

Reduced immunocompetence of nestlings in replacement clutches of the European magpie (*Pica pica*)

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SUMMARY

Laying date is one of the most important determinants of reproductive success and recruitment probability in birds. Late breeders usually fledge fewer chicks than individuals with earlier breeding dates, and fledglings produced late in the season have high mortality rates. Food availability and nestling mass have been evoked as the principal mechanistic links between laying date and offspring survival. Here we suggest that another factor may actually account for the difference in survival rate between early and late offspring: immunocompetence. We predicted that nestlings produced later in the season or in replacement clutches should have lower immune responses when challenged with an antigen, than early nestlings or nestlings produced in first clutches. This hypothesis was tested in a population of magpies (*Pica pica*), in which we experimentally induced breeding failure in a group of nests and compared the immune response of nestlings in replacement clutches with the immune response of first clutch nestlings. Cellular immune response, as measured by wing web swelling (a correlate of T-lymphocyte production after injection of phytohaemagglutinin-P), significantly decreased with hatching date and was significantly lower in nestlings of replacement clutches. Furthermore, coefficients of intraclutch variation in immune response were higher in nestlings of replacement clutches. This experiment demonstrates an inverse relationship between immune responsiveness and breeding date, and reduced recruitment probability of late nestlings may be a direct consequence of their inability to cope with parasites.

INTRODUCTION

Reproductive success (i.e. the number of fledged offspring) and recruitment probability (i.e. the probability to survive until the first reproductive event) of birds are known to depend on a number of environmental factors, such as nest predation rate, brood parasitism, food availability, and climate (Brittingham & Temple 1983; Martin 1987, 1995; Mertens 1987; Perrins 1991). Between-individual variation in reproductive success, or at least components of reproductive success (such as laying date), may however also reflect genetic heterogeneity (Blondel *et al.* 1990; van Noordwijk *et al.* 1981; van Noordwijk & van Balen 1988).

The study of the relationship between reproductive success, recruitment probability and laying date in birds dates back to the work of Lack (1950, 1968). He argued that there should be an optimal laying date since very early and late breeders experience low reproductive success. Some studies have confirmed that the

relationship between reproductive success and laying date is hump-shaped (Parson 1975; Findlay & Cooke 1982; Brinkhof *et al.* 1993). Other studies have, however, reported linear negative relationships between the two variables (Perrins 1965; Cavé 1968; Cooke *et al.* 1984; Daan *et al.* 1989; Newton 1989). Therefore, although the shape of the optimal laying date function may differ among species or populations, the common observation from all studies is that fledglings from late clutches always experience a very low probability of recruitment.

The mechanistic basis invoked to explain the correlation between laying date and recruitment rate is supposed to be food availability. For example, the great tit (*Parus major*) closely matches its timing of reproduction with the peak in caterpillar production (Perrins 1991; van Noordwijk *et al.* 1995). Several morphological characters of offspring are correlated with both laying date and recruitment probability. For example, body mass at fledging is usually a reliable predictor of recruitment probability (e.g. Lindén *et al.* 1992; but, see also, Newton 1989), and body mass obviously also depends on food availability. Despite

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good knowledge of the relationship between laying date, food availability, morphology and survival, we still have a poor knowledge of the physiological mechanisms responsible for reduced survival probability of late offspring. Fledglings produced late in the season may suffer from higher mortality for a number of reasons. They could experience difficulties catching prey or escaping predators, suffer from intraspecific and interspecific competition for food, especially when food is scarce, and be more exposed to macro- and microparasites.

The immune system is probably the most important defence system that vertebrates have evolved against parasites (Roitt *et al.* 1996; Wakelin 1996). Although recent work has pointed out that immune responses may be genetically variable (Wakelin 1996; Wakelin & Blackwell 1988), it has been known for a long time that environmental condition can significantly affect immune function. Nutritional status is one of the most important environmental determinants of the immune response. A large body of literature has emphasized that both the quality (e.g., a protein-rich diet) and the quantity of ingested food play an important role for developing and raising an efficient immune response (Chandra & Newberne 1977; Gershwin *et al.* 1985; Lochmiller *et al.* 1993). Therefore, we should expect a decrease in the efficiency of the immune system in fledglings produced under poor environmental conditions and a negative correlation between immunocompetence of nestling and laying date.

We tested this hypothesis in the magpie (*Pica pica*), which is a corvid rearing a single brood per year, although they may lay up to three replacement clutches if an early reproductive attempt fails, for instance due to predation on eggs or nestlings (Birkhead 1991). As for most passerine species, magpie reproductive success and recruitment probability of replacement clutches are significantly lower than that of first attempts (Birkhead 1991). We experimentally investigated the difference in the immune response of nestlings produced in first and repeat attempts. We provoked failure of the first breeding attempt in a random group of nests, whereas another random group was considered as controls. We then located the replacement clutches of the failed group and compared the immunocompetence of nestlings of the two groups.

When environmental conditions deteriorate parents may diversify their strategy of parental care because an equal distribution of resources among offspring could result in a lower fitness than a strategy where a limited number of offspring received most resources (Seger & Brockman 1987). This phenomenon should lead to higher intra- than interclutch variance in nestling phenotypic characteristics. Therefore, we tested whether nestlings from replacement clutches had higher intersib variation in immunocompetence than nestlings from first clutches.

We evaluated one component of immunocompetence, cell-mediated immune reaction, *in vivo* to intradermal injection of phytohaemagglutinin-P (PHA-P). The response to inoculation of PHA-P has been shown to be a reliable indicator of *in vivo* cellular immunity in poultry (Goto *et al.* 1978; McCorkle *et al.*

1980). Phytohaemagglutinin-P has a mitogenic effect on T-lymphocytes and subcutaneous administration induces macrophage infiltration and dense perivascular accumulation of T-lymphocytes.

2. MATERIALS AND METHODS

The experiments were carried out in Spring 1996 on a magpie population at La Calahorra, Hoya de Guadix (37°18' N, 3°11' W, southern Spain), a high altitude plateau, approximately 1000 m above sea level. At the beginning of April we searched extensively for magpie nests in the study area. A random subsample of nests were experimentally induced to fail by breaking the eggs (these nests served for another experiment on repeatability of magpie response to a model egg of a brood parasite (none of the nests used in this study were naturally parasitized by brood parasites), G. Sorci, personal observations), whereas another random subsample of nests was kept as a control and allowed to hatch. One week after breeding failure had been induced in the treatment group, we started to look for replacement clutches. Each nest (first and replacement clutches) of the treatment and control groups were visited several times to check for the start of laying and hatching. Nestlings were ringed when 15–18 days old, and we measured the length of left and right wing, tail, and right tarsus with a digital calliper (accuracy 0.01 mm), and body mass on a Pesola spring balance (accuracy 1 g). We also took a blood sample from the brachial vein for serological analyses.

(a) Measurement of immunocompetence

The immune system of birds consists of three major components: phagocytosis, T-cell mediated immunity, and antibody responses (Cheng & Lamont 1988). In this study we only gathered direct information on T-cell mediated immunity. When birds were ringed, we injected each nestling intradermally in the centre of the right wing web with 0.5 mg of phytohaemagglutinin-P (PHA-P; Sigma Chemical Co., St Louis, Missouri) in 0.1 ml of physiological saline solution (Bausch & Lomb Co.). The left wing web was used as a control by injecting 0.1 ml of saline solution. The thickness of each wing web was measured with a digital calliper (to the nearest 0.01 mm) at the injection site before and 24 ± 2 h after injection. We measured the wing web thickness three times. The measures were highly repeatable (all $p < 0.0001$), and therefore, we computed the mean value and used it for the statistical analyses. The immune response (wing web index) was estimated as the change in thickness of the right wing web from the day of injection with PHA-P until the following day, minus the change in thickness of the left wing web from the day of injection until the following day.

(b) Serological tests

We measured four serological traits, as follows.

- (i) Sedimentation rate—this was estimated from a blood sample collected in a microcapillary tube (32 mm, 9 μ l). The microcapillaries were placed vertically in a portable refrigerator for 2.5 ± 0.5 h. At the end of this period we measured, with a magnifying lens, the length (to the nearest 0.1 mm) of the capillary containing plasma. We considered the sedimentation rate as the percentage of the capillary containing blood plasma. Since the sedimentation rate is slower when the blood contains more erythrocytes, we regressed the sedimentation rate on

haematocrit and used the residuals of this regression model in the statistical analyses. Sedimentation rate increases during infectious and inflammatory diseases due to large quantities of blood-circulating fibrinogen and globulins (Gustafsson *et al.* 1994).

- (ii) Haematocrit—after having measured the sedimentation rate, we centrifuged the microcapillaries in a portable centrifuge for 3 min and measured the length of the microcapillary containing erythrocytes. We expressed the haematocrit as the percentage of the microcapillary containing red cells.
- (iii) Leucocytes—the amount of white cells was measured in the same capillaries and here again expressed as the percentage of the microcapillary containing white cells.
- (iv) Plasma colour—we scored the intensity of the yellow colour of the plasma following a colour atlas (Küppers 1982). The plasma colour was used as an index of the amount of immunoglobulin in the blood (L. Gustafsson, personal communication).

All physiological parameters were measured in two samples, which allowed us to estimate their repeatability. All traits were highly repeatable (all correlation coefficients $r > 0.85$, all $p < 0.0001$). We subsequently computed the mean which was used for the statistical analyses.

(c) Statistical analyses

Nestlings share a common environment and common genes. For this reason we should expect siblings to have similar phenotypic characteristics when compared with nestlings reared in different nests. We checked this assumption using a one-way ANOVA with nest as a factor. All variables considered showed high internest variation (body mass: $F_{45,126} = 4.04$, $p < 0.0001$; tarsus length: $F_{45,123} = 2.18$, $p = 0.0004$; wing web index: $F_{45,126} = 1.78$, $p = 0.007$; sedimentation rate: $F_{43,64} = 2.50$, $p = 0.0004$; haematocrit: $F_{41,47} = 2.72$, $p = 0.0005$; leucocyte concentration: $F_{41,47} = 2.13$, $p = 0.006$; plasma colour: $F_{41,47} = 5.86$, $p < 0.0001$). As a consequence, considering siblings as statistically independent observations would have inflated the degrees of freedom in the statistical analyses. We therefore used the mean nest value for each trait as the independent observation.

Intraclutch variability was estimated as the coefficient of variation. We compared coefficients of intraclutch variation between first and replacement clutches using non-parametric statistics.

All analyses were performed using SAS (SAS Release 6.4, SAS Institute 1990).

3. RESULTS

(a) Immunocompetence of nestlings of first and replacement clutches

Wing web index, which reflects T-lymphocyte production after injection of an antigen, is likely to depend on parental effort and nestling condition. For this reason, we first regressed wing web index on three potential correlates: body mass, tarsus length and brood size. A stepwise multiple regression showed that only brood size was a significant predictor of wing web index, nests with larger brood sizes having a larger wing web index than small broods (slope \pm s.e., $b = 2.037 \pm 0.722$, $n = 46$, $p = 0.007$, figure 1). The positive correlation between wing web index and brood size

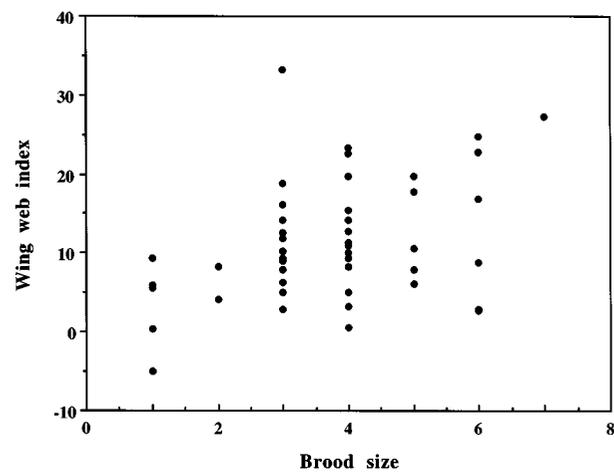


Figure 1. Mean wing web index in relation to brood size (number of nestlings per nest) in the magpie.

did not differ between first and replacement clutches (test of homogeneity of slopes: $F_{3,42} = 2.17$, $p = 0.149$).

Wing web index was significantly larger in nestlings of first clutches than in nestlings of repeat attempts (mean \pm s.e., first brood: 15.83 ± 1.72 ; replacement brood: 7.91 ± 1.73 ; $t = 3.93$, d.f. = 44, $p = 0.0003$). This difference was still present after correcting the wing web index for brood size (mean \pm s.e., first brood: 3.44 ± 1.55 ; replacement brood: -2.64 ± 1.23 ; $t = 3.11$, d.f. = 44, $p = 0.003$). The date when nestlings were injected with PHA-A, which reflects hatching date, was also significantly negatively correlated with the wing web index (slope \pm s.e., $b = -0.389 \pm 0.092$, $n = 46$, $p < 0.0001$, figure 2). However, if first and replacement clutches were analysed separately, the correlation between wing web index and date was only significant in first clutches, but not in replacement clutches (first clutches: $r = -0.607$, $n = 20$, $p = 0.005$; replacement clutches: $r = 0.058$, $n = 26$, $p = 0.779$).

Nestling body mass was negatively correlated with brood size and positively correlated with tarsus length (stepwise multiple regression: brood size, $b = -3.05 \pm 1.02$, $p = 0.004$; tarsus length, $b = 5.49 \pm 0.76$, $p < 0.0001$, $n = 46$). We computed the residuals of this regression and related them to wing web index corrected for brood size. Again first and replacement clutches differed. The correlation between residual body mass and residual wing web index was statistically significant in replacement clutches only (first clutches: $r = -0.224$, $n = 20$, $p = 0.343$; replacement clutches: $r = 0.422$, $n = 26$, $p = 0.032$).

(b) Serological tests

None of the serological traits differed significantly between first and replacement clutches (sedimentation rate: $t = 0.160$, $n = 42$, $p = 0.874$; leucocyte concentration: $t = -0.744$, $n = 42$, $p = 0.461$; haematocrit: $t = 0.082$, $n = 42$, $p = 0.935$; plasma colour: $t = -0.322$, $n = 42$, $p = 0.749$). These parameters were not significantly correlated with date (sedimentation rate: $r = -0.051$, $n = 42$, $p = 0.746$; leucocyte concentration: $r = 0.088$, $n = 42$, $p = 0.580$; haematocrit: $r = 0.058$, $n = 42$, $p = 0.712$; plasma colour: $r = 0.067$,

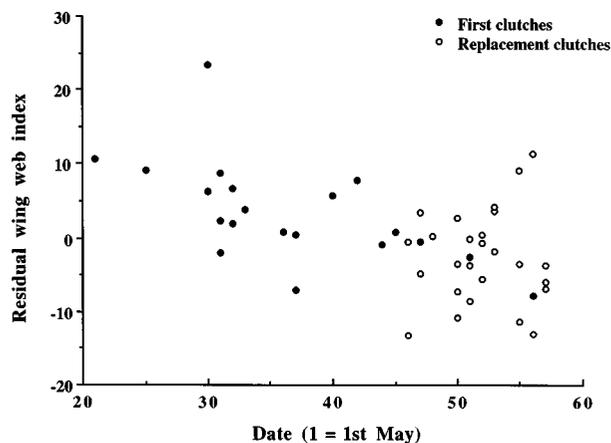


Figure 2. Negative correlation between residual wing web index and date for first and replacement clutches of the magpie. Wing web index was corrected for brood size.

$n = 42$, $p = 0.676$). Only plasma colour was significantly positively correlated with nestling body mass (sedimentation rate: $r = 0.154$, $n = 42$, $p = 0.329$; leucocyte concentration: $r = 0.146$, $n = 42$, $p = 0.357$; haematocrit: $r = 0.040$, $n = 42$, $p = 0.799$; plasma colour: $r = 0.526$, $n = 42$, $p = 0.0003$), and none of the variables was significantly correlated with brood size (sedimentation rate: $r = 0.291$, $n = 42$, $p = 0.062$; leucocyte concentration: $r = 0.151$, $n = 42$, $p = 0.339$; haematocrit: $r = -0.015$, $n = 42$, $p = 0.926$; plasma colour: $r = -0.067$, $n = 42$, $p = 0.673$).

(c) *Reproductive success*

Replacement nests had smaller broods than first clutch nests (mean \pm s.e.: first clutches, 4.25 ± 0.28 , $n = 20$; replacement clutches, 3.35 ± 0.31 , $n = 26$; $t = 2.11$, $p = 0.041$). However, neither body mass nor tarsus length differed significantly between reproductive attempts (residual body mass: first clutches, 1.53 ± 2.21 , $n = 20$; replacement clutches, -1.17 ± 1.89 , $n = 26$; $t = 0.931$, $p = 0.357$; tarsus length: first clutches, 46.74 ± 0.362 , $n = 20$; replacement clutches, 47.61 ± 0.433 , $n = 26$; $t = -1.48$, $p = 0.146$).

(d) *Intraclutch variability*

Intraclutch coefficients of variation for the wing web index were significantly larger in replacement than in first clutches (median of the coefficients of variation: first clutches, $M = 70.07$, $n = 20$; replacement clutches, $M = 119.48$, $n = 21$; Wilcoxon two-sample test, $z = -2.25$, $p = 0.024$). Intraclutch variation of the serological traits did not differ significantly between reproductive attempts (sedimentation rate: Wilcoxon two-sample test, $z = -0.36$, $p = 0.717$; leucocyte concentration: Wilcoxon two-sample test, $z = 0$, $p = 0.999$; haematocrit: Wilcoxon two-sample test, $z = 1.02$, $p = 0.305$; plasma colour: Wilcoxon two-sample test, $z = -1.57$, $p = 0.116$). Among the morphological characters, body mass was equally variable in first and replacement clutches (Wilcoxon two-sample test, $z = -1.03$, $p = 0.303$), whereas tarsus length, like wing web index, tended to be more variable in replace-

ment clutches than in first ones (median of the coefficients of variation: first clutches, $M = 4.05$, $n = 20$; replacement clutches, $M = 5.46$, $n = 21$; Wilcoxon two-sample test, $z = -1.99$, $p = 0.046$).

4. DISCUSSION

Magpie nestlings in replacement clutches were less immunocompetent, as measured by T-lymphocytes production, than nestlings of first clutches. The difference in immunocompetence between reproductive attempts was also apparent at the level of intraclutch variation, siblings of first clutches being more similarly immunocompetent than siblings of replacement clutches. Conversely, none of the serological traits (leucocyte concentration, haematocrit, plasma colour, sedimentation rate) differed between first and replacement clutches.

Several studies have investigated the effect of timing of reproduction on reproductive success and recruitment probability (e.g. Hatchwell 1991; Norris 1993; Wiggins *et al.* 1994), and they have generally provided supportive evidence for late breeders having lower reproductive success and nestlings with lower survival prospects than early breeders.

The mechanistic basis for this general finding has been suggested to be reduced food availability late in the season. Reduced food availability results in a decrease in nestling body mass and thus recruitment probability, since survival prospects are positively related to body mass at fledging. Even though this scenario has received supportive evidence, differences in body mass between reproductive attempts, and the strength of the correlation between mass and survival, are relatively small compared to the large differences usually observed in recruitment rate between clutches. For example, magpies which have a failed first reproductive attempt and produce a replacement clutch have lower reproductive success than pairs with a successful first clutch, and nestlings produced in replacement clutches have lower survival rates (Birkhead 1991), although the difference in nestling body mass between reproductive attempts is small. Birkhead (1991) studied a population of magpies in England and showed that (i) repeat nests fledged 0.5 chicks less than first clutch nests; (ii) chicks hatched late in the season had less than a 20% chance of still being alive in September, whereas chicks hatched earlier had a 40% survival rate; and (iii) hatching date accounted for less than 5% of the variation in nestling body mass. In this study we also found that replacement clutches had a brood size significantly smaller than first clutch nests and that the difference in body mass between first and replacement broods was small and non-significant.

The probability to recruit in a given year depends on the probability of surviving after fledging. Juvenile survival in passerines is usually very low (Saether 1989). Mortality may occur a few days after fledging because of food shortage or exposure to predators, or during the first winter. Another possible source of juvenile mortality is parasitic infections. Several examples of age-dependent mortality induced by parasites exist. For instance, haematzoa can have severe pathogenic

effects on naive non-immune birds, which in most cases are young individuals (e.g. Atkinson & van Riper 1991). Immunocompetence may therefore represent a key factor determining the probability of surviving parasitic infections and, hence, determining the probability of recruitment. Moreover, immunosuppressed birds are also exposed to opportunistic infections which may be an additional significant source of mortality (Korschgen *et al.* 1978). In accordance with this line of reasoning, a positive correlation between immunocompetence and survival has been recently reported for male barn swallows (*Hirundo rustica*) (Saino *et al.* 1997a).

Magpies may differ in their probability of breeding failure. Low quality individuals in territories of poor quality may often fail their first attempt and produce low quality nestlings in a repeat attempt (Birkhead 1991). This could account for the observed correlation between immune response and brood sequence. However, in this study magpies were experimentally induced to fail and therefore should not differ from those which successfully reared a first brood.

The magpie is the second passerine species where a negative correlation between brood sequence and immune response has been documented. Saino *et al.* (1997b) studied the relationship between laying date and immune response (after injection of PHA-P) in nestling barn swallows. As in this study, they found that nestlings from second broods had reduced immune responses as compared to first brood nestlings, and that late breeders produced immunosuppressed offspring. Furthermore, the immune response was negatively correlated with brood size in both first and second broods. This result suggests that offspring quality is traded against offspring number in the barn swallow. In contrast, we found that immune response and brood size were positively correlated in magpies. A positive correlation between major fitness components may arise because of among-individual heterogeneity in phenotypic and/or genetic quality: high quality individuals are likely to produce a large number of high quality offspring (van Noordwijk & de Jong 1986). Magpies usually defend all-purpose territories where all breeding and feeding activities (at least during the breeding season) occur (Birkhead 1991). Territories differ considerably in the amount of resources which gives rise to large differences in reproductive success (Birkhead 1991). Testing the trade-off between immune response and brood size thus requires an experimental approach.

Brood reduction (in its broad definition, see Mock 1994) is a common strategy in birds where females lay more eggs than they are able to rear. When resources are abundant and temporally readily available, parents may succeed in fledging all young. If the environment deteriorates and becomes temporally unpredictable, parents may adopt an alternative strategy with resources not being equally distributed among nestlings, resulting in brood reduction. This strategy, so-called bet-hedging (Seeger & Brockman 1987), should lead to higher intraclutch phenotypic variance in poor and unpredictable environments as compared to good and relatively predictable ones. In agreement with this prediction we found that coeffi-

cients of intraclutch variation in immune response were significantly larger in replacement broods than in first broods. Tarsus length, which reflects growth rate, also tended to be more variable in nestlings of replacement than first broods.

Ecological immunology is becoming a major field of investigation in evolutionary biology (Zuk 1990; Sheldon & Verhulst 1996). The expression and the costs of immune function have been evoked to explain female choice in sexually selected species (e.g. Folstad & Karter 1992; Saino *et al.* 1997a), the trade-offs among life history traits (Gustafsson *et al.* 1994), the evolution of senescence (Miller 1996), and host population dynamics (Lochmiller 1996). This study provides further evidence for the potential role of immune function in one of the best studied subjects of bird ecology: the relationship between laying date and survival probability.

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