Sperm velocity in a promiscuous bird across experimental media of different viscosities

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Running head: Sperm velocity in viscous media

Abstract

In species with internal fertilisation, the female genital tract appears challenging to sperm, possibly resulting from selection on for example ovarian fluid to control sperm behaviour and, ultimately, fertilisation. Few studies, however, have examined effects of swimming media viscosities on sperm performance. We quantified effects of media viscosities on sperm velocity in promiscuous willow warblers *Phylloscopus trochilus*. We used both a reaction-norm and a character-state approach to model phenotypic plasticity of sperm behaviour across three experimental media of different viscosities. Compared to a standard medium (Dulbecco's Modified Eagle Medium, DMEM), media enriched with 1% or 2% w/v methyl cellulose decreased sperm velocity by up to about 50%. Spermatozoa from experimental ejaculates of different males responded similarly to different viscosities, and a lack of covariance between elevations and slopes of individual velocity-by-viscosity reaction norms indicated that spermatozoa from high- and low-velocity ejaculates were slowed down by a similar degree when confronted with high-viscosity environments. Positive cross-environment (1% *versus* 2% cellulose) covariances of sperm velocity under the character-state approach suggested that sperm performance represents a transitive trait, with rank order of individual ejaculates maintained when expressed against different environmental backgrounds. Importantly, however, a lack of significant covariances in sperm velocity involving a cellulose concentration of 0% indicated that pure DMEM represented a qualitatively different environment, questioning the validity of this widely used standard medium for assaying sperm performance. Enriching sperm environments along ecologically relevant gradients prior to assessing sperm performance will strengthen explanatory power of *in vitro* studies of sperm behaviour.

Keywords:

cryptic female choice, ovarian-fluid viscosity, phenotypic plasticity, *Phylloscopus trochilus*, sperm competition, sperm motility

1. Background

In species with internal fertilisation, spermatozoa typically have to migrate through the female genital tract to reach and eventually fertilise eggs. On this long and challenging journey, spermatozoa face a highly complex and selective environment [1, 2]. While the original adaptive function of such an environment was possibly rooted in pathogen defence, selection may have favoured any extension of sophisticated discrimination and control mechanisms of non-self cells to include control over sperm behaviour and thereby, ultimately, fertilisation. In particular in promiscuous species, sexually antagonistic co-evolution of loci coding for male *versus* female traits in control of fertilisation [3] predicts the evolution of cryptic female choice enabling females to select spermatozoa between and within ejaculates [reviewed in 4, 5].

Obstacles impeding spermatozoa from reaching the egg may include sperm ejection by females [6-8], immunological (e.g. phagocytosis) as well as physico-chemical barriers [e.g. acidic pH and ovarian-fluid viscosity and composition, structure of the cervix, 1, 9]. As a result, only a tiny proportion of the usually vast number of spermatozoa inseminated will ever get close to the site of fertilisation. In galliform birds, for example, it has been shown that only about 1-2% of the inseminated spermatozoa enter the sperm-storage tubules located at the uterovaginal junction of the female genital tract [10, 11]. Traversing the vagina thus seems to represent a major barrier for avian sperm to overcome and recent evidence suggest that the vagina is indeed an important site for sperm selection [12]. A non-random sub-population of fast-swimming spermatozoa reaches the ovum in the zebra finch *Taenopygia guttata*, suggesting that sperm swimming velocity is a highly important trait that enables fast swimming spermatozoa to have a greater chance of migrating through the vagina and entering the sperm-storage tubules [13]. Selection for high sperm velocity is expected to be stronger in more promiscuous species [cf. 14, 15], since sperm in this case will have to compete more intensely with spermatozoa from other males. Sperm selection is likely mediated by a multitude of different filter mechanisms [16], yet detailed knowledge about these mechanisms is currently very limited [reviewed in 17].

One potential mechanism likely to affect sperm transit through the female genital tract that so far has received little attention is ovarian-fluid viscosity. The sperm swimming

environment within the female genital tract is highly viscous, although there may be considerable temporal and spatial variation [18, 19]. Ovarian-fluid viscosity may have a strong impact on sperm swimming performance [e.g. 18, 19, 20, 21] and could thus greatly influence the ability of spermatozoa to migrate through the vagina, to enter the sperm-storage tubules, to leave the tubules at the optimal time and to ultimately fertilise eggs. Furthermore, given the potentially strong effect of ovarian-fluid viscosity on sperm velocity and the potential for ovarian-fluid viscosity to vary in time and space, there may be selection on spermatozoa to be able to perform in different viscosity environments.

While there is a growing body of evidence indicating taxonomically widespread phenotypic plasticity in sperm morphology [e.g. 22, 23, 24], relatively few studies have addressed plasticity in sperm behaviour. In fish, for example, males were found to produce slower sperm when experimentally promoted to higher ranks in social dominance hierarchies [25] or males responded to perceived sperm competition intensity by producing longer-lived [26] or faster sperm [27]. In birds, sperm motility traits varied in a phenotypically plastic manner with male social status [28], female attractiveness [29] or season [30]. Even fewer studies have quantified reaction norms in sperm performance in response to experimentally modified swimming environments, for example across gradients of temperature [31] or pH [32, 33] or when contrasting sperm activated in ovarian fluid *versus* water [34]. In mice, spermatozoa chemo-attracted by progesterone showed erratic trajectories and non-progressive movement in low-viscosity media, but linear trajectories and more progressive movement in high-viscosity media, suggesting the latter should be used for *in vitro* assessment of mammal sperm behaviour to better simulate conditions experienced by sperm *in vivo* [35]. In birds, advanced sperm mobility assays in poultry science made use of media of different traversability to more efficiently discriminate between males with different siring potential [e.g. 36].

Vernon and Woolley [20] used media of two different viscosities (standard medium *versus* medium enriched with 2% w/v methyl cellulose) for an analysis of sperm swimming behaviour in selected wild bird species, including a passerine bird, the starling *Sturnus vulgaris*. Their analyses were purely qualitative, however, and focused on an in-depth description of the functional morphology of sperm propulsion in non-passerine *versus*

passerine birds [20]. Interestingly, nevertheless, the authors stated that "… the actual shape of the sperm head is adapted for the screw-like motion seen in high viscosity media rather than for propulsion through low viscosity salines" [20]. This emphasises the need of incorporating ecologically relevant properties of the sperm swimming environment like viscosity into the quantitative analysis of sperm behaviour. We are, however, unaware of any studies that have examined effects of media viscosity on sperm performance quantitatively in natural bird populations.

Female extra-pair mating is common and widespread in passerine birds [reviewed in 37, 38, 39]. As a consequence, passerines have become the most widely used vertebrate model system to study the ecology and evolution of male reproductive traits under post-copulatory sexual selection. Passerine birds are particularly well suited to study sperm traits due to the ease of non-invasive sperm sampling via cloacal massage [40]. We here used wild willow warblers *Phylloscopus trochilus* to study phenotypic plasticity in sperm velocity in response to three experimental sperm swimming environments characterised by different viscosities. The willow warbler is a widespread and common socially monogamous passerine bird with a high frequency of extra-pair paternity [41-43], suggesting significant post-copulatory sexual selection on sperm competitiveness.

We took two different statistical approaches allowing complementary inference; a reaction-norm (RN) and a character-state (CS) approach [44]. Our RN approach fits one parameter for individual variation in average trait values (in the form of a random intercept variance) and one parameter for individual variation in response to different viscosities (in the form of a random slope variance). Both parameters (i.e. elevation and slope of the reaction norm) are interpretable in evolutionary terms, if we think of phenotypic plasticity as a trait that itself can be the target of natural or sexual selection [44]. Our CS approach, by contrast, treats phenotypes expressed in different environments as different characters/traits and fits separate inter-individual variance parameters for each environment (in the form of random intercept variance). Individual variation in phenotypic plasticity is then modelled indirectly as cross-environmental correlations of sperm performance from individual ejaculates. A perfect correlation among environments suggests no variation in plasticity, while perfect independence of performance in different environments is characterised by zero correlation

among environments and reflects maximum variation in plasticity. The evolutionary motivation for the CS approach is the conception that the trait might have been selected in different environments and phenotypic plasticity arises as a consequence of differential selection in different environments [44]. Despite these different conceptual perspectives on variation in plasticity, the two approaches are mathematically interchangeable in the case of two discrete environments [44].

2. Materials and methods

(a) Study population and field methods

Field work was carried out in the Pasvik Valley (69°28'N, 29°50'W) in northern Norway at the onset of the breeding season on June 15th and 16th in 2012. Male willow warblers (n = 28) were captured with playback and mist-nets. To avoid inadvertent re-sampling of individuals, each male was ringed with a uniquely numbered aluminium ring provided by the Norwegian Bird Ringing Centre at Stavanger Museum. One sperm sample per male was obtained by gently massaging the cloacal protuberance as described in detail elsewhere [40], and immediately diluted in 20 µl pre-warmed (38°C) Dulbecco's Modified Eagle Medium (advanced DMEM, Invitrogen)**.** From this stock solution, 4.5 µl was transferred to either i) 20 µl pre-warmed (38°C) DMEM with 1% w/v methyl cellulose (sodium carboxymethyl cellulose, Sigma-Aldrich; hereafter: cellulose), or ii) 20 µl pre-warmed (38°C) DMEM with 2% w/v cellulose or iii) 20 µl pre-warmed (38°C) DMEM without cellulose. An aliquot of 4.5 µl of each of the three sperm solutions was then deposited on a pre-heated (38°C) microscope count slide (2 chambers, 20 µm, Leja, Nieuw-Vennep, The Netherlands) mounted on a MiniTherm stage warmer (Hamilton Thorne, Beverly, MA, USA) set to 38°C.

Sperm velocity declines with increasing time interval since sampling. Thus to avoid any systematic bias in sperm velocities due to the order in which treatment levels (i.e. 0%, 1% or 2% cellulose) of any experimental ejaculate were recorded, their sequence was alternated and randomised with respect to male/ejaculate identity. Sperm velocity was recorded within two minutes of sampling the experimental ejaculate from a male using a CCD black and white video camera (XCST50CE PAL, Sony) mounted on a negative phase-contrast microscope (CH30, Olympus) with a 10x objective. For each slide chamber/video recording,

multiple independent video takes (between one and ten), each lasting for up to a maximum of five seconds, were recorded in quick succession to increase the number of different spermatozoa measured. Representative video recordings (one for each of three cellulose concentrations) are provided in the electronic supplementary material (ESM; ESM video files 1-3). Birds were released immediately after video recording was finished.

(b) Computer-assisted sperm analysis (CASA)

Videos were analysed using the sperm tracker software HTM-CEROS v.12 (Hamilton Thorne, Beverly, MA, USA). The image analyser was set at a frame rate of 50 Hz and 25 frames (i.e. spermatozoa were tracked for 0.5 seconds). Each video recording was visually examined and cell-detection parameters were adjusted using two interactive quality control plots as well as directly from visual examination of each recording. Video recordings were analysed with MiniDV with a resolution of 720 x 576 (PAL). The minimum size setting for sperm detection was set to nine pixels. The CASA system recorded by default curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL). Spermatozoa with VSL < 15 µm s^{-1} were counted as static and excluded from the motility analyses, along with spermatozoa tracked for < 15 frames. We also excluded from analysis any tracked objects that were spherical (elongation value > 60) as willow warblers have highly elongated sperm heads, see ESM Figure 1. VCL, VAP and VSL were highly correlated (Pearson's r for VCL/VAP: 0.94; VCL/VSL: 0.84; VAP/VSL: 0.96); we therefore decided to focus on just one of these. With no attractant (e.g. egg or chemical gradient) present in our *in vitro* assays, swimming trajectories of spermatozoa were not expected to be straight and we therefore used VCL as the least derived variable [45]. Sperm video recordings were analysed blindly with respect to experimental treatment and ejaculate identity by a single observer (GR).

(c) Statistical analysis

The multi-level hierarchical structure of the data and our specific interest in estimating variance components required a mixed effects modelling approach, the rationale of which we explain in detail below. We took two complementary statistical modelling approaches to analyse phenotypic plasticity of sperm behaviour across experimental environments. First, we adopted a reaction-norm perspective that models sperm velocity as a function of environmental variation in a linear mixed effects model random regression framework [46].

Second, we took a character-state approach, which treats sperm velocities at different cellulose concentrations as different characters/traits and allows the estimation of sperm velocity variances within and covariances among the three different media [47].

Besides cellulose concentration as our fixed treatment effect, we were mostly interested in inter-ejaculate random variation in sperm velocity in order to test the idea that spermatozoa from ejaculates of different males may specialise in their performance in different swimming environments and thereby trade off high velocity in a low-viscosity environment with their ability to show high velocity in a high-viscosity environment, or vice versa. In the following, we therefore focus on analysis of random effects. Note that we had sampled only a single experimental ejaculate per male, such that our ejaculate identity variance term includes both among-individual variation but also among-ejaculate variation caused by uncontrolled environmental effects (e.g. seasonal plasticity in sperm phenotype). We used log-transformed VCL as our dependent variable in all analyses.

Reaction-norm approach

Under the RN approach, we tested for effects of experimental media viscosities on sperm velocity by means of linear mixed effects models and used random regression analyses to test for variation in phenotypic plasticity among ejaculates. Linear mixed effects models were fitted in R 3.5.1 [48] using the function *lmer* from the package *lme4* [49].

As explanatory fixed effects, we included *cellulose* concentration (ranging from 0% over 1% to 2% w/v) as a continuous variable which we mean-centred to obtain biologically meaningful estimates for the intercept and corresponding intercept variances of the random effects described below. As a cellulose concentration of 1% represents the average experimental environment, the intercept of the models thus describes the mean velocity-by-cellulose concentration reaction norm elevation (defined as the predicted phenotype in the average environment experienced) and the corresponding random intercept variances describe the among-ejaculate variation in reaction norm elevations. Furthermore, we included *order* of measurement as an ordered factor (including a linear and quadratic term) to account for slightly different time intervals between obtaining an experimental ejaculate and the start of the three corresponding video recordings (one recording for each of the

three cellulose concentrations). We defined the second order position to be used for estimation of the intercept. Furthermore, we included a *cellulose-by-order* interaction term.

As random effects, we included video *take* identity nested within video *recording* identity nested within experimental *ejaculate* identity as random intercept effects to account for the non-independence in the hierarchical data structure and to estimate the respective variance components (random intercept model). To test for differences in slopes of sperm velocity of spermatozoa from different ejaculates across the range of experimental environments, we added a *cellulose-by-ejaculate* random slope term (random intercept and slope model). To test for potential trade-offs between average sperm velocity and the response in sperm velocity to the cellulose gradient, we evaluated the covariance between the *ejaculate* random intercept term (reflecting random variation in the reaction norm elevation) and the *cellulose-by-ejaculate* random slope term in our random intercept and slope model.

Significance of fixed effects was determined by likelihood ratio tests after removing the focal term from a maximum likelihood (ML) fit of our random intercept model. Significance of random effects (random intercepts, random slopes and covariance among random intercepts and slopes) was determined by likelihood ratio tests comparing models before and after removing the focal terms from restricted maximum likelihood (REML) fits of the respective more complex models. All statistical tests were two-tailed and we rejected the null hypothesis at $p < 0.05$.

Character-state approach

The CS approach treats sperm velocity at different cellulose concentrations as different characters/traits and allows the estimation of velocity variances and covariances within and among the three different cellulose concentrations. We therefore fitted a multi-response mixed effects model that controls for average sperm velocity at each cellulose concentration and for the order of measurement in the fixed effects part of the model separately for the three environments (thus effectively including a *cellulose-by-order* interaction effect).

The random effects part includes the variances within environments and covariances among environments. For each of the three random effects, these are estimated as 3 x 3 variance-covariance matrices for the three cellulose concentrations. Covariances can be easily

converted to correlations by dividing the covariance by the geometric mean of the two respective variances. The random effect for which all variances and covariances could be estimated was *ejaculate* identity. Furthermore, we fitted video *take* identity as another random effect. Note, however, that for video *take* identity the cross-environment covariances were undefined, because each video *take* was nested within a single video *recording* and thus viscosity treatment level. For our experimental design video *recording* identity was confounded with video *take* identity in a model that treats the environments separately and therefore fitting video *recording* identity was not necessary under the CS approach. Residual covariances were undefined by design. We fitted the multi-response models using the software ASReml 3.0 [50]. The significance of fixed and random effects was tested by likelihood ratio tests comparing nested models.

Comparison of approaches

While the three different cellulose concentrations were treated as snapshots of a continuous environmental gradient in the RN approach, they represent three discrete environments in the CS approach (with three cross-environmental covariances). Thus the two approaches are mathematically not interchangeable for our experimental design and the CS approach estimates three more parameters than the RN approach. In contrast to the CS approach, our RN model makes the assumption that the relationship between cellulose concentrations and sperm velocity is linear, which appears to be approximately true for our data after log-transformation. The two approaches also differ in their null hypotheses for significance testing. In the RN approach, the null hypothesis for the random slope term is no individual variation in plasticity while in the CS approach the null hypothesis for the covariance term is no correlation among environments (i.e., the opposite). While it is technically possible to test against a null hypothesis of a perfect correlation between environments (e.g. by likelihood ratio tests), such tests suffer from the fact that both sampling variation and measurement error tend to reduce any actually existing correlation, rendering a significance test against H_0: r = 1 largely meaningless; we therefore refrain from applying such a test under the CS approach. Finally, we note that the multi-response model estimates separate residual variances for each environment, unlike the random slope model of the RN approach

that fits a single residual variance and thus assumes residuals to be identically distributed across all observations.

3. Results

(a) Fixed effects

The final sample size consisted of 10,908 individual spermatozoa originating from 582 video takes of 84 video recordings of 28 experimental ejaculates of 28 males. On average (± SD), 19 ± 18 spermatozoa were tracked per video take, 130 ± 125 per video recording and 390 ± 239 per experimental ejaculate. Fixed effects estimates turned out to be very similar whether estimated under the RN or the CS approach and we therefore only report details from the RN approach in the main text (see ESM Tables 1 and 2 for comprehensive model outputs from both approaches). There was no significant effect of *order* of measurement on sperm velocity (random intercept model: $\chi^2 = 3.33$, df = 2, p = 0.19, Figure 1, see ESM Table 1a for detail). Sperm velocity, however, decreased by nearly 50% from the lowest to the highest cellulose concentration (random intercept model: $\chi^2 = 183.1$, df = 1, $p < 0.001$, Figures 1 and 2, ESM Table 1a; note that this decline represents the mean population-wide sperm *velocity-by-cellulose* concentration reaction norm slope). The strength of the observed decrease in velocity was independent of the order in which video recordings associated with specific cellulose concentrations were taken within experimental ejaculates (random intercept model: *cellulose-by-order* interaction: $\chi^2 = 0.87$, df = 2, p = 0.65, Figure 1). Subsequent analyses of random effects were therefore based on a fixed effects structure represented by the additive effects of cellulose concentration and order of measurement.

(b) Random effects under a reaction-norm approach

We found significant between-video *take* variance ($\chi^2 = 47.9$, df = 1, $p < 0.001$), between-video *recording* variance ($\chi^2 = 33.8$, df = 1, $p < 0.001$) and between-*ejaculate* variance ($X^2 = 9.5$, df = 2, p = 0.002) for the sperm *velocity-by-cellulose* concentration reaction norm elevation (ESM Table 1a). However, conditional on the fixed effects *cellulose* concentration and *order* of measurement, these variance components explained only 2.3%, 2.8% and 2.8% of the total phenotypic variance, respectively. Including a *cellulose-by-ejaculate* random slope term did not significantly increase model fit ($\chi^2 = 0.13$, df = 2, p = 0.94; ESM Table 1b).

This indicates that spermatozoa from different ejaculates respond in a similar way to changes in cellulose concentrations (see Figure 2). The correlation between the random intercept term for *ejaculate* identity and the *cellulose-by-ejaculate* random slope term in our random intercept and slope model was negative (r = -0.27) but not significantly different from zero (χ^2 = 0.08, df = 1, p = 0.77; ESM Table 1b). This suggests that spermatozoa from ejaculates with different elevations of the sperm *velocity-by-cellulose* reaction norm did not differentially decrease in sperm velocity across the experimental cellulose gradient. Thus spermatozoa from ejaculates with high mean sperm velocity in the mean environment experienced (cellulose concentration = 1%) were affected by changing cellulose concentrations in a similar way to spermatozoa from ejaculates with low mean sperm velocity.

(c) Random effects under a character-state approach

Controlling for the fixed effects of *cellulose* concentration and *order* of measurement (see ESM Table 2 for details), there was significant between-ejaculate variation in sperm velocities, accounting for 5-11% of the variance in the three different cellulose concentrations (lowest between-ejaculate variation at 1%, highest at 0%, Table 1). Furthermore, we found low, but significant, variation in sperm velocities among different video *takes* within ejaculates, accounting for 1-4% of the phenotypic variance (after controlling for fixed effects, Table 1). All between-environment correlations were estimated positive, but only the correlation in sperm velocities between cellulose concentrations of 1% and 2% was strong (r = 0.88 ± 0.11) and highly significant (χ^2 = 12.14, df = 1, p = 0.0005, Table 2). By contrast, both correlations involving a cellulose concentration of 0% were substantially weaker (r = 0.17 ± 0.26 and 0.47 ± 0.22, respectively) and not significantly different from zero (0% *versus* 1%, χ^2 = 0.36, df = 1, p = 0.55, 0% *versus* 2%, χ^2 = 2.94, df = 1, p = 0.09). This indicates that a cellulose concentration of 0% (i.e. pure DMEM without dissolved cellulose) represented a qualitatively different environment compared to the other two treatments. By allowing for separate residual variances in the three cellulose concentrations, our CS approach also confirmed a decrease in the residual variance from lower to higher cellulose concentrations (χ^2 = 6.00, df = 2, p < 0.05) when comparing the full model to a model where

residual variances were constrained to be identical for the three environments (Figure 1; see also Table 1).

4. Discussion

Although ovarian-fluid viscosity may exert a strong influence on sperm swimming behaviour (see introduction) few studies have examined this relationship empirically, in particular in birds. In the poultry industry, sperm mobility tests used media of different traversability with the goal to better discriminate between males with different siring potential [e.g. 36]. Vernon and Woolley [20] used media of two different viscosities (standard medium *versus* 2% cellulose) for their analysis of sperm swimming behaviour in selected bird species, including a passerine, the starling *Sturnus vulgaris*. But their analyses were purely qualitative and focused on a detailed description of the functional morphology of sperm propulsion in non-passerine *versus* passerine birds [20]. Our study, therefore, represents the first quantitative analysis of sperm behaviour as a function of media viscosities in any passerine bird. By combining a reaction-norm with a character-state approach, our study revealed four major findings: First, sperm velocity sharply decreased with increasing levels of media viscosities; second, ejaculates of different males responded in a very similar way when exposed to different viscosities; third, a lack of covariance between elevations and slopes of reaction norms indicated that spermatozoa from high-velocity ejaculates were not slowed down more strongly than spermatozoa from low-velocity ejaculates; and fourth, spermatozoa from different ejaculates demonstrated positive cross-environment correlations in velocity in 1% *versus* 2% cellulose but none with 0% cellulose.

Main effects of media viscosities on sperm velocity

Sperm velocity decreased substantially with increasing media viscosities, featuring a 50% decline in sperm swimming velocity from our lowest to highest experimental media viscosities. This has important methodological and biological implications.

In internally fertilising species, spermatozoa are unlikely to be confronted with swimming environments that resemble, in terms of viscosity, the standard cell culture media commonly used for assaying sperm performance (velocity, motility). Several authors have speculated

that the female genital tract, portrayed as challenging to sperm in general [1], must possibly represent a high-viscosity environment [18, 20]. In fact, the nearly universal cork-screw design of the passerine sperm head [ESM Figure 1, see also 51, 52] and the highly distinct style of twist-drill swimming of passerine sperm [20] are strongly suggestive for high-viscosity fluids being a defining feature of the selective environment to which passerine spermatozoa are exposed [15, 53]. Thus enriching sperm environments along potentially relevant gradients prior to assessing their performance will help strengthening the reliability and explanatory power of *in vitro* studies of sperm performance in birds and other taxa [for mammals see e.g. 35]. Indeed, recently this step has sometimes been incorporated into experimental designs. For example, Cramer et al. [54] used experimental media containing female genital tract fluid to test for differential sperm performance across (sub-) species boundaries of closely related *Ficedula* flycatchers.

The striking changes in sperm velocity in response to the viscosity of the medium might also be of substantial evolutionary significance, in particular in the context of cryptic female choice [5, 55]. The willow warbler is a highly promiscuous species [41-43] and patterns of variation in sperm length suggest that sperm competition is generally high across the wide distribution ranges of two subspecies [56]. Thus a predicted high ovarian-fluid viscosity in the female genital tract may contribute to better informed cryptic female choices of preferred spermatozoa within ejaculates but in particular among competing ejaculates [the latter in a similar way poultry science has used media of different traversability with the goal to better differentiate between males with different siring potential, see 36]. High fluid viscosity may here serve as one of several filter mechanisms weeding out spermatozoa with inferior swimming performance and/or enable females to generally slow down sperm swimming velocity in order to allow other probing mechanisms, for example the immune system, to function best. Furthermore, our results show that with only small changes in ovarian-fluid viscosity females may be able to impose large effects on sperm behaviour (cf. Figure 2), potentially allowing them to fine-tune selectivity in response to e.g. specific copulation partners or general social and environmental conditions. Unfortunately, to our knowledge, no information is available with respect to fluid viscosity in the genital tract of female birds. Examining this represents an important line for future research including the study of potential temporal dynamics for example in relation to the female reproductive

cycle. Theoretical considerations and the visual inspection of individual reaction norms in our results suggest that the effects of media viscosities on sperm velocity may not be linear, but rather a decelerating function (Figure 2). Only three experimental cellulose concentrations are, however, insufficient to allow further inference regarding the potential curvature of this effect. Future work should expose spermatozoa to a better resolved and also wider range of experimental cellulose concentrations. Furthermore, as cellulose concentration may not scale linearly with viscosity, it would also be informative to measure the actual viscosity of experimental (and of course natural, see above) sperm swimming environments.

Sources of random variation and co-variation in sperm velocity

In both statistical frameworks, we found low, but significant, between-video take variance within video recordings in sperm velocity. This pattern suggests small-scale variation e.g. in cellulose concentration within slide chambers or artefacts due to edge effects, for example when the slide chamber is not fully or not equally filled such that some of the probed grids start drying from the margins earlier than others. While such variance components are often not accounted for in statistical models applied to CASA data, our results suggest they should. For any split-ejaculate designs, the same reasoning applies to the low but significant between-video recording variance within experimental ejaculates detected under the RN approach.

Interestingly, we found significant between-ejaculate variance in sperm velocity both in our random slope model and in each of the three environments under the CS approach. In promiscuous species in particular, sperm velocity is a potentially highly relevant trait in determining competitive fertilisation success [15] and testing for consistent differences among individual males in natural populations is therefore important. In our data set, however, male and ejaculate identity are confounded as just one experimental ejaculate per male had been sampled. Thus it remains unclear at present whether between-ejaculate variance in our models actually maps to individual male phenotypes (or even genotypes) rather than individual ejaculates. While some evidence for consistent among-individual variation in sperm velocity suggests the former [e.g. 40, 57], only routinely sampling

replicate ejaculates within males will allow evaluating the relative contribution of between-ejaculate and between-male variation in this key sperm trait.

In our RN approach, we found no evidence that spermatozoa from different ejaculates responded differentially to changes in media viscosity. This corresponds well with the visual representation of our aggregated raw data (Figure 2) and may suggest biomechanical constraints on cross-environment sperm performance and/or selection acting on the slope of the velocity-by-viscosity reaction norm, resulting in little genetic and/or phenotypic variation in this trait. It remains unclear, however, whether any random slope variation would refer to individual male phenotypes (or even genotypes) rather than individual ejaculates (as discussed above). An extended sampling regime with higher replication on under-sampled grouping levels would be necessary to firmly exclude the possibility of between-male random slope variation. Such a sampling regime may be more feasible in captive rather than natural bird populations. Under the RN approach, we found that spermatozoa from ejaculates with different elevations of their *velocity-by-cellulose* reaction norm (i.e. that have relatively fast or slow spermatozoa in the mean experimental environment experienced) did not differentially decrease in sperm velocity across the experimental cellulose concentration gradient. Thus there is also no evidence for any kind of trade-off in sperm performance (which could lead to specialisation), for example such that generally fast spermatozoa tire out sooner in environments where they meet with more resistance.

The CS approach estimated all correlations in sperm velocity among cellulose concentrations to be positive, but both correlations involving the lowest cellulose concentration (i.e. 0%) were low and non-significant. This indicates that sperm performance in a medium without cellulose was strongly independent from sperm performance in the presence of cellulose, which questions the validity of inference from sperm performance assays in such standard media. In a within-species context, for example, a reported lack of significant associations between i) sperm morphology and sperm velocity as assessed in standard media [e.g. 58] or ii) sperm velocity as assessed in standard media and competitive fertilisation success [e.g. 59] may then represent false negative results. The correlation among viscosities of 1% and 2%, however, was strong and highly significant, emphasising the importance of among-

ejaculate variation in overall swimming velocity rather than specialisation to these different media viscosities. This result suggests that sperm velocity of individual ejaculates/males represents a transitive trait, the rank order of which is maintained when expressed across different environmental backgrounds. In line with this and the results from the RN approach, since none of the correlations was estimated to be negative, there is no indication for specialisation, which would be the case if different ejaculates contained sperm that perform well in some environments, but poorly in others.

Ethics

Permits to capture, handle and ring the birds were issued by the Norwegian Directorate for Nature Management to OK (A-license, 1082). Sperm was collected in adherence with the Norwegian regulations for the use of animals in research.

Data accessibility

Data including R and ASReml code are available as electronic supplementary materials.

Authors' contribution

OK and GR conceived the study and conducted the field work. GR performed the computer-assisted sperm analysis. TS analysed the data (reaction-norm approach) and led the writing of the manuscript. HS analysed the data (character-state approach). All authors contributed critically to the drafts and gave final approval for publication.

Conflict of interest

We declare we have no competing interests.

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Tables

Table 1: Variance components and ratios of variance components (± SE) of curvilinear sperm velocity of 10,908 spermatozoa from 582 video takes from 84 video recordings of 28 experimental willow warbler *Phylloscopus trochilus* experimental ejaculates/males in three experimental media of different viscosities (cellulose concentrations). Estimates are from a multi-response mixed model under a character-state approach (see methods for details).

	Phenotypic variance V_P	Among *ejaculate* variance V_{EJ}		Among video *take* variance V_{VT}		Residual (within video *take*) variance V_R	
Cellulose (w/v)	V_P	V_{EJ}	V_{EJ}/V_P	V_{VT}	V_{VT}/V_P	V_R	V_R/V_P
0%	731.51 ± 31.23	78.75 ± 26.65	0.11 ± 0.03	22.33 ± 7.07	0.03 ± 0.01	630.43 ± 16.29	0.86 ± 0.03
1%	455.97 ± 13.12	24.63 ± 8.97	0.05 ± 0.02	6.27 ± 2.62	0.01 ± 0.01	425.07 ± 9.64	0.93 ± 0.02
2%	277.36 ± 9.76	21.40 ± 7.76	0.08 ± 0.03	11.91 ± 2.91	0.04 ± 0.01	244.05 ± 5.77	0.88 ± 0.03

le 2: Within-ejaculate, cross-environment phenotypic covariances (± SE; lower left) and correlations (± SE; upper right) of curvilinear sperm velocity of 908 spermatozoa from 582 video takes from 84 video recordings of 28 experimental willow warbler *Phylloscopus trochilus* experimental culates/males in three experimental media of different viscosities (cellulose concentrations). Estimates are from a multi-response mixed model under a racter-state approach (see methods for details).

ellulose (w/v)	0%	1%	2%
0%	–	0.168 ± 0.255	0.467 ± 0.215
1%	7.40 ± 11.56	–	0.876 ± 0.108
2%	19.18 ± 11.12	20.11 ± 7.29	–

Figure legends

Figure 1: Mean curvilinear sperm velocity per video recording as a function of cellulose concentration (% m/v) and order of measurement (within ejaculates) for N = 84 video recordings of 28 experimental willow warbler ejaculates/males. Boxplots show median, interquartile range (box) and data within 1.5 times the interquartile range (whiskers). Note that boxplots are based on aggregated raw data (per recording) while statistical analyses were based on log-transformed values of sperm velocity for individual spermatozoa.

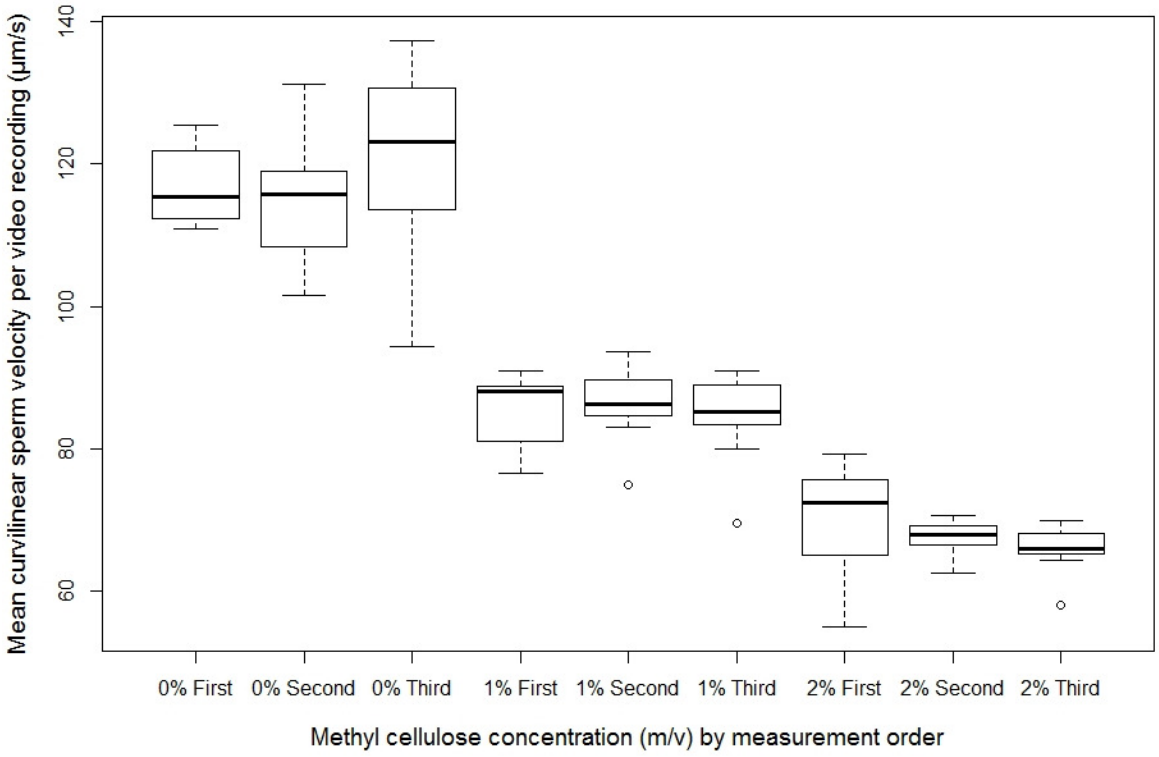

Figure 2: Mean curvilinear sperm velocity per video recording as a function of cellulose concentration for N = 28 experimental willow warbler ejaculates/males (identified by ring numbers). Note that on display are aggregated raw data (per recording) which in contrast to statistical analysis are not controlled for the (non-significant) effects of order of measurement.