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Male disturbance, repeated insemination and sperm competition in the damselfly *Coenagrion scitulum* (Zygoptera: Coenagrionidae)

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Abstract. Before mating, all male odonates translocate sperm from the testes (IX segment) to the penis (II segment), but in *Coenagrion scitulum* this behaviour is repeated up to six times during copulation. The aim of this study was to find an explanation for this unusual behaviour. Copulation behaviour consists of three to seven cycles, each of which includes one intra-male sperm translocation, one stage I and one stage II (i.e. the complete copulatory sequence typical of zygopterans). The duration of pre-copulatory tandem and cycle 1 of copulation was negatively correlated with time of day and positively with male disturbance. Males captured during stage I of any copulatory cycle always had the seminal vesicle full of sperm, thus indicating that they do translocate sperm during sperm translocation behaviour. Females were inseminated at stage II. The volume of ejaculate in females in the field interrupted during stage I of the first copulatory cycle was not significantly different from the volume stored by pre-copula females indicating that males cannot remove a significant amount of sperm from the female's genital tract at this stage. Spines on the horns of the penis, which in other damselflies help remove the sperm stored by females, are, however, absent in *C. scitulum*. Experiments with virgin females that received up to five inseminations indicated that the sperm are progressively packed, and therefore the volume is not directly proportional to the number of inseminations. Male *C. scitulum* thus has a poor sperm removal ability and multiple intra-male sperm translocation and insemination of the female during the same copulatory act seems to be a mechanism of sperm competition by which the male achieves a greater fertilization success.

Competition between males to achieve greater fertilization success does not end after mating. When females mate with more than one male and there is a delay between mating and fertilization, as is the case in most insects, sexual selection should favour mechanisms during and after mating that promote greater fertilization success (Parker 1970). The potential for sperm competition has favoured the evolution of the ability to remove rival sperm, which was first described in damselflies (Waage 1979). The recent finding of sperm removal in at least two other insect orders, the Orthoptera and the Coleoptera (Ono et al. 1989; von Helversen & von Helversen 1991; Gage 1992) suggests that this might be much more common than assumed thus far.

Among the insects the Odonata are unique in that they possess a secondary genitalia in the second abdominal segment, which has no internal communication with the testes. For this reason, before every mating, males must bring in contact the abdominal segments IX and II in order to translocate sperm from the primary reservoir to the (secondary) seminal vesicle (Fig. 1; the intra-male sperm translocation behaviour of Bick & Bick 1970). This characteristic behaviour is performed while the male holds the female's head or prothorax with his cerci in the so-called tandem position, a linkage that is maintained throughout the entire copulation and often (part of) the oviposition (Fig. 1). The tandem position probably functions as a mechanism by which the male

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Figure 1. The intra-male sperm translocation behaviour of *C. scitulum*. While holding the female with his terminal appendages, the male (upper position) brings his genital opening (segment IX) to contact the penis (segment II).

prevents other males from mating with his female, since this would cause the loss of precedence in fertilizing the female's eggs (Parker 1970; Waage 1979). During copulation the female brings in contact the tip of her abdomen with the male's secondary genitalia forming the copulatory wheel.

In contrast to most odonates so far studied, in *Coenagrion scitulum* (Coenagrionidae) intra-male sperm translocation is repeated up to seven times per mating. We know only one other species in which this occurs as a rule (*Megaloprepus coerulatus*, Pseudostigmatidae; Fincke 1984a).

In some Odonata, particularly in the Zygoptera, copulation can be divided into several stages, according to the movements and position of male and female abdomens. Miller & Miller (1981) divided the copulation of *Enallagma cyathigerum* into three stages. In stage I the male moves the abdomen at a rhythm of 10–60 cycles per min. The second phase, or stage II, is much shorter, and the movements appear at 9–22 cycles per min. After this stage, there is a phase in which the male's abdomen remains motionless (stage III). Since Miller & Miller's (1981) paper, many authors have reported the existence of different stages in the copulation of other Zygopteran species: Coenagrionidae (*Argia fumipennis*: Bick & Bick 1982; *Cercion sieboldii*: Naraoka 1986; *Ischnura elegans*: Miller 1987a; *Ischnura verticalis*: Fincke 1987; *Ischnura graellsii*: Cordero 1989, 1990a), Lestidae (*Lestes virens* and *L. barbarus*: Utzeri et al. 1987), Calopterygidae (*Mnais pruinosa*: Siva-Jothy & Tsubaki 1989), Protoneuridae (*Nosoticta kalumburu*: Thompson 1990).

All these observations in members of four families of Zygoptera suggest that there is a functional explanation for the existence of these different stages. In the species where copulation has been studied in detail, the male removes rivals' sperm from the female genital tract in stage I and inseminates the female during stage II, and probably also during stage III (Naraoka 1986; Miller 1987b; Perry & Miller 1991; Cordero & Miller 1992).

Utzeri & Sorce (1988a) first described the copulatory activity of *C. scitulum*. They reported the existence of two phases. In the first phase, the male makes a series of rocking movements, intercalated with periods of no movements. During this phase the copulatory wheel breaks up to seven times leaving the pair in tandem for periods of 1–10 min. In the second phase, no pauses are observed and the copulatory wheel is interrupted up to five times at regular intervals while he translocates sperm to the seminal vesicle.

Our aim in the present study was to determine the occurrence and duration of the copulatory stages (in the sense of Miller & Miller 1981) of *C. scitulum* in order to find an explanation for this repeated intra-male sperm translocation. We studied the reproductive behaviour of this species in field and laboratory experiments. In addition, we interrupted copulation at different moments and measured the volume of sperm stored by males and females. We specifically tested the following hypotheses for the repetition of sperm translocation put forward by Utzeri & Sorce (1988a): (1) a single sperm translocation act may

not be enough for full insemination; (2) the amount of sperm available cannot be translocated in one go; (3) only seminal fluids are transferred at first to facilitate removal of rival sperm by diluting it; (4) a large amount of sperm is transferred to the female because the male is able to remove most of the stored sperm; and (5) alternatively, the male has only a limited sperm displacement ability, so a large ejaculate is needed to dilute rival sperm with self sperm.

METHODS

Field Observations

We studied two natural populations of *C. scitulum* in Castel Porziano, Roma, Italy, during 10 days in May–July 1991 and 9 days in June–July 1992. Observations were carried out in a portable net insectary ($2 \times 2 \times 2$ m), into which individually marked specimens were introduced. Most individuals performed normal behaviour after a brief period of acclimatization. All records were made by two to four observers, usually between 1000 and 1300 hours, on pairs spontaneously formed in the insectary. After the introduction of males and females into the insectary, we recorded the time of formation of pre-copulatory tandem, the identification codes of specimens involved, and the time and duration of copulatory phases (intra-male sperm transfer, breaks of the copulatory wheel, stages of copulation). We also recorded the number of disturbances to the copulating pair by single males present inside the insectary. In 1992, using a standard VHS camera in close-up position, we video-recorded the copulatory movements of some pairs outside the insectary, to allow a detailed analysis of copulatory activity.

Phases of copulation were defined according to the movements and position of the male's abdomen. The second phase (stage II) was of very short and constant duration (see Results) and difficult to detect in the field. In stage III there was little or no observable movement, but the transition from stage II was gradual and inconspicuous. Therefore we divided field copulations into cycles (stage I + stage II) using an unambiguous cue: the repetition of intra-male sperm translocation. We assumed that this behaviour indicated that the male had just inseminated the female.

To see if the ejaculate volume increases with the number of sperm translocations, some matings were experimentally interrupted at different moments during the first few minutes of copulation (before the repetition of sperm translocation; $N = 25$), and after one or more sperm translocations (and probably inseminations; $N = 27$). The specimens from these interrupted copulations were preserved in vials containing 70% ethanol with some drops of formaldehyde, and kept at 4°C until dissection. The storage organs of the males (seminal vesicle) and females (bursa copulatrix and spermatheca) were dissected out and the sperm mass was compressed to the uniform thickness of 0·05 mm under a supported coverslip on a slide (Miller 1987b; Cordero & Miller 1992). Camera lucida drawings were made of every sperm mass, at magnifications of 50, 60, 125 or 150 times, depending on the size of each mass. The area of every drawing was measured twice with a planimeter Placom KP90, and the mean of the two measurements was used in the analysis. The ejaculate volumes were estimated as the products of each mean area and the thickness of the preparations (0·05 mm for all). Sperm appeared of homogeneous density in most preparations. In a few cases the sperm mass expanded, and these were excluded from the analysis. The morphology of the penis was observed by scanning electron microscopy.

Laboratory Observations

Final instar larvae and 1-day teneral adults of *C. scitulum* were collected from a natural population in Corrubedo (A Coruña, Galicia, Spain) during April–May 1992. These larvae were reared in the laboratory in plastic containers filled with tap water until adult emergence. They were fed ad libitum with freshwater invertebrates. The adults were marked and put into insectaries of $50 \times 50 \times 50$ (or 70) cm, where they were fed daily with *Drosophila* (see Cordero 1990a, b).

The first adults we obtained, maintained at 21–23°C and 15:9 h light:dark, did not develop the typical blue coloration of field specimens and failed to mate, probably because intraspecific recognition in odonates is based on visual cues (Miller 1987c). We had the same problem when we put the insectaries under natural indirect light (photoperiod of May–June) and room temperature (about 20°C). We therefore painted, using water colours, two small blue post-ocular spots and two antehumeral lines on the females to make

them resemble field-collected females. These painted females mated successfully.

A total of 36 matings involving 19 males and 36 females were obtained in the laboratory. Twenty-two copulations were experimentally interrupted after up to four sperm translocations, and the remaining 14 copulations ended spontaneously after three to five sperm translocations. The ejaculate volumes in the females were measured as previously described. Females were used in just one mating (therefore all were virgin), but eight males mated two to eight times, because there was no attempt to induce or to prevent a particular male from mating. We believed it is unlikely that this depleted the sperm reserves transferred to the female, because the interval between consecutive copulations was 2·5 days (SE = 0·7, $N = 8$ males) and most of the previous copulations of these males had been interrupted (only two males completed more than one copulation). To obtain matings, mature painted females (9–15 days of age) were introduced into the insectary containing males, and the males themselves started the matings.

To test Utzeri & Sorce's (1988a) hypothesis 1 (a single sperm translocation may not be enough for full insemination) we allowed eight virgin females interrupted after the first copulatory stage II (after one insemination) and six virgin females upon ending copulation to oviposit. If one insemination is enough, then no difference should be found in the proportion of fertile eggs laid by the two samples. To test hypotheses 3, 4 and 5 we examined ejaculate volumes in first-mated (virgin) females.

Values are presented as $\bar{X} \pm$ SE. In parametric analyses integer variables (number of disturbances, age, etc.) were transformed as square root of (variable + 0·5).

RESULTS

Pre-copulatory Tandem

In the field, pre-copulatory tandem (from the seizure of the female to the start of copulation) lasted 10·1 ± 2·6 min ($N = 55$; range: 0·03–81·3 min). In the laboratory, males remained less time in tandem with non-receptive females (those that did not accept copulation, 19·2 ± 6·7 min, $N = 14$) than with receptive ones (24·3 ± 5·0 min, $N = 36$; Mann–Whitney test: $U = 4·7$, $P < 0·001$).

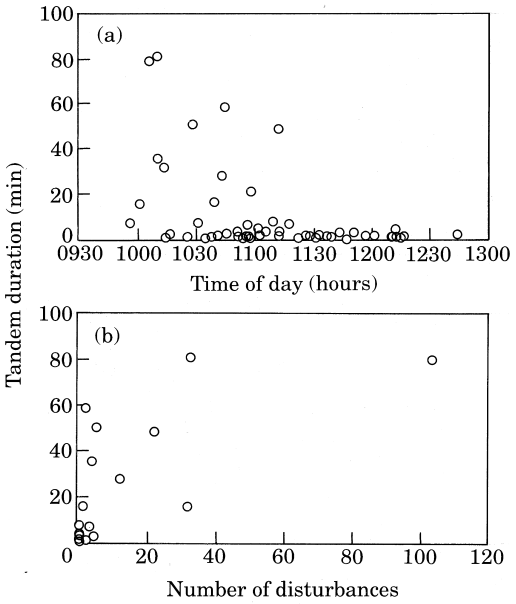

Figure 2. The relationship between tandem duration in the field and (a) time of day ($N = 55$ pairs) and (b) the number of male disturbances ($N = 30$ pairs).

We excluded two very long tandems (one lasted 23 h 33 min), which were probably caused by the females dying while still in tandem (Cordero et al. 1992).

The duration of pre-copulatory tandem in the field was negatively correlated with time of day ($r = -0·45$, $N = 55$, $P < 0·001$; Fig. 2a), and positively with the number of times single males disturbed the copulating pair ($r = 0·79$, $N = 30$, $P < 0·001$; Fig. 2b). Using a step-wise multiple regression procedure, we tested the effect of time of day and number of disturbances on tandem duration: the number of disturbances explained 62% of variance in tandem duration ($P < 0·001$), but time of day had no significant effect.

In the laboratory, tandem duration was also negatively correlated with time of day ($r = -0·48$, $N = 35$, $P = 0·004$). A step-wise multiple regression procedure, with tandem duration as the dependent variable and time of day, male and female age, and male and female length as the independent variables, indicated that both time of day and male age had a significant and negative effect on tandem duration ($P = 0·001$ and 0·004, respectively). Taking into account that male disturbance was not measured in the

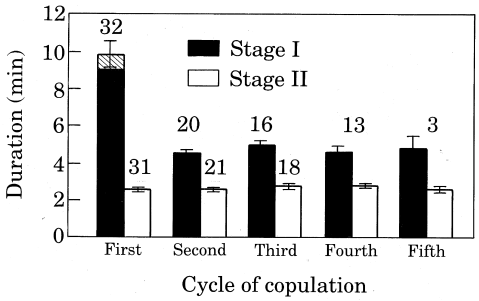

Figure 3. The duration ($\bar{X} \pm$ SE) of stages of copulation in the laboratory. □: Duration of spontaneous interruptions of copulation. Numbers are sample sizes.

laboratory it is not clear if this effect of male age on tandem duration is real or a statistical artefact.

The Stages of Copulation

Copulation took place 12·9 ± 3·03 s (N = 17) after the first sperm translocation (although in one case it was delayed 19 min after sperm translocation). Complete copulations in the field (including breaks) lasted 51·6 ± 7·7 min (N = 17; range: 13·6–141·2 min) and in the laboratory 35·2 ± 1·5 min (N = 14; Mann–Whitney test: U = 1·49, P = 0·137).

Our results indicate that copulation in *C. scitulum* is a cyclic behaviour. There were three to seven cycles, each cycle consisting of one intramale sperm translocation (3–8 s), one Miller & Miller's (1981) stage I and one stage II (i.e. the complete copulatory sequence typical of Odonates).

Stage I

Analyses of video-recordings of field pairs indicated that copulation started with rapid and energetic movements of the male's abdomen, at a rhythm of 10–24 movements per min, which became progressively less marked. During the first stage I there were periods (9–27 s) without movements, intercalated with sequences of five to seven movements. The first stage I was the longer and more variable phase of copulation (range: 4·9–23·0 min in the laboratory, and 5–50 min in the field; Fig. 3). The movements and position of the male's abdomen during this phase (Fig. 4a) are similar to the stage I of other coenagrionids

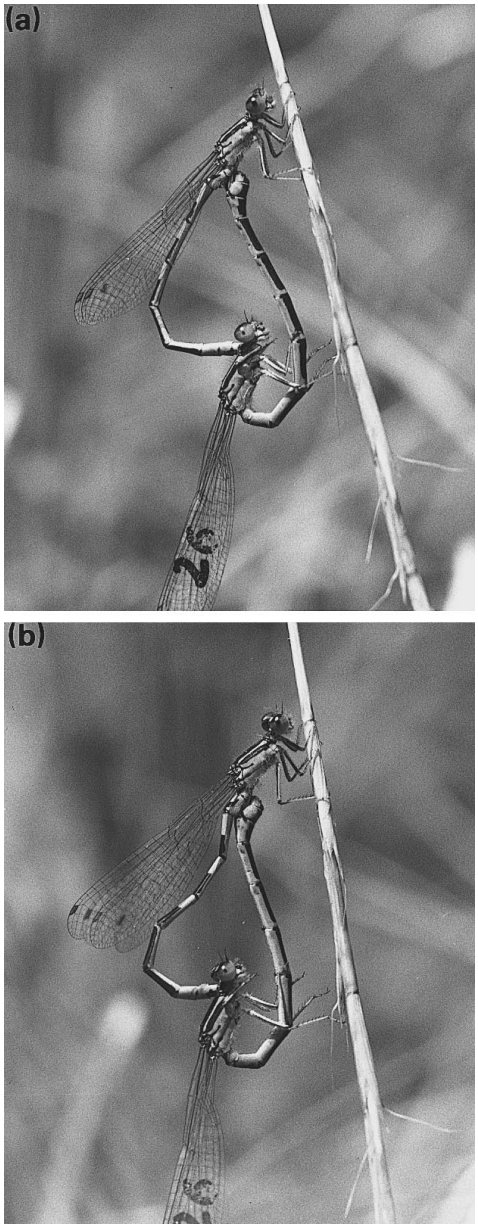

Figure 4. The stages of copulation in *C. scitulum*. The active phase of (a) stage I (note the elevation of the second abdominal segment in the male) and (b) stage II (note the flexion of the male abdomen).

(Miller & Miller 1981; Naraoka 1986; Miller 1987b; Cordero 1989). Similar (but not identical) movements are observed in the stage I of subsequent cycles (4·5–5 min).

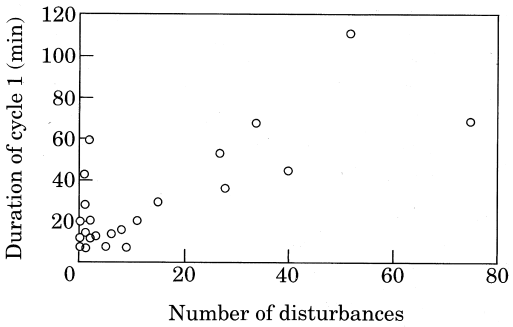

Figure 5. The relationship between the gross duration (including interruptions) of cycle 1 in the field and the number of disturbances by single males to the copulating pair.

The copulatory wheel was spontaneously interrupted during the first stage I in most field pairs (0–7 times, mode = 1, $N = 38$ matings) but rarely in the laboratory (0–2 times, mode = 0, $N = 31$ matings; Mann–Whitney U-test: $U = 3{\cdot}58$, $P < 0{\cdot}001$). The positive correlation between the number of spontaneous interruptions of cycle 1 in the field and the net duration (excluding interruptions) of this phase ($r = 0{\cdot}45$, $N = 34$, $P = 0{\cdot}007$) indicates that in longer copulations the breaks were more frequent. This relationship was not significant in the laboratory ($r = 0{\cdot}17$, $N = 31$, $P = 0{\cdot}349$). There were no interruptions during stage I of subsequent cycles.

The gross duration (i.e. including breaks) of the first stage I in the laboratory was negatively correlated with time of day ($r = -0{\cdot}37$, $N = 32$, $P = 0{\cdot}035$), but when breaks were excluded the correlation was only marginally significant ($r = -0{\cdot}35$, $N = 32$, $P = 0{\cdot}05$). Similarly, the correlation between the duration of cycle 1 and time of day in the field was also negative and significant when breaks were included ($r = -0{\cdot}36$, $N = 32$, $P = 0{\cdot}043$), but not when they were excluded ($r = -0{\cdot}27$, $N = 32$, $P = 0{\cdot}130$). When a single male approached a mating pair of *C. scitulum*, usually both partners responded by vibrating their wings, and occasionally they made a short flight in tandem following such an 'attack'. Nevertheless, we never saw a pair separate as a consequence of male disturbance. The number of interruptions was not correlated with the number of male disturbances ($r = 0{\cdot}19$, $N = 26$, $P = 0{\cdot}346$), but the duration of cycle 1 in the field was (duration with breaks: $r = 0{\cdot}75$, $N = 26$, $P < 0{\cdot}001$; Fig. 5; duration excluding breaks: $r = 0{\cdot}61$, $N = 26$, $P < 0{\cdot}001$). In a step-wise multiple regression analysis with the gross duration of cycle 1 (with breaks) as dependent variable and time of day and male disturbance as independent variables, male disturbance explained 55% of variance in cycle 1 duration ($P < 0{\cdot}001$), and time of day had no significant effect.

Stage II

In the laboratory, stage II lasted 2·5–2·7 min (Fig. 3) in all cycles. In the first 15–30 s of stage II the male made four to seven pumping movements, then the position of the male's abdomen gradually changed, and finally there was a series of subtle oscillatory movements at a rate of 30–55 per min (this may be considered to be stage III, but given that the change was gradual we included all this phase in stage II). The position of the male's and female's abdomens during the first 15–30 s of stage II (Fig. 4b) was similar to that in other Coenagrionidae (Miller & Miller 1981; Naraoka 1986; Miller 1987a; Cordero 1989).

In the field the duration of cycles 2–7 together was negatively correlated with time of day ($r = -0{\cdot}55$, $N = 19$, $P = 0{\cdot}015$) owing to a reduction in the duration, rather than the number, of cycles (correlation between number of cycles and time of day, $r = -0{\cdot}15$, $N = 17$, $P = 0{\cdot}557$). The duration of cycles 2–7 was not correlated with the number of disturbances during these cycles ($r = 0{\cdot}08$, $N = 15$, $P = 0{\cdot}783$).

In the laboratory, complete matings included three to five cycles. To investigate the factors that might explain this variability, we entered the number of cycles as the dependent variable in a step-wise multiple regression procedure, and male and female age, male and female body length and time of start of copulation as the independent variables. Only male size explained a significant amount of variance in the number of cycles of copulation (38%), small males making more cycles (regression coefficient, $b = -0{\cdot}096$, $t = -2{\cdot}701$, $N = 14$ matings, $P = 0{\cdot}0193$).

Anatomy of Genitalia

Females of the Coenagrionidae have two sperm storage organs, the bursa copulatrix and the spermatheca (Fig. 6a). In *C. scitulum* their morphology is similar to that in *Argia* and *Ischnura*

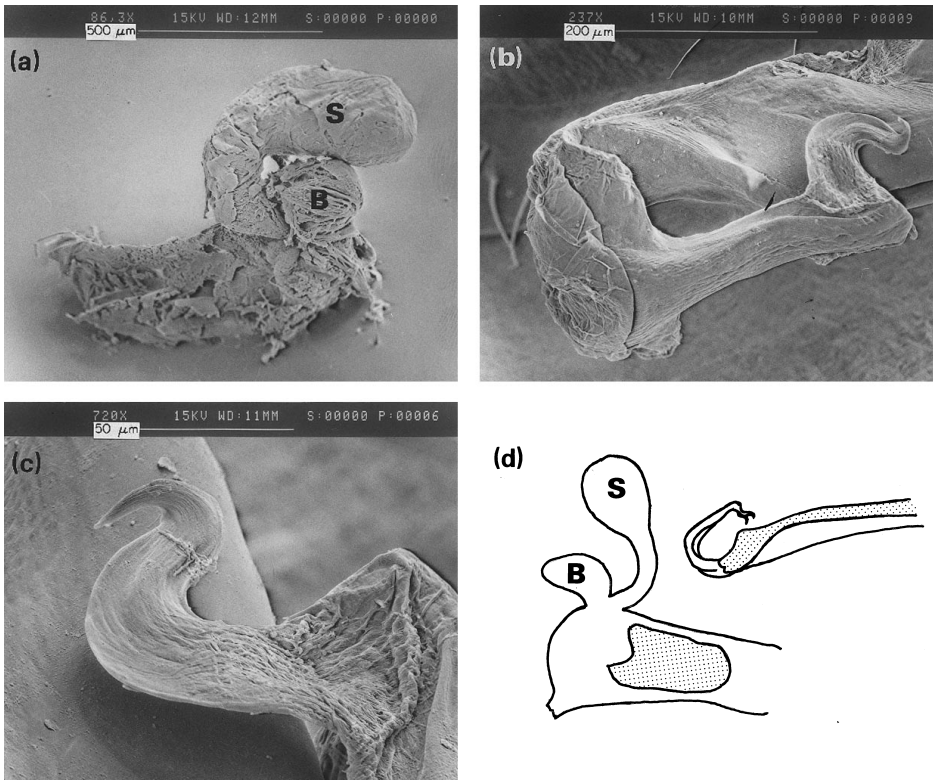

Figure 6. The genitalia of *C. scitulum*. (a) Female reproductive tract, showing the bursa (B) and the spermatheca (S); (b) distal part of the penis; (c) detail of the smooth horns of the penis. Note the absence of spines. (d) Schematic representation of male and female genitalia drawn to scale.

(Waage 1986; Miller 1987a; Cordero & Miller 1992), but the spermathecal duct is shorter and broader than in these species, which is consistent with the absence of thin flagella on the penis (Fig. 6b, c). In females dissected after completing copulation in the field, the bursa stored 20% of total sperm volume.

In *C. scitulum* males, the distal part of the penis carries two horn-like structures (Fig. 6b, c), which are similar to (but shorter than) those described for other Coenagrionidae (Waage 1986; Miller 1987a). While all damselflies known to remove sperm have spines on these horns (Waage 1986; Miller 1987a; Siva-Jothy & Tsubaki 1989; Thompson 1990), the horns on the penis of *C. scitulum* are smooth and have no spines at all, suggesting poor sperm removal ability (but see Waage 1982). Also, because they are short, the penis horns may only be capable of entering the bursa copulatrix, not the spermatheca (Fig. 6d). Unfortunately we were unable to obtain in-copula preparations, because the male and female genitalia immediately disengaged upon capture.

Ejaculate Volumes

Males

All field males captured during the first stage I of a copulation bout had full vesicles ($0{\cdot}0252 \pm 0{\cdot}0027$ mm^3, $N = 30$), as well as all males captured during stage I of subsequent cycles ($0{\cdot}0255 \pm 0{\cdot}0050$ mm^3, $N = 8$). In contrast, seminal vesicles were empty in 13 out of 14 males captured at the end of copulation ($0{\cdot}0002 \pm 0{\cdot}0002$ mm^3) as well as in all five males captured at the end of stage II of an intermediate cycle. These results indicate that insemination took place during every stage II, and that a similar volume of ejaculate was transferred in every sperm translocation. As a

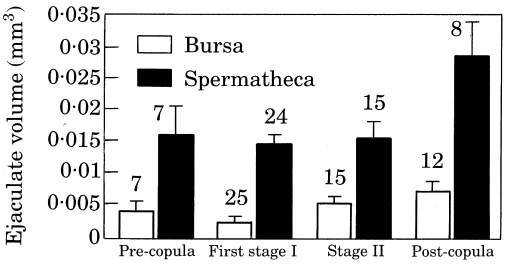

Figure 7. The volume of ejaculate ($\bar{X}$ + SE) stored in the bursa and spermatheca of field females captured at different moments of the copulatory sequence. Pre-copula: females captured before copulation; first stage I, females whose copulation was interrupted during stage I of the first cycle; stage II, females whose copulation was interrupted after a variable number of inseminations; post-copula, females captured at the end of copulation. Numbers are sample sizes.

rule, all males made the first sperm translocation before every mating, but in nine out of 66 field matings and four out of 36 laboratory matings the male did not translocate sperm before starting copulation. This occurred only (but not always) when the preceding copulation of the same male was experimentally interrupted during stage I of either cycle, even when the delay between the interrupted mating and the following copulation was greater than 1 day. The same has been observed in *I. graellsii* (Cordero 1989). This indicates that a male has information about the presence of sperm in his seminal vesicle, and that sperm translocated to that vesicle is still viable for a copulation 24 h after, and corroborates that males do not inseminate during stage I of copulation.

Field females

The ejaculate volumes stored in the genital tract of field females captured before, during and after copulation (Fig. 7) were significantly different (bursa: Kruskal–Wallis one-way ANOVA = 15·46, $N = 59$, $P = 0·001$; spermatheca: Kruskal–Wallis test = 9·15, $N = 55$, $P = 0·027$; we assume that seven females without sperm in both organs were virgins, and therefore were excluded from the analysis). Because sperm are used to fertilize eggs after each copulation, we had a priori reasons to expect that the volume of ejaculate in pre-copula females should be smaller than the volume in post-copula females. Although this is true, the difference is significant only for the spermatheca (one-tailed Mann–Whitney test: bursa: $U = 0·25$, $P = 0·403$; spermatheca: $U = 1·68$, $P = 0·047$). In the same way, if males remove a significant amount of sperm during stage I, then the volume of sperm in females interrupted during the first stage I should be smaller than the volume stored in pre-copula females. The volume of ejaculate was smaller in the interrupted females, but given the sample size available the difference is not significant (one-tailed Mann–Whitney test: bursa: $U = 1·35$, $P = 0·088$; spermatheca: $U = 0·54$, $P = 0·294$).

For females interrupted during stage I of the first cycle there was no significant relationship between the ejaculate volume and the net duration of stage I (excluding breaks; bursa: Spearman $r_S = -0·29$, $N = 24$, $P = 0·157$; spermatheca: $r_S = -0·10$, $N = 23$, $P = 0·625$).

The ejaculate volume in the vesicle of males captured during stage I of the first cycle (0·0252 mm^3) was only slightly smaller than the volume stored in the bursae plus spermathecae of females captured at the end of copulation after up to seven inseminations (0·0346 mm^3). This apparent anomaly could be explained if the majority of the sperm are removed at every stage I and if compaction occurs during insemination (also Waage 1986; Miller 1987b), or if some of the sperm are expelled by the female, and indicates the existence of a maximum volume that the female can store.

The correlation between the number of inseminations and the volume of ejaculate stored in field females is not significant (Fig. 8a; spermatheca: $r_S = 0·04$, $N = 22$, $P = 0·867$; bursa: $r_S = 0·22$, $N = 22$, $P = 0·312$).

Laboratory females

None of four females interrupted before stage II of the first copulatory cycle contained sperm, which confirms that males do not inseminate during stage I. The ejaculate volume in the spermatheca increased with the number of copulatory cycles (Spearman $r_S = 0·49$, $N = 34$, $P = 0·008$), while it remained more or less constant in the bursa ($r_S = 0·22$, $N = 34$, $P = 0·158$; Fig. 8b). After five copulatory cycles the volume in the spermatheca was about five times that in the bursa. If males inseminate the same amount of sperm irrespective of the copulatory cycle, as they seem to do (see above), then the total volume of ejaculate should

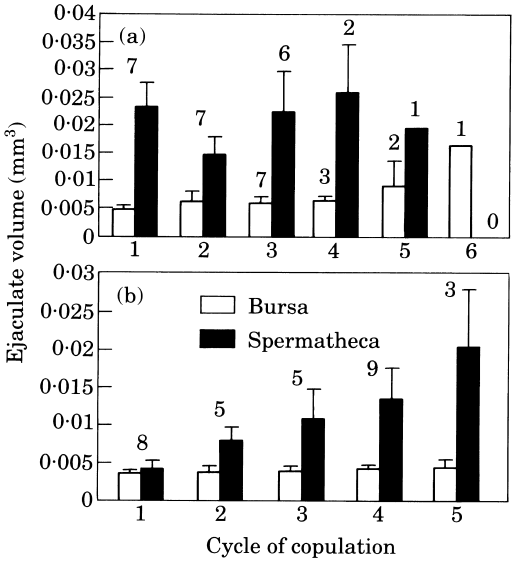

Figure 8. The volume of ejaculate ($\bar{X}$+SE) stored in the bursa and spermatheca of (a) field females and (b) virgin females in the laboratory after completing a variable number of copulatory cycles. Numbers are sample sizes.

be directly proportional to the number of inseminations. Comparing the volume of ejaculate measured in females that received two to five inseminations with that expected if males inseminated at every cycle the average volume found in once-inseminated females, we found that in 20 females the observed volume was smaller than that expected, with great deviations in many cases, while only 10 females had slightly more sperm than expected (Mann–Whitney test: $U=2{\cdot}75$, $P=0{\cdot}006$). Three alternatives can explain this result, namely sperm removal or packaging by the male, or sperm ejection by the female.

Of the eight virgin females interrupted after the first copulatory stage II (after one insemination) and six virgin females interrupted upon ending copulation, only two oviposited. One interrupted female laid 25 eggs, all fertile. The other female, which oviposited after one complete copulation, laid just two eggs, both fertile.

DISCUSSION

Male Disturbance and Mating Activity

In some insects the duration of copulation is long and negatively correlated with time of day, which has been interpreted as an 'in-copula' guarding strategy (Sillén-Tullberg 1981; Robertson 1985; Miller 1987a; Svärd & Wiklund 1988; McLain 1989; Cordero 1990a). In these species males do not guard during oviposition, and copulation ends when it is unlikely that the female will remate that day. Utzeri & Sorce (1988b) first showed that pre-copulatory tandem and copulation duration are negatively correlated with time of day in *C. scitulum*, and suggested a guarding function for this phenomenon. In our observations, pre-copulatory tandem and cycle 1 of copulation were not only negatively correlated with time of day, but also positively correlated with the number of disturbances by single males to the pair. Furthermore, only male disturbance explained a significant amount of variation in tandem and copula duration when both variables were taken into account. This suggests that males prolong tandem and cycle 1 of copulation as a response to disturbance by other males. Males could be guarding the female by prolonging tandem and copulation, but male *C. scitulum* guards strongly during oviposition, and therefore there is no need for a pre-copulatory or in-copula guarding. A possible explanation for these correlations could be based on the effect of temperature: as the day advances temperature rises, and this could produce both shorter copulations and greater male disturbance. Nevertheless, although in the laboratory temperature was fairly constant, the duration of pre-copulatory tandem and cycle 1 of copulation were still negatively correlated with time of day. Therefore this interesting result is not yet fully explained. Perhaps males are assessing male competition, the risk of sperm competition or female quality, delaying oviposition until few single males are around. The fact that the duration of cycles 2–7 was negatively correlated with time of day, but not with male disturbance, indicates that male harassment affects only the pre-insemination phase of copulation. The duration of post-copulatory tandem in *L. virens* was positively correlated with male disturbance (Utzeri & Ercoli 1991) and negatively with time of day (Utzeri & Sorce 1988b) in a probable case of post-copulatory guarding. In contrast, Perry & Miller (1991) found no relationship between copulation duration and the frequency of male interference in *E. cyathigerum*, a species that also oviposits in tandem.

Mating Activity and the Stages of Copulation

Copulation in *C. scitulum* consisted of three to seven cycles, with one sperm translocation, one stage I and one stage II per cycle. We interpret this as the repetition of a normal (complete) copulatory act, which is unique among the Odonata. Additional intra-copula sperm translocation has also been reported in *Cercion sieboldii* (four out of 14 pairs translocated sperm a second time during copula, Naraoka 1986), *L. barbarus* (in which it seems to occur just occasionally, Utzeri et al. 1987; Utzeri & Falchetti 1990), and *M. coerulatus* (Fincke 1984a gave a brief report of it in this species without additional details).

Our results indicate that insemination occurs at stage II of every copulatory cycle. First, virgin females whose copulation was interrupted before the first stage II had received no sperm. Second, males have full seminal vesicles during stage I and empty vesicles after stage II, irrespective of the copulatory cycle. Third, in most cases when males re-mated after one interrupted mating, they did not repeat sperm translocation if the interruption was during stage I, presumably because their seminal vesicle was still full. Finally, the volume of ejaculate stored by first-mated females increased with the number of copulatory cycles.

The functional significance of stage I is less clear. Stage I of the first copulatory cycle was always longer and more variable than stage I of subsequent cycles (Fig. 3). First stage I was also distinguished by the presence of pauses in abdominal activity and spontaneous breaks in which the genitalia were disengaged. Furthermore, during this phase, the female has not yet been inseminated, while in stage I of subsequent cycles an increasing number of inseminations has been made. Therefore it is likely that the function of stage I of cycle 1 is different to that of stage I of subsequent cycles.

Several lines of evidence suggest that sperm are displaced from the female genital tract during stage I of cycle 1. First, the movements of the male's abdomen during this phase are identical to the removal movements of *Ischnura* (Miller 1987a; Cordero & Miller 1992) and *Cercion* (Naraoka 1986). Furthermore, the first stage I has spontaneous breaks of the copulatory wheel that could be used to expel the removed sperm from the female (Perry & Miller 1991; E. González Soriano, personal communication). There is some evidence in favour of this hypothesis: (1) laboratory copulations (with virgin females) had fewer interruptions than field copulations (this could also be attributable to the laboratory environment); (2) in our field observations, interruptions were more frequent in longer copulations, and it is likely that the removal of sperm is associated with long copulations, as occurs in *I. graellsii* (Cordero 1990a); and (3) the second mating of females whose first copulation was interrupted at the end of cycle 1 had fewer interruptions than the second mating of females that completed the first copulation, perhaps because the interrupted females had fewer sperm to remove (Utzeri & Sorce 1988a).

The preceding observations suggest that males are trying to remove sperm during the first stage I, but other results indicate that the sperm removal ability of male *C. scitulum* is poor. First, the horns of the penis lack the spines observed in other species that remove sperm (but lack of spines does not impede the removal of sperm, Waage 1982). Second, field females captured during stage I of cycle 1 did not have significantly fewer sperm than those captured in the pre-copulatory tandem, although the difference is marginally significant for the bursal volume (Fig. 7). Third, in field females interrupted before the first insemination, there is no correlation between the amount of sperm stored and the net duration of stage I (i.e. the duration of sperm removal). Some of these results could be explained by the existence of a variable amount of sperm in these field females, which obscured the sperm displacement ability. For instance, the fact that the ejaculate volumes in field females dissected after one to four copulatory cycles did not show a clear pattern (Fig. 8a) is probably because of the variable amounts of sperm they received during earlier copulations. The use of virgin females is therefore essential in future experiments (Cordero & Miller 1992).

Stage I of cycles two to seven was of very short and constant duration (about 5 min) and was never spontaneously interrupted in contrast to stage I of the first cycle. The volume of ejaculate stored by virgin females after a variable number of inseminations was less than that expected if sperm were simply added at every copulatory cycle. This could arise from sperm removal, but taking into account the short duration of stage I of cycles two

to seven and the morphology of the penis, this result is likely to be caused by sperm compaction. This makes sense because after the first copulatory cycle the female has already been inseminated, and it seems surprising that the male removes his own sperm. It is unlikely that females could expel sperm during this phase because there are no interruptions of the copulatory wheel (other than the interruptions to repeat sperm translocation). Further experiments are needed to clarify this question.

Repetition of Sperm Translocation

Five hypotheses might explain the occurrence of multiple sperm translocations.

(1) Full insemination may require several sperm translocations. One virgin female that received just one insemination laid 25 eggs, all fertile. These represent only a small fraction of a clutch of eggs normally laid by a Coenagrionid female (Banks & Thompson 1987; Gribbin & Thompson 1990; Cordero 1991), but demonstrate that viable sperm had been transferred to the female in the first cycle of copulation. Unfortunately we were unable to elicit oviposition in the laboratory, even in females that completed copulation. Two field females captured during oviposition and transported to the laboratory did not oviposit the same day of capture, but they laid several hundred eggs the day after. One insemination is not enough to fill up the spermatheca of a virgin female (Fig. 8b) but perhaps enough sperm are received to fertilize a normal clutch (female *E. hageni* shows full sperm loads after fertilizing a full clutch of eggs; Fincke 1984b). Taking into account that complete copulations in the field included three to seven inseminations, we conclude that several inseminations are not necessary to fertilize all the eggs of a female.

(2) The male may be unable to transfer the large volume of ejaculate produced by the testes to the male seminal vesicle in one go. This hypothesis is difficult to test. Assuming that males inseminate 0·0252 mm^3 of sperm at every cycle (the average volume found in the seminal vesicle of males interrupted during the first stage I), the total volume transferred to the female in a complete copulation should be 0·0756–0·1764 mm^3. It seems likely that the male's seminal vesicle cannot accommodate an ejaculate of this size, but this hypothesis does not explain why the males repeat insemination given that the volume stored by post-copula females averaged only 0·0346 mm^3 ($N=8$; range: 0·0202–0·0598 mm^3). Therefore, males could transfer most of the volume that the female can store in just one act of insemination. Hypothesis 2 alone seems unable to account for repeated sperm translocation.

(3) Only seminal fluids may be transferred first, to facilitate ulterior sperm dilution or removal. The seminal vesicle of males that had been interrupted during stage I of different cycles was always full of sperm. This result, together with the fact that one virgin female laid fertile eggs after just one insemination, indicates that this hypothesis cannot explain multiple sperm translocation in *C. scitulum*.

(4) A large amount of sperm may be transferred to the female because the male is able to remove most of the sperm from previous matings. The results of the experiments carried out in the laboratory using virgin females, together with the absence of spines on the horns of the penis, and the comparisons of volumes between pre-copula and interrupted females, indicate that the sperm removal ability of this species is poor compared with other Coenagrionid species. Since apparently sperm are not removed at all from the spermatheca, which stores the greater amount (80%), hypothesis 4 can be rejected.

(5) The male may have a limited sperm displacement ability, and a large ejaculate may be needed to overcome dilution with rival's sperm. If males are unable to remove a significant number of sperm, an alternative way to obtain a greater fertilization success is to inseminate more. Although sperm number was not estimated, it is not unlikely that a male *C. scitulum* transfers very many sperm to his female. From the preceding discussion we concluded that the copulatory activity of *C. scitulum* consists of a first cycle during which the male makes removal attempts, then in the following two to six cycles the male repeatedly repositions the sperm and inseminates the female. The reason why some males inseminate three times while others up to seven times is still unclear but our preliminary data from laboratory matings suggest that small males tend to make more inseminations. This is consistent with the behaviour of small *Scatophaga* males which copulate for longer and inseminate more sperm (Simmons & Parker 1992).

Conclusion

Waage (1979) first showed that male damselflies remove sperm from the female genital tract before transferring their own. This behaviour is one of the most specialized among those selected by sperm competition and is probably widespread in the Zygoptera (Waage 1986). Besides sperm removal, dragonfly males increase fertilization success in two other ways: (1) by guarding their females (in tandem, in copula, or without male–female contact; Waage 1984) to reduce sperm competition; and (2) by transferring large numbers of sperm to their females, to displace or outnumber rival sperm, as was suggested for the libellulids *Crocothemis* (Siva-Jothy 1984) and *Sympetrum* (Waage 1984). Our paper provides evidence that sperm competition by abundant insemination is also adopted by *C. scitulum* (hypothesis 5 in Utzeri & Sorce 1988a). It is unclear why *C. scitulum* amongst its close relatives is the only species in which males are unable to remove a rival's sperm, but because *C. scitulum* oviposit in tandem, the last male must be able to fertilize most eggs in order to explain the maintenance of this behaviour. Future work should measure fertilization success using irradiated males or genetic markers, in order to make a more powerful test of this hypothesis. Given the above interpretation of the behaviour of *C. scitulum*, it would be interesting to examine the copulatory behaviour of *M. coerulatus* (Fincke 1984a), to see if this species also has a limited sperm removal ability.

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