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POSTMATING SEXUAL SELECTION: ALLOPATRIC EVOLUTION OF SPERM COMPETITION MECHANISMS AND GENITAL MORPHOLOGY IN CALOPTERYGID DAMSELFLIES (INSECTA: ODONATA)

A. CORDERO RIVERA,[1,2*] J. A. ANDRÉS,[1,3*] A. CÓRDOBA-AGUILAR,[4,5] AND C. UTZERI[6,7]
[1] *Grupo de Ecoloxía Evolutiva, Departmento de Ecoloxía e Bioloxía Animal, Universidade de Vigo, EUET Forestal, Campus Universitario, 36005 Pontevedra, Spain*
[2] *E-mail: acordero@uvigo.es*
[4] *Centro de Investigaciones Biológicas, Universidad Autónoma del Estado de Hidalgo, Apdo. P. 69-1, Plaza Juárez, 42001 Pachuca, Hidalgo, Mexico*
[5] *E-mail: acordoba@uaeh.reduaeh.mx*
[6] *Dipartimento di Biologia Animale e dell'Uomo, Università ''La Sapienza'', Viale dell'Università 32, 00185 Roma, Italy*
[7] *E-mail: carlo.utzeri@uniroma1.it*

Abstract.—Postmating sexual selection theory predicts that in allopatry reproductive traits diverge rapidly and that the resulting differentiation in these traits may lead to restrictions to gene flow between populations and, eventually, reproductive isolation. In this paper we explore the potential for this premise in a group of damselflies of the family Calopterygidae, in which postmating sexual mechanisms are especially well understood. Particularly, we tested if in allopatric populations the sperm competition mechanisms and genitalic traits involved in these mechanisms have indeed diverged as sexual selection theory predicts. We did so in two different steps. First, we compared the sperm competition mechanisms of two allopatric populations of *Calopteryx haemorrhoidalis* (one Italian population studied here and one Spanish population previously studied). Our results indicate that in both populations males are able to displace spermathecal sperm, but the mechanism used for sperm removal between both populations is strikingly different. In the Spanish population males seem to empty the spermathecae by stimulating females, whereas in the Italian population males physically remove sperm from the spermathecae. Both populations also exhibit differences in genital morphometry that explain the use of different mechanisms: the male lateral processes are narrower than the spermathecal ducts in the Italian population, which is the reverse in the Spanish population. The estimated degree of phenotypic differentiation between these populations based on the genitalic traits involved in sperm removal was much greater than the differentiation based on a set of other seven morphological variables, suggesting that strong directional postmating sexual selection is indeed the main evolutionary force behind the reproductive differentiation between the studied populations. In a second step, we examined if a similar pattern in genital morphometry emerge in allopatric populations of this and other three species of the same family (*Calopteryx splendens*, *C. virgo* and *Hetaerina cruentata*). Our results suggest that there is geographic variation in the sperm competition mechanisms in all four studied species. Furthermore, genitalic morphology was significantly divergent between populations within species even when different populations were using the same copulatory mechanism. These results can be explained by probable local coadaptation processes that have given rise to an ability or inability to reach and displace spermathecal sperm in different populations. This set of results provides the first direct evidence of intraspecific evolution of genitalic traits shaped by postmating sexual selection.

Key words.—*Calopteryx haemorrhoidalis*, cryptic female choice, damselfly, genital evolution, phenotypic differentiation, sexual selection.

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During the last 30 years the study of postmating sexual selection processes has grown exponentially. Now it is accepted that the ejaculates of several males are commonly involved in postcopulatory battles for fertilization in almost all taxonomic groups (Smith 1984; Birkhead and Møller 1998; Simmons 2001). There is also an increasing evidence that postmating sexual selection arises not only as the result of the fertilization battles among males in an inert environment but as the consequence of interactions between the offensive and defensive male traits and the female reproductive tract and physiology (Eberhard 1996; Wilson et al. 1997; Andrés and Arnqvist 2001; Brown and Eady 2001; Nilsson et al. 2002). One key prediction of postmating sexual selection is the existence of variance among allopatric populations in those male traits related to fertilization success. This variance is likely to arise as the result of local coevolution driven by male-male competition (sperm competition hypothesis) or male-female interactions (cryptic female choice hypothesis). However, only the cryptic female choice hypothesis, driven by antagonistic coevolution between the sexes (Holland and Rice 1998) or by a process parallel to conventional female mate choice (Andersson 1994; Eberhard 1996) predicts the existence of adaptive variation among populations in female responsiveness (e.g., Andrés and Arnqvist 2001; Brown and Eady 2001; Nilsson et al. 2002, 2003) and in those female traits that modulate the response. Given enough time, this covariation between the sexes may lead to a rapid differentiation of sperm competition mechanisms, promoting eventually the evolution of reproductive isolation and, thus, speciation. Indirect evidence from at least three different lines of research support this point (Brown and Eady 2001; Nilsson et al. 2002, 2003). However, to our knowledge there is no direct evidence for the existence of incipient differentiation of sperm competition mechanisms in allopatry, which may

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* E-mail: jaa53@cornell.edu. The first two authors have contributed equally to this paper.

[3] Present address: Department of Ecology and Evolutionary Biology, Cornell University, Corson Hall, Ithaca, New York 14853.

be the consequence of our inability to fully understand sperm competition mechanisms. In this paper, we address the microevolutionary aspects of genital morphology and function in insects by studying the geographic variation of one of the best-known sperm competition mechanisms, the physical displacement of rivals' sperm in damselflies of the family Calopterygidae.

Possibly the most conspicuous male adaptation to prevent sperm competition is sperm removal. Since Waage's (1979) pioneering work on odonates, researchers have found that the ability to physically remove the stored sperm has evolved in several insect orders and other animals (Ono et al. 1989; Yokoi 1990; vonHelversen and Helversen 1991; Haubruge et al. 1999; Kamimura 2000). Indirect evidence of postmating sexual selection being the main engine responsible for the evolution of odonate sperm competition mechanisms comes from the detailed studies in closely related species of the genus *Calopteryx*, the family in which sperm removal was first reported (Waage 1979). Calopterygid damselflies can be divided into three groups according to their copulatory mechanisms: (1) species whose males have physical access to the spermathecae (Waage 1979; Adams and Herman 1991; Córdoba-Aguilar 2002);(2) species whose males cannot physically remove sperm from the spermathecae (Siva-Jothy and Tsubaki 1989; possibly because the spermathecal lumen is too narrow to allow the entry of the male genital processes; Siva-Jothy and Hooper 1995); and (3) species whose males elicit sperm ejection from the spermathecae via sensory stimulation (Córdoba-Aguilar 1999b). This remarkable variation among very closely related species suggests that postmating sexual selection may have played an important role during speciation events in this family (Córdoba-Aguilar 2002). However, this pattern is also compatible with differentiation mechanisms arising after speciation was completed. One way to study the role of postmating sexual selection on the microevolution of sperm competition mechanisms that may lead to differentiation between closely related species or populations is to study sperm competition mechanisms in different populations of the same species.

In this paper we have investigated this in two different steps. First, we show how differences in genital morphology might be associated to differences in sperm competition mechanisms in allopatric populations of one calopterygid species, *Calopteryx haemorrhoidalis*. We describe the sperm displacement mechanism of an Italian population of this species and compare it with the mechanism previously described for this species in a Spanish population (Córdoba-Aguilar 1999b). In this latter population, male genitalia stimulate the female sensory system to achieve ejection of rival sperm. Because our results indicated an allopatric divergence in sperm competition mechanisms in this species, in a second step we examined if a similar pattern in genital morphometry may emerge in allopatric populations of this and other three species of the same family, *Calopteryx splendens*, *C. virgo*, and *Hetaerina cruentata*, whose copulatory mechanisms have been described: male inability to displace spermathecal sperm in *C. splendens* (Siva-Jothy and Hooper 1996; Lindeboom 1998) and *H. cruentata* (Córdoba-Aguilar 2002); spermathecal physical sperm removal in *C. virgo* (Córdoba-Aguilar 2002). *Calopteryx* species are widely distributed in Europe (Askew 1988), whereas *H. cruentata* is present mainly in Central America (Garrison 1990). In the phylogeny of this family, the genus *Hetaerina* appears to have a basal position with respect to *Calopteryx*, and the European *Calopteryx* species are closely related (Misof et al. 2000). We show that allopatric populations of *C. haemorrhoidalis* have diverged in genital morphology and function, giving rise to different sperm competition mechanisms. These results also seem to apply to other calopterygid species given the significant geographic variation in both male and female genital traits. Our results suggest that postmating sexual selection may be contributing to speciation events within this family of damselflies.

MATERIALS AND METHODS

Genital Morphology and Sperm Competition Mechanisms in Calopteryx haemorrhoidalis

To compare whether *C. haemorrhoidalis* populations have diverged in genital morphology and sperm competition mechanisms with respect to that in Spain, we studied a population of *C. haemorrhoidalis* at the river Forma Quesa, Frosinone province, in central Italy (UTM: 33TUF 8787) in August 1999–2001. We approached the study of the copulatory mechanisms of this species by investigating the possible occurrence of sperm physical removal, sensory stimulation, and/or inability to displace sperm.

Anatomy of genitalic traits as indicators of sperm competition mechanisms

To test whether a male ability to physically remove the sperm from the spermathecae is present, we measured the width of the common duct of the spermathecae, as the curved processes of male distal genitalia must enter this duct to remove sperm from these organs. The spermathecae were not narrower than the common duct (see Fig. 1B, C). The width and length of left distal process were measured in 27 males (the width at the median point - its maximum width).

The length of spermathecae was measured in postcopula and interrupted females, from their insertion at the bursa to their tips (i.e., including the common duct). To investigate whether male genital processes have physical access to the spermathecal ducts, we preserved eight pairs during stage I of copulation (the stage when sperm removal takes place; Miller and Miller 1981) by cutting female's abdomen and conserving the engaged genitalia in ethanol. To investigate whether males may use a sensory stimulation mechanism, we counted the number of mechanoreceptor sensilla on the vaginal plates of these dissected females and estimated body size by measuring right hindwing (from the basis to tip). The width of aedeagus was measured in the region that makes contact with the vaginal plates, because this trait is related to male ability to elicit sperm ejection by females: the wider the aedeagus, the greater the quantity of sperm ejected (Córdoba-Aguilar 1999b, 2002c).

Tests of sperm competition mechanisms based on experimental couples and sperm volume measurements

In 1999 we investigated the sperm removal ability of male *C. haemorrhoidalis* by measuring sperm volumes in inter-

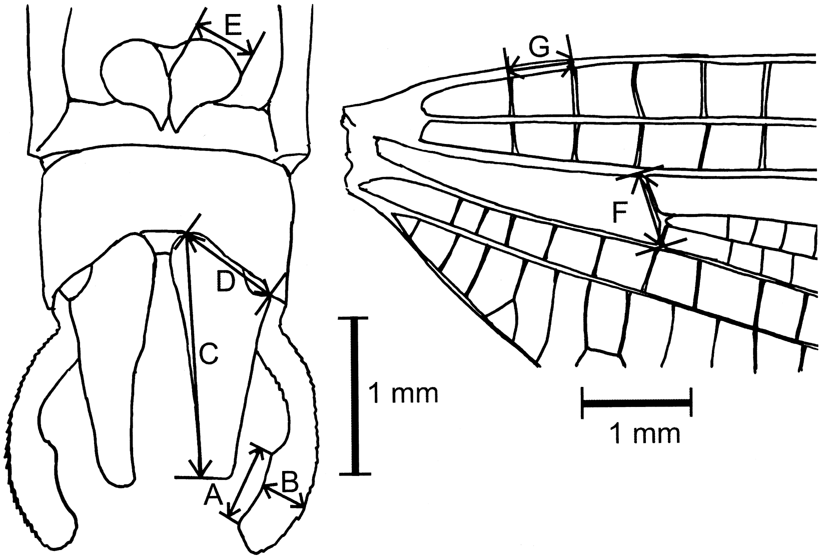

FIG. 1. Morphological characters measured to compare phenotypical differentiation between populations of *Calopteryx haemorrhoidalis*. Ventral view of male abdomen and right wing: A, Length of distal part of superior appendages; B, width; C, length of inferior appendages; D, width; E, width of primary genitalia (genital valve); F, length of arculus; G, distance between first and second antenodal.

rupted pairs (*n* = 26) at fixed intervals (10, 20, 40, 60 abdominal, sperm displacement-related movements; Miller and Miller 1981) during stage I. The average number of stage I movements was 63 ± 6.0 (21) in courtship-preceded copulations (Cordero Rivera and Andrés 2002). Females were immediately preserved in 70°C ethanol until dissection. We also captured females immediately after complete copulation at different times of day (71 postcopula females) and oviposition (22 postoviposition females), and obtained a third sample by capturing females early in the morning (0900 h), before mating activity (six precopula females). Final sample sizes differ from these figures due to damaging of some bursae or spermathecae during dissections. To measure sperm volume, the sperm storage organs were compressed to a uniform thickness under a supported cover-slide, and the sperm area was obtained using an image analysis software. Sperm volume was estimated by multiplying the area by the thickness (Cordero and Miller 1992). Sperm volume estimations were made blindly in relation to the experimental group of each female. We measured the sperm volume of each spermathecae, to test for the potential lateral relationships between sperm volume and number of vaginal sensilla: if the sensory stimulation is taking place, the spermathecae whose vaginal plate had more sensilla should eject more sperm (Córdoba-Aguilar 1999b).

The experimental design described above allows the investigation of sperm removal pattern; however, because females probably entered the experiment with different residual amounts of sperm from previous matings, to test if males displace sperm via sensory stimulation we carried out an experiment in 2001 with double-mated females. These females (*n* = 31) were introduced into an outdoor insectary (1.6 × 2.0 × 1.5 m) and were hand-paired to a male (Ophenheimer and Waage 1987), to start with females having full volumes of stored sperm. After this first copulation, females were immediately hand-paired to a second male but were interrupted after 60 abdominal movements of stage I. To minimize the number of animals used in the experiments, when possible males were used twice, once as the first mate and once as the second mate (therefore they were sperm donors or removers only once). All experimental individuals were preserved in 70°C ethanol until dissection. Another experiment tested this hypothesis directly by using dissected aedeagi to stimulate females (the lateral processes of the aedeagus were removed to avoid physical displacement of sperm; Córdoba-Aguilar 1999b). Eight females were captured in the field immediately after an undisturbed copulation and maintained in a cold dark box for 2–4 h. Each female was then stimulated with a dissected aedeagus (three aedeagi were used) introduced in the vagina, mimicking 60 pumping movements of stage I of copulation, for 2–4 min. Control females (*n* = 5) were treated identically, except for aedeagus stimulation. After the experiment, both the experimental and control females were preserved in ethanol until dissection.

Phenotypic Differentiation between Calopteryx haemorrhoidalis Populations

Given enough time, all characters will diverge among isolated populations unless they are under strong stabilizing selection for the same optimum. Thus, only showing that different populations have indeed diverged in their genitalic traits does not inform us about the evolutionary forces involved in the differentiation process. This is especially important when populations are likely to have been isolated for long periods of time. If strong directional postmating sexual selection is the main force driving the differentiation of the reproductive traits, one would expect greater phenotypic differentiation in genitalic characters relatively to the mean phenotypic differentiation among populations. Additionally, odonates show an extraordinarily modified mode of sperm transfer, so that the intromittent organ (secondary genitalia;

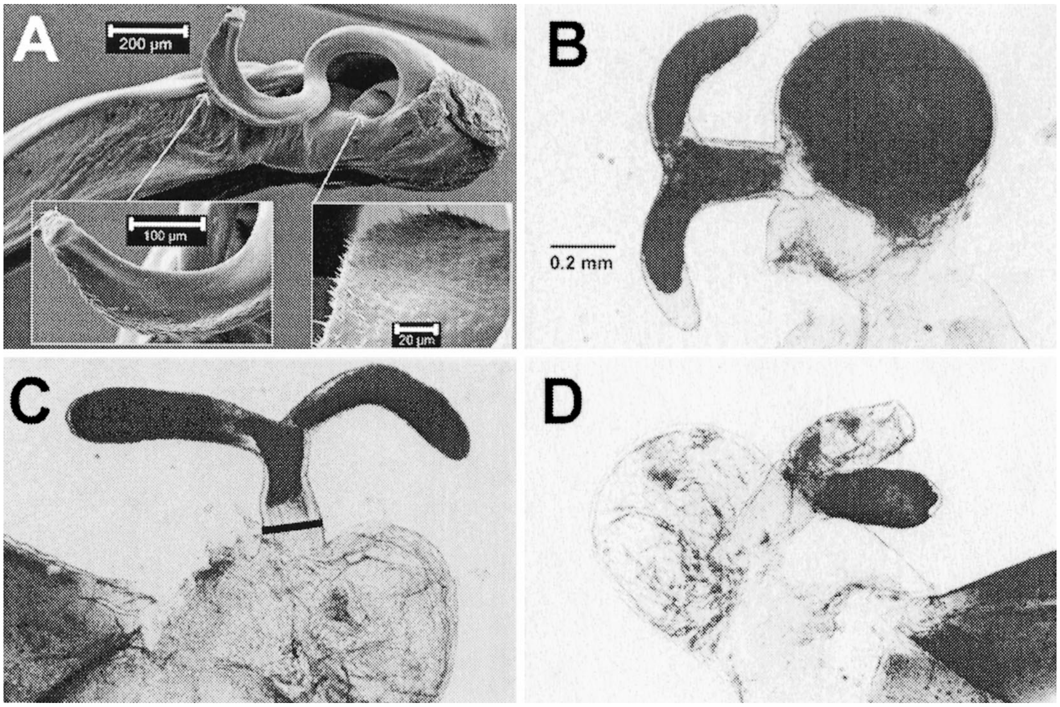

FIG. 2. Male and female genitalia in *Calopteryx haemorrhoidalis*. (A) Male aedeagus showing the distal spiny processes (used to remove sperm from the spermathecae), and a detail of the scooplike flap (used to remove sperm from the bursa); (B, C) two examples of female genitalia after 10 pumping movements of stage I (note that in C the bursa is completely empty, while in B the tips of the spermathecae are empty.). The line in C indicates the width of spermatheca. (D) Female genitalia after 60 pumping movements of stage I (bursa and one spermatheca empty).

Fig. 2) is independent of the primary genitalia (genital valves; see Fig. 1E). Hence, in this case a second independent test of the sexual selection, hypothesis could be made because only the secondary genitalic traits are related to the variance in male fertilization success. If the male intromittent organ is under directional sexual selection we would expect phenotypic differentiation between populations to be greater when based on secondary genitalic traits than when estimated on the primary genitalic traits.

To test the above predictions, the degree of phenotypic differentiation between the *C. haemorrhoidalis* populations of Frosinone (Italy) and Pontevedra (Spain) were estimated for two different set of characters: (1) two of the main traits involved in sperm removal (width of the male aedeagus distal process [see Fig. 2A] and female spermatheca [Fig. 2C]); and (2) a set of six traits not related to sperm removal (see Fig. 1). Phenotypic differentiation estimates of each trait were obtained using Spitze's (1993) Q_{ST}, calculated as follows:

$$Q_{ST} = \frac{\sigma_B}{\sigma_B + 2\sigma_W}, \qquad (1)$$

where σ_B and σ_W are the between- and withinpopulation components of the variance for the trait. Each of these components were calculated by means of the Variance Components ANOVA of Biomstat software (Sokal and Rohlf 1995). All traits were measured using ImageTool software (available via http://ddsdx.uthscsa.edu/dig/itdesc.html).

Genital Morphometry in Allopatric Populations of the Family Calopterygidae

Our results suggested that both the Spanish and Italian *C. haemorrhoidalis* populations differed in genital morphology and sperm displacement mechanisms. Thus, we indirectly explored the widespread nature of these results by measuring the genital morphology in allopatric populations of other calopterygids including *C. haemorrhoidalis*. We obtained specimens of the following species: *C. haemorrhoidalis* (three populations: Vidourle River, southern France; Frosinone, central Italy; Pontevedra, northern Spain), *C. splendens* (three populations: Vidourle River; Pontevedra and Valencia, southern Spain), *C. virgo* (three populations; Vidourle River; Valencia and Macerata, central Italy), and *H. cruentata* (two populations: Xalapa, eastern Mexico; Molango, central Mexico). Individuals were collected in the same week. We only used fully mature individuals (those that have already produced or stored sperm) because immature animals are not amenable for dissection and genitalic measurements (Córdoba-Aguilar 2003a).

We used these species given that their copulatory mechanisms (ability or inability of spermathecal sperm displacement) and genital morphometry have been fully documented (see references in introduction) so a priori expectations can be formulated. As a general rule, in those calopterygid species in which there is spermathecal sperm displacement, the male lateral processes are narrower than the spermathecal ducts (e.g., *C. virgo*), with the reverse occurring in species in which

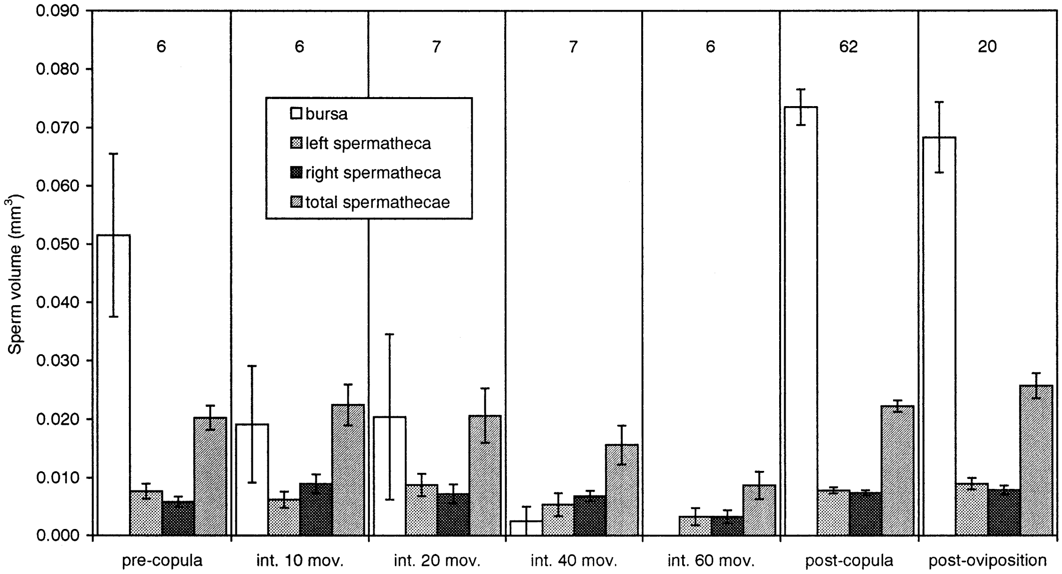

FIG. 3. Sperm removal by male *Calopteryx haemorrhoidalis*. The figure shows the mean sperm volume in the bursa and spermathecae either separately or together (including the common duct). Copulations were interrupted at fixed intervals during stage I, after 10, 20, 40, and 60 pumping movements of male genitalia. Precopula females were collected early in the morning before mating activity, postcopula females after a complete copulation, and postoviposition females after spontaneously ending of egg laying. Numbers on top indicate sample sizes. Note the great variability in the bursal sperm removed after 10–20 pumping movements.

males are not able to access spermathecal sperm (e.g., *C. haemorrhoidalis*, *C. splendens*, and *H. cruentata*; Córdoba-Aguilar 2003b). Given this generality and the knowledge we have on the copulatory mechanisms of the studied species, we examined to what extent this applied to the studied species in different populations. The genital structures measured were those involved in spermathecal sperm storage and spermathecal sperm removal and included the following: width (medial part) of right and left spermatheca and width (medial part) of right and left genital process. Additionally, we measured wing size as an indicator of body size. For each species, we used an ANCOVA to investigate differences for right and left genital structures in males and females among and within populations. We entered wing size as a covariate to investigate whether it had a role explaining genitalic differences.

The genitalic structures were measured using image analysis software (Optimas ver. 6.1, available via http://www.optimas.com; and GlobalLab 3.0 available via http://www.datx.com) via a video camera attached to a compound microscope. Genitalic measurements are presented in millimeters. Means are presented with their standard errors and (sample size). Analyses were made with GenStat, NAG Statistical Add-Ins for Excel (available via http://www.nag.co.uk), and xlStat (available via http://www.xlstat.com).

RESULTS

Description of Sperm Competition Mechanisms in Italian C. haemorrhoidalis

Genital morphometry and sperm displacement

Similar to other *Calopteryx* species, females store sperm in the bursa copulatrix and two spermathecae (Fig. 2B–D). Nevertheless we found two females (of 200) with only one spermatheca. Male genitalia are similar to those of other *Calopteryx* species known to physically remove sperm from both the bursa and spermathecae (Waage 1979; Fig. 2A): the penis head has two lateral processes with proximally oriented spines. The width of the penis at the region of contact with vaginal sensilla is 0.178 ± 0.002 mm $n = 27$.

Comparisons of width between distal genital processes and spermathecal ducts indicated that male processes (0.122 ± 0.001, $n = 27$) are narrower than the spermathecal duct (0.182 ± 0.002, $n = 114$; $t = 21.8$, $p < 0.001$). Furthermore, we found one of the male genital processes inside the left spermatheca in one out of eight pairs interrupted during stage I, confirming that, at least in some cases, males do have physical access to the spermathecae. Nevertheless, the spermathecae are longer and more variable in length (mean length of both spermathecae: 0.768 ± 0.015, $n = 88$, range: 0.468–1.095) than the penis processes (0.716 ± 0.008, $n = 27$, range: 0.628–0.809; $t = 3.48$, $P < 0.001$). Only 4% of males have penis processes longer than 0.8 mm, but 38% of spermathecae are longer than this value, indicating that the tips of spermathecae are inaccessible to most males. Interestingly, larger females have longer spermathecae ($r = 0.23$, $n = 88$, $P = 0.033$), but larger males do not have longer processes ($r = 0.07$, $n = 20$; $P = 0.780$).

Females have an average of 25.6 ± 0.3 ($n = 174$) sensilla per plate (range: 16–39) and the distribution of the number of sensilla in (right-left) sides is normal with a mean not significantly different from zero (i.e., fluctuating asymmetry; $t = -0.37$, $P = 0.711$). The number of sensilla was positively correlated with wing length (GLM with Poisson errors and log-link [Crawley 1993]; left side: $P < 0.001$; right side: $P = 0.003$).

Sperm removal

Figure 3 shows the amount of sperm in experimental females. There are clear differences between treatments in the

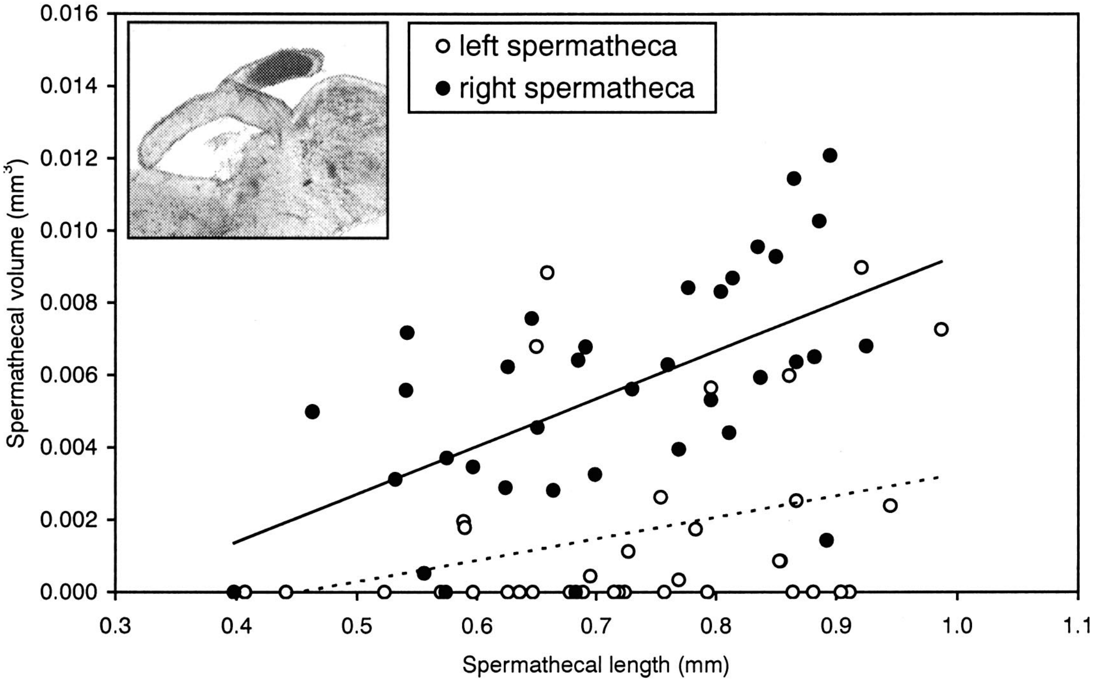

FIG. 4. The relationship between spermathecal length and the amount of sperm remaining in the spermathecae of females interrupted after 60 pumping movements during stage I of their second copulation. The upper left figure shows one example where the left spermatheca is empty.

volume of sperm stored (bursa: $F_{6,107} = 23.34$, $P < 0.001$; total spermathecae: $F_{6,103} = 3.10$, $P = 0.008$). Postcopula and postoviposition females have similar sperm volumes. Males are able to remove all the sperm stored in the bursa after 60 pumping movements, the average number in normal copulations (Cordero Rivera and Andrés 2002). None of the 26 females interrupted during stage I had both spermathecae empty.

In postcopula females ($n = 61$), a GLM analysis with the total volume of spermathecae as the dependent variable and wing length and mean number of sensilla as predictor variables indicates that larger females do not store more sperm ($t_{58} = 0.84$, $P = 0.405$), but the spermathecal volume is positively related to the number of sensilla ($t_{58} = 2.38$, $P = 0.021$). The volume in the bursa copulatrix is not predicted by wing length ($t_{60} = 0.99$, $P = 0.318$) or number of sensilla ($t_{60} = 0.52$, $P = 0.607$).

Twice-mated females interrupted after 60 pumping movements of stage I of their second copulation had the bursa almost empty, in agreement with the previous experiment. The left spermatheca was empty in 19 of 31 females but the right spermatheca was empty in only three females. We compared the volume of sperm of both spermathecae, using an ANOVA with left or right side as the factor and length of spermatheca and number of sensilla per plate as covariates. Results indicate a significant effect of side ($F_{1,50} = 59.83$, $P < 0.001$) and spermathecal length ($F_{1,50} = 20.83$, $P < 0.001$), but no effect of sensillum number ($F_{1,50} = 0.19$, $P = 0.665$). If sensillum number is entered in the model before spermathecal length, the effect of sensilla is almost significant ($F_{1,50} = 3.08$, $P = 0.088$), but the other factors remain as significant as before. These contrasting results are likely due to the correlation between number of sensilla and spermathecal length ($r = 0.46$, $P < 0.05$). Therefore the predictive power of number of sensilla is entirely related to this correlation, whereas length of spermatheca has additional predictive power independent of the correlation. Longer spermathecae had greater volumes of sperm, suggesting that they are not easily emptied (correlation between length and volume: left spermatheca, $r = 0.50$, $P = 0.007$; right spermatheca, $r = 0.59$, $P = 0.001$; Fig. 4). The difference in sperm volume between spermathecae in the same female is not due to a difference in their length (paired t-test, mean difference: 0.009 ± 0.019 mm, $t_{26} = -0.48$, $P = 0.633$). We analyzed the genitalia of the 36 males used in this experiment. There is no significant relationship between wing length and the length of the genital processes (left, $r = -0.22$, $P = 0.194$; right, $r = 0.13$, $P = 0.446$), but the left process was significantly longer (paired t-test, mean difference: 0.01 ± 0.004 mm, $t_{35} = -2.45$, $P = 0.019$).

There were no significant differences in the average volume of sperm of the spermathecae of females stimulated with dissected aedeagi and control females (total spermathecae, $t_{12} = 0.90$, $P = 0.389$).

Phenotypic Differentiation between C. haemorrhoidalis Populations

As predicted by the sexual selection hypothesis, the degree of phenotypic differentiation for two of the traits involved in the sperm competition mechanism (width of male aedeagus distal process and female spermatheca) was greater than the phenotypic differentiation averaged over all other traits (Table 1). This difference is statistically significant: the Q_{ST}-

TABLE 1. Comparison of phenotypic differentiation estimates between populations of *Calopteryx haemorrhoidalis* from Spain and Italy based on traits related to the sperm competition mechanism and a set of randomly chosen characters not related with sperm competition. For this latter dataset, we present the arithmetic mean (99.9% confidence intervals).

Dataset	Q_{ST}
Sperm competition related traits	
Width of male aedeagus distal process	0.997
Width of female spermatheca (Fig. 2)	0.965
Primary genitalia (Fig. 1E)	0.074
All seven traits not related to sperm competition (Fig. 1)	0.150 (−0.045 to 0.347)

values based on the reproductive traits did not overlap with the 99.9% confidence intervals for the random set of traits. In addition, the estimate of phenotypic differentiation between the studied population based on a secondary genitalic trait; width of male genital process ($Q_{\mathrm{ST}} = 0.998$) was much greater than the estimated for a primary genitalic trait (width of genital valve: $Q_{\mathrm{ST}} = 0.074$; Fig. 1E).

Geographic Variation of Genitalic Traits

There were significant differences among populations in all *Calopteryx*, but not in *Hetaerina* (Table 2). The comparison between spermathecal width and male genital processes suggests a male ability to physically remove spermathecal sperm (via entry of lateral processes) in Frosinone but not in Pontevedra and Vidourle in *C. haemorrhoidalis*, in Valencia and Pontevedra but not in Vidourle in *C. splendens*, and in Vidourle and Macerata but not in Valencia in *C. virgo*; and a male inability to remove sperm in Xalapa and Molango in *H. cruentata*.

Discussion

Differences in Genital Form and Function in C. haemorrhoidalis

We have found clear differences in copulatory mechanisms between Italian and Spanish *C. haemorrhoidalis*, as was previously predicted based on genital measurements (Córdoba-Aguilar 2003b). Our results suggest that, unlike the Pontevedra population (Córdoba-Aguilar 1999b), male stimulation does not take place in Frosinone or has a limited effect on sperm ejection. We tried to replicate the experiment carried out by Córdoba-Aguilar (1999b), who used dissected aedeagi to stimulate females to eject spermathecal sperm, but found no evidence of the effectiveness of this stimulation in Italian *C. haemorrhoidalis* (Fig. 4). Furthermore, in the experiment with double-mated females, we found no relationship between spermathecal volume and the number of sensilla in females whose second copulation was interrupted after 60 pumping movements of stage I, a relationship predicted if sensory stimulation was the prevailing mechanism (Córdoba-Aguilar 1999a). These results are suggestive of very limited role for male stimulation in this population.

The key character that allows males to elicit spermathecal sperm ejection in *C. haemorrhoidalis* is the aedeagus width in the part where it makes contact with the vaginal plates. In *C. haemorrhoidalis* from Pontevedra, the aedeagus is significantly wider than in other calopterygids and can elicit sperm ejection also in females of *C. xanthostoma*, *C. virgo*, and *H. cruentata*, which, nevertheless, do not eject sperm when stimulated by conspecific aedeagi (Córdoba-Aguilar 2002, 2003b). Paradoxically, the Frosinone *C. haemorrhoidalis* aedeagus is not only considerably wider (0.178 mm)

TABLE 2. Geographic variation on genitalic traits associated with sperm removal in Calopterygids. All measurements are given in millimeters (mean ± SE [*N*]). Results of ANCOVA examining structure × population interaction. All *F*-values are significant at $P < 0.0001$, even after Bonferroni correction, with the exception of *Hetaerina cruentata*. Body size was entered as covariate, but its effect was never significant.

	Length of spermathecae		Length of horns		*F*	
Population	Right	Left	Right	Left	Right	Left
Calopteryx haemorrhoidalis					203.9	252.1
Vidourle, France	0.35 ± 0.003 (12)	0.35 ± 0.003 (12)	0.45 ± 0.002 (12)	0.44 ± 0.002 (12)		
Frosinone, Italy	0.14 ± 0.006 (15)	0.16 ± 0.005 (15)	0.13 ± 0.002 (15)	0.13 ± 0.002 (15)		
Pontevedra, Spain	0.35 ± 0.003 (15)	0.35 ± 0.003 (15)	0.45 ± 0.002 (15)	0.44 ± 0.002 (15)		
C. splendens					2076.8	2106.7
Vidourle, France	0.34 ± 0.005 (13)	0.34 ± 0.005 (13)	0.43 ± 0.003 (13)	0.43 ± 0.003 (13)		
Valencia, Spain	0.46 ± 0.004 (10)	0.46 ± 0.004 (10)	0.25 ± 0.003 (10)	0.25 ± 0.004 (10)		
Pontevedra, Spain	0.64 ± 0.006 (12)	0.64 ± 0.005 (12)	0.17 ± 0.004 (12)	0.17 ± 0.005 (12)		
C. virgo					1102.9	1783.4
Vidourle, France	0.34 ± 0.003 (11)	0.35 ± 0.004 (11)	0.15 ± 0.003 (11)	0.15 ± 0.002 (11)		
Valencia, Spain	0.25 ± 0.003 (10)	0.25 ± 0.004 (10)	0.31 ± 0.003 (10)	0.31 ± 0.002 (10)		
Macerata, Italy	0.66 ± 0.011 (13)	0.65 ± 0.008 (13)	0.13 ± 0.002 (11)	0.13 ± 0.003 (11)		
H. cruentata					0.77	0.22
Xalapa, Mexico	0.32 ± 0.004 (10)	0.32 ± 0.004 (10)	0.54 ± 0.004 (10)	0.57 ± 0.005 (10)		
Molango, Mexico	0.31 ± 0.008 (10)	0.32 ± 0.006 (10)	0.54 ± 0.003 (10)	0.53 ± 0.003 (10)		

than that of Pontevedra (0.142 mm; Córdoba-Aguilar 2002) but also significantly wider than the aedeagus of the above cited three calopterygid damselflies (*C. xanthostoma* = 0.108 mm, *C. virgo* = 0.109 mm, *H. cruentata* = 0.101 mm; Córdoba-Aguilar 2002), presumably suggesting a great potential for sensory exploitation. Another contrasting difference with the *C. haemorrhoidalis* from Pontevedra and other calopterygids whose females are asymmetric in the number of vaginal sensilla in both vaginal plates (Córdoba-Aguilar 1999a, 2003b), is that most females of Italian *C. haemorrhoidalis* are symmetrical in sensillum number, and larger females have more sensilla.

Another source of evidence not supporting the sensory exploitation hypothesis for Italian *C. haemorrhoidalis* is our results of the sperm volumes stored by postcopula females; after ending egg-laying, females preserve the same amount of sperm as after copulation, suggesting a highly efficient sperm use for fertilization (a result that is actually contrasting compared to other odonates; e.g., in *C. maculata* [Waage 1980] and *Argia moesta* [Waage 1986], postoviposition females had 77–90% and around 60%, respectively, of spermathecal sperm volume compared to postcopula females). If sperm ejection had been elicited by male sensory exploitation in the Frosinone *C. haemorrhoidalis*, females should have ejected approximately the same amount of sperm as for egg fertilization, and no appreciable change in spermathecal volume would occur. The fact that spermathecal sperm volume greatly diminishes following male genital stimulation in *C. haemorrhoidalis* from Pontevedra (Córdoba-Aguilar 1999b) could be explained if males produced a super-stimulus, that is, one much more intense than egg friction during egg laying.

Our results suggest a great potential of female control over sperm reserves and several lines of evidence can illustrate this. Males are physically removing sperm from the spermathecae (which do not discard that they also simultaneously stimulate females), but it seems that females have retained control over sperm reserves by having evolved longer spermathecae (as indicated by shorter penis lateral processes than spermathecal ducts). This structural change may have impeded males to have access to all sperm. In accordance with this hypothetical adaptation, we found a male asymmetry in removal ability between the paired spermathecae: most males are unable to empty both spermathecae during a normal copulation (this occurred in only three females of 31). Actually, the left spermatheca was more frequently emptied, perhaps because the left process of the penis head was longer. Such directional asymmetry might be much more common in insects that previously thought (Pither and Taylor 2000).

Still in line with the female control hypothesis, in many cases, spermathecal sperm masses were not continuously distributed, and the fact that the sperm starts to disappear from the tip (Fig. 2B) or has a patchy distribution inside the spermatheca is suggestive evidence of a female-mediated process: if males were physically removing sperm from the spermathecae, their tips would be emptied last (Córdoba-Aguilar 1999b). Finally, some postcopula females also showed partially or completely empty spermathecae, and some females expelled sperm after copulation (but this behavior was too cryptic to be recorded at each copulation in the field), again suggesting that females control the amount of sperm they store in these organs (but see Lindeboom 1998). In summary, our study of the intricacies of the sperm competition mechanism used by the Italian *C. haemorrhoidalis* population has revealed a set of possible adaptations aimed to provide females with great control over sperm reserves. However, recognizing that females are not passive objects of male sperm competition is not a new situation for odonates (e.g., Waage 1984), although the widespread occurrence of the sperm removal ability may suggest otherwise.

Further evidence for female control of the use of sperm reserves in odonates was previously found in another damselfly (*Ceriagrion tenellum*), in which males obtained greater fertilization success by long than by short copulations, even if they were unable to remove sperm from the spermatheca (Andrés and Cordero Rivera 2000). There is also molecular evidence that *C. splendens xanthostoma* females are able to use the sperm from either the spermathecae or the bursa (Siva-Jothy and Hooper 1996) and that their spermathecal sperm are more genetically diverse than their bursal sperm (Siva-Jothy and Hooper 1995). In the Frosinone *C. haemorrhoidalis*, males can completely empty the bursa and at least part of the spermathecae, but females maintain a partial control over their sperm reserves due to the length of their spermathecae.

Córdoba-Aguilar (2002c) has interpreted calopterygid copulatory mechanisms as the outcome of a coevolution between the sexes in which the central tenet is the spermathecal sperm. According to this, *C. haemorrhoidalis* females prevented male sperm removal by reducing the width of spermathecal ducts, which is evidenced by the genital morphometry and absence of penetration in the Pontevedra population. A second evolutionary step was the male sensory stimulation to gain access to spermathecal sperm. This would have been followed by a reduction in sensillum numbers, which has some supporting evidence. Experimental aedeagal stimulation induced considerably larger volumes of sperm ejected in *C. splendens*, a species with large sensillum numbers (around 70), than in *C. haemorrhoidalis* from Pontevedra, a species with reduced sensilla numbers (about 45; A. Córdoba-Aguilar, unpubl. data). Possibly, the Frosinone population is one step ahead in this coevolutionary scenario; males have the potential for sensory exploitation (as judged by the relatively large aedeagal width), but a subsequent reduction in female responsiveness might have rendered this mechanism little effective.

Sperm competition studies in odonates have indicated high last-male sperm precedence in the short term, but sperm mixing seems to take place after a few days if females do not remate (Siva-Jothy and Tsubaki 1989; Cordero and Miller 1992; Hadrys et al. 1993; Siva-Jothy and Tsubaki 1994; Simmons 2001). In other insects, the number of spermathecae is variable and females seem able to partition the ejaculates between different spermathecae (Ward 1998, 2001). Zygopterans have none, one or two spermathecae, and the evolution of more spermathecal reservoirs could represent a counter-adaptation to increase female control over the fertilization process. As far as we know, we have found for the first time a variable number of spermathecae in odonates. The fact that *H. americana*, a primitive calopterygid (Misof et al. 2000), and most zygopterans have only one very small spermatheca (for *H. cruentata*, A. Córdoba-Aguilar, pers. obs.; for zygopterans in general, Waage 1984, 1986) suggests that the duplication of spermathecae in calopterygids is a derived character. Future research should address the question of the adaptive function of multiplicity of sperm storage organs in these insects.

Allopatric Evolution of Sperm Competition Mechanisms in the Calopterygidae: Speciation by Sexual Selection?

Despite the fact that all the different postmating sexual selection hypotheses predict the existence of significant geographic variation in those traits related to sperm competition (e.g., Eberhard 1996; Parker and Partridge 1998; Gavrilets 2000), the variability among populations in these traits has remained largely unexplored. Furthermore, though some previous studies showed phenotypic variability among populations for genitalic traits (see Hribar 1994; Jennions and Kelly 2002), these studies have failed to relate this variation to sperm competition abilities or male fertilization success (but see Tatsuta and Akimoto 1998).

In this paper we have provided evidence that in three (of four) different species of calopterygids there are indeed significant differences among allopatric populations in key genitalic traits likely affecting sperm removal ability. In some populations males potentially reach rivals' sperm stored in the spermathecae, whereas in other populations males seem to have no access to these sperm storage organs.

We have also found that in at least one of the studied species, *C. haemorrhoidalis*, sperm competition mechanisms have indeed diverged in allopatric populations (see above). Allopatric differentiation in the studied reproductive traits might not be surprising. Based on the European glacial history, these populations are likely to have been isolated for several thousand years, and given enough time geographically, isolated populations diverge so that different genes will become fixed either by differential selection pressures or drift. Thus, to be able to demonstrate that postmating sexual selection is the main evolutionary force behind this divergence one should demonstrate that the traits involved in sperm competition mechanisms have diverged more quickly than others. Our results strongly suggest that this is the case. First, the degree of phenotypic differentiation on sperm competition related traits was significantly greater than the phenotypic differentiation averaged over traits that are not related with fertilization success. Second, male aedeagus processes directly involved in sperm competition (i.e., secondary genitalic traits) showed a stronger degree of phenotypic differentiation than the primary genitalic traits, which are not involved in sperm removal. Therefore, we believe that our results show, for the first time, direct evidence of within species genitalic differentiation of traits shaped by postmating sexual selection.

A potential criticism to our interpretation arises from the fact that our results might not reflect the divergence between allopatric populations of the same species but the divergence between two closely related sister species. In fact, based in morphological traits we could assign the individuals of the studied populations to members of different subspecies (*C. haemorrhoidalis asturica*, Pontevedra, Spain [Ocharán 1983], and *C. h. haemorrhoidalis*, [Frosinone, Italy]). However, studies based on allozymes (Maibach 1985) and noncoding regions (ITS) of rDNA (Weekers et al. 2001) were unable to detect subspecific differentiation in *C. haemorrhoidalis*. In fact, the mean ITS estimated genetic distance based on the Kimura two-parameter model between European sister species (*C. haemorrhoidalis* vs. *C. virgo*: 0.0263; *C. xanthostoma* vs. *C. splendens*: 0.008) is higher than the estimated intraspecific variation in *C. haemorrhoidalis*, including European and North African populations (0.003). Among the surveyed European populations there was no differentiation, but all of them were different from North African populations (for further details see Weekers et al. 2001). Nevertheless, it can always be argued that genetic distances are at best weakly related with post- and premating isolation (Coyne and Orr 1997; Butlin and Tregenza 1998; Tregenza 2002), and therefore these populations might still represent two different sister subspecies. Laboratory mating experiments are the only safe way to establish this possibility. Unfortunately, the complex life history of *Calopteryx* species makes it practically impossible.

Recent experimental and comparative evidence suggests that both sexes might be coevolving at the level of genitalic traits (Arnqvist and Rowe 1995; Rice 1996; Arnqvist 1998; Presgraves et al. 1999; Arnqvist and Rowe 2002; Pitnick et al. 2002). This coevolution has the potential of eventually producing new species (Simmons 2001). Our experimental and morphometric evidence indicates that males in some populations have access to the spermathecae, while in other populations males are unable to do so. Furthermore, some characters that are different between populations were recently documented as targets of sexual selection in Calopterygidae, such as body size, wing pigmentation (e.g., Dumont 1972; Maibach 1985), and courtship flight parameters (Anders and Rüppell 1997). These differences along with divergences in sexual behavior (cf. behaviors of Spanish and Italian *C. haemorrhoidalis* described by Cordero 1999; Córdoba-Aguilar 2000; and Cordero Rivera and Andrés 2002) and sperm removal traits allow us to suggest that sexual selection might be operating as the promoter of speciation events in this family.

Interspecific differences in male sperm displacement ability in odonates have already been documented (e.g., Waage 1984, 1986), but a situation in which postmating sexual selection has been the motor of speciation deserves further research. Our results suggest that postmating sexual selection acting within populations might be one of the evolutionary forces promoting reproductive isolation in calopterygids. It is not difficult to envisage how populations might differentiate due to, for example, a female adaptation preventing males from displacing sperm. With such a situation, another population having a different evolutionary route would differentiate from the former given enough time and little genetic between-population intromission. The relatively large distribution areas of *Calopteryx* species (e.g., the range of *C. virgo* is from western Europe to Japan; Misof et al. 2000) is also another variable that might facilitate events of reproductive isolation. It would not be surprising to find differences in genital morphometry and copulatory mechanisms in those extremes of a species' distribution.

Finally, the mere fact of local coadaptations leads to the question of what drives copulatory diversification. Parker (1979) and Parker and Partridge (1998) have suggested that females may act as a selective force during speciation, and recent findings of sperm-female coevolution in *Drosophila melanogaster* agree with this scenario (Miller and Pitnick 2002). Our results in *C. haemorrhoidalis* indicate that females have been evolving a variable suite of means to keep sperm unreachable: reduction in the width of spermathecal duct and sensillum number and longer spermathecae. This suggests a great potential of female control and a role for females (Eberhard 1996). What exactly is driving females to evolve these adaptations and males to follow them remains the fundamental question in the sperm competition puzzle of calopterygids.

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