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METHODS

Sperm viability staining in ecology and evolution: potential pitfalls

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Abstract The causes and consequences of variation in sperm quality, survival and ageing are active areas of research in ecology and evolution. In order to address these topics, many recent studies have measured sperm viability using fluorescent staining. Although sperm viability staining has produced a number of interesting results, it has some potential pitfalls that have rarely been discussed. In the present paper, I review the major findings of ecology and evolution studies employing sperm viability staining and outline the method's principle limitations. The key problem is that the viability assay may itself kill sperm, which is likely to confound many common experimental designs in addition to producing artificially low estimates of sperm viability. I further suggest that sperm number should be routinely measured in sperm viability studies, as it may be an important but overlooked source of spurious results. I provide methodological advice on sperm viability staining aimed at minimising artefacts and producing robust conclusions, and discuss possible avenues for future research.

Keywords Sperm competition · Sperm aging · Sperm survival · Live/dead kit · Spermicide · Sexual conflict

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Introduction

Post-copulatory sexual selection, which comprises sperm competition (Birkhead and Møller 1998) and cryptic female choice (Eberhard 1996), occurs throughout the animal kingdom and has produced a wealth of adaptations in both males and females. These adaptations are particularly apparent in the evolution of the ejaculate: Empirical and theoretical studies have shown that post-copulatory sexual selection influences both the number (Parker 1982; Gage and Morrow 2003) and quality of sperm (Miller and Pitnick 2002; Snook 2005) and affects the composition and quantity of seminal fluid (Fiumera et al. 2005; Linklater et al. 2007; Crudgington et al. 2009). Recently, there has been increasing interest in the evolutionary causes and consequences of sperm survival and ageing, phenomena that may affect sexual selection, impact male and female fitness and select for novel adaptations in both sexes (e.g. Greeff and Parker 2000; Siva-Jothy 2000; Jones and Elgar 2004; Reinhardt and Siva-Jothy 2005; Holman and Snook 2006; Reinhardt 2007; Pizzari et al. 2008; White et al. 2008). Accordingly, there has been a rapid increase in the number of studies motivated by evolutionary questions that have measured sperm viability (Table 1). Sperm viability is defined as the number of live sperm divided by the total number (expressed as either a proportion or percentage) and therefore provides an additional way to assess ejaculate quality, functionality and longevity. Here, I define sperm survival as the change in the proportion of live sperm over time.

Sperm viability is measured using fluorescent staining. This method has been used for >50 years in the study of human and domestic animal reproduction, fertility and artificial insemination (e.g. Swanson and Bearden 1951), but has only recently been applied to ecology and evolution

Table 1 List of ecology and evolution studies using sperm viability staining

Year	Study species	Reference	Main conclusion
1995	*Pave cristatus*	Birkhead and Petrie (1995)	SV does not differ amongst males
1999	*Homo sapiens*	Moore et al. (1999)	SV is not reduced by rival ejaculates
2001	*Apis mellifera*	Collins and Pettis (2001)	SV is not lowered by parasitism
2002	*Scathophaga stercoraria*	Bernasconi et al. (2002)	SV is lower in females than males
	Two chalcidoid wasps	Damiens et al. (2002)	SV differs between sexes and species
	14 insect species	Hunter and Birkhead (2002)	SV is higher in promiscuous species
2004	*Tribolium castaneum*	Fedina and Lewis (2004)	Treating females with CO_2 does not affect survival of stored sperm
	Cyrtodiopsis whitei	Fry and Wilkinson (2004)	SV of meiotic drive males is reduced by rival seminal fluid
	Apis mellifera	Lodesani et al. (2004)	SV decreases with increasing time in storage
	Nauphoeta cinerea	Montrose et al. (2004)	Multiply-mated males transfer lower viability sperm
	Nauphoeta cinerea	Moore et al. (2004)	SV has low heritability; SV is negatively genetically correlated with testis mass
	Drosophila melanogaster	Snook and Hosken (2004)	SV is not reduced by rival seminal fluid
2005	Two *Photinus* sp.	Demary (2005)	SV is lower in females than males
	Teleogryllus oceanicus	Garcia-Gonzalez and Simmons (2005a)	SV does not predict hatching success
	Teleogryllus oceanicus	Garcia-Gonzalez and Simmons (2005b)	SV is correlated with paternity success
	Calopterygid damselflies	Hayashi and Tsuchiya (2005)	Variation in SV between female storage organs
	Cervus elaphus hispanicus	Malo et al. (2005)	SV is not correlated with male fertility; it is uniformly high
	Teleogryllus oceanicus	Simmons and Roberts (2005)	SV is genetically correlated with immune measures
	Ceratitis capitata	Twig and Yuval (2005)	Inferences about the function of multiple sperm stores
2006	*Poecilia reticulata*	Locatello et al. (2006)	SV is correlated with male colouration
2007	*Callosobruchus maculatus*	Dowling et al. (2007)	SV is affected by male cytoplasmic (not nuclear) genotype
	Two gobiid fish	Locatello et al. (2007)	'Sneaker' males have better sperm survival
	Ischnura senegalensis	Nakahara and Tsubaki (2007)	No difference in SV between female storage organs
	Teleogryllus oceanicus	Simmons et al. (2007)	Males adjust SV depending on female mating history and presence of rivals
	Teleogryllus oceanicus	Thomas and Simmons (2007)	Males adjust SV depending on female mating history
2008	*Drosophila simulans*	Angelard et al. (2008)	Meiotic drive males have lower SV
	Dinarmus basalis	Bressac et al. (2008)	Sperm can be kept viable by males for >30 days
	Drosophila melanogaster	Civetta et al. (2008)	Sperm survival varies between male strains; not affected by female strain
	Atta colombica	den Boer et al. (2008)	Seminal fluid preserves SV
	Bombus terrestris	Greeff and Schmid-Hempel (2008)	SV lower in females than males
	Dermestes maculatus	Hale et al. (2008)	SV is not correlated with male age or mass
	Drosophila pseudoobscura	Holman and Snook (2008)	SV of eusperm is correlated with proportion of parasperm
	Gambusia holbrooki	Locatello et al. (2008)	SV is not correlated with body size
	Ischnura senegalensis	Nakahara and Tsubaki (2008)	No effect of inbreeding on SV
	Litoria peronii	Sherman et al. (2008a)	Large inter-male variation in sperm survival
	Litoria peronii	Sherman et al. (2008b)	Sperm viability does not affect siring success
	Teleogryllus oceanicus	Simmons and Thomas (2008)	Males do not adjust SV when mating with relatives
2009	*Apis mellifera*	den Boer et al. (2009)	Seminal and spermathecal proteins preserve SV
	Drosophila melanogaster	Holman (2009)	Sperm survival is increased by rival seminal fluid
	Litoria peronii	Sherman et al. (2009)	Sperm viability does not affect siring success
	Teleogryllus oceanicus	Thomas and Simmons (2009)	Males use chemical cues to assess female mating status and adjust SV

This list includes only empirical studies explicitly motivated by evolutionary questions and not those focusing on applied topics such as sperm cryostorage and artificial insemination (although many of these have interdisciplinary interest). The main conclusion column lists the primary conclusion from the part of the paper using sperm viability staining

SV sperm viability

(Table 1). Typically, two stains that differentially colour live and dead sperm are used, the most common choices being SYBR-14 (which stains live cells green) and propidium iodide (which stains dead cells red). A dead cell stain followed by a count of the total number of sperm may also be used. Live and dead sperm are discriminated by the integrity of their cell membranes, which become more permeable after death. The method is relatively quick to learn and uses widely available equipment.

Although sperm viability staining has potential to provide new advances in ecology and evolution, it can sometimes produce experimental artefacts that are not immediately apparent, and sperm viability data have frequently been misinterpreted. The aims of the present paper are (a) to provide a critique of sperm viability staining in the field of ecology and evolution and clarify what it can and cannot do for us and (b) to offer practical advice for researchers using sperm viability staining. I begin by reviewing recent findings from studies using this technique.

What has sperm viability staining told us about ecology and evolution?

Sperm viability staining has facilitated a number of interesting results. In a sample of seven pairs of insect species, Hunter and Birkhead (2002) showed that the viability of sperm in males' seminal vesicles is higher in promiscuous species than in their monogamous relatives, suggesting that post-copulatory sexual selection has selected for higher sperm viability. Consistent with this idea, natural variation in sperm viability correlates with success in sperm competition in the cricket *Teleogryllus oceanicus* (Garcia-Gonzalez and Simmons 2005b). Subsequent work in this species indicated that the viability of sperm transferred by a male is affected by both contact with other males and the female's mating history (Simmons et al. 2007; Thomas and Simmons 2007, 2009), which also suggests that viability plays a role in sperm competition.

Investment in adaptations that affect sperm survival is predicted to vary between taxonomic groups with different mating systems and mechanisms of fertilisation (Johnson and Yund 2004; Reinhardt 2007). These traits are likely to affect the costs and benefits of investing in sperm survival, and viability staining has contributed to testing this prediction. In the polyandrous insects *Drosophila melanogaster* (Holman 2009), *Apis mellifera* honey bees (den Boer et al. 2009) and *Atta colombica* leafcutter ants (den Boer et al. 2008), seminal fluid was found to positively influence sperm survival, suggesting that its composition has evolved to help keep sperm alive following insemination as in mammals (e.g. Ashworth et al. 1994; Garner et al. 2001; Hernandez et al. 2007). Female processes have also been found to increase sperm survival. Queen *A. mellifera*, which must keep stored sperm viable for several years, produce a spermathecal fluid that improves sperm survival in vitro. The proteins responsible for this effect probably differ from those found in seminal fluid, suggesting that males and females may use different strategies to improve sperm survival (den Boer et al. 2009). Future studies may reveal additional male and female adaptations that increase sperm survival, test how these co-vary with mating system and other ecological parameters and measure selection on traits affecting sperm survival.

Just as males may evolve adaptations to protect their own sperm, they may sometimes be selected to harm the sperm of their competitors. In *Drosophila* sp., initial results indicated that males might produce seminal fluid that selectively kills sperm from other males that previously mated with the same female (reviewed in Holman 2009), but this was not supported by later experiments using viability staining (Snook and Hosken 2004; Angelard et al. 2008; Holman 2009). The "kamikaze sperm" hypothesis (Baker and Bellis 1988) was similarly not supported by sperm viability staining experiments in humans (Moore et al. 1999). However, viability staining suggested that sperm are indeed killed by seminal fluid in the stalk-eyed fly *Cyrtodiopsis whitei*, but only if the sperm belong to a meiotic drive male (Fry and Wilkinson 2004). The reduced viability of meiotic drive-carrying sperm could potentially oppose the spread of drive alleles through populations (Fry and Wilkinson 2004; Angelard et al. 2008). Females are also predicted to sometimes harm sperm; such harm might either be adaptive, e.g. if the sperm come from a disfavoured male (Eberhard 1996; Pizzari and Birkhead 2000), or a non-adaptive by-product of other processes such as immunity (Birkhead et al. 1993). Viability staining studies have suggested that the reproductive tract of female *Drosophila pseudoobscura* is damaging to sperm (Holman and Snook 2008), although no evidence of this was found in the dung fly *Scathophaga stercoraria* (Bernasconi et al. 2002) or *D. melanogaster* (Civetta et al. 2008; Holman 2009).

The viability of ejaculated sperm was shown to have a heritable component in *T. oceanicus* (Simmons and Roberts 2005), suggesting that some of the factors affecting it are genetically variable and could respond to selection, although heritability did not differ from zero in the cockroach *Nauphoeta cinerea* (Moore et al. 2004). In *D. melanogaster*, there is an effect of male genotype on sperm viability sampled after 4 days of storage in the female, implying a genetic basis of viability at insemination and/or survival (Civetta et al. 2008). Sperm viability has also been found to have genetic (Simmons and Roberts 2005) and phenotypic (Moore et al. 2004; Locatello et al. 2006, 2007) correlations

with a range of traits. For example, sperm viability and testis size are negatively correlated in *N. cinerea*, implying that selection for sperm quality may represent a constraint in the evolution of testis size (Moore et al. 2004). By contrast, sperm viability is positively related to male carotenoid colouration in *Poecilia reticulata* guppies, suggesting that colour honestly signals sperm quality and there is no detectable trade-off between these primary and secondary sexual traits (Locatello et al. 2006). Lastly, an introgression study using the weevil *Callosobruchus maculatus* found that sperm viability was affected by mitochondrial genotype but not nuclear genotype, supporting the hypothesis that the maternal inheritance of mitochondria constrains selection on sperm function (Dowling et al. 2007).

In summary, sperm viability staining has identified male and female traits that influence the survival of sperm, suggested that the proportion of live sperm in the ejaculate has fitness consequences and is heritable and hinted at the wider ecological and evolutionary consequences of variation in sperm survival. Although sperm viability staining has produced informative and interesting results, it has several limitations which have rarely been made explicit. I now discuss these with the aims of assisting in the planning of future experiments and highlighting some common pitfalls in the interpretation of sperm viability data.

Limitations of sperm viability staining

The "true" sperm viability value cannot be known

The principal drawback of sperm viability staining is that the protocols used to extract, dilute, stain and mount sperm for counting under the microscope may kill some of them. To my knowledge, there is no direct method for quantifying the membrane integrity of a sample of sperm, the criterion most commonly used to score viability, other than by fluorescent staining; this means that one cannot directly measure the mortality caused by the assay itself. Sperm viability counts are therefore best viewed as potential underestimates, which may in some cases be far below the true value. This issue was raised by a study which examined progeny production to indirectly estimate the number of fertilisation-competent sperm in mated female *D. melanogaster* (Stewart et al. 2007). The results of Stewart et al. implied a higher proportion of live sperm than the viability staining study they had set out to test (Snook and Hosken 2004), leading them to conclude that some sperm had been killed during the viability assay (numerous differences between the studies make the amount of death unclear). Few authors have acknowledged that the viability assay itself could be the main source of dead sperm; for example, Damiens et al. (2002) remarked that the seminal vesicles of two wasp species contained a lower proportion of live sperm than expected (20–30%). The authors suggested that these values must be genuine because the dissections were performed carefully, but I argue that it is not possible to ascertain how close a sperm viability count is to the "true" value without additional data.

Additionally, sperm which are impermeable to the dead cell stain are not necessarily capable of fertilisation, or free from non-lethal deleterious effects. The viability staining protocol only measures membrane integrity and therefore should not be expected to provide a full description of senescence and fertilisation competence. Studies seeking a holistic estimate of ejaculate function may need to quantify additional parameters, such as sperm motility, ATP content (Locatello et al. 2007) and acrosome integrity (Moore et al. 1999; Malo et al. 2005), or use functional assays, e.g. quantifying fertilisation ability and zygote fitness of sperm from different treatments (White et al. 2008).

Despite imposing the limitation that sperm viability values must be interpreted with caution, sperm death caused by the assay is not necessarily a problem for most common empirical studies and questions. For example, in experiments comparing sperm viability between controls and treatment groups, the difference in sperm viability is of more interest than the absolute values. Additionally, sperm viability can be repeatable within males (Garcia-Gonzalez and Simmons 2005b), implying that sperm mortality during the assay can be minimal (or at least consistent within males). However, more serious problems arise when the viability assay kills unequal amounts of sperm in different groups under study.

Viability counts of sperm from different sources are usually not comparable

Sperm mortality during the assay may confound estimates of sperm viability obtained from different sources, such as different internal organs, spermatophores or the external environment. For example, seven studies have compared the viability of sperm obtained from males and females, or from different organs in the female (Table 1). The problem with this approach is that the amount of sperm killed during the viability assay may differ depending on where sperm are removed from, e.g. because removing sperm from one site may be more time-consuming or damaging than another, or because sperm are more numerous in some organs than others (see next section).

To illustrate, three studies (Bernasconi et al. 2002; Demary 2005; Greeff and Schmid-Hempel 2008) found that sperm viability values were lower when sperm were removed from females' spermathecae relative to male seminal vesicles in various insect species and concluded that the difference was due to mortality of sperm after

mating (caused by either intrinsic or female-mediated factors). However, these studies cannot rule out the possibility that some or all of the reduced viability can be explained by additional damage incurred during dissection of the female compared to the male; there is no direct way to measure how many sperm were killed during the two different assays.

Similarly, two studies measured sperm viability in the cockroach *N. cinerea*, using sperm from the seminal vesicles (Hunter and Birkhead 2002) and the spermatophore (Montrose et al. 2004), respectively. Mean sperm viability was recorded as 50% in the seminal vesicles but only 2% in the spermatophore, which is a gelatinous mass that needs to be "ground" to extract sperm (Montrose et al. 2004); both of these figures are likely to be large underestimates, and removal of sperm from the spermatophore is probably more damaging. Also, sperm obtained from male *Dermestes maculatus* hide beetles were predominantly alive, whilst all sperm in females' spermathecae stained as though they were dead; the authors noted that this was likely an artefact because the females were fertile (Hale et al. 2008). These results suggest that some dissection protocols kill more sperm than others and that it is not informative to compare viability values amongst them. Comparisons of sperm viability between different species are likely to suffer from similar problems; it will be difficult to convincingly demonstrate that the sperm viability assay caused similar mortality for all species in the dataset, which may limit the usefulness of sperm viability staining in inter-specific comparative studies.

Non-independence of sperm viability and sperm number

Although the relationship between sperm survival and the number of sperm a male produces or ejaculates has rarely been tested experimentally (except in external fertilisers; Manríquez et al. 2001; Johnson and Yund 2004), there are several biological reasons to expect these traits to be correlated. A positive relationship between the number and survival of a male's sperm could be caused by a shared dependence of both traits on common environmental and genetic factors; for example, males in good condition might have both large testes (more sperm) and large accessory glands (more protective seminal fluid). Alternatively, males have been predicted to produce large numbers of sperm to minimise various kinds of intrinsic and extrinsic mortality, such as the metabolic activity of the sperm (Reinhardt 2007) or a hostile female reproductive tract or external medium (Greeff and Parker 2000; Manríquez et al. 2001; Holman and Snook 2006, 2008). Conversely, the predicted evolutionary trade-off between sperm quality and quantity (e.g. Parker 1993) could result in a negative relationship between sperm number and viability within or between species.

Hypotheses such as these suggest that we may expect to find interdependence between sperm number and viability caused by biologically relevant factors. However, I propose that sperm number and viability are also likely to be correlated as a consequence of mortality during the viability assay. If the number of sperm killed by the viability assay (e.g. by damage during dissection, dilution or exposure to artificial media) is non-linearly related to the number of sperm being tested, then it is mathematically inevitable that number and viability will be correlated, all else being equal (Fig. 1). This potentially serious artefact has never been made explicit and is the subject of the present section.

The strength of the sperm number–viability relationship produced by experimental artefacts alone depends on the shape of the relationship between the number of sperm being sampled and the number killed by the assay (Fig. 1). The shape of this relationship is largely speculative, because sperm viability studies have not presented data that could be used to measure it; almost all have either measured sperm viability only or have measured both sperm number and viability but not statistically tested their interdependence. An exception is Sherman et al. (2008a), in which a positive correlation between sperm number and viability was found (consistent with Fig. 1b). Although data are lacking, several lines of evidence suggest that the relationship between sperm number and mortality during the assay is usually non-linear and could therefore confound estimates of sperm viability (Fig. 1b). In the field of mammalian sperm storage and artificial insemination, it is well-established that sperm survive better in vitro when they are more concentrated (e.g. Ashworth et al. 1994; Garner et al. 1997, 2001). Similarly, the sperm of external fertilisers survive better in seawater at higher concentrations (Manríquez et al. 2001; Johnson and Yund 2004). The concentration of seminal fluid in the buffer has also been shown to interact with sperm number and affect the survival of bull sperm (Garner et al. 2001); this represents another potentially widespread confounding effect because seminal fluid has been shown to improve sperm survival in vitro in several mammals and insects (e.g. Ashworth et al. 1994; Hernandez et al. 2007; den Boer et al. 2008, 2009; Holman 2009). Also, studies of beetles (Dowling et al. 2007) and fish (Locatello et al. 2008) have found that sperm survive better in vitro when aggregated in bundles, perhaps because the individualised sperm were exposed to buffer over a greater part of their surface. These results all suggest that the ratio of sperm and/or seminal fluid to buffer could produce density-dependent mortality during the viability assay as shown in Fig. 1b.

Another way in which the number of sperm could affect mortality during the viability count is via the dissection protocol. In a study of *Cimex lectularius* bedbugs, I found that the number of sperm in the female sperm storage

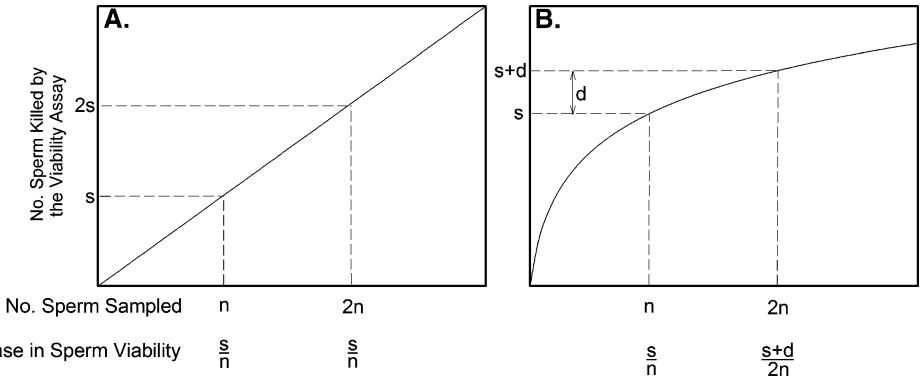

Fig. 1 The relationship between sperm number and viability depends on the shape of the correlation between the number of sperm being sampled and the number that are killed during the viability assay (e.g. by damage or exposure to artificial media). **a** If the relationship increases linearly over the range of sperm numbers being sampled in the study, then the decrease in sperm viability during the viability assay is independent of sperm number. For example, if $n=100$ and $s=10$, the proportion of live sperm is always 0.1 lower than the true value. Sperm number therefore does not confound estimates of sperm viability. The slope of the line could be any positive number. **b** If the relationship is non-linear over the range of sperm numbers being sampled, then sperm viability depends on the number of sperm. The difference d is never equal to s, so the viability estimate is inevitably correlated with the number of sperm under test through experimental artefacts alone. Note that any function other than a positive linear one also produces a spurious correlation between sperm viability and sperm number

organs was strongly and non-linearly correlated with the viability measurement (L. Holman, unpublished data; Fig. 2). During dissections, it was more difficult to remove all the sperm from the storage organs when only a small number were present. Small numbers of sperm needed to be scraped out of the organ, whilst larger volumes flowed out with minimal manipulation. Therefore, some or all of the number–viability correlation may be an artefact of the way in which sperm were collected (compare Figs. 1b and 2), rather than an effect of biologically interesting processes.

Previous studies may have missed information by not considering the potential confounding effect of the sperm number artefact described here. For example, sperm viability has been linked to immunocompetence (Simmons and Roberts 2005), a secondary sexual trait (Locatello et al. 2006) and mitochondrial genotype (Dowling et al. 2007). However, it is possible that only sperm number was correlated with those traits, and the correlations with viability arose because the number of sperm dying from the viability assay depends on sperm number. The same is true of Hunter and Birkhead's (2002) result that sperm viability is higher in promiscuous species; such species often produce more sperm (Birkhead and Møller 1998), which could produce a spurious correlation between sperm viability and mating system. Studies testing whether males adjust the viability of inseminated sperm following experimental manipulation (in the style of Thomas and Simmons 2007, 2009) should consider that males might also alter the number of sperm inseminated, which could confound the viability count if not controlled for. Similarly, when examining how sperm viability changes with time, e.g. as sperm are stored in females (Lodesani et al. 2004; Greeff and Schmid-Hempel 2008), as males mature (Garcia-Gonzalez and Simmons 2005b), or over successive matings (Montrose et al. 2004), it is important to test and control for concurrent changes in sperm number.

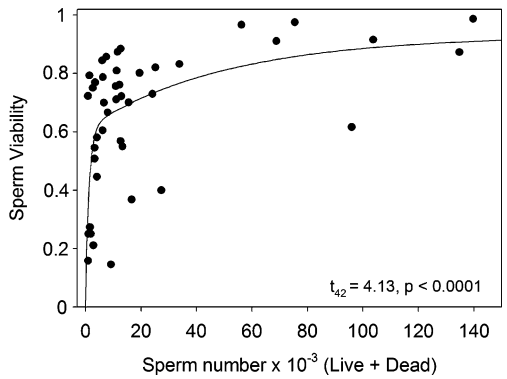

Fig. 2 The number of sperm stored in the seminal conceptacles (storage organs) of female *C. lectularius* bedbugs was strongly correlated with their viability. There is a high probability that some or all of this relationship was an artefact of the dissection protocol, because sperm were much easier to extract when they were plentiful. A parsimonious interpretation of this dataset is that sperm viability was both higher and less variable before dissection (e.g. 0.9–1), and the remaining variation and the correlation with sperm number were caused by artefacts associated with sperm number as shown in Fig. 1b. See Supplementary material for methods

There are at least two ways to control for the potential confounding effect of sperm number. The first is to count both sperm number and viability for each sample (this does not necessarily require additional effort; see below) and then statistically account for the number–viability relationship if it exists, e.g. by entering sperm number as an independent variable in the model. This approach cannot determine how much of the sperm number–viability relationship is caused by the assay rather than by biologically relevant factors, but it

does ensure the robustness of the study's other conclusions (e.g. the effect of treatment on sperm viability). The second method is to attempt to standardise the number of sperm in each treatment. This approach is especially tractable for experimental designs examining sperm survival in vitro after experimental manipulation (e.g. Bernasconi et al. 2002; den Boer et al. 2008; Holman and Snook 2008; den Boer et al. 2009; Holman 2009). The number of sperm (and perhaps seminal fluid) in each experimental treatment can be standardised by starting with a single sample, e.g. the ejaculate of one male, and then dividing it into equal parts, each of which is then subjected to the experimental treatment (e.g. Bernasconi et al. 2002; Holman and Snook 2008). For in vivo studies, it may be possible to minimise sperm number variation by controlling the length of copulation and standardising male age (e.g. Garcia-Gonzalez and Simmons 2005b).

Practical advice on sperm viability staining

Generally, sperm from a range of species can be stained clearly by starting with the concentrations recommended by the manufacturer of the stain and adjusting them as required. The optimal buffer medium for preserving sperm viability probably varies across taxonomic groups such that general advice may not be helpful; for insect sperm, Grace's Insect Medium (Grace 1962) provides good results relative to phosphate buffered saline or Beadle saline (personal observation). For mammals, there are several commercially available buffers designed to maximise in vitro sperm survival. However, as mentioned above, it is not always essential to maintain high viability, e.g. when comparing sperm viability between experimental groups and appropriate controls.

Core tenets of experimental design such as blocking, randomisation, blind data recording and repeatability measurements should always be applied to sperm viability studies, but have often been neglected. For blocking, researchers should design rotations to ensure that measurements of sperm viability in different groups (e.g. treatments) are not separated in time, as the assay is sensitive to extraneous factors. Examples of blocked experimental designs in sperm viability studies are provided by Thomas and Simmons (2007) and Holman and Snook (2008); this approach prevents many potentially confounding factors associated with time, such as the dissection skill of the researcher (Holman 2009), from affecting the results. Regarding randomisation, it is important that the sperm that are counted to estimate viability are selected without bias. One way of doing this is through the use of haemocytometers or other gridlines to count sperm in randomly chosen squares; this may be important because the spatial distribution of live and dead sperm can differ throughout the sample (Dowling et al. 2007; personal observation). Sperm counts should always be performed blind with respect to the source of the sperm; only nine of the 32 papers in Table 1 which would have benefitted from blind counts stated that they were blind. Blind sperm viability studies will often necessitate two researchers: one to collect and prepare the sperm and one to do the viability count. If there is only one primary researcher, he or she may photograph sperm for later counting after the photographs have been re-labelled with a code by a second person; photographs also allow rapid recording of sperm before they begin to die. Lastly, researchers should attempt to verify that their measurement of sperm viability is repeatable wherever possible, e.g. by repeated counts of the same samples, in order to verify that measurement error is within acceptable limits. Measurement error can be reduced by counting a greater number of sperm in each viability count and by working to standardise dissection protocols (incubation time can have particularly strong effects; Holman and Snook 2006).

Regarding data analysis, sperm viability is a proportion, meaning that sperm viability data will not meet the assumptions of many standard statistical tests. Proportion data should generally be either transformed prior to analysis or analysed with a statistical test that accounts for their non-normal errors. The standard transformation applied to proportion data is the arcsine transformation ($\sin^{-1}(\sqrt{x})$), but a superior alternative is to use generalised linear models with binomial errors and the logit link function. These models explicitly assume that the response variable is strictly bounded and allow input of the number of observations used to calculate the proportion, i.e. the number of sperm counted to estimate viability (see Crawley 1993; Bolker et al. 2009).

For reasons described above, sperm number and viability should generally be measured together for each sample in the dataset. An efficient method of doing this is to use a disposable haemocytometer. The experimenter can count the number of live and dead sperm in a standard number of haemocytometer squares (typically after diluting the original sample of sperm by a known amount) and thereby calculate both total number and viability from a single sample. Another potential advantage of haemocytometers is that the sperm are counted in a shallow chamber, rather than pressed between two pieces of glass as with a microscope slide, which appears to reduce and standardise the damage received when sperm are mounted (personal observation). Flow cytometry may also be used to count sperm number and viability simultaneously and has been widely used for this purpose in studies of fertility in mammals (e.g. Garner et al. 1997; Grundler et al. 2004). Flow cytometry has several potential advantages over manual counting: Many more sperm may be counted per

sample and blind counting and randomisation are easier to implement.

Perspectives

Sperm viability staining has multiple applications in ecology and evolution as a measure of ejaculate quality and sperm survival. However, there is room for improvement both in how sperm viability studies are conducted and how their results are interpreted. Mortality of sperm during the assay is likely to be common, resulting in artificially low viability values across the board. Of greater concern is that this mortality can produce artefacts if it differs between different groups under study (e.g. species, organs or treatment groups). A related problem is that the number of sperm under test represents a previously unacknowledged confounding factor in estimates of sperm viability. Some experimental artefacts can be eliminated using methods described here, but others cannot be avoided and should be kept in mind when designing studies and forming conclusions. There is also a need for greater experimental rigor in this field, particularly in the use of blind sperm counts.

Inter-specific comparative studies are an initially attractive way of studying the evolution of sperm viability and survival, but interpretation of such studies may be complicated by the possibility of correlations between the number of sperm dying during the viability assay and other variables in the dataset (e.g. sperm number and its correlates, such as mating system). Experimental approaches are therefore preferable for the majority of evolution and ecology questions. However, for some questions, a valid comparative tool may be to perform the same experiment in multiple species and then test whether the response is related to biological factors. For example, one could experimentally measure the effects of the female reproductive tract on sperm viability in species with different mating systems to elucidate the ecological factors determining how much females harm (Holman and Snook 2008) or protect (den Boer et al. 2009) sperm.

A notable paucity in the literature is the application of sperm viability staining to non-insect model organisms (32 out of 41 studies in Table 1 used insects). There are also only two studies providing empirical evidence that inter-male variation in sperm viability affects the outcome of sperm competition (Fry and Wilkinson 2004; Garcia-Gonzalez and Simmons 2005b), although in neither case was sperm viability experimentally manipulated. Also, sperm viability was twice found to have no relationship to siring success in the externally fertilising frog *Litoria peronii* (Sherman et al. 2008b; Sherman et al. 2009). Studies of the genetic architecture of sperm viability at the time of collection have produced interesting but inconsistent results (Moore et al. 2004; Simmons and Roberts 2005; Dowling et al. 2007), and the genetic architecture of sperm survival (i.e. the change in viability over time) remains largely unexplored. Experimental evolution and knockout studies may represent the most powerful tools for investigating the causes, consequences and genetic basis of variation in sperm viability and survival.

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