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

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

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34 **Abstract**

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

47

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

49

50

51 Male-male competition is a key component of sexual selection and can lead to the evolution of
52 exaggerated traits that are used as weapons in battles (Andersson 1994). Deer antlers and beetle horns
53 are examples of such weapons (Siva-Jothy 1987, Moczec and Emlen 2000, Okada and Miyatake 2004,
54 2007). Many beetles often show inter-sexual dimorphism, in which only male beetles develop
55 exaggerated weapons for male-male battles (Siva-Jothy 1987, Moczec and Emlen 2000, Okada and
56 Miyatake 2004, Okada et al. 2008). A winner of a battle succeeds at getting mates, resulting in his high
57 fitness. Thus, males' exaggerated weapons have evolved to win battles and to achieve mating success
58 via sexual selection. Males of stag beetles also have exaggerated mandibles to grasp and remove a rival
59 from a feeding site to which females later come to feed (Siva-Jothy 1987, Inoue and Hasegawa 2012).
60 Usually, larger males with large weapons tend to win in battles (Inoue and Hasegawa 2012, Hongo and
61 Okamoto 2013, Goyens et al. 2015).

62

63 Previous studies have shown the existence of trade-offs between weapon size and other body parts
64 (Emlen and Nijhout 2000, Emlen 2001). When the weapon becomes large, other body parts become
65 small to compensate for resource investments into the weapon. For example, in Onthophagini beetles,
66 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

100 seems to be related with battle tactics because the foretibia is not an organ adjacent to the horns.

101

102 The Japanese stag beetle *Prosopocoilus inclinatus* shows an inter-sexual dimorphism, in which only
103 males have exaggerated mandibles. Male beetles fight with rival males to defend sap-exuding areas of
104 trees (territory); these areas are used by males to copulate with females that come to feed on the sap.
105 Additionally, this species display an intra-sexual dimorphism in mandible size (Okada et al. 2008). The
106 minor males show an alternative strategy in that they are more insistent than the major males when
107 approaching females (Okada and Hasegawa 2005). Small males also secure mates by arriving earlier to
108 the sap-exuding areas; large males tend to arrive later and tend to hesitate to fight, resulting in the small
109 male holding the area (Inoue and Hasegawa 2012). Although small males show these alternative tactics,
110 they still have weapons, and they do not hesitate to fight even with large males (Inoue and Hasegawa
111 2012). Thus, the directly supportive traits for weapons (e. g., head and prothorax) should be correlated
112 with the weapon size in both morphs. However, the battle tactics of both morphs seem to be different
113 (see Materials and Methods). Thus, morph-specific traits should have coevolved with the weapons to
114 increase the probability of winning.

115

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

123

124 **Materials and Methods**

125

126 *Study organism*

127 *P. inclinatus* is the common Japanese stag beetle. Sampling was conducted from 20:00 to 23:00 on July
128 7, 2011 in Hobetsu-cho, Hokkaido, Japan (the same site reported in Okada et al. 2008). One hundred
129 and fifty adults were collected under streetlights. Males were kept individually in plastic containers
130 without food for more than 24 hours to evacuate all foods present in the digestive organs. This
131 treatment was conducted to control for the effect of feeding timing differences. After this treatment, we
132 froze all samples at -20°C to enable further morphometric measurements. The two male morphs of *P.*

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

139

140 *Battle tactics of both the morphs*

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

145

146 *Measurements*

147 Figure 1 shows the measured traits. The width of 2 traits (head (HW), thorax (TW)) and the length of 3
148 traits (right mandible, prothorax (FTL), and elytra) were measured to 0.01 mm using a digital vernire
149 caliper (Digimatic caliper^{TM}, Mitutoyo, Kanagawa, Japan). The length of 4 traits (right foreleg (FL),
150 right middle leg (ML), right hindleg (HL), and left antenna (ANT)) and the diameter of the right eye
151 (EYE) were measured to the nearest 0.001 mm on digital photographs of individuals with a scale using
152 computer software (ImageJ, ver. 1.45). An area of the right hindwing (WA) was measured on a digital
153 photograph to the nearest 0.001 mm² using the Polygon Section tool of the ImageJ. After the
154 measurements were taken, the samples were dried (80°C, 24 hours) in a drying oven (Do-450, AZONE,
155 Osaka, Japan), and the dry weight of the right mandible (WS), head (HW), thorax (TW) and abdomen
156 (AbW) were measured to the nearest 0.01 mg using a digital scale (Sartorius Research R200,
157 Goettingen, Germany). The wet weight may be considered to be more adequate to represent muscle
158 mass or reproductive organs because an insect's exoskeleton mainly determines its dry weight.
159 However, if a strong correlation exists between wet and dry weights, the use of dry weights is not a
160 problem in a multivariate regression (especially one that uses standardized variables) from the
161 statistical viewpoint. To confirm this viewpoint, we calculated correlations between wet and dry
162 weights for the above 4 traits. Very strong correlations existed ($r=0.947-0.988$; $n=37$; for all 4 traits,
163 $p<0.0001$; see Supp. Figure S1). The slopes of the allometric line would differ between wet and dry
164 weights (the slopes would be steeper in wet-weight regressions). However, we used the standardized
165 residuals from the allometric line to the TW (the index of body size) as variables (see the next section).

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

168

169 *Residuals calculation for each trait*

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

177

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

185

186 We used the residuals of each trait from the regression line of a trait's values on the TW as the relative
187 trait size from which the effect of size difference was removed. For each male morph, the residuals of a
188 trait were obtained from the regression line of each trait on the TW. All data were log transformed
189 before the analysis. Furthermore, the residuals were standardized by the mean and standard deviation
190 for each trait. Then, for each morph, we conducted a multivariate regression by setting the weapon dry
191 weight (WS) as the dependent variable and setting the 9 remaining traits (HW, ANT, EYE, TA, FL, ML,
192 HL, AbW and WA) as the independent variables. When the same independent traits were chosen in
193 both morphs, we removed these traits from the following analyses because such traits were not useful
194 to find morph-specific traits that correlated with the WS. Then, we conducted a new multivariate
195 regression of the WS on the remaining traits with a model selection based on a Bayesian Information
196 Criterion (BIC). Model selection is conducted frequently based on an Akaike Information Criterion
197 (AIC), but an AIC is developed to optimize the predictability of the model for the dependent variable
198 (Anderson and Burnham 2002). Thus, a model selection by an AIC may select another model that is

199 better at predicting the dependent variables than the true model, whereas a model selection by a BIC
200 selects a model that has the highest probability of being the true model (Sober 2008). In this study, our
201 aim is to construct testable hypotheses for correlated traits with battle tactics in each morph. Thus,
202 variable selection by a BIC is more adequate than by an AIC. We calculated a BIC for all possible
203 models (all possible combinations of traits as independent variables), and selected a model that showed
204 the least BIC as the best model.

205

206 **Results**

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

218

219 **Discussion**

220 Consistent with several previous studies, positive correlations between the weapon size and a
221 supportive trait (HW) were observed in both morphs, indicating that males with relatively large
222 weapons also possessed large supportive traits. Weapon size is one of the most important traits allowing
223 males to win in male-male combats (Moczek and Emlen 2000). Thus, supportive traits also have an
224 important function allowing beetles that possess large supportive traits to display extreme abilities in
225 male-male combats (Okada et al. 2012). In fact, males of the stag beetle *Cyclommatus metallifer* have
226 hypertrophied muscles in their prothorax to help raise the head while lifting opponents (Goyens et al.
227 2015). Although minor *Prosopocoilus inclinatus* males adopt several alternative reproductive tactics
228 that do not involve fights (Okada and Hasegawa 2005, Inoue and Hasegawa 2012), they still have
229 exaggerated mandibles and do not hesitate to fight even with major males (Inoue and Hasegawa 2012).
230 The positive correlation between the WS and the supportive HW is thus maintained in both morphs.
231 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

267 a battle. Thus, having large eyes in addition to large weapons may be beneficial when defending
268 territory. Although a previous study showed that vision does not affect the results of battles in the stag
269 beetle *Cyclommatus metallifer*, its battle mode is different from that of *P. inclinatus* (Goyens et al
270 20015). Thus, the above hypothesis is worth testing in *P. inclinatus* in the future.

271
272 In contrast, the battle tactic in *P. inclinatus* is quite different in the minor males than it is in the major
273 males. The minor males bite somewhere (e.g., mandible or forelegs) from the ventral side of rivals
274 using their straight mandibles (see Figure 1), lift the rivals up by planting their own legs firmly on the
275 tree surface, and expel the rivals from the territory. Because a small male attacks a rival from every
276 direction, the WS does not have to correlate with sensory organ size in the minor morph. A previous
277 study has shown that males of *P. inclinatus* close mandibles that are touched from the upper side
278 (Hongo and Okamoto 2013) and that this physiological response enables them to use the above battle
279 tactic of the minor morph. For this tactic of the minor males, long forelegs (FL) would be advantageous
280 for lifting the rival up. We are now testing this hypothesis. Of course, the eyes of the small morph may
281 be important for their alternative reproductive strategies (Okada and Hasegawa 2005), but there is no
282 expectation of a correlation between weapon and eye size from this viewpoint because a small male
283 does not use mandibles when approaching females (Okada and Hasegawa 2005).

284
285 This study did not include female trait measurements. It may be suggested that without female data, we
286 cannot detect exaggerated traits in males because the degree of a trait's exaggeration is estimated by
287 comparing it with the female homologous trait as a baseline. However, the aim of this study is to find
288 male-morph specific traits that coevolved with weapon size. Thus, we did not provide female
289 morphological data in this study. Of course, female data are important to understand how sexual
290 selection drives the evolution of male-exaggerated traits, but for such a purpose, we need the
291 inheritance mode of each trait. Currently, we are estimating the heritability of each trait from the
292 mother and the father by rearing many larvae from many females. In addition, we are trying to
293 differentiate sons of a singly mated mother between major and minor males. We can understand genetic
294 correlations of traits between the male morphs by comparing traits between these brothers. These
295 studies reveal important information for understanding morphological evolutions in this impressive
296 insect.

297
298 In this study, we identified a set of linked traits in two male morphs of a stag beetle. These traits may
299 confer distinct advantages to each morph depending on different battle tactics and/or basic reproductive
300 strategies. Several studies have focused on the relationship between weapons and reproductive

301 strategies, but to understand the evolution of phenotypes as a whole, the linked traits that maximize the
302 efficiency of the adaptive strategies adopted by each morph should be considered. The hypotheses
303 obtained by this study will be tested in the near future, and these studies will bring us new insights into
304 understanding the evolution of morphologies in insects under sexual selection.

305

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312

313 **Competing interests**

314 The authors declare no competing financial interests.

315

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377

378

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

388

389 **Figure Captions**

390

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

393

394

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-value=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)