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Multiple mating in natural populations of a simultaneous hermaphrodite, *Lymnaea stagnalis*

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ABSTRACT

In field populations, it is largely unknown how many mating partners simultaneous hermaphrodites have, although their promiscuous mating is often observed in captivity. Documenting the number of mates in natural populations can expand the understanding of postcopulatory sexual selection and the maintenance of sexual systems. Here we estimated the number of mates in natural populations of the great pond snail *Lymnaea stagnalis* by estimating the minimum number of fathers within siblings from a given mother. Based on the microsatellite genotypes of mothers and siblings, we found a moderate number of siring fathers, ranging between one and three individuals per clutch. We did not find any offspring produced by self-fertilization, indicating extensive outcrossing. The number of mates was positively correlated with density of potential mates, suggesting that mate availability is an important determinant of their genetic mating success. Although our survey only provides an initial insight into the situation in the field, the results do highlight the potential for postcopulatory sexual selection in natural populations of this simultaneous hermaphrodite.

INTRODUCTION

There are very few species that are strictly monogamous. As a consequence, when females mate with multiple partners, they can store sperm from several partners in their reproductive tracts, providing the opportunity for sperm competition, cryptic female choice and thus postcopulatory sexual selection (e.g. Birkhead, Hosken & Pitnick, 2008). Hence, the number of mates is an important determinant for the potential for postcopulatory sexual selection, as supported by theoretical predictions (e.g. Bateman, 1948; Parker, 1970; Jones, 2009).

Furthermore, the number of mates is especially fascinating to examine in simultaneous hermaphrodites (which are male and female at the same time, hereafter called hermaphrodites). On the one hand, it has been predicted that hermaphrodites have a limited number of mates in the wild, based on their low chance to find a mate and/or low mobility (Charnov, Maynard Smith & Bull, 1976; Charnov, 1982). Under such circumstances, being a hermaphrodite is advantageous, because each individual can copulate with any conspecific partner encountered and, if necessary, self-fertilize (Ghiselin, 1969; Jarne & Auld, 2006; Tian-Bi *et al.*, 2008; Ramm *et al.*, 2015). On the other hand, hermaphrodites can potentially have a substantial number of mates, since some hermaphroditic species are observed to copulate multiply (see below). When there are many rivals' and/or own sperm cells competing for fertilizing a limited number of eggs, this leads to local sperm competition and the average male fitness gain curve per resource is expected to saturate, making hermaphroditism evolutionarily stable (Schärer, 2009; Schärer & Pen, 2013). In both scenarios, male reproductive success is limited, although the number of mates is, respectively, low or high. Therefore, documenting the number of mates that hermaphrodites have can provide additional information for our understanding of postcopulatory sexual selection as well as of how their sexual system is maintained.

In previous studies, multiple matings of hermaphrodites have often been observed in captivity (sea slugs: Yusa, 1996; Angeloni, Bradbury & Burton, 2003; Lange *et al.*, 2012, snails: Koene & Ter Maat, 2007; planarians: Pongratz & Michiels, 2003; Janicke & Schärer, 2009; reviews by Baur, 1998; Michiels, 1998; Jordaens, Dillen & Backeljau, 2007; Nakadera & Koene, 2013; Schärer, Janicke & Ramm, 2014). Consistently, in these cases, multiple fathers sire offspring with a given mother, as supported by genetic paternity testing (sea slug: Angeloni *et al.*, 2003, snails: Mulvey & Vrijenhoek, 1981; Wethington & Dillon, 1991; Baur, 1994; Städler, Weisner & Streit, 1995; Coutelle-Vreto, Madec & Guiller, 1997; Viard, Doums & Jarne, 1997; Evanno, Madec & Arnaud, 2005; Henry *et al.*, 2005; Chase & Blanchard, 2006; Koene *et al.*, 2009; Garefalaki *et al.*, 2010; Pélissié, Jarne & David, 2012; Pélissié *et al.*, 2014; planarian: Marie-Orleach *et al.*, 2016; polychaete: Lorenzi, Schleicherová & Sella, 2013), even though in gastropods a single insemination suffices for long-term fertilization (Chen & Baur, 1993; Ludwig & Walsh, 2004; Nakadera, Blom & Koene, 2014). However, animals in mass culture tend to be maintained at high density (*c.* 200 snails per 1 m^2 in VU University Amsterdam), so that they have plenty of potential mates. Also, cultured animals are expected to have a longer lifespan than animals in the field, which is mainly due to low predation rate and provision of more than sufficient food. These conditions are expected to elevate their mating frequency and the number of mates in a cultured population (Janicke *et al.*, 2013; Auld & Henkel, 2014; Auld & Houser, 2015; Janicke, David & Chapuis, 2015; Janicke & Chapuis, 2016). Therefore, measuring the number of mates in natural populations is necessary to see how many mates hermaphrodites have in the field and to permit comparison with laboratory situations.

In contrast with their well-documented promiscuity in the laboratory, to the best of our knowledge, attempts to quantify the number of mates in natural populations of hermaphrodites are scarce; we found such studies for only one species of gastropod, the land snail *Arianta arbustorum* (Kupfernagel, Rusterholz & Baur, 2010; Kupfernagel & Baur, 2011; Janssen & Baur, 2015; but note several reports in barnacles: Kelly, Grosberg & Sanford, 2012; Barazandeh *et al.*, 2013; Barazandeh, Davis & Palmer 2014; Plough, Moran & Marko, 2014; ascidians: Johnson & Yund, 2007; corals: Yeoh & Dai, 2010; Warner, Willis & van Oppen, 2016). To expand the scope of knowledge about multiple mating in hermaphroditic gastropods, we investigated multiple paternity in natural populations of the great pond snail *Lymnaea stagnalis* (L.). In the laboratory, this species copulates repeatedly (Koene & Ter Maat, 2007) and the number of siring fathers within a clutch can be more than one (Cain, 1956; Koene *et al.*, 2009). In addition, this species usually uses sperm from its mates for fertilizing its own eggs (called outcrossing), although they do not show explicit inbreeding depression or self-fertilizing depression (Coutellec & Lagadic, 2006; Puurtinen *et al.*, 2004, 2007; Koene, Loose & Wolters, 2008; Koene *et al.*, 2009). In order to estimate the number of sperm donors (i.e. fathers) that sired siblings of a given mother, we collected *L. stagnalis* from several locations, allowed them to lay eggs in the laboratory and genotyped the offspring and mothers using microsatellite markers. The resulting estimated number of siring fathers indicates the minimum number of mates that successfully transferred sperm and fertilized eggs of a given mother (Jones, 2005). With this approach, we find a relatively low number of fathers within progenies, implying that in the field, these hermaphrodites have a limited number of mates.

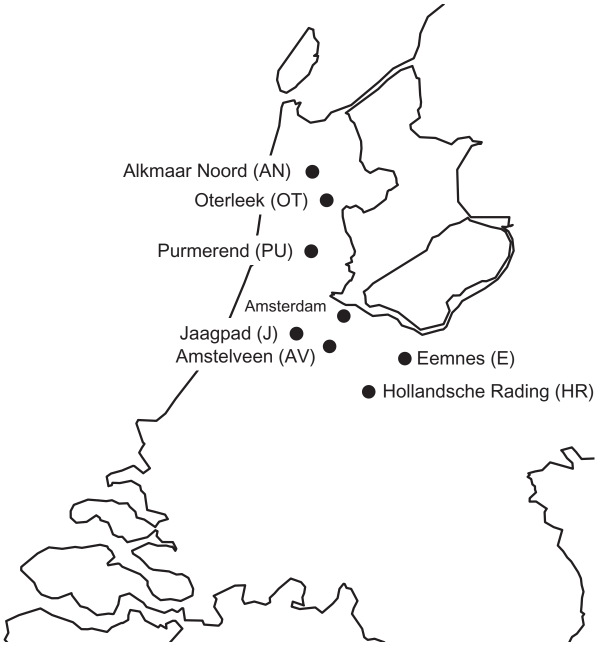

Figure 1. Geographical location of collection sites in The Netherlands. AN, Alkmaar Noord; AV, Amstelveen; E, Eemnes; HR, Hollandsche Rading; J, Jaagpad in Amsterdam; OT, Oterleek; PU, Purmerend.

MATERIAL AND METHODS

The freshwater snail *Lymnaea stagnalis* is abundantly distributed in Europe. Major habitats are stagnant ponds, ditches and rivers. We collected adult *L. stagnalis* (>20 mm shell length; Koene *et al.*, 2008) from seven localities in the Netherlands in 2011 and 2012 (Fig. 1; Table 1): Alkmaar Noord (AN), Amstelveen (AV), Eemnes (E), Hollandsche Rading (HR), Jaagpad in Amsterdam (J), Oterleek (OT) and Purmerend (PU). The minimum and maximum distance between two sites are 4.15 km (AV and J) and 60.53 km (AN and HR), respectively. Upon collection, we put each snail into a plastic bottle to avoid further copulation between collected individuals. Our searching effort was *c.* 1 min per 1 m^2 per person in all localities, except for the two extremely low- and high-density populations (AN and OT, respectively). The habitat type of AN is a deep and stagnant ditch, and two persons spent 90 min searching the whole area, due to the extremely low density. Locality OT is a shallow ditch and here two persons spent 2 min, but the abundance was too high to collect all the snails in sight (*c.* 50 per m^2; Table 1). Note that snails from OT did not show enough genetic polymorphism, so they were omitted from further analysis (see Results). Given that most of the sampling sites were shallow ditches (except AN), the large body size of this species allows us to spot them readily. Because the temporal fluctuation of density may be considerable, the estimated density indicates the mate availability at least at the moment of sampling. For comparison between populations, we refer to this estimate as relative density.

The collected snails were transferred to our breeding facility at VU University Amsterdam and each of them was isolated in an perforated container within a large flow-through tank to provide low-copper water at 20 ± 1 °C, constant O$_2$ supply and a light: dark cycle of 12:12 h. We provided a lettuce disc (19.6 cm^2) to each snail each day (Zonneveld & Kooijman, 1989). We checked for the presence of egg masses laid daily, because some snails are

Table 1. Description of sampled populations.

Locality name	GPS coordinates (°N, E)	Sampled area (m^2)	*N*	Relative density	Collection date
Alkmaar Noord (AN)	52.6850, 4.8118	3,933	22	0.006	14 August 2011
Amstelveen (AV)	52.3159, 4.8907	77	11	0.143	19 May 2011
Eemnes (E)	52.2539, 5.2879	213	*36.5*	*0.172*	14 June 2011 (*N* = 46), 25 July 2012 (*N* = 27)
Hollandsche Rading (HR)	52.1799, 5.1422	57	*22*	*0.385*	16 August 2011 (*N* = 25), 25 July 2012 (*N* = 19)
Jaagpad (J)	52.3328, 4.8364	84	*30.5*	*0.363*	18 May 2011 (*N* = 41), 23 July 2012 (*N* = 20)
Oterleek (OT)	52.6283, 4.8298	50	50	1.000	24 July 2012
Purmerend (PU)	52.5235, 4.8983	159	31	0.195	24 July 2012

Sampled area indicates approximate searching surface area. *N* is the number of snails collected. Relative density is the number of collected snails per 1 m^2 sampled area. Italic numbers indicate average number and relative density of snails collected over 2 years.

expected to be immature or infected by parasites, such as trematodes (McClelland & Boums, 1969; Ter Maat *et al.*, 2007). For each individual, we collected the egg masses laid within the first few days after collection, which is usually triggered by the clean water stimulus (Ter Maat, Lodder & Wilbrink, 1983). To obtain sufficient total DNA from an embryo, we incubated these egg masses for 14 d (close to hatching) and freeze-dried them. Note that this sampling timing of offspring allowed us to estimate the paternity distribution without the effect of early mortality after hatching (e.g. Escobar *et al.*, 2007). In order to genotype the mothers, we collected a haemolymph sample in 1.5-ml Eppendorf tubes by gently poking their foot with a blunt plastic rod. These haemolymph and egg mass samples were stored at −20 °C until further analyses.

We focused on genetic variation in five microsatellite loci, including three new markers developed by us (Kopp, Wolff & Jokela, 2012; Supplementary Material Table S1). To extract DNA from haemolymph samples, after thawing the samples, we collected the cell fraction by centrifuging for 15 min at maximum speed. The supernatant was discarded and total DNA was extracted from the pellet according to the protocol of Nakadera *et al.* (2014). To genotype offspring in egg masses, we picked individual eggs out of the dried egg masses one by one, using fine forceps and an antistatic mat (ROMEX). Then, we added 50 μl of 0.5 M NaOH to each sample and crushed it. After 10 min of incubation at room temperature, the reaction was neutralized with 5 μl of 1 M Tris buffer. The extracted whole DNA samples were stored at −20 °C.

To amplify DNA fragments containing target microsatellites by PCR, we prepared the master mix, including 13.3 μl of H_2O, 5 μl of 5× colourless reaction buffer (Promega), 1.5 μl of 25 mM $MgCl_2$, 2 μl of 10 mM dNTP (2.5 mM each), 1 μl of 5 μM forward (labelled) and reverse primers, 0.2 μl of GoTaq® DNA polymerase (Promega), 0.02 μl of *pfu* (Promega) and 1 μl of DNA extract. The thermocycling program was initiated with hot-start at 80 °C, then initial denaturation at 95 °C for 5 min and 30 cycles of [95 °C for 5 s (58 °C for 2k11, 52 °C for 2k68, 56 °C for Lsc1014, Lsc2678 and Lsc3163) and for 45 s, 72 °C for 60 s] and final extension at 72 °C for 10 min in a thermal cycler (MJ Research). After the PCR products were confirmed on 2.5% agarose gels, we made loading samples by mixing 2 μl of each PCR product per individual. Then, we added 1 μl of loading sample to 8.1 μl of HiDi formamide (Applied Biosystems) and 0.9 μl of SizeStandard (500LIZ™, Applied Biosystems). After denaturing for 5 min at 95 °C, we ran the samples for DNA fragment analysis in the sequencer, a 3730 DNA analyser (Applied Biosystems). We measured microsatellite-containing DNA fragment lengths using GeneMapper® (Applied Biosystems), allowing us to distinguish each genotype of each locus based on both length and colour signal of the labelled primers.

Statistics

We tested the genetic structure of sampled populations by first estimating the degree of heterozygosity in parental individuals using GenAlEx (Peakall & Smouse, 2012). Here, we reported the unbiased expected heterozygosity, which is adjusted by sample size. To examine genetic differentiation between populations, we used F_{ST} calculated by Fstat v. 2.9.3. (Goudet, 2002). Then, to test whether these populations significantly diversified, we carried out a permutation test without assuming Hardy-Weinberg equilibrium. To visualize genetic structure between populations, we calculated genetic distance, D_A, to construct a phylogenetic tree using the neighbour-joining method in Populations (Nei, Tajima & Tateno, 1983; Saitou & Nei, 1987; Langella, 1999). Since null alleles influence the estimate of F_{ST} values and multiple paternity (Dakin & Avise, 2004; Jones *et al.*, 2010), we examined whether our samples deviated from Hardy-Weinberg equilibrium (i.e. random mating) using GenoDive (Meirmans & Van Tienderen, 2004). We tested heterozygosity-based G_{IS} statistics and F_{IS} statistic with 999 permutation steps, but both tests yielded essentially the same outcomes. Our progeny-array data did not contain missing data or any mismatch of mother's and offspring's genotype, as confirmed in the process of parentage analysis in GERUD v. 2.0 (Jones, 2005).

To evaluate multiple paternity in the sampled populations, we estimated the number of siring fathers in each egg mass that was laid in the laboratory. Based on genotypes of siblings and mothers, we calculated the least number of different fathers that were required to explain the variation of genotypes in siblings, using GERUD v. 2.0 (Jones, 2005). Since this method cannot discriminate potential fathers with the same genotype, the output is interpreted as the minimum number of fathers. We also calculated exclusion probabilities—the probability that an unrelated candidate parent will be eliminated from consideration as a true parent by the locus in question (Chakraborty, Meagher & Smouse, 1988; Jones & Ardren, 2003; Jones *et al.*, 2010). Thus, this value indicates the detectability of a given progeny array. All the mothers were unambiguously genotyped for the five microsatellite loci, except for three sibling arrays (one case each in AN, HR and J) where the mothers died before haemolymph collections. We report the data including these progeny arrays with unknown mothers, since excluding them did not affect the overall result. To explain the variation of promiscuity between populations, we tested the relationship with relative density (i.e. whether the number of fathers correlates with mate availability), heterozygosity (i.e. whether the number of fathers correlates with overall genetic differentiation) and exclusion probability (i.e. whether the number of fathers relates to the detectability of potential siring fathers) using Pearson's correlation tests. We carried out all the statistical analyses in R v. 3.0.3 (R Core Team, 2014).

RESULTS

Even within a small geographic range, we found a well-defined genetic structure across populations, based on the genotypes of adult snails (N = 53; Fig. 2, Table 2). Most populations showed genetic variation in our microsatellite loci and population E showed significant deviations from Hardy-Weinberg equilibrium (2k11, P = 0.003; Lsc3163, P = 0.001; overall, P = 0.001: Supplementary Material Table S2). The latter suggests the potential existence of null alleles, nonrandom mating and/or inbreeding in population E. Also, pairwise F_{ST} tests indicated significant genetic differentiation between populations, as represented by the phylogenetic tree based on genetic distance (Fig. 2; Table 2). This suggests distinct evolutionary histories of each study population.

Our progeny-array data include genotypes of 34 mothers and 380 offspring in total (Table 3). Based on exclusion probabilities, two populations (OT and PU) did not have high enough resolution to estimate reliably the number of paternal genotypes, due to the low genetic variation of these populations (Table 3). Thus, these two populations were excluded from further analyses. The estimated minimum number of fathers averaged 1.77 within mothers (range: 1–3 fathers; Table 3) and 20 out of 34 mothers (59%) showed multiple paternity. In this survey, we did not find any self-fertilized offspring, i.e. all the siblings had at least one allele from other individuals than their mother. Also, it should be noted that our analysis is limited by the small sample size and the inability to discriminate offspring sired by different fathers with the same genotype. Therefore, our estimates indicate the minimal degree of multiple paternity. Lastly, we found that high-density populations show higher numbers of fathers than low-density populations (r = 0.91, t_3 = 3.81, P = 0.032; Fig. 3), while neither exclusion probability (r = 0.83, t_3 = 2.55, P = 0.084) nor heterozygosity (r = 0.61, t_3 = 1.33, P = 0.275) were found to be significantly correlated with the number of fathers. Thus, the number of fathers estimated is unlikely to be explained by genetic differences between populations, or by the detectability of different siring fathers.

DISCUSSION

We found that *Lymnaea stagnalis* mates multiply in the field, thus suggesting that sperm competition could play an important role in natural populations. The number of siring fathers in the sampled populations was moderate (1–3 fathers per mother), even though this species has been reported to copulate quite promiscuously in the laboratory (Koene & Ter Maat, 2007). Additionally, we found a positive correlation between the number of fathers and relative density. Although the estimated multiple paternity might be underestimated due to our limited sample size, the observation of multiple mating in natural populations sheds light on postcopulatory processes in simultaneous hermaphrodites.

Consistent with the previous studies in the laboratory, our results show that natural populations of *L. stagnalis* undergo a moderate degree of sperm competition (Parker, 1970; Koene *et al.*, 2009), although their actual mating frequency is unknown. We expect that the mating frequency in the field is higher than the number of siring fathers we estimated, based on biological and technical arguments. First, several biological mechanisms lead to underestimation of mating frequency from progeny-array data. For instance, unsuccessful matings (e.g. no sperm transfer, no sibling sired) and repeated matings with the same partner are not reflected in the estimated number of mates, although these events contribute to mating frequency. Also, cryptic female choice potentially further distorts the degree of multiple paternity and mating frequency in the wild. Given that this species has efficient sperm storage capacity that allows it to store and use sperm from mates for a few months, it is at least able to choose between using allo- and autosperm for fertilization of its own eggs; whether such ability to choose also extends to the choice of sperm from specific partners (i.e. cryptic female choice) remains unclear (Cain, 1956;

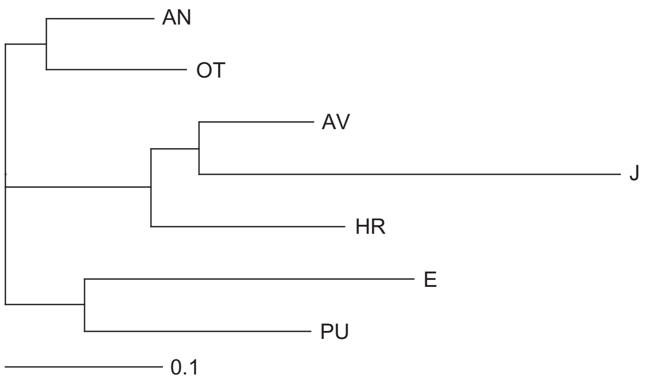

Figure 2. Genetic relationship between populations of *Lymnaea stagnalis*. The tree is based on genetic distance, D_A, and constructed using the neighbour-joining method.

Table 2. Pairwise F_{ST} values (below diagonal) and P values from pairwise tests of differentiation between populations of *Lymnaea stagnalis* (above diagonal).

	AN	OT	AV	J	HR	E	PU
AN	0	0.212	0.012	**0.002**	**0.002**	**0.002**	0.007
OT	0.021	0	0.010	**0.002**	**0.002**	**0.002**	0.012
AV	0.168	0.191	0	0.012	0.005	**0.002**	0.010
J	0.324	0.356	0.117	0	**0.002**	**0.002**	0.005
HR	0.217	0.255	0.106	0.208	0	**0.002**	**0.002**
E	0.216	0.220	0.118	0.246	0.213	0	**0.002**
PU	0.238	0.210	0.276	0.422	0.370	0.222	0

Bold font indicates significant genetic differentiation after Bonferroni corrections and 420 permutations (without Hardy-Weinberg equilibrium assumption, adjusted type 2 error threshold = 0.002).

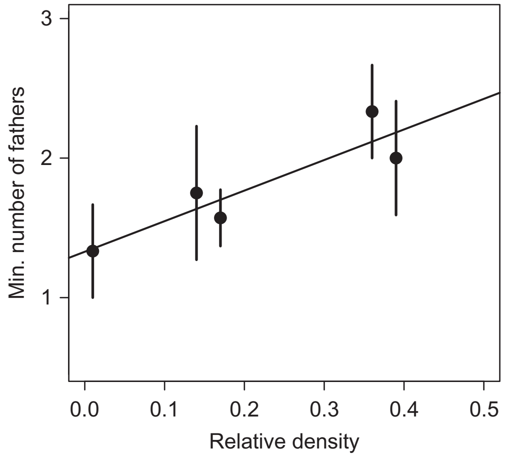

Figure 3. Correlation between relative density and the estimated number of fathers (mean ± SE) in populations of *Lymnaea stagnalis*. Two populations, OT and PU, were excluded due to low detectability of multiple-siring fathers.

Table 3. Multiple paternity in wild populations of *Lymnaea stagnalis*.

Population	Expected exclusion probability[*]	*N* mother	*N* offspring[†]	Min. *N* father[‡]
AN	0.747 (0.440)	3	24 (8.33)	1.333 (0.333)
AV	0.916 (0.734)	4	47 (11.75)	1.750 (0.479)
E	0.804 (0.609)	7	71 (10.14)	1.571 (0.202)
HR	0.942 (0.803)	4	46 (11.50)	2.000 (0.408)
J	0.914 (0.720)	6	75 (12.50)	2.333 (0.333)
OT	**1.000 (1.000)**	**5**	**63 (12.60)**	**1.800 (0.374)**
PU	**1.000 (1.000)**	**5**	**54 (10.80)**	**1.400 (0.245)**

These exclusion probabilities are based on allele frequencies of all five loci (GERUD v. 2.0). The last two populations, indicated in bold, did not give reliable estimates (see Results).

[*] Exclusion probability when genotype of one parent is known (and when neither genotype known).

[†] Total number of offspring genotyped (mean per mother).

[‡] Mean (SE).

Puurtinen *et al.*, 2007; Nakadera *et al.*, 2014). Second, there are technical issues related to estimating the number of mates. Our limited sample size might have prevented us from sampling all the potential siring fathers for a given mother snail, although our random sampling within egg masses should at least provide the relative variation of multiple paternity between mothers and populations. In addition, more variable genetic markers, such as SNPs, could improve the detectability of siring fathers in populations with low genetic polymorphism (OT and PU in our case). Given these two lines of reasoning, the mating frequency of *L. stagnalis* in the field is expected to be higher than the estimated number of one to three siring fathers.

Due to the limited sample size, the positive correlation between the number of mates and relative density of potential mates is rather preliminary, but it is intuitively plausible (see also Kupfernagel *et al.*, 2010). That is, when *L. stagnalis* has more mates around, they produce offspring fathered by more mates. Furthermore, mate availability is a very powerful factor for altering reproductive output and sex allocation of hermaphrodites in the laboratory (e.g. Trouvé *et al.*, 1999; Tan, Govedich & Burd, 2004; Lorenzi *et al.*, 2005; Koene, Montagne-Wajer & Ter Maat, 2006; Janicke *et al.*, 2013). Thus, it seems convincing that mate availability is a major determinant of sexual-selection processes in field populations of hermaphrodites.

Comparing our findings of multiple paternity with other hermaphroditic species is both fascinating and challenging, due to the diversity of reproductive traits among taxa. For example, compared with our study in the freshwater snail *L. stagnalis*, the number of siring fathers seems higher in the land snail *A. arbustorum*, in which there are 3.7 fathers on average (range: 2–6, estimated by GERUD v. 2.0, Kupfernagel *et al.*, 2010; but see also Kupfernagel & Baur, 2011; Janssen & Baur, 2015). While investigations in the latter species may have yielded more reliable estimates of mating frequency, the differences in reproductive traits between these two species cannot be dismissed. First, many land snail species mate reciprocally, thus both partners transfer and receive sperm at the same time. Compared with the unilateral mating in freshwater snails, their reciprocal mating inevitably increases the number of partners from which sperm needs to be accepted, since this is a condition for also donating sperm. Second, populations of *A. arbustorum* usually have quite high density, *c.* 1–10 snails per m^2 (Kupfernagel *et al.*, 2010). Third, their characteristics of egg production substantially differ: *A. arbustorum* lays a few clutches (with 20–50 eggs) per year but, as in many freshwater snail species, *L. stagnalis* keeps laying egg masses (each containing 50–150 eggs) after maturation until winter sets in (e.g. Hoffer *et al.*, 2012; Nakadera *et al.*, 2015). Furthermore, it is interesting to compare the multiple paternity in sperm-casting and sessile hermaphrodites (Johnson & Yund, 2007; Yeoh & Dai, 2010; Kelly *et al.*, 2012; Barazandeh *et al.*, 2013, 2014; Plough *et al.*, 2014; Warner *et al.*, 2016; sequential hermaphrodites: Dupont *et al.*, 2006; Brante, Fernández & Viard, 2011). Given that sperm-casting species are predicted to experience different postcopulatory sexual-selection pressures compared with copulating species (e.g. Pizzari & Parker 2008), we believe that documenting multiple paternity in copulating hermaphrodites, which has scarcely been done, will eventually allow recognition of a general pattern in genetic mating system in diverse hermaphroditic species.

While previous studies provided observational (i.e. F_{IS}-value based) and experimental support for this species' preference to outcross (Cain, 1956; Puurtinen *et al.*, 2007; Koene *et al.*, 2009; Nakadera *et al.*, 2014), our study further confirms that *L. stagnalis* in the wild predominantly use sperm from their mating partners. This is surprising, given that this species does not seem to suffer from inbreeding depression (e.g. Puurtinen *et al.*, 2007). One of the potential mechanisms could be the bottlenecks arising from frequent extinction and range expansion, which can efficiently purge unfavourable alleles from a gene pool (e.g. Pujol *et al.*, 2009; Facon *et al.*, 2011). Such a dynamic population structure is supported by the disappearance of study populations during our monitoring (YN and JMK, personal observation) and the presumably recently established population (OT with extremely high density and low genetic variation). Thus, the dynamics in the populations implies that there must be clear advantage(s) in use of allosperm for fertilization. Obviously, there are many possible hypotheses based on indirect benefits or sexual conflict (e.g. Griffith, Owens & Thuman, 2002; Uller & Olsson, 2008), but these remain to be examined for *L. stagnalis*.

In summary, field populations of *L. stagnalis* experience sperm competition, as indicated by the moderate degree of promiscuity we detected. Most notably, the positive correlation of the number of siring fathers with relative density emphasizes the importance of mate availability for potential postcopulatory sexual selection. These results are informative for further understanding of sexual-selection processes across the diverse sexual systems of vertebrates and invertebrates.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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