Stable isotopic responses of otoliths of juvenile sardine *Sardinops melanostictus* experimentally cultured at different temperatures

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Stable oxygen isotope ($\delta^{18}O$) of $CaCO_3$ in fish otolith is a useful indicator as ambient water temperature through fish growth, thus, the high resolution δ18O analysis of otolith contributes to understanding of fish migratory history. To improve the estimation of their migration route, the otolith-$\delta^{18}O$ thermometer of each species should be determined precisely through the reliable culture experiment. In previous study, Sakamoto et al. (2017) clarified the relationship between $\delta^{18}O$ of otoliths and temperature by using juvenile sardine *Sardinops melanostictus* reared in different water temperatures. Their findings would be robust by a further experiment including (1) the rigorous marking of otoliths for definition of the newly precipitate areas during the rearing period, (2) the extension of rearing period for the sufficient growth of otolith, and (3) high-frequency sampling of the rearing water for fine-scale analysis of the environmental $\delta^{18}O$. In this study, we improved the culture experiment for evaluating the previous work, and examined the growth and stable isotopes of the otoliths of juvenile specimen of *S. melanostictus* reared at the three different temperatures for longer experimental period (60 days) by using the otolith marking method (Alizarin complexone: ALC), and by increasing the water sampling intervals for $\delta^{18}O$ analysis under the rearing environment. In addition, stable carbon isotope ($\delta^{13}C$) is known to reflect $\delta^{13}C$ of seawater dissolved inorganic carbon and metabolic derived carbon, and therefore, temperature-related physiological changes could appear in otolith $\delta^{13}C$ of our experimental specimens. We compared the otolith $\delta^{13}C$ and temperature and growth for further understanding of a metabolic effect on otolith $\delta^{13}C$. 29 specimens of *S. melanostictus* caught in Suzaki Bay, Kochi Prefecture were cultured for 60 days with a flow-through experimental tank system at the Hakatajima station (Ehime Prefecture) of the National Research Institute of Fisheries and Environment of Inland Sea, FRA, Japan. Before the temperature experiment, the experimental specimens had been treated in ALC to mark a distinct red ring in each otolith under UV light. During the experimental period, water temperature in each tank was recorded every 30 minutes by digital data loggers, and was 13.8 ±0.4, 19.2 ±0.6, 24.2 ±0.5°C (mean ±1SD). The experimental fishes were fed to satiation daily with 2-6 % of their body weight (g) of commercial dry pellets in relation to temperature regimes. Carbonate powder was collected from newly-formed areas during the experiment by using a high-precision micromilling system (GEOMILL326, Izumo-web, Japan), and $\delta^{18}O$ and $\delta^{13}C$ of these samples were determined using a continuous-flow isotope ratio mass spectrometry system (MICAL3c with IsoPrime 100) at the National Institute of Technology, Ibaraki College.

The average growth increments of individual specimens for 60 days were 71 ±16 μm, 168 ±34 μm, and 229 ±47 μm for treatments at temperatures of 14, 19, 24°C, respectively; thus the otolith growth rate tended to be higher at higher water temperature. From the measured $\delta^{18}O$ values of otoliths and seawater, we obtained the following relationships between water temperature and aragonite–water fractionation in *S. melanostictus*:

$$\delta^{18}O_{otolith} - \delta^{18}O_{seawater} = -0.16 \times T + 2.56\ (R^2 = 0.96,\ P < 0.01)\ (N = 17)$$

where *T* is water temperature. Our equation is close to the equation reported by Sakamoto et al. (2017); thus our study evaluated their reported equations by the rigorous experimental methods. Therefore, these equations can be a useful temperature proxy for understanding the migration history. The otolith $\delta^{13}C$ values showed a significant negative correlation with water temperatures, and also had a significant negative correlation with otolith growth rates. Thus, the physiological variation affected by water temperature might be recorded in δ13C of *S. melanostictus* otoliths.

Keywords: Otolith, Stable oxygen isotope, Stable carbon isotope, Sardine, Water temperature, Fish