MEASURING SPERM SWIMMING PERFORMANCE IN BIRDS: EFFECTS OF DILUTION, SUSPENSION MEDIUM, MECHANICAL AGITATION, AND SPERM NUMBER

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Abstract: Sperm swimming performance, including swimming speed and the proportion of motile cells, may strongly affect fertilization success. However, little is known about how methodological factors affect *in vitro* measurement of these parameters. We compare the swimming performance of sperm from House and Spanish Sparrows (*Passer domesticus* and *P. hispaniolensis*) in two standard suspension media, at two different dilutions, and with different degrees of cell agitation. Further, we conduct a re-sampling analysis to investigate sample size effects. Sperm performance was generally reduced when sperm were diluted, or when suspended in phosphate-buffered saline (PBS) rather than a medium containing additional nutrients (DMEM). Sperm performance was particularly low when they were diluted more and suspended in PBS, suggesting that seminal fluid may provide compounds that enhance performance but that these are less available following dilution. Mechanical agitation of the cells by vigorous pipetting increased the proportion of motile cells. Between-male repeatability, assessed on the single sample measured in multiple conditions, was moderate and significant, suggesting that similar results may be obtained regardless of the methodology used to assess sperm motion. We found no evidence of biased results when low numbers of cells per male were used in analysis, though precision increased substantially as sample size increased from 5 to 20 or more cells per male. We recommend using the same suspension media and similar sperm concentrations and levels of agitation, to the greatest degree possible, and including as many individuals as possible in analyses, even when some individuals are represented by few sperm cells.

Introduction

In many bird species, females copulate with multiple males (Griffith et al. 2002), which implies that sperm and ejaculate characteristics can strongly affect male reproductive success, via competition between sperm from different males and/or via cryptic female choice for certain

sperm traits (Parker 1970; Eberhard 1996). Accordingly, there has been a recent surge of interest in examining the evolution of post-copulatory traits in birds, particularly wild passerines (e.g., (Calhim et al. 2007; Lifjeld et al. 2010; Rowe et al. 2013). Of primary consideration have been the morphology of sperm cells, the speed at which sperm swim, and associations between morphology and swimming speed (Lüpold et al. 2009; Kleven et al. 2009; Cramer et al. 2015), with swimming speed often hypothesized to be a key functional aspect of sperm performance (Bennison et al. 2014). Because of the putative importance of sperm swimming speed to fertilization success, it is important to assess methodologies for measuring sperm swimming speed, as has been done with sperm morphology (Laskemoen et al. 2007; Schmoll et al. 2016).

Most studies on passerine sperm have recorded videos of swimming cells through a microscope, then tracked individual cells through multiple video frames using specialized commercial or open-access software (e.g., Wilson-Leedy and Ingermann 2007; Lüpold et al. 2009; Kleven et al. 2009). To conduct such studies, ejaculates must first be diluted sufficiently that individual cells can be visualized on the video recording, without diluting to a point where too few cells are visible. In this study, we focused on four factors that are likely to arise in any study performing video recording of sperm cells: 1) the choice of the suspension medium, 2) the degree to which the ejaculate is diluted, 3) the vigor with which sperm cells and the suspension medium are mixed before being deposited on the microscope slide, and 4) the number of individual sperm cells tracked per male. Degree of dilution and type of suspension medium affect sperm swimming parameters in several mammal species (Farrell et al. 1996; Rijsselaere et al. 2003), and studies optimizing video recording have been conducted in some wild vertebrates (Fasel et al. 2015; Humann-Guilleminot et al. 2018). However, systematic data are currently lacking for passerine birds. Here, we compare swimming performance in two suspension media, two degrees of dilution, and two levels of agitation testing the sperm of two closely-related

79 species, the House Sparrow (*Passer domesticus*) and the Spanish Sparrow (*P. hispaniolensis*).
80 We then use a re-sampling analysis to investigate the impact of including different numbers of
81 sperm cells per male.
82
83 **Methods**
84 *Study subjects and general methods*
85 We conducted experiments on 25 April, 2014 using populations of House and Spanish
86 Sparrows that had been in captivity in Oslo, Norway since 2010 (see full details in Cramer et al.
87 2014). Birds were confirmed to be in breeding condition, as 19 of the 23 males captured
88 produced sperm samples, and courtship and copulation behaviors were regularly observed. For all
89 experiments, we collected sperm samples into a capillary tube via cloacal massage (Wolfson
90 1952) and deposited the sample onto a sheet of parafilm; this procedure typically yielded at least
91 3 μL of sample per male. Sperm were then immediately pipetted into suspension media for
92 Experiment 1 (see details below), where we tested the effects of suspension medium and degree
93 of dilution in a fully crossed design. The effects of mechanical agitation via pipetting were
94 examined separately in Experiment 2, which we conducted immediately after Experiment 1,
95 using excess from the Experiment 1 treatment that we judged to have the best sperm cell density
96 for video analysis. Therefore, we conducted Experiments 1 and 2 sequentially on each sample,
97 before progressing to the next male (Fig. S1, S2). Excess sperm samples were placed in 300 μL
98 of 5% formaldehyde to allow for morphological analysis, and these samples and video recordings
99 were accessioned to the University of Oslo Natural History Museum (NHMO) sperm collection
100 (Table S1).
101 For both experiments, we filmed sperm swimming behavior using a HDR-HC1E Sony
102 camera attached to an Olympus CX41 microscope with a heated Tokai Hit TP-S glass stage (TP-

S, Gendoji-cho, Shizuoka Japan). All solutions and glassware were pre-warmed to 40 ºC before contact with the sperm, and were maintained at 40 ºC throughout recording. We used Leja 4-chambered microscope slides (20 μm depth; Nieuw-Vennep, Netherlands), which fill via capillary action.

Experiment 1: Effects of dilution and suspension medium

For the different suspension media, we compared DMEM (Dulbecco's modified Eagle medium, a commonly-used medium for sperm swimming analysis; e.g., Kleven et al. 2009) to PBS (Phosphate-Buffered Saline, a medium that lacks the sugars and amino acids in DMEM, but that has been used as a neutral medium in several studies, e.g. Laskemoen et al. 2008; Cramer et al. 2014b, 2016a, b). For the degrees of dilution, we created one treatment (termed concentrated) where sperm cells would be as dense as possible while still allowing analysis software to identify separate sperm cells. The other treatment (termed diluted) was a 2:7 dilution of the concentrated treatment. We chose this dilution ratio to match methods used in previous studies (Cramer et al. 2014, 2016b, a) and to achieve a substantial increase in dilution while still obtaining movement data on a large number of sperm cells. The exact concentration of sperm cells from each sample, however, was not counted.

To conduct experiments, we pipetted 1.2 μL sperm from the parafilm into 74 μL of suspension medium (DMEM or PBS). After mixing thoroughly by pipetting up and down 4-6 times, we transferred 17 μL of this concentrated-sperm solution into 42 μL of the same medium and mixed by pipetting up and down 4-6 times. To equalize pipetting between the concentrated and dilute treatments, we then re-pipetted the concentrated sperm 4-6 times (note that our dilution scheme creates approximately equal end volumes in the concentrated (57 μL) and dilute (59 μL) treatments, so that pipetting an equal number of times should have similar effects in each

treatment). We then repeated this procedure with the other medium. Finally, we loaded 2.9 µL of each mixture into a chamber on a 4-chambered Leja slide, and filmed sperm swimming in each chamber. We alternated which solution was mixed first, and we rotated which treatment was loaded into each slide chamber among males, to reduce potential biases. Mixing cells and loading the slide fully took approximately 1.5 min.

Each slide chamber was filmed in four different locations, to increase the number of sperm cells filmed. To allow us to investigate how sperm behavior changed over time, filming of the different slide chambers was interspersed (i.e., in the order a b c d d c b a a b c d d c b a, where each letter represents a slide chamber; Fig. S2). Approximately 5 seconds elapsed between each successive filming, as we switched chambers, found filming locations without air bubbles or other imperfections or contaminants, and paused to ensure a sufficiently long still period for analysis (approximately 1 second). We therefore recorded the single sample in all four treatments (DMEM concentrated, DMEM diluted, PBS concentrated, and PBS diluted) in rapid succession, with filming typically lasting approximately 1.5 minutes in total.

Experiment 2: Effects of mechanical agitation

Immediately after filming a sample in Experiment 1, we chose the treatment from Experiment 1 that we judged to be best for video analysis, based on visual inspection of cell density on the video recording, and we used that treatment as the source of sperm for Experiment 2. In all but two cases, the diluted treatment from Experiment 1 was judged a better cell density for analysis and thus was the source of sample for Experiment 2; to simplify analysis, we excluded those two experiments where concentrated sperm was used. By chance, we chose only DMEM-diluted sperm from Spanish Sparrow males, but chose both DMEM and PBS diluted sperm from House Sparrow males.

For the low agitation treatment, we transferred 2.9 µL from the original treatment tube to one chamber on a 4-chambered Leja slide. These cells had been in suspension at 40 °C without agitation during filming for Experiment 1, with a total duration of approximately 3.5 minutes between collecting the sample and beginning to video record for Experiment 2. For the high agitation treatment, we mixed the solution in the same tube by pipetting up and down 5-10 more times, with pipette volume set above 15 µL. We then loaded 2.9 µL of suspended sperm onto an adjacent chamber on the microscope slide and filmed each chamber in an interspersed fashion, as above (e.g., a b b a a b), in 3 – 5 locations (filming in more locations when we judged that we had filmed fewer cells). While the low-agitation treatment was thus always loaded onto the microscope slide a few seconds before the high-agitation treatment, we rotated which treatment was filmed first by alternating assignment of treatments to slide chambers.

Analysis of sperm swimming velocity and the proportion of motile sperm

All videos were analyzed with the software Hamilton Thorne CEROS II Sperm Analyzer (Hamilton Thorne Research, Beverly, MA). We analyzed 0.5 sec of video, at a frame rate of 50 Hz, from each of the recording locations. To exclude air bubbles and contaminants, we excluded all detections with an elongation score > 50 from the data set. Detections with a straight-line velocity (VSL) < 25 or average-path velocity (VAP) < 30 were typically cells moving by drift, rather than swimming. These cells were considered immotile in calculating the proportion of motile cells and were excluded from analyses on sperm swimming speed (see also Cramer et al. 2014).

For our velocity measure, we used the curvilinear velocity (VCL), which follows the motions of the sperm cells most closely, following the logic of Laskemoen et al. (2010) that more-derived measurements may be less informative in *in vitro* calculations. To exclude

inaccurate tracks (for example, tracks where the software switched sperm cells between successive detections), we excluded tracks that failed to detect a cell at each successive timepoint, where track straightness was less than 80 or track linearity was less than 35. Only tracks with at least 10 detection points were included. Further, we excluded a track if any single movement between successive detections was greater than 5 interquartile ranges for the other movement distances in that track.

Statistical analysis

A summary of statistical analyses is in Table S2. To assess treatment effects, we constructed linear mixed models. Models concerning sperm swimming speed used the measures from individual sperm cells as data points, while models on the proportion of motile cells used the proportion of motile cells in each recording location as data points. Velocity results were similar when averages for each recording location were used instead of individual cells (not shown). We included a random effect of slide chamber nested within male identity to account for having multiple data points per chamber per male. In velocity measures, we further included a random effect of filming location nested within chamber, to account for possible non-independence of cells filmed together; this was not possible for proportion motile as each filming location was used as a data point. The nested random effect structure significantly improved model fit in all cases (assessed via a likelihood ratio test on models fit with REML estimation, and full parameterization of fixed effects; Zuur et al. 2009). Models initially included an interaction term between time since the beginning of recording and other variables of interest (below), because previous work (Cramer et al. 2016a, b) shows that sperm behavior changes over the time it takes to obtain video recordings for each sample. Specifically, for Experiment 1, we began with a four-way interaction between degree of dilution, type of suspension medium,

species and time since the beginning of filming, as well as all constituent lower-order interactions (Analysis 1, Table S2). For Experiment 2, as indicated above, we began with an unbalanced subset of dilution and suspension-medium treatments. Therefore, we began with a three-way interaction between agitation treatment, suspension medium, and time (as well as constituent pairwise interactions), and pairwise interactions of species with agitation treatment and with time. A four-way interaction was not possible because all Spanish Sparrow males were recorded in DMEM for experiment 2 (Analysis 2, Table S2). To reduce the issue of "cryptic" multiple testing in model selection, we followed Forstmeier and Schielzeth (2011)'s recommendation to first compare the global model (with all fixed effects and interactions) to a null model. As the likelihood ratio tests were significant in all cases ($p < 0.0001$, not shown), we proceeded to simplify models by removing non-significant interactions ($p > 0.05$). We began by removing the highest-order interactions, until only significant interactions, or lower-order interactions supporting a higher-order significant interaction, remained. Finally, we applied false discovery rate correction to F-test results for each test (Verhoeven et al. 2005; Forstmeier and Schielzeth 2011). We base interpretations on corrected values and report the raw values. Because model residuals approximated normality for both velocity and the proportion of motile cells, we modeled each response variable as normally distributed.

During analysis, we noticed that the initial values of proportion motile for experiment 2 seemed higher than the same males' final values for experiment 1, which were measured approximately 30 seconds prior. To test whether this effect was real, and to assess whether it also occurred in velocity, we calculated the average velocity of sperm cells and the overall proportion of motile cells, in the second half of experiment 1 and the first half of experiment 2 (Analysis 3, Table S2). We compared these values using paired non-parametric Wilcoxon tests. Only data

from the appropriate treatment in Experiment 1 was included (i.e., the treatment that was used for Experiment 2).

For Analyses 1-3, we included data only from males with at least 5 well-tracked motile cells in the first and second half of the video recording for velocity models, and only males with at least 15 total cells detected in both the first and second half of the video recordings for proportion motile models. These cut-off values were chosen to include data from a large number of males, while also having data from a moderate to high number of cells for each male. To best allow within-male comparisons, only males that met these criteria for all experimental conditions were included.

To assess whether males' overall sperm performance rank relative to other males was robust to different measurement conditions, we analyzed repeatability, defined as the percent of variance that could be attributed to a random effect of male identity in a mixed effects model (Nakagawa and Schielzeth 2010; Analysis 4, Table S2). For this analysis, in order to simplify the dataset, we calculated the average swimming speed or total proportion of motile cells in each of the six experimental conditions (four combinations of dilution and suspension medium from Experiment 1 and agitation combinations from Experiment 2). We used only males with at least 20 detected cells (proportion motile) or at least 10 well-tracked motile cells (VCL) and fit a linear mixed effects model with REML estimation in the package nlme (Pinheiro et al. 2013). Following the recommendation of Zuur et al. (2009) that all possible fixed effects of interest should be included when assessing significance of random effects, we included an interaction between species and the six-category variable describing the recording conditions. To determine significance of repeatability, we compared the mixed effect model to a model including only the fixed effects using a likelihood ratio test (Zuur et al. 2009; Pinheiro et al. 2013). Note that only a single sample was measured per male. Further, we calculated the coefficient of variation

(standard deviation / mean, *100) in the proportion of motile cells and in VCL among males. Bias-corrected and accelerated (BCa) confidence intervals around the coefficient of variation were calculated using 1000 replicates using the package boot (Canty and Ripley 2017).

Finally, we conducted a re-sampling analysis to assess the impact of the number of sperm cells recorded for each male on sperm swimming speed analysis, by drawing cells from each male from both the concentrated DMEM and PBS treatments of Experiment 1 (Analysis 5, Table S2). On each re-sampled data set, we assessed repeatability as above, with a three-way fixed effect interaction between species, time, and suspension medium, and constituent pair-wise interactions. Additionally, for each re-sampled data set, we compared males' mean speed in DMEM and PBS using a paired t-test. Finally, we calculated the difference between the re-sampled mean and the male's grand mean (including all measured cells) in the DMEM treatment only (chosen arbitrarily). In order to include individuals with a large number of cells to resample from, and to minimize additional variation that we would need to account for, we included only males with at least 150 cells detected in both the concentrated PBS and concentrated DMEM treatments in Experiment 1. We did not include recordings from diluted treatments nor recordings from Experiment 2. We randomly sampled with replacement 5, 10, 20, 30, 40, 50, 100, or 125 cells from each treatment and each male, 100 times for each sample size.

All analyses were conducted in R (3.3.0, R Development Core Team). Unless otherwise noted, mixed effects models were constructed using package lme4 (Bates et al. 2014) with statistical significance assessed via package lmerTest (Kuznetsova et al. 2014). Marginal (r^2_m) and conditional (r^2_c) r^2 values were calculated in package MuMIn (Barton 2016), which reflect variation explained by fixed effects only and by fixed and random effects together, respectively. Trendlines (with 95% confidence intervals for visualization purposes) and other graphs were

269 constructed in ggplot2 (Wickham 2009). Normality of residuals was assessed visually, following
270 Zuur et al. (2009).
271
272 **Results**
273 *Experiment 1: Effects of dilution and suspension medium (Analysis 1)*
274 *Effects on sperm speed-* Swimming speed depended on the interaction between
275 suspension medium and concentration (Fig. 1, $F_{1,205.1} = 10.06$, $p = 0.002$; n = 8 house sparrow
276 and 4 Spanish sparrow males, 9201 cells, $r^2_m = 0.21$, $r^2_c = 0.39$; Table S3). At the start of filming
277 (i.e., estimated intercept from the statistical models), swimming speed was 6-9 μm/sec slower in
278 the diluted PBS treatment than in any of the other treatments ($|t| > 2.7$, $p < 0.01$), while
279 differences among other treatments were not significant ($|t| < 0.81$, $p > 0.4$). Spanish sparrow
280 sperm swimming speed tended to be reduced in PBS compared to the reduction in PBS for house
281 sparrows, though this difference was not significant following correction for multiple testing
282 (corrected $p = 0.054$).
283 Sperm swimming speed declined over time for all treatment combinations (all $|t| > 8.2$, p
284 <0.001), and the rate of decline over time depended on the suspension medium (interaction
285 $F_{1,205.4} = 5.78$, $p = 0.02$). Swimming speed declined more quickly in PBS than in DMEM ($t_{205.4} =$
286 2.4, $p = 0.02$), and they tended to decline faster when sperm were diluted than when concentrated,
287 though this effect was not significant following correction for multiple testing (corrected $p =$
288 0.054).
289 *Effects on the proportion of motile cells-*For the proportion of motile cells, the simplified
290 model included a three-way interaction between time, suspension medium, and concentration
291 (Fig. 1, $F_{1277.58} = 5.52$, $p = 0.02$) as well as significant pairwise interactions of species with time
292 ($F_{1,272.84} = 4.42$, $p = 0.01$; n = 12 house sparrow and 5 Spanish sparrow males, 343 filming

293 locations, $r^2_m = 0.44$, $r^2_c = 0.79$; Table S4). The proportion of motile cells was lower in dilute than
294 concentrated treatments when cells were suspended in PBS ($t_{113.8} = -5.97$, $p < 0.001$) but not in
295 DMEM ($t_{122.3} = -1.02$, $p = 0.31$). Cells suspended in DMEM had a higher proportion motility than
296 cells suspended in PBS for both degrees of dilution ($t > 2.3$, $p < 0.03$).

297 The proportion of motile cells decreased over time in all treatments ($|t| > 4.5$, $p < 0.001$).
298 Decline in the proportion of motile cells over time was greater in Spanish sparrows than house
299 ($t_{272.8} = 2.48$, $p = 0.01$). Decline in the proportion of motile cells was faster in dilute cells
300 suspended in DMEM compared to diluted cells suspended in PBS ($t_{276.7} = 2.48$, $p = 0.01$). Other
301 pairwise comparisons were not significant ($|t| < 1.4$, $p > 0.15$), though the decline tended to be
302 faster in diluted than concentrated cells suspended in DMEM ($t_{276.9} = -1.93$, $p = 0.055$).

303

304 *Experiment 2: Effects of mechanical agitation (Analysis 2)*

305 *Effects on sperm swimming speed.* – The simplified model included a significant pairwise
306 interaction between time and suspension medium (Fig. 2, $F_{1,99.0} = 10.89$, $p = 0.001$) and between
307 time and species ($F_{1,85.9} = 20.27$, $p < 0.001$, n = 9 house sparrow and 4 Spanish sparrow males,
308 2749 cells; $r^2_m = 0.17$, $r^2_c = 0.42$; Table S5). The effect of agitation level was not significant
309 ($F_{1,87.1} = 0.2$, $p = 0.66$). Initial sperm swimming speed was faster for Spanish sparrow sperm than
310 house sparrow sperm ($t_{33.58} = 5.41$, $p < 0.001$), and was faster for sperm suspended in PBS ($t_{40.0} =$
311 2.37, $p = 0.02$). Sperm swimming speed declined over time in all treatments ($|t| > 4.0$, $p < 0.001$)
312 except for house sparrow sperm in DMEM ($t_{86.0} = -0.51$, $p = 0.61$). Comparisons between
313 suspension media in this experiment were between-male tests.

314 *Effects on the proportion of motile cells.* –The final model included significant pairwise
315 interactions between suspension medium and time ($F_{1,84.51} = 5.52$, $p = 0.02$); and species and time
316 ($F_{1,84.49} = 8.34$, $p = 0.004$; n = 9 house sparrow and 5 Spanish sparrow males, 15 analysis frames;

317 $r^2_m = 0.23$, $r^2_c = 0.73$; Table S6). The initial proportion of motile cells was higher in the more
318 highly agitated treatments ($t_{12.55} = 3.78$, $p = 0.002$). The proportion of motile cells declined more
319 quickly in Spanish sparrows than in house sparrows ($t_{84.5} = 2.89$, $p = 0.004$), and more quickly in
320 PBS than in DMEM for both species ($t_{84.5} = 2.35$, $p = 0.02$). Specifically, the proportion of motile
321 cells did not decline significantly over time for cells suspended in DMEM ($t_{85.2} = 0.01$, $p = 0.99$),
322 but it did decline significantly cells suspended in PBS ($t_{84.2} = -2.86$, $p = 0.005$).
323
324 *Comparison of Experiments 1 and 2 (Analysis 3)*
325 In paired tests, the proportion of motile cells at the beginning of Experiment 2 was higher than at
326 the end of Experiment 1 (Wilcoxon V = 96 and V = 104, $p < 0.005$, n = 14 males, Fig. 3), despite
327 the apparent decline over time in cell motility during filming in Experiment 1 and the fact that we
328 filmed Experiment 2 later in time than Experiment 1. We saw no similar "recovery" of cells in
329 velocity: swimming speed in the second half of Experiment 1 did not differ significantly from
330 swimming speed in the first half of Experiment 2 (Wilcoxon V = 22 and 30, $p > 0.6$, Fig. 3).
331
332 *Consistency of male ranking across recording conditions (Analysis 4)*
333 Male identity (which is equivalent to sperm sample identity in our dataset) explained 52.5% of
334 the variation in mean sperm swimming speed and 59.4% of the variation in proportion of motile
335 cells across treatments. These repeatability scores were significant (VCL: likelihood ratio 31.62,
336 $p < 0.001$, n = 18 males, 102 recordings; proportion motile, likelihood ratio 36.96, $p < 0.001$; n =
337 19 males, 111 recordings). In this dataset, samples were measured in all six treatments for 18
338 (proportion motile) and 13 males (VCL); in five treatments for four males (VCL), in four
339 treatments for one male (VCL) and in three treatments for one male (proportion motile).

The between-male CV in proportion of motile cells for highly agitated samples was noteably lower than other measures for proportion motile, though 95% confidence intervals overlapped with intervals for other conditions (Table 1).

Re-sampling analysis: Impact of number of cells analyzed (Analysis 5)

Including a larger number of cells per male per treatment increased the precision of estimated values of between-male repeatability in sperm swimming speed, of the difference in swimming speed between PBS and DMEM, and of the mean speed for each male, but there was no apparent bias when low numbers of cells were included (Fig. 4). In the full data set from which re-sampling was conducted, between-male repeatability in sperm swimming speed was low but significant (with each male represented by a single sample measured in two conditions; Likelihood ratio 757, $p < 0.0001$, 14.87% of variance attributable to male identity, n = 12 males and 7174 sperm cells). For each number of cells re-sampled, the mean repeatability across the 100 re-samples closely approximated the value from the full data set (Fig. 4a). In re-sampled datasets, repeatability was significant ($p < 0.05$) for 56/100 tests using 5 cells per male per treatment, for 96/100 tests using 10 cells per male per treatment, and for all tests using more than 10 cells per male per treatment. In the full data set, swimming speed was higher in DMEM than in PBS ($t_{11} = 2.791$, $p = 0.02$, mean difference = 4.84 μm/s, based on the mean value for each male across all cells in concentrated treatments, Experiment 1). Similarly, cells tended to be faster in DMEM than in PBS, for all re-sampled sets except for some of the smaller data subsets where cells tended to be faster in PBS (9 5-sperm sets, 2 10-sperm sets, and 1 20-sperm set, all with non-significant t-tests; Fig. 4b). T-test results were significant and in the expected direction for 17, 24, 38, 44, 52, 57, 73, and 79 of 100 tests (for 5, 10, 20, 30, 40, 50, 100, and 125 cells per

male per treatement, respectively). The precision of the estimated mean VCL for each male improved with increasing sample size of cells (Fig. 4c).

Discussion

Here, in two congeneric species of passerine birds, we show that sperm performance differs depending on how a sample is prepared for measurement. Specifically, sperm swimming velocity and the proportion of motile cells was typically higher when sperm were concentrated (having been diluted to a lesser degree) and when cells were suspended in a medium that contained nutrients (DMEM), compared to a medium without nutrients (PBS), a result that has also been seen in some mammal species (Farrell et al. 1996; Rijsselaere et al. 2003). This result might suggest an important role for other components of the ejaculate, such as seminal fluid proteins, sugars, or ions, in affecting sperm performance, since these other factors would also be diluted by the suspension medium. Factors such as pH and the concentration of calcium ions are known to affect *in vitro* sperm performance in poultry (Holm and Wishart 1998; Wishart and Wilson 1999), highlighting the ability of sperm to respond to their chemical environment. A similar interactive effect was observed in a study on rooster (*Gallus gallus domesticus*) sperm using a different measure of sperm quality; more diluted sperm exhibited lower sperm performance, particularly when dilution was conducted in saline solution similar to PBS (Parker and McDaniel 2006). While PBS appeared to be a harsh medium in our study, sperm from Bluethroats (*Luscinia svecica*) swim faster in PBS than in a medium derived from conspecific blood plasma (Laskemoen et al. 2008). Which suspension medium and degree of dilution is most representative of conditions sperm face within the female reproductive tract is unclear, making it difficult to determine which sperm measurements are the most biologically relevant.

Surprisingly, we found that mechanical agitation increased the proportion of motile sperm cells. Samples that were subjected to a high degree of agitation before being applied to the microscope slide showed a higher proportion of motile cells, compared to cells from the same sample that had been subjected to minimal agitation. Moreover, the proportion of motile cells was higher at the beginning of Experiment 2, when cells were newly introduced to a microscope slide (which involved agitation as the cells were pipetted to the slide and filled the chamber via capillary action, as well as additional agitation for cells in the high agitation treatment), compared to the same sample at the end of Experiment 1, by which time cells had been on the microscope slide for several minutes. This latter comparison could also be partly due to different conditions cells experienced during incubation in the microscope slide chamber, compared to in the microcentrifuge tube where excess sample was stored between experiments (e.g., differences in availability of oxygen). While biological relevance, if any, of this responsiveness to mechanical agitation is unknown, we can speculate that mechanical agitation of the cells during ejaculation could increase sperm movement as they enter the female reproductive tract; or that mechanical agitation of sperm cells stored in the female's sperm storage organs, for example when a fully-shelled egg is laid, facilitates the exit of sperm from storage (as hypotheized by Grigg 1957, though biochemical stimulation also plays a role in release, e.g., Ito et al. 2011; Hiyama et al. 2014).

The effects of methodological factors on sperm swimming speed and the percentage of motile sperm were not always parallel, for example, with mechanical agitation affecting the proportion of motile cells but not the swimming speed of cells. Similarly, velocity and the proportion of motile cells showed different treatment effects in studies on how sperm respond to conspecific versus heterospecific female fluids (Cramer et al. 2016b), and in methodological optimizations in mammals (Farrell et al. 1996). Together these results strongly suggest that

conditions that affect whether an individual sperm cell is motile or not will not necessarily also affect the rapidity with which it moves.

In this study, we standardized the degree to which we diluted a sample, rather than standardizing the final concentration of sperm cells in the video recording. Which approach provides more reliable and/or biologically relevant results remains to be determined. Moreover, different experimental approaches tended to result in differing levels of between-male variation in sperm performance, which may affect statistical power to detect patterns. Nonetheless, sperm performance was moderately repeatable across experimental treatments among males. In this study we examined only a single sample per male, but previous work in the same captive populations showed low but significant between-male repeatability in sperm swimming speed in a dataset that included multiple samples per male, collected weeks to years apart and recorded following different protocols (Cramer et al. 2015). The same study found near-zero between male repeatability for the proportion of motile cells. Lower repeatability in the study that examined multiple samples per male may be expected if sperm performance changes depending on, for example, social, evironmental, or physiological factors that could differ among sampling events.

Resampling analysis showed little evidence for a bias when low cell numbers were used, and precision of estimates did not improve dramatically when cell numbers increased beyond 20 cells per male. In contrast, recommendations from studies on livestock and humans suggest that at least 200 cells per male be assessed (ESHRE Andrology Special Interest Group 1998). While we agree that including more cells per male is beneficial, we have found it exceptionally difficult to record large numbers of cells for each individual in some species, particularly under field conditions and when trying to assess speed in multiple experimental treatments (pers. obs.). For example, when designing our resampling analysis, we initially included only males with 200 cells per treatment (rather than 150, resulting in a data set of 9, rather than 12, males). However, in this

reduced data set, the paired comparison between DMEM and PBS became marginally non-significant, presumably due to the lower number of males (data not shown). Given the lack of bias with low sperm numbers, we argue that it may be better for studies on evolutionary biology and behavioral ecology to include more males, despite having low cell numbers for some males, than to exclude males with few cells.

This study highlights the need to be consistent within a study in suspension medium, degree of dilution, and the vigor of pipetting applied to samples. Other studies performed in domestic mammals further suggest that factors such as the type of microscope slide is important (e.g., Hoogewijs et al. 2012; Gloria et al. 2014). However, given the significant and moderate repeatability between males across experimental treatments, results of between-male studies examining how sperm characteristics relate to factors such as timing in the breeding season (Cramer et al. 2013b), male age and ornamentation (Sætre et al. 2018), and paternity success (Cramer et al. 2013a; Edme et al. 2017) should be robust to methodological decisions, as long as researchers are consistent within a study. While *in vitro* conditions fail to capture many aspects of the complexity of biological reality (e.g., architecture of the oviduct, viscosity and biochemical milieu of the female reproductive tract) and thus may not provide an accurate picture of sperm behavior (Lüpold and Pitnick 2018), we currently lack the technology to readily conduct *in vivo* experiments in internally fertilizing vertebrates such as birds. Nonetheless, we suggest that until such technology becomes available, we can still capture ecologically and evolutionarily relevant information from carefully conducted *in vitro* studies.

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562
563

Table 1. Coefficient of variation (CV) and 95% confidence intervals (CI) in sperm performance among House and Spanish Sparrow males, in different measurement conditions

Treatment	CV for swimming speed (CI, n males)	CV for proportion motile (CI, n males)
DMEM, Concentrated	5.46 (3.94-7.73, n =18)	33.52 (20.09 – 73.74, n = 19)
DMEM, Dilute	7.12 (5.54 – 9.31, n =18)	39.57 (27.16 – 59.82, n = 19)
PBS, Concentrated	7.08 (6.49 – 10.25, n = 18)	38.60 (22.83 – 71.07, n = 19)
PBS, Dilute	10.03 (7.03 – 15.29, n =16)	42.69 (31.73 – 64.08, n = 18)
Low Agitation	7.27 (6.25 - 9.11, n = 15)	37.54 (27.26 – 50.98, n = 18)
High Agitation	7.76 (6.58 – 9.42, n =17)	21.28 (13.12 – 35.91, n = 18)

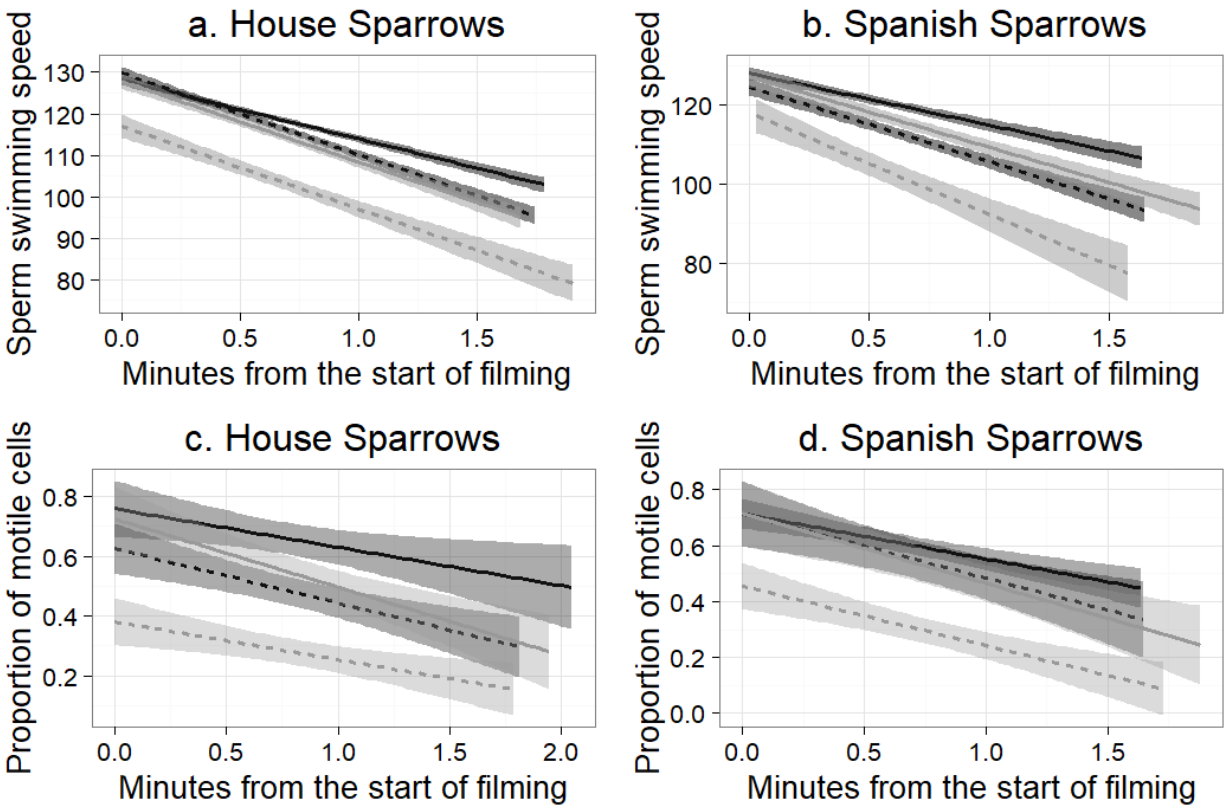
564

565 **Figure Legends**

566 **Fig. 1** Effects over time of suspension medium and the degree of dilution on sperm swimming
567 speed (a,b) and the proportion of motile cells (c,d) in House (a,c) and Spanish (b,d) Sparrows.
568 Details on the methods are under Experiment 1. Each individual was represented in all four
569 treatments, allowing within-individual tests. Dark grey shows the concentrated treatment, light
570 grey line shows the dilute treatment, solid lines show cells suspended in DMEM, and dashed
571 lines show cells suspended in PBS. Shaded areas represent the 95% confidence intervals
572
573 **Fig. 2** Effects over time of mechanical agitation on sperm swimming speed (a,b) and the
574 proportion of motile cells (c,d) in House (a,c) and Spanish (b,d) Sparrows. Details on the methods
575 are under Experiment 2. High agitation level is represented by dark grey, and low agitation level
576 is light grey; solid lines are cells suspended in DMEM and dashed are suspended in PBS. Each
577 individual sample was measured in high and low levels of agitation treatments, but only by a
578 single suspension medium and degree of dilution for this experiment, and Spanish Sparrows were
579 tested only in DMEM. Shaded areas represent the 95% confidence intervals
580
581 **Fig. 3** Overall changes in sperm swimming speed (a,b) and the proportion of motile cells (c,d) for
582 lightly agitated (a,c) and highly agitated (b,d) treatments, combined across Experiments 1 (grey-
583 shaded) and 2 (unshaded). Data from individual males are connected by lines. Cells suspended in
584 DMEM are with solid lines, and cells suspended in PBS are in dotted lines. To simplify
585 visualization, we averaged values within four time periods: the first and second half of
586 Experiment 1 (grey shading, periods 1 and 2, with each half encompassing approximately 1 min),
587 and the first and second half of Experiment 2 (no shading, periods 3 and 4, with each half
588 encompassing approximately 30 seconds). Approximately 30 seconds elapsed between the end of

589 data collection for Experiment 1 and the beginning of Experiment 2. For this figure and analysis,
590 each sample was represented in both agitation treatments but only a single concentration and
591 suspension medium, such that time periods 1 and 2 are the same for lightly and vigorously
592 agitated cells
593
594 **Fig. 4** Results from re-sampling cells from 12 males, from concentrated treatments in Experiment
595 1. The box and midline are the 25^{th}, 50^{th}, and 75^{th} percentiles; whiskers extend to the most
596 extreme value within 1.5 interquartile ranges beyond the 25^{th} and 75^{th} percentiles, and outliers
597 beyond that value are dots. a) Repeatability was assessed as the percent of variance attributable to
598 male identity (equivalent to sample identity) in a mixed-effect model that also included a three-
599 way interaction among fixed effects of species, time, and treatment. The horizontal line indicates
600 repeatability in the full dataset including all measured cells for those males. b) The estimated
601 difference between mean swimming speed in PBS and in DMEM (concentrated cells only) in
602 paired testing. The horizontal line indicates the paired difference in the full dataset. c). The
603 difference between the mean swimming speed of a male's sperm in DMEM in a randomly-
604 chosen subset of cells, compared to his overall mean in DMEM
605

606 Fig 1



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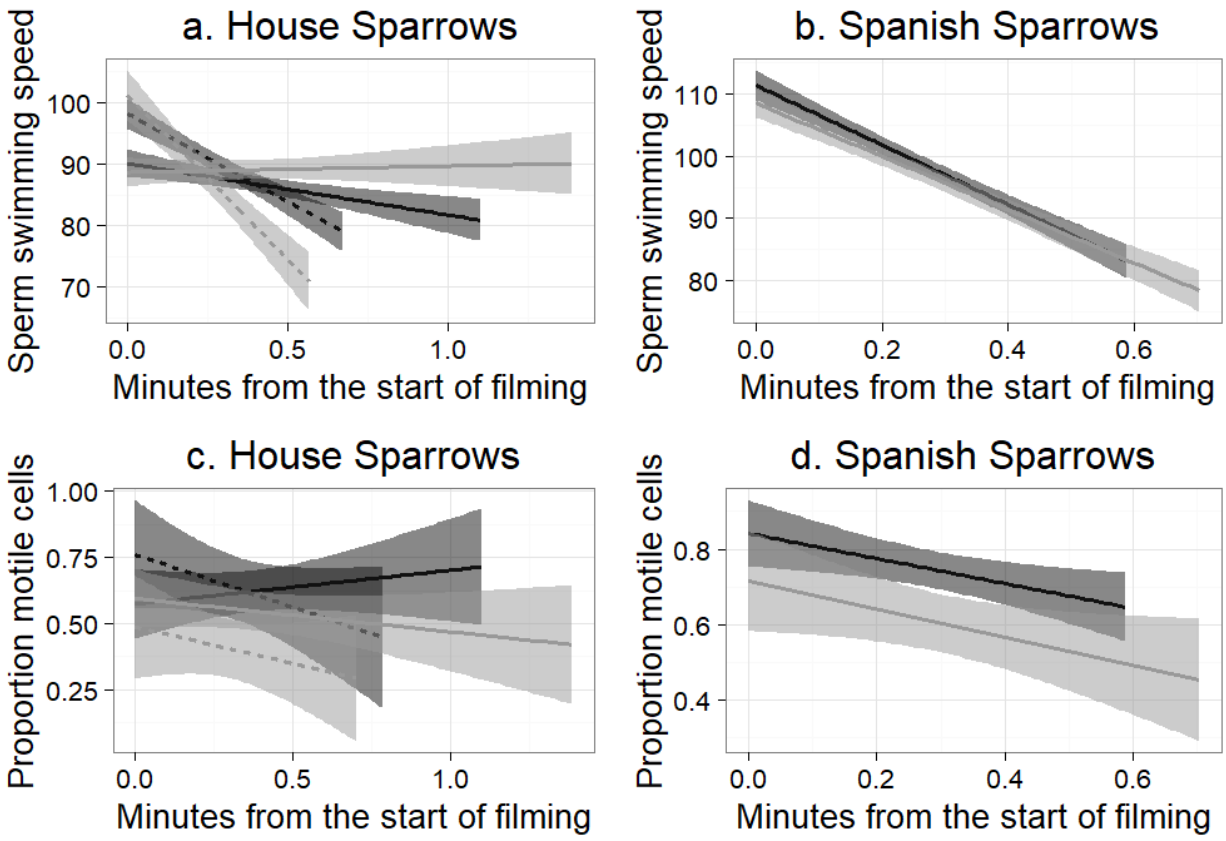
609 Fig 2



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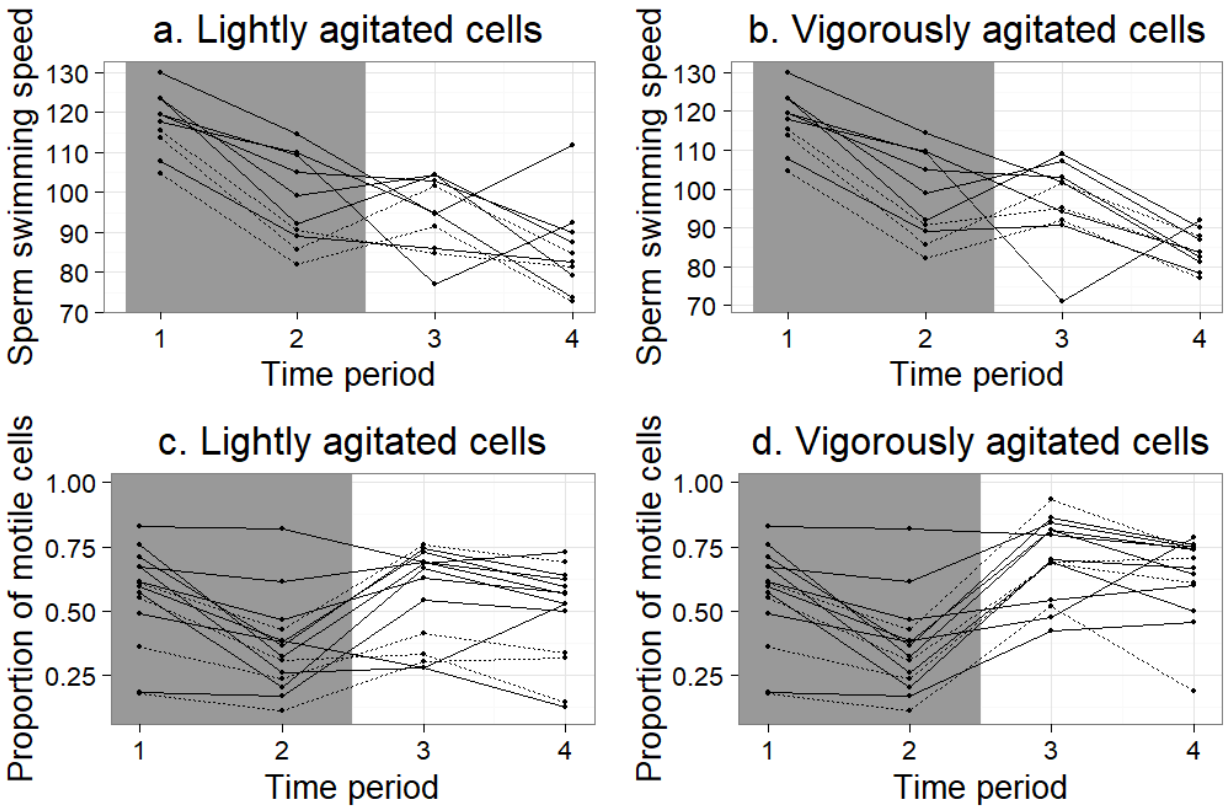
612 Fig3



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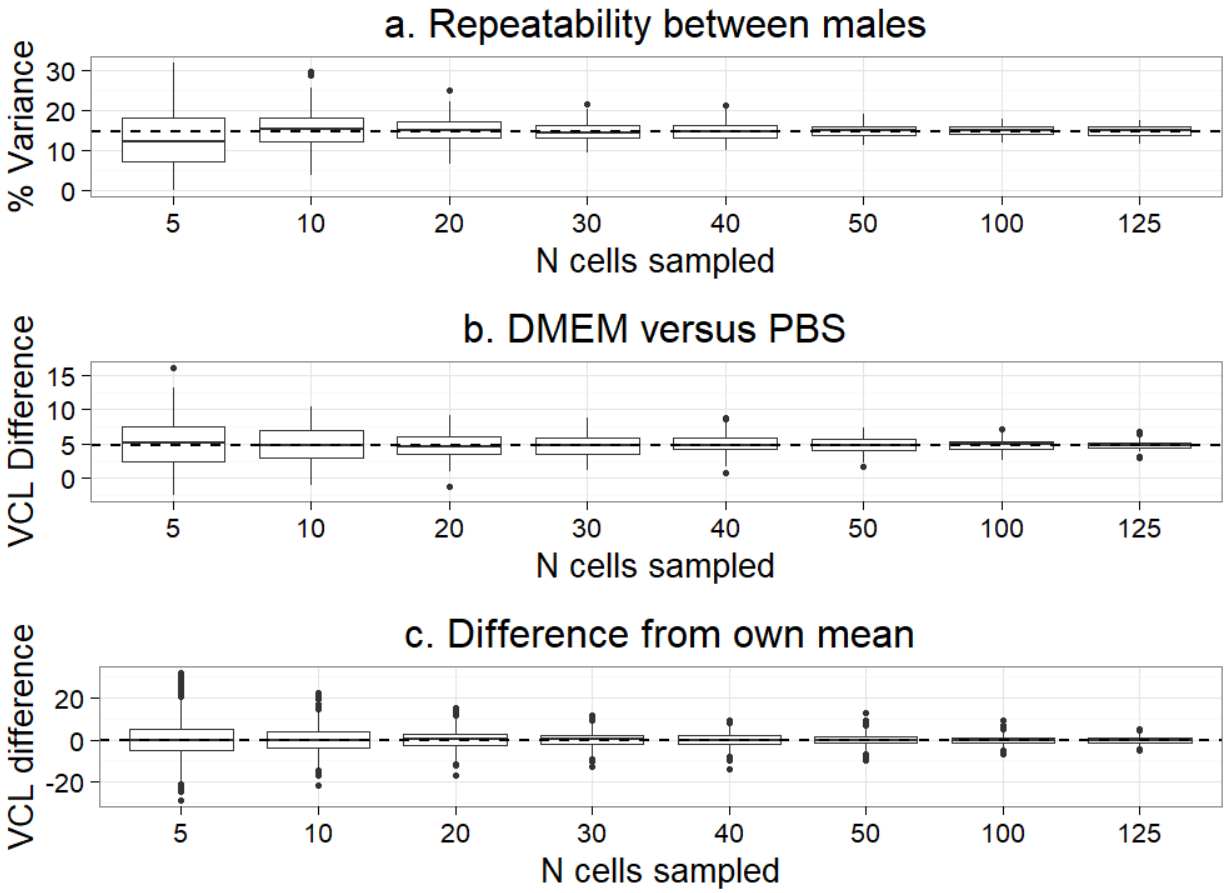
a. Repeatability between males
% Variance
30
20
10
0
5
10
20
30
40
50
100
125
N cells sampled
b. DMEM versus PBS
VCL Difference
15
10
5
0
5
10
20
30
40
50
100
125
N cells sampled
c. Difference from own mean
VCL difference
20
0
-20
5
10
20
30
40
50
100
125
N cells sampled

615