Post-mortem stability of blubber retinoids in by-caught harbour porpoises (*Phocoena phocoena*): implications for the design of biomarker studies

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ABSTRACT

The effect of post-mortem time (0-48 hours) on retinoid concentrations in the blubber and liver of harbour porpoises under natural conditions is investigated to assess the stability of samples collected from animals after death. Organochlorine compounds and lipid content were also determined to assess their potential effects on retinoid status. Organochlorine concentrations remained low throughout the post-mortem period and were considered unlikely to influence retinoid body dynamics. Retinoid concentrations in liver were 5-6 times higher than those in blubber and both were highly correlated. In contrast with liver, blubber can be easily sampled from live individuals using non-destructive biopsy techniques and is therefore considered an alternative tissue to assess retinoid status in marine mammals. Neither significant differences nor trends were detected in the concentration of retinoids over the studied period, indicating that degradation agents (ultraviolet rays, oxygen exposure and heat) did not affect them. Blubber can thus be regarded as a reliable tissue for the assessment of the retinoid status of unpreserved specimens kept up to 48 hours in conditions similar to those of this study.

KEYWORDS: HARBOUR PORPOISE; ORGANOCHLORINES; INCIDENTAL CATCHES; BIOMARKERS; POLLUTANTS; BIOCHEMISTRY

INTRODUCTION

Retinoids comprise both natural molecules with vitamin A activity and synthetic analogues of retinol with or without biological activity (Blomhoff *et al.*, 1992). They are essential in a number of biological processes including vision, reproduction, growth, immune function, differentiation, embryonic development and general health maintenance (Blomhoff, 1994). Organochlorine compounds are known to alter metabolism and the accumulation of retinoids in the body (Brouwer *et al.*, 1989; Chu *et al.*, 1995; 1998; Rolland, 2000; Käkelä *et al.*, 2002; Nyman *et al.*, 2003), although the level at which this effect takes place may be species-specific (Håkansson *et al.*, 1991; Zile, 1992). In general, it is observed that exposure to polychlorinated biphenyls (PCBs), dioxin (TCDDs) and dichloro-diphenyl-trichloroethanes (DDTs) leads to depletion of retinoid reserves in mammalian tissues due to increased mobilisation of retinoids from storage sites, especially the liver and a subsequent increase in their degradation rate (Kelley *et al.*, 1998). Because of the sensitivity of retinoids to organochlorines, they have been proposed as biomarkers of the impact of this group of pollutants (Simms and Ross, 2000; Borrell *et al.*, 2002).

In mammals, retinoids are mainly stored in the liver (Blomhoff, 1994) and thus their body status is commonly assessed through determination of hepatic concentrations (Schweigert and Buchholz, 1995; Käkelä *et al.*, 2002). However, retinoids are lipophilic and they also accumulate in other fatty tissues. Marine mammals have a thick, extremely lipid-rich hypodermis, commonly known as blubber that acts as a thermoregulatory barrier and reserve depot. Blubber is the largest body fat compartment and represents a significant proportion of body mass: approximately 40% in pinnipeds (Schweigert *et al.*, 1987) and 15-45% in cetaceans (Aguilar *et al.*, 1999; Tornero *et al.*, 2004a). Therefore, it is an important body site for retinoid deposition in this group of animals (Schweigert *et al.*, 1987; Mos and Ross, 2002; Tornero *et al.*, 2004a; Tornero *et al.*, 2004b).

Retinoids are unstable compounds with extreme sensitivity to light, oxygen, trace metals, strong acids and excess heat (Blomhoff, 1994; Barua and Furr, 1998). Therefore, the appropriate conditions for the storage and treatment of samples can only be decided after conducting stability studies under controlled field and laboratory conditions. Earlier data suggest that samples must be kept frozen and shielded from light to prevent retinoid oxidation and/or isomerisation (Kishi *et al.*, 1981; Driskell *et al.*, 1985; Comstock *et al.*, 1993; Tanumihardjo *et al.*, 1996; Albalá-Hurtado *et al.*, 2000a; Gatti *et al.*, 2000; Dupertuis *et al.*, 2002). However, no information is available on the stability of retinoids during the time period from death to sample collection.

Bycaught cetaceans are a good source of samples for ecological studies because compared with those found stranded, they are relatively fresh and are representative of the overall population. Thus, they are expected to be neither affected by severe disease nor emaciated, which are common conditions in specimens washed ashore. Moreover, bycaught cetaceans provide biological data and allow the examination of tissues and organs, which are used to determine the main biological traits (age, sex, reproductive condition) and assess their toxicological status. However, in field conditions, a long interval of time between death and sample collection is often unavoidable. Tissue retinoid levels may vary owing to physiological alterations and breakdown. The quantitative determination of these changes

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is essential to calibrate the effect of post-mortem time on retinoid tissue concentrations and thus assess the validity of bycatches as a source of samples for evaluating retinoid status.

The harbour porpoise (*Phocoena phocoena*) is one of the most vulnerable cetaceans to incidental capture in fishing gear, particularly in the North Atlantic (e.g. Donovan, 1994), thus allowing samples to be readily obtained from a relatively large number of individuals. Taking advantage of this and also because this species inhabits waters ranging from pristine to highly polluted, the harbour porpoise was selected with the bottlenose dolphin (*Tursiops truncatus*) as target species for the International Whaling Commission's (IWC) POLLUTION 2000+ programme, an initiative that aims to elucidate pollutant cause-effect relationships in cetaceans (Reijnders *et al.*, 1999; Reijnders *et al.*, 2002). A first step in this project is the design and validation of sampling protocols. A previous paper (Borrell *et al.*, 2002), reviewed overall information on retinoids in cetaceans and the use of these compounds as biomarkers of organochlorine exposure in this group of animals. The objective of the present study, which is part of the IWC POLLUTION 2000+ project, is to calibrate the effect of post-mortem time on retinoid concentrations in the blubber and liver of harbour porpoises to assess the reliability of samples collected from dead animals. To our knowledge, this is the first time such a study has been undertaken for any cetacean.

Organochlorine compounds and lipid content were concurrently determined to evaluate their potential effect on the retinoid status of the sampled individuals. The correlation between blubber and liver retinoid concentrations was also investigated to determine whether the former is a reliable alternative for monitoring retinoids in this species.

MATERIAL AND METHODS

Sample collection

Six freshly by-caught harbour porpoises (5 males and 1 female) of known time of death were examined during the summer of 2001 in the weir fishery in Grand Manan, Bay of Fundy (Canada). They were measured and sexed, and a series of samples were collected from them with the objective of creating sequential replicates of each of the main tissues. Tissue collection, time intervals and preservation conditions followed the methods described in the 'Field Protocol for POLLUTION 2000+' (Reijnders *et al.*, 2002). Thus, an initial blubber sample was collected immediately following death. To mimic natural conditions, animals were then placed in a tank, at a depth of 2m underwater, and suspended beside the dock. Blubber was periodically re-sampled at 3, 9, 24 and 48 hours. Liver samples were not collected at all timepoints to maintain the integrity of the carcasses but, in all animals, a liver sample was collected at 48 hours. Water and carcass temperatures were monitored throughout the holding period. Carcass temperatures were measured using a needle temperature probe. After excision, samples were immediately wrapped in aluminium foil and stored at –20°C until analysis, a temperature at which retinoids in plasma and tissues are known to be stable for up to 10 years (Thomas *et al.*, 1998; Barua and Furr, 1998).

Retinoid analysis

Samples, analysed in triplicate, were treated at room temperature and under red light. The replicates, weighing about 100mg each, were saponified overnight in an ethanolic KOH solution (1g KOH, 2ml distilled H_20, 2ml ethanol, 20mg ascorbic acid) under nitrogen in a mechanical shaker. Retinoids were extracted by adding 8ml di-isopropyl ether and shaking for 30min. After separation from the aqueous phase, the organic extract was cleaned three times with 4ml of aqueous phosphate buffer (pH 7.4) [$KH_2PO_4 10^{-2}$M/KOH6*10^{-3}M]. The extract was dried under nitrogen and reconstituted with 1ml methanol, 0.05% butylated hydroxy toluene (BHT) as an antioxidant and retinyl acetate as an internal standard. Reconstituted samples were filtered (0.20μm mesh) and a 20μl subsample was automatically injected (Waters 700 Satellite wisp) onto a high performance liquid Chromatography (HPLC) system (Waters 600 E System Controller Pump) equipped with a Restek column (Tracer Excel 120 ODS-A, 10cm length, 5μm bed, 0.46cm internal diameter) and a UV detector (Waters 486 Tuneable absorbance D) set at 326nm. Retinoids were eluted at a flow rate of 1ml min^{-1} using a mobile phase of methanol/water (80/20 V/V) for 1min followed by a linear gradient over 3min to 100% methanol. The column was then cleaned and equilibrated with 100% methanol for 14min at the same flow rate.

Organochlorine pollutant analysis

Lipids were extracted from blubber (samples weighing 0.2-1g) using n-hexane in Soxhlet apparatus, and lipid content was determined gravimetrically. Analysis of organochlorine compounds was carried out using capillary gas chromatography-electroncapture detector (GC-ECD) and following the procedures described by Borrell *et al.* (2001). The samples were analysed for the following compounds: HCB, α-HCH, β-HCH, γ-HCH, *pp'*-DDE, *op'*-DDE, *pp'*-DDD, *op'*-DDT, *pp'*-DDT and PCBs. Total hexachlorocyclohexane (tHCH) was the sum of all three isomers (α,β,γ). tDDT concentration was calculated as the sum of the five DDT compounds and total PCB concentration (tPCB) as the sum of 22 individual peaks (IUPAC number 28, 52, 95, 101, 151, 149, 118, 153, 105, 138, 187, 183, 128, 174, 156, 180, 170, 201, 195, 194, 206, and 209). Recoveries of organochlorine compounds ranged from 82-101% (n=12). The laboratory participated in interlaboratory calibration exercises for OCs in biota organised by Quasimeme (1998) and NIST/NOAA (2000 and 2003), obtaining fitting results.

Statistical analysis

The correlation between retinoid concentrations in liver (collected at 48h post-mortem) and blubber (mean of all blubber samples) was analysed by correlation/regression analysis. To compensate for the undesired variability between individuals, the analytical results of retinoids from each porpoise were standardised by calculating the proportion that the concentration at each timepoint (mean of the three replicates) represented in relation to the mean concentration of all timepoints (mean of the 15 replicates: three replicates × five timepoints). The proportions obtained by this method were used in the statistical comparisons. Data were tested for normality by a Kolmogorov-Smirnov test of goodness of fit. As the data distributed normally, differences in retinoid and pollutant levels were determined using analysis of variance (ANOVA) followed by the Tukey t-test to identify distinct sample pairs at $p<0.05$. The standardised retinoid values were also analysed for potential time trends using correlation/ regression analysis. All calculations were carried out using the SPSS-x statistical package.

RESULTS AND DISCUSSION

Table 1 shows the biological characteristics of the sampled porpoises. The POLLUTION 2000+ field protocols (Reijnders *et al.*, 2002) require collection of tissues immediately after the porpoise's death and impose very strict sampling procedures. Proper protocol validation required adjustment to these conditions, and this made field sampling extremely laborious and demanding (see above in 'Sample Collection' section). As a consequence, the number of specimens studied was necessarily limited, though considered sufficient for the study.

Table 1

Sex, body length (cm), blubber lipid content (BLC) (%), and blubber pollutant concentrations of the harbour porpoises studied. Concentrations are determined at several times (hours) after death of the individuals and expressed as μg/g calculated on the basis of the lipids extracted.

ID code	Sex	Body length	BLC	Post-mortem time	tHCH	HCB	tPCB	tDDT
69	M	139	85.07	24	0.12	0.12	4.62	2.27
				48	0.13	0.14	5.45	3.18
84	M	119	80.15	0	0.15	0.11	5.33	2.69
				48	0.14	0.10	4.71	2.44
85	M	126	56.41	24	0.08	0.10	3.13	1.79
				48	0.09	0.09	3.92	1.88
184	M	109	80.62	0	0.10	0.10	3.85	1.67
				48	0.14	0.12	5.42	2.47
191	M	129	69.58	0	0.11	0.10	5.42	2.95
				48	0.10	0.11	4.98	2.65
199	F	150	77.81	0	0.09	0.04	4.76	2.00
				48	0.11	0.06	5.35	2.61

The sex ratio of the animals studied was skewed towards males. However, this was not considered to affect the calibration study because the harbour porpoise does not present significant sexual dimorphism either in body size or in any other anatomical trait that could affect retinoid decomposition. For example, the body surface area to volume ratio, which would affect thermal inertia of internal tissues after death and thus retinoid decomposition rates, is comparable in both sexes. Confirming this, the rate of decrease in the internal temperature of the only female sampled was not significantly different ($p>0.3$) to that of the males. Also, previous studies have showed that retinoid loads in harbour porpoises of comparable ages are also similar in both sexes (Borrell *et al.*, 2002).

The blubber lipid content ranged 56.41-85.07%, suggesting that sampled individuals were overall in good nutritive condition (Lockyer, 1995). The results of the blubber organochlorine analyses are also presented in Table 1. The concentrations of all compounds were higher than those found in harbour porpoises from Greenland (Borrell *et al.*, 1999; Bruhn *et al.*, 1999), of the same order of magnitude as those from Ireland (Smyth *et al.*, 2000), and lower than those from the Baltic Sea (Kannan *et al.*, 1993; Berggren *et al.*, 1999), the North Sea (Wells *et al.*, 1994), Denmark (Berggren *et al.*, 1999) and the United Kingdom (Law, 1994). As compared to studies carried out in the same population in 1989-1991, current organochlorine levels are significantly (approximately four times) lower (Tilbury *et al.*, 1997; Westgate *et al.*, 1997), in agreement with the trend of decreasing organochlorine pollution observed in most temperate regions of the Northern Hemisphere during the last decade (Aguilar *et al.*, 2002). The low concentrations found in this study seem unlikely to have influenced the retinoid dynamics of the porpoises studied. As expected, organochlorine concentrations in blubber did not vary during the 48 hour post-mortem period ($p>0.05$).

Table 2 shows the retinoid concentrations found in the blubber and liver of the specimens studied. Blubber concentrations varied widely between individuals, ranging from 42.6-224μg g^{-1}. These values were slightly higher than those reported in the same tissue in other cetaceans, such as harbour porpoises from West Greenland (Borrell *et al.*, 1999) and common dolphins (*Delphinus delphis*) (Tornero *et al.*, 2004a; b). Liver concentrations were, as expected, very high (131-1680μg g^{-1}) and similar to the highest values recorded in cetaceans, e.g. blue whales (*Balaenoptera musculus*), fin whales (*B. physalus*) and sperm whales (*Physeter macrocephalus*) (Schmidt-Nielsen *et al.*, 1934; Klem, 1935; Braekkan, 1948; Schweigert *et al.*, 1987).

Table 2

Blubber retinoid concentrations (0, 3, 9, 24 and 48 hour post-mortem replicates: mean ± SD) and liver retinoid concentrations (48 hour post-mortem replicates: mean ± SD) in the harbour porpoises studied. Concentrations are expressed as μg/g tissue.

Identification code	*n*	Blubber	*n*	Liver
69	15	140.04 ± 41.12	3	877.83 ± 88.82
84	15	74.23 ± 15.98	3	575.91 ± 118.95
85	12	103.10 ± 26.00	3	170.98 ± 25.27
184	15	42.60 ± 10.46	3	252.21 ± 10.57
191	15	224.03 ± 42.64	3	1,679.46 ± 643.92
199	14	88.26 ± 19.70	3	131.24 ± 52.59

As mentioned above, liver was only sampled at the 48 hour timepoint in order to preserve the integrity of the carcass. Given the absence of trends in blubber retinoid levels, it is assumed that liver was similarly unaffected by post-mortem times. Liver retinoid levels were approximately 5-6 times higher than those in the blubber. Similar studies on marine mammals have also described higher retinoid concentrations in liver than in blubber: more than 10 times higher in adult males and juveniles of grey seals (*Halichoerus grypus*) (Schweigert *et al.*, 1987), 7-8 times higher in ringed seals (*Phoca hispida*) (Käkëla *et al.*, 1997), eight times higher in precocious harbour seals (*Phoca vitulina*) (Mos and Ross, 2002) and approximately three times higher in common dolphins (Tornero *et al.*, 2004a; b).

Retinoid concentrations in blubber and liver were positively correlated (Fig. 1; $p<0.05$, $R^2=0.8$), suggesting that retinoid deposition in both tissues is subject to similar processes. This result concurs with that of Mos and Ross (2002), who reported a similar correlation in harbour seals. Therefore, both liver and blubber are equally reliable tissues for monitoring body retinoid status in these animals. However, access to the liver is not possible in free-ranging individuals, and the tissue decomposes rapidly post–mortem, so liver is in most cases an unsuitable tissue to monitor. As blubber can be easily sampled from both free-ranging and captured individuals using non-destructive biopsy techniques (Aguilar and Borrell, 1994), it is a reliable alternative to assess the retinoid status of marine mammals.

Fig. 2 shows the variation of the mean temperature of carcasses and seawater at various timepoints during the 48 hour post-mortem period. Holding water temperature ranged from 11.1-14.5°C (mean: 12.9°C). Carcass internal temperatures decreased drastically from the moment of

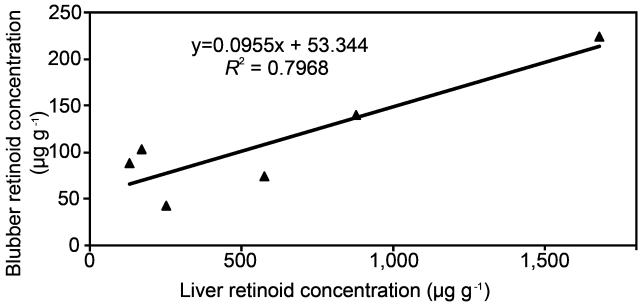

Fig. 1. Correlation between liver and blubber retinoid concentrations (µg g^{-1} tissue).

death (35.4-36.6°C) to the 48 hours timepoint (12.0-12.4°C). Fig. 3 shows the mean relative retinoid blubber concentrations at each sampling time in each studied individual. We did not find significant differences or trends in the concentration of retinoids over the studied period, neither in the ANOVA nor in the correlation/regression analyses (p>0.05). This indicates that the potential degradation agents did not affect blubber retinoid levels.

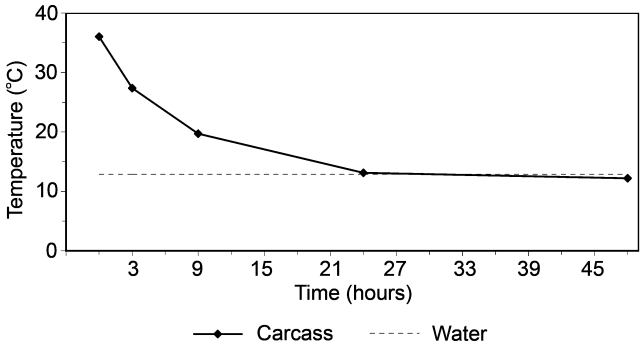

Fig. 2. Means of seawater and carcass temperatures at each sampling time: 0, 3, 9, 24 and 48h.

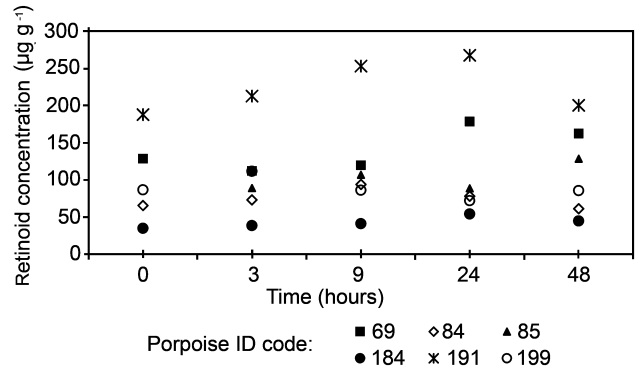

Fig. 3. Blubber retinoid concentrations at the different sampling times (0, 3, 9, 24 and 48h) in each harbour porpoise.

Three main agents have been reported to influence retinoid levels: ultraviolet (UV) rays, oxygen and heat. Although no information is available on the physicochemical stability of retinoids contained within the cellular structure of a tissue, direct exposure to the UV rays present in sunlight causes severe degradation of retinoids (Allwood and Plane, 1984; Chen *et al.*, 1996; Gatti *et al.*, 2000). Here, the skin cover apparently provided an effective barrier to UV penetration into the carcass. However, it should be noted that the corpse was kept 2m below the water surface and that the seawater in the Bay of Fundy is quite opaque owing to strong tidal mixing coupled with ample sediment sources. Thus, sunlight exposure at timepoints was strongly mitigated by the prevailing environmental conditions. From the moment of tissue collection to analysis at the laboratory, samples were protected from light by wrapping with aluminium foil and deep freezing, thus avoiding the effect of UV rays.

Exposure to oxygen also induces the loss of retinoids (Le Maguer and Jackson, 1983; McCarthy *et al.*, 1986). In previous studies, oxidative degradation had been prevented through special handling and preservation procedures, including storage under nitrogen or argon and addition of antioxidants like ascorbic acid or butylated hydroxy-toluene (BHT) (Wyss, 1990). Anoxia in the corpse occurs within hours of death and as a consequence, oxygen degradation did not appear to affect retinoid tissue concentrations before sampling. When the specimen was retrieved, the samples were quickly wrapped in aluminium foil to avoid dehydration and prevent direct exposure to the atmosphere; the analysis was performed under nitrogen and BHT was added. These precautions were sufficient to prevent retinoid oxidation.

Finally, the temperature of seawater also seemed to be sufficiently low to ensure the stability of retinoids during the study period. This is in agreement with previous studies reporting the stability of retinoids during storage in the dark at 2-8°C (Gatti *et al.*, 2000; Sforzini *et al.*, 2001), at room temperature (Nierenberg, 1985; Halbaut *et al.*, 1997; Albalá-Hurtado *et al.*, 2000b; Gatti *et al.*, 2000) and even at 20-40°C (Albalá-Hurtado *et al.*, 2000b; Sforzini *et al.*, 2001; Dupertuis *et al.*, 2002). In contrast, Sforzini *et al.* (2001) found high instability at room temperature, Chen *et al.* (1996) at 4, 25 and 35°C, and Halbaut *et al.* (1997) at 30°C. At higher temperatures, numerous authors have described considerable losses of retinoid content: the higher the temperature, the greater the loss (Albalá-Hurtado *et al.*, 2000a; Gatti *et al.*, 2000). Retinoid loss depends on the chemical nature of both the retinoids and the other species present in the sample (Albalá-Hurtado *et al.*, 2000a). The variable amount of molecular oxygen, the fat protective effect, and the possible synergetic effect between retinoids and other components, such as tocopherols, ascorbic acid and lipids, may account for the differences between authors' results (Billion-Rey *et al.*, 1992; Albalá-Hurtado *et al.*, 2000b; Dupertuis *et al.*, 2002). The protein-bound retinoids and the richness in natural antioxidants (Barua and Furr, 1998), as well as the high lipid content of cetacean blubber, 35-90% (Lockyer *et al.*, 1985; Aguilar and Borrell, 1990; Lockyer, 1991; 1993; 1995), may also have contributed to the stability of the retinoids.

We can conclude that, in the conditions of this study, retinoids remain stable at least during a 48 hour post-mortem period and, more generally, that retinoids are present in the blubber of harbour porpoises in such a state that they are not easily affected by degradation. As a consequence, blubber can be considered a reliable tissue for the assessment of the retinoid status of unpreserved specimens during periods and in conditions similar to those reported here. There are no grounds to extend this conclusion to longer periods, higher environmental temperatures or specimens of different body size. This is critical for the use of stranded individuals because corpses are often found over 48 hours post-mortem, directly exposed to sunlight and may have been subjected to temperature rises due to sun irradiation. As a consequence, the current

protocol validation should not be unreservedly extended to stranded individuals. Given that this is the only attempt to calibrate sampling protocols so far undertaken for a cetacean species, further comparable studies are required to determine the actual range of conditions acceptable for retinoid monitoring in these animals.

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