Genital variation in a dimorphic moth
Selenia tetralunaria (Lepidoptera, Geometridae)

MARKO MUTANEN* and ARJA KAITALA
Department of Biology, University of Oulu, PO Box 3000, FIN-90014 University of Oulu, Finland

Received 16 August 2004; accepted for publication 9 March 2005

INTRODUCTION

Insect genitals vary greatly among species and provide a key tool for species-level taxonomy. Insects differing in the genitalia are often treated as discrete, reproductively isolated species. This principle dates back to the lock-and-key hypothesis, which states that genitalia vary between species in order to provide a mechanical reproductive isolation system. Thus, the hypothesis assumes low within-species variability in genital traits. However, recent studies suggest that sexual selection may be responsible for the evolution of insect genitalia. We studied allometry and genital size and shape variation in a dimorphic moth Selenia tetralunaria. We found that the genitalia showed negative allometry in relation to body size as reported in many insect and spider species. This allometry was stronger in internal genital structures than it was in external genitalia. We also found that there was more variation in internal compared with external genitalia. Finally, we found that the shape of genital structures differed between morphs in all three examined areas. S. tetralunaria is among the first reported cases of genitally dimorphic insect species. Considerable variation in internal genitalia and especially the presence of genital shape differences between morphs were not consistent with the predictions of the lock-and-key hypothesis. © 2006 The Linnean Society of London, Biological Journal of the Linnean Society, 2006, 87, 297–307.


*Corresponding author: E-mail: marko.mutanen@oulu.fi

© 2006 The Linnean Society of London, Biological Journal of the Linnean Society, 2006, 87, 297–307
morphological variation in any trait, including genital characteristics, but rather they predict it in certain circumstances (Jocque, 2002). If genital differences have not evolved to prevent sperm transfer from one species to another and if reproductive isolation does not occur incidentally as a by-product of genital diversification, genital polymorphism could well also be found between temporally or geographically isolated populations. Despite the supposed occurrence, there are few documented observations of genitally polymorphic insects species.

*Selenia tetralunaria* (Hufnagel, 1767) is, like its close relatives, a nonsexually dimorphic geometrid moth (Mikkola, Jalas & Peltonen, 1989; Skou, 1991). The morphs are similar genetically but differ morphologically. This is due to their different life histories; specimens of the partial second generation lack their final larval instar (Mikkola et al., 1989). The second generation specimens are smaller and differ also in wing shape and patterns (Mikkola et al., 1989; Skou, 1991) (Fig. 1). Genital differences of the morphs have never been studied systematically. Since some larvae of the second generation may have a full number of instars and some pupae of the second generation overwinter, both morphs can occasionally occur contemporarily (Mikkola et al., 1989).

In this study, we tested the relevance of the ‘one species–one genitalia’ assumption using dimorphic *S. tetralunaria* as a test organism. We explored both size and shape variation of the genitalia in both sexes. At the same time, we tested the lock-and-key hypothesis, which assumes the correct size and especially shape of genitalia to be vital for successful fertilization. In all analyses, we kept external and internal genitalia separated. More specifically, we first studied whether the morphs could be distinguished by their genital size characteristics. Second, we explored whether genital traits showed negative allometry in relation to body size as observed in many other insects and spiders (Eberhard et al., 1998; Palestrini, Rolando & Laiolo, 2000; Schmitz, Reinhold & Wagner, 2000; Tatsuta, Mizota & Akimoto, 2001; Bernstein & Bernstein, 2002; Eberhard, 2002; Ohno et al., 2003). Strong negative allometry between body size and genital size is assumed by the lock-and-key hypothesis, but also by the cryptic female choice hypothesis (Eberhard et al.,

---

**Figure 1.** The two morphs of *Selenia tetralunaria*. The left column represents the larger morph (normally the first generation) and the right column the smaller morph (normally the second generation). Males are above, females below.
and may be produced by other mechanisms of sexual selection as well (Bonduriansky & Day, 2003). Third, we compared the amount of intraspecific variation between external and internal genitalia, because especially internal genitalia are claimed to work as reproductive isolation systems between species (Mikkola, 1992), and are therefore assumed to show little variation. These analyses addressed patterns related to size rather than shape of genital characteristics. Consequently, in these three analyses, we applied traditional distance morphometrics.

Traditional morphometrics is well-suited to explorations of size, but can capture only a very limited amount of information about the shape of structures (Rohlf & Marcus, 1993). Modern geometric morphometric methodology serves as an appropriate way with which to quantify shape properties (Bookstein, 1991; Rohlf & Marcus, 1993). We conducted geometric morphometric analyses to see whether the morphs of *S. tetralunaria* differed in genital shape. As it is genital shape rather than size that tends to evolve rapidly, we considered such characters more important compared with size variables in terms of the lock-and-key hypothesis. We assumed that if differences in the shape of genital structures work as mechanical reproductive isolation systems between species, then their correct shape should be tightly determined genetically and genital shape variation should be stabilized by selection (see Artnqvist, 1997). Consequently, there should be no genital shape differences between the morphs.

### MATERIAL AND METHODS

The samples were gathered from the Zoological Museums of Oulu and Helsinki Universities as well as from private collections. With the exception of two samples from northern Germany, all specimens were collected from central and southern Finland. The study material consisted of 50 male and 18 female specimens. Both morphs were represented equally (25 specimens of each morph) in male samples, while in females there were 12 larger and six smaller morphs. The total number of females was smaller because females are difficult to catch and are therefore under-represented in collections, especially females of the partial second generation.

Genital structures were divided into external and internal genitalia (Fig. 2). Male internal genitalia consist of intromittent genital structures. The male aedeagus was considered as part of the external genitalia, although it is sometimes considered an intromittent part. However, it does not always couple intensively with female internal tracts in moths (Callahan & Chapin, 1960). During copulation its caudal end is positioned on the female ostium bursae but is not placed in the female ductus bursae. The retractable internal genitalia are then inversely intruded (everted) into the female tracts (Callahan & Chapin, 1960). Moreover, the aedeagus is strongly sclerotized like other external genitalia, while internal genitalia are soft except for the dozens of long and stout, strongly sclerotized cornuti. As in males, the external female genitalia consist of sclerotized areas that are not in close contact with the male intromittent genital parts. The internal genitalia are softer, the most significant parts being the ostium bursae, ductus bursae and corpus bursae. There are some sclerotized areas in the female internal genitalia as well.

Hind femura were first boiled in water and then washed with ethanol. They were then prepared between microscope slides in Euparal fixative. Male abdomens were boiled in 10% caustic potash after which the genitalia were removed and cleaned. The aedeagus was removed and measured prior to preparation of the internal genitalia, which were prepared mainly according to the technique described by Dang (1993). The external genitalia were prepared in the same way as the hind femura. Male internal genitalia were stored in liquid Euparal fixative to avoid changes in their three-dimensional shape. Female genitalia were prepared as were those of males; external genitalia were placed in Euparal fixative between slides and internal genitalia were stored in liquid Euparal. Prior to fixation, all parts were stained, the sclerotized parts with eosin red and membranous parts with chlorazol black.

After preparation, the genitalia were photographed through a microscope using an Olympus C-5050ZOOM (USA) digital camera.

### TRADITIONAL MORPHOMETRIC ANALYSES

We took five measurements from the external and three from the internal genitalia of males, plus two in each of the corresponding areas of females. All genital measurements are shown in Figure 2. The measurements were made using an ImageJ 1.34 Java image processing program. To evaluate measurement error, ten measurements of each measured trait were repeated nonconsecutively.

Before statistical analyses, we tested for normality of the traits. The distributions of genital traits did not deviate from normal curves after Bonferroni correction in either sex. The only deviation from normality was the male nongenital femur length of the smaller morph, which was skewed to the right. Since this was not observed in the larger morph, for the purposes of this study we considered the skewing to be unimportant. Genital differences between morphs were at first quantified separately for each trait by *t*-test. To indicate the overall size difference between morphs, the
same test was carried out for femur lengths. We then reduced the number of original genital variables with principal component analysis (PCA) for both morphs and for external and internal genitalia separately. Due to the small number of female measurements, this analysis was performed only on males.

For allometric analysis, each value was first log-transformed. We calculated the regression slopes for each trait with linear ordinary least squares (OLS) regression analysis. There has been some debate about the most appropriate regression analysis (Eberhard, Huber & Rodriguez, 1999; Green, 1999). A comparison by Bernstein & Bernstein (2002) indicated that the regression method applied does not have a great effect on the results. This was also found by Kawano (2004), who found the OLS to be the most appropriate method for allometric analysis of dimorphic beetles. We used the right femur length as an overall indicator of size. This is not known to show allometric patterns to other body-size variables in moths. Meanwhile, in noctuid moths it describes reliably the overall size of individuals and is not allometrically scaled to other nongenital size variables (M. Mutanen, A. Kaitala & M. Mönkkönen, unpubl. data). To distinguish positive or negative allometry, we examined the confidence limits for each regression slope and compared whether or not they overlapped with unity ($b = 1$).

For variability comparisons between genital groups, we calculated standardized values for each measurement by subtracting the mean of the trait from the original measurement and then dividing this deviation by the mean. We then calculated the means of standardized values of both genital groups for each individual and these means were compared using repeated measures ANOVA.

**GEOMETRIC MORPHOMETRIC ANALYSES**

Geometric morphometric methodology is based on the two- or three-dimensional coordinates of landmark points of structures (Bookstein, 1991; Rohlf &
Normal distance measurements can be computed from these coordinates, but the revolutionary feature of this method is that it also enables quantitative exploration and comparison of shapes. The shape variables are geometric properties invariant to changes in the translation, rotation and scale of an object. Therefore, when only shape variation is addressed, landmark changes due to these properties must be removed from the data matrix (superimposition). The relationships between the coordinate points can be described with the aid of an appropriate mathematical function. So-called thin-plate spline functions can be used for superimposition (Bookstein, 1991). The estimates of parameters of this function can be used as variables in statistical, primarily multivariate, analyses (Rohlf & Marcus, 1993).

In order to explore possible genital shape differences between the two morphs of *S. tetralunaria*, two male and one female genital area were analysed using geometric morphometric methodology. In males, the shape of the right valva of the external genitalia and a section of the outline of the endophallus of the internal genitalia were selected. In females, we explored the shape of the bursa copulatrix outline. In these analyses, we used the combination of true landmarks and so-called sliding semilandmarks. The latter can be used for outline explorations when there is a lack of homologous landmarks (Bookstein, 1997; Adams, Slice & Rohlf, 2004), as was the case in our study. Sliding semilandmarks are allowed to slide along the outline to fit maximally with the corresponding landmarks of the reference (i.e. average) specimens. Both true and sliding landmarks are shown in Figure 3. The landmark digitations were carried out by tpsDig software (Rohlf, 2004).

For statistical analyses, we first calculated the partial warps for each landmark and specimen. Partial warps are shape variables that can be used in multivariate statistical analyses for comparisons between groups. They were calculated using tpsRelW software (Rohlf, 2003a). We explored the possible shape differences by analysing partial warps by multivariate ANOVA and discriminant function analysis. The former analysis was conducted by tpsRegr (Rohlf, 2003b) software and the latter by SPSS statistical.
RESULTS

TRADITIONAL MORPHOMETRIC ANALYSES

The average measurement error was 1.47% (range 0.02–5.54%). In male external and internal genitalia the values were 1.81% and 1.17% and in females they were 0.74% and 3.22%, respectively, while for the femora, the measurement error was only 0.07% (both sexes combined). Since the measurement errors were low and did not differ dramatically between compared groups, we used the original values in the analyses (see Yezerinac, Lougheed & Handford, 1992).

The comparison of femur lengths between morphs showed them to differ significantly in size. The results were parallel in both sexes; those of male specimens of the second generation (smaller morph, mean = 2.70 mm) were on average 20.0% smaller than were those of the first generation (larger morph, mean = 3.38 mm) ($t = 11.27$, d.f. = 46, $P < 0.001$). In females, the corresponding value was 13.6% (means: larger morph = 3.47 mm, smaller morph = 3.00 mm) ($t = 3.05$, d.f. = 16, $P = 0.008$). The same tests in genital traits showed that the male external genitalia of the second generation were on average 9.22% smaller compared with those of the first generation (range 3.08–15.01%, all $P < 0.035$). The same pattern recurred in females, in which external genitalia of the second generation were on average 11.71% (range 10.12–13.30%) smaller compared with those of the first generation ($P = 0.004–0.013$). In internal genitalia, there was no size variation between morphs. In males, internal genitalia were the same size, the second generation having on average 0.07% larger internal genitalia compared with the first generation (range 0.85% smaller–0.80% larger, $P = 0.781–0.917$). The difference was also not significant in females, in which internal genitalia of the second generation were on average 1.26% smaller compared with those of the first generation (range 1.33% smaller–1.20% smaller, $P = 0.665–0.771$).

The two most significant principal components explained 81.11% of the total variance in male external genitalia and 80.23% in male internal genitalia. The PCA analysis supported the previous results, but also showed that there may be differences in shape in the external genitalia in both sexes, as the scatter plots of the two main components overlapped less than did the size component (PC1) alone (Fig. 4A). The same graphs of internal genitalia produced dissimilar patterns; the plots of the morphs did not form separate clusters but were totally mixed (Fig. 4B).

The OLS regression slopes indicated the male external genital traits to be in negative allometric relation to body size in both morphs (average slope = 0.283, range = $-0.027$ to 0.588, $P < 0.05$ in all cases) (Fig. 5). Possibly as a result of the smaller sample size, this pattern was less pronounced in the female external genitalia (average slope = 0.409, range = $-0.015$ to 0.827, $P < 0.05$ in two out of four cases). The slopes of the internal genitalia were also negative.
allometric in both sexes and morphs (male average 0.530, range = 0.342 to 0.839; female average 0.205, range = 0.008 to 0.313), but differed significantly from an isometric relationship in only two cases out of six in males and three cases out of four in females.

In males, the average deviation of measurements from the mean was significantly larger in internal (0.762) than it was in external genitalia (0.484) ($P = 0.000$). This pattern was not observed in females, the corresponding values being 0.563 and 0.611 ($P = 0.874$). These patterns were the same in both morphs (male $P = 0.359$, female $P = 0.245$). Thus, despite size stability between morphs, the internal genitalia vary at least as much as do the external genitalia within morphs.

**GEOMETRIC MORPHOMETRIC ANALYSES**

Multivariate ANOVA of partial warps showed the morphs to differ in their genital shapes in all analysed areas. The shape of the male valvae differed significantly ($F = 13.399$, d.f. = 30, 1410, $P < 0.001$). This was also confirmed by discriminant function analysis of the partial warps, which classified all the specimens correctly into two morphs. Examination of the discriminant function 1 showed that the morph distributions did not overlap (Fig. 6A). The most remarkable difference between morphs was the length of the distal half of the valva in relation to its proximal half, but there were also differences, for example, in valva acuteness and bending strength of the proximal part of the valva. Differences between average valvae of both morphs are shown exaggerated by a factor of three in thin-plate spline deformation grids in Figure 7A. The studied outline section of the male internal...
genitalia showed consistent differences between morphs ($F = 6.701$, d.f. = 22, 638, $P = 0.000$) and no specimens were categorized incorrectly by discriminant function analysis, indicating no overlap between morphs in genital shape in its function 1 (Fig. 6B). Here, the most significant difference was caused by the length–height ratio of the studied genital area (Fig. 7B). The result was the same in female bursa shape, which showed significant differences between the morphs ($F = 1.880$, d.f. = 30, 480, $P = 0.004$). Again, all specimens were classified correctly by the discriminant function analysis and no overlap was observed in its function 1 (Fig. 6C). Visualization of differences in bursa copulatrix shape is shown in Figure 7C.

**DISCUSSION**

The lock-and-key hypothesis predicts low within-species variability in genitalia. It assumes only one correct size and shape of genitalia for each species that permits fertilization, otherwise either copulation or sperm transfer will fail. In contrast, sexual selection has traditionally been considered as showing positive allometric tendencies by exaggeration of sexually selected traits (Green, 1992) as well as by maintaining a relatively high degree of morphological variation (Arnqvist, 1997). Recently, negative allometry between genital traits and body size has been reported in a number of insect and spider taxa (Eberhard et al., 1998; Palestrini et al., 2000; Schmitz et al., 2000; Tatsuta et al., 2001; Bernstein & Bernstein, 2002; Eberhard, 2002; Ohno et al., 2003). It seems that low degrees of genital size variation are almost universal among these arthropod groups. Since these findings, size variation in terms of sexual selection has been under revision and debate (Eberhard et al., 1998; Eberhard et al., 1999; Green, 1999; Bonduriansky & Day, 2003). It has been suggested that cryptic female choice may also favour an average male genital size (one-size-fits-all hypothesis) and thus express negative allometric relationships between genital and body size (Eberhard et al., 1998). The finding that genitalia show negative allometry has been claimed to contradict predictions of the sexual conflict and sperm competition hypotheses (Eberhard et al., 1998). However, modelling has recently shown that sexual selection may produce any pattern of allometry or isometry, depending on selective regimes and the precise nature of net selection (Bonduriansky & Day, 2003). Therefore, the present understanding is that allometric patterns alone cannot be interpreted straightforwardly as evidence for any genital evolution hypothesis. Thus, the most obvious distinction between the lock-and-key and sexual selection hypotheses regarding morphological variation in genitalia is what they predict about...
shape variation. While the lock-and-key hypothesis predicts low intraspecific shape variation, sexual selection hypotheses predict relatively high degrees of variation in genital shapes (Arnqvist, 1997).

In *S. tetralunaria*, all measured genital traits were scaled in negative allometric relation to body size, supporting previous findings of allometric scaling of genital traits in relation to body size. The allometry was stronger in internal than in external genitalia so that, despite an approximately 20% change in body size, internal genital structures were of the same size in both morphs. We also found that external genitalia differed in size between morphs. Surprisingly, this pattern was not found in internal genitalia, which were of exactly the same size in both morphs. As stated above, with current knowledge these findings cannot be interpreted as support for any of the genital evolutionary hypotheses. Nevertheless, reduced size variability in genital traits is a striking phenomenon and its evolutionary causes are insufficiently understood. Therefore, size explorations should not be ignored in genital variation studies. Male internal genitalia and female internal genitalic tracts are in closer contact during copulation than are external genitalia, which may affect their size stability between morphs. It seems obvious that the size of the genitalia is determined genetically, especially in the case of the internal genitalia. Size invariance may be due to stabilizing selection, but this possibility cannot be proven with morphometric methods alone.

We have documented discontinuous intraspecific genital shape dimorphism in our moth study species. The shapes of all analysed genital areas differed significantly between morphs and no overlap was present. Moreover, we have shown that structures of the internal genitalia varied more than did those of the external genitalia. The result that coupling genital traits varied more than did external genital ones as well as the presence of different shapes of genital structures between morphs are not in good concordance with the predictions of the lock-and-key hypothesis (Arnqvist, 1997). Several studies show that genitalia vary a lot within species (Goulson, 1993; Eberhard et al., 1998; House & Simmons, 2003). If genital characters function as a mechanical reproductive isolation system, differently shaped genitalia might prevent successful sperm transfer between the sexes of different morphs of *S. tetralunaria* as the morphs may occur contemporarily. The lock-and-key hypothesis predicts correct genital size and shape to be tightly determined genetically (Arnqvist, 1997). Meanwhile, sexual selection theories suggest relatively high variation in sexually selected traits (Arnvist, 1997). Genital dimorphism in *S. tetralunaria* is possibly a direct consequence of overall dimorphism between morphs and not a consequence of sexual selection. The differences in genital shape may be a result of different ontogenic histories between morphs, but it is also possible that there are adaptive reasons for them. This possibility cannot be evaluated on the basis of the results of this study. The possibility of accidental dimorphism does not exclude the possibility that genital traits are under sexual selection and that genital evolution may be due to sexual selection. It is not known why there are two different life-history strategies in *S. tetralunaria*, but this is possibly an adaptation that enables bivoltinism as in the northern part of the range the species is univoltine. The summer would generally be too short for two annual generations with complete life histories in the southern part of the range as well.

Knowledge about genital polymorphism in insects is practically absent. The only data reported were in a thrip species, in which winged and apterous male specimens differ remarkably in their genital sizes (Mound, Crespi & Tucker, 1998), and in geometrid moths, which show polymorphism in genital sternites (Hausmann, 1999). The other few observations on genital polymorphism refer to either molluscs or arachnids (Schrag et al., 1994; Huber & Gonzalez, 2001; Jocque, 2002), which differ from insects essentially in their reproductive systems. In most of these cases, genital polymorphism is probably caused by sexual selection (Jocque, 2002). Although rarely documented, we consider our observations regarding genital dimorphism in genitalia to be quite expected. We believe that the scarcity of observations may be partly as a result of circular reasoning as species are often delimited by differences in genital characteristics.

Since intraspecific genital dimorphism is reported rarely in insects, the genital dimorphism and size stability that we found in *S. tetralunaria* morphs could be viewed as being negligible, exceptional phenomena. As stated above, the scarcity of observations on genital polymorphism may be partly artificial. It may be incorrect to treat automatically genetically distinguishable populations as reproductively isolated species. This concerns temporarily or geographically isolated populations in particular, which traditionally are allowed to differ in their external appearance but not in their genitalia. Such practice is not necessarily expected even by the lock-and-key hypothesis, because it suggests that genital differentiation is due to character displacement in sympatry (Shapiro & Porter, 1989). Thus, even if there is evidence for a lock-and-key hypothesis, it would be incorrect to extrapolate its predictions to cases in which differentiation has developed in allopatry. Furthermore, as sexual selection may maintain dimorphism in nongenital traits, sympatric genital polymorphism maintained by sexual selection may also exist. Sympatric genital polymorphism could be found at least in such externally sim-
ilar sibling species in which genital differences are not linked with nongenital differences.

Internal genitalia are often conspicuously complicated in moths, but cryptic in routine preparations. Due to progress in preparation techniques, their use in taxonomic studies has increased rapidly in recent decades. Literature comparisons make it clear that their evolution is generally extremely rapid and divergent, particularly in males (Mikkola, 1992; Dang, 1993; Fibiger, 1997; Sihvonen, 2001; Yela, 2002). This is apparently the case in many other insect taxa as well (Roig-Alsina, 1993; Arnaud, Haubrege & Gage, 2001). Internal, retractable genitalia are still little studied in an evolutionary context. In moths, they are exceptionally large, and the male genital tube is generally enlarged in the part that settles behind the female genital opening during copulation. There are often many projections and structures that are apparently meaningless to sperm transfer. Similarly, if there are long sclerotized cornuti, they are placed almost regularly inside female genital tracts and are directed backwards (for examples, see Fibiger, 1997). Such structures may cause injuries to female genital tracts (see Johnstone & Keller, 2000). Moreover, backward-directed thorns may make it difficult for a female to regulate the duration of copulation. Similar structures have been reported in other insect taxa (Sota & Kubota, 1998; Fairbairn et al., 2003). As a possible female counteradaptation (see Arnvist & Rowe, 2002a; Arnvist & Rowe, 2002b; Rowe & Arnvist, 2002) there may be sclerotized plates near the genital opening. This is the case in S. tetralunaria. As shown in Figure 2, there is a number of long, stout and backward-directed cornuti in an enlarged area of the male endophallus. Correspondingly, the female ductus bursae is strongly sclerotized near the genital opening but is softer elsewhere. Genital correspondence in moth internal genitalia was shown in a number of species by Mikkola (1992), who interpreted this as evidence for the lock-and-key hypothesis. However, as stated above, sexual conflict may also produce such a correlation.

ACKNOWLEDGEMENTS

We are indebted to F. Bokma, M. Monkkonen, M. Pentinsaari, S. Rytkonen and P. Valimaki for help and valuable comments on the manuscript and Lauri Simonson for revising the English language. We also thank T. Leinonen for advising us in geometric morphometrics analyses. The staff of the Zoological Museum of Helsinki University is thanked for loaning material to us. Finnish Cultural Foundation and Academy of Finland (grant #53899) financially supported the study through grants to M.M. and A.K., respectively.

REFERENCES


© 2006 The Linnean Society of London, Biological Journal of the Linnean Society, 2006, 87, 297–307


