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Experimental Animal Research Longitudinally Investigating the Onset Timing of Left-Right Asymmetry in the Femur and Pelvis

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Abstract

Background: The external appearance of the human body appears to be a bilaterally symmetrical structure, whereas most internal organs are distributed asymmetrically. Although previous studies have demonstrated skeletal asymmetry, few studies have described the causes and timing of left-right differences. The aim of this study was to explore when left-right differences in the femur occur and discuss why they occur.

Methods: SD rats (n = 14) were divided into a complex activity (CA) group and a general activity (GA) group, according to the activity environment. In data collected every 2 weeks from 4 to 16 weeks of age using digital mammography, femoral length was measured at 4 positions and pelvic length at 2 positions. Statistical processing was conducted in 2 groups to investigate left-right differences at each age and the timing of the onset of change.

Results: In terms of femoral length, the left femur was longer than the right in both the CA and GA groups at all ages. However, no difference was found in the timing of left- right differences between the 2 groups, even in the presence of different amounts of exercise. Furthermore, this asymmetry did not widen or diminish halfway through but had already occurred at 4 weeks of age.

Conclusions: This study found that asymmetry of the femur occurred as early as 4 weeks of age in rats, and this asymmetry did not change due to the environment. The extent to which asymmetry affects movement may indicate an opportunity to consider that asymmetry exists and is a natural phenomenon.

Keywords: Asymmetry; Left-right difference; Femur; Digital mammography; Rats

Abbreviation: AD: Acetabular Diameter; APD-FC: Anteroposterior Diameter of the Femoral Condyles; CA: Complex Activity; DFH: Diameter of the Femoral Head; GA: General Activity; LF: Total Length of the Femur; LP: Length of the Pelvis; LT-FC: Distance of the Lesser Trochanter- Femoral Condyle

Introduction

The human body appeared bilaterally symmetrical. However, the locations of these organs are often not bilaterally symmetrical. The segmental structure of the lungs is such that the left lung is divided into 2 lobes by 1 oblique fissure, and the right lung is divided into 3 lobes by 1 oblique and 1 horizontal fissure, with the right lung being slightly larger in volume than the left lung.

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The location and structure of many organs such as the liver and heart are not bilaterally symmetrical [1]. The asymmetric structure of the body arises through 3 major steps and is determined by the direction in which the cilia in the nodes of the embryo turn. First, the symmetry in the midline of the embryo is broken. Second, the signal generated at the midline is laterally transmitted. Third, the shape of an organ becomes asymmetric [2-5]. In breaking this first symmetry, the rotation of the cilia in the nodes creates an asymmetric organ structure. Although the body appears bilaterally symmetrical, the structure of the organ is asymmetrical. In addition, the center-of-mass line of the body is slightly displaced to the right of the midline axis in the frontal plane [6,7]. Thus, visceral asymmetry is formed during development, and the position of the center of mass is also laterally asymmetric, leading us to conjecture that similar laterally asymmetric structures exist in other body components, such as bones.

Some studies have measured the left-right difference of the femur. However, the results are not uniform, with some studies stating that there is a left-right difference, while others state that there is no left-right difference [8-11]. In addition, scattered studies have reported left-right differences in pelvic studies [12,13]. To trace the growth process in human subjects, very long time periods may be required, and it cannot be ignored that there is a large variation in changes in bone morphology due to changes in the load placed on bones due to changes in height, weight, and living environment.

Therefore, the aforementioned human studies have drawn inconsistent conclusions. In addition, it is difficult to determine the point at which asymmetry occurred in the above- mentioned cross-sectional studies. Therefore, it is necessary to track changes in growth and bone morphology longitudinally.

In this study, 2 groups of male SD rats were allowed to live in different environments. Digital mammography (hereinafter referred to as mammography) was used to regularly observe the changes in the femoral and hip bones with growth and to examine whether changes in the living environment affected the left-right differences.

Subjects and Methods Animals

The experimental animals used in the study were 2 pregnant Sprague-Dawley (SD) rats purchased from Japan SLC, which gave birth naturally, and 14 male rats were used in this study. After 4 weeks of age, 14 male rats were weaned and randomly divided into 2 groups: the complex activity group (CA group (n = 6) and the general activity group (GA group (n = 8). These groups were housed in different cages. In addition, for the CA group, the number of animals in the cages was adjusted in a timely manner according to the growth of the animals, and the rearing environment was considered so that the growth of the rats was not affected.

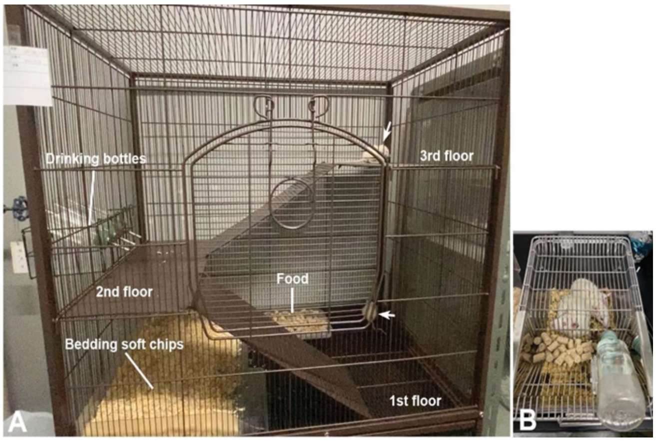

Figure 1: The special rearing environment (large cage) for the CA group (A) and the general rearing cage for the GA group (B).

The large cages used in the CA group were metal products purchased from the market, with a height of 60 cm, depth of 52.1 cm, and width of 78.7 cm (Figure 1A). The cage was divided into 3 levels of platforms, with a 30° slope between the platforms. Drinking bottles were placed at the second level. Containers with heat-dried laboratory animal bedding soft chips (heat-dried) (hereinafter referred to as soft chips) were prepared in the rearing space at the bottom as a space for stress relief and sleep. Food for the rats was also placed at the bottom of the cages and provided *ad libitum* by the Japan National Agricultural Industrial Co., Ltd. Laboratory Animal Feed Lab MR Stock. The CA group had a water hole placed on the second step up the ramp to allow movement of the rats within the cage. Cage housing was limited to a maximum of 3 animals. The GA group used conventional housing cages with soft chips that were well spread under the cages, and water bottles were placed on the cage lids for access to water (Figure 1B). Considering the size of the rats, they were kept in 1 cage with 2 rats until 12 weeks of age, after which 1 rat was housed in 1 cage. The temperature of the rearing room was controlled at an average of 25°, with a 12-h light-dark cycle.

This study was approved by the Institutional Animal Care and Use Committee of Tokyo Metropolitan University (approval numbers A31-27, A2-4, A3-21, A4-18, and A5-13), and all experiments were conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals.

Measurement Methods

From 4 to 16 weeks of age, the growth of the rat femur and pelvis was photographed, measured, and examined every 2 weeks for a total of 7 times (Figure 2A). Imaging was performed using a digital mammography machine (The MGU-1000D MAMMOREX, Canon Medical Systems, Otawara, Japan). With the aim of unifying the posture of the rats during

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photography, shooting was performed under anesthesia. The anesthetics were medetomidine hydrochloride (7.5 μg/kg), midazolam (0.4 mg/kg), and butorphanol tartrate (0.5 mg/kg) mixed with normal saline and administered by intraperitoneal injection [14]. To achieve an optimal anesthetic effect, the dosage was appropriately adjusted every week according to the changes in the rats' weight.

The posture of the rats at the time of mammography was 90° of hip flexion, maximum external displacement, and internal rotation of both hindlimbs. A polystyrene base was created and photographed to keep the table and femur parallel during measurement (Figure 2). At that time, the knee and ankle joint angles were not specified and each rat was photographed twice. In addition, for use as an indicator during measurement of the femur, an aluminum gauge and an indicator in which the thickness was changed by stacking 20 pieces of 1-mm aluminum plates to follow the change in bone density were placed next to the rat so that they were reflected together when the individual was photographed (Figure 2B).

To confirm the movement of the rats in the cage, infrared videography was used to record the behavior of the CA group from 5 to 15 weeks of age, a total of 6 times.

Recording was performed once every 2 weeks, with continuous recording during day and night for 72 h (Supplemental Figure 1).

Based on previous studies [8,10-12,15-18], 6 positions on the right and left sides of the femurs and pelvis were measured using ImageJ. The 6 measurements were as follows:

1. Total length of the femur (LF): The length between the most salient point of the femoral head and the most salient point of the mediolateral condyles (Figure 3B-a)

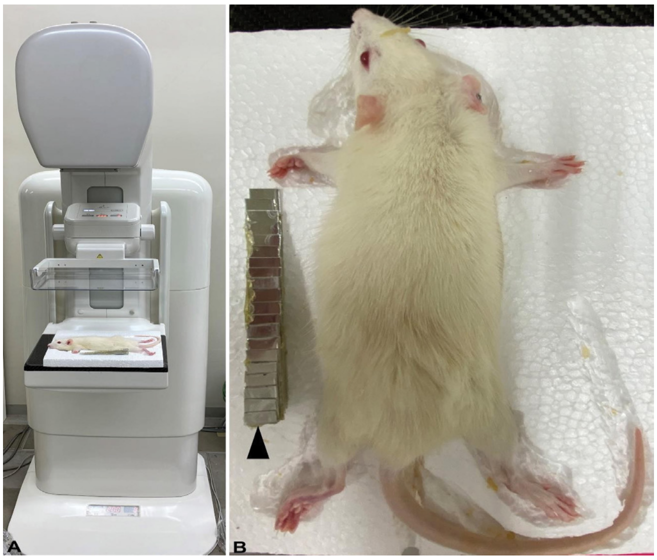

Figure 2: Imaging using mammography (A) and the imaging posture of the rat under anesthesia (B). ▲, aluminum plate.

2. Distance of the lesser trochanter–femoral condyle (LT-FC): The distance between the most prominent point of the lesser trochanter and the most distal end of the femoral condyles on the image (Figure 3B-b).
3. Diameter of the femoral head (DFH): Transverse diameter of the femoral head (Figure 3B-d)
4. Anteroposterior diameter of the femoral condyles (APD-FC) (Figure 3B-c)
5. Length of the pelvis (LP): Distance between the most protruding end of the iliac crest and the ischial tuberosity (Figure 3C-e)
6. Acetabular diameter (AD) (Figure 3D-f): Distance between the upper and lower outer edges of the acetabulum on the image.

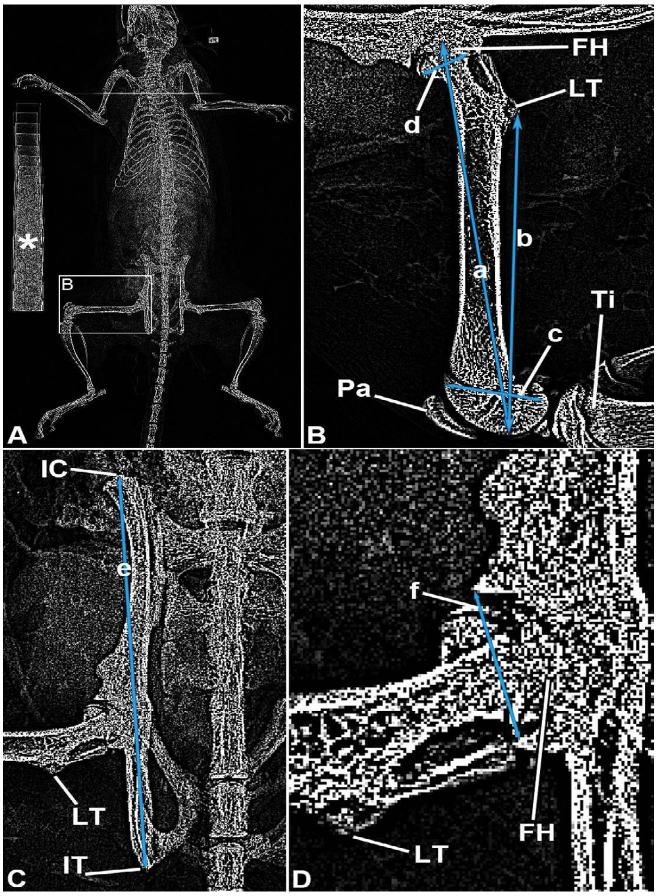

Figure 3: An overall image of the bones of a rat's entire body (A). Measurement of the femur (B) and pelvis (C and D) in mammographic images. B: Enlarged view after rotating the frame in A 90° counterclockwise. a, Total length of the femur (LF). b, Distance of the lesser trochanter-femoral condyle (LT-FC). c, Anteroposterior diameter of the femoral condyles (APD-FC). d, Diameter of the femoral head (DFH). e, Length of the pelvic (LP). f, Acetabular diameter (AD). FH, femoral head; Pa, patella; LT, lesser trochanter; IT, ischial tuberosity; IC, iliac crest; Ti, tibia; * (A), aluminum plate.

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Image Analysis

Images of the femurs and pelvis of rats at each age were analyzed using ImageJ (ver. 1.52a). When measuring the length and width of the bones, because the edge of the bone was unclear on simple radiography, an image was prepared in which smoothing was performed 3 times by replacing the mean value in the range of 3 × 3 at each position of the image (Figure 4A) and an original image without smoothing (Figure 4B). An image in which the edge of the bone was enhanced was prepared by calculating the difference between the 2 images (Figure 4C). The brightness of Figure 4C was adjusted (Figure 4D) and the measurement was performed (Figure 4). The process of creating Image D was recorded using a macro so that the same process was performed on all images.

When performing the analysis, an aluminum measure to be used as an indicator was applied at the same time as imaging, and the measurement was performed after setting 10 mm on the software program. In addition, to ensure accuracy when measuring each part, the magnification was increased to 1600 times to determine the measurement point. When the magnification was increased to 1600 times, 1 dot was 0.082 mm. The indicator points for measurement were marked at 7 locations on the femur and 4 locations on the pelvis, and the X and Y coordinates of each point were measured. The length and width of each part were measured by calculating the X- and Y-coordinates using trigonometric functions.

Data were obtained by measuring each site in 14 rats at 12 sites (6 sites on the left and right sides) in one animal at each of the 2 imaging sessions. Imaging and analyses were performed using the same method 7 times from 4 to 16 weeks of age to obtain data for a total of 2352 points.

Statistical Processing

To investigate left-right differences between the femur and pelvis at each age, Wilcoxon signed-rank sum tests were performed for LF, LT-FC, DFH, APD-CF, LP, and AD at each time point from 4 to 16 weeks of age. The tests were performed in the CA and GA groups to confirm whether left-right differences occurred in length.

Left-to-right ratios were obtained at each age and site to investigate whether differences in exercise quantity due to rearing environments occurred as growth changed at each site. Growth changes up to 16 weeks, using 4 weeks of age as the standard, were tested by a 2-way ANOVA with a mixed model. For testing, left-to-right ratios were used as dependent variables, and Bonferroni correction was used to adjust for multiple comparisons.

Because of differences in exercise load due to weight gain or loss, an unpaired t-test of body weight was performed from 4 to 16 weeks of age to compare the CA and GA groups.

All statistical analysis were performed using IBM SPSS Statistics version 27 (IBM, Armonk, NY, USA). P values of <0.05 were considered to indicate statistical significance.

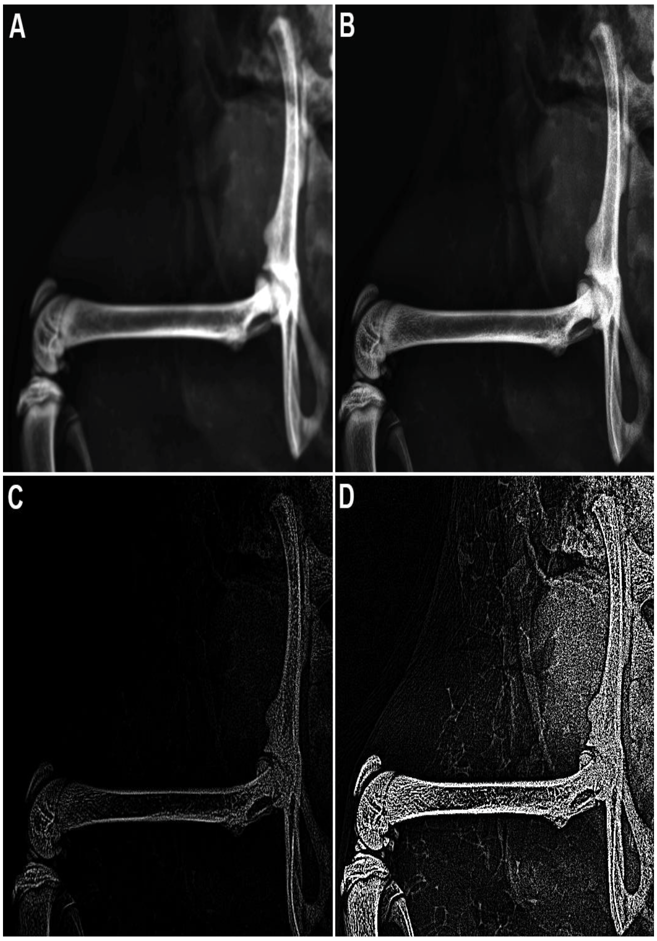

Figure 4: Image processing method. A, 3× smoothed image. B, Original image. C, Image obtained by subtracting image A from image B. D, Image with adjustment of brightness.

Results

Measurements of the femurs and pelvis at various ages

1. Total length of the femur (LF): The left side was significantly longer than the right side at all ages from 4 to 16 weeks of age in both groups (Figure 5A; Figure 6A).
2. Distance of the lesser trochanter–femoral condyle (LT-FC): The left side was significantly longer than the right side in all epochs in the CA and GA groups (Figure 5B; Figure 6B).
3. Diameter of the femoral head (DFH): No left/right differences were observed at any age in the two groups (Supplemental Figure 2A; Supplemental Figure 3A).
4. Anteroposterior diameter of the femoral condyle (APD-

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FC): No asymmetry differences were observed in the CA group at all ages (Supplemental Figure 2B), but the GA group had a significantly longer left side at 10 and 12 weeks of age (Supplemental Figure 3B).

5. Length of the pelvis (LP): The CA group showed no left-right differences at all ages (Supplemental Figure 2C); in the GA group, the right side was longer than the left side at 16 weeks of age (Supplemental Figure 3C).
6. Acetabular diameter (AD): The CA group showed no left/right difference at any age (Supplemental Figure 2D). In the GA group, the left side was longer than the right side at 8 and 16 weeks of age (Supplemental Figure 3D).

Changes in growth relative to 4 weeks of age

1. Total length of the femur (LF): A significant difference in the amount of change in asymmetry occurred at 4 and 12 weeks of age in the CA group. No difference was found in the amount of change in asymmetry in the GA group (Figure 7A).
2. Distance of the lesser trochanter–femoral condyle (LT-FC): No difference was found in the amount of change in asymmetry in either group (Supplemental Figure 4A).
3. Diameter of the femoral head (DFH): No discernible difference was noted in the magnitude of asymmetry change between the CA and GA groups (Supplemental Figure 4B).
4. Anteroposterior diameter of the femoral condyle (APD-FC): Both the CA and GA groups showed a similar degree of change in asymmetry, with no significant difference between them (Supplemental Figure 4C).
5. Length of the pelvis (LP): In the CA group, the amount of change in asymmetry was significantly different at 4 weeks of age and at 10, 12, and 14 weeks of age. In the GA group, the amount of change in asymmetry was significantly different at 4 weeks of age and at 8, 10, 12, 14, and 16 weeks of age (Figure 7B).
6. Acetabular diameter (AD): The change in asymmetry was comparable between the CA and GA groups (Supplemental Figure 4D).

Weekly body weight change

The body weight increased from 4 to 16 weeks of age. There was no significant difference in body weight between the CA and GA groups in any of the weeks (Figure 8).

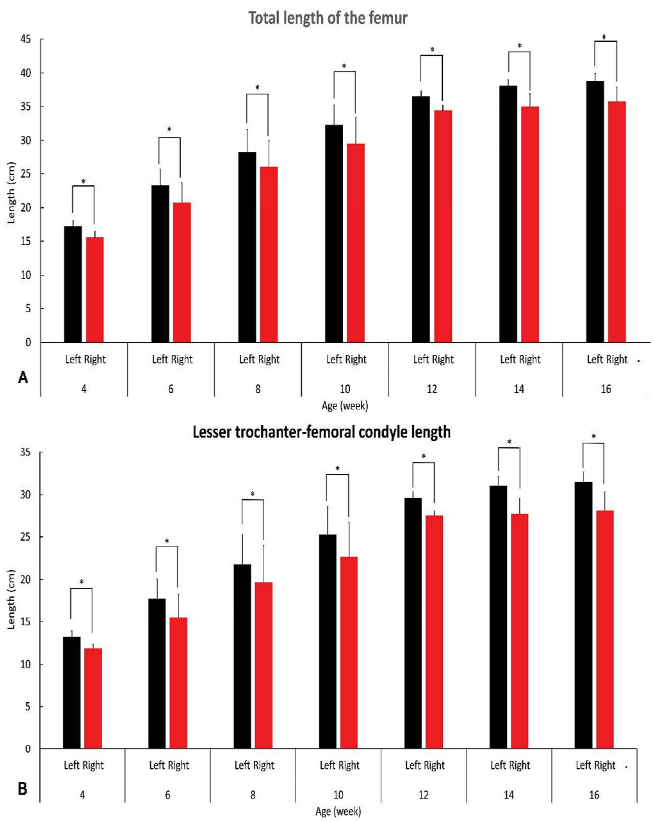

Figure 5: Measurement of the left-right length difference in the femur (A) and LT-FC (B) in the CA group at different ages. * P <0.05.

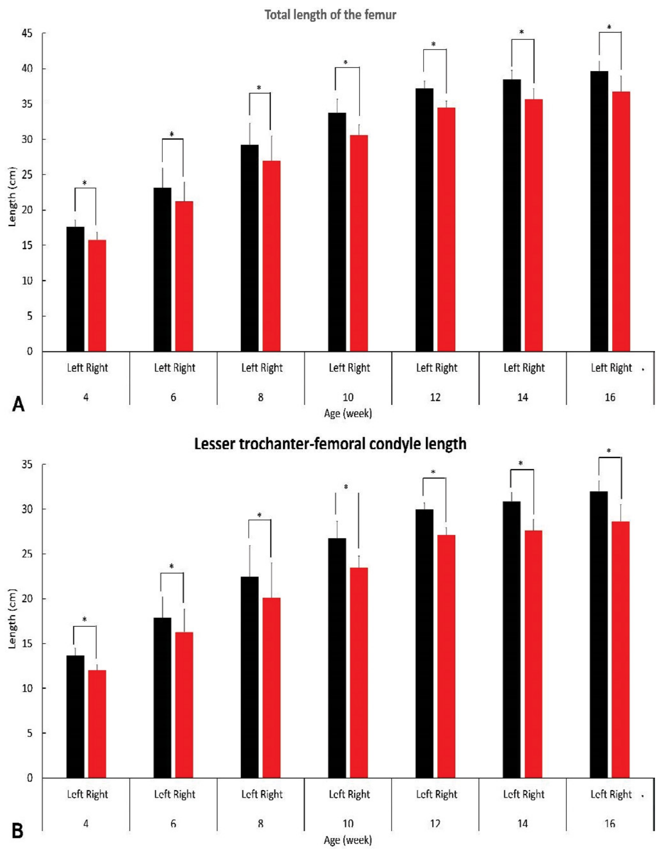

Figure 6: Measurement of the left-right length difference in the femur (A) and LT-FC (B) in the GA group at different ages. * P <0.05.

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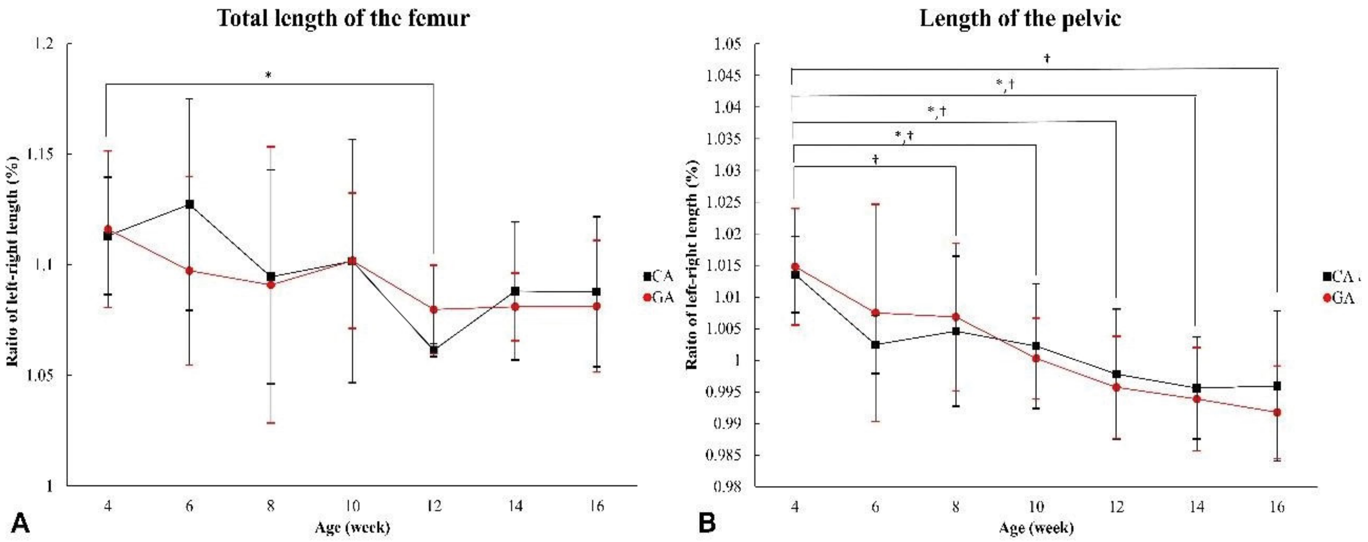

Figure 7: Measurement of the left-right difference of the femur (A) and pelvis (B) at each age. CA, complex activity group; GA, general activity group. * $P < 0.05$.

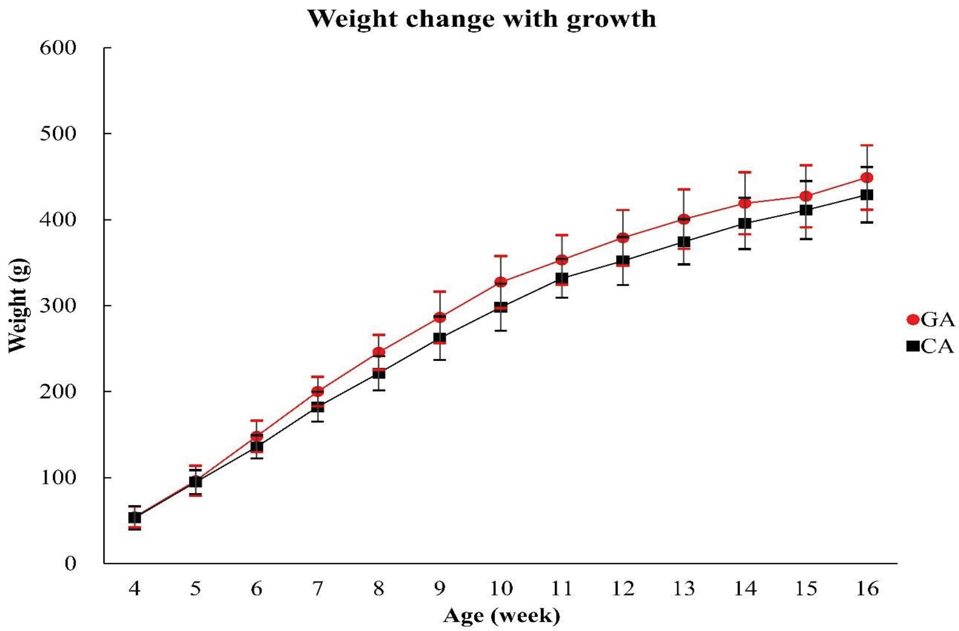

Figure 8: Changes in body weight at different ages. CA, complex activity group; GA, general activity group.

Discussion

In this study, we used mammography to examine and measure changes in bone morphology with the growth of femurs and pelvises in SD rats. Regarding the growth of the LF and LT-FC, the results showed that the left side was longer than the right side from 4 to 16 weeks of age in both the CA and GA groups. However, at 4 weeks, the left-right difference did not expand or shrink, and the results showed that the left side was consistently longer than the right side with growth. Regarding LP in both the CA and GA groups, the left-right difference was reversed after 12 weeks of age in the CA group and after 6 weeks of age in the GA group, and there was no significant difference in any of the age groups. However, the right side was still longer than the left side.

Studies in humans have shown variations in left-right differences, but none of the studies measured the left and right femurs of the same individual. The average length of the femur was slightly longer on the right than on the left in 250 femurs from different colleges in South India [9]. The total length of the left femur was longer on the left, but no significant difference was observed between the right and left femurs [10]. On the other hand, Young et al. [11], examined asymmetry, sex, and racial differences in the proximal femurs and found no laterality, sex, or racial differences in the

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proximal femurs; however, there was a negative correlation between height and asymmetry. In a study examining the asymmetry and the position of bones, such as bone length and width/rotation in children and adults, the femurs of 100 cadavers were dried and measured, and the results showed that left femurs were on average 0.93 cm longer than right femurs [8], which is consistent with our study of quadrupedal gait subjects.

Regarding transverse radial bone growth in the femora and pelvic bones, the left-right difference appeared and disappeared only in the GA group as a result of occasional left- right differences at some ages, and there was no consistency between ages. The growth of long bones on the horizontal axis is due to intramembranous ossification, which is a growth mode in which osteoblasts invade the fibrous membrane and are generated by the secretion of collagen fibers and bone matrix. This growth mode is considered to be involved in bone formation in flat bones, such as the pelvis [19]. The results of the present study showed that intramembranous ossification did not contribute in a left/right difference in bone formation.

Dangneaux et al. [17], measured the lengths and widths of the femoral epiphysis and patella and claimed that there was no left-right difference in the mediolateral condylar width. In addition, Boulay et al. [12], measured the lengths, widths, and angles of the pelvic bones at 71 locations, but found no left-right difference in the total pelvic length. These results are consistent with ours and suggest that no left/right difference was in growth by intramembranous ossification.

Bone mineral density and longitudinal bone formation and repair in long bones involve insulin-like growth factor (IGF-1) and growth hormones (GH) [20-24]. With regard to the growth of long bones on the horizontal axis, it has been proposed that leptin acts on the sympathetic nervous system as a factor that regulates the actions of osteoclasts and osteoblasts [25]. In addition, the hormone status also changes according to the nutritional status and amount of body water. In the present study, we observed that the growth process in living environments was as unified as possible and did not limit food and water. Although individual differences were observed, no significant differences in body weight were observed between the GA and GA groups. No left/right differences were observed at any age with respect to transverse radial bone growth. Since we did not measure hormone levels at each age, we were not able to clarify causality. Further research should be conducted to investigate whether the effects of hormones such as IGF-1 and GH are among the factors that cause bone asymmetry and to examine the differences in bone mineral density and bone formation when the levels of hormones (e.g., IGF-1) are altered.

Furthermore, Sakai et al. [22], reported that differences in the loads placed on the bones affect bone growth and density. Foster [26], compared SD rats with and without bipedal locomotion and found that SD rats with bipedal locomotion had increased physical activity and significantly increased bone density in the femur and tibia. Liu et al. [27], reported that treadmill exercise in SD rats resulted in dense trabecular and compact bone architecture. Thus, the relationship between load bearing and changes in bone density and morphology is well known.

In this study, there were differences in the load capacity between the CA and GA groups, depending on their activities. The amount of activity in the CA group also differed greatly from the observation records, indicating an increased burden on the hind legs. In the CA group, the left-right difference increased at 4 and 12 weeks of age; however, in the GA group, no increase in the left-right difference was observed at any age. There were no significant differences in body weight between the CA and GA groups. Accordingly, it is unlikely that the difference in body weight affected the bone load. Previous studies have shown that differences in load bearing affect bone formation [27], but in this study, differences in physical activity seem to be 1 of the factors causing the difference in bone size between the left and right sides.

Another possible factor affecting the bone is the influence of the vestibular function. Vignoux et al. [28], reported that vestibular dysfunction affects bone remodeling. In addition, research on the relationship between vestibular function and the sympathetic nervous system has found that it can cause differences in homeostasis, such as the effects of body temperature [29]. Alternatively, it has been reported that physiological changes, such as vestibular system dysfunction, enhance central nervous system activity, and bone loss is common in space [30]. In this study, the GA group showed fewer vertical and horizontal movements from the active environment, and the direction of gravity applied to the vestibular system was usually vertically downward. However, in the CA group, the direction of gravitational acceleration applied to the vestibule was different from that in the GA group (e.g., the animals were observed clinging to the cage and turning upside down). Although this may cause a left-right difference, no significant difference between the CA and GA groups was found in this study. This suggests that changes in bone may occur in special environments, such as weightlessness, or from excessive gravity acceleration. However, the degree of change in gravity acceleration in normal life may not be a sufficient factor to promote bone growth.

In this study, although there were differences in the amount of exercise load between the CA and GA groups, the effect on the vestibular system, and the stress imposed by the living environment, there was a difference in the total femur

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length between the left and right sides, with the left side being significantly longer. Furthermore, no significant difference was observed between the CA and GA groups in terms of bone length because of the difference in exercise load. This led us to believe that the difference between the left and right bones is determined by inherent structural influences rather than external factors (e.g., the environment) or internal factors (e.g., hormones).

Asymmetry of the viscera has been reported to be caused by the rotation of cilia at the nodes of the fertilized egg [31]. The rotation of the spinal column varies depending on the position of the viscera, and the position of the viscera may be a factor that generates bone asymmetry [32]. The liver, weighing approximately 1.2–1.5 kg, is located on the right side of the body, while the heart, spleen, and sigmoid colon are located on the left side. The resulting difference in the load on the body between the left and right sides may be a topic for future research (e.g., how it affects the left-right difference in body limb morphology).

In this study, we found that the left side was significantly longer in terms of the asymmetry of the femur in the long-axis direction, but no obvious left-right asymmetry was found in terms of the growth of the transverse diameter. We could not exclude the influence of internal factors or find any information about the differences in bone morphology depending on the position of the organ. However, we found that bone asymmetry occurred as early as 4 weeks of age, and that the asymmetry was not affected by the environment, and that it always occurred. Further studies are needed to investigate the changes that occur when stimulation is applied to the vestibular apparatus by changing gravitational acceleration, as well as whether left-right asymmetry in bone occurs in rats with visceral positions. It is unclear how much asymmetry affects movement, and this study suggests an opportunity to consider the existence of asymmetry as a natural phenomenon.

Author Contributions: Conceptualization: S.U. and S.Y.; Methodology: S.U. and T.N.; Software: T.N.; Validation: Y.N., M.Z., and S.Y.; Formal Analysis: S.U. and T.N.; Investigation: S.U. and T.Y.; Resources: T.Y.; Data Curation: S.U. and T.N.; Writing – Original Draft Preparation: S.U.; Writing – Review and Editing: S.Y. and Y.N; Supervision: S.Y.; Project Administration: S.Y.; Funding Acquisition: S.Y.

Conflict-of-interest statement: The authors declare no conflicts of interest in association with the present study.

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