THE EFFECTS OF SELECTION FOR HIGH VOLUNTARY WHEEL-RUNNING BEHAVIOR ON NUTRIENT CANAL ABUNDANCE AND SIZE

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A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

by
Nicolas Lawrence Schwartz

December 2017
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Approved by:

Angela Horner, Committee Chair, Biology
Tomasz Owerkowicz, Committee Member
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Biren Patel, Committee Member
ABSTRACT

Variations in skeletal morphology have often been used to interpret an organism’s overall activity level when direct observation is not possible. Although skeletal change in response to exercise is well documented, the skeleton’s response to mechanical loading is modulated by several factors (e.g. age, hormones, sex). Additionally, variation in skeletal morphology is partially a result of genetic variation, which is rarely accounted for in inferences of locomotor activity from skeletal remains. However, blood flow to long bones serves as a proxy for bone metabolic activity, which can be used to infer locomotor activity. Long bones receive blood from three sources, with the nutrient artery supplying the bulk of total blood volume in mammals (50-70%). The size of the nutrient artery can be estimated from the dimensions of the nutrient canal, which is present long after the vascular tissue has degenerated. The literature on nutrient canals is sparse, with most studies consisting of anatomical descriptions from surgical proceedings, and only a few studies investigating the links between nutrient canals and physiology or behavior. Moreover, no study to date has accurately modelled the size and shape of the nutrient canal. For this study, mice from an artificial selection experiment for high voluntary wheel-running behavior were used. High Runner mice from the experiment are known to differ in both metabolic and locomotor activity, with mice from HR lines having increased VO₂max and increased voluntary wheel-running behavior when compared to controls. 137 femora from mice selected over 11 generations for high voluntary
wheel-running behavior were µCT scanned. Three-dimensional reconstructions of nutrient canals were measured for minimum cross-sectional area (an index of blood flow). Nutrient canals varied far more in number and shape than prior descriptions would indicate. Canals adopted non-linear shape and pathing as they traversed from the periosteum to the medullary cavity, occasionally even branching within the cortical bone. Additionally, mice from both HR and control lines had more than four nutrient canals per femur. Mice from HR lines had significantly larger nutrient canal area than controls, which was not the result of an increase in the number of nutrient canals, but rather an increase in their average size. This study demonstrates that mice with an evolutionary history of increased locomotor activity and metabolic rate have a concomitant increase in the size of their nutrient canals.
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CHAPTER ONE

INTRODUCTION

Locomotion and the Skeleton

The evolutionary history of vertebrate life on earth has frequently been interpreted by reference to the remains of extinct organisms. The techniques used to interpret that history vary depending on the material present. Skeletal remains are often the only recourse to infer the biology of extinct organisms, and have been used to ascertain aspects of locomotor behavior (J. Hutchinson, Anderson, Blemker, & Delp, 2005) and physiology (Allan, Cassey, Snelling, Maloney, & Seymour, 2014; Farlow, Hayashi, & Tattersall, 2010; Main, de Ricqlès, Horner, & Padian, 2005; Seymour, Smith, White, Henderson, & Schwarz-Wings, 2012) of fossil taxa. While skeletal morphology is currently used to infer the physical activity of organisms, the efficacy of using skeletal morphometrics should be questioned due to compounding variables affecting the skeleton. Although the vertebrate skeleton is composed of rigid, mineralized tissue, bone is dynamic, responding to changing mechanical needs during growth, load bearing, and locomotion via osteoblast/osteoclast mediated bone modeling and remodeling (Frost, 1987). These types of skeletal changes in response to exercise are well documented (Eliakim, Raisz, Brasel, & Cooper, 1997; Lieberman, 2003; Rector, Rogers, Ruebel, & Hinton, 2008; Rubin & Lanyon, 1984); however, the skeleton’s training response to loading also varies in relation to age (Pearson & Lieberman, 2004), hormones (Devlin & Lieberman,
2007), sex (Robling, Warden, L Shultz, Beamer, & Turner, 2007), exercise frequency (Rubin & Lanyon, 1984), and magnitude (Rector et al., 2008). Additionally, differences in skeletal morphology may be a result of genetic variation, and this effect is rarely accounted for (Middleton et al., 2008; I. J. Wallace et al., 2010; I. J. Wallace, Tommasini, Judex, Garland, & Demes, 2012). In mice selected for high voluntary wheel running behavior, selected mice exhibit increased femoral cortical areas (I. J. Wallace et al., 2010), decreased hindlimb asymmetry (T Garland & Freeman, 2005), and increased diameters and masses of select long bones (Kelly, Czech, Wight, Blank, & Garland, 2006), even without long term access to exercise wheels. However, skeletal changes, whether they be plastic or genetic, must be enacted by osteoblast/osteoclast activity. Osteoblast/osteoclast bone deposition and resorption are a major source of oxygen consumption within bone (Knothe Tate, 2003), and their activity contributes to the overall aerobic metabolism of an organism (Schirrmacher, Lauterbach, & Bingmann, 1997). Increases in bone cell activity necessitates increased blood flow to the skeleton (Gross, Marcus, & Heistad, 1981; Sim & Kelly, 1970), and the resulting increases in blood perfusion necessitate larger blood vessels. Thus, as blood flow to bone serves as an indication of bone’s metabolic activity, and bone metabolism contributes to an organism’s overall metabolic rate, nutrient foramen size is suggested to be a suitable proxy for the metabolic activity of an organism (Seymour et al., 2012).
Blood Flow to Long Bones

The long bones of amniotes receive blood from three sources: nutrient vessels, epiphyseal-metaphyseal arteries, and periosteal arteries (Rhinelander, 1972). Nutrient vessels supply a majority of the blood volume, accounting for 50-70% of total blood supply for the cortical and medullary regions in mammals (J Trueta, 1963). Previous observations suggest that a single nutrient artery enters the diaphysis of long bones, penetrating the cartilaginous primordium at a right angle during bone formation. The ensuing growth of long bones, however, is asymmetrical; thus, the nutrient foramen of adults is neither centrally placed nor at a right angle to the long axis of the bone, but rather develops an oblique orientation (Brookes, 1958; Brookes & Harrison, 1957; Greene, 1935; Henderson, 1978; Rogers & Gladstone, 1950). Additionally, nutrient arteries do not appear to branch within the nutrient canal, and upon reaching the medullary cavity, the artery typically divides into ascending and descending branches that approach the metaphyseal ends (Singh, Sandhu, & Herskovits, 1991). Arteries of the medullary vessels end in medullary sinusoids, which drain into a central venous channel. The central vein of these channels ends in one or more veins that retrace the path of the nutrient artery out of the bone, though they may also exit independently (Brookes, 1971). Arterial vessels are dynamic, adjusting to changing blood flow requirements by thickening their walls in response to greater blood pressures, and increasing circumference in relation to the velocity of blood (Langille, 1996). The size of arterial vessels is therefore subject to requirements
of systemic cardiac output as well as variations in regional blood flow. The size of
blood vessels for long bones can be interpreted from the dimensions of the
nutrient canals they traverse, as the blood vessel is limited in maximum size by
the bone surrounding it. The nutrient foramen has previously been expected to
accommodate maximal blood flow, as opposed to resting rates (Seymour et al.,
2012). Perfusion through blood vessels is normally laminar, and can be
described by the Hagen-Poiseuille equation:

\[ Q = \frac{(P\pi r^4)}{(8L\eta)} \]

where \( Q \) is flow rate (cm\(^3\) s\(^{-1}\)), \( P \) is the difference in blood pressure (dynes
cm\(^{-2}\)), \( L \) is vessel segment length (cm), \( \eta \) is blood viscosity (dynes cm\(^{-2}\) s\(^{-1}\)), and \( r \)
is the radius of the vessel (cm) (Seymour et al., 2012). Although blood pressure
cannot always be determined, blood flow rates are assumed to be proportional to
an index of blood flow:

\[ Q_i = r^4/L \text{ (cm}^3\text{)} \]

which is calculated from the foramen radius and the length of the bone
(Seymour et al., 2012).

Gaps in Knowledge

Most previous literature on nutrient arteries/foramina is limited to
anatomical descriptions (Campos, Pellico, Alias, & Fernandez-Valencia, 1987;
Carroll, 1963; Payton, 1934), with only a few studies which have investigated the
potential link between nutrient foramina and an organism’s physiology. Though
the nutrient foramen has previously been suggested to be a suitable proxy for
maximal metabolic activity (Allan et al., 2014; Seymour et al., 2012), the inability to determine the direction of the nutrient canal from external observations suggests that previous reports on nutrient foramen size are arguably overestimates of the nutrient artery’s true size. The oblique orientation of the nutrient foramen and inability to visually determine the direction of the nutrient canal from the external surface of bone prevents accurate determination of nutrient foramen area based on external dimensions alone, as measurements of the ovoid surface area of the nutrient foramen in any orientation other than perpendicular would cause overestimation of its size. Additionally, the nutrient foramen is presumed to be the location with the minimum cross-sectional area (i.e., the structure whose size limits blood flow) of the nutrient canal, despite no prior observations as to whether the canal widens or narrows in its journey from the periosteal surface to the medullary cavity. As a result, the existing link between nutrient foramen size and maximal metabolic intensity (Allan et al., 2014; Seymour et al., 2012) requires additional verification using methods which account for the orientation of the nutrient foramen and the ability to measure the minimum cross-sectional area along the entire length of the nutrient canal. Although studies that describe nutrient arteries and their foramina have been scarce, no studies to date have accurately modeled both the size and shape of nutrient canals.
Aims and Model Organism

Although studies investigating potential links between nutrient canals and physiological correlates are needed, a guideline to the accurate representation and measurement of nutrient canal size is a critical foundation for future research. Therefore, a primary objective of the present study is the recording of nutrient canal abundance and size in femora via virtual 3-dimensional modelling of the nutrient canals. In addition, I aim to verify whether nutrient canal size is an appropriate indicator of locomotor or metabolic activity by use of a well-established model system: mice that have been selectively bred for high voluntary wheel-running behavior. These four replicate High Runner (HR) lines of mice have been produced by Theodore Garland’s lab at the University of Wisconsin-Madison, and then at the University of California, Riverside. Since 1993, Garland and colleagues have been using the selection experiment to study potential correlations between high levels of locomotor activity and other behavioral, morphological, and physiological traits (T Garland., 2003; Swallow, Carter, & Garland., 1998). As adults, HR mice differ from Control mice in behavior, morphology, and physiology (E. M. Dlugosz et al., 2013; T Garland. & Freeman, 2005; Kelly et al., 2006; J. Malisch, Breuner, Gomes, Chappell, & Garland., 2008; J. L. Malisch et al., 2009; Rezende, Garland., Chappell, Malisch, & Gomes, 2006; Rezende, Gomes, Malisch, Chappell, & Garland., 2006). If metabolic activity affects nutrient canal dimensions, then more active individuals, populations, or species are predicted to have relatively larger canals (Seymour et
al., 2012), and this should be reflected in differences between the High Runner and Control lines, given the known differences in daily activity levels and maximum aerobic capacity (E. M. Dlugosz et al., 2013; Girard, McAleer, Rhodes, & Garland., 2001; Kolb et al., 2010; Rezende, Garland., et al., 2006; Rezende, Gomes, et al., 2006; Swallow, Garland., Carter, Zhan, & Sieck, 1998), as well as endurance capacity (Meek, Lonquich, Hannon, & Garland., 2009).
CHAPTER TWO

METHODS

Selection Protocol

Laboratory house mice (*Mus domesticus*) from a long-term artificial selection experiment on voluntary wheel-running behavior were used for this study (T Garland., 2003; Swallow, Carter, et al., 1998). These mice originated from a base population of the outbred Hsd:ICR strain. Base population mice were randomly paired for two generations and the resulting offspring were used to establish eight closed lines. Lines were established by randomly selecting one male and female from each litter, pairing these individuals randomly, with the exception that sibling mating was prohibited, and randomly assigning 10 pairs to each line and four lines to each linetype (Swallow, Carter, et al., 1998). These were designated generation 0. Subsequent generations were reared using 13 pairs, to ensure each line propagated with 10 families per generation, and breeder selection differed for High Runner and Control lines (Figure 1). For all lines, six to eight-week-old mice were housed individually with access to Wahman-type activity wheels (1.12-m circumference) for six days, and daily revolutions were recorded with a computer-automated system in one-minute bins. For each High Runner line, the highest-running males and females on days five and six of the six-day trial were chosen as breeders for each of the 10 families composing each HR line. Additionally, three of the second highest running males and females from the highest running families were also chosen to
create backup pairings to ensure 10 families were produced for the next
generation. The 13 males and females were randomly bred, except that sibling
pairing was not allowed. For each Control line, one male and female were
chosen randomly from each of the 10 families composing each Control line.
Three additional males and females were also chosen randomly to ensure 10
families were produced for the next generation. The 13 males and females were
randomly bred, except that sibling pairing was not allowed (T Garland., 2003;
Swallow, Carter, et al., 1998). Lines are termed "closed" as breeding between
lines is not allowed by experimental design. This blocks any exchange of genetic
information between lines, and results in each line acting as a distinct replicate.
Thus, the effects of the selection protocol are tested statistically by comparison of
all four HR lines with all four Control lines by nested mixed models in which
replicate line is a random effect nested within linetype. In addition, comparisons
of the replicate lines with the HR selection treatment allows for the discovery of
"multiple solutions" (T Garland., 2003; Theodore Garland. et al., 2011; I. Wallace
& Garland., 2016).

Mini-Muscle Mice
Two of the HR lines and one of the Control lines were observed to have
individuals with hindlimb muscles that were reduced by ~50% as compared with
normal-muscle individuals (Theodore Garland. et al., 2002; Houle-Leroy,
Guderley, Swallow, & Garland., 2003). This ‘mini-muscle’ phenotype is caused
by an autosomal, Mendelian recessive, single nucleotide polymorphism (Kelly et
The mini-muscle phenotype was originally present at low frequency in the base population, and has increased in frequency in subsequent generation in both HR lines (Theodore Garland et al., 2002), resulting in fixation in HR line three and ~50% frequency in HR line six (Syme, Evashuk, Grintuch, Rezende, & Garland, 2005). Pleiotropic effects of this condition relevant to the present study include higher average running speeds on wheels (E. Dlugosz, Chappell, McGillivray, Syme, & Garland, 2009), higher maximal aerobic capacity in hypoxia (Rezende, Garland, et al., 2006) and sometimes during normoxia (Hiramatsu et al., 2017), reduced hind limb bone diaphyseal diameters and reduced femoral polar moments of area and cortical areas (Kelly et al., 2006; I. J. Wallace et al., 2012). In the present sample of 137 mice, a total of 8 mice had the mini-muscle phenotype; six were present in HR line 3 (four males, two females) and two were present in HR line 6 (one male, one female).

Measuring Nutrient Canals

137 femora from mice of generation 12 of the selection experiment were used. These mice had previously been measured in another study, where measures of body size were recorded and femoral morphometrics were measured with digital calipers (T Garland. & Freeman, 2005). Mice were euthanized via carbon dioxide at 232 days of age, frozen, and de-fleshed by dermestid beetles. The right femur of each mouse was scanned at a resolution of 10µm (70kV, 60µA, 300-ms exposure time, 0.5 mm Aluminum filter) with a µCT scanner (SCANCO Medical) housed in the Molecular Imaging Center at the
University of Southern California. Output DICOM files were imported into Amira three-dimensional visualization and analysis software, where models of femora were generated (Figure 2). Utilizing Amira’s segmentation view, transverse sections of each femur were examined for the presence of nutrient canals, defined here as a continuous absence of cortical bone starting from the periosteal border and traversing the entire cortex to the medullary cavity of the femur (Figure 3). These observations were restricted to transverse sections composing the diaphyseal region of the femur, distal to the femoral neck and proximal to the edge of the patellar groove (Figure 4). This restriction was implemented to prevent the inclusion of metaphyseal and periosteal vessels. Upon identification, a digital mask was manually applied to the empty space in the cortical bone of each serial slice, filling the empty space entirely (Figure 4). This mask was applied to all slices that depicted a nutrient canal, and to all canals present within the defined region of the diaphysis. From each individual mask, a three-dimensional model of the nutrient canal was generated. Each canal was then isolated from the femur, and oriented such that a cross-section could be taken perpendicularly to the long axis of the canal. Multiple measurements were taken for each canal, and only the minimum cross-sectional area was recorded (Figure 5). Because blood flow is limited by the smallest cross-sectional area in a cylindrical pipe, only the minimum cross-sectional area represents a meaningful estimate of blood flow. Additionally, any measurement not perpendicular to the long axis of the canal would be an overestimate of
nutrient canal cross-sectional area, as the oblique angle would distort and lengthen the ellipsoid shape of the canal.

Accounting for Unforeseen Variability

In the process of modeling canals, it became clear that nutrient canals differed more in size and shape than prior observations would indicate. Often the canal would adopt a non-linear shape along its intracortical path from the periosteal surface to the medullary cavity (Figure 6). To ensure the minimum cross-sectional area of each canal was being taken, and overestimation caused by measuring at an oblique angle was minimized, the long axis of the canal was re-adjusted at each curvature such that a perpendicular cross-section was always being taken (Figure 7). This process was repeated for every distinctive change in the long axis of the nutrient canal, along the entire length of the canal from the periosteum to the medullary cavity, with only one minimum cross-sectional area being recorded per nutrient canal. Additionally, the nutrient canal occasionally branched within the cortical bone (Figure 6). When this occurred, all potential branches were measured for minimum cross-sectional area in accordance with the methods described above; however, not all branches were recorded. If the sum of the minimum cross-sectional areas of the branches was greater than their source trunk, then the branches were not considered to be blood flow limiting structures and were ignored, while only the trunk was recorded. If the cross-sectional area of the source trunk was greater than the sum of its branches, then the branches were the blood-flow limiting structures
and both branches counted as distinct nutrient canals, while the trunk was ignored (Figure 8). In addition to the minimum cross-sectional area of each nutrient canal, the total number of canals and their relative location (proximal or distal) on the femur were recorded to determine their abundance.

Statistics

All statistical analyses were performed using SAS Procedure Mixed v. 12. Control and HR lines were compared by a two-way (linetype, sex) mixed model nested analysis of variance (ANCOVA), with line as a random effect nested within linetype, and body length, body mass, and femoral length considered as potential covariates, based on prior studies demonstrating that these variables correlated with femoral morphometrics (Kelly et al., 2006; Middleton et al., 2008; Seymour et al., 2012). A main effect of the mini-muscle phenotype was also included, as mini-muscle individuals are known to differ in femoral morphology (Kelly et al., 2006). Statistical significance was judged at P < 0.05. Data on nutrient canal area were square-root transformed to normalize residuals, but this was unnecessary for canal numbers. Least-squares (adjusted) means and standard errors for dependent variables were taken from SAS Procedure Mixed.
Figure 1. Schematic of Within-Family Selection Used to Propagate High Runner and Control Lines.
**Figure 2.** Reconstruction of Mouse Femur From μ-CT Scans. Red Arrow Points to a Nutrient Foramen in the Distal Half of the Femur. Panels Progress in Magnification From Left to Right to Visualize the Nutrient Foramen.
Figure 3. DICOM Files From µ-CT Scans of Mouse Femora Scanned at a Resolution of 10 µm. Panels From Left to Right are in Series, Depicting the Progression of the Nutrient Canal From the Outer Surface to the Medullary Cavity. The Nutrient Canal is Highlighted by a Green Mask.
Figure 4. Measurements Were Restricted to the Region Proximal to the Patellar Groove and Distal to the Femoral Neck, Defined Above, to Prevent Inclusion of Metaphyseal and Periosteal Vessels Which Typically Penetrate the Femur Outside This Defined Area.
Figure 5. Transparent Mouse Femur Demonstrating the Reconstructed Nutrient Canal in its Natural Location at the Proximal End of the Femur. Nutrient Canal Without the Femur in View, Re-Oriented Such That a Perpendicular Cross-Section can be Taken.
Figure 6. Reconstruction of Mice Femora With Nutrient Canals That Were Modelled for Each Femur. Modeling of Entire Nutrient Canal Displays the Wide Variability in Size and Shape That Nutrient Canals Adopt.
Figure 7. Nutrient Canal With Non-Linear Pathing From the Periosteal Surface to Medullary Cavity. Canal is Re-Oriented at Each Change in Curvature Such That a Perpendicular Cross-Section is Always Being Taken. Only the Minimum Cross-Sectional Area is Recorded for Each Canal.
Figure 8. Nutrient Canal Which Branches Within Cortical Bone. Black Indicates Cross-Section Taken From Source Trunk, While Red Indicates Cross-Sections Taken From Branches.
CHAPTER THREE

RESULTS

No significant effects of linetype were detected for body mass, body length, or femur length (P = 0.2326, P = 0.4899, P = 0.6905 respectively, Table 1). Sex specific effects were detected for body mass and femur length, with males being significantly heavier and possessing shorter femora than females (P <0.0001, P = 0.0007, Table 1). Additionally, a significant effect of mini-muscle phenotype was detected for body mass, with mini-muscle mice being significantly lighter than mice without the phenotype (P = 0.0297, Table 1).

Body mass, body length, and femur length were identified as potential covariates in early analyses, however, none of these covariates was statistically significant for any canal trait measured, and thus were excluded from final analyses (Table 2). Additionally, nutrient canals were far more variable in number, size, and structure than predicted from prior literature (Figure 6).

Effects of Selection

Mice from the High Runner lines had significantly higher total nutrient canal area than Control lines (P = 0.0304, Figure 11, Table 3), as a result of significantly higher proximal canal area (P = 0.0434, Figure 9, Table 3), but not differences in distal canal areas (P = 0.1522, Figure 10, Table 3). Higher cross-sectional areas observed in High Runner lines were not attributable to increases in the number of canals, as no significant differences in canal number were detected in either the proximal (P = 0.2941, Figure 12, Table 3) or distal regions.
(P = 0.4924, Figure 13, Table 3), nor in the total number of canals (P = 0.256, Figure 14, Table 3).

Effects of Sex

Male mice tended to have higher total nutrient canal area than females (P = 0.0515, Figure 11, Table 3), driven by a significant difference in distal canal area (P = 0.0491, Figure 10, Table 3), but not by differences in proximal canal area (P = 0.2215, Figure 9, Table 3). Higher areas in the distal region observed in male mice were not caused by increases in the number of distal canals, as no significant differences in nutrient canal number were detected in the proximal (P = 0.4278, Figure 12, Table 3) or distal regions (P = 0.9068, Figure 13, Table 3), nor in total canal number (P = 0.5622, Figure 14, Table 3).

Effects of the Mini-Muscle Phenotype

Distal canal area was the only variable significantly affected by the presence of the mini-muscle phenotype, with mini-muscle mice having higher nutrient canal areas than normal mice (P = 0.0488, Figure 10, Table 3). This increase in distal canal area was not the result of increases in the number of distal canals, although there was a non-significant trend for mini-muscle mice to have more distal canals than normal mice (P = 0.0752, Figure 13, Table 3). No significant differences of the mini-muscle phenotype were detected in proximal canal area (P = 0.9857, Figure 9, Table 3) or number (P = 0.5407, Figure 12,
Table 3), nor in total canal area (P = 0.2158, Figure 11, Table 3) or number (P = 0.1033, Figure 14, Table 3).

**Table 1.** Results From Two-Way Nested Analysis of Variance. Least Square Means (± Standard Error) of Body Mass, Body Length, and Femoral Length are Displayed for Male and Female Mice of High Runner and Control Lines, Along With Statistical Results. Significant Values (P<0.05) are in Bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>High Runner</th>
<th>Normal</th>
<th>Mini-muscle</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Body Mass</td>
<td>44.51±1.45</td>
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<td>Femur Length</td>
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<table>
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<th>Sex Effects</th>
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<th>Mini-muscle effects</th>
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<td></td>
<td>N</td>
<td>df F P</td>
<td>df F P</td>
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<td>1.119 4.84 0.0297</td>
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<tr>
<td>Body Length</td>
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<td>1.6 0.54 0.4899</td>
<td>1.6 0.68 0.4411</td>
<td>1.123 0.39 0.5342</td>
</tr>
<tr>
<td>Femur Length</td>
<td>139</td>
<td>1.6 0.17 0.6905</td>
<td>1.6 0.55 0.4878</td>
<td>1.122 2.71 0.1024</td>
</tr>
</tbody>
</table>
Table 2. In Preliminary Analyses, a Nested Analysis of Covariance was Performed for All Canal Traits with Body Mass, Body Length, or Femur Length Identified as Potential Covariates. As Shown in the Table, None of These Covariates was Ever Statistically Significant for Any Canal Trait, so they were Removed From Final Models.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Body Mass</th>
<th>Body Length</th>
<th>Femur Length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>df</td>
<td>F</td>
</tr>
<tr>
<td>Proximal Area (mm²)</td>
<td>133</td>
<td>1, 6</td>
<td>2.74</td>
</tr>
<tr>
<td>Distal Area (mm²)</td>
<td>134</td>
<td>1, 6</td>
<td>0.05</td>
</tr>
<tr>
<td>Total Area (mm²)</td>
<td>134</td>
<td>1, 6</td>
<td>0.32</td>
</tr>
<tr>
<td>Proximal Number</td>
<td>137</td>
<td>1, 6</td>
<td>1.34</td>
</tr>
<tr>
<td>Distal Number</td>
<td>137</td>
<td>1, 6</td>
<td>0.07</td>
</tr>
<tr>
<td>Total Number</td>
<td>137</td>
<td>1, 6</td>
<td>0.34</td>
</tr>
</tbody>
</table>
Table 3. Results from Two-Way Nested Analysis of Variance. Least Square Means (± Standard Error) of Nutrient Canal Area and Number Displayed for Male and Female Mice of High Runner and Control Lines, Along with Statistical Results. Significant Values (P<0.05) are in Bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th></th>
<th>High Runner</th>
<th></th>
<th>Normal</th>
<th></th>
<th>Mini-muscle</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal Area (mm²)</td>
<td>133</td>
<td>0.0899±0.0071</td>
<td>0.0852±0.0071</td>
<td>0.1046±0.0059</td>
<td>0.0961±0.0065</td>
<td>0.0940±0.0024</td>
<td>0.0939±0.0101</td>
<td></td>
</tr>
<tr>
<td>Distal Area (mm²)</td>
<td>134</td>
<td>0.1242±0.0064</td>
<td>0.1108±0.0065</td>
<td>0.1290±0.0054</td>
<td>0.1212±0.0059</td>
<td>0.1119±0.0023</td>
<td>0.1307±0.0091</td>
<td></td>
</tr>
<tr>
<td>Total Area (mm²)</td>
<td>134</td>
<td>0.1537±0.0073</td>
<td>0.141±0.0075</td>
<td>0.1678±0.0062</td>
<td>0.1561±0.0068</td>
<td>0.1479±0.0025</td>
<td>0.1615±0.0106</td>
<td></td>
</tr>
<tr>
<td>Proximal Number</td>
<td>137</td>
<td>2.069±0.1585</td>
<td>2.062±0.1606</td>
<td>1.842±0.1347</td>
<td>2.033±0.1467</td>
<td>1.930±0.0557</td>
<td>2.073±0.2273</td>
<td></td>
</tr>
<tr>
<td>Distal Number</td>
<td>137</td>
<td>2.531±0.2480</td>
<td>2.361±0.2513</td>
<td>2.214±0.2099</td>
<td>2.425±0.2291</td>
<td>2.052±0.0864</td>
<td>2.714±0.3580</td>
<td></td>
</tr>
<tr>
<td>Total Number</td>
<td>137</td>
<td>4.905±0.3437</td>
<td>4.723±0.3483</td>
<td>4.279±0.2910</td>
<td>4.747±0.3176</td>
<td>4.243±0.1198</td>
<td>5.084±0.4962</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Selection Effects</th>
<th>Sex Effects</th>
<th>Sex*Linetype</th>
<th>Mini-muscle effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N df F P</td>
<td>df F P</td>
<td>df F P</td>
<td>df F P</td>
</tr>
<tr>
<td>Proximal Area (mm²)</td>
<td>133 1, 6 6.51 0.0434</td>
<td>1, 6 1.86 0.2215</td>
<td>1, 6 0.16 0.7065</td>
<td>1, 116 0 0.9857</td>
</tr>
<tr>
<td>Distal Area (mm²)</td>
<td>134 1, 6 2.69 0.1522</td>
<td>1, 6 6.05 0.0491</td>
<td>1, 6 0.42 0.5396</td>
<td>1, 117 3.97 0.0488</td>
</tr>
<tr>
<td>Total Area (mm²)</td>
<td>134 1, 6 7.94 0.0304</td>
<td>1, 6 5.88 0.0515</td>
<td>1, 6 0.01 0.9233</td>
<td>1, 117 1.55 0.2158</td>
</tr>
<tr>
<td>Proximal Number</td>
<td>137 1, 6 1.32 0.2941</td>
<td>1, 6 0.72 0.4278</td>
<td>1, 6 0.83 0.3969</td>
<td>1, 120 0.38 0.5407</td>
</tr>
<tr>
<td>Distal Number</td>
<td>137 1, 6 0.53 0.4924</td>
<td>1, 6 0.01 0.9068</td>
<td>1, 6 1.28 0.3003</td>
<td>1, 120 3.22 0.0752</td>
</tr>
<tr>
<td>Total Number</td>
<td>137 1, 6 1.58 0.2560</td>
<td>1, 6 0.38 0.5622</td>
<td>1, 6 1.96 0.2115</td>
<td>1, 120 2.69 0.1033</td>
</tr>
</tbody>
</table>
Figure 9. (A) Average Minimum Cross-Sectional Area (Least Squares Means ± Standard Error) of Nutrient Canals Present in the Proximal Region of the Diaphysis for Male and Female Mice of High Runner and Control Lines. Significant Differences (P<0.05, N=133) are Indicated with a Red Asterisk; High Runner Mice had Higher Proximal Canal Areas than Control Mice. (B) Average Minimum Cross-Sectional Area in the Proximal Region of the Diaphysis for Mice Without Versus With the Mini-Muscle Phenotype.
Figure 10. (A) Average Minimum Cross-Sectional Area (Least Squares Means ± Standard Error) of Nutrient Canals Present in the Distal Region of the Diaphysis for Male and Female Mice of High Runner and Control Lines. Significant Differences (P<0.05, N=134) are Indicated with a Red Asterisk; Male Mice had Higher Distal Canal Areas than Females. (B) Average Minimum Cross-Sectional Area in the Distal Region of the Diaphysis for Mice Without Versus With the Mini-Muscle Phenotype; Mice With the Mini-Muscle Phenotype had Higher Distal Canal Areas than Mice Without.
Figure 11. Average Minimum Cross-Sectional Area (Least Squares Means ± Standard Error) of Nutrient Canals Present in Both Regions of the Diaphysis for Male and Female Mice of High Runner and Control Lines. Significant Differences (P<0.05, N=134) are Indicated with a Red Asterisk; High Runner Mice had Higher Total Canal Areas than Control Mice. (B) Average Minimum Cross-Sectional Area in Both Regions of the Diaphysis for Mice Without Versus With the Mini-Muscle Phenotype.
Figure 12. (A) Average Number (Least Squares Means ± Standard Error) of Nutrient Canals Present in the Proximal Region of the Diaphysis for Male and Female Mice of High Runner and Control Lines. (B) Average Number of Nutrient Canals in the Proximal Region of the Diaphysis for Mice Without Versus With the Mini-Muscle Phenotype.
Figure 13. Average Number (Least Squares Means ± Standard Error) of Nutrient Canals Present in the Distal Region of the Diaphysis for Male and Female Mice of High Runner and Control Lines. (B) Average Number of Nutrient Canals in the Distal Region of the Diaphysis for Mice Without Versus With the Mini-Muscle Phenotype.
Figure 14. (A) Average Number (Least Squares Means ± Standard Error) of Nutrient Canals Present in Both Regions of the Diaphysis for Male and Female Mice of High Runner and Control Lines. (B) Average Number of Nutrient Canals in Both Regions of the Diaphysis for Mice Without Versus With the Mini-Muscle Phenotype.
CHAPTER FOUR

DISCUSSION

Effects of Selective Breeding for Wheel Running on Nutrient Canals

Mice from High Runner and Control lines of the present study have statistically similar body mass, body length, and femoral length (Table 1), consistent with reports for mice of generations 10 and 11 of the selection experiment (Castro & Garland, 2017; Swallow, Garland, et al., 1998). However, High Runner mice evolved to be significantly smaller in body mass (Swallow, Koteja, Carter, & Garland, 1999, 2001) and body length (Kelly et al., 2006), along with lower growth rates (Girard & Garland, 2002; J. L. Malisch et al., 2006), in subsequent generations. High Runner mice have a higher maximal rate of oxygen consumption during forced exercise ($\text{VO}_2\text{max}$) (E. M. Dlugosz et al., 2013; Kolb et al., 2010; Rezende, Garland, et al., 2006; Rezende, Gomes, et al., 2006; Swallow, Garland, et al., 1998) and higher voluntary wheel running behavior (Meek et al., 2009; Swallow, Carter, et al., 1998) in generations prior and subsequent to the generation studied herein. Although prior experiments have demonstrated the importance of controlling for size-related variables when examining femoral morphology (Middleton et al., 2008; Seymour et al., 2012), surprisingly nutrient canal area and number varied independently of the metrics of body size examined here (Table 2).

High Runner lines of mice with a history of high locomotor activity and maximal metabolic rate exhibit a concomitant increase in the size of their nutrient
canals (Figure 11, Table 3). This increase in nutrient canal size is not the result of increases in nutrient canal number (Figure 14, Table 3), although there are more nutrient canals than would be expected based on prior literature (Campos et al., 1987; Carroll, 1963; Payton, 1934). Blood vessels supplying the skeleton are limited in maximum size by surrounding bone, and thus nutrient canals must accommodate maximal blood flow (Seymour et al., 2012), it is currently unclear whether blood flow through nutrient arteries is determinant of bone morphology (i.e. blood flow dictates bone size and/or structure), or if bone metabolic need determines blood flow through the nutrient artery (i.e. osteoblast/osteoclast bone activity dictate blood flow to bone). Additionally, the size of long-bone nutrient canals may be related to multiple factors. In terms of function, larger canals would allow greater maximal rates of blood flow. In turn, higher rates of blood flow might allow more rapid growth rates (Carano & Filvaroff, 2003; J Trueta, 1963), more rapid modeling and remodeling (Sim & Kelly, 1970), and more rapid fracture repair (Carano & Filvaroff, 2003; Joseph Trueta, 1974). Although it is not clear which of these, if any, causes maximal blood flow to long bones, bone is a highly aerobic tissue (Schirrmacher et al., 1997) and an estimated 11% of total cardiac output is directed towards the skeleton at rest (Gross et al., 1981).

In mice selectively bred for high voluntary wheel running behavior, nutrient canal size is linked to locomotor activity. If body size alone determined nutrient canal area, consistent with mass dependent increases in metabolic activity (White & Seymour, 2005), then we would predict canal area to be smaller in High
Runner lines than Control lines. However, the metabolic rate of an organism is dependent on factors in addition to body size, with locomotor speed being an important factor (Taylor, Heglund, & Maloiy, 1982). Mice bred for high voluntary wheel running exhibit increased activity levels, running more revolutions per day than control counterparts, and this increase in wheel running is attributable primarily to an increase in running speed rather than an increase in overall time spent running (Theodore Garland. et al., 2011; Swallow, Carter, et al., 1998).

Furthermore, HR mice have greater maximal metabolic rates than Control mice (E. M. Dlugosz et al., 2013; Rezende, Garland., et al., 2006; Rezende, Gomes, et al., 2006; Swallow, Garland., et al., 1998). These, coupled with High Runner mice possessing greater total nutrient canal area than Control mice (Figures 11, Table 3), suggest nutrient canal area is related to locomotor activity and maximal metabolic rate for house mice. However, at least in later generations, High Runner mice also have elevated home-cage activity when housed without wheels (Copes et al., 2015; J. L. Malisch et al., 2009).

Effects of Sex on Nutrient Canals

In addition to an effect of selective breeding, males tended to have larger total canal area than females (Figure 11, Table 3). Female mice exhibit higher voluntary wheel running behavior than males, in terms of total distance run, running speed, and time spent running (Theodore Garland. et al., 2011; Swallow, Carter, et al., 1998). However, male mice have increased VO2max compared to females (Rezende, Garland., et al., 2006), introducing the possibility that
differences in nutrient canal area observed between the sexes is associated more with VO$_2$max than running behavior per se. Further study would be needed to differentiate sex-related variation in nutrient canal size from variation due to maximal metabolic rate and locomotor activity.

Effects of the Mini-Muscle Phenotype on Nutrient Canals

Mini-muscle mice are distinct in several ways from other mice of the selection experiment; e.g. mini-muscle mice have much smaller hindlimb muscles, but have enlarged livers, kidneys, and ventricular masses (Theodore Garland. et al., 2002), elongated femoral length (Kelly et al., 2006), and increased mass-specific mitochondrial enzyme activity in hindlimb muscle (Houle-Leroy et al., 2003). However, mini-muscle variants are not normally known to differ from normal mice in VO$_2$max (Rezende, Garland., et al., 2006), though they have been reported to differ in later generations (Hiramatsu et al., 2017), or in total distance run (T Garland., 2003; Houle-Leroy et al., 2003), though mini-muscle mice run faster than mice without the phenotype in later generations (E. Dlugosz et al., 2009). As such, they would not be predicted to differ from other mice in nutrient canal area, and no significant differences were found in total nutrient canal area or number between normal mice and those possessing the mini-muscle phenotype (Figures 11, 14, Table 3).
Notes on Methodology

A primary objective of this study was to develop methods for accurate modeling and quantification of nutrient canals. Although descriptions of nutrient arteries and their foramina have been published previously, there are currently no precedents for modelling the major vessels supplying blood to the skeleton. Thus, a novel methodology was developed to acquire accurate measurements. Three-dimensional modelling of nutrient canals revealed novel morphologies and branching patterns previously undescribed (Figure 6). The methodology of this study was designed with two primary objectives; the first was to reduce potential overestimation of nutrient canal size due to the oblique orientation of the canal in the femur and its varied shape as it travels from the periosteum to the medullary cavity. The second was to establish a descriptive protocol to act as a standard guiding future research on vessels supplying blood to the skeleton. As such, it is important to note the advantages and limitations of using the methods described herein.

Micro-computed tomography was utilized to obtain the sectional slices of femora needed to generate models of nutrient canals. This was necessitated by the size of femora, yet nutrient canal analysis can and presumably will, be performed on organisms that are not constrained in methodology by size. µ-CT scanning offers a range of benefits over traditional surface-based measurements. The resolution of sectional slices is high, and can be tailored to specific needs. 10 microns was chosen as the slice thickness for this study, as some of the
smallest nutrient canal openings were measured to be slightly less than 50µm in diameter during preliminary analysis. Slice thickness could be reduced for analysis of smaller vessels, or increased to process individual femora more rapidly and at lower costs. Yet perhaps the most valuable aspect of μ-CT scanning is the preservation of specimens. By choosing to use CT scanning, femora were preserved and can be used in future experimentation. Furthermore, files from CT scans host a repository of femoral morphometric data which can be made available in online repositories and accessed by any individual trained in digital analysis.

Although CT scanning offers distinct benefits, the ability to view and take measurements along the entire length of the nutrient canal is a prime advantage of the methodology used. The benefits are compounded when considering the substantial variation in nutrient canal size observed (Figure 6). If the nutrient artery showed little variation in diameter along its path from the periosteum to the medullary cavity, then measurements at the nutrient foramen opening would suffice to determine blood flow. However, the nutrient canal shows considerable variation in both size and shape. Blood flow through the nutrient artery is restricted by the smallest cross-sectional area of the vessel, and hence only measurements taken at the minimum cross-sectional area of the nutrient canal can be used as predictive values of potential blood flow. Measurements of 137 femora demonstrated that the nutrient foramen opening was never a site of minimum cross-sectional area for the nutrient canal, and hence does not serve
as a blood flow limiting structure, thus necessitating measurements taken from within the nutrient canal.

Although the entirety of the nutrient canal can be modelled, the precise angle that individual sections were taken from the nutrient canal could not be quantified using the methodologies described here. As a result, a precise 90-degree transverse section cannot be verified, but rather must be estimated by eye. The potential overestimation this may cause is mitigated by measurements of each nutrient canal being taken in multiplicate, and only recording the minimum area that was achieved. It is important to note that this issue would not be present in a perfectly linear shape, and is instead a direct result of the variability in nutrient canal size and shape.

On the Variability of Nutrient Canals

Although previous observations of nutrient foramen are limited, most describe a single nutrient artery penetrating long bones at a right angle to the long axis of the bone, developing an oblique orientation over time due to asymmetrical bone growth, with no branching detected in route to the medullary cavity (Brookes, 1958; Brookes & Harrison, 1957; Greene, 1935; Henderson, 1978; Rogers & Gladstone, 1950; Singh et al., 1991). Variation in nutrient foramen number is not unheard of, but anatomical descriptions of nutrient foramen have noted that while most long bones possess one nutrient foramen, some may possess two, and some have none at all (Campos et al., 1987; Carroll, 1963; Payton, 1934). Despite the long history of external descriptions on
nutrient arteries, and the foramina they leave behind, there has been no previous study which accurately models the size and shape of the nutrient canal. In over 137 observations, mice averaged between 4 and 5 nutrient canals per femur! This difference may be the result of variations in interpretation of what constitutes a ‘nutrient canal’; here defined as a continuous absence of cortical bone breaching the periosteum and traversing to the medullary cavity of the femur within the range below the femoral neck and above the patellar groove. The condition that a nutrient canal must traverse the entirety of the cortical bone eliminates potential confusion with periosteal vessels, while limiting the range of observations to the diaphyseal region between the femoral neck and patellar groove eliminates confusion with metaphyseal vessels found above and below this range. Although values for the total number of canals were not significantly correlated with selection history, sex, or the mini-muscle phenotype (Figures 12, 13, 14, Table 3), the display of variability in nutrient canal structure is unprecedented (Figure 6). Modeling of nutrient canals revealed an additional novelty in that nutrient arteries can, and occasionally do, branch within cortical bone. The findings of this study warrant meaningful, quantitative observations of nutrient canals, to help move towards a broad-scale understanding of the physiology behind the major vessels supplying blood to long bones.

Future Directions

The power of these observations is dependent on their universal nature. Before meaningful discussion can begin on extinct taxa, the universal scope of
this study must be thoroughly tested in representative members of a wider sample of taxa that vary in body size, locomotor activity, and metabolic rate. For *Mus domesticus*, the methodology of this study reduces overestimation of nutrient canal size, and suggests nutrient canal dimensions are related to locomotor activity. However, the mechanism of this relation has not been tested. Correlation of individual metabolic data to individual nutrient canal size is still needed to definitively link the physiology of an organism to its skeletal remains. This is not an issue in studies where sample sizes are relatively large, however, studies of extinct taxa are often limited to single digit sample sizes. Thus, correlation of the physiology and skeletal remains of an organism at the individual level would significantly increase the confidence of observations on extinct taxa. Additionally, the interplay between maximal metabolic rate and locomotor activity is apparent, yet the distinct individual effects of metabolic rate and locomotor activity on nutrient canal size remain obscured. Future studies considering the effects of training, with wheel access and denial groupings for mice of High Runner and Control lines, would aid immensely in discerning the independent effect of exercise on the relationship between nutrient canal size and maximal metabolic activity.

Although the present study concludes that nutrient canal area is related to locomotor and metabolic activity, there is a distinction to be made between the metabolic rate of an organism and the metabolic rate of its organs. The metabolic activity of the skeleton can be relegated to the following actions: growth, bone
modeling/remodeling, hematopoiesis, and its function as a mineral reservoir. The current contributions of each action to bone’s specific metabolic rate, as well as their contribution to an organism’s overall metabolic rate, are unknown. However, it has been estimated that approximately 11% of total cardiac output was directed towards the skeleton under resting conditions (Gross et al., 1981). The large volume of blood supplied towards the skeleton at rest may indicate that nutrient canal dimensions may be more reflective of bone’s specific metabolic needs as opposed to systemic cardiac output during exercise. As a result, insights into the relation between the metabolic actions of bone (bone growth, modeling/remodeling, and mineral deposition/acquisition) and the major vasculature system supplying nutrients to feed those actions (nutrient vessels) opens the possibility of gaining further inferences towards the physiology of extinct taxa. Investigation into physiological correlates to nutrient canal size would ultimately be a boon in exposing the evolutionary physiology (T Garland. & Carter, 1994) of extinct taxa (e.g., see Seymour et al., 2012).
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