Regional femoral bone blood flow rates in laying and non-laying chickens estimated with fluorescent microspheres

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Summary statement: Regional femoral bone blood flow rates, measured using fluorescent microspheres, are approximately two times higher in laying hens than in non-laying hens and roosters.

ABSTRACT

The metabolic rate of vertebrate bone tissue is related to bone growth, repair and homeostasis, which are all dependent on life stage. Bone metabolic rate is difficult to measure directly, but absolute blood flow rate ($\dot{Q}$) should reflect local tissue oxygen requirements. A recent 'foramen technique' has derived an index of blood flow rate (Q_i) by measuring nutrient foramen sizes of long bones. Q_i is assumed to be proportional to $\dot{Q}$, however, the assumption has never been tested. This study used fluorescent microsphere infusion to measure femoral bone $\dot{Q}$ in anaesthetised non-laying hens, laying hens and roosters. Mean cardiac output was 338 ± 38 ml min^{-1} kg^{-1}, and the two femora received 0.63 ± 0.10 % of this. Laying hens had higher wet bone mass-specific $\dot{Q}$ to femora (0.23 ± 0.09 ml min^{-1} g^{-1}) than the non-laying hens (0.12 ± 0.06 ml min^{-1} g^{-1}) and roosters (0.14 ± 0.04 ml min^{-1} g^{-1}), presumably associated with higher bone calcium mobilization during eggshell production. Estimated metabolic rate of femoral bone was 0.019 ml O_2 min^{-1} g^{-1}. Femoral $\dot{Q}$ increased significantly with body mass, but was not correlated with nutrient foramen radius (r), probably due to a narrow range in foramen radius. Over all 18 chickens, femoral shaft $\dot{Q}/r$ was 1.07 ± 0.30 ml min^{-1} mm^{-1}. Mean Q_i in chickens was significantly higher than predicted by an allometric relationship for adult cursorial bird species, possibly because the birds were still growing.

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KEY WORDS: allometry, blood flow rate, bone, calcium mobilization, metabolic rate, nutrient foramen

INTRODUCTION

Contrary to their commonly perceived status as inert, bones are highly vascularized to support processes that alter bone mass and structure such as modelling (growth) and remodelling (repair). In addition to cortical and trabecular bone that function for support and locomotion, birds develop medullary bone, particularly in the legs, to act as a labile calcium source for eggshell formation around the onset of sexual maturity (Whitehead, 2004). Commercial layer chickens require a lot of calcium for intense egg production, and about 10% of their total body calcium is cycled into the shell of every egg produced (Bar, 2009). Blood carries calcium as well as oxygen, hormones and nutrients to bones to satisfy varied metabolic demands. Studying regional blood flow rates improves the understanding of physiological processes that occur in different organs, because the oxygen demand of an organ determines the blood supply to it (Wolff, 2008). Studying bone blood flow in chicken femoral bones can thus provide insight into chicken femur metabolic demand associated with physiological processes.

Measuring regional perfusion is challenging, but techniques such as Doppler ultrasound and microsphere infusion have been developed over the last century. Microsphere infusion has been particularly useful to quantify regional blood flow and blood flow distribution. This technique requires injection of microspheres into left ventricle or atrium of an animal, and it relies on a principle that the microspheres are distributed evenly within the blood stream after the injection and lodged in the microcirculation. The number of microspheres that are trapped in tissue capillaries or small arteries is proportional to the regional perfusion rate (Anetzberger and Birkenmaier, 2016). Absolute regional blood flow rates can be measured after the invention of the arterial reference sampling technique, which uses a pump as an artificial organ to withdraw arterial blood with a constant, known rate from the same experimental animal during microsphere injection (Kaihara et al., 1968; Makowski et al., 1968; Neutze et al., 1968). Fluorescent microspheres have been specifically used

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to quantify blood flow in bones (Anetzberger et al., 2004b; Aref et al., 2017; Serrat, 2009).

Another method to estimate regional blood flow rate is to measure the sizes of foramina that contain blood vessels in bones. This 'foramen technique' relies on a theory that the foramen sizes are proportional to the sizes of the occupying vessels. This technique has been developed to evaluate the blood supply to femora through nutrient foramina (Allan et al., 2014; Hu et al., 2018; Schwartz et al., 2018; Seymour et al., 2012) and to brains through carotid foramina (Boyer and Harrington, 2018; Boyer and Harrington, 2019; Seymour et al., 2015; Seymour et al., 2016; Seymour et al., 2019). Blood flow rates estimated from human, rat and mouse carotid foramina match direct blood flow measurements (Seymour et al., 2015), suggesting this technique can provide accurate regional perfusion values in some cases by simply measuring the sizes of foramina. However, no nutrient foramen studies have ever related the femoral nutrient foramen sizes with absolute blood flow of femoral bone, because the nutrient artery does not completely fill the nutrient foramen. Therefore, the foramen technique results in a blood flow index (Q_i) only, but it is assumed to be proportional to absolute blood flow rate.

The present study has two objectives. The first is to correlate absolute blood flow rates through femoral nutrient foramina and Q_i estimated from foramen size, by comparing the microsphere infusion technique and foramen technique. The second objective was to evaluate femoral bone perfusion in chickens with both techniques. Three chicken groups (i.e. non-laying hens, laying hens and roosters) with similar ages were chosen as experimental animals as only a few studies have looked into femoral bone blood flow in birds (Boelkins et al., 1973). Laying hens and non-laying hens were compared to test the hypothesis that layers exhibit higher rates of femoral bone blood flow due to the role of medullary bone in eggshell formation (Whitehead, 2004).

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MATERIALS AND METHODS

Animal preparation

Crossbreed ISA brown hens and roosters aged from 4 to 8 months old were used in this study. Animals were obtained under Animal Ethics Committee approval (S-2017-058). Chickens were divided into three groups: non-laying hens, laying hens and roosters. Each group consisted of six individuals. Chickens were kept in a constant temperature room (25°C) with a 16 h day and 8 h night cycle before operations. All chickens had free access to water and calcium-rich food. Hens that were sexually immature and had not developed any eggs in their reproductive organs were designated as non-laying hens, and usually they were not older than 5 months. Hens that had begun to lay eggs regularly were selected as laying hens, and their ages ranged from about 6 to 8 months old. Ages of roosters ranged from 4 to 7 months old.

Microsphere standard curve

Polystyrene, green fluorescent (excitation wavelength: 450 nm; emission wavelength: 480 nm), 15 μm microspheres (FluoSpheres, Thermo Fisher Scientific, Victoria, Australia) were used for determination of cardiac output and blood flow rate of femoral bone. Before using each microsphere vial for injections, the relationship between fluorescence intensity and microsphere number/concentration was determined by dissolving known amounts of microsphere suspensions in cellosolve acetate (2-Ethoxyethyl acetate, 98%, Sigma, cat. No. 109967-1L), and by analysing fluorescence intensities of a series of cellosolve acetate solutions with different concentrations of dissolved microspheres using a Cary Eclipse Fluorescence Spectrophotometer (Varian Australia Pty Ltd, Victoria, Australia). An excitation wavelength of 450 nm and a slit width of 10 nm were chosen.

Procedures

A 2 ml plastic syringe with a 25 ga needle was prepared for microsphere injection. This syringe and the needle were weighed separately to 0.0001 g. A 2 ml glass syringe was filled with 1 ml heparinized saline (125 i.u. ml^{-1}) and placed on a syringe pump (Harvard Universal Infusion Pump, Harvard Apparatus, Holliston, Massachusetts, USA), modified to withdraw reference blood during the microsphere injection.

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Before each operation, chickens were weighed to 1 g. They were anesthetized with a combination of ketamine (40 mg kg^{-1}) and xylazine (4 mg kg^{-1}). Under anaesthesia, they were placed on their right side and were stabilized on a dissection table using a wooden frame with Velcro. Chickens were rested on a thick, dry towel to keep them warm, and the room temperature was 25 °C. Feathers at the left humerus region were plucked, and the skin next to the wing brachial vein was removed. A scalpel was used to separate biceps and triceps next to the brachial vein to expose the brachial artery underneath the muscles. The brachial artery was then isolated and blocked at the proximal region with a temporary ligature. The brachial artery was cannulated and sutured distal to the ligature and toward the heart using heparinized clear vinyl tubing (internal diameter: 0.5 mm; outer diameter: 0.8 mm) with a heparinized 25 ga needle connected to the end.

Right after the artery cannulation, a microsphere vial was vortexed for 10 s and sonicated for 4 min in an ultrasonic cleaner (Bransonic B-221, Branson Cleaning Equipment Company, Shelton, USA). During the sonication, the chicken pericardium was exposed by cutting into the left pectoralis major muscle and opening the gap between the first second and third ribs. A heparinized 20 ga Venocan pencil style IV catheter (Cat. No. 121931, Kruuse, Denmark) was inserted into the left ventricle and connected to a pressure transducer (P23Dc, Statham Instruments, Hato Rey, Puerto Rico) and amplifiers (Model 79D EEG, Grass Instruments, Quincy, Massachusetts, USA). The output of the equipment was recorded to a computer with an analog-digital converter and software (DI-145, WinDaq version 3.98, DATAQ Instruments, Akron, Ohio, USA). As the catheter needle tip reached the left ventricle, a typical left ventricular tracing wave could be observed. Flow in the brachial artery was then restored at the proximal region by removing the temporary ligature. More heparinized saline was injected into the brachial artery if blood did not flow out to the cannulated vinyl tubing because of blockage. The other end of the tubing with the 25 ga needle was then connected to the 2 ml glass syringe on the syringe pump. Blood was continuously withdrawn from the brachial artery from 30 s before microsphere injection until 2 min after it. The withdrawal rate was set at either 0.28 or 0.46 ml min^{-1}, depending on the size of the chicken. 1.5 ml of sonicated microsphere suspension (~1.5×10^6 microspheres) was withdrawn into the preweighed 2 ml plastic

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syringe. The syringe was weighed again to 0.0001 g, and the needle was removed for later weighing. The catheter needle was removed from the catheter inside the left ventricle, and the 1.5 ml microsphere suspension was slowly injected into the left ventricle over 15 s. Chickens were sacrificed by injecting excessive anesthetic into the left ventricle through the catheter 2 min after the injection. To account for uninjected microspheres, the 2 ml plastic syringe was rinsed out with 2% Tween 80 into a 40 ml glass vial, and the needle with uninjected microspheres was weighed again and placed into another 4 ml glass vial for later fluorescent intensity analysis.

Sample processing

Some of our processes referred to a recent protocol, which describes in detail a method to measure relative bone blood supply in mice with fluorescent microspheres (Serrat, 2009).

Reference withdrawal blood in the glass syringe was poured into a 100 ml glass bottle. The glass syringe was rinsed three times using about 20 ml 2% Tween 80, and all rinse liquid was also poured into the 100 ml glass bottle. A further 2 ml of heparinized saline was added into the bottle to prevent blood from clotting.

Spleen and kidneys of five laying hens were harvested, weighed and kept in phosphate buffered saline (PBS) in the dark before tissue digestion and microsphere analysis to determine organ blood flows for comparisons with the literature. Femora were harvested, and nutrient foramen microphotographs were taken using a microscope set up. Fiji (Open Source, www.fiji.sc) was used to measure the foramen areas to calculate foramen radii. Methods that measure foramen size microphotographically are described in detail elsewhere (Hu et al., 2020). Femur lengths were measured to 1 mm. Nutrient arteries support the whole femur shaft regions while the metaphyses and epiphyses receive perfusion from other arteries (Trueta, 1963). Therefore, we expect that nutrient foramen sizes may be more related to flow to the shaft region rather than the whole femur. Femora were therefore sectioned into three parts, as previously illustrated (Aref et al., 2017; Colleran et al., 2000). The sections were proximal end (25%), shaft (42%) and distal end (33%) measured in relation to total femur length. Bone marrow was retained in all sections.

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Each bone section was weighed to 0.001g and then placed into Cal-Ex decalcifying solution in the dark for 4–5 days. After decalcification, bone samples were rinsed three times with PBS and placed into 100 ml glass bottles. Freshly prepared 100 ml quantities of 2M ethanolic KOH with 2% Tween 80 were used to digest all blood, soft tissue, and decalcified bone samples. Samples were digested in the glass bottles placed on a swirling shaker (No. 436, Penetron Mark III, Sunkay Laboratories, Inc., Tokyo, Japan) in the dark for 2–3 days. The digestion process was completed when there were no large particles remaining in the bottles.

Digested tissues and blood were filtered using a glass vacuum filtration apparatus with glass microfiber 1.2 μm filter paper (Grade 333, 47 mm DIA, Filtech, NSW, Australia). During filtration, 2% Tween 80 was used to rinse the sample bottles three times and at least 100 ml potassium phosphate buffer rinse solution was used for the final rinse of the filtration unit and to adjust pH. After filtration, filter papers were moved and pushed into 70 ml, flat bottom vials (Diameter: 44; height: 57 mm) using forceps and polyethylene plungers. The plungers remained inside the vials and the vials were placed in dark before fluorescence intensity analysis. On the day of analysis, 12 ml cellosolve acetate was added into each vial to dissolve the microspheres and release fluorescent dye. Vials were vortexed well and placed in the dark for 2–4 h before analysis. Three replicates of 3 ml solutions from each sample vial were pipetted into glass cuvettes for analysis. If the fluorescence intensity was higher than the upper record limit of the spectrophotometer, the samples were quantitatively diluted with cellosolve acetate in the cuvette to make up 3 ml solutions. Uninjected microspheres in the injection syringe and needle were also quantified.

Microsphere analysis

A pilot study was conducted to investigate whether our experimental setups would cause severe microsphere loss during processing. Known numbers of microspheres were placed in different glass vials with either Cal-Ex or 2M ethanolic KOH solutions for 2–4 days, and the microspheres were filtered and dissolved in cellosolve acetate solutions. The number of recovered microspheres was quantified. We found that almost all microspheres (> 95 %) can be successfully recovered. Fluorescence intensities detected in samples were converted to numbers of microspheres. Numbers

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of injected microspheres for each chicken were calculated and calibrated based on microsphere suspension density (1.005 g ml^{-1}), the weight difference between the syringe and needle with and without microspheres and the amount of uninjected microspheres in the injection syringes and needles. Cardiac output from the left ventricle ($\dot{Q}_v$, ml min^{-1}) of each chicken was calculated as: $\dot{Q}_v = (\dot{V}_{with} \times N_{inj}) / N_{blood}$, where $\dot{V}_{with}$ is pump withdrawal rate (ml min^{-1}), N_{inj} is number of injected microspheres, and N_{blood} is the number of microspheres in the reference blood sample. Absolute blood flow rates ($\dot{Q}$, ml min^{-1}) to different tissues were calculated by the equation: $\dot{Q} = (\dot{V}_{with} \times N_{tis}) / N_{blood}$, where N_{tis} is the number of microspheres recovered from the target tissue. Femoral bone blood flow rates were averaged from both left and right femora for each individual chicken.

Statistical analysis

All error statistics refer to 95% confidence intervals (CI) calculated in GraphPad (Prism 6.0; GraphPad Software, La Jolla, CA, US).

Mass-specific cardiac output (ml min^{-1} kg^{-1}) and tissue blood flow rates (ml min^{-1} g^{-1}) were calculated by dividing the absolute blood flow rates by body mass (kg) and individual wet tissue mass (g), respectively. Foramen area (mm^2) and radius (mm) of each chicken were averaged from both femora. Body masses, cardiac output, mass-specific blood flow rates, foramen areas and radii among three chicken groups were compared using ANOVA. If ANOVA showed a significant difference among groups, Tukey's multiple comparisons test was used for comparing means between two groups. The 95% confidence intervals of the differences between the two groups were also calculated. Mass-specific blood flow rates are commonly used in literature, so we use them here. We recognize that most biological factors scale with body mass in non-linear ways, thus mass-specific values may be still dependent on body mass. True mass-independent data (raw values divided by body mass raised to the exponent of the allometric equation describing the relationship between the values and body mass) are ideally used, and some are presented in the supplementary information. However, in this study, there is little difference between mass-specific and mass-independent data.

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Femoral bone blood flow index (Q_i) was calculated using an equation derived from Poiseuille's Law: $Q_i = r^4/L$, where r (mm) is the foramen radius and L (mm) is an arbitrary length, measured as femur length (Allan et al., 2014; Hu et al., 2018; Seymour et al., 2012). It was assumed that the arbitrary units of Q_i (mm^3) are proportional to absolute blood flow rate. To compare Q_i between chickens and other cursorial birds interspecifically, nutrient foramen sizes of adult chickens would be required. However, our chickens were not mature enough to be considered as fully-grown adults, especially non-laying hens. Therefore the analysis is based on body mass, not age. To present chicken Q_i more precisely, only laying hens and roosters were selected to estimate Q_i as they were generally older than the non-laying hens and were close to being adults. Nutrient arteries mainly supply the femur shaft. To test whether foramen sizes are associated with blood flow rates, Pearson's correlation coefficient (Pearson's r) was calculated to measure the strength of a linear correlation between foramen radius and femur shaft blood flow rates in all 18 chickens.

RESULTS

Body mass of 18 chickens ranged from 1.1 to 2.7 kg, and the mean body mass was 1.67 ± 0.22 kg. Body mass was significantly different among three groups ($F_{2, 15} = 8.4$, $P = 0.004$). Non-laying hens (1.25 ± 0.11 kg) were significantly lower than laying hens (1.78 ± 0.20 kg) ($P = 0.03$, the 95% confidence interval (CI) of the difference being -0.54 ± 0.48 kg), and roosters (2.0 ± 0.54 kg) ($P = 0.004$, the 95% CI of the difference being = -0.73 ± 0.48 kg). The mean and 95% confidence intervals of body mass-specific cardiac output of three chicken groups was 338 ± 38 ml min^{-1} kg^{-1}. ANOVA showed no significant differences in mass-specific cardiac output among three groups ($F_{2, 15} = 0.10$, $P = 0.90$). Average spleen and kidney masses of five laying hens were 1.8 ± 0.4 and 10.7 ± 2.9 g, respectively. Data collected incidentally showed that the spleen of laying hens received 1.6 ± 1.0 % of cardiac output and required 5.6 ± 5.3 ml min^{-1} g^{-1} of blood flow. The kidneys received 4.2 ± 1.0 % of cardiac output and required 2.4 ± 1.4 ml min^{-1} g^{-1} of blood flow.

Blood flow rates to the femoral bone were not significantly related to body mass when all three groups of chickens were considered, as the confidence interval of the exponent (1.32 ± 0.93) includes 1.0 (Fig. 1). The slope is significantly different from 0 ($R^2 = 0.36$; $P = 0.008$). If the scaling relationships among three chicken groups are

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compared, ANCOVA indicated that there were no significant differences in both the scaling exponents ($F_{2, 12} = 1.2$, $P = 0.33$) and scaling factors ($F_{2, 14} = 1.8$, $P = 0.20$) among all three groups. However, mass-specific values showed that laying hens had significantly higher blood flow rates to the whole femoral bone and to the shaft than the non-laying hens (Whole femur: $P = 0.02$, the 95% CI of the difference being -0.11 ± 0.10 ml min^{-1} g^{-1}. Femur shaft: $P = 0.005$, the 95% CI of the difference being -0.11 ± 0.08 ml min^{-1} g^{-1}) (Fig. 2A & B). Femoral and regional femoral bone blood flows between the non-laying hens and roosters were not significantly different from each other (Table 1). Mass-independent femoral bone blood flow and shaft blood flow rate values compared among three chicken groups showed results similar to the mass-specific value comparisons (Fig. S1 & Table S1).

Averaged across all chickens, the two femora received a combined total of 0.63 ± 0.10 % cardiac output. Within the individual femur of six non-laying chickens, the proximal end received 35.7 ± 1.6 %, the shaft 15.7 ± 2.7% and the distal end 48.6 ± 3.2 % of the total femoral bone blood flow. Laying hens received 30.5 ± 4.8%, 20.9 ± 3.4 % and 48.6% ± 5.5%, respectively, and roosters received 31.7 ± 4.6%, 21.1% ± 5.8% and 47.1 ± 6.4%, respectively.

Mean foramen radius was 0.33 mm, and foramen area was 0.36 mm^2 in 12 laying hens and roosters. Average femoral bone blood flow index (Q_i) of these chickens was 1.50×10^{-4} mm^3. Mean foramen area of non-laying hens, laying hens and roosters was 0.36 ± 0.09, 0.27 ± 0.07 and 0.45 ± 0.08 mm^2, respectively. Mean foramen radius of all three groups was 0.34 ± 0.04, 0.29 ± 0.04 and 0.38 ± 0.03 mm, respectively. Laying hens had significantly smaller nutrient foramen sizes than the roosters (Area: $P = 0.003$, the 95% CI of the difference being -0.18 ± 0.11 mm^2; Radius: $P = 0.003$, the 95% CI of the difference being -0.09 ± 0.06 mm), but foramen sizes were not significantly different between non-laying hens and laying hens (Area: $P = 0.13$, the 95% CI of the difference being 0.09 ± 0.11 mm^2; Radius: $P = 0.10$, the 95% CI of the difference being -0.05 ± 0.06 mm) or between non-laying hens and roosters (Area: $P = 0.16$, the 95% CI of the difference being -0.09 ± 0.11 mm^2; Radius: $P = 0.20$, the 95% CI of the difference being -0.04 ± 0.06 mm). (Fig. 3A & B).

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Nutrient foramen radius and femur shaft blood flow rate have no correlation in our study because the Pearson correlation coefficient (Pearson's r) is -0.11 and the P-value is 0.68 (Fig. 4). In all 18 chickens, mean blood flow rate to the shaft bone was 0.34 ± 0.10 ml min^{-1} and mean nutrient foramen radius was 0.33 ± 0.02 mm. The mean ratio of these values is 1.07 ± 0.33 ml min^{-1} mm^{-1}.

DISCUSSION

Chicken cardiac output

Cardiac output of our roosters (337.9 ± 39.7 ml min^{-1} kg^{-1}) is significantly higher than the 150.4 ± 28 ml min^{-1} kg^{-1} measured using radioactive microspheres in adult roosters under local anaesthesia (Merrill et al., 1981). Cardiac output of our laying hens (327.2 ± 104.7 ml min^{-1} kg^{-1}) is also significantly higher than the 177 ± 11 ml min^{-1} kg^{-1} and the 218 ml min^{-1} kg^{-1} reported by Boelkins et al. (1973) and Sapirstein and Hartman (1959) using an indicator dilution technique in adult laying hens. However, Boelkins et al. (1973) used two different dyes to measure cardiac output, and the 277 ± 16 ml min^{-1} kg^{-1} measured using Evans Blue dye is not markedly different from our value. Therefore, methodological differences may cause a wide range of cardiac output values. Interspecifically, the scaling of cardiac output on the body mass of avian taxa interspecifically is $CO = 290.7M^{0.69}$, where CO is cardiac output (ml min^{-1}), and M is body mass (kg) (Grubb, 1983). Grubb's cardiac output values were collected by measuring arteriovenous oxygen content difference and oxygen consumption rate (Fick Principle) under local anaesthesia. According to the equation, chicken cardiac output is estimated to be 241 ml min^{-1} kg^{-1} using our average chicken body mass (1.82 kg). This estimated cardiac output value is significantly lower than our estimated cardiac output, but significantly higher than all the literature values of laying hens described above.

It is not clear why mass-specific cardiac output was somewhat high in this study, but it may be related to the fact that previous studies involve older chickens in which cardiac output would be expected to be lower. In humans, mass-specific cardiac output tends to decrease as body mass increases (de Simone et al., 1997). Body-mass-specific cardiac output of broilers also decreases with aging from 4 to 6 weeks, although not statistically significant (Wideman, 1999). The decline is mainly

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associated with decreasing mass-specific metabolic rate with growth, but it also may be influenced by body composition. Additionally, anesthetics can affect animal heart rates and cardiac output, although the impacts on birds are not fully understood. Ketamine alone can retain or increase heart rate in birds while Xylazine alone can reduce heart rate, respiration rate and may cause hypoxemia, hypercarbia and death in birds (Abou-Madi, 2001; Varner et al., 2004). Ketamine/xylazine combination has been widely used to anesthetize animals including birds, as xylazine well relaxes muscles along with ketamine (Abou-Madi, 2001). However, the ketamine/xylazine combination still has side effects. For example, lower doses of the combination (Ketamine: 15 mg kg^{-1}; Xylazine: 0.15 mg kg^{-1}) can reduce heart rate of great horned owl (Raffe et al., 1993). Lowering body temperature may also affect cardiac output. For instance, spinal cord cooling and ambient cooling can increase heart rate and cardiac output of pigeons by raising compensatory thermogenesis (Barnas et al., 1984). However, all of our surgical procedures were performed at room temperature, which is within the thermal neutral zone of chickens (Van Kampen et al. 1979). The time duration from chest opening to microsphere injection was controlled to be as short as possible, however, exposing organs to a lower ambient temperature may still have influenced cardiac output.

Absolute rate of blood flow in the chicken femoral bone

The percentages of blood flow to three femoral regions in chickens are similar to the blood flow proportions in rats (Aref et al., 2017), with two ends receiving more blood flow than the shaft. Wet bone mass-specific blood flow in the femoral bone of laying hens is 0.23 ± 0.09 ml min^{-1} g^{-1}, which is much lower than the 0.77 ± 0.09 ml min^{-1} g^{-1} measured using radioactive microspheres in laying hens (Boelkins et al., 1973). Despite this difference, spleen and kidneys of our laying hens received 5.6 ± 5.3 ml min^{-1} g^{-1} and 2.4 ± 1.4 ml min^{-1} g^{-1} of wet tissue mass-specific blood flow, which are not significantly different from the 4.81 ± 0.95 ml min^{-1} g^{-1} and 2.48 ± 0.26 ml min^{-1} g^{-1} reported by Boelkins et al. (1973). Wet bone mass-specific blood flow rates in the femoral bone of non-laying hens (0.12 ± 0.06 ml min^{-1} g^{-1}) and roosters (0.14 ± 0.04 ml min^{-1} g^{-1}) are not significantly different from the 0.13–0.15 ml min^{-1} g^{-1} measured in rabbit femora using both radioactive and fluorescent microspheres (Anetzberger et al., 2004a). Wet bone mass-specific flow rates in proximal end, shaft and distal end of rabbit femora are approximately 0.16–0.17 ml min^{-1} g^{-1}, 0.15 ml min^{-1} g^{-1} and 0.11–

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0.12 ml min^{-1} g^{-1}, respectively (Anetzberger et al., 2004a), and are not significantly different from most of our regional femoral bone blood flow values in chickens, the shaft in non-laying hens and the distal ends in roosters, being the only exceptions (Table 1).

Blood flow rates can roughly estimate metabolic rates of the supplied tissues. The haemoglobin content of chicken blood is about 0.18 g ml^{-1} (Elagib and Ahmed, 2011). Assuming each gram of haemoglobin carries 1.34 ml of oxygen (Hüfner's constant), and assuming half of blood oxygen is consumed by bone tissue, every milliliter of blood then supplies (1.34 × 0.18) / 2 = 0.12 ml of oxygen to the bone tissue. Mean absolute blood flow rate of femoral bone is 1.75 ml min^{-1} and femur mass is 10.9 g. Therefore, the femur metabolic rate is estimated to be (0.12 × 1.75) / 10.9 = 0.019 ml O_2 min^{-1} g^{-1}. This is about twice the metabolic rate (0.0095 ml O_2 min^{-1} g^{-1}) of adult guinea pig calvarial bone (Schirrmacher et al., 1997), but this is expected given that the chickens were measured *in vivo* at normal body temperature and the guinea pig bone was measured *in vitro* at room temperature. We are unaware of any published estimates of avian bone metabolic rate or oxygen extraction ratio.

Bone blood flow and egg production

Laying hens have significantly higher wet bone mass-specific femoral bone blood flow and femur shaft blood flow rates than the non-laying hens. Femoral bone blood flows of roosters are not significantly different from non-laying hens and laying hens, suggesting gender alone does not affect femoral bone blood flow in chickens around the onset of sexual maturity. However, egg production in laying hens may affect bone perfusion. The mean mass-specific blood flow rate of femoral bone is approximately 1.9 times higher, and femur shaft blood flow rate is 2.7 times higher, in laying hens than in non-laying hens (Fig. 2). The major cause of this blood flow difference may relate to calcium homeostasis. Laying hens may require extra blood flow to transport calcium from their bone reserves to form eggshells. As hens reach sexual maturity, osteoblasts in chicken leg bones start to form medullary bone, which is a special bone type that exists only in birds and crocodylians, and it acts as a labile calcium reserve for eggshell formation (Whitehead, 2004). During egg production, calcium can be removed and regained rapidly in medullary bone. To maintain calcium balance, laying hens need to consume a great amount of calcium from their diet. During the daytime

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when chickens are active, calcium from their diet is absorbed from intestines and used directly for eggshell formation and stored in bone for later use. Almost no calcium is left in intestines 6 to 10 h after feeding (Bar, 2009). Eggshell formation of laying hens is high during the night, when calcium is obtained from bone. The calcium loss in bones can then be regained the next day when layers absorb calcium from their food source. Laying hens need to use 2.2 g of calcium, which represents about 10% of total body calcium volume, for daily egg production (Bar, 2009; Bouvarel et al., 2011). Almost all this calcium goes into eggshell, as egg yolk contains negligible amount of calcium (Etches, 1987). The 2.2 g eggshell calcium mostly comes directly from the food source, but 20–40% comes from bone (Bar, 2009). Therefore, laying hens need to export about 0.44–0.88 g calcium from their skeleton in every laying cycle. Medullary bone is absorbed and renewed rapidly (Bain et al., 2016), and mobilizes calcium at a much higher rate than the cortical bones and femur ends (Hurwitz, 1965). Most bone-sourced eggshell calcium is therefore from medullary bone. Hurwitz (1965) fed laying hens using Ca^{45}-labeled diet and found that calcium turnover rate of medullary bone is about 10 times higher than cortical bones. Moreover, he discovered that about 70% calcium in femur and tibia medullary segments were renewed within a 12-day period in laying hens. The high calcium turnover rate of medullary bone is associated with its structure. Compared to cortical bone, medullary bone has a lower mineral concentration, lower mineral crystallinity, and has thinner, shorter and less organized mineral particles (Kerschnitzki et al., 2014; Nys and Le Roy, 2018).

Bone blood flow for growth and locomotion

Femoral nutrient foramen sizes in relation to femoral bone blood flow were previously correlated with bone growth in growing animals. Intraspecifically, growing animals may require a relatively higher blood flow rate to long bones to support primary bone modelling. Long bones of younger mammals have higher relative blood flow rates than in older ones, consistent with higher metabolic rates during growth (Nakano et al., 1986; Pasternak et al., 1966; Whiteside et al., 1977). Femoral nutrient foramen sizes of in-pouch kangaroo joeys are many-fold larger than the adult marsupial species of similar body mass (Hu et al., 2018). All chickens in our study were still growing to some extent, so they may have been influenced by elevated bone perfusion for growth. Younger non-laying hens tend to have relatively larger foramen sizes than older laying hens (Fig. 3), and the mass-independent values are

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significantly different (Fig. S2). Although the chickens in this study were selected to have similar ages around the onset of sexual maturity, in order to avoid bone growth differences among groups, the ages among three chicken groups were still slightly different and were all much younger than the chickens studied previously. Greater perfusion for bone growth is also implied by higher wet bone mass-specific blood flow to the femur ends, compared to the shaft (Table 1), as the secondary ossification centres are located at the ends of long bones (Brookes and Revell, 1998).

Femora are responsible for absorbing stresses from weight-bearing and locomotion. The microfractures on bones caused by the stresses can be repaired by energy-driven Haversian remodelling (Lieberman et al., 2003). Femoral bone blood flow is therefore also related to animal locomotor activity levels in adult terrestrial vertebrates. Interspecifically, terrestrial vertebrates with higher maximum metabolic rates and higher activity levels tend to have relatively larger femoral nutrient foramen sizes (Allan et al., 2014; Seymour et al., 2012). The femoral bone perfusion rates between the non-laying hens and roosters are not significantly different. This probably suggests that they have no great difference in locomotion intensity, without considering the minor effects of calcium mobilization and growth rate differences between the two groups. The higher perfusion rates in chicken femur ends than the shaft may relate to higher oxygen demand in these regions. Femur ends include both metaphyses and epiphyses, which are supplied by a great number of arteries. Some foramina at the ends are larger than the shaft nutrient foramen in mammals (Brookes and Revell, 1998), suggesting long bone ends contain larger arteries and thus require higher blood flow rates. The femur ends may require more energy for undergoing the remodelling process than the shaft, as they locate near the joints and experience more intense stress during daily activity.

Relating nutrient foramen size with femoral bone blood flow

Despite the microsphere experiment showing that laying hens have higher blood flow rates of femoral bone and higher blood flow rates of femur shaft than the non-laying hens, laying hens have relatively smaller absolute and mass-independent foramen sizes than the non-laying hens (Fig. 3 & Fig. S2). No significant differences in

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foramen sizes occur between non-laying hens and roosters. We expected that the higher femoral bone blood flows in laying hens would correlate with larger, rather than smaller foramen sizes. All previous studies on long bone nutrient foramina assumed that the foramen size is proportional to the occupying artery size, which represents the amount of supplying blood flow. Previous foramen studies also assumed the size ratio between the foramen and artery remains constant interspecifically and intraspecifically. In this study, no correlation was found between nutrient foramen radius and femoral bone blood flow (Pearson's r = -0.11, P = 0.68) (Fig. 4). In contrast, our companion study involved another group of chickens in which imaging of nutrient arteries filled under physiological pressure with a contrast medium (BriteVu) showed a significant positive correlation between foramen and artery lumen size (Hu, 2021). It should be pointed out that the 18 chicken samples in this study only cover a 2.5-fold body mass range. Coupled with high variability in the data, this range is too narrow to reveal any correlation between the foramen sizes and blood flow rates, as allometric studies generally require body mass ranges of 100-fold or more. However, if we consider the results for chickens in this study to represent a single value for *Gallus domesticus*, we can compare it to an interspecific allometric analysis of over 1000-fold range of body mass in adult cursorial birds (Fig. 5). The mean Q_i is 1.50×10^{-4} mm^3, which is higher than the 95% confidence bands for adult birds in general. This probably relates to the fact that our chickens were actually still growing, because bone growth is associated with relatively larger nutrient foramina (Hu et al., 2018). At present, the best correlation between bone shaft blood flow rate ($\dot{Q}$, ml min^{-1}) and nutrient foramen radius (r, mm) is $\dot{Q}/r$ = 1.07 ml min^{-1} mm^{-1} (Fig. 4). This absolute value may replace the relative bone perfusion index, Q_i, in future evaluations of femoral blood flow rate based on nutrient foramen size alone.

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Competing interests

No competing interests are declared.

Author contributions

Q. H. conceived the study, conducted the experiments, analysed data and wrote the initial manuscript draft as part of her PhD research. T. J. N. assisted in the experiments, helped with data collection and literature research. R. S. S. provided advice and helped with the experiments. All authors contributed to the data analysis and writing of the manuscript.

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Abbreviations:

L: femur length (mm)

M_b: body mass (g)

N_{blood}: number of microspheres in the reference blood sample

N_{inj}: number of injected microspheres

N_{tis}: number of microspheres recovered from the target tissue

$\dot{Q}$: absolute blood flow rate (ml min^{-1})

Q_i: blood flow index (mm^3)

$\dot{Q}_v$: cardiac output from the left ventricle (ml min^{-1})

r: nutrient foramen radius (mm)

$\dot{V}_{with}$: pump withdrawal rate (ml min^{-1})

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Figures

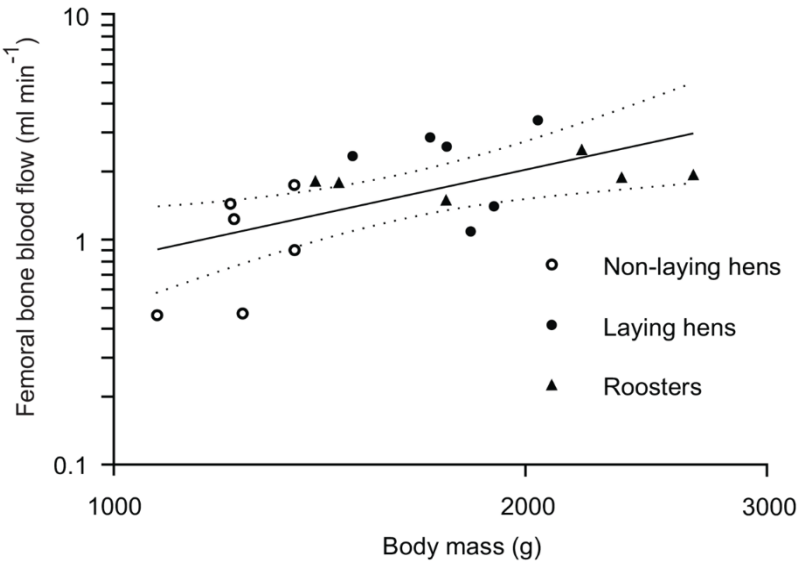

Figure 1. Relationship between femoral bone blood flow rate ($\dot{Q}$, ml min^{-1}) and chicken body mass (M_b, g). Three different symbols represent three different chicken groups. The equation set to all groups is $\dot{Q} = 9.1 \times 10^{-5} M_b^{1.32\pm0.93}$ ($R^2 = 0.36$; P = 0.008). Dashed lines represent the 95% confidence belts for the regression mean. Data are plotted on logarithmic scales.

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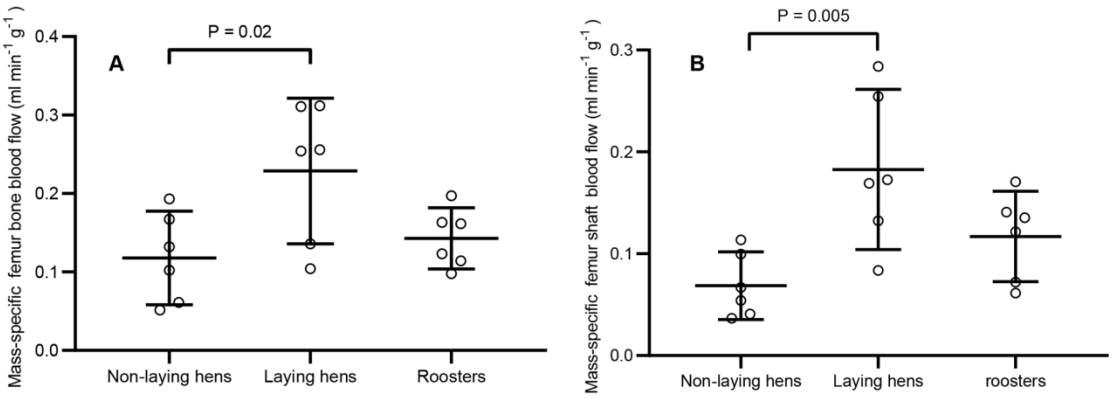

Figure 2. Mass-specific blood flow rates to the entire femur (A) and to the femur shaft (B) among non-laying hens, laying hens and roosters. Mass is wet bone mass. Error bars represent 95% confidence interval of the means of 6 replicates.

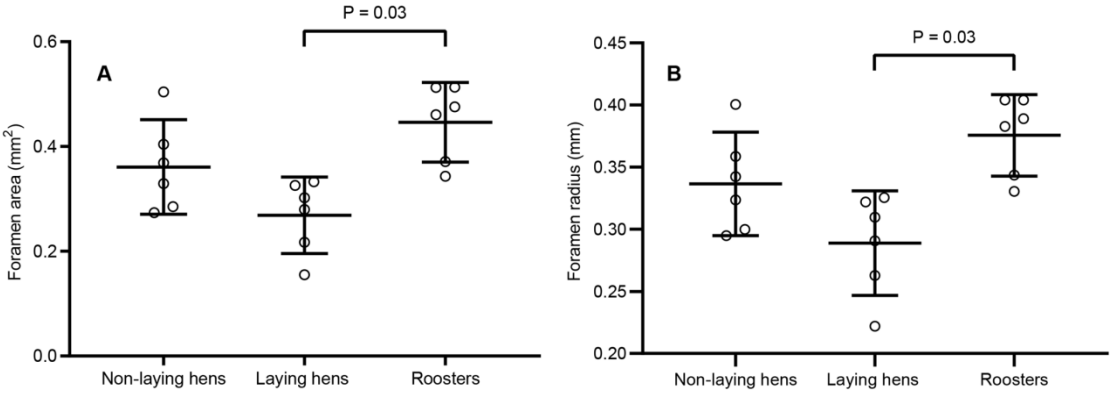

Figure 3. Nutrient foramen areas (A) and radii (B) of non-laying hens, laying hens and roosters. Error bars represent 95% confidence interval of the means of 6 replicates.

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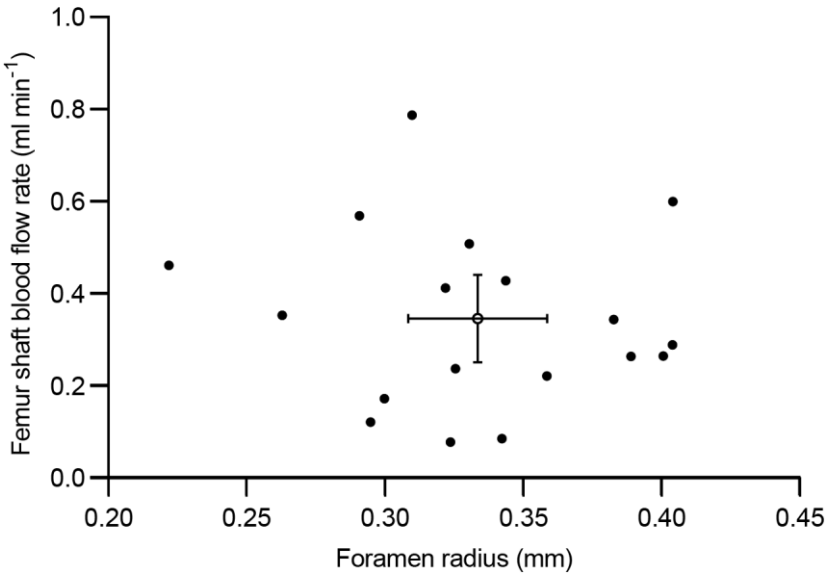

Figure 4. Individual values of foramen radius (mm) and femur shaft blood flow (ml min^{-1}) in 18 chickens (Pearson's r = -0.11, P = 0.68**).** Solid dots represent individual chickens. The open circle represents the mean values and 95% confidence intervals of foramen radius and femur shaft blood flow rate in 18 chickens.

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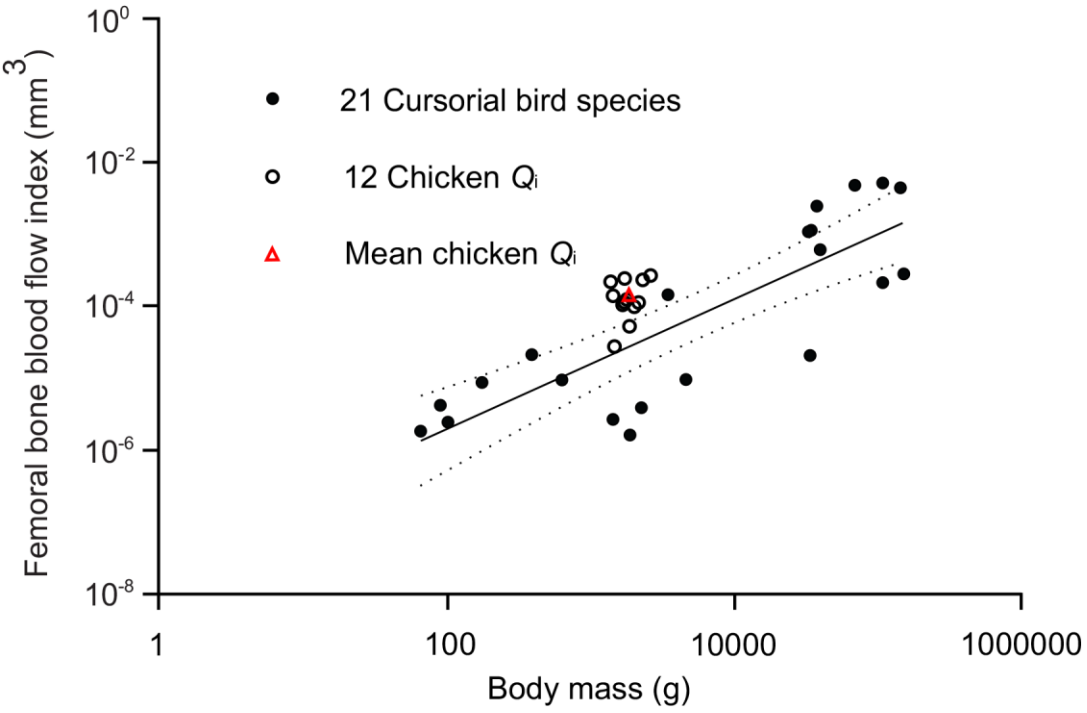

Figure 5. Scaling of femoral bone blood flow index (Q_i, mm^3) on body mass (M_b, g) of 22 cursorial birds including chickens. Solid circles represent mean Q_i values of 21 cursorial bird species. Open circles present individual Q_i value of 12 subadult chickens in this study, and the red triangle represents the mean Q_i of these 12 data points. The equation of the regression is $Q_i = 3.2 \times 10^{-8} M_b^{0.90 \pm 0.29}$ ($R^2 = 0.68$; P = 0.03), and it includes the single mean value for chickens (red triangle). The dotted lines represent 95% confidence interval for regression mean. Data other than chicken are collected from Allan et al. (2014).

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Table

Table 1. Mean and 95% confidence intervals of femoral bone blood flow rates and regional femoral bone blood flow of non-laying hens, laying hens, roosters and all 18 chickens. Mass-specific blood flow rate is the blood flow rate per gram of wet bone tissue. Each datum is the mean from both left and right femora of each chicken, giving n = 6 for each group.

	Tissue wet weight (g)	Absolute blood flow rate (ml min^{-1})	Mass-specific blood flow rate (ml min^{-1} g^{-1})	Proportion of cardiac output (%)
Non-laying hens				
Average femur	8.76 ± 0.66	1.05 ± 0.55	0.12 ± 0.06	0.26 ± 0.13
Femur proximal end	2.78 ± 0.25	0.38 ± 0.20	0.13 ± 0.07	0.09 ± 0.05
Femur shaft	2.28 ± 0.22	0.16 ± 0.08	0.07 ± 0.03	0.04 ± 0.02
Femur distal end	3.69 ± 0.25	0.51 ± 0.28	0.14 ± 0.07	0.13 ± 0.07
Laying hens				
Average femur	10.07 ± 0.74	2.29 ± 0.93	0.23 ± 0.09	0.39 ± 0.07
Femur proximal end	3.24 ± 0.29	0.70 ± 0.31	0.22 ± 0.10	0.12 ± 0.03
Femur shaft	2.61 ± 0.24	0.47 ± 0.20	0.18 ± 0.08	0.08 ± 0.02
Femur distal end	4.22 ± 0.33	1.11 ± 0.46	0.27 ± 0.11	0.19 ± 0.04
Roosters				
Average femur	13.89 ± 3.34	1.91 ± 0.35	0.14 ± 0.04	0.30 ± 0.06
Femur proximal end	4.42 ± 1.12	0.61 ± 0.13	0.14 ± 0.04	0.10 ± 0.03
Femur shaft	3.61 ± 0.84	0.41 ± 0.14	0.12 ± 0.04	0.06 ± 0.01
Femur distal end	5.87 ± 1.47	0.90 ± 0.21	0.16 ± 0.04	0.14 ± 0.03
All 18 chickens				
Average femur	10.91 ± 1.43	1.75 ± 0.39	0.16 ± 0.04	0.31 ± 0.05
Femur proximal end	3.48 ± 0.47	0.56 ± 0.12	0.16 ± 0.04	0.10 ± 0.02
Femur shaft	2.84 ± 0.37	0.34 ± 0.10	0.12 ± 0.03	0.06 ± 0.01
Femur distal end	4.59 ± 0.62	0.84 ± 0.20	0.19 ± 0.05	0.15 ± 0.03

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