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The Accumulation of Lead and Mercury from Seawater and Their Depuration by Eggs of the Spotted Dogfish *Scyliorhinus canicula* (Chondrichthys)

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Abstract Radiotracer experiments using ^{210}Pb and ^{203}Hg demonstrated that eggs of the spotted dogfish *Scyliorhinus canicula* absorbed lead and inorganic mercury directly from seawater over 21 days of experimental exposure, attaining total egg concentration factors (CFs) relative to water of approximately 400 for Pb and 180 for Hg, predominantly (≥98%) due to their accumulation by the collagenous egg case. The rates of accumulation of both Pb and Hg by the total egg were significantly ($P < 0.0001$) reduced by its increasing age since parturition, whereas only the rate of depuration of Pb was reduced ($P < 0.0001$) with increasing age; these effects indicate a declining chemical reactivity of the egg case that may be due to the continued tanning of the case following parturition. The egg case per se, attained average CFs of about 1,500 and 850 for Pb and Hg, respectively. Both Pb and Hg showed declining concentration gradients from the exterior to the interior membranes of the wall of the egg case; CFs for Pb declined from 3,500 to 2,000 and for Hg from 5,000 to 500. Comparison of concentrations in separate membranes also demonstrated significant ($P \leq 0.01$) depurations of Hg from the external and internal membranes during the loss experiments. The presence of radiotracers of Pb and Hg in the internal components of the egg at the end of uptake phase, and prior to the opening of the apertures, confirmed the permeability of the egg case wall to them, consistent with their observed gradients in it. The average CFs for all embryos at the end of the uptake experiment were 34 and 44 for Pb and Hg, respectively, but were significantly ($P < 0.001$) enhanced for Hg by a factor of 6 in the older eggs. The accumulatory and kinetic characteristics of the egg-case may operate to optimize the exposure of embryos to Pb and Hg following episodic contaminant events in coastal habitats.

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Introduction

The cartilaginous or Chondrichthyan fishes are characterised by lifecycle attributes that result in low intrinsic rates of increase (r), making their populations particularly susceptible to environmental impacts. These qualities include low fecundity that is expressed in the production of a relatively small number of large, heavily yolked eggs in the oviparous species, which comprise about 40% of the sharks and all the skates (Wourms and Demski 1993). These Chondrichthyan embryos develop slowly following their laying in nursery grounds, commonly found in shallow coastal waters that are increasingly under threat from land-based contaminants (GESAMP 2001), including the submarine groundwater discharge of Hg, for example (Bone et al. 2007). The location of Chondrichthyan eggs in such habitats could enhance their exposure to elevated contaminant levels during this long early phase of their lifecycle, which is most susceptible to environmental contaminants in teleost fish, particularly for metals (Weis and Weis 1991). Hence the susceptibility of Chondrichthyan eggs to pollutant exposure is worthy of investigation.

Recent experimental studies (Jeffree et al. 2006a and b; Jeffree et al. 2007) have been conducted on eggs of the dogfish *Scyliorhinus canicula* commonly known to lay its eggs in shallow coastal waters (Wood 2002; Knight et al. 1996). These initial studies using suites of radio tracers

have demonstrated that they have accumulatory behaviours that indicate prima facie an enhanced susceptibility to contamination by metal pollutants present in the aqueous phase. The eggs of *S. canicula* absorb ^{241}Am, ^{109}Cd, ^{57}Co, ^{134}Cs, ^{54}Mn, and ^{65}Zn from seawater during short-term exposures (14–15 days), reaching concentration factors (CFs) in the total egg exceeding 10^3 for some of these contaminants, the majority of these being associated with the case itself. The egg case wall was demonstrated to be permeable to ^{241}Am, ^{60}Co, and ^{134}Cs to varying degrees and the opening of apertures in the egg case by embryonic secretions led to enhanced concentrations of ^{241}Am and ^{60}Co in its internal medium that also enhanced concentrations of ^{241}Am in the embryo. For ^{57}Co and ^{241}Am these short-term exposures give even higher CFs in the outer membrane of the egg case that, coupled with linear rates of accumulation, suggest that the accumulatory potential of the eggs may be even higher over the 5+ month period of embryonic development prior to the emergence of the hatchling.

Gamma-emitting environmental radionuclides such as ^{241}Am and ^{60}Co that are absorbed to high CFs and retained by the egg case indicate that these would be the major source of radiotoxic exposure to the embryo. Some of these accumulated radiotracers also represent reservoirs of potentially chemotoxic stable metals that may subsequently transfer to the embryo following their relatively quick accumulation during episodic contamination events, for example.

To further understand the susceptibility of this Chondrichthyan embryonic model to metal and radionuclide pollutants in its aquatic medium, we have experimentally investigated the biokinetics of two other environmental contaminants of recognised heightened chemotoxicity, viz. lead and inorganic mercury (Nierboer and Richardson 1980).

Materials and Methods

Acclimation and Experimental Exposure of Encased Embryos

The eggs used in these experiments were harvested from a breeding colony of spotted dogfish *S. canicula* established in the MEL Radioecology Laboratory. Each egg's date of parturition was recorded, it was weighed and then maintained under conditions similar to those of the experimental exposure, i.e. Mediterranean seawater at 17.0 ± 0.5°C salinity 38 PSU, pH 8.05, and light/dark cycle of 10 h/14 h. Egg masses varied between 4.0 and 7.9 g at the beginning of the experimental exposure, and in age since parturition between 24 and 53 days for those exposed to ^{203}Hg, and 24–46 days for those exposed to ^{210}Pb. They could be grouped into three clutches based on the following ranges of ages since parturition, as of the first day of experimental exposure, viz: G1 ($n = 62$); 46–53 days; G2 ($n = 41$); 35–38 days; and G3 ($n = 63$), 24–28 days.

During the uptake experiments of 21 days' duration, the stages of embryological development that were passed through by all three cohorts precede the pre-hatching 'P stage' as described in Mellinger et al. (1986) and 'stage 31' of Ballard et al. (1993), within which the apertures in the egg case are opened by the embryo's secretions from its head gland. Therefore any radioisotopes found in the embryo at the end of the uptake experiment will only be those that have passed through the wall of the egg case.

However, the depuration experiment continued for a subsequent 85 days that encompassed the 'pre-hatching' phase of development, when the apertures in the wall of the egg case would open, leading to direct access by seawater to the interior of the egg case, as previously demonstrated experimentally (Jeffree et al. 2007).

Tissue Distributions of Radioisotopes

At the completion of both the uptake and loss experiments eggs were dissected into the following four components to determine their radioisotope content and concentrations following wet weighing: egg case, glycosaminoglycan jelly, yolk, and embryo. The case of *S. canicula* is constructed of approximately 30 lamellae, mainly of collagenous composite (Knight and Feng 1992). To further investigate the bioaccumulation and transfer of these radioisotopes directly through the wall of each egg case, at the end of the uptake and loss experiments, vertical sections of the egg case wall were removed and separated into three broad regions (Knight et al. 1996) that could be easily dissected, i.e., the thin outer membrane, the thick middle membrane, and the thin inner membrane. The outer layers were peeled away using forceps and scalpel, and then the internal layers were removed in the same way, leaving the median membranes. For every sample each of these three membranes was then weighed and gamma-counted for total activity of each radioisotope, and then their CFs, absolute activity concentrations, and percentage distributions were calculated.

Radioisotopes and Their Radioanalysis

Radioactivity levels in seawater, eggs, and their dissected components were determined using a high-resolution γ-spectrometry system consisting of four coaxial Germanium (N- or P-type) detectors (EGNC 33-195-R, Intertechnique; 40–70% efficiency) that were connected to a multichannel

analyzer and a personal computer employing spectral analysis software (Interwinner 6, Intertechnique). The radioactivity levels of the samples were determined by comparison with known standards of appropriate geometry and were corrected for background and isotope physical decay. Counting times were adapted to obtain count rates with relative propagated errors less than 5%, viz. typically 10–30 min for whole organism radioanalyses and 1–12 h for seawater and dissected body components.

Data Analysis

Fitting of Uptake and Loss Equations

The uptake of radioisotopes from seawater by eggs over 21 days of exposure to radiolabelled water was expressed as the change in concentration factors (CF = Bq g^{-1} wet organism divided by the time-integrated Bq g^{-1} sea water) over time. Uptake kinetics in whole individuals was described by either using a single-component first-order kinetic model

$$CF_t = CF_{SS}(1 - e^{-k_e t}),$$

where CF_t and CF_{ss} represent the concentration factors at time t (d) and at steady state, respectively, and k_e is the biological depuration rate constant (d^{-1}) (Whicker and Schultz 1982).

If individual eggs did not tend to reach a steady state during the exposure time course or their loss rate constant was not statistically significant ($P > 0.05$), then a simple linear regression model was also fitted,

$$CF_t = k_u t,$$

where k_u is the regression slope (i.e., the rate of increase in CF, d^{-1}).

Loss kinetics were expressed in terms of the percentage of remaining radioactivity, i.e., the radioactivity at time t divided by initial radioactivity (times 100) measured in the eggs at the beginning of the depuration (loss) period. The kinetics were described by a double-component exponential model

$$A_t = A_{0s}e^{-k_{es}t} + A_{0l}e^{-ke_l t}$$

where A_t and A_0 are the remaining percentage activities at time t (d) and 0, respectively, and k_e is the biological depuration rate constant (d^{-1}).

The determination of k_e allows the calculation of the radioisotopic biological half-life ($T_{b1/2} = \ln 2/k_e$). The "s" subscript refers to a short-lived component (loss of the fraction of radioisotope pool that is weakly associated to the egg) while the "l" subscript refers to a long-lived component (loss of the fraction of the radioisotope pool that is tightly bound to the egg).

Constants of the uptake and loss equations and their statistics were estimated by iterative adjustment of the models and Hessian matrix computation, respectively, using the nonlinear curve-fitting routines in the STATISTICA©5.1 software (StatSoft Inc.; Tulsa, Oklahoma, USA). These procedures were used to fit curves and/or straight lines to data for each cohort separately and for the total data, for both the uptake and loss experiments.

Multiple Regression Analysis

The fitting of the curves to each dataset suggested that there were appreciable effects of egg age on the uptake and loss rates (Figs. 1 and 2). To determine if an age effect on rate of accumulation was statistically significant ($P < 0.05$) for either radioisotope, the CF of each egg was regressed against both (1) the period of experimental exposure and (2) the age of the egg from parturition, using multiple linear regression, so that most of the experimental data could be used in a single analysis. Although the fitting of

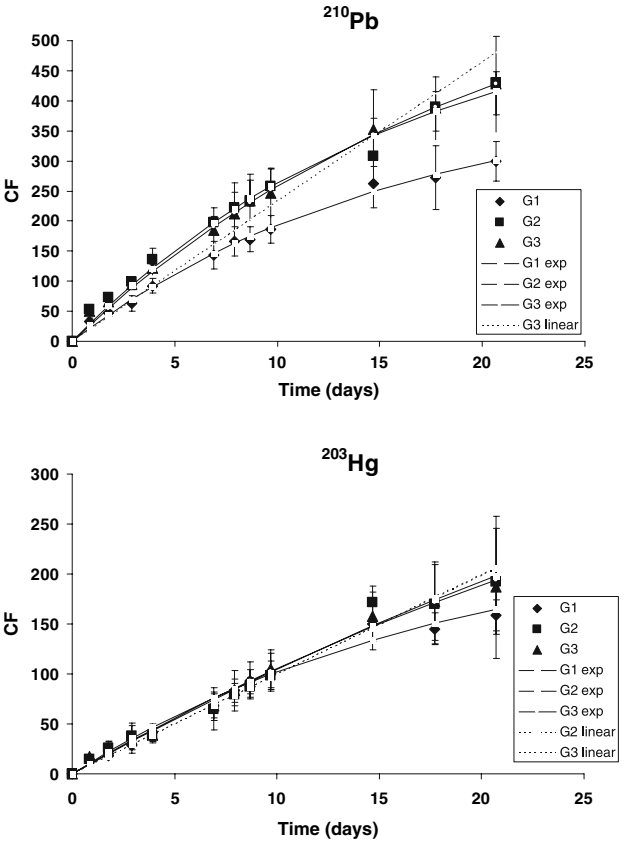

Fig. 1 Concentration factors for ^{210}Pb and ^{203}Hg in three clutches of dogfish eggs of varying age since parturition, as a function of period of exposure to radiolabelled water over 21 days. Clutch G1 was laid between 9–17 February; G2 between 24–27 February to 3 March; and G3 between 06–10 March, 2006

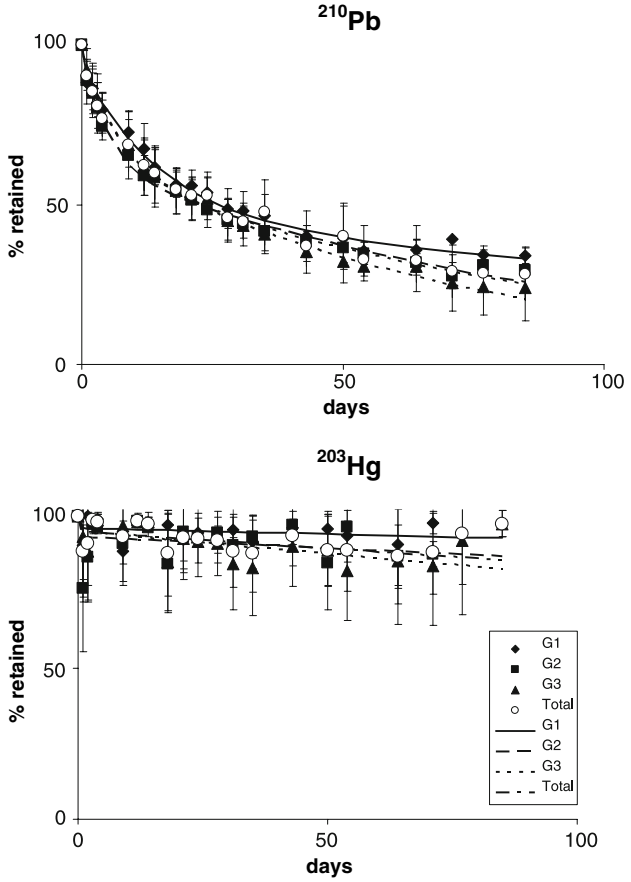

Fig. 2 Depuration kinetics in three clutches of dogfish eggs of varying age previously exposed to ^{210}Pb and ^{203}Hg in water for 21 days

regression lines to the G2 and G3 cohorts for ^{210}Pb indicated some slight curvilinearity, this regression analysis was performed on data for all three cohorts, where the simplest model is a linear one.

For the data plotted in Fig. 2, the percentage loss values were $\log_{-10}$ transformed to more closely approximate linearity (particularly for ^{210}Pb) and for homogeneity of variance in percentage values among the periods of loss. Then multiple linear regression analysis was used to regress the percentage retention (log) of each radioisotope against (1) period of exposure and (2) age at parturition of the egg.

Chemical Speciation Modelling for Pb and Hg in Experimental Seawater

The physicochemical parameters of seawater used to model the speciation of ^{210}Pb and ^{203}Hg in the Mediterranean seawater used in this study are given in Jeffree et al. (2006c) and the approach outlined here is identical to that previously used to model the speciation of seven other metals, as summarised below. For major ions, seawater is a one-component system, and the concentration ratios among the major ions, and between salinity and any major ion, are constant. Thus, only a salinity measurement is required to determine the concentrations of the major ions. The major ion concentrations at the average seawater salinity of 35‰ (Pilson 1998) were multiplied by a factor of 1.086 to reflect the major ion concentrations at a salinity of 38‰ used in this study. The mean concentrations of dissolved Pb and Hg were taken from Millero (1996), i.e., 1.025×10^{-11} mol L^{-1} and 5.124×10^{-12} mol L^{-1}, respectively. A redox potential (pe) of 7.0 reflects oxidised surface waters (7 mg L^{-1} dissolved oxygen) at 16.5°C. Dissolved organic carbon (DOC), was assigned a value of < 0.1 mg L^{-1} for modelling purposes, in accord with the prefiltering of the experimental waters through activated charcoal.

The speciation of Pb and Hg were calculated using the HARPHRQ speciation code (Brown et al.1991). The input parameters were based on the physicochemical data (pH, pe, and ion concentrations) that were used previously (Jeffree et al. 2006c). The equilibrium constants included in the thermodynamic database used for the speciation modelling were derived from only reliable and quality-assured data; for Pb the sources were Markich and Brown (1999) and Smith and Martell (2004) and in the case of Hg data they were taken from Powell et al. (2004). The activity coefficients were corrected using a truncated Davies equation (Falck et al.1996), which uses the Davies expression up to an ionic strength of 0.3 M and then fixes the charge up to ~0.75 M (38‰). Since most equilibrium constants used by HARPHRQ are standardised to 25°C, the van't Hoff relationship (Smith 2004) was used to correct for the small reduction in temperature to 16.5°C.

Results

Chemical Speciation Modelling for Pb and Hg in Seawater

The speciation calculations indicate that Pb occurs in the +2 oxidation state and forms strong complexes predominantly with carbonate [47% $PbCO_3$, 16% $PbOHCO_3^-$ and 1.9% $Pb(CO_3)_2^-$] and chloride [10% $PbCl^+$, 11% $PbCl_2$ and 3.0% $PbCl_3^-$] in oxic seawater. The free lead ion (Pb^{2+}) forms only a very small (2.8%) component of dissolved Pb. Oxidation–reduction reactions for Pb in oxic surface waters are not significant (Baxter and Frech 1995). Based on these results, the bioavailability of inorganic Pb in seawater is expected to be very low.

The speciation calculations indicate that Hg occurs in the +2 oxidation state in oxic seawater and forms very strong complexes with chloride. The predominant species are $HgCl_4^{2-}$ (88%) and $HgCl_3^-$ (12%). The free mercuric

ion (Hg^{2+}) forms only <0.01% of dissolved Hg. However, Hg has a more complex aquatic chemistry than the thermodynamic calculations suggest. Mercuric forms of Hg can be transformed through abiotic and biotic (microbial) processes to form alkylmercury compounds, such as methylmercury [CH_3Hg^+ or $(CH_3)_2Hg$] (Ebinghaus et al. 1994). Typically, methylmercury represents less than 10% of total dissolved mercury in oxic surface waters (CCME 2003). It is well established that methylmercury is considerably more bioavailable than inorganic mercury (Boening 2000) because of its capacity to traverse phospholipids membranes of cells. However, in this experiment it is the initial interaction between the chemical species and the collagenous egg case that is of primary significance for subsequent bioaccumulation with regard to the encased embryo. Previous studies have indicated that the wall of the egg case of *S. canicula* is highly permeable to small ions and small-molecular-weight polar solutes (Hornsey 1978) and much less permeable to higher-molecular-weight molecules. Reduction of mercuric (Hg^{2+}) forms to elemental mercury (Hg^0) may also be initiated by microorganisms, direct photolysis or by organic matter (Ravichandran 2004).

Uptake of ^{210}Pb and ^{203}Hg by Whole Eggs

Figure 1 shows the CFs for ^{210}Pb and ^{203}Hg in whole eggs as a function of their period of exposure to the experimental water labelled with these isotopes over a period of 21 days, and the fitted uptake regression lines for the three cohorts. The results of the associated statistical analyses are given in Table 1.

Lead–210 CFs in whole eggs continue to increase over the experimental period, for each cohort, attaining levels of between 250 and 400. The significant ($P < 0.05$) fitted regression lines explain 86–93% of the variability between individual measurements within cohorts. These regressions indicate some curvilinearity that is concave to the *X*-axis for the separate cohorts, and that is more pronounced for the oldest cohort. Mercury-203 CFs increase up to about 150–180 in a more consistently linear fashion among the cohorts over the exposure period, although the oldest cohort showed some slight curvilinearity. The fitted regressions explain 89–92% of the variance between measurements within the three cohorts. The loss rate constant is significant ($P < 0.01$) only for the oldest cohort.

The results of the multiple regression analyses to evaluate the effect of egg age on uptake rates are shown in Table 2 to confirm a highly significant ($P < 0.0001$) and inverse effect of egg age on the CF for ^{210}Pb. The same analysis was also performed on the ^{203}Hg CF data but no

Table 1 Parameter values for fitted uptake curves based on a single compartment, first-order loss kinetic model $\left[CF_t = CF_{SS}(1 - e^{-k_e t})\right]$ or a linear regression model ($CF_t = k_u t$; in italics) for the three egg clutches. Standard errors are given in brackets. G1 is the oldest clutch and G3 is the youngest

	^{203}Hg					^{210}Pb				
	CF_{ss}	*p*-level	k_e or k_u (in italics)	*p*-level	R^2	CF_{ss}	*p*-level	k_e or k_u (in italics)	*p*-level	R^2
G1 ($n = 62$)	244.7 (60.09)	<0.001	0.05 (0.02)	<0.01	0.91	G1 ($n = 57$)	409.19 (64.34)	<0.001	0.06 (0.02)	0.002	0.90
G2 ($n = 41$)	641.3	ns	0.02	*Ns*	0.92	G2 ($n = 41$)	584.15 (106.55)	<0.001	0.06 (0.02)	0.001	0.93
			9.96 (0.29)	*< 0.01*	*0.92*					
G3 ($n = 62$)	505.4	ns	0.02	Ns	0.85	G3 ($n = 57$)	690.6 (316.4)	0.003	0.05	Ns	0.89
			9.00 (0.30)	*< 0.01*	*0.89*				*23.24 (0.59)*	*< 0.01*	*0.86*

Table 2 Regression analysis of the effect of egg age on the accumulation and depuration on ^{210}Pb and ^{203}Hg

Predictor	Regression coefficient	t	P for t	F ratio	P for F	R^2
Uptake experiment						
^{210}Pb ($n = 144$)						
Age (days)	–3.40	−7.3	<0.0001	441	<0.0001	0.86
Experimental exposure (days)	17.6	28.8	<0.0001			
^{203}Hg ($n = 134$)						
Age	−0.3	0.20	0.20	338	<0.0001	0.83
Experimental exposure	8.83	26.0	<0.0001			
Loss experiment						
^{210}Pb ($n = 262$)						
Age	0.0026	4.14	<0.0001	4.33	<0.0001	0.78
Experimental exposure	−0.0062	−29.3	<0.0001			
^{203}Hg ($n = 235$)						
Age	−0.13	0.83	0.41	1.57	<0.21	0.01
Experimental exposure	−0.08	−1.47	0.14			

significant ($P = 0.2$) effect of the age of the egg was found.

Loss of ^{210}Pb and ^{203}Hg from Whole Eggs

Figure 2 shows the percentage of the ^{210}Pb and ^{203}Hg activities retained in the eggs as a function of days of exposure to radiotracer-free seawater, where the mean data for samples from (a) each of three cohorts and (b) for the total samples are plotted. The loss curves are also fitted and the results of the associated statistical analyses are given in Table 3. The patterns of loss are distinctly different between the radioisotopes with little or no loss of ^{203}Hg over the whole experimental exposure of 85 days, but with more than 60% of the ^{210}Pb being lost over the same period. The plot for ^{210}Pb suggested an age-related effect and to assess its statistical validity its CF was multiply regressed against (1) age and (2) period of exposure to tracer-free water. The results of these analyses are shown in Table 2 to confirm that the retention of ^{210}Pb is significantly greater ($P < 0.0001$) in older eggs. A similar analysis for ^{203}Hg gave no significant ($P = 0.41$) effect of egg age on its retention rate.

Distribution of ^{210}Pb and ^{203}Hg Among Components of the Egg During the Uptake and Loss Experiments

Table 4 shows the average percentage distributions of both radioisotopes among the four dissected components of all exposed eggs, viz. egg case, jelly, embryo, and yolk, to confirm that the egg case itself accumulates more than 98% of their total activities for the total egg, with very low percentages associated with the embryo, yolk, and jelly. Therefore the uptake patterns shown in Fig. 1 for the total

Table 3 Parameter values for fitted loss curves for ^{210}Pb and ^{203}Hg based on a double-component exponential model $\left(A_t = A_{0s}e^{-k_{es}t} + A_{0l}e^{-ke_lt}\right)$ for the three egg clutches. Standard errors are given in brackets

	A_{0s}	p-level	k_{es}	p-level	R^2	A_{0l}	p-level	k_{el}	p-level
^{210}Pb									
G1	46.66 (12.30)	<0.001	0.07 (0.03)	0.011	0.93	47.03 (13.25)	<0.001	0.004 (0.004)	0.28
G2	35.26 (3.16)	<0.001	0.21 (0.07)	0.002	0.95	62.63 (3.63)	<0.001	0.01 (0.001)	<0.001
G3	30.55 (4.86)	<0.001	0.17 (0.07)	0.016	0.92	67.30	<0.001	0.01 (0.002)	<0.001
Total	34.90 (5.43)	<0.001	0.13 (0.05)	0.012	0.92	61.49 (6.83)	<0.001	0.01 (0.002)	<0.001
^{203}Hg									
G1	3.95 (4.36)	0.37	23.3 (nd)	1.0	0.04	96.05 (1.59)	<0.001	0.0004 (0.001)	0.46
G2	6.72 (6.22)	0.28	24.8 (nd)	1.0	0.06	93.28 (2.15)	<0.001	0.0008 (0.0006)	0.23
G3	4.81 (5.24)	0.36	22.2 (nd)	1.0	0.09	95.19 (1.91)	0.008	0.0017 (0.0006)	0.008
Total	4.88 (3.19)	0.13	24 (nd)	1.0	0.07	95.12 (1.13)	<0.001	0.001 (0.0003)	0.001

Table 4 Mean percentage distributions of ^{203}Hg and ^{210}Pb in egg components at (a) the end of the uptake phase, (b) loss phase at 31 days, and (c) the end of the loss experiment for each hatched dogfish*. Standard errors are given in brackets

Egg components	End of uptake		Loss experiment at 31 days		End of loss phase*	
	^{203}Hg	^{210}Pb	^{203}Hg	^{210}Pb	^{203}Hg	^{210}Pb
Case	98.23 (0.9)	98.96 (0.79)	99.26	99.66 (0.36)	98.96 (0.38)	99.63 (0.85)
Embryo	0.58 (0.66)	0.21 (0.18)	0.21	0.14 (0.15)	1.04 (0.38)	0.37 (0.85)
Yolk	0.76 (0.36)	0.43 (0.64)	0.45	0.17 (0.21)	—	—
Jelly	0.43 (0.23)	0.40 (0.32)	0.08	0.03 (0.01)	—	—

egg actually represent the accumulatory behaviour of the egg case. Consequently, the differences in the uptake rates of eggs among cohorts (Fig. 1) are predominantly determined by the varying accumulation rates of the egg case itself. Dissections of eggs at two periods during the loss experiment also show the same distributions of Pb and Hg among egg components, again indicating that their patterns of loss (Fig. 2) are dominated by the depuration behaviours of the egg case.

Table 5 shows the mean (±SD) CFs for the dissected egg components in the oldest and youngest cohort, i.e., 1 and 3 (group 1; 46–53 days; group 3, 24–28 days of age at the beginning of the uptake experiment) and also their grouped averages. For the embryos the grouped averages are similar between isotopes, viz. 44 for ^{203}Hg and 34 for ^{210}Pb. Comparison of the mean CFs for ^{203}Hg between the two cohorts by an independent t test showed that the oldest cohort had a significantly elevated mean CF by a factor of 6 compared to the youngest cohort ($t = 7.1$, $df = 5$, $P < 0.001$). For ^{210}Pb the mean CF is also higher for the older cohort compared to the youngest but the means were not significantly different ($P > 0.05$).

With regard to the average distributions of ^{203}Hg among egg components, the CFs of the jelly and yolk are comparable, differing little from unity, whereas the embryo is an order of magnitude higher, with the case being more than a factor of ten greater than the embryo. A very similar pattern is seen for ^{210}Pb CFs among egg components.

Table 5 Mean CFs for ^{203}Hg and ^{210}Pb in egg components at the end of uptake phase

Component	G1 Mean (SD)	G3 Mean (SD)	Total Mean (SD)
(a) ^{203}Hg			
Case	672 (159)	974 (403)	844 (340)
Embryo	84 (5)	14 (16)	44 (39)
Yolk	7 (4)	4 (2)	5 (3)
Jelly	2 (1)	3 (2)	3 (2)
(b) ^{210}Pb			
Case	1452 (25)	1533 (303)	1501 (219)
Embryo	47 (22)	26 (24)	34 (23)
Yolk	3 (4)	7 (12)	5 (9)
Jelly	2 (2)	10 (9)	6 (8)

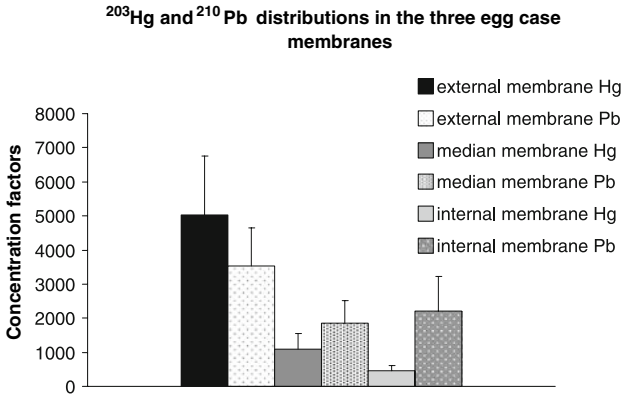

Fig. 3 Concentration factors for ^{210}Pb and ^{203}Hg in the three dissected layers of the egg case, after exposure for 21 days

^{210}Pb and ^{203}Hg Gradients in the Wall of the Egg Case

Figure 3 shows the CFs of the radiotracers in the three egg case membranes at the end of the uptake experiment. These are highest in the outer membrane for both radioisotopes, attaining means of 5000 for ^{203}Hg and about 3500 for ^{210}Pb, with reductions in the two inner membranes for ^{210}Pb to about 2000. The gradient is particularly steep for ^{203}Hg, which declines systematically to a CF of 500 for the innermost membrane.

Figure 4 shows a comparison of the mean activity concentrations of ^{203}Hg and ^{210}Pb in these three membranes at the end of both the uptake and loss experiments. For ^{203}Hg, comparisons of the mean activity concentrations for each of the three membranes of the egg case by a two sample t test for independent variables showed that both the external and internal membranes significantly declined over the loss phase ($t = 3.35$, $df = 12$, $P < 0.01$; $t = 5.22$, $df = 12$, $P < 0.001$, respectively). However, there was no significant reduction ($P = 0.25$) in the activity concentration of the median membrane during the loss period. These results are still consistent with the slow depuration indicated in Fig. 2, as the median membrane is much thicker

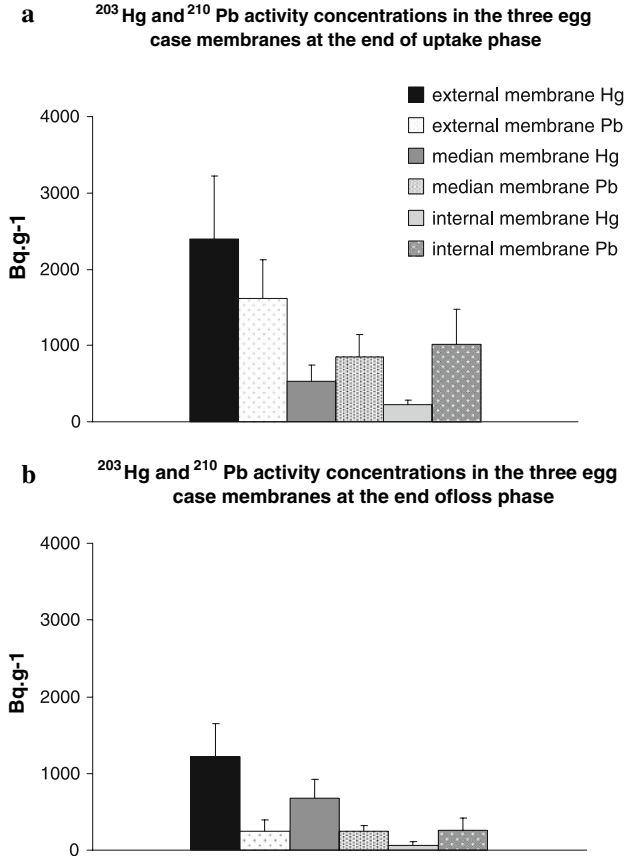

Fig. 4 Activity concentrations of ^{210}Pb and ^{203}Hg in egg case layers at the end of the (**a**) uptake and (**b**) loss experiments

than the other two membranes, containing the majority of ^{203}Hg in the egg case. A similar set of statistical analyses showed that over the loss phase the activity concentration of ^{210}Pb significantly declined in the external membrane ($t = 5.85$, $df = 11$, $P < 001$) and by more than a factor of three. The median membrane significantly declined in mean activity concentration over the loss phase ($t = 5.25$, $df = 11$, $P < 0.001$) by a factor of three, as did the inner membrane ($t = 4.04$, $df = 11$, $P < 0.01$) by about a factor of 4.

Discussion

^{203}Hg and ^{210}Pb Kinetics in Total Eggs

The patterns of accumulation of ^{203}Hg and ^{210}Pb by the total egg (Fig. 1) indicate that equilibrium CFs have not been attained during the 21-day period of experimental exposure, particularly for ^{203}Hg in the younger cohorts where the rates of increase approximate to linearity. Hence the total egg is likely to have a greater capacity to absorb both radioisotopes over longer periods of exposure. These patterns of increasing CF with period of exposure are also consistent with those of a previous experiment showing approximately linear rates of accumulation of seven other radioisotopes (^{241}Am, ^{109}Cd, ^{57}Co, ^{134}Cs, ^{54}Mn, and ^{65}Zn) in the eggs of *S. canicula* over 15 days of exposure under very similar experimental conditions (Jeffree et al. 2006a).

The association of the vast majority (>98%) of ^{210}Pb and ^{203}Hg accumulated by the total egg with the case itself (Table 5) was also virtually identical to the results of this previous experiment for ^{241}Am, ^{109}Cd, ^{57}Co, ^{54}Mn, and ^{65}Zn, with ^{134}Cs being the slight exception in that about 70% was accumulated by the case (Jeffree et al. 2006a). The average concentration factors in the case attained at the end of the uptake experiment for ^{210}Pb and ^{203}Hg, viz. c. 1500 and 850, respectively, were also comparable to those for ^{241}Am, ^{109}Cd, ^{57}Co, ^{54}Mn and ^{65}Zn, that ranged between about 550 and 1800, after a 15 day experimental exposure.

Kinetics of ^{203}Hg and ^{210}Pb in Egg Components

Egg Case

Our results have also demonstrated for measurements on total eggs that the rates of uptake of both radioisotopes, and the rate of depuration of ^{210}Pb, are significantly reduced ($P < 0.001$) with increasing age of the egg (Table 2) following parturition. The indicated curvilinearity in the rates of accumulation of the oldest cohort (Fig. 1; Table 1) combined with results of the multiple regression analysis demonstrating the inverse effect of age on uptake rate (Table 2) suggest that equilibrium CFs may be achieved during longer experimental exposures. This is particularly indicated for ^{210}Pb, which also has a significant rate of depuration ($P \leq 0.05$), based on measurements on the total egg case and its three membranes that all show significant ($P \leq 0.01$) reductions by the end of the loss phase. For ^{203}Hg, this age-dependent effect on its kinetics in the case is less obvious, being only discerned as significant ($P \leq 0.01$) in the thin outer and inner membranes of the case, by the end of the loss phase.

However, such extrapolations of the accumulatory behaviours of the egg case for these two radioisotopes over longer periods based on exposures restricted to the initial phases of a long embryological developmental process assume no appreciable changes in the patterns of its accumulation kinetics. This may well be an unreliable assumption given that qualitative changes take place further in this cycle, for example, the opening of the apertures in the egg case wall and the digestion of the jelly, due to embryonic secretions from its transient head gland.

The reduced rates of accumulation and depuration of ^{210}Pb by the total egg with increased age (Figs. 1 and 2;

Table 2) since parturition is predominantly due to differential rates of accumulation by the case, as it represents the repository of about 99% of the total activity associated with the egg. These demonstrated effects of age on the rate kinetics predominantly of ^{210}Pb, may be due to the following factors:

(1) *Tanning of the egg case*. Earlier histochemical studies on the egg case of the grey bamboo shark *Chiloscyllium griseum* indicated the presence of phenol oxidase in the outer and inner layers of the wall indicative of the tanning of the collagen, making the case harder and more chemically resistant (Krishnan 1959). This is consistent with more detailed information on the sclerotization of both the skate (*Raja erinacea*) and dogfish (*S. canicula*) egg cases that occurs after secretion of capsule precursors from their shell glands during its construction (Koob and Cox 1993; Knight et al. 1996). This process involves a type of quinone tanning in which catechols are introduced in utero and subsequently oxidized to quinones by catechol oxidase, with a latent form of enzyme incorporated in the capsular matrix during secretion. The function of this tanning process in the egg case is interpreted as enhancing its resistance to enzymatic attack by predators and chemical degradation from seawater exposure. If this chemically based tanning process of the egg case of *S. canicula* continues during the post-laying period, consistent with continued darkening following oviposition that is typical in egg-laying sharks and rays (Wourms and Demski 1993), then its radiotracer kinetic properties could also to be modified. Hence the reductions in the rate kinetics of the egg case observed in this study for ^{210}Pb with increasing time following parturition may be explained by its increased tanning and reduced chemical reactivity over this period.

(2) *Effect of embryonic secretions*. Within the later prehatching phase of embryonic development secretions of the embryonic hatching gland of *S. canicula* digest both the jelly content inside the egg case and the cement that seals the apertures in the egg case, leading to direct access by seawater to its interior (Mellinger et al. 1986; Ballard et al. 1993). So it may be possible that as the embryo develops following parturition, that its secretions could influence the kinetics of ^{210}Pb in the egg case. Previously we investigated both the effect of the presence of the embryo within the egg case and the aperture in the wall of the egg case on the CFs of ^{241}Am, ^{60}Co, and ^{134}Cs in the three dissected membranes of the egg case (Jeffree et al. 2007). For only ^{60}Co there was a single significant ($P = 0.038$) effect where the embryo's presence enhanced its CF in the median layer of the egg case, but no detected effect of the aperture itself on accumulation of these radioisotopes in the three layers of the egg case. In the present study only in the depuration phase had embryonic development proceeded to the prehatching phase where the apertures in the cell wall were opened by the embryonic secretions; whereas age-dependent effects on the rate kinetics of ^{210}Pb are also observed during the uptake phase, before any obvious effects of embryonic secretions on the egg case are known to occur.

Distributions of ^{210}Pb and ^{203}Hg in the Wall of the Egg Case

The high CFs attained in the egg case by both radioisotopes, particularly in its external layer (Fig. 3), show declining gradients towards the inner membrane, suggesting their movement to the interior of the egg capsule. This interpretation is consistent with the presence of both radioisotopes in the embryo, yolk, and jelly (Table 5) for eggs in which the apertures in the case have not yet opened. The differences in the CFs among the three layers, also previously demonstrated for ^{241}Am and ^{57}Co (Jeffree et al. 2007), may also be influenced by the microanatomical differences between them. In *S. canicula* the external layer of the case differs anatomically from the two internal layers in that it contains numerous granules within a dense matrix of collagen molecules. These granules contain catechol groups that are hypothesised to be associated with hydrophobicity and chemical resistance of the external membrane (Knight et al. 1996).

The comparison of activity concentrations of both radioisotopes between case membranes sampled at the end of the uptake and loss experiments (Fig. 4) also reveals a lability in the ^{203}Hg accumulated in both the external and internal membranes that was not discerned when its CFs in the whole egg case were statistically assessed for loss (Table 2). Of course the significant ($P < 0.05$) depuration of both radioisotopes during the loss phase, particularly that for the inner membrane, is a prerequisite for them being available for bioaccumulation by the embryo, with the consequent potential for chemotoxic effects on it.

Embryo

The mean embryonic CF's attained at the end of the uptake phase for ^{203}Hg and ^{210}Pb are both much higher (see Table 5) than those attained by embryonic *S. canicula* in a previous experiment of 15 days exposure where they were 0.14–7.38 for radioisotopes of Mn, Co, Zn, Cd, Cs, and

Am. This difference may be explained by the longer exposure period with ^{203}Hg and ^{210}Pb; however, egg-case CFs were comparable among all these radioisotopes, as discussed above. This would suggest that there is not a strong correlation between the capacities of the case and the embryo to accumulate this range of elements. Similarly, our results show that older embryos accumulate ^{203}Hg to an appreciably greater degree than younger individuals (Table 5), but this was not found for its rate of accumulation by the case. This increasing effect of age on the embryonic CF for ^{203}Hg is similar to that previously demonstrated for ^{57}Co and ^{65}Zn (Jeffree et al. 2006a).

The case apertures were not open during the uptake phase in the present study, but were so in our previous experiment where their presence increased the internal water concentration of ^{241}Am and ^{57}Co and the embryonic CF for ^{241}Am (Jeffree et al. 2007). The high embryonic CFs for ^{203}Hg and ^{210}Pb show both the ability of these elements in solution, most likely as carbonate and chloride complexes for ^{210}Pb and as chlorides for ^{203}Hg, to traverse the wall of the case and the affinity of the embryo to accumulate them from its labelled internal medium (Table 4). Although the wall of the egg case may be a barrier to bacteria and larger molecules (Knight et al. 1996), our results show that it does not guard against exposure to these more toxic metals during the earliest stages of embryonic development in *S. canicula.* In contrast, a field-based study of foetal Hg load in the spiny dogfish *Squalus suckleyi* indicated its limited maternal transfer to the embryo (Childs et al. 1973).

Factors Contributing to the Accumulatory Capacity of the Egg Case

The results of this study have demonstrated the capacity of the egg-case to accumulate both ^{203}Hg and ^{210}Pb reaching high CFs relatively quickly, particularly in the external layer of the egg-case with CFs of 3.5–5 $\times$ 10^3. Similar accumulatory capacity and behaviour were also previously demonstrated experimentally for ^{241}Am and ^{57}Co in the egg case layers (Jeffree et al. 2007). Structural and functional characteristics of the egg-case that are summarised in Knight et al.'s review (1996) suggest that the following attributes may contribute to its high accumulatory capacity:

(1) the indicated oxidative phenolic cross-linking of the case, in combination with bound catechol groups in the outermost layer, to facilitate the chelation of metals from seawater.
(2) the presence of disulphide bonds that act in the stabilisation of the egg-case collagen could be expected to provide strong binding sites for metal chelation, particularly of inorganic Hg; this interpretation is also in accord with the strong retention of ^{203}Hg during the depuration experiment (Fig. 2). Lead-210 would also be expected to be chelated by the case because of its sulphur-seeking behaviour (Nieboer and Richardson 1980).
(3) the high internal surface area that is created by the open meshwork structure of the collagen fibres that make up the laminae of the egg-case layers would increase sites for the potential adsorption of elements passing through the transverse channels in the wall.

The reported capacity of the skate egg case to bind Ca and Mg during the tanning process and following exposure to seawater suggests that the tanning process generates an affinity of the egg case for divalent cations (Hamlett and Koob 1999), which may also be present in the egg case of dogfish.

Conclusions

This experimental study has shown the capacity of the egg case of *S. canicula* both to accumulate very high concentrations of both ^{203}Hg and ^{210}Pb in its surface and internal layers and also its permeability to them, which results in exposure of the embryo. The lability of these metals accumulated by the egg case, demonstrated through the results of the loss experiments with total eggs and comparisons of its dissected layers, indicates the capacity of the egg case to both purge its contaminant load but also to continue to make these contaminants available to the embryo over the longer term during its relatively long period of embryonic development. Such accumulatory and kinetic capacities of the egg case would be particularly significant for heightening embryonic exposure. Following short-term episodic contaminant events in coastal environments, the rapid accumulation by the egg case and its longer-term delivery to the enclosed embryo would maximise both the embryo's chronic and acute exposure regimes for these chemotoxic metals. The results of our study warrant that more field-based measurements be undertaken of these toxic metals in Chondrichthyan eggs and their embryos, particularly in those coastal environments more likely to be exposed to their elevated concentrations.

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