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Sperm Accumulated Against Surface: A novel alternative bioassay for environmental monitoring

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ABSTRACT

Forecasting the impacts of changes in water quality on broadcast spawning aquatic organisms is a key aspect of environmental monitoring. Rapid assays of reproductive potential are central to this monitoring, and there is a need to develop a variety of methods to identify responses. Here, we report a proof-of-concept study that assesses whether quantification of "Sperm Accumulated Against Surface" (SAAS) of tissue culture well-plates could be a rapid and simple proxy measure of fertilisation success. Our results confirm that motile sperm (but not immotile sperm) actively accumulate at surfaces and that the pattern of accumulation reflects fertilisation success in the model oyster species *Crassostrea gigas*. Furthermore, we confirm these patterns of SAAS for another marine species, the polychaete *Galeolaria caespitosa*, as well as for a freshwater species, the fish *Gasterosteus aculeatus*. For all species considered, SAAS reflected changes in sperm performance caused by experimentally manipulated differences in water quality (here, salinity). These findings indicate that SAAS could be applied easily to a range of species when examining the effects of water quality. Measurement of SAAS could, therefore, form the basis of a rapid and reliable assay for bioassessments of broadcast spawning aquatic organisms.

1. Introduction

Developing bioassays to assess the effects of environmental conditions on organisms is a focus of many scientific disciplines including aquaculture (Kime et al., 2001), ecotoxicology (Macken et al., 2008) and climate change research (Falkenberg et al., 2013; Gazeau et al., 2010). For organisms that release gametes freely into the water column – so-called "broadcast spawning organisms" – the sensitivity and ecological relevance of reproductive endpoints to modified environmental conditions is especially relevant, and has led to their widespread use in bioassays (Lewis and Watson, 2012; Nipper et al., 1993; Watanabe et al., 2007). Many bioassays exploit reproductive endpoints such as fertilisation, which requires the collection of both male and female gametes from the species of interest, as well as successfully fertilising those gametes *in vitro* (Caldwell et al., 2011; Gopalakrishnan et al., 2007; Ross and Bidwell, 2001). These requirements can be difficult to meet as viable eggs may be hard to collect and fertilisation usually requires the careful application of species-specific protocols (Strathmann, 1987), which may not exist for many species of interest (Fabbrocini et al., 2010). Consequently, the use of simpler measures, such as those which focus solely on assessing the motility of sperm, are increasingly being used as reproductive endpoints (Lewis and Watson, 2012).

A number of methods exist for assessing sperm motility, a proxy for fertilisation success. One of the most common and easily applied of these is visual assessment and classification according to a pre-defined scale (see, for example, Griffin et al., 1998; Haddy and Pankhurst, 2000; Nissling et al., 2006; Westin and Nissling, 1991). While these measures are quick and require little in the way of microscopy equipment they are also subjective and observer bias can markedly influence the results (Dunphy et al., 1989). A more reliable alternative is computer assisted sperm analysis (CASA), which provides objective, quantitative measures of motility (reviewed in Amann and Waberski, 2014; Kime et al., 2001). CASA typically requires sophisticated and expensive microscopy and digital video analysis resources, as well as expert personnel to determine and measure the relevant motility parameters (e.g. percent motile sperm, velocity [multiple metrics], flagella beat frequency and distance travelled [multiple metrics]) (Amann and Waberski, 2014; Boryshpolets et al., 2013a; Valeanu et al., 2015).

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There are, however, several artefacts associated with CASA that can influence the results (e.g. frame rate of recordings, duration of recordings, inadvertent re-sampling, and focal position inside the drop of swimming sperm) (Boryshpolets et al., 2013b; Davis and Katz, 1993). Furthermore, some of these measured parameters can show very weak correlations with fertilisation success (Wu et al., 2002). Given these issues, alternative sperm-based bioassays are being developed. One promising method is the assessment of the sperm mitochondrial membrane integrity, although this method also requires specialised equipment (flow cytometry) and expertise (Binet et al., 2014; Schlegel et al., 2015). Consequently, there is a need for a simpler, yet still objective, approach to assess sperm motility that has a clear relationship with fertilisation success.

We propose an alternative method to quantify sperm motility (and a potential proxy measure of fertilisation success) based on the observation that motile sperm move toward, and accumulate at, solid surfaces (see, for example, Berke et al., 2008; Boryshpolets et al., 2013a; Rothschild, 1963). The method is based on our expectation that for sperm suspensions held in three dimensional spaces (such as tissue culture well-plates), the rate of accumulation of sperm against the lower surface of the well will reflect the relative motility of sperm in that suspension. Accumulation should reflect activity levels of motile sperm because swimming will increase contact rate with the surfaces of the well, where they are likely to be captured by hydrodynamic forces (Berke et al., 2008; Makler et al., 1993; Woolley, 2003). This process is likely to be greater at the bottom surface of a well because gravity often causes the heads of sperm to passively turn downward, leading to a strong downward bias in active movement (Makler et al., 1993). While these effects should result in the accumulation of motile sperm against lower surfaces, their effect on non-motile sperm is expected to be negligible because sperm sinking rates are very slow, leading to greatly reduced accumulation of non-motile sperm on lower surfaces (Makler et al., 1993). Given these expectations, the rate of accumulation of sperm against the lower surface of a well should provide a reliable measure of sperm motility (the integral of percent motility and swimming speed).

Here we test whether the accumulation of sperm against surfaces (Sperm Accumulation Against Surfaces – SAAS) could be exploited as an alternative metric of sperm motility and hence as an indicator of fertilisation success. For this metric to be useful, it is required that: 1) sperm accumulate at surfaces in such a way that enables their measurement; 2) accumulation correlates with fertilisation success; 3) accumulation can be measured in diverse taxa; and 4) accumulation at surfaces co-varies with conditions in the water column. Successful demonstration of these characteristics would indicate the potential for SAAS to be used as an additional metric for future assessments of water quality.

2. Material and methods

2.1. Sperm Accumulated Against Surface (SAAS)

We first determined whether Sperm Accumulated Against Surface (SAAS) could be measured reliably. Adult Pacific oysters (*Crassostrea gigas*) were collected from around the island of Tjärnö, Sweden, in December of 2014 and transferred to Ostrea Sverige AB (Koster, Sweden) where they were conditioned to maturity. Experiments using these oysters were conducted at the Sven Lovén Centre for Marine Sciences (Tjärnö, Sweden) in August of 2015. All experiments were conducted at 20 ± 1 °C (the typical ambient seawater temperature in this region during the spawning season). Concentrated sperm were extracted from each of five males using a Pasteur pipette inserted through a hole drilled in the shell above the gonad (Havenhand and Schlegel, 2009). Sperm were stored in separate Eppendorf tubes on ice to maximise longevity. These sperm were combined and diluted with filtered seawater (FSW) (30 PSU) to a concentration of 2×10^6 sperm ml^{-1} in each of three replicate wells in a 6-well plate. Concentrations were verified by haemocytometer counts of samples dyed and immobilised with Lugol's solution. These sperm were then exposed to the treatment seawater for 10 min (in this case 30 PSU). Following this exposure period 1.5 ml of these suspensions were pipetted into wells of a new multi-well plate from which the pattern of accumulation over time was quantified for sperm that had been exposed to treatment conditions. The bottom surface of each well was observed using a phase-contrast inverted microscope (Leica, DMIL, Germany) equipped with a digital camera (PixeLINK, PL-D725CU, Canada). To quantify accumulation, still images of different (central) areas of the lower surface of the wells were taken 20, 60, 180 and 540 s after the addition of the sperm suspension to the well ('well-time'; $n = 3$ images per well per time point). Digital images were post-processed and the number of sperm that had accumulated at the lower surface of the well counted manually. This procedure was repeated for sperm that had been warmed to ~50 °C for 5 min, after which time no motile sperm could be detected when viewed under the microscope (these were regarded as 'dead sperm'). Number of sperm accumulated in the live and dead treatments at each 'well-time' was analysed with a repeated measures (split-plot design) ANOVA (with factors; 'Treatment' [fixed, two levels], 'Time' [fixed, four levels]). Wells were used as replicates ($n = 3$), and data were square root transformed to equalise variances.

2.2. Correlation between Sperm Accumulated Against Surface (SAAS) and fertilisation success

To determine the extent to which SAAS is a useful proxy measure for fertilisation success, sperm and eggs were obtained from 5 male and 5 female sexually mature adult *C. gigas* using methods described above. Following standard practice in established bioassays, the gametes of five individuals were pooled (Environment Agency UK, 2007). In this study, such pooling was advantageous for two key reasons: 1) we were interested in the 'average' response for the species rather than the influence of individual variability, and, 2) mixed batches of gametes minimise sperm-egg incompatibilities in fertilisation tests (as in Havenhand and Schlegel, 2009). Extracted sperm were stored in separate Eppendorf tubes on ice before being combined and diluted with FSW to 1×10^7 sperm ml^{-1} (a higher concentration than used in other parts of the experimental design because pilot studies indicated this would allow us to better quantify the smaller changes in motility between data points obtained here) ($n = 3$ replicate wells). After 10 min exposure time, the diluted sperm were then pipetted into new wells. Our initial experiment showed that SAAS plateaued after ~540 s on the well-plate, therefore we photographed each well-plate 600 s after addition of sperm ($n = 3$ replicate photos of each well). Images were post-processed as detailed above.

To quantify fertilisation success, eggs were placed in 10 ml FSW in an incubator at 20 °C and left for an hour before fertilisation to allow their hydration. During this time, egg suspensions were adjusted to yield a final concentration of ~300 oocytes ml^{-1} for each female and then combined to form a pooled batch. Similarly, the final concentrations of sperm in the mixed batches were adjusted such that eggs were fertilised with 5×10^8 sperm ml^{-1} (this concentration yields ~ 90% fertilisation success in 1 h old gametes; Havenhand pers. obs.). Gametes were left to fertilise for 12 min after which time fertilisations were halted by separating sperm and eggs via centrifugation for 5 min at 2000 rcf and 20 °C. The supernatant containing the sperm was discarded and the lightly