# Egg colour reflects the amount of yolk maternal antibodies and fledging success in a songbird

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**Blue–green colours in avian eggs have been proposed as post-mating signals of female phenotypic quality to their mates. Egg colour may not only indicate female value, but also the quality of the eggs themselves and of resulting offspring. To date, there has been no demonstration of an association between egg colour and egg or offspring immune quality. We here show that the intensity of blue–green colour of pied flycatcher *Ficedula hypoleuca* eggs reflects the amount of maternal antibodies in the yolk, a form of passive immunity crucial for offspring performance. Moreover, egg colour predicts fledging success. Also, incubating females in better condition lay more colourful eggs. The immunoglobulin level in incubating females is positively associated with that in the eggs. These results support the signalling hypothesis of eggshell coloration, underlining its role as an indicator of trans-generational transmission of immune defences in birds.**

**Keywords:** immunocompetence; maternal effects; nestling survival; IgY

## 1. INTRODUCTION

A recent hypothesis proposes that blue–green base colours of the eggshells of many avian species with biparental care may function as post-mating sexually selected signals of female phenotypic or genetic quality to their mates (Moreno & Osorno 2003). The hypothesis is based on the antioxidant properties of the pigment biliverdin (Kaur *et al.* 2003), whose allocation to eggshells may indicate female antioxidant capacity.

Offspring could inherit maternal traits indicating antioxidant capacity (Moreno & Osorno 2003). The development of the immune system of nestlings may depend on antioxidant capacity inherited or derived from maternal transmission (Saino *et al.* 2003a). Alternatively, offspring immune function could be directly associated with maternal transfer of antibodies to the egg (Apanius 1998). Maternally derived antibodies (IgY) provide the first form of humoral immunity for offspring early in life and improve offspring performance and survival (Apanius 1998). Since breeding females may differ in immunocompetence (Grindstaff *et al.* 2003; Morales *et al.* 2004), it should be expected that immunologically high-quality females should benefit by signalling their allocation of immune factors to eggs to promote male investment.

Egg IgY level is positively associated with that in maternal serum (Gasparini *et al.* 2002; Saino *et al.* 2002). Hence, egg colour could reflect egg IgY level and offspring quality as a product of maternal immunocompetence as proposed by Moreno *et al.* (2004).

In the present study, we investigated whether the blue–green egg coloration of the first egg in pied flycatcher clutches reflected egg quality in terms of maternally transferred immunity (IgY). Furthermore, we explored whether egg coloration predicted fledging success. Also, we studied whether it was associated with female condition and serum IgY level.

## 2. MATERIAL AND METHODS

The study was conducted in a population of pied flycatchers (*Ficedula hypoleuca*) in Sierra de Guadarrama, central Spain (40°48′ N, 3°54′ E). Most clutches contain five or six eggs. We studied egg coloration and IgY level of the first egg from 25 clutches. Egg volume was calculated following Ojanen *et al.* (1981). In order to exclude the possibility that first eggs were exceptional in IgY level, we also collected the fourth egg in eight nests. The reasoning behind choosing the fourth egg for this comparison was that there might be physiological limitations for the laying females in the transfer of either biliverdin or antibodies, so that differences in the amount of transferred components are likely to be found between first and last eggs in the clutch. But the very last egg in a clutch is consistently either larger or smaller than the average size of the clutch (Ojanen *et al.* 1981). Consequently, it might also be anomalous in its content. Therefore, as there are clutches of five eggs in our population, choosing the fourth one is a compromise between prolonging the period from laying of the first egg and avoiding including last eggs. Also, a previous study on barn swallows has shown that the level of maternally transferred antibodies could differ between first and fourth eggs in a clutch (Saino *et al.* 2002). For ethical reasons and due to legal restrictions, samples of removed eggs were kept to a minimum, especially for nests where two eggs were removed. Eggs were removed on the laying day. The range of laying dates of the study nests was of one week.

Egg colour was measured with a portable spectrophotometer (Minolta CM-2600d, Japan) as soon as collected. Eggs were placed directly with their broad pole on a target mask (diameter of 8 mm), so that eggs completely filled the measuring port. Reference calibrations with respect to zero and white standards were performed according to apparatus specifications. Reflectance spectra for each egg are automatically produced as means of three sequential measurements. The spectrophotometer covers the reflectance spectrum above 360 nm in intervals of 10 nm. Following Siefferman *et al.* (2006), blue–green chroma (BGC) was calculated as the proportion of total reflectance in the blue–green region ($R_{400-580}/R_{360-700}$) of the spectrum, which corresponds to the region of greatest reflectance of biliverdin (Falchuk *et al.* 2002) and because pied flycatcher eggs reflect light maximally in it (Moreno *et al.* 2005a).

Clutch size could affect first egg IgY level. Assuming that pied flycatchers are indeterminate egg-layers when their clutch size is experimentally reduced (Kern *et al.* 2000), clutch size can be estimated as the number of eggs laid minus the eggs removed. On the assumption that they are determinate layers (Kennedy 1991), clutch size can be estimated as the number of eggs laid by including the eggs that were removed. As the degree of indeterminacy of clutch size in pied flycatchers is uncertain, we have estimated clutch size as the average of both highly correlated values ($r = 0.86$, $p < 0.001$).

The concentration of yolk IgY was assessed by means of an indirect enzyme-linked immunosorbent assay following Müller *et al.* (2004). The linear range of the antibody–antigen response had been previously determined for this species. The dilution used was 1 : 1000 for both the yolk samples and the secondary antibody (Sigma, anti-chicken IgY). We used absorbance as the final value of IgY concentration or level.

Incubating females were captured at the nest on the 10th day after the first egg was laid. They were weighed (accuracy 0.25 g) and their tarsus length was measured (accuracy 0.01 mm). As a measure of body condition, we used mass divided by the cube of tarsus length. A blood sample was collected from the brachial vein and centrifuged (Labnet, Cat.N°1201-220V, USA). The serum was separated from the cellular fraction and maintained in a cool box until being frozen on the same day. We estimated female serum IgY
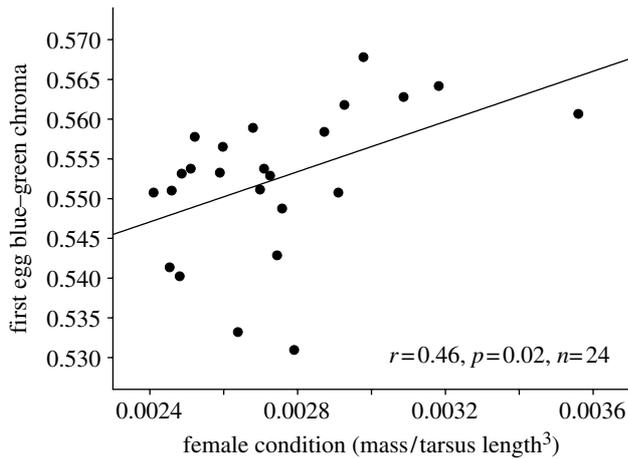
Figure 1. Association of female condition with first egg BGC.



Figure 2. Association of first egg IgY level with first egg BGC.

level following Martínez *et al.* (2003). One female could not be captured. Blood samples of four females were too small to be analysed.

Fledging success was calculated as the number of offspring that survived until day 13 divided by the incubated clutch size. The number of offspring on day 13 is strongly associated with the number of fledglings in another population (8 years data: $r=0.94$, $p<0.001$, $n=418$ broods).

All variables were normally distributed (Kolmogorov–Smirnov test for continuous variables, $p>0.05$).

## 3. RESULTS

Although the sample size available to compare first and fourth eggs is small, first eggs did not differ in IgY level compared with fourth eggs (paired $t_8=0.001$, $p=0.999$). There was no association between first egg volume and IgY level ($r=-0.02$, $p=0.96$, $n=25$). Female condition was positively associated with first egg BGC (figure 1). Female IgY level was positively associated with first egg IgY level, when correcting for the estimated clutch size (female IgY level: $F_{1,17}=12.17$, $p=0.003$; clutch size: $F_{1,17}=5.04$, $p=0.038$), but it was not associated with first egg BGC (all $p>0.2$).

First egg IgY level was positively associated with BGC when correcting for the estimated clutch size (figure 2; BGC: $F_{1,22}=4.74$, $p=0.042$; clutch size: $F_{1,22}=0.36$, $p=0.55$). Fledging success was positively and strongly associated with first egg BGC ($r=0.64$, $p<0.001$, $n=25$). Controlling additionally for egg volume did not alter any of the results.

## 4. DISCUSSION

First eggs did not differ in IgY level compared to fourth eggs. We therefore assume that first eggs are representative in IgY level with respect to the rest of the clutch. Females in better condition laid more colourful eggs. This is in accordance with that both T-cell mediated and humoral immunocompetence of females were positively associated with egg colour in another study performed in a neighbouring population (Moreno *et al.* 2005a). This result confirms that found by Siefferman *et al.* (2006) in another species with blue–green eggs. Also, females with higher IgY level laid eggs with higher IgY level. This concurs with previous studies (Gasparini *et al.*
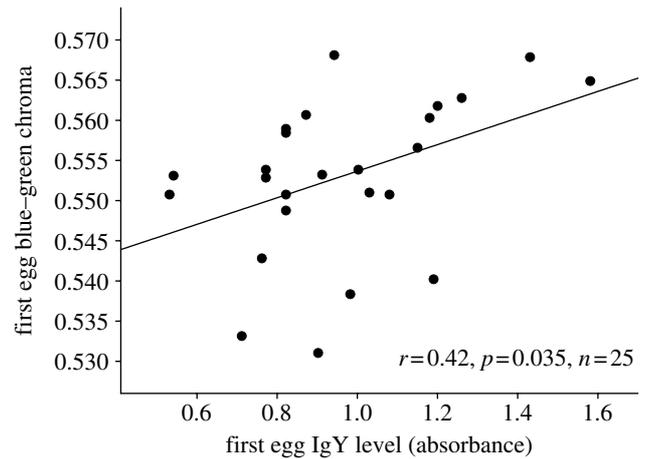
2002; Saino *et al.* 2002), and indicates that only females in a good immunological state were able to allocate more immune defences to eggs. However, we did not find an association between first egg colour and female serum IgY level. Female health may vary after laying according to unknown factors like relapses of chronical infections, new infections or male contribution in incubation feeding. Egg colour seems to better reflect induced immune responses to foreign antigens (Moreno *et al.* 2005a) than IgY levels measured some time after laying. Unfortunately, females cannot be sampled at laying due to the risk of desertion.

First egg BGC reflected accurately the egg IgY level. First eggs with more maternal antibodies reflected more in the blue–green part of the spectrum, which coincides with the reflectance peak of the pigment biliverdin (Falchuk *et al.* 2002). Maternal antibodies can adaptively influence the chick phenotype via non-genetic inheritance (Mousseau & Fox 1998) and may continue to affect offspring during growth and development (Grindstaff *et al.* 2003). This suggests that this form of passive immunity may have important implications for offspring performance and survival (Blount *et al.* 2002; Saino *et al.* 2003b; Müller *et al.* 2004). Indeed, first egg BGC predicted fledging success. This result shows that immune defences in nestlings as derived from maternally transferred antibodies may affect survival in the nest. The best predictor of local recruitment of pied flycatcher fledglings is immune capacity (Moreno *et al.* 2005b), so it should be expected that if maternal antibodies affect nestling immune system development positively, egg colour might in fact signal the future recruitment prospects of offspring. Therefore, maternal allocation of antibodies to the eggs enhances the reproductive value of the brood. According to Moreno *et al.* (2004), male effort in caring for the brood may also contribute to offspring survival, but we cannot separate the effects of male input and egg immune quality in the present study.

In conclusion, this is the first study to show that an external egg characteristic such as blue–green base coloration predicts egg immune quality and a crucial fitness trait (i.e. fledging success). These results give

support to the hypothesis proposed by Moreno & Osorno (2003) and Moreno *et al.* (2004) about the signalling function of certain egg colours. As egg IgY content remains cryptic to the male, a way females would have to make it conspicuous to their mates is to use an external signal. Future studies are needed to experimentally demonstrate that blue–green egg colour is indeed a signal subjected to post-mating sexual selection. For instance, cross-fostering of eggs of different colours between nests would demonstrate that males respond to egg colour and not to another female trait that is in turn associated with egg colour. This trait seems a good candidate for evaluating egg quality in the field and to monitor the health status of avian populations without capturing adults or destroying eggs. This would be especially important for endangered populations, where manipulation is not recommended.

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