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Comparative thyroidology: thyroid gland location and iodothyronine dynamics in Mozambique tilapia (*Oreochromis mossambicus* Peters) and common carp (*Cyprinus carpio* L.)

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Summary

In teleosts, the thyroid gland is mostly found in the subpharyngeal region. However, in several species thyroid follicles are found in, for example, heart, head kidney and kidney. Such heterotopic thyroid follicles are active, and considered to work in concert with the subpharyngeal thyroid. In Mozambique tilapia (*Oreochromis mossambicus*) thyroid activity is, indeed, restricted to the subpharyngeal region; in common carp (*Cyprinus carpio*) the functional endocrine thyroid is associated with renal tissues. The subpharyngeal follicles of carp comprise only 10% of the total thyroid tissue, and these follicles neither accumulate iodide nor synthesize or secrete thyroid hormones to a significant degree. Although the shape and size of carp subpharyngeal and renal follicles vary, the epithelial cell height of the thyrocytes and thyroxine immunoreactivity do not differ, which suggests that the activity of the carp subpharyngeal thyroid follicles is dormant. Differences in thyroid physiology between the two fish species were further assessed at the level of peripheral thyroid hormone metabolism. Carp clears plasma of thyroid hormones faster than tilapia does. Furthermore, a significant amount of conjugated thyroid hormones was observed in the plasma of tilapia, which was preceded by the occurrence of thyroid hormone conjugates in the subpharyngeal region and coincides with the appearance of conjugates in the surrounding water. Apparently, plasma thyroid hormone conjugates in tilapia originate from the thyroid gland and function in the excretion of thyroid hormones. Our data illustrate the variability in teleostean thyroidology, an important notion for those studying thyroid physiology.

Supplementary material available online at
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Key words: thyroid gland, iodothyronines, kidney, carp, tilapia, follicle, heterotopic, conjugates, excretion.

Introduction

The main products of the thyroid gland are thyroid hormones, the actions of which are pleiotropic and involve the regulation of metabolism, growth and development, including metamorphosis. Thyroid hormones are synthesized in thyroid follicles, the functional units of the thyroid gland, composed of thyrocytes enclosing a protein-filled colloid matrix. Thyroid-stimulating hormone (TSH) from the pituitary gland is the major stimulus for thyroid hormone synthesis and release (Blanton and Specker, 2007; Eales and Brown, 1993). Plasma thyroid hormone levels are not only determined by thyroid hormone synthesis and secretion but also by peripheral metabolism (*viz.* deiodination and conjugation), clearance and excretion of thyroid hormones. Thyroid hormones are generally excreted as glucuronide or sulphate conjugates via the bile (Finnson and Eales, 1996). Unlike the compact mammalian thyroid gland, the thyroid gland of most teleostean fish consists of non-encapsulated follicles scattered in the subpharyngeal region surrounding the ventral aorta (Gudernatsch, 1911). In several species of fish, however, heterotopic thyroid follicles, i.e. follicles located outside the typical subpharyngeal region, have been reported (Baker, 1958).

Heterotopic thyroid follicles can be found near or in the heart, spleen, liver, oesophagus, brain and choroid rete mirabile of fish, but are generally restricted to tissues that ontogenetically derive from renal primordia, *viz.* the head kidney (pronephros) and the adult kidney (opistonephros) (Baker, 1958). Thyroid heterotopia has been described in species throughout the Teleostei infraclass; it is found in representatives of the order of anchovies and herrings (Clupeiformes, 1 species), catfish (Siluriformes, 4 species), killifish (Cyprinodontiformes, 3 species), swamp eels (Synbranchiformes, 1 species), perch-like fish (Perciformes, 3 species), rainbow fish and silversides (Atheriniformes, 1 species), and minnows and suckers (Cypriniformes, 14 species). Interestingly, 13 of the 27 fish species in which heterotopic thyroid follicles have been

described belong to the family of carp and minnows (Cyprinidae), including species such as goldfish (*Carassius auratus*) and common carp.

Because of their ectopic nature, heterotopic thyroid follicles have often been interpreted as resulting from metastases (Berg et al., 1953; Blasiola et al., 1981; Nigrelli, 1952). Although thyroid hyperplasia and neoplasia have been described in teleostean fish (Fournie et al., 2005; Leatherland and Down, 2001), normal heterotopic thyroid follicles do not follow the diagnostic criteria for thyroid hyperplasia, adenoma or carcinoma as proposed by Fournie et al. (Fournie et al., 2005).

Most reports on heterotopic thyroid follicles in fish only describe the presence of heterotopic thyroid follicles without consideration as to quantitative or functional aspects (Agrawala and Dixit, 1979; Baker, 1958; Qureshi, 1975; Qureshi et al., 1978; Qureshi and Sultan, 1976; Sathyanesan, 1963). Extra-pharyngeal thyroid follicle populations have been reported to be less active than (Bhattacharya et al., 1976), of equal activity to (Frisén and Frisén, 1967) or more active than (Chavin and Bouwman, 1965; Peter, 1970; Srivastava and Sathyanesan, 1971) the subpharyngeal thyroid tissue. The general opinion is that these heterotopic follicles work in concert with the subpharyngeal thyroid and contribute to the thyroid status of an animal.

Since iodide is exclusively incorporated into thyroid hormones and its metabolites, the use of radioactive isotopes of iodide offers unique possibilities for the investigation of thyroid hormone dynamics. Autoradiography of the thyroid gland in Mozambique tilapia (*Oreochromis mossambicus* Peters) and common carp (*Cyprinus carpio* L.) serendipitously revealed differences in iodide metabolism, and pointed to the presence, in carp, of heterotopic thyroid tissue that is functionally different from that in the subpharyngeal region. This was the motivation for the studies described here.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) of the all-male E4×R3R8 isogenic strain (Bongers et al., 1998) were obtained from the Department of Fish Culture and Fisheries of Wageningen University, The Netherlands. Mozambique tilapia (*Oreochromis mossambicus* Peters) were obtained from laboratory stock. Fish were kept in 150 l tanks with aerated, recirculating city of Nijmegen tap water, at a photoperiod of 16 h light and 8 h darkness at 23°C for carp and 27°C for tilapia. Fish were fed commercial fish food (Trouvit, Trouw, Putten, The Netherlands) at a daily ration of 1.5% of their estimated body weight. Animal handling followed approved university guidelines.

Whole-body autoradiography

Juvenile carp and tilapia (standard length 6–8 cm) were exposed for 16.5 h to 250 μCi $Na^{125}I$, which was added to the aerated water in a 3 l tank, at 23 and 27°C, respectively. Thyrostatic potassium perchlorate ($KClO_4$) was added at a concentration of 1 mmol l^{-1}, and its effect on ^{125}I uptake was compared with that in an untreated group. After exposure, fish were deeply anaesthetized with 0.1% (v/v) 2-phenoxyethanol and killed by immersion in melting isoflurane (–70°C). Animals were embedded in 5% carboxymethyl cellulose and stored at –20°C, and whole-body crysosections were obtained according to a method described by Rijntjes et al. (Rijntjes et al., 1979). In short, a carboxymethyl cellulose block containing a specimen was mounted on the stage of a LKB 2250-PMV cryomicrotome (LKB, Stockholm, Sweden). Sections were collected with the aid of cellulose tape that was applied to the upper surface of the carboxymethyl cellulose block, and were freeze dried in the microtome for 24 h. Sections 30 μm thick were taken every 90 μm. Freeze-dried whole-body sections of the whole fish were placed on Biomax MR-1 X-ray film (Eastman Kodak Company, Rochester, NY, USA); films were exposed for 3 days at –70°C after which they were developed according to the manufacturer's protocol.

Injection procedure and sampling

Carp (102±14 g; *N*=24) and tilapia (117±17 g; *N*=24) were injected intraperitoneally (i.p.) with 20.3 μCi carrier-free $Na^{125}I$ (Amersham Biosciences, Amersham, Bucks, UK) per 100 g body weight. The ^{125}I specific activity was 82×10^{15} Bq mol^{-1} and the radiotracer was dissolved in 0.9% NaCl. After injection, fish were immediately transferred to individual tanks with 3.5 l aerated city of Nijmegen tap water. During the experiment, water was sampled and radioactivity was measured. Fish were sampled at set times after injection by adding 0.1% (v/v) 2-phenoxyethanol to the individual tanks to induce anaesthesia. Blood was sampled by puncture of the caudal vessels with a heparinized syringe fitted with a 23 G needle and plasma was collected after centrifugation at 4°C (4000 ***g***, 15 min). The fish were then killed by spinal transection and selected organs and tissues, as indicated in the figure legends, were collected. All tissues and the remaining carcass were weighed and the volume and weight of total bile and the collected plasma sample were determined. The radioactivities of bile and plasma were measured in an LKB 1272 Clinigamma γ-counter (Wallac, Turku, Finland) and immediately thereafter subjected to Sephadex LH-20 chromatography (see below). All tissues were homogenized according to Chopra et al. (Chopra et al., 1982) with an all-glass Potter-Elvehjem homogenization device in ice-cold 0.1 mol l^{-1} Tris-HCl buffer (pH 8.7), added at 6 ml g^{-1} tissue. Total radioactivity of the homogenates was determined as described for bile and plasma. The carcass was microwaved for 3 min at 800 W and homogenized in a blender in 200 ml 0.1 mol l^{-1} Tris-HCl buffer (pH 8.7). The resulting total volume was assessed and the radioactivity of sextuplicate 1 ml subsamples was determined.

Histochemistry

The subpharyngeal region, head kidney and kidney of four carp and tilapia (39.3±0.5 g) were fixed in Bouin's solution for 24 h. Tissues were dehydrated in a graded series of ethanol, embedded in paraplast and sectioned at 7 μm. Every 140 μm, two serial sections were collected and mounted on glass slides. Sections were stained with a modified Crossmon's connective tissue stain (Crossmon, 1937) as follows: 1.3 g l^{-1} Light Green SF yellowish (Chroma-Gesellschaft, Stuttgart, Germany), 1.7 g l^{-1} Orange G (Searle Diagnostic, High Wycombe, Bucks, UK) and 2 g l^{-1} acid fuchsin (Fuchsin S from Chroma-Gesellschaft) were dissolved in distilled water at 80°C. The solution was cooled

to room temperature, and 1 g of phosphotungstic acid hydrate was added to a 50 ml volume, followed by 2 ml glacial ethanoic acid and 100 ml absolute ethanol. The final solution was filtered and stored. Crossmon's trichrome stain was followed by a haematoxylin counterstain. Using this procedure, the colloid in thyroid follicles stains bright orange-red, which facilitates digital analysis of images.

Immunocytochemistry

Serial sections were incubated with 2% H_2O_2 and 10% normal goat serum in ice-cold phosphate buffer to inactivate endogenous peroxidase activity and to block non-specific antigenic sites. Sections were then incubated overnight with a polyclonal antiserum against human thyroxin (MP Biomedicals, Irvine, CA, USA) at a dilution of 1:5000. Then, sections were incubated for 1 h with a 1:200 dilution of biotinylated goat anti-rabbit secondary antibody (VectaStain, Vector Laboratories, Burlingame, CA, USA) and incubated for 30 min with VectaStain ABC reagent. Antibody binding was detected with 0.025% 3,3-diaminobenzidine (Sigma, St Louis, MO, USA) in the presence of 0.02% H_2O_2.

Morphological data analysis

Sections were analysed with a Leica DM-RBE light microscope (Leica, Wetzlar, Germany). Each thyroid follicle in the section was digitally photographed at 20-times magnification. The colloid in every follicle was manually selected using Adobe Photoshop 7.0 software and quantified using MetaMorph 6 software (Universal Imaging, Downingtown, PA, USA). The epithelial cell height of three thyrocytes per follicle in five follicles per tissue per fish was digitally determined. The area, perimeter, maximal diameter, length and width of every single colloid cross-section were measured. The shape of the colloid was described with three dimensionless shape descriptors: form factor, roundness and aspect ratio, which were calculated as follows (Ponton, 2006):

$$\text{Form factor} = 4\pi\ \text{Area} / \text{Perimeter}^2 ,$$

where Area and Perimeter are the measured area (μm^2) and perimeter (μm) of a colloid, respectively. The form factor expresses the evenness of the colloid's outline; as its value approaches 1, so the outline resembles more the outline of a circle.

$$\text{Roundness} = 4\text{Area} / \pi(\text{Maximal diameter})^2 ,$$

where Area and Maximal diameter are the measured area (μm^2) and maximal diameter (μm), respectively, of a colloid. A colloid with a maximum roundness value of 1 perfectly resembles a circle.

$$\text{Aspect ratio} = \text{Maximal length} / \text{Maximal width} .$$

The larger the aspect ratio, the more elongated the colloid is; a ratio of 1 corresponds to a perfectly circular colloid.

An initial analysis of frequency distributions revealed that form descriptors were not Gaussian distributed, and we therefore chose the mode as a descriptive statistic. To avoid subjective selection of bin width and endpoint, we determined the frequency distribution by kernel density estimation (Parzen, 1962) using an add-in utility (version 1.0e) for Microsoft® Excel from the Royal Society of Chemistry (Thompson, 2006) (see Fig. S1 in supplementary material).

In vitro incubations

Subpharyngeal tissue between the second and fourth gill arches, head kidney and kidney was dissected from 14 carp (61±15 g). Tissues were weighed and diced into approximately 1 mm^3 fragments and immediately transferred to a microtitre plate in 3 ml Hepes-Tris-buffered medium (pH 7.4) saturated with carbogen (95% O_2–5% CO_2) and allowed to recover for 1 h. Then, tissues were transferred to a clean plate in 3 ml of the aforementioned buffer and exposed to 10 mIU ml^{-1} bovine TSH (bTSH; Sigma) or saline vehicle. Tissues were incubated for 24 h at 23°C, after which the incubation medium was sampled. Total T4 (thyroxine, or 3,5,3′5′-tetraiodothyronine) in the medium was determined using a commercially available enzyme-linked immunoassay (Research Diagnostics, Inc., Flanders, NJ, USA) according to the manufacturer's instructions. Thyroxine-spiked samples gave representative readouts.

Thyroid hormone extraction

Several different methods based on extraction with Tris-HCl buffer, ethanol, methanol, butanol or chloroform were tested. We found a combination of Tris-HCl buffer and chloroform to be the most efficient in extracting radioactivity. Homogenates from the subpharyngeal region, head kidney and kidney tissues were obtained from intraperitoneally ^{125}I-injected animals, as described above. Then, 25 mg of pancreatin (Merck, Darmstadt, Germany), suspended in 0.1 mol l^{-1} Tris-HCl buffer (pH 8.7), was added to 1 ml of tissue homogenate as described by Tong and Chaikoff (Tong and Chaikoff, 1957), which was then incubated for 17 h at 35°C. Chloroform (1.5 ml) was added and the incubate was vigorously mixed for 2 min and centrifuged at 4°C (4000 ***g***, 15 min). The water phase was collected by aspiration and stored at –20°C until further analysis; 1 ml of 0.1 mol l^{-1} Tris-HCl buffer (pH 8.7) was added to the remainder of the chloroform–pancreatin mixture, and it was mixed for 10 min, and incubated for 48 h at 4°C. The mixture was then centrifuged at 4°C (4000 ***g***, 15 min) and the water phase was aspirated and stored at –20°C until further analysis. The extraction procedure was repeated once, after which the radioactivity of the three pooled water phases and of the remaining chloroform–pancreatin mixture was determined.

Sephadex LH-20 column chromatography

Sephadex LH-20 column chromatography was performed as described by Mol and Visser (Mol and Visser, 1985). In short, glass pipettes were filled with 1 ml Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) suspension in water (10% w/v) and equilibrated with 3× 1 ml volumes of 0.1 mol l^{-1} HCl. Samples (100 μl) of plasma, bile and extracts of the subpharyngeal region, head kidney and kidney were deproteinized with 4 volumes of methanol and centrifuged at 4°C (4000 ***g***, 15 min). The supernatants were acidified with 1 volume of 1 mol l^{-1} HCl and loaded on to the column. The samples were then eluted from the column with 2× 1 ml volumes of 0.1 mol l^{-1} HCl to separate free iodide, 6× 1 ml volumes of H_2O to separate water-soluble conjugated forms of iodothyronines, and 3× 1 ml volumes of 1 mol l^{-1} NH_3/ethanol

to separate native iodothyronines. The radioactivity of the collected fractions was measured in a γ-counter.

Statistics

All data are presented as mean values ± s.d. Differences between groups were assessed by one-way ANOVA and Tukey's *post hoc* test. Statistical significance was accepted at $P<0.05$ (two-tailed) and probabilities are indicated by asterisks (*$P<0.05$; **$P<0.01$; ***$P<0.001$) and plus signs (+ $P<0.05$; ++ $P<0.01$; +++ $P<0.001$).

Results

Autoradiography

Autoradiography demonstrated ^{125}I in the subpharyngeal region of tilapia. The radioactivity we observed in the intestinal tract was most probably caused by drinking (Fig. 1A,B). The inhibitory effect of perchlorate (Fig. 1H) hints at the involvement of the sodium–iodide symporter in the accumulation of radioiodide in tilapia intestine. In carp, ^{125}I was evident in the kidney but not in the subpharyngeal region (Fig. 1C,D). Furthermore, the gall bladder of carp contained radioactivity (Fig. 1E,F), which is in contrast with tilapia. Exposure to perchlorate blocked iodide accumulation in the subpharyngeal region of tilapia (Fig. 1G,H) and carp kidney, although radioactivity was still present in carp gall bladder (Fig. 1I,J).

^{125}Iodide tissue distribution

We retrieved 97±14% of the nominal amount of injected radioactivity from the tissue extracts. Measured 2 h after injection, the plasma ^{125}I radioactivity in tilapia was $719(\pm290)\times10^3$ c.p.m. g^{-1}, which decreased to $151(\pm110)\times10^3$ c.p.m. g^{-1} at 96 h. In carp, these values were $1190(\pm230)\times10^3$ c.p.m. g^{-1} and $22(\pm12)\times10^3$ c.p.m. g^{-1}, respectively. The subpharyngeal region in tilapia maximally accumulated ^{125}I 31-fold over the plasma level at 96 h into the chase (Table 1). All tissues other than the subpharyngeal region showed a decrease in radioactivity during this period (Fig. 2A). In carp, kidney, head kidney and bile were the only compartments where radioactivity accumulated (Fig. 2B). In carp, kidney tissue was able to accumulate ^{125}I more than 500-fold relative to plasma radioactivity at 96 h. The head kidney and subpharyngeal region of carp also accumulated iodide, 91-

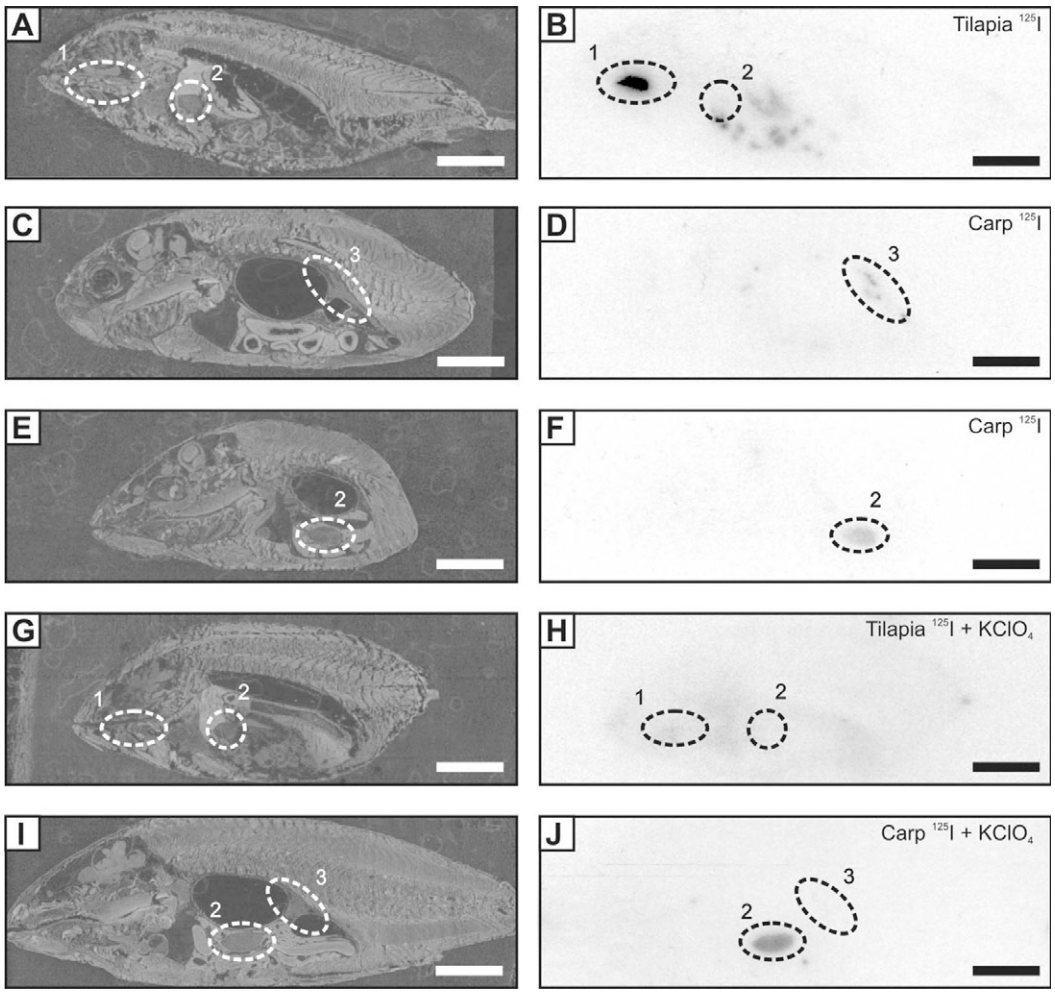

Fig. 1. Representative autoradiographs of 30 μm, whole-body cryosections of juvenile tilapia (A,B) and juvenile carp (C–F) exposed to ^{125}I, and of tilapia (G,H) and carp (I,J) exposed to ^{125}I and $KClO_4$. Broken circles indicate the position of the subpharyngeal region (1), the gall bladder (2) and the kidney (3). Scale bars, 1 cm.

Table 1. *Fold increase in ^{125}I radioactivity relative to plasma ^{125}I radioactivity 96 h after i.p. injection of radioiodide*

	Tilapia	Carp
Subpharyngeal region	31±27	20±13
Head kidney	0.5±0.07	91±54
Kidney	0.6±0.05	544±490
Bile	2±3	37±16

^{125}I radioactivity is given as Bq g^{-1} tissue (means ± s.d., N=6).

and 20-fold, respectively, compared with plasma (Table 1). While carp bile showed a 37-fold accumulation of ^{125}I at 96 h, tilapia bile radioactivity increased only twofold (Table 1).

Histology and morphological analysis

We could only detect thyroid follicles in the subpharyngeal region of tilapia, and this observation is corroborated by the ^{125}I tissue distribution shown in Fig. 2A. In carp, thyroid follicles were observed in kidney, subpharyngeal region and head kidney (Fig. 3A–C). The bright red stain of the lumen of follicular

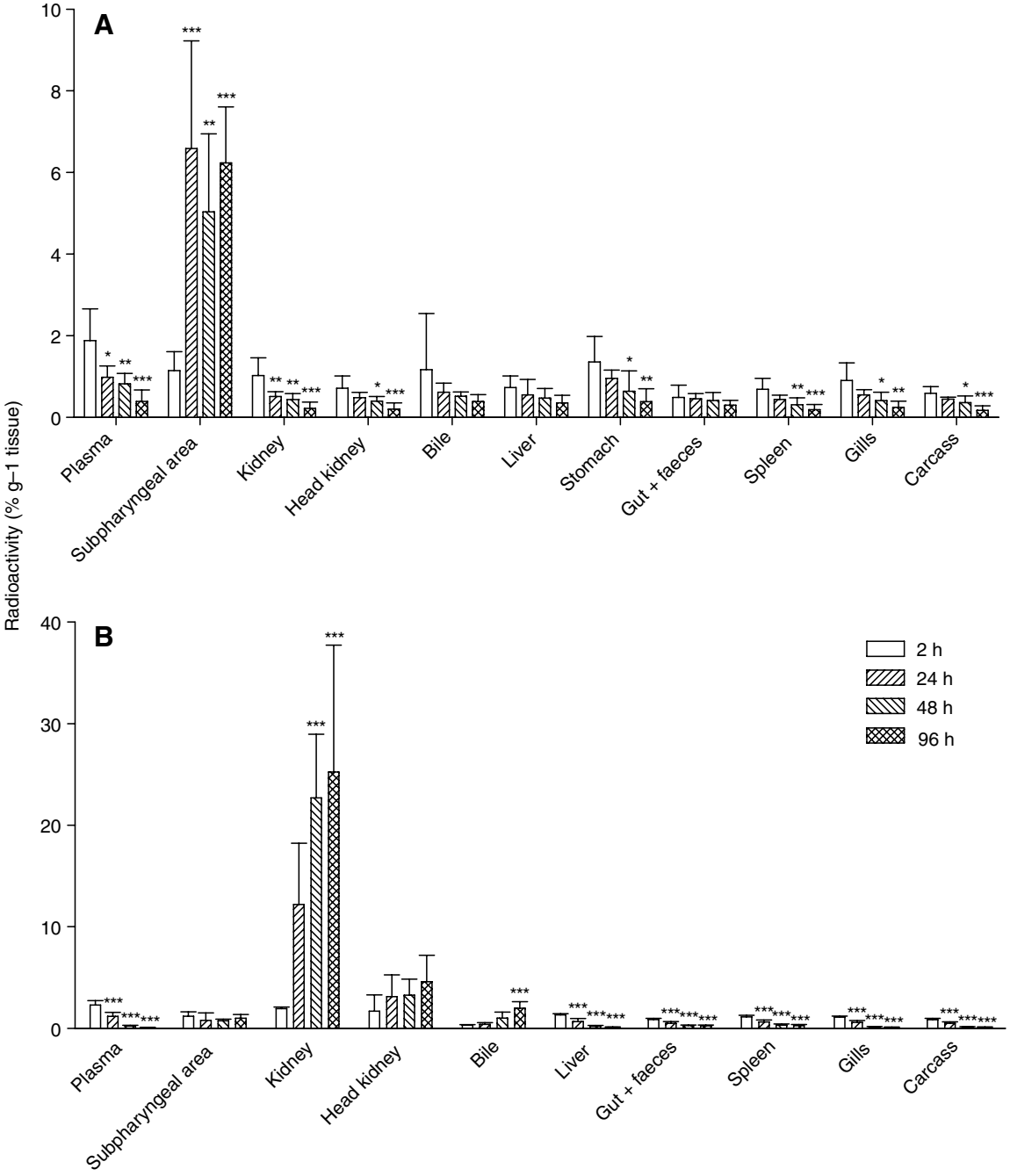

Fig. 2. Tissue distribution of the radioactivity, shown as the percentage of the total dose injected in the fish per gram of tissue at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (A, N=6) and carp (B, N=6). Results are means ± s.d. One-way ANOVA was used for statistical evaluation. Asterisks show significant difference compared with the radioactivity level at 2 h (*P<0.05, **P<0.01, ***P<0.001).

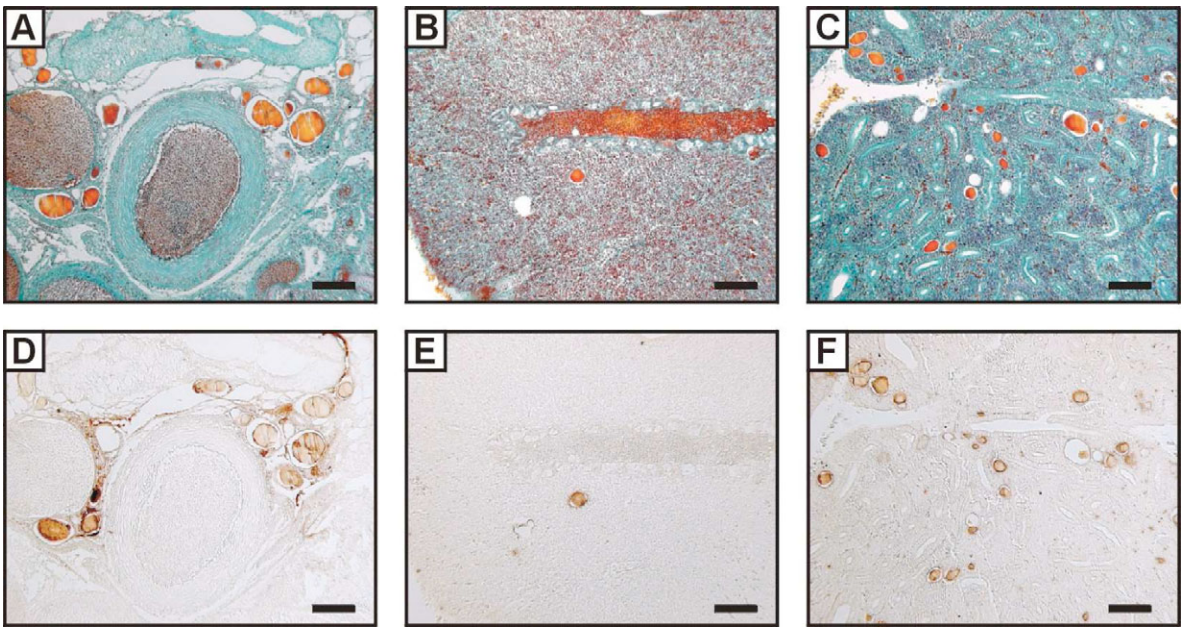

Fig. 3. Crossmon's staining of 7 μm thick sections of carp subpharyngeal region (A), head kidney (B) and kidney (C). Thyroxine immunohistochemistry on serial sections of carp subpharyngeal region (D), head kidney (E) and kidney (F). Scale bars indicate 200 μm.

structures obtained with Crossmon's trichrome colocalized with a specific thyroxine immunoreactivity, confirming that the follicles found in all three tissues were indeed thyroid follicles (Fig. 3D–F). On average, 1391±196 (N=4) follicle cross-sections were observed in carp, of which 87±2% was located within the kidney, 3±1% within the head kidney and 10±2% within the subpharyngeal region.

The modal value of the area of the kidney colloid was significantly smaller than that of the colloid in the subpharyngeal region and head kidney (Table 2). Also, the mode of the perimeter of the colloid was significantly smaller in the kidney follicles as compared with head kidney follicles (Table 2). Although the mode of the form factor of the colloid did not differ between the tissues, the mode of the roundness and aspect ratio did; the colloid in the subpharyngeal region was significantly rounder and less elongated than the colloid in the head kidney (Table 2). No differences (P=0.192) were observed between the epithelial cell height of the subpharyngeal region (6.04±0.12 μm, N=4), head kidney (6.67±0.68 μm, N=4) and kidney follicles (6.54±0.43 μm, N=4).

TSH-mediated T4 release in vitro

Exposure to 10 mIU ml^{-1} bTSH for 24 h significantly stimulated the release of T4 from carp kidney and head kidney tissue, 1.7- and 3.6-fold, respectively (Fig. 4). Tissue from the subpharyngeal region was unresponsive to 10 mIU ml^{-1} bTSH.

^{125}Iodide pulse chase

Radiolabelled compounds were extracted with very high recoveries from the subpharyngeal region (92±2% of total radioactivity), head kidney (77±11%) and kidney tissue (92±5%) in tilapia. Similar efficiencies were obtained for carp tissues (99±1%, 96±2% and 96±2%, respectively). In previous studies, extraction of radiolabelled thyroid hormones from tissues with ethanol or methanol combined with chloroform usually yielded recoveries of less than 70%, and ranging from 45% to 94% (Crane et al., 2004; Krysin, 1990; Szisch et al., 2005; Tagawa and Hirano, 1987).

Chromatographic analysis of plasma revealed that the decrease in total radioactivity, as observed in Fig. 2, can mainly be attributed to a decrease of the tracer injected (^{125}I), which occurs at a faster rate in carp than in tilapia; between 2 and 48 h after injection, the ^{125}I plasma level had decreased by 90±6% in carp, whereas in tilapia, it had decreased by 48±31% (Fig. 5). Radiolabelled, i.e. *de novo* synthesized, thyroid hormones appeared in increasing amounts in tilapia plasma during the experimental chase. In carp, however, newly synthesized thyroid hormones decreased from 2 h onwards. Conjugated

Table 2. *Mode of size and morphology descriptors for carp thyroid follicle colloid*

	Area (μm^2)	Perimeter (μm)	Form factor	Roundness	Aspect ratio
Subpharyngeal region	1403±226^a	151±13^{a,b}	0.80±0.06^a	0.75±0.01^a	1.18±0.01^a
Head kidney	1446±283^a	166±30^a	0.83±0.05^a	0.70±0.02^b	1.27±0.03^b
Kidney	963±91^b	125±8^b	0.88±0.01^a	0.72±0.02^{a,b}	1.23±0.04^{a,b}

One-way ANOVA was used for statistical evaluation. Different superscript letters indicate significant differences within the column, P<0.05. Modes are shown ± s.d. (N=4).

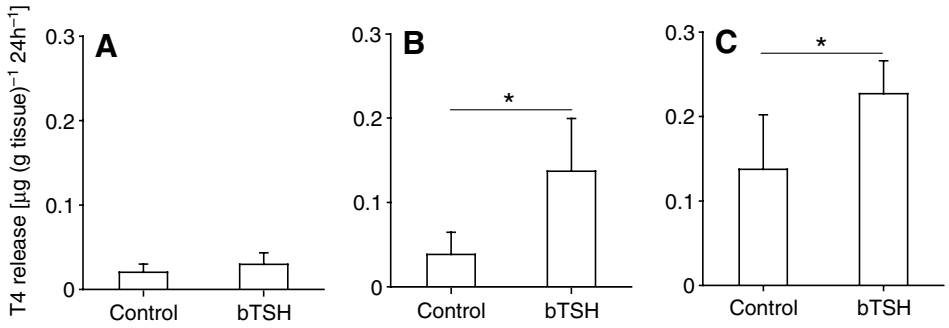

Fig. 4. bTSH (10 mIU ml^{-1})-mediated T4 release by carp subpharyngeal region (A, *N*=7), head kidney (B, *N*=7) and kidney (C, *N*=7). Results are means ± s.d. **P*<0.05 (Student's *t*-test).

forms of thyroid hormones appeared in tilapia plasma following the appearance of newly synthesized thyroid hormones.

The chronology of the appearance of labelled thyroid hormone metabolites in the subpharyngeal region, the head kidney and the kidney differed markedly between tilapia and carp. After an initial accumulation, the iodide level remained essentially constant as of 24 h in the subpharyngeal tissue of tilapia, whereas levels of labelled thyroid hormones and conjugates increased after 24 h (Fig. 6A). In kidney (Fig. 6B) and head kidney tissue (Fig. 6C) of tilapia no accumulation of iodide was observed. Small amounts of labelled thyroid hormones and conjugates appeared in these tissues at 48 h, which corresponds with the chronology seen in plasma, suggesting that these compounds originate from plasma.

In carp, maximum radioiodide accumulation in the kidney was reached at 48 h, after which the iodide level remained stable at 96 h (Fig. 6B). Thyroid hormones and thyroid hormone conjugates increased from 24 h onwards, reaching maximum levels at 48 h. Also in carp head kidney, newly synthesized thyroid hormones were observed as of 24 h, while thyroid hormone conjugates were essentially absent in this tissue (Fig. 6C). The subpharyngeal region of carp did not accumulate detectable radioiodide and virtually no radiolabelled thyroid hormones and thyroid hormone conjugates were observed (Fig. 6A).

Chromatographic analysis of bile revealed an increase in radioiodide content in carp bile, whereas in tilapia bile, the radioiodide level did not change significantly (Fig. 7A). Thyroid hormones and conjugated forms of thyroid hormone accumulated in the bile of both species. The average total volume of the bile of both fish species did not decrease during the experiment, indicating that the gall bladder had not emptied in the intestinal tract during the chase period of the experiment.

In the ambient water of both fish, iodide, thyroid hormones and thyroid hormone conjugates were observed (Fig. 7B). Equal amounts of iodide and thyroid hormones were excreted by the two fish species. Conjugated thyroid hormones, however, were only excreted by tilapia.

Discussion

We observed remarkable differences in the location of active endocrine thyroid tissue between carp and tilapia. In carp, three thyroid follicle populations, active and inactive, were identified in the subpharyngeal region, head kidney and kidney. Also, differences at the level of the peripheral metabolism of thyroid hormones were observed. Not only did plasma clearance differ

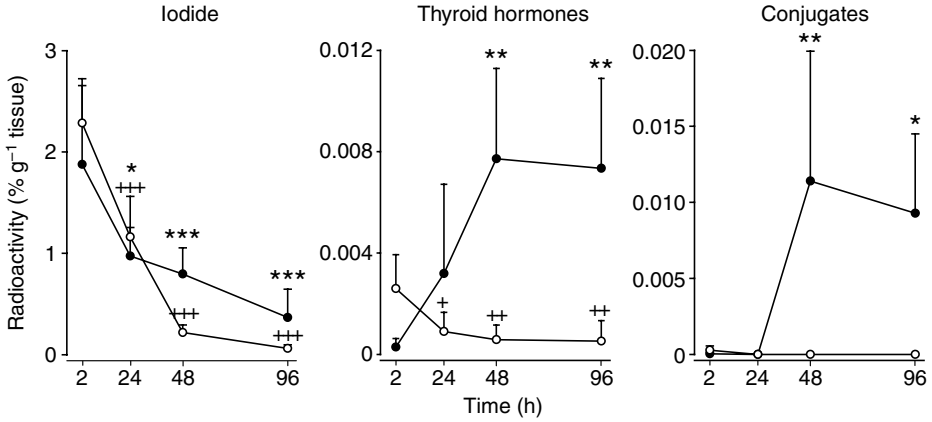

Fig. 5. Radioactivity of iodide, thyroid hormone and thyroid hormone conjugates fractions, shown as the percentage of the total dose remaining in the fish at the time of sampling per gram of plasma, after Sephadex LH-20 chromatography at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (●, *N*=6) and carp (○, *N*=6). Results are means ± s.d. One-way ANOVA was used for statistical evaluation. Significantly different levels of radioactivity compared with that at 2 h are indicated for tilapia (**P*<0.05, ***P*<0.01, ****P*<0.001) and carp (+*P*<0.05, ++*P*<0.01, +++*P*<0.001).

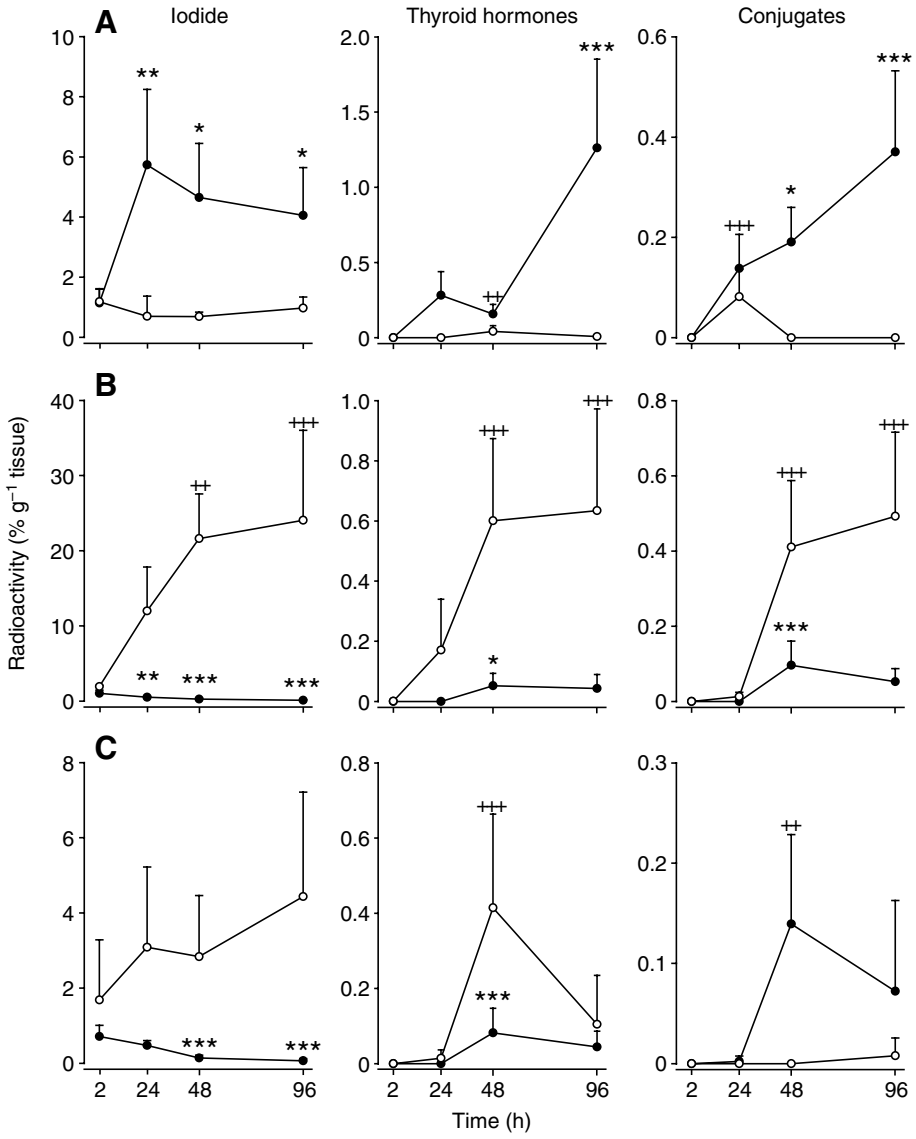

Fig. 6. Radioactivity of iodide, thyroid hormone and thyroid hormone conjugates fractions, shown as the percentage of the total dose remaining in the fish at the time of sampling per gram of tissue, in the subpharyngeal region (A), kidney (B) and head kidney (C), after Sephadex LH-20 chromatography at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (●, N=6) and carp (○, N=6). Results are means ± s.d. One-way ANOVA was used for statistical evaluation. Significantly different levels of radioactivity compared with that at 2 h are indicated for tilapia (*P<0.05, **P<0.01, ***P<0.001) and carp (+P<0.05, ++P<0.01, +++P<0.001).

but also the route of thyroid hormone excretion varied between carp and tilapia.

In tilapia, the subpharyngeal region was the only site in which thyroid follicles were found, where perchlorate-sensitive iodide accumulation was observed, and where thyroid hormones were synthesized *de novo*. This demonstrates, for tilapia, a location and activity typical for the teleostean thyroid gland. The anatomical location of the thyroid gland in carp, however, deviates from that in tilapia. In carp, the renal tissues display thyroid activity as evidenced by iodide accumulation, confirming the observations of Leray and Febvre (Leray and Febvre, 1968) and Lysak (Lysak, 1964). Although both head kidney and kidney in carp synthesized thyroid hormones and secreted thyroid hormones following TSH stimulation, the head kidney can have only a moderate share in total thyroid output. Not only was the kidney the foremost iodide-accumulating tissue in carp, which was inhibited by perchlorate, it also harbours the largest amount of thyroid tissue: 87% of the total thyroid follicle population, as opposed to 3% in the head kidney. This suggests a significant role for the kidney thyroid follicles in thyroid economy of carp.

The most striking aspect of this study is the absence of iodide accumulation and thyroid hormone synthesis in the subpharyngeal region of carp, despite the presence of thyroid follicles. Furthermore, we found that carp subpharyngeal thyroid follicles do not have the capacity to release thyroid hormones upon stimulation with TSH *in vitro*, whereas renal thyroid follicles do. This, together with the high prevalence of thyroid follicles, establishes the kidney as the anatomical site of the thyroid gland in this species. These results may pose questions as to whether to (re-)consider the term ‘heterotopic’ in conjunction with ‘thyroid follicles’ in common carp. In goldfish (*Carassius auratus*), a species closely related to common carp, the subpharyngeal thyroid follicles are active and are responsible for 11–40% of total iodide accumulation (Chavin and Bouwman, 1965; Peter, 1970), leaving a considerable role for subpharyngeal thyroid follicles in the uptake of iodide in this species. However, the subpharyngeal follicles in goldfish appear not to be responsive to T4 treatment; changes in thyroid activity, i.e. iodide accumulation and epithelial cell height, are primarily mediated through inter-renal

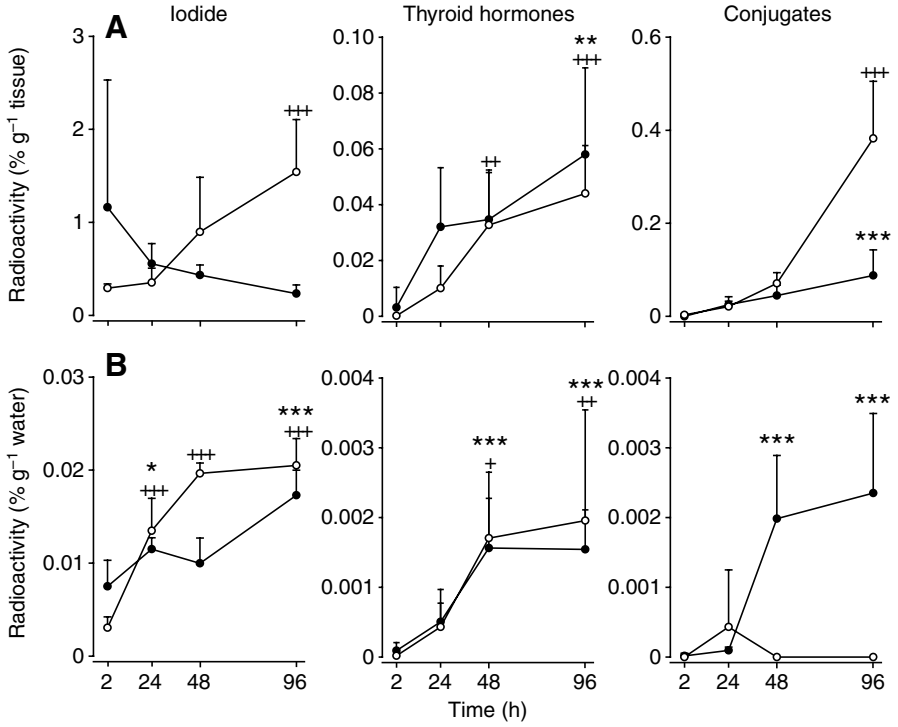

Fig. 7. Radioactivity of iodide, thyroid hormone and thyroid hormone conjugates fractions, shown as a percentage of the total dose remaining in the fish at the time of sampling per gram of tissue, in the bile (A) and the ambient water (B), following Sephadex LH-20 chromatography at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (●, N=6) and carp (○, N=6). Results are means ± s.d. One-way ANOVA was used for statistical evaluation. Significantly different levels of radioactivity compared with that at 2 h are indicated for tilapia (**P<0.01, ***P<0.001) and carp (+P<0.05, ++P<0.01, +++P<0.001).

thyroid follicles, which shows that the thyroid populations are not physiologically equivalent (Peter, 1970).

Histologically, the subpharyngeal follicles in carp appear normal, active and similar to kidney thyroid follicles, as evidenced by the epithelial cell height, which does not differ significantly from that of kidney follicles. Differences in size and shape were observed between the thyroid follicle populations, *viz*. the colloid of kidney follicles is the smallest, and subpharyngeal follicles appear to be more round than renal follicles. It is tempting to interpret the small colloidal area of renal thyroid follicles as an indication of increased colloidal resorption, which, again, is indicative of increased hormonogenesis in these follicles. The latter is corroborated by our observation of increased iodide accumulation and the presence of *de novo* synthesized thyroid hormones in this region.

Studies from Raine and colleagues (Raine and Leatherland, 2000; Raine et al., 2005) show that in embryonic rainbow trout (*Oncorhynchus mykiss*) the functional unit of the thyroid gland appears tubular, not follicular, and that this morphology is retained in the juvenile stages. Despite the fact that carp thyroid tissues were sectioned in varying planes, we did not detect tubular structures in our serial sections. A significant proportion of tubular colloid-filled structures would also have been made apparent by a larger variation in the morphology descriptors of the thyroidal colloid. Instead, they show little variation. Of course, it remains to be determined whether measurement of thyroidal colloid accurately reflects thyroid follicle morphology. Still, one can speculate that differences in morphometrics might reflect differences in organogenesis between species.

Although the subpharyngeal thyroid follicles in carp did not incorporate radioiodide to a significant degree or synthesize thyroid hormones *de novo*, these subpharyngeal follicles do show T4 immunoreactivity. Apparently these follicles do have an intrinsic capacity to synthesize thyroid hormones. Whether the rate of thyroid hormone synthesis is too slow to detect within the 96 h of the experimental chase, or whether these follicles were active during earlier life stages and are now dormant (and still contain T4) remains to be determined. Interestingly, neotenic urodeles are able to complete a full life cycle without metamorphosis (Rosenkilde and Ussing, 1996). Because of an impaired thyroid system these amphibians are unable to release a surge of thyroxine, necessary to initiate metamorphosis. Although intact and functional, their thyroid system is relatively inactive at several levels of the thyroid axis, from the central regulation of the thyroid gland to the peripheral deiodination of thyroid hormones. Neoteny has also been described in fish; during adult life the ice goby (*Leucopsarion petersii*) exhibits several larval characteristics, indicative of an impaired metamorphosis. During its development, the thyroid follicles are smaller and have a lower epithelial cell height when compared with a metamorphic goby species; also no TSH immunoreactivity was observed in the pituitary (Harada et al., 2003). Although carp are not neotenic, further research on the carp subpharyngeal thyroid follicles may provide more insight into the mechanisms controlling the non-pharmacologically induced inactivity of the thyroid gland as observed in neotenic organisms. A possible mechanism could be the temporal expression of active and/or inactive splice variants of key regulators of thyroid hormone synthesis, e.g. TSH receptor, sodium–iodide symporter or thyroglobulin.

It is unclear why the functional endocrine thyroid tissue is located in the kidney and not in the subpharyngeal region. We hypothesize that two, potentially functional, thyroid populations with different sensitivities to thyrotropic factors, or with different synthesizing properties, confer an accurate regulation of thyroid gland output in response to a demand for systemic

thyroid hormone. It can also be hypothesized, regarding the close juxtaposition of the extra-pharyngeal thyroid follicles to specific cell types in the head kidney and kidney, that thyroid hormones have a paracrine effect on inter-renal (cortisol-producing), chromaffin (catecholamine-producing), and/or haematopoietic cells or on nephron structures. Paracrine relationships between the stress axis and immune system have already been demonstrated in the multifunctional carp head kidney (Metz et al., 2006). Attempts to demonstrate a direct *in vitro* effect of thyroid hormones on the release of cortisol in carp head kidney have not been successful yet, even though treatment of carp with thyroxine resulted in a decrease in the level of plasma cortisol (Geven et al., 2006).

The presence of ^{125}I radioactivity in carp bile and its absence in tilapia bile suggests a faster turnover rate of thyroid hormones in carp than in tilapia. This is corroborated by the faster clearance of iodide and thyroid hormones from plasma, and the accumulation of iodide and thyroid hormone conjugates in bile of carp compared with tilapia. Not considering differences between species, these results appear to contradict the general idea that higher temperatures result in increased thyroid activity (Eales et al., 1982), as our carp were held at a temperature that was 5°C lower than that for tilapia.

The appearance of thyroid hormone conjugates in the plasma of tilapia is consistent with the observations of DiStefano et al. (DiStefano et al., 1998), who found a significant amount of T3 glucuronides in plasma of Mozambique tilapia after i.p. injection with [^{125}I]T3 ([^{125}I]3,5,3′-triiodothyronine). Although thyroid hormone sulphates are found in the sera of several mammals (Santini et al., 1993; Wu et al., 1992; Wu et al., 1993), and indirect evidence exists for thyroid hormone conjugates in the plasma of European plaice (*Pleuronectes platessa* L.) (Osborn and Simpson, 1969), the Mozambique tilapia appears to be the only vertebrate in which plasma thyroid hormone glucuronides are observed. By injection of trace amounts of radioiodide instead of radiolabelled thyroid hormones, we circumvented the possibility of altering the thyroid status of the fish. The injection of radioiodide, as opposed to radiolabelled thyroid hormones, also allowed us to speculate on the anatomical site at which conjugated thyroid hormones are produced. Whereas thyroid hormone conjugates were observed in tilapia plasma at 48 and 96 h after i.p. injection, they were already present in the subpharyngeal region at 24 h, suggesting that the glandular thyroid itself may be responsible for the production of thyroid hormone conjugates in tilapia plasma. In this respect, the finding of conjugated forms of thyroid hormones in the kidney of common carp, which harbours the functional thyroid, is remarkable. However, we cannot exclude the possibility that cell types other than thyrocytes, or tissues other than thyroid tissue, are responsible for the presence of thyroid hormone conjugates.

The thyroid hormone conjugates in tilapia plasma have been suggested to function as a pool of thyroid hormones from which, by deconjugation, a rapid mobilization of bioactive thyroid hormones is available (DiStefano et al., 1998). Our results, however, suggest that the thyroid hormone conjugates in tilapia plasma are involved in the excretion of thyroid hormones, through routes other than bile. The appearance of thyroid hormone conjugates in the ambient water coincides with the appearance of thyroid hormone conjugates in the plasma, suggesting that thyroid hormone conjugates are excreted via plasma, possibly through the gill or kidney. Since the volume of the gall bladders did not decrease during the experiment and leakage of thyroid hormone conjugates over the gall bladder wall in fish is negligible (Collicutt and Eales, 1974), the thyroid hormone conjugates in the ambient water are unlikely to originate from bile. It is striking that in channel catfish (*Ictalurus punctatus*), 8.1% of all injected [^{125}I]T4 is excreted via routes other than the gall bladder (Collicutt and Eales, 1974) and that in rainbow trout (*Oncorhynchus mykiss*), 8.2% and 6.7% of injected [^{125}I]T4 and [^{125}I]T3, respectively, was excreted via urine (Parry et al., 1994). These reported percentages are of the same order of magnitude as the observed percentage (8.3%) of thyroid hormone conjugates found in tilapia plasma, supporting the idea that plasma thyroid hormone conjugates in tilapia may function in the excretion of thyroid hormone metabolites (DiStefano et al., 1998).

In summary, we have shown that thyroid hormone synthesis, anatomical location and activity of thyroid tissue, and thyroid hormone excretion in teleost fish differ greatly between two species. The most distinct feature of teleost thyroid physiology observed in this study is the presence of a completely functional endocrine thyroid gland in the renal tissues of common carp. This finding may open new possibilities for *in vitro* studies on fish thyroid.

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