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Coevolution of male and female genitalia in stalk-eyed flies (Diptera: Diopsidae)

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Abstract The present study investigates the coevolution of a particular male genital process and the female spermathecal ducts in a clade of stalk-eyed flies (Diptera, Diopsidae) and debates the underlying evolutionary mechanisms. The fine morphology and interaction of the male and female genitalic structures are reconstructed from serial sections of mating pairs in one of the species. It is found that the male genital process traverses the common spermathecal duct to enter the base of one of the separate spermathecal ducts during the mating. Spermatozoa and accessory secretions are not transferred through the male genital process but can be discharged only from the male gonopore near its base. A detailed morphometric study reveals low intraspecific variation and hypoallometry of the male genital process. Across 17 species studied comparatively, the lengths of the male genital process and the female common and separate spermathecal ducts are highly variable. The length of the male genital process is correlated significantly with that of the female common spermathecal duct, but not with that of the separate spermathecal ducts. Based on the combined evidence it is concluded that the male genital process and the female common spermathecal duct have coevolved, and that sexual selection by cryptic female choice constitutes a possible and parsimonious explanation for their coevolution. Alternative or additional explanations in terms of sexually antagonistic coevolution cannot be ruled out conclusively, but are not supported by the available evidence.

Keywords Aedeagus · Sexual selection · Female choice · *Teleopsis* · *Cyrtodiopsis* · Reproduction

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Introduction

Male genitalia are typically subject to rapid and divergent evolution resulting in morphological differences between closely related species (Eberhard 1985, 2009; Sirot 2003; Hosken and Stockley 2004; Joly and Schiffer 2010 and references cited therein). The classic lock and key hypothesis suggests that this diversity evolves to establish a species-specific fit between male and female genitalia, thus avoiding interspecific mating and hybridisation (reviewed in Eberhard 1985; Mikkola 2008). More recent concepts are based on aspects of sexual selection, such as intrasexual competition, sexually antagonistic coevolution and cryptic female choice (Eberhard 1985, 1996; Arnqvist and Rowe 2002, 2005; Sirot 2003; Hosken and Stockley 2004), and there has been much debate over the relative importance of one or the other (reviewed in Cordero and Eberhard 2003; Eberhard 2010). Along with this came an increased interest in the study of the coevolution of male and female genitalia, e.g. in flies (Ilango and Lane 2000; Joly and Schiffer 2010; Minder et al. 2005), beetles (Dybas and Dybas 1981; Gack and Peschke 2005; Rodriguez et al. 2004; Cayetano et al. 2011; Simmons and Garcia-Gonzalez 2011), bugs (Tadler 1999), spiders (Kuntner et al. 2009) and water fowl (Brennan et al. 2007). Nevertheless, the underlying evolutionary mechanisms remain poorly understood (Cayetano et al. 2011).

The present study investigates the coevolution of a particular male genital process and the female spermathecal ducts in the closely related stalk-eyed fly genera *Teleopsis*, *Cyrtodiopsis* and *Megalabops* (Diptera, Diopsidae, generic concepts according to Feijen 2011), and debates the underlying evolutionary mechanisms. Stalk-eyed flies constitute a favoured model organism for the study of sexual selection. Studies focussed first and foremost on aspects of precopulatory sexual selection based on the spectacular and often sexually dimorphic stalked eyes (de la Motte and Burckhard 1983; Burkhardt and de la Motte 1985, 1988; Burkhardt et al. 1994; Wilkinson and Dodson 1997; Warren and Smith 2007; Rogers et al. 2008; Cotton et al. 2010 and references cited therein). Females of many diopsid species are highly promiscuous (Kotrba 1996; Harley et al. 2010) and studies of their reproductive system soon revealed additional rewarding research fields. A detailed study of all aspects of reproduction in *Cyrtodiopsis whitei* Curran (Kotrba 1993) was followed by a number of comparative studies, e.g. on the internal female genital organs of *Chaetodiopsis* and *Diasemopsis* (Kotrba 1995), sperm transfer by spermatophore (Kotrba 1996) or the coevolution of spermatozoa and certain features of the male and female reproductive tracts (Presgraves et al. 1999).

In *Cyrtodiopsis whitei*, two spermathecal ducts originate close together from a short pouch in the dorsal vagina wall (genital papilla) and bear apically single and double spermathecae, respectively. Kotrba (1993) reports that, during mating, the tip of a rod-shaped process of the male aedeagus enters, and possibly serves to pry open, the entrance of the female spermathecal ducts (Fig. 1a). Spermatozoa and accessory secretions are not transferred through this process but discharged from the male gonopore near its base, forming a spermatophore within the vagina. The male process helps to mould the neck portion of the spermatophore, which fits like a funnel into the entrance of the spermathecal ducts. Spermatozoa and accessory secretions are then transferred from the spermatophore to the spermathecae, which constitute the primary sperm storage organs.

Subsequent comparative studies showed that in an undescribed *Teleopsis* species from Singapore (*Teleopsis* spec. A), a long common duct connects the vagina and the two separate spermathecal ducts (Fig. 1b). A similar structure was illustrated and briefly discussed for *T. quinqueguttata* and *T. discrepans* by Tan (1965). Concurrently, in *Teleopsis* spec. A as well as some other *Teleopsis* species the process of the male aedeagus is much longer than in *C. whitei* (Fig. 1c, Feijen 1998, 2011; Feijen and Feijen 2011). It is suggested here that the respective male and female genitalic structures have coevolved. A detailed study of the genital morphology, mating, and intraspecific allometry in *Teleopsis* spec. A, as well as a comparative study in 16 additional species of *Teleopsis*, *Cyrtodiopsis* and *Megalabops*, was performed to test this hypothesis and to gain insight into the underlying evolutionary mechanisms such as lock and key, intrasexual competition, sexually antagonistic coevolution and cryptic female choice.

Materials and methods

In total, 3 *Cyrtodiopsis*, 3 *Megalabops* and 11 *Teleopsis* species were studied. The provenance of the material is listed in Table 1. The name *C. dalmanni* has been used in recent literature for a *Cyrtodiopsis* from West Malaysia. We refer to this species as *C.* nr *dalmanni*, because the type of *dalmanni* Wiedemann, 1830 originates from Java and is unlikely to be conspecific with the former. One *Megalabops* from India and one *Teleopsis* from Singapore represent undescribed species.

Fresh specimens of *Teleopsis* spec. A were available from a culture in the laboratory of the first author. Larval development in different substrates such as breeding dishes with different qualities and quantities of corn or the rotting floor tissue resulted in a wide size range of specimens to be included in the study of intraspecific variation. The detailed morphology of the male and female genitalia of *Teleopsis* spec. A and their interaction was studied in serial sections of mating pairs frozen in liquid nitrogen and subsequently dissected, fixed in glutaraldehyde, embedded in Epon resin, cut at 1.5 μm and stained according to Richardson et al. (1960). For the study of intraspecific variation (Table 2) body length, wing length, and eye span were measured by standard procedure (e.g. Kotrba 2004) using a Leica MZ 8 dissecting microscope. The male external and female internal genital organs were subsequently dissected and mounted on microscopic slides in polyvinyllactophenol with an admixture of chlorazol black E. All dissections were examined and documented with a Zeiss Axioplan 2 compound microscope equipped with a drawing tube and a Zeiss AxioCam digital camera. The length of the studied genitalic structures was measured by tracing their course in line drawings. The genitalia of the remaining species (Table 3) were studied from pinned or ethanol preserved specimens after maceration in KOH. Data on the genitalia of *C. whitei* were taken from Kotrba (1993). Data on the average male eye span and body length of species other than *Teleopsis* spec. A were taken from literature or from unpublished studies of the third author.

The male genitalia of Schizophora are often highly variable, sometimes involving the development of spines, lobes or other processes of the aedeagus. These cannot be homologized unambiguously across families and no agreed terminology is available for these structures. We use the general term "male genital process" for a particular process of the aedeagus, which was studied here. Although its morphology varies, this process could be clearly identified and homologized in all studied species based on its ventroapical position on the dorsal part of the everted aedeagus (Figs. 1a, c and 4).

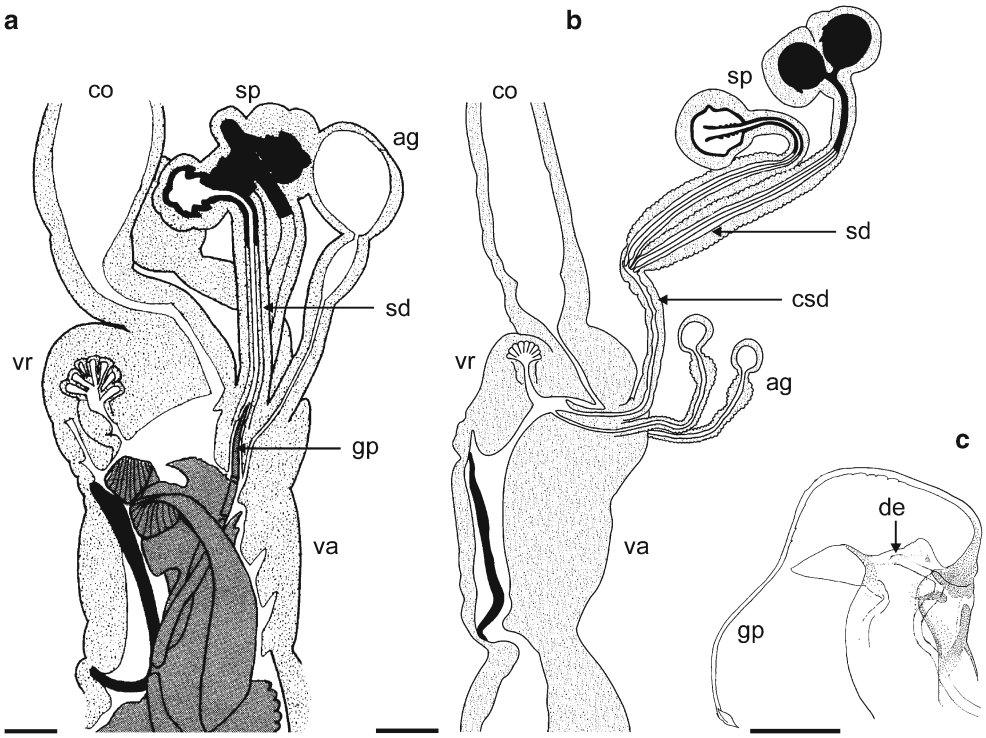

Fig. 1 **a**–**c** Female internal and male external genitalia. **a** *Cyrtodiopsis whitei* female internal and male external genitalia interacting during copulation, modified from Kotrba (1993), male genitalia shaded in *grey*. **b** *Teleopsis* spec. A female internal genitalia. **c** *Teleopsis* spec. A dorsal part of aedeagus. *ag* Accessory glands, *co* common oviduct, *csd* common spermathecal duct, *de* external opening of ejaculatory duct, *gp* male genital process, *sd* separate spermathecal ducts, *sp* spermathecae, *va* vagina, *vr* ventral receptacle. Schematic illustrations: *black structures* indicate dark brown, strongly sclerotized cuticle. *Bar* 100 μm

Statistical analyses were computed with MS Excel 1997. Least squares regressions were computed after $\log_{10}$ transformation of both variables (Tables 2 and 3). Statistic values are indicated as: mean, cv (coefficient of variation), sd (standard deviation), SE (standard error), n (number of observations), r^2 (coefficient of determination), P (probability of error), and b (allometric slope).

Results

Morphology of the male genital process and female spermathecal ducts in Teleopsis spec. A and their interaction during mating

The male genital process of *Teleopsis* spec. A consists of a long and narrow cuticular tube (Figs. 1c, 2a,b and 3l). Its wall is of uneven thickness and sclerotisation in cross section along parts of its length (Fig. 2a). Reconstruction from serial sections shows that the process has no external opening anywhere along its length and no internal open connection with the ejaculatory duct. The ejaculatory duct opens into a pouch next to the base of the male genital process (Fig. 1c).

The female internal genital organs (Fig. 1b) of *Teleopsis* spec. A are basically similar to those of *C. whitei* (Fig. 1a, Kotrba 1993). However, in *Teleopsis* spec. A, a long common duct is inserted between the vagina and the two separate spermathecal ducts. The wall of the common spermathecal duct consists of epithelium lined internally by unsclerotised, slightly wrinkled cuticle (Fig. 2a). No muscular component was detected. Contrarily, the two separate spermathecal ducts are equipped with a prominent layer of longitudinal muscles along most of their length, and are lined internally by thick cuticle with a smooth internal surface (Fig. 2a). Only their apical portions lack the musculature, and here their cuticle is strongly sclerotised. They are deeply inserted into the bases of the spermathecal capsules. Basally, the separate spermathecal ducts open side by side through narrow cuticular valves into the distal end of the common spermathecal duct (Fig. 2b).

The interaction of the male and female structures in *Teleopsis* spec. A was reconstructed from four mating pairs fixed in copula after flash freezing in liquid nitrogen. While the male rests on top of the female, the male genitalia are folded anteroventrally and inserted into the vagina posteriorly. The male genital process thus ends up in a dorsal orientation within the vagina, where it enters the orifice of the common spermathecal duct. In two pairs the process was found to traverse the common spermathecal duct and enter the base of the separate spermathecal duct leading to the double spermathecae with its tip stretched out straight (Fig. 2a). In the other two pairs, the process was found inserted in the common spermathecal duct with its apical portion folded back upon itself at about half the way in one and directly before the opening of the separate spermathecal ducts in the other pair (Fig. 2b). In none of the four pairs were spermatozoa found in the male ejaculatory duct or the female spermathecal ducts, indicating that sperm transfer had not yet begun. However, in one of the females all three spermathecae contained

Table 1 Provenance of studied specimens: *coll ZSM* Zoologische Staatssammlung München, *coll Feijen* H. R. Feijen, personal collection. *C*, Cyrtodiopsis; *M*, Megalabops; *T*, Teleopsis

	Provenance of dissected specimens
C. nr. *dalmanni* (Wiedemann, 1830)	Culture, University of Maryland at College Park, Gerald Wilkinson, from specimens from West Malaysia, Ulu Gombak
C. thaii (Földvári and Carr, 2007)	Culture, University College London, Pomiankowski et al., from specimens from Thailand, Doi Suthep National Park
*C. whitei (*Curran, 1936)	Culture, University of Regensburg, Burckhard & de la Motte, from specimens from West Malaysia, Ulu Gombak
M. bigotii (Hendel, 1914)	Taiwan, Hueisuen (coll. ZSM)
M. quadriguttata (Walker, 1857)	Culture, University of Maryland at College Park, Gerald Wilkinson, from specimens from West Malaysia, Ulu Gombak
Megalabops spec. A	India, West Bengal (coll. Feijen)
T. adjacens Brunetti, 1928	West Malaysia, Ulu Gombak (coll. ZSM)
T. boettcheri Frey, 1928	Philippines, Mindanao (coll. Feijen)
T. ferruginea (Röder, 1893)	Sri Lanka, Udawattekele (coll. Feijen)
T. freyi Feijen, 2011	Philippines, Palawan (coll. Feijen)
T. pallifacies Feijen and Feijen, 2011	Malaysia, Sabah, near Ranau (coll. ZSM)
T. quinqueguttata (Walker, 1857)	Culture, University of Maryland at College Park, Gerald Wilkinson, from specimens from West Malaysia, Ulu Gombak
T. rubicunda van der Wulp, 1897	Indonesia, Java (coll. Feijen)
T. sexguttata Brunetti, 1928	West Malaysia, Ulu Gombak (coll. ZSM)
T. sykesii (Westwood, 1837)	India, Anamalai Hills (coll. Feijen)
T. trichophora de Meijere, 1916	Indonesia, Sumatra (coll. Feijen)
Teleopsis spec. A	Culture, ZSM, from specimens from Singapore

spermatozoa, which must have been deposited during a previous mating.

Several of the other *Teleopsis* spec. A females dissected for the study of intraspecific variation contained a large teardrop-shaped spermatophore in their vagina, its neck portion aligned with, and inserted into, the orifice of the common spermathecal duct.

Intraspecific variation of male and female structures in Teleopsis spec. A

To obtain good coverage of the intraspecific size range in *Teleopsis* spec. A, some of the largest and smallest specimens from the laboratory culture were included in the study. Accordingly, the body length (cv=12 % in males, 11 % in females) and wing length (cv=12 % in males, 10 % in females) vary considerably within the sample (33 males, 54 females, Table 2, Fig. 4a). The species is highly dimorphic with respect to the eye span, which varies more in males (cv=28 %) than in females (cv=13 %). Eye span is correlated tightly with body length (r^2=0.89, P<0.001, b=2.46 in males, r^2=0.96, P<0.001, b=1.19 in females). It is the only one of the studied traits that exhibits positive allometry. Wing length is likewise tightly correlated with body length, albeit with slight hypoallometry (r^2=0.83, P<0.001, b=0.90 in males, r^2=0.91, P<0.001, b=0.88 in females).

The length of the male genital process varies comparatively little (cv=6 %, Table 2, Fig. 4b). It is not correlated with the body length (r^2=0.01, P=0.650, b=0.04), eye span (r^2=0.01, P=0.594, b=0.02), or wing length (r^2=0.01, P=0.584, b=0.04).

The length of the female common spermathecal duct (cv=12 %) and the lengths of the separate ducts to the single (cv=10 %) and double spermathecae (cv=9 %) vary to a similar degree as the external features (Table 2, Fig. 4b). The lengths of the separate spermathecal ducts are correlated tightly with body length (duct to single spermatheca: r^2=0.46, P<0.001, b=0.58; duct to double spermathecae: r^2=0.53, P<0.001; b=0.60), eye span (duct to single spermatheca: r^2=0.45, P=0.001; b=0.47; duct to double spermathecae: r^2=0.52, P<0.001, b=0.49) and wing length (duct to single spermatheca: r^2=0.40, P<0.001, b=0.59; duct to double spermathecae: r^2=0.48, P<0.001, b=0.62). The tendential increase of the length of the common spermathecal duct with the body length (r^2=0.09, P=0.028, b=0.33) and eye span (r^2=0.07, P=0.049, b=0.24) is small, but still significant; its correlation with the wing length is not (r^2=0.06, P=0.075, b=0.30).

Interspecific variation of male and female structures

The length of the male genital process varies greatly across the compared species (cv=56 %, n=17, Table 3, Figs. 3 and 4c–f). It is not correlated with the average body length of the

Table 2 Intraspecific variation of body length, eye span, wing length and genitalic measurements in *Teleopsis* spec. A. *n* Number of observations, *sd* standard deviation, *cv* coefficient of variation. Log transformed correlation values: r^2 coefficient of determination; *P* probability of error, *b* allometric slope

	Teleopsis spec. A males				*Teleopsis* spec. A females					
	Body length (mm)	Eye span (mm)	Wing length (mm)	Genital process length (μm)	Body length (mm)	Eye span (mm)	Wing length (mm)	Common spermathecal duct (μm)	Duct to single spermatheca (μm)	Duct to double spermatheca (μm)
1	6.4	13.2	4.7	540	6.4	6.5	4.2	397	474	527
2	6.7	14.1	4.5	500	5.6	5.5	3.8	354	474	507
3	6.5	13.5	4.6	484	6.2	6.2	4.1	447	494	557
4	6.3	12.8	4.2	548	6.4	6.5	4.2	340	484	527
5	6.4	14.0	4.1	492	6.5	6.3	4.2	334	474	484
6	5.6	9.9	3.8	500	6.3	6.2	4.3	344	477	507
7	6.4	12.7	4.5	504	5.7	5.5	3.9	313	440	477
8	6.4	13.1	4.6	548	5.8	5.5	3.9	323	420	440
9	6.5	13.8	4.4	556	6.4	6.2	4.2	364	450	470
10	6.2	12.3	4.5	588	6.2	6.5	4.2	424	484	507
11	6.4	13.2	4.6	512	6.4	6.5	4.3	387	470	487
12	5.1	7.4	3.7	548	6.4	6.3	4.2	394	534	574
13	4.6	5.0	3.1	520	6.2	6.2	4.2	340	467	510
14	5.4	8.6	3.7	536	6.3	6.2	4.2	367	560	560
15	5.5	10.6	4.0	584	6.2	6.3	4.2	367	420	507
16	5.8	11.2	4.2	536	5.9	5.8	4.1	454	464	500
17	5.8	11.9	4.1	508	5.7	5.7	3.9	374	454	487
18	5.8	10.6	4.0	536	5.4	5.2	3.9	320	447	470
19	5.8	10.5	4.1	516	6.5	6.5	4.3	367	504	534
20	4.7	5.9	3.3	524	5.5	5.3	3.8	273	420	470
21	4.6	4.4	2.9	488	5.2	5.1	3.6	330	430	470
22	4.6	6.5	3.3	552	4.9	4.6	3.4	300	374	394
23	5.0	6.9	3.3	520	5.3	5.1	3.7	337	387	434
24	6.0	11.8	4.1	556	5.7	5.7	3.8	427	427	440
25	4.2	4.9	3.2	544	5.7	5.5	3.8	350	440	480
26	5.6	10.4	4.0	528	6.3	6.0	4.1	424	457	474
27	4.6	8.2	3.8	516	6.3	6.2	4.2	300	490	487
28	5.5	10.3	3.7	524	7.1	6.9	4.8	327	414	457
29	6.4	13.6	4.6	548	4.8	4.4	3.4	397	404	410
30	5.6	10.9	4.2	512	5.9	5.9	4.2	313	484	500
31	6.3	13.3	4.4	572	4.9	4.2	3.2	370	407	464
32	5.1	9.6	4.0	452	6.3	6.2	4.2	370	434	504
33	5.4	9.4	3.7	556	5.7	5.7	4.0	286	411	451
34					5.6	5.3	3.7	291	394	457
35					6.3	6.7	4.5	309	417	434
36					4.5	4.2	2.9	320	377	383

Table 2 (continued)

	Teleopsis spec. A males				*Teleopsis* spec. A females					
	Body length (mm)	Eye span (mm)	Wing length (mm)	Genital process length (μm)	Body length (mm)	Eye span (mm)	Wing length (mm)	Common spermathecal duct (μm)	Duct to single spermatheca (μm)	Duct to double spermatheca (μm)
37					4.8	4.6	3.3	320	366	394
38					4.4	4.1	3.0	326	354	389
39					6.1	6.0	3.9	326	434	446
40					5.5	5.7	3.9	326	434	463
41					5.4	5.4	3.9	326	474	514
42					5.7	5.7	3.9	337	429	491
43					5.9	5.9	4.2	343	451	469
44					5.6	5.8	3.9	343	383	429
45					5.8	5.9	3.8	343	434	491
46					4.7	4.3		366	366	400
47					4.5	4.2	3.2	371	411	411
48					5.4	5.4	3.9	383	400	451
49					6.5	6.4	4.3	389	469	503
50					6.4	6.2	4.1	389	417	486
51					5.9	5.8	4.0	406	463	491
52					6.2	6.3	4.1	423	423	474
53					6.4	6.5	4.1	434	469	474
54					6.6	6.6	4.5	457	434	497
n	33	33	33	33	54	54	53	54	54	54
mean	6	10	4	529	5.8	5.7	4.0	358	440	474
sd	1	3	0	30	0.6	0.7	0.4	45	42	43
cv%	12	28	12	6	11	13	10	12	10	9
Correlation with:										
Body length		r^2=0.89, P<0.001, b=2.46*	r^2=0.83, P<0.001, b=0.90*	r^2=0.01, P=0.650, b=0.04		r^2=0.96, P<0.001, b=1.19*	r^2=0.91, P<0.001, b=0.88*	r^2=0.09, P=0.028, b=0.33	r^2=0.46, P<0.001, b=0.58*	r^2=0.53, P<0.001, b=0.60*
Eye span			r^2=0.91, P<0.001, b=0.36*	r^2=0.01, P=0.594, b=0.02			r^2=0.93, P<0.001, b=0.73*	r^2=0.07, P=0.049, b=0.24	r^2=0.45, P<0.001, b=0.47*	r^2=0.52, P<0.001, b=0.49*
Wing length				r^2=0.01, P=0.584, b=0.04				r^2=0.06, P=0.075, b=0.30	r^2=0.40, P<0.001, b=0.59	r^2=0.48, P<0.001, b=0.62*
Common spermathecal duct									r^2=0.10, P=0.020, b=0.24*	r^2=0.12, P=0.011, b=0.26*
Duct to single spermatheca										r^2=0.79, P<0.001, b=0.92*

* Significant values

species (r^2=0.03, P=0.496, Fig. 4c) or its average male eye span (r^2=0.04, P=0.460, Fig. 4d).

C. nr *dalmanni* (Fig. 3a), C. *whitei* and *C. thaii* (Fig. 3b) share a comparatively short rod-shaped male genital process, while *T. freyi*, *T. trichophora* (Fig. 3k) and *Teleopsis* spec. A (Fig. 3l) have a long whip-shaped process. The remaining species have processes of intermediate length. Among these, *T. adjacens* (Fig. 3e) and *T. rubicunda* (Fig. 3h) have a small knob at the end of the process. In *Megalabops bigotii* (Fig. 3c) and *M. quadriguttata* (Fig. 3d), the process has a prominent, strongly sclerotised base. In *M. bigotii,* the tip consists of two filaments rather than one. Moreover, *T. adjacens* (Fig. 3e), *T. boettcheri*, *T. pallifacies* (Fig. 3f), *T. rubicunda* (Fig. 3h) and *T. sexguttata* share the presence of a second prominent process in a different, more ventral position.

In females of *C.* nr *dalmanni*, *C. thaii* and *C. whitei*, the separate spermathecal ducts open jointly into a pouch within the genital papilla (Fig. 1a), while they open separately into a wider pouch in *M. bigotii* and *M. quadriguttata*. In all *Teleopsis* species and in *Megalabops* spec. A, the separate spermathecal ducts open jointly into a common spermathecal duct of variable length (Fig. 1b). Based on its position it can be concluded that this structure evolved from the pouch within the genital papilla. The pouch and the common duct, therefore, constitute different states of the same character and the term "common spermathecal duct" is here applied to the entire range of it. The length of this structure varies greatly across the compared species (cv=68 %, n=17, Table 3, Fig. 4e). It is not correlated with the average body length of the species (r^2=0.00, P=0.887).

The lengths of the separate spermathecal ducts vary much less than that of the common spermathecal duct (duct to single spermatheca: cv=17 %, n=17; duct to double spermathecae cv=23 %, n=17, Table 3, Fig. 4f). Their correlation with the average body length of the species is weak to insignificant (duct to single spermatheca: r^2=0.25, P=0.043; duct to double spermathecae: r^2=0.11, P=0.192). They are not correlated with the length of the common spermathecal duct (duct to single spermatheca: r^2=0.01, P=0.774; duct to double spermathecae: r^2=0.07, P=0.292), but correlated significantly with each other (r^2=0.73, P<0001). In general, the duct leading to the double spermathecae is as long as or longer than that leading to the single one. In the three species of *Megalabops* the separate spermathecal ducts have a short, strongly convoluted portion just before they enter the base of the spermathecal capsules. This portion could not be measured exactly and was excluded from the measurements. It might, however, constitute a synapomorphy of the genus *Megalabops* and should be investigated further.

Interspecific correlation of male and female structures

The lengths of the male genital process and the female common spermathecal duct are correlated significantly across the species (r^2=0.69, P<0.001, Table 3, Fig. 4e). In all species the male genital process is longer than the common spermathecal duct (above the 1:1 line in Fig. 4e). The length of the male genital process and the lengths of the separate spermathecal ducts are not correlated (duct to single spermatheca: r^2=0.10, P=0.210, duct to double spermathecae: r^2=0.01, P=0.694, Fig. 4f).

Discussion

The lengths of the studied male external and female internal genitalia differ considerably across the 17 species studied. The length of the male genital process is correlated significantly with the length of the female common spermathecal duct, which it traverses during mating, but not with that of the separate spermathecal ducts, which it enters only basally. The main objective of this study was to test the hypothesis that the male genital process and the common spermathecal duct have coevolved and to gain insight into the underlying evolutionary mechanisms.

Because neither the average length of the male genital process nor that of the female common spermathecal duct is correlated with average body size or eye span across the species (Table 3), the correlation between the two structures cannot be explained as a secondary effect of general size differences. A pleiotropic effect involving the lengths of the male genital process and the female common spermathecal duct, but not other closely associated genitalic size characters such as the length of the separate spermathecal ducts, also seems unlikely. Unfortunately, an independent contrast analysis is impossible, because a fully resolved phylogenetic hypothesis for the studied taxa is not available. Moreover, due to unresolved deficiencies in diopsid taxonomy the congruence of the species *Cyrtodiopsis dalmanni*, *Teleopsis breviscopium*, and *Megalabops rubicunda*, in phylogenetic hypotheses of other authors (Földvári et al. 2007; Husak et al. 2011) with these species in our study is uncertain. Nevertheless, the available phylogenetic information is sufficient to rule out phylogenetic crowding. First, *T. quinqueguttata*, which has a moderately long male genital process and common spermathecal duct, is known to be sister to all other species in the present study (Földvári et al. 2007; Feijen 2011; Husak et al. 2011; *Tq* in Fig. 4e). Second, the generic concept of Feijen (2011) implies that *C. whitei*, *dalmanni* and *thaii*, all of which have short male genital processes and common spermathecal ducts, form one monophyletic clade among the remaining species (*Cw, Cd* and *Ct* in Fig. 4e). A second monophyletic clade is formed by *M. bigotii* and *quadriguttata* (*Mb* and *Mq* in Fig. 4e), both with short male

Table 3 Interspecific variation of body length, eye span, wing length and genitalic measurements in *Cyrtodiopsis*, *Megalabops* and *Teleopsis*. *n* number of observations, *sd* standard deviation, *SE* standard error, *cv* coefficient of variation. Log transformed correlation values: r^2 coefficient of determination, *P* probability of error, *b* allometric slope

	Male body length			Male eye span			Genital process length			Common spermathecal duct			Duct to single spermatheca			Duct to double spermathecae		
	(mm)	SE	*n*	(mm)	SE	*n*	(μm)	SE	*n*	(μm)	SE	*n*	(μm)	SE	*n*	(μm)	SE	*n*
C. nr *dalmanni*[a]	6.2[b]		93	7.5[b]		93	120	16	2	58	3	5	376	12	5	458	23	5
C. thaii	8.0[b]		20	11.2[b]		20	157	10	2	68	5	2	380	27	2	463	2	2
C. whitei	6.4[b]		86	8.0[b]		86	77[c]		1	52[c]		1	304[c]		1	442[c]		1
M. bigotii	5.1	0.0	87	3.4	0.0	87	304	9	2	77	5	2	429	21	2	554	18	2
M. quadriguttata	4.9	0.1	10	3.4	0.1	10	283	6	2	56	0	2	501	23	6	576	22	6
M. spec. A	4.9	0.5	2	3.3	0.3	2	450		1	267		1	520		1	713		1
T. adjacens	6.1	0.2	12	6.6	0.3	12	212	8	2	131		1	356		1	400		1
T. boettcheri	5.0[c]	0.1	15	4.2[2)]	0.1	15	138		1	101	15	2	343	5	2	346	24	2
T. ferruginea	5.8	0.1	19	5.0	0.2	19	385		1	249		1	340		1	425		1
T. freyi	5.4[c]	0.1	14	4.7[2)]	0.1	14	655		1	442	27	2	320	20	2	333	29	2
T. pallifacies	5.1	0.1	44	7.5	0.4	44	222		1	109		1	457		1	478		1
T. quinqueguttata	7.0[b]		102	4.3[b]		102	393	22	3	337	16	4	326	17	4	342	27	4
T. rubicunda	5.2	0.1	14	6.5	0.4	15	166		1	138		1	340		1	340		1
T. sexguttata	6.6	0.1	8	4.9	0.2	8	206	6	2	173		1	322		1	378		1
T. sykesii	6.8	0.1	58	8.3	0.3	58	290	2	2	232	4	2	360	12	2	404	4	2
T. trichophora	6.5	0.2	13	10.2	0.6	13	560		1	360		1	377		1	402		1
T. spec. A	5.6	0.1	53	10.0	0.4	53	539	4	12	388	11	12	469	7	12	497	9	12
n	17			17			17			17			17			17		
mean	5.7			6.4			303			191			384			444		
sd	0.9			2.6			169			129			67			100		
cv%	15			40			56			68			17			23		
Correlation with:																		
Male body length				r^2 0.35, P=0.012, b=1.64			r^2 0.03, P=0.496, b=−0.73			r^2 0.00, P=0.887, b=0.19			r^2 0.25, P=0.043, b=−0.57			r^2 =0.11, P=0.192, b=−0.48		
Male eye span							r^2 =0.04, P=0.460, b=−028			r^2 =0.00, P=0.955, b=0.03			r^2 =0.03, P=0.507, b=−0.07			r^2 =0.04, P=0.420, b=−0.11		
Genital process length										r^2 =0.69, P<0.001, b=1.02*			r^2 =0.10, P=0.210, b=0.09			r^2 =0.01, P=0.694, b=0.04		
Common spermathecal duct													r^2 =0.01, P=0.774, b=−0.02			r^2 =0.07, P=0.292, b=−0.08		
Duct to single spermatheca																r^2 =0.73, P<0.001, b=1.07*		

*Significant values

[a] *C.* = *Cyrtodiopsis*, *M.* = *Megalabops*, *T.* = *Teleopsis*

[b] Data from Földváry et al. (2007)

[c] Data from Feijen (2011)

[d] Data from Kotrba (1993)

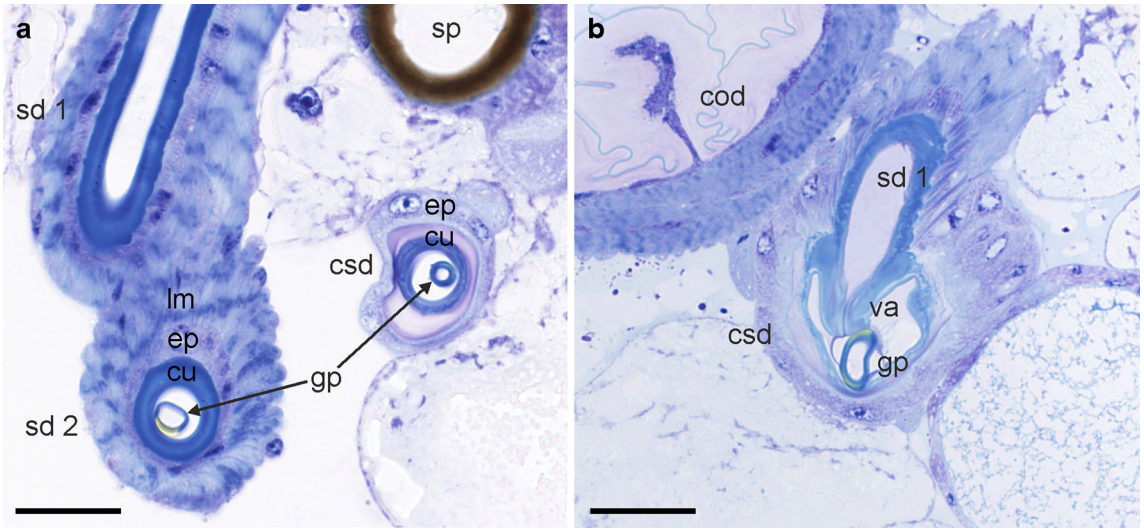

Fig. 2 **a**, **b** Spermathecal ducts with male genital process inserted during mating in *Teleopsis* spec. A. **a** Male genital process enters separate spermathecal duct. **b** Male genital process folded back upon itself next to separate spermathecal duct valve. *cod* Common oviduct, *csd* common spermathecal duct, *cu* cuticle, *ep* epithelium, *gp* male genital process, *lm* longitudinal musculature, *sd 1* and *2* separate spermathecal ducts to single and double spermatheca, *sp* spermatheca, va valve. Semithin sections, light microscopy. *Bar* 10 μm

genital processes and common spermathecal ducts, together with *Megalabops* spec. A which has a moderately long male genital process and common spermathecal duct. In the remaining *Teleopsis* species, the male genital processes and common spermathecal ducts range from short to very long. The observed correlation therefore cannot be traced back to a single evolutionary concurrence, but must be due to a number of correlated evolutionary events, i.e. coevolution.

Coevolution of long male genital structures and long spermathecal ducts has also been discovered in other insect taxa. In phlebotomine sand flies (Psychodidae) both structures can become extremely long, with the length of the aedeagal filaments generally exceeding that of the spermathecal ducts (Hertig 1949; Ilango and Lane 2000). Spermatophores are transferred through the hollow filaments directly into the spermathecae or their ducts. The authors suggest that intrasexual competition among the males occurs through sperm competition or displacement and that an evolutionary arms race between males and females occurs over the control of fertilisation (Ilango and Lane 2000).

Rodriguez et al. (2004) found a strong correlation between the length of the male genital process (flagellum) and that of the spermathecal duct across 56 species of chrysomelid beetles. The flagellum is inserted into the spermathecal duct and has to traverse several reversals in coiling direction as well as, in some species, an ampulla. In most of these species, the flagellum length exceeds the distance to the spermathecal capsule. In *Chelymorpha alternans,* it measures nearly four times the average spermathecal duct length and, at least sometimes, enters the spermatheca. Sperm is transferred by means of a spermatophore formed at the base of the flagellum, but also through the flagellum itself. The authors presume that especially long spermathecal ducts evolved to enable females to reduce the paternity of some males. In response, long genitalic flagella of males evolved in order to enable them to improve their chances of paternity when mating with females that possessed such ducts.

Gack and Peschke (2005) report the repeated convergent evolution of long flagella and long spermathecal ducts in alecharine beetles. In *Aleochara tristis* the flagellum traverses most of the spermathecal duct, but does not enter the spermatheca. Sperm is not transferred through the flagellum. Instead, the flagellum serves as guiding structure for a tube growing out of the spermatophore, which is produced at the base of the process. The authors suggest that greater flagellum length enables males to position their sperm closer to the site of storage than other males, and that such benefits might have contributed to the evolution of extreme length.

The cited findings of convergent evolution of long male genital processes and long spermathecal ducts are superficially similar to, but differ in detail from, the findings in Diopsidae. The present study provides detailed results on the morphology of the structures involved and their interaction during mating, as well as their inter- and intra-specific variation, which allows some educated speculation on the underlying evolutionary mechanisms.

In *C. whitei*, the male genital process enters, and possibly serves to pry open, the entrance of the female spermathecal ducts (Kotrba 1993). Spermatozoa and accessory secretions are not transferred through this process but discharged from the male gonopore near its base, forming a spermatophore within the vagina. The male genital process helps to mould the neck of the spermatophore through which sperm is transferred into the spermathecal ducts. In *Teleopsis* spec. A, the male

Fig. 3 **a**–**l** Dorsal portion of aedeagus in lateral view. Species in alphabetic order. **a** *C.* nr *dalmanni*, **b** *C. thaii*, **c** *M. bigotii*, **d** *M. quadriguttata*, **e** *T. adjacens*, **f** *T. ferruginea*, **g** *T. pallifacies*, **h** *T. rubicunda*, **i** *T. sykesii*, **k** *T. trichophora*, **l** *T.* spec. A. *Arrows* Male genital process. Genitalic dissections, light microscopy. All figures to same scale. *Bar* 100 μm

genital process traverses the female common spermathecal duct and enters the base of the separate spermathecal ducts, likely serving a similar purpose. Reconstruction from serial sections ascertains that the cavity of the male genital process has no external opening anywhere along its length and no internal connection with the ejaculatory duct. Instead, as in *C. whitei*, the ejaculatory duct opens into a pouch next to the base of the male genital process and sperm is transferred by means

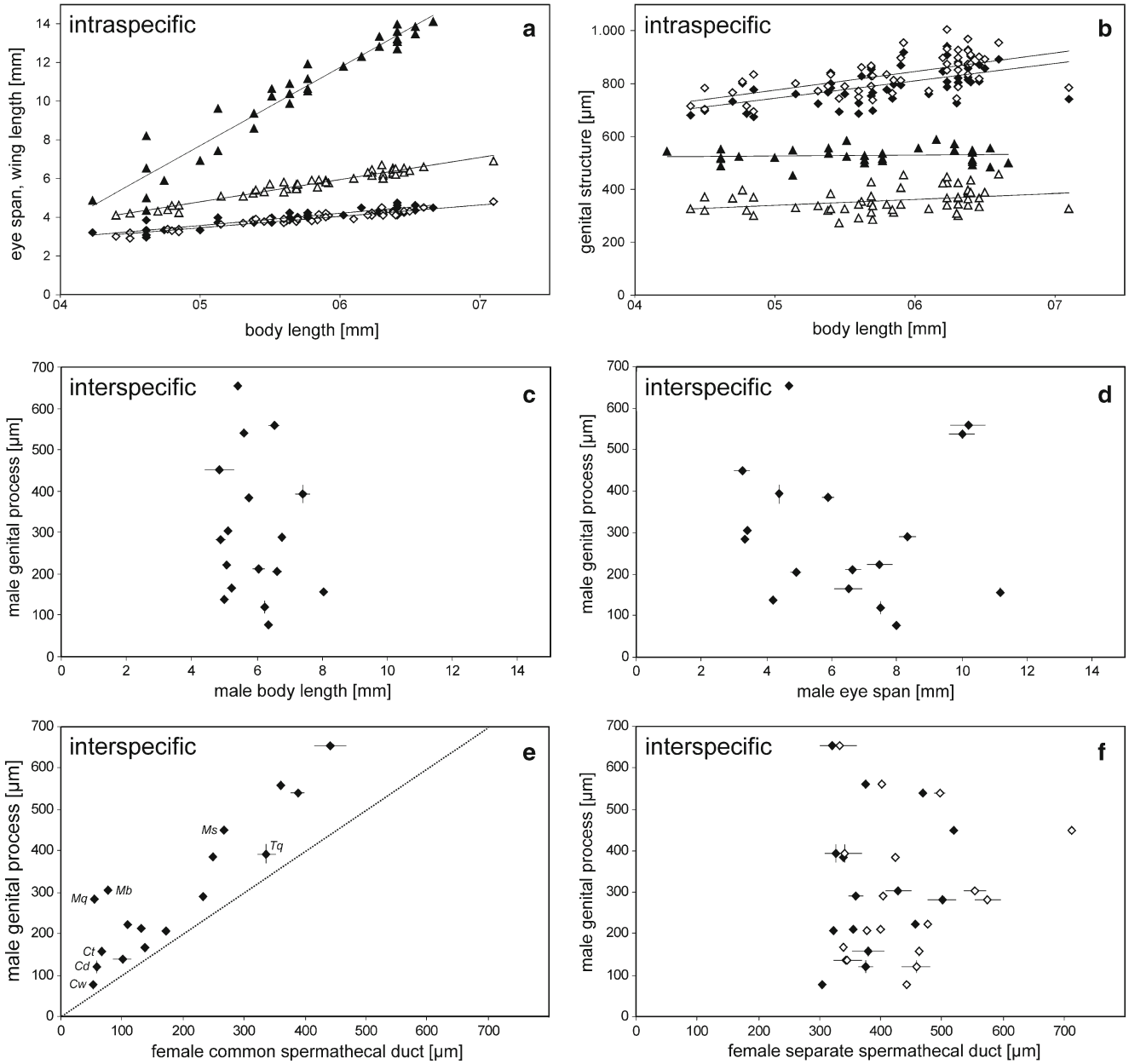

Fig. 4 **a**–**f** Intra- and inter-specific variation, allometry and correlation of male and female genital structures. *Bars* indicate ± SE where available. **a** Intraspecific correlation of male and female eye span and wing length with body length in *Teleopsis* spec. A. *Filled triangles* Male eye span, *empty triangles* female eye span, *filled diamonds* male wing length, *empty diamonds* female wing length. **b** Intraspecific correlation of male and female genital structures with body length in *Teleopsis* spec. A. *Filled triangles* Male genital process, *empty triangles* female common spermathecal duct, *filled diamonds* female common and separate spermathecal duct leading to single spermatheca combined, *empty diamonds* female common and separate spermathecal duct leading to double spermathecae combined. **c**–**e** Interspecific correlation of male genital process length with average male body length (**c**), average male eye span (**d**) and female common spermathecal duct length (**e**). *Cd C.* nr *dalmanni*, *Cw C. whitei*, *Ct C. thaii*, *Mb M. bigotii*, *Mq M. quadriguttata*, *Ms Megalabops* spec. A, *Ta T. adjacens, Tq T.quinqueguttata, Ts T. sexguttata*, all others *Teleopsis. Dotted line* 1:1 relation. **f** Interspecific correlation of male genital process length with female separate spermathecal duct length. *Filled diamonds* Female separate spermathecal duct leading to single spermatheca; *empty diamonds* female separate spermathecal duct leading to double spermatheca

of a spermatophore. Because it can be excluded that spermatozoa and/or any accessory secretions are transferred through the male genital process directly into the spermathecal ducts, the function of the male genital process must be strictly mechanical in these and, in all likelihood, all of the studied species.

Passing the genital process through the valves at the base of the separate spermathecal ducts very likely constitutes a mechanical prerequisite for successful insemination. The length of the male genital process and the length of the common spermathecal duct, which it

needs to traverse for that purpose, are thus functionally related.

In two of the pairs fixed during mating, the male genital process was inserted in the common spermathecal duct with its apical portion folded back upon itself. This suggests that the male genital process is inserted with a kink first, similar to the way a seamstress inserts a thread into a needle's eye. Doubling up the thread serves to stabilize the leading end, which might otherwise be deformed or deflexed instead of entering the narrow hole. Similarly, doubling up the male genital process might help to insert it into the valves at the entrance of the separate spermathecal ducts.The uneven thickness and sclerotization of the male genital process wall in cross section probably causes it to fold primarily in certain regions and in a certain direction. Doubling up the tip of the process requires some extra length. Indeed the male genital process is distinctly longer than the common spermathecal duct in all species studied (above the 1:1 line in Fig. 4e), and this extra length might constitute a prerequisite for inserting the process into the separate spermathecal ducts.

In the other two pairs, apparently fixed at a later stage, the process was found inserted into the base of a separate spermathecal duct with its tip stretched out straight. The separate spermathecal ducts are lined by thick cuticle with a smooth internal surface uniform along their length. No cuticular mechano-receptive structures were detected by light microscopy and it appears unlikely that the thick cuticle allows mechanic stimulation of the underlying tissue. Therefore there is no evidence that it could make a difference exactly how deeply the tip is inserted, once it has passed the valve at the entrance, and as long as it does not reach near the spermathecal capsules, where it could mechanically interact with sperm deposited by previous matings. Neither in *Teleopsis* spec. A (Fig. 4b), nor in any of the other species, does the male genital process reach the length of the common and separate spermathecal ducts combined.

Because direct mechanical or chemical interaction of the male genital process with sperm deposited by other males is impossible, it can be ruled out that direct intrasexual competition among males drives the evolution of long male genital processes in these species. Moreover, male competition alone would not explain a correlated evolutionary response in the female morphology. Instead, the observed coevolution must be driven by some kind of intersexual selection, i.e. a lock and key mechanism, sexually antagonistic coevolution, or cryptic female choice.

Lock and key

If inserting the male genital process into the separate spermathecal ducts constitutes a necessary prerequisite for successful insemination, then the length of the common spermathecal duct together with the valves at the entrance of the separate spermathecal ducts constitutes a potent selective device, excluding all males with genital processes shorter than required. In the event of sympatric species with an existing risk of interspecific matings, any pre-existing, species specific difference in average male genital process length could be utilized by females of the species with the longer process to reduce the risk of interspecific insemination by excluding males with shorter processes, e.g. by the suggested selective process. Intraspecifically this might result in selection for males with longer processes and thus an increase in the length difference between the two species. However, such a selective process would continue only until the difference was large enough to exclude hybridization and then stabilize the difference as long as the risk of hybridization persists.

The sympatric occurrence of species is best documented for the locality of Ulu Gombak, Malaysia. All *Cyrtodiopsis*, *Megalabops* and *Teleopsis* species known to coexist in this locality were included in the present study (*C. whitei*, *C. dalmanni*, *M. quadriguttata*, *T. adjacens*, *T. quinqueguttata* and *T. sexguttata*). Among these, *T. quinqueguttata* has the longest common spermathecal duct (337 μm, Table 3, *Tq* in Fig. 4e). Only males of the same species have genital processes long enough (393 μm) to exceed this length. Out of all other sympatric species, the males of *M. quadriguttata* come closest to matching this length requirement. But their genital process (283 μm) is already 110 μm (28 %) shorter than that of *T. quinqueguttata* males and not nearly long enough to traverse the common spermathecal duct of that species, let alone enter the separate spermathecal ducts. The length of the common spermathecal duct in *T. quinqueguttata* therefore considerably exceeds what would be necessary to exclude hybridization by sympatric species. It cannot be explained by a lock and key mechanism.

Sexually antagonistic coevolution

Males with longer genital processes might be more successful at manipulating the insemination success and/or allocating their sperm to a more advantageous storage site within the female, e.g. the single or double spermatheca. Alternatively, males with longer genital processes might be more successful at manipulating females, e.g. to emit sperm from their spermathecae, resulting in indirect sperm removal. Although there is no morphological evidence, such processes cannot be ruled out and, apart from intrasexual selection (see above), could result in some reproductive disadvantage to the female. Females with a longer common spermathecal duct may in turn regain some control over such processes. However, a merely larger distance males need to traverse will not reduce manipulation in general, but selectively reduce the range of males by which these females are manipulated and inseminated. In fact it will primarily exclude less manipulative males with shorter genital processes to the advantage of males with long

processes. Such "selective female cooperation" would arguably fall into the category of cryptic female choice rather than sexual antagonism (Cordero and Eberhard 2003; Eberhard 2010).

Cryptic female choice

In *C. whitei* and *C.* nr *dalmanni*, females exert precopulatory choice preferring males with longer eye stalks (Burkhardt and de la Motte 1985, 1988; Wilkinson and Dodson 1997; Cotton et al. 2010). Male eye span is an indicator of male quality in terms of the absence of a detrimental meiotic drive gene (Wilkinson et al. 1998), the size of male testes and accessory glands and male fertility (Rogers et al. 2008; Cotton et al. 2010), as well as male general phenotypic and/or genetic quality (Burkhardt et al. 1994; David et al. 2000; Cotton et al. 2010; Pomiankowski and Bellamy 2011). However, male eye span is also correlated tightly with general body size parameters such as body and wing length, and, together with these, highly condition-dependent, i.e. sensitive to environmental stress during larval development (David et al. 2000; Cotton et al. 2010 and references therein). So females exert precopulatory female choice based on a size character of only limited reliability regarding male genetic qualities.

It is suggested here that females might also exert cryptic (internal) female choice based on the length of the male genital process. This character shows no correlation with male body length (Table 2, Fig. 4b), wing length or eye span in *Teleopsis* spec. A. Conclusively, the length of the male genital process is not condition-dependent. Instead, it might constitute an independent indicator of male superiority, either with respect to other favourable life history traits ("good genes") or in terms of adaptation to said female choice itself.

Provided that the length of the male genital process is controlled genetically, females with short common spermathecal ducts that allow insemination by males with short genital processes may invest their resources into sons with likewise short genital processes unable to successfully inseminate the full range of their prospective mates. Because the female common spermathecal duct length is correlated with body size, these sons would particularly fail to inseminate the larger and potentially fitter females of the population. Conversely, females with longer common spermathecal ducts, will specifically exclude males with short genital processes. Because they are promiscuous, such females will not remain uninseminated. Instead, by allowing insemination by males with long genital processes only, they may allocate their resources to sons with likewise long genital processes. These "sexy sons" will be able to inseminate the entire size range of prospective mates, irrespective of their own body size, because the male genital process length is not condition-dependent.

Elimination of males with shorter genital processes probably causes a selective pressure on minimizing the condition-dependence of this trait and ontogenetically developing long processes also in phenotypically small males, as observed in *T.* spec. A. Consequently, continued female choice will predominantly address the remaining condition-independent, i.e. genetic, variability of the trait, resulting in directional selection. This scenario is consistent with the prediction that sexual selection sometimes increases the allometric intercept without affecting the slope (Bonduriansky 2007). Low intraspecific variability and low static allometry are characteristic in insect male genitalia due to stabilizing selection (Eberhard et al. 1998; Eberhard 2009; Bertin and Fairbairn 2007; Bonduriansky 2007). According to the "one size fits all" scenario (Eberhard et al. 1998), stabilizing selection favours male genitalia of intermediate size, which are able to suit most females. The scenario suggested here for *Teleopsis* spec. A could be termed "the longest fits all" because the longest male genital processes will allow to suit even the largest females of the population without the disadvantage of losing out on the smallest ones.

Conclusion

The presented results confirm that the male genital process and the female common spermathecal duct have coevolved in the studied taxa. The suggested mechanism of directional sexual selection by cryptic female choice constitutes a possible and parsimonious explanation. Alternative or additional explanations in terms of sexually antagonistic coevolution cannot be ruled out conclusively, but are not supported by the available evidence.

We strongly encourage further research on this fascinating clade of stalk-eyed flies. In particular, we would like to stress the need for a well resolved phylogeny within the family and for more extensive intraspecific studies in additional species, also taking into account their sympatric or allopatric occurrence. With new methods such as microtomography it will become easier to investigate the functional interaction of male and female genitalia in detail. We hope for continued progress in our understanding of male and female strategies, their potential conflicts, and the evolutionary processes involved.

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