Shield colours of the Moorhen are differently related to bacterial presence and health parameters

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Parasites have the potential to be important forces driving the evolution of honest signals (e.g. visual communication in the context of sexual selection) because they can affect the expression of colours in the host. Birds are affected by a variety of parasite taxa, but despite their acknowledged importance in epidemics, the role of bacteria in sexual selection in birds has been poorly investigated. In this study, we examined the expression of colours (red and yellow) in the frontal shield of Moorhens and compared the results with the cloacal bacterial presence. We also measured several health parameters (body condition, PHA immune response, heterophils/lymphocytes ratio, haematocrit) that could be related to the expression of frontal shield colour. We found a negative correlation between frontal shield redness and bacterial presence, but red colouration was not related to any other health index. In contrast, the colouration of the yellow portion of the bill was positively related to health (haematocrit and H/L ratio) and immune (PHA) parameters, but not to bacterial presence. These correlational data are consistent with the recent hypothesis that different kinds of colouration within an individual may provide different units of information. In the Moorhen, the frontal shields could act as a twofold honest signal of health status, where red reflects cloacal bacterial presence and yellow is related to blood parameters.

KEY WORDS: bacteria, colour expression, honest signal, immune system, Moorhen, parasites.

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INTRODUCTION

The evolution of conspicuous signals is viewed as a way in which individuals can assess each other's quality. Visual signals, and particularly colours, have received the greatest attention as reliable signals of health and condition. In this light, it is assumed that as signals are costly, colours honestly indicate the bearer's quality (ZAHAVI & ZAHAVI 1997). Carotenoids, the substances responsible for most non-structural colours (from yellow to red), must be obtained through foraging and cannot be synthesized via metabolism. The effect of carotenoids on colouration depends on many factors, in addition to their environmental availability and individual foraging abilities (ENDLER 1980): for example, the redness of a bird's plumage may be influenced by the previous nutritional state (HILL 1995) or by its physiological condition, especially the immune status (LOZANO 1994, BLOUNT et al. 2003, FAIVRE et al. 2003) and parasite load (THOMPSON et al. 1997). Moreover, carotenoids have themselves a direct boosting activity on the immune system (CHEW 1993).

Several studies have shown a relationship between the parasite load and the colour of feathers, bill or comb (ZUK & JOHNSEN 1998, MCGRAW & HILL 2000, HATCHWELL et al. 2001). However, even if the prediction of HAMILTON & ZUK (1982) concerning the role of parasites as important forces driving the evolution of sexual ornament is now widely accepted (CLAYTON & MOORE 1997, MØLLER et al. 1999), when avian biologists have searched directly for parasites, they have looked mostly for macroparasites (BUCKHOLZ 1995, DUFVA & ALLANDER 1995, POTTI & MERINO 1995, MERILA et al. 1999, HÕRAK et al. 2001). In studying the role of parasites in their hosts' sexual selection, few authors have considered the effect of other parasites such as viruses, fungi (LINDSTRÖM & LUNDSTRÖM 2000, POIANI & WILKS 2000) or pathogenic bacteria (NUTTALL 1997, NOLAN et al. 1998, POIANI & GWODZ 2002).

The study of the interactions between a bird's colouration, health state and parasites is also complicated by the fact that (a) various stressors (not only parasites) or different parasite taxa can affect colours differently (FITZE & RICHNER 2002), (b) bacterial infections are controlled mainly by innate immunity, while acquired immunity is crucial to control mainly viruses and ectoparasites (ROITT et al. 1998, NORRIS & EVANS 2000), (c) in several bird species there are different colours and it is conceivable that each one could provide different information to interacting individuals (BADYAEV & HILL 2000, SENAR et al. 2002).

The object of our study was the Moorhen (*Gallinula chloropus*), a species with a conspicuous frontal shield that represents an important element of intraspecific communication (PETRIE 1988). We examined two colours (red and yellow) in the frontal shield and compared their expression with the cloacal bacterial presence. We also measured other health (PHA immune response, heterophils/lymphocytes ratio, haematocrit) and nutritional descriptors (body condition), likely to be related to colour expression. The aim of the study was to assess if the expression of the two frontal shield colours is consistent with the hypothesis that different ornamental traits in an individual provide different units of information (BADYAEV et al. 2001).

METHODS

The study was conducted at Racconigi in Piedmont, NW Italy (44°48'N, 7°40'E; FENOGLIO et al. 2002a). Twenty-seven resident adult Moorhen were captured by funnel traps in

January and February of 2001. For each individual, we measured the structural size, bill frontal shield colours, mass, cloacal bacterial presence and some haematic indices.

Structural size and colour indices

After measuring the body mass (to 0.1 g accuracy), three standard biometrical measures were taken: wing and tail lengths (to the nearest 0.5 mm), tarsus length (to the nearest 0.1 mm). Since the wing, tail and tarsus lengths were highly correlated to each other, they were compacted in an index of structural size calculated as the first factor from a Principal Components Analysis (PCA). Factor 1 explained 64.0% of the variance of the original variables and was highly correlated to them (loadings: wing = 0.86, tarsus = 0.83, tail = 0.70). We measured the colours of the yellow and red portions of the shield using a portable X-Rite spectrodensitometer. Colours were acquired in a continuous scale using the L*a*b CIE method (L = Luminance and two chromatic components, a = green to magenta, and b = blue to yellow) (FIGUEROLA et al. 1999). Because hue, saturation and brightness have normally been the parameters utilized when studying the relationship between colouration and other biological variables (ANDERSSON 2000, SENAR et al. 2002), the L*a*b values were directly converted to the HSB (H = Hue, S = Saturation and B = Brightness) system for statistical analyses.

Haematic assays

Blood was drawn from the brachial vein into two 75 mm heparinized capillaries. The first capillary was placed in a vertical position in a refrigerator for four hours at 4 °C, after which the erythrosedimentation rate (ES rate) was measured as the ratio between the length of the capillary tube with blood cells and the total length (SAINO & MØLLER 1996). The second capillary was centrifuged for 10 min at 4000 rpm to assess haematocrit. We also collected blood smears: they were air-dried, fixed with absolute ethanol and stained with the May-Grünwald Giemsa method. Smears were scanned at 630 × magnification until 100 white blood cells were counted. Heterophils, eosinophils, basophils, lymphocytes and monocytes were differentiated and the erythrocytes were counted. We then calculated the relative frequency of the leukocytes of each family with respect to the total population of leukocytes (relative counts) and the number of leukocytes of the different families per 10,000 red blood cells (absolute counts). This method has been shown to give significantly repeatable relative and absolute leukocyte counts (SAINO et al. 1995). Absolute values were log10 transformed for statistical analysis.

Immunocompetence was measured by means of the T-lymphocyte response (LOCHMILLER et al. 1993). We injected 0.25 mg of PHA (Sigma L-8754) diluted in 0.05 ml PBS into the right wing web area. Subcutaneous injection with PHA produces a local inflammation, and its relative thickness (wing-web index) is directly related to immunological conditions (MERINO et al. 1999). The thickness of the right wing web was measured after 24 hr with a spessimeter (Alpha spa, Milan, code SM112) with an accuracy of 0.01 mm.

Microbial isolation and identification

A sterile cotton swab (Bio-Merieux) was inserted into the cloaca for 5 sec, then the terminal 1 cm of the swab was cut with scissors and placed in a tube containing 3 ml of sterile Thioglycollate broth (Difco). The tube was kept at 5-10 °C in a cool-box before being transported to the laboratory and then refrigerated at 4 °C. Each sample was processed within 5 hr of collection. These temperatures are sufficiently low to inhibit further microbial reproduction but are not considered cold enough to cause mortality (LOMBARDO et al.

1996). For microbial isolation, the Thioglycollate medium was subjected to serial dilutions at a 1:10 ratio and 100 µl of each dilution was plated out onto different selective media (see Appendix 1). Bacterial counts were expressed as colony-forming units (CFU/ml). For *Salmonella-Shigella* enrichment, 500 µl of each sample were used to inoculate 5 ml of Rappaport broth and incubated at 42 °C for 24 hr. Ten microliters of Rappaport medium were subsequently plated out onto SS medium. Incubation of all media was carried out at 37 °C for 24-48 hr, except for Campylosel medium which was carried out at 42 °C in an anaerobic jar. Randomly collected bacterial colonies from each selective media were subsequently isolated on Trypticase soy agar and then stored at – 80 °C in Trypticase soy broth plus 20% glycerol until identification. Bacterial identification was performed by preliminary Gram-staining and then with the Analytical Profile Index kits (API E, API NE, API Staph-Bio-Merieux) (KONEMANN et al. 1997, ALEXANDER & STRETE 2001). A tentative index of bacterial infection was established as the cumulative number of pathogenic taxa found (bacterial richness: the index theoretically ranges from 0 to 19, with an observed range of 0-7. Taxa included: *Pseudomonas putida*, *P. aeruginosa*, *P. fluorescens, Staphylococcus xylosus*, *S. sciuri*, *S. lentus*, *S. aureus*, *S. cohni cohni, Salmonella-Shigella* spp., *Helicobacter* sp., *Vibrio parahaemoliticus*, *Klebsiella oxytoca*, *Aeromonas hydro-caviae*, *Pasteurella aerogenes*, *Pantoea* sp., *Enterobacter amnigenus*, *Buttiauxella agrestis*, *Hafnia alvei*, *Escherichia coli*). Most of these species are well known agents of pneumonia (*Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Pasteurella aerogenes*, *Streptococcus aureus*) and enteric diseases (*Salmonella-Shigella* spp., *Helicobacter* sp., *Vibrio parahaemoliticus*, *Hafnia alvei*, *Escherichia coli*) in birds (NUTTAL 1997). Beneficial or non-pathogenic bacterial taxa were not considered.

RESULTS

The highly pathogenic *Salmonella-Shigella* bacteria were never found in the sampled individuals, while the other microbes were present in 22-100% of Moorhens. The prevalence and abundance of microbes in the sampled birds are reported in Appendix 1.

Table 1 shows the relationships between colour expression (red and yellow saturations) and structural, microbial and health indices. The two colour indexes were not correlated (r_{27} = 0.138; P = 0.49 NS).

The structural variables (mass and body size) were not correlated with either of the two colours. The saturation of the yellow portion of the shield was positively correlated to three health indices: haematocrit, heterophils/lymphocytes ratio and PHA immune reaction (Fig. 1). Neither the bacterial richness index nor the bacterial concentration were significantly related to yellow saturation.

The saturation of the red portion of the shield was significantly correlated to the bacterial infection index. Individuals with a higher bacterial richness showed a duller red colour, with lower saturation values (Fig. 2). However, the bacterial concentration was not related to red saturation (probably because in this case pathogenic, neutral and beneficial taxa were all considered). None of the health indices was significantly correlated to the expression of red in the shield.

The same statistical analysis reported in Table 1 for the red and yellow saturations was calculated separately with hue and brightness as the dependent variables and the ten structural, microbial and health indices as independent variables. However, hue and brightness were not correlated to any of the independent variables (all P > 0.05 after Bonferroni correction); thus for the sake of brevity, single statistical values are not tabulated.

Table 1.

Relationships [a] between shield size and colours (saturation values in the HSB system) and microbial and health indices.

	Shield colour	
	Yellow (HSB saturation)	Red (HSB saturation)
n = 27 individuals		
Mass	0.137	0.299
Body size (PCA factor 1)	0.204	0.028
Heterophils/lymphocytes ratio	0.652**	0.111
ES rate	0.271	0.104
Haematocrit	0.500*	0.266
Immune reaction (PHA index)	0.608*	0.249
Bacterial presence index	0.196	– 0.817**
n = 14 individuals		
Total bacterial concentration	0.008	0.011
G-dark fermentors concentration	0.030	0.226
G-red fermentors concentration	0.078	0.349

[a] Pearson's r. Because multiple non-independent tests were conducted, the Bonferroni correction was applied. Only correlations with $P < 0.005$ (i.e. 0.05/10 tests) were considered significant. * $P < 0.05$, ** $P < 0.01$.
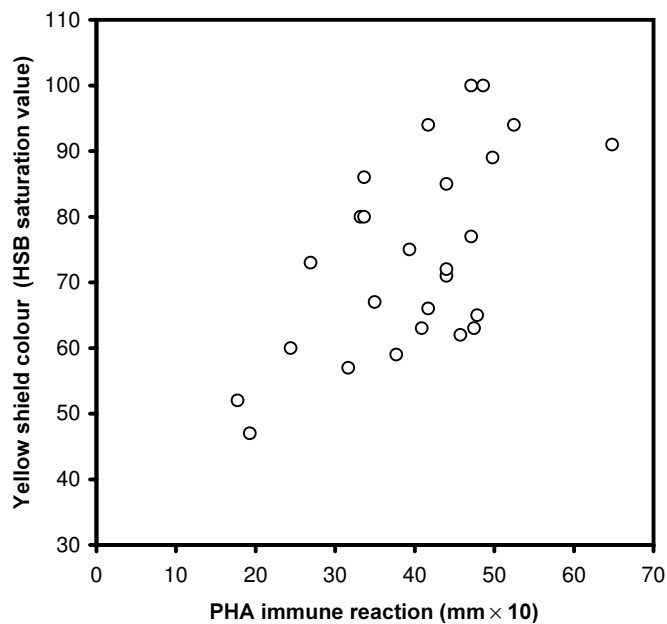

Fig. 1. — Relationship between PHA immune reaction and colour of the yellow portion of the shield.
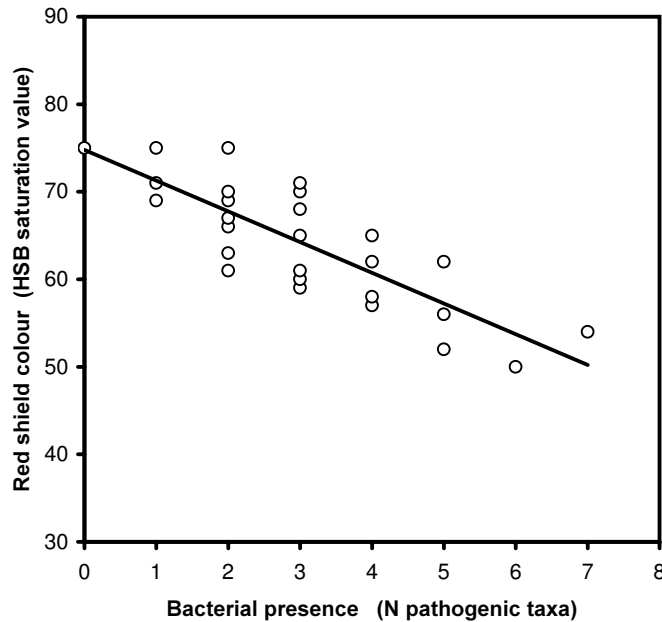
Fig. 2. — Relationship between bacterial presence index and colour of the red portion of the shield.

DISCUSSION

The first main result of this study is that one of the Moorhen shield colours (red) was negatively related to the pathogenic bacterial presence. The effect of bacterial infections on the health and body condition of natural bird populations has been neglected until recently (NUTTALL 1997, POIANI & WILKS 2000). However, the results of the available studies contrast: many bacteria are pathogenic, but others are beneficial (MORENO et al. 2003; e.g. *Lactobacillus* spp.), and it is even supposed that female-cuckoldry in birds could arise as a means to obtain beneficial cloacal parasites (MILLS et al. 1999). In our study, the contribution of beneficial bacteria was probably substantial when the colony-forming units concentration was assessed in three standard but poorly selective media. This non-selective method could have prevented the finding of a negative effect of microbial colony-forming units concentration on colour parameters. On the other hand, negative effects of microbial colonizations are commonly reported in avian medical care textbooks (RUPLEY 1997). Less data are available on free-living bird populations, although an effect of microbes has been found on growth (POTTI et al. 2002) and even on less studied characters, such as wing symmetry during growth (LOMBARDO et al. 1996). Concerning the relationship between bacteria and colours, the strongest negative effect of a bacterial infection on colour (feather redness) was documented in the House Finch *Carpodarcus mexicanus*: in 1990, the bacterium *Mycoplasma gallisepticum* spread from chickens to house finches, causing a massive die-off among finches on the east coast of the USA, and birds with brighter

plumage were more likely to have survived the disaster (NOLAN et al. 1998). Interestingly, the coccidid *Isospora* (Protozoa) also negatively influences carotenoid-based colouration of male house finches, although *Mycoplasma* appears to produce a greater decrease in plumage redness than coccidiosis (TYCZCKOWSKI et al. 1991, BRAWNER et al. 2000).

Our second main result is that the expression of the other shield colour (yellow) was not related to the bacterial abundance or richness but rather to the heterophils/lymphocytes ratio (in agreement with previous data: FENOGLIO et al. 2002b), haematocrit and cell-mediated immunocompetence (response to PHA). According to NORRIS & EVANS (2000), this could be explained by the different functions of the two immune system components, i.e. innate and acquired immunity. Bacterial infections are generally controlled by the innate immune system, while acquired immunity (humoral and cell-mediated) is crucial to control a range of other pathogens and parasites, from viruses to ectoparasites (ROITT et al. 1998).

Our correlational results generally agree with the hypothesis that different kinds of colouration provide different units of information (MØLLER & POMIANKOWSKI 1993, BRAWNER et al. 2000, BLANCO & DE LA PUENTE 2002). This hypothesis is corroborated by findings in the House Finch, in which melanin-based plumage colouration was unaffected by a Coccidia parasite (HILL & BRAWNER 1998), while carotenoids were strongly affected (MCGRAW & HILL 2000). Our study represents the first time that two carotenoid-based colours (red and yellow) could potentially convey complementary information about health state and bacterial presence. Experimental work is needed to support this perspective, both to investigate the possibility that yellow and red reflect different physiological states, and to see if energy that would have been used to produce ornamental colour was used instead to combat infection (HILL 1996, 2000).

Future studies should employ infections with different pathogens (from virus and bacteria to macroparasites) and/or pharmacological sanitation techniques (antibiotic, antihelmints, etc.) to experimentally investigate to what extent the colours can mirror different aspects of health status (LINDSTRÖM et al. 2001, POTTI et al. 2002).

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APPENDIX 1

The prevalence and average colony count of cloacal microbes among the studied Moorhens.

Growth media	Microbial groups	Percent positive (n = 27), or average count ± SE (n = 14)
SAB	Fungi (*Candida* spp.)	33.3%
EMB	G-dark lactose fermentors	44.4%
MAC	G-red lactose fermentors	100%
CTR	Pseudomonadales	74.1%
CHA	*Staphylococcus* MF	70.4%
CHA	*Staphylococcus* MNF	22.2%
SS	*Salmonella-Shigella* spp.	0%
ROG	*Lactobacillus* spp.	22.2%
BHI	Aerobic and facultative anaerobic bacteria	$8.89 * 10^5 \pm 2.67$ CFU/ml
EMB	G-dark lactose fermentors	$7.68 * 10^5 \pm 3.26$ CFU/ml
MAC	G-red lactose fermentors	$6.55 * 10^5 \pm 3.43$ CFU/ml