Tesis doctoral

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Retinoides como biomarcadores de contaminación en pequeños cetáceos

Memoria presentada por Mª Victoria Tornero Álvarez para optar al título de Doctora en Biología por la Universidad de Barcelona, bajo la dirección del Dr. Alex Aguilar Vila y la Dra. Assumpció Borrell Thió.

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1. INTRODUCCIÓN

El desarrollo de la moderna actividad industrial y agrícola ha derivado en una creciente producción y utilización por el hombre de un amplio rango de compuestos químicos. Estos productos, sus derivados y los subproductos generados durante su fabricación, son usualmente depositados en el reservorio final de la mayoría de los deshechos de nuestra sociedad: el mar. El problema que se presenta entonces es conocer si la presencia de estos compuestos supone un riesgo para el ser humano y para la biota del medio marino. Entre los contaminantes ambientales, los más peligrosos para la vida salvaje son aquellos con alto potencial para acumularse gradualmente y permanecer en los organismos, incorporándose y persistiendo a lo largo de las cadenas tróficas. A mediados de los años 60, nuevos representantes de compuestos recalcitrantes de este tipo fueron añadidos a la lista de contaminantes orgánicos persistentes cuando se descubrió que diversos compuestos organoclorados, principalmente los DDTs y los bifenilos policlorados (*polychlorinated biphenyls* o PCBs), se acumulaban hasta alcanzar concentraciones que podían resultar tóxicas para la fauna silvestre.

Los mamíferos marinos, un amplio grupo de animales que incluye a cetáceos, pinnípedos y sirénidos, resultan particularmente vulnerables a estos contaminantes debido a su biología, fisiología y ecología. No sólo muchos de estos animales son predadores finales de las redes alimentarias, sino que, debido a sus necesidades de almacenamiento de energía y termorregulación, una larga proporción de su cuerpo está compuesta por tejido adiposo, rico en lípidos, que acumula eficientemente los compuestos organoclorados, fuertemente lipófilos (Aguilar *et al*., 1999). Los cetáceos, además, carecen de ciertos sistemas de detoxificación comunes en aves y otros mamíferos, por lo que en ellos los procesos de acumulación y toxicidad son más intensos (Tanabe *et al*., 1988). Por todo ello, no resulta una sorpresa que, entre los vertebrados, los odontocetos cetáceos sean los animales en los que se han registrado las mayores concentraciones de contaminantes organoclorados (O`Shea & Aguilar, 2001).

Aunque existe muy poca información acerca del impacto tóxico de estos compuestos en las poblaciones salvajes de mamíferos marinos, la exposición crónica a DDTs y PCBs ha sido asociada con una serie de efectos fisiológicos adversos, tales como daños en el sistema reproductor, alteración del crecimiento y desarrollo óseo, hepatotoxicidad, generación de tumores o depresión del sistema inmunitario (Addison *et al*., 1989; Béland *et al*., 1993; De Swart *et al*., 1995; Lahvis *et al*., 1995; Zakharov *et*

al., 1997; Schwacke *et al*., 2002). Asimismo, ciertas epidemias infecciosas que han devastado diversas poblaciones de delfines y focas en las últimas décadas han sido relacionadas con las altas concentraciones de organoclorados presentes en los ejemplares que sucumbieron a la enfermedad (Aguilar & Borrell, 1994; Simmonds & Mayer, 1997). Obviamente, estos compuestos pueden influir significativamente en las poblaciones afectadas, lo cual puede resultar especialmente grave en aquellos casos en que la supervivencia de los individuos se encuentre ya amenazada por otras actividades humanas, como la captura incidental en artes de pesca, la explotación, la destrucción o deterioro del hábitat o la sobreexplotación pesquera. Por tanto, la conservación y protección de los mamíferos marinos requieren, de un modo muy particular, el conocimiento del efecto tóxico de estos contaminantes en las especies más susceptibles de resultar expuestas a ellos.

Una cuestión crucial y aún no resuelta en ecotoxicología es la relación causa-efecto de las concentraciones de contaminantes en los organismos. Hasta el momento, la mayoría de los estudios de contaminación realizados en cetáceos únicamente han determinado las concentraciones de compuestos químicos presentes en sus tejidos. Por sí sola, esta información es insuficiente para establecer si los niveles de exposición detectados son relevantes y están relacionados con algún efecto sobre el metabolismo, fisiología o la salud de los animales. Durante los últimos años, el concepto de biomarcadores ha sido introducido para evaluar tales relaciones. Un biomarcador se define como "cualquier variación inducida por uno o varios agentes contaminantes en los componentes celulares o bioquímicos de un proceso, estructura o función que puede ser medida en un sistema biológico" (NRC, 1987). Es esperable que los biomarcadores proporcionen una medida integral de la respuesta de un organismo a la exposición a la contaminación y a partir de aquí, una medida del riesgo ecológico (Mc Carthy & Shugart, 1990). No obstante, el uso de biomarcadores en mamíferos marinos está en sus inicios y todavía se necesita un considerable esfuerzo investigador para poder validar su aplicabilidad.

Un biomarcador ideal debe ser específico, sensible y fácil de usar en el análisis de muestras obtenidas de una manera no destructiva. Para su uso, es fundamental definir cuidadosamente las especies y las áreas donde sea más probable lograr los mejores resultados. Sin embargo, una de las principales dificultades para la utilización de biomarcadores en poblaciones salvajes de mamíferos marinos, y específicamente en cetáceos, es la recolección de muestras adecuadas. En otros grupos animales, la mayor

parte de este tipo de estudios ha sido llevado a cabo en condiciones experimentales de laboratorio, empleando animales que se sacrifican específicamente para la investigación. Este tipo de estudio resulta imposible en mamíferos marinos, en los que la mayor parte de las especies se halla en recesión o amenazada. Es necesario, por tanto, que las muestras sean obtenidas usando técnicas no destructivas, perturbando lo mínimo posible a los animales, o bien que procedan de ejemplares que mueren por causas ajenas al estudio.

La ausencia de trabajos experimentales en cetáceos ha obligado a los investigadores a depender, para la evaluación de la toxicidad, de la extrapolación de datos encontrados en otros grupos de animales o de las correlaciones entre los efectos observados y los niveles de contaminantes detectados en los tejidos sin ser capaces de confirmar una relación causa-efecto. De este modo, varios biomarcadores que aparentemente responden a la exposición a contaminantes en otras especies, en estudios con pinnípedos en cautividad o en experimentos de laboratorio, han sido propuestos para su aplicación en cetáceos. Por ejemplo, resultan particularmente prometedores en este sentido los aductados de ADN, las hormonas sexuales, la inducción enzimática, las hormonas tiroideas, los indicadores del estatus del sistema inmune, las porfirinas, la luciferasa y los retinoides (Peakall, 1992).

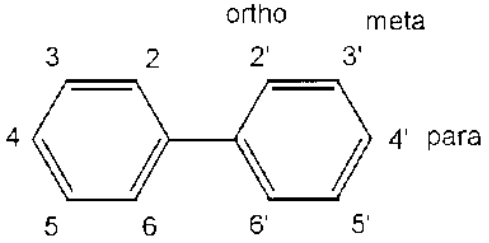
Por estos motivos, en la actualidad, los estudios toxicológicos en cetáceos se centran en dos aspectos principales: la identificación de las poblaciones más afectadas por los contaminantes percibidos como más tóxicos y abundantes, y el desarrollo y validación de biomarcadores. En esta última tendencia se ubica la presente tesis, cuyo eje central es la investigación del potencial uso de los niveles de retinoides como biomarcadores de la exposición a contaminantes organoclorados en cetáceos.

2. COMPUESTOS ANALIZADOS

2.1. CONTAMINANTES

A la hora de seleccionar los compuestos contaminantes prioritarios para identificar efectos sobre cetáceos, hay que considerar sus niveles de producción, su toxicidad, su estabilidad en el medio y su potencial de bioacumulación. Este estudio se centra en los compuestos organoclorados, concretamente PCBs y DDTs, puesto que su origen es exclusivamente antropogénico, sus concentraciones en los tejidos de algunos cetáceos son extraordinariamente elevadas y sus negativos efectos sobre la vida salvaje son ampliamente reconocidos. Además, debido a su gran producción y uso, estos compuestos se hallan dispersos por todo el planeta y existe una sustancial información acerca de sus modelos de variación y su cinética en los tejidos (Reijnders *et al*., 1999). Así, es sabido que las concentraciones de organoclorados y la susceptibilidad al compuesto varían considerablemente entre individuos de diferente sexo, edad, estado nutritivo o condición reproductiva. Por tanto, la interpretación de las concentraciones de contaminantes requiere un conocimiento sustancial de las características biológicas del animal muestreado (Aguilar *et al*., 1999).

Los bifenilos policlorados o PCBs están formados por un sistema de dos anillos bencénicos (bifenilo), en los que un número variable de hidrógenos ha sido sustituido por átomos de cloro. Teóricamente pueden formarse 209 isómeros y congéneres de PCB, variando el número y posición de los átomos de cloro sustituidos.



bifenilos policlorados (PCBs)

Aunque los PCBs fueron introducidos en el mercado al comienzo del siglo XX, su producción alcanzó el máximo en las décadas de los 1960 y 1970, cuando fueron utilizados en una gran diversidad de aplicaciones industriales: fluidos de transferencia

de calor, fluidos hidráulicos, transformadores, aislantes o fluidos dieléctricos. Esta inusual versatilidad de los PCBs está directamente relacionada con sus propiedades físicas, las cuales incluyen resistencia a los ácidos, a las bases y a la oxidación, estabilidad térmica y fuerte carácter lipófilo. Su amplio uso, unido a una eliminación inadecuada de los productos de deshecho, ha conllevado una significativa contaminación ambiental de estos compuestos. A finales de los años 70, se tomó conciencia de que la exposición a los PCBs acarreaba importantes efectos negativos a los organismos, por lo que su uso y comercialización se prohibió en la mayoría de países desarrollados.

El DDT (dicloro bifenil tricloroetano) ya había sido sintetizado a finales del siglo XIX, pero no fue utilizado como insecticida hasta la 2ª guerra mundial, cuando se empleó para combatir la malaria entre las tropas norteamericanas. En los años 50 fue comercializado para usos agrícolas, y a mediados de los 60 se usaba extensivamente para el control de plagas en agricultura. Debido a la creciente resistencia que adquirieron los insectos al DDT y al progresivo convencimiento de que se estaba produciendo un daño ecológico irreparable, su uso fue igualmente prohibido en los años 70 en muchas partes del mundo.

A pesar de esta prohibición, la recalcitrante naturaleza de estos contaminantes y, por tanto, su larga vida media en el ambiente, ha favorecido su transporte global desde la tierra al medio marino, donde se acumulan a través de las redes tróficas (Tanabe, 2002). Además, en algunos países continúan aún hoy siendo fabricados y usados (en particular, el DDT), y redistribuidos al ecosistema marino global, por lo que su importancia como contaminantes de alto riesgo no puede.menospreciarse.

2.2. RETINOIDES

El término “retinoide” incluye tanto las formas naturales de la vitamina A como otros compuestos sintéticos análogos del retinol sin actividad biológica (Blomhoff *et al*., 1994). Esta definición comprende compuestos como retinol, retinal, ácido retinoico y retinil palmitato. El retinol, o vitamina A, es un alcohol primario insaturado de bajo peso molecular (286.46 daltons) y de fórmula empírica $C_{20}H_{30}O$, el 3,7-dimetil-9-(2,6,6-trimetil-1-ciclohexen-1-il)-2,4,6,8-nonatetraen-1-ol. La forma aldehído, retinal, se encuentra en la retina del ojo y el ácido retinoico es altamente activo en muchas funciones fisiológicas (Wolf, 1984). Los retinoides son prácticamente insolubles en

agua y muy solubles en grasas y disolventes orgánicos. Son compuestos inestables, extremadamente sensibles al oxígeno, a los agentes oxidantes, a los metales, la luz y el excesivo calor (Barua & Furr, 1998).

Los retinoides son esenciales para muchos procesos biológicos, como la visión, la reproducción, el crecimiento, el desarrollo y la diferenciación celular. Sin embargo, son tóxicos cuando se toman en exceso y pueden provocar pérdida de peso y apetito, irritabilidad, disfunciones renales o dolores de huesos y articulaciones. El interés de los investigadores en los retinoides aumentó considerablemente desde que la vitamina A fue asociada con la prevención y el tratamiento del cáncer y otras enfermedades (Goodman, 1984). Desde un punto de vista ambiental, los retinoides han recibido una creciente atención porque algunos contaminantes químicos, especialmente los compuestos organoclorados, alteran su metabolismo y concentraciones en los tejidos. Debido a esto, Peakall (1992) clasificó a los retinoides entre los biomarcadores apropiados para el control de los contaminantes ambientales. Por otra parte, dado que los retinoides juegan un papel crítico en los sistemas reproductor e inmunitario, dos funciones a través de las cuales se cree que los organoclorados han afectado a las poblaciones de mamíferos marinos, la identificación de anomalías en las concentraciones de vitamina A es relevante a la hora de valorar el impacto de estos contaminantes en las poblaciones expuestas (Aguilar *et al*., 1999; O`Shea & Aguilar, 2001).

3. OBTENCIÓN DE MUESTRAS

3.1 FUENTES

Para la evaluación de un potencial biomarcador es imprescindible disponer de un número de muestras relativamente elevado y, en la medida de lo posible, de información acerca de las variables biológicas necesarias para la interpretación de los resultados toxicológicos. Además, como se ha comentado anteriormente, conviene que dichas muestras sean obtenidas mediante técnicas no destructivas. Debe reconocerse que, en cetáceos, la recolección de muestras apropiadas constituye una tarea difícil que supone un gran esfuerzo y, generalmente, un alto coste económico. Dadas las limitaciones éticas y prácticas, el material necesario para la investigación en estos animales puede provenir de tres fuentes principales: los varamientos de animales muertos en la costa, las capturas incidentales en artes de pesca y las biopsias realizadas a ejemplares vivos.

En el caso de los varamientos, los datos tienden a estar sesgados y deben ser interpretados con precaución. Es muy probable que un animal varado haya sufrido una enfermedad y, si ésta fue larga y severa, presentará un pobre estado nutritivo, lo cual puede afectar tanto a los niveles de contaminantes como a los niveles del biomarcador objeto del estudio, en este caso de los retinoides. Al mismo tiempo, la composición por edades de los animales varados más bien refleja la composición por edad de mortalidad que la composición por edad de la población, ya que las crías y los animales mayores suelen varar más frecuentemente que los juveniles y los animales maduros. Por otra parte, cuando se encuentra el cadáver de un animal varado, normalmente no se conoce el tiempo transcurrido desde su muerte y es esperable que la descomposición y los cambios térmicos a los que ha estado sometido (p.e. exposición intensa al sol) hayan producido variaciones en las concentraciones de los distintos compuestos. La ventaja de esta fuente de muestras es que se puede acceder a todos los tejidos y así conocer con precisión la información biológica necesaria.

Las capturas incidentales son aquellas que se producen en ciertos artes de pesca en los que los delfines quedan atrapados, sin que exista una intención de captura por parte del pescador. Ésta es, probablemente, la amenaza más grave para muchas poblaciones de cetáceos a escala mundial. Sin embargo, y obviando su adverso impacto ecológico, representan una excelente fuente de muestras para estudios del tipo aquí propuesto ya que, comparadas con los varamientos, las muestras son más frescas y

representativas de la población. Además, los ejemplares así obtenidos también permiten el examen de todos los tejidos y órganos y, por ello, obtener la información biológica necesaria. No obstante, la disponibilidad de ejemplares obtenidos de este modo está limitada a un reducido número de especies y áreas geográficas.

Las biopsias son pequeñas secciones de epidermis y grasa hipodérmica que se obtienen de animales vivos y aparentemente sanos. La recolección de biopsias, efectuada con dardos equipados con un cabezal en forma de pequeño taladro, resulta inofensiva para el animal pues se realiza a distancia y no precisa su captura. Las muestras que se recogen son totalmente frescas y se asume que constituyen una representación no sesgada de la población. Mediante técnicas moleculares y genéticas, es posible determinar el sexo, la condición reproductiva y el estado nutritivo de los individuos muestreados. Desafortunadamente, esta técnica también tiene sus limitaciones. Su principal desventaja reside en que a partir de los tejidos recolectados no es posible establecer la edad del animal, una variable de gran importancia en las evaluaciones toxicológicas. Por otro lado, para que una muestra sea representativa de la carga de contaminantes del animal debe incluir todas las capas de grasa (Aguilar *et al*., 1991; Koopman *et al*., 1996). Esto es posible en marsopas y delfines de pequeño o medio tamaño, pero no en los grandes cetáceos, ya que los dardos empleados habitualmente son capaces de extraer sólo la capa de grasa más superficial.

3.2. ESPECIES/ÁREAS

Tres especies de cetáceos, todos ellos del suborden *Odontoceti*, fueron seleccionadas para llevar a cabo el presente estudio: el delfín común (*Delphinus delphis*), el delfín mular (*Tursiops truncatus*) y la marsopa común (*Phocoena phocoena*). Estas especies fueron escogidas porque su distribución y estratégica posición en lo alto de la cadena trófica las convierten en particularmente susceptibles a la presencia de contaminantes y, fundamentalmente, porque la obtención de manera adecuada de un número razonable de muestras resultaba factible.

3.2.1. Delfín común (*Delphinus delphis*)

Se trata de un pequeño delfín (hasta 2.5 metros de longitud), de la familia *Delphinidae*, que se caracteriza por el dibujo que presentan sus costados, a modo de reloj de arena, con la parte anterior amarillenta y la parte posterior de color gris. Se encuentra en las aguas oceánicas de todas las áreas tropicales, subtropicales y templadas. En el océano Atlántico, aparece en lugares tan lejanos como Islandia y Nueva Escocia y también se observa en el mar Mediterráneo, aunque en este mar sus poblaciones han sufrido una importante disminución en las últimas décadas, posiblemente debido a la reducción de las fuentes de alimento y a la polución (Universidad de Barcelona, 1994).

El delfín común es una especie frecuentemente capturada de modo incidental en diversas artes de pesca. En España, este problema parece ser particularmente importante en Galicia, y en concreto en la pesquería de arrastre por parejas. La red empleada en este tipo de pesca tiene forma de embudo de grandes dimensiones que es arrastrada por dos barcos a modo de filtro y captura de modo accidental los delfines que se introducen en ella para aprovecharse de la pesca obtenida. Estudios previos indicaban que unos pocos centenares de estos delfines mueren cada año de esta manera en la costa nor-occidental de Galicia. Por tanto, durante los años 2000-2003, se planificaron y efectuaron varias campañas en esta zona con el propósito de recoger el suficiente número de muestras de tejidos de esta especie.

3.2.2. Delfín mular (*Tursiops truncatus*)

Este delfín, que también pertenece a la familia *Delphinidae*, se caracteriza por su aspecto robusto y su corto morro. Pueden llegar a medir casi 4 metros de longitud y su color varía de gris claro o gris pizarra en la parte superior del cuerpo a gris claro y rosáceo en la parte ventral. Habita todos los océanos del mundo excepto en las regiones polares. Suele aparecer cerca de la costa, en zonas como bahías, lagunas o estuarios, aunque se ha demostrado que existen dos formas de esta especie, una costera y otra oceánica. Debido al importante descenso que han sufrido las poblaciones, esta especie está catalogada por la Unión Internacional para la Conservación de la Naturaleza (IUCN), como sujeta a amenazas en su conservación. La Directiva Habitats de la Unión Europea la considera asimismo una especie de conservación prioritaria.

En la bahía de Sarasota (Florida, EEUU), habita la población de delfines mulares mejor estudiada del mundo. Ha sido objeto de un estudio continuado e intensivo durante más de treinta años, y la mayoría de los individuos que componen la población han sido capturados con fines científicos, y liberados a continuación, en repetidas ocasiones desde el año 1970 (Scott *et al*., 1990). Esta población es de particular interés no sólo por el elevado grado de conocimiento general disponible sobre ella, sino también porque permite el acceso a delfines perfectamente identificados, facilitando la recolección de muestras y ofreciendo un profundo conocimiento acerca de las características biológicas de los ejemplares sometidos a estudio.

3.2.3. Marsopa común (*Phocoena phocoena*)

Perteneciente a la familia *Phocoenidae*, es el más pequeño de los cetáceos, con una longitud de unos 1.5 metros. Tiene un aspecto robusto y la cabeza pequeña. Su color varía de gris a negro en el dorso y aletas, gris más claro en las zonas laterales y color crema en la parte inferior. Habita únicamente el hemisferio Norte, en aguas costeras, frías y subárticas, de profundidad inferior a los 150 metros. Esta marsopa ha estado sometida a numerosas presiones humanas que han acarreado un decremento drástico de muchas de sus poblaciones. Las principales amenazas a las que está sometida son las excesivas capturas y la contaminación química. La marsopa común es uno de los cetáceos más frecuentemente capturada de manera incidental en las actividades pesqueras, principalmente en el Atlántico Norte (Donovan 1994). Consecuentemente, se consideró que era viable conseguir muestras no sesgadas, con su información biológica asociada, de un número relativamente alto de individuos. Así, se utilizaron para este estudio muestras procedentes de marsopas capturadas incidentalmente en la industria pesquera de Grand Manan, en la Bahía de Fundy (Canadá).

4. OBJETIVOS

El objetivo principal de esta tesis es evaluar la aplicabilidad de los niveles de retinoides como biomarcadores de exposición a contaminantes organoclorados en poblaciones de cetáceos. Para ello, se llevaron a cabo distintos estudios con los siguientes objetivos específicos:

1) Identificar, a partir de la recopilación y evaluación crítica de la información disponible en la literatura, los patrones generales de la fisiología de los retinoides aplicables a los Odontocetos, con especial énfasis en su absorción, transporte, acumulación en los tejidos, factores de variación natural y respuestas toxicológicas.
2) Establecer un protocolo de recogida de muestras adecuado para la evaluación del estatus de retinoides en cetáceos. Este protocolo requiere en particular:

   2.1.) El conocimiento de la distribución corporal de los retinoides en delfines con el propósito de seleccionar el tejido más apropiado para evaluar los niveles de retinoides en cetáceos.

   2.2.) La identificación de la posición corporal de la grasa de los delfines que debe ser utilizada en el análisis de las concentraciones de retinoides con el fin de asegurar la fiabilidad a la hora de comparar resultados de diferentes estudios.

   2.3.) La evaluación de los cambios sucedidos post-mortem en las concentraciones de retinoides presentes en los tejidos recomendados para el control de los retinoides en cetáceos y el establecimiento de umbrales de tiempo seguros.
3) Establecer los patrones de variación natural de los retinoides en poblaciones de cetáceos mediante la evaluación de las relaciones entre las concentraciones de estos compuestos y las principales variables biológicas: edad, sexo y contenido lipídico.
4) En las poblaciones estudiadas, y con la finalidad de evaluar de modo apropiado el impacto de los organoclorados en las concentraciones de retinoides corporales, conocer las diferencias en las concentraciones de

DDTs y PCBs que existen entre los delfines de una misma población en función de su edad, sexo, condición nutritiva y éxito reproductivo.

5) Establecer los efectos que los niveles de contaminantes organoclorados producen sobre las concentraciones de retinoides una vez que puede ser minimizada la influencia de las variables biológicas sobre ambos compuestos.

5. ARTÍCULOS INCLUIDOS

Esta tesis es el resultado de la compilación de los siguientes trabajos de investigación, los cuales se encuentran publicados, aceptados o enviados para publicar en diversas revistas científicas:

Art. 1. Borrell, A. Tornero, V. Aguilar, A. 2002. Retinoids as biomarkers of organochlorine compounds in marine mammals. Journal of Cetacean Research and Management 42(2): 203-211.

Art. 2. Victoria Tornero, Asunción Borrell, Jaume Forcada, Eva Pubill, Alex Aguilar. 2004. Patterns of retinoid and lipid concentrations in the blubber of common dolphins (*Delphinus delphis*): implications for monitoring vitamin a status. Comparative Biochemistry and Physiology Part B. Vol 137/3: 391-400.

Art. 3. Victoria Tornero, Asunción Borrell, Jaume Forcada, Alex Aguilar. Tissue distribution of retinoids in common dolphins (*Delphinus delphis*). Marine Ecology Progress Series 280: 275-283.

Art. 4. Victoria Tornero, Asunción Borrell, Alex Aguilar, Randall S. Wells, Jaume Forcada, Teri K. Rowles and Peter J.H. Reijnders. 2005. Effect of organochlorine pollutants and individual biological traits on blubber retinoid concentrations in bottlenose dolphins (*Tursiops truncatus*). Journal of Environmental Monitoring. 7: 109-114.

Art. 5. Tornero, V., Borrell, A., Pubill, E., Read, A., Reijnders, P.J.H. and Aguilar, A. Post-mortem stability of blubber retinoids in by-caught harbour porpoises (*Phocoena phocoena*): implications for biomarker design studies. Journal of Cetacean Research and Management. Submitted.

Art. 6. Randall S. Wells, Victoria Tornero, Asuncion Borrell, Alex Aguilar, Teri K. Rowles, Howard L. Rhinehart, Suzanne Hofmann, Walter M. Jarman, Aleta A. Hohn, Jay C. Sweeney. Integrating life history and reproductive success data to examine potential relationships with organochlorine compounds for bottlenose dolphins (*Tursiops truncatus*) in Sarasota Bay, Florida. The Science of the Total Environment. Submitted.

Art. 7. Victoria Tornero, Asunción Borrell, Alex Aguilar, Jaume Forcada, Christina Lockyer. Influence of organochlorines and natural factors on blubber retinoids in common dolphins (*Delphinus delphis*). Environmental Pollution. Submitted.

Retinoids in marine mammals and their use as biomarkers of organochlorine compounds

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ABSTRACT

Retinoids, also known as vitamin A, are non-endogenous molecules that are essential for a number of physiological processes in mammals. Imbalance of retinoids has been associated with reproductive impairment, embryonic mortality, growth retardation and bone deformities, pathologies in skin and the nervous system, and immune suppression. Mammals cannot produce retinoids so their primary source is dietary. They are absorbed by the small intestine and packaged as retinyl esters in chylomicrons, which enter the circulation and end up mostly in the liver and fatty tissues. Plasma retinoid levels are homeostatically regulated, so they remain constant despite variations in dietary supply or tissue stores. Therefore body depletion of retinoids cannot be reliably assessed through levels in blood, and should be evaluated through concentrations in depot tissues. In marine mammals, the main storage sites for retinoids are liver and blubber. Although not a universal rule, the concentration of retinoids often increases with age in both sexes because of progressive build-up of retinyl esters. In addition, sex often affects retinoid levels, but the nature and magnitude of this effect varies between species and populations. Taxonomic, life-style (particularly dietary) and climatic differences may explain dissimilarities in the effect of age and sex on retinoid levels. For this reason, retinoids can be used to distinguish populations or population components showing distinct dietary, behavioural, or other traits. Disease, particularly when affecting organs of physiological importance or inducing malnutrition, may affect retinoid tissue levels, so care should be taken when studying concentrations in stranded animals. Organochlorine compounds, particularly PCBs, dioxin (TCDDs) and DDTs, increase mobilisation of retinoids from hepatic and extrahepatic storage sites into serum, accompanied by enhanced degradation and elimination of retinoids through urine. In terrestrial mammals, this effect increases retinoid concentration. Conversely, in some species of marine mammals plasma retinoid levels have been reported to decrease when exposure to organochlorines increases, although the physiological mechanisms are unclear. However, given the homeostatic regulation of retinoids in blood, variation in plasma is expected to be less than that in liver or blubber. Because retinoid tissue levels vary in marine mammals even at moderate exposure to organochlorines, and original levels are restored when such exposure decreases or disappears, retinoids may be used as a biomarker of the impact of pollutants on populations. Further research is needed to validate their use, particularly in cetaceans.

KEYWORDS: MARINE MAMMALS; RETINOL; ORGANOCHLORINES; BIOMARKERS

INTRODUCTION

Retinoids, also known as vitamin A, are a family of essential molecules involved in a number of physiological functions in mammals. They are not produced endogenously and can only be acquired from external sources (Blomhoff, 1994); therefore, the capacity of the organism for regulation is limited (Green and Green, 1994). From an environmental perspective, retinoids have attracted attention because some xenobiotic compounds, particularly organochlorine compounds such as PCBs and dioxins, have been found to cause their depletion in mammalian body tissues. This effect may induce severe physiological dysfunction (e.g. Thompson, 1976; Brouwer *et al.*, 1989a; b; Jurek *et al.*, 1990; Håkansson *et al.*, 1991a; b; 1992; Ikegami *et al.*, 1991; Chu *et al.*, 1995; 1996; Kelley *et al.*, 2000). As a result of their physiological role and reactivity to certain chemicals, retinoids have been proposed as a biomarker for exposure to organochlorine and perhaps other pollutants (Peakall, 1992; Jensen *et al.*, 1995; Murk *et al.*, 1998). Given that marine mammals (particularly small predators such as seals, dolphins and porpoises) are subject to extremely high levels of organochlorine exposure, this type of pollution is a potential threat to the conservation of populations (Aguilar *et al.*, 1999; O'Shea and Aguilar, 2001).

Although the liver and other tissues of some large whales have long been recognised as a profitable source of retinoids (see below), information on these compounds in marine mammals is limited. This paper reviews current information on retinoid physiology, natural patterns of variation, and effect of organochlorine exposure in marine mammals. When no information is available, literature on terrestrial mammals is referred to.

CHEMICAL STRUCTURE OF RETINOIDS

Retinoid is a general term referring to a group of closely related compounds whose molecular structure consists of four isoprenoid units joined in a head-to-tail manner. This definition includes compounds such as retinol and retinol derivatives, retinal, retinyl palmitate and retinoic acid.

All-trans-retinol (vitamin A alcohol) is the parent vitamin A compound. It is a fat-soluble primary alcohol of low molecular weight (mw = 286). The aldehyde form, retinal, is found in the retina of the eye, and retinoic acid, a metabolite of vitamin A, is highly active in a number of physiological processes (Wolf, 1984).

PHYSIOLOGY OF RETINOIDS

Although retinoids can be toxic in high concentrations and the adverse effects of hypervitaminosis are documented in both man and animals (Armstrong *et al.*, 1994), in natural conditions the most frequent disorders and pathological effects are produced by low availability of the compound.

Retinoids play an important role in: vision (xerophthalmia and night blindness are both symptoms of its deficiency); the maintenance of the reproductive, endocrine and immune systems; growth and foetal development; and the regulation of the proliferation and differentiation of many cell types. Thus, imbalance of retinoids has been associated with a diversity of anomalies, including reproductive impairment, embryonic mortality, growth retardation and bone deformities, pathologies in skin and the nervous system, and immune suppression (Thompson, 1976; Peakall, 1992). Many of these effects are mediated by the action of retinoic

acid on gene expression (Blomhoff *et al.*, 1991). In addition, retinoids have a protective effect against the development of various cancers (Wolf, 1984).

For mammals, none of which can synthesise retinoids, vitamin A is an essential nutrient. Dietary retinoids are available from two sources: from plants in the form of provitamin A precursor compounds, namely β- (mainly), α- and γ-carotene and cryptoxanthin; and from animal tissues as long-chain retinyl esters.

Once in the digestive system, retinoids are absorbed by the small intestine and packaged as retinyl esters in chylomicrons, which enter the circulation and are taken mostly by the liver. In this organ, chylomicrons are metabolised and retinyl esters are processed for hepatic storage or for secretion as retinol bound to retinol binding protein or RBP (mw = 21000) (Blomhoff, 1994; Green and Green, 1994).

RBP delivers plasma retinoids to target tissues throughout the body (Soprano and Blaner, 1994). Over 95% of RBP-retinol circulates in the blood as a 1:1 molar complex with a second transport protein called transthyretin or TTR (mw = 54980), which also transports thyroid hormones TT4 (Blomhoff, 1994; Green and Green, 1994). It has been established that retinoids recycle among plasma, liver and extrahepatic tissues, since the plasma retinoid turnover is more than one order of magnitude greater than the utilisation rate. The vehicle for retinoid recycling is RBP (Blomhoff *et al.*, 1992; Sommer and West, 1996).

Plasma retinoid levels are constant despite great variation in dietary supply or in liver and extrahepatic tissues stores. Thus, it appears that plasma retinoid levels are homeostatically regulated, ensuring that retinoids are continuously available to vitamin A-dependent cells (Wolf, 1984). As a consequence, body depletion of retinoids cannot be assessed through circulating levels in blood, but should be evaluated through concentrations in depot tissues such as liver and fat. The excretion of retinoids in the urine does not appear to be affected by the retinoid status of the animal itself but by the amount of retinoids available through the diet (Raila *et al.*, 2000).

STORAGE OF RETINOIDS IN TISSUES

The comparative tissue distribution of retinoids in mammals has not been studied systematically. However, surveys available for terrestrial species usually point to the liver as the main storage site, with 50-80% of the body load commonly present in this organ. Extrahepatic tissues such as kidneys, adipose tissue, lung or testis, can also play a significant role in the storage and mobilisation of these compounds (Blaner and Olson, 1994). However, there are dissimilarities among species and/or taxonomic groups. For example, in the Canidae and Mustelidae families, retinoid concentrations in plasma are about 10-50 times higher than in other mammals; indeed, in many mammals such a high level would reflect hypervitaminosis A (Schweigert *et al.*, 1990; 1991b). Kidney retinoid concentration in canids is also high and far exceeds those in the liver; such low hepatic levels would normally be considered an indication of severe vitamin A deficiency in other mammals (Underwood, 1984; Schweigert and Buchholz, 1995). It should be pointed out that the urine of canids contains both retinol and retinyl esters (Schweigert *et al.*, 1991a), while that of human and rats only contains metabolic forms of retinoids, such as retinoic acid (Schweigert and Buchholz, 1995). Therefore, the high level of retinoids observed in the kidney of at least the canids can be associated with this particular form of excretion (Schweigert and Buchholz, 1995). As stated above, generally in terrestrial mammals, the concentration of retinoids in blood is kept constant homeostatically and it decreases only when storage tissues are severely depleted (Wolf, 1984; Blomhoff *et al.*, 1992).

Information on the distribution of retinoids in the body of marine mammals is limited to a few studies that report the concentration in selected tissues from the same individuals (Table 1). There are some data on concentrations in isolated tissues, but these cannot be compared between studies because of substantial variation at individual, population and species levels (see below). The information available suggests that, as is usual in terrestrial mammals, retinoids are extensively stored in the form of retinyl esters in the liver. Indeed, it has long been known that the liver of cetaceans is extremely rich in retinoids (Schmidt-Nielsen *et al.*, 1934), and the interest in obtaining this compound for commercial production of vitamin A led a number of researchers during the first half of the century to investigate its contents in the tissues of large whales (e.g. Klem, 1935; Wetlesen, 1938; Braekkan, 1948; Ishikawa *et al.*, 1948; 1951; Kaneko, 1948; Mori and Saiki, 1950; Tawara and Fukazawa, 1950a; b). A similar richness in hepatic retinoids was later confirmed in pinnipeds (Rodahl and Davies, 1949; Schweigert *et al.*, 1987; Ball *et al.*, 1992; Schweigert and Buchholz, 1995; Käkëla and Hyvärinen, 1997; Käkëla *et al.*, 1997).

However, in marine mammals, blubber is also a significant storage site of retinoids and the concentration of retinoids in the blubber of at least some marine mammals appears to be higher than in comparable fatty tissues of man and other terrestrial mammals (Schweigert *et al.*, 1987). Thermoregulatory and lipid storage needs render fatty tissues of marine mammals to be a substantial proportion of body mass, usually in the range 15-55% and, given the lipophilic nature of retinoids, this allows for massive accumulation of these compounds. The blubber/body mass ratio in marine mammals is inversely scaled, so smaller species tend to have a larger contribution of fatty tissues, and therefore larger relative retinoid stores, than larger species (Ryg *et al.*, 1990; 1993; Aguilar *et al.*, 1999). In grey seals (*Halichoerus grypus*), Schweigert *et al.* (1987) have estimated that blubber accounts for about 40% of total body reserves of retinoids. Borrell *et al.* (1999) found that blubber is also a significant site for retinoid deposition in harbour porpoises (*Phocoena phocoena*) from West Greenland.

Information on retinoid levels in tissues or body organs other than liver and blubber is fragmentary. Mori and Saiki (1950) reported concentrations in the intestine of sperm whales (*Physeter macrocephalus*), Iida *et al.* (1998) in muscle of Antarctic minke whales (*Balaenoptera acutorostrata*), Gregory *et al.* (1955) in the milk of blue whales (*B. musculus*), and Rosas and Lehti (1996) in the milk of Amazon river dolphins (*Inia geoffrensis)*. However, the sample size in these studies was extremely small, often limited to a single individual, and they offer no reliable insight into individual variation. Studies in harp seals (*Pagophilus groenlandicus*), grey seals and common seals (*Phoca vitulina*) indicate that other tissues such as kidneys, lung, retina, pancreas and spleen also have minor shares of the retinoid body content (Rodahl and Davies, 1949).

MAIN FACTORS AFFECTING VARIATION IN TISSUE CONCENTRATIONS

As stated above, retinoids are regulated within individual organisms. However biological traits (e.g. sex, age, diet and body condition, incidence of disease, occurrence of

Table 1

Distribution of retinoids (mean ± SD) in plasma (μg/ml) and other tissues (μg/g tissue) of marine mammals. Only surveys reporting concentrations in more than one tissue have been included (see text).

Species	Location	*n*	Age/Sex (M/F)	Liver	Blubber	Serum	Kidney	Lung	Reference
Harp seal (*Pagophilus groenlandicus*)	Newfoundland	1	Adult	720	3.6	-	1.8	0.9	Rodahl and Davis, 1949
Grey seal (*Halichoerus grypus*)	Pembrokeshire	1	Juvenile	465	1.074	-	4.725	0.75	Rodahl and Davis, 1949
Grey seal (*Halichoerus grypus*)	Sable Island	12	Adult M	502.6 ± 314.9	33.7 ± 10.9	0.26 ± 0.057	-	-	Schweigert *et al.*, 1987
Grey seal (*Halichoerus grypus*)	Sable Island	5	Adult F	264.9 ± 118.4	62.4 ± 3.7	0.41 ± 0.085	-	-	Schweigert *et al.*, 1987
Grey seal (*Halichoerus grypus*)	Sable Island	21	Juvenile	375.7 ± 320.6	21.9 ± 14.8	0.21 ± 0.068	-	-	Schweigert *et al.*, 1987
Grey seal (*Halichoerus grypus*)	Sable Island	6	Adult M	609 ± 395	45 ± 10	0.2 ± 0.1	8 ± 3 (all)	-	Schweigert and Buchholz, 1995
Harbour seal (*Phoca vitulina*)	Wash	1	Juvenile	27	Not detected	-	0.27	0.18	Rodahl and Davis, 1949
Ringed seal (*Pusa hispida*)	Baltic Sea	7-9	Adult	175.3 ± 32.6 (*n*=7)	21.6 ± 3.4 (*n*=9)	-	-	-	Käkelä *et al.*, 1997
Ringed seal (*Pusa hispida*)	Lake Ladoga	4	Juvenile	36.1 ± 7.6	3.1 ± 0.5	-	-	-	Käkelä *et al.*, 1997

lactation) and anthropogenic influences (e.g. environmental pollutants) have a substantial effect on tissue levels and body content of retinoids.

Age

The influence of ageing on retinoids status in terrestrial mammals has been widely studied. Many authors reported an increase in concentrations with age: e.g. liver and blood of rats (Blomhoff *et al.*, 1988); plasma of Florida panthers, *Felis concolor oryi* (Dunbar *et al.*, 1999) and man (Malvy *et al.*, 1993; Stephenson and Gildengorin, 2000), and in the kidney of dogs (Schweigert *et al.*, 1998). However, other surveys revealed either no trend in retinoid levels between age classes, or even decreasing ones. For example, Garry *et al.* (1987) found similar plasma retinoid levels in young and old humans, and Savage *et al.* (1999) reported that age did not affect plasma levels of retinoids in free-ranging African elephants (*Loxodonta africana*). A decrease in serum retinoids was observed by Succari *et al.* (1991) in humans and by Shrestha *et al.* (1998) in female Nepalese elephants (*Elephas maximus*).

Similarly, studies on pinnipeds and cetaceans (Table 2) do not produce consistent results. While many populations showed, both in the liver and in the blubber, an increasing trend in retinoid concentrations with age, others revealed no apparent trend or even a decreasing tendency with age. This variation could not be explained by inter-specific, inter-population or even inter-tissue differences. For example, the studies on ringed seals (*Pusa hispida*) from Lake Saimaa by Käkelä *et al.* (1997) showed a significant positive age-related trend in the blubber and a negative trend in the liver, while those conducted on the same species by the same research group and with an identical sample size ($n = 12$) in Spitsbergen showed the opposite result: a negative trend in the blubber and a positive trend in the liver, although in this case the correlation was non-significant (Table 2).

However, although a general, consistent pattern cannot be deduced from the information available, an increasing trend was the most common finding. This relationship appears to be the result of a decrease in the circulatory clearance of retinoids and other liposoluble compounds with age, coupled with an excess intake of retinoids via diet, which leads to a

Table 2

Age trends in retinoid concentrations observed in tissues of marine mammals. * = only females; ** = significative $p<0.05$; " = statistics not performed; ↑ = positive trend; ↓ = negative trend.

Species	Location	*n*	Liver	Blubber	Reference
Australian fur seal (*Arctocephalus forsteri*)	Australia	24*	↑**		Southcott *et al.* 1974
Grey seals (*Halichoerus grypus*)	Sable Island	65	↑ "	↑ "	Schweigert *et al.* 1987
Hooded seals (*Cystophora cristata*)	Newfoundland	60	↑ "		Rodahl and Davis, 1949
Harp seal (*Pagophilus groenlandicus*)	Newfoundland	145	↑ "		Rodahl and Davis, 1949
Ringed seals (*Pusa hispida*)	Lake Saimaa	12	↓**	↑**	Käkelä *et al.* 1997
Ringed seals (*Pusa hispida*)	Spitsbergen	12	↑	↓	Käkelä *et al.* 1997
Ringed seals (*Pusa hispida*)	Baltic Sea	9	↓	↑	Käkelä *et al.* 1997
Harbour porpoise (*Phocoena phocoena*)	Greenland	100		↑	Borrell *et al.*, 1999

build-up of retinyl ester concentrations with age (Maiani *et al.*, 1989; Krasinski *et al.*, 1990). Although it is not clear why some species or populations do not show this general trend, taxonomic, life-style (particularly dietary) and climatic differences may be responsible.

Sex

Information on sex-related variation in retinoids is even more sparse and less consistent than that for age. In terrestrial mammals, no gender-related differences were observed in circulating concentrations of retinoids in black rhinoceros, *Diceros bicornis* (Ghebremeskel *et al.*, 1988), serum levels in free-ranging African elephants (Savage *et al.*, 1999) or liver and serum concentration in humans (Raica *et al.*, 1972; Succari *et al.*, 1991). Conversely, circulating retinoid levels were reported to be higher in female Florida panthers (Dunbar *et al.*, 1999) but lower in females in some human populations (Krasinski *et al.*, 1989; Stephenson and Gildengorin, 2000). These inter-specific differences may be produced by dissimilarities in types of diet and source of retinoids.

In marine mammals, studies on pinnipeds have often suggested sex-related differences although these varied among tissues and species (Fig. 1). Levels of retinoids were found to be higher in the blubber of adult female grey seals (Schweigert *et al.*, 1987) and in the liver of adult female Australian fur seals (*Arctocephalus forsteri*) (Southcott *et al.*, 1974) than in the corresponding tissues of adult males. However, other surveys have shown the reverse trends. Thus, Rodahl and Davies (1949) found higher concentrations in the liver of male hooded and harp seals than in those of females, and Schweigert *et al.* (1987) found a similar difference in the liver of grey seals. In cetaceans, the only available survey refers to harbour porpoises, in which no significant differences were found between the blubber retinoid concentrations of males and females (Borrell *et al.*, 1999).

It has been suggested that mothers transfer retinoids to their calves during lactation (Simms and Ross, 2000), which would explain the lower levels in the liver of adult females (Schweigert *et al.*, 1987). Milk is a source of essential nutrients, including retinoids. Although studies are limited, marine mammals appear to have relatively higher levels of retinoids in their milk than terrestrial mammals. However, this appears to be due to the high lipid content of the milk in pinnipeds and cetaceans because, when concentrations are expressed as quantity per unit lipid, levels are of the same order of magnitude or even lower than in terrestrial mammals (Schweigert and Stobo, 1994; Debier *et al.*, 1999). Irrespective of this, during lactation, females of both cetaceans and pinnipeds mobilise a large proportion of their blubber reserves, including the blubber-associated retinoid stores. This explains why during lactation, unlike humans, marine mammals may have high levels of circulatory retinoids coupled with lowered stores of retinoids in the blubber and probably other tissues (Schweigert *et al.*, 1987). However, no explanation has been put forward to explain the higher concentrations of males reported in some studies.

Similarly to the age-related variation, it is likely that taxonomic, dietary and life-style dissimilarities between sexes are responsible for sex-related variations. Reproductive activity may be particularly significant in adult individuals because it often involves changes in hormone levels, behavioural traits and diet (see below).

Diet and nutritive condition

Since retinoids are incorporated via food, diet affects tissue levels. However, it is unknown, even in man and laboratory animals, whether body stores of retinoids change as a function of long-term intake of these compounds (Ascherio *et al.*, 1992; Booth *et al.*, 1997; Scrofano *et al.*, 1998). As mentioned above, retinoids in blood are homeostatically controlled when liver stores are sufficient and therefore they only respond to extreme situations, for which reason diet has not been observed to have an effect on them (Blaner and Olson, 1994).

In marine mammals, information on the influence of diet on retinoid status is limited to the study by Käkelä *et al.* (1997), who reported differences in liver and blubber levels between freshwater and marine ringed seals and attributed them to food quality. Differences in diet, as well as climatic or photoperiod dissimilarities may explain variations in retinoid levels between allopatrid populations of the same species. However, such differences may also occur between different components within a single population. For example, variation in diet associated with age, sex or

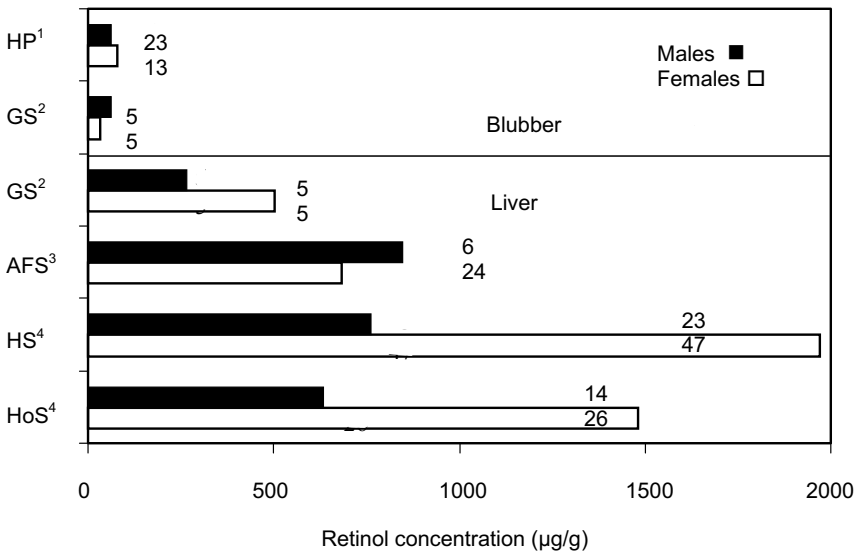

Fig. 1. Sex related variation in retinoid levels (µg/g) in liver and blubber of different marine mammal species. Key: HP = Harbour porpoise; GS = Grey seal; AFS = Australian fur seal; HS = Harp seal; HoS = Hooded seals. References: [1]Borrell *et al.*, 1999; [2]Schweigert *et al.*, 1987; [3]Soutcott *et al.*, 1974; [4]Rodahl and Davies, 1949.

reproductive condition has been reported for many cetaceans and pinnipeds (Seaman *et al.*, 1982; Perez and Mooney, 1986; Stewart and Murie, 1986; Bernard and Hohn, 1989; Recchia and Read, 1989; Rodhouse *et al.*, 1992; Smith and Read, 1992; Clarke *et al.*, 1993). Retinoids thus have the potential to be used to distinguish populations or population components with distinct dietary, behavioural or other traits, provided that the natural sources of variation are properly controlled.

The effect of nutritive condition on retinoid levels is difficult to assess. Neither Rodahl and Davies (1949) or Borrell *et al.* (1999), found any significant effect of condition on the retinoids present in the liver and blubber of hooded and harp seals, or in the blubber of harbour porpoises, respectively. Nevertheless, this conclusion should be treated with caution. The sample examined by Borrell *et al.* (1999) was mainly composed of healthy individuals. While in these conditions, retinoid tissue distribution may remain unaltered. It may require situations of food shortage, massive fat mobilisation (e.g. during migration in large baleen whales or during intensive lactation in some phocids), or starvation caused by disease or other condition, for retinoids to be significantly mobilised, redistributed or excreted. This may be particularly relevant for stranded cetaceans, often found in poor nutritive condition.

The tissue vitamin concentration reflects the essential amounts of these substances necessary for enzymatic and metabolic pathways, coupled with any excess picked up from the environment. The establishment of baseline values of retinoid concentrations is a requisite for the understanding of the chronic effects of toxicity and deficiency (Gelatt *et al.*, 1999).

Disease

Disease, particularly when it affects organs of physiological importance or induces malnutrition, may affect tissue levels of retinoids. However, the information available on this is restricted to humans. Patients suffering from acute or chronic diseases of the liver such as hepatitis, cirrhosis and hepatic tumours have markedly reduced serum levels of RBP, TTR and retinol. Those affected by significant renal disease also show disorders in RBP and retinoid transport, since the kidneys are a major site for RBP catabolism; thus, levels of retinoids increase when excretion is reduced as a consequence of renal tubular damage or reduced glomerular filtration rate of retinol RBP (Goodman, 1984). In addition, sub-normal serum concentrations of RBP and retinoids have been found in patients with a variety of cancers, but it is not clear whether this is a result of protein or energy denutrition (Soprano and Blaner, 1994).

No information is available on disease and retinoids in marine mammals. Given that disease may affect retinoid tissue levels, data from stranded animals in which disease is suspected should not be included in surveys of retinoid status.

EFFECT OF ORGANOCHLORINE POLLUTANTS ON RETINOIDS

Organochlorine compounds can alter retinoid metabolism. However, the biochemical pathway and intensity of the toxic effect appears to vary among species (Håkansson *et al.*, 1991a; Zile, 1992). In general, exposure to PCBs, dioxin (TCDDs) and DDTs leads to depletion of retinoid reserves in mammalian tissue due to increased mobilisation of retinoids from storage sites, especially the liver, and a subsequent increase in their degradation rate (Kelley *et al.*, 2000).

In terrestrial mammals (e.g. rats, otters, minks) feeding on a diet containing toxic organochlorine compounds, the retinol and retinyl ester concentrations in several body organs (liver, depot fat, intestine, lungs and adrenals) have been found to be lower in sample groups exposed to organochlorines than in non-polluted groups. (Brunström *et al.*, 1991; Håkansson *et al.*, 1992; Zile, 1992; Chu *et al.*, 1996; 1998; Murk *et al.*, 1998; Käkëla *et al.*, 1999; Nilsson *et al.*, 2000; Rolland, 2000; Simpson *et al.*, 2000). In contrast, the concentration of retinoids in kidney and, to a lesser extent, in serum, generally increased (Brouwer *et al.*, 1989a; Jurek *et al.*, 1990; Håkansson *et al.*, 1991a; b; Van Birgelen *et al.*, 1994a; b; Chu *et al.*, 1995; Nilsson *et al.*, 2000). This indicates that organochlorines increase mobilisation of retinoids from hepatic and extrahepatic storage sites into serum, accompanied by enhanced degradation and renal elimination of retinoids through urine (Kelley *et al.*, 1998; 2000). Studies on coplanar PCBs and TCDDs have shown that the toxic effect of these compounds is positively correlated with their ability to bind the Ah (arylhydrocarbon) receptor, causing the induction of cytochromes P-450 1A1 and 1A2 (Pelissier *et al.*, 1992; Brouwer, 1995). Thus, it appears that the mixed-function oxidases containing the cytochrome P450s are particularly active in metabolising retinoic acid (Roberts *et al.*, 1979; Ikegami *et al.*, 1991). Moreover, Roberts *et al.* (1992) reported that many rabbit liver cytochrome P-450 isoforms including 2A4, 1A2, 2E1, 2E2, 2C3, 2G1 can catalyse the 4-hydroxylation of both retinol and retinaldehyde. These findings indicate that the decrease in hepatic retinoids storage is related to the induction of cytochrome P-450 and retinoid metabolisation. In laboratory animals exposed to individual PCB congeners, the order of potency in causing reductions in the hepatic contents of retinoids was: PCB 126 > PCB 77 > PCB 153. This order of potency was found to be positively correlated with the ability of each congener to induce cytochrome P450 and with its toxicity measured as weight loss and thymic involution (Chen *et al.*, 1992; Håkansson *et al.*, 1994). In addition, exposure to organochlorines also inhibits the intestinal absorption of ingested vitamin A, thus exacerbating the imbalance produced by the previous effects (Bank *et al.*, 1989).

However, the retinoid depletive effect of these toxic organochlorines can not simply be extrapolated to all organochlorine forms or derivatives. For example, long-term (1 year) experiments conducted with mink fed with methylsulfonyl-PCBs, which are not very AhR-active, did not reveal any effect on retinoid concentrations in tissues (Lund *et al.*, 1999).

Given the evolutionary basis of the physiological processes involved, most of these effects can probably be extended to marine mammals. However, the specific pathways or dynamics may be somewhat different. Thus, most of the studies so far undertaken in three species of pinnipeds and the polar bear (Table 3) have shown a decrease in plasma retinoids when PCB or other organochlorine (OCs) loads increased (Brouwer *et al.*, 1989b; De Swart *et al.*, 1994; Jensen *et al.*, 1995; Beckmen *et al.*, 1997; Skaare *et al.*, 2001). These results originate from studies in both captive and wild populations. In experiments with captive seals, retinoid concentrations returned to normal when animals were fed with slightly contaminated fish (Brouwer *et al.*, 1989b). Unfortunately, only plasma was analysed, so the mechanisms of this decrease were unclear. Given the homeostatic regulation of retinoids in blood, variation in plasma is expected to be lower than in other tissues such as liver or blubber. The only exception (Table 3)

Table 3

Details of studies reporting observed effects of organochlorine pollutants on plasma retinol levels (1) or plasm a retinoid levels (2) in marine mammals, including the polar bear.

Species	Location	*n*	Pollutant	Study type	Effect on concentration	References
Harbour seal (*Phoca vitulina*)	-	24	Organochlorines	Experimental	(1) Decrease	Brouwer *et al.*,1989
Harbour seal (*Phoca vitulina*)	-	22	Organochlorines	Experimental	(2) Decrease	De Swart *et al.*, 1994
Northern elephant seal (*Mirounga angustirostris*)	California	31	Organochlorines	Wild	(1) Decrease	Beckmen *et al.*, 1997
Grey seal (pups) (*Halichoerus grypus*)	Norway	51	PCBs	Wild	(2) Decrease	Jenssen *et al.*, 1995
Harbour seal (pups) (*Phoca vitulina*)	British Columbia/ Washington State	61	PCBs	Wild	(1) Decrease (between populations)	Simms *et al.*, 2000
Harbour seal (pups) (*Phoca vitulina*)	British Columbia/ Washington State	37	PCBs	Wild	(1) Increase (in non-nursing pups)	Simms *et al.*, 2000
Polar bear (*Ursus maritimus*)	Svalbard/ Russian Arctic	79	PCBs	Wild	(2) Decrease	Skaare *et al.*, 2001

appears to be the study by Simms *et al.* (2000), which showed that, although retinoid levels in more polluted populations of harbour seal pups were lower than those in a cleaner population, in non-nursing pups levels were positively correlated with organochlorine levels in the blubber. This correlation was explained by the mobilisation of hepatic stores of retinoids into blood and the disruption of the vitamin A transport complex following exposure to milk-derived pollutants, as previously observed in laboratory and terrestrial mammals.

In pinnipeds, hydroxylated PCBs, which are metabolites produced by phase I enzymes, have also been shown to disrupt retinoid transport complexes in plasma, reducing delivery of retinoids to target tissues (Brouwer *et al.*, 1989b; 1998; Ross and Troisi, 2001) as has been seen in terrestrial mammals.

Given that variation in retinoid tissue levels in marine mammals appears to occur even at moderate exposure to organochlorines (Håkansson *et al.*, 1992; Jensen *et al.*, 1995) and that original levels are restored when pollutants disappear or significantly decrease (Brouwer *et al.*, 1989b), retinoids are potentially sensitive biomarkers of organochlorine exposure. However, it is likely that this sensitivity is higher for retinoid reserve tissues, such as blubber, than for blood. In addition, retinoids play a critical role in reproduction and immune competence, two functions through which organochlorines have allegedly impacted marine mammal populations (e.g. see Reijnders *et al.*, 1999). Thus the identification of any potential imbalance of these compounds is relevant to the assessment of the pollutants impact on the involved populations. However, prior to the use of retinoids as biomarkers in ecotoxicological studies, further research is needed to clarify the dynamics of retinoids and their degradation pathways in the tissues of marine mammals, particularly cetaceans.

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Retinoid and lipid patterns in the blubber of common dolphins (*Delphinus delphis*): implications for monitoring vitamin A status

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Abstract

We determined retinoid concentrations in various body positions of the blubber of 25 common dolphins (*Delphinus delphis*) to study topographical variation in concentrations. Specimens were obtained from incidental catches and were apparently healthy. We found concentrations to be high and therefore conclude that blubber represents a significant contribution to total retinoid body load. Consequently, blubber is proposed as a tissue of choice for monitoring retinoid status in this species. Anterior-ventral blubber had the highest vitamin A concentration and posterior-dorsal the lowest. Therefore, when assessing retinoid status, topographical variation should be taken into account to ensure consistent sampling. This pattern appeared to be explained by a parallel variation in lipid content. Thus, the dynamics and body distribution of retinoids appear to be basically governed by the lipophilicity of the molecules. The highest lipid richness found in the anterior-ventral region might indicate that this region is comparatively more important for insulation and lipid storage than the dorsal posterior region. Retinoid levels did not appear to vary according to sex, but they did vary with lipid content. This should be taken into account when designing sampling protocols; for monitoring purposes, biopsies from healthy, free-ranging individuals should be preferred to samples from stranded animals.

Keywords: Retinoids; Lipid content; Common dolphin; Marine mammals; Blubber; Lipophilicity; Biomarker; North-western Spain

1. Introduction

Retinoids, a group of compounds collectively known as vitamin A, are fat-soluble molecules that play a key role in mammal physiology, particularly in vision, reproduction, growth, immune function and cellular differentiation (Blomhoff, 1994). Retinoids are not produced endogenously and can thus only be acquired through food intake (Blomhoff et al., 1991). Liver is usually the main body site for retinoid storage, but extrahepatic tissues, mainly kidney, adipose tissue, lung or testis also play a significant role in the storage and mobilization of these compounds (Borrell et al., 2002). Biological traits such as age, sex, diet, incidence of disease, occurrence of lactation and lipid composition affect retinoid levels of tissues in varying degrees (Borrell et al., 2002). However, in many species, plasma retinoid levels are homeostatically regulated, ensuring that retinoids are constantly available to vitamin A-dependent cells (Blomhoff et al., 1991).

Exposure to organochlorine pollutants, particularly PCBs, dioxins and DDTs, is known to significantly affect vitamin A dynamics (Borrell et al.,

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2002). In several land mammals such as rats, minks and otters, exposure to these contaminants has been shown to lead to depletion of retinoid reserves (Brunström et al., 1991; Håkansson et al., 1992; Zile, 1992; Chu et al. 1995, 1998; Murk et al., 1998; Käkelä et al., 1999, 2002, 2003; Kelley et al., 2000; Rolland, 2000; Simpson et al., 2000). Few studies have been carried out on this regard in marine mammals, but disruption of plasma retinoids in harbour seals (*Phoca vitulina*), Northern elephant seals (*Mirounga angustirostris*), grey seals (*Halichoerus grypus*) and the polar bear (*Ursus maritimus*) has been demonstrated in both captive and wild individuals (Brouwer et al., 1989; De Swart et al., 1994; Jenssen et al., 1995; Beckmen et al., 1997; Simms et al., 2000; Skaare et al., 2001). The disruption of retinoids may induce several physiological dysfunctions, such as reproductive impairment, embryonic mortality, growth retardation and decreased resistance to infections (Thompson, 1976; Peakall, 1992). Disruption of retinoids appears to occur even at moderate levels of organochlorine exposure (Håkansson et al., 1992; Jenssen et al., 1995) and the original concentrations are restored when pollutants disappear or significantly decrease (Brouwer et al., 1989). Therefore, retinoids have been proposed as sensitive biomarkers of environmental exposure to organochlorine pollutants (Simms and Ross, 2000; Borrell et al., 2002). Since plasma retinoid concentrations are, in general, tightly controlled by homeostasis, changes in retinoid concentration in depot tissues (e.g. fat and liver) may be expected to better reflect overall retinoid status than blood, making them optimal targets for environmental monitoring studies. However, baseline research in this regard in marine mammals is restricted to the work of Nyman et al. (2003), who studied retinoid concentration in the liver of ringed seals (*Phoca hispida*) and grey seals, and found a significant decrease with increasing organochlorine concentrations.

Retinoid status in mammals is commonly assessed through liver concentrations (Schweigert and Buchholz, 1995; Käkelä et al., 2002; Higashi and Senoo, 2003). However, in cetaceans, liver is in most cases inappropriate for monitoring; access to the organ is not possible in free-ranging individuals and the tissue decomposes rapidly postmortem, thus rendering dead animals found stranded of limited use. Blubber, or hypodermic fatty layer, has been proposed as an alternative (Schweigert et al., 1987, 2002; Käkelä et al., 1997; Borrell et al., 1999; Mos and Ross, 2002). Blubber represents a considerable proportion of the total body mass, usually 20–40% (Bryden, 1972), and given its high lipid content, 35–90% (Lockyer et al., 1985; Aguilar and Borrell, 1990; Lockyer, 1991, 1993, 1995), and the lipophilic nature of retinoids, it has been found to be a significant site for retinoid storage (Schweigert et al., 1987). Moreover, hypodermic fat can easily be sampled from both free-ranging and captured individuals using biopsy techniques (Aguilar and Borrell, 1994).

However, the use of blubber for monitoring retinoid status may be hampered by the heterogeneous structure of the tissue. Blubber is structured in layers, of which the biochemical composition varies according to body location (Aguilar and Borrell, 1990, 1994; Lockyer, 1995; Olsen and Grahl-Nielsen, 2003). Since lipophilic compounds distribute in tissues following molecular affinities, this heterogeneity may potentially affect patterns of retinoid deposition within the fatty tissues. Blubber is a massive compartment that practically constitutes the whole body cover of cetaceans; therefore, an understanding of distribution patterns is critical to identifying appropriate sampling locations to be used for monitoring.

Information on retinoid concentrations in the hypodermic fat of cetaceans is extremely limited. Mori and Saiki (1950) reported retinoid values in the blubber of sperm whales (*Physeter macrocephalus*), Iida et al. (1998) in that of minke whales (*Balaenoptera acutorostrata*), and Borrell et al. (1999) in that of harbour porpoises (*Phocoena phocoena*). However, there are no studies on the distribution of retinoids within the tissue. In the present paper, we examine retinoid concentrations in different body locations of the blubber of common dolphins (*Delphinus delphis*) and investigate the effect of sex, body size and body condition on these concentrations.

2. Materials and methods

2.1. Sample collection

We examined and sampled 25 fresh carcasses of common dolphins (15 males and 10 females) incidentally caught by fishing vessels in Northwestern Spanish waters in 2001 and 2002. Necropsies were carried out onboard the boats less than

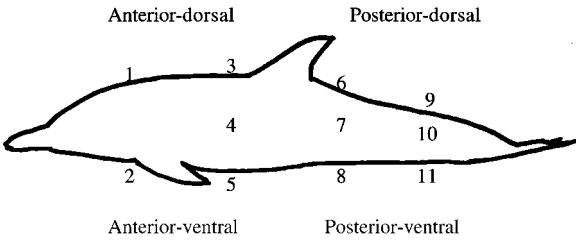

Fig. 1. Blubber sampling locations examined in common dolphins (*D. delphis*).

12 h post-mortem in all cases. Dolphins were measured and sexed and, to avoid the effect of blubber stratification (Aguilar and Borrell, 1990) a sample including all blubber layers was excised from a total of 11 body locations from each individual (Fig. 1). To examine differences in the distribution of retinoids and lipid content between the main body regions, we grouped these locations into four different regions: anterior-dorsal (locations 1,3), posterior-dorsal (locations 6,9), anterior-ventral (locations 2,5) and posterior-ventral (locations 8,11). Samples were transported to the laboratory on dry ice and stored at −20 °C in the dark for analysis.

2.2. Chemical analysis

The methods for retinoid analysis are described in Borrell et al. (1999). Samples were treated at room temperature and under red light. The samples, weighing approximately 100 mg each, were saponified overnight in an ethanolic KOH solution (1 g KOH, 2 ml distilled H_2O, 2 ml ethanol, 20 mg ascorbic acid) in a mechanical shaker under a nitrogen atmosphere. Retinoids were extracted by adding 8 ml diisopropyl ether and shaking for 30 min. After separation from the aqueous phase, an internal standard (retinyl acetate) was added and the organic extract was cleaned three times with 4 ml aqueous phosphate buffer (pH 7.4). The extract was dried under nitrogen and reconstituted with 1 ml methanol and 0.05% butylated hydroxy toluene (BHT) as antioxidant. Reconstituted samples were filtered (0.20 μm mesh) and a 20 μl subsample automatically injected (Waters 700 Satellite wisp) on an HPLC (Waters 600 E System Controller Pump) equipped with a Restek column (Tracer Excel 120 ODS-A, 10-cm length, 5 μm beds, 0.46-cm internal diameter) and a UV detector (Waters 486 Tuneable absorbance D) set at 326 nm. The retinoid was eluted at a flow rate of 1 ml/min using a mobile phase of methanol/water (80/20 by volume) for 1 min followed by a linear gradient of 3 min to methanol 100% for 14 min.

Lipids were extracted from blubber samples with methanol–chloroform (Folch et al., 1957). Tissue lipid content was determined gravimetrically from the extract and expressed as a percentage of the tissue fresh mass (blubber lipid content, BLC%).

2.3. Statistical analysis

The study was designed to obtain a sequence of blubber samples from different dolphins. Each dolphin was treated as an experimental unit and, in each dolphin, blubber samples from different locations in the body were considered as repeated measures of retinoid concentration and lipid content. Thus, this study fitted a repeated-measures design; because dolphins were expected to vary independently, measures of retinoid concentrations, as well as of lipid content, within a dolphin were expected to be correlated.

We first investigated the variation in retinoid concentrations between sample locations with an ANOVA design. The design considered that the response, retinoid concentrations, depended on factors sex, sample location and the interaction between them. To account for random variation between dolphins, and the correlation of samples within dolphins, each individual was defined by an error stratum (Chambers and Hastie, 1992).

This analysis assumed that the variance of the differences in retinoid measurements at different locations was constant. To avoid the effect of departures from this assumption on the interpretation of the ANOVA tests (e.g. by having a too small P value in the F test, and analogous to Bonferroni corrections) we followed a conservative approach (Mathsoft, 1999) by dividing the degrees of freedom, in both the numerator and denominator of the F test, by the number of repeated measures minus one, i.e. 11 retinoid samples minus one.

Differences in retinoid concentrations between sampling locations were sorted out with a multiple comparison analysis (e.g. Hsu, 1996). Depending on the results of the previous ANOVA, we used the factors found to be significant to test pair-wise comparisons. The results were expressed in simultaneous 95% confidence intervals of all pair-wise differences in the mean of retinoid concentrations

Table 1
Date of capture, sex, length, retinoid concentrations (mean ± S.D.) and blubber lipid content (BLC) (mean ± S.D.) of common dolphins (*D. delphis*)

Dolphin	Date	Sex	Length (cm)	Retinoids ($\mu g\ g^{-1}$)	BLC (%)
1	28-03-2001	M	187	75.16 ± 32.72	60.58 ± 8.57
2	11-07-2001	M	202	67.40 ± 28.72	55.79 ± 8.24
3	11-07-2001	M	206	56.01 ± 23.25	58.02 ± 7.57
4	18-07-2001	F	189	47.59 ± 14.96	56.26 ± 8.89
5	19-07-2001	F	179	33.48 ± 6.59	59.50 ± 8.77
6	17-07-2001	F	197	59.91 ± 13.97	62.36 ± 7.15
7	23-07-2001	M	204	53.56 ± 18.64	52.85 ± 6.78
8	23-07-2001	M	175	29.04 ± 8.82	64.12 ± 6.33
9	23-07-2001	M	204	49.95 ± 23.88	59.63 ± 10.30
10	23-07-2001	M	208	64.20 ± 17.61	53.25 ± 8.27
11	23-07-2001	M	205	45.23 ± 13.91	58.60 ± 4.34
12	23-07-2001	M	200	48.50 ± 13.30	63.07 ± 9.68
13	23-07-2001	M	190	58.97 ± 17.82	49.84 ± 10.61
14	04-07-2002	F	176	50.50 ± 14.71	67.00 ± 5.41
15	18-07-2002	F	206	80.31 ± 24.79	63.42 ± 4.64
16	18-07-2002	F	187	45.98 ± 16.34	67.55 ± 7.53
17	18-07-2002	F	180	53.55 ± 6.47	68.32 ± 5.96
23	25-07-2002	F	200	29.65 ± 10.62	55.38 ± 5.98
18	31-01-2000	M	176	44.47 ± 10.55	67.47 ± 4.21
20	31-07-2002	M	207	44.27 ± 24.09	65.29 ± 7.29
21	31-07-2002	M	183	30.86 ± 9.48	68.94 ± 5.47
22	31-07-2002	M	182	52.98 ± 18.14	58.64 ± 11.77
24	31-07-2002	M	201	70.81 ± 27.60	67.60 ± 5.37
19	22-08-2002	F	194	104.20 ± 28.81	62.25 ± 6.30
25	10-09-2002	F	198	77.12 ± 27.56	62.53 ± 6.06

Note: M, male; F, female; S.D., standard deviation.

based on the different sampling locations. The same repeated-measures and multiple comparison analysis were used to assess differences in lipid content between sampling locations.

Due to the lipophilic character of vitamin A, the lipid content of a sample could be a confounding factor in the understanding of retinoid distribution in the dolphin blubber. To investigate the possible association of highest retinoid concentrations to samples of highest lipid content, we compared differences in retinoid concentration among samples with differences in lipid content among samples with another ANOVA design with repeated measures. The response was retinoid concentration and depended on lipid content and the interaction of sample location and lipids, with lipid contents nested on sample, and additional effects were sex and body size. To account for random variation, each individual was defined by an error stratum.

Table 2
Retinoid concentrations (mean ± S.D.) and blubber lipid content, BLC (mean ± S.D.), in the blubber locations examined in common dolphins (*D. delphis*)

Blubber location	*n*	Retinoids ($\mu g\ g^{-1}$)	BLC (%)
1	25	50.01 ± 22.73	60.74 ± 9.83
2	25	57.02 ± 22.16	66.23 ± 6.03
3	25	46.88 ± 16.75	62.81 ± 6.72
4	25	67.90 ± 25.47	62.75 ± 9.35
5	25	81.51 ± 33.66	65.18 ± 6.92
6	25	40.85 ± 12.68	58.33 ± 9.58
7	25	50.14 ± 17.91	60.59 ± 8.56
8	25	66.59 ± 26.74	61.45 ± 9.18
9	25	47.63 ± 17.03	59.51 ± 7.81
10	25	49.27 ± 28.97	57.81 ± 10.60
11	25	45.31 ± 20.59	57.01 ± 8.65

Note: S.D., standard deviation.

Differences in retinoid levels and lipid content between the main body blubber regions were determined using a repeated-measure design analysis of variance (ANOVA) and multiple comparisons, as used for the 11 sampling locations explained above.

3. Results

Table 1 details the biological characteristics of each dolphin sampled. The length of the individuals ranged from 175 to 208 cm, so they compose

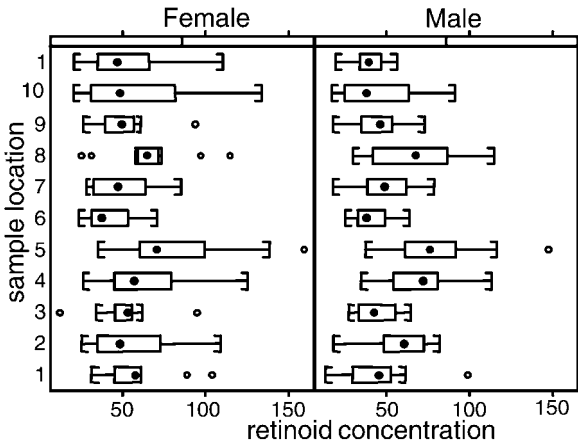

Fig. 2. Box-plots of blubber retinoid concentration ($\mu g\ g^{-1}$) in different sampling locations by sexes in common dolphins (*D. delphis*).

Table 3
Significant differences of the means of retinoid concentrations and of the lipid content between sampling locations in common dolphins (*D. delphis*), as determined by the multicomparison analysis

Sampling location	1	2	3	4	5	6	7	8	9	10	11
1					R						
2					R					L	L
3					R						
4						R					R
5						R	R		R	R	RL
6								R			
7											
8											R
9											
10											
11											

R: significant differences on retinoid concentrations at $P<0.05$.L: significant differences on lipid content at $P<0.05$.

a reasonably homogenous group. Mean retinoid values varied greatly among dolphins, ranging from 29 to 104 $\mu g\ g^{-1}$. Table 2 summarizes the concentrations of retinoids in the different blubber locations; mean concentrations ranged from 41 to 81 $\mu g\ g^{-1}$. Retinoid concentration presented a very high variation among locations both within and between individuals. This variation masked the potential differences between sexes (Fig. 2) and the ANOVA indicated that the effect of sex on retinoid concentrations was not significant either between individuals or as an interaction with sample location. Therefore, in subsequent analysis males and females were computed together. In contrast, sample location was found to be a highly significant source of variation within-individuals (conservative F-test: $P<0.001$). Table 3 shows the results of the multicomparison analysis detailing levels of significance between body locations.

Mean blubber lipid content (BLC) in individuals ranged from 50 to 69% (Table 1); mean BLC in each body location ranged from 35 to 83% (Table 2). An ANOVA with percent lipid content as response, sex and sample location as factors, and an error stratum associated to individual dolphin, indicated no significant variation in lipid content by sex. However, sampling location was a significant source of within-individual variation (conservative F-test: $P=0.048$). Similarly to the relation between retinoid concentration and sample location, individual variability in lipid content was high enough to mask potential differences between sexes (Fig. 3). The result of the multiple comparison analyses of BLC is shown in Table 3. Since the lipid content was highly variable between individuals, most of the differences between locations were not significant.

The concentration of retinoids was associated with the lipid content of the blubber samples. The relation was not very linear, as indicated by a simple linear model of retinoid on lipid content (F-statistic: 3.352; $r^2=0.012$; $P=0.0682$) and again, this was mostly due to high individual variation in both parameters. The results of the ANOVA indicated that between dolphins, neither sex, body size or lipid contents were significant

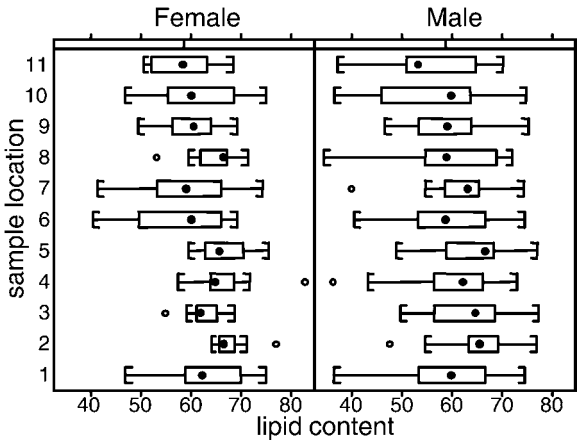

Fig. 3. Box-plots of blubber lipid content (%) in different sampling locations by sexes in common dolphins (*D. delphis*).

Table 4
Significant differences of the means of retinoid concentrations and of the lipid content between body regions in common dolphins (*D. delphis*), as determined by the multicomparison analysis

Body region	Anterior-dorsal	Posterior-dorsal	Anterior-ventral	Posterior-ventral
Anterior-dorsal			R	
Posterior-dorsal			RL	
Anterior-ventral				L
Posterior-ventral				

R: significant differences on retinoid concentrations at $P<0.05$.L: significant differences on lipid content at $P<0.05$.

sources of variability. However, within dolphins, retinoid concentration was positively related to lipid contents (conservative *F*-test: $P=0.012$), and the interaction of sample location and lipid contents was highly significant (conservative *F*-test: $P=0.001$). Within dolphins, neither sex nor body size explained significant variation in retinoid concentration.

Body region was a significant source of variation of retinoid concentration within-individuals (conservative *F*-test: $P=0.013$), as well as of lipid content (conservative *F*-test: $P=0.046$) (Table 4). We found the highest concentrations of both retinoids and lipid content in the anterior-ventral region and the lowest in the dorsal-posterior region.

4. Discussion

Retinoids are lipophilic and thus concentrate in lipid-rich tissues. Since blubber is the largest fat compartment in marine mammals, in pinnipeds it has been proposed as the main body depot for retinoids (Schweigert et al., 1987, 2002). In cetaceans, information available is comparatively much more limited (Borrell et al., 2002). We found retinoids to be present in the blubber of common dolphins at concentrations averaging 52.76 $\mu g\ g^{-1}$ (females) and 57.93 $\mu g\ g^{-1}$ (males). These values are comparable to those reported for harbour porpoises (range: 30–80 $\mu g\ g^{-1}$; Borrell et al., 1999), grey seals (range: 20–74 $\mu g\ g^{-1}$; Schweigert et al., 1987, 2002; Schweigert and Buchholz, 1995; Nyman et al., 2003), slightly higher than those found in ringed seals (range: 21–32 $\mu g\ g^{-1}$; Käkelä et al., 1997; Nyman et al., 2003), and some terrestrial mammals, such as canines (range: 15–30 $\mu g\ g^{-1}$; Schweigert and Buchholz, 1995), but much higher than those found in the blubber of harp seals, *Phoca groenlandica* (3.6 $\mu g\ g^{-1}$; Rodahl and Davies, 1949) and in the visceral fat of humans (1.5 $\mu g\ g^{-1}$; Raica et al., 1972).

Taking into account the large proportion that blubber represents of the body of cetaceans and the high blubber retinoid concentrations found in the common dolphins here investigated, as well in that of harbour porpoises found in a previous study (Borrell et al., 1999), it is reasonable to conclude that blubber also represents a significant contribution to total retinoids in small odontocetes. Therefore, blubber is proposed as tissue of choice for monitoring retinoid status in these animals. However, the large mass of the blubber compartment in marine mammals, as well as its distribution over practically the whole body surface, makes it necessary to define precise sampling protocols to avoid inconsistencies. Understanding within-blubber topographical variation in retinoid concentrations is relevant in this context.

Marine mammal blubber is far from being a homogeneous, single-function tissue. It is commonly accepted that it plays four main roles: to supply insulation to the body core, to serve as energy store, to contribute to streamline the body, and to regulate buoyancy (Ryg et al., 1988; Iverson, 2002). However, the information available on topographical variation in histological structure, composition, and physicochemical properties of blubber indicate that the importance of each function varies among body regions. In balaenopterid cetaceans the anterior ventral blubber forms the ventral feeding grooves, which are semi-elastic to permit distension of the mouth and the throat during feeding. As a consequence, the blubber from this region is composed of abundant structural collagen and its lipid content in fin whales (*Balaenoptera physalus*), sei whales (*Balaenoptera borealis*) and minke whales (*B. acutorostrata*) is much lower than that of the dorsal posterior region (Pedersen, 1950; Watanabe and Suzuki, 1950a; Ackman et al., 1975; Lockyer et al., 1984,

1985; Kvadsheim et al., 1996). This, together with the fact that the dorsal posterior muscle is also richer in lipids than the ventral (Næss et al., 1998), indicates that this latter region is the major body site for energy storage (Lockyer et al., 1984). Although in sperm whales (*P. macrocephalus*) the anterior ventral region has no specialized feeding functions, the dorsal posterior and lateral regions of the body also display the highest lipid contents (Watanabe and Suzuki, 1950b; Lockyer, 1991). It has been suggested that since the blubber layer is also thicker in the latter area in balaenopterid whales and sperm whales, lipids accrue in this region to shape the body and possibly to regulate buoyancy (Lockyer et al., 1984; Iverson, 2002).

Information on small cetaceans is comparatively much more limited. In pilot whales (*Globicephala melas*), topographical variation in lipid content is non-existent or at least masked by seasonal variation (Lockyer, 1993). In harbour porpoises the pattern of variation of lipid content has been found to be either non-existent (Tilbury et al., 1997) or to be the opposite of that found in large cetaceans; this is lowest in the blubber of the dorsal posterior region and highest in the anterior ventral one (Calambokidis, 1986; Ishaq et al., 2000). It has been suggested that in harbour porpoises the tissue is divided into two functional components, one anterior and one posterior, with the line of division occurring at the level of the anus; thus, both parts would play insulatory and buoyancy activities, but the posterior section would not be significant for energy storage and would mainly serve as a structural element to streamline the body (Koopman, 1998). In the present study, we found that concentration of lipids progressively decreased from head to tail and from belly to back, confirming the pattern observed in harbour porpoises. This suggests that in common dolphins the anterior-ventral region is also comparatively more important for insulation and lipid storage than the dorsal posterior region, perhaps to provide thermal protection to the viscera.

Retinoids followed an almost identical pattern, which is not surprising given their lipophilicity. Thus, it appears that after the incorporation of retinoids into the organism via food, the dynamics and body distribution of these compounds are greatly controlled by the physicochemical properties of their molecule. As a consequence, not only retinoids accumulate in lipid-rich tissues, but also their concentration within a given tissue is proportional to the lipid content of the different components of this tissue. While retinoid concentration in blubber can be considered a reliable indicator of vitamin A status in common dolphins, it should be stressed that sampling location must be considered as a significant source of variability and taken into account to design sampling protocols. Thus, consistency in sampling body location is critical to ensure comparability of results.

In all large cetacean species, and independently of body location, blubber shows a stratified structure that also reflects heterogeneous function of the various layers (Ackman et al., 1975; Lockyer et al., 1985; Aguilar and Borrell, 1990; Lockyer, 1991). Since blubber thickness in these animals can be up to 3–50 cm depending on the species (Iverson, 2002), added difficulties are posed to representative sampling as the suitable tissue mass for analysis is only a few grams. However, in small cetaceans, such as common dolphins or harbour porpoises, this effect, although existing is limited (Koopman et al., 1996) and can be readily overcome by collecting and analysing chunks of blubber containing all blubber strata. In this way, all layers are pooled into a single analytical sample providing a composite average of the whole blubber thickness, as it has been done in the present study.

No significant differences in blubber retinoid concentrations were found between males and females. This is in agreement with the only comparable study so far undertaken in cetaceans (Borrell et al., 1999). However, sex-related differences in blubber vitamin A concentrations have been reported for adult grey seals (Schweigert et al., 1987) and harbour seals (Nyman et al., 2003). The reasons for such interspecific differences in sex-related patterns are unclear, but may be related to taxonomic, dietary or life-cycle dissimilarities between species.

The positive relationship found in this study between retinoid concentration and lipid content apparently differs from results of previous studies by Rodahl and Davies (1949) on harp and hooded seals (*Cystophora cristata*), Borrell et al. (1999) on harbour porpoises, and Mos and Ross (2002) on harbour seal pups. Fat is mobilized by seasonal variation in food intake or the increased energy expenditure caused by migration, lactation, disease or other factors. This mobilization may also induce retinoid mobilization from blubber. The fate of these retinoids is unclear. They may either be

redistributed or excreted, but significant alteration in blubber concentrations is to be expected. This effect should be taken into account when designing sampling protocols. Moreover, it points out that stranded cetaceans, often found in poor nutritive condition, are likely to present altered retinoid conditions and should therefore not be used to monitor retinoid status of populations. Blubber biopsies can be obtained from healthy, free-ranging individuals using non-destructive methods and are already a standard technique used in ecotoxicological surveys of cetaceans (Aguilar and Borrell, 1994). The biopsy includes all blubber layers and thus avoids potential bias due to stratification of retinoids, lipid content or pollutants, thus constituting a more reliable alternative.

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Tissue distribution of retinoids in common dolphins *Delphinus delphis*

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ABSTRACT: Exposure to organochlorines induces retinoid deficiency in mammals; hence, retinoids are potential biomarkers of the impact of these pollutants. Appropriate target tissues to monitor retinoids in cetaceans have not been properly identified because of a lack of information on the contribution of each tissue to total body retinoids. Therefore, we have addressed this issue by studying the contribution of the main body tissues to retinoids in 21 common dolphins obtained from incidental catches and in apparent good health and nutritive condition. Although concentrations in the liver were highest, those in blubber were also high and accounted for 43% of the total retinoid load of the compartments examined. As blubber can be obtained using non-invasive biopsy techniques, this tissue is proposed as a reliable indicator of retinoid status in cetaceans. However, blubber topographical variation in structure and composition requires standardization of sampling sites. Retinoid concentrations did not differ significantly between sexes or with body size for any of the tissues, but the lipid content of blubber strongly influenced these concentrations. Biopsies from healthy, free-ranging individuals are preferred to samples from stranded animals. Further research on the influence of factors (age, sex, reproductive condition, diet) that potentially affect retinoid levels is required to implement the use of retinoids as biomarkers of pollutant exposure in cetaceans.

KEY WORDS: Retinoids · Common dolphin · Compartmentation · Blubber · Biomarker · Northwestern Spain

INTRODUCTION

Linking the tissue concentration of a given contaminant with its effect on an organism is a major challenge in environmental toxicology. Biomarkers, defined as contaminant-induced variations in the cellular or biochemical components of a process, structure or function that can be assessed in a biological system (NRC 1989), are being developed and applied to establish such a relationship. Biomarkers are expected to provide an integrated measure of the response of an organism to exposure to a chemical or group of chemicals and, hence, a measure of toxicological risk (McCarthy & Shugart 1990, Depledge & Fossi 1994). Pollution by organochlorines is a source of concern for top predator cetaceans and pinnipeds (Reijnders & Aguilar 2002). Substantial literature reports the monitoring of these compounds in this group (O'Shea & Aguilar 2001). However, the application of biomarkers remains incipient.

In mammals, body levels of retinoids are disrupted by organochlorine compounds, particularly polychlorinated biphenyls (PCBs). Consequently, retinoids have been proposed as sensitive biomarkers (Peakall 1992, Murk et al. 1998) and several studies have addressed their potential application to marine mammals (Rolland et al. 2000, Simms & Ross 2000, Borrell et al. 2002). Retinoids are a family of lipid-soluble substances that possess Vitamin A-like biological activity, including compounds such as retinol and retinol derivatives, retinal, retinyl palmitate and retinoic acid (Blomhoff et al. 1992). In mammals, they can be provided only through diet (Blomhoff et al. 1991) and are crucial for vision, growth, bone development, reproductive success, immune function, normal differentiation and proliferation of cells, and

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maintenance of the general health of the organism (Wolf 1984, Favennec & Cals 1988, Blomhoff 1994). Retinoid deficiency is associated with a diversity of anomalies, such as reproductive impairment, embryonic mortality, growth retardation, and decreased resistance to infections (Thompson 1976, Peakall 1992). These symptoms are similar to those that some environmental pollutants, particularly PCBs, dioxins (2,3,7,8-tetrachlorodibenzo-p-dioxin: TCDDs) and DDTs, produce in mammals (Arnold et al. 1995, Colborn & Smolen 1996), including marine mammals (Busbee et al. 1999, Respess et al. 1999). Therefore, retinoid deficiency may also enhance the toxicity of these compounds.

Traditionally, disruption of body retinoids has been assessed through plasma levels in terrestrial (Brouwer & Van der Berg 1986, Bank et al. 1989, Brouwer et al. 1989a, Håkansson et al. 1991, Käkelä et al. 1999) and marine mammals (Brouwer et al. 1989b, De Swart et al. 1994, Jenssen et al. 1995, 2003, Beckmen et al. 1997, Simms et al. 2000, Skaare et al. 2001, Nyman et al. 2003). However, their concentration in plasma is, in general, regulated homeostatically (Wolf 1984, Blomhoff et al. 1992). Thus, plasma may not represent a stable measure of retinoid body deficit and is therefore of limited diagnostic use. Body retinoid status should be assessed through concentration in other tissues (Borrell et al. 2002).

Exposure of experimental animals, such as rats, mink and otters, to organochlorines leads to depletion of hepatic retinoid levels (Brunström et al. 1991, Håkansson et al. 1992, Chu et al. 1995, 1998, Murk et al. 1998, Käkelä et al. 1999, 2002, 2003, Kelley et al. 2000), while those in the kidney generally increase (Brouwer et al. 1989a, Jurek et al. 1990, Nilsson et al. 2000). This indicates that the pollutants mobilize retinoid storage forms, which is followed by an increase in their degradation and renal elimination through the urine (Kelley et al. 1998, 2000). Study on the disruption of retinoids induced by pollutants in tissues other than plasma in marine mammals is restricted to the work of Nyman et al. (2003). They found that increasing concentrations of PCBs and DDTs led to a significant decrease in hepatic retinoids of ringed seals *Phoca hispida* and grey seals *Halichoerus grypus*.

Body distribution of retinoids has not been studied in detail. In terrestrial mammals, the liver holds 70 to 90% of total body retinoids (Wolf 1984). In marine mammals, information is practically restricted to pinnipeds. In grey, ringed, harbour (*Phoca vitulina*), and harp seals *Pagophilus groenlandicus*, the liver presented the highest retinoid concentrations, although those of the blubber were also elevated (Rodahl & Davies 1949, Schweigert et al. 1987, Schweigert & Buchholz 1995, Käkelä et al. 1997, Mos & Ross 2002, Schweigert et al. 2002, Nyman et al. 2003). Lower concentrations are found in other tissues, such as kidney, lung, retina, pancreas, skin and spleen (Rodahl & Davies 1949, Schweigert & Buchholz 1995, Mos & Ross 2002). In cetaceans, data on retinoid concentrations are available only for some tissues, but results are not comparable between studies because of significant variation at individual, population and species levels. The liver of cetaceans is also extremely rich in retinoids (Schmidt-Nielsen et al. 1934), and several decades ago researchers studied hepatic concentrations in large whales with the aim to industrially extract Vitamin A from them (Klem 1935, Wetlesen 1938, Wagner 1939, Braekkan 1948, Ishikawa et al. 1948, 1951, Kaneko 1948, Mori & Saiki 1950, Tawara & Fukazawa 1950a,b). Other tissues have received little attention. Mori & Saiki (1950) measured retinoid concentrations in the blubber and intestine of sperm whales *Physeter macrocephalus*, Gregory et al. (1955) in the milk of blue whales *Balaenoptera musculus*, Rosas & Lehti (1996) in the milk of Amazon river dolphins *Inia geoffrensis*, Iida et al. (1998) in the muscle and blubber of minke whales *Balaenoptera acutorostrata*, and Borrell et al. (1999) in the blubber of harbour porpoises *Phocoena phocoena*. The scarce information on blubber retinoid in cetaceans is surprising, as this tissue can easily be sampled using non-destructive biopsy techniques (Aguilar & Borrell 1994).

Here we studied retinoid distribution in the main body components of common dolphins *Delphinus delphis* in order to evaluate the representativeness of the various tissues in the assessment of body retinoid status in cetaceans. We also examined the effects of sex, body size and blubber lipid content on retinoid concentrations in these tissues.

MATERIALS AND METHODS

Sample collection. We examined and sampled 21 common dolphin carcasses (11 males and 10 females) that were incidentally caught by fishing boats in northwestern waters of Spain in 2001 and 2002. Necropsies were performed onboard no more than 12 h post mortem. Thus, the individuals sampled were fresh and were considered to be representative of the population. Dolphins were measured and sexed, and samples of liver, kidney, lung, heart, muscle, and blubber were collected from each individual. Blubber samples were excised from the region posterior to the dorsal fin. To avoid the potential effect of lipid stratification on retinoid concentrations (Aguilar & Borrell 1990), each sample was carefully taken to include all blubber layers, from the skin to the fascia adjacent to the muscle. The various body components of 1 of the dolphins was carefully excised and weighed separately to calculate total body retinoid loads. All samples for retinoid

determinations were transported to the laboratory on dry ice and stored in darkness at –20°C until analysis.

Chemical analysis. For retinoid analysis, samples were treated at room temperature and under red light. The samples, which weighed ca. 100 mg each, were saponified overnight in an ethanolic KOH solution (1 g KOH, 2 ml distilled H_2O, 2 ml ethanol, 20 mg ascorbic acid) in a mechanical shaker under a nitrogen atmosphere. Retinoids (retinol and retinyl esters) were extracted by adding 8 ml diethyl ether and shaking for 30 min. After separation from the aqueous phase, an internal standard (retinyl acetate) was added and the organic extract was cleaned 3 times with 4 ml of aqueous phosphate buffer (pH 7.4). The extract was dried under nitrogen and reconstituted with 1 ml methanol and 0.05% butylated hydroxy toluene (BHT) as antioxidant. Reconstituted samples were filtered (0.20 µm mesh) and a 20 µl subsample was injected automatically (Waters 700 Satellite wisp) into a HPLC (Waters 600 E System Controller Pump) equipped with a Restek column (Tracer Excel 120 ODS-A, 10 cm length, 5 µm beds, 0.46 cm internal diameter) and a UV detector (Waters 486 Tuneable absorbance D) set at 326 nm. The retinoid was eluted at a flow rate of 1 ml min^{-1} using a mobile phase of methanol/water (80/20 by volume) for 1 min, followed by a linear gradient of 3 min to 100% methanol for 14 min.

For lipid content analyses of blubber samples, a subsample of the tissue was extracted with methanol-chloroform (Folch et al. 1957). Tissue lipid content was determined gravimetrically from the extract and expressed as a percentage of the tissue fresh weight (blubber lipid content, BLC%).

Calculation of retinoid load. One dolphin was thoroughly necropsied by excising the main body tissues, which were then weighed separately. The total amount (load) of retinoids in each tissue was calculated by multiplying its weight by its retinoid content, as determined by the analysis of a subsample. Total body retinoid load was estimated by addition of the tissue.

Statistical analysis. The analysis aimed at investigating systematic differences in retinoid concentration between different tissues (liver, kidney, heart, muscle, lung, and dorsal blubber), but nutritive condition (as measured by lipid content), sex and individual effects were other potential sources of retinoid variability. Therefore, in order to model variability in retinoids as predicted by these variables, we considered tissue as the main experimental factor, and sex, lipids and individual as potential covariates.

In usual linear models, individual effects would be treated as factors in which each dolphin would be a different level. However, such complex models were not supported by our sample size. Instead, we considered individual variability as random deviates of the population mean. In this way, different organ effects modelled across dolphins were considered to be fixed or population mean effects, and individual deviates from the population mean were considered to be random effects. Thus, we modelled variability in retinoid concentration using linear mixed-effects models (Pinheiro & Bates 2000). Retinoid concentration was transformed to natural logarithm scale to reduce the large differences between the concentration in liver and the rest of the tissues and to normalise the data.

Models with different combinations of predicting variables were fitted to investigate the main source of variability in retinoids. The modelling also considered different variance functions to reduce heteroscedasticy, and thus improve the fit and the assessment of variability. To test assumptions about covariate effects and homoscedasticity, we used the Akaike's information criterion (AIC) (Burnham & Anderson 1998) and ANOVA methods specifically developed for mixed-effects model comparisons (Pinheiro & Bates 2000). Models with comparably lower AIC provided the best fit in terms of a good compromise between model parsimony and a good description of the data.

RESULTS

Body lengths ranged between 176 and 207 cm (mean = 195.1 cm) in males and 176 and 206 cm (mean = 190.6 cm) in females (Table 1), which indicates that our sample was mostly composed of adult animals

Table 1. *Delphinus delphis*. Date of capture, sex and length. M: male; F: female; RIV: Riveira (sampling location)

Dolphin	Date (dd/mm/yy)	Sex	Length (cm)
RIV 2001–1	28/03/01	M	187
RIV 2001–2	11/07/01	M	202
RIV 2001–3	11/07/01	M	206
RIV 2001–4	18/07/01	F	189
RIV 2001–5	19/07/01	F	179
RIV 2001–6	17/07/01	F	197
RIV 2001–7	23/07/01	M	204
RIV 2001–10	23/07/01	M	208
RIV 2001–13	23/07/01	M	190
RIV 2002–14	04/07/02	F	176
RIV 2002–15	18/07/02	F	206
RIV 2002–16	18/07/02	F	187
RIV 2002–17	18/07/02	F	180
RIV 2002–23	25/07/02	F	200
RIV 2002–18	31/01/02	M	176
RIV 2002–20	31/07/02	M	207
RIV 2002–21	31/07/02	M	183
RIV 2002–22	31/07/02	M	182
RIV 2002–24	31/07/02	M	201
RIV 2002–19	22/08/02	F	194
RIV 2002–25	10/09/02	F	198

(Collet 1981). Individual retinoid values ranged from concentrations below analytical detection limit for heart and lung to 460 µg g^{-1} for liver, while mean values ranged between 1.5 and 134 µg g^{-1}, with extreme figures for lung and liver, respectively (Table 2).

The largest variation in mean retinoid concentration (unmodelled) was between different tissues, and the lowest variation was by sex (Fig. 1). Individual variation was moderate in comparison, but the boxplot of the residuals (Fig. 2) of mixed-effects models (Table 3; Model 6) highlighted individual variability and outlier effects. The residuals were centred at zero, indicating a relatively good model fit, but also a larger variability in males than in females.

Models allowing different variances first by tissue and by sex reduced heteroscedasticity (Table 3; Models 2 and 3), and also reduced the number and dispersion of outliers towards normality. A model with different variance by sex fit less well than a model with different variance by tissue, suggesting that individual deviations from the mean retinoid concentration in tissues masked possible sex effects. Therefore, the next comparisons were based on models with different variance by tissue, including different covariates as independent linear predictors of retinoid concentration.

Table 2. *Delphinus delphis*. Mean, associated SD and ranges of retinoid concentrations in the tissues of the dolphins examined. Concentrations are expressed as µg g^{-1} calculated in relation to the fresh weight of the tissue. nd: not detected

Tissue	n	Retinoid concentration			
		Mean	SD	Max.	Min.
Liver	21	134.13	131.76	459.68	14.94
Blubber	21	41.38	13.43	70.86	23.47
Kidney	21	7.76	4.05	15.92	1.91
Muscle	21	2.98	3.29	13.21	0.78
Heart	20	2.06	2.39	10.94	nd
Lung	21	1.51	1.09	4.71	nd

Model 6, which had blubber lipid content as a covariate, provided a significant improvement in relation to Model 2, as shown by a lower AIC. Although the explanatory power of the 2 models was comparable, the latter result was in agreement with the lipophilic nature of retinoids, suggesting that retinoid concentration was strongly related to lipid content.

Models 4 and 5, with sex and body size as covariates respectively, did not provide a better fit than Model 6, but a model with sex, lipid content and the interaction between the 2 (Model 8) provided the best fit among models. The ANOVA tests for this model indicated that differences in retinoid concentration were highly significant between tissues (F = 219.640; df = 5; $p < 0.0001$), that retinoid covaried with lipid content (F = 6.440; df = 1; p = 0.0219), and that the interaction between sex and lipid content affected retinoid concentration (F = 8.134; df = 1; p = 0.0115).

The maximum likelihood estimates of variance components ($\hat{\sigma}$) were 0.111 for between-dolphin variation ($\hat{\sigma}$, approximately 95% CI: 0.034 to 0.368) and

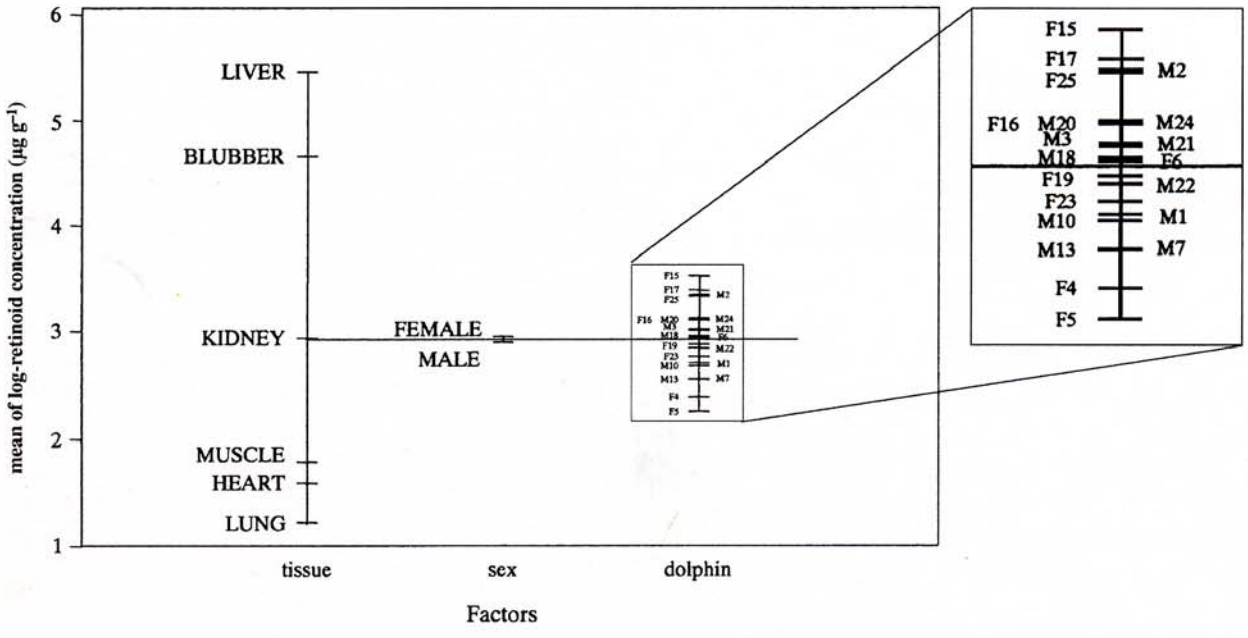

Fig. 1. *Delphinus delphis*. Variation in mean log-retinoid concentration (µg g^{-1}) among tissues, sexes and dolphins

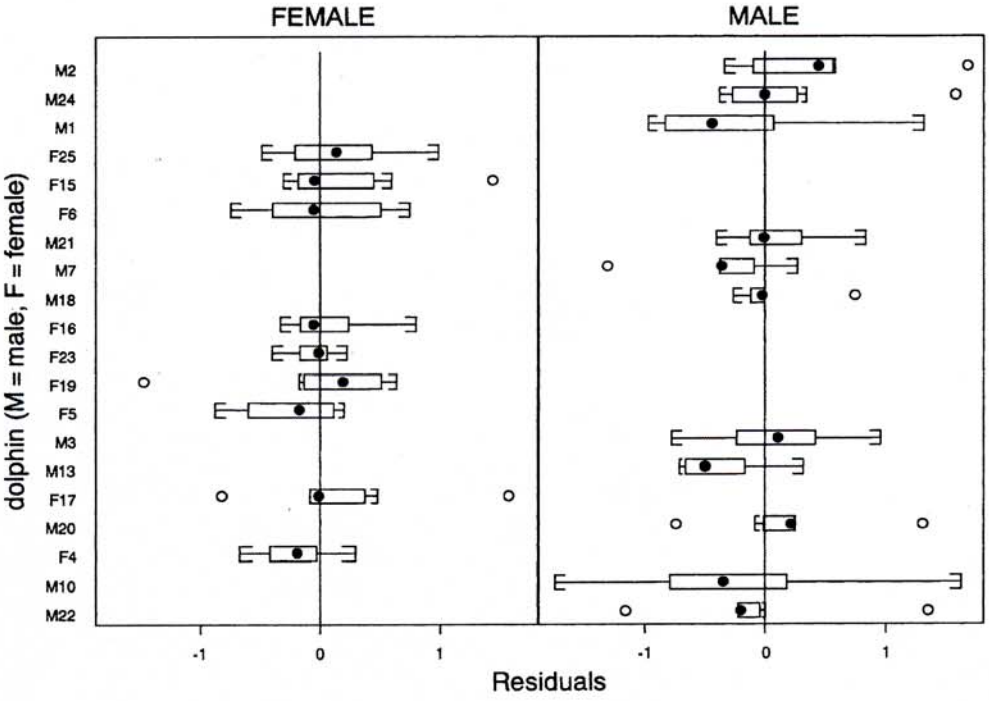

Fig. 2. *Delphinus delphis*. Boxplot of the residuals by individual dolphin and split by sex, with outliers as open circles; resulting from the fit of model 1

Table 3. *Delphinus delphis*. Mixed-effects model selection and testing. The basic model (1) is as Eq. (1) (see text) and more complicated models add structure to the variance model and additional covariates sex (*S*), body size (*BS*), and the overall lipid content (*LP*) of retinoid variation. Model testing is based on ANOVA methods specifically developed for mixed-effects models (Pinheiro & Bates 2000). In this test, a p-value below 0.05 indicates a significantly better fit of the model with more structure, i.e. larger df. In the model notation α_j was the population mean retinoid concentration, also known as fixed effects; b_i was a random variable representing the deviation from the population mean α_j of the mean retinoid concentration in the *i*th dolphin, also known as random effects; ε_{ij} was the residual variance, which measured unexplained within-dolphin variability in retinoid concentration among tissues; β_i represents the effect corresponding to the covariate name. Heteroscedasticity was modelled using different residual variance functions. These functions considered different variability for different tissues and sex; for instance, for sex, $var(\varepsilon_{ij}) = \sigma^2\ \delta^2_{sex}$ with a different variance parameter δ_l corresponding to each of the *l* sexes. Interactions between covariates, for instance, between sex and lipid content, are noted as *LP-in-S*. Df: degrees of freedom; AIC: Akaike's information criterion; LogL: Model log-likelihood; LRT: likelihood ratio statistic; Test: indicates which models are compared

Model		df	AIC	LogL	Test	LRT	p
1	$y_{ij} = \alpha_j + b_i + \varepsilon_{ij}$	8	259.60	−121.80			
2	$y_{ij} = \alpha_j + b_i + \varepsilon_{ij}$, $var(\varepsilon_{ij}) = \sigma^2\delta^2_{tissue}$	13	242.71	−108.35	1 vs 2	26.89	0.0001
3	$y_{ij} = \alpha_j + b_i + \varepsilon_{ij}$, $var(\varepsilon_{ij}) = \sigma^2\delta^2_{sex}$	9	260.57	−121.29	2 vs 3	25.87	<0.0001
					1 vs 3	1.03	0.3104
4	$y_{ij} = \alpha_j + b_i + \beta_i S_{ij} + \varepsilon_{ij}$, $var(\varepsilon_{ij}) = \sigma^2\delta^2_{tissue}$	14	244.65	−108.33	2 vs 4	0.06	0.8117
5	$y_{ij} = \alpha_j + b_i + \beta_i BS_{ij} + \varepsilon_{ij}$, $var(\varepsilon_{ij}) = \sigma^2\delta^2_{tissue}$	14	243.07	−107.54	2 vs 5	1.63	0.2016
6	$y_{ij} = \alpha_j + b_i + \beta_i LP_{ij} + \varepsilon_{ij}$, $var(\varepsilon_{ij}) = \sigma^2\delta^2_{tissue}$	14	240.44	−106.22	2 vs 6	4.27	0.0389
7	$y_{ij} = \alpha_j + b_i + \beta_i LP_{ij} + \beta_i BS_{ij} + \varepsilon_{ij}$, $var(\varepsilon_{ij}) = \sigma^2\delta^2_{tissue}$	15	241.97	−105.98	6 vs 7	0.47	0.4917
8	$y_{ij} = \alpha_j + b_i + \beta_i S_{ij} + \beta_i LP_{ij} + \beta_i LP\text{-}in\text{-}S_{ij} + \varepsilon_{ij}$, $var(\varepsilon_{ij}) = \sigma^2\delta^2_{tissue}$	16	237.34	−102.67	6 vs 8	7.10	0.0287
					1 vs 8	11.34	0.0099
9	$y_{ij} = \alpha_j + b_i + \beta_i LP\text{-}in\text{-}S_{ij} + \varepsilon_{ij}$, $var(\varepsilon_{ij}) = \sigma^2\delta^2_{tissue}$	15	241.93	−105.97	6 vs 9	0.51	0.4753
					8 vs 9	6.59	0.0102

0.996 of within-dolphin variation ($\hat{\sigma}$, approximately 95% CI: 0.725 to 1.368). These estimates confirmed that a higher variability in retinoid concentration was explained by differences between tissues, rather than additional covariates or individual differences. However, the large confidence interval of $\hat{\sigma}$ highlighted the effects of a few dolphins, mostly males, with extreme differences in values of retinoids. For instance, dolphin RIV 2001–1 had a total body retinoid load estimated at 1306 mg (Table 4). Considering this

Table 4. *Delphinus delphis*. Weights (kg), retinoid concentrations (μg g^{-1}) and loads (mg) of the main body tissues of the common dolphin RIV2001–1

	Blubber	Liver	Muscle	Kidney	Lung	Heart	Total
Weight	22.34	2.2	34.87	3.4	3.2	0.9	
Retinoid concentration	25.21	319.18	0.78	2.67	0.77	2.26	
Retinoid load	563.19	702.20	27.20	9.08	2.46	2.03	1306
% of total retinoids	43.12	53.76	2.08	0.69	0.19	0.16	100

value as 100%, it was calculated that the body tissues stored from 53% (liver) to 0.16% (heart) of retinoid body load.

DISCUSSION

We found that variability among organs within the same individual was much higher than among distinct individuals for the same organ. Consequently, the choice of tissue to monitor retinoids in common dolphins is a central issue.

As established for most terrestrial and marine mammals, the liver presented the highest retinoid concentrations; However, interspecific variation was large. The concentrations found were lower than those found in the liver of other cetacean species, such as blue, fin (*Balaenoptera physalus*) and sperm whales (Schmidt-Nielsen et al. 1934, Klem 1935, Wagner 1939, Braekkan 1948). In comparison to other marine mammals, our concentrations were higher than those reported for harbour and freshwater ringed seals (Rodahl & Davies 1949, Käkelä et al. 1997, Mos & Ross 2002), similar to those in hooded and marine ringed seals and California sea lions *Zalophus californianus* (Rodahl & Davies 1949, Ball et al. 1992, Käkelä et al. 1997), and lower than those reported for harp, grey, fur (*Arctocephalus pusillus doriferus*) and bearded seals *Erignathus barbatus* and polar bears *Ursus maritimus* (Rodahl & Moore 1943, Rodahl & Davies 1949, Southcott et al. 1974, Ball et al. 1986, Schweigert et al. 1987, 2002).

Although these high concentrations of retinoids in principle suggest that the liver may provide an important measure of body retinoid status, the contribution of this organ to total retinoid reserves in common dolphins was estimated to be only 53%, while in terrestrial mammals it accounts for up to 90% (Blomhoff 1994). It should be taken into account that the retinoid load of liver, as well as those of the other analysed tissues, was obtained from only 1 common dolphin and, therefore, the obtained values must be interpreted with caution. In addition to this, liver is not a practical tissue for monitoring free-ranging populations as it requires the capture, immobilization and handling of the individual, and the use of highly invasive biopsy techniques or, alternatively, necropsy when the individual is sacrificed or found dead.

In mammals, kidneys constitute another large reserve for retinoids (Bomhoff et al. 1991). Kidney values for common dolphins were similar to those found in grey seals (Rodahl & Davies 1949, Schweigert et al. 2002) and higher than those in harp seals (Rodahl & Davies 1949). Nevertheless, all kidney concentrations were several orders of magnitude lower than those of the liver and, occasionally, almost bordered analytical detection limits. The retinoid concentrations in the muscle, lung, and heart of the common dolphins were also insignificant compared to those of the liver. The contribution of these 4 tissues to the total retinoid load was lower than 4%; therefore, none appear to be representative of retinoid status in marine mammals.

As retinoids are fat-soluble, they accumulate in lipid-rich tissues. Blubber is the fattest compartment in marine mammals and it has been proposed that this tissue is an important body depot for retinoids in pinnipeds (Schweigert et al. 1987, 2002, Käkelä et al. 1997). In cetaceans, information is more limited, but the high blubber retinoid levels found in the present study, and those reported in a similar study on harbour porpoises (Borrell et al. 1999), are comparable to those found in grey seals (Schweigert et al. 1987, 2002, Schweigert & Buchholz 1995, Nyman et al. 2003) and ringed seals (Käkelä et al. 1997), but much higher than those reported in harp seals (Rodahl & Davies 1949).

Blubber constitutes a significant proportion of the total body mass of marine mammals, approximately 40% in pinnipeds (Schweigert et al. 1987) and 15 to 45% in cetaceans (Aguilar et al. 1999). Consequently, although blubber retinoid concentrations are lower than those in liver, the contribution of the former to the total retinoid reserves is comparatively as high as that of the latter. Schweigert et al. (1987) and Mos & Ross (2002) determined that ca. 40 and 66% of body retinoids were stored in the blubber of grey and harbour seals, respectively. In the common dolphin studied here, blubber was estimated to contribute 43% of body retinoids, although this is probably an underestimate because the blubber location sampled (posterior to the

dorsal fin) is likely to contain slightly lower retinoid concentrations than other blubber regions (Tornero et al. 2004). Given that blubber can be readily obtained from both free-ranging and captured individuals using biopsy techniques (Aguilar & Borrell 1994), we propose blubber as a tissue of choice for monitoring retinoid status in delphinid populations.

However, blubber is a massive compartment that covers the whole body surface of cetaceans and presents substantial heterogeneities in structure and composition between body sites (Iverson 2002). In a previous study, Tornero et al. (2004) found significant within-blubber topographical variation in retinoid concentrations in common dolphins. Protocols for monitoring retinoids through this tissue must ensure consistency in body sample location to guarantee comparability of results.

In all large cetacean species, and independently of body location, blubber presents a stratified structure that reflects the various functions of the distinct layers (Lockyer et al. 1985, Aguilar & Borrell 1990, Lockyer 1991). Because blubber thickness in these animals can be between 3 and 50 cm depending on the species (Iverson 2002), representative sampling is difficult because only a few grams of the blubber thickness can be used in the analysis. However, in small cetaceans, such as common dolphins, this effect is limited (Koopman et al. 1996) and can be easily overcome by collecting and analysing sections of blubber that contain all blubber layers, as in the procedure followed in the present study.

Blubber lipid content (BLC) was a strong determinant of overall retinoid concentration. We found a positive relationship between retinoid concentration and BLC, which is in agreement with Tornero et al. (2004). The body distribution of retinoids is significantly affected by the physicochemical properties of their molecules; thus, not only do retinoids concentrate in lipid-rich tissues, but also their concentration within a given tissue is proportional to the lipid content of this tissue. However, this relationship was not observed by Rodahl & Davies (1949) in hooded seals *Cystophora cristata* and harp seals, by Borrell et al. (1999) in harbour porpoises, or by Mos & Ross (2002) in harbour seal pups. Lactation, migration, disease and other factors cause the mobilization of lipids and, presumably, that of the blubber-associated retinoid reserves. In these situations, retinoids may be either redistributed or excreted, and significant variation in tissue concentrations is expected. Consequently, stranded cetaceans, which are often in poor nutritive condition, are a poor sample group to assess retinoid status of populations because they are likely to show altered retinoid values.

Other individual traits (e.g. age, sex, diet, pollutant concentrations) also generate variability in retinoid status (Borrell et al. 2002). We did not find significant differences in retinoid concentrations between males and females for any of the tissues, which is in agreement with the results obtained by Borrell et al. (1999) in blubber of harbour porpoises and by Mos & Ross (2002) in blubber of young harbour seals. However, Schweigert et al. (1987) and Nyman et al. (2003) found sex-related differences in blubber of adult grey seals and harbour seals, respectively. Rodahl & Davies (1949), Southcott et al. (1974) and Schweigert et al. (1987) also reported these differences in the liver of hooded, harp, fur and grey seals. Taxonomic, dietary, life-cycle and reproductive status dissimilarities between the individuals sampled could explain these sex-related variations; However, we found that the interaction between sex and lipid content affected tissue retinoid concentration. This appears to indicate that the relationship between retinoid levels and BLC in males and females differs. Reproductive activity may explain this difference, as it often involves changes in behavioral traits and diet. Further research into the influence of factors or conditions (age, sex, reproductive condition, diet) inducing variation in retinoid status and deposition in blubber is required to implement the use of retinoids as biomarkers of pollutant exposure in cetaceans.

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PAPER

Effect of organochlorine contaminants and individual biological traits on blubber retinoid concentrations in bottlenose dolphins (*Tursiops truncatus*)

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Here we assessed retinoids as biomarkers of contaminant exposure by studying whether the sex, age, lipid content and organochlorine concentrations of bottlenose dolphins induced variation in retinoid status and its deposition in blubber. Blubber samples were collected from 47 individuals of known age and gender from Sarasota Bay in June 2000 and 2001. The sample included a representative cross-section of the resident dolphin community, with ages ranging from 2 to 50 years. Organochlorine levels showed the age- and sex-related variation commonly observed in other species, with concentrations increasing in youngsters of both sexes and in adult males, and decreasing in adult females after the onset of maturity. Blubber lipid content was low in the overall population and significantly decreased with age in adult males. Retinoid blubber concentrations were comparable to other odontocete species previously studied, and were strongly determined by lipid content. As a consequence of the latter, retinoid concentration was observed to decrease with age in adult males. This effect could not be statistically dissociated from the negative correlation observed between levels of organochlorines and retinoid blubber concentration. Consequently, we could not clarify whether high organochlorine loads in this population lowered retinoid concentrations or, conversely, whether depleted lipid reserves were indeed responsible for the high organochlorine concentrations and the low retinoid levels detected in blubber. With the current knowledge, both options should be considered and investigated, with initial focus on male dolphins.

Introduction

Organochlorine compounds (OCs) are man-made chemicals that have become ubiquitous pollutants in recent decades, particularly in the marine environment. Among these, polychlorinated biphenyls or PCBs, a family of molecules with a basic structure composed of two phenyl rings with a variable number of chlorine substitutions, have become widespread and have reached almost all natural environments. Although PCBs were introduced into the market early in the twentieth century, their production peaked in the 1960s and 1970s. At the end of this period it was determined that they were having a deleterious effect on man and wildlife alike and, consequently, their use was banned in most industrialized countries. However, the recalcitrant nature of PCBs and their consequent long half-life in the environment has favoured their global transport from land to the marine environment.[1,2] Because of their persistence and lipophilicity, OCs concentrate in biota through the food chain.[3] Small odontocetes are particularly vulnerable to these pollutants; not only are they top predators, but a large proportion of their body is composed of fatty tissues that accumulate lipophilic compounds. Moreover, they show a low capacity to decompose certain OC molecules.[4,5] Although OCs produce serious pathological disorders, such as reproductive impairment[6–10] and depression of the immune function,[11,12] the links between pollutant toxicity and adverse biological effects in organisms have yet to be determined. Biomarkers, defined as contaminant-induced variations in the cellular or biochemical components of a process, structure or function that can be evaluated in a biological system,[13] have been developed to assess the true impact of exposure to pollutants in wild populations. Biomarkers are expected to provide an integrated measure of the response of an organism to exposure to a chemical or group of chemicals and, hence, a measure of toxicological risk.[14,15] However, the use of these markers in cetaceans is in its early days, and significant research is required to validate their applicability.[16]

Retinoids (vitamin A) are a group of fat-soluble molecules that are indispensable for many mammalian functions.[17] Because OCs and other pollutants disrupt metabolism and tissue concentrations of retinoids in mammals, retinoids have been proposed as potential biomarkers of exposure to this group of pollutants, particularly to PCBs.[18–20] Imbalance in retinoid concentrations may lead to diverse anomalies, such as reproductive impairment, embryonic mortality, growth retardation and decreased resistance to infections.[18,21] In marine mammals, most of the studies to date on the disruption of retinoids caused by exposure to OCs have been performed in pinnipeds,

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and have used plasma as the monitoring tissue.[11,19,22–26] Given that plasma retinoid concentrations are subject to homeostatic regulation,[27] they are not reliable indicators of retinoid body status or retinoid disruption. In marine mammals, the bulk of body retinoids is stored in the liver, which carries very high concentrations but its mass is comparatively small, and in the blubber, where concentrations are lower but tissue mass is extensive.[26,28,29] For monitoring retinoid status, blubber is a more practical alternative than liver because it can be sampled easily from live animals using non-destructive biopsy techniques.[30]

The concentrations of retinoids and OCs vary substantially among individuals depending on their age, sex, reproductive status or nutritive condition.[5,31,32] Therefore, the use of retinoids as biomarkers of OC exposure requires the study of the natural patterns of variation within populations in order to avoid confounding effects caused by biological traits. The bottlenose dolphins (*Tursiops truncatus*) off the coast of central western Florida have been studied since 1970, and are one of the most analysed small cetacean populations in the world.[33–35] This population is of particular interest because (i) it is possible to identify and sample individual dolphins, mostly with known associated biological data and (ii) it lives within a relatively small bay (Sarasota Bay), which presents moderate to high levels of pollution as a result of its densely inhabited coastline.[36] Here we analysed the relationships between OC and retinoid concentrations in the blubber of live bottlenose dolphins from Sarasota Bay in order to assess the usefulness of retinoids as biomarkers of OC exposure. To gauge the potential incidence of individual biological traits on this variation, we also studied the effect of sex, age and lipid content on retinoids.

Materials and methods

Sampling

As part of the International Whaling Commission's (IWC) Pollution 2000+ program[16,37] matched blubber samples were collected from 47 live bottlenose dolphins from Sarasota Bay in June 2000 and 2001. Dolphins were captured for examination and sampled by encircling them with a 500 m × 4 m seine net in shallow waters where handlers could stand safely and support the dolphins as necessary.[38] Initially, adult females underwent ultrasound examination for pregnancy before bringing them aboard the vessel. Each dolphin considered suitable for further examination was transferred to foam pads on the shaded deck, where it was weighed and a standard series of length and girth measurements were collected. Throughout the examination, behavior and respiratory patterns were closely monitored, and water was applied liberally externally. Small blubber wedges (4 cm long × 3 cm wide × up to 1.5 cm deep) from a standard location below the dorsal fin were obtained for contaminant and retinoid analyses after local anesthesia.

Most of the dolphins in Sarasota Bay were of known age through observation, but a tooth was collected under local anesthesia from individuals of unknown age for sectioning and counting of growth layer groups.[39] A full examination and sampling program typically required about one hour, and the dolphin was then released back into the bay. The sample included a representative cross-section of the resident dolphin community (~140 individuals), with dolphins ranging in age from 2 to 50 years (Table 1).

Blubber OC concentrations

Blubber samples were placed in liquid nitrogen immediately after collection, stored in an ultra-cold freezer (−80 °C), and then shipped in a batch from Florida to Spain in a liquid nitrogen-shipping container. In Spain, the samples were stored at −30 °C until analysis. Samples weighing about 0.2–1 g were ground with anhydrous sodium sulfate and extracted with n-hexane (residue-free quality) in a Soxhlet apparatus for five hours. The solution obtained was concentrated to 10 ml. A portion of this extract (2 ml) was used to determine the amount of extractable fat per gram of blubber. A further amount was mixed with sulfuric acid for the clean up, following the procedures described by Murphy,[40] and the resulting extract was concentrated to 0.1–1 ml and centrifuged for 5 min.

Chromatographic analysis was performed on a Hewlett-Packard 5890-II gas chromatograph equipped with an electron capture detector (ECD) at 350 °C. A fused silica capillary column (length 60 m, 0.25 mm id) coated with SPB-1 was used as the stationary phase (0.25 μm film thickness). The splitless technique was used to inject 1 μl of the purified extract. Pure nitrogen gas (99.9%) at a flow rate of 1 ml min^{-1} was used as a carrier. The temperature was programmed as follows: injection at 40 °C for one minute and increased to 170 °C at a rate of 25 °C min^{-1}; one minute constant, to 250 °C at a rate of 2 °C min^{-1} and then to 280 °C, at 5 °C min^{-1}. A preliminary screening of the samples revealed that heptachlor and congener 199 were not present in the samples analysed. Therefore, these compounds were used as internal standards. Total polychlorinated biphenyls (tPCB) were the sum of 22 congeners (IUPAC # 28, 52, 95, 101, 151, 149, 118, 153, 105, 138, 187, 183, 128, 174, 156, 180, 170, 201, 195, 194, 206, 209). Total dichlorodiphenyl-trichloroethane (tDDT) included *p,p'*-DDT, *o,p*-DDT, *p,p'*- DDE, *o,p*-DDE, *p,p'*-DDD and *o,p*-DDD. Blanks of pure n-hexane, with all lab reagents, were periodically run to ensure the purity of the system. Recoveries of organochlorine compounds ranged from 82–101% (n = 12). The detection limit was 1 μg kg^{-1} wet weight. The laboratory participated in interlaboratory calibration exercises for OCs in biota organized by Quasimeme (1998) and NIST/NOAA (2000–2003), obtaining satisfactory results.

Table 1 Concentrations, expressed as mean ± SD, of blubber retinoid (μg g^{-1} wet weight) and organochlorine (μg g^{-1} lipid weight) and blubber lipid content (BLC, %) of the bottlenose dolphins sampled sorted by sex and age class

	Age class				
	0–10	11–20	21–30	31–40	41–50
Females	(n = 10)	(n = 5)	(n = 2)	(n = 3)	(n = 4)
Retinoids	14.85 ± 6.31	8.22 ± 3.21	17.47 ± 4.52	12.58 ± 3.51	9.67 ± 2.86
BLC	25.80 ± 17.38	30.60 ± 18.38	31.17 ± 25.16	30.70 ± 18.94	30.08 ± 12.88
tDDT	15.56 ± 11.29	1.27 ± 0.34	2.15 ± 1.81	5.64 ± 3.72	4.05 ± 3.80
tPCB	22.97 ± 12.59	3.72 ± 1.89	4.11 ± 2.67	8.57 ± 5.19	6.22 ± 4.36
Males	(n = 10)	(n = 5)	(n = 2)	(n = 3)	(n = 2)
Retinoids	12.38 ± 6.20	12.10 ± 5.21	8.03 ± 2.62	6.92 ± 2.11	7.79 ± 5.86
BLC	27.80 ± 10.91	23.13 ± 7.67	6.91 ± 1.39	8.27 ± 4.68	7.36 ± 7.64
tDDT	21.40 ± 8.88	38.23 ± 25.52	56.81 ± 18.42	73.68 ± 31.88	404.07 ± 484.76
tPCB	31.95 ± 11.92	67.18 ± 47.15	102.16 ± 53.49	138.81 ± 68.21	483.67 ± 544.10

Blubber retinoid concentrations

The methods for retinoid analysis are described in Borrell *et al.*[31] Samples were treated at room temperature and under red light. The samples, weighing about 100 mg each, were saponified overnight in an ethanolic KOH solution (1 g KOH, 2 ml distilled H_2O, 2 ml ethanol, 20 mg ascorbic acid) in a mechanical shaker under a nitrogen atmosphere. Retinoids (retinol and retinyl esters) were extracted by adding 8 ml diisopropyl ether and shaking for 30 min. After separation from the aqueous phase, an internal standard (retinyl acetate) was added and the organic extract was cleaned three times with 4 ml of aqueous phosphate buffer (pH 7.4). The extract was dried under nitrogen and reconstituted with 1 ml methanol containing 0.05% w/v butylated hydroxy toluene (BHT) as antioxidant. Reconstituted samples were filtered (0.20 μm mesh) and a 20 μl subsample automatically injected (Waters 700 Satellite wisp) into an HPLC (Waters 600 E System Controller Pump) equipped with a Restek column (Tracer Excel 120 ODS-A, 10 cm length, 5 μm beds, 0.46 cm id) and a UV detector (Waters 486 Tuneable absorbance D) set at 326 nm. Retinoid was eluted at a flow rate of 1 ml min^{-1} using a mobile phase of methanol–water (80 : 20 by volume) for 1 min followed by a linear gradient of 3 min to 100% methanol for 14 min. Calibration curves, using matrix-free standard solutions, were used for the determination of retinoid levels in blubber. The correlation coefficient for retinol was $r^2 = 0.99$ from a range of concentrations of 1–15 μg ml^{-1} (all injections were within such range; more concentrated samples were diluted). Recoveries for retinol in the whole extraction and saponification procedure (calculated using a standard addition technique) ranged from 83 to 102% (mean = 94%; SD = 9.5%, $n = 12$). Every 5 samples, a duplicate was run to ensure the quality of the system.

Statistics

We studied the variables related to retinoids that should be accounted for in a model to examine the relationship between retinoids and OC pollutants. Given the anticipated differences in sex and age trends of OC pollutants,[5,41] the analyses for males and females were performed separately.

We first analysed the relationships between blubber retinoid concentrations and lipid content (BLC) using a robust linear regression model.[42] This method was chosen because: (i) the number of dolphins was low and the potential for outlier and extreme values was high; (ii) the robust regression model is minimally influenced by outliers in the response and predictors' space; (iii) the model fit minimizes the bias in coefficient estimates caused by non-Gaussian errors; and (iv) statistical inference is based on large sample size approximations and is comparable to that obtained with the least squares method. The relationships between BLC and OCs (PCBs and DDTs) were also analysed by robust regressions. In these analyses, PCBs and DDTs (as lipid weight values) were log-transformed to reduce the effect of the extreme between-individual differences.

This preliminary analysis indicated that most variables that predicted retinoid concentration, except in the male data set which was dominated by the effects of the age, would have poor explanatory power. Therefore, we fitted multiple regression models with reduced combinations of variables and adequate structure to accommodate age effects in males. We used these models to assess the explanatory power of these variables and the uncertainty in their power to explain retinoid variability. For this purpose, we used the AIC (Akaike Information Criterion).[43] Models with lower AIC values and high AIC weights provided the best fit in terms of a good compromise between model parsimony and a good description of the data.

Results

The concentrations of blubber retinoids and OCs, and the blubber lipid content (BLC) of the bottlenose dolphins are shown in Table 1. We found large differences between sexes in the age trends of retinoids, lipids and OC pollutant blubber loads (Table 1). Retinoid concentration was positively correlated with the lipid content in males (Fig. 1: $r^2 = 0.27$; $p < 0.001$). The oldest males had lower retinoid concentrations and lower lipid content. In contrast, the relationship between retinoids and lipid content in females was not significant.

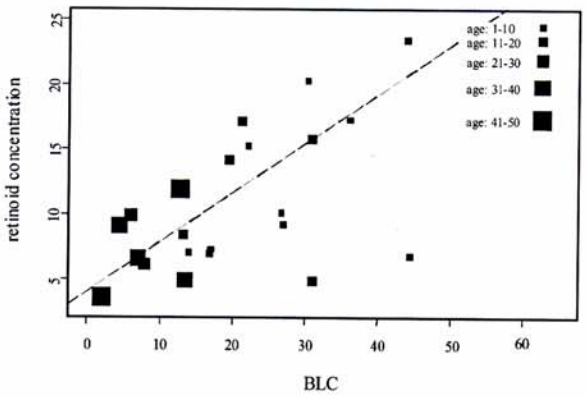

Fig. 1 Relationship between retinoid concentrations (μg g^{-1} wet weight) and BLC (%) in male bottlenose dolphins.

Fig. 2 shows the relationships between DDTs and PCBs and BLC in males. Both $\log_e$(tDDT) (Fig. 2a: $r^2 = 0.40$; $p < 0.001$) and $\log_e$(tPCB) (Fig. 2b: $r^2 = 0.44$; $p < 0.01$) were negatively correlated with lipid content. In females, however, the relationship between these variables was not significant and was independent of age. Old males had high levels of DDTs and PCBs, while old female counterparts had much lower concentrations.

The relationships between DDTs and PCBs and retinoids in males are presented in Fig. 3. Retinoids had a significant relationship with tDDT (Fig. 3a: $F = 3.957$; on 2 and 18 degrees of freedom; $p = 0.037\ 65$) and with tPCB (Fig. 3b: $F = 2.964$; on 2 and 18 degrees of freedom; $p = 0.077\ 13$). In females, there was no significant relationship either between retinoids and tDDT or between retinoids and tPCB. The data indicate that below concentrations of approximately 40 μg g^{-1} of DDT and 50 μg g^{-1} of PCB, the relationship between retinoid and OC concentrations was uncertain for both sexes. Male OC concentrations exceeded these values, thus we observed that the higher the pollutant levels, the lower the retinoid concentrations. However, the OC levels in females did not exceed these thresholds, therefore we did not fit further regression models in females.

Table 2 shows the multiple regression models used to assess the effect of BLC, age, tPCB and tDDT on retinoid concentrations in male bottlenose dolphins. A model with BLC as the single linear predictor provided the best fit, indicating that retinoid concentration was strongly related to this parameter, in agreement with the robust regression analysis. The AIC weights showed that other models showed a poorer fit, except a model with tDDT as linear and quadratic predictor.

Discussion

Here we studied whether sex, age, BLC and OC concentrations induced variation in retinoid status in the blubber of bottlenose dolphins. Relationships between these variables must be established and interpreted with caution in order to ascertain the usefulness of retinoids as biomarkers of contaminant exposure.

As expected, older females had lower OC concentrations in blubber than males, as they transfer substantial amounts of pollutants to their offspring during gestation and lactation.[5] In this species, lactation is long (18 to 20 months),[34] and thus reproductive transfer of OC is high.[44] Juveniles already display relatively high OC tissue levels, which tend to increase with age as, in this early stage of growth, intake exceeds degradation or

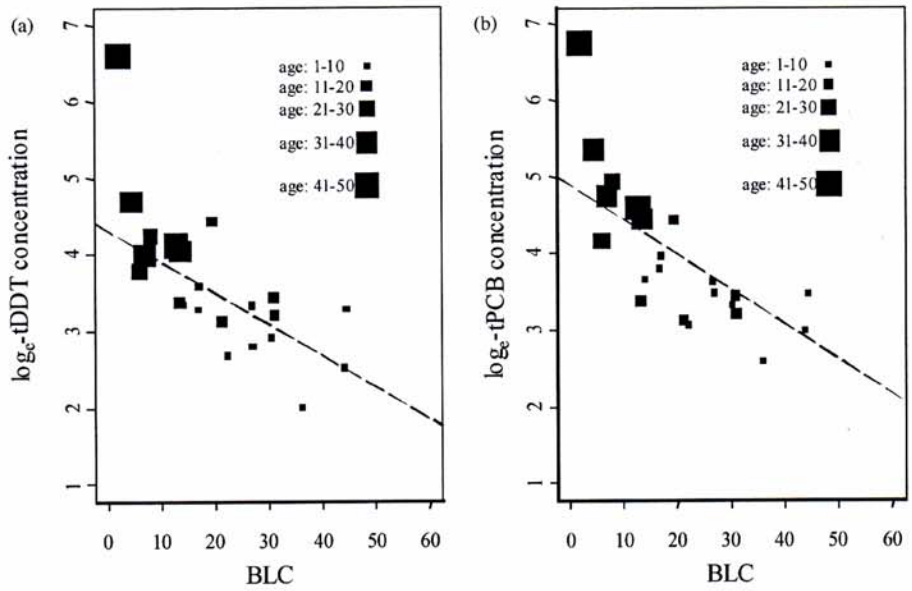

Fig. 2 (a) Relationship between $\log_e$(tDDT) concentrations (μg g^{-1} lipid weight) and BLC (%) in male bottlenose dolphins. (b) Relationship between $\log_e$(tPCB) concentrations (μg g^{-1} lipid weight) and BLC (%) in male bottlenose dolphins.

excretion rates. In females, this age-related pattern shifts at an age of 8–12 years, when they start reproducing,[34] and concentrations start to decrease because of OC reproductive transfer. However, three of the older females (34, 38 and 43 years old, respectively) displayed higher pollutant concentrations than other females in their age class, but lower values than old males. Wells *et al.*[45] propose that these apparently high PCB values may be due to increased calving intervals in these old females, as has been previously suggested to occur in other odontocete species.[46,47] In males, concentrations of DDTs and PCBs tended to increase throughout the whole life span, as expected.[5] However, some of the older males had higher OC concentrations than the remaining population of the same age. In Sarasota, adult males are known to occasionally leave the usual home range of the community,[34,35] and it is likely that these highly polluted individuals temporarily visited other, presumably more contaminated waters.

Overall OC concentrations detected in this study (Table 1) were in the same order of magnitude as those reported by Schwacke *et al.*[10] in bottlenose dolphins from the same area and those described in other water masses along the US Southeast coast (Beaufort, NC, Matagorda Bay, TX, Charleston, SC, and Indian River Lagoon, FL).[10,48]

Overall BLC of the dolphins (24.5%) was similar to that reported by Hansen *et al.*[48] in Beaufort and Charleston, but substantially lower than that observed in bottlenose dolphins in other parts of the world, such as the Indian River Lagoon,[48] the Bay of Bengal,[49] Scotland,[50] or the Mediterranean Sea.[51,52] The Sarasota community is believed to have a good nutritive status and the low BLC generally found in this study may be due to the warmer environment in which these dolphins live and the consequent less stringent thermoregulatory needs. Indeed, an inverse relationship between water temperature and blubber depth has been reported in bottlenose dolphins

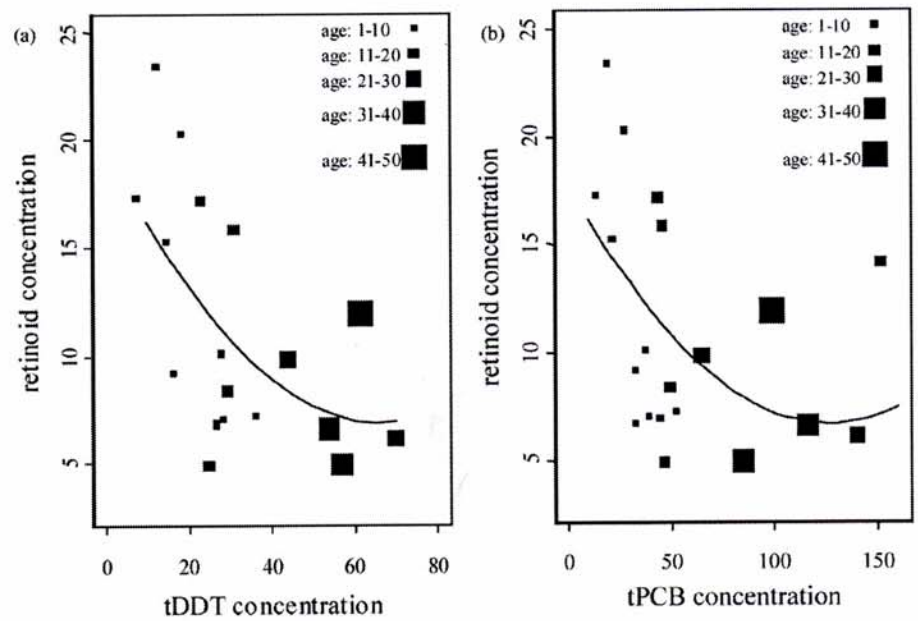

Fig. 3 (a) Relationship between retinoid (μg g^{-1} wet weight) and tDDT concentrations (μg g^{-1} lipid weight) in male bottlenose dolphins. (b) Relationship between retinoid (μg g^{-1} wet weight) and tPCB concentrations (μg g^{-1} lipid weight) in male bottlenose dolphins.

Table 2 Models of multiple regression to assess the effect of pollutants on retinoid concentration in male bottlenose dolphins. AIC_c is the small sample Akaike Information Criterion, w_i are the Akaike weights of the i models measuring the likelihood of each model given the data, and R^2 is the fraction of variance explained by the model. The "~" relates the response variable, retinoid concentration, with the predictors

	Model	AIC_c	w_i	R^2
1	Retinoids ~ lipids	130.08	0.437	0.25
2	Retinoids ~ tDDT + $tDDT^2$	131.57	0.208	0.31
3	Retinoids ~ lipids + age	133.12	0.096	0.25
4	Retinoids ~ tPCB + $tPCB^2$	133.24	0.090	0.25
5	Retinoids ~ lipids + tDDT + $tDDT^2$	133.65	0.073	0.35
6	Retinoids ~ age	134.10	0.056	0.09
7	Retinoids ~ lipids + tPCB + $tPCB^2$	134.98	0.034	0.30

from this and other communities.[53,54] Furthermore, the effect of seasonality of food availability should also be considered.[55] Hansen *et al.*[48] found higher BLC in samples collected in July than in those taken in April and November. Our samples were collected in June, after significant blubber thinning, so a lower BLC is to be expected. As a further potential confounding factor, the sampling method may also affect BLC. Cetacean blubber is vertically stratified and the various layers may differ in lipid content and composition.[56,57] On the other hand, Tornero *et al.*[58] found significant within-blubber topographical variation in % BLC in common dolphins (*Delphinus delphis*). Also, studies on other species[59] have indicated that blubber sampled by biopsy darts and that obtained from blubber wedges may show dissimilarities in lipid content.

In this study, BLC was significantly much lower in adult males than in immature individuals of both sexes and adult females. Hansen *et al.*[48] also reported higher BLC in adult females than in adult males in Charleston and in the Indian River Lagoon, although these differences were smaller than those detected in the present study. Effect of age and sex on BLC have also been reported in some cetaceans,[5] including bottlenose dolphins.[60–62] The sampling period for the present study coincided with the birthing period of most Sarasota dolphins (late spring to early summer)[63] and Scott *et al.*[33] found that females with neonates and juveniles occupy different geographic areas in Sarasota Bay than the rest of the population, thus possibly explaining the observed differences with adult males.

As OCs are lipophilic pollutants, changes in BLC are expected to affect their concentration. In general, the mobilisation of lipid stores produces an increase in OC levels.[5] Expressing the OC data on a lipid weight basis instead of on a fresh weight basis compensates for this effect to some extent, but does not completely remove it. Indeed, the relationship between BLC and OCs in males demonstrates that even taking into account the lipid content of the tissue, OC concentrations were inversely related to lipid content. This observation is in agreement with the findings of Aguilar and Borrell,[64] who reported the same negative relationship for male Mediterranean striped dolphins (*Stenella coeruleoalba*). Lipids are mobilised from the blubber faster than lipophilic chemicals and, therefore, the decrease in lipid content is not coupled with a corresponding reduction in pollutant loads.[53] Conversely, in our study pollutant concentrations in females were not affected by changes in blubber lipids. While lipid content remained constant over age, contaminant levels decreased progressively and markedly from the first calf.

Like OCs, retinoids are also lipophilic and concentrate in lipid-rich tissues. Blubber is the main body fat depot and body site for retinoid deposition in marine mammals.[28,29,65,66] Despite the advantages that this tissue offers for monitoring retinoid status, the information available on cetaceans in this regard is limited. Indeed, the data from the present study are the first for bottlenose dolphins. We found overall retinoid concentrations averaging 12.52 $\mu g\ g^{-1}$ in females and 10.76 $\mu g\ g^{-1}$ in males. These values are much lower than those reported for other cetacean species, such as harbour porpoises (*Phocoena phocoena*)[31] and common dolphins.[58]

Retinoid concentrations were positively correlated with BLC in males, a finding consistent with the marked lipophilicity of these compounds. In fact, BLC was the variable that best described the differences in male retinoid concentrations. Tornero *et al.*[29,58] also reported that BLC was a strong determinant of overall retinoid concentration in common dolphins. However, the studies by Rodahl and Davies[67] in hooded seals (*Cystophora cristata*) and harp seals (*Phoca groenlandica*), by Borrell *et al.*[31] in harbour porpoises and by Mos and Ross[28] in harbour seal (*Phoca vitulina*) pups, did not find this relationship. Mobilization of lipids from blubber and, most probably, of the blubber-associated retinoid reserves, can be caused by lactation, migration, disease and other events. In these situations, retinoids may be redistributed or excreted, and considerable variation in tissue concentrations is likely to occur. Unlike males, the relationship between retinoid concentration and BLC in Sarasota females was non-significant. Sex-related differences were also reported by Tornero *et al.*[29] Similarly, as in the case of the abnormally high OC levels detected in some old females, reproductive activity may explain these dissimilarities as it may involve alterations in diet and behaviour.

The effect of ageing on body retinoid concentrations has been extensively studied in terrestrial mammals. Concentrations have often been found to increase with age, although a decrease or a lack of trend between age-classes has also been reported.[32] Studies on marine mammals show similarly inconsistent results. Käkelä *et al.*[68] found a significant positive age-related trend in the blubber of ringed seals (*Phoca hispida*) from the Baltic Sea and Lake Saimaa, and the opposite result in ringed seals from Spitsbergen. Positive age trends have also been reported in harbour porpoises[31] and grey seals (*Halichoerus grypus*).[65] However, we found that ageing did not account for the variations observed in retinoid levels in bottlenose dolphins, which is consistent with the results on ringed and grey seals.[26] These conflicting results could reflect differences in overall health status, diet, taxonomic relationship or habitat occupied by the populations studied.

Preliminary studies on the disruption of blubber retinoids by OCs in marine mammals is limited to the work of Nyman *et al.*,[26] who found a negative correlation between retinyl palmitate and OCs in the blubber of grey seals. Consequently, these authors proposed retinyl palmitate levels as biomarkers for the depletion of vitamin A reserves. Our observations in male and female bottlenose dolphins differed. In females, the lack of significance in the retinoid–OC relationship could be attributable either to the fact that contaminant concentrations were not high enough to exert any effect or to a lack of retinoid sensitivity to a low level of exposure. In contrast, males, with higher OC loads, showed a significant negative correlation between OC and retinoid concentrations. This observation indicates that effects on blubber retinoid levels occurred at high OC exposures: over 40 $\mu g\ g^{-1}$ of DDT and 50 $\mu g\ g^{-1}$ of PCB. Below these thresholds, retinoid values are expected to correspond to the natural variability of the population.

In summary, on the basis of our results we conclude that a number of biological factors may have influenced the retinoid levels detected in our sample of bottlenose dolphins. There is evidence of a non-linear effect of OCs on blubber retinoids in male dolphins, but results do not clarify whether high OC loads lowered retinoid concentrations or, conversely, whether depleted lipid reserves were indeed responsible for both the high OC concentrations and the low retinoid levels observed in blubber. Further research is required, focusing initially on male dolphins and with higher sample size, to answer this latter question, which is key to clarifying the potential cause–effect relationship between high levels of OC exposure and retinoid depletion in bottlenose dolphins.

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

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ABSTRACT

We investigated the effect of post-mortem time (0-48 hours) on retinoid concentrations in the blubber and liver of harbour porpoises under natural conditions to assess the reliability of samples collected from animals after they died. Organochlorine compounds and lipid content were also determined to test whether they affected 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. As opposed to 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 tissue retinoids. 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 the study.

Keywords: harbour porpoises; retinoids; organochlorines; incidental catches; biomarker

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). Since their metabolism and tissue concentration are affected by exposure to organochlorine compounds, like PCBs and dioxins (TCDDs) (Brouwer *et al*., 1989; Chu *et al*., 1995, 1998; Käkelä *et al*., 2002; Rolland, 2000; Nyman *et al*., 2003), they have been proposed as biomarkers of the impact of this group of pollutants (Simms and Ross, 2000; Borrell *et al*., 2002). Some biological traits such as age, sex, diet, incidence of disease, occurrence of lactation and lipid composition are also known to affect tissue retinoid levels in varying degrees (Borrell *et al*., 2002). Therefore, these factors should be taken into account when attempting the use of retinoids as biomarkers.

In mammals, retinoids are mainly stored in the liver (Blomhoff *et al*., 1992) and thus their body status is commonly assessed through hepatic concentrations (Schweigert and Buchholz, 1995; Käkelä *et al*., 2002). However, retinoids are lipophilic and they also accumulate in fatty tissues. Marine mammals have a thick, extremely lipid-rich hypodermis, commonly known as blubber that acts as 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*., in press). 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*., 2004).

Retinoids are unstable compounds with extreme sensitivity to light, oxygen, trace metals, strong acids and excessive 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 retinoids from being oxidized and/or isomerised (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 from death to sample collection.

By-caught 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 healthy population. Thus, they are expected to be neither affected by severe disease nor emaciated, which are common conditions in washed ashore specimens. Moreover, by-caught cetaceans provide biological data and allow the examination of tissues and organs, which are used to determine main biological traits (age, sex, reproductive condition) and assess the 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 is essential to calibrate the effect of post-mortem time on retinoid tissue concentrations and thus assess the validity of by-catches 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), and so samples can be readily obtained from a relatively large number of individuals. In this work, we calibrated 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 animals after they died. Organochlorine compounds and lipid content were concurrently determined to evaluate their potential effect on the retinoid status of the sampled individuals. We also investigated the correlation between blubber and liver retinoid concentrations 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 by the "Field Protocol for Pollution 2000+" (Anonymous, 2002). Thus, an initial blubber sample was collected at the moment of death. To mimic natural conditions, animals were then placed in a tank, at a depth of two meters 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 aluminum foil and stored at -20ºC until analysis, a temperature at which retinoids in plasma and tissues are known to be safe 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) in a mechanical shaker under nitrogen atmosphere. Retinoids were extracted by adding 8ml diisopropyl 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). The extract was dried under nitrogen and reconstituted with 1ml methanol, 0.05% butylated hydroxy toluene (BHT) as antioxidant and retinyl acetate as internal standard. Reconstituted samples were filtered (0.20σm mesh) and a 20σl subsample was automatically injected (Waters 700 Satellite wisp) on a HPLC (Waters 600 E System Controller Pump) equipped with a Restek column (Tracer Excel 120 ODS-A, 10cm length, 5σm beds, 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 using a mobile phase of methanol/water (80/20 by volume) for 1min followed by a linear gradient of 3min to methanol 100% for 14min.

Organochlorine pollutant analysis

Samples weighing 0.2-1g were ground with anhydrous sodium sulphate and extracted with n-Hexane (residue-free quality) in a Soxhlet apparatus for 5h. The resulting solution was concentrated to 10ml. A portion of this extract (2ml) was used to determine the quantity of

extractable fat per gram of blubber. A further quantity was mixed with sulphuric acid for the clean up, following the procedures described by Murphy (1972), and the resulting extract was concentrated to 0.1-1ml and centrifuged for five minutes.

Chromatographic analysis was carried out on a Hewlett-Packard 5890-II gas chromatograph equipped with an electron capture detector (ECD) at 350ºC. A fused silica capillary column (length 60m, 0.25mm ID) coated with SPB-1 was used as the stationary phase (0.25um film thickness). The splitless technique was used to inject 1σl of the purified extract. Pure Nitrogen gas at a flow rate of 1ml/min was used as a carrier. Temperature was programmed as follows: injection at 40ºC for one minute and increased to 170ºC at a rate of 25ºC/minute; one minute constant, to 250ºC at a rate of 2ºC/minute and then to 280ºC, at 5ºC/minute. Heptachlor and congener 199 were used as internal standards. Total polychlorinated biphenyls (tPCB) were the sum of 22 congeners (IUPAC # 28, 52, 95, 101, 151, 149, 118, 153, 105, 138, 187, 183, 128, 174, 156, 180, 170, 201, 195, 194, 206, and 209). Total dichloro-diphenyl-trichloroethane (tDDT) included *p,p´*-DDT, *o,p*-DDT, *p,p´*- DDE, *o,p*-DDE, *p,p´*-DDD and *o,p*-DDD. Total hexachlorocyclohexane (tHCH) was the sum of all three isomers (ζ,η,θ). Hexachlorobenzene (HCB) was also measured.

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 each porpoise sampled. The body length of the individuals (ranging from 109 to 150cm) was used to estimate the age class distribution. The blubber lipid content ranged from 56.41 to 86.07%, 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 sensibly 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 showed a considerable decrease (approximately four times) (Tilbury *et al*., 1997; Westegate *et al*., 1997), in agreement with the decreasing trend of organochlorine pollution observed in most temperate regions of the northern hemisphere during the last decades (Aguilar *et al*., 2002). The low concentrations found in this study seem unlikely to influence 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. Liver concentrations were, as expected, very high (range: 131-1680mg/kg) and similar to the highest values recorded in cetaceans, e.g. blue whales (*Balaenoptera musculus*), fin whales (*Balaenoptera physalus*) and sperm whales (*Physeter macrocephalus*) (Schmidt-Nielsen *et al*., 1934; Klem, 1935; Braekkan, 1948; Schweigert *et al*., 1987). Blubber concentrations varied widely between individuals, ranging from 42.6 to 221.5mg/kg. 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*., 2004). Liver levels were approximately 5-6 times higher than those found in the blubber. Similar studies on marine mammals have also described higher 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*) (Käkelä *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*., in press).

Retinoid concentrations in blubber and liver were positively correlated (Fig. 1; $p<0.05$, $r^2=0.80$), 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, in cetaceans, access to the liver is not possible in free-ranging individuals, and the tissue decomposes rapidly post–mortem, so liver is in most cases a non-suitable tissue for monitoring. 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 to 14.5ºC (mean: 12.9ºC). Carcass internal temperatures decreased drastically from the moment of death (35.4-36.6ºC) to the 48 hours timepoint (12-12.4ºC). 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.

In this regard, three main agents have been reported to influence retinoid levels: ultraviolet 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 ultraviolet 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 ultraviolet penetration into the carcass. However, it should be noted that the corpse was kept two meters 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 aluminum foil and deep freezing, thus avoiding the effect of ultraviolet 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 ascorbate or BHT (Wyss, 1990). In our case, anoxia in the corpse is to be expected in a few hours after death and, as a consequence, oxygen did not appear to affect retinoid tissue concentrations before sampling. When the specimen was retrieved, the samples were quickly wrapped in aluminum foil to avoid dehydration and prevent direct exposure to the atmosphere; the analysis was performed under Nitrogen atmosphere and BHT was added as antioxidant. 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 over storage in the dark at 2-8ºC (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 20-40ºC (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 35ºC, and Halbaut *et al*. (1997) at 30ºC. 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 nature of both the retinoids and the other compounds 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 (Billion-Rey *et al*., 1992; Albalá-Hurtado *et al*., 2000b; Dupertuis *et al*., 2002). The proteins bound to 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 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 manner that they are not easily affected by degradation processes. As a consequence, blubber is reliable for the assessment of the retinoid status of unpreserved specimens kept up to 48 hours in conditions similar to those of the study. Nevertheless, this conclusion should not be unreservedly extended to stranded individuals. In this latter case, corpses are usually found over 48 hours post-mortem, they are directly exposed to sunlight and may have been subjected to temperature rises due to sun irradiation.

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Table 1. Sex, body length (cm), age class, 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 σg/g calculated on the basis of the lipids extracted.

harbour porpoise	sex	body length	age class*	BLC	time	tHCH	HCB	tPCB	tDDT
69	male	139	mature	85.07	24	0.12	0.12	4.62	2.27
					48	0.13	0.14	5.45	3.18
84	male	119	immature	80.15	0	0.15	0.11	5.33	2.69
			(1-3)		48	0.14	0.10	4.71	2.44
85	male	126	immature	56.41	24	0.08	0.10	3.13	1.79
			(1-3)		48	0.09	0.09	3.92	1.88
184	male	109	{8	80.62	0	0.10	0.10	3.85	1.67
					48	0.14	0.12	5.42	2.47
191	male	129	immature	69.58	0	0.11	0.10	5.42	2.95
			(1-3)		48	0.10	0.11	4.98	2.65
199	female	150	mature	77.81	0	0.09	0.04	4.76	2.00
			(pregnant)		48	0.11	0.06	5.35	2.61

*Estimate based on length

Table 2. Blubber retinoid concentrations (0, 3, 9, 24 and 48 hour post-mortem replicates: mean ∂ SD) and liver retinoid concentrations (48h post-mortem replicates: mean ∂ SD) in the harbour porpoises studied. Concentrations are expressed as σg/g tissue.

harbour porpoise	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	1679.46 ± 643.92
199	14	88.26 ± 19.70	3	131.24 ± 52.59

Fig. 1. Correlation between liver and blubber retinoid concentrations (σg/g tissue).

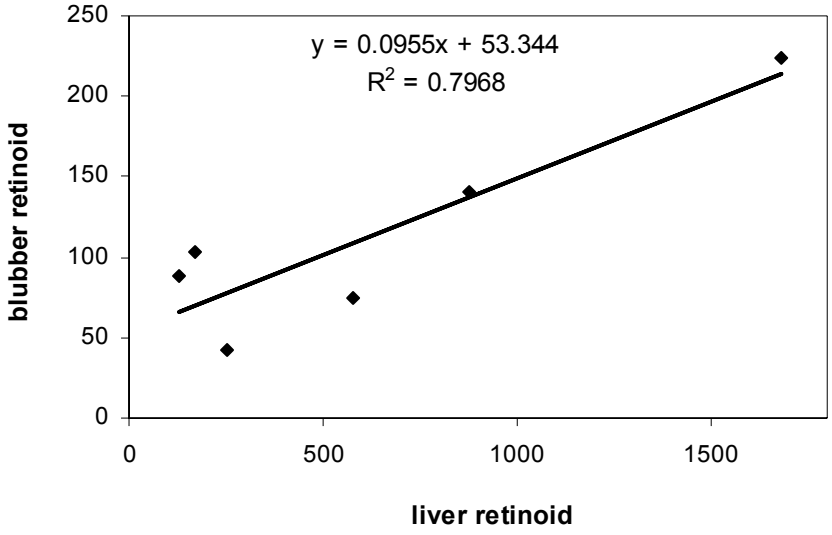


Fig. 2. Means of seawater and carcass temperatures at each time point: 0, 3, 9, 24 and 48h.

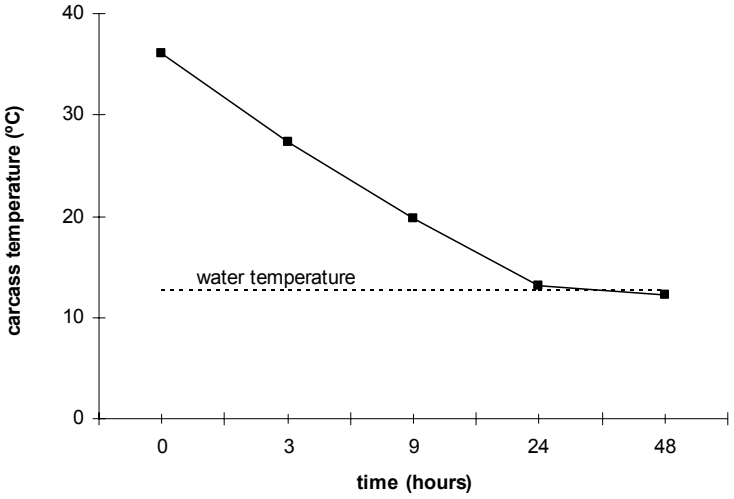
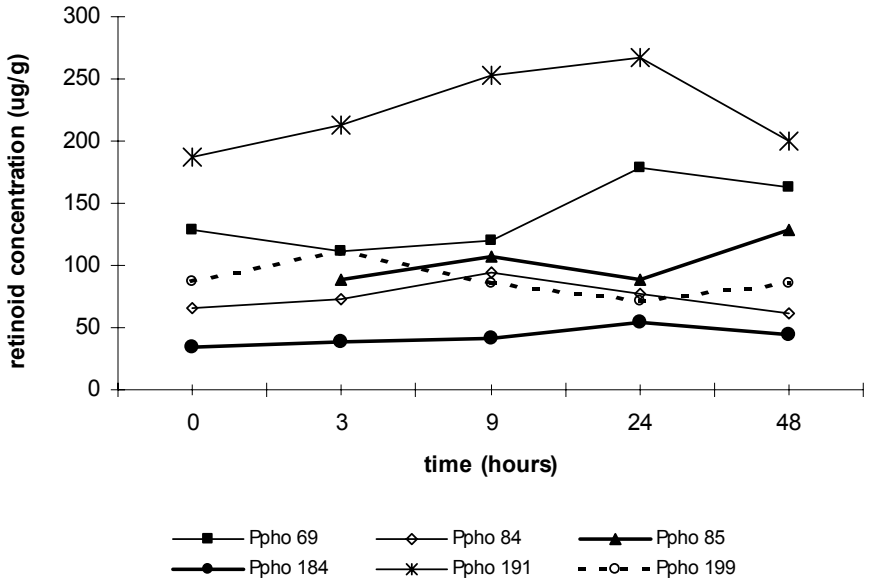


Fig. 3. Blubber retinoid concentrations at the different sampling times (0, 3, 9, 24 and 48h) in each harbour porpoise.



Integrating life history and reproductive success data to examine potential relationships with organochlorine compounds for bottlenose dolphins (*Tursiops truncatus*) in Sarasota Bay, Florida

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ABSTRACT

Research initiated in 1970 has identified a long-term, year-round resident community of about 140 bottlenose dolphins (Tursiops truncatus) in Sarasota Bay, Florida, providing unparalleled opportunities to investigate relationships between organochlorine contaminant residues and life history, health and reproductive parameters. Many individual dolphins are identifiable, and of known age, sex, and maternal lineage ($\leq$ 4 generations). Observational monitoring provides data on dolphin spatial and temporal occurrence, births and fates of calves, and birth order. Capture-release operations conducted for veterinary examinations provide biological data and samples for life history and genetic analyses, health assessment, and contaminant residue measurement. Organochlorine concentrations in blubber, milk, and blood (plasma) can be examined relative to

age, sex, body condition, lipid content, birth order, and health parameters. Reproductive success is evaluated through tracking of individual female lifetime calving success.

For the current study, 47 blubber samples collected during June 2000 and 2001 were analyzed for PCB concentrations relative to life history factors and reproductive success. Prior to sexual maturity, males and females exhibited similar concentrations. Classical patterns of accumulation with age were identified in males, but not in females. Subsequently, males accumulated higher concentrations of PCBs through their lives, whereas females begin to depurate with their first calf, reaching a balance between contaminant intake and lactational loss. In primiparous females, PCB concentrations in blubber and plasma and the rates of first-born calf mortality were both high. While high PCB concentrations may decrease the fitness of calves, absence or early interruption of lactation may also hamper transfer of pollutants, making it difficult to clarify cause and effect. First-born calves had higher concentrations than subsequent calves of similar age. Maternal burdens were lower early in lactation, and increased as calves approached nutritional independence. Empirical data were generally consistent with a published theoretical risk assessment, and supported the need for incorporation of threats from indirect anthropogenic impacts such as environmental pollutants into species management plans. Long-term observational monitoring and periodic biological sampling provide a powerful, non-lethal approach to understanding the correlations of organochlorine concentrations and health or reproductive parameters for coastal dolphins.

Keywords: pollutants; organochlorines; monitoring; conservation; survivorship; reproduction; age at sexual maturity; parturition; lactation

INTRODUCTION

More than three decades have passed since the initial recognition of possible health and reproductive effects of persistent organic pollutants on marine mammals, but much of the literature available today is still limited to chemical residue data, with little information on the toxicokinetics and impacts of the chemicals relative to the animals (see reviews by O'Shea, 1999; O'Hara and O'Shea, 2001; Reijnders and Aguilar, 2002; O'Shea and Tanabe, 2003). In recent years, high concentrations of environmental contaminant residues in carcasses recovered during large scale marine mammal mortality events (*e.g.*, Geraci, 1989; Aguilar and Borrell, 1994) have led to increasing interest in identifying the potential contribution of anthropogenic contaminants to these and other cetacean mortalities (Kuehl and Haebler, 1995). Such events are increasingly reported around the world, and there is a need to be proactive in understanding and responding to morbidity and mortality that might result from, or be exacerbated by, organochlorine contaminants (OCs). To this end, efforts are underway to measure and compare contaminant concentrations in free-ranging populations of bottlenose dolphins (Tursiops truncatus) at multiple sites in the southeastern United States (Hansen et al., 2004; Schwacke et al., 2004; Wells et al,. in press), and to perform risk assessments using available information on effects of these chemicals on surrogate mammalian models (Schwacke et al., 2002, 2004). These preliminary models would benefit from improved information on the dynamics and effects of OCs on dolphins.

Evaluation of individual and population-level risks from OCs requires knowledge of modes and variability in accumulation and excretion patterns, especially relative to age, gender, and life history. Based largely on compilations of single sampling events, descriptions of OC residue accumulation and excretion have been inferred for several species of small odontocete cetaceans, beginning with the work by Gaskin et al. (1971) with harbor porpoises (Phocoena phocoena). Because of the persistent and lipophilic properties of many OCs, they are accumulated through the food chain, into tissues of high lipid content (such as blubber), where they can reach concentrations of concern for health and reproduction in some apex predators such as some marine mammals (O'Shea, 1999). In males, accumulation of some of the more persistent OCs continue through life, but in females, concentrations appear to decline with reproductive activity,

presumably through transfer across the placenta (Aguilar and Borrell, 1994; Salata et al. 1995) and via lactation (Tanabe et al. 1994). The transfer of OC residues from blubber through the circulatory system to milk has been described for grey seals (Halichoerus grypus, Addison and Brodie, 1987), but comparable studies remain to be completed for dolphins.

Data on apparent variations in female OC residue concentrations relative to reproductive activity were provided by the work of Cockcroft et al. (1989). They measured concentrations of polychlorinated biphenyl compounds (PCBs) and DDT and its metabolites in bottlenose dolphins killed in shark nets off South Africa. Based on blubber concentrations of OCs relative to occurrence of ovarian scars indicative of sexual maturity, they proposed that females transferred 80% of their body burden of OCs to calves through lactation. They calculated that this redistribution occurred predominantly during the first seven weeks of life, with first-born offspring receiving the majority of the mother's body burden. Further delineation of this pattern in the absence of matched samples from the calves of the sampled females or serial samples from the females themselves was not possible.

Access to captive bottlenose dolphins provided Ridgway and Reddy (1995) with opportunities to measure OC concentrations in serially-collected milk samples from living females. The authors noted relationships between female age and lactation history, along with a decline in OC concentrations in one case through the course of lactation, that supported the concept of mammary-based transfer and excretion. Several factors confounded interpretation of these data, however, including the unusual circumstances of induced lactation and re-lactation, much-delayed onset of female reproduction as compared to the wild, and inconsistent results across females. Subsequent work examined OC concentrations in maternal blubber prior to parturition relative to calf survivorship (Reddy et al. 2001). Sum PCB concentrations for mothers with calves surviving less than 12 days were about 2.5 times those in mothers with surviving calves, and many of the lost calves of mothers with high concentrations were first-borns.

For the purposes of small cetacean risk assessments, empirical evidence of rates of OC accumulation in blubber and depuration from living members of a wild population, in combination with details of gender, age, and reproductive history, would be most beneficial.

Measures of sublethal levels of OCs are crucial for defining accumulation patterns. Based on the findings of Cockcroft et al. (1989) and others, knowledge of parity is important for identifying the mother's OC legacy relative to subsequent environmental contributions to calf concentrations. For males, single samplings of blubber matched with information on age and birth order are sufficient for defining patterns. For females, OC concentration data must be examined in the context of their reproductive cycle and the temporal proximity of tissue sampling to lactation. A variety of measures would help to determine the impact of mother's OC body burden on her reproductive success. Serial sampling of the mother before and after lactational transfer to the calf, or of the calf prior to weaning would provide a measure of the rate of OC exposure, and measures of subsequent calf survivorship could be used in weight of evidence evaluations. To date, there have been few opportunities to obtain this level of information in the wild.

In one of the most detailed studies of its kind, Ylitalo et al. (2001) analyzed biopsy dart samples of blubber from more than 70 free-ranging killer whales (Orcinus orca) known from years of observational and genetic studies in Alaska. These authors found that reproductive females contained lower levels of OCs than immature whales or mature males of the same age class, and that first-recruited whales contained much higher levels than non-first-recruited whales, providing additional support for the proposed patterns of accumulation and depuration. However, the lack of serial samples from individuals, incomplete data on ages, reproductive histories, lactational status, and birth orders, and the use of remote biopsy sampling techniques that may not fully sample blubber through its entire thickness (O'Hara and O'Shea, 2001), limited the interpretation of results.

Another promising field site for obtaining empirical information on small cetacean patterns of OC accumulation, excretion, and potential effects on reproductive success exists for bottlenose dolphins along the central west coast of Florida. Research on the year-round resident Sarasota Bay dolphin community has been ongoing since 1970, and about 140 identifiable individuals, mostly of known gender, age, and genetic relationships (Duffield and Wells 1991, 2002), are currently under study (Irvine and Wells, 1972; Irvine et al., 1981; Scott et al., 1990a; Wells, 1991, 2003). The resident community is composed of at least four generations, and includes

about one third of the dolphins first identified in the early 1970's. Using tagging, tracking, and photographic identification techniques (Scott et al., 1990b) it has been possible to define individual home ranges, monitor female reproductive histories including documenting birth order, measure female reproductive success, and determine population-level trends in abundance, losses, and other vital rates (Wells and Scott, 1990). In addition, capture and release operations have been conducted in which dolphins have been examined for health and reproductive status (Wells et al., in press), and full-depth blubber samples and other tissues have been collected for analyses of OC residues. In combination, these features of the Sarasota Bay dolphin research program provided a unique opportunity to relate accurate measures of concentrations of OCs to precise measures of life history parameters and reproductive success, leading to a more refined understanding of the processes of OC accumulation and excretion, and their potential effects.

Ultimately, our desire to understand the processes of accumulation and depuration is based on a need to evaluate the risks from OCs to individuals, and assess the expected impacts of documented exposures, as this relates to conservation of dolphin populations. A probabilistic risk assessment of reproductive effects of PCBs has been performed by Schwacke et al. (2002) for Sarasota Bay and several other bottlenose dolphin populations in the southeastern United States. This analysis found a high likelihood that reproductive success, especially of primiparous females, is being severely impaired by chronic exposure to PCBs. Previous observational studies have documented disproportionately high mortality rates for first-born calves in Sarasota Bay (Wells 2003), consistent with the model of Schwacke et al. (2002) and the suggestion of Cockcroft et al. (1989). By integrating empirical data on life history parameters, reproductive success, and OC concentrations from the Sarasota Bay dolphin community we have been able to provide a test of aspects of the model as a step toward refinement.

METHODS

Study area and field sampling

Dolphins sampled for this project resided in and around Sarasota Bay, Florida (27^{o}N, 82^{o}W). Sarasota Bay and associated waters extend for about 30km along the central west coast of Florida, south of Tampa Bay. Sarasota and associated shallow bays (generally 1m to 4m deep)

are separated from the Gulf of Mexico by a series of narrow barrier islands, and they communicate with the Gulf through narrow passes up to 10m deep. The shallow, sheltered bay waters facilitated capturing small groups of selected dolphins with a 500m long x 4m deep seine net for sampling, measurements, and marking (Wells et al., in press). Efforts were made to obtain a representative cross-section of the local dolphin population, though dolphins less than two years old or more than 45 years old were avoided for animal safety reasons. Individual dolphins were placed in a sling, and lifted aboard a veterinary examination vessel. The animals were weighed, measured for lengths and girths and ultrasonically for blubber thickness, given a physical examination, and were evaluated ultrasonically for reproductive and organ condition. Microbiological samples were obtained from the blowhole, anus, feces, and urine. Milk samples were obtained as possible. Blood samples for health assessment were drawn from the fluke into evacuated tubes via butterfly catheter.

Sampling for contaminant residues involved specially prepared tools. Plasma and whole blood samples for contaminant residue analyses were transferred from the collection tubes via acid washed glass pipettes into Teflon vials, and then placed in liquid nitrogen until they could be transferred to a -80^{o}C freezer at the laboratory. Wedge-shaped, full-thickness blubber samples were obtained from a site below the caudal insertion of the dorsal fin. Prior to biopsy, blubber thickness was determined ultrasonically, the site was rinsed with water and lidocaine hydrochloride was administered for local anesthesia. Surgical instruments, foil, and sample receptacles that had been hexane and acid washed and autoclaved were used to obtain and handle blubber wedges of up to approximately 5cm x 2.5cm x 2cm deep. Blubber samples were cut into subsamples for various studies, then placed in Teflon vials and stored in liquid nitrogen until they could be transferred to a -80^{o}C freezer at the laboratory. Milk samples were obtained via a specially-designed suction device consisting of the barrel of a 12cc syringe attached via plastic tubing to a 60cc syringe. Samples were transferred into Teflon vials and stored in liquid nitrogen until they could be transferred to a -80^{o}C freezer at the laboratory. Blanks of lidocaine administration and blood and milk sampling gear were retained for evaluation of their possible contributions to measured residue concentrations.

Upon completion of sampling, dolphins were marked with freezebrands or tagged as appropriate (Scott et al., 1990b), photographed, involved in a variety of other physiological or acoustic experiments, and released. The total time from capture to release was typically about 1h to 3h, depending on the number of procedures performed and the number of animals captured concurrently. There were no adverse impacts on the animals from the handling and sampling; most of the dolphins sampled were experienced with the capture-release process.

Life history and reproductive success data

Information on the gender, age, maturity, and reproductive histories of the sampled dolphins was obtained through long-term observation and monitoring, and through hands-on examinations and sampling when necessary. Gender was confirmed through direct examination of the genital region of the dolphins during capture-release. The ages of most of the sampled dolphins were known from observations since birth to identifiable mothers. Ages of others were determined from examination of growth layer groups in a tooth extracted under local anesthesia (Hohn et al., 1989). Sexual maturity was evaluated through measurements of reproductive hormone concentrations, through ultrasonic examination of reproductive organs, and through observations of presumed first births (Wells, 2003). Births to well-known, identifiable females were documented through regular, systematic photographic identification surveys through the dolphins' home range, and relationships were confirmed through genetic analyses (Duffield and Wells, 1991, 2002). Efforts were made to capture, mark, sample, and release two and three year old calves if they lacked naturally-distinctive markings, in order to facilitate monitoring of calves of known maternal lineages and birth order. Birth order data were considered to be relative data within a maternal lineage rather than absolute values, because it was possible for calves to be born and lost before observers had opportunities to detect them, thereby potentially biasing parity values downward. Birth order was assigned only when evidence suggested that monitoring of the mother began prior to her first parturition. Survivorship and reproductive success data were obtained through photographic identification surveys that documented presence and absence of individuals from the resident population, and through recovery of carcasses by the Mote Marine Laboratory Stranding Investigations Program (Wells and Scott, 1990).

Contaminant residue sample analyses

The analyses that follow result from OC analyses of blubber samples collected during June 2000 and 2001 as part of a project to evaluate biomarkers of OCs and their effects (Reijnders et al., 1999), and of blood samples collected during 1988-1999. Blubber samples were analyzed at the University of Barcelona, and plasma samples were analyzed at the University of California, Santa Cruz and the University of Utah.

Organochlorine analyses of blubber

Samples weighing 0.2g to 1.0g were ground with anhydrous sodium sulphate and extracted with n-Hexane (residue-free quality) in a Soxhlet apparatus for 5h. The resulting solution was concentrated to 10ml. A portion of this extract (2ml) was used to determine the quantity of extractable fat per gram of blubber. A further quantity was mixed with sulphuric acid for the clean up, following the procedures described by Murphy (1972), and the resulting extract was concentrated to 1.0ml and centrifuged for 5min.

Chromatographic analysis was carried out on a Hewlett-Packard 5890-II gas chromatograph equipped with an electron capture detector (ECD) at 350ºC. A fused silica capillary column (length 60m, 0.25mm ID) coated with SPB-1 was used as the stationary phase (0.25µm film thickness). The splitless technique was used to inject 1σl of the purified extract. Pure Nitrogen gas at a flow rate of 1ml/min was used as a carrier. Temperature was programmed as follows: injection at 40ºC for 1min and increased to 170ºC at a rate of 25ºC/min; 1min constant, to 250ºC at a rate of 2ºC/min and then to 280ºC, at 5ºC/min. Heptachlor and congener 199 were used as internal standards.

Organochlorine analyses of blood

Plasma samples were thawed and gently vortexed. Aliquots of 3ml were placed into 10ml culture tubes and the weights were recorded. The samples were then spiked with PCB 103, PCB 207 [F1 surrogates], and pentachloro-nitrobenzene (PCNB) [F2 surrogate] to facilitate internal standard quantitation. To denature serum proteins, an equal volume of formic acid was added to each sample and the mixture was vortexed. Varian Mega Bond Elut⊇ 1g, 6cc C18 columns were prepared on a vacuum manifold by rinsing twice with 6ml of methanol, and twice with 6ml of Optima⊇ water. The rinses were eluted by applying vacuum at 3psi to 5psi, and the final

rinse was not completely eluted. The serum/formic acid mixtures were loaded onto the columns and eluted at a rate of 6ml/min to7ml/min. The columns were dried thoroughly with vacuum for ~20min and were placed under streams of purified nitrogen. The analytes were eluted from the columns with two 3ml portions of 1:1 hexane/diethyl ether. The extracts were dried with anhydrous $NaSO_4$, transferred into 2ml amber screw cap vials with at least six rinses of hexane, and concentrated to 500σl for Florisil™ fractionation.

Fractionation was performed on a 10mm ID x 40mm glass column with 27cm of Florisil (and 1cm of anhydrous $NaSO_4$ on top and bottom). The Florisil was rinsed with 100ml of hexane prior to loading the sample. When the hexane rinse reached the head of the column, the sample (1ml) was loaded with four (1ml) rinses of the vial. The first fraction [F1] (containing >90% p,p, DDE, and <10% p,p, DDT) was eluted with 60ml of hexane. The second fraction [F2] (containing >90% p,p, DDT, and >10% p,p, DDE) was eluted with 50ml of 30% methylene chloride in hexane. The third fraction [F3] (containing >70% HE and all of Dieldrin) was eluted with 70ml of 50% methylene chloride in hexane. All fractions were concentrated on a rotary evaporator and were transferred to 2ml amber screw cap vials and prepared for GC/ECD analysis. A dual-column gas chromatograph equipped with electron capture detectors was used to look for halogenated compounds. Standards were run through the same process for calibration.

QA/QC

Analytical methods for blubber were evaluated in multi-lab comparisons to standard reference materials provided by the U.S. National Institute of Standards and Technology. For blood analyses, a method blank was analyzed with every eight samples to monitor laboratory contamination. A Certified Reference Material sample (BCR-CRM 349 cod liver oil), and a sample duplicate were analyzed with every sixteen samples to ensure that method precision and accuracy were within the required EPA limits.

OC data presentation and statistical tests

Polychlorinated biphenyl data are presented as sums of analyzed congeners. For blubber samples, ∑PCBs was the sum of 22 congeners (IUPAC # 28, 52, 95, 101, 105, 118, 128, 138, 149, 151, 153,

156, 170, 174, 180, 183, 187, 194, 195, 201, 206, and 209). For blood samples, ∑PCBs was the sum of 58 congeners (IUPAC # 3/30, 5/8, 15, 17, 18, 27, 28, 29, 31, 44, 46, 49, 52, 60, 64, 66, 70, 74, 84, 85, 87, 89, 95, 97, 99, 101, 105, 110, 118, 128, 132, 137, 138, 141, 146, 149, 151, 153, 156, 157, 158, 167, 170, 174, 177, 178, 180, 183, 184, 187, 189, 190, 194, 196, 198, 200, 203, 206, 209). In order to control for variability in OC concentrations relative to lipid composition of blubber, contaminant concentrations are expressed as µg/g or parts per million (ppm) on a lipid weight basis (Hall et al., 2004). The blubber contaminant concentrations were log-normally distributed (Hall et al., 2004). Thus, descriptive blubber data are presented without transformation, but all statistical analyses were carried out on log.transformed data to normalize them for parametric tests. Because of variability introduced through consideration of lipid composition of blood, plasma OC concentrations are presented as wet weight, in ng/g or parts per billion (ppb). Statistical significance was evaluated at the $p \leq 0.05$ level.

RESULTS

Bottlenose dolphin capture and release operations conducted in Sarasota Bay during June 2000 and June 2001 resulted in the sampling of 47 dolphins, including 25 females and 22 males, ranging in age from two to 50 years (Table 1). This sample included 14 mother-offspring pairs (including dependent calves and independent offspring) sampled within the same year, with offspring ranging in age from two to 17 years. Data on presumed birth order were available for 16 dolphins, including four first-born offspring, ranging from in age from two to 13 years.

Relationships between sex, age, and blubber ∑PCBs are depicted in Figure 1. Males and females exhibited similar concentrations of about 15ppm to 50ppm until they reached sexual maturity, then diverged through the remainder of their lives. Concentrations in males increased with age from about 10 years of age to approximately 860ppm in the oldest male sampled, at 43 years of age, suggesting a pattern in which the rate of absorption exceeded excretion through life. Female concentrations of PCBs tended to decline to relatively low levels beyond sexual maturity, typically at five to 10 years of age (Wells and Scott, 1999), and remained low in females up to 50 years of age. Log transformation of concentration data clarified these divergent patterns relative

to gender (Figure 2). Mean concentration of PCBs for males $\geq$ 10 years of age were significantly different from those of females $\geq$ 10 years age (t-test, unequal variance, $p < 0.001$).

Concentrations of PCBs in blubber of females appeared to be related to reproductive condition, as indicated when blubber concentrations of PCBs were depicted relative to age and timing of parturition (Figure 3a). Similar patterns were reflected in plasma samples collected during summer months, when circulating levels of contaminants were likely to be highest due to thinning blubber (Figure 3b). Females in Sarasota Bay produced calves every three to six years on average, from about six to eight years of age through 48 years (Wells and Scott, 1999; Wells, 2003). The highest concentrations were found in females that had not yet given birth. Concentrations of PCBs in blubber declined with the birth of the first calf, and remained generally lower thereafter. Blubber OC concentrations increased with time since most recent parturition, but still remained below pre-parturient levels, even after ten years (Figure 4). Blubber PCB concentrations for nulliparous females (mean = 27.7ppm $\pm$ 10.67 s.d.) were significantly greater (t-test, unequal variances, $p < 0.001$) than those of parous females (mean = 6.8ppm $\pm$ 5.45 s.d.). Within the category of females that had given birth to more than one calf, females sampled within four years of giving birth, including the period of greatest lactation activity (mean = 3.2ppm $\pm$ 1.37 s.d.) had significantly lower concentrations of blubber PCBs (t-test, unequal variances, $p < 0.05$) than did multiparous females more than four years post-parturition (mean = 8.8ppm $\pm$ 4.34 s.d.). Declines in maternal load as lactation began, and increases as lactation declined suggested the occurrence of the process of excretion unique for the mature females.

The significant differences in PCB concentrations between nulliparous and parous females, with a precipitous decline for primiparous females, provided support for higher exposure to PCBs via nursing for first-born as compared to subsequent offspring. Further support for increased PCB exposure was derived from examination of the concentrations of blubber PCBs for calves of known birth order (Figure 5). PCB concentrations were grouped in age classes in order to detect potential age-related variation in sources of contaminants (*e.g.* proportion of milk *vs*. prey fish). In each case, PCB concentrations were higher in the blubber of earlier calves as compared to subsequent calves. Two year olds provided the best indication of maternal contributions to calf

body burdens, because they better reflected the recent results of nursing, whereas older calves were obtaining most of their PCBs from prey they captured, in combination with accumulated PCBs of maternal origin. First-born two year olds exhibited blubber concentrations of 28ppm to 46ppm, greater than the values for the third-born or fifth-born calves included in the age class. Even though concentrations in older calves were likely influenced by their increased nutritional independence from mother, the within-age class patterns still held for four year olds, suggesting a possible maternal legacy in body burdens.

The impacts of elevated concentrations of PCBs in first-born offspring were difficult to evaluate given the small sample size available for this category. Only one first-born calf was lost within three years of sampling, and its blubber PCB load was less than that of the surviving first-borns. Given that the concentrations for living first-born calves presented here were for dolphins at least two years old, and Cockcroft *et al.* (1989) suggested that most contaminant transfer occurred during the first few weeks of life, ours was likely a biased sample of calves that had already survived the period of highest exposure to these contaminants. Overall, at least 50% (n=22, 95% confidence interval = 0.29 – 0.71) of first-born calves in Sarasota Bay during 1982 through 2002 were lost during the first year of life. For comparison, less than 30% of calves born to multiparous mothers were lost during their first year of life in Sarasota Bay (Wells, 2000). PCB concentrations in blubber of primiparous females with offspring surviving the first year were lower than those in an unsuccessful primiparous female. Four successful females with calves of two to four years of age had an average blubber PCB concentration of 6.4ppm ($\pm$ 1.93 s.d.). In contrast, an unsuccessful primiparous female sampled nine months after the loss of its first calf, which had survived and presumably nursed for about 1.5mo, had a concentration of 23.1ppm. In all of these cases blubber concentrations were measured after the birth of the calf, and the successful mothers were sampled after much longer periods of excretion through lactation, so comparisons must be treated with caution.

The data presented above suggest mechanisms for OC accumulation and transfer, but they do not address the potential health effects of these contaminant concentrations on non-calves. Anecdotally, one of the four males lost during this time was the oldest male sampled, with a blubber PCB concentration of 868ppm at 43 years of age. Unfortunately, the carcass of this

animal was not able to be recovered for more detailed examination. A preliminary study by Lahvis et al. (1995) involving a small sample of male Sarasota Bay dolphins showed a decline in immune system function with increasing concentrations of OCs in blood.

DISCUSSION

The findings of this study support previously-described patterns of accumulation and depuration of OCs by small cetaceans (Aguilar, et al. 1999), and they add new details regarding the influence of life history parameters on these patterns for Sarasota Bay bottlenose dolphins. The concept of excretion via production of offspring and to a larger extent via lactation is supported by: 1) decline in female blubber PCB concentrations with age in contrast to the continuing increase in male PCB concentrations with age, 2) the onset of the female decline in PCB concentrations at about the time of first calving and lactation, 3) continuing low levels of PCBs through the 40^{+} year reproductive lifespan of the females, 4) increased concentrations in mothers with increasing time since parturition, and 5) sustained higher concentrations in first-born calves as compared to subsequent calves.

Changes in OC concentrations in mothers and milk during the period of rearing a given calf provided additional evidence of depuration. Mothers and calves typically remained in close association for three to six years, with calving intervals increasing with female age (Wells and Scott, 1999; Wells, 2000, 2003). Significantly lower PCB concentrations were noted for mothers during the first four years of this association as compared to subsequent years, indicating that loss of pollutants from mother can be larger when reproductive intervals are shorter. Nutritional dependence on the mother declined over this period as the calf learned to capture prey fish. Calves often began to catch small fish within the first few months of life, but nutritional independence was not achieved typically until the second year of life (Wells and Scott, 1999). In one case, a calf orphaned at 16mo of age survived without being adopted, presumably by capturing its own prey (Wells, 2003). However, lactation commonly occurred at some level through much of the period of association between mother and calf, suggesting that the calves continued to suckle beyond the time of nutritional need. In the sample considered here, milk samples were obtained from all but one of the mothers examined with calves of two to five years

of age. These samples remain to be analyzed for PCB concentrations, but Vedder (1996) measured PCB concentrations in earlier samples of milk from multiparous Sarasota Bay dolphins with calves at least two years old, and noted that concentrations increased with calf age for females sampled in multiple summers. She attributed this trend to the likely decrease in nursing frequency with increasing nutritional independence, and continued accumulation of contaminants by the mothers. Weaning is likely not abrupt in bottlenose dolphins. Continued nursing at reduced levels for several years may moderate mother's contaminant accumulation. Simultaneous pregnancy and lactation are unusual for Sarasota Bay bottlenose dolphins, but continuing reproduction may effectively limit the period during which contaminants are accumulated without lactational-based excretion, between calves.

Further support for our findings from live Sarasota Bay dolphins derives from the work of Küss (1998), who measured OCs in the blubber of 20 stranded resident Sarasota Bay dolphins ranging in age from three weeks to 50yr. Differences in PCB congeners examined, and a lack of inter-laboratory calibration preclude direct comparisons of concentrations across studies, but general patterns within her study were consistent with patterns from the current study. Immature males and females showed similar levels of OC concentrations. Males accumulated OCs with age, declines in OCs with age were noted for females, and increased levels in females later in life were correlated with increased calving intervals. Küss (1998) was able to sample calves at much younger ages than was possible with the live dolphins, and the only first-born sampled, a 5.3mo old calf of a nine year old female, had the highest PCB concentrations of any calf in her study.

Similar general relationships between life history parameters and OC concentrations have been reported for several odontocete cetaceans. Increases in OC concentrations up to sexual maturity for both sexes, with lower concentrations for females than males once they become reproductively active have been noted for stranded bottlenose dolphins (Kuehl and Haebler, 1995; Salata et al., 1995) and pilot whales (Globicephala melaena, Tilbury et al., 1999), free-ranging killer whales (Ylitalo et al., 2001), and white whales (Delphinapterus leucas, Krahn *et al*., 1999). In some cases, elevated OC levels were found in older females of species that exhibit marked post-reproductive senescence, such as killer whales and pilot whales (Tanabe et al., 1987; Tilbury et al. 1999; Ross et al. 2000), but this relationship was not supported by other

studies of pilot whales (Borrell et al. 1995). Bottlenose dolphins have not yet been demonstrated to exhibit reproductive senescence (Wells and Scott, 1999). Ylitalo et al. (2001) reported consistent findings relative to the influence of birth order on contaminant concentrations for male killer whales, with first-recruited individuals exhibiting higher concentrations of PCBs than subsequent whales.

In Sarasota Bay, female reproductive success improved with age, potentially reflecting depuration and correlating with changes in mother's PCB load. Only 50% of first-born calves survived through their first year, whereas more than 70% of calves born to multiparous mothers survived (Wells, 2000). However, the measured 50% loss of first-born calves in Sarasota Bay likely included losses from other causes in addition to PCBs and associated effects, such as predation and human interactions. The relative contributions of other factors such as other environmental contaminants or physiological or behavioral development of the mother must be considered as well when evaluating calf losses. PCBs are often found to correlate with other persistent and widespread contaminants, including pesticides such as DDT or chlordane, which may also affect reproductive success. First-time mothers in Sarasota Bay were significantly smaller (t-test, $p < 0.002$) than more experienced mothers in average length (235cm *vs*. 251cm) and average mass (167kg *vs*. 195kg), raising the possibility that they were not fully mature and may have been less capable, physiologically, to successfully rear offspring. Owen (2001) observed that more experienced mothers were significantly better, behaviorally, than first-time mothers in controlling the calf's environment, and therefore could provide better protection to the calf.

Reddy et al. (2001) found higher pre-parturient OC levels in mothers whose calves were lost as compared to mothers who had surviving calves. In their sample of 14 captive mothers, half were primiparous, and 86% of these had a stillborn calf or lost the calf within 12 days of birth. This level of loss is higher than that recorded from Sarasota Bay, in spite of the fact that numerous threats to wild animals (*e.g*., predation, entanglement in fishing gear, boat strikes, etc.) were eliminated in the captive environment, suggesting a greater risk from OC exposure.

Schwacke et al. (2002) integrated measured blubber concentrations of PCBs with a surrogate dose-response relationship to develop predictions of reproductive health risks. The authors modeled the excess risk for reproductive failure, including stillbirth and calf mortality, due to chronic PCB exposure for Sarasota Bay bottlenose dolphins. Overall, they calculated an expected excess risk of reproductive failure of 0.79 for primiparous females (95% CI = 0.44 – 0.89) and 0.03 for multiparous females (95% CI = 0.01 – 0.48). The calculated risk was in excess of background incidence, indicating that the expected overall reproductive failure, accounting for all other factors, would be even higher. Their predicted risk is difficult to compare with the observed rates of calf loss because: 1) background mortality (unrelated to PCB exposure) cannot be directly estimated from the current data, 2) not all endpoints of reproductive failure (*e.g.* stillbirth or abortion) included in the risk calculations would necessarily have been observed and 3) confidence intervals for both the estimated risk and the observed calf mortality rates were relatively wide. The confidence limits for the calculated reproductive risk determined by Schwacke et al. (2002) were wide primarily due to the uncertainty introduced by extrapolating from measured blubber concentrations to the actual dose at a target tissue. Similarly, the confidence intervals for the observed first-born mortality rate of 50% (0.29-0.71) were relatively wide due to the limited sample size of calves (n=22). Further monitoring of the Sarasota dolphin population and additional analyses, integrating empirical observations into the estimation of dose-response relationships, will contribute to the refinement of the reproductive risk model.

Based on theoretical assessments of risk for Sarasota Bay and other dolphins, Schwacke et al. (2002) suggested that the increased risk of reproductive failure for primiparous females would effectively increase the age at first birth, which in turn would likely impact the future growth potential or stability of the population. In light of this potentially large cumulative impact on dolphin populations in addition to natural sources of mortality and direct losses in fisheries, the authors encouraged consideration of indirect anthropogenic stressors such as contaminant pollution in management schemes such as the U.S. Marine Mammal Protection Act. Though empirical data suggest that risks for Sarasota Bay dolphins may be towards the lower end of the theorized risk intervals, the weight of evidence suggests that environmental contaminants still pose an important threat to the population, and such a precautionary approach would be

warranted. Of particular concern in this regard is the increase in female OC blubber concentrations between calves, suggesting that these restricted compounds remain available in the environment, and may continue to impact populations for years to come (see also Reijnders and Aguilar, 2002).

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The long-term data and sample collection represented in this study reflects the work of many dedicated Sarasota Dolphin Research Program staff members and volunteers. We would like to especially thank Larry Fulford, Jocelyn Greene, Kim Hull, Blair Irvine, Stephanie Nowacek, Michael Scott, Forrest Townsend, and Kim Urian for their efforts in the field and laboratory. Ailsa Hall, Todd O'Hara, and Lori Schwacke provided important input on the analyses and manuscript. We are grateful to Peter Reijnders and Greg Donovan for their efforts to develop and sustain the International Whaling Commission's Pollution 2000+ Programme, which provided some of the basic support for this research. Additional crucial support for sample collection and analyses was provided by the NOAA Marine Mammal Health and Stranding Response Program, the National Marine Fisheries Service, and Dolphin Quest. Partial funding for the blubber organochlorine analyses was received from the Fundació pel Desenvolupament Sostenible (FDS). While doing this work, A. Borrell was supported by a contract under the "Ramón y Cajal" Program. Earthwatch Institute, the Chicago Zoological Society, and Mote Marine Laboratory provided support for the long-term monitoring of the Sarasota Bay dolphins. Research was conducted under National Marine Fisheries Service Scientific Research Permit No. 522-1569 issued to R.S. Wells.

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Table 1. Summary of Sarasota Bay bottlenose dolphins sampled during 2000-2001.

Age Class	**Females**	**Males**
0 - 10	11	10
11 - 20	5	5
21 - 30	1	2
31 - 40	4	3
41 - 50	4	2
Total	**25**	**22**

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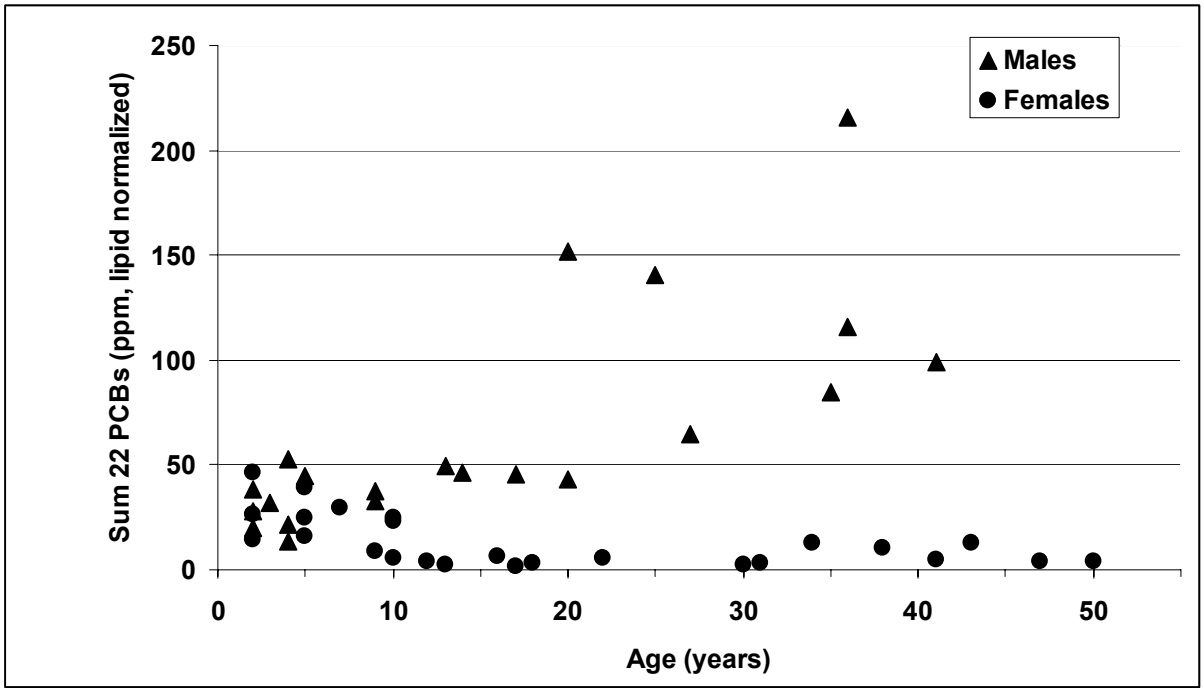

Figure 1. Sum of blubber PCBs relative to dolphin sex and age, showing general patterns of accumulation and depuration. Data for 43-yr-old male (not shown) exceeds scale (868 ppm).

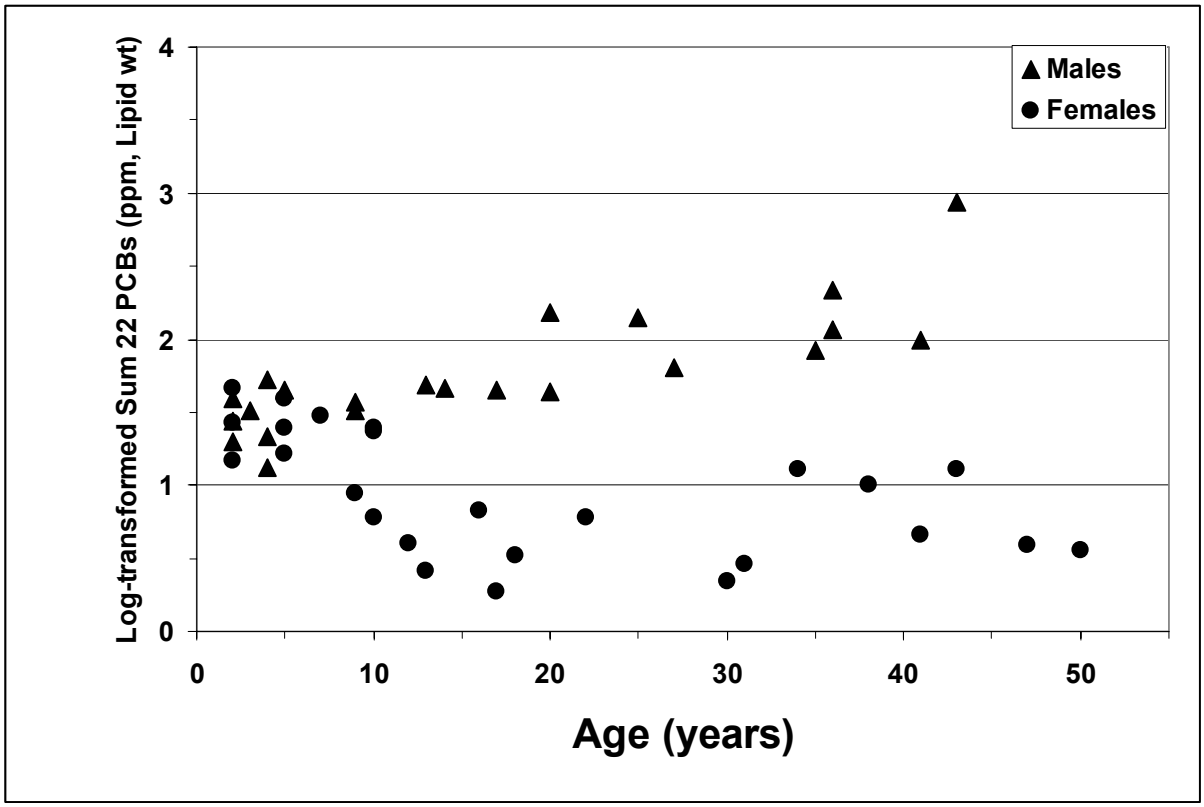

Figure 2. Log-transformed sum of blubber PCBs relative to dolphin sex and age, showing general patterns of accumulation.

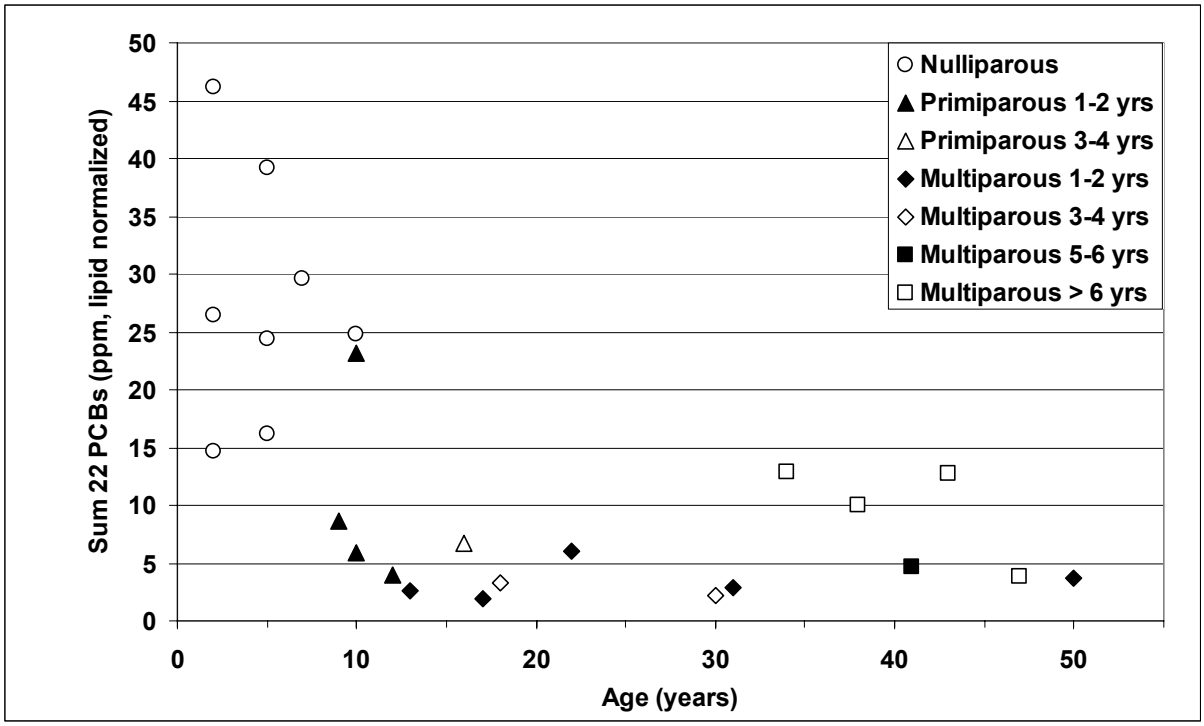

Figure 3a. Sum of 22 PCBs in blubber of female dolphins relative to age and timing of parturition. Nulliparous females had not yet been documented has having given birth. Primiparous females were believed to be accompanied by their first calf; multiparous females had been observed with at least one previous calf. Years refer to the time since the most recent known parturition.

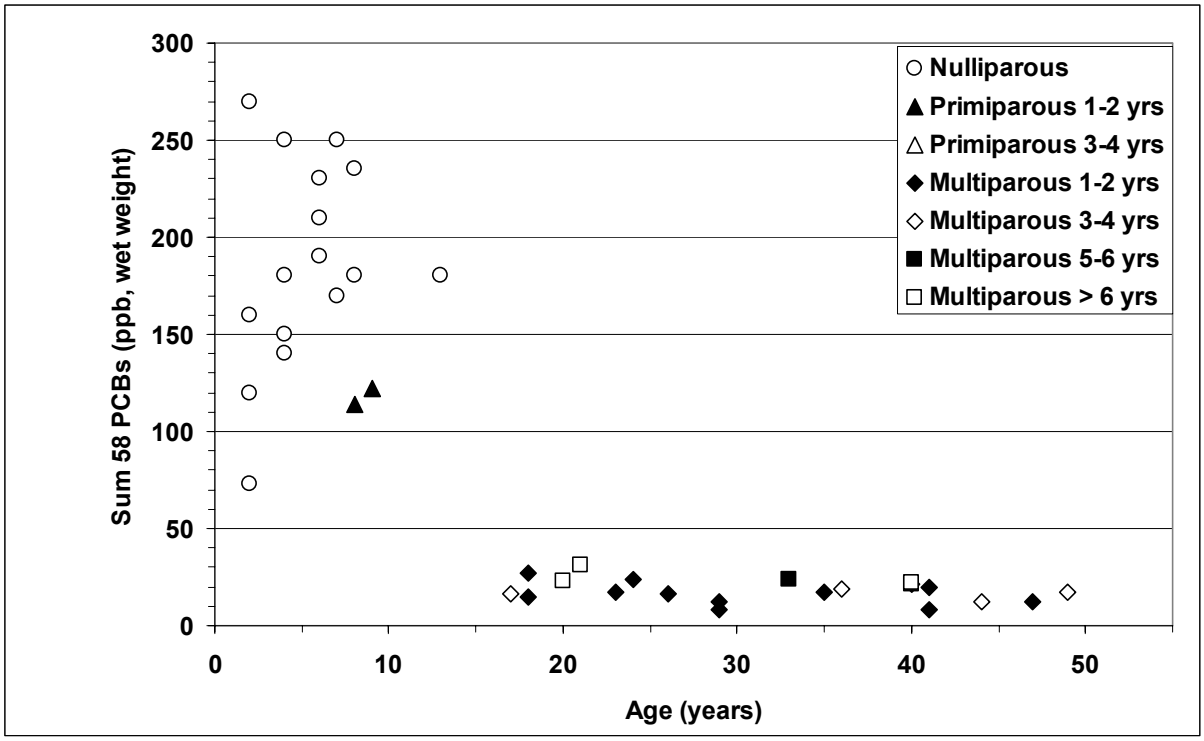

Figure 3b. Sum of 58 PCBs in plasma of female dolphins relative to age and timing of parturition.

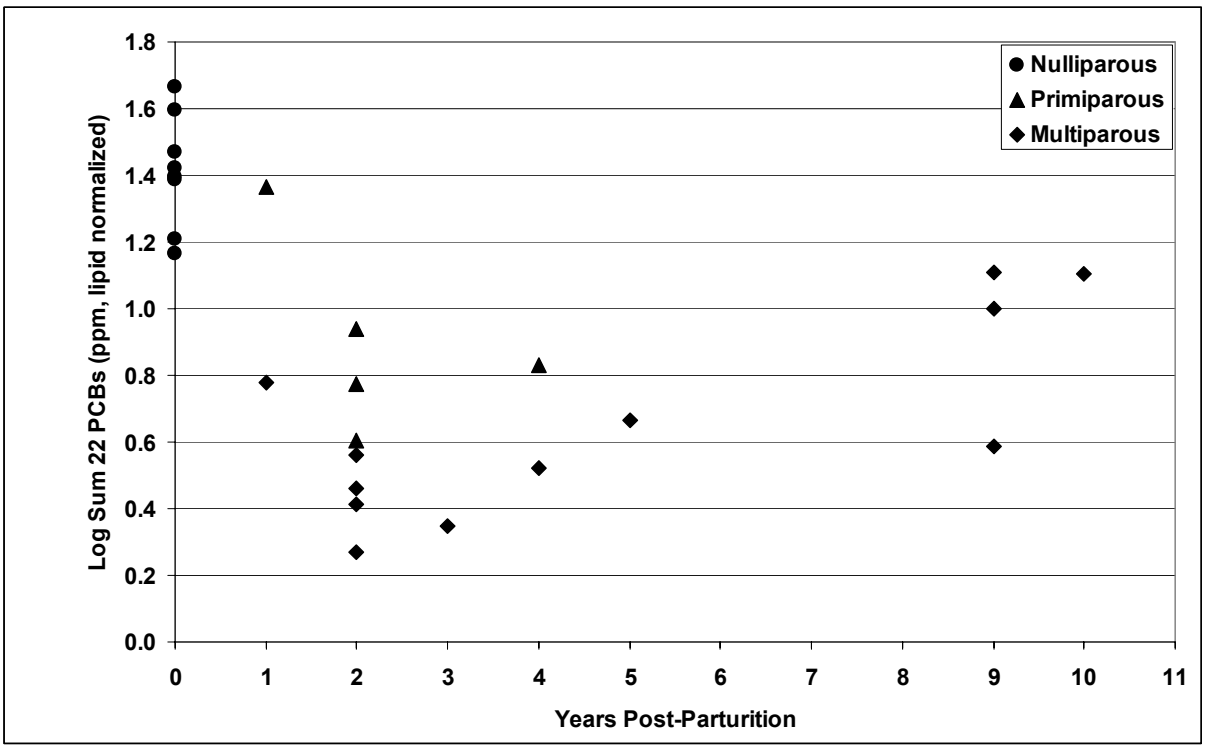

Figure 4. Sum of 22 PCBs in blubber of female dolphins relative to timing of parturition. Years refer to the time since the most recent known parturition.

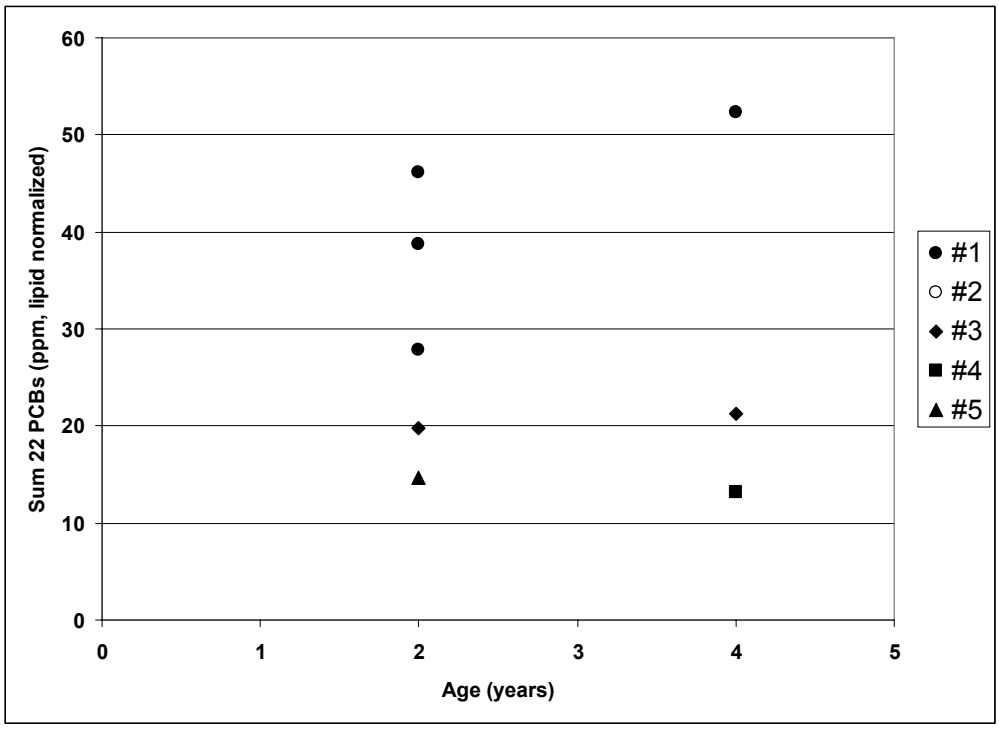

Figure 5. Comparison of sum 22 PCBs in blubber by birth order. Birth order is depicted by different symbols. Age refers to age of calf at time of sampling.

INFLUENCE OF ORGANOCHLORINES AND NATURAL FACTORS ON BLUBBER RETINOIDS IN COMMON DOLPHINS (*Delphinus delphis*)

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"Capsule": The use of blubber retinoids as biomarkers of organochlorine compounds requires knowledge on the biological traits of individuals.

ABSTRACT

The effect of age, sex, nutritive condition (as determined through the lipid content of the blubber) and organochlorine concentration on blubber retinoid concentrations was examined in 74 common dolphins incidentally caught off northwestern Spain. Age and blubber lipid content were strong determinants of the retinoid concentrations in males, while these variables did not account for the variation found in females. Some of these associations may be also reflecting dissimilarities in influential non-controlled factors, such as diet, behaviour or ecology of the various segments of the population. Retinoids were positively correlated with organochlorines in males and negatively in females. However, pollution levels were moderate and likely to be below threshold levels above that a toxicological response is to be expected. Thus, a cause-effect relationship between organochlorine and retinoid concentrations could not be properly established and the observed correlation may be the result of an independent association of the two variables with age. Further research on the influence of the best predictor variables on retinoid dynamics is required to implement the use of retinoids as biomarkers of pollutant exposure in cetaceans.

Keywords: retinoids; common dolphin; organochlorine; blubber; biomarker

INTRODUCTION

Organochlorine compounds (OCs) such as DDTs and PCBs biomagnify through the food web due to their persistence and lipophilicity (Tanabe, 2002). Small odontocetes are particularly vulnerable to these contaminants as they are top predators of marine ecosystems, their food intake rate is high, their capacity to decompose some OC molecules is low and their adipose tissue, rich in lipids, accumulate efficiently lipophilic pollutants (Tanabe et al., 1988; Aguilar et al., 1999). In fact, odontocete cetaceans are the vertebrates in which the highest OC concentrations have been so far reported (O´Shea and Aguilar, 2001). Despite these alarming levels, the actual impact of OCs in cetaceans has not been determined although exposure to these compounds has been associated with adverse effects on reproduction and immune function (Martineau et al., 1987; Addison et al., 1989; Béland et al., 1993; De Swart et al., 1995; Lahvis et al., 1995; Schwacke et al., 2002). Biomarkers, defined as contaminant-induced variations in the cellular or biochemical components of a process, structure or function that can be evaluated in a biological system (NRC, 1989), may provide an insight into the actual effect of contaminants on the health of cetaceans. However, information available on biomarkers in this group of vertebrates is extremely limited.

A number of studies have demonstrated that environmental chemicals, particularly PCBs, DDTs and dioxins, interact with retinoid metabolism pathways and affect tissue concentrations in mammals (Brunström et al., 1991, Håkansson et al., 1992; Zile, 1992; Chu et al., 1995, 1998; Murk et al., 1998; Käkelä et al., 1999; Nilsson et al., 2000; Rolland, 2000; Simpson et al., 2000). Considering this interaction, retinoids have been proposed as potentially useful biomarkers of exposure to these pollutants (Peakall, 1992; Jenssen et al., 1995; Murk et al., 1998; Simms and Ross, 2000; Borrell et al., 2002). The term “retinoids” includes the natural forms with vitamin A activity as well as the synthetic analogues of retinol, with or without biological activity (Blomhoff et al., 1992). Retinoids are essential for normal vision, growth, reproduction, immune function, and cellular division and differentiation in mammals (Blomhoff, 1994; Sporn et al., 1994; Ross et al., 2000). Imbalance in retinoid concentrations may lead to severe dysfunctions, such as reproductive impairment, embryonic mortality, growth retardation, and increased vulnerability to infections (Thompson, 1976; Peakall, 1992;

Leonards et al., 1996). Consequently, effects of the anthropogenic pollutants on retinoid levels may be contributory to their toxicity.

In marine mammals, studies undertaken in captive harbour seals (*Phoca vitulina*) (Brouwer et al., 1989a, De Swart et al., 1994), and in wild populations of northern elephant seals (*Mirounga angustirostris*) (Beckmen et al., 1997), grey seal pups (*Halichoerus grypus*) (Jenssen et al., 1995, 2003) and polar bears (*Ursus maritimus*) (Skaare et al., 2001) have shown decreased plasma retinoids when organochlorine loads increased. In contrast, Simms et al. (2000) and Nyman et al. (2003) reported a positive correlation between plasma vitamin A and organic contaminants in non-nursing pups of harbor seals and grey seals, respectively. Since plasma retinoid levels are controlled by homeostasis (Wolf, 1984) they may not be reflective of actual retinoid body status; rather, retinoid concentration in depot tissues is likely to better gauge body retinoid disruption. The lipid-rich hypodermic fat, commonly known as blubber, is a choice tissue for monitoring retinoids in marine mammals; it stores high retinoid loads (Borrell et al., 1999; 2002; Mos and Ross, 2002; Nyman et al., 2003; Tornero et al., 2004), represents a substantial proportion of total body mass (Schweigert et al., 1987; Aguilar et al., 1999) and can be easily sampled from live animals using non-destructive biopsy techniques (Aguilar and Borrell, 1994a).

Biological traits (e.g. age, sex, diet, incidence of disease, reproductive status, occurrence of lactation, and lipid composition) are known to have a significant influence on tissue concentrations of both OC pollutants and retinoids (Aguilar et al., 1999; Borrell et al, 2002). Validation of the use of retinoids as biomarkers of OC exposure requires baseline knowledge on the intrapopulation variability an interaction of both groups of compounds in natural conditions.

The common dolphin (*Delphinus delphis*) is a frequent small cetacean in the temperate waters of the eastern north Atlantic. Here we examine the effect of age, sex, nutritive condition (as determined through the lipid content of the blubber) and organochlorine concentration on blubber retinoid concentrations in this species with the ultimate aim of assessing the applicability of retinoids as a biomarker of OC pollutant exposure.

MATERIAL AND METHODS

Sample collection

We examined and sampled 74 fresh carcasses of common dolphins (48 males and 26 females) that were incidentally caught in pair-trawling fishing operations off northwestern Spain in 2001, 2002 and 2003. We measured and sexed the dolphins, and collected teeth from the lower jaw for age determination and a blubber sample from the region behind the dorsal fin for OCs, lipid and retinoid analysis. Blubber samples included all tissue layers to avoid stratification effects (Aguilar and Borrell, 1990, Krahn et al. 2003). Necropsies were in all cases carried out onboard the boats before 12h post-mortem. Samples were transported to the laboratory on dry ice and stored at –20º C in the dark until analysis.

Age determination

The teeth were cleaned (but not boiled) and fixed in 10% neutral buffered formalin. In the laboratory they were decalcified in Rapid Bone Decalcifier for the Preparation of Histological Materials (RDO), a commercially prepared mixture of acids, for 2-8 hours depending on tooth volume. Teeth were then longitudinally sectioned in a freezing microtome, stained with hematoxylin, blued in weak ammonia solution, and mounted onto gelatin-coated slides. Age was determined by counting growth layer groups (GLG) in dentine, assuming that each GLG corresponded to 1 year. More detail on the techniques used can be found in Lockyer (1995a, 1995b).

Blubber OC Concentrations

Samples weighing about 0.2-1 g were ground with anhydrous sodium sulphate and extracted with n-Hexane (residue-free quality) in a Soxhlet apparatus for 5 h. The solution obtained was concentrated to 10 ml. A portion of this extract (2 ml) was used to determine the quantity of extractable fat per gram of blubber (blubber lipid content, BLC). A further quantity was mixed with sulphuric acid for the clean up, following the procedures described by Murphy (1972), and the resulting extract was concentrated to 0.1-1 ml and centrifuged for five minutes.

The samples were analysed for the following compounds: p,p´-DDT, o,p-DDT, p,p´- DDE, o,p-DDE, p,p´-DDD and o,p-DDD. Total DDT (tDDT) was calculated as the sum of the six DDT compounds analysed. Total PCB (tPCB) was the sum of 19 individual congeners (IUPAC # 28, 52, 95, 101, 151, 149, 118, 153, 105, 138, 187, 183, 128, 174, 180, 170, 201, 195, 194).

Four standard solutions of different concentrations were prepared by weighing different amounts of reference materials (Laboratory Dr. Ehrenstorfer-Schäfers, Germany).

Chromatographic analysis was performed using a Thermo-Finnigan TRACE DSQ GC/MS instrument, equipped with an AS 3000 autosampler. A fused silica capillary column HP-5 (Crosslinked 5% Phe Me Silicone, Hewlett-Packard) with a length of 25m, 0.25 mm ID and a film thickness of 0.5σm was used as the stationary phase. Helium at a flow rate of 1 ml/min was used as a carrier gas. Temperature was programmed as follows: Initial oven temperature of 90ºC held for 2min, ramped at 20ºC/min to 180ºC; one minute constant, ramped at 2.5ºC/min to 300ºC and held for 5 min. Injector and transfer line temperatures were set at 250ºC and 290ºC, respectively. Standards and samples were injected (2σl) in splitless mode (purge off time: 1min).

Mass spectra were acquired in the electronic ionisation mode (70eV) with an ion source temperature of 250ºC. Analysis was carried out in selected ion monitoring mode (SIM) at m/z values of 256 and 258, 290 and 292, 324 and 326, 360 and 362, 394 and 396, 428 and 430 for tri-, tetra-, penta-, hexa-, hepta- and octachlorobiphenyls, respectively, and values of 235 and 237 for DDD and DDT and 246 and 318 for DDE. Heptachlor was used as the internal standard by monitoring at m/z values of 272 and 274.

Individual organochlorines were quantified from a four-point calibration curve generated from the standard solutions.

Blubber Retinoid Concentrations

The methods for retinoid analysis are described in detail by Borrell et al. (1999). Handling of tissues was always made at room temperature and under red light. The

samples, weighing about 100 mg each, were saponified overnight in an ethanolic KOH solution (1 g KOH, 2 ml distilled H_20, 2 ml ethanol, 20 mg ascorbic acid) in a mechanical shaker under a nitrogen atmosphere. Retinoids (retinol plus retynil esters) were extracted by adding 8 ml diisopropyl ether and shaking for 30 min. After separation from the aqueous phase, an internal standard (retinyl acetate) was added and the organic extract was cleaned three times with 4 ml of aqueous phosphates buffer (pH 7.4). The extract was dried under nitrogen and reconstituted with 1 ml methanol and 0.05% butylated hydroxy toluene (BHT) as antioxidant. Reconstituted samples were filtered (0,20 σm mesh) and a 20 σl subsample automatically injected (Waters 700 Satellite wisp) on a HPLC (Waters 600 E System Controller Pump) equipped with a Restek column (Tracer Excel 120 ODS-A, 10 cm length, 5 σm beds, 0.46 cm internal diameter) and a UV detector (Waters 486 Tuneable absorbance D) set at 326 nm. The retinoid was eluted at a flow rate of 1 ml/min using a mobile phase of methanol/water (80/20 by volume) for 1 min, followed by a linear gradient of 3 min to methanol 100% for 14 min.

Statistics

For statistical analyses, retinoid and OC concentrations were transformed to natural logarithm scale to reduce the variability between the concentrations of the different individuals and to normalise the data.

We first examined the relationships between retinoid concentrations and the biological variables (sex, age and BLC) by multiple regression analysis. Given that sex-related differences were found age trends of retinoids, subsequent analyses were performed separately for males and females. The effects of age and BLC on OC concentrations were investigated by linear regression.

We fitted multiple regression models with reduced combinations of variables and adequate structure to investigate the effects of OCs on retinoids. We used these models to assess the explanatory power of these variables and the uncertainty in their power to explain retinoid variability. For this purpose, the following statistics were computed: AICc, the small sample Akaike´s Information Criterion (Burnham and Anderson, 2002) and wi, the AICc weight. From the initial set of M competing models, the AICc weight

for model Mi was computed. Models with AICc weight above 0.1 provided the best fit in terms of a good compromise between model parsimony and a good description of the data. Models with weights below 0.1 had very low or no empirical support, and were not considered good for prediction.

RESULTS

Given the observed patterns in OC concentrations, and because no sex-related differences in blubber concentrations were detected for dolphins 7 years of age or less (p}0.05), we combined samples from both males and females of these ages in an unique group of immature individuals. Table 1 summarizes the results obtained on blubber lipid content (BLC) and retinoid and OC concentrations. Statistics relative to the multiple regression of $\log_e$(retinoids) and several variables (sex, age, BLC and their interactions) are detailed in Table 2.

Age trends of BLC and retinoid varied with sex. In males, BLC showed a significant decrease and retinoid concentrations a significant increase, while these variables did not show any significant trend in females. Moreover, the effect of the interaction between age and BLC on retinoids was significant in males: old males had a negative relationship between retinoids and BLC, but this relationship was not apparent in the younger animals (Figure 1). The relationship between retinoids and BLC in females was not significant and was independent of age.

In males, both loge(tDDT) and loge(tPCB) were negatively correlated with lipid content (p{0.01 and p=0.05, respectively) and positively correlated with age (p{0.01). In contrast, both loge(tDDT concentration) and loge(tPCB concentration) were negatively correlated with age (p{0.01) and were not correlated with BLC (p}0.05) in females.

Table 3 shows the results of the various multiple regression models used to assess the effect of age, BLC, tPCB and tDDT on retinoid concentrations in females (3a) and males (3b). We found than the best model describing blubber retinoid concentrations in males was that with $\log_e$(tDDT) as single linear predictor. The AIC weights indicated that other models including the interaction of BLC and $\log_e$(tDDT)

and the interaction between $\log_e$(tDDT) and $\log_e$(tPCB) provided comparatively similar good fits. This indicates that retinoid concentrations were strongly related with these parameters. Figure 2 shows the relationship between $\log_e$(tPCB) and $\log_e$(tDDT) and $\log_e$(retinoid) in males.

In females, the best models included $\log_e$(tPCB) as single linear predictor, the interaction of age and $\log_e$(tPCB), the interaction of lipids and age on $\log_e$(tPCB) and $\log_e$(tDDT) as single linear predictor. Figure 3 shows the relationship between $\log_e$(tPCB) and $\log_e$(tDDT) and $\log_e$(retinoid) in females.

DISCUSSION

Despite the advantages of using blubber as choice tissue for monitoring retinoid status in cetaceans, there are very few published data on retinoid concentrations in this tissue in dolphins. Overall concentrations in the population here studied averaged 46.32 $\sigma g \cdot g^{-1}$ in males and 38.81 $\sigma g \cdot g^{-1}$ in females. These values are similar to those reported for harbour porpoise (*Phocoena phocoena*) from Greenland (Borrell et al., 1999) and much higher that those found in bottlenose dolphins (*Tursiops truncatus*) from the west coast of Florida (Tornero et al., in press).

We found in males a positive relationship between retinoid levels and age, being age the most important predictor of retinoid variability in this gender. In females, conversely, age did not account for the variation in tissue retinoid concentrations, which indeed remained rather constant throughout lifespan. In mammals, retinoid concentrations frequently increase along lifespan as a consequence of two factors: an age-related decrease in the circulatory clearance of these compounds, and their progressive accumulation due to a dietary intake higher than the consumption rate (Maiani et al., 1989; Krasinski et al., 1990). Increased tissue levels of retinoids with age have also been associated with a regulatory process of adaptation, possibly as a counter-regulation against oxidative tissue damage (van der Loo et al., 2004). However, despite these general patterns, a decrease in concentrations and no age-related differences have also been observed in some cases (Borrell et al., 2002) possibly due to disparity in overall nutritional status, diet, health, stress, reproductive history, feeding habitats or environmental conditions of the studied populations. Surveys conducted on marine

mammals also show this lack of uniformity. Thus, absence of age trends in blubber retinoid concentrations was observed by Tornero et al. (in press) in bottlenose dolphins from Florida and by Nyman et al. (2003) in ringed seals (*Phoca hispida*) and grey seals from the Baltic. An age-related decrease was found by Käkelä et al. (1997) in ringed seals from Spitsbergen, and an increase was observed by Käkelä et al. (1997) in ringed seals from both the Baltic Sea and the Saimaa Lake, by Schweigert et al. (1987) in grey seals from Sable Island and by Borrell et al. (1999) in harbour porpoises from Greenland. Collet (1981) did not find qualitative differences in the stomach content analysis of North Atlantic common dolphins of different sex or age, although the scope of prey consumed was large and the sample size probably too limited to detect such differences. Indeed, it is likely that some age- and sex-related dissimilarities in diet composition do occur in the common dolphin, as has been reported to be the case in other delphinid species of similar morphology and ecology (*e.g.* Bernard and Hohn, 1989). These dissimilarities, if actually occurring, would be likely to affect retinoids intake.

Besides this, being retinoids lipohilic substances, a significant portion of retinoid loads may be transferred by females to offspring during reproduction. The placenta restricts the intrauterine transport of retinoids during fetal development, but lactational transfer appears to be significant and of critical importance to offspring (Debier et al., 2002). While no information is available in this respect on cetaceans, studies on pinnipeds have shown that substantial mobilization of fat-soluble vitamins occurs when lipids are mobilized from the blubber during lactation (Schweigert et al., 1987, 2002; Schweigert and Stobo, 1994; Debier et al., 2002). In North Atlantic common dolphins, the peak of reproduction appears to take place in May-June (Collet, 1981). As most samples for this study were collected during the period July-September, retinoid and OC concentrations are likely to be affected by lactation and associated lipid mobilization. Unfortunately, reproductive status could not be determined in the individuals sampled because of logistical limitations onboard the fishing boats. Therefore, its influence on the variables analyzed cannot be properly evaluated.

Common dolphins in this study were in apparent good health and nutritive condition. Overall BLC (61.02%) was slightly higher than that reported by Borrell et al. (2001) in blubber biopsies of common dolphins of the same area, but of the same order of magnitude of that observed in common dolphins entangled in driftnets in the

Mediterranean (Borrell et al., 2001). The sampling method may originate these differences as it has been shown that lipid content of blubber sampled by biopsy darts is usually slightly lower than that obtained from blubber wedges (Krahn et al., in press).

Age- and sex-related dissimilarities in nutritive condition have been reported in some cetaceans and associated with variations in diet or reproductive status (Aguilar et al., 1999). While no age-trend was observed in females, we found that old males had lower BLC than the other population components, a difference that has also been reported in bottlenose dolphins by Hansen et al. (2004) and Tornero et al. (in press). As mentioned above, sex- and age-related variation in diet composition may account for this difference. Moreover, the foraging behaviour, as well as the daily amount of food ingested and its absorption rate is also expectable to vary with growth and the engagement of reproductive activities (Aguilar et al., 1999). For example, adult males of many ungulates and certain cetaceans, such as sperm whales (*Physeter macrocephalus*), are known to segregate from juveniles and adult females, at least partially to leave optimal foraging habitats to the reproductive segment of the population (Clutton-Brock et al., 1982; Rice, 1989; Main and Coblentz, 1990). A behaviour of this type may at least partially explain the low nutritive condition here observed in older common dolphin males.

Given the lipophilicity of retinoids, BLC was found to be a strong determinant of overall retinoid concentration (Borrell et al., 2002; Tornero et al., 2004). Thus, retinoids were negatively correlated with BLC in old males, probably as a result of the also negative correlation that BLC followed with age in this gender. Results of previous investigations are not consistent. While Nyman et al (2003) also found a negative relationship between retinoid concentrations and blubber lipid content in grey seals, Borrell et al. (1999) and Mos and Ross (2002) did not find any relationship between these variables in harbour porpoises and harbour seal pups, respectively. In contrast, Tornero et al. (in press) reported a positive relationship in male bottlenose dolphins. Migration, reduced food intake caused by disease or other condition, and other potential events can cause the mobilization of lipids and, presumably, that of the blubber-associated retinoid stores. In these situations, retinoids may be either redistributed or excreted, and significant variation in tissue concentrations is to be expected. In females, the relationship between retinoids and BLC was non-significant and independent of the

age, similarly to results reported by Tornero et al. (in press) in bottlenose dolphins. Again, differences in diet and food intake, as well as in reproductive condition may explain the dissimilarities observed between males and females.

Matching the usual pattern of OC accumulation observed in most cetaceans (Aguilar et al., 1999), concentrations found in juveniles of both sexes were similar, while in adult males they were significantly higher than in adult females. In immature individuals, OC levels increase with age as intake exceeds degradation or excretion rates. This accumulation process continues in males through lifespan, but in females the pattern shifts after the onset reproductive activities at an age of 7-9 years (Collet, 1981). Thus, in adult females concentrations tend to stabilize or decrease as a consequence of the transfer of lipophilic contaminants to offspring during gestation and lactation. As compared to a study carried out in the same population in 1984 and 1996 (Borrell et al., 2001), overall OC concentrations here observed in 2001-2003 were about twofold lower. This substantiates the decreasing trend of OC pollution that has been reported to occur in the temperate regions of the northern hemisphere during the last two decades (Aguilar et al., 2002).

OC pollutants are also lipophilic, so changes in BLC are expected to affect their tissue concentrations. In general, lipid mobilization results in an increase in OC concentrations (Aguilar et al., 1999). If data are expressed on a lipid weight basis instead of on a fresh weight basis this effect can be somewhat compensated, but not totally removed. Thus, we found that OC concentrations expressed on a lipid weight basis were negatively correlated with BLC in males. This negative relationship had also been reported for male striped dolphins (*Stenella coeruleoalba*) (Aguilar and Borrell, 1994b), male ringed and grey seals (Nyman et al., 2003) and male bottlenose dolphins (Tornero et al., in press) and it is commonly attributed to the fact that lipids are more readily mobilized from blubber than OCs, so the reduction in lipid is not coupled with a parallel decrease in pollutants loads (Aguilar et al., 1999). However, because BLC negatively correlates with age, and OCs do it positively, the BLC-OC correlation may simply reflect the dependence of both variables to age. In females, conversely, a BLC-OC correlation was neither observed in our survey nor in the above mentioned studies; here, BLC of females remained constant over age, but OC levels decreased progressively and manifestly from the first calf as a result of reproductive transfer.

Regarding the influence of OC pollutants on retinoid concentrations, a central issue to validate the use of retinoids as biomarkers of exposure to these pollutants, we observed different patterns in males and females. In males, retinoids showed a positive correlation with OCs and, indeed, OCs had a much greater statistical contribution to retinoid variation than age. The only comparable study so far undertaken in cetaceans, a survey on bottlenose dolphins from Sarasota, Florida, reported the opposite result, *i.e.* a significant decrease in blubber retinoid levels with OCs in males. The OC concentrations determined in this latter population (over 40 σg/g of DDT and 50σg/g of PCB) (Tornero et al., in press) were between 2 and 8 times higher than those here determined.

Although sensibility to retinoid interference by OCs may be species-specific (Fletcher et al., 2001), it is also possible that the OC concentrations reached by the male common dolphins here studied do not reach the threshold over which a physiological response is elicited. If this is so, the observed increase in the concentrations of both OCs and retinoids may simply reflect positive and strong dependence of both variables to age.

However, recent studies on endocrine disrupters have shown effects at both low and high concentrations. This might be associated with hormetic effects, where responses are stimulated at low levels due to an overcompensation following disruptions in homeostasis (Calabrese and Baldwin, 2003). Thus, the increase of retinoids in blubber may result from the mobilisation of liver retinoid stores after exposure to contaminants, as seen to occur in some terrestrial mammals (Brouwer et al., 1989b; Kelley et al., 1998; Nilsson et al., 2000; Fletcher et al., 2001). Nevertheless, this latter hypothesis appears to be contradicted by results by Nyman et al. (2003), who found a significant decrease in retynil palmitate levels in blubber of grey seals exposed to OC concentrations similar to our study.

As in males, we found in females that retinoid concentrations were also correlated with those of OCs, although in this gender the trend for both variables was a decrease with age. Again, the significance of this finding is difficult to interpret but it may also be the result of the independent relationship –though this time of negative sign- of both variables with age. Moreover, as mentioned above, sampling was

undertaken at the peak of the reproductive season and it is likely that some individuals were post-pregnant and/or lactating. It is unclear whether the decrease in retinoids observed may be also reflecting the effect of lipid mobilization associated to these stages. Whatever the case, judging from information from other species (Tornero et al 2004), the very low OC concentrations found in the females here studies seem unlikely to affect retinoid dynamics.

Conclusions

Knowledge on the biological traits of individuals is essential to assess the effects of pollutant exposure and the potentially associated changes in the retinoid status of dolphins. In particular, sex, age and lipid content have been observed to bear a significant effect on blubber retinoid concentrations in common dolphins. As a consequence, the use of retinoids as biomarkers of organochlorine exposure requires proper assessment of the role of these variables in natural, non-polluted, conditions. We cannot conclude whether in our survey OC loads influenced retinoid concentrations or, conversely, whether the variations observed in the latter variable were the result of natural factors only. It is remarked that extrapolation of toxicological responses between species should be treated with caution as physiological response to chemical exposure may be highly species-specific or even population-specific. Sources for variation may not only be taxonomic or evolutionary, but may also be a reflection of the particular behavioural, dietary or ecological traits of the concerned species or populations. For future research on retinoid disruption by OCs in dolphins, we recommend: i) to focus on old males because they display the highest OC levels in the population, and ii): to investigate the effects of lactation and reproductive status on retinoid levels in females.

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Table 1. Concentrations of blubber retinoids (σg/g wet weight) and organochlorines (σg/g lipid weight), and blubber lipid content (BLC, %) of the common dolphins split by sex and age-class.

	Males and Females < 7years (n=30)				Males ≥ 7 years (n=31)				Females ≥ 7 years (n=13)			
	Mean	S.D.	Max.	Min.	Mean	S.D.	Max.	Min.	Mean	S.D.	Max.	Min.
BLC	64.08	11.78	95.60	37.91	59.11	8.28	71.53	35.19	58.21	11.55	82.12	42.60
Retinoids	38.98	13.52	70.71	14.19	50.37	14.76	87.49	22.21	42.21	16.77	71.23	23.47
tPCB	13.83	7.26	39.34	5.25	24.64	16.11	82.67	7.67	7.10	4.81	15.10	1.03
tDDT	3.52	1.58	9.24	1.44	6.27	3.80	22.99	2.32	2.07	1.69	5.48	0.55

Table 2. Multiple regression of log(retinoid concentration) on sex, lipids, age by sex, interaction of lipids on sex, and separate effects of the interaction of lipids on age by sexes. M:males; F:females.

	Value	Std. Error	t value	Pr (> |t|)
(Intercept)	2.937	0.531	5.535	0.000
sex	0.037	0.5307	0.0695	0.9448
BLC	0.0095	0.0085	1.1245	0.2647
sexFage	0.0488	0.0984	0.4957	0.6217
sexMage	0.1544	0.0664	2.3247	0.0231
sex:BLC	-0.0002	0.0085	-0.0238	0.9811
sexFageBLC	-0.0006	0.0016	-0.3791	0.7058
sexMageBLC	-0.0021	0.001	-2.0192	0.0474

Table 3. Models of multiple regression to assess the effects of pollutants on retinoid concentrations in female (3a) and male (3b) common dolphins. AIC is the small sample Akaike Information Criterion, w_i are the Akaike weights of the I models measuring the likehood of each model given the data, and R^2 is the fraction of variance explained by the model. The "~" relates the response variable, log(retinoid concentration), with the predictors.

3a

Model	Females AIC_c	w_i	R^2
log(retinoids) ~ log(tPCB)	25.39	0.183	0.23
log(retinoids) ~ log(tPCB) + age:log(tPCB)	26.17	0.124	0.28
log(retinoids) ~ log(tDDT)	26.20	0.122	0.21
log(retinoids) ~ log(tPCB) + age:BLC:log(tPCB)	26.27	0.118	0.28
log(retinoids) ~ age + log(tPCB)	27.65	0.059	0.27
log(retinoids) ~ log(tDDT) + age:BLC:log(tDDT)	27.86	0.053	0.24
log(retinoids) ~ log(tDDT) + age:log(tDDT)	27.98	0.050	0.23
log(retinoids) ~ log(tPCB) + $log(tPCB)^2$	28.01	0.049	0.23
log(retinoids) ~ log(tPCB) + BLC:log(tPCB)	28.01	0.049	0.23
log(retinoids) ~ log(tDDT) + log(tPCB)	28.15	0.046	0.23
log(retinoids) ~ log(tDDT) + $log(tDDT)^2$	28.64	0.036	0.22
log(retinoids) ~ age + log(tDDT)	28.86	0.032	0.24
log(retinoids) ~ log(tDDT) + BLC:log(tDDT)	28.97	0.030	0.21
log(retinoids) ~ BLC + log(tPCB)	29.03	0.030	0.23
log(retinoids) ~ BLC + log(tDDT)	29.90	0.019	0.21
log(retinoids) ~ age*BLC*log(tPCB)	39.13	0.000	0.42
log(retinoids) ~ age*BLC*log(tDDT)	41.43	0.000	0.37

3b

Model	AIC_c	Males w_i	R^2
log(retinoids) ~ log(tDDT)	22.42	0.254	0.21
log(retinoids) ~ log(tDDT) + BLC:log(tDDT)	22.96	0.194	0.25
log(retinoids) ~ log(tDDT) + log(tPCB)	24.27	0.101	0.23
log(retinoids) ~ log(tDDT) + $log(tDDT)^2$	24.59	0.086	0.22
log(retinoids) ~ log(tDDT) + age:BLC:log(tDDT)	25.01	0.070	0.22
log(retinoids) ~ log(tDDT) + age:log(tDDT)	25.08	0.067	0.21
log(retinoids) ~ BLC + log(tDDT)	25.20	0.063	0.23
log(retinoids) ~ age + log(tDDT)	25.67	0.050	0.22
log(retinoids) ~ log(tPCB) + BLC:log(tPCB)	26.53	0.033	0.19
log(retinoids) ~ log(tPCB)	26.85	0.028	0.14
log(retinoids) ~ BLC + log(tPCB)	28.22	0.014	0.18
log(retinoids) ~ log(tPCB) + age:log(tPCB)	28.46	0.012	0.16
log(retinoids) ~ age + log(tPCB)	28.64	0.011	0.17
log(retinoids) ~ log(tPCB) + $log(tPCB)^2$	29.02	0.009	0.15
log(retinoids) ~ log(tPCB) + age:BLC:log(tPCB)	29.61	0.007	0.14
log(retinoids) ~ age*BLC*log(tDDT)	33.88	0.001	0.36
log(retinoids) ~ age*BLC*log(tPCB)	35.19	0.000	0.34

Figure 1. Relationship between retinoid concentrations (σg/g wet weight) and BLC (%) in male common dolphins. The fitted line shows the trend in individuals older than 7 years.

Figure 2.1. Relationship between retinoid (σg/g wet weight) and DDT (σg/g lipid weight) concentrations in male common dolphins.

Figure 2.2. Relationship between retinoid (σg/g wet weight) and PCB (σg/g lipid weight) concentrations in male common dolphins.

Figure 3.1. Relationship between retinoid (σg/g wet weight) and DDT (σg/g lipid weight) concentrations in female common dolphins.

Figure 3.2. Relationship between retinoid (σg/g wet weight) and PCB (σg/g lipid weight) concentrations in female common dolphins.

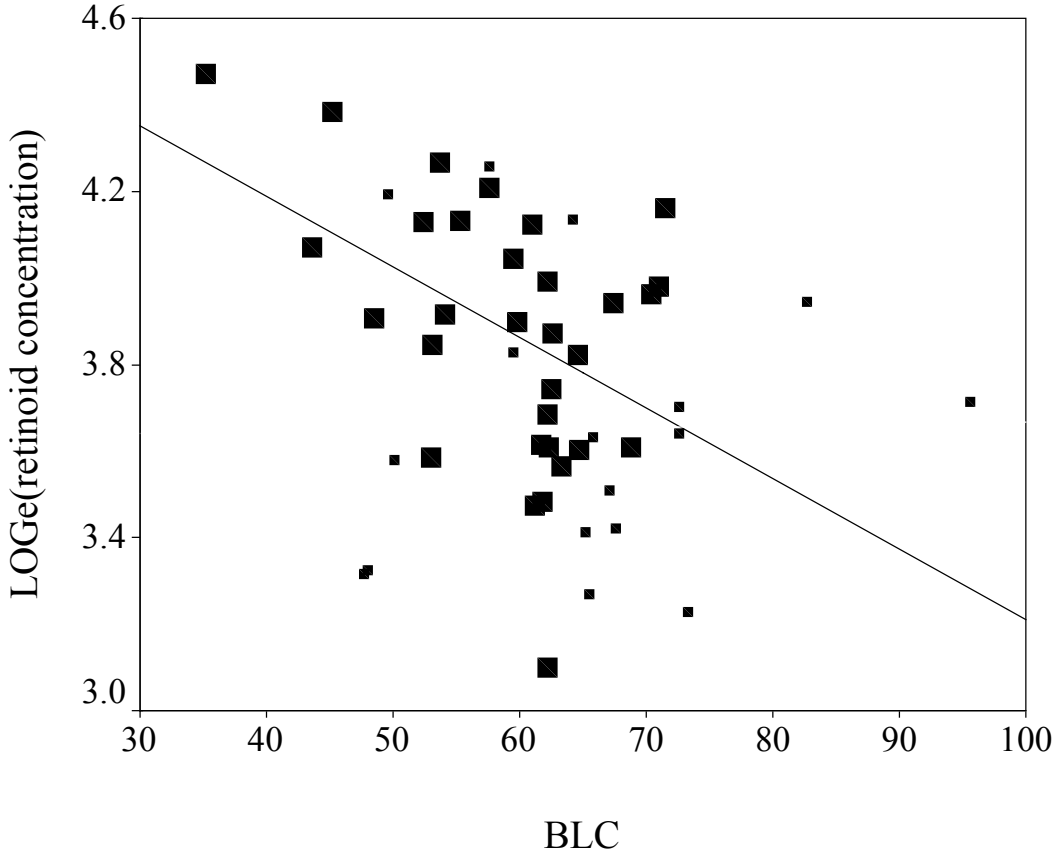
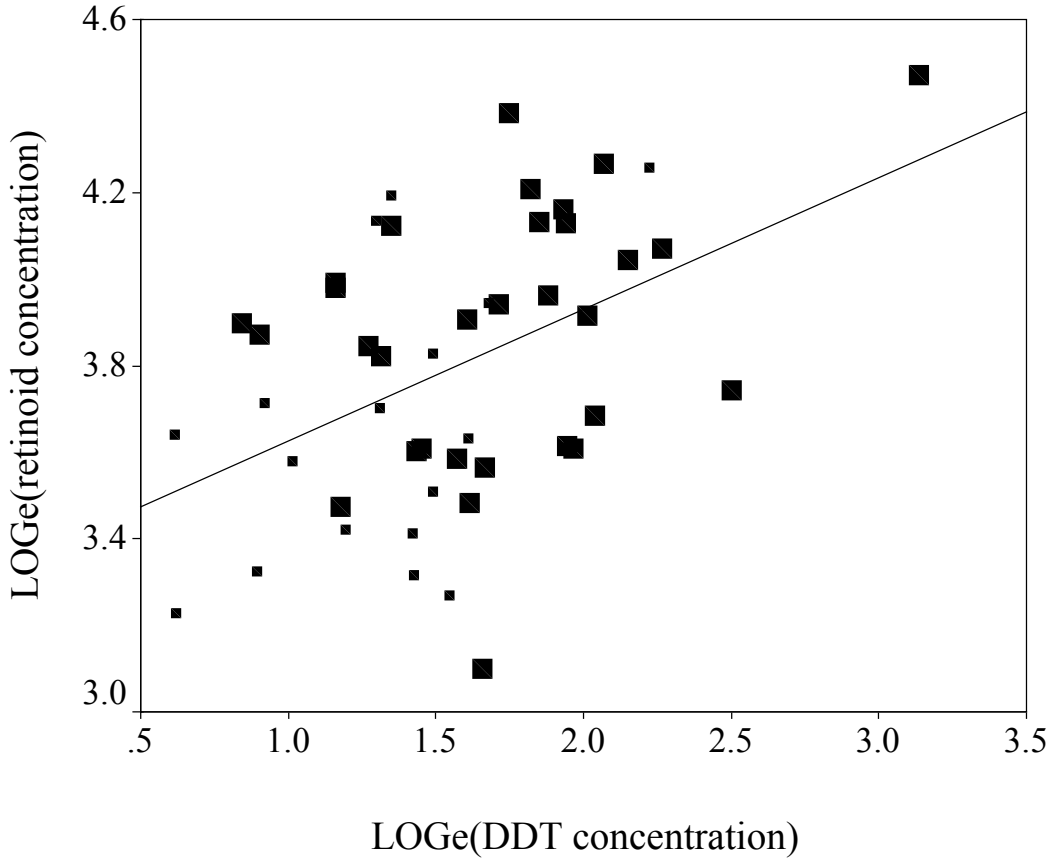
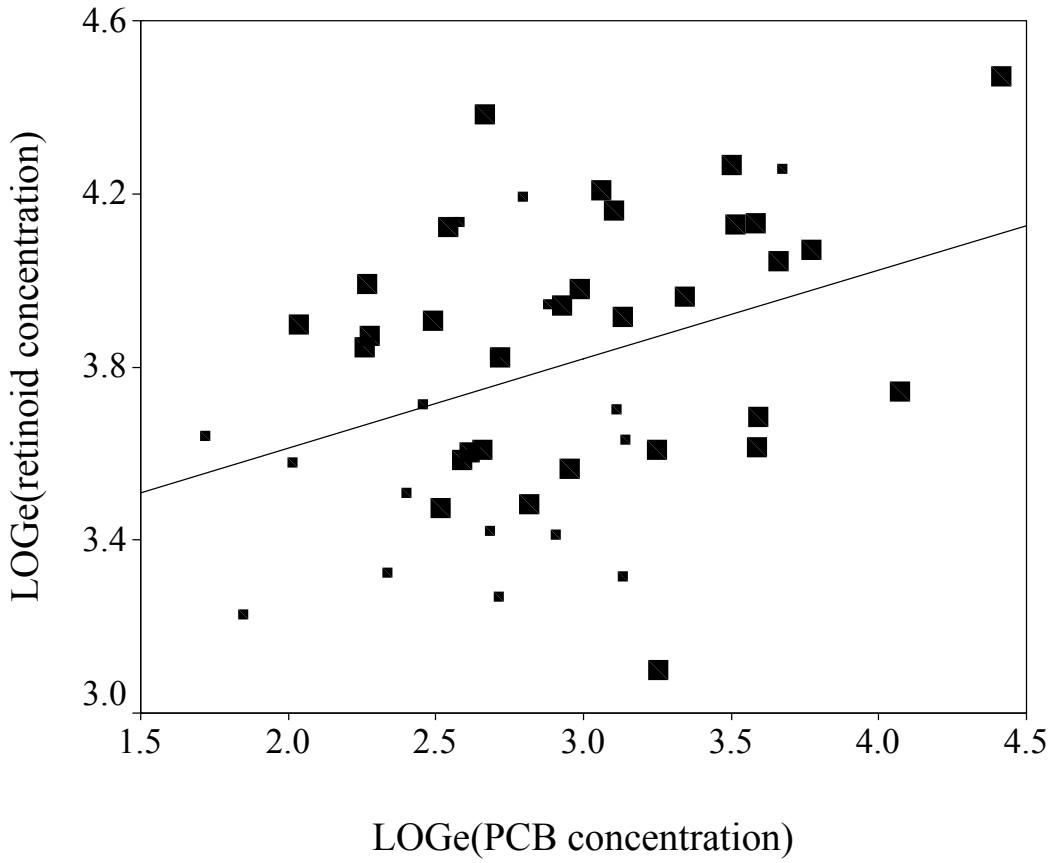

4.6
4.2
3.8
3.4
3.0
LOGe(retinoid concentration)
30
40
50
60
70
80
90
100
BLC
individuals 1-7 years old
individuals older than 7 years

Figure 1



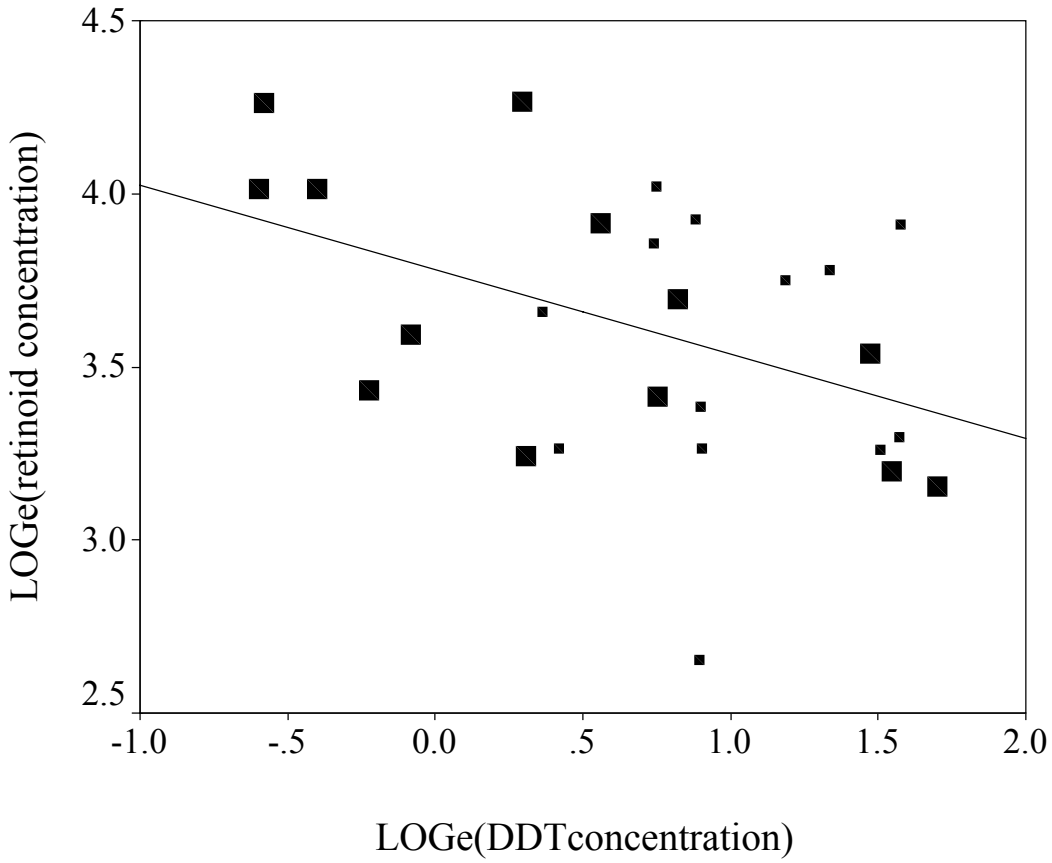
- individuals 1-7 years old
- individuals older than 7 years

Figure 2.1



- individuals 1-7 years old
- individuals older than 7 years

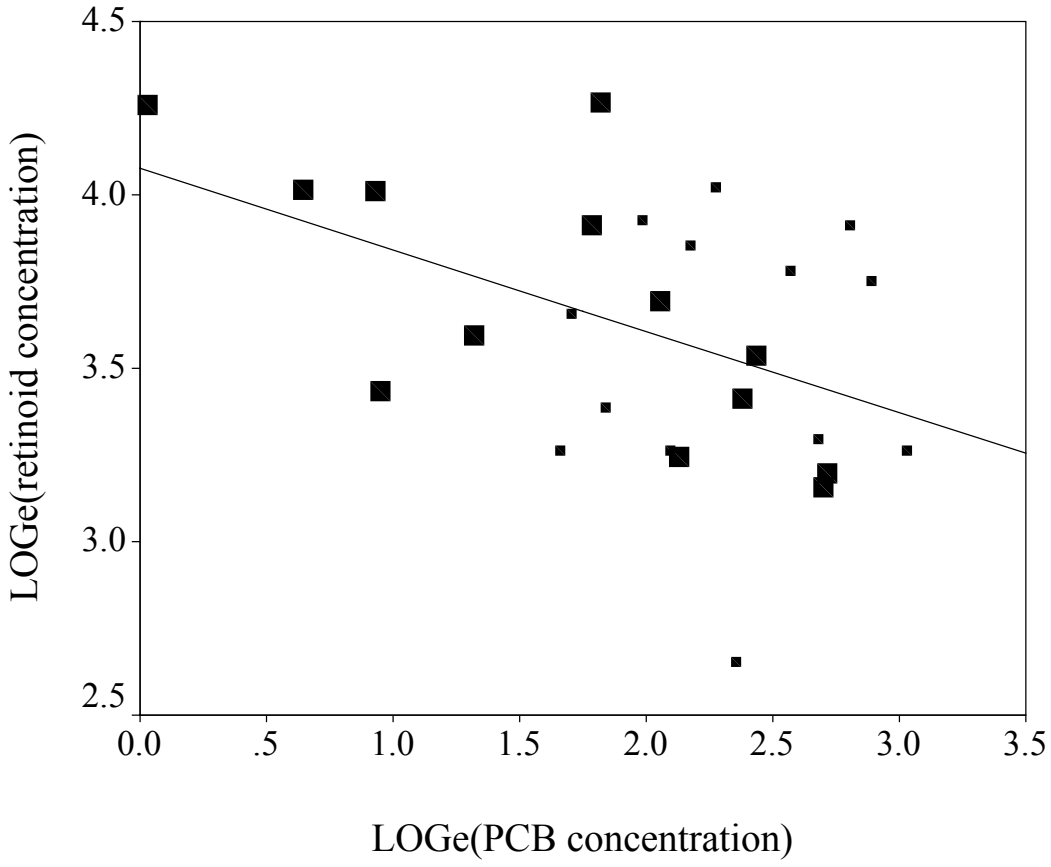
Figure 2.2



▪ individuals 1-7 years old

■ individuals older than 7 years

Figure 3.1



- individuals 1-7 years old
- individuals older than 7 years

Figure 3.2

6. RESULTADOS GLOBALES Y DISCUSIÓN

El conocimiento de los retinoides ha avanzado sustancialmente durante los últimos años. Sin embargo, en los mamíferos marinos, y especialmente en cetáceos, la información sobre estos compuestos es muy escasa. Por este motivo, la potencial utilidad de los retinoides como biomarcadores de contaminación en cetáceos ha sido deducida a partir de información existente en otros mamíferos. En este contexto, muchos de los aspectos estudiados en esta tesis se refieren y comparan a trabajos efectuados en pinnípedos y mamíferos terrestres y, por este motivo, y como paso previo a las investigaciones experimentales desarrolladas en odontocetos, se consideró necesario realizar una evaluación bibliográfica y una interpretación y revisión de resultados previos en mamíferos para así poder establecer los patrones generales de la fisiología y metabolismo de los retinoides que pueden ser aplicados a los odontocetos.

Los retinoides y su evaluación en los mamíferos marinos

En resumen, los retinoides componen un grupo de compuestos estrechamente relacionados que se hallan presentes de forma natural en los animales, desempeñando funciones indispensables en prácticamente cada tejido y órgano. El término vitamina A debe emplearse para describir genéricamente aquellos retinoides que exhiben la actividad biológica del retinol (Blomhoff *et al*., 1992). Aunque su exceso puede resultar tóxico, su deficiencia produce mayores desórdenes y efectos patológicos, entre los que cabe destacar una reducción del crecimiento, inmunosupresión, lesiones dérmicas y fallos reproductivos (Håkansson *et al*., 1992; De Swart *et al*., 1994), síntomas que se asemejan a muchos de los efectos tóxicos que caracterizan la exposición a ciertos contaminantes, como los organoclorados. Para humanos y otros mamíferos, la vitamina A es un nutriente esencial que, ingerida como tal o producida a partir de la provitamina A, se absorbe intestinalmente, se incorpora a la circulación sanguínea en forma de ésteres de retinol y se transporta mayoritariamente hacia el hígado. En este órgano, los ésteres de retinol son procesados para el almacenamiento hepático o bien se va liberando a la sangre retinol sin esterificar unido a la proteína fijadora de retinol (RBP=Retinol Binding Protein) conforme es requerido por el resto de los tejidos, de manera que la concentración en sangre permanece más o menos constante (Blomhoff, 1994). La secreción hepática de vitamina A debe estar fuertemente controlada; una

liberación insuficiente no permitiría cubrir las necesidades de los demás tejidos y una liberación excesiva podría causar efectos tóxicos. Es decir, los niveles de retinol en plasma se mantienen constantes a pesar de que se produzcan variaciones sustanciales en la dieta y en las reservas corporales. Por tanto, debe existir un mecanismo homeostático que establezca un nivel de retinol en plasma "normal" para una especie, individuo o circunstancia dada (Blomhhoff *et al*., 1994). Por este motivo, aunque el estado de poblaciones que se hallan en riesgo de deficiencia de vitamina A ha sido frecuentemente evaluado a través de las concentraciones de retinoides en suero y plasma, esta variable no resulta adecuada, pues las concentraciones sanguíneas no comienzan a disminuir hasta que los niveles en los órganos de reserva son peligrosamente bajos. En este punto surge uno de los aspectos más significativos para la presente investigación, ya que es necesario buscar otros tejidos que reflejen mejor las alteraciones corporales en la disponibilidad de retinoides en cetáceos y que resulten por ello más apropiados en estudios medioambientales.

El examen de la literatura revela que la vitamina A se acumula masivamente en el hígado, tejido que presenta las concentraciones más altas tanto en mamíferos terrestres (Wolf, 1984), como en pinnípedos (Schweigert *et al*., 1987; Mos & Ross, 2002; Nyman *et al*., 2003). Por este motivo, el estatus de retinoides ha sido tradicionalmente estimado a partir de las concentraciones encontradas en este órgano (Schweigert & Buchholz, 1995; Käkelä *et al*., 2002). En el caso de los cetáceos, es sabido desde hace muchas décadas que el hígado es enormemente rico en retinoides (Schmidt-Nielsen *et al*., 1934) y varios investigadores concentraron sus esfuerzos en extraer vitamina A del hígado de ballenas con fines comerciales en la primera mitad del siglo pasado (Klem, 1935; Wetlesen, 1938; Wagner, 1939; Braekkan, 1948; Ishikawa *et al*., 1948, 1951; Kaneko, 1948; Mori & Saiki, 1950; Tawara & Fukazawa, 1950a, 1950b). Así, parecería lógico utilizar el hígado como indicador del estatus de retinoides también en cetáceos. Sin embargo, este tejido no resulta indicado para monitorear poblaciones salvajes. En los ejemplares hallados muertos, el tejido hepático es de los primeros en iniciar el proceso de descomposición debido a su elevada riqueza enzimática y, en el caso de animales vivos, se requiere la captura, inmovilización y manejo del ejemplar, así como el empleo de técnicas de biopsias altamente invasoras.

Aparte del hígado, el estudio de los retinoides en otros tejidos de cetáceos se halla casi inexplorado y los trabajos realizados recientemente en mamíferos marinos, no muy abundantes, están prácticamente restringidos a pinnípedos (Schweigert *et al*., 1987;

2002; Käkelä *et al*., 1997; Mos & Ross 2002; Nyman *et al*., 2003). No obstante, estos estudios nos han permitido identificar la grasa hipodérmica como una excelente alternativa al uso del hígado, puesto que constituye una gran proporción de la masa corporal de los mamíferos marinos, aproximadamente 40% en pinnípedos (Schweigert *et al*., 1987) y 15-45% en cetáceos (Aguilar *et al*., 1999), y su elevado contenido lipídico acumula eficazmente compuestos lipófilos, como es el caso de los retinoides. Por ejemplo, Schweigert *et al*. (1987) y Mos & Ross (2002) encontraron que la foca gris (*Halichoerus grypus*) acumulaba aproximadamente el 40% de los retinoides totales en la grasa, y que en la foca común (*Phoca vitulina*) este valor alcanzaba el 66%. Además, como la grasa puede ser fácilmente obtenida, tanto en animales capturados como salvajes, utilizando técnicas de recolección de biopsias no destructivas y ampliamente experimentadas (Aguilar & Borrell, 1994a), este tejido puede considerarse, a priori, como potencialmente muy útil para valorar los niveles de retinoides en cetáceos.

La grasa hipodérmica como indicador del estatus de retinoides en cetáceos

Precisamente el hecho de que la grasa hipodérmica recubra todo el cuerpo de los cetáceos puede dificultar su aplicación en la evaluación de retinoides en estos animales. Se ha demostrado que la estructura y composición de la grasa varían dependiendo de la parte del cuerpo que es examinada (Iverson, 2002), por lo que las concentraciones de retinoides también podrían reflejar dicha heterogeneidad. En ese caso, los protocolos de recogida de muestras tendrían que definir cuidadosamente la posición de grasa que debe ser analizada para evitar inconsistencias a la hora de confrontar resultados de diferentes estudios. Por este motivo, es absolutamente relevante el conocimiento de la distribución topográfica de los niveles de retinoides en la grasa de cetáceos y, con este objetivo, se llevó a cabo un estudio de validación empleando muestras de delfín común (*Delphinus delphis*) obtenidas de ejemplares capturados en la pesca de arrastre por parejas en aguas del noroeste de la Península Ibérica.

Como se había previsto, se observó que las concentraciones de retinoides diferían significativamente entre las distintas posiciones y secciones de grasa, siendo la región anterior-ventral la que presentaba las concentraciones más altas y la posterior-dorsal las más bajas. Estas diferencias fueron relacionadas con la naturaleza lipófila de los retinoides, ya que el patrón de distribución de estos compuestos era prácticamente idéntico al de los lípidos. Es decir, la parte anterior-ventral de los delfines también

presentaba el mayor contenido en lípidos. Estudios similares realizados en marsopa común también encontraron una mayor riqueza lipídica en la parte anterior ventral de los animales (Calambokidis, 1986; Ishaq *et al*., 2000). Estos resultados sugerían que la región anterior-ventral es más importante para las funciones de aislamiento y almacenamiento de lípidos que la región posterior-dorsal, probablemente para proporcionar una mayor protección térmica a las vísceras, y que esta gran riqueza lipídica es, seguramente, la responsable de la extensiva acumulación de retinoides en dicha región. De cualquier forma, la influencia de los lípidos sobre las concentraciones de retinoides se discute más adelante y, de momento, sólo se subraya la importancia de utilizar siempre la misma posición corporal para poder asegurar fiabilidad al comparar los niveles de retinoides en grasa de cetáceos.

Por otro lado, independientemente de la región del cuerpo de donde se extraiga la muestra que va a ser analizada, la grasa de los cetáceos se halla dividida en varias capas que se distinguen por su composición bioquímica y función (Aguilar & Borrell, 1990, 1994; Lockyer, 1995; Olsen & Grahl-Nielsen, 2003). Esta estructura estratificada de la grasa también podría suponer una fuente de heterogeneidad a la hora de evaluar los retinoides en este tejido puesto que, en función de la capa analizada, los resultados de retinoides podrían variar. Teniendo en cuenta que la masa de tejido necesaria para el análisis es de unos pocos gramos y que el espesor de la grasa en grandes cetáceos es de 3-50 cm dependiendo de las especies (Iverson, 2002), conseguir una muestra representativa de todas las capas en esos animales resulta muy complicado. En cambio, en pequeños cetáceos, como delfines y marsopas, esta limitación se resuelve fácilmente recolectando y analizando muestras de grasa que contengan todos los estratos, de manera que se obtenga una media de la totalidad de los estratos que componen la capa de grasa, como se hizo en este estudio y como puede conseguirse en el caso de usar biopsias para el análisis.

Distribución corporal de retinoides en pequeños cetáceos

Una vez establecidas las condiciones requeridas para garantizar la representatividad de las muestras de grasa, se planteó la necesidad de estudiar la distribución corporal de retinoides y comprobar si, como se había sugerido, este tejido era realmente idóneo para valorar el estatus de retinoides en cetáceos.

Para tal fin, se analizaron y compararon las concentraciones de retinoides presentes en los principales tejidos corporales del delfín común, utilizando para ello muestras obtenidas de ejemplares capturados en la pesca de arrastre de parejas en el noroeste peninsular antes mencionada. El estudio permitió determinar que, al igual que ocurre en la mayoría de los mamíferos, las concentraciones calculadas en el hígado son muy elevadas (entre 15-460 $\mu g\ g^{1}$) y mucho mayores que en el resto de los tejidos. Sin embargo, a diferencia de los mamíferos terrestres, en los que el hígado constituye en términos cuantitativos la principal reserva de vitamina A del organismo ya que almacena entre 70-90% de los retinoides totales, el hígado del delfín común acumula solamente un 53% del total corporal. Comparativamente, la grasa mostraba concentraciones más bajas que el hígado pero suficientemente altas como para ser detectadas con facilidad (entre 23-70 $\mu g\ g^{-1}$) y, a pesar de estas menores concentraciones, se vio que constituía un sustancioso depósito corporal de retinoides ya que su elevada contribución a la masa corporal hacía que almacenara hasta el 43% de la reserva total de estos compuestos. Ello nos permite afirmar que, en los delfines, los retinoides se distribuyen de manera comparable entre la grasa y el hígado, en consonancia con los resultados previamente obtenidos en otros grupos de mamíferos marinos (Schweigert *et al*., 1987).

Aunque el riñón es considerado importante para la acumulación de retinoides en mamíferos terrestres, especialmente en cánidos y mustélidos (Bomhoff *et al*., 1991; (Schweigert & Buchholz, 1995), las concentraciones en este tejido en el delfín común eran muy bajas, de hecho varios órdenes de magnitud menores que las del hígado y la grasa. Igualmente, las concentraciones de retinoides en los demás tejidos analizados (músculo, pulmón y corazón) eran insignificantes comparadas con las del hígado y, en ocasiones, se encontraban por debajo de los límites de detección analíticos. La suma de las contribuciones de estos cuatro tejidos a los retinoides corporales totales resultaba ser inferior al 4%, de modo que ninguno de ellos puede considerarse útil para monitorear el estatus de estos compuestos en delfines.

El hecho de que tanto las concentraciones en hígado como en grasa son igualmente válidas para la evaluación de retinoides en cetáceos fue también demostrado en el estudio efectuado con muestras de marsopa común. En este trabajo se determinó que las concentraciones en ambos tejidos estaban significativa y positivamente correlacionadas, sugiriendo que la deposición de retinoides se halla sujeta a similares procesos en uno y otro. Sin embargo, como consecuencia de las restricciones

mencionadas para obtener muestras de hígado en poblaciones salvajes de cetáceos, y las también indicadas ventajas que exhibe la obtención de muestras de grasa, puede concluirse que este último tejido es, ciertamente, el más aconsejable para estimar el estatus de retinoides en este grupo de animales.

Cambios en las concentraciones de retinoides tras la muerte de los individuos

Las características moleculares de los retinoides abren una serie de interrogantes acerca de los procedimientos correctos de recolección, manejo y almacenamiento de las muestras utilizadas en la investigación de estos compuestos. Los retinoides son moléculas muy inestables, con extrema sensibilidad a la luz, oxígeno, metales, ácidos fuertes y excesivo calor (Barua & Furr, 1998). Por tanto, es preciso realizar estudios de estabilidad controlando estos parámetros y definir las condiciones que deben cumplirse en el tratamiento de las muestras para garantizar que los niveles de retinoides no sufran una alteración significativa previa al análisis. Los trabajos hasta aquí presentados ya habían apuntado la necesidad de preservar las muestras congeladas y alejadas de la luz para prevenir la degradación de los retinoides, pero nunca se había determinado qué sucedía con las concentraciones de estos compuestos durante el tiempo transcurrido entre la muerte de un individuo y la recolección de sus tejidos. Éste es un aspecto esencial en los estudios con cetáceos ya que, cuando se trabaja con ejemplares muertos, a menudo pasa un largo intervalo de tiempo desde que el ejemplar muere hasta que el muestreo es posible. Ello también es aplicable a las capturas incidentales en actividades pesqueras, una de las fuentes de muestras de tejidos considerada como más adecuada para estudios ecotoxicológicos. Por esta razón, se llevó a cabo un estudio sobre el efecto que el tiempo post-mortem producía sobre las concentraciones de retinoides en la grasa de seis marsopas que habían sido capturadas incidentalmente y que quedaron sometidas a unas condiciones de conservación representativas de este tipo de situaciones.

Para el estudio se trabajó en colaboración con un programa de rescate de marsopas enmalladas en artes de pesca en la Bahía de Fundy (Canadá), obteniendo muestras de ejemplares que eran hallados en estado agonizante. Los ejemplares fueron muestreados inmediatamente después de su muerte, las muestras conservadas en nitrógeno líquido, y el cadáver devuelto a las aguas para que así experimentara los cambios post-mortem usuales en una captura incidental. Posteriormente, el cadáver fue muestreado a distintos intervalos de tiempo y las muestras recolectadas se conservaron

igualmente en nitrógeno líquido, realizándose después los análisis de retinoides a fin de evaluar los potenciales cambios sufridos con el tiempo.

Una vez extraídas las muestras de las marsopas, se demostró que los niveles de retinoides se mantenían estables durante el tiempo que duró el experimento (48 horas tras la muerte del animal). La penetración de los rayos ultravioleta en el cuerpo de las marsopas quedaba minimizada por la barrera natural que representa la piel y, también, por el hecho de que éstas quedaban sumergidas a unos dos metros bajo el agua y que ésta era bastante opaca debido a las fuertes mareas y aportación de sedimentos propias de la zona. La oxidación fue probablemente evitada por las condiciones de anoxia producidas tras la muerte del animal. Finalmente, la temperatura del agua era lo suficientemente baja como para asegurar la estabilidad térmica de los retinoides.

Además de estos factores, la degradación de los retinoides puede depender de la naturaleza de otros compuestos presentes en la muestras, como tocoferoles, ácido ascórbico o lípidos (Billion-Rey *et al*., 1992; Albalá-Hurtado *et al*., 2000a; 2000b; Dupertuis *et al*., 2002). Por ejemplo, el alto contenido lipídico de la grasa de los cetáceos, 35-90% (Lockyer *et al*., 1985; Aguilar & Borrell, 1990; Lockyer, 1991, 1993, 1995), podría contribuir a la estabilidad encontrada.

En cualquier caso, el estudio dejó claro que los retinoides se hallan presentes en la grasa de las marsopas de tal forma que no son afectados por los procesos de degradación en las condiciones ambientales usuales en una captura incidental, al menos durante el período de tiempo evaluado. Por tanto, puede afirmarse que las muestras de grasa procedentes de animales capturados muertos, no preservados, pueden emplearse con fiabilidad para monitorear retinoides en cetáceos, siempre que sean recogidas en un tiempo inferior a las 48 horas post-mortem.

En el caso de los cetáceos hallados varados, otra potencial fuente de obtención de muestras para estudios ecotoxicológicos, la situación es bastante más compleja. Como se ha explicado en la introducción, el uso de estos animales presenta serias limitaciones, pues un animal varado es normalmente localizado pasadas las 48 horas desde su muerte y después de que haya permanecido un tiempo fuera del agua y, por ello, haber resultado expuesto directamente a la luz solar y sufrido variaciones marcadas de temperatura debido a la irradiación. Además, los ejemplares varados frecuentemente han padecido largas enfermedades que han desencadenado su muerte, lo que es susceptible de haber variado no sólo sus constantes fisiológicas sino también otras variables capaces de influir en el status corporal de retinoides, como son la dieta, la

reproducción o la condición nutritiva. Por este motivo, el estudio concluye que, a diferencia de las capturas incidentales, los animales varados no constituyen una fuente fiable de muestras para monitorear los niveles poblacionales de retinoides.

Influencia de las variables biológicas sobre los niveles de contaminantes (DDTs y PCBs) y de retinoides

De acuerdo con la información disponible en la literatura, las concentraciones de retinoides y de contaminantes organoclorados pueden variar entre los individuos de una misma población en función de sus particulares características biológicas (Aguilar *et al*., 1999; Borrell *et al*., 1999). Por tanto, para evaluar de manera adecuada la aplicabilidad de los niveles de retinoides en grasa como biomarcadores de exposición a PCBs y DDTs en las poblaciones estudiadas, es necesario elucidar primero las diferencias que existen en las concentraciones de esos compuestos entre los delfines dependiendo de su edad, sexo, condición nutritiva o éxito reproductivo. Cuantos más potenciales factores influyentes puedan ser minimizados, más se ajustará la búsqueda de la utilidad de los retinoides como biomarcadores.

Con la finalidad de establecer la significación de estos factores, se procedió a estudiar su influencia en el status de retinoides en dos especies de odontocetos: los delfines comunes que eran objeto de captura incidental en la pesca de arrastre por parejas en el noroeste peninsular, y los delfines mulares (*Tursiops truncatus*) que son objeto de seguimiento y control en la bahía de Sarasota, Florida (EEUU). La primera población permitía la recolección de ejemplares muertos de los que se podía obtener la información y las muestras necesarias (dientes para determinar la edad, talla, sexo, etc) para establecer sus principales características biológicas. La segunda ha sido objeto durante más de tres décadas de un seguimiento continuado que incluye la captura y posterior liberación de ejemplares de manera periódica. Ello, combinado con técnicas de marcaje y fotoidentificación, así como con la recolección de tejidos mediante técnicas de biopsias no destructivas, permite igualmente conocer con precisión los rasgos biológicos propios de cada individuo muestreado. A continuación se detallan los resultados obtenidos para las principales variables.

Contaminantes

Sexo y edad

En ambas especies de delfines se observó que las concentraciones de PCBs y DDTs aumentaban con la edad en los machos mientras que disminuían en hembras, coincidiendo con el patrón de acumulación y depuración de contaminantes lipófilos descrito usualmente en cetáceos (Aguilar & Borrell, 1994; Tanabe *et al*., 1994; Aguilar *et al*., 1999). Así, se sabe que, en los animales inmaduros, los niveles de organoclorados aumentan con la edad debido a que la tasa de absorción de estos compuestos excede la capacidad de excreción o degradación del organismo. Este proceso de acumulación continúa en los individuos machos a lo largo de toda su vida, pero se atenúa o invierte en las hembras desde el comienzo de su actividad reproductiva: a los 7-9 años de edad en el caso del delfín común (Collet, 1981) y a los 8-12 años en el delfín mular (Scott *et al*., 1990). Como resultado de este proceso, en las hembras adultas las concentraciones tienden a estabilizarse o disminuir debido a la transferencia de cantidades variables de su carga de contaminantes a sus crías durante la gestación y la lactancia. En consecuencia, las concentraciones de PCBs y DDTs en los individuos inmaduros de ambos sexos son similares, mientras que en las hembras adultas son mucho menores que en los machos adultos.

Actividad reproductiva

El posible impacto negativo que provoca la carga de contaminantes en el éxito reproductivo de las hembras y el riesgo que supone para las crías el traspaso maternal fue evaluado en el delfín mular, ya que gracias a la constante investigación que se está llevando a cabo en la población de Sarasota se posee suficiente información acerca de la historia reproductiva de los individuos (Scott *et al*., 1990; Wells, 2003).

Así, se encontró que las concentraciones de PCBs disminuían en las hembras desde que se producía la primera lactancia, continuaban siendo bajas mientras duraba su época reproductora (aproximadamente hasta los 40 años de edad) y aumentaban en las hembras más viejas, lo cual reflejaba una disminución en la transferencia de contaminantes conforme se alargaban, al envejecer la hembra, los intervalos de tiempo entre nacimientos. Además, se observó que el éxito reproductivo de las hembras se incrementaba con la edad y que los niveles de contaminantes traspasados a las crías variaban dependiendo del orden en que éstas nacían, siendo mayores en las primeras crías que en las siguientes. Se considera probable que la alta carga de contaminantes

transferida por las madres a sus primeras crías influya significativamente en el hecho de que la tasa de mortalidad de éstas durante su primer año de vida alcance el 50%, pues debe señalarse que dicha tasa en las subsiguientes crías no sobrepasa el 30% (Wells, 2000). No obstante, hay que tener en cuenta que la presencia de otros contaminantes en el medio, así como otros factores relacionados con las actividades humanas o con el comportamiento de las madres, podrían tener también una influencia en la alta tasa de mortalidad de las primeras crías observada.

Condición nutritiva

La riqueza lipídica de la grasa hipodérmica puede considerarse un efectivo indicador de la condición nutritiva de los cetáceos (Aguilar & Borrell, 1990; Lockyer, 1995). Aunque se cree que la población de Sarasota se encuentra en un aparente buen estado nutritivo y de salud, el contenido lipídico medio de la grasa de los delfines mulares analizados era muy bajo (24.5%). Parece ser que las menores necesidades de termorregulación de los individuos que habitan medio cálidos, como la bahía de Sarasota, junto con la reducción del espesor de la capa de grasa que se produce en los meses de primavera-verano (época del año en que los delfines fueron muestreados) son los responsables de la reducida cantidad de lípidos hallada en estos delfines. En cambio, el contenido lipídico medio de los delfines comunes, también considerados en buen estado nutritivo, fue bastante elevado (61.02%) y considerado como el usual en esta especie.

En ambas poblaciones se observó que el contenido lipídico de los machos disminuía conforme aumentaba la edad, mientras que en las hembras se mantenía constante a lo largo de su vida. Así, los machos adultos presentaban menor cantidad de lípidos que los animales inmaduros de ambos sexos y las hembras adultas. Los cambios en el contenido lipídico relacionados con la edad y el sexo han sido descritos en numerosos cetáceos y asociados a las variaciones producidas en la dieta y estado reproductivo de los individuos (Aguilar *et al*., 1999).

Dado el fuerte carácter lipófilo de los PCBs y DDTs, es de esperar que las variaciones en el contenido lipídico afecten a la dinámica de estos contaminantes. En general, se ha observado que la movilización de lípidos produce un incremento de las concentraciones de organoclorados en cetáceos (Aguilar *et al*., 1999). Normalmente, este efecto puede compensarse en cierta medida si las concentraciones de contaminantes son expresadas en base lipídica en lugar de en base peso fresco, aunque no puede

obviarse por completo. En nuestro estudio, aun teniendo en cuenta el contenido lipídico de la grasa, las concentraciones de PCBs y DDTs en este tejido estaban inversamente relacionadas con la cantidad de lípidos en los delfines machos de las dos poblaciones estudiadas. Esta relación negativa también ha sido hallada en los individuos machos de otros mamíferos marinos, como delfines listados (*Stenella coeruleolaba*) (Aguilar & Borrell, 1994b) y focas grises y anilladas (*Phoca hispida*) (Nyman *et al*., 2003), y se atribuye a que los lípidos se movilizan desde la grasa más rápidamente que los organoclorados, de modo que la disminución del contenido lipídico no va acompañada de una paralela reducción de la carga de contaminantes (Aguilar *et al*., 1999). Por el contrario, en ninguno de estos estudios se observó una relación similar en las hembras: sus concentraciones de contaminantes disminuían progresivamente desde la primera cría, mientras que su proporción de lípidos en grasa era similar en todas las edades.

Retinoides

Sexo y edad

A diferencia de los organoclorados, los datos disponibles sobre la influencia del sexo y la edad sobre los retinoides son extremadamente dispersos y varían mucho de unas especies a otras, lo cual dificulta el establecimiento de patrones generales. En el caso de la edad, la tendencia más habitual parece ser una progresiva acumulación de retinoides a lo largo de la vida de los individuos como resultado de dos factores: una disminución del transporte de retinoides a través de la sangre al aumentar la edad y un mayor incremento de la tasa de ingestión de estos compuestos en comparación con su tasa de eliminación (Maiani *et al*., 1989; Krasinski *et al*., 1990). Además, los niveles de retinoides pueden aumentar en los individuos más viejos como consecuencia de un proceso de adaptación, posiblemente para regular la oxidación y desgaste de los tejidos (van der Loo *et al*., 2004). Sin embargo, varios estudios también han demostrado que las concentraciones de retinoides pueden disminuir con la edad o bien no depender de esta variable. La ausencia de una tendencia consistente podría asociarse con las diferencias en el estado de salud, dieta, condición nutritiva o condiciones ambientales de las poblaciones examinadas.

La poca información existente en mamíferos marinos también muestra esta aparente inconsistencia. Por ejemplo, se ha descrito que los niveles de retinoides en grasa aumentaban con la edad en focas anilladas del mar Báltico y del lago Saimaa, mientras que disminuían en focas de la misma especie de Spitsbergen (Käkelä *et al.*,

1997). Por tanto, los resultados pueden variar dependiendo de la zona de la que provengan los individuos e incluso, dentro de una misma zona, puesto que Nyman *et al*. (2003) no encontró ninguna tendencia ni en focas anilladas ni grises del Báltico. En otras especies, como la marsopa común de Groenlandia (Borrell *et al*., 1999) y la foca gris de Sable Island (Schweigert *et al*., 1987), han sido encontradas tendencias positivas con la edad.

Los resultados obtenidos en la presente tesis tampoco manifestaron uniformidad. Así, mientras la edad no parecía influir sobre las concentraciones de retinoides en la población de machos de delfín mular de Sarasota, sí se observaba un incremento significativo en los delfines comunes machos del noroeste peninsular. En el caso de las hembras, se observó que en ambas poblaciones dichos niveles se mantenían más o menos constantes en todas las edades.

La información sobre las diferencias de retinoides entre machos y hembras es todavía menos consistente que en el caso de la edad. La naturaleza y la magnitud del efecto del sexo sobre los niveles de retinoides varía sustancialmente dependiendo de las especies y poblaciones investigadas (Borrell *et al*., 1999). Probablemente, estas variaciones sean producidas por diferencias en la dieta y las fuentes de retinoides entre los dos sexos. En el caso de los ejemplares adultos, la actividad reproductiva puede tener también una influencia, puesto que frecuentemente implica cambios en los niveles hormonales, la dieta y el comportamiento de los individuos.

En el presente estudio, las concentraciones de retinoides medias calculadas en el delfín común fueron significativamente mayores en machos (46.32 $\mu g\ g^{-1}$) que en hembras (38.81 $\mu g\ g^{-1}$), mientras que no se observaron diferencias significativas entre los dos sexos en el delfín mular (12.52 $\mu g\ g^{-1}$ en machos y 10.76 $\mu g\ g^{-1}$en hembras). Aunque no hay información en la literatura acerca de diferencias en la composición de la dieta de machos y hembras de delfín común, es probable que éstas existan, al igual que ocurre en otras especies de delfínidos de similares características morfológicas y ecológicas (Bernard & Hohn, 1989).

En el caso del delfín mular, a pesar de que se observó que los valores medios de retinoides no diferían entre sexos, la influencia de la condición nutritiva sobre estos compuestos sí variaba de un modo significativo, tal como se detalla en el siguiente apartado. Las diferencias en la dieta asociadas al sexo descritas en esta especie por

varios autores (Barros & Odell, 1990; Cockcroft & Ross, 1990; Blanco *et al*., 2001) podrían ser el origen de tales variaciones.

Condición nutritiva

Como los retinoides son compuestos lipófilos, sus concentraciones en los tejidos pueden verse afectadas por las variaciones en el contenido lipídico, tal como sucedía con las de compuestos organoclorados. Los estudios realizados mostraron que, efectivamente, la cantidad de lípidos presente en las distintas regiones corporales de la grasa de delfín común parecía ser un fuerte determinante de la deposición de retinoides en cada una de ellas. Igualmente, la distribución de retinoides en los tejidos estaba significativamente afectada por las propiedades fisicoquímicas de sus moléculas, de modo que no sólo los retinoides se acumulaban en los tejidos ricos en lípidos, sino que su concentración dentro de un tejido dado era proporcional al contenido lipídico del mismo. No obstante, los delfines comunes machos más viejos presentaban una correlación negativa entre la concentración de retinoides en grasa y el contenido lipídico. Esta aparente contradicción se debía probablemente a las tendencias opuestas de ambas variables con la edad: mientras que los niveles de retinoides aumentaban, la cantidad de lípidos disminuía. En el delfín mular, en cambio, sí se observó una correlación positiva entre retinoides y contenido lipídico de la grasa, aunque dicha relación sólo se demostró como significativa en el caso de los machos. De hecho, en este colectivo, el contenido lipídico era la variable que mejor explicaba las diferencias en los niveles de retinoides. Sin embargo, se vio que las relaciones entre esas dos variables no eran significativas ni en las hembras de delfín común ni en las de delfín mular. Como se ha mencionado anteriormente, las diferencias en la dieta y en la tasa de ingestión de alimento, así como la condición reproductiva de los individuos son probablemente los factores que explican las diferencias encontradas entre machos y hembras.

Al igual que sucedía en el caso de la edad y el sexo, la influencia del contenido lipídico sobre los retinoides parece ser considerablemente heterogénea en las especies y poblaciones hasta ahora estudiadas. Así, en los estudios realizados en marsopa común (Borrell *et al*., 1999) y en crías de foca común (Mos & Ross, 2002) no se observó ninguna relación significativa entre esas variables, mientras que en los machos de foca gris (Nyman *et al*., 2003), retinoides y lípidos estaban inversamente relacionados. Las diferencias inter-específicas podrían vincularse a la movilización de lípidos desde la

grasa y, presumiblemente, de las reservas de retinoides asociadas que tiene lugar durante algunos eventos, como la lactancia o la migración. En esas situaciones, los retinoides pueden ser redistribuidos o excretados, por lo que es esperable que sus concentraciones en los tejidos experimenten una sustancial variación. A pesar de los variables resultados obtenidos en los diversos estudios en poblaciones salvajes de mamíferos marinos, las concentraciones de retinoides y lípidos en los tejidos de cetáceos parecen estar íntimamente relacionadas, lo que explica los resultados aquí obtenidos.

Retinoides como biomarcadores de contaminación en pequeños cetáceos

A pesar de que el metabolismo, el transporte y la acumulación de los retinoides en los mamíferos están sujetos a estrictos procesos de regulación homeostática, numerosos estudios experimentales han demostrado que la dinámica de estos compuestos resulta alterada como consecuencia de la exposición a los contaminantes ambientales, particularmente organoclorados como PCBs, DDTs y dioxinas (Brunström *et al*., 1991; Håkansson *et al*., 1992; Zile, 1992; Chu *et al*., 1996; 1998; Murk *et al*., 1998; Käkelä *et al*., 1999; Nilsson *et al*., 2000; Rolland, 2000; Simpson *et al*., 2000). En general, la exposición a estos compuestos químicos provoca una movilización de la carga de retinoides desde los órganos de reserva, especialmente el hígado, acompañada de un incremento en su tasa de degradación y eliminación a través de la orina (Kelley *et al.,* 2000), aunque los mecanismos de acción y la intensidad de los efectos tóxicos parecen variar entre las especies (Håkansson *et al*., 1991; Zile, 1992).

Si bien todos esos estudios están referidos a mamíferos terrestres, dada la base evolutiva de los procesos fisiológicos implicados, muchos de los efectos encontrados pueden ser extendidos a los mamíferos marinos. En éstos, sin embargo, el número de trabajos disponible es muy escaso y casi todos ellos, llevados a cabo en pinnípedos y en el oso polar (*Ursus maritimus*), emplearon los niveles en plasma para evaluar las respuestas toxicológicas de los retinoides (Brouwer *et al*., 1989; De Swart *et al*., 1994; Jenssen *et al*., 1995; Beckmen *et al*., 1997; Skaare *et al*., 2001). Como ya se ha indicado, el plasma no es un tejido indicado para tal fin, pues debido a la regulación homeostática de sus niveles éstos no constituyen una medida estable del déficit de retinoides. Únicamente Nyman *et al*. (2003) analizaron otros tejidos aparte del plasma. En este trabajo se determinó que los niveles de retinil pamitato en el hígado y en la grasa de focas grises y anilladas disminuían

al aumentar las concentraciones de organoclorados, por lo que dichos niveles fueron propuestos como potenciales biomarcadores de la disminución de las reservas de vitamina A.

Aunque el indicador más sensible y consistente de los efectos asociados a los contaminantes sea, probablemente, la reducción de las reservas hepáticas, la estimación de las variaciones de los niveles en la grasa resulta, por las razones esgrimidas a lo largo de la presente tesis, mucho más conveniente en las poblaciones salvajes de mamíferos marinos. Así, se investigaron las relaciones entre PCBs y DDTs y retinoides en la grasa de delfín mular de Sarasota y de delfín común del noroeste peninsular, siendo los datos aportados los primeros disponibles en la literatura sobre la alteración de retinoides por exposición a contaminantes en cetáceos.

Los resultados obtenidos difirieron entre las especies estudiadas y, dentro de cada una de ellas, entre individuos de distinto sexo. En el caso de las hembras de delfín mular, las relaciones entre contaminantes y retinoides no fueron halladas significativas. Esto podría atribuirse a que las concentraciones de contaminantes (medias de 8.63 μg g-1de DDT y 13.46 μg g-1de PCB) no eran lo suficientemente elevadas como para provocar una alteración en los niveles de retinoides. En contraste, los machos de esta especie, con una mayor carga de contaminantes (medias de 70.36 μg g-1de DDT y 101.97 μg g-1de PCB), presentaban una relación cuadrática negativa y significativa entre ambas variables, lo cual parece indicar que las concentraciones de retinoides no resultaban afectadas a concentraciones bajas de contaminantes pero sí lo eran, disminuyendo, cuando los contaminantes alcanzaban ciertos umbrales, concretamente a partir de 40 μg g-1de DDT y 50 μg g-1de PCB. Así, por debajo de estas concentraciones de organoclorados, los valores de retinoides encontrados en ambos sexos corresponderían a la variabilidad natural de la población.

En el delfín común, en cambio, en los machos se observa una relación significativamente positiva entre retinoides y contaminantes. Nuevamente, es probable que las concentraciones determinadas en estos animales (medias de 5.41 μg g^{-1}de DDT y 21.31 μg g^{-1}de PCB) fueran insuficientes para mostrar efecto alguno en los retinoides, al hallarse muy por debajo de los umbrales de respuesta fisiológica anteriormente citados para el delfín mular. En este contexto, la relación positiva observada sería en realidad el resultado de la dependencia de ambas variables (concentraciones de OCs y retinoides) con la edad, y las tendencias de incremento detectadas reflejarían patrones

naturales de variación, sin que existiera necesariamente una interacción potenciadora entre ellas. Sin embargo, recientes estudios han mostrado que los efectos depresores de las concentraciones de retinoides pueden aparecer a niveles de exposición tanto altos como bajos (Nyman *et al*., 2003), lo que podría asociarse con un proceso de hormesis, es decir, con las sobrecompensaciones que siguen a la alteración de la homeostasis. Así, la hormesis se caracterizaría por una estimulación de la respuesta a dosis bajas y una inhibición a dosis altas (Calabrese & Baldwin, 2003).

Los resultados obtenidos en las hembras de delfín común son todavía más difíciles de interpretar, puesto que se encontró una relación negativa entre retinoides y contaminantes, aún a pesar de que los niveles de contaminantes detectados son extremadamente bajos (medias de 2.53 $\mu g\ g^{-1}$de DDT y 9.42 $\mu g\ g^{-1}$de PCB). Atendiendo a los umbrales antes mencionados, dichos niveles son aparentemente incapaces de producir una alteración notoria en los niveles de retinoides. Por este motivo, la explicación más plausible a los resultados aquí obtenidos debería buscarse en el contexto del ciclo reproductivo de los individuos y de la proximidad temporal del muestreo de los tejidos con relación a la lactancia. Aunque no hay información sobre la transferencia de retinoides en delfines, los estudios en pinnípedos han mostrado que durante la lactancia se traspasan grandes cantidades de esta vitamina a las crías (Schweigert *et al*., 1987, 2002; Debier *et al*., 2002). Igualmente, como se ha comentado, existe un importante traspaso de organoclorados y otros contaminantes lipófilos de madres a crías (Aguilar *et al*., 1999). Dado que los mecanismos de transferencia a la leche materna de ambos tipos de compuestos son diferentes (Schweigert *et al*., 1987; Schweigert & Stobo, 1994), es posible que las tasas de transferencia difieran sustancialmente. Como el principal periodo de reproducción de los delfines comunes del Atlántico ocurre durante los meses de Mayo y Junio (Collet, 1981), y la mayoría de las muestras utilizadas en esta tesis fueron recogidas entre Julio y Septiembre, es muy probable que las concentraciones de retinoides y organoclorados estén afectadas por la actividad reproductiva, un extremo que no pudo determinarse debido a limitaciones logísticas durante el muestreo a bordo de los barcos pesqueros, pero que merece ser objeto de futuras investigaciones.

Por otro lado, dadas las aparentes diferencias halladas en la respuesta de las dos especies de delfines, habría que considerar si existen diferencias sustanciales específicas a cada especie en la sensibilidad a la interferencia de los retinoides por la exposición a PCBs y DDTs, como ha sido demostrado en otros animales (Fletcher et al., 2001;

Nyman et al., 2003). De ser así, la extrapolación de los resultados entre especies debería tratarse con especial precaución.

Puede de este modo concluirse que los resultados de estas investigaciones no permiten afirmar de manera inequívoca que exista una relación causa-efecto entre exposición a contaminantes organoclorados y disminución de retinoides corporales. Este efecto sólo ha podido observarse en machos de delfines mulares y a partir de un umbral moderado-alto de exposición a los organoclorados. En los otros grupos muestrales investigados este efecto no se observó, aunque no ha podido clarificarse si la ausencia del mismo fue debida a una insuficiente exposición a los contaminantes o a una diferente sensibilidad a éstos por parte de las especies y colectivos analizados. En este sentido, se recomienda extender las investigaciones a otras especies de odontocetos, concentrándolas en los individuos machos adultos (con concentraciones más altas) y en poblaciones sometidas a unos niveles de exposición superiores a los umbrales de respuesta aquí determinados. Únicamente de este modo será posible discernir si las variaciones de retinoides observadas son originadas por factores naturales o son un efecto directo de la contaminación, una cuestión central que debe responderse previamente a la utilización de los retinoides como biomarcadores de exposición a los contaminantes organoclorados.

7. CONCLUSIONES

1) En los mamíferos, los niveles sanguíneos de retinoides no son buenos indicadores de las alteraciones corporales de estos compuestos puesto que, debido a la regulación homeostática a la que están sometidos, sólo comienzan a disminuir cuando las concentraciones en los órganos de reserva son anormalmente bajas.
2) En cetáceos, al igual que se ha observado en pinnípedos y en mamíferos terrestres, el hígado es el tejido que presenta las concentraciones de retinoides más altas. Sin embargo, el hígado de los cetáceos sólo almacena algo más del 50% de las reservas corporales totales de retinoides mientras que, en mamíferos terrestres, este órgano acumula hasta el 90%.
3) En los cetáceos, la grasa hipodérmica constituye un depósito de retinoides tan importante como el hígado debido a que sus concentraciones de estos compuestos son suficientemente elevadas y a que este tejido representa una considerable proporción de la masa corporal. Ambos tejidos son buenos indicadores del estatus de retinoides de los delfines, puesto que sus concentraciones están estrechamente correlacionadas.
4) Los tejidos de otros órganos corporales, como el riñón, el pulmón, el músculo y el corazón no son adecuados para evaluar el estatus de retinoides en cetáceos debido a que sus concentraciones de estos compuestos son extremadamente bajas.
5) La grasa es el tejido más aconsejable para evaluar el estatus de retinoides en cetáceos ya que, a diferencia del hígado, es más resistente a la descomposición y puede ser fácilmente obtenido a partir de ejemplares vivos por medio de técnicas no destructivas de biopsia, hoy ampliamente experimentadas.
6) Los niveles de retinoides varían sustancialmente entre las distintas regiones de la grasa hipodérmica de los delfines, de la misma manera que lo hace su contenido lípidico. La relación entre ambas variables parece consecuencia clara del carácter lipófilo de los retinoides. Así, la mayor cantidad de lípidos presente en la región anterior-ventral del cuerpo, probablemente debida a la necesidad de proteger a las vísceras, parece ser responsable de la mayor deposición de retinoides en esa zona.

7) Para garantizar la consistencia entre diferentes estudios, los protocolos de recolección de muestras de grasa deben ser diseñados teniendo en cuenta la significativa fuente de variabilidad que supone la posición corporal de donde se extrae el tejido. Igualmente, las muestras de grasa deben incluir todas las capas para evitar las heterogeneidades provocadas por la estratificación del tejido.
8) Las muestras de grasa procedentes de delfines capturados muertos son válidas para el análisis de retinoides al menos hasta 48 horas post-mortem y siempre que las condiciones ambientales no permitan que los potenciales agentes de degradación (luz, temperatura y oxígeno) afecten significativamente las concentraciones de estos compuestos.
9) Los cetáceos hallados varados no son recomendables para evaluar el estatus de retinoides, ya que estos animales son normalmente localizados con posterioridad a las 48 horas desde su muerte y después de haber pasado un tiempo expuestos directamente a la luz solar, lo que puede afectar la estabilidad de los retinoides. Además, los animales varados pueden haber sufrido enfermedades largas y que hayan comportado variaciones en la dieta y el estado nutritivo, habiéndose por este motivo alterado los niveles de retinoides.
10) Las variables biológicas estudiadas (sexo, edad y condición nutritiva) influyen en las concentraciones de retinoides, aunque de manera distinta según la especie y el colectivo examinados. Por este motivo, al igual que ocurre en otros mamíferos, es difícil establecer patrones generales para los odontocetos. A pesar de los variables resultados, sí puede concluirse que las concentraciones de retinoides y lípidos en los tejidos de cetáceos están íntimamente relacionadas, que existen importantes variaciones entre machos y hembras que están probablemente asociadas a diferencias en la dieta, y que la tendencia más consistentemente observada es la de un aumento de los niveles de retinoides con la edad.
11) El efecto de las variables sexo y edad sobre las concentraciones de contaminantes organoclorados se ajusta al patrón observado habitualmente en cetáceos: un aumento de los niveles con la edad en los machos y una disminución en las hembras, aunque en éstas los niveles pueden aumentar de nuevo al envejecer debido al progresivo incremento de los intervalos de tiempo entre ciclos reproductivos. En cuanto al contenido lipídico, disminuye con la edad en los machos por lo que, a pesar de la lipoficilidad de los PCBs y los

DDTs, la reducción de la cantidad de lípidos no va acompañada de una disminución de la carga de contaminantes.

12) La alta carga de contaminantes traspasada de madres a crías durante la lactancia parece ser una de las principales causas de la elevada tasa de mortalidad observada en las primeras crías en la población de delfines mulares de Sarasota.

13) Cuando las concentraciones de los contaminantes estudiados son relativamente bajas (menores de 40 μg g^{-1}de DDT y de 50 μg g^{-1}de PCB), éstos no parecen afectar los niveles de retinoides presentes en la grasa de los pequeños cetáceos. No obstante, puede especularse que la movilización de las reservas hepáticas de retinoides producida tras la exposición a los contaminantes podría conllevar un aumento de los niveles de retinoides en otros tejidos, como la grasa. Sólo cuando las concentraciones de DDT y PCB superan los umbrales citados, los retinoides comenzarían también a movilizarse desde la grasa, observándose entonces una disminución de sus concentraciones en este tejido.

14) Es probable que existan diferencias especie-específicas en la respuesta a la exposición a los contaminantes, por lo que la extrapolación de los resultados entre especies debería tratarse con especial precaución.

15) No puede afirmarse con certeza que los contaminantes organoclorados afecten los niveles de retinoides de las poblaciones estudiadas. Se recomienda que los futuros estudios sobre la respuesta toxicológica de retinoides en cetáceos se centren en los machos adultos, cuyas concentraciones de contaminantes son mayores que las de los otros colectivos poblacionales y su actividad reproductiva no implica tantas variaciones como en las hembras. Además, el esfuerzo investigador debería concentrarse en poblaciones sometidas a unos niveles de exposición superiores a los umbrales de respuesta antes mencionados.

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