Selection of a conspicuous male genitalic trait in the seedbug *Lygaeus simulans*

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Phenotypic selection of a male genitalic trait based on insemination success in the seedbug *Lygaeus simulans*, Deckert 1985, was demonstrated in this study. In a laboratory experiment, 97 pairs of virgin animals copulated once. The females consisted of six groups of full sisters, all sisters of a group having been raised in the same box. Related females were used in order to lower the variance of female preferences and to thereby increase the chances of detecting selection of male traits by female choice. The males were independent, i.e. each male descended from a unique pair of parents. Multiple regressions of insemination success on linear and nonlinear terms of a male genitalic trait (processus gonopori length) and male weight revealed stabilizing selection on the genitalic trait. According to functional considerations this selection is most probably the result of female choice of male processus length.

Keywords: genitalia; sexual selection; sperm competition; female choice

1. INTRODUCTION

Genitalic extravagance includes two phenomena: first, the genitalia of many animal groups are often much more complex than necessary for sperm transfer and, second, they evolve more rapidly than other structures (Eberhard 1985). The most reasonable explanations for this evolutionary pattern assume that genitalia underlie sexual selection, either via male competition (sperm competition) or by cryptic female choice (for reviews, see Eberhard 1985, 1996, 1997; Arnqvist 1997). So far, these hypotheses have been developed and tested mainly on the basis of comparative data. Experiments pertaining to actual selective processes are rare and badly needed (Arnqvist 1997; Møller 1998; but see Rodriguez 1993; cited in Dickinson 1997; Arnqvist *et al.* 1997; Arnqvist & Danielsson 1999). The aim of the present study was to test whether phenotypical selection of male processus gonopori length exists in the seedbug *Lygaeus simulans*.

The processus gonopori is a characteristic male genitalic trait of lygaeid bugs. It is a sclerotized, tube-like extension of the aedeagus, shaped for the transfer of sperm into the female receptaculum seminis (figure 1). The processus is an impressive example of the rapid and divergent evolution of genitalic structures. Within the family Lygaeidae its length is highly variable: in some species it is only a short spine, whereas it reaches spectacular lengths in several other species, e.g. three times the body length in *Kleidocerus resedae* (Carayon 1989; Deckert 1990). In *L. simulans* the processus measures ca. 60% of the body length. The female counterpart of the processus is the ductus receptaculi, an elastic tube which connects the bursa copulatrix with the receptaculum. The receptaculum itself is tube like, its proximal part being wound like a corkscrew; it is separated from the ductus by a valve-like structure supported by muscles (Ludwig 1926;

Micholitsch 1997; see also Bonhag & Wick (1953) for the genitalic anatomy of the closely related species *Oncopeltus fasciatus*).

L. simulans is a promiscuous species. Females lay a series of egg clutches and usually copulate with one or more males between each oviposition. There is no particular courtship behaviour prior to copulation, only a brief pre-copulatory struggle where the male grasps the female dorsally with his legs and tries to insert his aedeagus into the female bursa copulatrix (Ludwig 1926; Sillén-Tullberg 1981; Tadler *et al.* 1999). When this has been achieved the male performs complicated genitalic manoeuvres in order to introduce the processus gonopori into the female ductus receptaculi (Ludwig 1926). For successful insemination the tip of the processus has to penetrate the valve-like structure between the ductus and receptaculum and must be inserted into the proximal part of the receptaculum (figure 1a). There are no spermatophores; the filiform sperm can move freely, but often they form twisted strands within the receptaculum (Ludwig 1926; Micholitsch 1997). Males may prolong copulation in order to reduce sperm competition (Sillén-Tullberg 1981), but there is no post-copulatory mate guarding or obligate remating with the same male. Females may remate with another male immediately after finishing a copulation (Ludwig 1926; A. Tadler, unpublished data).

In a laboratory study, Tadler *et al.* (1999) found that only around 60% of *L. simulans* copulations led to insemination. However, no relationship between insemination or fertilization success and processus gonopori length was detected.

In this study I attempted to lower the genetical as well as the phenotypical variance of female preferences by using groups of full sisters raised in the same cage. According to the female choice hypothesis of genitalia (Eberhard 1985), females discriminate between males on the basis of male genitalic traits. Male traits and the

Figure 1. (a) Male processus introduced into female receptaculum. The drawing was made from a freeze-fixed preparation. Female parts: bc, bursa copulatrix; dr, ductus receptaculi; od, oviduct; rs, receptaculum seminis; va, valve between the receptaculum and ductus. Male parts: ae, aedeagus; pg, processus gonopori. (b) Whole-mount preparation of the processus for length measurements (along the dotted line). (c) Three examples of phenodeviant processus, excluded from the analysis.

corresponding female preferences underlie a genetic feedback mechanism, e.g. the Fisher–Lande process (Lande 1981). One of the most general assumptions of these genetic models is that female preferences are variable and heritable (Fisher 1930). Since the selection of male traits by female choice becomes stronger if females are more similar in their preferences (for an illustration see Arnold (1983, fig. 4.4)), it is possible to increase the chances of detecting the selection of male traits by female choice by lowering the environmental and/or genetical variance in female preferences.

2. MATERIAL AND METHODS

(a) *Animal breeding and copulation experiment*

The study was performed with laboratory-bred bugs. The animals were kept singly or in pairs in plastic Petri dishes (9 cm diameter) or in groups in boxes (8 cm × 14 cm × 10 cm) provided with sunflower seeds at 31 °C under an 18 L:6 D regime. A glass vial with a perforated lid served as a source of drinking water. To guarantee virginity, all laboratory-bred animals were sexed

Figure 2. Processus length of males (mm) assigned at random to six groups of female full sisters. Open symbols, unsuccessful males; filled symbols, successful males.

and separated as instars according to the pattern of black spots on the ventral rear end of their abdomens.

The grandmothers of the experimental animals were 54 females collected on 3 and 10 July 1997 near Hundsheim, Lower Austria. The bugs were sexed and separated in the field and the females reared individually in the laboratory so that they had no opportunity to copulate after capture. The parental generation consisted of four to five male and female offspring from each grandmother. They were assigned at random into separately kept pairs. One male offspring was isolated in a Petri dish at the fourth or fifth instar from each of these pairs (if fertile). To obtain the females, the largest egg batches (more than 60 eggs) from six different parental females were selected. Each of these batches was transferred to a separate box. Only 19–32 adult females developed from each of these egg batches due to the high mortality of first instars. These females remained together in their boxes until two days before copulation, when they were separated into Petri dishes.

On 7 November 1997, 124 randomly assigned pairs were allowed to copulate. The pairs were put into plastic Petri dishes (diameter 5.5 cm) and observed continuously. Interactions between the animals were scored as copulations if a stable connection between the genitalia and the 'tail-to-tail' position typical of copulating Lygaeidae was reached. The animals were separated immediately after copulation so that only one copulation per pair was possible. Non-copulating pairs were removed 6 h after the beginning of the experiment while copulating pairs were allowed to finish copulation before they were separated. All animals involved were weighed one day before copulation on an electrobalance to the nearest 0.1 mg. One day after copulation the animals were killed and preserved in 75% ethanol. The male and female internal genitalia were dissected and whole-mount preparations were made of the male processus gonopori and the female receptaculum seminis. The male processus gonopori was spread (figure 1b) between the microscopic slide and the cover glass in two dimensions so that its length could be accurately measured, using a drawing tube on a microscope, a digitizing tablet and the program Sigma Scan (Jandel Scientific, SPSS, Chicago, IL, USA). Female receptacula were checked for sperm load using an interference contrast microscope.

Table 1. Selection coefficients ($\pm$ s.e.) of processus length and male weight with regard to insemination success

(p -values were obtained by 10 000 randomizations of the dependent variable. An asterisk denotes significance at the 0.05 level after Bonferroni correction.)

	univariate		multivariate	
	linear (β)	nonlinear (γ)	linear (β)	nonlinear (γ)
length of processus gonopori	0.079 ± 0.077 $p = 0.3008$	-0.282 ± 0.047 $p = 0.0023^*$	0.055 ± 0.085 $p = 0.5318$	-0.260 ± 0.049 $p = 0.0057^*$
cube-root of male weight	0.079 ± 0.077 $p = 0.3084$	-0.079 ± 0.058 $p = 0.5116$	0.056 ± 0.085 $p = 0.4997$	0.039 ± 0.067 $p = 0.7768$

(b) Statistical analysis

The mode and magnitude of the selection of male traits was determined by applying multiple regression as described by Lande & Arnold (1983). This standard method consists of multivariate regressions of relative fitness (w' , mean value = 1) on z -standardized trait values (z). The selection coefficients correspond to the regression coefficients. I included two male trait measures into the analysis, namely processus gonopori length and the cube root of the body weight. Male weight can be regarded as a measure of overall body size, since it is closely correlated to a series of other measures (including head capsule and thorax width and wing and body length) in *L. simulans* (A. Tadler, unpublished data).

Univariate linear selection coefficients (β_i) were derived from the model $w' = c + \beta_i z_i$ and univariate nonlinear selection coefficients for quadratic terms (γ_{ii}) were derived from the model $w' = c + \beta_i z_i + (\gamma_{ii}/2) z_i^2$. Multivariate linear selection gradients (β_i') were estimated in a model including only the two linear terms, whereas the multivariate nonlinear (quadratic) selection gradients were derived from the full model including linear, squared and cross-product terms. The univariate linear and nonlinear selection coefficients estimate the net (direct + indirect) selection acting on a trait. In the multivariate selection coefficients the effect of indirect selection via those correlated traits which have been measured is removed (Lande & Arnold 1983; Endler 1986; Brodie *et al.* 1995).

Insemination success (as assessed by the presence of sperm within female receptacula, regardless of the number of sperm) was used as a fitness measure. Since this is a binary variable, the assumptions of conventional parametric significance testing are violated (Lande & Arnold 1983; Brodie *et al.* 1995). I therefore tested the significance of selection coefficients with 10 000 randomizations of the dependent variable using the linear regression module of the RT program (Manly 1996).

Whether a female factor causes differences in the selection of processus gonopori length was tested using a one-way ANOVA and Levene's test, with female families as the factor and processus gonopori length of successful males as the dependent variable. Because the variances of processus lengths of successful males were unequal between female families (figure 2), these tests were carried out with 10 000 randomizations, using the analysis of variance module of the RT program. This routine reallocates the original observations at random to factor levels and computes the test statistics (F - and Levene's statistic) for each randomization. The p -value is the proportion of the values from the randomizations exceeding the original test statistic value (Manly 1996).

3. RESULTS

In the 124 pairs involved, 102 copulations were observed. The processus gonopori of eight males showed phenodeviations (for examples see figure 1c); four of these males copulated. None of them led to insemination, so all phenodeviant males were excluded from the analysis. One processus was damaged during preparation so that 97 pairs were included in the analyses. Of these pairs 62 (63.9%) were successful in that sperm were found in female receptacula after copulation. The mean value ($\pm$ s.d.) of processus gonopori length was 6.80 mm (0.15 mm, $n = 62$) in successful males and 6.76 mm (0.26 mm, $n = 35$) in unsuccessful males. Successful copulations were considerably longer (mean $\pm$ s.d. 183.80 $\pm$ 71.01 min) than unsuccessful copulations (62.57 $\pm$ 63.70 min). The duration of the shortest successful copulation was 35 min.

Male body weight and processus length were significantly correlated (Pearson $r = 0.412$, $p < 0.01$, $n = 97$). The nonlinear (quadratic) selection coefficients of processus length were negative and significant in both univariate and multivariate analyses (table 1), indicating stabilizing selection of processus length. The coefficient of determination of the full multiple regression model was significant ($R^2 = 0.132$, $p = 0.0198$). As one would expect (remembering that males were assigned at random to females), there were no significant differences in male processus length between female families (figure 2) if all copulating males were considered (one-way ANOVA $F_{5,91} = 0.71$, $p = 0.6172$ and Levene's statistic = 0.52, $p = 0.7448$). If only successful males were included, no significant differences in the mean values ($F_{5,56} = 0.75$, $p = 58.77$) were detected, but Levene's test indicated significant heterogeneity of variances in processus lengths between female families (Levene's test statistic = 2.52, $p = 0.0139$).

4. DISCUSSION

The results of this study show that there is selection of male processus length during copulation. This can be explained by male malfunctions or cryptic female choice. Another important explanation for selection of genitalia, namely male competition, can be excluded as the animals copulated only once and selection was based on insemination failures, so that no interaction between males or different ejaculates was possible.

Male malfunctions (e.g. the inability of a male to produce and transfer sperm) constitute a form of natural selection and are independent of any female properties and preferences (Eberhard 1996). In contrast, where cryptic female choice is involved, fertilization success depends on both male traits as well as female preferences (Birkhead 1998; Birkhead & Møller 1998). The premise of this experiment is that groups of full sisters should be more similar in their preferences than non-related females. As the main purpose of this study was to test whether selection of male genitalia exists at all, I attempted to lower the variance in preferences within all females but made no effort to increase the differences between female families, e.g. by keeping the different families under different conditions. Nevertheless, the variances in process length of successful males differed significantly between female families. Since male malfunctions are by definition independent of females they cannot explain the significant differences in the distribution of traits of successful males between female families if males are assigned to females at random. However, one can expect such differences, if female choice occurs and if female preferences differ between female families. This makes female choice the most probable explanation for the selection of process length.

In this study each female was mated with one male only. A possible objection to this design could be that a meaningful evaluation of cryptic female choice must be based on measurements of the P_2 -value, the proportion of eggs fathered by the second male after double mating (Birkhead & Møller 1998). One could argue that insemination failures may occur only in females copulating for the first time and would influence male fitness only if the P_2 -value is low. However, it is easy to show that insemination failures of the first male will influence male fitness, as long as the P_2 -value is not always exactly unity.

Consider a scenario where insemination failures occur exclusively in the first copulation of females so that the second male is always successful and where these insemination failures do not influence the probability of a female remating. If the first male fails, his fertilization success (P_1') will be zero and the success of the second male (P_2') will be unity. On the other hand, if the first male is successful, the proportion of eggs fertilized by the second male will be $P_2'' = 1 - P_1''$. Insemination failures will influence male fertilization success if $P_2' \neq P_2''$, i.e. if $P_2'' < 1$. It is fair to assume that complete precedence of the sperm of the second male does not exist in *L. simulans*. Complete precedence is generally rare in insects and has never been found in Hemiptera (Simmons & Siva-Jothy 1998). Sillén-Tullberg (1981) reported a P_2 -value close to 0.9 in *Lygaeus equestris* (a sibling species of *L. simulans*). One has to consider, however, that this value may not necessarily correspond to P_2'' . If insemination failures are actually more frequent in the first copulation, P_2'' would be lower.

Another important question is whether the selection of process length is direct or indirect. Indirect selection occurs if a trait is correlated with the true target of selection, but is not selected itself (Endler 1986). Indirect selection of genitalia is predicted by the pleiotropy hypothesis, which maintains that genitalic structures are not influenced by direct selection but are genetically correlated with

selected external characters (Mayr 1963; Arnqvist 1997; Arnqvist *et al.* 1997). The regression analysis of the present study indicates direct selection of process length. However, multiple regression can reveal the actual target of selection only if the selected trait is included in the analysis (Lande & Arnold 1983). Therefore, it might seem wise to measure all traits probably related to the fitness measure (Endler 1986; Brodie *et al.* 1995). On the other hand, the inclusion of a number of additional independent variables would obscure the results of the analysis by inflating the statistical α -error (Rice 1989). Since the aim of this study was to test whether selection of a genitalic trait can be demonstrated at all, only body mass as a measure of size was included additional to process length.

The following functional considerations make direct selection of process length via female choice more probable than indirect selection and pleiotropy. The analysis included copulating pairs only; the observed selection was therefore most probably caused by mechanisms acting during copulation. There is little reason to assume that, during copulation, the external morphological traits (e.g. head capsule width, tibia length or external parts of the male genitalia) contributed more to insemination success than the internal genitalic structures shaped for insemination. Micholitsch (1997) freeze-fixed pairs of *L. simulans* 30 and 60 min after the beginning of copulation. With virgin females the process was fully inserted into the receptaculum in only two out of 16 pairs after 30 min and in seven out of 18 pairs after 60 min. In several other experiments, where bugs were allowed to terminate copulation at will, around 30% of the copulations did not result in insemination. These unsuccessful copulations were normally terminated within 60 min (Micholitsch 1997; Tadler *et al.* 1999; this study). This suggests that insemination failures were most often caused by insertion failures.

There is no rigid connection between the aedeagus and the rest of the body and there are no muscles on the aedeagus or the process. The aedeagus can be moved by hydraulic pressure but the process lacks both hydraulic structures and sensory organs (Ludwig 1926; Bonhag & Wick 1953). Its length is probably of importance in two situations. First, in order to insert the process into the ductus receptaculi the male has to rotate the aedeagus within the female bursa copulatrix, so that the tip of the process slips into the proximal opening of the ductus (see Ludwig (1926) for a detailed description of these manoeuvres). The tip could thereby fail to enter the opening of the ductus if the length of the process is not appropriate. Second, the process must be pushed against the valve between the ductus and receptaculum. If the process is too short it would not be able to enter the receptaculum. From a functional point of view it therefore seems not surprising that successful insertion depends on the appropriate length of the process.

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