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Corresponding author: E. Hasegawa

Laboratory of Animal Ecology,
Department of Ecology and Systematics,
Graduate School of Agriculture,
Hokkaido University, Sapporo
060-8589, Japan.
Phone: +81-11-706-3690
Fax: +81-11-706-2494
E-mail: ehase@res.agr.hokudai.ac.jp

Running head:
Ito et al.: Morph-specific morphological correlations with weapons

# Morph-specific weapon-correlated traits in a male dimorphic stag beetle Prosopocoilus inclinatus, (Coleoptera: Lucanidae)

J. Ito, Y. Ohkubo and E. Hasegawa

Laboratory of Animal Ecology, Department of Ecology and Systematics, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

## Abstract

Several male beetles that fight with rival males show dimorphic weapons. Major males have large weapons and fight aggressively with rival males. Minor males have small or no weapons and often adopt an alternative reproductive tactic without fighting. Suitable body shapes are likely to differ depending on the tactic. Thus, the set of body parts that are compatible with distinct weapons may be different in major and minor males according to their battle tactics. Many studies have reported correlations between weapons and some morphological traits, but few studies have shown the difference in the correlation patterns between major and minor males. Here, we show that in a male dimorphic stag beetle, *Prosopocoilus inclinatus,* several morph-specific traits are correlated with weapon size. Mandible size correlates positively with eye size in major males only but correlates positively with the length of forelegs and negatively with abdomen weight in minor males only. These results suggest that the correlated trait sets are different between the morphs because each morph adopts different battle tactics and mating strategies.

**Key Words:** weapon dimorphism, morph-specific correlated evolution, stag beetles, morphology

Male-male competition is a key component of sexual selection and can lead to the evolution of exaggerated traits that are used as weapons in battles (Andersson 1994). Deer antlers and beetle horns are examples of such weapons (Siva-Jothy 1987, Moczec and Emlen 2000, Okada and Miyatake 2004, 2007). Many beetles often show inter-sexual dimorphism, in which only male beetles develop exaggerated weapons for male-male battles (Siva-Jothy 1987, Moczec and Emlen 2000, Okada and Miyatake 2004, Okada et al. 2008). A winner of a battle succeeds at getting mates, resulting in his high fitness. Thus, males' exaggerated weapons have evolved to win battles and to achieve mating success via sexual selection. Males of stag beetles also have exaggerated mandibles to grasp and remove a rival from a feeding site to which females later come to feed (Siva-Jothy 1987, Inoue and Hasegawa 2012). Usually, larger males with large weapons tend to win in battles (Inoue and Hasegawa 2012, Hongo and Okamoto 2013, Goyens et al. 2015).

Previous studies have shown the existence of trade-offs between weapon size and other body parts (Emlen and Nijhout 2000, Emlen 2001). When the weapon becomes large, other body parts become small to compensate for resource investments into the weapon. For example, in Onthophagini beetles, negative correlations exist between weapon size and neighboring organ sizes (eyes, antennae and wings) (Emlen 2001). Similarly, in *Gnatocerus cornutus*, males with large mandibles have small eyes (Okada and Miyatake 2008). Thus, exaggerated weapons bring some costs to the owner.

Male beetles with large weapons sometimes show positive correlations with other traits. For example, *G. cornutus* shows a genetic correlation between the size of the weapon and the relative size of the head and prothorax (Okada and Miyatake 2008). Traits such as the head and the prothorax are likely to provide direct support for the weapon and are important for realizing large weapons (Okada et al. 2012). In fact, the muscles in the prothorax of a stag beetle are hypertrophied to help raise the head while lifting opponents (Goyens et al. 2015). Therefore, a positive correlation is expected between the weapon and its direct supportive traits.

Some other species of beetles show intra-sexual dimorphism among males. Major males with large weapons engage in aggressive battles as a tactic to acquire mates, whereas minor males with small weapons often adopt alternative reproductive tactics that may not involve fighting (Siva-Jothy 1987, Emlen 1997, Moczec and Emlen 2000, Okada and Miyatake 2004, 2007, Okada and Hasegawa 2005, Inoue and Hasegawa 2012). Depending on these differences in reproductive tactics, body shape may differ between major and minor males. In *Onthophagus taurus*, major males have a high fighting ability but show low tunnel agility, which is used in an alternative tactic (sneaking) by small males (Moczec and Emlen 2000).

Many of the small morph of male dimorphic species still have exaggerated weapons and fight with a rival occasionally. Usually, the size advantage has been observed in a battle between males with different body sizes (large males tend to win; see Inoue and Hasegawa 2012). One exception is in the case of interspecific battles between two stag beetles, *Lucanus maculifemoratus* and *Prosopocoilus inclinatus* (Hongo and Okamoto 2013). *P. inclinatus* tends to win over the larger *L. maculifemoratus* male because battle tactics are different between the two species (Hongo and Okamoto 2013). This suggests that the small morph of male dimorphic species may have different battle tactics from the large morph to overcome this size disadvantage. In fact, 27.8% of small males of *P. inclinatus* won when fighting with conspecific larger males (Inoue and Hasegawa 2012). In *P. inclinatus*, battle tactics seem to be different between morphs (see below). If so, traits that do not support weapons directly might have evolved with weapon size in each morph to increase the probability of winning. For example, the dung beetle *Onthophagus taurus* shows a positive phenotypic correlation between the relative size of the foretibia and the size of the horns (Tomkins et al. 2005). This positive correlation seems to be related with battle tactics because the foretibia is not an organ adjacent to the horns.

The Japanese stag beetle *Prosopocoilus inclinatus* shows an inter-sexual dimorphism, in which only males have exaggerated mandibles. Male beetles fight with rival males to defend sap-exuding areas of trees (territory); these areas are used by males to copulate with females that come to feed on the sap. Additionally, this species display an intra-sexual dimorphism in mandible size (Okada et al. 2008). The minor males show an alternative strategy in that they are more insistent than the major males when approaching females (Okada and Hasegawa 2005). Small males also secure mates by arriving earlier to the sap-exuding areas; large males tend to arrive later and tend to hesitate to fight, resulting in the small male holding the area (Inoue and Hasegawa 2012). Although small males show these alternative tactics, they still have weapons, and they do not hesitate to fight even with large males (Inoue and Hasegawa 2012). Thus, the directly supportive traits for weapons (e. g., head and prothorax) should be correlated with the weapon size in both morphs. However, the battle tactics of both morphs seem to be different (see Materials and Methods). Thus, morph-specific traits should have coevolved with the weapons to increase the probability of winning.

Although many studies have demonstrated correlations between weapons and morphological traits (Tomkins et al. 2005, Okada et al. 2012), few studies have explored differences in the correlation patterns among traits in major and minor males. In this study, we examined the difference in morphological trait sets that correlate morph specifically with weapons in both the morph of *P. inclinatus*. The aim of this study is to construct testable hypotheses for morph-specific correlational traits other than direct supportive ones with the weapons from the viewpoint of each morph’s battle tactics. The obtained hypotheses will be tested in further studies.

## Materials and Methods

### *Study organism*

*P. inclinatus* is the common Japanese stag beetle. Sampling was conducted from 20:00 to 23:00 on July 7, 2011 in Hobetsu-cho, Hokkaido, Japan (the same site reported in Okada et al. 2008). One hundred and fifty adults were collected under streetlights. Males were kept individually in plastic containers without food for more than 24 hours to evacuate all foods present in the digestive organs. This treatment was conducted to control for the effect of feeding timing differences. After this treatment, we froze all samples at -20□ to enable further morphometric measurements. The two male morphs of *P.*

*inclinatus* can be classified on the basis of elytra length. Males showing elytra lengths over 19.70 mm are classified as major males, whereas males with elytra lengths shorter than 19.70 mm are classified as minor males because allometric patterns of the weapons on elytra length are different based on this threshold (see Okada et al. 2008). We confirmed this threshold for the present samples and obtained the same result. Thus, we measured the elytra lengths of all samples to distinguish the morphs. Then, 50 individuals of each morph were randomly selected to measure morphological traits.

### *Battle tactics of both the morphs*

The large morphs grasp rivals from the front and above by their downward curved mandibles (see Figure 1), lift rivals up and throw rivals to the air. In contrast, the small morphs bite somewhere (e.g., a mandible or forelegs) from the ventral side of rivals using their straight mandibles (see Figure 1), lift rivals up by planting their own legs firmly on the tree surface, and throw rivals into the air.

### *Measurements*

Figure 1 shows the measured traits. The width of 2 traits (head (HW), thorax (TW)) and the length of 3 traits (right mandible, prothorax (FTL), and elytra) were measured to 0.01 mm using a digital vernire caliper (Digimatic caliper$^{\text{TM}}$, Mitutoyo, Kanagawa, Japan). The length of 4 traits (right foreleg (FL), right middle leg (ML), right hindleg (HL), and left antenna (ANT)) and the diameter of the right eye (EYE) were measured to the nearest 0.001 mm on digital photographs of individuals with a scale using computer software (ImageJ, ver. 1.45). An area of the right hindwing (WA) was measured on a digital photograph to the nearest 0.001 mm$^2$ using the Polygon Section tool of the ImageJ. After the measurements were taken, the samples were dried (80□, 24 hours) in a drying oven (Do-450, AZONE, Osaka, Japan), and the dry weight of the right mandible (WS), head (HW), thorax (TW) and abdomen (AbW) were measured to the nearest 0.01 mg using a digital scale (Sartorius Research R200, Goettingen, Germany). The wet weight may be considered to be more adequate to represent muscle mass or reproductive organs because an insect’s exoskeleton mainly determines its dry weight. However, if a strong correlation exists between wet and dry weights, the use of dry weights is not a problem in a multivariate regression (especially one that uses standardized variables) from the statistical viewpoint. To confirm this viewpoint, we calculated correlations between wet and dry weights for the above 4 traits. Very strong correlations existed (r=0.947–0.988; n=37; for all 4 traits, p<0.0001; see Supp. Figure S1). The slopes of the allometric line would differ between wet and dry weights (the slopes would be steeper in wet-weight regressions). However, we used the standardized residuals from the allometric line to the TW (the index of body size) as variables (see the next section).

Thus, the slope of the allometric line does not affect the degree of variance of a trait, meaning that the use of dry weights is not problematic in a multivariate regression using standardized variables.

*Residuals calculation for each trait*

*P. inclinatus* males show a large degree of variation in body size. Thus, a body size index for each individual was calculated to solve the analytical problem arising from trait-size differences in polymorphic species. In general, the main principal component (PC1) is used as a body-size index in monomorphic species. However, the regression between each trait and PC1 presents a statistical problem because each trait in itself has been included in the PC1 calculation. Thus, we used the trait that was most isometric with the PC1 as an index of body size (see Harvey and Pagel 1998. pp203-205).

In many cases, the correlation curves between morphological traits are expressed as a power function ($Y=aX^b$); thus, the measured trait values had to be log transformed before further analyses. We calculated the PC1s of principal component analyses (PCA) on each log-transformed trait values for each morph. The most isometric trait with PC1 was the TW in both morphs; thus, we used the TW as an index of body size in the following analyses. The PCA loadings were 0.816 and 0.914 for the minor and major morphs, respectively. The obtained PC1s explained 96.54% and 96.36% of variance in the traits in the minor and major morphs, respectively.

We used the residuals of each trait from the regression line of a trait's values on the TW as the relative trait size from which the effect of size difference was removed. For each male morph, the residuals of a trait were obtained from the regression line of each trait on the TW. All data were log transformed before the analysis. Furthermore, the residuals were standardized by the mean and standard deviation for each trait. Then, for each morph, we conducted a multivariate regression by setting the weapon dry weight (WS) as the dependent variable and setting the 9 remaining traits (HW, ANT, EYE, TA, FL, ML, HL, AbW and WA) as the independent variables. When the same independent traits were chosen in both morphs, we removed these traits from the following analyses because such traits were not useful to find morph-specific traits that correlated with the WS. Then, we conducted a new multivariate regression of the WS on the remaining traits with a model selection based on a Bayesian Information Criterion (BIC). Model selection is conducted frequently based on an Akaike Information Criterion (AIC), but an AIC is developed to optimize the predictability of the model for the dependent variable (Anderson and Burnham 2002). Thus, a model selection by an AIC may select another model that is better at predicting the dependent variables than the true model, whereas a model selection by a BIC selects a model that has the highest probability of being the true model (Sober 2008). In this study, our aim is to construct testable hypotheses for correlated traits with battle tactics in each morph. Thus, variable selection by a BIC is more adequate than by an AIC. We calculated a BIC for all possible models (all possible combinations of traits as independent variables), and selected a model that showed the least BIC as the best model.

## Results

The full models (including all 9 traits) show that the HW was chosen in both morphs (see Table 1). These results suggest that males that possess relatively large weapons also possess a relatively large HW (a direct supportive traits of weapons). Next, we removed the HW from independent variables and conducted a multivariate regression with a model selection by a BIC. In the major morph, the EYE and ML are chosen and these traits have positive effects on the WS. However, a partial regression coefficient of the ML is not significant (p=0.056), and the effect of this trait on the WS is weak. None of these traits is chosen in the minor morph. In the minor morph, the FL and AbW are chosen, and their effects on the WS are positive and negative, respectively (Table 1). Thus, major males that possess relatively large weapons also possess relatively large eyes, whereas minor males that have relatively large weapons also possess relatively long forelegs and small abdomens (Table 1). In conclusion, different traits were correlated with weapon size in each of the two morphs.

## Discussion

Consistent with several previous studies, positive correlations between the weapon size and a supportive trait (HW) were observed in both morphs, indicating that males with relatively large weapons also possessed large supportive traits. Weapon size is one of the most important traits allowing males to win in male-male combats (Moczec and Emlen 2000). Thus, supportive traits also have an important function allowing beetles that possess large supportive traits to display extreme abilities in male-male combats (Okada et al. 2012). In fact, males of the stag beetle *Cyclommatus metallifer* have hypertrophied muscles in their prothorax to help raise the head while lifting opponents (Goyens et al. 2015). Although minor *Prosopocoilus inclinatus* males adopt several alternative reproductive tactics that do not involve fights (Okada and Hasegawa 2005, Inoue and Hasegawa 2012), they still have exaggerated mandibles and do not hesitate to fight even with major males (Inoue and Hasegawa 2012). The positive correlation between the WS and the supportive HW is thus maintained in both morphs. After removing this common supportive trait of weapons, the EYE and ML are chosen only in the major male, and both traits have positive effects on the WS. The other traits (FL and AbW) are chosen only in the minor males, although the directions of effect are different (the FL is positively and the AbW is negatively correlated with the WS).

The results showed that small males with relatively small weapons also possess a relatively large abdomen. The males with small weapons (and supportive traits) find it more difficult to hold a territory and have reduced copulation chances when engaging in male-male combats. However, the large abdomen may allow for larger testes, which may give these males an increased sperm quantity as in *Gnatocerus cornutus* (Okada et al 2016). A previous study has shown that minor males of *Onthophagus binodis* have relatively larger testes and larger sperm ejaculation volumes than major males do (Simmons and Emlen 2006). In *Gnatocerus cornutus*, males with relatively small mandibles also have relatively large abdomens (Okada and Miyatake 2008). Minor males of *P. inclinatus* have been shown to approach females more persistently than major males do to secure mating chances (Okada and Hasegawa 2005). When a minor male with small weapons succeeded in mating with a female, it may ejaculate a larger volume of semen than the other males. Copulation numbers of a *P. inclinatus* female are unknown, but if multiple copulations occur, the large abdomen (with a larger semen volume) may benefit small males with small weapons via a sperm competition. Copulation numbers in females should be confirmed in future studies.

The relative sizes of eye showed positive correlations with the WS only in the major morph (Table 1). A previous study has shown a size trade-off between the size of weapons and sensory organs in *G. cornutus* (Okada and Miyatake 2008). A negative correlation also exists between the size of weapons and eyes in *Onthophagus* beetles, and this phenomenon is interpreted as a developmental trade-off between the two organs (Emlen 2001). However, such a trade-off is absent in *P. inclinatus* (Table 1). Thus, the positive correlation observed in major males of *P. inclinatus* may reflect morph-specific battle tactics. The *P. inclinatus* major males are generally stronger than the minor males (Inoue and Hasegawa 2012). The major males grasp rivals from the front and above by their downward curved mandibles (see Figure 1), pick rivals up and throw them to the air from the tree surface (territory). In this tactic, the weapons of large males are effective only in frontal attacks. In some instances, a major male may lose when a minor male attacks from the side. In fact, 27.8% of *P. inclinatus* smaller males won in intraspecific battles (Figure 3a in Inoue and Hasegawa 2012). The main reproductive tactic of the major males is holding a territory (feeding site) and defending it from nearby rivals. In this situation, it may be advantageous to develop eyes that can quickly detect the movements of rivals, particularly when they attack from the side. In fact, we repeatedly observed that a major male in a battle quickly turned his body direction to set a rival at his frontal side. This behavior is rare in the small males during a battle. Thus, having large eyes in addition to large weapons may be beneficial when defending territory. Although a previous study showed that vision does not affect the results of battles in the stag beetle *Cyclommatus metallifer*, its battle mode is different from that of *P. inclinatus* (Goyens et al 20015). Thus, the above hypothesis is worth testing in *P. inclinatus* in the future.

In contrast, the battle tactic in *P. inclinatus* is quite different in the minor males than it is in the major males. The minor males bite somewhere (e.g., mandible or forelegs) from the ventral side of rivals using their straight mandibles (see Figure 1), lift the rivals up by planting their own legs firmly on the tree surface, and expel the rivals from the territory. Because a small male attacks a rival from every direction, the WS does not have to correlate with sensory organ size in the minor morph. A previous study has shown that males of *P. inclinatus* close mandibles that are touched from the upper side (Hongo and Okamoto 2013) and that this physiological response enables them to use the above battle tactic of the minor morph. For this tactic of the minor males, long forelegs (FL) would be advantageous for lifting the rival up. We are now testing this hypothesis. Of course, the eyes of the small morph may be important for their alternative reproductive strategies (Okada and Hasegawa 2005), but there is no expectation of a correlation between weapon and eye size from this viewpoint because a small male does not use mandibles when approaching females (Okada and Hasegawa 2005).

This study did not include female trait measurements. It may be suggested that without female data, we cannot detect exaggerated traits in males because the degree of a trait's exaggeration is estimated by comparing it with the female homologous trait as a baseline. However, the aim of this study is to find male-morph specific traits that coevolved with weapon size. Thus, we did not provide female morphological data in this study. Of course, female data are important to understand how sexual selection drives the evolution of male-exaggerated traits, but for such a purpose, we need the inheritance mode of each trait. Currently, we are estimating the heritability of each trait from the mother and the father by rearing many larvae from many females. In addition, we are trying to differentiate sons of a singly mated mother between major and minor males. We can understand genetic correlations of traits between the male morphs by comparing traits between these brothers. These studies reveal important information for understanding morphological evolutions in this impressive insect.

In this study, we identified a set of linked traits in two male morphs of a stag beetle. These traits may confer distinct advantages to each morph depending on different battle tactics and/or basic reproductive strategies. Several studies have focused on the relationship between weapons and reproductive strategies, but to understand the evolution of phenotypes as a whole, the linked traits that maximize the efficiency of the adaptive strategies adopted by each morph should be considered. The hypotheses obtained by this study will be tested in the near future, and these studies will bring us new insights into understanding the evolution of morphologies in insects under sexual selection.

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## Competing interests

The authors declare no competing financial interests.

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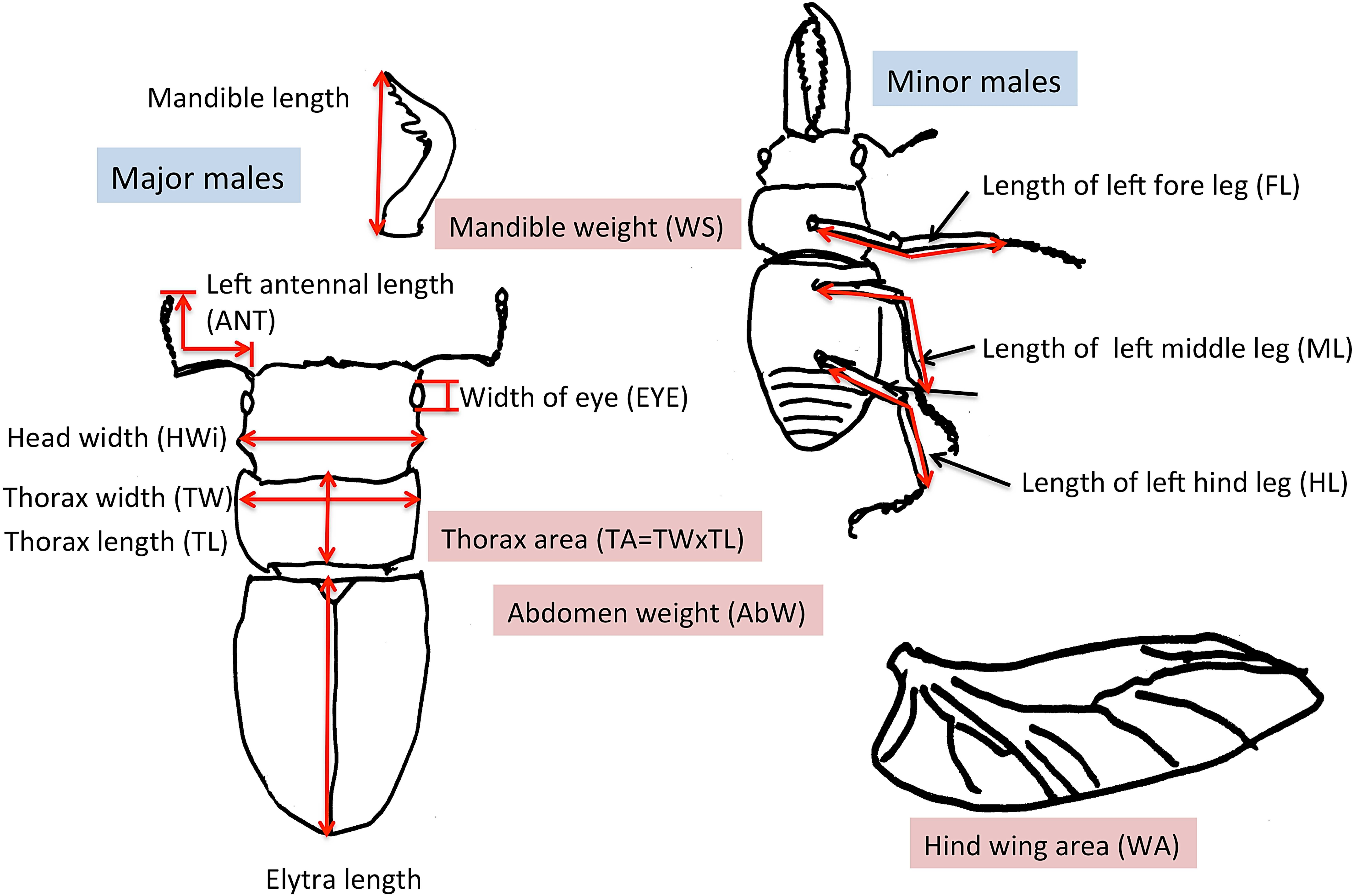
Table 1. The results of the multivariate regressions. The data for each trait were standardized by the mean and the standard deviation before analyzing. For each morph, we conducted a model selection based on a BIC for all models that consisted of possible combinations of traits. First, we used all 9 variables as independent variables to the WS (the dependent variable), and the HW was commonly selected in both morphs. This trait could be interpreted as a trait that directly supported weapons (mandibles), and it thus was removed from the 2nd analysis. Second, we used the remaining 8 traits to make models. The eye and ML were selected for the major morph, and the FL and AbW were selected for the minor morph. Partial regression coefficients and its statistical significances were presented for each selected trait.

## Figure Captions

Figure 1. Measured traits for each of the 50 major and minor *P. inclinatus* males. Pink frames around traits indicate the dry weight or the area of those traits.

Table 1. The results of the multivariate regressions. The data for each trait were standardized by the mean and the standard deviation before analyzing. For each morph, we conducted a model selection based on a BIC for all models that consisted of possible combinations of traits. First, we used all 9 variables as independent variables to the WS (the dependent variable), and the HW was commonly selected in both morphs. This trait could be interpreted as a trait that directly supported weapons (mandibles), and it thus was removed from the 2nd analysis. Second, we used the remaining 8 traits to make models. The eye and ML were selected for the major morph, and the FL and AbW were selected for the minor morph. Partial regression coefficients and its statistical significances were presented for each selected trait.

| Analysis | Morph | | HWi | TA | ANT | EYE | FL | ML | HL | AbW | WA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1st | Major | Intercept | 6.608e$^{-11}$ (Std. Error=1.026e$^{-01}$, t-value=0.000, p=1.000) | | | | | | | | |
| | | Estimate | 6.959e$^{-01}$ | - | - | - | - | - | - | - | - |
| | | Sta.Error | 1.307e$^{-01}$ | - | - | - | - | - | - | - | - |
| | | t-value | 6.713 | - | - | - | - | - | - | - | - |
| | | p value | 2.02e$^{-08}$ | - | - | - | - | - | - | - | - |
| | Minor | Intercept | -5.724e$^{-12}$ (Std. Error=8.330e$^{-02}$, t-value=0.000, p=1.000) | | | | | | | | |
| | | Estimate | 4.776e$^{-01}$ | - | - | - | 2.695e$^{-01}$ | - | - | -4.033e-$^{01}$ | - |
| | | Sta.Error | 9.187e$^{-02}$ | - | - | - | 8.696e$^{-02}$ | - | - | 8.693e$^{-02}$ | - |
| | | t-value | 5.199 | - | - | - | 3.005 | - | - | -4.639 | - |
| | | p value | 4.50e$^{-06}$ | - | - | - | 0.00429 | - | - | 2.92e$^{-05}$ | - |
| 2nd | Major | Intercept | 1.180e$^{-10}$ (Std. Error=1.262e$^{-01}$, t-vale=0.000, p=1.000) | | | | | | | | |
| | | Estimate | - | | | 4.124e$^{-01}$ | | 2.495e$^{-01}$ | | | |
| | | Sta.Error | - | | | 1.275e$^{-01}$ | | 1.275e$^{-01}$ | | | |
| | | t-value | - | | | 3.234 | | 1.957 | | | |
| | | p value | - | | | 0.00223 | | 0.05636 | | | |
| | Minor | Intercept | -5.291e-12 (Std. Error=1.038e$^{-01}$, t-value=0.000, p=1.000) | | | | | | | | |
| | | Estimate | - | | | | 4.215e$^{-01}$ | | | -5.028e$^{-01}$ | |
| | | Sta.Error | - | | | | 1.057e$^{-01}$ | | | 1.057e$^{-01}$ | |
| | | t-value | - | | | | 3.988 | | | -4.757 | |
| | | p value | - | | | | 0.000231 | | | 1.91e$^{-05}$ | |


Mandible length
Major males
Mandible weight (WS)
Left antennal length (ANT)
Width of eye (EYE)
Head width (HWi)
Thorax width (TW)
Thorax length (TL)
Thorax area (TA=TWxTL)
Abdomen weight (AbW)
Elytra length
Minor males
Length of left fore leg (FL)
Length of left middle leg (ML)
Length of left hind leg (HL)
Hind wing area (WA)