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Sexual ornamentation reflects antibacterial activity of ejaculates in mallards

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Bacteria present in ejaculates can impair sperm function and reduce male reproductive success. Thus, selection should favour the evolution of antimicrobial defences to limit the detrimental effects of sperm-associated bacteria. Additionally, current hypotheses suggest that ornamental traits may signal information about the infection status of an individual or the ability of an individual to resist bacterial-induced sperm damage. However, despite the evolutionary implications of ejaculate antimicrobials, and the putative importance of pathogens for the evolution of male ornamentation, tests of these hypotheses are lacking. We examined the antibacterial activity of semen from mallard ducks (*Anas platyrhynchos*) and tested whether the bactericidal capacity of semen was associated with bill coloration, a sexually selected trait. We show that mallard semen exhibits significant antibacterial activity, as measured by the *in vitro* capacity to kill *Escherichia coli* and *Staphylococcus aureus*. Furthermore, we demonstrate that males with more colourful bills have semen with superior bacterial-killing ability. These results suggest that females could use male phenotypic traits to avoid sexually transmitted pathogens and acquire partners whose sperm suffer less bacteria-induced damage.

Keywords: *Anas platyrhynchos*; bacteria; sexual selection; sperm; sexually transmitted diseases

1. INTRODUCTION

Animals are constantly exposed to a variety of pathogens, and the ability of individuals to combat microbial attack is an important component of fitness [1]. Sperm cells are not immune to microbial exposure; indeed the ejaculate is colonized by a range of potential pathogens [2,3]. Moreover, sperm may be exposed to pathogens in the female reproductive tract, an environment characterized by a diversity of microbial species (e.g. [4]). Importantly, microbial exposure can impact sperm form and function (e.g. [5–7]) with consequences for male fitness.

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By reducing male fitness, micro-organisms present in the ejaculate may impose strong selection on males to develop antimicrobial protection of sperm. Antibacterial activity of semen has been observed in a variety of taxa [3,8–10] and appears effective against a wide range of bacterial species [10]. Sperm protection via antibacterial substances would maintain male fertilization efficiency, and may also allow sperm to remain protected in the female reproductive tract [11] and storage organs [8]. Thus, antibacterial activity is likely to benefit males, even under conditions of monogamy, and selection should favour individuals with greater antimicrobial defences.

Pathogens can also be transferred during copulation (sexually transmitted diseases, STDs; [3,4]) and generate significant costs for females [3]. Thus, females would benefit from being able to identify males with high STD loads and avoid copulations with such males. The notion that ornamental traits may evolve to signal aspects of general immunity and that females use these traits to select a superior mate has received considerable attention (e.g. [12–14]). By contrast, the suggestion that ornaments reflect the likelihood that an individual will transmit STDs [15,16] or the ability of an individual to resist bacterial-induced sperm damage remains to be tested.

In this study, we investigated the antibacterial activity of semen from mallard ducks (*Anas platyrhynchos*). Additionally, to test the hypothesis that sexual ornaments may reveal information about a male's ability to resist ejaculate microbial attack, we examined the relationship between bill coloration, a sexually selected trait [17], and the bacterial-killing activity (BKA) of semen against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus*, two potentially pathogenic micro-organisms found in the avian cloaca and ejaculate [18,19].

2. MATERIAL AND METHODS

Ejaculates were collected from 11 adult mallards during May 2009 using cloacal massage [20]. Samples were frozen at -80°C for 12 months prior to antibacterial analysis. All males were part of a captive-bred population maintained at Arizona State University (see the electronic supplementary material). We recorded body mass ($\pm 1\text{ g}$) and quantified reflectance of the bill from $\lambda = 320\text{--}700\text{ nm}$ using an Ocean Optics (Dunedin, FL, USA) USB2000 spectrophotometer and PX-2 pulsed xenon light source. Following Butler *et al.* [21], we calculated three tristimulus scores related to the carotenoid content of the mallard bill: brightness (B1), saturation in the blue region (S1B) and hue (H4b). Additionally, we calculated ultraviolet (UV) reflectance (S1U) as it reflects immune responsiveness and sperm velocity in mallards [14].

We assessed BKA of semen against *E. coli* (ATCC no. 8739) and *S. aureus* (ATCC no. 6538P) using a liquid growth inhibition assay modified from Otti *et al.* ([9]; see the electronic supplementary material). Briefly, semen samples were diluted 1 : 1 with Tryptic Soy Broth (TSB; no. CM0129, Oxoid Deutschland GmbH). Next, $5\text{ }\mu\text{l}$ of diluted semen and $45\text{ }\mu\text{l}$ of bacteria (concentration corresponding to an attenuation (*D*) of 0.01) were added to a 96-well plate. Controls were TSB with bacteria and TSB with diluted semen. The plate was incubated overnight at 37°C , and *D* at 595 nm determined using a spectrophotometer (Biotek). Finally, after controlling for the contribution of sperm cells, the BKA of semen for each microbial strain was defined as the per cent of bacteria killed, where $\text{BKA} = 1 - (\text{sample } D / \text{control bacterial solution } D)$.

To test whether semen BKA was predicted by male coloration or condition, we fitted linear models with the four colour metrics and male condition (i.e. body mass) as independent variables (similar results were obtained when condition was estimated as the residuals of body mass/tarsus length). We then simplified the models, by removing terms in order of least significance, and identified the best model using Akaike Information Criterion (AIC) values.

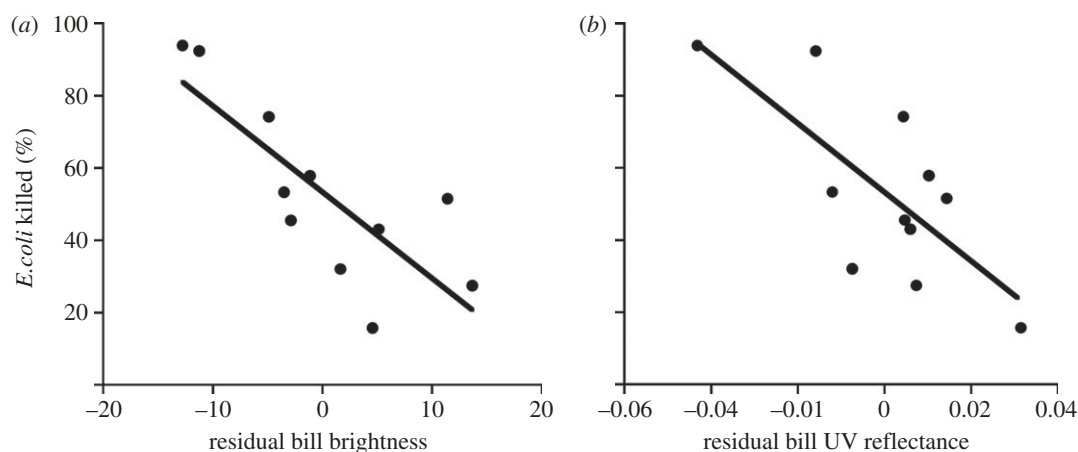


Figure 1. Partial residual plots showing the relationship between semen BKA against *E. coli* and (a) bill brightness (corrected for S1U), and (b) bill UV reflectance (corrected for B1). Line is a simple regression line.

Separate models were fitted for *E. coli* and *S. aureus*. Modelling assumptions were validated through visual inspection of residual plots and normality testing of residuals (see the electronic supplementary material). All analyses were performed using R (v. 2.12.0) software package [22], and residuals from all analyses were normally distributed.

3. RESULTS

All semen samples showed bactericidal capacity against both *E. coli* (mean 51%, range 16–94%) and *S. aureus* (mean 40%, range 8–60%), but there was no association between *E. coli* and *S. aureus* BKA of individual samples ($r = -0.07$, $p = 0.84$). For *E. coli*, only bill brightness and UV reflectance explained any variation in semen BKA: both B1 ($t = -3.77$, $p = 0.006$; figure 1a) and S1U ($t = -3.58$, $p = 0.007$; figure 1b) were significantly negatively correlated with the capacity of a male's ejaculate to kill *E. coli*. By contrast, bill saturation ($t = -0.53$, $p = 0.61$), bill hue ($t = -0.32$, $p = 0.76$) and body mass ($t = -0.04$, $p = 0.97$) were not related to semen *E. coli* BKA and were removed from the final AIC-selected model. Bill coloration did not significantly predict semen BKA against *S. aureus* and all colour metrics were removed from the final model (B1: $t = -0.11$, $p = 0.92$; S1B: $t = 0.48$, $p = 0.65$; S1U: $t = 1.18$, $p = 0.27$; H4b: $t = -0.34$, $p = 0.75$). Only body mass was retained in the final model for *S. aureus*: there was a trend for BKA to increase with increasing body mass ($t = 2.26$, $p = 0.0502$).

4. DISCUSSION

Evolutionary pressures associated with fertility and post-copulatory sexual selection can result in the evolution of ejaculate components that influence fitness [3,23]. In this study, we found that semen from mallards possessed significant antibacterial activity against both *E. coli* and *S. aureus*, and that the capacity of semen to kill *E. coli* was associated with bill coloration. Specifically, males with darker bills and those that were less reflective in the UV range had greater antibacterial defences against *E. coli*. As brightness is negatively correlated with carotenoid content of the bill [21], our results demonstrate that male mallards with more

elaborate carotenoid-enriched ornaments have semen with superior antibacterial activity towards *E. coli*.

Escherichia coli is probably a biologically relevant bacterial threat to sperm quality: *E. coli* has been found in the semen and cloaca of birds [18,19], and in humans has been shown to damage sperm ultra-structure [24] and reduce motility through sperm adhesion and agglutination [5,25] and the secretion of sperm immobilization factor [26]. Thus, *E. coli* may contribute to variation in paternity success among male birds, and the antibacterial properties of semen observed in this study are probably the result of selection for non-sperm components (e.g. antimicrobial proteins, amines [10]) of the ejaculate that mitigate the presence of *E. coli*.

Because the costs of STDs are thought to be greater for females [11], females should be under strong selection to avoid STD exposure. Our results suggest that females may be able to use ornamentation to identify males with high ejaculate antibacterial activity, and that by doing so they may be able to avoid sexual transmission of pathogenic bacteria. In a previous study of mallards, Peters *et al.* [14] showed that males possessing bills with lower UV reflectance produced faster sperm. Thus, mate choice based on bill coloration in mallards may allow females to both avoid costs associated with STDs and increase the likelihood of receiving high-quality sperm. Furthermore, our results offer a potential mechanism for the result found by Peters *et al.*: the reduced sperm velocity of males with bills exhibiting high UV reflectance may result from low antibacterial protection of sperm in ejaculates.

It is unclear why we did not observe an association between coloration and *S. aureus* BKA. One possibility is that the prevalence and the pathogenicity of *S. aureus* are low relative to that of *E. coli*. There were also differences in the ability of semen to kill specific microbes, *E. coli* was more susceptible than *S. aureus*, and we found no relationship between *E. coli* and *S. aureus* BKA. These results suggest that different components of the immune system respond to the various bacterial threats in the ejaculate and that different microorganisms will generate varying costs and thus differentially influence the evolution of ejaculate antimicrobial defences.

In summary, our findings offer novel insight into ejaculate evolution and transmission-avoidance models of parasite-mediated sexual selection. We show for the first time, to our knowledge, that the ejaculates of birds possess significant antibacterial activity. Furthermore, we show that bill coloration predicts the ability of a male mallard's semen to kill *E. coli*, thus providing a potential signal to females of their ability to protect sperm from microbial damage. Future studies should investigate the generality of this relationship in birds and other taxa, the components underlying specific bacteriolytic activities, and the prevalence and the pathogenicity of specific ejaculate and reproductive tract micro-organisms. In fact, ejaculate-borne bacteria may be beneficial to females, and ornamental traits may evolve to signal the potential for transmission of these beneficial microbes [27].

All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Arizona State University (protocol no: 08-979R).

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