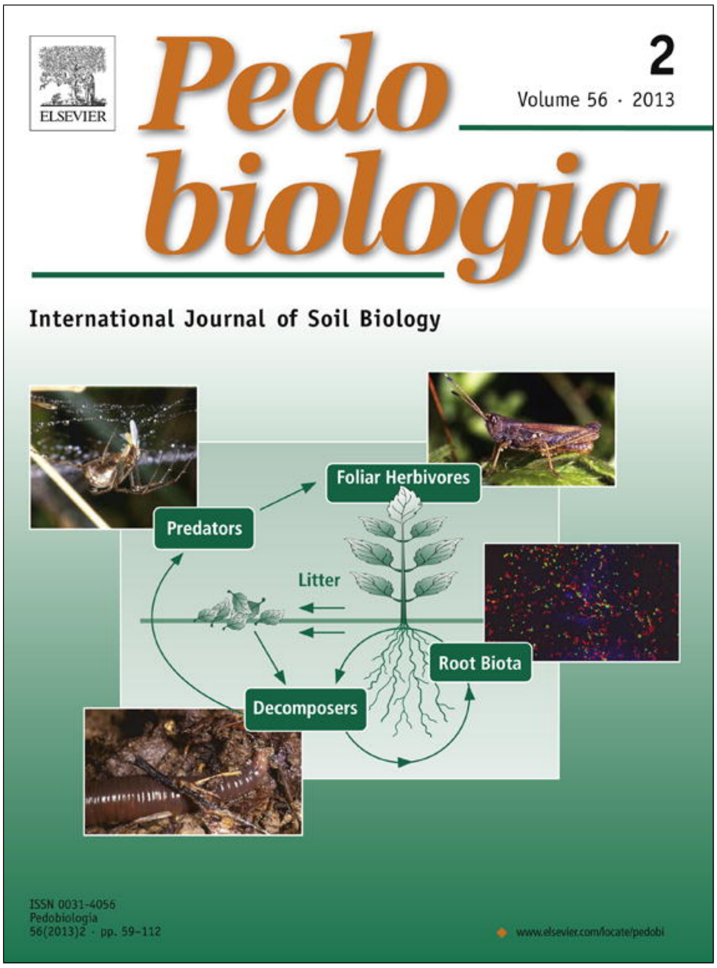
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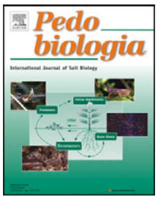

Does the order of copulation matter? Experimental paternity analyses in the earthworm *Hormogaster elisae* (Annelida: Hormogastridae)

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ABSTRACT

Multiple mating and sperm storage organs allow postcopulatory sexual selection to occur. This is the first time that a paternity test has been performed in an earthworm species. Microsatellite markers are used in *Hormogaster elisae* to trace paternity for multiple sires. Multiple paternity was detected in some cases, showing sperm mixture even within one cocoon, suggesting the eventual mixture of the allosperm within the spermathecae. Order of copulation influences paternity, the first (P1) and third partner (P3) being the most successful. Interestingly the second partner (P2) almost lacks paternity. The most plausible hypothesis suggests the existence of sperm displacement by flushing out older sperm once the spermathecae are full, which seems to occur when they contain sperm from two partners and P3 donates. At that moment sperm is still stratified thus removing sperm from P2. Afterwards sperm from P1 and P3 would be mixed provoking an equivalent paternity. Given the endogeic nature of the earthworm, copulation was not observed, making the lack of copulation with P2 (due to the need of a recovery time) or copulation without sperm transfer (due to low sperm production rate) possible alternative hypotheses. The weight of the earthworms is related to the number of cocoons they produce but not to their viability, which in all the cases was low, probably due to laboratory conditions. The sperm was kept viable inside the spermathecae for a maximum of two years and three months, suggesting a very effective nourishment system in the epithelium of the storage structures.

Introduction

Hermaphrodites exhibit multiple mating to increase reproductive success for the male function (e.g. Baur 1998; Michiels 1998; Pongratz and Michiels 2003). When females receive sperm from multiple males, paternity success (sperm precedence) is determined by the underlying processes of sperm storage and sperm utilization. Therefore how mating success translates into fertilization success is difficult to predict in simultaneous hermaphrodites with internal fertilization (Pongratz and Michiels 2003). It is also difficult to distinguish the relative importance of sperm competition and cryptic female choice over postcopulatory mechanisms (Birkhead and Møller 1993; LaMunyon and Eisner 1993; Simmons et al. 1996; Otronen et al. 1997). Numerous studies have detected an effect of mating order on paternity. Although documented for a wide range of taxonomic groups (Smith 1984; Birkhead and Møller 1998; Simmons 2001), including sessile marine invertebrates (Bishop et al. 2000), and even free-spawning marine invertebrates (Marshall et al. 2004) the influence of mating order on sperm use has never been described in any earthworm species.

The literature offers a great variety of examples on last-male precedence, although most examples are based on studies involving only two partners (Eady and Tubman 1996). A sperm stratification hypothesis (Parker 1970) could be used to explain non-random usage of sperm from different males and last male sperm precedence. Under this hypothesis, the last sperm to enter the spermathecae (female storage organs) would be the first to exit and fertilize the eggs. Moreover, some adaptations for males have been promoted by sperm storage such as volumetric displacement of the sperm of previous mates, its active removal or blocking the deposition of sperm by subsequent mates (e.g. Diesel 1991).

Different reproductive strategies are present in earthworms (Díaz Cosín et al. 2011), the most common being simultaneous hemaphroditism with cross fertilization (i.e., simultaneously transferring and receiving sperm during copulation). This is the case for *Hormogaster elisae*, endogeic and endemic to the central area of the Iberian Peninsula where it plays an important ecological role. *H. elisae* presents a long life cycle when compared to other earthworm species, needing on average 484 days for clitellum development from hatchling and showing a cocoon production rate between 0.9 and 2.29 per specimen per year (Díaz Cosín et al. 2009).

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Several factors have been explored as possible influences in the sexual selection of earthworms. Regarding the selection before mating, we could cite body size (Monroy et al. 2005; Novo et al. 2010) as it is related to female fecundity (Michiels et al. 2001), reciprocity (Michiels 1998), the quality of the place where cocoons will be deposited after mating (Grigoropoulou et al. 2008), and the distance between potential partners (Sahm et al. 2009), for example. But in spite of the existence of selection before mating, multiple mating has been observed in earthworms (Monroy et al. 2003; Sahm et al. 2009) and specifically in *H. elisae* (Novo et al. 2010) and therefore selection after mating and/or a multiple paternity could be expected. Postcopulatory sexual selection is possible because sperm remains viable within the spermathecae until fertilization. Indeed, in *H. elisae* nourishment of the sperm by spermathecal epithelium has been described (Novo et al. 2012). Mechanisms of sexual selection proposed for earthworms at this level are sperm digestion (Richards and Fleming 1982), differential storage in different spermathecae or maybe in different cavities of complex spermathecae (Van Praagh 1995; Butt and Nuutinen 1998), an adjustment of the reproductive effort depending on the relatedness degree of the partner (Velando et al. 2006), a control over the donated sperm volume (Velando et al. 2008) or allohormone injection (Koene et al. 2002).

Differential storage of sperm in different spermathecae was discarded in *H. elisae* by Novo et al. (2010), who found no differences in the origin of the allosperm contained in the four spermathecae of this earthworm, proposing that if sperm competition was present in these animals, it should be orchestrated by mechanisms inside each spermatheca. Sperm digestion has been observed in *H. elisae* but it is unknown if it was for sexual selection (Novo et al. 2012). If this is the case, we could hypothesize that the oldest sperm would be digested and therefore the last male should win the paternity. The spermathecae of *H. elisae* are tubular and even though no tissue subdivisions have been observed, stratification could be expected because of their shape, therefore also predicting last male precedence. This species has normally been shown to store the sperm of at least two partners (62.66% of the analyzed individuals; with less cases storing sperm from one or three partners), preferably of the same size that are found in close proximity thereby eliminating the need for long-distance dispersion (Novo et al. 2010).

As mentioned above, several authors have studied postcopulatory sexual selection in earthworms but earthworm paternity has not yet been explored. Here we present the first paternity study in earthworms taking the endogeic species *H. elisae* as a model. The objectives were to test firstly whether the paternity was multiple or single and secondly whether the order of copulation had an influence on paternity by means of cryptic female choice or sperm competition processes.

Material and methods

Earthworms were collected in El Molar (Madrid, Spain) (GPS: 40°44′ 22.9″ N, 3°33′ 53.1″ W). The climatic and edaphic characteristics of the site are fully described in Hernández et al. (2007). Prior to the main experiment, preliminary assays were performed in order to obtain some necessary information.

Assuring virginity of experimental individuals

The difficulty of raising hormogastrids in the laboratory (Díaz Cosín et al. 2009) obliged us to use a different strategy and collect immature earthworms in the field for further use in the experiments. Although it may sound trivial, it was neccessary to assure that these immature individuals had not received sperm. Therefore, we dissected under the stereomicroscope 52 immature earthworms and checked for the presence of spermathecae and sperm inside them. These individuals were collected in previous studies of our group in spring and autumn of 2001 and 2002 and were preserved in formalin (Hernández et al. 2007). The spermathecae (when they were present) were placed on a glass slide and compressed with a cover slip to check for sperm presence/absence. All of these individuals lacked the clitellum and most of them did not show tubercula pubertatis. Only in a few of them (14) was this structure starting to develop. The immature individuals examined (measures of preserved specimens) presented a length of 3.7–13.6 cm (mean = 9.02 cm) and weight of 0.2–2.19 g (mean = 1.16 g). In 14 of the individuals, spermathecae were not even found, in three specimens only small signs were observed in the place where the spermathecae were supposed to be located and in two of the individuals only the posterior pair was detected. In the remaining 33 individuals both spermathecae pairs were present (the larger was posterior) but we did not observe sperm content in any case. Therefore, individuals collected in the field with no clitellum could be reliably used as virgin earthworms in the experiments. To assure virginity without any doubt, we only collected immature individuals that showed no signs of tubercula pubertatis. We cultured these immatures isolated in individual microcosms in *ad libitum* laboratory conditions controlling their growth and maturity acquisition. This provided our virgin stock for the given experiments.

Assuring copulation of the virgin individuals under laboratory conditions

Another limitation when working with an endogeic species is that copulation occurs below the soil surface and thus it cannot be observed. Therefore we attempted to design flat transparent microcosms where we could observe the earthworms and detect copulation events. However, they were not successful because these microcosms, even though supplied with sufficient water, triggered aestivation processes.

In order to check if *H. elisae* copulates under laboratory conditions we set up six PVC cylinders, filled with 2400 g of superficial soil from El Molar, previously dried and then moistened by layers until the water content was 20%. Some grass was included in the upper part. In each of the cylinders four virgin mature individuals (showing clitellum and tubercula pubertatis) from our stock were introduced (allowing them to burrow). The experiments were maintained for one month at 16 °C in culture chambers. After this period, cocoons were collected by wet-sieving the soil (2 mm) and the specimens were fixed in absolute ethanol and dissected to check for sperm content. Presence of sperm within the spermathecae indicated that copulation had taken place, which occurred in five of the six cylinders. Only one (non-viable) cocoon was found in one of the cylinders.

We know, from the previous experiment, that in one month four individuals copulate and most probably they do not have time to lay cocoons (only one was found) so if a consecutive three-partner experiment with no cocoons output before isolation of the earthworms needs to be set up, this time should be divided into three. Therefore we were interested to know if 10 days are enough for two virgin individuals placed together to copulate. For that purpose, we set up four fresh PVC cylinders as previously described. In each cylinder we introduced two individuals of similar size, one of them previously marked with a pink visual implant elastomer (VIE), in order also to test the durability of those markers for the main experiment. We cut away a caudal portion (ca. 25 mg) one week before starting the experiment (to test if this was affecting the individuals). In the main experiment (order of copulation) this portion of the earthworm tail would act as a DNA reserve in case some of the earthworms died during the experimental period. No cocoons were recovered after the ten days. The earthworms were dissected and we observed sperm in the spermathecae of the earthworms from three of the four replicates, which gave us a 75% assurance of copulation in future experiments. When sperm was present, it was in the four spermathecae, although posterior ones were filled with a higher quantity of sperm as previously observed by Garvín et al. (2003). This is compatible with the observations of Novo et al. (2010) that showed a mixture of the received sperm from different partners in the four spermathecae with no differential storage.

Once assured of virginity and copulation of experimental individuals under laboratory conditions, we performed the main experiment of this study as described below.

Order of copulation experiment

We collected 48 immature earthworms and raised them isolated (to assure their virginity) in the laboratory until they accomplished sexual maturity (i.e. appearance of tubercula pubertatis and clitellum), approximately after one year (see Díaz Cosín et al. 2009). We organized twelve groups of individuals including four earthworms each, of approximately the same size. Each of these four individuals was tagged with visual implant elastomer (VIE) of different colours (pink, blue, yellow, orange). According to Butt and Lowe (2007) this tagging method does not affect growth, mating or cocoon production, which made it suitable for our study. Within each group, we performed the controlled-mating experiment by pairs in a way that all the earthworms of the group copulate with the other three, in a sequential order.

Mating pairs were together for ten days after which partners were changed. Once every earthworm had been with three different partners, it was isolated. Every two months, the soil in the cylinders containing the isolated individuals was changed and cocoons were collected by wet-sieving (2 mm). The recovered cocoons were incubated in separate labelled Petri dishes on moisturized filter paper (Whatman No. 1) and checked every two days for hatching. The newborn earthworms were preserved in ethanol for subsequent DNA analyses. The experiments were started in two batches according to virgin availability. The earthworms from the first half of the experiment were isolated in December 2008 and the second half in November 2009 and all were maintained and cocoons searched for until September 2011, when several months with no cocoons had passed and several earthworms had died.

The microcosms consisted of PVC cylinders of 10.5 cm diameter and 30 cm depth filled with 2400 g of moisturized soil from El Molar (previously dried) of 20% moisture content following the procedure of Díaz Cosín et al. (1996). The microcosms were prepared 24 h before introducing the earthworms and maintained at 16 °C in culture chambers.

At least one month before starting the experiment, a set of caudal segments was cut off every earthworm, which would be our source of DNA, thus preventing the loss of samples in case of death. The tips were carefully cleaned and preserved in absolute ethanol at −20 °C for DNA analyses.

Microsatellite analyses of paternity

DNA from the tips of the mating individuals and from the newborn earthworms was isolated using the DNeasy Tissue Kit (QIAGEN) and stored at 4 °C. We selected four loci (Hem07, Hem188, Hem193, Hem194b) among the microsatellite markers developed by Novo et al. (2008) because of their high degree of polymorphism and their good performance in the study by Novo et al. (2010), where the conditions for PCR can be found. The amplified products were analyzed on a 3730 DNA Analyzer (Applied Biosystems) and the alleles were sized using the GS-500 LIZ size standard and Peak Scanner Software v. 1.0 (Applied Biosystems).

The four loci permitted us to assign with certainty the paternity (sperm donor) for each neonate, except for one case, in which the paternity could not be determined. We excluded this case from the analyses.

Statistical analyses

We investigated the influence of the order of copulation in paternity success by means of generalized linear mixed-effects models (GLMM). Models including a random term are appropriate to analyze clustered and therefore potentially autocorrelated data (Pinheiro and Bates 2000). In this experiment, random variability in paternity success may arise from differences caused by earthworm identity and group assignment (i.e. differences caused by the size of the earthworms). Following Pinheiro and Bates (2000), model selection proceeded in two steps: finding the optimal structure of the random component and finding the optimal structure of the fixed component. Different GLMMs, with Poisson error distribution and a log link function, were compared. The most complex model was stated as follows:

$$\text{Number of offspring} = \text{Copulation} + \text{MotherId}\ \ (\text{Group})$$

where *Copulation* is an ordered factor with three levels specifying the order of copulation in each group and *MotherId* (*Group*) is a random term specifying the effect of the “mother” (earthworm laying the cocoon), nested within the copulation group. Only “mother” earthworms producing at least one viable cocoon were included in this analysis. Model selection was based on Akaike’s Information Criterion (AIC). We fitted all linear mixed-effects models using the ‘lme4’ package (Bates et al. 2011) in R 2.15 (http://www.rproject.org).

We used simple correlations in order to investigate the effect of the weight of the “mother” in fecundity and cocoon viability.

Results

Out of the 48 earthworms used in the experiment, 149 cocoons were collected, with eleven the maximum produced by a single individual (Table 1). Of those, 94 cocoons (63%) were not viable whereas 55 cocoons (37%) successfully hatched. The number of newborns was 56 because one pair was born from the same cocoon (Table 1). The maximum number of descendants that a single earthworm had was eight. A total of 17 earthworms did not produce viable cocoons and nine did not produce any cocoons at all (Table 1). Paternity was assigned to only one parent in 16 cases (eleven with one descendant and five with multiple descendants) out of the 22 earthworms producing viable cocoons, and to more than one parent in the remaining six cases (Table 1). Regarding the 16 cases of single paternity, seven earthworms had descendants with the first parent (P1), three with the second (P2), five with the third (P3) and one could not be assigned to any parent. Regarding the six cases of multiple paternity, three earthworms had descendants with two sperm donors, and three with three partners. In no case had P2 more than one descendant with the same “mother” (Table 1) In the case of the two earthworms that hatched from the same cocoon, each of them was conceived with sperm from different donors (P1 and P3).

The order of copulation influenced paternity (Table 2). More precisely, the second partner contributed to the paternity to a lesser degree than the first and third partners (Table 2), who had a similar contribution (Fig. 1).

The last viable cocoon collected was laid after the earthworms had been isolated for two years and three months, indicating that the sperm was kept viable within the spermathecae.

There was a significant correlation between the weight of the earthworms and the total number of cocoons they produced

Table 1
Established groups for copulation experiments, weight of the earthworms in grams, number of total cocoons, non-viable cocoons and offspring produced per earthworm and number of offspring produced by each partner. P1: first partner; P2: second partner; P3: third partner. Totals are shown below.

Group	Individual weight (g)	Total cocoons	Non-viable cocoons	*N* offspring	*N* offspring P1	*N* offspring P2	*N* offspring P3
1	7.78	7	2	5	1	0	4
	7.4	11	6	5	3	1	1
	6.34	6	3	3	3	0	0
	6.24	4	0	4	1	1	2
2	6.13	7	3	4	0	0	4
	5.93	0	0	0	0	0	0
	5.83	1	1	0	0	0	0
	5.62	8	1	8[a]	2	1	5
3	5.53	3	0	3	1	0	2
	5.46	7	4	3	3	0	0
	5.39	4	2	2	0	0	2
	5.37	3	2	1	0	1	0
4	5.3	0	0	0	0	0	0
	5	6	2	4	0	1	3
	4.92	9	5	4	0	0	4
	4.4	1	1	0	0	0	0
5	4.4	2	1	1	1	0	0
	4.2	5	4	1	1	0	0
	4.04	0	0	0	0	0	0
	4	0	0	0	0	0	0
6	3.76	1	1	0	0	0	0
	3.44	1	1	0	0	0	0
	3.4	0	0	0	0	0	0
	3.2	1	1	0	0	0	0
7	7.98	3	2	1	1	0	0
	7.77	3	3	0	0	0	0
	7.45	2	2	0	0	0	0
	7.44	2	1	1	0	0	1
8	6.9	6	6	0	0	0	0
	6.78	6	6	0	0	0	0
	6.27	0	0	0	0	0	0
	6.51	1	1	0	0	0	0
9	6.21	5	4	1	1	0	0
	5.92	1	1	0	0	0	0
	5.92	0	0	0	0	0	0
	5.65	2	1	1	0	1	0
10	5.65	3	3	0	0	0	0
	5.5	1	1	0	0	0	0
	5.05	3	3	0	0	0	0
	4.98	3	2	1	[b]	[b]	[b]
11	4.72	3	2	1	1	0	0
	4.72	1	1	0	0	0	0
	4.66	0	0	0	0	0	0
	4.66	3	2	1	0	1	0
12	4.65	5	4	1	0	0	1
	4.63	4	4	0	0	0	0
	4.46	0	0	0	0	0	0
	4.53	5	5	0	0	0	0
Total		149	94	56	19	7	29

[a] 8 neonates coming from 7 cocoons.

[b] Sperm donor could not be inferred from microsatellite profiles in this case.

($r = 0.35$, $P = 0.02$). No significant correlation was found between the weight of the earthworms and the proportion of viable cocoons ($r = 0.25$, $P = 0.09$).

Discussion

Sperm coming from different partners are mixed in the four spermathecae of *H. elisae* (Novo et al. 2010), and therefore postcopulatory sexual selection processes such as sperm competition or cryptic female choice could be expected to occur within each spermatheca of this species. Here, we investigated whether the order of copulation influences the paternity of *H. elisae*, when placed together with three different partners. A typical last male precedence pattern (Silberglied et al. 1984; Simmons 2001; Friedlander et al. 2005) could be expected given the previous knowledge of the species because of two reasons: (i) the digestion processes observed within its spermathecae (Novo et al. 2012), which, if for sexual selection, would be presumably directed to eliminate the oldest sperm (Villavaso 1975; Hellriegel and Bernasconi 2000; Snook and Hosken 2004) therefore favouring the fertilization by the newest one (i.e., last male precedence) or to

Table 2
Results of the generalized linear mixed-effects models to examine the influence of the order of copulation in paternity success. Models included data from earthworms producing at least one viable cocoon with assigned paternity ($n = 21$). The estimated variance and standard deviation are shown for the random effects. For the fixed terms, the estimated coefficient and its standard error, the z-value and P-value are shown. Best fitted model [*Number of offspring* = *Copulation* + *MotherId* (*Group*)]. The intercept corresponds to the first copulation as the reference group. Actual effects of the estimated coefficients have to be calculated through the exponential function (i.e. the inverse function to the log link function), meaning that the contribution to paternity of the first and third copulations was similar whereas the contribution of the second was smaller.

Random effects		Variance		SD
Group		0.316		0.562
Fixed effects	Estimate	SE	z value	P (>|z|)
(Intercept)	−0.309	0.308	−1.003	0.316
Second	−0.999	0.446	−2.238	0.025
Third	0.423	0.298	1.420	0.156

maintain healthier sperm (Jones et al. 2000; Siva-Jothy 2000); (ii) at the same time, the tubular structure of these spermathecae would presumably provoke a stratified storage, making it easier for the last sperm entering the spermathecae to be released for fertilization. However, the results observed in this experiment present some complications that need to be taken into account if the previous assumptions are to be accepted. Indeed the order of copulation influences the paternity, but interestingly the most successful partners are first (P1) and third (P3) whereas the second partner (P2) almost lacks paternity. What could be happening with the second mate?

Passive sperm displacement?

The first hypothesis we propose is the existence of sperm displacement, which seems to better explain the results obtained. Removal of previously deposited sperm from the female reproductive tract has been observed in other animals (Pair et al. 1977; Silberglied et al. 1984; Diesel 1991), and in the case of earthworms would involve passive displacement of one ejaculate by a subsequent one, since they lack structures for such removal. Therefore, resident sperm from the previous male would be flushed out by the incoming ejaculate of the last male. Nevertheless, this passive displacement would only work when the spermathecae are full or almost full. The capacity of *H. elisae* spermathecae is unknown but most of the spermathecae studied by Novo et al. (2010) contained the sperm from two individuals. In this context, the sperm of *H. elisae* would be stratified in the short-term (immediately after the matings) and when P3 is copulating (spermathecae already full with sperm from two partners), its sperm passively displaces the sperm of the previous partner (P2) by increased pressure. Eventually, since there are no more copulations in our experimental design, the sperm of P1 and P3 (and small quantities of P2 if left) would be mixed and therefore cocoon production would be done with remaining types of sperm leading to multiple paternity. This initial sperm stratification followed by later mixing is predicted to occur in species with tubular spermathecae (Walker 1980; Lewis and Jutkewicz 1998). The homogeneous mixture of sperm in the spermathecae is supported in the case we found of one cocoon producing two individuals from different sperm sources. Moreover, Porto et al. (2012) found that a similar amount of sperm was donated to six successive partners when studying behaviour in *Eisenia andrei*, which explains the almost equivalent contribution of P1 and P3 to the progeny. Under this hypothesis we do not expect an active sperm removal or ejection from storage by the female to accept the sperm from the last male (as observed in e.g. Villavaso 1975; Hellriegel and Bernasconi 2000; Xu and Wang 2010) since in both cases the pattern would be P3 > P2 > P1.

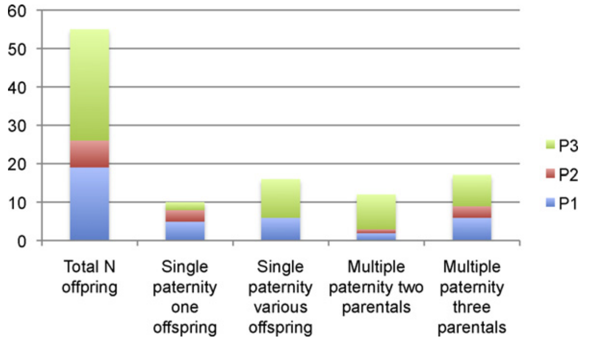

Fig. 1. Histogram representing the number of offspring coming from the first (P1), second (P2) and third partner (P3) (total N offspring) depending on different paternity scenarios. Single paternity: one partner is responsible for all the offspring from one "mother". Multiple paternity: more than one parent donated sperm for the production of offspring.

The paternity pattern found in the present work has some similarities to the one observed in *Tribolium castaneum* (Lewis et al. 2005). The authors found that in this beetle, when females remated before sperm mixing occurred, sperm stratification resulted in differential loss of sperm from the most recent mate. They made experiments with double and triple matings and in both they found the last male precedence pattern. This pattern was not so clear when the space for the sperm in the spermathecae was sufficient (i.e. double matings), however in triple matings, the third partner displaced the sperm from the second partner, placing the second partner as the least successful, as in the present study.

Sperm production time?

The second possible explanation of low paternity for P2 is that sperm production (or its transfer to seminal funnels) needed more time between copulation events. In *T. castaneum*, for example, the time interval between competitive matings had a significant effect upon subsequent paternity precedence (Arnaud et al. 2001). It is unknown if the seminal funnels of *H. elisae* became totally depleted after a copulation but other earthworms such us *E. andrei* have shown under experimental conditions that copulations between mate pairs were within approximately 31 h and there was sperm transfer (Velando et al. 2008), indicating that sperm was available for usage in these earthworms within this short period of time. Also Porto et al. (2012) suggested that *E. andrei* probably replenish their reservoirs of mature spermatozoa between consecutive copulations. However, the cycle of *E. andrei* (Domínguez 2004) is much more rapid and shorter than that of *H. elisae* (Díaz Cosín et al. 2009) and both species present very different life strategies. Indeed, it has been demonstrated that male gametic strategies can vary greatly, even among closely related species (Pitnick and Markow 1994) and it has been shown in other animals that males can be depleted of sperm in subsequent matings (Nakatsuru and Kramer 1982; Pitnick and Markow 1994), so in some species the production rate of sperm or other ejaculatory materials is limiting male reproductive success, and the number of ejaculates is restricted because of the time necessary to restore the depleted reserves (see review in Dewsbury 1982).

There is no information on the time needed to produce sperm in earthworms and specifically in *H. elisae.* The annual cycle of gametogenesis of this species was studied in the field by Garvín et al. (2003), who showed that gametogenic processes occur mainly during autumn and winter, being interrupted in summer because of quiescence. Our experiments were performed with virgin individuals that had been maintained in experimental cylinders with favourable and constant conditions, so gametogenesis should have been occurring all the time. Moreover, the matings were performed in November–December, which is within the most favourable period detected for sperm production.

In this particular case it should be considered that virgin individuals had been isolated for more than one year (sometimes much longer) until their first copulation. Therefore it is possible that they were in need to release their mature sperm. In this scenario, virgin individuals would hypothetically donate all the sperm that was prepared in the seminal funnels, needing a new sperm batch production to fill them. According to Velando et al. (2008), *E. andrei* can adjust its ejaculate depending on the mating status of the partner and it triplicates the donated sperm when the partners are not virgin. If this were true also for *H. elisae*, there is no reason why they should donate all the sperm they have available in the first mating, where all the earthworms are virgin. Nevertheless, there are other examples of animals doing the contrary. For instance, the butterfly *Papilio machaon* provides more sperm and secretions to initial versus subsequent mates (Svard and Wiklund 1986; Svard and Wiklund 1991) and the authors attributed this to males donating more sperm to virgin than to non-virgin females. Also female flies *Drosophila melanogaster* and fishes *Hyphessobrycon pulchripinnis* choose not to copulate with recently mated males since this is negatively correlated with sperm volume (Markow et al. 1978; Nakatsuru and Kramer 1982). Regarding sperm production rate, current evolutionary theory predicts that males should produce a higher quantity of sperm when the sperm competition is high (Parker 1998) and in this case, given that only two earthworms were located in the cylinder, competition was absent. Also, the densities in the field for *H. elisae* are not as high as for other earthworms such as *E. andrei*. Schärer and Vizoso (2007) showed that the free-living flatworm *Macrostomum lignano* modulated the speed of spermatogenesis in response to group size and not only by increasing the testis size (normally an indicator of higher sperm production rates: e.g. Schärer et al. 2004), but also by a higher sperm production rate per unit testicular tissue. Again, it is worth remembering that *H. elisae* individuals had been isolated since immaturity, and for a period longer than a year so they may have adjusted their sperm production rate to a low-density scenario.

Recovery time needed after copulation?

The last hypothesis of the low paternity of the second partner derives from our limitations in the direct observation of the endogeic earthworms. They may need a recovery time after the first copulation and therefore mating with the second partner never occurs, is shorter or not as efficient. Copulation is very costly (Daly 1978) and in earthworms it involves sperm and mucus production, coupling with tubercula and setae and long periods of time. Thus this extra mating would reduce the energy available for cocoon production (e.g. Sprenger et al. 2008), although it has been shown that in *E. andrei* multiple mating in fact increases cocoon hatching success and does not reduce cocoon number and size (Porto et al. 2012). However, the benefits that these authors found seem to be the same from two to six partners, which means that two matings would be enough to avoid sperm limitation. There is no information on the mating time for *H. elisae*, but copulations of between 69 and 200 min have been observed in *Lumbricus terrestris* (Díaz Cosín et al. 2011). Also, courtships have been recorded to last 90 min in *L. terrestris* (Nuutinen and Butt 1997). Copulation time could also be increased in order to assure reciprocation, which may involve even more energy. We did not observe lack or degeneration of clitellum when changing partners so clitellum recovery could not be the reason for this hypothetical delay in copulation. Evanno et al. (2005), who performed similar experiments with *Helix aspersa*, found that the second partner took between 8 and 47 days to copulate. They also state that snails could adjust their time to the next mating depending on the amount of sperm they had in the spermathecae, as snails with filled spermathecae were reluctant to remate (see Simmons and Siva-Jothy 1998). One of our problems and challenges is that we could not observe copulation events between the pairs, because of the endogeic nature of this earthworm so we do not know if the low paternity for the second partner means either a complete lack of copulation (recovery time), or a copulation with no sperm transfer (sperm production time) or less quantity of sperm transfer, as in the snail example. Neither can we assure that sperm for the second partner was not inside the spermathecae and not used, since the spermathecae content was not evaluated. Thus it remains unclear if the cases of single paternity found here can be justified by single individual sperm content, which would contradict the results found by Novo et al. (2010) in *H. elisae*, who found sperm from at least two partners in its spermathecae.

Further remarks

Sperm digestion of the oldest sperm can be discarded as a plausible hypothesis for paternity determination, since the last and the first partner are the ones gaining the paternity. If there was digestion of the oldest sperm by the spermathecal wall, the paternity would follow the pattern P3 > P2 > P1, and if digestion of low quality sperm was the cause of reduced paternity we would not always find the same pattern of P2 being the worst affected.

Therefore, we propose that the sperm digestion by the spermathecal wall of *H. elisae* observed previously by Novo et al. (2012) could eliminate random sperm cells (the ones located near the wall), and that the purpose of that digestion would be gaining energy rather than digesting old sperm cells. Indeed the transfer of nutritious ejaculates or nuptial gifts has been described in other animals (Eberhard 1996; Vahed 1998; Arnqvist and Nilsson 2000) as a source of nutrients invested in the production of eggs (Greeff and Michiels 1999).

We showed that the heavier the earthworms, the more cocoons they lay, a relationship that had not been found before (Butt and Nuutinen 1998; Tato et al. 2006). Nevertheless the viability is not significantly affected by the weight of the earthworms and therefore, the heavier the earthworms, the larger offspring they have. Other studies had identified this relationship before (Michiels et al. 2001).

Aditionally, our observations indicate that *H. elisae* can maintain viable sperm in the spermathecae for long periods of time, with the maximum observed in one earthworm being two years and three months. This is possible due to the nourishment provided by the spermathecal walls in *H. elisae* and the environment created by this wall within the storage organ (Novo et al. 2012). Butt and Nuutinen (1998) observed that *L. terrestris* was capable of successfully maintaining the received sperm for up to six months and Butt (2011) showed that the same species could produce viable cocoons after multiple matings for over 12 months after isolation. Meyer and Bowman (1994) reported that *Eisenia fetida* continued cocoon production for up to 12 months after the earthworms were isolated from their partner, although these authors did not measure viability. This means that to our knowledge, this is the longest time of maintenance of viable sperm found in an earthworm, which would imply that the nourishment mechanisms are very efficient and should be continuous.

It is unfortunate and surprising that the viability of the collected cocoons was very low (37%). This could be attributable either to the experimental conditions or the low quantities of sperm in the spermathecae when time passes. However, the non-viable cocoons are not always the last ones, meaning that viable sperm is still present in the spermathecae. Future research should be conducted on the amounts of sperm used for fertilization since, at the time of writing, no information is available for earthworms.

The results of this experiment open interesting questions such as; how much time is needed for sperm formation in between copulation events? The sperm production rate in testicles, their maturation in seminal vesicles or their allocation to the seminal funnels would be an interesting topic of research given different times in between copulation events. It would also be interesting to perform experiments involving different numbers of partners (double and multiple), to further test the hypothesis for sperm removal and capacity of spermathecae. This would follow the suggestion of Eady and Tubman (1996) who stated that sometimes different numbers of partners can result in different proportions of last-male precedence, after looking into the findings of Zeh and Zeh (1994).

This is the first study of this nature on earthworms and the challenge of working with endogeic species makes this information even more valuable. There is a considerable lack of information on reproduction in this animal group and this study could be the basis for future research. It would be interesting to compare the results presented here with those of similar studies on anecic and epigeic earthworms.

Conclusions

This is the first time that paternity has been investigated in earthworms. Multiple paternity was detected, in the sense that one mother produced descendants from more than one partner. This probably indicates that the sperm is mixed within the spermathecae. The order of copulation has been demonstrated to be important, with the second partner adversely affected. Most of the descendants came from the first and third partners, which we hypothesized was a result of sperm displacement where the sperm from the third partner coming in flushes out the sperm from the second, because of lack of space. Another possibility we considered is that recovering time is needed either for copulation or for sperm production provoking a lack of copulation with P2, which we can not confirm because of the endogeic nature of these earthworms. The weight of the earthworms is related to the number of cocoons they produce but not to their viability, which in all the cases was low, probably due to laboratory conditions. Sperm inside the spermathecae is viable for up to two years and three months, suggesting an effective nourishment system in these storage structures. This study raises interesting questions on the usage of sperm stored in multiple mated earthworms and further research would be desirable to clarify some of its aspects.

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