Gape coloration reliably reflects immunocompetence of barn swallow (Hirundo rustica) nestlings

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In some passerines, parents allocate more food to offspring with the brightest red gapes, but the function of parental decisions based on offspring gape coloration is unknown. We hypothesize that gape coloration is part of a communication system where nestlings reveal their condition to attending parents, which may thus base their decisions on reliable signals of offspring reproductive value. We analyze the effects of brood size manipulation, injection with an immunogen and food deprivation, on gape coloration, morphology, and T-cell-mediated immunocompetence of nestling barn swallows (Hirundo rustica). For each gape we measured three components of coloration (hue, saturation, and brightness) and obtained an overall color score by principal component analysis. Enlargement of brood size and injection with an antigen resulted in less red and less saturated and brighter gape color. Nestlings in enlarged broods had smaller body mass and T-cell-mediated immunocompetence compared to those in reduced broods. A positive covariation existed between redness and saturation of gape color and T-cell-mediated immunocompetence. Gape color siblings raised in different nests did not depend on parentage. Thus, condition-dependent gape coloration can reveal different components of nestling state on which parents may base their adaptive decisions about allocation of care to the offspring. Key words: begging; food provisioning, parental food allocation, parent–offspring conflict, reproductive value. [Behav Ecol 14:16–22 (2003)]

Gape coloration is considered a key component of solicitation display that offspring of altricial bird species show off to their parents to obtain care (Kilner, 1999; Kilner and Davies, 1998; Kilner et al., 1999). Bright red gapes have been shown to causally affect decisions of parents about allocation of food among their progeny in a few experiments (Götmark and Ahlström, 1997; Kilner, 1997; Saino et al., 2000a). Such parental preference for red gapes raises several issues about its adaptive function and the mechanisms that generate variance in gape coloration among siblings in a brood. Because nestlings that display at a high level would ensure high intake of food, a high level of display would be rewarded by natural selection in terms of enhanced viability (Martin, 1987). However, it remains unclear which factors prevent such high levels of display.

Individual parents and offspring of sexually reproducing species can be envisaged as the actors in a complex evolutionary game. These actors have interests that are only partly overlapping because offspring are selected to obtain larger parental investment than parents are selected to provide (Dawkins and Krebs, 1979; Godfray, 1991, 1995; MacNair and Parker, 1979; Parker et al., 1989; Trivers, 1974). To adaptively allocate limiting resources, parents should have evolved the ability to assess individual condition of their offspring, thus differentially investing in those with the largest reproductive value. Parents are expected to base their decisions only on signals that reliably reflect the particular component(s) of offspring state relevant to their decisions (i.e., offspring signals that cannot be faked; see Mock and Parker, 1997).

Immunity is a good candidate as a crucial component of nestling fitness, given the pervasive nature of parasitism, the primary role of acquired immunity in host defense against parasites and pathogens in general, and the intense effects of parasitism for young birds with a relatively naïve immune system in particular (Lowe and Zuk, 1991; Möller, 1997; Pastoret et al., 1998; Price, 1980; Roitt et al., 1996; Tizard, 1991). A link between gape coloration and parasitism is also suggested by several studies showing that infected birds have reduced carotenoid levels and thus pigmentation of the integument (reviewed in Möller et al., 2000).

One mechanism potentially linking infection to pigmentation is a trade-off with antioxidative and immunostimulant actions of carotenoid pigments, which has been documented mostly in mammals (Ames, 1983; Bendich, 1989; Chew, 1993; Kim et al., 2000a,b; Olson and Owens, 1988), but also in birds (e.g., Haq et al., 1996a,b; Tengerdy et al., 1990), and fish (e.g., Christensen et al., 1995; see also Lozano, 1994; Shykoff and Widmer, 1996; Möller et al., 2000 for a review). Competition for limiting dietary carotenoids could enforce honesty on gape coloration as a signal of current infection and immune status, as carotenoids allocated to immunity and other physiological processes would be unavailable for pigmentation (Lozano, 1994; Olson and Owens, 1998; Shykoff and Widmer, 1996). These speculations obviously rely on the assumption that carotenoids, which can only be acquired through the diet (Goodwin, 1984), are available in limiting amounts (Möller et al., 2000; but see also Hill, 1999).

One way to test this assumption is to simulate conditions where food is available in different amounts, for example, by altering brood size and thus the amount of food available per individual offspring and measuring the effect of food limitation on pigmentation (Saino et al., 1997b, 1999a, 2000b).
However, we are aware of no studies where this relationship has been experimentally investigated.

The first aim of the present study of barn swallow nestlings was to analyze the effects of brood size manipulation and short-term food deprivation on gape coloration under field experimental conditions. One of the major effects of manipulating brood size is to increase (brood reduction) or decrease (brood enlargement) the availability of food per nestling (Saino et al., 1997b, 2000b). To the best of our knowledge, this is the first experimental test of the effect of brood size on the expression of gape coloration as a component of nestling begging display in any bird species. We also tested the effect of temporary food deprivation, which simulated conditions where nestlings markedly differed in the amount of food received for a limited amount of time (3 h), on gape coloration. In addition, we injected nestlings with sheep red blood cells (SRBC), which are an antigen commonly used to elicit a T-cell-dependent humoral immune response (see Methods), to mimic an infection by a relatively innocuous pathogen. Obviously, this experiment differs from infection with parasites in one important respect, as the antigen does not replicate.

Our second aim was to test the hypothesis that gape coloration can convey reliable information on nestling state and immunity by analyzing its covariation with body mass and size, feather development, and T-cell-mediated immunity. We are aware of no studies in which the potential of gape coloration to act as a signal of nestling immune response has been analyzed.

Third, for the first time we tested for an effect of parentage on gape coloration, indicating additive genetic and/or maternal effects on phenotypic variation of this trait, by partially cross-fostering nestlings between broods. A significant resemblance between nestlings reared in different nests, and, thus, environmental conditions, could be taken as evidence of gape coloration being partly determined by additive genetic or early maternal effects as mediated, for example, by difference in allocation of carotenoids to the eggs.

We predicted that (1) brood enlargement rendered the gape less red (more yellow) compared to brood reduction because the biochemical determinants of gape redness might be limiting under severe intrabrood competition, and (2) food deprivation had no effect on gape coloration because contingent need affects gape coloration in seed-regurgitating finches but not in insectivorous birds like the barn swallow we studied (Kilner and Davies, 1998). In a previous study where we visually evaluated gape coloration (Saino et al., 2000a), we showed that nestlings injected with SRBC had less red and less saturated gape coloration. Hence, part of the present study allowed us to replicate the analysis of the effect of SRBC injection with the advantage of using a less subjective measure of gape coloration. In the present study we therefore also predicted less red, less saturated, and less bright coloration of SRBC-injected nestlings.

Under the hypothesis that gape coloration reveals components of offspring condition, we formulated the general prediction that nestlings with relatively red, highly saturated, and bright coloration were in relatively good condition and thus had larger body mass and more efficient T-cell-mediated immunity.

The barn swallow is a semicolonial, aerially insectivorous passerine. Both parents feed two to seven nidicolous nestlings (Møller, 1994). Offspring solicit food provisioning by giving typical calls and opening their yellow-to-red gape (Lotem, 1998; Sacchi et al., 2002; Saino et al., 2000b). Begging rate is enhanced by contingent need of food (Saino et al., 2000b). Parents preferentially feed hungry nestlings and preferentially feed nestlings with an experimentally reddened gape (Lotem, 1998; Saino et al., 2000a,b). Food provisioning is also affected by injection with SRBC; inoculated nestlings are fed less than controls and particularly so when food deprived (Saino et al., 2000b). Gape coloration is reduced by injection with SRBC but can be restored by supplementing the diet with the main circulating carotenoid (lutein) in barn swallow plasma (Saino et al., 1999b).

METHODS

We studied barn swallows breeding in four colonies located in cow stables near Milano (northern Italy) during spring–summer in 1998 and 1999. When we found two broods in the same colony where hatching was completed in the same day, on the day of hatching completion we swapped an unbalanced number of individually marked nestlings so that post-manipulation brood size was either increased or decreased by one nestling with respect to the original size of the brood. The brood to be enlarged or reduced within a pair (hereafter “dyad”) of matched synchronous broods and nestlings to be cross-fostered were chosen randomly. The number of nestlings that were cross-fostered between nests depended on the original size of both broods in the dyad and the size of the brood that was to be enlarged or reduced and was decided according to a predetermined scheme aimed to minimize the difference in the number of cross-fostered and resident nestlings within each brood (see also Saino et al., 2000b).

Six days after hatching, we intraperitoneally injected half (or half the number of nestlings + 0.5 in the case of an odd brood size) with 3.5 × 10⁷ SRBC in 30 μl phosphate-buffered saline (PBS); the remaining nestlings were sham-inoculated with the same amount of PBS. SRBC is a multigenic antigen frequently used to test the ability to raise a T-cell-dependent humoral immune response (e.g., Deerenberg et al., 1997; Hudson and Hay, 1980; Lochmiller et al., 1991; Pinard-van der Laan et al., 1998; Roitt et al., 1996; Ros et al., 1997), and it has also been shown to elicit an antibody response in the barn swallow (Saino and Møller, 1996; Saino et al., 1997a). Hence, inoculation with SRBC was assumed to simulate an infection by a foreign antigen because it elicits a T-cell-dependent humoral immune response similar to that elicited by real pathogens (Roitt et al., 1996). During the morning of the sixth day after injection, we subjected one or two randomly chosen nestlings from groups of SRBC-injected and sham-inoculated nestlings to 3 h of food deprivation by placing them in the same part of the nest and covering them with a thin, metal net. Hence, in our factorial experiment there were eight groups of nestlings resulting from the three treatments × two levels for each treatment, and a blocking factor, the dyad.

After the end of the food deprivation procedure one experimenter took a full-format frontal photograph of the nestling’s head while another experimenter held its beak wide open. We standardized the conditions under which photographs were taken by using the same macro-lenses (Vivitar 100 f/3.5 Macro, C/FD) with the same type of ring flash (Starblitz 1000 Auto Macro-Lite), type and sensitivity of photographic film (Kodak Gold ISO 200), diaphragm (f 8) and shutter speed (1/50 s), and the angle of the lens relative to the opened mouth. In addition, all photographs of nestlings in a colony were taken in the same darkened room in the farm buildings. Later in the same day we measured body mass, tarsometatarsus length, and length of the left innermost rectrix. We also did a cutaneous test to assess the ability to raise an in vivo T-cell-mediated cellular immune response. We measured the thickness of both wing webs by a pressure-sensitive micrometer (precision of 0.01 mm; Alpa S.p.A., Milano). The right wing web was then injected with 0.2 mg phytohemagglutinin (PHA; Sigma) dissolved in 0.04 ml PBS, whereas the left wing...
web was injected with the same amount of PBS. PHA is a lectin that is mitogenic to T lymphocytes, and its inoculation results in thickening of the wing web. The difference between change in the thickness of the right wing web measured 24 h after injection of PHA minus the change in the thickness of the left wing web, which controlled for the effect of inoculation per se, is considered as an index of T-cell-mediated immunocompetence (Lochmiller et al., 1993; Moreno et al., 1999; see also Saino et al., 1997b, 1998a; Sorci et al., 1997). Measures of wing web thickness have been shown to be highly repeatable (repeatability computed according to Falconer, 1981: \( R = .95 \); see also Sorci et al., 1997 for similar results).

After film processing, pictures of gapes were transferred onto an electronic support, and gape coloration was measured using the AdobePhotoshop 5.0 package. For each gape we selected three small sampling areas on the palate. Two areas, one on each side of the bird, were approximately halfway between the tip of the beak and the limit between the upper and lower flange and halfway between the bird’s median line and the flange. The third area was along the median line in the front part of the palate. For each of the sampling areas we measured three components of color: hue, saturation, and brightness (see Endler, 1990, for a definition of these variables). Hue is the actual color and is expressed in degrees. Values ranging between 330° and 30°, according to this scale, correspond to red, values between 30° and 50° to orange, and values between 50° and 70° to yellow. Hence, increasing values between 45° and 65°, the range of values recorded in barn swallow nestling gapes, correspond to decreasing red and increasing yellow coloration. Saturation is the purity of color and is expressed as a percentage ranging from 0 to 100. Brightness is a measure of the relative amount of light reflected by an object. Values of the three coloration variables recorded in the three regions of the gape were highly repeatable within individuals (ANOVA for hue: \( F_{2,99,540} = 13.64 \), repeatability \( R = .81 \), saturation: \( F_{2,99,540} = 5.48 \), \( \text{R} = .60 \), brightness \( F_{2,99,540} = 12.97 \), \( \text{R} = .80 \), \( p < .0001 \) in all cases). Hence, gape coloration measured at the selected regions was likely to reflect these color components also of other gape areas. We therefore used mean values of the three coloration variables calculated between gape regions. It should be emphasized that the measures of coloration we used, although being highly repeatable and not biased by subjective judgments, do not necessarily represent what birds would perceive but are based on humans’ perception of colors (Cuthill et al., 2000; Endler, 1990; Fleishman and Endler, 2000).

We entered mean values of gape color components in a principal component analysis to obtain an overall score (PC1 score) of gape coloration in the human-visible spectrum and assumed that this score is correlated with color differences that can be perceived by birds. To test whether gape coloration measured in this way reflected gape coloration exhibited by nestlings when they beg for food, in summer 2001 we took a picture of the gape of resting nestlings (i.e., nestlings that were not begging and had not just ended a begging event), as described above. Approximately 10 min later, we stimulated the nestlings to beg and then immediately took a second picture of their gape. There was no difference in principal component gape coloration score before and after the begging event (paired \( t \) test, \( t = 0.09, \text{df} = 32 \)), indicating that gape coloration measured on resting nestlings was a reliable measure of gape coloration presented by nestlings when they beg for food. This result was expected because rapid change of gape coloration during begging events has been documented only in a few species (e.g., the canary, *Serinus canaria*; Kilner, 1997).

We investigated 33 dyads of broods. The mean difference in postmanipulation brood size between enlarged and reduced broods was 1.8 nestlings. Our sample consisted of 270 nestlings. In enlarged broods we measured 56 SRBC-injected and food-deprived, 30 SRBC-injected and non-food-deprived, 42 sham-inoculated and food-deprived, and 32 sham-inoculated and non-food-deprived nestlings (\( N = 160 \) nestlings). In reduced broods, sample sizes of experimental groups, listed in the same order as for enlarged broods were 56, 29, 30, and 21, respectively (\( N = 110 \)).

In statistical analyses of nestling morphology and immune response, we used the sequential Bonferroni correction to assess the tablewise rate of type I statistical errors by adjusting the significance level downward in relation to the number of tests (hereafter \( k \)) made (Holm, 1979; Wright, 1992).

**RESULTS**

Hue, saturation, and brightness data were first subjected to a principal components analysis to obtain a single score for coloration of each gape. The principal component associated with the largest eigenvalue (PC1) summarized 48.3% of the variation in the three gape coloration variables, and the score of each gape on the PC1 was therefore used in subsequent analyses. Gape hue and brightness positively contributed to the PC1 (correlation coefficients = .804 and .736, respectively), whereas the contribution of saturation was negative (−.0736). Hence, large scores on the PC1 were associated with less red (more yellow), less saturated, and more bright gape coloration to a human eye.

**Effect of experimental manipulations on gape coloration**

The first aim of this study was to test the effects of experimental treatments on gape coloration. ANOVAs with dyad as a factor showed that PC1 gape coloration scores were significantly affected by SRBC injection (\( F_{1,231} = 13.81, \ p < .001 \)), and brood size manipulation (\( F_{1,231} = 4.19, \ p = .042 \)), whereas no effect of food deprivation was found (\( F_{1,231} = 0.14 \)). Nestlings that received an injection with SRBC and those in enlarged broods had more yellow (less red) and bright, and less saturated gape coloration (Figure 1) compared to those in reduced broods and those that received an injection with a control solution. A significant interaction existed between brood size manipulation and food deprivation (\( F_{1,231} = 6.15, \ p = .014 \)), with the difference between food-deprived and non-food-deprived nestlings being larger in enlarged compared to reduced broods.

To test for the existence of additive genetic and/or early maternal effects on gape coloration, we also included the nest of origin as a two-state factor within each dyad in the analysis reported above. Origin had no significant effect gape coloration scores (\( F_{3,198} = .60 \)).

**Effect of experimental manipulations on nesting T-cell-mediated immune response and morphology**

We analyzed the effect of brood size manipulation, injection with SRBC, and food deprivation on body mass, on tarsus and rectrix length, and on the index of T-cell-mediated immunity in ANOVAs with dyad and experimental treatments as factors, together with their two-way interactions. Nestling body mass was significantly affected by brood size manipulation (\( F_{1,231} = 29.30, \ p < .001 \) after Bonferroni correction with \( k = 4 \)), with nestlings in enlarged broods being lighter than those in reduced ones (Figure 2). No significant effect of the other treatments or interactions existed. Tarsus length and rectrix length, reflecting body size and, respectively, feather development, were unaffected by brood size manipulation, SRBC injection, and food deprivation (Figure 2). T-cell-mediated
Gape coloration and condition in nestling swallows

Johnstone, 1997). However, the function of conspicuous signals in parent–offspring communication (see Kilner and Davies, 1998) study, but coloration was found to be less red (more yellow), and saturated, and brighter in nestlings that had their immune system challenged with an immunogen and in nestlings belonging to enlarged broods, where sib–sib competition was likely to be relatively intense (Saino et al., 1997b, 2000b). The interpretation of the mechanisms generating the observed effects of experimental manipulations largely relies on assumptions and previous knowledge about the determinants of gape coloration. In a previous study (Saino et al., 2000a), injection with SRBC resulted in a reduction in redness and saturation of gape coloration (Saino et al., 2000a). Moreover, we showed that lutein, which is the main carotenoid pigment in barn swallow plumage and plasma (Stradi, 1998; Saino et al., 1999b), at least partly determines gape coloration, since artificial provision of the pigment resulted in enhanced gape redness and color saturation (Saino et al., 2000a). In the present study, by manipulating brood size we demonstrated that reduced intake of food by individual nestlings after brood enlargement (see Saino et al., 1997b, 2000b) also affected gape coloration. We interpret these findings as evidence for determinants of gape coloration being limiting in the diet of barn swallow nestlings. However, the observed effect of brood size manipulation might also be mediated by other mechanisms, such as larger levels of parasitism and infection in crowded nests (Saino et al., unpublished results) or increased stress, resulting from larger levels of competition for food among nest mates. In addition, the effect of food deprivation depended on previous manipulation of brood size, as shown by larger effect of food deprivation among nestling of enlarged compared to reduced broods (Figure 1). This finding suggests that food deprivation, while not having an effect per se, affected gape coloration more under relatively severe conditions such as those experienced by nestlings in enlarged broods.

Carotenoids play a crucial role in protection against oxidative stress because they act as scavengers of free radicals and stimulate immune function (see Lozano, 1994; Möller et al., 2000). However, to exert their physiological action, carotenoids may have to be sequestered from peripheral tissues (e.g., integument and visible gape tissues), thus paving the way for a trade-off between allocation of limiting amounts of these biochemicals to the competing demands of gape coloration and antioxidative and immunological actions. The idea that carotenoids may be limiting immune function and gape coloration is corroborated in the present study by the negative effect of brood enlargement on one important component of acquired immunity (i.e., the ability to raise a T-cell-mediated immune response), combined with the negative relationship we found between gape redness and intensity of brood size manipulation might also be mediated by other mechanisms, such as larger levels of parasitism and infection in crowded nests (Saino et al., unpublished results) or increased stress, resulting from larger levels of competition for food among nest mates. In addition, the effect of food deprivation depended on previous manipulation of brood size, as shown by larger effect of food deprivation among nestling of enlarged compared to reduced broods (Figure 1). This finding suggests that food deprivation, while not having an effect per se, affected gape coloration more under relatively severe conditions such as those experienced by nestlings in enlarged broods.

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Figure 2
Mean (+ SE bars) tarsus length reflecting skeletal size, left innermost rectrix length reflecting feather development, body mass, and index of T-cell-mediated immunocompetence measured as the swelling of the right wing web 24 h after a subcutaneous injection of a lectin (PHA), which is mitogenic to T-lymphocytes, in relation to brood size manipulation, injection with SRBC, and short-term food deprivation. Nestlings in enlarged broods had smaller body mass and T-cell-mediated immunocompetence compared to those in reduced broods.

In enlarged broods, where dietary carotenoids may be relatively more limiting because of intense competition for food (see Figure 2), T-cell-mediated immunocompetence was lower, and nestlings with less red gape had weaker immune responsiveness (Saino et al., 1997a, 2000b).

Experimentally increasing brood size also had a negative effect on nestling body mass and T-cell-mediated immunocompetence, consistent with the effect of brood size manipulation that we have previously documented for the barn swallow and with several other studies, and with the documented negative effect of poor food intake and nutritional conditions on acquired immunity (e.g., Chandra and Newberne, 1977; Dietert et al., 1994; Gershwin et al., 1985; Klasing, 1988; Martin, 1987; Saino et al., 1997b, 1999a). Injection with SRBC did not influence response to PHA, which is assumed to reflect T-cell-mediated immune response. Hence, we found no evidence for a negative effect of response to SRBC on cell-mediated response to PHA. This lack of effect might be due to stimulation of proliferation of T cells, which are involved in humoral response to SRBC and cellular response to the mitogenic PHA.

We found statistically highly significant covariation between the ability to raise a T-cell-mediated immune response and gape coloration. This suggests that gape coloration is affected by current immunological conditions and brood size and that it reliably reflects a component of acquired immunity. Obviously, the existence of these relationships does not necessarily imply that gape coloration serves the function of reliably signaling components of nestling general state, nor that parents base their parental decisions on the expression of this condition-dependent trait. However, we have previously shown that nestling barn swallows are fed more when the redness of their gape is experimentally increased but are fed less when they are injected with SRBC (Saino et al., 2000a,b).

Immunity probably cannot be assessed directly, and parents may thus have to rely on signals reliably reflecting this trait. Apparently, several spectrographic features of barn swallow nestlings begging calls are not affected by injection with SRBC (Sacchi et al., 2002), suggesting that parents do not rely on acoustic signals to assess this component of nestling state. This corroborates the idea that expression of gape coloration is one of the mechanisms mediating the effect of injection with SRBC on parental feeding decisions (Saino et al., 2000b). Parent barn swallows might actually allocate more food to nestlings that by displaying a red gape honestly reveal their high phenotypic quality, in terms of T-cell-mediated immunity.

By partially cross-fostering nestlings between broods we tested whether part of the variance in gape coloration could be explained by nestling parentage. If that was the case, either additive genetic variation in gape coloration, or early maternal effects (or both) could be invoked to account for the similarity in coloration between nestlings sharing the same origin (Mousseau and Fox, 1998). However, we found no statistically significant evidence for an effect of parentage on this component of begging behavior.

In conclusion, brood enlargement and a challenge of the
immune system, but not contingent need of food, affected gape coloration, whereas brood enlargement negatively affected body mass and depressed an important component of acquired immunity. Gape coloration significantly predicted an important component of offspring quality such as immunity. The present results suggest that gape coloration as a whole is a condition-dependent character that can convey to attending parents information on a diverse array of offspring fitness-related traits.

We are grateful to many people who helped during field work. This project was supported by C.N.R. and M.U.R.S.T. COFIN 40% grants to N.S.

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