


# Paternity success depends on male genetic characteristics in the terrestrial isopod *Armadillidium vulgare*

Sylvine Durand<sup>1</sup>  · Aurélie Cohas<sup>2</sup> · Christine Braquart-Varnier<sup>1</sup> · Sophie Beltran-Bech<sup>1</sup>

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## Abstract

Mate choice for compatible partners has been gaining interest during the past years, which implies that what represents a suitable male varies between females and that genetic diversity in offspring should be increased. In this context, mechanisms of mate choice for heterozygous and/or genetically dissimilar partners can be expected. To test for the presence of such mechanisms in the gregarious crustacean *Armadillidium vulgare*, we performed experimental crosses allowing a female to reproduce with two males. More heterozygous males had a higher paternity success whatever the number of males (only one or both males) participating in reproduction, which could result from better competing abilities or from female preference for such males. When only one male fathered a brood, this male was the most genetically dissimilar to the female, suggesting the existence of a female choice for dissimilarity. Additionally, the more genetically similar both males were to the female, the fewer offspring were produced per brood. Genetic diversity is thus likely to be involved in

both pre- and post-copulatory processes, as well as to have an impact on fitness in this terrestrial isopod. This work improves our understanding of the genetic characteristics involved in mating behaviours, and provides insights on the mechanisms at the basis of genetic diversity maintenance.

## Significance statement

Amongst the many parameters that can be taken into account during mate choice, genetic characteristics must not be neglected, as more and more studies highlight that what is best for one female may not be best for another. Here, we show that both heterozygosity and genetic similarity can influence paternity success in a gregarious terrestrial isopod. This could have strong implications in terms of fitness as a high genetic similarity has a negative impact on offspring number in this species. This work reflects the growing interest for genetic variables, and is positioned at the interface between molecular biology and behavioural ecology.

**Keywords** Genetic diversity · Heterozygosity · Mate preference · Inbreeding avoidance · Multiple mating · Compatibility

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✉ Sylvine Durand  
durand.sylvine@gmail.com

<sup>1</sup> Laboratoire Ecologie et Biologie des Interactions, Equipe Ecologie, Evolution, Symbiose, UMR CNRS 7267, Université de Poitiers, F-86073 Poitiers, France

<sup>2</sup> Université de Lyon F-69000, Lyon; Université Lyon 1; CNRS, UMR 5558, Laboratoire de Biométrie et Biologie Evolutive, F-69622 Villeurbanne, France

## Introduction

For a long time, the idea of a single best male for all females was widespread. Indeed, during male-male competition, it is expected that some phenotypic traits such as body weight (Wedell 1997) or weapon size (Karino et al. 2005) are determinant for male reproductive success, and that males possessing higher values for these traits are more likely to outcompete rivals. Regarding female choice, the Fisherian runaway theory (Fisher 1930) and the good-gene hypothesis (Fisher 1915; Garcia-Gonzalez and Simmons 2011) predict

that female preference for given male traits should evolve whenever exaggerated ornaments (under the Fisherian runaway theory) or traits advertising higher fitness (under the good-gene hypothesis) can be transmitted to offspring. These male-male competition and female choice theories imply sexual selection acting on a specific set of alleles and consequently predict a reduction of genetic diversity in populations. However, studies on genetic compatibility between partners (Zeh and Zeh 1996; Tregenza and Wedell 2000) have more recently undermined this “single best male for all females” paradigm. Several examples of mate choice for compatible partners have been empirically highlighting different best males depending on the female and an increased genetic diversity of offspring (Foerster et al. 2003; Beltran et al. 2008; Kamiya et al. 2014).

The effects of genetic diversity on fitness at the individual level have been investigated through numerous studies on heterosis (increased trait values for hybrid offspring resulting from a cross between inbred lines) (Zhang et al. 2008) or inbreeding depression (Keller and Waller 2002; Coltman and Slate 2003; Charlesworth and Willis 2009). In a wide range of organisms from plants to invertebrates and vertebrates, individuals presenting low levels of heterozygosity often experience lower survival (Rossiter et al. 2001) and/or lower reproductive success (Slate et al. 2000).

In this context, we can expect heterozygosity to be a sexually selected trait (Brown 1997; Hoffman et al. 2007; Kempenaers 2007). Indeed, if some phenotypic traits determining male-male competition are correlated with individual genetic diversity, more heterozygous males are expected to outcompete other males in both pre- and post-copulatory processes. In the black grouse, heterozygous males are more likely to gain territories compared to their competitors, which increases their lifetime reproductive success (Hoglund et al. 2002), and in the clam shrimp, inbred (consequently less heterozygous) males produce half the amount of sperm than outbred males, which is likely to lead to a strong disadvantage in sperm competition (Weeks et al. 2009).

Heterozygosity could also be the target of female mating preferences. As stated in the “good-gene-as-heterozygosity” theory (Brown 1997), females can benefit from having more heterozygous offspring, which should have a better fitness due to positive heterozygosity-fitness correlations (Zeh and Zeh 1996; Kempenaers 2007; Kamiya et al. 2014). Three different strategies can lead to a genetically diverse progeny. First, a female can prefer to mate with heterozygous males. Theoretical studies predict a positive correlation between parental and offspring heterozygosity when allelic frequencies are uneven, a situation likely to occur in natural populations (Mitton et al. 1993; Fromhage et al. 2009; Nietlisbach and Hadfield 2015), and such correlation has been reported in several empirical studies (Mitton et al. 1993; Garcia-Navas et al. 2009). Hence, mate choice for heterozygous partners to

produce heterozygous offspring can be expected (Lehmann et al. 2007), and has been highlighted in natural populations, such as in the wire-tailed manakin *Pipra filicauda*, where females select more heterozygous males as mating partners (Ryder et al. 2010). According to the disassortative mating hypothesis, also called genetic compatibility (Tregenza and Wedell 2000), a second possibility is a choice for genetically dissimilar partners, as in the Australian sleepy lizard *Tiliqua rugosa* (Bull and Cooper 1999) or in the parasite *Schistosoma mansoni* (Beltran et al. 2008). Mating with an individual that possesses other alleles for a given locus ensures that offspring will inherit different alleles from its parents, leading to an increase in offspring heterozygosity. Finally, a third strategy to improve genetic diversity in offspring is through multiple matings (Yasui 1998; Jennions and Petrie 2000). In addition to ensure that more paternal alleles are present in the progeny as a whole (genetic diversity hypothesis) (Yasui 1998; Jennions and Petrie 2000), polyandry may also evolve to ensure that most offspring will be sired by genetically dissimilar males, provided that post-copulatory mechanisms can favour the use of sperm from the more dissimilar males, or favour female investment in embryos sired by the more dissimilar males (Tregenza and Wedell 2000; Simmons 2005).

The woodlouse *Armadillidium vulgare* (Crustacea, Oniscidea) is a gregarious terrestrial isopod (Takeda 1984). Mating strategies to avoid inbred matings and their deleterious effects on individual's fitness might have evolved in this species, as a preference for dissimilar mates has already been highlighted in *A. vulgare* males when the available females present a large range of genetic similarity (Durand et al. 2015). A classic prediction in sexual selection theory is that the choosy sex should be the one investing the most in the offspring, whether in terms of gamete production or parental care (Bateman 1948; Trivers 1972; Kokko and Jennions 2008). Female isopods further invest heavily in reproduction and suffer costs from carrying offspring in their marsupial pouch for 1 month (Kight and Ozga 2001). Though to this day, no female choice has ever been described in this species, *A. vulgare* females nevertheless can be expected to perform both pre- and post-copulatory mate choice. They store the sperm of the different males they mated with in their spermatheca before egg fertilisation (Moreau and Rigaud 2002; Ziegler and Suzuki 2011). This structure could allow both sperm competition and cryptic female choice to take place, two nonexclusive post-copulatory mechanisms from which the outcome could be potentially impacted by genetic characteristics (Tregenza and Wedell 2002; Gage et al. 2006; Frasier et al. 2013). Moreover, multiple mating occurs in the wild in *A. vulgare* (Valette et al. 2017), and females are able to mate with a second male within a few hours after a first mating, even though only 25% of females remate if they are later presented to a new male (Moreau et al. 2002). To this day, the factors conditioning the acceptance of a second mating are

unknown, and we ignore whether females might mate with several males which can be assessed simultaneously. Multiple paternity patterns (i.e. offspring repartition between multiple fathers) are not determined by sperm precedence (Moreau et al. 2002) but might be influenced by the genetic characteristics of partners (Olsson et al. 1996; Bretman et al. 2004; Fitzpatrick and Evans 2014). All these elements make *A. vulgare* a relevant model to study the importance of heterozygosity in sexual selection.

In this paper, we searched for mechanisms that favour genetic diversity amongst offspring for *A. vulgare* females. To investigate this question, we performed experimental crosses involving one female and two males. To allow both pre- and post-copulatory processes to occur, we consider the resulting progeny. Microsatellite markers were used to perform paternity tests and to investigate whether sexual selection could promote genetic diversity. We here search for evidence of (1) multiple mating performed by females when several males are available at the same time, (2) a female preference for the most genetically dissimilar male and (3) an advantage for the most heterozygous mate in sexual selection (via either female mate choice or male-male competition). As these hypotheses are independent, we can make three nonexclusive predictions: (1) both males should participate in reproduction, (2) the male which is the most dissimilar to the female within a pair of males should father more offspring than the other male and/or (3) the most heterozygous male of a pair should father more offspring than the other male.

## Material and methods

### Biological model

The common pillbug *A. vulgare* (Latreille, 1804) is a terrestrial isopod belonging to the suborder Oniscidea. This arthropod is a widespread detritivore that lives in groups in a variety of open environments such as grasslands, but also in anthropised sites like gardens. Reproduction takes place between April and October (Vandel 1962), and woodlouse reproductive cycle is tightly linked with moulting cycle. Matings occur during the week preceding female parturial moult (Moreau et al. 2002), characterised by the apparition of a marsupial pouch on the ventral side of the female. The parturial moult is followed by fertilisation as eggs cross the spermatheca whilst entering the oviduct. Eggs are afterwise laid in the marsupial pouch, and incubated for 1 month until fully developed juveniles leave the marsupium (Surbida and Wright 2001).

## Experimental procedure

### Experimental population

The animals used in our experiments originate from a wild population collected in Helsingør (Denmark) in 1982, and since reared in laboratory under the natural photoperiod of Poitiers, France (46° 35' N, 0° 20' E) at 20 °C on moistened soil and fed with dried lime tree leaves (*Tilia* sp.) and fresh carrot slices. The absence of symbiotic bacteria of the genus *Wolbachia*, which commonly infect *A. vulgare* and have an impact on woodlouse reproduction and sexual behaviour (Bouchon et al. 1998; Moreau et al. 2001), has been controlled regularly. Crosses are performed each year between animals of known pedigree to minimise inbreeding. Once born, siblings are kept together in boxes, then sexed as soon as possible (approximately 3 months) to separate males and females before sexual maturity, so as to obtain groups of virgin brothers and virgin sisters.

### Experimental setup

We performed our experiments on 40 females and 80 males, all of them being 1-year-old virgin individuals that never encountered individuals of the other sex. Each female was placed together with two males in a small cylindrical plastic box on moistened soil with lime tree leaves and carrot slices under a 18:6-h light:dark photoperiod to stimulate reproduction. Males were randomly assigned to females, but all the individuals were of approximately the same size and belonged to different sibships. As males in moulting period are not sexually receptive, we weekly checked the moulting status of individuals, and removed both males of the box if one of them appeared to begin moult. Males were placed back with the female after the moult, so that each female was always presented to two sexually receptive males at the same time. Once a female laid eggs in her marsupial pouch (usually between 2 and 6 weeks after being placed with the males), she was separated from males to ensure a good development of offspring. After approximately 1 month of gravidity, females released completely formed juveniles that were all counted and collected within 3 days after birth. Thirty randomly chosen juveniles of each clutch were collected separately for genetic analyses. As data in our study result from molecular analysis and not from behavioural observations, there is no possibility for observer bias necessitating blinded methods.

This protocol allows to determine a posteriori which male fathered each offspring, but the behavioural, physiological and cellular processes conditioning the paternity cannot be determined as they occur prior to birth. Indeed, this experiment was not designed to disentangle pre- and post-copulatory mechanisms.

## Genetic analyses

### Adult genotyping

Right before crosses were performed, a leg was collected from each adult using thin tweezers. We performed phenol-chloroform DNA extraction on those samples, consisting in digestion of sample with proteinase K, purification with phenol and chloroform isoamyl washing and then precipitation with sodium acetate and isopropanol (Durand et al. 2015). DNA was afterwards diluted by 1/20th. We then performed genotyping on DNA extracts using 11 microsatellite markers gathered in three multiplexes (Appendix 1) (Verne et al. 2006; Giraud et al. 2013). PCR reactions, product separation in a sequencer and determination of product size were conducted as indicated in Appendix 1.

### Paternity analyses on juveniles

Due to the high number of offspring to genotype (30 per brood, for a total of 840 offspring from 28 broods), we selected a Chelex DNA extraction which presents the advantage to be faster than phenol-chloroform extraction. Animals were placed without crushing in 120  $\mu\text{L}$  of sterile water, 10  $\mu\text{L}$  of proteinase K (20 mg/mL) and 20  $\mu\text{L}$  of a 5% Chelex solution (Sigma-Aldrich). Samples were placed at 56 °C for 3 h, then at 95 °C for 10 min.

Knowing the genotype of adults, we selected for each triad a locus (or a set of loci if necessary), allowing us to determine the paternity of offspring by detecting which allele was inherited from the father without ambiguity.

For each of the 30 extracted DNA of collected juveniles of a brood, PCRs were performed on the selected locus in the same PCR mix using amplification conditions as in adults (Appendix 1), but in simplex condition (i.e. only one primer pair was used at a time). Then, PCR products were gathered in poolplex for separation in the sequencer; in the same way as in a multiplex, we avoided any size overlap between markers, so that we could assemble products from different individuals in the same solution. Genotyping was then performed as in adults. Once the genotype of each juvenile was obtained, we compared it to the genotypes of the adults in the triad to determine which allele was inherited from the father and then assign a father to each offspring. Once paternity was assessed, we qualified the type of brood as “unique paternity” when only one father was detected, and as “multiple paternity” when two fathers were detected, and we named “major male” the male who fathered the majority of offspring in a given brood (i.e. either more offspring than the other male of the dyad or all offspring in the brood) and “minor male” the other one.

## Genetic variables

For each adult, mean heterozygosity ( $H$ ) was estimated by dividing the total number of heterozygous loci by the number of genotyped loci.  $H$  ranges from 0 for individuals with no heterozygous locus to 1 for individuals with all heterozygous loci. We also calculated the  $r$  coefficient of Wang (2002) between each male and the female to which he was proposed using SPAGeDi 1.2 (Hardy and Vekemans 2002). This coefficient estimates genetic similarity between individuals, more precisely the probability that two individuals share a given allele, taking into account allelic frequencies in the population. It can range from  $-1$  for completely genetically dissimilar individuals to 1 for completely genetically similar individuals.

We calculated the difference in heterozygosity between the major and the minor male ( $\Delta H$ ).  $\Delta H$  was thus positive if the major male was more heterozygous than the minor male, and negative otherwise. Similarly, the difference in genetic similarity to the female between the major and the minor male ( $\Delta r$ ) was calculated.  $\Delta r$  was positive if the major male was more similar to the female than the minor male. Those two last coefficients were calculated because we can expect that a substantial difference between males is needed for a choice to occur.

All those genetic variables were standardised prior to including them into statistical models by subtracting the mean of the variable to each value and dividing it by the standard deviation of the variable, in order to get variables centred on zero with a standard deviation of 1. This allows to compare the effect size of different variables whenever they are included simultaneously in a statistical model.

## Statistical analyses

1. Does the number of males participating in reproduction depend on their genetic characteristics?

To determine whether a female’s brood was fathered by a single or by two males depending on the males’ genetic characteristics, generalised linear models (GLMs) were constructed with a logit link and a variance given by a binomial distribution and with the type of brood (0 for “unique paternity” brood and 1 for “multiple paternity” brood) as the dependent variable. Female heterozygosity, major and minor male heterozygosities and genetic similarity for each male-female pair were included as explanatory variables in a first model, as were the differences between males in terms of heterozygosity and genetic similarity to the female in a second model.

2. Is the male with the highest reproductive success the most dissimilar and/or the most heterozygous?

We used a conditional regression with matched pairs constituted of the major male and the minor male. Such regression allowed us to model the probability for a male to father a given offspring within a brood as a function of its heterozygosity and genetic similarity to the female knowing the same genetic characteristics of the other male (i.e. equivalent to work on the difference between males). To take into account the fact that 30 offsprings were considered for a given trio (one female and two males), the identity of the trio was entered as a cluster into the model and the within-cluster correlation was used to estimate the variance parameter (Kleinbaum and Klein 2005).

### 3. Do male characteristics affect brood size?

To determine whether male genetic characteristics or the type of brood could affect brood size, GLMs were constructed with a log link and a variance given by a negative binomial distribution to account for the overdispersion of brood sizes. The size of the brood was set as the dependent variable; the type of brood was set as explanatory variable in a first model. Then, female heterozygosity, major and minor male heterozygosities and genetic similarity for each male-female pair were included as explanatory variables in a second model. To investigate more in depth the impact of genetic similarity on brood size, two additional models were built. In the first, mean genetic similarity for male-female pairs within a triad was included as explanatory variable; in the second, a new explanatory variable was used, designated as “father(s) genetic similarity”, namely either the mean genetic similarity if two males produce offspring, or similarity of the single father if only one male produces offspring. Those two models were built because we ignore if a male that did not father offspring (in case of unique paternity) can influence brood size or not, so we constructed a model to consider each of those possibilities. AICc values were then calculated for each model to determine which one was the most parsimonious.

Statistical analyses were performed with R 3.0.1 (R Development Core Team 2013), the function “glm.nb” in the package “MASS” was used to fit GLMs with a negative binomial distribution (Venables and Ripley 2002), the function “glm” in the package “stats” was used to perform GLMs with other distributions (R Development Core Team 2013), the function “coxme” in the package coxme was used to construct Cox’s proportional hazard models with clusters (Therneau 2015), and the function “AICc” in the package “AICcmodavg” was used to calculate AICc values (Mazerolles 2016). We verified that genetic variables were not correlated so that they could be included as explanatory variables in the same statistical models without generating collinearity issues (see Appendix 2 for correlations). Only simple additive effects were included in our models, as no interaction between variables corresponds to tested

hypotheses. We built full models including all potential explanatory variables, then in a second step removed the non-significant terms all at once to obtain the minimal model containing only significant variables. In the “Results” section, the estimates obtained from the full models regarding the nonsignificant terms and the estimates from the minimal models regarding the significant terms are provided. Estimates from both the significant and nonsignificant terms obtained from the full models are presented in Appendix 3. We tested for linear effects of the variables using type III ANOVA, therefore testing for a significant effect once the effects of all other variables present in the model being taken into account. For all models, we performed goodness-of-fit tests according to Agresti (2002), in which the sum of squared Pearson residuals of fitted models follows a chi-squared distribution with degrees of freedom equal to the degrees of freedom of the model residual deviance and all models adequately fitted the data. We set the level of significance to  $\alpha = 0.05$ , and parameter estimates are given as mean  $\pm 1$  SE on the logit and log-scales. For all models, we provided odd ratios (exponentiated betas) to ease interpretation of the effect size.

## Results

We performed 40 crosses with one female and two males. Only 28 broods were analysed because three females died; one was never gravid; four aborted; and in four cases, we could not manage to assign paternity (either due to issues when genotyping or mismatch between paternal and offspring alleles). Detailed genetic characteristics and brood characteristics for each cross can be found in Appendix 4. Brood size ranged from 61 to 186 offsprings (first quartile  $Q_1 = 100$ , median  $\tilde{x} = 114$ , third quartile  $Q_3 = 130.5$ ). The genotype of 20 offsprings could not be determined, leading to a total of 820 offsprings for which we were able to assess paternity, gathered in broods of 26 to 30 analysed offsprings ( $Q_1 = 29$ ,  $\tilde{x} = 29.5$ ,  $Q_3 = 30$ ). The major male of each brood fathered 57 to 100% of the offspring ( $Q_1 = 72.4$ ,  $\tilde{x} = 100$ ,  $Q_3 = 100$ ).

- 1) Does the number of males participating in reproduction depend on their genetic characteristics?

Amongst the 28 broods, 15 had a unique paternity whilst 13 had a multiple paternity. The type of brood did depend neither on female ( $\beta \pm \text{SE} = -0.32 \pm 0.48$ , exp. ( $\beta$ ) = 0.72,  $N = 28$ ,  $p = 0.50$ ) or male heterozygosity (major male heterozygosity  $\beta = -0.13 \pm 0.45$ , exp. ( $\beta$ ) = 0.88,  $N = 28$ ,  $p = 0.77$ ; minor male heterozygosity  $\beta = 0.13 \pm 0.41$ , exp. ( $\beta$ ) = 1.14,  $N = 28$ ,  $p = 0.74$ ), nor on male-female genetic similarity (similarity with major male  $\beta = 0.49 \pm 0.56$ , exp. ( $\beta$ ) = 1.63,  $N = 28$ ,  $p = 0.39$ ; similarity with minor male  $\beta = 0.40 \pm 0.40$ , exp. ( $\beta$ ) = 1.49,  $N = 28$ ,  $p = 0.32$ ). There was no influence of

the difference between males in terms of heterozygosity ( $\beta = 0.13 \pm 0.39$ , exp. ( $\beta$ ) = 1.14,  $N = 28$ ,  $p = 0.75$ ) or of genetic similarity to the female ( $\beta = 0.16 \pm 0.38$ , exp. ( $\beta$ ) = 1.18,  $N = 28$ ,  $p = 0.67$ ) on the type of brood.

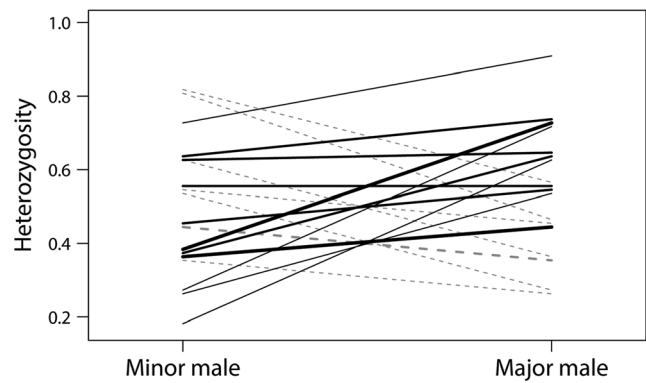
2) Is the male with the highest reproductive success the most dissimilar and/or the most heterozygous?

For all 820 offsprings in the 28 broods, an increase in difference between males' heterozygosity leads to a greater probability for the most heterozygous male to father a given offspring ( $\beta = 0.32 \pm 0.05$ , exp. ( $\beta$ ) = 1.38,  $N = 820$ ,  $p < 0.001$ ; Fig. 1), whilst male-female genetic similarity had not effect ( $\beta = -0.10 \pm 0.06$ , exp. ( $\beta$ ) = 0.91,  $N = 820$ ,  $p = 0.09$ ). In trials where only one male sired the brood, the chance for a male to be the father increased for the most heterozygous male with an increase in heterozygosity difference ( $\beta = 0.58 \pm 0.08$ , exp. ( $\beta$ ) = 1.78,  $N = 442$ ,  $p < 0.001$ ), but decreased for the most similar male with an increase in male-female similarity difference ( $\beta = -0.41 \pm 0.10$ , exp. ( $\beta$ ) = 0.66,  $N = 442$ ,  $p < 0.001$ ). For broods with multiple paternity, paternity success only increased with difference in heterozygosity between males ( $\beta = 0.23 \pm 0.09$ , exp. ( $\beta$ ) = 1.26,  $N = 378$ ,  $p = 0.008$ ) whilst no effect of genetic similarity was observed (male-female similarity  $\beta = -0.03 \pm 0.08$ , exp. ( $\beta$ ) = 0.97,  $N = 378$ ,  $p = 0.68$ ).

3) Do male characteristics affect brood size?

Total brood size was not influenced by the type of brood (unique or multiple,  $\beta = 0.09 \pm 0.11$ , exp. ( $\beta$ ) = 1.09,  $N = 28$ ,  $p = 0.39$ ). Female ( $\beta = 0.04 \pm 0.05$ , exp. ( $\beta$ ) = 1.04,  $N = 28$ ,  $p = 0.41$ ) and male heterozygosities (major male  $\beta = 0.02 \pm 0.05$ , exp. ( $\beta$ ) = 1.02,  $N = 28$ ,  $p = 0.70$ ; minor male  $\beta = -0.02 \pm 0.05$ , exp. ( $\beta$ ) = 0.98,  $N = 28$ ,  $p = 0.65$ ) did not impact total brood size, but male genetic similarity did; there was a negative effect of both major male ( $\beta = -0.12 \pm 0.06$ , exp. ( $\beta$ ) = 0.89,  $N = 28$ ,  $p = 0.049$ ) and minor male genetic similarity to the female on brood size ( $\beta = -0.08 \pm 0.04$ , exp. ( $\beta$ ) = 0.92,  $N = 28$ ,  $p = 0.042$ ).

More specifically, when the mean genetic similarity between the female and the two males of a triad increased of one unit (whether the minor male fathered offspring or not), the brood size decreased by 0.86-fold ( $\beta = -0.15 \pm 0.05$ , exp. ( $\beta$ ) = 0.86,  $N = 28$ ,  $p = 0.001$ , AICc = 274.2; Fig. 2). Similarly, brood size decreased when father(s) genetic similarity with the female increased ( $\beta = -0.14 \pm 0.05$ , exp. ( $\beta$ ) = 0.87,  $N = 28$ ,  $p = 0.01$ , AICc = 277.3), even if this last model is less



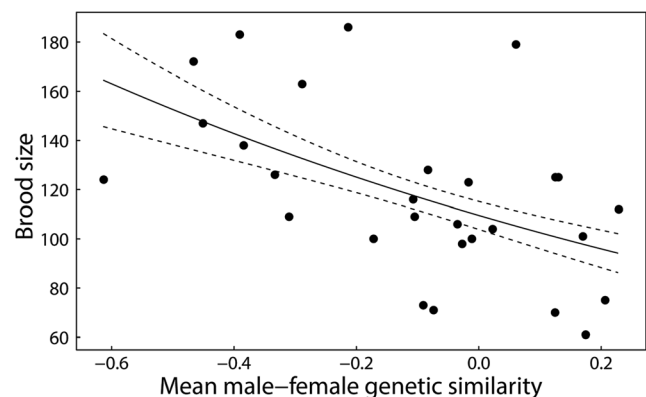
**Fig. 1** Heterozygosity of male dyads presented to the same female according to their paternity success. *Solid black line* the major male was the most heterozygous ( $N = 20$ ); *dashed grey line* the major male was the least heterozygous ( $N = 8$ ). The width of the line is proportional to the number of male pairs (from one to three dyads in the same configuration)

parsimonious than the previous one including the two males of the triad.

## Discussion

In the context of mate choice for genetic compatibility, mechanisms that favour mating with heterozygous and/or genetically dissimilar partners are expected. In this manuscript, we highlight the presence of such mechanisms in *A. vulgare*, where half of the females produced broods with more than one father. When only one male fathered the brood, this male was more likely to be the most heterozygous and the most genetically dissimilar to the female of the two males present. In broods with multiple fathers, only a higher heterozygosity predicted a higher paternity success. Since brood size depended on male-female genetic similarity, mate choice for genetic diversity is likely to have strong fitness consequences on individuals.

Half of the broods were fathered by both males, independently of male genetic characteristics, which implies that those



**Fig. 2** Effect of the mean male-female genetic similarity of a triad on brood size

females mated twice. Remating under laboratory conditions has already been observed in *A. vulgare*. Moreau et al. (2002) showed that 25% of females remate within hours after a first copulation and explained this low percentage by the presence of a refractory period in females. In our case, several days could have elapsed between the first and the second copulations. This delay could be sufficient for the refractory period to end and could explain the 50% of multiple paternity observed. These results thereby suggest that multiple paternity could be widespread in the wild as the result of remating and using stored sperm from previous copulations (Howard 1943). Indeed, after sampling gravid *A. vulgare* females in the wild and determining the number of males contributing to their broods, multiple paternity was effectively observed in 100% of the broods (Valette et al. 2017), making multiple mating a likely female mating strategy and not an artefact of our experiment being performed under laboratory conditions.

If genetic characteristics did not predict the number of males contributing to the broods, they predicted paternity success. Indeed, a higher heterozygosity led to a higher paternity success whether one or both males fathered offspring. Additionally, when one male monopolised paternity, this male was also the most genetically dissimilar to the female. The male genetic characteristics involved differing between unique and multiple paternity suggest that the mechanisms underlying paternity success differ between the two types of broods. However, we ignore whether unique paternity results from mating with only one male or from mating with both males followed by extreme selection for the sperm of a single male. Such drastic sperm selection has been highlighted in the feral fowl, in which the female can reject the sperm of a subordinate male right after mating (Pizzari and Birkhead 2000), but no similar mechanism has ever been suggested in *A. vulgare*, though sperm ejection by females has already been documented in *Drosophila melanogaster* (Manier et al. 2010). For this reason, it is difficult to disentangle between mechanisms taking place before and after copulation, namely pre- and post-copulatory mechanisms. This study was not designed for this purpose, as we wanted to focus on paternity patterns in the progeny as a result of all mechanisms occurring freely as they would under natural conditions. Other protocols are necessary to distinguish pre- and post-copulatory mechanisms, including for example behavioural observations to investigate pre-copulatory mechanisms and genotyping of spermatozoa in the spermatheca after mating to investigate post-copulatory mechanisms.

Whatever the number of males participating in reproduction, their relative paternity success is predicted by their genetic diversity, with more heterozygous males in trials obtaining more offspring. This could be due to a positive link between male heterozygosity and competing abilities. More diverse males could perform better than the others before mating (Hoglund et al. 2002) or after mating during spermatic

competition, especially in the context of inbreeding (Gage et al. 2006; Weeks et al. 2009). Even though in *A. vulgare* male contests have been suggested to be unusual (Lefebvre et al. 2000), we cannot dismiss the hypothesis that paternity success depends on better pre-copulatory capacities related to male genetic characteristics. Moreover, *A. vulgare* spermatozoa are not motile and are grouped in bundles (Cotelli et al. 1976), but nothing is currently known about males' spermatic performances. On the other hand, we cannot exclude the possibility that females exerted a choice for more heterozygous males, either before or after copulation (Ryder et al. 2010; Laloï et al. 2011).

When only one male was involved in offspring production, this male was on average the one presenting the lowest genetic similarity with the female. Male-male competition cannot be responsible for this pattern as genetic similarity is always relative to the female. Hence, other processes such as female choice may be involved. Females could bias paternity success towards the more dissimilar male by pre-copulatory means, either by controlling sperm transfer through asymmetric copulation duration with each partner (Sillén-Tullberg 1985) or by deciding to mate only with the most dissimilar. Male monopolisation of paternity could also result from post-copulatory assessment of genetic similarity with their partner(s). As such, cryptic female choice could favour the more dissimilar sperm, like in the red junglefowl *Gallus gallus* (Lovlie et al. 2013). Alternatively, females could assess male similarity only once a first mating has been performed, and decide to remate with the second male only if the first was not dissimilar enough. Hence, the less similar to the female the first male is, the less likely the female mates again and the more likely the second male is to be more similar to the female than the first male. This could explain why the type of brood is not predicted by male similarity to the female since the effect of similarity on the type of brood could be obscured by the order of mating. Interestingly, a pre-copulatory male preference for genetic dissimilarity has already been shown in *A. vulgare* (Durand et al. 2015). Together with the current study, those results suggest that mutual mate choice for genetically dissimilar partners in *A. vulgare* is likely to occur under natural conditions. Besides, mutual mate choice has been demonstrated in *Blattella germanica*, another arthropod species with a similar gregarious behaviour (Lihoreau et al. 2008). Conversely, a last hypothesis to consider is a differential offspring production for both males independently of female choice, resulting from genetic incompatibility between gametes (Palumbi 1999) or mortality during embryo development (Jin et al. 1995) for the more similar male.

In line with this last hypothesis, genetic similarity does influence brood size; the more genetically similar both males were to the female, the smaller the brood size, similarly to what is observed in other arthropod species (Lihoreau et al. 2008; Müller and Müller 2016). Further work is needed to

determine if the small brood size produced by similar parents results from a low total number of eggs laid by the female or from a decrease in oocyte fertilisation or embryo survival. The decrease in offspring number is likely to impose a selective pressure towards a choice for dissimilar partner, and could explain a female choice for dissimilarity. If so, one can be surprised by the fact that we do not observe a ubiquitous choice for dissimilarity, whereas there is always a strong effect of heterozygosity. It seems legitimate to postulate that when only one male fathered the entire brood, the most dissimilar male was chosen by the female. The co-occurring effect of heterozygosity in this case could be explained either by a simultaneous female choice for male dissimilarity and heterozygosity, as in the fur seal *Arctocephalus gazella* (Hoffman et al. 2007), or by pre-mating male-male competition based on heterozygosity. However, we cannot reach such definitive conclusions about why only heterozygosity (and not dissimilarity) predicts paternity success when both males father offspring. Indeed, we are limited by our knowledge of the role played by both pre- and post-copulatory processes in our experimental design, and cannot distinguish between female choice and male-male competition. Any conclusion would thus be very speculative. In order to go further on this question, additional experiments are required, such as Y-choice tests to investigate female pre-copulatory preferences, or forced copulation assays to determine which factor determines paternity success when controlling for the order of mating for example.

Our study suggests that female choice is likely to be involved in male paternity success. This implies the existence of phenotypic differences between individuals reflecting their genetic characteristics, as in the lekking wire-tailed manakin *Pipra filicauda*, where female preference for heterozygous mates could be based on male wing and tarsus length (Ryder et al. 2010). In our model, sexual interactions have been suggested to be based on chemical cues (Beauche and Richard 2013), as often in arthropods (Howard and Blomquist 2005). Olfactive cues could thereby reflect male genetic characteristics and be implied in a potential female choice for heterozygosity and/or genetic dissimilarity.

Overall, the impact of genetic characteristics on paternity success in *A. vulgare* seems to involve complex processes, which are likely to be even more complex in the wild where females may encounter many more males. This could lead to complicated patterns of multiple paternity, favouring both offspring number and offspring genetic quality through male competition and female choice selecting the “best” male(s) from a broad range of competitors. It is thus likely that operational sex ratio (Kvarnemo and Ahnesjö 1996; Moreau and Rigaud 2000) might strongly constrain the capacity of females to exert a mate choice.

We further highlighted mechanisms that are expected to increase heterozygosity in offspring. This process prevents

fixation of alleles (Neff and Pitcher 2005) and is thus likely to contribute to the persistence of genetic diversity in populations. As intraspecific genetic diversity is required for evolutionary processes to take place, understanding sexual selection in relation to genetic characteristics might provide insights on the adaptive potential of populations and the capacity of species to face environmental changes.

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