

**Title**

Lines selected for different durations of tonic immobility have different leg lengths  
in the red flour beetle *Tribolium castaneum*

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## **Abstract**

Tonic immobility is an adaptive anti-predator behaviour observed in many species. This anti-predator behaviour is often correlated with a species' movement motivation, so a relationship between the duration of tonic immobility and morphological traits supporting movement would be expected. Using the red flour beetle *Tribolium castaneum*, we carried out two-way artificial selection for the duration of tonic immobility over more than 43 generations, establishing populations with longer (L) and shorter (S) tonic immobility durations compared to those of a non-selected control (C) population. Here, we investigated differences in walking motivation and leg length between the selected populations. Walking motivation was significantly higher in beetles from the S population than that in those from the L population. Moreover, S-population beetles of both sexes had significantly longer legs than those from L and C populations. The present results suggest the evolution of longer legs in response to selection pressure for a shorter duration of tonic immobility in *T. castaneum*.

## **Keyword**

anti-predator strategy, tonic immobility, artificial selection, leg, *Tribolium castaneum*

## Introduction

Predation exerts selective pressure on the traits of many prey species, often affecting the evolution of behavioural, morphological, physiological, and life-history traits in prey (Lima & Dill, 1990; Abrams, 2000; Caro, 2005). Many animals display tonic immobility (TI) as an adaptive anti-predator strategy (Edmunds, 1974; Miyatake et al., 2004; Humphreys & Ruxton, 2018). However, the duration of TI may vary greatly within a population, which suggests that TI imposes fitness costs in other situations. Because TI is a motionless posture, individuals who adopt TI decrease their investment in activity. In contrast, individuals who do not adopt TI often invest more energy in activity. That is, TI may be correlated with an individual animal's motivation for movement. For example, in the adzuki bean beetle *Callosobruchus chinensis*, individuals with longer TI durations showed significantly lower flight motivation than individuals with shorter TI durations (Ohno & Miyatake, 2007), and females with longer TI durations exhibited increased investment in life-history traits because they devoted more resources to reproduction than to activity (Nakayama & Miyatake, 2009). Moreover, in the red flour beetle *Tribolium castaneum*, individuals with higher genetically determined levels of walking motivation showed significantly shorter TI duration than individuals with lower genetically determined

levels of walking motivation (Matsumura et al., 2016). These previous studies suggest a negative correlation between resource allocation for TI and movement motivation, leading to a fitness trade-off between longer and shorter TI duration.

Movement is often affected by morphological traits (Weihmann & Blickhan, 2009; Cummings et al., 2018). For example, wing size is positively correlated with flight speed in birds (Moreno-Rueda, 2003). Moreover, in some cricket species, dispersers have larger wings than non-dispersers (e.g., Dingle, 2006; Roff & Fairbairn, 2007). In lizards, leg length is positively correlated with sprinting speed (Losos, 1990; Irschick & Losos, 1998). If anti-predator behaviour is correlated with movement motivation, morphological traits supporting insect movement may also be affected by investment in anti-predator behaviours. Some previous studies have reported correlations between anti-predator strategies and morphological traits. For example, lizards that adopt a running escape strategy have longer legs than those that adopt a hiding strategy (Bonine & Garland, 1999; Zamora-Camacho et al., 2014), suggesting correlations between anti-predator strategies and leg length at the phenotypic level in lizards. However, few studies have investigated the genetic basis of the relationships among anti-predator strategies, movement motivation, and morphological traits.

In this study, we investigated the relationships among anti-predator behaviour, movement motivation, and morphological traits supporting movement in *T. castaneum*. This species can fly when dispersing over a relatively long distance but tends to walk for short and medium dispersal distances and routine movements (Ridley et al. 2011; Drury et al. 2016). *Tribolium castaneum* often displays TI as an adaptive anti-predator behaviour when encountering the jumping spider *Hasarius adansoni* as a model predator (Miyatake et al., 2004). We previously carried out artificial selection for TI duration in *T. castaneum* and established populations with longer (L population) and shorter (S population) genetically determined TI durations (Miyatake et al., 2004; Matsumura & Miyatake, 2018). Beetles from the L population show significantly lower walking motivation than those in the S population (Miyatake et al., 2008; Matsumura et al., 2017). If the walking motivation of *T. castaneum* is affected by leg morphology, then leg length and/or width may differ between the L and S populations. In the present study, we compared walking motivation and leg morphology between the L and S populations of *T. castaneum*.

## **Materials & Methods**

### **(1) Insects and artificial selection**

The lines of *T. castaneum* used in this study have been maintained in the laboratory for more than 40 years. The beetles used in this study were reared on a mixture of whole wheat flour (Nisshin Seifun Group, Tokyo, Japan) and brewer's yeast (Asahi Beer, Tokyo, Japan) in a chamber (Sanyo, Tokyo, Japan) maintained at 25°C under a 16L:8D photoperiod (lights on at 7:00, off at 23:00). These beetles have been artificially selected for TI duration for more than 15 years (Matsumura & Miyatake, 2018). Details of the methods are described in Miyatake et al. (2004). In brief, 100 virgin males (14–21 days old) and 100 virgin females (14–21 days old) were chosen at random from the stock population (base population) and placed into individual wells of 48-well tissue culture plates with food. The next day, each beetle's TI duration was recorded following the method described below. The 10 males and 10 females with the longest TI durations were collected and propagated as the L population, while the 10 males and 10 females with the shortest TI durations were collected and propagated as the S population. The offspring of each population were reared in a chamber as described above. When the next generation of adults emerged, we measured the duration of TI and selected those with the longest and shortest TI durations in the same manner as before. We repeated this procedure for more than 43 generations (Matsumura & Miyatake, 2018). We simultaneously

created two replicates of each line in this selection experiment (LA and SA, LB and SB). We also used the base population from which the L and S populations were originally derived as a control (C) population. This C population has been maintained in the laboratory over the same period as the L and S populations without undergoing artificial selection for TI. Therefore, we were able to assume that the C population represents the ancestral state of the L and S populations. All trials in the present study were conducted between 12:00 and 17:00 in a room maintained at 25°C.

## **(2) Observation of TI**

Virgin beetles (40 males and 40 females; 21–35 days post-eclosion) were randomly collected from the L and S populations, approximately the 44<sup>th</sup>–47<sup>th</sup> generations, and were placed, with food, in individual wells with 48-well tissue culture plates (Falcon; Becton–Dickinson and Co., Franklin Lakes, NJ, USA). We also randomly selected 61 virgin males and 64 virgin females from the C population. To examine TI duration, an individual beetle was gently moved onto a small white China saucer (140-mm diameter, 15 mm deep). We induced TI by touching the beetle's abdomen with a wooden stick. A trial consisted of provoking TI and recording its duration with a

stopwatch (the duration of the behaviour was defined as the length of time between touching the beetle and detecting its first visible movement). If the beetle did not become immobile, the touch was repeated up to 3 times. If the beetle did not respond to this stimulus, its TI time was recorded as zero.

### **(3) Walking motivation**

To assess walking motivation, we measured walking distance using an image tracker system (Digimo, Tokyo, Japan; Matsumura & Miyatake, 2015). One day before measurements, virgin males (L population:  $N = 80$ ; S population:  $N = 91$ ; C population:  $N = 80$ ) and virgin females (L population:  $N = 95$ ; S population:  $N = 96$ ; C population:  $N = 69$ ; 21–35 days post-eclosion) were randomly collected from the 44<sup>th</sup>–47<sup>th</sup> generation of L and S populations and placed in one well of a 48-well tissue culture plate with food. The next day, each individual was gently placed on a piece of filter paper in a plastic petri dish (35 mm in diameter, 10 mm in height). Walking distance was recorded for 30 min using a CCD monochrome camera. Analysis software (2D-PTV Ver. 9.0, Digimo, Tokyo, Japan) was used to measure the walking distance of each individual from the recorded images.



#### **(4) Morphological traits**

Measurements of morphological traits were conducted using a dissecting microscope monitoring system (VM-60, Olympus, Japan). Adults from the 44<sup>th</sup>–47<sup>th</sup> generations of each population (L population:  $N = 83$ ; S population:  $N = 80$ ; C population:  $N = 123$ ) were frozen at  $-20^{\circ}\text{C}$ . Each leg (foreleg, middle leg, and hind leg) was detached from the right side of the beetle, and we measured femur width as well as the length of each segment (tarsus, tibia, and femur). Each leg was observed with a microscope (SZX-12, Olympus, Japan) including a camera (CS220, Olympus, Japan) with an adaptor (U-CMAD-2, Olympus, Japan), and the image was displayed on a video monitor (TM-I 50S, Victor, Japan). We measured the lengths and widths using a calibrated image. We also measured body length as an indicator of body size. Each length was measured as a straight-line distance.

#### **(5) Statistical analysis**

To compare the TI duration and walking distance between L and S populations, we used a generalized linear mixed model (GLMM) with a Gaussian distribution. In these analyses, population (i.e., L, S, and C) and sex (male and female) were used as fixed effects, with replicate lines (A and B) as a random effect. The duration of TI (+1

sec) was log transformed. For the analysis of body size (length and width), we used analysis of variance (ANOVA) with population and sex as fixed effects and replicate lines as a random effect. For the analysis of leg morphology, we used a multivariate analysis of variance (MANOVA). Analysis of covariance (ANCOVA) was used as a post hoc test for MANOVA. ANCOVA was conducted with population and sex as the fixed effects, replicate lines as a random effect, and body length as a covariate. We also used ANCOVA to analyse the total length of each segment (i.e., tarsus + tibia + femur). We conducted Tukey's HSD as a post hoc test when ANCOVA indicated significant effects. All analyses were conducted in JMP version 12.2.0 (SAS, 2015).

## **Results**

We were able to find the assumed effect of artificial selection on TI again in the present experiment (Fig. 1a). The assumption that our artificial selection protocol would affect beetle behaviour was met.

There was a significant effect of population on TI ( $\chi^2 = 522.38, p < 0.001$ ). Beetles from L populations showed longer TI durations than those from S and C populations (Fig. 1a). There were no significant effects of sex and no interaction between population and sex on TI duration (sex:  $\chi^2 = 0.05, p = 0.825$ ; population  $\times$  sex:  $\chi^2 =$

0.66,  $p = 0.720$ ). Beetles from the S population showed significantly walking longer distance than those from L and C populations ( $\chi^2 = 88.49$ ,  $p < 0.001$ ; Fig. 1b). There was a significant effect of sex on walking distance ( $\chi^2 = 5.39$ ,  $p = 0.020$ ). There was a significant effect of the interaction between population and sex on walking distance ( $\chi^2 = 21.38$ ,  $p < 0.001$ ).

There was a significant difference in body length between populations (Fig. S1, Table S2). MANOVA showed significant effects of population and sex on morphological traits (Table 1). Post hoc ANCOVA with body length as a covariate showed significant effects of population on the length of the tarsus and tibia of the middle leg and the length of the tibia of the hind leg (Table S1). Significant effects of sex were shown in the tarsi of all legs (Table S1). The results of ANCOVA showed that the total leg length of the S population was significantly longer than that of the L population for all legs (Fig. 2, Table 2). There was no significant difference in leg length between the L and C populations (Fig. 2). Males had significantly longer forelegs and middle legs than females (Fig. 2, Table 2).

## **Discussion**

In the present study, *T. castaneum* beetles from populations selected for shorter TI

durations displayed higher walking motivation than beetles from populations with longer genetically determined TI durations. This result is consistent with the results of previous studies (Miyatake et al., 2008; Matsumura et al., 2017), suggesting a genetic relationship between anti-predator behaviour and movement motivation. We found that beetles from the S population had longer legs than those from the L population, providing support for our hypothesis. Of particular interest, the S population was more different from the C population than the L population (Figure 2). This result shows that leg length had responded to selection pressure for shorter TI durations. These results suggest that *T. castaneum* beetles with less investment in anti-predator behaviour showed increased movement motivation and longer leg length.

Some previous studies have reported correlations between anti-predator strategies and morphological traits. For example, lizards that adopt a running escape strategy have longer legs than those adopting a hiding strategy (Bonine & Garland, 1999; Zamora-Camacho et al., 2014). This phenomenon strongly depends on environmental conditions; lizards in environments with many hiding places evolved short legs, whereas leg length was increased in lizards living in environments with few hiding places (Losos, 1990; Irschick & Losos, 1998). Moreover, birds with

greater flight ability have larger wings, increasing their success in avoiding predators (Moreno-Rueda, 2003). Thus, predation pressure may affect the evolution of wing morphology in birds (Møller & Nielsen, 1997). Although the coevolutionary relationships of anti-predator strategies and morphological traits have been considered in some previous studies, few have observed the response of morphological traits to selection pressure for anti-predator traits over many generations. To our knowledge, the present study is the first report on the response of leg length to artificial selection for anti-predator behaviour. In particular, it is important to note that leg length was correlated to selection for a shorter duration of death-feigning. That is, it is suggested that leg length is sensitive to the effects of mobility rather than predation pressure in *T. castaneum*.

*T. castaneum* can fly over longer distances and can often walk for short to intermediate distances. A previous study revealed that walking motivation was positively correlated with leg length in this species (Arnold et al., 2017). Thus, we expected that individuals with higher walking motivation that also adopt an escape strategy to avoid predation would have longer legs. The results of the present study reveal that beetles from the S population have longer legs than those from the L population, which supports this hypothesis.

In lizards, leg length is positively correlated with sprinting speed. Thus, individuals who adopt a running escape strategy are thought to have evolved longer legs (Losos, 1990; Irschick & Losos, 1998). In the present study, we did not reveal why *T. castaneum* individuals from the S population had longer legs than those of the L population. Moreover, rates of change in leg length (compared with those of the C population) differed among front (L: 0.50%, S: 2.70%), middle (L: -0.68%, S: 3.52%), and hind legs (L: -0.76%, S: 2.08%). Although the length of the middle leg in the S population showed the largest degree of change, we did not discover the factors underlying this finding. Additional research is needed to examine the mechanisms of the relationship between leg morphology and walking motivation in *T. castaneum*.

In the results of the width of the hind leg, we could not find a significant difference between populations. This result suggested that selection pressure on death-feigning behaviour did not affect leg width. In the house mouse *Mus domesticus*, artificial selection for higher levels of wheel-running activity resulted in a wider distal femur (Castro & Garland Jr. 2018). Therefore, there are differences in the effects of leg width on mobility among species, and the present study suggests that leg widths may not be important for walking mobility in *T. castaneum*.

We found significant effects of sex on leg length, with males having longer tarsi

than females (Table S1). This sex difference in the length of the tarsus suggests some degree of sexual selection. During copulation in *T. castaneum*, males mount females' backs and rapidly rub their legs against the sides of the females' bodies (Fedina & Lewis, 2008). Previous studies have reported a positive relationship between the intensity of leg-rubbing behaviour and paternity success in *T. castaneum* (Edvardsson & Arnqvist, 2000, 2005). The results of the present study suggest that tarsal length is important for this male rubbing behaviour. Future research should investigate the effects of tarsal length on rubbing behaviour in male *T. castaneum*. However, there were sex differences in walking distance only in the C population (no artificial selection) (Fig. 1b). Sexual differences in the walking distance of *T. castaneum* have already been reported in a previous study (Matsumura et al., 2016). Therefore, if individuals with shorter legs (especially tarsal) show greater walking ability in *T. castaneum*, shorter leg lengths may be shown in females than males.

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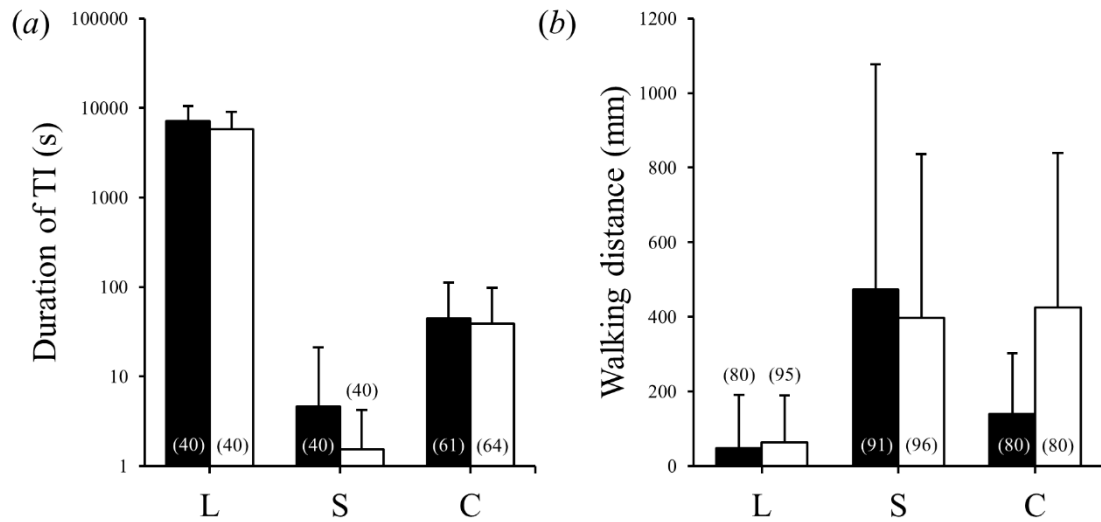
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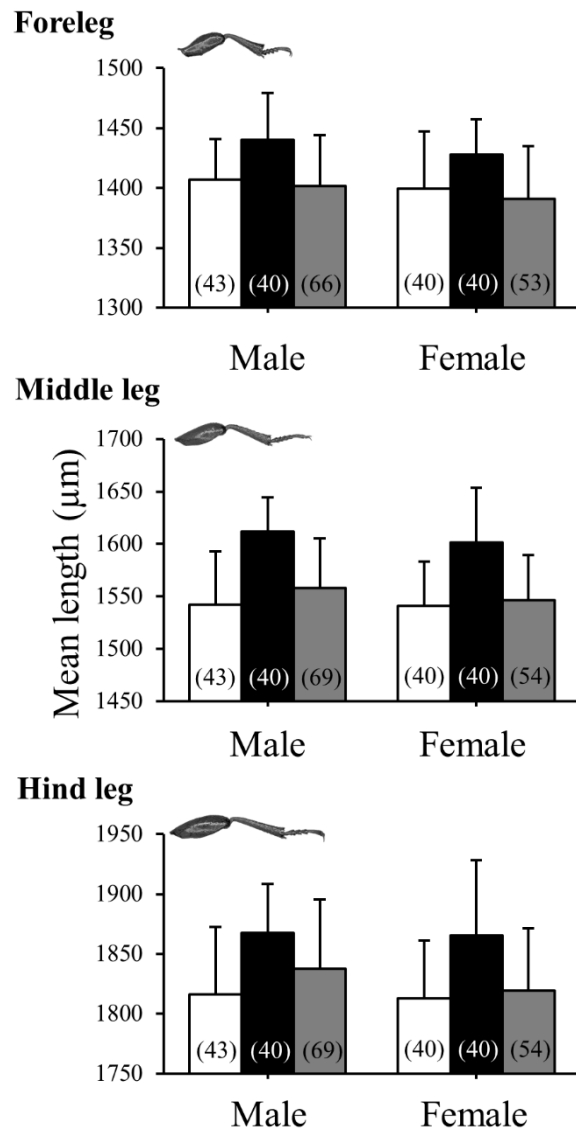
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## Figures and Tables



**Figure 1.** Duration of TI (a) and walking distance (b) of the beetles from L, S and C populations. Black and white bars show males and females, respectively. Error bars show standard deviation. Numbers in parentheses indicate the sample size.



**Figure 2.** Length of each leg of *T. castaneum* from L (white), S (black), and C (gray) populations. Error bars show standard deviation. Numbers in parentheses indicate the sample size.

**Table 1.** Result of MANOVA for each segment (tarsus, tibia, and femur) of each leg (foreleg, middle leg, and hind leg) and body size.

Factor	Test	Value	Num df	<i>p</i>
Population	Wilks' lambda	0.147	28	< 0.0001
Sex	<i>F</i>	0.415	14	< 0.0001
Population × Sex	Wilks' lambda	0.906	28	0.5417

**Table 2.** Results of ANCOVA for the differences in leg length between *T. castaneum* from the L, S, and C populations.

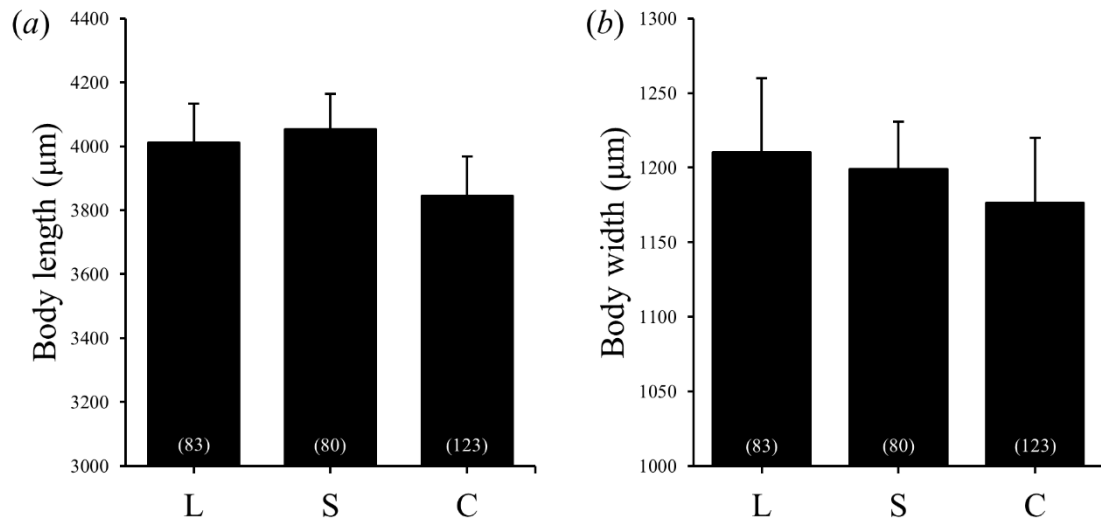
Trait	Factor	df	<i>F</i>	<i>p</i>
Foreleg length	Population	2	8.12	0.032
	Sex	1	8.35	0.004
	Population × Sex	2	0.19	0.828
	Body size	1	310.72	< 0.001
	Error	276		
Foreleg width	Population	2	6.45	0.053
	Sex	1	0.83	0.365
	Population × Sex	2	0.91	0.404
	Body size	1	152.75	< 0.001
	Error	276		
Middle leg length	Population	2	13.40	0.014
	Sex	1	3.96	0.048
	Population × Sex	2	0.71	0.493
	Body size	1	280.17	< 0.001
	Error	280		



Middle leg width	Population	2	0.10	0.903
	Sex	1	0.86	0.353
	Population × Sex	2	0.75	0.472
	Body size	1	0.07	0.787
	Error	280		
Hind leg length	Population	2	7.67	0.036
	Sex	1	3.19	0.075
	Population × Sex	2	1.71	0.182
	Body size	1	339.65	< 0.001
	Error	280		
Hind leg width	Population	2	2.68	0.181
	Sex	1	3.19	0.075
	Population × Sex	2	1.88	0.154
	Body size	1	156.20	< 0.001
	Error	280		

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## Supplemental data



**Figure S1.** Body length (a) and body width (b) of each population. Error bars show standard deviation. Numbers in parentheses indicate the sample size.

**Table S1.** Results of post-hoc ANCOVA for MANOVA.

Leg	Segment	Factor	df	<i>F</i>	<i>p</i>
Front	Tarsus	Population	2	5.31	0.064
		Sex	1	26.35	< 0.001
		Population × Sex	2	0.40	0.673
		Body size	1	29.54	< 0.001
		Error	276		
	Tibia	Population	2	4.70	0.086
		Sex	1	1.82	0.179
		Population × Sex	2	0.27	0.765
		Body size	1	213.17	< 0.001
		Error	276		
	Femur length	Population	2	1.21	0.374
		Sex	1	2.80	0.095
		Population × Sex	2	2.09	0.126
		Body size	1	223.57	< 0.001
		Error	276		
Femur width	Population	2	6.45	0.053	

		Sex	1	0.83	0.365
		Population × Sex	2	0.91	0.404
		Body size	1	152.75	< 0.001
		Error	276		
Middle	Tarsus	Population	2	11.31	0.017
		Sex	1	21.21	< 0.001
		Population × Sex	2	1.77	0.172
		Body size	1	37.83	< 0.001
		Error	280		
	Tibia	Population	2	10.08	0.024
		Sex	1	4.14	0.043
		Population × Sex	2	2.40	0.093
		Body size	1	167.90	< 0.001
		Error	280		
Femur length	Population	2	2.12	0.230	
	Sex	1	2.06	0.153	
	Population × Sex	2	1.52	0.220	
	Body size	1	263.34	< 0.001	

		Error	280		
	Femur width	Population	2	0.10	0.903
		Sex	1	0.86	0.353
		Population × Sex	2	0.75	0.472
		Body size	1	0.07	0.787
		Error	280		
Hind	Tarsus	Population	2	5.30	0.062
		Sex	1	10.49	0.001
		Population × Sex	2	0.15	0.859
		Body size	1	50.54	< 0.001
		Error	280		
	Tibia	Population	2	9.52	0.027
		Sex	1	0.53	0.466
		Population × Sex	2	0.93	0.397
		Body size	1	218.37	< 0.001
		Error	280		
	Femur length	Population	2	0.49	0.642
		Sex	1	1.48	0.225

	Population × Sex	2	3.52	0.031
	Body size	1	314.68	< 0.001
	Error	280		
Femur width	Population	2	2.68	0.181
	Sex	1	3.19	0.075
	Population × Sex	2	1.88	0.154
	Body size	1	156.20	< 0.001
	Error	280		

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**Table S2.** Mean values ( $\mu\text{m}$ ) with standard deviation (SD) of body length and body width.

Trait	Population	Male		Female	
		Mean	SD	Mean	SD
Body length	L	3985.35	98.96	4039.65	135.61
	S	4019.10	119.40	4087.20	89.35
	C	3816.13	126.98	3882.24	109.50
Body width	L	1197.42	49.45	1224.13	46.19
	S	1192.93	34.30	1205.20	27.79
	C	1171.03	44.25	1184.04	41.25