Behavioral Ecology and Sociobiology (2021) 75:84
https://doi.org/10.1007/s00265-021-03013-4

METHODS PAPERS

Sperm Sizer: a program to semi-automate the measurement of sperm length

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Received: 28 December 2020 / Revised: 22 March 2021 / Accepted: 29 March 2021

Abstract

Research on sperm is incorporated into many areas of ecology and evolution including including sexual selection, reproductive physiology and ecotoxicology, as well as comparative studies in evolution and phylogenetics. Currently, producing data on sperm morphology involves several time-consuming steps, particularly photographing sperm and measuring their length (e.g. head, midpiece, tail and total sperm length). Here, we present *Sperm Sizer*, a freely available Java program that semi-automates the process of measuring sperm length along the centre of the sperm (including head, midpiece, tail and total length). We compare sperm measurements made with *Sperm Sizer* to those made with the widely used non-automated software ImageJ, for sperm from a single bird species (the long-tailed finch *Poephila acuticauda*), eight species of passerine bird and eight species of lizard, and provide examples demonstrating that the program can measure at least some mammalian, fish and mollusc sperm. Sperm length measurements from *Sperm Sizer* are highly correlated to those made using ImageJ, demonstrating that *Sperm Sizer* produces high quality sperm length data while taking drastically less time. Our data suggests that *Sperm Sizer* measurements could possibly be incorporated into existing large datasets with a small correction, although this will need to be assessed on a case-by-case basis. We suggest that generally, sperm image quality (high contrast, minimal overlap of sperm, etc.) will be more important than the shape of the sperm for whether or not *Sperm Sizer* is for whether or not *Sperm Sizer* can be employed for a given project.

Keywords Sperm morphology · Sperm length · Sperm competition · Spermatozoa

Introduction

Sperm size varies across species, within a species, and even within and between the ejaculates of individual males (Pitnick et al. (Pitnick et al. 2009)). For sexually reproducing taxa, male fitness relies on having functionally competent and competitive sperm, and sperm morphology has been shown to play a role in determining fertilisation success under both non-competitive and competitive conditions (Simmons and Fitzpatrick (Simmons and Fitzpatrick 2012); Bennison et al. (Bennison et al. 2015)). Consequently, much attention has been directed to understanding the evolution of sperm morphology and the functional significance of variation in sperm length (Fitzpatrick et al. (Fitzpatrick et al. 2010); Rowe et al. 2015a). While there have been prudent suggestions to consider a more diverse array of sperm morphological measures (Støstad et al. (Støstad et al. 2018); Hook and Fisher (Hook and Fisher 2020)), there remains considerable interest in quantification of sperm length and particularly the ratios between the lengths of different sperm components that may influence sperm velocity (Humphries et al. (Humphries et al. 2008); Hook and Fisher (Hook and Fisher 2020)).

Currently, one of the barriers to insightful ecological and evolutionary work on sperm is that quantifying sperm morphology requires time-consuming and often costly approaches. For instance, recommendations to measure the length of 5–30 sperm per male (Bennison et al. (Bennison et al. 2015); Kahrl and Cox (Kahrl and Cox 2015)) require a

Communicated by S. Pruett-Jones

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Published online: 27 April 2021

considerable time investment to both photograph the sperm cells and to measure their length (i.e. head, midpiece, tail length), which may constrain the sample sizes that can reasonably be assessed. Here, we focus on improving the process of sperm measurements, as while there are already programs available to speed up or semi-automate quantification of sperm head morphology (e.g. Yániz et al. (Yániz et al. 2015); Skinner et al. (Skinner et al. 2019)), there are no widely adopted programs to rapidly measure sperm length in non-model taxa (see Hook and Fisher (Hook and Fisher 2020), for other available approaches). Some sperm motility programs offer a service that automatically estimates some components of sperm morphology (e.g. Microptic Sperm Class Analyzer 5.4.0.0 SCA®), but these systems are costly, and software is optimised for use with mammalian sperm, especially model taxa such as humans.

To overcome these limitations, we developed *Sperm Sizer*, a freely available semi-automated program to measure the length of sperm components. Here, we present data to confirm the validity of *Sperm Sizer* for measuring sperm length by comparing measurements obtained using *Sperm Sizer*to those obtained using ImageJ Version 1.52a (Schneider et al. (Schneider et al. 2012)) for (i) a dataset of 75 sperm cells collected from 10 male long-tailed finches, (ii) a dataset of 82 sperm cells from 8 passerine bird species and (iii) a dataset of 85 sperm cells from 8 species of lizard (using on average 3 males per species for both i and ii). Finally, we also use*Sperm Sizer* to measure sperm from broader taxonomic groups (mammals, a fish, a mollusc) to demonstrate the broad potential of the program.

Methods

Software overview

Sperm Sizer is a Java program that is openly available for download and use on both PC and Mac (https://github.com/wyrli/sperm-sizer/). *Sperm Sizer* employs user-selected points to identify the beginning and end of the sperm components (head, midpiece and tail; Figs. 1 and 2b). The program then ('behind the scenes') automatically converts the image into a binary image (black and white, or red and 'not red' in Fig. 2c) by increasing the threshold value (Fig. 2d–e) until the four points are connected by one continuous selection (Fig. 2e). The program then finds the centre line along the selection ('skeletonization') and measures lengths along that line between the user selected points (Fig. 2f). Once completed, the user then sees the traced lines between the points they selected, as well as the measurement lengths (in pixels; Fig. 2f), which can be exported as an image. The measurements are saved as a CSV file ready for conversion from pixels to micrometres.

Users have the opportunity to manipulate a number of specifications of the program in the *config.ini* file, including line thickness, marker size, labels and degree of line smoothing. Changing the line smoothing may be appropriate for some biological systems but may alter measurement lengths and so needs to be reported. Below, we use standard settings. Due to its reliance on contrast to identify sperm cells, *Sperm Sizer* may not work where whole sperm or parts of the sperm are extremely faint and not strongly contrasted with the image background. Hence, before generating a large dataset of microscope images to measure with *Sperm Sizer*, it is important to try *Sperm Sizer* with a few photos. There are likely to be some relatively straightforward adjustments to how the photographs are taken (e.g. contrast level) that will maximise how well *Sperm Sizer* works. In addition, for challenging photos where the tips of sperm head or tail are too faint to be picked up *by Sperm Sizer*, the user can select slightly inwards (away from sperm tips) so that the sperm is recognised and then use the Straight-line Extension feature to extend the existing measurements in a straight line to reach those faint tips of the sperm (not used in the current study). While *Sperm Sizer* still requires user input when demarcating sperm components, a fully automatic system would likely be either highly species specific or introduce error to the data, or both. *Sperm Sizer* also likely increases repeatability of measurements by eliminating the requirement of ImageJ that users select many points along a curved sperm cell, as the number of points selected by different users may introduce a source of error in the measurements.

Examples — ground-truthing *Sperm Sizer*

Sample preparation and photography

Sperm fixation followed slightly different methods for long-tailed finches *Poephila acuticauda* and the other passerines (as per Rowe et al. (Rowe et al. 2015), (Rowe et al. 2015)) and for the lizards (as per Kahrl et al. (Kahrl et al. 2019)), and three different imaging systems were used. Long-tailed finch samples were images at 200× magnification using a camera (14MP Aptima COMS, RisingCam) attached to a brightfield microscope (Olympus BX50, Olympus Japan). The other passerine species were captured at magnifications of 160× or 320× (depending on species and total sperm length) using a camera (DFC420, Leica) attached to a digital microscope (DM6000 B Leica). The lizards were imaged with differential interference contrast microscopy using an Olympus Magnafire Camera (Olympus America, Melville, NY) at 100× magnification. The examples of mammal and insect sperm are from the San Diego Zoo Sperm Atlas (https://ielc.libguides.com/sdzg/databases/spermatlas), an online database with photos of sperm collected for a variety of species. These photographs were taken mostly using light microscopy but some with electron microscopy.

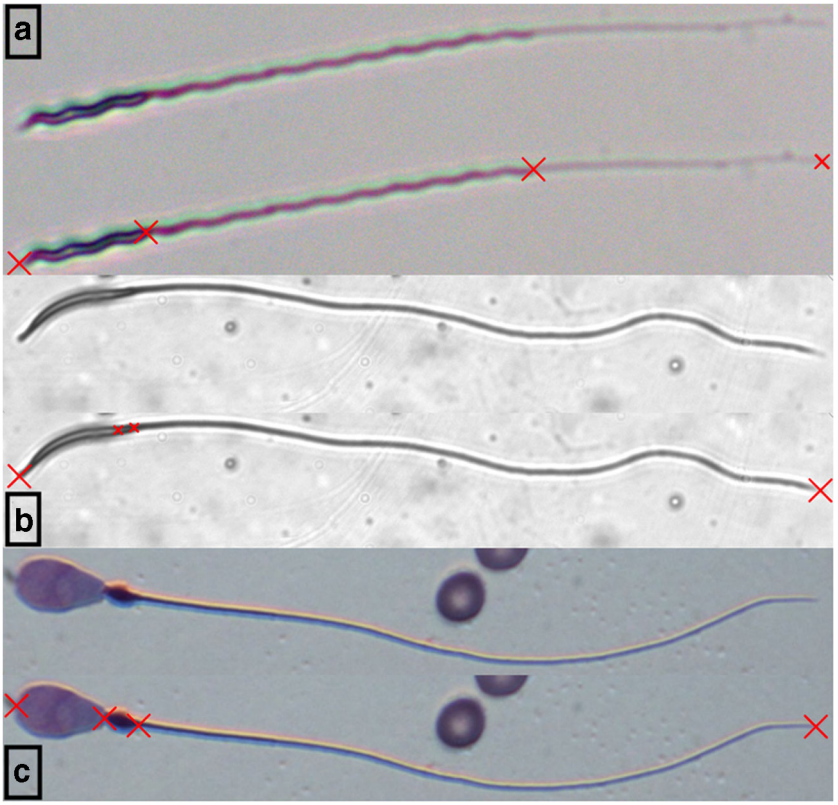

Fig. 1 Typical sperm of the different taxa measured in this paper. **a** Long-tailed finch, typifying passerine birds; **b** brown anole *Anolis Sagrei*; **c** desert bighorn sheep *Ovis canadensis nelson* demonstrating the rounder head of many mammal sperms

Ground-truthing datasets

For all three datasets, individual sperm cells were measured with both *Sperm Sizer* and manually with ImageJ, blind to the measurements taken by the other software. We used photographs that had been taken for analysis with ImageJ, as there were typically plenty sperm in the photos that were not crossing over. Before measurement was taken, all the sperm cells were marked using the 'pen' tool in ImageJ to specify points between the sperm components (head, midpiece and tail). These marks were then used as a point of reference for both ImageJ and *Sperm Sizer* measurements to try and keep the start and end points of sperm selection as consistent as possible, minimising noise introduced into the data due to how the user selected these points. To measure sperm with ImageJ, we used the segmented line tool to click multiple points along the length of each sperm cell component, following the curvature of the sperm, and report the summed length between these points for each component (sensu Kahrl and Cox (Kahrl and Cox 2015)). In lizards, the midpiece is a short section at the base of the head (Kahrl and Cox (Kahrl and Cox 2015)), whereas in passerines, the midpiece is typically much longer and winds around the flagellum (Fig.1). With the exception of the long-tailed finch, where 75 sperm cells (from 10 males) were measured, a set of 9–12 sperm cells (average = 10.5) were measured for each species using *Sperm Sizer*. Using R Version 3.5.1 (R Core Team (R 2018)), for each sperm component (head, midpiece, tail, total) of the long-tailed finch, we performed a linear model using the lme4 package (Bates et al. (Bates et al. 2015)) with the ImageJ measurement as the predictor and*Sperm Sizer* measurement as the response variable. For the multispecies datasets (passerines and lizards), we performed a linear mixed model (LMM) with 'species' as a fixed effect for each sperm component, with separate models run for lizards and passerines. From each of these models, we extracted the multiple R^2 values using the summary() function to examine how tightly correlated the measurements of sperm components were between *Sperm Sizer* and ImageJ. Next, we assessed the appropriateness of combining measures from the two systems into a single study and to see whether a correction could facilitate combining datasets. To do this, we calculated the average difference in length between *Sperm Sizer* and ImageJ for each sperm component for every sperm cell and report this difference as a percentage of the *Sperm Sizer* measurements of that individual sperm cell.

Finally, to assess whether *Sperm Sizer* increases within-measurer repeatability by removing the need for users to select multiple points along a curve as required for ImageJ, we measured a set of 35 long-tailed finch sperm cells twice using ImageJ and twice using *Sperm Sizer*and compared the repeatability between the two using the rptR package (Stoffel et al. (Stoffel et al. 2017)).

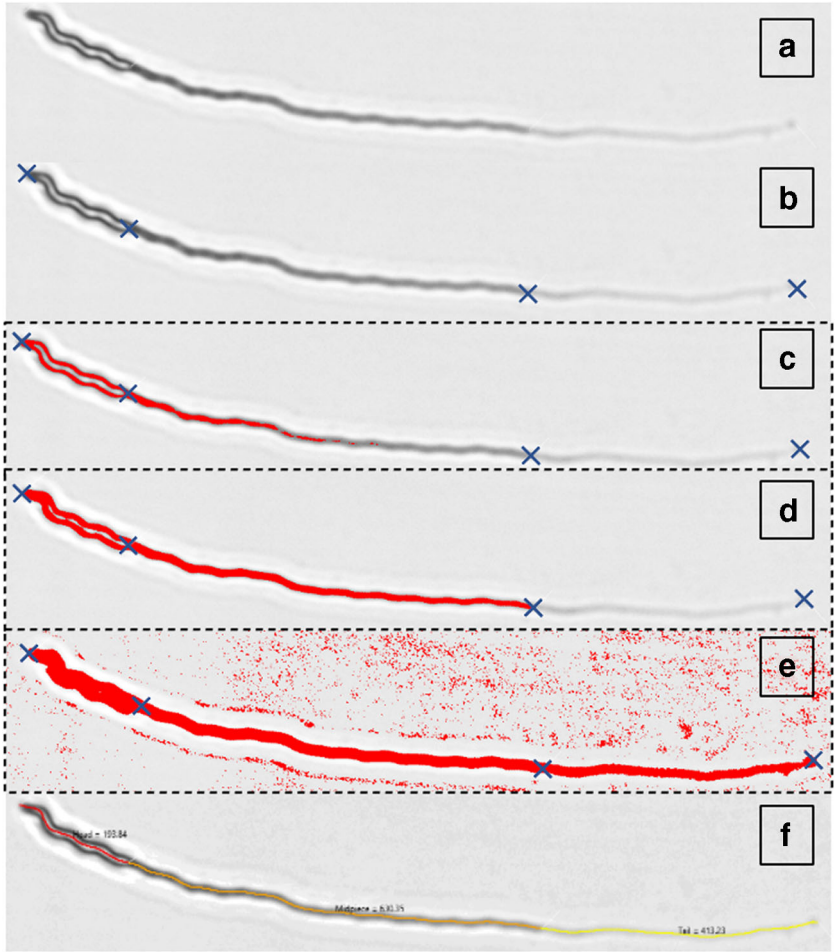

Fig. 2 *Sperm Sizer* in action, with **c**-**e** happening 'behind the scenes'. A sperm (**a**) is measured by first the user specifying points separating sperm components (the blue Xs in **b**). The program (without the user seeing) then performs thresholding (**c**, **d**, **e**) until all four points are connected (**e**). *Sperm Sizer* then measures the distance between the selected points along the centre of the highlighted area (**e**) and then shows the output (**f**)

Estimates of time required for sample preparation, photographing and measuring

Once researchers have collected sperm samples, there are several time-consuming steps to generate sperm morphology data, including preparation of microscope slides, photographing sperm under the microscope and measuring sperm. The time required for these steps will all vary depending on the biological system and methodology employed. We here recorded the time required for these steps in our long-tailed finch system, as an example for the time scales involved and the potential efficiencies of using the *Sperm Sizer* tool.

Results

Measurements obtained from single sperm cells using *Sperm Sizer* and ImageJ were highly correlated for long-tailed finches, passerines more generally and lizards (Table 1; Fig. 3a–c). Measurements of all sperm components (head, midpiece, tail and total sperm length) obtained using *Sperm Sizer* were typically 5% longer than measurements obtained using Image J (Fig. 4). The exceptions to this were the measures of sperm head length in the passerine dataset and sperm midpiece length in the lizard dataset, where the difference was 0.5% and 8.9% (passerine head length and lizard midpiece

Table 1 Linear mixed model (LMM) testing the relationship between measurements made using ImageJ and *Sperm Sizer*. For the other passerines and lizard species comparisons, species is included as a fixed effect in the model

	Long-tailed finch ($N = 75$)		Other passerines ($N = 82$, 8 species)		Lizards ($N = 85$, 8 species)	
	R^2	Mean % difference (95% CIs)	R^2	Mean % difference (95% CIs)	R^2	Mean % difference (95% CIs)
Head	0.95	4.8% (4.3–5.2)	0.98	0.5% (− 0.3–1.2)	0.99	4.7% (4.4–5.0)
Mid	0.99	4.5% (4.2–4.8)	0.99	5.1% (4.8–5.4)	0.97	8.9% (7.8–10.1)
Tail	0.99	5.5% (5.1–5.8)	0.99	4.9% (4.2–5.4)	0.99	5.1% (4.9–5.3)
Total	0.97	4.8% (4.6–5.1)	0.99	4.7% (4.4–5.0)	0.99	5.1% (5.0–5.3)

Table 2 Repeatability and 95% confidence intervals (CIs) when a set of 35 long-tailed finch sperm were measured twice using ImageJ and twice using *Sperm Sizer*

	R [CIs], using ImageJ	*R* [CIs], using *Sperm Sizer*
Head	0.983 [0.966, 0.992]	0.989 [0.978, 0.994]
Mid	0.997 [0.994, 0.999]	0.999 [0.998, 0.999]
Tail	0.993 [0.986, 0.996]	0.996 [0.991, 0.998]
Total	0.996 [0.992, 0.998]	0.998 [0.997, 0.999]

length, respectively). We found that *Sperm Sizer* performed well with a range of other taxa, including mammals, fish and molluscs (see Supplementary Fig. 1) and that *Sperm Sizer* measurements were more slightly repeatable than ImageJ measurements, but not significantly so (Table 2).

For our estimate of sample preparation duration, we found that our slide preparation (creating smears, then rinsing them 24 h later) took 1 m 35 s per slide (average of 20 slides). Photographing sperm took 22 s per sperm (average of 519 sperm cells). We had two people measure sperm with both

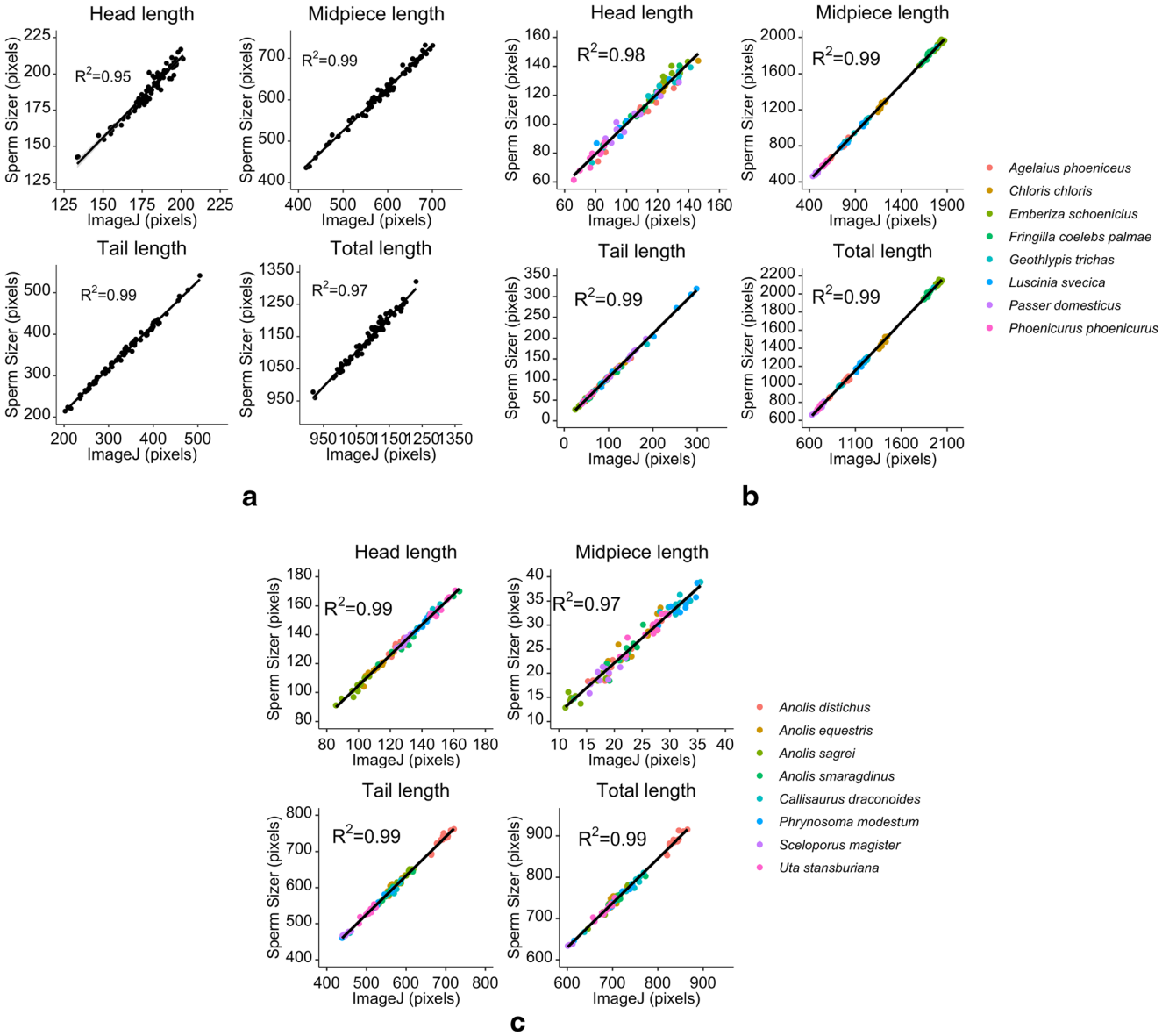

Fig. 3 The correlation between *Sperm Sizer* and ImageJ measurements of the same sperm cells. Sperm photos had been previously marked with points indicating the start and end of each sperm component, in an attempt to eliminate most human error and focus on measurement differences between the two softwares. **a** Long-tailed finch, **b** other passerine birds and **c** species of lizard. 95% CIs around the line of best fit are shown in grey

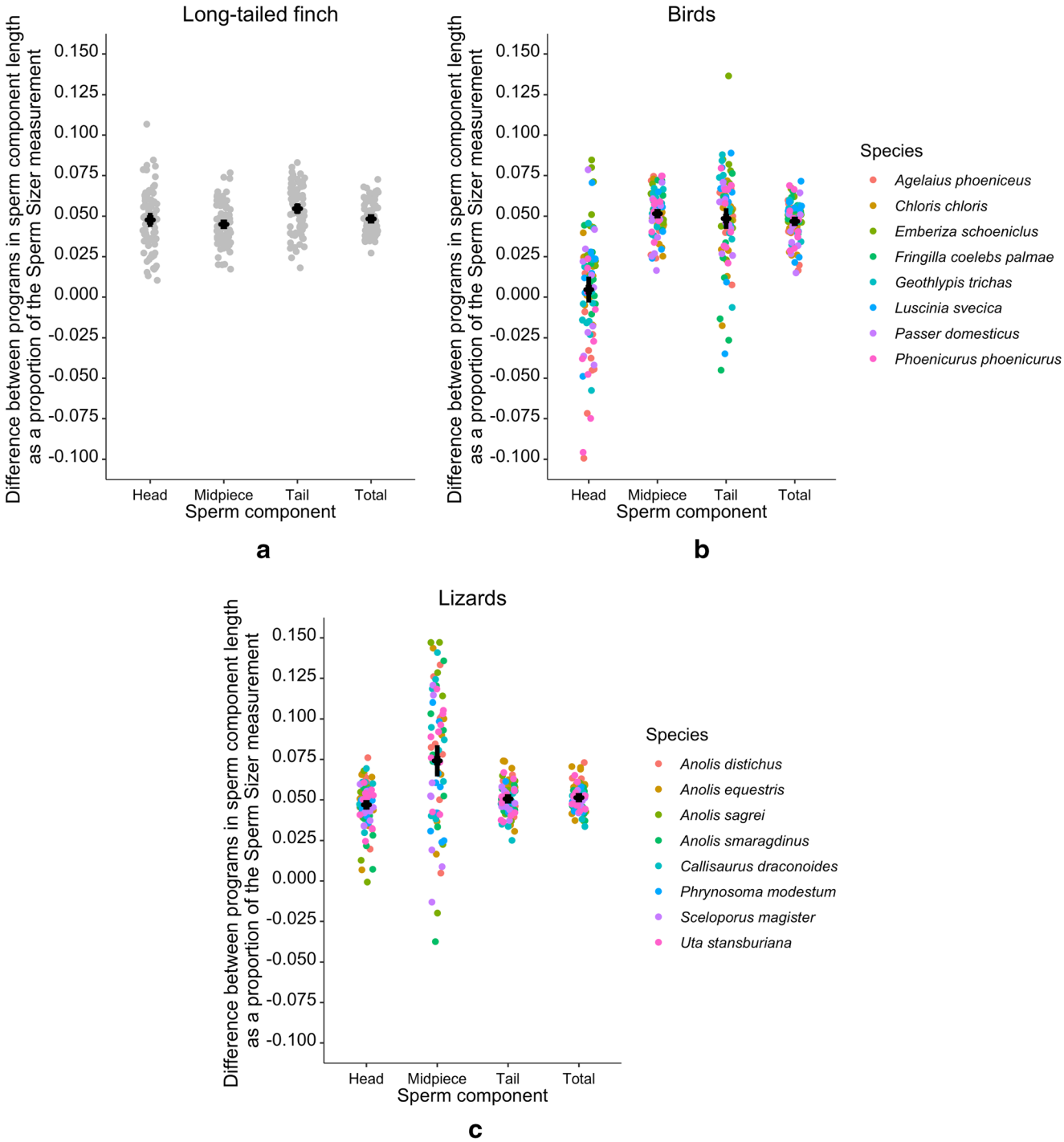

Fig. 4 The difference between the *Sperm Sizer* and ImageJ measuremements of each sperm component as a proportion of the *Sperm Sizer* measurement, where positive values indicate that the *Sperm Sizer* measurement was longer. **a** Long-tailed finch, **b** other passerine birds and **c** species of lizard. The mean and 95% confidence interval are shown in black

ImageJ and *Sperm Sizer* (either measuring 50 sperm or measuring for an hour, whichever came first), and in both cases, ImageJ took at least three times as long (time taken per sperm; Person 1: ImageJ 1 m 34 s, *Sperm Sizer* 26 s, Person 2: ImageJ 1 m, *Sperm Sizer* 19 s, Average: ImageJ 1 m 17 s, *Sperm Sizer* 23 s). Thus, to process one male (10 sperm) would take 1 m 35 s to create the slide, 4 m 45 s to photograph (13 sperm as we usually do a couple extra) and 12 m 50 s to measure with ImageJ or 3 m 50 s to measure with *Sperm Sizer*. So once sperm samples have been collected (which can be time consuming but varies greatly depending on the study organism), the total processing time per male with our long-tailed finch

samples was 19 m 10 s using ImageJ and 10 m 10 s using *Sperm Sizer*. We emphasise that this is an example, and the time requirements for these different stages will vary with organism and set-up.

Discussion

Here, we introduce *Sperm Sizer* and demonstrate that it generates large amounts of accurate data on sperm lengths faster than traditional methods. There was a high degree of correlation between *Sperm Sizer* and ImageJ measurements for all sperm components, both within a single species and across multiple passerine and lizard species. We suggest that the broad applicability of this program should facilitate research on a variety of non-model species by saving valuable researcher time. We suggest it is not surprising that *Sperm Sizer* measurements were consistently slightly longer, as the measurements made with ImageJ used a series of short straight lines to measure the length of the curved cell, whereas *Sperm Sizer* follows along the curve itself. This slight but relatively consistent difference between the programs should not be a concern, as the relative difference in length between sperm components, individuals and species will be consistent. Furthermore, existing programs that measure sperm morphology may also consistently differ from one another depending on their measurement algorithm, for example, whether they follow the curve of the cell or use straight lines. Additionally, given the relatively consistent differences we observed in the data generated by the two systems (i.e. measures differed by 5%), we suggest it may therefore be possible to combine *Sperm Sizer* data with existing datasets collected with other systems; however, this will require confirmation and calculation of a correction factor on a case-by-case basis. We found no statistically significant support for *Sperm Sizer* increasing repeatability of measurements by a single user, as we might expect if the multiple points selected using ImageJ was a source of error, but our test may be lacking the power to detect a small but existing effect (Table 2).

One exception to the observed consistent ~5% difference between *Sperm Sizer* and ImageJ was lizard sperm midpiece length, which we suggest is likely due to the shortness of the midpiece in the species we examined. The lizard midpiece differences were variable and an average of 8.9% different, but this 8% represents only 1–3 pixels. Thus, a very small difference in number of pixels translates into a moderate difference in actual length. The other exception was sperm head length across passerine bird species, for which measurements were, on average, only 0.5% different. When processing images, *Sperm Sizer* performs thresholding 'behind the scenes' until the whole sperm is highlighted (to connect the full length of the sperm). When there is a lack of contrast between the head and the background, or a 'halo' around the head, the whole area around the head can be selected rather than just the curves around head itself (illustrated in Supplementary Fig. 2). This can result in *Sperm Sizer* measuring in a straight line down the head rather than following the curves of the head (Supplementary Fig. 2). This causes *Sperm Sizer* to underestimate the length that would have been calculated if it had followed the curves of the head more, resulting in the *Sperm Sizer* measurement being more similar to the shorter ImageJ measurement. Importantly, this is visible to users as they are making measurements (e.g. Fig. 2f), allowing the user to judge whether or not *Sperm Sizer* is accurately following complex curves of the sperm or not. The photos used here were not produced specifically for *Sperm Sizer*, so with some optimization of photography conditions prior to using *Sperm Sizer,* tracking the waviness of the head could be improved for those species where *Sperm Sizer* struggled (i.e. those with low values in Fig. 4).

It is worth noting that how the photo is taken is likely to be more important for being able to employ *Sperm Sizer* than is, for example, unusual sperm morphology (e.g. Fig. 1 and Supplementary Fig. 1). *Sperm Sizer* is not as effective when the sperm cells crossover other structures, including other sperm cells, non-sperm items, or if there is not enough contrast between the sperm and the background. If there is a strong 'halo' effect around the sperm, this can result in missing subtleties in tracking along the centre of the sperm (as discussed above). In our experience, cells originally photographed for use with other programs could typically also be used with *Sperm Sizer* without issue, but as this may not always be the case, we advise new users to trial *Sperm Sizer* with a few photos to ensure it is working before photographing their full dataset. In cases of highly dense sperm, it may be beneficial to reduce the density of sperm cells in each frame, by using a higher dilution. New users can also use the 'Threshold' tool in ImageJ to explore what areas of the sperm are selected with increasing contrast of the image (e.g. Fig. 2 and Supplementary Fig. 2).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00265-021-03013-4.

Acknowledgements The authors would like to thank two anonymous reviewers for valuable feedback on the manuscript, Jan Lifjeld for providing access to the Olso sperm library and feedback on the manuscript, Gaute Grønstøl and Madiline Le Mesurier for feedback after using *Sperm Sizer*, Laura Hurley for help with sperm sampling the long-tailed finch and Carina Lau for coining the name *Sperm Sizer*. The authors would also like to thank Nicole Ravida and Barbara Durrant at the San Diego Zoo for permission to use photographs from the San Diego Zoo Sperm Atlas, a valuable online resource.

Availability of code, data and material *Sperm Sizer* is freely available to download at https://github.com/wyrli/sperm-sizer/, where user instructions and more information can be found (also see Supplementary Materials 1). The data analysed here as ground-truthing data, and the corresponding R script has been uploaded to an online

repository (Open Science Framework) and can be accessed via this link osf.io/j5dqe/.

Author contribution CM and RL came up with the idea for *Sperm Sizer*, and RL conceived and developed the software. AK, MR and CM collected and imaged sperm samples for the analyses. CM measured the sperm, performed analyses and led the writing of the manuscript with MR and SG. All authors contributed critically to the drafts and gave final approval for publication.

Funding Funding was provided by a Student Research Award from the Australian Ecological Society and a Macquarie University Research Excellence Scholarship to CSM, and ARC funding DP180101783 to SCG and MR.

Declarations

Ethics approval All applicable international, national and/or institutional guidelines for the use of animals were followed. All animal sampling was done with approval from the animal ethics committees at the respective institutions, including Macquarie University for the long-tailed finch samples, Oslo Natural History Museum for other passerine species and University of Virginia for lizard samples.

Conflict of interest The authors declare no competing interests.

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