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3 SPERM ACCUMULATED AGAINST SURFACE: A NOVEL ALTERNATIVE BIOASSAY

4 FOR ENVIRONMENTAL MONITORING

5

6 Running title: Sperm Accumulated Against Surface

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16

17 **Abstract**

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

33

34 **Key-words:** bioassay; bioassessment; ecotoxicology; oyster; polychaete; sperm movement;
35 spermatozoa; stickleback

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37

38 ## 1. Introduction

39 Developing bioassays to assess the effects of environmental conditions on organisms is a
40 focus of many scientific disciplines including aquaculture (Kime et al. 2001), ecotoxicology
41 (Macken et al. 2008) and climate change research (Falkenberg et al. 2013; Gazeau et al.
42 2010). For organisms that release gametes freely into the water column – so-called "broadcast
43 spawning organisms" – the sensitivity and ecological relevance of reproductive endpoints to
44 modified environmental conditions is especially relevant, and has led to their widespread use
45 in bioassays (Lewis and Watson 2012; Nipper et al. 1993; Watanabe et al. 2007). Many
46 bioassays exploit reproductive endpoints such as fertilisation, which requires the collection of
47 both male and female gametes from the species of interest, as well as successfully fertilising
48 those gametes *in vitro* (Caldwell et al. 2011; Gopalakrishnan et al. 2007; Ross and Bidwell
49 2001). These requirements can be difficult to meet as viable eggs may be hard to collect and
50 fertilisation usually requires the careful application of species-specific protocols (Strathmann
51 1987), which may not exist for many species of interest (Fabbrocini et al. 2010).
52 Consequently, the use of simpler measures, such as those which focus solely on assessing the
53 motility of sperm, are increasingly being used as reproductive endpoints (Lewis and Watson
54 2012).
55
56 A number of methods exist for assessing sperm motility, a proxy for fertilisation success. One
57 of the most common and easily applied of these is visual assessment and classification
58 according to a pre-defined scale (see, for example, Griffin et al. 1998; Haddy and Pankhurst
59 2000; Nissling et al. 2006; Westin and Nissling 1991). While these measures are quick and
60 require little in the way of microscopy equipment they are also subjective and observer bias
61 can markedly influence the results (Dunphy et al. 1989). A more reliable alternative is
62 computer assisted sperm analysis (CASA), which provides objective, quantitative measures

63 of motility (reviewed in Amann and Waberski 2014; Kime et al. 2001). CASA typically
64 requires sophisticated and expensive microscopy and digital video analysis resources, as well
65 as expert personnel to determine and measure the relevant motility parameters (e.g. percent
66 motile sperm, velocity [multiple metrics], flagella beat frequency and distance travelled
67 [multiple metrics]) (Amann and Waberski 2014; Boryshpolets et al. 2013a; Valeanu et al.
68 2015). There are, however, several artefacts associated with CASA that can influence the
69 results (e.g. frame rate of recordings, duration of recordings, inadvertent re-sampling, and
70 focal position inside the drop of swimming sperm) (Boryshpolets et al. 2013b; Davis and
71 Katz 1993). Furthermore, some of these measured parameters can show very weak
72 correlations with fertilisation success (Wu et al., 2002). Given these issues, alternative sperm-
73 based bioassays are being developed. One promising method is the assessment of the sperm
74 mitochondrial membrane integrity, although this method also requires specialised equipment
75 (flow cytometry) and expertise (Binet et al. 2014; Schlegel et al. 2015). Consequently, there
76 is a need for a simpler, yet still objective, approach to assess sperm motility that has a clear
77 relationship with fertilisation success.
78
79 We propose an alternative method to quantify sperm motility (and a potential proxy measure
80 of fertilisation success) based on the observation that motile sperm move toward, and
81 accumulate at, solid surfaces (see, for example, Berke et al. 2008; Boryshpolets et al. 2013a;
82 Rothschild 1963). The method is based on our expectation that for sperm suspensions held in
83 three dimensional spaces (such as tissue culture well-plates), the rate of accumulation of
84 sperm against the lower surface of the well will reflect the relative motility of sperm in that
85 suspension. Accumulation should reflect activity levels of motile sperm because swimming
86 will increase contact rate with the surfaces of the well, where they are likely to be captured by
87 hydrodynamic forces (Berke et al. 2008; Makler et al. 1993; Woolley 2003). This process is

88 likely to be greater at the bottom surface of a well because gravity often causes the heads of
89 sperm to passively turn downward, leading to a strong downward bias in active movement
90 (Makler et al. 1993). While these effects should result in the accumulation of motile sperm
91 against lower surfaces, their effect on non-motile sperm is expected to be negligible because
92 sperm sinking rates are very slow, leading to greatly reduced accumulation of non-motile
93 sperm on lower surfaces (Makler et al. 1993). Given these expectations, the rate of
94 accumulation of sperm against the lower surface of a well should provide a reliable measure
95 of sperm motility (the integral of percent motility and swimming speed).
96
97 Here we test whether the accumulation of sperm against surfaces (Sperm Accumulation
98 Against Surfaces – SAAS) could be exploited as an alternative metric of sperm motility and
99 hence as an indicator of fertilisation success. For this metric to be useful, it is required that: 1)
100 sperm accumulate at surfaces in such a way that enables their measurement; 2) accumulation
101 correlates with fertilisation success; 3) accumulation can be measured in diverse taxa; and 4)
102 accumulation at surfaces co-varies with conditions in the water column. Successful
103 demonstration of these characteristics would indicate the potential for SAAS to be used as an
104 additional metric for future assessments of water quality.
105

106 2. Material and Methods

107 *2.1 Sperm Accumulated Against Surface (SAAS)*

108 We first determined whether Sperm Accumulated Against Surface (SAAS) could be
109 measured reliably. Adult Pacific oysters (*Crassostrea gigas*) were collected from around the
110 island of Tjärnö, Sweden, in December of 2014 and transferred to Ostrea Sverige AB
111 (Koster, Sweden) where they were conditioned to maturity. Experiments using these oysters
112 were conducted at the Sven Lovén Centre for Marine Sciences (Tjärnö, Sweden) in August of

113 2015. All experiments were conducted at 20 ± 1°C (the typical ambient seawater temperature
114 in this region during the spawning season). Concentrated sperm were extracted from each of
115 five males using a Pasteur pipette inserted through a hole drilled in the shell above the gonad
116 (Havenhand and Schlegel, 2009). Sperm were stored in separate Eppendorf tubes on ice to
117 maximise longevity. These sperm were combined and diluted with filtered seawater (FSW)
118 (30 PSU) to a concentration of 2×10^6 sperm ml^{-1} in each of three replicate wells in a 6-well
119 plate. Concentrations were verified by hemocytometer counts of samples dyed and
120 immobilised with Lugol's solution. These sperm were then exposed to the treatment seawater
121 for 10 min (in this case 30 PSU). Following this exposure period 1.5 ml of these suspensions
122 were pipetted into wells of a new multi-well plate from which the pattern of accumulation
123 over time was quantified for sperm that had been exposed to treatment conditions. The
124 bottom surface of each well was observed using a phase-contrast inverted microscope (Leica,
125 DMIL, Germany) equipped with a digital camera (PixeLINK, PL-D725CU, Canada). To
126 quantify accumulation, still images of different (central) areas of the lower surface of the
127 wells were taken 20, 60, 180 and 540 s after the addition of the sperm suspension to the well
128 ('well-time'; n = 3 images per well per time point). Digital images were post-processed and
129 the number of sperm that had accumulated at the lower surface of the well counted manually.
130 This procedure was repeated for sperm that had been warmed to ~50°C for 5 minutes, after
131 which time no motile sperm could be detected when viewed under the microscope (these
132 were regarded as 'dead sperm'). Number of sperm accumulated in the live and dead
133 treatments at each 'well-time' was analysed with a repeated measures ANOVA (with factors;
134 'Treatment' [fixed, two levels], 'Time' [fixed, four levels]). Wells were used as replicates
135 (n = 3), and data were square root transformed to equalise variances.
136

137 *2.2 Correlation between Sperm Accumulated Against Surface (SAAS) and fertilisation*
138 *success*

139 To determine the extent to which SAAS is a useful proxy measure for fertilisation success,
140 sperm and eggs were obtained from 5 male and 5 female sexually mature adult *C. gigas* using
141 methods described above. Following standard practice in established bioassays, the gametes
142 of five individuals were pooled (Environment Agency UK, 2007). In this study, such pooling
143 was advantageous for two key reasons: 1) we were interested in the 'average' response for
144 the species rather than the influence of individual variability, and, 2) mixed batches of
145 gametes minimise sperm-egg incompatibilities in fertilisation tests (as in Havenhand and
146 Schlegel, 2009). Extracted sperm were stored in separate Eppendorf tubes on ice before
147 being combined and diluted with FSW to 1×10^7 sperm ml^{-1} (a higher concentration than
148 used in other parts of the experimental design because pilot studies indicated this would allow
149 us to better quantify the smaller changes in motility between data points obtained here) ($n = 3$
150 replicate wells). After 10 min exposure time, the diluted sperm were then pipetted into new
151 wells. Our initial experiment showed that SAAS plateaued after ~540 seconds on the well-
152 plate, therefore we photographed each well-plate 600 seconds after addition of sperm ($n = 3$
153 replicate photos of each well). Images were post-processed as detailed above.
154

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

162 separating sperm and eggs via centrifugation for 5 min at 2000 rcf and 20 °C. The
163 supernatant containing the sperm was discarded and the lightly pelleted eggs were
164 resuspended in 6.5 ml FSW. Eggs were left to develop for 2 h at 20 ± 0.5 °C following which
165 fertilisation success was determined by recording the proportion of eggs that displayed
166 cleavage in each of three replicate plates (n = 3). Embryos had typically reached the 4 cell
167 stage after this time. Preliminary data showed that *C.gigas* gametes (both eggs and sperm)
168 held at 20°C progressively lost viability and were incapable of fertilisation after ~ 6 h (P
169 Mooney & J Havenhand, pers. obs.). Therefore, to obtain a range of different SAAS and
170 fertilisation success, the fertilisation and SAAS protocols were repeated once every 30 min
171 for 7.5 h, resulting in 16 data points. The relationship between mean estimates of SAAS and
172 fertilisation success was assessed using linear regression and single-factor ANOVA where
173 'Response variable' was treated as a fixed factor with two levels (SAAS *v*. fertilisation
174 success) after SAAS was first square root transformed and fertilisation success arc-sine
175 transformed to equalise variances.

176

177 *2.3 Sperm Accumulation Against Surface (SAAS) as an indicator of the responses of*
178 *different species to modified salinity*

179 To assess the general usefulness of SAAS, we tested the change in this metric for a range of
180 species in response to modified salinities. In addition to responses for the oyster *C. gigas*
181 (collected from western Sweden – see above), we also measured SAAS in the marine
182 polychaete *Galeolaria caespitosa* (collected from South Australia) and the euryhaline
183 stickleback (*Gasterosteus aculeatus*, collected from freshwater ponds, western Sweden).
184 Oyster and stickleback experiments were conducted at the Sven Lovén Centre for Marine
185 Sciences (Tjärnö, Sweden), while polychaete experiments were conducted at the Australian
186 campus of University College London (Adelaide, Australia). In all cases we tested SAAS in

187 ambient salinity as well as in lower salinities (ambient diluted with freshwater). Given the
188 ambient salinities at different locations, levels used for *C. gigas* and *Gasterosteus aculeatus*
189 were 30, 25, 20, 25, 20, 15, 10, 5 and 0 PSU, while for *Galeolaria caespitosa* levels were 35,
190 30, 25, 20, 25, 20, 15, 10, 5 and 0 PSU. Treatment levels were verified using a conductivity
191 meter (WTW, Cond 3210, Germany) and hand refractometer (ATAGO, S-10_E, Japan).
192

193 Sperm were extracted from the oyster *C. gigas* following the procedure described above. To
194 extract sperm from the polychaete, dislodged clusters of *Galeolaria caespitosa* were broken
195 apart and individuals carefully removed from their calcareous tubes with fine forceps.
196 Individuals were then rinsed with artificial seawater (ASW) and placed in a well-plate to
197 which ~ 1 ml of ASW was added to stimulate sperm release. Release of gametes began
198 almost immediately and was allowed to continue for 15 min. For the stickleback, sperm were
199 extracted by dissection directly from the testes. For all species, concentrated sperm from five
200 males were combined and diluted with FSW to the appropriate salinity (0, 5, 20, 15, 20, 25,
201 30 or 35 PSU) to 2×10^6 sperm ml^{-1} (n = 3 replicate wells per salinity per species). As
202 detailed above, these suspensions were then transferred to new well-plates after 10 min, with
203 three replicate photos taken of different areas of the bottom surface of each well after 600 s
204 well-time, and the number of accumulated sperm determined using the methods outlined
205 earlier. The response of each species to salinity was analysed with a separate one-way
206 ANOVA (with the factor of 'Salinity', fixed, 8 or 9 levels) with wells as replicates (each
207 datum the mean of the 3 images taken at each time-point). Data were square root transformed
208 to equalise variances.
209

210 **3. Results**

211 *3.1 Sperm Accumulated Against Surface (SAAS)*

212 Sperm accumulated against the lower surface of well-plates in a consistent and measurable
213 way. Accumulation of live sperm was initially rapid and then slowed exponentially
214 (Figure 1). Accumulation of dead sperm over time showed a very different pattern, and few
215 sperm (~25 per well) were observed at the lower surface, even after 540 s (Figure 1).
216 Statistically, SAAS differed among these treatments for all time periods, even after just
217 20 seconds in the well-plate, with the differences increasing with well-time (Figure 1;
218 Treatment × Time interaction $F_{3,12} = 25.71$, $P = < 0.001$, alive > dead for all well times).

219

220 *3.2 Correlation between Sperm Accumulated Against Surface (SAAS) and fertilisation*
221 *success*

222 Comparison of SAAS with fertilisation success in oysters revealed a strong positive
223 correlation between the two variables (Figure 2, $R^2 = 0.8473$; $F_{1,30} = 5122.3$, $p = <0.001$).
224 The slope of a model I regression through these data showed that fertilisation success
225 increased by 15.84 % for every 100 sperm accumulated.

226

227 *3.3 Sperm Accumulated Against Surface (SAAS) as an indicator of the responses of*
228 *different species to modified salinity*

229 Salinity influenced SAAS in all three species tested (Figure 3). For both the marine species,
230 SAAS was strongly positively related with salinity although the highest SAAS was observed
231 at the next to highest salinities (Figure 3a, 3b; $F_{7,16} = 10.01$, $p = <0.001$; $F_{8,18} = 41.73$, $p =$
232 <0.001 respectively). In contrast, for the euryhaline stickleback collected from freshwater
233 environments, SAAS showed a strong negative relationship with increasing salinity (Figure
234 3c; $F_{7,16} = 63.27$, $p = <0.001$). For all species, SAAS of treatments containing live sperm

235 were substantially (and always significantly) greater than the dead sperm controls.
236 Comparing the SAAS of dead sperm across species revealed that the greatest accumulation
237 occurred for the polychaete (mean SAAS ± SE, 35 ± 2 after 540s) with intermediate
238 accumulation for the oyster (25 ± 1) and least for the stickleback (14 ± 3).
239

240 ## 4. Discussion

241 Our findings show that Sperm Accumulated Against Surface (SAAS) – a novel and
242 standardised metric of the accumulation of sperm at surfaces – is a repeatable measure of
243 sperm motility and (by proxy) fertilisation success. We also show the potential of SAAS as a
244 rapid and reliable bioassay under a range of environmental conditions and in three very
245 different broadcast-spawning species. As outlined in the Introduction, demonstrating the
246 utility of SAAS as a new bioassay was dependent on meeting four key requirements, which
247 the experiments outlined in this manuscript were designed to assess. The first of these was
248 that swimming sperm actively and rapidly accumulated at surfaces over time in a repeatable
249 and measurable way. This was indeed the case and – importantly – we also demonstrated that
250 accumulation of dead sperm controls was consistently and substantially lower than in
251 suspensions with active sperm (Figure 1). This result indicates clearly that SAAS is a reliable
252 measure of active, motile sperm. The second requirement was that SAAS correlated
253 significantly with fertilisation success, which our results also demonstrated clearly (Figure 2).
254 The third requirement was that the bioassay could be applied to diverse species, which we
255 met by successfully measuring SAAS in a range of marine and aquatic organisms (a marine
256 bivalve, a marine polychaete, and a freshwater fish). Finally, the fourth requirement was
257 sensitivity of SAAS to environmental variables. Our finding that SAAS did indeed change
258 gradually and dependably with increasing salinity (Figure 3) indicates the potential sensitivity
259 and utility of the metric in this respect. Together, these results show considerable potential for

260 SAAS as a rapid and reliable bioassay to assess the biotic effects of toxicants and
261 environmental change. Further testing will, however, be required to demonstrate responses to
262 the range of toxicants and environmental change conditions of interest, and to indicate the
263 general reliability of this method. The following discussion examines in more detail the
264 capacity of SAAS to meet these key requirements, and presents important issues for
265 consideration when implementing this new measure.
266
267 Sperm accumulation occurred in a measurable way with SAAS easily quantifiable, indicating
268 the potential utility of this metric. Furthermore, the negligible accumulation of dead sperm
269 that we observed shows this metric is almost exclusively measuring active sperm (Figure 1).
270 The mechanism underlying differences between accumulation rates of live and dead sperm
271 was apparent from watching the accumulation process: live sperm swam actively before the
272 head collided with, and adhered to, the well surface, whereas dead sperm drifted very slowly
273 toward the surface and did not obviously collide or stick (see video in Electronic Supporting
274 Material). As with most sperm motility metrics, SAAS is clearly time-dependent – the rate of
275 sperm accumulation changed over even short periods. For motile sperm, accumulation was
276 initially rapid within the first few minutes but then slowed and began to plateau (Figure 1).
277 This pattern likely reflects an exponential decline in the number and speed of sperm
278 remaining in suspension in the well through time; that is, as active sperm accumulate at
279 surfaces, fewer remained in suspension. The effect of time would have been amplified
280 because faster-swimming sperm are more likely to accumulate first, and therefore late
281 accumulating sperm may not only have been more distant from the surface, but may also
282 have swum more slowly either because they were inherently slower or because they had
283 depleted their energy stores (as in Rahman et al. 2009). These findings also highlight the
284 potential for bias in sperm tracking measurements with common experimental setups that

285 involve small volumes of water on mounted glass slides (e.g. Havenhand et al. 2008; Styan et
286 al. 2005; Styan and Butler 2000). Unless such measurements are made quickly, the effect we
287 have documented here could easily bias measures of the proportion of immotile and slowly
288 moving sperm made using CASA systems (also discussed in Boryshpolets et al. 2013b).
289
290 The ecological relevance of sperm motility measures is strengthened when they correlate well
291 with fertilisation success. The strength of the relationship between SAAS and fertilisation
292 success we observed ($R^2 = 0.85$, $P < 0.001$, Figure 2) is comparable to that found for
293 relationships identified between 'traditional' CASA-derived measures of sperm motility and
294 fertilisation success; Wu et al. (2002) summarised R^2 values of the best models for a variety
295 of CASA-determined parameters (e.g. VSL, % motile sperm, VAP, VCL) and fertilisation
296 success and found that they ranged between 0.59 and 0.95. Clearly the relationship between
297 SAAS and fertilisation success we found is toward the upper end of this range, indicating the
298 potential for SAAS to predict reproductive success and subsequent ecological impacts as
299 effectively as CASA-derived measures of sperm motility.
300
301 Currently, many bioassay endpoint metrics are species-specific. A key advantage of SAAS
302 was proposed to be the relative ease with which it could be applied to a diverse range of
303 species. This generality was demonstrated here for marine organisms (the bivalve
304 *Crassostrea gigas* and polychaete *Galeolaria caespitosa*), as well as a euryhaline organism
305 (the freshwater fish *Gasterosteus aculeatus*) (Figures 3a, b, c respectively).
306
307 To be useful, bioassays need to be able to detect and measure the influence of modified
308 environmental conditions (e.g. changes in salinity, temperature, toxicant concentration) on
309 fertilisation success. Here, we evaluated the change in SAAS for the sperm of three species

310 exposed to a range of salinities. For the marine species (*Galeolaria caespitosa*, *C. gigas*)
311 SAAS was higher in more saline conditions, results which align with previous studies
312 indicating the sperm of marine species are more motile at high salinities (e.g. Atlantic cod,
313 *Gadus morhua*, Litvak and Trippel et al., 1998; striped mullet *Mugil cephalus*, Lee et al.,
314 1992). Interestingly, in these marine species the very highest salinities did not elicit the
315 highest SAAS, perhaps indicating that a threshold, or (more likely) optimum, salinity may
316 exist for sperm motility (Figure 3a, b). Interestingly, more traditional motility measures have
317 also shown optimal salinities for sperm of marine species (e.g. sea bass, *Dicentrarchus*
318 *labrax*, Billard, 1978; Baltic cod, *Gadus morhua*, Westin and Nissling, 1991). In contrast, in
319 the euryhaline stickleback (*Gasterosteus aculeatus*), which were collected from a freshwater
320 environment, higher salinities were associated with lower sperm motility – a result which
321 again aligns with results previously obtained from other species of similar environments
322 (sooty grunter, *Hephaestus fuliginosus*, Hogan and Nicholson, 1987; zebra mussel, *Dreissena*
323 *polymorpha*, Fong et al., 1995). Importantly, for all species and in all treatments SAAS of
324 live sperm was always greater than the dead sperm controls, indicating that substantial
325 numbers of motile sperm were still present at the end of the assay (typically 9 minutes). The
326 accumulation rates of dead sperm did differ among the species. While we did not test the
327 mechanism underlying this pattern, we suggest it was likely driven by species-specific
328 differences in sinking rates arising from contrasting sperm size, shape (either head or tail)
329 and/or buoyancy. Together, our results demonstrate that the number of sperm accumulating at
330 a surface over a period of 9 minutes can be used reliably to distinguish between waters of
331 differing salinities, that this method is applicable in a range of aquatic organisms, and that the
332 results are a good proxy for fertilisation success.
333

334 The potential benefits of using SAAS as a rapid, high-precision bioassay are considerable.
335 We were able to detect differences between motile and immotile sperm just 20 seconds after
336 transferring sperm-rich solutions to measuring wells, and extending the duration to nine
337 minutes amplified this difference (Figure 1). Furthermore, the precision of this measure was
338 such that the effects of even relatively slight changes in environmental conditions (5 PSU)
339 were readily detectable (Figure 3). Further benefits derive from the fact that preparation time
340 for the process is minimal (diluting test suspensions to an appropriate concentration, pipetting
341 into a well, starting a timer), such that even with relatively long 'well-times' of nine minutes
342 numerous tests could be run sequentially or in parallel-overlapping series. Short run times for
343 bioassays are important where environmental impacts must be assessed rapidly and
344 incorporated quickly into the decision-making process; for example, deciding whether to add
345 dispersants after an oil spill (Chapman et al. 2007). Moreover, the simple, small, and
346 inexpensive equipment and short run times of SAAS would allow for far more treatments
347 and/or replicates to be run than could be achieved using established approaches. This rapidity
348 may, in turn, facilitate more complex tests of combinations of toxicants and environmental
349 factors in order to determine toxicity and bioavailability across a range of present and future
350 conditions (Falkenberg and Styan 2015; Ross and Bidwell 2006). While the advantages of
351 being able to consider a wide range of species and conditions are self-evident, these are likely
352 to become more important in the future as human activities impact a wider range of species
353 and modify an increasing array of environmental conditions.
354
355 As with all metrics of sperm motility, the reliability, quality and comparability of results from
356 SAAS will depend on careful control of experimental conditions (see Boryshpolets et al.
357 2013b, Amann & Waberski 2014 for equivalent comment on CASA). Following protocols
358 that ensure results reflect differences among the samples, rather than time-dependent changes

359 in sperm motility, will be crucial in this regard (as discussed for CASA in Boryshpolets et al.
360 2013b). For SAAS, consistency of measurement and observation can be achieved by ensuring
361 that the timing of dilution on the well-plate, and subsequent collection of images, is uniform.
362 To achieve this consistency, we established rigid schedules for timing of dilution and
363 recording initiation, and maintained these throughout all replicates using timers. Choice of
364 appropriate recording quality may also be a crucial factor in some systems as this can
365 influence the time required to record and analyse the samples. Consequently, only one
366 recording system (i.e. camera, computer) and quality (resolution) was used for each species.
367 Strict adherence to relevant protocols will be of particular importance for species in which
368 sperm are motile for only short periods, such as some fish (Browne et al. 2015). Where
369 consistent protocols are followed (as was done here) SAAS may be an easy, convenient and
370 reliable alternative to more complex sperm motility metrics.
371
372 In conclusion, patterns of sperm motility have a pervasive influence on fertilisation and
373 reproductive success, which are invaluable in biomonitoring. Given the growing need for
374 assessments of environmental impacts on reproduction, the new SAAS-based method we
375 describe here shows considerable potential utility. Specifically, our findings that: 1)
376 swimming sperm accumulate predictably and repeatedly against surfaces; 2) their
377 accumulation correlates closely with fertilisation success; 3) the method works for
378 taxonomically diverse species; and 4) the rate of accumulation co-varies with a key
379 environmental variable, indicate that SAAS could be a reliable, rapid, inexpensive, and
380 sensitive tool for future bioassays. More work is needed to demonstrate the broader
381 applicability of SAAS and to further develop protocols. Specifically, additional testing is
382 required to determine SAAS responses to a range of toxicants, pollutants and environmental
383 change conditions. Similarly, documenting the responses of a greater range of species,

384 particularly those with different life history strategies, is also required. Such developments
385 may open the way for SAAS to be used in a broader scientific context, for example, as a
386 viable method for detecting the effects of water quality on the interaction between
387 chemoattractants and sperm swimming. A priority, however, must be tests that compare the
388 sensitivity of SAAS to existing standardised approaches (e.g. OECD test guidelines). Results
389 from such comparisons will ultimately reveal the utility of SAAS as a novel, rapid,
390 inexpensive, and reliable bioassay for environmental monitoring.
391
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528

529 **Figure Legends**

530

531 **Figure 1.** The a) Sperm Accumulated Against Surface (SAAS) (+/- SE), specifically the
532 lower surface of a well-plate, over time following deposition of aliquots containing live
533 (filled circles) and dead sperm (empty circles). Also shown are subsets of images taken from
534 the bottom surface of the well plates for bi) live, 60 seconds, bii) live, 540 seconds, biii) dead,
535 60 seconds, biv) dead, 540 seconds.

536

537 **Figure 2.** The Sperm Accumulated Against Surface (SAAS) of a well-plate after 600 seconds
538 of well-time, compared to fertilisation success (%) (both +/- SE) (see text for further details);
539 Regression: y = 0.0016x – 0.442, R^2 = 0.8473. Note that the x-axis starts at 300.

540

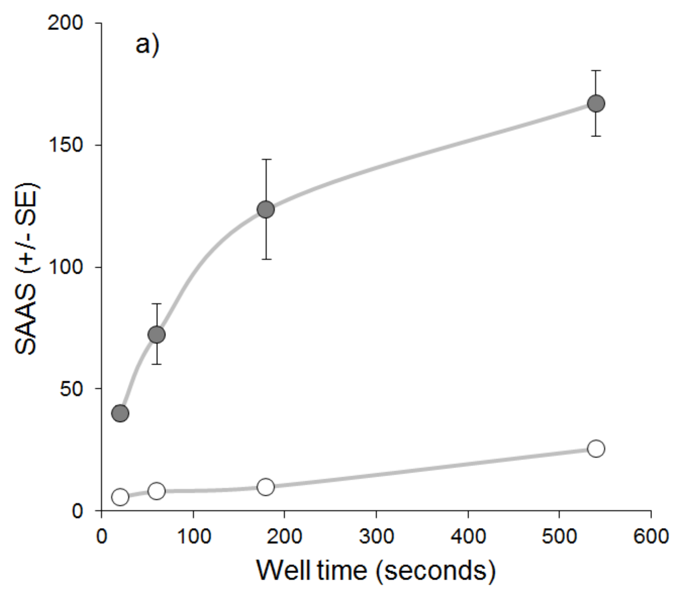
541 **Figure 3.** The Sperm Accumulated Against Surface (SAAS) (+/- SE) of a well-plate after 600
542 seconds of well-time for live sperm exposed to salinity treatments (PSU) (filled circles) and a
543 dead control (empty circle) for a) oyster *Crassostera gigas,* b) polychaete *Galeolaria*
544 *caespitosa*, and c) stickleback fish *Gasterosteus aculeatus*. Note different scales on the axes.

545

546

547 **Figure 1.**

548



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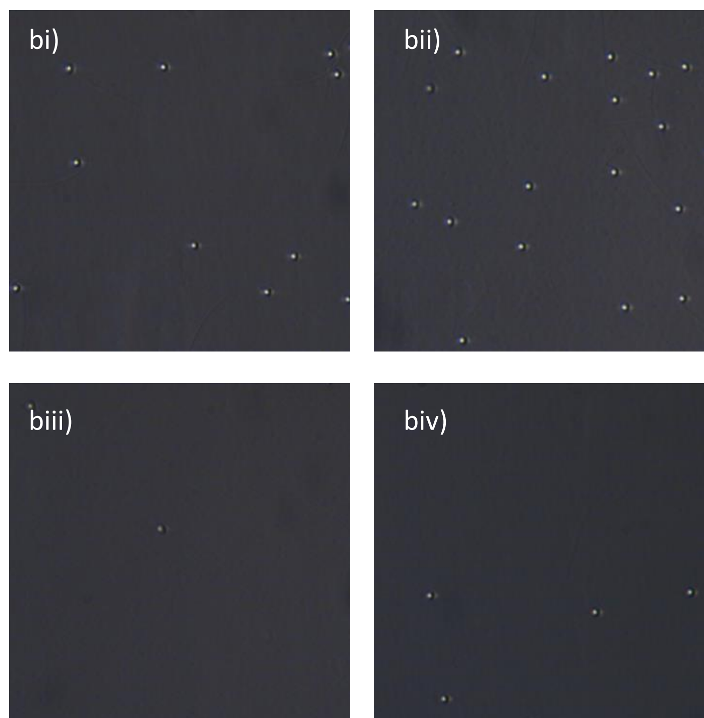

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553 **Figure 2.**

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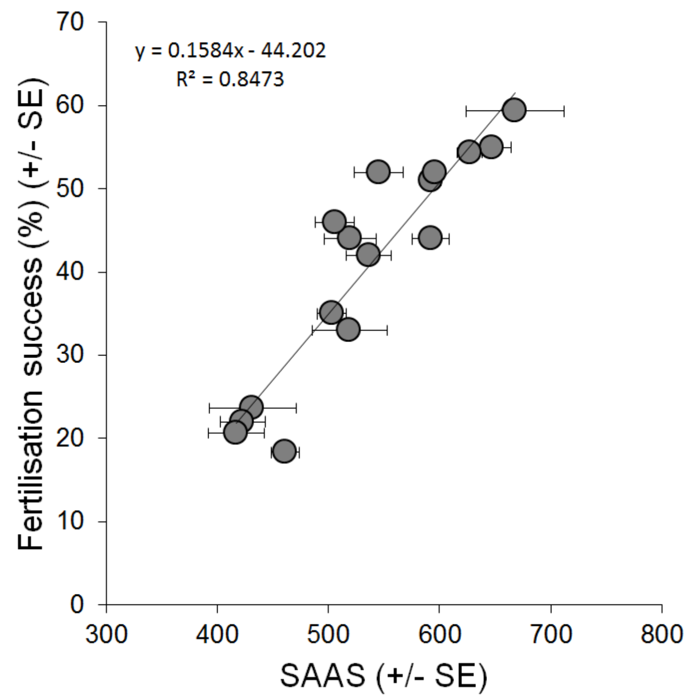

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557 **Figure 3.**

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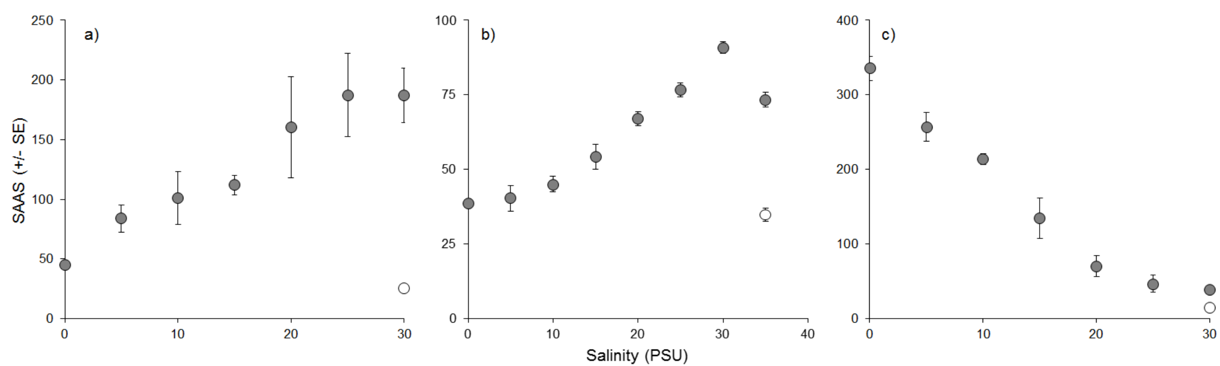

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