Brainless but not clueless: earthworms boost their ejaculates when they detect fecund non-virgin partners

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In many animals in which females store sperm, males may detect female mating status and, in order to outcompete rival sperm, increase ejaculate size when copulating with non-virgin females. Although most studies have been restricted to organisms with separate sexes, theoretical models suggest that sperm competition should also be an important selective agent shaping life-history traits in simultaneous hermaphrodites. Nevertheless, the empirical support for ejaculate adjustment in a mating opportunity is scarce in hermaphrodites. In the present study, we performed a double-mating experiment to determine whether earthworms (*Eisenia andrei*) detect the mating status of their partners and whether they respond by adjusting their ejaculate. We found that earthworms triplicated the donated sperm when mating with a non-virgin mate. Moreover, such increases were greater when the worms were mated with larger (more fecund) partners, indicating that earthworms perform a fine-tune control of ejaculate volume. The results of the present study suggest that, under high intensity of sperm competition, partner evaluation is subject to intense selection in hermaphrodite animals, and donors are selective about to whom they donate how much sperm.

Keywords: sperm competition; hermaphrodites; partner assessment; sensory abilities; earthworms

1. INTRODUCTION

In ch. IX of his book 'The descent of man, and selection in relation to sex', Charles Darwin (1871) considered sexual selection to be restricted to higher animals and pointed out, for example, that 'Annelids apparently stand too low in the scale, for the individuals of either sex to exert any choice in selecting a partner, or for the individuals of the same sex to struggle together in rivalry'. Although the cognitive abilities needed to assess mate rivalry may be weak in hermaphroditic invertebrates (e.g. Charnov 1987), the theory predicts that sexual selection and especially sperm competition may also be an important selective force shaping the mating behaviour in hermaphrodites (Charnov 1996; Michiels 1998).

Simultaneous hermaphrodites have both functional female and male reproductive organs and multiple matings are common (Baur 1994; Angeloni *et al.* 2003; Monroy *et al.* 2003), and sperm storage organs have evolved in many species (Michiels 1998). It is assumed that hermaphrodites have a limited amount of reproductive resources for both sexual functions (Charnov 1996; Schärer *et al.* 2005). Given that the costs of sperm production are often non-trivial (Dewsbury 1982), hermaphrodites should optimize the amount of sperm allocated to the current partner while reserving enough sperm for future matings (Wedell *et al.* 2002). Since increased sperm production may reduce resources that can be allocated to egg production (De Visser *et al.* 1994; Lorenzi *et al.* 2007) and decrease somatic growth or maintenance (Van Voorhies 1992; Sella & Lorenzi 2003), hermaphrodites are expected to be prudent with their expensive male reserves (Koene & Ter Maat 2007).

However, when males compete with rivals for fertilization, larger ejaculates are predicted, especially to high-quality mates (Cook & Gage 1995; Martin & Hosken 2002; Wedell *et al.* 2002; Friberg 2006). Thus, opportunistic mating decisions should be selected in hermaphrodites, but empirical support for ejaculate adjustment according to sperm competition intensity is scarce (but see Anthes *et al.* 2006).

Some recent evidence supports increased sperm production in hermaphrodites when raised in large groups (Trouvé *et al.* 1999; Schärer & Ladurner 2003; Tan *et al.* 2004) and also in individuals exposed to enlarged mating groups (Brauer *et al.* 2007; but see Lorenzi *et al.* 2005), which indicates that hermaphrodites adjust sex allocation to the number of potential rivals (but see Locher & Baur 2000). The increased investment in male function with group size may be attributed to higher mating rates, or to the ability of individuals to differentiate the mating history of their partners and adjust sperm production accordingly. Nevertheless, there is little evidence that hermaphrodites can evaluate the mating history of their partners to estimate the intensity of sperm competition that their ejaculates will face in a particular mating event (Baur *et al.* 1998; but see Anthes *et al.* 2006), as occurs in many insects (Wedell *et al.* 2002; Uhía & Cordero 2005; Friberg 2006) and vertebrates (del Barco-Trillo & Ferkin 2004).

In the present study, we performed a double-mating experiment to determine whether redworms respond to the mating status of their partners by adjusting their ejaculate. In this simple situation over the sperm competition continuum (no competition and one competitor), an increase in the ejaculate size is predicted (Wedell *et al.* 2002). The redworm, *Eisenia andrei*, is a simultaneously
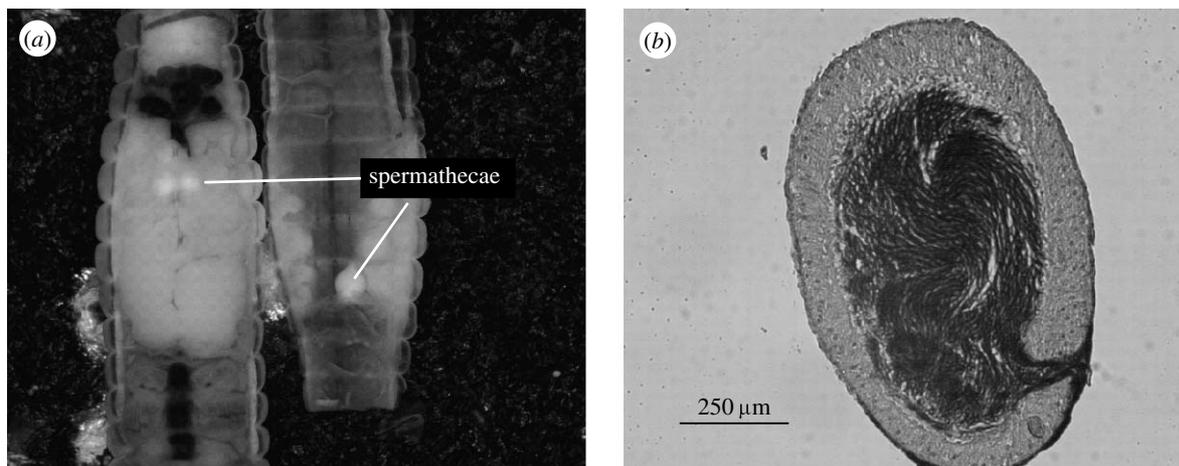
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Figure 1. Spermathecae of the redworm, *E. andrei*. (*a*) Spermathecae are located on the dorsal side of ninth and tenth body segments. The photography shows one dissected earthworm with the dorsal body wall removed to the right. Spermathecae can be observed as two pairs of brilliant spherical sacs. The remaining white mass corresponds to the seminal vesicles. (*b*) Freshly dissected spermatheca showing a bundle of sperm stored after two copulations.

hermaphroditic earthworm that lives at high densities where multiple mating is common (Monroy *et al.* 2003). Consequently, strategies to overcome strong sperm competition are expected. Indeed, redworms display a prolonged courtship that involves short and repeated touches between partners before mating attachment; they also spend a long time in copulation (Grove & Cowley 1926), offering possibilities for partner evaluation. Although sperm digestion is widespread in hermaphrodites, which makes it difficult to distinguish sperm competition and mating investment (Michiels 1998; Greeff & Michiels 1999*a*), redworms are unable to digest received allosperm (Richards & Fleming 1982). Redworms therefore constitute an excellent model for testing the effect of sperm competition on mating behaviour.

2. MATERIAL AND METHODS

(a) *Study animal*
Eisenia andrei (Bouche 1972) is an epigeic hermaphrodite earthworm (Oligochaeta, Lumbricidae) with a worldwide distribution due to its tolerance to a wide range of temperature and moisture conditions (Domínguez *et al.* 2005). During mating, both worms are attached in an inverse position by their ventral sides and there is transfer of sperm from the male pores up to the spermathecae of the partner with the help of epidermal organs such as *tubercula pubertatis*, which together with the mucus secreted, ensure perfect fixation of the earthworms during mating (Grove & Cowley 1926). During copulation, sperm are exchanged simultaneously and reciprocally and stored in the two pairs of spermathecae, located on the dorsal side of ninth and tenth body segments; these are spherical sacs that store the sperm until cocoon laying (figure 1).

(b) *Collection and maintenance*
Sixty hatchlings of *E. andrei* were obtained from a laboratory stock cultured at $20 \pm 2°C$. The laboratory stock was large enough (more than 10 000 individuals) to prevent endogamy. Hatchlings were placed in separate plastic Petri dishes to ensure that they were virgins at the time of experimental matings. Vermicompost and cow manure were supplied ad libitum as breeding medium. The dishes were kept in the darkness in a scientific incubator at $25°C$ and high humidity.

Earthworms were inspected weekly until sexual maturity was attained, detected as the development of *tubercula pubertatis* and *clitellum*.

(c) *Mating experiments*
We manipulated earthworm-mating history by performing a double-mating experiment. Forty-two mature and virgin earthworms were randomly assigned to three experimental groups (first partner, second partner and focal recipient). One week before the start of the experiment, earthworms were marked by a tiny light burn on different segments behind the *clitellum*, so that they could be recognized after matings. The body mass of each earthworm was recorded prior to the experiment, and there were no significant differences among experimental groups ($F_{2,41} = 0.62$, $p = 0.55$).

First, we placed 14 pairs of mature virgin earthworms (first partner × focal recipient) housed in a Petri dish with vermicompost and cow manure. All the pairs were examined twice daily until the appearance of spermatophores, indicative of copulation (Monroy *et al.* 2003). When the pairs had completed mating, one earthworm was removed (focal recipient) and placed with another mature virgin earthworm (second partner) for a second mating ($n = 14$) and examined twice daily. In both cases, the earthworms were randomly assigned to the mating pairs. The estimated time elapsed between two matings was 31 ± 6 hours, within the natural range of multiple matings (Monroy *et al.* 2003). After matings, all earthworms were fixed in formaldehyde 4% : ethanol 96% (1 : 1) and preserved with formaldehyde 4% in plastic tubes until their later dissection.

(d) *Estimation of sperm volume*
Spermathecae (figure 1) were dissected under a Nikon SMZ1500 stereomicroscope at various magnifications and were compressed to a uniform thickness of 66.3 μm under a supported cover-slip on a slide. The sperm mass was photographed with a Nikon Digital Camera DXM1200F. The sperm area of each spermatheca was measured twice by the use of ANALYSIS software and the mean of the two measurements was used to estimate the sperm volume as the area multiplied by the separation between cover-slip and slide (66.3 μm; see Cordero & Miller 1992). Sperm showed homogeneous density in all preparations (measured by colour

intensity in the ANALYSIS software). Thus, the total volume of sperm received in the matings was estimated as the sum of the volume of the four spermathecae. The earthworms included in the experiment contained sperm in the four spermathecae, except for two earthworms that showed three spermathecae with sperm and an empty one.

(e) *Statistical analysis*
Intraclass correlation coefficients (ICC) were used to estimate the correlation in the volume of sperm in spermathecae of the same earthworm. Paired *t*-tests were used to determine the effect of the mating status of the focal recipient on the volume of donated sperm to the first and second partner.

Homogeneity of the variance of sperm volume was tested by Levene's test for equality of variances. Mean differences in the sperm volume received after a single copulation and two consecutive copulations were analysed using a generalized linear mixed model (GLMM) with experimental group (first partner and focal recipient) as fixed factor and individual earthworms within mating pairs as repeated measure factor, controlling for heterogeneous treatment variances (PROC MIXED in SAS; Littell *et al.* 1996). The sperm stored by the first partner was used to estimate the volume of sperm transferred after a single copulation. Since earthworms were randomly assigned to experimental groups, we assumed that the distribution of sperm donated was similar in both groups of partners (first partner and focal recipient) in the first mating. The volume of sperm transferred by the second partner to the focal recipient was estimated by randomization (Monte Carlo; 1000 simulations) as the total volume of sperm in the spermathecae of the focal recipient minus the volume of sperm in the spermathecae of one randomly selected first partner. The results obtained by the randomization procedure are robust to any assumption about the exact amount of sperm that a earthworm (focal earthworm) has received during the first copulation.

The statistical significance of the difference in the volume of sperm in the first and second partners was estimated by means of Monte Carlo analysis (Manly 1997). After 10 000 simulations, the distribution of the *t*-test statistic in the original data, which measures the discrepancy in the sperm volume donated by the first and second partners (estimated by randomization), was calculated. The observations were then randomly allocated to the two samples and the *t*-test statistic was recomputed. After 10 000 simulations, the distribution of the *t*-test statistic under the null hypothesis was calculated. Finally, the estimated distribution of the *t*-test statistic in the original data was compared with the randomized *t*-test distribution (null hypothesis). Approximate *p*-values were calculated as the probability of randomized *t*-test values exceeding the distribution of *t*-test statistics based on estimated data. Similar results were achieved when the volume of sperm transferred by the second partner to the focal recipient was estimated by subtracting the amount of sperm present in the first partner from the total amount of sperm present in its focal partner after two matings, and the significance was tested by a paired *t*-test (data not shown). Data are expressed as mean ± s.e.

3. RESULTS
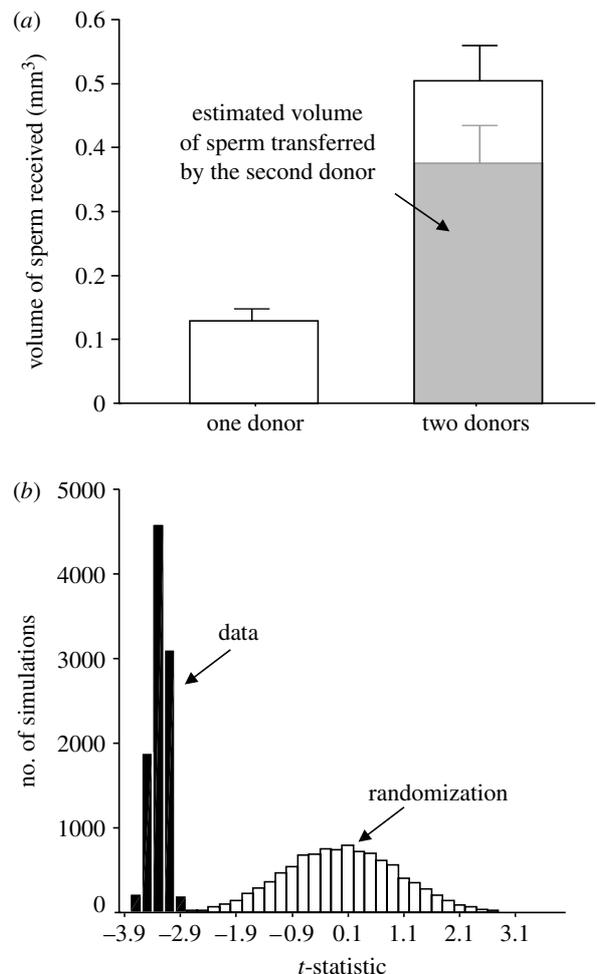Sperm volume stored in the spermathecae was similar in the first and second partners of the focal redworms (paired

Figure 2. Comparison of the sperm stored in relation to the number of matings. (*a*) Total volume of sperm (mm³) received after copulation with one or two donors. In grey, the sperm volume donated by the second donor to non-virgin recipients, as estimated by randomization. (*b*) Distribution of Monte Carlo simulations of the *t*-statistic values for the original data, which measures the discrepancy in the volume of sperm donated by the first and second donors (estimated by randomization) and the *t*-statistic values for randomly allocated observations (null hypothesis).

t-test, $t_{13} = 0.10$, $p = 0.91$), which indicated that there was no sperm depletion after two consecutive copulations. Sperm was similarly distributed in the four spermathecae. Thus, after a single copulation, sperm volume was correlated among the four spermathecae within individuals (ICC, $r = 0.86$, $p < 0.001$), and the same occurred in the focal redworms after two copulations (ICC, $r = 0.80$, $p < 0.001$). Interestingly, the total sperm volume stored in the focal redworm after two consecutive copulations was 3.9 times greater and more variable than that after a single copulation (figure 2*a*; Levene's test for homogeneity of variance, $p = 0.003$; GLMM, $F_{1,13} = 43.96$, $p < 0.0001$).

Since earthworms were randomly assigned to the experimental groups, we estimated the sperm volume transferred by the second partner as the total sperm volume in the spermathecae of the focal recipient minus the randomized sperm volume in the spermathecae of the first partner. Thus, the estimated sperm volume transferred by the second partner was $0.375 \pm 0.06 \text{ mm}^3$ ($n = 14$), 3.3 times greater than that transferred in the first copulation (Monte Carlo analysis, $p = 0.0001$; figure 2*b*).
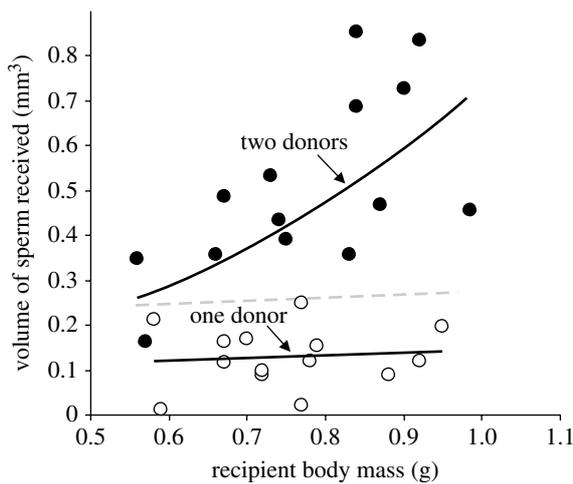
Figure 3. Total volume of sperm (mm^3) stored in the spermathecae after copulation with one or two donors in relation to the recipient (own) body mass (interaction recipient body mass $\times$ number of donors, $F_{1,24} = 5.925$, $p = 0.02$). Dashed line indicates the expected sperm volume stored in the recipient if the sperm volume donated by the second donor be similar to that donated by the first donor.

The total sperm volume stored after two copulations was related to the recipient body mass (figure 3; $r_{14} = 0.65$, $p = 0.018$), but not to the body mass of donors (first partner: $r_{14} = 0.37$, $p = 0.19$; second partner: $r_{14} = 0.42$, $p = 0.13$). This effect was not found in the first copulation, where the total sperm volume stored was related neither to the receiver body mass (figure 3; $r_{14} = 0.11$, $p = 0.71$) nor to the donor mass ($r_{14} = 0.06$, $p = 0.83$).

4. DISCUSSION

In this study, we found that earthworms responded to the mating status of their partners and triplicated the donated sperm when they mate with a non-virgin mate. Moreover, such increases were greater when the worms were mated with larger partners, indicating that earthworms perform a fine-tune control of ejaculate volume. These results indicate that redworms have evolved mechanisms to detect and adjust ejaculate investment in order to maximize fertilization opportunities (Wedell *et al.* 2002). Our study suggests that sperm competition (Parker 1970) is a powerful evolutionary force that has influenced the mating behaviour in earthworms.

Sperm competition occurs when multiple matings lead to the simultaneous presence of live sperm from two or more individuals within a female's reproductive tract. In this multiple-mating experiment, we found that redworms equally distributed the sperm among the four spermathecae and that the volume of sperm donated by the second partner, although more variable, was probably added to the sperm stored from the preceding copulation. Therefore, when redworms copulate with a mated partner, their ejaculates compete with the rival sperm to fertilize the ova. Under sperm mixing from multiple donors, precedence rules probably approximate to 'fair raffles' mixing, and sperm competition will select for an increased amount of transferred sperm (Parker 1998).

Accordingly, we found that redworms are able to perceive partner mating status and triplicate their ejaculates after detecting a risk of sperm competition (a previously mated partner). Interestingly, this finding

also reveals that under no sperm competition risk red-worms are prudent in their ejaculate expenditure, even when paired with high-quality mates, as that occurs in the first copulation of earthworms reared in isolation. These results indicate that they may regulate the duration of copulation or, alternatively, that they may have mechanisms that prevent all sperm being released in a single mating event. Although we did not find sperm depletion after two consecutive ejaculations, the prudent sperm allocation shown by redworms is in line with the models suggesting non-trivial costs of ejaculate production (Greeff & Michiels 1999*b*).

Our results indicate that redworms boost their ejaculate when paired with recently mated partners. However, interestingly, the volume of sperm donated to non-virgin partners was more variable than that transferred to virgin recipients. This variability may indicate that under high sperm competition intensity, resource allocation to sperm may become expensive (Greeff & Michiels 1999*b*), and sperm donors become more selective about to whom they donate sperm (Michiels *et al.* 2003). To test this prediction, we examined the relationship between sperm volume donated and partner quality, estimated as body mass. As in many hermaphrodites, egg production in earthworms increases with body mass (Domínguez *et al.* 1997) and larger partners are preferred (Monroy *et al.* 2005). Thus, if sperm is costly, redworms should boost their ejaculates under high sperm competition intensity, especially when paired with larger partners, since the potential benefits are greater. According to this prediction, we found that the total sperm volume stored after two copulations was related to the recipient body mass and this effect was not found in the first copulation. Thus, the estimated volume transferred to larger non-virgin partners (above 0.85 g) was five times greater than that transferred to virgin partners (figure 3). These results indicate that redworms bias potential sperm competition in their favour, particularly when they copulate with more fecund partners.

Phenotypic plasticity, as we have found in sperm allocation, is expected to be favoured when the environment is unpredictable on a time scale that is short relative to generation time (de Jong 1995). Redworms live in populations in which multiple matings are common, but density and mating rates oscillate frequently and probably unpredictably (Monroy *et al.* 2006). Opportunistic ejaculate expenditure according to the fluctuations in mating rates should be advantageous, favouring the allocation of resources to egg production under low mating rates, and higher allocation to male reproduction in a scenario of higher sperm competition. As it has been pointed out, this plasticity may explain why hermaphroditism is maintained in fluctuating large and dense populations (Brauer *et al.* 2007).

We do not know the cues that redworms use to assess partner mating status, but tactile and chemical cues are probable sources of this information (see Schleicherová *et al.* 2006). Earthworms show a prolonged courtship with short and repeated touches between partners before mating and they spend a long time in copulation with constriction movements between partners, which provides ample opportunity for partner assessment (e.g. Michiels *et al.* 2001). Earthworms are sensitive to chemicals having large numbers of chemoreceptors all over their bodies, most of them concentrated in the prostomium and

anterior segments (Wallwork 1983). In addition, the epithelium in the mouth region accommodates groups of sensory cells that are associated with the detection of mucus secretions from other earthworms (Edwards & Lofty 1972). In his last work, Charles Darwin devoted himself entirely to the investigation of earthworm biology (Darwin 1881). Contrary to his previous thinking (Darwin 1871) but in accordance with his observations, he concluded that earthworms possess more cognitive potential than was generally assumed. The results of the present study are consistent with this view and show that the hermaphroditic redworm recognition system is actually highly efficient in terms of mate evaluation and is important to the reproductive success of these animals.

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