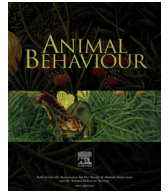




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Different underlying mechanisms drive associations between multiple parasites and the same sexual signal

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Associations between parasite loads and sexual signal expression have long been the focus of research. However, our understanding of how sexual selection operates in the context of multiple parasite infections within the same host is still quite limited. We examined the expression of plumage coloration, which is sexually selected in North American barn swallows, *Hirundo rustica erythrogaster*, and tail streamer length, which is sexually selected in other barn swallow subspecies, in the context of eight different parasite infections. We found that two parasites, nest mites and *Plasmodium*, were associated with colour expression, but in opposite directions. Attractive males were less likely to have mites in their nests, but more likely to be infected with *Plasmodium*. We found that different underlying mechanisms were generating the links between these different parasites and the same colour signal. Males that invested more in colour expression were less likely to survive when infected with *Plasmodium*, suggesting a physiological mechanism linking colour to *Plasmodium* infections. However, we found no clear cost to males when examining nest mites, which primarily infect offspring. Instead, using experimental mite manipulations, we provide evidence for a behavioural mechanism, where territorial behaviour and male–male competition likely drive the association between colour expression and nest mite infections. We did not find associations between parasites and streamer length in this subspecies, suggesting that the links between divergent sexual signals and parasites may be quite flexible. These results suggest that the evolution of a single sexual signal can be shaped by several, even opposing, links with different parasite infections.

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Hamilton and Zuk (1982) first proposed a coevolutionary mechanism whereby sexual signals could provide females with information about parasites, predicting that healthier individuals would have more attractive signals. Since this work, studies in a diversity of taxa have uncovered associations between the expression of sexual signals and parasite infections (Balenger & Zuk, 2014; Clayton, 1991; Milinski, 2006; Moore & Wilson, 2002). However, the direction of these associations is not always consistent. While studies in many species have revealed a negative relationship between sexual signal expression and parasite loads, supporting the original intraspecific predictions of Hamilton and

Zuk (e.g. Martin & Johnsen, 2007; Molnár, Bajer, Mészáros, Török, & Herczeg, 2013; Zuk, Thornhill, Ligon, & Johnson, 1990), other studies have found a positive relationship, where attractive males actually have more parasites (e.g. Beltran-Bech & Richard, 2014; Kurtz & Sauer, 1999; Mougeot, Irvine, Seivwright, Redpath, & Piertney, 2004; Roberts, Buchanan, & Evans, 2004). Additionally, research in many species has demonstrated no clear relationship between sexual trait expression and the parasites that were measured (e.g. Martin & Johnsen, 2007; Ottová et al., 2005; Reid et al., 2005).

One way that we may gain new insights into these variable results across studies is by examining sexual signals in the context of the broader parasite community that infects a given host (Clayton, 1991; Karvonen & Seehausen, 2012). Indeed, these different relationships between infection and signal expression suggest that parasites can interact with sexual signals in different ways, opening

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up the intriguing possibility that multiple parasites within a host could be influencing the evolution of the same signal in different ways. The idea that sexual signals can be shaped by several different, and even opposing, selective pressures is not a new one (Hunt, Breuker, Sadowski, & Moore, 2009; Safran, Scordato, Symes, Rodríguez, & Mendelson, 2013; Sinervo, Miles, Frankino, Klukowski, & DeNardo, 2000), and this concept could now be applied to understand the evolution of sexual signals in the context of multiple parasite species.

In nature, hosts are typically infected with a whole community of different parasites (Hellard, Fouchet, Vavre, & Pontier, 2015; Roberts, Janovy, & Schmidt, 2000). Yet, it remains largely an open question whether and how sexual signals are influenced by multiple parasite infections. Accordingly, the need to examine these signals in the context of the parasite community or co-infection has long been suggested (Clayton, 1991; Karvonen & Seehausen, 2012). The examples that we do have where studies have measured multiple parasites suggest that a sexual signal can simultaneously be correlated positively, negatively, or not at all with infection depending on the focal parasite (Doucet & Montgomerie, 2003; Folstad, Hope, Karter, & Skorping, 1994; Martin & Johnsen, 2007; Moore & Wilson, 2002; Weatherhead, Metz, Bennet, & Irwin, 1993). However, in most of these cases, it is unknown why certain parasites are associated with sexual signals and not others, and what the underlying mechanisms are that connect parasites in different ways (i.e. positively and negatively) to the same signal trait. Answering these questions will be important steps forward in our understanding of parasite-mediated sexual selection and how signals evolve in the context of multiple parasite infections.

When we consider the parasite community of a given host population, it is clear that each parasite species is unique in how it interacts with its host. Parasites can differ greatly in their transmission, life cycles, pathology, how they infect hosts (e.g. ectoparasites versus endoparasites), how common and how costly they are, and what host life stage they infect (e.g. adults versus offspring) (Budischak, Jolles, & Ezenwa, 2012; Rynkiewicz, Pedersen, & Fenton, 2015). These factors likely influence which parasites are relevant to females in the context of mate choice. For example, we predict that females may use sexual signals to preferentially gain information about parasites that are common and/or have the greatest fitness costs. Differences in parasite life history may also influence how infections are associated with signal expression. However, our view of this has been somewhat limited as the study of parasite-mediated sexual selection has largely focused on parasites that directly infect adult males (Beltran-Bech & Richard, 2014; Clayton, 1991; Møller, Christe, & Lux, 1999; Roberts et al., 2004). We propose expanding our view to also include parasites that infect other life history stages, such as offspring. Parasites that infect offspring can often have significant impacts on fitness, and gaining information about them can be adaptive for females who are using sexual signals for mate choice (Owen, Nelson, & Clayton, 2010). Given that parasites that infect males directly may be functionally quite different from parasites that infect offspring, it is likely that they are interacting with sexual signals in different ways.

The majority of proposed mechanisms linking sexual signal expression to parasites focus on the physiological consequences of, or trade-offs imposed by, parasite infections. These include parasite resistance, which allows for greater investment in sexual signals (Andersson, 1994; Hamilton & Zuk, 1982; Müller, Heylen, Eens, Rivera-Gutierrez, & Groothuis, 2013), immunosuppression and resource trade-offs as a consequence of investment in sexual trait expression (Folstad & Karter, 1992; Foo, Nakagawa, Rhodes, & Simmons, 2017; Møller & Saino, 1994), or variation in the stress response during sexual trait development (Buchanan, 2000; Leary & Knapp, 2014; Mougeot, Martínez-Padilla, Bortolotti, Webster, &

Piertney, 2010; von Schantz, Bensch, Grahn, Hasselquist, & Wittzell, 1999). These mechanisms work for parasites that infect males directly, thus interacting with male physiology and immunity when sexual traits are developed or expressed. However, for parasites that infect offspring, we must consider alternative mechanisms linking signals and infections. Behaviours such as territoriality and male–male competition, which have been studied widely in the context of sexual selection (e.g. Cowlshaw, 1996; Mathis, 1990; Seddon, Amos, Mulder, & Tobias, 2004; Wolfenbarger, 1999), may also be important for linking sexual signals to parasites that do not infect males directly. For example, if top signalling individuals can acquire and defend territories with fewer parasites, then parasite infections of offspring could be linked with sexual signal expression.

To advance our understanding of how sexual signals are evolving in the context of multiple parasites within the same host, and what mechanisms could be linking multiple parasites to the same signal, we asked the following questions. (1) Is sexual signal expression associated with multiple types of parasites, including those that infect adults and offspring, and what are the directions of those associations? (2) What are the costs of different parasite infections, and are females using sexual signals to gain information specifically about parasites that impact fitness? (3) What mechanisms connect different parasites to the same sexual signal, and how might this influence the evolution of that signal? Overall, our study tests the hypothesis that sexual signals may reflect the unique and variable costs of different types of parasites, both within and between different age classes of individuals (offspring and adults). Evidence to support this hypothesis could include, for example, that a single sexual signal has unique cost-associated relationships with different parasites. If supported, this framework provides a broader scope for understanding the evolution of parasite-mediated sexual signals.

METHODS

Study System

Barn swallows, *Hirundo rustica*, are a small migratory songbird that have played a prominent role in the study of sexual selection (Møller, 1994; Romano, Costanzo, Rubolini, Saino, & Møller, 2017; Safran, Neuman, McGraw, & Lovette, 2005; Scordato & Safran, 2014). Indeed, studies demonstrating female preference for longer tail streamers in the European subspecies (*H. r. rustica*) have even become a textbook example (Møller, 1988, 2001; Møller et al., 1998). However, in the North American subspecies (*H. r. erythrogaster*), experiments have shown that females do not prefer males with longer streamers (Safran et al., 2016); instead, they prefer males with darker redder ventral plumage (Safran et al., 2005, 2016). While associations between tail streamer length and parasites are well established in the European subspecies (Møller, 1990b; Saino & Møller, 1994), whether variation in streamer length or ventral colour signals any information about parasites in the North American subspecies was previously unknown. Given this, we compared the expression of both of these traits in North American barn swallows to a number of different parasite infections in both adults and offspring.

In *H. r. erythrogaster*, males arrive first to the breeding grounds in mid- to late April where they nest in human structures (i.e. barns, bridges). They then establish and defend breeding territories that typically contain one or more previously constructed mud-cup nests (Brown & Brown, 2020; Safran, 2006). Barn swallows prefer to reuse an old nest rather than construct a new one in order to maximize breeding timing and output (Safran, 2006). Females then arrive, choose a mate and use a nest in that male's territory to raise

offspring. Both males and females participate in parental care, and social pairs typically remain together throughout the breeding season and have one to three broods consisting of three to five nestlings each (Brown & Brown, 2020). Barn swallows are short-lived; typically surviving 1–3 years (Brown & Brown, 2020; Costanzo et al., 2017). Both tail streamer length and ventral coloration are known to increase with age in the North American subspecies (Lifjeld et al., 2011). Given this, we examine the relationships between parasites and sexual signals with known-age individuals.

General Field Methods

We surveyed barn swallows during May–September 2012 at 27 breeding colonies or sites (i.e. barns and bridges) in Boulder, Jefferson and Weld counties, Colorado, U.S.A. We attempted to capture all adults at each site using mist nets. We banded each adult with a numbered aluminium band (U.S. Geological Survey) and a unique combination of colour bands, measured morphology (wing length, tail streamer length and mass), collected a blood sample (venal puncture, stored in 1% SDS lysis buffer) and a ventral plumage sample (Hubbard, Jenkins, & Safran, 2015; Jenkins, Vitousek, Hubbard, & Safran, 2014) and quantified parasites (see below). Using behavioural observations, we assigned social pairs to a nest. All nests were monitored every 3 days unless they were near the end of incubation, at which point, nests were checked daily to determine the date that nestlings hatched. Nestlings were then measured and banded when they were 12 days old. As we have consistently studied barn swallows at these sites for several years, we were able to estimate age for most individuals. Barn swallows are highly site-faithful, so birds that were new to a site (unbanded) were assumed to be in their first year of breeding (Safran, 2004). Given the challenges of field work on wild birds, we had missing pieces of information for some of the males included our study; thus, we report sample sizes for each of the results.

Measurement of Parasites

We quantified parasites for adult males ($N = 172$) and their nestlings ($N = 444$ nestlings). We counted ectoparasites on adults using a loupe ($3.5\times$ magnification) and a headlamp at the start of the breeding season (May–June). We systematically searched the body, tail, wings and head of each bird for parasites (Møller, 1991a). We note that lice are often difficult to accurately quantify on birds without using methods that remove them (Clayton & Drown, 2001). We did not want to remove the parasites, as we were interested in the fitness consequences of parasites across the breeding season and thus wanted to leave infections intact. We therefore quantified lice damage to feathers as a proxy for lice infection load (Clayton & Drown, 2001; Moulia, Le Brun, Loubes, Marin, & Renaud, 1995; Price, Hellenthal, Palma, Johnson, & Clayton, 2003). Parasite counts were done by the same person (A. K. Hund).

Molecular techniques were used to detect the presence or absence of blood parasite infections (haemosporidians): *Plasmodium*, *Leucocytozoon* and *Haemoproteus*. DNA was extracted from blood samples using the Qiagen Blood and Tissue Kit (Qiagen Inc., Germantown, MD, U.S.A.). Haemosporidians were detected via nested PCR targeting cytochrome *b* following the protocol of Hellgren, Waldenström, and Bensch (2004). Parasite presence was evaluated by running PCR products on a 2% agarose gel and all positive samples were Sanger-sequenced by Macrogen (Macrogen, Inc., Seoul, South Korea). Because the nested PCR method is very sensitive and parasite DNA occurs at low concentrations in blood samples, low-intensity bands can sometimes occur on gels. To

ensure that these were true signals and not nonspecific host DNA amplification, each sample was tested three times to reduce the number of false negatives. Only samples that showed a clear signal at least two times were scored as positive infections. Haplotypes were assigned to known haemosporidian lineages using the MalAvi database (Bensch, Hellgren, & Pérez-Tris, 2009). Given that this method cannot tell us about infection intensity, we coded haemosporidian data for each individual as present or absent in our analyses. Blood slides were also created for each individual and were screened using a light microscope for other blood parasites outside our molecular analysis (e.g. *Trypanosoma*, microfilariae).

We quantified parasites in nests and on nestlings 12 days posthatching. Nestlings were removed and placed in a clean container. Nests were then visually inspected for large parasites such as fly larvae. We quantified other parasites, such as mites, by placing a cupped hand in the nest for 30 s and counting any parasites that were found on the hand. This method has been used to estimate mite loads in other studies (Møller, 1990; Saino, Ferrari, Romano, Ambrosini, & Møller, 2002) and was found to correlate well with the population of mites recovered from swallow nests using Berlese funnels (Hund, Blair, & Hund, 2015; Møller, 1990). We also quantified parasites on each nestling and in the container used to hold the nestlings (nestlings were transferred to a second container after they were counted). All of these counts were then added such that each nest had a single count for each type of parasite.

We use the following parasitology terminology for reporting results: (1) prevalence, or the proportion of infected hosts in the population; (2) abundance, or the average number of parasites across all hosts (including zero counts); (3) intensity, or the average number of parasites for infected individuals (excluding zero counts).

Measuring Sexual Signal Expression

Ventral plumage samples were collected and stored in envelopes until they could be mounted on cards and analysed in the laboratory using a spectrometer (USB 4000, Ocean Optics, Dunedin, FL, U.S.A.), with a pulsed xenon light (PX-2, Ocean Optics) and Spectrasuite software (v.2.0.151), following (Hubbard et al., 2015; Jenkins et al., 2014; Safran, McGraw, Wilkins, Hubbard, & Marling, 2010). Each measurement was an average of 20 scans, and each feather sample was measured three times and averaged. This process produced three measures of colour: brightness, hue and chroma. Brightness describes the amount of pigment (how light or dark when viewed in greyscale, with lower values indicating darker plumage), while hue describes the ratio of the two pigments underlying melanin coloration: eumelanin and pheomelanin (McGraw, Safran, & Wakamatsu, 2005). Feathers with more eumelanin appear redder (higher hue), while feathers with more pheomelanin appear yellower (lower hue). Chroma is measured as the brightness at the dominant hue and is often closely correlated with the brightness measure, as it was in our data set. Given this, we choose to analyse brightness and hue of the breast (largest and most visible ventral patch) as measures of sexual trait expression. Tail streamer length was measured with a flexible ruler from the base of the feather to the tip. This measurement was taken three separate times on each streamer and averaged. If streamers were broken or growing, they were not measured.

Measures of Male Condition, Immunity, Survival and Reproductive Success

For each male, we calculated body condition as a residual from the regression of mass on wing length (body size measure)

(Labocha & Hayes, 2012; Schulte-Hostedde, Zinner, Millar, & Hickling, 2005) at the time of capture (when parasites were quantified). We also created blood smears in order to quantify immune parameters. We note that blood samples were taken within 1 h of capture (typically <30 min) to ensure that leukocyte profiles were not affected by handling stress (Davis, 2005). Blood smears were air-dried and stored in the dark at room temperature for up to 2 weeks until they were stained at the Colorado State University Veterinary Pathology Lab with a Wright-Giemsa Quick stain. Slides were analysed using a light microscope (1000 × with oil immersion) to quantify a white blood cell differential (100 leukocytes) (Vinkler, Schnitzer, Munclinger, Votýpka, & Albrecht, 2010). From this leukocyte profile, we calculated the heterophil-to-lymphocyte ratio (H/L ratio). This measure has been used widely in wild birds (Davis, Maney, & Maerz, 2008), where a larger H/L ratio is commonly associated with increased stress and mortality (Clark, 2015; Goessling, Kennedy, Mendonça, & Wilson, 2015). There is also evidence that parasite infections and stress can directly influence the H/L ratio (Davis & Maney, 2018; Wojczulanis-Jakubas, Jakubas, Czujkowska, Kulaszewicz, & Kruszewicz, 2012).

We also quantified several measures of reproductive success: onset of breeding, where earlier breeding is associated with increased offspring quality, longevity and survival (Dubiec & Cichoń, 2001; Gurney, Clark, & Slattey, 2012; Saino et al., 2012; Öberg, Pärt, Arlt, Laugen, & Low, 2014); the number of nestlings that fledged; and whether a male carried out a second breeding attempt during that season. To quantify male survival, we returned to breeding sites during the spring and summer of 2013. We attempted to capture all individuals and used observations of banded birds to determine whether males were present or absent. Adult barn swallows generally have high breeding site fidelity from year to year, so presence the following year is considered a good measure of survival (Møller & De Lope, 1999; Safra, 2004; Schaub & von Hirschheydt, 2009; Shields, 1984; Turner, 2006).

Testing for Associations between Parasites and Measures of Sexual Signals, Condition, Immunity, Survival and Reproductive Success

We built mixed effects models with breeding site as a random effect to test for associations between the abundances of different parasites and each of our response variables: sexual signal expression (breast brightness, breast hue, tail streamer length), breeding success (breeding onset, nestlings fledged, second breeding attempt) and measures of male condition (body condition, H/L ratio, survival). The type of mixed model that we constructed was based on the distribution of the response variable (linear mixed models, LMM; general linear mixed models, GLMM). These models included each type of parasite (nest mites, feather mites, lice, *Plasmodium*, bat bugs) and male age as fixed effects, as well as all two-way interactions between parasites (to test for the role of co-infection) and between each type of parasite and age (to test for age-related impacts of infection). For many of our models, we could not include all interactions in the same model because our power was limited. In these cases, we tested co-infection interactions (parasite × parasite) and age interactions (age × parasite) separately. When these two-way interactions were not significant ($P > 0.05$), they were removed from the model in order to more easily interpret the main effects and improve model fit and stability. In all models we found that no interactions were significant; thus, all results are from models with just additive fixed effects (each parasite type and age). All significant associations are reported in the main text and full model results are reported in the Appendix. We also report the conditional R^2 value (R^2_c , associated with the fixed effects plus the random effects) for each model using the 'MuMIn' package (Bartoń, 2020).

In our initial data exploration, we found that there were no significant co-infection patterns between parasites (parasites occurring together within hosts more or less than expected, such that abundances were correlated); thus, we were able to include different parasites together in the same models as fixed effects. We also checked for the independence of all our fixed effects (different parasite types and age) using Spearman rank correlations before running models and information about the correlation of fixed effects in model summaries. Additionally, we centred and scaled all fixed effects to have a mean of zero and a standard deviation of one, which allowed us to directly compare parameter estimates between fixed effects within a model (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). To meet model assumptions and improve model stability, data for feather mites, nest mites, lice and H/L ratio were log-transformed. We checked each model that we constructed for overdispersion, heteroscedasticity and outliers, examined residuals for appropriate fit and confirmed other necessary model assumptions. All analyses were performed with R v.4.0.2 (R Core Team, 2020), the 'nlme' package (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2020), 'lme4' package (Bates, Maechler, Bolker, & Walker, 2015) and the 'MuMIn' package (Bartoń, 2020).

Analysis of Trade-offs between Investment in Sexual Signal Expression and Parasite Defence

We found that nest mites and *Plasmodium* were both associated with breast colour (see Results). We were then interested in what mechanisms connected these parasites to colour expression. To explore this, we first tested whether a greater investment in colour put males at risk for higher costs associated with infections from these two parasites (an interaction effect). To do this, we built models with measures of body condition (LMM), immunity (LMM) and survival (GLMM: binomial) as the response variables and the pairwise interactions between colour expression (brightness and hue) and both nest mites and *Plasmodium* as fixed effects. We also included male age as a fixed effect in these models. These interactions allowed us to test whether attractive males who invested in sexual signal expression faced increased or decreased cost with infection relative to less attractive males. All fixed effects were centred and scaled to have a mean of zero and a standard deviation of one before models were run. Site was included as a random effect in these models.

Nest Mite Manipulation Experiment

As our results indicated that attractive males had fewer mites in their nests (see Results), we experimentally manipulated nest mites to examine the mechanism linking colour expression to this parasite. Because mites are present in nests during spring settlement, we hypothesized that males avoid parasitized nests and that more attractive males are better able to secure and defend mite-free nests. To test this, we conducted an experiment at six breeding sites during March–August 2014.

Nest mites that parasitize the North American barn swallow overwinter in nests. This allowed us to collect mites in late March before swallows had returned from migration by removing nests from sites not used in our experiment. These nests were dissected and live mites were vacuumed into tubes and stored at 1.6 °C in a humidified refrigerator. Before birds arrived at breeding sites (4–6 April), we removed the old lining (feathers) from all nests and removed any partial and broken nests at each of our six sites. Every nest was then disinfected by heating the nest and a 0.5 m radius of substrate where the nest was attached to 120 °C using an industrial heat gun. This method kills all parasites in the nest but allows for reinfection, unlike chemical disinfection methods (Hund, Blair, &

Hund, 2015). At three of these sites (hereafter referred to as 'experimental'), we randomly assigned 50% of these nests to a parasite treatment and added 100 live nest mites ($N = 94$ nests, 44 males). At the remaining three sites (hereafter referred to as 'control'), we did not add mites to any of the nests, leaving them all disinfected at the start of the breeding season ($N = 132$ nests, 76 males). For the rest of the breeding season, breeding biology for all swallows at our experimental and control sites was monitored using the field methods described above. We were able to capture most, but not all, of the males at our experimental and control sites to quantify plumage colour.

Testing for Associations between Settlement Patterns, Parasites and Coloration

Settlement patterns (whether a nest was used for breeding: yes/no) were compared between treatment groups at experimental sites using a GLMM with a binomial distribution. We compared the plumage colour (breast brightness and hue) and clutch initiation dates (analysed as number of days since 1 January) of males who settled in nests of each treatment group at experimental sites using linear mixed effects models. These models also included male age as a fixed effect and site as a random effect.

At control sites, where all nests were disinfected at the start of the breeding season, we built models to test whether male colour predicted whether nests became infected with mites after settlement. We compared colour expression (breast brightness and hue) to the number of mites in a male's nest (new transmission after heat treatment) at the completion of the male's first brood using a GLMM with a Poisson distribution. We also compared the infection levels in nests at control sites to natural 'overwintering' mite populations from our 2012 data set using a GLMM with a Poisson distribution. We included site as a random effect in these models.

Ethical Note

This work followed the ASAB/ABS Guidelines for the treatment of animals in behavioural research. Capturing and handling of birds was done in accordance with the guidelines set by the University of Colorado Institutional Animal Care and Use Committee (IACUC, permit number 1303.02). All procedures in this study were approved by IACUC, and birds did not appear to suffer from measurements, feather or blood collection procedures. Barn swallows are quite robust to handling and to nest disturbances, as they nest in human-made structures where human traffic is common (i.e. barns). Abandonment after nests are checked or nestlings are measured is very rare and did not occur with any of the pairs included in this study. Nest mite manipulations that were performed for the settlement study were targeted to be a low or

moderate infection level relative to natural infections (typically sublethal to nestlings).

RESULTS

Parasite Community Measured on Adult and Nestling Barn Swallows

We identified eight different parasites that infect barn swallow adults ($N = 172$) and nestlings ($N = 444$ nestlings). The prevalence and mean intensity for each parasite are reported in Table 1.

Associations between Parasites and Sexual Signal Expression

Both measures of plumage colour were associated with nest mite abundance. Darker, redder males had nests with fewer nest mites (brightness: LMM: $\beta \pm SE = 5.07 \pm 1.23$, $F = 17.88$, $P = 0.0001$, $R^2c = 0.19$, $N = 162$; hue: LMM: $\beta \pm SE = -23.93 \pm 3.88$, $F = 38.00$, $P < 0.0001$, $R^2c = 0.30$, $N = 162$; Fig. 1a and b), resulting in a negative relationship between sexual signal expression and nest parasites. However, males with darker plumage were also more likely to be infected with *Plasmodium* compared to males that were lighter in colour (brightness: LMM: $\beta \pm SE = -4.55 \pm 1.70$, $F = 7.55$, $P = 0.008$, $R^2c = 0.19$, $N = 162$; Fig. 1c), resulting in a positive relationship between *Plasmodium* and sexual trait expression. Breast hue was not significantly associated with *Plasmodium* infections (hue: LMM: $\beta \pm SE = -0.68 \pm 5.32$, $F = 0.02$, $P = 0.90$, $R^2c = 0.30$, $N = 162$; Fig. 1d). As expected, breast brightness was also associated with age, where older males were darker on average (brightness: LMM: $\beta \pm SE = -1.18 \pm 0.60$, $F = 4.08$, $P = 0.05$, $R^2c = 0.19$, $N = 162$; Appendix, Fig. A1), however, breast hue was not significantly associated with age (hue: LMM: $\beta \pm SE = 3.35 \pm 1.98$, $F = 2.88$, $P = 0.09$, $R^2c = 0.30$, $N = 162$). Breast colour was not significantly associated with any of the other parasites that we measured, demonstrating a neutral relationship between those parasites and sexual trait expression (see Appendix, Tables A1-A2).

Male tail streamer length was not significantly associated with any of the parasites that we measured (see Appendix, Table A3) but was associated with male age, where older males had, on average, longer streamers (LMM: $\beta \pm SE = 1.84 \pm 0.52$, $F = 12.45$, $P < 0.001$, $R^2c = 0.088$, $N = 170$; Appendix, Fig. A2).

Associations between Parasites and Measures of Condition, Survival, Immunity and Reproductive Success

Reproductive performance was significantly negatively associated with two parasites: nest mites and *Plasmodium*. High numbers of nest mites were associated with fewer fledglings (LMM: $\beta \pm SE = -1.56 \pm 0.27$, $F = 32.01$, $P < 0.0001$, $R^2c = 0.23$, $N = 155$) and a decreased likelihood that a male would initiate a second brood,

Table 1

Parasites found on adults and in nests for our study population of North American barn swallows.

Parasite	Infects	Parasite type	Prevalence	Mean intensity (SD)
Feather lice (<i>Acrionirmus gracilis</i>)	Adults	Ectoparasite: Feeds on feathers	72.9%	3.63 (5.2)
Feather mites (<i>Trouessartia</i> sp.)	Adults	Ectoparasite or commensal: Feeds on preen oil, microbes	95.9%	166.5 (203.4)
<i>Plasmodium</i> (DENPET03, LAIRI01)	Adults	Endoparasite: Intracellular, infects red blood cells	15.5%	—
<i>Leucocytozoon</i> (CB1)	Adults	Endoparasite: Intracellular, infects red and white blood cells	1.4% ^a	—
<i>Trypanosoma avium</i>	Adults	Endoparasite: Extracellular, infects blood stream	0.58% ^a	—
Bat bugs (<i>Cimex adjunctus</i>)	Nestlings/Adults	Ectoparasite: Feeds on blood	3.2%	13.8 (23.7)
Nest mites (<i>Ornithonyssus sylvianum</i>)	Nestlings	Ectoparasite: Feeds on blood	58.1%	101.09 (167.95)
Blow flies (<i>Protocalliphora</i> sp.)	Nestlings	Ectoparasite: Feeds on blood	1.2% ^a	4.0 (1.4)

Each parasite is identified to genus and species (or MalAvi lineage for blood parasites) when possible. 'Infects' indicates whether the parasite primarily infects adults or nestlings. Parasite type gives information about parasite life history and how it feeds on or infects its host.

^a Indicates that the parasite was excluded from further analyses because it infected <2% of the population and sample sizes were too small. For haemosporidians, we were unable to measure infection intensity.

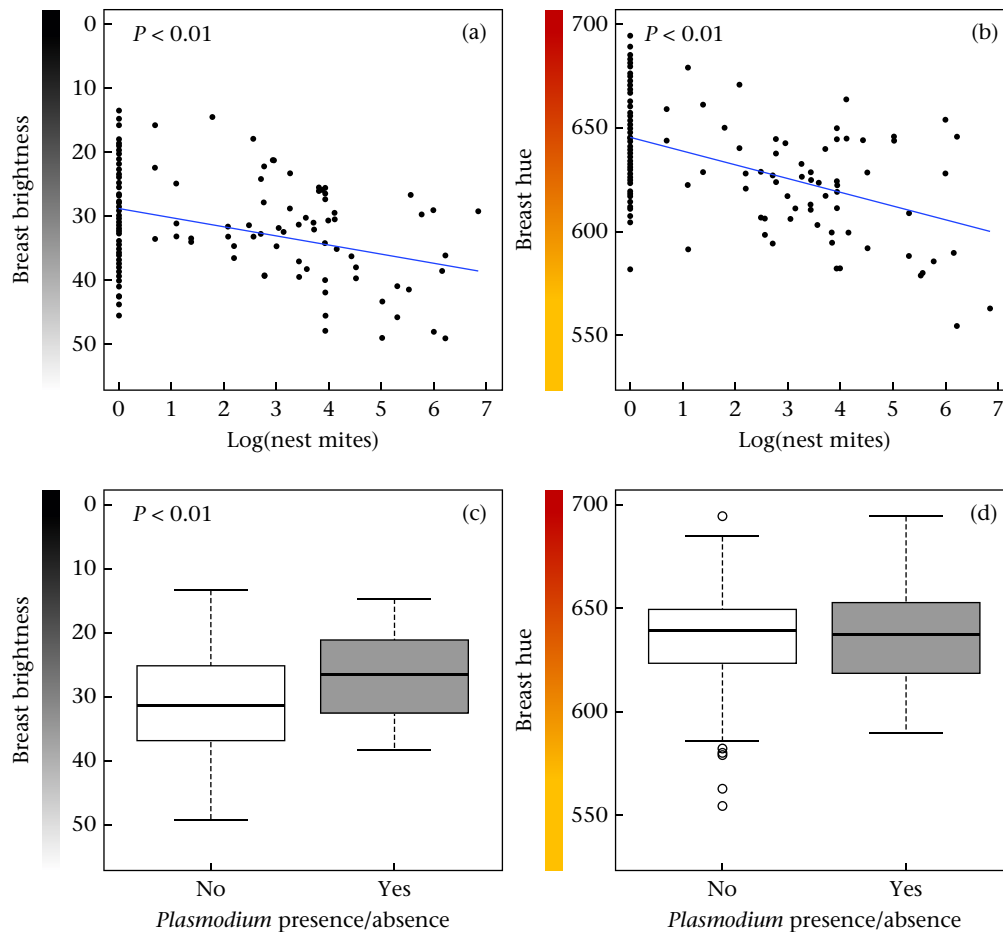


Figure 1. Sexual signal expression (breast colour) and parasite load. Relation between nest mite infestation and (a) breast brightness and (b) breast hue. Relation between *Plasmodium* infection and (c) breast brightness and (d) breast hue. Brightness axes (a, c) are reversed, and colour gradients are added for ease of interpretation. Graphs and trend lines were made from raw data; statistics presented in the text are from mixed effects models with site as a random effect, $N = 162$ adult males. Box plots (c, d) show 25% and 75% quartiles (boxes), medians (lines in the boxes), outermost values within the range of 1.5 times the respective quartiles (whiskers) and outliers (circles).

thus lowering total seasonal reproductive success (GLMM: $\beta \pm SE = -0.77 \pm 0.35$, $F = 6.65$, $P = 0.03$, $R^2c = 0.19$, $N = 170$). *Plasmodium* infections in males were also associated with fewer fledglings (LMM: $\beta \pm SE = -0.84 \pm 0.39$, $F = 4.59$, $P = 0.03$, $R^2c = 0.23$, $N = 155$). *Plasmodium* was not significantly associated with whether males attempted a second brood (GLMM: $\beta \pm SE = -0.13 \pm 0.53$, $F = 0.07$, $P = 0.80$, $R^2c = 0.18$, $N = 170$). H/L ratio was not significantly associated with any of the parasites that we measured, but it was associated with age (LMM: $\beta \pm SE = 0.21 \pm 0.04$, $F = 26.71$, $P < 0.0001$, $R^2c = 0.21$, $N = 131$; Appendix, Fig. A3), where older males tended to have higher H/L ratios. Measures of breeding onset, survival and body condition were not significantly associated with any of the parasites that we measured or with male age (full results reported in the Appendix, Tables A4, A8, A9).

Analysis of Trade-offs between Investment in Sexual Signal Expression and Infection Costs

The interaction between *Plasmodium* infection and breast brightness significantly predicted male survival to the next breeding season ($\beta \pm SE = 2.19 \pm 0.85$, $F = 6.04$, $P = 0.01$, $R^2c = 0.23$, $N = 162$). For males that were uninfected, brightness did not predict survival ($\beta \pm SE = -0.04 \pm 0.023$, $F = 2.16$, $P = 0.10$, $R^2c = 0.07$, $N = 140$; Fig. 2a); however, for males that were infected

with *Plasmodium*, brightness significantly predicted survival ($\beta \pm SE = 0.23 \pm 0.11$, $F = 5.24$, $P = 0.03$, $R^2c = 0.49$, $N = 22$; Fig. 2b), where darker males were less likely to survive relative to lighter males. Survival was not significantly associated with the interaction between hue and *Plasmodium* infection, with male age, or with colour \times nest mite interaction (see Appendix, Table A10). We found no evidence of trade-offs between colour expression and either parasite for body condition or H/L ratio (Appendix, Table A10).

Behavioural Mechanism Linking Sexual Signal Expression to Nest Parasites

Settlement patterns differed significantly between disinfected and parasitized nests at experimental sites, where males were more likely to settle in nests without parasites (nests settled in parasitized treatment = 13 out of 47 (27.6%); nests settled in disinfected treatment = 31 out of 46 (67.4%); odds ratio = 2.65, CI = 1.07, 6.97, $\beta \pm SE = 0.97 \pm 0.47$, $F = 4.35$, $P = 0.039$, $N = 93$). Males who settled in disinfected nests had significantly darker, redder breast plumage compared to those who settled in parasitized nests (brightness: $\beta \pm SE = -6.27 \pm 2.41$, $F = 6.80$, $P = 0.02$, $R^2c = 0.21$, $N = 35$; disinfected: LSM = 25.35 \pm 1.41; parasitized: LSM = 31.64 \pm 1.95; Fig. 3a; hue (treatment): $\beta \pm SE = 22.45 \pm 9.19$, $F = 5.97$, $P = 0.02$, $R^2c = 0.18$, $N = 35$; disinfected: LSM = 639.60 \pm 5.51; parasitized: LSM = 617.22 \pm 7.39; Fig. 3b). In

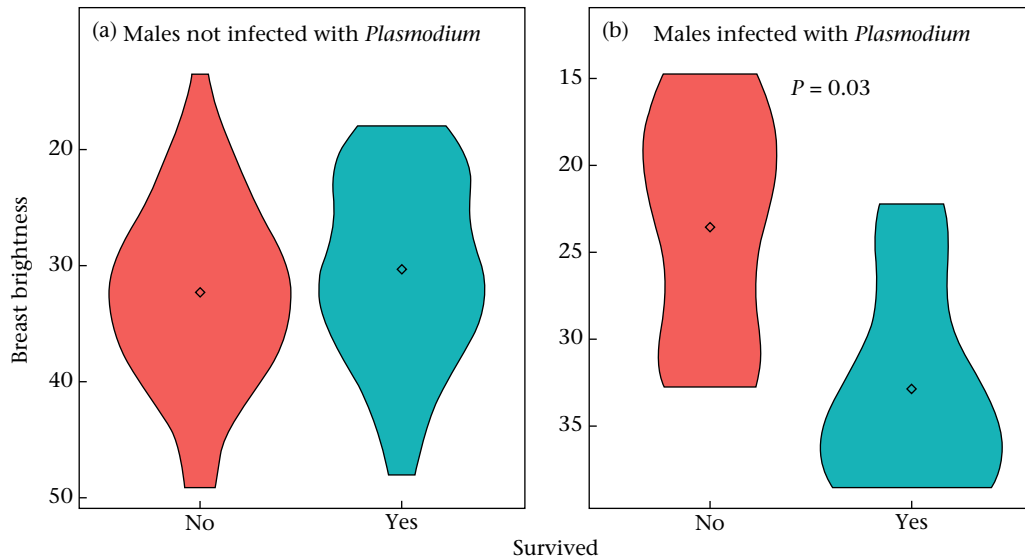


Figure 2. Violin plots illustrating the relationship between breast brightness and survival for (a) males ($N = 140$) not infected with *Plasmodium* and (b) males ($N = 22$) infected with *Plasmodium*. Diamonds indicate the mean of each group. Brightness axes are flipped for ease of interpretation (darker feathers have lower brightness). Graphs were created with raw data; statistics presented in the text are from a mixed effects model with site as a random effect.

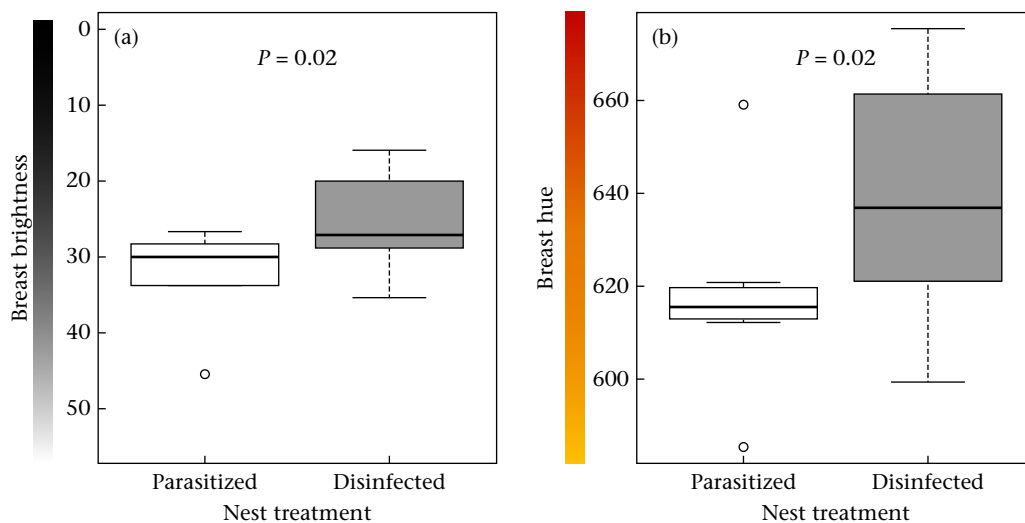


Figure 3. Ventral plumage colour measures for males who settled in parasitized and disinfested nests at the start of the breeding season for experimental sites: (a) breast brightness values for males that settled in parasitized territories versus disinfested territories; (b) breast hue values for males that settled in parasitized territories versus disinfested territories. Colour gradients are added for ease of interpretation. Graphs were created with raw data; statistics presented in the text are from mixed models with site as a random effect, $N = 35$ males. Box plots show 25% and 75% quartiles (boxes), medians (lines in the boxes), outermost values within the range of 1.5 times the respective quartiles (whiskers) and outliers (circles).

this data set, male age was not significantly associated with either measure of colour (see Appendix, Tables A11–A12). Clutch initiation date (onset of breeding) did not differ between treatment groups and was not predicted by male age (see Appendix, Tables A13). These results indicate that males avoided settling in nests infected with nest mites and that males with lighter plumage colour were more likely to settle in infected nests compared to attractive males.

At control sites, where all nests started as disinfested, 32.08% of the nests that were occupied ($N = 76$) later became infected with nest mites over the course of the breeding season. However, new nest mite infestations at control sites were significantly lower compared to sites with naturally overwintering mite infestations (control sites: average \pm SE abundance = 7.97 ± 3.05 mites; average \pm SE intensity = 18.05 ± 6.27 ; natural overwintering infestations:

44.2% of nests infected; average abundance = 40.32 ± 8.87 mites; average intensity = 162.09 ± 20.22 ; comparison: GLMM: $\beta \pm$ SE = 1.73 ± 0.06 , $F = 879.35$, $N = 216$, $P < 0.0001$). Male ventral colour at control sites did not predict the likelihood of a nest becoming newly infected with nest mites during the breeding season (brightness: GLMM: $\beta \pm$ SE = -0.11 ± 0.11 , $F = 1.05$, $N = 54$, $P = 0.31$; hue: GLMM: $\beta \pm$ SE = -0.50 ± 0.82 , $F = 0.82$, $N = 54$, $P = 0.37$). Thus, while dark-coloured males selected and defended parasite-free nests during spring settlement, male plumage colour did not predict whether a nest would later become infested with nest mites (new transmission). However, dark males that can secure a nest without mites at the start of the breeding season face significantly lower parasite loads compared to males that start the breeding season with mites in their nests.

DISCUSSION

Given that, in nature, hosts are infected by a diversity of different parasites, we set out to test whether multiple parasites could be associated with the same sexual signal. We asked the following questions. (1) Is sexual signal expression associated with multiple parasites, including those that infect adults and offspring, and what are the directions of those associations? (2) What are the costs of different parasites, and are females using sexual signals to gain information specifically about parasites that impact fitness? (3) What mechanisms connect different parasites to the same sexual signal and how might this influence the evolution of that signal?

We found support for the hypothesis that a sexual signal can reflect the unique and variable costs of different types of parasites for both adults and offspring, and that unique mechanisms can connect these parasites to signal expression. Specifically, we found that sexually selected colour was associated with two different parasites, nest mites and *Plasmodium*. When we examined the direction of these associations, we were able to recover all three patterns found in past studies (positive, negative, neutral associations) within one host population: attractive males had fewer nest mites and were more likely to be infected with *Plasmodium*, and there were no associations between colour and the other parasites that we measured. Both nest mites and *Plasmodium* had significant impacts on reproductive success relative to the other parasites that we quantified, suggesting that females could be using sexual signals to preferentially gain information about parasites that are costly. Furthermore, we found evidence for different mechanisms underlying these associations. By examining trade-offs, we found that males infected with *Plasmodium* who also invested in colour expression were less likely to survive to the following breeding season. However, for nest mites, which primarily infect offspring, we identified a behavioural mechanism where attractive males were able to obtain and defend higher-quality nesting territories with fewer mites. Our study suggests that sexual selection within one host population can be shaped by the balanced effects of multiple parasites that interact with the same sexual signal in unique ways.

It is well established in barn swallows that both ventral coloration and tail streamers tend to increase with age (Bradley, Hubbard, Jenkins, & Safran, 2014; Lifjeld et al., 2011; Møller, 1991b; Møller & De Lope, 1999; Turner, 2006), and we were able to replicate these findings in our study population. While age was a significant predictor in our model for breast brightness, nest mites and *Plasmodium* both had a larger effect (age: -1.18; nest mites: 5.07; *Plasmodium*: -4.55), suggesting that colour variation within and across age classes was associated with parasites. Our other measure of colour, hue, was associated with nest mites but not with *Plasmodium* or age. This suggests that different components of signal expression could be shaped by age and parasites in unique ways. As our study was conducted in only 1 year, it takes a population level view of the relationship between age and signal expression and does not account for how individuals change as they age. It would be revealing for future studies to track individuals and parasites over several years to better understand how signal expression is associated with different parasites through time and further partition age-related effects. Barn swallows are also relatively short-lived birds with age-independent mortality rates (Costanzo et al., 2017), so it would also be interesting to see how age in longer-lived species may influence relationships between sexual signals and multiple parasites.

In addition to changes in signal expression with age, we also detected a change in our immune measure. Older males tended to have higher H/L ratios – indicating a shift towards a more

corticosterone-mediated response (Davis et al., 2008). This could be a mark of increased stress experienced by older birds, or immunosenescence (Haussmann et al., 2005; Lavoie, 2005). In general, immune system function tends to peak in early adulthood and then wane or shift in character (often becoming more inflammatory) towards the end of life (Peters, Delhey, Nakagawa, Aulsebrook, & Verhulst, 2019). However, we failed to find associations between this particular immune measure and the parasites we measured. In the future, it would be informative to use other methods to quantify immune capacity, or even experimental infections paired with immune measures, that may tell us more about how birds are responding to infection and how this may be influenced by investment in sexual signal expression and with age.

We acknowledge that our study is limited to only one population of barn swallows in 1 year, is largely based on correlations, is limited by small sample sizes for some comparisons and that we were only able to measure a subset of the full parasite community. Including more types of parasites (i.e. bacteria, viruses, gut parasites) could add further nuance into how infections are shaping sexual selection in this system. More work is also needed in other systems to understand how common it is for multiple parasites to be associated with the same sexual signal and whether these associations are typically in the same or opposite directions. However, the importance of multiple parasite infections has gained recent and widespread attention in disease ecology and ecoimmunology. It is increasingly recognized that different species of parasites compete within hosts for limited resources (Budischak et al., 2015; Mideo, 2009) and can shift the optimal defence strategies of hosts who face trade-offs when combatting multiple infections (Bordes & Morand, 2011; Sofonea, Alizon, & Michalakakis, 2017). While mechanisms underlying the interactions between hosts and multiple parasites have been worked out in some model systems (e.g. Budischak et al., 2018; Reynolds, Finlay, & Maizels, 2015), our understanding of how multiple parasites interact and influence host evolution for most systems is still in its infancy (Hellard et al., 2015). Our results indicate that these interactions may play an important, and underappreciated, role in shaping host sexual selection.

The two parasites that were associated with colour expression in our system, nest mites and *Plasmodium*, are quite different from each other. Nest mites are blood-feeding ectoparasites that primarily feed on nestlings in *H. r. erythrogaster* (Hund, Aberle, & Safran, 2015) and are found in 58.1% of nests. *Plasmodium*, on the other hand, is an intercellular blood parasite that is transmitted by mosquitos and infects 15.5% of adults in our population but does not appear to infect nestlings before they fledge (Hund, 2012). We found that both of these parasites influenced reproductive success, but that nest mites may be more important in this regard as they had a more substantial impact on the number of fledglings (coefficient value of -1.56 versus -0.85), are more common in the population and reduced the likelihood that a male had a second breeding attempt, which *Plasmodium* did not.

Given the differences between nest mites and *Plasmodium*, it follows that these parasites can represent different selective pressures shaping the evolution of plumage colour in barn swallows. Females who choose dark males benefit by gaining access to territories with fewer nest mites, which are relatively common and have an important impact on reproductive success. This benefit may outweigh the risk that dark males are also more likely to be infected with *Plasmodium*, which is less common and has a smaller effect on reproductive success. However, males who invest in sexual trait expression may face increased susceptibility to *Plasmodium*, as we found that dark males were more likely to be infected, and when infected, had reduced annual survival. This suggests that the costs associated with *Plasmodium* may be more important for

males than they are for females. Thus, parasite-mediated sexual selection in this system may lie at the intersection between benefits to females, who are choosing males based on colour, and costs to males who face trade-offs with parasites and investment in sexual signal expression. More experimental work in this system that manipulates parasites and measures female preferences is needed to better test these predictions. However, the idea that both sexual selection, driven by female preferences and male–male competition, and natural selection, driven by costs associated with investment in sexual traits, interact to shape the evolution of sexual traits has been discussed broadly in the literature (Hunt et al., 2009; Safran et al., 2013; Sinervo et al., 2000; Zahavi, 1975). An expanded view of how costs are measured across multiple parasite infections within hosts may reveal that the patterns detected here are relatively common.

To further understand the evolution of breast colour in our system, it is important to consider the underlying mechanisms that generate the different associations between signal expression and multiple parasites. *Plasmodium* seems to fit well into established hypotheses that predict a trade-off between sexual signal investment and parasite defence, such as the immunocompetence handicap hypothesis (ICCH) (Folstad & Karter, 1992; Mougeot, Redpath, Piertney, & Hudson, 2005; Sheldon & Verhulst, 1996). We found that infected males who invested in plumage colour had lower survival, and evidence from other studies suggests that *Plasmodium* infections interact with immune function and physiology (Asghar, Hasselquist, & Bensch, 2011; Valkiunas, 2004). Previous studies in our system have also demonstrated that darker males have higher levels of circulating testosterone (Eikenaar, Whitham, Komdeur, van der Velde, & Moore, 2011; Safran, Adelman, McGraw, & Hau, 2008), following predictions of the ICCH, where testosterone increases sexual trait expression but suppresses the immune system (Folstad & Karter, 1992). From our study, it is not clear what is generating variation in *Plasmodium* infections and the subsequent mortality associated with male colour. There are several possibilities: darker males could differ in their exposure to vectors carrying *Plasmodium*, they could be more susceptible to infection, or they could be less able to clear *Plasmodium* once exposed and thus end up with chronic infections. Both acute and chronic infections combined with the stress of migration could lead to increased mortality (Asghar et al., 2011; Emmenegger et al., 2018; Hegemann et al., 2018). Manipulative experiments with *Plasmodium* in this system would help to further clarify this relationship and hopefully will be the focus of future research.

For nest mites, however, we found little evidence for physiological mechanisms or trade-offs within adult males. Instead, our mite manipulation experiment supported a behavioural mechanism linking sexual signal expression to this parasite. We found that darker males secured and defended territories without nest mites, while lighter males settled in territories with mites, although we caution that the sample size for this study was relatively small. There is a clear benefit for males to obtain parasite-free nests: infection status of a nest at the start of the breeding season predicts whether nestlings will later be exposed to large nest mite infestations during development (McCulloch & Owen, 2012; Møller, Allander, & Dufva, 1990). This is relevant as costs associated with ectoparasites are typically intensity dependent (Roberts et al., 2000). In alignment with previous studies that show that darker males have higher levels of circulating testosterone at the start of the breeding season (Eikenaar et al., 2011; Safran et al., 2008) and are more interactive in social networks (Levin et al., 2018), we infer that male competition is the likely mechanism linking colour expression to nest mites. It is possible that darker males arrive earlier on the breeding grounds and are thus able to first occupy

higher-quality territories, but we found no difference in the timing of breeding between treatment groups or between light and dark males. It is also not clear from our experiment exactly how males are detecting whether nests are infested with mites, and more experimental work is required to tease apart what specific cues they may be using. Male competition has been linked to sexual trait expression and territory quality in other avian studies (e.g. Seddon et al., 2004; Wolfenbarger, 1999) and across a diversity of taxa (e.g. Cowlshaw, 1998; Mathis, 2006), although studies of parasites in territories are relatively rare. Behaviour may, therefore, be a widespread but overlooked mechanism underlying the associations between sexual traits and parasites, particularly for parasites that do not directly infect adult males.

Studies in the European subspecies (*H. r. rustica*) have also found associations between tail streamers and a similar species of blood-feeding mite (Møller, 1990, 1994; Saino & Møller, 1994). In this case, it is suggested that physiological mechanisms, including heritable resistance to mites and immunocompetence, are driving the association between streamer length and mite loads in *H. r. rustica* (Møller & Erritzøe, 1996; Møller & Saino, 1994; Saino et al., 2002; Saino & Møller, 1995), although it would be interesting to test whether there is also a behavioural component to this association. We found no significant associations between any parasites that we measured and tail streamer length in the North American subspecies, suggesting that links between parasites and divergent signal traits across these relatively young subspecies may be quite flexible (Hund et al., 2020).

Conclusion

Our results support the need to examine the information content and evolution of sexual traits in the context of multiple parasite infections. Within one population, we found that sexually selected colour was associated with multiple parasites and that the direction of those associations was parasite specific. The parasites associated with colour also had a significant impact on reproductive success relative to the other parasites we measured. Finally, we found evidence for different underlying mechanisms connecting parasites to sexual trait expression, one physiological and one behavioural, that were acting simultaneously to shape the evolution of sexual selection within one host population. An analysis of the variable ways that different parasite infections interact with their hosts not only expands our view of how parasite-mediated evolution shapes variation in sexual signals within a population, but also may be fruitful for understanding the divergence of sexual signals among closely related taxa.

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Author Contributions

A.K.H. and R.J.S. came up with the conceptual design and secured funding required for field and laboratory work, A.K.H. and J.K.H. performed the field and laboratory work, and S.K. and P.M. performed and funded molecular work for blood parasite detection.

A.K.H. performed all analyses and wrote the manuscript, and R.J.S provided feedback and mentorship. All authors provided feedback on the manuscript.

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Appendix

Table A1

Model results for breast brightness, including each parasite type and male age as fixed effects and breeding site as a random effect

Term	Coefficient	SE	F	P
Nest mites	5.07	1.23	17.88	0.0001
Feather mites	-0.75	0.79	0.94	0.34
<i>Plasmodium</i>	-4.55	1.69	7.55	0.008
Lice	-0.76	0.94	0.69	0.42
Bat bugs	-0.09	0.56	0.03	0.87
Age	-1.18	0.60	4.08	0.05

No two-way interactions were significant for this model, so they were removed to make it easier to interpret the main effects and improve model fit and stability. The conditional R^2 for this model was 0.19, $N = 162$, $df = 128$.

Table A2

Model results for breast hue, including each parasite type and male age as fixed effects and breeding site as a random effect

Term	Coefficient	SE	F	P
Nest mites	5.07	1.23	17.88	0.0001
Feather mites	-0.75	0.79	0.94	0.34
<i>Plasmodium</i>	-4.55	1.69	7.55	0.008
Lice	-0.76	0.94	0.69	0.42
Bat bugs	-0.09	0.56	0.03	0.87
Age	-1.18	0.60	4.08	0.05

No pairwise interactions were significant for this model, so they were removed to make it easier to interpret the main effects and improve model fit and stability. The conditional R^2 for this model was 0.19, $N = 162$, $df = 128$.

Table A3

Model results for tail streamer length, including each parasite type and male age as fixed effects and breeding site as a random effect

Term	Coefficient	SE	F	P
Nest mites	-0.73	1.06	0.47	0.49
Feather mites	-0.37	0.68	0.30	0.58
<i>Plasmodium</i>	-0.79	1.49	0.28	0.60
Lice	-0.67	0.79	0.71	0.40
Bat bugs	0.38	0.50	0.57	0.45
Age	1.84	0.52	12.45	0.0006

No pairwise interactions were significant for this model, so they were removed to make it easier to interpret the main effects and improve model fit and stability. The conditional R^2 for this model was 0.09, $N = 170$, $df = 135$.

Table A4

Model results for clutch initiation date (days since 1 January), including each parasite type and male age as fixed effects and breeding site as a random effect

Term	Coefficient	SE	F	P
Nest mites	3.13	2.58	1.47	0.23
Feather mites	0.76	1.71	0.20	0.66
<i>Plasmodium</i>	2.14	3.56	0.36	0.55
Lice	-0.45	1.89	0.06	0.81
Bat bugs	0.09	1.17	0.01	0.94
Age	-0.98	1.37	0.51	0.48

No pairwise interactions were significant for this model, so they were removed to make it easier to interpret the main effects and improve model fit and stability. The conditional R^2 for this model was 0.30, $N = 162$, $df = 127$.

Table A5

Model results for nestlings fledged, including each parasite type and male age as fixed effects and breeding site as a random effect

Term	Coefficient	SE	F	P
Nest mites	-1.56	0.27	32.12	<0.0001
Feather mites	-0.08	0.19	0.17	0.68
<i>Plasmodium</i>	-0.84	0.39	4.59	0.03
Lice	-0.12	0.21	0.35	0.55
Bat bugs	0.06	0.13	0.23	0.63
Age	0.11	0.15	0.54	0.46

No pairwise interactions were significant for this model, so they were removed to make it easier to interpret the main effects and improve model fit and stability. The conditional R^2 for this model was 0.23, $N = 155$, $df = 120$.

Table A6

Model results for second breeding attempt, including each parasite type and male age as fixed effects and breeding site as a random effect

Term	Coefficient	SE	F	P
Nest mites	-0.77	0.35	6.65	0.03
Feather mites	0.25	0.25	0.70	0.32
<i>Plasmodium</i>	-0.13	0.53	0.07	0.80
Lice	0.15	0.29	0.31	0.61
Bat bugs	0.33	0.91	0.12	0.71
Age	0.12	0.20	0.39	0.53

No pairwise interactions were significant for this model, so they were removed to make it easier to interpret the main effects and improve model fit and stability. The conditional R^2 for this model was 0.19, $N = 170$, $df = 165$.

Table A7

Model results for heterophil–lymphocyte ratio (H/L ratio), including each parasite type and male age as fixed effects and breeding site as a random effect

Term	Coefficient	SE	F	P
Nest mites	-0.08	0.10	0.54	0.47
Feather mites	-0.01	0.05	0.08	0.78
<i>Plasmodium</i>	0.20	0.12	2.79	0.10
Lice	-0.01	0.06	0.05	0.82
Bat bugs	-0.01	0.04	0.15	0.70
Age	0.21	0.04	26.71	<0.0001

No pairwise interactions were significant for this model, so they were removed to make it easier to interpret the main effects and improve model fit and stability. The conditional R^2 for this model was 0.21, $N = 131$, $df = 98$.

Table A8

Model results for survival, including each parasite type and male age as fixed effects and breeding site as a random effect

Term	Coefficient	SE	F	P
Nest mites	-0.002	0.34	0.04	0.99
Feather mites	-0.02	0.24	0.05	0.95
<i>Plasmodium</i>	-0.19	0.49	0.10	0.69
Lice	-0.16	0.26	0.45	0.55
Bat bugs	0.33	0.56	0.37	0.55
Age	-0.18	0.18	1.07	0.30

No pairwise interactions were significant for this model, so they were removed to make it easier to interpret the main effects and improve model fit and stability. The conditional R^2 for this model was 0.09, $N = 172$, $df = 165$.

Table A9

Model results for body condition, including each parasite type and male age as fixed effects and breeding site as a random effect

Term	Coefficient	SE	F	P
Nest mites	-0.28	0.21	1.83	0.18
Feather mites	-0.08	0.14	0.38	0.53
<i>Plasmodium</i>	-0.25	0.29	0.72	0.40
Lice	0.23	0.15	2.38	0.13
Bat bugs	-0.08	0.10	0.57	0.45
Age	-0.002	0.10	<0.001	0.99

No pairwise interactions were significant for this model, so they were removed to make it easier to interpret the main effects and improve model fit and stability. The conditional R^2 for this model was 0.07, $N = 170$, $df = 135$.

Table A10

Results from general linear mixed models for three measures of cost to males (survival, body condition, and heterophil–lymphocyte ratio (H/L ratio)), and the interaction between colour expression (brightness and hue) and two parasites, nest mites and *Plasmodium*

Term	Coefficient	SE	F	P
Survival ($R^2_c = 0.23$, $N = 162$, $df = 151$)				
Brightness: <i>Plasmodium</i>	2.19	0.85	6.04	0.01
Hue: <i>Plasmodium</i>	-0.26	0.76	0.15	0.73
Brightness: Nest mites	-0.58	0.44	0.48	0.19
Hue: Nest mites	0.51	0.35	2.10	0.15
Age	-0.23	0.19	1.45	0.23
Body condition ($R^2_c = 0.07$, $N = 159$, $df = 122$)				
Brightness: <i>Plasmodium</i>	0.004	0.31	<0.001	0.99
Hue: <i>Plasmodium</i>	0.06	0.30	0.05	0.83
Brightness: Nest mites	0.05	0.21	0.06	0.80
Hue: Nest mites	0.07	0.16	0.19	0.66
Age	-0.01	0.10	0.005	0.94
H/L ratio ($R^2_c = 0.22$, $N = 121$, $df = 87$)				
Brightness: <i>Plasmodium</i>	-0.13	0.29	0.19	0.67
Hue: <i>Plasmodium</i>	-0.25	0.31	0.64	0.43
Brightness: Nest mites	-0.06	0.21	0.08	0.77
Hue: Nest mites	0.19	0.25	0.57	0.45
Age	0.44	0.10	20.50	<0.001

Age was also included as a fixed effect and breeding site as a random effect in these models.

Table A11

Model results for breast brightness and whether males settled in parasitized or disinfected nests at experimental sites

Term	Coefficient	SE	F	P
Treatment (disinfected)	-6.29	2.41	6.80	0.02
Age	-1.02	1.09	0.86	0.36

This model also included male age as a fixed effect and breeding site as a random effect. The conditional R^2 for this model was 0.21, $N = 35$, $df = 28$.

Table A12

Model results for breast hue and whether males settled in parasitized or disinfected nests at experimental sites

Term	Coefficient	SE	F	P
Treatment (disinfected)	22.44	9.19	5.97	0.02
Age	0.05	4.17	<0.001	0.99

This model also included male age as a fixed effect and breeding site as a random effect. The conditional R^2 for this model was 0.18, $N = 35$, $df = 28$.

Table A13

Model results for clutch initiation date and whether males settled in parasitized or disinfested nests at experimental sites

Term	Coefficient	SE	F	P
Treatment (disinfested)	5.58	3.14	3.16	0.09
Age	2.16	1.42	2.29	0.14

This model also included male age as a fixed effect and breeding site as a random effect. The conditional R^2 for this model was 0.16, $N = 35$, $df = 28$.

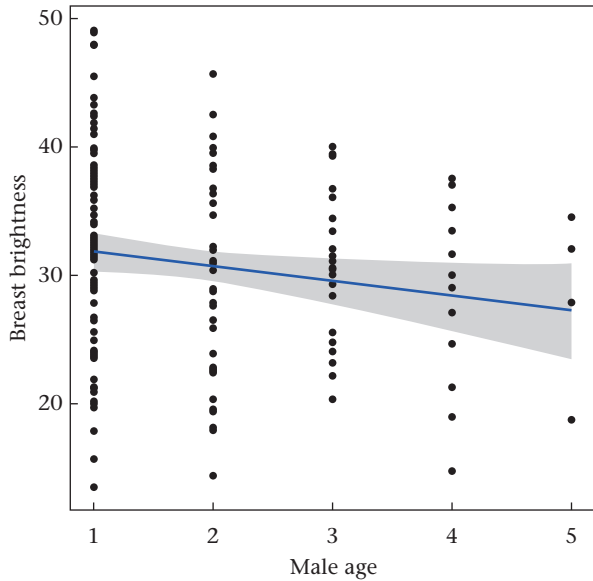


Figure A1. Darkness of ventral plumage (darker feathers have lower brightness) and age for breeding males in our study population. Graph was created with raw data; statistics presented in the text are from mixed models with site as a random effect, $N = 162$ males.

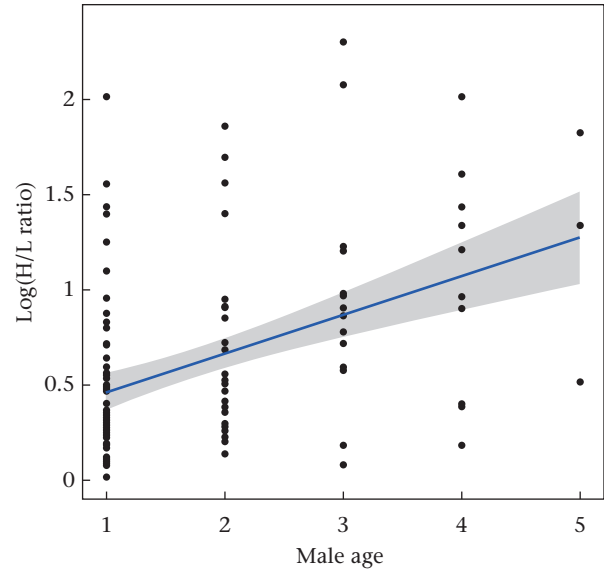


Figure A3. H/L ratio (heterophil/lymphocyte, immune measure) and age for breeding males in our study population. Graph was created with raw data; statistics presented in the text are from mixed models with site as a random effect, $N = 131$ males.

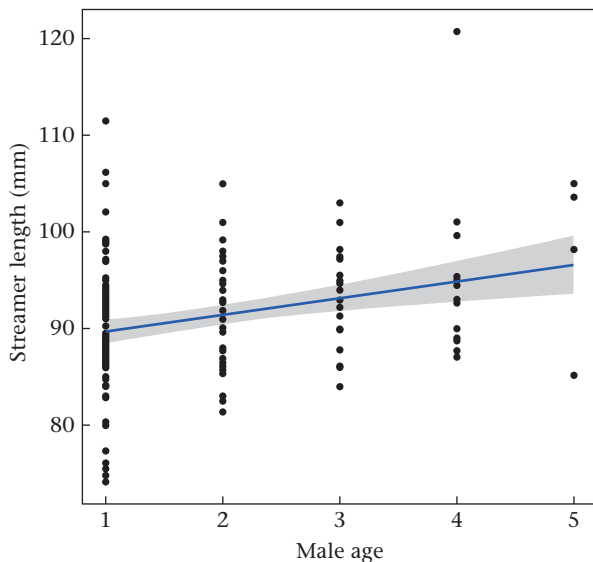


Figure A2. Tail streamer length and age for breeding males in our study population. Graph was created with raw data; statistics presented in the text are from mixed models with site as a random effect, $N = 170$ males.