Evaluation of Body Size Indicators for Morphological Analyses in Two Sister Species of Genus *Dorcus* (Coleoptera, Lucanidae)

Itsuiki Ohtsu,1 Yasuhiko Chikami,2,3 Taichi Umino,3 and Hiroki Gotoh1,4,5

1Department of Science, Graduate School of Integrated Science and Technology, Shizuoka University, Suruga-ku, Shizuoka, 422-8529, Japan, 2Division of Evolutionary Developmental Biology, National Institute for Basic Biology, Okazaki, Aichi, 444-8585, Japan, 3Sugadaira Research Station, Mountain Science Center, University of Tsukuba, Ueda, Nagano, 386-2204, Japan, 4Department of Biological Science, Faculty of Science, Shizuoka University, Suruga-ku, Shizuoka, 422-8529, Japan, and 5 Corresponding author, e-mail: goto.hiroki@shizuoka.ac.jp

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Abstract

The relationship between trait and body size, i.e., the scaling relationship or static allometry, is an essential concept for investigating trait size. However, usage of an inappropriate body size indicator can lead to misinterpretation of morphology. In this study, we examined several possible body size indicators in two closely related stag beetle species, *Dorcus rectus* and *Dorcus amamianus*. We raised animals in captivity and used pupal weight as a measure of true, or overall body size, and then evaluated six adult morphological traits to test whether these traits could be reliably used as body size indicators in static scaling relationship comparisons. We analyzed two comparisons, between sexes in same species and between species in same sex. We showed that the most appropriate body size indicators differ depending on the comparisons. Our results indicated that the scaling relationship of focal traits could be over- or under-estimated depending on which body size indicators are used.

Key words: body size, stag beetle, morphometry, static allometry, weaponed beetle

Many insect species possess ‘weapon’ traits to combat among individuals over resources such as food and mating partners (Emlen 2008, Rico-Guevara and Hurme 2019). The weapon traits are often used in male–male competitions and show striking sexual dimorphism due to sexual selection (Andersson 1994, Kawano 2006, Zinna et al. 2018). Coleoptera includes many species with male weapons, including rhinoceros (Dynastinae) and dung beetles (Scarabaeinae) with various shapes of large horns (Eberhard 1982, Moczek 2005, Emlen et al. 2007, McCullough et al. 2015, Moczek 2005) stag beetles (Lucanidae) with enlarged mandibles (Kawano 2006, Gotoh et al. 2014), long-armed scarabs (Euchirinae) with long forelegs (Kawano 2004) and the enlarged hindlegs of frog-legged beetles (O’Brien et al. 2017). These exaggerated beetle traits have long-time fascinated biologists, inspiring more than a century of evolutionary and morphological studies (Darwin 1871, Inukai 1924, Huxley 1931, Arrow 1951; Eberhard 1979, 1982). More recently, developmental genetics has examined the mechanisms of weapon growth in several beetle groups (Moczek and Rose 2009, Emlen et al. 2012, Kijimoto et al. 2012, Gotoh et al. 2017, Ohde et al. 2018, Okada et al. 2019). It is well known that the expression of weapon traits is highly condition-dependent (Emlen 1994, Moczek 1998, Gotoh et al. 2011, Okada et al. 2019). In most cases, males that grow large under good nutritional conditions express the largest weapon traits. All of these studies apply the methodology of scaling relationships or static allometry, i.e., two-dimensional scatter plots with the x-axis as body size and the y-axis the focal trait size, to compare the size of weapon traits across populations or species (Huxley and Teissier 1936, Stern and Emlen 1999, Kawano 2000, Moczek 2003). For scaling relationships to accurately capture relative trait size, it is critical that these studies use an appropriate metric for body size.

Measurement of overall body size is difficult in insects despite its significance for accurately comparing traits among species or sexes. Adult body weight is a poor indicator of body size because it is confounded by fluctuations in the feeding history and body condition of the animals and is often unavailable when studies are conducted on dried museum specimens. Pupal weight would be an ideal measure of overall body size because it can be sampled across individuals at the time of metamorphosis when animals are not feeding. However, this trait is generally impossible or impractical to collect in most cases. Hence, biologists typically estimate body size by measuring a simple...
body part instead of overall body size using these traits. For example, prothorax width is often used as an indicator of body size in insects (Green 1999), including weaponed beetles (Emlen 1994, Gotoh et al. 2012, McCullough et al. 2015). The validity of using prothorax width as a body size indicator has been examined for some species, e.g., after using principle components analyses of a number of different body size traits (See Emlen 1997, McCullough et al. 2015). But most of the time, prothorax width is simply used because it is easy to measure and well established in the literature. However, for some beetles and weapons, other metric traits besides prothorax width may be more appropriate. If the relative value of prothorax width to body size varies among the groups being compared (e.g., between sexes, populations, species, or experimental groups), then using prothorax width as a body size indicator may lead to erroneous morphological conclusions (Fig. 1A and B). Indeed, it is known that the conclusions of morphological studies can vary depending on which trait size is used as a body size indicator (Nava-Bolaños et al. 2014). However, which adult traits are an appropriate indicator of body size has not been verified for any species of stag beetle.

In this study, we used two species of stag beetles, *Dorcus rectus* (Motschulsky 1857) and *Dorcus amamianus* (Nomura 1964), to evaluate which adult morphological traits are the best indicators of body size. We raised these two species in captivity and weighted their pupae to provide a robust estimate of the overall body size of each individual. We then used this to compare the efficiency of several linear morphological measurements collected on these same individuals as adults, to test which would be the most effective trait to use as an indicator of body size for subsequent scaling analyses.

We defined two conditions for adult traits as a good body size indicator. First, the indicator trait should be isometrically correlated with overall body size, i.e., pupal weight, across the full range of body sizes. That is, traits with disproportionally larger (hyper allometric) or smaller (hypo allometric) sizes in the largest individuals were unsuitable as body size indicators. Second, the relationship of indicator trait size against pupal weight should not change among the groups. Such traits will meet those three criteria, that is, 1) strongly correlates with pupal weight, 2) regression model is not affected by sex or species, and 3) regression model does not show significant interaction effect against pupal weight between comparing groups.

We examined the appropriate body size indicators when comparing traits between sexes in the same species. In addition, to determine if these body size indicators can be used across species, we compared measures from the same sex between species. Our results showed that the appropriate body size indicators differed for each comparison. This result suggests that evaluation of body size indicators is essential when comparing morphology among different groups, such as between sexes and between species.

### Materials and Methods

**Measuring ‘Reliable’ Body Size**

We used pupal weight as reliable measure of overall body size because 1) it is constant compared to the dry weight of adults, which may increase or decrease depending on feeding and egg-laying conditions (Le Roux et al. 2008), 2) it can be used regardless of morphological differences between species or sexes, 3) it can be used...
even when experimental treatments affect specific morphological traits, such as with inhibition of morphogenetic genes and hormonal treatments. In fact, pupal weight has been used as a body size indicator when analyzing morphogenetic genes via gene knockdown in various coleopteran groups (Moczek and Rose 2009, Gotoh et al. 2014, 2017; Okada et al. 2019).

Model Species
Two closely related species of stag beetles Dorcus rectus and Dorcus amamianus were used. Adult D. rectus were sampled from Shizuoka prefecture, Japan, in the summers of 2020 and 2021. Adults of D. amamianus were purchased in 2020 from Mushikichi (Fukuoka, Japan). For both species, eggs and larvae were obtained in laboratory. The laboratory colonies were started from more than twenty adult pairs (D. rectus) and four adult pairs (D. amamianus), respectively. Third instar larvae were transferred from the breeding log to a Kinshi-cup (Dorcus hyper, Fujikon, Japan) and reared until pupation. The Kinshi-cup size was 430 or 200 ml for males and 200 ml for females. By using different size of cup (male larvae were randomly assigned to 200 or 430 ml cup), we were able to obtain small to large size males. The rearing temperature was 25°C ± 3°C. Pupal weights were measured after larvae pupated, and adults were frozen and fixed after eclosion. Pupal weights were almost constant during the first 14 days of pupal stage (less than 1% variation in pupal weight in every preliminary weighed individual, N = 4, two D. rectus male, one D. rectus female, and one D. amamianus female). We measured pupae within ten days from pupation using a digital balance (400-TST008, Sanwa Supply, Okayama Japan) to 0.01 g. Morphological traits were measured using a digital caliper (Shinwa Rules, Niigata, Japan) to 0.01 mm. Six morphological traits were measured: head width, prothorax width, prothorax length, elytra width, elytra length, and foreleg tibia length. Sample sizes were as follows: D. rectus (Female; N = 14, Male; N = 14), D. amamianus (Female; N = 13, Male; N = 11).

Intraspecific Comparison Between Sexes
First, for each species, comparisons between the sexes were performed. We used general linear models (GLMs) to detect effects of sex and pupal weight on adult morphological traits. In this study, we employed models with pupal weight (PW), sex (sex), and their interaction (PW × sex) as explanatory variables, with adult traits as the objective variables. Then, gaussian distributions and gamma distributions were set for the error structure, and identity or logarithm and inverse or logarithm for the link function, respectively. To investigate the best fitting error distribution and link function, we used Akaike information criteria (AIC) and selected the models with lowest AIC for further analyses (Supp Table S1 [online only]). Subsequent likelihood tests were performed using ANOVA function of ‘car’ package on the R (Version 4.2.1). Except head width comparison between sexes in D. amamianus, linear regression model was selected as best fit model. In that case, if the effect of sex is significant, then the intercept of the regression line is different; if the effect of the PW × sex interaction is significant, then the slope of the regression line is different. Significant level is P < 0.05. In head width comparison between sexes in D. amamianus, logarithm model was selected. In this case, if the effect of sex is significant, then the intercept of the log-transformed regression line is different; if the effect of the PW × sex interaction is significant, then the slope of the log-transformed regression line is different (significance level is P < 0.05).

Interspecific Comparison Between the Same Sexes
We compared the four trait values between D. rectus and D. amamianus. We did not evaluate head width and foreleg tibia length, which showed a large sexual dimorphism in both species. Comparisons between species were made for both sexes. As linear regression model was selected as best fit model in all of comparisons (Supp Table S1 [online only]), statistical tests for differences in the scaling relationship between species were performed as described in the previous section.

Results and Discussion
Intraspecific Comparison Between Sexes
In D. rectus, all six of the putative indicator traits of body size were highly correlated with overall body size, i.e., pupal weight, in both sexes (Fig. 2A). In particular, prothorax width, prothorax length, and elytra width showed coefficients of determination, R², greater than 0.93 in both sexes (Fig. 2A). This result suggests that these three traits highly correlate with overall body size. Also, the individual variations within same-size individuals and measurement errors are minor.

When comparing the scaling relationship between males and females, there were apparent differences in head width and foreleg tibia length (Fig. 2A, Supp Table S2 [online only]). In both traits, the regression of the scaling relationship was significantly different between sexes, and males had completely larger trait sizes than females.

In addition to these two traits, prothorax length, elytra length, and elytra width also showed significant differences in regression slopes between sexes. Although the differences between regressions were relatively small, these three traits also showed completely larger trait sizes in females than males (Fig. 2A, Supp Table S2 [online only]). In contrast, in prothorax width, the interaction between pupal weight and sex, indicating the slope of the regression lines, was not significantly different between sexes. However, there was a significant effect of sex on the prothorax width (Fig. 2A, Supp Table S2 [online only]), indicating that the intercept of regression lines is different between the sexes.

The same trend was observed in the sister species D. amamianus (Fig. 2B). That is, males had larger traits size in head width and foreleg tibia length (Fig. 2B). Prothorax length, elytra length, and elytra width showed significantly larger trait sizes in females than males in the observed body size range (Fig. 2B). The effect of the interaction of pupal weight and sex on these traits was not significantly different for prothorax width, indicating that the slope of the regression lines is not different between sexes. In contrast, the significant effect of sex on prothorax width cannot be detected, demonstrating that the intercepts of the regression lines were distinct between sexes (Fig. 2B, Supp Table S2 [online only]).

In many studies on insect species, head width is a commonly used cue for instar identification (Nijnbout 1981, Daly 1985), and tibia length is a trait used as a body size indicator (Green and Extavour 2014). However, these traits are rarely used as a body size indicator in stag beetles because they are known to be sexually dimorphic, consistent with our results. In particular, as the head contains the muscles controlling mandibular movements, sexual dimorphism is particularly large (Goyens et al. 2014). Such traits with large sexual differences are not suitable as body size indicators, especially in comparisons between sexes.

Although there were statistically significant differences in prothorax width, prothorax length, elytra width, and elytra length between sexes, the differences were relatively smaller than those
observed in head width or foreleg tibia length. Prothorax width and elytra length are often used as body size indicators in stag beetles (Gotoh et al. 2012) and other weaponed beetles (McCullough et al. 2015). Considering the small difference between sexes, those four traits can be used as a body size indicator in both *D. rectus* and *D. amamianus*. On the other hand, it should be noted that, although the differences are small, the prothorax width of females is smaller than that of males, and the prothorax length, elytra width, and elytra length of females is larger than that of males in same-size individuals, in both species. When comparing specific traits between males and females, the use of prothorax width as a body size indicator may lead to an overestimate of female trait size, while the use of the prothorax length, elytra width, and elytra length may cause an overestimate of male trait size.

**Interspecific Comparison Between the Same Sexes**

Body size indicators were also evaluated for comparison between species (Fig. 3). Then, we cannot detect significant effects of species and interaction of species and pupal weight on prothorax width, prothorax length, and elytra width in males, indicating that there are no differences in the slope or intercept of the regression line between the two species in males (Fig. 3A, Supp Table S2 [online only]). Also, female prothorax width between the two species was not significantly affected by species and the interaction of species and pupal weight (Fig. 3B, Supp Table S2 [online only]). On the other hand, despite the close relationship between the two species, the elytra length was significantly larger in *D. amamianus* than in *D. rectus* in both sexes. These results suggest that prothorax width is a suitable body size indicator for comparison between the two species and if elytra width is used as a body size indicator, all focal trait sizes may be overestimated in *D. rectus* and underestimated in *D. amamianus*.

**Conclusion**

In this study, we examined appropriate body size indicators in two species of stag beetles. We propose that pupal weight is an ideal trait that most accurately reflects overall body size. However, pupal weight is a difficult trait to measure depending on the study design. It is impossible to know the pupal weight from adult insects in the field. Even in laboratory studies, it is also difficult to measure pupal weight, because some beetle species show high mortality when the pupal cell or cocoon, is broken in order to collect measurements (Kikumoto 2013). Pupal weight can be used as a body size indicator only in limited situations. Therefore, investigating versatile
indicators of body size has been required for accurately evaluating the scale relationship in stag beetles.

In this study, we confirmed that prothorax width is a better trait as body size indicators, at least for the two species used in this study. Our data also clearly indicated that the scaling relationship of focal traits could be over- or underestimated depending on traits used as the body size indicator. Since this problem occurs even in the two closely related sister species shown in this study, it should be noted that the results and interpretation of scaling relationship comparisons between more distantly related species may vary greatly depending on the body size indicators used.

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Authors’ Contributions

IO: Data curation, Investigation, Formal analysis, Resources, Writing – review & editing. YC: Formal analysis, Writing – review & editing. UT: Formal analysis, Writing – review & editing. HG: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supplementary Data

Supplementary data are available at Journal of Insect Science online.

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