

Genital shape correlates with sperm transfer success in the praying mantis *Ciulfina klassi* (Insecta: Mantodea)

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Abstract The rapid divergence of male genitalia is a widely observed evolutionary phenomenon. Although sexual selection is currently regarded as providing the most likely driving force behind genital diversification, the mechanisms responsible are still debated. Here, we investigate the relationship between male genital morphology and sperm transfer in the praying mantid *Ciulfina klassi* using geometric morphometrics. The shape of male genitalia in *C. klassi* influenced sperm transfer duration and the number and proportion of sperm transferred, suggesting that genital morphology is under sexual selection in this species. Genital size however was not correlated with any aspect of sperm transfer. Intriguingly, two of the major genital shape components correlated positively with the number of sperm transferred, but negatively with sperm transfer duration. Hence, males that most effectively transfer sperm to the female spermatheca do so in a relatively short period of time. A direct negative relationship was also found between sperm transfer duration and sperm transfer success. Overall, our study suggests that the

variable genital shape of *Ciulfina* may have been selected for more efficient sperm transfer.

Keywords Genital evolution · Sexual selection · Copulation duration

Introduction

The morphology of male genitalia can be elaborate and diverse, even among closely related species. This is one of the most widely observed evolutionary patterns for animals with internal fertilisation and promiscuous mating systems (Arnqvist 1997, 1998). Three general hypotheses to explain the rapid divergent evolution of genitalia have been debated: the species isolation, pleiotropy and sexual selection hypotheses (Eberhard 1985). The species isolation or ‘lock and key’ hypothesis proposes that genital traits are under divergent selection due to their role in preventing costly heterospecific matings (Shapiro and Porter 1989), while the pleiotropy hypothesis suggests that genital traits are selectively neutral and diverge due to genetic linkage with correlated non-genital traits (Mayr 1963). Today, the general consensus is that sexual selection is responsible for the pattern of rapid genital evolution (Arnqvist 1998; Eberhard 1985; Hosken and Stockley 2004; House and Simmons 2003), although some support for both the species isolation (Sota and Kubota 1998; Usami et al. 2006) and pleiotropy hypotheses (Arnqvist et al. 1997; Arnqvist and Thornhill 1998) exists for some species.

The mechanisms involved in sexual selection on genital traits, however, are unclear (Hosken and Stockley 2004). Among the possible mechanisms are (1) male competition, including sperm competition (Parker 1970; Simmons 2001; Waage 1979) and adaptations to avoid displacement during

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mating, (2) female choice, either through Fisherian or good genes models of selection (Eberhard 1993), and (3) sexual conflict generated by opposing selection on male and female reproductive strategies (Arnqvist and Rowe 2005; Crudgington and Siva-Jothy 2000; Rönn et al. 2007; Kuntner et al. 2009). Distinguishing between these potential mechanisms is difficult (Hosken and Stockley 2004), reflecting current difficulties in distinguishing between mechanisms of sexual selection in a range of contexts. Recent models (Gavrilets 2000) and comparative data accounting for phylogeny (Arnqvist et al. 2000) are consistent with higher rates of speciation driven by sexual conflict. Conversely, some large-scale comparative surveys (Eberhard 2004a, b, 2006) dismiss sexual conflict as a general driver of genital diversification, supporting models of female choice.

One possible approach to understanding the mechanisms involved in genital evolution is to establish how male genitalia can influence sperm transfer. Although the transfer of sperm is assumed to be the major function of male genitalia, data linking variation in genital shape and size to sperm transfer success are rare. How genital structure relates to sperm transfer is particularly intriguing in animal groups with very complex genitalia comprising multiple structural elements. Identifying components of genital structure that influence the number of sperm eventually being stored in the female sperm storage organ is essential to understanding the evolution of these traits and how sexual selection acts upon them.

Praying mantids in the genus *Ciulfina* are small, fast-moving mantids found on the bark of trees in northern Australia. Males stealthily approach females, mount swiftly and insert their genital complex immediately. During copulation, they insert a small, simple spermatophore into the female genital opening. Part of the spermatophore, which is roughly spherical with no obvious projections, protrudes visibly from the female, who later removes the spermatophore with her mouthparts and consumes it. Sperm transfer starts within 5 min of the onset of copulation in *C. klassi*, during which the majority of sperm is transferred (unpublished data). Copulation duration in *Ciulfina* is variable among and within species and can potentially influence male reproductive success by (a) influencing the number of sperm transferred, (b) increasing copulatory mate-guarding and reducing the opportunity for other males to mate with the female or (c) increasing the duration of copulatory courtship, which may influence subsequent female behaviour (e.g. propensity to remate—Dickinson 1986; Holwell 2008a). Laboratory observations and molecular estimates of natural paternity indicate that both male and female *C. klassi* will mate multiply (Holwell 2007; Umbers 2006), suggesting that sperm competition is likely to be important in their mating

system. Male adaptations to reduce sperm competition or influence the number of sperm transferred to a female are likely to be under strong selection in such polyandrous species (Simmons 2001).

In *Ciulfina* praying mantids, the asymmetric male genitalia are twisted 90° and inserted so as to splay open the female genital opening, separating her supra-anal plate, ovipositor complex and sub-genital plate. While a detailed investigation of the functional morphology of genitalia in *C. klassi* is not complete, preliminary observations suggest that the positioning of the three major genital structures in males follows the same pattern as that observed for the congeneric *Ciulfina baldersoni*. The male genitalia of praying mantids consist of a right phallomere and a left complex comprising dorsal and ventral components (Klass 1997; for detail in *Ciulfina*, see Holwell et al. 2007). In *C. baldersoni*, the ventral left complex fits laterally into the female genital opening and splays apart the female dorsal and ventral structures. The right phallomere grips onto the female ovipositor and its major lobe is partially inserted into the female reproductive tract. The dorsal left complex is inserted deeper into the female reproductive tract along with the spermatophore (Evans and Holwell, unpublished). The positioning of these structures suggests that each may have an influence over copulation duration, spermatophore attachment or sperm transfer success (Fig. 1).

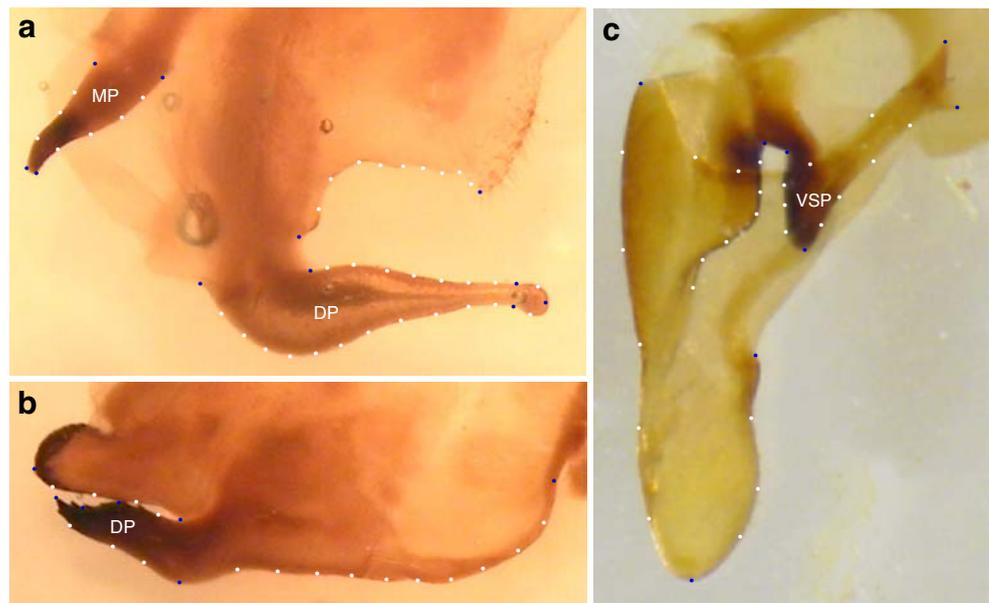
We investigate the relationship between the duration of sperm transfer and the number of sperm transferred to the female spermatheca in *C. klassi*. We use geometric morphometric measures of variation in the shape and size of the three male genital components for *C. klassi* to test the hypothesis that male genital morphological traits influence sperm transfer duration and the number of sperm transferred to the female spermatheca.

Methods

Collection and housing

Populations of *C. klassi* were identified and collected in January 2005. *C. klassi* (35 male, 35 female) were collected from eucalypt woodland in Giringun National Park, approximately 30 km south of Cardwell (Queensland, Australia, 18°31'S 146°11'E). All mantids were collected as sub-adult nymphs and housed individually in the laboratory in inverted plastic cups (10 cm diameter) containing bark to perch on and a fabric window to allow airflow. They were fed *Drosophila melanogaster* three times per week and watered daily. Mantids were collected under permit ATH 05/006 from the Queensland Parks and Wildlife Service and the Environmental Protection Authority.

Fig. 1 Position of fixed landmarks (*blue*) and sliding semi-landmarks (*white*) for the dorsal left complex (**a**), ventral left complex (**b**) and right phallomere (**c**). Abbreviated labels refer to the distal processes (*DP*), medial process (*MP*) and the ventral sclerotised process (*VSP*) of the respective structures



Mating observations

Observations of mating behaviour were made in a semi-natural laboratory environment. The mating arena consisted of a section of tree trunk (30 cm diameter, 60 cm length), sawn in half and screwed onto a Perspex® board. This prevented mantids from moving out of the observer's range of view, as mantids show strong fidelity to the tree trunk once introduced and rarely leave it. Adult virgin males and females (between 2 and 4 weeks after final eclosion) were weighed (to 0.01 mg) and prothorax length was measured (to 0.1 mm) using electronic callipers prior to observations. Females were introduced to the tree trunk 10 min before males to allow them to adjust to the novel environment. Mantids were observed throughout and copulation duration and attachment duration of the spermatophore prior to female removal were recorded using a stopwatch. Copulation duration was defined as the interval between genitalic contact between the pair and the point when the male removed his genitalia from the female genital opening and dismounted. Postcopulatory spermatophore attachment duration was defined as the interval between the end of copulation and removal of the spermatophore from her genital opening by the female. All pairs that were placed together as part of this study successfully mated. For a detailed description of the mating behavioural repertoire in *Ciulfina*, see Holwell (2007).

Sperm counting

Following copulation and the period of postcopulatory spermatophore attachment, females were anaesthetised with carbon dioxide at the moment they attempted to remove the

spermatophore. The spermatophore was removed from the female with a paintbrush and placed in a 0.5-ml microcentrifuge tube containing 20 μ l of 0.5% Triton® X-100 (Sigma Aldrich Pty Ltd, Castle Hill, Australia) in distilled water. The female was then weighed again without the spermatophore.

Under a stereomicroscope (Olympus SZ-40), the anaesthetised female was decapitated posterior to the pronotum, which was stored in 70% ethanol. The pronotum was subsequently measured with electronic digital callipers. The male was anaesthetised, and the pronotum was similarly measured. In distilled water, the spermatheca was severed at the spermathecal duct by a pair of fine entomological scissors and placed into a 0.5-ml microcentrifuge tube containing 20 μ l of 0.5% Triton® X-100 in distilled water. The spermatheca was broken apart using fine entomological scissors and an entomological pin. The contents were macerated in the tube for 5 min and the tube was vortexed for 3 min. Using a pipette, the contents of the spermatheca were placed onto two slides, and each was covered by an 18 \times 18-mm coverslip. The coverslip was glued down in each corner using a drop of nail polish. The slides were left to dry overnight, and the sperm were counted under a phase contrast microscope (Olympus BH-2) at a magnification of \times 200. The slides were systematically viewed to ensure all sperm were counted. The same procedure was followed to assess the sperm in the spermatophore.

Preparation of genitalia and geometric morphometrics

After copulation, males were killed by freezing and genitalia were dissected out and submerged in 10% KOH for 1 h to dissolve the muscle and fat bodies. This method

of preparation has been successfully applied to these structures in previous studies (Holwell et al. 2007, 2008b).

The right phallomere and the dorsal and ventral components of the left complex were separately mounted in one drop of glycerol between a glass slide and cover slip. While some minor variation due to differences in angle of view will undoubtedly remain, attempts were made to position each genital structure in a similar way so as to reduce this variation. Digital images were taken of each genital structure using a Moticam 480® microscope-mounted camera. These images were then imported into the tpsDig2 (Rohlf 2004) digitisation program.

Landmark-based geometric morphometric analysis was used to quantify the variation in genital shape for the right phallomere and both the dorsal and ventral left complex of *C. klassi* (Adams et al. 2004). Compared to traditional morphometrics which analyse variation in pre-determined linear measurements of morphological structures, landmark-based geometric morphometrics assigns landmarks to recognisably equivalent points. Analyses of total morphological variation between samples are then based on the relative positions of assigned landmarks, after the images are normalised for position, orientation and scale. For this study, a combination of both fixed landmarks and sliding semilandmarks were used. This approach combined landmark- and outline-based methods allowing a comprehensive analysis of both homologous points and the shape of curved outlines between these points. The position of seven fixed landmarks and 17 sliding semilandmarks were recorded onto the dorsal view of the ventral left complex (Fig. 1). The position of 11 fixed landmarks and 35 sliding semilandmarks were recorded onto a dorsal view of the dorsal left complex (Fig. 1). The position of 12 fixed landmarks and 21 sliding semilandmarks were recorded onto the dorsal view of the right phallomere (Fig. 1). Fixed landmarks were essentially type-two landmarks, defined as extremes of curvature around the shape of these genital structures. Landmarks were superimposed onto images using the program tpsDig2 (Rohlf 2004). Variations in the position of the *X–Y* coordinates of landmarks for all samples of the right phallomere and both the dorsal and ventral left complex were analysed using the program tpsRelw (Rohlf 1997). This program normalised all specimens for position, orientation and scale so as to remove ‘non-shape variation’ (Adams et al. 2004) and reduced the data to a series of relative warp scores (RWS) using a principal components analysis. tpsRelw also allowed us to visualise variation in the shape of each genital component as shape deformations of the thin plate spline (Zelditch et al. 2004) so as to compare the genitalia of males that varied in their copulation duration or capacity for sperm transfer. Centroid size, which is the square root of the sum of squared distances of landmarks from the centroid, was calculated using tpsRelw (Rohlf

1997) for the right phallomere and both the dorsal and ventral left complex and was used as a measure of genital size in further analyses. A detailed mathematical explanation of geometric morphometrics, along with general concepts and methods, can be found in Adams et al. (2004), Rohlf and Marcus (1993) and Zelditch et al. (2004).

Statistical analyses

Data were checked for normal distribution and homogeneity of variance prior to analyses. Pearson's correlation tests were used to determine if any of the relative warp scores used for each genital structure were correlated to the centroid size of that structure. The absence of any significant correlations between these variables indicated that all relative warp scores were independent of genital size, as predicted (Zelditch et al. 2004). We used multilinear forward regression models to analyse the effects of male mass, male prothorax length, right phallomere morphology (centroid size and RWS 1–5), ventral left complex morphology (centroid size and RWS 1–5) and dorsal left complex morphology (centroid size and RWS 1–5) on (a) copulation duration, (b) duration of spermatophore attachment, (c) the combined duration of the two (representing total potential sperm transfer duration), (d) the total number of sperm transferred to the female spermatheca and (e) the proportion of sperm transferred from the spermatophore to the spermatheca for *C. klassi*. Conservative Bonferroni adjustment (to adjust for probability of type 1 errors through having conducted five tests) produced a critical significance value of 0.01, which was used when ascribing significance for these analyses. The percentage of overall shape variation explained by each relative warp score for each genital component was determined by relative warp analysis using the program tpsRelw (Rohlf 1997) and is presented in Table 1. Individual linear regressions were performed to investigate the relationship between sperm transfer and (a) copulation duration, (b) duration of spermatophore attachment and (c) the combined duration. Bonferroni adjustments produced a critical significance value of 0.0167, which was used when ascribing significance for these analyses. These analyses were conducted using SPSS 11 for Mac OS X.

Results

Genital morphology and sperm transfer

For the observed matings, *C. klassi* copulated for $2,414 \pm 1,703$ s (mean \pm SD), the spermatophore remained attached for $2,816 \pm 1,643$ s and the combined duration of potential

Table 1 Percentage of shape variation explained by relative warp scores 1–5 for each genital structure

Genital structure	RWS1	RWS2	RWS3	RWS4	RWS5	Total
Right phallomere	43.93%	28.20%	13.08%	6.48%	2.27%	93.96%
Dorsal left complex	57.32%	20.01%	6.38%	4.61%	3.67%	92.00%
Ventral left complex	53.68%	20.35%	9.83%	5.35%	4.20%	93.40%

sperm transfer was $5,230 \pm 2,026$ seconds ($n=15$). The number of sperm successfully transferred from the spermatophore to the spermatheca was $1,312 \pm 781$. While the number of sperm transferred to the spermatheca was not significantly influenced by copulation duration ($R^2=0.201$; CI (95%)= $-0.124-0.526$; $F=3.025$; $t=-1.739$; $df=14$; $p=0.108$) or postcopulatory spermatophore attachment duration ($R^2=0.131$; CI (95%)= $-0.155-0.417$; $F=1.806$; $t=-1.344$; $df=14$; $p=0.204$), it was significantly associated with the combined duration of copulation and postcopulatory spermatophore attachment ($R^2=0.449$; CI (95%)= $0.113-0.784$; $F=9.796$; $t=-3.130$; $df=14$; $p=0.009$). Copulation duration was not significantly associated with postcopulatory spermatophore attachment duration ($R^2=0.071$; CI (95%)= $-0.154-0.296$; $F=0.922$; $t=-0.960$; $df=14$; $p=0.356$).

A number of significant regression models were generated from the analyses of genital shape variation and its influence on sperm transfer. Components of genital shape

were found to influence copulation duration (dorsal left complex=RWS1), total duration of potential sperm transfer (dorsal left complex=RWS1 and 3; right phallomere=RWS5) (Figs. 2 and 3) and the total number of sperm transferred to the spermatheca (dorsal left complex=RWS1; right phallomere=RWS1 and 5; Figs. 2 and 3). The outputs of these analyses are presented in Table 2.

We employed the program tpsRelw (Rohlf 1997) to visualise variation in genital shape for the range of warp score values significantly influencing aspects of sperm transfer. Deformations of the thin plate spline for each relative warp score that significantly related to the variables measured are shown in Figs. 2 and 3. The mean consensus genital shape along with the minimum and maximum values for the individuals used in this study show the variation in genital shape as it relates to the number of sperm transferred and total sperm transfer duration (Figs. 2 and 3). Shape components of both the dorsal left complex

Fig. 2 The influence of dorsal left complex (DLC) shape (relative warp score 1) on sperm transfer (blue squares) and total sperm transfer duration (red circles). Thin plate spline (TPS) diagrams represent a visualisation of the mean DLC shape and the shape of DLC from males with low and high values of RWS1

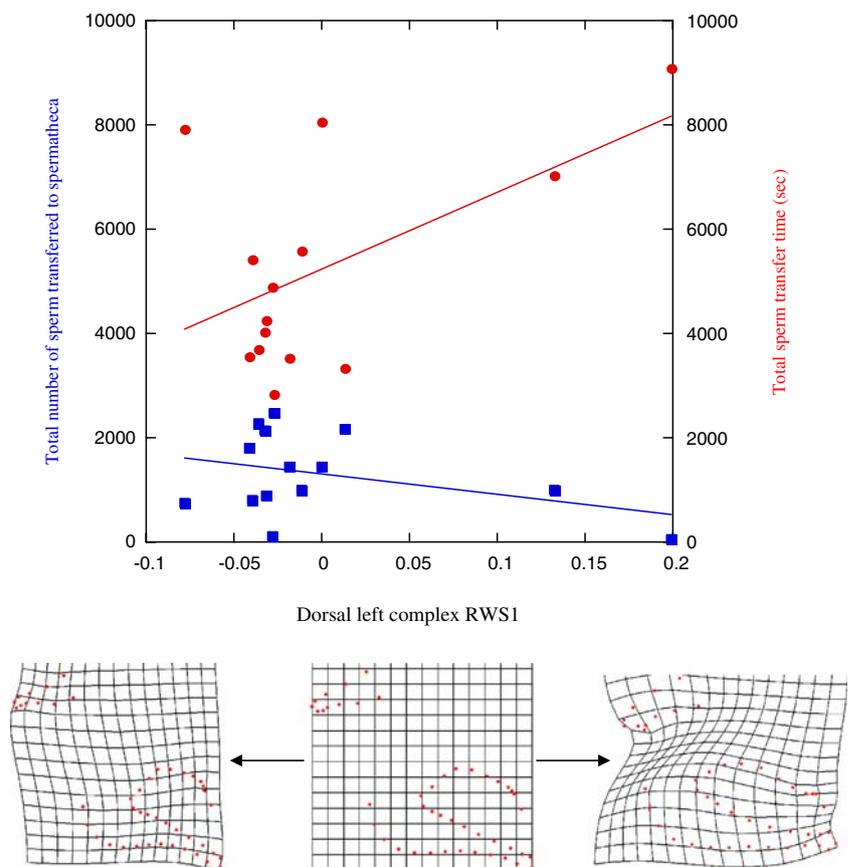
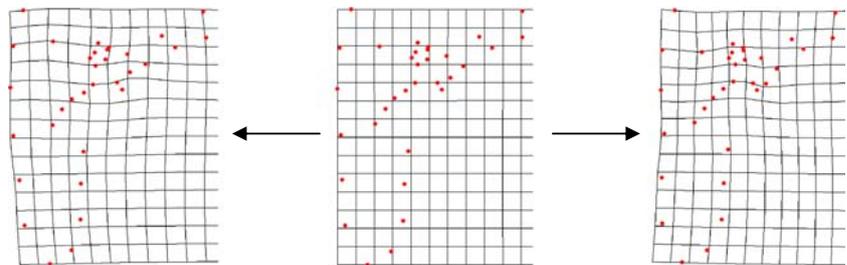
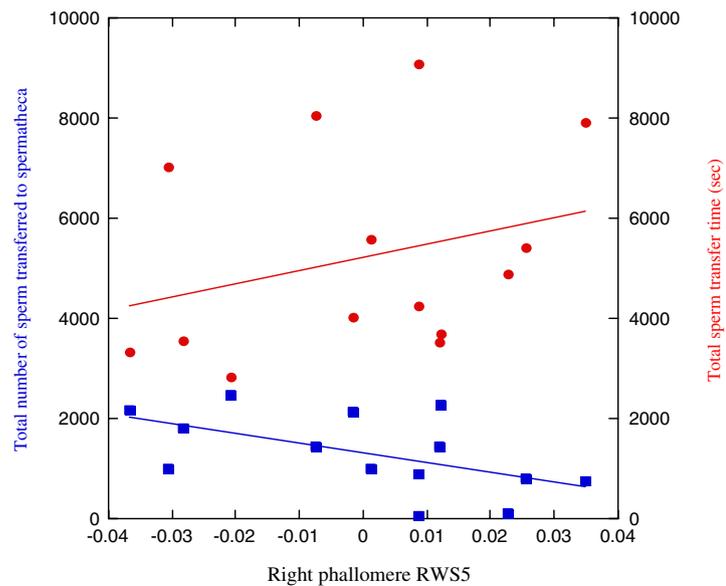


Fig. 3 The influence of right phallomere shape (relative warp score 5) on sperm transfer (blue squares) and total sperm transfer duration (red circles). Thin plate spline (TPS) diagrams represent a visualisation of the mean right phallomere shape and the shape of right phallomeres from males with low and high values of RWS1



(RWS1) and the right phallomere (RWS1 and 5) influenced the number of sperm transferred to the female spermatheca. Generally, for the dorsal left complex, the morphological variation relating to sperm transfer occurred in the relative position of the distal and medial processes and in the curve of the distal process (Figs. 1 and 2). For the right phallomere, the shape variation relating to the number of sperm transferred occurs in the position of the ventral sclerotised process (Figs. 1 and 3).

Discussion

Genital morphology, sperm transfer success and duration

Our results show a direct correlation between male genital morphology and the number of sperm transferred to the female spermatheca in *C. klassi*. The shape, but not size, of two components of the male genital apparatus correlated with both sperm transfer success and the duration of sperm transfer. Although genital shape is generally assumed to influence sperm transfer, rarely have elements of genital shape been shown to directly correlate to the number of sperm reaching the sperm storage organs of females. We have also demonstrated that sperm transfer duration is

negatively related to sperm transfer success for *C. klassi*. Parker and Simmons (2000) found a similar relationship between the diameter of the aedeagal duct, sperm displacement and copula duration in the yellow dung fly *Scatophaga stercoraria*. In male *S. stercoraria* that possessed wider aedeagi, sperm displacement was more successful and was achieved more quickly. Similarly, for *C. klassi*, the shape of genitalia that corresponded with the highest sperm transfer success also corresponded with the shortest sperm transfer duration. In fact, two unrelated genital shape components (dorsal left complex RWS1 and right phallomere RWS5) correlated positively with sperm transfer duration but negatively with the number of sperm transferred. It appears that males possessing genitalia of a particular shape are able to transfer more sperm and do so in a shorter time.

These data shed some light on the function of extended copulations in *Ciulfina*, which varies widely within and among species (Holwell 2007). Extending the duration of copulation and sperm transfer can influence a male insect's reproductive success by increasing (a) the number of sperm transferred (Dickinson 1986; Simmons 2001), (b) the duration of copulatory mate-guarding (Carroll 1991) and/or (c) the duration of copulatory courtship (Eberhard 1996). Firstly, our results clearly demonstrate that, for *C. klassi*,

Table 2 Results of regression analyses, investigating the relationship between genital morphology and aspects of sperm transfer in *C. klassi*

Model	Dependent	Overall model					Contribution of individual variables to model				
		R^2	CI (95%)	f^2	F	p	B	SE	t	P	
1	Sperm transferred	0.759	0.717– 0.801	3.15	10.48	0.002*	Intercept	1,312.643	116.941	11.225	<0.0001
							DLC	–7,457.734	1,788.613	–4.170	0.002
							RWS1				
							RP RWS1	–3,200.106	1,302.337	–2.457	0.034
							RP RWS5	–26,699.646	5,750.348	–4.643	0.001
2	Proportion transferred	0.388	0.312– 0.464	0.63	7.61	0.017	Intercept	0.582	0.044	13.181	<0.0001
							RP RWS5	–5.700	2.066	–2.759	0.017
3	Copulation duration	0.909	0.892– 0.926	9.99	119.70	<0.0001*	Intercept	2,414.214	143.002	16.882	<0.0001
							DLC	21,815.886	1,993.963	10.941	<0.0001
							RWS1				
4	Attachment	0.337	0.260– 0.414	0.51	6.109	0.029	Intercept	2,816.000	372.251	7.565	<0.0001
							VLC	61,761.181	24,988.136	2.472	0.029
							RWS4				
5	Total transfer duration	0.691	0.640– 0.742	2.24	7.470	0.007*	Intercept	5,230.214	343.035	15.247	<0.0001
							DLC	19,659.540	5,022.568	3.914	0.003
							RWS1				
							DLC	–35,567.247	14,841.316	–2.397	0.038
							RWS3				
						RP RWS5	55,790.227	17,393.681	3.207	0.009	

Each regression model was determined using a forward stepwise model incorporating centroid size and the first five relative warp scores (RWS) for the dorsal left complex (DLC), right phallomere (RP) and ventral left complex (VLC), along with male mass and male pronotum length. The statistics associated with each overall model and the contribution of each variable contributing significantly to the model are presented. f^2 is the standard measure of effect size for multilinear regression analyses. Asterisked significance values indicate models that were considered statistically significant after conservative Bonferroni adjustment ($\alpha=0.01$)

increased sperm transfer duration does not improve sperm transfer success. Secondly, because all males were maintained in the laboratory under the same conditions, no variation in the perceived risk of sperm competition existed; however, males may inherently differ in their propensity to mate-guard or may be more likely to mate-guard to compensate for reduced competitive ability. Thirdly, it is possible that males may copulate for longer to extend the period of copulatory courtship and influence female utilisation of sperm (Eberhard 1996). Our results suggest that males that transferred fewer sperm were indeed compensating by increasing the time in which mate-guarding and/or female stimulation took place.

Sexual selection and genital morphology

Recent studies have shown the effect of sexual selection on genital morphology by demonstrating a relationship between male genital morphology and fertilisation success (Otronen 1998; Arnqvist and Danielsson 1999; Danielsson

and Askenmo 1999; House and Simmons 2003; Rodriguez et al. 2004; Wenninger and Averill 2006; Hotzy and Arnqvist 2009). Whilst there is now general support for sexual selection driving genital evolution, the exact mechanisms are unclear and the alternative hypotheses are not mutually exclusive (Hosken and Stockley 2004). Firstly, sexually antagonistic coevolution is important in driving genital adaptations in some insect groups (bruchid beetles—Rönn et al. 2007; water striders—Arnqvist and Rowe 2002). However, the results of this and previous studies do not suggest sexual conflict to be important in genital diversification in *Ciulfina*. Females show no resistance to successfully mounted males and do not reject them once copulation has begun (Holwell 2007), and the male genitalia of *C. klassi* do not possess harmful spines such as those of bruchid beetles (Hotzy and Arnqvist 2009). Secondly, male genitalia may also be under sexual selection via cryptic female choice, through their ability to stimulate females to differentially utilise their sperm to fertilise eggs (Eberhard 1985, 1993; Briceño and Eberhard 2009). It is

possible that female responses to variation in male genital shape have led to the pattern of genital evolution in *Ciulfina* through postcopulatory female choice. Female genitalia possess sensory hairs in *Ciulfina* (Holwell et al. 2007) and could potentially respond to variation in male genital shape via the differential uptake of sperm to the spermatheca. The muscular structure of the female spermatheca in *C. klassi* also suggests that females can influence sperm utilisation and storage (Winnick et al. 2009).

Finally, male genitalia may be under sexual selection through their ability to influence sperm competition via enhanced sperm transfer or positioning (Tadler 1999; Parker and Simmons 2000). The patterns revealed in our study strongly suggest that the shape of the complex male genital structures of *C. klassi* have indeed been selected for efficient sperm transfer, possibly in the context of sperm competition. This is perhaps the most parsimonious explanation for the observed patterns, and emphasises that while the merits of both the sexual conflict and female choice models of genital evolution are debated (Eberhard 2004a, b, 2006; Arnqvist and Rowe 2005; Rönn et al. 2007), male–male competition should not be dismissed as an important factor in selection on male genital morphology.

Although an influence on fertilisation success provides the most unambiguous evidence for the influence of sexual selection in promiscuous species, the ability of male genitalia to influence sperm transfer is likely to be under strong selection. We have shown that sperm transfer success is variable and relates to genital shape in *C. klassi*. We also suggest that, to fully understand the influence of sexual selection on genital morphology, it is important to investigate the influence of genital shape on key components of reproductive success such as sperm transfer, sperm storage and finally fertilisation.

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