Gait changes in a line of mice artificially selected for longer limbs

Leah M. Sparrow [1] , **Emily Pellatt** [1] , **Sabrina S Yu** [2] , **David A. Raichlen** [3] , **Herman Pontzer** [4] , **Campbell Rolian**
Corresp. 1, 5

[1] Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada
[2] Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada
[3] School of Anthropology, University of Arizona, Tucson, Arizona, United States
[4] Department of Anthropology, City University of New York, Hunter College, New York, New York, United States
[5] McCaig Institute for Bone and Joint Health, University of Calgary, Calgary, Alberta, Canada

Corresponding Author: Campbell Rolian
Email address: cprolian@ucalgary.ca

In legged terrestrial locomotion, the duration of stance phase, i.e., when limbs are in contact with the substrate, is positively correlated with limb length, and negatively correlated with the metabolic cost of transport. These relationships are well documented at the interspecific level, across a broad range of body sizes and travel speeds. However, such relationships are harder to evaluate within species (i.e., where natural selection operates), largely for practical reasons, including low population variance in limb length, and the presence of confounding factors such as body mass, or training. Here, we compared spatiotemporal kinematics of gait in Longshanks, a long-legged mouse line created through artificial selection, and in random-bred, mass-matched Control mice raised under identical conditions. We used a gait treadmill to test the hypothesis that Longshanks have longer stance phases and stride lengths, and decreased stride frequencies in both fore- and hind limbs, compared with Controls. Our results indicate that gait differs significantly between the two groups. Specifically, and as hypothesized, stance duration and stride length are 8-10% greater in Longshanks, while stride frequency is 8% lower than in Controls. However, there was no difference in the touch-down timing and sequence of the paws between the two lines. Taken together, these data suggest that, for a given speed, Longshanks mice take significantly fewer, longer steps to cover the same distance or running time compared to Controls, with important implications for other measures of individual variation in whole-organism performance, such as the metabolic cost of transport.

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Rolian[1, 5]

Author Affiliations:

[1] Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine,

University of Calgary, Calgary, Alberta, Canada

[2] Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada

[3] School of Anthropology, University of Arizona, Tucson, Arizona, United States

[4] Department of Anthropology, City University of New York, Hunter College, New York, New York, United

States

[5] McCaig Institute for Bone and Joint Health, University of Calgary, Calgary, Alberta, Canada

Corresponding Author: Campbell Rolian

Email address: cprolian@ucalgary.ca

16 **ABSTRACT**

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18 with the substrate, is positively correlated with limb length, and negatively correlated with the

19 metabolic cost of transport. These relationships are well documented at the interspecific level, across a

20 broad range of body sizes and travel speeds. However, such relationships are harder to evaluate within

21 species (i.e., where natural selection operates), largely for practical reasons, including low population

22 variance in limb length, and the presence of confounding factors such as body mass, or training. Here,

23 we compared spatiotemporal kinematics of gait in Longshanks, a long-legged mouse line created

24 through artificial selection, and in random-bred, mass-matched Control mice raised under identical

25 conditions. We used a gait treadmill to test the hypothesis that Longshanks have longer stance phases

26 and stride lengths, and decreased stride frequencies in both fore- and hind limbs, compared with

27 Controls. Our results indicate that gait differs significantly between the two groups. Specifically, and as

28 hypothesized, stance duration and stride length are 8-10% greater in Longshanks, while stride frequency

29 is 8% lower than in Controls. However, there was no difference in the touch-down timing and sequence

30 of the paws between the two lines. Taken together, these data suggest that, for a given speed,

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32 compared to Controls, with important implications for other measures of individual variation in whole-

33 organism performance, such as the metabolic cost of transport.

34

35   **INTRODUCTION**

36   In terrestrial legged locomotion, a stride is divided into two phases: stance, when the foot/paw

37   is in contact with the ground, and swing, when the foot leaves the substrate as the limb transitions to

38   the next stance phase. Stance duration is influenced by a number of anatomical and biomechanical

39   factors, including limb length. Limb length is positively correlated with step length, the distance traveled

40   while a foot is in contact with the ground. As stance duration is simply step length divided by travel

41   speed, for a given travel speed, organisms with relatively longer limbs typically have longer stance

42   durations [1]. Stance duration is itself an important determinant of whole-organism performance,

43   especially in relation to the metabolic cost of moving the body. During level walking and running, muscle

44   forces produced by the limbs, integrated over the stance phase, must equal bodyweight integrated over

45   the whole stride. Longer stance phases reduce muscle force production rates per unit of body mass, in

46   turn reducing the overall metabolic cost of supporting the body during locomotion [2-5]. Hence, it

47   follows that organisms with longer limbs and stance durations also tend to have a relatively lower mass-

48   specific metabolic cost per unit of distance traveled, often called the cost of transport or COT (J kg^{-1} m^{-1})

49   [2, 4].

50   The relationships between limb length, stance duration (also known as "contact time") and COT

51   are well documented at the interspecific level, e.g., among terrestrial quadrupedal mammals in which

52   limb length and COT data were sampled from the same individuals [1-4, 6-8]. In contrast, these

53   relationships have been more equivocal at the population level, where differences among individuals in

54   whole organism performance due to variation in these factors have the potential to lead to differential

55   reproductive success (i.e., where natural selection operates [9]). Several studies in human populations,

56   have shown a correlation between limb length and gait variables such as stance duration [10-12] , as

57   well as between limb length and COT in walking and running, after controlling for other factors that

58   influence COT, such as body mass (e.g., Refs [13-18], but see also Ref. [19]). Fewer intraspecific studies

59   have examined the relationships between hind limb length, kinematics, and COT within quadrupedal

60   species, and these are limited to comparisons among breeds of dogs or horses. Longer limbs are

61   associated with greater stance duration in dogs [20, 21] and horses [22, 23], but while hind limb length

62   can be predictive of COT in dogs [13], taller horses do not necessarily have lower COT [24].

63   One of the practical challenges in relating limb length to locomotor mechanics among

64   individuals *within* populations, is that only a limited range of limb lengths can realistically be sampled,

65   meaning that either variation in skeletal anatomy is too small to detect subtle effects of leg length on

66   gait (i.e., a low signal-to-noise ratio), or impractically large samples are necessary to do so.

67   Another important limitation of the comparative approach to studying the relationship between

68   limb anatomy and locomotor mechanics - both within and between species - is the complexity of teasing

69   apart the relative impact on gait of body mass, skeletal anatomy, posture and other biological factors

70   (e.g., proportions of muscle fiber types [25]). Within species, many of these factors are genetically and

71   phenotypically correlated, and interact with each other, and with other factors such as age, body

72   composition (e.g., lean mass), and training, which makes it harder to quantify the relative contributions

73   of limb length alone to gait variation among individuals. For example, among artificially bred dog and

74   horse species, differences in size and limb length are typically correlated with other changes in body

75   proportions and conformation can affect kinematics and may influence metabolic cost [21, 22].

76   Similarly, among species, fundamental differences in posture (e.g., quadrupedalism vs bipedalism, limb

77   joint extension angles, limb design (e.g., plantigrady vs unguligrady) and orders of magnitude of

78   differences in body mass may also obscure the effects of limb skeletal anatomy on locomotor mechanics

79   [26].

80   Here, we use an experimental evolution model to explore the relationship between limb length

81   and locomotor kinematics. Longshanks mice have been selectively bred for increases in tibia length

82   independently of body mass [27, 28]. After 14 generations, Longshanks tibiae were on average 14%

83   longer than a random-bred wild-type cohort of the same genetic background (hereafter Controls), but

84   average body masses were the same in both groups. By pooling Longshanks and Control mice, we have

85   created a new population of mice in which the range of variation in limb length is artificially increased,

86   thereby circumventing sample size and resolution issues. More importantly, in this sample the

87   potentially confounding effects of body mass, diet, training, somatic growth, age and even genetic

88   background, are more rigorously controlled. We used the unique Longshanks mouse to investigate the

89   proximate relationships between limb morphology and locomotor mechanics within species. We test

90   the hypothesis that, at a given speed, increased limb length produces predictable changes in gait

91   parameters. Specifically, we predict an increase in stance duration and stride length, and a parallel

92   decrease in stride frequency.

93   **METHODS**

94   *1. Samples*

95   All animal procedures were approved by the Health Sciences Animal Care Committee at the

96   University of Calgary (protocol AC13-0077), and were conducted in accordance with best practices

97   outlined by the Canadian Council on Animal Care. We used a sex-balanced sample of adult mice (156 ±

98   17 days, mean ± SD) selected at random from 12 Longshanks families (hereafter LS, n= 11 females, 11

99   males) and 12 Control families (hereafter C, n=12 females, 11 males) lines at generation F14. Details of

100   the selective breeding protocol are given elsewhere [27]. Mice were housed individually and kept under

101   similar environmental conditions, in a separate room from the main colonies of the selective breeding

102   experiment, maintained at 22-25C, humidity 50-70% on a 12-hour light/dark cycle. Mice were given food

103   and water ad libitum. ~~The food provided was a low-fat food (low-fat Pico-Vac lab rodent chow (20%~~

104   ~~protein, 4.5% fat).~~

105   *2. Gait data collection and analysis*

106   *a) Gait treadmill procedure*

107   *Training phase:* Beginning at 10 weeks old, each mouse completed a total of eight training trials

108   on a closed chamber treadmill (Columbus Instruments, Columbus, OH) over the course of five months.

109   In the first two trials, the treadmill was inactive, as these trials allowed the mice to acclimate to the

110   novel treadmill environment. For the remaining six trials, mice completed an initial 5-10-minute

111   habituation period, followed by a running schedule as follows: four minutes at 5 m/min, three minutes

112   at 10m/min, three minutes at 15m/min, three minutes at 20m/min, and a 90 second cool-down period.

113   Within three days of completing the final training trial, gait data for each mouse were collected

114   using a TreadScan gait treadmill (Cleversys, Reston, VA). Mice were weighed to the nearest 0.01g prior

115   to being placed in the treadmill. The TreadScan apparatus comprises a transparent, variable-speed

116   treadmill belt (190mm L, 38mm W) enclosed within a Plexiglas chamber. While mice run on the

117   treadmill, their paw placements are recorded on the ventral surface through the use of a mirror placed

118   at 45 degrees to a high-speed camera recording at 100 frames per second. To improve digital tracking of

119   footprints, the contrast between paws and fur was enhanced by applying red food coloring to the

120   plantar surfaces of the paws. The mice were run for approximately three minutes during which three 20-

121   second digital video recordings were taken at each of three test speeds: 10, 15, and 20 m/min. After the

122   first video was captured, each foot was manually traced for approximately 20 frames (equivalent to a

123   full locomotor cycle) to create a foot model for all subsequent analyses.

124

125   *b) Gait variables and analysis*

126   The TreadScan software automatically tracks stance and swing phases of each paw and

127   subsequently generates multiple gait variables from the video footage. A Matlab script (v.2012b, Reston,

128   MA) was first used to extract gait variables from the TreadScan output spreadsheet from all individual

129   strides recorded for each mouse at each speed. For consistency, and because lighting and visibility of

130   kinematics was better for the animal's right side, only gait variables for the right forelimb and hind limb

131   paws were used for analysis, with the exception of the touch-down sequence of the four paws (see

132   below).

133   The following variables were obtained for the right fore- and hind limb paws from the TreadScan

134   output spreadsheets: (1) Stance Time: Time (ms) during which that foot was in contact with the belt; (2)

135   swing time (ms): time during which that foot is not in contact with the treadmill, (3) Stride Length (mm):

136   distance travelled between two stance phases of the same foot. Note that, as the mice are stationary

137   relative to the treadmill, stride length is estimated as the product of stride time (i.e., stance time plus

138   swing time) and treadmill belt speed (4) stride Frequency (Hz): 1/mean stride duration. To determine

139   whether the increased limb length of Longshanks resulted in a change in its gait sequence relative to

140   Controls, we also obtained timing data for each paw during a full stride cycle. Specifically, using the

141   initiation of stance in the right forepaw as a reference, we obtained the relative timing of touch-down

142   for the other paws, expressed as a percentage of a full stride cycle.

143   A second Matlab (v.2012b, Reston, MA) script was used to obtain means for each gait parameter

144   in each individual's right fore- and hind limb paws, based on one or more sets of at least three

145   consecutive steps. Outliers, defined as data points that were greater than two standard deviations away

146   from their respective means, were removed and the mean was recalculated. Individuals' gait variable

147   means were based on a minimum of 3 steps in each limb type and speed condition (mean: 17 steps,

148   range 3 – 63). Videos of the slowest speed, 10m/min, were excluded from our analyses due to the

149   difficulty in selecting a section of video with enough consecutive strides to obtain reliable gait patterns

150   ~~at this relatively slow speed~~.

151   Statistical analyses of the gait and gait sequence variables were performed using four separate

152   sets of generalized linear models (GLM). In the first two sets, GLMs were used to test for mean

153   differences between lines (LS1 vs C) and speeds (20 and 25 m/min) in each gait variable *within* a limb

154   type. In the third set of GLMs, we compared mean fore- and hind limb gait variables between the mouse

155   lines at the fastest speed. Finally, we used a GLM to test for mean differences between lines and speeds

156   in gait sequence variables. In all GLMs, we first used a full factorial model, in which line, speed or limb

157   type were treated as categorical predictors, and body mass was included as a continuous predictor

158   (covariate). The models included interaction terms for the respective categorical factors (i.e., Line x

159   Speed, or Line x Limb Type), as well as covariate-by-factor interaction terms (i.e., homogeneity of slopes

160   tests). In all but one variable (see below), none of the covariate-by-factor interaction terms were

161   significant, i.e., the effect of the categorical factors (e.g., speed) on the responses (gait variables) was

162   not dependent on the magnitude of body mass. As argued by Engqvist [29], in the absence of significant

163   covariate-by-factor interaction terms, GLM analyses should be re-run without these terms, as failing to

164   exclude them implies that the main effects of the factors cannot be generalized over the range of the

165   covariate. Conversely, however, a significant covariate-by-factor interaction term indicates that any

166   significant mean differences among the factors are only true at the intercept, i.e., where the covariate –

167   body mass in this case - is equal to 0.

168   In the fourth set of GLM analyses, we found a significant interaction between mouse line and

169   body mass in the timing of touch-down of the left hind paw relative to the right forepaw ($F(7,82) = 4.04$,

170    $p=0.047$). However, the inclusion of this interaction term had only a small effect on the least squares

171 (LS) means among groups. For example, at the covariate mean (42.93g), the difference in LS means

172 when the interaction term was excluded vs. included was less than 1% across all groups (range: 0.4-

173 0.9%), while at the 10th and 90th percentile of body mass this difference was less than 10% (range: 4.5-

174 9.5%). Hence, given its modest impact on the actual gait variable response over the covariate range, and

175 to be consistent with the remaining analyses, we excluded all covariate x factor interactions from this

176 GLM. In all GLM analyses, relevant post-hoc pairwise comparisons on all gait variables were made using

177 Tukey's HSD tests. All analyses were carried out in Statistica software (v.10.0, Statsoft, Tulsa, OK).

178 *3. Morphometric Data Collection and Analysis*

179 After completing the gait trials, mice were euthanized by CO_2 inhalation, weighed and

180 immediately frozen at -20C. As part of an ongoing digital tomography archive of the Longshanks

181 experiment, full body micro-CT scans were made using a Skyscan 1173 µCT scanner at a resolution of 45

182 µm (65-70 kV, 75-105 µA). One of the Longshanks bodies was not recovered following euthanasia. 3D

183 isosurfaces of the scans were produced using Amira v.5.4.2 (Visage Imaging, Berlin, Germany). Bone

184 lengths were determined by calculating the linear distance between 3D digital landmarks placed on

185 specific anatomical features of the limb long bones. Micro-CT measurements are superior to linear

186 measurements from dissected limbs, as soft tissues are not visible on the µCT scans, and the placement

187 of digital landmarks on homologous anatomical features across individuals is highly repeatable[28].

188 The following anatomical features were used for landmark placements: (1) humerus length –

189 from the center of the proximal articular surface to the distal-most point on the medial epicondyle, (2)

190 ulna length – from the tip of the olecranon process to the tip of the styloid process, (3) carpo-

191 metacarpus – from the proximal articular facet of the central carpal to the tip of the distal articular

192 facet, (4) scapula length – from the caudal end of the spine of the scapula to the tip of the center of the

193 glenoid (5) third manual proximal phalanx - from the center of the proximal to the center of the distal

194   articular facets, (6) femur length - from the center of the medial condyle to the tip of the greater

195   trochanter, (7)) tibia length – from the anterior-most lip on the proximal epiphysis to the most distal

196   point on the medial malleolus, (8) tarso-metatarsus – from the proximal dorsal border of the centrale

197   tarsal bone to the dorsal border of the distal articular facet of the third metatarsal, (9) third pedal

198   proximal phalanx - from the center of the proximal to the center of the distal articular facets. Limb bone

199   lengths were also summed within limb to obtain forelimb and hind limb anatomical lengths. Two-tailed

200   t-tests were used to compare mean body mass and limb bone lengths between the groups.

201   **RESULTS**

202   *Morphometric differences*

203   Mean body mass was not significantly different between Control and Longshanks mice in either

204   the test trials or the *ex vivo* CT scanning (**Table 1**). In the forelimb, all bones were significantly longer in

205   Longshanks when compared to Control mice. Longshanks scapulae were on average 6.6% longer, humeri

206   were ~12.5% longer, ulnae ~10.7%, and the hand bones (carpo-metacarpus and third proximal phalanx)

207   were ~4% longer. When summed across the elements, the anatomical length of the forelimb in

208   Longshanks, including the scapula [30, 31] was on average 9.1%, or approximately 4.2 mm, longer than

209   Control forelimbs. Hind limb bones were all significantly longer in Longshanks mice compared to the

210   Control mice: Longshanks mice had a 7.4% longer femur, 14.3% longer tibia (**Figure 1**), 9.5 % longer

211   tarso-metatarsus, and 9.3% longer pedal proximal phalanx (Table 1). When summed across elements

212   (femur + tibia + foot elements), the Longshanks hind limb was on average 10.7%, or 5.2 mm, longer than

213   Control hind limbs. Selection for relative tibia length in Longshanks thus caused disproportionate

214   changes in the other limb bones, such that overall the Longshanks forelimb increased in length slightly

215   less than the hind limb, although the mean difference in length between the limbs is not significantly

216   different between Longshanks (mean ± SD = 2.84±2.85mm) and Control (1.89±2.08mm) (**Table 1**, t-test,

217   t(42) = 1.27, p = 0.21).

218   *Gait differences*

219   *Line and speed effects on gait within limbs*: The first two sets of GLMs indicate that body mass

220   was not significantly correlated with any gait variable in the forelimb, but in the hind limb was

221   significantly negatively correlated with swing duration, and positively correlated with stance duration

222   (**Table 2**). Both speed and line type had significant effects on forelimb and hind limb stance duration,

223   stride length and stride frequency (Table 2, all standardized slopes significantly different from zero,

224   p<0.001). In the forelimb, speed had a significant effect swing duration, while in the hind limb there was

225   no change in swing duration due to speed (Table 2). There was no significant interaction between line

226   type and speed in either limb, indicating that running faster did not affect gait variables in Longshanks

227   and Control mice differently (0.19<p<0.92).

228   Pairwise comparisons between lines indicate that at the lower speed, mean stance duration was

229   significantly longer, by 10.7% in the forelimb and 9.1% in the hind limb, ~~respectively,~~ in Longshanks

230   compared to Controls (**Table 3**, **Figure 2**). At the faster speed, the difference between lines in mean

231   forelimb stance duration (7.5%) trended towards significance (Tukey's HSD, p=0.087), while in

232   Longshanks, hind limb stance duration was over 12.7% longer (Tukey's HSD, p<0.001). Swing durations

233   did not differ between the lines in either limb, however forelimb swing times were significantly shorter

234   at the faster speed (Table 3, Figure 2). Mean forelimb and hind limb stride lengths are greater by 7-8% in

235   Longshanks at both speeds, while ~~similarly~~ mean stride frequency in both limbs at both speeds is

236   decreased by 7-8% in Longshanks.

237   *Line and limb effects on gait within speed:* The GLM analysis with limb type and line as factors

238   (fast speed only) revealed a significant effect of limb type on swing and stance durations in the

239   Longshanks mouse, with the forelimb having a relatively longer swing phase and shorter stance phase

240   compared to the hind limb (**Table 4**, Tukey's HSD, p<0.05). As expected, however, stride duration, stride

241   length and stride frequencies are the same between the fore- and hind limb within line. In other words,

242   in Longshanks, stance duration as a percentage of stride duration (i.e., duty factor) is different in the

243   forelimb and hind limb, but fore- and hind-limb cycles are of equal duration.

244   *Line and speed effects on gait sequence:* The mean relative timing of paw touch-downs is shown

245   in **Table 5**, and gait sequences are shown in **Figure 3**. The GLM analysis showed no effect of line on gait

246   sequence data, but speed had a significant effect on the gait sequence of the forepaws (standardized

247   beta = -0.23, $F_{4,85}$ = 4.21, p=0.03), with contact of the contralateral forepaw occurring 4-5% earlier at the

248   faster speed in both lines (Tukey's post-hoc HSD, p = n.s.). Combining the touch-down sequence and

249   stance duration data at 20 m/min, both lines have very similar gait sequence profiles, although

250   Longshanks mice have relatively longer hind limb stance phases (greater duty factors, Figure 3).

251   **DISCUSSION AND CONCLUSION**

252   Gait is influenced by a number of anatomical (e.g., mass, skeletal size) and biomechanical

253   factors (e.g., speed, bipedal vs. quadrupedal locomotion). Limb length is positively correlated with step

254   length, and hence with stance duration, in terrestrial species across a broad range of body sizes and limb

255   lengths [1-3]. Whether this relationship also holds true within populations is less clear, however, largely

256   due to the challenge of sampling adequate variation among individuals in limb bone length to detect

257   subtle differences in gait, and sampling limb bone lengths that are not also correlated with other

258   variables that influence gait.

259   We used the long-limbed Longshanks mouse line to test the hypothesis that these mice would

260   have longer strides, longer stance durations and thus lower stride frequencies when compared to mice

261   from a random-bred control group. Crucially, these two groups have been raised under the same

262   conditions and were trained on the treadmill using identical protocols. In addition, they have the same

263   average body mass, and come from the same genetic background. Hence, we were able to isolate the

264   effects of limb bone length on gait parameters from other potentially confounding factors such as

265   training, speed and body mass.

266   Our results provide strong support for our hypothesis, in both limbs: at the lower speed

267   Longshanks had 9-10% longer stance durations, 7-8% longer strides and stride durations, and thus 7-8%

268   lower stride frequencies. At the faster speed, swing durations in the forelimb were reduced in both

269   lines, while Longshanks' stance duration for the hind limb was longer by 12.7%. Overall, however, the

270   differences in stride length and duration, and stride frequency between the lines in both limbs remained

271   7-8% at 20 m/min. Interestingly, there was no difference between Longshanks and Control in swing

272   times of either limb, at either speed. Equivalent swing times suggest that, despite its increase in bone

273   lengths, the natural swing period of the limbs in Longshanks have not changed substantially relative to

274   Controls, although this could also be due to greater muscle work to move the limb during the swing

275   phase in Longshanks. Similar natural periods could result from a similar distribution of muscle mass in

276   both lines, despite the increased limb length in Longshanks. Future work will more carefully assess ~~in~~

277   ~~these populations to determine~~ why their mean swing phase durations do not differ.

278   The gait sequence data show that the increase in limb length in Longshanks did not impact the

279   relative timing and sequence of stance initiation in the four limbs at 20 m/min (Table 5, Figure 3).

280   Moreover, the forelimb duty factors are very similar in both lines at this speed (~0.59, Table 4).

281   However, the relatively longer stance duration of the Longshanks hind limbs at 20 m/min increases its

282   duty factor slightly relative to Controls (0.647 vs 0.612, Table 4). The reasons for the longer stance

283   duration in the Longshanks hind limb are unclear, but may relate to the fact that overall the Longshanks

284   hind limb increased in length slightly more than the forelimb as a result of selection on tibia length

285   (Table 1).

286   One limitation of this study is the absence of kinematic data from lateral views of the limbs in

287   these mice, which precludes us from determining precisely if and how limb protraction, retraction, and

288   joint angles have changed in Longshanks as a consequence of changes in the relative lengths of its limb

289   bones (Table 1). As a first approximation, we used our morphometric and gait data to estimate

290   differences in joint angles in the hind limbs of Longshanks vs Controls, based on preliminary limb angular

291   excursion data. Step length is the horizontal distance the hip travels while the paw is in contact with the

292   substrate. On a treadmill, the proximal joints are effectively stationary, and step length represents

293   instead the distance traveled by the paw while in contact with the treadmill belt. Stance on a treadmill

294   can thus be modeled as a "support triangle", described by the hip joint (vertex) and the points of paw-

295   belt contact at touch-down and toe-off (Figure 4). The base of the triangle represents step length, the

296   height is the vertical distance between the hip and substrate. The triangle's sides represent the hind

297   limb at touch-down and toe-off, and their lengths at these time points are determined by the limb's

298   joint angles and bone lengths.

299   When the limb angular excursion (i.e., the vertex angle) is known, the lengths of the triangle's

300   sides can be estimated. We obtained hind limb protraction and retraction angles from a small sample of

301   Longshanks and Control mice from generation F22 (n = 5 each), running at 20 m/min (**Figure S1**). The

302   hind limb protraction, retraction, and excursion angles did not differ significantly between the lines

303   (**Table S1**), and the overall mean excursion angle was 74.7° ±2.8 (mean ±SEM). Thus, even though step

304   length in Longshanks hind limbs was 12.7% longer, the angles in the support triangle in both lines were

305   the same, implying that the hind limb support triangle's sides in Longshanks are all also ~12.7% longer.

306   Using the sine rule, we estimated the mean triangle side length at touch-down (i.e., protraction) to be

307   38.91mm in Controls, and 43.85mm in Longshanks, while at toe-off (retraction) mean lengths were

308   28.09mm and 31.72mm, respectively (Figure S1).

309   Combining these support lengths with the mean lengths of the femur, tibia and tarso-

310   metatarsus in each line (Table 1), and assuming that the hind paw at touch-down is horizontal and at

311   toe-off the tarso-metatarsus is perpendicular to the treadmill (Figure S1), we solved graphically for

312   mean knee and ankle joints in Longshanks and Control (**Figure 4**). Mean angles in Longshanks hind limbs

313   are all very similar to Controls. The greatest difference is in the knee at touch-down, which is ~5°, or 4%,

314   more extended in Longshanks than in Control mice. This small difference may be due to the fact that the

315   tibia and femur in Longshanks did not increase in length proportionately (+14.3% vs +7%). Overall, the

316   10.7% increase in hind limb length in Longshanks likely accounts for much of the 12.7% increase in its

317   hind limb step length, and necessitates only minor joint extensions at the knee and ankle in Longshanks

318   (Figure 4). Limb angular changes in the forelimb are likely equally small, however given that the mean

319   increase in forelimb step length (+7.5%) is less than the overall increase in forelimb length (+9%), the

320   joint angular changes in that limb in Longshanks more likely entailed flexion rather than extension,

321   absent any changes in forelimb excursion angles between the lines. More precise limb kinematics will

322   clearly be required to confirm these data and determine the combination of changes adopted by

323   Longshanks to increase step and stride lengths in both limbs.

324   Our findings have implications with respect to the cost of transport in Longshanks. The derived

325   morphology of the Longshanks mouse altered its gait in a predictable fashion, resulting in increased

326   stance durations and stride lengths relative to Controls. Both of these characteristics contribute to the

327   cost of transport in terrestrial organisms. Increased stance time distributes the muscle forces necessary

328   to support body weight over a longer interval, lowering the *rate* of muscle force production, i.e., the

329   volume of muscle recruited per distance traveled [2-5]. Complementary to this, increasing stride length

330   reduces the number of steps required to cover a given distance, reducing activation costs [4]. Thus, our

331   gait data predict that, all else being equal, including limb and joint angles as estimated above, the mass-

332   specific cost of transport in Longshanks should be lower when compared with Control mice. We are

333   currently testing this hypothesis with the use of a metabolic treadmill in Longshanks and Control.

334   Variation in whole organism performance directly impacts evolutionary fitness, and is an

335   important driver of adaptive evolution [9, 32-34]. In the process of selecting for a target morphological

336   trait (longer tibiae relative to body mass), we have "forward engineered" a morphology in Longshanks

337   with a quantifiable impact on whole organism *biomechanical* performance. We do not yet know the

338   impact of the observed change in gait on *physiological* performance (e.g., metabolism, especially cost of

339   transport), and it is challenging to relate any differences in these measures of whole organism

340   performance to differences in survival and reproductive success of Longshanks and Control mice in the

341   wild, as these are laboratory-reared animals raised in highly controlled and homogeneous

342   environments. Nevertheless, this study demonstrates the relationship of selectable phenotypic variation

343   in skeletal anatomy to variation among individuals in whole organism performance, in the form of

344   locomotor mechanics. Hence, it provides an important link between population-level, microevolutionary

345   processes and the adaptive origins of macroevolutionary diversity in limb musculoskeletal anatomy

346   among terrestrial mammals.

348 **ACKNOWLEDGEMENTS**

355 **FIGURE CAPTIONS**

356 **Figure 1**: μCT scans of two individuals from the study sample closest to mean raw tibia length in Control

357 (C, 18.85mm) and Longshanks (LS, 21.45mm).

358 **Figure 2**: Comparison of gait variables in Longshanks and Control mice at 15 and 20 m/min. Boxplots of

359 swing duration (A, in milliseconds), stance duration (B, in milliseconds), stride length (C, in mm), and

360 stride frequency (D, in seconds^{-1}) in Longshanks (shaded box) and Control mice (open box). Horizontal

361 lines within boxes represent medians, boxes indicate interquartile ranges, whiskers indicate non-outlier

362 ranges, and outliers are indicated with black dots. Solid brackets below boxplots denote statistically

363 significant mean differences between the lines within a speed, dotted brackets above boxplots indicate

364 statistically significant differences between speeds within lines (at the $p < 0.05$ level). For clarity,

365 differences between limbs within speed are not shown (see Table 4).

366 **Figure 3**: Mean gait sequence in Control (grey boxes) and Longshanks (thin black boxes). The solid boxes

367 represent the stance phases of each paw, the white boxes the swing phases. Total length, from 0 to 1,

368 represents a full stride cycle of the right fore paw, where 0 touch-down and 1 = touch-down of the

369 following cycle. The stance phase durations of the left paws are duplicated from the right side, as only

370   the latter were analyzed (see methods). Dashed boxes highlight the slightly longer stance time relative

371   to stride time in the hind limbs of Longshanks. Abbreviations: LH = left hind paw, LF = left forepaw, RF =

372   right forepaw, RH, right hind paw.

373   **Figure 4**: Diagram of the support triangle in Control (black) and Longshanks (red), obtained using mean

374   step length and the mean protraction, retraction and excursion angles from an unrelated sample of mice

375   from both groups (n = 5 each). Step length (base of the support triangle) and these angles were used to

376   obtain the lengths of sides of the support triangle in each group. Using the mean long bone lengths in

377   each group (Table 1), we then solved graphically for angles at the ankle and knee, on the assumption that

378   the tarso-metatarsus was approximately flat at touch-down, and perpendicular to the treadmill at toe-off

379   (Figure S1).

380   **Figure S1**: Sample video footage of a mouse running on a treadmill, showing the frame at the initiation

381   of stance for the right hind paw (A, touch-down), and the last frame before the initiation of the swing

382   phase for the same limb (B, toe-off). A stick model of the right hind limb is superimposed on each frame,

383   showing the approximate location of the hip, knee, ankle and metatarsophalangeal joints (white circles),

384   as well as the femur, tibia, tarso-metatarsus and forefoot (phalanges) (black lines). This model was used

385   to estimate the protraction angle at touch down (P), and the retraction angle at toe-off (R). The sum of

386   the protraction and retraction angles is the excursion angle.

387   **TABLE CAPTIONS**

388   **Table 1**: Morphometric Data: Body masses at the gait (TreadScan) and μCT scanning stages, and fore-

389   and hind limb bone lengths between Control and Longshanks mice, expressed as means (SEM).

390   Significance of the difference in means for all variables was determined using two-tailed t-tests. One

391   Longshanks body was not recovered from euthanasia prior to scanning (n=21).

392   **Table 2**: Standardized coefficients (betas, with standard errors) for each predictor variable in the linear

393   model for the gait variables in each limb. For Line, factor levels are Control = 0, Longshanks = 1, for

394   Speed, factor levels are 15 m/min = 0, 20 m/min = 1. Covariate-by-factor interaction terms were

395   excluded from the analyses (see Methods). The gait sequence data are shown as a fraction of the full

396   stride cycle for the right forepaw (from 0 to 1).

397   **Table 3**: Limb gait parameters at 15 and 20 m/min in Longshanks (LS, N=22) vs Control (C, N=23) mice.

398   Data reported as least squares means ± SEM, based on a full factorial linear model, with Line and Speed

399   as categorical factors, and body mass as a continuous predictor (see Table 2). Significance of differences

400   in pairwise comparisons of means were determined using post-hoc Tukey's HSD tests. Statistical

401   significance (p<0.05) of mean differences between lines within speed is indicated in bold, and between

402   speeds within line with an asterisk. (p<0.05).

403   **Table 4**: Comparison of fore- and hind limb gait parameters at 20 m/min in Longshanks (LS, N=22) vs

404   Control (C, N=23) mice. Data reported as least squares means ± SEM, based on a full factorial linear

405   model, with Line and Limb Type as categorical factors, and body mass as a continuous predictor.

406   Significance of differences in pairwise comparisons of means were determined using post-hoc Tukey's

407   HSD tests. Statistical significance (p<0.05) of mean differences between lines within limb type is

408   indicated in bold, and between limb types within line with an asterisk. (p<0.05).

409   **Table 5:** Comparison of gait sequences in Longshanks and Control. Data reported as means ± SEM, based

410   on a full factorial linear model, with Line and Speed as categorical factors, and body mass as a

411   continuous predictor. Means represent the proportion of a full stride cycle of the right forepaw (from 0

412   = stance initiation to 1 = stance initiation of the next cycle) at which the other paws initiate their stance

413   phases. No significant differences were found between lines within speed, or between speeds within

414   line (Tukey's HSD tests).

415   **Table S1:** Comparison of limb excursion angles during stance in Control and Longshanks mice. Data

416   reported as means ± SEM (in degrees), in a sample of 10 mice unrelated to the mice used in this study.

417   The angle values for each individual were based on 4-5 steps. No significant differences were found

418   between the lines in any of the angles (two-tailed t-tests, 0.13<p<0.83).

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501

Figure 1

Figure 1 - µCT scans of Longshanks and Control tibiae

µCT scans of two individuals from the study sample closest to mean raw tibia length in Control (C, 18.85mm) and Longshanks (LS, 21.45mm).

C

LS

5 cm
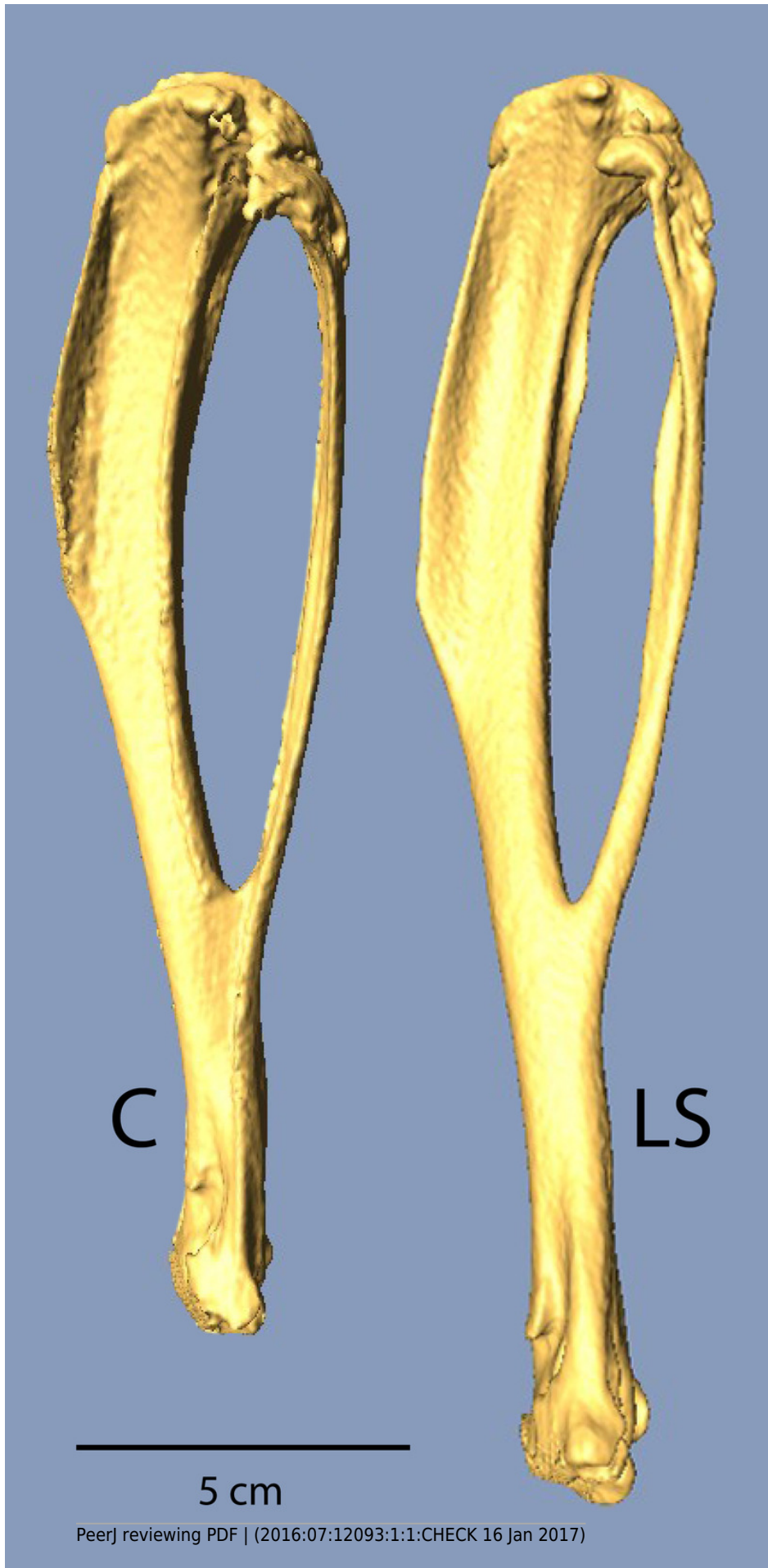
Figure 2

Figure 2 - Boxplots

Comparison of gait variables in Longshanks and Control mice at 15 and 20 m/min. Boxplots of swing duration (A, in milliseconds), stance duration (B, in milliseconds), stride length (C, in mm), and stride frequency (D, in seconds^{-1}) in Longshanks (shaded box) and Control mice (open box). Horizontal lines within boxes represent medians, boxes indicate interquartile ranges, whiskers indicate non-outlier ranges, and outliers are indicated with black dots. Solid brackets below boxplots denote statistically significant mean differences between the lines within a speed, dotted brackets above boxplots indicate statistically significant differences between speeds within lines (at the $p<0.05$ level). For clarity, differences between limbs within speed are not illustrated (see Table 4).

A. Swing Time (ms)

B. Stance Time (ms)

C. Stride Length (mm)

D. Stride Frequency (1/s)
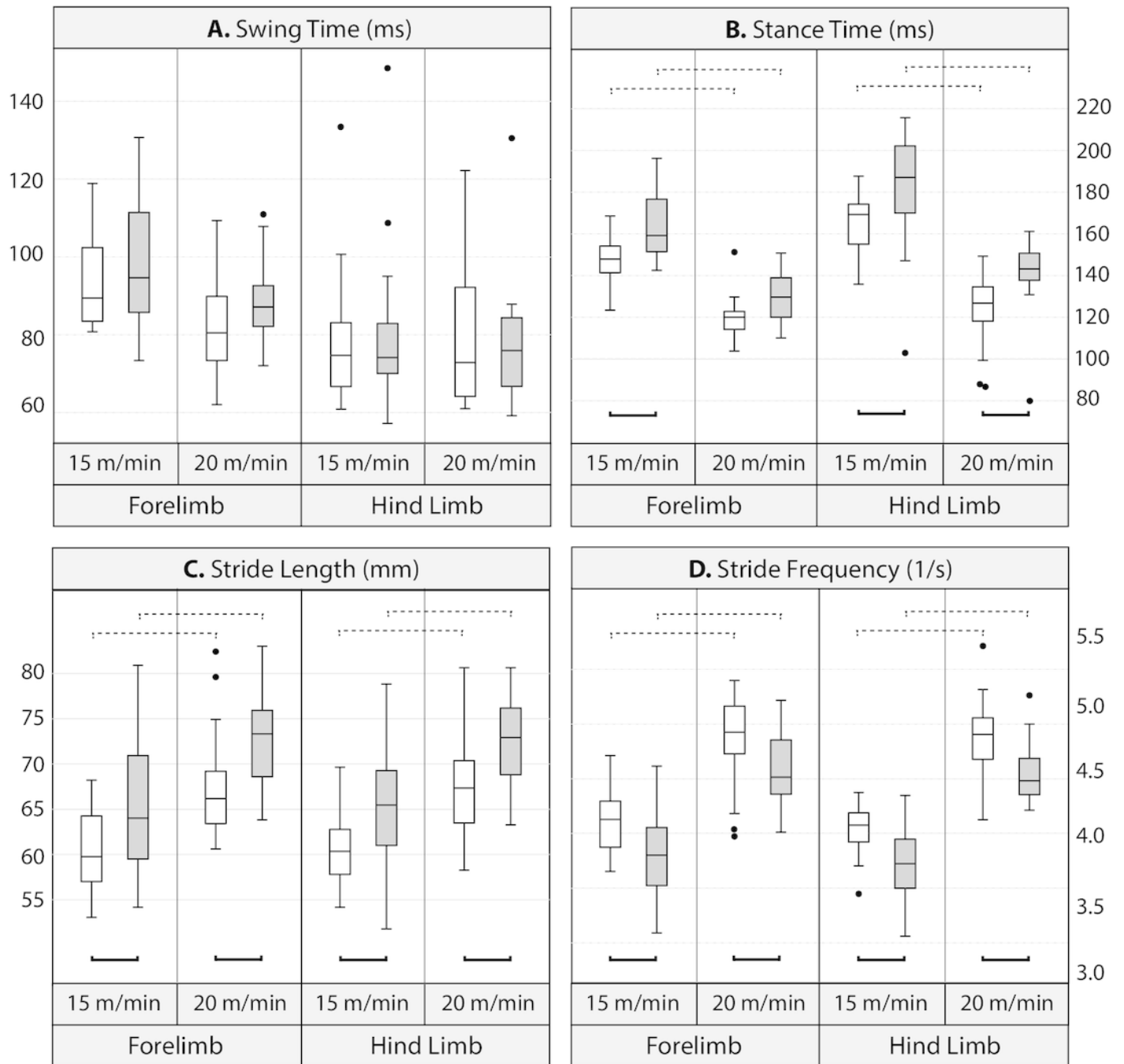
Figure 3

Figure 3 - Gait Sequence

Mean gait sequence in Control (grey boxes) and Longshanks (thin black boxes). The solid boxes represent the stance phases of each paw, the white boxes the swing phases. Total length, from 0 to 1, represents a full stride cycle of the right fore paw, where 0 touch-down and 1 = touch-down of the following cycle. The stance phase durations of the left paws are duplicated from the right side, as only the latter were analyzed (see methods). Dashed boxes highlight the slightly longer stance time relative to stride time in the hind limbs of Longshanks. Abbreviations: LH = left hind paw, LF = left forepaw, RF = right forepaw, RH, right hind paw
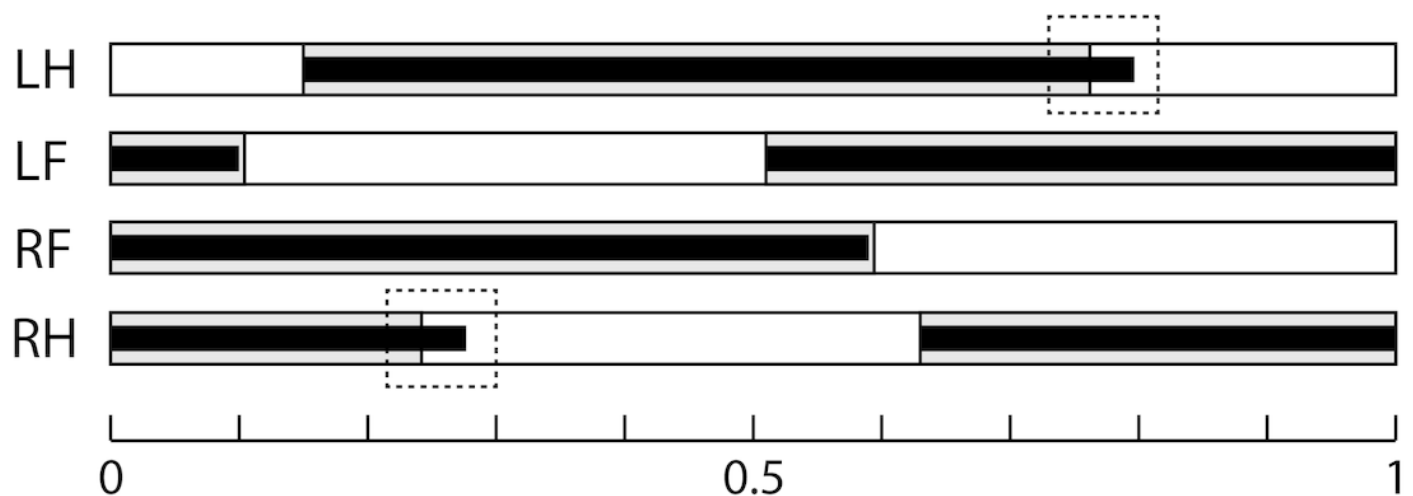
Figure 4

Figure 4 - Support Triangle

Diagram of the support triangle in Control (black) and Longshanks (red), obtained using mean step length and the mean protraction, retraction and excursion angles from an unrelated sample of mice from both groups (n = 5 each). Step length (base of the support triangle) and these angles were used to obtain the lengths of sides of the support triangle in each group. Using the mean long bone lengths in each group (Table 1), we then solved graphically for angles at the ankle and knee, on the assumption that the tarso-metatarsus was approximately flat at touch-down, and perpendicular to the treadmill at toe-off (Figure S1).
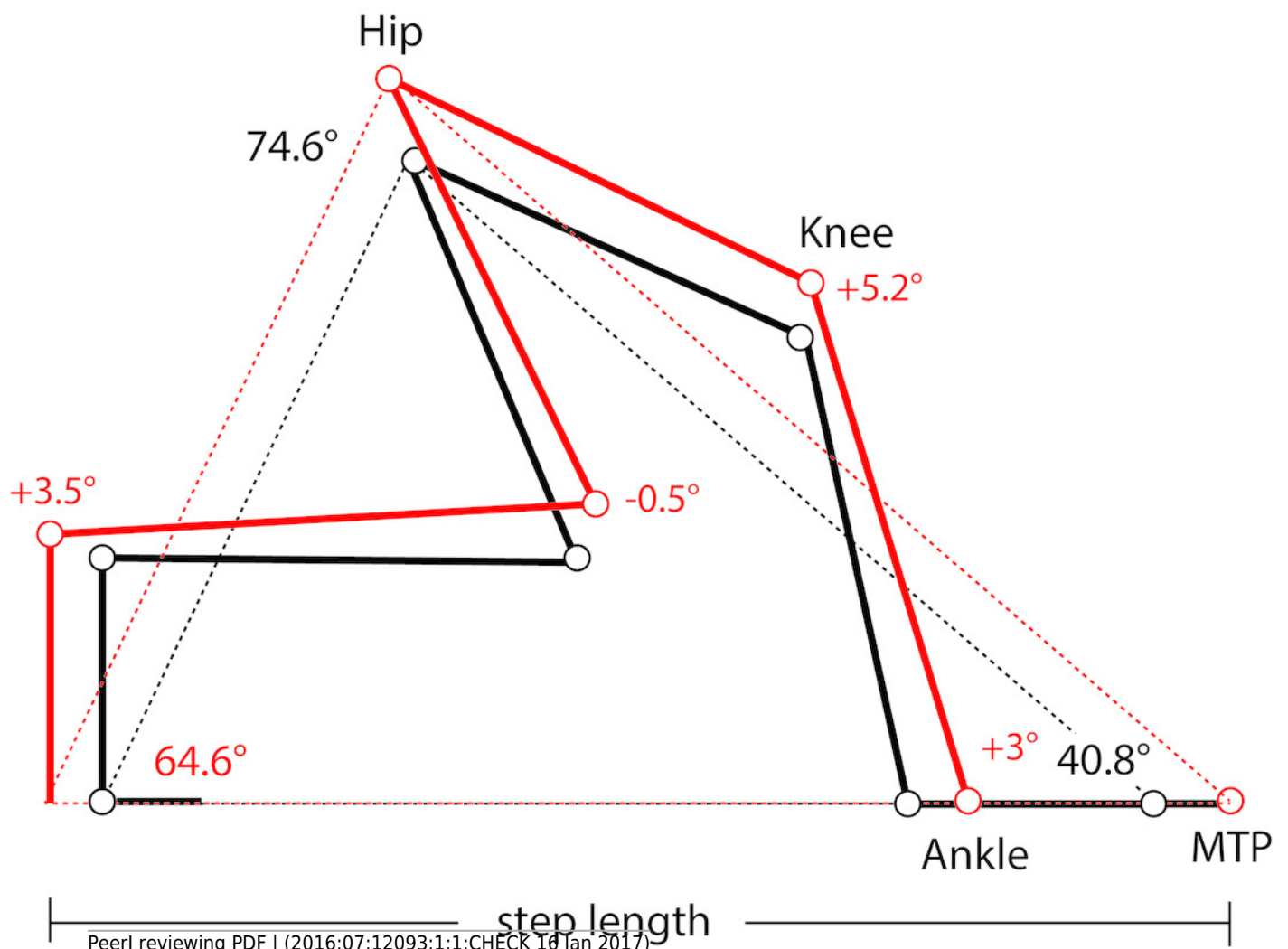
Table 1 (on next page)

Table 1

Morphometric Data: Body masses at the gait (TreadScan) and µCT scanning stages, and fore- and hind limb bone lengths between Control and Longshanks mice, expressed as means (SEM). Significance of the difference in means for all variables was determined using two-tailed t-tests. One Longshanks body was not recovered from euthanasia prior to scanning (n=21).

1   **Table 1**: Morphometric Data: Body masses at the gait (Treadscan) and µCT scanning stages, and fore-

2   and hind limb bone lengths between Control and Longshanks mice, expressed as means (SEM).

3   Significance of the difference in means for all variables was determined using two-tailed t-tests.. One

4   Longshanks body was not recovered from euthanasia prior to scanning (n=21).

	Longshanks (n=21)	Control (n=23)	Statistic
Body mass (gait trials) (g)	43.73 (1.31) (n=22)	42.18 (1.27)	t = 0.84, df = 43, p = 0.40
Body mass (µCT scan) (g)	45.81 (1.43)	44.55 (1.60)	t = 0.58, df = 42, p = 0.56
Scapula	13.83 (0.15)	12.97 (0.08)	t = 5.07, df = 42, p < 0.001
Humerus	13.65 (0.11)	12.17 (0.07)	t = 12.02, df = 42, p < 0.001
Ulna	17.02 (0.13)	15.36 (0.08)	t = 11.34, df = 42, p < 0.001
Carpo-metacarpus	4.43 (0.04)	4.26 (0.03)	t = 3.69, df = 42, p < 0.001
Manual proximal phalanx 3	2.14 (0.02)	2.06 (0.02)	t = 2.89, df = 42, p < 0.01
Femur	18.5 (0.25)	17.2 (0.17)	t = 4.39, df = 42, p < 0.001
Tibia	21.44 (0.33)	18.75 (0.18)	t = 7.37, df = 42, p < 0.001
Tarso-metatarsus	10.62 (0.17)	9.7 (0.10)	t = 4.79, df = 42, p < 0.001
Pedal proximal phalanx 3	3.35 (0.06)	3.06 (0.03)	t = 4.47, df = 42, p < 0.001
Forelimb	51.07 (0.38)	46.82 (0.20)	t = 10.11, df = 42, p < 0.001
Hind limb	53.91 (0.76)	48.71 (0.43)	t = 6.11, df = 42, p < 0.001

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Table 2(on next page)

Table 2

Standardized coefficients (betas, with standard errors) for each predictor variable in the linear model for the gait variables in each limb. For Line, factor levels are Control = 0, Longshanks = 1, for Speed, factor levels are 15 m/min = 0, 20 m/min = 1. Covariate-by-factor interaction terms were excluded from the analyses (see Methods). The gait sequence data are shown as a fraction of the full stride cycle for the right forepaw (from 0 to 1).

1   **Table 2**: Standardized coefficients (betas, with standard errors) for each predictor variable in the linear

2   model for each gait variable in each limb. For Line, factor levels are Control = 0, Longshanks = 1, for

3   Speed, factor levels are 15 m/min = 0, 20 m/min = 1. Covariate-by-factor interaction terms were

4   excluded from the analyses (see methods). The gait sequence data are shown as a fraction of the full

5   stride cycle for the right forepaw.

	Effect	Swing (ms)	Stance (ms)	Stride Length (mm)	Stride Freq. (1/s)
FORELIMB	Mass	0.102 (0.098)	0.006 (0.064)	0.068 (0.085)	-0.057 (0.068)
	Line	0.183 (0.098)	0.296 (0.064)**	0.362 (0.085)**	-0.293 (0.068)**
	Speed	-0.373 (0.098)**	-0.752 (0.063)**	0.503 (0.084)**	0.720 (0.067)**
	Line*Speed	0.051 (0.098)	-0.082 (0.063)	0.010 (0.084)	-0.034 (0.067)
HIND LIMB	Mass	-0.308 (0.104)*	0.2 (0.065)*	0.023 (0.084)	-0.030 (0.064)
	Line	0.033 (0.104)	0.27 (0.065)**	0.358 (0.084)**	-0.312 (0.064)**
	Speed	-0.021 (0.103)	-0.72 (0.065)**	0.522 (0.083)**	0.745 (0.064)**
	Line*Speed	-0.06 (0.103)	0.006 (0.065)	0.015 (0.083)	-0.002 (0.064)

6   * p <0.05, ** p<0.001

Table 3(on next page)

Table 3

Limb gait parameters at 15 and 20 m/min in Longshanks (LS, N=22) vs Control (C, N=23) mice. Data reported as least squares means ± SEM, based on a full factorial linear model, with Line and Speed as categorical factors, and body mass as a continuous predictor (see Table 2). Significance of differences in pairwise comparisons of means were determined using post-hoc Tukey's HSD tests. Statistical significance (p<0.05) of mean differences between lines within speed is indicated in bold, and between speeds within line with an asterisk. (p<0.05).

1   **Table 3**: Limb gait parameters at 15 and 20 m/min in Longshanks (LS, N=22) vs Control (C, N=23) mice.

2   Data reported as least squares means ± SEM, based on a full factorial linear model, with Line and Speed

3   as categorical factors, and body mass as a continuous predictor (see Table 2). Significant differences in

4   pairwise comparisons of means were determined using post-hoc Tukey's HSD tests. Statistical

5   significance (p<0.05) of mean differences between lines within speed are indicated in bold, and between

6   speeds within line with an asterisk. (p<0.05).

	Speed	Line	Swing (ms)	Stance (ms)	Stride Length (mm)	Stride Freq. (1/s)
FORELIMB	15m/min	C	94.07 (2.66)*	**147.91 (2.64)***	**60.50 (1.15)***	**4.15 (0.07)***
		LS	97.67 (2.72)*	**163.8 (2.7)***	**65.35 (1.18)***	**3.87 (0.07)***
	20m/min	C	82.39 (2.66)*	119.77 (2.64)*	**67.31 (1.15)***	**4.98 (0.07)***
		LS	88.83 (2.72)*	128.75 (2.7)*	**72.45 (1.18)***	**4.61 (0.07)***
HIND LIMB	15m/min	C	77.07 (3.48)	**166.42 (3.67)***	**60.87 (1.11)***	**4.12 (0.06)***
		LS	80.25 (3.56)	**181.57 (3.76)***	**65.44 (1.13)***	**3.78 (0.07)***
	20m/min	C	78.4 (3.48)	**124.73 (3.67)***	**67.63 (1.11)***	**4.95 (0.06)***
		LS	77.5 (3.56)	**140.55 (3.76)***	**72.61 (1.13)***	**4.60 (0.07)***

7

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Table 4(on next page)

Table 4

Comparison of fore- and hind limb gait parameters at 20 m/min in Longshanks (LS, N=22) vs Control (C, N=23) mice. Data reported as least squares means ± SEM, based on a full factorial linear model, with Line and Limb Type as categorical factors, and body mass as a continuous predictor. Significance of differences in pairwise comparisons of means were determined using post-hoc Tukey's HSD tests. Statistical significance (p<0.05) of mean differences between lines within limb type is indicated in bold, and between limb types within line with an asterisk. (p<0.05).

1 **Table 4:** Comparison of fore- and hind limb gait parameters at 20 m/min in Longshanks (LS, N=22) vs

2 Control (C, N=23) mice. Data reported as least squares means ± SEM, based on a full factorial linear

3 model, with Line and Limb Type as categorical factors, and body mass as a continuous predictor.

4 Significant differences in pairwise comparisons of means were determined using post-hoc Tukey's HSD

5 tests. Statistical significance (p<0.05) of mean differences between lines within limb type are indicated

6 in bold, and between limb types within line with an asterisk. (p<0.05).

Limb Type	Line	Swing (ms)	Stance (ms)	Stride (ms)	Stride Length (mm)	Stride Freq. (1/s)
FORE	C	82.04 (3)	120.12 (2.76)	**202.16 (3.32)**	**67.31 (1.11)**	**4.97 (0.07)**
	LS	89.2 (3.07)*	128.39 (2.82)*	**217.59 (3.39)**	**72.45 (1.13)**	**4.61 (0.07)**
HIND	C	78.88 (3)	**124.37 (2.76)**	**203.25 (3.32)**	**67.68 (1.11)**	**4.95 (0.07)**
	LS	77 (3.07)*	**140.93 (2.82)***	**217.92 (3.39)**	**72.56 (1.13)**	**4.61 (0.07)**

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Table 5 (on next page)

Table 5

Comparison of gait sequences in Longshanks and Control. Data reported as means ± SEM, based on a full factorial linear model, with Line and Speed as categorical factors, and body mass as a continuous predictor. Means represent the proportion of a full stride cycle of the right forepaw (from 0 = stance initiation to 1 = stance initiation of the next cycle) at which the other paws initiate their stance phases. No significant differences were found between lines within speed, or between speeds within line (Tukey's HSD tests).

1   **Table 5: Comparison of gait sequences in Longshanks and Control.** Data reported as means ± SEM,

2   based on a full factorial linear model, with Line and Speed as categorical factors, and body mass as a

3   continuous predictor. Means represent the proportion of a full stride cycle of the right forepaw (from 0

4   = stance initiation to 1 = stance initiation of the next cycle) at which the other paws initiate their stance

5   phases. No significant differences were found between lines within speed, nor between speeds within

Speed	Line	Ipsilateral (right) hind paw	Contralateral (left) forepaw	Contralateral (right) hind paw
15m/min	C	0.63 (0.01)	0.53 (0.01)	0.16 (0.01)
	LS	0.63 (0.01)	0.54 (0.01)	0.16 (0.01)
20m/min	C	0.63 (0.01)	0.51 (0.01)	0.15 (0.01)
	LS	0.63 (0.01)	0.51 (0.01)	0.14 (0.01)

6   line (Tukey's HSD tests).

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