

Correlated sexual selection on male genitalia, copulatory performance and nuptial gifts in a bushcricket (Orthoptera: Tettigoniidae) indicated by allometric scaling

GERLIND U. C. LEHMANN^{1,*}, SINA KUCHENREUTHER¹, ARNE W. LEHMANN² and THORSTEN DICKHAUS³

¹*Evolutionary Ecology, Department of Biology, Humboldt University Berlin, Invalidenstrasse 110, 10115 Berlin, Germany*

²*Friedensallee 37, 14532 Stahnsdorf, Germany*

³*Institute for Statistics, University of Bremen, PO Box 330 440, 28344 Bremen, Germany*

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We adopt an allometric framework of scaling relationships for comparison between mating-related traits in the middle European bushcricket *Roeseliana roeselii* (Hagenbach, 1822). Eight characters, covering ontogenetic fitness (size traits; fixed at final moult), male condition (mass traits) and mating motivation (reproductive behaviours), were analysed in unrestricted matings and in matings involving genital manipulation. Shortening the male titillators had no effect on mating-related traits in males. However, titillators, known to be under sexual selection, scale hyperallometrically, with larger males possessing proportionally longer titillators, performing more titillator movements and exhibiting a reduced duration of copulation. Scaling was also hyperallometric for spermatophore mass, with larger males being heavier and transferring heavier nuptial gifts. Both titillator length and spermatophore mass might be condition-dependent indicators, because their variances were nearly twice as large those of body size or body mass. Mass traits were also dynamic, increasing by 11% for male body mass and 17% for spermatophore mass between the first and second matings. Sexual selection by female choice seems to favour larger trait size in the bushcricket *R. roeselii*, acting in concert on titillator length, intensity of titillator movements and spermatophore mass.

ADDITIONAL KEYWORDS: allometric scaling – condition dependent – female choice – genitalia – hyperallometry – nuptial gifts – sexual selection – spermatophores – titillators – variances.

INTRODUCTION

Mating choice and behaviours are central for the understanding of evolution and are strong forces for the development of sex-related characters. Traits under sexual selection are linked to the genotype and expressed in the phenotype of their bearer (Andersson, 1994; Johnstone, 1995). Furthermore, handicap models predict that the phenotypic expression of such traits in males depends on their condition (Kotiaho, 2001; Cotton *et al.*, 2006; Tazzyman *et al.*, 2014). Despite the evidence for sexual selection as a driving force in evolution, studies seldom examine

multiple phases of the mating process. Mate choice by both sexes is exhibited in the precopulatory phase, mainly by sensory input of long- and short-ranging signals (Endler, 1992; Greenfield, 2002), followed by close interaction during copulation (Andersson, 1994) and concluded by postcopulatory factors (Eberhard, 1996; Simmons, 2001). These different phases vary in the type of social contact involved, and therefore, the interaction between the sexes (Arnqvist & Rowe, 2005).

Sexual selection is mostly studied by behavioural experiments manipulating the traits of interest and measuring the effect on mating success (Andersson, 1994). An allometric framework can complement such experiments to help identify characters that are under directional selection. Scaling of individuals in

*Corresponding author. E-mail: gerlind.lehmann@biologie.hu-berlin.de

the same developmental stage, in our case adults, is called static allometry, and it is proposed that condition-dependent development creates slopes of different steepness between a trait and body size (Warton *et al.*, 2006; Klingenberg, 2016; Mirth *et al.*, 2016; Stillwell *et al.*, 2016). Individuals in better condition have the resources to develop larger traits, and sexual selection is thought to favour larger trait size when its expression benefits the bearer during sexual competition and female choice (Bonduriansky & Day, 2003; Bonduriansky, 2007; Eberhard *et al.*, 2009, 2018). A large number of studies report a relative increase in size of sexually selected traits with increasing body size, a phenomenon known as hyperallometry (Calder, 1984; Anichini *et al.*, 2017; Shingleton, 2019) or positive allometry (Kodric-Brown *et al.*, 2006; Voje, 2016; Eberhard *et al.*, 2018; Rodríguez & Eberhard, 2019). However, hyperallometric scaling and sexual selection do not demonstrate one-to-one correspondence; hyperallometry is not restricted to sexually selected characters, and not all sexual characters scale hyperallometrically. In consequence, allometries are shaped by multiple factors, combining sexual and viability selection and trade-offs in resource allocation in trait and body size (Bonduriansky, 2007). Therefore, demonstrating the role of hyperallometry in sexual selection requires the characters to be manipulated experimentally. In such cases, hyperallometry indicates a directional rather than stabilizing effect of sexual selection.

We use the middle European bushcricket *Roeseliana roeselii* (Hagenbach, 1822) of the subfamily Tettigoniinae for allometric correlations between mating traits. This species has been studied intensively for its reproductive behaviour (Zippelius, 1949) and aspects of sexual selection (Wulff & Lehmann, 2016, 2020). The species exhibits a polygamous mating system, with up to five female matings throughout the lifetime (Vahed, 2006; Kañuch *et al.*, 2013). Of particular interest for this study are the sclerotized titillators of the males (Wulff & Lehmann, 2014; Wulff *et al.*, 2015, 2017), which are sexually selected through cryptic female choice (Eberhard & Lehmann, 2019) and of average complexity compared with other bushcrickets (Vahed *et al.*, 2011; Lehmann *et al.*, 2017). During copulation, the titillators are inserted rhythmically into the female genital chamber (Wulff & Lehmann, 2016; Wulff *et al.*, 2017; Wulff & Lehmann, 2020), thereby stimulating the female (Wulff *et al.*, 2018) to accept the attachment of the spermatophore (Wulff & Lehmann, 2016, 2020). Intact titillators suppress female mate-rejection behaviours, because females reject males with experimentally shortened titillators (Wulff & Lehmann, 2016, 2020), and mate rejection increases when female genitalia are sensorily blinded (Wulff *et al.*, 2018). Genital titillators in our

model bushcricket species are one of the best-studied characters to be selected by cryptic female choice (Eberhard & Lehmann, 2019). In the majority of insect species the genitalia appear to be under stabilizing allometric selection (Eberhard *et al.*, 2009, 2018), whereas several genitalia scale hyperallometrically (Simmons, 2014).

Along with the study of titillator allometry, we analyse a set of eight characters, which cover body size, body mass and the behaviour of *R. roeselii* males. The two body size parameters are static characters fixed at adult ecdysis and capture the male fitness during development. In contrast, body mass data reflect the condition of an individual. Given that many bushcrickets invest heavily into the nutritious spermatophore (Wedell, 1993a; Vahed & Gilbert, 1996), heavier males show condition-dependent mating investment by transferring bigger spermatophores (Lehmann & Lehmann, 2008, 2009). Larger spermatophores are beneficial for males, because females require more time to consume the sperm-protective spermatophylax, increasing the amount of sperm transferred and leading to higher fertilization success under sperm competition (reviewed by Lehmann, 2012). Although it is often argued that condition might not be measured by body mass alone, in most bushcricket species body mass is a better predictor of spermatophore size than body size or a combination of both (Lehmann & Lehmann, 2009).

The largest set of characters we studied was behavioural characters, in particular their plasticity (Stamps, 2016). These covered mating motivation and mating decisions. We measured four parameters of the mating sequence, two during the pre-mating approach (duration of approach and duration to reach copula position) and two during copulation (copula duration and the number of copulatory titillator movements). Previous research indicates that these four mating characters are under control of the females (approach duration) or the males (copulatory movements and copula duration) or are influenced by both sexes (reach copula position) (see Wulff *et al.*, 2016; Wulff & Lehmann, 2020).

All eight characters were analysed in unrestricted matings, followed by controlled matings using a split design that tested the influence of titillator manipulation in the second mating. Female choice in the second mating was measured as resistance behaviours during copulation and failed spermatophore transfers, analogous to previous experiments (Wulff & Lehmann, 2016, 2020).

We hypothesize that the sexually selected characters, spermatophores and titillators, scale hyperallometrically owing to directional selection. Mating-related behaviours under male control are also hypothesized to scale hyperallometrically, due to males

in better condition having greater resources available to optimize their mating behaviours.

MATERIAL AND METHODS

Bushcrickets of *R. roeselii* were caught between May and June 2013 as fourth and fifth nymphal instars near Berlin (Stahnsdorf, 52°23'06"N, 13°13'03"E). Animals were taken to the laboratory and raised for 2–3 weeks to adulthood individually in transparent 0.5 L plastic containers covered with gauze. Every vial contained a stick for crawling and an *ad libitum* diet of garden-collected fresh grass, oat flakes, bee pollen and dried fish food pellets (ASTRA Aquaristik, Hameln, Germany). Food was replaced daily, and water was sprinkled three to five times a day on the brim of the containers. Ambient temperature in the laboratory was 22–25 °C, with a light–dark cycle of 16 h–8 h.

As an indicator of male body size, hind femur length was measured with digital sliding callipers (Mitutoyo; accuracy ± 0.01 mm). Male body mass was weighed before each mating using an electronic precision balance (Kern EG 300-3M; accuracy ± 0.1 mg).

MATING TRIALS

Five days after final ecdysis, we allowed 39 adult males to mate with a virgin female in a mating box covered with gauze (length 30 cm \times width 30 cm \times height 20 cm). After this first mating, males were randomized to one of two treatments: either sham-treated controls or having their titillator shortened 2 days after their initial mating. In line with previous experiments, we ablated both processing titillator arms at the base using fine scissors, identical to the symmetrical treatment classified initially as T_{-2} (Wulff & Lehmann, 2016) and later refined as T_{-2}^p (paired titillators both arms removed; Wulff & Lehmann, 2020). The treatment group comprised 19 males and the control group 20 males. After a refractory period of 5 days, males in both treatment groups were mated a second time with a virgin female, 7–9 days after eclosion.

The males showed considerable variation in body size, ranging in hind femur length from 14.19 to 16.24 mm, with a mean (\pm SD) of 15.38 ± 0.55 mm ($N = 39$). Male body mass ranged from 211 to 379 mg, with a mean (\pm SD) of 287 ± 40 mg ($N = 38$). This variation was maintained for the second matings; hind femur length in sham-treated controls was 15.24 ± 0.57 mm ($N = 20$) vs. males with shortened titillators (T_{-2}^p) 15.51 ± 0.52 mm ($N = 19$; Student's *t*-test: $t = -1.55$, $P = 0.13$), and body mass of sham-treated controls was 313.75 ± 31.86 mm ($N = 20$) vs. males with shortened titillators 325.79 ± 40.38 mm ($N = 19$; Student's *t*-test: $t = -1.04$, $P = 0.31$).

MATING BEHAVIOURS

All mating pairs were observed and timed with a stopwatch to analyse the duration of several mating behaviours (see Wulff & Lehmann, 2016, 2020). The 'approach duration' is defined by the time from the first antennae contact until females crawl onto the back of the male. The mating continues with the behaviour 'reach copula position', whereby the female stays on the back of the male until fixation of the grasping cerci at the base of the ovipositor of the female and the head of the male is turned towards the ventral side of the female. The 'copula duration' is defined as the time between the establishment of a firm grip of the cerci of the male at the base of the ovipositor of the female until spermatophore transfer is completed.

During copulation, the titillators are inserted rhythmically into the female genital chamber (Wulff *et al.*, 2017, 2018). The frequency of this male 'copulatory titillator movements' in and out of the genitals of the female was counted during the first 8 min of copulation and averaged per minute. In instances where males showed arrhythmic movements interrupted by pauses > 20 s, counting time was prolonged until an uninterrupted period of 5 min was recorded.

Female behaviours during copulation were also recorded. The association between 'female resistance behaviour' (defined as struggling by jumping, fast walking, kicking or biting the male) in combination with the 'success of spermatophore transfer' was studied (for details, see Wulff & Lehmann, 2016).

TITILLATOR MORPHOLOGY

Male bushcricket titillators are chitinized and concealed inside the genital chamber of the male (Fig. 1). Analysis of the titillator morphology involved killing males (by freezing at -18 °C) and surgically extracting the titillators from the abdomen under a binocular microscope.

The greater part of the titillator is firmly fixed in the male tissue; therefore, only the free extending left titillator process was measured from the apical tip to the part where tissue attaches (Fig. 2). All measurements were made using a digital microscope (VHX-1000; Keyence, Neu-Ilsenburg, Germany; zoom-lens: VH-Z00W). Owing to the experimental design, with one group having their titillators cut, titillator lengths were measured only in 19 unmanipulated males.

SPERMATOPHORE MASS

For both the first and second matings, we removed the spermatophore from the female genitalia with

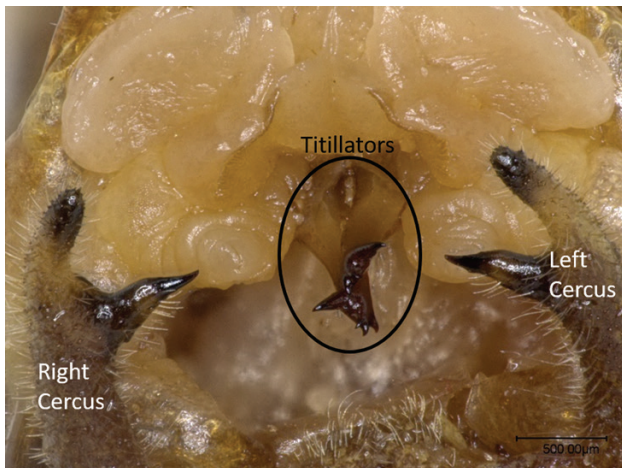


Figure 1. End of a male *Roeseliana roeselii* bushcricket abdomen, showing the cerci and titillator processes (original by Nadja Wulff).



Figure 2. Left titillator of *Roeseliana roeselii* dissected. The apical process is marked by a black line; it has a length of 842 µm in this example.

forceps after the spermatophore had been transferred by the male. Spermatophores were weighed on an electronic precision balance (Kern EG 300-3M; accuracy ± 0.1 mg). After the experiments, females were returned to the field.

MEASURED PARAMETERS

To analyse a set of mating-related parameters, we measured ten variables, which can be classified into three groups: body size, mass data and behavioural mating characters. As body size parameters, we chose male hind femur length (as a proxy for overall male

size) and titillator length. However, titillator size was accessible only in the unmanipulated males, resulting in a reduced dataset ($N = 19$). Both body size parameters are static characters, because they are fixed at adult ecdysis. In contrast, individual fitness is often visible in body mass data; therefore, we measured male body mass before mating, and postcopulation the mass of the transferred spermatophore. Motivation and individual decisions are expressed in behaviours. We measured four parameters of the mating sequence: the duration of approach, the duration to reach copula position, the total copula duration and the number of copulatory titillator movements.

In addition to the eight continuous characters, we tested for the influence of titillator manipulation in the second mating by counting the number of females exhibiting resistance behaviour during copulation and the number of failed spermatophore transfers. The association analysis for these two characters was performed by Fisher's exact test.

REPEATED-MEASURES ANOVA

Given that we had tested all males twice, at the first and at the second mating, we used a repeated-measures ANOVA on the eight continuous variables to test whether mating (first or second), titillator status (sham-treated or manipulated before second mating) or the interaction between both factors influenced the results.

ALLOMETRIC RELATIONSHIPS

Given that the relative size of a trait is a potential indicator of selection, we regressed each of the eight continuous characters to analyse allometric relationships. In an allometric relationship, the observed variables are related by the power law equation: $y = \gamma \times x^\beta$, with y being a dependent variable, γ a proportionality coefficient, x a measure of body size and β a scaling exponent. Logarithmic transformation of the data (Warton *et al.*, 2006) normalizes the distribution and reduces heteroscedasticity (Kerkhoff & Enquist, 2009; Packard, 2009; Packard *et al.*, 2011). It also allows easy comparison of slopes, including visual inspection (Glazier, 2013; Klingenberg, 2016; Mirth *et al.*, 2016). After \log_{10} -transformation, our equation becomes linear: $\log y = \log \gamma + \beta \log x$. The scaling exponent (β) is equivalent to the slope of the fitted line (Falster *et al.*, 2006). Allometric relationships were analysed using the standardized major axis (SMA) method (Supporting Information, Table S1), because this method assumes no direction of the relationship and covers the variances along the x - and the y -axes (Warton *et al.*, 2006; Smith, 2009; Stillwell *et al.*, 2016). Given that many studies regress their data using

ordinary least squares, we include this method for comparison (Supporting Information, Table S2).

Traits may vary in how they scale with each other: when trait size scales proportionally with another trait, with a slope value of $|1|$, the relationship is called isometric. This isometry can be positive (slope = 1) or negative (slope = -1). Disproportionate scaling of traits is classified as hyperallometry (slope > 1 for positive hyperallometry and < -1 for negative hyperallometry) or hypoallometry (slope between zero and one for positive hypoallometry and between zero and minus one for negative hypoallometry) (Bonduriansky & Day, 2003; Mirth *et al.*, 2016; Stillwell *et al.*, 2016). The usage of positive and negative hyper- and hypoallometric scaling relationships differs from usage in relationship to allometry. Negative allometries (slope < 1) equate to positive hypoallometries in our definition, whereas positive allometries (slope > 1) equate to positive hyperallometry (Anichini *et al.*, 2017; Rebrina *et al.*, 2020).

For testing the hypothesis H0 of isometry, we calculated the value of the slope ($\pm\beta$) based on the dimensions of the two traits in question. Assuming that a body grows equally in all three dimensions, body volume/mass grows as a cube of body length (Small, 1996; Rebrina *et al.*, 2020). Thus, when male body mass or spermatophore mass was analysed in relationship to hind femur length, the slope value to test for isometry was equal to the slope of three, representing a cubic vs. a linear relationship. The value of the SMA slope could vary between zero and three in the case of hypoallometry and could be greater than three in the case of hyperallometry. In contrast, when titillator length was analysed for isometry in relationship to femur length, or spermatophore mass in relationship to body mass, the factor equals one. The resulting pairwise coefficients of determinations, denoted R^2 , between all eight continuous traits are presented in a matrix for the SMA (Supporting Information, Table S1) and the ordinary least squares correlations (Supporting Information, Table S2). Using linear regressions with only one explanatory variable, the coefficient of determination, R^2 , describes the proportion of variance of the response variable (y) explained by the explanatory variable (x), ranging between zero (or 0%) and one (or 100%). This is a widely used measure of the strength of regressions (Kasuya, 2019), with effect sizes conventionally interpreted as high ($R^2 > 0.25$), medium ($R^2 > 0.09$) or low ($R^2 > 0.01$) in agreement with Cohen (1992).

VARIANCES

Selection, both sexual and natural, acts on phenotypically expressed differences between

individuals (Nakagawa *et al.*, 2015). Therefore, we compared the variances for the eight continuous traits. The morphological characters (male body size and titillator length) are fixed at ecdysis; hence, they were not compared between matings. In contrast, male body mass and spermatophore mass depend on food intake and food conversion and might differ between matings. Consequently, they were analysed separately for the first and the second matings. Mating behaviours are generally considered highly dynamic traits, because they show high levels of variation (Gerhardt, 1991; Gerhardt & Huber, 2002; Stamps, 2016; Patricelli *et al.*, 2016). They were, therefore, also analysed separately for the first and second matings. Given that the steepness of an allometric relationship also influences the variance (Eberhard *et al.*, 1998; Cuervo & Møller, 2001), we provide the modified coefficient of variation (CV'), calculated as the variance of data dispersion around the regression line for male body mass, spermatophore mass and titillator length against body size (Supporting Information, Table S1).

STATISTICAL ANALYSIS

For statistical analysis, the R software package v.3.6.1 (R Core Team, 2019) was used, with standardized major axis regression using the R package 'smatr' v.3.4.8 (Falster *et al.*, 2006).

DATA AVAILABILITY

The data underlying this article will be shared on reasonable request to the corresponding author.

RESULTS

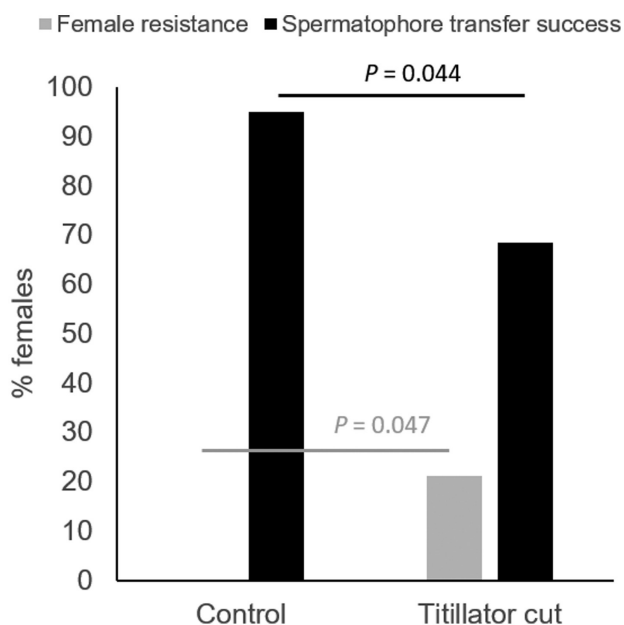
TITILLATOR MANIPULATION

When comparing males in the second mating, titillator manipulation had no significant effect on any of the male mating-related traits (Table 1). Titillator ablation did not influence the mass of the nuptial gift (spermatophore mass), the number of male genital stimulations (copulatory titillator movements) or the duration of mating (repeated-measures ANOVA, titillator cut vs. control: $F_{1,36} = 0.20-3.09$, $P = 0.09-0.66$; see Table 1). However, the interaction term for spermatophore mass and approach duration was significant, indicating some influence of titillator manipulation on the change from first to second matings (Table 1).

Despite the lack of influence on male traits, cutting the titillator tips resulted in altered female mating behaviour. Females mated with control males showed no resistance during copulation, but four females mated to titillator-manipulated males struggled

Table 1. Differences in male traits of *Roeseliana roeselii* analysed for the first vs. the second mating and for titillator-manipulated vs. control males in the second mating by repeated-measures two-way ANOVA with interaction

Parameter	First vs. second mating		Titillator cut vs. control	Interaction		
Male body mass mg	$F_{1,36} = 50.50$	$P < 0.001$	$F_{1,36} = 0.50$	$P = 0.49$	$F_{1,36} = 0.80$	$P = 0.38$
Spermatophore mass mg	$F_{1,36} = 18.53$	$P < 0.001$	$F_{1,36} = 0.78$	$P = 0.38$	$F_{1,36} = 13.89$	$P < 0.001$
Approach min	$F_{1,36} = 6.64$	$P = 0.014$	$F_{1,36} = 1.75$	$P = 0.19$	$F_{1,36} = 7.06$	$P = 0.0091$
Reach copula position min	$F_{1,36} = 7.58$	$P = 0.0092$	$F_{1,36} = 0.20$	$P = 0.66$	$F_{1,36} = 0.00$	$P = 1.00$
Copula total duration min	$F_{1,36} = 0.28$	$P = 0.60$	$F_{1,36} = 2.60$	$P = 0.12$	$F_{1,36} = 2.59$	$P = 0.12$
Copulatory movements (N/min)	$F_{1,36} = 6.83$	$P = 0.013$	$F_{1,36} = 3.09$	$P = 0.09$	$F_{1,36} = 0.96$	$P = 0.33$

**Figure 3.** Percentage of *Roeseliana roeselii* females showing resistance behaviour (orange column) and success of spermatophore attachment (green columns) for females mated either with a sham-treated control male (left) or with a titillator-manipulated male (T_{-2} ; right). Significant difference using Fisher's exact tests: P values.

against their partners (Fig. 3), which represented a significant difference (Fisher's exact test: $P = 0.047$). Spermatophore transfer was successful for 19 control males, but for only 13 titillator-manipulated males (Fisher's exact test: $P = 0.044$).

ALLOMETRIC RELATIONSHIPS

When analysing correlations between male traits, several of these turned out to be significant (Fig. 4), with coefficients of determination reaching $\leq 45\%$

(Supporting Information, Table S1). Hereafter, we concentrate on the 13 significant allometries, of which 11 showed hyperallometric scaling. The full table for all allometric relationship is presented for completeness (Supporting Information, Table S1).

Male body mass (mbm) was significantly correlated with body size at first mating, at both the first and second mating (Figs 4, 5A). The scaling had a positive hyperallometric correlation at the first mating (SMA: $y_{\log \text{mbm}} = 3.88x_{\log \text{body size}} - 2.15$, $R^2 = 0.45$, $N = 39$) and scaled isometric at the second mating (SMA: $y_{\log \text{mbm}} = 3.11x_{\log \text{body size}} - 1.18$, $R^2 = 0.39$, $N = 39$). Larger males also transferred heavier spermatophores (Fig. 5B), exhibiting positive hyperallometry for the first (SMA: $y_{\log \text{spermatophore}} = 8.73x_{\log \text{body size}} - 8.92$, $R^2 = 0.17$, $N = 39$) and second mating (SMA: $y_{\log \text{spermatophore}} = 1.49x_{\log \text{body size}} - 3.97$, $R^2 = 0.21$, $N = 39$). This positive hyperallometry was even stronger when relating spermatophore mass to male body mass (Fig. 5C), resulting in large effect sizes of 25% (SMA_{first mating}: $y_{\log \text{spermatophore}} = 2.2x_{\log \text{mbm}} - 4.08$, $R^2 = 0.25$, $N = 38$) and 29% of the explained variance, respectively (SMA_{second mating}: $y_{\log \text{spermatophore}} = 1.49x_{\log \text{mbm}} - 2.21$, $R^2 = 0.29$, $N = 39$).

Another significant relationship was found for the length of the free titillator process, which showed a medium effect size for positive hyperallometric correlation with male body size (SMA: $y_{\log \text{titillator}} = 1.93x_{\log \text{body size}} + 0.66$, $R^2 = 0.15$, $N = 18$), such that larger males possessed proportionally longer titillators (Fig. 6A). Larger males not only had longer titillators, but they also performed more titillator movements during copulation at first mating (SMA: $y_{\log \text{copulatory movements}} = 7.50x_{\log \text{body size}} - 7.90$, $R^2 = 0.11$, $N = 39$), although the correlation was not significant for the second mating (SMA: $y_{\log \text{copulatory movements}} = 11.42x_{\log \text{body size}} - 12.62$, $R^2 = 0.017$, $N = 39$; Fig. 6B).

Males with longer titillator processes spent less time in copula (Fig. 7A), exhibiting significant negative hyperallometry for the first mating (SMA: $y_{\log \text{copula}} = -3.24x_{\log \text{titillator}} + 11.00$, $N = 18$), with a medium

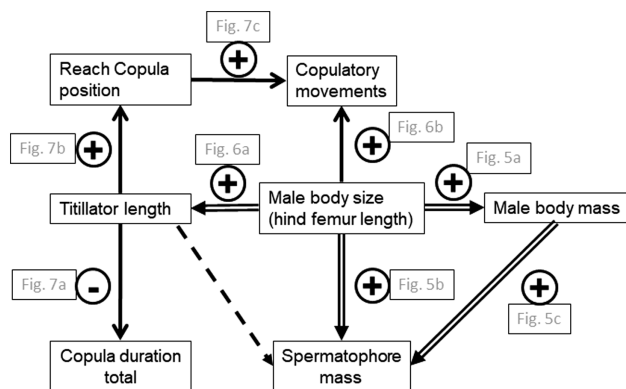


Figure 4. Schematic overview of significantly correlated male traits, at both first and second mating (double-lined arrows, pink), at only first mating (single-lined arrow, dark blue) or at only second mating (dashed-lined arrow, light blue). We also indicate whether the allometric relationship was positive hyperallometric (+) or negative hyperallometric (-). The correlation matrix for all relationships is presented in the [Supporting Information](#) (Table S1). For comparison, we cross-refer to the correlation plots presented in [Figures 5–7](#).

effect size ($R^2 = 0.18$). Although the slope was also negative, this correlation was not significant for the second mating (SMA: $y_{\log \text{ copula}} = -3.03x_{\log \text{ titillator}} + 10.38$, $R^2 = 0.044$, $N = 18$). The duration that mating pairs needed to reach the copula position was greater for males with longer titillator processes ([Fig. 7B](#)). It exhibited a positive hyperallometric relationship for the first mating of a male (SMA: $y_{\log \text{ reach copula position}} = 3.51x_{\log \text{ titillator length}} - 10.32$, $R^2 = 0.13$, $N = 18$) but was not significant in second matings (SMA: $y_{\log \text{ reach copula position}} = 16.77x_{\log \text{ titillator length}} - 49.03$, $R^2 = 0.024$, $N = 18$). Males that spent more time reaching the copula position moved their titillators more frequently afterwards ([Fig. 7C](#)), as shown by positive hyperallometry for the first mating of a male (SMA: $y_{\log \text{ copula movements}} = 1.86x_{\log \text{ reach copula position}} - 1.80$, $R^2 = 0.12$, $N = 39$). This correlation was not significant, probably owing to the large variation in times to reach copula position for second matings (SMA: $y_{\log \text{ copula movements}} = -1.26x_{\log \text{ reach copula position}} + 3.37$, $R^2 = 0.0034$, $N = 39$).

VARIANCES

There were substantial differences in the extent to which male traits varied ([Fig. 8](#); [Supporting Information](#), Table S1). The two static morphological characters fixed at final moult (the length of the male hind femur and the length of the free titillator process) showed the lowest levels, with 3.6 and 7.0% variance, respectively. Intermediate levels of variance, ranging from 11 to 30%, were found for male body mass and spermatophore mass. The greatest variance was seen

for the behavioural characters, with intermediate levels for the number of copulatory movements and the copula duration and high levels found for the approach duration and the time to reach the copula position ([Fig. 8](#)). Percentage of variances were similar for the first and the second matings for all characters. However, the variance was slightly reduced for male body mass and spermatophore mass in the second matings. Inter-mating variance was especially high for the time to reach the copula position, which increased threefold in the second mating.

DISCUSSION

Sexual selection generally favours the evolution of hyperallometry by directional selection on trait size ([Bonduriansky & Day, 2003](#); [Kodric-Brown *et al.*, 2006](#); [Eberhard *et al.*, 2009, 2018](#)). It should be noted that this relationship is not bijective; hyperallometry is not restricted to sexually selected characters, nor do all sexual characters scale hyperallometrically. However, scaling relationships add correlational evidence for sexual selection on phenotypic trait expression. Individuals with superior condition might be able to invest more into exaggerated traits ([Emlen & Nijhout, 2000](#); [Johnstone *et al.*, 2009](#)), linking hyperallometry with handicap models of sexual selection ([Andersson, 1994](#); [O'Brien *et al.*, 2018](#); [Rodríguez & Eberhard, 2019](#)).

Male body mass, spermatophore mass and titillator length all scale hyperallometrically with body size in *R. roeselii*, indicative of characters under sexual selection. Body mass as a conditional trait was hyperallometrically correlated with body size. As a consequence, males with larger body size were absolutely and relatively heavier than smaller males. Body mass might not be under sexual selection directly but reflect the condition of a male. Heavier male bushcrickets can outcompete lighter rivals acoustically ([Anichini *et al.*, 2018, 2019](#)), and females from several bushcricket species prefer heavier males ([Gwynne, 1982](#); [Wedell & Sandberg, 1995](#); [Lehmann & Lehmann, 2008](#)). Moreover, heavier male bushcrickets transfer larger spermatophores in many species ([Gwynne, 1982](#); [Bailey & Gwynne, 1988](#); [Wedell & Arak, 1989](#); [Simmons & Bailey, 1990](#); [Wedell, 1993b](#); [Heller & Reinhold, 1994](#); [Wedell & Sandberg, 1995](#); [Wedell & Ritchie, 2004](#); [Gao & Kang, 2006a, b](#); [Reinhold & Sevgili, 2007](#); [Lehmann & Lehmann, 2008, 2009](#); [McCartney *et al.*, 2010](#); [Ortiz-Jiménez & Cueva del Castillo, 2015](#); [Uma & Sevgili, 2015](#)). As expected under a conditional model, heavier males invest more resources than lighter ones and can therefore bear the increased costs of enlarged spermatophores ([Lewis *et al.*, 2014](#)). As with body mass, spermatophore mass

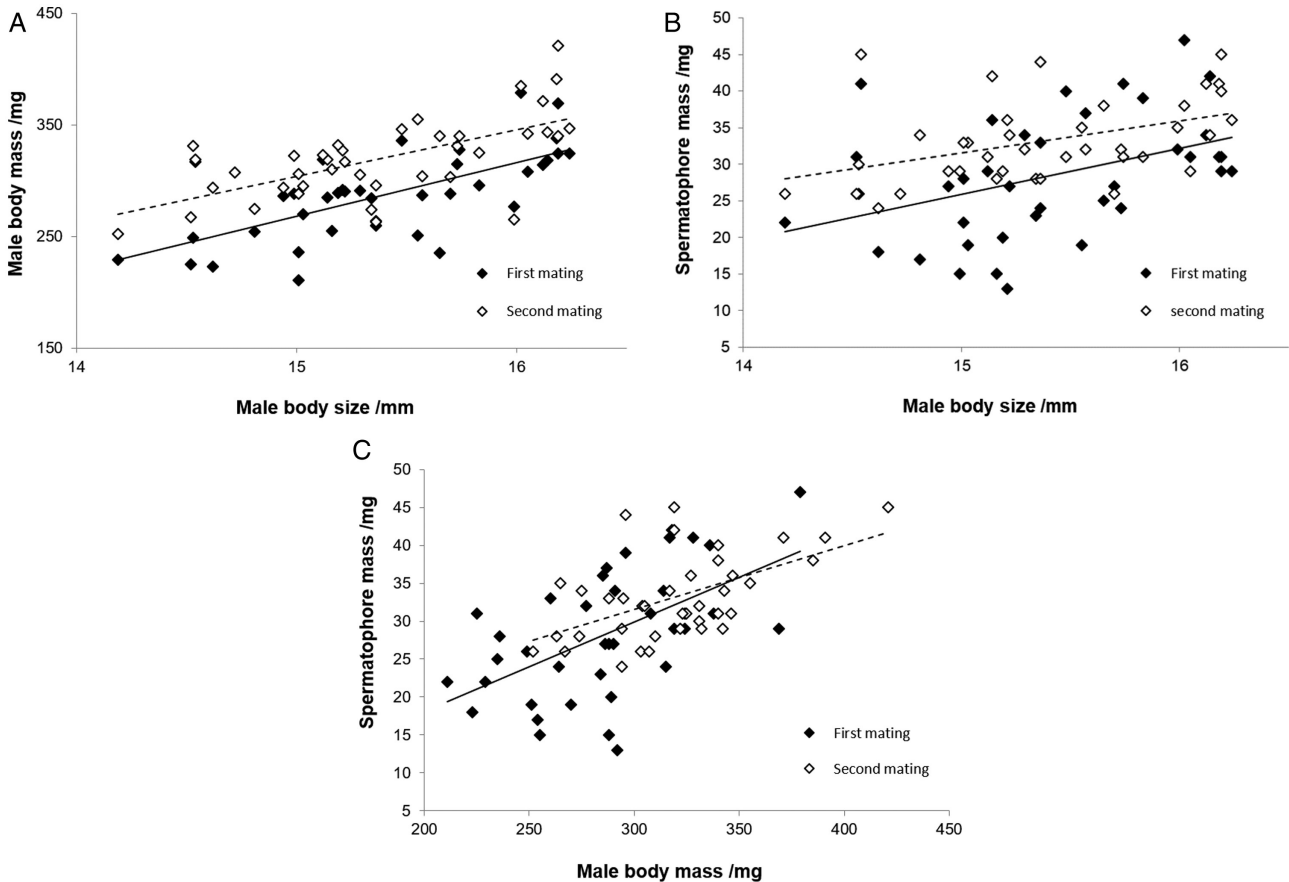


Figure 5. Correlations of *Roeseliana roeselii* male body size, male body mass and spermatophore mass for the first (dark blue diamonds, continuous lines) and second matings (light blue diamonds, dashed lines). A, male body mass in correlation with male body size. The correlation was positive hyperallometric at the first mating (SMA: $y_{\log \text{mbm}} = 3.88x_{\log \text{body size}} - 2.15$, $R^2 = 0.45$, $N = 39$) and isometric at the second mating (SMA: $y_{\log \text{mbm}} = 3.11x_{\log \text{body size}} - 1.18$, $R^2 = 0.39$, $N = 39$). B, spermatophore mass in correlation with male body size. Larger males transferred heavier spermatophores, exhibiting positive hyperallometry for the first (SMA: $y_{\log \text{spermatophore}} = 8.73x_{\log \text{body size}} - 8.92$, $R^2 = 0.17$, $N = 39$) and second mating (SMA: $y_{\log \text{spermatophore}} = 1.49x_{\log \text{body size}} - 3.97$, $R^2 = 0.21$, $N = 39$) of a male. C, spermatophore mass in correlation with male body mass. Heavier males transferred heavier spermatophores, exhibiting positive hyperallometry for the first (SMA: $y_{\log \text{spermatophore}} = 2.25x_{\log \text{mbm}} - 4.08$, $R^2 = 0.25$, $N = 38$) and second mating (SMA: $y_{\log \text{spermatophore}} = 1.49x_{\log \text{mbm}} - 2.21$, $R^2 = 0.29$, $N = 39$) of a male.

had positive hyperallometric scaling against body mass in *R. roeselii* for both the first and second matings of the male (Fig. 5). That spermatophore size is under sexual selection is reflected in the proportionally greater increase in mass between the first and second matings; while male body mass increased on average by 11%, spermatophore mass was on average 17% heavier in the second mating.

Finding that spermatophore mass is under strong directional selection is in some respects surprising, because the bushcricket spermatophore is a large and energetically expensive nuptial gift (Gwynne, 2008; Lehmann, 2012; Lewis *et al.*, 2014). However, males benefit from larger spermatophylaxes owing to the prolonged duration of consumption by the female, which increases the amount of sperm transferred

(Gwynne, 1986; Simmons & Gwynne, 1991; Reinhold & Heller, 1993; McCartney *et al.*, 2013; Reinhold & Ramm, 2013) and reduces the risk of future sperm competition (Wedell, 1991; Simmons, 2001). Furthermore, the ejaculate contains a rich cocktail of proteins (Lehmann *et al.*, 2018), prolonging the post-mating sexual refractory period of the female in a dose-dependent manner (Gwynne, 1986; Wedell & Arak, 1989; Simmons & Gwynne, 1991). Comparative evidence also suggests that substances in large ejaculates decrease the lifetime degree of polyandry in female bushcrickets (Vahed, 2006).

Females, in turn, prefer heavier males that transfer larger spermatophores (Lehmann & Lehmann, 2008, 2009). Spermatophores provide direct benefits to females because the material is a fast-uptake

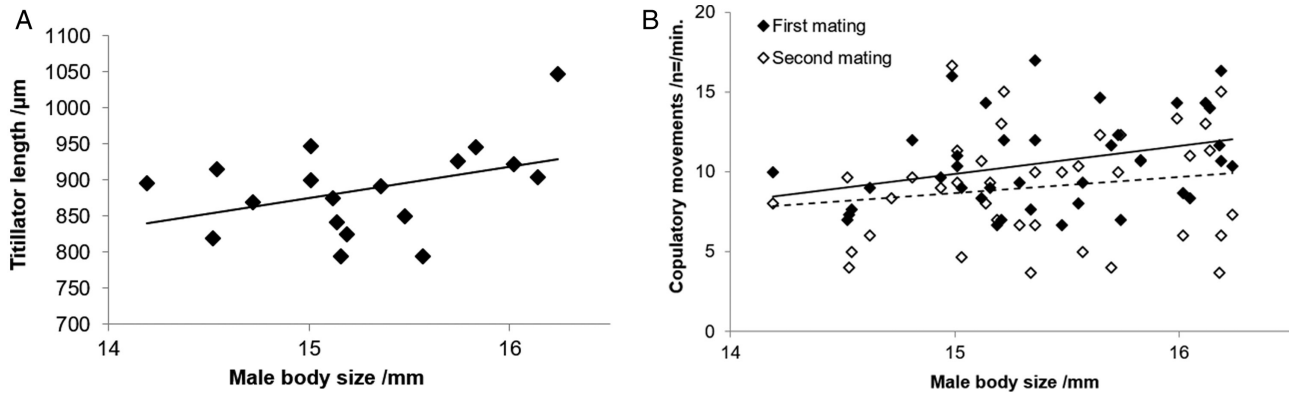


Figure 6. Correlations of male body size with titillator length and copulatory movements in *Roeseliana roeselii* for the first (dark blue diamonds, continuous lines) and second matings (light blue diamonds, dashed lines). A, length of the free titillator process in correlation with male body size. The correlation was positive hyperallometric (SMA: $y_{\log \text{ titillator length}} = 1.93x_{\log \text{ body size}} - 0.66$, $R^2 = 0.15$, $N = 18$). Given that both traits are static characters, they are identical for the first and the second matings. B, number of copulatory movements per minute in correlation with male body size. Larger males titillated hyperallometrically more during their first mating (SMA: $y_{\log \text{ copulatory movements}} = 7.50x_{\log \text{ body size}} - 7.90$, $R^2 = 0.11$, $N = 39$), but this correlation was not significant for second matings (SMA: $y_{\log \text{ copulatory movements}} = 11.42x_{\log \text{ body size}} - 12.62$, $R^2 = 0.017$, $N = 39$).

food (Lehmann & Lehmann, 2016), contributing to body homeostasis (Voigt *et al.*, 2006, 2008). Larger spermatophores increase fecundity, egg size, hatching success, growth rate of sons, and help offspring to survive starvation (summarized by Gwynne, 2001; Lehmann, 2012).

Animal genitalia are often sexually selected traits (Eberhard, 1985, 2009; Hosken & Stockley, 2004; Simmons, 2014), with multiple functions during mating, especially in facilitating sperm transfer (Simmons, 2001). The bushcricket titillators of *R. roeselii* function as copulatory courtship devices (Wulff *et al.*, 2015, 2017), stimulating females (Wulff *et al.*, 2018) and suppressing female resistance (Wulff & Lehmann, 2016, 2020; Wulff *et al.*, 2018). The cumulative evidence indicates that titillators are sexually selected by cryptic female choice (Eberhard & Lehmann, 2019), and there is also evidence for sexual agonistic co-evolution (Wulff & Lehmann, 2020). Animal genitalia tend to scale isometrically with body size across a great variety of arthropods and mammals, a fact described by the ‘one-size-fits-all’ expression (Eberhard *et al.*, 1998, 2009; Eberhard, 2009). This contrasts with many sexually selected traits, especially those used as threat signals, such as weapons (Eberhard *et al.*, 2018; Rodriguez & Eberhard, 2019). However, we found hyperallometric scaling of the length of the free titillator processes in relationship to male body size, meaning that larger males possessed proportionally longer titillators. Given that the titillators do not function as grasping devices or have a role in sperm competition (Wulff *et al.*, 2015; Wulff & Lehmann, 2016), their hyperallometric scaling suggests that they are sexually selected traits

(Kodric-Brown *et al.*, 2006; Eberhard *et al.*, 2018). In combination with the number of movements during copulation, titillator length might convey information about the quality of the male and act as an honest signal to females. Indeed, the number of titillator movements was hyperallometric in relationship to body size, at least for the first mating. In consequence, larger males titillated their females more frequently during copulation. Such increased titillation probably stimulates females in a way (Wulff *et al.*, 2018) that leads to a greater acceptance of the spermatophore from the male (Wulff & Lehmann, 2016, 2020). One behavioural outcome of longer titillators was reduced time spent in copula, with a significant negative hyperallometry for the first mating, but a non-significant allometry for the second mating. Interestingly, this is in line with results from the carabid beetle *Carabus maiyasanus*, in which males with longer genitalia also had shorter copula durations (Okuzaki & Sota, 2014).

Titillator manipulation in *R. roeselii* was established as a tool to test for genitalia-related behaviours (Wulff & Lehmann, 2016). When comparing males at the second mating, cutting both titillator processes led to changes in female behaviour, and one in five of them struggled with their mates. Consequently, only three in four of the titillator-manipulated males could transfer their spermatophores successfully, whereas for the males in the control group the success rate was 95%. The occurrence and frequency of this resistance behaviour corresponds well to the results from previous experiments (Wulff & Lehmann, 2016, 2020) and supports our hypothesis that titillators in this species influence the decision by the female to accept the mating and the spermatophore transfer

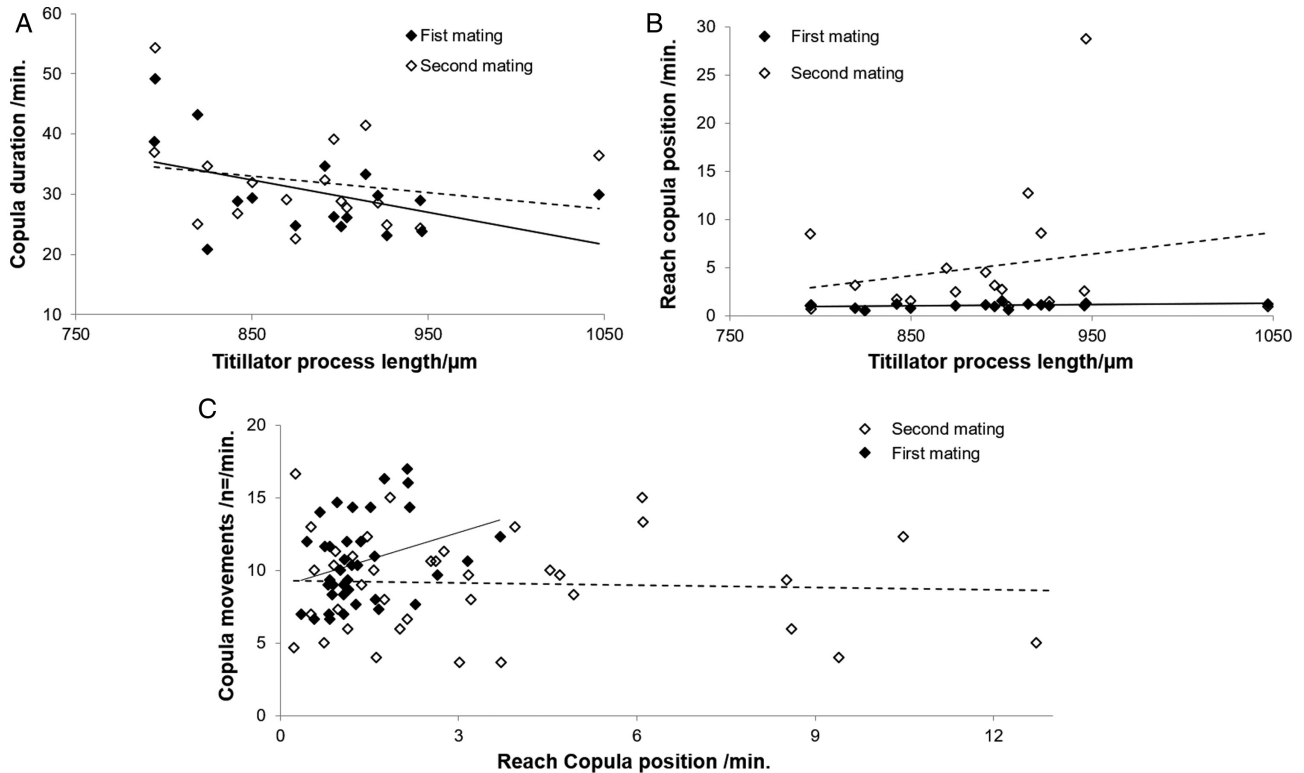


Figure 7. Correlations of mating behaviours with titillator process length in *Roeseliana roeselii* males at their first (dark blue diamonds, continuous lines) and second mating (light blue diamonds, dashed lines). A, copula duration in correlation with titillator length, negative hyperallometric for the first mating of a male (SMA: $y_{\log \text{ copula}} = -3.24x_{\log \text{ titillator}} + 11.00$, $R^2 = 0.18$, $N = 18$), but non-significant for second matings (SMA: $y_{\log \text{ copula}} = -3.0x_{\log \text{ titillator}} + 10.38$, $R^2 = 0.044$, $N = 18$). B, time to reach the copula position in correlation with the titillator length; positive hyperallometric for the first mating of a male (SMA: $y_{\log \text{ reach copula position}} = 3.51x_{\log \text{ titillator length}} - 10.32$, $R^2 = 0.13$, $N = 18$), but non-significant for the second mating (SMA: $y_{\log \text{ reach copula position}} = 16.77x_{\log \text{ titillator length}} - 49.03$, $R^2 = 0.024$, $N = 18$). C, males needing longer to reach the copula position moved their titillators more frequently afterwards, as shown by positive hyperallometry for the first mating of a male (SMA: $y_{\log \text{ copula movements}} = 1.86x_{\log \text{ reach copula position}} - 1.80$, $R^2 = 0.12$, $N = 39$), but non-significant for second matings (SMA: $y_{\log \text{ copula movements}} = -1.26x_{\log \text{ reach copula position}} + 3.37$, $R^2 = 0.0034$, $N = 39$). For better visibility, the x-axis is truncated, which excludes two outliers reaching copula position at 28 and 42 min.

(Wulff *et al.*, 2017, 2018). In contrast, males were unaffected by the titillator manipulation in their mating-related traits. Titillator-manipulated males transferred similar large nuptial gifts, exhibited a similar number of copulatory titillator movements (see also Wulff & Lehmann, 2016, 2020) and reached all behavioural landmarks at similar times compared with unmanipulated control males. Previously, we also found unchanged male mating traits when females were sensorially blinded (Wulff *et al.*, 2018). The finding that only female behaviours are affected by titillator manipulation is in line with evidence that titillators in *R. roeselii* are under sexual selection by cryptic female choice (Eberhard & Lehmann, 2019).

Variation is central for any model of sexual selection (Darwin, 1871), with its genetic basis (Prokuda & Roff, 2014) reflected in phenotypic plasticity

(West-Eberhard, 2003). Higher variance is typically observed in sexually selected rather than non-sexually selected traits (Eberhard *et al.*, 1998, 2018; Cotton *et al.*, 2006; Reinhold, 2011). Mating-related behaviours exhibited the highest variances between individuals (23–160%), reflecting the highly dynamic nature of behavioural characters and contrasting with the rather static nature of size (3.6–7%). Variance in mass-related traits (body mass and spermatophore mass) lay between (11–30%) these traits.

There is the need to stratify the data, not only for function (Rodríguez & Eberhard, 2019) but also according to the type of data (such as size, mass and behavioural traits). Applying this approach to our data confirmed that the titillators had twice as much variance compared with the femur length (7.0 vs. 3.6%) and that variance in spermatophore mass

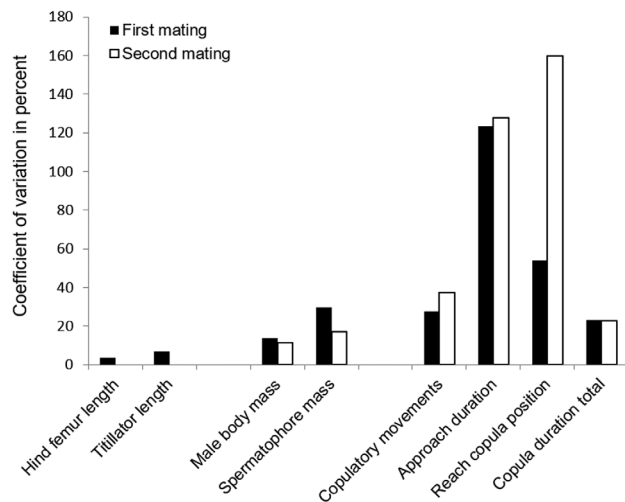


Figure 8. Variances of *Roeseliana roeselii* male mating-related characters analysed by the coefficient of variance (CV = standard deviation divided by mean size). Data are grouped into the static body sizes, which are fixed at the final moult and therefore identical for first and second mating (left), and the dynamic body masses (middle) and behaviours (right), which are separated for the first (dark blue) and second mating (light blue).

was nearly twice that for body mass for the first (30 vs. 14%) and the second matings (17 vs. 11%). These comparisons were influenced only marginally by whether we used the coefficient of variation (CV) or the modified CV', taking the dispersion of data around the regression line into account (see [Supporting Information, Table S1](#)).

Although not tested directly, the hyperallometric scaling along with higher variance provides correlational indications that spermatophore mass and titillator length are both under directional selection attributable to female choice. Furthermore, larger males with longer titillators mate more quickly and titillate with a higher frequency, which suggests the potential for selection based on condition-dependent trait expression. Our results are in line with indicator models of sexual selection acting in *R. roeselii* on multiple sets of traits spanning the precopulatory, copulatory and postcopulatory periods of mating.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Pairwise standardized major axis (SMA) correlation matrix between eight \log_{10} -transformed characters in male *Roeseliana roeselii*, separated for the first and the second mating. Top row, coefficient of determination (R^2) describing the strength of the linear relationship, given in bold if significant. Middle row, simple linear regression, represented as $\beta(\text{slope}) \times x + \alpha$ (intercept), shown in bold when deviating from an isometric allometry. Third row, coefficient interval of the slope, shown in bold when deviating from an isometric allometry. Abbreviations: CV, coefficient of variation (as a percentage); CV' , modified coefficient of variation, calculated as the variance of data dispersion around the regression line of the traits against body size; N , sample size; SD, standard deviation.

Table S2. Pairwise ordinary least squares (OLS) correlation matrix between eight \log_{10} -transformed characters in male *Roeseliana roeselii*, separated for the first and the second mating. Top row, coefficient of determination (R^2) describing the strength of the linear relationship, given in bold if significant. Bottom row, simple linear regression, represented as $\beta(\text{slope}) \times x + \alpha$ (intercept). Abbreviations: CV, coefficient of variation (as a percentage); N , sample size; SD, standard deviation.