SPECIFIC ACCUMULATION OF HYDROXYLATED POLYCHLORINATED BIPHENYLS (HO-PCBs) AND PCBs IN GREAT CORMORANT (*PHALACROCORAX CARBO*) AT DIFFERENT STAGE OF EMBRYONIC DEVELOPMENT

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Introduction

Persistent organic pollutants (POPs) have been spread globally even to the polar region, and found in various trophic organisms. A wide variety of toxic effects may occur in human and wildlife due to the exposure to these chemicals. It is reported that some kinds of pathological effects appear to occur in thyroid of wild great cormorants in Japan, with indication that the issues may have caused by exposure to dioxins and/or dioxin-like PCBs [1)]. Hydroxylated polychlorinated biphenyls (HO-PCBs) are known as thyroid hormone modulator and we speculated that they might be one of causative compounds. PCBs accumulated in organisms are metabolized to HO-PCBs as one of the major metabolic products. HO-PCBs are known to disturb thyroid hormone action by potentially interfering with the receptor-mediated transcription [2)]. Some HO-PCB congeners inhibit thyroid hormone-dependent dendritic development of cerebellar Purkinje cells in vitro [3)]. In human study, the materno-fetal transfer for HO-PCBs should be more significant during the period of pre-born child in a mother body rather than intake through breast milk after birth [4)]. Since the adult great cormorants accumulate relatively high levels of HO-PCBs [5, 6)], the effects of these compounds to during the period of pre-hatch for this species are particularly concerned. The purpose of this study was to determine the levels and profiles of HO-PCBs and to understand their behavior during embryonic development of great cormorants.

Materials and Methods

The eggs of great cormorants were collected in Lake Biwa, Shiga, Japan in May, 2007. Six egg samples were opened and divided into embryo, yolk, albumen and amnion. These samples were stored at -20℃ until analysis.

The sample (2~8g) was homogenized and acidified with addition of hydrochloric acid (1mL) and isopropanol (1mL), and then $^{13}C_{12}$-labeled HO-PCB standards {4'-HO-CB 29, 4'-HO-CB61, 4'-HO-CB120, 4'-HO-CB159, 4-HO-CB187} and $^{13}C_{12}$-labeled PCBs (CB3, CB15, CB28, CB52, CB118, CB153, CB180, CB194) were spiked onto sample. HO-PCBs and PCBs were extracted in three times with acetonitrile (20mL) under acidic condition.

Hexane was added to acetonitrile extract for partition. Less polar constituents dissolved in the acetonitrile extract was transferred into hexane layer (20mL). The hexane layer, which contained PCBs, was treated with sulfuric acid treatment in order to remove the interfering substances. Acetonitrile layer was mixed in 400mL of 5% NaCl solution (less than pH2) and 50ml hexane. HO-PCBs was recovered in hexane layer and subjected to further clean-up procedures. The HO-PCBs was concentrated and then was passed through silica-gel column (3g; 5% water, w/w). PCBs were recovered with elution of 60mL hexane and put together hexane layer after sulfuric acid, and the PCBs fraction was concentrated under nitrogen gas flow for GC-MS analysis. HO-PCBs were recovered with elution of 100mL dichloromethane/hexane (30% v/v). HO-PCBs in this fraction were methylated with trimethylsilyl diazomethane. After the derivatization, the solution was purified with double layer column containing deactivated (5% water, w/w) silica-gel (2g; upper layer) and activated Florisil column (5g; lower layer). Methylated HO-PCBs were recovered with elution of 50mL dichloromethane/hexane (20% v/v) and concentrated under nitrogen gas flow for GC-MS analysis. The $^{13}C_{12}$-labeled standards (CB138) were spiked onto samples as syringe spike. HO-PCBs and PCBs were analyzed using HRGC (6890 series, Agilent, USA) / HRMS (JMS-800D, JEOL, Japan) at high resolution of 10,000 and selected-ion monitoring (SIM) mode.

HO-PCBs were quantified using MeO-PCB standards derivatized by the same procedure as samples. The peaks that matched the retention times and isotopic ratios of primary and secondary ions of those compounds in the standard solutions were quantified as "identified HO-PCBs" {(4'-HO-CB72, 4'-HO-CB26, 4-HO-CB107, 3-HO-CB153, 4-HO-CB146, 3'-HO-CB138, 4'-HO-CB130, 4-HO-CB187, 4'-HO-CB172}. The peaks that have different retention times from authentic standards but have the same isotopic ratios of primary and secondary

ions were considered as "unidentified HO-PCB congeners". The sum of identified HO-PCBs and unidentified HO-PCBs was referred to total HO-PCBs. Recovery rate of the $^{13}C_{12}$- HO-PCBs and $^{13}C_{12}$- PCBs congeners were 47-88% and 57-104%, respectively, throughout the whole procedures.

Results and Discussion

HO-PCBs and PCBs were detected in all samples analyzed. Sample weight and the HO-PCBs and PCBs concentrations are presented in Table 1. In the study, embryo weight was regarded as an indicator of growth development. Each egg numbers were given in the order of development, for example egg 1 was the earliest stage of growth development, and egg 6 was the latest stage of one. It should be noted that egg 1 consisted of only yolk and albumen, which indicates nearly fresh-laid or unfertilized egg.

Table 1. Concentrations of HO-PCBs (pg/g ww) and PCBs (ng/g ww) in eggs of great cormorant.

		Egg 1		Egg 2				Egg 3			
		Yolk	Albumen	Amniotic fluid	Yolk	Embryo	Albumen	Amniotic fluid	Yolk	Embryo	Albumen
	weight	10.8	12.2	2.16	9.22	2.61	6.98	5.03	6.98	3.8	7.4
3Cl	4'HO-CB26	27	<1.0	<1.0	190	12	<1.0	<1.0	61	<1.0	<1.0
4Cl	4'HO-CB72	49	2.6	31	69	7.4	2.9	22	150	30	<1.0
5Cl	4HO-CB107	75	7.6	32	<1.0	72	9.1	14	<1.0	24	<1.0
6Cl	3HO-CB153	1,800	281	1,100	5,000	4,200	150	370	1,100	820	50
	4HO-CB146	290	51	150	710	450	16	66	180	130	9.4
	3'HO-CB138	300	54	160	1,100	580	39	46	260	110	8.4
	4'HO-CB130	92	16.4	100	320	370	12	34	54	58	3.1
7Cl	4HO-CB187	960	106	550	3,000	1,600	83	190	330	470	26
	4'HO-CB172	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Total HO-PCB(pg/g wet wt.)		8,600	970	3,900	29,000	12,000	720	1,900	8,100	4,000	190
Total PCB(ng/g wet wt.)		700	20.8	20	2,400	330	170	35.9	1,000	99	1.1
HO-PCB/PCB		0.0123	0.0466	0.195	0.0121	0.0364	0.00424	0.0529	0.0081	0.0404	0.173

		Egg 4				Egg 5			Egg 6		
		Amniotic fluid	Yolk	Embryo	Albumen	Amniotic fluid	Yolk	Embryo	Amniotic fluid	Yolk	Embryo
	weight	12.8	7.7	7.84	2.9	11.7	9.21	10.4	4.96	6.8	15.2
3Cl	4'HO-CB26	<1.0	<1.0	<1.0	<1.0	5.3	250	19	12	140	23
4Cl	4'HO-CB72	2.9	120	23	1.8	2.5	81	13	9.7	67	17
5Cl	4HO-CB107	13	25	81	2.1	44	<1.0	250	33	<1.0	150
6Cl	3HO-CB153	170	1,900	1,300	2.9	870	6,600	6,600	540	4,400	4,100
	4HO-CB146	56	130	370	63	190	690	1200	95	260	650
	3'HO-CB138	21	430	140	10	110	2,200	830	81	1,200	400
	4'HO-CB130	16	50	110	9.5	72	360	640	40	97	360
7Cl	4HO-CB187	130	350	980	39	810	2,300	3,200	170	440	2,200
	4'HO-CB172	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Total HO-PCB(pg/g wet wt.)		820	8,100	6,400	210	3,000	30,000	30,000	2,300	19,000	15,000
Total PCB(ng/g wet wt.)		96.9	2,710	419	76.8	89	29,000	430	190	33,000	1,100
HO-PCB/PCB		0.00846	0.00299	0.0153	0.00274	0.0337	0.00103	0.0698	0.0121	0.000576	0.0136

*The sum of identified HO-PCBs (4'-HO-CB72, 4'-HO-CB26, 4-HO-CB107, 3-HO-CB153, 4-HO-CB146, 3'-HO-CB138, 4'-HO-CB130, 4-HO-CB187, 4'-HO-CB172) and unidentified HO-PCBs was referred to total HO-PCBs.

Median and range of total HO-PCBs and PCBs concentrations in each part of eggs were shown in Fig.1. The concentrations of both HO-PCBs and PCBs were in the order of yolk > embryo > amniotic fluid > albumen. The levels of PCBs in the yolk were considerably higher than other parts of eggs. This is likely attributed to the fact that PCBs are lipophilic and yolk contains relatively high amount of lipids. Similar trend of distinct deposition of PCBs in egg yolk have been reported in the domestic chicken [7, 8]. In contrast, the levels of HO-PCBs in the yolk were comparable to the embryo. Previously, the occurrence of HO-PCBs in eggs of wild birds such as gulls and raptors have been reported [9, 10], but the results were based on analysis of whole egg homogenate. To date, the data about distribution of HO-PCBs in avian egg have not been reported as far we are concerned. HO-PCBs was detected in the yolk of egg 1 not identified embryo, indicating the maternal transfer to the eggs. HO-PCBs burden in the yolk of egg 1 accounted for approximately 90% of the total burden in the whole egg (yolk plus albumen). Interestingly, it was found that 95% of thyroxine mass of chicken fresh-laid egg is contained in yolk [11]. It has been suggested that thyroid hormone were transferred from maternal blood into oocytes with bound to lipoproteins and thyroxine transport protein such as transthyretin (TTR) during the avian ovogenesis [11]. It is assumed that some HO-PCBs which structurally resemble to thyroid hormone would be transferred into egg by means of similar mechanism.

Thus, it seems that not only lipophilicity but also other factors such as protein binding should be accounted for the distribution and behavior of HO-PCBs in egg. HO-PCBs are phenolic weak acids, and it is expected that some fractions of them are present as ionized form in vivo according to their pK_a values [12].

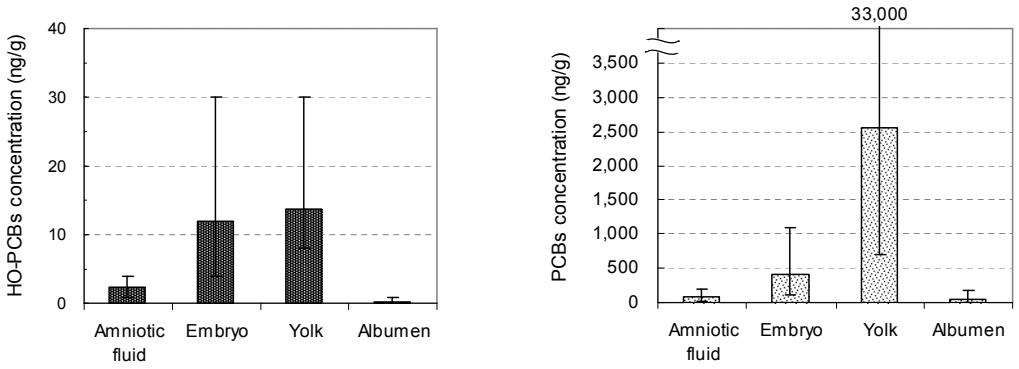

Fig. 1. Median and range of total HO-PCBs and PCBs concentrations in each part of eggs.

And the fact that some HO-PCBs have strong affinity to TTR is well known [12]. Nevertheless, HO-PCBs have certain degree of lipophilic character, for example log K_{ow} values of 4-HO-CB146 and 4-HO-CB187 were estimated at 5.9 ± 0.4 and 6.9 ± 0.4, respectively [12]. Meanwhile, Sandermann [13] investigated partitioning between non-polar triglycerides and polar phospholipids for 20 xenobiotics using trioleoylglycerol (TG) and egg yolk phosphatidylcholine (PC). The results showed that the partitioning coefficients ($K_{TG/PC}$) values of penta-PCB and 2,4,6-trichlorophenol were calculated as 5.9 and 0.48, respectively. Therefore, it is suspected that HO-PCBs particularly lower chlorinated ones possibly have relatively higher affinity to phospholipids rather than triglycerides. The described presumption might be attributed to the distribution of HO-PCBs in egg and their absorption to embryo, as egg yolk contains relatively abundant phospholipids.

The composition of both HO-PCBs and PCBs were mainly consisted of penta, hexa and hepta-chlorinated congeners in all samples. The ratio of HO-PCBs/PCBs was 1/100 -1000, which is similar to the case of organ tissues of adult great cormorant [5]. In the previous paper, we reported that HO-PCBs were mainly distributed in blood with an average concentration was 2 ng/g wet weight in adult great cormorants at same region [5]. Meanwhile, the concentrations of HO-PCBs in the embryo analyzed in the present study ranged from 4 to 30 ng/g wet weight. These results imply that the developing embryos of this species could be more exposed to the high levels of HO-PCBs than adults.

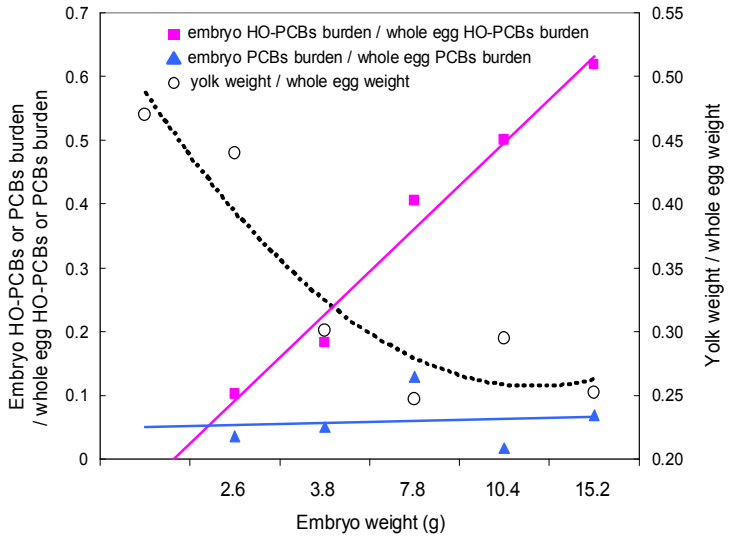

Fig. 2. Variation of ratios of embryo HO-PCB /whole egg HO-PCBs burdens and ratios of embryo PCB /whole egg PCBs burdens at different embryonic growth stages.

Variation of ratios of embryo HO-PCBs /whole egg HO-PCBs burdens, ratios of embryo PCBs /whole egg PCBs burdens and ratios of yolk /whole egg weight at different embryonic growth stages were shown in Fig. 2. The ratios of yolk weight were obviously decreasing according to embryonic development. A gradual uptake and exponentially accumulation of PCBs with increasing lipid absorption in chicken embryo especially during the late stage of development have been observed [7, 8]. As shown in Table 1, the concentration of PCBs in the cormorant embryo at latest growth stage was elevated compared to other growth stage. In contrast, the ratios of PCBs burdens in embryo were no significantly different through during these growth stages. It appears that a significant amount of PCBs mass remained external to the embryo peculiarly yolk until these stage

of development. Bargar et al. [7] reported that greater than 70% of the PCB (PCB105, 156, and 189) burden in the chicken whole egg remained with in the yolk through 19 day of embryonic development (90% of incubation).

On the other hand, the ratios of HO-PCB burden in embryo were increased gradually according to the embryo development. There are two possible explanations for this process: firstly, HO-PCBs moves to embryo from other parts in egg mainly yolk, and secondly, embryo acquires metabolic capability during the growth and more HO-PCBs were formed from PCBs. In ovo exposure experiment using chicken embryo, it has been shown that PCB77-induced hepatic aryl hydrocarbon hydroxylase activity was significantly increased from early incubation stage (embryonic day 7) through the hatching (day 21), with a maximum at middle stage (day 14). Actually, some congeners of hydroxylated metabolites have been found in the gallbladder of the late stage of chicken embryo exposed to PCB77 in ovo [15]. It is assumed that the formation of HO-PCBs could be more accelerated in the late growing stage, since substantial amount of PCBs is transferred into embryo during the period as described above. However, it must be noted that the involvement of individual variability is not negligible for the observation in the present study because our sample size was quite limited, and more research is needed.

Only few studies have been reported on the toxic effects of HO-PCBs on avian embryo. It was found that Japanese quail exposed to 4-HO-CB107 and 4-HO-CB187 in ovo was neither affected reproductive variables nor plasma thyroid hormone levels despite considerably high dose levels (up to 750 μg/egg), but high mortality of early embryo was evidently exhibited at the higher dose [16]. On the other hand, in ovo exposure to 4'-HO-CB50 (10-1,000 ng/g egg), which known to be possess relatively high estrogenic activity, caused dose-dependent reduction of reproductive tract size after sexual maturation of hatched quail chicks [17]. Thus the toxic effects of HO-PCBs seem to depend on its chemical structure. Since it was confirmed that the cormorant embryo analyzed in this study accumulated various HO-PCB congeners consisted of many unidentified ones, possible multiple effects are notably concerned.

Our results provided the valuable information to consider HO-PCBs exposure in avian embryonic growth stage, and suggested that the distribution and behavior of HO-PCBs in the egg is quite different from those of PCBs. Further research is necessary to identify individual congeners for understanding toxic effect of HO-PCBs.

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