Male genital titillators and the intensity of postcopulatory sexual selection across bushcrickets

Gerlind U.C. Lehmann[1(*)], James D. J. Gilbert[2], Karim Vahed[3], Arne W. Lehmann[4]

[1] Humboldt-University Berlin, Department of Biology, Behavioral Physiology, Invalidenstrasse 43, 10115 Berlin, Germany.

[2] School of Biological, Biomedical and Environmental Sciences, University of Hull, UK.

[3] Environmental Sustainability Research Centre, Department of Natural Sciences, University of Derby, Derby DE22 1GB, UK.

[4] Friedensallee 37, 14532 Stahnsdorf, Germany.

Abbreviated title: Genital titillators and polyandry in bushcrickets

[(*)] Corresponding author

Gerlind U.C. Lehmann

Humboldt-University Berlin, Department of Biology, Behavioral Physiology,

Invalidenstrasse 43, 10115 Berlin, Germany.

Tel.: ++49-30-2093-9006

e-mail: *gerlind.lehmann@t-online.de*

24 **Abstract**

25 Animal genitalia are diverse and a growing body of evidence suggests that they evolve

26 rapidly under post-copulatory sexual selection. This process is predicted to be more intense

27 in polyandrous species, although there have been very few comparative studies of the

28 relationship between the complexity of genital structures in males and measures of the

29 degree of polyandry. In some bushcricket families, males possess sclerotised copulatory

30 structures known as titillators, which are inserted into the female's genital chamber and

31 moved rhythmically. Like other genital structures, bushcricket titillators are widely used as

32 important taxonomic characters and show considerable variation across species in structure,

33 shape and the extent to which they are spined. Here, we examine relationships between the

34 presence/absence of titillators, titillator complexity and both mating frequency and the

35 degree of polyandry in bushcrickets, using phylogenetic comparative analyses. Using

36 published sources combined with original observations, data were obtained for the mean

37 level of polyandry, the duration of the male and female sexual refractory periods and the

38 level of complexity of titillators. To analyse data, we fitted phylogenetic generalised least

39 squares models. No significant relationships were found between titillator presence or

40 complexity and either the level of polyandry, duration of the male's sexual refractory

41 period or the ratio of the female and male sexual refractory periods. The duration of the

42 female's refractory period, however, was positively associated with titillator presence and

43 negatively associated with titillator complexity. The data therefore partially support the

44 hypothesis that post-copulatory sexual selection drives genital evolution in this taxon.

45
46

47   Genital structures are extremely diverse and are therefore often used as taxonomic

48   characters to separate closely related species (Eberhard 1985, 2010). Genital traits appear to

49   diverge more rapidly than non-genital traits (Rowe and Arnqvist 2012). The selective

50   pressures that drive such rapid evolutionary change in genitalia have been the subject of

51   considerable debate. These include selection for species isolation (the "Lock and Key"

52   hypothesis, reviewed in Mikkola 2008), neutral evolution (Pleiotropy hypothesis) and

53   sexual selection (reviewed in Eberhard 2010; Simmons 2014). The sexual selection

54   hypothesis potentially involves three closely inter-related processes of post-copulatory

55   sexual selection: sperm competition, sexually antagonistic co-evolution and cryptic female

56   choice (Simmons 2014). Strong support for the role of sexual selection in genital evolution

57   comes from comparative studies that have found a positive relationship between indices of

58   the degree of polyandry (and hence the intensity of sexual selection) and the degree of

59   evolutionary divergence or elaboration of genital structures in mammals (Ramm 2007; Orr

60   and Brennan 2016) and insects (Arnqvist 1998, Rowe and Arnqvist 2012).

61

62   Bushcrickets (Orthoptera: Tettigoniidae) are an excellent model taxon in which to test

63   hypotheses relating to the evolution of copulatory structures. They are a diverse and

64   relatively well studied family of insects, which exhibit varying degrees of polyandry

65   (Gwynne 2001; Vahed 2006, 2007). Two types of sclerotized copulatory structures occur in

66   male bushcrickets: the cerci and titillators. The cerci act as genital claspers (Rentz 1972,

67   Vahed et al. 2014). Comparative evidence suggests that sexual conflict over the duration of

68   ejaculate transfer has affected the form and function of the cerci in bushcrickets (Vahed et

69   al. 2014, Lehmann et al. 2016). Titillators (Figure 1) are concealed structures that are

70   inserted into the female's genital chamber prior to spermatophore transfer and are moved

71   rhythmically with contractions of the male's abdomen and phallic complex (Wulff et al.

72   2015, 2017; Wulff & Lehmann 2016). The tips of the male's titillators contact the sensilla-

73   rich, un-sclerotised, soft tissues of the opening of the female's genital chamber during

74   copulation, prior to spermatophore transfer (Wulff et al. 2015, 2017). Like other genital

75   structures, bushcricket titillators are widely used as important taxonomic characters

76   (Chamorro-Rengifo and Lopes-Andrade 2014) and show considerable variation across

77   species in structure, shape and the extent to which they are spined (Vahed et al. 2011).

78   Comparative evidence indicates that the presence of titillators is associated with longer

79   copulation durations (prior to spermatophore transfer) in bushcrickets (Vahed et al 2011),

80   but the relationship between titillator complexity and polyandry has not previously been

81   examined. In fact, we are not aware of any previous studies that have tested for a

82   relationship across species between direct measures of the degree of polyandry and genital

83   complexity in any animal taxon (for studies that have used in-direct measures of the degree

84   of polyandry or used a binary "monandrous versus polyandrous" classification, see Ramm

85   2007; Arnqvist 1998; Rowe and Arnqvist 2012; Orr and Brennan 2016; Kuntner et al.

86   2016). Bushcrickets are one of the few animal groups in which data on the lifetime degree

87   of polyandry are available for a range of species (Vahed 2006).

88

89   Here, we use phylogenetic comparative analyses to test the prediction that genital

90   complexity in males will be positively related to the intensity of sexual selection in

91   bushcrickets. We examine the relationship between titillator complexity and two related

92   measures of the intensity of sexual selection: the lifetime degree of polyandry and the

93   relative potential reproductive rates (the reciprocal of the sexual refractory period) of males

94   and females. Where the male's potential reproductive rate exceeds that of the female (i.e.

95   where the operational sex ratio is male biased), males are assumed to be under stronger

96   sexual selection (Clutton-Brock & Parker 1992, Ahnesjö et al. 2001: see also Kokko et al.

97   2014 for a critical review).

98

99   It should be noted that our study is not designed to distinguish between the different

100   mechanisms of postcopulatory sexual selection (i.e. sperm competition, cryptic female

101   choice and sexually antagonistic co-evolution). One mechanism of postcopulatory sexual

102   selection, however, may be distinguishable because it potentially makes the opposite

103   prediction: If complex genitalia cause damage to the female's reproductive tract (see

104   Crudginton and Siva-Jothy 2000) and thereby delay the female from re-mating, then more

105   elaborate genitalia could be associated with a longer "time out" from mating (and therefore

106   a lower potential reproductive rate in females and a lower degree of polyandry, see

107   Stockley 2002; Kuntner et al. 2016).

108

Methods

Data on the mean lifetime degree of polyandry were obtained for 29 species of tettigoniid bushcrickets from published sources including our own studies (Supplementary Table S1). For the majority of these species, data on polyandry were obtained from counts of spermatodoses within the spermatheca of tettigoniids sampled at the end of the season in the field (Gwynne 1984, Vahed 2006, Vahed et al. 2011, Gwynne & Lorch 2013, Kaňuch et al. 2015; Gao Yong, pers. comm.). Spermatodoses are capsules of sperm that are formed within the female's sperm storage organ (spermatheca) each time she mates (Vahed 2003, 2006, Parker et al. 2017). Microsatellite analysis of sperm from spermatodoses in the bushcricket *Pholidoptera griseoaptera*, which had mated under natural field conditions, revealed that in over 80% of females, each spermatodose within the spermatheca was from a different male (Parker et al. 2017). Re-mating between pairs in bushcrickets is unlikely due to the long sexual refractory periods of both males and females that occur after each mating, which are associated with the transfer of the large spermatophores that are typical of this family (Vahed 2007). Because of this, spermatodose counts can be used to estimate the degree of polyandry in bushcrickets (Gwynne 1984, Vahed 2006, Vahed et al. 2011, Gwynne & Lorch 2013, Kaňuch et al. 2015). Complementary data were obtained from assessments of caged populations (in one species, *Lluciapomaresius stalii*, Bateman 1998), or marked field populations (in six taxa, Heller and von Helversen 1991, McCartney 2010) and, in two species, molecular analysis of sperm in the spermatheca and offspring of field-mated females (Hockham et al. 2004, Simmons et al. 2007). Caged populations could yield an exaggerated degree of polyandry (females may not be able to evade the males as

131 effectively as they would in the field). It should be noted, however, that in the bushcricket

132 species in this study which was examine in caged populations, *L. stalii*, the males are

133 unable to force the female to copulate and the females play an active role in pair formation

134 and in mounting the male to initiate copulation (Bateman 1998). In addition, the degree of

135 polyandry for this species was at the lower end of the spectrum (mean of 2.1 mates), rather

136 than high. Conversely, studies of marked individuals in field populations could under-

137 estimate the degree of polyandry because matings could be missed. The large

138 spermatophore, which remains attached to the female for several hours after copulation,

139 however, means that matings are unlikely to have been overlooked in these studies (Heller

140 and von Helversen 1991, McCartney 2010).

141

142 Data on refractory periods for males and females came from individuals assessed for the

143 time to re-mating in the laboratory (Supplementary Table S1). Individual pairs were

144 assigned to observation cages at a time of day corresponding to the peak activity period for

145 that species (see Vahed 2007 for further details). Data from two Australian species were

146 from mating observations in field cages (Lehmann 2007, Lehmann and Lehmann 2007).

147 These refractory periods correspond with 'time-outs from matings' and are therefore tightly

148 linked with the concept of potential reproductive rate (= 1/time-out, Clutton-Brock &

149 Parker 1992, Ahnesjö et al. 2001).

150

151 **Titillator structure**

152 Data on titillator morphology (Figure 1) were obtained from taxonomic sources, chiefly

153 Harz (1969) for the majority of European species (Supplementary Table S1). We developed

154 a ranked classification system to reflect titillator complexity, with emphasis on the

155 complexity (e.g. presence/absence of visible spines; clumping of spines at the tip; single or

156 double pair of projections) of the apical part of the titillators (median projection) which

157 makes contact with the female during copulation (Wulff et al. 2015, 2017) (see Figure 1,

158 Table 1). Titillators were scored blind (by both the first and the last authors) for a previous

159 study (Vahed et al. 2011), i.e. without knowledge of polyandry or duration of male or

160 female refractory periods.

161

162 **Analysis**

163 We split "titillator complexity" into two components analysed in parallel: presence/absence

164 of titillators, analysed as a binary variable (henceforth titillator presence), and complexity

165 of titillators where present (henceforth titillator complexity), analysed as a continuous

166 variable. This is because it would make little sense to treat species without titillators

167 effectively as bearing "titillators of zero complexity".

168

169 All analyses were conducted in R 3.2.0 (R Core Team 2015). To account for non-

170 independence due to phylogenetic relatedness, we used comparative-phylogenetic statistical

171 methods that account for this non-independence by modelling errors in the model residuals

172 as a function of the phylogenetic distance between species and the underlying model of

173 how traits evolve along branches. We analysed continuous data (refractory periods) using

174 phylogenetic generalized least squares models (PGLS; Grafen, 1989, Martins and Hansen,

175 1997) using the pgls() function in the *caper* package (Orme et al. 2013), simultaneously

176 estimating Pagel's λ, i.e. how closely the tree structure can predict covariance among the

177   model's residuals (Pagel 1999, Freckleton et al. 2002). For analysis of binary or Poisson

178   data (titillator presence/absence and polyandry, respectively) we fitted Generalised

179   Estimating Equation (GEE) models (Paradis & Claude 2002), which are more appropriate

180   for non-normally distributed data (Paradis 2006), using the compar.gee() function in the

181   *ape* package (Paradis et al 2004). We used standard residual plots to assess model fit by

182   eye.

183

184   We initially tested whether, within our dataset, polyandry was associated with the duration

185   of the female or male refractory periods and whether female and male refractory periods

186   were associated, as would be predicted (Vahed 2006, 2007). We first fitted a PGLS model

187   of polyandry with female or male refractory period as a predictor variable, then a PGLS

188   model with female refractory period as a response and male refractory period as a predictor.

189   Data on polyandry were log-transformed before analysis to improve model fit. Then, for

190   each analysis of titillator structure (binary GEE models of presence; continuous PGLS

191   models of complexity) we fitted models against predictor variables including polyandry,

192   female refractory period and male refractory period. We performed analyses of potential

193   reproductive rate directly on male and female reproductive rate, as this improved model fit.

194   We additionally ran analyses including the reciprocal of the ratio of the male and female

195   refractory period as a predictor variable. This represents the relative reproductive rates of

196   males and females (a measure of the operational sex ratio).

197

198   To avoid confounds due to variable measuring techniques, we re-ran all analyses excluding

199   species where polyandry was assessed using molecular rather than field-based methods (*R.*

200   *verticalis, E. ephippiger*) or where field rather than lab-based methods were used to assess

201   the male refractory period (*P. affinis, P. v. veluchianus*) or the female refractory period (*P.*

202   *affinis, P. v. veluchianus, P. v. minor*). See Supplementary Table S1 for details.

203

204   The phylogeny used for all analyses was based primarily on the molecular phylogeny by

205   Mugleston et al. (2013) supplemented by the molecular phylogenetic information derived

206   from barcoding of Central European species (Hawlitschek et al. 2016), combined with the

207   morphological phylogeny developed by Naskrecki (2000) (this phylogeny did not use

208   titillators as a character). For the sub-family Tettigoniinae, we used the morphological

209   phylogeny provided by Rentz and Coless (1990) (majority consensus tree of 50 equally

210   short cladograms) because many of the genera were neither included in Mugleston et al.

211   (2013) nor in Naskrecki (2000). For phylogenetic relationships within the genus

212   *Anonconotus* (Tettigoniinae), we used an unpublished molecular phylogeny based on

213   mtDNA (R. Szabo, G. Carron, K. Vahed & M. Ritchie). For the genus *Poecilimon*

214   (Phaneropterinae), we used the molecular phylogeny given in Ullrich et al. (2010), for the

215   *Poecilimon propinquus*-group the mtDNA tree of Lehmann (1998). Branch lengths were

216   not available and so we ran all analyses twice, arbitrarily scaling branches according to

217   node depth, following Grafen (1989), or setting all branch lengths to 1. We conducted our

218   analyses on the datasets for which relevant data were available (n=48). For analyses

219   involving subsets of the full dataset, the full tree was pruned to the appropriate set of taxa

220   only after arbitrary branch lengths had been assigned, in order to preserve the node depths

221   in the full tree. The datasets supporting this article have been uploaded as Supplementary

222   Table S1.

223

Results

225 Our analyses returned similar results regardless of whether we excluded minority methods,

226 or how we assigned branch lengths (arbitrarily to 1 or scaling according to node depth).

227 Thus, we present only results using all available data regardless of the method used to

228 obtain them, and using Grafen's (1989) node-depth scaling to assign arbitrary branch

229 lengths to the tree.

230

231 *Polyandry*

232 There was a high variation in polyandry in bushcricket females, ranging from as low as a

233 mean of 1.5 matings up to a mean of 25 to 28 matings per female in *Anonconotus* species

234 (Figure 2). The data were largely biased towards species with relatively low polyandry, i.e.

235 between 1.5 and 3.1 numbers of matings over the female lifespan. Polyandry was

236 significantly negatively associated with the duration of the female refractory period (GEE,

237 df=1, P=0.013, n=12) and the male refractory period (GEE, df=1, P=0.047, n=14). Female

238 and male refractory periods were positively associated with each other ($F_{1,25}$=18.23,

239 p<0.001, n=27); in this model Pagel's λ had wide confidence intervals (0 to 0.906),

240 indicating low certainty in the estimate of phylogenetic signal.

241

242 In the full dataset, titillators were absent in 15 species and present in 32; titillator

243 complexity in species with titillators was distributed as shown in Figure 2. Polyandry was

244 related neither to titillator presence (PGLS: $F_{1,\ 27}$=0.0142, P=0.91, n=29; Figure 3a) nor to

245 titillator complexity (PGLS, $F_{1, 20}$=2.56, P=0.13, n=22; Figure 3b) and vice versa:

246 polyandry was a good predictor neither of titillator presence (GEE, df=1, p=0.98, n=29) nor

247 complexity (PGLS, $F_{1, 20}$=2.56, P=0.13, n=22). Estimates of Pagel's λ ranged from 0.94 to

248 1.00 [95% CIs ~0.7 − 1] for these models, indicating a high degree of phylogenetic signal,

249 i.e. covariance in model residuals could be predicted by phylogenetic distance.

250

251 *Male refractory period*

252 The male refractory period can span over several days, the most extreme mean data coming

253 from *Antaxius hispanicus* with 7 days, and *Poecilimon thessalicus* with 6 days. In contrast,

254 several species are able to re-mate within an hour down to a few minutes. Titillator

255 presence did not reliably depend on the male refractory period (PGLS: $F_{1, 30} = 0.51$, P =

256 0.41, n=32, Figure 3c) but titillator complexity showed a marginal trend suggesting that

257 species with longer male refractory periods have more complex titillators (PGLS:

258 $F_{1,18}$=3.71, P=0.069, n=20, Figure 3d). The phylogenetic signal estimate had wide

259 confidence intervals (λ~0.81 [CI 0.28, 0.96]). The male refractory period did not predict

260 titillator presence (GEE: df=1, P=0.49, n=32) but again showed a marginally positive

261 association with titillator complexity (PGLS: $F_{1, 18}$=3.66, P=0.072, n=20, λ=0.77[CI 0.33,

262 0.95]).

263

264 *Female refractory period*

265 The female refractory period in most species was typically longer than that of the male,

266 ranging from 1.07 h to 19 days. Unlike the male refractory period, the female refractory

267   period was positively associated with titillator presence (PGLS: $F_{1, 26}$=9.04, P=0.005, n=28;

268   λ=0.62, Figure 3e) and negatively associated with titillator complexity (PGLS:

269    $F_{1,13}$=18.052, n=15, P<0.001; λ=0, Figure 3f). Analysing the inverse relationships, the

270   female refractory period did not predict titillator presence (GEE, df=1, P=0.15, n=28) but

271   was a good predictor of titillator complexity ($F_{1,13}$=18.052, P<0.001, n=15; λ=0).

272

273   *Male vs female potential reproductive rates*

274   The Operational Sex Ratio (measured as the reciprocal of the ratio of female and male

275   refractory periods) was not associated with titillator presence (PGLS: $F_{1, 25}$=1.164, P=0.16,

276   n=27, λ=0.85; Figure 3g) nor with titillator complexity (PGLS: $F_{1, 13}$=0.94, P=0.35, n=15;

277   λ=0.60; Figure 3h). The inverse relationships were similarly nonsignificant, as the

278   Operational Sex Ratio predicted neither titillator presence (GEE: df=1, P=0.73, n=27) nor

279   complexity (PGLS: $F_{1, 13}$=1.00, P=0.34, n=15; λ=0.66).

280

281

Discussion

No significant relationships were found between titillator complexity and any of the degree of polyandry in bushcrickets, the duration of the sexual refractory period in males, or the relative potential reproductive rates of males and females. The duration of the female's sexual refractory period, however, was associated with both titillator presence and complexity. The results, therefore, provide partial support for the hypothesis that post-copulatory sexual selection is a driving force in titillator evolution. It should be noted, however, that while there was a high degree of variation between species in the degree of polyandry (ranging from a mean of 1.5 to a mean of 28 matings per lifetime), there were no entirely monandrous species in this data set. Any effect of post-copulatory sexual selection on genital evolution should be most detectable by comparing monandrous with polyandrous species (Arnqvist 1998). On the other hand, previous studies have found evidence for a positive relationship between indicators of the degree of polyandry, measured on a continuous scale, and the complexity or degree of development of the males' genitalia in both insects and mammals (Ramm 2007, Rowe and Arnqvist 2012, Orr and Brennan 2016).

The duration of the female's sexual refractory period was significantly longer in species in which titillators were present. On the face of it, this appears to be opposite to the prediction of the sexual selection hypothesis, which predicts that titillators should be more complex in species in which females are more polyandrous (and therefore have *shorter* sexual refractory periods, since there was a significant negative relationship between the duration of the female's sexual refractory period and the lifetime degree of polyandry in our present

14

304   study). The results are, however, consistent with the hypothesis that complex genitalia

305   delay or deter the female from remating, which can be driven by various mechanisms of

306   post-copulatory sexual selection. Stockley (2002), for example, found that in primates,

307   relatively high penile spinosity was associated with lower potential reproductive rates in

308   females and interpreted this in terms of internal damage caused to the female by the spines.

309   Kuntner et al. (2016) similarly found that in nephilid spiders, the male's genitalia were

310   more complex in polyandrous species than in monandrous species. Titillators in some

311   bushcrickets have spines (see Figure 1) that contact the soft, un-sclerotised lining of the

312   female's bursa copulatrix (Wulff et al. 2015, 2017). However, we found no evidence for

313   any damage by the titillators to the female's reproductive tract (Wulff et al. 2015, 2017;

314   Wulff and Lehmann 2016), in contrast to the action of penile spines in *Callosobruchus*

315   beetles (Hotzy and Arnqvist 2009). Comparative evidence suggests that genital damage

316   selects for females to evolve a thicker wall of the bursa copulatrix to minimise damage by

317   the male's spines in seed beetles (Coleoptera: Bruchidae) (Rönn et al. 2007). The thickness

318   of the parts of the female contacted by the male's genitalia was not measured in the present

319   study. There is strong evidence from other comparative studies of other insect taxa that co-

320   evolution between male and female genital structures does occur (Rönn et al. 2007, Yassin

321   and Orgogozo 2013, reviewed in Simmons 2014) but for several reasons female genitalia

322   are still understudied (Ah-King et al. 2014, Brennan and Prum, 2015) even in bushcrickets.

323

324   Amongst species with titillators, shorter sexual refractory periods were associated with

325   more complex titillators. These results appear to be in contrast to the analysis based on the

326   presence/absence of titillators (see above). Shorter sexual refractory periods were

327 associated with a higher degree of polyandry (this study), so may be used as an indicator of

328 the degree of polyandry. Comparative studies of water-striders (Heteroptera: Gerridae)

329 have similarly found relationships between indices of the degree of polyandry and genital

330 complexity (Rowe and Arnqvist 2012). The results of the present study should be

331 interpreted with caution, however. The relationship between the duration of the female's

332 sexual refractory period and titillator complexity appear to be driven by the two species in

333 the sub-family Zaprochilinae in our dataset, which have unusually long female sexual

334 refractory periods (Simmons and Gwynne 1991; Lehmann and Lehmann 2007) and simple

335 titillators (Rentz, 1993). This subfamily is phylogenetically distinct from the majority of

336 other Tettigoniid families. It should also be noted that we did not find a significant

337 relationship between the actual degree of polyandry itself (rather than the female's sexual

338 refractory period) and titillator complexity in the present study (the two analyses were

339 based on slightly different subsets of species, see Figure 2).

340

341 It is possible that the classification scheme of titillators used in the present study does not

342 reflect adequately the characters of the titillators that are subject to sexual selection. An

343 alternative approach would be to use more complex morphometric analyses to quantify the

344 shape and/or to measure the relative sizes of parts of the titillators (see, for example Rowe

345 and Arnqvist 2012). In addition, denser sampling of species within selected titillator-

346 possessing genera would allow for a more fine-scale examination of the relationship

347 between titillator morphology and polyandry. In the present study, most titillator-possessing

348 genera were represented by only three species or fewer.

349

350   Although the present study provided mixed support for sexual selection hypotheses for

351   genital evolution, experimental manipulations, in which either one or both titillators were

352   removed, have provided some support for the role of cryptic female choice (Wulff et al.

353   2015, Vahed 2015, Wulff and Lehmann 2016). The results of these manipulations indicated

354   that correct stimulation by titillators is necessary for the proper insertion of the

355   spermatophore and to avoid resistance by females during copulation (Wulff et al. 2015,

356   2017; Wulff and Lehmann 2016). Such results, however, are also consistent with the "Lock

357   and Key" hypothesis, a hypothesis that was not tested in the present study. Simmons (2014)

358   pointed out that female choice that enforces species isolation and female choice that targets

359   variation in male quality within populations may be seen as part of the same continuum.

360   To conclude, the present comparative study provided only partial support for the hypothesis

361   that post-copulatory sexual selection has driven the evolution of titillator complexity in

362   bushcrickets. The inclusion of monandrous species in the sample, examination of the parts

363   of the female's reproductive tract that are contacted by the titillators and denser sampling of

364   selected genera, however, would be necessary to test the sexual selection hypotheses more

365   fully.

366

367

371

372

373   **References**

374   Ah-King M, Barron AB, Herberstein ME. 2014. Genital evolution: why are females still

375   understudied? PLOS Biol 12(5):e1001851

376   Ahnesjö I, Kvarnemo C, Merilaita S. 2001. Using potential reproductive rates to predict

377   mating competition among individuals qualified to mate. Behav Ecol 12:397-401.

378   Arnqvist G. 1998. Comparative evidence for the evolution of genitalia by sexual selection.

379   Nature 393:784-786.

380   Arnqvist G, Rowe L. 2005. Sexual Conflict. Princeton, New Jersey: Princeton University

381   Press.

382   Bateman PW. 1998. Does size matter? The function of the large spermatophore of

383   *Steropleurus stali* Bolivar (Orthoptera: Tettigoniidae: Ephippigerinae). J Orthoptera

384   Res 7:209-212.

385   Brennan P L, Prum RO. 2015. Mechanisms and evidence of genital coevolution: the roles

386   of natural selection, mate choice, and sexual conflict. Cold Spring Harbor Perspect

387   Biol 7(7): a017749.

388   Chamorro-Rengifo J, Lopes-Andrade C. 2014. The phallus in Tettigoniidae (Insecta:

389   Orthoptera: Ensifera) a revision of morphology and terminology, and discussion on its

390   taxonomic importance and evolution. Zootaxa 3815:151-199.

391   Clutton-Brock TH, Parker GA. 1992. Potential reproductive rates and the operation of

392   sexual selection. Quart Rev Biol 67:437-456.

393   Crudginton HS, Siva-Jothy MT. 2000. Genital damage, kicking and early death. Nature

394   407:855-856.

395   Eberhard WG. 1985. Sexual Selection and Animal Genitalia. Cambridge: Harvard

396   University Press.

397   Eberhard WG. 1996. Female Control: Sexual Selection by Cryptic Female Choice.

398   Princeton, New Jersey: Princeton University Press.

399   Eberhard WG. 2010. Evolution of genitalia: theories, evidence, and new directions.

400   Genetica 138:5-18.

401   Freckleton RP, Harvey PH, Pagel M. 2002. Phylogenetic Analysis and Comparative Data:

402   A Test and Review of Evidence. Am Nat160:712-726. doi:10.1086/343873

403   Grafen A. 1989. The Phylogenetic Regression. Phil Trans R Soc B 326:119-157.

404   doi:10.1098/rstb.1989.0106.

405   Gwynne DT. 1984. Sexual selection and sexual differences in mormon crickets

406   (Orthoptera: Tettigoniidae, *Anabrus simplex*). Evolution 38:1011-1022.

407   Gwynne DT. 2001.Katydids and Bush-crickets: reproductive behavior and the evolution of

408   the Tettigoniidae. Ithaka, Cornell University Press.

409   Gwynne DT, Lorch PD. 2013. Mating failures as a consequence of sexual selection on

410   females. Entomol Exp Appl 146:3-10.

411   Harz K. 1969. Die Orthopteren Europas/The Orthoptera of Europe I. Gravenhage,

412   Netherlands: Dr. W. Junk.

413   Hawlitschek O, Morinière J, Lehmann GUC, Lehmann AW, Kropf M, Dunz A, Glaw F,

414   Detcharoen M, Schmidt S, Hausmann A, Szucsich NU, Caetano-Wyler SA,

415   Haszprunar G. 2016. DNA barcoding of crickets, katydids, and grasshoppers

416   (Orthoptera) from Central Europe with focus on Austria, Germany, and Switzerland.

417   Mol Ecol Resour, doi: 10.1111/1755-0998.12638

418   Heller K-G, Helversen D. 1991. Operational sex ratio and individual mating frequencies in

419   two bushcricket species (Orthoptera, Tettigonioidea, Poecilimon). Ethology 89:211-

420   228.

421   Helversen D von, Helversen O von. 1991.Pre-mating sperm removal in the bushcricket

422   *Metaplastes ornatus* Ramme 1931 (Orthoptera, Tettigonioidea, Phaneropteridae).

423   Behav Ecol Sociobiol 28:391-396.

424   Hockham LR, Graves JA, Ritchie MG. 2004. Sperm competition and the level of polyandry

425   in a bushcricket with large nuptial gifts. Behav Ecol Sociobiol 57:149-154.

426   Hotzy C, Arnqvist G. 2009. Sperm competition favours harmful males in seed beetles. Curr

427   Biol 19:404-407.

428   Kaňuch P, Kiehl B, Low M, Cassel-Lundhagen A. 2013.On variation of polyandry in a

429   bush-cricket, *Metrioptera roeselii,* in northern Europe. J Insect Sci13(1):16. doi:

430   10.1673/031.013.1601

431   Kokko H, Klug H, Jennions MD. 2014.Mating systems. In Shuker DM & Simmons LW,

432   editors: The Evolution of Insect Mating Systems. Oxford: Oxford University Press. p.

433   42-58.

434   Kuntner M, Cheng RC, Kralj-Fišer S, Liao CP., Schneider JM, Elgar MA. 2016. The

435   evolution of genital complexity and mating rates in sexually size dimorphic spiders.

436   BMC Evol Biol 16(1): 242.

437   Lehmann AW. 1998. Artbildung, akustische Kommunikation und sexuelle Selektion bei

438   griechischen Laubheuschrecken der Poecilimon propinquus-Gruppe (Orthoptera:

439   Phaneropteridae). Doctoralthesis, Univ Erlangen, Germany.

440   Lehmann GUC. 2007. Density-dependent plasticity of sequential mate choice in a

441   bushcricket (Orthoptera: Tettigoniidae). Aust J Zool 55:123-130. doi:

442   10.1071/ZO06105

443   Lehmann GUC, Lehmann AW. 2007. Sex differences in "time out" from reproductive

444   activity and sexual selection in male bushcrickets (Orthoptera: Zaprochilinae:

445   *Kawanaphila mirla*). Insect Behav 20:215-227. doi: 10.1007/s10905-007-9076-1

446   Lehmann GUC, Heller K-G, Mai R. 2016.Mate guarding as an alternative to male nuptial

447   gift investment in the bushcricket *Letana inflata* (Orthoptera: Tettigoniidae). J Ethol

448   34:191-194. doi:10.1007/s10164-016-0457-9

449   Martins EP, Hansen TF.1997. Phylogenies and the Comparative Method: A General

450   Approach to Incorporating Phylogenetic Information into the Analysis of Interspecific

451   Data. Am Nat 149:646-667. doi: 10.1086/286013

452   McCartney J. 2010. Spermatophore size variation in the bush-cricket genus *Poecilimon*.

453   PhD Massey University, New Zealand.

454   McCartney J, Lehmann AW, Lehmann GUC. 2010. Lifetime spermatophore investment in

455   natural populations of two closely related bush-cricket species (Orthoptera:

456   Tettigoniidae: *Poecilimon propinquus*-group). Behaviour 147:285-298. doi:

457   10.1163/000579509X12529331375383

458   Mikkola K. 2008. The lock-and-key mechanisms of the internal genitalia of the Noctuidae

459   (Lepidoptera): How are they selected for? Eur J Entomol105:13-25.

460   Mugleston JD, Song H, Whiting MF. 2013. A century of paraphyly: A molecular

461   phylogeny of katydids (Orthoptera: Tettigoniidae) supports multiple origins of leaf-like

462   wings. Mol Phylogen Evol 69:1120-1134.

463   Naskrecki P. 2000. The phylogeny of katydids (Orthoptera: Ensifera: Tettigoniidae) and the

464   evolution of their acoustic behaviour. PhD thesis, Univ Connecticut, USA.

465   Orme D, Freckleton R, Thomas G, Petzoldt T, Fritz S, Isaac N, Pearse W. 2013. caper:

466   Comparative Analyses of Phylogenetics and Evolution in R. R package version 0.5.2.

467   http://CRAN.R-project.org/package=caper

468   Orr TJ, Brennan PLR. 2016. All features great and small: the potential roles of the baculum

469   and penile spines in mammals. Integr Comp Biol 56: 635-643. doi:

470   10.1093/icb/icw057

471   Pagel M. 1999. Inferring the historical patterns of biological evolution. Nature 401:877-

472   884.

473   Paradis E, Claude J. 2002. Analysis of comparative data using generalized estimating

474   equations. J Theor Biol 218: 175-185.

475   Paradis E, Claude J, Strimmer K. 2004. APE: an R package for analyses of phylogenetics

476   and evolution. Bioinformatics 20: 289–290.

477   Parker DJ, Zaborowska J, Ritchie MG, Vahed K. 2017. Paternity analysis of wild-caught

478   females shows that sperm package size and placement influence fertilization success

479   in the bushcricket *Pholidoptera griseoaptera*. Mol Ecol 26:3050-3061. doi:

480   10.1111/mec.14089

481   Parker GA. 1979. Sexual selection and sexual conflict. In: Blum MS, Blum NA, editors.

482   Sexual Selection and Reproductive Competition in Insects. London, UK: Academic

483   Press. p. 123-166.

484   R Core Team. 2015. R: A language and environment for statistical computing. R

485   Foundation for Statistical Computing, Vienna, Austria. www.R-project.org.

486   Ramm S. 2007.Sexual selection and genital evolution in mammals: a phylogenetic analysis

487   of baculum length. Am Nat 169:360-369.

488   Rentz DCF. 1972. The Lock and Key as an isolating mechanism in katydids. Am Sci

489   60:750-755.

490   Rentz DCF. 1993. A monograph of the Tettigoniidae of Australia. Volume 2:

491   Austrosaginae, Zaprochilinae and Phasmodinae. Melbourne, Australia, CSIRO

492   publishing.

493   Rentz DCF, Birchim JD. 1968. Revisionary studies on the Nearctic Decticinae. Memoirs of

494   the Pacific Coast Entomological Society 3: 1-173.

495   Rentz DCF, Colless DH. 1990. A classification of the shield-back katydids (Tettigoniinae)

496   of the world. In: Bailey WJ, Rentz DCF, editors. The Tettigoniidae: biology,

497   systematics and evolution. Bathurst: Crawford House Press. p. 352-377

498   Rönn J, Katvala M, Arnqvist G. 2007. Coevolution between harmful male genitalia and

499   female resistance in seed beetles. PNAS 104:10921-10925.

500   Rowe L, Arnqvist G. 2012. Sexual selection and the evolution of genital shape and

501   complexity in water striders. Evolution 66:40-54.

502   Simmons LW. 2001. Sperm Competition and its Evolutionary Consequences in the Insects.

503   Princeton, New Jersey: Princeton University Press.

504 Simmons LW. 2014. Sexual selection and genital evolution. Aust Entomol 53:1-17.

505 Simmons LW, Beveridge M, Kennington WJ. 2007. Polyandry in the wild: temporal

506   changes in female mating frequency and sperm competition intensity in natural

507   populations of the tettigoniid *Requena verticalis*. Mol Ecol 16:4613-4623.

508 Simmons LW, Gwynne D T. 1991. The refractory period of female katydids (Orthoptera:

509   Tettigoniidae): sexual conflict over the remating interval? Behav Ecol 2: 276-282.

510 Stockley P. 2002. Sperm competition risk and male genital anatomy: comparative evidence

511   for reduced duration of female receptivity in primates with penile spines. Evol Ecol

512   16:123-137.

513 Ullrich B, Reinhold K, Niehuis O, Misof B. 2010.Secondary structure and phylogenetic

514   analysis of the internal transcribed spacers 1 and 2 of bush crickets (Orthoptera:

515   Tettigoniidae: Barbitistini). J Zool Syst Evol Res 48:219-228.

516 Vahed K. 2003. Structure of spermatodoses in shield-back bushcrickets (Tettigoniidae,

517   Tettigoniinae). J Morphol 257:45-52.

518 Vahed K. 2006. Larger ejaculate volumes are associated with a lower degree of polyandry

519   across bushcricket taxa. Proc R Soc B 273:2387-2394.

520 Vahed K. 2007. Comparative evidence for a cost to males of manipulating females in

521   bushcrickets. Behav Ecol 18:507-512.

522 Vahed K. 2015. Cryptic female choice in crickets (Orthoptera: Ensifera). In: Peretti AV,

523   Aisenberg A, editors. Cryptic Female Choice in Arthropods-Patterns, Mechanisms and

524   Prospects. Heidelberg: Springer.

525 Vahed K, Lehmann AW, Gilbert JDJ, Lehmann GUC. 2011. Increased copulation duration

526   before ejaculate transfer is associated with larger spermatophores, and male genital

527   titillators, across bushcricket taxa. J Evol Biol 24:1960-1968. doi: 10.1111/j.1420-

528   9101.2011.02325.x

529   Vahed K, Gilbert JDJ, Weissman DB, Barrientos-Lozano L. 2014. Functional equivalence

530   of grasping cerci and nuptial food gifts in promoting ejaculate transfer in katydids.

531   Evolution 68:2052-2065.

532   Wulff NC, Lehmann GUC. 2016. Function of male genital titillators in mating and

533   spermatophore transfer in the tettigoniid bushcricket *Metrioptera roeselii*. Biol J Linn

534   Soc 117:206-216. doi: 10.1111/bij.12661

535   Wulff NC, Lehmann AW, Hipsley CA, Lehmann GUC. 2015. Copulatory courtship by

536   bushcricket genital titillators: functional morphology, μCT scanning for 3D

537   reconstruction and female sense structures. Arthropod Struct Dev 44:388-397. doi:

538   10.1016/j.asd.2015.05.001

539   Wulff NC, van de Kamp T, dos Santos Rolo T, Baumbach T, Lehmann GUC. 2017.

540   Copulatory courtship by internal genitalia in bushcrickets. Sci Rep 7:42345. doi:

541   10.1038/srep42345.

542   Yassin A, Orgogozo V (2013) Coevolution between male and female genitalia in the

543   *Drosophila melanogaster* species subgroup. PLoS ONE 8:e57158.

544

545

Figure legends

Figure 1: Left hand side: Electron micrograph of Titillator tips with spines in the tettigoniid

bushcricket *Metrioptera saussuriana*; Right hand side: Variation in titillator morphology in

the Tettigoniidae. The numbers represent the system of titillator classification used in the

present study (see Table 1). Images adapted from Rentz and Birchim (1968) and Rentz

(1993). Note that titillator types 2 and 3 are shown together because they are similar in

overall structure (although the apical part that contacts the female, the median projection, is

much less strongly projecting in type 2 than in type 3).

Figure 2: Variation of male refractory period (MRP), female sexual refractory period

(FRP), their difference, and lifetime degree of polyandry, across the bushcricket phylogeny.

Data have been scaled such that open and closed circles represent the minimum and

maximum in the dataset, respectively. For raw data, see Supplementary Table S1.

Figure 3: a) Lifetime polyandry in bushcricket species lacking titillators ("No") and with

titillators ("Yes"); b) Polyandry against titillator complexity (ordinal ranked scale, see

Table 1); c) The duration of the male sexual refractory period in bushcricket species lacking

titillators ("No") and with titillators ("Yes"); d) The duration of the male sexual refractory

period against titillator complexity; e) The duration of the female sexual refractory period

in bushcricket species lacking titillators ("No") and with titillators ("Yes"); f) The duration

of the female sexual refractory period against titillator complexity; g) Operational sex ratio

(measured as the reciprocal of the ratio of the female and male refractory periods) in

568   bushcricket species lacking titillators ("No") and with titillators ("Yes"); h) Operational sex

569   ratio against titillator complexity.

570

571

572 **Table 1**: The titillator classification scheme used in this study (adapted from Vahed et al.

573 2011).

574

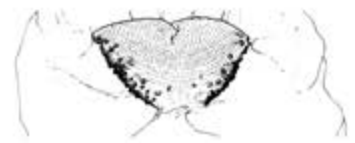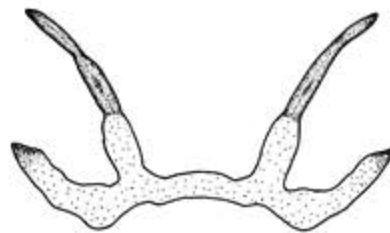| Numerical classification	Explanation	Examples
0	Titillators absent	*Poecilimon*
1	No sclerotised titillators, but a densely covered field of small tubercles	*Kawanaphila*
2	One pair of sclerotised titillators, apical part (median projection) conical and not strongly projecting, may have minute teeth	*Ruspolia, Yersinella*
3	One pair of sclerotised titillators: apical part strongly projecting, with no teeth (the tip however can be hooked)	*Metrioptera roeselii*
4	One pair of sclerotised titillators: apical part strongly projecting with clearly visible teeth	*Anonconotus, Decticus*
5	One pair of sclerotised titillators: apical part strongly projecting with teeth concentrated on the club shaped tip	*Metrioptera saussuriana*
6	Two pairs of sclerotised titillators: apical part strongly projecting with teeth on one or both pairs.	*Gampsocleis, Antaxius*

575

576

577 **Supplementary Table S1**: Data base for levels of titillator complexity, polyandry and male
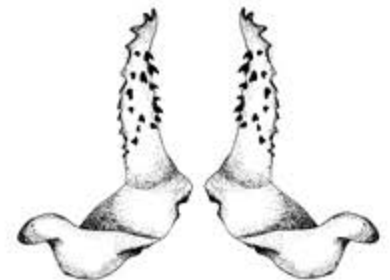
578 and female refractory periods.
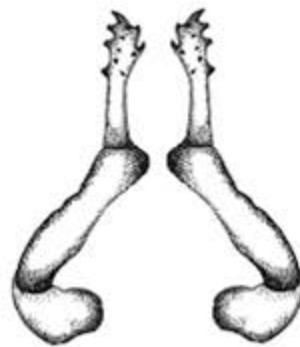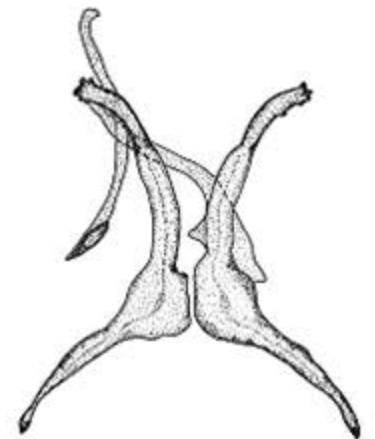
Titillators absent (0)   Small tubercles (1)

One pair, no teeth (2-3)   One pair, with teeth (4)

One pair, spines on club shaped tip (5)   Double pairs of titillators (6)

Most complex titillators
Longest MRP
Longest FRP
Longest F–M difference
Greatest polyandry

Phaneroptera nana
Phaneroptera falcata
Poecilimon thessalicus
Poecilimon veluchianus minor
Poecilimon veluchianus veluchianus
Poecilimon mariannae
Poecilimon ampliatus
Poecilimon laevissimus
Poecilimon erimanthos
Poecilimon schmidtii
Poecilimon affinis
Leptophyes punctatissima
Leptophyes laticauda
Ruspolia nitidula
Conocephalus fuscus
Metrioptera saussuriana
Metrioptera brachyptera
Metrioptera roeselii
Platycleis affinis
Platycleis albopunctata albopunctata
Sepiana sepium
Gampsocleis gratiosa
Gampsocleis glabra
Decticus albifrons
Decticus verrucivorus
Anonconotus ghilianii
Anonconotus alpinus
Anonconotus pusillus
Anonconotus occidentalis
Eupholidoptera chabrieri
Pholidoptera griseoaptera
Antaxius pedestris
Antaxius hispanicus
Yersinella raymondi
Anabrus simplex
Tettigonia viridissima
Xederra charactus
Metaballus litus
Ephippiger ephippiger
Lluciapomaresius stalii
Lluciapomaresius asturiensis
Uromenus rugosicollis
Acanthoplus discoidalis
Requena verticalis
Phasmodes ranatriformis
Kawanaphila nartee
Kawanaphila mirla