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Lysozyme-associated bactericidal activity in the ejaculate of a wild passerine

MELISSAH ROWE[1]*, GÁBOR ÁRPÁD CZIRJÁK[2], JAN T. LIFJELD[1] and MATHIEU GIRAUDEAU[3]

[1]*Natural History Museum, University of Oslo, NO-0318 Oslo, Norway*
[2]*Leibniz Institute for Zoo and Wildlife Research, D-10315 Berlin, Germany*
[3]*School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA*

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Numerous antibacterial substances have been identified in the ejaculates of animals and are suggested to protect sperm from bacterial-induced damage in both the male and female reproductive tracts. Lysozymes, enzymes that exhibit bactericidal activity through their ability to break down bacterial cell walls, are likely to be particularly important for sperm defence as they are part of the constitutive innate immune system and are thus immediately available to protect sperm from bacterial attack. Birds are an ideal model for studies of ejaculate antimicrobial defences because of the dual function of the avian cloaca (i.e. waste excretion and sperm transfer), yet the antibacterial activity of avian ejaculates remains largely unexplored, and data on ejaculate lysozyme levels are only available for the domestic turkey (*Meleagris gallopavo*). Moreover, ejaculate lysozyme levels have not been reported for any species in the wild; which many argue is necessary to gain a comprehensive understanding of the function and dynamics of immune responses. Here, we show that lysozyme is present in the ejaculate of a wild passerine, the superb fairy-wren (*Malurus cyaneus*), and that the concentration of lysozyme in ejaculates varies substantially among males. This variation, however, is not associated with male condition, sperm quality or plumage coloration. Nevertheless, we suggest that lysozyme-associated antibacterial activity in ejaculates may be the target of natural and sexual selection and that these enzymes are likely to function in defending avian sperm from bacterial-induced damage. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, **109**, 92–100.

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INTRODUCTION

For sexually reproducing taxa, normal sperm function is essential for successful reproduction. Consequently, factors affecting sperm production and performance have the potential to significantly impact an individual's fitness. One such factor is exposure of sperm to bacteria. Bacteria can directly damage sperm form and function through their action on sperm cells; a range of Gram-positive and Gram-negative bacterial species are known to significantly reduce sperm motility and viability (Althouse & Lu, 2005; Eley *et al*., 2005; Zan Bar *et al*., 2008; Kaur *et al*., 2009; Moretti *et al*., 2009). For example, in humans, *Escherichia coli* damages sperm ultrastructure (e.g. membrane defects, cytoplasmic vacuoles; Diemer *et al*., 2000) and reduces motility through sperm adhesion and agglutination (Wolff *et al*., 1993; Diemer *et al*., 1996) and through the secretion of sperm immobilization factor (Prabha *et al*., 2010). Moreover, exposure to bacteria can lead to indirect damage via reactive oxygen species produced as a result of a bacterial-induced acute inflammatory response (Fraczek *et al*., 2007; Tremellen, 2008).

A range of antimicrobial proteins have been identified in the ejaculates of animals, including lactoferrin, phospholipase A_2, lysozyme, secretory leukocyte protease inhibitor (SLPI), amines and defensins (Hall, Hamil & French, 2002; Bourgeon *et al*., 2004; Poiani,

*Corresponding author. E-mail: melissah.rowe@nhm.uio.no

2006). Importantly, antimicrobial proteins exhibit significant antibacterial activity against a broad range of Gram-positive and Gram-negative bacteria (Shivaji, 1984; Bourgeon *et al*., 2004) and are suggested to protect sperm from bacterial-induced damage in both the male and female reproductive tracts (Hall *et al*., 2002; Poiani, 2006). Of particular interest are the lysozymes (muramidase or *N*-acetylmuramide glycanhydrolase), a group of enzymes characterized by their ability to hydrolyse the β-(1,4)-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine residues of bacterial peptidoglycan, and cause rapid cell lysis (Callewaert & Michiels, 2010). Lysozymes are part of the constitutive innate immune system and are therefore capable of mounting an immediate response to bacterial infections (Millet *et al*., 2007). Thus, lysozymes offer an important first response to the threat of bacterial-induced sperm damage. Furthermore, lysozymes appear to exhibit non-enzymatic bactericidal activity (Masschalck & Michiels, 2003) and play a role in the amelioration of oxidative stress (Liu *et al*., 2006).

Lysozyme activity in ejaculates has been reported for a range of animal taxa [e.g. mammals (Hankiewicz & Swierczek, 1974), insects (Otti *et al*., 2009), fish (Lahnsteiner & Radner, 2010), and domestic turkey (*Meleagris gallopavo*; Sotirov, Dimitrov & Jeliazkov, 2002)] and appears to be associated with sperm quality and male fertility (Kuzmin *et al*., 1991; Lahnsteiner & Radner, 2010). Birds are an ideal model for studies of ejaculate antibacterial defences for several reasons. First, the avian cloaca functions in both excretion and sperm transfer; thus intestinal pathogens may become incorporated into ejaculates and pathogen loads are thought to be relatively high, compared with other taxa (Sheldon, 1993). Second, bacteria have been isolated from the cloaca and semen in a range of species (Sheldon, 1993; Lombardo & Thorpe, 2000; Westneat & Rambo, 2000; Poiani & Gwozdz, 2002; Hupton *et al*., 2003) and recent studies demonstrate the sexual transmission of bacteria (Lombardo *et al*., 1996; Stewart & Rambo, 2000; White *et al*., 2010). Finally, bacteria have been shown to adhere to rooster sperm (*Gallus gallus*; Vizzier-Thaxton *et al*., 2006), although the consequences for sperm function are currently unknown. To date, however, lysozyme activity in semen has only been reported for one domestic avian species (i.e. *Meleagris gallopavo*; Sotirov *et al*., 2002). Furthermore, no data are available for any animal species in the wild, which is argued to be vital to our understanding of the function and dynamics of immune responses (Pederson & Babayan, 2011).

The aim of the present study was to determine whether lysozyme is present in the ejaculates of wild passerines, and, if present, whether variation in ejaculate lysozyme concentration is associated with male condition. Next, given the suggestion that antibacterial proteins protect sperm from bacterial-induced damage (Poiani, 2006), we tested for an association between ejaculate lysozyme concentration and sperm quality (i.e. sperm swimming speed and percentage of motile sperm). We chose to study the superb fairy-wren (*Malurus cyaneus*) because it is a relatively common passerine, and standard avian ejaculate collection techniques allow the collection of a sufficiently high volume of semen for analysis. Moreover, a number of potentially pathogenic bacterial species have been isolated from the cloaca of both male and female *Malurus cyaneus* (Poiani & Gwozdz, 2002). Finally, a recent study of captive mallards (*Anas platyrhynchos*) reported a correlation between male carotenoid-based bill coloration and total antibacterial activity of ejaculates, suggesting that sexual ornamentation may reveal information about a male's ability to resist ejaculate microbial attack (Rowe *et al*., 2011). Consequently, we tested for an association between male coloration and the lysozyme-associated antibacterial activity of male ejaculates in order to examine the generality of this relationship across taxa and ornament types (e.g. pigment-based versus structural coloration).

MATERIAL AND METHODS

Study species and general methods

Malurus cyaneus is a small (~8–11 g), insectivorous, cooperatively breeding passerine endemic to Australia (Rowley & Russell, 1997). Males exhibit seasonal dichromatism: moulting into a nuptial plumage consisting of azure blue feathers on the crown, collar and ear tufts, and dark black–blue throat feathers during the breeding season. We studied *Malurus cyaneus* in the vicinity of the University of New South Wales Smiths Lake Field Station, located near Bungwahl, New South Wales, Australia (32°24′S, 152°28′E) during the spring of 2011 (15–24 November). We trapped adult male birds using mist nets and song playback. Upon capture, birds were weighed (±0.1 g) using a Pesola spring balance and banded with a numbered Australian Bird and Bat Banding Scheme (ABBBS) aluminium leg band. We also took a series of standard morphological measurements, including tarsus length and three measures of the cloacal protuberance (CP): length (l), width (w) and height (h). We calculated CP volume using the formula $\pi(h/2 \times w/2) \times l$ (Tuttle, Pruett-Jones & Webster, 1996). All measurements were taken by the same person (M.R.) to avoid observer effects.

Sperm quality analysis

We collected fresh ejaculate samples (~1–2 μL) from males using cloacal massage (Wolfson, 1952; Rowe & Pruett-Jones, 2011), and immediately measured sperm swimming speed following the principles of Lifjeld *et al*. (2013). Specifically, exuded semen was collected in a 10 μL capillary tube and mixed with (~400–600 μL) pre-heated (40 °C) Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Ltd). Next, we placed 6 μL of the diluted semen in a pre-heated microscopy counting chamber (depth 20 μm; Leja, Nieuw-Vennep, the Netherlands) mounted on a MiniTherm slide warmer (Hamilton Thorne Inc.) maintained at a constant temperature of 40 °C. Sperm movement was then recorded using a phase-contrast microscope (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.

The resulting videos were later analysed using computer-assisted sperm analysis (CASA) (HTM-CEROS sperm tracker, CEROS 12; Hamilton Thorne Research). The CASA 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 adapted to exclude non-sperm particles: minimum contrast, 100; minimum cell size, eight pixels; sperm head elongation (i.e. width/length), < 45%. We also excluded non-continuous sperm tracks or sperm tracked for less than ten 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, sperm cells with 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 an average path velocity (VAP, i.e. average velocity over a smoothed sperm track) < 30 μm s^{-1} were considered immotile. The total number of motile sperm that were recorded ranged from 29 to 371 (172.6 ± 24.3, mean ± SE) per individual male, and we recorded curvilinear velocity (VCL, i.e. velocity over the actual sperm track), VSL and VAP for each tracked sperm.

Ejaculate antimicrobial assays

When possible, we also collected a second ejaculate to search for lysozyme-based bactericidal activity. Importantly, the second ejaculate was collected immediately after the assessment of sperm swimming speed and thus within 2 minutes of the collection of the first ejaculate. As before, ejaculates were collected using cloacal massage, and the exuded semen was collected in a 10 μL capillary tube. Semen samples ($N = 19$) were then immediately placed in a sterile (buffer-free) Venoject vacuum glass tube and stored at –20 °C until transport to the Leibniz Institute for Zoo and Wildlife Research (Germany), where they were kept at –20 °C until further analysis; all samples were analysed within 6 months of sample collection, and all samples were subject to identical storage periods.

To measure the lysozyme concentration of ejaculates, we used a modified version of the standard lysoplate assay method of Osserman & Lawlor (1966). Briefly, 8 mL of 1% agar gel (A5431; Sigma-Aldrich) containing 50 mg per 100 mL of lyophilized *Micrococcus lysodeikticus* (M3770; Sigma-Aldrich) was pipetted into sterile Petri dishes and, after solidification, 1 μL of diluted semen (1 : 1 *v/v* in phosphate-buffered saline, PBS) was inoculated in test holes (1.7 mm in diameter). Standard dilutions of crystalline hen egg white lysozyme (0.5, 1, 5, 10 and 25 μg mL^{-1}; L6876; Sigma-Aldrich) were used to prepare a standard curve in each plate. Plates were then incubated at 37 °C for 18 h. During this period, a clearing zone developed in the area of the gel surrounding the sample inoculation site as a result of bacterial lysis: the diameter of this cleared zone is proportional to the log of lysozyme concentration. Cleared zones were measured and converted into hen egg lysozyme equivalents (expressed in μg mL^{-1}) according to the standard curve from each plate. Repeatability analysis on a subset of samples ($N = 15$) showed that the assay was highly repeatable across plates (repeatability, $R = 0.99$; confidence interval, CI = 0.98–1.0; $P < 0.0001$; Nakagawa & Schielzeth, 2010). Lysozyme concentrations were ln-transformed prior to analysis to achieve normality.

Coloration

At the time of capture, we also removed between six and eight feathers from both the throat and crown of males and stored these feathers in glassine envelopes. We chose to focus on throat and crown coloration because these colour patches are visible during the courtship display known as the 'Sea Horse Flight' during this display the neck is extended upwards and the feathers on the top of the head are held erect (Rowley & Russell, 1997). It should be noted, however, that it is not currently known whether plumage coloration functions as a sexual signal in this species.

For each region (throat and crown), we mounted feathers in an overlapping pattern on standard black cardboard and measured reflectance using an Ocean Optics USB2000 UV-VIS spectrophotometer (Dunedin, FL, USA) with a UV-VIS probe and a PX-2 pulsed xenon light source. The probe was mounted in

a metal block that excluded all ambient light from a standardized measurement area (approximately 3 mm^2) and maintained the probe perpendicular to the feather surface. Reflectance (R) was measured at five overlapping locations in each plumage patch for each individual, and after every five samples we recalibrated the spectrometer against both a dark and a white (Ocean Optics WS-1) standard. Using CLR 1.05 (Montgomerie, 2008), we assessed coloration across all wavelengths (λ) of the avian visual system (320–700 nm). All spectrophotometric values were binned into 1-nm increments. Repeat measures were highly repeatable ($R = 0.83$–0.98, all $P < 0.005$; Nakagawa & Schielzeth, 2010), thus we collapsed our five measures into a single average spectrum per individual and plumage patch. Finally, we summarized reflectance spectra using the following colour metrics: B1, brightness (R_{total} = sum of R values between 320 and 700 nm); H1, hue (λ_{Rmax}); S1U, UV chroma (i.e. saturation in the ultraviolet range of the spectrum, $R_{320–400\ nm}/R_{total}$); and S5b, blue chroma (i.e. saturation in the blue range of the spectrum, $R_{400–475\ nm}/R_{total}$; crown only). We measured both UV and blue chroma for blue crown feathers because reflectance spectra from these feathers displayed a peak involving both spectral regions. In contrast, the predominately black feathers of the throat showed low reflectance outside of the UV region of the spectrum.

Statistical analysis

We conducted statistical analyses using the statistical package R 2.14.1 (R Core Team, 2012) and, where appropriate, the R packages 'nlme' (Pinheiro *et al*., 2012) and 'car' (Fox & Weisberg, 2011). Modelling assumptions were validated through the visual inspection of model evaluation plots.

We first examined whether variation in ejaculate lysozyme concentration was associated with either male condition or CP volume using a linear model, with lysozyme level as the dependent variable and both male condition and CP volume as predictor variables. We modelled body condition as size-adjusted body mass by including both body mass and tarsus length as covariates in our analyses (Darlington & Smulders, 2001; Green, 2001). Size-adjusted body mass is a commonly used index of energy reserves (Green, 2001; Peig & Green, 2010) and has been shown to correlate with fat stores in the congeneric red-backed fairy-wren (*Malurus melanocephalus*; Lindsay *et al*., 2009).

Next, the relationship between ejaculate lysozyme concentration and sperm swimming speed was examined using a restricted maximum-likelihood generalized linear mixed model (REML-GLMM); this approach allowed us to incorporate data from all motile sperm in our analysis. Thus, lysozyme concentration was included as a fixed factor and individual identity as a random factor. We choose to use VCL (square-root transformed to meet modelling assumptions) for statistical analyses because this metric measures the actual path of sperm movement and thus represents sperm movement better than simpler approximations. Nonetheless, sperm motility parameters were strongly intercorrelated (all $r > 0.61$, $P < 0.006$), and analyses using VAP and VSL returned qualitatively similar results (data not shown). We also examined the effect of ejaculate lysozyme concentration on the percentage of motile sperm (arcsine transformed to meet modelling assumptions) in an ejaculate using a linear model; in this instance we did not use GLMM as each male was represented by just one measure of the percentage of motile sperm.

We tested for associations between ejaculate lysozyme concentration and male coloration for each colour patch (i.e. crown and throat) separately. For throat coloration, we examined the relationship between ejaculate lysozyme levels and plumage brightness, hue and UV chroma using a linear model. We did not perform the same analysis using tristimulus colour metrics for crown coloration because of the strong collinearity between these variables, as reflected by variance inflation factors (VIFs: brightness, 13.3; hue, 3.3; UV chroma, 24.6; blue chroma, 31.5) generally exceeding the suggested threshold of 10 (Marquardt, 1970; Kleinbaum, Kupper & Muller, 1998). Consequently, we used a principal component analysis to collapse tristimulus colour variables into fewer independent variables (principal components) of crown coloration. Principal components analysis (PCA) of crown feather coloration generated two principal components that explained 87% of the total variation in crown colour. Thus, we examined the relationship between ejaculate lysozyme concentration and crown colour PC1 and PC2 using a linear model. Crown colour PC1 loaded positively with crown UV chroma and blue chroma, thus we interpret increasing PC1 as indicating increased saturation in both the blue and UV regions of the spectrum. Crown colour PC2 loaded positively with crown brightness and hue, thus we interpret increasing PC2 values as indicating brighter feathers reflecting at a higher maximum wavelength (see Table S1 for a summary of PCA results). Finally, we examined the relationships between coloration and individual condition using linear models, with coloration as the dependent variable and male condition as the predictor variable. Separate models were run for each colour variable of interest (i.e. throat brightness, hue, UV chroma, and crown colour PC1 and PC2), and, as before, we modelled body condition as size-adjusted

body mass by including both body mass and tarsus length as covariates in our analyses.

RESULTS

We successfully collected ejaculates for measurement of sperm quality and lysozyme concentration from 19 adult male *Malurus cyaneus*. All 19 individuals exhibited lysozyme activity in their ejaculate, although concentrations varied more than 30-fold, ranging from 1.12 to 33.73 μg mL^{-1} (mean ± SD: 9.14 ± 9.8 μg mL^{-1}). This variation, however, was not associated with male condition when condition was expressed as size-adjusted body mass (body mass, $\beta = -0.12$, $t = -0.16$, $P = 0.87$; tarsus, $\beta = 0.13$, $t = 0.25$, $P = 0.81$), or with the volume of the cloacal protuberance ($\beta = 0.007$, $t = 0.45$, $P = 0.66$).

Sperm quality also varied substantially among males, with the percentage of motile sperm ranging from 47 to 100% (mean ± SD: 86 ± 12%) and VCL ranging from 81.9 to 97.8 μm s^{-1} (mean ± SD: 89.00 ± 4.0 μm s^{-1}). We found no relationship between the concentration of lysozyme in a male's ejaculate and sperm swimming speed ($\beta = -0.05$, $t = -1.20$, $P = 0.25$). There was also no relationship between the percentage of motile sperm and ejaculate lysozyme concentration ($\beta = 0.02$, $t = 0.36$, $P = 0.73$).

Finally, we found no association between throat coloration and the concentration of lysozyme in the ejaculates of males (B1, $\beta = 0.06$, $t = 0.49$, $P = 0.63$; H1, $\beta = -1.80$, $t = -0.11$, $P = 0.91$; S1U, $\beta = -2.12$, $t = -0.34$, $P = 0.74$). Similarly, we found no association between ejaculate lysozyme concentration and crown coloration PC1 or PC2 (crown PC1, $\beta = 0.10$, $t = 0.54$, $P = 0.60$; crown PC2, $\beta = 0.12$, $t = 0.54$, $P = 0.60$). Likewise, individual condition was not associated with either throat coloration (body mass, $\beta = -0.06$ to -5.23, all $P > 0.09$; tarsus, $\beta = -0.02$ to -5.99, all $P > 0.05$) or crown coloration (body mass, $\beta = 0.06$ to 0.67, all $P > 0.4$; tarsus, $\beta = -0.11$ to 0.55, all $P > 0.3$; see Table S2 for the full results of these analyses).

DISCUSSION

Lysozymes have been identified in a variety of body tissues (e.g. blood, urine, saliva, tears, and gastric juices) and are widely recognized for their antibacterial activity (Hankiewicz & Swierczek, 1974; Callewaert & Michiels, 2010), although the relative contribution of lysozymes to total antibacterial activity is currently unknown. Lysozymes have also been identified in semen from a range of animal taxa (Hankiewicz & Swierczek, 1974; Sotirov *et al*., 2002, 2006; Otti *et al*., 2009; Lahnsteiner & Radner, 2010), although to date most reports concern humans or domestic species of agricultural importance, and studies focus on medical and agricultural applications of lysozymes. Here, we show for the first time that lysozyme is also present in the ejaculate of a wild passerine, *Malurus cyaneus*. In this species, the average concentration of lysozyme in the ejaculates of males was comparable with levels observed in the ejaculates of humans (mean ± SD, 13.4 ± 5.4 μg mL^{-1}; Hankiewicz & Swierczek, 1974) and *Meleagris gallopavo* (mean only, 6.8 μg mL^{-1}; Sotirov *et al*., 2002), but far exceeded those reported for the domestic boar (*Sus scrofa domesticus*; mean, <0.05 μg mL^{-1}; Sotirov *et al*., 2006). Moreover, ejaculate lysozyme concentrations in *Malurus cyaneus* varied substantially among males.

Given the antibacterial activity of lysozymes, it has been suggested that these enzymes may be present in the ejaculates of males to protect sperm from bacterial attack (Hall *et al*., 2002). In brown trout (*Salmo trutta*), high seminal plasma lysozyme levels are associated with higher sperm velocities and a greater percentage of motile sperm (Lahnsteiner & Radner, 2010). Similarly, in humans, studies show that decreased semen lysozyme levels are associated with poor sperm motile performance and oligospermia (Hankiewicz & Swierczek, 1974; Kuzmin *et al*., 1991; Kuzmin, Ivanov & Bukharin, 1998), although a further study reported opposite results with higher lysozyme levels observed in the semen of infertile males (Ulèová-Gallová *et al*., 1999). A similar positive association between semen lysozyme levels and sperm quality has been suggested for *Meleagris gallopavo* (Sotirov *et al*., 2002); in this instance, however, the data underlying this assertion are unclear.

In the current study, we found no association between ejaculate lysozyme levels and either the swimming velocity of sperm or the percentage of motile sperm in an ejaculate. There are a number of potential explanations for these results. First, although both Gram-negative and Gram-positive bacteria have been isolated from the cloaca and ejaculates of birds (Westneat & Rambo, 2000; Poiani & Gwozdz, 2002; Hupton *et al*., 2003), the pathogenicity of these bacteria is largely unknown. In poultry, bacteria are generally thought to be detrimental to sperm quality during cryopreservation (Donoghue & Wishart, 2000), and as a consequence antibiotics are a common additive to cryopreservation diluents. Moreover, ejaculate-borne pathogens have been shown to attach themselves to sperm (Vizzier-Thaxton *et al*., 2006) and have been linked to widespread flock infertility (Stipkovits *et al*., 1983). Generally, lysozymes are less effective against Gram-negative bacteria compared with their effect on Gram-positive bacteria (Hall *et al*., 2002). Moreover, lipopolysaccharides (LPSs), a major constituent of the bacterial outer membrane, from Gram-negative

bacteria can inhibit lysozyme activity in a dose-dependent fashion (Ohno & Morrison, 1989). Thus, under conditions of a primarily Gram-negative bacterial threat to sperm quality, an association between ejaculate lysozyme activity and sperm quality might not be expected.

Next, the ejaculate bacterial loads of males examined in this study are unknown. Although a component of the constitutive innate immune system, lysozymes also appear to be regulated in response to need; administration of an LPS challenge resulted in elevated plasma lysozyme concentrations in chickens (Millet *et al*., 2007). Thus, high ejaculate lysozyme activity may reflect male investment in constitutive innate immunity or current/recent levels of infection. Consequently, infection history may obscure any potential correlation between male sperm quality and lysozyme activity. Finally, lysozymes may be minor contributors to antibacterial defence in ejaculates. On the other hand, lysozyme-based antibacterial activity can be significantly increased by synergistic and additive interactions with other antimicrobial molecules (Singh *et al*., 2000; Garcia *et al*., 2001). Thus, sperm quality may be correlated with the overall antibacterial activity of ejaculates, but not with any one specific component of antibacterial defence.

Finally, in the current study it was necessary to sample an ejaculate for the measurement of sperm velocity first and then collect a second ejaculate sample for the measurement of lysozyme concentration. Thus, any differences between the two samples have the potential to influence our results. However, we collected ejaculate samples within 2 minutes of one another. Moreover, both samples were drawn from the same pool of sperm stored in the seminal glomera. Consequently, differences between samples in terms of sperm quality and lysozyme concentration are likely to be minimal or absent entirely. Nonetheless, we suggest that studies examining within-male (i.e. across season, between-ejaculate) variation in ejaculate lysozyme concentrations would be a fruitful avenue of research, although in this instance we recommend examination of natural ejaculates produced at time intervals reflecting the natural copulation frequency of the species under study.

The results of this study also show there is no association between male plumage coloration and ejaculate lysozyme levels in *Malurus cyaneus*, which contrasts with a recent study of *Anas platyrhynchos* in which males with more colourful bills had semen with superior bacterial-killing ability (Rowe *et al*., 2011). In that study, however, the total antibacterial activity of ejaculates was examined, which may be more relevant information to convey to potential mates than the activity of any one single antibacterial substance. Moreover, bill coloration in male *Anas platyrhynchos* can be rapidly adjusted in response to immune system activation (Butler & McGraw, 2011), whereas the plumage of *Malurus cyaneus* is likely to be relatively static because the timing and extent of feather replacement (i.e. moult) is often fixed by temporal, phylogenetic or physiological constraints (Payne, 1972). Finally, in *Malurus cyaneus*, female mate choice appears to be based on the timing of the prenuptial moult, rather than coloration *per se* (i.e. females prefer males that acquire breeding plumage earlier in the season; Dunn & Cockburn, 1999). Thus, it appears unlikely that plumage coloration functions to signal lysozyme-based antibacterial defence of sperm in *Malurus cyaneus*. We therefore suggest that, given that lysozyme levels may reflect either investment in immunity or infection status, future studies examining the relationship between ejaculate antibacterial activity and male ornamentation may be best directed towards species exhibiting flexible secondary signals, such as skin, eye or bill coloration.

In conclusion, our results demonstrate that the ejaculate of *Malurus cyaneus* exhibits lysozyme-associated bactericidal activity. Our findings also show that there is considerable variation between males in ejaculate lysozyme levels, upon which selection could act. Under the assumption that lysozymes contribute to the total antibacterial defence of sperm, perhaps via synergistic interactions with other proteins, we suggest that these enzymes may be a target of natural or sexual selection for antibacterial sperm protection. Consequently, we recommend that future studies investigating the presence and function of antibacterial proteins in avian ejaculates be undertaken, and suggest that such studies will provide considerable insight into the evolution of animal ejaculates.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Principal component analysis (PCA_ loadings, with contributing factors set in bold, and the cumulative proportion of variance explained by the first two principal components extracted by PCA of four measures of crown-feather coloration in *Malurus cyaneus*.

Table S2. Relationship between male plumage coloration (crown and throat coloration) and individual condition, where condition is modelled as size-adjusted body mass by including both body mass and tarsus length as covariates in the analyses.