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Subspecific variation in sperm morphology and performance in the Long-tailed Finch (*Poephila acuticauda*)

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Abstract

Background: Evolutionary biology endeavours to explain biological diversity, and as such it is critical to develop an understanding of the adaptive and functional significance of trait variation. Spermatozoa exhibit remarkable levels of morphological diversification. However, our understanding of the evolutionary causes and functional significance of this variation is limited, especially at the intraspecific level.

Methods: We quantified variation in sperm morphology and performance between two subspecies of Long-tailed Finch (*Poephila acuticauda acuticauda* and *P. a. hecki*), a small grassfinch found in tropical northern Australia. Despite a zone of secondary contact, these subspecies are maintained as two distinct forms: *P. a. acuticauda* occurs in the western part of the species' range and has a yellow bill, while *P. a. hecki* exhibits a red bill and is found in the eastern part of the range.

Results: We found small, but significant differences in sperm size between these subspecies (*P. a. acuticauda* had longer and narrower sperm than *P. a. hecki*), which was surprising given the recent evolutionary origins of these two taxa (i.e. 0.3 million years ago). Additionally, both subspecies exhibited high values of between- and within-male variation in sperm morphology, though in the case of sperm midpiece length this variation was significantly lower in *P. a. acuticauda* relative to *P. a. hecki*.

Conclusions: We suggest these observed differences in sperm morphology are the result of genetic drift and reflect historical processes associated with divergence between the eastern and western populations of these two subspecies. Finally, we discuss the potential implications of our findings for the process of population divergence and reproductive isolation.

Keywords: Passerine, Reproductive barriers, Sperm evolution, Structure-function relationships

Background

Despite their common role as fertilisers of ova, sperm cells exhibit remarkable interspecific variability in size and shape (reviewed in Pitnick et al. 2009). Sperm also show considerable variation among subspecies and between populations of the same species in a range of taxa. For example, significant intraspecific differences in sperm length have been reported for *Drosophila* (Pitnick et al. 2003), frogs (Hettyey and Roberts 2006), land snails (Minoretti and Baur 2006) and birds (Schmoll and Kleven 2010; Lüpold et al. 2011; Hogner et al. 2013; Laskemoen et al. 2013). In fact, sperm cells are the most variable cell type known (Pitnick et al. 2009). Surprisingly, however, our understanding of the evolutionary causes and adaptive significance of this variation remains somewhat limited. Moreover, our knowledge of sperm morphological and functional variation within-species is relatively poor compared to what is known at the interspecific level. Thus studies describing variation in sperm morphology and quantifying how variation in sperm morphology corresponds to variation in sperm function are warranted, especially at the intraspecific level.

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Sperm competition is generally thought to be an important driver of evolutionary change in sperm phenotype (Snook 2005; Pizzari and Parker 2009). For example, sperm competition is thought to favour the evolution of faster swimming sperm in a range of taxa, including fish (Fitzpatrick et al. 2009), mammals (Tourmente et al. 2011a; Lüpold 2013), and birds (Kleven et al. 2009). Similarly, comparative studies have reported a positive association between sperm competition strength and sperm length (e.g. Fitzpatrick et al. 2009; Lüpold et al. 2009; Tourmente et al. 2011a), though this pattern is far from universal and the nature of this relationship appears to be variable (reviewed in Simmons and Fitzpatrick 2012; and see Immler et al. 2011) for a non-linear relationship in birds. Variation in sperm size has also been linked to among-species variation in body size (Pitnick et al. 2009; Lüpold et al. 2011; Immler et al. 2011), mass-specific metabolic rate (Tourmente et al. 2011b) and the degree of genetic divergence among subspecies and populations (Hogner et al. 2013; Laskemoen et al. 2013). Finally, sperm traits also appear to evolve as an adaptation to the female reproductive environment; sperm length is correlated with the length of sperm storage organs or their associated ducts in a variety of taxa (reviewed in Pitnick et al. 2009), including birds (Briskie et al. 1997).

Divergence in sperm morphology among closely related taxa suggests that sperm form may evolve rapidly (Pitnick et al. 2003; Landry et al. 2003; Hogner et al. 2013), as has been shown for other ejaculatory traits (e.g. seminal fluid proteins, Swanson and Vacquier 2002). Other studies, however, have shown that total sperm size can remain relatively constant among populations over time (Hosken et al. 2003; Manier and Palumbi 2008). In birds, variable rates of evolutionary change in sperm size have been linked to variation in the intensity of postcopulatory sexual selection. For example, subspecies of Bluethroat (*Luscinia svecica*) exhibit significant differences in sperm length, despite being a group of relatively 'young' taxa (*c.* 0.15–0.35 million years since divergence), and appear to experience relatively strong sperm competition (Hogner et al. 2013). In contrast, sperm competition is presumed absent in both the Eurasian (*Pyrrhula pyrrhula*) and Azores (*P. murina*) Bullfinch, and sperm length does not differ between the sister species despite the longer time since divergence (*c.* 0.6–1.5 mya) (Töpfer et al. 2011; Lifjeld et al. 2013). More recently, a comparative study of passerine birds found a significant, positive association between the rate of evolutionary divergence in sperm size between closely related taxa and the strength of sperm competition (Rowe et al. 2015). Further investigation of variation in sperm morphology between closely related taxa is thus likely to contribute to our understanding of the evolutionary processes responsible for the remarkable evolutionary diversification of sperm form.

Variation in both sperm length (Bennison et al. 2015) and sperm motile performance (Birkhead et al. 1999; Denk et al. 2005; Pizzari et al. 2008) has been linked to fertilization success in birds. Here, we examine intraspecific variation in sperm morphology and sperm swimming speed in the Long-tailed Finch (*Poephila acuticauda*), a small grassfinch (family: Estrildidae) endemic to tropical northern Australia. Two subspecies of Long-tailed Finch, *P. a. acuticauda* and *P. a. hecki*, are recognized. Although phenotypically similar, the subspecies can be distinguished on the basis of bill color; The nominate *P. a. acuticauda* exhibits a yellow bill and occurs in the western part of the species' range, while *P. a. hecki* has a red bill and is found in the eastern part of the range. Differences in song structure have also been identified between the two subspecies (Zann 1976). A recent analysis based on multiple nuclear loci suggests that *P. a. acuticauda* diverged from *P. a. hecki* approximately 0.3 million years ago across the Ord Arid Intrusion, a minor biogeographic barrier splitting Arnhem Land and the Kimberley Plateau (Jennings and Edwards 2005). Recent mtDNA data supports the presence of these two distinct groups (western *P. a. acuticauda* and eastern *P. a. hecki*), along with the occurrence of a central region in the vicinity of the Ord Arid Intrusion where the subspecies are in contact (Rollins et al. 2012). We therefore tested for differences in sperm length (i.e. total sperm length and length of the individual sperm components—head, midpiece and flagellum) and sperm swimming speed between the two subspecies. We also explored variation in a relatively poorly studied aspect of sperm morphology—the ratio of acrosome length to nuclear length (A:N ratio). Although the adaptive significance of the A:N ratio is unknown, this trait appears variable in the small number of passerine species for which it has been investigated (Jamieson 2007), suggesting further investigation of the trait may be informative. Finally, we quantified how within-subspecies variation in sperm morphology corresponds to variation in sperm performance (i.e. sperm swimming speed).

Methods

Bird maintenance and sampling

We compared sperm morphology and sperm performance in the two subspecies of Long-tailed Finch (*P. a. acuticauda* and *P. a. hecki*) using both wild-caught birds held in captivity and first generation (F1) captive bred birds. Sperm samples were collected from 43 sexually mature males over 2 days: 21 December 2011 and 9 January 2012. At the time of sampling, all birds were held in aviaries located at Macquarie University in Sydney. All individuals from *P. a. hecki* ($n = 23$) were initially captured from a breeding population in October Creek, Northern Territory (eastern region population

sensu, Rollins et al. 2012; 16°37′S, 134°51′E) in October 2010 and subsequently held in captivity. For *P. a. acuticauda* ($n = 20$), we sampled both wild-caught birdsheld in captivity ($n = 9$) and F1 captive bred birds ($n = 11$). Wild-caught birds were initially captured from two sites (Mt House: 17°02′S, 125°35′E and Nelson's hole: 15°49′S, 127°30′E) in Western Australia in September 2009 and subsequently held in captivity. These sites were located 250 km apart, but comprise a single breeding population (western region population *sensu*, Rollins et al. 2012). F1 captive bred birds were hatched during January 2010 and March 2011 in the Macquarie University aviaries. Finches were maintained in captivity in mixed-sex aviaries under male-biased sex-ratio conditions and provided with food and water *ad lib*. The subspecies were housed separately. Nest boxes and nesting material was placed in aviaries approximately 4 weeks prior to sampling to encourage breeding activity. We noticed that eggs were found more frequently in aviaries containing *P. a. hecki* individuals, which may reflect inter-individual variation in the reproductive state of females. Alternatively, it may suggest that *P. a. hecki* were mating more actively than *P. a. acuticauda* males, which may have implications for variation in sperm function. Importantly, however, all males had the opportunity to breed (i.e. females and nesting materials were present in all aviaries) and were actively producing sperm at the time of sampling.

We captured males from their aviaries using mist nets and hand nets, and placed birds in small holding cages prior to sampling. Fresh sperm samples were collected from males using cloacal massage (Wolfson 1952), and we immediately measured sperm swimming speed. Specifically, we collected exuded semen in a 10 µL capillary tube and immediately mixed it with (*c.* 20–40 µL) pre-heated (40 °C) Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Ltd). Next, we pipetted 6 µL of the diluted semen into a pre-heated microscopy counting chamber (depth 20 µm, Leja, Nieuw-Vennep, Netherlands) mounted on a MiniTherm slide warmer (Hamilton Thorne Inc) maintained at 40 °C. Finally, sperm movement was recorded using a phase contrast scope (CX41, Olympus, Japan) connected to a digital video camera (Legria HF S200 Canon, Japan). For each male we recorded six different fields of view for 5 s, for a total recording time of 30 s.

Videos of sperm motion were analysed at a later date using computer-assisted sperm analysis (CASA; HTM-CEROS sperm tracker, CEROS v.12, Hamilton Thorne Research), and all analyses were conducted without knowledge of the males' subspecies identity. In each field of view, sperm were tracked for 0.5 s and the image analyser was set with a frame rate of 50 frames/s. We also set the following cell detection parameters to exclude non-sperm particles: minimum contrast, 80–120; minimum cell size, 8–12 pixels; sperm head elongation (i.e. width/length), <70. Additionally, we excluded non-continuous sperm tracks or sperm tracked for less than 10 frames, as well as tracks for which the maximum frame-to-frame movement exceeded the average frame-to-frame movement by 4 SDs for the same track, as such tracks tended to represent tracking errors in the software. Finally, to exclude the effects of drift in the chamber, sperm cells having a straight-line velocity (VSL, i.e. average velocity on a straight line between the start and end point of the sperm track) <25 µm s^{-1}, or a average path velocity (VAP, i.e. average velocity over a smoothed sperm track) <30 µm s^{-1} were counted as immotile and excluded from calculations of sperm swimming speed. These criteria were based on visual inspection of cells in all analyses, which was undertaken to optimize the detection of motile sperm, and are in line with previous studies of passerine sperm (e.g. Lifjeld et al. 2013; Cramer et al. 2015). Finally, following this filtering process, males with fewer than 20 motile sperm tracks were excluded from all analyses of sperm motile performance.

The total number of motile sperm that were tracked for each male ranged from 23 to 465 (median = 219, mean = 218.4 ± 18.5 s.e.). For each sperm we recorded curvilinear velocity (VCL, i.e. velocity over the actual sperm track), VSL and VAP. However, we choose to use VCL for statistical analyses because this metric measures the actual path of sperm movement and thus is likely to represent sperm velocity better than simpler approximations. Nonetheless, sperm motility parameters were strongly intercorrelated (all $r > 0.77$, $p < 0.001$), and analyses using VAP and VSL returned qualitatively similar results (data not shown). Finally, we calculated the proportion of motile sperm as the number of motile tracks divided by the total number of cells.

After assessing sperm swimming speed, we fixed the remainder of the sperm sample in 5 % buffered formaldehyde solution. For examination of sperm morphology, we placed an aliquot of the fixed sperm sample on a microscope slide and allowed it to air dry. We then captured high magnification (320×) digital images of sperm using a light microscope (DM6000 B Leica digital microscope) fitted with a digital camera (DFC420, Leica Microsystems) and measured sperm morphology using digital image analysis (Leica Application suite v. 2.6.0 R1). Following recommendations in the literature (Laskemoen et al. 2007; Bennison et al. 2015), 10 morphologically normal and undamaged sperm were analysed from each individual to obtain measurements (to the nearest 0.1 µm) of the following sperm traits: (1) head length, (2) midpiece length, (3) flagellum length, and (4) total sperm length. All measurements were taken blind to the subspecies identity of individuals. Additionally, these measures

were used to calculate the following composite measures: (5) ratio of flagellum length to head length and (6) ratio of midpiece length to flagellum length.

We also used scanning electron microscopy (SEM) to obtain high-resolution images of sperm from 10 individuals from each subspecies. To prepare sperm for SEM, we first attached sperm cells to glass coverslips precoated with poly-lysine (1 mg mL^{-1}; Sigma P1274) by placing a small (*c*. 10 µL) aliquot of formalin-fixed sperm onto each coverslip and incubating samples overnight in a wet chamber at room temperature. Next, sperm were dehydrated using a graded ethanol series consisting of a 10-min treatment with 70, 80, 90, and 96 % ethanol, followed by 4 × 15-min treatments with 100 % ethanol. Samples were then critical point dried (BAL-TEC CPD 030 Critical Point Dryer) and coverslips were mounted on SEM stubs using carbon tape. Finally, samples were sputter coated with 6 nm platinum using a Cressington 308R coating system, and samples were examined and digital images recorded using a Hitachi S-4800 Field Emission Scanning Electron Microscope operated at 5.0 kV.

SEM images were used toobtain the following additional measures: (1) sperm head width, and (2) ratio of acrosome length to nuclear length (A:N ratio). For these measures, images were taken at 9000× magnification and, for each individual, 10–15 intact and undamaged sperm cells were selected for imaging and measurement by systematic uniform random sampling. We then used standard stereological methods to obtain accurate measures of head width, total head length (HL) and the length of the sperm nucleus (NL). More specifically, measurements were obtained using a counting grid and the Buffon formula for length of a line trace $L = (\pi/4) \times l \times d$, in which L = length in micrometres, l = the sum of the intersections between L and the grid lines, and d = the grid line spacing in micrometres (Cruz-Orive and Weibel 1990). Sperm head width was measured at the boundary between the acrosome and the nucleus, and the A:N ratio was obtained using the formula, A:N ratio = (HL − NL)/NL.

Statistical analysis

Data for *P. a. acuticauda* included samples from both wild-caught and captive bred (F1) birds. Thus we first tested for potential differences in sperm morphology and swimming speed between these groups using t tests or Wilcoxon rank sum tests. However, we found no significant differences in any of the sperm traits measured (all $p > 0.21$), and therefore these birds were treated as a single group in all subsequent analyses.

To test for differences in sperm morphology and swimming speed (i.e. VCL) between *P. a. acuticauda* and *P. a. hecki*, we used linear mixed-effects models with subspecies included as a fixed factor and male identity as a random factor, running separate models for each sperm trait. To account for heteroskedasticity in some models, we specified the variance structure of models: inter-factor variation was modeled using the VarIdent variance structure in the sperm midpiece length and A:N ratio models and we applied the varExp variance structure using the fitted values to the model of VCL. The decision to include variance structure in models and choose between potential variance structures was based on likelihood ratio tests and AIC values, and modeling assumptions (i.e. heterogeneity of variance, normality of residuals) were validated through visual inspection of residual plots (Zuur et al. 2009). Additionally, we used Fisher's F test to compare variance in sperm traits between the subspecies using the mean values within individuals, and tested for differences between species in the proportion of motile sperm using a two-sample t test.

Next, we calculated the within-male coefficient of variation (CV) in sperm traits as CV_{wm} = (standard deviation [SD]/mean) × 100, and compared values between subspecies using a two-sample t test. We also calculated the among-male CV in total sperm length for both subspecies as CV_{am} = (SD/mean) × 100, and adjusted these values for small sample size according to the formula: adjusted $CV_{am} = (1 + 1/4n) \times CV_{am}$ (Sokal and Rohlf 1995). Finally, separately for each subspecies, we used linear models to determine the relationships between sperm morphology and sperm swimming speed. Because the number of sperm cells tracked varied considerably between individuals (see above) we also included the number of tracked sperm as a covariate in all models, and, as before, model validity was assessed through inspection of residual plots. All analyses were performed with R (v. 3.0.2; R Core Team 2013) and, where appropriate, the R package 'nlme' (Pinheiro et al. 2014). All proportion data was arcsine square-root transformed and, when necessary, other data were ln-transformed to meet modeling assumptions.

Results

Scanning electron microscopy showed that sperm from both subspecies exhibit the typical helical shaped head of passerine sperm, with a helical membrane restricted to the acrosome and the mitochondrial helix extending along a large proportion of the flagellum (Fig. 1). However, sperm size differed slightly, but significantly, between the subspecies. Specifically, *P. a. acuticauda* had longer sperm (i.e. total sperm length) and narrower sperm (i.e. sperm head width) than *P. a. hecki*, while the ratio of midpiece to flagellum length (i.e. relative midpiece length) was greatest in *P. a. hecki* (Table 1). In contrast, there was no significant difference between the

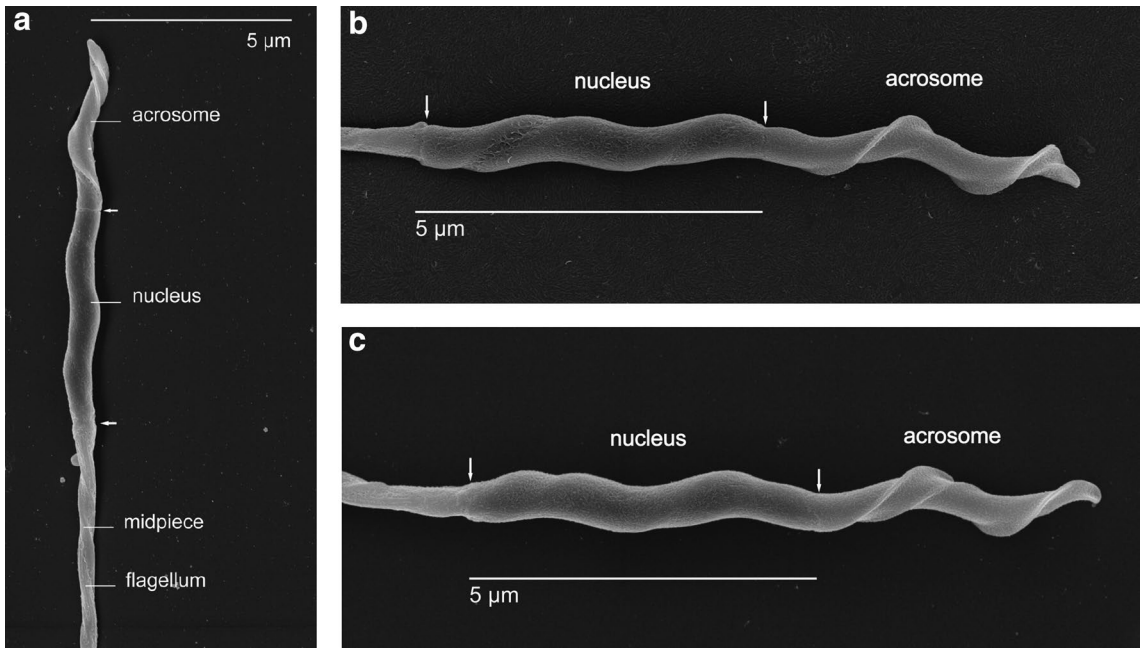

Fig. 1 Scanning electron micrographs of sperm cells from the Long-tailed Finch. Typical sperm of the Long-tailed Finch showing **a** the head and anterior portion of the flagellum with the helical midpiece (*P. a. acuticauda* shown here), and sperm head morphology of **b** *P. a. hecki*and and **c** *P. a. acuticauda*. *Arrows* indicate the junctions between the acrosome and nucleus and the nucleus and flagellum

Table 1 Data on sperm morphology and performance for male Long-tailed Finches for each of the two subspecies, *P. a. acuticauda* and *P. a. hecki*

Trait	*P. a. acuticauda* Mean ± SD	*P. a. hecki* Mean ± SD	*t*	*p*
Total length (µm)	75.50 ± 4.35 ($n = 19$)	72.02 ± 5.85 ($n = 23$)	*2.15*	*0.038*
Head length (µm)	12.80 ± 0.73 ($n = 19$)	12.77 ± 0.59 ($n = 23$)	0.19	0.85
Midpiece length (µm)	39.98 ± 4.41 ($n = 19$)	40.97 ± 5.30 ($n = 23$)	−0.65	0.52
Flagellum length (µm)	62.70 ± 4.61 ($n = 19$)	59.25 ± 6.17 ($n = 23$)	2.01	0.051
Head width (µm)	6.61 ± 0.27 ($n = 10$)	7.03 ± 0.49 ($n = 10$)	*−2.43*	*0.026*
Midpiece:flagellum	0.64 ± 0.07 ($n = 19$)	0.69 ± 0.06 ($n = 23$)	*−2.79*	*0.008*
Flagellum:head	4.92 ± 0.51 ($n = 19$)	4.66 ± 0.62 ($n = 23$)	1.43	0.16
Acrosome:nucleus	0.75 ± 0.09 ($n = 10$)	0.77 ± 0.09 ($n = 10$)	−0.45	0.66
Total sperm length CVwm	2.71 ± 0.89 ($n = 19$)	3.00 ± 1.45 ($n = 23$)	0.54	0.60
VCL (µm s^{-1})	91.33 ± 15.00 ($n = 18$)	86.70 ± 14.88 ($n = 22$)	0.99	0.33
Proportion of motile sperm	0.70 ± 0.15 ($n = 18$)	0.75 ± 0.16 ($n = 22$)	0.98	0.33

Descriptive statistics are based on mean values calculated from ten sperm cells for each male, while significance testing used linear mixed-effects models with subspecies included as a fixed factor and male identity as a random factor

VCL curvilinear velocity in µm s^{-1}

Significant values shown in italics ($p < 0.05$)

subspecies in sperm head, midpiece or flagellum length; though in the case of flagellum length there was a nearly significant difference between the subspecies (*P. a. acuticauda* tended to have a longer flagellum compared to *P. a. hecki*; Table 1). There was also no difference between the subspecies in the ratio of flagellum length to head length, and no difference in sperm swimming speed or the proportion of motile sperm in ejaculates (Table 1). Similarly, there was no difference between the subspecies in the mean A:N ratio (Table 1), though in both subspecies, males showed considerable variation in A:N ratio: values in *P. a. acuticauda* ranged from 0.60 to 0.85, while

values ranged from 0.65 to 0.94 in *P. a. hecki*. Finally, in the comparison of variance in trait mean values between the subspecies we found no difference between *P. a. acuticauda* and *P. a. hecki* in sperm morphology (head: $F = 0.66$, $p = 0.35$; midpiece: $F = 1.45$, $p = 0.43$; flagellum: $F = 1.79$, $p = 0.22$; total: $F = 1.81$, $p = 0.21$; width: $F = 0.32$, $p = 0.10$; midpiece:flagellum: $F = 0.88$, $p = 0.76$; flagellum:head ratio: $F = 1.48$, $p = 0.41$; A:N ratio: $F = 0.93$, $p = 0.92$), sperm performance (VCL: $F = 0.99$, $p = 0.97$) or the proportion of motile sperm in ejaculates ($F = 1.21$, $p = 0.70$).

The subspecies showed different values of among-male coefficient of variation in total sperm length (CV_{am}: *P. a. acuticauda*, 5.82; *P. a. hecki*, 8.21); though as there was no significant difference in variance in total sperm length between the two subspecies (see above) this difference cannot be considered statistically significant. Within-male coefficient of variation in sperm total length also differed between the subspecies, and as before values for *P. a. hecki* exceeded those for *P. a. acuticauda* (CV_{wm}: *P. a. acuticauda*, 2.71; *P. a. hecki*, 3.00), though again this difference was not significant (Table 1). Similarly, there was no difference between the subspecies in within-male variation in sperm head length ($t = 0.38$, $p = 0.71$) or flagellum length ($t = 1.27$, $p = 0.21$). In contrast, within-male variation in sperm midpiece length was (marginally) significantly greater in *P. a. hecki* compared to *P. a. acuticauda* ($t = -2.00$, $p = 0.05$).

The relationship between sperm morphology and swimming performance also differed between the subspecies. In *P. a. acuticauda*, sperm swimming speed was significantly, positively related to both absolute and relative midpiece length, but was not associated with sperm head length, flagellum length, total sperm length or the ratio of flagellum length to head length (Table 2; Fig. 2a). In contrast, sperm swimming speed was significantly, positively related to sperm midpiece and flagellum length, as well as total sperm length and the ratio of flagellum length to head length, but unrelated to either sperm head length or relative midpiece length in *P. a. hecki* (Table 2; Fig. 2b). Finally, sperm swimming speed was positively associated with the number of tracked sperm in *P. a. hecki* (all $p < 0.01$), whereas these traits showed no association in *P. a. acuticauda* (all $p > 0.35$).

Table 2 Results from linear models testing the relationship between sperm swimming speed (i.e. VCL) and a number of sperm morphological traits in (a) *P. a. acuticauda* and (b) *P. a. hecki*

Sperm trait	Coefficient (±SE)	*t*	*p*
(a) *P. a. acuticauda*			
Head length	−1.03 ± 0.8	−1.28	0.22
Midpiece length	*1.14 ± 0.27*	*4.20*	*0.0008*
Flagellum length	0.44 ± 0.55	0.80	0.44
Total sperm length	0.44 ± 0.72	0.61	0.55
Flagellum:head	0.44 ± 0.38	1.16	0.26
Midpiece:flagellum	*1.59 ± 0.52*	*3.04*	*0.008*
(b) *P. a. hecki*			
Head length	−1.27 ± 0.70	−1.80	0.09
Midpiece length	*1.03 ± 0.09*	*11.13*	*<0.0001*
Flagellum length	*1.10 ± 0.16*	*6.79*	*<0.0001*
Total sperm length	*1.44 ± 0.21*	*6.73*	*<0.0001*
Flagellum:head	*0.82 ± 0.15*	*5.60*	*<0.0001*
Midpiece:flagellum	78.57 ± 39.56	1.99	0.06

Sperm count data was also included in each model, but for simplicity and because the results of the covariate are not relevant to the relationships being tested, we present only the results of the correlation between sperm morphology and velocity

Significant relationships shown in italics ($p < 0.05$)

Discussion

In the current study, we show that the two subspecies of Long-tailed Finch exhibit typical passerine sperm morphology: sperm are filiform, the acrosome bears a helical membrane (or keel) and the single fused mitochondria twists along much of the length of the flagellum (i.e. mitochondrial helix; Jamieson 2007). However, we also found significant differences in sperm size between the two subspecies. Specifically, *P. a. acuticauda* had slightly longer, but narrower sperm relative to *P. a. hecki*.

Variation in sperm size may be attributed to either selection or genetic drift over evolutionary time. In the case of the Long-tailed Finch, we suggest that the subspecies differences in sperm morphology are likely to have primarily resulted from genetic drift. Though it is perhaps surprising to observe any difference in sperm size given the relatively short time since these taxa split (i.e. 0.3 Mya, Jennings and Edwards 2005), there is some evidence that the western *P. a. acuticauda* has experienced a genetic bottleneck due to a small founding population size (Rollins et al. 2012). Under such a scenario, it is possible that sperm size in individuals in the founder population represented values from the upper portion of the distribution (i.e. sperm tended to be longer), allowing the differences observed between the contemporary taxa to evolve relatively rapidly via genetic drift. Interestingly, the trend towards lower phenotypic variation in sperm morphology observed between males (i.e. CV_{am}) in *P. a. acuticauda* is also indicative of a bottleneck followed by genetic drift. Specifically, given the strong genetic basis of sperm length (Birkhead et al. 2005; Simmons and Moore 2009), reduced among-male variance in sperm size is predicted under conditions of small effective population sizes with reduced genetic variation due to drift. Thus, we suggest the subspecies differences observed here may

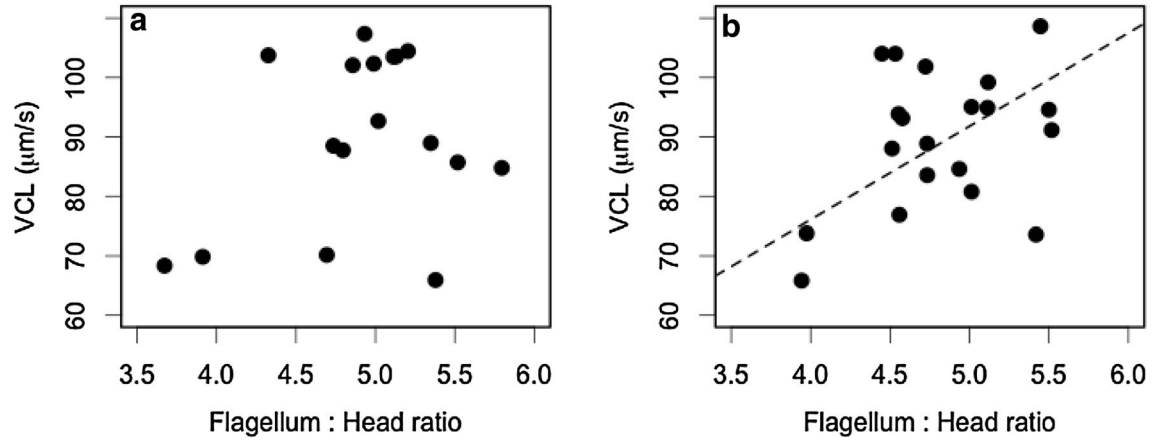

Fig. 2 Relationship between sperm swimming speed (VCL) and the ratio of the lengths of the sperm flagellum and head in the two subspecies of Long-tailed Finch. **a** *P. a. acuticauda*, and **b** *P. a. hecki*. In **b** *line* represents a simple regression line. See main text for full statistical details

simply reflect the historical processes associated with the divergence of the western and eastern populations.

An alternate explanation for the differences observed between the subspecies is that sperm size has diverged rapidly as a result of high levels of sperm competition experienced by the two taxa (Rowe et al. 2015) or because the two populations vary in the strength or direction of selection. However, we consider these explanations unlikely for the following reasons. First, the one available estimate of extra-pair paternity in the Long-tailed Finch (in *P. a. acuticauda*) indicates low to moderate rates of extra-pair paternity (i.e. 12.8 %, Griffith, pers comm), implying sperm competition is not especially strong in this species. Second, relative testes size is extremely low in both subspecies (just 0.26 and 0.34 % of male body mass, *P. a. acuticauda* and *P. a. hecki* respectively; see Additional file 1), suggesting sperm competition is in fact extremely low or absent in these taxa (cf. Møller 1991; Harcourt et al. 1995; Hosken 1997; Stockley et al. 1997; Birkhead et al. 2006; Rowe and Pruett-Jones 2011). Finally, in both subspecies, we found high phenotypic variance in sperm morphology both among- (CV_{am}) and within-males (CV_{wm}). Moreover, while values of CV_{am} and CV_{wm} tended to be higher in *P. a. hecki* relative to *P. a. acuticauda*, in general these differences were not significant (with the exception of CV_{wm} for midpiece length). In a range of taxa, including birds, comparative studies have shown that among- and within-male variation in sperm length decreases with increasing values of extra-pair paternity rates and relative testes size (Calhim et al. 2007; Kleven et al. 2007; Immler et al. 2008; Lifjeld et al. 2010; Fitzpatrick and Baer 2011; Varea-Sánchez et al. 2014). Consequently, sperm competition appears to be low in both subspecies, and data on phenotypic variance in sperm length suggests the potential for selection via sperm competition is unlikely to differ between *P. a. acuticauda* and *P. a. hecki*.

In this study, we also examined the ratio of acrosome length to nucleus length, a trait that has received relatively little attention in studies of avian sperm biology to date. Available data, however, suggests this ratio is variable, ranging from <0.1 to 4 across passerine species (Jamieson 2007). Jamieson (2007) suggested that the Passerida are characterized by having an acrosome that is longer than the nucleus (i.e. A:N ratio > 1). In contrast to this, however, we found A:N ratio was less than 1 (i.e. the acrosome was shorter than the nucleus) in both subspecies of Long-tailed Finch. Though the adaptive significance of this trait is unclear, we also found that there was considerable variability in this trait: values across both subspecies ranged from 0.6 to 0.94. Thus, there appears to be sufficient variation in this trait on which selection could potentially act, and we suggest it may be valuable to investigate the relative roles of phylogeny, drift and selection in shaping variation in this trait in future comparative studies of avian sperm biology.

In order to understand the remarkable evolutionary diversification of sperm morphology, it is helpful to develop an understanding of the adaptive and functional significance of sperm trait variation. Sperm swimming speed has been linked to fertilization success under non-competitive and competitive mating scenarios in a broad range of species (Simmons and Fitzpatrick 2012, but see Smith 2012; Lüpold et al. 2012), including birds (Birkhead et al. 1999; Denk et al. 2005; Pizzari et al. 2008). Similarly, a recent study found that longer sperm fertilize more eggs under competitive mating conditions in the Zebra Finch (*Taeniopygia guttata*, Bennison et al. 2015). Moreover, sperm length is generally thought to be an important determinant of sperm swimming speed, and indeed

theoretical (Humphries et al. 2008) and both intra- and inter-specific empirical studies provide evidence for such a link (e.g. in birds Lüpold et al. 2009; Mossman et al. 2009). However, further studies show no relationship (e.g. Kleven et al. 2009; Laskemoen et al. 2010) or a negative relationship between these traits (e.g. Cramer et al. 2015; reviewed in Humphries et al. 2008; Simmons and Fitzpatrick 2012; Fitzpatrick and Lüpold 2014). Thus no clear patterns are evident regarding how sperm length influences swimming speed. It has therefore been suggested that, rather than the absolute length of a sperm's constituent parts, the ratio of flagellum length to head size may be a better predictor of sperm swimming speed as this trait balances the drag produced from the head with the propulsive thrust of the flagellum (Humphries et al. 2008). Yet even this trait appears to be inconsistently associated with sperm velocity, even within a single species (e.g. Helfenstein et al. 2010; Cramer et al. 2015). Similarly, in the current study we found the ratio of flagellum to head length was positively correlated with sperm velocity in *P. a. hecki*, but not *P. a. acuticauda*. Thus, our results add to the growing body of evidence suggesting that the mechanism linking sperm structure and function may not be straightforward and that simply incorporating the flagellum to head length ratio in studies of sperm performance is unlikely to fully resolve sperm structure–function relationships. At least for passerines, we suggest future investigations of sperm shape may help further elucidate how sperm morphology translates into sperm performance.

The differences in sperm morphology observed in the current study, combined with knowledge of the general breeding ecology of the Long-tailed Finch, suggest this species may be an interesting system for investigations into processes associated with population divergence and reproductive isolation. Specifically, although the subspecies currently meet and interbreed in a zone of secondary contact in the vicinity of the Ord Arid Intrusion (Schodde and Mason 1999), mitochondrial data do not support the idea of contemporary gene flow between the eastern and western regions (Rollins et al. 2012). Moreover, there is little variation in the expression of both yellow and red bill color within each of the subspecies' ranges (Griffith, unpublished data) until the area of the relatively narrow contact zone where intermediate phenotypes have been observed (Schodde and Mason 1999; also Griffith unpublished data), suggesting that the genes underlying this trait are not being readily introgressed from one subspecies to another. The subspecies are thought to have diverged 300,000 years ago when separated by an arid intrusion through the savannah belt that runs across the top end of Australia in the Pleistocene Ord Arid Intrusion (Jennings and Edwards 2005; Bowman et al. 2010). The aridification of the Australian continent occurred in cycles throughout the Quaternary period with the last major event occurring over 10,000 years ago (De Deckker 2001), and the subspecies of Long-tailed Finch have likely been in contact for around that period of time. The maintenance of the two subspecies despite the length of time that they are likely to have been in contact suggests the existence of reproductive isolating mechanisms that have prevented, or at least slowed, the rate of admixture between the genes of the two lineages.

Significant differences between the subspecies in the expression of both song (Zann 1976) and bill color (Higgins et al. 2006) imply that pre-copulatory mate choice may play a role in the maintenance of the subspecies in the contact zone. However, we suggest that post-copulatory processes, including those involving sperm traits, are also worth investigating in this system for a number of reasons. First, preliminary evidence suggests assortative mating based on bill color may be weak (Van Rooij and Griffith 2012; Griffith unpublished data). Next, given the positive correlation between sperm length and the length of female sperm storage organs (reviewed in Pitnick et al. 2009; see Briskie and Montgomerie 1993 for evidence in birds), it has been widely hypothesized that divergence in sperm traits between allopatric populations may lead to incompatibilities between males and females upon secondary contact (Howard et al. 2009), and there is growing empirical evidence that divergence in sperm traits can have implications for the generation and maintenance of reproductive barriers in closely related taxa (e.g. *Drosophila*, Lüpold et al. 2012; Manier et al. 2013a, b; mice, Dean and Nachman 2009; Albrechtová et al. 2012). Finally, variation in sperm length has been linked to fertilization success in birds (Bennison et al. 2015). Thus it is possible that the differences in sperm size observed in the subspecies of Long-tailed Finch may influence the breeding dynamics of the species, especially in the current contact zone. For example, heterosubspecific pairings (i.e. *P. a. acuticauda* × *P. a. hecki*) might experience a sperm–sperm storage tubule mismatch leading to lower sperm fertilization success and reduced fertility, which could in turn generate selection for elevated levels of extra-pair paternity in such heterosubspecific pairs, equivalent to the pattern observed in *Ficedula* flycatcher species in a contact zone (Veen et al. 2001). Consequently, we recommend future studies investigate the fertility effects of heterosubspecies pairings (relative to consubspecific pairings) and examine the potential role of sperm variation in individual fitness in this system.

Conclusion

In summary, we examined differences in sperm morphology, sperm swimming speed and sperm

structure-function relationships between two genetically distinct subspecies of Long-tailed Finch. We found significant differences in sperm size between the closely related taxa and suggest that these differences may have arisen via drift during a period of allopatry. Though these differences were relatively small, we discuss the potential implications of our findings for the process of population divergence and reproductive isolation and suggest that these taxa would make an interesting model system for the study of how post-copulatory processes, and sperm traits in particular, may contribute to reproductive isolation between intergrading populations.

Additional file

Additional file 1. Relative testes mass in the Long-tailed Finch.

Authors' contributions

MR conceived the study, collected and analyzed the sperm samples, analyzed the data and wrote the paper. SCG conceived the study, contributed reagents/material/analysis tools and helped to draft the manuscript. AH performed electron microscopy, implemented stereological methods for obtaining measurements and helped draft the manuscript. JTL contributed reagents/material/analysis tools and helped to draft the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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