

Genital variation within and between three closely related *Euxoa* moth species: testing the lock-and-key hypothesis

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Keywords

Euxoa; genital evolution; lock-and-key hypothesis; one-size-fits-all; sexual selection.

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Abstract

Genital morphology shows peculiar patterns of variation among insect species. Traditionally, genital species specificity has been assumed to serve as a mechanical isolation system between species (the lock-and-key hypothesis). Most recent studies suggest, however, that such variation may also be because of sexual selection. These two hypotheses give different predictions on genital variation within and between species. We tested the lock-and-key hypothesis by morphometrically exploring variation and allometry in male genitalia in three closely related *Euxoa* moth species. Single genital characteristics usually show overlap between species. As a whole, internal genitalia distinguish species better than external genitalia. The size of genitalia is generally correlated with body size, but the relationship is strongly negatively allometric so that the size of internal genitalia increases least with body size. These findings support the lock-and-key hypothesis. Both external and internal male genitalia show morphometric variation both within and between species, but the variation is significantly smaller in external genitalia. As internal genitalia are assumed to work as 'keys' in moths, this finding does not support the predictions of the lock-and-key hypothesis. Therefore, we cannot unambiguously support or reject this hypothesis. Our results agree well with the sexual selection hypotheses, particularly, the one-size-fits-all concept of the cryptic female choice hypothesis, which suggests stabilizing selection on genital size, but at the same time allows genital shape to vary relatively considerably.

Introduction

Rapid and divergent evolution of genitalia is common in several insect taxa, but evolutionary causes for differences are insufficiently understood (Eberhard, 1985, 1990, 1993; Shapiro & Porter, 1989; Alexander, Marshall & Cooley, 1997; Arnqvist, Thornhill & Rowe, 1997; Arnqvist & Thornhill, 1998; Hosken & Stockley, 2004). Differences in genitalia have led taxonomists to utilize genital characteristics in species-level classification; insects differing in genital morphology are considered biologically distinct species. Dufour (1844), according to Mayr (1963), was probably the first to pay attention to high genital variability. He assumed that species-specific genital differences function as reproductively isolating mechanisms in insects (i.e. the lock-and-key hypothesis) and the belief that genital differences imply biological isolation is based on this assumption.

The lock-and-key hypothesis makes several predictions about genital morphology (Eberhard, 1985; Arnqvist, 1997). First, between-species divergence in genitalia should be high in both sexes. Second, stabilizing selection on genitalia is assumed and therefore, within-species variation should be reduced. Third, as a consequence of low within-species variation, genital traits should be less condition dependent and their size should correlate less strongly with body size

than other traits. Finally, fertilization is not possible without the exact match of female and male genitalia. These predictions concern sympatrically living species in which reproductive character displacement is expected as a reproductive isolation mechanism against hybridization (Shapiro & Porter, 1989). Maximum attention has been paid to the variability of genitalia between species (Eberhard, 1985; Proctor, Baker & Gwynne, 1995), but studies on the other three predictions are rare.

Several hypotheses based on sexual selection have also been proposed to explain peculiar patterns of genital evolution. According to these hypotheses, genital evolution may be explained by cryptic female choice (Eberhard, 1985), sperm competition (Waage, 1979, 1984; Birkhead & Hunter, 1990; Birkhead & Møller, 1998; Haubruge *et al.*, 1999) or sexual conflict (Thornhill, 1984; Arnqvist, 1989; Alexander *et al.*, 1997; Arnqvist & Thornhill, 1998; Arnqvist & Rowe, 2002a, b). According to the cryptic female choice hypothesis, sperm of males with superior stimulating ability during copulation is preferred by females and this occurs either via Fisherian or good genes selection mechanisms. The sperm competition hypothesis assumes genital evolution to be because of male–male competition over egg fertilization. The sexual conflict hypothesis emphasizes different interests between males and females over reproductive decisions like

copulation time and duration. The sexual conflict is basically a consequence of different investments in gamete quality between the sexes. In theory, this conflict may easily lead to competition on superior ability over copulating decisions and thus evolutionary arms races of genital morphologies between sexes. Unlike the lock-and-key hypothesis, all sexual selection hypotheses assume polyandry and therefore do not predict rapid evolution in monogamous organisms. Similarly, contrary to the lock-and-key hypothesis, sexual selection hypotheses assume relatively high levels of genotypic and phenotypic variation in sexually selected traits. They do not require discrete interspecific variation between closely related species in genital characteristics either as does the lock-and-key hypothesis. Meanwhile, understanding the underlying processes affecting size variability in sexually selected traits has developed rapidly because of recent empirical findings (Eberhard *et al.*, 1998; Bernstein & Bernstein, 2002; Kawano, 2004) and theoretical considerations (Bonduriansky & Day, 2003).

Eberhard (1985) reviewed studies of genital morphology, and concluded that the majority of evidence was against the lock-and-key hypothesis. The evidence was largely based on scarcity of complicated structures in female genitalia in different taxa as well as theoretical difficulties of the hypothesis (e.g. natural selection should favour species recognition early rather than late in courtship). Subsequently, several morphometric tests of the lock-and-key hypothesis have also yielded negative results (Ware & Opell, 1989; Porter & Shapiro, 1990; Goulson, 1993; Arnqvist *et al.*, 1997; Arnqvist & Thornhill, 1998). Similarly, many observations concerning functional morphology and copulatory behaviour as well as different comparisons among taxa have not supported the lock-and-key hypothesis, but rather sexual selection hypotheses (Eberhard, 1985, 1991, 1992, 1993*a,b*, 2001; Heming-van Battum & Heming, 1989; Weislo & Buchmann, 1995; Tadler, 1996; Arnqvist, 1998; Arnqvist & Danielsson, 1999; Cordoba-Aguilar, 1999; Haubruge *et al.*, 1999; Tadler, Nemeschkal & Pass, 1999). Direct support for the lock-and-key hypothesis is presented only by Sota & Kubota (1998), who showed experimentally that the lack of mechanical correspondence causes injuries to the copulatory pieces of internal genitalia in two parapatric carabid beetles and by Kawano (2004), who found that beetle genital differentiation is the most noticeable under conditions where several morphologically similar species coexist.

Eberhard *et al.* (1998) showed that the relation of genital size to body size is generally strongly negatively allometric and that genital traits tend to be less variable than other traits in insects and spiders, patterns so far contradictory to sexual selection hypotheses (Green, 1992), but well consistent with the lock-and-key hypothesis (Arnqvist, 1997). Eberhard *et al.* (1998) did not interpret their results as support for the lock-and-key hypothesis, but designated a new hypothesis explaining the low slopes as a consequence of cryptic female choice. This hypothesis suggests that sexual selection favours males with genitalia of average size and is called the 'one-size-fits-all' hypothesis. Contrary to the lock-and-key hypothesis, one-size-fits-all allows overlap

in genital structures between species and allows variation in genital traits. Later, Bernstein & Bernstein (2002) provided data supporting Eberhard's *et al.* (1998) observations. However, it has recently been suggested that any form of sexual selection may produce any allometric pattern depending on selective regimes (Bonduriansky & Day, 2003), indicating that allometric patterns alone cannot be used safely in distinguishing between hypotheses.

It is known that sexual pheromones often show high divergence between species in moths and therefore might alone form a safe reproductive isolation barrier. However, there are many examples showing that these systems can fail. For example, females of the European moths *Furcula bicuspis* (Borkhausen, 1790) and *Dicallomera fascelina* (Linnaeus, 1758) regularly attract heterospecific males of related *Odontotia carmelita* (Esper, 1799) and *Calliteara abietis* (Denis & Schiffermüller, 1775), respectively (Marttila *et al.*, 1996). It is therefore possible that genital mechanisms might form backup devices against heterospecific fertilizations (Eberhard, 1985). In this paper, we specifically addressed the first three predictions of the lock-and-key hypothesis. We used three closely related *Euxoa* moth species as study organisms. Species of the genus *Euxoa* are polyandrous (Byers, 1978) and hence provide good opportunities for sexual selection. We explored whether the male genital structures of *Euxoa* differ enough to form safe lock-and-key mechanisms among three closely related, but undoubtedly biologically distinct species, which occur sympatrically on sandy meadows in coastal Finland. Within the subfamily Noctuidae, the genital differences are generally distinct especially in internal genitalia, but not so within the genus *Euxoa* (Hardwick & Lefkovich, 1973; Fibiger, 1990, 1997). We studied whether genital characteristics differ between species and to what extent they overlap. Secondly, we studied whether male genital structures show less variation than other morphological traits as predicted by the lock-and-key hypothesis. To accomplish this, we compared phenotypic variation of male external and internal genitalia with the variation of non-genital traits. Furthermore, we studied the correlation and allometry in different areas of genitalia in relation to body size, as different hypotheses predict differences in genital size dependence (Arnqvist, 1997; Eberhard *et al.*, 1998). Because of their rapid and divergent evolution in most Lepidoptera (e.g. Mikkola, 1992; Yela, 2002) as well as their exclusion from most studies, we also included traits of the endophallus (vesica) of the male internal genitalia in the analysis.

Materials and methods

The study organisms

Noctuid moths (Noctuidae) with about 25 000 known species (Fibiger, 1990) represent one of the most species-rich families of Lepidoptera. As is common in noctuid moths, characteristics of the male genitalia play the most important role in their taxonomic classification (Lafontaine, 1987; Fibiger, 1990; Fibiger, 1997). As the technique for artificial

endophallus eversion was developed, several new sibling species were described. The study species, *Euxoa cursoria* (Hufnagel, 1766), *E. obelisca* (Denis & Schiffmüller, 1775) and *E. tritici* (Linnaeus, 1761), are easily distinguishable by wing patterns and genitalia and there has never been doubt that they represent reproductively isolated species. The flight period of each species starts at the end of July and lasts until the beginning of September (Mikkola & Jalas, 1977). They are frequently found occurring together in the sandy coastal areas (Huldén *et al.*, 2000; Kullberg *et al.*, 2001), and thus provide favourable circumstances for testing the lock-and-key hypothesis.

Structure of genitalia

As in most moth species, genital structures of *Euxoa* moth males are elaborate. The strongly sclerotized male external genitalia consist of two bilaterally symmetrical clasping organs, valvae, by which the male attaches to the female abdomen at the beginning of copulation (Fig. 1). Valvae have two long horn-shaped projections: the clasper and the sacculus extension. These structures are derivations of abdominal tergites and sclerites. The penis (aedeagus) is situated in the middle of external genitalia. Internal genitalia are less rigid. At rest, the most conspicuous structure of internal genitalia, the endophallus, is folded inside the phallus (= penis or aedeagus). It is a retracted and membra-

nous structure, which does not become visible during normal genital dissection, likely the reason it has been neglected in most studies. It is everted into the female genital tract only during copulation. The size of the endophallus is several times the size of the phallus (see Fig. 1). The match between the special structures of the male endophallus and the female corpus bursae is argued to form an isolation mechanism in moths (Callahan & Chapin, 1960; Mikkola, 1992), although without supporting experimental data. The endophallus of *Euxoa* consists of an endophallus tube and its appendages (Fig. 1). Despite its less rigid structure, the shape of the single endophallus is constant when fully everted. This was confirmed by carrying out several successive everting and emptying operations.

Sample origin

All the specimens studied were collected from the southwestern archipelago of Finland, from the island of Örö (59°N 22°E). The samples are from a very abundant and somewhat isolated population, in which individuals of each species live together in the same habitat: sandy coastal meadows. The samples (110 specimens in total) were collected with sugar bait and light traps. All samples were collected in July–September 2001. The sampling period covered the entire flight period of all the species.

Preparation procedure

The body parts and the external genitalia were dissected in a standard way. The abdomen was first removed and boiled with 10% caustic potash. Genitalia were then removed from the softened surrounding tissues. The aedeagus was removed, and the external genitalia were dehydrated with ethanol and mounted on Euparal between the microscope slides and cover slips. In the endophallus eversion, Dang's (1993) procedure was followed. Because of their three-dimensional shape and fragile structure, the endophalli were stored in liquid Euparal.

Morphological traits and measurements

Only males were analysed. Females were not included here, because rapid genital evolution particularly concerns male genitalia (Eberhard, 1985), and because the three first predictions (see Introduction) can be tested using males only. In this study, we did not concentrate on the fourth prediction of the lock-and-key hypothesis, that corresponding structures should occur between sexes. Morphological traits were divided into three groups: non-genitalia, external genitalia and internal genitalia. The genital traits were divided into two groups, because male internal (intromittent) genitalia, as compared with external genitalia, are in closer contact with female genitalia during copulation. Parts of genitalia showing species specific characteristics were especially analysed. Five traits in each trait group were measured, so the total number of measurements per specimen was 15. In *E. cursoria* and *E. obelisca*, 30 males were analysed, whereas in *E. tritici* there were more specimens

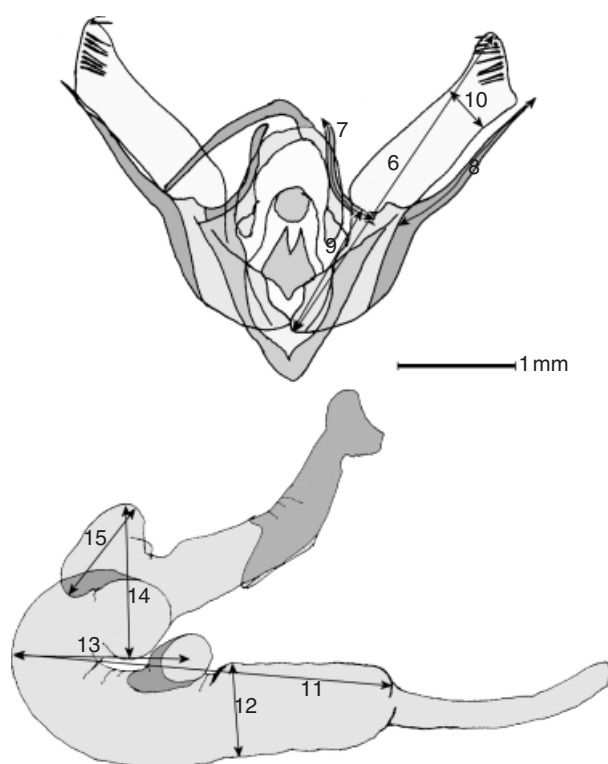


Figure 1 External (top) and internal (phallus: dark grey, endophallus: light grey) genitalia of *Euxoa tritici* and measured genitalic traits with their numbers as referred to in the text.

available and the number was 50. The five non-genitalic traits measured were the lengths of the second and the third segment of the right labial palpus, the lengths of the femur of the right hind leg, the second tibial spur of the right hind leg and the CuA2 vein of the right fore wing. In external genitalia, the measured traits were as follows: the length of the right valva (trait 6), the length of the right clavus (trait 7), the length of the right sacculus extension (trait 8), the distance from the base of the right valva to the clasper (trait 9) and the minimum width of the valva (trait 10). In internal genitalia, the following measurements were taken: the length of the vesica tube (trait 11), the maximum width of the vesica tube (trait 12), the distance from the curve of the vesica tube to the middle of the median diverticulum (trait 13), the maximum distance between the subbasal diverticulum and the additional subbasal diverticulum (trait 14) and the length of the subbasal diverticulum (trait 15). For clarity, the measured genitalic traits are shown with arrows in Fig. 1.

The genital preparations were separately photographed with an Olympus Camedia C-3030ZOOM (Japan) digital camera. The position of the endophallus was standardized, placing it laterally so that it lay freely in Euparal liquid. All measurements were performed by computer, using the ImageJ Java 1.34 image-processing program. To estimate measurement error, each measurement was taken twice but not consecutively to avoid systematic error.

Measurement error

To evaluate the significance of measurement error to the results, the percentage measurement error was calculated as follows: (Yezerinac, Lougheed & Handford, 1992):

$$\%ME = \frac{s_{\text{within}}^2}{s_{\text{within}}^2 + s_{\text{among}}^2} \times 100$$

The repeatability of measurements was then calculated as $100 - \%ME$.

The percentage measurement error values were mostly less than 1% of the total variance (mean 0.566, range 0.017–5.663), so the measurements were highly repeatable (99.4% on average). The measurements of different trait groups were equally repeatable (one-way ANOVA, $F_{2, 42} = 0.15$, $P = 0.864$). For these reasons, variables for further analyses were calculated averaging the repeated measurements.

Species specificity of genital morphology

To see whether each measured male genital trait differs between *E. cursoria*, *E. obelisca* and *E. tritici s. l.*, we used one-way ANOVA and Tukey's *post hoc* tests. This was studied by comparing whether the ranges of the measured variables overlap among species. Mechanical isolation between species may also be formed by interactions of several morphological traits, even in cases in which single characters overlap. To observe whether genital morphology as a whole differs among species and to estimate the amount of possible

overlap, we analysed the data with discriminant function analysis (DFA). It reanalyses the original traits and constructs new functions, which differentiate the given species as well as possible (Sokal & Rohlf, 1995). Because DFA by definition minimizes the overlap between species, it provides an effective way of testing the prediction of the lock-and-key hypothesis that species are morphologically dissimilar. It also enables comparison between reclassification and the given classification. The discriminant analysis was performed separately for external and internal genitalia. The patterns of genital differences, variation and overlap are presented with scatterplots.

Intraspecific variation in genital and non-genital traits

To compare the amount of variation between non-genital, external genital and internal genital traits, original variables were standardized by first subtracting the species-specific mean of each variable from the original measures and then dividing this deviation by the mean. Finally, absolute values were returned. We divided deviations by the mean because we wanted to retain differences in variance among measurements while removing the effects of differing mean values. The data now consisted of values that describe the deviation of the original measures from the species mean for that variable and that are independent of the mean. The null hypothesis is that deviations in the three trait groups come from the same underlying process, resulting in equal amounts of variability in each of the three trait groups. To test this null hypothesis we ran repeated measures ANOVA with five measures within each trait group as repeated measures. Repeated measures ANOVA was applied because variation of a measurement is not independent of variation in other measurements within a trait group as they were taken from the same individuals and partly from the same organs.

Correlation between body size and genital size

We derived a variable representing body size by principal component analysis (PCA) on non-genitalic traits. PCA reduces information of several correlated variables into fewer orthogonal principal components. Generally, with morphological data, the first principal component can be used as a size variable, particularly if the original variables correlate approximately equally well and in the same direction as the component (Ricklefs & Travis, 1980). This is the case in our data. The original variables were all highly positively correlated with the first PC-axis (range 0.703–0.961, $n = 5$). The first component of the PCA of the non-genitalic traits explained 75.45% (eigenvalue 3.77) and the second explained 12.00% (eigenvalue 0.60) of the total variance. Thus, in the statistical study, it was reasonable to apply PCA factor 1 as a single non-genitalic size factor instead of five separate non-genitalic traits.

The correlations between the body size and the genital traits were then calculated one by one using Pearson's product-moment correlation coefficient. The size distribution of none of the traits appeared significantly different from a normal distribution (Kolmogorov-Smirnov, P -values between 0.111 and 0.998), so no transformation was needed prior to analyses.

To see whether the relationship between the body size and size of genital traits is isometric, we used linear ordinary least squares regression analysis to test whether or not the slope of a trait (log transformed) on body size (log transformed) deviated from the expected value (unity = isometric relationship). There has recently been a debate as to which regression methods should be used to evaluate isometry or allometry of genitalia (Eberhard *et al.*, 1998; Eberhard, Huber & Rodriguez, 1999; Green, 1999). As claimed by Eberhard *et al.* (1999) and later shown by Bernstein & Bernstein (2002), the different regression methods do not seem to have significant influences on the results and the eventual conclusions. We used the length of the right hind femur as a body size variable in allometry analyses. It is a good proxy for body size as its correlation with the PCA size factor was very high ($r = 0.925$).

We also compared whether or not observed negative allometry was equal in both genital trait groups. We first derived PCA size factors independently for each trait group and for each species based on five traits. We then calculated regression slopes between non-genital size factor and genital size factors by linear regression. These slope values were then subjected to repeated measurements ANOVA to test whether there is a difference between the two genital trait groups in how they relate to body size.

Results

Species specificity of genital morphology

According to the ANOVA, each measured genital trait shows clear differences between *E. tritici*, *E. cursoria* and *E. obelisca* (Table 1). In seven out of 10 cases, this was caused by differences between two out of three species. In

five out of these seven cases, the lack of difference was detected between *E. cursoria* and *E. obelisca* and in two cases differences between *E. obelisca* and *E. tritici*. *Euxoa cursoria* and *E. tritici* were distinguishable at $P \leq 0.005$ in all cases. The lengths of valvae, vesica tubes and subbasal diverticuli (traits 6, 11 and 15) differed significantly among all three species. Although the means of traits differed in many cases, a significant overlap was regularly found.

The discriminant analysis gave two functions, of which the first covered 67.2% (eigenvalue 3.845) and the second covered 32.8% (eigenvalue 1.876) of the variance in external genitalia and 85.2 and 14.8% in internal genitalia, respectively. In reclassification of the individuals, five specimens were incorrectly classified on the basis of functions of external genitalia, while only one specimen was misclassified according to internal genital functions. Thus, despite overlap in many single characters in both genital areas, the species were relatively well distinguished by discriminant functions and markedly better by internal than by external genitalia (see Fig. 2).

Intraspecific variation in genital and non-genital traits

Comparison of trait groups indicated that the amount of variation differs significantly among them (repeated measurements ANOVA, $F_{2,73} = 7.87$, $P = 0.001$). Pairwise comparisons revealed that this is mainly caused by the external genitalia, in which the amount of variation deviated from that of non-genitalia ($P = 0.006$) as well as from that of internal genitalia ($P = 0.012$). Variation in internal genitalia was equal to non-genitalia ($P = 1.00$). Comparison of means shows that variation in external genitalia is lower than in non-genital traits and internal genitalia (Fig. 3). As the interaction term between trait groups and species was not significant ($F_{4, 148} = 0.44$, $P = 0.782$), the pattern is similar in all three species.

Correlation between body and genital trait sizes and tests of isometry

In general, genital traits correlated positively with body size, particularly in *E. tritici* in which all 10 correlations were

Table 1 ANOVA test statistics and Tukey's *post hoc* tests of genital trait differences between species

	ANOVA		<i>Euxoa cursoria</i> versus <i>E. obelisca</i>	<i>E. cursoria</i> versus <i>E. tritici</i>	<i>E. obelisca</i> versus <i>E. tritici</i>
	<i>F</i>	<i>P</i>			
Trait 6	$F_{2,106} = 28.69$	<0.001	0.003	0.001	<0.001
Trait 7	$F_{2,104} = 24.73$	<0.001	0.206	<0.001	<0.001
Trait 8	$F_{2,107} = 21.82$	<0.001	0.199	<0.001	<0.001
Trait 9	$F_{2,106} = 101.90$	<0.001	0.203	<0.001	<0.001
Trait 10	$F_{2,107} = 92.67$	<0.001	<0.001	<0.001	0.838
Trait 11	$F_{2,107} = 46.77$	<0.001	<0.001	<0.001	<0.001
Trait 12	$F_{2,94} = 90.88$	<0.001	0.295	<0.001	<0.001
Trait 13	$F_{2,107} = 11.19$	<0.001	0.516	0.005	<0.001
Trait 14	$F_{2,101} = 76.09$	<0.001	<0.001	<0.001	0.516
Trait 15	$F_{2,97} = 50.87$	<0.001	<0.001	<0.001	<0.001

For traits, see Fig. 1.

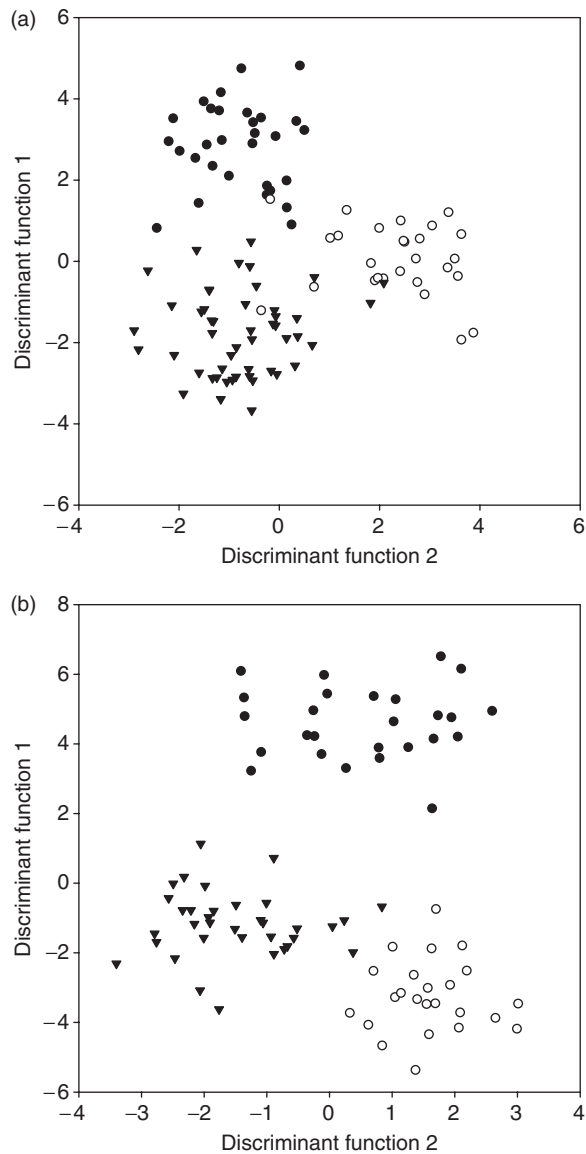


Figure 2 Morphometric overlap in genital structure according to discriminant functions in external (top) and internal (bottom) genitalia among three *Euxoa* species. Black dots, *E. cursoria*; open circles, *E. obelisca*; triangles, *E. tritici*.

significant ($r = 0.29\text{--}0.79$, all $P < 0.05$). In *E. obelisca*, seven out of 10 possible correlations were significant ($r = 0.15\text{--}0.74$) but in *E. cursoria* only five significant correlations were found ($r = 0.06\text{--}0.64$). When a correlation was observed, it was typically statistically highly significant ($P \leq 0.001$ in 12 cases and $P \leq 0.01$ in 16 cases out of 22 significant values). Correlations were statistically highly significant ($P \leq 0.001$) for male genital length and width (traits 6 and 10) and the distance from the base of the right valva to the clasper (trait 9) in all three species. The most remarkable exception from the general line was the internal genitalia of *E. cursoria*, where only one significant correlation was found (trait 14, $P = 0.046$).

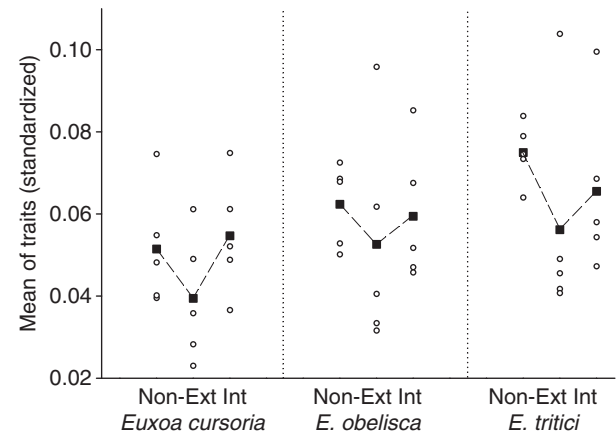


Figure 3 Standardized means of traits (open circles) and means of five traits of each trait group (black squares) of the study species. Non, non-genitalic traits; Ext, external genitalic traits; Int, internal genitalic traits.

Table 2 Regression slopes and their 95% confidence limits (in parentheses) of log-transformed genital traits on log-transformed body size

	<i>Euxoa cursoria</i> <i>n</i> = 30	<i>E. obelisca</i> <i>n</i> = 30	<i>E. tritici</i> <i>n</i> = 50
External genitalia			
Trait 6	0.377 (0.163–0.591)	0.554 (0.380–0.728)	0.487 (0.360–0.613)
Trait 7	0.207 (–0.141–0.554)	0.268 (–0.060–0.597)	0.466 (0.284–0.649)
Trait 8	0.665 (0.166–1.164)	0.143 (–0.322–0.609)	0.262 (0.046–0.477)
Trait 9	0.492 (0.283–0.701)	0.460 (0.272–0.648)	0.390 (0.236–0.543)
Trait 10	0.900 (0.382–1.418)	1.304 (0.777–1.831)	1.115 (0.761–1.470)
Internal genitalia			
Trait 11	0.237 (–0.128–0.603)	0.387 (0.053–0.721)	0.290 (0.088–0.493)
Trait 12	0.426 (–0.169–1.021)	0.404 (0.060–0.749)	0.356 (0.039–0.674)
Trait 13	0.235 (–0.224–0.695)	0.538 (0.185–0.890)	0.383 (0.178–0.588)
Trait 14	0.524 (0.016–1.032)	0.481 (0.060–0.901)	0.547 (0.301–0.792)
Trait 15	0.259 (–0.572–1.091)	0.544 (–0.098–1.186)	0.571 (0.079–1.064)

Slopes significantly different from unity (isometric relationship) are shown in bold type.

The relationship between body size and genital traits was in general strongly negative allometric (i.e. slopes significantly below unity) (Table 2 and Fig. 4). The only clear deviation from the general pattern was the width of male valva (trait 10), where the slopes were isometric. Moreover, although less than one, the deviation from isometry was not

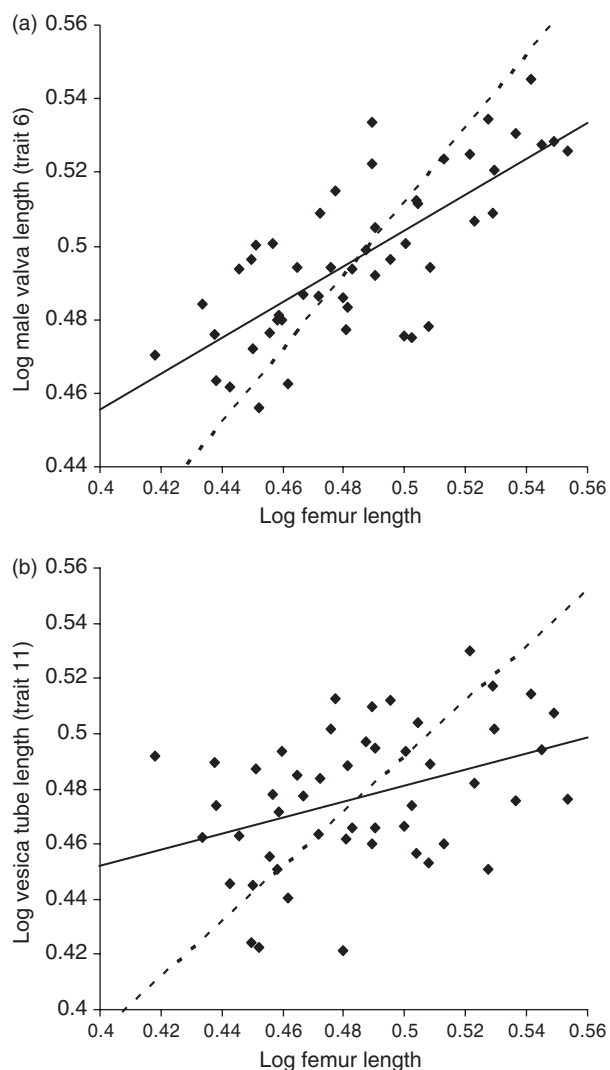


Figure 4 Negative allometry between body size and male valva length (trait 6) of external genitalia (a) and trait 11 of internal genitalia (b) in *Euxoa tritici*.

significant in the length of sacculus extension (trait 8) of *E. cursoria*, traits 12 and 14 of internal genitalia of *E. cursoria* and the length of the subbasal diverticulum (trait 15) in all three species. The relationship between body size (hind femur length) and other non-genital traits was isometric in all cases ($n = 12$), except in two traits in *E. cursoria*, in which the slopes showed slight negative allometry.

The comparison of the strength of negative allometry among the genital trait groups may indicate that it was more pronounced in internal genitalia. The difference was nearly significant between the genital trait groups (repeated measurements ANOVA, $F_{1,73} = 3.42$, $P = 0.068$), suggesting that regression slopes between internal genital size and body size were less steep (i.e. negative allometry more pronounced) than slopes between external genital size and body size (for specific instances, see Fig. 4).

Discussion

This study clarifies patterns of morphometric variation and allometry in different parts of male genitalia in moths. The results show that there is an overlap in genital features among closely related *Euxoa* species. As a whole, internal genitalia distinguish the species nearly unambiguously into morphologically discrete clusters and make this clearly superior to external genitalia in distinguishing species. Genital morphology varies within *Euxoa* species, but the variation in external genitalia is significantly lower than in the other trait groups. The size of genitalia is generally positively correlated with body size, but the relation between body size and sizes of genital traits is not isometric, the traits of external genitalia being negatively allometric and the traits of internal genitalia being the least dependent on body size. Low variation was found in external genitalia, and although the decreased size dependence (negative allometry) was found in all parts of genitalia, the pattern was most pronounced in internal genitalia.

Our results are consistent with the predictions of the one-size-fits-all hypothesis, but they are not fully inconsistent with the lock-and-key hypothesis either. They both predict negative allometry slopes in genitalia. We found that variation in external genital structures was reduced, but also that they showed overlap between species. They probably cannot form barriers for successful sperm transfer as they do not act in this process. On the other hand, we did not find reduced variation in internal genitalia, which, however, provided more reliable diagnostic characteristics than external genital ones. Corresponding structures between the sexes have been reported in internal genitalia in moths (Mikkola, 1992). This match could therefore function as a reproductive barrier against heterospecific sperm transfer. Although it is unknown how much genital divergence is required for mechanical isolation in terms of the lock-and-key hypothesis, we consider it quite improbable that the divergence in the study species might allow safe mechanical isolation, because extreme genital types within species are clearly more different than closest types between the species. There is also further evidence that supports this opinion. Byers & Hinks (1978) experimentally studied mating discrimination between three genitally similar, but distinguishable *Euxoa* species. They found that in the absence of homogamic males, females mated readily with genitally different, heterogamic males. More importantly, they found that the lack of full genital compatibility between sexes did not lead to unsuccessful sperm transfer, but frequently yielded viable offspring. They also found that in the presence of both heterogamic and homogamic males, females regularly chose homogamic males. This strongly emphasizes the importance of premating behaviour as an isolation mechanism, and on the other hand indicates that small genital differences do not necessarily cause failure in sperm transfer. Despite the finding of some overlap in genital morphology, the lock-and-key mechanisms could still be explained by the invariability of female genitalia and that extreme male genital types would represent extreme limits that are still compatible with

morphologically stable female genitalia. However, there is evidence of high intraspecific variation in female genitalia in *E. tritici* (Mutanen, 2005), suggesting that they are not under stabilizing selection, either.

Sexual selection is assumed to be directional in form and should therefore favour exaggeration of sexually selected traits. Also, it has been assumed that sexually selected traits should express positive allometric slopes in relation to body size (Alatalo, Höglund & Lundberg, 1988; Green, 1992; Eberhard *et al.*, 1998; Cuervo & Møller, 2001). This holds true especially in sexual conflict and sperm competition hypotheses, because males with small genitalia are assumed to be less capable of fertilizing eggs than males with large genitalia. However, this reasoning was recently questioned by Bonduriansky & Day (2003), who showed by modelling experiments that positive allometry in sexually selected traits is favoured only under a very limited set of conditions and that sexual selection may produce any pattern of allometry or isometry, depending on the relative fitness of body size and trait size. Thus, negative allometry in rapidly evolving genital structures might not only be because of stabilizing selection towards genital size (lock-and-key and one-size-fits-all hypotheses) but also because of sexual conflict and sperm competition. House & Simmons (2003) also emphasized that size scaling of genitalia is still relatively little discussed and studied in the framework of sexual selection or sperm competition. Available data in a variety of taxa, including *Euxoa* moths, indicate that genital size is rarely, if ever, positively allometric in relation to body size (Eberhard *et al.*, 1998; Bernstein & Bernstein, 2002; Kawano, 2004). Most often, genitalia show negative allometry. For example, Eberhard *et al.* (1998) found that in all 20 studied taxa, the median allometric coefficient was lower than the median non-genitalic value. In many studies concentrating on size variability, including the present one, the generality of negative allometry in genitalia could partly be explained by the close relatedness and hence statistical independence of the organisms. It is possible that in the study moths, negative allometry in genitalia was already present in their common ancestor and therefore the three species cannot be treated as independent evidence for the generality of low variation in genital size. The generality of this pattern across the variety of insect and spider taxa, however, suggests that either there is a common pattern of stabilizing selection towards intermediate genital size or, alternatively, directional selection towards a larger body size in relation to genital size. As we are unaware of theories suggesting directional selection towards a larger overall body size in insects, we consider the latter possibility as less probable, but emphasize that we are not able to directly show stabilizing selection.

Our result concerning the positive correlation between genital traits and body size supports earlier findings of a general positive relationship between the size of genitalia and body size (Coyne, 1983; Goulson, 1993). This correlation is sometimes interpreted as evidence against the lock-and-key hypothesis. The significance of a correlation coefficient is, however, dependent on sample size and even in the

case of very strong negative allometry, highly significant positive correlations may emerge between a trait and body size. We found that even though genital traits were positively correlated with body size, scaling of genital size to body size was far from being isometric but showed significant negative allometry. Therefore, studies on size scaling should concentrate on allometric patterns, not correlation coefficients.

An essential problem in criticism against the lock-and-key hypothesis is that internal genital structures are excluded in many studies. Goulson (1993) and Sota & Kubota (1998) emphasized that morphological studies should be aimed at all functional areas of genitalia. The retractable endophallus is almost always neglected although the sperm is transferred into the female tracts through it. Eberhard (1985) concluded the evolution of internal genitalia to be less rapid and less divergent. However, this is not the case in Lepidoptera or in some other insect orders, as modern taxonomic revisions are often predominantly based on the differences in male endophalli. In taxonomy, the male endophallus provides helpful characteristics, but whether genital differences tell something about the biological relationships of species living in sympatry or allopatry remains to be tested. The endophallus is assumed to work as a lock-and-key mechanism in moths, because of its generally very rapid and divergent evolution as well as many apparent structural correspondences between male endophalli and female genital tracts (Callahan & Chapin, 1960; Mikkola, 1992). It is, however, important to note that match in genitalia is not evidence for the lock-and-key hypothesis, because other hypotheses, like sexual selection and sperm competition, may also yield correlated evolution of genitalia between the sexes (Thornhill, 1984; Waage, 1984; Arnqvist & Rowe, 2002*a, b*).

The lock-and-key hypothesis is favoured by taxonomists, although apparently sometimes unconsciously. This is also the case in moth taxonomy as even many recent revisions rely on genital invariability and genital differences as an isolation system (see e.g. Fibiger, 1990, 1997; Troubridge, 1997; Hacker, 1998), and few data on genital variability are presented in this context. For example, allopatric populations with only slight genital differences and very restricted material may be described as 'good' species. Our results indicate that there is evidently much morphological variation in genitalia. As the lock-and-key hypothesis has remained poorly supported, utmost care in morphological taxonomy should be extended to also include genital characteristics.

In conclusion, it can be stated that the low allometric slopes of invertebrate genitalia against body size are probably a common pattern and that genital morphology shows not only interspecific but also clear intraspecific variation. There is increasing evidence that sexual selection may be responsible for genital variability (for a review, see Hosken & Stockley, 2004). Most existing data suggest the sexual selection to play a major role in genital divergence, but it is still inadequately known whether and how much the mechanical correspondence between sexes is necessary for

successful sperm transfer. Studies concentrating on experimentally testing sperm transfer from one species to another would be of particularly great importance.

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References

- Alatalo, R.V., Höglund, J. & Lundberg, A. (1988). Patterns of variation in tail ornament size in birds. *Biol. J. Linn. Soc.* **34**, 363–374.
- Alexander, R.D., Marshall, D.C. & Cooley, J.R. (1997). Evolutionary perspectives on insect mating. In *The evolution of mating systems in insects and arachnids*: 4–31. Choe, J.C. & Crespi, B.J. (Eds). Cambridge: Cambridge University Press.
- Arnqvist, G. (1989). Multiple mating in a water strider: mutual benefits or intersexual conflict? *Anim. Behav.* **38**, 749–756.
- Arnqvist, G. (1997). The evolution of animal genitalia: distinguishing between hypotheses by single species studies. *Biol. J. Linn. Soc.* **60**, 365–379.
- Arnqvist, G. (1998). Comparative evidence for the evolution of genitalia by sexual selection. *Nature* **393**, 784–786.
- Arnqvist, G. & Danielsson, I. (1999). Copulatory behavior, genital morphology, and male fertilization success in water spiders. *Evolution* **53**, 147–156.
- Arnqvist, G. & Rowe, L. (2002a). Correlated evolution of male and female morphologies in water striders. *Evolution* **56**, 936–947.
- Arnqvist, G. & Rowe, L. (2002b). Antagonistic coevolution between the sexes in a group of insects. *Nature* **415**, 787–789.
- Arnqvist, G. & Thornhill, R. (1998). Evolution of animal genitalia: patterns of phenotypic and genotypic variation and condition dependence of genital and non-genital morphology in water strider (Heteroptera: Gerridae: Insecta). *Genet. Res.* **71**, 193–212.
- Arnqvist, G., Thornhill, R. & Rowe, L. (1997). Evolution of animal genitalia: morphological correlates of fitness components in a water strider. *J. Evol. Ecol.* **10**, 613–640.
- Bernstein, S. & Bernstein, R. (2002). Allometry of male genitalia in a species of soldier beetle: support for the one-size-fits-all hypothesis. *Evolution* **56**, 1707–1710.
- Birkhead, T.R. & Hunter, F.M. (1990). Mechanisms of sperm competition. *Trends Ecol. Evol.* **5**, 48–52.
- Birkhead, T.R. & Møller, A.P. (1998). *Sperm competition and sexual selection*. London: Academic Press.
- Bonduriansky, R. & Day, T. (2003). The evolution of static allometry in sexually selected traits. *Evolution* **57**, 2450–2458.
- Byers, J.R. (1978). Biosystematics of the genus *Euxoa* (Lepidoptera: Noctuidae) X. Incidence and level of multiple mating in natural and laboratory populations. *Can. Entomol.* **110**, 193–200.
- Byers, J.R. & Hinks, C.F. (1978). Biosystematics of the genus *Euxoa*. XI. Mating discrimination between three closely related species of the *declarata* group. *Can. J. Zool.* **56**, 1981–1987.
- Callahan, P.S. & Chapin, J.B. (1960). Morphology of the reproductive systems and mating in two representative members of the family Noctuidae, *Pseudaletia unipuncta* and *Peridroma margaritosa*, with comparison to *Heliothis zea*. *Ann. Ent. Soc. Am.* **53**, 768–782.
- Cordoba-Aguilar, A. (1999). Male copulatory sensory stimulation induces female ejection of rival sperm in a damselfly. *Proc. R. Soc. Lond. B* **266**, 779–784.
- Coyne, J.A. (1983). Genetic basis of differences in genital morphology among three sibling species of *Drosophila*. *Evolution* **37**, 1101–1118.
- Cuervo, J.J. & Møller, A.P. (2001). Components of phenotypic variation in avian ornamental and non-ornamental feathers. *Evol. Ecol.* **15**, 53–72.
- Dang, P.T. (1993). Vesicas of selected tortricid and small lepidopterous species, with descriptions of new techniques of vesica eversion (Lepidoptera: Tortricidae, Oecophoridae, Gelechiidae, and Nepticulidae). *Can. Entomol.* **125**, 785–799.
- Dufour, L. (1844). Anatomie Générale des Dipteres. *Ann. Sci. Nat.* **1**, 244–264.
- Eberhard, W.G. (1985). *Sexual selection and animal genitalia*. Massachusetts: Harvard University Press.
- Eberhard, W.G. (1990). Animal genitalia and female choice. *Am. Sci.* **78**, 134–140.
- Eberhard, W.G. (1991). Copulatory courtship and cryptic female choice in insects. *Biol. Rev. Camb. Philos. Soc.* **66**, 1–31.
- Eberhard, W.G. (1992). Species isolation, genital mechanics, and the evolution of species-specific genitalia in three species of *Macroductylus* beetles (Coleoptera, Scarabaeidae, Melolonthinae). *Evolution* **46**, 1774–1783.
- Eberhard, W.G. (1993). Evaluating models of sexual selection: genitalia as a test case. *Am. Nat.* **142**, 564–571.
- Eberhard, W.G. (1993a). Copulatory courtship and morphology of genitalic coupling in seven *Phyllophaga* species (Coleoptera: Melolonthidae). *J. Nat. Hist.* **27**, 683–717.

- Eberhard, W.G. (1993b). Copulatory courtship and genital mechanics of three species of *Macrodactylus* (Coleoptera Scarabaeidae Melolonthinae). *Ethol. Ecol. Evol.* **5**, 19–63.
- Eberhard, W.G. (2001). Species-specific genitalic copulatory courtship in sepsid flies (Diptera, Sepsidae, *Microsepsis*) and theories of genitalic evolution. *Evolution* **55**, 93–102.
- Eberhard, W.G., Huber, B.A. & Rodriguez, R.L. (1999). Don't forget the biology: a reply to Green. *Evolution* **53**, 1624–1627.
- Eberhard, W.G., Huber, B.A., Rodriguez, R.L.S., Briceno, R.D., Salas, I. & Rodriguez, V. (1998). One size fits all? Relationships between the size and degree of variation in genitalia and other body parts in twenty species of insects and spiders. *Evolution* **52**, 415–431.
- Fibiger, M. (1990). *Noctuidae I. Noctuidae Europaeae*. Sorø: Entomological Press.
- Fibiger, M. (1997). *Noctuidae III. Noctuidae Europaeae*. Sorø: Entomological Press.
- Goulson, D. (1993). Variation in the genitalia of the butterfly *Maniola jurtina* (Lepidoptera: Satyrinae). *Zool. J. Linn. Soc.* **107**, 65–71.
- Green, A.J. (1992). Positive allometry is likely with mate choice, competitive display and other functions. *Anim. Behav.* **43**, 170–172.
- Green, A.J. (1999). Allometry of genitalia in insects and spiders: one size does not fit all and reply. *Evolution* **53**, 1621–1624.
- Hacker, H. (1998). Revision der Gattungen *Hadula* Staudinger, 1889 (= *Discestra* Hampson, 1905; = *Aglossastra* Hampson, 1905; = *Cardiastrea* Boursin, 1963), *Anartomorpha* Alphéraky, 1892, *Trichanarta* Hampson, 1895, *Anarta* Ochsenheimer, 1816 und *Cardepija* Hampson, 1905 mit Beschreibung einer neuen Gattung *Hadumorphia* gen. n. (Lepidoptera, Noctuidae). *Esperiana* **6**, 577–843.
- Hardwick, D.F. & Lefkovitch, L.P. (1973). Species separation in the *declarata* group of the genus *Euxoa*, a computer analysis based on structural characters. *Can. Entomol.* **105**, 501–508.
- Haubruege, E., Arnaud, L., Mignon, J. & Gage, M.J.G. (1999). Fertilization by proxy: rival sperm removal and translocation in a beetle. *Proc. R. Soc. Lond. B* **266**, 1183–1187.
- Heming-van Battum, K.E. & Heming, B.S. (1989). Structure, function, and evolutionary significance of the reproductive system in males of *Hebrus ruficeps* and *H. pusillus* (Heteroptera, Gerromorpha, Hebridae). *J. Morphol.* **202**, 281–323.
- Hosken, D.J. & Stockley, P. (2004). Sexual selection and genital evolution. *Trends Ecol. Evol.* **19**, 87–93.
- House, C.M. & Simmons, L.W. (2003). Genital morphology and fertilization success in the dung beetle *Onthophagus taurus*: an example of sexually selected male genitalia. *Proc. R. Soc. Lond. B* **270**, 447–455.
- Huldén, L., Albrecht, A., Itämies, J., Malinen, P. & Wettenhovi, J. (2000). *Atlas of Finnish Macrolepidoptera*. Helsinki: Suomen Perhostutkijain Seura-Luonnontieteellinen keskusmuseo.
- Kawano, K. (2004). Developmental stability and adaptive variability of male genitalia in sexually dimorphic beetles. *Am. Nat.* **163**, 1–15.
- Kullberg, J., Albrecht, A., Kaila, L. & Varis, V. (2001). Checklist of Finnish Lepidoptera. *Sahlbergia* **6**, 45–190.
- Lafontaine, J.D. (1987). *Noctuoidea: Noctuidae*. In *The moths of America North of Mexico*. Dominick, R.B. (Ed.). Washington: The Wedge Entomological Research Foundation.
- Marttila, O., Saarinen, K., Aarnio, H. & Ojalainen, P. (1996). *Suomen kiitäjät ja kehrääjät*. Helsinki: Kirjayhtymä.
- Mayr, E. (1963). *Animal species and evolution*. Massachusetts: Harvard University Press.
- Mikkola, K. (1992). Evidence for lock-and-key mechanisms in the internal genitalia of the *Apamea* moths (Lepidoptera, Noctuidae). *Syst. Entomol.* **17**, 145–153.
- Mikkola, K. & Jalas, I. (1977). *Suomen Perhoset. Yökköset I*. Helsinki: Otava.
- Mutanen, M. (2005). Delimitation difficulties in species splits: a morphometric case study on the *Euxoa tritici* complex (Lepidoptera, Noctuidae). *Syst. Entomol.*, **30**, 632–643.
- Porter, A.H. & Shapiro, A.M. (1990). Lock-and-key hypothesis: lack of mechanical isolation in a butterfly (Lepidoptera: Pieridae) hybrid zone. *Ann. Ent. Soc. Am.* **83**, 107–114.
- Proctor, H.C., Baker, R.L. & Gwynne, D.T. (1995). Mating behaviour and spermatophore morphology: a comparative test of the female-choice hypothesis. *Can. J. Zool.* **73**, 2010–2020.
- Ricklefs, R.E. & Travis, J. (1980). A morphological approach to the study of avian community organization. *Auk* **97**, 321–338.
- Shapiro, A.M. & Porter, A.H. (1989). The lock-and-key hypothesis: evolutionary and biosystematic interpretation of insect genitalia. *Ann. Rev. Entomol.* **34**, 231–245.
- Sokal, R.R. & Rohlf, F.J. (1995). *Biometry*. New York: W. H. Freeman.
- Sota, T. & Kubota, K. (1998). Genital lock and key as a selective agent against hybridization. *Evolution* **52**, 1507–1513.
- Tadler, A. (1996). Functional morphology of genitalia of four species of julidan millipedes (Diplopoda: Nemasomatidae; Julidae). *Zool. J. Linn. Soc.* **118**, 83–97.
- Tadler, A., Nemeschkal, H.L. & Pass, G. (1999). Selection of male traits during and after copulation in the seedbug *Lygaeus simulans* (Heteroptera, Lygaeidae). *Biol. J. Linn. Soc.* **68**, 471–483.
- Thornhill, R. (1984). Alternative hypotheses for traits believed to have evolved by sperm competition. In *Sperm competition and the evolution of animal mating systems*: 151–178. Smith, R.L. (Ed.). Orlando: Academic Press.
- Troubridge, J.T. (1997). Revision of the nearctic species of the genus *Entephria* Hübner (Lepidoptera, Larentiinae). *Entomol. Scand.* **28**, 121–139.

- Waage, J.K. (1979). Dual function of the damselfly penis: sperm removal and transfer. *Science* **203**, 916–918.
- Waage, J.K. (1984). Sperm competition and the evolution of Odonate mating systems. In *Sperm competition and the evolution of animal mating systems*: 251–290. Smith, R.L. (Ed.). Orlando: Academic Press.
- Ware, A.D. & Opell, B.D. (1989). A test of the mechanical isolation hypothesis in two similar spider species. *J. Arachnol.* **17**, 149–162.
- Wcislo, W.T. & Buchmann, S.L. (1995). Mating behaviour in the bees, *Dieunomia heteropoda* and *Nomia tetrazonata*, with a review of courtship in Nomiinae (Hymenoptera: Halictidae). *J. Nat. Hist.* **29**, 1015–1027.
- Yela, J.L. (2002). The internal genitalia as a taxonomic tool: description of the relict endemic moth, *Coranarta restricta* sp. n., from the Iberian Peninsula (Lepidoptera: Noctuidae: Hadeninae). *Entomol. Fenn.* **13**, 1–12.
- Yezerinac, S.M., Lougheed, S.C. & Handford, P. (1992). Measurement error and morphometric studies: statistical power and observer experience. *Syst. Biol.* **41**, 471–482.