THE UNIVERSITY OF HULL

Male gamete morphology in relation to swimming velocity and fertilisation microenvironment

being a Thesis submitted for the Degree of

Doctor of Philosophy, Biological Sciences

in the University of Hull

by

Julia Louise Simpson, BSc(Hons), MSc

November 2012

Candidate declaration

I declare that the work recorded in this thesis is entirely my own, except where otherwise stated and that it is of my own composition. Some of the material included in this thesis as been produced in co-authorship with others and has been presented for publication. My personal contribution to each results chapter is as follows:

2. Sperm swimming behaviour in the presence of mature oocytes: comparison between fresh and freeze-thawed bovine samples. The idea was jointly developed by JLS, SH and Roger Sturmey.JLS performed all experimental work and wrote the chapter, SH assisted with analysis.

3. No link between sperm morphology and velocity across maternal mouthbrooding cichlid fish from Lake Malawi. The idea was jointly developed by JLS, SH, Domino Joyce and Chris Venditti. JLS performed all experiemntal work and wrote the chapter, SH assisted with analysis, DJ and CV assisted with phylogeny.

4. *Submitted to Evolution as:* Simpson J.L., Fitzpatrick J.L., Evans J.P, Simmons L.W. and Humphries S. Relationships between sperm length and speed differ among three internally and three externally fertilizing species. The idea was jointly developed by all authors.Analysis carreid out by JLS and SH with manuscript preperationpreformed joinly by JLS, JLF and SH.

I further declare that no part of this work has been submitted as part of any other degree.

Julia L. Simpson

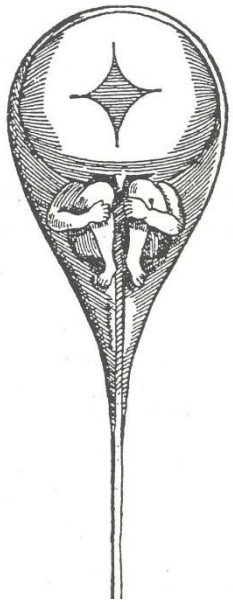
November 2012

Acknowledgements

Firstly of course I have to thank Stuart for his support and supervision over the last three and a bit years. It has not always been a smooth journey but hopefully the end result is something we can take pride in. Thanks also to the academics within the department, and those I have met at conferences for helpful discussions about my project. This thesis would not have been possible without funding support from NERC.

Exceptional support came from Hull York Medical School in the shape of Roger Sturmey and Paul Mckeegan. Thanks to their knowledge, skills and enthusiasm it was possible for me to look at bovine sperm-egg interactions. Thanks to the invaluable technical support from Marlean who ensured cichlid DNA extractions were processed extremely efficiently. Special thanks need to go out to Alan for his endless cichlid knowledge and technical expertise. The cichlid chapter would not have been possible without his help. Finally support from collaborators at the Centre for Evolutionary Biology at the University of Western Australia has been tireless. Special thanks to John Fitzpatrick for refusing to give up on trying to improve my writing style!

Heartfelt thanks go to my friends and family for their relentless support and encouragement. Gayle, Dawn and Jenny have been always been amazing friends but have excelled themselves over the last few years. You have listened to the ranting, provided wonderful distractions to the madness and have been endlessly patient and loving. Thanks also to the support network of fellow PhD students at Hull for keeping me as sane as I will ever be. The coffee breaks and brisk walks round campus will be missed. Special thanks to Richard Lindsay for continuing to support and inspire me; for always finding the time to reply to my emails with reassuring words that never fail to make me smile. Eternal gratitude also goes to Anglesey Monkey, thank you for your patient proofreading, inspirational phone calls and general outlook on life! Last but not least extra special thanks go to James for his love, patience, IT support and Swiss Miss care packages.



Homunculus

– the little pre-formed person in the sperm

by Nicolaus Hartsoeker in Essai de diotropique, 1694.

The sperm seems never to transgress the few rules which govern the production of its fundamental parts, but in the arrangement of these parts every sperm (flagellate or non-flagellate) seems to be a law unto itself.

Robert H. Bowen 1925

Table of Contents

List of Tables ..... **ix**

List of Figures ..... **xi**

Abstract **1**

Chapter One: General Introduction **4**

1.1. Aim of thesis **4**

1.2. Traditional methods rely on mean values **5**

1.3. A new approach **6**

1.4. Sperm morphology **9**

1.4.1. Head 11

1.4.2. Midpiece 12

1.4.3. Flagellum 13

1.5. Sperm velocity **15**

1.6. The hydrodynamics of propulsion **16**

1.7. Linking sperm morphology to velocity **16**

1.8. Measuring sperm morphology **17**

1.9. Selection pressures – sexual selection **19**

1.9.1. Postcopulatory sexual selection 20

1.9.2. Sperm competition 21

1.9.3. Cryptic female choice 23

1.10. Internal versus external fertilisation **24**

1.11. Mating strategy **25**

1.12. Aims **26**

1.13. Thesis outline **26**

Chapter Two: Sperm swimming behaviour in the presence of mature oocytes: comparison between fresh and freeze-thawed bovine samples **28**

Abstract **28**

2.1. Introduction **29**

2.1.1. Bovine spermatozoa; motility and chemotaxis 30

2.1.2. Cryopreserved versus fresh bovine spermatozoa 31

Hypothesis **33**

2.2 Methods **34**

2.2.1. Freeze-thawed sperm preparation 34

2.2.2. Fresh sperm preparation 35

2.2.3. Oocyte preparation 35

2.2.4 Capturing motility 35
2.3 Analysis 37
2.3.1 Tracking individual sperm speed 37
2.3.2. Track complexity 40
2.4. Results 42
2.4.1. Velocity 42
2.4.2. Directionality 48
2.4.2 Track complexity 52
2.5. Discussion 56
2.5.1. Is there any evidence for chemotaxis? 57
2.5.2. Cryopreservation – effect on sperm motility 58
2.5.3. Capacitated versus non-capacitated sperm 59
2.5.4. Fractal dimension analysis elucidates track complexity 61
2.5.5. The missing component - sperm morphology 62
2.6. Conclusion 63
Chapter Three: No link between sperm morphology and velocity across maternal mouthbrooding cichlid fish from Lake Malawi 64
Abstract 64
3.1. Introduction 65
3.1.1. Sperm characteristics of teleost fish 65
3.1.2. Mouthbrooding 68
3.1.3. Sexual selection 69
3.1.4. Mating sequence and fertilisation location 70
3.1.5. Sperm competition 71
3.1.6 Cichlids of Lake Malawi 73
3.1.6.a. Cichlid clades as indicated by mtDNA 74
Hypothesis 75
3.2. Methods 76
3.2.1. Stripping milt 77
3.2.2. Recording motility 78
3.2.3. Sequence data 79
3.2.4. Analysis of videos 79
3.2.5. Controlling for phylogeny 80
3.2.6. Statistical analysis 81
3.3. Results 83
3.3.1 Phylogenetic analysis 83
3.3.2. Velocity measurements 84

3.3.3. Sperm morphology – velocity across cichlid species 84
3.3.4. Mixed effect analysis 86
3.4. Discussion 93
3.4.1. Within-male variation reveals functional links 94
3.4.2. Direction of correlation alters between species 94
3.4.3. Functional links between sperm morphology and swimming speed 95
3.4.4.Mode of fertilisation and polyandry 95
3.5. Conclusion 96

Chapter Four: Relationships between sperm length and speed differ among three internally and three externally fertilizing species 99

Abstract 99
4.1. Introduction 100
4.2. Methods 103
4.2.1. Internally fertilizing species 103
i) Human – *Homo sapiens* 103
ii) Emu – D*romaius novaehollandiae* 103
iii) Guppy - *Poecilia reticulata* 104
4.2.2. Externally fertilizing species 104
iv) Rainbowfish – *Melanotaenia australis* 104
v) Mussel – *Mytilus galloprovincialis* 105
vi) Quacking frog – *Crinia georgiana* 105
4.2.3. Analysis of videos 106
4.2.4. Statistical Analyses 107
4.5. Results 108
4.5.1. Internally fertilising species 112
i) Human 112
ii) Emu 112
iii) Guppy 112
4.5.2. Externally fertilising species 112
iv) Mussel 112
v) Rainbowfish 112
vi) Frog 113
4.6. Discussion 116
4.6.1. Assessing the prevalence of sperm length-speed relationships 116
4.6.2. Flagellum/total length-speed relationships differ in internal and external fertilizers 117
4.6.3. Sperm head morphology and sperm swimming speed 118

4.6.4. The importance of accounting for intra-male variance in sperm traits 119
4.6.5. Frogs and guppies: species-specific considerations 120
4.7. Conclusion 121

Chapter Five: General Discussion 123
5.1. Overview 123
5.2. Putting the findings into context 124
5.3. Future work 129

Chapter Six: Literature Cited 132

Appendix One: Fractal analysis of bovine sperm swimming tracks 148

Appendix Two: Cichlid sample information. 208

Appendix Three: Technique for measuring individual sperm in ImageJ 211

Appendix Four: Information provided as supplemental data when Chapter four was submitted to the journal Evolution 214
Details of video analysis 215
Head volume calculations 215

List of Tables

Table 1.1 Published studies investigating links between sperm size and velocity. Correlation key: -, negative correlation between morphology and velocity; +, positive correlation between morphology and velocity; 0, no correlation found between morphology and velocity. * refers to studies using individual sperm measurements, all others use mean measures. **8**

Table 2.1. Summary of directional plots from Figures 2.11-2.13 based on mean direction calculation (as described in Section 2.3.1) per experiment. **49**

Table 2.2. Tukey-Kramer HSD results for all significant ANOVA results obtained from fractal analysis. COC=cumulus oocyte complex. Significant comparisons are shown in bold (p=<0.05). **55**

Table 3.1. Review of published studies considering the characteristics of sperm morphology across cichlid species. Tang=Lake Tanganyika; N.Cam=North Cameroon; EA=East Africa; CA=Central America; VCL=curvilinear velocity; NT=non-territorial males; T=territorial males. **67**

Table 3.2. Details of the 33 cichlid fish species used to compare sperm characteristics. +=Lake Chilingali (satellite lake to Lake Malawi); ++=Lake Rovuma, feeds into Lake Malawi (Figure 3.2); *=breeding stock sourced from pet shop; S=substrate; B=buccal cavity. The suggestion that most haplochromine eggs are fertilised inside the buccal cavity (Mrowka 1987) was not found to be consistent. Gaps in the table represent absence of detailed, species specific information available from the literature (Fryer and Iles 1972; Konings 2007). **76**

Table 3.3. Results for sperm length-VAP from mixed model centring, allowing random effect intercepts for between and within male analysis. HW=head width, HL=head length, HV=head volume, FL=flagella length, TL=total length, HL:FL=ratio of head length to flagellum length, and $n_{t=}$total number of males. F=degrees of freedom within and between males. 95% confidence intervals (95% CI) calculated for effect size (r). Significant correlations (p≤0.05) in bold **90**

Table 4.1. Results for sperm length-VAP from mixed model centering allowing random effect intercepts for between and within male analysis. HW=head width, HL=head length, HV=head volume (no HV measure for mussel data), FL=flagella length, TL=total length and HL:FL=ratio of head length to flagellum length, $n_{t=}$total number of males. F = degrees of freedom within and between males. 95% confidence intervals (95% CI) calculated for effect size (r). Significant correlations (p=<0.05) in bold. **109**

Table 2A.1. Information on sequence data used in creation of phylogenetic tree and species mean measurements for average path velocity (VAP) and all aspects of sperm morphology. HL=head length; HW=head width; FL=flagellum length; HL:FL= ratio of head length to flagellum length; TL=total length (measure as tip of head to end of flagellum). SL=standard length (distance from the tip of the snout to the posterior end of the vertebral column measured to nearest mm). Species without GenBank accession numbers were sequenced at the University of Hull from fin-clips taken from males used as part of the sperm analysis research. **208**

Table 4A.1. Summary of specific computer assisted sperm analysis (CASA - http://rbs.info.nih.gov/ij/plugins/casa.html) Sperm Tracker values used to record individual cell speed using the ImageJ v. 1.44o plugin (Wilson-Leedy and Ingermann 2007). In the Sperm Tracker window of CASA in ImageJ the following parameters were altered from their default values: a, Minimum sperm size (pixels); b, Maximum sperm size (pixels); c, Minimum track length (frames); d, Maximum sperm speed between frames (pixels) and q, Frame Rate (frames per second). **214**

Table 4A.2. Loadings for PC 1 for each speed measure for all species, eigenvalues with cumulative % variance explained by PC1. VAP was consistent across species as the speed which explained correlations with length components for all species. As such VAP was chosen as the main speed measure reported here. **216**

Table 4A.3. Results for all species from mixed model centering allowing random effect intercepts for curvilinear velocity (VCL) and straight line velocity (VSL). HW=head width, HL=head length, HV=head volume (no HV measure for mussel data), FL=flagella length, TL=total length and HL:FL=ratio of head length to flagellum length, $n_{t=}$total number of males. F = degrees of freedom within and between males. 95% confidence intervals (95% CI) calculated for effect size (r). Significant correlations (p=<0.005) in bold. **217**

List of Figures

Figure 1.1.Examples of some of the diversity of sperm morphology across species. Mammalian sperm in box are to scale, with the human sperm at 40μm (adapted from Brennen and Winet 1977) **4**

Figure 1.2.Basic structural elements found across spermatozoa from most taxa. Head contains male DNA, midpiece contains mitochondria, which provide energy to the cell, and flagellum provides the propulsive motility of the cell. **10**

Figure 1.3.Head shape diversity (not to scale) representing species with diverse sperm morphologies.i) Normal mouse sperm displaying the apical hook commonly found in rodent sperm (after Kishikawa et al. 1999); ii) typical paddle-shaped human sperm; iii) Eupyrene sperm of the snailPomacea canaliculata (after Gamarra-Luques et al. 2006); iv) almost-spheroid head, as commonly found in (cichlid) fish; v) typical spiral head of several bird species (Birkhead and Montogmerie 2009); vi) typically elongated head for frogs from the family Myobatrachidae. **10**

Figure 1.4.Schematic showing elements of the 9+2 doublet microtubule structure of an axoneme in cross-section. Dynein arms are the molecular motors which induce axoneme bending; Nexin bridges connect doublets, preventing adjacent microtubules from moving; Inner sheath and central microtubules provide a core which resists bending; Radial spokes are involved in the mechanical movement of the flagellum. **14**

Figure 1.5. Schematic and cross-section of typical fish sperm flagella, with two fins along the axoneme. Axoneme cross-section contains a 9+2 microtubule doublet, as described in Figure 1.4. Purple outline represents bending of flagellum as in swimming motion, where central rod and fins bend together via sliding of **14**

Figure 1.6. Illustration of the difference in velocity measurement from start point to end point of the swimming trajectory of sperm cells tracked using CASA. Solid line represents actual path of tracked sperm cell; grey area represents VCL, reflecting velocity across the actual path taken; dashed line represents the average path of the cell as calculated for VAP; and dotted line is path used to calculate VSL. Paths tracked by CASA using head centroid position. **16**

Figure 2.1.Schematic of a DunnChemotaxis Chamber indicating relative locations of male and female gametes. The red box indicates the area on which the microscope was focused in order to record sperm motility. **36**

Figure 2.2. Focal area in ImageJ. Red dashed line indicates line used for angle measurements to assess swimming direction of sperm in relation to source of COC. Each coloured track represents an individual sperm's swimming trajectory as tracked in MTrackJ. **38**

Figure 2.3. An example of standardized tracks. Axes represent relative x, y coordinates from the central start point. Each coloured line represents the track of an individual spermatozoa, with points indicating frame by frame progression of the sperm (equivalent to step-length parameter in fractal dimension analysis, see Section 2.4.2). Tracks were standardised in this way for all spermatozoa, grouped by experiment, as shown in Figures 2.11-2.13. ..... **39**

Figure 2.4. Schematic for the calculation of VSL based on x, y coordinates. The red line represents track of sperm head from start to end of video. **39**

Figure 2.5. Example graphs of different sperm characteristics generated from fractal analysis. Each sperm tracked has a comparable bank of five graphs (see Appendix 1 for all individual sperm graphs). Looking for subpopulations of sperm responding to the presence of COC in the above tracks potentially reveal sperm responding differently to *in vitro* conditions. The relatively straight path of track 36, for example, suggests that the flagella is beating symmetrically and the cell is therefore not hyperactivated, whilst track 10 has the circular trajectory indicative of the asymmetrical beating of hyperactivated sperm (Chang and Suarez 2010). **41**

Figure 2.6. Comparison of real speed measures across treatments with standard error bars. Large error bars for freeze-thawed sperm in the presence of one and three COC suggest there is a lot more variation within these samples which could be related to tract complexity (see Figure 2.13). Fresh capacitated (C); freeze-thawed (FT) and fresh non-capacitated (NC)..... **44**

Figure 2.7. Sperm speed a) VSL and b) VCL as a function of number of COC treatment. Error bars are for standard errors. Numbers at the base of each bar indicate individual sperm sample size. Colours represent: fresh capacitated; fresh non-capacitated; freeze-thawed sperm samples. **45**

Figure 2.8. Box and whisker plot from one-way ANOVA indicating significant difference (p=<0.0001) in two measures of velocity a) VSL and b) VCL across treatments depending on sperm type and number of COC. 0=control, no COC, 1-3 refers to the number of COC present in the treatment. C=fresh capacitated sperm, (sample size control sperm n=24; 1 COC sperm n=36; 2 COC sperm n=34; 3 COC sperm n=47). FT=freeze-thawed sperm, (sample size control sperm n=24; 1 COC sperm n=43; 2 COC sperm n=24; 3 COC sperm n=15), NC=fresh, non-capacitated sperm, (sample size Control sperm n=73; 1 COC sperm n=60; 2 COC sperm n=42; 3 COC sperm n=34). Error bars indicate 95% confidence limits. **46**

Figure 2.9. Interaction plot from least squares regression with standard error bars fori) VSL against number of COC for each sperm type and ii) effect of sperm type for fresh capacitated (c) freeze-thawed (ft) and fresh non-capacitated (nc) sperm. Numbers on plots indicate number of sperm per sperm type colour coded to match the key. **47**

Figure 2.10. Interaction plot from least squares regression with standard error bars fori) VCL and number of COC and ii) effect of number of COC iii) effect of sperm type for fresh capacitated (c) freeze-thawed (ft) and fresh non-capacitated (nc) sperm. Numbers on plots indicate number of sperm colour coded to match the key for sperm type where applicable. . **48**

Figure 2.11.Plots of expected trajectories (top two plots) if sperm are not responding (left plot) or are responding to the presence of COC . Sperm velocities (VSL) relative to the direction of the source of attractant for all treatment using freeze-thawed sperm. All tracks were centralised to central start point (as per Section 2.3.1). The chamber line (red bar on the left side of each plot) represents the direction of the COC relative to the sperm start point. Each black line represents the trajectory of an individual sperm with the red dashed

line indicating the mean velocity and direction of all sperm tracked in each treatment. Control sperm n=24; 1 COC sperm n=43; 2 COC sperm n=24; 3 COC sperm n=15. ..... **50**

Figure 2.12. Sperm velocities (VSL) relative to the direction of the source of attractant for all treatment using fresh capacitated sperm. All tracks were centralised to central start point (as per Section 2.3.1). The chamber line (red gradient on the left side of each plot) represents the direction of the COC relative to the sperm start point. Each black line represents the trajectory of an individual sperm with the red dashed line indicating the mean velocity and direction of all sperm tracked in each treatment. Control sperm n=24; 1 COC sperm n=36; 2 COC sperm n=34; 3 COC sperm n=47. ..... **51**

Figure 2.13. Sperm velocities (VSL) relative to the direction of the source of attractant for all treatment using fresh non-capacitated sperm. All tracks were centralised to central start point (as per Section 2.3.1). The chamber line (red bar on the left side of each plot) represents the direction of the COC relative to the sperm start point. Each black line represents the trajectory of an individual sperm with the red dashed line indicating the mean velocity and direction of all sperm tracked in each treatment. Control sperm n=73; 1 COC sperm n=60; 2 COC sperm n=42; 3 COC sperm n=34. ..... **52**

Figure 2.14.Box-and-whisker plots of fractal dimension (FD) for i) freeze-thawed sperm(number of sperm per treatment: 0 COC=24; 1 COC=43; 2 COC=24; 3 COC=15) ii) fresh non-capacitated - number of sperm per treatment: 0 COC=73; 1 COC=60; 2 COC=42; 3 COC=34; and iii) mean velocity (MV) for fresh capacitated sperm (number of sperm per treatment: 0 COC=24; 1 COC=36; 2 COC=34; 3 COC=47)..... **54**

Figure 3.1. Female *Astatotilapia calliptera* brooding fry, highlighting location of buccal cavity. Females can brood up to 80 fry at a time. **72**

Figure 3.2. Location of Lake Malawi (365 miles long by 52 miles wide), identifying areas around the lake where cichlids were originally collected. Lake Chilwa (24 miles long, 17 miles wide according to Kirk) is isolated from the main body of Lake Malawi. Lake Chilingali (~3 miles long by ~0.6 mile wide according to Genner et al (2007)) is only seasonally connected to Lake Malawi..... **73**

Figure 3.3. Progression of the scaling parameter, with λ reflecting strength of phylogenetic signal from strong signal (λ=1) to moderate signal (λ=0.5) and no signal (λ=0). **81**

Figure 3.4. Phylogenetic tree based on mtDNA D-loop sequence data. Numbers at junctions represent posterior percentage of the posterior sample of 1,000 trees containing the base change, with red numbers 50% or below, blue numbers above 50%, and absence of number representing base changes present in all trees (100%). Scale line refers to number of nucleotide substitutions per site in the alignment. The tree is congruent with published trees for Lake Malawi cichlids. Colours refer to mtDNA clades: Red='sand-dwellers'; Blue='mbuna'; Orange=*Rhamphochromis*; Green=*Astatotilapia calliptera*. Species without colour are from outgroups. **83**

Figure 3.5. Scatterplot created after linear regression, illustrating the correlation between velocity measurements for VAP and VCL (individual sperm n=243, p=<0.0001)..... **84**

Figure 3.6. Scatter plots from linear regression analysis, p-values indicat no significant relationships between sperm morphology and velocity across species. Each point represents mean sperm measurement from one male (species n=33). a) HL=head length (p=0.82); b) HW=head width (p=0.29); c) FL=flagella length (p=0.87); d) TL=total sperm length (p=0.86) and e) H:F= ratio of head length to flagella length (p=0.83). mtDNA clades – Red='sand-dwellers', with triangles indicating bower building species; Blue='mbuna'; Orange=*Rhamphochromis*; Green=*Astatotilapia calliptera,* and Black=outgroups. **85**

Figure 3.7. Scatter plots from linear regression analysis for a) whole fish standard length (p=0.49) and b) male weight (p=0.30) against sperm velocity (species n=33)..... **86**

Figure 3.8. (a) and (b) *Astatotilapia calliptera* Rovuma (n=8 males with a total of 44 sperm); (c) *Astatotilapia sp. Calliptera chizumulu*(n=9 males with a total of 63 sperm). Between-male effect only – correlations for the relationship between sperm flagellum length (FL), total length (TL), head width (HW) and velocity (VAP) within and between males. Regression slopes for within-male variation in black. Gray dashed line depicts the estimate from a standard mixed model of the effect of length measures on the speed measure (guide only). Solid black lines depict individual regression slope for between-male data. All data log transformed. **87**

Figure 3.9. (a-c) *Rhamphochromis cf. longiceps*(n=7 males with a total of 41 sperm); (d) and (e) *Rhamphochromis sp. chillingali*(n=7 males with a total of 36 sperm). Correlations for the relationship between sperm flagellum length (FL), total length (TL), head length to flagella length ratio (HL:FL) and velocity (VAP) within-males. Gray dashed line depicts the estimate from a standard mixed model of the effect of length measures on the speed measure (guide only). All data log transformed. **88**

Figure 3.10. *Nyassachrmis cf. microcephalus*(n=4 males with a total of 32 sperm). Correlations for the relationship between sperm for a) flagellum length (FL) and b) total length (TL) and velocity (VAP) within-males. Gray dashed line depicts the estimate from a standard mixed model of the effect of length measures on the speed measure (guide only). All data log transformed. **89**

Figure 4.1. Relationships between sperm flagellum length (FL) and velocity (VAP) within and between males across internally (left column) and externally (right column) fertilising species. Thick black lines describe significant between-male effects from the mixed effect model, while thin solid lines depict individual within-male slopes from linear regressions as a visual guide to significant within-male effect. The dashed grey line depicts the estimate from a standard mixed model of the effect of the length measure on the speed measure (as a guide only)..... **114**

Figure 4.2. Differences in absolute effect size (within male – between male) for the relationship between sperm length and speed (VAP) for each sperm trait analyzed in each of the species examined. Positive values indicate that the effect size for the relationship between sperm length and speed was greater in the within male analyses and negative values indicate larger effect sizes in the between male analyses. These data are for

illustrative purposes only: effect sizes and statistical models are presented in full in Table 1. 115
Figure 1A.1. Individual sperm track characteristics for freeze-thawed sperm in the control group without COC (n=24). **151**
Figure 1A.2. Individual sperm track characteristics for freeze-thawedsperm in the presence of one COC (n=43) **156**
Figure 1A.3. Individual sperm track characteristics for freeze-thawed sperm in the presence of two COC (n=24) **159**
Figure 1A.4. Individual sperm track characteristics for freeze-thawed sperm in the presence of three COC (n=15) **161**
Figure 1A.5. Individual sperm track characteristics for fresh capacitated sperm in the control group without COC (n=24) **164**
Figure 1A.6. Individual sperm track characteristics for fresh capacitatied sperm in the presence of one COC (n=36) **169**
Figure 1A.7. Individual sperm track characteristics for fresh capacitated sperm in the presence of two COC (n=34) **173**
Figure 1A.8. Individual sperm track characteristics for fresh capacitated sperm in the presence of three COC (n=47) **179**
Figure 1A.9. Individual sperm tracks characteristics for fresh non-capacitated sperm in the control group without COC (n=73) **189**
Figure 1A.10. Individual sperm track characteristics for fresh non-capacitated sperm in the presence of one COC (n=60) **196**
Figure 1A.11. Individual sperm track characteristics for fresh non-capacitated sperm in the presence of two COC (n=42) **202**
Figure 1A.12. Individual sperm track characteristics for fresh non-capacitated sperm in the presence of three COC (n=34) **206**
Figure 3A.1.Protocol for isolating and measuring individual spermatozoa using ImageJ **213**

Abstract

The heterogeneity of spermatozoa has been widely used to inform phylogenetic relationships among taxa, yet the reason such diversity has evolved, in relation to the sperms primary functional role in fertilisation and reproduction, is not well understood. Research into the evolutionary significance of sperm morphology is concomitant with the study of sexual selection and the evolution of the biological diversity of life. The formulation of sperm competition theory in the 1970s provided a new insight for the study of sexual selection and the development of the field of sperm biology. The idea that males not only competed for access to females but that, through direct competition, spermatozoa of individual males were also in competition to fertilise the maximum number of eggs was a revolutionary concept.

An integral part of many sperm competition models is the assumption that there is a relationship between sperm morphology and swimming velocity in terms of fertilisation success. In addition to this it is further assumed that longer sperm swim faster than shorter sperm. During competitive mating, when the ejaculate from two or more males compete to fertilise a given set of eggs, longer and therefore faster, sperm should have a fertilisation advantage as they can reach and fertilise eggs first. However identifying the traits that make individual sperm more or less capable of successfully fertilising an egg remain largely unknown for most species. There has so far been little empirical support for a link between sperm morphology and swimming speed and mixed results regarding the importance of swimming speed when it comes to increasing fertilisation success.

Here, three aspects of sperm biology were investigated in order to clarify functional links between sperm morphology and velocity. Firstly, the influence that female gametes have on the swimming behaviour of both fresh and cryopreserved bovine sperm was examined. Bovine samples were used because it is known that in the bovidae family, female gametes release chemoattractans which appear to play a role in sperm guidance towards the oocyte (egg). Additionally, a large literature base regarding damage caused by cryopreservation of bovine sperm exists. The second topic of research focuses on cichlid fish. Cichlids from Lake Malawi have been underrepresented in the published literature in sperm biology despite being extensively studied in relation to other aspects of their biology. A phylogenetically controlled study into links between sperm morphology and

velocity across mouthbrooding cichlids from Lake Malawi was therefore undertaken. All endemic cichlids in Lake Malawi are maternal mouthbrooders in which the female broods both eggs and fry inside her buccal cavity (a cavity inside the mouth of the female between the jaw and cheek). The functional significance of the buccal cavity is also important as the point of fertilisation for some species. Variation in sperm length has been found in cichlid species in Lake Tanganyika where substrate fertilisers have significantly longer sperm than species fertilising inside the buccal cavity. However, links between morphology and velocity have found little support when using traditional methods that rely on the calculation of mean measurements of sperm morphology and velocity. Here an experimental approach focusing on measurements from individual sperm whilst accounting for within-male variation was employed to investigate the extent to which links between sperm morphology and velocity to be revealed across species. The third area expands on the idea that fertilisation point will have an influence on sperm form and function and compares sperm data from three internally and three externally fertilising species. Once again measurements from individual sperm were used and within-male variation was included in the analysis.

The influence of chemoattractants on sperm swimming speed and direction are frequently omitted in the sperm competition literature, as are comparisons of the effects of sperm preservation methods such as cryopreservation. Such considerations are, in a large part left to articles on animal breeding and artificial insemination. It is however, widely acknowledged that chemoattractans do influence sperm motility in a range of taxa and that cryopreservation damages sperm. Whilst chemotaxis in bovine sperm was not detected in this study, variation in swimming characteristics of bovine sperm related to the method of preservation was observed. As cryopreservation appears to alter sperm behaviour it seems unlikely that observations of swimming velocity of fresh and cryopreserved sperm can be compared when considering relationships between sperm length and velocity. Information from both fresh and cryopreserved sperm is useful when considering links between sperm traits and fertilisation success but need to be considered independently.

Using comparative methods to investigate sperm traits across closely related species of cichlid fish no phylogenetic correlation for sperm traits was found.

Furthermore, sperm morphology did not show any correlation with velocity across species. However, when within-male variation was accounted for, a relationship between sperm morphology and velocity was revealed. Interestingly the relationship was frequently a negative one, a novel finding despite numerous studies into sperm morphology-speed relationships. When the results from data comparing internal and externally fertilising species was considered negative relationships tended to be more prevalent in internally fertilising species. If this is a general trend, that shorter sperm swim faster in internally fertilising species, we could extrapolate that the negative relationships between sperm length and velocity found in the cichlid data set exhibit sperm morphology adapted for internal fertilisation. A larger sample size would be required to conclusively support this suggestion that could confirm Lake Malawi cichlids share a common point of fertilisation, within the buccal cavity, despite high levels of diversity found in other aspects of the speciation of these cichlids.

The findings of the work presented here highlight the negative influence cryopreservation has on sperm motility and that measurements of individual spermatozoa are vital if links between morphology and velocity are to be detected. The importance of sperm preservation method on the swimming behaviour, and by association the ability of the individual sperm to successfully complete fertilisation, requires further investigation to link the morphology and velocity of individual sperm to the method of preservation. This link would provide valuable insight into the structure of sperm subpopulations which are the most resistant to cryopreservation and therefore of most value for techniques such as artificial insemination. Using a new method for measuring individual sperm morphology and velocity whilst accounting for intra-male variation significant links between sperm morphology and velocity in a number of vertebrates and invertebrates were revealed. The data gathered here suggests that such links could be widespread across more species than current empirical work indicates. Furthermore, the direction of relationships between morphology and velocity appear to be revealing disparity between internally and externally fertilising species.

Chapter One: General Introduction

Spermatozoa, the mature male germ cells which fuse with and fertilise the female oocyte in sexually reproducing species, are the most diverse cell type in the animal kingdom. Sperm are notably smaller and more numerous than the female gametes in most multicellular species and they are usually motile. The primary function of all sperm is to transfer the male's DNA to as many oocytes as possible (Birkhead and Møller 1998). In fulfilling this task, a vast diversity of sperm morphologies have evolved across taxa (Figure 1.1.). However, the functional significance of this diversity is still largely unknown (Birkhead and Møller 1998).

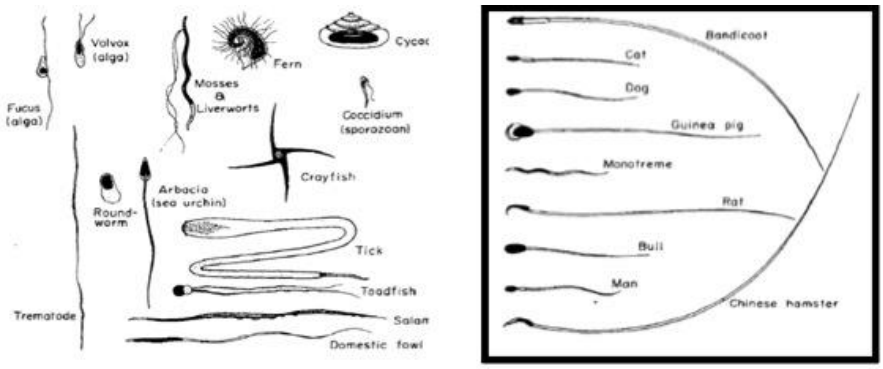


Figure 1.1.Examples of some of the diversity of sperm morphology across species. Mammalian sperm in box are to scale, with the human sperm at 40µm (adapted from Brennen and Winet 1977)

1.1. Aim of thesis

Theory suggests that increased ejaculate expenditure via more numerous or faster sperm should be the male's response to increasing levels of sperm competition. However, in relation to producing faster sperm, the question of whether longer sperm swim faster has received little empirical attention, with the relationship between length and velocity largely unexplained for most species so far studied (see examples in Table 1.1).

It is the relationship between sperm morphology and velocity that forms the underlying theme of this thesis. There is evidence that longer sperm are selected for in the presence of sperm competition when closely related polygamous and monogamous species are compared, but associations between sperm length and

velocity are less clear (Humphries et al. 2008). Assuming that sperm length influences swimming velocity there would then be a clear relationship between sperm length and fertilisation success (Gomendio and Roldan 1991; Oppliger et al. 2003) as faster sperm fertilise more eggs (Gomendio and Roldan 1991). However, links between sperm length, velocity and fertilisation success expected by sperm competition theory (Snook 2005; Gomendio et al. 2006) are not well supported by empirical studies. The assumption that longer sperm swim faster (Gomendio and Roldan 1991) and that faster sperm have a fertilisation advantage (but see Dziminski et al. 2009; Fitzpatrick et al. 2012) does not hold for all taxa (Table 1.1.). The primary aim of the following thesis was to establish the prevalence of a link between morphology and velocity for individual spermatozoa across a range of species and fertilisation types.

1.2. Traditional methods rely on mean values

Sperm morphology is important in determining male fertility (Mossman et al. 2009), however traditional methods employed to investigate relationships between sperm morphology and velocity have tended to used mean values of sperm measurements from an ejaculate (for example Gomendio and Roldan 1991; Briskie et al. 1997 and most of the references in Table 1.1). It has been suggested that this in itself poses a problem, as any link between morphology and velocity needs to be viewed in terms of individual spermatozoa (Fitzpatrick et al. 2010). Sperm of many species exhibit different phenotypes and subpopulations of sperm within an ejaculate have been identified as having varying motility (Abaigar et al. 1999; Quintero-Moreno et al. 2003; Dorado et al. 2010) and morphological characteristics (for example see Valle et al. 2012). This suggests that the use of mean values suppresses the variation found within ejaculates and can therefore potentially mask important relationships at the level of individual sperm.

Within-male variation of individual spermatozoa may reveal links between sperm morphology and velocity that have not been found using intraspecific data for mean ejaculate sperm quality (morphology-velocity). According to theory, stabilising selection acting within species is linked to the level of sperm competition. When there is little or no competition, there is increased variation within a male's ejaculate; however, increasing sperm competition decreases

within-male variation towards an optimum design (Calhim et al. 2007). The identification of subpopulations, subsets of sperm with divergent morphology or swimming kinematics, within an ejaculate (Abaigar et al. 1999) can be key to providing information on functional relationships across different sperm phenotypes and highlights the importance of accounting for the heterogeneous nature of ejaculates. Variation in sperm phenotypes can be related to i) fertilisation mode, ii) phylogeny, and/or iii) post-copulatory sexual selection (Birkhead and Immler 2007), each of which will be considered in more detail in later sections.

Various measures of morphology have been investigated in the search for a link with velocity but such a link has rarely been found (Snook 2005; Humphries et al. 2008). One consideration is whether or not the element of morphology measured is mechanistically related to the velocity of the cell. Much of the current literature uses sperm or flagellum length and velocity as variables, in combination with some manipulation of sperm competition, but often fails to find a relationship between morphology and velocity (Table 1.1). Fitzpatrick and Balshine (2009) compared sperm from cichlids and found that sperm competition led to an increase in both sperm length and velocity; however the intraspecific link between length and velocity was less conclusive. This apparent inconsistency complicates our ability to understand how sperm competition has influenced the evolution of sperm traits. The answer is not likely to be a simple one, as there are numerous variables to account for. However, it has been proposed that a ratio of head length to flagellum length might give a better correlation with velocity than simply overall length (Humphries et al. 2008), and Holt et al. (2010) have suggested that a ratio of head measures might be key to understanding velocity.

1.3. A new approach

As we have seen, an alternative methodology to using mean values for sperm traits needs to be investigated in order to establish which morphological trait, if any, can be related to the velocity of spermatozoa. Recent work by Fitzpatrick et al. (2010) has highlighted the importance of using individual measurements for each cell. Using sea urchin *(Heliocidaris erythrogramma)* sperm, it was found that samples which matched morphology and velocity for the same cell revealed significant

relationships when compared to mismatched sample pairs (Fitzpatrick et al. 2010).

Fertilisation environment is also likely to have an impact on the way selection pressures act on sperm traits, and will be considered in more detail later in this chapter (Section 1.10), as well as in Chapter Four. Competition between males to release their sperm closest to the ova has been considered an important selection pressure in the evolution of internal fertilisation (Parker 1970). Males that can get their gametes closer to the ova are at a fertilisation advantage (Parker 1970). The evolution of spermatophores long before the evolution of copulation allowed males to release sperm as a more concentrated mass than had previously been possible, and remains a functional solution for many species. As an intermediary strategy between broadcast spawning, where males and females release thousands of gametes into water columns, and internal fertilisation via copulation, spermatophores are bundles of semen which are deposited by males and subsequently taken up by females, often without the need for copulation (Parker 1970). The evolution of copulation would, in turn, lead to morphological changes of females and males, as those with the most successful traits pass on their adaptive genes (Parker 1970).

Work in each chapter was designed to address a specific question relating to the measurement of sperm morphology in relation to swimming velocity, and as such each chapter deals with a species, group of species and/or system that is amenable to such tests. Briefly, **Chapter Two** will consider the influence of a chemoattractant on swimming behaviour of bovine sperm and of sperm storage - fresh versus freeze-thawed - for both capacitated and non-capacitated sperm. Bovine sperm is a good model for this type of work as the literature contains well-documented accounts of the potential for chemotaxis and the impact of cryopreservation of semen for domestic cattle. **Chapter Three** focuses on cichlid fish from Lake Malawi, investigating the potential link between sperm morphology and velocity across these closely related species. Malawi cichlids have been extensively studied because of their divergent evolution, yet little is known about the details of their reproductive biology, especially with respect to sperm traits. **Chapter Four** is a comparative study assessing the relationships between morphology and velocity of sperm cells from internally and externally fertilising

species. The influence of microenvironment on sperm motility is considered across human, emu, guppy, mussel, rainbowfish and frog spermatozoa. Finally, **Chapter Five** is a general discussion, where the main findings of the previous chapters are brought together and used as the foundation for recommendations for future work. The rest of the current chapter focuses on the specifics of sperm morphology, before reviewing the mechanics of sperm swimming. It ends by considering the different selection pressures that act on sperm morphology. Throughout this thesis, the terms 'sperm' and 'spermatozoa' will be used interchangeably.

Table 1.1. Published studies investigating links between sperm size and velocity. Correlation key: -, negative correlation between morphology and velocity; +, positive correlation between morphology and velocity; 0, no correlation found between morphology and velocity. * refers to studies using individual sperm measurements, all others use mean measures.

Species	Morphology measured	Correlation with velocity	Study/ Studies
Land snail *Arianta arbustorum*	Total length	0	(Minoretti and Baur 2006)
Frog *Crinia georgiana*	Head length	0	(Dziminski et al. 2009)
	Flagellum length	0	
*Sea urchin *Heliocidaris erythrogramma*	Flagellum length	+	(Fitzpatrick et al. 2010)
	Flagellum length	0	
*Mussel *Mytilus galloprovincialis*	Head length	0	(Fitzpatrick et al. 2012)
	Head width	0	
	HV calculated	0	
Atlantic salmon *Salmo salar*	Head length	-	(Gage et al. 2002)
	Flagellum length	-	
Bluegill *Lepomis macrochirus*	Total length	0	(Burness et al. 2004)
	Flagellum length	0	(Stoltz and Neff 2006)
Black goby *Gobius niger*	Tail length	0	(Locatello et al. 2007)
	Total length	0	
Grass goby *Zosterisessor ophiocephalus*	Tail length	0	
	Total length	0	
Guppy *Poecilia reticulata*	Head length	+	(Pitcher et al. 2007)
	Flagellum length	0	
Shell brooding cichlid *Telmatochromis vittatus*	Total length	0	(Fitzpatrick et al. 2007)
Cichlid fish – 29 species from Lake Tanganyika	Total length	+ (for 2 species)	(Fitzpatrick and Balshine 2009)

Species	Morphology measured	Correlation with velocity	Study/ Studies
Primates and rodents	Sperm length	+	(Gomendio and Roldan 1991)
Red deer *Cervus elaphus hispanicus*	Head length	+	(Malo et al. 2006)
	Midpiece length	-	
	Flagellum length	0	
	Total length	0	
House mice *Mus domesticus*	Head length	0	(Firman and Simmons 2010)
	Head width	0	
	Midpiece length	+	
	Flagellum length	0	
	Total sperm length	0	
Zebra finch *Taeniopygia guttata*	Midpiece length	0	(Birkhead et al. 2005)
	Flagellum length	0	
	Tail length	0	
	Total length	+	(Mossman et al. 2009)
	Flagellum length (midpiece +tail)	+	
	Tail length	+	
	Flagellum:head length	+	
Passerine birds	Head length	0	(Lüpold et al. 2009)
	Midpiece length	0	
	Midpiece length	+	
	Flagellum length	+	
	Total length	+	
	Midpiece : flagellum	+	
	Flagellum : head	+	
Pied flycatcher *Ficedula hypolecua*	Total length	0	(Kleven et al. 2009)
	Total length	0	(Lifjeld et al. 2012)
House sparrow *Passer domesticus*	Total length	0	(Helfenstein et al. 2009)
	Head length	0	
	Midpiece length	0	
	Flagellum length	0	
	Head:flagellum	-	

1.4. Sperm morphology

Although diverse, sperm morphology exhibits essentially the same basic elements (Figure 1.2.) for most species. Comprising a head, a midpiece and a tail (flagellum), diversity is found in the relative size and shape of these primary components. Sperm can be flagellate, multi-flagellate or aflagellate, in both plants (Southworth and Cresti 1997) and animals (Morrow 1999).Here, animal sperm with a single flagellum will be considered, all of which typically comprise a head, midpiece and tail (flagellum).

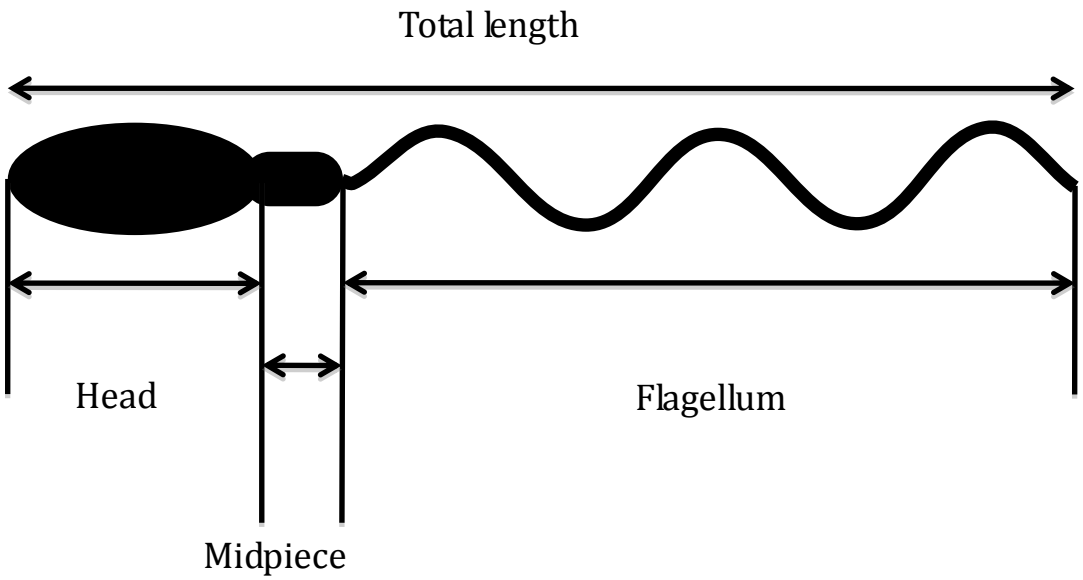


Figure 1.2. Basic structural elements found across spermatozoa from most taxa. Head contains male DNA, midpiece contains mitochondria, which provide energy to the cell, and flagellum provides the propulsive motility of the cell.

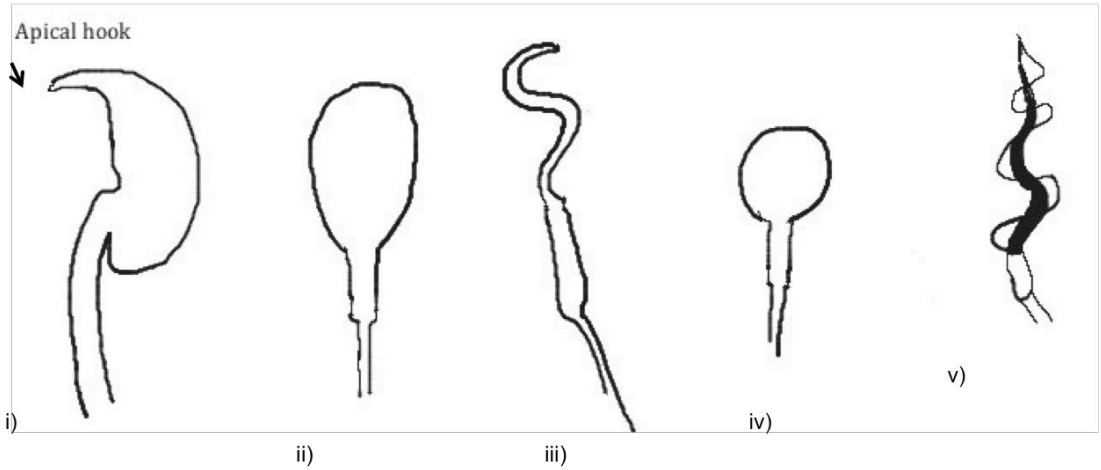


Figure 1.3. Head shape diversity (not to scale) representing species with diverse sperm morphologies. i) Normal mouse sperm displaying the apical hook commonly found in rodent sperm (after Kishikawa et al. 1999); ii) typical paddle-shaped human sperm; iii) Eupyrene sperm of the snail *Pomacea canaliculata* (after Gamarra-Luques et al. 2006); iv) almost-spheroid head, as commonly found in (cichlid) fish; v) typical spiral head of several bird species (Birkhead and Montogmerie 2009); vi) typically elongated head for frogs from the family Myobatrachidae.

1.4.1. Head

A great diversity of head shapes exist (Figure 1.3 and see Roldan et al. 1992 for mammalian sperm head diversity). The classic head shape of eutherian sperm is round/oval but some taxa have developed extremely complex head morphologies. Spiral heads can be found in the sperm of centipedes, beetles and other insects, some frogs, and passerine birds. Marsupials and rodents have apical hooks on the head of their sperm. These hooks have been linked to sperm cooperation in wood mice, where 'trains' of sperm from the same male link together to increase forward propulsion (Moore et al. 2002). There is some suggestion that the shape of the sperm's head might be an important factor in sperm motility for some species (Gillies et al. 2009). Head shape is often mentioned in relation to streamlining (Taggart et al. 1995; Maden et al. 1996; Lopez-Smith and Renzaglia 2008; but see Werner and Simmons 2008) but others state that streamlining is not an important factor on account of the microscopic environment spermatozoa occupy (Humphries et al. 2008). The criteria associated with streamlining reduce its application as a useful concept (Vogel 1996) when considering the movement of such small cells. To date, variation in head shape observed across a range of externally and internally fertilising fish species has failed to be linked to any modification in swimming behaviour (Lahnsteiner and Patzner 2008).

Differences in head morphology may be important, however, when considering Stokes flow in relation to rotation of the cell. Stokes flow refers to the type of flow associated with conditions in which viscosity dominates over inertia, as it does in the microscopic world in which sperm live (Kirkman-Brown and Smith 2011). The scaling parameter of Reynolds number (*Re*) has been used to create scaled models to simulate what is likely to happen when a solid and a fluid interact when inertia is dominated by viscosity (Vogel 1996). When fluid 'particles' in viscosity-dominated fluids move in parallel layers to each other, the flow is described as laminar (Vogel 1996). Very specific wave propagation patterns are required order to create propulsion when flows are laminar.

Having noted that streamlining is unlikely to influence the velocity of cells at low *Re*, there are other hydrodynamic considerations to take into account in relation to head morphology. In high viscosity situations, like those inside the female reproductive tract, the side-to-side movement (yaw) of the head is greatly reduced

(Kirkman-Brown and Smith 2011). Whilst sperm from externally fertilising species seem unable to maintain velocity under highly viscous conditions, the sperm of internally fertilising species are able to achieve this (Kirkman-Brown and Smith 2011). This seems to indicate that head yaw has different functional significance to velocity, depending on the fertilisation environment.

Another consideration is that head size and shape may not be linked to velocity as much as they are to ensuring compatibility with the female gamete. Levitan (1998) compared gamete size across three species of sea urchin, finding that egg size correlated with width of sperm head, swimming velocity and longevity. Gamete trait pairings were found in sea urchins, in which females producing small eggs have males which produce fast, short lived sperm compared to species producing larger eggs where males produced slower but longer lived sperm (Levitan 1998). Such results are a reminder that sperm traits are not under selection pressures in isolation, something that will be discussed further in Chapter 4. The eggs of many externally fertilising teleost fish have a micropyle (a funnel shaped opening on the surface of the egg), through which the sperm must swim in order to complete fertilisation (Amanze and Iyengar 1990). In species with micropyles, there is therefore a necessity for the head of spermatozoa to be complementary to the opening through which they must swim.

1.4.2. Midpiece

The midpiece contains mitochondria that provide the sperm cell with energy via the production of adenosine triphosphate (ATP). Although the energetics of the midpiece are not fully understood, the volume of the midpiece appears to be influenced by sperm competition, with spermatozoa of polyandrous primates exhibiting midpieces with greater volume than monogamous species (Anderson and Dixson 2002). Furthermore, midpiece size appears to be heritable. In a small scale study, no relationship was found between the number or arrangement of mitochondria and the length of sperm in externally fertilising species (Lahnsteiner and Patzner 2008). However, previous work across a range of mammalian species has found a correlation between allometric measures of the midpiece (length and volume) and flagellum length, which the researchers found could be used to predict flagellar beat frequency (Anderson et al. 2005). Malo et al. (2006) then

found a negative correlation between midpiece size and sperm swimming velocity in Iberian red deer (*Cervus elaphus hispanicus*). Complex, species specific selection on midpiece morphology appears to be emerging from empirical data (Firman and Simmons 2010). The midpiece also shows a diversity of form, including spirals (in some passerine birds, molluscs and insects) and lateral membranes (in fish and amphibians) (Pitnick et al. 2009a). As with many aspects of sperm morphology, the adaptive significance of such variation is unclear.

1.4.3. Flagellum

The maximum power output of a single flagellum is proportional to its length (McNeill 1971). In addition, changes in flagellar orientation can double the amount of drag (Vogel 1994), since ATP produced by the mitochondria is often transported down the flagellum using a PCr (phosphocreatine) shuttle to the dynein arms, which are located in the axoneme (Figure 1.4). This, in turn, generates waves along the flagellum (Cosson 2008) which, when propagated down the flagella rod, create the propulsive force for the cell (Brennen and Winet 1977; Kirkman-Brown and Smith 2011). The locus of wave generation is the axoneme (Figure 1.4), located inside the flagellum, with the typical axoneme exhibiting a 9+2 microtubule pattern (Cummins 2009).

The flagellar morphology of teleost fish differs from the morphology typical of other taxa (Figure 1.5). Although there has been little modelling of the specific flagellar morphology of teleost fish spermatozoa, generalisation to the non-circular cross-section of flagella would remain applicable to cylindrical flagella with fin-like projections along the length(Batchelor 1970). It has been suggested that the lateral fins found along the flagella of teleost fish sperm could increase the force created during flagella beating (Lahnsteiner and Patzner 2008). However, the adaptive significance of the lateral fins has yet to be investigated or modelled in terms of their effect on sperm motility. Given the low *Re* environment of sperm, it is likely that any increased drag which might be created by the fins would only have a very weak impact on motility (E. A. Gaffney, pers. comm.).

Cosson et al.(2008) calculated that a cylindrical turbot sperm flagellum with fins has a 25% greater surface area than a cylindrical flagellum without fins. This increased surface area is likely to be important in ionic and osmotic interactions of

the flagellum with the water into which they are released (Cosson 2008), with the possibility that the primary function of the fins is not exclusively concerned with motility.

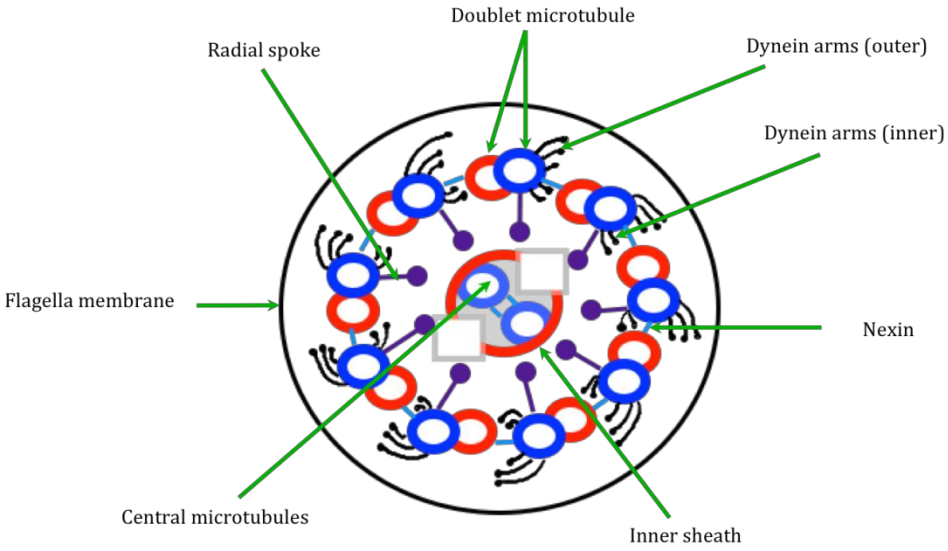


Figure 1.4. Schematic showing elements of the 9+2 doublet microtubule structure of an axoneme in cross-section. Dynein arms are the molecular motors which induce axoneme bending; Nexin bridges connect doublets, preventing adjacent microtubules from moving; Inner sheath and central microtubules provide a core which resists bending; Radial spokes are involved in the mechanical movement of the flagellum.

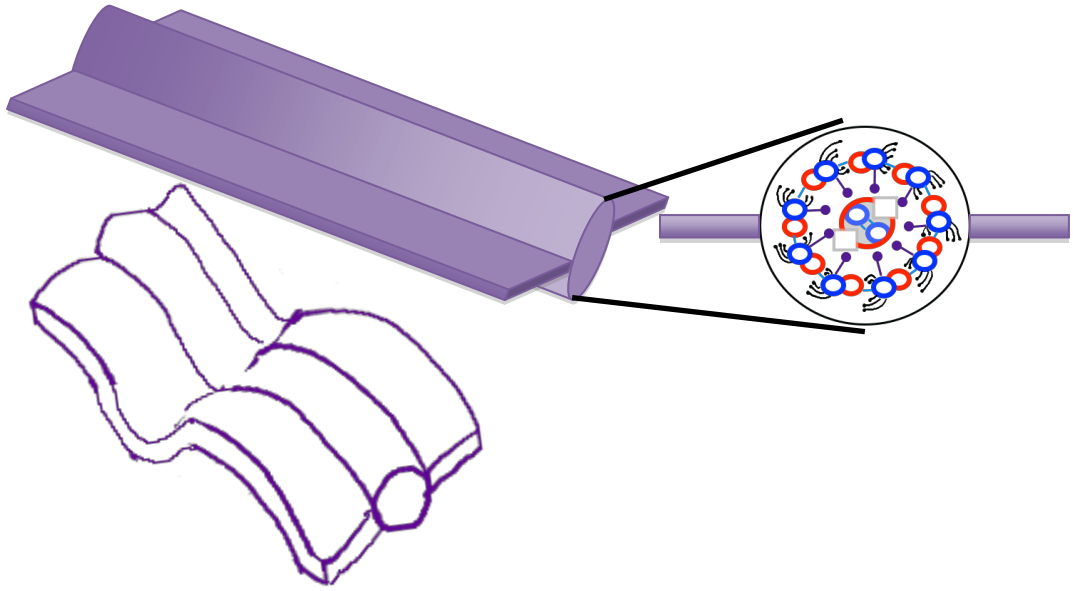


Figure 1.5. Schematic and cross-section of typical fish sperm flagella, with two fins along the axoneme. Axoneme cross-section contains a 9+2 microtubule doublet, as described in Figure 1.4. Purple outline represents bending of flagellum as in swimming motion, where central rod and fins bend together via sliding of microtubules to propel the sperm (see Sections 1.4.3 and 1.5 for details of sperm motility).

1.5. Sperm velocity

Sperm velocity, as measured in computer assisted sperm analysis (CASA) systems, can be described using three measures: velocity along the average path (VAP) – this is point-to-point velocity using an average of the cell's actual trajectory; velocity curvilinear (VCL) – here point-to-point velocity reflects the total distance travelled; and velocity along a straight line (VSL) – calculated by taking the start point and end point and averaging the path in between (Figure 1.6). The propagation of flagellar waves requires a balance between the shear force created internally from microtubule sliding, the elastic resistance of the flagellum, and viscous drag (Gadelha et al. 2010).

Much of the early work on sperm flagellar movement was carried out by Gray and Hancock (1955), who used sea urchins as a study species to assess flagellar waveform and forward movement of sperm. Sperm motility is achieved through the bending of the flagellum, using energy from the mitochondria in the midpiece. The number of mitochondria present in the midpiece varies from species to species. For example, in mammals, ~15 mitochondria are typical in human sperm whilst 350 are typical in rat sperm (Cummins 2009). Within the flagellum, the axoneme (Figure 1.4) is a structure typically comprising an archetypal pattern of nine microtubule pairs surrounding a central pair, referred to as '9+2' doublet microtubule pattern (Gaffney et al. 2011). Bending of the flagella is created when the inner and outer dynein arms hydrolyse ATP into adenosine diphosphate (ADP), thereby transforming chemical energy into shearing force. Shearing results in axonemal bending as a product of the sliding force between the microtubule doublets (Cosson 1996; Gaffney et al. 2011). This bending moment propagates the length of the flagellum (Machin 1958), propelling the sperm forwards.

A symmetrical flagellar waveform produces a relatively straight trajectory whilst asymmetrical waveforms result in curved trajectories. However, the way in which asymmetrical beating is produced remains unclear (Gadelha et al. 2010).

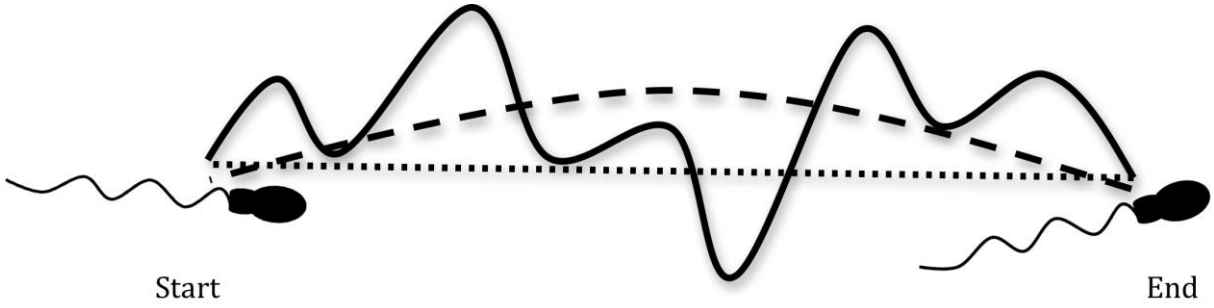


Figure 1.6. Illustration of the difference in velocity measurement from start point to end point of the swimming trajectory of sperm cells tracked using CASA. Solid line represents actual path of tracked sperm cell; grey area represents VCL, reflecting velocity across the actual path taken; dashed line represents the average path of the cell as calculated for VAP; and dotted line is path used to calculate VSL. Paths tracked by CASA using head centroid position.

1.6. The hydrodynamics of propulsion

It is important to remember that at the microscopic scale of sperm, there are environmental conditions that are counterintuitive to the physical laws we are familiar with. At the small (~0.03) *Re* at which sperm operate, the dominant drag force at low *Re* is that of viscosity rather than inertia (Vogel 1994). Viscosity influences sperm propulsion by affecting the movement of the head and flagellum (Kirkman-Brown and Smith 2011). As soon as propagation of waves down the flagellum stops, the movement of the spermatozoa ceases as there is little or no inertia to continue the progressive movement of the cell. It is interesting to note that increased viscosity appears to impact the swimming velocity of external fertilisers more than decreased viscosity influences internal fertilisers (Gaffney et al. 2011).

1.7. Linking sperm morphology to velocity

Links between morphology and velocity are often contradictory to theoretical predictions (Table 1.1.), which is important as the way in which we consider the evolution of sperm traits is based mainly on theory. Knowledge of sperm biology is fundamental to our understanding of the biological processes involved in reproduction. Agriculture relies heavily on artificial insemination (Gravance et al.

1998), with the semen from the best quality bulls often being extremely valuable. It is important that sperm selected artificially are the ones best able to fertilise the egg and exhibit the same traits that would be selected for under natural conditions. Sperm which are not fit to fertilise the egg or which would produce less viable offspring are naturally filtered out during the selection process within the female reproductive tract (Suarez 2005). The criteria by which the fittest sperm can be identified therefore requires updating as the field of sperm biology develops and the factors that make certain sperm fitter than others become evident. As we have seen, the literature contains little directly comparable evidence of a link between sperm length and swimming velocity (Humphries et al. 2008), implying a need to establish criteria by which the morphology of sperm can be consistently assessed.

1.8. Measuring sperm morphology

Each of the components of sperm morphology could potentially reveal important correlations with velocity. Traditionally, total sperm length has been the measurement most often cited in the literature, which is typically measured from the front of the head to the tip of the tail. Until recently, these measurements have been taken from a subset of sperm for which measures of velocity are not taken (for logistical reasons). It is the appropriateness of measuring total length for one set of sperm and then comparing such measurements against velocity measures taken from a different subset that shall be reviewed in this section.

It has been predicted that sperm morphology should be optimised independently of the phenotype of any individual male and that the selective forces relating to fertilisation environment and level of competition exert the greatest selection pressures (Pitnick et al. 2009b). Several studies across taxa link length to fertilisation success (Mossman et al. 2009), finding both positive (Lüpold et al. 2009) and negative (Stockley et al. 1997) correlations, but few link morphology to velocity (for a link across mammals see Tourmente et al. 2011)(Table 1.1). If length is not directly related to velocity but velocity is correlated to fertilisation (for a review see Simmons and Fitzpatrick 2012), are the most appropriate elements of morphology being measured?

It has been suggested that a ratio of components may be a more reliable indicator of velocity than single measurements of length (Humphries et al. 2008). This has been demonstrated empirically (Mossman et al. 2009) to be the case using a flagellum:head length ratio. It has also been established that when comparing within-species ratios of morphological measures, these composite measures relate to velocity measures more accurately than single morphology measures alone (Gomendio and Roldan 2008). Furthermore, selection is likely to act on the individual component parts of spermatozoa rather than on overall length (Humphrieset al. 2008).

Variation in sperm morphology has been identified both within and between males and the extent of the variation found has been linked to sperm competition (see Section 1.9.2). Increasing levels of sperm competition were found to reduce variation in within-male sperm traits in passerine birds (Calhim et al. 2007). However, when between-male variation was investigated in *Drosophila melanogaster,* it was found that measuring only two individual sperm could account for up to 91% of the variation found in sperm traits between-males (Pattarini et al. 2006). If within-male variation is present but not accounted for, it should not be surprising that no correlation between morphology and velocity can be found. It is well documented that males from several species produce pleomorphic sperm, which often represent fertilising and non-fertilising types. The Kamikaze sperm hypothesis (Parker and Begon 1993) allows for the possibility that different sperm phenotypes within an ejaculate have specific functions, some of which increase the overall fertilisation success of the ejaculate by incapacitating sperm from other males. Sperm heteromorphism, where males produce both short and long sperm, has been observed in *Drosophila obscura,* where the short sperm compose half of the ejaculate but do not fertilise the ova and do not seem to be linked to increasing levels of sperm competition - a unique feature which is not found in other *Drosophila* species (Snook 1998).

Different sperm types can be more or less obvious, depending on the species (Till-Bottraud et al. 2005), making functional significance of morphological traits difficult to interpret. Small scale variation, which makes it difficult to identify different sperm types in mammals, has been related to production errors during sperm development (Harcourt 1991). The production of high quality sperm is

costly and an absence of sperm competition appears to increase production errors. A recent phylogenetically controlled comparative study of Australian Maluridae (passerine birds) found that the level of sperm competition was positively related to the production of more motile and morphologically normal sperm than were produced when sperm competition was reduced (Rowe and Pruett-Jones 2011). Heteromorphism in invertebrate sperm, however, has not been related to production errors but rather reflects consistency of specific developmental origins (sperm types are produced in different cysts within the testis), with large percentages of ejaculate forming discrete sperm types (Till-Bottraud et al. 2005). Whilst it has been suggested that the existence of multiple sperm types within an ejaculate is selectively neutral, it could also be adaptive (Till-Bottraud et al. 2005), with the functional significance yet to be fully understood.

Calhim et al. (2007) note that sperm morphology is highly heritable, and is not restricted by environment or condition, suggesting that selection should act to decrease within-species variation. There are several evolutionary mechanisms that might factor in the attainment of optimum sperm morphology: i) sperm competition ii) cryptic female choice and iii) genetic heritability. The selection pressures on sperm traits are considered in the following sections.

1.9. Selection pressures – sexual selection

Whilst this thesis does not directly deal with the manipulation of selection pressures to investigate the influence they have on sperm evolution, it is useful to outline these pressures as they are fundamental to an understanding of sperm form and function. As such, the processes outlined below form integral parts of the discussions in all chapters of this thesis.

The ability of organisms to adapt to their environment in order to produce numerous, viable offspring forms an essential part of natural selection (Darwin 1859). As part of natural selection, it is sexual selection which exerts pressure on gametes of both sexes (Darwin 1871). Sexual selection, which can be expressed as a preference of one sex for characteristics of the other sex, can work at the whole-animal level or the level of the gametes, and can be expressed via male-male competition as well as by female choice. Sexual selection via male nuptial colour has been found across many species to demonstrate male fitness and assist

females in mate choice (Kodric-Brown and Brown 1984). Pattern can also be important. There is some evidence, for example, that in cichlids living in deep-water it is the male's pattern rather than its colour which is selected for, as the range of visible light is reduced with water depth (Genner et al. 2007b). In guppies evidence has been found for phenotype-linked fertility through sexual dimorphism which can reflect male sperm traits (Pitcher et al. 2007).

Investment in reproduction is a fundamental aspect of the life history of every species. Sexual selection can be defined as "selection that arises from intrasexual differences in the proportion of an individual's gametes that fuse to become zygotes" (Levitan 1998). In accordance with Bateman's principle, an individual male's reproductive success will vary considerably as competition increases (Arnold 1994). Males have a greater potential to produce offspring than females (Arnold 1994) as females look to increase the quality of offspring whilst males, by-and-large, seek to produce high numbers of offspring, resulting in a conflict of interest between males and females. In addition, the fitness priorities of the haploid genome expressed by the spermatozoa may be in "evolutionary conflict" with that of the diploid genome expressed by the male (Pizzari and Foster 2008). Such conflicting selection pressures could explain why there is so much variation in sperm form, as reproductive fitness is different for individual male versus individual sperm.

1.9.1. Postcopulatory sexual selection

Postcopulatory sexual selection is considered here in the form of sperm competition, occurring when more than one male's sperm compete to fertilise a specific female's eggs, and represents cryptic female choice. Postcopulatory sexual selection has the potential to have a significant impact on the evolution of sperm morphology. Both direct and indirect benefits for female polyandry have been identified, leading to population and molecular level evolutionary implications (Birkhead and Pizzari 2002). Cryptic female choice and male-male competition are the key elements that influence fertilisation success in polyandrous species (Birkhead and Pizzari 2002). Of interest to this thesis is the finding that sperm length tends to correlate with morphology and length of the female reproductive tract and/or sperm-storage organs (Pitnick et al. 2009a). Miller and Pitnick (2002)

have shown that in *Drosophila melanogaster,* the length of the female sperm-storage organ influences the length of sperm that males produce.

1.9.2. Sperm competition

Sperm competition is a widespread form of sexual selection found throughout the animal kingdom as well as in the plant kingdom, where it takes the form of pollen competition (Birkhead and Pizzari 2002). Defined as "competition between sperm from two or more males for the fertilization of a given set of ova" (Parker 1998), sperm competition imposes a significant selection pressure on the structure and function of sperm (Birkhead and Pizzari 2002; Evans and Simmons 2008).

Sperm competition increases as the number of males vying to fertilise a limited number of eggs increases (Levitan 1998). There is an important distinction to be made here between sperm competition and sperm limitation, as there are different consequences in relation to sexual selection in each scenario. Sperm competition implies an abundance of sperm in competition, whereas sperm limitation refers to the availability of sperm (Levitan 1998). According to sperm competition theory, sperm traits are predicted to be a reflection of the level of sperm competition. This has often been broadly interpreted to suggest that longer sperm swim faster, therefore enhancing competitive edge and reproductive success (Snook 2005; Humphries et al. 2008).

Another way to view sperm competition is as intra-ejaculate competition, where competition exists between different genotypes within an ejaculate (Parker and Begon 1993). Developmental noise and production errors have been identified as reasons for some levels of within and between-male variation in sperm traits (Pitnick et al. 2009). Birkhead and Pizzari (2002) have highlighted that the variation within a male's ejaculate, once considered to be the result of 'production errors', may instead be adaptations to increase fertilisation success. Snook and Karr (1998) found that the *Drosophila obscura* group produce two size classes of sperm, with only the long sperm fertilising eggs despite the shorter sperm possessing all the attributes necessary to fertilise ova. Some molluscs, annelids and lepidopterans (Jamieson 1987; Brakefield et al. 2001) produce different classes of sperm, only some of which can fertilise ova and with the rest used as a bulk defence against sperm from other males. Fisher and Hoekstra (2009) found evidence of co-operation in genetically-related spermatozoa in ejaculates of male

deer mice (genus *Peromyscus*), which increased their swimming velocity by forming motile groups with conspecific sperm. A similar phenomenon has also been observed in wood mice, *Apodemus sylvaticus*, where spermatozoa link up using their apical hooks so that the group swims with an increased velocity (Moore et al. 2002). Unlike sperm competition, wherein sperm of different males compete for fertilisation success, sperm cooperation occurs within an individual male's ejaculate to enhance the fertilisation success of that male. Such cooperation may be advantageous in species with internal fertilisation and multiple mating. The genetic variation within one male's sperm may thus have phenotypic and functional implications for sperm form.

Trade-offs between sperm traits and the production of different sperm phenotypes within a single ejaculate are also considered to be an important factor in sperm competition. The cost incurred by the male of producing competitive sperm may be a limiting factor in the evolution of sperm optima (Mossman et al 2009). Selection through sperm competition can erode variation in sperm phenotype (Immler et al. 2008). There is evidence to indicate that males can produce an ejaculate that is composed of different types of sperm, with each type having specific roles in order to increase the fertilisation success of the individual male. *Drosophila pseudoobuscura* males produce at least two different sizes of spermatozoa (Snook and Karr 1998). Evidence of killer sperm phenotypes, as suggested by the Kamikaze sperm hypothesis (Baker and Bellis 1988), has not found support in humans (Moore et al. 2006), but some evidence supporting the hypothesis has been found in the rat *Rattus norvegicus* (Bellis et al. 1990). Whilst the idea of their function - to prevent sperm from other males fertilising the ova by sacrificing themselves - seems plausible in the light of other known strategies, there is little empirical evidence that this is truly so. Gomendio and Roldan (1993) suggest that trade-offs between sperm size and longevity require further consideration. The implicit requirement of sperm competition is that all sperm are capable of completing fertilisation. However, it has been suggested that sperm which can remain motile in the cervix despite having lost fertilising capacity can reduce the number of sperm reaching the oviduct from a rival male (Baker and Bellis 1995).

Møller (1998) considers that sperm competition needs to be viewed as one element of sexual selection, with its importance being related to other aspects of sexual selection, such as mating system (section 1.11). Only then can the relative importance of sperm competition be elucidated. Sperm competition has been related to male parental care in some bird species, with greater risk of sperm competition correlated to a reduction in male involvement in raising the offspring (Møller and Birkhead 1993). In the absence of sperm competition, theory predicts a reduction in the investment in sperm design (Parker 1998), quality control (Birkhead and Immler 2007) and number. The influence of the female induced selection on sperm form could be even more important in species with little sperm competition.

1.9.3. Cryptic female choice

Cryptic female choice is a process by which females can affect the reproductive success of males after copulation, which in turn means that male mating success will not automatically equate to fertilisation success (Eberhard 1996). Sperm competition requires females to be promiscuous, which can be costly for the female (Birkhead 2000). The costs to females of mating with multiple males range from investment of extra time/energy in finding additional mates, to exposure to sexually transmitted disease and increased risk from predation (Jennions and Petrie 2000). However, if the quality of males cannot be accurately assessed, it may be in the female's interest to mate with additional males. This bet-hedging strategy (Philippi and Seger 1989) provides the female with the opportunity to have the male with the best genes fertilise her eggs. As part of the good genes hypothesis, the assumption is that gametes produced by the fittest males will outcompete gametes produced by less genetically fit males, resulting in most offspring being fathered by the fittest male (Parker 1983). As an extension to the good genes hypothesis, the good-sperm and sexy-sperm hypotheses reviewed by Evans et al. (2007) consider female polyandry to not only be beneficial to females, indirectly through their sons, but to also influence sperm competition.

Cryptic female choice may also be displayed through chemical signalling, especially through the release of chemoattractants that guide sperm to the egg (Kaupp et al. 2008). Such attractants can activate sperm motility (Zimmer and Riffell 2011),

guide sperm towards the egg (Eisenbach and Giojalas 2006; Krug et al. 2009) and ensure species-specific gamete encounter in order to reduce cross-fertilisation and hybridisation (Ram et al. 1996). For example, the egg jelly surrounding eggs of the quacking frog contain chemoattractants which have been found to influence sperm activation and swimming velocity (Simmons et al. 2009). Ovarian fluid can also increase sperm longevity and swimming velocity in salmonids (Lahnsteiner and Weismann 1999; Turner and Montgomerie 2002).

1.10. Internal versus external fertilisation

Models of sperm competition use evolutionarily stable strategies (ESS), which can be used to simulate evolutionary games between rival males considering various ejaculate strategies, as part of evolutionary game theory (Parker 1998). Parker (1970) originally described sperm competition as a raffle, which can be fair or loaded. In a fair raffle, a male's probability of fertilisation success is equal to the number of his sperm in the female tract divided by the total number of sperm in the female tract. In contrast, a loaded raffle involves the situation where one male's sperm are at a disadvantage when compared to the sperm from a competing male (Ball and Parker 1996).

Evolutionarily stable strategy models can be used to outline strategies males should adopt when faced with sperm competition, depending on whether fertilisation is internal (Parker and Begon 1993) or external (Ball and Parker 1996). The continuous fertilisation model, in which sperm size is predicted to increase with sperm competition (Ball and Parker 1996), is generally used for externally fertilising species. However, this model may not be appropriate for the mouthbrooding cichlid species considered in Chapter 3 (Section 3.4). For species of mouthbrooding cichlids, it is possible that the instantaneous model is more appropriate as mouthbrooding has been considered akin to internal fertilisation (Konings 2007). Selection pressures on internally fertilising species include female tract length (Miller and Pitnick 2002) and female storage organ morphology (Simmons and Kotiaho 2007).

The instantaneous fertilisation model, usually considered most appropriate for internally fertilising species, predicts sperm size does not alter with level of sperm competition (Parker 1993). However, it is important to note that in non-

competitive contexts there may be little selection for increasing sperm size and neither model applies in the absence of sperm competition.

External fertilisation involves the release of gametes into open water and is considered to be the ancestral state of sexual reproduction. Externally fertilising species therefore produce sperm which are not adapted to swim through highly viscous fluid (Kirkman-Brown and Smith 2011). Internally fertilising species not only need to navigate the highly viscous female tract but also need to be able to free themselves from epithelium cells.

A model of external fertilisation developed by Ball and Parker (1996) assumes a relationship between size and longevity, further assuming that velocity increases with length, with velocity viewed as an indicator of competitiveness (Birkhead and Møller 1998). The fertilisation success of externally fertilising species is influenced by gamete encounter rates (Levitan and Petersen 1995), which in turn depends on water currents (Zimmer and Riffell 2011). Male fertilisation success increases if he mates <1m away from the female (Levitan and Petersen 1995). The effect of water hardening means that once gametes are released, they only have a short amount of time before eggs become unfertilisable. Liley et al. (2002), for example, suggest a 10-20 second window of opportunity before water hardening of rainbow trout eggs prevents sperm being able to penetrate.

High levels of interspecific variation in sperm traits are often linked to differences in mating system. The next section will therefore look at different mating strategies and consider how this might influence sperm form and function.

1.11. Mating strategy

Internal fertilisation is a prerequisite for species to have life-histories fully independent of the need for an aquatic medium to release gametes into, and so to become truly terrestrial (Parker 1970). The mating strategy of individuals is important when considering how to allocate resources to ejaculate characteristics in order to maximise fertilisation success. It has been suggested that, in some species, males of less attractive phenotypes might invest in more competitive ejaculates to compensate for a reduction in mating opportunities (Birkhead and Pizzari 2002). Whilst this seems a valid trade-off, such a strategy may be condition

dependent. There is evidence to suggest individual males can adjust ejaculate characteristics according to mating conditions, but how this may influence evolution of individual spermatozoa is unclear (Parker 1998; Birkhead and Møller 1998). When different mating strategies are used in a population, males can adapt the ejaculate qualities to an optimum for their chosen mating strategy (Gage et al. 1995).

Many species of birds are socially monogamous and sexually promiscuous, meaning that females that have paired with phenotypically suboptimal males may seek out extra-pair mating opportunities with more attractive males (Birkhead and Pizzari 2002). However, Evans (2010) found that in guppies there may be an evolutionary trade-off between sexual attractiveness and investment in sperm competition, finding that males engaged in sneaker behaviour were better equipped in the presence of sperm competition than males preoccupied with courtship. When there are extra pair copulations, the order in which males mate can be related to fertilisation success. In birds, the last male to mate fathers most of the brood, but in the sierra dome spider (*Linyphia litigiosa*) the reverse situation is true, with the first male to mate fathering 60-70% of the offspring (Watson 1991).

1.12. Aims

The aim of this thesis was to assess the likelihood that relationships between sperm morphology and sperm swimming velocity would be found to be widespread across species if appropriate measurement and analysis techniques were applied. The next section will introduce how this was carried out, on a chapter-by-chapter basis.

1.13. Thesis outline

With the broad background in place, the remaining chapters cover the following specific areas of research, with details regarding chapter-specific themes outlined in the introduction to each chapter. In **Chapter Two,** the influence of mature oocytes on the swimming behaviour of bovine sperm is considered, with particular focus on velocity and direction, and comparing fresh and freeze-thawed sperm samples. As with spermatozoa from other internally fertilising species, bovine

sperm are transported much of the way through the female's reproductive tract by muscular contractions of the uterus (Suarez 2005), and may not rely on their own propulsion until they are in relatively close proximity to the oocyte. This chapter aims to investigate the variation in velocity achieved by sperm cells in the presence of a source of attractant. **Chapter Three** then considers the question of whether sperm morphology can predict velocity across closely related species. This chapter also discusses the influence of mating strategies and shared ancestry. Despite cichlids generally being a well-studied group in relation to such aspects of their ecology as speciation, little information has been gathered on sperm morphology of cichlid fish from Lake Malawi, and on how this might relate to the speciation of the flock. Using methods adopted in the previous chapter for measuring sperm traits, **Chapter Four** focuses on the potential advantage of taking measurements of sperm morphology and velocity from the same cell. As outlined above, traditional methods of comparing sperm morphology to velocity tend to use mean measures from different subsets of cells, which could potentially mask correlations between individual cell morphologies and velocities. This chapter also considers the importance of within-male variation in sperm traits in relation to fertilisation point, i.e. internal vs. external, using a broad taxonomic sample (emu, frog, guppy, human, mussel and rainbowfish). A general discussion forms **Chapter Five,** which reviews the findings of the previous chapters in order to summarise the data, and brings the thesis to a close by suggesting future directions that could further elucidate links between sperm form and function.

Chapter Two: Sperm swimming behaviour in the presence of mature oocytes: comparison between fresh and freeze-thawed bovine samples.

Abstract

Chemotaxis in sperm has been well documented in externally fertilising marine species, however sperm-egg chemical communication in mammals has only recently been identified, appears to be complex, and remains unclear. Hyperactivated mammalian sperm have been shown to undergo chemotaxis as an integral aspect of navigation towards an oocyte. Furthermore, the presence of the cumulus-oocyte complex has been shown to influence the swimming behaviour of mammalian sperm. Changes in sperm swimming behaviour suggest a chemo-attractant released by the cumulus-oocyte complex could be important in mammalian sperm guidance to the oocyte. The concentration gradient of the attractant required to induce changes in sperm motility appears to be variable and species-specific. This study therefore sought to investigate the influence of the number of mature oocytes present on the swimming behaviour of bovine sperm, with particular focus on speed and direction. Sperm from two, high value stud bulls were analysed. Sperm from one male had been frozen using industry standard cryopreservation techniques. The sperm from the second male was used fresh, having been stored and transported in an ambient extender. Bovine oocytes were harvested from abattoir-derived ovaries and matured *in vitro*. Results indicate that fresh sperm had faster straightline velocities than freeze-thawed sperm, with fresh non-capacitated sperm having the fastest curvilinear velocities. In addition, the effect of the cumulus-oocyte complex varied depending on sperm preservation technique (fresh versus freeze-thawed), as revealed through the complexity of swimming trajectory and sperm velocity.

2.1. Introduction

The process of chemotaxis is used to refer to the movement of sperm towards a source of chemical attraction (Eisenbach and Giojalas 2006). Investigations of sperm chemotaxis have largely been focused on marine species (Miller 1997; Morita et al. 2009; Zimmer and Riffell 2011; for example Binks et al. 2012), especially broadcast spawning species that have been found to release species-specific attractants which appear to reduce hybridisation and increase gamete encounter rates (for example Riffell et al. 2004). The existence and functional significance of chemotaxis in internally fertilising species was not appreciated until relatively recently. Chemotaxis was largely ignored in internally fertilising species because sperm are released in large volumes directly into the female tract (Eisenbach and Ralt 1992), thereby negating the need for eggs to attract spermatozoa in the manner of marine species.

There is now, however, increasing evidence that chemotaxis is a widespread phenomenon across a range of species, including insects (Yang and Lu 2011), plants (Paolillo 1981) and mammals (Cohen-Dayag et al. 1995; Fabro 2002; Gakamsky et al. 2008; Armon and Eisenbach 2011), and plays a vital role in sperm-egg interactions (for a review see Eisenbach and Giojalas 2006). The difficulty in recognising chemotaxis in mammals relates to the small number of sperm which are receptive to chemoattractants at any one time within an ejaculate (Eisenbach 1999). A further limitation has been the spatial scale detectable by tracking software (Gakamsky et al. 2008). The difficulty lies in determining how to track the small number of chemoreceptive sperm when faced with an ejaculate in which a majority of motile but non-receptive sperm mask the receptive sperm movements (Gakamsky et al. 2008).

Longer sperm are often assumed to swim faster than short sperm (Gomendio and Roldan 1991) and, with all other things being equal and under conditions of sperm competition, faster sperm should reach and fertilise the oocyte first (Snook 2005). However, all things are rarely equal and models based on evolutionarily stable strategies (ESS) do not predict that sperm size alters as a result of sperm competition unless assumptions about the functional role of sperm length are made (Parker and Begon 1993; Ball and Parker 1996; Parker 1998). There remains

little empirical data supporting assumptions about the functional significance of sperm traits for most species (Snook 2005).

The potential for the presence of a chemoattractant to alter swimming velocity of a spermatozoon (Eisenbach 1999) whilst the length of the cell remains the same would be further evidence that a simplistic link between sperm length-speed-fertilisation success is unlikely to be valid for many species. Stages of activation may be also be common characteristic of sperm motility, with slow swimming velocity during the early stages of activation having been recorded for marine fish (Lahnsteiner and Patzner 1998). Mammalian sperm can apparently switch between activated and hyperactivated motility (Suarez and Osman 1987) in order to respond to gradients of attractant produced by oocytes (Sun et al. 2005).

In the present study, bovine gametes were used to investigate the interactions of sperm and the mature oocytes surrounded by cumulus cells. Cumulus cells are granulose cells which form around the oocyte as a part of its maturation (Tanghe et al. 2002). Female gametes will hereafter be referred to as the cumulus-oocyte complex (COC) and were used as the source of chemical attractant to investigate sperm chemotaxis. The definition of chemotaxis used in the following sections follows that of Eisenbach (1999), where chemotaxis is described as a change in the directional movement of sperm responding to a chemical attractant.

2.1.1. Bovine spermatozoa; motility and chemotaxis

The state of hyperactivation in mammals accompanies capacitation and is vital for fertilisation success (Armon and Eisenbach 2011). For mammalian spermatozoa to become chemoreceptive, they must have been capacitated (Eisenbach and Ralt 1992). The process of capacitation involves a change in the plasma membrane of the sperm cell (Watson 2000), and alters flagella waveform and swimming trajectory (Gadelha et al. 2010). Hyperactivation and high-amplitude flagella beat frequency accompany capacitation (Yanagimachi 1970; Gaffney et al. 2011) and precede the acrosome reaction, which is vital for fertilisation (Marquez 2004). The process of acrosome reaction involves the release of proteolytic enzymes, which in turn enable the sperm to penetrate the oocyte (Eisenbach and Giojalas 2006).

The presence of a bovine COC has been found to influence both capacitation and acrosome reaction of bovine spermatozoa (Chain et al. 1995). The cumulus cells appear to be the source of attractant for cattle (Chain et al. 1996) in which mature oocytes release progesterone (Tanghe et al. 2002). Chemotaxis may guide sperm towards the site of fertilisation (Eisenbach and Giojalas 2006), however it has also been suggested that chemotaxis may be a release mechanism allowing only capacitated sperm to move from storage sites (Eisenbach 1999). Sperm bind to the oviductal epithelium, creating storage reservoirs from which only hyperactivated sperm can release themselves (Suarez 2006; 2008). Oviductal epithelium lengthens sperm viability (Satake et al. 2006), which appears to be achieved by reducing sperm motility (Holt 2011). Bovine cumulus cells stimulate sperm motility (Bronson and Hamada 1977) and appear to be important in the capacitation of spermatozoa (Chain et al. 1995). This in turn affects the ability of the sperm to penetrate the COC and fertilise the oocyte (Chain et al. 1996).

2.1.2. Cryopreserved versus fresh bovine spermatozoa

Cryopreservation is an important tool in artificial insemination (AI) for livestock breeding (Vishwanath 2003). The main benefit of cryopreservation is that it provides long-term storage of male semen. Maintaining desirable livestock genetics for traits such as improved milk yield, calving rates, overall health, and longevity of a herd is big business, with the semen of high quality stud bulls in much demand (Vishwanath 2003). Technology which improves the delivery of high quality livestock, such as cryopreservation of semen, is of vital importance to global agriculture. The cryopreservation of bovine semen typically involves freezing straws containing 10-15 million live spermatozoa (0.25ml) using liquid nitrogen or controlled-rate freezing machines (Holt 2000).

Despite 60 years of refinement (Curry 2000), the process of cryopreservation continues to have the effect of reducing the fertility of individual sperm cells (Shannon and Vishwanath 1995; Cormier and Sirard 1997). Cryopreservation has been shown to negatively impact sperm viability as freeze-thawed sperm show reduced motility (Nijs and Ombelet 2001). Cryopreservation has also been found to alter the ultrastructure of the sperm's plasma membrane, which reduces the cell's ability to adapt to fluctuations in calcium levels (Cormier and Sirard 1997)

and this in turn influences motility. Shannon and Vishwanath (1995) found that it took eight times as many freeze-thawed bovine spermatozoa to reach the same fertilisation levels as fresh sperm *in vivo* as a result of cryoinjury.

The discovery and use of glycerol as a cryoprotectant has proved extremely beneficial to the preservation of bovine semen (Holt 2000) and improved the survival rates of cattle sperm (Trimeche et al, 1999). Cryoprotectants are added to the sperm samples as they are cooled for storage in liquid nitrogen, but damage to the sperm cell still occurs (Creemers et al. 2011). Most of the damage to sperm during the freezing process is caused by the formation of ice crystals at the intracellular level, as well as osmotic damages caused by the addition and/or removal of cryoprotectants (Hammadeh et al. 2001; Nijs and Ombelet 2001).

As a functional link between sperm morphology and fertilisation success, chemotaxis could provide insight into why speed alone may not increase fertilisation rates. The ability to detect and swim towards a chemoattractant released by the female or her gametes (Eisenbach 1999) could in fact be key to fertilisation success (Riffell et al. 2004; Guerrero et al. 2010). Morphological modifications to enhance swimming skill might therefore be expected to increase fertilisation success. In mammals, when a gradient of chemoattractant is released by oocytes it has been found that the form of flagellar beating of hyperactivated sperm alters in such a way that the path of the sperm moves towards the attractant (Chang and Suarez 2010).

In the present study, bovine gametes were used to assess the swimming behaviour of conspecific sperm from two individual bulls with proven fertility across different levels of activation (capacitated/non-capacitated) in the presence of increasing numbers of COC. Samples of fresh spermatozoa were collected from one bull and samples subsequently cryopreserved were collected from the other bull. The limitations of this small sample size in terms of data comparisons and interpretation will be discussed below (in Section 2.5), however problems with supply of oocytes meant that sperm samples from only two bulls could be used before the oocyte supply ran out unexpectedly. Both animals had comparably high fertility based on non return rates (NRR). This measurement represents the percentage of heifers that did not return to oestrus by day 49 after AI (i.e. the number that were pregnant). Sperm samples were from bulls with NRR in the

range of 70-83%. Both males were part of livestock artificial insemination breeding programmes and as such, of proven fertility.

Freeze-thawed sperm exhibit capacitation-like characteristics (Green and Watson 2001), and were compared with both fresh sperm that had been incubated under capacitation conditions and fresh sperm that were not capacitated. It was expected that comparing capacitated and non-capacitated sperm would correlate with hyperactivated and activated motility as there is some suggestion that hyperactivated motility is important in mammalian chemotaxis (Chang and Suarez 2010). Whilst the study had intended to record flagella morphology for individual spermatozoa as well as velocity, as work progressed it became evident that focusing on both sperm head (for tracking velocity) and flagella (for beat amplitude/morphology measurement) was not going to be consistently achievable across samples. It was therefore decided that it would be most practical to concentrate on the velocity of the spermatozoa rather than flagellum characteristics, as identifying alternate track characteristics could potentially be used as a proxy for the flagella's wave pattern since trajectory is a result of flagellar movement (Eisenbach 1999; Gadelha et al. 2010).

Hypothesis

Bovine sperm velocity will alter in speed and direction in the presence of a conspecific cumulus oocyte complex, depending on spermatozoa preservation method (fresh versus freeze-thawed) and level of activation (capacitated versus non-capacitated).

2.2 Methods

Freeze-thawed and fresh bovine spermatozoa samples were obtained from two individual Holstein-Friesian stud bulls (all semen samples were obtained from Genus/ABS, Ruthin, Wales, a subsidiary of Genus plc.). Each bull was considered to have high fertility and had previously provided semen for use in artificial insemination. Male sample size for each set sperm type was one. The semen of one bull, having been cryopreserved, was used for the freeze-thawed samples and a second male provided the fresh samples.

2.2.1. Freeze-thawed sperm preparation

Sperm were selected using a discontinuous Percoll (colloidal silica coated with polyvinylpyrrolidone) gradient (Rosenkrans et al. 1993). Briefly, Percoll gradients were prepared by layering 2ml of 45% Percoll on top of 2ml of 90% Percoll. Straws containing frozen bull semen were removed from liquid nitrogen and thawed at 37°C before being laid on top of the Percoll gradient. The Percoll gradient was then placed in a centrifuge for 30 min at 2200 rpm. The supernatant was then carefully removed, leaving the pellet of sperm at bottom of the centrifuge tube, which was then suspended in 4ml of Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) TALP (tyrode-albumin-lactate-pyruvate) and centrifuged for 5 min at 1200rpm. The supernatant was again removed and the remaining sperm resuspended in 200µl Hepes TALP. Sperm were kept in the Hepes TALP at 39°C to maintain the pH of the samples and keep the sperm viable.

Experiments were run over a number of weeks, and each used freshly prepared sperm samples from the same bull. The order of treatments was randomised across weeks to ensure no effect of treatment order. For each treatment, data across weeks were pooled so that each treatment used sperm from every fresh preparation. Sperm counts were performed by taking 10µl of the sperm suspension and diluting it with 190µl of tap water to kill the sperm. 10µl of diluted dead sperm was then placed on a haemocytometer and the total number of sperm in a specific pattern of 10 squares was recorded before being divided by two to get sperm concentration per ml. A sperm count was carried out for each sample for both freeze-thawed and fresh sperm samples.

2.2.2. Fresh sperm preparation

Fresh semen samples were received via next day special delivery and used within 36 hours of ejaculation. Samples of 100µl raw semen were stored in 1ml of 'Ruthin' ambient temperature extender for transportation. Sperm were selected using a discontinuous Percoll gradient, as previously described for freeze-thawed sample preparation (2.2.1.). Selected samples were divided into two equal aliquots. One aliquot was left to capacitate, whilst the second aliquot was used straight away (non-capacitated). Capacitation was carried out by incubating the sample in Fert TALP for four hours in a CO_2 incubator. Fert TALP contains heparin, which has been found to capacitate bovine sperm (Parrish et al. 1988). The sample was then spun down at 1200rpm for 5 min, the supernatant discarded and sperm re-suspend in Hepes TALP. After this stage, the process was the same as for freeze-thawed sperm. Once removed from the incubator, sperm were used as quickly as possible, with all recordings taken within 60 min.

2.2.3. Oocyte preparation

Bovine oocytes were harvested from abattoir-derived ovaries and matured *in vitro*. The oocytes were placed in maturation medium and matured for 24 hours at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. To ensure the COC used were viable, i.e. could be fertilised and would develop into embryos, COC were selected from batches prepared as part of a bovine embryo generation project (Sturmey lab, Hull-York Medical School). As it has been suggested that it is the cumulus cells which release an attractant (Chain et al. 1996), only mature COC were selected. Chemoattraction is normally observed at low concentrations of attractant (Adler 1973; Eisenbach and Ralt 1992). Increasing the numbers of COC was therefore used to reflect increasing levels of attractants, as neither the amount of attractant released by an individual COC nor the amount required for sperm chemotaxis has been published.

2.2.4 Capturing motility

The Dunn chemotaxis chamber was used to capture sperm motility as it provided a known and consistent depth for sperm motility to be accurately replicated across

treatments (Zicha et al. 1991). Before samples were loaded, the Dunn chemotaxis chamber and cover slide were coated with 1% polyvinyl alcohol to reduce the chance of trapping due to cells being attracted to the edges of the sides (Wilson-Leedy and Ingermann 2007).

Both the outer and inner wells of the chamber were filled with Hepes buffer before any samples were loaded. For treatments using COC, the COC was placed in the outside well. Sperm count information was used as a guide to the concentration of sperm for each sample and to decide the volume of sperm added. All treatments used 2-4µl of the sperm solution, which was placed in the inner well (Figure2.1). Once the cover-slide was added, the chamber was placed on a heated stage at 37°C and allowed to settle for ~2min before motility was recorded. This time allowed the fluid in the wells to settle and avoid the effects of flow created when the cover-slide was added, as this would have influenced sperm movement. As relatively few sperm per ejaculate have the ability to capacitate and therefore to react to an attractant, concentrations of sperm were kept as high as possible. Sperm motility was recorded at 5 frames per second for 100 frames, at a frame size of 2208 x 3000 pixels using a phase contrast microscope (Olympus BX40) with a 10x PLN objective and a CMOS camera (PixeLink PL-B686CF).

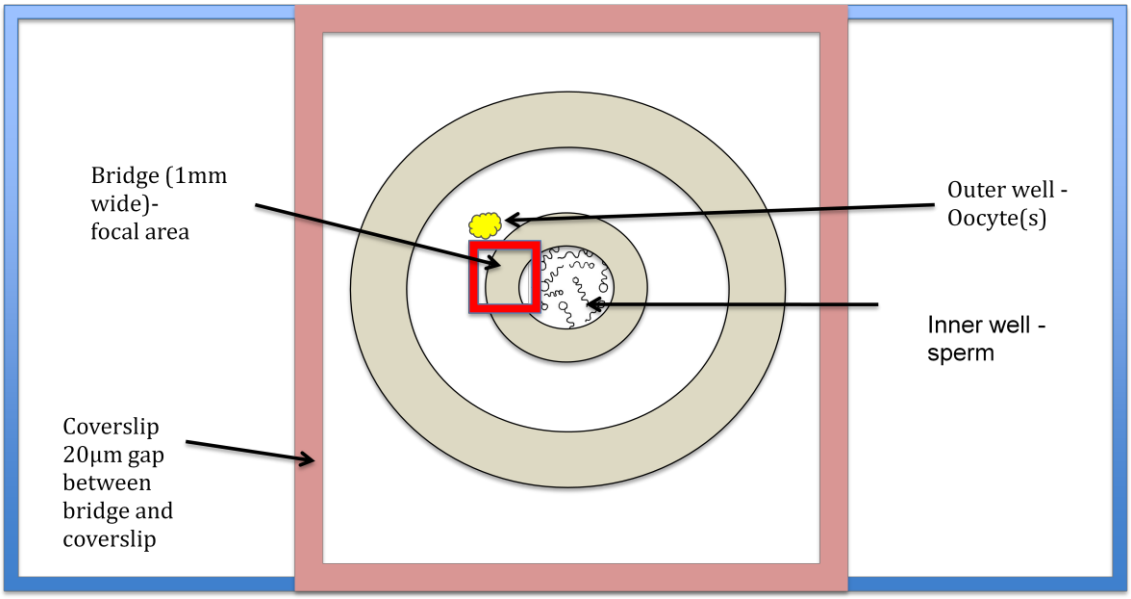


Figure 2.1. Schematic of a Dunn Chemotaxis Chamber indicating relative locations of male and female gametes. The red box indicates the area on which the microscope was focused in order to record sperm motility.

2.3 Analysis

2.3.1 Tracking individual sperm speed

Individual, morphologically normal sperm (one head and one tail) showing progressive motility were tracked manually frame by frame for each experiment as follows. In ImageJ (version 1.44o, National Institutes of Health, USA http://imagej.nih.gov/ij), a scale was added in order to calibrate the tracking software with the microscope magnification used. Measurements taken from a micrometer were used to represent a known distance under the conditions in which videos were recorded, and added to ImageJ using the 'set scale' function. Previously recorded videos could then be loaded into MTrackJ (Meijering et al. 2012) to measure tracks of individual sperm frame-by-frame (Figure 2.2). The output files this created were then exported into MS Excel (version 12.3.3). Coordinate data (x, y) were used to calculate relative track positions for each sperm in each frame of the video. The relative coordinates were calculated by sequentially dividing each absolute coordinate by the first coordinate, thereby centring each tracked sperm (Figure 2.3). Tracks were standardised to allow comparison by ensuring that the starting coordinates for each sperm were the same whilst keeping the individual track characteristics of speed and direction. The straight-line distance travelled by each tracked sperm was calculated using Pythagoras' theorem for the lengths of the sides of a right-angled triangle a, b, and c:

$$a^2+b^2=c^2$$

where a and b are the horizontal and vertical components of the triangle formed by the start point, end point, and the point described by the x coordinate from the initial point and the y coordinate from the final point (Figure 2.4).

Straight line velocity (VSL) was calculated by dividing the number of frames in each track by the number of frames per second, then dividing this by the distance as calculated using the equation given above. The directional assay then used the VSL measurements to calculate the mean swimming speed and direction of sperm across treatments and plotted each trajectory relative to the source of attractant (Figures 2.11-2.13).

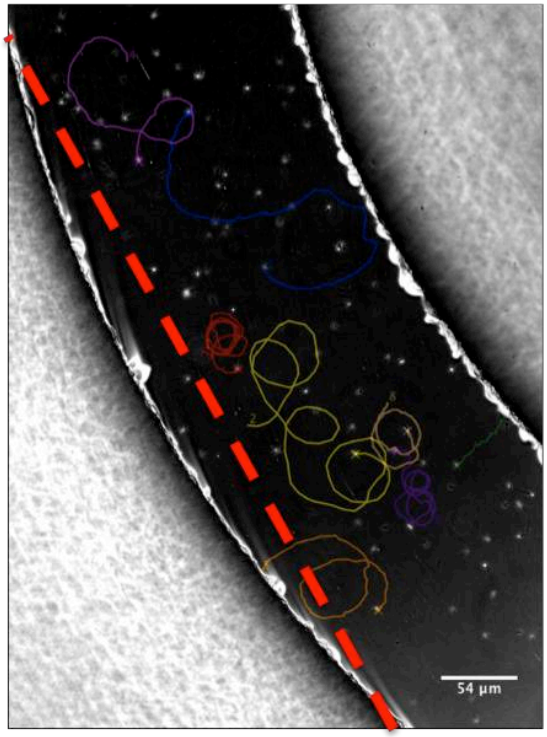


Figure 2.2. Focal area in ImageJ. Red dashed line indicates line used for angle measurements to assess swimming direction of sperm in relation to source of COC. Each coloured track represents an individual sperm's swimming trajectory as tracked in MTrackJ.

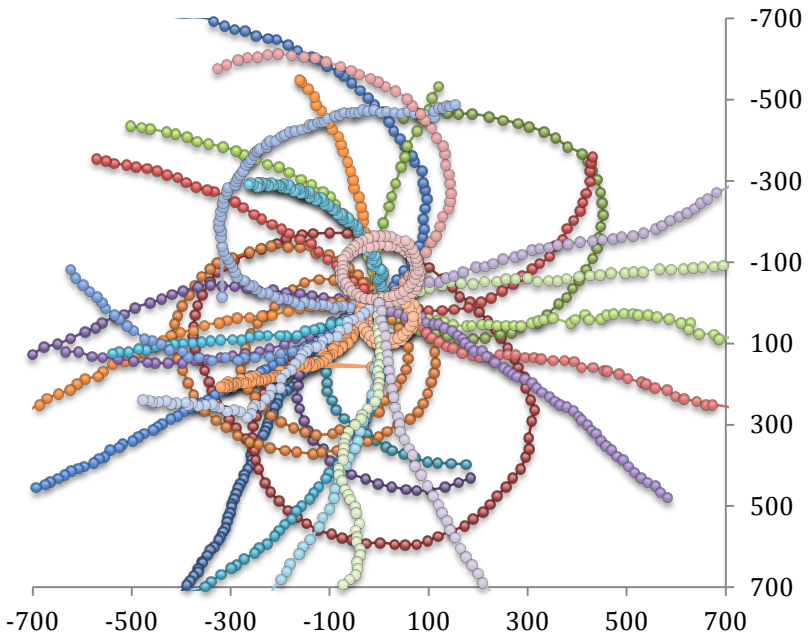


Figure 2.3. An example of standardized tracks. Axes represent relative x, y coordinates from the central start point. Each coloured line represents the track of an individual spermatozoa, with points indicating frame by frame progression of the sperm (equivalent to step-length parameter in fractal dimension analysis, see Section 2.4.2). Tracks were standardised in this way for all spermatozoa, grouped by experiment, as shown in Figures 2.11-2.13.

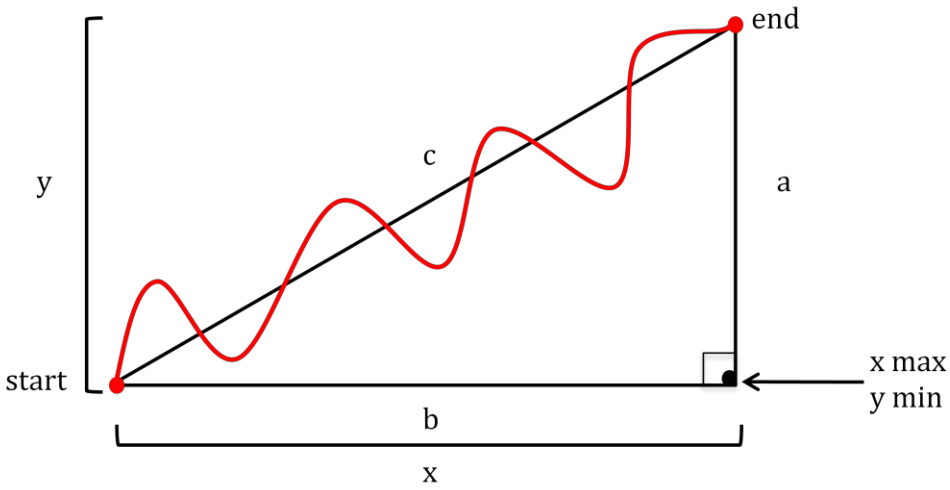
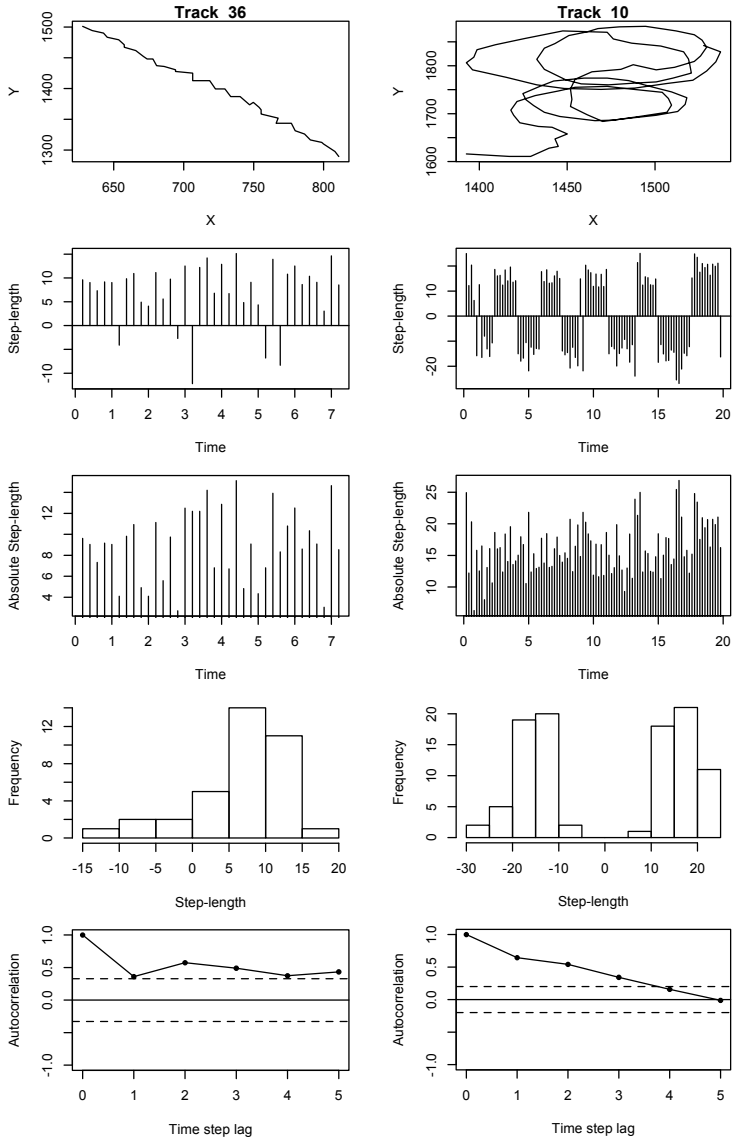


Figure 2.4. Schematic for the calculation of VSL based on x, y coordinates. The red line represents track of sperm head from start to end of video.

2.3.2. Track complexity

Fractal analysis was used to classify the complexity of the trajectory of each sperm (Abaigar et al. 2012) and was carried out using R (version 2.13.0R Development Core Team, 2011). Fractal analysis involved calculation of mean speed, which equates to curvilinear velocity (VCL), track complexity (fractal dimension), and the variation in step-length (VAR). The fractal dimension describes how much 2D space the sperm track occupied, giving an indication of its complexity on a scale from one (straight line) to infinity. However, an upper limit of three (described by Katz and George as a constrained path) is a more useful limit for the scale (Katz and George 1985). Mortimer et al (1996) describe how a small variation (from a fractal dimension of 1.03 being relatively smooth to a fractal dimension of 2.05 representing a highly complex trajectory, as per Figure 3 in Mortimer et al 1996) in fractal dimension can in fact indicate a large increase in track complexity. The variation in step-length is large as the sampling frequency used here is low (5Hz), and as such VAR may not be an informative parameter for these data. However, VAR was calculated as it has the potential to be informative in relation to sperm subpopulations (Abaiger et al. 2012), even if not comparable to the wider literature for reasons of sampling frequency (see below). The amount of time across the tracked trajectory that movement could not be detected as a consequence of the spatial resolution of the tracking methods was also calculated (IMR). The fractal dimension of a sperm trajectory is used to describe the track's complexity in relation to the amount of space filled by the trajectory (Mortimer et al. 1996).

Sampling frequency was 5Hz for all recordings, which can reduce the observable track complexity (Mortimer et al. 1988). The data were not normally distributed and since non-parametric post hoc tests would rank data, which is not appropriate given the nature of fractal dimension data, the data were therefore logit transformed to normalise them before carrying out an ANOVA on the results from the fractal analysis. Means per treatment were calculated and one-way ANOVA performed across treatments for each sperm type (see Figure 2.5 for explanations of parameters and Appendix 1 for all individual sperm track characteristics).



Reconstructed trajectory – path of the tracked cell based on x, y coordinates

Time series step-length – direction of sperm at each time interval where gaps indicate no movement

Absolute step-length – all step-lengths flip to positive to compare all step-lengths across time

Histogram – frequency of each step-length where skew indicates directionality

Correlogram – used to infer sperm behaviour and identify subpopulations. Coefficients between dashed lines are not significantly different from zero

Figure 2.5. Example graphs of different sperm characteristics generated from fractal analysis. Each sperm tracked has a comparable bank of five graphs (see Appendix 1 for all individual sperm graphs). Looking for subpopulations of sperm responding to the presence of COC in the above tracks potentially reveal sperm responding differently to *in vitro* conditions. The relatively straight path of track 36, for example, suggests that the flagella is beating symmetrically and the cell is therefore not hyperactivated, whilst track 10 has the circular trajectory indicative of the asymmetrical beating of hyperactivated sperm (Chang and Suarez 2010).

2.4. Results

2.4.1.Velocity

Velocity was measured for individual spermatozoa to examine the potential for chemokinesis in the presence of COC across sperm types. Across treatments, fresh sperm, especially non-capacitated samples, exhibited VSL (Figure 2.6) similar to published velocities for Holstein bulls (VSL=100-151μm/sec depending on the age of the male Farrell et al. 1998; VSL=82-86μm/sec Hallap et al. 2006). Similar VSL (~108μm/sec) from cloned Holstein-Friesian bulls have also been recorded (Hoflak et al. 2007). VSL of freeze-thawed sperm varied greatly across treatments (Figure 2.6). VCL, in contrast, was more uniform across treatments, with fresh non-capacitated sperm consistently showing faster VCL that the other sperm types. Nevertheless, all sperm had lower VCL than reported in the literature (VCL 116-176μm/sec Farrell et al. 1998; VCL ~150μm/sec Hallap et al. 2006). However, as will be discussed below, direct comparisons with data from the literature may not be straightforward.

As a consequence of the low frame rate (5Hz) at which the sperm trajectories were recorded, it is important to keep in mind that the VCL values reported here (Figure 2.6) will be lower than the VCL for bovine sperm if recorded at higher frame rates (Mortimer et al. 1988). VCL data across treatments are, however, comparable within this study. For VSL, as this is not affected by recording frame rate (Mortimer et al. 1988), VSL measures are comparable outside the present study. *In vitro* VSL and VCL varied across treatments (Figure 2.7). The results from the two-way ANOVA indicated significant variation in VSL and VCL in relation to pairing of sperm type and COC number ($p=<0.001$ Figure 2.8). A Tukey-Kramer HSD test indicated significant differences across treatments, however no clear pattern was evident.

In order to explore the relationships between independent variables of sperm type and COC number suggested by the two-way ANOVA and Tukey-Kramer HSD tests, a standard least squares regression was performed on logit transformed data. This analysis revealed a clearer pattern, with the VCL for fresh capacitated sperm appearing to be different from other sperm types for 0 and 3 COC. With VSL as the dependent variable, there was a significant effect of sperm type ($p=<0.0001$) and interaction between sperm type and number of COC ($p=0.02$). There was no effect

of number of oocytes (p=0.34). The Tukey-Kramer HSD test indicates that, in general, VSL in fresh capacitated and fresh non-capacitated sperm do not differ with number of COC (Figure 2.9 (i)). Both fresh capacitated and fresh non-capacitated sperm are faster than freeze-thawed sperm until in the presence of 3 COC, when there is no difference between the three groups (Figure2.9(i)). Freeze-thawed sperm had the least straight trajectory of all three sperm types (Figure2.9(ii)), indicated by their having the slowest VSL, with VCL faster than fresh capacitated but slower than fresh non-capacitated sperm (Figure2.10 (iii)).

The interaction between sperm type and number of COC indicated that there was an increase in VSL for freeze-thawed sperm in the presence of 3 COC, whilst VSL peaked for both fresh capacitated and fresh non-capacitated sperm in the presence of 2 COC and dropped off in the presence of 3 COC (Figure 2.10 (i)). Applying the standard least squares regression on log transformed data with VCL as the dependent variable indicated significant interaction between sperm type and number of COC (p=0.008 (Figure 2.10 (i)), COC number (p =<0.0001) (Figure 2.10 (ii)) and sperm type (p=<0.001) (Figure 2.10 (iii)).

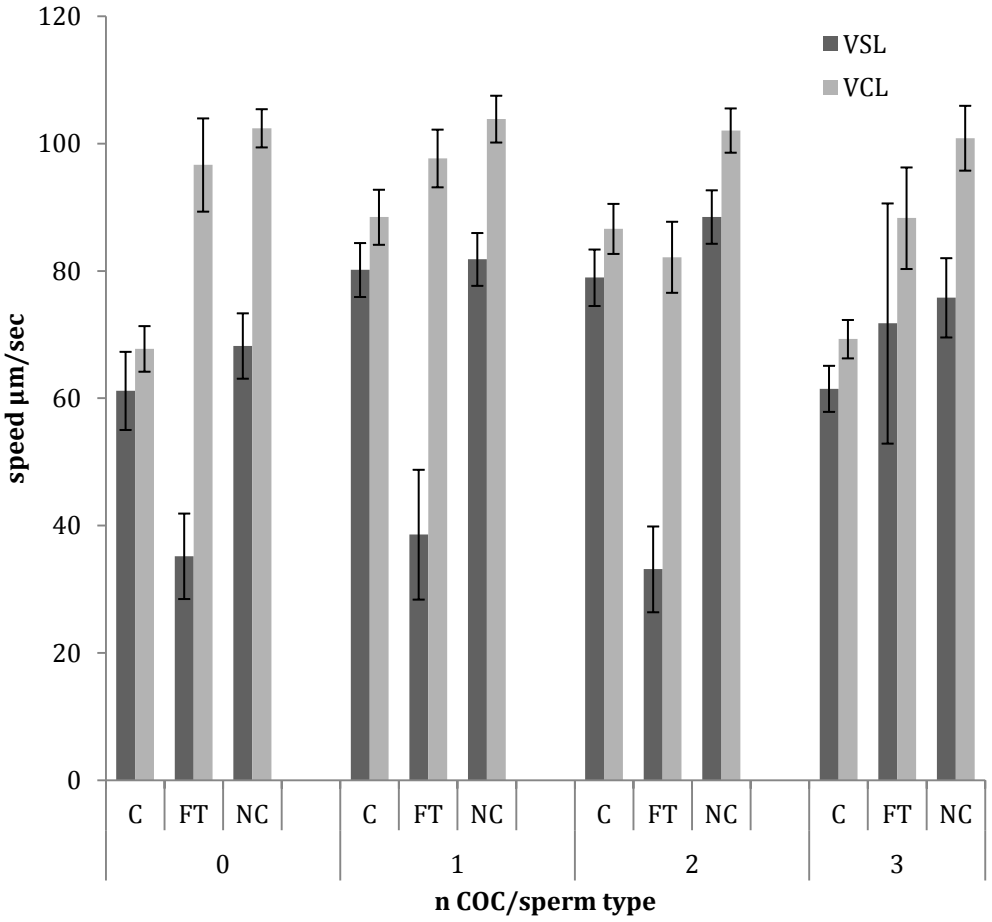
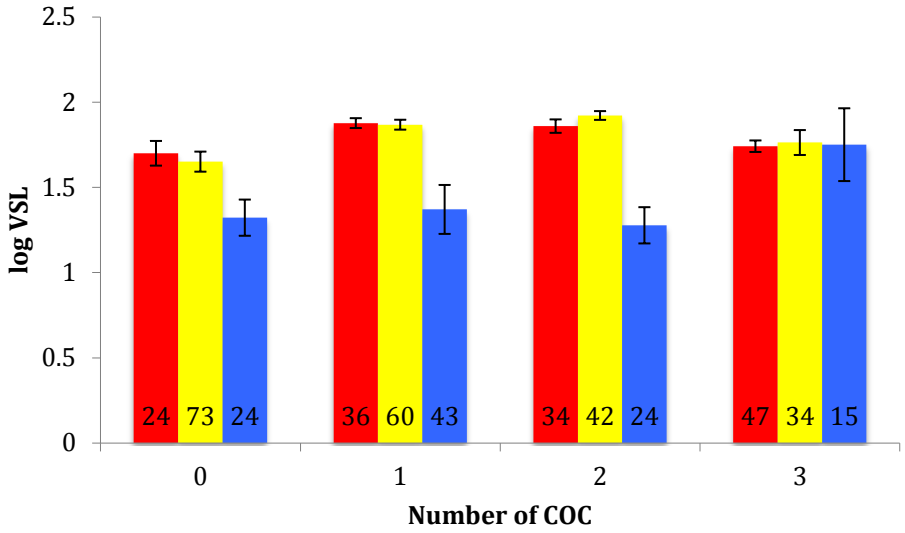


Figure 2.6. Comparison of real speed measures across treatments with standard error bars. Large error bars for freeze-thawed sperm in the presence of one and three COC suggest there is a lot more variation within these samples which could be related to tract complexity (see Figure 2.13). Fresh capacitated (C); freeze-thawed (FT) and fresh non-capacitated (NC)

a)



b)

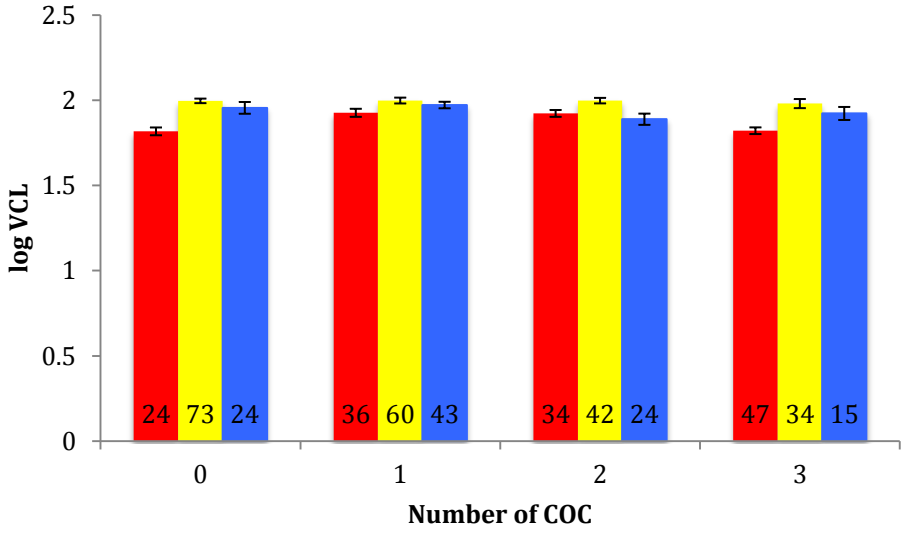
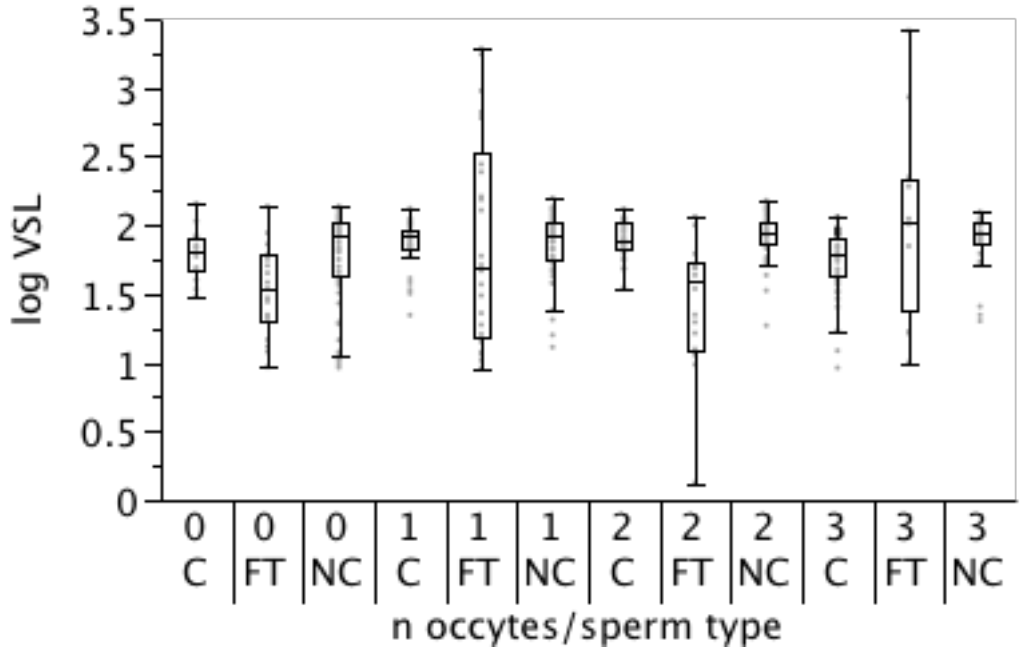


Figure 2.7. Sperm speed a) VSL and b) VCL as a function of number of COC treatment. Error bars are for standard errors. Numbers at the base of each bar indicate individual sperm sample size. Colours represent: fresh capacitated; fresh non-capacitated; freeze-thawed sperm samples.

a)



b)

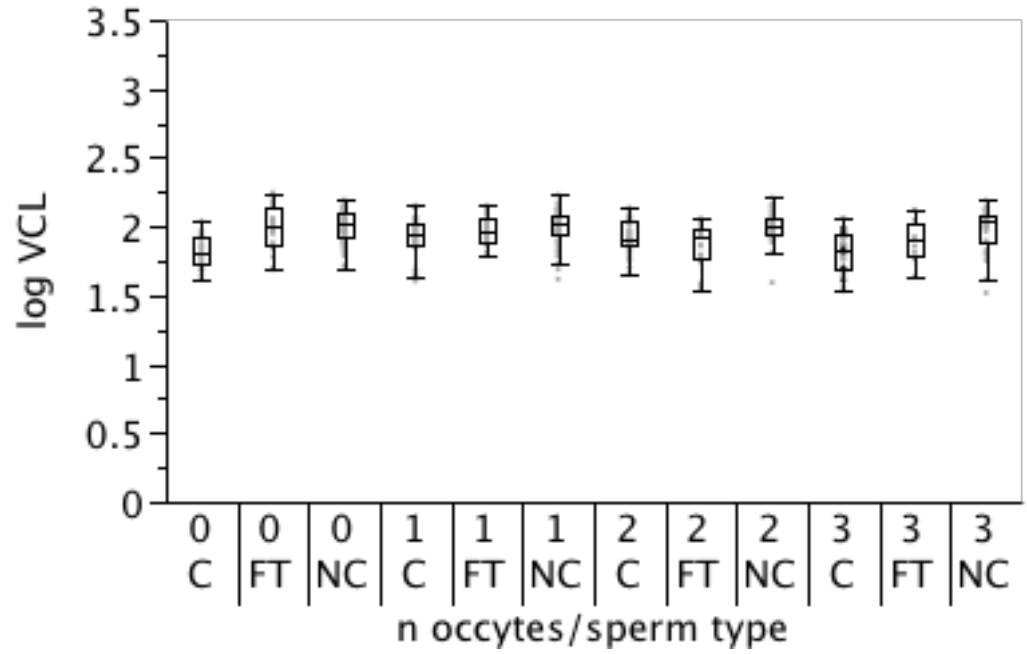


Figure 2.8. Box and whisker plot from one-way ANOVA indicating significant difference (p=<0.0001) in two measures of velocity a) VSL and b) VCL across treatments depending on sperm type and number of COC. 0=control, no COC, 1-3 refers to the number of COC present in the treatment. C=fresh capacitated sperm, (sample size control sperm n=24; 1 COC sperm n=36; 2 COC sperm n=34; 3 COC sperm n=47). FT=freeze-thawed sperm, (sample size control sperm n=24; 1 COC sperm n=43; 2 COC sperm n=24; 3 COC sperm n=15), NC=fresh, non-capacitated sperm, (sample size Control sperm n=73; 1 COC sperm n=60; 2 COC sperm n=42; 3 COC sperm n=34). Error bars indicate 95% confidence limits.

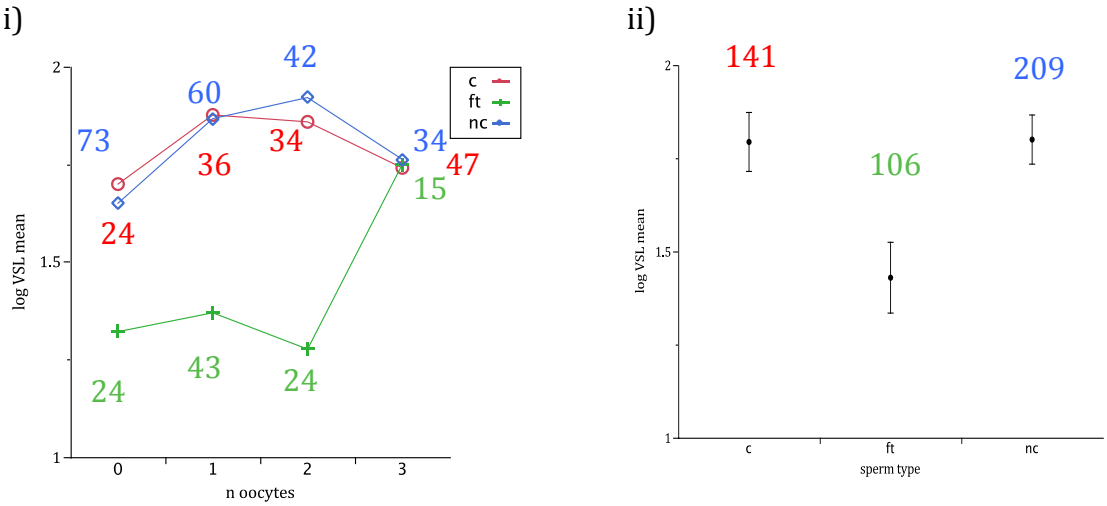


Figure 2.9. Interaction plot from least squares regression with standard error bars for i) VSL against number of COC for each sperm type and ii) effect of sperm type for fresh capacitated (c) freeze-thawed (ft) and fresh non-capacitated (nc) sperm. Numbers on plots indicate number of sperm per sperm type colour coded to match the key.

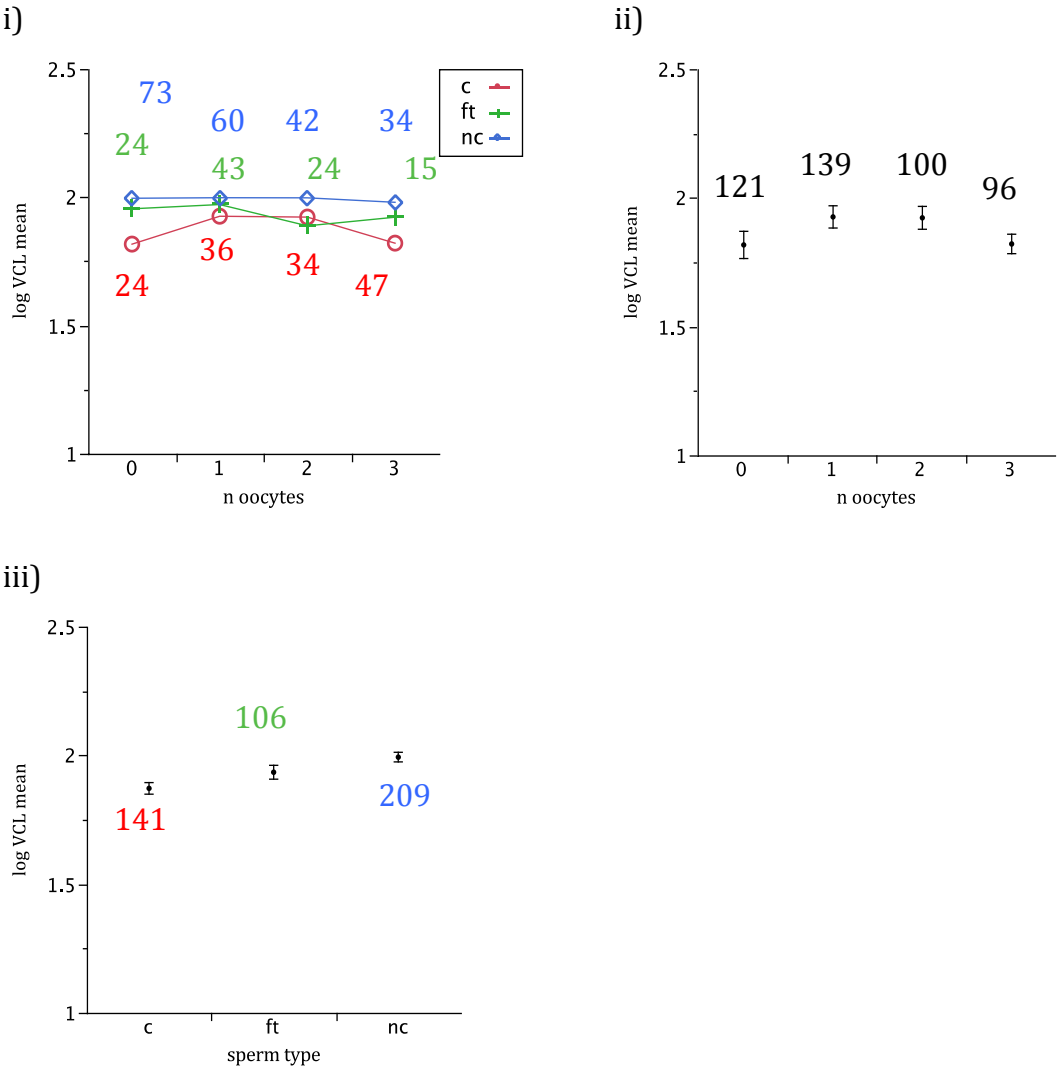


Figure 2.10. Interaction plot from least squares regression with standard error bars for i) VCL and number of COC and ii) effect of number of COC iii) effect of sperm type for fresh capacitated (c) freeze-thawed (ft) and fresh non-capacitated (nc) sperm. Numbers on plots indicate number of sperm colour coded to match the key for sperm type where applicable.

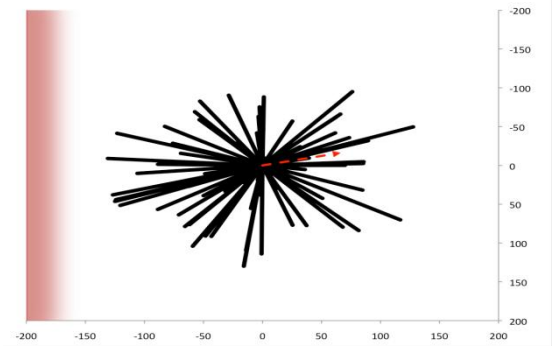
2.4.2. Directionality

There was no indication that sperm were responding to the presence of COC(s). The mean direction of sperm tracked (as calculated in 2.3.1) across treatments did not indicate sperm of any type were swimming towards the source of attraction (i.e. the outer edge of the bridge section of the chamber)(Figures 2.11-2.13). There were instances where the mean direction indicated that sperm were swimming towards the attractant (Table 2.1), but in each case the same directionality was also observed in the control group, where no attractant was present. The VSL

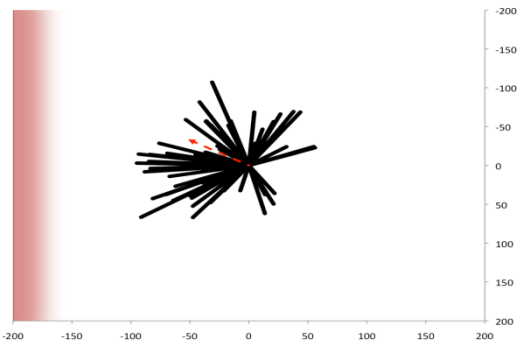
tracks for the freeze-thawed sperm (Figure 2.11) were noticeably shorter than those for the fresh sperm (Figure 2.12 and 2.13). The sperm within the freeze-thawed samples exhibited circular trajectories more frequently than was observed in any of the fresh sperm treatments (personal observation, but see top plots in Appendix 1).

Table 2.1. Summary of directional plots from Figures 2.11-2.13 based on mean direction calculation (as described in Section 2.3.1) per experiment.

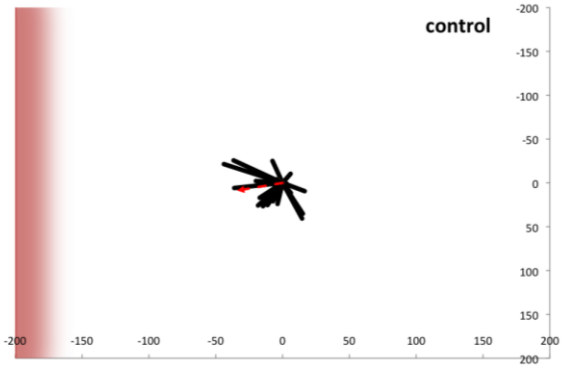
Sperm type	n COC	Swimming towards attractant (y/n)
Freeze-thawed	0	Y
	1	N
	2	N
	3	Y
Fresh capacitated	0	Y
	1	Y
	2	Y
	3	Y
Fresh non-capacitated	0	N
	1	N
	2	N
	3	N

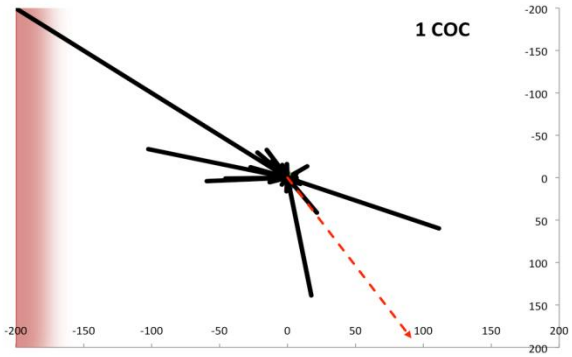


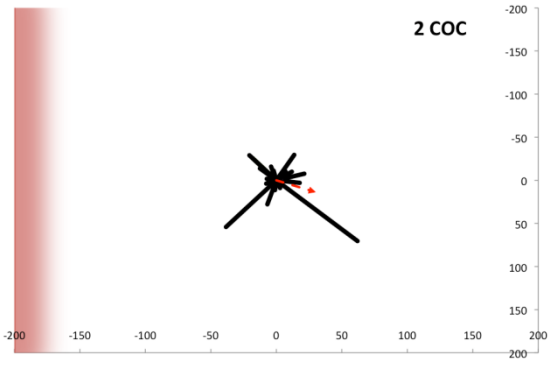
Expected plot if sperm are not responding to attractant. Mean direction (red dashed line) away from COC (red bar on left) and trajectories indicaterandom directionality.



Expected plot if sperm are responding to COC. Mean direction (red dashed line) towards COC (red bar on left). Not every sperm would not be expected to respond and some trajectories will be away from the COC.







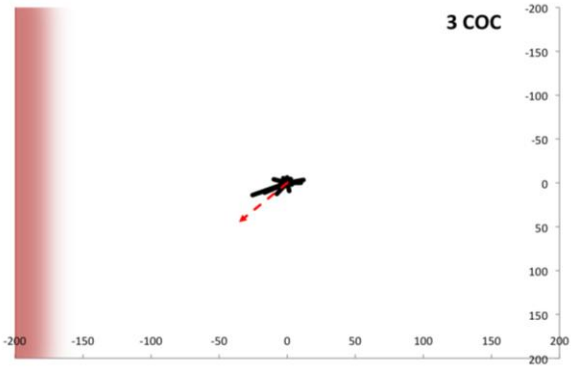


Figure 2.11. Plots of expected trajectories (top two plots) if sperm are not responding (left plot) or are responding to the presence of COC . Sperm velocities (VSL) relative to the direction of the source of attractant for all treatment using freeze-thawed sperm. All tracks were centralised to central start point (as per Section 2.3.1). The chamber line (red bar on the left side of each plot) represents the direction of the COC relative to the sperm start point. Each black line represents the trajectory of an individual sperm with the red dashed line indicating the mean velocity and direction of all sperm tracked in each treatment. Control sperm n=24; 1 COC sperm n=43; 2 COC sperm n=24; 3 COC sperm n=15.

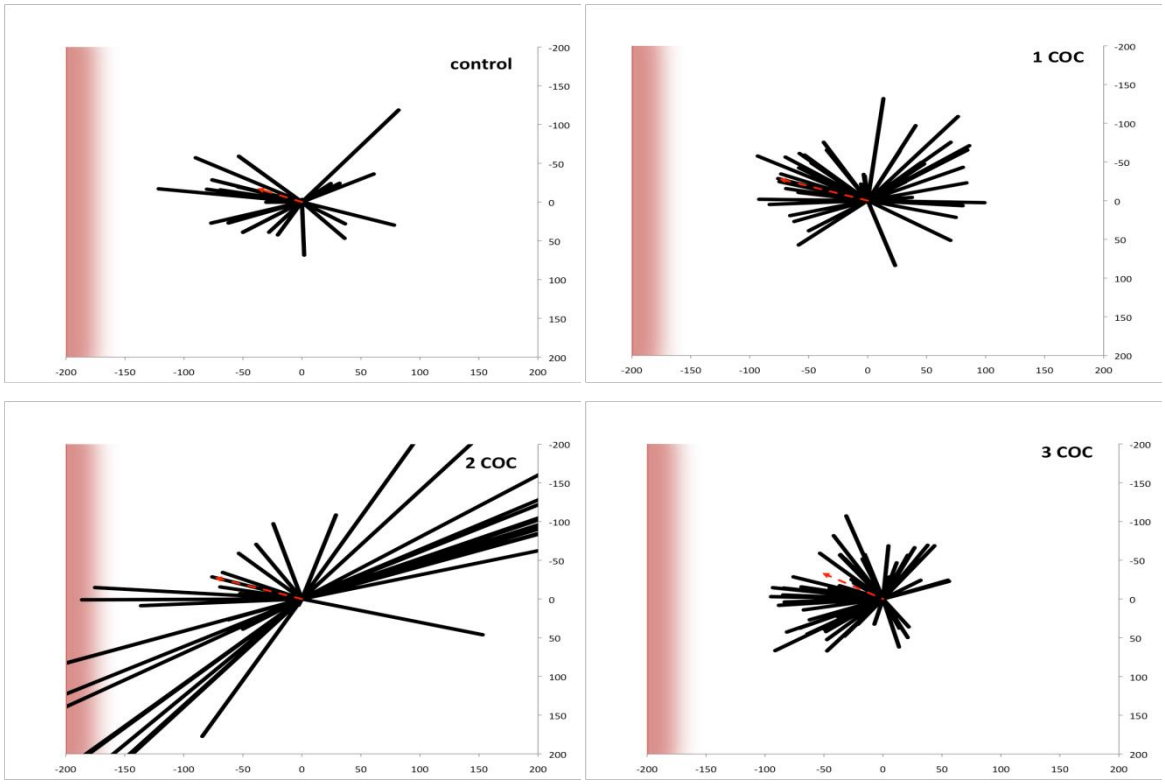


Figure 2.12. Sperm velocities (VSL) relative to the direction of the source of attractant for all treatment using fresh capacitated sperm. All tracks were centralised to central start point (as per Section 2.3.1). The chamber line (red gradient on the left side of each plot) represents the direction of the COC relative to the sperm start point. Each black line represents the trajectory of an individual sperm with the red dashed line indicating the mean velocity and direction of all sperm tracked in each treatment. Control sperm n=24; 1 COC sperm n=36; 2 COC sperm n=34; 3 COC sperm n=47.

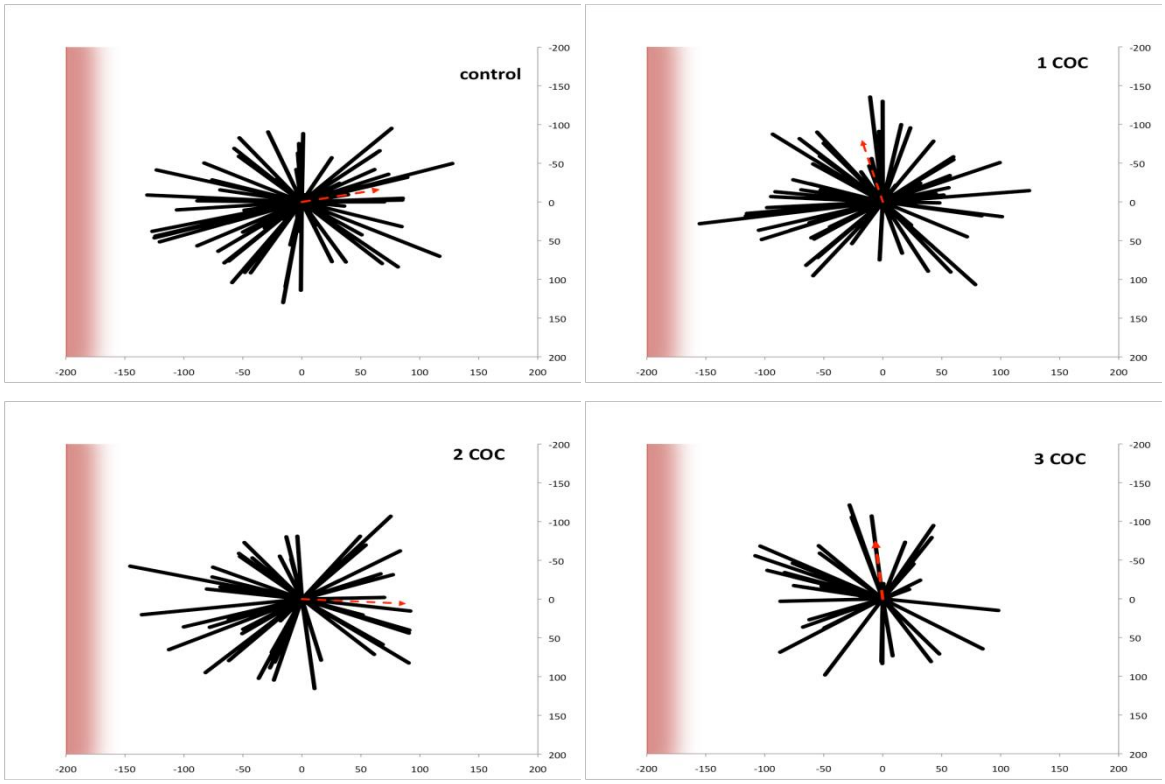


Figure 2.13. Sperm velocities (VSL) relative to the direction of the source of attractant for all treatment using fresh non-capacitated sperm. All tracks were centralised to central start point (as per Section 2.3.1). The chamber line (red bar on the left side of each plot) represents the direction of the COC relative to the sperm start point. Each black line represents the trajectory of an individual sperm with the red dashed line indicating the mean velocity and direction of all sperm tracked in each treatment. Control sperm n=73; 1 COC sperm n=60; 2 COC sperm n=42; 3 COC sperm n=34.

2.4.2 Track complexity

The five parameters calculated as part of the fractal analysis (Figure 2.5.) were used to describe variation in swimming behaviour of sperm across treatments largely based on the complexity of their swimming trajectories. Variance in step-length (VAR) was not significantly different across treatments for any sperm type (Freeze-thawed p=0.30, fresh capacitated p=0.10, fresh non capacitated p=0.91), which may in part be related to the low sampling frequency used when the original videos were recorded. Mean velocity and fractal dimension were the only parameters that were not affected by sampling frequency (Abaigar et al. 2012). Mean velocity is comparable to VCL parameters presented in the velocity and directionality assays. Fractal dimension alone cannot be used to explicitly identify

hyperactivation (Mortimer et al. 1996) but will be used to indicate variation in track complexity across treatments according to sperm type. Fractal analysis was also carried out to provide information on subpopulations within each treatment based on information gathered from the distance the sperm head moves, frame to frame, over the duration of the track as reported in the correlograms in Appendix 1 (Abaigar et al. 2012). Across sperm types the freeze-thawed sperm had the highest fractal dimension (FD=2.52 in the control treatment Figure 2.14) indicating that across treatments cryopreserved sperm had the most consistently complex trajectories compared to fresh sperm. As mentioned previously, the sampling frequency videos were recorded at was much lower that typically reported in the literature (for example 30 and 60Hz Mortimer et al, 1996; 50Hz Abaigar et al. 2012). The data presented here was recorded at 5Hz which will reduce the detail of movement recorded and as a consequence fractal dimension will be smaller than it would be if a higher sample frequency had been used. All recordings were taken at 5Hz making it possible to compare fractal dimensions within the present data but extrapolation to the wider literature must be done with caution.

The one-way ANOVA for the fractal analysis of the freeze-thawed sample did not show significant differences for mean speed ($p=0.21$) and variation in step length ($p=0.30$) but was significant for fractal dimension ($p=0.02$, Figure 2.14(i)). Fresh capacitated one-way ANOVA indicated significant variation for mean velocity for fresh capacitated sperm ($p=<0.001$, Figure 2.14(iii)) with non-significant correlations for fractal dimension ($p=0.27$) and variation in step length ($p=0.10$). Fresh non-capacitated one-way ANOVA was significant for fractal dimension ($p=<0.001$, Figure 2.14(ii)) with mean velocity and variation in step length non-significant ($p=0.96$ and $p=0.92$ respectively). Tukey-Kramer HSD test on significant ANOVA results form fractal analysis indicated there were significant differences between the control and 1 COC treatment (Table 2.2). However freeze-thawed sperm was the only sperm type that only had the control-1 COC treatment as the significant pair both fresh capacitated and fresh non-capacitated sperm also indicated significant differences between other treatments.

An immobility ratio (IMR) was also calculated as part of the fractal analysis to provide an indication of the resolution available for this analysis. Calculated as the percentage of total track time which is missed as an artefact of the sensitivity of

the tracking system, IMR represents the minimum velocity detectable between step lengths. As previously mentioned the step-lengths were large because of the low sampling frequency however IMR was <10% across all samples with the sampling resolution for detectable velocity being 3.4μm s^{-1}.

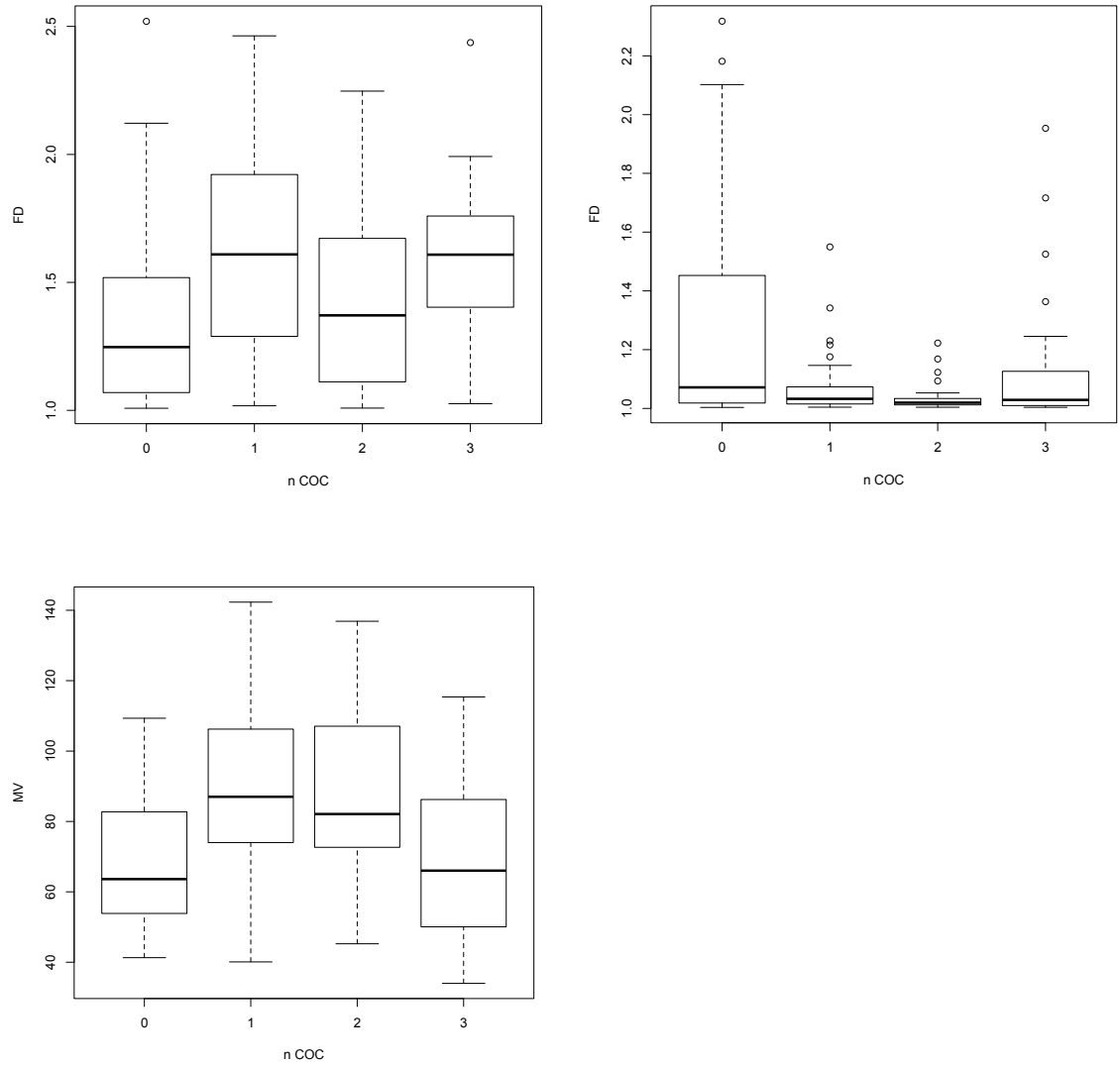


Figure 2.14. Box-and-whisker plots of fractal dimension (FD) for i) freeze-thawed sperm (number of sperm per treatment: 0 COC=24; 1 COC=43; 2 COC=24; 3 COC=15) ii) fresh non-capacitated - number of sperm per treatment: 0 COC=73; 1 COC=60; 2 COC=42; 3 COC=34; and iii) mean velocity (MV) for fresh capacitated sperm (number of sperm per treatment: 0 COC=24; 1 COC=36; 2 COC=34; 3 COC=47).

Table 2.2. Tukey-Kramer HSD results for all significant ANOVA results obtained from fractal analysis. COC=cumulus oocyte complex. Significant comparisons are shown in bold (p=<0.05).

Sperm type	Parameter	Treatment pair (n COC)	P adj
Freeze-thawed	Fractal dimension	1-0	**0.04**
		2-0	0.98
		3-0	0.24
		2-1	0.11
		3-1	0.99
		3-2	0.43
Fresh capacitated	Mean velocity	1-0	**0.003**
		2-0	**0.01**
		3-0	0.99
		2-1	0.99
		3-1	**<0.001**
		3-2	**0.004**
Fresh non-capacitated	Fractal dimension	1-0	**<0.001**
		2-0	**<0.001**
		3-0	**0.03**
		2-1	0.89
		3-1	0.58
		3-2	0.27

2.5. Discussion

The main aim of this chapter was to identify alterations in sperm velocity and direction in the presence of a source of chemoattractant. It was expected that the method by which the sperm had been preserved and whether or not sperm had been capacitated would affect individual sperm velocity and trajectory. However, limitations of the experimental design only allow for tentative conclusions to be drawn in terms of the effect of preservation technique. Variation in sperm velocity was observed across treatments and fractal analysis proved informative in describing track complexity of individual sperm.

Reduced motility has been found as a consequence of cryopreservation (for example see Medeiros et al. 2002), although there are several other factors which could affect the comparison of velocity across the sperm analysed in the present study, which will be reviewed in the following discussion. However, VCL and fractal dimension results suggest freeze-thawed sperm have a more complex trajectory than fresh sperm. Whilst there was no indication of directional swimming, an increase in swimming speed, as seen in these results for VSL of freeze–thawed sperm in the presence of three COC, could indicate chemokinesis, which is typically observed in combination with chemotaxis but is not related to the chemical gradient (Eisenbach 1999; Riffell et al. 2004).

The findings presented here indicate that, overall, the fresh sperm samples analysed exhibited higher VSL than the freeze-thawed sperm across all treatments. Fresh sperm VSL was not significantly different across treatments, regardless of level of activation, with both fresh sperm types showing significantly straighter trajectories than freeze-thawed sperm. Freeze-thawed VSL was consistently lower than fresh sperm, until in the presence of three COC, when the VSL for all sperm types converged. The VCL was less variable across sperm types than VSL, but VCL measurements were dependent on sampling frequency. This was consistent throughout this study but has to be taken into consideration when comparing these results to the wider literature, and is a limitation that will be discussed in greater detail below.

There are two important limitations of the data presented here: the sample size in relation to males and the frame rate at which the videos were recorded. Sperm were analysed from only one bull whose sperm had been cryopreserved and

another bull whose sperm were used as the fresh samples. Despite both bulls being of a proven fertility and used for commercial AI, it is possible that the observed variation in velocity is related to individual variation between males rather than storage method. Variation in the freezability of boar sperm has been demonstrated to vary between males (Thurston et al. 2001) and can reduce the fertility of even the highest quality males differently, depending on the individual male's sperm's susceptibility to cryoinjury (this will be discussed further in Section 2.5.2). Variation in sperm quality could be accounted for if sperm from both males had been examined as fresh and cryopreserved samples. However, the original freeze-thawed data were gathered from a bull that had been subsequently slaughtered, making such a comparison impossible. Later problems with oocyte availability reduced the experimental time available for this project, resulting in the restriction of data collection to just one bull for fresh samples.

An additional limitation was the low sampling frequency (5Hz). Low rate of frames per second are a problem when considering track complexity as much of the track detail could been missed in the time between frames. The frames per second recorded were unavoidably low for reasons outlined below in Section 2.5.1. The following discussion will therefore only make tentative links with existing literature in an effort not to over-interpret complex interactions which have many confounding variables.

2.5.1. Is there any evidence for chemotaxis?

Although it has been established that bovine COC release an attractant that affects sperm motility (Chain et al. 1995), chemotaxis has been found to be a difficult phenomenon to accurately identify. Chemotaxis can cause sperm to abruptly alter their swimming direction and path (Eisenbach and Giojalas, 2006) as a result of alterations in flagella beating (Eisenbach 1999). The data presented here do not explicitly provide evidence for chemotaxis of bovine sperm in the presence of COC, when chemotaxis is defined as a change in direction of movement towards the source of a chemical gradient/attractant (Eisenbach 1999). Fresh sperm appear to swim in the straightest trajectory, which is reflected in fresh sperm having the higher VSL when compared to cryopreserved samples. The analysis of interactions between sperm and COC revealed a significant impact of COC number across

treatments. However, the mean swimming direction analysis did not indicate directionality towards the COC for any sperm type across any treatment. Sperm do not appear to be actively swimming towards the source of attractant any more that sperm swim in that direction for the control treatments, but sperm do respond differently depending on preservation method.

It had been expected that the COC would release an attractant, possibly progesterone (Kaupp et al. 2008), which would diffuse across the bridge area of the Dunn Chemotaxis chamber (Zicha et al. 1991) creating a gradient which the sperm would detect and swim towards. Although it is possible that any attractant released filled both chambers, obscuring any gradient and the potential for any directional swimming, this does not seem likely because progesterone released by cumulus cells is at very low concentrations. However, what seems more likely is that the subpopulation of sperm capable of responding was so small that its members were not tracked.

2.5.2. Cryopreservation – effect on sperm motility

The difference in fractal dimension across sperm preparations could reflect the damage to freeze-thawed sperm that can occur as part of the cryopreservation process. Up to 50% of bovine sperm do not survive the process of cryopreservation (Martinez et al. 2006). Whilst sperm which survive the process of cryopreservation are still viable and capable of fertilising *in vitro*, their ability to respond to chemoattractants produced by the cumulus cells or the oocyte could be damaged. The fractal dimension for both capacitated and non-capacitated fresh sperm suggest they have much straighter trajectories than freeze-thawed sperm. A significant variation in trajectory complexity between the control and the three COC treatments for the fresh non-capacitated sperm could indicate that capacitation and/or hyperactivation is induced by proximity to COC. However, circling trajectories of sperm could also be caused by low linearity rather than hyperactive motility (Mortimer et al. 1996). High VCL in combination with low linearity of tracks would be a more convincing indication of hyperactive motility.

Individual males show great variation in the level of damage that cryopreservation has on their semen and consequently on the viability of individual sperm post-

thaw, which can be addressed with some modification of the process of cryopreservation (Parkinson and Whitfield 1987). There are also variations across cattle breeds in sperm morphological traits (Morrow and Gage 2001). Despite such male-specific variation, AI using frozen bovine semen has been more successful than the use of similar techniques in other domestic species (Curry 2000). Both males in the data presented here were Holstein-Friesian bulls, selected for AI as high quality sires. As AI bypasses the vagina and cervix and deposits spermatozoa into the uterus (Vishwanath 2003), sperm are not filtered by cervical mucus, which is known to act as a mechanism to select the highest quality sperm (Suarez 2006). The uterotubal junction can also act as a barrier to sperm that do not have the correct expressions of proteins on the plasma membrane of the sperm head (Suarez 2008), an important fact when considering the cryoinjury caused to the plasma membrane of freeze-thawed spermatozoa. The ability of sperm to fertilise an oocyte can be increased by the epithelial cells that line the lumen, which have been found to prolong sperm viability in boar (Suarez et al. 1991) and bovine (Pollard et al. 1991).

As sperm from the same bull could not be analysed across all three sperm types, it is not possible to state categorically that variation in swimming behaviour between sperm types was a result of preservation technique. However, capacitation is an important stage in preparing sperm to fertilise oocytes and a comparison between capacitated and non-capacitated sperm can be made within the same male.

2.5.3. Capacitated versus non-capacitated sperm

Previous studies suggest that a capacitation medium containing heparin is sufficient to induce capacitation *in vitro* (Parrish et al. 1988). Capacitation has to be achieved for sperm to respond to a chemoattractant (Cohen-Dayag et al. 1995). The capacitation of individual spermatozoa within an ejaculate does not occur at the same time (Curry 2000), with less than 10% of the total ejaculate capable of capacitation at all (Kaupp et al. 2008). It is therefore possible that the noise created by the majority of unresponsive sperm (Eisenbach and Giojalas 2006) masks the behaviour of the small number of sperm that are responding. Capacitated sperm would be expected to exhibit hyperactive motility (Suarez

1996) as this allows capacitated sperm to free themselves from epithelium cells at storage sites and progress up the oviduct (Chang and Suarez 2010). However, it is possible for sperm to be capacitated when incubated with heparin without hyperactive motility (Marquez and Suarez, 2004). The fractal analysis would seem to indicate that the fresh sperm did not become hyperactivated, as the fractal dimension did not get above 1.3 in any treatment.

Fresh capacitated sperm had the slowest VCL, with fresh non-capacitated sperm consistently swimming in the most curved trajectories across all treatments. The curvature of the trajectories could indicate changes in direction as a response to chemoattractant (Eisenbach 1999). The increasing level of track complexity for fresh non-capacitated sperm in the presence of increasing number of COC when compared to the control also suggests some level of response to *in vitro* conditions, possibly hyperactivated motility. Track complexity was confirmed in the fractal analysis, which indicated that within freeze-thawed and fresh non-capacitated sperm types there was a significant difference in fractal dimension across treatments.

Whilst spontaneous capacitation has been related to the freeze-thaw process (Cormier and Sirard 1997; Green and Watson 2001) and so could explain the track complexity of the cryopreserved sperm, it was surprising that the fresh non-capacitated sperm also had significantly different track complexities in the presence of COC when compared to the control. However, it has been suggested that COC release chemicals that initiate capacitation in bovine sperm (Chain et al. 1995), so some level of oocyte-induced capacitation may have been possible. Although capacitation is an important aspect of chemotaxis (Cohen-Dayag et al. 1995), the underlying mechanisms which control capacitation and cryopreservation-induced capacitation are not fully understood. Nevertheless, the ability to identify subpopulations of sperm with alternate swimming behaviour within an ejaculate is an important aspect of evaluating male fertility (for example see Holt et al. 1989). The following section will review the potential for fractal analysis to identify variation in sperm kinematics within an ejaculate.

2.5.4. Fractal dimension analysis elucidates track complexity

Fractal analysis allowed the complexity of the sperm's movements to be visualised in terms of how the trajectory of the individual sperm filled a three-dimensional space (Mortimer et al. 1996). Widely used for semen analysis, computer assisted sperm analysis (CASA) software does not differentiate hyperactive swimming (Dunson et al. 1999) subpopulations from the rest of the sperm. It has been suggested that fractal analysis could provide the resolution required to identify individual sperm swimming differently from the rest (Abaigar et al. 2012). The fractal analysis in the present study did reveal significant variation in track complexity (fractal dimension) for freeze-thawed and fresh non-capacitated sperm, as well as significant variation in mean velocity (equivalent to CASA VCL) for fresh capacitated sperm. Track complexity was significantly different for all treatments when compared with that of the control for fresh non-capacitated sperm, suggesting that any number of COC alters the complexity of swimming trajectory. Freeze-thawed sperm only indicated a significant increase in fractal dimension (track complexity) in the presence of one COC, compared to the control. It had not been expected that freeze-thawed sperm would exhibit alternate swimming behaviour in the presence of an attractant, as the process of cryopreservation causes damage to sperm membranes (for example Martinez et al. 2006), which can also effect motility.

Whilst mean velocity was the only parameter from the fractal analysis which indicated significant variation across treatments for the fresh capacitated sperm, there was no clear indication of an effect of number of COC. However, the fresh capacitated sperm were the only sperm type to indicate significant variation in the parameter of mean velocity, and velocity alternations can indicate chemokinesis, which is described as an alternation of velocity in the presence of an attractant (Eisenbach and Giojalas 2006). The mean velocity (VCL) increased significantly in the presence of one and two COC when compared to the control, which could be interpreted as a chemokinetic response to the presence of an attractant. There was also a significant difference in mean velocity for fresh capacitated sperm in the presence of three COC when compared to one and two COC, which could suggest saturation of chemoattractant. However, in the absence of track complexity indicating hyperactivation, it is difficult to attribute the observed variation in VCL

detected by fractal analysis to being a consequence of sperm detecting an attractant.

The velocity measurement calculated for sperm trajectories is influenced by the parameters at which the original recordings are taken. Methodological limitations in the present study required sperm trajectories to be recorded at 5Hz, which is likely to have smoothed the sperm track thus resulting in the VCL data presented here being lower than if recordings had been taken at higher frame rates (Mortimer et al. 1988), for example 50Hz (Abaigar et al. 2012). The directionality of sperm trajectory was the initial focus of this chapter, and as such a trade-off between longer tracks (100 frames), and resolution (2208 x 3000 pixels) was made at the expense of using a high frame rate. Unlike VCL, VSL is a more consistent measure of sperm velocity as it is not frame rate dependent (Mortimer et al. 1988). Although sperm motility is traditionally used as a measure of male fertility, sperm morphology is also important if gamete fusion is to result in a healthy embryo.

2.5.5. The missing component - sperm morphology

It had initially been intended that individual sperm morphology would be measured and linked to velocity as part of this study. However, as the work progressed it became evident that it would not be possible to get consistently clear images of live sperm morphology. Morrow et al. (2001) did not find significant between-breed variation in head morphology of bovine sperm, but head morphology appears to be an important factor in the swimming characteristics of sperm in internally-fertilising species (Gillies et al. 2009). When moving through mucus, bovine sperm appear to be able to modulate their velocity without significantly altering the waveform along the flagellum (Katz et al. 1981), which could have significant implications for selection criteria for AI. Significant variations in sperm length between cattle breeds has previously been reported, however there was no difference in head length (Morrow and Gage 2001). It would have been ideal to have morphology data to relate to velocity for individual spermatozoa. This would be an interesting aspect for future work as it could reveal much about the relative importance of sperm morphology on swimming velocity of cryopreserved and fresh sperm across cattle breeds.

2.6. Conclusion

The results presented here support the suggestion that the method of preservation can influence sperm velocity. However, as the comparisons made here are based on data from one male for fresh and one male for freeze-thawed sperm, within-male variation cannot be discounted as a reason for variation in velocity. Despite the use of two bulls, each providing a different type of sperm, being far from ideal, the results do seem to indicate some differences that are more likely to be due to preservation than to between-male differences. Fractal analysis has been a useful tool to indicate the level of sperm activation through track complexity. The findings of these experimental data support the hypothesis previously outlined (Section 2.1.) to some extent. There was evidence of alterations in sperm swimming velocity (chemokinesis), however there was no indication of directional movement in the presence of the COC. There was also evidence of altered sperm velocity in relation to method of sperm preservation, in support of the final aspect of the hypothesis.

The specific morphology of the chemoreceptive spermatozoa and those which best survive cryopreservation requires further study to identify the relative influence of sperm form on function. As only a small number of sperm in any one ejaculate are capable of responding to a chemical attractant, there are problems associated with identifying responding spermatozoa. However, they represent an important subpopulation in terms of fertilisation success, and as such their functional morphology requires further investigation to clarify links between morphology, velocity and fertility.

Limitations imposed on this study by the availability of samples of both oocytes and semen prevented the exploration of variation in sperm velocity in relation to sperm preservation and within- and between-male variation across a larger sample size. However, within- and between-male variation in terms of sperm velocity and morphology is potentially an important factor influencing male fertility and highlights the links between sperm form and function. This will be the focus of the remaining chapters, beginning with a phylogenetically-controlled study examining sperm traits in cichlid fish.

Chapter Three: No link between sperm morphology and velocity across maternal mouthbrooding cichlid fish from Lake Malawi

Abstract

Sperm competition theory predicts that when sperm from more than one male compete to fertilise a given set of eggs, males optimise sperm traits in order to maximise fertilisation rates. In addition to this, it is often assumed that longer sperm swim faster and therefore increase fertilisation rates for males that produce them. However, many studies report conflicting data which do not always support this aspect of sperm trait optimisation, and even fewer report links between velocity and morphology. Differences in sperm morphology between internally and externally fertilising species have been reported though, reflecting the importance of the fertilisation microenvironment. One strategy – mouthbrooding - can be viewed as intermediate between external and internal fertilisation, allowing insights into the relative importance of different selection pressures, depending on point of fertilisation.

Sperm characteristics from 29 species of maternal mouthbrooding cichlid fish from Lake Malawi were examined for an association between sperm morphology and velocity. Two velocity measures (curvilinear velocity and average path velocity) and six morphology measures (head width, head length, head volume, flagellum length, total length and ratio of head length: flagellum length) were taken for individual spermatozoa within the ejaculates of individual males. When shared ancestry was accounted for, no relationship was detected between sperm swimming velocity and any element of morphology across species. However, within-male correlations between morphology and velocity were found for the five species, which could be analysed. Compared with work on Tanganyikan cichlids, the data from this study suggest that there may be little opportunity for sperm competition within cichlids from Lake Malawi.

3.1. Introduction

The Lake Malawi cichlid species flock evolved from a single common ancestor 2.3 – 4.6Ma (Genner et al. 2007a),which has since speciated into the estimated 450-600 species of cichlid now endemic in the lake (Genner et al. 2004).The cichlid radiation in Lake Malawi is dominated by the haplochrominetribe, which is the most species-rich, ecologically diverse tribe of cichlids (Turner 2007). Although generally considered a well-studied system, there is surprisingly little detailed information on the reproductive biology of cichlids from Lake Malawi. The field of sperm biology seems to have largely focused on cichlids from Lake Tanganyika (Table 3.1), presumably because of the array of mating behaviour displayed by the Tanganyikan radiation (Fryer and Iles 1972). Of the cichlid species studied, the literature reveals mixed or no correlation between sperm size and velocity for cichlid spermatozoa (Table 3.1), with only one study out of ten involving species from Lake Malawi. As table 3.1 shows, social status and mating system have been found to influence sperm quality in several species of cichlid from Lake Tanganyika. There is, however,a lower diversity of mating systems in the Malawi species flock than is seen in cichlids found in other African Lakes (Balshine et al. 2001).

The morphology of teleost sperm can reflect mode of fertilisation (Coward et al. 2002). Sperm from internally fertilising teleost species typically have smaller, elliptical heads, with a larger midpiece containing numerous mitochondria as compared to the simple aquasperm produced by externally fertilising species (Lahnsteiner and Patzner 2008). This study sought to investigate whether measurements of sperm morphology or swimming velocity can shed any light on the apparently homologous reproductive biology of cichlids from Lake Malawi.

3.1.1. Sperm characteristics of teleost fish

The morphology of teleost sperm differs from other groups of animals in that there is no acrosome on the head of the sperm. The loss of the acrosome has been linked to the appearance of the micropyle in teleost eggs, which is the only point on the chorion of the egg through which sperm can penetrate to complete fertilisation (Amanze and Iyengar 1990; Jamieson and Leung 1991). Teleost sperm also differ from other species in that most have fin-like projections (Figure 1.5) along the

length of the flagellum (Cosson et al. 2008). The ribbon like fins are extensions of the plasma membrane which surrounds the flagella, and it has been suggested that the fins may provide a propulsive benefit to the spermatozoa through increased propulsive force (Lahnsteiner and Patzner 2008) and/or be involved in water exchange and osmotic regulation (Alavi and Cosson 2006), which could also influence sperm motility. However, the precise functional significance of the flagella fins found in many species of teleost remains to be investigated. Whist the fine structure of sperm (number and location of mitochondria, arrangement of centrioles etc.) can affect the velocity of the cell and vary greatly across species (Lahnsteiner and Patzner 2008), it is the morphological shape of the sperm head, the length of the flagella and the size of these components relative to each other that will be the focus of this chapter.

Table 3.1. Review of published studies considering the characteristics of sperm morphology across cichlid species. Tang=Lake Tanganyika; N.Cam=North Cameroon; EA=East Africa; CA=Central America; VCL=curvilinear velocity; NT=non-territorial males; T=territorial males.

Species	Lake	Trait considered	Findings	Reference
Ophthalmotilapia ventralis	Tang	'Sperm shopping'	Mixed paternity, sperm competition in buccal cavity	(Haesler et al. 2011)
O. ventralis	Tang	'Sperm shopping'	Female benefits by increasing genetic quality of offspring	(Immler and Taborsky 2009)
Telmatochromis vittatus	Tang	Longevity Flagellum length	-Territorial males produce longer lived sperm in presence of competition -Flagellum length same across reproductive tactic -Male standard length not related to sperm velocity	(Ota et al. 2010)
Telmatochromis vittatus	Tang	Sperm velocity vs. male reproductive tactic	-Sperm in sneakers swims faster -no variation in tail length among tactics -sperm length not related to velocity -FL not correlated with longevity or male body size	(Fitzpatrick et al. 2007)
Astatotilapia burtoni	Tang	Territorial vs. non territorial	-Percentage of motile sperm related to social status -VCL same across NT &T -NT males increase sperm motility once	(Kustan et al. 2011)

Species	Lake	Trait considered	Findings	Reference
			becoming T Velocity correlated with length among but not within species	
29 closely related species	Tang	Level of sperm competition vs. mating system		(Fitzpatrick and Balshine 2009)
21 species	Tang	Sperm size vs. sperm competition level	-Polygamous sp. have significantly longer sperm than closely related monogamous sp. -substrate longer sperm than mouthbrooders	(Balshine et al. 2001)
13 species	Israel x5 EAx7 CA x1	Spermatogenesis	-Sperm structure similar to other perciform teleosts -longer sperm in substrate brooders compared to mouth brooders	(Fishelson 2003)
Pelvicachromis taenioithus	N.Cam	Total sperm length – within male variation	Sperm 70um, very long – positive correlation between HL and TL	(Thünken et al. 2007)

3.1.2. Mouthbrooding

All but one species (the non endemic *Tilapia rendalli*) of cichlid found in Lake Malawi exhibit maternal mouthbrooding (Konings 2007). Mouthbrooding resembles internal fertilisation and involves a female taking eggs and sperm into her buccal cavity, where she then broods eggs and fry, and is considered to be an adaptation to high predation risk to eggs (Fryer and Iles 1972). This adaptation provides the opportunity for there to be greater female choice than in other, traditionally externally fertilising species (Genner and Turner 2011). Females that may not be able to assess male quality adequately could mate with several males in

succession in order to increase the chance of sperm competition inside the buccal cavity. This form of bet-hedging is in the interest of the female if it increases the quality of offspring, and is an important factor considering the amount of resources the female invests in spawning and care of offspring (Avis 2012). Maternal care may also increase the pressure on male courtship (Turner 2007), encouraging male-male competition for mates.

The buccal cavity is akin to the cheek area of mammals (Figure 3.1). Whilst conditions inside the buccal cavity have not been explicitly described for mouthbrooding cichlids, some level of through water flow, as the female breathes and oxygenates the eggs, could be in play at the time of fertilisation. Water velocities within the buccal cavity could create hydrostatic pressure gradients (Holeton and Jones 1975), creating flow conditions moving sperm and eggs around and thus influencing encounter rates. The water currents created by the female's breathing have been shown to be important in keeping the eggs viable by cleaning their surface through a process known as 'churning' (Fryer and Iles 1972).

3.1.3. Sexual selection

Sexual selection can work via female choice as well as male-male competition (Malo et al. 2006) and has been an important force in the speciation of cichlids (Fryer and Iles 1972; Parker and Kornfield 1996). Together with multiple invasions, hybridisations and the ability to breed several times a year (Fryer and Iles 1972), female choice is typically considered to be key to the speciation of Lake Malawi cichlids (Fryer and Iles 1972; Parker and Kornfield 1996; Knight and Turner 1999). Sexual selection acts on specific male traits in response to female preference for specific characteristics (Konings 2007). For example, rock dwelling (mbuna) species use colour and/or pattern to identify conspecifics, with sand-dwelling species exhibiting species-specific bower designs and courtship displays (Konings 2007). In lekking species of cichlids, males expend energy creating elaborate sand castle structures (bowers) which, in accordance with the genetic capture hypothesis (Tomkins et al. 2004), are costly to produce and likely to reflect the male's genetic condition. A lek is an area in which reproductively mature males congregate to build their bowers for the purpose of attracting females (McKaye 1983), and females only visit the lek to spawn with males. Each male establishes a

territory within the lek, where he then builds a bower. Male-male competition for the best locations within the lek could show the reproductive fitness of individuals, minimising the need for physical conflicts. Not all females choose to spawn once they reach the male's bower, suggesting that although male attractiveness initially interests the female, she retains the final choice over whether to lay eggs or not, possibly depending on courtship activities or bower traits (Fryer and Iles 1972).

3.1.4. Mating sequence and fertilisation location

The cichlids found in Lake Malawi are largely thought to be polyandrous (Konings 2007). Polyandry is often considered to be male-biased as there are numerous costs for the female associated with mating with multiple males (Simmons 2005). However, as an adaptive trait, female promiscuity appears to be widespread (Simmons and Fitzpatrick 2012), suggesting that the advantages for females mating with multiple males outweigh the associated risks (Birkhead 2000).

The mating sequence for many cichlids has been described as follows: female mouths the anal fin area thus collecting the sperm before laying and collecting a small batch of eggs. This is repeated until the female has laid all her eggs or moves onto spawn with another male. An alternative process is that the female lays the batch of eggs, which is then fertilised by the male on the substrate before the female picks up the eggs and, potentially, moves to another male, where the process starts again (Fryer and Iles 1972; Konings 2007). Depending on the speed at which egg collection occurs, it can be hard to confirm the point at which the male releases his milt and therefore to confirm likely point of fertilisation. The lack of clarity in relation to fertilisation point has important repercussions for determining the level of sperm competition possible in mouthbrooding cichlids. The mating sequence could create conditions of sperm competition if females are polyandrous, and with sperm competition there may be an associated reduction in the variation of sperm morphology (Calhim et al. 2007; Scharer et al. 2011). As will be seen in the following section, sperm competition requires sperm from two or more males to be in competition for the same eggs (Parker 1970), a situation which may be less likely for many Lake Malawi cichlids.

3.1.5. Sperm competition

The level of sperm competition should be reflected in sperm traits (Parker and Pizzari 2010). Theory predicts that intense sperm competition will select for longer sperm, largely based on the assumption that longer sperm swim faster and are therefore more competitive (Parker 1998). There are several reasons to suggest that there is likely to be little or no sperm competition in mouthbrooding cichlids (Stockley et al. 1997). As small numbers of eggs are released during anyone spawning with a male, sperm competition is unlikely because available eggs are fertilised as soon as they are released (Mrowka 1987). Sequential polyandry could result in sperm competition but would require sperm to be long-lived (Fitzpatrick 2005) and females to find and spawn with additional males in quick succession (Haesler et al. 2011).

Nevertheless, work by Balshine et al suggests that there is the potential for sperm competition in the buccal cavity of mouthbrooding cichlids. In species with sequential mating, in which males coat sperm in a mucus, sperm longevity increases, resulting in the potential for viable sperm from more than one male to be in the female's buccal cavity with unfertilised eggs at the same time (Balshine et al. 2001). Furthermore, the same study found sperm traits to be associated with the point of fertilisation. Tanganyikan cichlids that fertilise eggs within the female buccal cavity had significantly shorter sperm than those which fertilised eggs on the substrate. We might therefore expect that sperm traits from cichlids from Lake Malawi could also be associated with the point of fertilisation.

The fertilisation point is not always clear in mouthbrooding cichlids from Lake Malawi but it has been established that, in general, the eggs of most haplochromine are fertilised in the buccal cavity (Mrowka 1987). Not only do fish sperm then have to find the egg, they then need to swim around the surface of the egg to find the micropyle. No data on the amount of time the micropyle remains open for on eggs of Lake Malawi cichlids were available, but in other teleosts it can be between 10-20 seconds. However, if male and female gametes are inside the confines of the buccal cavity, sperm-egg collisions would be greatly increased compared to substrate fertilisation (Denny and Shibata 1989). Fertilisation inside the buccal cavity could be considered to resemble internal fertilisation, as both male and female gametes are confined within the female's body. However, internally

fertilising teleost generally produce longer sperm, which would seem to contradict the findings of Balshine et al. (2001), where shorter sperm were found to be linked to fertilisation inside the buccal cavity of cichlids. Considering the number and diversity of species covered by the infraclass Teleostei, it should perhaps not be surprising that generalisations may lose robustness as the quantity of species specific data is increased. The data gathered in this chapter present, for the first time, morphology and velocity information on the sperm characteristics of 33 cichlid species.

Figure 3.1. Female *Astatotilapia calliptera* brooding fry, highlighting location of buccal cavity. Females can brood up to 80 fry at a time.

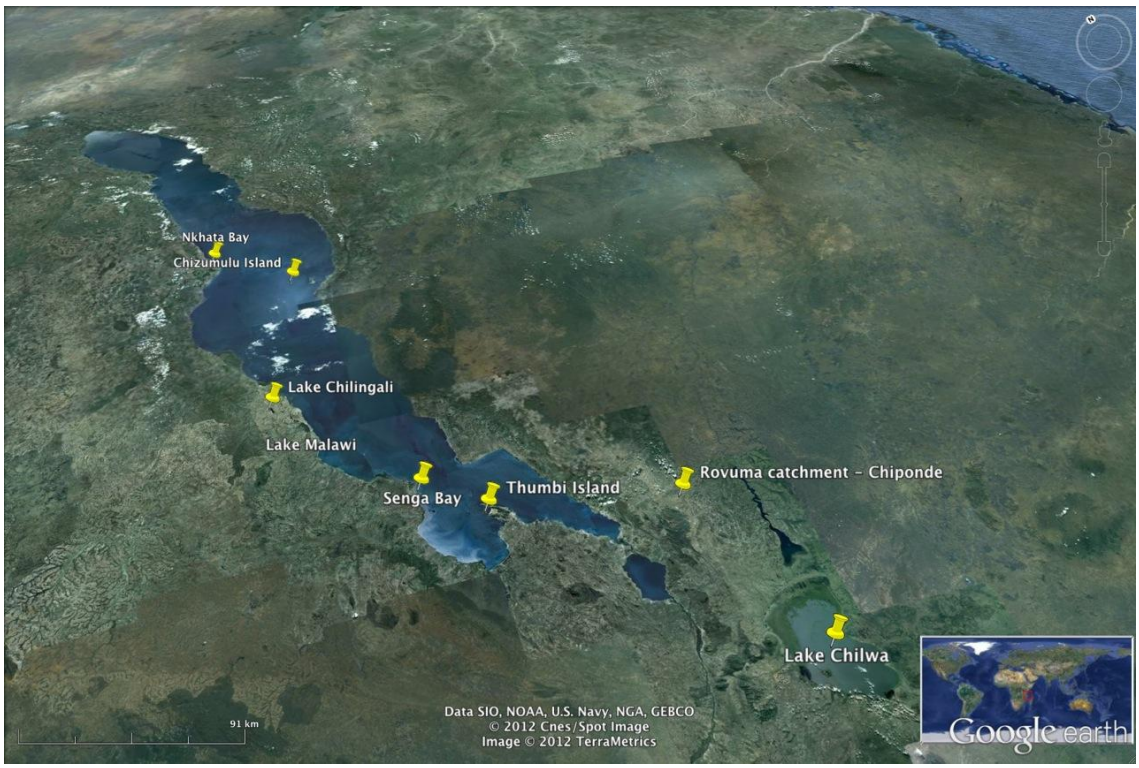


Figure 3.2. Location of Lake Malawi (365 miles long by 52 miles wide), identifying areas around the lake where cichlids were originally collected. Lake Chilwa (24 miles long, 17 miles wide according to Kirk) is isolated from the main body of Lake Malawi. Lake Chilingali (~3 miles long by ~0.6 mile wide according to Genner et al (2007)) is only seasonally connected to Lake Malawi.

3.1.6 Cichlids of Lake Malawi

It has been established that the radiation of Lake Malawi cichlids is likely to have occurred sequentially in three stages, involving divergence in habitat niche, trophic morphology and phenotypic colour variation (Kocher 2004). The first stage of habitat divergence can be seen in the allocation of mtDNA clades described below. Trophic morphology represents a functional trade-off for mouthbrooding haplochromines, as a recent investigation indicated that the evolution of an extended buccal cavity reduced the efficiency with which females feed as compared to males from the same species. It has been suggested that the diversity of male colour within clades is an example of divergence by sexual selection, with female preference for male colour driving the co-evolution of male trait and female selection (Kocher 2004).

3.1.6.a. Cichlid clades as indicated by mtDNA

All species from Lake Malawi described here are from the haplochromine tribe (the descriptions of the species used here will be outlined in section (3.2)), which has previously been classified into mtDNA clades (Joyce et al. 2011). These clades broadly reflect variation in ecological adaptations. In the absence of detailed information on reproductive biology, cichlids used here were grouped using ecological variation as a general proxy for possible divergence in reproductive behaviour as a consequence of environmental conditions. Clades represented here include benthic sand-dweller, pelagic (*Rhamphochromis*), and rock-dweller (mbuna). The remaining mtDNA clade of *Astatotilapia calliptera*and the outgroups are defined by phylogeny rather than habitat specificity. All species names used throughout follow those used by Konnings (2007).

Here, measurement of individual sperm size (head length, head width, head volume, flagella length, total length and a ratio of head length: flagella length) and velocity were taken from closely related cichlids from Lake Malawi. Functional links between sperm size and velocity for Lake Malawi cichlids have not previously been explored. A robust phylogeny based on a mtDNA control region was constructed to assess the amount of variation found across sister groups (Barraclough et al. 1998). In addition, the elaborate extension to the male phenotype found in some cichlids – bower building – was considered. In line with reproductive trade-off theories (Parker and Pizzari 2010), it was predicted that species in which the male expends part of his reproductive budget on constructing and defending elaborate bowers would have distinct sperm traits compared to closely related, non-bower building species. The 29 species of Malawi cichlid were chosen to represent the morphological and ecological diversity found in the Lake Malawi radiation.

Hypothesis

It has been shown that the length of cichlid sperm varies with fertilisation location. If the point of fertilisation varied across the cichlid species sampled here, shorter sperm would indicate eggs are fertilised inside the buccal cavity, with longer sperm suggesting external fertilisation (substrate or water column). Furthermore, links between velocity and morphology will only be evident if both measurements are taken from an individual sperm cell and analysed whilst accounting for intra male variation.

3.2. Methods

All the cichlids used in this investigation were housed in the fresh water aquarium at the University of Hull. The fish were kept in a re-circulatory system with a daily water change of 10% and 12 hour light cycle, at 23-25°C in stock tanks measuring 180x45x40cm. Although the ancestors of most species of cichlid used here were originally caught from wild populations, there were six species which originated from UK pet shops (Table 3.2).

Table 3.2. Details of the 33 cichlid fish species used to compare sperm characteristics. +=Lake Chilingali (satellite lake to Lake Malawi); ++=Lake Rovuma, feeds into Lake Malawi (Figure 3.2); *=breeding stock sourced from pet shop; S=substrate; B=buccal cavity. The suggestion that most haplochromine eggs are fertilised inside the buccal cavity (Mrowka 1987) was not found to be consistent. Gaps in the table represent absence of detailed, species specific information available from the literature (Fryer and Iles 1972; Konings 2007).

Species	Lake Malawi mtDNA clade/outgroup Lake	Species spawning characteristics	Fertilisation location	n male (sperm)	Year breeding stock caught in wild
Nyassachromis cf. microcephalus	Sand-dweller	Bower building		4(32)	2007
Otopharynx lithobates	Sand-dweller	Cave dweller		1(7)	*
Cyrtocara moorii	Sand-dweller		S	1(10)	*
Lethrinops furcifer	Sand-dweller	Bower building	S	1(5)	2010
Hemitilapia oxyrhynchus	Sand-dweller	Bower building		1(10)	*
Lethrinops sp. chilingali+	Sand-dweller	Bower building	S	1(9)	2010
Rhamphochromis sp. Chilingali+	Rhamphochromis	Pelagic	B	7(36)	2007
Rhamphochromis cf. longiceps	Rhamphochromis	Pelagic	B	7(41)	2005
Astatotilapia calliptera Rovuma++	Astatotilapia calliptera	Spawning pits	S	8(44)	2007
Astatotilapia sp. 'calliptera chizumulu'	Astatotilapia calliptera		S	9(63)	2002
Metriaclima kingsizei Nkhata Bay	Mbuna			1(4)	2007
Metriaclima 'zebra gold'	Mbuna	Spawning pit under rocks		1(9)	1998
Metriaclima estherae	Mbuna			1(18)	2003
Metriaclima fanizilberi	Mbuna			1(3)	2004
Mertiaclima zebra Nkhata Bay	Mbuna			1(5)	1998
Metriaclima zebra Thumbi west	Mbuna			1(9)	2007
Metriaclima emmiltos	Mbuna			1(7)	2004

Species	Lake Malawi mtDNA clade/outgroup Lake	Species spawning characteristics	Fertilisation location	n male (sperm)	Year breeding stock caught in wild
Metriaclima sp. 'elongatus chewere'	Mbuna			1(10)	2007
Metriaclima zebra Thumbi east	Mbuna			1(13)	2007
Cynotilapia sp. 'lion' Sanga	Mbuna			1(10)	2007
Cynotilapia afra Nkhata Bay	Mbuna			1(11)	1998
Cynotilapia axelrodi	Mbuna			1(10)	2007
Cynotilapia afra Thumbi west	Mbuna			1(6)	2007
Tropheops sp. 'olive'	Mbuna			1(11)	1998
Pseudotropheus sp. 'acei'	Mbuna			1(3)	1998
Labidochromis caeruleus	Mbuna			1(7)	*
Neochromis omnicaeruleus	Lake Victoria – Makobe Island			1(10)	2003
Paralaabidochromis chilotes	Lake Victoria - Ruti Island			1(5)	2003
Lipochromis melanopterus	Lake Victoria - Makobe Island			1(8)	2001
Haplochromis sp therutereon	Lake Victoria - Senga			1(4)	2003
Oreochromis shiranus	Lake Chilwa	Spawning craters		1(8)	2006
Stomatepia pindu	Barombi mbo			1(3)	*
Sarotherodon steinbachi	Barombi mbo			1(8)	*

3.2.1. Stripping milt

Reproductively-mature dominant males from the species listed in Table 3.2 were identified on the basis of size and nuptial colour. They were then removed from stock tanks and anesthetised in a bath of MS-222 (3 litres of tank water to 0.3g MS-222) before being stripped of milt by applying gentle pressure to the abdomen. To avoid activation of sperm via contamination with excreta/water, the area around the genital pore was dried prior to stripping, with any samples which could have been contaminated discarded from further analysis (Trippel 2004). The standard length (SL) was measured to the nearest mm and body mass to the nearest 0.01g for each male. When all measurements had been taken, males were placed into a recovery tank. Once fully conscious males were returned to communal tanks, they

were observed for several minutes to ensure they had fully recovered. The milt stripping procedure was carried out under license and in accordance with UK Home Office regulations (project licence PPL 60/4036 personal licence PIL 60/12760). Ethical approval was also received from the University of Hull (U008) for all experiments.

Collected spermatozoa were placed in Ependorff tubes, in which sperm were then activated and diluted using tank water (Fitzpatrick et al. 2007). Volume and density of milt samples varied between individuals and was diluted on a case-by-case basis to allow individual spermatozoa morphology to be measured. The amount of water added depended on a visual inspection of the opacity of the sample and ranged from 100-500 µl. All samples were diluted and analysed by the author to ensure consistency in sample preparation.

3.2.2. Recording motility

For each male, 1µl of diluted sample was placed in individual wells of 12 cell multitest slides (MP Biomedicals, Aurora, OH, USA), with a cover-slide placed over each set of wells. Before sperm samples were added, both the multitest slide and cover-slide were coated in 1% polyvinyl alcohol (PVA) to reduce the attraction between spermatozoa and the edges of the slides (Wilson-Leedy and Ingermann 2007). All recordings were taken using a phase contrast microscope (Olympus BX40) with a heated stage at 23°C, and filmed for one second using a 400× PLN objective with a CMOS camera (PixeLink PL-B686CF) at 97 frames per second (fps). Each video clip was assigned a relative clip time to ensure motility recordings taken from all species were used after comparable 'time since activation' points.

For most species, data from one male were used to represent the species, as variation among-species has been found to be greater than within-species variation in sperm traits from 21 species of cichlid from Lake Tanganyika (Balshine et al. 2001). However, data from multiple males were also gathered for five species, which were then used to assess within-male variation.

3.2.3. Sequence data

Sequence data for the mtDNA control region were gathered because, as a rapidly evolving region, it has been shown to indicate distinct haplogroups which cannot be resolved using nuclear markers due to the recent and rapid evolutionary history of cichlids (Genner and Turner 2011). Sequences for 14 species were taken from Genbank (Appendix Two), and the remaining 19 species were fin clipped in accordance with Home Office Licence procedures (project licence 30/4331). DNA was extracted from ethanol preserved fin clips using the hotshot method (Truett et al. 2000). Next, a 1.2kb base pair region of the mtDNA D-loop was amplified using primers and conditions as used by Joyce et al. (2005), and sequenced by Macrogen (http://www.macrogen.com). Mitochondrial sequences were aligned using CodonCodeAligner (v.3.7.1.1) and ClustalX in MEGA 4.0 (Tamura et al. 2007), and by eye, with the final alignment ranging from 571-1124 base pairs in length (see Appendix Two for species specific alignment lengths).

3.2.4. Analysis of videos

Measurements from individual sperm were taken for morphology and velocity. Focal sperm were selected when and if morphology was clearly visible throughout the video and the flagellum exhibited high amplitude beat frequency waves that propagated along the entire length of the flagellum and therefore indicated that the sperm was fully activated. All videos were analysed using NIH ImageJ (v. 1.42q) with a computer assisted sperm analysis (CASA) plugin (Wilson-Leedy and Ingermann 2007). Using the ImageJ function 'threshold', individual cells were isolated (Fitzpatrick et al. 2010) before velocity measures could be recorded. Once all other cells and any background noise had been removed from the area around the focal spermatozoa (by selecting areas for removal and using 'clear outside' to remove the same area across each frame in the clip), the velocity of the isolated cell was recorded using CASA. In the Sperm Tracker window of CASA, the following parameters were altered from their default values: a) Minimum sperm size (pixels); b) Maximum sperm size (pixels); c) Minimum track length (frames); d) Maximum sperm velocity between frames (pixels); and q) Frame Rate (frames per second). The same cell's morphology was then measured, i.e. head width (HW), head length (HL) and flagella length (FL). Total length (TL) was calculated by adding head length and flagella length. In addition, the head length: flagellum

length ratio (HL:FL) and head volume (HV) were also calculated for the mixed effects analysis. After importing the original video into ImageJ and zooming in 200%, each element of morphology was traced over before using 'set scale' and 'measure' functions to measure each individual cell at three points along its path. Measurements were taken to the nearest 0.001mm and an average of the three measures was used for each cell in further analyses (see Appendix 3 for full details of ImageJ techniques). A minimum of two spermatozoa per male was required for the male to be included in subsequent analysis. Whilst this is a low number, two to three sperm per male were found to capture 91.2% of the among-male variation in *Drosophila melanogaster* (Pattarini et al. 2006).

3.2.5. Controlling for phylogeny

Bayesian mixture model likelihood methods (Pagel et al. 2004) were used to produce a posterior sample of 1,000 phylogenetic trees. Briefly, a number of independent Markov chains were allowed to run to convergence (at least 500,000 iterations) before sampling trees at widely spaced intervals to ensure independence among successive trees in the sample. A single majority rule consensus tree was calculated for future analyses.

The consensus tree was then used to test whether there was any phylogenetic signal in sperm velocity and morphology measures. If these traits evolve according to the phylogenetic tree (i.e. there is phylogenetic signal) it is important to take into account their shared ancestry when testing hypotheses regarding the evolution of adaptive traits such as sperm quality, as will be described by the phylogeny in subsequent statistical analyses (Harvey and Pagel 1991).

A phylogenetic generalised least-squares approach (Pagel 1999), implemented in the program BayesTraits (Pagel et al. 2004), was used to estimate the parameter λ (lambda) to quantify the phylogenetic signal. The parameter λ scales the branches of a phylogeny to assess signal strength (see Freckleton et al. 2002 and Figure 3.3). Values for λ range from 1-0, with 0 indicating that the trait under investigation, here sperm form, evolved separately from the between-species evolution indicated by the phylogeny. Lambda values indicating a strong signal (i.e. approaching λ=1) reveal that the trait and phylogeny are evolutionarily linked.

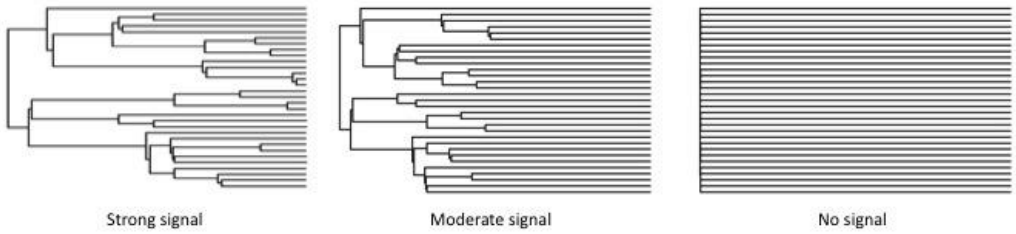


Figure 3.3. Progression of the scaling parameter, with λ reflecting strength of phylogenetic signal from strong signal (λ=1) to moderate signal (λ=0.5) and no signal (λ=0).

3.2.6. Statistical analysis

Phylogenetic generalised least-squares analysis indicated no signal (λ=0), allowing linear regressions to be carried out without phylogenetic control. Linear regression analysis was carried out in JMP (9.0.2) using logged velocity and morphology data.

To distinguish within-male effects from between-male effects in sperm morphology-velocity relationships, within-subject centring was performed (van de Pol and Wright 2009). To achieve this, a mixed effect analysis (van de Pol and Wright 2009) was carried out on five species for which data from multiple males had been collected. The analysis was carried out using R v 2.13.0 (R Development Core Team, 2011), using the nlme package (Pinheiro et al. 2011). Following van de Pol and Wright (2009), mixed-effects models were used with intra- and inter-male sperm length components fitted as fixed effects and, to use their terminology, 'within' and 'between' male effects. Male identity was entered as a random effect and fitted both a random intercept and a random intercept and slope model for all datasets. The random intercept model (equation 2 of van de Pol and Wright 2009) allowed the magnitude of velocity measures to vary between males, and in the simplest case this model accounts for the possibility that two males could have sperm that are of similar lengths, but that one of the males might have generally faster sperm. In contrast, the random slopes and intercepts model (equation 4 in van de Pol and Wright 2009) allows both the magnitude of the velocity measure and its relationship to length components to vary between males. Data were

logged (natural log) to help account for positive correlations between means and variances. An ANOVA was performed to distinguish between the two models for each dataset, choosing the one that explained the most variance, or the simpler model if the amount of variance explained did not differ. This analysis indicated that the random intercept model explained most of the variance for all data comparing sperm length to VAP. Consequently, all of the VAP results described below include only random effect intercepts; allowing random slopes and intercepts provides no additional information. In recognition of the small sample sizes, Bonferroni corrections were not used when analysing subsets of the data, as this can increase type II errors. Instead, calculated effect size (*r*) and 95% confidence intervals (*CI*) were calculated for the mixed effects models using the methods given in Nakagawa and Cuthill (2007), allowing effect size to be quantified for both fixed effect components of the model (within- and between-male variation).

3.3. Results

3.3.1 Phylogenetic analysis

Phylogenetic generalised least-squares indicated no phylogenetic signal in relation to sperm velocity or morphology (λ=0). This implies that the evolution of male gametes is not related to the phylogeny of the cichlid radiation (Figure 3.4.).

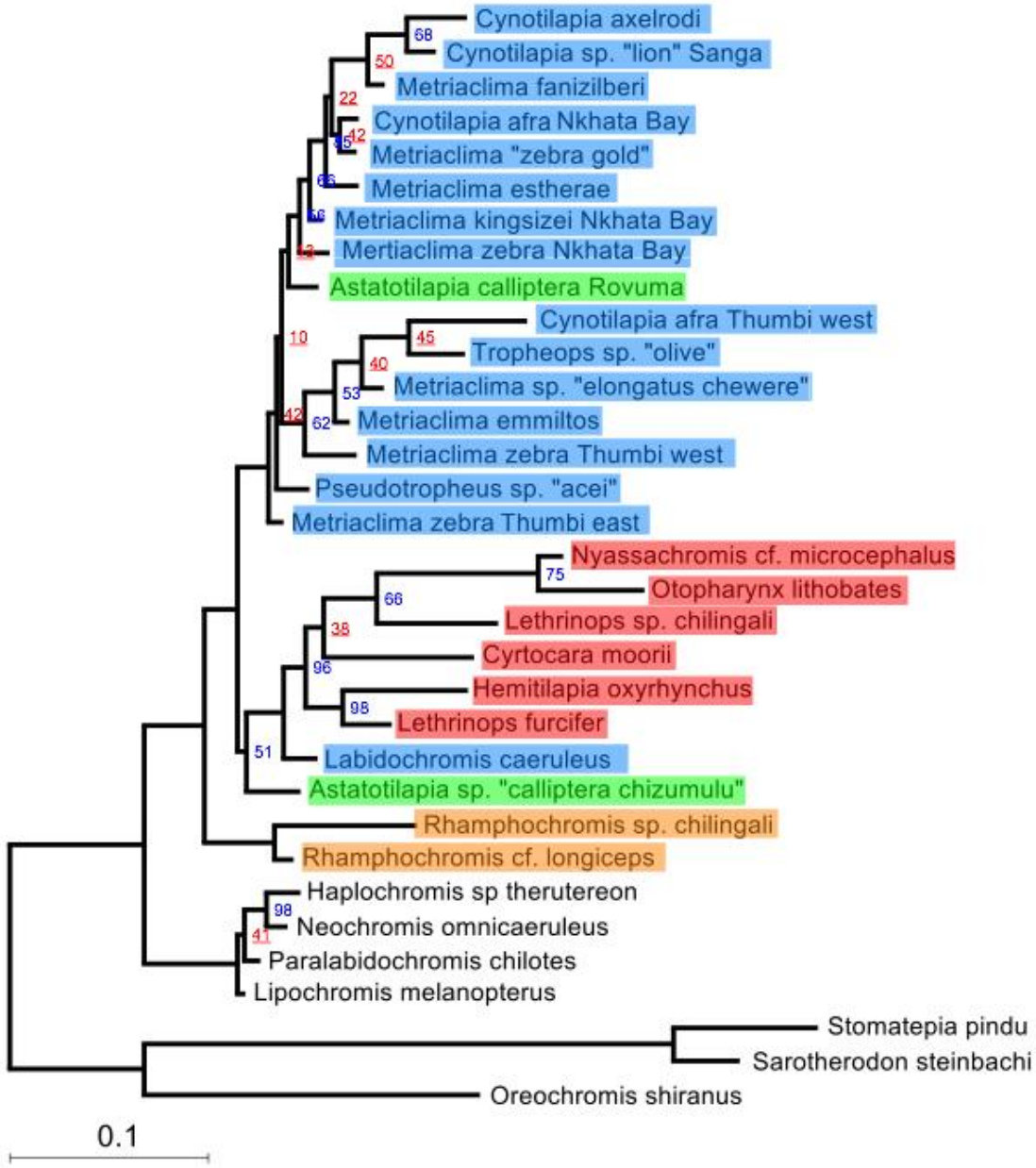


Figure 3.4. Phylogenetic tree based on mtDNA D-loop sequence data. Numbers at junctions represent posterior percentage of the posterior sample of 1,000 trees containing the base change, with red numbers 50% or below, blue numbers above 50%, and absence of number representing base changes present in all trees (100%). Scale line refers to number of nucleotide substitutions per site in the alignment. The tree is congruent with published trees for Lake Malawi cichlids. Colours refer to mtDNA clades: Red='sand-dwellers'; Blue='mbuna'; Orange=*Rhamphochromis*; Green=*Astatotilapia calliptera*. Species without colour are from outgroups.

3.3.2. Velocity measurements

Linear regression analysis was performed on log transformed VAP and VCL data and the two speed measurements were found to be correlated (p=<0.0001, Figure 3.5), as expected (Fitzpatrick and Balshine 2009). To avoid repetition, only VAP results will be presented here.

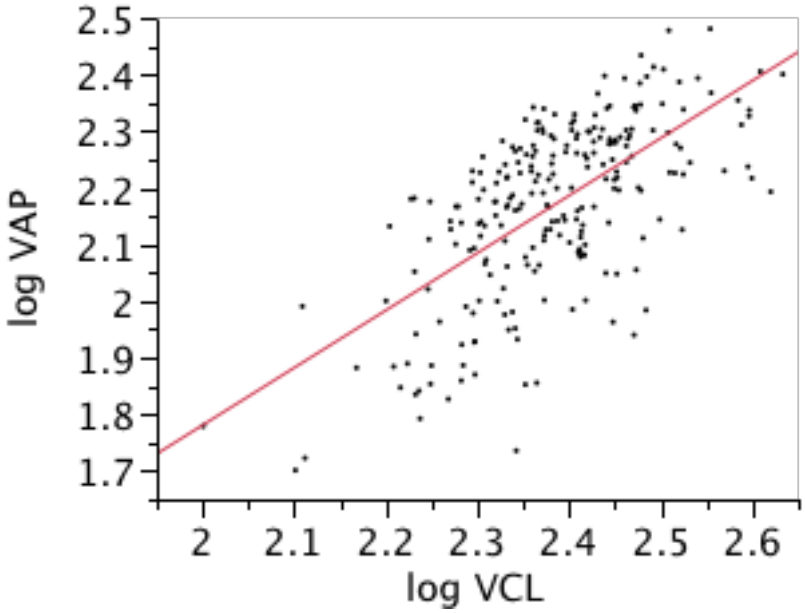


Figure 3.5. Scatter plot created after linear regression, illustrating the correlation between velocity measurements for VAP and VCL (individual sperm n=243, p=<0.0001).

3.3.3. Sperm morphology – velocity across cichlid species

Linear regression revealed no relationships across cichlid species for any measured aspect of sperm morphology and velocity (species n=33, minimum p=0.29, Figure 3.6.). Accounting for male standard length (Figure 3.7-a) or weight (Figure 3.7-b) does not reveal any link between sperm size and velocity in relation to male size across species.

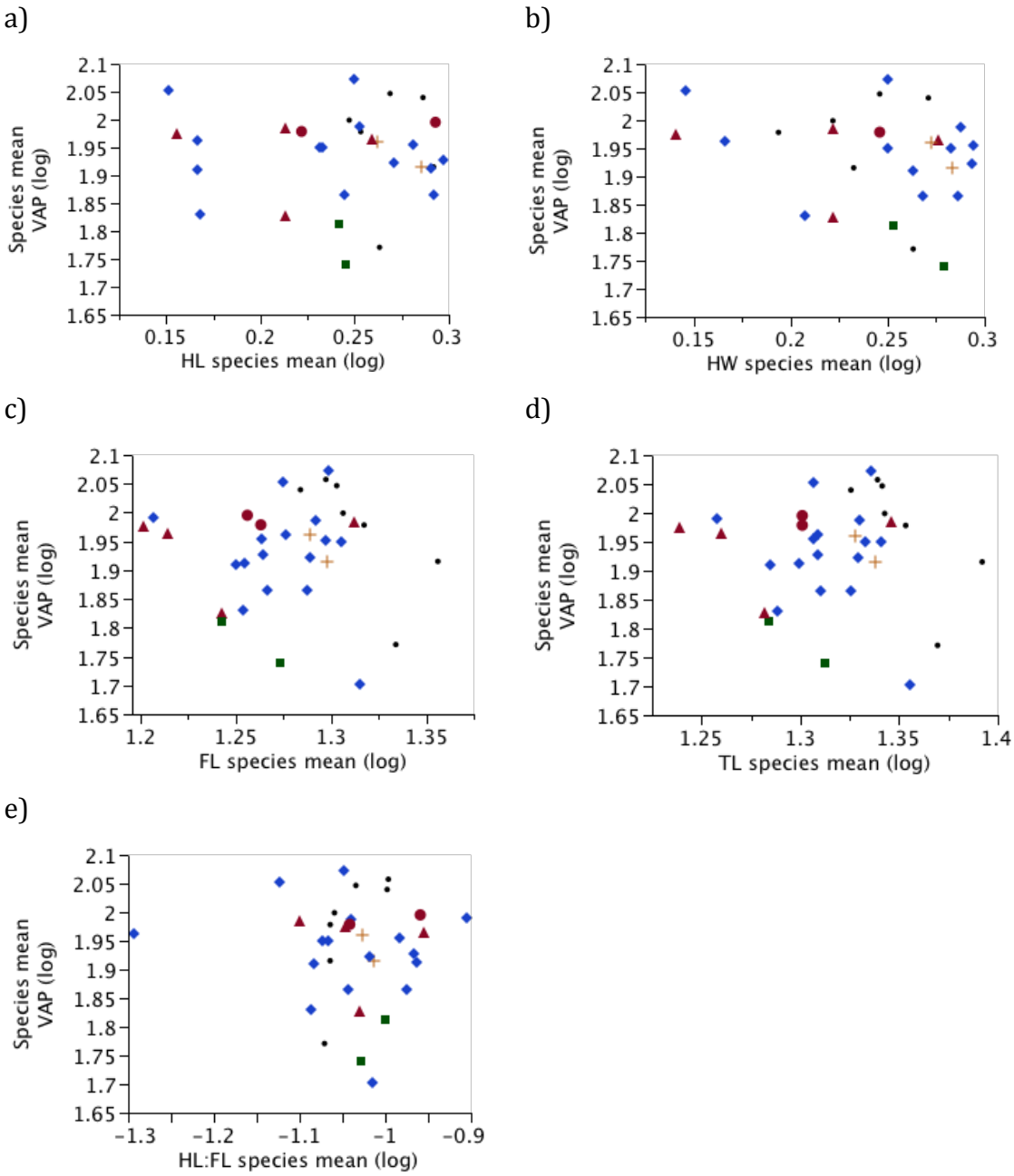


Figure 3.6. Scatter plots from linear regression analysis, p-values indicate no significant relationships between sperm morphology and velocity across species. Each point represents mean sperm measurement from one male (species n=33). a) HL=head length (p=0.82); b) HW=head width (p=0.29); c) FL=flagella length (p=0.87); d) TL=total sperm length (p=0.86) and e) H:F= ratio of head length to flagella length (p=0.83). mtDNA clades – Red='sand-dwellers', with triangles indicating bower building species; Blue='mbuna'; Orange=*Rhamphochromis*; Green=*Astatotilapia calliptera,* and Black=outgroups.

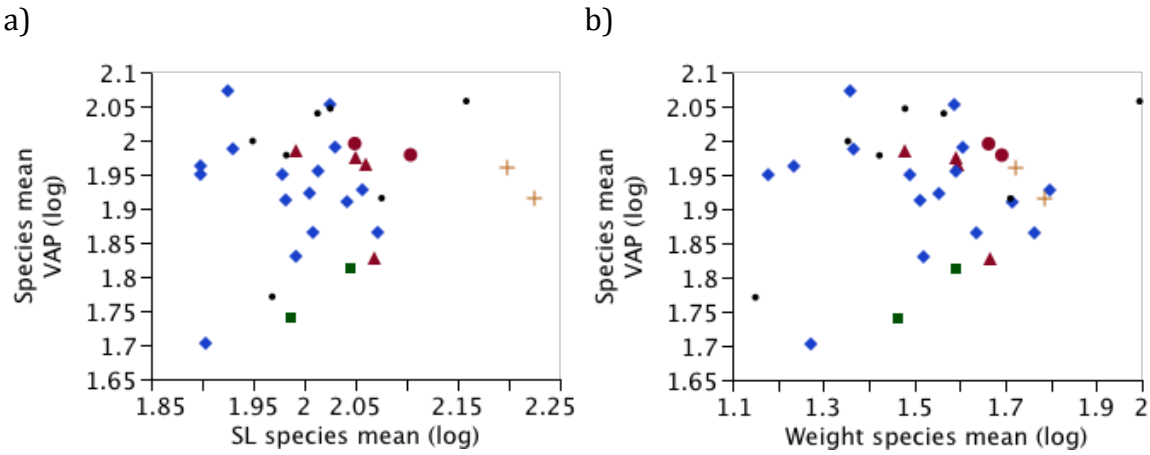


Figure 3.7. Scatter plots from linear regression analysis for a) whole fish standard length (p=0.49) and b) male weight (p=0.30) against sperm velocity (species n=33).

3.3.4. Mixed effect analysis

The mixed effects models indicated that equation 2 explained most of the variation, and that allowing random slopes and intercepts (equation 4 Van de Pol 2009) did not explain any additional variance. The analysis revealed significant within- and between-male correlations between some elements of morphology and VAP for all five species examined (Table 3.3). Significant results from the mixed effect model are presented in pairs for sister-species *Astatotilapia calliptera* Rovuma and *Astatotilapia sp. 'calliptera chizumulu',* then for *Rhamphochromis cf. longiceps* and *Rhamphochromis sp. Chillingali*. Within-male correlations for flagellum length and total length with VAP were found for *Astatotilapia calliptera Rovuma*(Figure 3.8), *Rhamphochromis cf. longiceps* and *Rhamphochromis sp. Chillingali* (Figure 3.9), and *Nyassachrmis cf. microcephalus* (Figure 3.10). Between-male correlations for head width and VAP were found in *Astatotilapia sp. calliptera chizumulu* (Figure 3.8) and for total length-VAP for *Astatotilapia calliptera Rovuma* (Table 3.2).

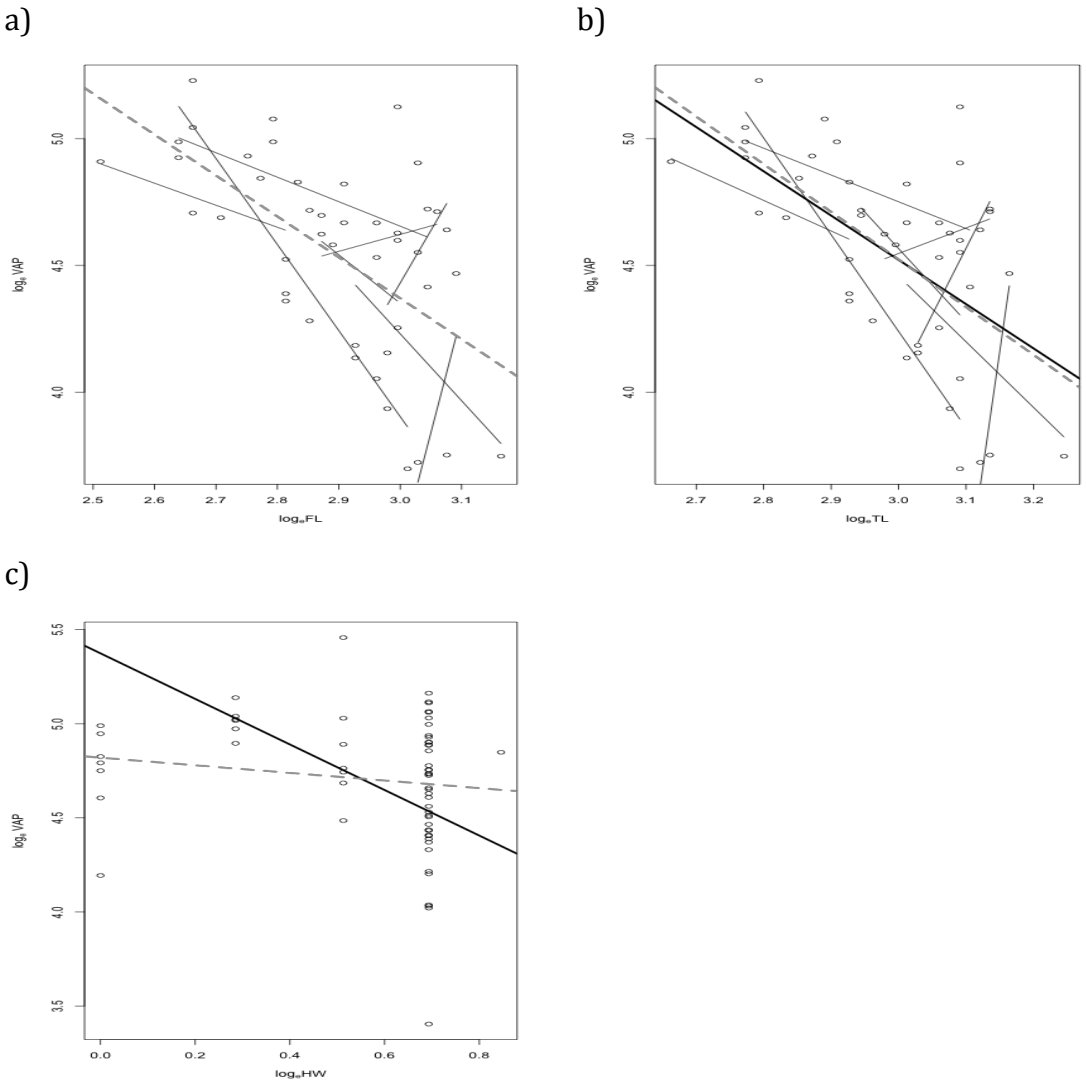


Figure 3.8. (a) and (b) *Astatotilapia calliptera* Rovuma (n=8 males with a total of 44 sperm); (c) *Astatotilapia sp. Calliptera chizumulu* (n=9 males with a total of 63 sperm). Between-male effect only – correlations for the relationship between sperm flagellum length (FL), total length (TL), head width (HW) and velocity (VAP) within and between males. Regression slopes for within-male variation in black. Gray dashed line depicts the estimate from a standard mixed model of the effect of length measures on the speed measure (guide only). Solid black lines depict individual regression slope for between-male data. All data log transformed.

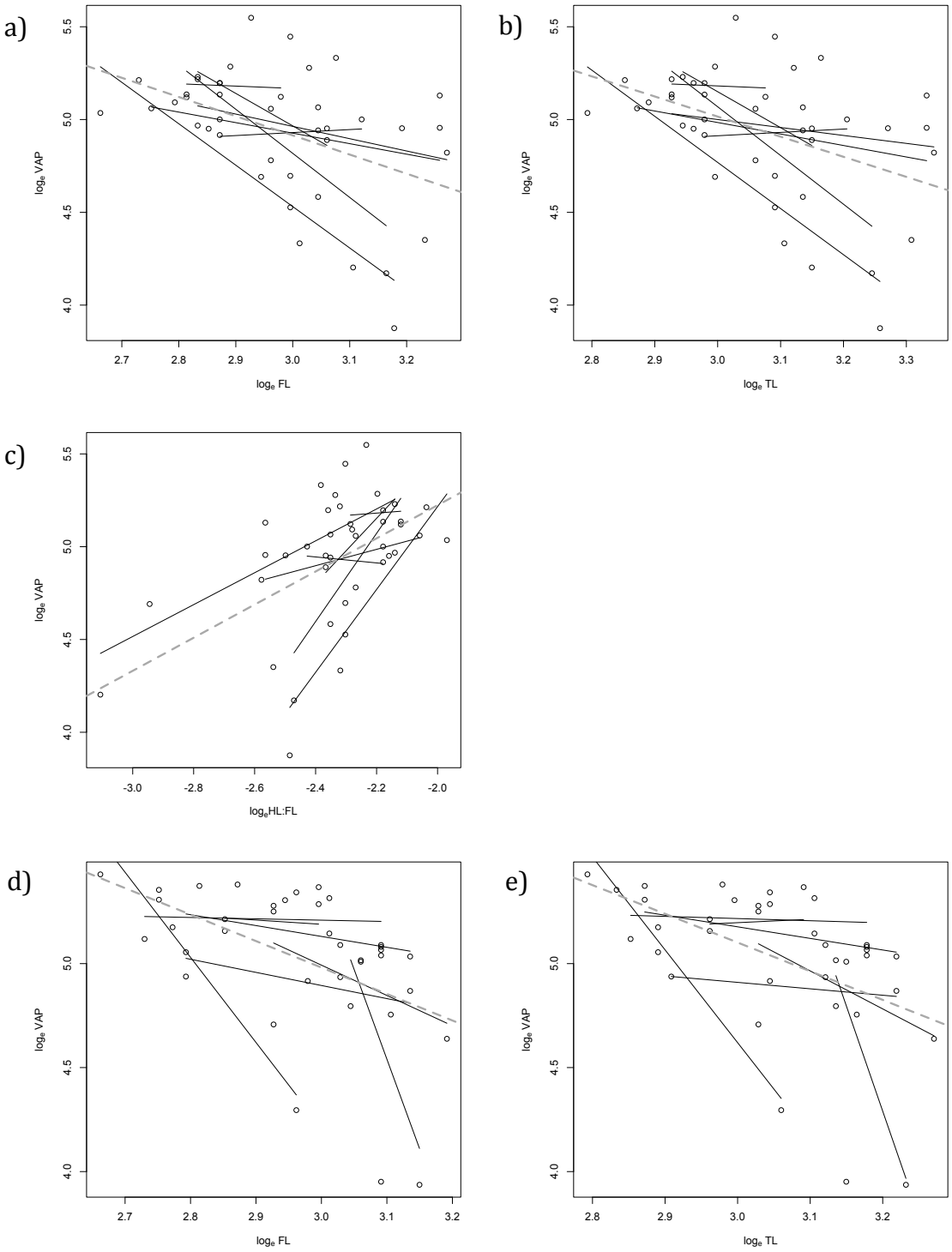


Figure 3.9. (a-c) *Rhamphochromis cf. longiceps* (n=7 males with a total of 41 sperm); (d) and (e) *Rhamphochromis sp. chillingali* (n=7 males with a total of 36 sperm). Correlations for the relationship between sperm flagellum length (FL), total length (TL), head length to flagella length ratio (HL:FL) and velocity (VAP) within-males. Gray dashed line depicts the estimate from a standard mixed model of the effect of length measures on the speed measure (guide only). All data log transformed.

a) b)

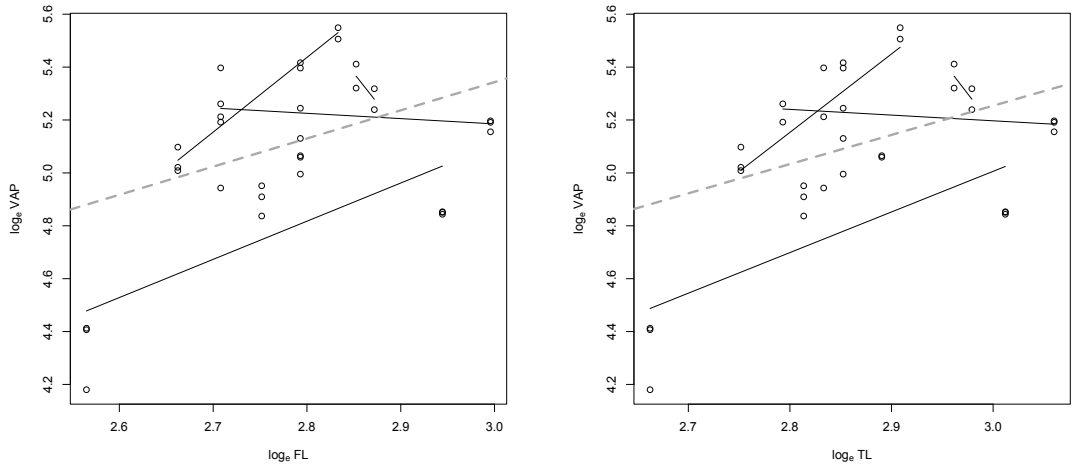


Figure 3.10. *Nyassachrmis cf. microcephalus* (n=4 males with a total of 32 sperm). Correlations for the relationship between sperm for a) flagellum length (FL) and b) total length (TL) and velocity (VAP) within-males. Gray dashed line depicts the estimate from a standard mixed model of the effect of length measures on the speed measure (guide only). All data log transformed.

Table 3.3. Results for sperm length-VAP from mixed model centring, allowing random effect intercepts for between and within male analysis. HW=head width, HL=head length, HV=head volume, FL=flagella length, TL=total length, HL:FL=ratio of head length to flagellum length, and n_t=total number of males. F=degrees of freedom within and between males. 95% confidence intervals (95% CI) calculated for effect size (r). Significant correlations (p≤0.05) in bold.

Species	Sperm trait	n_t	Between-male effects				Within-male effects			
			$F_{(within, between)}$	p	r	95% CI	$F_{(within, between)}$	p	r	95% CI
Astatotilapia calliptera Rovuma	HW	8	$F_{1,6}$=1.00	0.357	0.18	-0.19 to 0.47	$F_{1,35}$=0.03	0.873	0.03	-0.31 to 0.35
	HL		$F_{1,6}$=0.12	0.738	0.06	-0.27 to 0.37	$F_{1,35}$=0.01	0.933	0.01	-0.31 to 0.33
	HV		$F_{1,6}$=0.68	0.442	0.15	-0.21 to 0.44	$F_{1,35}$=0.04	0.848	0.03	-0.30 to 0.36
	FL		$F_{1,6}$=5.79	0.053	-0.40	-0.64 to 0.01	$F_{1,35}$=13.53	**0.001**	-0.55	-0.71 to -0.27
	TL		$F_{1,6}$=6.25	**0.046**	-0.41	-0.65 to -0.01	$F_{1,35}$=14.53	**0.001**	-0.56	-0.71 to -0.29
	HL:FL		$F_{1,6}$=2.19	0.189	0.26	-0.12 to 0.53	$F_{1,35}$=2.31	0.137	0.26	-0.09 to 0.53
Astatotilapia sp. Calliptera chizumulu	HW	10	$F_{1,8}$=6.54	**0.034**	-0.36	-0.58 to -0.03	$F_{1,52}$=0.16	0.694	-0.06	-0.33 to 0.23
	HL		$F_{1,8}$=0.45	0.522	-0.09	-0.35 to 0.18	$F_{1,52}$=<0.001	0.958	0.01	-0.26 to 0.27

Species	Sperm trait	n_t	Between-male effects				Within-male effects			
			$F_{(within, between)}$	p	r	95% CI	$F_{(within, between)}$	p	r	95% CI
	HV		$F_{1,8}$=4.67	0.063	-0.30	-0.53 to 0.02	$F_{1,52}$=0.09	0.762	-0.05	-0.32 to 0.24
	FL		$F_{1,8}$=1.20	0.305	-0.15	-0.40 to 0.14	$F_{1,52}$=1.68	0.201	-0.18	-0.42 to 0.10
	TL		$F_{1,8}$=1.20	0.305	-0.15	-0.40 to 0.14	$F_{1,52}$=1.06	0.211	-0.18	-0.41 to 0.10
	HL:FL		$F_{1,8}$=0.05	0.828	-0.03	-0.29 to 0.24	$F_{1,52}$=0.49	0.486	0.10	-0.17 to 0.35
Rhamphochromis cf. longiceps	HW	7	$F_{1,5}$=0.44	0.535	0.13	-0.26 to 0.47	$F_{1,33}$=1.34	0.255	0.23	-0.16 to 0.53
	HL		$F_{1,5}$=0.05	0.835	-0.04	-0.40 to 0.33	$F_{1,33}$=3.70	0.063	0.36	-0.02 to 0.67
	HV		$F_{1,5}$=0.25	0.638	0.10	-0.29 to 0.44	$F_{1,33}$=3.41	0.074	0.35	-0.04 to 0.61
	FL		$F_{1,5}$=0.13	0.737	-0.07	-0.42 to 0.31	$F_{1,33}$=9.82	**0.004**	-0.53	-0.72 to -0.20
	TL		$F_{1,5}$=0.17	0.699	-0.08	0.43 to 0.30	$F_{1,33}$=8.64	**0.006**	-0.51	-0.71 to -0.17
	HL:FL		$F_{1,5}$=0.00	0.998	<0.001	-0.36 to 0.36	$F_{1,33}$=16.85	**<0.001**	0.63	0.35 to 0.78
Rhamphochromis sp. chillingali	HW	7	$F_{1,5}$=0.79	0.415	-0.18	-0.50 to 0.23	$F_{1,29}$=0.75	0.393	-0.17	-0.50 to 0.22
	HL		$F_{1,5}$=0.03	0.862	-0.04	-0.39 to 0.34	$F_{1,29}$=0.18	0.674	-0.09	-0.43 to 0.30
	HV		$F_{1,5}$=0.48	0.521	-0.14	-0.47 to 0.26	$F_{1,29}$=0.55	0.464	-0.15	-0.48 to 0.24

Species	Sperm trait	n_t	Between-male effects				Within-male effects			
			$F_{(within, between)}$	p	r	95% CI	$F_{(within, between)}$	p	r	95% CI
	FL		$F_{1,5}$=5.09	0.074	-0.43	-0.69 to 0.04	$F_{1,29}$=5.17	**0.031**	-0.44	-0.67 to -0.05
	TL		$F_{1,5}$=5.34	0.069	-0.44	-0.70 to 0.04	$F_{1,29}$=5.19	**0.030**	-0.44	-0.67 to -0.05
	HL:FL		$F_{1,5}$=1.37	0.295	0.24	-0.20 to 0.55	$F_{1,29}$=0.37	0.547	0.13	-0.27 to 0.48
Nyassachrmis cf. microcephalus	HW	4	$F_{1,2}$=0.16	0.729	0.05	-0.22 to 0.3	$F_{1,28}$=0.05	0.823	-0.03	-0.28 to 0.23
	HL		$F_{1,2}$=0.65	0.506	0.12	-0.19 to 0.37	$F_{1,28}$=0.05	0.818	-0.03	-0.30 to 0.24
	HV		$F_{1,2}$=0.26	0.661	0.07	-0.21 to 0.32	$F_{1,28}$=0.19	0.669	-0.06	-0.31 to 0.21
	FL		$F_{1,2}$=0.53	0.541	0.09	-0.18 to 0.32	$F_{1,28}$=11.62	**0.002**	0.38	0.16 to 0.52
	TL		$F_{1,2}$=0.93	0.437	0.13	-0.17 to 0.37	$F_{1,28}$=10.55	**0.003**	0.38	0.15 to 0.55
	HL:FL		$F_{1,2}$=0.34	0.619	0.08	-0.20 to 0.32	$F_{1,28}$=4.07	0.053	-0.26	-0.45 to <0.001

3.4. Discussion

The results presented here did not indicate any evidence that sperm traits in the 33 species of cichlid investigated have evolved according to the phylogenetic tree structure. This suggests that closely related species were no more or less likely to have similar sperm traits than species which were less related. The cichlids found in the Lake Malawi catchment are mainly representatives of the Haplochromine tribe (Salzburger et al. 2005; Turner 2007), with the similarity in sperm size across species likely reflecting this shared ancestry (Barraclough et al. 1998). Whilst sperm length in mammals has been shown to have strong phylogenetic dependence (Gage and Freckleton 2003), the comparatively close ancestry of these cichlids appears to remove any such dependence. Another explanation could be that the similarity of sperm traits reflects the same point of fertilisation (Balshine et al. 2001).

When compared across species, the lack of correlation in sperm traits is consistent with findings from a phylogenetically-controlled study of cichlids from Lake Tanganyika, where no overall links between sperm size and velocity were found (Fitzpatrick and Balshine 2009). Variation in VAP more than doubles from the fastest (*Metriaclima kingsizei* Nkhata Bay, 113μm/s) to the slowest sperm (*Astatotilapia calliptera* Rovuma, 55μm/s), with very little variation in total sperm length (20μm and 21μm respectively, see Appendix 2). This suggests that factors other than the size of morphological components are important in dictating sperm velocity.

When intra-male variation was considered, however, correlations were found between sperm size and velocity, indicating that correlations within-males may be lost at the species level. The lack of a correlation between sperm length and velocity across species is in contradiction to sperm competition theory, but is in accordance with many empirical studies which have also failed to find a link between morphology and velocity of fish sperm (Burness et al. 2004; Locatello et al. 2007; Fitzpatrick et al. 2007). These results therefore highlight the importance of considering the level at which analysis takes place, implying that links may be found across more species than currently indicated in the literature.

3.4.1. Within-male variation reveals functional links

The assumed links between sperm velocity and length (Gomendio and Roldan 1991) received ambiguous support in empirical studies (see Table 1.1). Of the five species for which there was sufficient data to account for within-male variation, links between sperm morphology and velocity were found in four species. Furthermore, on all but one occasion within-male effects were stronger than between-male effects, which were mostly absent.

Flagellum length and total sperm length were consistently significantly correlated with velocity over four of the five species analysed whilst accounting for intra-male variation. Overall, nine significant correlations were found within-males, with only two significant correlations found between-males. These results support those found by Fitzpatrick at el. (2010) in sea urchins, where within-male correlations were found whilst between-male correlations were absent or correlations were weaker.

3.4.2. Direction of correlation alters between species

It is perhaps interesting to note that the direction of the correlation between sperm morphology and velocity is not consistent across species. The sister-species analysed from mtDNA clades *Astatotilapia calliptera* and *Rhamphochromis* have all evolved in different habitats after their divergence from a common ancestor, and reveal negative correlation between sperm length and swimming velocity. However, a positive correlation was found for the *Nyassachrmis cf. microcephalus*data. *Nyassachrmis cf. microcephalus*males build elaborate bowers out of sand, which they use to attract females. However, males initiate courtship away from the bower, suggesting that females do not use the bower as the primary indicator of male fitness (Genner et al. 2008). Non territorial males potentially attempt sneak copulations in some bower building species, and, though this has not been directly reported for this species, sperm motility was found to be related to social status in *Aststotilapia burtoni* (Kustan et al. 2011), and cichlids adopting sneak mating strategy have been found to have different sperm traits from other cichlid species (Fitzpatrick et al. 2007). A positive relationship between sperm length and velocity would be expected by

sperm competition theory, potentially suggesting dominant *Nyassachromis cf. microcephalus* males do experience competition from sneaker males. Whilst a larger sample size and extensive behavioral studies would be required to prove sperm competition via sneaker males, it is an interesting difference between the species.

3.4.3. Functional links between sperm morphology and swimming speed

There is little theoretical basis to expect flagellum length to be related to velocity at the microscopic scale of spermatozoa; it is wave length, wave amplitude, and wave speed that have been identified as having the greatest effect on the swimming velocity of spermatozoa (Gray and Hancock 1955). In human sperm, for example, at a constant flagella length, increases in swimming velocity were related to variation in wave speed and wave length (Smith et al. 2009). Despite this, traditional assumptions in sperm biology have related sperm length to velocity on the basis that longer sperm swim faster (Gomendio and Roldan 1991).

The assumption that sperm are swimming at optimal speed when first activated and when measurement of velocity is taken may not be valid for all species. For example, it has been found that the sperm of freshwater fish can exhibit low initial swimming velocity (Lahnsteiner and Weismann 1999). The present study aimed to link morphology and velocity, but it was not possible to record individual sperm throughout their activation, nor was it possible to record the entire activation period of an ejaculate in a manner that would allow analysis of longevity to be performed. However, sperm from some species did remain motile for over 7mins (personal observation). Sperm motility in fish has been correlated with fertility, suggesting that as long as fish sperm remain motile they are capable of fertilisation (Cosson 2004).

3.4.4.Mode of fertilisation and polyandry

The sperm traits of the 33 species analysed here revealed little morphological variation with which to identify alternate modes of fertilisation. However,

extrapolation from data gathered across a range of cichlids from Lake Tanganyika (Balshine et al. 2001) is tentatively possible. The total length of spermatozoa from all species in the dataset presented for this chapter fit in the range of data Balshine et al. (2001) gathered for the fertilisation point to be within the buccal cavity. The validity of this comparison across cichlid radiations from different lakes requires further investigation to explicitly examine mode of fertilisation in combination with level of sperm competition as sperm length has also been related to level of sperm competition (Balshine et al. 2001; Fitzpatrick and Balshine 2009). Without data on both aspects of reproductive biology the mode of fertilisation cannot be explicitly correlated with sperm length.

Although polyandry is considered to be widespread in maternaly mouthbrooding cichlids (Fryer and Iles 1972; Konings 2007), and is reflected in high levels of mixed paternity in some sand-dwelling species (Kellogg et al. 1995), not all species produce offspring from multiple males. Genner et al. (2007b) found no evidence of mixed paternity in laboratory mate preference trials involving *R.longiceps* and *R.'chilingali'* and only 7% of *Astatotilapia burtoni* broods were found to have multiple paternity (Theis et al. 2012) when females were presented with a choice of males with differing ornamentation (anal fin egg spots). Multiple paternity has been found in *Ophthalmotilapia ventralis* (Immler and Taborsky 2009) a lekking, mouthbrooding species which has been found to coat sperm in a sticky mucous apparently to increase sperm longevity inside the buccal cavity (Haesler 2007 PhD thesis; Immler and Taborsky 2009). There was no evidence of a mucous coating on the sperm across any of the species examined as part of the data gathered for this chapter. In cichlids which exhibit maternal mouthbrooding, multiple mating could increase offspring quality (Parker and Kornfield 1996) but a mechanism to facilitate longevity of sperm would be required for sperm competition to occur.

3.5. Conclusion

Pre-mating sexual selection is important in the speciation of cichlids (Seehausen 2006), which could occur via sperm competition (Kocher 2004). However, the extent of sperm competition has not been established in the species of cichlids

used here. It is important to note that mixed paternity does not equate to sperm competition. As female mouthbrooding cichlids only lay a proportion of the brood with any one male, it is quite feasible that multiple paternity can occur in the absence of sperm competition. The longevity of sperm therefore requires further detailed, species-specific work if the potential for sperm competition to influence sperm evolution is to be discussed. Increased sperm longevity appears to be achievable via a mucus secreted over the sperm, for example by the male *Ophthalmotilapia ventralis* (Haesler et al. 2011). However, there was no evidence of a mucus coating found for any of the species used in the present study. Closer investigation of the point of fertilisation and the potential for sperm competition in these cichlids would be beneficial. Multiple paternity does not *ipso facto* indicate that criteria for sperm competition are being met, as females lay fresh eggs with each male they spawn with (Kellogg et al. 1995). Additionally, sperm from a previous male may no longer be viable to compete with sperm from subsequent males.

A relatively small sample size, both of males and individual sperm, was collected here for most species. It is possible that a larger sample size could reveal a more consistent relationship for sperm morphology to velocity. The associations seen for the species with a larger sample size did indicate significant relationships between morphology and velocity of sperm cells, as the increased sample size provided the opportunity to apply mixed effect model analysis.

A more detailed investigation into the fine structure of cichlid sperm is required, firstly to establish whether there are ribbons along the flagella and, secondly, if ribbons are present, then to establish their functional significance. Knowledge of the function of flagella ribbons may clarify links between sperm morphology and velocity, and would certainly allow for greater confidence when comparing traits across species which do and do not have ribbons along the flagella. Flagella beating can be affected by many aspects of the fine structure of sperm (Lahnsteiner and Patzner 2008), and the diversity and function of sperm form therefore requires further detailed research to extrapolate the relative contribution the elements of sperm morphology have on swimming velocity.

By far the most important finding revealed by these data is that correlations between sperm size and velocity can be found if intra-male variation is accounted for in the analysis. Therefore, unlike traditional methods of taking mean ejaculate information, measurements of individual sperm are required in order to accurately match morphology data with velocity data. The potential to find within-male correlations of sperm morphology and velocity will be developed in Chapter 4.

Chapter Four: Relationships between sperm length and speed differ among three internally and three externally fertilizing species

Submitted as an original article to Evolution

Julia L. Simpson[1], John L. Fitzpatrick[2], Jonathan P. Evans[2], Leigh W. Simmons[2] and Stuart Humphries[1]

1. Physical Ecology Group, Department of Biological Sciences, The University of Hull, Hull, HU6 7RX, UK
2. Centre for Evolutionary Biology, School of Animal Biology (M092), The University of Western Australia, WA 6009, Australia

Abstract

It is often assumed that longer sperm, by virtue of their increased swimming speed, have a fertilization advantage over shorter sperm when in competition to fertilize eggs. However, there is surprisingly little evidence for a positive correlation between sperm length and speed. In addition, the mode of fertilization (internal vs. external fertilization) is likely to determine the selective forces driving the evolution of sperm length and speed, with possible implications for length-speed relationships across taxa. Here we provide a prospective analysis of the relationships between sperm length and sperm speed across a broad range of species, including three internally fertilizing (humans Homo sapiens; emus *Dromaius novaehollandiae*; and guppies *Poecilia reticulata*) and three externally fertilizing species (rainbowfish *Melanotaenia australis*; mussels *Mytilus galloprovincialis*; and frogs *Crinia georgiana*). Importantly, our methods include the prescribed approach of accounting for within-male variation when assessing these relationships among species. Our results reveal significant associations between sperm flagellum length and speed in the majority of our species, but also that the sign of this relationship differs between internal and external fertilizers. We suggest that such relationships may be prevalent across many more species than previously thought.

4.1. Introduction

Sperm competition, where sperm from two or more males compete to fertilize a female's eggs (Parker 1970), and cryptic female choice, where females influence the outcome of this competition (Eberhard 1996), are powerful selective forces influencing sperm evolution (Birkhead and Møller 1998). Sperm competition is generally thought to select for an increase in the length of sperm, with the assumption that there is a relationship between sperm length and speed (Gomendio and Roldan 1991). In the context of sperm competition, increased sperm swimming speed is expected to be selectively advantageous due to the enhanced success of relatively fast sperm (Gomendio and Roldan 1991; Ball and Parker 1996). However, while sperm swimming speed positively predicts competitive fertilization success in many externally and internally fertilizing species (reviewed by Simmons and Fitzpatrick 2012), evidence of a widespread positive association between sperm length and speed is limited (Snook 2005; Humphries et al. 2008). Possible reasons for this lack of evidence for associations between sperm length and speed include the fact that most studies have not considered (1) the environment in which fertilization takes place (internal vs. external fertilization: Eberhard 2009), (2) the complexities surrounding sperm hydrodynamics (Humphries et al. 2008), and (3) the extensive within-male variation in sperm traits that typically characterises ejaculates, and can obscure length-speed relationships at the intra-specific level (Fitzpatrick et al. 2010; Gadelha et al. 2010). Consideration of these factors is important when assessing functional links between sperm length and sperm swimming speed (Humphries et al. 2008).

The contrasting environments in which fertilization takes place, broadly categorised as internal and external to the female's body, have the potential to exert strong selective pressures on the functional morphology of sperm (Parker 1993; Ball and Parker 1996). In internally fertilizing species, sperm often have to travel relatively long distances through a viscous mucus (Kirkman-Brown and Smith 2011), and often complex female reproductive tract (Gaffney et al. 2011). In such species, a key factor determining sperm swimming speed can include hydrodynamic 'wall effects' (Winet 1973; Cosson et al. 2003), which influence the sperm's speed near surfaces of the female's reproductive tract. Wall effects may therefore be an important environmental factor influencing the selection of sperm

phenotypes that are better suited to manoeuvring through such a constrained environment. Such physical constraints are absent in the environment faced by sperm from externally fertilizing species.

Movement in the sperm microenvironment is dominated by viscosity and hydrodynamic forces that are very different to those experienced by larger organisms (Humphries et al. 2008). At the microenvironment scale, streamlining is irrelevant and so the adaptive reasons for a particular shape are much less clear than for large organisms (Humphries et al. 2008). As there is currently little empirical evidence linking sperm length to speed using single length measures, one suggestion is that selection may not be acting independently on individual elements of sperm morphology (Humphries et al. 2008). Thus, just as a car's speed is in part determined by its mass and engine size, so a sperm's speed might be determined by the length of its flagellum ('engine+wheels') and the size of its head ('mass' or drag). Thus, the specifics of the sperm microenvironment have led to the suggestion that a ratio of head length to flagellum length could be a more accurate predictor of sperm speed (Humphries et al. 2008).

Intra-male variation in sperm traits could also mask length-speed relationships, as within-male variation is hidden when assigning average values to sperm length and speed. In contrast to the traditional methods for assessing sperm length-speed relationships, where mean values of speed and length of different groups of sperm are used, Fitzpatrick et al.(2010) highlighted the importance of accounting for intra-male variation by matching sperm speed and length measurements to individual cells. By measuring multiple morphological traits for individual sperm cells and accounting for intra-male variation, length-speed relationships might be revealed.

To date, no definitive patterns have been reported regarding fertilization system and sperm length across taxa (Pitnick et al. 2009a). Here we use six species – three internal and three external–to assess relationships between sperm length and speed. We use the recently developed approach that assesses length and speed of individual sperm cells within a single ejaculate (Fitzpatrick et al. 2010), measuring various sperm components including ratios of head length to flagellum length (Humphries et al. 2008). Our analysis of length-speed relationships for internal and externally fertilizing species spans a broad taxonomic range (a mollusc, two

fishes, an amphibian, a bird and two mammals) and reveals that whilst fertilization environment has a contradictory effect on correlations between sperm length-speed traits there are some general trends among species in relation to internal or external fertilization.

4.2. Methods

The methods employed to collect samples for the current analysis reflect the best available practice for the individual species concerned, some of which was recorded prior to this study(Humans: Kilgallon and Simmons 2005; Frogs: Dziminski et al. 2009; Emu: Sood et al. 2011; 2011). Digital video recordings of activated sperm samples were collected from three internally fertilizing (humans *Homo sapiens*, guppies *Poecilia reticulata* and emu *Dromaius novaehollandiae*) and three externally fertilizing species (mussels *Mytilus galloprovincialis*, rainbowfish *Melanotaenia australis* and frogs *Crinia georgiana*). From the videos, sperm swimming speed and the different components of sperm length were measured from the same individual sperm cells using the species-specific methods outlined below. Videos were considered of sufficient quality when they showed clearly intact, motile sperm swimming with no evidence of bulk water flow in the sample (Wilson-Leedy and Ingermann 2006). Any videos not meeting these requirements were not analyzed.

4.2.1. Internally fertilizing species

i) Human – *Homo sapiens*

Male volunteers from the University of Western Australia were recruited to donate semen samples (for details see Kilgallon and Simmons 2005). Samples were collected and analysed in accordance with protocols from the World Health Organization (1999). Briefly, 10µl of semen were mounted on a microscope slide under a coverslip, and viewed at 400x magnification, at a temperature of 23^{o}C. Video recordings were made on a Sony Videocassette recorder via a camera mounted onto a compound microscope. Of 52 male subjects, the quality of video recording for 29 individuals met the above criteria for analysis with 3-6 individual sperm measured per male.

ii) Emu – D*romaius novaehollandiae*

Digital video recording of motile emu sperm were taken using fresh emu semen collected using an artificial cloaca (for details see Sood et al. 2011). For video-recording, the concentration of spermatozoa was adjusted to 17–20 × 10^6 sperm/mL with Dulbecco's Modified Eagle's Medium containing 0.03% BSA at 37°C. For each sample, videos were recorded for 10s each using 10× phase

contrast objective (Olympus Australia Pty. Ltd.) and a digital camera (Olympus DP 71/25, Olympus Australia Pty Ltd). The videos for nine males had sufficient resolution to measure lengths and speed. Ten individual sperm were measured per male.

iii) Guppy - *Poecilia reticulata*

Captive-bred guppies used in this study were descendants of fish caught in 2006 from a feral population in the Alligator Creek River in Queensland, Australia. Sexually mature males were anesthetised and placed on a glass slide under a dissecting microscope (see Evans 2009). The ventral side of each male was gently dried before 60 µl of an extender medium (207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.49 mM MgCl2, 0.41 mM MgSO4, and 10 mM Tris with pH 7.5) was added to the base of the male's gonopodium. This extender solution is designed to ensure sperm bundles stay intact and the sperm within remain dormant (Gardiner 1978). Light pressure was then applied to the male's abdomen to release the sperm bundles into the extender medium (Matthews et al. 1997). The sperm were activated with 60 µl of a 150mM KCL solution (Billard and Cosson 1990) containing 2mg/l bovine serum albumin (BSA) to prevent sperm from sticking to the glass slide (Pitcher et al. 2007). From each activated sample, individual sperm bundles were taken up in 3 µl of solution and placed into individual wells of 12-cell multi test slide (MP Biomedicals, Aurora, OH, USA). Slides and cover slips had been coated with 1% polyvinyl alcohol (PVA) to further prevent sperm from sticking to the slides (Wilson-Leedy and Ingermann 2007). Recordings of motile sperm were taken using phase contrast microscope (Olympus BX41) at 97 frames per second (fps) under 400× magnification for one second using a Prosilica EC-650 digital camera (resolution 640x480 px) and Norpix StreamPix 3.4 image capture software. Several one-second clips were taken in quick succession. Data were gathered and analysed from 18 males, with 3-10 individual spermatozoa measured for speed and lengths per male.

4.2.2. Externally fertilizing species

iv) Rainbowfish – *Melanotaenia australis*

Rainbowfish used in this study were captured in 2006 from a wild population in the Fortescue River near Wittenoom, Western Australia. Fish were returned to the lab and maintained in mixed-sex aquaria. Sexually mature males were taken from

stock aquaria, given a lethal dose of anaesthetic (clove oil), and the testes were removed. Dissected testes were used in this analysis as sperm could not be manually stripped from mature males. Testes were macerated and a ~1 µl sample of flowing sperm was activated through dilution with 0.5 ml distilled water (for similar methods applied to other fish see Fitzpatrick et al. 2007). A 2µl sample of the activated sperm was immediately placed on a 12-cell multi test slide for recording motility, which was assessed using the video collection methods described above for guppies. From the 31 males sampled, motility and length data could be gathered from 14 males, and from these samples between three and 10 individual sperm were measured per male.

v) Mussel – *Mytilus galloprovincialis*

Mussels were collected from the Claremont Jetty, Western Australia, in July 2010, returned to the laboratory and maintained in seawater at 18-20°C. After collection, approximately 100 mussels were given a heat shock by placing them in a warm water bath (30°C) to stimulate gamete release. Following the onset of gamete release, males were removed from the warm water bath, washed in clean seawater to prevent contamination from other gametes, and placed in individual containers with 250ml of sea water (following methods in Fitzpatrick et al. 2012). Males continued to release sperm in their individual containers, and fresh sperm was collected from individual males. A 2µl sample from each male was placed on a 12-cell multi test slide for recording motility, which was assessed using the video collection methods described above for guppies. Data were collected from 20 individual males. For each male, length components and speed of 7-21 individual spermatozoa were recorded.

vi) Quacking frog – *Crinia georgiana*

Frogs were collected during the winter breeding season from populations in Western Australia. Sperm extractions were carried out following the methods of Dziminski et al. (2009). Briefly, male frogs were killed (double pithing) and their testis were removed and crushed in Petri dishes containing chilled simplified amphibian ringer (SAR) to prevent activation of the sperm (Dziminski et al. 2009). Sperm were activated by the addition of fresh pond water, and motility was recorded using a Leica DICOMAR 3CCD digital video recorder mounted on a Leica DME compound microscope, using 100 X magnification at 37°C (Dziminski et al.

2009). Sperm from 16 individual males could be measured adequately from the frog videos, with 3-10 sperm per male.

4.2.3. Analysis of videos

Where appropriate, digital videos of motile sperm were edited into 1-2 second clips using QuickTime Pro (v 10.0), then converted into image stacks so that sperm motility could be analysed using NIH ImageJ (v. 1.42q) with the computer-assisted sperm analysis (CASA) plugin (Wilson-Leedy and Ingermann 2007). Once imported into ImageJ, image stacks were prepared by applying the 'find edges' and 'threshold' functions before isolating the focal cell and running CASA. Because only one cell was analysed at a time, all other sperm were cleared from the clip by isolating the focal cell and using the 'clear outside' function to remove all other cells from the clip. Once all other cells and any background noise had been removed from around the focal spermatozoa, the speed of the isolated cell could be recorded using CASA. Three sperm speed measurements were recorded: average path velocity (VAP), curvilinear velocity (VCL) and straight line velocity (VSL). Principal component analysis indicated all speed measures were co-linear (see Table 4A.2Appendix Four). Therefore for clarity we only report VAP here (see Appendix Four Table 4A.3 for details of all speed measures). To measure sperm length, the zoom function was used to magnify the focal cell (up to 200%) with the 'brightness' and 'contrast' altered as necessary to allow the head and flagellum to be seen clearly. A minimum of three spermatozoa per male were measured at three points along their path (at the start, middle and towards the end). Limited focal depths mean that as the flagellum moves, its tip might not be visible in each frame. An average of the three measures were used for each cell in all analyses.

Specifically, three components of sperm length were measured; head length (HL) head width (HW) and flagellum length (FL). From these measurements it was possible to estimate total sperm length (TL = HL + FL) and head to flagellum ratio (HL:FL = HL/FL). Head shape can also be an important determinant of swimming trajectory (Gillies et al. 2009; Gadelha et al. 2010) so we therefore calculated head volume (HV) for all species based on the assumption that the head was an ellipsoid (Humphries et al. 2008) (see Appendix 4 pg 215 for more details). HV was not

calculated for mussels as limitations in video resolution meant it was not possible to measure HW for this species.

4.2.4. Statistical Analyses

We explored the relationships between sperm length (composite measures, ratios and individual components) and sperm swimming speed using within-subject centering (Van de Pol and Wright 2009) which enables the separation of within- from between-male effects when assessing sperm-length relationships as outlined in the previous chapter (3.2.6. see also Fitzpatrick et al. 2010).

4.5. Results

The mixed-effects models revealed relationships between sperm length and speed, but these depended on whether relationships were assessed at the between- or within-male level. Significant correlations between length components and speed were found in both internally and externally fertilizing species in four of the six species examined in this study (Table 1, also see Appendix 4 Table 4A.3 for all results). Among these length components, sperm length (either total length or flagellum length) was the most consistent trait exhibiting a correlation with speed. Specifically, VAP and flagellum length exhibited significant correlations in humans, emus, rainbowfish, and mussels (Figure 1, Table 1). Neither guppies nor frogs exhibited any significant correlations between any measure of sperm length and sperm swimming speed (Figure 1, Table 1, Appendix 4 Table 4A.3). Interestingly, the correlation between flagellum length and speed appeared to differ between internal and external fertilizers, with negative correlations between flagellum length and speed in internally fertilizing species but positive correlations in externally fertilizing species. However, correlations between flagellum length and speed were absent in guppies (internal) and frogs (external). Overall within-male effects were stronger than between-male effects (Figure 2).

Table 4.1. Results for sperm length-VAP from mixed model centering allowing random effect intercepts for between and within male analysis. HW=head width, HL=head length, HV=head volume (no HV measure for mussel data), FL=flagella length, TL=total length and HL:FL=ratio of head length to flagellum length, n_t=total number of males. F = degrees of freedom within and between males. 95% confidence intervals (95% CI) calculated for effect size (r). Significant correlations (p=<0.05) in bold.

Species	Sperm trait	n_t	Between male effects				Within male effects			
			$F_{(within, between)}$	p	r	95% CI	$F_{(within, between)}$	p	r	95% CI
(a) internally fertilizing species										
Human	HW	29	$F_{1,27}$=2.58	0.120	-0.21	-0.44 to 0.06	$F_{1,79}$=19.63	**<0.001**	-0.51	-0.65 to -0.30
	HL		$F_{1,27}$=0.48	0.496	-0.10	-0.34 to 0.18	$F_{1,79}$=0.71	0.403	0.12	-0.15 to 0.36
	HV		$F_{1,27}$=2.60	0.118	-0.22	-0.44 to 0.06	$F_{1,79}$=16.69	**<0.001**	-0.49	-0.63 to -0.27
	FL		$F_{1,27}$=1.90	0.179	-0.19	-0.42 to 0.09	$F_{1,79}$=11.22	**0.001**	-0.42	-0.59 to -0.18
	TL		$F_{1,27}$=0.32	0.578	-0.08	-0.33 to 0.19	$F_{1,79}$=5.61	**0.020**	-0.31	-0.51 to -0.05
	HL:FL		$F_{1,27}$=0.09	0.766	-0.04	-0.30 to 0.22	$F_{1,79}$=1.98	0.164	0.19	-0.08 to 0.42
Emu	HW	9	$F_{1,7}$=5.95	**0.045**	0.27	0.01 to 0.47	$F_{1,80}$=13.27	**<0.001**	0.38	0.18 to 0.54
	HL		$F_{1,7}$=3.87	0.090	0.21	-0.03 to 0.41	$F_{1,80}$=45.52	**<0.001**	0.58	0.44 to 0.68
	HV		$F_{1,7}$=9.83	**0.017**	0.34	0.06 to 0.54	$F_{1,80}$=33.17	**<0.001**	0.55	0.38 to 0.66
	FL		$F_{1,7}$=0.54	0.487	-0.08	-0.28 to 0.14	$F_{1,80}$=7.89	**0.006**	-0.29	-0.46 to -0.09
	TL		$F_{1,7}$=0.14	0.717	0.04	-0.17 to 0.25	$F_{1,80}$=0.57	0.453	0.08	-0.13 to 0.28
	HL:FL		$F_{1,7}$=3.05	0.124	0.18	-0.05 to 0.38	$F_{1,80}$=55.98	**<0.001**	0.61	0.48 to 0.70
Guppy	HW	18	$F_{1,6}$=1.21	0.313	-0.14	-0.36 to 0.12	$F_{1,47}$=0.03	0.871	-0.02	-0.26 to 0.22

Species	Sperm trait	n_t	Between male effects				Within male effects			
			$F_{(within, between)}$	p	r	95% CI	$F_{(within, between)}$	p	r	95% CI
	HL		$F_{1,15}$=2.28	0.152	-0.16	-0.35 to 0.06	$F_{1,83}$=0.50	0.482	0.08	-0.14 to 0.28
	HV		$F_{1,6}$=0.78	0.412	-0.11	-0.33 to 0.14	$F_{1,47}$=1.10	0.299	0.13	-0.11 to 0.34
	FL		$F_{1,16}$=1.50	0.239	0.12	-0.08 to 0.31	$F_{1,95}$=0.15	0.696	-0.04	-0.23 to 0.16
	TL		$F_{1,16}$=0.05	0.828	-0.02	-0.21 to 0.17	$F_{1,95}$=0.46	0.500	-0.07	-0.25 to 0.13
	HL:FL		$F_{1,15}$=2.69	0.122	-0.18	-0.37 to 0.05	$F_{1,95}$=0.63	0.431	0.09	-0.13 to 0.29
(b) externally fertilizing species										
Mussel	HL	20	$F_{1,18}$=0.10	0.753	-0.02	-0.12 to 0.09	$F_{1,255}$=1.78	0.183	-0.07	-0.17 to 0.03
	FL		$F_{1,18}$=13.15	**0.002**	0.21	0.08 to 0.32	$F_{1,255}$=2.85	0.093	0.10	-0.02 to 0.21
	TL		$F_{1,18}$=14.16	**0.001**	0.22	0.08 to 0.33	$F_{1,255}$=2.66	0.104	0.10	-0.02 to 0.21
	HL:FL		$F_{1,18}$=5.60	**0.029**	-0.13	-0.24 to -0.01	$F_{1,255}$=4.85	**0.029**	-0.12	-0.23 to -0.01
Rainbowfish	HW	14	$F_{1,12}$=0.27	0.613	-0.05	-0.22 to 0.13	$F_{1,70}$=3.22	0.077	-0.16	-0.32 to 0.02
	HL		$F_{1,12}$=2.03	0.179	0.14	-0.06 to 0.31	$F_{1,70}$=3.98	**0.050**	-0.19	-0.35 to 0.00
	HV		$F_{1,12}$=<0.01	0.985	0.00	-0.18 to 0.17	$F_{1,70}$=4.00	**0.049**	-0.18	-0.33 to 0.00
	FL		$F_{1,12}$=8.26	**0.014**	0.29	0.06 to 0.45	$F_{1,70}$=7.54	**0.008**	0.27	0.08 to 0.43
	TL		$F_{1,12}$=9.11	**0.011**	0.30	0.07 to 0.47	$F_{1,70}$=5.83	**0.018**	0.25	0.04 to 0.41
	HL:FL		$F_{1,12}$=0.44	0.520	-0.06	-0.23 to 0.12	$F_{1,70}$=10.40	**0.002**	-0.27	-0.41 to -0.11
Frog	HW	16	$F_{1,14}$=0.94	0.348	-0.10	-0.29 to 0.11	$F_{1,68}$=0.13	0.725	0.04	-0.17 to 0.24
	HL		$F_{1,14}$=0.27	0.615	-0.05	-0.25 to 0.15	$F_{1,68}$=0.54	0.463	0.08	-0.13 to 0.27
	HV		$F_{1,14}$=0.86	0.371	-0.10	-0.29 to 0.11	$F_{1,68}$=0.29	0.595	0.06	-0.15 to 0.25

Species	Sperm trait	n_t	Between male effects				Within male effects			
			$F_{(within, between)}$	p	r	95% CI	$F_{(within, between)}$	p	r	95% CI
	FL		$F_{1,14}$=0.53	0.480	0.08	-0.13 to 0.27	$F_{1,68}$=1.72	0.194	0.14	-0.07 to 0.32
	TL		$F_{1,14}$=<0.01	0.961	0.01	-0.19 to 0.20	$F_{1,68}$=2.11	0.151	0.15	-0.05 to 0.33
	HL:FL		$F_{1,14}$=0.57	0.463	-0.08	-0.27 to 0.13	$F_{1,68}$=0.01	0.908	-0.01	-0.21 to 0.19

4.5.1. Internally fertilising species

i) Human

Within-males VAP was negatively correlated to all length measures except head length and head length-flagellum length ratio (Table 1, Figure 1). In contrast, there were no significant between-male correlations (Table 1).

ii) Emu

VAP and flagellum length were significantly negatively correlated within males, but not between males (Table 1, Figure 1). VAP was also significantly positively correlated with head length, head width, head volume and to the ratio of head length to flagellum length within males, but of these only head width and head volume were significantly correlated between males. There were no significant relationships between total sperm length and VAP when assessed either between or within males.

iii) Guppy

The guppy data did not reveal any significant correlations between length components and any speed measure (VAP or otherwise) either between or within males. (Table 1, Appendix 4 Table 4A.3).

4.5.2. Externally fertilising species

iv) Mussel

In mussels, the ratio of head length to flagellum length was significantly negatively correlated both within- and between males. However, both flagellum length and total length were significantly positively correlated with VAP between, but not within, males (Table 1, Figure 1). We detected no relationship between head length and VAP (Table 1).

v) Rainbowfish

In rainbowfish, flagellum length and total length were significantly positively correlated with VAP at both levels of analysis (within and between males; Table 1, Figure 1). We also detected a significant positive correlation between the ratio of head length to flagellum length and VAP, although this relationship was only apparent for the within-male relationships (Table 1). Head length and head

volume were significantly negatively correlated with VAP within but not between males (Table 1).

vi) Frog

In frogs, we detected no significant relationships between any of the sperm length measures and VAP (Table 1), or any other speed measure (Table 4A.3 Appendix 4).

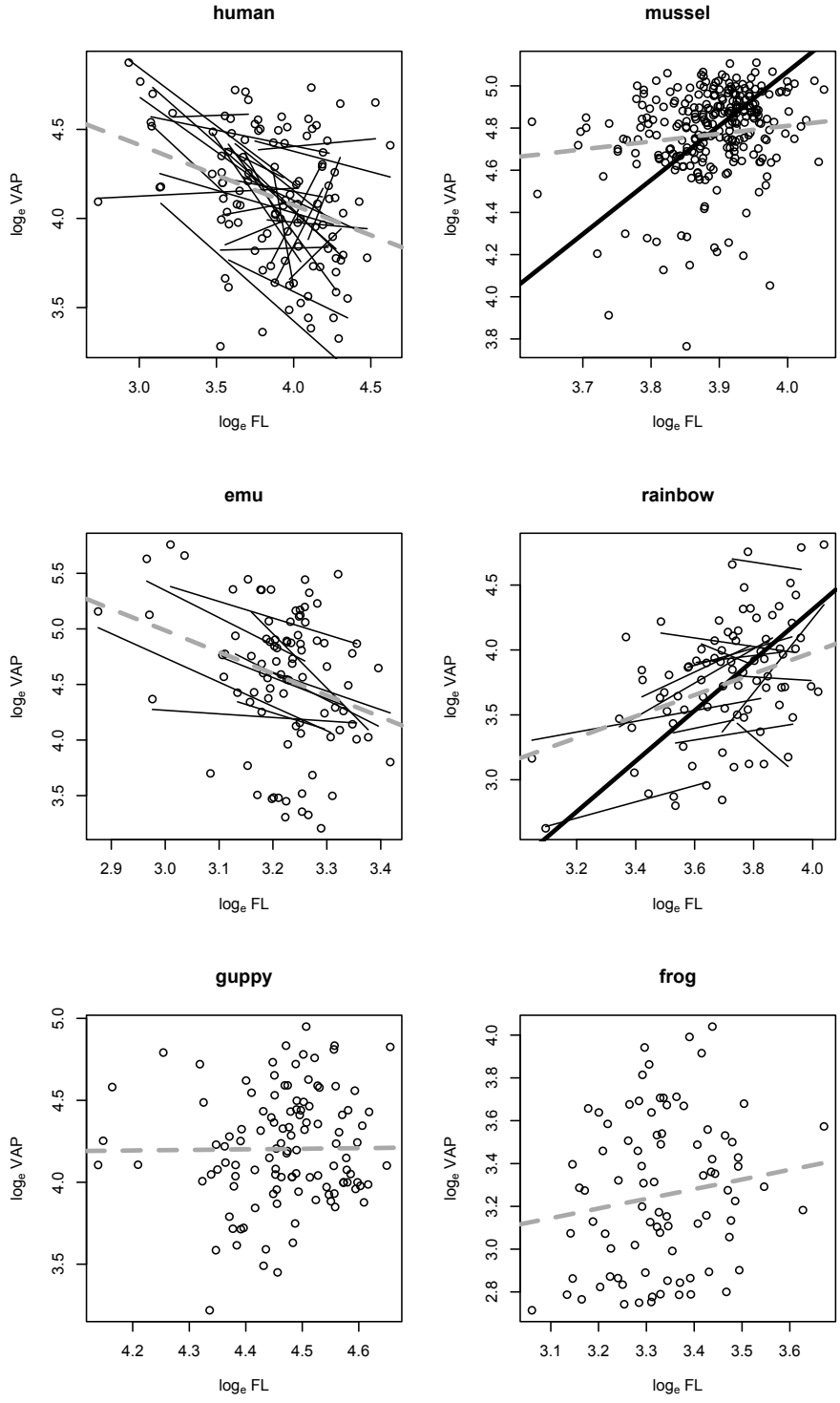


Figure 4.1. Relationships between sperm flagellum length (FL) and velocity (VAP) within and between males across internally (left column) and externally (right column) fertilising species. Thick black lines describe significant between-male effects from the mixed effect model, while thin solid lines depict individual within-male slopes from linear regressions as a visual guide to significant within-male effect. The dashed grey line depicts the estimate from a standard mixed model of the effect of the length measure on the speed measure (as a guide only).

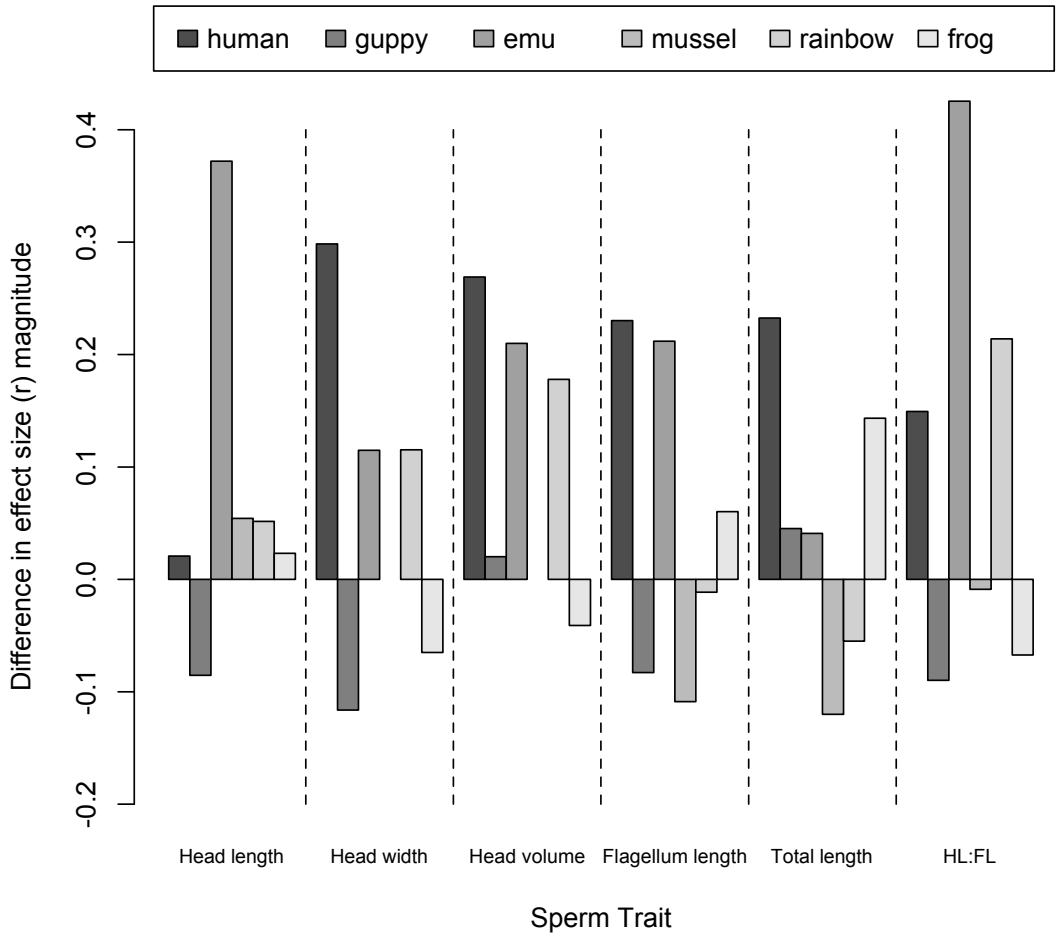


Figure 4.2. Differences in absolute effect size (within male – between male) for the relationship between sperm length and speed (VAP) for each sperm trait analyzed in each of the species examined. Positive values indicate that the effect size for the relationship between sperm length and speed was greater in the within male analyses and negative values indicate larger effect sizes in the between male analyses. These data are for illustrative purposes only: effect sizes and statistical models are presented in full in Table 1.

4.6. Discussion

In applying a new method to the analysis of sperm form and function, our results provide fresh insights into the presence of length-speed correlations in sperm. Specifically, our findings of correlations between various sperm morphological components and sperm swimming speed in four of the six species examined provides further support for the idea that sperm length-speed correlations are more prevalent than suggested by current evidence (reviewed by Humphries et al. 2008). Our ability to more readily detect sperm length-speed correlations likely stems from the methodology employed here, which accounts for the often-high levels of intra-male variance in sperm traits. Underscoring this point, we found that within-male effects, which account for intra-male variance in sperm traits, were generally stronger than between-male effects (Figure 2), the latter of which is typically used to assess sperm length-speed correlations. Moreover, our finding that the direction of sperm length-speed correlations differs between internally and externally fertilizing species suggest that these relationships are influenced by the microenvironment in which sperm operate.

4.6.1. Assessing the prevalence of sperm length-speed relationships

Despite the presumed functional link between sperm size and swimming speed (Gomendio and Roldan 1991), detecting correlations between sperm length and speed at the intra-specific level has proven problematic. For example, in a recent review Humphries et al. (2008) highlighted that sperm length-speed correlations were only evident in two of nine species examined at the time. Although several recent studies have subsequently demonstrated sperm length-speed correlations (e.g. Mossman et al. 2009; Helfenstein et al. 2009; Lüpold et al. 2009; Fitzpatrick et al. 2010; Firman and Simmons 2010; Lüpold et al. 2012) the overall relationship between sperm size and speed remains unclear. This ambiguity in the link between sperm size and speed is illustrated by three recent studies of zebra finches (Mossman et al. 2009), blackbirds (Lupold et al. 2009) and mussels (Fitzpatrick et al. 2012), where correlations between sperm size and speed were either weak or non-existent despite extensive sampling efforts (i.e. n>100 individuals). Thus, the primary issue highlighted by Humphries et al. (2008)

remains, as sperm morphology is either not, or weakly, linked with sperm swimming speed in the majority of species examined to date. Therefore, our findings that sperm length-speed relationships are evident where we accounted for intra-male variance in sperm traits in four of the six species examined in this study suggests that relationships between sperm size and speed may be more prevalent than currently thought. As sperm swimming speed is an important determinant of competitive fertilization success in many species (reviewed by Simmons and Fitzpatrick 2012) these findings highlight how selection on sperm morphology can influence male competitive fertilization success.

4.6.2. Flagellum/total length-speed relationships differ in internal and external fertilizers

The traditional view of how sperm competition shapes sperm length and speed rests on the assumption that sperm with a longer flagellum are capable of achieving greater swimming speeds (Gomendio and Roldan 1991). We found evidence to support this view in externally fertilizing mussels and rainbowfish where positive correlations were detected between flagellum/total sperm length and sperm swimming speed when assessed between males (in mussels and rainbowfish) and within males (in rainbowfish). However, the direction of the significant correlations between sperm flagellum/total length and swimming speed detected in this study differed for internally and externally fertilizing species. In internally fertilizing humans and emus, within male effects revealed that sperm with longer flagellum lengths (in humans and emus) and total lengths (in humans) swam more slowly than shorter sperm produced in the same ejaculate. These unexpected results provide some of the first evidence that sperm length and speed are *negatively* correlated in some species and to our knowledge there is no theoretical basis for predicting such relationships. The only other evidence of a negative relationship between sperm length and speed that we are aware of is from a recent study of *Drosophila melanogaster*, another internally fertilizing species, where Lüpold et al. (2012) demonstrated both that longer sperm swim more slowly and that long and slow sperm experience a fertilization advantage in competitive matings.

While the negative relationship between sperm length and speed in internally fertilizing species was unanticipated, it seems probable that such a relationship could be linked to how sperm operate in the microenvironment of the female reproductive tract. In particular, the physics of motion in viscoelastic fluids, such as those fluids found in the female's reproductive tract, can influence sperm performance as the elasticity in the medium introduces new forces acting on a moving flagellum as these media push back on the sperm in a way that other liquids do not (Lauga 2007). The complex microstructure of mucus in the female reproductive tract has been show to influence flagella waveform and sperm swimming trajectory (Lauga 2007). The relationship between sperm flagella beat frequency, swimming speed and reproductive tract mucus remains to be fully understood, but it is clear that relationships are complex and may be species-specific. While there is ample evidence that the female's reproductive tract influences the evolution of sperm morphology (Pitnick et al. 2009a), our results suggest that the underlying relationship between sperm morphology and performance is also influenced by the females' reproductive tract. Thus, provided negative correlations between sperm size and speed are prevalent in internally fertilizing species, the influence of the female's reproductive tract on sperm may help to explain the short sperm advantage observed in competitive matings in other internally fertilizing species (including mice, dung beetles, crickets and fruit flies; reviewed by Simmons and Fitzpatrick 2012).

4.6.3. Sperm head morphology and sperm swimming speed

Sperm head shape is also important in governing sperm hydrodynamics (Humphries et al. 2008). For example, head morphology has been linked to lateral head movement in internally fertilizing species (Kirkman-Brown and Smith 2011) as short sperm heads have larger lateral movement when compared to longer heads of the same volume (Gillies et al. 2009). In the current study we report that sperm head morphology exhibited both significantly positive and negative correlations with sperm swimming speed in humans, emus and rainbowfish. For both human and rainbowfish, sperm head morphologies were significantly negatively related to speed when assessed within-males while head

morphology was positively related with speed both within and between males in emu. Thus, sperm head morphology does not appear to differ in a consistent manner between external and internal fertilizing species.

In three species the ratio of sperm head length to flagellum length was significantly correlated with speed when assessed within males (in emus, mussels and rainbowfish) and between males (in mussels). Although previous studies have also found relationships between sperm head to flagellum ratios in internally fertilizing birds (Mossman et al. 2009; Helfenstein et al. 2010), the results of the present study suggest that a link between speed and the ratio of head length to flagellum length was more evident in externally than internally fertilizing species, in keeping with theoretical perditions (Humphries et al. 2008). However, as argued by Humphries et al. (2008), the use of this ratio of length components was not explicitly better than single length measures at predicting swimming speed in the internally fertilizing species.

4.6.4.The importance of accounting for intra-male variance in sperm traits

Correlations between sperm length and speed were more prevalent when assessed at the within male level: 15 significant within-male correlations were detected compared with only seven between-male correlations. This bias of sperm length-speed correlations within males, compared to between males, confirms the finding of Fitzpatrick et al. (2010) who found within-male correlations for different speed measures to be more prevalent than between male correlations in sea urchin (*Heliocidaris erythrogramma*). By avoiding the use of mean speed and length measures taken from different subpopulations of sperm within the ejaculate, both our study and that of Fitzpatrick et al. (2010), demonstrate that substantial correlations do exist. Thus, our results generated with mixed model centering underscore the need to use appropriate levels of analysis to detect length-speed correlations.

The within-subject centering approach taken here also allows us to draw inferences on the general pattern of length-speed relationships within as compared to between males. The random slopes and intercepts model (equation

4 in van de Pol and Wright 2009) is consistently unable to explain any more variation than the simpler random intercepts model (equation 2 in van de Pol and Wright 2009) for any significant trait-pairs. In addition, equation 3 of van de Pol and Wright (2009, results not shown) yields little evidence that slopes differ in their magnitude or sign. The only exceptions are for flagellum length and total length in mussels where the indication is that the slopes differ between males, and these are also the only two instances were we find between-male effects but no within-male effect (Table 1). Taken together, these results indicate that in all the species where a relationship was found, sperm length-speed relationship share a common slope and differences are only apparent in the magnitude of speed for a given length. Thus, the way in which speed is correlated to length is essentially invariant between males of a species, and only in mussels is there any indication that within-male relationships are different from the between- male ones. While such a pattern is implicit in the majority of studies of sperm speed, this is the first empirical evidence of the effect and suggests a lack of different selection pressures at different levels of organisation (within males versus within species).

4.6.5. Frogs and guppies: species-specific considerations

Sperm length was not correlated with swimming speed in either frogs or guppies. The lack of clear relationships between sperm length and speed in frogs may stem from differences in sperm function and fertilization dynamics in this species. Frog sperm differ dramatically from the 'typical' sperm morphology and swimming mechanism seen in the other species sampled here. In the myobatrachid frog studied here, sperm are propelled by an undulating membrane that is supported by a longitudinal axial fiber that stretches from the base of the head to the tip of the 'tail' (Jamieson et al. 1993). We might therefore not necessarily expect any length-speed relationship in frog sperm to be like that of other species with more typical sperm morphology and swimming mechanism. Additionally, frog sperm must penetrate a highly viscous jelly surrounding frog eggs, which likely means that the selective pressures on sperm morphology differ in frogs compared with other externally fertilizing species, such as mussels and rainbowfish. Indeed, frogs that produce relatively slow

swimming sperm have increased fertilization success compared to those producing faster sperm (Dziminski et al. 2009). In contrast, guppy sperm conforms with the 'typical' sperm morphology and it is therefore unclear why sperm length-speed correlations were absent in this species. We did not find correlations between head length and speed in guppy sperm as described by Pitcher et al. (2007) where head length was found to be related to speed. Yet, in accordance with our results Pitcher et al. (2007) also did not find correlations between flagellum length and speed in guppies. The sample size in the present study (3-10 individual sperm measured per male from 18 males) may also limit interpretation of the data. Considering the extended periods for which female guppies can store sperm (Houde 1997) and the concomitant trade-offs between speed and longevity which are becoming apparent (Higginson et al. 2012; Smith 2012), incorporating a more complete view of interaction between sperm performance and the reproductive environment (i.e. female storage) will help future analysis to characterise how selection acts on sperm traits.

4.7.Conclusion

The difficulties in detecting sperm length-speed correlations have been vexing for evolutionary biologists as a growing number of comparative studies have demonstrated that longer sperm swim faster and that longer and faster sperm, which presumably offer a fertilization advantage, are selected for in species experiencing a greater risk of sperm competition (Gomendio and Roldan 1991; Fitzpatrick and Balshine 2009; Lüpold et al. 2009; Tourmente et al. 2011; Gómez Montoto et al. 2011). Thus, explaining how selection has generated the macroevolutionary patterns observed in comparative studies becomes difficult without the underlying support of sperm length-speed correlations in intra-specific studies. Our results emphasise that correlations between sperm morphological traits and measures of swimming speed may be more common than previously thought. Consequently, we seem to be further towards the goal of understanding the variation we see in sperm form and function. We expect that future work will reconcile results from studies using mean values with our prescribed approach of focusing on individual sperm in order that we might

begin to understand levels of selection acting to produce different trends seen at these different scales. Moreover, reconciling the negative relationship between sperm morphology and speed observed in internal fertilizing species with the macroevolutioanry patterns of increasing sperm size in response to sperm competition represents a major research challenge in future studies.

Chapter Five: General Discussion

5.1. Overview

The functional significance of the observed diversity of sperm morphology is complex. Polyandry is proving to be widespread throughout taxa (Simmons 2005) and this provides the conditions for the prevalence of sperm competition to be a selective force on sperm characteristics (Parker 1970; Parker and Begon 1993; Parker 1998). There may be no unifying resolution to the relationships between sperm morphology and velocity because of the intricate, often species-specific selection pressures faced by spermatozoa. In addition, the evolutionary arms race between haploid and diploid genome place different, though not necessarily conflicting selection pressures on sperm traits (Pizzari and Foster 2008). However, the results presented in the previous chapters have shown that correlations between sperm morphology and velocity can be detected across species when using measurements from individual sperm and accounting for within-male variation.

The hypotheses tested within the preceding chapters were designed to address some fundamental aspects of sperm biology. The original aims and specific areas of research (Sections 1.13 and 1.14) for each experimental chapter found mixed support. There was some corroboration for the hypothesis that cryopreservation can effect sperm swimming velocity, (Chapter Two) but there was no evidence of chemotaxis. Validation for the hypothesis that links between sperm morphology and velocity could be revealed when measurements from individual sperm were used was found. There was also some indication that fertilisation site might be inside the buccal cavity across all cichlid species analysed based on comparisons with published data (Balshine et al. 2001). When variation within-males was accounted for (Chapter Three) links between sperm length and velocity were found in both externally and internally fertilising species (Chapter Four). As expected (Eberhard 2009), the variation in the direction of the correlations were related to fertilisation point; externally fertilising species indicated positive relationships and internally fertilising species showing negative relationships between sperm morphology and velocity.

The overriding theme of this thesis was to assess the potential for links between sperm morphology and velocity to be elucidated across taxa. Using several model species an insight into the methods which might detect such relationships have been identified. The following section will place the results of each of the preceding chapters into the context of existing literature before the final section discusses how the findings of this work can inform future directions in research into sperm biology.

5.2. Putting the findings into context

Whilst the results in chapter two did not explicitly reveal evidence of chemotaxis the data has shown that differences in sperm swimming behaviour can be found as a result of preservation technique and may be an example of chemokinses. However the potential for the variation to be consequence of limited sample size (samples were from one male for the freeze-thawed sperm and one male for the fresh sperm) should not be overlooked. Cryodamage to sperm plasma membrane is known to alter the morphology of bovine sperm heads (Gravance et al. 1998) motility and level of fertility (reviewed by Bailey et al. 2000) and is lethal to ~50% of sperm in a typical semen sample (Holt 2000). The ability to choose the fittest sperm based on biologically relevant criteria is vital for AI. Differences in the "freezability" of the sperm of specific males may reflect some genetically inherited resistance to cryoinjury, which may, or may not be linked to fitness of fresh semen (Holt 2000). Under natural conditions only hyperactivated spermatozoa can fertilise an oocyte and this is the state in which sperm are found to respond to chemical cues (Eisenbach and Ralt 1992). Therefore the hyperactive swimming speed of bovine spermatozoa might be required in order to match sperm morphology with velocity.

Unfortunately technical restrictions meant that bovine sperm morphology could not be measured but this would seem to be an interesting line of investigation. Longer recording times, would allow more sperm to be tracked increasing the possibility of identifying the hyperactivated subpopulation. However, the most instructive method may be to use three-dimesnsional microchannels (Denissenko et al. 2012) which can more accurately replicate the internal

geometry of the female reproductive tract, manipulate viscosity and record the swimming behaviour of sperm subpopulations. Microchannel tracking could be used in combination with sperm from males whose sperm have been stained to allow sperm from each male can be identified, for example as in genetically isogenitc lines of *Drosphila melanogaster* (Lüpold et al. 2012). Interactions of sperm in competition would allow sperm morphology to be more precisely linked to swimming velocity and progress through the 'female reproductive system', proving insight into the interactions of sperm in a in vitro system more closely replicating internal fertilisation.

Little variation in sperm morphology was found across cichlid species analysed. When compared to measurement of sperm length of cichlids form Lake Tanganyika (Balshine et al. 2001) the Lake Malawi cichlids, and the seven species used as outgroups, appear to reflect fertilisation within the buccal cavity. Allowing for the slight variation expected as measurements of total length were not taken in exactly the same way, the data in chapter three non the less fits within the range of buccal cavity fertilisation much more than that of substrate fertilisation (see Figure 2 of Balshine et al. 2001). However assignment to a mating system based on data from Lake Tanganyika cichlids is less convincing on the basis of sperm length for the cichlids for which data is presented here.

Cichlid reproductive biology may not allow much opportunity for sperm competition even in polygamous species as only small numbers of eggs laid with any single male. The traditional definition of sperm competition may not be applicable to cichlid species in which fertilisation takes place inside the buccal cavity. Sperm longevity, water hardening of eggs and the length of time it can take for a whole clutch of eggs to be laid may not result in sperm from different males competing to fertilise a given set of ova as required by sperm competition (Parker 1998). Female sand-dwelling cichlids for example, lay batches of eggs on the bowers of several different males (Kellogg et al 1995) until the whole clutch has been laid and fertilised, a process which could take up to 65 mins (Kellogg et al 2000). An individual male may therefore, only have direct access to a small number of eggs from the whole clutch laid by a female in any one breeding cycle. Water hardening of the eggs once the female has laid them can mean eggs are

only viable for very short periods of time, potentially less that 40 seconds, presenting sperm with a very brief window of opportunity to get to the micropyle.

Mouthbrooding females take sperm and eggs into the buccal cavity for brooding (and possible fertilisation) leaving room for the potential that the female produces a substance that can prolong sperm-egg viability. However the intricacies of the buccal cavity have received little attention despite its importance in cichlid ecology and reproduction. For example, in a recent study of the feeding mechanics of maternally mouthbrooding haplochromine cichlids from Lake Victoria sexual dimorphism has been found; females are less efficient feeders as a result of the evolution of the buccal cavity when compared to males from the same species (tkint et al 2012). It is well established that the females of many cichlid species do not feed when they are brooding eggs or young (Fryer and Iles 1972; Konings 2007) but the work of tkint et al. (2012) indicated there are potentially greater fitness implications as a result of morphological adaptations intrinsic to mouthbrooding.

There are methodological problems associated with attempting to link sperm morphology to velocity. Measuring sperm swimming velocity in vitro is know to present problems in relation to sperm being attracted to the edges of slides (Wilson-Leedy and Ingermann 2007). The point at which sperm are swimming optimally after activation could conceivably vary across species leading to functional disparity between morphology and velocity depend on time since activation (Fitzpatrick and Balshine 2009). Furthermore, there has often been insufficient account taken for the within male covariance between individual sperm phenotypes (Fitzpatrick et al. 2010). Even if there has been postcopulatory sexual selection to optimise sperm phenotype within males (Immler et al. 2008) from which links between sperm length and velocity can be found (Gomendio and Roldan 1991) data analysed across males, may not reveal links, or they may become non significant (Fitzpatrick et al. 2010). The results in chapter three have provided an indication that applying a new methodological and statistical approached to sperm size-velocity relationships can reveal otherwise hidden links between sperm morphology and velocity. Only when

within-male variation in sperm characteristics was accounted for did the relationships between sperm morphology and velocity become apparent.

Building upon investigations from the previous two chapters, chapter four confirmed that advances in the field of sperm biology could be made by considering individual sperm, intra-male variation and fertilisation environment. Often overlooked in previous work, within-male variation could be the key variable in resolving relationships between sperm morphology and velocity. If nothing else, this highlights the importance of selecting a method for analysis which is robust and can detect both within- and between-male variation in sperm traits. Here within subject centering has been shown to provide such discrimination when applied to data collected from individual spermatozoa.

Chapter four revealed extensive relationships between sperm morphology and velocity across multiple taxa. Sperm morphology and velocity have revealed inconsistent correlations across taxa both in terms of relationship with each other, and to fertilisation success generally (for a review see Simmons and Fitzpatrick 2012). Data presented her revealed directional variation in relation to correlations between morphology and velocity depending on fertilisation microenvironment. Internally fertilising species showing negative relationships between sperm morphology and velocity whilst externally fertilising species showed positive correlations with longer sperm swimming faster than shorter sperm. The finding that short sperm swim faster in internally fertilising species is in contradiction to the findings of some comparative studies which have found longer sperm to swim faster across internally fertilising species (for example Gomendio and Roldan 1991; Tourmente et al. 2011). However the findings that longer sperm swim faster in externally fertilising species is in support of some of the existing data (Fitzpatrick and Balshine 2009; Fitzpatrick et al. 2010). The literature reviewed in Table 1.1 however emphasises that there are numerous contradictory data for correlations between sperm length and velocity across taxa (also see Humphries et al. 2008).

Inter-ejaculate competition indicates the difference in morphological and behavioural traits that are selected for by haploid and diploid genome; every

sperm is in competition with every other sperm released during a mating event but for each male every spermatozoa is an important investment in his reproductive fitness (Pizzari and Foster 2008).This extra level of competition could be being expressed by the variation in sperm phenotypes found within ejaculates which is becoming evident across a wide range of species (for example Snook 1997; 1998; Swallow and Wilkinson 2002; Oppliger et al. 2003; Valle et al. 2012; Maroto-Morales et al. 2012). The deliberate production of sterile sperm (Till-Bottraud et al. 2005) would appear to be a waste of resources however *Drosophila pseudobsura* females' have been found to prefer ejaculates containing a high number of infertile 'parasperm' (Holman and Snook 2006).

The factors such as female reproductive tract morphology (for example Higginson et al. 2012)and filtering barriers such as cervical mucus (Katz et al. 1978; for example 1981) are important selection obstacles which only the 'best' sperm can overcome to reach the egg. Fields of AI for human fertility, agriculture and species conservation require a deep understanding of the quality of sperm naturally selected in order to improve assisted reproduction fertilisation rates. Recent work by Denissenko et al (2012) tracking the migration of human spermatozoa through different shaped microchannels found sperm tend to swim along the sides of the channel with their head in very close proximity to the wall and the shape of the channel influenced sperm swimming behaviour. Typically empirical studies looking at sperm swimming velocity attempt to reduce the attraction of sperm to the edges of slides and cover-slips but this could be having previously unrecognised influences on the motility characteristics of spermatozoa. The relative head shape-flagella wave form influences the motion of the spermatozoa when following walls (Satake et al. 2006; Denissenko et al. 2012) which has implications for comparisons between sperm morphology-velocity. This effect could be more notable for internally fertilising species as the architecture of the female reproductive tract would provide species specific wall geometry.

Sperm swimming velocity may reflect a males fertility and competitive ability during sperm competition (for a review see Snook 2005) but duration of sperm storage can be related to swimming velocity with a decrease in speed associated

with storage in several species (Ball and Parker 1996; Levitan 2000; Kleven et al. 2009). In addition, individual males can alter the composition of their ejaculate depending on his perception of sperm competition risk/intensity and the quality of competing males can be rapid and in response to different cues. Male cichlids for instance have been found to rapidly alter their ejaculate. When the dominant male was removed the subordinate males ejaculate increased in quality to similar levels of the previously dominant male after only 24 hours (Kustan et al. 2011). A preliminary investigation made as part of the present study attempted to address variation in sperm morphology and swimming velocity in relation to social status in the bower building cichlid *Nyassachromis cf. microcephalus*. A trial of five replicates using size matched males and artificial bowers indicated a sample size beyond the resources available would be required to gather sufficient data for meaningful statistical comparison to be undertaken. However, as outlined above, the work by Kustan et al. (2011) suggests that this type of investigation would be worth pursuing. A combination of behavioural observation of male-male and male–female interactions, sperm quality analysis and paternity investigations would allow much more detailed interpretations of how selection might be influencing sperm traits in *Nyassachromis cf. microcephalus.*

To understand the significance of sperm size it is necessary to understand how the size of individual sperm relate to function. The previous chapters have highlighted that the simplistic assumptions of a homogenous ejaculate and the use of mean calculations of sperm morphology and velocity can hide biologically relevant factors which influence fertilisation success. As fertilisation success is the primary function of sperm it is vital that links between sperm form and function are elucidated on a species by species level if necessary.

5.3. Future work

Whilst progress is being made in the relative selection pressure imposed on sperm quality by sperm competition (for example Lüpold et al. 2012) the functional significance of sperm size in relation to velocity and the importance of velocity as a factor of fertilisation success receives less empirical investigation.

This thesis has shown that relationships between sperm size and velocity may be more prevalent than the existing literature suggests. However, evidence for links between sperm form and function remain ambiguous. Even though data presented here indicated correlations between sperm morphology and velocity, the importance of such relationships in terms of fertilisation are not clear.

The data presented here provides validation for the importance of considering within-male variation (Fitzpatrick et al. 2010) in sperm traits when looking for functional links between morphology and velocity in future studies. But the data presented here also makes us question how and why do shorter sperm swim faster than longer sperm in internally fertilising species? The mechanics of sperm function in terms of energy transfer (Burness et al. 2004; 2005), flagella propulsion and motility (Gaffney et al. 2011), and the influence of viscosity (Kirkman-Brown and Smith 2011) require continued investigation to better understand the functional significance of the diversity of sperm morphology found across and within species. The main suggestion for future work is that focused, holistic and species-specific studies, investigating sperm-egg interactions, within-male variation, fertilisation microenvironment and the identification of sperm subpopulations are necessary to explain sperm trait evolution. Correlations with motility and fertilisation success do not necessarily suggest absolutely faster sperm win the race to fertilise the egg.

Technological advances and improvements in modelling sperm motility in more realistic setting such as 3D tracking (Corkidi et al. 2008) and simulating the female reproductive tracks using computer modes (Burkitt et al. 2012) will provide detailed information on the motility of sperm which could give insight into the functional aspects which continue to be so elusive. The importance of ensuring sperm motility is measured as accurately as possible in vitro, or that compensation can be made and limitations of tracking can be compensated for have been highlighted in a recent study using fish sperm (Boryshpolets et al. 2012). Boryshpolets et al. (2012) found that sperm velocity dropped by 16% when sperm were recorded at the liquid-solid boundary (i.e. the bottom of the slide and the activation medium) when compared to sperm recorded at the liquid-gas boundary (i.e. activation medium and slide well which was not

covered with a cover-slide). Finding links between morphology and velocity can only be usefully interpreted in relation to species reproductive biology if it is a truly biologically relevant link. Future work must incorporate the effect surfaces have on spermatozoa swimming behaviour if biologically meaningful data is to be gathered for analysis.

The results presented here provide support for the use of a novel methodology and analysis which can be used to find previously elusive links between sperm morphology and velocity. If these techniques can be implemented across the field of sperm biology, relationships across more species could be found and the importance of intramale variation can be robustly investigated. Crucially sperm do not act alone, the fluid characteristics of the ejaculate (Simmons and Fitzpatrick 2012) and female choice (Eberhard and Cordero 2003) must be considered as having an influence over sperm motility. As we are finding out, sperm morphology is not the only determinant of sperm velocity, even if a link between the two traits is found to be widespread, interactions between these microswimmers and their mircroenvironment remains complex and largely unknown.

Chapter Six: Literature Cited

Abaigar, T., J. Barbero and W. V. Holt. 2012. Trajectory variance and autocorrelations within single-sperm tracks as population-level descriptors of sperm track complexity, predictability, and energy-generating ability. J Androl 33:216–228.

Abaigar, T., W. V. Holt, R. A. Harrison and G. del Barrio. 1999. Sperm subpopulations in boar (Sus scrofa) and gazelle (Gazella dama mhorr) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol Reprod 60:32–41.

Adler, J. 1973. A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by Escherichia coli. J. Gen. Microbiol. 74:77–91.

Alavi, S. M. H. and J. Cosson. 2006. Sperm motility in fishes. (II) Effects of ions and osmolality: a review. Cell Biol Int 30:1–14.

Amanze, D. and A. Iyengar. 1990. The micropyle: a sperm guidance system in teleost fertilization. Development 109:495–500.

Anderson, M., J. Nyholt and A. Dixson. 2005. Sperm competition and the evolution of sperm midpiece volume in mammals. J Zool 267:135–142.

Armon, L. and M. Eisenbach. 2011. Behavioral Mechanism During Human Sperm Chemotaxis: Involvement of Hyperactivation. PLoS ONE 6(12): e28359. doi:10.1371/journal.pone.0028359.

Arnold, S. J. 1994. Bateman's Principles and the Measurement of Sexual Selection in Plants and Animals. Am Nat 144:S129–S149.

Avis, J. C. 2012. Molecular markers, natural history and evolution. Chapman and Hall, New York.

Bailey, J. L., J. F. Bilodeau and N. Cormier. 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl 21:1–7.

Baker, R. and M. Bellis. 1988. “Kamikaze” sperm in mammals? Animal Behaviour 36:936–939.

Baker, R. R. and M. A. Bellis. 1995. Human sperm competition: copulation, masturbation and infertility. Chapman & Hall, London.

Ball, M. and G. A. Parker. 1996. Sperm competition games: external fertilization and “adaptive” infertility. J Theor Biol 180:141–150.

Balshine, S., B. Leach, F. C. Neat and N. Werner. 2001. Sperm size of African cichlids in relation to sperm competition. Behav Ecol 12:726–731.

Barraclough, T. G., A. P. Vogler and P. H. Harvey. 1998. Revealing the factors that promote speciation. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 353:241–249.

Batchelor, G. K. 1970. Slender-body theory for particles of arbitrary cross-section in Stokes flow. J Fluid Mech 44:419–440.

Bellis, M. A., R. R. Baker and M. J. G. Gage. 1990. Variation in rat ejaculates consistent with the kamikaze-sperm hypothesis. Journal of Mammalogy 71:479–480.

Billard, R. and J. Cosson. 1990. The energetics of fish sperm motility. Pp. 153–173 *in* C. Gagnon, ed. Controls of sperm motility: biological and clinical aspects. CRC, Boca Raton.

Binks, R. M., J. Prince, J. P. Evans and W. J. Kennington. 2012. More than bindin divergence: reproductive isolation between sympatric subspecies of sea urchin by asynchronous spawning. Evolution 66:3545-3557.

Birkhead, T. R. 2000. Promiscuity: an evolutionary history of sperm competition and sexual conflict. Faber, London.

Birkhead, T. R. and A. P. Møller (eds). 1998. Sperm competition and sexual selection. Academic Press, London.

Birkhead, T. R. and S. Immler. 2007. Making sperm: design, quality control and sperm competition. Soc Reprod Fertil Suppl 65:175–181.

Birkhead, T. R. and T. Pizzari. 2002. Postcopulatory sexual selection. Nature Reviews Genetics 3:262-273.

Birkhead, T. R., E. J. Pellatt, P. Brekke, R. Yeates and H. Castillo-Juarez. 2005. Genetic effects on sperm design in the zebra finch. Nature 434:383–387.

Boryshpolets, S., J. Cosson, V. Bondarenko, E. Gillies, M. Rodina, B. Dzyuba and O. Linhart. 2012. Different swimming behaviors of sterlet (Acipenser ruthenus) spermatozoa close to solid and free surfaces. THE http://dx.doi.org/10.1016/j.theriogenology.2012.09.011.

Brakefield, P., E. El Filali, R. Van der Laan, C. Breuker, I. Saccheri and B. Zwaan. 2001. Effective population size, reproductive success and sperm precedence in the butterfly, Bicyclus anynana, in captivity. J Evol Biol 14:148–156.

Brennen, C. and H. Winet. 1977. Fluid mechanics of propulsion by cilia and flagella. Annu. Rev. Fluid Mech. 9:339–398.

Bronson, R. and Y. Hamada. 1977. Gamete interactions in vitro. Fertility and Sterility 28:570–576.

Burkitt, M., D. Walker, D. M. Romano and A. Fazeli. 2012. Using computational modeling to investigate sperm navigation and behavior in the female reproductive tract. THE 77:703-716.

Burness, G., C. D. Moyes and R. Montgomerie. 2005. Motility, ATP levels and metabolic enzyme activity of sperm from bluegill (Lepomis macrochirus). Comp Biochem Phys A 140:11–17.

Burness, G., S. Casselman, A. I. Schulte-Hostedde, C. D. Moyes and R. Montgomerie. 2004. Sperm swimming speed and energetics vary with sperm competition risk in bluegill (Lepomis macrochirus). Behav Ecol Sociobiol 56:65–70.

Chain, R. C., C. K. Park and M. A. Sirard. 1996. Cumulus cells act as a sperm trap during in vitro fertilization of bovine oocytes. Theriogenology 45:258.

Calhim, S., S. Immler and T. R. Birkhead. 2007. Postcopulatory sexual selection is associated with reduced variation in sperm morphology. PLoS ONE 2:e413.

Chain, R. C., K. Okuda and K. Niwa. 1995. Influence of cumulus cells on in vitro fertilization of bovine oocytes derived from in vitro maturation. Animal Reproduction Science 38:37–48.

Chang, H. and S. S. Suarez. 2010. Rethinking the Relationship Between Hyperactivation and Chemotaxis in Mammalian Sperm. Biol Reprod 83:507–513.

Cohen-Dayag, A., I. Tur-Kaspa and J. Dor. 1995. Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. Proc. Natl. Acad. Sci. 92:11039-11043.

Corkidi, G., B. Taboada, C. D. Wood, A. Guerrero and A. Darszon. 2008. Tracking sperm in three-dimensions. Biochem Bioph Res Co 373:125–129.

Cormier, N. and M. Sirard. 1997. Premature capacitation of bovine spermatozoa is initiated by cryopreservation. J Androl 18:461-468.

Cosson, J. 1996. A moving image of flagella: News and views on the mechanisms involved in axonemal beating. Cell Biol Int 20:83-94.

Cosson, J. 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aquaculture International 12:69–85.

Cosson, J. J. 2008. The Motility Apparatus of Fish Spermatozoa. Pp. 281–316 *in* S. M. H. Alavi, J. J. Cosson, K. Coward, and G. Rafiee, eds. Fish Spermatology. Alpha Science International Ltd, Oxford.

Cosson, J., A.-L. Groison, M. Suquet, C. Fauvel, G. Dorange and R. Billard. 2008. Marine fish spermatozoa: racing ephemeral swimmers. Reprod 136:277–294.

Cosson, J., P. Huitorel and C. Gagnon. 2003. How spermatozoa come to be confined to surfaces. Cell Motil Cytoskeleton 54:56–63.

Coward, K., N. Bromage, O. Hibbitt and J. Parrington. 2002. Gamete physiology, fertilization and egg activation in teleost fish. Rev Fish Biol Fisheries 12:33–58.

Creemers, E., M. Nijs, E. Vanheusden and W. Ombelet. 2011. Cryopreservation of human sperm: efficacy and use of a new nitrogen-free controlled rate freezer versus liquid nitrogen vapour freezing. Andrologia 43:392–397.

Cummins, J. 2009. Sperm motility and energetics. Pp. 185–206 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm Biology: An Evolutionary Perspective. Elsevier.

Curry, M. R. 2000. Cryopreservation of semen from domestic livestock. Rev.

Reprod. 5:46–52.

Darwin, C. 1859. On the origins of species by means of natural selection. London: Murray.

Darwin, C. 1871. The Descent of Man and Selection in Relation to Sex. Murray, London.

Denissenko, P., V. Kantsler, D. J. Smith and J. Kirkman-Brown. 2012. Human spermatozoa migration in microchannels reveals boundary-following navigation. Proc Nat Acad Sci 109:8007–8010.

Denny, M. W. and M. Shibata. 1989. Consequences of Surf-Zone Turbulence for Settlement and External Fertilization. Am Nat 134:859–889.

Dorado, J., I. Molina, A. Muñoz-Serrano and M. Hidalgo. 2010. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Florida goats. THE 1–10.

Dunson, D. B., C. R. Weinberg, S. D. Perreault and R. E. Chapin. 1999. Summarizing the motion of self-propelled cells: applications to sperm motility. Biometrics 55:537–543.

Dziminski, M. A., J. D. Roberts, M. Beveridge and L. W. Simmons. 2009. Sperm competitiveness in frogs: slow and steady wins the race. Proceedings: Biological Sciences 276:3955–3961.

Eberhard, W. G. 1996. Female control. Princeton University Press.

Eberhard, W. G. 2009. Postcopulatory sexual selection: Darwin's omission and its consequences. Proceedings of the National Academy of Sciences 106:10025–10032.

Eberhard, W. G. and C. Cordero. 2003. Sexual conflict and female choice. Trends Ecol Evol 18:438–439.

Eisenbach, M. 1999. Sperm chemotaxis. Reprod 4:56-66.

Eisenbach, M. and D. Ralt. 1992. Precontact mammalian sperm-egg communication and role in fertilization. American Journal of Physiology- Cell Physiology 262:C1095.

Eisenbach, M. and L. Giojalas. 2006. Sperm guidance in mammals—an unpaved road to the egg. Nature Reviews Molecular Cell Biology 7:276–285.

Evans, J. P. 2009. No evidence for sperm priming responses under varying sperm competition risk or intensity in guppies. Naturwissenschaften 96:771–779.

Evans, J. P. 2010. Quantitative genetic evidence that males trade attractiveness for ejaculate quality in guppies. P R Soc B 1–8.

Evans, J. P. and L. W. Simmons. 2008. The genetic basis of traits regulating sperm competition and polyandry: can selection favour the evolution of good- and sexy-sperm? Genetica 134:5–19.

Fabro, G. 2002. Chemotaxis of Capacitated Rabbit Spermatozoa to Follicular Fluid Revealed by a Novel Directionality-Based Assay. Biol Reprod 67:1565–1571.

Firman, R. C. and L. W. Simmons. 2010. Sperm midpiece length predicts sperm swimming velocity in house mice. Biol Lett 6:513–516.

Fishelson, L. 2003. Comparison of testes structure, spermatogenesis, and spermatocytogenesis in young, aging, and hybrid cichlid fish (Cichlidae, Teleostei). Journal of Morphology 256:285-300.

Fisher, H. S. and H. E. Hoekstra. 2009. Competition drives cooperation among closely related sperm of deer mice. Nature 463:801-803.

Fitzpatrick, J. 2005. Male reproductive suppression in the cooperatively breeding fish Neolamprologus pulcher. Behav Ecol 17:25–33.

Fitzpatrick, J. and S. Balshine. 2009. Female promiscuity promotes the evolution of faster sperm in cichlid fishes. PNAS 106:1128-1132.

Fitzpatrick, J., F. Garcia-Gonzalez and J. P. Evans. 2010. Linking sperm length and velocity: the importance of intramale variation. Biol Lett 6:797-799.

Fitzpatrick, J., J. K. Desjardins, N. Milligan, R. Montgomerie and S. Balshine. 2007. Reproductive-tactic-specific variation in sperm swimming speeds in a shell-brooding cichlid. Biol Reprod 77:280–284.

Fitzpatrick, J., L. W. Simmons and J. P. Evans. 2012. Complex patterns of multivariate selection on the ejaculate of a broadcast spawning marine invertebrate. Evolution 66:2451-2460.

Fryer, G. and T. D. Iles. 1972. The Cichlid Fishes of the Great Lakes of Africa. Oliver & Boyd, Edinburgh.

Gadelha, H., E. A. Gaffney, D. J. Smith and J. C. Kirkman-Brown. 2010. Nonlinear instability in flagellar dynamics: a novel modulation mechanism in sperm migration? J Roy Soc Interface 7:1689–1697.

Gaffney, E. A., H. Gadelha, D. J. Smith, J. R. Blake and J. C. Kirkman-Brown. 2011. Mammalian Sperm Motility: Observation and Theory. Annu Rev Fluid Mech 43:501–528.

Gage, M. J. G. and R. P. Freckleton. 2003. Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length. P R Soc B 270:625–632.

Gage, M. J. G., C. Macfarlane, S. Yeates, R. Shackleton and G. A. Parker. 2002. Relationships between sperm morphometry and sperm motility in the Atlantic salmon. J Fish Biol 61:1528–1539.

Gage, M. J. G., P. Stockley and G. A. Parker. 1995. Effects of alternative male mating strategies on characteristics of sperm production in the Atlantic salmon (Salmo salar): theoretical and empirical investigations. Phil Trans Roy Soc Lond B 350:391–399.

Gakamsky, A., E. Schechtman, S. R. Caplan and M. Eisenbach. 2008. Analysis of chemotaxis when the fraction of responsive cells is small - application to mammalian sperm guidance. Int J Dev Biol 52:481–487.

Gardiner, D. M. 1978. Utilization of extracellular glucose by spermatozoa of two viviparous fishes. Comparative Biochemistry and Physiology Part A: Physiology 59:165–168.

Genner, M. J. and G. F. Turner. 2011. Ancient Hybridization and Phenotypic Novelty within Lake Malawi's Cichlid Fish Radiation. Molecular Biology and Evolution 29:195–206.

Genner, M. J., K. A. Young, M. P. Haesler and D. A. Joyce. 2008. Indirect mate choice, direct mate choice and species recognition in a bower-building cichlid fish lek. J Evol Biol 21:1387–1396.

Genner, M. J., O. Seehausen, D. F. R. Cleary, M. E. Knight, E. Michel and G. F. Turner. 2004. How does the taxonomic status of allopatric populations influence species richness within African cichlid fish assemblages? Journal of biogeography 31:93–102.

Genner, M. J., O. Seehausen, D. H. Lunt, D. A. Joyce, P. Shaw, G. Carvalho and G. F. Turner. 2007a. Age of Cichlids: New Dates for Ancient Lake Fish Radiations. Mol Biol Evol 24:1269–1282.

Genner, M. J., P. Nichols, G. Carvalho, R. Robinson, P. Shaw, A. Smith and G. F. Turner. 2007b. Evolution of a cichlid fish in a Lake Malawi satellite lake. P R Soc B 274:2249.

Gillies, E. A., R. M. Cannon, R. B. Green, A. A. Pacey, undefined author, undefined author, undefined author and undefined author. 2009. Hydrodynamic propulsion of human sperm. J Fluid Mech 625:445.

Gomendio, M. and E. R. S. Roldan. 2008. Implications of diversity in sperm size and function for sperm competition and fertility. Int J Dev Biol 52:439–447.

Gomendio, M. and E. R. S. Roldan. 1991. Sperm competition influences sperm size in mammals. Proc Roy Soc Lond B 243:181–185.

Gomendio, M., J. Martin-Coello, C. Crespo, C. Magaña and E. R. S. Roldan. 2006. Sperm competition enhances functional capacity of mammalian spermatozoa. Proc Nat Acad Sci 103:15113–15117.

Gómez Montoto, L., C. Magaña, M. Tourmente, J. Martín-Coello, C. Crespo, J. J. Luque-Larena, M. Gomendio and E. R. S. Roldan. 2011. Sperm Competition, Sperm Numbers and Sperm Quality in Muroid Rodents. PLoS ONE 6:e18173.

Gravance, C. G., R. Vishwanath, C. Pitt, D. L. Garner and P. J. Casey. 1998. Effects of cryopreservation on bull sperm head morphometry. J Androl 19:704–709.

Gray, J. and G. Hancock. 1955. The propulsion of sea-urchin spermatozoa. Journal of Experimental Biology 802-814.

Green, C. E. and P. F. Watson. 2001. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. Reprod 122:889–898.

Guerrero, A., T. Nishigaki, J. Carneiro, Y. Tatsu, C. D. Wood and A. Darszon. 2010. Tuning sperm chemotaxis by calcium burst timing. Developmental Biology 344:52–65.

Haesler, M. P., C. M. Lindeyer, O. Otti, D. Bonfils, D. Heg and M. Taborsky. 2011. Female mouthbrooders in control of pre- and postmating sexual selection. Behav Ecol 22:1033–1041.

Hammadeh, M. E., S. Greiner, P. Rosenbaum and W. Schmidt. 2001. Comparison between human sperm preservation medium and TEST-yolk buffer on protecting chromatin and morphology integrity of human spermatozoa in fertile and subfertile men after freeze-thawing procedure. J Androl 22:1012. Am Soc Andrology.

Harcourt, A. 1991. Sperm competition and the evolution of nonfertilizing sperm in mammals. Evolution 314–328.

Harvey, P. H. and M. D. Pagel. 1991. The comparative method in evolutionary biology. Oxford University Press, Oxford.

Helfenstein, F., M. Podevin and H. Richner. 2009. Sperm morphology, swimming velocity, and longevity in the house sparrow Passer domesticus. Behav Ecol Sociobiol 1–9.

Higginson, D. M., K. B. Miller, K. A. Segraves and S. Pitnick. 2012. Female reproductive tract form drives the evolution of complex sperm morphology. Proceedings of the National Academy of Sciences, doi: 10.1073/pnas.1111474109.

Holeton, G. F. and D. R. Jones. 1975. Water flow dynamics in the respiratory tract of the carp (*Cyprinus carpio* L.). Journal of Experimental Biology 62:537–549.

Holman, L. and R. R. Snook. 2006. Spermicide, cryptic female choice and the evolution of sperm form and function. J Evol Biol 19:1660–1670.

Holt, W. V. 2000. Basic aspects of frozen storage of semen. Animal Reproduction Science 62:3–22.

Holt, W. V. 2011. Mechanisms of Sperm Storage in the Female Reproductive Tract: an Interspecies Comparison. Reproduction in Domestic Animals 46:68–74.

Holt, W. V., F. Shenfield, T. Leonard, T. D. Hartman, R. D. North and H. D. Moore. 1989. The value of sperm swimming speed measurements in assessing the fertility of human frozen semen. Hum. Reprod. 4:292–297.

Holt, W. V., M. Hernandez and L. Warrell. 2010. The long and the short of sperm selection in vitro and in vivo: swim-up techniques select for the longer and faster swimming mammalian sperm. Journal of Evolutionary Biology 23:598-608.

Houde, A. E. 1997. Sex, color, and mate choice in guppies. Princeton University Press, Princeton.

Humphries, S., J. P. Evans and L. W. Simmons. 2008. Sperm competition: linking form to function. BMC Evolutionary Biology 8:319.

Immler, S. and M. Taborsky. 2009. Sequential polyandry affords post-mating sexual selection in the mouths of cichlid females. Behav Ecol Sociobiol 63:1219–1230.

Immler, S., S. Calhim, T. R. Birkhead and undefined author. 2008. Increased postcopulatory sexual selection reduces the intramale variation in sperm design. Evolution 62:1538–1543.

Jamieson, B. 1987. A biological classification of sperm types, with special reference to annelids and molluscs, and an example of spermiocladistics. New horizons in sperm cell research. 311.

Jamieson, B. G. M. and L. K.-P. Leung. 1991. Introduction to fish spermatazoa and the micropyle. *in* B. G. M. Jamieson, ed. Fish Evolution and Systematics: Evidence from Spermatazoa. Cambridge University Press, Cambridge.

Jamieson, B. G. M., M. S. Y. Lee and K. Long. 1993. Ultrastructure of the spermatozoon of the internally fertilizing frog Ascaphus truei (Ascaphidae: Anura: Amphibia) with phylogenetic considerations. Herpetologica 52–65.

Jennions, M. D. and M. Petrie. 2000. Why do females mate multiply? A review of the genetic benefits. Biol Rev Camb Philos Soc 75:21–64.

Joyce, D. A., D. H. Lunt, M. J. Genner, G. F. Turner, R. Bills and O. Seehausen. 2011. Repeated colonization and hybridization in Lake Malawi cichlids. Curr Biol 21:R108–9.

Katz, D. F., R. N. Mills and T. R. Pritchett. 1978. The movement of human spermatozoa in cervical mucus. Reprod 53:259–265.

Katz, D. F., T. D. Bloom and R. H. BonDurant. 1981. Movement of bull spermatozoa in cervical mucus. Biology of Reproduction 25:931-937.

Kaupp, U. B., N. D. Kashikar and I. Weyand. 2008. Mechanisms of Sperm Chemotaxis. Annu. Rev. Physiol. 70:93–117.

Kellogg, K. A., J. A. Markert, J. R. Stauffer and T. D. Kocher. 1995. Microsatellite variation demonstrates multiple paternity in lekking cichlid fishes from Lake Malawi, Africa. Proceedings: Biological Sciences 260:79–84.

Kilgallon, S. J. and L. W. Simmons. 2005. Image content influences men's semen quality. Biol Lett 1:253–255.

Kirkman-Brown, J. C. and D. J. Smith. 2011. Sperm motility: is viscosity fundamental to progress? Mol. Hum. Reprod. 17:539–544.

Kleven, O., F. Fossøy, T. Laskemoen, R. J. Robertson, G. Rudolfsen and J. T. Lifjeld. 2009. Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage duration in passerine birds. Evolution 63:2466–2473.

Knight, M. and G. F. Turner. 1999. Reproductive isolation among closely related Lake Malawi cichlids: can males recognize conspecific females by visual cues? Animal Behaviour 58:761–768.

Kocher, T. D. 2004. Adaptive evolution and explosive speciation: the cichlid fish model. Nat Rev Genet 5:288–298.

Kodric-Brown, A. and J. H. Brown. 1984. Truth in advertising: the kinds of traits favored by sexual selection. American Naturalist 309–323.

Konings, A. 2007. Malawi cichlids in their natural habitat. Cichlid Press.

Krug, P. J., J. A. Riffell and R. K. Zimmer. 2009. Endogenous signaling pathways and chemical communication between sperm and egg. Journal of Experimental Biology 212:1092–1100.

Kustan, J. M., K. P. Maruska and R. D. Fernald. 2011. Subordinate male cichlids retain reproductive competence during social suppression. P R Soc B 1–11.

Lahnsteiner, F. and R. A. Patzner. 2008. Sperm Morphology and Ultrastructure in Fish. Pp. 1–162 *in* S. M. H. Alavi, J. J. Cosson, K. Coward, and G. Rafiee, eds. Fish Spermatology. Alpha Science International Ltd., Oxford, UK.

Lahnsteiner, F. and R. Patzner. 1998. Sperm motility of the marine teleosts Boops boops, Diplodus sargus, Mullus barbatus and Trachurus mediterraneus. J Fish Biol 52:726–742.

Lahnsteiner, F. and T. Weismann. 1999. Physiological and biochemical parameters for egg quality determination in lake trout, *Salmo trutta lacustris*. Fish Physiology and Biochemistry 20:375-388.

Lauga, E. 2007. Propulsion in a viscoelastic fluid. Phys. Fluids 19:083104–083104–13. American Institute of Physics.

Levitan, D. 1998. Does Bateman's principle apply to broadcast-spawning organisms? Egg traits influence in situ fertilization rates among congeneric sea urchins. Evolution 52:1043–1056.

Levitan, D. R. 2000. Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin Lytechinus variegatus. Proc Roy Soc Lond B 267:531–534.

Levitan, D. R. and C. Petersen. 1995. Sperm limitation in the sea. Trends Ecol Evol 10:228–231.

Lifjeld, J. T., T. Laskemoen, O. Kleven, A. T. M. Pedersen, H. M. Lampe, G. Rudolfsen, T. Schmoll and T. Slagsvold. 2012. No Evidence for Pre-Copulatory Sexual Selection on Sperm Length in a Passerine Bird. PLoS ONE 7:e32611.

Liley, N., P. Tamkee, R. Tsai and D. Hoysak. 2002. Fertilization dynamics in rainbow trout (*Oncorhynchus mykiss*): effect of male age, social experience, and sperm concentration and motility on in vitro fertilization. Can. J. Fish. Aquat. Sci. 59:144–152.

Locatello, L., A. Pilastro, R. Deana, A. Zarpellon and M. B. Rasotto. 2007. Variation pattern of sperm quality traits in two gobies with alternative mating tactics. Func Ecol 21:975–981.

Lopez-Smith, R. and K. Renzaglia. 2008. Sperm cell architecture, insemination, and fertilization in the model fern, Ceratopteris richardii. Sexual Plant Reproduction 21:153–167.

Lüpold, S., M. K. Mainier, K. S. Berben, K. J. Smith, B. D. Daley, S. H. Buckley, J. M. Belote and S. Pitnick. 2012. How Multivariate Ejaculate Traits Determine Competitive Fertilization Success in *Drosophila melanogaster*. Curr Biol 1–6.

Lüpold, S., S. Calhim, S. Immler and T. R. Birkhead. 2009. Sperm morphology and sperm velocity in passerine birds. P R Soc B 276:1175–1181.

Machin, K. 1958. Wave propagation along flagella. J Exp Biol. 796-806.

Maden, A., K. Renzaglia and D. Whittier. 1996. Ultrastructure of the spermatozoid of Lycopodium obscurum (Lycopodiaceae). American Journal of Botany 419–429.

Malo, A. F., M. Gomendio, J. Garde, B. Lang-Lenton, A. J. Soler and E. R. S. Roldan. 2006. Sperm design and sperm function. Biol Lett 2:246–249.

Maroto-Morales, A., M. Ramón, O. García-Álvarez, A. J. Soler, M. R. Fernández-Santos, E. R. S. Roldan, M. Gomendio, M. D. Pérez-Guzmán and J. Garde. 2012. Morphometrically-distinct sperm subpopulations defined by a multistep statistical procedure in Ram ejaculates: intra- and interindividual variation. THE 1–11.

Marquez, B. 2004. Different Signaling Pathways in Bovine Sperm Regulate Capacitationand Hyperactivation. Biol Reprod 70:1626–1633.

Martinez, C. O., M. Juarez-Mosqueda, J. Hernandez and J. Valencia. 2006. Cryopreservation of bull spermatozoa alters the perinuclear theca. Theriogenology 66:1969–1975.

Matthews, I., J. P. Evans and A. E. Magurran. 1997. Male display rate reveals ejaculate characteristics in the Trinidadian guppy, Poecilia reticulata. Proc. R. Soc. Lond. B. 264:695–700.

McKaye, K. R. 1983. Ecology and breeding behaviour of a cichlid fish, Cyrtocara eucinostomus, on a large lek in Lake Malawi, Africa. Env. Biol. Fish. 8:81–96.

McNeill, A. R. 1971. Size and Shape. Arnold, London.

Medeiros, C., F. Forell, A. Oliveira and J. Rodrigues. 2002. Current status of sperm cryopreservation: why isn't it better? THE 57:327–344. Elsevier.

Meijering, E., O. Dzyubachyk and I. Smal. 2012. Methods for cell and particle tracking. Methods in Enzymology.

Miller, G. T. and S. Pitnick. 2002. Sperm-Female Coevolution in Drosophila. Science 298:1230–1233.

Miller, R. L. 1997. Specificity of sperm chemotaxis among great barrier reef

shallow-water Holothurians and Ophiuroids. Journal of Experimental Zoology 279:189–200.

Minoretti, N. and B. Baur. 2006. Among- and within-population variation in sperm quality in the simultaneously hermaphroditic land snail Arianta arbustorum. Behav Ecol Sociobiol 60:270–280.

Møller, A. P. 1998. Sperm competition and sexual selection. Pp. 55–91 *in* T. R. Birkhead and A. P. Møller, eds. Academic Press, London.

Moore, H. D. M., K. Dvořáková, N. Jenkins and W. G. Breed. 2002. Exceptional sperm cooperation in the wood mouse. Nature 418:174–177.

Moore, H., M. Martin and T. Birkhead. 2006. No evidence for killer sperm or other selective interactions between human spermatozoa in ejaculates of different males in vitro. Sperm Competition in Humans 213–227.

Morita, M., M. Kitamura, A. Nakajima, E. Sri Susilo, A. Takemura and M. Okuno. 2009. Regulation of sperm flagellar motility activation and chemotaxis caused by egg-derived substance(s) in sea cucumber. Cell Motil Cytoskeleton 66:202–214.

Morrow, E. and M. J. G. Gage. 2001. Consistent significant variation between individual males in spermatozoal morphometry. J Zool 254:147–153.

Morrow, E. H. 1999. How the sperm lost its tail: the evolution of aflagellate sperm. Biol Rev 79:795–814.

Mortimer, D., C. Serres, S. T. Mortimer and P. Jouannet. 1988. Influence of image sampling frequency on the perceived movement characteristics of progressively motile human spermatozoa. Gamete Res 20:313–327.

Mortimer, S., M. Swan and D. Mortimer. 1996. Fractal analysis of capacitating human spermatozoa. Human Reproduction 11:1049–1054.

Mossman, J., J. Slate, S. Humphries, T. Birkhead, undefined author and undefined author. 2009. Sperm morphology and velocity are genetically codetermined in the zebra finch. Evolution 1–8.

Mrowka, W. 1987. Oral fertilisation in a mouthbrooding cichlid fish. Ethology 74:293-296.

Nakagawa, S. and I. Cuthill. 2007. Effect size, confidence interval and statistical significance: a practical guide for biologists. Biol Rev 82:591–605.

Nijs, M. and W. Ombelet. 2001. Cryopreservation of human sperm. Hum Fertil (Camb) 4:158–163.

Oppliger, A., Y. Naciri-Graven, G. Ribi and D. J. Hosken. 2003. Sperm length influences fertilization success during sperm competition in the snail Viviparus ater. Mol Ecol 12:485–492.

Organisation, W. H. 1999. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Press.

Ota, K., D. Heg, M. Hori and M. Kohda. 2010. Sperm phenotypic plasticity in a cichlid: a territorial male's counterstrategy to spawning takeover. Behav Ecol 21:1293–1300.

Pagel, M., A. Meade and D. Barker. 2004. Bayesian Estimation of Ancestral Character States on Phylogenies. Systematic Biology 53:673–684.

Paolillo, D., Jr. 1981. The swimming sperms of land plants. Bioscience 31:367–373.

Parker, A. and I. Kornfield. 1996. Polygynandry inPseudotropheus zebra, a cichlid fish from Lake Malawi. Environ Biol Fish 47:345–352.

Parker, G. 1998. Sperm competition and the evolution of ejaculates: towards a theory base. Pp. 3–49 *in* T. R. Birkhead and A. P. Moller, eds. Sperm competition and sexual selection. Academic Press, London.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in the insect. Biol Rev 45:525–567.

Parker, G. A. 1993. Sperm competition games - sperm size and sperm number under adult control. Proc Roy Soc Lond B 253:245–254.

Parker, G. A. and M. Begon. 1993. Sperm competition games - sperm size and number under gametic control. Proc Roy Soc Lond B 253:255–262.

Parker, G. A. and T. Pizzari. 2010. Sperm competition and ejaculate economics. Biol Rev 85:897–934.

Parkinson, T. J. and C. H. Whitfield. 1987. Optimisation of freezing conditions for bovine spermatozoa. THE 27:781–797.

Parrish, J. J., J. Susko-Parrish, M. A. Winer and N. L. First. 1988. Capacitationof Bovine Sperm by Heparin. Biol Reprod 38:1171–1180.

Pattarini, J. M., W. T. Starmer, A. Bjork and S. Pitnick. 2006. Mechanisms underlying the sperm quality advantage in Drosophila melanogaster. Evolution 60:2064–2080.

Philippi, T. and J. Seger. 1989. Hedging One's Evolutionar Bets, Revisited. TREE 4:41–44.

Pinheiro, J., D. S. Sarkar and R. D. C. Team. 2011. nlme: Linear and Nonlinear Mixed Effects Models.

Pitcher, T. E., F. H. Rodd, L. Rowe, undefined author, undefined author and undefined author. 2007. Sexual colouration and sperm traits in guppies. J Fish Biol 70:165–177.

Pitnick, S., M. F. Wolfner and S. S. Suarez. 2009a. Ejaculate-female and sperm - female interactions. Pp. 247–301 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm Biology: An evolutionary Perspective. Elsevier, London.

Pitnick, S., R. Dobler and D. J. Hosken. 2009b. Sperm length is not influenced by haploid gene expression in the flies Drosophila melanogaster and Scathophaga

stercoraria. P R Soc B 1–7.

Pizzari, T. and K. R. Foster. 2008. Sperm Sociality: Cooperation, Altruism, and Spite. PLoS Biol 6:e130.

Quintero-Moreno, A., J. Miró, A. Teresa Rigau and J. E. Rodríguez-Gil. 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. THE 59:1973–1990.

Ram, J., P. Fong and D. Garton. 1996. Physiological aspects of zebra mussel reproduction: Maturation, spawning and fertilization. Pp. 326–338 *in* American Zoologist.

Riffell, J. A., P. J. Krug and R. K. Zimmer. 2004. The ecological and evolutionary consequences of sperm chemoattraction. Proc Nat Acad Sci 101:4501–4506.

Rosenkrans, C., Jr, G. Zeng, G. McNamara, P. Schoff and N. First. 1993. Development of bovine embryos in vitro as affected by energy substrates. Biol Reprod 49:459–462.

Rowe, M. and S. Pruett-Jones. 2011. Sperm Competition Selects for Sperm Quantity and Quality in the Australian Maluridae. PLoS ONE 6:e15720.

Salzburger, W., T. Mack, E. Verheyen and A. Meyer. 2005. Out of Tanganyika: Genesis, explosive speciation, key-innovations and phylogeography of the haplochromine cichlid fishes. BMC Evolutionary Biology 5:17.

Satake, N., R. Elliott, P. F. Watson and W. V. Holt. 2006. Sperm selection and competition in pigs may be mediated by the differential motility activation and suppression of sperm subpopulations within the oviduct. Journal of Experimental Biology 209:1560.

Scharer, L., D. T. J. Littlewood, A. Waeschenbach, W. Yoshida and D. B. Vizoso. 2011. Mating behavior and the evolution of sperm design. Proceedings of the National Academy of Sciences 1–6.

Seehausen, O. 2006. African cichlid fish: a model system in adaptive radiation research. P R Soc B 273:1987–1998.

Shannon, P. and R. Vishwanath. 1995. The effect of optimal and suboptimal concentrations of sperm on the fertility of fresh and frozen bovine semen and a theoretical model to explain the fertility differences. Animal Reproduction Science 39:1–10.

Simmons, L. W. 2005. The Evolution of Polyandry: Sperm Competition, Sperm Selection, and Offspring Viability. Annu. Rev. Ecol. Evol. Syst. 36:125–146.

Simmons, L. W. and J. L. Fitzpatrick. 2012. Sperm wars and the evolution of male fertility. Reprod, doi: 10.1530/REP-12-0285.

Simmons, L. W. and J. S. Kotiaho. 2007. Quantitative genetic correlation between trait and preference supports a sexually selected sperm process. Proc Nat Acad Sci 104:16604–16608.

Simmons, L. W., J. D. Roberts and M. A. Dziminski. 2009. Egg jelly influences sperm motility in the externally fertilizing frog, Crinia georgiana. J Evol Biol 22:225–229.

Smith, C. C. 2012. Opposing effects of sperm viability and velocity on the outcome of sperm competition. Behav Ecol, doi: 10.1093/beheco/ars036.

Smith, D. J., E. A. Gaffney, H. Gadelha, N. Kapur and J. C. Kirkman-Brown. 2009. Bend propagation in the flagella of migrating human sperm, and its modulation by viscosity. Cell Motil Cytoskeleton 66:220–236.

Snook, R. R. 1997. Is the Production of Multiple Sperm Types Adaptive? Evolution 51:797–808.

Snook, R. R. 2005. Sperm in competition: not playing by the numbers. Trends Ecol Evol 20:46–53.

Snook, R. R. 1998. The risk of sperm competition and the evolution of sperm heteromorphism. Animal Behaviour 56:1497–1507.

Sood, S., A. Tawang, I. A. Malecki and G. B. Martin. 2011. Artificial insemination technology for the emu - improving sperm survival. Reprod Biol 11 Suppl 3:43–49.

Sood, S., I. A. Malecki, A. Tawang and G. B. Martin. 2011. Response of spermatozoa from the emu (Dromaius novaehollandiae) to rapid cooling, hyperosmotic conditions and dimethylacetamide (DMA). Animal Reproduction Science 1–26.

Southworth, D. and M. Cresti. 1997. Comparison of flagellated and nonflagellated sperm in plants. American Journal of Botany 84:1301.

Stockley, P., M. J. G. Gage, G. A. Parker and A. P. Møller. 1997. Sperm competition in fishes: the evolution of testis size and ejaculate characteristics. Am Nat 149:933–954.

Stoltz, J. A. and B. D. Neff. 2006. Sperm competition in a fish with external fertilization: the contribution of sperm number, speed and length. J Evol Biol 19:1873–1881.

Suarez, S. S. 1996. Hyperactivated motility in sperm. J Androl. 17:331-335.

Suarez, S. S. 2006. Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction. Reprod Fertil Dev 19:103–110.

Suarez, S. S. 2008. Regulation of sperm storage and movement in the mammalian oviduct. Int J Dev Biol 52:455–462.

Suarez, S. S. 2005. Sperm transport in the female reproductive tract. Hum Reprod Update 12:23–37.

Suarez, S. S. and R. A. Osman. 1987. Initiation of hyperactivated flagellar bending in mouse sperm within the female reproductive tract. Biol Reprod 36:1191–1198.

Suarez, S., K. Redfern, P. Raynor, F. Martin and D. M. Phillips. 1991. Attachment of Boar Sperm to Mucosal Explants of Oviduct In Vitro: Possible Role in Formationof a SpermReservoir. Biol Reprod 44:998–1004.

Sun, F., A. Bahat, A. Gakamsky, E. Girsh and N. Katz. 2005. Human sperm chemotaxis: both the oocyte and its surrounding cumulus cells secrete sperm chemoattractants. Human Reproduction 20;761-767.

Swallow, J. G. and G. S. Wilkinson. 2002. The long and short of sperm polymorphisms in insects. Biol Rev Camb Philos Soc 77:153–182.

Taggart, D. A., C. M. Leigh, D. Schultz and W. G. Breed. 1995. Ultrastructure and motility of spermatozoa in macropodid and potoroidid marsupials. Reprod Fertil Dev 7:1129–1140.

Tamura, K., J. Dudley, M. Nei and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution 24:1596–1599.

Tanghe, S., A. Van Soom, V. Sterckx, D. Maes and A. de Kruif. 2002. Assessment of different sperm quality parameters to predict in vitro fertility of bulls. Reprod. Domest. Anim. 37:127–132.

Theis, A., W. Salzburger and B. Egger. 2012. The function of anal fin egg-spots in the cichlid fish Astatotilapia burtoni. PLoS ONE 7:e29878.

Thurston, L. M., P. F. Watson, A. J. Mileham and W. V. Holt. 2001. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl 22:382–394.

Thünken, T., T. Bakker and H. Kullmann. 2007. Extraordinarily long sperm in the socially monogamous cichlid fish Pelvicachromis taeniatus. Naturwissenschaften 94:489–491.

Till-Bottraud, I., D. Joly, D. Lachaise and R. R. Snook. 2005. Pollen and sperm heteromorphism: convergence across kingdoms? J Evol Biol 18:1–18.

Tomkins, J. L., J. Radwan, J. S. Kotiaho and T. Tregenza. 2004. Genic capture and resolving the lek paradox. Trends Ecol Evol 19:323–328.

Tourmente, M., M. Gomendio and E. R. Roldan. 2011. Sperm competition and the evolution of sperm design in mammals. BMC Evolutionary Biology 11:12.

Trippel, E. A. 2004. Estimation of Male Reproductive Success of Marine Fishes. 1–34.

Truett, G. E., P. Heeger, R. L. Mynatt, A. A. Truett, J. A. Walker and M. L. Warman. 2000. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). BioTechniques 29:52–54.

Turner, E. and R. Montgomerie. 2002. Ovarian fluid enhances sperm movement in Arctic charr. J Fish Biol 60:1570–1579.

Turner, G. F. 2007. Adaptive radiation of cichlid fish. Curr Biol 17:1–5.

Valle, R. R., P. L. Nayudu, C. L. V. Leal and M. García-Herreros. 2012. Sperm head

morphometry in ejaculates of adult marmosets (Callithrix jacchus): A model for studying sperm subpopulations and among-donor variations. THE 78:1152–1165.

Van de Pol, M. and J. Wright. 2009. A simple method for distinguishing within- versus between-subject effects using mixed models. Animal Behaviour 77:753–758.

Vishwanath, R. 2003. Artificial insemination: the state of the art. THE 59:571–584.

Vogel, S. 1996. Life in moving fluids: the physical biology of flow. Princeton University Press.

Watson, P. F. 2000. The causes of reduced fertility with cryopreserved semen. Animal Reproduction Science 60-61:481–492.

Watson, P. J. 1991. Multiple paternity and first mate sperm precedence in the sierra dome spider, Linyphia litigiosa Keyserling (Linyphiidae). Animal Behaviour 41:135–148.

Werner, M. and L. W. Simmons. 2008. Insect sperm motility. Biol Rev Camb Philos Soc 83:191–208.

Wilson-Leedy, J. G. and R. L. Ingermann. 2007. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 67:61–672.

Wilson-Leedy, J. G. and R. L. Ingermann. 2006. imageJ CASA instructions. http://rsbweb.nih.gov/ij/plugins/casa.html.

Winet, H. 1973. Wall drag on free-moving ciliated micro-organisms. J Exp Biol 59:753-766.

World Health Organisation. 1999. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Press.

Yanagimachi, R. 1970. The movement of golden hamster spermatozoa before and after capacitation. J Reprod Fertil 23:193–196.

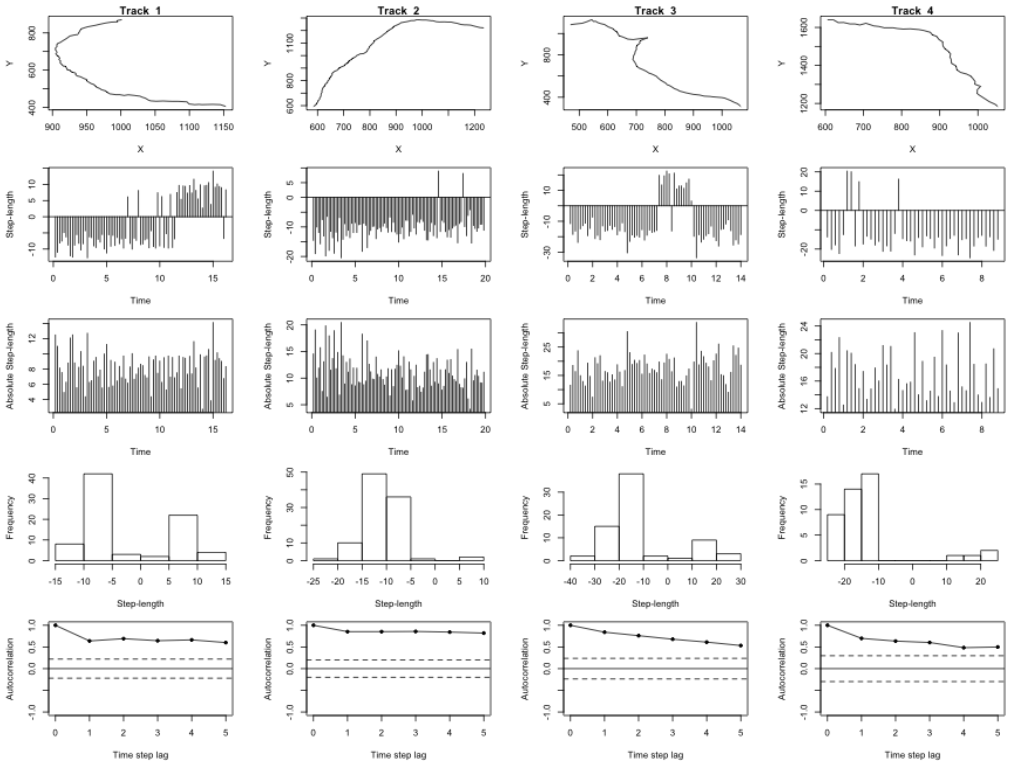
Yang, Y. and X. Lu. 2011. Drosophila Sperm Motility in the Reproductive Tract. Biol Reprod 1–25.

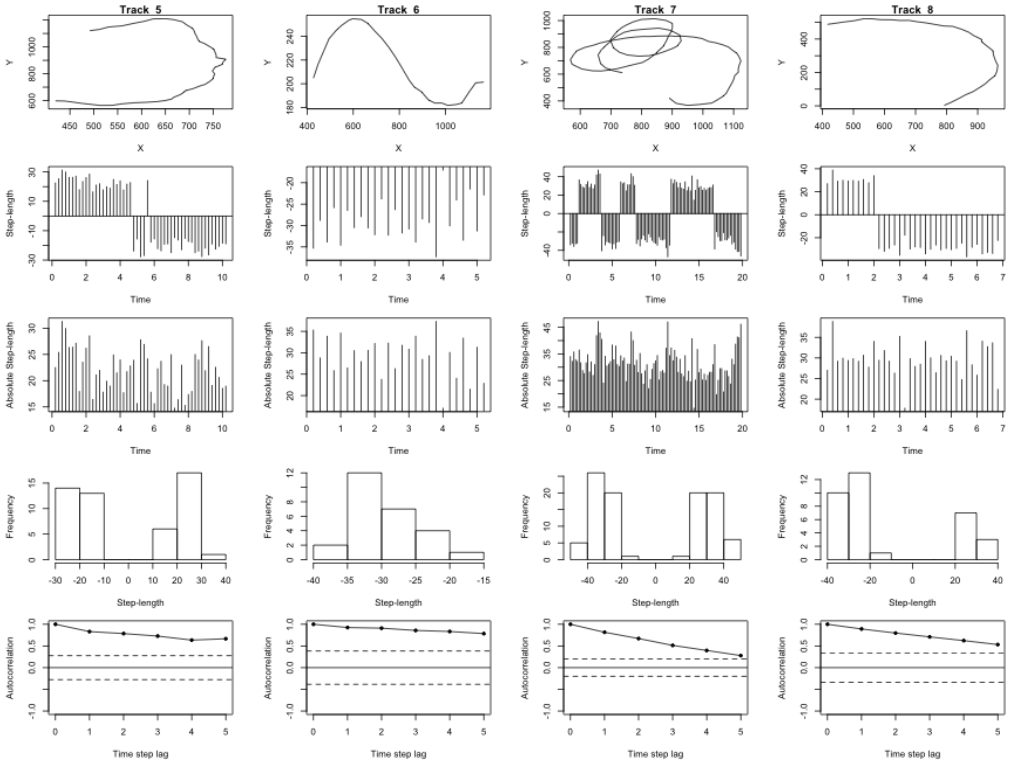
Zicha, D., G. A. Dunn and A. F. Brown. 1991. A new direct-viewing chemotaxis chamber. Journal of Cell Science 99:769-775.

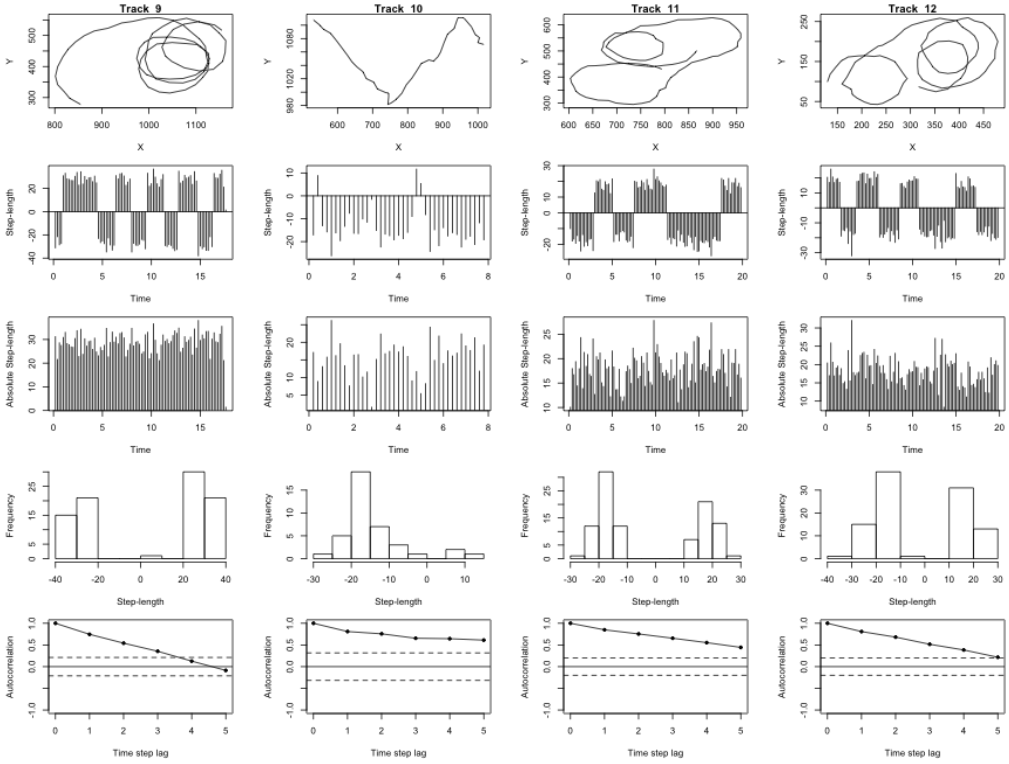
Zimmer, R. K. and J. A. Riffell. 2011. Sperm chemotaxis, fluid shear, and the evolution of sexual reproduction. Proceedings of the National Academy of Sciences 1–6.

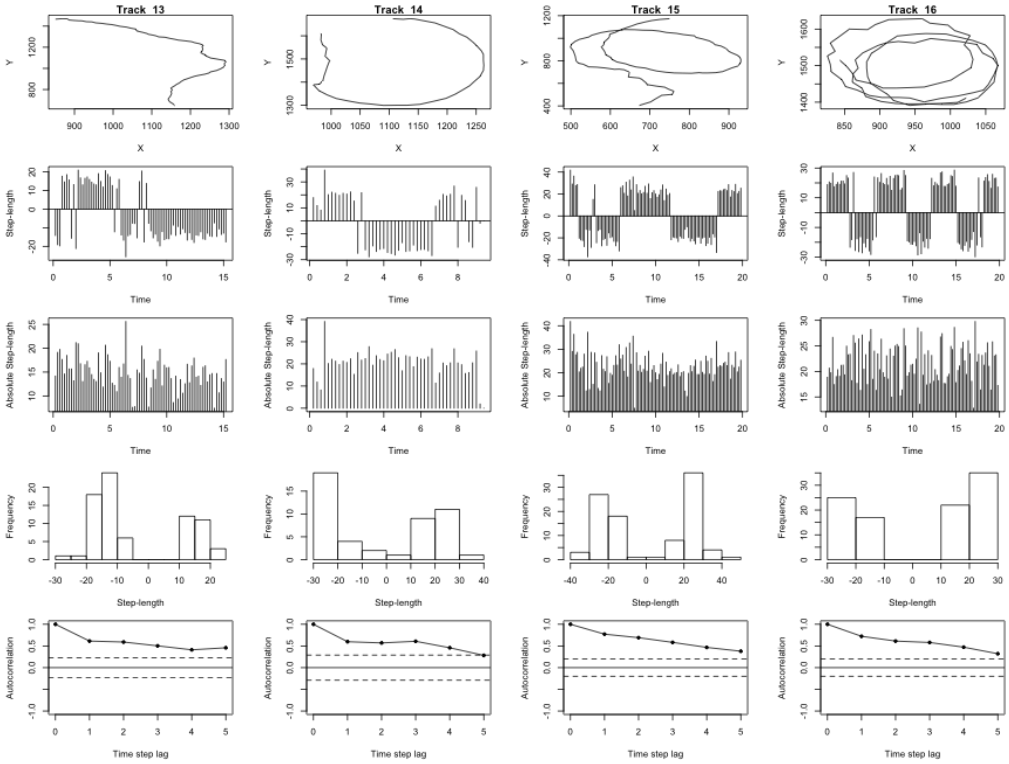
Appendix One: Fractal analysis of bovine sperm swimming tracks

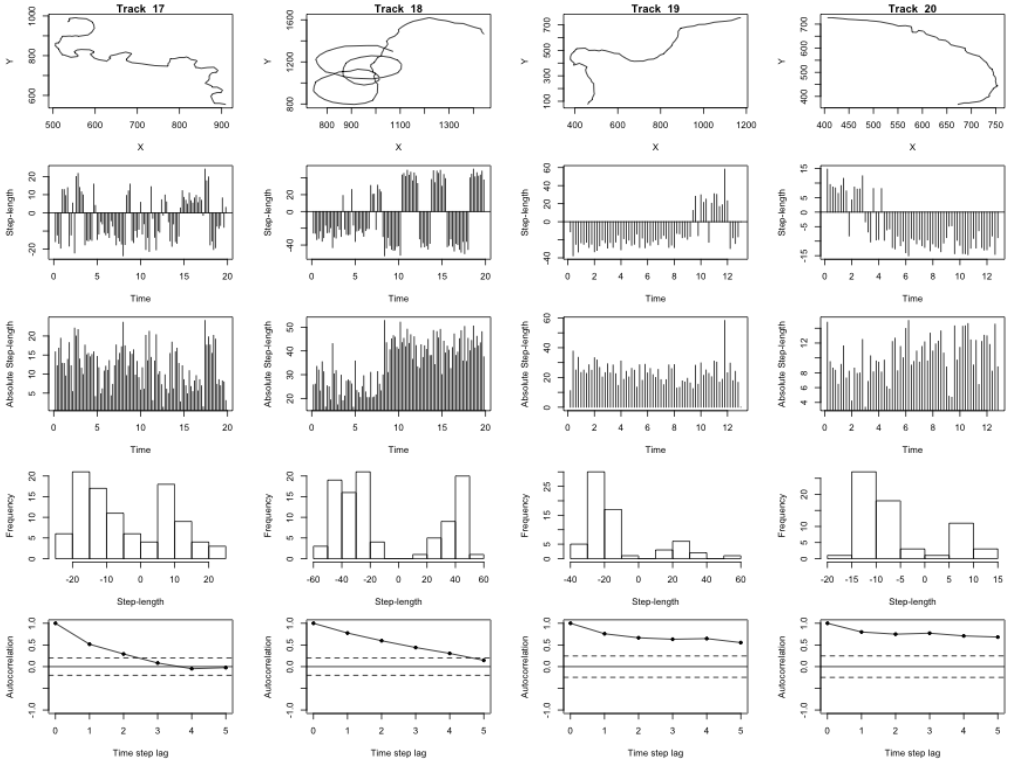
Data from the fractal analysis for each individual sperm is presented. Each sperm has a bank of 5 graphs which represent, from top to bottom; the path of the tracked cell, the direction of the cell over time; step lengths; the histograms indicate the frequency of each step length with bimodal skews indicating directionality; the last graph is a correlogram which shows the orientation of step lengths (after Abaigar et al. 2012). All sperm from two individual bulls (one for each preservation method, bull one freeze-thawed spermatozoa vs bull two fresh spermatozoa).




Track 5
Track 6
Track 7
Track 8
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 9
Track 10
Track 11
Track 12
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 13
Track 14
Track 15
Track 16
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag


Track 17
Track 18
Track 19
Track 20
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag

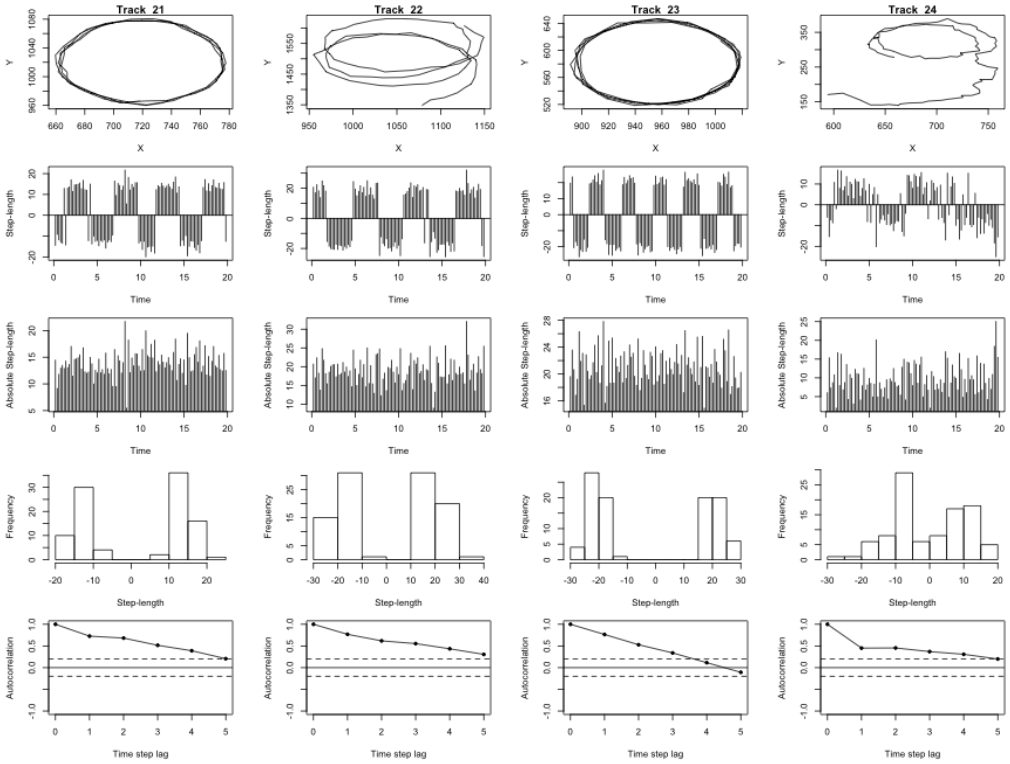
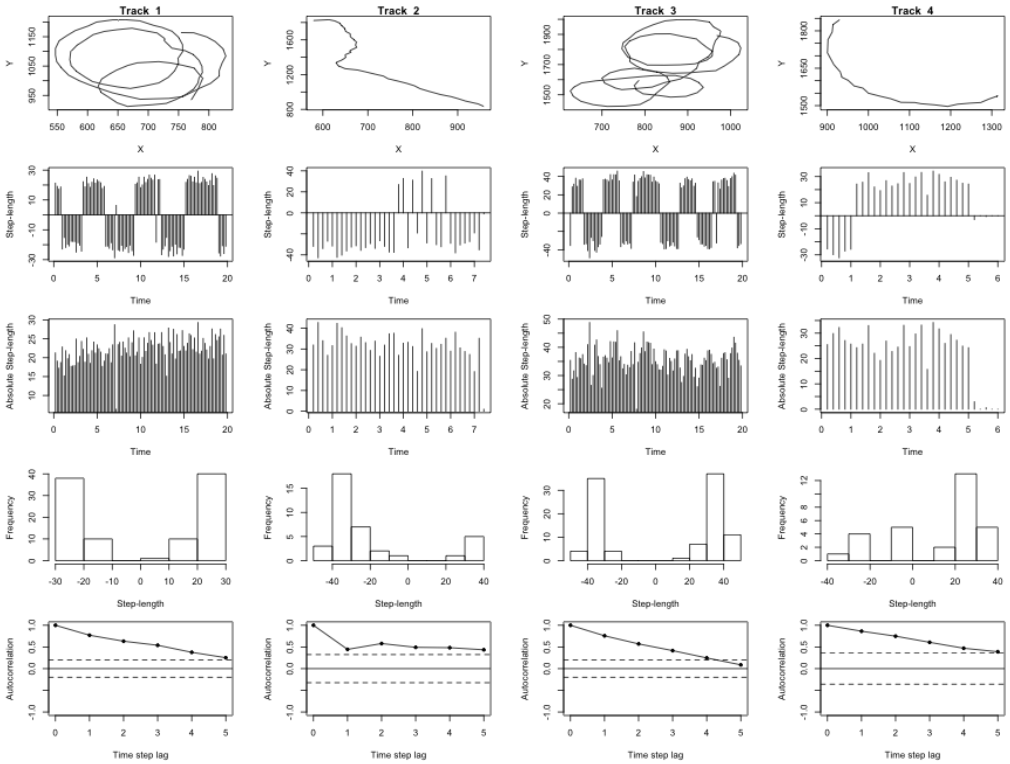
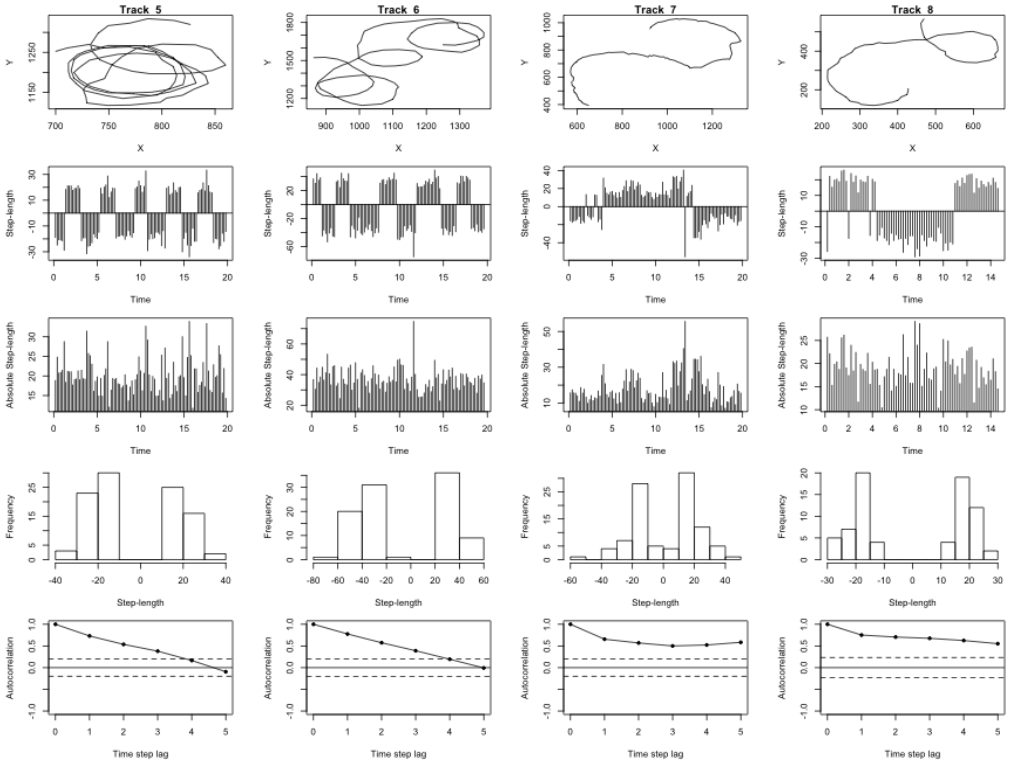
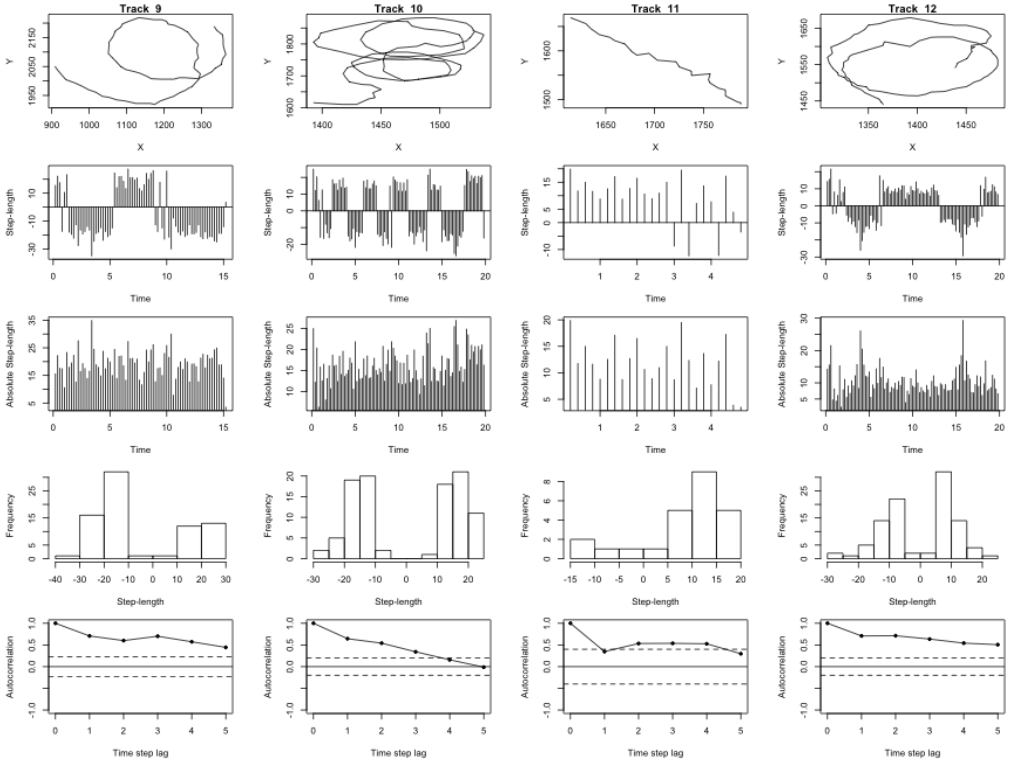
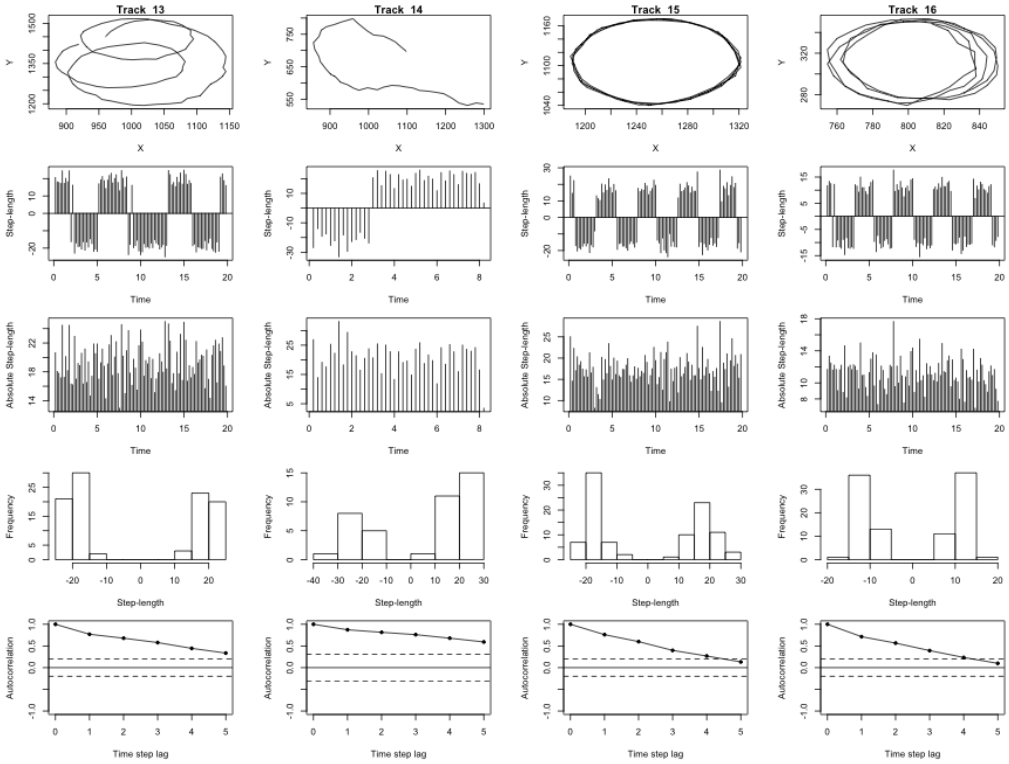


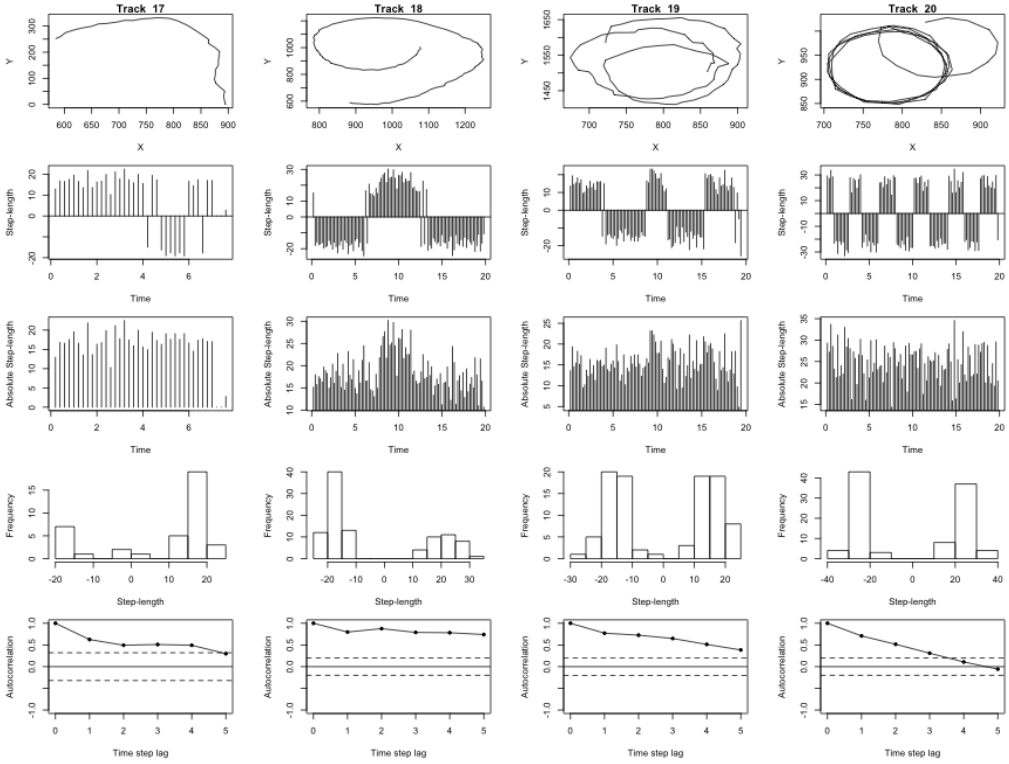
Figure 1A.1. Individual sperm track characteristics for freeze-thawed sperm in the control group without COC (n=24).

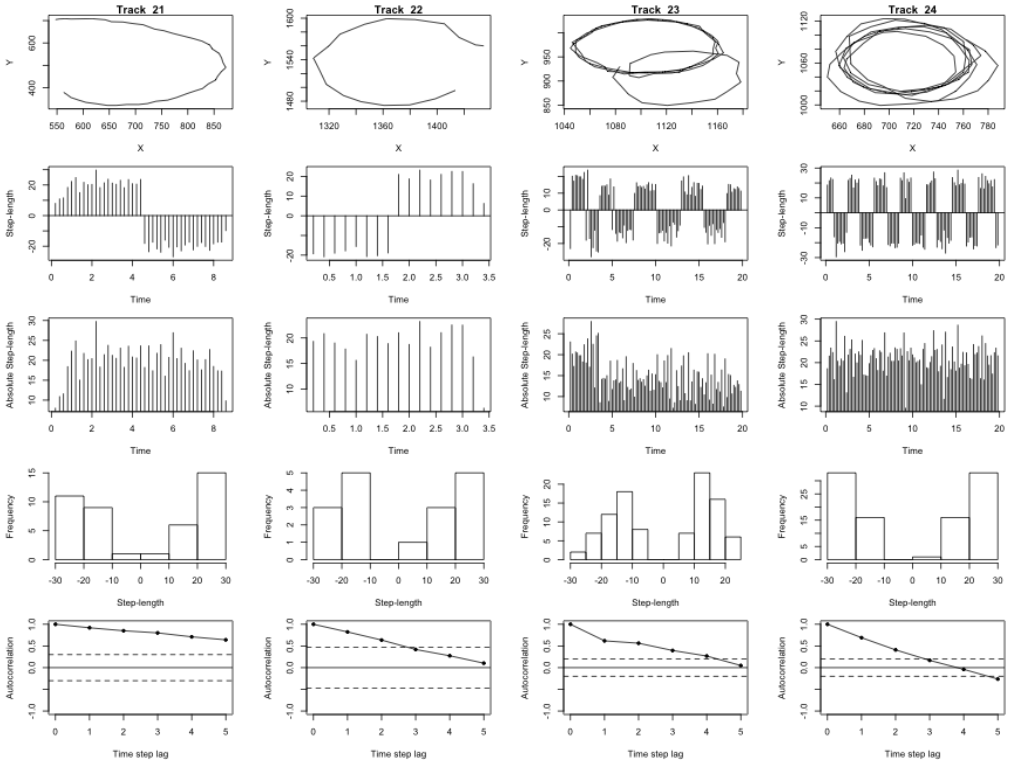


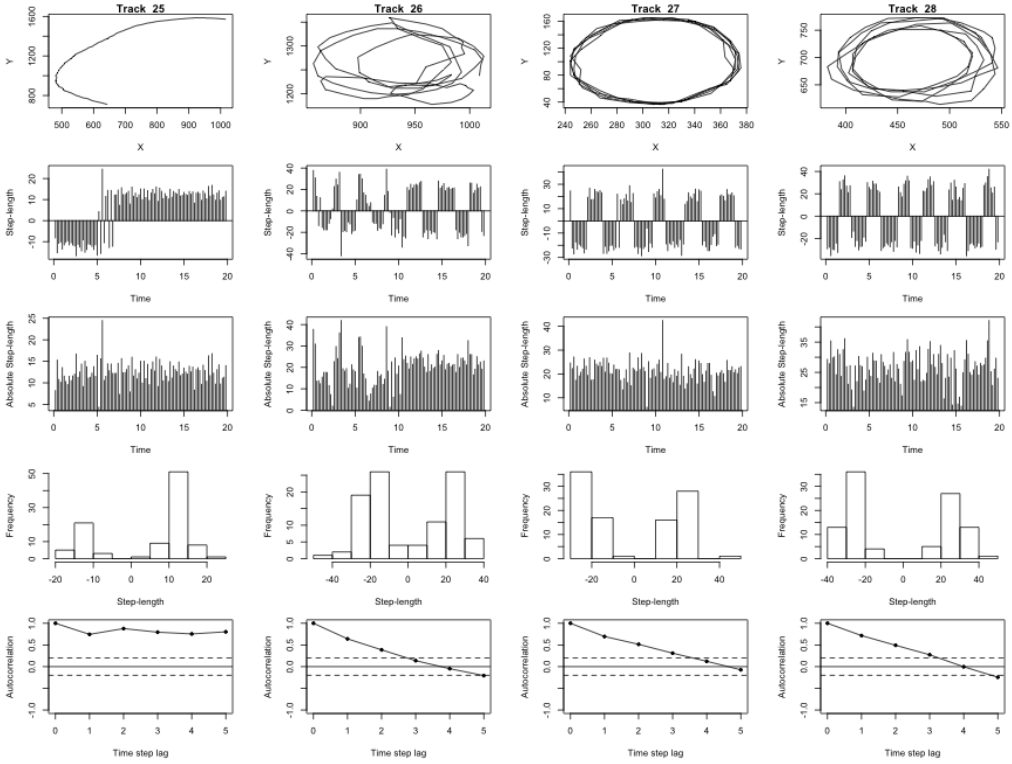

Track 5
Track 6
Track 7
Track 8
X
Y
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

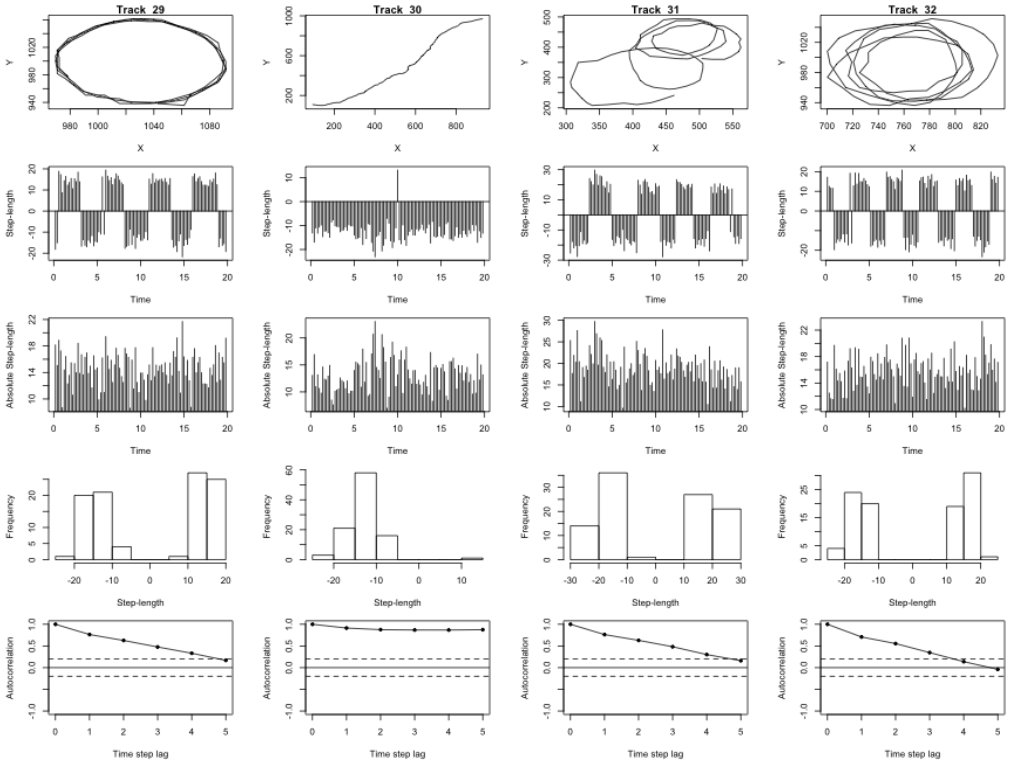

Track 9
Track 10
Track 11
Track 12
X
Y
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

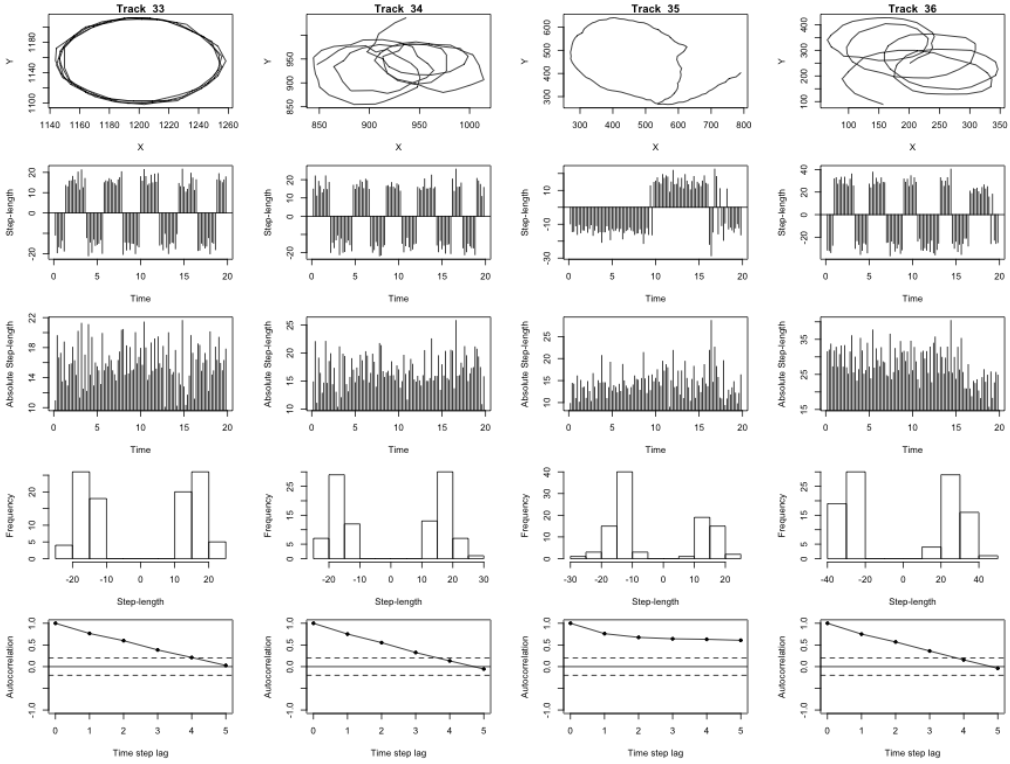

Track 13
Track 14
Track 15
Track 16
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag


Track 17
Track 18
Track 19
Track 20
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag


Track 21
Track 22
Track 23
Track 24
X
Y
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag


Track 25
Track 26
Track 27
Track 28
X
Y
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag


Track 29
Track 30
Track 31
Track 32
Y
X
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag


Track 33
Track 34
Track 35
Track 36
Y
X
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag

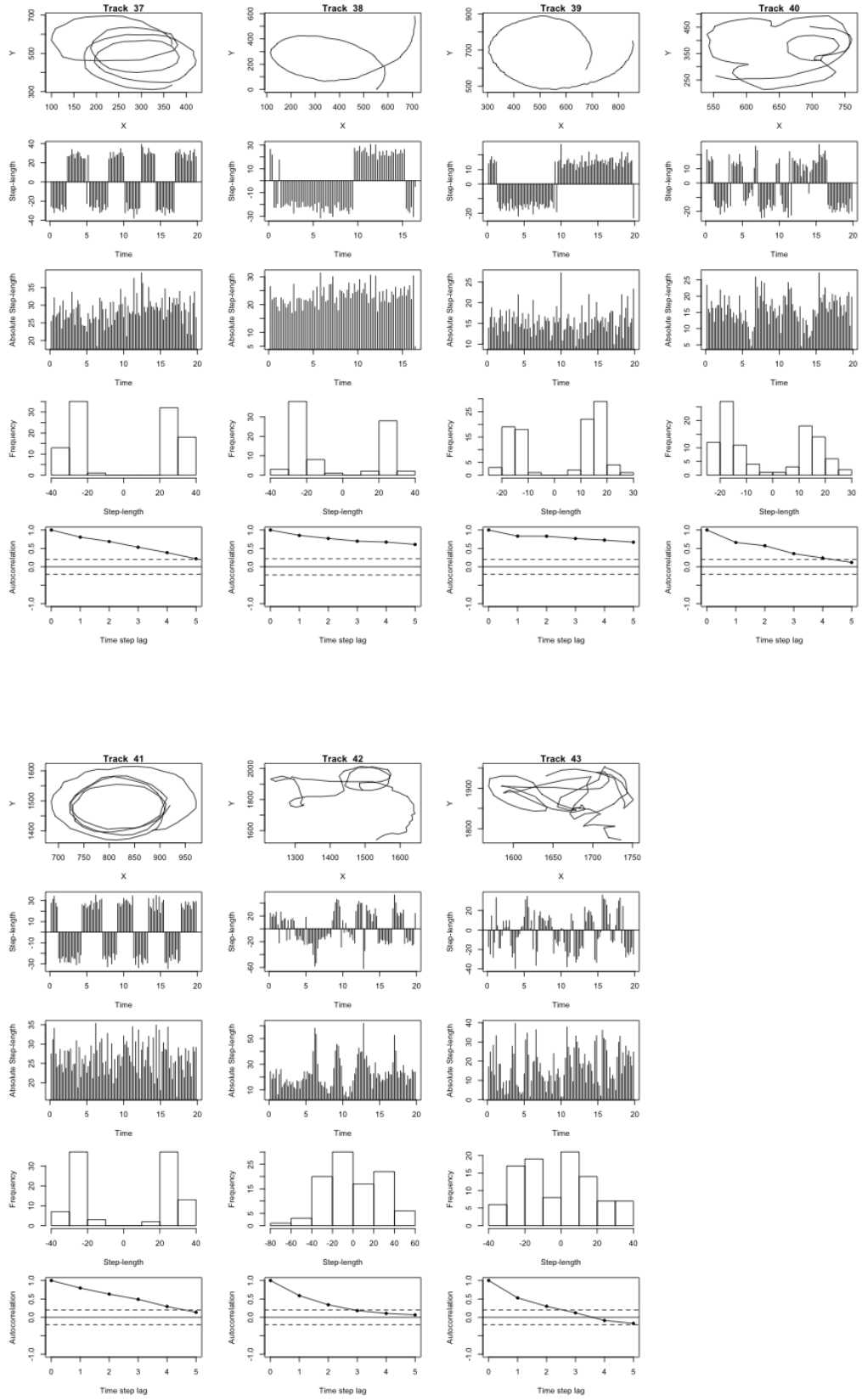
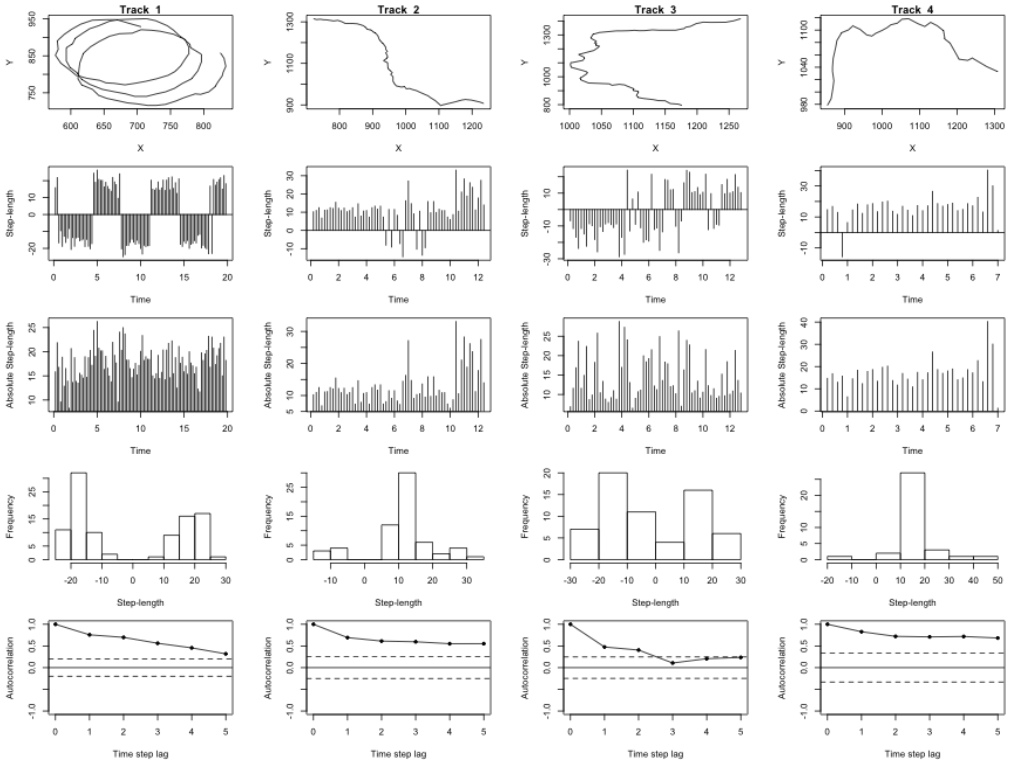
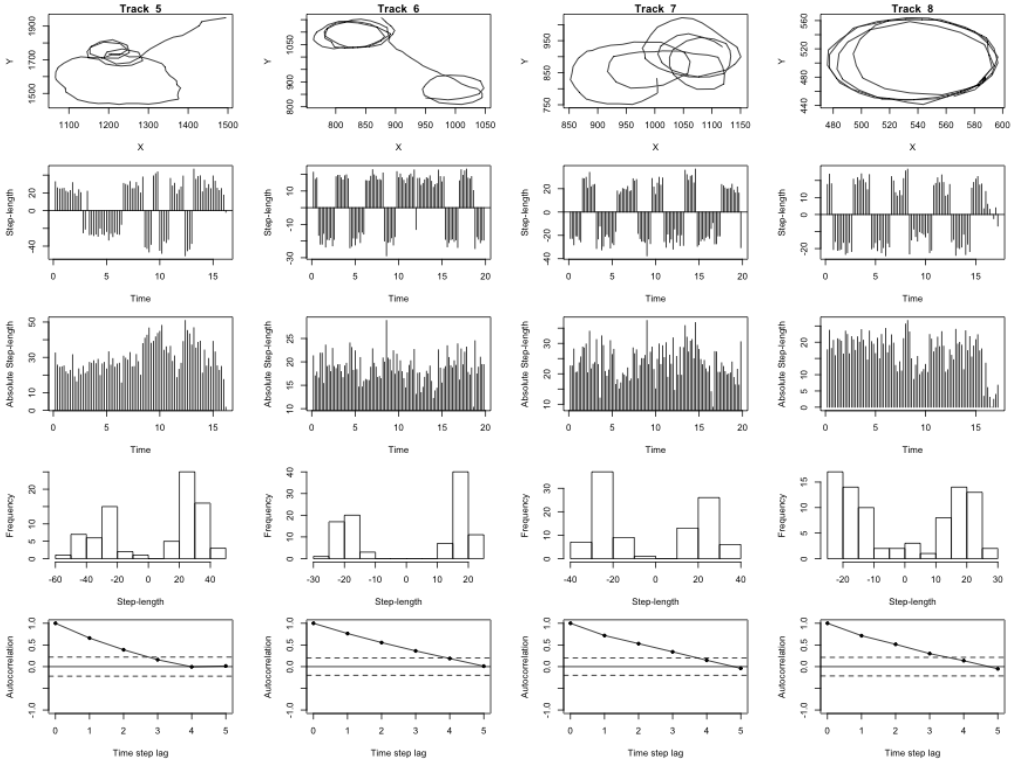
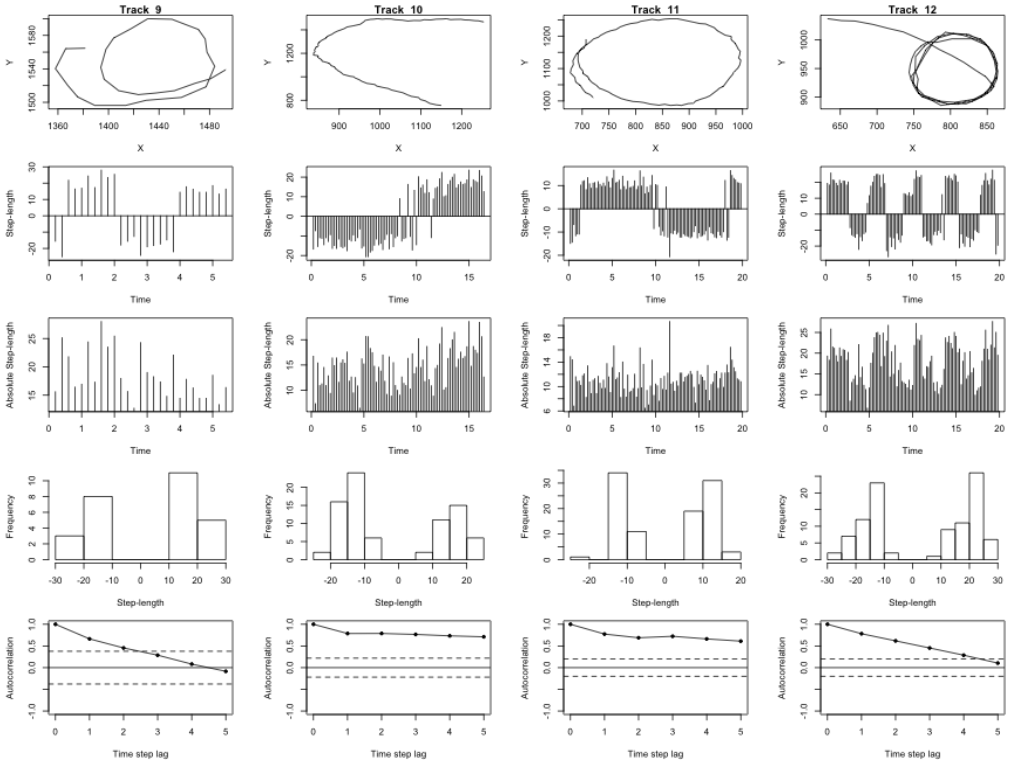
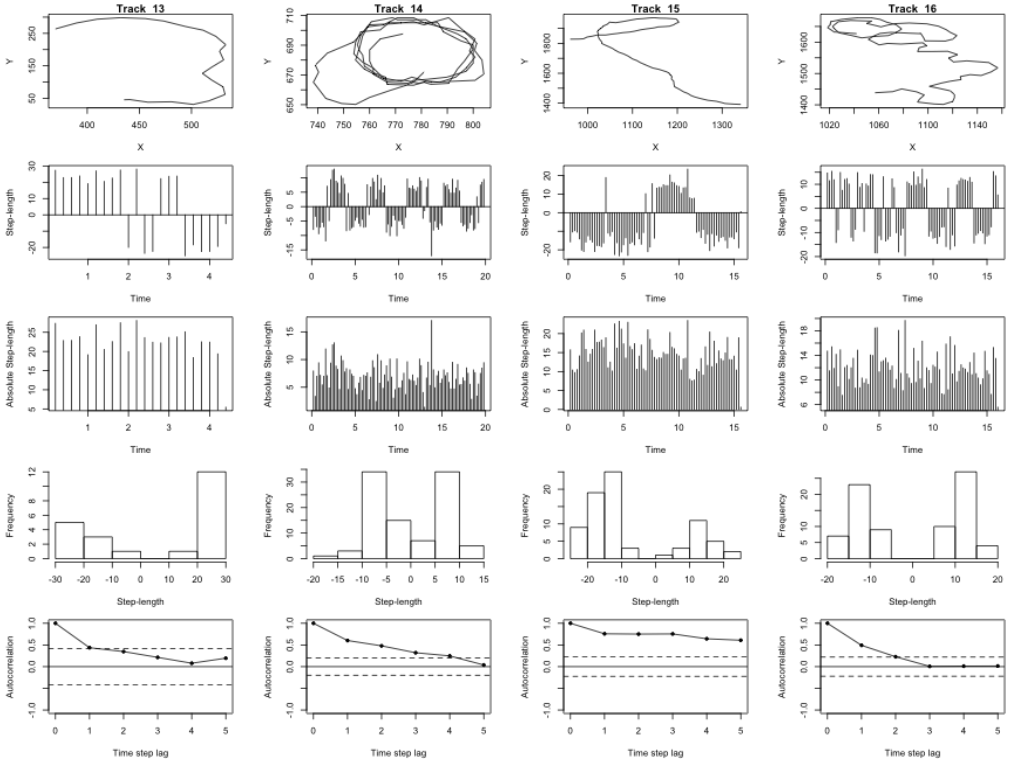


Figure 1A.2. Individual sperm track characteristics for freeze-thawedsperm in the presence of one COC (n=43)


Track 1
Track 2
Track 3
Track 4
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 5
Track 6
Track 7
Track 8
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 9
Track 10
Track 11
Track 12
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 13
Track 14
Track 15
Track 16
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

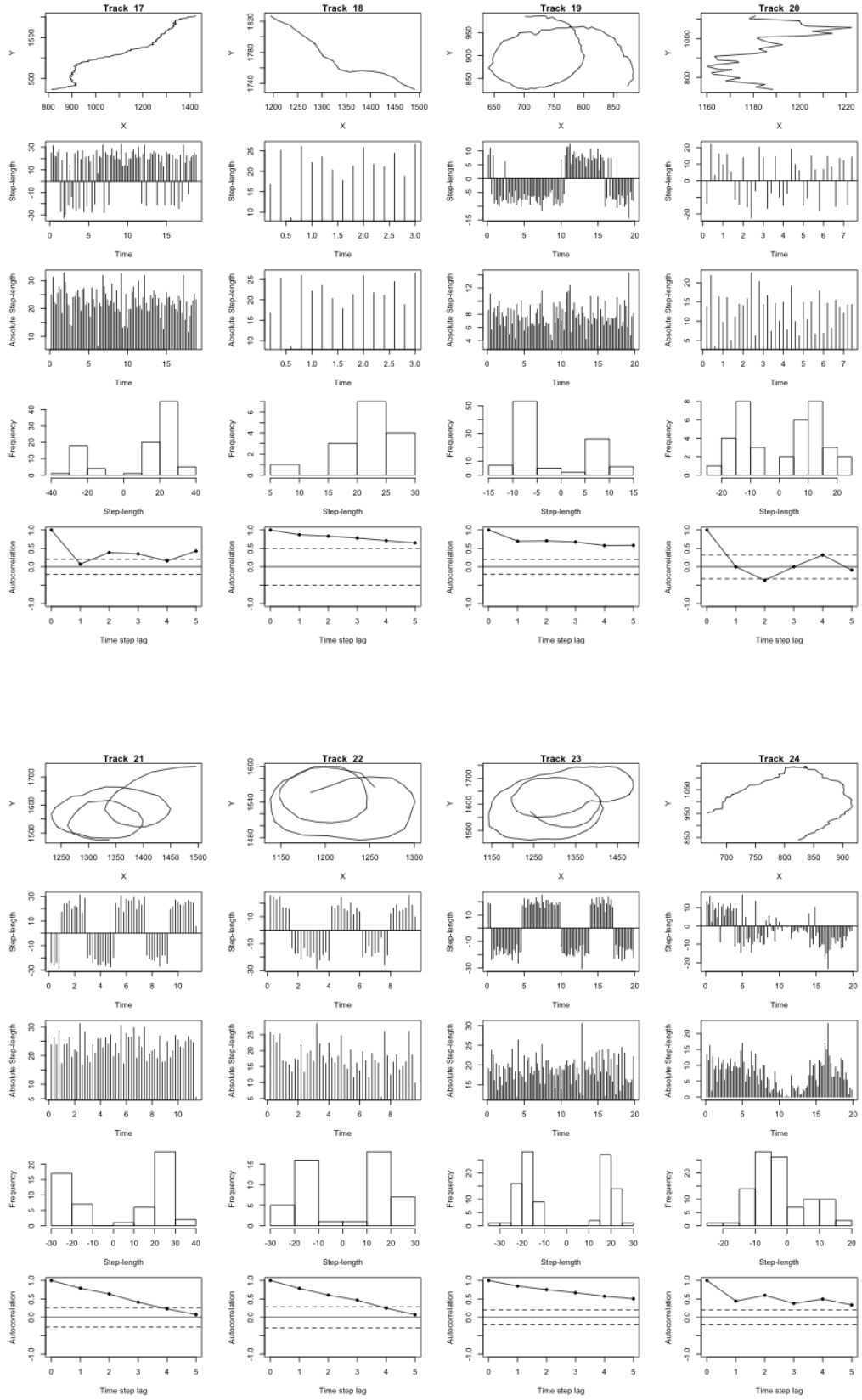
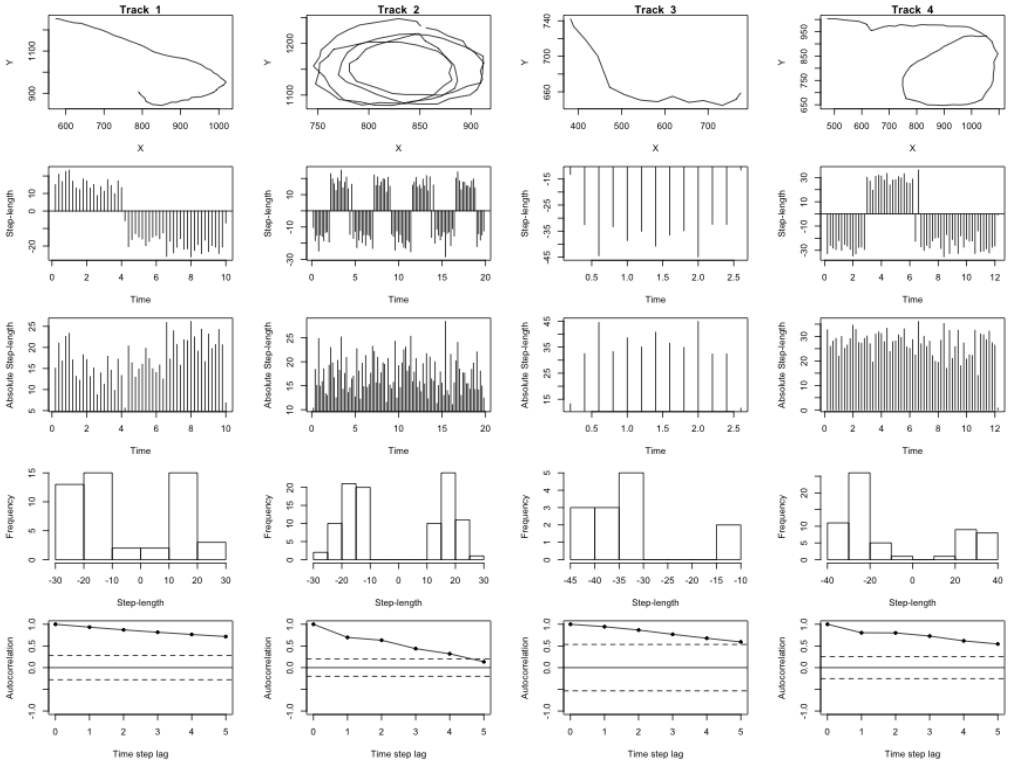
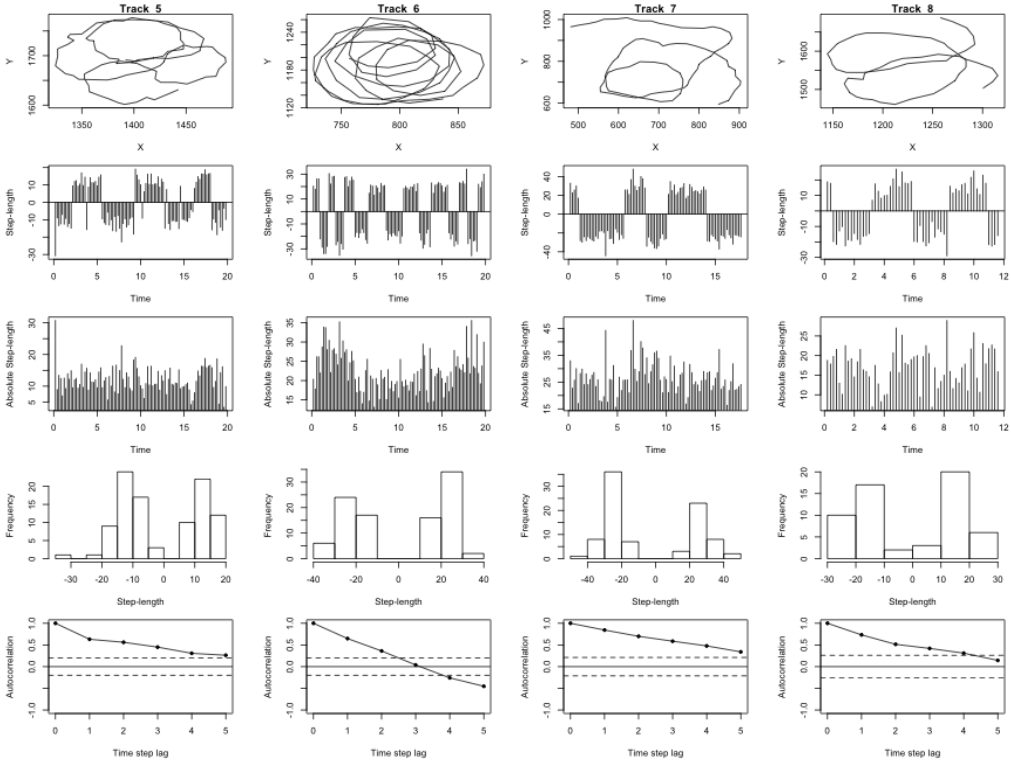


Figure 1A.3. Individual sperm track characteristics for freeze-thawed sperm in the presence of two COC (n=24)


Track 1
Track 2
Track 3
Track 4
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 5
Track 6
Track 7
Track 8
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

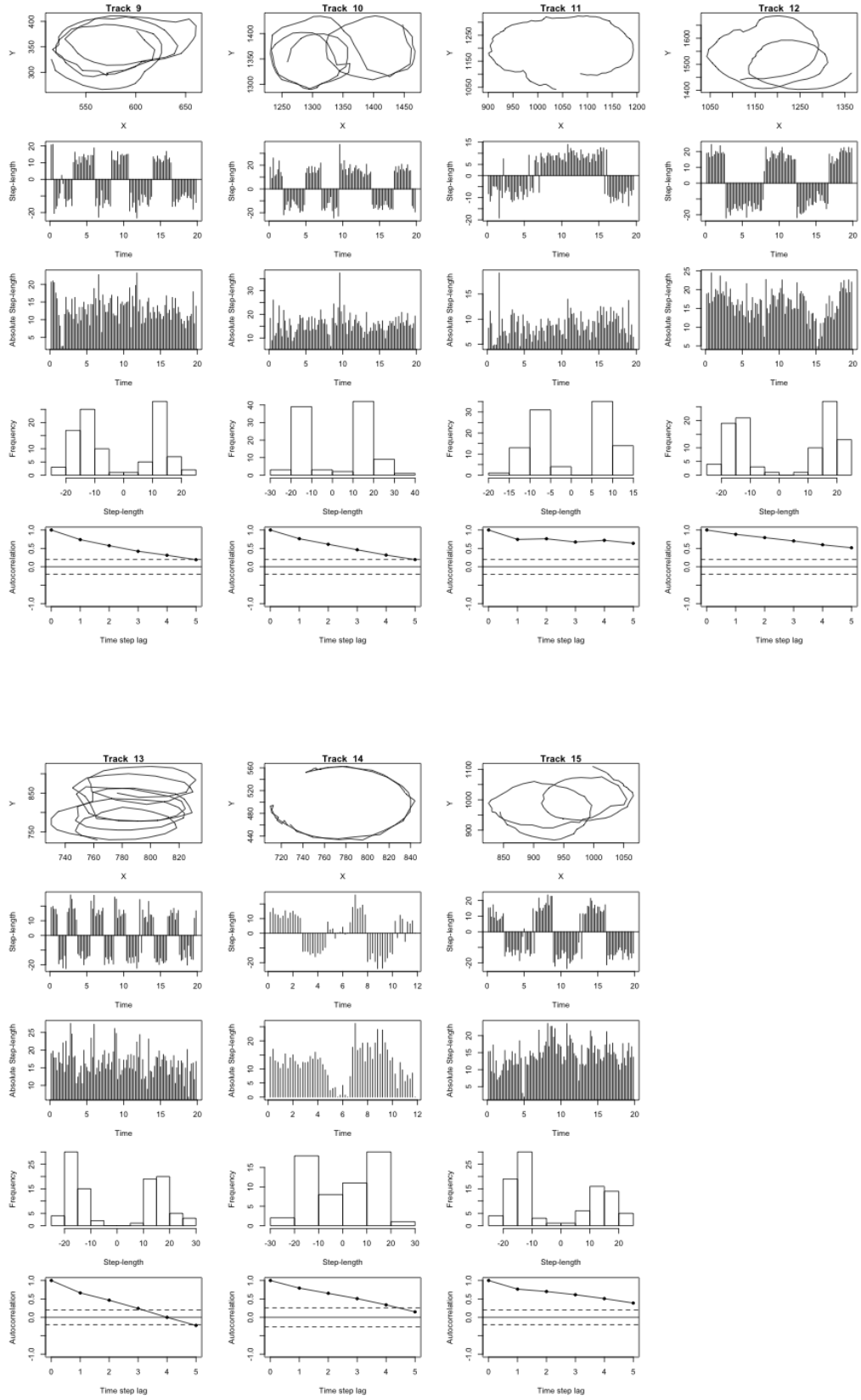
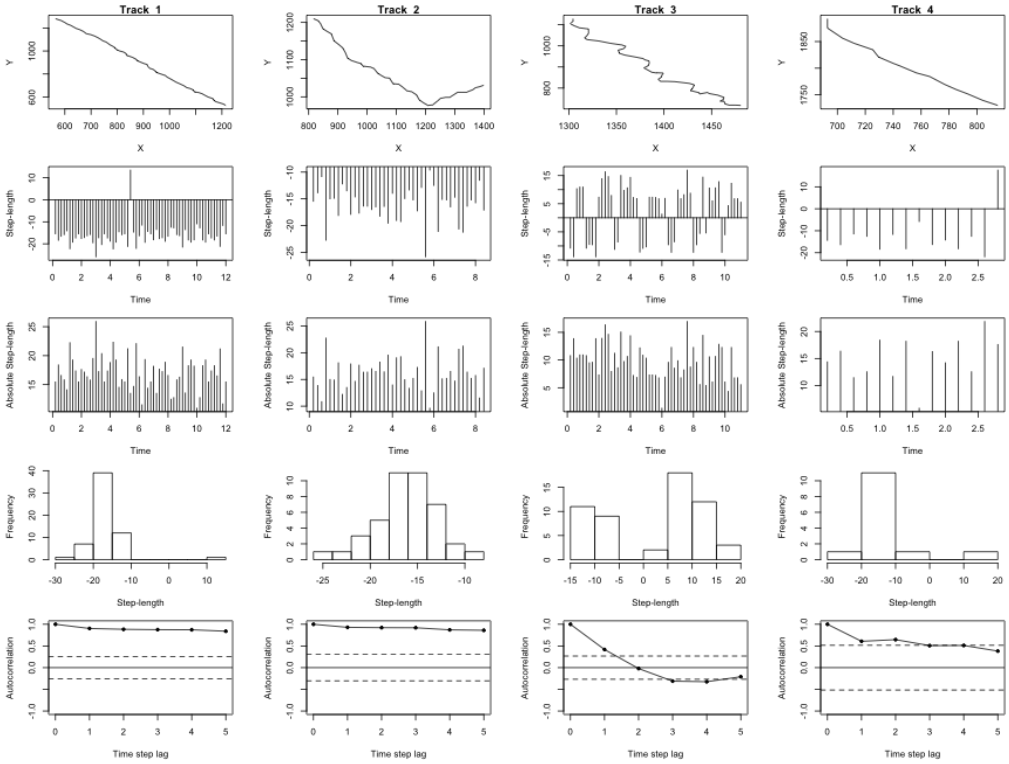
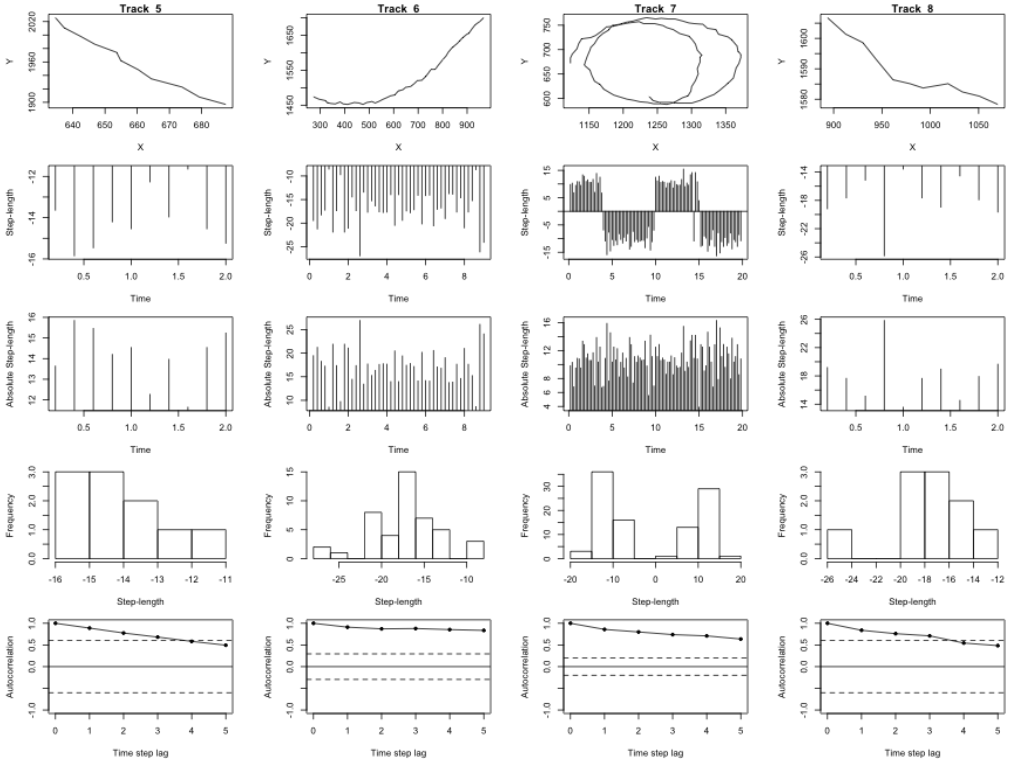
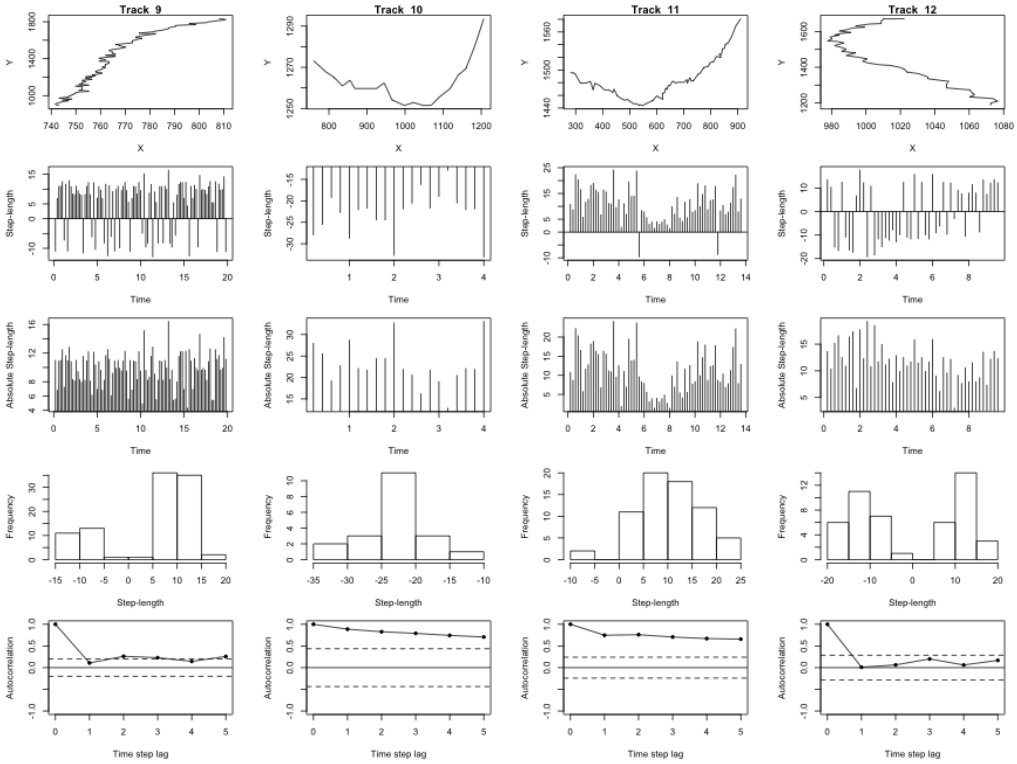
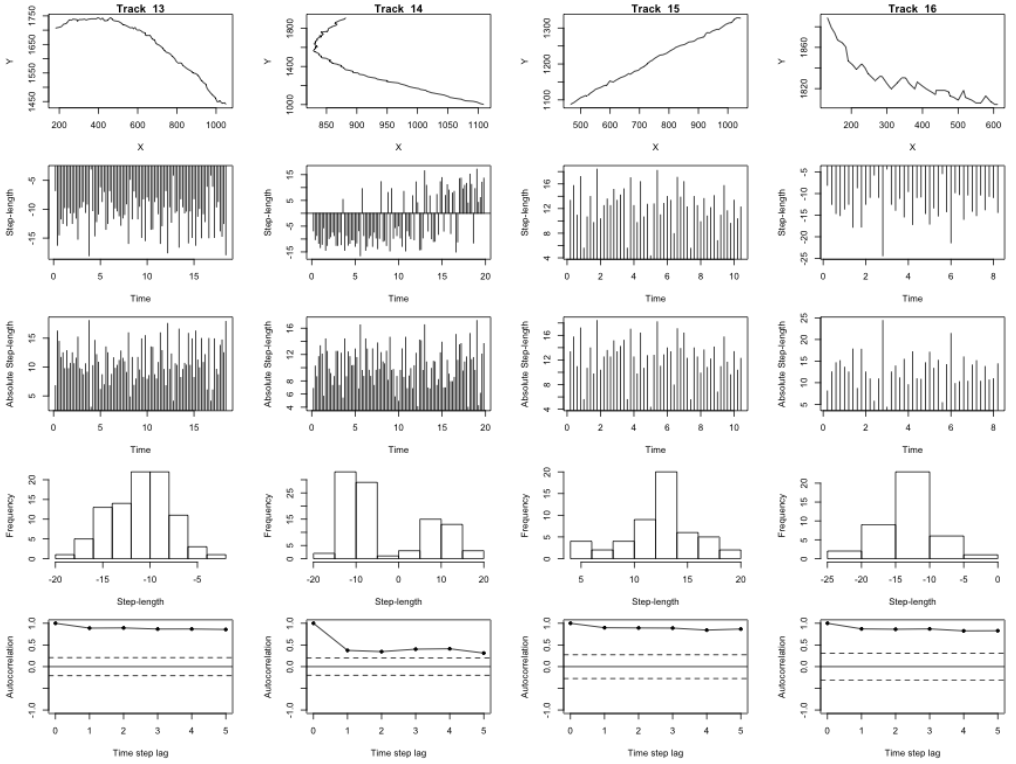


Figure 1A.4. Individual sperm track characteristics for freeze-thawed sperm in the presence of three COC (n=15)


Track 1
Track 2
Track 3
Track 4
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag


Track 5
Track 6
Track 7
Track 8
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag


Track 9
Track 10
Track 11
Track 12
X
Y
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 13
Track 14
Track 15
Track 16
X
Y
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

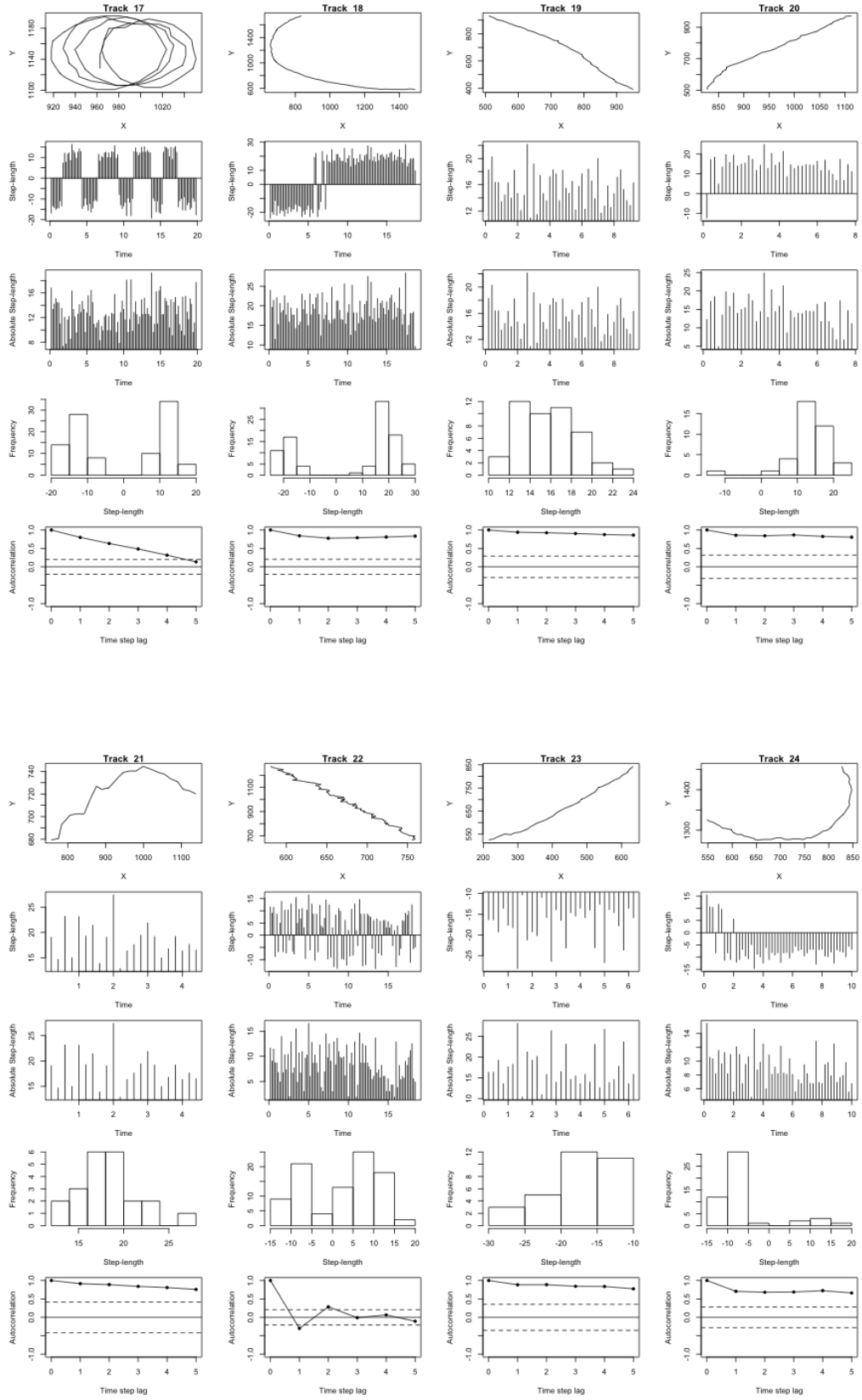
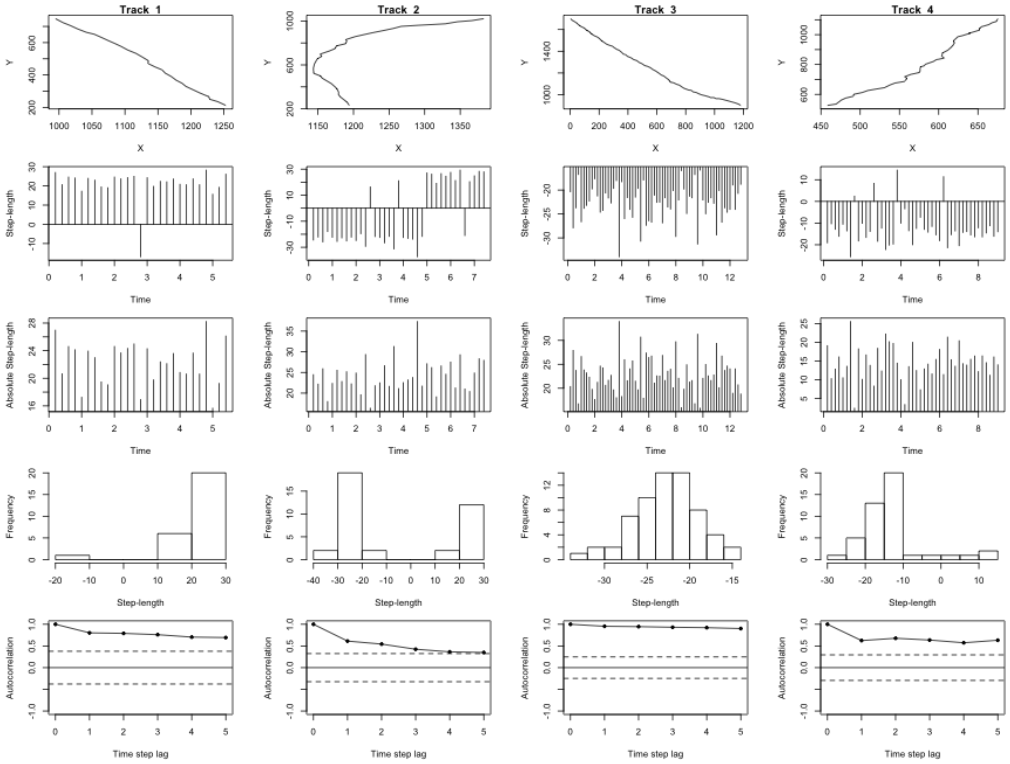
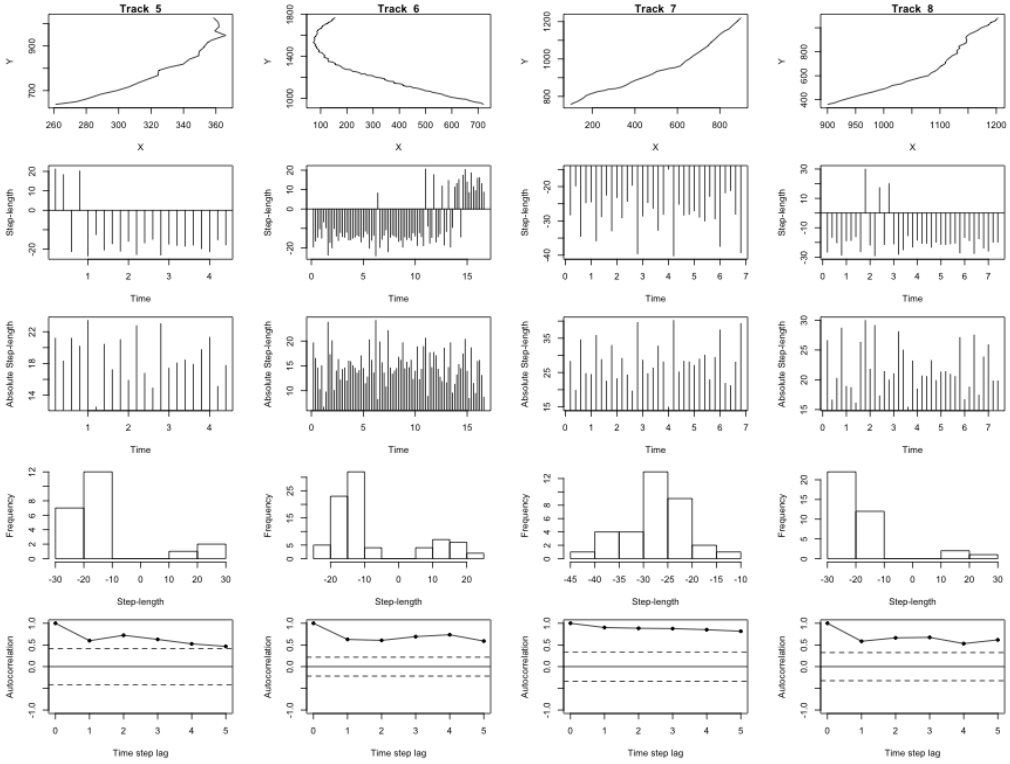
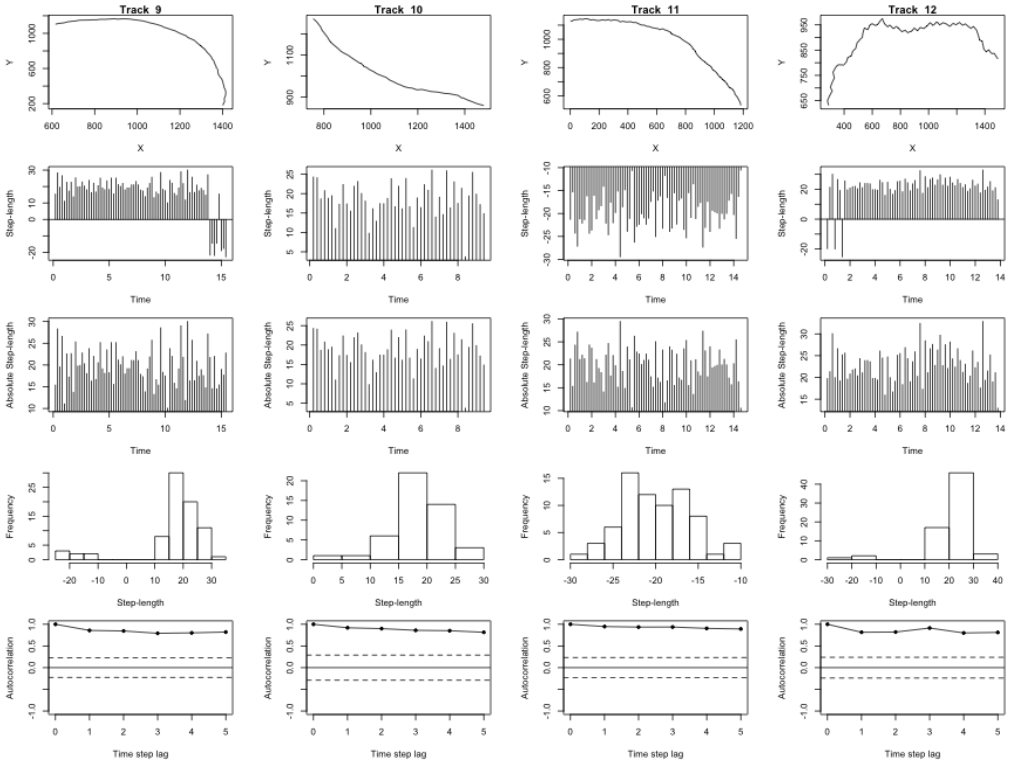
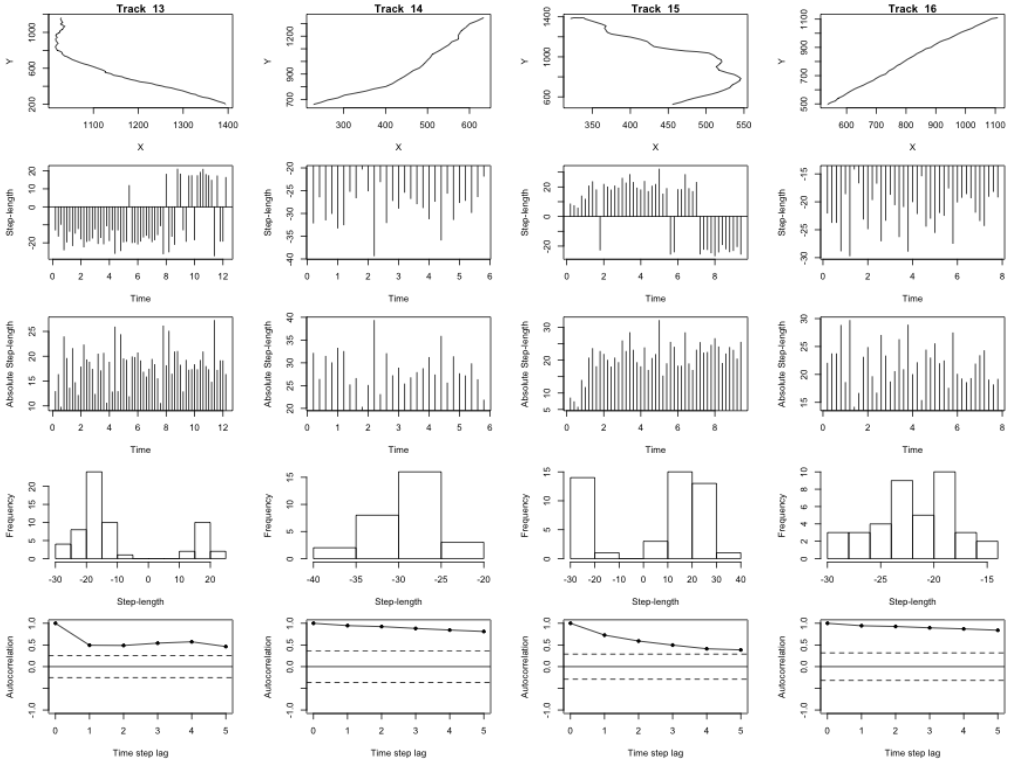


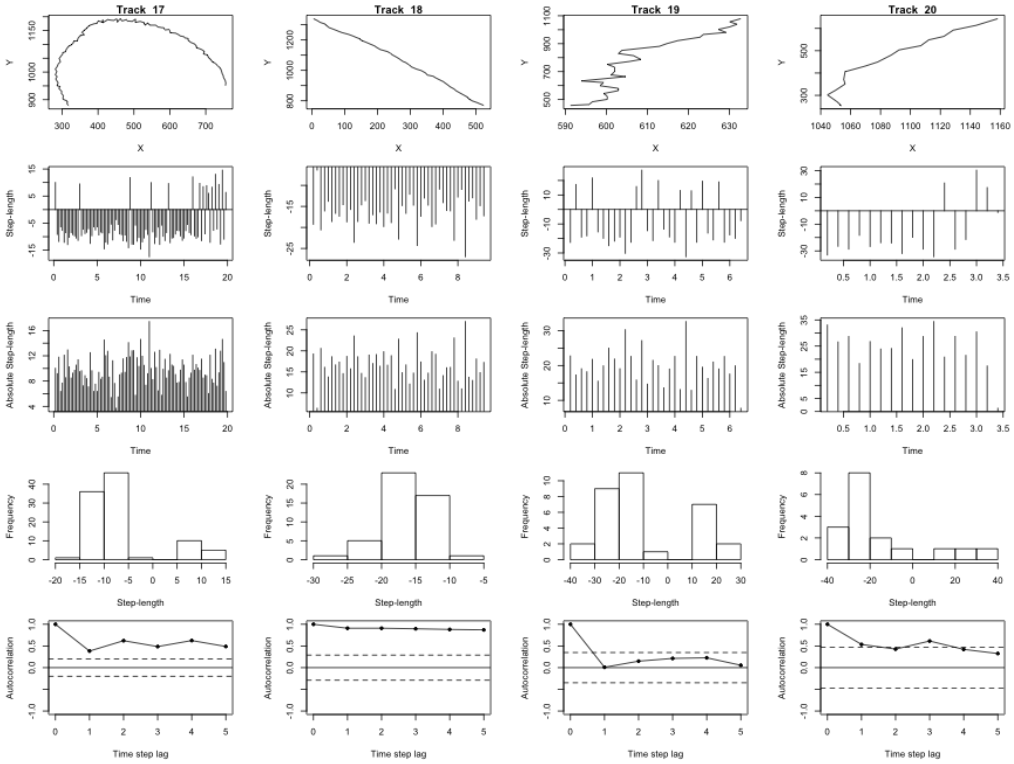
Figure 1A.5. Individual sperm track characteristics for fresh capacitated sperm in the control group without COC (n=24)

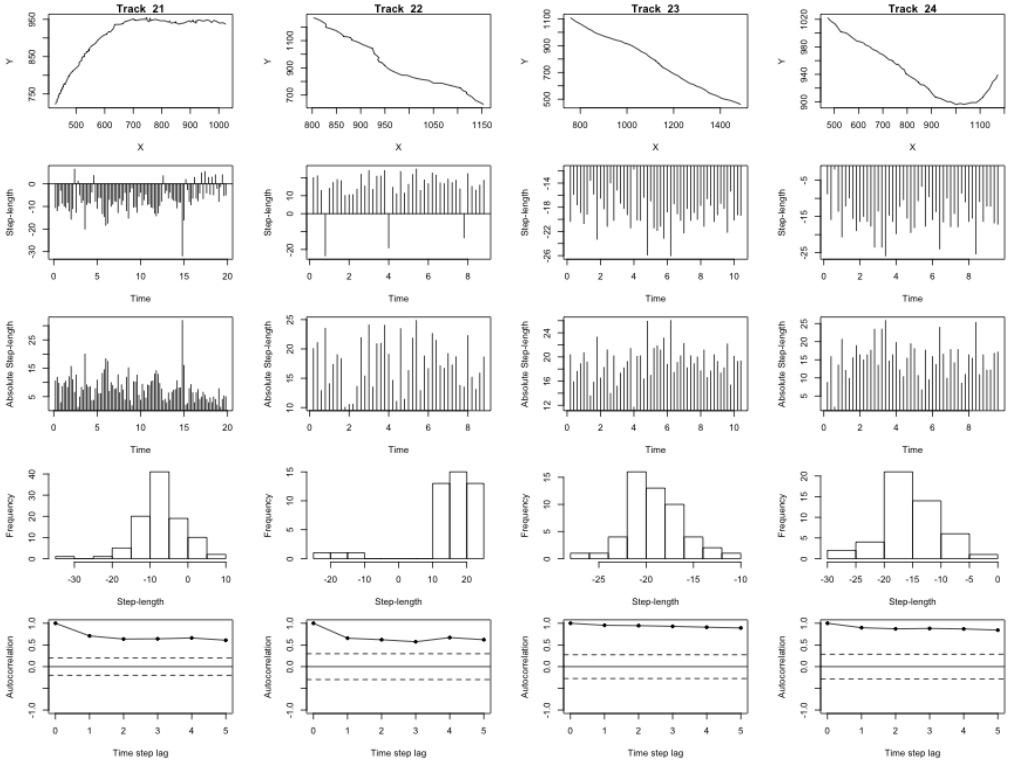

Track 1
Track 2
Track 3
Track 4
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

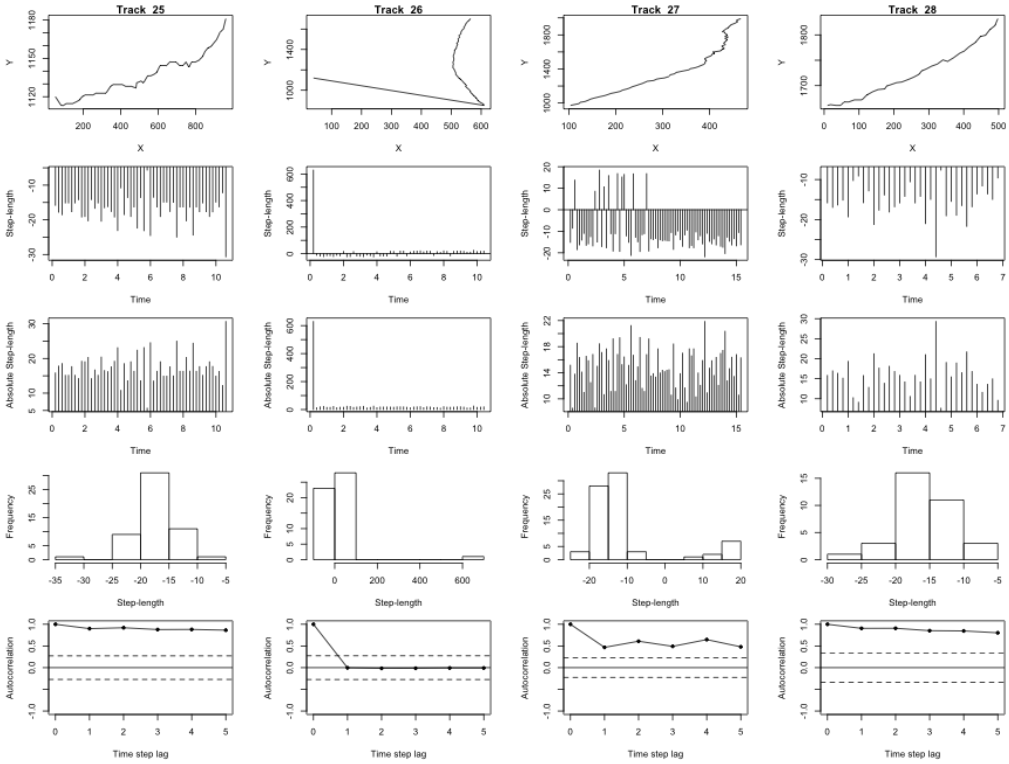

Track 5
Track 6
Track 7
Track 8
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

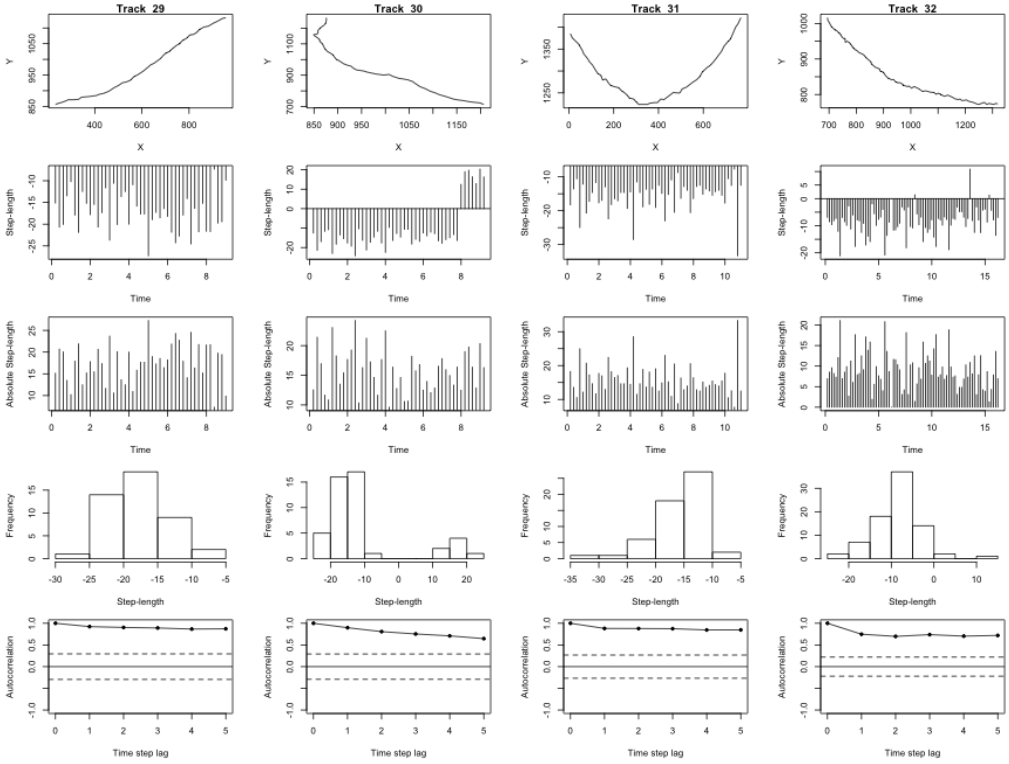

Track 9
Track 10
Track 11
Track 12
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 13
Track 14
Track 15
Track 16
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 17
Track 18
Track 19
Track 20
X
Y
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag


Track 21
Track 22
Track 23
Track 24
X
Y
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag


Track 25
Track 26
Track 27
Track 28
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 29
Track 30
Track 31
Track 32
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

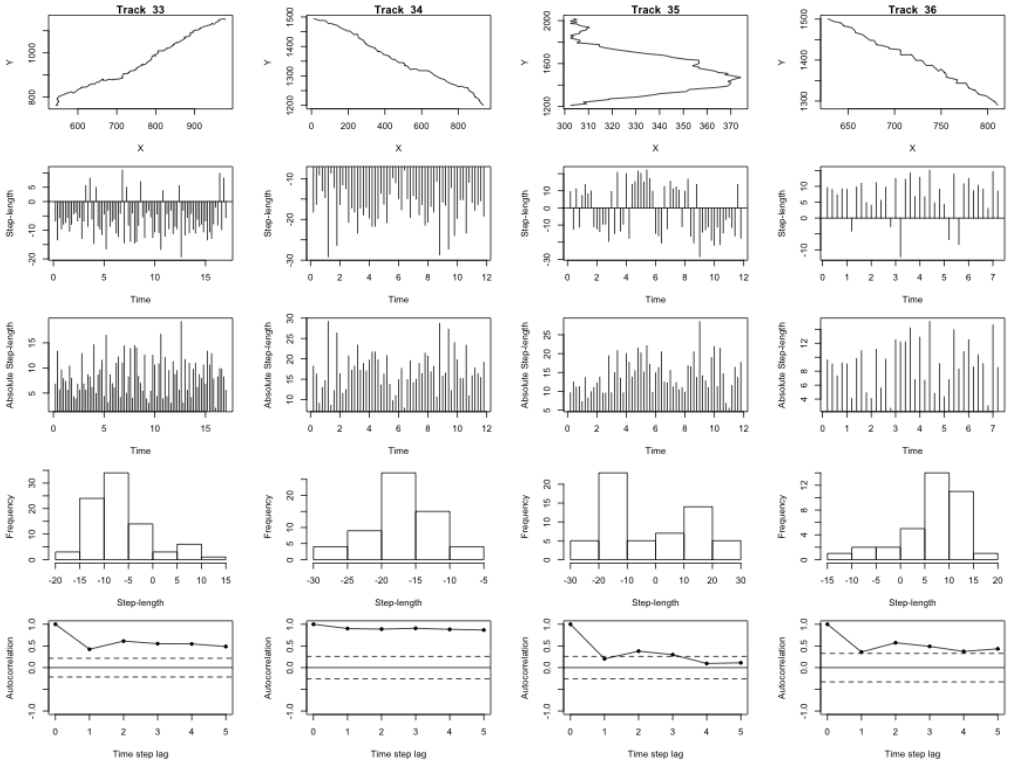
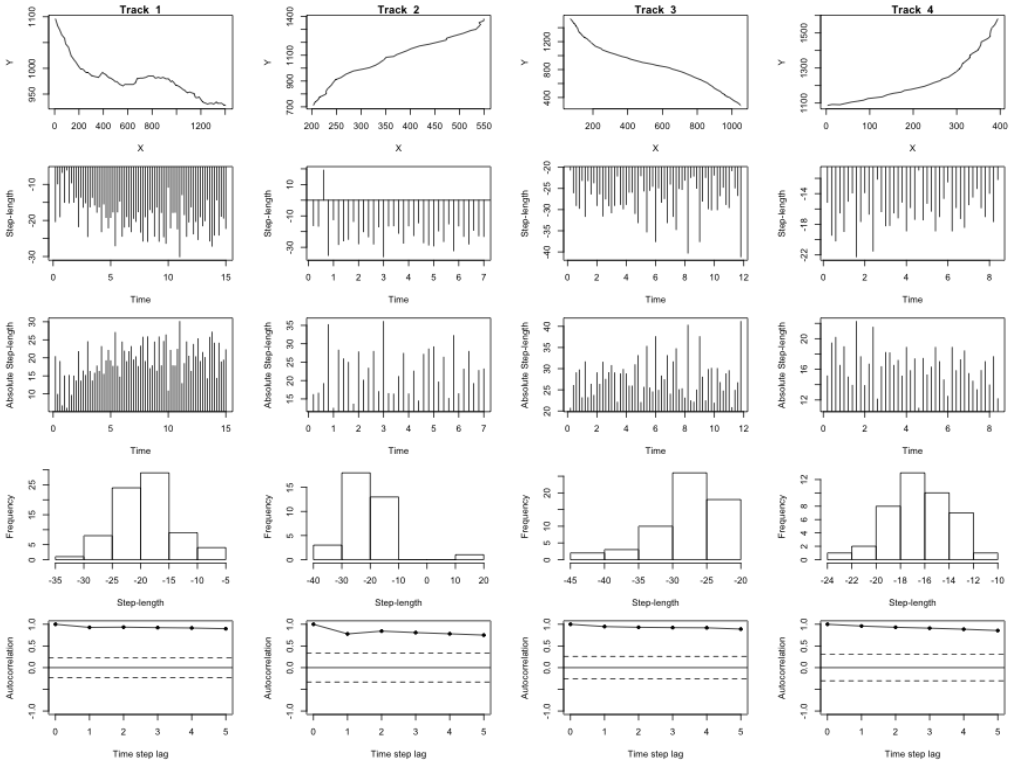
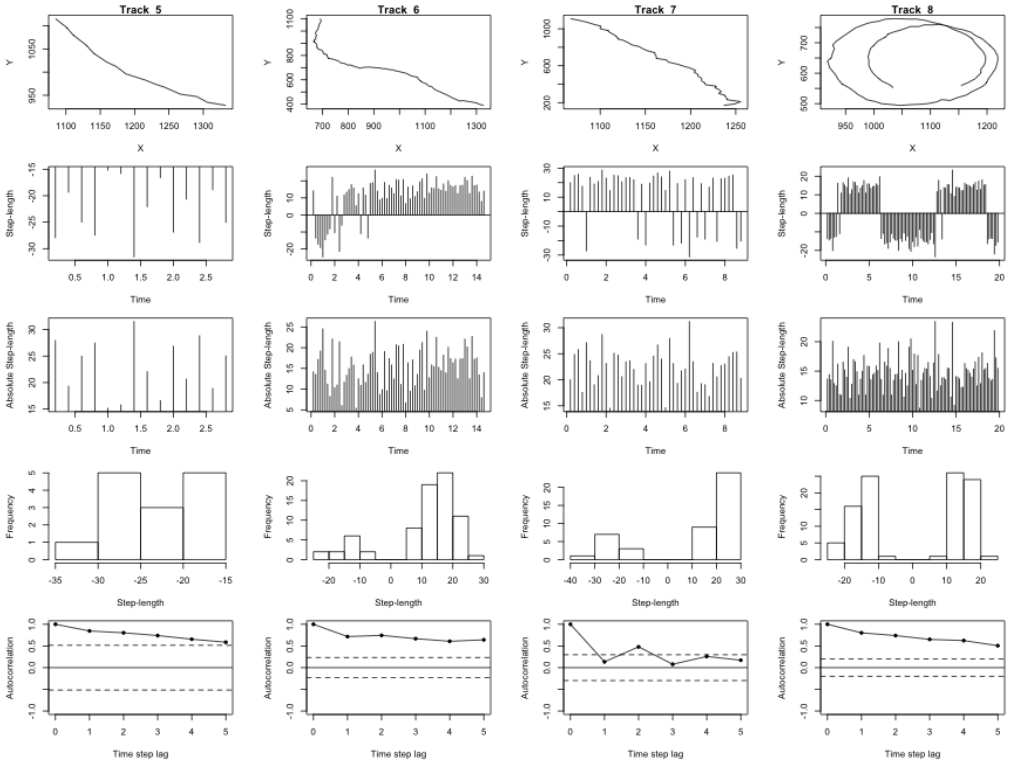
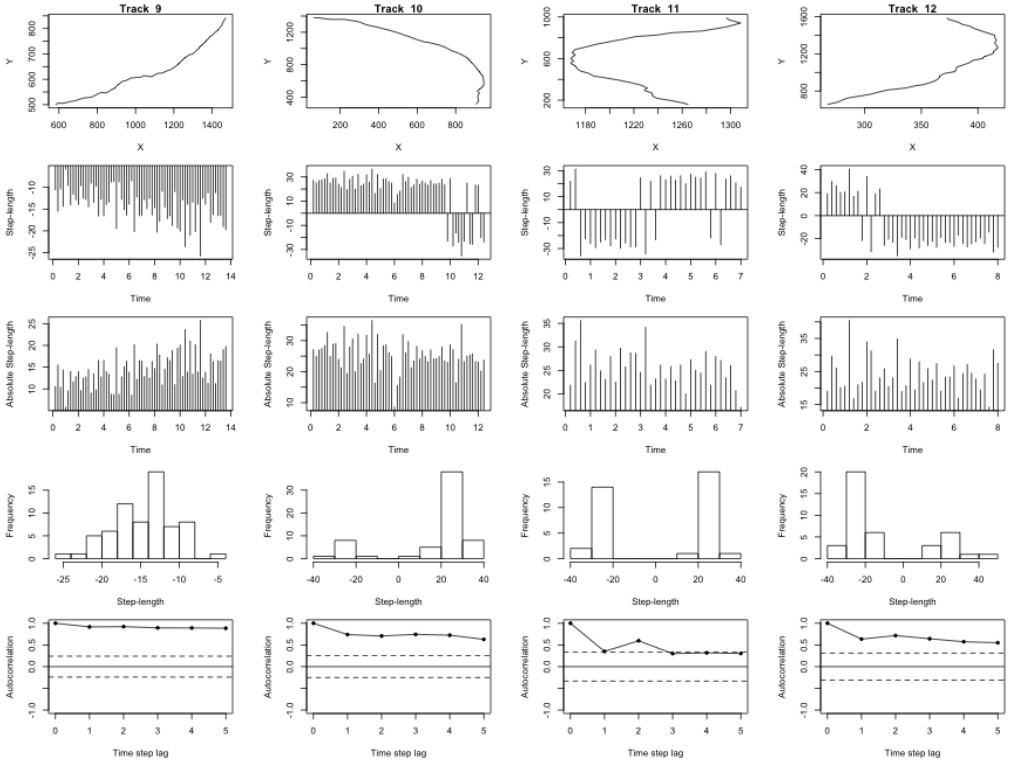
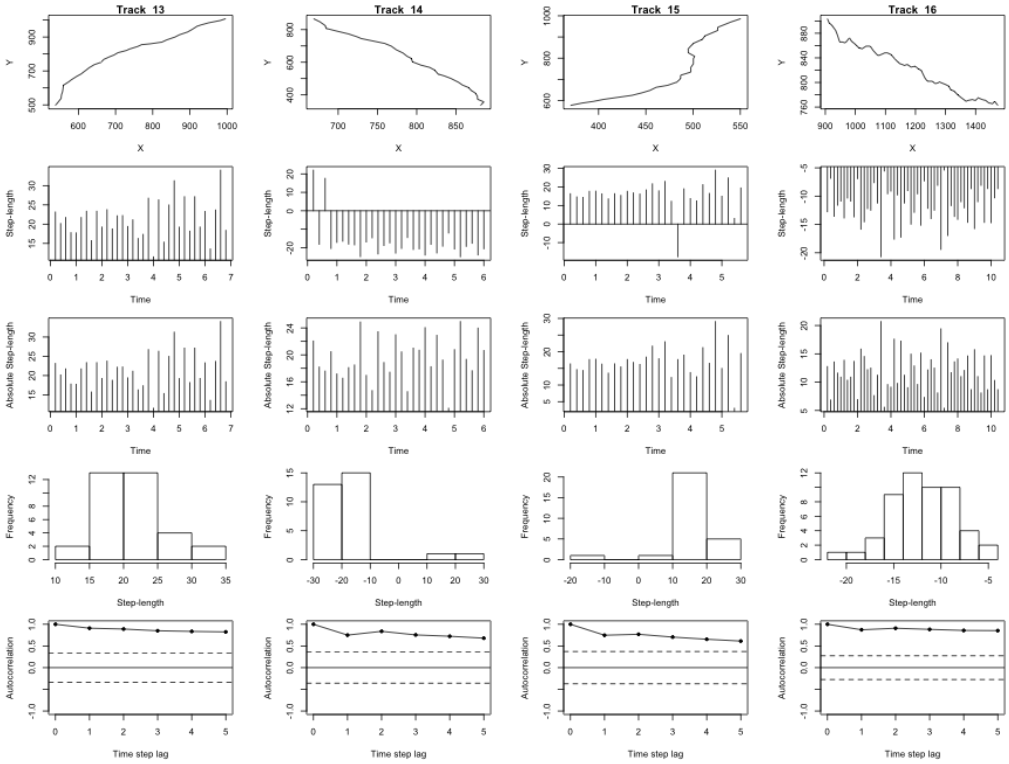


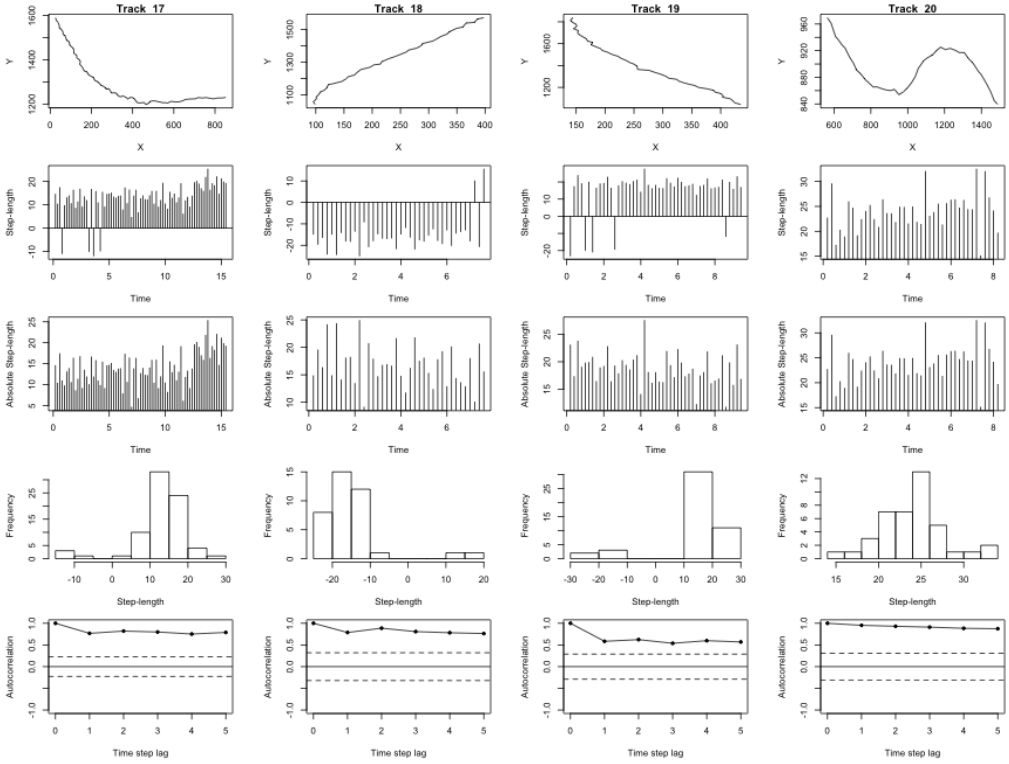
Figure 1A.6. Individual sperm track characteristics for fresh capacitatied sperm in the presence of one COC (n=36)

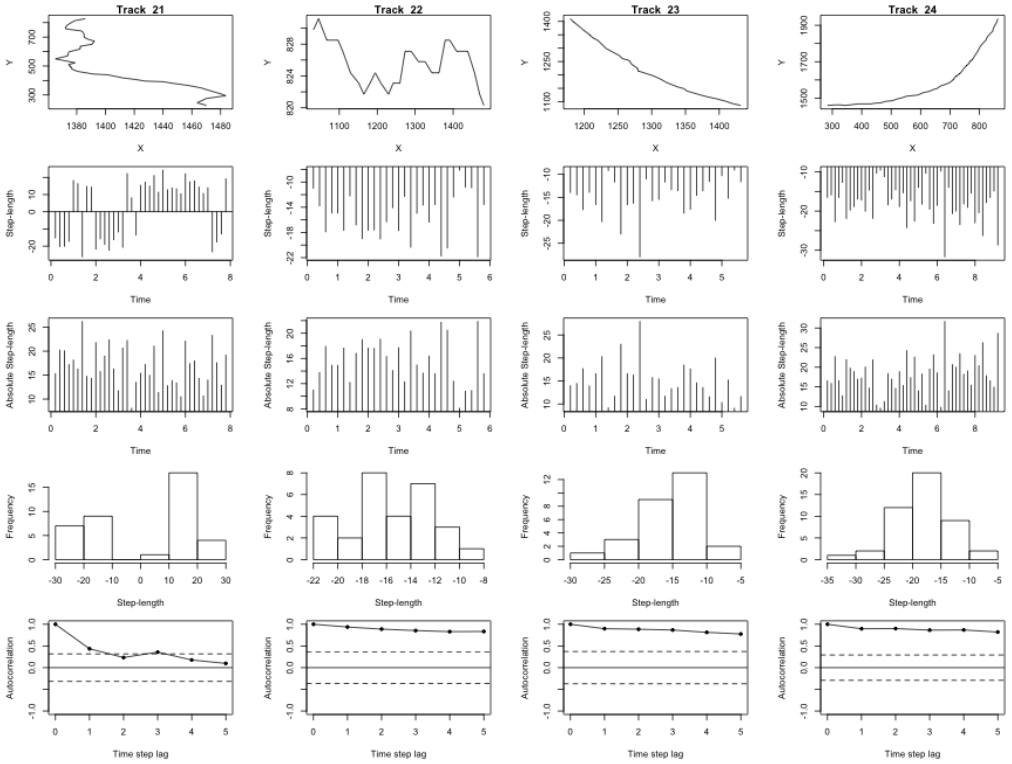


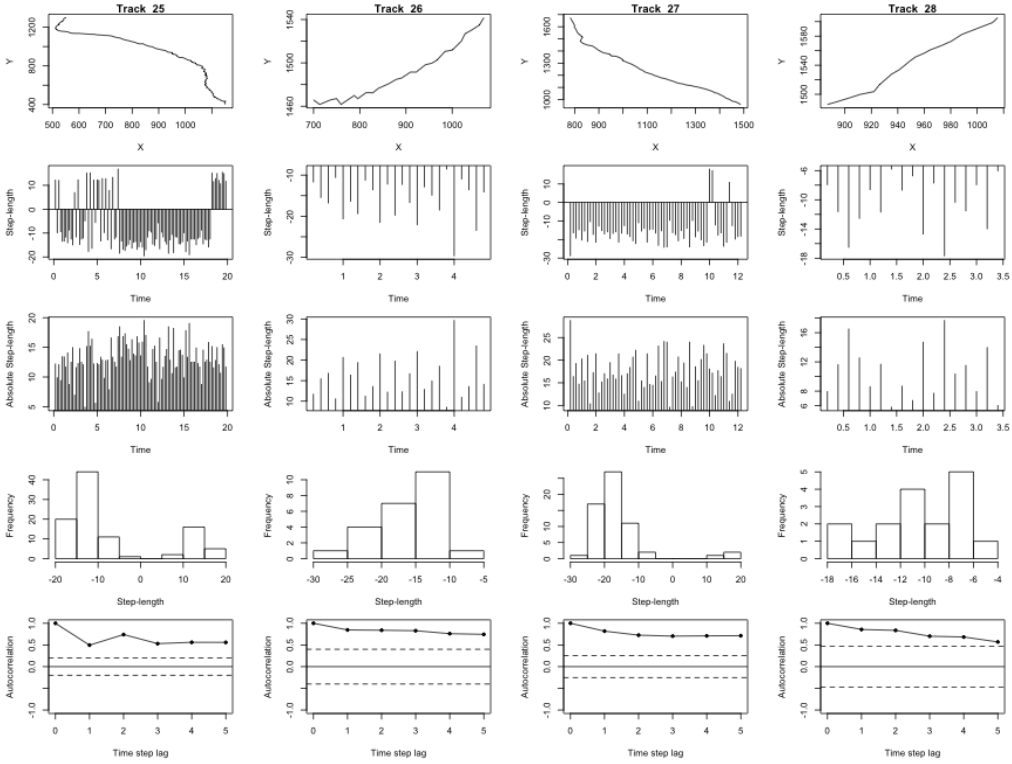

Track 5
Track 6
Track 7
Track 8
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 9
Track 10
Track 11
Track 12
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 13
Track 14
Track 15
Track 16
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 17
Track 18
Track 19
Track 20
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 21
Track 22
Track 23
Track 24
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag


Track 25
Track 26
Track 27
Track 28
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag

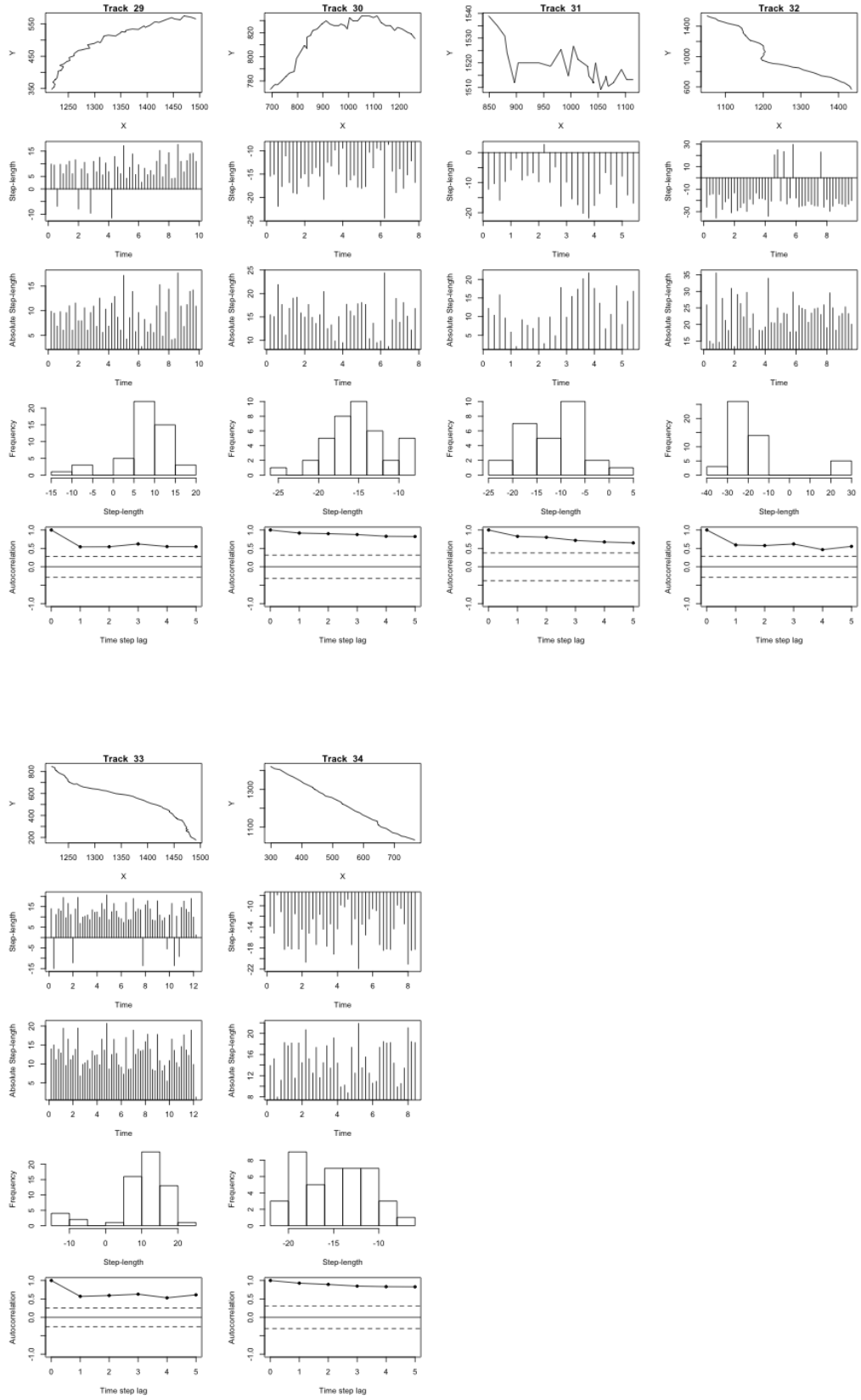
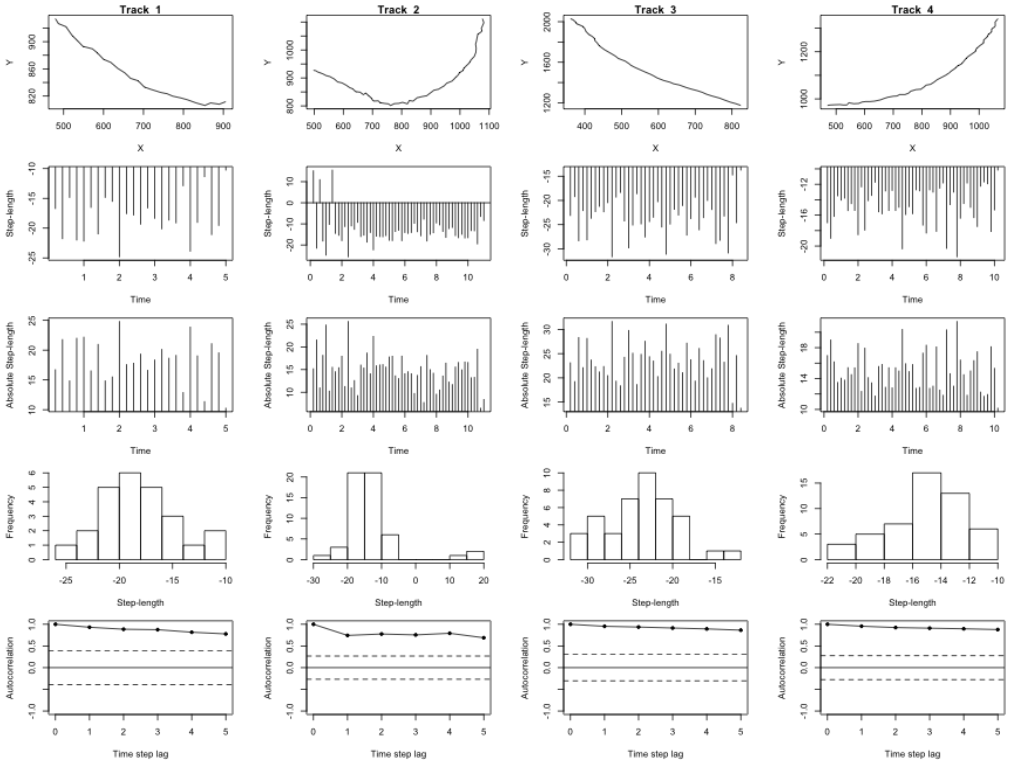
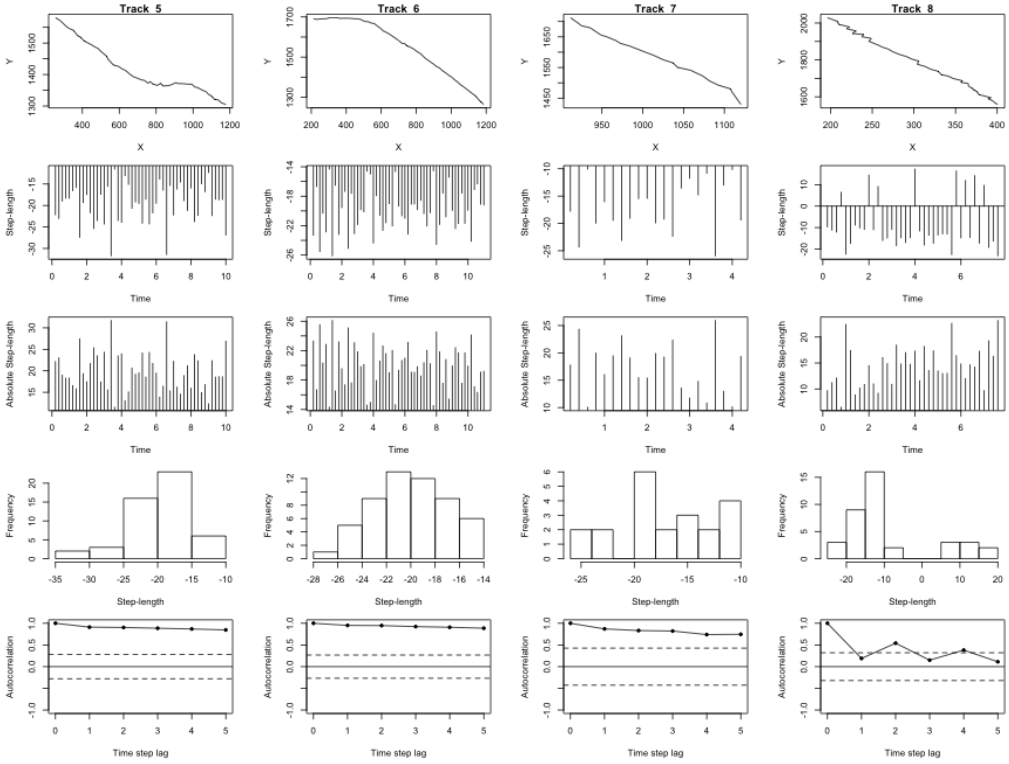
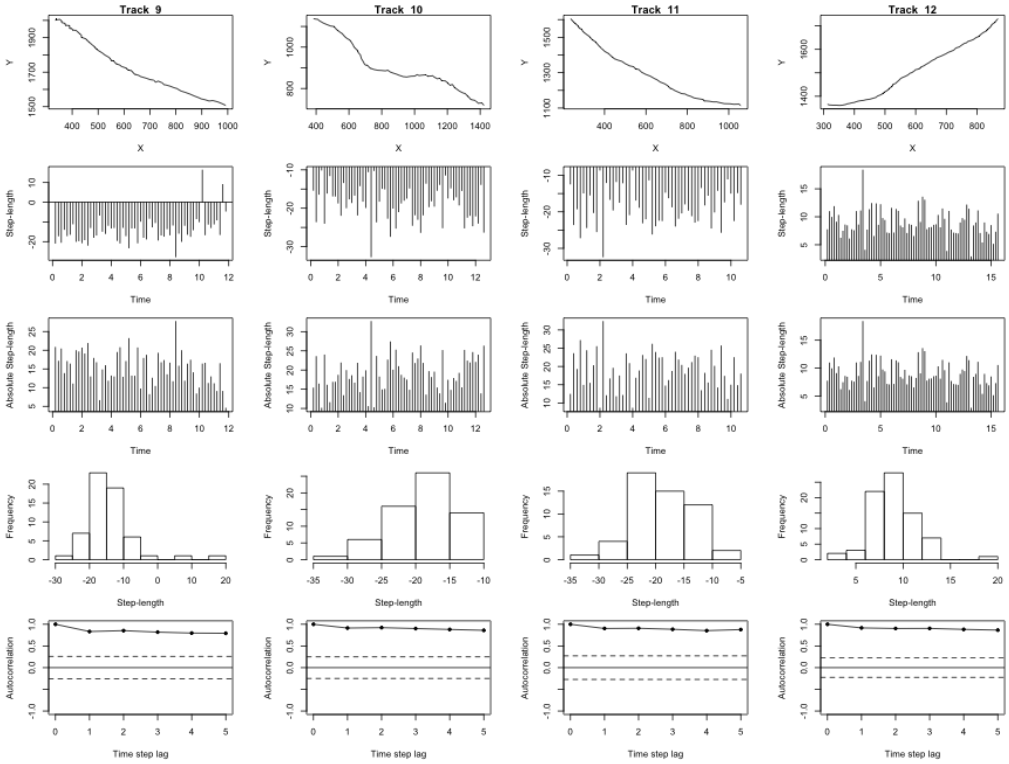
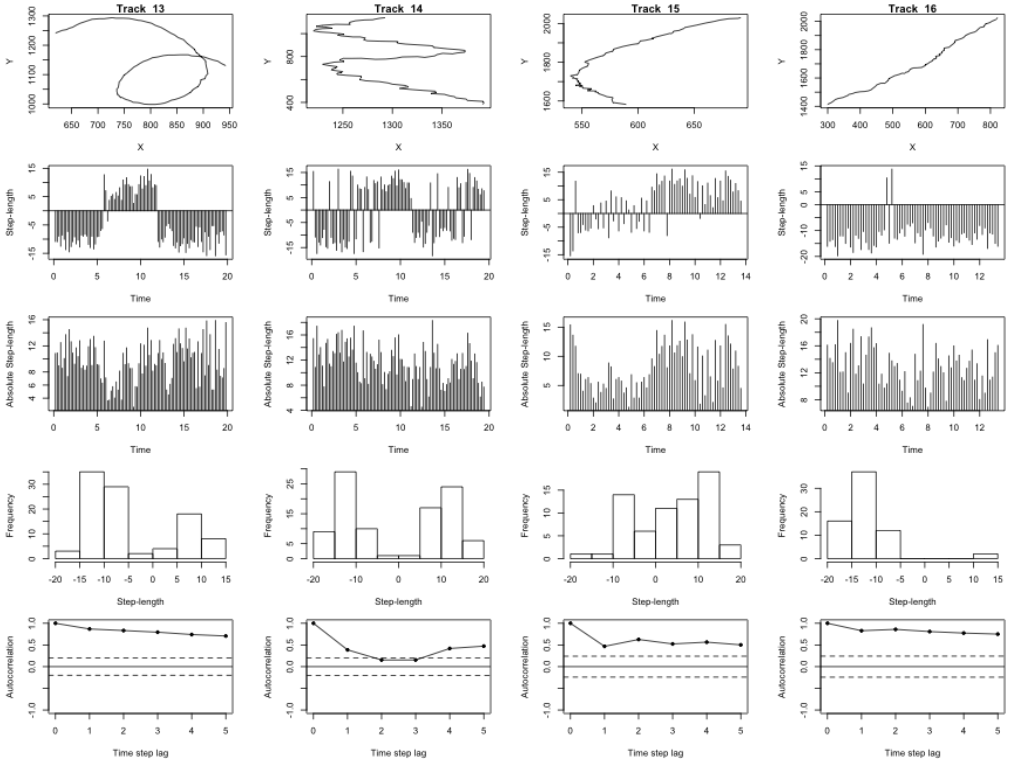


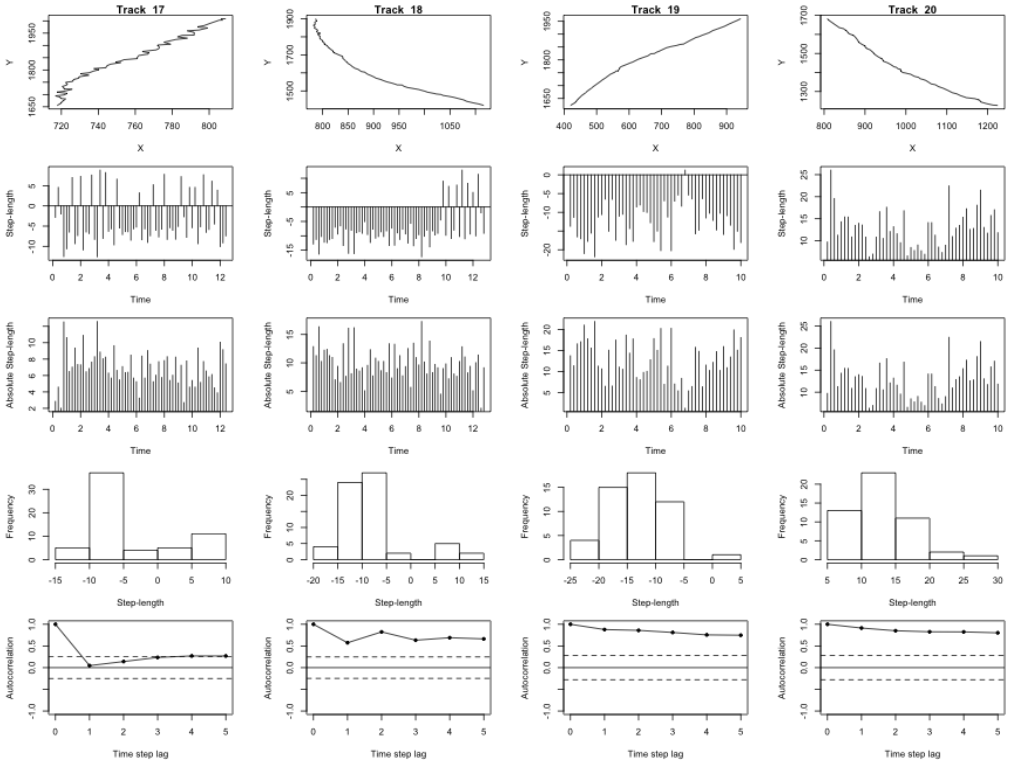
Figure 1A.7. Individual sperm track characteristics for fresh capacitated sperm in the presence of two COC (n=34)

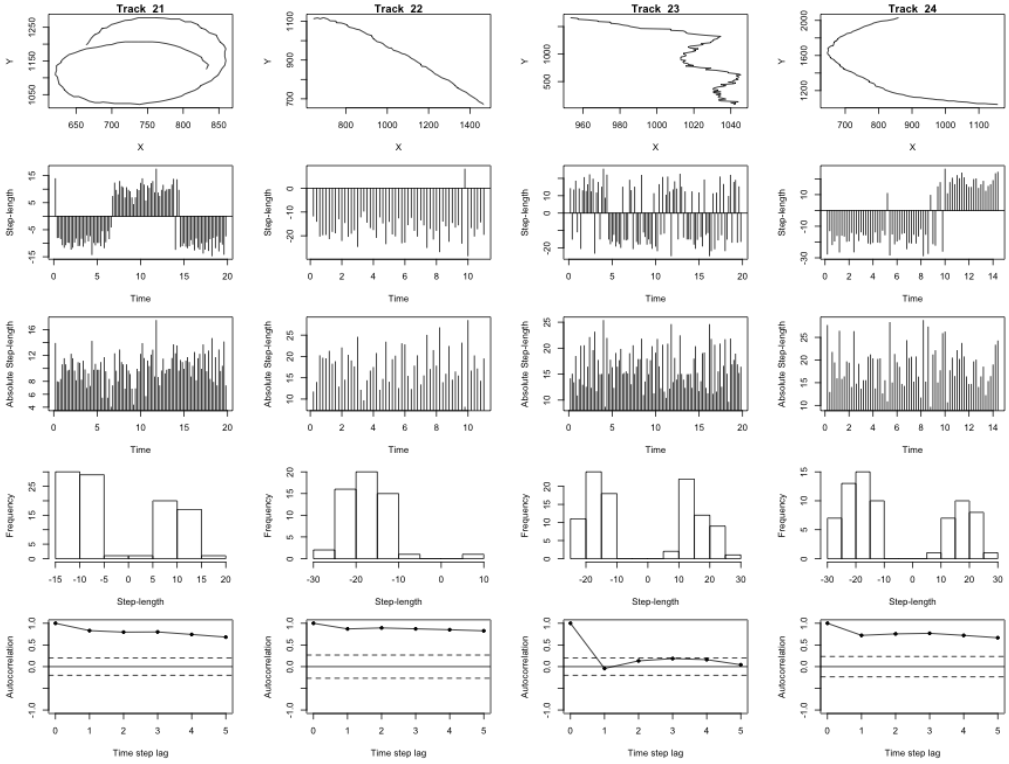

Track 1
Track 2
Track 3
Track 4
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag

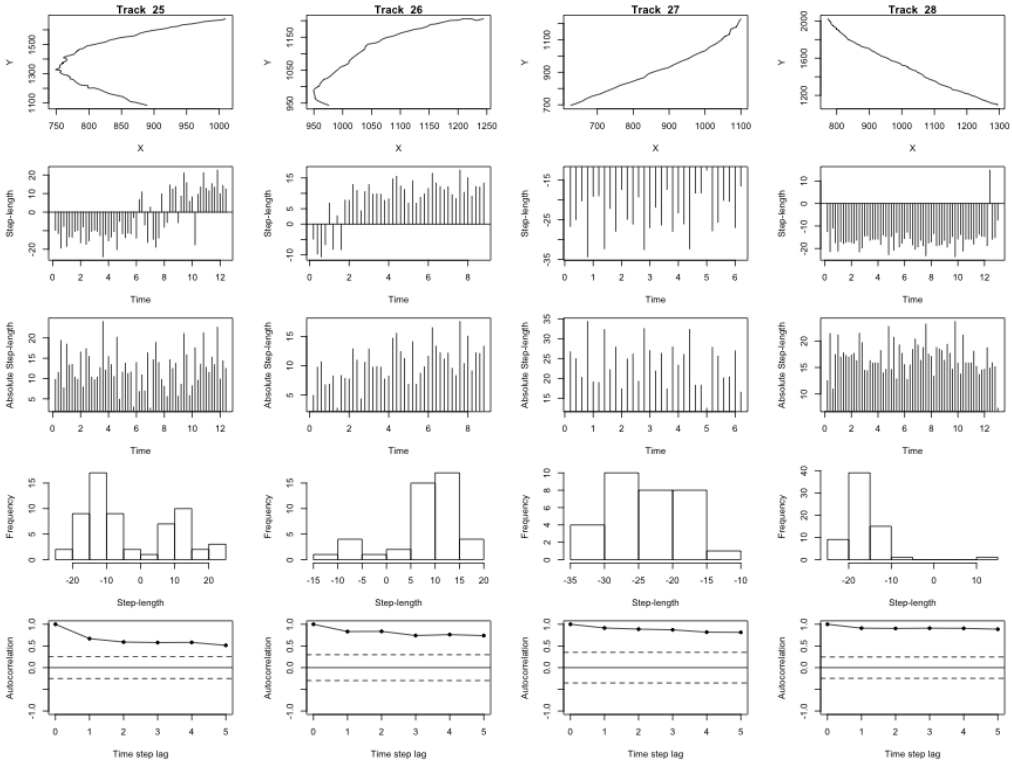

Track 5
Track 6
Track 7
Track 8
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag

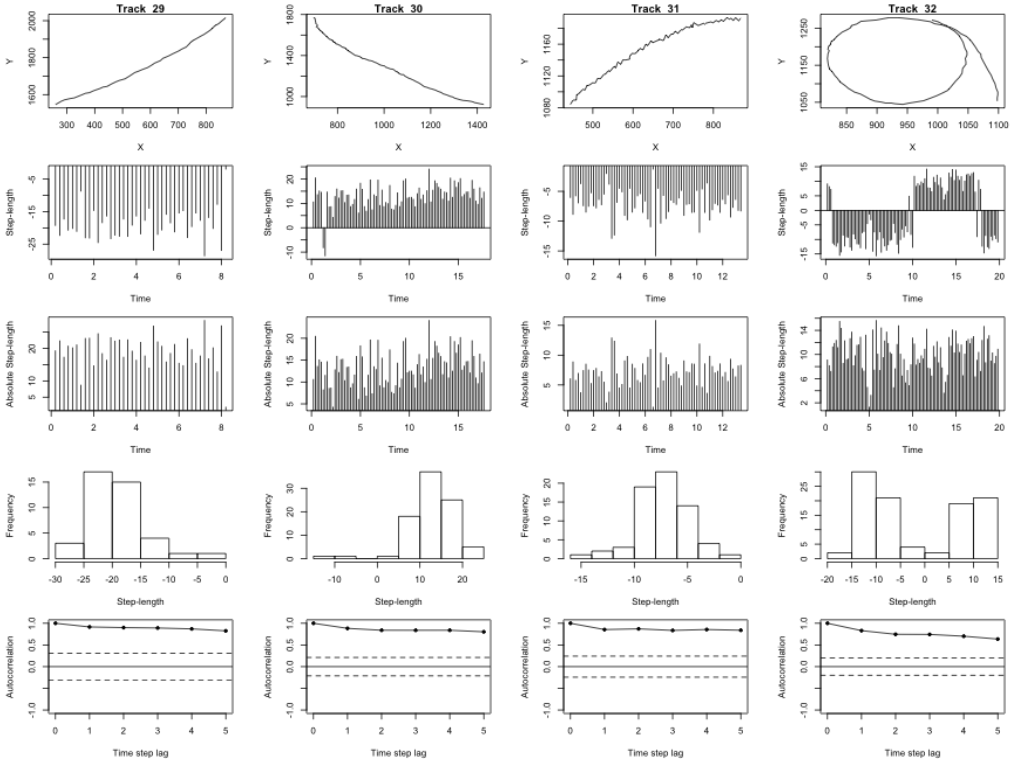

Track 9
Track 10
Track 11
Track 12
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

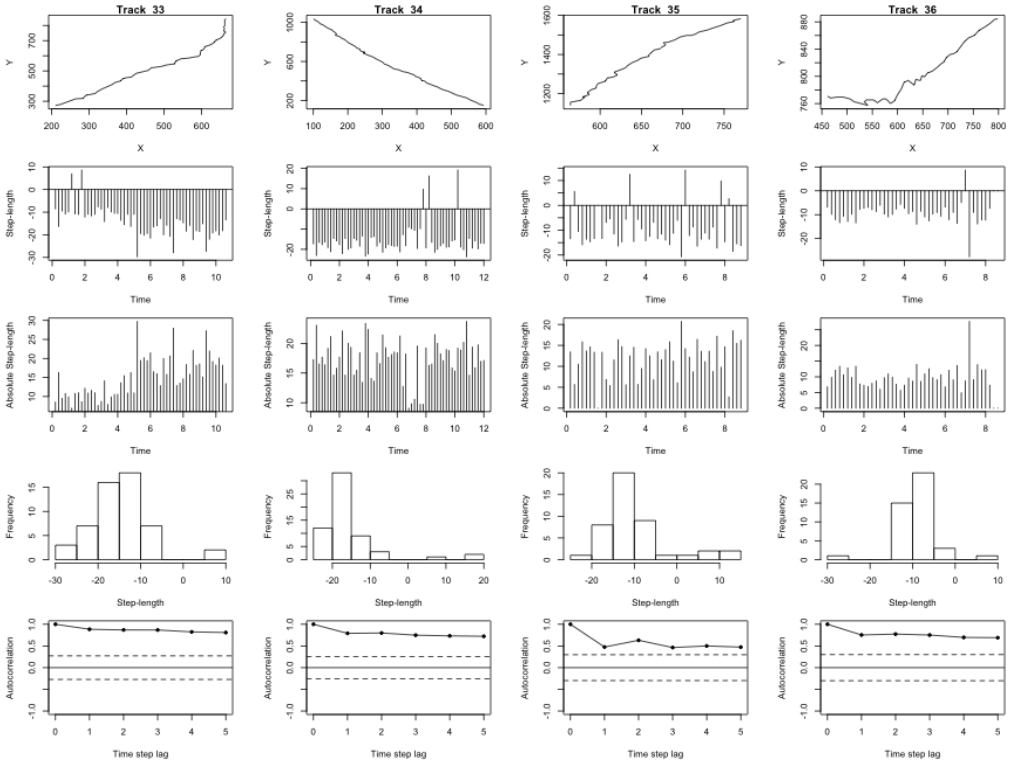

Track 13
Track 14
Track 15
Track 16
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

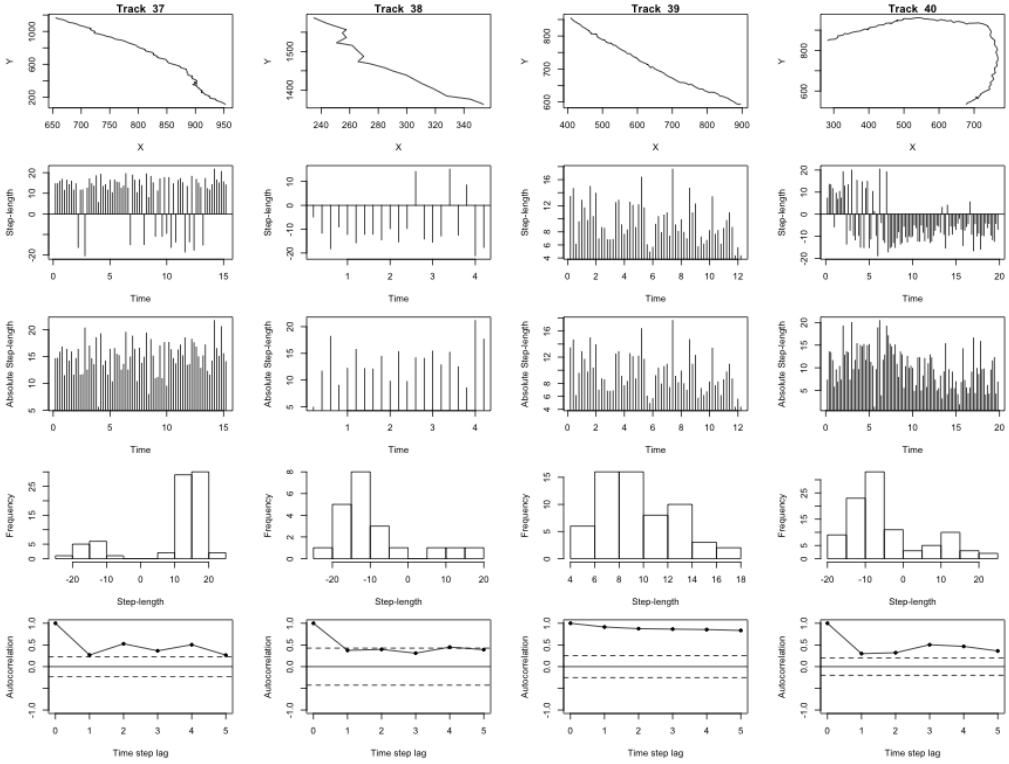

Track 17
Track 18
Track 19
Track 20
X
Y
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag


Track 21
Track 22
Track 23
Track 24
X
Y
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag


Track 25
Track 26
Track 27
Track 28
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 29
Track 30
Track 31
Track 32
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 33
Track 34
Track 35
Track 36
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag


Track 37
Track 38
Track 39
Track 40
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag

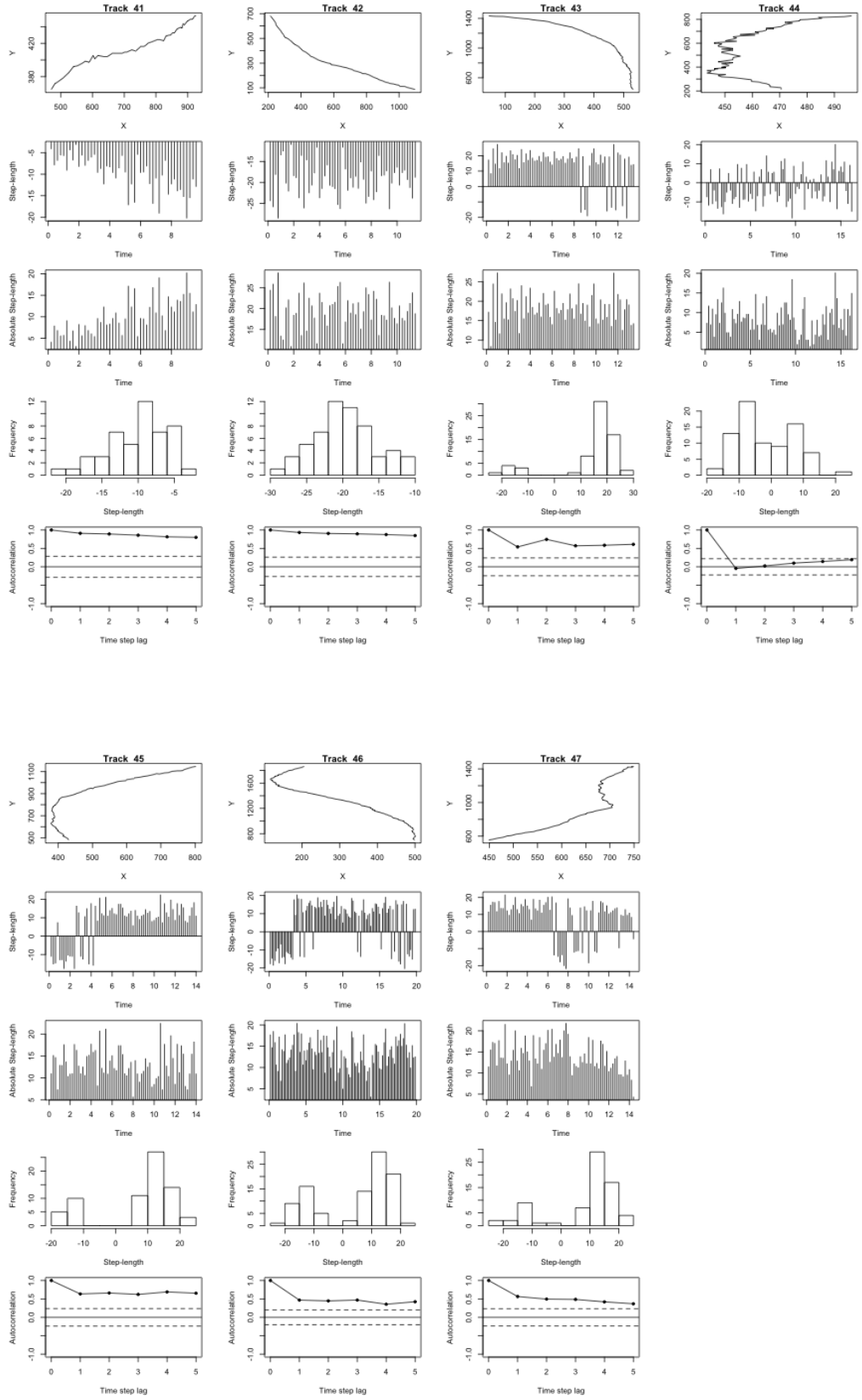
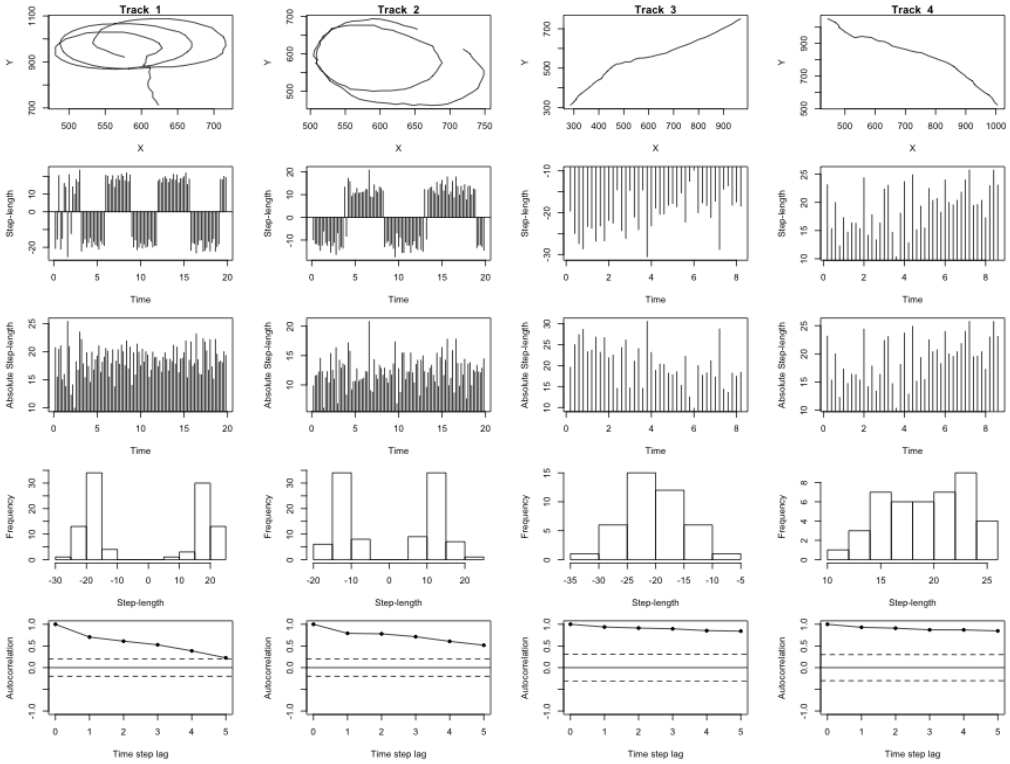
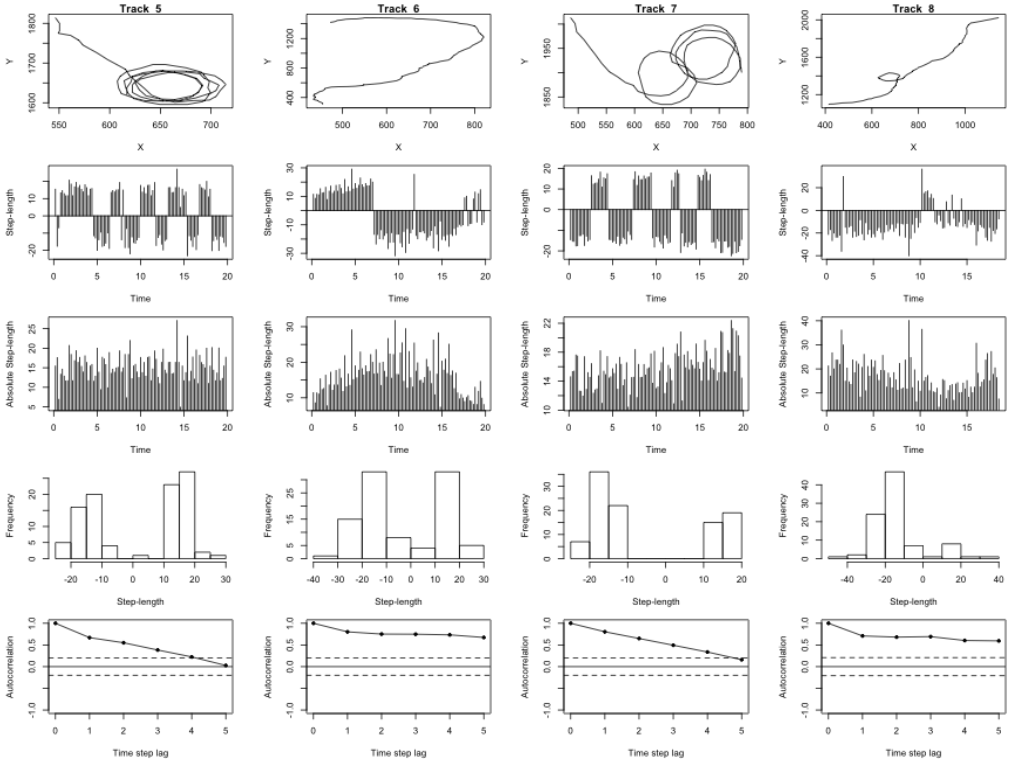
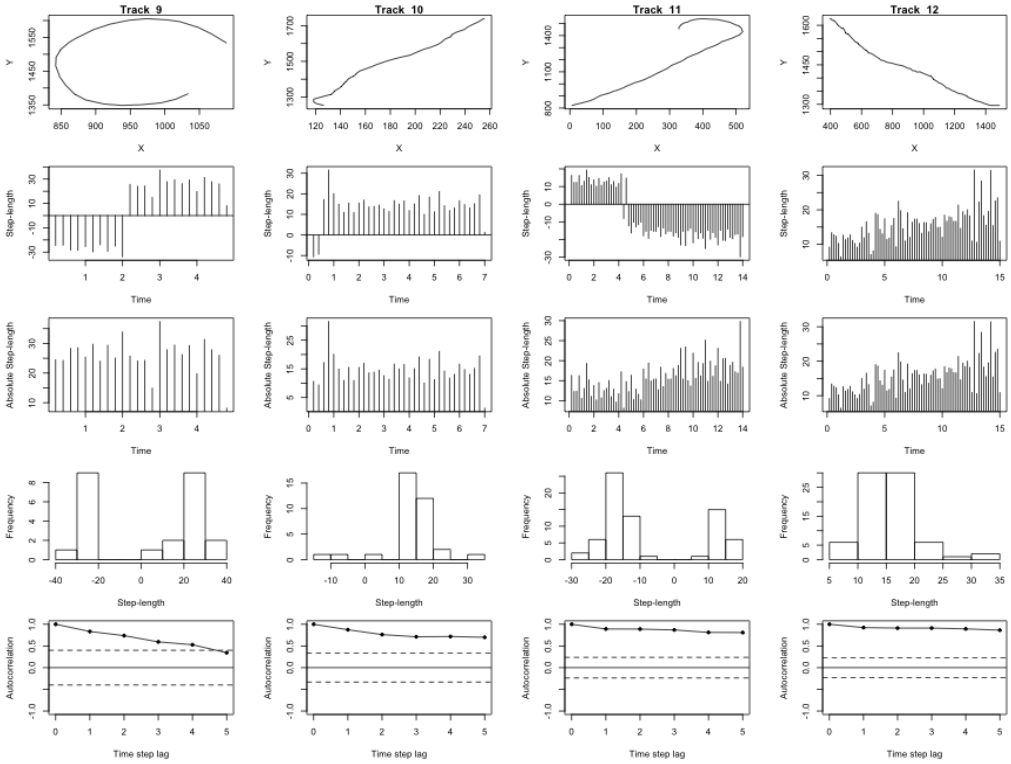
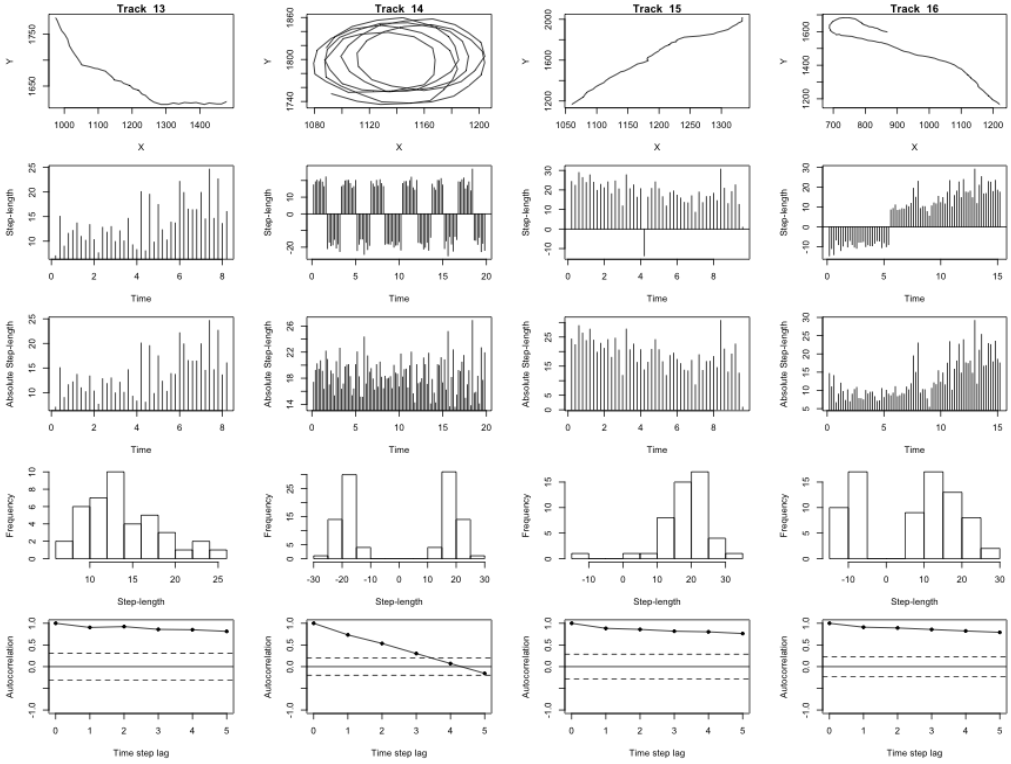


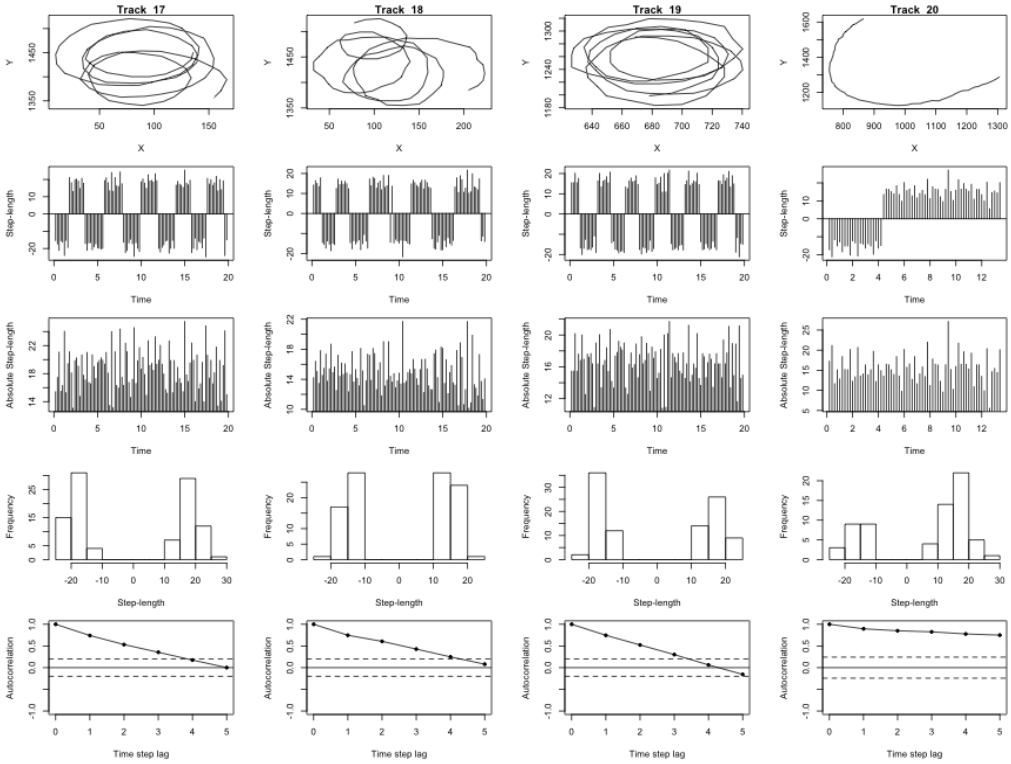
Figure 1A.8. Individual sperm track characteristics for fresh capacitated sperm in the presence of three COC (n=47)

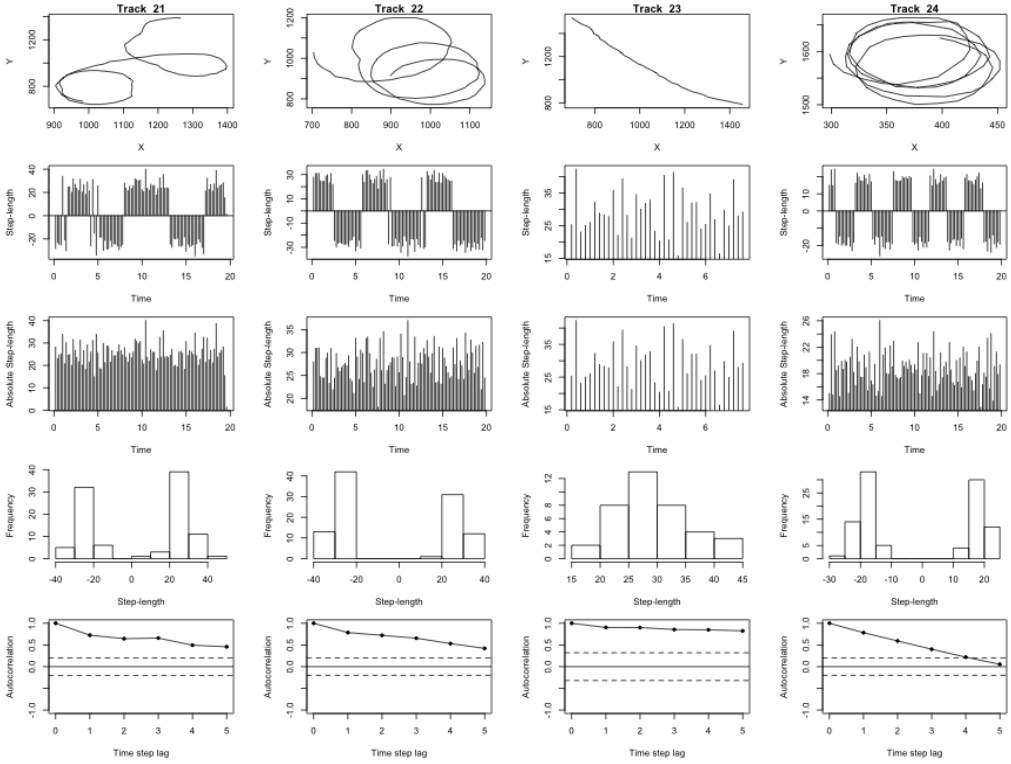

Track 1
Track 2
Track 3
Track 4
X
Y
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

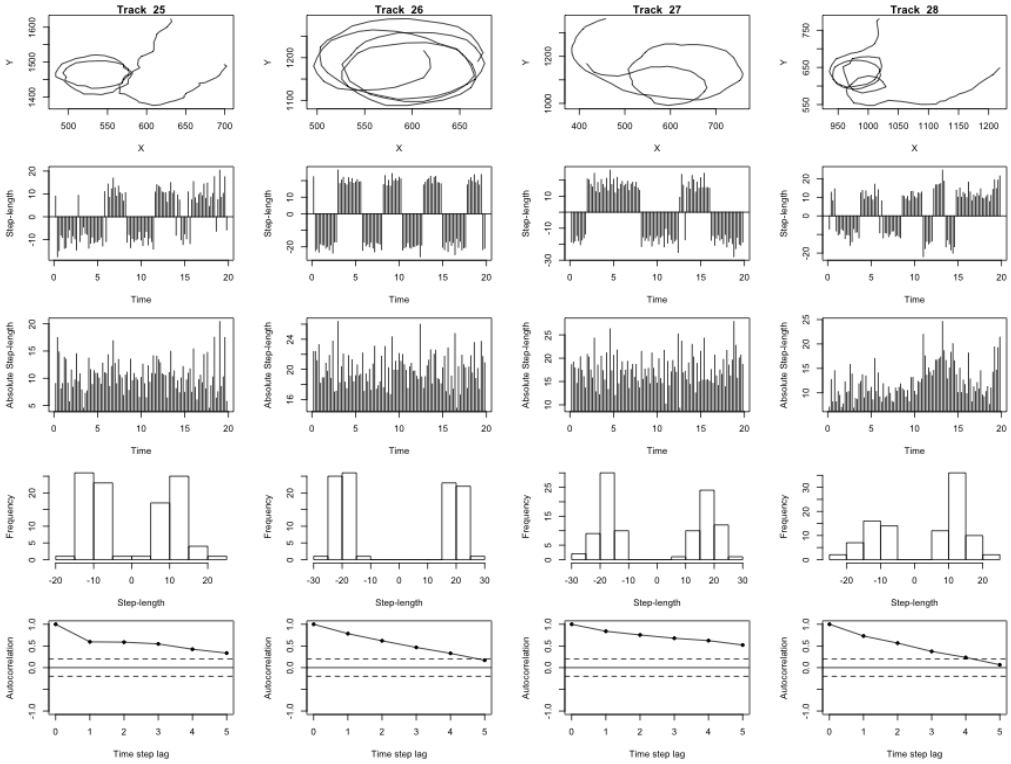

Track 5
Track 6
Track 7
Track 8
X
Y
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

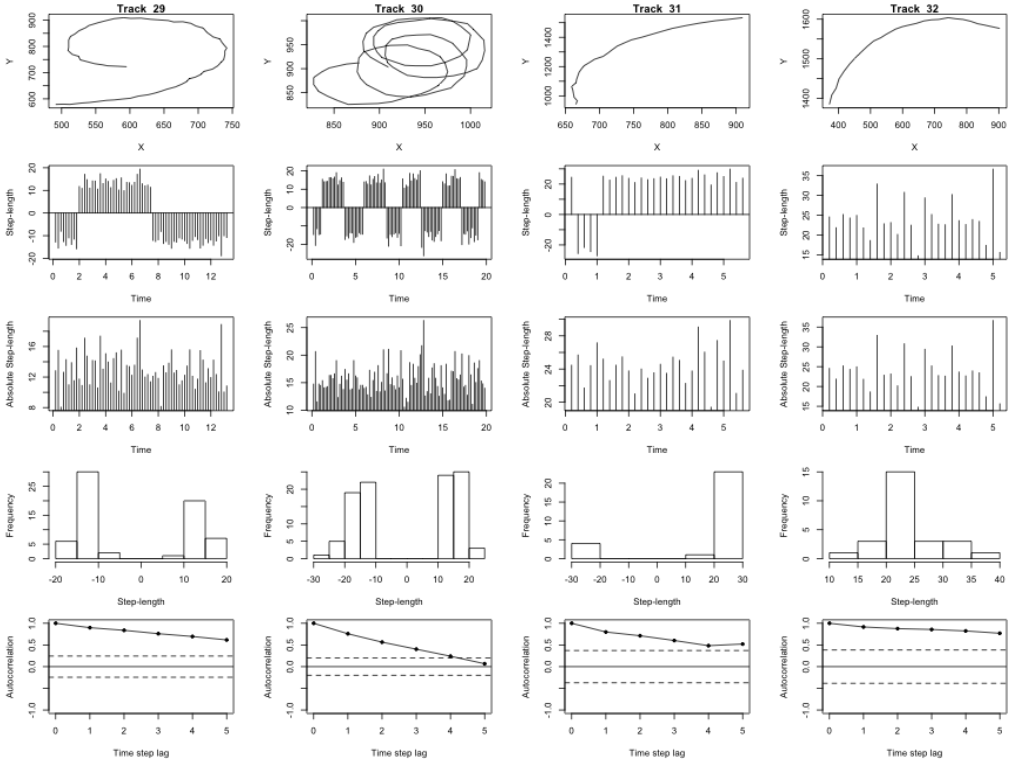

Track 9
Track 10
Track 11
Track 12
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

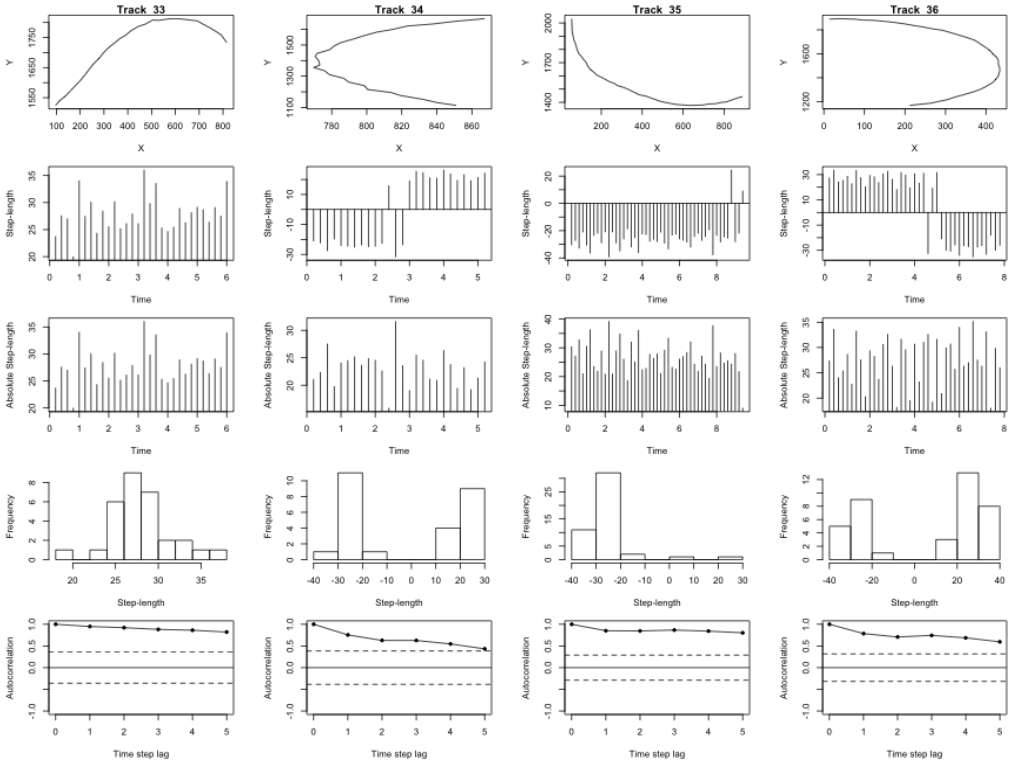

Track 13
Track 14
Track 15
Track 16
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

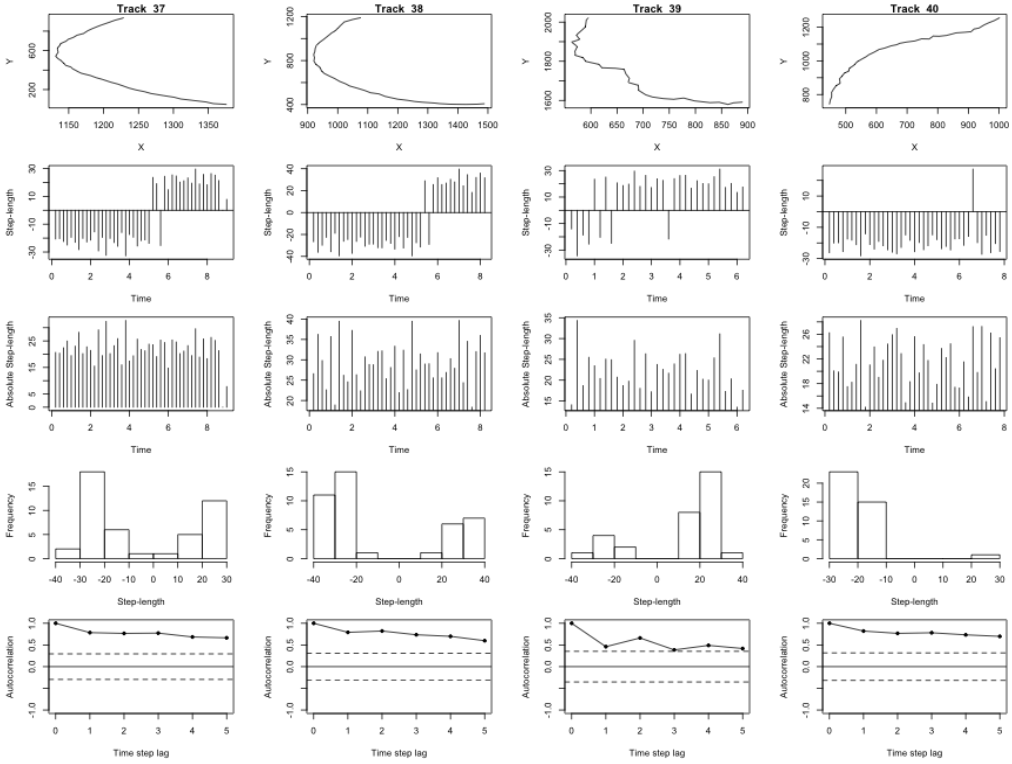

Track 17
Track 18
Track 19
Track 20
X
Y
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag

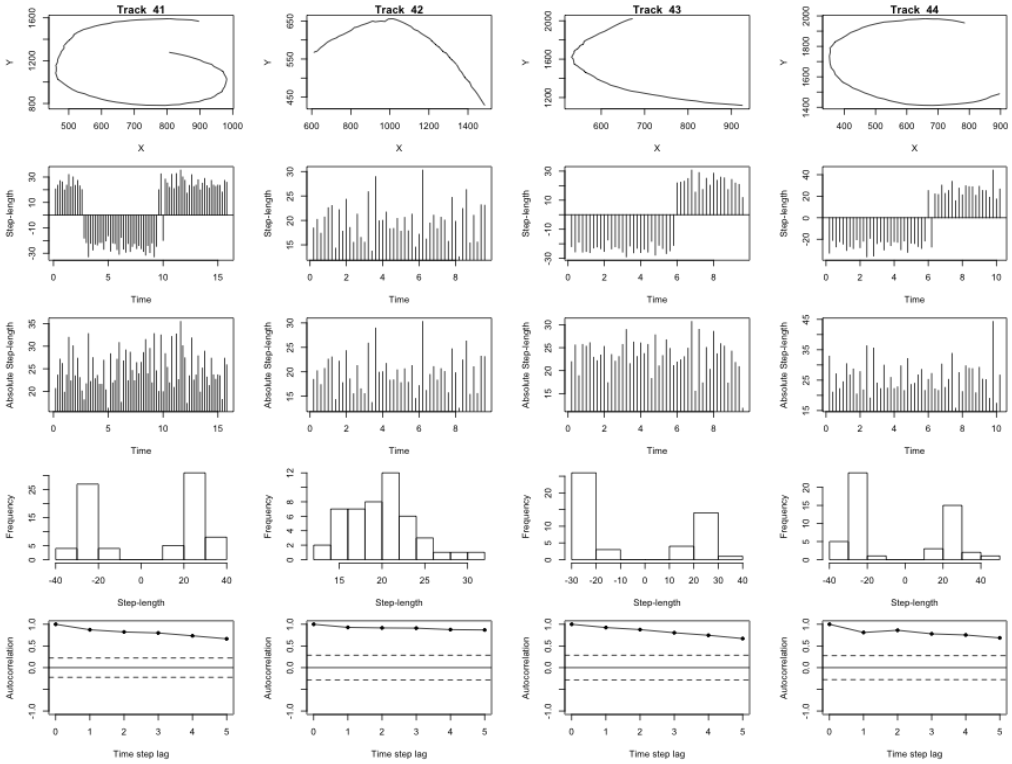

Track 21
Track 22
Track 23
Track 24
X
Y
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag

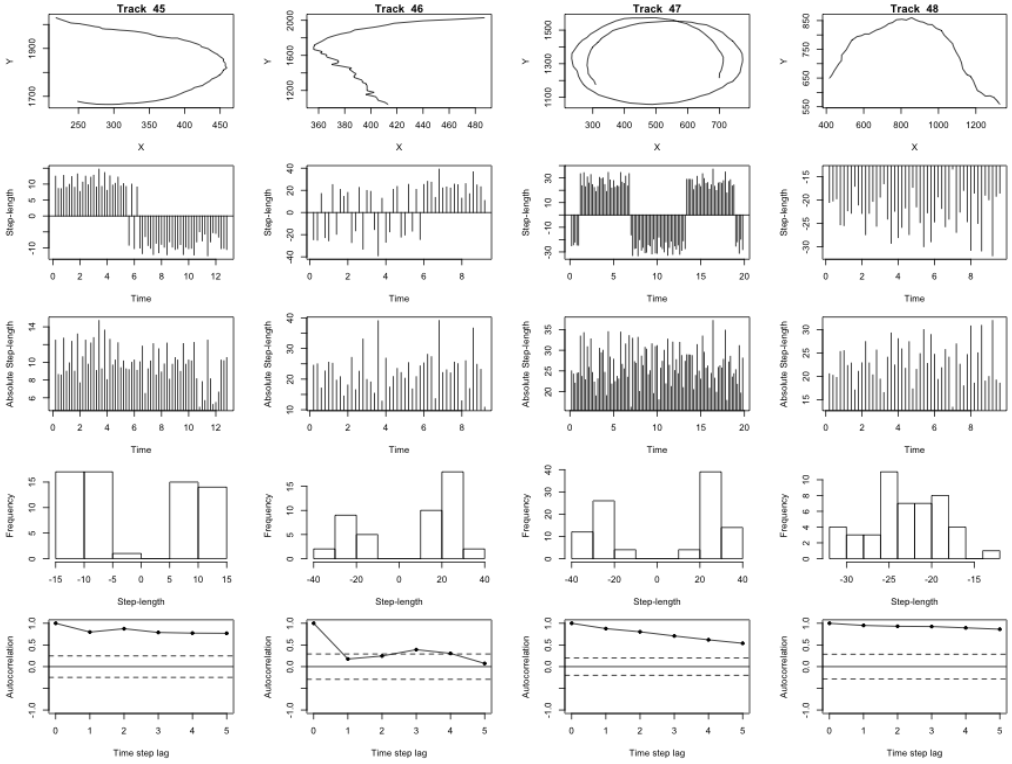

Track 25
Track 26
Track 27
Track 28
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

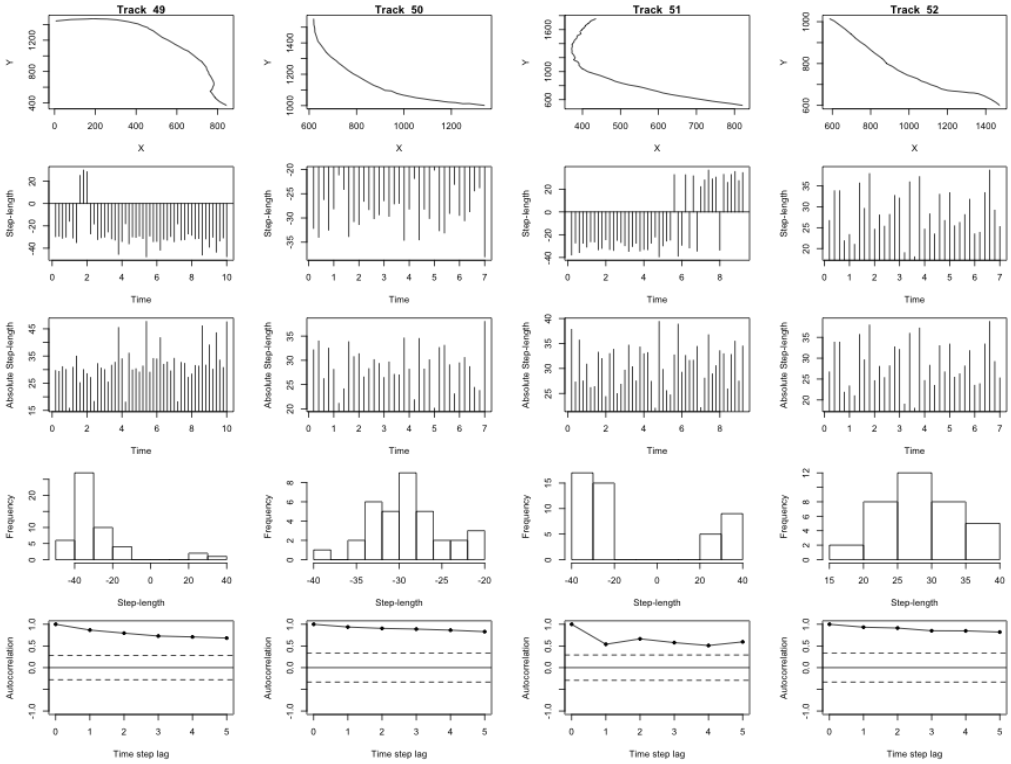

Track 29
Track 30
Track 31
Track 32
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

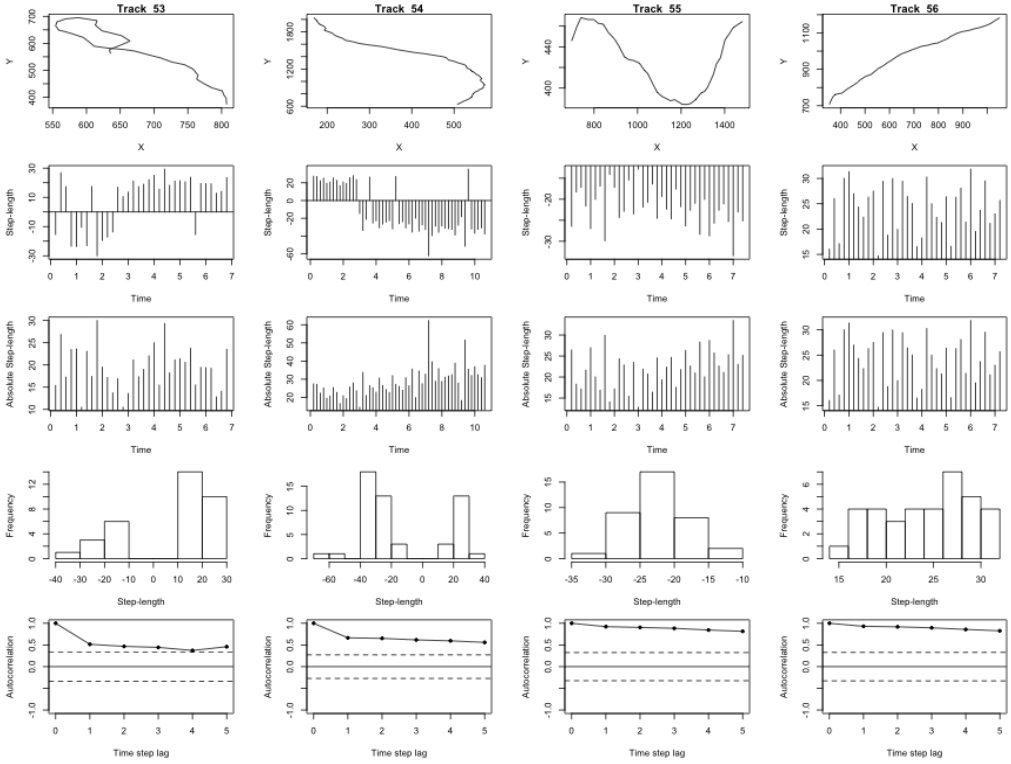

Track 33
Track 34
Track 35
Track 36
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag

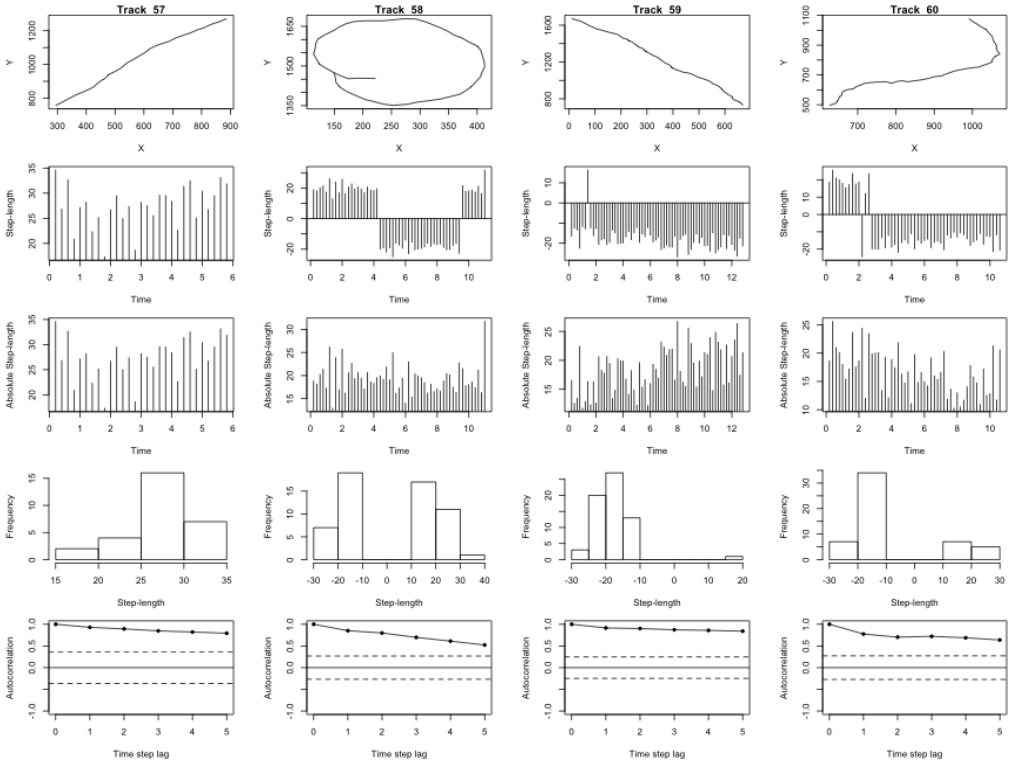

Track 37
Track 38
Track 39
Track 40
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag

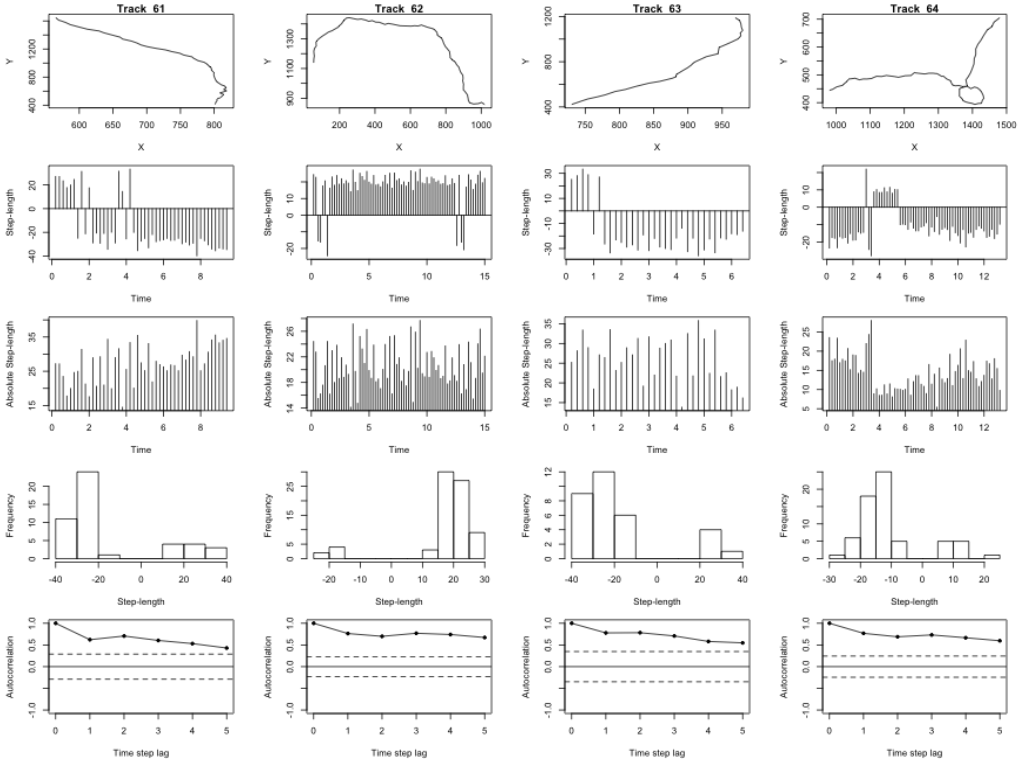

Track 41
Track 42
Track 43
Track 44
Y
X
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag

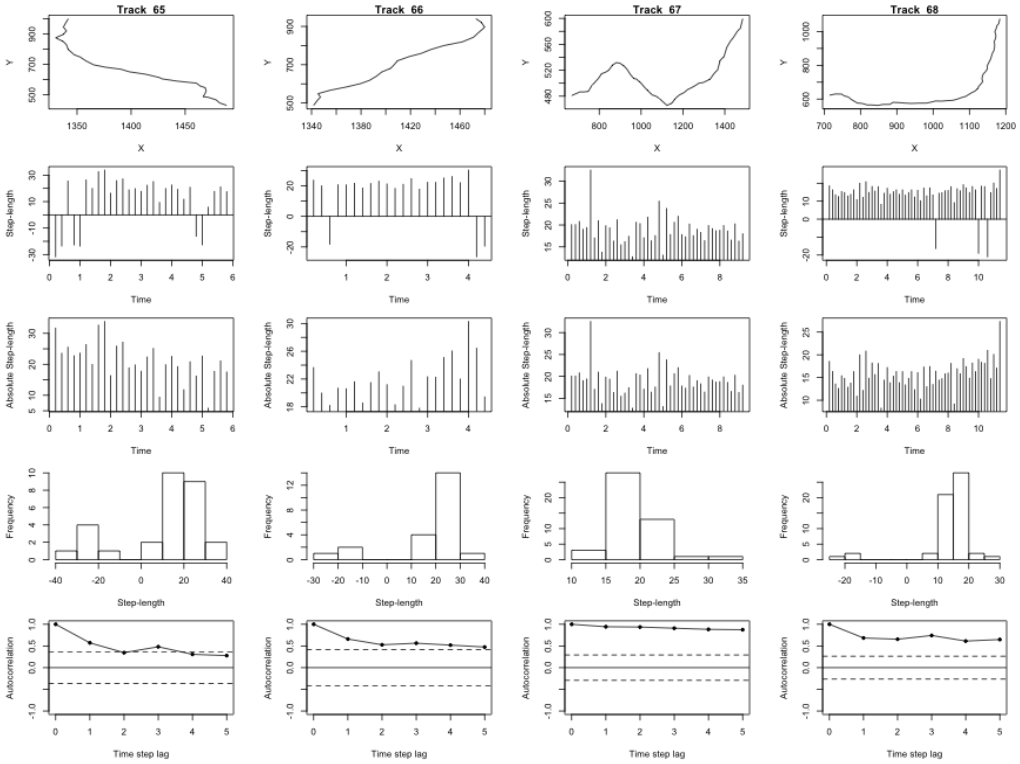

Track 45
Track 46
Track 47
Track 48
Y
X
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag

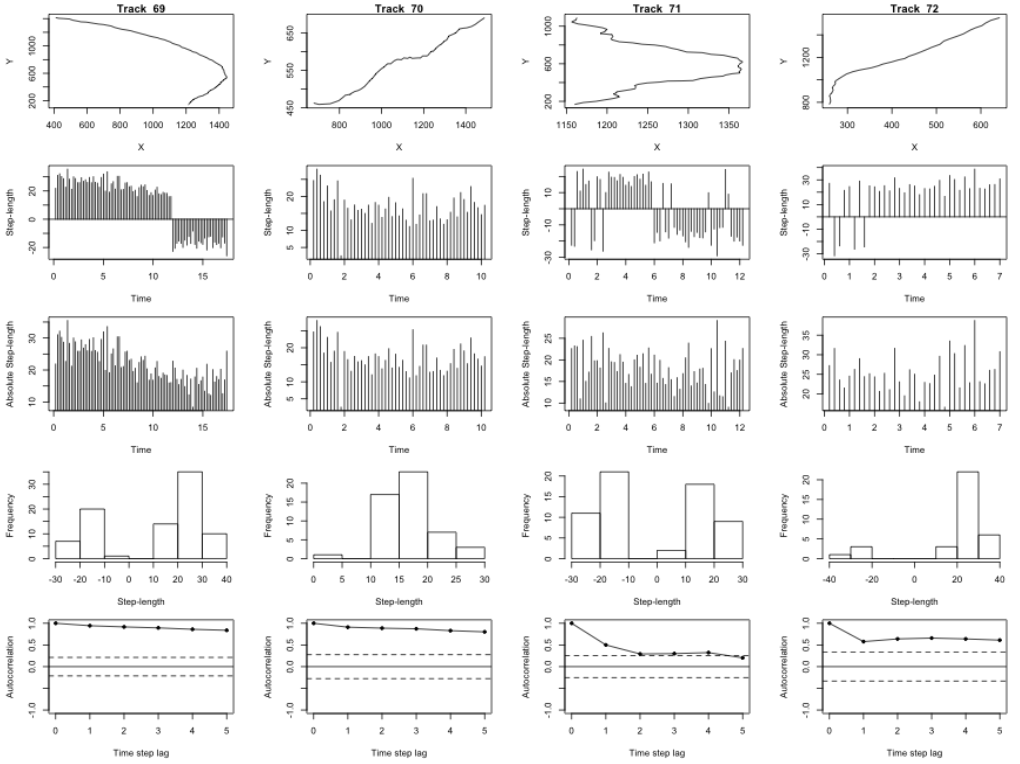

Track 49
Track 50
Track 51
Track 52
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 53
Track 54
Track 55
Track 56
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 57
Track 58
Track 59
Track 60
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 61
Track 62
Track 63
Track 64
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 65
Track 66
Track 67
Track 68
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 69
Track 70
Track 71
Track 72
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

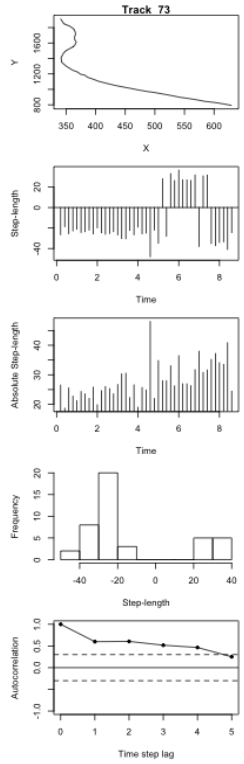
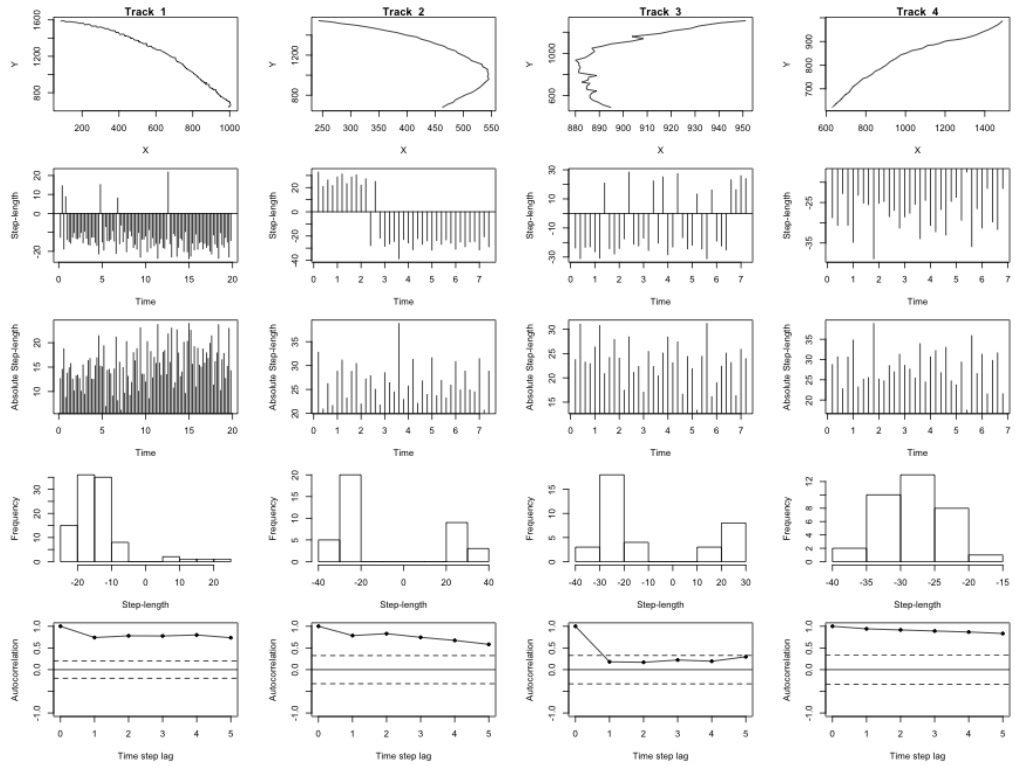
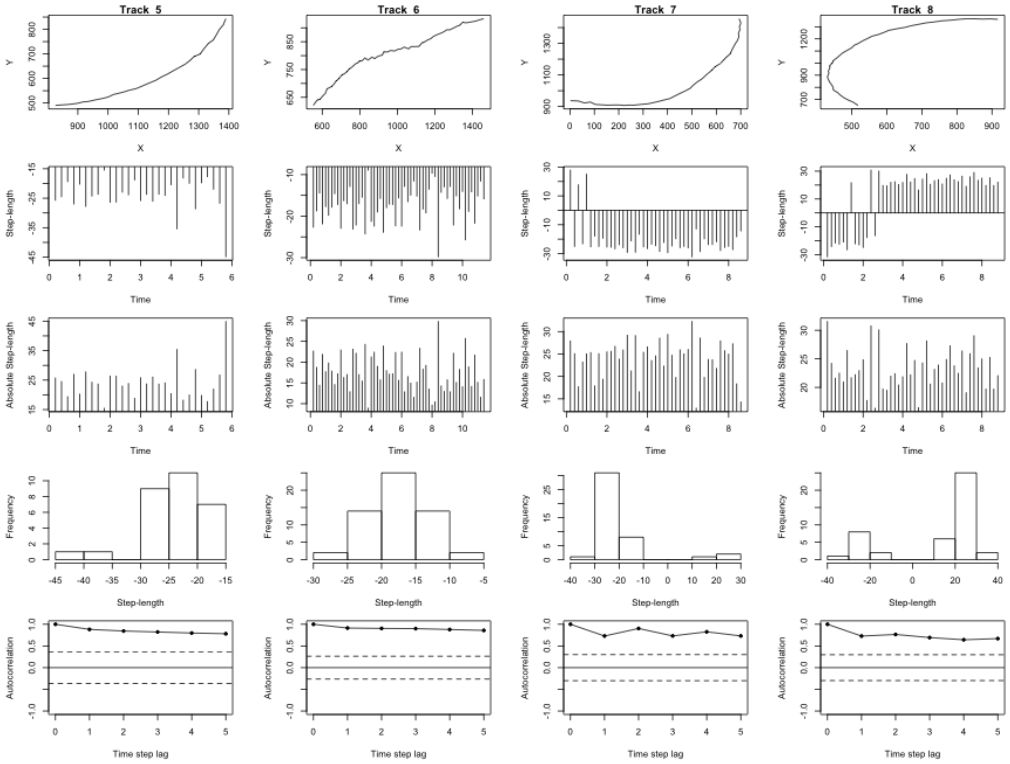
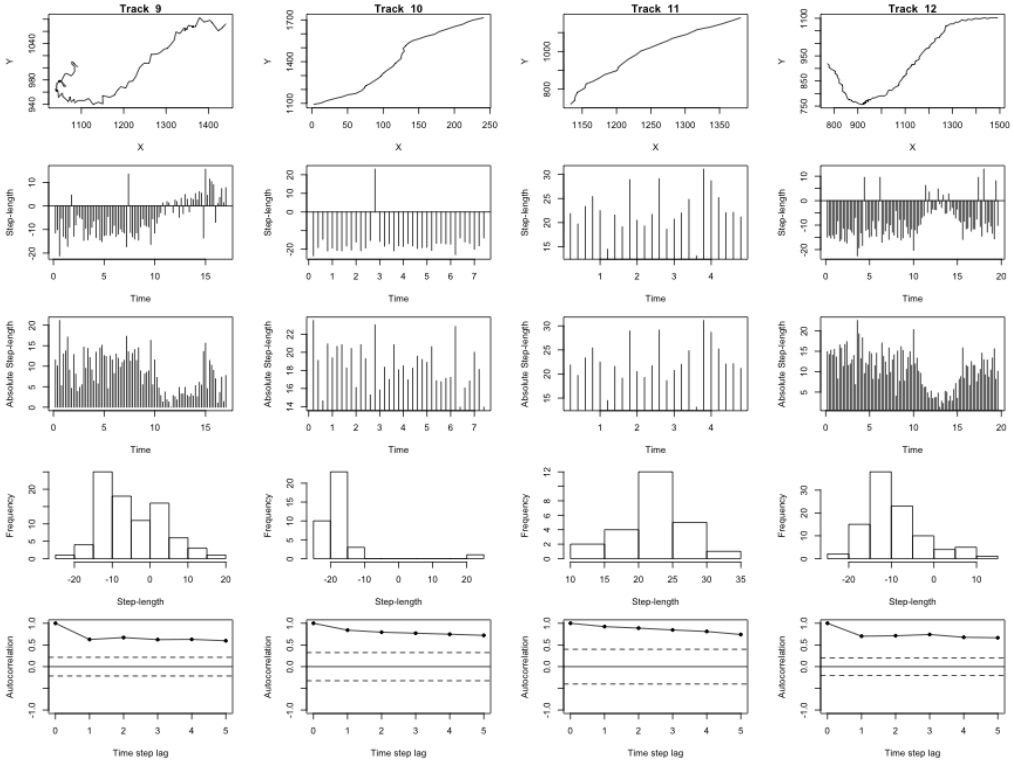
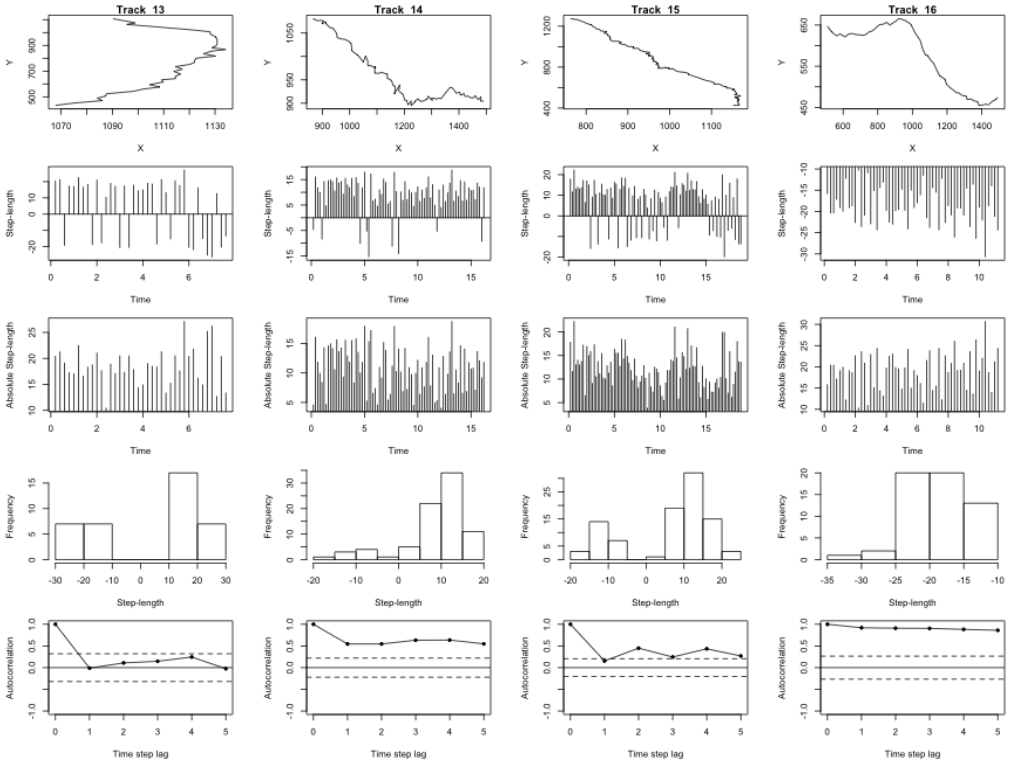


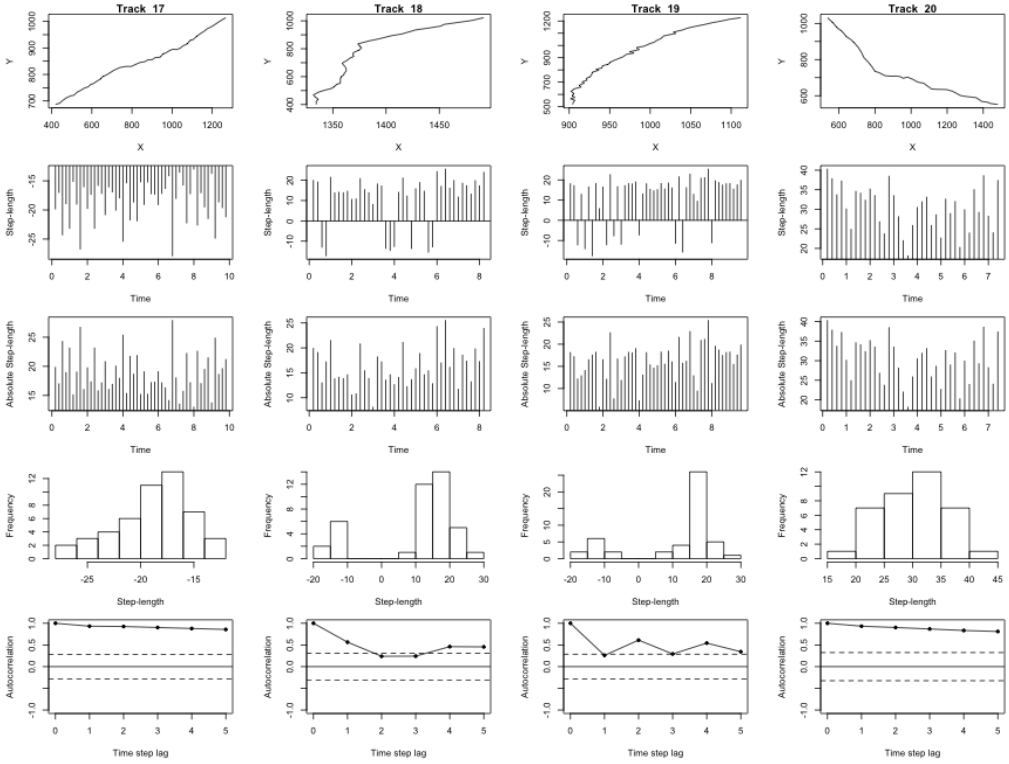
Figure 1A.9. Individual sperm tracks characteristics for fresh non-capacitated sperm in the control group without COC (n=73)

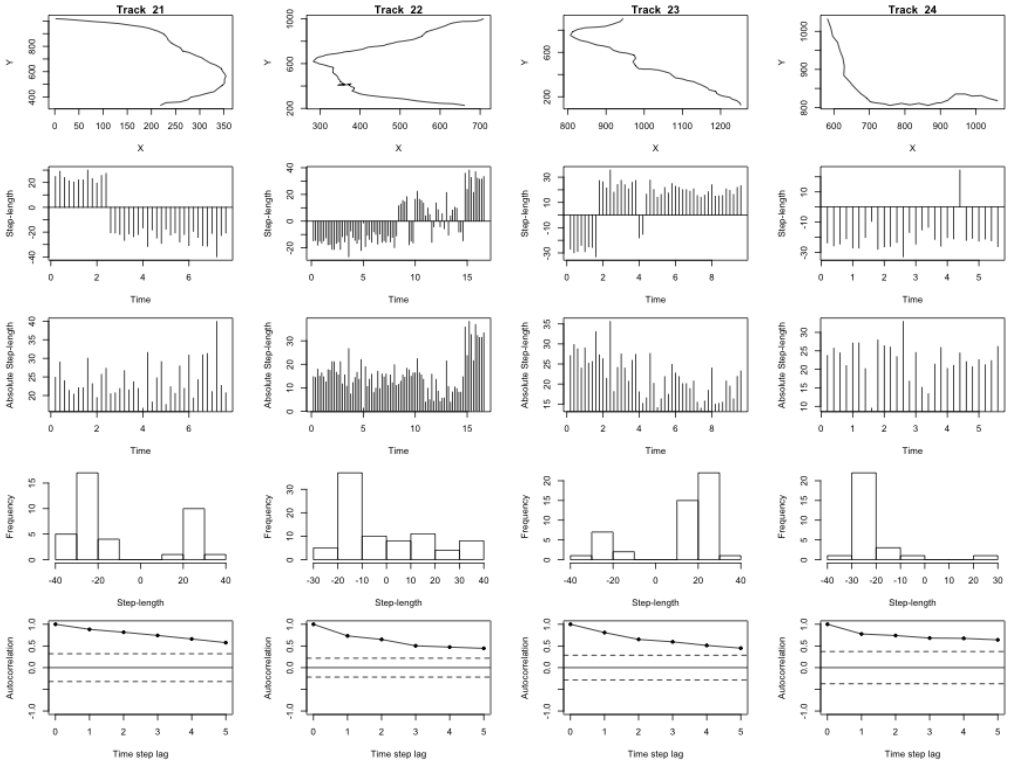


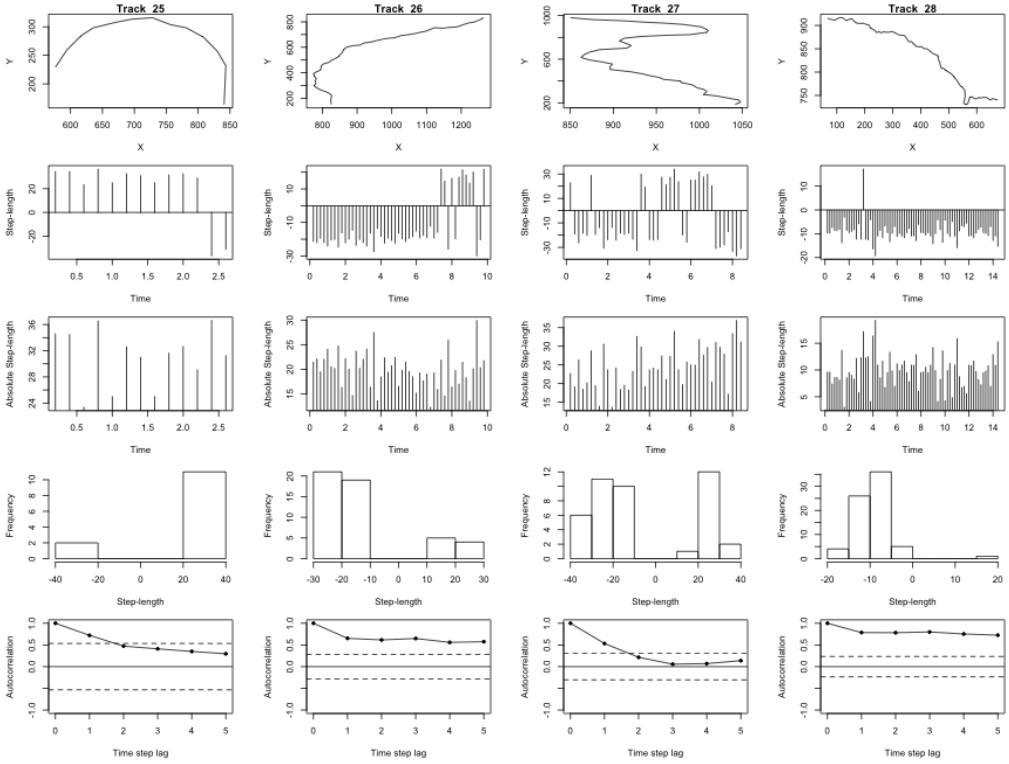

Track 5
Track 6
Track 7
Track 8
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag

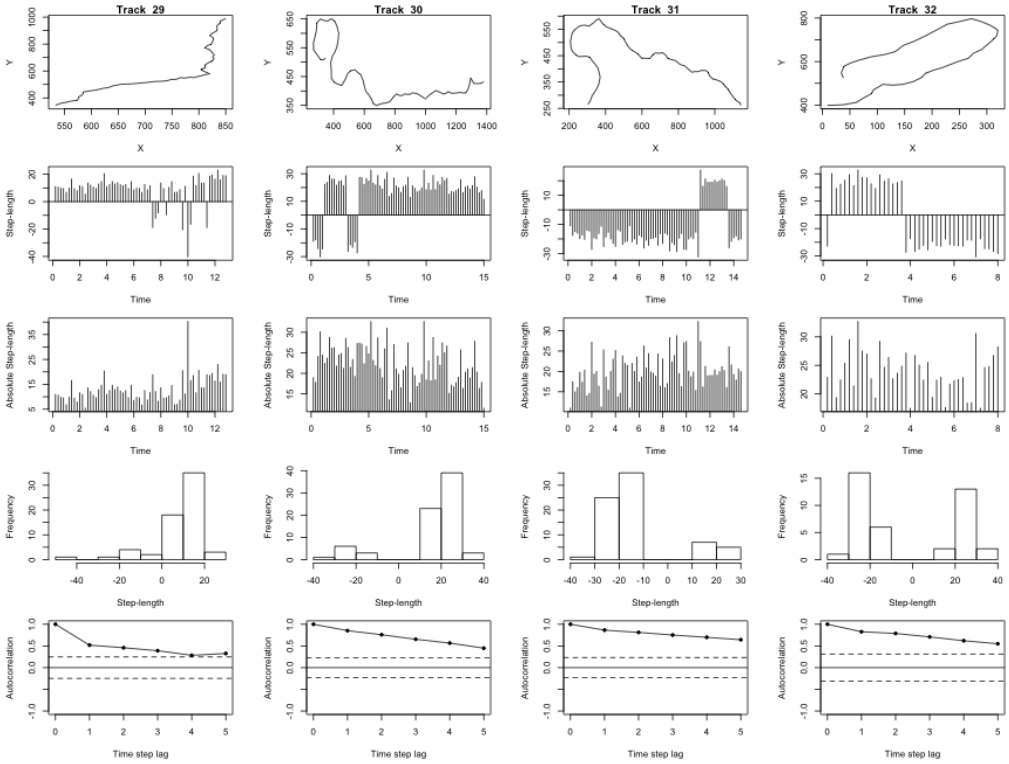

Track 9
Track 10
Track 11
Track 12
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag

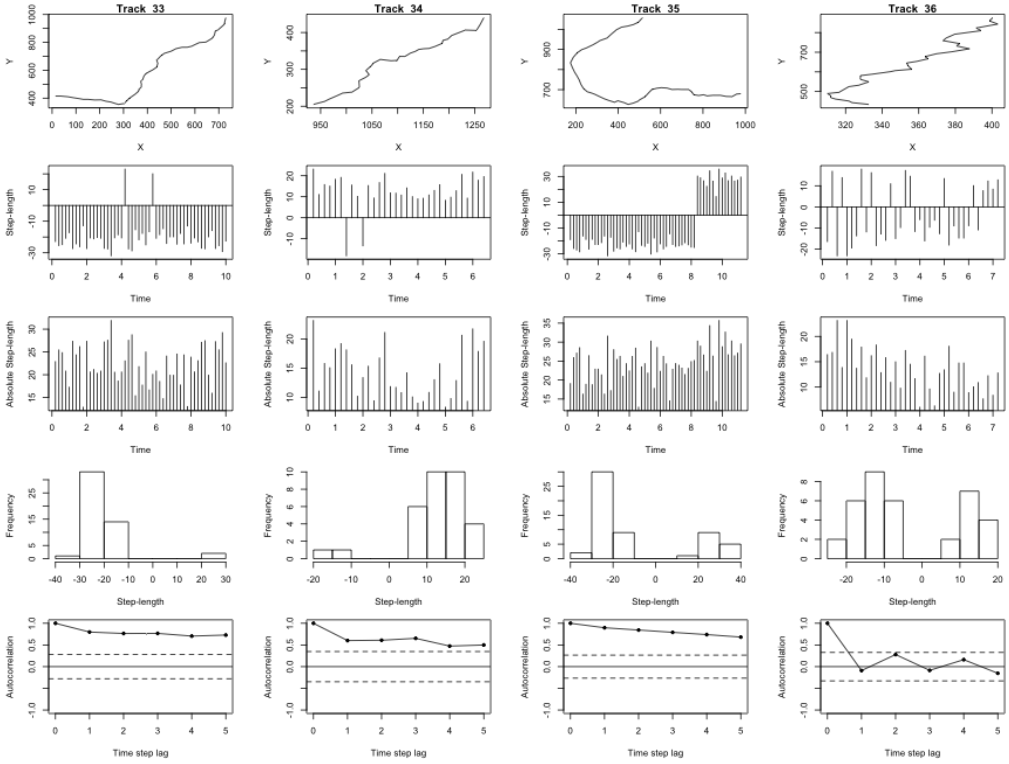

Track 13
Track 14
Track 15
Track 16
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

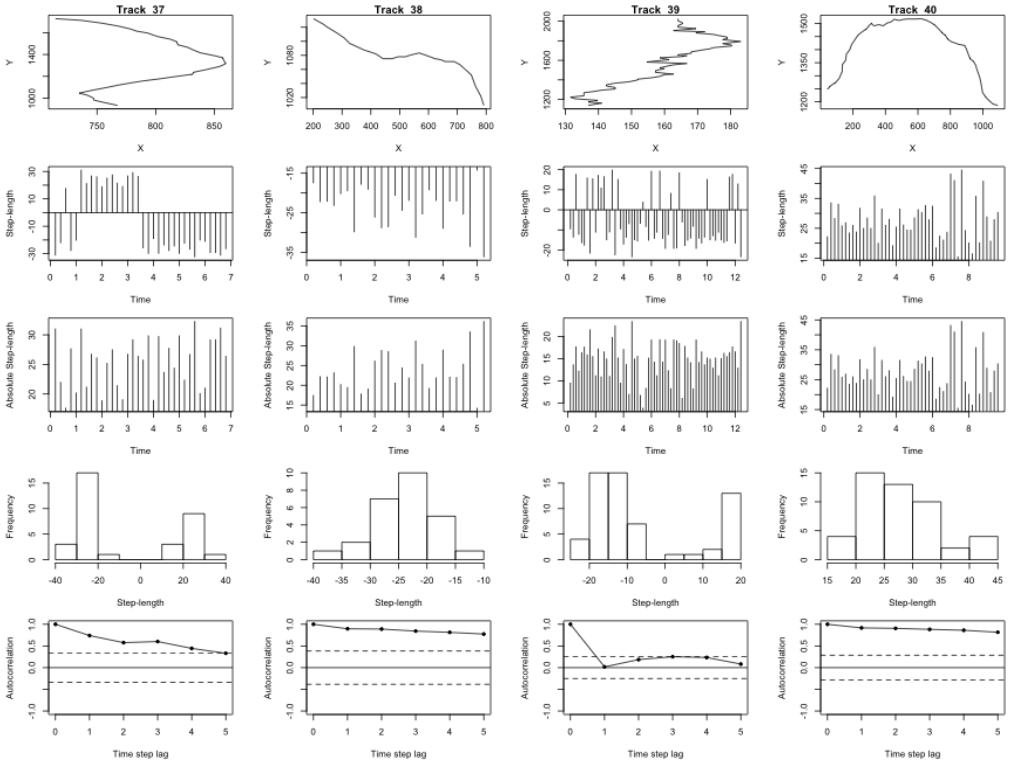

Track 17
Track 18
Track 19
Track 20
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

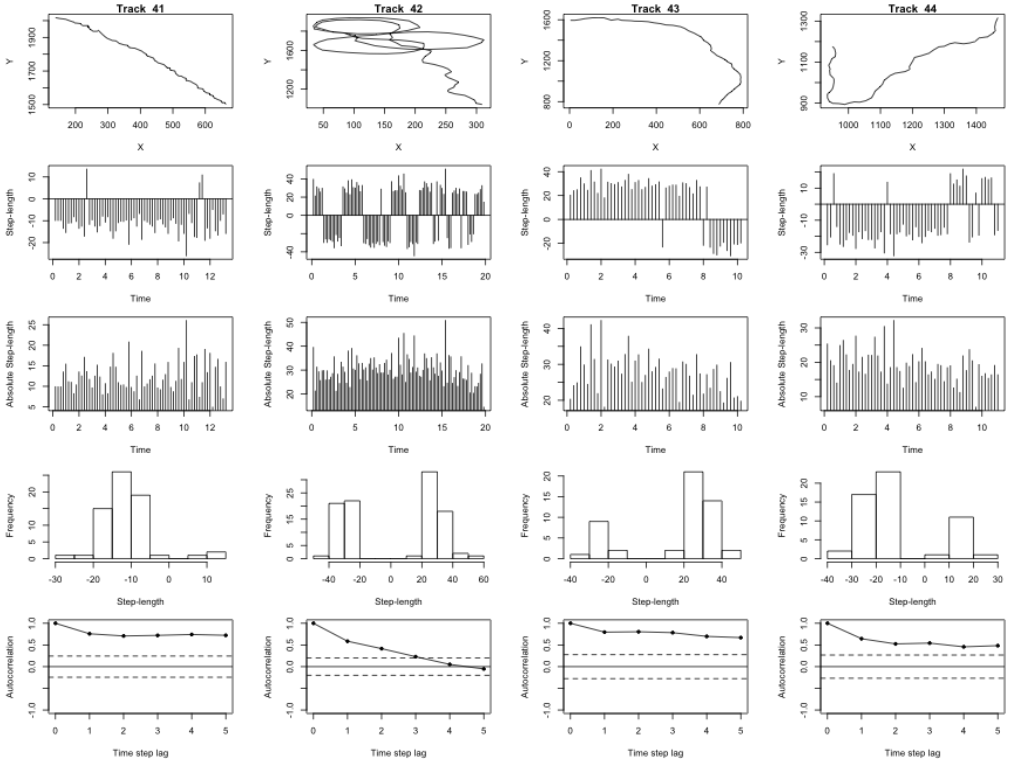

Track 21
Track 22
Track 23
Track 24
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

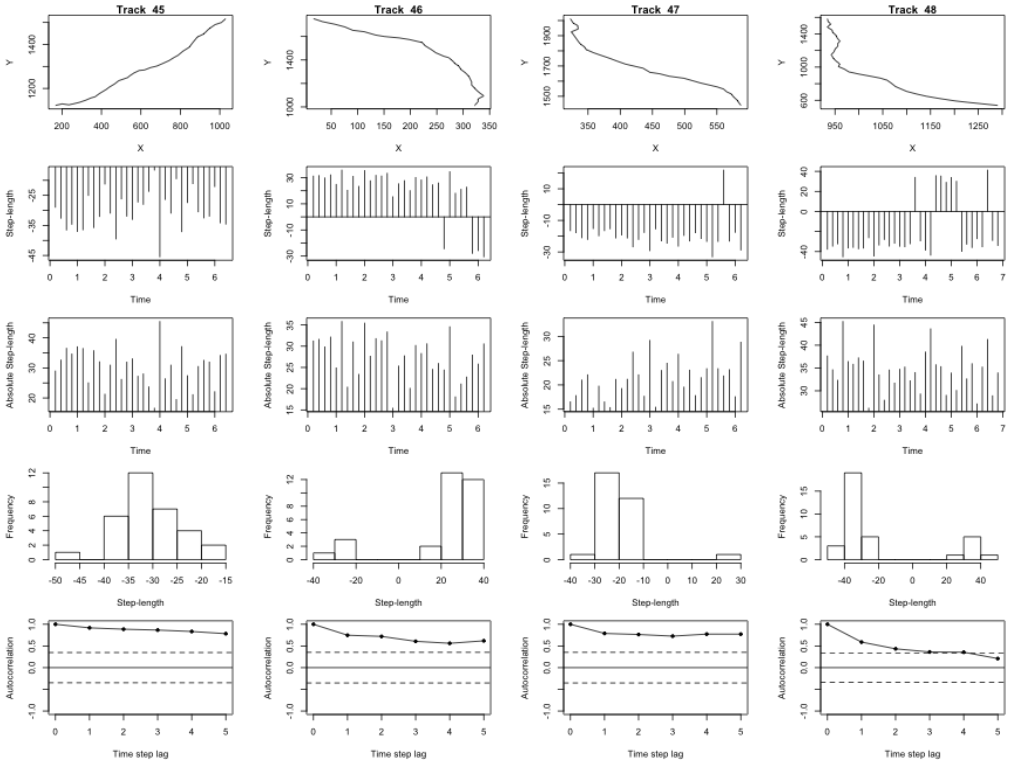

Track 25
Track 26
Track 27
Track 28
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

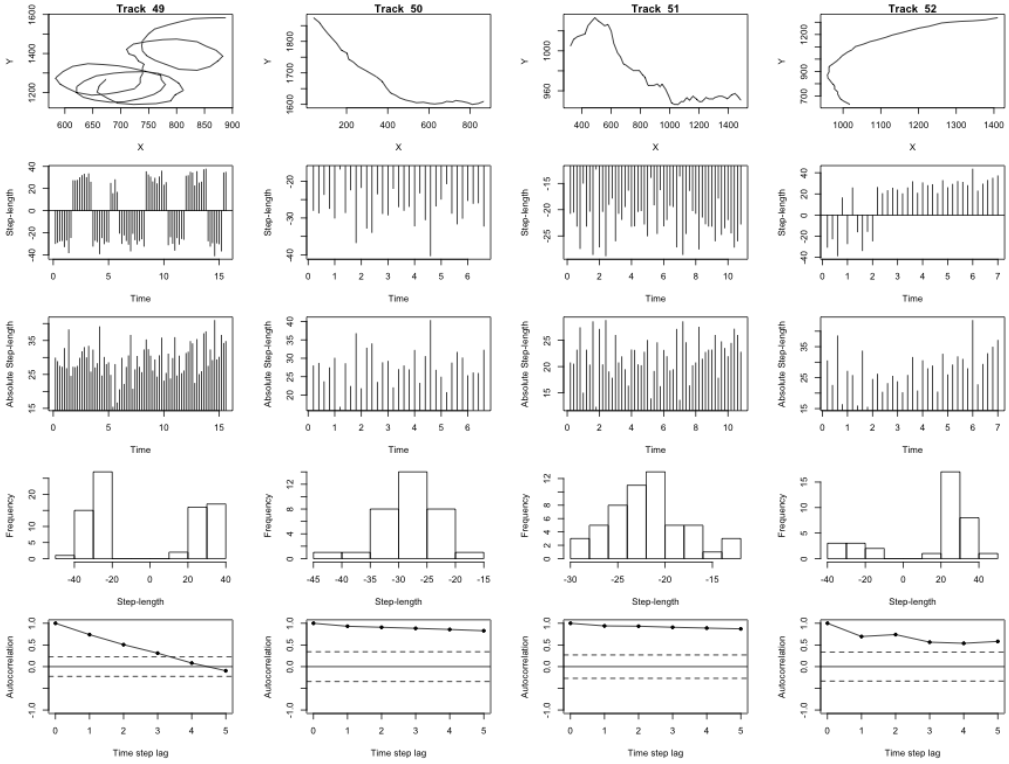

Track 29
Track 30
Track 31
Track 32
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag


Track 33
Track 34
Track 35
Track 36
Y
X
Step-length
Time
Absolute Step-length
Frequency
Autocorrelation
Time step lag


Track 37
Track 38
Track 39
Track 40
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 41
Track 42
Track 43
Track 44
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 45
Track 46
Track 47
Track 48
X
Y
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag


Track 49
Track 50
Track 51
Track 52
X
Y
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag

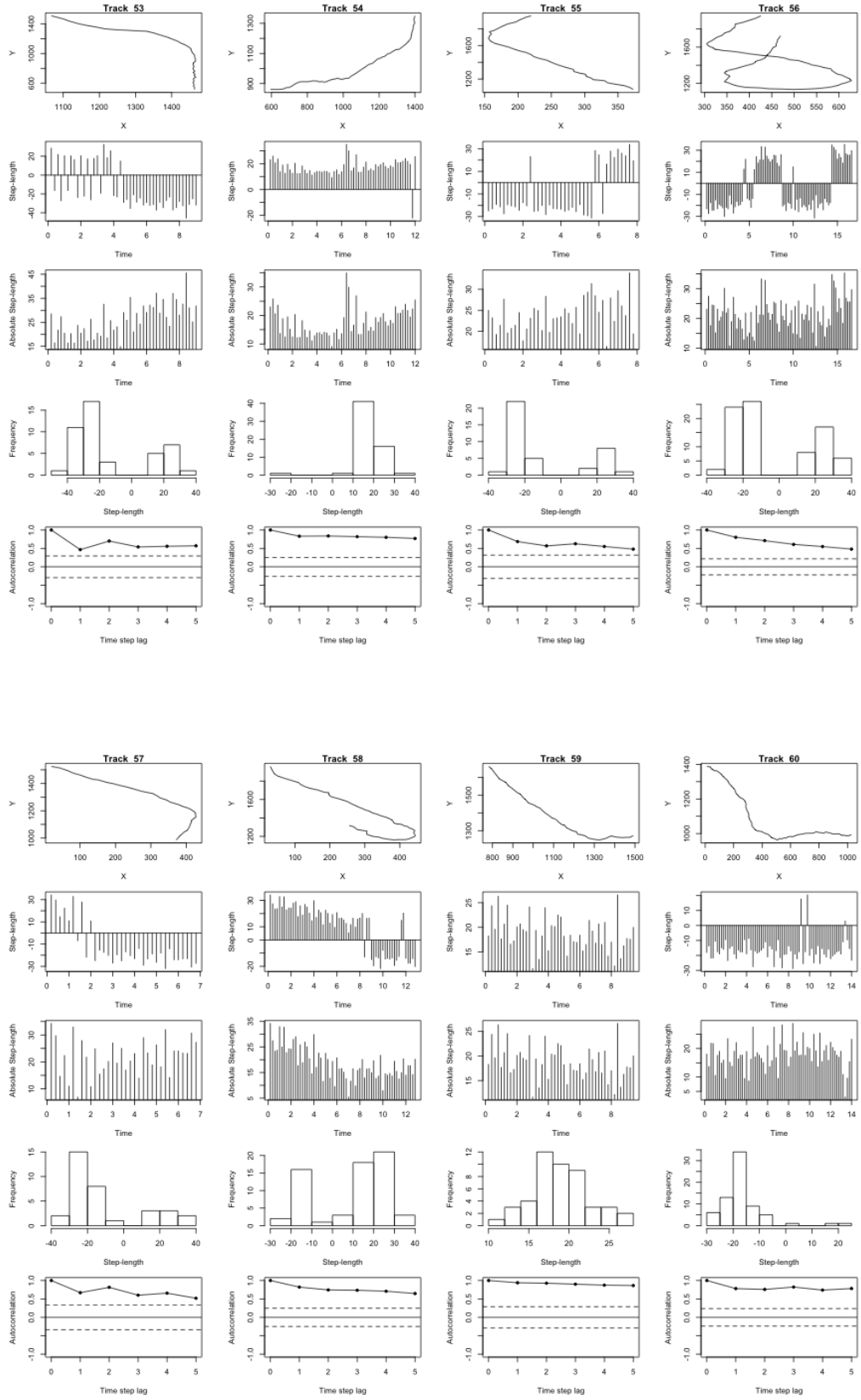
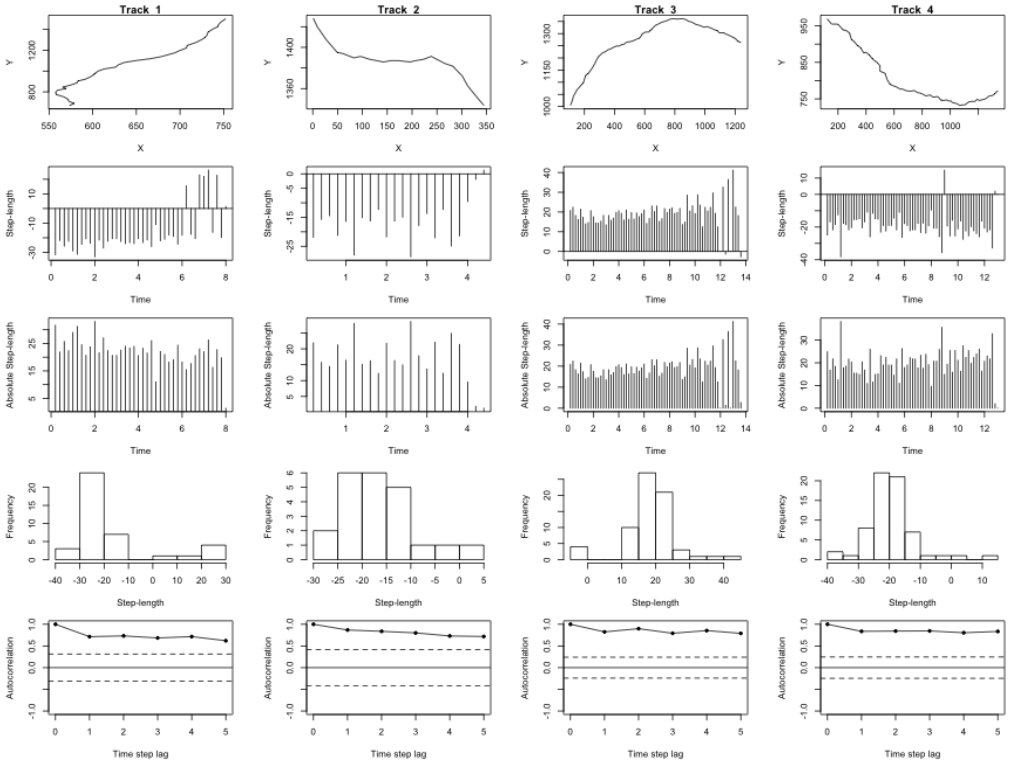
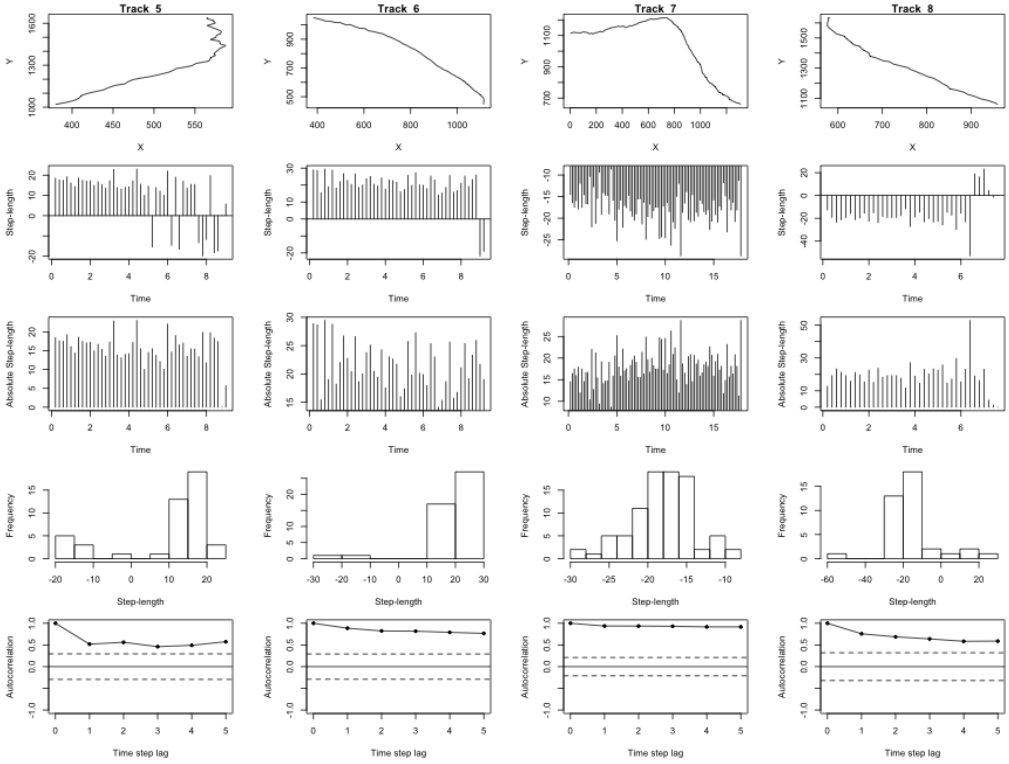
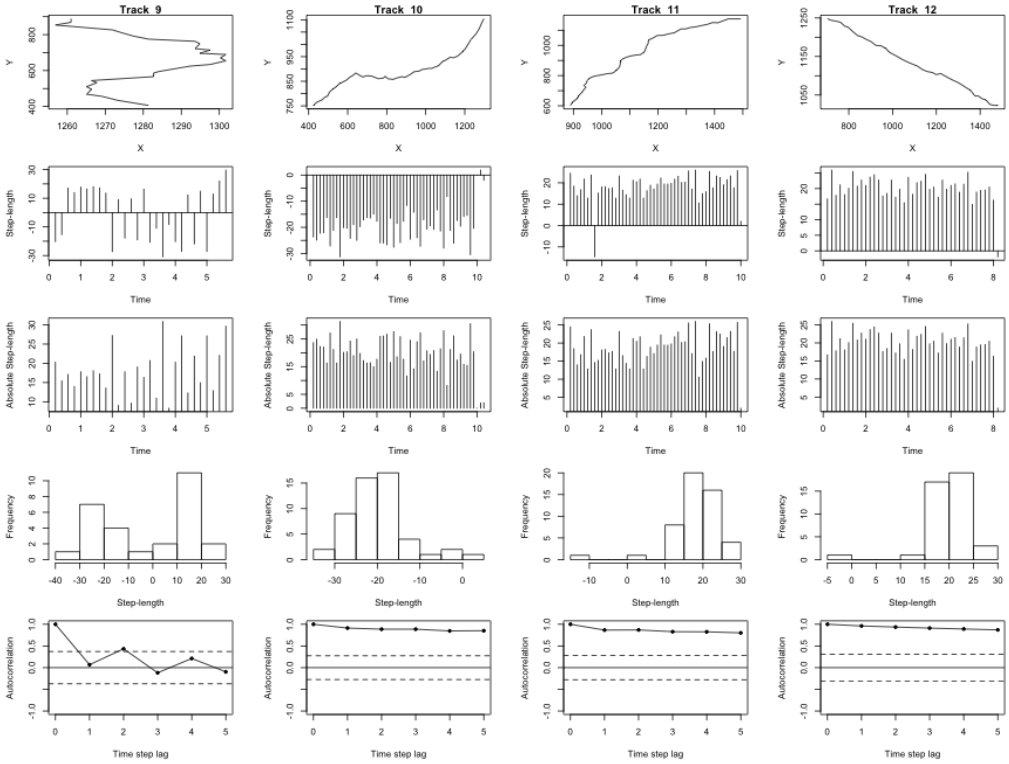
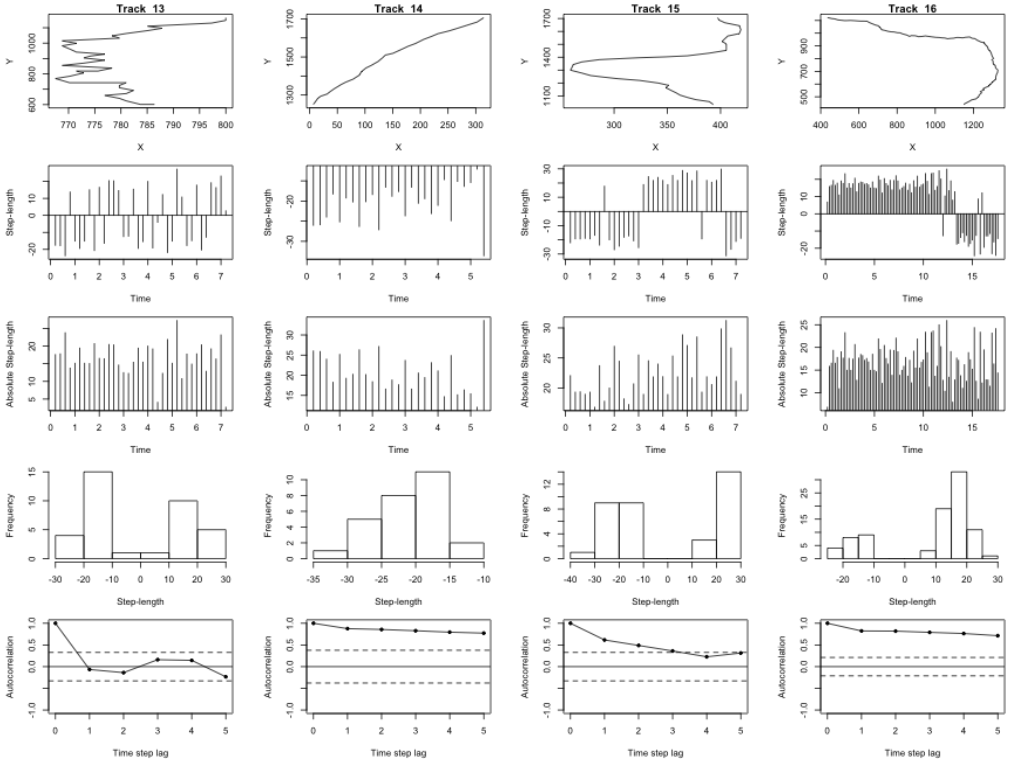


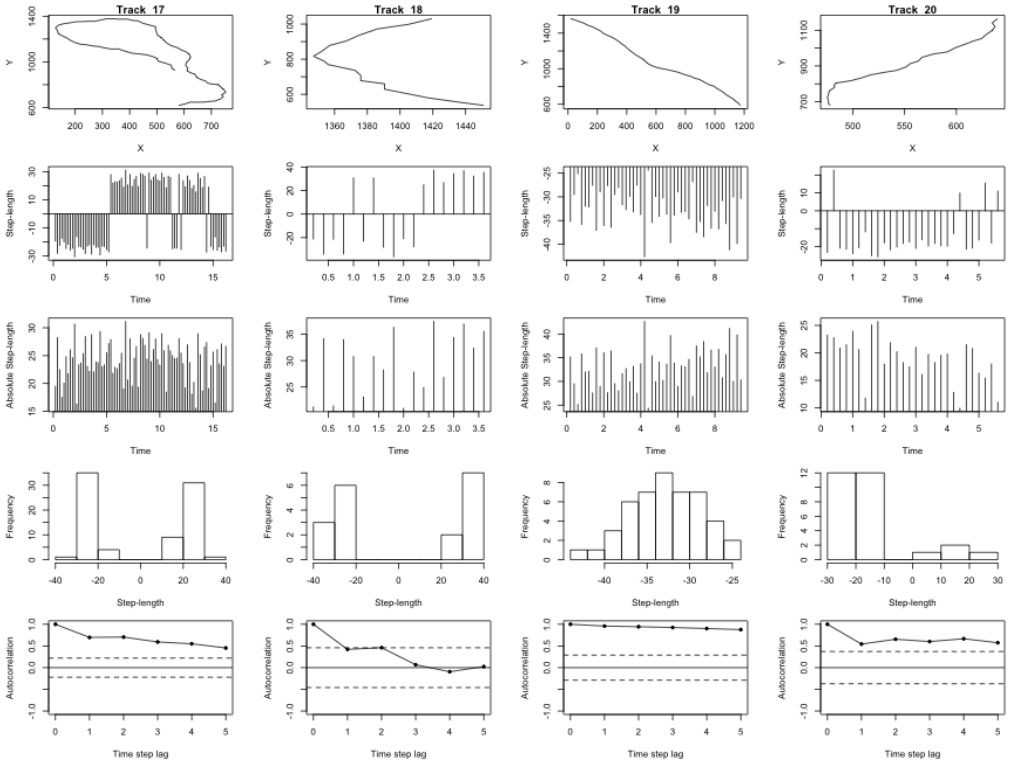
Figure 1A.10. Individual sperm track characteristics for fresh non-capacitated sperm in the presence of one COC (n=60)

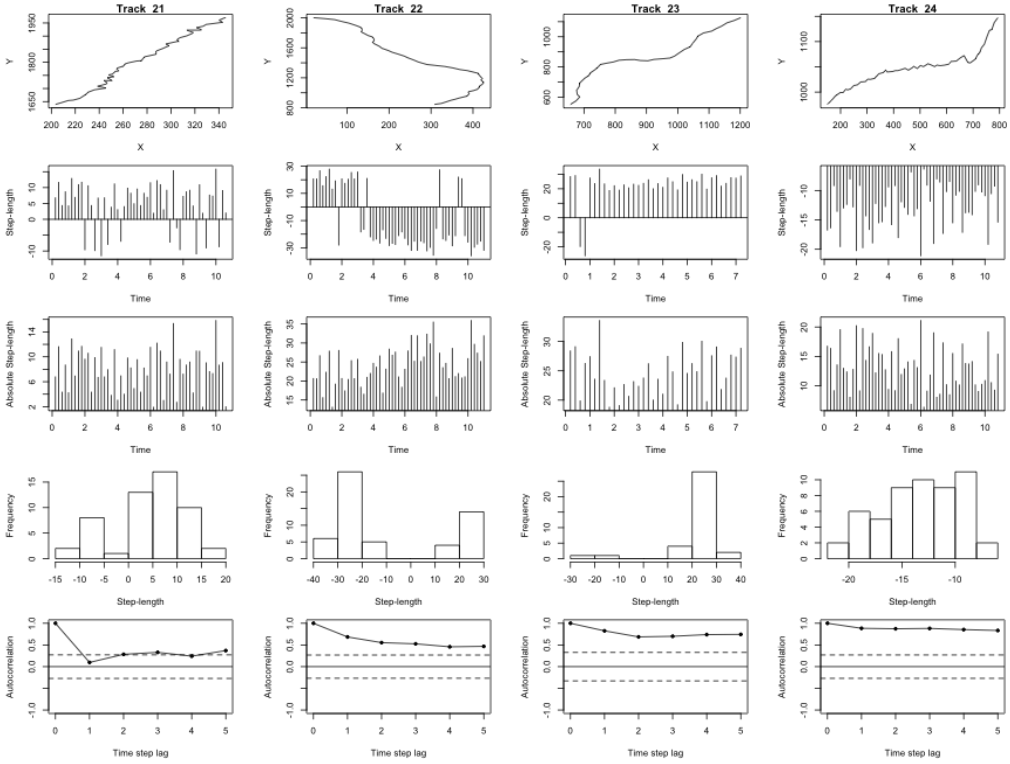

Track 1
Track 2
Track 3
Track 4
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

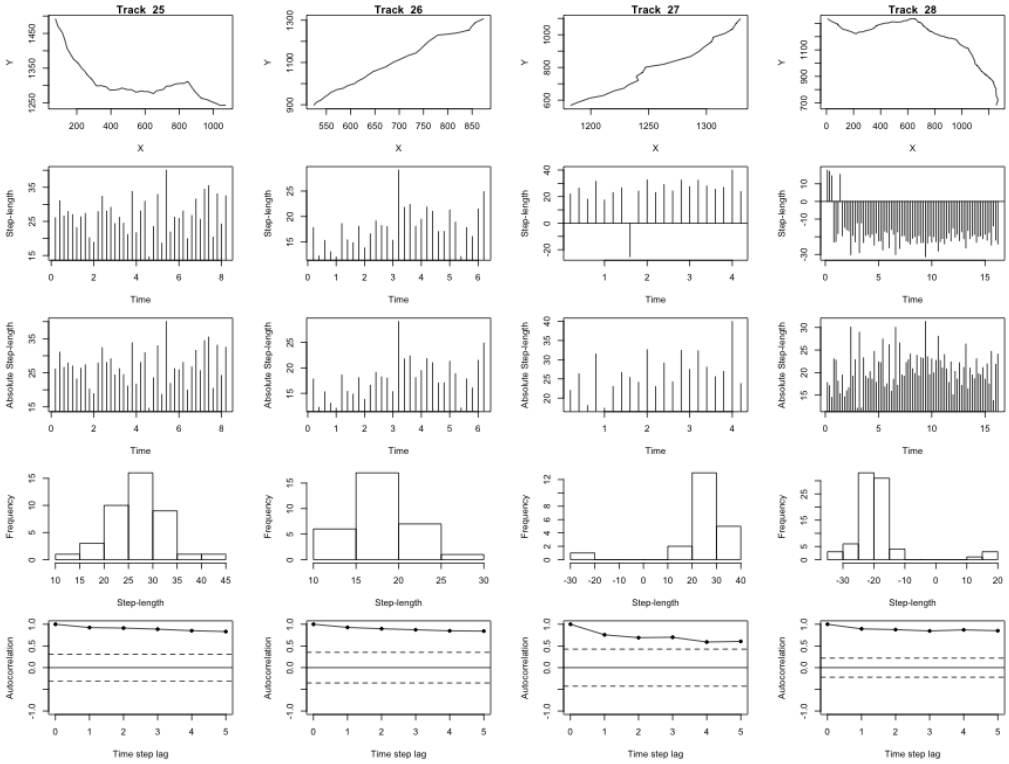

Track 5
Track 6
Track 7
Track 8
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

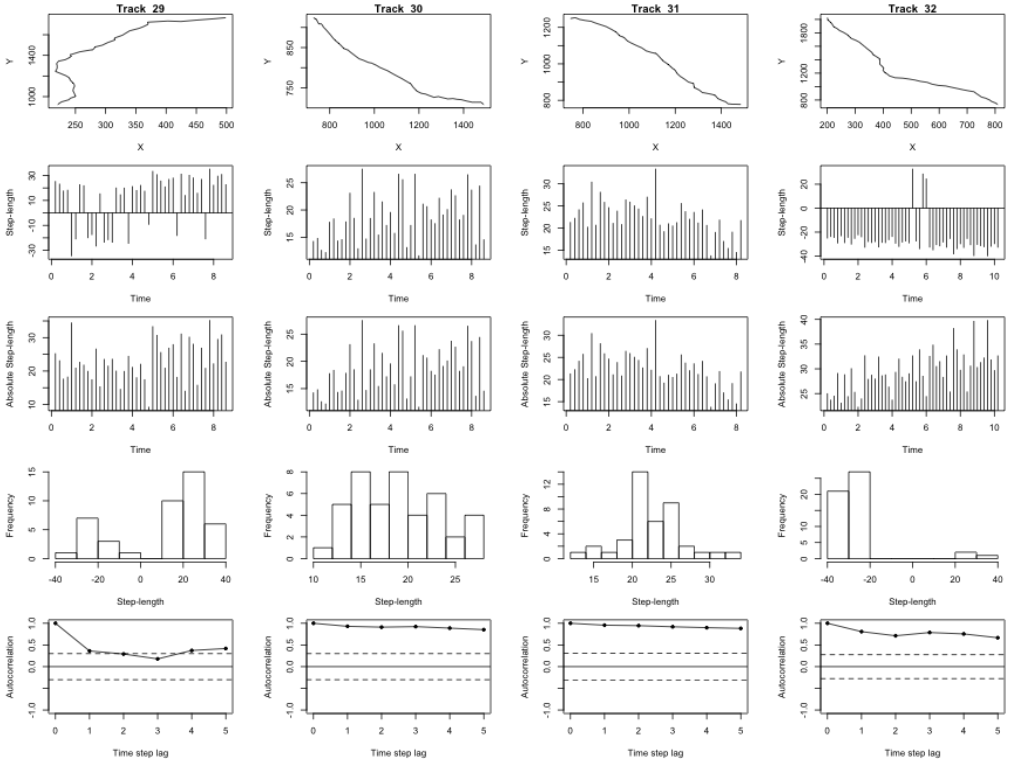

Track 9
Track 10
Track 11
Track 12
X
Y
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

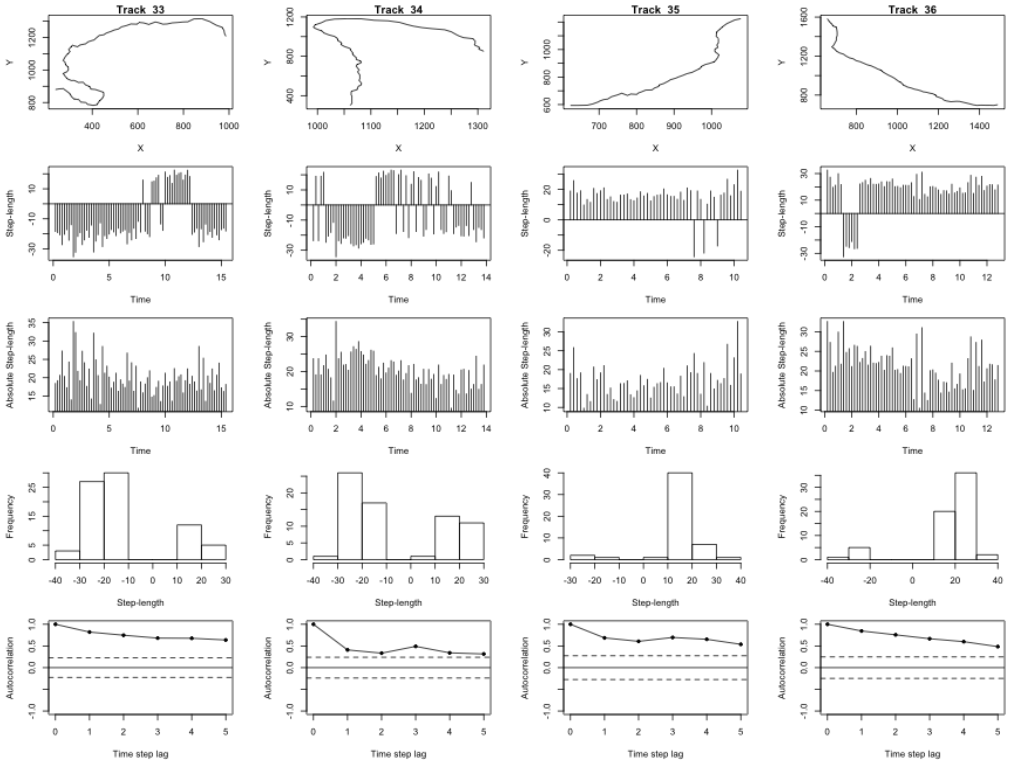

Track 13
Track 14
Track 15
Track 16
X
Y
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

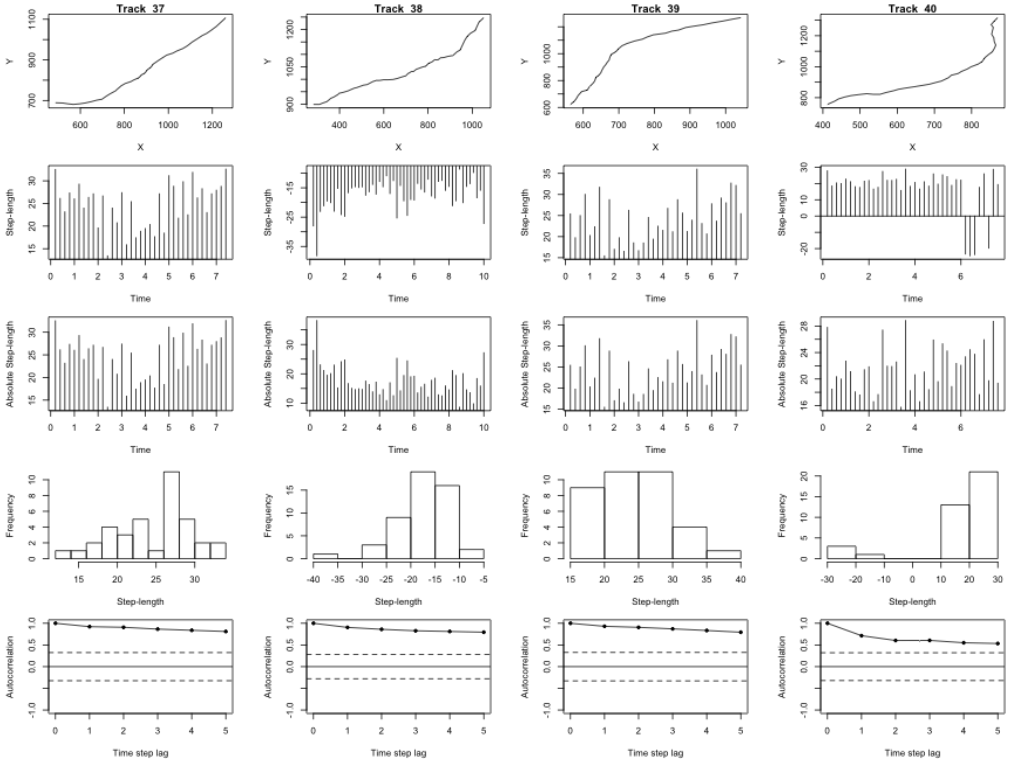

Track 17
Track 18
Track 19
Track 20
X
Y
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag


Track 21
Track 22
Track 23
Track 24
X
Y
Step-length
Absolute Step-length
Time
Frequency
Step-length
Autocorrelation
Time step lag


Track 25
Track 26
Track 27
Track 28
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 29
Track 30
Track 31
Track 32
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 33
Track 34
Track 35
Track 36
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 37
Track 38
Track 39
Track 40
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

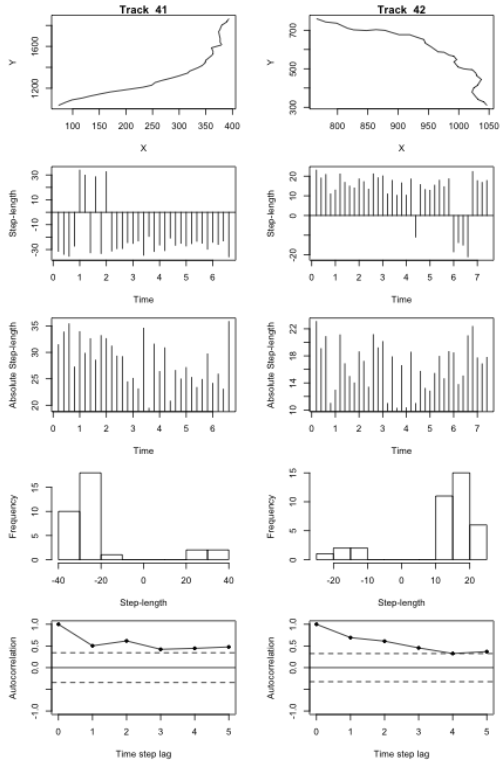
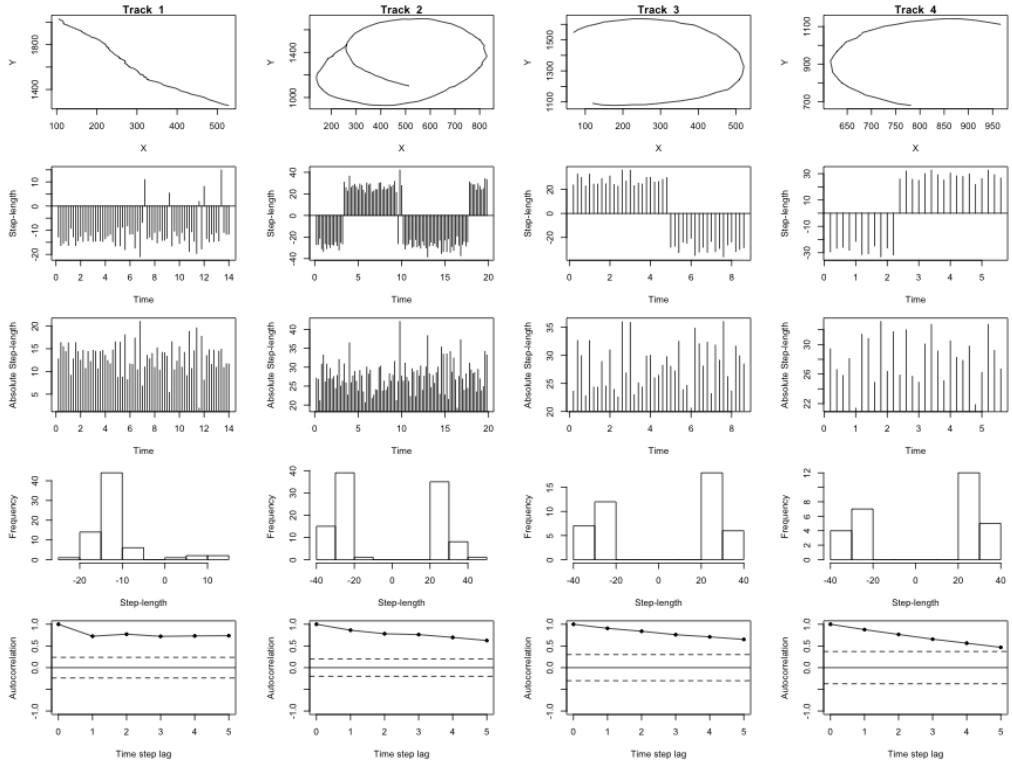
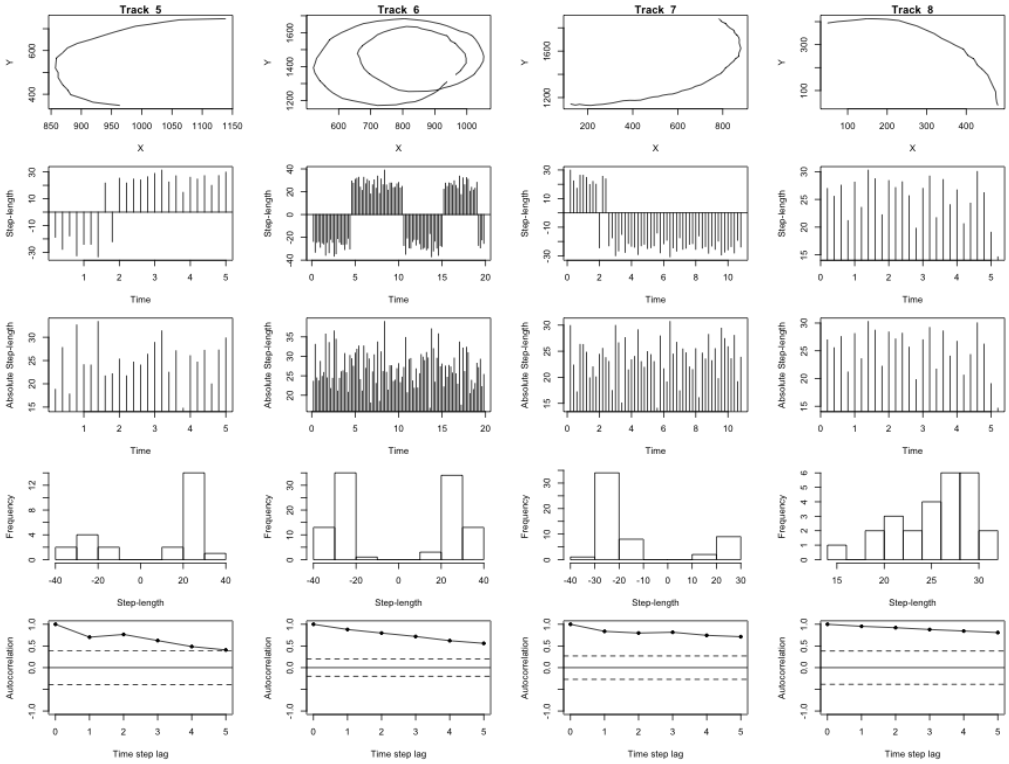
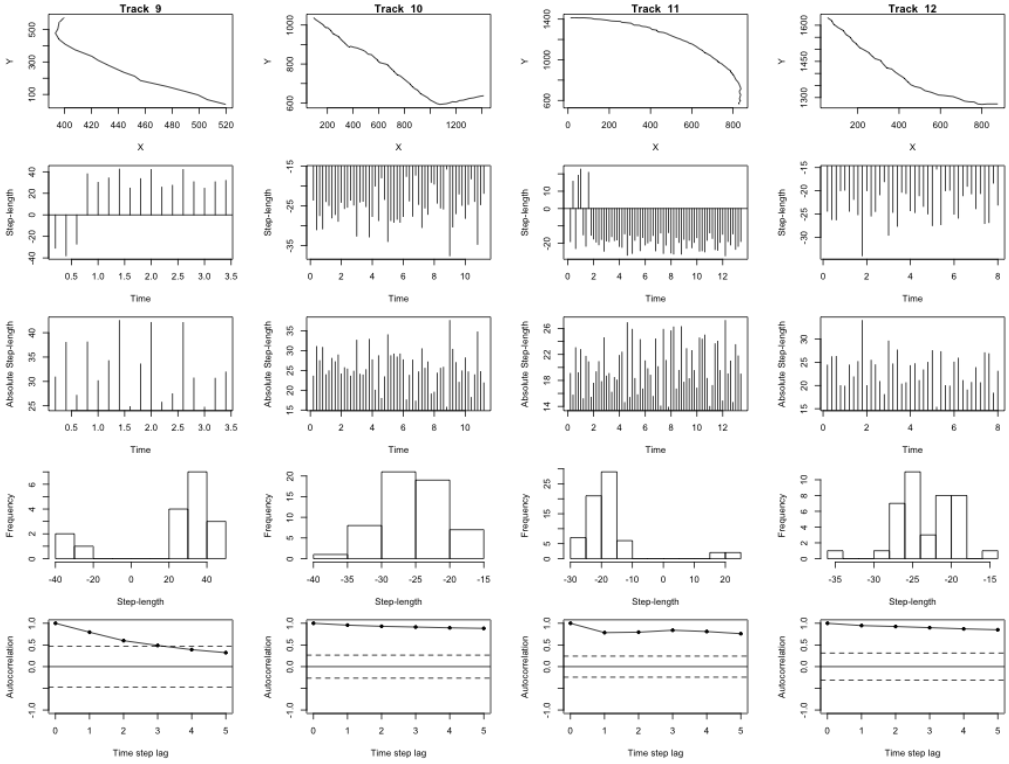
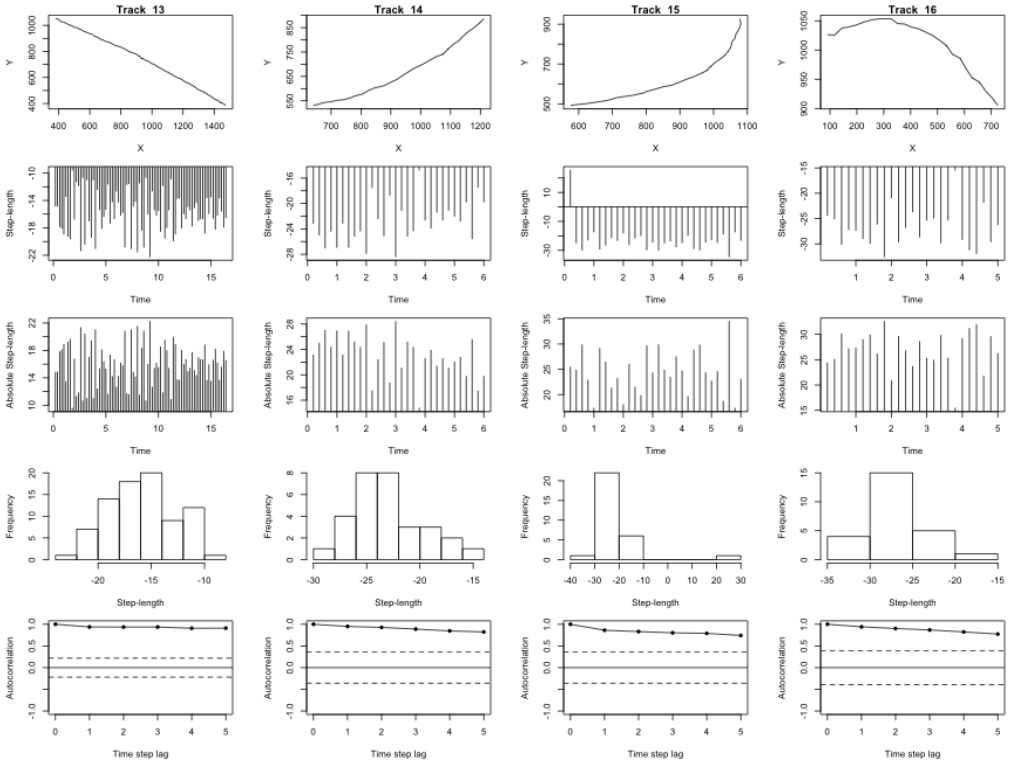


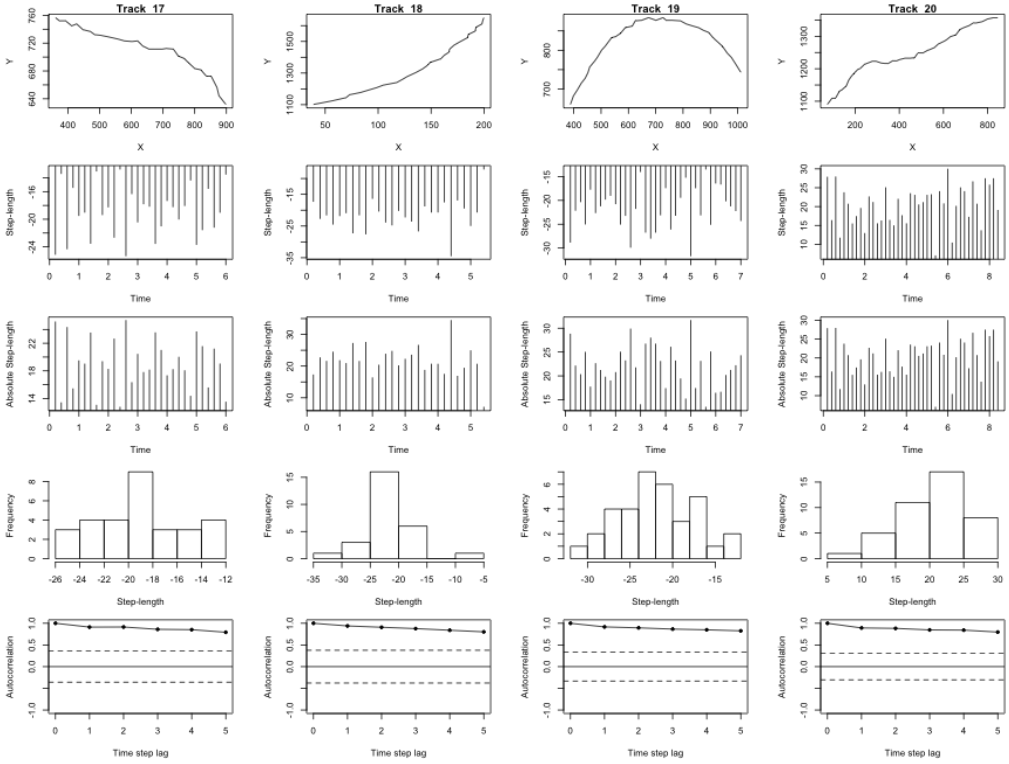
Figure 1A.11. Individual sperm track characteristics for fresh non-capacitated sperm in the presence of two COC (n=42)

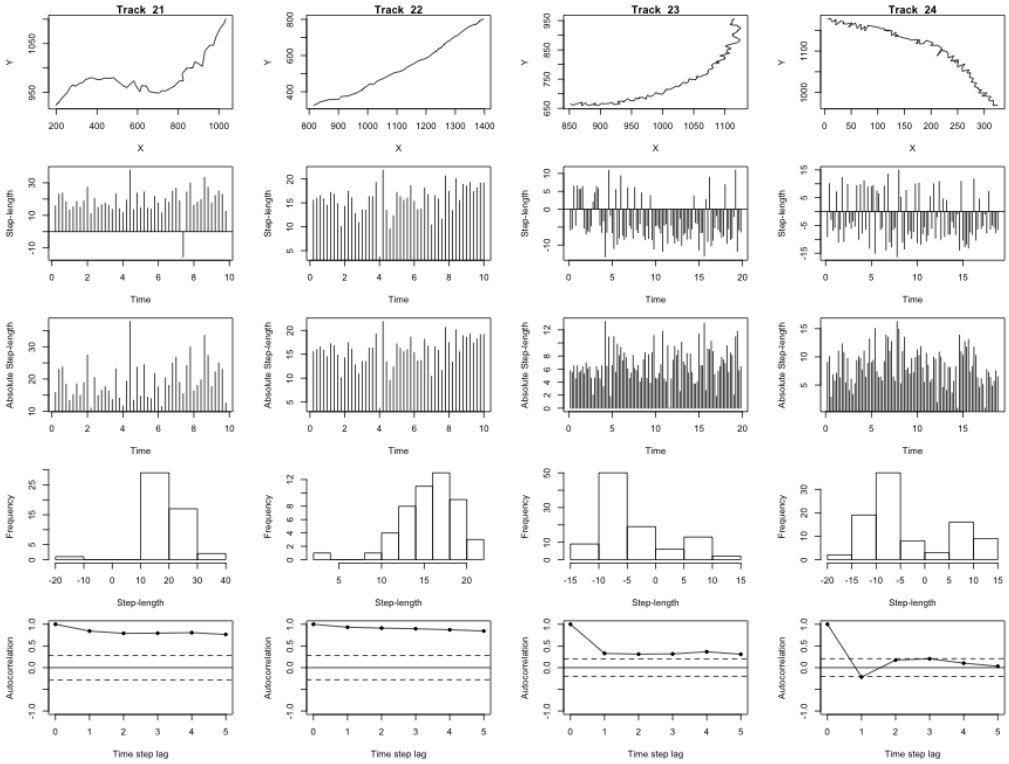


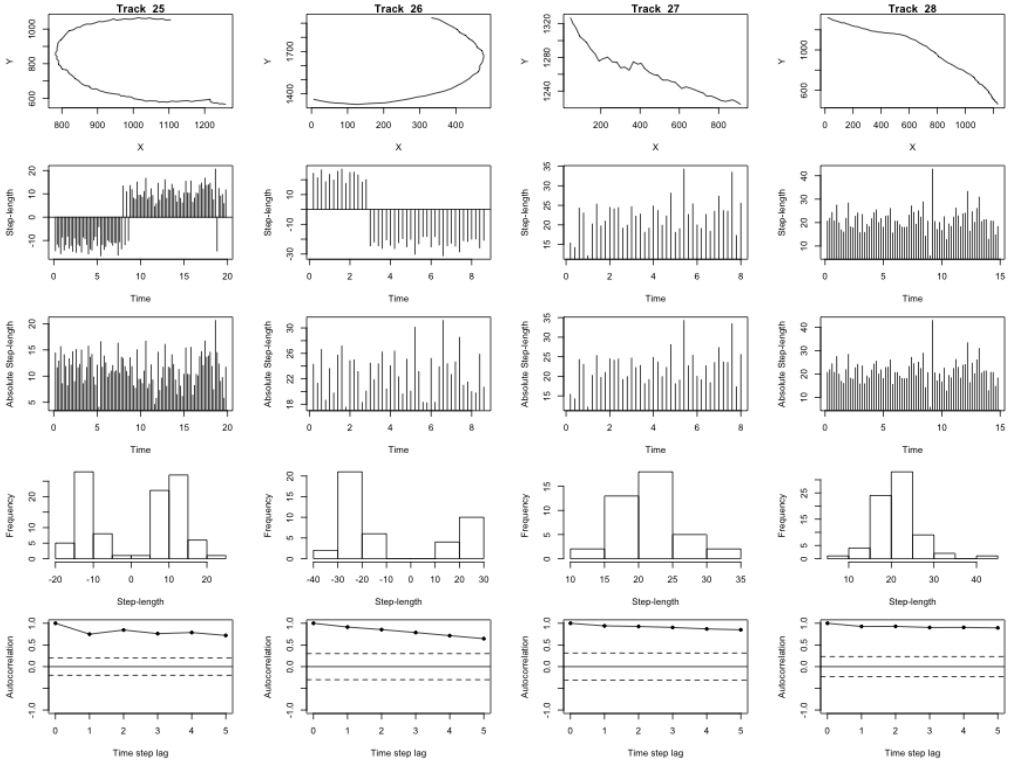

Track 5
Track 6
Track 7
Track 8
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 9
Track 10
Track 11
Track 12
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 13
Track 14
Track 15
Track 16
X
Y
Step-length
Absolute Step-length
Time
Frequency
Autocorrelation
Time step lag


Track 17
Track 18
Track 19
Track 20
X
Y
Step-length
Absolute Step-length
Time
Frequency
Autocorrelation
Time step lag


Track 21
Track 22
Track 23
Track 24
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag


Track 25
Track 26
Track 27
Track 28
Y
X
Step-length
Time
Absolute Step-length
Frequency
Step-length
Autocorrelation
Time step lag

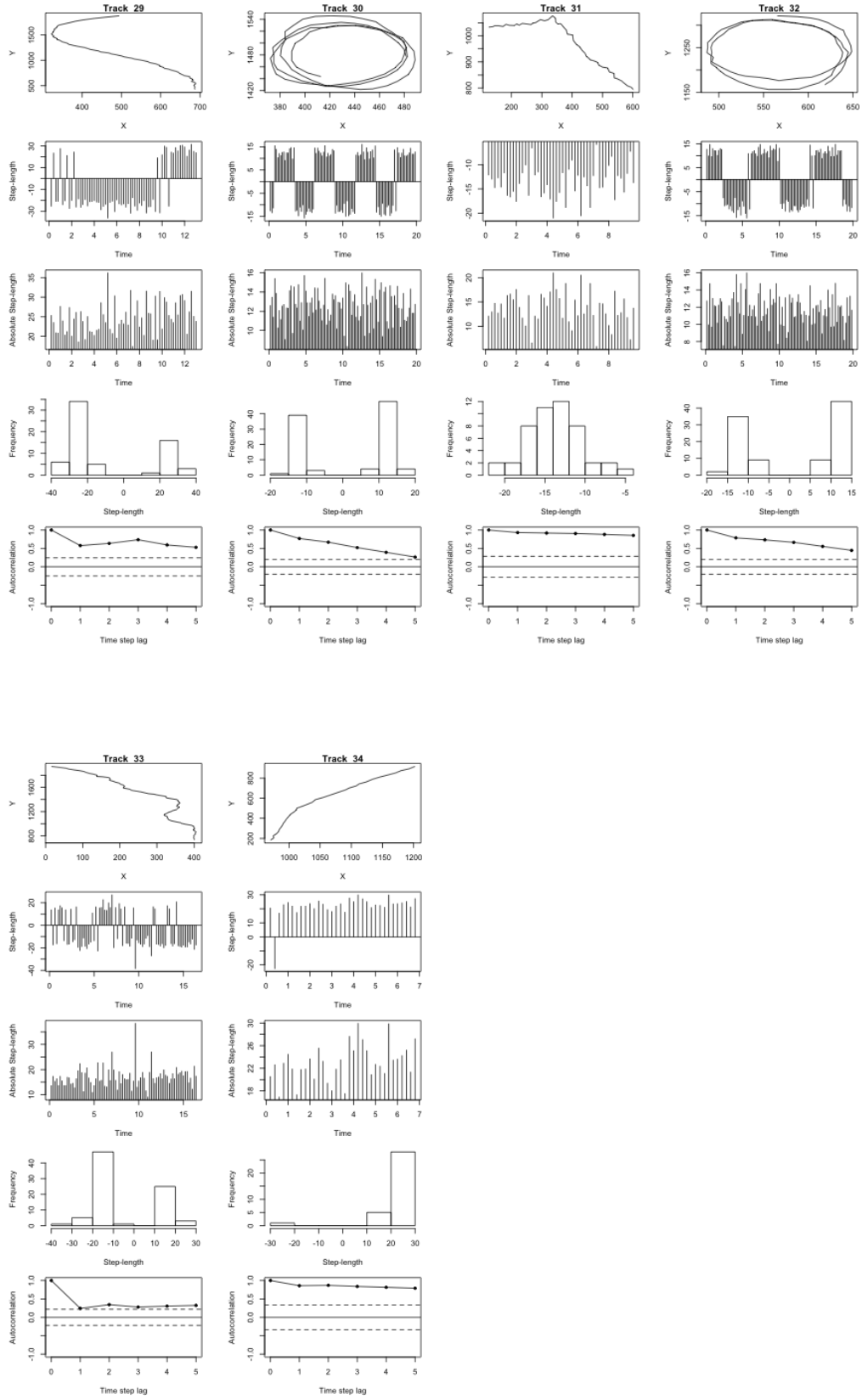


Figure 1A.12. Individual sperm track characteristics for fresh non-capacitated sperm in the presence of three COC (n=34)

References

Abaigar, T., J. Barbero and W. V. Holt. 2012. Trajectory variance and autocorrelations within single-sperm tracks as population-level descriptors of sperm track complexity, predictability, and energy-generating ability. J Androl 33:216–228.

All fractal analysis carried out in R (v. 2.13.0 – R Development Core Team, 2011).

Appendix Two: Cichlid sample information.

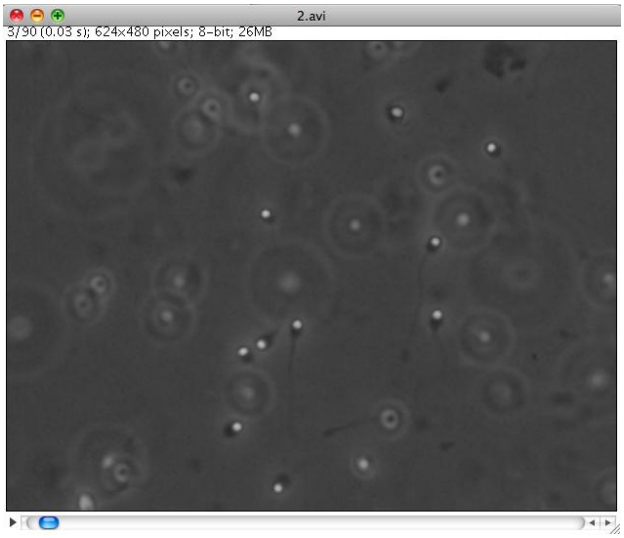
Table 2A.1. Information on sequence data used in creation of phylogenetic tree and species mean measurements for average path velocity (VAP) and all aspects of sperm morphology. HL=head length; HW=head width; FL=flagellum length; HL:FL= ratio of head length to flagellum length; TL=total length (measure as tip of head to end of flagellum). SL=standard length (distance from the tip of the snout to the posterior end of the vertebral column measured to nearest mm). Species without GenBank accession numbers were sequenced at the University of Hull from fin-clips taken from males used as part of the sperm analysis research.

			Species mean sperm traits						Species mean body mass	
Species	**GenBank accession number**	**Alignment length (bp)**	**VAP (µm/s)**	**HL (µm)**	**HW (µm)**	**FL (µm)**	**HL:FL (µm)**	**TL (µm)**	**SL (mm)**	**Weight (g)**
Nyassachromis cf. microcephalus	GQ422530	304	94.51	1.43	1.38	15.91	0.09	17.35	112	39
Otopharynx lithobates	Fin-clip	1099	95.20	1.67	1.76	18.33	0.09	20.00	127	49.22
Cyrtocara moorii	Fin-clip	937	98.90	1.96	2.00	18.04	0.11	20.00	112	46.03
Lethrinops furcifer	AF305316	981	96.90	1.63	1.67	20.53	0.08	22.17	98	30.09
Hemitilapia oxyrhynchus	Fin-clip	1117	67.05	1.63	1.67	17.50	0.09	19.13	117	46.55
Lethrinops sp. chilingali	Fin-clip	1117	92.21	1.81	1.89	16.37	0.11	18.19	115	39.22
Rhamphochromis sp. Chilingali	EF683285	482	91.29	1.83	1.87	19.45	0.09	21.28	158	52.62
Rhamphochromis cf. longiceps	AF298907	857	82.25	1.93	1.92	19.85	0.10	21.78	168	61.15
Astatotilapia calliptera Rovuma	HQ335175	804	54.90	1.76	1.90	18.78	0.09	20.54	97	29.1
Astatotilapia sp. 'calliptera chizumulu'	AF298938	851	64.82	1.74	1.79	17.48	0.10	19.22	111	39.14
Metriaclima kingsizei Nkhata Bay	Fin-clip	1122	112.69	1.42	1.40	18.83	0.08	20.25	106	38.64
Metriaclima 'zebra gold'	Fin-clip	1122	73.22	1.96	1.85	18.48	0.11	20.44	118	58.26

Species	GenBank accession number	Alignment length (bp)	Species mean sperm traits						Species mean body mass	
			VAP (µm/s)	HL (µm)	HW (µm)	FL (µm)	HL:FL (µm)	TL (µm)	SL (mm)	Weight (g)
Metriaclima estherae	Fin-clip	1123	84.72	1.98	2.00	18.37	0.11	20.35	114	62.59
Metriaclima fanizilberi	GU128786	481	118.02	1.78	1.78	19.89	0.09	21.67	84	22.77
Mertiaclima zebra Nkhata Bay	Fin-clip	1118	81.36	1.47	1.83	17.80	0.08	19.27	110	51.76
Metriaclima zebra Thumbi west	Fin-clip	1119	88.93	1.71	1.92	20.21	0.08	21.92	95	30.85
Metriaclima emmiltos	GU128786	481	97.99	2.00	2.00	16.10	0.12	18.10	107	40.36
Metriaclima sp. 'elongatus chewere'	Fin-clip	1124	83.44	1.87	1.97	19.47	0.10	21.33	101	35.91
Metriaclima zebra Thumbi east	AY930025	830	73.22	1.76	1.93	19.40	0.09	21.16	102	43.37
Cynotilapia sp. 'lion' Sanga	Fin-clip	1124	91.68	1.47	1.47	18.88	0.05	20.35	79	17.24
Cynotilapia afra Nkhata Bay	Fin-clip	1124	97.11	1.79	1.94	19.61	0.09	21.39	85	23.34
Cynotilapia axelrodi	Fin-clip	571	89.23	1.70	1.78	19.83	0.09	21.54	79	15.03
Cynotilapia afra Thumbi west	Fin-clip	608	67.50	1.47	1.61	17.94	0.08	19.42	98	33.25
Tropheops sp. 'olive'	Fin-clip	775	90.06	1.91	1.97	18.36	0.10	20.27	103	39.15
Pseudotropheus sp. 'acei'	HQ335189	846	50.41	2.00	2.00	20.67	0.10	22.67	80	18.76
Labidochromis caeruleus	HQ335181	849	81.72	1.95	2.00	17.98	0.11	19.93	96	32.56
Neochromis omnicaeruleus	Fin-clip	1103	99.75	1.77	1.67	20.25	0.09	22.02	89	22.59
Paralaabidochromis chilotes	AF213540	832	109.52	1.93	1.87	19.23	0.10	21.17	103	36.69

Species	GenBank accession number	Alignment length (bp)	Species mean sperm traits						Species mean body mass	
			VAP (μm/s)	HL (μm)	HW (μm)	FL (μm)	HL:FL (μm)	TL (μm)	SL (mm)	Weight (g)
Lipochromis melanopterus	AF213527	832	95.11	1.79	1.56	20.77	0.09	22.56	96	26.50
Haplochromis sp therutereon	Fin-clip	1102	58.99	1.83	1.83	21.58	0.08	23.42	93	14.15
Oreochromis shiranus	Fin-clip	1078	111.33	1.86	1.76	20.10	0.09	21.95	106	30.16
Stomatepia pindu	Fin-clip	942	114.13	2.00	2.00	19.83	0.10	21.83	144	98.85
Sarotherodon steinbachi	AJ845161	287	82.26	1.96	1.71	22.71	0.09	24.67	119	51.39

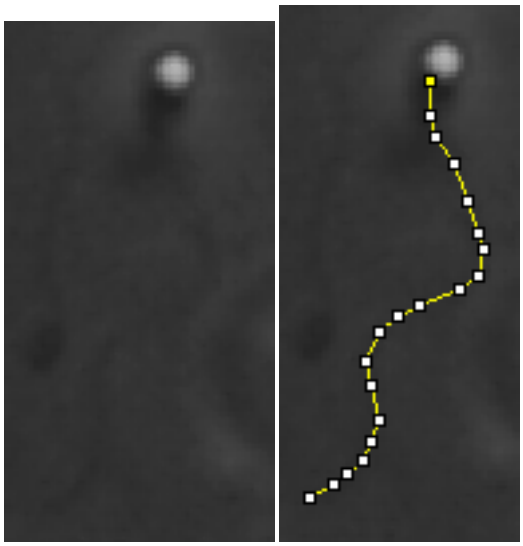
Appendix Three: Technique for measuring individual sperm in ImageJ



Videos were imported into ImageJ as AVI files. The image to the left represents they typical level of image clarity and resolution of video data.

Bright circles indicate sperm heads.

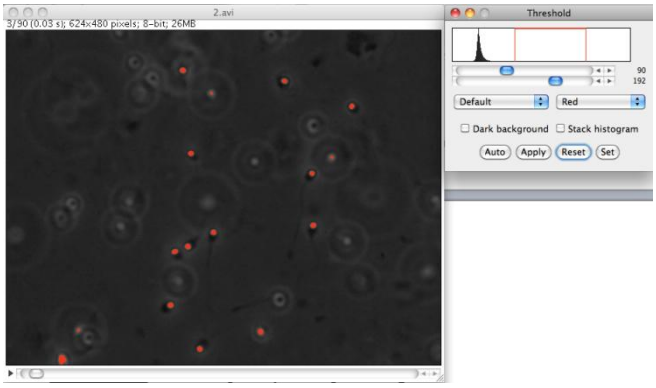
Each video was played back repeatedly so that focal cells with clear morphology and progressive motility could be selected for morphology and velocity analysis.



Once selected, the start coordinates for focal cells were recorded to prevent duplication.

The zoom function was applied so that flagellum length could be traced as indicated in the images to the left. Morphology could then be measured and recorded.

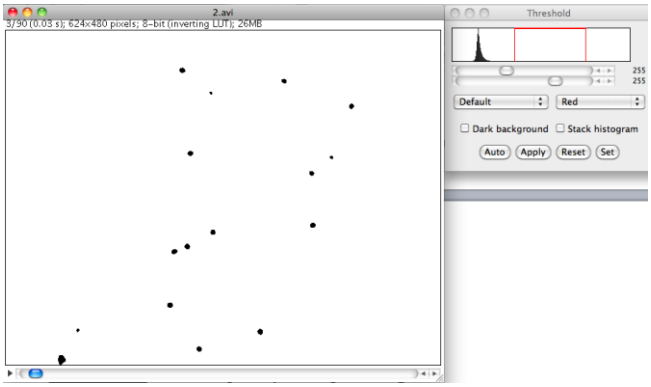
Head length, head width and flagellum length were all measured in this way at the beginning, middle and end of the recorded track for each focal sperm.



In order to measure the speed of the focal cell using computer assisted sperm analysis (CASA) for ImageJ all other cells needed to be removed.

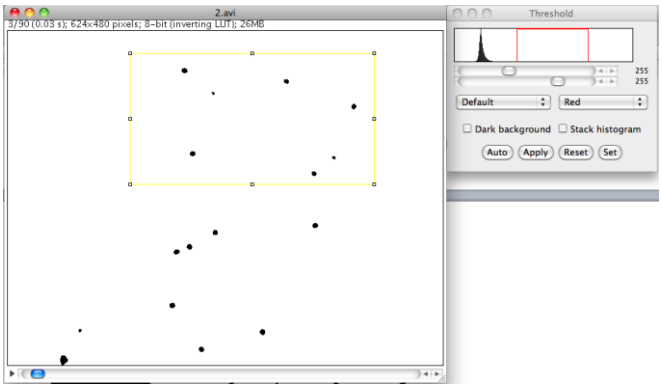
The threshold function was applied which allowed sperm heads to be highlighted in red (see left).

This process helped eliminated much of the information that would otherwise create noise in the CASA output.

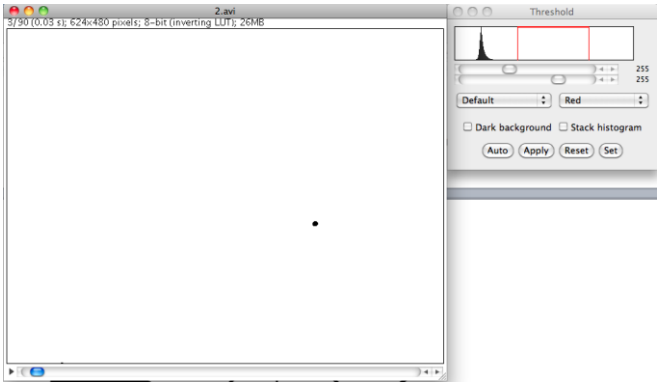


On applying the threashold function previously red heads become black and all other noise from the original video is removed (see left).

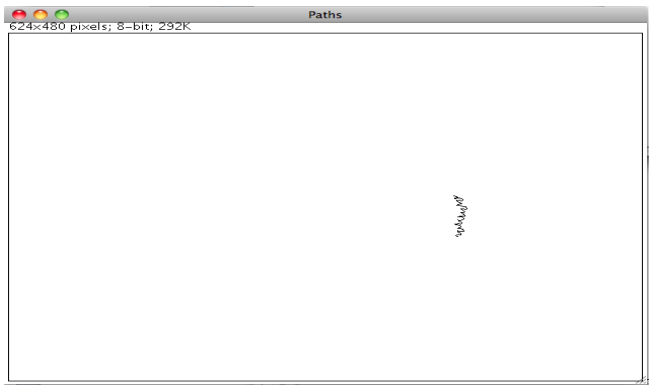
Next all sperm heads other than the focal cell needed to be removed from each frame of the video.



Selections could be made of varying sizes (yellow box shown opposite) and the content within could be deleted frame by frame until only one sperm head (black circle) remained across all frames.



Once the whole video had been cleared of noise (as shown to the left), the path of this focal cell could then be traced using parameters in CASA.



CASA output gives visualization of the actual path of the sperm head (as shown opposite) as well as the numerical data on velocity (Wilson-Leedy and Ingermann 2007).

This method of isolating the focal cell and tracking the path of the head of the sperm cell was repeated for every individual sperm measured in this thesis.

Figure 3A.1.Protocol for isolating and measuring individual spermatozoa using ImageJ

References

NIH ImageJ (v. 1.42q)

Wilson-Leedy, J. G. and R. L. Ingermann. 2007. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 67:61–672.

Wilson-Leedy, J. G. and R. L. Ingermann. 2006. imageJ CASA instructions.

Appendix Four: Information provided as supplemental data when Chapter four was submitted to the journal Evolution

Table 4A.1. Summary of specific computer assisted sperm analysis (CASA - http://rbs.info.nih.gov/ij/plugins/casa.html) Sperm Tracker values used to record individual cell speed using the ImageJ v. 1.44o plugin (Wilson-Leedy and Ingermann 2007). In the Sperm Tracker window of CASA in ImageJ the following parameters were altered from their default values: a, Minimum sperm size (pixels); b, Maximum sperm size (pixels); c, Minimum track length (frames); d, Maximum sperm speed between frames (pixels) and q, Frame Rate (frames per second).

Species	Minimum sperm size (pixels)	Maximum sperm size (pixels)	Minimum track length (frames)	Maximum sperm velocity between frames (pixels)	Frame rate (frames per second)
Human	5	150	25	40	26
Emu	10	300	30	40	31-38
Guppy	10	400	97	150	97
Rainbowfish	5	200	97	40	97
Frog	5	400	65	20	65
Mussel	5	40	97	40	97

Details of video analysis

In order to standardise the length of clip and compensate for varying frame rates, videos with less frames per second were cropped into clips with more frames in total. For example, the frame rate for frog videos were low compared to other species (See Table 1 Appendix 4). As it was not possible to increase the number of frames per second for existing videos longer clips were analysed, 2 second clips were used (43-70 frames) to standardise the number of frames isolated sperm could be tracked for. Frames rates were also low for emu therefore clips of 4 seconds were used which gave total frames per clip of between 31 and 38 frames. Frame rates for human data could not be increased in this was due to constrains of the original recordings.

Head volume calculations

Head volume calculated assuming shape of head to be an ellipsoid with rotational symmetry about its longest axis (front to back: $\frac{3}{4}\ \pi a\ b^2$where a = head length and b = head width) therefore working out the volume for an ellipsoid. While frog sperm heads are not ellipsoid, and are more cone-like, the two geometries are similar enough (cone: $\frac{1}{3}\pi r^2 h$ where r = radius of circle at base and h = distance from base to tip) that the volume of a cone (essentially half an ellipsoid) scales linearly with that of an ellipsoid given the same linear measurements.

Table 4A.2. Loadings for PC 1 for each speed measure for all species, eigenvalues with cumulative % variance explained by PC1.VAP was consistent across species as the speed which explained correlations with length components for all species. As such VAP was chosen as the main speed measure reported here.

	Human	Emu	Guppy	Mussel	Rainbow	Frog
VCL	-0.58	0.58	0.54	-0.62	-0.51	0.47
VAP	-0.60	0.59	0.60	-0.63	-0.63	0.64
VSL	-0.56	0.57	0.59	-0.47	-0.58	0.61
Eigenvalue	2.67	2.88	2.66	2.16	2.28	2.33
%	89	96	89	72	77	78

Table 4A.3. Results for all species from mixed model centering allowing random effect intercepts for curvilinear velocity (VCL) and straight line velocity (VSL). HW=head width, HL=head length, HV=head volume (no HV measure for mussel data), FL=flagella length, TL=total length and HL:FL=ratio of head length to flagellum length, n_t=total number of males. F = degrees of freedom within and between males. 95% confidence intervals (95% CI) calculated for effect size (r).Significant correlations (p=<0.005) in bold.

				between male effects				within male effects			
Species	**Speed**	**Sperm trait**	**n_t**	**F**	**p**	**r**	**95 % CI**	**F**	**p**	**r**	**95 % CI**
Human	VCL	HW	29	$F_{1,27}$=0.45	0.507	-0.09	-0.34 to 0.18	$F_{1,79}$=16.06	**<0.001**	-0.48	-0.63 to -0.26
		HL		$F_{1,27}$=<0.01	0.952	0.01	-0.26 to 0.27	$F_{1,79}$=0.91	0.343	0.13	-0.14 to 0.38
		HV		$F_{1,27}$=0.36	0.555	-0.08	-0.33 to 0.19	$F_{1,79}$=13.39	**<0.001**	-0.45	-0.61 to -0.22
		FL		$F_{1,27}$=0.70	0.409	-0.12	-0.36 to 0.16	$F_{1,79}$=10.09	**0.002**	-0.40	-0.58 to -0.16
		TL		$F_{1,27}$=0.23	0.633	-0.07	-0.32 to 0.20	$F_{1,79}$=5.79	**0.018**	-0.32	-0.52 to -0.06
		HL:FL		$F_{1,27}$=0.13	0.720	0.05	-0.22 to 0.31	$F_{1,79}$=2.17	0.144	0.20	-0.07 to 0.43
	VSL	HW		$F_{1,27}$=4.14	0.052	-0.26	-0.47 to 0.00	$F_{1,79}$=20.14	**<0.001**	-0.51	-0.65 to -0.31
		HL		$F_{1,27}$=0.79	0.384	-0.12	-0.36 to 0.15	$F_{1,79}$=2.74	0.102	0.22	-0.05 to 0.44
		HV		$F_{1,27}$=4.21	**0.050**	-0.27	-0.48 to 0.00	$F_{1,79}$=15.34	**<0.001**	-0.47	-0.62 to -0.24
		FL		$F_{1,27}$=3.42	0.075	-0.24	-0.46 to 0.03	$F_{1,79}$=16.41	**<0.001**	-0.48	-0.62 to -0.26
		TL		$F_{1,27}$=1.12	0.300	-0.14	-0.38 to 0.13	$F_{1,79}$=10.29	**0.002**	-0.40	-0.57 to -0.16

Species	Speed	Sperm trait	n_t	between male effects				within male effects			
				F	**p**	**r**	**95 % CI**	**F**	**p**	**r**	**95 % CI**
		HL:FL		$F_{1,27}$=0.01	0.914	0.01	-0.24 to 0.27	$F_{1,79}$=6.22	**0.015**	0.32	0.07 to 0.51
Emu	VCL	HW	9	$F_{1,7}$=7.01	**0.033**	0.29	0.02 to 0.49	$F_{1,80}$=13.40	**<0.001**	0.38	0.18 to 0.54
		HL		$F_{1,7}$=1.98	0.202	0.14	-0.08 to 0.34	$F_{1,80}$=50.30	**< 0.001**	0.58	0.44 to 0.67
		HV		$F_{1,7}$=8.95	**0.020**	0.32	0.05 to 0.52	$F_{1,80}$=34.58	**< 0.001**	0.55	0.38 to 0.66
		FL		$F_{1,7}$=0.31	0.593	-0.06	-0.26 to 0.15	$F_{1,80}$=6.59	**0.012**	-0.26	-0.43 to -0.06
		TL		$F_{1,7}$=0.12	0.741	0.04	-0.17 to 0.24	$F_{1,80}$=1.12	0.293	0.11	-0.10 to 0.31
		HL:FL		$F_{1,7}$=1.62	0.244	0.13	-0.09 to 0.32	$F_{1,80}$=56.65	**< 0.001**	0.59	0.46 to 0.68
	VSL	HW		$F_{1,7}$=5.01	0.060	0.25	-0.01 to 0.46	$F_{1,80}$=8.42	**0.005**	0.32	0.10 to 0.49
		HL		$F_{1,7}$=3.49	0.104	0.21	-0.04 to 0.41	$F_{1,80}$=38.50	**<0.001**	0.56	0.41 to 0.67
		HV		$F_{1,7}$=8.03	**0.025**	0.31	0.04 to 0.52	$F_{1,80}$=22.65	**< 0.001**	0.48	0.30 to 0.62
		FL		$F_{1,7}$=0.37	0.560	-0.07	-0.28 to 0.15	$F_{1,80}$=6.90	**0.010**	-0.28	-0.45 to -0.07
		TL		$F_{1,7}$=0.23	0.649	0.05	-0.17 to 0.26	$F_{1,80}$=0.65	0.422	0.09	-0.13 to 0.30
		HL:FL		$F_{1,7}$=2.59	0.152	0.17	-0.06 to 0.37	$F_{1,80}$=46.61	**<0.001**	0.59	0.45 to 0.69

Species	Speed	Sperm trait	n_t	between male effects				within male effects			
				F	**p**	**r**	**95 % CI**	**F**	**p**	**r**	**95 % CI**
Guppy	VCL	HW	18	$F_{1,6}$=0.52	0.500	-0.10	-0.35 to 0.18	$F_{1,47}$=0.19	0.666	-0.06	-0.32 to 0.21
		HL		$F_{1,15}$=0.16	0.698	-0.04	-0.25 to 0.17	$F_{1,83}$=0.93	0.338	0.11	-0.11 to 0.31
		HV		$F_{1,6}$=0.22	0.654	0.07	-0.21 to 0.32	$F_{1,47}$=1.07	0.306	0.14	-0.13 to 0.38
		FL		$F_{1,16}$=0.34	0.567	0.06	-0.14 to 0.27	$F_{1,95}$=0.18	0.675	-0.04	-0.24 to 0.16
		TL		$F_{1,16}$=<0.01	0.984	0.00	0.00 to 0.20	$F_{1,95}$=0.04	0.849	-0.02	-0.22 to 0.18
		HL:FL		$F_{1,16}$=0.26	0.618	-0.06	-0.26 to 0.16	$F_{1,83}$=1.19	0.278	0.12	-0.10 to 0.32
	VSL	HW		$F_{1,6}$=1.58	0.256	-0.16	-0.37 to 0.11	$F_{1,47}$=0.15	0.704	-0.05	-0.28 to 0.19
		HL		$F_{1,15}$=1.41	0.254	-0.13	-0.33 to 0.09	$F_{1,83}$=0.72	0.400	0.09	-0.12 to 0.29
		HV		$F_{1,6}$=0.78	0.411	-0.11	-0.32 to 0.14	$F_{1,47}$=0.76	0.387	0.11	-0.13 to 0.32
		FL		$F_{1,16}$=0.87	0.366	0.09	-0.11 to 0.28	$F_{1,95}$=0.15	0.697	-0.04	-0.23 to 0.16
		TL		$F_{1,16}$=0.18	0.679	-0.04	-0.23 to 0.16	$F_{1,95}$=0.41	0.523	-0.06	-0.25 to 0.13
		HL:FL		$F_{1,15}$=1.67	0.216	-0.14	-0.34 to 0.08	$F_{1,83}$=0.97	0.327	0.11	-0.11 to 0.31
Mussel	VCL	HL	20	$F_{1,18}$=0.04	0.843	-0.01	-0.12 to 0.10	$F_{1,255}$=0.03	0.868	-0.01	-0.12 to 0.10
		FL		$F_{1,18}$=3.64	0.072	0.11	-0.01 to 0.23	$F_{1,255}$=2.98	0.085	0.10	-0.01 to 0.21

Species	Speed	Sperm trait	n_t	between male effects				within male effects			
				F	**p**	**r**	**95 % CI**	**F**	**p**	**r**	**95 % CI**
Rainbow fish	VSL	TL	14	$F_{1,18}$=3.55	0.076	0.11	-0.01 to 0.23	$F_{1,255}$=3.86	**0.050**	0.12	0.00 to 0.22
		HL:FL		$F_{1,18}$=1.80	0.197	-0.08	-0.19 to 0.04	$F_{1,255}$=2.04	0.154	-0.08	-0.19 to 0.03
		HL		$F_{1,18}$=0.72	0.407	-0.05	-0.15 to 0.06	$F_{1,255}$=<0.01	0.959	0.00	-0.10 to 0.11
		FL		$F_{1,18}$=1.40	0.252	0.06	-0.04 to 0.17	$F_{1,255}$=5.53	**0.019**	0.12	0.02 to 0.22
	VCL	TL		$F_{1,18}$=1.05	0.319	0.05	-0.05 to 0.16	$F_{1,255}$=5.93	**0.016**	0.13	0.02 to 0.23
		HL:FL		$F_{1,18}$=1.60	0.222	-0.07	-0.17 to 0.04	$F_{1,255}$=2.29	0.131	-0.08	-0.18 to 0.02
		HW		$F_{1,12}$=2.35	0.151	-0.17	-0.37 to 0.06	$F_{1,70}$=3.52	0.065	-0.21	-0.39 to 0.01
		HL		$F_{1,12}$=0.20	0.664	0.05	-0.17 to 0.26	$F_{1,70}$=1.94	0.168	-0.15	-0.34 to 0.06
		HV		$F_{1,12}$=1.07	0.322	-0.11	-0.31 to 0.11	$F_{1,70}$=3.42	0.069	-0.20	-0.39 to 0.02
		FL		$F_{1,12}$=0.78	0.394	0.10	-0.13 to 0.30	$F_{1,70}$=<0.01	0.987	0.00	-0.21 to 0.22
	VSL	TL		$F_{1,12}$=0.78	0.395	0.10	-0.13 to 0.30	$F_{1,70}$=0.02	0.899	-0.01	-0.23 to 0.20
		HL:FL		$F_{1,12}$=0.05	0.832	-0.02	-0.23 to 0.19	$F_{1,70}$=1.25	0.268	-0.12	-0.32 to 0.09
		HW		$F_{1,12}$=0.09	0.764	-0.03	-0.22 to 0.17	$F_{1,70}$=3.29	0.074	-0.18	-0.35 to 0.02
		HL		$F_{1,12}$=1.40	0.260	0.12	-0.09 to 0.31	$F_{1,70}$=3.40	0.070	-0.19	-0.36 to 0.02

Species	Speed	Sperm trait	n_t	between male effects				within male effects			
				F	**p**	**r**	**95 % CI**	**F**	**p**	**r**	**95 % CI**
		HV		$F_{1,12}$=0.01	0.931	0.01	-0.19 to 0.20	$F_{1,70}$=3.85	0.054	-0.20	-0.36 to 0.00
		FL		$F_{1,12}$=4.72	0.051	0.23	0.00 to 0.41	$F_{1,70}$=4.19	**0.044**	0.22	0.01 to 0.40
		TL		$F_{1,12}$=5.14	**0.043**	0.24	0.01 to 0.43	$F_{1,70}$=3.12	0.082	0.19	-0.02 to 0.38
		HL:FL		$F_{1,12}$=0.34	0.572	-0.06	-0.25 to 0.14	$F_{1,70}$=6.99	**0.010**	-0.26	-0.41 to -0.06
Frog	VCL	HW	16	$F_{1,14}$=0.09	0.773	0.04	-0.21 to 0.27	$F_{1,68}$=0.44	0.507	0.08	-0.16 to 0.31
		HL		$F_{1,14}$=0.12	0.733	0.04	-0.20 to 0.28	$F_{1,68}$=2.08	0.154	0.18	-0.08 to 0.39
		HV		$F_{1,14}$=0.12	0.731	0.04	-0.20 to 0.28	$F_{1,68}$=1.04	0.311	0.13	-0.12 to 0.35
		FL		$F_{1,14}$=<0.01	0.972	0.00	-0.24 to 0.24	$F_{1,68}$=0.66	0.420	0.10	-0.14 to 0.33
		TL		$F_{1,14}$=0.07	0.801	0.03	-0.21 to 0.27	$F_{1,68}$=2.32	0.132	0.19	-0.06 to 0.40
		HL:FL		$F_{1,14}$=0.06	0.808	0.03	-0.21 to 0.27	$F_{1,68}$=0.63	0.429	0.10	-0.15 to 0.33
	VSL	HW		$F_{1,14}$=1.10	0.313	-0.12	-0.31 to 0.11	$F_{1,68}$= 0.52	0.475	0.08	-0.14 to 0.28
		HL		$F_{1,14}$=0.18	0.680	-0.05	-0.25 to 0.17	$F_{1,68}$=0.01	0.931	0.01	-0.20 to 0.22
		HV		$F_{1,14}$=0.89	0.361	-0.10	-0.30 to 0.12	$F_{1,68}$=0.47	0.496	0.08	-0.14 to 0.28
		FL		$F_{1,14}$=0.61	0.447	0.09	-0.13 to 0.29	$F_{1,68}$=0.24	0.626	0.05	-0.16 to 0.26

Species	Speed	Sperm trait	n_t	between male effects				within male effects			
				F	**p**	**r**	**95 % CI**	**F**	**p**	**r**	**95 % CI**
		TL		$F_{1,14}$=0.04	0.850	0.02	-0.19 to 0.23	$F_{1,68}$=0.25	0.621	0.05	-0.16 to 0.26
		HL:FL		$F_{1,14}$=0.50	0.492	-0.08	-0.28 to 0.14	$F_{1,68}$=0.05	0.830	-0.02	-0.23 to 0.19