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

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Abstract

The function of nuptial gifts has generated long standing debate. Nuptial gifts consumed during ejaculate transfer may allow males to transfer more ejaculate than is optimal for females. However, gifts may simultaneously represent male investment in offspring. Evolutionary loss of nuptial gifts can help elucidate pressures driving their evolution. In most katydids (Orthoptera: Tettigoniidae), males transfer a spermatophore comprising two parts: the ejaculate-containing ampulla and the spermatophylax - a gelatinous gift that females eat during ejaculate transfer. Many species, however, have reduced or no spermatophylaces and many have prolonged copulation. Across 44 katydid species, we tested whether spermatophylaces and prolonged copulation following spermatophore transfer are alternative adaptations to protect the ejaculate. We also tested whether prolonged copulation was associated with (i) male cercal adaptations, helping prevent female disengagement, and (ii) female resistance behaviour. As predicted, prolonged copulation following (but not before) spermatophore transfer was associated with reduced nuptial gifts, differences in the functional morphology of male cerci and behavioural resistance by females during copulation. Furthermore, longer copulation following spermatophore transfer was associated with larger ejaculates, across species with reduced nuptial gifts. Our results demonstrate that nuptial gifts and the use of grasping cerci to prolong ejaculate transfer are functionally equivalent.

"Gifts are like hooks."

Marcus Valerius Martialis (40-103 AD).

Introduction

Nuptial gifts occur in a wide range of animal taxa and take numerous forms, including gifts synthesised or collected by the male and parts of the male's body (reviewed in Vahed 1998, 2007a; Gwynne 2008; Lewis and South 2012).The evolution and maintenance of nuptial gifts and the extent to which they represent intersexual conflict or co-operation have long been a focus for debate. Nuptial gifts that are consumed by the female during ejaculate transfer could function to prolong ejaculate transfer. By transferring a larger ejaculate, the male could transfer larger doses of allohormones that manipulate female reproduction to favour the male, while imposing direct fitness costs on the female (reviewed in Parker and Simmons 1989; Arnqvist and Nilsson 2000; Vahed 2007a). In katydids or bushcrickets (Orthoptera: Ensifera: Tettigoniidae), for example, substances in large ejaculates delay females from re-mating (Wedell 1993; Vahed 2006, 2007b) and may shorten female lifespan (Wedell *et al.* 2008). Nuptial gifts could also be counter to the female's interests because, by maximising ejaculate transfer, they may circumvent female mating preferences (reviewed in Vahed 2007a). On the other hand, nuptial gifts might additionally function to provide nutrients to the female for use in egg production (the Paternal Investment hypothesis, reviewed in Vahed 1998; Gwynne 2008). Nuptial gifts could thereby benefit both sexes, although the nutritional benefits of nuptial gifts have been questioned (reviewed in Vahed 2007a). The evolutionary loss of nuptial gifts offers a special opportunity to gain insight into these selective pressures, especially via comparative studies (Lewis and South 2012). South *et al.* (2011), for example,

79 concluded that factors associated with the presence or absence of nuptial gifts within
80 the Lampyridae (fireflies) provided support for the paternal investment hypothesis.
81
82 Katydids are a model clade for studying nuptial gifts (reviewed in Gwynne 2001;
83 Vahed 2007a; Lehmann 2012). Male katydids transfer a spermatophore towards the
84 end of copulation consisting of two parts: an ampulla, containing the ejaculate, and a
85 spermatophylax, a gelatinous nuptial gift (Fig S1). After copulation, the female
86 consumes the spermatophylax while the ejaculate is transferred from the ampulla
87 (Boldyrev 1915). The spermatophylax may prolong ejaculate transfer by delaying the
88 female from eating the ampulla (reviewed in Vahed 2007a). For example,
89 spermatophylax size is adjusted within species to occupy females long enough to
90 ensure complete ejaculate transfer (Sakaluk 1984; Reinhold and Ramm 2013); species
91 with relatively larger ejaculates and more sperm also produce larger spermatophylaces
92 (Wedell 1993; Vahed and Gilbert 1996). Additionally, female katydids (and Ensifera
93 generally) routinely eat ampullae before complete sperm transfer, unless prevented by
94 males (Boldyrev 1915; Alexander and Otte 1967). Large spermatophylaces might
95 additionally function as paternal investment: spermatophylax feeding can increase
96 female reproductive output in some species (reviewed in Gwynne 2001; Vahed
97 2007a; Lehmann 2012).
98
99 Katydids represent an ideal opportunity to test for factors associated with the loss of
100 nuptial gifts (see Lewis and South 2012). Although the spermatophylax appears to be
101 ancestral in this clade (Gwynne 2001), in a variety of species spermatophylaces are
102 reduced or absent. The hypothesis that the spermatophylax functions to protect the
103 ejaculate predicts that, in taxa where the spermatophylax is evolutionarily lost, its loss

will be associated with other methods of ejaculate protection. One such possible candidate method is prolonged copulation following spermatophore transfer, a behaviour that occurs in a variety of katydids, whereby the body of the male itself acts as a barrier to prevent the female from consuming the ampulla before complete ejaculate transfer (Boldyrev 1915; Vahed 1996, 1997; Wedell 1998).

Prolonged copulation is predicted to select for male ability to grasp and hold the female via "copulatory structures" (both genital and non-genital grasping and intromittent organs) to prevent the female from uncoupling (Alexander and Otte 1967). Male copulatory structures diverge more rapidly than other morphological traits (Eberhard 1985; Rowe and Arnqvist 2012). Sexual selection is currently regarded as the main hypothesis accounting for this (reviewed in Eberhard 2004, 2006; Arnqvist and Rowe 2005; Simmons 2014), with three main mechanisms: sperm competition, cryptic female choice and sexually antagonistic co-evolution, which need not be mutually exclusive (Kokko *et al.* 2003; Simmons 2014). Under the sexually antagonistic co-evolution hypothesis, male and female copulatory structures co-evolve in an arms race over mating, each sex being selected to achieve different optimum mating rates or copulation duration, for example (reviewed in Eberhard 2004, 2006; Arnqvist and Rowe 2005). Although evidence for sexually antagonistic co-evolution in genital evolution is compelling in some species (reviewed in Eberhard 2004, 2006; Arnqvist and Rowe 2005; Perry and Rowe 2012), its general applicability has been questioned (Eberhard 2004, 2006, 2010). For example, even where potentially sexually antagonistic co-evolution is evident, parts of the female contacted by male copulatory structures mostly lack evidence of counter-adaptations to resist

copulation (Eberhard 2004, 2006). Female resistance to male coercion, however, may be behavioural rather than morphological (Eberhard 2004).

In katydids, males generally possess two different types of copulatory structures: the cerci and the titillators (Hartz 1969). Cerci are generally used to clasp the female during mating (Rentz 1972; Hartley and Warne 1984), while the titillators are inserted into the female's reproductive tract prior to spermatophore transfer (Vahed *et al.* 2011). Vahed *et al.* (2011) found that the presence of titillators was associated with prolonged copulation prior to spermatophore transfer in katydids, but the functional morphology of cerci with respect to copulation duration is as yet unstudied.

In this paper we test the hypothesis that prolonged copulation following spermatophore transfer functions in the same way as the spermatophylax, i.e. to prolong ejaculate transfer by protecting the ampulla of the spermatophore from being eaten by the female (Boldyrev 1915, Vahed 1996, 1997; Wedell 1998). As a result, sexual conflict over prolonged copulation should have led to sexually antagonistic co-evolution between male copulatory structures and female behaviour (Alexander and Otte 1967; Arnqvist and Rowe 2005). By contrast, prior to spermatophore transfer, prolonged copulation is less likely to result in sexual conflict (it may represent a mutual period of mate assessment, Vahed *et al.* 2011). Thus, the following predictions should be true for copulation duration after, but not before, spermatophore transfer:

(1) Prolonged copulation following spermatophore transfer should typically appear in species in which the spermatophylax is reduced in size, or absent, and thus should correlate negatively with spermatophylax mass (Vahed 1996, 1997).

(2) Because relatively larger ampullae contain more sperm (Vahed and Gilbert 1996) and take longer to transfer their content (Reinhold and Ramm 2013), copulation duration following spermatophore transfer should correlate positively with ampulla mass, but only in species in which the spermatophylax is absent or reduced (ie. is a small percentage of the spermatophore) (Vahed 1996; Wedell 1998).

(3) Prolonged copulation following spermatophore transfer should have led to: a) the evolution of modified morphology and/or use of the male's cerci (to prevent the female from dis-engaging, Alexander and Otte 1967), which in turn should have selected for: b) behavioural resistance by the female.

Materials and methods

The form and use of the male's cerci during copulation

In order to study the morphology of the male's cerci and the parts of the female contacted by the male's cerci, specimens for the majority of the 44 species in this study (Tables S1 & S2) were obtained from the field. Collection localities for the majority of European species were the same as those given in Vahed *et al.* (2011). Specimens of *Dichopetala* and *Pterophylla beltrani* were collected from near Victoria, Tamaulipas, Mexico by L. Barrientos-Lozano. Specimens of *Decticita brevicauda* were collected by D. B. Weissman from near Fairfax, Marin County, California, U.S.A.. Specimens of *Coptaspis* spp., from Bawley Point, New South Wales, Australia, were supplied by D.C.F. Rentz, specimens of *Docidocercus gigliotosi* from Panama were supplied by H. ter Hofstede, while K-G. Heller supplied

specimens of *Poecilimon veluchianus* and *P. affinis* from Florina, Vernon, Greece. For two species (*Kawanaphila nartee* and *Phasmodes ranatriformis*) the morphology of the male's cerci was not observed first hand, but was taken from the taxonomic literature (Rentz 1993). Specimens were preserved in 75% ethanol and were stored at 5ºC. A minimum of three males and females of each species were examined under a dissecting microscope. The right cercus of each male was removed using watch-maker's forceps. Cerci were then air-dried, gold coated using an Emitech K550X (EM Technologies Ltd, Ashford, UK) and examined using a scanning electron microscope (SEM; Leo 1450 VP, Zeiss Ltd, Oxford, UK).

In order to observe how the male's cerci were used to contact the female during copulation, pairs were observed closely during laboratory mating trials in which the duration of copulation was timed (see below). For six of the species in this study (Tables S1 & S2), copulation was not observed first hand and details were taken from the literature (*Docidocercus gigliotosi*, *Coptaspis* spp 2, 5 & 10, *Kawanaphila nartee* and *Phasmodes ranatriformis*, see Table S1). For a range of species, macro-photographs of copulating pairs were taken using a digital camera (Nikon D3000, 10.2 MP). For selected species (*Leptophyes punctatissima*, *Dichopetala castanea*, *D. pollicifera*, *Pterophylla beltrani*, *Pholidoptera griseoaptera*, *Decticita brevicauda*, *Conocephalus fuscus*, *Coptaspis* sp. 6), a minimum of three pairs were also preserved in the copulatory position by placing copulating pairs in a freezer (at either -80 or -18ºC) for 5 min before immersing them in 75 % ethanol. The parts contacted by the male's cerci were examined under a dissecting microscope. Electron micrographs of the parts of the female contacted by the males' cerci were taken for *Leptophyes*

punctatissima, *Conocephalus fuscus* and *Platycleis albopunctata* using methods described for the cerci, above. For the statistical analysis (see below), we developed a classification system of the morphology of the male's cerci and the different ways in which they contact the female during copulation (Table 1).

Copulation duration, male body mass, spermatophylax mass and ampulla mass

Data on the duration of copulation following spermatophore transfer were obtained for 44 species (all data used in this study are given in Table S1). Novel data for this variable were obtained for 24 of these species, following methods outlined in Vahed *et al.* (2011), while data for the remainder of the species were taken from the literature. Data on the duration of copulation prior to the secretion of the spermatophore, were obtained for 39 species (Table S1). Data were taken primarily from Vahed *et al.* (2011), with the addition of data for *Dichopetala* spp. Data on spermatophylax mass, ampulla mass and male body mass (Table S1) were obtained from the literature for most species (primarily from Vahed and Gilbert 1996, Vahed 2006 and Vahed 2007b), while novel data for these variables and/or additional replicates were obtained for nine of the species following methods described in Vahed and Gilbert (1996) and Vahed *et al.* (2011).

Resistance by females during copulation

For each species, the occurrence of resistance behaviour by the female (consisting of kicking at the male, rapid locomotion during copulation and/or bending to bite at the male) during copulation was recorded (Table S1). For five of the 44 species included

in this part of the study (*Kawanaphila nartee*, *Phasmodes ranatriformis* and *Coptaspis* sp 2, 5 and 10), we did not observe copulation first hand, so relied instead upon accounts of copulation behaviour for these species in the literature (Simmons and Bailey 1990; Bailey and Lebel 1998; Wedell 1998).

The phylogeny

The phylogeny used in the analyses (Fig 1) was derived from the morphological phylogeny developed by Naskrecki (2000). For the subfamily Tettigoniinae, we used the morphological phylogeny developed by Rentz and Colless (1990). For the genus *Poecilimon*, we used the phylogeny developed by Ullrich *et al.* (2010), while for the genus *Anonconotus*, we used an unpublished molecular phylogeny based on mtDNA (16S and *cyt b*; R. Szabo, G. Carron, K. Vahed and M. Ritchie, unpublished). There was no overlap between the source phylogenies, so tree-combining algorithms were unnecessary and trees were assembled jigsaw fashion. Branch lengths on the complete phylogeny were not available and so were assigned the arbitrary value of 1.

Statistical analyses

We used the program MultiState under a Maximum Likelihood framework implemented in the program BayesTraits (Pagel and Meade 2006), to reconstruct ancestral male cercal forms for the whole phylogeny and for each subfamily within it.

To test for correlated evolutionary transitions between male cercal functional morphology and female resistance behaviour, we collapsed our classification of male cercal forms (Table 1) into a binary variable: "Unmodified" (including species with purely "lock and key"-based systems and those in which the cerci do not engage with the female: states LK1, LK2, LK3 and N) versus "Modified" (states P, T, MP, MP/P/LK3 and MP/LK1), and used the program Discrete, again implemented in BayesTraits under Maximum Likelihood.

To test predictions 1-3 with respect to factors associated with prolonged copulation, we modelled copulation duration before and after spermatophore transfer using a Phylogenetic Generalized Least Squares approach (PGLS; Pagel 1999; Martins and Hansen 1997) with the package "ape" version 3.0-9 (Paradis *et al.* 2004). We included the predictors (1) ampulla mass as an absolute index of investment by males in the ejaculate, (2) spermatophylax mass, (3) whether the proportional contribution of the spermatophylax to the spermatophore exceeded 0.30 (i.e. spermatophylax/[spermatophylax+ampulla] > 0.30) as an indicator of evolutionary reduction of the nuptial gift (breakpoint determined visually based on data; see Fig S2), (4) presence of male modified cerci, fitted as a binary variable, and (5) male body mass as a covariate. Prior to analysis, data for pre- and post-transfer copulation duration and all mass variables were ln-transformed.

Initial data exploration revealed strong pairwise collinearity among spermatophylax mass, spermatophylax contribution to spermatophore and the presence of modified cerci, taking into account body mass as a covariate (PGLS with male body mass as

covariate, dropping predictor variable, $p < 0.0001$ in all cases) whereas none of these variables was strongly correlated with relative ampulla mass (PGLS as above, $p > 0.1$ in all cases) except for spermatophylax mass (PGLS as above, $p < 0.01$). Yet, each of these collinear variables had distinct and specific relevance to our predictions (see Introduction). As recommended by Zuur *et al.* (2009), to test our predictions in the light of this collinearity, we did not include collinear predictors in the same analysis. Instead we first conducted three separate analyses, each with "copulation duration pre- or post-spermatophore transfer" as the response. In each of these separate analyses, the full model had four terms: (i) one of the three strongly collinear predictor variables (spermatophylax mass, whether spermatophylax > 30% of spermatophore, or modified cerci), (ii) ampulla mass, (iii) the interaction of these two terms, and (iv) male body mass as a covariate. For each analysis we fitted multiple models including all possible combinations of terms (all models fitted are given in Table 2). We compared models under an information-theoretic framework, using corrected Akaike's Information Criterion (AICc) as a criterion for model selection (with an AICc difference of 2 as a selection threshold; Burnham and Anderson 2002). This approach is less sensitive to multicollinearity than alternative methods (Graham 2003) and allows model averaging, a way of providing more meaningful parameter estimates, and also comparing of non-nested models. We interpreted each of the three analyses separately with respect to the relevant predictions. Finally, we asked which of the separately-fitted models was best at explaining copulation duration after spermatophore transfer, by combining all models from the separate analyses and comparing all fitted models in a single information-theoretic analysis, again using AICc as a criterion for model selection.

Results

Evolution of pre- and post spermatophore-transfer copulation duration

Analyses of pre- spermatophore transfer copulation duration

In all three analyses of pre-spermatophore transfer copulation duration, the top two models were identical (ΔAICc < 2; Table 2a); the overall top model was the model with no terms, i.e. simply an intercept; the next-best model (ΔAICc =1.37 in all cases) was the model with simply male body mass. Models containing variables relevant to our predictions always had ΔAICc > 3, and dropping the variable of interest from these models never resulted in significant reductions in model fit (PGLS, Δdf=1, P>0.1 in all cases). When the analyses were combined, the overall top two models were, identically as above, the intercept alone, followed by male body mass alone. We conclude that the candidate predictor variables had very limited capacity to explain variation in pre-transfer copulation duration.

Post- transfer copulation duration and spermatophylax mass

There were two best PGLS models of copulation duration with respect to spermatophylax mass (ΔAICc < 2; Table 2b). The top model (Akaike weight 0.568) contained, in addition to male body mass, main effects of spermatophylax mass and ampulla mass only. Dropping either term from this model significantly reduced model performance (spermatophylax, $F_{1,40}$=27.5, P<0.0001; ampulla, $F_{1,40}$=8.62, P<0.01). The second-best model (ΔAICc=0.66, Akaike weight=0.408) additionally contained their interaction. After model averaging, post-spermatophore transfer copulation duration was associated negatively with spermatophylax mass, indicating that males invested less in spermatophylaces where copulation was prolonged after spermatophore transfer (prediction 1) (Fig 2a). In some species in which copulation

following spermatophore transfer was prolonged (e.g. *Dichopetala castanea*, Fig S1, *D. pollicifera, Meconema thalassinum* , *M. meridionale, Decticita brevicauda* and *Pterophylla beltrani*, Fig S3c), the spermatophylax was absent altogether. Post-spermatophore transfer copulation duration was associated positively with ampulla mass in the final averaged model, indicating that, across species, males tend to spend longer in copulation after transferring a larger ampulla (prediction 2), but the interaction of spermatophylax mass and ampulla mass was not different from zero (Table 2b).

Post- transfer copulation duration and spermatophylax contribution to the spermatophore

There were three best PGLS models of copulation duration with respect to the proportional contribution of the spermatophylax to the spermatophore (ΔAICc<2; Table 2b). The top model, with Akaike weight of 0.462, was the full model, containing, in addition to male body mass, the interaction between spermatophylax contribution to the spermatophore and ampulla mass. In this model, the slope of the relationship between post-transfer copulation duration and ampulla mass was positive in species where the spermatophylax comprised less than 30% of the spermatophore, but was not different from zero in species where this was not the case (prediction 2). Dropping the interaction from this model resulted in a marginally significant reduction in explanatory power ($F_{1, 39}$=3.59, P=0.06). The second-best of the top models (ΔAICc=0.74; Akaike weight 0.319) contained male body mass and a negative main effect of the spermatophylax contribution to the spermatophore only, while the third (ΔAICc=1.49; Akaike weight 0.219) contained a positive main effect of ampulla mass and a negative main effect of the spermatophylax contribution to the

343 spermatophore, but not their interaction. Combining these models using model
344 averaging, the interaction was important (i.e. its confidence intervals did not overlap
345 zero; Table 2b), indicating that the relationship between post-transfer copulation
346 duration and ampulla mass depended upon whether the spermatophylax was reduced
347 or absent (prediction 2; Fig 2b).
348
349 *Post- transfer copulation duration and male cercal form*

350 The form and use of the male's cerci during copulation is summarised in Table 1 and
351 the accompanying figs (Figs. 3, 4 & S3) and is described for each species in Table S2.
352 In the majority of species, each of the male's cerci has a single tooth which engages
353 with a sclerotised pit or groove on the female. Some species within each sub-family,
354 however, depart from these patterns (Fig. 1, Table S2). In such species, the cerci show
355 a variety of modifications in morphology and in the way in which they contact the
356 female (Tables 1 & S2, Figs. 4 & S3).
357
358 There were two best models of post-spermatophore transfer copulation duration with
359 respect to cercal form (ΔAICc < 2; Table 2b). The first, with Akaike weight 0.688,
360 contained, in addition to male body mass, both main effects of cercal form and
361 ampulla mass but without their interaction. In this model, longer copulation times
362 following spermatophore transfer were associated with modified cerci (prediction 3a;
363 Fig 5) and larger ampullae. In clades where females additionally resisted male
364 copulation attempts, copulation durations were in fact marginally statistically shorter
365 after spermatophore transfer than in those species where females did not resist (PGLS,
366 planned contrast between "modified/non-resisting females" and "modified/resisting
367 females", t= -1.73, p=0.08; Fig 5), indicating that female resistance may be somewhat

effective in reducing the duration of copulation after transfer. The second-best model (ΔAIC=1.63, Akaike weight 0.305) was the full model, containing the interaction of male cercal form with ampulla mass. In this model, copulation duration following spermatophore transfer was more strongly positively related to ampulla mass in species with modified cerci than in those with unmodified cerci. In the averaged model, only the main effects were different from zero (Table 2b).

Combined analysis of post-transfer copulation duration

Comparing all fitted models of post-spermatophore transfer copulation duration, all the models including "modified cerci" were superior to all other models. Thus, as above, the two overall best models were (1) "modified cerci" and "ampulla mass" but not their interaction (Akaike weight 0.672) and (2) "modified cerci" and "ampulla mass" plus their interaction (Akaike weight 0.326; Table 3b).

Evolution of female resistance behaviour

There were no taxa in the dataset in which females resisted copulation by males with "unmodified" cerci. Thus, female resistance behaviour was, superficially, entirely contingent upon the presence of modified cerci (prediction 3b). We therefore amalgamated the two traits into one trait with three extant states, and used MultiState to model transitions between these three states. This analysis indicated no detectable transitions between "modified cerci/non-resisting females" and "modified cerci/resisting females" (Fig S4; note that re-running the model using Discrete did not produce appreciably different results). This is consistent with a scenario where female resistance to copulation is ubiquitous in some entire clades where males carry modified cerci, but is entirely absent from others, as was the case for our data (see Fig

1). This lack of variation meant that our phylogeny was not finely resolved enough for us to ascertain which of the two traits evolved first. Thus, in those clades where females all resisted copulation (e.g. in the genus *Anonconotus*; see Fig 1), modified cerci may have evolved to counteract female resistance to copulation, or *vice versa*. More fine-grained data will be required to resolve this issue, although the fact that modified cerci occurred independently, whereas female resistance behaviour appeared to be dependent upon the presence of modified cerci, circumstantially supports the idea that female resistance follows evolutionary modification of male cerci.

Ancestral character states of male cerci

Figure 1 shows the phylogeny with the extant states of all traits analysed. We were statistically unable to resolve which type of cerci was ancestral to katydids as a whole. In this case outgroup comparison was unhelpful, since in related families within the Ensifera, such as the Anostostomatidae, Stenopelmatidae, Gryllacrididae, Rhaphidophoridae, and Gryllidae , males carry simple cerci with a sensory function that are not typically used in mating, and so are uninformative in resolving ancestral character states (Alexander and Otte 1967; Weissman 2001; Field and Jarman 2001; Eades *et al*. 2013). Across the whole phylogeny, collapsing cerci into "modified" vs. "unmodified", a maximum of 7 origins of modified cerci were evident if unmodified cerci were treated as ancestral, whereas a maximum of 11 origins of unmodified cerci were evident if modified cerci were treated as ancestral.

Within each subfamily, the cercal form ancestral to the Phaneropterinae was most likely to be "LK3" (Probability, Pr, =0.73) or "MP & P & LK3" (Pr=0.22), for

417 Bradyporinae, “LK1” (Pr=0.93); for Meconematinae, “T” (Pr=0.65), or “N”

418 (Pr=0.31); for Tettigoniinae, “MP” (Pr=0.51), “LK2” (Pr=0.21) or “MP & LK1”

419 (Pr=0.19).

420

421

Discussion

The present study provides the first comparative evidence that prolonged copulation during ejaculate transfer and nuptial feeding are functionally analogous (Boldyrev 1915; Vahed 1996, 1997; Wedell 1998). Both predictions 1 and 2 were supported: prolonged copulation following spermatophore transfer was associated with a loss or reduction in size of the spermatophylax (both in absolute terms and relative to the rest of the spermatophore); and larger ejaculates were associated with an increase in the duration of copulation following spermatophore transfer, but only in species in which the nuptial gift (spermatophylax) was reduced or absent. Prolonged copulation following spermatophore transfer, with associated loss or reduction in the size of the spermatophylax, appears to have evolved independently numerous times. In the Tettigoniidae, the spermatophylax appears to be the ancestral character state as it occurs in virtually all subfamilies of katydids studied so far (Gwynne 2001), so it appears that prolonged copulation has replaced the spermatophylax in function. This finding supports the hypothesis that the main function of nuptial feeding relates to enhancing the male's mating or fertilisation success, rather than providing the female with nutrients for egg production. If nuptial gifts evolved, or currently function, as a form of paternal investment (reviewed in Gwynne, 2001; Lewis and South 2012), then there is no reason to expect any association between nuptial gift size and the duration of copulation following spermatophore transfer.

The prediction that prolonged copulation following spermatophore transfer would be associated with a change in the functional morphology of cerci in males (prediction 3a), was supported. In species with brief copulation following spermatophore transfer, the majority of species in this study, the cercal tooth generally engaged with

447 specialised pits or grooves at the base of the ovipositor or on the female's sub-genital
448 plate. This mechanism is consistent with inter-sexual co-operation over copulation
449 rather than conflict. In contrast, prolonged copulation following spermatophore
450 transfer was associated with three different types of "modified" cerci: those that
451 contact the female in multiple places, those that encircle the female's abdomen, and
452 those that pierce the female's abdominal cuticle. Few previous studies have taken
453 copulation duration into account when seeking to explain inter-specific variation in
454 the morphology of copulatory structures in males (for insects, see Takami and Sota
455 2007; Vahed *et al.* 2011; Ronn and Hotzy 2012; for mammals, see Dixson 1995;
456 Larivière and Ferguson 2002).
457
458 The prediction that prolonged copulation and modified cerci will be associated with
459 behavioural resistance by the female (prediction 3b), was also supported. Similar
460 resistance behaviour has been reported in various insect taxa with prolonged or
461 coercive copulation (reviewed in Arnqvist and Rowe 2005; see also Edvardsson and
462 Canal 2006; Kuriwada and Kasuya 2009; Mazzi *et al.* 2009). Studies in which
463 resistance by females during copulation has been prevented have demonstrated that
464 resistance behaviour can shorten copulations (eg. in *Callosobruchus* beetles and in
465 *Drosophila montana*; Crudgington and Siva-Jothy 2000; Mazzi 2009). Whether such
466 behavioural resistance can lead to sexually antagonistic co-evolution has been
467 questioned: Eberhard (2004, 2006) suggested that species-specific differences in
468 female resistance behaviour would have to be shown to be effective (i.e. adaptations)
469 against particular details of male grasping traits. Although our findings cannot satisfy
470 these strict requirements, we suggest that even general resistance behaviour by the
471 female, when accompanied by selection on males to prolong ejaculate transfer (i.e. for

sperm competition avoidance), can select for copulatory structures in males that are more effective in maintaining a firm hold of the female.

While we interpret behaviour such as biting the male, rapid locomotion during copulation and kicking the male as reflecting inter-sexual conflict over copulation duration/ ejaculate transfer, we cannot exclude the possibility that it reflects a means by which females assess their mates (e.g. Eberhard 1996). If such behaviour is a form of mate screening, however, it is hard to explain why in the present study this behaviour only occurred during prolonged copulation *following* spermatophore transfer and not before spermatophore transfer. Prolonged copulation *prior to* spermatophore transfer was not associated with either resistance behaviour by the female or with modified cerci. This could suggest that it is generally not in the female's interest to break off from copulation *before* receiving the spermatophylax (in order to gain any nutritional benefits from spermatophylax consumption; reviewed in Lehmann 2012).

In species in which males showed "modified" cerci and prolonged copulation in this study, it is perhaps surprising that females did not appear to show any morphological adaptations to resist the grasping or piercing male cerci. This may be because resistance was behavioural rather than morphological. Where females did possess specialised structures in parts contacted by males, these tended to occur in species in which copulation following spermatophore transfer was brief and apparently functioned to *facilitate* copulation (Fig. 3). The tendency for such structures in females to facilitate rather than to resist copulation is seen in a wide range of

arthropod taxa (Eberhard 2004, 2006), although with notable exceptions (reviewed in Arnqvist and Rowe 2005; Perry and Rowe 2012).

The present study demonstrates that comparative analyses involving species in which nuptial gifts have been lost or reduced can provide valuable insights into the selective pressures underlying gift evolution (South *et al.* 2011; Lewis and South 2012). This study also underscores the importance of behavioural data in understanding male copulatory structure evolution. Furthermore, it demonstrates that emphasizing morphology alone could be misleading: we cannot expect sexually antagonistic co-evolution always to lead to increases in complexity of male copulatory structures (as is sometimes implied, e.g. Eberhard 2006); there may be several different evolutionary pathways by which males can increase grasping efficiency in the face of resistance by females, not all of which necessarily involve an increase in morphological complexity.

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668

669 **Figure and table legends**

670

671 **Figure 1.** Phylogeny used in this study showing extant states for all analysed traits.
672 Branch lengths have been scaled to make the tree ultrametric and are not
673 representative of those used in the analysis. For each binary trait, character states are
674 true (black circles) or false (white circles). "Spx" = spermatophylax; "post-transfer
675 copulation duration" is the duration for which the male maintains hold on the female
676 with his cerci following spermatophore transfer. Post-transfer copulation duration has
677 been scaled so that totally white and black circles represent, respectively, the
678 minimum and maximum observed in the dataset (see supplementary information for
679 all raw data). "Male modified cerci" refers to the functional morphology of the cerci
680 and primarily includes cases which depart from purely "lock and key" based systems
681 (see text for further details).

682

683 **Figure 2.** (a) Relationship between post-spermatophore transfer copulation duration
684 and spermatophylax mass. Line shows model-averaged regression ±s.e. from PGLS
685 models of post-spermatophore transfer copulation duration. (b) Relationship between
686 post-spermatophore transfer copulation duration and ampulla mass in species with
687 varying spermatophylax contribution to spermatophore (open circles: spermatophylax
688 < 30% of spermatophore; closed circles: spermatophylax > 30% of spermatophore).
689 Fitted lines are model-averaged regressions from PGLS models of post-
690 spermatophore transfer copulation duration, and show best-fit lines ±s.e. for species
691 where spermatophylax < 30% of spermatophore (thin line, solid area) and > 30% of
692 spermatophore (thick line, hatched area).

693

694

695 **Figure 3.** The end of the female's abdomen in a. *Leptophyes punctatissima*
696 (Phaneropterinae), b. *Platycleis albopunctata* (Tettigoniinae), c. *Conocephalus fuscus*
697 (Conocephalinae) and d. *Steropleurus stalii* (Bradyporinae), showing the structures
698 that receive male's the cercal tooth during copulation (p = pit into which the male's
699 cercal tooth engages; s= lateral sclerite; l = lamella). The base of the ovipositor (ov) is
700 visible on the left. See Table 1 & S2 for the accompanying text.

701

Figure 4. Cerci in male katydids with brief copulation following spermatophore transfer in comparison with those in which copulation following spermatophore is prolonged (and coercive, in the case of *Anonconotus pusillus* and *A. baracunensis*) (see Tables 1 & S2 for the accompanying text). a. *Leptophyes punctatissima*; b. *Poecilimon affinis*; c. *P. veluchianus*; d. *Dichopetala castanea*; e. *D. pollicifera* (Phaneropterinae) f. *Docidocercus gigliotosi*, showing the tip of the abdomen (photo by P. Naskrecki); g. *Pterophylla beltrani*, showing the tip of the abdomen (Pseudophyllinae). Note the three projections on each cercus (ve = ventral arm; ce = central tooth; do = dorsal arm); g1. Enlargement of the dorsal arm in *P. beltrani* to show the sharply pointed hook that grips the female's abdominal cuticle; h. *Yersinella raymondi*; i. *Pholidoptera griseoaptera*; j. *Metrioptera roeselii*; k. *Platycleis albopunctata*; l. *Anonconotus pusillus*; m. *A. baracunensis*; n. *Decticita brevicauda* (Tettigoniinae); o. *Conocephalus fuscus*; p. *Ruspolia nitidula*; q. *Coptaspis* sp. 6 (Conocephalinae); r. *Cyrtaspis scutata*, showing the tip of the abdomen; s. *Meconema thalassinum*, showing the tip of the abdomen; t. *M. meridionale*, showing the tip of the abdomen (Meconematinae). t1. Enlargement of the tip of a cercus in *M. meridionale*. The arrow indicates the apical tooth (to), which is absent in *M. thalassinum*; u. *Kawanaphila nartee*, showing the tip of the abdomen; *Phasmodes ranatriformis*, showing the tip of the abdomen (adapted from Rentz 1993) (Zaprochilinae/ Phasmodinae); w. *Ephippigerida taeniata*; x. *Steropleurus stalii*; y. *Uromenus rugosicollis* (Bradyporinae).

Figure 5. Pre-spermatophore transfer copulation duration plotted against post-spermatophore transfer copulation duration in species with unmodified (white circles) and modified (black circles) cerci, and species with modified cerci where females resist mating (ringed circles).

Table 1. Classification of the functional morphology of cerci of male katydids used in the analysis (see also Table S2).

Table 2. Tables of coefficients and AIC selection criteria for PGLS models of (a) pre- and (b) post-spermatophore transfer copulation duration. In each case three separate analyses were carried out with respect to each of three collinear predictor variables (see text for details). Key: K, number of parameters; w_i, Akaike weight; INT, Intercept; M, male body mass; AMP, ampulla mass; SPX, spermatophylax mass; PSPX, proportional contribution of spermatophylax to spermatophore (binary; greater or less than 30%); MOD, modified cerci (binary, yes or no). X:Y denotes the interaction of term X and term Y.

Supplementary online material legends.

Figure S1. a.) Female *Ephippiger diurnus* carrying the spermatophore (photo by S. Dourlot). Note the large spermatophylax (am = ampulla; spx = spermatophylax); b.) Female *Dichopetala castanea* carrying a spermatophore (am = ampulla). Note the lack of a spermatophylax in this species.

Figure S2. Frequency distribution of the proportional contribution of the spermatophylax to the spermatophore across species.

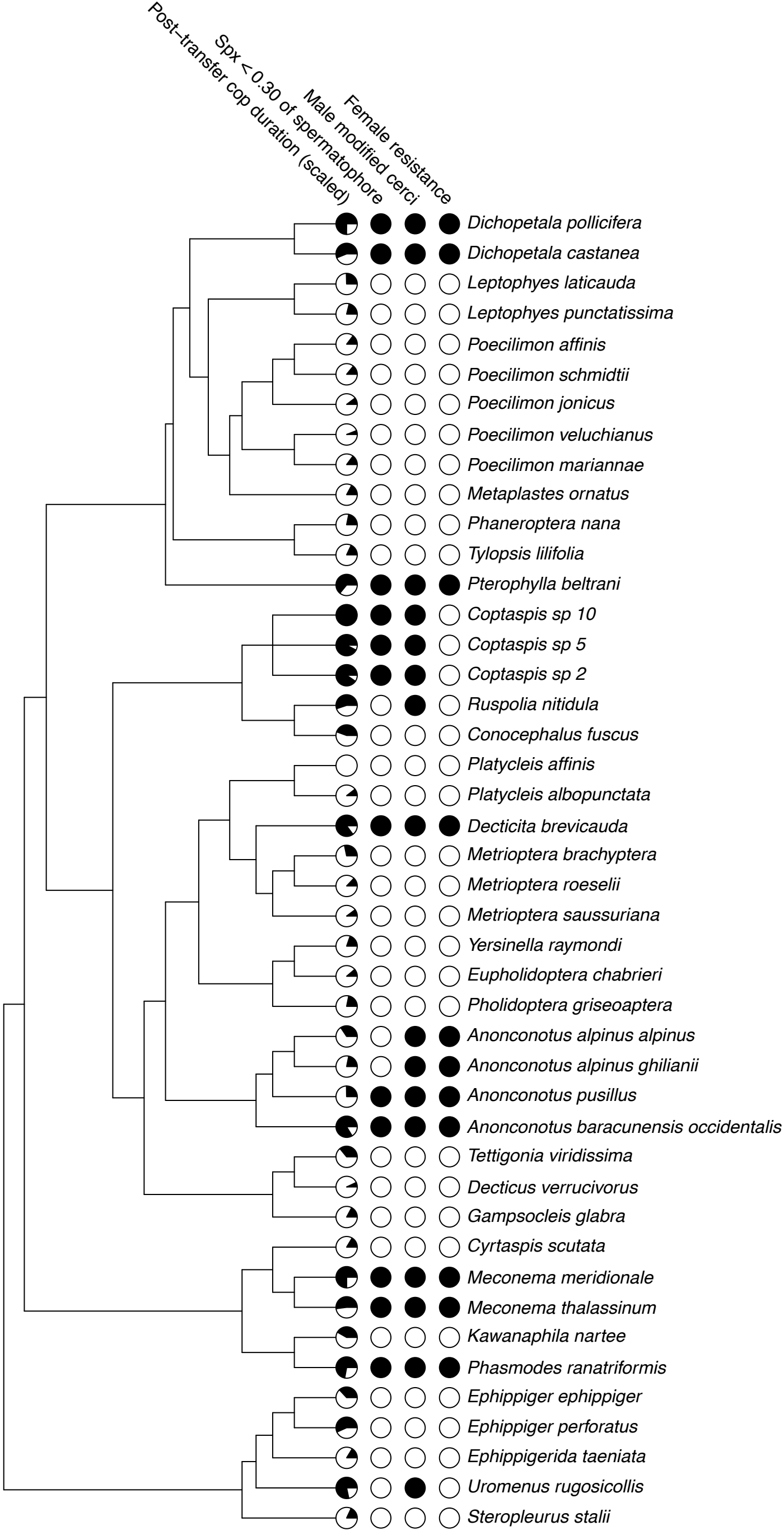
Figure S3. Examples of copulating pairs of tettigoniid species in which copulation is prolonged following spermatophore transfer. See Table S2 for the accompanying text. The male is upside-down on the left (ce = the male's cercus; am = ampulla of the spermatophore). a. *Anonconotus baracunensis* (Tettigoniinae) (modified from a photo by C. Roesti). Note that the cerci (Fig 4m) grip the sides of the female's abdomen. The insert shows melanised scarring (sc) on the sides of the female's abdomen cause by the apical teeth of the male's cerci; b. *Uromenus rugosicollis* (Bradyporinae) (photo by. G. Carron). The insert shows melanised scarring (sc) on the ventral surface of the female's abdomen from puncture wounds caused by the sharp teeth of the male's cerci (Fig 4y); c. *Pterophylla beltrani* (Pseudophyllinae) (photo by L. Barrientos-Lozano) (the cerci of this species are shown in Fig 4g); d. *Dichopetala pollicifera* (Phaneropterinae) (photo by L. Barrientos-Lozano). The cerci (Fig 4e) grip the sides of the female's abdomen, causing it to indent; e. *Meconema meridionale* (Meconematinae) (modified from a photo by B. Baur). Note that the male's cerci (which have been darkened digitally to make them visible, see also Fig 4t) enclose the end of the female's abdomen and cross over one another on the other side.

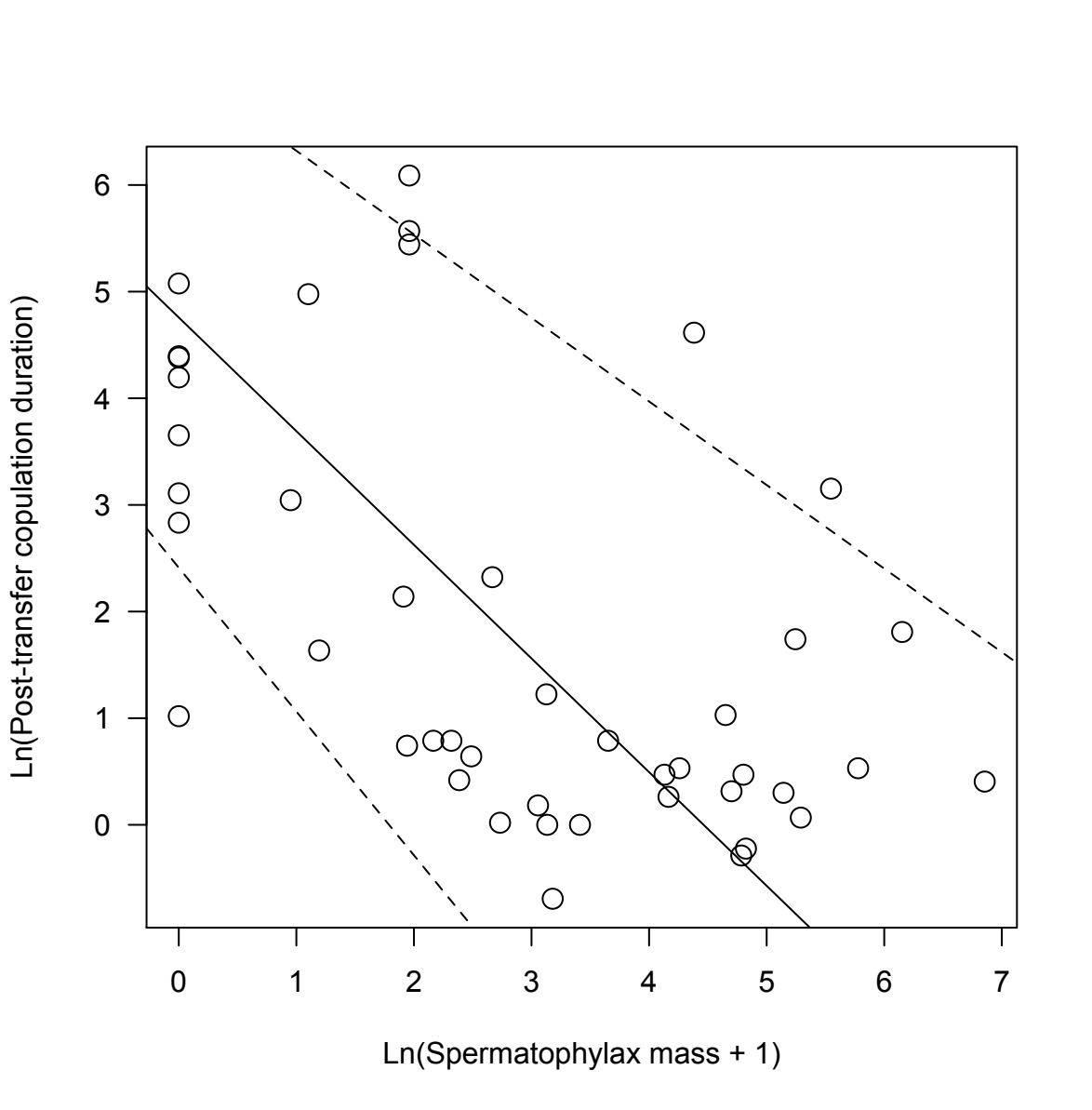
Figure S4. Reconstructed evolutionary transitions between cercal states (Unmodified, U, versus modified, M) and female resistance (no resistance, NR, versus resistance, R) using the program MultiState. Transition rate parameters represent the relative probability of a given evolutionary transition along a branch of the phylogeny (Pagel and Meade 2006). Arrow weights are scaled according to transition rates. Dashed arrows indicate transition rates that were not different from zero, i.e. which did not

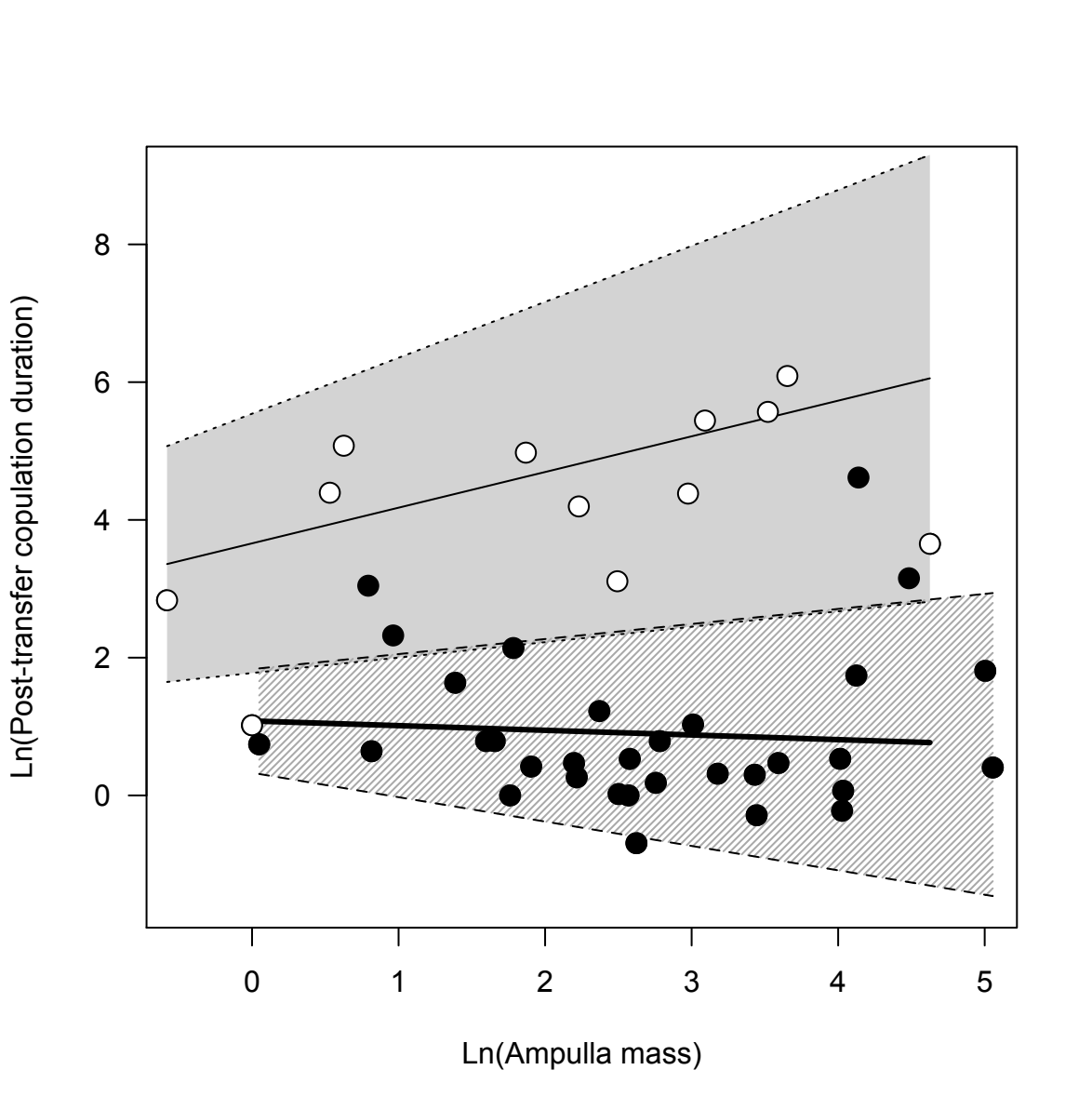
reduce the model's explanatory power when restricted to zero. Greyed-out state combinations did not occur on the phylogeny.

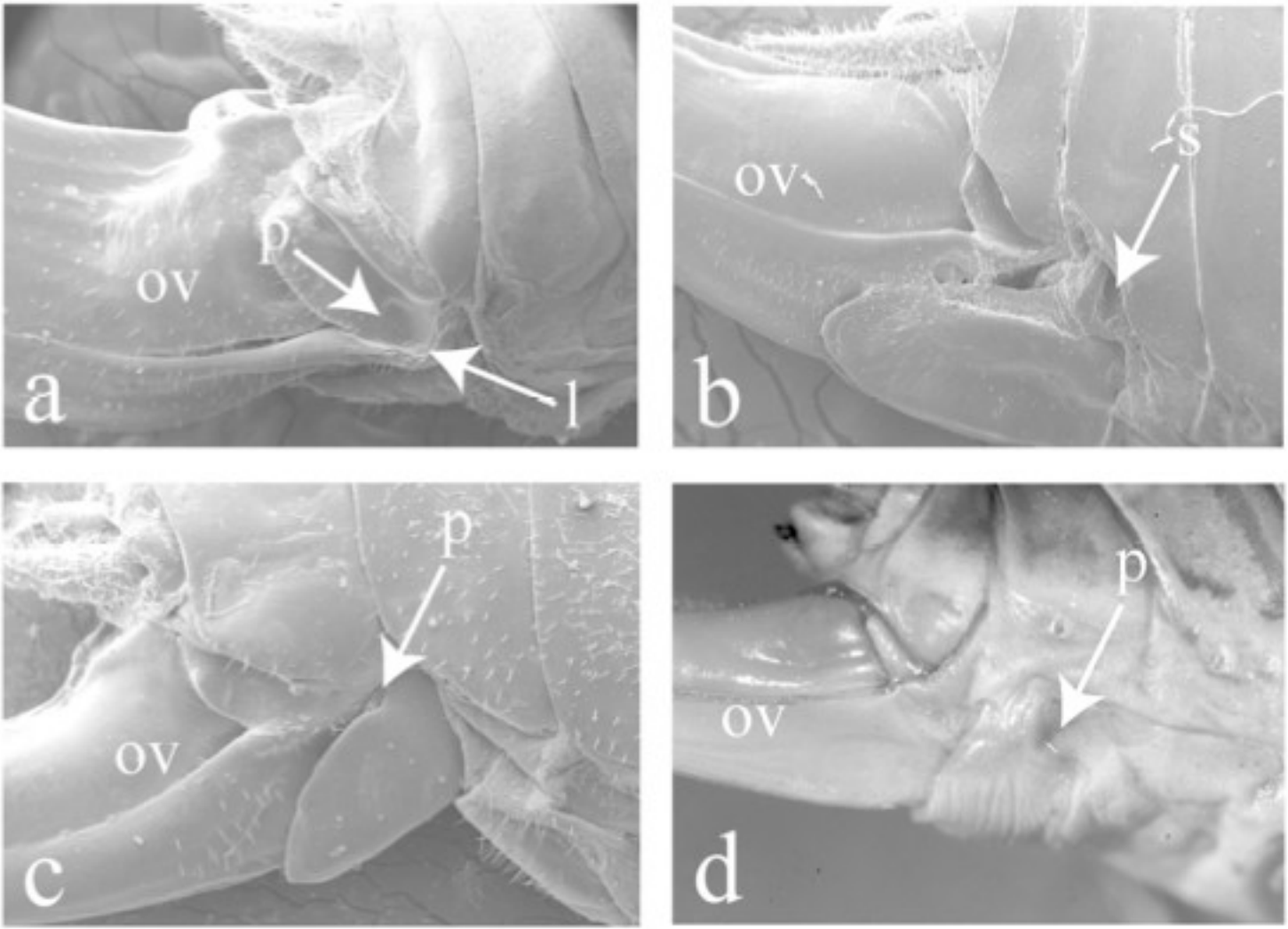
Table S1. Raw data used in the analyses.

Table S2. The form and use of the male's cerci in tettigoniid males in which copulation following spermatophore transfer is brief in comparison with species in which copulation following spermatophore transfer is prolonged. "Code" is the classification of the functional morphology of the cerci used in the analysis (see also Table 1). For the purposes of this table, species with "prolonged copulation following spermatophore transfer" include those in which the mean duration of copulation following spermatophore transfer (see Table S1) is greater than 15 min and/or those in which ejaculate transfer is likely to occur largely during copulation (because the female typically eats the ejaculate-containing ampulla within 5 min. following the end of copulation).


Post–transfer cop duration (scaled)
Spx < 0.30 of spermatophore
Male modified cerci
Female resistance
Dichopetala pollicifera
Dichopetala castanea
Leptophyes laticauda
Leptophyes punctatissima
Poecilimon affinis
Poecilimon schmidtii
Poecilimon jonicus
Poecilimon veluchianus
Poecilimon mariannae
Metaplastes ornatus
Phaneroptera nana
Tylopsis lilifolia
Pterophylla beltrani
Coptaspis sp 10
Coptaspis sp 5
Coptaspis sp 2
Ruspolia nitidula
Conocephalus fuscus
Platycleis affinis
Platycleis albopunctata
Decticita brevicauda
Metrioptera brachyptera
Metrioptera roeselii
Metrioptera saussuriana
Yersinella raymondi
Eupholidoptera chabrieri
Pholidoptera griseoaptera
Anonconotus alpinus alpinus
Anonconotus alpinus ghilianii
Anonconotus pusillus
Anonconotus baracunensis occidentalis
Tettigonia viridissima
Decticus verrucivorus
Gampsocleis glabra
Cyrtaspis scutata
Meconema meridionale
Meconema thalassinum
Kawanaphila nartee
Phasmodes ranatriformis
Ephippiger ephippiger
Ephippiger perforatus
Ephippigerida taeniata
Uromenus rugosicollis
Steropleurus stalii


Ln(Post-transfer copulation duration)
Ln(Spermatophylax mass + 1)
0
1
2
3
4
5
6
7


Ln(Post-transfer copulation duration)
8
6
4
2
0
0
1
2
3
4
5
Ln(Ampulla mass)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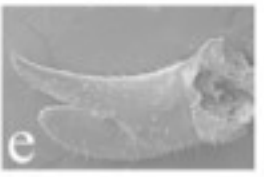
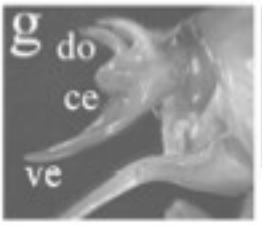
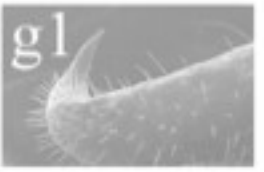
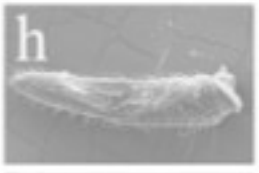
Brief copulation

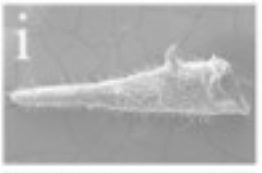
Prolonged copulation

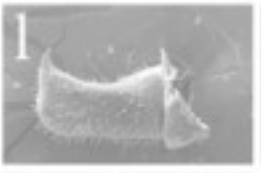


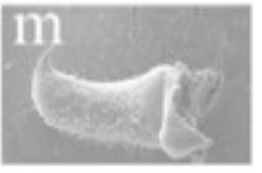


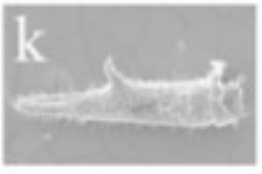


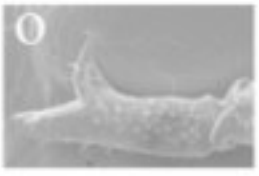


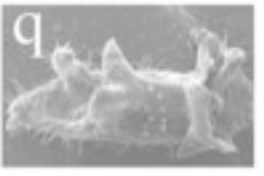


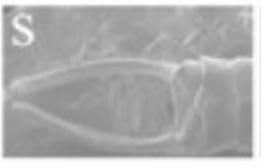


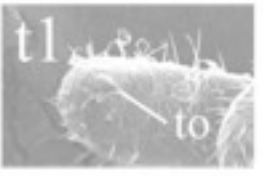


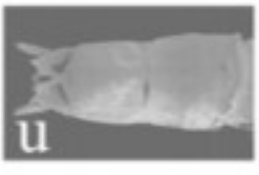


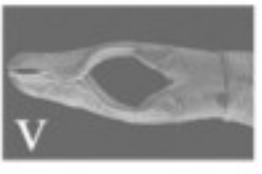


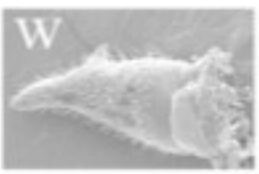


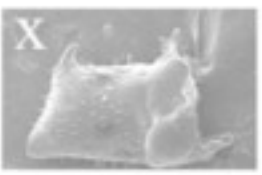


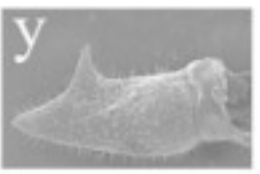


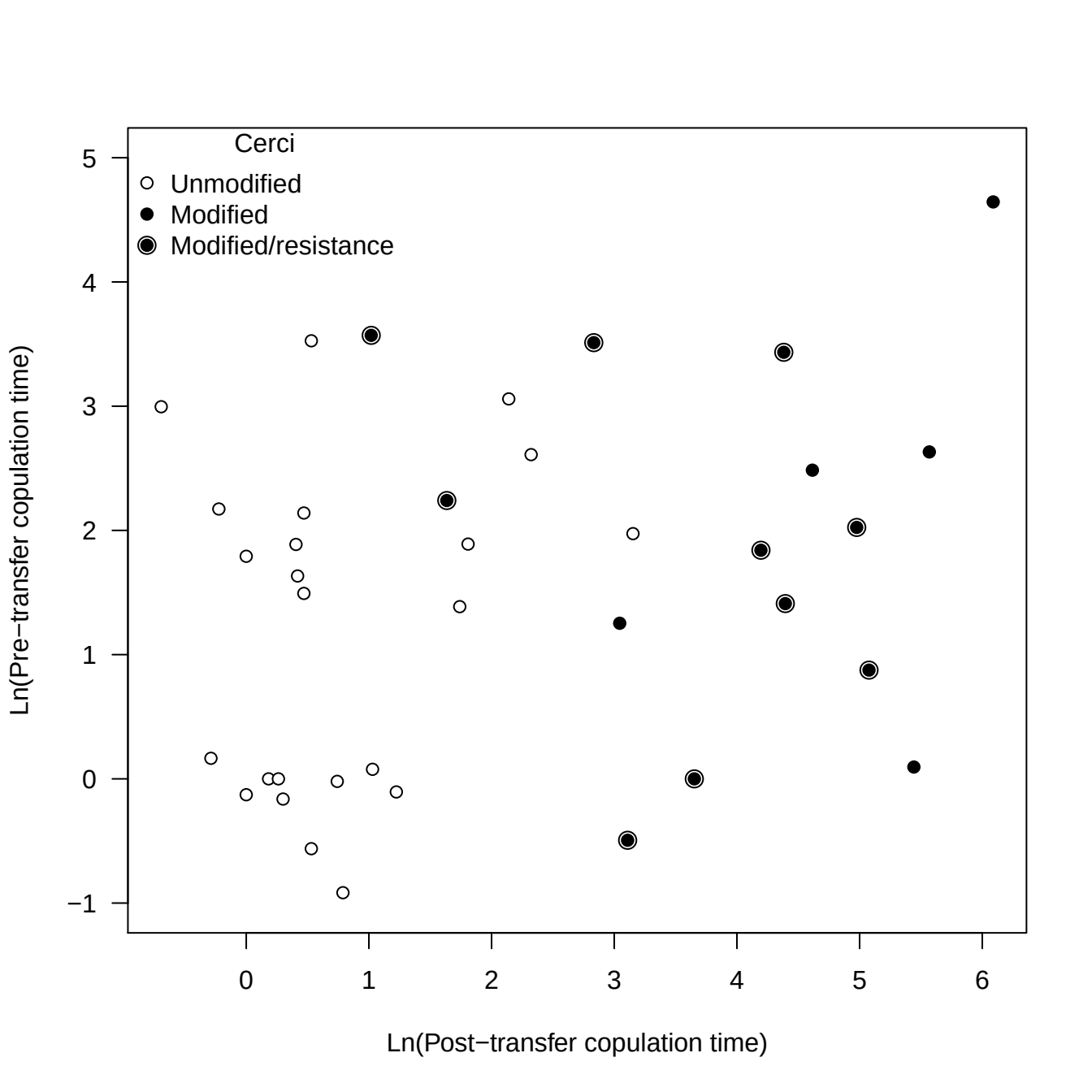








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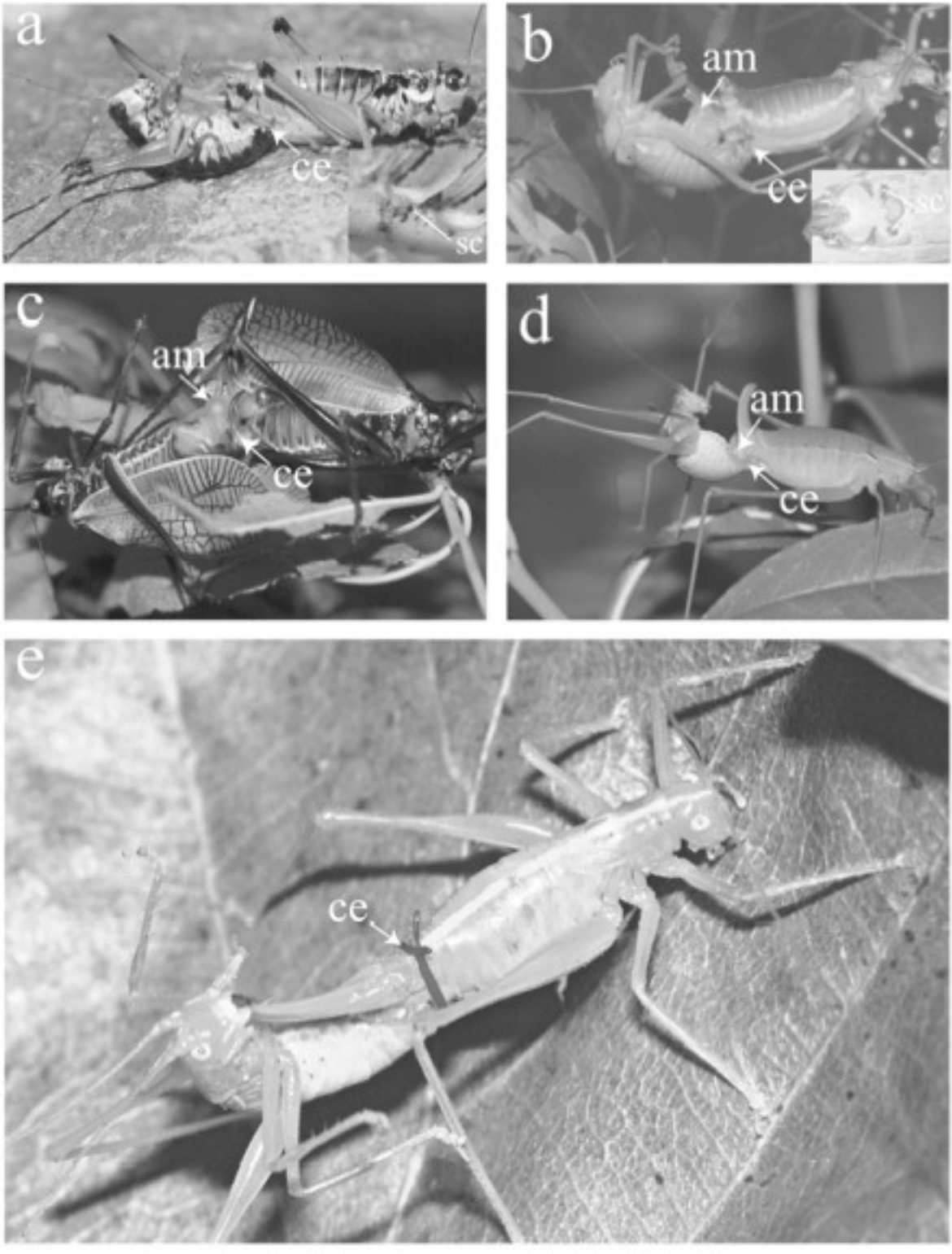
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