DURATION OF SPERM STORAGE IN THE SIMULTANEOUS HERMAPHRODITE *LYMNAEA STAGNALIS*

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(Received 6 June 2013; accepted 25 October 2013)

ABSTRACT

Reproductive success is a crucial variable to understand the action of sexual selection, but its quantification is not straightforward because several factors lead to nonrandom paternity gain, i.e. internal fertilization, multiple and polyandrous mating, and female sperm storage (hereafter, termed simply ‘sperm storage’) (Parker, 1970; Eberhard, 1996; Nakadera & Koene, 2013; Shuster, Biggs & Dennis, 2013). In many studies where male RS has been estimated, the aspect of sperm storage was ignored or compensated for by taking a limited amount of samples (e.g. Angeloni, 2003; Chase & Blanchard, 2006; Koene et al., 2009; Garefalaki et al., 2010; Kupfermangel & Baur, 2011; Lombordo et al., 2013; Pelissié, Jarne & David, 2012). A short time window for measuring male RS could lead to a biased estimation, especially if the species has evolved to store sperm efficiently, which is often the case (Racey, 1979; Page, 1986; Birkhead & Moller, 1993, 1998; Neubauer & Wollner, 1999; Pearse & Avise, 2001; Boomsma, Baer & Heinze, 2005; Dillon, McCullough & Earnhardt, 2005; Jordaens, Dillen & Backeljau, 2007; Leonard & Cordoba-Aguilar, 2010). Thus, to measure RS reliably, the long-term paternity gain via sperm storage needs to be investigated.

Given these considerations, paternity longevity can be seen as a candidate trait for sexual selection, not only natural selection. Paternity longevity is defined as the duration of paternity success through the sperm storage ability of one or more mating partners. In iteroparous species with long-term sperm storage, variation in paternity longevity can be a determinant of RS, since longer paternity longevity leads to higher male RS (Olsson et al., 2007, 2009; Uller et al., 2013), even after a male’s death (Zamudio & Sinervo, 2000; López-Sepulcre et al., 2013). Although paternity longevity has been implicitly included in measurements of male RS in terms of the proportion of offspring sired by a female’s second mate (P2 value; e.g. Boorman & Parker, 1976; Simmons & Siva-Jothy, 1998), where all eggs from mates were counted, this has not been the case in recent studies. Particularly in gastropods, P2 values have often been calculated from the first egg clutch only, even though these animals would produce multiple egg clutches afterwards (Pelissié et al., 2012; Lombardo et al., 2013; Nakadera & Koene, 2013). Given this deficiency, and the high potential as a candidate trait of sexual selection, measuring paternity longevity is a promising approach in diverse animal groups.

INTRODUCTION

Reproductive success (RS) is one of the essential variables for theoretical studies of sexual selection (Bateman, 1948; Wade, 1979; Arnold, 1994; Arnold & Duval, 1994; Jones, 2009; Anthes et al., 2010). However, its empirical quantification is far from straightforward, because several factors lead to nonrandom paternity gain, i.e. internal fertilization, multiple and polyandrous mating, and female sperm storage (hereafter, termed simply ‘sperm storage’) (Parker, 1970; Eberhard, 1996; Nakadera & Koene, 2013; Shuster, Biggs & Dennis, 2013). In many studies where male RS has been estimated, the aspect of sperm storage was ignored or compensated for by taking a limited amount of samples (e.g. Angeloni, 2003; Chase & Blanchard, 2006; Koene et al., 2009; Garefalaki et al., 2010; Kupfermangel & Baur, 2011; Lombordo et al., 2013; Pelissié, Jarne & David, 2012). A short time window for measuring male RS could lead to a biased estimation, especially if the species has evolved to store sperm efficiently, which is often the case (Racey, 1979; Page, 1986; Birkhead & Moller, 1993, 1998; Neubauer & Wollner, 1999; Pearse & Avise, 2001; Boomsma, Baer & Heinze, 2005; Dillon, McCullough & Earnhardt, 2005; Jordaens, Dillen & Backeljau, 2007; Leonard & Cordoba-Aguilar, 2010). Thus, to measure RS reliably, the long-term paternity gain via sperm storage needs to be investigated.

Given these considerations, paternity longevity can be seen as a candidate trait for sexual selection, not only natural selection. Paternity longevity is defined as the duration of paternity success through the sperm storage ability of one or more mating partners. In iteroparous species with long-term sperm storage, variation in paternity longevity can be a determinant of RS, since longer paternity longevity leads to higher male RS (Olsson et al., 2007, 2009; Uller et al., 2013), even after a male’s death (Zamudio & Sinervo, 2000; López-Sepulcre et al., 2013). Although paternity longevity has been implicitly included in measurements of male RS in terms of the proportion of offspring sired by a female’s second mate (P2 value; e.g. Boorman & Parker, 1976; Simmons & Siva-Jothy, 1998), where all eggs from mates were counted, this has not been the case in recent studies. Particularly in gastropods, P2 values have often been calculated from the first egg clutch only, even though these animals would produce multiple egg clutches afterwards (Pelissié et al., 2012; Lombardo et al., 2013; Nakadera & Koene, 2013). Given this deficiency, and the high potential as a candidate trait of sexual selection, measuring paternity longevity is a promising approach in diverse animal groups.
Paternity longevity is of significance for RS in sperm-storing, mainly out-crossing animals irrespective of gender system (gonochoristic or hermaphroditic). However, simultaneous hermaphrodites potentially have a unique reproductive mode—self fertilization or selving. (Note that in an extensively selving system, neither male RS nor paternity longevity is worthwhile to quantify, since most RS arises from the female function; Jarne & Charlesworth, 1993; Jarne, Vianey-Liaud & Delay, 1993; Jarne & Auld, 2006.) For instance, many pulmonates are capable of selving (Jarne & Auld, 2006; Escobar et al., 2011). Even if they are inseminated only once, they have two different sperm types for fertilizing eggs, their own sperm (autosperm) and their mating partner’s sperm (allosperm). Thus, even if they have used up the stored allosperm, they can continue to reproduce by using autosperm. Alternatively, despite the presence of allosperm, they could selectively use autosperm for their own eggs (Janicke et al., 2013). Therefore, knowledge about the usage pattern of auto- and allosperm is pivotal for quantifying RS and investigating the action of sexual selection in hermaphrodites (Koene et al., 2009; Schärer & Pen, 2013).

A ‘single-insemination experiment’ under controlled conditions would be the first step towards expanding our understanding about paternity longevity in a simultaneous hermaphrodite. Here, we have focused on the great pond snail, Lymnaea stagnalis (L.), as a model species. According to Cain (1956), this species can store and use allosperm for up to 116 days. Her study has already provided valuable information, but several factors remain untested. First, it remains unclear how many times the snails had mated before monitoring started, for this snail is known to be promiscuous (Koene & Ter Maat, 2007). This is very relevant, because a recent study has shown that multiple mating induces a change in investment in eggs, although the implications for sperm storage were not investigated (Hoffler et al., 2012). Second, Cain’s (1956) study used albino mutants which, although convenient as a genetic marker of paternity, might cause a reproductive deficiency or advantage (as is known to be the case for other freshwater snail species; Vianey-Liaud, Joly & Dussart, 1996). Moreover, from Cain’s figure and description, we cannot access the details of variation in paternity longevity, although this can be precisely the target for sexual selection. To improve knowledge about sperm storage in this species, we conducted an experiment in which we allowed the snails to mate once in their lifetime (i.e. single-insemination experiment), using a neutral genetic marker (microsatellite) and measuring variation in paternity longevity of each individual.

In addition, an analytical methodology needs to be developed for comparing paternity longevity between individuals. Clearly, paternity longevity after mating shows variation due to various, largely unknown, factors (Baur, 1994; Angeloni, 2003; Ludwig & Walsh, 2004; Dillon et al., 2005). To study the mechanism causing this variation and to assess its significance for RS, a general metric of paternity longevity is necessary. One option would be the maximum duration of sperm storage, which is the best documented feature of sperm storage (Birkhead & Møller, 1993, 1998; Jordansen et al., 2007). However, sperm storage duration requires considerable effort (and patience) to document and is highly dependent on egg-laying activity and/or offspring production. Therefore, we recently proposed an alternative metric, paternity longevity 50 (PL50), which is the time point when the paternity ratio of a focal sperm donor declines to 50% (Nakadera & Koene, 2013). PL50 is useful if the study animals show a clear change in paternity ratio of autosperm and/or sperm of rival males over time. To generalize this type of metric to different study animals and conditions, PL50 may require further modifications (e.g. using relative paternity ratio). To date, however, the scarce knowledge of paternity gain over time prevents us from proposing a more general methodology.

Therefore, in this study, we adopt this approach to establish its utility for further investigations.

Given the above, we re-examined sperm storage in L. stagnalis. First, we describe how they store and use allosperm, which is important information in order to estimate where and how sexual selection can act on internal fertilization events. Then, we test whether paternity longevity and male RS are correlated. We expected that individuals that have a longer paternity longevity (i.e. sire offspring for a longer time after insemination) should be more reproductively successful as males, in terms of fertilized eggs. We also examine whether paternity longevity correlates with other traits, which could shed light on the underlying mechanism of sperm storage.

MATERIAL AND METHODS

We used Lymnaea stagnalis, which is a simultaneous hermaphrodite and has been bred at VU University Amsterdam for about 50 years. To date, explicit inbreeding depression or self-fertilization depression in this species have not been reported (Coutelle & Lagadic, 2006; Puurtinen et al., 2007; Koene et al., 2008). They copulate unilaterally, meaning that one individual plays the male role and the other the female role. When both are motivated to mate as male, they can swap mating roles right after the first mating (Van Duivenboden & Ter Maat, 1985; Koene & Ter Maat, 2005). This species is quite fecund; snails from our mass culture produce 2–3 egg masses per week and each egg mass contains 100–150 eggs on average. Snails were kept in low-copper water, which was maintained at 20 ± 1°C and in a light:dark cycle of 12:12 h. Each isolated snail was supplied with a lettuce disc (about 19.6 cm²) each day as food, which is slightly less than their maximum intake (Zonneveld & Kooijman, 1989).

First, we made three homozygous strains for each of the three alleles of microsatellite A16 (GenBank accession code: AY225956, Knott, Puurtinen & Kaitala, 2003; Note that the reverse primer is displayed in 3’–5’ orientation, Koene et al., 2009). About 100 snails were isolated in perforated jars well before their maturation (shell height about 1 cm). When they started laying eggs by self-fertilization, we anaesthetized them, by injecting c. 2 ml of 50 mM MgCl₂ and, using fine scissors, collected a small piece of their foot tissue for genotyping (see below). We selected and incubated the selfed egg masses of 3–4 homozygous individuals per strain (A, B and C) for the next generation. We provided lettuce and fish food (Tetraphyll) ad libitum to these juveniles. Before maturation, about 80 of these homozygous snails were isolated, raised to maturity and used for the mating experiment.

Most of our genotyping protocol followed Koene et al. (2009), except for the DNA extraction method and PCR condition. First, we extracted the total DNA from foot tissues. We added 100 µl of 0.5 M NaOH to samples and crushed them. These were incubated for about 10 min at room temperature. Then, we added 10 µl of 1 M Tris buffer and centrifuged them for 10 min at maximum speed to collect the supernatant. These DNA extracts were stored at −20°C. Next, we amplified the target DNA fragments by PCR. We made a master mix solution [1.5 µl of H₂O, 5 µl of 5X Go Taq® reaction buffer (Promega), 1.5 µl of 25 mM MgCl₂ (Promega), 2 µl of 2.5 mM dNTP, 1 µl of 5 µM forward and reverse A16 primers, 0.2 µl of Go Taq® DNA Polymerase (Promega), 0.02 µl of pfu (Promega) per sample] adding 1 µl of DNA extracts and ran the thermal cycler (MJ Research) using the following program: denaturation at 95°C for 5 min, [95°C for 15 s, 55°C for 45 s, 72°C for 60 s] × 35 cycles, final extension time at 72°C for 10 min. After the confirmation of PCR products on 3% agarose gel, they were visualized on Spreadex EL 600 Wide Mini Gels (S-2×25
when the outcrossing rate reached zero. Because one recipient calculated the maximum duration of sperm storage by using the day of an alleles. As far as we could detect, there were no missing material. To deal with this problem, we quantified the paternity longevity of donors in terms of PL50. We modelled the declining pattern of factors (e.g. egg availability or need to reach 100% selfing). To above (see Introduction), it is costly and depends on various causes 50% of response) in toxicology. For this calculation, we emplaced by the EC50 (median effective concentration, which represents the threshold is that it does not depend on a fitted model, as ex-

This traditional quantification of sperm storage duration is useful for comparison with previous studies but, as mentioned above (see Introduction), it is costly and depends on various factors (e.g. egg availability or need to reach 100% selfing). To deal with this problem, we quantified the paternity longevity of donors in terms of PL50. We modelled the declining pattern of outcrossing rate and calculated the time point (day) when the paternity ratio of a donor declined to 50% (= PL50). The reason why we chose the middle point of the outcrossing rate as the threshold is that it does not depend on a fitted model, as exemplified by the EC50 (median effective concentration, which causes 50% of response) in toxicology. For this calculation, we entered the paternity data of each pair in a generalized linear model with binomial distribution (outcrossed/selfed). We used PL50 as a reference point for calculating the average paternity ratio. So, the average paternity ratio before PL50 was multiplied by the dry weight of each egg mass laid before PL50.

The same procedure was also applied to the dataset after PL50, and the sum of these two values was used as male RS. We entered PL50 as dependent variable and male RS as explanatory variable in a linear regression model.

Furthermore, to take the variation of fecundity between recipients into account, we divided this estimate of male RS by the total dry weight of egg masses laid by a recipient snail, referred to as the adjusted male RS. In addition, to explore the factors that might explain variation in paternity longevity, we used shell length of donors and recipients, copulation duration and total dry weight of egg masses as the female RS of recipients. We also calculated overall size of pairs by using shell lengths of recipient and donors in a principal component analysis. The first principal component (PC1) explains 90% of the variation and correlates positively with recipient size but negatively with
donor size (see Fig. 2). We examined whether these variables could explain the variation in PL50, using a linear regression model. Finally, we tested whether egg production changed during our monitoring, using an analysis of variance of dry weight of egg masses ($n = 91$), with time after mating (Days) as a fixed factor and nested by recipient identity (Recipients). All statistical tests were conducted in R (v. 2.11.1).

RESULTS

We obtained 12 out of 25 recipients, which were virgin until they received sperm from one donor and then produced outcrossed offspring; the remaining recipients did not mate during our observation. For four of these 12 we could not determine the paternity longevity of the donors, because recipients did not survive until they started selfing (they died on days 10, 13, 22 and 48, respectively). For the remaining eight, we monitored their usage of allosperm until they stopped outcrossing and switched to 100% selfing.

By genotyping 684 offspring, we examined the usage of allosperm in recipients. Immediately after snails mated, most of them started to use received allosperm for fertilization extensively (Fig. 1). One recipient laid a selfed egg mass first and then started outcrossing (Fig. 1). This confirms that allosperm can travel through the female reproductive organs while selfed eggs are being packaged and transported in the opposite direction (Jarne et al., 2010). Furthermore, since the egg-laying process takes 2 h on average (from ovulation to oviposition; Ter Maat, Lodder & Wilbrink, 1983), allosperm should take at least 2 h to reach the sperm storage site and become ready to be used for fertilization. Consistent with the previous study (Cain, 1956), most snails showed a sudden decline in outcrossing at some time point. However, sperm storage duration was much shorter than 90 d (zero out of eight cases, binomial test, $P = 0.008$). The maximum sperm storage duration was $62.14 \pm 5.59$ d (mean $\pm$ SE, range $48–87$ d).

Using our other metric for paternity longevity, PL50, we also found considerable variation between donors/pairs, measured at $43.09 \pm 6.07$ d (Fig. 1). However, this variation in PL50 could not be explained by the estimate for male RS of donors ($F_{1,6} = 0.005, P = 0.947$), copulation duration ($F_{1,6} = 0.125, P = 0.736$) or female RS of recipients ($F_{1,6} = 0.833, P = 0.397$). Even after adjusting male RS by the recipients’ fecundity, we did not detect a relationship with paternity longevity ($F_{1,6} = 0.476, P = 0.516$). Furthermore, we found a strong relationship between PL50 and recipient and donor body size ($F_{1,6} = 6.99, P = 0.038; F_{1,6} = 12.28, P = 0.0128$; Fig. 2). In addition, overall body size of pairs (PC1) explained variation in PL50 ($F_{1,6} = 11.31, P = 0.015$; Fig. 2C), but residuals did not ($PC2, F_{1,6} = 0.239, P = 0.642$). Therefore, larger pairs achieved longer paternity longevity, but not higher fecundity ($F_{1,6} = 0.399, P = 0.551$). Finally, dry weight of egg masses declined over time ($F_{6} = 3.62, P = 0.001$; Fig. 2D).

DISCUSSION

We found phenotypic variation in paternity longevity and male RS of simultaneously hermaphroditic snails after they received sperm only once. Even though our strain of Lymnaea stagnalis has been bred in the laboratory for several decades, and our experiment was done under standardized conditions, paternity...
longevity and RS still showed considerable variation. This implies the potential for sexual selection acting on these reproductive traits.

Intuitively, it seems important that in order for a sperm donor to increase its RS it should extend its paternity longevity but, in this experiment, we were unlikely to detect a direct correlation with male RS. There are several possible explanations for this. First, our sample size might have been too small to detect the correlation between paternity longevity and male RS. Second, our experimental setup was far removed from the natural situation for _L. stagnalis_, but it was necessary to standardize mating times. In this experiment, snails mated only once, but the species is known to be highly promiscuous and able to mate as often as twice per day (Koene & Ter Maat, 2007). Hence, both donors and recipients invest in, or have been selected for, obtaining further RS from multiple matings, e.g. via sperm competition (Koe ne et al., 2009). Therefore, paternity longevity based on a single mating may not predict overall male RS very well. Third, we observed a decline in the dry weight of egg masses during the experiment (Fig. 2D). This decline was not very steep and may simply represent an aging effect of recipients (see Hoffer et al., 2012, where an increase in egg production with age was observed). Nonetheless, it could also indicate that donors may not gain as much benefit with older recipients as when recipients are younger. Therefore, these considerations imply that the two traits are unlikely to correlate with each other in the current experimental design.

Another intriguing finding that emerged from our data was that the variation of paternity longevity strongly correlated with the body size of donors and recipients (Fig. 2A–C), although these two traits could not be disentangled from the potential genetic correlation of laboratory strains. Previous studies have shown that the number of sperm transferred does not correlate with absolute body size of donor or recipient snails (body weight, Loose & Koene, 2008; shell length, Hoffer, Ellers & Koene, 2010). To explain this, one of the possible factors is seminal fluid. As widely known in many animal taxa, seminal fluid proteins have various functions, to protect sperm and manipulate recipients (reviewed by Swan son & Vacquier, 2002; Gillott, 2003; Chapman & Davis, 2004; Ravi Ram & Wolfler, 2007), including a role in sperm storage (Collins, Williams & Evans, 2004; reviewed by Wolfler, 2011). In particular, _L. stagnalis_ also has a seminal fluid protein, Ovipostatin, which suppresses the egg-laying activity of recipients (Koene et al., 2010).

So, this species may have other seminal fluid proteins that determine paternity longevity. For instance, given that a larger donor has a larger prostate gland (which produces seminal fluid; Y. Nakadera, unpub.; see also Koene & Ter Maat, 2004), it should be able to transfer a larger amount of these proteins along with its sperm. Assuming a ‘dosage effect’ upon body size, this would also explain the correlation with recipients’ shell length that suggests that larger recipients have a shorter PL50 (Fig. 2A). Hence, the influence of seminal fluid might depend on the body size of recipients. Although the proximate mechanism (e.g. male property or cryptic female choice) remains to be examined, this scenario seems plausible.

In _L. stagnalis_, we found that most of the general characteristics of sperm storage correspond with those described in previous studies, except for average paternity longevity (Cain, 1956; Koene et al., 2009). This deviation of sperm storage duration could be due to differences in genetic background or inbreeding depression, although Cain (1956) also used lines that had been inbred for over 10 years (Noland & Carriker, 1946). After an ejaculate has been transferred to a recipient, allosperm actively swim (or are transported) to the recipient’s sperm-storage organ. These processes seem to take at least 2 h before the sperm are capable of fertilizing eggs. There are two reasons supporting this: (1) We found that one snail laid selfed eggs just before it started outcrossing, as was also observed in a previous study (Cain, 1956). (2) The production of an egg mass, which starts with the fertilization of the unpackaged, ripe oocytes, takes at least 2 h from the time of ovulation to oviposition, and these processes are induced by environmental factors (e.g. oxygen-rich water, clean surface, light) rather than copulation (Ter Maat et al., 1983; Ter Maat, Pieneman & Koene, 2012). This time window of sperm transportation is matched by direct observations of sperm distribution after copulation (Koene et al., 2009). In addition, even if eggs are being packaged and transported through the female tract, received sperm can travel in the opposite direction through the allosperm duct. This duct is an evaginated channel that runs along the oviduct and can be physically separated from the lumen of the oviduct by contractions of muscles within the tract (Jarme et al., 2010). This implies that, within the oviduct, allosperm remain separated from packaged eggs because the lumen of the oviduct is actively divided into two ducts. Furthermore, this species can use both allo- and auto-sperm within one egg mass (Fig. 1), suggesting female control over these types of sperm (Eberhard, 1996). The above information about sperm storage, utilization and transport could be useful to estimate where sexual selection could potentially act in the female reproductive tract.

In summary, we found considerable variation in paternity longevity in simultaneously hermaphroditic snails, which has so far been overlooked (Cain, 1956). Although we expected that greater paternity longevity would result in increased male RS, our data did not bear this out. Therefore, in the future it will be of great interest to investigate the paternity longevity and male RS of this species under an experimental scenario with multiple inseminations, to better simulate sperm competition. Furthermore, we know hardly anything about the mechanisms that determine paternity longevity and the switch from outcrossing to selfing (e.g. sperm depletion and cryptic female choice). However, the strong correlation between paternity longevity and body size of donors and recipients appears to indicate an important role of seminal-fluid protein concentrations for sperm storage. In addition, the genetic bases of these reproductive traits need to be discovered in order to understand the action of sexual selection. These perspectives will help to further improve our understanding of sexual selection in simultaneous hermaphrodites.

ACKNOWLEDGEMENTS

We thank C. Popelier and J. Mariën for technical assistance and N. M. van Straalen, D. Giese, E. Swart and four anonymous referees for fruitful discussions and valuable comments. This research was supported by a Schure-Beijerinck-Popping research grant of the Royal Netherlands Academy of Arts & Sciences (KNAW) to J.M.K. and a PhD grant from the Japan Student Services Organization (JASSO) to Y.N.

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