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Male damselflies detect female mating status: importance for postcopulatory sexual selection

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The existence of postcopulatory sexual selection is now clearly established in many animal species. In Odonates, males remove sperm during copulation from the bursa copulatrix and (when physically accessible) from the spermatheca. We used these model organisms to test the relative importance of sperm competition and cryptic female choice for copulation duration in laboratory experiments. If long copulations evolved only because of sperm competition, males should prolong copulation with previously mated females, and use this extra time to remove/displace the stored sperm. In species without a spermatheca (or when it is physically inaccessible), copulation duration should be similar in mated and virgin females. The cryptic female choice hypothesis predicts that copulations should be prolonged (acting as copulatory courtship) when males cannot physically remove sperm from the spermatheca but not if females do not have a spermatheca. We found that male damselflies can detect the mating status of females probably using chemical sensilla in their genitalia. Copulation duration with mated females was almost twice as long as with virgins in species with a spermatheca, but this behaviour was probably not the result of sperm competition, because in our model species, males could not remove sperm from this organ. The duration of copulation did not increase in species without a spermatheca. We conclude that even in odonates, where sperm removal is widespread, females have retained control over sperm reserves in their spermatheca(e), and males prolong copulation with mated females to elicit rival sperm ejection and/or to induce females to use their sperm in fertilization.

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Male and female interests do not necessarily coincide in reproductive decisions. This is the basis for the current interest in sexual conflict as an evolutionary force (Parker 1979; Alexander et al. 1997; Chapman et al. 2003; Pizzari & Snook 2004). In sexual species, copulation marks the end of conspicuous behaviours such as courting, and fighting, but it is also the start of concealed competition between ejaculates (Parker 1970; Smith 1984; Birkhead & Møller 1998; Simmons 2001) and cryptic female decisions (Eberhard 1996). Both processes are the basis for the existence of postcopulatory sexual selection (Birkhead & Pizzari 2002), which is usually based on a conflict of interests. This conflict is best viewed as 'discord between the genetic interests of alleles expressed in the two sexes' (Arnqvist 2004, page 1384), which might produce sexually antagonistic adaptations.

In many animals, there is a delay between copulation and fertilization (Eberhard 1985), which allows intense sperm competition (Parker 1970). As a consequence, in some groups male genitalia have evolved to allow males to manipulate sperm reserves inside female sperm storage organs. The first demonstration of this mechanism was by Waage (1979), who found a dual function for male genitalia in odonates: both sperm transfer and removal of rivals' sperm. This is a male adaptation that can have negative consequences for females (e.g. if a 'low' quality male is able to remove the sperm of a 'high' quality male). Therefore, we expect females to show counteradaptations (Córdoba-Aguilar et al. 2003). In odonates, females usually have two sperm storage organs: the bursa copulatrix and the spermatheca(e). Copulatory mechanisms have been studied in detail by Córdoba-Aguilar (1999, 2002, 2003) in the damselfly *Calopteryx haemorrhoidalis asturica*: males stimulate females with the aedeagus and elicit sperm ejection from the paired spermathecae. This allows males to 'remove' indirectly sperm from the spermathecae, which are inaccessible to the male aedeagus. The sperm stored in the spermatheca is therefore considered to be the main source of sexual conflict in odonates, because bursal sperm seems to be easily removed in all species (Córdoba-Aguilar et al. 2003).

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The reproductive behaviour of odonates has therefore been interpreted as the result of extreme sperm competition pressures, and the importance of female behaviour and control over reproductive decisions has often been minimized (Fincke 1997), even if some behaviours suggest that cryptic female choice mechanisms are likely (Eberhard 1996; Cordero Rivera 2002). Similarly, in *Drosophila*, sperm ejection by females is common, and might explain patterns of sperm precedence previously thought to be the result of sperm incapacitation by ejaculates (Snook & Hosken 2004). This situation parallels the development of sexual selection studies, from a predominantly male perspective to a greater interest in female behaviour and sources of sexual conflict (Birkhead & Pizzari 2002; Zeh & Zeh 2003).

We used odonates as a model to test the importance of sexual conflicts on the evolution of reproductive behaviour. By comparing copulation duration in species with and without a spermatheca, we tested the relative importance of sperm competition and cryptic female choice on copulation duration. Some odonates engage in very long copulations, more than 7 h in *Ischnura* (Miller 1987; Cordero 1990; Cordero & Andrés 1999), which have classically been interpreted as the result of male–male competition, and therefore as a kind of in-copula guarding (Cordero 1990; Alcock 1994). Alternative explanations for this behaviour are that the extra time is used in sperm removal or insemination, or that prolonged copulation is a mechanism of copulatory courtship and males use it to 'show' their quality to females (Eberhard 1994). Many insects show conspicuous copulatory courtship, and in some cases it seems to be related to cryptic female choice (Rodríguez 1995; Santolamazza Carbone & Cordero Rivera 1998). There is now evidence that copulatory courtship affects male mating and paternity success in beetles (Edvardsson & Arnqvist 2000; Shuker et al. 2002) and spiders (Watson & Lighton 1994; Schäfer & Uhl 2002).

We tested the above ideas by examining copulatory behaviour and genital morphology, looking for structures similar to chemical sensilla that would allow sperm detection, in damselfly species of the families Platycnemididae, Lestidae and Coenagrionidae. Specifically, we predicted that: (1) males should be able to detect female mating status; (2) if long copulations have evolved to allow more efficient sperm removal, copulations with virgin females should be shorter than copulations with mated females, except in species whose females do not have a spermatheca (where sperm removal should be fast), or in species whose males cannot remove sperm physically from the spermatheca; (3) if long copulations are mainly a mechanism of copulatory courtship (as defined by Eberhard 1994) in a scenario of cryptic female choice, copulations should be prolonged particularly if males cannot physically remove sperm from the spermatheca(e) but not if females do not have a spermatheca.

METHODS

Copulatory Behaviour

Teneral (=newly emerged) specimens of *Platycnemis acutipennis*, *P. latipes*, *Lestes virens*, *L. viridis*, *L. barbarus* and *Enallagma cyathigerum* were collected from natural populations in Galiza, northwest Spain during June and July 1998–2000 with permission from the Galician Government (Xunta de Galicia). We individually marked damselflies by writing a number on their wings with a permanent marker. They were maintained in insectaries ($50 \times 50 \times 50$ cm) with regulated moisture (by adding water to covered containers), temperature (25.2 ± 1.18°C) and indirect natural light (see Cordero 1994; Van Gossum et al. 2003). Food was added ad libitum (*Drosophila* sp.). Male density affects copulation duration, especially when one compares very low (less than five) and very high (more than 25) male densities (Cordero 1990; Andrés & Cordero Rivera 2000), but is less important for intermediate densities. We therefore maintained damselflies at medium density ($\bar{X} \pm \mathrm{SE} = 9.9 \pm 0.9$ individuals/insectary). After the study, they were kept for further analyses.

In many damselflies, copulation duration is negatively related to time of day (Cordero 1990, 1999; Perry & Miller 1991; Cordero et al. 1995; Cordero & Andrés 1999; Andrés & Cordero Rivera 2000). We measured copulation duration at the time when each species shows mating activity in the field, and explored the effect of time of day on reproductive behaviour with regression analysis.

The duration of complete copulation and its component stages (Miller & Miller 1981; see Miller 1987), i.e. tandem, sperm transfer, stage I, stage II (and stage III for *E. cyathigerum*), were recorded for all species. Sample sizes vary across each phase of copulation within a species (Table 1), because in some pairs we were unable to record all copulatory phases, and some females refused a second copulation. Males remove sperm during stage I and inseminate during stage II (and probably stage III; Miller 1987). For *E. cyathigerum* we calculated a gross value of copulation duration (including the times when the pair interrupted genital contact but remained in tandem position) because these interruptions during stage I seem to be an essential part of copulation in this species (see Perry & Miller 1991).

To test male ability to detect female mating status, we measured copulatory behaviour in virgin and mated females. Time between matings was usually only 24–48 h (median value: *L. viridis:* 23.5 h; *L. barbarus:* 48.1 h; *L. virens:* 23.7 h; *P. latipes:* 24.5 h; *P. acutipennis:* 24.6 h; *E. cyathigerum:* 23.5 h) to limit any confounding effect of female age. When possible, males were used for only one copulation, but females were used twice (first and second copulation). Nevertheless, in some cases the same male was used more than once, owing to a limited number of specimens, although these repeated observations with the same male were not included in statistical analyses (but are presented in the figures). Values are given as means $\pm$ SE.

In a first analysis, the durations of copulatory phases for the first and second copulation of the same female (mated by different unique males) were analysed by repeated measures ANOVA, with the female as the experimental unit, including the presence/absence of a spermatheca and the species identity as fixed factors (using families instead of species to control for phylogeny produces similar results). We further analysed differences between the first and second copulation for each species separately

Table 1. Duration of copulatory phases in Zygoptera (min)

Species	Tandem	Sperm transfer	Stage I	Stage II	Total copulation
P. acutipennis	*N*=42/16	*N*=45/15	*N*=41/13	*N*=41/13	*N*=17/17
Virgin	6.54±1.79	0.35±0.01	25.57±3.82	1.77±0.09	27.87±2.21
Mated	6.26±2.66	0.40±0.03	65.65±5.81***	2.00±0.14	65.99±4.89***
P. latipes	*N*=40/11	*N*=41/13	*N*=49/16	*N*=39/12	*N*=19/19
Virgin	3.23±0.70	0.24±0.00	29.20±1.8	1.55±0.11	30.94±1.82
Mated	1.55±0.47	0.29±0.03	73.42±7.67***	1.80±0.19	71.37±6.16***
L. virens	*N*=18/8	*N*=20/8	*N*=30/8	*N*=32/7	*N*=13/13
Virgin	5.58±2.14	0.32±0.02	8.97±0.73	1.17±0.09	12.16±2.18
Mated	3.07±1.37	0.32±1.52	12.05±1.52***	0.99±0.17	13.59±1.75
L. viridis	*N*=40/30	*N*=47/30	*N*=49/27	*N*=46/20	*N*=16/16
Virgin	5.77±1.53	1.03±0.11	6.31±0.4	1.28±0.06	9.11±1.29
Mated	4.87±1.01	1.01±0.09	19.15±1.9***	1.36±0.15	19.59±2.00***
L. barbarus	*N*=13/15	*N*=13/17	*N*=19/18	*N*=20/18	*N*=12/12
Virgin	22.54±5.44	0.63±0.14	5.71±0.84	3.01 8±0.29	8.76±0.87
Mated	7.22±2.00*	0.48±0.03	9.89±1.59*	2.51±0.14	12.43±1.57
E. cyathigerum	*N*=6/7	*N*=6/7	*N*=10/8	*N*=10/8	*N*=8/8
Virgin	2.79±1.40	0.28±0.02	27.85±1.93	1.39±0.13 3.97±1.93 (stage III)	33.21±2.00
Mated	1.90±0.87	0.26±0.02	44.48±6.05*	1.20±0.10 3.79±0.3 (stage III)	49.47±6.03***

Values are presented as mean ± SE. Sample sizes are indicated for virgin/mated females and include females that did not mate a second time. For total copulation, means were calculated using only data from females mated by two different males. *$P < 0.05$; ***$P < 0.001$, means significantly different, from a repeated measures ANOVA.

(Table 1), because we predicted different results for species without a spermatheca. Analyses were performed with GenStat 6th edition (GenStat 2002).

Genital Morphology

Inspired by the results of behavioural observations, we examined male genitalia by scanning electron microscopy (SEM) to search for structures potentially able to be used as chemical sensilla. Species studied include *P. acutipennis*, *P. latipes*, *P. pennipes*, *L. virens*, *L. viridis*, *L. barbarus*, *L. dryas*, *Sympecma fusca* and *E. cyathigerum*. Optical microscopy was used to study female genitalia.

RESULTS

Copulation Duration

Table 1 gives the duration of complete copulation for all females that mated twice with different males. Copulation with mated females was longer than with virgins in all species (repeated measures ANOVA, time factor: $F_{1,88} = 82.59$, $P < 0.001$), and the presence of a spermatheca(e) is highly significant (spermatheca factor: $F_{1,88} = 21.41$, $P < 0.001$). There was also a significant effect of species identity on copulation duration ($F_{4,88} = 9.08$, $P < 0.001$). These differences were due to stage I, which was shorter with virgins (time: $F_{1,69} = 99.09$, $P < 0.001$; spermatheca: $F_{1,69} = 27.94$, $P < 0.001$; species: $F_{4,69} = 10.94$, $P < 0.001$). The duration of stage II was similar in virgin and mated females (time: $F_{1,58} = 0.08$, $P = 0.778$) but was shorter in species with a spermatheca ($F_{1,58} = 7.88$, $P = 0.007$). Species identity did not affect stage II duration ($F_{4,58} = 0.41$, $P = 0.799$). The duration of precopulatory tandem and intramale sperm transfer was not affected by female mating status, presence of a spermatheca or species identity ($P = 0.14$–0.99). The duration of stage III in *E. cyathigerum* was not affected by female mating status (time: $F_{1,7} = 0.02$, $P = 0.890$).

Figure 1 shows copulation duration for the first and second mating of all females (including those mated by the same male, and those that did not mate a second time) in relation to time of day. Copulations were shorter at the end of the period of activity, but a regression analysis with order of copulation (first/second) and time of day indicates that time had a significant effect on copulation duration only in *P. latipes* ($t_{44} = -2.27$, $P = 0.028$; Fig. 1b).

Genital Morphology

All studied species stored sperm in the bursa copulatrix, but *L. virens* and *L. barbarus* did not have a spermatheca, all *Platycnemis*, *S. fusca* and *E. cyathigerum* had only one spermatheca, and *L. viridis* had two small spermathecae (Fig. 2).

For species with both storage organs, the relative size of the bursa and spermatheca(e) differed between genera: in once-mated females the amount of sperm stored in the spermatheca(e) was 13% in *L. viridis*, 22% in *P. acutipennis*, 15% in *P. latipes* and 23% in *E. cyathigerum*.

Male genitalia in *P. acutipennis* (see Figure 2 in Córdoba-Aguilar et al. 2003) have four small cornua, covered with spiny structures, whereas *P. latipes* and *P. pennipes* had only two cornua (Fig. 3). Lestids had a spoon-shaped penis head without appendages (Fig. 3), as previously described for *Lestes vigilax* (Waage 1982). The aedeagus in *L. viridis* and *S. fusca* had a complex morphology (Fig. 3), and these were the only lestids with a spermatheca in our sample. *Enallagma cyathigerum* males had typical coenagrionid genitalia, with two short horns (Fig. 3). Lestids showed

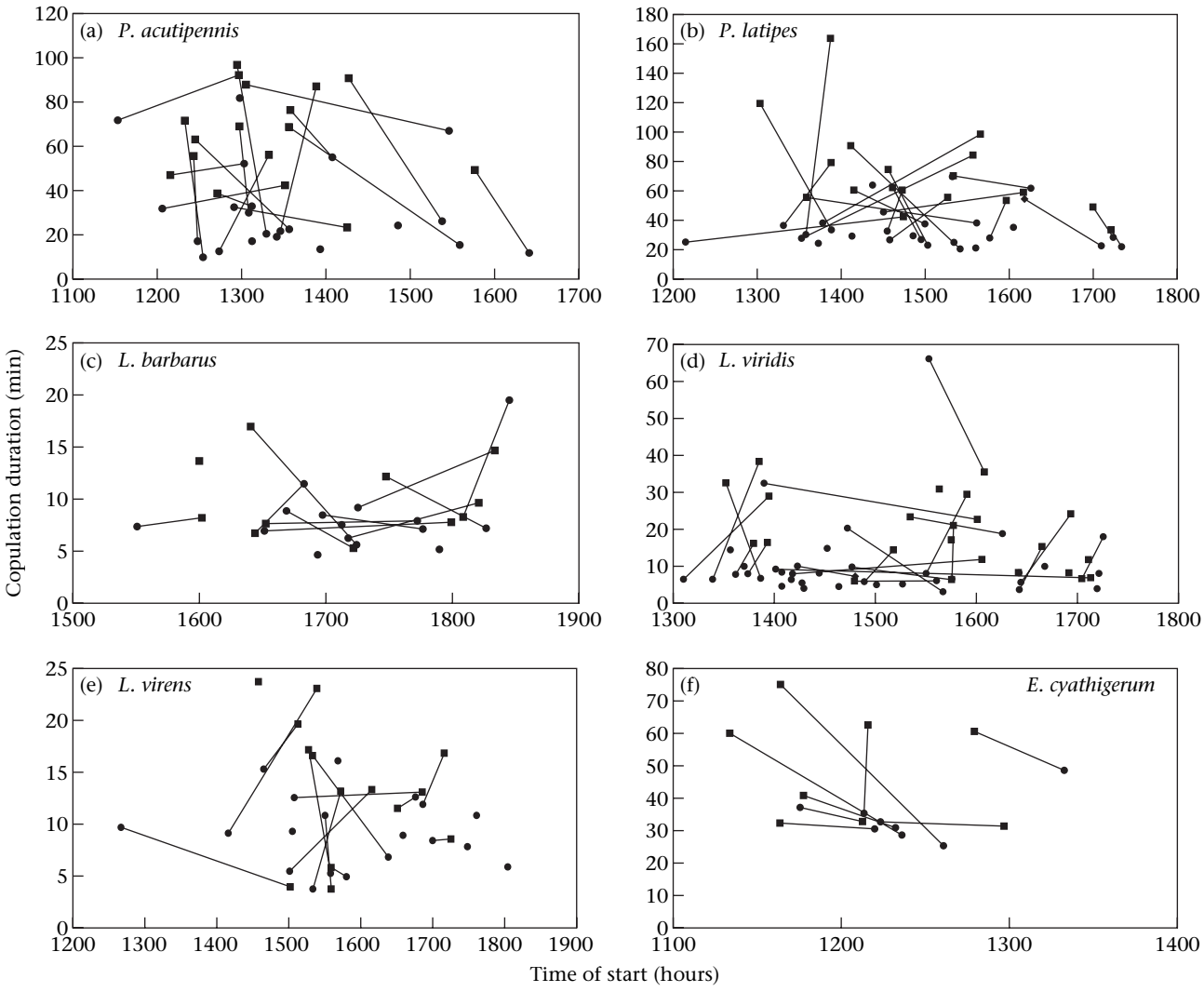


Figure 1. The relation between time of day and copulation duration for first (●) and second (■) matings of females of the studied species. Copulations by the same female are united by a line. (a) *Platycnemis acutipennis*, (b) *P. latipes*, (c) *Lestes barbarus*, (d) *L. viridis*, (e) *L. virens* and (f) *Enallagma cyathigerum*.

abundant structures similar to chemical sensilla in the ventral side of the aedeagus head (Fig. 3). These putative chemical sensilla were less evident in the other species, but some spiny structures found in all species might also function as sensilla (Fig. 3).

DISCUSSION

We found that the ability to adjust copulation duration in relation to female mating status is widespread in male damselflies of three families. Our results therefore corroborate previous findings in three further coenagrionid damselflies, *Ischnura graellsii* (Cordero 1990), *I. elegans* (Cooper et al. 1996) and *Ceriagrion tenellum* (Andrés & Cordero Rivera 2000). The ability to modulate copulation duration in relation to female mating status is common in insects and arachnids (see references in Andrés & Cordero Rivera 2000). Usually copulation is prolonged with mated females, because the last male to mate with a particular female tries to remove the sperm from previous matings. In some cases, when the risk of sperm competition is high, males increase the amount of sperm inseminated (strategic ejaculation; Gage & Baker 1991; Gage & Barnard 1996; Wedell & Cook 1999), and this might also prolong copulation with nonvirgins. An interesting exception is the bedbug, where males reduce copulation duration and sperm numbers if the female has recently copulated, probably because the first male's ejaculate is subjected to disproportionate phagocytic attack in this species with 'traumatic' insemination (Siva-Jothy & Stutt 2003). Long copulations are nevertheless not strictly necessary for sperm removal or strategic ejaculation. For instance, some calopterygids have very short copulations (3–6 min) but nevertheless remove most of the rivals' sperm (Waage 1979; Córdoba-Aguilar 2001) and inseminate the full content of their sperm vesicle. Earwigs, *Euborellia plebeja*, have developed long penises apparently as a response to the elongation of the spermatheca and remove sperm in just 4.6 min (Kamimura 2000).

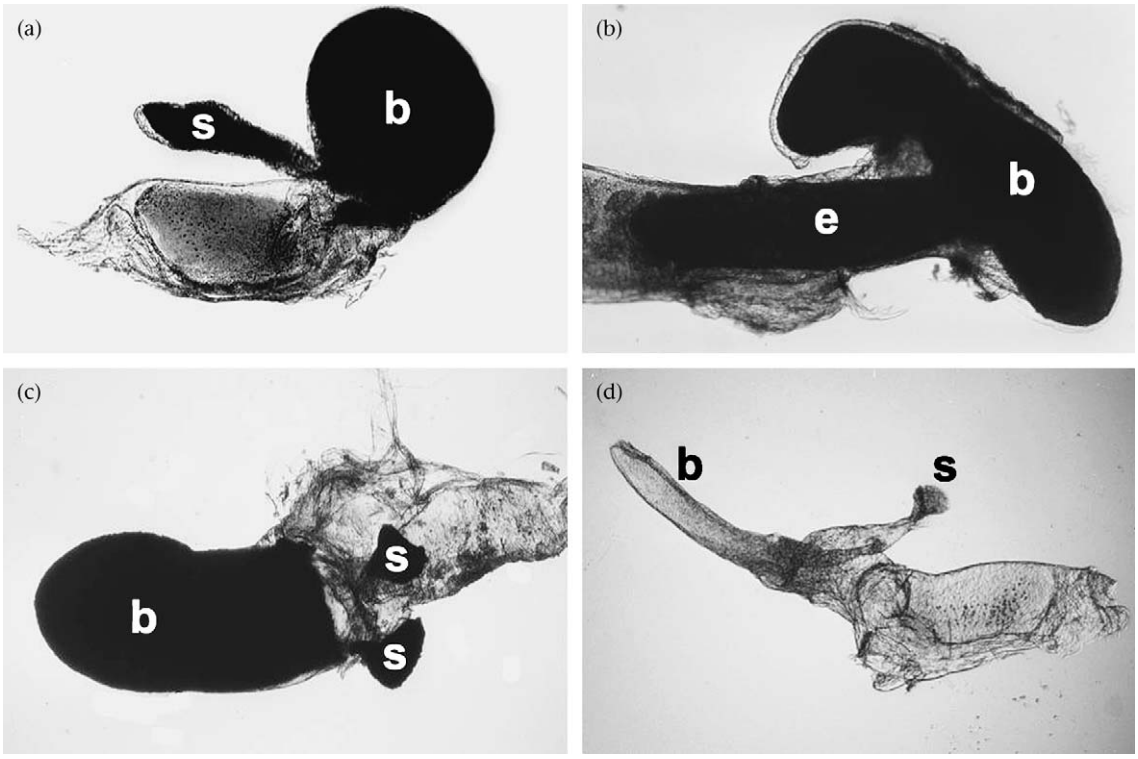


Figure 2. Female genitalia in platycnemids and lestids, showing the relative size of the bursa (b) and spermatheca (s). (a) *Platycnemis latipes*, (b) *Lestes virens*, (c) *L. viridis* and (d) *S. fusca*. The sperm is visible as dark masses in all cases except *Sympecma fusca*, because it was a virgin female. One egg (e) was found in the genitalia of *L. virens*.

Therefore, our results suggest that sperm competition alone is unlikely to explain the difference in mating duration between virgins and mated females. As far as we know, this study is the first to show that copulation duration with previously mated females is related to the presence/absence of a spermatheca(e): the second copulation lasted for twice as long as the first one in species with a spermatheca, but was only slightly longer in species without a spermatheca (Table 1). This behaviour could be explained by sperm competition if males use the extra time to remove sperm. This is unlikely, however, because (1) male genitalia do not have flagella in any of the species we analysed, suggesting that they are unable to remove sperm physically from the spermatheca and (2) the bursa copulatrix is emptied in about 10–15 min in all species except *P. latipes*, which needs about 1 h (unpublished data). The alternative explanation of long matings as copulatory guarding (Cordero 1990) is also unlikely because males do not prolong copulation with virgins.

Our results therefore suggest that males prolong copulation with mated females for reasons other than sperm removal or mate guarding. We suggest that males engage in long matings with mated females because they are courting, and trying to stimulate females to use their sperm for egg fertilization instead of rivals' sperm stored in the spermatheca; alternatively, females might expel rivals' sperm from the spermatheca because of male stimulation (Andrés & Cordero Rivera 2000; Córdoba-Aguilar 2002). Both mechanisms might be considered examples of copulatory courtship (Eberhard 1994) selected by cryptic female choice, and clearly suggest that female control over fertilization in damselflies has been underestimated, probably because of the widespread male ability to remove rivals' sperm (Waage 1984; Córdoba-Aguilar et al. 2003). In fact, there is evidence that female damselflies are able to fertilize the eggs using the sperm from either the bursa or the spermatheca (Siva-Jothy & Hooper 1996), and, in *C. tenellum*, males that engage in longer copulations fertilize more eggs (Andrés & Cordero Rivera 2000).

Our results clearly show that even in species where male control over reproductive decisions was considered prevalent, there is ample 'female control' (Eberhard 1996). There is currently great interest in female behaviour in sexual selection studies, because molecular techniques have revealed that polyandry is widespread (Zeh & Zeh 2003), and odonates are not an exception. Our interpretation of copulatory behaviour is in agreement with reproductive behaviour in lestids. In this family most species do not have a spermatheca, but *L. viridis* has two small spermathecae. Copulation was very short in species without a spermatheca, and was longer in *L. viridis*, again suggesting that the sperm stored in the spermatheca is the main cause of this difference. Given the genital morphology of females and males, we predict that *S. fusca* should show a strong effect of female mating status on copulation duration.

Our results on copulation duration and its component stages in *E. cyathigerum* coincide with those of other authors (Perry & Miller 1991). We used the gross values of copulation duration (including the time in which the male disengages its genitalia but remains in tandem) because in *E. cyathigerum* the interruptions in stage I seem

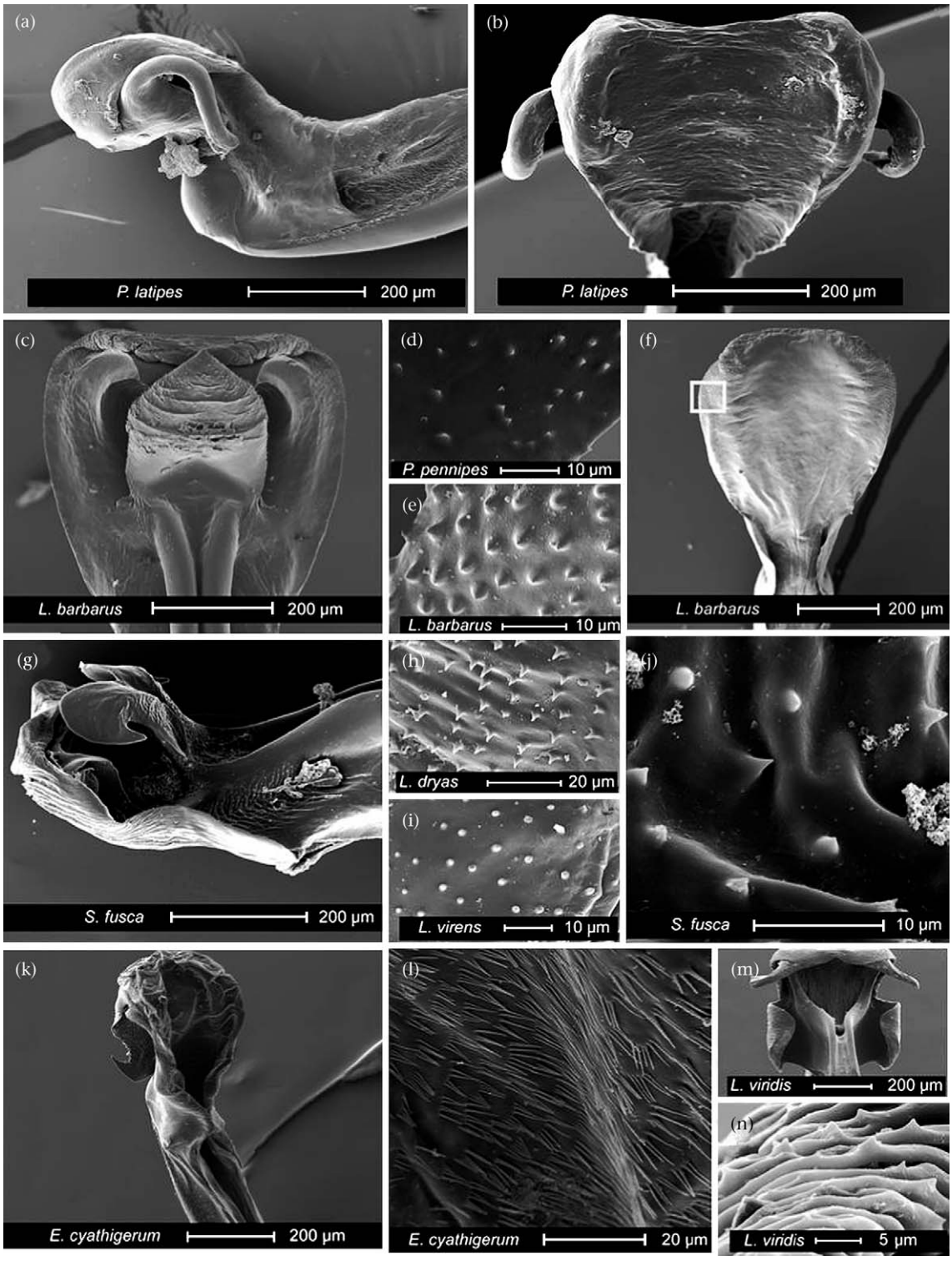


Figure 3. Male genitalia observed under a scanning electron microscope (a) lateral and (b) ventral view of the aedeagus in *Platycnemis latipes*, showing one of the paired horns, probably used to remove sperm from the bursa. A detail of putative chemical sensilla in *P. pennipes* is shown in (d). (c) Dorsal and (f) ventral view of the aedeagus in *Lestes barbarus*, with details of putative sensilla in (e). The region where these sensilla are more common is indicated with a square in (f). (g) Lateral view of the aedeagus in *Sympecma fusca*. Putative sensilla are shown in (h) *L. dryas*, (i) *L. virens* and (j) *S. fusca*. (k) Lateral view of the aedeagus head in *Enallagma cyathigerum*, with (l) the spines that are probably used to remove sperm. (m) Dorsal view of the aedeagus in *L. viridis*, the only lestid with two spermathecae. Note the absence of flagellae to remove sperm from the spermathecae. This species also bears putative chemical sensilla (n).

to be an essential part of copulation. Perry & Miller (1991) suggested that these interruptions may facilitate sperm removal. This hypothesis would be supported by the fact that the number of interruptions is positively correlated with the duration of stage I (Perry & Miller 1991). Alternatively, if males are unable to remove spermathecal sperm physically (which seems to be the case given the aedeagus morphology; Fig. 3), then the interruptions during stage I might facilitate the expulsion of the sperm ejected by the female from the spermatheca instead of the sperm removed by the males from the bursa (or both).

There is growing evidence for the evolution of animal genitalia by sexual selection processes (Eberhard 1985). Postcopulatory sexual selection, either by sperm competition or by cryptic female choice, is widespread and has strong effects on reproductive behaviour and genital evolution (Arnqvist 1997, 1998; Otronen 1998; Tadler 1999; Arnqvist et al. 2000; House & Simmons 2003; Cordero Rivera et al. 2004; Hosken & Stockley 2004). In extreme cases male genitalia can even damage females during copulation (Crudgington & Siva-Jothy 2000), which is indicative of high sexual conflict (Parker 1979; Rowe et al. 1994; Brown et al. 1997; Johnstone & Keller 2000; Chapman et al. 2003). Our results suggest that prolonged copulations in damselflies are probably the result of cryptic female choice, but sperm competition has clearly influenced these behaviours, as the effect of rival density on copulation duration shows (Cordero Rivera 2002). Furthermore, given that we did not measure female fitness, we do not know whether prolonged copulations are detrimental or beneficial for females, and this merits further study.

Finally, we have found that male genitalia bear structures similar to chemical sensilla. These structures would allow males to detect female mating status, although we cannot discount the existence of an external chemical mark (but see Andrés & Cordero Rivera 2000). In lestids these structures are very conspicuous and are concentrated on the ventral side of the head of the aedeagus (Fig. 3), the area that is most likely to be in contact with the sperm stored in the bursa when copulation starts. Preliminary experiments with *L. viridis* indicate that the penis head has neural connections with the ganglion on the second abdominal segment (E. Uhía, unpublished data). There is now evidence for the presence of sensilla in male genitalia in several insect orders (Spiegel et al. 2000; Siva-Jothy & Stutt 2003). We predict that chemical receptors should be common in male genitalia of odonates, given the widespread ability of males to detect female mating status, and a detailed inspection of other orders would be illuminating.

In summary, this study shows that copulation duration is affected by sperm competition and cryptic female choice in odonates, and suggests that the spermatheca is a key organ in the subtle conflict between male and female insects.

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