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Current Sperm Competition Determines Sperm Allocation in a Tephritid Fruit Fly

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Abstract

Sperm competition (SC) occurs when the sperm of two or more males compete for the same set of ova. Theoretical models and experimental observations indicate that the presence of rival males causes focal males to adjust sperm allocation in a given copulation. Males allocate more sperm when they perceive the presence of one rival male (SC risk), either before or during mating, or when they perceive the presence of multiple rival males before mating (previous SC intensity). Conversely, males are expected to allocate fewer sperm when they perceive the presence of rival males during mating (current SC intensity). Here, we varied male perception of SC by manipulating the number of rival males, both before mating (from emergence to mating) and during mating (at the time of mating) to examine their effects on mating latency, copulation duration, and sperm allocation in the South American fruit fly *Anastrepha fraterculus*. We showed that exposure to rival males at the time of mating decreased mating latency. However, in contrast to the theory, exposure to multiple rivals at the time of mating increased sperm allocation. Female and male size were significant predictors of mating latency, copulation duration, and sperm allocation. Our results showed that there is a plastic response of males to the level of perceived SC through the number of rival males. Current levels of SC intensity are important in shaping male responses to SC, although the patterns in this species are opposite to predictions from the existing theory. We propose that female preference for males forming leks could explain lower sperm counts when encountering only one or two males.

Introduction

Sperm competition (SC) occurs when sperm from two or more males compete for a female's eggs (Parker 1970; Simmons 2001) and can have important implications for the evolution of ejaculate characteristics such as sperm velocity, sperm morphology, and other components of the ejaculate. For example, males that have evolved with high levels of SC have greater mating effort and fitness compared to males evolving with low levels of SC (Klemme & Firman 2013). SC theory predicts that males will vary sperm allocation according to the number of rival males with two possible situations: 0 vs. 1 rival male, defined as *SC risk*; and 1 vs. several males, defined as *SC intensity*. SC risk is the probability that the male's sperm will compete against the sperm from other males for a given set of ova while SC intensity is related to the number of competing ejaculates (Parker 1998; Engqvist & Reinhold 2005; Kelly & Jennions 2011).

The SC model predicts an increase in sperm allocation both in cases of long-term exposure to a single or multiple males and in the presence of a rival male at the time of mating, but a decrease in sperm allocation in the presence of rival males at the time of mating.

The latter effect is due to the fact that as the number of competitors increases, the rate of return per sperm released decreases (Parker et al. 1996; Engqvist & Reinhold 2005). Larval rearing density affects number of apyrene sperm (He & Miyata 1997; McNamara et al. 2010), while long-term exposure to rival males is likely to affect sperm production through increased spermatogenesis or investment in larger testes size (Awata et al. 2006, 2008; Firman et al. 2013). The sudden presence of rivals affects how much of the current sperm reserves should be allocated to a specific copulation (Engqvist & Reinhold 2005). Optimal sperm allocation could thus be achieved via different mechanisms in different scenarios (Garbaczewska et al. 2013).

Two recent meta-analytic reviews across taxa empirically support the predictions of SC risk theory. That is, there was a significant increase in sperm allocation when males mated in the presence of a rival male (i.e., high risk of SC), compared to an absence of a rival male (i.e., low risk of SC) (delBarco-Trillo 2011; Kelly & Jennions 2011). For example, males of the beetle *Tenebrio molitor* accompanied by a rival male for 5 d before mating, 5 min before mating or during mating inseminated more sperm per ejaculate than unaccompanied males (Gage & Baker 1991). There is, however, no consensus for the general prediction that sperm allocation decreases as the number of competitors increases from one to several males (i.e., high intensity of SC) (Kelly & Jennions 2011). While some cases are in agreement with the theory (Pilastro et al. 2002; Schaus & Sakaluk 2001 [*Gryllus veletis*]; Simmons et al. 2007; Thomas & Simmons 2007 [the last two examples measured sperm viability and not sperm allocation]), in others, males increased the number of sperm transferred while in the presence of other males (Gage & Barnard 1996; Garbaczewska et al. 2013). Finally, some studies have found no effect of the presence of a rival (SC risk) or rivals (SC intensity) on the number of sperm allocated by males (Cook & Gage 1995; Schaus & Sakaluk 2001 [*Gryllus texensis*]; Worthington et al. 2013). This evidence suggests that the response to increasing SC could be related to the species-specific mating system and the frequency of multiple mating.

A further factor confounding evidence for sperm allocation under SC, and traits related to the reproductive success of the males such as mating frequency, mating latency, and copulation duration is the influence of either the previous or the current social context. There is empirical evidence that the social context early in adult life can influence male mating behavior and success. For example, *Drosophila melanogaster* flies housed in isolation from emergence showed higher mating frequencies and shorter latencies to mate than did group-housed flies (Ellis & Kessler 1975) and the larger space available per fly seems to be the key factor in increasing their mating success (Dukas & Mooers 2003). Similarly, in the Mexican fruit fly *Anastrepha ludens* males that experienced low densities during early adulthood obtain more copulations than male flies housed in higher densities (Díaz-Fleischer et al. 2009), while males of *D. melanogaster* held singly during early adult life have shorter copulations than males held in groups (Bretman et al. 2009, 2010). Based on male perception of the previous level of SC (number of males prior to the time of mating), *D. melanogaster* males can vary copulation duration (Nandy & Prasad 2011), can vary seminal fluids (ovulin and sex peptide) transferred during mating and can modify the ratio of seminal fluid genes transcribed (Wigby et al. 2009; Fedorka et al. 2011), while in praying mantids *Pseudomantis albofimbriata*, males can vary the number of sperm transferred when reared with either males or females (Allen et al. 2011). Furthermore, in the Mediterranean fruit fly *Ceratitis capitata*, males under high male–male competition during 2 wks (one focal male with five rival males) court more but gain lower paternity than when under lower competition (one focal male with one rival male) (Leftwich et al. 2012).

When the perceived current level of SC is high, that is, when there are rival males present in the mating arena, there is also evidence that mating latency and copulation duration are significantly shorter than when no rivals are present (Gage & Baker 1991; Bretman et al. 2009), while in the soldier fly *Merosargus cingulatus* males respond to current SC by prolonging copulations when rival density at the oviposition site is high (Barbosa 2011), and fertilizing more eggs than when under low SC (Barbosa 2012). However, few studies have simultaneously manipulated previous and current number of competitors with increasing levels of SC.

Here, we manipulated the perceived levels of SC by exposing focal males to rival males from adult emergence until mating (previous level of SC), and then exposing males to rivals during mating (current level of SC). Furthermore, we varied the intensity of SC by exposing males to different number of rivals during mating. If males respond predominately to previous SC levels, then we expected higher sperm allocation as SC increased. If males responded to current levels of SC, we expected higher sperm allocation as SC risk increases, but lower sperm allocation as SC intensity increased, in line with theoretical predictions (Parker et al. 1997; Engqvist & Reinhold 2005; Bretman et al.

2009) (Table 1). We used the South American fruit fly *Anastrepha fraterculus* (Diptera: Tephritidae) as a model organism to investigate the responses by males to the number of rival competitors, both prior to and during mating. In other tephritids, males appear to prudently allocate sperm between consecutive matings (Pérez-Staples & Aluja 2006) and increase sperm numbers with increasing SC risk (Gage 1991). In addition, we also tested the hypothesis that male behavioral response to SC affects mating latency and copulation duration, while controlling for male and female body size.

Methods

Anastrepha fraterculus adults were obtained from a laboratory colony established at the Agricultural Zoology laboratories of the Estación Experimental Agroindustrial Obispo Colombres, Tucumán, Argentina. This colony was initiated in 1997 with pupae obtained from infested guavas, collected in the vicinity of Tafí Viejo, Tucumán province, north-western Argentina (Jaldo 2001). Rearing followed methods described by Jaldo et al. (2001) and Vera et al. (2007). Experiments were carried out at the laboratories of Cátedra de Terapéutica Vegetal, Facultad de Agronomía y Zootecnia, Tucumán, Argentina.

Experimental Procedures

On the day of emergence, flies were sorted by sex. Females were transferred to 750-ml plastic containers in groups of 25 adults. Males were kept in 750-ml plastic containers but under two conditions to vary previous levels of SC: in groups of 25 (crowded condition) or singly (isolated condition). The containers had a voile cloth at the top with a small opening which was closed with a piece of cotton to allow fly removal. Both sexes were fed with water and a standard adult diet consisting of sugar (57.9%), hydrolyzed yeast (14.5%) (Yeast Hydrolyzated Enzymatic, MP Biomedicals®), hydrolyzed corn (27.3%) (Gluten Meal, ARCOR® Argentina), and vitamin E (0.3%) (w/w) (Jaldo et al. 2001). Flies were tested 10–21 d after adult emergence. This ensured that all individuals were sexually mature (Jaldo 2001; Petit-Marty et al. 2004; Jaldo et al. 2007).

On the day of testing at 07:30–08:00 h, virgin males and females were released into a plastic cage (14 l) with an artificial plastic branch inside. Released males were from both previous levels of SC: either crowded or isolated. To vary current levels of SC, males from each of the two previous treatments were randomly assigned to groups of 1, 2, or 10 males immediately before mating. Number of males included the focal male; thus, there were no rivals in group 1, one rival in group 2, and 9 rivals in the last group. Two females were released in each cage to increase the probability of obtaining at least one mated female. A total of six treatments resulting from the combination of previous and current levels of SC were set up (crowded.one, crowded.two, crowded.ten, isolated.one, isolated.two, and isolated.ten) in different cages (Table 1). Cages were checked for copulating pairs at 5-min intervals for 3 h after flies were released. Argentinean populations of *A. fraterculus* exhibit a narrow period of mating activity early in the morning (Petit-Marty et al. 2004; Vera et al. 2006). Copulating pairs were care-

Table 1: Summary of experimental design, associated predictions, and results obtained with *Anastrepha fraterculus*. Previous treatment refers to the presence (crowded) or absence (isolated) of rival males early in adult life (from emergence to mating). Current treatment refers to the presence or absence of rival male/s at the time of mating including the focal male. Arrows indicate sperm allocation increase (↑), or decrease (↓) with respect to one male

Previous treatment	If males respond predominately to the Previous level of SC, we expected:	Result	Current treatment	If males respond predominately to the Current level of SC, we expected:	Results
Crowded: 25 ♂ per cage (SC intensity)	Higher sperm allocation in the crowded treatment	Similar sperm allocation in the Crowded and Isolated treatment	Ten males (SC intensity)	Sperm allocation ↓	Higher sperm allocation with ten males at the time of mating compared with one male
			Two males (high SC risk)	Sperm allocation ↑	
			One male (low SC risk)	Sperm allocation	
Isolated: 1 ♂ per cage (low SC risk)	Lower sperm allocation in the isolated treatment		Ten males (SC intensity)	Sperm allocation ↓	Similar sperm allocation with two and one males at the time of mating
			Two males (high SC risk)	Sperm allocation ↑	
			One male (low SC risk)	Sperm allocation	

fully coaxed into test tubes (20 ml), which were then plugged and numbered. After the formation of each pair *in copula*, both females and males were replaced to maintain the same sex ratio during the 3 h of observation. Mating latency was calculated from the time each new pair was placed inside the cage to the time mating started. The time at which copulations started was recorded to calculate copulation duration. Pairs were checked every 5-min until copulations finished and end time was recorded. Mated females were anesthetized with ice and dissected for sperm counts. Both males and females were anesthetized with ice and preserved at −20°C for morphometric analyses. This procedure was repeated eleven times in different days throughout 3 wks until 11–22 mated females were obtained per treatment.

Sperm Counts

Females of the different treatments were randomly dissected between 2 and 8 h after copulation under a dissecting microscope (Arcano ZTX 1065) using a 60× magnification, following Abraham et al. (2011b). Dissections were carried out blind to the observer. In a separate pilot study, we found no significant difference in sperm stored by females 2 or 24 h after copulation ended (*t*-test for independent samples, $t = 0.27$, $df = 45$, $p = 0.785$). Reproductive tracts were removed and placed over a slide with a 50-μl drop of sterilized water. Spermathecae were dissected and placed separately on slides with 7 μl of sterilized water containing 0.1% of soap (Triton®). Each of the three spermathecae was broken apart with fine forceps and a 3-μl drop of acetic orcein was added to allow the staining of spermatozoa. The drop was stirred quickly with entomological pins for 1 min. A 18 × 18 mm coverslip was then placed on top of each of the storage organs and secured on each corner with a drop of transparent nail polish. Spermatozoa were allowed to stain for at least 5 d and then were counted under a light microscope (Leica DM 500) at 400× magnification. The whole slide was covered by counting all spermatozoa in 204 randomly selected fields, which corresponds to 10% of the total area. To obtain the total number of sperm for each storage organ, a conversion factor of 10 was applied to the sperm counted for each storage organ (Pérez-Staples & Aluja 2006).

Male and Female Size

Male and female head widths were measured following Rodriguero et al. (2002) and Sciurano et al. (2007) using a dissecting microscope (Arcano ZTX 1065) fitted with an ocular micrometer. Males were measured to account for a potential effect of male size on the number of sperm allocated to female. Females were measured to account for potential male strategic sperm allocation in relation to female size (Ingleby et al. 2010; Pérez-Staples et al. 2014).

Statistical Analyses

Generalized linear mixed effects models (GLMMs) implemented in the *lme4* library (Bates et al. 2011) of the R statistical software were used for all analyses (R Development Core Team 2008).

Sperm allocation (total number of sperm stored in the three spermathecae of the females, excluding females that did not store sperm), was analyzed with a GLMM using previous level of SC (factor with two levels: crowded or isolated), current level of SC (factor with three levels: one, two, or ten males) and their interaction as explanatory variables. Male size, female size, and copulation duration were included as covariables (fixed factors) in the analyses, while the cage (as repeated measure) was defined as a random factor. A Poisson error distribution was declared in the statistical model. The probability of sperm storage (females with or without sperm stored in their spermathecae) was also analyzed with a GLMM with the same factors as above except a binomial error distribution was defined for this model.

Mating latency and copulation duration were analyzed with GLMMs, where previous level of SC, current level of SC, their interaction, male size, and female size were fixed factors, while cage was set as a random component of the model. We also defined a Poisson error distribution for these two models.

Significance of main factors and interactions for all tests were obtained via model simplification and ANOVA tests between models based on the chi-squared distribution of the deviance. p-Values were obtained by likelihood ratio tests of the full model of the effect in question against the model without the effect in question. To minimize interference of non-significant factors, standard errors were calculated from simple models including only one significant factor or co-variable (Crawley 2002).

Lastly, following Kelly & Jennions (2011) and Cooper et al. (2009) (equations 12.12 and 12.13), we calculated the effect size Hedge' *d* and the variance in *d*, which is designed to compare two independent groups. We compared first the current treatment with one male compared with two males and then one male compared with ten males. To calculate the 95% CI, we used equation (15) in Nakagawa & Cuthill

(2007). In this case, all the mated females (with and without sperm stored) were included in the analysis but one outlier was deleted from the current.one treatment. Nakagawa & Cuthill (2007) highlight that effect size calculated with nonparametric data are likely to be biased and the CI are likely to be inaccurate. To resolve this problem, we sqrt ($x + 1$)-transformed the variable to achieve normality.

Results

The number of sperm allocated was significantly affected by the current level of SC, female size, male size, and copulation duration, but not by previous level of SC or the interaction between previous and current levels of SC (Table 2). Contrary to our prediction, sperm allocation was higher for females mated with males facing high intensity of current SC (ten males at the time of mating), compared with males facing low levels of current SC (two or one males at the time of mating) (Fig. 1). Sperm allocation showed a positive relationship with female size and a negative one with male size. On the other hand, sperm allocation positively increased with copulation duration.

The calculated effect size for the comparison between 0 vs. one rival at the time of mating was not statistically significant ($d = -0.47$, 95% CI: −0.95 to 0.01). When we compared 0 vs. several rivals (one male at the time of mating compared to ten males), the difference was statistically significant ($d = 0.77$, 95% CI: 1.21–0.32).

The percentage of spermless females was 9% (2/22) and 9.5% (2/21) for the crowded.ten and isolated.ten treatments, respectively, 18.2% (2/11) and 29.4% (5/17) for the crowded.two and isolated.two treatments, respectively. For the crowded.one and isolated.one treatments, 30% (6/20) and 18.2% (4/22) of females stored no sperm. The percentage of spermless females was not affected by the previous level of SC, the current level of SC, their interaction, female size, male size nor copulation duration (Table 2).

Mating latency was significantly affected by the current level of SC, the interaction between current and previous level of SC, female size, and male size. Mating latency was shorter for males from the crowded condition and when faced with high intensity of SC at the time of mating (i.e., ten males at the time of mating) compared to males from the crowded and isolated condition facing lower levels of SC at the time of mating (i.e., two or one males at the time of mating). Males from the isolated condition and with high intensity of SC at the time of mating had intermediate values (Fig. 2). Mating latency showed a positive relationship with female size but a negative one with male size. Latency was not significantly affected by previous level of SC (Table 2).

Copulation duration was not affected by the current level of SC, the previous level of SC nor the interaction between previous and current level of SC) (Fig. 3, Table 2). However, copulation duration showed a positive relationship with female and male size.

Table 2: Predictors (fixed effects) of mating latency, copulation duration, sperm number and sperm storage in females of *Anastrepha fraterculus*

Analysis	Fixed factor	χ^2	df	p
Mating latency	Previous level of SC	6.959	3	0.073
	Current level of SC	22.50	4	**<0.0001**
	Previous * Current	6.948	2	**0.030**
	Female size	62.841	1	**<0.0001**
	Male size	110.37	1	**<0.0001**
Copulation duration	Previous level of SC	0.009	1	0.923
	Current level of SC	1.324	2	0.515
	Previous * Current	1.037	2	0.595
	Female size	23–221	1	**<0.0001**
	Male size	42.161	1	**<0.0001**
Sperm number	Previous level of SC	3.420	1	0.064
	Current level of SC	15.449	2	**<0.0001**
	Previous * Current	5.253	2	0.072
	Female size	774.74	1	**<0.0001**
	Male size	528.91	1	**<0.0001**
	Copulation duration	82.555	1	**<0.001**
Sperm storage	Previous level of SC	0.055	1	0.814
	Current level of SC	3.768	2	0.151
	Previous * Current	1.345	2	0.510
	Female size	0.674	1	0.411
	Male size	0.851	1	0.356
	Copulation duration	0.178	1	0.672

Values in bold letter are significant at the 0.05 level.

Discussion

Anastrepha fraterculus males had a strong response to currents levels of SC. Mating latency was shorter and sperm allocation increased when there were several rival males at the time of mating (current intensity of SC). These results confirm the plastic capacity of males to modulate the number of sperm transferred in order to maximize their paternity when faced with SC.

Sperm Allocation

SC theory predicts that sperm allocation increases with (1) previous risk of SC, (2) previous intensity of SC, and (3) current risk of SC, but decreases with current intensity of SC, given that as the number of competitors increases, the rate of return per sperm release

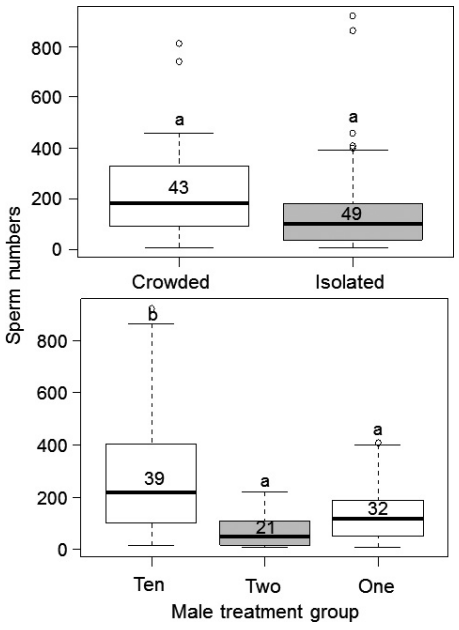

Fig. 1: Sperm allocation (Q1-median-Q3) of males exposed to different levels of sperm competition. Males were kept from emergence either alone or in groups of 25 males (previous treatments: Isolated or Crowded) and then placed with a total of 10, 2, or 1 (no rival) male per cage at the time of mating (current treatments). Different letters indicate significant differences among groups predicted by the generalized linear mixed effects model. Numbers within boxes represent sample sizes.

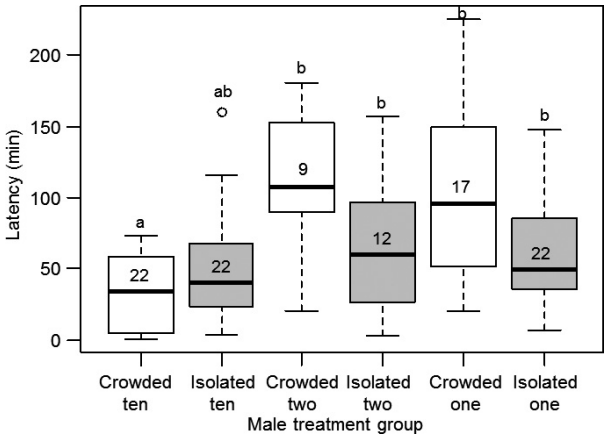

Fig. 2: Mating latency (Q1-median-Q3) of mating pairs with different levels of sperm competition. Males were kept from emergence either alone or in groups of 25 males (previous treatments: Isolated or Crowded) and then placed with a total of 10, 2, or 1 (no rival) male per cage at the time of mating (current treatments). Different letters indicate significant differences among groups predicted by the generalized linear mixed effects model. Numbers within boxes represent sample sizes.

decreases (Parker et al. 1996; Engqvist & Reinhold 2005). However, our findings were not strictly in agreement with predictions from SC risk or intensity, but did, in general, support the idea that SC intensity was the more important force in shaping male responses to SC (Bretman et al. 2009).

Contrary to predictions on current levels of SC, sperm allocation increased with increasing intensity of SC. Given that *A. fraterculus* males form leks of up to five males (Malavasi et al. 1983), finding many rivals at lekking sites could be perceived by males as the 'rule' in this species, and they would respond to increased intensity of competition as other species respond to risk of SC. In *Anastrepha suspensa* leks of up to nine males have been observed (Sivinski 1989). In the golden egg bug *Phyllomorpha laciniata*, the presence of rival males during mating leads to an increase in the number of sperm transferred per unit of time. However, it seems that this response implies that SC levels are not always high and that the increase of male ejaculate expenditure in the presence of rivals makes sense if this situation is not usually the norm (García-González & Gomendio 2004). Overall, our findings showed that the presence and number of rival males during mating was the key factor determining the number of sperm allocated by males, and this is possibly the cue used to assess the level of SC in *A. fraterculus*.

Nevertheless, it is perplexing why males respond so strongly to the presence of other males, given that *A. fraterculus* females have a low remating rate (45–51% of females mate more than once, according to the strain), the remaining females never remate in a period of 35 d, and the refractory period has a range of 7–20 d (Abraham et al. 2011a). If finding many males at the lekking and mating site is the 'rule' rather than the exception, perhaps males do not increase the number of sperm in the presence of rivals, but rather decrease the number of sperm transferred when they perceive little or no competition. Alternatively, females could be storing less sperm when mating in a small lek (two males) or with a single male. A recent meta-analysis for lekking species across taxa revealed that female visits generally increased when lek size increased (Isvaran & Ponkshe 2013). Indeed, in another lekking tephritid, females preferred large artificial leks over small ones (Shelly 2001). This female preference could explain the lower number of sperm found in females mated with males in small leks. However, this hypothesis is only feasible if sperm storage is under female control by cryptic female choice, and this has not yet been tested in this species. Nevertheless, there is evidence that sperm storage is under considerable female control in other related tephritids (Fritz & Turner 2002; Pérez-Staples et al. 2010).

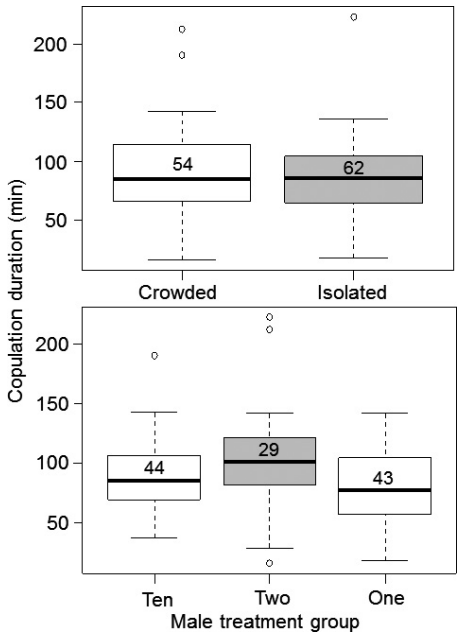

Fig. 3: Copulation duration (Q1-median-Q3) of mating pairs with different levels of sperm competition. Males were kept from emergence either alone or in groups of 25 males (previous treatments: Isolated or Crowded) and then placed with a total of 10, 2, or 1 (no rival) male per cage at the time of mating (current treatments). Numbers within boxes represent sample sizes.

On the other hand, male size, female size, and copulation duration affected sperm allocation. If larger females mate more frequently than do smaller females, SC intensity may co-vary with female body size (Simmons & Kvarnemo 1997; reviewed by Wedell et al. 2002). Kelly & Jennions (2011) found a positive relationship between ejaculate size and female body size (a measurement of female quality) in their meta-analysis, as in *C. capitata* (Taylor et al. 2000) and *A. ludens* (Pérez-Staples et al. 2014). Similarly, in our study, we also found a positive relationship between these two variables. It remains to be tested if female size is correlated with remating propensity. While in *C. capitata* and *Bactrocera tryoni* females mated to larger males store more sperm than females mated to smaller ones (Taylor & Yuval 1999; Pérez-Staples et al. 2007), here females mated with bigger males had less sperm stored. Contrary to previous results (Abraham et al. 2011b), total sperm stored was positive correlated with copulation duration, probably due to a more sensitive analysis used in this study.

The effect size Hedge' *d* was calculated to compare our data with the data for insects provided by Kelly & Jennions (2011) in their meta-analysis. Our effect size was statistically significant for the comparison between one vs. ten males at the time of mating. This is comparable to results above where sperm allocation increased with SC intensity. Similarly, this occurs in six of nine insect species studied (Kelly & Jennions 2011, appendix 1, section X, supporting information).

Mating Latency

The effects of current SC intensity were also apparent in mating latency, where there was an interaction between the competition perceived by males prior to and during mating. Pairs mated earlier when males faced current SC intensity and when there were from the crowded condition. Similarly, in *A. ludens* early adulthood exposure to a high male density affects sexual performance later in life (Díaz-Fleischer et al. 2009). In *D. melanogaster*, the presence of just one rival was enough to decrease mating latency (Bretman et al. 2009), while in our case, this response was observed only in the presence of several rival males prior to and during mating. This difference may be due to the lekking mating system of *A. fraterculus* where males encounter several rivals at the time of mating and respond to this stimulus strongly. Alternatively, mating latency could be under female control (Abraham et al. 2014). Females could be detecting and copulating earlier when there are more males to choose from at the mating site, or they could be copulating earlier as a way to avoid male harassment at higher male densities. In the sandfly *Lutzomyia longipalpis*, for example, female latency to mate varied with lek size, and females copulate with males sooner in bigger leks, compared with smaller aggregations (Jones & Quinnell 2002).

Mating latency was also affected by male and female size. Smaller females and bigger males had shorter latencies. Mating latency could be a measure of female acceptance or it could be a measure of male manipulation. Perhaps bigger males were more able to circumvent female resistance when females were small, thus resulting in shorter mating latencies for bigger males and smaller females. A faster ability to mate may pay off in terms of sperm precedence. While we have no information on whether there is first male sperm precedence in this species, studies from other tephritids have found second male sperm precedence but increasing first male sperm precedence as females oviposit throughout time (Opp et al. 1990; Bertin et al. 2010; Collins et al. 2012).

One possibility for the lack of an effect of previous SC intensity on mating latency and also sperm allocation is that the isolated treatment perhaps was not effective because males were held in individual

containers but the containers were next to each other. Males could not see each other but the detection of other males using olfactory and/or auditory cues cannot be discarded. For example, in the butterfly *Pieris napi*, males assess male density through the recognition of the sex pheromone citral (Larsdotter Mellstrom & Wiklund 2009). In *D. melanogaster*, males use auditory, olfactory, and tactile modalities to determine the presence of potential rivals (Bretman et al. 2011; Garbaczewska et al. 2013).

Copulation Duration

Copulation duration was not affected by the presence of rival males, neither prior to nor during mating. In *Drosophila bifasciata*, for example, when competitors are present before mating, males do not exhibit plasticity in copulation duration, as opposed to *D. subobscura* and *D. acanthoptera* (Lizé et al. 2012a,b). In contrast, the presence of rivals both prior to and during mating led to an increase in copulation duration in *P. laciniata* (Garcia-Gonzalez & Gomendio 2004), while in *D. melanogaster*, there is a plastic response of males to the presence of competitors in both situations and males are able to adjust mating duration optimally (Bretman et al. 2009, 2010, 2012). The interpretation of the results in the case of *D. melanogaster* assumes that copulation duration is under male control (MacBean & Parsons 1967), while in our case, crosses between different morphotypes of *A. fraterculus* have demonstrated that copulation duration is affected by female origin but not by male origin (Abraham et al. 2014), suggesting that copulation duration is under female control. Female-mediated effects could have a stronger effect on copula duration than exposure to rival males.

On the other hand, copulation duration was affected by female and male size. Larger females copulated longer, and the same tendency was observed in *C. capitata* (Taylor & Yuval 1999; Taylor et al. 2000). The authors hypothesized that small females may have less resistance to intromission or that more extensive penetration is required in large females to reach the bursa copulatrix (Solinas & Nuzzaci 1984). Similarly, in our case, larger males copulated longer, while in *C. capitata* shorter males copulated longer (Taylor & Yuval 1999) or had no effect in copulation duration (Taylor et al. 2000). In our case, larger males may be able to remain *in copula* longer to exert a kind of mate guarding, with or without female consensus.

In conclusion, *A. fraterculus* males strategically transferred sperm to females according to the number of perceived competing males at the time of mating. How males perceive the number of surrounding males, and whether they can regulate other components of the ejaculate such as accessory gland proteins remains to be tested.

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