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In ovo temperature manipulation influences embryonic motility and growth of limb tissues in the chick (*Gallus gallus*)

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Summary

The chick embryo, developing in the egg, is an ideal system in which to investigate the effects of incubation environment on the development of the embryo. We show that raising the temperature of the eggs by just one degree, from 37.5°C to 38.5°C, during embryonic days (ED) 4–7 causes profound changes in development. We demonstrate that embryonic movement is significantly increased in the chicks raised at 38.5°C both during the period in which they are at the higher temperature but also 4 days after their return to the control temperature. Concomitant with this increase in embryonic activity, the embryos raised at higher temperature grow to significantly heavier weights and exhibit significantly longer leg bones (tibia and tarsus) than the controls from ED12 onwards, although mineralization occurs normally. Additionally, the number of leg myonuclei is increased from ED12 in the embryos raised at the higher temperature. This is likely to promote greater leg muscle growth later in development, which may provide postural stability to the chicks posthatch. These changes are similar to those seen when drugs are injected to increase embryonic activity. We therefore believe that the increased embryonic activity provides a mechanism that can explain the increased growth of leg muscle and bone seen when the eggs are incubated for 3 days at higher temperature.

Key words: chick, movement, temperature, growth, incubation, muscle, bone.

Introduction

It is well established that the embryonic environment can influence the growth of the embryo in many species. In particular, the effect of temperature on subsequent growth has been studied for a long time in fish (reviewed by Johnston, 2006) but also, more recently, in the development of avians (Maltby et al., 2004) and reptiles (Booth, 1998; Booth, 2006). The effects of incubation temperature have been examined on the growth of long bones in chick (Brookes and May, 1972) and on muscle in fish and turkeys (Johnston, 2006; Maltby et al., 2004). Whilst it is clear that temperature can influence the development both of muscle and of bone, the mechanisms by which this is occurring are unclear.

In addition to the general developmental biology interest in studying the effects of environment on the development of the chick musculoskeletal system, there are important welfare implications in this work. A huge and growing number of chicks are bred each year for their meat, with 860 million chicks per year in the UK (Defra, 2006). The majority are intensively farmed in order to elicit the highest weight gain in the shortest time. There have been a number of reports showing the correlation between rapid growth rates and the incidence of leg disorders (Kestin et al., 2001; Kestin et al., 1992), with further reports showing that this affects the behaviour of the birds and their ability to walk (Vestergaard and Sanotra, 1999; Weeks et al., 2000). It would therefore be of great potential benefit to manipulate the growth and development of support tissues such as leg muscle and bone by making simple changes to incubation regimes such that chickens are better able to support greater weights and thereby reduce the incidence of abnormalities.

A problem with investigating the effects of temperature in chicks is that the scope for altering the incubation temperature of poultry eggs is relatively small. In turkeys, increasing the incubation temperature by 1°C throughout the duration of incubation causes a significant reduction in hatchability, whereas shorter periods of around 4 days spent at the higher temperature do not cause decreases in viability (French, 2000). In chick embryos, the period between embryonic day 4 (ED4), roughly HH23 on the Hamburger and Hamilton staging series (Hamburger and Hamilton, 1951), and ED7 (roughly HH30) corresponds to the time when the primary muscle fibres have been laid down in the trunk and is just prior to the formation of the secondary muscle fibres, which begins at ED8 (Crow and Stockdale, 1986; Lee et al., 2004). It is a time when there is a great deal of proliferation among myoblasts, followed by differentiation. It is also the time that the limb buds form and grow and when the undifferentiated cells of the limb mesenchyme start to differentiate as muscle, cartilage and skin (reviewed in Christ and Brand-Saberi, 2002). From ED5, the limb muscle masses undergo cleavage, and the final muscle pattern is apparent by ED7.5 or HH30 (Kardon, 1998; Pautou et al., 1982). Thus, it is a critical time in limb development, and as such we hypothesize that intervention at this time would give the greatest changes to the development of limb tissues.

Interestingly, it has been shown that raising temperature by 1°C from ED5 to ED8 in turkeys caused an increase in semitendinosus muscle fibre number at 16 days posthatch, without a concomitant decrease in hatchability (Maltby et al., 2004). Comparison of the stages of turkey development (Mun and Kosin, 1960) with those of chick development (Hamburger and Hamilton, 1951) showed that ED5 in turkeys corresponds to chick HH20, approximately ED3.5, while ED8 in turkeys corresponds to HH29, which is approximately ED6.5. We therefore chose ED4–7 as the time period that best corresponded to the time reported to be most successful in turkeys and also the time likely to have the greatest effect on limb development. We hypothesize that temperature might control the balance between proliferation and differentiation not only in the muscles but also in the growing bones of the limb, supplying more cells to the tissues to promote future growth.

Small differences in incubation temperature, applied throughout incubation, have been previously shown to influence growth of the long bones in the chick leg (Brookes and May, 1972). Additionally, short-term changes in temperature, in which high deviations from normal temperature are applied, influence embryonic bone development in the rat, probably through activation of heat shock (Harrouk et al., 2005; Kimmel et al., 1993). However, it is not known whether a small change in temperature applied during early incubation would be sufficient to change bone growth throughout development or whether any change would be diminished by the later stages of development. Importantly, the choice of our experimental time period (ED4–7), coming at a time when, in the trunk, the sclerotome of the somites has condensed and the cartilage model for the vertebrae has been laid down (Christ et al., 2004), suggests that our manipulations may be unlikely to interfere with trunk skeletal patterning.

One way in which temperature might influence the growth and development of the limb tissues could be by changing the motility of the chick, such that differentiation of myoblasts might be promoted through muscle stretch (Otis et al., 2005). It has been reported that decrease of temperature reduces metabolic activity and diminishes motor activity (Oppenheim and Levin, 1975). It was therefore of interest to investigate whether the converse is also true; whether increased temperature could increase the motor activity of the chick.

In this study, we demonstrate that increasing the temperature of chick incubation by as little as 1°C for 3 days during early incubation has a significant effect on the motility and body mass of the chick embryo and on the growth of limb muscle and bone, coupled with a decrease in adipocyte size. We propose that the increased movement in the chicks raised at higher temperature may explain the effects seen on the growth of the musculoskeletal system.

Materials and methods

Animals

A total of 126 fertile White Leghorn eggs (Joice and Hill Poultry Ltd, Norfolk, UK) were incubated in an LMS 301 forced draft incubator (Wolf Laboratories, York, UK) at 37.5°C and relative humidity of ~60–70% throughout embryonic growth in the case of controls. Approximately half of the eggs were transferred to an identical incubator set to 38.5°C from ED4 to ED7 before being returned to the control incubator (Fig. 1). Temperatures within the incubators were measured with a Squirrel Logger (Grant Instruments Ltd, Cambridge, UK), with 10 probes each recording the temperature at a different position within the incubator every 5 min. Prior to experiments, incubators were mapped to see if any positions within the incubator deviated by more than 0.3° from the set temperature at any time over a 3-day period. Any positions that showed deviation, such as the corners of the lowest shelf, were not utilised for further experiments. During the experiments, temperatures continued to be monitored, and any eggs judged to have experienced a temperature that was out of the range of set temperature ±0.3°C were removed from the experiment.

A number of eggs from ED5 onwards were windowed and sealed with adhesive tape to limit infection. Movement was measured for 5 min per embryo per day. Each egg was removed from the incubator in turn and placed in an insulated support to prevent cooling, with a light source placed close to the blunt end of the egg to illuminate the inside of the egg in order to aid observation. The total number of movements was recorded, as was the incidence of different categories of movement as follows: whole-body movement, head movement and limb movements. Amnion contractions were not counted, since it has been shown that amniotic contractions do not affect overall embryonic motility (Oppenheim, 1966). After observation, the egg was resealed and replaced in the appropriate incubator. All measurements in this paper were taken by the same researcher, although a random sample were blinded and recorded by an independent researcher to ensure validity of the measurements.

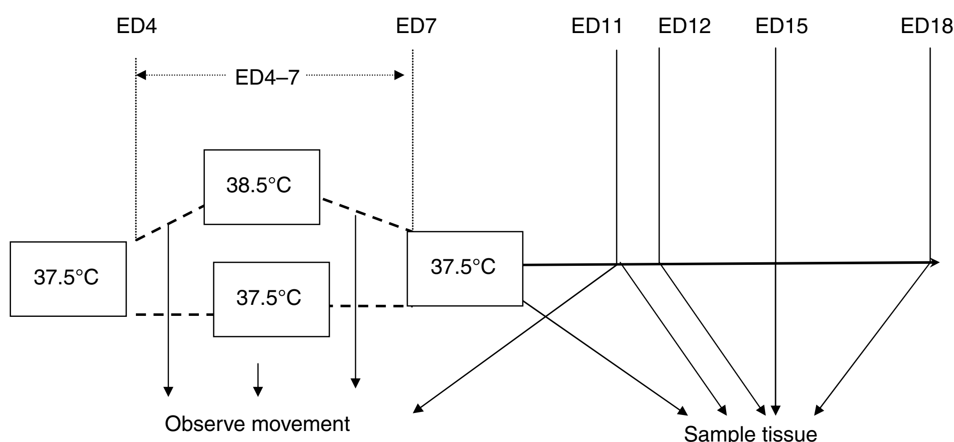

Fig. 1. Schematic to show the incubation temperature regime and tissue collection schedule of experiments

Sampling

Embryos from ED7 to ED18 were killed by decapitation, weighed and

samples taken as per the scheme in Fig. 1. The tissues were isolated and processed as detailed below.

Skeletal development

Embryos for skeletal staining were fixed in 96% ethanol for 2 days, then stained with Alcian Blue and Alizarin Red to show cartilage and mineralized bone, respectively, as previously described (Lamb et al., 2003). The length of the bones was measured on a dissecting microscope, with the measurements calibrated using a 1 mm graticule.

Adipocyte morphology

Pectoral fat pads from chicks at ED15 and ED18 were fixed in buffered formalin (BDH, Poole, UK) and were processed for wax embedding in a Shandon Citadel 2000 automatic tissue processor (Shandon Scientific Ltd, Shandon, UK) to preserve their delicate morphology. 5 μm-thick sections were cut using a Microm HM360 microtome (Microm International, Bicester, UK). Sections were stained with haematoxylin and eosin for subsequent measurements.

The Kontron image analysis software (Zeiss, Oberkochen, Germany) was used to determine mean adipocyte cross-sectional areas by taking high-magnification pictures, picking frames at random and counting the number of adipocytes per frame. The total number of frames was such that more than 30% of the adipocytes in the pectoral fat pad would be measured (more than 1000 cells were measured per age per chick).

Muscle morphology

Tissue for histological analysis, with the exception of fat, which was processed as described above, was snap frozen in isopentane cooled in liquid nitrogen. Cryosections at 15 μm were taken on a Bright Crysotat at –20°C (Bright Instruments, Huntingdon, UK). Sections were adhered to Superfrost slides (Fisher Scientific, Loughborough, UK), airdried and stored at –20°C until required.

Haematoxylin and eosin staining was performed as previously described (Heywood et al., 2005). Within cross sections of the leg, the gastrocnemius muscle was identified. For myofibre and myonuclear quantification, high-magnification pictures were taken, choosing frames at random but discarding any that contained large blood vessels, as blood vessels have more haematoxylin-stained nuclei per area than do muscle. The number of myofibres and myonuclei were counted in a number of frames, which totalled 8–12% of the total area of the muscle. The numbers for each animal were averaged to a number per unit area, then the number of myonuclei was divided by the number of myofibres to give a ratio of nuclei per fibre.

Immunohistochemistry

Immunohistochemistry was carried out essentially as previously described (Groves et al., 2005). Briefly, sections were rehydrated in PBS+0.1% Tween20 (PBST), blocked in PBST+5% goat serum and incubated in 1/200 CB-1 Antibody (DSHB, Iowa City, IA, USA) diluted in the blocking reagent overnight at 4°C. The primary antibody was detected with biotin-conjugated goat-derived anti-mouse IgG (Vector, Peterborough, UK), Vectastain ABC Elite Peroxidase kit (Vector) and visualized using 0.5 mg ml^{-1} of diaminobenzidine.

Photography

Light photomicrographs were taken using the KS300 system and sections were analysed using Zeiss KS300 image analysis software (Image Associates, UK).

Micro computer tomography (Micro-CT)

Bones for microCT analysis were stripped of adherent muscle and other tissue, then fixed in neutral buffered formalin. Scans were performed on a SkyScan 1172 high resolution micro-CT (SkyScan, Kontich, Belgium), using the software provided by the company. The bones were scanned with a pixel size of 13 μm, with the picture size set to 1024×768 pixels, the camera position set to near, and with a 0.5 μm filter. Scans were reconstructed in the NRecon 14.4.0 software supplied with the SkyScan and analysed with the CTAnalyse software supplied by the manufacturer.

Statistical analysis

Statistics were performed using SPSS software (Chicago, IL, USA). Data was analysed to ensure a normal distribution within groups, then Student's *t*-tests were used to compare differences in each parameter between the two temperature groups. All data displayed on graphs are presented as means ± s.e.m.

Results

Chicks raised at the higher temperature are more motile than controls

The total number of movements made by the embryo (*in ovo*) during a 5-min period, independent of amnion contractions, was counted on ED5, 6, 7 and 11. Measurements were not made after ED11 since from this period onwards the movements of the embryo can be constrained by the space in the shell (Sharp et al., 1999), which would lead to the introduction of another variable, that of shell size, to the experiments. On ED6 and ED7, the embryos incubated at 38.5°C made significantly more movements over the measured period than the controls (Fig. 2). Moreover, those embryos that had been incubated at the higher temperature continued to move more than the controls on ED11, 4 days after the eggs had been returned to the control incubator (Fig. 2). Therefore, not only does higher temperature induce the

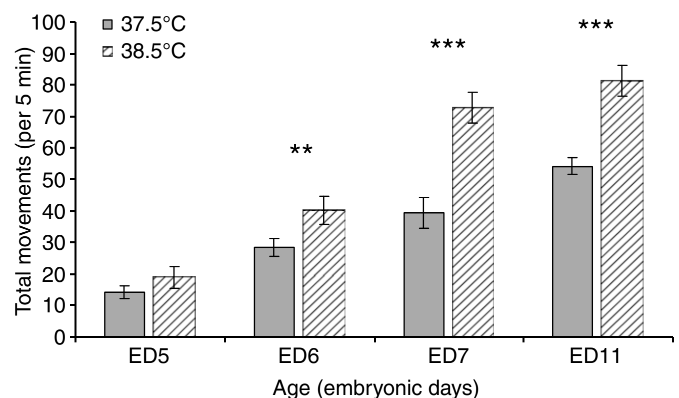

Fig. 2. Mean number of embryonic movements, not including amnion contractions, per embryo in a 5 min period. ***P*<0.05, ****P*<0.001. Sample sizes: ED5, *N*=10; ED6, *N*=22; ED7, *N*=18; ED11, *N*=14.

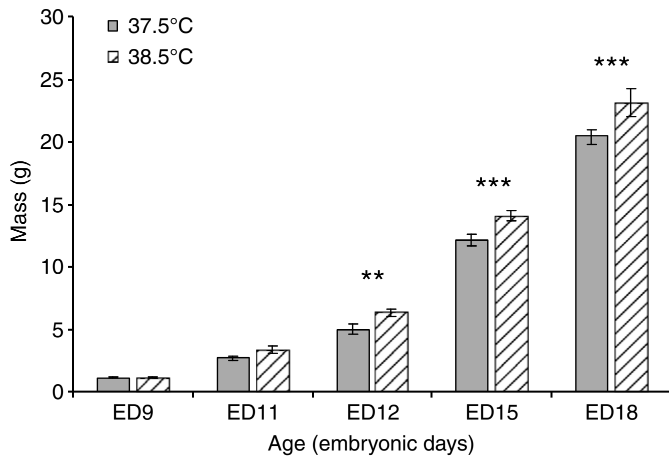

Fig. 3. Total embryo wet mass of chicks sampled between ED9 and ED18. ***P*<0.05, ****P*<0.001. Sample sizes: ED9, *N*=14; ED11, *N*=14; ED12, *N*=15; ED15, *N*=32; ED18, *N*=24.

embryos to move more during the period spent at high temperature but also somehow makes changes to the behaviour of the embryo such that they continue to be more active long after their return to control conditions. We therefore wanted to see what effect these sustained movements were having on the growth and development of the chick.

Chicks raised at the higher temperature are heavier than controls

We were interested to see if the increased movement of the embryos had any impact on the growth of the chicks. We therefore measured the whole body mass of chicks raised under each regime at different stages (Fig. 3). Although chicks raised under both temperature regimes were the same weight up to ED11, those that had spent 4 days at 38.5°C weighed significantly more than control chicks from ED12 onwards. As the increase in temperature impacted on the whole body mass of the chicks and continues to diverge from controls throughout development, we wanted to establish whether this

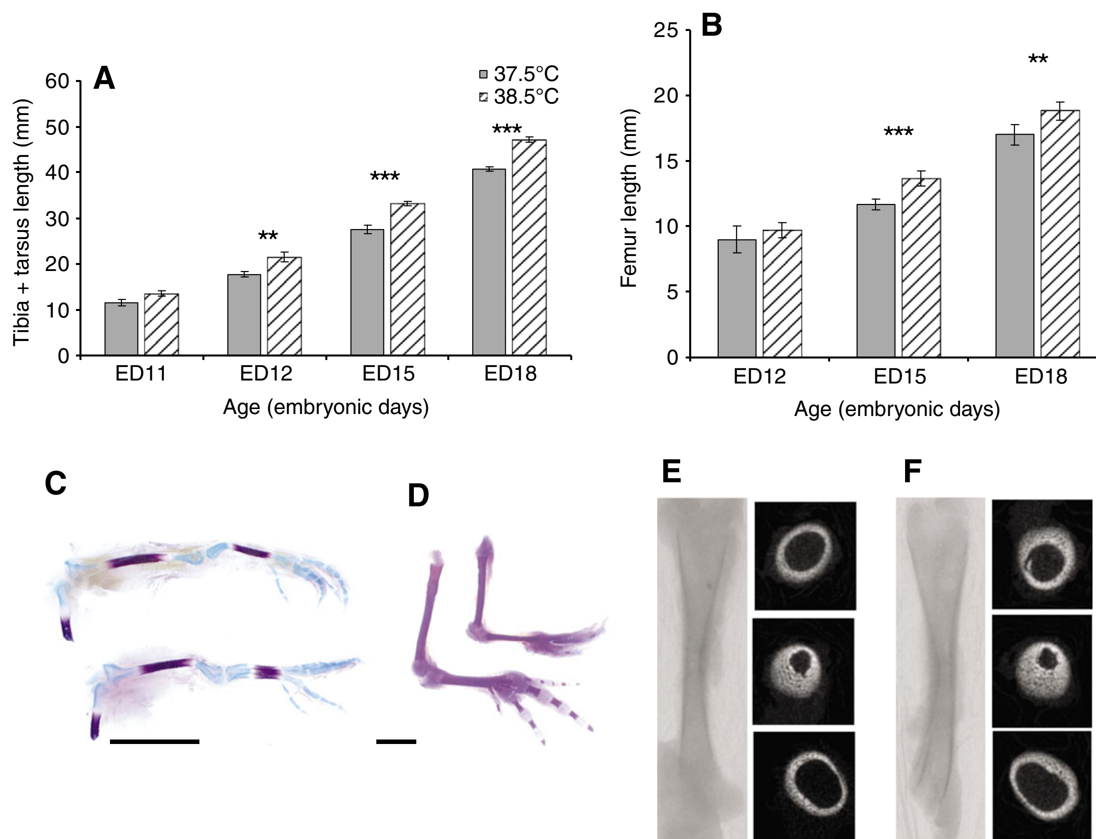

Fig. 4. (A) Mean combined length of the tarsus and tibia in chicks between ED11 and ED18. ***P*<0.05, ****P*<0.001. Sample sizes: ED11, *N*=14; ED12, *N*=16; ED15, *N*=32; ED18, *N*=24. (B) Mean femur length between ED12 and ED18. Sample sizes: ED12, *N*=16; ED15, *N*=28; ED18, *N*=22. ***P*<0.05, ****P*<0.001. (C,D) Alcian Blue/Alizarin Red-stained chick hind limbs; red staining shows mineralized bone, while blue staining shows the unmineralized cartilage. (C) Representative hind limbs at ED12; the top limb comes from a chick raised at 38.5°C while the lower limb comes from a control chick. (D) Representative hind limbs at ED15; the limb on the left comes from a chick raised at 38.5°C, with a control limb on the right. Scale bar in C and D represents 5 mm. Sample sizes were ED12, *N*=12; ED15, *N*=22. (E,F) Images taken from Micro-CT scans of ED15 femurs. (E) ED15 femur of a chick raised at 37.5°C; left image shows the whole bone while the three right images show, top to bottom, individual scans at 25%, 50% and 75% length of the mineralized portion of the bone (as calculated by the programme); the white portion in the cross-sectional scan represents mineralized bone. (F) As E but for a chick raised on the experimental temperature regime.

was reflected by an increase in the growth rates of the leg bones and limb muscle.

Chicks raised at the higher temperature have longer leg bones than controls

Whole legs were stained for bone and cartilage with Alcian Blue/Alizarin Red (Fig. 4). The femur, tarsus and tibia were measured in a number of embryos from each regime on days ED9–18. From ED12, we saw a significant increase in the combined length of the tarsus and tibia bones (Fig. 4A) and in the length of the femurs at ED15 (Fig. 4B). In chick tibias, the first time at which osteoid starts to become mineralized is between ED7.5 and ED8 (Hall, 1987), after which point the extent of the mineralized portion of the bone increases towards the joints. At none of the stages examined did we see any differences in the timing of the onset or the extent of mineralization (measured as a percentage of the length of the bone stained red by Alizarin Red) between the two temperature regimes (Fig. 4C,D and data not shown). In addition to looking at the length and mineralization of the bone, we investigated the structure of the mineralized portions of the tibia and femur bones later in development at ED15 and ED18 by micro-CT. We saw no difference in the extent, or the structures, of the mineralized portion of the femur and tibia bones between the two groups (Fig. 4E,F and data not shown).

Chicks raised at the higher temperature have more muscle fibres and nuclei in the gastrocnemius than do controls

We examined the number of myofibres per 150 000 μm^2, the number of myonuclei (per 150 000 μm^2) and the ratio between the two in the gastrocnemius muscle at ED18. The gastrocnemius is an important muscle in chickens both for postural stability and for walking. Both the mean number of myofibres and the mean number of myonuclei per cross-sectional area at ED18 were higher in the chicks raised at the higher temperature than in the chicks raised at 37.5°C throughout (Fig. 5A,D,E). This appears to be due to two factors: a small decrease in the size of the myofibres and a small reduction in the amount of connective tissue seen in each frame (Fig. 5D–G). In addition to the numbers of myofibres and myonuclei per unit area, the total mass of the gastrocnemius muscle is significantly higher in the chicks raised on the experimental temperature regime (Fig. 5B). Together, these results suggest that the total number of myonuclei and myofibres in the gastrocnemius muscle will be much higher in the temperature-manipulated chicks than in the controls. Interestingly, the ratio of nuclei per fibre was also increased in

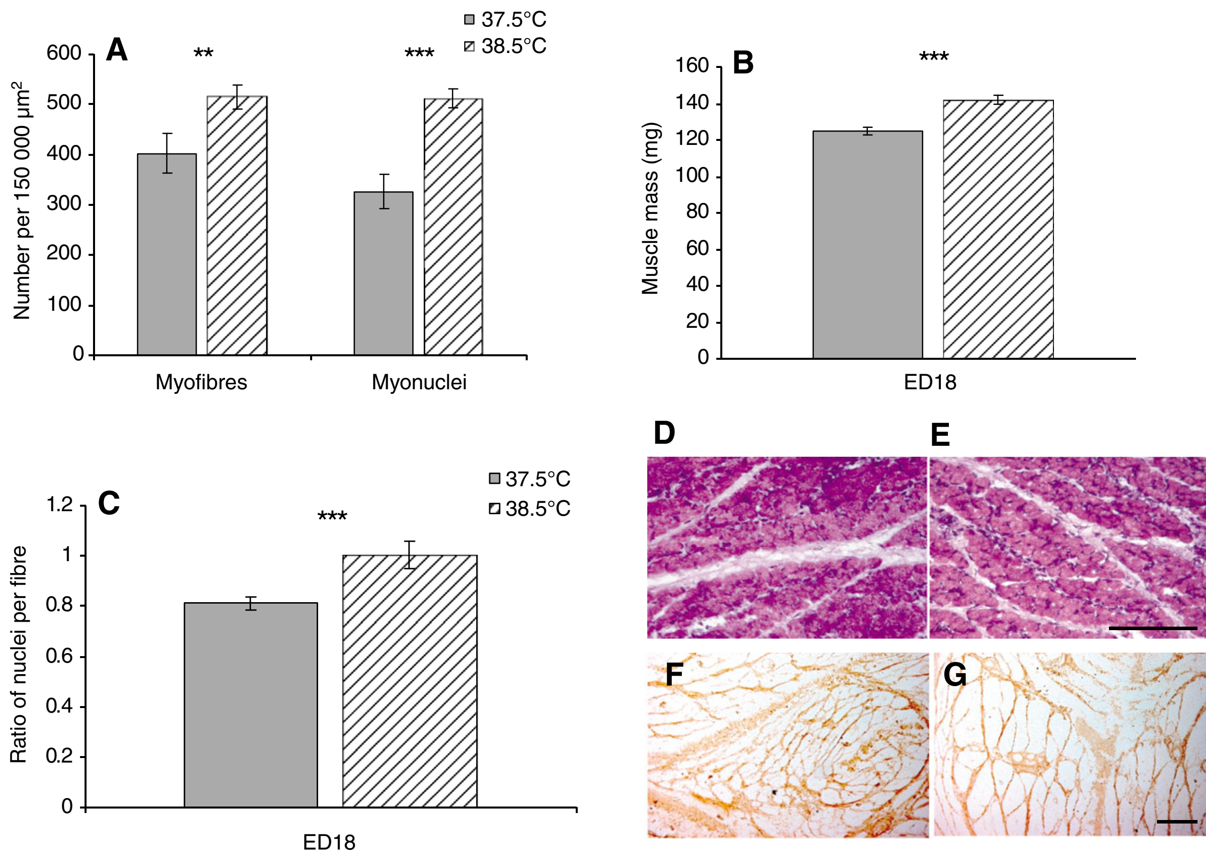

Fig. 5. (A) Mean number of myofibres and myonuclei per 150 000 μm^2 of cross-sectional area of the gastrocnemius muscle at ED18 (*N*=14). (B) Mean mass of the gastrocnemius at ED18 (*N*=14). (C) Mean ratio of myonuclei:myofibre in the gastrocnemius at ED18 (*N*=14). In A–C, ***P*<0.05, ****P*<0.001. (D,E) Representative haematoxylin- and eosin-stained cross sections through the gastrocnemius of ED18 chicks raised at 37.5°C (D) and 38.5°C (E). Scale bars, 50 μm. (F,G) Representative Decorin antibody stained cross sections, through the gastrocnemius at ED18 of chicks raised at 37.5°C (F) and 38.5°C (G).

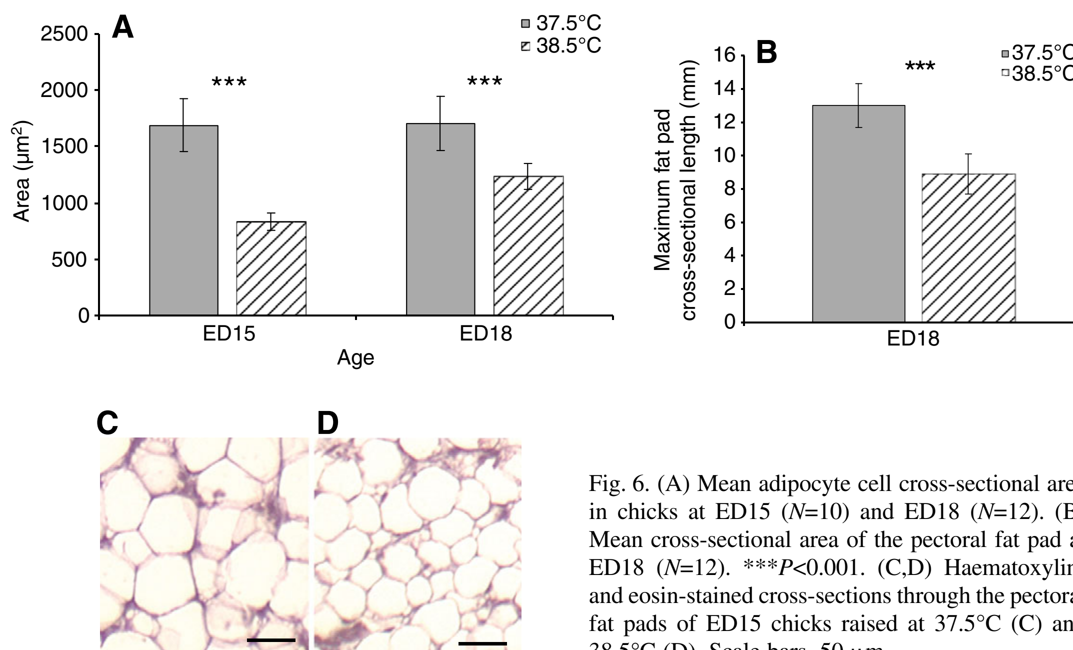

Fig. 6. (A) Mean adipocyte cell cross-sectional area in chicks at ED15 (N=10) and ED18 (N=12). (B) Mean cross-sectional area of the pectoral fat pad at ED18 (N=12). ***P<0.001. (C,D) Haematoxylin- and eosin-stained cross-sections through the pectoral fat pads of ED15 chicks raised at 37.5°C (C) and 38.5°C (D). Scale bars, 50 µm.

the chicks raised at 38.5°C, from a mean of 0.81 nuclei visibly associated with each fibre to just over 1 nucleus per fibre (Fig. 5C).

Chicks raised at the higher temperature have smaller fat pads and adipocytes than controls

We measured the size of adipocytes in the pectoral fat pads of chicks from both temperature regimes at ED15 and ED18. The mean cross-sectional size of adipocytes was significantly larger at both time points in the chicks raised at the control temperature throughout (Fig. 6A,C,D). Additionally, the maximum cross-sectional area of the pectoral fat pad was higher in the control than the temperature-manipulated chicks at ED18 (Fig. 6B).

Discussion

Temperature and embryonic movement

While temperature is known to affect the growth and differentiation of a number of different tissues, the way in which it could be doing so remains unclear. We hypothesize that one way that temperature could affect the musculoskeletal system is by an increase in the number of embryonic movements made by the chick. Measurements were made between ED5 and ED11 and were not made after ED11 since from this period onwards the movements of the embryo can be constrained by the space in the shell (Sharp et al., 1999), which would lead to the introduction of another variable to the experiments. Additionally, the number of limb movements appears to peak around this period (ED11–12) before declining dramatically by ED15 (Bradley, 1999; Bradley et al., 2005).

The effects of movement have been widely studied during the *in ovo* development of the chick because of the ability to control the environment of the chick independently of maternal influence and because of the ease of studying movements *in ovo* by windowing eggs. The first muscle contractions in chicks begin early in development, around ED3 (Bekoff, 1981; Bekoff, 1992; Bekoff, 2001). A number of different drugs have been used, by injection, to control the movement of the chick. Two drugs have been widely utilized to induce paralysis, namely pancurium bromide (PB), to induce flaccid paralysis, and decamethonium bromide (DMB), to induce rigid paralysis, while a third drug, 4-aminopyridine (4-AP), has been used to induce hyperactivity (Pitsillides, 2006). With respect to temperature, it has been reported that decrease of temperature reduces metabolic activity and diminishes motor activity (Oppenheim and Levin, 1975). Conversely, in another paper, it was stated that cooling embryos between days 5 and 15 resulted in no change in embryonic motor activity unless the embryos are cooled to 22°C, although a cessation in amnion contractions was noted (Nechaeva and Turpaev, 1991). Our results are more in agreement with those of Oppenheim and Levin and show that a small rise in the temperature of incubation can have a significant and sustained effect on the motility of the embryo. Indeed, the fact that the embryos show increased activity after a return to the control temperature suggests that the time spent at higher temperature programmes the later motility of the chick. This could be through changes to the pattern of muscle innervation or to the metabolism of the muscles. Interestingly, a recent paper on Atlantic salmon has demonstrated that changing the incubation temperature of salmon larvae during the period between fertilization and hatching leads to changes in the motility of the fish, which are maintained until at least 21 weeks after first feeding (the latest stage examined) (Albokhadaim et al., 2007). Since, in this paper, fish activity was stimulated by feeding, the changes in the motility between the groups could be related either to changes in muscle development or to changes in appetite regulation (Albokhadaim et al., 2007).

Temperature, movement and long bone growth

Increased temperature throughout the incubation period has been demonstrated to cause increased long bone length in chicks (Brookes and May, 1972). While it has also been demonstrated that short periods spent at high temperature can cause changes to bone development in rodents (Harrouk et al., 2005; Kimmel et al., 1993), these studies have used non-physiological temperatures of 42°C, which cause rib fusions and vertebrae truncations and are likely to be due to the effects of heat shock during segmentation. It still remained unclear whether a relatively short exposure to a temperature within a physiological range could have effects on bone growth that would be sustained throughout later development. That our embryos did not show a significant difference in leg bone length until ED12, 5 days after a return to the control temperature, is interesting and, as in the case of the increased motility, suggests that the early time spent at higher temperature is programming later bone development. This could be achieved by changes in the balance between proliferation and differentiation in the long bone cartilage model, with different proportions of chondrocytes in the different zones of the growth plate at each stage, which are under the control of various signalling pathways (reviewed in Kronenberg, 2003). It is possible that an extra round of proliferation at an early stage, such as during the period spent at higher temperature, would not become apparent until much later in development when those extra cells have already differentiated. The other possibility is that we do not see any changes in leg bone length in the earlier stages because extra leg growth could be secondary to another effect that is sustained through later development, of which one possible candidate might be movement.

Our results showing that the embryos that were more motile had longer leg bones than controls also concur with previous reports showing that paralysis of chicks during embryonic development by either PB or DMB leads to long bones that are shorter than controls (Bertram et al., 1997; Hogg and Hosseini, 1992; Hosseini and Hogg, 1991a; Hosseini and Hogg, 1991b; Lamb et al., 2003). The effects of increased motility on chick development have been less well characterized, although treatment of embryos with the hyperactivity-inducing drug 4-AP led to increased chick body mass at ED15 and ED16, along with increased tibial length at these times, although the differences were not significant at ED20 (Heywood et al., 2005). Similar experiments showed that the balance of insulin-like growth factors (IGFs) can be altered in leg muscles by motility-inducing drugs (McEntee et al., 2006). It is interesting that our temperature experiments have yielded results that closely correlate with the results of inducing motility. Indeed, in our temperature-treated embryos, the differences in both body mass and long bone length appear to continue to diverge throughout development and are larger than the effects seen following 4-AP treatment, despite the early nature of our intervention. This may suggest that the chicks are more susceptible to alterations to their incubation environment early in development than they are at later stages. The mechanism behind the increased motility remains unclear, but possibilities could be a change in the timing and pattern of muscular innervation in the embryonic limbs or an acceleration in the establishment of functional motor units within the muscle.

Temperature and increased embryonic movement leads to increases in the number of leg myonuclei

It has been shown that raising the temperature of incubation for a short period during the development of turkeys can lead to an increase in the number of myonuclei in the semitendinosus posthatch (Maltby et al., 2004). We show that increased incubation temperature leads to increased numbers of myonuclei and increased myofibre number. Since there is an apparent decrease in the size of the myofibres in addition to an increase in their numbers in the chicks raised at 38.5°C, it suggests that there may be a temperature-dependent change in the balance between the numbers of myoblasts undergoing proliferation and those undergoing differentiation and hypertrophy, with differentiation and growth delayed in favour of increased proliferation. Interestingly, a recent paper on the effects of incubation temperature on muscle development in larval haddock showed that incubating the fish at higher temperature resulted in an increase in the number of myofibres in the deep myotome 28 days after hatch (Martell and Kieffer, 2007). Taken together, this suggests that diverse species may exhibit similarities in their effects to altered incubation temperature, which are sustained for long periods after the time spent at higher temperature. The effects of altered temperature could perhaps be leading to subtle changes in the balance between the phases of proliferation and differentiation in the different cell populations.

However, an alternative explanation for the fact that changes to the muscle and bone are seen much later in development may be offered by the fact that this correlates with increased embryonic motility. The hyperactivity of the embryos could provide a link between the changes that we are making early and the effects, which we only see manifested later. Indeed, the only effect that we see both during the time spent at increased temperature and during later development is the hyperactivity of the embryos raised at higher temperature. This would suggest that the change in movement could be driving the changes in the development of the muscle and bone. This idea fits with the results of Heywood et al., who demonstrated that artificially increasing embryonic movement by *in ovo* injection of 4-AP leads to increased numbers of myonuclei at ED20 (Heywood et al., 2005). This may be caused by motility-driven changes in IGF expression in the chick, as has been shown following injection of 4-AP (McEntee et al., 2006). However, there are numerous signalling pathways and other growth factors operating in the chick limb during embryonic development such as the FGF (fibroblast growth factor), Shh (Sonic hedgehog), Wnt and BMP (bone morphogenic protein) signalling pathways, with their associated growth factors, which could also be influenced by either temperature or mechanical stretch (reviewed in Duprez, 2002).

While the results are in agreement with those of Heywood et al. (Heywood et al., 2005), it is interesting to speculate as to whether the effect of movement on muscle development is a primary effect or is secondary to the effect of temperature/ movement on long bone development. Indeed, in papers where the effect of paralysis on long bone development has been studied, it has been postulated that the main force in driving the increase in muscle fibre length is the growth of the skeletal elements (Hall and Herring, 1990). This is interesting given that

stretch in muscles is linked to muscle growth. Stretch-induced hypertrophy has been linked to changes in IGF signalling (Goldspink, 1999) and leads to increases in the expression of myogenic regulatory factors (MRFs) (Lowe and Alway, 1999). In culture systems, biaxial or uniaxial, stretch of the muscle cultures has been demonstrated to increase myoblast proliferation through a number of pathways (Kumar et al., 2004; Otis et al., 2005). It is possible that the increased bone length subjects the muscle fibres to greater stretch forces and therefore promotes myogenic proliferation. If so, an increase of temperature during early development leads to increased embryonic motility, which in turn promotes increased growth of the bone and then the muscle of the limb. In avian mutants that show defective motility, such as the *cn/cn* mutant, muscle growth is severely limited (Oppenheim et al., 1997). Conversely, it has been reported that skeletal muscle can itself model bone development. In *Myf5*$^{-/-}$;*Myod*$^{-/-}$ double-mutant mice, lacking striated muscle, a number of bone defects have been characterized, including fused vertebrae and long bone truncations (Rot-Nikcevic et al., 2006). This suggests that the forces that muscle exerts on the bone, as well as the forces that bone exerts on muscle are required for normal growth and patterning of both tissues.

The increase both in the number of myofibres and the nuclei:fibre ratio in the temperature-treated chicks has interesting implications for the long-term growth, posthatch, of these chicks. As the number of muscle fibres in amniotes is fixed at the time of birth or hatch (Goldspink, 1972), an increase in the number of fibres in a particular muscle will allow that muscle to grow more by hypertrophy than controls. Increases in hypertrophy of these fibres are also likely to be driven by the increased nuclei:fibre ratio for two reasons. Firstly, because it is likely that a proportion of the extra nuclei seen in the group raised at higher temperature are satellite cells, the proliferation of which provides most of the additional myonuclei required posthatch for muscle growth (Campion, 1984). Secondly, because typically after birth/hatch, most fibres tend to have a set nuclear domain size, which is the volume of cytoplasm that can be supported by each nucleus (Brack et al., 2005). The increase in the number of nuclei in each fibre should increase the size to which the fibre can grow. Taken together, these changes suggest that the chicks grown at this brief higher temperature regime are likely to continue to show increased leg muscle growth later in life.

Adipose tissue begins to form in the chick around ED12 (Speake et al., 1996). Since the chick egg is a sealed system, the amount of fat and protein available for the growth of the embryo is fixed at the time the egg is laid. Since those embryos raised at the higher temperature for a number of days show increased motility and growth during the stages of development studied, it would seem apparent that in order to achieve these phenomena the embryo must be expending more energy than the control embryos. It is therefore unsurprising that that these embryos have smaller adipocytes in the latter stages of development. It might also be predicted, from these results, that the mass of the yolk might be reduced in the later stages of development. It would be interesting in future studies to examine whether this is the case and, if so, whether this would have any effect on the timing of hatching. Previous studies have shown that decreasing temperature can cause delayed hatch times (Suarez et al., 1996), so it might be predicted that our temperature regime might subtly reduce the length of incubation.

In conclusion, increasing the temperature of incubation by 1°C for 3 days in early development affects a number of parameters during embryonic growth, namely embryonic motility, body mass, long bone length and gastrocnemius size and nuclear number. The fact that such a small change in temperature in early development leads to continued divergence between the groups throughout later development suggests that the stage between ED4 and ED7 may be a critical window during which the tissues of the limb are more susceptible to change. This study demonstrates that small temperatures can cause significant changes that persist long after their application in chick, as is the case in fish species such as haddock and salmon (Martell and Kieffer, 2007; Albokhadaim et al., 2007). Additionally, these results also illustrate the importance of keeping a constant incubation environment when carrying out other experiments in developmental biology, otherwise the effect of the experiment could be masked by changes caused by a fluctuation in the temperature of incubation.

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