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TISSUE-SPECIFIC EFFECTS OF HYPOTHYROIDISM ON POSTNATAL MUSCLE DEVELOPMENT IN THE BARNACLE GOOSE

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Summary

The hypothesis that tissue-specific levels of thyroid hormones may be required for normal locomotor muscle development was investigated in the barnacle goose *Branta leucopsis*. Hypothyroidism was induced in goslings by treatment with methimazole from either 3 days or 2 weeks of age, and birds were killed at 7 weeks of age. The masses of the pectoralis, iliofibularis, semimembranosus and cardiac ventricle muscles were measured, and samples from these tissues were analysed for the mass-specific activity of the mitochondrial enzyme citrate synthase (CS). An ultrastructural electron micrograph analysis of the pectoralis was also carried out. No significant differences were found between the two hypothyroid groups except for the effect on the relative mass of the iliofibularis muscle. Developmental responses to hypothyroidism were found to be tissue-specific. Hypothyroidism resulted in a significantly lower relative cardiac ventricle mass (by 17 %) and CS activity of the leg muscles (by 34 %), while absolute leg muscle mass was not affected. The relative mass of the pectoralis was significantly lower (by 57 %) in hypothyroid birds and showed a significant, uniformly lower CS activity (by 60–83 %) as a result of a lower mitochondrial fractional volume. Haematocrit and capillary-to-fibre ratio in the pectoralis were also significantly lower in hypothyroid birds, and skeletal growth and plumage development were affected.

Key words: thyroid hormones, citrate synthase, muscle development, bird, haematocrit, haemoglobin, hypothyroidism, growth, bone barnacle goose, *Branta leucopsis*.

Introduction

Just 12 weeks after hatching, the precocial young of the barnacle goose (*Branta leucopsis*) migrate approximately 2500 km from their Arctic summer breeding site in Svalbard to overwinter in southwest Scotland. Therefore, they must undergo relatively rapid development to meet the aerobic demands of this arduous activity. Between 5 and 7 weeks of development, both pectoralis mass and mass-specific activity of citrate synthase (CS, an indicator of oxidative capacity) increase rapidly (Bishop *et al.* 1995, 1996, 1998), in association with an increase in the capillary density and fractional volume of mitochondria in the pectoralis (Egginton *et al.* 1997) and a similar rise in circulating levels of thyroxine (Bishop *et al.* 1998). Both wild and captive birds show this developmental profile (Bishop *et al.* 1998). As thyroxine has been shown to be important for normal body and skeletal muscle development in both birds and mammals (King and May, 1984), it has been suggested that alterations in circulating thyroxine concentrations may be involved in the control of aerobic muscle development in the barnacle goose (Deaton *et al.* 1997; Bishop *et al.* 1998).

Altered thyroid state in adult rats can effect changes in the activity of CS with parallel changes in fibre composition of the muscle. Hypothyroidism results in a reduction in the proportion of fast oxidative glycolytic (FOG) fibres (Nwoye *et al.* 1982; Sillau, 1985), while hyperthyroidism exhibits greatest effect in muscles containing slow oxidative (SO) fibres, the proportion of these fibres decreasing while the proportion of fast glycolytic (FG) and FOG fibres increases (Winder, 1979; Fitts *et al.* 1980; Nicol and Bruce, 1981; Nicol and Johnston, 1981; Capo and Sillau, 1983). However, it has been shown that the effect of hyperthyroidism on the activity of CS in the quadriceps muscle of the rat is greatest during development, as treatment to induce hyperthyroidism for 6 days after birth results in a threefold increase in activity of CS (Baldwin *et al.* 1978), whereas treatment for 6 weeks at a higher dose is required before an effect is shown in adult rats (Winder *et al.* 1975). Also, chickens treated with tri-iodothyronine (T_3) during postnatal development showed an increase in the activity of CS in the anterior and posterior latissimus dorsi muscles that is not seen after T_3 treatment in adults (Snyder *et al.* 1991). Thus, thyroid hormones appear to exert their greatest influence on mitochondrial enzymes (such as CS) in neonates, and these effects may diminish as the animal reaches maturity (Baldwin *et al.* 1978).

Altered thyroid state in developing barnacle geese has been shown to have muscle-specific effects. Mild hypothyroidism, induced by the administration of methimazole (2 mg 100 g^{-1} body mass), from 2 weeks of age, reduced the

muscle mass and mass-specific activity of CS in the pectoralis but had little effect on the relatively early-maturing leg and cardiac musculature (Deaton *et al.* 1997). Thus, it was suggested that thyroid hormones may be involved in controlling the tissue-specific timing of the maturation of locomotor and cardiac muscles in the barnacle goose, and this hypothesis was investigated further in the present study. Thyroid hormone production was reduced from either 3 days of age or 2 weeks of age to test the hypothesis that the activity of CS in cardiac and selected leg muscles would be affected to a greater extent by hypothyroidism induced at the relatively earlier age. In addition, a more effective hypothyroidism than that described in the study of Deaton *et al.* (1997) was induced by increasing the dose of methimazole used (to 6 mg 100 g^{-1} body mass). The effects of hypothyroidism on other factors associated with aerobic capacity, such as haematocrit, haemoglobin concentration and the capillary and mitochondrial volume density of the pectoralis muscles were also investigated.

Materials and methods

Experimental design

Eggs of barnacle geese (*Branta leucopsis*) were obtained from captive birds and hatched by artificial incubation at 37.7 °C at the University of Birmingham. Goslings were kept indoors in large groups with a photoperiod of 18 h:6 h L:D, the light period being from 06:00 to 22:00 h. Food and water were available *ad libitum*.

The birds were divided into three groups. Hypothyroidism was induced in two groups by treatment with the thyroid-inhibiting drug methimazole (Sigma M8506). One group (N=5) began treatment at 3 days of age (M@3days) with 3 mg 100 g^{-1} body mass day^{-1}, which was increased to 6 mg 100 g^{-1} body mass day^{-1} at 1 week of age. The second group (N=6) began treatment with 6 mg 100 g^{-1} body mass day^{-1} at 2 weeks of age (M@2weeks). The final group served as controls (N=6). The methimazole compound was suspended in 200 µl of 1 % gelatine solution and was administered orally at 15:00 h daily. The control group was given 200 µl of 1 % gelatine solution per day. Doses were delivered onto the back of the bird's tongue using a 1 ml syringe with an attachment of 2 cm of soft plastic tubing. A separate group of 12 euthyroid birds was raised for an investigation into the changes in haematocrit and haemoglobin concentration during development.

Data collection

Blood samples were taken each week at 10:00 h. Until 4 weeks of age, blood was taken from a leg vein, using a heparinized hypodermic syringe. From 4 weeks onwards, blood samples were taken from the brachial (wing) vein. For analysis of thyroid hormones, blood was centrifuged at 7300 ***g*** for 3 min to obtain the plasma, which was stored at –20 °C for subsequent analysis. Whole blood was used for the analysis of haematocrit and haemoglobin concentration at 7 weeks of age in the experimental birds and weekly from 2 to 9 weeks in the additional euthyroid birds. All birds were weighed weekly.

The three groups of birds were killed at 7 weeks of age, towards the end of the most rapid period of growth (Bishop *et al.* 1995, 1996), by an intravenous injection of sodium pentabarbitone (200 mg kg^{-1}). Dissection was carried out immediately and as quickly as possible. The cardiac ventricles (left and right together), pectoralis and supracoracoideus muscles were dissected free and weighed. All tissue subsamples (0.1–0.3 g) were taken within 40 min of death, from the left ventricle of the heart, the anterior region of the pectoralis from both peripheral and deep sites (see Deaton *et al.* 1996), and the central portions of the semimembranosus and iliofibularis muscles of the leg. Samples were immediately placed in preweighed, perforated Eppendorf tubes, reweighed and then frozen in liquid nitrogen (–196 °C) for storage, awaiting enzyme analysis. O'Connor and Root (1993) showed that the activity of CS in the pectoralis of the house sparrow (*Passer domesticus*) is not affected up to 60 min after death, and these findings have been confirmed in the barnacle goose (Bishop *et al.* 1995). Using Vernier callipers, measurements were made of the length of the sternum keel, head (posterior of head to tip of beak), tibiotarsus, tarso-metatarsus, femur, humerus, radius and ulna together, and the ninth primary wing feather. To investigate whether the relative growth of the bones was affected by hypothyroidism, using equations describing growth relative to the body mass in euthyroid goslings (Deaton, 1997), the body measurements of the 7-week-old birds were predicted from their body mass and compared with the actual measurements made at 7 weeks of age.

Citrate synthase assay

The mass-specific activity of CS was measured as described by Deaton *et al.* (1997). Samples from identical tissues were always homogenised and assayed as a batch, and the enzyme assay was carried out 1–3 days after sample homogenisation. Each sample was assayed in triplicate, at 41 °C, and results are expressed as µmol substrate min^{-1} g^{-1} wet mass of tissue.

Electron microscopy

A strand of muscle (0.5 mm×0.5 mm×5 mm) was taken along the striations of the pectoralis muscle of control birds and those given methimazole from 2 weeks of age onwards, within 30 min of death. The tissue was placed in fixative (25 % glutaraldehyde, 2 % paraformaldehyde, in a 0.1 mol l^{-1} phosphate buffer at pH 7.2 at 25 °C) and stored at 4 °C. The samples were then rinsed in phosphate buffer and postfixed in OsO_4 (phosphate-buffered to pH 7.2) for 1 h before being dehydrated through a series of alcohols up to absolute, cleared in propylene oxide (1,2-epoxypropane) and vacuum-embedded in resin. Transverse semi-thin sections (0.5 µm) were cut on an ultramicrotome, stained with Toluidine Blue, and examined under a light microscope to check the orientation and position of the sample. Ultrathin transverse sections (70 nm) were then cut using an ultramicrotome with a diamond knife and stained with Reynold's lead citrate and 30 % uranyl acetate in methanol. Sections were viewed under a Joel 1200 ex electron microscope at 60 kV.

Capillary-to-fibre ratio was estimated by calculating the ratio of the number of capillaries to the number of fibres, using

a non-biased counting technique (S. Egginton, personal communication). Samples from control and hypothyroid birds were viewed at ×500 and ×250 magnification, respectively, to ensure a similar number of fibres (approximately 110) was represented in the photographs taken of each group.

Morphometric analysis was carried out using the stereological techniques of Weibel (1973). Samples from control and hypothyroid birds were viewed at ×5000 and ×2500 magnification, respectively, and photographic negatives of four unbiased sampled views of each bird were printed up to 24 cm×18 cm. A 13 mm grid was placed over the photographs.

Fractional volume (Vv) was estimated for subsarcolemmal mitochondria, myofibrillar mitochondria, lipids and capillaries, by point counting:

$$V\text{v} = P\text{i}/P\text{T}, \quad (1)$$

where Pi is the number of points on a variable and PT is the total number of points.

Surface density (Sv) was also estimated for the above variables by counting the number of intersections formed along both horizontal (H) and vertical lines (V) on the grid:

$$S\text{v} = I\text{i}_\text{H}/L\text{T}_\text{H} + I\text{i}_\text{V}/L\text{T}_\text{V}, \quad (2)$$

where Ii is the number of intersections formed and LT is the total length of the lines.

Thyroid hormone assays

Total plasma thyroxine (T_4) and T_3 were measured by radioimmunoassay using standard double antibody kits from Immunodiagnostic Systems Ltd and Kodak Clinical Diagnostics Ltd, respectively.

Haematology

Haematocrit was measured in fresh whole blood by a standard technique using a Hawksley microhaematocrit centrifuge and reader. Each sample was measured in duplicate. Haemoglobin concentration was measured by spectrophotometry with a commercial assay kit (Sigma Procedure no. 525).

Statistics

Analysis was carried out to determine differences in the parameters measured between the three experimental groups. As muscle masses are expressed as a percentage of body mass (see below), and therefore do not follow a normal distribution, these data underwent an arcsine transformation. A logarithmic transformation was carried out on data sets where variance was found to be unequal between the treatment groups (Zar, 1984). One-way analysis of variance (ANOVA) was then carried out and followed by *post-hoc* Fisher's least significant difference test. The acceptance level of $P<0.05$ was used. The F ratio and P value from the ANOVA are shown in the figure legends. The P values of the *post-hoc* Fisher test are shown in the text and symbolised on the figures. Two-way ANOVA was carried out to assess the effects of age and body mass, and age and plasma T_4 concentration. Student's independent t-tests were carried out between control and hypothyroid groups in the electron microscopy study, and paired t-test were carried out between the predicted and actual skeletal measurements. The acceptance level of $P<0.05$ was used throughout.

Values given in the text are means ± S.E.M.

Results

Hormones

There were no significant differences between the two hypothyroid groups in plasma T_3 or T_4 concentrations at any age. Plasma T_4 concentration of control birds increased (by 173%) between 5 and 7 weeks of age, from 6.3 nmol l^{-1} to 17.2 nmol l^{-1} (Fig. 1), while plasma T_3 concentration showed little change during this period (data not shown). Both hypothyroid groups showed plasma T_4 concentrations significantly below those of control birds throughout development from 3 weeks onwards. By 7 weeks of age, both plasma T_3 (data not shown) and T_4 (Fig. 1) concentrations in both groups of methimazole-treated birds were significantly ($P<0.001$) lower at 14% and 26% (M@2weeks), and 8.2% and 21% (M@3days), respectively, of the concentrations shown by control birds.

Body mass, citrate synthase activity and muscle mass

Two-way ANOVA showed both age and experimental group to have a significant effect on body mass ($P<0.001$). At 1 week of age, M@3days showed a significantly lower body mass than control birds and M@2weeks (Fig. 2). From 5 weeks of age onwards, there was a significant difference between control birds and M@2weeks, but no significant differences in body mass were found between the two hypothyroid groups once methimazole treatment had begun. As body mass was significantly affected by hypothyroidism, muscle masses are expressed as a percentage of body mass to allow comparison of the proportionality of different muscles during development when under the influence of thyroid hormone manipulation.

Pectoralis muscle

No significant differences in relative mass of the pectoral muscle or in mass-specific CS activity were found between the

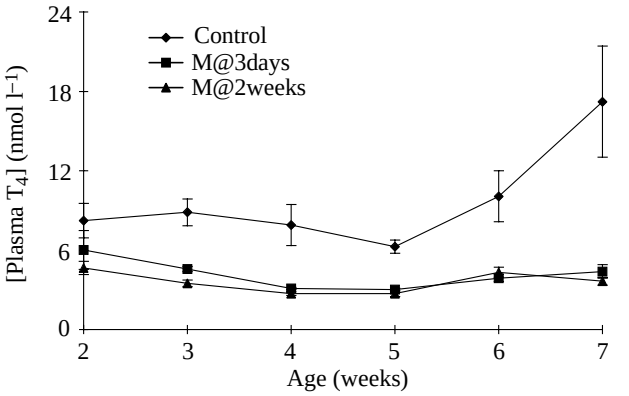

Fig. 1. Plasma T_4 hormone concentrations (means ± S.E.M., N=5–6) measured through development of birds treated with methimazole (M) from either 3 days of age (M@3days) or 2 weeks of age (M@2weeks) and in control birds.

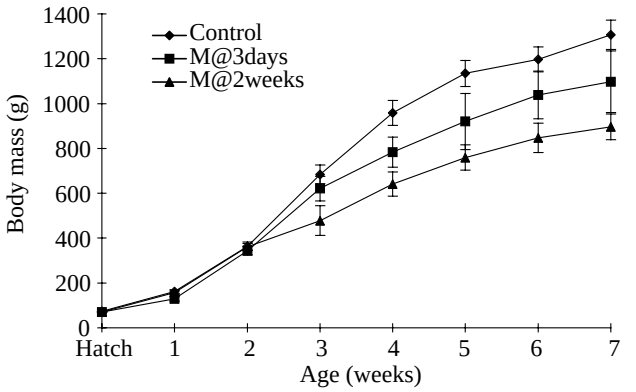

Fig. 2. Body mass (means ± S.E.M., N=5–6) measured through development of birds treated with methimazole (M) from either 3 days of age (M@3days) or 2 weeks of age (M@2weeks) and in control birds.

two hypothyroid groups. Both hypothyroid groups showed both significantly lower (P<0.001) relative pectoralis masses (by 49 % for M@3days and 65 % for M@2weeks, Fig. 3A) and mass-specific activity of CS in the peripheral pectoralis sample (by 60 % for M@3days and 83 % for M@2weeks, Fig. 3B) compared with control birds (relative mass 10.5±0.1 % body mass, mass-specific CS activity 123.2±9.8 μmol min^{-1} g^{-1}). Mass-specific CS activities in the deep sites were very similarly affected, showing activities lower by 61 % and 83 %, respectively, compared with those in control birds (177.0±19.6 μmol min^{-1} g^{-1}), and there was a tendency for these values to be higher (40 %) than those of the peripheral site, although this was not significant because of the large variance. A significant relationship (y=8.24x–3.09, r^2=0.77, P<0.001) was found between mass-specific activity of CS and relative pectoralis mass as a percentage of body mass across the three groups.

Heart

No significant differences in relative masses or mass-specific activities of CS were found between the two hypothyroid groups. In both hypothyroid groups, the relative mass of the heart ventricles (Fig. 3C) was significantly lower (by 16 % for M@3days and 17 % for M@2weeks, P<0.01) than that of control birds (0.62±0.02 % body mass). Mass-specific activity of CS did not differ from control values (P=0.74, control 208.1±15.5 μmol min^{-1} g^{-1}, Fig. 3D).

Leg muscles

In both hypothyroid groups, the relative masses of both the iliofibularis and semimembranosus muscles (Fig. 4A) were found to be significantly greater (by 18 %, P=0.05, and 25 %, P<0.001, respectively, for M@3days, and by 44 %, P=0.01, and 33 %, P<0.001, respectively, for M@2weeks) than those of control birds (0.72±0.0 % body mass and 0.78±0.04 % body mass, respectively). However, the absolute masses of these muscles in both hypothyroid groups (Fig. 4B) did not differ (P=0.76 and P=0.91, respectively) from those of control birds (9.2±0.4 g and 9.9±0.9 g, respectively). No significant difference was found between the two hypothyroid groups for absolute mass of the semimembranosus or iliofibularis or for the relative mass of the semimembranosus. However, the relative mass of the iliofibularis in M@3days was found to be significantly smaller (P=0.04) than that in M@2weeks.

The mass-specific activity of CS in the semimembranosus (Fig. 4C) was significantly lower in both hypothyroid groups (by 33 % for M@3days and 34 % for M@2weeks, P<0.05) compared with that of control birds (87.0±8.5 μmol min^{-1} g^{-1}), with no significant difference between the two hypothyroid groups. The mass-specific activity of CS in the iliofibularis (Fig. 4C) of M@2weeks was found to be significantly lower (26 %, P=0.02) than that in control birds (103.7±7.7 μmol min^{-1} g^{-1}). Activity of CS in M@3days was not significantly different from that in control birds (P=0.2).

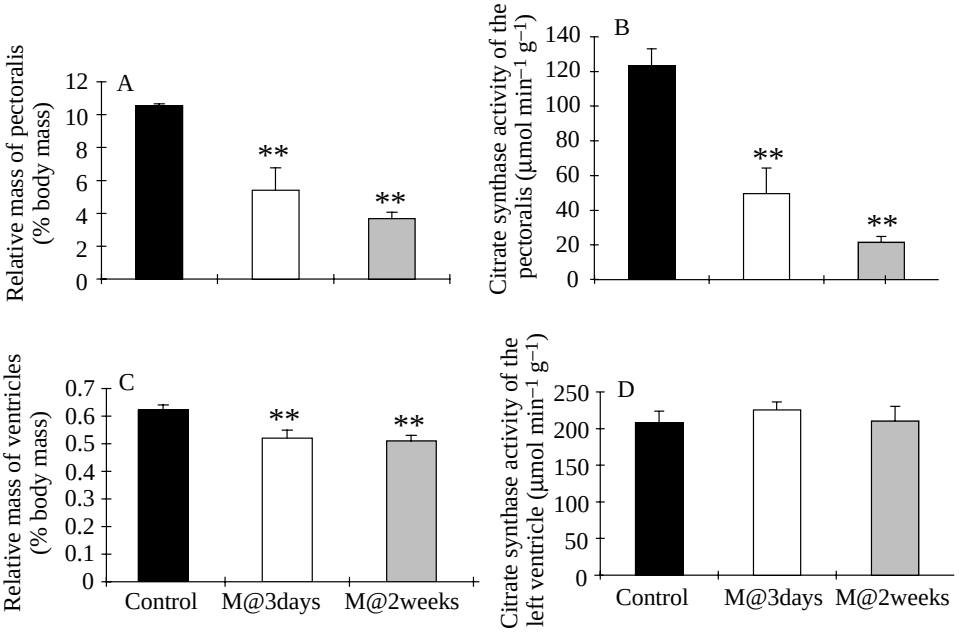

Fig. 3. The effect of treatment with methimazole from either 3 days of age (M@3days, open columns) or 2 weeks of age (M@2weeks, stippled columns) on (A) relative mass of the pectoralis muscle (ANOVA F=68.159, P<0.001), (B) mass-specific activity of citrate synthase (CS) in the pectoralis in samples taken from peripheral sites (ANOVA F=0.003, P=0.001, N=6), (C) relative mass of the ventricles (ANOVA F=8.432, P=0.004) and (D) mass-specific activity of CS in the left ventricle (ANOVA F=0.307, P=0.74) of 7-week-old goslings. Values are means + S.E.M., N=5–6. Significant differences from control birds (filled columns) are marked: **P<0.01.

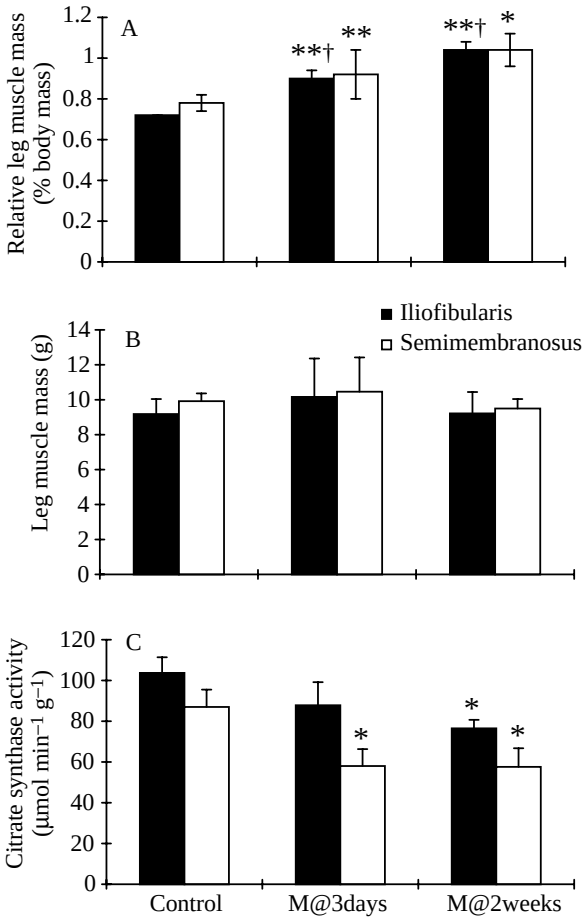

Fig. 4. The effect of treatment with methimazole from either 3 days of age (M@3days) or 2 weeks of age (M@2weeks) on (A) relative mass of the iliofibularis muscle (filled column, ANOVA F=31.354, P<0.001) and semimembranosus muscle (open column ANOVA F=6.8, P=0.014), (B) absolute mass of the iliofibularis muscle (filled column, ANOVA F=0.282, P=0.76) and semimembranosus muscle (open column, ANOVA F=0.282, P=0.76) and (C) mass-specific activity of citrate synthase (CS) in the iliofibularis muscle (filled column, ANOVA F=3.214, P=0.071) and semimembranosus muscle (open column, ANOVA F=3.834, P=0.047) of 7-week-old goslings. Values are means ± S.E.M., N=5–6. Significant differences from control birds are marked: *P<0.05; **P<0.01. † indicates a significant difference between M@3days and M@2weeks, P<0.05.

Ultrastructure of the pectoralis

This analysis was only carried out on the M@2wk hypothyroid birds.

The fractional volume of myofibrils in the hypothyroid birds was significantly greater than that in control birds (by 25 %, P=0.002, Fig. 5A), while the capillary-to-fibre ratio (Fig. 5B) was significantly lower in hypothyroid birds (by 76 %, P=0.02) compared with that of control birds (1.27±0.17).

Hypothyroidism resulted in lower fractional volumes (Fig. 6C) of subsarcolemmal mitochondria (by 78 %, P=0.001), myofibrillar mitochondria (by 71 %, P<0.001) and capillaries (by 64 %, P=0.007) compared with values for control birds (0.05±0.01, 0.16±0.05 and 0.11±0.04, respectively). There was no significant difference (P=0.57) in the ratio of CS activity to total fractional volume of mitochondria between hypothyroid (532±138 μmol min^{-1} g^{-1}) and control birds (625±70 μmol min^{-1} g^{-1}). No significant difference from control values was seen in the fractional volume of myonuclei (P=0.805) and lipids (P=0.055).

The hypothyroid group showed lower surface densities (Fig. 5D) of subsarcolemmal mitochondria (67 %, P<0.001), myofibrillar mitochondria (by 51 %, P=0.01) and lipids (by 45 %, P=0.03) in comparison with the values found in control birds (166.0±17.4 cm^{-1}, 744.5±66.4 cm^{-1} and 106.4±14.6 cm^{-1}, respectively). The surface density of capillaries in the hypothyroid group was not significantly different from that of the control group (P=0.087).

Bone length and plumage development

The growth of all bones measured was slower in hypothyroid geese (Fig. 6). Both hypothyroid groups showed significantly shorter sternum (by 22 % in M@3days, P=0.004, and by 33 %, P<0.001 in M@2weeks), humerus (by 16 %, P<0.001, and 29 %, P<0.001, respectively), radius and ulna (by 16 % in M@3days, P=0.01, and by 25 % in M@2weeks, P<0.001) and head (by 8 % in M@3days, P=0.02, and by 12 % in M@2weeks, P<0.001) measurements. The lengths of the leg bones were less affected than those of the wings, with only M@2weeks showing a significantly (P<0.01) shorter femur (by 11 %, P<0.001), tibiotarsus (by 9 %, P=0.005) and tarso-metatarsus (by 9 %, P=0.007) than those of control birds. There were no significant differences in bone length measurements between the two hypothyroid groups. The growth of the tibiotarsus and tarso-metatarsus relative to body mass was not affected by hypothyroidism (P=0.5 and P=0.2, respectively). However, the relative growth of the head, sternum and radius/ulna was significantly affected (P<0.01).

Hypothyroid birds showed retarded plumage development. The ninth primary feather (Fig. 6) was significantly shorter in length in both hypothyroid groups (by 31 % in M@3days, P=0.03, and by 55 % in M@2weeks, P<0.001). Feathers were not only slow to develop but their morphology was also affected, the primary feathers being narrow and fringed.

Haematocrit and haemoglobin concentration

The haematocrit of the 12 developing non-experimental euthyroid goslings remained constant (32.8±0.2 %) until 5 weeks of age, when it began to increase. It reached levels similar to those seen in adult birds (46±1.0 %) by 9 weeks of age (Fig. 7A). Haemoglobin concentration showed a similar developmental profile (data not shown), approaching adult values of 16.1±0.2 g dl^{-1}.

At 7 weeks of age, haematocrit was significantly lower (by 18 %, P<0.05) in both hypothyroid groups compared with that of control birds (35.3±1.3 %), and no significant difference was found between the two hypothyroid groups (Fig. 7B). Haemoglobin concentration was significantly lower (by 22 %, P<0.01) in M@2weeks compared with that in control birds

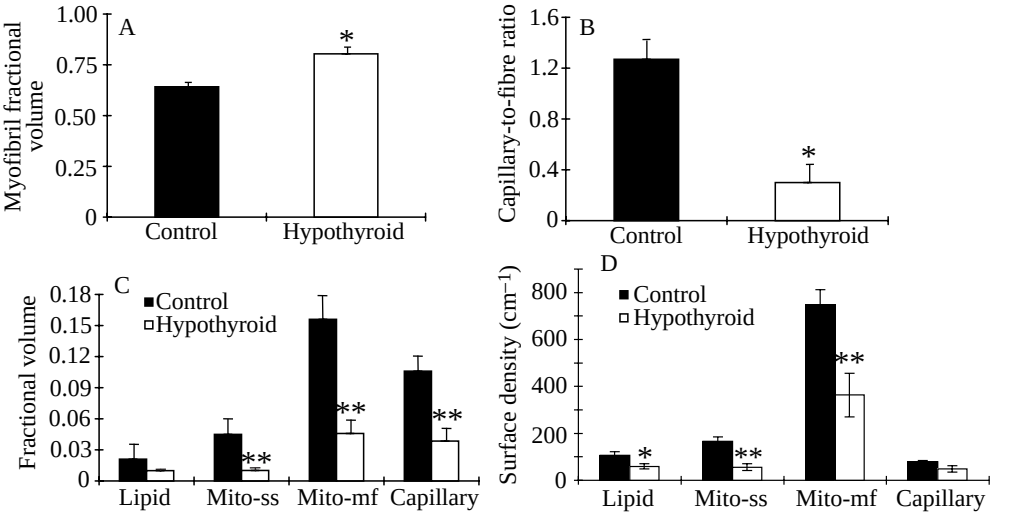

Fig. 5. The effect of treatment with methimazole from 2 weeks of age on (A) myofibril fractional volume, (B) capillary-to-fibre ratio, (C) fractional volume of lipid, subsarcolemmal mitochondria (Mito-ss), myofibrillar mitochondria (Mito-mf) and capillaries and (D) surface density of lipid, subsarcolemmal mitochondria (Mito-ss), myofibrillar mitochondria (Mito-mf) and capillaries of 7-week-old goslings. Filled columns represent control birds, open columns represent hypothyroid birds. Values are means ± S.E.M., N=6. Significant differences from control birds are marked: *P<0.05; **P<0.01.

(13.4±0.7 g dl^{-1}). The haemoglobin concentration of M@3days showed no significant difference from that of control birds or from that of the other hypothyroid group (Fig. 7C).

Discussion

Methimazole treatment was effective in rendering the goslings hypothyroid, and this tended to induce negative effects on the relative development of a number of the parameters studied. While no significant differences were found between the two hypothyroid groups, except for the effect on the relative mass of the iliofibularis muscle, there was a trend for the group commencing treatment with methimazole at 2 weeks of age to show a greater degree of developmental retardation. This was the opposite effect from that expected. The original hypothesis being tested was that birds beginning methimazole treatment at 3 days of age would show a greater effect than those commencing treatment at 2 weeks of age. Plasma thyroid hormone concentrations were very similar in these two groups, suggesting that this result was not due to any variability in the effect of dosing. One possible explanation for these findings is that an early onset of hypothyroidism results in an upregulation of the receptors for thyroid hormones. Hypothyroid piglets showed a higher muscle maximal T_3-binding capacity than that of euthyroid piglets (Duchamp *et al.* 1994). This result therefore merits further investigation.

Hypothyroidism during development resulted in lower mass-specific activities of CS both in the pectoralis muscle and in the two leg muscles studied. It appears that thyroid hormones may be required for the maturation of these skeletal muscles. Studies on rats (Gambke *et al.* 1983; Butler-Browne *et al.* 1984; d'Albis *et al.* 1990) and turkeys (Maruyama *et al.* 1991, 1993, 1995*a*,*b*) have found that, during postnatal development of skeletal muscle, the neonatal myosin heavy chains (MHCs) differentiate into the fast myosin heavy chains of type IIa and IIb (which correlate with muscle fibre types; Delp and Duan, 1996), and this process requires thyroid hormones. The differentiation of neonatal myosin to adult fast types is thought to involve the direct action of thyroid hormones (Gambke *et al.* 1983), whereas the differentiation of neonatal myosin into slow myosin (type I/SO)

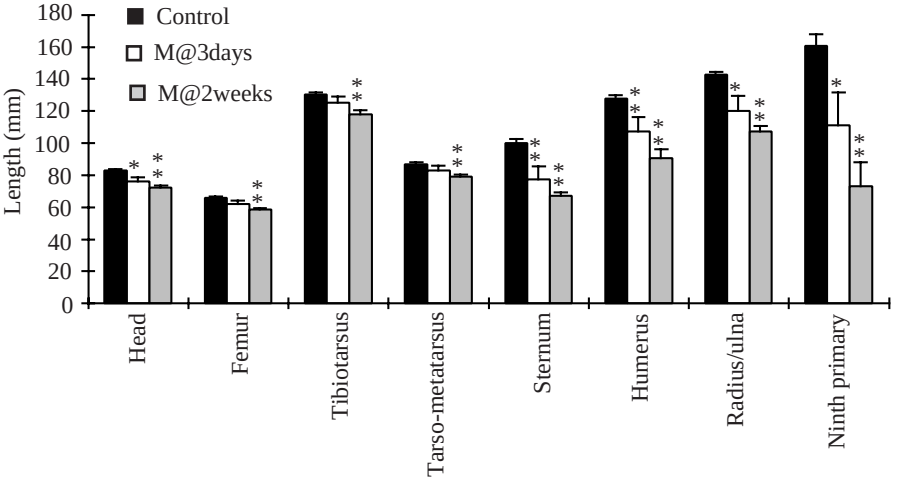

Fig. 6. The effect of treatment with methimazole from either 3 days of age (M@3days, open columns) or 2 weeks of age (M@2weeks, stippled columns) on measurements of the head (ANOVA F=9.581, P=0.002), femur (ANOVA F=7.48, P=0.006), tibiotarsus (ANOVA F=5.5, P=0.017), tarso-metatarsus (ANOVA F=5.02, P=0.023), sternum (ANOVA F=15.3, P=0.0003), humerus (ANOVA F=11.24, P=0.001), radius/ulna (ANOVA F=11.59, P=0.001) and ninth primary feather (ANOVA F=10.0, P=0.002) of 7-week-old goslings. Filled columns represent values for control birds. Values are means + S.E.M., N=6–7. Significant differences from control birds are marked: *P<0.05; **P<0.01.

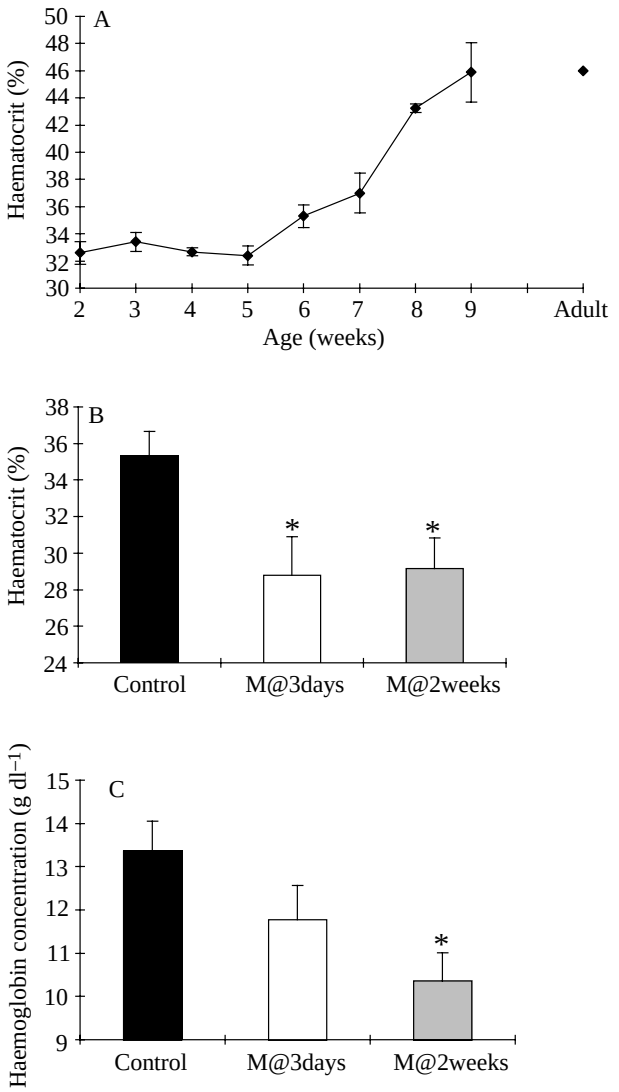

Fig. 7. (A) Measurement of haematocrit throughout the development of euthyroid goslings (N=12). (B,C) The effect of treatment with methimazole from either 3 days of age (M@3days, open columns) or 2 weeks of age (M@2weeks, stippled columns) on (B) haematocrit (ANOVA F=4.814, P=0.026) and (C) haemoglobin concentration (ANOVA F=4.806, P=0.026) of 7-week-old birds. Filled columns represent control birds. Values are means ± S.E.M., N=5–6. Significant differences from control birds are marked: *P<0.05.

is thought to be more dependent on innervation, although thyroid hormones seem to switch off the expression of the neonatal myosin genes (Gambke *et al.* 1983). It is possible that hypothyroidism has prevented fibre type maturation in the present study and that this is reflected in the lower activity of CS.

The effect of hypothyroidism on the activity of CS was greater in the pectoralis muscle than in the leg muscles, and this difference may be due to fibre type maturation occurring at tissue-specific times. Goslings are unable to fly until they are 7 weeks old, whereas they are able to walk immediately after hatching, unlike altricial animals such as rats which are unable to bear weight immediately after hatching. Bishop *et al.* (1995) found that mass-specific activity of CS in the leg muscles of barnacle geese is maximal at 1 week of age; therefore, maturation of the leg muscles is likely to be under way prior to hatch (birth), as was found in foetal sheep (Finkelstein *et al.* 1991*a*,*b*). The pectoralis matures much later and the inhibitory effects of hypothyroidism are likely to be greater.

The volume density of mitochondria was also significantly lower in the pectoralis of hypothyroid birds and remained at a level associated with a much earlier stage of development (Egginton *et al.* 1997), while the ratio of CS activity to fractional volume of mitochondria was the same in both control and hypothyroid birds. Thus, the lower mass-specific activity of CS seen in the flight muscles of hypothyroid birds is due to a lower volume density of mitochondria. This suggests that thyroid hormones may act primarily on the formation of mitochondria rather than on the synthesis of CS within existing mitochondria.

The mass-specific activity of CS in samples taken from the deeper region of the pectoralis was 40 % greater than that of the peripheral samples. This difference in CS activity has previously been observed in the barnacle goose (Deaton *et al.* 1996, 1997), and histochemical studies on various avian and mammalian muscles (Armstrong and Laughlin, 1985; Rosser and George, 1985; Turner and Butler, 1988; Torrella *et al.* 1995) also show that there is usually a greater proportion of aerobic fibres in the deeper regions of muscles. It appears that, in hypothyroid barnacle geese, the aerobic capacity of the pectoralis is uniformly lower than that of control birds, the percentage difference from respective control sites being the same for both sites within each group, and the percentage difference between the two sites being similar in all three groups.

As has previously been found in the barnacle goose (Deaton *et al.* 1997), mass-specific activity of CS in the left ventricle was not affected by hypothyroidism. However, the relative mass of the ventricles was lower in the hypothyroid birds, as was found in the rat (Canavan *et al.* 1993). Protein turnover is very high during growth and development and decreases with age (Brown *et al.* 1981), and hypothyroidism is thought to reduce the rate of protein synthesis (Flaim *et al.* 1978; Brown *et al.* 1981; Carter *et al.* 1981; see Lompre *et al.* 1991; Canavan *et al.* 1993). Thus, as the rate of protein degradation is very high in cardiac muscle (Brown *et al.* 1981), a reduction in the rate of protein synthesis as a result of hypothyroidism could result in lower cardiac muscle mass.

The pectoralis of hypothyroid goslings also had a lower relative mass, probably also as a result of reduced rates of protein synthesis. The number of fibres in a muscle is thought to be fixed at birth, hypertrophy occurring when satellite cells fuse with existing adjacent muscle fibres and donate their nuclei to them. In the pectoralis of the domestic chicken, this results in an increase in fibre cross-sectional area (McFarland *et al.* 1993; Moss, 1968*a*) proportional to the total number of nuclei (Moss, 1968*a*). In the present study, the number of nuclei was not affected, while the myofibril cross-sectional area was lower than that in control birds. A similar observation was made in the domestic chicken, in which muscle mass and fibre cross-sectional area were reduced, as a result of growth reduction (induced by

food restriction during development), but no loss of nuclei was observed compared with the situation in control birds (Moss, 1968*b*). Thus, it seems that, during restricted growth, myotubules continue to fuse but they are of reduced cross-sectional area.

A linear relationship was found between relative pectoralis mass and the mass-specific activity of CS in the pectoralis muscle, as has previously been found in the barnacle goose (Deaton *et al.* 1997; Bishop *et al.* 1998), which suggests that these two factors are co-regulated during development. Hypothyroidism delays the developmental changes in both relative mass and mass-specific activity CS in the pectoralis muscle, but appears to retain the relationship between these two variables; consequently, hypothyroid birds occupy a relatively lower position along the regression line.

In contrast to the flight and cardiac muscles, the absolute mass of the two leg muscles in the hypothyroid birds appears to have continued developing at the same absolute rate as those in the control birds, despite body mass (Fig. 2) and leg bone length (Fig. 6) being negatively affected. The leg muscles were therefore disproportionately large. Perhaps functional leg muscles are highly selected for in precocial birds such as the barnacle goose, as failure to forage with the family group would result in predation or starvation. Thus, the insensitivity of leg muscle growth to the effects of hypothyroidism would be highly adaptive. The aerobic capacity of the leg muscles would be less critical for survival and so may be under less intense selection. It would be interesting to determine whether the leg muscles of altricial species of birds are more susceptible to the effects of hypothyroidism.

The differential effects seen between the leg and flight muscles are also paralleled to some extent in the effects on bone growth. Hypothyroidism had less effect on the length of the leg bones than it did on the bones associated with flight (sternum and wing bones). The linear growth relative to body mass of the tibiotarsus and tarso-metatarsus of the hypothyroid birds was not affected by hypothyroidism; these bones therefore grew in proportion to body mass. However, hypothyroidism did affect the relative growth of the head, sternum and wing bones, as well as retarding the absolute growth. Thus, the hypothyroid birds retained the 'leggy' characteristic of younger birds, as found in thyroidectomized starlings (*Sturnus vulgaris*; Dawson and McNaughton, 1994).

It is well known that hypothyroidism during human development results in retarded linear growth and bone maturation (Mazzaferri, 1980). It is thought that thyroid hormones are required for the stimulation of bone formation by osteoblasts, and nuclear receptors for T_3 have been found on osteoblast cells in the rat (Allain and McGregor, 1993). It is also thought that thyroid hormones are involved in cartilage growth and maturation (Auwerx and Bouillon, 1986). Hypothyroidism in postnatal development of birds also results in stunted skeletal growth (King and May, 1984). Hall (1973) found that the tibias of hypothyroid embryonic chicks were shorter and lighter than those of euthyroid embryos, and showed delayed maturation of chondrocytes and in the formation of the cartilage matrix, resulting in eroded epiphyses, and Burch and Lebovitz (1982) showed that the maturation and growth of cartilage in the embryonic chick is directly stimulated by T_3.

Plumage development was retarded in hypothyroid goslings, and primary feathers showed altered morphology. Thyroid hormones are known to be involved in the moulting process and to stimulate feather regeneration. The feather regrowth of hypothyroid adult birds shows thinner, elongated, fringed feathers due to affected barbule development (Dawson and McNaughton, 1994; Assenmacher, 1973; Payne, 1973). Hypothyroidism induced during embryonic development of chicks also affects the growth of down (Payne, 1973).

Capillary-to-fibre ratio and the fractional volume of capillaries were lower in the pectoralis of hypothyroid birds and were at a level characteristic of goslings at a much earlier stage of development (Egginton *et al.* 1997). During normal growth in the rat, the number of capillaries increases as the diameter of the muscle fibres increases (Ripoll *et al.* 1979), while Sillau (1985) found that, at a given fibre cross-sectional area, the capillary-to-fibre ratio of adult hypothyroid rats did not differ from that of euthyroid control rats. Thus, the lower capillary-to-fibre ratio seen in hypothyroid goslings could be a consequence of the general retardation of muscle fibre cross-sectional area. However, Capo and Sillau (1983) found that, taking into account the effect of fibre cross-sectional area, hyperthyroid adult rats did show an increase in capillarity.

Distinct postnatal increases in haematocrit and haemoglobin concentrations, rising to adult levels, were observed in the developing euthyroid goslings. This agrees with previous studies suggesting that juvenile birds usually have a lower haematocrit and haemoglobin concentration than adults (D'Aloia *et al.* 1995). In the barnacle goose, the increases in haematocrit and haemoglobin concentration are correlated with an increase in heart mass (Bishop *et al.* 1996) and with an increased oxygen demand as the pectoralis muscles develop and the birds begin flight activity. Haematocrit and haemoglobin concentration were lower in hypothyroid birds. This could be interpreted as an adaptive change rather than a pathological change. Studies on humans have shown that hypothyroid patients often show a reduced haematocrit and haemoglobin concentration that are not due to lack of iron, vitamin B_{12} or folate, and this is termed hypoplastic anaemia (Horton *et al.* 1976; Herbert, 1986). It is well known that hypothyroidism causes a reduction in basal metabolic rate in both mammals (Hardy, 1981) and birds (Ringer, 1976), and it is thought that hypoplastic anaemia is an adaptive response to this decrease in oxygen consumption (Bomford, 1938). Erythropoietin production is usually triggered by tissue hypoxia and stimulates the differentiation of red blood cells from precursor cells; however, as a result of the decreased oxygen requirement of the tissues during hypothyroidism, erythropoiesis is reduced (Herbert, 1986).

Hypothyroidism had its greatest influence on the pectoralis, and the variables measured in hypothyroid birds were characteristic of birds at an earlier stage of development. Thus, it appears that thyroid hormones are needed for the maturation process of the pectoralis muscles to be completed. The generality of the effect of hypothyroidism within a specific

muscle suggests that thyroid hormones may act on genes controlling the differentiation of myoblasts. However, it appears that hypothyroidism may have little direct effect on haematocrit, haemoglobin concentration and capillary-to-fibre ratio in the pectoralis, but that the changes observed in these parameters may actually be adaptations to the changes in metabolism brought about by hypothyroidism.

In general, the nature and timing of the effects of hypothyroidism on developing goslings were tissue-specific; both relative mass and CS activity were lower in the pectoralis, while in the heart ventricles only relative mass was affected, and only mass-specific CS activity was affected in the leg muscles. This tissue specificity is probably reflected in the fibre composition of each muscle. Izumo *et al.* (1986) studied the expression of six genes in the MHC multigene family in several muscles of the rat. The study showed that each muscle expresses a specific set of genes which characterise that muscle, and that all six genes and all the muscles studied could respond to thyroid hormones, but that the same gene can be regulated by thyroid hormones in different modes, and in opposite directions, depending on the tissue in which it was expressed and at what stage of development thyroid state was altered. These effects are likely to be mediated by the temporal and tissue-specific expression of thyroid hormone receptor isoforms (Dainat *et al.* 1984, 1986; Forrest *et al.* 1990; Schmidt *et al.* 1992; Yen and Chin, 1994).

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