Long-lived sperm in the geothermal bryophyte *Pohlia nutans*

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Non-vascular plants rely on sperm to cross the distance between male and female reproductive organs for fertilization and sexual reproduction to occur. The majority of non-vascular plants have separate sexes, and thus, this distance may be a few millimetres to many metres. Because sperm need water for transport, it has been assumed that sperm lifespans are short and that this type of sexual reproduction limits the expansion of non-vascular plants in terrestrial environments. However, little data is available on the lifespan of sperm in non-vascular plants, and none is available for bryophytes, the group thought to have first colonized terrestrial habitats. Here, we documented the lifespan of sperm of *Pohlia nutans*, collected from a geothermal spring's area, and tested the effects of variation under environmental conditions on this lifespan. Surprisingly, 20 per cent of the sperm were still motile after 100 h, and sperm lifespan was not significantly affected by temperature variation between 22 and 60°C. Lifespan was significantly affected by sperm dilution and temperatures above 75°C. These results suggest the need to reconsider the importance of sperm motility in bryophyte fertilization.

Keywords: geothermal; bryophyte; plant; reproduction; sperm

1. INTRODUCTION

For organisms with motile gametes, understanding gamete lifespan and the effects of environment on lifespan is often the key to understanding variation in reproductive success. For organisms in which motile gametes disperse (e.g. algae, bryophytes and many marine invertebrates), such information is critical to understanding the evolution of mating and sexual systems and predicting the role of global change on reproductive success. While we have a large body of knowledge on sperm motility, survival and stress in terrestrial animal species (e.g. Turner 2006; Stewart *et al.* 2007) and marine invertebrates (e.g. Caldwell *et al.* 2004; Johnson & Yund 2004), very little is known about this important life-history stage in algae and non-vascular plants (Rice & Laetsch 1967; Paolillo 1981). As taxa with bryophyte-type morphology were probably the first land plants (Kenrick & Crane 1997; Nickrent *et al.* 2000), reliance on motile sperm

is theorized to have limited their ability to spread in the terrestrial environment, particularly in comparison with seed plants that disperse gametes as pollen (Shaw & Renzaglia 2004). However, little data are available on bryophyte sperm longevity (Showalter 1926; Cronberg *et al.* in press) or on sperm dispersal distances (Shaw 2000; Van der Velde *et al.* 2001), making it difficult to compare among bryophyte taxa.

Here, we examine sperm longevity and the effect of temperature on sperm lifespan in the moss *Pohlia nutans* growing in geothermal soils at Lassen Volcanic National Park, CA, USA. *Pohlia nutans* grows across a range of temperatures at Lassen from 11 to 52°C, and thus we expect thermal stress to influence reproductive success in this system. Further, studies in marine invertebrates suggest that sperm dilution (fewer sperm per volume of water) reduces sperm lifespan (for review see Chia & Bickell 1983; Levitan *et al.* 1991), and we hypothesize that similar dilution effects occur in bryophytes. Using controlled laboratory experiments, we examine the impacts of high temperature stress and dilution on *P. nutans* sperm lifespan.

2. MATERIAL AND METHODS

(a) Study system

Pohlia nutans (Bryaceae) is a nearly cosmopolitan species, with combined sexes (Grout 1933). Plants were collected from seven populations at Lassen Volcanic National Park, CA, USA, growing at a range of temperatures (20.1–45.8°C pre-dawn and 26.8–54.6°C mid-day rhizoid temperatures). Individuals from the seven populations were established in a greenhouse at Portland State University and produced antheridia (October 2007 to May 2008).

(b) Sperm collection

For each replicate in a series of experiments, ripe antheridia (10.5 ± 1.3 s.e.) were dissected from three individuals (one from each of three populations), placed on a glass slide in 40 µl of water, either rainwater or deionized water (DI water) and gently agitated. Rainwater was collected locally, sterile filtered and frozen until needed. Tetracycline ($15 \mu\text{g ml}^{-1}$) was added before use; this concentration had no significant effect on sperm lifespan, but significantly reduced bacterial growth. After 30 min, the swim solution was transferred to a centrifuge tube using a Pipetman and briefly centrifuged (10g) to remove antheridial material. The sperm containing supernatant was recovered using a Pipetman and stored in a closed 0.5 ml centrifuge tube wrapped in aluminium foil to limit evaporation; storage conditions varied with experiment (discussed subsequently).

(c) Lifespan estimations

To estimate sperm lifespan, motile and non-motile sperm were counted at various times using a phase-contrast microscope (400× magnification; 2 µl of solution per slide with a coverslip). We took the average of two counts of 100 sperm per treatment. Live–dead cell stains (L-7011, Molecular Probes, Eugene, OR, USA) were used to confirm the accuracy of the motility protocol (data not shown).

(d) Experiments: variation in water source and sperm dilution

We varied water source and volume added to collected sperm and monitored motility at 24, 48, 96, 192, 384 and 768 h. Sperm were released into either 40 µl DI or rainwater (referred to as 1×), and a subset of rainwater samples were subsequently diluted with rainwater to 10 times (10×) and 100 times (100×) the original volume. We repeated the experiments eight times, with four replicates of each water source and sperm dilution per experiment. We included 'start date' in our statistical models as sperm lifespan varied seasonally (figure S1, electronic supplementary material). Samples were maintained at ambient room temperatures (22–25°C) or 29°C, but temperature (22–29°C; $p = 0.8783$) and interactions with temperature were not significant and not included in our analysis of water treatment.

(e) Experiments: sugar additions

To examine the impacts of carbohydrates on sperm lifespan, sperm were suspended in rainwater solutions containing either 0.2 or

Electronic supplementary material is available at <http://dx.doi.org/10.1098/rsbl.2009.0380> or via <http://rsbl.royalsocietypublishing.org>.

Received 15 May 2009

Accepted 9 July 2009

0.4 M sucrose or sorbitol, and motility was assayed at 24, 48 and 96 h. These values were chosen to reflect 1–2× concentrations of sugars (90–250 mM) measured in sperm-free (14 k × G) antheridial exudates using a colorimetric assay (Chow & Landhäuser 2004).

(f) Experiments: temperature variation

To compare the effects of temperature on sperm lifespan, we varied temperature over a broad range. Populations with mid-day temperature ranging from 26.8 to 31.8°C were used for these experiments: experiment 1 (four replicates of 1× and 10× rainwater treatments at 22 and 60°C), experiment 2 (four replicates of 1× and 100× treatments at 22 and 60°C) and experiment 3 (four replicates of 1× and 100× treatments at 22 and 75°C).

(g) Data analyses

Because tubes were repeatedly assayed, we used repeated-measures ANOVAs to analyse motility data. We used such an analysis to determine whether water treatment, start date, time and interactions affected motility (SAS Institute 2007). *Post hoc* analyses were applied to test for significance among treatments. We used regression, with time as a continuous variable, to determine half lives for each treatment. We log-transformed time, as sperm lifespan is expected to decline exponentially (Johnson & Yund 2004). Repeated-measures ANOVAs were used to determine the effects of sugar concentrations, start date, time and interactions on sperm motility, as well as to determine the effects of temperature, dilution, time and interactions on sperm motility. We analysed the results for experiments comparing 22 and 60°C separately from those comparing 22 and 75°C.

3. RESULTS

In *P. nutans*, sperm lifespan was significantly affected by water treatment (figure 1a; $n = 71$; $F_{3,62} = 64.07$; $p < 0.0001$), time ($F_{5,58} = 338.11$; $p < 0.0001$) and the interaction between time and water treatment ($F_{5,60} = 64.07$; $p < 0.0001$; table S1, electronic supplementary material). Mean motility was significantly higher in 1× rainwater than in DI, and mean motility significantly differed between dilutions. Lifespan for sperm in dilute water treatments was less, on average, than that in more concentrated water treatments. Sperm half lives were estimated from logarithmic regression, and the resulting half lives were: 70.8 h in DI, 108.6 h in 1×, 49.0 h in 10× and 7.0 h in 100×.

Additions of sugars significantly affected sperm lifespan. Dilution and start date were significant effects in our analysis of experiments with added sugars (figure 1b; $n = 63$; $F_{4,55} = 8.85$; $p < 0.0001$ and $F_{3,55} = 3.35$; $p = 0.0255$, respectively). Contrast analysis showed no significant differences between sugar treatments, but a significant difference was found between the addition of sugar (either type) to rainwater and pure rainwater ($p < 0.001$). There was no significant effect of time, but time by start date was significant in this analysis ($F_{2,54} = 2.07$; $p = 0.1358$ and $F_{3,55} = 8.65$; $p < 0.0001$, respectively).

Temperature significantly affected motility of sperm; however, only at temperatures over 60°C (figure 2; $n = 15$; $F_{1,9} = 0.09$; $p < 0.7762$ for comparisons between 22 and 60°C; $n = 6$; $F_{1,9} = 140.83$; $p < 0.0070$ for comparisons between 22 and 75°C). In temperature experiments, dilution, time, the interaction between dilution and time and start date were all significant (see table S2, electronic supplementary material). The interaction between temperature and time was not significant.

Figure 1. The per cent of motile *P. nutans* sperm in (a) DI water and three dilution treatments over 768 h. Open circles, 1× DI H₂O; filled squares, 1× rain; filled triangles, 10× rain; inverted triangles, 100× rain. (b) Rainwater with varying sucrose concentrations over 96 h. Sorbitol additions had the same effect as that of sucrose. Filled squares, 1× rain; filled asterisks, 0.2 M sucrose; filled diamonds, 0.4 M sucrose.

4. DISCUSSION

Our data suggest that *P. nutans* sperm are remarkably long-lived, even when highly diluted by rainwater (half lives of approx. 7–109 h depending on dilution and 20% survival after 100 h for all dilutions). Previous reports of lifespans for bryophyte sperm, primarily anecdotal, range from 1 to 144 h (for review see Showalter 1926; Cronberg *et al.* in press). Although few detailed paternity studies have been carried out to determine sperm dispersal distances (e.g. Van der Velde *et al.* 2001), our data support the idea that bryophyte sperm may be able to disperse relatively long distances (perhaps metres rather than centimetres, even in species without specialized sperm dispersal structures), as longer lifespans allow time for sperm to be carried via water between plants and for sperm to swim to fertile archegonia once they reach a potential mate. Longer lifespans may also allow transport via animal vectors, when water is limited (Cronberg *et al.* 2006). However, further experiments are needed to determine whether long-lived sperm are functional (able to fertilize ovules) over this time.

Sperm lifespan may vary with dilution of sperm for two reasons: (i) high concentrations of sperm may

Figure 2. The mean per cent of motile *P. nutans* sperm over 96 h. Comparisons were made between (a) 22 and 60°C and (b) 22 and 75°C. Unfilled bar, 1×; filled bar, 100×.

cause sperm to remain quiescent and thus conserve energy or (ii) high concentrations of antheridia serum in rainwater may allow sperm to metabolize or reduce osmotic stress. In free-spawning benthic marine invertebrates (e.g. echinoderms and colonial ascidians), the 'respiratory dilution effect' has been observed and suggests that when sperm are in high concentrations, they are forced to remain still, conserving energy, and living longer than those in more dilute suspensions (Chia & Bickell 1983). Concentrations of moss sperm in natural systems during dispersal remain unknown. Animal systems also suggest that sperm can live longer in suspensions with added nutrients than without (Fishel *et al.* 1985). For bryophyte sperm, there is the potential that such energy gains could occur during sperm dispersal. In systems with regular wetting/drying cycles, sugar release from bryophytes (up to 122 kg ha⁻¹ in one study; Coxson *et al.* 1992) may be high enough to provide energy gains for dispersing sperm. Furthermore, archegonia release sugars into the bryophyte canopy when fertile (Ziegler *et al.* 1988), potentially providing a beacon for sperm and/or additional energetic resources, consistent with our observations of increased sperm lifetime with exogenous carbohydrates.

Increased temperature should both increase cellular energy use and decrease the viscosity of swim media, potentially having a large effect on the

energetics of small organisms such as sperm. However, surprisingly, we found that *P. nutans* sperm were unaffected by temperature variation between 22 and 60°C. We are unable to find evidence of such thermal stability in sperm from other systems. It is unclear whether this dramatic thermal tolerance is unique to populations of *P. nutans* in geothermal areas, or whether bryophyte sperm are generally thermotolerant. The ability of bryophyte sperm to tolerate extreme stress is an unexpected discovery and has implications for the population dynamics of bryophytes under stressful environments and potentially the first colonization of land by plants.

We are grateful to C. Graves, E. Llana Garcia, C. Haaning, K. Halpin and especially Lee Hyejin for assistance. We are thankful to J. Christy and J. Shaw for help with bryophyte identification. Research was supported by 3M Corporation.

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