Distinctive developmental variability of genital parts in the sexually dimorphic beetle, *Prosopocoilus inclinatus* (Coleoptera: Lucanidae)

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Recent comparative studies have revealed that the rapid diversity of genitalia is closely related to sexual selection and that genital development interacts with the development of different body parts. Hypotheses about developmental stability due to selection to genital parts were tested by estimating allometric relations in a sexually dimorphic stag beetle *Prosopocoilus inclinatus*. All genital parts of males scaled to body size with a slope of less than 1 and all but the median lobe (male intromittent organ) showed smaller variability than other body parts. This supported the 'one-size-fits-all' hypothesis, which suggests broad copulation opportunity by males of any size with females within a population. Nevertheless, we found large variation among different genital parts in coefficients of variation and in values of the switch point where the allometric relations varied significantly. These results strongly support the view that developmental trajectories of genital traits are not necessarily integrated. Among the genitalic traits, male intromittent organ and female genitalia exhibited large variability, suggesting a high responsiveness to the selective regimes and physical interaction during copulation. This may account for rapid diversification of genital morphology, even in closely-related populations in beetle species. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, **90**, 573–581.

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INTRODUCTION

Most recent studies on genital variation agree that sexual selection processes such as female choice and male–female interaction, rather than traditional 'lock-and-key' or pleiotropy mechanisms, play an important role in the divergence of genital shape and functions (for a review, see Hosken & Stockley, 2004). The best way to unravel the pathway of genital evolution will be to measure selective pressures acting directly on the genitalia. However, this approach is restricted to very few species because of the difficulty in rearing large amounts of animals. Instead, comparative morphological studies on genital features have been applied to indirectly infer the relationship between genital characteristics and selective regimes. For example, comparison of variation in genital features has revealed that genitalia of males were more diverged within a polyandrous clade than those within a monoandrous clade, suggesting that selective opportunity differs between the two clades (Arnqvist, 1998).

A further indirect tool to disentangle hypotheses on the evolution of genital structures is to study the allometry of genitalia. Two existing hypotheses based on sexual selection (i.e. the good genes hypothesis and

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the weapon hypothesis) were tested by Eberhard *et al*. (1998) using 20 species of insects and spiders. They found that the allometric slopes of male genitalia tended to be lower than the slopes of nongenital parts. Consequently, an alternative hypothesis was proposed, namely the one-size-fits-all hypothesis. This hypothesis predicts that females perceive tactile signals from the male genitalia and bias their fertilization decision accordingly. Selection therefore tends to decouple genitalia from the development of other general body parts (Eberhard *et al*., 1998).

Accordingly, stabilizing selection results in a similar size of male genitalia regardless of the body size of males. As a result, low variation should be expected across males, implying that stabilizing selection itself cannot explain why genital parts have acquired complicated shapes. Recent evolutionary studies on sexual selection have provided an insight into this puzzle: some genitalic traits may evolve independently from one another (i.e. mosaic evolution) by way of male and female interactions (Cordoba-Aguilar, 2002; Cordero Rivera *et al*., 2004; Hosken, Minder & Ward, 2005). Moreover, although allometric coefficient of the genitalia as a whole is constant and lower than 1 in many cases (Eberhard *et al*., 1998; Tatsuta, Mizota & Akimoto, 2001; Bernstein & Bernstein, 2002; Mutanen & Kaitala, 2006), a positive allometry is sometimes found in a part of genitalia, presumably due to postcopulatory sexual selection (Lupold, Mcelligott & Hosken, 2004; Hosken, Minder & Ward, 2005). These facts lead to a contradiction in terms of developmental stability and rapid diversification of genital structures. Comparative studies on phenotypic variation would contribute to distinguishing these conjectures.

In the present study, comparison of phenotypic variability is attempted using the external morphology of male and female body parts of a lucanid beetle, *Prosopocoilus inclinatus*. In this species, male genitalia consist of four fundamental parts: sternite IX and tergite IX, which constitute the abdominal plate, and the aedeagus and the median lobe (penis), which show a complex structure and are directly related to the copulatory process (Fig. 1). Female genitalia are relatively simple and consist of bursa copulatrix and spermatheca (Fig. 1). Males of the *P. inclinatus* species group often show dimorphism in mandible size (Tatsuta, Mizota & Akimoto, 2004). Because such a dimorphism can result in different behavioural tactics (Gage, Stockley & Parker, 1995; Shiokawa & Iwahashi, 2000), the alternative behaviour may exert different selective pressures on different body parts. The developmental threshold at which allometric relations switch can be analysed from allometric equations (Kotiaho & Tomkins, 2001). Comparison of the inflection points on the allometric curve between different body parts will provide valuable insights that allow

assessment of the selective regimes and developmental constraints in a particular character set (Knell, Pomfret & Tomkins, 2004). If traits are under selection and tend to be subject to physiological constraint, the developmental patterns would be similar with each other. By contrast, if sexual selection (i.e. antagonistic sexual selection; Arnqvist & Rowe, 2002) drives genitalia evolution, not only the inflection points on the allometric curve differ across genitalic parts, but also the disparity pattern around the character mean would differ. Finally, comparing how the respective variability of male and female genital parts matches may also allow functional considerations (Hosken & Stockley, 2004; Mendez & Cordoba-Aguilar, 2004).

MATERIAL AND METHODS

MATERIALS AND MEASUREMENTS OF BODY PARTS

A total of 133 males and 142 females of *P. inclinatus* from a locality in Hidaka region, Hokkaido, northern Japan was used for the analysis. All specimens were collected by light trap in the summers of 1998 and 1999 and killed immediately by ethyl acetate. Prior to the measurement of each body part in males and females, five parts of each individual were separated at the membranous joints: left and right mandible, head, prothorax, fused segments of mesothorax-abdomen (SMA) (*sensu* Tatsuta, Mizota & Akimoto, 2001, 2004) (Fig. 1). Previous studies adopted morphometric analysis to evaluate shapes of body parts, but this method is not always feasible, especially when body parts have a three-dimensional shape and tend to induce substantial measurement errors due to small size (cf. Bernstein & Bernstein, 2002). Thus, each shape feature is represented as the weight of dry mass, except female genitalia whose majority are constituted by membrane. Male genitalia were partitioned into the four fundamental parts: tergite IX, sternite IX, the tegmen (comprising the parameres and the basal piece), and the median lobe (Fig. 1). To measure each part, after softening the whole genitalia in hot water (95 °C) for a few minutes, each part was separated and then dried at 65 °C for 24 h using a drying oven and weighed on a 1.0×10^{-5} g scale using a fine electric balance (ISO9001, Sartorius). The dry weight of a character was shown to be governed by the resource allocation during development (Tatsuta, Mizota & Akimoto, 2001, 2004), and also reduces scale differences between body parts and overcomes difficulties in measuring dimensional complex body parts. We eliminated damaged body parts from the subsequent analysis.

For analysis of female genitalia, the sperm storage organs, comprising the bursa copulatrix and the spermatheca (Fig. 1), were surgically removed from SMA that had been softened in hot water (95 °C) before
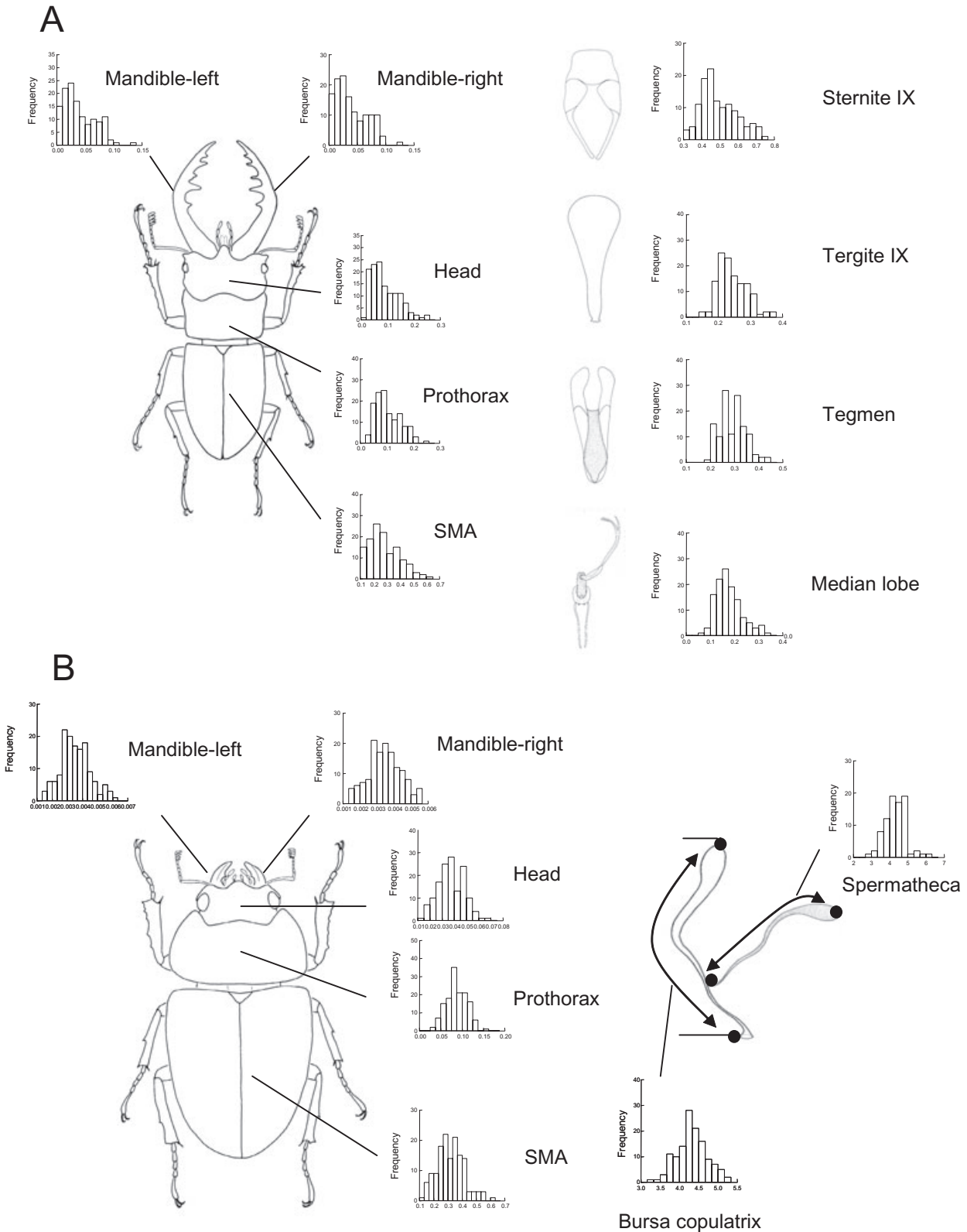
Figure 1. Characters measured in males (A) and females (B) and their frequency distributions. The unit of measurement is grams for nongenital parts in both sexes and genital parts in males and pixel for genital parts in females, respectively. Landmarks for the length of female genitalia are indicated by the overlays.

surgery. Because the genital parts of females are made of thin membrane and are not suitable for weighing, we quantified the length of genital organs (Fig. 1). For the length measurement of female genitalia, the whole genitalia, comprising the bursa copulatrix and spermatheca, was mounted in Hoyer's medium on a grass slide and then spread by careful placement of a cover slip so as not to deform original features. Images for each part of genitalia were captured using a digital camera (TK-1283, Victor) linked to a stereoscope (SZH-ILLD, Olympus, Japan). For all characters, images were magnified to the exact same scale on a computer screen to minimize measurement errors. The measurement (where the scale unit was one pixel) of female genitalia was conducted using Scion Images for Windows, version 4, (Scion Corp., 2000) as shown in Figure 1. To determine measurement precision of each part, measurements were repeated three times in each character and the proportion of within-individual variance to the total variance was estimated using a nested analysis of variance model. The proportion of within-individual variance to the total variance was conspicuously small (< 1% in nongenital characters in males and females, < 4% in genital characters in males, < 2% in genital characters in females). Because this indicates that our measures of each character were highly repeatable, the mean weights or lengths of characters for each individual were used in the subsequent analysis.

VARIABILITY AND ALLOMETRY OF BODY PARTS

To assess the relationship between body parts, we first calculated Pearson's product-moment correlation coefficients between SMA and other body parts for each sex. The trend of relative growth of a body part was evaluated using the least-square fitting of the second-order polynomial (Eberhard & Gutierrez, 1991). If the partial regression coefficient of the quadratic term (b_2 in Table 1) significantly deviated from zero, this part was considered as having at least one inflection point on allometric curve. We then searched for the switch point of the allometric coefficient. In general, to determine the optimal switch point in the allometry of a character, a conditional regression model with least-square fashion (Eberhard & Gutierrez, 1991), in which the dependent variable is regressed on the independent variable (i.e. body size), was adopted. However, as noted by Kotiaho & Tomkins (2001), the accuracy of the model estimate is questionable when substantial variance is primarily induced by environmental and/or genetic origin around the 'realized' switch point. Therefore, we first estimated the switch point in the dimorphic parts (instead of finding the switch point in body size first) by use of the modified least-square model implemented by Kotiaho & Tomkins (2001):

$$\ln X = \alpha + \beta_1 \cdot \ln Y(1 - D) + \beta_2(\ln Y - \ln Y_D)D + \varepsilon$$

in which $\ln X$ is log-transformed body size; $\ln Y$ is the log-transformed response variable; $\ln Y_D$ is a log-transformed optimal switch point where the total residual variance $(1 - R^2)$ of the above equation is the smallest; $D = 0$ if $Y < Y_D$, $D = 1$ if $Y > Y_D$; α is a constant; β is the partial regression coefficient; and ε is the error term. Based on the value of Y_D, the data were partitioned into subsets and the range of SMA (shown as an untransformed scale; Table 1) of each subset was estimated. We finally estimated regression slope of a trait on SMA. For comparison of regression coefficients estimated by different methods (for a detailed discussion, see Green, 1999), ordinary least-square (OLS) regression and major axis (MA) regression were used to estimate the allometry slope. Confidence intervals were also given to test the regression slope for a particular allometric coefficient.

The coefficient of variation (CV) is entangled with the change of allometric slope and the increase of variability (Eberhard *et al*., 1998: fig. 1A, B). To distinguish between the two, a modified estimator CV′, calculated as $CV(Y) \times (1 - r^2)^{1/2}$ (Eberhard *et al*., 1998), was estimated for each regression line when significant switching of allometric curve was found. Correlation coefficients of residuals around the OLS regression lines were also calculated between characters. All calculations were implemented using code developed for statistical program R, version 1.90 (R Development Core Team, 2004) and JMP for windows, version 5.01 (SAS Institute Inc., 2002).

RESULTS

Figure 1 shows the frequency distribution of each body part. In males, most body parts showed a significant departure from normality (Table 1). By contrast, although all nongenital parts of the females conformed to normality, two genital parts were significantly deviated from normality (Table 1).

Basic statistics and allometric relations of each body part are summarized in Table 1. Because the correlation between the length of spermatheca (with a very large CV of 86.451%) and SMA was not significant ($F = 2.93$, $P = 0.091$), further analysis of allometry was not carried out. In males and females, all body parts, except the median lobe, had at least one inflection point on the allometric curve. Two meaningful switch points were found only in sternite IX.

Overall, although allometric coefficients calculated from OLS regression tended to be smaller than those from MA regression, the results were consistent irrespective of regression methods. In males, allometric slopes for the left and right mandible changed at a very similar body size (left = 0.253; right = 0.254):

Table 1. Basic statistics and allometric relationships for genitalic and nongenitalic body parts for male and female stag beetles, *Prosopocoilus inclinatus*

Character	N	Mean ± SD	CV	D_{max}	r	b_2	Range of SMA	Allometric coefficients		
								OLS (CI)	MA (CI)	CV†
Male										
Nongenital parts										
Mandible: left	133	39.93 ± 27.21	68.094	0.137**	0.907	0.626**	< 0.253	1.400 (1.361, 1.439)	1.978 (1.675, 2.379)	46.139
							> 0.253	0.418 (0.374, 0.462)	0.527 (0.364, 0.715)	27.202
Mandible: right	133	39.43 ± 26.90	68.171	0.133**	0.907	0.622**	< 0.254	1.428 (1.388, 1.468)	2.063 (1.742, 2.494)	47.092
							> 0.254	0.402 (0.359, 0.444)	0.497 (0.342, 0.673)	25.980
Head	133	90.89 ± 52.39	57.607	0.118**	0.962	0.510**	< 0.252	0.435 (0.396, 0.473)	0.900 (0.508, 1.541)	32.141
							> 0.252	1.224 (1.203, 1.244)	1.320 (1.212, 1.440)	33.339
Prothorax	133	105.99 ± 46.80	44.196	0.114**	0.961	0.392**	< 0.270	0.845 (0.828, 0.862)	0.991 (0.852, 1.152)	25.162
							> 0.270	0.374 (0.342, 0.407)	0.440 (0.320, 0.571)	22.826
SMA‡	133	280.27 ± 118.47	40.733	0.113**						
Genital parts										
Sternite IX	124	0.497 ± 0.101	33.204	0.159**	0.794	0.160**	< 0.172	0.045 (0.028, 0.061)	0.048 (−0.094, 0.192)	6.828
							0.172–0.187	0.033 (0.029, 0.037)	0.033 (0.002, 0.064)	2.136
							> 0.187	0.343 (0.328, 0.357)	0.374 (0.307, 0.444)	15.090
Tergite IX	124	0.242 ± 0.046	32.255	0.175**	0.700	0.139**	< 0.201	0.191 (0.178, 0.204)	0.212 (0.139, 0.288)	11.824
							> 0.201	0.084 (0.054, 0.114)	0.097 (−0.073, 0.272)	8.154
Tegmen	124	0.290 ± 0.054	31.986	0.145**	0.651	0.114**	< 0.266	0.131 (0.118, 0.144)	0.136 (0.068, 0.206)	8.304
							> 0.266	0.189 (0.175, 0.203)	0.201 (0.142, 0.263)	11.644
Median lobe	122	0.175 ± 0.055	43.617	0.088*	0.447	0.084		0.262 (0.154, 0.371)	0.390 (0.236, 0.562)	42.700

Table 1. *Continued*

| | | | | | | | | Allometric coefficients | | |
Character	N	Mean ± SD	CV	D_{max}	r	b_2	Range of SMA	OLS (CI)	MA (CI)	CV†
Female										
Nongenital parts										
Mandible: left	142	3.53 ± 0.99	28.119	0.063	0.836	−0.389**	< 0.467	0.699 (0.682, 0.716)	0.802 (0.712, 0.901)	22.711
							> 0.467	0.212 (0.246, 0.278)	0.278 (−0.033, 0.650)	8.512
Mandible: right	142	3.49 ± 0.99	28.434	0.049	0.840	−0.349**	< 0.417	0.680 (0.663, 0.696)	0.769 (0.673, 0.875)	20.848
							> 0.417	0.265 (0.238, 0.291)	0.360 (0.156, 0.595)	11.616
Head	142	37.62 ± 11.13	29.586	0.074	0.970	−0.142*	< 0.450	0.935 (0.925, 0.945)	0.979 (0.926, 1.034)	25.766
							> 0.450	0.511 (0.469, 0.554)	0.575 (0.313, 0.912)	11.262
Prothorax	142	86.60 ± 23.96	27.663	0.053	0.940	−0.259**	< 0.503	0.877 (0.866, 0.888)	0.927 (0.871, 0.987)	25.345
							> 0.503	0.391 (0.366, 0.415)	0.413 (0.223, 0.631)	6.651
SMA‡	142	320.05 ± 95.72	29.907	0.049						
Genital parts										
Bursa copulatrix‡	128	4.33 ± 0.39	34.513	0.302**	0.121	−0.193**	< 0.163	0.046 (0.012, 0.079)	0.050 (−0.133, 0.237)	2.902
							> 0.163	0.061 (0.033, 0.090)	0.159 (−0.277, 0.664)	6.121
Spermatheca§¶	83	4.35 ± 0.59	86.451	0.292**	0.155					

*$P < 0.05$, **$P < 0.001$.

†Modified coefficient of variation (see text).

‡Segments of mesothorax-abdomen (SMA) (see text) was used as an indicator of body size, and thus information on allometry was not obtained.

§Measurements cubed were used for allometric analysis.

¶No significant correlation with SMA ($F = 1.964$, $P = 0.147$), and thus further analysis was not implemented.

N, number of individuals; SS, standard deviation; CV, coefficient of variation; D_{max}, statistic of Kolmogorov–Smirnov test for normality; r, Pearson's product-moment correlation coefficient with SMA; b_2, partial regression; coefficient of quadratic term in second-order polynormial (see text); OLS, cefficient estimated by ordinary least-square regression; MA, coefficient estimated by major-axis regression; CI, 95% confidence interval of each coefficient.

individuals with relatively smaller body size developed disproportionately larger mandibles but the allometric slope changed into 'negative' in males with relative large body size. By contrast, the allometry of the head was slightly negative when SMA was small, but was positive when SMA was larger than a threshold (> 0.252, Table 1). The prothorax showed an almost isometric relationship to small SMA, but strongly negative relationships to large SMA. All allometric coefficients of genital parts were negative and did not contradict the prediction of a one-size-fits-all hypothesis. However, compared with nongenital parts, genital parts showed a wider range of switch points (0.253–0.270 in nongenital parts vs. 0.172–0.266 in genital parts). Among genital parts, the tergite IX showed the opposite pattern to these parts: a relatively large allometry coefficient was found in smaller individuals, whereas sternite IX and tegmen tended to be larger in allometry as the body size increased.

In females, all body parts tended to show 'negative' allometry despite the existence of dimorphism. Moreover, a decrease of the relative growth of genital parts was observed in individuals with a larger body size. As in males, large variation in switch point of allometry was found between genital and nongenital parts.

In both sexes, dispersion around the mean (CV′) was relatively larger in nongenital parts than in genital parts, except for the median lobe that showed substantial large variation around the mean (Table 1). Residuals between nongenitalic traits showed significant correlations in both sexes (Table 2). Significant correlation between genitalic and nongenitalic traits was found only in females (mandible right–bursa copulatrix and prothorax–bursa copulatrix) whereas no correlation was significant in males.

DISCUSSION

The present study revealed that most genital parts possessed much lower allometric coefficients than nongenital parts. This is consistent with the prediction by Eberhard *et al*. (1998), in which individuals with intermediate genital size are favourably selected by stabilizing sexual selection. Stabilizing selection appears to play an important role in maintaining restricted size of genitalia in many organisms (Eberhard *et al*., 1998; Bernstein & Bernstein, 2002). Under this hypothesis, a unimodal distribution and a small amount of variation were predicted in the frequency distribution of characters. However, in *P. inclinatus*, the variation in genitalia did not agree with the expectations.

If developmental pattern of each genital part is canalized by stabilizing selection, the range of inflection points, as well as phenotypic variation, would be more restricted in genital parts than in nongenital parts that are considered to be free from such a selective regime. Indeed, the present study showed that genitalia were much less variable than other body parts as a whole, but also revealed that ranges of inflection points on allometric curve varied more in genital parts than in nongenital parts. To our knowledge, these are the first findings of this kind. Furthermore, the deviations from the expected mean (CV′) varied in both genital and nongenital parts. The latter two findings cannot be reconciled with stabilizing selection and

Table 2. Correlations of residuals of ordinary least-square regression between genitalic and nongenitalic body parts of male and female stag beetles, *Prosopocoilus inclinatus*

	Mandible left	Mandible right	Head	Prothorax	Sternite IX	Tergite IX	Tegmen
Mandible right	0.962*						
Head	0.621*	0.611*					
Prothorax	0.812*	0.785*	0.613*				
Sternite IX	0.088	0.134	−0.002	0.231			
Tergite IX	0.251	0.277	0.218	0.171	0.202		
Tegmen	0.126	0.173	0.097	0.150	0.321*	0.095	
Median lobe	−0.154	−0.188	−0.154	−0.048	0.267	0.139	−0.107
Female†							
Mandible right	0.493*						
Head	0.512*	0.342*					
Prothorax	0.705*	0.485*	0.771*				
Bursa copulatrix	0.203	0.271*	0.177	0.268*			

*Significant at 5% level after Bonferroni correction.
†Female spermatheca did not correlate with the size of the segments of mesothorax-abdomen (SMA) and thus the residuals were not estimated. Further details are provided in the text.

physiological constraints on genitalia. Recent theoretical and empirical studies have highlighted the importance of complex mating systems being associated with a change in genital morphology (Arnqvist, 1998; Cordoba-Aguilar, 2002). Provided that the functional differentiation of genital parts during or after coupling causes the difference in their relative growth patterns, the developmental mechanisms inducing this differential variability among genitalic parts are most interesting.

In the present study, although all allometric coefficients of genital parts were less than 1, some critical differences were found among genital parts. Relatively large CV and CV′ were found in the median lobe (43.62 and 42.70, respectively) compared with other genital parts (31.99–33.20). Interestingly, a large CV was also found in female genitalia (spermatheca: 86.45). Because the median lobe is inserted into the female genital opening, these two organs are very likely to interact mechanically and/or sensory during copulation. Such an interaction has been suggested to involve a coevolutionary arms race between sexes (Hosken & Stockley, 2004). A large CV, which might result from directional sexual selection (Pomiankowski & Moller, 1995), provides substantial potential for a rapid change that is necessary to cope with coadaptation of the opposite sex (Arnqvist & Rowe, 2002). Furthermore, the allometric curve of the median lobe did not have any inflection point whereas the spermatheca did not correlate with body size (SMA). These lines of evidence underpin the potential for a unique change by sexual selection in any of these characters.

The phenotypic correlations demonstrated that the male sternite and tergite, which belong to abdominal plates, were more strongly associated with body size than other genital parts such as the median lobe. Experimental evidence suggests that the presence/absence of an adjacent character affects the fate of the developmental pattern of other characters because of trade-offs in resource allocation (Nijhout & Wheeler, 1996; Klingenberg & Nijhout, 1998; Nijhout & Emlen, 1998). However, the results of the present study suggest that the growth of genitalic parts is not necessarily phenotypically correlated and support the view that resource competition for development is not necessarily restricted between traits developing from spatially close areas of the epithelium (for further discussion, see Moczek & Nijhout, 2004; Pigliucci & Preston, 2004; Tomkins, Kotiaho & LeBas, 2005).

Another finding worthy of note is that developmental patterns expressed as an allometric curve are more similar among male nongenital parts than female nongenital parts. One possible interpretation for such a sexual difference may be that the genetic architecture necessary for developing organs is different between sexes, and thus the pattern and timing of consuming nutritional resources are consequently different (Tatsuta, Mizota & Akimoto, 2004). Incorporative studies of developmental and genetic architecture of morphology will be useful to assess the prospects of the evolution of phenotypic integration.

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