5.1 mm LS in *M. opercularis*] were transferred to a spawning tank (58.0 × 41.0 × 34.5 cm) with plants (*Spirodela polyrrhiza*) at the water surface to provide shelter for the fish. After spawning, the fertilized eggs were transferred to another aquarium (27 × 17 × 11 cm) in the incubator (orbital shaking incubator, TKS, Japan). The temperature of the aerated water was controlled at 29 °C for the experimental procedure.

2.4. Scanning electron microscopy

Larvae at different dph were anesthetized with 0.4 g/L MS-222 (3-aminobenzoic acid ethyl ester, Sigma, USA) and prefixed in a phosphate buffer (0.1 M $NaH_2PO_4 \cdot 2H_2O$ + 0.1 M $Na_2HPO_4 \cdot 2H_2O$, pH = 7.2) with 2% paraformaldehyde and 0.5% glutaraldehyde for 8 to 12 h at 4 °C, pH 7.4. These samples were rinsed three times with 0.1 M phosphate buffer before being post-fixed with phosphate-buffered 1% osmium tetroxide for 1 h. The tissues were dehydrated in 7 ascending concentrations of ethanol (30% to 100%) and in 100% acetone and subsequently dried using a critical-point drier (HCP-2, Hitachi, Japan). After sputter coating for 3 min with a gold–palladium complex using an ion coater (Eiko IB-2, Hitachi, Japan), the gills were examined under a scanning electron microscope (S-2300, Hitachi, Japan). All chemicals used in the experiment were obtained from Sigma (USA) and Merck (Germany).

2.5. Histological sections

Larvae at different dph were anesthetized with MS-222, sacrificed and fixed in Bouin's solution (Sigma, USA) for 48 h at 4 °C. These

samples were washed with 70% ethanol several times, followed by an ethanol–xylene series for dehydration. After paraffin embedding, tissue sections were prepared at a thickness of 10 μm (RM2025RT, Leica, Germany) and placed on slides that were pre-coated with poly-L-lysine solution. The samples were de-waxed and rehydrated before staining with hematoxylin and eosin. They were dehydrated again, mounted, and examined under a light microscope (E600, Nikon, Japan).

2.6. Immunohistological staining

All the samples that were placed on slides pre-coated with poly-L-lysine solution (Sigma) were de-waxed and rehydrated. They were subsequently immersed in 3% H_2O_2 (in 100% methanol) for 10 min to remove any endogenous reaction, followed by three 5 min rinses in PBS (containing in mM: NaCl 136.9, KCl 2.68, $Na_2HPO_4 \cdot 2H_2O$ 6.39, and KH_2PO_4 1.76, pH 7.4). The samples were incubated with an NKA primary antibody (α5, Na^+/K^+–ATPase monoclonal antibody, Developmental Studies Hybridoma Bank, University of Iowa, USA, 1:10,000) for 1 h in the dark at room temperature (RT, 26 °C). After three more rinses with PBS for 5 min each, the samples were incubated with the secondary antibody (HRP/Fab polymer conjugate, Zymed) and the color reagent (aminoethyl carbazole signal solution chromogen (AEC kit), Zymed) for 30 and 15 min, respectively. Finally, the samples were stained with hematoxylin (Zymed) for 2 min. The samples were mounted (GVA mounting solution, Zymed) and examined using a light microscope (Eclipse E600, Nikon, Japan). Pictures were taken using a digital camera (D1, Nikon, Japan). The slides of the negative control without the application of the primary antibody were prepared simultaneously.

2.7. Whole mount immunofluorescence staining

The larvae were prefixed in a phosphate buffer (0.1 M $NaH_2PO_4 \cdot 2H_2O$ + 0.1 M $Na_2HPO_4 \cdot 2H_2O$, pH = 7.2), 2% paraformaldehyde and 0.5% glutaraldehyde for 3 h at 4 °C, pH 7.4. After three 5 min washes with PBS, fixed larvae were maintained in a solution (PBST) containing 1% bovine serum antigen (BSA) and 0.5% Tween 20 in PBS (pH 7.8) at 4 °C for 48–72 h. This step will improve the permeability of the epithelial tissue for later uptake of NKA and 5-HT. After three 5 min washes with PBS, the samples were incubated with 5% BSA for 2 h to block nonspecific binding. The samples were then kept for 24 h at 4 °C with a primary antibody against NKA (α5 monoclonal antibody; 1:400, ATPase, Na^+, K^+ alpha-subunit, cytosolic epitope for all isoforms, DSHB, USA) and 5-HT (polyclonal antibody, 1:200, serotonin, Sigma, USA). The antibody of NKA was used to examine cell distribution in several species, such as medaka (*Oryzias dancena*) and zebrafish (*Danio rerio*) (Pan et al., 2005; Yang et al., 2013). After washing with PBS three times for 30 min, larvae were further incubated in goat anti-mouse secondary antibody conjugated to IgG Alexa Fluor 488 (1:400, Molecular Probes, USA) or goat anti-rabbit secondary antibody conjugated to fluorescein isothiocyanate (FITC, 1:200, Jackson ImmunoResearch Laboratories Inc., USA) for 2 h at RT in darkness. After washing with PBS three times for 1 h, the whole-mount larvae preparations were examined in the longitudinal plane using an upright microscope (Eclipse E600, Nikon, USA) and a confocal scanning system (LSM 510, Zeiss confocal laser scanning microscope, Germany). The confocal image software was Zeiss LSM image browser. The slides of the negative control without the application of the primary antibody were prepared simultaneously.

2.8. The density of MRCs

Eight larvae were randomly chosen to examine the density of NKA-IR cells in three different regions per larva, and each region represented 250 × 250 μm on the skin surface.

2.9. Protein extraction and relative abundance of CAII and NKA

Two procedures performed in this study were the same as in previous studies by our group (Huang and Lin, 2011a,b, Huang et al., 2011b), unless otherwise noted.

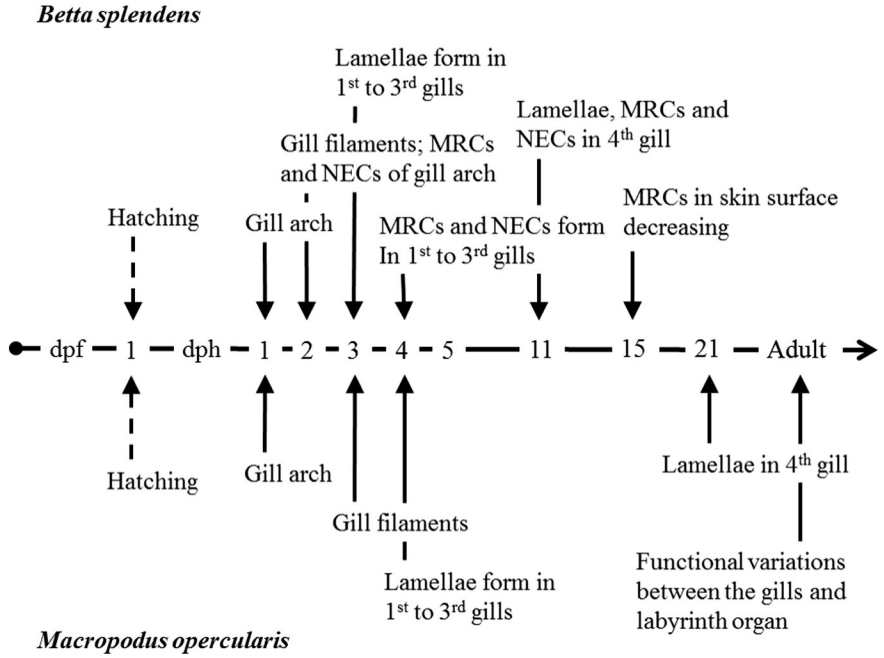

Fig. 1. The schematic diagram of the growth of the gills was summarized in the early stage of *Betta splendens* and *Macropodus opercularis*. dpf: days post-fertilization; dph: days post-hatching.

2.10. Effect of hypoxia on air-breathing frequency

For the hypoxic treatment, 100% nitrogen was continuously bubbled through the water. After a two-day period of acclimation to normoxic conditions, adult fish were videotaped (DCR-HC 46, Sony, Japan) to record their air-breathing frequency. Subsequently, the fish were transferred to either normoxic or hypoxic water, and the air-breathing frequency was recorded over a standardized 45 min period for the following 2 d (N = 7–9). The oxygen level was 1.85 ± 0.37 mg/L, and the pH was 7.57 ± 0.14. The water temperature was controlled at 28 °C.

2.11. Statistical analyses

All the data are presented as the mean ± SEM. Two-way ANOVA for multiple comparisons was conducted to analyze the relative abundance of CAII and NKA between days and tissues. If a significant effect was detected, Tukey's test was used to examine the further differences. Student's t-test was used to compare the difference in the air-breathing frequencies between the normoxic and hypoxic groups; $p < 0.05$ was considered statistically significant. All statistical analyses were conducted using SAS 8e for Windows (SAS Institute, Cary, NC, USA).

3. Results

3.1. Description of a growing gill

The developmental processes of the gills are summarized in Fig. 1. In both *B. splendens* and *M. opercularis* at 7 dph, filaments and lamellae were found in the first 3 gills but not in the 4th gill [Figs. 2 and 3(A and E)]. In *B. splendens*, lamellae were observed for the first time in the 4th gill between 11 and 15 dph [Figs. 2 and 3(B to C)]. At 21 dph, the 4th gill had a similar appearance to the first 3 gills [Figs. 2 and 3(D)]. In *M. opercularis*, filaments and lamellae were present in the first 3 gills but not in the 4th gill at 7 dph [Figs. 2 and 3(E)]. Lamellae were not observed in the 4th gill until 21 dph [Figs. 2 and 3(F, G, H)].

3.2. MRCs and NECs in the gills or skin surface

The MRCs and the NECs in the gills or skin surface were examined at 1, 11, and 21 dph in *B. splendens*. At 1 dph, there were no NKA-IR cells in any gill arch (Fig. 4A); the NKA-IR cells were found in the trunk (Fig. 4B), yolk sac (Fig. 4C), and tail regions (Fig. 4D). There were no 5-HT-IR cells in any gill arch (Fig. 4E). At 11 dph, the NKA-IR cells in the filaments were first found in the 4th gill (Fig. 4F), and the number of NKA-IR cells in the three skin regions clearly decreased [Fig. 4(G to I)]. In addition, 5-HT-IR cells were observed in the gills and filaments (Fig. 4J). At 21 dph, the NKA-IR cells in the filaments were found in all gills and the inner opercular membrane (Fig. 4K), and there were almost no NKA-IR cells in all three skin regions [Fig. 4(L to N)]. The 5-HT-IR cells in the 4 gill arches and filaments were still apparent (Fig. 4O). Pictures are not shown for other sampling times, such as 4, 7 and 15 dph. The densities of NKA-IR cells in the three different skin regions were obtained at six sampling times (Fig. 4P). The density of the NKA-IR cells at 4 dph clearly decreased in the trunk and tail regions but increased in the yolk sac region, and at 7 dph, the number of the NKA-IR cells in the yolk sac region also decreased. Almost no NKA-IR cell was found by 21 dph in the three skin regions. From our results, the distributions of NKA-IR cells in the filaments and lamella in the gills or in the body surface at larvae stage were also observed in medaka, zebrafish, tilapia (*Oreochromis mossambicus*) and other fish species (Pan et al., 2005; Hwang and Lee, 2007; Hwang et al., 2011; Yang et al., 2013).

3.3. Relative protein abundance of CAII and NKA

Two CAII-IR bands at approximately 29 kDa and one NKA-IR band at approximately 95 kDa were detected in the gills and labyrinth organs when *M. opercularis* was acclimated to control, hypoxic, or restricted conditions for 7 d (Fig. 5A). The NKA expression was higher in the 1st gill in the restricted group and in the 4th gill in the hypoxic group, but no difference was observed in the labyrinth organ among the three groups (two-way ANOVA, treatments, $F_{2,63} = 8.44$, $p < 0.01$; tissues, $F_{2,63} = 229.40$, $p < 0.001$; treatment and tissue interaction, $F_{4,63} = 4.18$, $p = 0.005$; Tukey's test, $p < 0.05$, for comparing different treatments in each tissue; n = 8) (Fig. 5B). The CAII expression in the labyrinth organ was the highest in the hypoxic group and significantly lower in the restricted group (two-way ANOVA, treatments, $F_{2,63} = 5.33$, $p < 0.01$; tissues, $F_{2,63} = 11.15$, $p < 0.001$; treatment and tissue interaction, $F_{4,63} = 4.10$, $p = 0.005$; Tukey's test, $p < 0.05$, for comparing different treatments in each tissue; n = 8) (Fig. 5C). The air-breathing frequency of fish in the hypoxic group was significantly higher than that in the normoxic group at 56, 240, and 365 dph (at 56 dph, t-test, d.f. = 6, t-value = 4.63, $p < 0.001$; 240 dph, t-test, d.f. = 8, t-value = 5.21, $p < 0.001$; 365 dph, t-test, d.f. = 7, t-value = 4.88, $p < 0.001$) (Fig. 5D). At 56 dph, the air-breathing frequency increased by 2.2-fold in the hypoxic group. At 240 dph and 365 dph, the air-breathing frequency increased by 1.74- and 1.63-fold, respectively.

4. Discussion

This study described the development of the gills in young *B. splendens* and *M. opercularis* to partially explain the morphological differences of the adult stages. The MRCs switched their distribution and numbers from the skin to the gills at approximately 11 dph, and the oxygen-sensing ability of the gills was concordant with the ion regulatory function in young *B. splendens*. The relative abundance of CAII and NKA in the gills and labyrinth organ changed in response to the application of various oxygen uptake stresses in adults of *M. opercularis*.

This study is one of the few studies focusing on the growth of each gill in aquatic air-breathing fish. Several previously studies have reported the different cell morphologies of the gills at certain post-hatching stages (Galman and Avtalion, 1989; González et al., 1996; Rojo and González, 1999; Varsamos et al., 2005; Fridman et al., 2011; Blank and Burggren, 2014). The results of this study support the hypothesis that unequal gill growth occurs in the early stages of development. From our studies on *B. splendens* and *M. opercularis*, we conclude that the morphological variations among the gills in adult anabantoid fish are established in early gill development. The smaller 4th gill in adult *M. opercularis* and this might result from its early development processes. From Fig. 1, the development of the lamellae in the 4th gill became apparent from 21 dph. The gill development in aquatic air-breathing anabantoid species is distinct from that of other fish, and the additional question as to whether the gill developmental differences have any relationship with ecological habitats or air-breathing ability warrants further study.

In the embryos and larvae, the epithelia of the body surface are for ion regulation and gas exchange in freshwater systems (Evans et al., 2005; Hwang and Lee, 2007). Extrabranchial MRCs mainly distributed in the yolk sac, trunk, and tail regions during early stages of development and considered responsible for ion regulation in Japanese flounder (*P. olivaceus*) (Hiroi et al., 1998). The distribution of the MRCs between the gills and the skin surface was examined in the early embryo of killifish (*Fundulus heteroclitus*). The embryos and larvae were sampled at 2, 4, 6, 8, 10, 15, 20, and 25 dph, and the yolk-sac membrane and body skin were for ion regulation before branchial MRCs began to function (Katoh et al., 2000). Jonz and Nurse (2006) studied the relationship between epithelial MRCs and associated innervation in the gills of zebrafish and also found that the MRCs and the NECs in the gills were

both found at 5 dph (Jonz and Nurse, 2006). The distributions of the MRCs shifted from the skin to the gills starting at 4 dph in this species. The present study on *B. splendens* provides evidence to clearly describe the distribution of the MRCs and the NECs in the gills and the timing on the shift of MRC distribution from the skin to the gills.

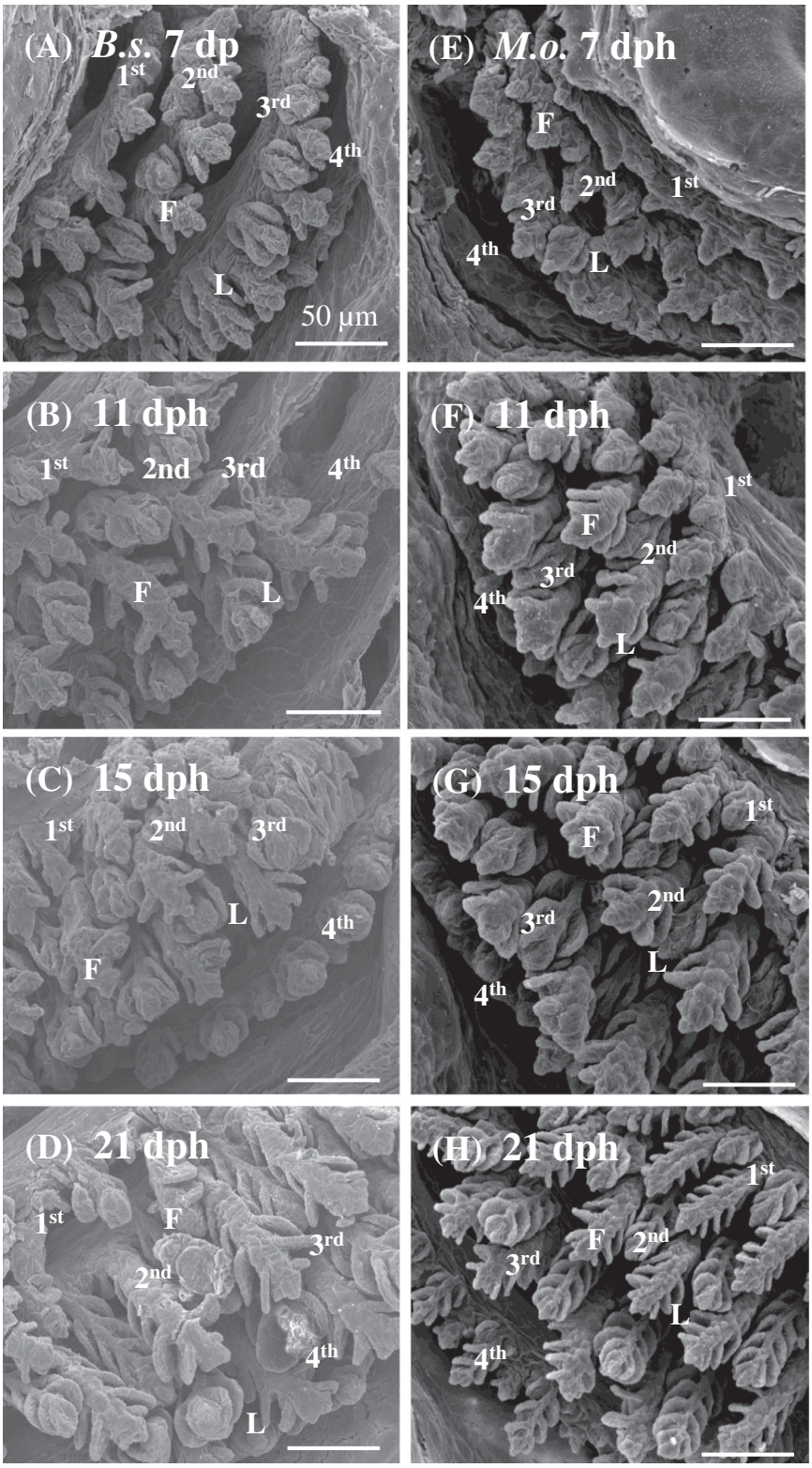

Fig. 2. Scanning electron microscope (SEM) images of gills were examined in *B. splendens* (A) to (D) and *M. opercularis* (E) to (H) at different sampling times: (A) and (E) At 7 dph, filaments and lamellae were in the first 3 gills but not in the 4th gill in two species. (B) and (F) At 11 dph, lamellae were observed for the first time in the 4th gill in *B. splendens* but not observed in *M. opercularis*. (C) and (G) At 15 dph, lamellae were observed for the 4th gills in *B. splendens* but not observed in *M. opercularis*. (D) and (H) At 21 dph, the 4th gill had a similar appearance to the other 3 gills in *B. splendens*, and lamellae were first observed in the 4th gill in *M. opercularis*. 1st: first gill; 2nd: second gill; 3rd: third gill; 4th: fourth gill; F: filament; L: lamella; *B.s.*: *Betta splendens*; *M.o.*: *Macropodus opercularis*.

Rombough proposed the hypothesis that the gills of fish larvae primarily serve as an ion regulation structures before they engage in respiration (Rombough, 1999, 2007). In this study, at 1 dph, no MRCs were present in any of the gills, but the densities of the MRCs in the yolk sac, trunk and tail regions were higher than those at other days. At 11 dph, the MRCs and the NECs of the filaments were found in all the gills, and the density of cutaneous MRCs clearly decreased in the fish body at the same time. According to the distribution of branchial MRCs, the gills had low ion regulatory function until 11 dph, during which the fish body surface may play a role in ion uptake prior to this period. These phenomena were also found in other species; for example, in rainbow trout (*Oncorhynchus mykiss*) larvae, a shift in the MRC distribution was reported at 27 dph (Rombough, 1999), and this change was found at 21 dph in Japanese flounder (*P. olivaceus*) larvae (Hiroi et al., 1998). In the present investigation, the MRCs and NECs appeared almost simultaneously in the filaments at 4 dph in *B. splendens*, an indication that its gills were for both gas exchange and ion regulation at the same time after hatching, and this phenomenon contrasts Rombough's hypothesis.

The air-breathing frequency in *M. opercularis* also significantly increased after 2 days in the hypoxic group, ranging from 1.63- to 2.2-fold among the three sampling times. *M. opercularis* performed more air breathing under hypoxic conditions at 56 dph. When there is a low dissolved oxygen condition, *M. opercularis* could increase its survival rate at the young stage by performing so.

The gas exchange ability was further examined in the gills and labyrinth organ in adults of *M. opercularis*. It is more likely that the variation in the relative abundance of CAII in the 4th gill and the labyrinth organ in *M. opercularis* was for CO_2 excretion in the hypoxic group (Evans et al., 2005). In the European flounder (*Platichthys flesus*), hypoxia led to a significant decrease in the mRNA levels and enzyme activity of NKA, suggesting a response for energy conservation in teleosts (Lundgreen et al., 2008). The theme behind conserving energy in metabolic efficiency is known as the down-regulation of energy

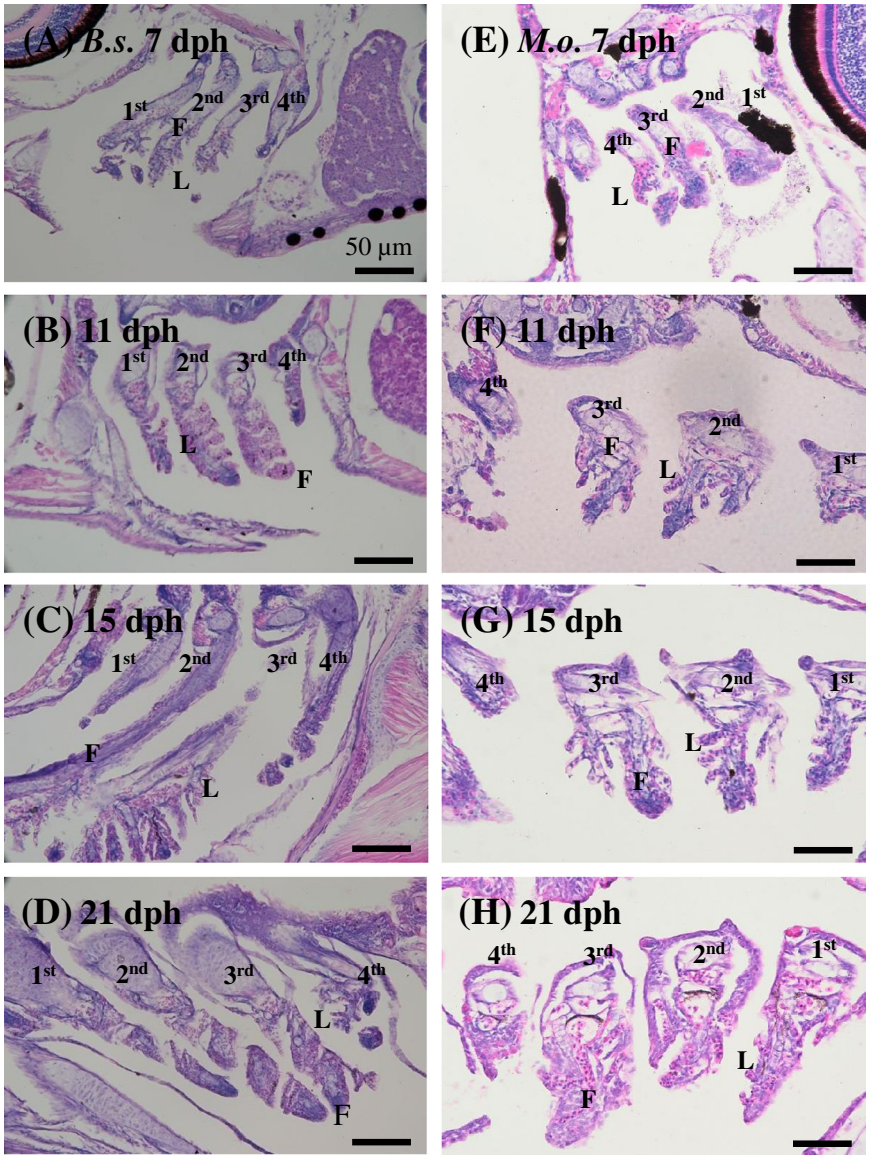

Fig. 3. Histological section (HS) images of gills were examined in *B. splendens* to (D) and *M. opercularis* (E) to (H) at different sampling times: (A) and (E) At 7 dph, filaments and lamellae were in the first 3 gills but not in the 4th gill in the two species. (B) and (F) At 11 dph, lamellae were observed for the first time in the 4th gill in *B. splendens* but not observed in *M. opercularis*. (C) and (G) At 15 dph, lamellae were observed for the gills in *B. splendens* but not observed in *M. opercularis*. (D) and (H) At 21 dph, the 4th gill had a similar appearance to the other 3 gills in *B. splendens*, and lamellae were first observed in the 4th gill in *M. opercularis*. 1st: first gill; 2nd: second gill; 3rd: third gill; 4th: fourth gill; F: filament; L: lamella; *B.s.*: *Betta splendens*; *M.o.*: *Macropodus opercularis*.

production and consumption (Bickler and Buck, 2007). Ion regulatory suppression is one of the examples of metabolic efficiency (Bickler and Buck, 2007). Therefore, the present study used NKA to evaluate the effect of the ion regulatory suppression in the gills and the labyrinth organ for ambient oxygen changes. An increase in NKA expression was found in the hypoxic and restricted groups in the 1st and 4th gills, respectively, but not in the labyrinth organ. These results raise interesting questions compared to the data from *P. flesus* (Lundgreen et al., 2008), and the phenomenon requires further research on physiological and metabolic responses.

The protein quantity of both CAII and NKA in *M. opercularis* was not lessened even with the restriction of air-breathing behavior. This species may not be an obligate air breather and may be able to increase its gas exchange efficiency or perform other compensatory responses between the gills and labyrinth organ. In one of our previous studies on the aquatic air-breathing fish *Trichogaster microlepis*, the lengths of the filaments and lamellae between the 1st and 4th gills differed significantly even in the normoxic group. The mortality was significantly higher in the restricted group than in the control group (Huang and Lin, unpublished data). The discrepancy regarding the mortality in the control and restricted groups between *M. opercularis* and *T. microlepis* might be due to differences in the gill respiratory surface area and gas exchange ability. Although the two species are aquatic air-breathing fishes, they had different morphological variations in the gills and the capacity of oxygen uptake in the restricted treatment.

In conclusion, both species exhibited different developmental processes. The gills had the ability to perform both gas exchange and ion regulation at the same time in the early stages in *B. splendens*. The gills that exhibited morphological variation and that possessed the accessory air-breathing organ could respond to oxygen stresses accordingly in the adult stages in *M. opercularis*.

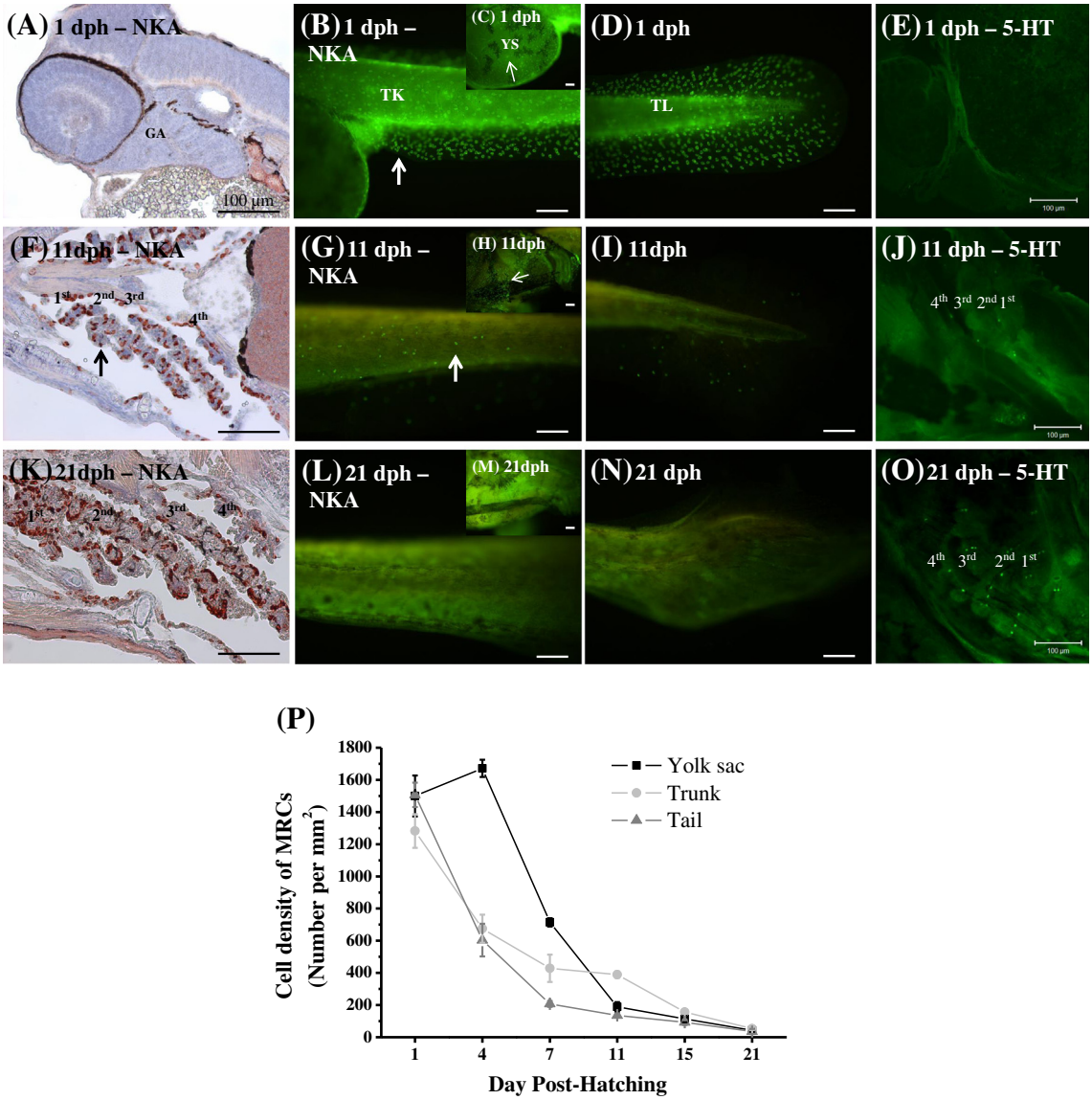

Fig. 4. The MRCs and the NECs in the gills or skin regions were examined at 1, 11, and 21 dph in *B. splendens*. At 1 dph, (A) there was no NKA-IR cell in any gill arch, and the NKA-IR cells were found in the trunk (B), yolk sac (C), and tail regions (D). (E) There were no 5-HT-IR cells in any gill arch. At 11 dph, (F) the NKA-IR cells in the filaments were first found in the 4th gill arches, and the NKA-IR cells were found in the trunk (G), yolk sac (H), and tail regions (I). (J) 5-HT-IR cells of the 4 gill arches and filaments were observed. At 21 dph, (K) the NKA-IR cells in the filaments were found in all gills and the inner branchial chamber, and there were almost no NKA-IR cells in the trunk (L), yolk sac (M), and tail regions (N). (O) 5-HT-IR cells of the 4 gill arches and filaments were still apparent. (P) The density of NKA-IR cells in the three different skin regions were obtained at these sampling times. Arrow: NKA-IR cell; 1st: first gill; 2nd: second gill; 3rd: third gill; 4th: fourth gill; GA: gill arch; TK: trunk; TL: tail; YS: yolk sac.

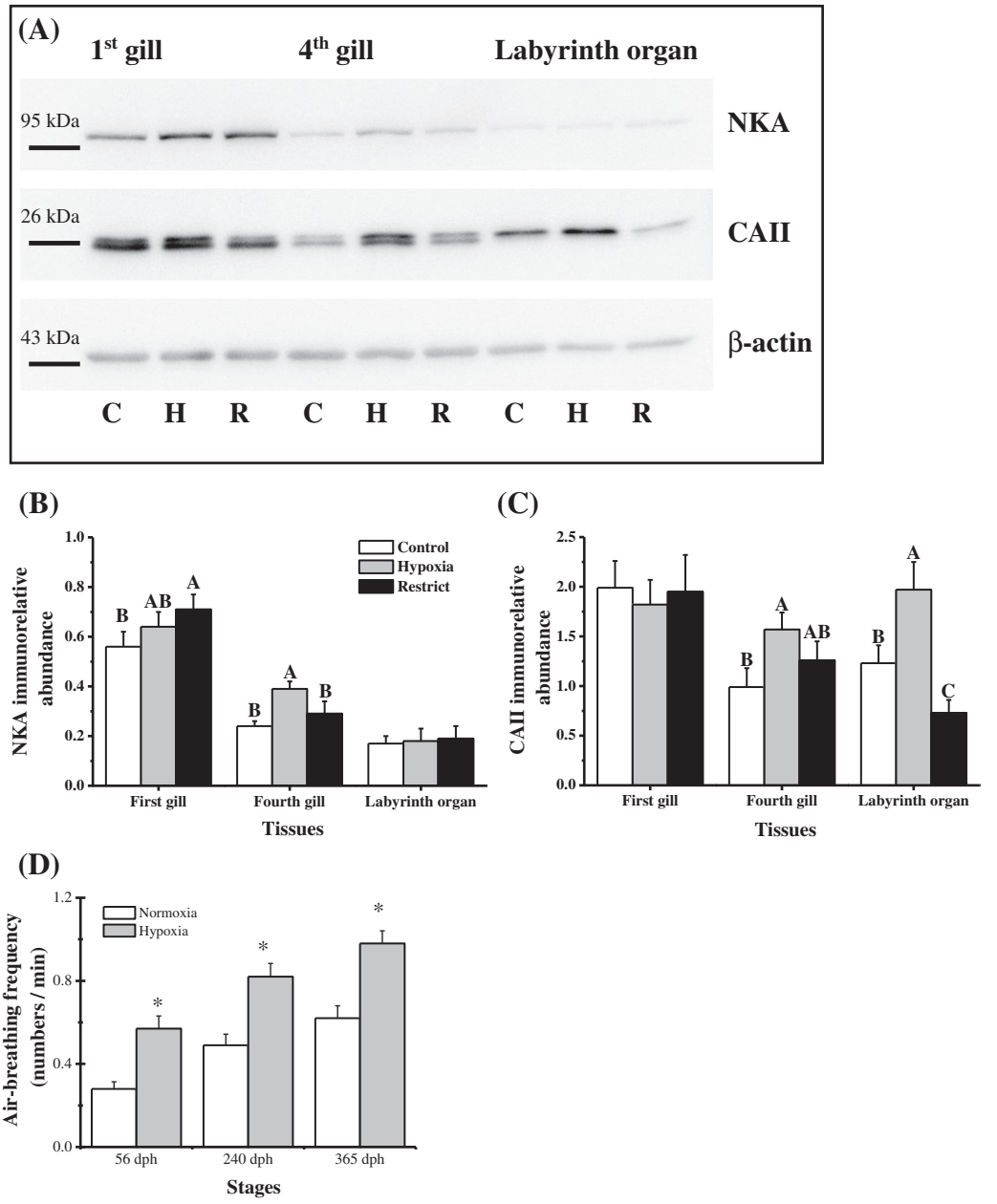

Fig. 5. Protein expressions of the gills and labyrinth organ in adult *M. opercularis* acclimated to control (C), hypoxic (H), and restricted (R) conditions for 7 days, and air-breathing frequency was examined in various stages. (A) Two CAII immunoreactive bands were found at approximately 29 kDa and one NKA immunoreactive band at approximately 95 kDa. (B) NKA protein was expressed at higher levels in the 1st and 4th gills in the hypoxic and restricted groups, respectively. (C) The relative abundance of CAII significantly increased in the labyrinth organ in fish subjected to hypoxia and decreased only in the labyrinth organ in the restricted group. (D) The air-breathing frequency in the hypoxic group was significantly higher than that of the normoxic group at 56, 240, and 365 dph, respectively. Significant differences (Tukey's test, $p < 0.05$) in the CAII and NKA expressions among tissues for each treatment are indicated using different symbols (A, B and C); differences between the hypoxic and normoxic groups at 56, 240, and 365 dph are indicated by asterisks (*: Student's t-test, $p < 0.05$).

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