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Dioxin type and mixed type induction of the cytochrome P-450 system of common eider ducklings (*Somateria mollissima*) by PCBs: with indications for biotransformation

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Abstract

In order to investigate the effects of PCBs on biotransformation enzymes and metabolism, 4-week-old common eider ducklings were exposed to single *ip* doses of 3,3',4,4' tetrachlorobiphenyl (CB 77) (5 or 50 mg/kg) or the technical mixture Clophen A50 (Clo A50) (50 or 200 mg/kg). The control group was injected with corn oil only (5 ml/kg). Comparison of CB patterns in adipose tissue of the Clo A50 injected groups with the original Clo A50 mixture revealed specific reduction of congeners with vicinal H atoms in the *meta* and *para* positions, suggesting biotransformation by the monooxygenase system (MO) as the most probable cause. For the group injected with 200 mg Clo A50, a difference in congener pattern was shown between liver and adipose tissue. This indicates either a saturation of the hepatic biotransformation capacity, or slow redistribution of the congeners from the adipose tissue to the site of metabolism (liver). Using only one adipose tissue concentration point in time, indicative biological half-lives for metabolisable congeners were calculated from congener pattern changes, ranging from 3.6 days for CB 44 to 16.1 days for CB 101. CB 77 caused a dose-dependent induction of total cytochrome P450, whole liver cytochrome P450, EROD and PROD activity. On the contrary, Clo A50 had no inducing effects after this specific incubation period. PROD activity measurements suggest that the PROD assay may not be a suitable indicator for CYP2B induction in common eider ducklings. Sex difference and parasitic infection had no influence on the biochemical responses measured. Internal dose–induction response curves were established; a very tentative maximum no effect level on toxic equivalents (TEQ) basis is suggested (7 ng TEQ/g lipid). Under these incubation conditions, exposure to CB 77 and

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Clophen A50 did not influence the infection rates of the intestinal parasite *Polymorphus botulus* (*Acanthocephala*).

Keywords: *Somateria mollissima*; Birds; PCB; 3,3',4,4'-tetrachlorobiphenyl; Cytochrome P-450; Induction; Biotransformation

1. Introduction

In the last decade the population of the common eider duck (*Somateria mollissima*) in the Dutch Wadden Sea has been affected by infections of goose virus hepatitis and infections of the parasitic worm *Polymorphus botulus*. Partly for this reason, the population development has stagnated (Swennen, 1991). The prevailing levels of contaminating components like polychlorinated biphenyls (PCBs) could be a possible cause. It has been suggested that PCBs reduce the resistance and render the birds more susceptible towards infections (Peakall, 1986). Using toxic equivalent values (TEFs) as an estimation for dioxin type toxicity, PCBs mainly were considered to be responsible for the reduced reproductivity of herring gulls (*Larus argentatus*) in Green Bay, Lake Michigan (Kubiak et al., 1989). In the Dutch Wadden Sea, PCBs were the main pollutants suspected to cause adverse effects as well. Seals (*Phoca vitulina*) showed a reduced reproductivity correlated with PCB contamination of the diet (Reijnders, 1986; Boon et al., 1987). Studies where American minks (*Mustela vison*) were dosed with the technical PCB mixtures Clophen A50 (Clo A50) and Aroclor 1248 confirmed these correlations (Kihlström et al., 1992).

The common eider could have reduced resistance towards infections due to PCB contamination. Two mechanisms could be applicable to explain this decrease. In one mechanism, unmetabolised PCBs may act to induce thymic atrophy and reduce immuno-responsiveness in a way similar to that demonstrated for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds in laboratory animals (Kubiak et al., 1989; Safe, 1990, 1993). These effects of TCDD are thought to be mediated by the Ah receptor (Safe, 1990, 1993).

The second mechanism is initiated by phase I metabolism by cytochrome P450 (CYP). Vitamin A and thyroxin are important in the development and maintenance of a functional immune system. Vitamin A is essential for the maintenance of the integrity of epithelia. A reduction of circulatory levels of vitamin A may result in an altered integrity, thereby changing transepithelial passage of micro-organisms. In addition, hypovitaminosis A reduces the effectiveness of the immune system to respond to infective agents. Hydroxylated metabolites of 3,3',4,4'-tetrachlorobiphenyl (CB 77) have been reported to reduce the transport of both vitamin A and thyroxin in the blood of rats (Brouwer and Van den Berg, 1986). For male quail (*Coturnix* sp.) fed on Aroclor 1242, vitamin A reduction was demonstrated as well. On the contrary, female quail remained unaffected (Peakall, 1986).

The difference in sensitivity to toxic effects of CBs between rats and birds could be the consequence of differences in the range of CB congeners that can be metabolised by CYP-dependent hepatic monooxygenase system (HMO). The oyster catcher

(*Haematopus ostralegus*) and other birds appeared to be unable to metabolise CB congeners with vicinal H atoms in the *ortho* and *meta* positions to the bond between both phenyl rings (*o,m* vic. H atoms) with one or more *ortho*-chlorines (*ortho*-Cl), whereas mammals like seals, cetaceans and rats are able to metabolise this type of CB congener when not more than one *ortho*-Cl is present. Birds, apparently, are able to metabolise CBs with vic. H atoms in the *meta* and *para* position (*m,p* vic. H). They share this capacity with seals, rats and the polar bear (Brouwer and Van den Berg, 1986; Borlakoglu et al., 1988; Tanabe et al., 1988; Boon et al., 1989, 1992b; Walker, 1990a; Everaarts et al., 1991).

CB 77 was selected as a TCDD type inducer (Safe, 1990) as well as to clarify whether the same mechanism of vitamin A reduction that was demonstrated in rats is applicable for common eider (Brouwer and Van den Berg, 1986). Clo A50 was selected to have an exposure pattern that is more or less similar to the pattern present in the diet of the common eider ducks (Borlakoglu et al., 1988; Boon et al., 1989). In addition, it is a mixed type of inducer in rats (Boon et al., 1992b). The goals of this study were:

- (i) to get indications for structure-related metabolism of PCBs in *S. mollissima*.
- (ii) to investigate the induction of the HMO system of the common eider duckling by several types of inducers (dioxin type and mixed type).
- (iii) to assess the influence of PCB exposure on the resistance against infection with the parasitic acanthocephalan *P. botulus*.

2. Material and methods

Chemicals

The technical mixture Clo A50 was obtained from Bayer AG (Leverkusen, Germany). CB 77 was obtained from Schmidt BV, Amsterdam (>99% pure, dioxin- and dibenzofuran-free). The 7-ethoxy- and 7-pentoxoresorufin and NADPH were obtained from Boehringer Mannheim (Germany). Resorufin was obtained from Aldrich. All other chemicals for biochemical use were of analytical grade and were obtained from Baker Chemicals. Chemicals used for PCB determination are described in Boon et al. (1985).

Capture and maintenance of the animals

On 21 and 22 May 1990, eider ducklings of approximately 1 day of age were captured in the 'Kroonpolders' on the island Vlieland in the northern part of the Netherlands. They were transferred to outdoor aviaries at the Netherlands Institute for Sea Research (NIOZ). Here, they were marked individually and kept in groups imitating normal raising behaviour. The animals were fed *ad libitum* with dried food pellets (Poultry pellets, Koopmans BV, Leeuwarden, Netherlands). Elaborate descriptions of maintenance are given by Murk et al. (1994).

Treatment

The acclimatisation period lasted 27 days before treatment was started. The ani-

Fig. 1. Chromatogram of the technical mixture Clo A50 under the GC-ECD conditions used. IUPAC numbers of CBs used are given above the peaks.

imals were injected *ip* with PCBs dissolved in corn oil. Two groups were treated with CB 77 at 5 mg/kg or 50 mg/kg body weight (group CB 77⁵, $n = 6$, and CB 77⁵⁰, $n = 16$, respectively). Two groups were treated with the technical mixture Clo A50 in concentrations of 50 and 200 mg/kg (group Clo A50⁵⁰, $n = 6$, and Clo A50²⁰⁰, $n = 16$, respectively). The control group was injected with corn oil only (5 ml/kg, $n = 16$).

To assess whether PCBs would reduce the resistance of the animals towards infections a part of the control group and of the CB 77⁵⁰ group and the Clo A50²⁰⁰ group ($n = 6$ for each) were infected with *P. botulus*, an intestinal parasite belonging to the Acanthocephala. Ripe cystacanths were collected from the shore crab *Carcinus maenas*, which is the intermediate host of the species. They were counted, and placed in emptied freshly killed crabs. These were force-fed to the ducklings (145 cystacanths/animal), 5 days after the injections with PCBs.

The incubation period with PCBs lasted for 10 days. Then, after sedation with diethyl ether, the ducklings were killed by exsanguination via cardiac puncture. Subsequently, the liver was removed and carefully rinsed with an ice-cold 1.15% KCl solution. Part of the liver was wrapped in aluminium foil and immediately frozen in liquid nitrogen for biochemical analyses. Tissues for PCB analyses were stored at -25°C .

PCB analyses

PCB analyses were done according to Duinker and Hillebrand (1978) and Boon et al. (1985, 1992a). Tissues were extracted with Soxhlet extraction using pentane as

solvent for at least 15 h. The column for GC analysis was a 50 m × 0.25 mm SE-54 column (Chrompack) with helium as carrier gas. In contrast to Schulz et al. (1989), the peaks of the *mono-ortho* CBs 105 and 118 were well separated from other CBs (Fig. 1). This enabled quantification on the basis of peak heights.

For adipose tissue analyses, a piece of abdominal fat near the bursa of Fabricius was removed (±60 mg). CB 88 was added before extraction of each sample as a recovery standard. In each series of five samples of extraction either a blank sample or an external standard mixture was incorporated and run through the entire procedure to check for pollution or recoveries. Recoveries of the standard solutions and CB 88 were 90 to 105% typically. If recoveries of CB 88 were less than 90%, the results were not used.

Calculation of CB patterns

To compare the pattern of individual CB congeners of the treated animals with the Clo A50 mixture, the concentration of each CB was expressed as a ratio to a reference congener. In this case, CB 180 was chosen, giving Ratio¹⁸⁰, as based on Boon et al. (1992b). The Ratio¹⁸⁰ of individual birds were combined group-wise to a mean value for each treatment group. The values of Ratio¹⁸⁰ were calculated for Clo A50 as well. With these data, relative ratios (R_{rel}) to Clo A50 were calculated (Eq. 1). Values of R_{rel} close to 1 indicate that the presence of a congener is proportional to its contribution in the technical mixture, while a value $\ll 1$ indicates that a congener is under-represented compared to the technical mixture (see also Boon et al., 1989, 1992b).

$$R_{rel} = \text{Ratio}^{180} (\text{CB in group}) / \text{Ratio}^{180} (\text{CB in Clo A50}) \quad (1)$$

Estimation of half-lives

It is possible to estimate apparent biological half-lives ($t_{1/2}$) for the metabolizable congeners, when the following three assumptions are made:

- (i) First order kinetics for elimination.
- (ii) A monophasic elimination.
- (iii) The reference congener CB 180 is not excreted during the experimental incubation period of 10 days.

Since the experimental incubation period is only 2% of the half-life (494 days), as calculated for CB 180 in the herring gull (*L. argentatus*, Norstrom, pers. comm.), it seems justified to neglect the loss of CB 180 during this period, and assumption (iii) is valid.

To calculate the values of $t_{1/2}$ the following equations were applied:

$$C_T = C_0 e^{(-kT)} \quad (2)$$

Substituting $C_T/C_0 = R_{rel}$ and $t_{1/2} = \ln 2/k$, this can be transformed to:

$$t_{1/2} = -(T \cdot \ln 2) / \ln(R_{rel}) \quad (3)$$

C_T = concentration after incubation period T , C_0 = concentration at start, k = elimi-

nation rate constant, T = incubation period in days ($= 10$ days), $t_{1/2}$ = half-life in days.

Based on Tanabe et al. (1988) and Boon et al. (1989), CB congeners were divided into four groups:

(I) CB congeners with at least *m,p* vic. H atoms, most likely metabolised by CYP2B (Mills et al., 1985). In this case, the number of *ortho*-Cls is irrelevant.

(II) CB congeners with only *o,m* vic. H atoms and one *ortho*-Cl. This class is probably biotransformed by CYP1A (Mills et al., 1985).

(III) CB congeners with only *o,m* vic. H atoms and two or more *ortho*-Cls. These appear to be persistent in Phocoibidae (seals) and Cetacea.

(IV) CBs without any vic. H atoms. These appear persistent in most animals.

The CBs actually considered are given in Table 1.

Absolute differences between theoretical and encountered amounts of CBs were calculated (δ). The theoretical amount in the group of animals (A_t) was assessed by multiplying the Ratio¹⁸⁰ of the CB in Clo A50 by the absolute concentration of

Table 1

Characterisation of the CBs which have been identified

IUPAC no.	Vicinal H atoms		<i>Ortho</i> -Cl	Cl-Substitution pattern
	<i>m,p</i>	<i>o,m</i>		
Group I: CBs with at least <i>m,p</i> vic. H atoms without distinction towards <i>ortho</i> -Cls				
44	+	+	2	2,2',3,5'-TCB
49	+	–	2	2,2',4,5'-TCB
52	+	–	2	2,2',5,5'-TCB
70	+	+	1	2,3',4',5-TCB
97	+	+	2	2,2',3',4,5-PeCB
101	+	–	2	2,2',4,5,5'-PeCB
136	+	–	4	2,2',3,3',6,6'-HxCB
141/179	+	–	2/4	2,2',3,4,5,5'-HxCB/2,2',3,3',5,6,6'-HpCB
151/82	+	+/-	3/2	2,2',3,5,5',6-HxCB/2,2',3,3',4-PeCB
Group II: CBs with only <i>o,m</i> vic. H atoms and 1 <i>ortho</i> -Cl				
105	–	+	1	2,3,3',4,4'-PeCB
118	–	+	1	2,3',4,4',5-PeCB
156/171	–	+/-	1/2	2,3,3',4,4',5-HxCB/2,2',3,3',4,4',5-HpCB
Group III: CBs with only <i>o,m</i> vic. H atoms and at least 2 <i>ortho</i> -Cls				
99	–	+	2	2,2',4,4',5-PeCB
128	–	+	2	2,2',3,3',4,4'-HxCB
138	–	+	2	2,2',3,4,4',5'-HpCB
170/190	–	+/-	2/2	2,2',3,3',4,4',5-HpCB/2,3,3',4,4',5,6-HpCB
Group IV: CBs without any vic. H atoms. CB 180 belongs to this group as well				
153	–	–	2	2,2',4,4',5,5'-HxCB
Reference CB				
180	–	–	2	2,2',3,4,4',5,5'-HpCB

CB 180 in the group ([CB 180], Table 2, Eq. 4). This theoretical amount was multiplied by R_{rel} yielding the encountered amount and subtracted from A_t yielding ∂ (Eq. 5).

$$A_t = \text{Ratio}_{(\text{Clo A50})}^{180} \cdot [\text{CB 180}] \quad (4)$$

$$\partial = A_t - A_t \cdot R_{\text{rel}} \quad (5)$$

TEQ calculations

TEF values were taken from Safe (1990, 1993). The most recent TEF value for each CB was applied. The concentration of each CB was multiplied by its TEF value yielding its toxic equivalent (TEQ). The GC conditions did not offer sufficiently high resolution to separate all CBs present in Clo A50 into independent quantifiable peaks. Therefore, a similar CB distribution was assumed for this batch of Clo A50 as described in Schulz et al. (1989). TEF values and TEQ were recalculated according to the partitioning of the different CBs to this peak and their individual TEF values.

Microsomal preparations

Microsomes were prepared according to Boon et al. (1992a). The final pellet was resuspended and homogenised in a 0.1 M Na-K-phosphate buffer (pH 7.40, containing 20% (v/v) glycerol, 1 mM EDTA, 1 mM dithio-threitol) frozen and stored in liquid nitrogen.

Spectral determinations for CYP

Total CYP (ΣCYP , nmol/mg microsomal protein) was measured according to Boon et al. (1992a). The assay had been optimised for temperature, pH, type of buffer and addition of reductor, for microsomes of *S. mollissima*. All CYP present in the liver (WL-CYP, nmol/liver) was determined by weighing the liver directly after removal and before rinsing it with ice-cold KCl. Using mg protein/liver, the WL-CYP (nmol/liver) could be calculated.

$$\text{WL-CYP} = [\Sigma\text{CYP}] \cdot [\text{mg protein/liver}] \quad (6)$$

HMO activity assays

Protein was determined according to the BIORAD method using bovine serum albumin (Sigma, fraction V) as a standard.

The EROD (as indicator of CYP 1A induction) and PROD (as indicator of CYP 2B induction) assays were used to characterise HMO activity (Burke and Mayer, 1983). The method was optimised for *S. mollissima*; final concentrations were: buffer, 0.1 M Na-K-HPO₄-buffer, pH 7.40; NADPH, 5 mM; substrate concentration, 1 μM ; and protein concentration, 1 mg/ml except for CB 77⁵⁰, 0.33 mg/ml. Since a stable temperature could not be achieved above 30°C, all measurements were performed at this temperature, using a Hitachi F2000 fluorimeter. Stock solutions of 7-ethoxy- and 7-pentoxoresorufin were made in ethanol (maximum addition to the assay 0.5%). Assays were linear for 3 min. This appeared sufficient to assess even PROD activity. Resorufin standards (molecular extinction coefficient used = $48.4 \cdot 10^{-3}$) were added after 3 min as an internal standard.

Parasite infections

Numbers of adult *P. botulus* were counted manually in the intestine.

Statistical treatment

Differences in CB ratios were tested with analysis of variance (ANOVA). TEQ levels and HMO data were log-transformed prior to testing with ANOVA to obtain homogeneity of variance. Covariations were established with the least squares sum method.

3. Results

Comparison of CB patterns

The absolute concentrations of the reference congener (CB 180) in each group are given on a pentane extractable lipid (PEL) basis for adipose tissues and liver (Table 2). The concentrations are averaged values of individual animals in each treatment group. Since parasite treatment had no influence on the absolute CB concentrations (ANOVA, $P = 0.14$, data not shown), the data on parasite and non-parasite treated Clo A50²⁰⁰ groups and CB 77⁵⁰ groups were pooled. In adipose tissue, the mean concentration of CB 180 was increased about twenty-fold for the Clo A50⁵⁰ group, and seventy-fold for the Clo A50²⁰⁰ group as compared to the control group. The standard deviations were large, indicating extensive individual variation. In control and Clo A50²⁰⁰ group, livers were analysed as well. In both cases, the mean concentration in the liver was similar to that in adipose tissue.

Furthermore, the concentration of CB 77 in the adipose tissue of the CB 77⁵⁰ group was about seven times the concentration of the CB 77⁵ group. The liver had a similar concentration on PEL basis as observed in the adipose tissue.

The CBs were grouped on the basis of presence and position of vic. H atoms and number of *ortho*-Cls (see Material and methods). The patterns were compared to each other as ratios (Ratio¹⁸⁰) of CB 180. For each individual animal, these ratios were

Table 2

Absolute concentration of CB 180 found in different tissues in $\mu\text{g/g}$ PEL (pentane extractable lipid) in all groups except the CB 77-treated groups where only the concentration of CB 77 (in $\mu\text{g/g}$ PEL) is given

Treatment	Tissue	mean	s.d.	<i>n</i>
Control	adipose tissue	0.030	0.014	13
	liver	0.031	0.008	3
Clo A50 ⁵⁰ Clo A50 ²⁰⁰	adipose tissue	0.57	0.21	4
	adipose tissue	2.17	2.12	13
	liver	1.80	0.5	4
CB 77 ⁵	adipose tissue	14.0	5.2	6
CB 77 ⁵⁰	adipose tissue	91.2	56.4	12
CB 77 ⁵⁰	liver	86.4	23.5	3

calculated. Subsequently, averages for each ratio were calculated. In the control animals, CBs of group I occurred to a lesser extent in the pattern than the other CB groups (data not shown). The pattern of CBs in the liver of the control group differed from the pattern in the adipose tissue (ANOVA, $P < 0.05$) in the sense that CBs of group I had lower values of Ratio¹⁸⁰ compared to adipose tissue (data not shown).

Under representation of CBs with m,p vic. H atoms was also demonstrated for the

Table 3

Ratios between concentrations of individual CB congeners and a selected reference congener (CB 180; Ratio¹⁸⁰) for the Clo A50 mixture in common eider ducklings

	Mixture	Adipose tissue of the birds								
		Clo A50 ⁵⁰					Clo A50 ²⁰⁰			
CB	Ratio ¹⁸⁰	Ratio ¹⁸⁰	R _{rel}	∂	t _{1/2}	Ratio ¹⁸⁰	R _{rel}	∂	t _{1/2}	
Group I										
44	6.62	0.99	0.15	3.2	3.6	2.09*	0.32	4.7	6	
49	3.64	2.12	0.58	0.9	12.7	3.48*	0.95	0.2	135	
52	14.2	6.78	0.48	4.2	9.4	12.4*	0.87	2.1	50	
70	9.7	5.73	0.59	2.3	13.1	9.16*	0.94	0.7	112	
97	7.26	1.55	0.21	3.3	4.4	3.98	0.55	2.8	12	
101	18.7	12.1	0.65	3.8	16.1	18.1*	0.97	0.9	228	
136	1.89	1.05	0.56	0.5	11.9	1.47*	0.78	0.7	28	
141/179	2.47	2.03	0.82	–		2.45	0.99	–		
151/82	2.13	1.38	0.64	–		1.38	0.83	–		
			Σ _∂	18.1			Σ _∂	12.1		
Group II										
105	9.24	9.77	1.06			10.85	1.18			
118	17.5	19.6	1.12			20.48	1.17			
156/171	2.04	2.11	1.04			2.21	1.09			
Group III										
99	8.69	10.4	1.2			11.15	1.28			
128	3.49	4.02	1.15			3.91	1.12			
138/158	13.8	14.8	1.07			15.23	1.1			
170/190	1.12	1.13	1.01			1.16	1.03			
Group IV										
153	8.44	8.69	1.03			9.07	1.07			
Reference CB										
180	1	1	1			1	1			

Groups of common eider ducklings were injected with 50 or 200 mg Clo A50/kg body weight (Clo A50⁵⁰ or Clo A50²⁰⁰ respectively). Ratio¹⁸⁰ is the average of the individual bird Ratio¹⁸⁰ for a congener. R_{rel} is the quotient of the average Ratio¹⁸⁰ of the animal group and the Clo A50 mixture. The difference in amounts between some congeners which are actually observed in 1 g of PEL and the theoretical amount on the basis of the mixture, is given as ∂ ($\mu\text{g/g}$ PEL). The apparent half-life for a congener is given as $t_{1/2}$ (days). (*Significantly different from CloA50⁵⁰, $P < 0.01$).

Fig. 2. Ratio¹⁸⁰ patterns of the Clo A50 mixture and of the adipose tissue and the liver of the Clo A50²⁰⁰-treated group (*significantly different from adipose tissue at $P < 0.05$). Data of Clo A50²⁰⁰ represent mean values with standard deviation (n is as in Table 2).

Clo A50 treated animals (both Ratio¹⁸⁰ and R_{rel} , Table 3). The R_{rel} indicates clearly whether CBs are underrepresented in the adipose tissue compared to the technical mixture. For both Clo A50⁵⁰ as well as Clo A50²⁰⁰, only CBs with m,p vic. H atoms showed lower values of R_{rel} than other CBs (Table 3). The Ratio¹⁸⁰ of the Clo A50²⁰⁰ group showed higher ratios for some CBs of group I than Clo A50⁵⁰ group (ANOVA, $P < 0.05$, Table 3). The amount of CBs (∂) which disappeared was calculated as well. Although the Clo A50²⁰⁰ animals had a higher R_{rel} than the Clo A50⁵⁰ group, still the summed amount of disappeared CBs is in the same range ($\Sigma\partial$, Table 3).

Also the liver of the Clo A50²⁰⁰ group showed lower Ratio¹⁸⁰ values for the CBs of group I compared to the technical mixture (Fig. 2). Interestingly, significant differences were observed between the adipose tissue and the liver of this group (Fig. 2). The CBs with m,p vic. H atoms showed lower Ratio¹⁸⁰ in the liver when compared to the adipose tissue (ANOVA, $P < 0.05$, Fig. 2). The values of Ratio¹⁸⁰ were similar to the values of the adipose tissue of the Clo A50⁵⁰ group (Table 3, Fig. 2). The summed amount of disappeared CBs of group I in the liver ($\Sigma\partial$) was much higher than the $\Sigma\partial$ of the adipose tissue (56 $\mu\text{g/g}$ PEL, data not shown).

Induction of HMO activities

Each HMO activity of individual eider ducklings was tested for three variables by

3-way ANOVA: (i) CB treatment, (ii) parasite infection and (iii) sex difference. This yielded a three-dimensional matrix of $3 \times 2 \times 2$ (3 types of PCB treatment: control, CB 77⁵⁰, Clo A50²⁰⁰, 2 of parasite infection and 2 sexes). Neither sex, nor parasites nor all their interaction factors had any effect on the response of Σ CYP, WL-CYP, EROD and PROD ($P > 0.4$ for each HMO activity). Since sex difference and parasite infection had no effect on the response for all variables, all results from the same dose level were combined and these final groups, together with CB 77⁵ and Clo A50⁵⁰, were tested with a 1-way ANOVA at 5% level for the effect of CB treatment. The post-hoc testing between sub-groups was restrained to twice. The comparisons selected were control with CB 77⁵ and control and CB 77⁵⁰. The significance level for each comparison to attain, was raised accordingly to 97.5%.

From the PCB treatments, only CB 77-dosed groups had a significant (dioxin type) induction of all variables (Table 4). For both Σ CYP and WL-CYP, values were about 1.5 times the control levels for the CB 77⁵ group and about 3.3-fold increased for the CB 77⁵⁰ group ($P < 0.01$). EROD was induced to a much greater extent: 6.8 and 57 times respectively ($P < 0.01$). Even PROD showed significantly elevated levels in both the CB 77-treated groups. The extent of induction, however, was much less (7-fold maximally). At the doses and time points studied, the mixed type inducer Clo A50 had no effect on any HMO activity.

In addition, the covariation was established between the log of individual CB levels of adipose tissue and the log of HMO variables. The CB levels were transformed to TEQ (see Material and methods) before log transforming. Both the low-dosed group and the high-dosed group of either CB 77 or Clo A50 were used in one covariation. The control group was not integrated because even on a log scale, a hiatus could be

Table 4

Induction of hepatic drug-metabolizing enzymes in common eider ducks after treatment with CB 77 (5 or 50 mg/kg, 77⁵ or 77⁵⁰ respectively) or the technical mixture Clophen A50 (50 or 200 mg/kg, Clo A50⁵⁰ or Clo A50²⁰⁰ respectively)

	Control	77 ⁵	77 ⁵⁰	CloA50 ⁵⁰	CloA50 ²⁰⁰
Σ CYP ^a	0.086 ± 0.027	0.142 ± 0.069	0.280 ± 0.098	0.087 ± 0.029	0.081 ± 0.021
Induction (N)	1 (15)	1.7 (6)*	3.3 (16)*	1 (5)	0.9 (12)
WL-CYP ^b	61.1 ± 18	104.9 ± 54	212.6 ± 124	64.0 ± 31	68.4 ± 24
Induction (N)	1 (14)	1.7 (12)	3.5 (15)*	1 (5)	1.1 (12)
EROD ^c	8.8 ± 3.6	60.2 ± 11	502.4 ± 362	9.7 ± 9.5	17.4 ± 12
Induction (N)	1 (15)	6.8 (6)*	57 (16)*	1.1 (5)	2.0 (14)
PROD ^c	0.40 ± 0.23	2.11 ± 0.27	3.02 ± 0.96	0.53 ± 0.49	0.93 ± 0.61
Induction (N)	1 (15)	5.3 (6)*	7.5 (16)*	1.3 (5)	2.3 (14)

All means are given with the 97.5% confidence interval, (*Significantly different from control, $P < 0.01$).

^a(nmol/mg microsomal protein).

^b(nmol/liver).

^c(pmol/min per mg).

Fig. 3. Covariation between the log of CB 77 TEQ ($\mu\text{g/g PEL}$) and the log of Clo A50 TEQ ($\mu\text{g/g PEL}$) in the adipose tissue vs. log EROD activity (pmol/min per mg protein). Log TEQ of the control group is given as well (not integrated). Regression equations are given in Table 5.

observed between the control data and the CB 77 data (Fig. 3). The covariations with their correlation coefficients (R) and probability (P) are given in Table 5. The levels of CB 77 showed a significant covariation with all HMO variables. Clo A50 showed no significant slope with any HMO variable. It appeared that tissue levels of CB 77 can explain 40% (log CB 77:log WL-CYP) to 74% (log CB 77:log EROD) of the variation. The covariations of both log CB 77 TEQ and log Clo A50 TEQ with log EROD are given in Fig. 3.

Infection rate

At the doses and time points studied, the number of *P. botulus* established was not influenced by any CB treatment. The quantity of parasites/animal showed no significant difference between exposed groups and control (Fig. 4). Covariations between log TEQ and number of parasites were not significant either (data not shown).

4. Discussion

Comparison of CB patterns

In the Clo A50-treated ducklings, the CB patterns from the adipose tissue and liver showed a relative decrease for only those congeners with *m,p* vic. H atoms in comparison to the technical mixture (Table 3). Values of R_{rel} for other congeners were all close to unity. This indicates that the uptake from the body peritoneal cavity was proportional to their concentration in the original mixture. The close relation between the extent of elimination and the presence of *m,p* vic. H atoms for the various CB congeners strongly suggest that enzymatic metabolism by the HMO is the most

Fig. 4. Number of adult *P. botulus* that were observed in the intestines of the control animals and the Clo A50²⁰⁰ and CB 77⁵⁰ group. Mean figures are given with s.d.; $n = 6$.

probable process. This is in agreement with the findings of others (Borlakoglu et al., 1988, 1991; Borlakoglu and Walker, 1989; Boon et al., 1989; Beyerbach and Heidmann, 1990; Everaarts et al., 1991). Some $t_{1/2}$ values were estimated as well. Since they were calculated on the basis of only one point in time, they should be considered as indicative values.

Surprisingly, a pattern difference was encountered between the liver and the adipose tissue of the Clo A50²⁰⁰ group. The liver had less CBs with *m,p* vic. H atoms and resembled more to that of the adipose tissue of the Clo A50⁵⁰ group. This difference was seen for the $\Sigma\delta$ as well (Table 3, Fig. 2). In general, pattern differences between organs are not found in environmentally exposed animals (Duinker et al., 1989; Kannan et al., 1993). The encountered differences could not be the result of destructive clean-up procedures (Kannan et al., 1993) since similar non-destructive methods were applied as Duinker et al. (1989) and Kannan et al. (1993). Under experimental conditions with relatively short incubation periods, however, body compartment differences have been shown before. Borlakoglu et al. (1991) demonstrated pattern differences between liver, gonads and adipose tissue in the pigeon.

This could indicate that the HMO capacity was saturated. Congeners that are transported from remote compartments, like muscle or adipose tissue, to the liver

may thus meet a saturated HMO system, and are subsequently redistributed over the body (Walker, 1987, 1990b).

Another possible explanation could be that rather than biotransformation, the redistribution from the adipose tissue via the blood to the liver is rate-limiting. In this manner, transport rate of CB congeners from the other compartments to the liver would regulate the concentration of a certain CB congener in the liver and thus indirectly its rate of metabolism as well (Spacie and Hamelinck, 1982). Since the pattern in the liver of the Clo A50²⁰⁰ group is rather similar to the pattern in adipose tissue of the Clo A50⁵⁰ group, not limited metabolism but limited transport seems the most likely explanation.

The *non-ortho*-Cl, *o,m* vic. H atoms CB 77 is probably metabolised *in vivo*. Klasson Wehler et al. (1990) showed that chickens were able to metabolise CB 77. Murk et al. (1993) confirmed that common eider microsomes from this experiment were able to metabolise CB 77 to hydroxy metabolites in an *in vitro* assay.

The properties required for hydroxy-CBs to reduce circulating thyroid hormone are a hydroxy group at a *meta* or *para* position and an adjacent Cl group (Lans et al., 1993). Some of the metabolised CBs can meet these structural requirements. The birds did show changes in thyroid hormone balances (Murk et al., 1994), possibly as a result of the metabolised CBs.

Induction of HMO characteristics

The ducklings showed an obvious dioxin type induction when exposed to CB 77. The mixed type induction of both CYP1A and CYP2B was less clear. No induction was observed by Clo A50 treatment after this incubation period. The levels of EROD activity and Σ CYP content of the control group of *S. mollissima* are low when compared to other bird species. This is a common feature for sea-birds and other birds with a specialised carnivorous diet (Ronis and Walker, 1989; Walker, 1990a). Even in comparison with sea-birds such as Leech's storm petrel (*Oceanodroma leucorhoa*), the guillemot (*Uria aalge*) and the puffin (*Fratercula arctica*), the activities are at the lower range (Peakall et al., 1987). The low HMO activities could be due to the food choice of *S. mollissima* (bivalves vs. fish, Livingstone and Farrar, 1984; Ronis and Walker, 1989; Broman et al., 1990; Swennen, 1991) or the life cycle stage (juvenile vs. adult, Peakall et al., 1987).

The PROD assay was included in the experiment because it was expected that the mixed type and pure CYP2B inducers in Clo A50 would increase the PROD activity in the ducklings treated with Clo A50. However, elevated levels of PROD activity were found in both the CB 77 treated groups (Table 4). Most likely the PROD activity is performed by CYP1A. The idea that PROD activity was exhibited by CYP1A was confirmed by the strong covariation between log EROD and log PROD ($R = 0.80$, $P = 0.0001$, data not shown).

The use of PROD as indicator for CYP2B activity in other species than rats or mice is disputable (Lubet et al., 1990; Soucet and Gut, 1992; Haasch et al., 1994). In addition, reconstituted systems of rat CYP1A1 and rat CYP1A2 exhibited a greater PROD activity than reconstituted systems of rat CYP2B1/2 (Yang et al., 1988). Furthermore, 3-MC induced mice and rats showed significantly increased PROD activi-

ties (Burke and Mayer, 1983; Yang et al., 1988). This is most likely due to the fact that pentoxyresorufin preferably assumes a planar configuration (Lubet et al., 1990), resulting e.g. in higher affinities of pentoxyresorufin for CYP1A (Burke and Mayer, 1983). Concluding, on the basis of PROD and EROD, one can only speak of CYP2B1/2 induction in birds if PROD activity and Σ CYP are induced whilst EROD activity is still comparable to control levels. The induction of CYP2B1/2 had better be assessed by other methods as e.g. testosterone metabolism profiles (Darby et al., 1986) or specific antibodies (Ronis et al., 1989a,b).

The variance in the CB 77⁵⁰ group, especially in the EROD activity, could partly be explained by the differences of CB 77 concentration in adipose tissue (Table 5, Fig. 3). WL-CYP showed a low regression coefficient which is not remarkable since it is a multiplication of several variables each with an uncertainty. Beside the fact that the levels were measured in the adipose tissue and not in the target organ (the liver), genetic differences in response to induction between the individual eiders caught in the wild could contribute to explain the rest of the variance (Ronis and Walker, 1989; Walker, 1990a).

Table 5

Regressions of the log of the adipose tissue TEQ levels versus the log of the HMO variables. TEQ levels were either calculated by multiplying CB concentrations in the adipose tissue with the TEF values of Safe (1990, 1993) resulting in TEQ, or with the TEF values for birds (Brunström and Andersson, 1988; Brunström, 1990; Bosveld et al., 1992b), given as TEQ_{birds}. *R* = correlation coefficient; *P* = probability, *n* = number of data

		<i>R</i>	<i>P</i>	<i>n</i>
Clo A50 TEQ				
Log Σ CYP	= 0.011 log [TEQ] - 1.12	0.084	0.76	15
Log WL-CYP	= 0.051 log [TEQ] + 1.71	0.187	0.49	15
Log EROD	= 0.227 log [TEQ] + 1.36	0.384	0.12	17
Log PROD	= 0.324 log [TEQ] + 0.169	0.373	0.13	17
CB 77 TEQ				
Log Σ CYP	= -0.446 log [TEQ] - 0.50	0.85	0.0001	16
Log WL-CYP	= -0.487 log [TEQ] + 2.37	0.636	0.0108	14
Log EROD	= 0.674 log [TEQ] + 2.32	0.857	0.0001	16
Log PROD	= 0.326 log [TEQ] + 0.57	0.754	0.0005	16
Clo A50 TEQ _{birds}				
Log Σ CYP	= -0.012 log [TEQ _{birds}] - 1.133	0.127	0.64	15
Log WL-CYP	= -0.470 log [TEQ _{birds}] + 1.66	0.237	0.37	15
Log EROD	= 0.095 log [TEQ _{birds}] + 1.29	0.214	0.39	17
Log PROD	= -0.109 log [TEQ _{birds}] - 0.006	0.028	0.50	17
CB 77 TEQ _{birds}				
Log Σ CYP	= -0.446 log [TEQ _{birds}] - 0.63	0.850	0.0001	16
Log WL-CYP	= -0.487 log [TEQ _{birds}] + 2.23	0.636	0.0108	14
Log EROD	= 0.674 log [TEQ _{birds}] + 2.12	0.857	0.0001	16
Log PROD	= 0.326 log [TEQ _{birds}] + 0.47	0.754	0.0005	16

The slope of the log TEQ CB 77 vs log EROD (0.67) was comparable to the slope that was found between the log TEQ derived from encountered *mono-ortho* CBs and the log EROD from the common tern (*S. hirundo*) and cormorants (*Phalacrocorax carbo*) (0.5, Bosveld et al., 1992a; Van den Berg et al., 1993).

To estimate the inducing potency of Clo A50 for CYP1A, the TEQ present in the adipose tissue was calculated. The Clo A50 groups showed no significant covariations for HMO activities. The incubation period of 10 days could have been either too long or too short to provoke a response.

Secondly, it could be argued that the inducing potency of Clo A50 is reduced by the metabolism and elimination of some congeners (see Table 3). The CBs, however, which contribute predominantly to the TEQ (CB 105, CB 118 and CB 156), belong to a group which is not readily metabolised (Table 3, Fig. 2). Therefore, a major reduction in TEQ and inducing potency is not likely to occur in the course of this short incubation time.

Thirdly, it should be emphasised that these TEQs are an approximation. This is because the TEF applied are derived from studies with rodents. Therefore, the TEQs and their effects should be used with caution, when applying them to birds. TEF values for chickens are also available (Brunström and Andersson, 1988; Brunström, 1990; Bosveld et al., 1992b). Here, only the *non-ortho* and the *mono-ortho*-Cl CBs have a TEF. Covariations were calculated between log TEQ_{birds} and the log HMO activities (Table 5). Once more, similar results were obtained. The covariations with CB 77 were significant whereas the relations with Clo A50 all had a $P > 0.36$ (Table 5). One should keep in mind that chickens are not especially representative for birds in general and common eiders in particular (Brunström, 1988, 1989; Brunström and Lund, 1988; Brunström et al., 1990; Engwall et al., 1994).

The absence of significant covariations with Clo A50 TEQ might suggest antagonistic behaviour of mixtures of CBs. Results on the American mink suggested antagonistic behaviour of the *di-ortho*-Cl fraction on the EROD induction by the *non-ortho* or *mono-ortho*-Cl fractions of Clo A50 (Brunström et al., 1991). A mixture of CB 77 and 2,2',5,5'-TCB resulted in less Ah-mediated induction than with CB 77 alone (Aarts et al., 1993). This was most likely due to a decrease in ligand-dependent complex formation between the Ah receptor and DNA. This effect differed in extent from (mammalian) species to species (Schalk et al., 1993).

The most likely explanation is that the level of the Clo A50 induction is around the no effect level (NOEL) for EROD induction in eider ducklings (Fig. 3). The Clo A50 data are at the lower, left-hand part of the figure and the CB 77 data at the higher end of the range. Plotting log TEQ vs EROD yields the sigmoid graph of a normal dose-response relation (not shown). The Clo A50 data points end where the inclining part of the curve begins. Also the fact that the covariation of the CB 77 data yields a much lower EROD level at control level TEQ than the control group actually has, indicates that the log-log transformation does not compensate for the no effect range. These make this option more likely than antagonism (see also Abraham et al., 1988; Bosveld et al., 1992b).

With most points of the Clo A50 data in the NOEL range, the covariation will be non-significant. A very tentative maximum NOEL can be obtained by calculating the

interception of the log CB 77 TEQ vs log EROD with log Clo A50 TEQ vs log EROD (Fig. 3). This yields a level of 7 ng TEQ/g PEL. This is rather insensitive compared to chickens and rats. Chicken embryos, treated in the egg with CB 77, showed an ED₅₀ for EROD induction at 1.7 µg/kg egg (Brunström and Anderson, 1988). Assuming a fat content of 4%, all CB 77 in the adipose tissue and no biotransformation, this yields an EC₅₀ of 0.43 ng TEQ/g adipose tissue. Rats injected with a single *sc* dose of TCDD had significantly increased EROD levels at an internal concentration of 0.014 ng TEQ/g adipose tissue (Abraham et al., 1988).

To explain the lack of CYP2B induction (as assessed by ΣCYP and WL-CYP, Tables 4, 5), the inducing potency of Clo A50 for CYP2B (in rodents) was estimated, using Schulz et al. (1989) and Boon et al. (1992b). The PB-type inducing congeners like CB 87, 99, 153, all together contributed 21% of the mixture. This means that at Clo A50⁵⁰, the animals received at least 10 mg/kg of PB-type inducing CBs and 40 mg/kg at the high doses. Still, an induction of CYP2B, as assessed by the PROD assay, ΣCYP and WL-CYP could not be observed. A lack of response (induction of CYP2B) of sea-birds and other birds towards PB-type inducers has been demonstrated before (Ronis and Walker, 1989; Walker, 1990a). As in field samples of sea-birds, CYP2B could not be detected while using antibodies for rat CYP2B (Ronis et al., 1989a,b). Quail showed no increase in PROD activity under the influence of PB and DDT. Aroclor 1254 showed a confined induction for PROD in quail (but an extensive for EROD, Lubet et al., 1990). As exceptions, the black-headed gull (*L. ridibendus*) as well as the black-tailed gull (*L. crassirostris*) showed both PROD activity and cross-reactivity with anti-rat CYP2B1 (Yamashita et al., 1992). Hence, the lack of CYP2B in *S. mollissima* is not unremarkable in this sense.

The sex difference had no effect on the responses measured. Knight et al. (1981) showed differences between male and female sea-birds on the basis of other substrate activities. Probably, these effects were coupled to the regulation of sex hormone-metabolising CYP enzymes in adult birds. These ducklings may not have developed any sexual differences in respect to CYP.

The combination of biotransformation of some CBs from Clo A50 and the lack of induction by this mixture creates some problems for the interpretation. In rats, CYP2B is responsible for the metabolism of poly-brominated biphenyls (PBBs) with *m,p* vic. H atoms and CYP1A for the PBBs with *o,m* vic. H atoms (Mills et al., 1985). For this discussion it is anticipated that the same is valid for CBs. Firstly, it is possible that *S. mollissima* does have CYP2B type enzymes but that they are not easily inducible. This has been demonstrated for cormorant and male Japanese quail (*C. c. japonica*, Ronis and Walker, 1989). Secondly, it is possible that *S. mollissima* does not have CYP2B but that the CBs are metabolised by other CYP families which have not been considered yet. For instance, birds have CYP forms which are strongly inducible by the model compounds Prochloraz and 2-acetylaminofluorene while rats have a weaker response towards these chemicals (Rivière et al., 1985; Darby et al., 1986; Ronis and Walker, 1989). More extensive in vitro metabolism assays with microsomes from the differently induced birds and selected CB congeners can bring more clarity in the metabolism of CB congeners by sea-birds.

5. Parasite infection

The changed levels of vitamin A and thyroid hormone (Murk et al., 1994) could have led to a less efficient immune system. Infection amount, however, was not related to type of CB treatment. Compared to adult eiders, the success percentage of established *P. botulus* was rather high (Swennen, pers. comm.). Probably, the incubation period of 5 days was too short to establish differences between CB treatments. The increased infection rate as encountered in the Wadden Sea is more likely due to forced changes in diet. Since bivalve fishery has increased, common eiders have altered their diet to predominantly shore crabs which carry high numbers of cystacanths (Swennen, 1991). Hence increased infections can be expected.

6. Conclusions

Common eider ducklings were able to metabolise CBs with *m,p* vic. H atoms. In this case, the number of *ortho*-Cl is irrelevant. They were not able to metabolise CBs with only *o,m* vic. H atoms and one *ortho*-Cl. Some of the metabolised CBs can meet structural requirements to reduce circulating thyroid hormone.

The eider ducklings responded to a dioxin type inducer (CB 77) by increasing values of Σ CYP, WL-CYP, EROD and PROD, after an incubation period of 10 days. Under the same circumstances, the mixed type inducer Clo A50 did not elicit any of these responses. For CYP1A induction, most likely, levels of Clo A50 in the liver were around NOEL. A tentative maximum NOEL for EROD induction was suggested (7 ng TEQ/g PEL) in common eider ducklings. A CYP2B1/2 induction (as assessed by Σ CYP and WL-CYP) could not be observed. This was explained by a lack of CYP2B as found in other birds. The applicability of the PROD assay to assess CYP2B1/2 induction in birds is discussed and found not suitable.

After an incubation period of 5 days with parasites, no differences could be observed in resistance against infections with the parasite *P. botulus*. Probably, the incubation period was too short.

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