Accelerated bioaccumulation of total mercury in red stingray, *Hemitrygon akajei*, by ontogenetic changes of feeding habits, and selective transfer of methylmercury to the aquatic animals located at higher trophic levels

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Abstract

Mercury is a trace element circulating in the biosphere on the earth, but nevertheless some of its organic forms are highly harmful to various living organisms. There is an increasing awareness of the progress of bioaccumulation of mercury concentration in various marine ecosystems in the world, since 7,400 tons of mercury is still annually being emitted into the environment throughout the world not only from anthropogenic sources, but also natural ones including volcanos, and finally discharged to the sea. Volcanic emission has a non-negligible impact on the mercury levels of the aquatic environment. In particular, the Japanese archipelago is located on the Circum-Pacific "ring of fire", and has with about 85 active volcanoes, which have erupted repeatedly, dispersing a large amount of mercury to the atmosphere surrounding them. Approximately 1,879,000 tons of fish and shellfish have been annually caught to obtain seafoods from the coastal seas in Japan. In this meaning, approximately one hundred and twenty-five million people living in the Japanese archipelago seem to be inevitable to face the risk of bioaccumulation of mercury in their daily lives through the consumption of sea foods.

This study deals with the trophic transfer of mercury species in the bentho-pelagic food web in the Isahaya Bay, focusing on the bioaccumulation process in one of the edible ray species, red stingray (*Hemitrygon akajei*). This bay is located in the western side of the inner part of Ariake Bay, Kyushu, western Japan. An active volcano, Mt. Unzen Fugendake, is located at the center of Shimabara Peninsula, which faces the southern side of the bay. In its last eruption over 1,928 days between 1990 and 1995, about 2.95 tons of mercury were emitted to the atmosphere around it. The volcano works as one of the natural discharging sources of mercury to the bay.

In this study, I collected 22 individuals of red stingray and six species of other fishes and invertebrates from Isahaya Bay, and aimed at clarifying the mechanisms how the total mercury (THg) content of the body tissues of red stingray markedly increases as it grows up although its trophic position tends to descend in the food web system and how the mercury is transferred to the animals located at the higher trophic positions, and evaluated the potential risk of mercury intake to the human health through the dietary intake of fishes captured in the coastal seas.

In the Isahaya Bay, the THg content of the muscles of red stingray acceleratedly following the growth ($y = 160.0e^{0.03x}$, x = the disc width, y = the THg content of the muscles). The THg contents of the immature female (disc width: 25 ± 5 cm, body weight: 623 ± 421 g) are 309 ± 76 ng g^{-1} d.w. (mean ± S.D., n=7), while those of the mature ones

(disc width: 60 ± 3 cm, body weight: 8,175 ± 1,023 g) increased to 869 ± 268 ng g^{-1} d.w. (n=10). The highest value, 1,370 ng g^{-1} d.w., was recorded in the individuals with the disc width of 63 cm and the body weight of 8,615 g. However, the results of stable analysis of carbon and nitrogen of the muscles of them revealed that the trophic positions of red stingray tended to descend in the food web system as it grew up. The analysis of the stomach content of these individuals indicated the presence of the ontogenetic changes of the feeding habits following the development of mouth. In mature female, it can feed on only epifauna macro-benthic animals with soft bodies such as shrimps and small crustaceans, while mature female tends to prefer to prey on infauna macro-benthic animals with hard shells such as short-neck clam, *Ruditapes philippinarum*, and some polychaetes. These main food items burrow the sediment, and contain much higher levels of THg to the epifaunal ones, since they are apt to expose to the mercury deposited and concentrated in the sediment.

In the Isahaya Bay, a large amount of mercury has been emitted from an active volcano, Mt.Unzen Fugendake, and a part of the mercury is absorbed by phytoplankton and deposited in the sediment of the sea floor in the bay, being concentrated in the body tissues of the phytoplankton and the sediment. The mercury deposited in the sediment was taken in to the body tissues of the macro-benthic animals on the sea floor though their feeding activities and exposure to the mercury, and transferred to the animals located at the higher trophic positions through the bentho-pelagic food web of the bay. In this transferring process of mercury between the prey and predator, inorganic mercury (InHg) tends to be excreted to outside the body of each animal, while methylmercury (MeHg) is apt to remain in the body tissues and selectively transferred to the predator side. Therefore, the results of the analysis on Trophic Magnification Factor (TMF) values of the mercury species in the seven species of animals liked with the bentho-pelagic food web system revealed that the biomagnification of MeHg of the muscles proceeded approximately 2.4 (6.22/2.62) times in the animals located at one step higher trophic position in the food web system. Consequently, MeHg of the muscles of short-neck clam as the primary consumer occupied approximately 22.5 % of THg, while it increased to more than 90 % in the predatory fishes as the intermediate consumers between the secondary and tertiary ones, such as *Pennahia argentata*, *H. akajei*, and *Nuchequula nuchalis*.

In the case of the red stingray, two large individuals of mature females (56 and 63 cm of the disc width) contained the highest values of MeHg of 0.26 μg g^{-1} w.w.. They are very close to the provisional reference value of MeHg for fish and shellfish in Japan, 0.3 μg g^{-1} w.w. in Japan, although their THg contents (0.28 and 0.27 μg g^{-1} w.w., respectively) were far lower than that of THg for fish and shellfish, 0.4 μg g^{-1} w.w.. These facts indicate

that we need to pay more attention to the effects of the selective transfer of MeHg among the mercury species in the bentho-pelagic food web system in the coastal seas.

Keywords: biomagnification, *Hemitrygon akajei*, total mercury, methylmercury, ontogenetic changes of feeding habits, red stingray, *Ruditapes phillipinarum*, selective transfer, short-neck clam

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1. Introduction

Bioaccumulation of hazardous substances such as heavy metals, polychlorinated biphenyls, dioxin, etc. has been reported from various marine ecosystems throughout the world. Among these harmful substances, this study has focused on the bioaccumulation processes of mercury to the animals occurring in the coastal water, since it has brought serious negative impacts on the health of not only the wildlife located at the higher trophic positions in the food chain, but also humans through dietary intake (Harada 1995; Atwell et al. 1998; Baeyens et al. 2003; Al-Reasi et al. 2007; Ward et al. 2010; Kim et al. 2012; Cardoso et al. 2014; Clayden et al. 2014; Harding et al. 2018; Yokoyama 2018). Mercury is a trace element circulating in the biosphere on the earth, but nevertheless some of its organic forms are highly harmful to various living organisms. The mercury exists primarily in the form of dissolved Hg, including elemental Hg (Hg^0) and inorganic species (Hg^{2+}) bound to the sediment in marine ecosystem, while that found in marine animals is almost entirely in the form of organic species, such as methylmercury (CH_3Hg^+) (Ullrich et al. 2001; Chen et al. 2008). Inorganic mercury (InHg) can be transformed to methylmercury (MeHg) by the metabolic activities of sulfate reducing bacteria in the marine sediments. MeHg is lipid-soluble, readily tends to pass through the blood-brain barrier, and accumulates in the brain of the animals, causing lysis of the cell of central nervous system and brings irreversible damages to the central nervous system. (Rabenstein 1978; Farrell et al. 1998; Mason et al. 1995; National Research Council (US) Committee on the Toxicological Effects of Methylmercury 2000; Yokoyama 2018). The presence of MeHg in coastal ecosystems poses a serious environmental hazard due to the bioaccumulation and toxicity to the animals located at the higher trophic positions in the food webs.

In the aquatic ecosystem, the mercury contaminated in the water tends to be absorbed by phytoplankton or aquatic animals such as fish (through their respiratory activities), which is referred to as “bioconcentration”. It is apt to be further biologically concentrated on the body tissues of animals in higher trophic levels linked with a food chain through their predatory activities, which is referred to as “biomagnification” (Arnot and Gobas 2006). Previous studies on the bioaccumulation of mercury, have described the discontinuous increase of mercury levels in the body tissues among the organisms located at the different trophic positions in a food chain in coastal waters (Baeyens et al. 2003; Kim et al. 2012; Cardoso et al. 2014; Harding et al. 2018; Jaingam et al. 2018a) and oceans (Atwell et al. 1998; Al-Reasi et al. 2007; Lavoie et al. 2013; Clayden et al. 2014). Consequently the mercury contents of the bodies of top predators such as carnivorous

fish, sea birds and marine mammals in aquatic ecosystem tend to be magnified 123 – 479 and 227 – 622 times, respectively, higher to those of the primary producers (Campbell et al. 2005; Di Beneditto et al. 2012; Seixas et al. 2014; Harding et al. 2018). There is an increasing awareness of the progress of bioaccumulation of mercury concentration in various marine ecosystems in the world (Baeyens et al. 2003; Lavoie et al. 2013; Gworek et al. 2016; Wu et al. 2019), since 7,400 tons of mercury is still annually being emitted into the environment not only from anthropogenic sources, but also natural ones including volcanos throughout the world, and finally discharged to the sea (Nriagu 1989; Mason et al. 2012; UN Environment 2019; Zhang et al. 2020, 2021)

Volcanic emission have a non-negligible impact on the mercury levels of the aquatic environment. In particular, the Japanese archipelago is located on the Circum-Pacific "ring of fire" with about 85 active volcanoes (Nakano and Hoshizumi 2005). Consequently, volcanic eruptions have occurred frequently, dispersing a large amount of mercury to the atmosphere surrounding them (Nriagu and Becker 2003). Approximately 1,879,000 tons of fish and shellfish have been annually caught to obtain seafoods from the coastal seas in Japan (Ministry of Agriculture, Forestry and Fishery 2018). In this meaning, approximately one hundred and twenty-five million people living in Japanese archipelago seem to be inevitable to face the risk of bioaccumulation of mercury in their daily lives through the consumption of the sea foods.

This study deals with the trophic transfer of mercury species in the bentho-pelagic food web in Isahaya Bay, focusing on the bioaccumulation process in one of the edible ray species, red stingray (*Hemitrygon akajei*). This bay is located in the western side of the inner part of Ariake Bay, Kyushu, western Japan. An active volcano, Mt. Unzen Fugendake, is located at the center of Shimabara Peninsula, which faces the southern side of the bay. In its last eruption over 1,928 days between 1990 to 1995, about 2.95 tons of mercury were emitted to the atmosphere (Nriagu and Becker 2003; Unzen Restoration Project Office 2007). Furthermore, there are many hot springs and fumaroles around the volcano. They also work as ones of natural discharging sources of mercury to the bay. In the previous studies on the bioaccumulation process of mercury of aquatic organisms occurring in Isahaya Bay, Jaingam et al. (2018b) noted that red stingray accumulated extremely high levels of the total mercury (THg) (max. 3,700 ng g d.w.$^{-1}$), although the trophic position of this species in the food web tended to descend from the tertiary consumer toward the secondary one as it grew. Jaingam et al. (2018a) found the presence of another bioconcentration of mercury not in the water but the bottom sediment in the bay, which is caused by the decomposition of the particulate organic matters (POM) such as dead bodies of phytoplankton that absorbed mercury from the surrounding water and

partly sank down on the sea floor. It tends to be decomposed to inorganic matters by bacteria and other micro-organisms, but the mercury contained in the particles is apt to be kept intact in the sediment. Therefore, the another type of bioconcentration of mercury proceeds in the sediment on the sea floor, where the THg contents of the sediment tends to be enhanced to several times higher levels to that of the POM suspended in the water. Some parts of THg concentrated in the sediment are transferred to the infaunal macro-benthic animals by their feeding activities that directly feed on the sediment or exposure of themselves to the THg-contaminated sediment (Jaingam et al. 2018a, Tsutsumi et al. 2019). In the bentho-pelagic food web in the bay, the further biomagnification process of THg occurs between the infaunal macro-benthic animals and carnivorous bentho-pelagic fishes such as red stingray, black headed seabream (*Acanthopagrus schlegelii*), grass puffer (*Takifugu niphobles*), etc. through their predations activities. Therefore, they far exceeded the THg contents of the tertiary consumers of fishes such as bastard halibut (*Paralichthys olivaceus*) and Japanese sea bass (*Lateolabrax japonicus*) (249 and 266 ng g^{-1} d.w., respectively) occurring in the pelagic system of the same bay (Jaingam et al. 2018b).

In this study, I tried to clarify the mechanisms how the THg content of the body tissues of red stingray markedly increases as it grows up, although its trophic position tends to descend, how the mercury tends to be transferred to the animals located at the higher trophic positions, and evaluate the potential risk of mercury intake to human health through the dietary intake of fishes collected in the coastal seas. The purposes of this study are to collect the red stingray with a wide variety of body size in Isahaya Bay, examine the stomach contents to identify its main food items, determine their stable isotope ratios of carbon and nitrogen to describe their trophic positions in the food web system and determine the THg contents of the muscles of the stingray, and clarify the mechanisms of the accelerated biomagnification of mercury to the body tissues of larger individuals of the red stingray. I also deal with the trophic transfer of mercury species among the aquatic organisms in this bentho-pelagic food web in Isahaya Bay. I aim at evaluating the potential risk of mercury to human health through the consumption of aquatic animals collected in the coastal seas.

2. Materials and methods

2.1 Study area

Isahaya Bay is located at the inner part of Ariake Bay. This bay is an enclosed bay with total area of 65 km^2 and an average depth of 10 m (Figure 1). An active volcano, Mt. Unzen Fugendake, is located at the center of Shimabara Peninsula. It is one of the major natural sources of discharging mercury into the bay, since this peninsula is located beside the southern side of Isahaya Bay.

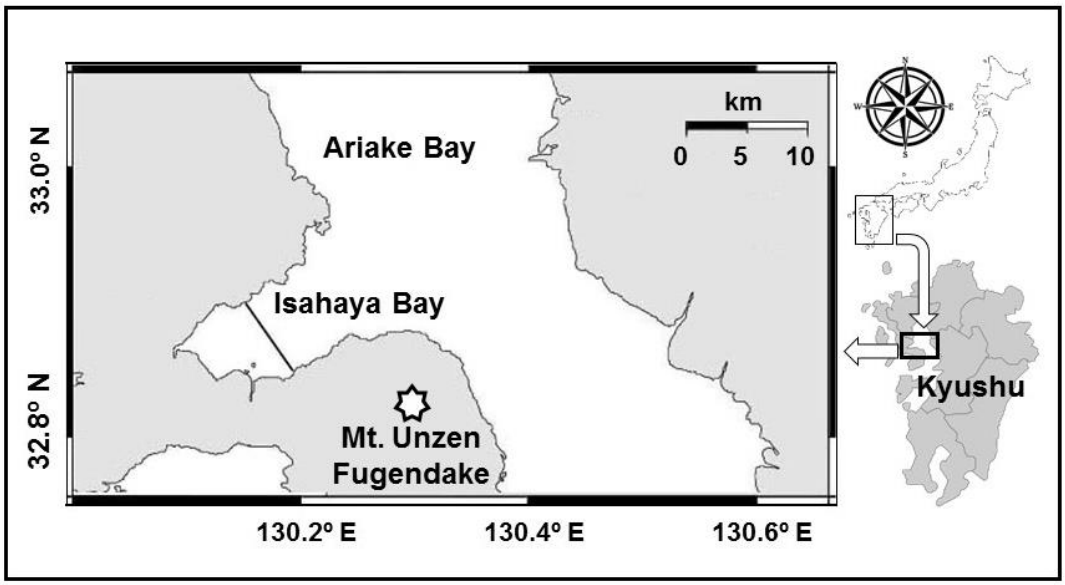

Figure 1 Study area, Isahaya Bay located in the western side of the inner parts of Ariake Bay, Kyushu, western Japan.

2.2 Sample collection and preparation

2.2.1 Red stingray collection

In total, 22 individuals of red stingray were captured from Isahaya Bay with a longline fleet on 27 April 2019 and a gill net on 15 May 2019. The specimens were weighed with a digital balance, and their disc widths were measured with a measuring tape. The gender was determined by the presence or absence of claspers. According to Furumitsu et al. (2019), the individuals whose disc widths were larger than 56 cm in females and 35 cm in males were regarded as sexually mature ones, the females with the disc width between 45 and 55 cm were treated as ones in transitional maturity, and those with the disc width or the less than 44 cm were regarded as immature. The ventral part of the body of each specimen was dissected to remove the stomach. Following the methods

noted by Bowen (1996) and Weidner et al. (2017), the stomach samples were put in plastic bottles and fixed with undiluted formalin solution to prevent decomposition, putrefaction, and autolysis of the stomachs for approximately one month, and the subsequent 70% ethanol preservation is done to maintain the integrity of fixed stomachs specimens for long-term storage. The muscles of each specimen were sampled from the dorsal part using a surgical knife, and kept in an icebox. At the laboratory, they were kept in a freezer at -30 °C prior to the chemical analyses. I captured one individual of immature female red stingray in Isahaya Bay to observe the teeth structure on 14 October 2020.

2.2.2 Collection of benthic invertebrates and bentho-pelagic fishes

Crab was captured with a fixed net in Isahaya Bay on 15 July 2020 and 14 October 2020, and clam was collected by hand picking on the shore of the bay on 14 October 2020. The bentho-pelagic fishes were captured from the bay with a gill net on 15 May 2019 and 15 July 2020. Additional specimens of red stingray were caught with a longline fleet in the bay on 27 April 2019. After collection, all of the specimens were identified, their body sizes were measured (total length for fish and benthic invertebrates, and disc width for red stingray), and their wet weights were weighed with a digital balance. The muscles collected from the dorsal part of fish and the soft parts of benthic invertebrates (without digestive tract) were sampled using a surgical knife, frozen at -30 °C, freeze-dried for 48 hours, and finally ground and homogenized using a pestle and mortar into a powder until further chemical analyses.

2.3 Stomach contents analysis

The stomach specimens of the red stingray were weighed, and dissected. Their contents were observed under a stereoscopic microscope. The remains of the preys in the stomach were identified, counted and weighed within each identifiable taxonomic group. The unidentifiable or highly digested items were just weight. The data on the identifiable prey items were used to calculate “Percentage of Index of Relative Importance, % IRI” with following three indices, Percentage by number (%N), Percentage by weight (%W), and Percentage of occurrence (%O).

Index of Relative Importance (IRI) = (%N + %W) × %O

%N = 100 X (total count of a given prey taxa) / (total count of all prey taxa) (Hyslop 1980)

%W = 100 X (total mass (g) of a given prey taxa) / (total mass of all prey taxa) (Hyslop 1980)

%O = 100 X (total number of stomachs containing a given prey taxa) / (total number of stomachs with any prey) (Bowen 1996)

$$\%\mathrm{IRI}_i = 100 \ \mathrm{X} \ (\mathrm{IRI}_i / \textstyle\sum_{i=1}^{n} \mathrm{IRI}_i)$$ (Cortés 1997)

n: the total number of identifiable taxonomic group, *i*: a given identifiable taxonomic group

2.4 Chemical analysis

2.4.1 Stable isotope analysis

Frozen specimens of the muscles of benthic invertebrates and bentho-pelagic fishes were treated with a chloroform-methanol mixture solution (2:1, V/V) to remove lipids from them, centrifuged at 10,000 rpm at 4 °C for 5 minutes twice, treated with 100% methanol, centrifuged at 10,000 rpm at 4 °C for 5 minutes again, and finally vacuum-dried for 48 hrs. They were also treated with 2N HCl, centrifuged at 35,000 rpm at 4 °C for 5 minutes twice, and vacuum-dried for 48 hours to remove $CaCO_3$. Prior to the analysis, the samples were ground and kept in plastic vials.

The stable isotope ratios of carbon and nitrogen of the samples were determined using an elemental analyzer (Flash Elemental Analyzer 1112 Series, Thermo Electron) and continuous flow isotope ratio mass spectrometer (Delta Plus, Thermo Electron). The data of stable isotope ratios of carbon and nitrogen are expressed with delta notations as parts per mill (in ‰) as follows:

$$\delta^{13}\mathrm{C} = [\frac{^{13}\mathrm{C} \ / ^{12}\mathrm{C}_{\mathrm{sample}}}{^{13}\mathrm{C}/^{12}\mathrm{C}_{\mathrm{standard}}} - 1] \times 1000 \ \ (‰) \qquad \delta^{15}\mathrm{N} = [\frac{^{15}\mathrm{N} \ / ^{14}\mathrm{N}_{\mathrm{sample}}}{^{15}\mathrm{N}/^{14}\mathrm{N}_{\mathrm{standard}}} - 1] \times 1000 \ \ (‰)$$

Pee Dee Belemnite (PDB) and atmospheric nitrogen were used as references for ^{13}C and ^{15}N, respectively. Glycine was used as a working standard in this study. The overall analytical error was within ± 0.2 ‰.

2.4.2 Total mercury content analysis

Prior to the determination of THg contents, the specimens of the muscles of red stingray were ground and homogenized. THg contents of these samples were determined using the MA-3000 mercury analyzer (Thermal vaporization atomic absorption, Nippon

Instruments). The detection was based on cold-vapor atomic absorption spectroscopy at a wavelength of 253.7 nm. The NIMJ CRM 7402-a (cod fish tissue) was used as standard reference for test the accuracy of the method.

2.4.3 Mercury species content analysis

THg and MeHg content of bentho-pelagic fishes and benthic invertebrates in the bentho-pelagic food web of Isahaya Bay was determined according to the analysis method for THg and MeHg in marine biological samples described by Yoshimoto et al. (2016), in brief, approximately 0.1 g of dry sample was weighed into 15 ml polypropylene (PP) tube and extracted with 0.1% L-Cysteine solution and 5 M NaOH at 80 °C for 1-2 hr. The digested solution was treated with distilled water, methyl isobutyl ketone (MIBK), and hexane to extracted THg. Then, for the extraction of MeHg, 2 ml of the THg extraction product was transferred and extracted with 5 M HBr, 2 M CuCl2, toluene, and 0.2% Cysteine - 2% NaOAc solution. The THg and MeHg concentrations of these extraction product were determined in triplicate using the MA-3000 mercury analyzer. The detection was based on cold-vapor atomic absorption spectroscopy at a wavelength of 253.7 nm. The values corresponding to the contents of inorganic mercury (InHg) were calculated as the difference between the values found of THg and MeHg (Fox et al. 2013; Seixas et al. 2014; Polak-Juszczack 2018).

The quality control was performed by the analysis of replicates, certified reference materials (CRM), and blank control. The CRM from the National Metrology Institute of Japan, NIMJ CRM 7402-a (cod fish tissue) No.452 with the certified value 0.61 $\pm$ 0.02 mg kg^{-1} for THg and 0.58 $\pm$ 0.02 mg kg^{-1} for MeHg was used as standard reference for test the accuracy of the method. The obtained values were 0.61 $\pm$ 0.02 mg kg^{-1} for THg and 0.59 $\pm$ 0.03 mg kg^{-1} for MeHg, the extraction recovery of THg and MeHg were 99.12 $\pm$ 3.51% and 101.51 $\pm$ 4.81%, respectively. The coefficient of variations for triplicate was lower than 5 % and the accuracy and precision of the methods of the method were larger than 95%. We confirmed the limits of detection of MA-3000 in this study is 0.1 ng Hg.

2.5 Trophic Magnification Slope (TMS), Trophic Magnification Factor (TMF) and Trophic Level (TL)

The biomagnification of mercury was determined by the correlations between $\log_{10}$ - transformed of mercury contents and corresponding values for the stable isotope ratios of nitrogen ($\delta^{15}N$),

Trophic Magnification Slope (TMS) is the regression slope (**b**) of following equation (Lavoie et al. 2013).

$$\text{Log}_{10}[\text{Hg}] = a + \mathbf{b}(\delta^{15}\text{N})$$

Trophic Magnification Factor (TMF) are calculated as (Borgå et al. 2012):

$$\text{TMF} = 10^{\mathbf{B}}$$

When B is the regression slope of following equation: $\text{Log}_{10}[\text{Hg}] = a + \mathbf{B}(\text{TL})$

The trophic level (TL) can be calculated from δ^{15}N using the following equation (Borgå et al. 2012):

$$\text{TL}_{\text{primary consumer}} = ((\delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{primary producer}}) / \Delta^{15}\text{N}) + 1$$

$$\text{TL}_{\text{consumer}} = ((\delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{primary consumer}}) / \Delta^{15}\text{N}) + 2$$

When Δ^{15}N is the enrichment factors (increase in ^{15}N from the diet to the consumer); 3.4 ‰

2.6 Statistical analysis

The statistical significances of the correlations between disc width and body weight, between disc width and THg content of red stingray, between the values of δ^{13}C and δ^{15}N of the muscle, and between mercury content of the organisms and δ^{15}N (for TMS) and TL (for TMF) were calculated with a nonparametric test, Spearman's correlation coefficient by rank. Those of the mean values of δ^{13}C and δ^{15}N of the muscles of red stingray were evaluated with Mann-Whitney's U test among the immature female, female in transitional maturity, and mature female. These statistics were conducted with Statview ver. 4.5 (Abacus Corporation, U.S.) for Apple Macintosh, and used throughout, using a significance level of $p < 0.05$.

3. Results

3.1 The mechanisms of biomagnification of THg in red stingray

3.1.1 Relationship between disc width and body weight of red stingray

Figure 2 indicates the relationship between the disc width and body weight of 22 red stingray individuals captured in this study (The data are noted in Table 1). A significant exponential curve is found between them ($BW=0.03DW^{3.09}$, DW: Disc width, BW: Body weight) (Spearman's correlation coefficient by rank, $z = 4.517$, $p < 0.0001$, $n = 22$). The growth rate and life span of red stingray are not clear in the previous studies. However, National Geographic reported that the red stingray reached max. 2 m in the whole body length and about 100 kg, and estimated that the max longevity was 15 to 25 years (National Geographic home page). I was able to collect a max. 9,540 g of the individual in this study, but found a further larger one although it was too heavy to capture with the longline fleet and gill net used in this study. Yokota (1952) estimated that the individuals with the disc width of about 18 cm were four years old, which is almost equivalent to the specimen No. 02 (immature female with the disc width of 19 cm and the body weight of 240 g). Therefore, mature females with the disc widths of 56 to 65 cm seem to be regarded as individuals at least 10 to 15 years old, and further elder ones occurred in the study area, Isahaya Bay.

The sex ratio of the specimens was extremely biased to female (20 individuals of the total 22 red stingrays). Since the period required to the size at sexual maturity is significantly different between males and females in red stingrays (Furumitsu et al. 2019), this study compared the food items, trophic positions in the food web system, and THg contents of the muscles among the specimens of immature females, ones in transitional maturity, and mature ones in this study.

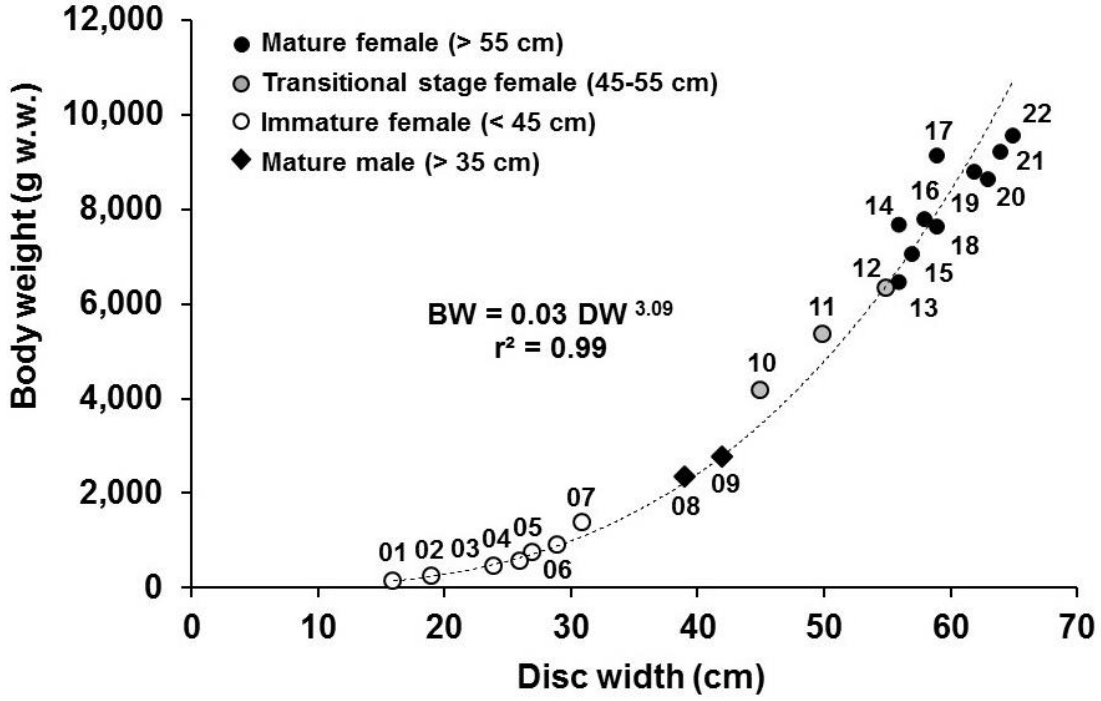

Figure 2 Relationship between disc width and whole body weight of 22 individuals of red stingray, *Hemitrygon akajei,* captured in this study in Isahaya Bay. Open circles: immature females, Gray circles: females in transitional maturity, Closed circles: mature females, Closed diamonds: mature males. The number of each plot indicates its specimen number noted in Table 1.

3.1.2 Changes of the stomach contents of female red stingray followed by its growth

The biological information of the 20 specimens of female red stingray used for the analysis are noted in Table 1. The disc widths of seven specimens of immature female, three ones of female in transitional maturity, and ten ones of mature female were 25 ± 5 cm (mean ± S.D.), 50 ± 5 cm, and 60 ± 3 cm, respectively. The stomach contents of these three different maturity level of females were compared with an index, %IRI, (Figure 3, Table 2). In this study, 71.7 %, 53.5 % and 58.0 % of the stomach contents in weight were identifiable to at least phylum in the specimens of immature female, female in transitional maturity, and mature female, respectively. In immature female, the identifiable preys of the stomach contents were mainly made up of Arthropoda (75.4 %) in %IRI, involving other crabs (42.3 %) and shrimp (33.1 %), small fishes as Chordata (16.5 %), and Annelida (8.1 %). The %IRI value of Arthropoda decreased to 34.6 % in female in transitional maturity, and 8.2% in mature female, while those of Mollusca and Annelida

increased to 2.1 % and 21.9 % in female in transitional maturity, and 45.2 % and 21.6 % in mature female, respectively. In particular, the mature females exclusively favored to feed on the short-neck clam, *Ruditapes phillipinnarum* (45.0 %) and a polychaete, *Nectoneanthes ijimai* (21.6 %).

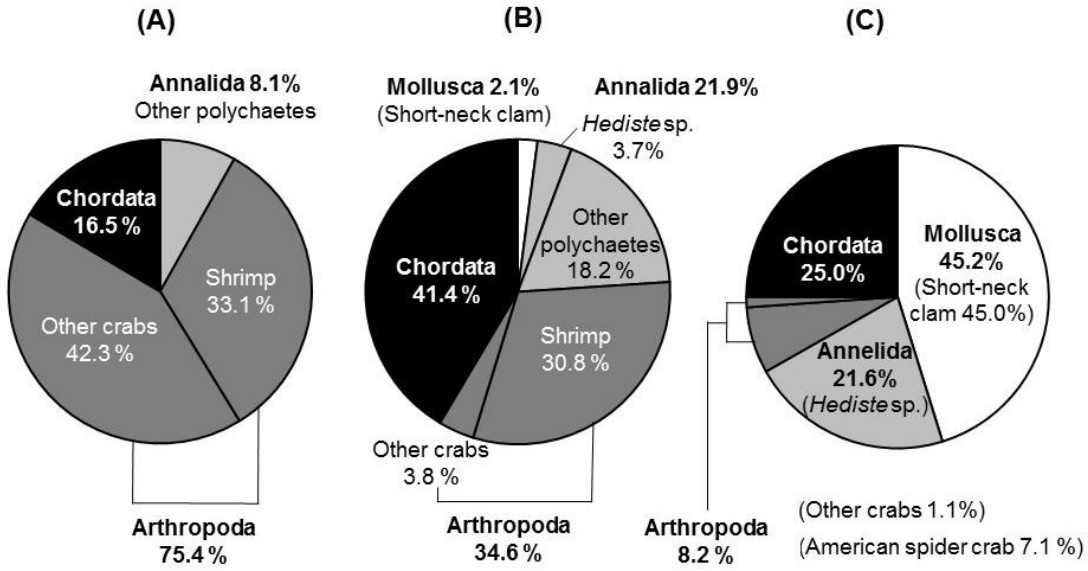

Figure 3 "Percentage of Index of Relative Importance (%IRI)" of the identifiable stomach contents of female red stingray, *Hemitrygon akajei*, caught in Isahaya Bay. (A) Immature individuals (Disc width: 25 ± 5 cm (mean ± S.D.) , n=7), (B) Females in transitional maturity individuals (Disc width: 50 ± 5 cm, n=3), (C) Mature individuals (Disc width: 60 ± 3 cm, n=10)

Thus, in the case of female red stingray, the food items markedly changed as it grew up to be sexually mature. The immature one tended to feed on mainly epifaunal macro-benthic animals such as crab and shrimp, while the diet preferences of the mature one drastically changed to infaunal macro-benthic animals burrowing the sediment such as short-neck clam, *R. philippinarum*, and polychaete, *N. ijimai*. These changes of feeding habits following the individual growth is referred to as "ontogenetic changes in the feeding habits" (Brickle et al. 2003).

3.1.3 Stable isotope ratios of carbon and nitrogen of female red stingray

I evaluated how the changes of the food items affected the female red stingray following the growth on its trophic position in the benthic food web system with the results of stable isotope analysis of carbon and nitrogen of the muscles of the specimens.

Figure 4 compares the relationship of the values of stable isotope ratios of carbon and nitrogen of the muscles among the immature female, female in transitional maturity, and mature female. The values of $\delta^{13}C$ and $\delta^{15}N$ of the muscles of the immature female and female in transitional maturity were -15.8 ± 0.8 ‰ and 15.7 ± 0.6 ‰ (n=7) and -16.5 ± 0.9 ‰ and 15.5 ± 0.5 ‰ (n=3), respectively (Table 1), and the value of $\delta^{15}N$ of these ten specimens significantly increased in proportion to that of $\delta^{13}C$ (r^2 = 0.679, n = 10) (Spearman's correlation coefficient by rank, z = 2.534, $p < 0.011$). In the mature female, all of the values of $\delta^{15}N$ were located below the regression line between the values of $\delta^{13}C$ and $\delta^{15}N$ of the immature female and female in transitional maturity.

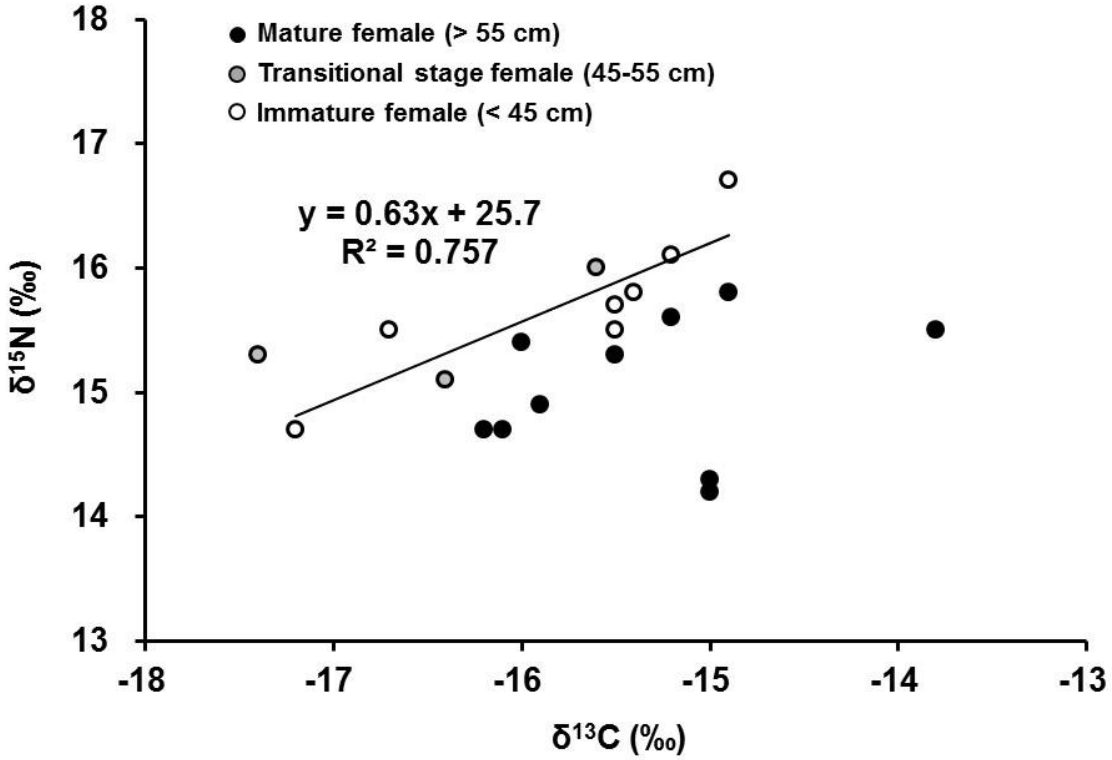

Figure 4 Relationships between $\delta^{13}C$ and $\delta^{15}N$ values of the body tissues of females red stingray, *Hemitrygon akajei,* collected in Isahaya Bay. Open circles: immature females (n=7), Gray circles: females in transitional maturity (n=3), Closed circles: mature females (n=10)

The mean value of $\delta^{15}N$ of the mature female was significantly lighter (-0.7 ‰) than that of the immature one (Mann-Whitney's U test, U = 13.50, p = 0.035). The mean value of $\delta^{13}C$ of the mature female was slightly heavier than those of the immature female (+0.4 ‰) and female in transitional maturity (+1.1 ‰), but the difference of $\delta^{13}C$ values between all ten individuals of the immature female and female in transitional maturity

and mature one was not statistically significant (Mann-Whitney's U test, U = 32.00, p = 0.173). These results indicate that all of the females in three different sexually mature levels preyed on the diets nutritionally sustained by the same primary producers, but the trophic position of the mature female was significantly lower than that of the immature one. This significant decrease of $\delta^{15}N$ value of the mature female was supported by the ontogenetic changes of main food items from shrimp (e.g., *Metapenaeus joyneri*: 14.4 ± 0.4 ‰ (Jaingam et al. 2018a) and crab as the secondary consumers (75.4 % in %IRI) in the immature female to short-neck clam (*R. philippunarum*: 9.7 ± 0.4 ‰ (Jaingam et al. 2018a) (45.2 % in %IRI) and polychaete, *N. ijimai*, (21.6 % in %IRI) as primary consumer in the mature one (Figure 3).

3.1.4 Accelerated biomagnification of THg

The THg contents of the muscles of 20 individual of females red stingray with the three different maturity levels (immature, transitional maturity, and mature) collected from Isahaya Bay ranged between 241 and 1,370 ng g^{-1} d.w. (Table 1). Figure 5 indicates the relationship between the disc width and THg content of all specimens. A statistically significant relationship was found between the disc width and THg content of the muscles ($y = 160.0e^{0.03x}$, $r^2 = 0.683$, n=20, Spearman's correlation coefficient by rank, z = 3.479, $p < 0001$). However, there were no significantly increasing trends of THg content of the muscles to the growth of immature female (Spearman's correlation coefficient by rank, n = 9, z = 1.349, p = 0.177), although they seem to be made up of the one with a variety of age at least for four years, judging from the growth rate reported by Yokota (1952). These results indicate that the bioaccumulation of THg tends to be accelerated as the females of the red stingray mature.

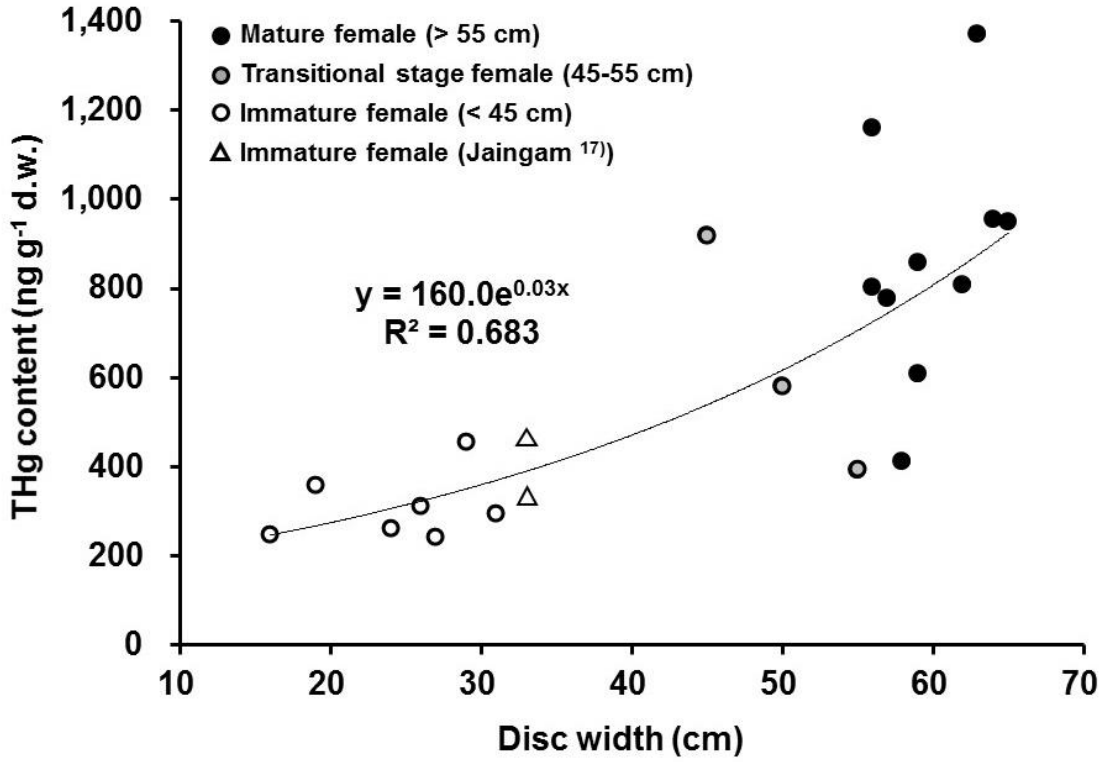

Figure 5 Relationships between Disc width and THg content of the body tissues of mature and immature females of red stingray, *Hemitrygon akajei,* collected in Isahaya Bay. Open circles: immature females (n=7), Gray circles: females in transitional maturity (n=3), Closed circles: mature females (n=10), Triangles: immature females from the previous study (n=2) (Jaingam et al. 2018b)

The biomagnification of THg of the muscles of female red stingray has a unique characteristic. Figure 6 shows the relationship between the $\delta^{15}N$ value and THg content of the muscles. The THg content of mature female was 869 ± 268 ng g^{-1} d.w.; n=10, which was significantly higher than that of the immature one (309 ± 76 ng g^{-1} d.w.; n=7) (Mann-Whitney's U test, U = 1.000, $p < 0.001$), although the $\delta^{15}N$ value of immature female (15.7 ± 0.6 ‰; n=7) indicates that it located at the higher trophic position than mature one ($\delta^{15}N$: 15.0 ± 0.6 ‰; n=10) in the food web system (Mann-Whitney's U test, U = 13.500, $p = 0.036$). Thus, the female red stingray tends to accumulate significantly higher levels of THg in their muscles (Figure 5), although they locate at the lower trophic position to the immature individuals (Figure 6).

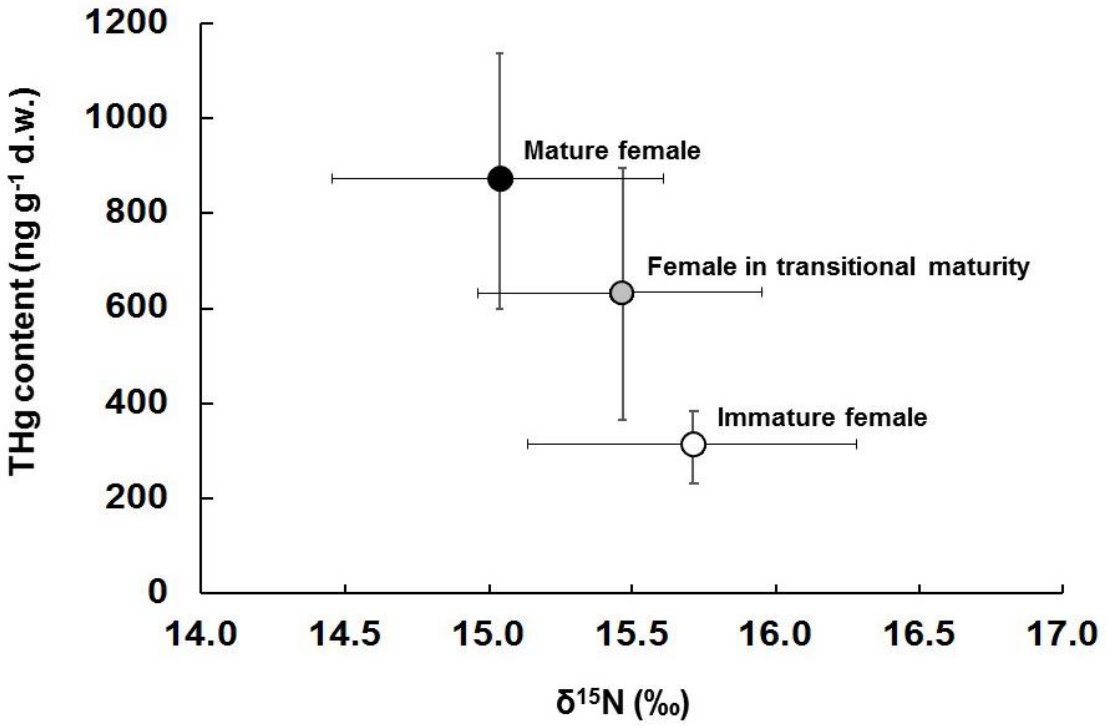

Figure 6 Relationships between $\delta^{15}N$ values and THg content of the body tissues of females red stingray, *Hemitrygon akajei,* collected in Isahaya Bay. Open circles: immature females (n=7), Gray circles: females in transitional maturity (n=3), Closed circles: mature females (n=10). Error bars represent standard deviation of the mean values.

3.2 Characteristics of mercury transfer to the animals located at higher trophic positions

3.2.1 Trophic structure of bentho-pelagic food web in Isahaya Bay

The relationship between $\delta^{13}C$ and $\delta^{15}N$ values of all seven species of fishes and invertebrates collected in Isahaya Bay was shown in Figure 7 (The data of $\delta^{13}C$ and $\delta^{15}N$ values of these animals are noted in Table 3). A significant positive relationship was found among them ($y=3.38x + 67.04$, $r^2 = 0.903$, Spearman's correlation coefficient by rank, $z = 2.165$, $p = 0.031$). The slope of the regression line is 3.38, which is almost coincided with the general trend on the isotopic shift of carbon and nitrogen isotope signature, 3.4, that appears among the organisms that prey on the diets nutritionally supported by the same primary producers (Wada et al. 1991). The lowest isotopic signature of $\delta^{13}C$ and $\delta^{15}N$ (-16.6 ± 0.3 ‰, 10.3 ± 0.2 ‰; n=4) was described in short-neck clams, which is regarded as the primary consumer, since it is one of the suspension-feeding bivalves (Nakamura 2004). American spider crab (*Pyromaia tuberculate*) is located at 0.3 ‰ higher positions in $\delta^{13}C$ and also 2.4 ‰ higher ones in $\delta^{15}N$ to the short-neck clam. It

seems to be close to the secondary consumer. The mean $\delta^{15}N$ values of other remaining five species of fishes range between 14.2 and 15.6 ‰. They seem to be located at the intermediate trophic position between the secondary consumer and tertiary ones.

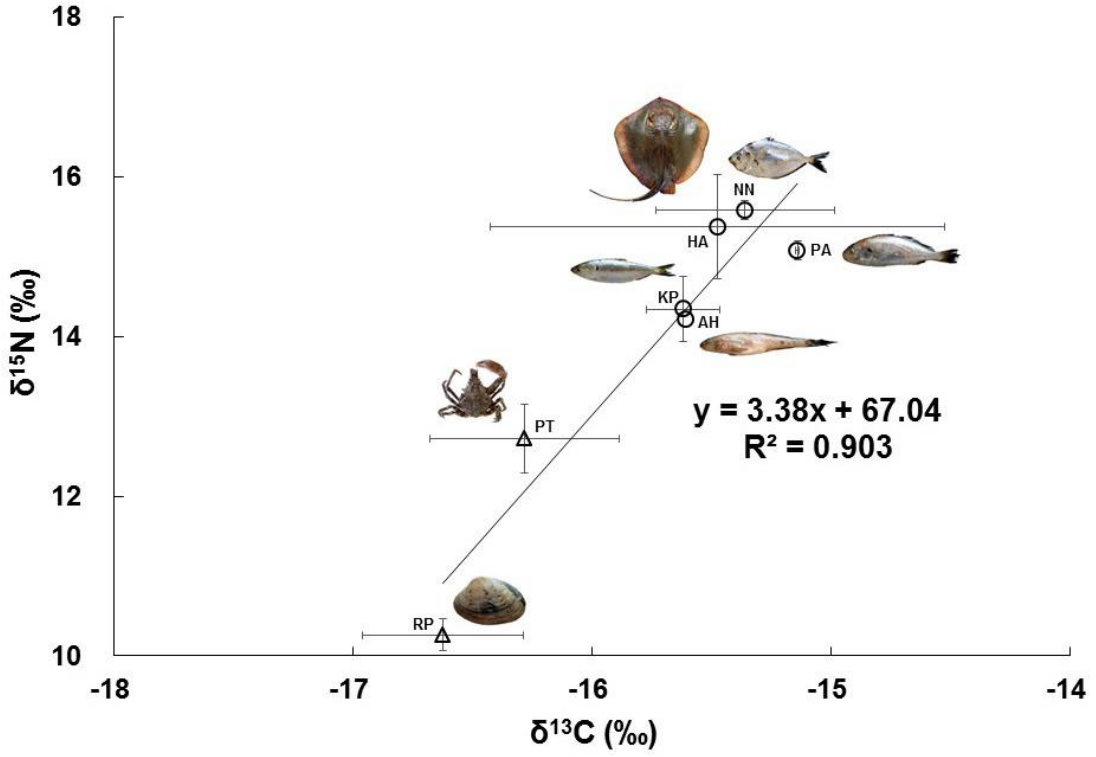

Figure 7 Relationships between $\delta^{13}C$ and $\delta^{15}N$ signatures of the body tissues of seven species of fishes and invertebrates collected in Isahaya Bay. The data of plots are noted in Table 3. Error bars represent standard deviation of the mean values.

3.2.2 Biomagnification of THg in bentho-pelagic food web

Figure 8 shows the relationship between the log-transformed THg contents and $\delta^{15}N$ values of their muscles (The data of THg contents and $\delta^{15}N$ values of the muscles are noted in Table 3). A significant positive linear relationship was found between them (log THg = 0.12 x + 0.40, x: $\delta^{15}N$ value, $r^2 = 0.402$, Spearman's correlation coefficient by rank, z = 2.012, p = 0.044). The trophic magnification slope (TMS) was estimated as 0.12, which indicates the increase rate of THg content to the increase of $\delta^{15}N$ value of the organism in the food web.

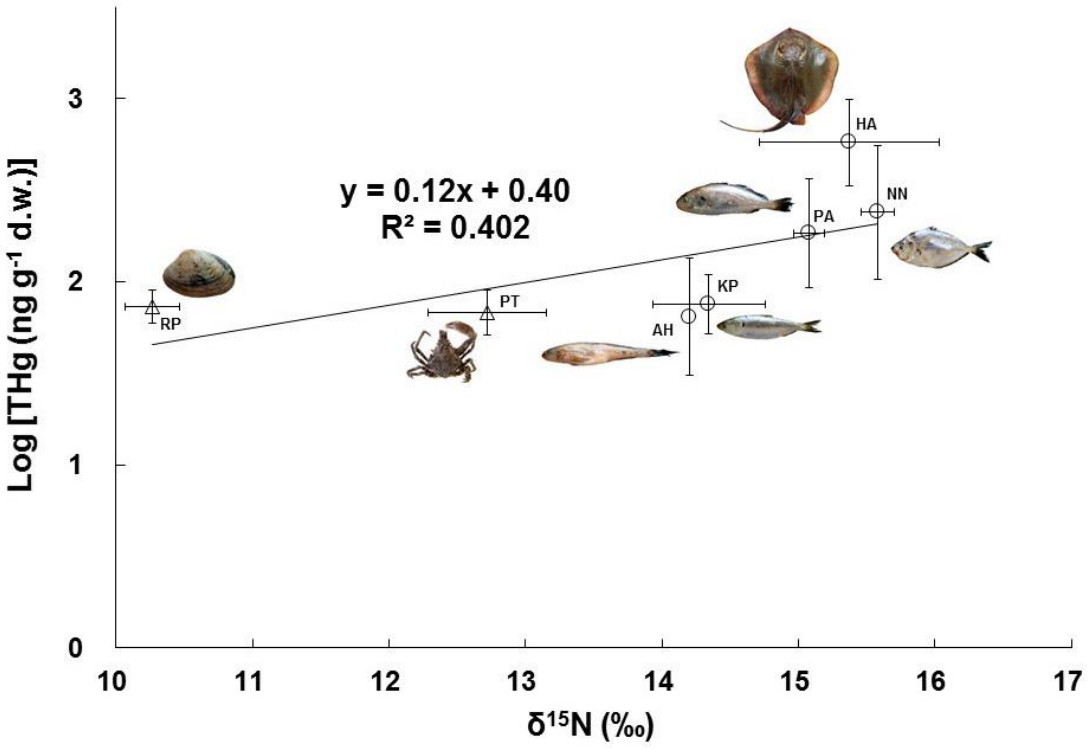

Figure 8 Relationships between $\delta^{15}N$ signatures and $\log_{10}$ - transformed of THg content of the body tissues of seven species of fishes and invertebrates collected in Isahaya Bay. The data of the plots are noted in Table 3. Error bars represent standard deviation of the mean values.

3.2.3 Accelerated bioaccumurate of MeHg in bentho-pelagic food web

Figure 9 shows the relationship between log-transformed values of the three different species of mercury (THg, InHg, and MeHg) and the TL of the seven species of fishes and invertebrates in the bentho-pelagic food web of Isahaya Bay. Significant positive linear relationships were found between both of the log-transformed THg and MeHg contents and the TL of these animals (THg: log THg = 0.42x + 0.83, x: TL, $r^2 = 0.402$, Spearman's correlation coefficient by rank, z = 2.012, $p = 0.044$; MeHg: log MeHg = 0.79x - 0.47, $r^2 = 0.761$, Spearman's correlation coefficient by rank, z = 2.275, $p = 0.023$), and the values of the slopes of these two regressions indicate that MeHg is trasfered to the animals located at the higher TL almost twice more efficiently to THg. However, the relationship between the log-transformed InHg contents and the TL was non-significant (log InHg = -0.40x + 2.39, x, $r^2 = 0.373$, Spearman's correlation coefficient by rank, z = -0.875, $p = 0.382$), and in contrast it appears that the InHg values tend to decrease in the animals located at the higher TL. It indicates that some parts of InHg are apt to be excreted from

the bodies of each animals.

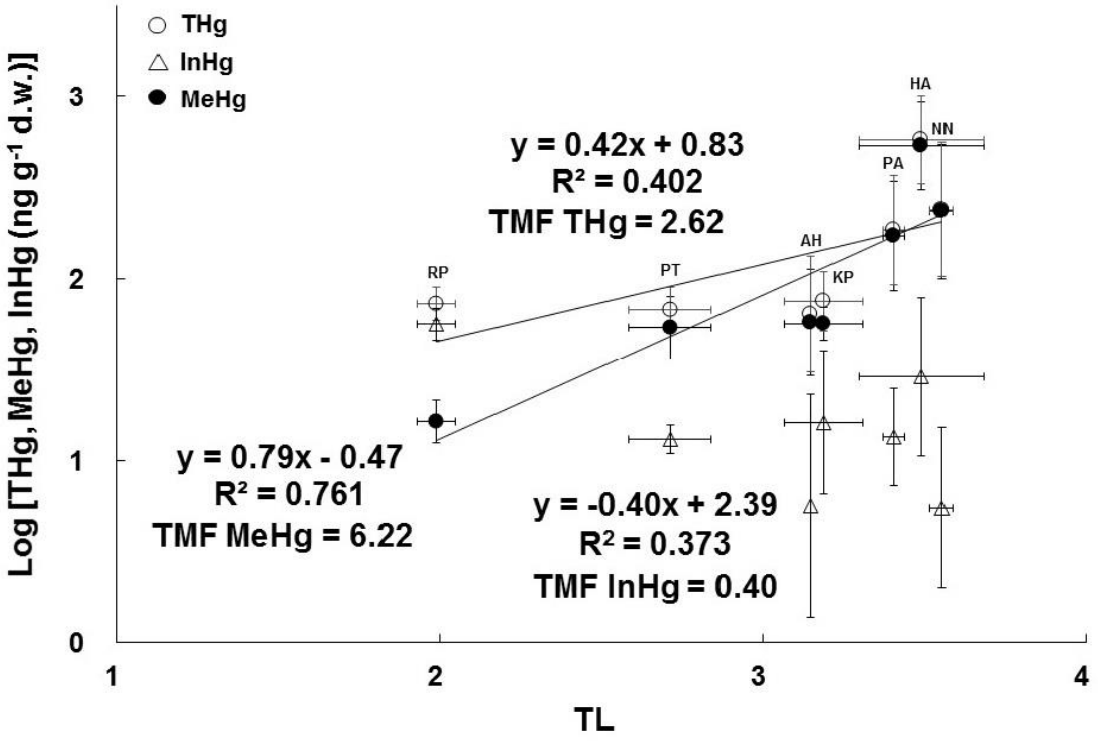

Figure 9 Relationships between TL and $\log_{10}$ - transformed of mercury species content of the body tissues of seven species of fishes and invertebrates collected in Isahaya Bay. The data of the plots are noted in Table 3. Error bars represent standard deviation of the mean values.

3.2.4 Selective transfer of MeHg to the animals at higher trophic positions

Figure 10 shows the relationship between the $\delta^{15}N$ signature and the ratio of the MeHg content to that of THg of the body tissues of the animals collected in Isahaya Bay. The ratio of the MeHg content to that of THg significantly increased to the $\delta^{15}N$ values (%MeHg = 12.92 x + 101.33, x: $\delta^{15}N$ value, $r^2 = 0.877$, Spearman's correlation coefficient by rank, z = 2.178, p = 0.03). The ratio is 22.5 ± 2.5% (n=4) in the bivalve, *R. philippinarum*, while the mean values of those of the fishes (*Pennahia argentata*, *H. akajei*, and *Nuchequula nuchalis*) with the $\delta^{15}N$ values over 15 ‰ exceeded 90 % (92.7 ± 1.0% to 97.6 ± 0.7%) (Table 3).

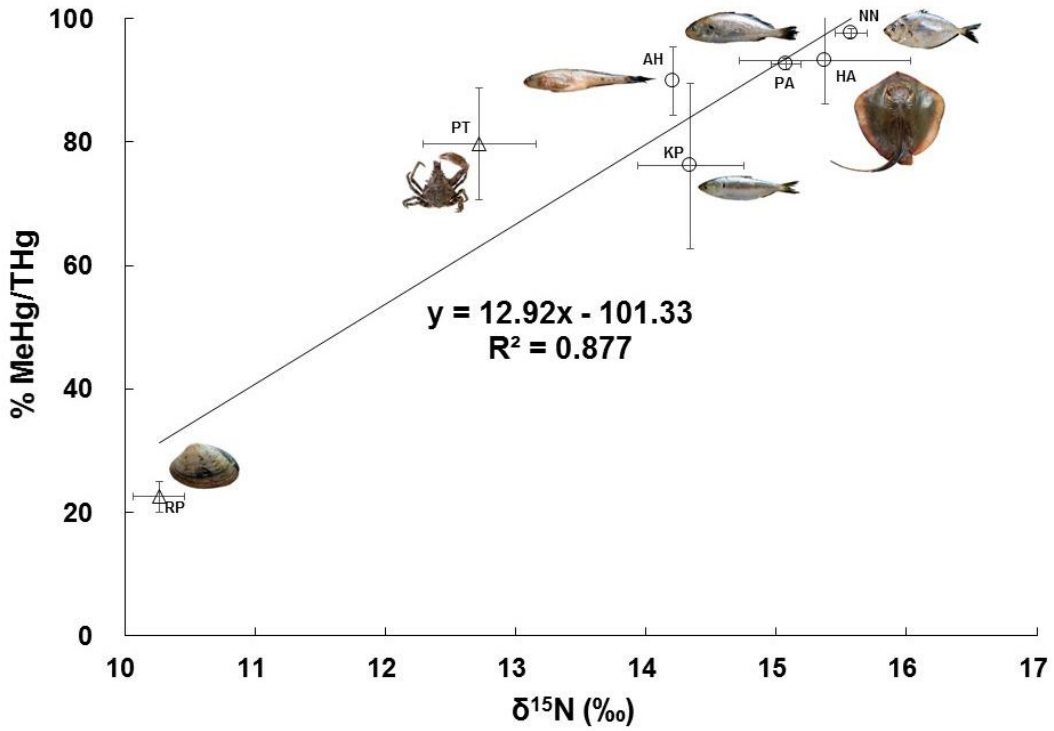

Figure 10 Relationship between the $\delta^{15}N$ signature and the ratio of the MeHg content to that of THg of the body tissues of the seven species of animals collected in Isahaya Bay. The data of plots are noted in Table 3. Error bars represent standard deviation of the mean values.

4. Discussion

4.1 Accelerated bioaccumulation of THg in red stingray by ontogenetic changes of feeding habits

According to the common knowledge of the biomagnification of heavy metals to the organisms in the aquatic ecosystem, the contents of the contaminants increase discontinuously at each step of the trophic levels in the food web systems (Atwell et al. 1998; Lavoie et al. 2010; Clayden et al. 2014; Braune et al. 2015). Aquatic predatory animals with large body sizes such as ray, shark, and dolphin are often located at the highest position. Extremely high levels (mg g^{-1} d.w. levels) of heavy metals including mercury tend to accumulate in the muscles and some organs such as gills and livers of the adults with large body sizes of 10 to 100 kg or more in dolphin and whale (Atwell et al. 1998), shark (Al-Reasi et al. 2007; Endo et al. 2008, 2009, 2013, 2015; Suk et al. 2009; Biton- Porsmoguer et al. 2018), ray (Gutiérrez-Mejía et al. 2009; Horvat et al. 2014; Murillo-Cisneros et al. 2018), skate (Taylor et al. 2014), ray and guitarfish (Murillo-

Cisneros et al. 2019). However, it is not easy to show the representative contents of these substances in the body of each species, nor easy to compare their species-specific values, since they have accumulated them in the body throughout their long lives, having accelerated them along their growth.

In some cases of rays and sharks, ontogenetic changes of the feeding habits occur following the growth, and they bring a further elevation of the trophic position in the food web systems, and increase of mercury contents in their bodies (Endo et al. 2008, 2009; Dale et al. 2011; Lyons et al. 2017). In this study, the results of the stomach content analysis of red stingray clearly showed the ontogenetic changes of the feeding habits between the immature and mature females, too (Figure 3). The immature individual has undeveloped small teeth of less than 500 μm in diameter, sparsely distributed in the mouth (Figure 11(A)), while the mature one has much larger teeth (about 5 mm in length), overlapping each other (Figure 11(B)) as reported by Taniuchi and Shimizu (1993). Therefore, the immature ones can only eat the animals with soft body structure such as shrimp, crab, small fish, etc., but the developed teeth of mature ones enable them to feed on bivalves with hard shells.

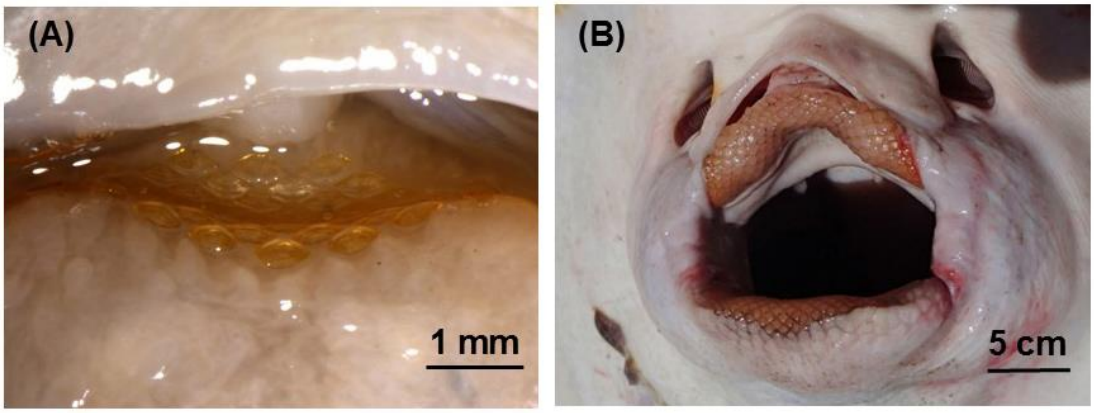

Figure 11 Teeth of female red stingray, *Hemitrygon akajei*, caught in Isahaya Bay.
(A) Immature individuals with the disc width of 12 cm, (B) Mature individuals with the disc width of 59 cm.

The ontogenetic changes of the feeding habits exceptionally led a descending order of the trophic position in the food web system to the mature females (Figure 4 and 6) since the immature ones tended to prefer epifaunal macro-benthic animals as the secondary consumers involving Arthropoda such as shrimps and crabs for diets, while the mature ones are apt to prey on infaunal macro-benthic animals as the primary consumers mainly consisting of short-neck clam, *R. philippinarum*, and polychaete, *N. ijimai* (Figure

3). Nevertheless, the mature females were followed by the accelerated accumulation of THg content in their muscles (Figure 5).

The key factor that raised the THg contents in the muscles of the mature females of red stingray occurring in Isahaya Bay is, therefore, the ontogenetic changes of feeding habits between the immature and mature females. The most preferred prey items by the mature females after the ontogenetic changes of the feeding habits have occurred, are infaunal macro-benthic animals, which belong to “High THg content group” of the primary consumers, while the ones by the immature females before the ontogenetic change are epifaunal macro-benthic animals, belong to “Low THg content group” of the secondary consumers. They have significantly lower THg contents than “High THg content group” of the infaunal primary consumers (Jaingam et al. 2018a).

The mercury contained in the muscles of the infaunal macro-benthic animals classified as members of “High THg content group” of the primary consumers is derived from one emitted from an active volcano via two steps of bioconcentration processes in the water and the sediment (Tsutsumi et al. 2019). It can, therefore, be concluded that the unique biomagnification of THg to the muscles of mature red stingray females observed in Isahaya Bay has been proceeded by the combined effects of the emission of mercury during volcanic activity, deposition of high levels of THg in the sediment in the bay by the two steps of bioconcentration processes by phytoplankton in the water and the bottom sediment and transference of the THg deposited on the sediment to the infaunal macro-benthic animals burrowing the sediment, and its ontogenetic changes of feeding habits from the epifaunal macro-benthic animals to the infaunal ones between the immature and mature females.

The ontogenetic changes of main food items of red stingray are not clearly observed from both males and females in the population occurring in Tokyo Bay (Taniuchi and Shimizu 1993). It mainly favors to prey on small fish, crustaceans including shrimp, mantis shrimp, and crab irrespective of sexual maturity and growth. The difference of feeding habits between Isahaya Bay and Tokyo Bay seems to be responsible for the topography of the shore. In Tokyo Bay, the sandy tidal flats where dense patches of clam are often established have disappeared widely due to reclamation in the past five decades, and the stock of clams has markedly declined in the bay (Toba 2004). Nowadays, it is difficult for red stingray to find clams on the shore in Tokyo Bay even if it favors to prey on the clams.

In this study, all of the 22 specimens of red stingray collected from Isahaya Bay contained THg between 241 and 1,370 ng g^{-1} d.w., and had an increasing tendency of THg content in the muscles to the growth (Figure 5). The provisional reference value of THg

for fish and shellfish is 400 ng g^{-1} w.w. in Japan (UNEP 2002; National Institute for Minamata Disease, Ministry of Environment 2013), which is equivalent to 2,000 ng g^{-1} d.w. (Jaingam et al. 2018b). According to the relationship between the disc width and THg content of red stingray in this study (Figure 5), the THg content of the muscles of the individual that grows up to a disc width of 84.2 cm reaches the reference value. Its body weight is estimated to 26,713 g w.w. from the equation between the disc width and body gained from the specimens of this study ($BW = 0.03DW^{3.09}$, BW: body weight, DW: disc width). Therefore, it seems to be safe to eat the market size (4 to 5 kg w.w., 50 to 60 cm in disc width; less than 10 kg w.w.) of red stingray from the standing point of the provisional reference value of THg.

4.2 Selective transfer of MeHg in the bentho-pelagic food web

The mean $\delta^{13}C$ and $\delta^{15}N$ value of the bentho-pelagic animals caught in the present study ranged from -16.6±0.3 ‰ to -15.1±0 ‰, and 10.3±0.2 ‰ to 15.6±0 ‰, respectively. The range of $\delta^{13}C$ (1.5 ‰) and $\delta^{15}N$ value (5.3 ‰) indicate that there are at least three trophic levels (TL) of animals found in the bentho-pelagic food web in the present study. (Tiews et al. 1973; Taniuchi and Shimizu 1993; Furota 1996; Nakamura 2004; Inoue et al. 2005; Han et al. 2013; Koh et al. 2014; Duangdee et al. 2021).

In the present study, TMF value was used for estimating the biomagnification of each of the three different mercury species (THg, InHg, and MeHg) in the food webs system. It represents an average rate of increase of the mercury species over multiple trophic levels in the food webs, and advantageous for comparing the degree of biomagnification among the mercury species in the food web and that among different food webs or different ecosystems (Borgå et al. 2012). The results of TMF values of the mercury species in this study revealed that the biomagnification of MeHg of the muscles proceeded approximately 2.4 (6.22/2.62) times faster to that of THg toward the animals located at the higher trophic positions linked with a bentho-pelagic food web system, while the InHg contents tended to be depressed as the trophic positions ascended (Fig. 9). These contrastive changes of the contents among the three different mercury species along the food web system seem to reflect the differences of their chemical characteristics and biochemical responses in the internal cells of the animals.

In fish, MeHg can migrate into the cells by binding to cysteine amino acid and sulphur-containing molecules such as glutathione (GSH), and transported by an L-neutral amino acid transporter system. Feeding activity is the major pathway of MeHg bioaccumulation, while uptake from water was at most 15%. About 10% of ingested MeHg is retained in the brain with the remainder transported to the liver and kidney where

it is excreted through bile and urine (Hall et al. 1997; Morcillo et al. 2017; Man et al. 2019). Thus MeHg tends to deposit in the inner parts of various organs of the fishes. In contrast, the InHg complexes (e.g., $HgCl_2$) are apt to be excreted from the body tissues. Some previous studies estimated that it excreted outside the bodies three folds faster than MeHg (Mason et al. 1995; Trudel and Rasmussen 1997). In this study, a statistically reliable attendance was not found between the TL values and the InHg content of the muscles among the seven species of the animals linked with the bentho-pelagic food web system (Fig. 9). However, it appears that the InHg content decreases approximately less than half as the trophic position of the animals ascends one step. Therefore, we need to pay more attentions to the risk of the-accumulation of MeHg to the fishes that have been utilized for sea foods.

4.3 Potential risk of mercury to human health through the consumption of fishes collected in the coastal seas

In Japan, the provision reference value for fish and shellfish is 0.4 μg g^{-1}w.w. in THg, while that is 0.3 μg g^{-1}w.w. in MeHg (UNEP 2002; National Institute for Minamata Disease, Ministry of Environment 2013). The value of THg is only approximately 1.3 times higher than that of MeHg. According to the results of this study, the MeHg content of the muscles of short-neck clam as the primary consumer occupied approximately 22.5 % of the THg content, while its ratio increased to more than 90 % in the predatory fishes such as silver croaker (*P. argentata*; TL= 3.4, 92.7 %), red stingray (TL= 3.5, 93.1 %), and spot nape ponyfish (*N. nuchalis*; TL= 3.6, 97.6 %) (Table 3). These facts indicate that we need to pay more attention to the effects of the selective transfer of MeHg in the food web system in the coastal seas on the mercury content of the fishes used for diets.

For example, in the present study, the highest mean THg and MeHg content was detected from the red stingray (Table 3). Its two large individuals of mature females (56 cm of the disc width and 6,450 g w.w. of body weight, 63 cm of the disc with and 8,615 g w.w. of the body weight) contained the max. THg of 1,280 and 1,430 ng g^{-1} d.w., respectively, which are equivalent to 0.28 μg g^{-1} w.w. and 0.27 μg g^{-1} w.w.). These values do not exceed the provisional reference value of THg for fish and shellfish in Japan, 0.4 μg g^{-1} w.w. (UNEP 2002; National Institute for Minamata Disease, Ministry of Environment 2013) (Figure 12). However, since 94.1 % and 96.8 % of the THg contents are made up of MeHg in these two individuals, their MeHg contents were 1,210 ng g^{-1} d.w. and 1,380 ng g^{-1} d.w., respectively, and equivalent to 0.26 μg g^{-1} w.w. of MeHg contents (Table 4). They are very close to the provisional reference value of MeHg for

fish and shellfish in Japan, 0.3 μg g^{-1} w.w.. This MeHg reference level is based on the Provisional Tolerable Weekly Intake: 0.17 mg MeHg (0.4 μg kg^{-1} body weight per day (UNEP 2002).

Fortunately, the red stingray is nowadays not a popular sea food, but we need to revise the present understanding to the simple application of the provision reference value of mercury to the fish and shellfish, in particular THg, and also consider various conditions of each species, such as body size, its trophic position in the food web for the safety as diet.

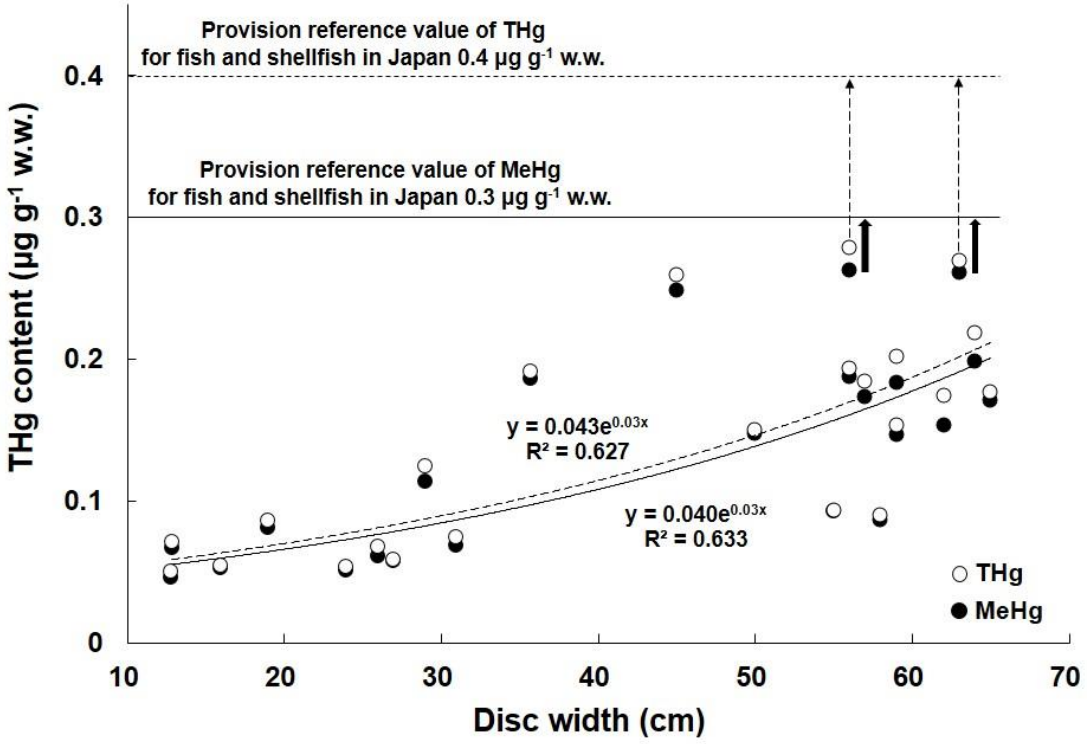

Figure 12 Relationships between Disc width and mercury species content of the body tissues of females of red stingray, *Hemitrygon akajei,* collected in Isahaya Bay. Open circles: THg content (ng g^{-1} w.w.), Closed circles: MeHg content (ng g^{-1} w.w.). The data of the plots are noted in Table 4.

5. Conclusions

In the present study, the exponential relationship was found between the disc width and body weight ($BW=0.03DW^{3.09}$, DW: Disc width, BW: Body weight) of 22 individuals of red stingray captured in the Isahaya Bay. The individual with the disc width of approximately 19 cm and the body weight of 240 g was estimated as one of four years old. The sex ratio of the specimens was extremely biased to female (20 individuals of the total 22 ones). The mature females with the disc widths between 56 and 65 cm seem to be regarded as individuals at least 10 to 15 years old. Since the period required to reach the size at sexual maturity is significantly different between male and female in red stingray, this study deal with food items, trophic positions in the food web system, and THg contents of the muscles among the specimens of immature females, ones in transitional maturity, and mature ones.

The stomach content analysis of the female red stingray exhibited the presence of ontogenetic changes of feeding habits, including marked changes of the food items following the growth. In the immature females, the preys were mainly made up of Arthropoda (75.4 %) in %IRI, involving other crabs (42.3 %) and shrimp (33.1 %), small fishes as Chordata (16.5 %), and Annelida (8.1 %). In the ones in transitional maturity, the %IRI value of Arthropoda decreased to 34.6 %, while those of Mollusca and Annelida increased to 2.1 % and 21.9 %. In the mature ones, the foods items mainly consisted of Mollusca (45.2 %) and Annelida (21.6 %). They exclusively favored the short-neck clam, *Ruditapes phillipinnarum* (45.0 %), and the polychaete, *Nectoneanthes ijimai* (21.6 %).

The ontogenetic changes of feeding habits of red stingray are responsible for the development of mouth. In immature females, the mouth has undeveloped small teeth of less than 500 μm in diameter, and they sparsely distributed in it, while that of the mature ones has much larger teeth (about 5 mm in length) overlapping each other. Therefore, the immature ones can only feed on the animals with soft body structure such as shrimp, crab, small fish, etc., which are epifaunal macro-benthic animals occurring mainly on the sediment surface. The developed teeth of mature ones enable them to prey on bivalves with hard shells which burrow in the sediment. Thus, the ontogenetic changes of feeding habits, which are derived from the development of the mouth structure, bring this species drastic changes of the main food items.

The results of stable isotope analysis of carbon and nitrogen of the muscles of the female red stingray revealed that all three different sexually mature levels of the individuals (immature, intermediate, mature) preyed on the diets nutritionally sustained by the same primary producers, but their trophic positions tend to significant descend in

the food web system from immature female ($\delta^{15}N$: 15.7 ± 0.6 ‰; n=7) to mature one ($\delta^{15}N$: 15.0 ± 0.6 ‰; n=10) following its growth. This phenomenon was caused by the ontogenetic changes of feeding habits, but it also brought this species another influence, an accelerated accumulation of mercury in its muscles.

The THg contents of the muscles of 20 individuals of females red stingray indicated that the THg content significantly increased with its disc width ($y = 160.0e^{0.03x}$, x = the disc width, y = the THg content of the muscles). The THg contents of the immature females with the disc widths of 16 to 31 cm were 309 ± 76 ng g^{-1} d.w. (n=7), while they increased to 869 ± 268 ng g^{-1} d.w (n=10) in the mature ones. The highest value of the THg content reached 1,370 ng g^{-1} d.w.. The results of the THg contents of the main food items of the red stingray indicated that the acceleration of THg content of the muscles following the growth derived from the high levels of mercury that were contained in the body tissues of the infaunal macro-benthic animals which were favored by the mature ones.

In the Isahaya Bay, seven species of bentho-pelagic animals (*R. phillipinnarum*, *Pyromata tuberculata*, *Amblychaeturichthys hexanema*, *Konosirus punctatus*, *Pennahia argentata*, *Hemitrygon akajei*, and *Nuchequula nuchalis*) caught in the present study indicated that they were liked in the single food web system nutritionally supported by the same primary producers and classified to three different trophic levels (primary, secondary, and tertiary consumers) with the results of stable isotope analysis of carbon and nitrogen. In this bentho-pelagic food web system, the mercury is originally discharged from the volcanic activities of Mt.Unzen Fugendake. It deposits in the sediment of the sea floor, being concentrated on the two steps of the bioconcentration process by the absorption of mercury from the water by phytoplankton and deposition of mercury contained in the dead bodies of the phytoplankton on the sediment. It is partly transferred to the infaunal macro-benthic animals, including the short-neck clam and polychaetes, in the sediment. In this trophic level, the majority of the mercury contained in the muscles is InHg (e.g. *R. philippinarum*: 77.5 %). However, MeHg tends to be selectively transferred to the animals located at the higher trophic positions ($y=12.92x - 101.33$, x = $\delta^{15}N$, y = %MeHg/THg, $r^2=0.877$). In the fishes located at the trophic level of 3.4, more than 90 % of the mercury contained in the muscles is occupied by MeHg (e.g. *P. argentata*: 92.7 %).

In the case of red stingray, which contain the highest levels of mercury among the fishes caught in the bay, mature females with the disc size of 60 ± 3 cm and 8,175 ± 1,023 g w.w. contained 946 ng gd.w.$^{-1}$ of THg in the muscles. 94.1 ±3.2 % of the THg content was occupied by MeHg. Therefore, the MeHg content was equivalent to 0.182 ± 0.052

μg gw.w.$^{-1}$, and the highest value reached 0.262 μg gw.w.$^{-1}$. This value is very close to the Japanese provisional reference one of MeHg for fish and shellfish, 0.3 μg gw.w.$^{-1}$. Even the natural release of mercury from a volcano has an effect that creates unsuitable conditions of the mercury content in some fishes through the accelerated bioaccumulation in the food web system in the coastal area.

Table 1 Morphometric characteristics, sex, maturity condition, carbon and nitrogen stable isotope and total mercury contents of red stingray, *Hemitrygon akajei,* collected in Isahaya Bay, Kyushu, western Japan. (T= Female in transitional maturity)

Specimen No.	Disc width (cm)	Weight (g w.w.)	Sex / Maturity	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	THg content (ng g^{-1} dw)
01	16	140	Female / Immature	-14.9	16.7	248
02	19	240	Female / Immature	-15.5	15.5	358
03	24	445	Female / Immature	-17.2	14.7	260
04	26	545	Female / Immature	-16.7	15.5	310
05	27	730	Female / Immature	-15.5	15.7	241
06	29	885	Female / Immature	-15.4	15.8	454
07	31	1,375	Female / Immature	-15.2	16.1	295
Mean ± S.D.	25 ± 5	623 ± 421	Female / Immature	- 15.8 ± 0.8	15.7 ± 0.6	309 ± 76
08	39	2,340	Male/ Mature	-15.1	16.2	455
09	42	2,775	Male/ Mature	-16.3	16.0	662
10	45	4,150	Female / T	-16.4	15.1	918
11	50	5,335	Female / T	-17.4	15.3	581
12	55	6,315	Female / T	-15.6	16.0	393
Mean ± S.D.	50 ± 5	5,267 ± 1,084	Female / T	-16.5 ± 0.9	15.5 ± 0.5	631 ± 266
13	56	6,450	Female/ Mature	-16.0	15.4	1,160
14	56	7,655	Female/ Mature	-15.0	14.2	802
15	57	7,030	Female/ Mature	-16.1	14.7	777
16	58	7,760	Female/ Mature	-15.5	15.3	411
17	59	7,600	Female/ Mature	-15.2	15.6	606
18	59	9,120	Female/ Mature	-13.8	15.5	858
19	62	8,775	Female/ Mature	-16.2	14.7	807
20	63	8,615	Female/ Mature	-15.9	14.9	1,370
21	64	9,200	Female/ Mature	-14.9	15.8	955
22	65	9,540	Female/ Mature	-15.0	14.3	949
Mean ± S.D.	60 ± 3	8,175 ± 1,023	Female/ Mature	-15.4 ± 0.7	15.0 ± 0.6	869 ± 268
23	33	1,677	Female / Immature	-14.8	12.3	457
24	33	2,016	Female / Immature	-15.0	13.6	336

* Specimen 23 and 24 were the individuals collected in Isahaya Bay and determined the THg content of the muscles with the same methods in the previous study (Jaingam et al. 2018b).

Table 2 Identifiable prey items found in the stomach contents of immature females (n=7), females in transitional maturity, (n=3), and mature ones (n=10) of red stingray collected in Isahaya Bay. The data are represented as Percentage number composition (%N), Percentage weight Composition (%W), Percentage of occurrence (%O), and as Phylum level for Percentage of the index of relative importance (%IRI). 71.7 %, 53.5 % and 58.0 % of the weights of the stomach contents were identifiable to at least phylum in the stomach specimens of immature, females in transitional maturity, and mature females, respectively.

	Immature individuals (n=7)				**Individuals in transitional maturity (n=3)**				**Mature individuals (n=10)**			
	%N	%W	%O	%IRI	%N	%W	%O	%IRI	%N	%W	%O	%IRI
Mollusca												
Ruditapes philippinarum					7.1	1.0	33.3	2.1	35.8	52.4	60.0	45.0
Theora lata									1.9	0	10.0	0.2
Total					**7.1**	**1.0**	**33.3**	**2.1**	**37.7**	**52.4**	**70.0**	**45.2**
Annelida												
Nectoneanthes ijimai					7.1	0.0	33.3	3.7	28.3	9.4	80.0	21.6
Other polychaete	23.1	1.6	42.9	8.1	35.7	0.0	33.3	18.2				
Total	**23.1**	**1.6**	**42.9**	**8.1**	**42.9**	**0.0**	**66.6**	**21.9**	**28.3**	**9.4**	**80.0**	**21.6**
Arthropoda												
Shrimps	15.4	89.0	14.3	33.1	7.1	53.2	33.3	30.8				
Pyromaia tuberculata									13.2	11.5	20.0	7.1
Other crabs	30.8	2.5	57.1	42.3	7.1	0.3	33.3	3.8	3.8	0.1	20.0	1.1
Total	**46.1**	**91.5**	**71.4**	**75.4**	**14.3**	**53.5**	**66.6**	**34.6**	**17.0**	**11.5**	**40.0**	**8.2**
Chordata												
Total	**30.8**	**6.9**	**57.1**	**16.5**	**35.7**	**45.5**	**66.7**	**41.4**	**17.0**	**26.7**	**80.0**	**25.0**

Table 3 Morphometric characteristic, stable isotope ratios of carbon and nitrogen, trophic level, and mercury contents of macro-benthic invertebrates and fishes collected in Isahaya Bay, Kyushu, western Japan. (mean ± S.D.) PC = primary consumers, SC = secondary consumers, IC = intermediate consumers

Species	Code	Group /Ecotype	Length (cm)	Weight (g)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	Trophic Level	THg (ng g^{-1} d.w.)	MeHg (ng g^{-1} d.w.)	InorHg (ng g^{-1} d.w.)	MeHg/ THg (%)	n
Ruditapes philippinarum	RP	Bivalve/PC	4.0±0.2	13.7±2.7	-16.6±0.3	10.3±0.2	2.0±0.1	74.3±16.7	16.9±5.00	57.5±12.2	22.5±2.5	4
Pyromata tuberculata	PT	Crab/SC	3.0±0.2	6.2±0.8	-16.3±0.4	12.7±0.4	2.7±0.1	69.4+19.3	56.2±21.7	13.2±2.3	79.7±9.0	3
Amblychaeturichthys hexanema	AH	Fish/IC	12.9±1.6	14.2±7.7	-15.6	14.2	3.1	76.6±53.6	67.2±44.4	9.50±9.2	89.8±5.5	3 (1)
Konosirus punctatus	KP	Fish/IC	16.3±1.6	39.5±13.5	-15.6±0.2	14.3±0.4	3.2±0.1	78.7±29.3	57.3±11.4	21.3±19.0	76.1±13.4	3
Pennahia argentata	PA	Fish/IC	17.5±2.4	69.7±26.9	-15.1±0.0	15.1±0.1	3.4±0.0	215±142	200±133	15.4±9.8	92.7±1.0	3
Hemitrygon akajei	HA	Fish/IC	42.5±17.8	4,364.9 ±3,580.7	-15.5±1.0	15.4±0.7	3.5±0.2	661±342	617±329	44.9±48.0	93.1±7.0	25
Nuchequula nuchalis	NN	Fish/IC	8.9±1.3	10.4±3.6	-15.4±0.4	15.6±0.1	3.6±0.04	291±185	284±180	7.20±4.7	97.6±0.7	3

Table 4 Morphometric characteristics, maturity condition, carbon and nitrogen stable isotope and mercury contents of female red stingray collected in Isahaya Bay. (I: immature; T: female in transitional maturity; M: mature)

No.	Disc width (cm)	Weight (g w.w.)	Maturity	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	THg (ng g^{-1} d.w.)	MeHg (ng g^{-1} d.w.)	% MeHg /THg	% d.w. / w.w.	THg (μg g^{-1} w.w.)	MeHg (μg g^{-1} w.w.)
01	13	73	I	14.2	-13.8	409	384	93.9	17.3	0.071	0.067
02	13	67	I	15.8	-13.8	299	274	91.5	16.7	0.050	0.046
03	16	140	I	-14.9	16.7	251	244	97.3	21.4	0.054	0.052
04	19	240	I	-15.5	15.5	406	379	93.5	21.2	0.086	0.080
05	24	445	I	-17.2	14.7	251	239	95.3	21.1	0.053	0.051
06	26	545	I	-16.7	15.5	346	311	89.8	19.5	0.067	0.060
07	27	730	I	-15.5	15.7	237	233	98.4	24.6	0.058	0.057
08	29	885	I	-15.4	15.8	519	472	91.0	23.9	0.124	0.113
09	31	1,375	I	-15.2	16.1	322	299	92.9	22.8	0.074	0.068
10	36	1967	I	15.4	-14.5	887	863	97.3	21.5	0.190	0.185
Mean ± S.D.	23 ± 8	646 ± 621	I	- 15.2 ± 1.1	15.5 ± 0.7	393 ± 135	370 ± 190	94.1 ± 2.9	21.0 ± 2.6	0.083 ± 0.044	0.078 ± 0.020
11	45	4,150	T	-16.4	15.1	1,040	995	95.7	25.0	0.259	0.248
12	50	5,335	T	-17.4	15.3	644	633	98.3	23.3	0.150	0.147
13	55	6,315	T	-15.6	16.0	403	400	99.2	23.0	0.093	0.092
Mean ± S.D.	50 ± 5	5,267 ± 1,084	T	-16.5 ± 0.9	15.4 ± 0.5	695 ± 321	676 ± 291	97.7 ± 1.8	23.8 ± 1.0	0.167 ± 0.079	0.167 ± 0.079
14	56	6,450	M	-16.0	15.4	1,280	1,210	94.1	21.7	0.278	0.262
15	56	7,655	M	-15.0	14.2	928	902	97.2	20.8	0.193	0.187
16	57	7,030	M	-16.1	14.7	893	840	94.1	20.6	0.184	0.173
17	58	7,760	M	-15.5	15.3	421	407	96.7	21.3	0.090	0.087
18	59	7,600	M	-15.2	15.6	721	691	95.9	21.2	0.153	0.147
19	59	9,120	M	-13.8	15.5	966	877	90.7	20.9	0.202	0.183
20	62	8,775	M	-16.2	14.7	871	766	87.9	20.0	0.174	0.153
21	63	8,615	M	-15.9	14.9	1,430	1,380	96.8	18.8	0.269	0.261
22	64	9,200	M	-14.9	15.8	1,030	940	91.1	21.1	0.218	0.199
23	65	9,540	M	-15.0	14.3	903	873	96.7	19.5	0.176	0.171
Mean ± S.D.	60 ± 3	8,175 ± 1,023	M	-15.4 ± 0.8	15.0 ± 0.6	946 ± 277	889 ± 266	94.1 ± 3.2	20.6 ± 0.9	0.194 ± 0.052	0.182 ± 0.052

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