Sperm competition, cryptic female choice and prolonged mating in the *Eucalyptus* Snout-Beetle, *Gonipterus scutellatus* (Coleoptera, Curculionidae)

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Abstract. The Eucalyptus weevil, *Gonipterus scutellatus*, shows a complex copulation behaviour whose duration is extremely variable, from less than one hour to more than two days. We tested different hypotheses that could explain the adaptive significance of prolonged copulations. Male genitalia is unable to reach to the interior of the espermatheca of the female, where the sperm of previous matings are stored, so prolonged copulation does not function to remove rivals' sperm. Neither the duration of the copulation nor the volume of the ejaculate were affected by the density of rivals, nor was there a significant relationship between copulation duration and volume of sperm ejaculated. The percentage of eggs fertilized by the second male to copulate with a female is very variable, even among different clutches of the same female. These results suggest, by a process of elimination, that prolonged copulation in *G. scutellatus* has evolved under sexual selection by cryptic female choice.

Key words: Coleoptera, Curculionidae, genitalia, prolonged copulation, sperm competition, cryptic female choice.

Resumen. *Competencia espermática, elección femenina críptica y cópula prolongada en el gorgojo del eucalipto,* Gonipterus scutellatus *(Coleoptera, Curculionidae)*. El gorgojo del eucalipto, *Gonipterus scutellatus*, presenta un complejo comportamiento de cópula, cuya duración es extremadamente variable, desde algo menos de una hora hasta más de dos días. Hemos puesto a prueba diferentes hipótesis que podrían explicar el significado adaptativo de las prolongadas copulaciones de esta especie. El análisis de la morfología genital indica que los machos son incapaces de acceder al interior de la espermateca de la hembra, donde ésta almacena el esperma de apareamientos previos. Este hecho excluye la posibilidad de que la cópula prolongada sirva para la remoción de esperma de los rivales. El efecto del riesgo de competencia espermática sobre la duración de la cópula se ha puesto a prueba mediante el mantenimiento de los machos a diferentes densidades. Si la duración de la cópula tiene un significado de vigilancia de la hembra, entonces los machos deben realizar apareamientos más largos cuanto mayor sea el riesgo de competencia espermática. Sin embargo los resultados indican que ni la duración de la cópula ni el volumen del eyaculado se ven afectados por la densidad de rivales. Tampoco se ha detectado una relación significativa entre duración de la cópula y el volumen del eyaculado, lo que excluye la posibilidad de que los machos estén prolongando la cópula para inseminar mayor número de espermatozoides. Mediante el uso de animales irradiados se ha comprobado que el porcentaje de huevos fertilizados por el segundo macho es muy variable, incluso entre diferentes puestas de la misma hembra. Estos resultados sugieren, mediante un proceso de eliminación de alternativas, que la cópula prolongada de *G. scutellatus* ha evolucionado bajo selección sexual por elección femenina críptica.

Introduction

Darwin (1871) proposed that sexual selection selects for the evolution of male mechanisms and behaviours to prevent interference from rival males, before or during the copulation. Parker (1970) recognized that sexual selection can also operate during the time between insemination and fertilization of the eggs, when the reproductive success of a male can be reduced or even annulled by the dilution, the removal or the substitution of his sperm by sperm from a rival male. Sperm competition can be defined therefore as the competition, inside a female, between the sperm from

two or more males for the fertilization of the eggs. The experimental evidence of such phenomenon is currently widespread in most animal groups (see reviews in Smith, 1984; Birkhead & Moller, 1998). Eberhard (1996) underlined the possibility of the existence of sexual selection operated by the female through a series of processes and structures that selectively favour the sperm of a particular male. Such selection is named "cryptic" in the sense that the traditional methods to determine the reproductive success of a male, based in the number of copulations with fertile females, are unable to detect it.

Gonipterus scutellatus is a weevil that feeds exclusively on eucalypts. Of Australian origin, in the last 70 years it has spread on almost all the continents, producing great damages to eucalypt plantations. Its has been intensively studied in biological control projects (Tooke, 1955), but nothing is known about its reproductive behaviour. This species was found in 1991 in NW Spain (Mansilla, 1992). Preliminary observations revealed that males remain in copula for very long times, even more than one day. One of the possible explanations of a prolonged copulation, under conditions of high density of individuals, is the attempt of the male to prevent the female from copulating again before laying the eggs, in order to prevent or to reduce the risk of sperm competition (Sillén-Tullberg, 1981; Svärd & Wiklund, 1988; McLain, 1989; Sutter & Parkhill, 1990; Watson, 1991; Alcock, 1994; Alonso Pimentel & Papaj, 1996). Prolonged copulation may also allow the male to more fully inseminate the female (Svensson et al., 1990). In damselflies, prolonged mating has been related to the time required to remove rivals's sperm from the female genital tract, but in some species it also has a guarding function (Robertson, 1985; Miller, 1987; Cordero, 1990; Sawada, 1995). In crabs it has been shown that males prolong their association with females as a response to predation risk (females mate shortly after moulting and are very vulnerable to predators) but also as a response to increased risk of sperm competition (Jivoff, 1997). An alternative explanation is that prolonged matings in the presence of other males are the result of sexual selection by cryptic female choice (Eberhard, 1996). This study represents an attempt to discriminate the relative roles of sperm competition and cryptic female choice in the evolution of the reproductive behaviour of *G. scutellatus*. We have tried to interpret the meaning of copulation duration and male-female association, designing experiments to test if prolonged copulation is related to the risk of sperm competition (the mate-guarding hypothesis, reviewed in Alcock, 1994). We also present a study of the sperm precedence in females mated by two males (P_2 value, or the proportion of eggs fertilised by the second male).

Materials and Methods

The adults used in the experiments were obtained from eggs laid in the laboratory by field-collected females, during 1997 and 1998. To obtain virgin individuals we developed a reliable system for sex recognition. Larvae and adults were maintained in 1-5 l plastic boxes and regularly fed in the laboratory using fresh leaves of *Eucalyptus globulus*. Larvae were placed in glass terraria provided with 10-15 cm of forest soil where they excavated the pupal cell. The

conditions of light, humidity and temperature during the breeding and during the experiments were those of the environment. The anatomy of the male and the female was studied by means of dissections of individuals preserved in 70° alcohol.

Reproductive behaviour

From 17 May to 13 August 1998 we observed 44 copulations of virgin individuals in transparent plastic boxes containing 10 males and 10 females of around 2 months of age, that were selected at random and individually marked with permanent ink. Pairs were isolated in small plastic containers to facilitate observation, and were observed continuously, but observations were interrupted for periods of 3-5 h if matings continued at night.

The effect of the risk of sperm competition on copulation duration and the volume of the ejaculate

Experiments to test the relationship between copulation duration and male density, between copulation duration and the volume of sperm ejaculated, and between male density and the volume of ejaculate were made from 11 to 24 June 1997. We established three groups of males, with 10 repetitions, maintained in 15 cm petri dishes at different density. Dishes contained the following treatments: a single male isolated from its emergence, 3 males grouped from their emergence, 10 males grouped from their emergence. Furthermore, inmediately before the experiment two more treatments, again with 10 repetitions, were made: one isolated male was put with 9 males (from mass cultures) creating a group, and one grouped male was isolated. If a male died before the experiment, it was substituted by a new male, also isolated or grouped from his emergence. For the experiment, we introduced two females in every dish. Males and females were about two months of age at the moment of the experiment. We obtained a total of 75 copulations by 54 males (range: 1-4 copulations/male; sample size by treatment is presented in figure 3). We treat several copulations by the same male as independent observations, because only 6 males remated in the same day. The duration of the copulation was measured from the introduction of the *aedeagus* to the moment in which the male climbed off from the female and the two individuals separated. Given the very long duration of some copulations, we observed mating pairs at about 30-60 min intervals. Pairs were maintaned in their dish, to allow male disturbance in the grouped treatments. Mated females were preserved in 70° ethanol.

To estimate the volume of the ejaculate we dissected the sperm mass from the spermatheca, and measured the area of the mass under a supported cover-slip (using two pieces of copper wire of 0.0235 mm of diameter) on a slide at 40-100 x with Global Lab 3.0 software (Cordero & Miller, 1992). The volume was calculated as the area multiplied by the separation between cover-slip and slide.

Sperm competition

Preliminary experiments with 500, 1500 and 3000 cGy as sterilizing doses indicated that only 3000 cGy produced significant sterility. Therefore, for the P_2 experiment, we obtained 20 sterilized males (R) by exposing them during

13.3 min to the gamma rays from a ^{60}Co source, giving a dose of 3000 cGy. As a control, we used 20 males that were not irradiated (N) but treated in the same way (both groups were transported to the Hospital Xeral de Galicia in Santiago de Compostela). Males and females were about 2 months of age. From 6 to 20 June 1998 we obtained six double matings in each group: N-N, R-R, R-N and N-R. Of the 24 mated females, 17 laid at least 50 eggs (range: 54-495) in the days following copulation and were used in the analyses. The P_2 value was calculated following Boorman & Parker (1976).

The cost of mating

Given the long-lasting association between males and females, and the fact that mated males are unable to feed, we estimated the cost of copulation for the male in terms of loss of body weight. Forty males were randomly assigned to two groups. One was maintained with food, the other without food, simulating the fasting during copulation. Males were weighed at the start of the experiment and after 6, 10, 24 and 48 hours. In all cases, results are presented as mean±SE (n).

Results

Sexual dimorphism

Tooke (1955) indicates that females of *G. scutellatus* are on average larger than males, but that otherwise the sexes are indistinguishable. Moutia & Vinson (1945) pointed out that the tip of the abdomen differs between males and females: it is straight in the male, and has a small central protuberance in the female. Nevertheless they note that this difference is visible only in KOH-cleared animals from which the setae have been removed.

A detailed observation of male and female morphology at 40x magnification indicated that in the male the penultimate sternite has a straight posterior margin and is covered by fine hairs that do not mask the dark coloration of the sternite. The last sternite is divided into two subtriangular chitinized plates separated by a membranous area. In ventral view, the reduced size of the last sternite leaves exposed the fine hairs on the distal part of the pygidium (fig. 1a). In the female, the posterior border of the penultimate sternite is rounded, densely covered with short hairs of constant diameter. It has a very conspicuous dark edge, that probably helps during oviposition to form the egg mass covering (fig. 1b). The last sternite is semicircular, densely covered by long hairs and joined by the spiculum ventrale to the distal end of the bursa copulatrix (fig. 1c, see also Vidal Sarmiento, 1955; Rosado-Neto, 1996).

Male and female genitalia

The *aedeagus* of the male (fig. 2a, b) is amber-coloured and cylindrical, but strongly flattened in its distal end, where it is opened on its dorsal face. It has two flat narrow proccesses in its proximal end, and a ring (*tegmen*) at its basis, with one apodeme (*spiculum gastrale*) in its inferior face that is prolonged between both parts of the aedeagus. Inside the aedeagus there is an internal sac (*endophallus*), that swolls during copulation, showing in its tip the armature composed of three chitinized elements that are also covered by dense spines. The external walls of this sac are covered by tiny sclerotized plates (see Snodgrass (1935) for a general description of genitalia in Coleoptera).

The female genitalia has an ample genitalic *bursa copulatrix* with a double covering, muscular on the outside and transparent inside (fig. 2). The bursa is prolonged to form the common oviduct and at its inner end is bifurcated in two lateral oviducts with two ovariola each (fig. 2). On the dorsal wall of the *bursa* there is the long thin spermathecal duct, the spermatheca and its accessory gland (fig. 2). The spermatheca is a curved, amber-coloured capsule with a rigid chitinous wall, on which a bundle of muscles is inserted (fig. 2). The *aedeagus* is about twice as long as the *bursa copulatri*
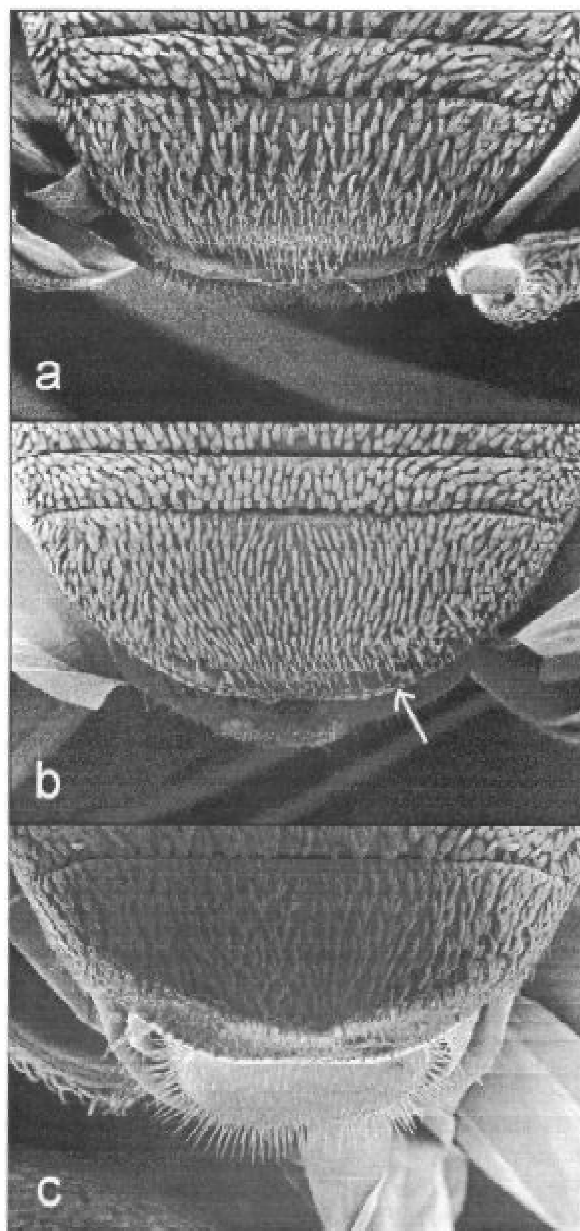

Figure 1. The end of abdomen of male and female *G. scutellatus* at SEM. (a) male, (b) female showing the distinctive edge that allows sex discrimination (arrow), (c) female with the last sternite (ovipositor) extruded.
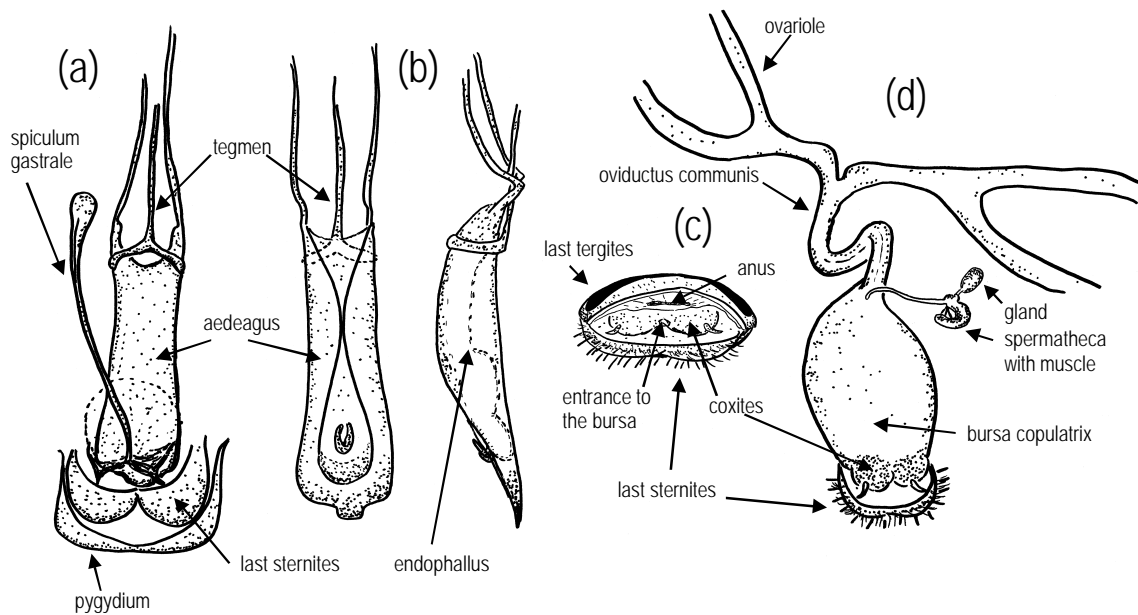
Figure 2. Male and female genitalia of *G. scutellatus*. (a) Male genital apparatus in ventral view; (b) the *aedeagus* in dorsal and lateral views; (c) a posterior view of the female genital opening; (d) female genitalia showing the long thin spermathecal duct.

Reproductive behaviour

The female possesses all the conditions that make possible sperm competition: she mates with more than one male before laying the eggs (pers. obs.). She starts to oviposit a minimum of two days after copulation, and can maintain the sperm alive during the whole duration of her life. One twice-mated female still laid fertile eggs 9 moths after mating, and contained 0.00531 mm^3 of sperm when she died after one year (after having laid 900), a value similar to the average volume after one mating (fig. 5 & 6).

The copulation of *G. scutellatus* lasts in average 6.98±0.49 h (n=75), but there is an exceptional variability between individuals, from a minimum of 0.7 to to a maximum of 55 h. The copulation of *G. scutellatus* is composed of two phases. In the «passive phase» (Parker, 1970) the male remains on the back of the female without introducing his aedeagus in her *bursa copulatrix*. The «active phase» is characterized by the introduction of the *aedeagus*. At the beginning of the copulation the male climbs onto the female's dorsum and remains there even if the female makes clear lateral movements in an apparent attempt to shake the male off. This phase, that we will define «pre-copulatory passive phase», lasts from a minimum of 10 minutes to a maximum of 24 hours. During this phase the male often everted the *aedeagus* repeteadly and in many cases touched the last sternite of the female with it.

In 7% of cases (n= 42 copulation attempts) the male did not succeed in eliciting the necessary cooperation from the female for the insertion of the *aedeagus*, and gave up before beginning the active phase. The active phase was preceded by a series of rubs by the male's thorax against the female's elytra, followed by the intromission of the *aedeagus* and then by rhythmic genitalic pushing. The «active phase» of copulation included three behaviours: the rubbing with the male's thorax; dorso-ventral movements of the male's fore-legs on the sides of female's thorax; and genitalic thrusts and pauses.

The sequence of movements was highly variable. In the beginning of a typical copulation, the sequence was first a bout of rubbing, and then one thrust and a pause. Later the number of thrusts increased progressively until to sometimes reaching over 270 consecutive thrusts. After having reached the maximum, the number of thrusts decreased and sometimes disappeared, leaving only rubbing and pauses. The duration of the pauses was highly variable (one minute to 12 hours). In some copulations thrusts resumed after having disappeared (fig. 3).

Once the *aedeagus* was withdrawn, the male remained absolutely immobile on the female's dorsum for up to a maximum of 24 h. At the end of this period it is possible that the pair separates, finishing copulation. In a group of 30 mating pairs observed in detail, 25 had only one copulation, in 2 cases the male reinserted his aedeagus and began a new copulation, and in the remaining cases copulation was repeated 3, 4 and 6 times.

The effect of other males on copulation duration and amount of sperm transfered

If the function of prolonged copulation is to guard the female, then its duration will be proportional to the density of the population. We therefore expected that isolated males would mate for shorter times than males in groups of three or ten. Results do not support this idea: copulation duration (active phase) does not depend on population density (Kruskal-Wallis test=8.865, gl=4, p=0.073; fig. 4; the same results are obtained considering only the first mating of males that mated more than once). Males that were isolated or grouped just before the experiment did not behave in differently from males isolated or grouped from their emergence. If the passive phase of the copulation
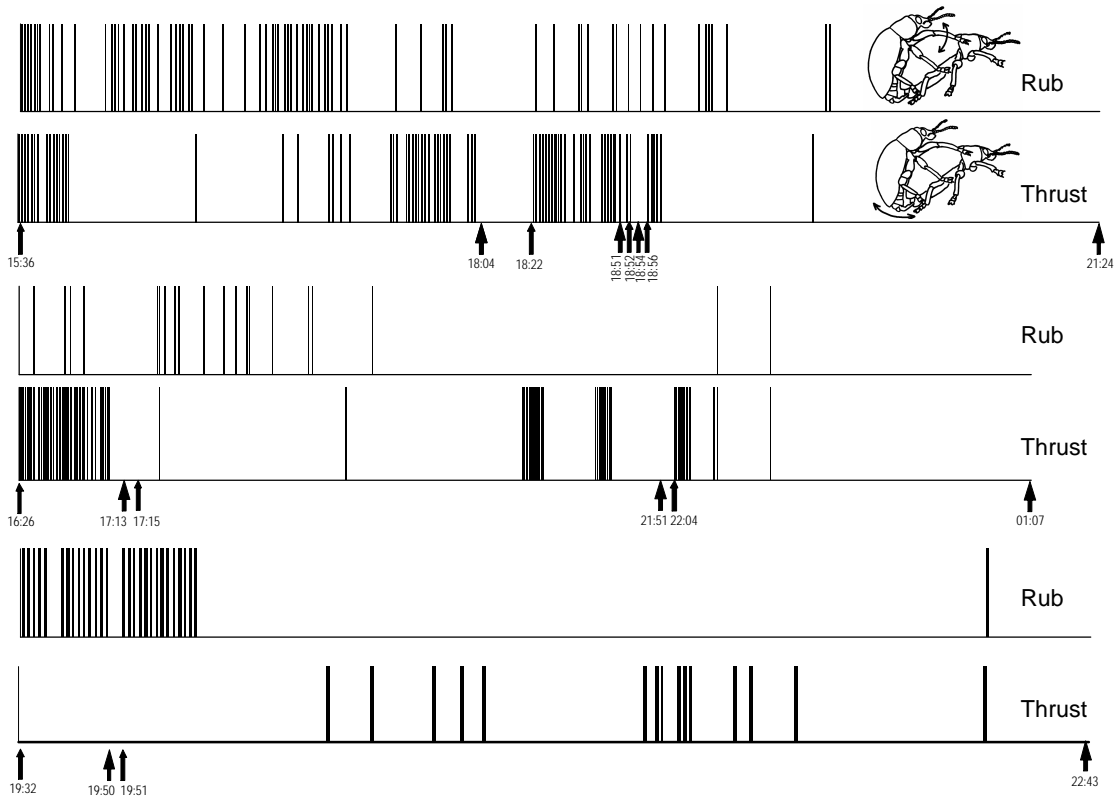
Figure 3. The temporal sequence of rubbing of female's elytra and thrusts of the adeagus in three copulations. Note the extreme variability. Arrow, start of intromission; triangle, end of intromission.
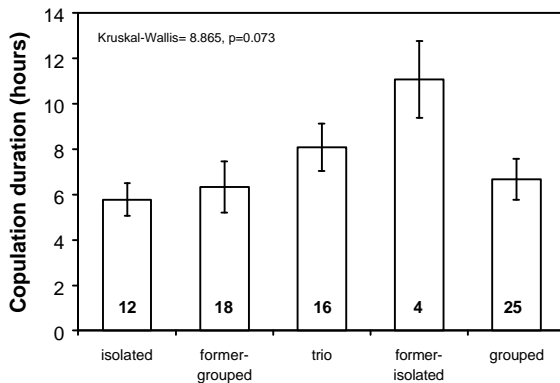

Figure 4. The relationship between male density and copulation duration. Number are sample size.

is also included in copulation duration, results are similar.

Alternatively, males may respond to the presence of other males by increasing the volume of the ejaculate (Gage & Baker, 1991). Again there was no significant effect of population density on ejaculate volume (Kruskal-Wallis test=4.591, gl=5, p=0.468; fig. 5; the same results are obtained considering only the first mating of males that mated more than once).

If the male employs the entire copulation to inseminate the female, then the volume of sperm ejaculated will be directly proportional to the duration of the copulation. There

was no significant relationship, however, between the volume ejaculated and the duration of copulation (r_s=-0.052, n=63, p=0.685, fig. 6). Note that in a few cases the volume of sperm transferred was very low, even after long copulations.

The cost of mating

The average weight of males maintained without food was not significantly different from control males at 6, 10 and 24 hours (when they had lost 10% of the initial weight), while it was significantly different at 48 hours (when the loss was of 18%, ANOVA, Fig. 7).

Sperm competition

Figure 8 presents the percentages of fertile eggs laid by double-mated females. The proportion of fertile eggs was 0.78±0.04 (5) in N-N matings and 0.10±0.03 (3) in R-R females, indicating that the treatment was successful in sterilizing experimental males. The mean P2 in N-R matings was 0.59±0.16 (4), but it ranged in these females from 0.10 to 0.93. In R-N matings P2 was 0.66±0.15 (5), but again was highly variable from 0.26 to 1.04 (the value higher than one is due to the fact that 79% of eggs were fertile, a value greater than the average in N-N females). Figure 9 shows the percentage of fertile eggs laid by females throughout their lives. There was great variability between females and also between clutches for the same female. Pulses of both low and high fertility are clear in R-N and
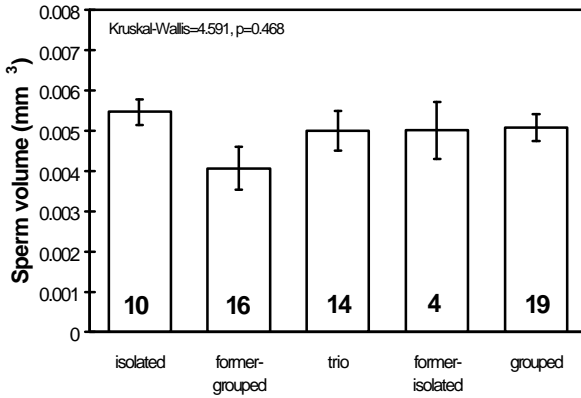
Figure 5. The relationship between male density and ejaculate volume.

Figure 7. The effect of fasting on male body weight loss. P after ANOVA.
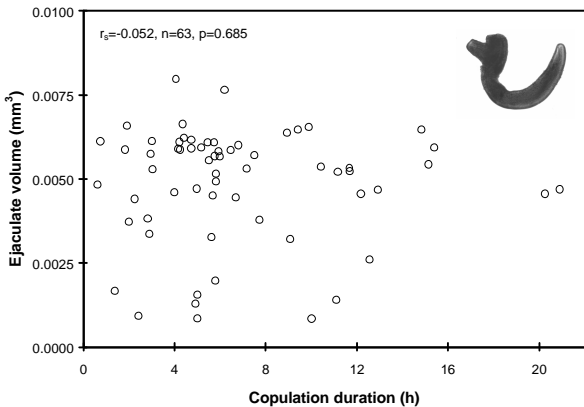

Figure 6. The relationship between copulation duration and ejaculate volume. Note that the sperm volume was very low in some long copulations, suggesting that the female did not cooperate to allow a complete insemination or the male refused to inseminate. The upper-right figure shows a typical sperm mass as dissected from the spermatheca.

Figure 8. The percentages (mean±SE) of fertile eggs laid by doubly-mated females, with normal (N) or irradiated (R) males.

N-R females (fig. 9). Two N-N females also laid some sterile clutches.

Discussion

Evolutionarily speaking, a brief copulation could have many advantages: reduce the risk of predation, leave more time for feeding, egg-laying, the search of other partners, and reduce the risk of suffering an interruption of the copulation before the sperm transfer has been completed. Despite these advantages copulation is very long in many species. From the male's point of view, prolonged copulation has some benefits that can explain its evolution (Dickinson, 1997). It reduces the risk of predation if the mating pair can respond more quickly to predators, if the pair succeeds in passing unnoticed, or if they combine their chemical defenses during copulation. Prolonged mating also provides phoresis in some species and can also increase the fertilization success of the male.

We do not know if predation on *G. scutellatus* is frequent in its country of origin. It is apparently scarce there due to the many parasitoids that attack its eggs, larvae and adults (Tooke, 1955). Surely the male takes advantage of the
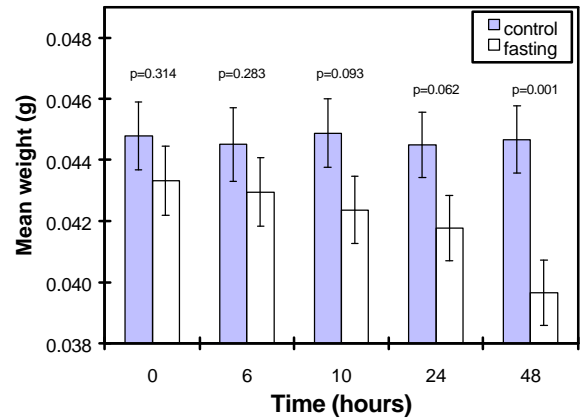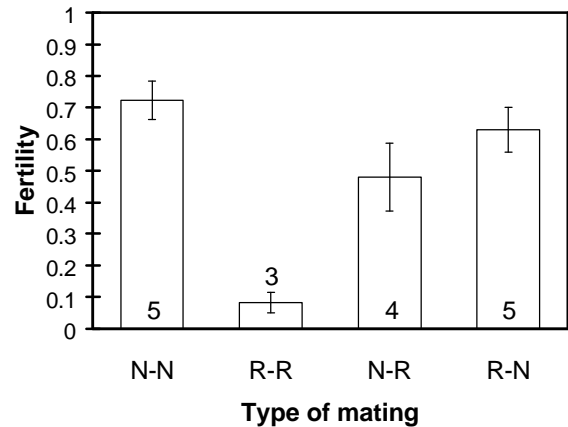
transport that the female offers to him, but these are very small-scale movements. It is possible that there is an increase in paternity when more time is spent in copula, and this should be analysed in future work. Certainly the male spends a lot of time in each copulation, and probably his frequency of copulation may be reduced in comparison to other similar species. Nevertheless given that this species is long-lived (up to one year in the laboratory), the problem of the frequency could be of little importance. Furthermore, prolonged copulation is not costly in terms of body weight lost, because most matings do not achieve 24 h, when the fasting period starts to have significant effects (which may be only temporary in any case).

In some insect species a very long copulation, much longer to the necessary time to completely fill the spermatheca of the female, has a meaning of mate guarding to reduce the risk of sperm competition (Alcock, 1994, but see Eberhard, 1996). Nevertheless, in *G. scutellatus* population density did not affect copulation duration or ejaculate volume, and longer matings did not result in greater ejaculate transmission. Long copulations were unlikely to have a guarding function, since the female did
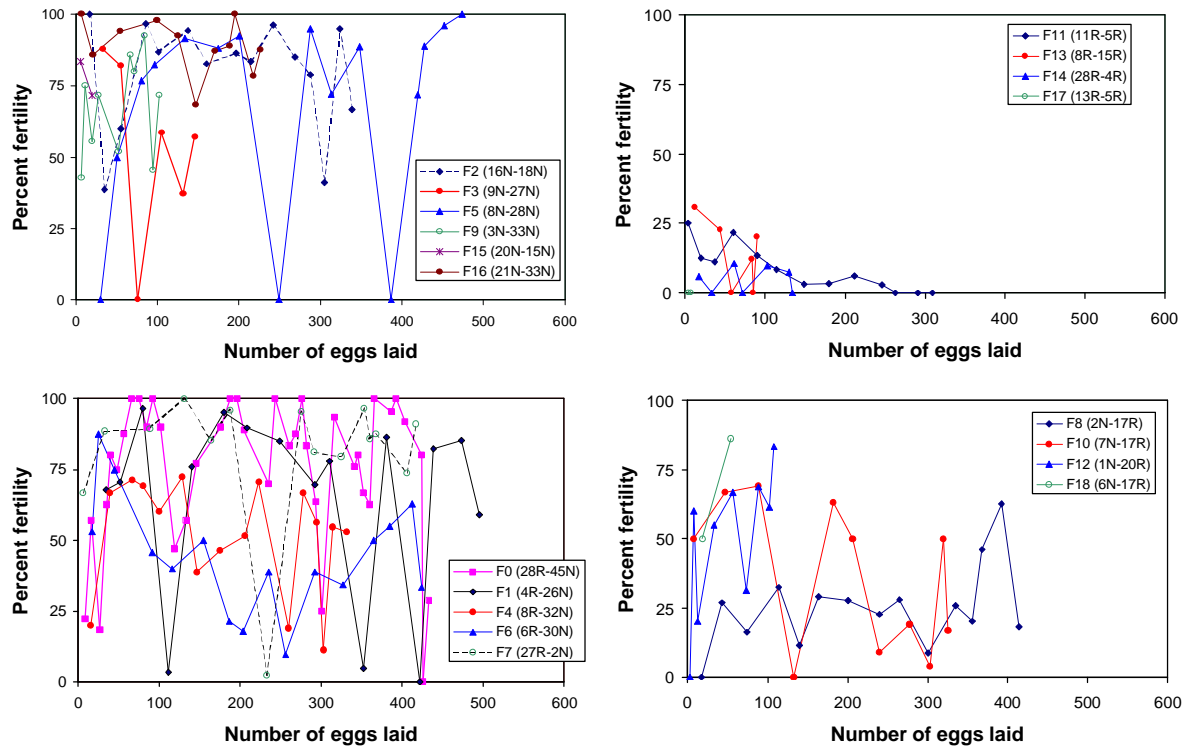
Figure 9. Changes in the percentage of fertile eggs laid by doubly-mated females over time. Note the extreme variability found in N-R and R-N females and lack of consistent trends over time.

not lay eggs at the end of the association, and was therefore free to mate again. The results of the P_2 experiment suggest that even if the second male may fertilize the majority of the eggs, females often use the sperm of the first male and cryptic choice is thus feasible. Figure 9 suggests that the female used the sperm from both males in a seemly random sequence: there were clutches with very low fertility in R-N matings and clutches with high fertility in N-R matings. This last finding is important because low fertility in R-N females might be due to unfavourable laboratory conditions, that perhaps have occasioned the low fertility of some N-N females. The sperm of the sterilized males is surely less competitive than that of the normal males, and therefore a more powerful test of this hypothesis using molecular techniques is needed. The alternate use of masses of sperm of different origin, or the preference towards one ejaculate rather than another, is already known in some orthopterans, dipterans and coleopterans (Eberhard, 1996).

The anatomy of the genitalia excludes the possibility that the *aedeagus* of the male reachs inside the spermateca to remove the sperm of the rivals, as is typical in odonates (Waage, 1986). Males are therefore not prolonging copulation to remove rivals' sperm. A large ejaculate to dilute rivals' sperm seems also absent in this species, given that males did not increase ejaculate volume at high population densities. These data lend us to wonder: what can be the reason for copulations lasting 20 hours under conditions of isolation? It is possible that the phases of the copulation and its duration must be interpreted as the signal that the male sends to «convince» her mate of his quality or to elicit in her the appropriate responses that allow him to introduce his *aedeagus* inside the bursa or his sperm

into the spermatheca. In some cases males remained during long periods with the female, even introduced the aedeagus, but the amount of sperm transferred to the spermatheca was very low (see fig. 6). These are pseudo-copulations, in the sense that males were either unable to inseminate the female, or the female did not transport the sperm to the spermatheca, perhaps due to a cryptic choice (Eberhard, 1996). If males were sperm limited, it is also possible that they were assesing female quality and refused to ejaculate to poor quality females. This nevertheless seems unlikely given that the volume of sperm transferred is very small. If similar pseudo-copulations occurred in the P_2 experiment, this could explain the high variability in P_2 values. It is also necessary to furnish an explanation to the repetition of the copulation up to six times in the same pair, even when they remained in complete isolation and the female was a virgin. We have not demonstrated cryptic sperm choice by female *G. scutellatus* using the criteria proposed by Birkhead (1998), but our results suggest that the long copulation of *G. scutellatus* is not explained by sperm competition, and might be a case of copulatory courtship that was selected by cryptic female choice (Eberhard, 1996). Males perform clear courtship behaviour during copulation (fig. 3). This idea can be tested in future work looking for associations between a male's behaviour and persistence and his fertilization success with singly- and double-mated females.

irradiation experiments.

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