Developmental and bone development indexes of medaka fish at 11 and 16 days of age

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Abstract:

The teleost fish medaka *(Oryzias latipes)* is currently used by laboratories worldwide as a model for many human diseases, including bone diseases. Our research group focuses on a medaka model for osteoporosis. To characterize the bone indexes of diseased animals, the bone development indexes of healthy wild-type fish are required for comparative analysis. Thus, this study examined the developmental and bone development indexes of wild-type medaka larvae at 11 and 16 days of age, the two developmental stages selected for the analysis of diseased patterns in osteoporosis-induced animals. The assessed parameters included the total body length of live larvae, number of caudal fin rays, vertebrae, and neural and hemal arches, as well as the total areas of mineralized vertebrae and lengths of mineralized arches. The obtained data are important for subsequent studies on bones using this fish model.

Keywords: **bone development indexes, medaka, mineralized bone, vertebrae.**

Classification number: **3.4**

Introduction

Medaka fish *(Oryzias latipes)* has recently become a valuable and favourable *in vivo* model to study human diseases [1]. Native to flooded rice fields in Japan, Taiwan and other areas of Southeast Asia, this small, egg-laying teleost has been brought to laboratory and examined for a long time [1, 2]. The biology of medaka fish indicates numerous advantages for a model organism: simple rearing, low-cost maintenance, short generation time, transparent embryo, and especially, easy techniques for live *in vivo* imaging, as well as for transgenesis and genetic manipulations [1-4]. More importantly, medaka shares a remarkable similarity, particularly at the cellular and molecular levels, with humans in mechanisms underlying biological processes [5]. Thus, medaka has been used as models in a wide variety of human diseases such as mental illnesses, neurodegenerative ailments [6, 7], cancer [8] and metabolism disorders [1, 9, 10], including bone diseases [11-13].

Osteoporosis, a common bone disease featured by reduced bone mass, destructed bone structures and high risk of bone fractures, is an important public health concern [14]. Finding better drugs and treatments for the disease is always of great interest to researchers [14, 15]. In our laboratory, we have used a transgenic medaka model for osteoporosis [13] and established methods and procedures for evaluating the anti-osteoporosis effect of tested substances [16]. In one of these methods, the bone indexes of experimental fish at 11 and 16 days of age have been protocolized for analysis. Therefore, we conducted this study to provide data on bone developmental hallmarks and characteristics for the fish at these two developmental stages that can be used as reference for further research. The analysed indexes included the total body length of live larvae, number of bone structures, including caudal fin rays, vertebrae and neural and hemal arches, as well as the areas of mineralized vertebrae and

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lengths of mineralized neural and hemal arches.

Materials and methods

Chemicals

The main chemicals used in this study were NaCl, KCl, Na_2HPO_4, KH_2PO_4, $MgCl_2$ (Sigma), PFA (paraformaldehyde Sigma P6148) and Alizarin red (Sigma A5533).

Fish lines

In this study, we used wild-type medaka fish originally provided by the Winkler's group from the National University of Singapore (NUS). The fish were raised and maintained at the Laboratory at the Department of Physiology and Human Biology, Faculty of Biology, VNU University of Science.

Fish maintenance and husbandry

Fish were raised in a small fish facility with temperature set at 26 to 28°C and a 14-h/10-h light/dark cycle to induce spawning. For the synchronous development of fish in the study, embryos were kept at a density of 20 individuals in 1×E3 medium in a 12-cm Petri dish and incubated in an incubator set at 30°C. Fish larvae were also raised at the same density at 30°C during experiments.

Live imaging of medaka larvae for the measurement of total length

The total length or body length of the analysed fish was measured on their live images. Live wild-type fish of 11 and 16 days post-fertilization (dpf) were immobilized by anaesthetizing with tricaine 0.01% and mounted in 3% methyl cellulose on a microscope slide. The images of live fish were obtained by an Optika B5 camera under a Zeiss Stemi 2000-C stereoscope (Carl Zeiss AG). Total length was measured on the obtained images using the "line function" of ImageJ software and defined as the length of the line drawn from the tip of the snout along the midline to the posterior edge of the caudal fin ray (see details in the results).

Alizarin red bone staining of PFA-fixed larvae

Fish were fixed and stained by Alizarin red that visualizes mineralized bone in purple following standard protocols as previously described [13, 17].

Imaging and measurement of the mineralization indexes of bone structures

Pictures were taken on Alizarin red-stained fish of 11 and 16 dpf as previously described [13] using an Axioplan Z microscope equipped with an Optika B5 camera. Bone

mineralization indexes need to be determined, including the length of neural arches (na), length of hemal arches (ha) and area of vertebral body (vb). Measurement was performed on images of Alizarin red-stained larvae using ImageJ software (https://imagej.nih.gov/ij/).

Statistical analysis

Statistical analysis was conducted using GraphPad software v5 for t-test to determine the significant difference in bone indexes between experimental fish groups.

Results and discussion

Total length of 11 dpf and 16 dpf fish

Total length or body length is an indicator for the development in fish. It is defined as the length of the line measured from the tip of the snout along the midline to the posterior edge of the caudal fin ray [18] (dark blue lines in Fig. 1A).

Total length measurement was performed for two live fish groups of 11 dpf and 16 dpf with over 30 individuals each (n=37 for 11 dpf and n=49 for 16 dpf group). The mean values of the total length between two groups were calculated and statistically compared by t-test (Fig. 1B).
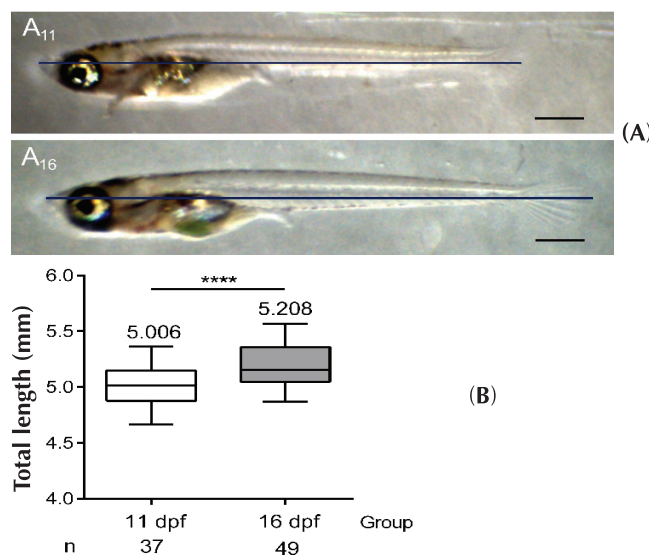

Fig. 1. Total length of fish at 11 dpf and 16 dpf: (**A**) Live images of fish at 11 dpf (A_{11}) and 16 dpf (A_{16}) and the measurement of their total lengths. Live larvae were mounted in methyl cellulose and imaged under a stereoscope with 1.5×magnification; dark blue lines drawn from the tip of the snout along the midline to the posterior edge of the caudal fin ray indicate the total/body length of the corresponding fish. Scale bar: 0.5 mm; (**B**) The mean values of the total length of fish form the two groups. Data are expressed as the mean values of total length, ****p<0.0001. Bars indicate standard deviation.

Representative images (Fig. 1A) illustrate the increase in the total length of 16 dpf compared to 11 dpf fish. The mean values of the total length were 5.006±0.027 mm and 5.208±0.029 mm for 11 dpf and 16 dpf fish, respectively; the difference between these two values was statistically extremely significant ($p < 0.0001$). Thus, from 11 to 16 dpf, the total length of fish increased approximately 0.2 mm; in other words, the fish grew roughly 4% in length during five days of development.

Several studies have used total length as a developmental hallmark for staging fish [19, 20]. Chatani, et al. reported the total body lengths of medaka from day 3 to day 22 post-hatching (dph) as a developmental indicator for assessing the appearance and development of osteoclasts in the TRAP-GFP transgenic medaka larva [19]. In this study, the total length of the 3 dph transgenic larva was roughly 5 mm [19], similar to the length of our 11 dpf wild-type fish. However, the hatching time of the fish in Chatani's study was not reported [19]; moreover, as the developmental rate of fish can vary significantly depending on rearing conditions, especially on the culturing temperature [21], our data can be referenced by research that raises the fish with similar protocols (see details in the methods).

Mineralized bone structures of the fish

The mineralized bone structures of 11 dpf and 16 dpf fish were visualized by Alizarin red staining on PFA-fixed larvae (Fig. 2). In both of these fish groups, mineralized structures were observed in the head, trunk and tail regions, namely, parasphenoid (ps), operculum (op), cleithrum (cl), anterior basicranial commissure (abc), supraoccipital (soc), basibranchial plt. (bp) in the head of 11 dpf (Figs. 2A, 2A'), and additionally dentary (den), quadate (qu), hyosymplectic (hys), ceratobranchial (cb) in the head of 16 dpf fish (Figs. 2B, 2B'); a vertebral column with a number of forming vertebrae each, if fully appeared, consisting of a vertebral body (vb) with a pair of forming neural (na) and hemal arches (ha) (Figs. 2A, 2B, 2A'', 2B'') in the trunk; and a number of caudal fin rays (fr) in the tail (Figs. 2A, 2B).

We previously reported the mineralized bone structures of 10 dpf and 15 dpf fish [22] compared to 11 dpf and 16 dpf fish, respectively, in this study; we did not observe any difference in the appearance of mineralized components (neither between 10 and 11 dpf nor between 15 and 16 dpf).

We subsequently quantified the indexes of some bone structures. First, we evaluated the number of certain types of mineralized structures that appeared in multiple units,
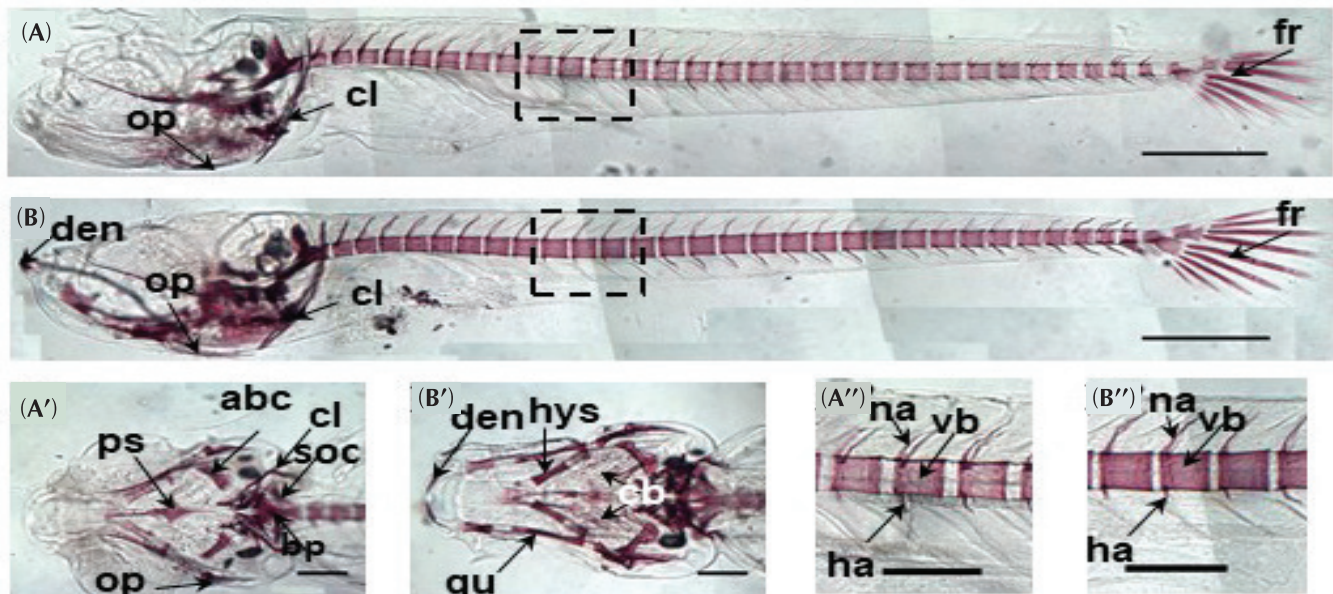

Fig. 2. Mineralized bone structures of fish larvae at 11 dpf and 16 dpf. Images of Alizarin red-stained mineralized bone structures of medaka fish at 11 dpf (**A**) and 16 dpf (**B**). (**A'**), (**B'**) ventral images of the head of 11 dpf and 16 dpf fish, respectively. (**A''**), (**B''**) zoomed-in images boxed in (**A**), (**B**), respectively; parasphenoid (ps), operculum (op), cleithrum (cl), anterior basicranial commissure (abc), supraoccipital (soc), basibranchial plt. (bp), dentary (den), quadate (qu), hyosymplectic (hys), ceratobranchial (cb); a vertebra consists of a vertebral body (vb), neural arch (na) and hemal arch (ha), fin ray (fr). Scale bars in A, B: 0.5 mm; in A'-B'': 0.2 mm.

including vertebrae with their vertebral bodies, neural arches, hemal arches and caudal fin rays. Results in Fig 3 indicated that the number of units of these bones varied to different extents in both fish groups. For the same bone structure, the number of units of 11 dpf fish had a wider variation than that of 16 dpf fish. The widest variation was observed in the number of hemal arches of the 11 dpf fish group that ranged from 0 to 26 units, and 50% of fish in the group (n=37) had the number of this bone ranging from 3 to 22 (median number was 13). This variation was reduced in 16 dpf fish to a range of 17 to 26 (median number was 23, and 50% of fish in the group (n=49) had 22 to 24 hemal arches). The increase in the number of bone units was also
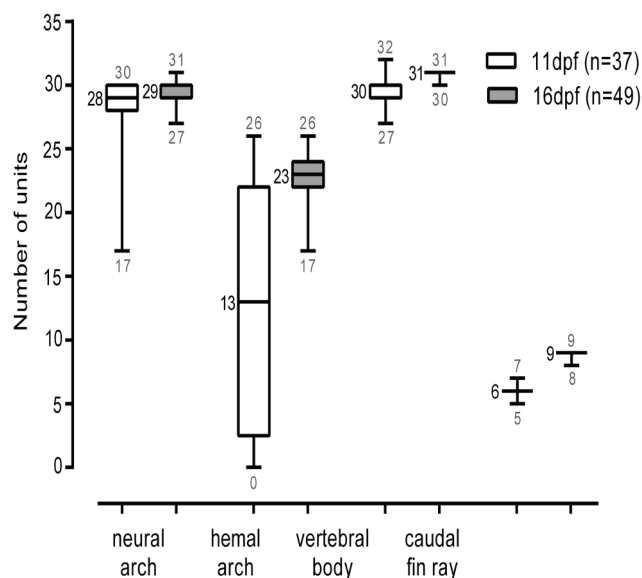

Fig. 3. The number of units of the analysed mineralized bone structures of 11 dpf and 16 dpf fish. Boxplot with whiskers from minimum to maximum and bands inside the box indicate medians (numbers denote the maximum, median and minimum median values of the corresponding groups). Boxes are interquartile ranges (IQR) or middle 50%.

most obvious for hemal arches (Fig. 3), from 13 in 11 dpf to 23 in 16 dpf fish. The variation and the increase in the number of neural arches of fish from 11 to 16 dpf could be observed at a lesser extent compared to those of hemal arches (Fig. 3).

High consistency in the number of units could be observed in the caudal fin rays and vertebral bodies of both fish groups, ranging from 5 to 7 with a median number of

6 fin rays for 11 dpf (30 in total 37 fish) and from 8 to 9 with a median number of 9 fin rays for 16 dpf fish (43 in total 49 fish). Moreover, the number of neural arches and vertebral bodies did not obviously increase (only 1 neural arch and 1 vertebral centrum were newly mineralized in 16 dpf compared to 11 dpf fish). This result demonstrated that in this period of development, the mineralized pattern classified by the number of neural arches and vertebral bodies was stabilized, whereas hemal arches were still highly mineralized and increased in number.

As we were searching for an indicator for the developmental stage of fish, based on these data, the number of caudal fin rays seemed to be the most reliable hallmark because it varied least among fish at the same age but differed significantly between 11 dpf and 16 dpf fish. Compared to the number of caudal fin rays, the number of vertebrae also did not considerably vary among fish at the same age, but the difference in quantity between the observed ages caused difficulty in differentiating one from the other as the number of vertebrae in fish can vary between individuals [23, 24]. Our results supported some previous studies that used the number of caudal fin rays as an indicator for medaka developmental staging [25].

Quantification of the mineralization of some bone structures

In our research, we selected vertebral column as the representative bone structure to be assessed for the level of mineralization; we therefore quantified the mineralization of bone components belonging to vertebrae, including the lengths of mineralized neural and hemal arches and the areas of mineralized vertebral bodies.

The mineralization level of the neural arches (I_{mn}) of a fish was defined as the total value of lengths of its mineralized neural arches (drawn by white lines in Fig. 4A) measured by ImageJ software. The total length of mineralized hemal arches (drawn by white lines in Fig. 4B) expressed the level of mineralization of these bone structures of one fish and denoted as I_{mh}. I_{ma} is the total value of all vertebral body areas of one fish (areas drawn by white lines in Fig. 4C). Measurement was performed for all fish of 11 dpf and 16 dpf groups. The mean values of I_{mn}, I_{mh} and I_{ma} of these two groups were calculated and statistically compared by t-test (Fig. 4D).
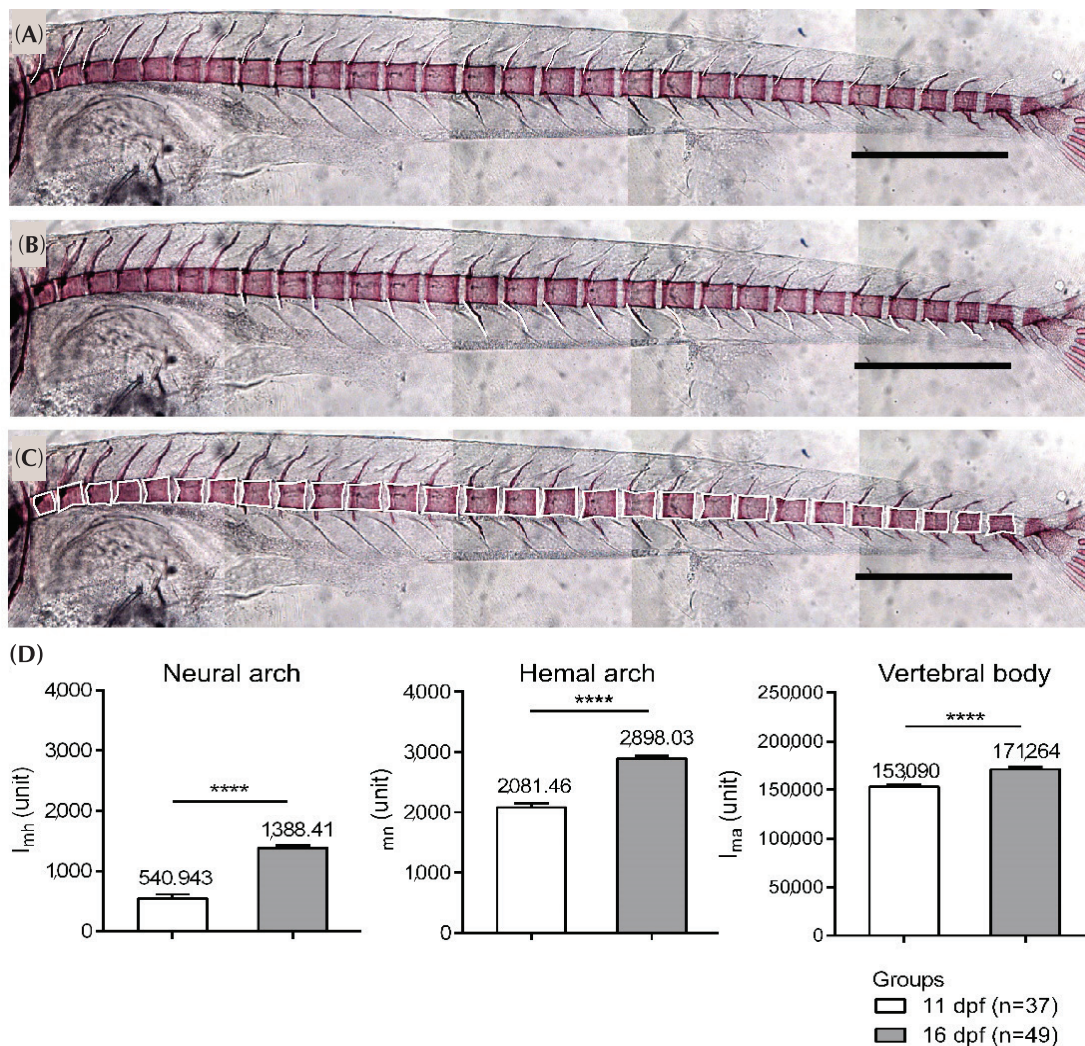
Fig. 4. Mineralization levels of neural bodies, neural and hemal arches of the fish. Neural (**A**) and hemal (**B**) arches are indicated by white lines for their length measurement (I_{mn} and I_{mh} indexes, respectively), vertebral bodies (**C**) are bordered by white lines for their area measurement (I_{ma} index). Lines and boxes are drawn and measured by ImageJ software. Scale bars: 0.5 mm, (**D**) mean values of I_{mh}, I_{mn} and I_{ma} of the 11 dpf and 16 dpf fish groups. ****p<0.0001.

The values of I_{mn} obtained are 2,081.46 and 2,898.03 for 11 dpf and 16 dpf fish, respectively. Thus, during five days of development, from day 11 to day 16 of age, the mineralization level of the neural arches (I_{mn}) of the fish increased about 39%. As the number of the neural arches increases only one (Fig. 3), the increase in the I_{mn} value was mainly due to the increase in their lengths and appropriate to indicate the development of the fish.

The results also suggested a sharp increase in the value of I_{mh} (Fig. 4D) (from 540.94 at 11 dpf to 1,388.41 at 16 dpf). Medaka fish gained an increase of 156% in the total length of hemal arches during five days of development, from day 11 to day 16 of age. As previously described, during this development period, hemal arches were rapidly mineralized, mostly by the formation of new ones in the posterior vertebrae of the fish. Along with the rise in number, the forming hemal arches also increased in length, thus sharply increasing the total length of these bones. The mineralized hemal arches are known to appear in chronological order after the mineralization of vertebral centrums and neural arches [26, 27]. Remarkable increases in lengths and number of mineralized hemal arches in the fish from day 11 to day 16 suggest for the use of hemal arches as sites or models to be assessed for studies on new or *de novo* mineralization, as occurred in research to evaluate the effects of bone anabolic substances.

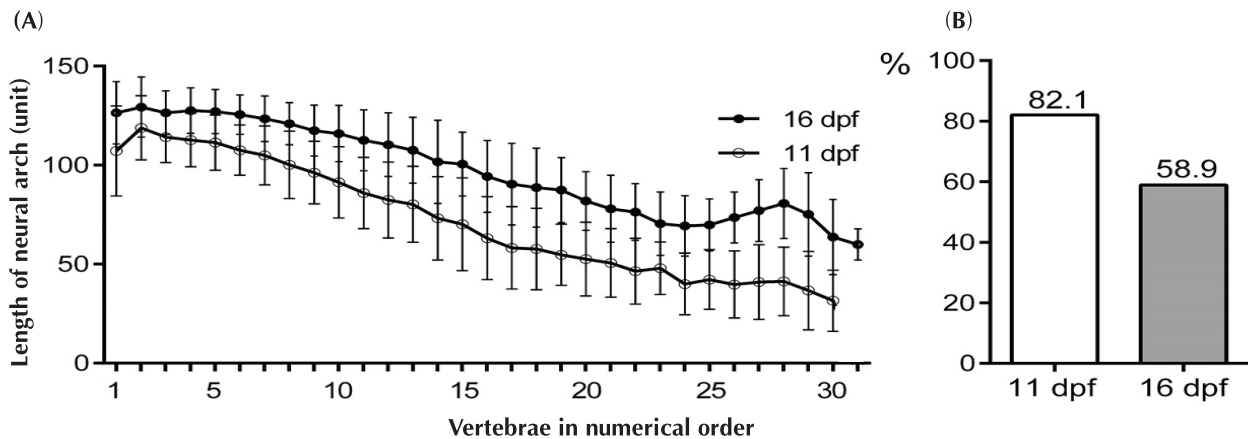Further mineralization was observed in the vertebral bodies of 16 dpf compared to 11 dpf fish; the mean values

Fig. 5. Mean length values of individual mineralized neural arches in numeric order of 11 dpf and 16 dpf fish. (A) Mean values of neural arches of each vertebra; (**B**) $I_{mn}15$ ratio: the ratio of the total length of the 15 first neural arches to the total length of all vertebral neural arches in 11 dpf and 16 dpf fish, (11 dpf: n=37 and 16 dpf: n=49). Bars indicate standard deviations, ****p<0.0001.

of the I_{ma} of 11 dpf and 16 dpf groups were 153,090 and 171,264 units, respectively, denoting an increase of 11.8% after five days. This result, together with the evidence of little increase in the number of vertebral bodies, suggest independent regulating processes for the mineralization of vertebral arches and bodies, which were also reported [11, 13].

Length of the 15 first neural arches relatively reflects total mineralization in 11 dpf fish

In recent years, our laboratory has been intensively screening for bioactive substances that have anti-osteoporosis potential. For this purpose, a medaka model for osteoporosis having damage in mineralized neural arches has been used [16, 28]. We therefore intended to evaluate the lengths of mineralized neural arches in normal wild-type fish to determine the possibility of using this bone structure as representative to assess the mineralization level of a fish (Fig. 5).

In both 11 dpf and 16 dpf fish groups, neural arches evidently shortened gradually along the anterior-posterior axis of the fish, except that the second neural arch was slightly longer than the first one (Fig. 5A). Moreover, as indicated in the data on 11 dpf fish, the total length of the 15 first neural arches contributed to 71% of the total length of all these bones; by contrast, this percentage in 16 dpf fish was merely 61% ($I_{mn}15$ ratios are 0.71 and 0.61 for 11 dpf and 16 dpf fish, respectively, Fig. 5B). These data suggest the establishment of a methodology for quantifying the level of mineralization in the osteoporosis fish model at 11 dpf by taking the value of the total lengths of 15 first neural arches as an index to indicate the mineralization level of the fish.

Conclusions

This study analysed the bone and developmental indexes in medaka larvae at 11 and 16 days of age, including the total body length of live larvae, number of mineralized bone structures, as well as the area of mineralized vertebrae and length of mineralized neural and hemal arches. Data obtained suggest the use of the number of caudal fin rays as a larva developmental hallmark and the length of the 15 first neural arches as an index that relatively indicates the level of mineralization of 11 dpf fish. These data are important for further research on bones in medaka.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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