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The Azores bullfinch (*Pyrrhula murina*) has the same unusual and size-variable sperm morphology as the Eurasian bullfinch (*Pyrrhula pyrrhula*)

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The Azores bullfinch is endemic to the island of São Miguel in the Azores archipelago and the sister species to the Eurasian bullfinch. Here we show that the spermatozoa of the two species have similar ultrastructure and gross morphology. Thus, the unusual and supposedly neotenous sperm morphology previously described for the Eurasian bullfinch appears to be an ancestral trait that evolved before the two taxa diverged. In addition, the coefficients of variation in total sperm length, both within and among males, were high in both species and exceed any previously published values for free-living passerines. Such high sperm-size variation is typically found in species with relaxed sperm competition. However, the high variance in mean sperm length among Azores bullfinches is surprising, because the trait has high heritability and this small, insular population shows clear signs of reduced genetic diversity at neutral loci. A possible explanation for this apparent contradiction is that the Azores bullfinch has retained more diversity at functional and fitness-related loci than at more neutral parts of the genome. Finally, we also present data on relative testis size and sperm swimming speed for the Eurasian bullfinch, and discuss the hypothesis that the small and putatively neotenous sperm in bullfinches has evolved in response to lack of sperm competition. *Biological Journal of the Linnean Society*, 2013, **108**, 677–687.

ADDITIONAL KEYWORDS: Fringillidae – Passeriformes – sperm competition – sperm length – sperm velocity – testis size.

INTRODUCTION

Sperm cells are extremely variable in shape and size across the animal kingdom (Cohen, 1977; Pitnick, Hosken & Birkhead, 2009). Phylogeny explains much of this variation, which makes sperm morphology an informative trait for phylogenetic inference and taxonomy (Jamieson, Ausió & Justine, 1995). In birds, the order Passeriformes has a unique and highly conserved sperm structure: sperm are filiform, the head (i.e. acrosome and nucleus) is corkscrew-shaped, the midpiece is elongated and wrapped around the flagellum, and a helical membrane (microtubule helix) is coiled along the length of the head and midpiece (Koehler, 1995). This is in contrast to the non-passeriform orders, in which sperm cells, while also filiform, typically have a smooth and cylindrical head, a short midpiece, and no helical membrane (Jamieson, 2006). There are also a number

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of ultrastructural details visible through scanning (SEM) and transmission electron microscopy (TEM) that make the sperm of oscine passerines (Passeri) unique among birds, including a single, fused mitochondrion, the lack of a proximal centriole (but see Aire & Ozegbe, 2012) and an elongated, pointed acrosome (Koehler, 1995; Jamieson, 2006). Importantly, passerine sperm vary greatly in size and dimensions (Koehler, 1995; Jamieson, 2006; Immler & Birkhead, 2007; Kleven *et al*., 2008): the mean total length of formalin-fixed sperm ranges between 40 and 280 μm among the more than 200 passerine species screened so far in our lab (Lifjeld *et al*., 2010; own unpublished data). Most of this variation is attributable to the length of the midpiece (Immler & Birkhead, 2007), which contains the fused mitochondria important for energy production and sperm movement (Cummins, 2009).

One species stands out as an exception to the typical passerine sperm, namely the Eurasian bullfinch *Pyrrhula pyrrhula* (Birkhead *et al*., 2006, 2007). In this species, spermatozoa are non-filiform and have a cylindrical head with a rounded, not pointed acrosome and a very short midpiece that lacks a helical membrane (Birkhead *et al*., 2006). In addition, ultrastructural studies reveal several primitive traits not found in other oscine passerines, including a proximal centriole and several small mitochondria instead of one large, fused mitochondrion (Birkhead *et al*., 2007). However, the deviant structural characteristics of the sperm of the Eurasian bullfinch are actually present in the spermatids (i.e. immature sperm) of other passerines. Thus, suppression of the final stages of spermatogenesis is thought to cause the unusual morphology of the Eurasian bullfinch sperm (Birkhead *et al*., 2007). It has been further hypothesized that the neotenous sperm of this species may ultimately result from a lack of sperm competition (Birkhead *et al*., 2006; Birkhead & Immler, 2007). Several lines of indirect evidence, including low relative testis mass, few sperm stored in the seminal glomera and low sperm swimming speed (Birkhead *et al*., 2006), as well as relatively short sperm with high coefficients of intermale variation in sperm total length and size components (Calhim, Immler & Birkhead, 2007), support this assumption. However, to date there are no paternity studies published on the species to confirm genetic monogamy.

The evolutionary origin of the unusual sperm morphology of the Eurasian bullfinch is poorly understood. According to the most recent classifications (del Hoyo, Elliott & Christie, 2010; Sangster *et al*., 2011; Gill & Donsker, 2012), the genus *Pyrrhula* consists of seven species. These species fall into three major phylogenetic clades: (1) the Eurasian clade with *P. pyrrhula* and the Azores bullfinch (*P. murina*); (2) the Himalayan clade with the orange bullfinch (*P. auranthiaca*), the red-headed bullfinch (*P. erythrocephala*), and the grey-headed (or Beavan's) bullfinch (*P. erythaca*); and (3) the Southeast Asian clade with the brown bullfinch (*P. nipalensis*) and the white-cheeked bullfinch (*P. leucogenis*) (Töpfer *et al*., 2011). The last-named clade is more basal in the bullfinch phylogeny, leaving the Himalayan clade as the sister to the Eurasian clade. To date, sperm morphology has not been described in these species, with the exception of one individual of the grey-headed bullfinch (see Birkhead *et al*., 2006). In that instance, sperm were found to have a pointed and spiral-shaped head (i.e. the typical passerine head shape contrasting markedly with that of the Eurasian bullfinch sperm), although sperm length was equally short with an extremely small midpiece. This finding would suggest that the neotenous sperm of the Eurasian bullfinch might be an autapomorphy (i.e. confined to the Eurasian bullfinch only). However, more species in this genus need to be analysed before any definitive conclusions on the evolution of the unusual sperm morphology in the Eurasian bullfinch can be made.

The Azores bullfinch is endemic to the island São Miguel in the Azores archipelago in the Atlantic Ocean (BirdLife International, 2010; del Hoyo *et al*., 2010). The breeding range of this species is restricted to a 100-km^2 area of native laurel forest on the eastern side of the island, and the current population size is estimated at about 1000–1600 individuals (Monticelli *et al*., 2010; Ceia *et al*., 2011). The species has recently been downlisted from 'Critically Endangered' to 'Endangered' on the IUCN Red List, as the population size is considered to be stable (BirdLife International, 2010). However, the species underwent a severe population bottleneck in the 20th century, and the current population shows clear signs of low genetic diversity. There seems to be only one mitochondrial haplotype in the current population (Töpfer *et al*., 2011; R. J. Lopes, unpubl. data). A microsatellite analysis also revealed very low levels of heterozygosity and allelic diversity relative to continental populations of the Eurasian bullfinch (R. J. Lopes, unpubl. data).

Here we present the first description of the sperm morphology and its variation in the Azores bullfinch. Our study has two main objectives. First, we wanted to know whether the neotenous sperm morphology of the Eurasian bullfinch is also present in the Azores bullfinch. If so, the trait might be a synapomorphy present in the last common ancestor of the entire Eurasian bullfinch clade, as opposed to a more recent event within the Eurasian bullfinch lineage. We studied the sperm morphology of both species using SEM and TEM for ultrastructural

details, and high-resolution light microscopy for gross morphology.

Second, we wanted to examine if the Azores bullfinch and the Eurasian bullfinch differ in sperm morphometry: sperm size and inter- and intramale variation in sperm length. Sperm length has a strong genetic basis (Birkhead *et al*., 2005; Simmons & Moore, 2009). Thus, high phenotypic variance in sperm morphology among males in a population indicates high additive genetic variance for the trait. Given the low population genetic diversity in the Azores bullfinch (R. J. Lopes, unpubl. data), one would expect reduced phenotypic variance in sperm length among males in the population. Knowledge of intermale variation in sperm length also provides insight into sperm competition levels faced by males within a species. Specifically, there is robust comparative evidence that the coefficient of variation in sperm length among males in a population decreases with increasing risks of sperm competition (Calhim *et al*., 2007; Kleven *et al*., 2008; Lifjeld *et al*., 2010). An interpretation of this association is that sperm competition acts as a stabilizing selection pressure on sperm length. Thus, both reduced genetic diversity and increased sperm competition are plausible and alternative explanations for reduced intermale variance in sperm length, and we examine this variation in order to discuss hypotheses of sperm evolution in bullfinches.

Similarly, we compared the levels of intramale variation in sperm length. Heterogeneous sperm within an ejaculate is thought to reflect developmental instability or imperfections in the sperm production machinery associated with relaxed sperm competition (Immler, Calhim & Birkhead, 2008; Kleven *et al*., 2008), inbreeding or low genetic diversity (Gage *et al*., 2006; Roldan & Gomendio, 2009), or environmental stressors or pollutants (Pitnick *et al*., 2009). In contrast to the Eurasian bullfinch, which exhibits no lower genetic diversity than other European species in the family Fringillidae (Durrant *et al*., 2010), the Azores bullfinch has significantly reduced genetic diversity (R. J. Lopes, unpubl. data). Consequently, we predicted that the Azores bullfinch should exhibit high levels of intramale variation in sperm morphology.

Finally, we also present some quantitative data on testis size and sperm swimming speed in the Eurasian bullfinch for comparison with published data from this and other species. Typically, bird species with low sperm competition have relatively small testes (Møller & Briskie, 1995) and slow-swimming sperm (Kleven *et al*., 2009). Thus, examination of these traits will help provide support for or against the hypothesis that the unusual sperm morphology of Eurasian bullfinch is due to an absence of sperm competition.

MATERIAL AND METHODS

The analyses of sperm morphology include samples from 11 male Azores bullfinches and 13 Eurasian bullfinches. The Azores bullfinches were caught in mist-nets erected at foraging sites throughout the distribution range, in São Miguel island, during 13–17 June 2011. The Eurasian bullfinches were caught in mist-nets at feeding trays baited with sunflower seeds at various locations in and around Oslo, south-east Norway, in April–June 2006–2012. The technique of sperm sampling followed the protocol of cloacal massage: collection of an ejaculate in a capillary tube, and subsequent dilution in phosphate-buffered saline before fixation in 5% formalin (Kleven *et al*., 2008). Four of the Eurasian bullfinches (all from 2012) were killed under licence for another study, and their testes dissected out and weighed (± 0.001 g). All other birds were released unharmed after sampling.

For SEM, sperm were attached to glass coverslips precoated with poly-lysine (1 mg mL^{-1}; Sigma P1274) by placing a 10-μL aliquot of formalin-fixed sperm onto each coverslip and incubating samples overnight in a wet chamber at room temperature. Next, sperm adhering to the coverslips were dehydrated using a graded ethanol series (5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 95 and 100% ethanol) and critical point dried (BAL-TEC CPD 030 Critical Point Dryer). Coverslips were then mounted on SEM stubs using carbon tape and sputter coated with 4–6 nm platinum using a Cressington 308R coating system. Finally, samples were examined and digital images recorded using a Hitachi S-4800 field emission scanning electron microscope operated at 5.0 kV.

For TEM (Azores bullfinch only), formalin-fixed sperm were further fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature (RT), and then post-fixed in 2% osmium tetraoxide in 0.1 M cacodylate buffer in the dark, for 1 h at RT. Samples were then processed for embedding in a Pelco BioWave following a multi-step protocol. First samples were processed in triplicate in 1% uranyl acetate in double distilled water (3 min at 37 °C/250 W; 3 min at RT; 3 min at 37 °C/250 W). Next sperm were rinsed three times in distilled water (each rinse for 60 s at 37 °C/250 W), and dehydrated using a graded acetone series (50, 70, 90, 100, 100 and 100%; each step for 60 s at 37 °C/250 W). Sperm were then infiltrated with resin at increasing concentrations (25, 50 and 75%, each for 3 min at 45 °C/350 W; followed by 100, 100 and 100% each for 3 min at 50 °C/350 W). Between each step in this process samples were centrifuged at 600 ***g*** for 15 min. Next, samples were polymerized overnight at 60 °C. Finally, sections (80 nm) were obtained with the Leica microtome, post-stained with lead citrate and

observed using a Philips CM200 transmission electron microscope operated at 80 kV.

For sperm morphometrics, a small aliquot of approximately 15 μL of the formalin–sperm solution was applied onto a microscope slide and was allowed to air-dry before inspection, digital imaging, and measurement under light microscopy. We took digital images of spermatozoa at magnifications of 640×, using a Leica DFC420 camera mounted on a Leica DM6000 B digital light microscope. The morphometric measurements were conducted using Leica Application Suite (version 2.6.0 R1). We measured the length of head (including the acrosome, nucleus, and midpiece) and the flagellum (± 0.1 μm) of ten intact spermatozoa per male. Total sperm length was calculated as the sum of head and flagellum length. We have previously shown that sperm length measurements have very low measurement error and high repeatability (Laskemoen *et al*., 2007, 2010). Standardized values of intra- and intermale variation was expressed as the coefficient of variation (CV = SD/mean × 100). For intermale variation, a correction factor for variation in sample size (N) was applied, namely $CV = SD/\text{mean} \times 100 \times (1 + 1/4N)$, as recommended by Sokal & Rohlf (1981).

For sperm velocity recordings, we collected fresh ejaculates from 11 Eurasian bullfinches and immediately diluted them in 30 μL of preheated Dulbecco's modified Eagle medium (advanced DMEM; Invitrogen, Carlsbad, CA, USA) and placed the tubes in a heating block set to 40 °C. Next, we carefully homogenized the solution using a pipette and within 20 s 5–6 μL of the diluted sperm was deposited on a preheated counting chamber (20 μm depth, two-chamber slide, Leja Products BV, Nieuw-Vennep, the Netherlands), mounted on a Hamilton Thorne MiniTherm stage warmer set to 40 °C (Hamilton Thorne Biosciences, Beverly, MA, USA). Sperm motion was recorded using a mini-DV camera (Sony HDR-HC1E or Canon Legria HF S200) mounted on an upright microscope (Olympus CX41) with a 4× objective. The camera was set to 10× optical zoom and infinite focus. For each male we recorded six different viewing fields of the counting chamber. Computer-assisted sperm analysis (HTM-CEROS sperm tracker, CEROS version 12, Hamilton Thorne Research) was used to analyse the recordings. The sperm analyser was set at a frame rate of 50 Hz and 25 frames (i.e. sperm cells were tracked for 0.5 s). Each analysis was visually examined and cell detection parameters were adjusted to optimize the detection of motile spermatozoa. Cell detection parameters thus varied slightly; minimum contrast 60–140, and minimum cell detection size 10–12 pixels. We recorded average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). Mean motility measurements were calculated from all motile spermatozoa to obtain one overall measurement for each ejaculate. Spermatozoa with VAP < 30 μm s^{-1} and/or VSL < 25 μm s^{-1} were considered static or drifting and excluded from the motility analyses, along with spermatozoa tracked for less than ten frames. We also removed any 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. Because the spermatozoa appeared to have size-variable, circular heads (see example video in Supporting Information), it was not possible to distinguish dead/immotile spermatozoa from faeces debris in the sample. Consequently, we did not estimate the percentage motile sperm in the samples. For logistical reasons, sperm velocity recordings could not be made for the Azores bullfinch. Raw data for the analyses of sperm morphometrics and sperm velocity can be downloaded from the Dryad database (Lifjeld *et al*. 2012).

RESULTS

The scanning electron micrographs revealed that the Azores bullfinch and the Eurasian bullfinch have the same sperm morphology (Figs 1, 2). Both have nonfiliform sperm showing a cylindrical, thickened, and tapering head with a rounded acrosome as a cap on top of the nucleus, an extremely small midpiece, and no helical membrane wound around the flagellum. There was, however, much variation in head elongation, even among sperm from the same ejaculate (e.g. Fig. 2), and for both species the length of the acrosome was generally shorter than the nucleus. There was also considerable variation in the surface texture of the head. In general, the acrosome had a smoother surface than the nucleus, which mostly had a very rugged and furrowed appearance. However, we cannot exclude the possibility that the surface structure has been altered during sample fixation (see below). The microtubular helix along the flagellum, being visible in both species (Figs 1, 2), reveals the only obvious diagnostic passerine trait of the external morphology of the spermatozoa.

Transmission electron micrographs of the Azores bullfinch spermatozoon confirmed the presence of multiple mitochondria in the midpiece (Fig. 3). There was also marked variation in the density of chromatin within the nucleus. The outer membrane of the nucleus and midpiece appeared broken in places (Fig. 3A–C), suggesting that the use of formalin-fixed samples may not be optimal for electron microscopy of these delicate structures. We were also not able to identify any centrioles, and thus we could not confirm

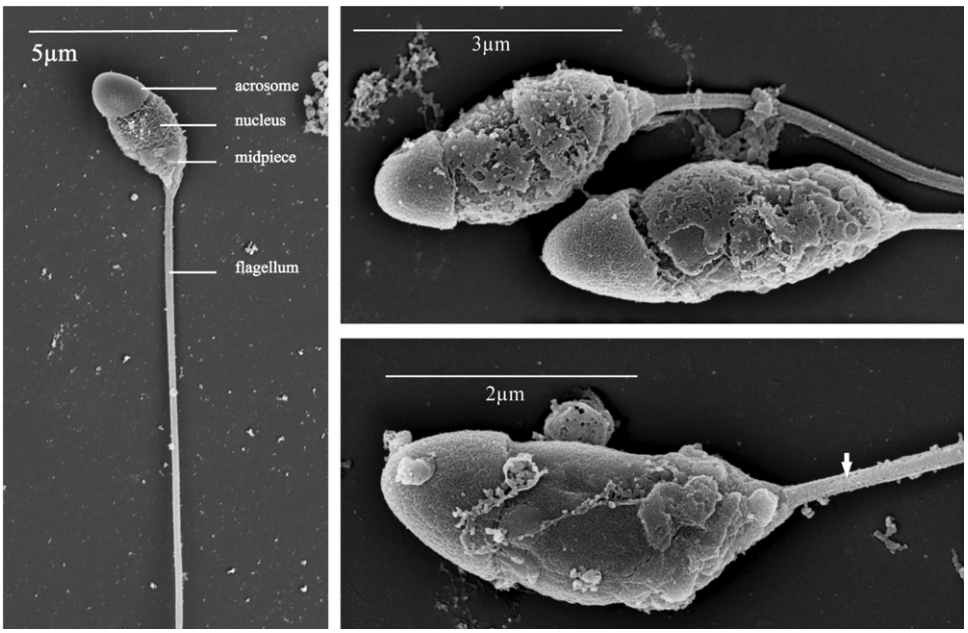

Figure 1. Scanning electron micrographs of the head and anterior part of the flagellum of the Azores bullfinch spermatozoon. The main structural parts are indicated with their names, and the arrow points to the microtubular helix on the flagellum.

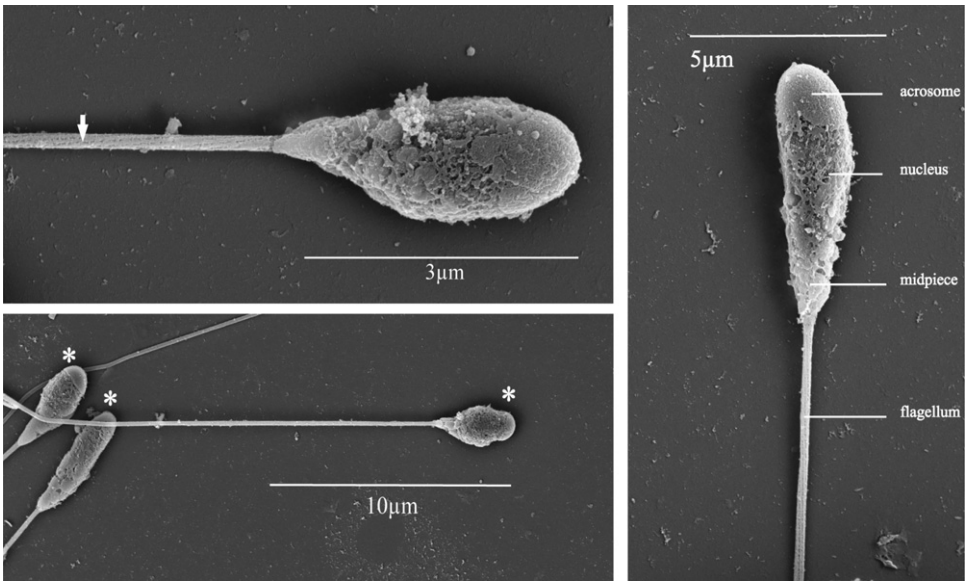

Figure 2. Scanning electron micrographs of the head and anterior part of the flagellum of the Eurasian bullfinch spermatozoon. The main structural parts are indicated by their names and the microtubular helix on the flagellum by an arrow. Note the variation in head shape among individual spermatozoa (indicated by *).

the presence of a proximal centriole in the Azores bullfinch spermatozoon.

Sperm size dimensions were also similar for the two species (Table 1). Total sperm length averaged about 46 µm in both, which is very similar to the value of 46.87 µm reported by Birkhead *et al*. (2006) for the Eurasian bullfinch. There was also a considerable variation in mean sperm length among males in the two species, with a size range of 39–52 µm for the 11 Azores bullfinches and 40–53 µm for the 13 Eurasian bullfinches (Fig. 4). The coefficient of inter-male variation in mean sperm length (CV_{bm}) was 9.6 and 8.6% (Azores bullfinch and Eurasian bullfinch, respectively), and the two variances did not differ

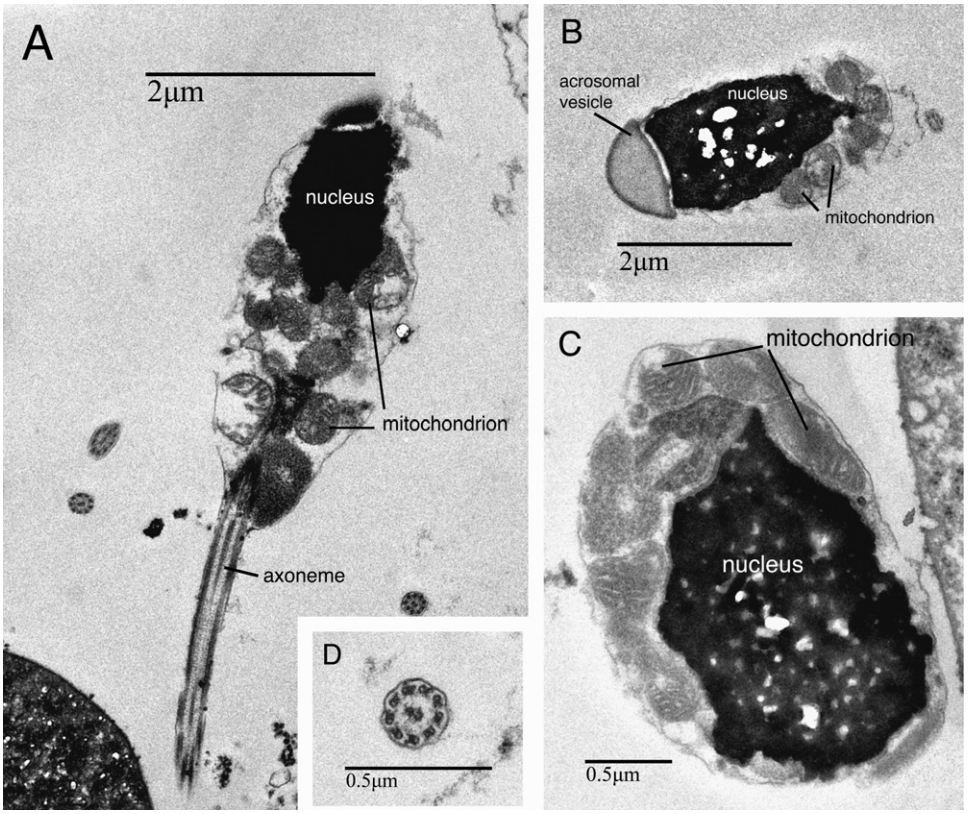

Figure 3. Ultrastructure of the spermatozoon of the Azores bullfinch by transmission electron microscopy (TEM). A, longitudinal section (LS) of a spermatozoon showing the midpiece consisting of multiple mitochondria grouped at the base of the nucleus. B, LS section of a spermatozoon showing the cap-like acrosome (acrosomal vesicle). C, transverse section (TS) of a spermatozoon showing several discrete mitochondria and the nucleus, in which the chromatin is loosely arranged. D, TS of the flagellum showing the archetypical 9 + 2 microtubule structure of the axoneme (two central microtubule singlets and nine outer microtubule doublets).

Table 1. Sperm morphometrics in the Azores bullfinch and the Eurasian bullfinch based on the measurement of ten sperm cells per male; all measurements are in μm, and mean values are given with ± SD

Sperm trait	Azores bullfinch *P. murina* ($N = 11$)	Eurasian bullfinch *P. pyrrhula* ($N = 13$)	Test of difference between means	Test of difference between variances
Head length*	5.60 ± 0.29	5.78 ± 0.37	$t = -1.35$, $P = 0.19$	F-ratio = 1.67, $P = 0.42$
Flagellum length	39.99 ± 4.23	40.49 ± 4.30	$t = -0.31$, $P = 0.76$	F-ratio = 1.20, $P = 0.75$
Total length	45.58 ± 4.29	46.28 ± 4.31	$t = -0.41$, $P = 0.68$	F-ratio = 1.22, $P = 0.73$
CV_{wm} of total length	6.07 ± 2.28	7.51 ± 1.80	$t = -1.72$, $P = 0.10$	F-ratio = 1.60, $P = 0.43$
CV_{bm} of mean total length†	9.62	8.62		

*Acrosome, nucleus, and midpiece.
†Adjusted for low sample sizes by the formula $CV_{bm} = SD/mean \times 100 \times (1 + 1/4N)$ (Sokal & Rohlf, 1981).

significantly (Table 1). Both values exceeded the highest value of CV_{bm} (= 6.2%) recorded among 55 passerine species reported by Lifjeld *et al*. (2010). The coefficients of intramale variation in total sperm length were also extremely high (mean CV_{wm} of 6.1 and 7.7%, Table 1), and exceeded the maximum value (= 3.6%) observed among 55 other passerine species (Lifjeld *et al*., 2010). Finally, neither head length nor flagellum length differed significantly between the two bullfinches, nor did their variances (Table 1).

For the four collected Eurasian bullfinches, the mass of the left and the right testis averaged 0.041 ± 0.016 g (mean ± SD) and 0.025 ± 0.006 g, respectively. With a mean body mass of 29.9 g for

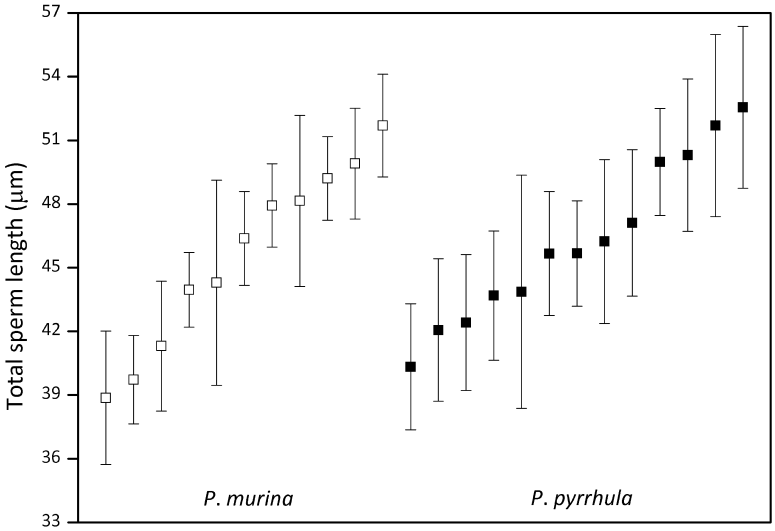

Figure 4. Variation in total sperm length among and within males of the Azores bullfinch and Eurasian bullfinch. Ten spermatozoa were measured for each male, and the mean and SD of total sperm length are indicated by squares and vertical bars, respectively. Within each species, individual males are ordered by increasing mean values.

Table 2. Sperm motility measurements in the Eurasian bullfinch from video recordings of semen samples of wild-caught birds ($N = 11$) at standard conditions of 40 °C, and using Computer-Assisted Sperm Analysis (CASA) with a filtered output (see Methods for details)

Parameter	Measurement
Velocity curvilinear (VCL) (μm s^{-1})	157.3 ± 22.8 (127.3–202.3)
Velocity average path (VAP) (μm s^{-1})	150.4 ± 21.0 (122.2–185.5)
Velocity straight line (VSL) (μm s^{-1})	144.8 ± 19.2 (119.8–169.7)
Amplitude of lateral head displacement (ALH) (μm)	3.2 ± 1.7 (1.4–7.0)
Beat cross frequency (BCF) (Hz)	21.6 ± 3.2 (14.9–25.8)

Measurements are given as mean ± SD (range) and were calculated from an average of 97 ± 113 SD sperm tracks per individual (range 8–353).

these males, the combined relative testis mass amounted to only 0.22%. This corresponds well with the value of 0.29% reported by Birkhead *et al*. (2006) for the same species, and implies extremely small testes for its body size compared with most other passerine species (Møller, 1991; Birkhead *et al*., 2006).

Values of sperm swimming speed for the 11 Eurasian bullfinches were surprisingly high (Table 2): mean curvilinear velocity (VCL) was 157 μm s^{-1}. Importantly, this value is among the highest records to date in passerines; mean VCL estimates published for 42 passerine species varied from 77 to 164 μm s^{-1} using the same methodology (Kleven *et al*., 2009). The three velocity parameters VCL, VAP and VSL were highly intercorrelated (Pearson's $r > 0.89$, $P < 0.001$), whereas the ALH and BCF parameters were not significantly correlated with any other motility measures. The ALH and BCF estimates lie within the normal range for passerines (J. T. Lifjeld *et al*., unpubl. data). A video clip of swimming sperm of the Eurasian bullfinch is available as Supporting Information.

DISCUSSION

Phylogeny of the unusual bullfinch spermatozoa

Our study showed that the spermatozoa of the Azores bullfinch and the Eurasian bullfinch have the same gross morphology and ultrastructure. The similarity is not only restricted to the unusual form and shape of the head and flagellum, as previously described for the Eurasian bullfinch (Birkhead *et al*., 2006, 2007), but there were also no differences in sperm morphometrics between the two species. Both species exhibit an extremely high variation in sperm size, both among individual sperm within an ejaculate and in mean sperm size among males. Hence, the two species seem indistinguishable based on their spermatozoa.

The lack of differentiation between the two sister taxa suggests an ancestral origin of this exceptional sperm morphology, as opposed to a more recent or

contemporary event in the Eurasian bullfinch lineage. The Eurasian bullfinch shows large geographical variation in plumage morphology, and 11 subspecies are currently recognized within its wide Palearctic range (Gill & Donsker, 2012). If it is correct that the Azores bullfinch is basal in the phylogeny of the Eurasian clade (Töpfer *et al*., 2011), then all Eurasian bullfinch populations and subspecies should share the same unusual sperm morphology. This prediction obviously warrants further empirical studies, especially of the eastern subspecies *griseiventris* and *cineracea*, both of which are morphologically and genetically distinct (del Hoyo *et al*., 2010; Töpfer *et al*., 2011). The Himalayan clade, with three species, is sister to the Eurasian clade (Töpfer *et al*., 2011), and one of the species, the Grey-headed bullfinch, has sperm with a helical head shape (Birkhead *et al*., 2006). This would suggest that the entire clade has retained the typical passerine sperm structure, and thus that the unusual bullfinch sperm evolved early in the Eurasian lineage after the split from the Himalayan clade. Nonetheless, more information about sperm morphology of all *Pyrrhula* species is needed to resolve this issue, and also to test whether there is an abrupt shift or a gradual change in sperm morphology across the *Pyrrhula* phylogeny.

Sperm competition in bullfinches

In both bullfinch species, the spermatozoa are very short. A mean value of 46 μm is towards the lower end of the length distribution for passerine birds. For example, total sperm length varies between 43 and 280 μm in a comparative study of 55 passerine species from 21 families (Lifjeld *et al*., 2010). Interestingly, the Fringillidae family spans almost the same range, from 46 μm in the Eurasian and Azores bullfinch (this study) to 275 μm in the common rosefinch (*Carpodacus erythrinus*; Lifjeld *et al*., 2010). This implies that there is also considerable variation in sperm competition risk within the family, as a positive association between sperm length and sperm competition risk has been documented for passerine birds (Briskie, Montgomerie & Birkhead, 1997; Kleven *et al*., 2009; Lüpold, Linz & Birkhead, 2009), and for the Fringillidae family (Immler & Birkhead, 2007). The short spermatozoa in the bullfinches are thus indicative of low sperm competition risk in these species.

Further evidence for low sperm competition in bullfinches comes from the very small relative testes size (Birkhead *et al*., 2006; this study), the small cloacal protuberance (Birkhead *et al*., 2006; own personal observations) and the high coefficients (CV) of both intra-male and inter-male variation in sperm total length compared with other passerines (Calhim *et al*., 2007; Immler *et al*., 2008; Kleven *et al*., 2008; Lifjeld *et al*., 2010). However, in contrast to expectations, we found that the sperm of the Eurasian bullfinch swim at very high speeds (157 μm s^{-1}), which is not only high compared with other passerine species (Kleven *et al*., 2009), but also with a previous report of only 22 μm s^{-1} in two captive-bred Eurasian bullfinches (Birkhead *et al*., 2006). However, the latter study also reported similarly low velocities for ten other passerine species (range 21–43 μm s^{-1}), and thus results do not appear comparable between the two studies, presumably due to different methodologies. Importantly, although the high sperm velocity in our Eurasian bullfinches might be taken as an indicator of high sperm competition risk from a comparative perspective (cf. Kleven *et al*., 2009), we would caution against such an interpretation due to the deviant morphology of the bullfinch sperm. Specifically, the ways in which variation in sperm morphology translates into variation in sperm velocity in passerine birds is poorly understood. Moreover, it should be noted that, under *in vitro* conditions, sperm swim in an artificial medium that may not accurately mimic the female environment. We therefore agree with Birkhead *et al*. (2006) that the current evidence seems to indicate that sperm competition is low or absent in the Eurasian bullfinch, and we suggest that it is similarly low or absent in the sister species, the Azores bullfinch.

Genetic diversity

Durrant *et al*. (2010) suggested that the unusual sperm morphology in the Eurasian bullfinch is an inbreeding effect caused by a reduction in genetic diversity. Abnormal sperm is typically more frequent in endangered or captive populations with reduced heterozygosity (Gage *et al*., 2006; Fitzpatrick & Evans, 2009; Roldan & Gomendio, 2009) and the Eurasian bullfinch may have undergone several population bottlenecks (Durrant *et al*., 2010). Deprivation of genetic diversity is also documented in the endangered Azores bullfinch (R. J. Lopes, unpubl. data). However, the analysis of microsatellite markers in the Eurasian bullfinch did not reveal any signature of a recent bottleneck or signs of reduced genetic variation when compared with three other fringillid finches (Durrant *et al*., 2010). Likewise, microsatellite allelic richness and heterozygosity levels are higher in the Eurasian bullfinch than in the Azores bullfinch (R. J. Lopes, unpubl. data). Therefore, it seems unlikely that the unusual sperm morphology in the two bullfinch species can be explained by inbreeding effects.

We would argue, however, that considering the typical bullfinch sperm as 'abnormal' is inappropriate. Rather, the term should be used to describe sperm that deviates markedly in shape or structure from the normal or average sperm phenotype within a given

species. In the two bullfinch species, the non-filiform sperm phenotype is universal and we saw no deviant types; thus for these species, a non-filiform, rounded sperm is normal and does not represent an abnormal sperm morphology. Moreover, the high intraspecific variation in sperm size and dimensions seen in bullfinches reflects a common phenomenon seen in every species, that sperm is variable both within and among males (Birkhead & Immler, 2007; Pitnick *et al*., 2009). What makes the bullfinches unique in this respect is that this normal variation is higher than in most other species, a result which is also consistent with the hypothesis of relaxed sperm competition in these species.

That the Azores bullfinch is deprived of genetic diversity while still maintaining high phenotypic diversity in sperm size, a genetically determined trait (Birkhead *et al*., 2005; Simmons & Moore, 2009), seems paradoxical. One possible explanation is that the estimates of low genetic diversity primarily reflect neutral genetic diversity and that functional and fitness-related loci may be less influenced by demographic processes. Such a pattern has been reported in the San Nicolas Island fox (*Urocyon littoralis dickeyi*), a species which has retained high genetic diversity at functional loci through balancing selection, despite a severe reduction in neutral diversity (Aguilar *et al*., 2004). It is commonly assumed that sperm competition exerts stabilizing selection on an optimal sperm size in passerine species (Calhim *et al*., 2007; Kleven *et al*., 2008; Lifjeld *et al*., 2010), which could explain why species with intense sperm competition have lower intermale variation in sperm size than those with little or no sperm competition. However, in species with small effective population size, one would also expect the genetic variation to be diminished due to drift. The fact that the Azores bullfinch has maintained the same intermale variation in sperm size as the Eurasian bullfinch despite much lower population size suggests that high phenotypic variance in sperm traits is somehow protected against genetic drift. A quantitative genetics study of sperm size components in the zebra finch (*Taenopygia guttata*), a species which also exhibits low sperm competition (Griffith *et al*., 2010), revealed both significant maternal genetic effects and strong negative genetic correlations between sperm components, which could serve to constrain the effects of selection and drift (Birkhead *et al*., 2005).

Evolution of neotenous spermatozoa

Although the bullfinch spermatozoon is unusual among passerines, it resembles the spermatids or immature testicular passerine sperm. It has therefore been suggested that this unusual morphology results from a suppression of the final stages of spermatogenesis (Birkhead *et al*., 2007). A similar phenomenon of evolution of simplified sperm is also known from other vertebrates. For example, in muroid rodents, several species have lost the ancestral apical hook of the sperm head and evolved a rounded acrosome, poor condensation of chromatin in the nucleus, and a shorter flagellum (Breed, 2005). Typically, these species have small relative testis size and high intramale variability in sperm components, which imply relaxed sperm competition (Breed, 2005; Breed *et al*., 2007). Similarly, in naked mole rats (*Heterocephalus glaber*), the sperm is simplified with several putatively neotenous features, and the mating system is characterized by low sperm competition (van der Horst *et al*., 2011). It therefore seems to be a common pattern that atypical and simplified sperm forms evolve in lineages with relaxed sperm competition as an adaptation reducing the cost of sperm production (Wedell, Gage & Parker, 2002). We encourage more comparative studies of sperm morphology in vertebrate taxa with contrasts in sperm competition levels to test the generality of this hypothesis for the evolution of simplified sperm.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Supplementary file. Example video of swimming sperm from an ejaculate of a Eurasian bullfinch as used in the CASA analysis of sperm velocity (for details see Methods).