Tissue-specific regulation of thyroid hormone receptor mRNA isoforms and target gene proteins in domestic ducks

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Abstract

Skeletal muscles are important target tissues for thyroid hormone action. The present study examines the influence of thyroid status on muscle growth and tissue-specific expression of thyroid receptor (TR) mRNA isoforms in a commercial strain of the domestic duck (*Anas platyrhynchos*). Four groups ($n=5$) of 1-week-old ducklings were rendered either hypothyroid by treatment with methimazole (6 mg 100 g⁻¹ body mass or 12 mg 100 g⁻¹ body mass), or hyperthyroid by treatment with methimazole (6 mg 100 g⁻¹ body mass) in combination with thyroid hormones (5 µg thyroxine (T₄) and tri-iodothyronine (T₃) 100 g⁻¹ body mass or 10 µg T₄ and T₃ 100 g⁻¹ body mass). Serum and tissue samples (cardiac, pectoralis and semimembranosus leg muscle, liver, pituitary and cerebral cortex) were collected from these four groups, and from a group of untreated controls, at 8 weeks of age. Development of duckling morphology was retarded in methimazole-treated birds compared with that in euthyroid controls, as evidenced by differences in skeletal dimensions, primary feather length, and body and muscle masses. Body mass was lower by 18%, and relative masses

of cardiac and pectoralis muscles were lower by 28% and 32% respectively. Heterologous oligonucleotides for TR α , TR β 0, TR β 2 and the housekeeping gene β -actin were derived from chicken sequences. RT-PCR showed that TR α mRNA was expressed in all tissues but was not significantly affected by any of the experimental treatments. TR β 0 mRNA expression was significantly lower in the leg muscles of ducklings treated with 12 mg methimazole 100 g⁻¹ body mass (0.109 ± 0.047 TR: β -actin ratio, $P<0.05$) compared with that in euthyroid controls (0.380 ± 0.202), but was unaltered in the pectoralis and cardiac muscles. Expression of TR β 0 mRNA was significantly higher in pectoralis (by 3.5-fold, $P<0.05$), cardiac (by 4.2-fold, $P=0.003$) and leg (by 4.0-fold, $P<0.001$) muscles of ducklings treated with thyroid hormones compared with those in euthyroid controls (0.098 ± 0.019 , 0.822 ± 0.297 and 0.38 ± 0.202 TR: β -actin respectively). Only the pituitary gland expressed significant levels of TR β 2 mRNA.

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Introduction

Thyroid hormones influence a large number of biological functions by modulating gene expression (Lazar 1993), and are essential for the normal development of brain (Dussault & Ruel 1987, Oppenheimer & Schwartz 1997) and muscle tissues (Everts 1996, Harrison *et al.* 1996) during ontogeny. Many species of wildfowl (ducks and geese) are migratory and their young undergo rapid growth during development in preparation for their first long distance flights (Sedinger 1986, Bishop *et al.* 1995). However, the locomotor and cardiac muscles of wildfowl show tissue-specific rates of maturation and different patterns of growth (Bishop *et al.* 1996), reflecting their various

functional requirements during development. In particular, the main flight muscles (the pectoralis) show an exponential increase in mass between 1 and 7 weeks of age, when the juveniles first begin to fly. In barnacle geese (*Branta leucopsis*), this rapid development of the pectoralis muscles occurs in parallel with an increase in circulating levels of thyroid hormone (Bishop *et al.* 1998).

Skeletal muscles are an important target tissue for thyroid hormones and, in birds, thyroid hormone deficiency has been shown to affect the mass of the developing locomotor muscles, and the maximum activities of muscle mitochondrial enzymes such as citrate synthase (CS) involved in oxidative phosphorylation of fuel substrates (Deaton *et al.* 1997, 1998). Hypothyroidism

in goslings from 2 to 7 weeks of age resulted in a significantly lower mass of the pectoralis and cardiac ventricle muscles (while the mass of the legs was not affected), and a lower activity of CS in the pectoralis and leg muscles, while the heart was not affected (Deaton *et al.* 1997, 1998). These results are consistent with the hypothesis that the muscle-specific responses of wildfowl to altered thyroid status may reflect differential expression of thyroid receptor (TR) mRNA, leading to altered expression, or function, of nuclear TR isoforms in target muscles.

The liver is also an important target tissue for thyroid hormones and a direct relationship between changes in thyroid status and the activity of the lipogenic enzymes of the liver (e.g. malate dehydrogenase and malic enzyme) has been reported in the rat (Diamant *et al.* 1972), and activation of the thyroid response elements for the malic enzyme gene has been studied *in vitro* (Petty *et al.* 1990, Hodnett *et al.* 1996). However, very little is known about TR isoform developmental expression in birds (Forrest *et al.* 1990, Sjöberg *et al.* 1992, Muñoz & Bernal 1997).

In chickens, the product of the TR α gene is a single TR α mRNA which is translated into two proteins by the use of two alternative start codons (Forrest *et al.* 1991). The TR β gene gives rise to two variant mRNAs which have been designated TR $\beta 0$ and TR $\beta 2$ (Sjöberg *et al.* 1992, Muñoz & Bernal 1997). TR $\beta 0$ is probably homologous to TR $\beta 1$ but the protein has a short amino terminal sequence (Sjöberg *et al.* 1992). In the present study, we have used a commercial strain of the domestic duck (*Anas platyrhynchos*), which has been selectively bred over hundreds of years from the wild mallard (Horton 1928). We developed a method for quantifying relative differences in avian TR α and TR β mRNA isoform expression by RT-PCR (Gittoes *et al.* 1997, 1998), and examined the effect of changes in thyroid status on tissue-specific pretranslational expression of TR isoforms.

Materials and Methods

Animals

Twenty-five 1-day-old Pekin ducklings were provided by a commercial supplier and kept indoors with food (chick starter crumbs) and water provided *ad libitum*, with a photoperiod of 18 h light:6 h darkness. Experimental treatments began at 1 week of age and were conducted in accordance with humane practice as specified by the Animal (Scientific Procedures) Act 1986 of the UK.

Treatment groups and protocol

At 1 week of age, ducklings were allocated at random into five groups of five. All birds between the ages of 1 and 8 weeks of age received daily treatment during the morning, by oral gavage, of the following mixtures. Two groups

were made hypothyroid by treatment with methimazole at 6 mg or 12 mg 100 g⁻¹ body mass (BM) respectively. Two further groups were both treated with methimazole at 6 mg 100 g⁻¹ BM and made hyperthyroid by the addition of 5 µg thyroxine (T₄) g⁻¹ BM and 5 µg triiodothyronine (T₃) g⁻¹ BM, or 10 µg T₄ g⁻¹ BM and 10 µg T₃ respectively. The concentrations of methimazole and T₄ and T₃ used in this experiment were partially based on earlier work on barnacle geese (Deaton *et al.* 1997, 1998). Two doses were used due to uncertainties in anticipating species-specific differences in the response to methimazole and T₄/T₃ treatment, and to provide preliminary information with regard to possible quantitative effects of these treatments. One group comprised control birds that were orally dosed with a carrier solution. The methimazole and thyroid hormone solutions were made up twice a week, using a dilute ammonia solution as a carrier. The carrier solution consisted of 5 ml 35% ammonia mixed with 5 ml water to which was added 20 ml sterile saline solution followed by 1 g bovine serum albumin (BSA). Once the BSA was dissolved the hormones were added and the pH lowered very gradually to pH 7.4. Finally, additional sterile saline was added to the mixture to a final volume of 50 ml.

Sample collection

At 8 weeks of age, the birds were given a final treatment dose around 0800 h, and a 1 ml serum sample was then collected from a peripheral leg vein 4 h later and frozen at -20 °C. The birds were then killed by intravenous injection of pentobarbitone, followed by immediate dissection, and 500 mg samples taken from the cardiac, pectoralis and semimembranosus muscles (Bishop *et al.* 1995, Deaton *et al.* 1996), and from the liver, pituitary and cerebral cortex for storage in liquid nitrogen until further analysis. Various morphometric measurements were made during dissection, including the total mass of the semimembranosus, pectoralis and cardiac muscles, and the total length of the sternum, tibiotarsus and radius bones and the length of the 9th primary feather of the wing. Serum T₄ and T₃ were assayed at Leicester Royal Infirmary using an established commercial radioimmunoassay.

Analysis of maximum enzyme activities

Muscle samples were homogenised on the day of assay in 9 volumes (wet weight) of ice-cold extraction buffer: 50 mM imidazole, 1 mM EGTA, 2 mM MgCl₂, 250 mM mannitol, pH 7.4. Homogenisation was at 13 500 r.p.m. for 15 s and repeated following a 2-min interval, using an Ultra-Turrax homogenizer with a 10 N shaft. Homogenate (300 µl) was then aliquoted into microcentrifuge tubes and 30 µl of a 10% Triton solution were added, followed by gentle mixing, to give an 11-fold final dilution (w/v).

Table 1 Fluorescence intensity (arbitrary units) of β -actin mRNA product following RT-PCR and separation on a 2% agarose gel, showing that there was no systematic or significant difference between treatments within tissues. Data are presented as means $\pm$ S.E.M.

Treatment	Pectoralis muscle	Cardiac muscle	Semimembranosus muscle
Control	463.0 $\pm$ 66.3	276.3 $\pm$ 87.3	340.3 $\pm$ 94.1
MMZ (12 mg)	501.8 $\pm$ 99.0	286.1 $\pm$ 59.8	284.3 $\pm$ 66.9
MMZ (6 mg)	537.4 $\pm$ 143.2	172.2 $\pm$ 35.1	303.5 $\pm$ 146.3
MMZ+T ₄ /T ₃ (5 μ g)	471.8 $\pm$ 25.7	272.5 $\pm$ 54.6	194.3 $\pm$ 72.7
MMZ+T ₄ /T ₃ (10 μ g)	494.7 $\pm$ 64.0	303.1 $\pm$ 89.0	294.8 $\pm$ 56.7
ANOVA	F=0.1, P>0.9	F=0.4, P>0.8	F=0.9, P>0.5

MMZ, methimazole.

Assay solutions were prepared on the day of assay and held at 41 °C in a water bath. Samples were assayed for CS (EC 4.1.3.7) activity in the following solution: 0.5 mM oxaloacetate (omitted from control cuvette), 0.25 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.3 mM acetyl-CoA in 50 mM triethanolamine-HCl, pH 8.0, and changes in spectral absorbance were read at 412 nm using a micro-mole extinction coefficient of 13.6 (Bishop *et al.* 1995). Malic enzyme (EC 1.1.1.40) activity was assayed in the following solution: 0.5 mM malate (omitted from control cuvette), 0.4 mM NADP⁺, 4 mM MgCl₂, 0.3 mM dithiothreitol in 50 mM imidazole, pH 7.4, and changes in spectral absorbance were read at 340 nm using a micro-mole extinction coefficient of 6.22. Changes in spectral absorbance were measured with a Shimadzu UV-160A ultraviolet-visible recording spectrophotometer fitted with a CPS-240 cell positioner and temperature control set to 41 °C. Volumes of assay solution in the cuvette were 1 ml with addition of 5 μ l aliquots of the crude homogenate. Samples were homogenised in batches of six at a time, placed on ice, and assayed immediately.

RNA extraction

Frozen samples of pectoralis, semimembranosus and cardiac muscle were ground to a fine powder in liquid nitrogen using a cold pestle and mortar, and the powder returned to storage in cryovials kept in liquid nitrogen until required. Total RNA was isolated from freshly thawed tissue using a commercially available kit (Tri-reagent; Sigma Co., Poole, Dorset, UK), based on the guanidinium phenol-chloroform extraction technique. Approximately 100 mg tissue, or powder, were homogenised in an Ultraturax homogeniser with a 10 N shaft at 8000 r.p.m. for 30 s, in the presence of 2 ml Tri-reagent. Total RNA was extracted by following the manufacturer's protocol.

Quantification of relative differences in mRNA by RT-PCR

RT-PCR and the method of cDNA quantification were based on methods previously described by Gittoes *et al.*

(1997, 1998). Reverse transcription was performed using a commercially available kit (Promega, Southampton, Hants, UK). Total RNA (2 μ g) was added to 60 pmol oligo(dT)₁₅ primer and the volume adjusted to 32 μ l with water. Primer annealing was encouraged by incubation of the solution for 10 min at 77 °C, and the mixture cooled to room temperature. Ten microlitres of 5 $\times$ avian myeloblastosis virus (AMV) reverse transcriptase buffer, 5 μ l dNTP mix (20 μ mol each), 50 units RNAsin and 15 units AMV reverse transcriptase were added, followed by incubation for 60 min at 42 °C. The RT reaction was terminated by heating to 90 °C for 5 min.

PCR was carried out in a total volume of 50 μ l and all samples underwent PCR at the same time in the same experiment. Each PCR tube contained 60 pmol each of forward and reverse primers (see Table 1), 1 μ l dNTP mix (20 μ mol each), 2–3 μ mol Mg²⁺ and 2–10% of the RT cDNA reaction product. Mineral oil was placed over the reaction mixture which was pulse centrifuged, heated to 95 °C for 6 min, cooled to 72 °C and the Taq polymerase added. PCR cycling was commenced using a melt temperature of 95 °C and extension at 72 °C. PCR products were visualised on 2% TBE agarose gels stained with ethidium bromide. PCR conditions for temperature and Mg²⁺ concentration were optimised for each primer pair and comparative kinetic analyses performed to determine that the selected PCR cycle number for each primer pair was within the exponential phase of product generation. PCR reactions and subsequent gel analysis were performed in duplicate. Ethidium bromide-stained 2% agarose gels were photographed under u.v. light, digitised and densitometry performed on the product bands. Quantitation of RT-PCR by analysis of ethidium-stained gels has yielded consistent and significant results in previous studies (Gittoes *et al.* 1997, 1998) and was sufficiently sensitive to provide quantifiable results in the present study.

Oligonucleotide primer design and PCR product sequencing

Oligonucleotide primer design was carried out on GCG-8 and DNAsar software, using chicken sequences for TR α

Table 2 Oligonucleotide sequences of β -actin and TR isoform PCR primers based on chicken mRNA sequences, their associated optimal PCR conditions and the % similarity of chicken sequences to those of domestic ducks (*Anas platyrhynchos*)

mRNA species	Chicken oligonucleotide sequences		Product size (bp)	Annealing temp. (°C)	Mg ²⁺ conc. (mmol)	Cycle no.	% similarity of chicken and duck product sequences
	5'	3'					
β -actin	5'	895 AACTACCTTCAACTCCATC 914	785	64	3	23	93.6
	3'	1679 CCTTCATTACATCTATCAC 1660					
TR α	5'	1321 CTGGCGTTTCGAGCACTACATCAA 1343	202	64	2	32	98.8
	3'	1522 GGGGGCCCTACACCTCCTG 1504					
TR β 0	5'	178 GGTGACAGAAGAAGAACAAAG 197	205	60	2	33	100
	3'	382 GAATAGGTTGGATGGAGG 365					
TR β 2	5'	CACCGAAGAAAAACCTAC	322	58	2	35	97.5
	3'	GTCATCCAACACCAAATC					

(accession number Y00987), TR β 0 (M65207) and TR β 2 (X62642) published by Genbank. Primers for β -actin (L08165) were designed and used as an internal standard to correct for sample to sample variation in RNA extraction, degradation and reverse transcription. All values for each PCR product for TRs were normalised and expressed as a ratio with respect to the quantity of β -actin. β -Actin has been used as an internal standard, or as a representative house-keeping gene, in brain and non-muscle tissues (Gittoes 1997, Slagboom *et al.* 1990). β -Actin is not part of the contractile process, so it might be expected to perform equally well as an internal standard in muscle tissues. To check that there was no systematic bias in the expression of β -actin as a result of the experimental treatment, fluorescence densitometry values for β -actin expression (arbitrary units) in cardiac, pectoralis and semimembranosus muscles were tested by ANOVA and found to exhibit no significant differences between experimental treatments (Table 1).

Table 2 lists oligonucleotide primer sequences, expected PCR product sizes, Mg²⁺ concentration, annealing temperatures and number of PCR cycles used for each primer pair. For each primer pair, a sample of the PCR product generated from the duck tissue was sequenced and the result compared with the published chicken sequence, excluding the primer sequences themselves (Table 2). PCR products were purified for sequencing after excision from TAE agarose gels and elution using QIAQuick gel purification kits (Qiagen, Hilden, Germany). Fragment concentration was gauged in relation to a DNA mass ladder (Pharmacia, Uppsala, Sweden). Fifty to one hundred nanograms of the resulting pure fragment was subsequently used per sequencing reaction, with 4 μ l sequence (Perkin Elmer, Foster City, CA, USA) and 3.2 pmol forward or reverse primer. Electropherograms were analysed using DNASTar software.

Statistics

Data in the text are presented as means $\pm$ S.E.M. Differences between groups were analysed by ANOVA fol-

lowed by Fisher's least significant difference post-hoc test. Where the variance between groups was found to be different, the data were first log transformed before applying the ANOVA. The level of significance was taken as $P < 0.05$.

Results

Chicken and duck sequence homology

Galliform birds (gamebirds such as grouse and chickens) had a common ancestor with the Anseriform birds (ducks and geese) around 70 million years ago (Sibley & Alquist 1990). Despite this, we have demonstrated that there is a very high degree of conservation between the TR isoforms of ducks and chickens. Table 2 shows that the degree of similarity between duck and chicken tissue TR mRNA sequences varies between 97 and 100%, while β -actin mRNA is around 93% conserved.

Relative TR mRNA tissue distribution by RT-PCR

The distributions and relative expressions of TR α , TR β 0 and TR β 2 mRNAs in the six tissues sampled from 8-week-old ducks are shown in Fig. 1. TR α mRNA was expressed in all tissues, but especially abundant expression was observed in the heart and pectoralis muscles. In contrast, TR β 0 mRNA was less abundant in skeletal and cardiac muscles but strongly expressed in liver, brain and pituitary tissues. TR β 2 mRNA was even more restricted in distribution, and was strongly expressed only in the pituitary, with mRNA also detectable in cerebral cortex.

Effect of changes in thyroid status on cardiac and locomotor muscles

Figure 2 shows that treatment with methimazole at both concentrations employed (12 mg or 6 mg 100 g⁻¹ BM) was effective in reducing serum levels of T₄ and T₃ below

Figure 1 Ratios of (a) TR α , (b) TR $\beta 0$ and (c) TR $\beta 2$ isoform mRNA expression normalised with respect to β -actin mRNA expression in six tissues of 8-week-old domestic ducklings. Data are presented as means $\pm$ S.E.M. Sections d and e are photographs of 2% agarose gels showing the fluorescence of RT-PCR products for TR α and TR $\beta 0$ mRNA purified from the same six tissues. Pec, pectoralis muscle; pit, pituitary.

Figure 2 (a) Total T₄ and (b) total T₃ hormone concentrations in serum from 8-week-old domestic ducklings treated with two concentrations of methimazole (MMZ; 6 or 12 mg 100 g⁻¹ BM), or MMZ (6 mg 100 g⁻¹ BM) in combination with two concentrations of both T₄ and T₃ (5 or 10 µg 100 g⁻¹ BM). Data are presented as means ± S.E.M. **P* < 0.05.

those in euthyroid control birds (7 and 2 nmol l⁻¹ respectively) at 8 weeks of age. Serum T₄ was reduced to below the level of assay sensitivity (<5 nmol l⁻¹), while serum T₃ concentrations were 1.3 and 1 nmol l⁻¹ respectively. Treatment with 5 µg or 10 µg 100 g⁻¹ BM of both T₄ and T₃ in combination with methimazole (6 mg 100 g⁻¹ BM) increased serum T₄ levels to three and six times that of euthyroid controls respectively, and increased serum T₃ levels to three and five times that of control birds.

Table 3 shows the impact of changes in thyroid status on muscle mass and the maximum activity of CS. The methimazole-treated hypothyroid ducklings had significantly smaller relative masses of both cardiac (*P* < 0.01) and pectoralis (*P* < 0.01) muscles compared with those of controls. Absolute leg muscle mass was lower in hypothyroid ducklings treated with 6 mg methimazole 100 g⁻¹ BM (*P* < 0.05) in line with the reduction in overall BM, while relative leg muscle mass was not affected. The hyperthyroid ducklings, as a result of treatment with methimazole in combination with both T₄ and T₃, had cardiac and

pectoralis muscle masses similar to those of control birds. Activity of CS was lower by 40% (*P* < 0.05) and 60% (*P* < 0.05) of control values in the pectoralis and leg muscles, respectively, of hypothyroid ducks, while CS activity of the cardiac muscles was not affected. In hyperthyroid birds, the activity of CS in the cardiac and pectoralis muscles was similar to that of controls birds.

RT-PCR analysis of relative TR α mRNA expression in pectoralis, semimembranosus and cardiac muscles yielded no significant differences between any of the experimental groups (data not shown), while TR β2 mRNA was not quantified due to its very restricted tissue distribution. Figure 3 shows the results for RT-PCR analysis of relative TR β0 mRNA expression. Hyperthyroidism resulted in a significantly higher TR β0 mRNA expression in cardiac (4.2-fold, *F* = 7.07, *P* = 0.003), pectoralis (3.5-fold, *F* = 2.05, *P* < 0.05) and leg (4.0-fold, *F* = 9.94, *P* < 0.001) muscles compared with those of euthyroid controls (0.822 ± 0.297, 0.098 ± 0.019 and 0.38 ± 0.202 respectively). However, only the leg muscle showed a different response to hypothyroidism, with a significantly lower TR β0 mRNA expression in birds treated with 12 mg methimazole 100 g⁻¹ BM (0.109 ± 0.047, *P* < 0.05).

Effect of changes in thyroid status on liver TR mRNA and enzyme expression

Quantitative analysis of relative TR α and TR β0 mRNA expression in liver samples showed no significant differences between any of the experimental groups (Fig. 4). However, Fig. 4 also shows that the maximum activity of malic enzyme in the liver was significantly lower (*P* < 0.001) in hypothyroid ducks (by 50–60%) compared with that of the control birds (15 µmol g⁻¹ min⁻¹). Hyperthyroid birds had significantly higher values (*P* < 0.05) for malic enzyme (by 25–30%). In contrast, the maximum activities of malic enzyme in the cardiac and locomotor muscles were relatively low (between 2 and 3 µmol g⁻¹ min⁻¹) and were not significantly different between any of the groups (data not shown).

Discussion

This study has established that the TR isoform mRNAs of 8-week-old ducklings are differentially distributed between different tissues. The general pattern of their expression is similar to that described for mammals (Lazar 1993), in that avian TR α mRNA was ubiquitous in distribution, while TR β2 mRNA was strongly expressed only in the pituitary gland. TR β2 mRNA expression was also detected in the cerebral cortex of the ducklings, and its expression has been reported in the retinal tissue of chick embryos (Sjöberg *et al.* 1992). TR β0 mRNA

Table 3 The effects of induced hypo- and hyperthyroidism on morphological characteristics of domestic ducks (*Anas platyrhynchos*) and maximum activity of CS in semimembranosus, pectoralis and cardiac muscles. Data are presented as means $\pm$ S.E.M.

Variable	Control	MMZ (12 mg)	MMZ (6 mg)	MMZ+T ₄ /T ₃ (5 µg)	MMZ+T ₄ /T ₃ (10 µg)
BM (g)	3424 $\pm$ 59	2946 $\pm$ 277	2818 $\pm$ 192**	3348 $\pm$ 133	3518 $\pm$ 81
Pectoralis muscle mass (g)	322 $\pm$ 11	201 $\pm$ 34**	174 $\pm$ 25**	327 $\pm$ 95	339 $\pm$ 8.1
Relative pectoralis mass (% BM)	9.42 $\pm$ 0.4	6.66 $\pm$ 0.68**	6.38 $\pm$ 0.48**	9.8 $\pm$ 0.4	9.66 $\pm$ 0.28
Leg muscle mass (g)	34.9 $\pm$ 1.8	29.4 $\pm$ 2.7	28.3 $\pm$ 3.2*	33.3 $\pm$ 0.8	37.4 $\pm$ 1.0
Relative leg mass (% BM)	1.02 $\pm$ 0.06	1.00 $\pm$ 0.02	1.04 $\pm$ 0.04	1.00 $\pm$ 0.04	1.06 $\pm$ 0.02
Heart muscle mass (g)	17.2 $\pm$ 0.7	10.1 $\pm$ 1.2**	9.7 $\pm$ 1.2**	18.1 $\pm$ 0.8	18.5 $\pm$ 0.8
Relative heart mass (% BM)	0.50 $\pm$ 0.015	0.34 $\pm$ 0.01**	0.36 $\pm$ 0.02**	0.54 $\pm$ 0.04	0.52 $\pm$ 0.02
9th primary feather length (mm)	136 $\pm$ 3.1	75.5 $\pm$ 23.4*	46.4 $\pm$ 1.2**	138.1 $\pm$ 4.0	130.8 $\pm$ 1.7
Tibiotarsus bone length (mm)	85.1 $\pm$ 0.3	81.6 $\pm$ 1.2*	81.3 $\pm$ 1.2*	84.9 $\pm$ 0.8	85.7 $\pm$ 1.1
Radius bone length (mm)	119 $\pm$ 1.0	104 $\pm$ 4.8**	99.9 $\pm$ 5.9**	116 $\pm$ 1.3	117 $\pm$ 1.2
Sternum length (mm)	145 $\pm$ 1.6	114 $\pm$ 5.3**	110 $\pm$ 4.0**	143 $\pm$ 4.6	146 $\pm$ 1.9
Head length (mm)	145 $\pm$ 0.7	127 $\pm$ 2.8**	125 $\pm$ 4.1**	143 $\pm$ 1.9	143 $\pm$ 1.8
Mass-specific activity of CS in pectoralis muscle (µmol/g per min)	26.8 $\pm$ 1.6	8.9 $\pm$ 1.7**	11.9 $\pm$ 2.0**	36.9 $\pm$ 2.2	30.0 $\pm$ 4.0
Mass-specific activity of CS in cardiac muscle (µmol/g per min)	93.5 $\pm$ 1.9	98.5 $\pm$ 5.3	82.8 $\pm$ 7.4	80.8 $\pm$ 3.1	88.7 $\pm$ 3.5
Mass-specific activity of CS in semimembranosus muscle (µmol/g per min)	24.1 $\pm$ 2.4	17.0 $\pm$ 1.6	13.0 $\pm$ 1.9**	30.6 $\pm$ 0.8	28.6 $\pm$ 2.5

MMZ, methimazole.

* $P < 0.05$, ** $P < 0.001$.

expression showed marked differences in abundance between tissue types but was relatively more abundant in cardiac than striated muscle tissue. Striated muscle appeared to express primarily TR α mRNA. A similar pattern of distribution for TR α and $\beta 1$ mRNA expression in cardiac and striated muscle tissue has recently been reported for the rat and the human (Haddad *et al.* 1998, Shahrara *et al.* 1999). TR $\beta 0$ mRNA expression was significantly modulated by changes in thyroid status, unlike TR α mRNA. In addition, the pattern of TR $\beta 0$ mRNA modulation by thyroid hormones was tissue specific.

The findings of this study demonstrate that there is not always a close association between the effect of changes in thyroid status on TR isoform mRNA expression and effects on tissue phenotype, such as tissue mass or the maximum activities of specific metabolic enzymes. Thus, these results support the hypothesis that mechanistic interpretations of target gene regulation by thyroid hormone receptor binding are likely to be tissue specific (Lin *et al.* 1997).

The major site for lipogenesis is the liver, and samples from the 8-week-old ducklings in the present study demonstrated a direct relationship *in vivo* between hypo- and hyperthyroidism and a subsequent decrease or increase, respectively, in the lipogenic activity of hepatic malic enzyme. However, the expression of TR α and TR $\beta 0$ isoform mRNAs in the liver of the ducks was not affected by thyroid status. Thus, the marked positive thyroid hormone regulation of the activity of hepatic malic enzyme did not require changes in the level of

expression of the TR α and TR $\beta 0$ isoform mRNAs. Assuming that there is little post-transcriptional regulation as a result of changes in thyroid status, a reduction in serum thyroid hormone concentration would result in a lower rate of ligand/receptor binding. This reduction in receptor occupancy could decrease the direct stimulation of transcription of the target gene, and may also result in increased ligand-independent repression of transcription (Chen & Evans 1995, Horlein *et al.* 1995, Wondisford 1996).

The activity of malic enzyme in the locomotor and cardiac muscles of the ducklings was not affected by thyroid status. This result is similar to that reported in neonatal rats where hepatic malic enzyme activity was positively regulated by thyroid status but brain malic enzyme activity was unaffected (Sood *et al.* 1996). Thus, our results suggest that tissue-specific factors in avian muscle impair thyroid hormone-induced expression of malic enzyme.

The effects of changes in thyroid status on the activity of CS and TR mRNA isoform transcription are more complex in the cardiac and locomotor muscles than in the liver. As a consequence of the exposure to a hyperthyroid state, TR α mRNA abundance, muscle mass measurements and CS enzyme activities were similar in all three muscles studied, while TR $\beta 0$ mRNA expression was significantly higher in these tissues. In the leg muscle, TR $\beta 0$ mRNA expression was lower during hypothyroidism along with a slightly lower activity of CS and absolute muscle mass, although relative leg muscle mass was unaltered. In the cardiac and pectoralis muscles, exposure

Figure 3 Ratio of TR $\beta 0$ mRNA isoform expression normalised with respect to β -actin mRNA expression in (a) pectoralis, (b) cardiac and (c) semimembranosus muscle tissue from 8-week-old domestic ducklings treated with two concentrations of methimazole (MMZ), or MMZ in combination with both T_4 and T_3 . For details see legend to Fig. 2. Data are presented as means $\pm$ S.E.M. * $P < 0.05$.

to hypothyroidism did not affect TR $\beta 0$ mRNA expression while cardiac and pectoralis mass and pectoralis CS activity were lower.

The finding that hyperthyroidism in the domestic duck did not lead to an increase in cardiac and locomotor muscle mass, or aerobic capacity, is consistent with previous findings in the barnacle goose (Deaton *et al.* 1997, 1998). This suggests that these tissues are already developing at a maximal rate in euthyroid birds with regard to stimulation of muscle growth by thyroid hormones. Conversely, induction of hypothyroidism significantly reduced the aerobic capacity of the locomotor muscles, and this action was effected independently of any pretranslational regulation of TR isoform mRNAs. It is possible that the alterations in muscle mass and mass-specific CS enzyme activity, as a result of changes in thyroid status, were

Figure 4 (a) Maximum mass-specific activity of malic enzyme ($\mu\text{mol g}^{-1} \text{min}^{-1}$) and (b) the ratio of TR α and TR $\beta 0$ mRNA isoform expression normalised with respect to β -actin mRNA expression in liver from 8-week-old domestic ducklings treated with two concentrations of methimazole (MMZ), or MMZ in combination with both T_4 and T_3 . For details see legend to Fig. 2. Data are presented as means $\pm$ S.E.M. * $P < 0.05$.

influenced by an independent effect of hyper- and hypothyroidism on the nutritional intake of the animals (cf. Harrison *et al.* 1996). In addition, deiodinase enzymes are expressed in muscle tissue (St Germain & Galton 1997, Hosoi *et al.* 1999) and their possible influence could be considered in further experiments.

The present study has highlighted the tissue-specific nature of the pretranslational regulation of TR mRNAs and associated target proteins to changes in the thyroid status of birds. In particular, modulation of the activity of thyroid-sensitive enzymes, such as malic enzyme and CS, are not necessarily associated with changes in TR α and TR $\beta 0$ mRNA expression. Future work should focus on the effect of thyroid status on the regulation of TR isoform translation and on changes in associated nuclear receptor species and their co-activators and co-repressors in avian tissues, since complex interactions between these proteins are likely to determine the transcriptional regulatory effects of thyroid hormones.

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