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HARBOR PORPOISE THYROIDS: HISTOLOGIC INVESTIGATIONS AND POTENTIAL INTERACTIONS WITH ENVIRONMENTAL FACTORS

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ABSTRACT: The thyroid plays an important role in development and is of primary importance in metabolism and heat loss for cetaceans, including the harbor porpoise (*Phocoena phocoena*). Several studies have demonstrated that environmental contaminants can alter various aspects of thyroid function in mammals and may contribute to various histologic changes. The present study completes the data set of a 2006 study by Das et al., by performing histological and immunohistologic investigations on thyroids of 36 harbor porpoises from Belgian and United Kingdom waters. The number and mean diameter of follicles (μm) and the relative proportion of follicular, connective, and vascular tissue (%) were quantified in the thyroid gland of each individual. Interfollicular fibrosis has been observed in these thyroid glands, and the collective findings support the hypothesis of an endocrine disruption of thyroid function through organochlorinated compounds. Our study aimed also to reveal potential relationships between thyroid morphometric data and metal levels (Cd, Fe, Zn, Cu, Se, and Hg) using multivariate statistical analysis. The multiple regressions revealed statistically significant relationships between trace elements (cadmium, selenium, and copper) and thyroid fibrosis. The largely negative relationships are interesting findings but do not support the hypothesis that these elements have an adverse effect on thyroid morphometry. Further research is needed to understand the nature of any relationship between organochlorine and trace element exposure and thyroid gland morphology and function in harbor porpoises.

Key words: Endocrine disruption, harbor porpoise, metals, organochlorine, *Phocoena phocoena*, thryroid.

INTRODUCTION

The harbor porpoise (*Phocoena phocoena*) is a native cetacean species in the North Sea (Benke et al., 1998). Along with the impacts of by-catch and reduced prey by overfishing, concern is growing about the adverse effects of environmental pollution on this marine mammal species (Reijnders, 1994; Siebert et al., 1999). The thyroid of marine mammals is a bilobated gland located on both sides of the larynx (Slijper, 1973). As with other vertebrates, the thyroid gland is composed of thyroid follicles that synthesize and store thyroid hormones (Bloom and Fawcett, 1975; Jubb et al., 1993; Junqueira et al., 1995; Feldman and Nelson, 1998). Several studies have demonstrated that environmental contaminants can alter various aspects of thyroid function (Hutchinson et al., 2000). The thyroid hormones contribute to the regulation of metabolism and growth, cell differentiation, and the development and function of the immune system (Woldstad and Jenssen, 1999). In cetaceans they are also believed to play an important role in controlling heat loss (Gregory and Cyr, 2003). Small cetaceans such as the harbor porpoise are obliged to remain active to maintain body temperature (Worthy and Edwards, 1990).

During recent years it has become evident that many xenobiotic chemicals may act as endocrine disrupters (Hutchinson et al., 2000). Endocrine-disrupting chemicals consist of synthetic and naturally occurring chemicals that affect the balance of normal hormonal functions in animals (Keith, 1997). Endocrine disrupters interfere with the functioning of the endocrine system in at least three possible ways (Damstra et al., 2002), including mimicking the action of a naturally produced hormone, such as oestrogen, testosterone, or thyroid hormones, and thereby setting off similar chemical reactions in the body (Hutchinson et al., 2000; Baker, 2001); blocking the receptors in cells receiving the hormones (hormone receptors), thereby preventing the action of normal hormones (Hutchinson et al., 2000; Gelbke et al., 2004); or by affecting the synthesis, transport, metabolism, and excretion of hormones, thus altering the concentrations of natural hormones (Zhou et al., 2000; Ishihara et al., 2003).

The thyroid gland represents one of the major target organs of endocrine disruptors (Brouwer et al., 1999). Synthetic chemicals and trace elements can disrupt nearly every step in the production and metabolism of thyroid hormones. They can interfere with uptake of iodine and cause inhibition of the peroxidase enzymes, displacements of the hormones from the transport proteins, and disruption of the hormone metabolism by influencing deiodinase, glucoronidase, and sulfatase activity (Howdeshell, 2002).

Organohalogens such as polychlorinated biphenyls (PCBs), pesticides (e.g., DDT, DDE), polybrominated diphenyl ethers (PBDEs), and chlorinated paraffins (CPs) are well-described endocrine disruptors (Damstra et al., 2002). Numerous studies have reported the presence of pesticide residues and metabolites, organochlorinated compounds, and other environmental compounds in a variety of tissues and species of marine mammals (Gregory and Cyr, 2003). Relationships between thyroid function and the concentration of organochlorine compounds are reported in wildlife animals. Significant decreases of T_3 and T_4 were found in sea lions in relation with PCBs and DDTs (Debier et al., 2005), in polar bears in relation with PCBs (Skaare et al., 2002), and in seals in relation with PCBs (Brouwer et al., 1989) and PBDEs (Hall et al., 2003).

Beside these results, several field studies reported alterations in thyroid gland morphology probably accompanied with impairment of thyroid function in marine mammals associated to exposure to persistent organic pollutants. Histologic examinations of 40 thyroid glands from harbor seals that died during the epizootic of phocine distemper infection in the North Sea (1988–89) exhibited colloid depletion and fibrosis that have been associated with chronic PCB exposure (Schumacher et al., 1993). Morphologic changes in thyroid gland have also been reported in beluga (*Delphinapterus leucas*) inhabiting the St. Lawrence estuary that have very high levels of organochlorine pollutants (De Guise et al., 1995). These results were similar to those of experiments with rats (Byrne et al., 1987) and seals (Brouwer et al., 1989) fed directly with PCBs. The effects of PBDEs, PCBs, and CPs on the thyroid are well documented in rats. Histopathological changes were reported to be associated the decrease of circulating thyroid hormones, especially T_4 (Hallgren and Darnerud, 2002). A relationship between PCBs, PBDE, DDE, and DDT compounds and interfollicular fibrosis has also been reported in the thyroids of harbor porpoises (Das et al., 2006b).

In contrast, fewer studies have focused on potential relationships between essential (zinc [Zn], copper [Cu], iron [Fe], selenium [Se]), and nonessential metals (cadmium [Cd] and mercury [Hg]) and thyroid histology. Essential and nonessential metals may also interact with the thyroid (Rolland, 2000). The cellular mechanisms involved in thyroid pathology are poorly understood. Generally the trace elements act at multiple sites via multiple mechanisms of action. These elements play a physiologic role in the metabolic regulation(s) of a thyroid disorder and can intervene in the secretion and distribution of thyroid hormones (Tsou et al., 1993;

Gupta et al., 1997b). They can stimulate or inhibit the secretion via the pituitary by inhibiting other hormones to connect with the corresponding receptors on the pituitary cell membranes (Oliver, 1975; Esipenko and Marsakova, 1990; Bedwal and Bhuguna, 1994; Goel et al., 1994; Kralik et al., 1996). Trace elements can also affect the hepatic iodothyronine deiodinase activity preventing the conversion of T_4 to T_3 (Arthur et al., 1991; Kralik et al., 1996; Gupta et al., 1997a; Gupta and Kar, 1998) or may accelerate the iodine depletion of thyroid (Wu et al., 1995). The adverse effect of these trace elements can be observed at several endpoints, such as a decreased thyroid hormone concentration in the plasma and peripheral tissues (Oliver, 1975; Kawada et al., 1980; Nishida et al., 1986; Esipenko and Marsakova, 1990; Ghosh and Bhattacharya, 1992; Goel et al., 1994; Nishijo et al., 1994; Pavia J'unior et al., 1997; Gupta and Kar, 1999; Zimmermann et al., 2000), reduced thyroid gland volume and weight, or the thyroid may show changes of atrophy and degeneration in the follicles (Oliver, 1975; Zimmermann et al., 2000).

There are indications that zinc is important for normal thyroid homeostasis. Its roles are complex and may include effects on both the synthesis and mode of action of the hormones. Thyroid hormone binding transcription factors, which are essential for modulation of gene expression, contain zinc bound to cysteine residues (Ruz et al., 1999). In the thyroid gland itself, transcription factor 2 (TF-2), which interacts with the promoters for the thyroglobulin and thyroperoxidase genes, is a zinc-containing protein (Tsou et al., 1993; Gupta et al., 1997b). Iron and copper status have also been linked to decreased plasma T_3 concentrations in animals and humans. It remains to be determined whether the changes in thyroid metabolism are a direct result of the iron and copper deficiencies or a nonspecific response to poor health (Oliver, 1975; Esipenko and Marsakova, 1990; Bedwal and Bhuguna, 1994; Goel et al., 1994; Kralik et al., 1996; Zimmermann et al., 2000). Selenium is a component of iodothyronine deiodinases, which transforms T_4 to T_3 in liver, kidney, muscle, and thyroid. It also plays a role in oxidative stress control at the thyroid as a component of the enzyme glutathione peroxidase (Arthur et al., 1991; Wu et al., 1995; Ruz et al., 1999). Cadmium alters the thyroid function at glandular as well as peripheral levels by preventing the conversion of T_4 to T_3 by inhibiting the iodothyronine deiodinase activity (Ghosh and Bhattacharya, 1992; Nishijo et al., 1994; Pavia J'unior et al., 1997; Gupta et al., 1997a; Gupta and Kar, 1998, 1999). Mercury is a toxic element with significant effects on many tissues, including the thyroid. It has been shown that moderate occupational exposure affects the enzyme deiodinase responsible for the deiodination of T_4 toT_3 (Kawada et al., 1980; Nishida et al., 1986; Ghosh and Bhattacharya, 1992).

Unlike their exposure to modern synthetic organic chemicals, the exposure of marine mammals to metals has occurred throughout history, during which they may have developed mechanisms either to control their concentration or to mitigate their toxic effects, such as the metallothioneins, which play an important role in the transport storage and detoxification of metals in vertebrates (Das et al., 2000, 2006a).

Recently Zn and Hg were found in high concentration in the livers of southern North Sea harbor porpoises; these high concentrations were linked to degrading body condition (Siebert et al., 1999; Bennett et al., 2001; Das et al., 2004). Questions arise about potential relationship between essential and nonessential metals and the thyroid histomorphometry.

The aims of the present study are 1) to evaluate the proportion of follicular, connective and vascular tissues in the thyroid of harbor porpoises collected around UK and Belgian waters by histomorphometry using the image acquisition software DP-

Soft; 2) to compare the observed histologic lesions with those previously observed in harbor porpoises from Germany, Norway, and Iceland (Das et al., 2006b); and 3) to use a multivariate analysis to investigate the potential relationships between thyroid histomorphometric parameters and previously described trace metal concentration in the liver (Zn, Fe, Cu, Se, Cd, and Hg) (Jepson, 2003; Das et al., 2004).

MATERIALS AND METHODS

Tissue sampling

Between 1998 and 2001 tissue samples (thyroid, liver) were collected from 113 porpoises from Belgian (n=46) and UK waters (n=67). Post mortem examinations were performed according to standard protocols (Law, 1994). For each histologic section of the thyroid the state of preservation, the presence of artifacts, and the presence of lesions such as congestion, cystic lesions, hyperplasia, and interfollicular fibrosis was assessed. The sections presenting signs of autolysis were discarded from this study. Of the 36 best-preserved animals included in this study, 22 were male and 14 were female (comprising 13 adults, 16 juveniles, and seven neonates). Thirteen harbor porpoises were by-caught and 23 animals stranded (Table 1). The age was determined for 24 porpoises by counting the dental growth layers (Lockyer, 1995) or were classified in age classes (neonate, juvenile, and adult) according to their size and development of the gonads.

Histology and immunohistochemistry

Samples of the thyroid glands were fixed in 10% formalin, processed by conventional techniques, then embedded in paraffin wax at 60 C for histologic and immunohistohistochemical investigations. Paraffin wax-embedded tissue sections (5 μm) were stained with haematoxylin and eosin (HE) and by elastic van Gieson for the detection of collagen (Siebert et al., 2002).

For immunohistochemistry a polyclonal rabbit antihuman thyroglobulin antibody (Code No. A 0251, DAKO Corporation Hamburg, Germany) and the Avidin-Biotin-Peroxidase complex method were used as described previously (Baumgärtner et al., 1989). The blocking serum used was from a goat (PAA Laboratories GmbH, Pasching, Austria). The polyclonal antibody against thyroglobulin was used in a solution of 1:2,600 in TBSc (900ml 0.85% NaCl, 100ml 0.05M Tris-Buffer, 37ml 1N HCL, 2.5ml Triton x-405, Aquadest, pH 7.6). A biotinylated anti-rabbit-immunoglobulin (Vector Laboratories Inc., BA 1000, Peterborough, UK) was used as a secondary antibody. The sections were then treated with avidin-biotin-peroxidase complex (Vector Laboratories Inc., PK 4000). As a negative control, thyroid gland sections were treated with a monoclonal antibody against the T-cell surface antigen of chicken lymphocytes (T1), which was used as control antibody. Previously positively stained sections were used as a control.

Scoring of the thyroid gland

For the histomorphologic analysis, images of 10 randomly selected visual fields in the microscope with a magnification of 200 of each section were observed. Thyroid histomorphology was measured using DP-Soft® software (version 3.2, Soft Imaging Systems GMBH) with a digital camera (Olympus C-4040 Olympus, Hamburg, Germany) connected to a light microscope (Olympus Statif CX 41 Olympus). The images showed a visual field of 633 μm in width and 475 μm in height. The proportion of different tissue types in the thyroid gland was determined by circumscribing the perimeter of the different tissue types (connective, follicular, and vascular tissue) present in the thyroids. The surface occupied by the follicular, vascular, and connective tissue was thus interactively measured, and the diameter and number of follicles present in each vision field were determined, and the mean value of these parameters from the 10 scored fields was used for statistical analyses.

Integration of previously published data

Thyroid histomorphometric measurements collected previously on porpoises from German (n=31; 24 from the Baltic Sea and seven from the North Sea), Norwegian (n=14), and Icelandic (n=11) waters and presented in Das et al. (2006b) were integrated in the study after intercalibration. This increased the sample size and statistical power for the analysis investigating potential relationships between thyroid parameters and trace element concentrations.

Trace metal results were extracted from larger studies presented previously (Das et al., 2003; Jepson, 2003; Das et al., 2004). Briefly, atomic absorption spectrophotometry (ARL 3510, Thermo Scientific, Breda, The Netherlands) was used to determine Cu, Zn, Fe, and Cd concentrations. Mercury was analyzed by flameless atomic absorption spectrophotometry (Perkin-Elmer MAS-50A Perkin-Elmer

Massachusetts, USA). Selenium was analyzed by fluorimetry. Concentrations are expressed as $\mu g\ g^{-1}$ dry weight.

Statistical analyses

Statistical analysis of the data was performed using Statistica® software (Statsoft Inc., version 7.1 Statsoft, Maison-Alfort, France). The Kolmogorov-Smirnoff test was used to test for normality of the statistically treated variables of thyroid morphology (the surface area occupied by follicular, vascular, and connective tissue and the diameter of follicles) and the hepatic trace metal concentrations (Zn, Cu, Fe, Se, Cd, and Hg). The trace metal concentrations had been log-transformed to normalize their distribution. The nonparametric Mann-Whitney *U*-test followed by Fisher's Omnibus post hoc tests were used to compare differences among sexes, age categories (neonate, juvenile, and adult), geographic origin (Belgian and UK waters), and cause of death (by-catch and stranding).

Sources of the potential differences between histologic quantification methods of thyroid tissues were analyzed by Wilcoxon test, which permitted us to evaluate systematic differences. The effect size, which in statistics is a measure of the strength of a relationship between two variables, has been extracted from the ANOVA method. It allowed us to know along with the statistically significance of the differences in evaluation methods the size of any observed effects. Thus we evaluated the size of the variance due to the quantification method, in other words, the size of the effect due to the differences in evaluation methods. A Spearman correlation permitted to evaluate the relation of the tissue definition concepts between the two evaluation methods.

Intersite comparison was realized using discriminant analysis to asses the ability of thyroid parameters and trace metals to discriminate among the different collection locations (Iceland, Norway, Germany [North Sea and Baltic Sea], UK, and Belgium). Multiple regressions were performed to examine the relationship between the hepatic trace metal concentrations and the thyroid parameters (connective tissue proportion and mean follicle size). Results were judged significant when $P<0.05$.

RESULTS

Histology

Using light microscopy, irregular or oval follicular lumens were seen in the parenchyma of the thyroid, surrounded by follicular epithelial cells. Follicular epithelial cells were often invaginated into the follicular lumen (Fig. 1). The follicular cells varied in height and shape and were commonly low cuboidal to flattened. Nuclei were spherical, central, poor in chromatin, and contained one or more nucleoli (Fig. 2). The follicles were surrounded by a variable layer of connective tissue and blood vessels. Three tissue compartments were distinguished in the thyroid gland: the follicular tissue (comprising follicular epithelial cells and colloid), the vascular tissue, and the connective tissue.

Rabbit polyclonal antibody human thyroglobulin, cross-reacted with the thyroid of harbor porpoises (Fig. 3). Thyroglobulin was detected in the lumen of the follicles and in the follicular epithelial cells. In some histologic sections the color was also disseminated in the parenchyma, probably a consequence of autolysis.

Tissue proportions

The follicular tissue occupied a mean surface of 70% of the total thyroid surface (ranging from 54% to 84%), whereas the vascular tissue occupied a surface of 20% (ranging from 2% to 33%) and the connective tissue a surface of 10% (ranging from 1% to 24%). The follicular lumens were larger in the central than in the peripheral regions of the gland. The diameter of follicular lumens ranged from 40 to 192 μm (Table 2). No differences in tissue proportions were observed between the harbor porpoises from Belgian and the UK waters.

Thyroid histomorphometric measurements collected previously on porpoises from German, Norwegian, and Icelandic waters (Das et al., 2006b) were integrated into this study to increase the sample sizes for the statistical analysis investigating potential relationships between these parameters and trace metal concentrations. To intercalibrate these two studies, we quantified and compared the three different tissues (follicular, connective, and

TABLE 1. General data of the sampled harbor porpoises (*Phocoena phocoena*) from Belgian and UK waters.

Collection country	Found date	Sex	Age	Age category
Belgium	23/06/2000	M	Unknown	Adult
Belgium	29/11/1999	M	8	Adult
Belgium	13/03/2000	F	1	Juvenile
Belgium	8/12/2000	F	Unknown	Juvenile
Belgium	24/03/2001	M	Unknown	Juvenile
Belgium	26/06/2002	F	Unknown	Neonate
Belgium	19/03/2002	F	Unknown	Juvenile
Belgium	2/09/2002	M	Unknown	Adult
Belgium	3/05/1999	M	Unknown	Juvenile
Belgium	30/06/1999	M	Unknown	Neonate
Belgium	15/02/1999	M	1	Juvenile
United Kingdom	29/01/1997	F	7	Adult
United Kingdom	13/02/1999	M	0	Juvenile
United Kingdom	04/03/1999	F	4	Adult
United Kingdom	05/11/2000	M	1	Juvenile
United Kingdom	21/11/2000	M	Unknown	Adult
United Kingdom	29/02/2000	F	1	Juvenile
United Kingdom	13/03/1998	F	0	Juvenile
United Kingdom	14/03/2000	F	Unknown	Adult
United Kingdom	13/04/1999	M	2	Juvenile
United Kingdom	09/04/1998	M	2	Adult
United Kingdom	01/06/1992	M	0	Neonate
United Kingdom	18/04/1997	F	0	Juvenile
United Kingdom	04/03/1993	M	1	Juvenile
United Kingdom	09/03/1993	M	1	Juvenile
United Kingdom	24/06/1992	M	0	Neonate
United Kingdom	06/06/1997	M	0	Neonate
United Kingdom	23/09/1992	M	15	Adult
United Kingdom	21/06/2001	M	Unknown	Adult
United Kingdom	07/07/1998	F	Unknown	Adult
United Kingdom	11/07/2000	M	0	Neonate
United Kingdom	23/07/1998	M	0	Neonate
United Kingdom	12/07/2001	F	Unknown	Adult
United Kingdom	05/12/1997	F	Unknown	Adult
United Kingdom	18/12/1995	M	1	Juvenile
United Kingdom	25/04/1991	F	1	Juvenile

vascular tissue) by DP-Soft in 10 thyroid sections that have been analyzed by the previous method (Table 3). Because vascular tissue was previously integrated in follicular tissue, only connective tissue proportions were comparable between the studies (Table 4). The approximate mean value from the follicle size was used to compare the mean follicle diameter of the harbor porpoises collected on Belgian, UK, German, Norwegian, and Icelandic waters.

Collection location differences were explored by discriminant analysis to simultaneously evaluate similarities in the thyroid parameters (connective tissue proportion and mean follicle size) and hepatic element concentrations (Zn, Cu, Fe, Se, Cd, and Hg) among the porpoises sampled at the six locations. Three porpoise groups could be distinguished by their connective tissue proportion, and the Cd, Zn, and Fe values (given in order of decreasing importance). The first discriminant function (root) explained 69% of the variations between groups involving mostly the connective tissue proportion, and the second discriminant function explained a further 17% of the variation between groups, involving the

TABLE 1. Extended.

Weight	Length	Blubber thickness	Cause of death	Place found	Location
58	160	5	Unknown	Nieuwpoort	Belgium
43.4	144	14	Unknown	Koksijde	Belgium
21.5	114	20	Unknown	Westende	Belgium
29	114	20	Unknown	Koksijde	Belgium
17	110	6	Unknown	Wenduine	Belgium
7	77	8	No bycatch	Knokke-Heist	Belgium
32	130	15	Infection	Oostende	Belgium
37	137	8	Infection	Oostende	Belgium
15	108	8	Unknown	Blankenberge	Belgium
7.4	80	6	Unknown	Middelkerke	Belgium
25.5	101	25	Unknown	Nieuwpoort	Belgium
50.5	154	14	Bycatch*	Bridlington Bay	Humberside
30	124	17	Bycatch	West Looe	Cornwall
50.8	143	21	Pneumonia, parasitic, and bacterial	Westminster Bridge	Greater London
24	109	22	Bycatch*	BYC off Bridlington	Humberside
38	148	7	Pneumonia, parasitic, and bacterial	Woolacombe Bay	Devon
18.2	109	9	Pneumonia, parasitic	Blyth	Northumberland
36	131	22	Live stranding	Westward Ho	Devon
48.5	148	22	Pneumonia, unknown aetiology	Battersea Bridge	Greater London
36.8	122	23	Bycatch*	BYC Minsmere Sluice	Suffolk
35.5	138	18	Bycatch*	Scarborough	North Yorkshire
8	81	12	Physical trauma	Withernsea Beach	Humberside
26	110	26	Bycatch*	Cromer Point	North Yorkshire
28	119	23	Bycatch	Sunderland	Tyne and Wear
24	113	16	Generalized bacterial infection	Bognor Regis	West Sussex
7.5	78	12	Starvation (neonate)	Isle of Sheppey	Kent
8	90	9	Starvation (neonate)	Gt. Yarmouth	Norfolk
40.5	136	19	Bycatch	Tresaith	Dyfed
43.8	136	19	Bycatch*	BYC off Bridlington	Humberside
45	154	10	Generalized bacterial infection	Sea Palling	Norfolk
9.5	88	12	Physical trauma	Rhos-on-Sea	Conwy
9	84	13	Starvation (neonate)	Snettisham	Norfolk
37	136	12	Bycatch*	BYC off Bridlington	Humberside
51	156	12	Starvation	Whitley Bay	Tyne and Wear
26.2	127	16	Bycatch	South Shields	Tyne and Wear
25	120	17	(Meningo) encephalitis	Mablethorpe	Lincolnshire

Cd, Zn, and Fe concentrations. Together, the two discriminant functions explained 86% of the variance (Table 5). As previously described, porpoises from Iceland are clearly separated from the other locations by the decreased proportion of connective tissue in their thyroids and the high Cd values (Das et al., 2004, 2006b). Porpoises from Norwegian and German North and Baltic Seas are situated close together in the diagram and could not be discriminated based on thyroid parameters and metal values. Porpoises from UK and Belgian waters were clearly discriminated from the other porpoises by their small connective tissue proportion and their high Zn values.

Relationships between histologic thyroid parameters and environmental factors

We evaluated different factors that could be implicated in the etiology of alterations of the thyroid histology. No significant relationship was observed between sex, age category, origin, cause of death, and the thyroid morphology (Mann-Whitney followed by Fisher's Omnibus test, $P>0.05$). No significant relationship was observed between sex, origin, cause of death, and trace element con-

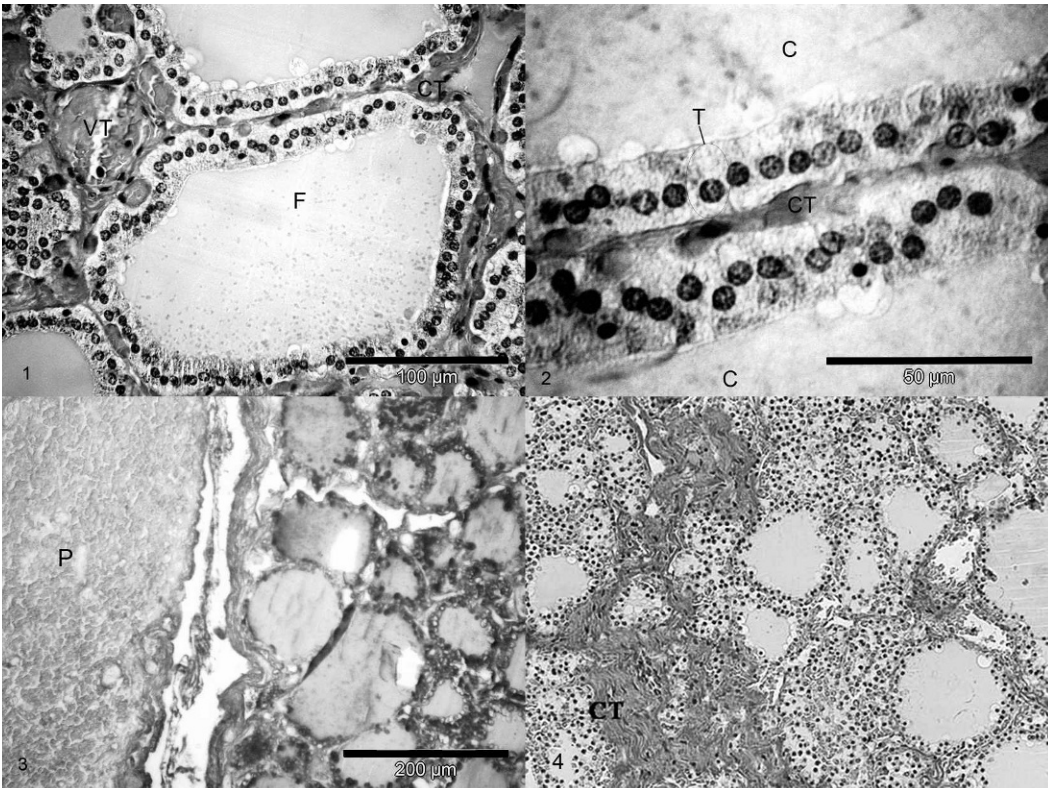

FIGURE 1. Thyroid gland of a harbor porpoise from Belgian waters. F: follicle is surrounded by connective tissue (CT) and some vascular tissue (VT) (Van Giesson staining, magnification 400×, scale bar = 100 μm). FIGURE 2. Follicular epithelium in a thyroid gland of a harbor porpoise from Belgian waters. C: colloid, CT: connective tissue, T: follicular epithelium (Van Giesson staining, magnification 1,000×, scale bar = 50 μm). FIGURE 3. Thyroglobulin specific reaction in the thyroid of harbor porpoise. Right: positive coloration of the thyroid tissue and left no coloration of the parathyroid tissues (immunohistochemical staining, magnification 200×, scale bar = 200 μm). FIGURE 4. Thyroid gland of a harbor porpoise showing a severe fibrosis (Van Giesson staining, magnification 200×, scale bar = 200 μm).

centration (Mann-Whitney followed by Fisher's Omnibus test, $P>0.05$). We observed a significant relationship between age category and trace element concentration (Mann-Whitney followed by Fisher's Omnibus test, $P<0.05$). The Se, Hg, and Cd concentrations increase with the age of the animals. A slight seasonal effect was observed with the thyroid parameters (Mann-Whitney followed by

TABLE 2. Morphometry of the thyroid gland in harbor porpoises from Belgian and UK waters.[a]

	n	Mean	Min	Max	SD
Follicular tissue (%)	36	71.6	53.9	83.1	7.4
Connective tissue (%)	36	10.2	0.5	23.5	4.8
Vascular tissue (%)	36	18.2	1.5	32.8	7.5
No. of follicles	36	15.7	4.7	34	7.7
Mean diameter of follicles (μm)	36	90.3	40.2	191.6	37.5

[a] Data are given as number of samples (*n*), mean, range (minimum [Min] and maximum [Max]), and standard deviation (SD).

TABLE 3. Intercalibration between the quantification method used by Das et al. (2006b) and the method used in this study.

		Quantification without DP-Soft (Das et al., 2006)			Quantification with DP-Soft (this study)		
Animal	Origin	Follicular tissue %	Connective tissue %	Solid tissue %	Follicular tissue %	Connective tissue %	Vascular tissue %
1318	Baltic Sea	19	33	48	64	27	9
1348	Baltic Sea	16	16	68	74	16	9
1423	Baltic Sea	13	13	74	72	10	18
1493	Baltic Sea	17	42	42	47	47	6
1638	Baltic Sea	2	61	37	34	62	4
1662	Baltic Sea	3	48	48	64	27	9
1666	Baltic Sea	3	48	48	58	37	8
1670	Baltic Sea	42	17	42	68	27	5
1681	Baltic Sea	3	48	48	43	51	6
1715	Baltic Sea	56	22	22	67	18	15

Fisher's Omnibus test, $P<0.05$). The porpoises that stranded in winter had a higher proportion of connective tissue.

Relationships between organohalogenated pollutants and thyroid histomorphometry have been observed in the 36 harbor porpoises collected along the Belgian and British coasts (Schnitzler, 2005). These results support the findings of Das et al. (2006b) and the hypothesis of a contaminant-induced thyroid fibrosis in harbor porpoises. In this study we examined the relationship between selected trace metals on thyroid histologic parameters, such as the proportion of connective tissue and mean follicle size by multiple regressions. This analysis revealed a negative correlation between the connective tissue proportion and the Cd, Cu, and Se concentrations in the livers of harbor porpoises from UK, Belgian, German, Norwegian, and Icelandic waters ($P=0.0004$, $R^2=0.348$; Table 6).

DISCUSSION

The morphology of the harbor porpoise thyroid was similar to thyroid glands of other mammal species (Bloom and Fawcett, 1975; Jubb et al., 1993; Junqueira et al., 1995; Cowan and Tajima, 2006). Although the follicular lumina were shrunken in size because of fixation, on average they were larger than that of goat but seem to be smaller in comparison to larger ruminants (Shimokawa et al., 2002; Table 7). The follicular tissue occupied around 70% of the thyroid surface on section, the vascular tissue 20%, and the connective tissue 10%.

TABLE 4. Analysis of the sources of differences between the tissue quantification methods used in these two studies.

		Follicular tissue	Connective tissue	Solid tissue
Wilcoxon	V	32	34	30
	P-value	0.693	0.557	0.846
	N	10	10	10
Effect size	SSeffect (=SCTr)	573.306	30.836	311.008
	SSerror (=SCE)	3,592.172	343.013	4,545.377
	Effect size	0.138	0.082	0.064
Spearman correlation	r_s	0.443	0.841	0.138
	P-value$_{app}$	0.203	0.009	0.705
	10 cases; 10,000 permutations			
	Determination coefficient	0.196	0.707	0.019

TABLE 5. Summary of the discriminant analysis results for the two first principal components (PCs).[a]

	Component 1	Component 2
Cadmium	0.14	**−0.85**
Iron	−0.31	**−0.33**
Zinc	−0.37	**−0.54**
Copper	0.06	**0.27**
Selenium	0.47	**0.32**
Mercury	−0.23	**0.49**
Connective tissue	**−1.1**	−0.15
Follicle diameter	**−0.43**	0.1
Cumulative variance	0.69	0.86

[a] Boldfaced values refer to the main correlation between the PCs and the variables.

TABLE 6. Results of the multiple regression between the hepatic trace elements concentration and the proportion of connective tissue in the thyroid.[a]

	t-Value	*P*-Value	
OrdOrig.	1.3	0.2	*N*
Cadmium	−4.6	**0.01**	73
Iron	−0.3	0.74	75
Zinc	0.3	0.75	80
Copper	−2.1	**0.04**	78
Selenium	2	**0.05**	69
Mercury	−1.6	0.11	77

[a] $R^2 = 0.35$; $F(6.56) = 4{,}98$; p-value $= 0.0004$.

Generally low variability in the concentration of the serum thyroid hormones is observed across age season and sex (Rosa et al., 2007). In our study the thyroid histomorphology was consistent among age classes (neonate, juvenile, and adult) and sex. This supports the existence of strong homeostatic mechanisms for maintaining thyroid hormone concentrations in healthy animals (Rosa et al., 2007).

A slight seasonal effect has been observed on the proportion of the connective tissue, with winter-stranded porpoises having a higher proportion of connective tissue in the thyroid. This can be related to the fact that older animals were more represented in winter-stranded porpoises and had a better nutritional status than porpoises found dead in summer. St. Aubin and Geraci (1989) observed marked seasonal differences in thyroid histology of beluga and suggested that these differences related to increased water temperatures of seasonal occupied area. Such changes may be species specific, because such effects were not observed in bottlenose dolphins (St. Aubin et al., 1996) and could be related to the fact that the belugas undergo a seasonal migration (Richard et al., 2001). Most of the studied porpoises were juveniles, which maintain an active thyroid appearance during the different seasons (Rosa et al., 2007).

Compared to the results of our former study (Das et al., 2006b), we observe several geographic differences. Icelandic porpoises were characterized by a very small proportion of connective tissue (3%) and small follicle size, whereas the German and Norwegian porpoises displayed a high proportion of connective tissue (35%; Fig. 4) and larger follicles. Based on these differences, the porpoises could be separated into three groups (Fig. 5); Belgian and UK porpoises take an intermediary position between those two extremes. The size of the follicles and the shape of the follicular cells give an indication of the secretary activity of the gland. Thyroid glands dominated by small follicles lined by cuboidal or columnar cells can be classified as highly active, whereas low active glands show large follicles lined by low or flattened epithelial cells (Hallgren and Darnerud, 2002). Thyroids of the porpoises from the Icelandic coast could have a higher secretory activity than those from the German and Norwegian coasts.

The follicles are normally separated from each other by a fine irregular layer of connective tissue, mainly formed by reticular fibers. Also, some connective tissue can be associated to the vascular tissue (Junqueira et al., 1995; Cowan and Tajima, 2006). In our study the connective tissue proportion varied widely (1–23%) in the thyroids of the harbor porpoises collected along the Belgian and British coasts and occupies an intermediate mean position of 10%. This accumulation of

TABLE 7. Diameter of thyroid follicles in different mammals.

Species	Follicle diameter (μm)	Source
Mouse	41.8–52.6	Shimokawa et al. (2002)
Cat	56.0–66.4	Shimokawa et al. (2002)
Goat	89.7–102.8	Shimokawa et al. (2002)
Harbor porpoise	48.3–127.4	This work
Risso's dolphin	98.1–120.3	Shimokawa et al. (2002)
Cattle	169.1–192.0	Shimokawa et al. (2002)
Camel	155.3–240.7	Shimokawa et al. (2002)

connective tissue could be of pathologic origin. The collagen deposition and reduction of the initial vascularization in the newly formed scar tissue is a long process that takes weeks to months (Schumacher et al., 1993). Schumacher et al. (1993) showed that no morphologic signs of an increase in the collagen content occurred in his autolysis experiments, so that artificial swelling of the connective tissue due to autolysis can be excluded (Schumacher et al., 1993).

Schumacher et al. (1993) and Das et al. (2006b) related an observed colloid depletion and interfollicular fibrosis in seals and harbor porpoises, respectively, to elevated concentrations of organic contaminants (mainly PCBs, DDTs, and PBDEs). This relationship between organohalogenated pollutants and thyroid histomorphometry has also been observed in the 36 harbor porpoises collected along the Belgian and British coasts (Schnitzler, 2005). The Belgian and British harbor porpoises presented a lower concentration of organochlorinated pollutants (Mean sum of seven ICES PCBs±SD) in their blubber (1,780±1,370 and 1,990±1,553 ng g^{-1} lipid weight, respectively; Covaci et al., 2002; Jepson, 2003) compared to those from the German North and Baltic Sea coasts (7,664±5,075 and 8,247±7,949 ng g^{-1} lipid weight, respectively; Siebert et al., 2002) but still higher than those from Icelandic coasts (1,550±1,517 ng g^{-1} lipid weight; Siebert et al., 2002). These findings were therefore considered to support the hypothesis of an endocrine disruption of thyroid function mediated through chronic exposure to organochlorinated compounds (Covaci et al., 2002; Siebert et al., 2002; Jepson, 2003).

In contrast, the results of this study also demonstrated that hepatic Cd, Se, and Cu concentrations were negatively correlated with the proportion of connective tissue, thus failing to support the hypothesis that these metals may influence histologic changes in porpoise thyroid in a dose-dependent manner.

Our results also suggest that interfollicular fibrosis of the thyroid affect not only porpoises from the highly polluted Baltic Sea but also porpoises from other locations, including the Belgian and UK coasts. We have to emphasize that the high proportion of connective tissue measured in thyroid glands of the harbor porpoises from the German and Norwegian coasts indicates a severe pathologic dysfunction that, in other animals, results in a reduction of the thyroid function (Jubb et al., 1993; Schumacher et al., 1993). The effect of such dysfunction in the harbor porpoise remains poorly understood.

Thirty of the 36 analyzed Belgian and UK harbor porpoise thyroids had an interfollicular fibrosis. When these data were combined with earlier studies on thyroids of harbor porpoises from the German, Norwegian, and Icelandic coasts, the collective findings support the hypothesis of an endocrine disruption of thyroid function through organochlorinated compounds. The largely negative relationships found in this study between trace element (Cd, Se, and Cu) concentrations and histologic thyroid gland parameters (especially fibrosis) are interesting findings but do not support the hypothesis that these metals have an adverse affect on thyroid morphometry. Further research is needed to better understand the nature of any relationships between organochlorine and metals exposure and thyroid gland morphology and function in harbor porpoises.

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