Immune Challenge Affects Female Condition and Egg Size in the Grey Partridge

MARCO CUCCO*, IRENE PELLEGRINO, AND GIORGIO MALACARNE
University of Piemonte Orientale, DISAV, Alessandria, Italy

ABSTRACT

As maintenance of the immune system is energetically costly and resource-limited, trade-offs among immune function, body condition, and reproductive allocation are expected. In this study, we experimentally test the possible trade-off between immune response, self maintenance, and reproductive output in breeding grey partridges Perdix perdix. Before laying, half the females were immune challenged with a novel antigen vaccine (Newcastle disease virus, NDV). The challenged females showed a higher erythrosedimentation rate, a serum parameter related to worsened health conditions, but their cell-mediated immune reaction (PHA test) did not differ from that of controls. The NDV-treated females laid smaller eggs (mass, length, and breadth), while the concentrations of antibacterial substances (lysozyme and avidin, two enzymes that confer innate antibacterial immunity) were unrelated to the hen's immune challenge. Our study suggests that an immune challenge can have physiological consequences in terms of self-maintenance and reproductive allocation to the egg. J. Exp. Zool. 313A:597–604, 2010. © 2010 Wiley-Liss, Inc.


As parasites have the potential to reduce the fitness of their hosts, animals have evolved elaborate immune defences against infections (Price, ’80; Loye and Zuk, ’91). Ecological immunology deals with proximate and ultimate explanations of differences in immune investment (Schulenburg et al., 2009). The life history theory has offered the evolutionary theoretical framework within which to analyze self-defence strategies, as well as the concept of trade-offs between immune function, condition and reproductive allocation. In this light, immunoeconomics models assume that mounting an immune response requires resources and/or is energetically demanding (Sheldon and Verhulst, ’96; Norris and Evans, 2000) at the expenses of other vital functions. However, the immune apparatus of animals is not a linear system and can reveal complex interactions where one component can have contrasting or even antagonistic effects on the other (Martin et al., 2006; Allen et al., 2009).

Experimental approaches have demonstrated the presence of trade-offs between some immune component and reproduction in many species, both via an experimental increase of reproductive investment or a manipulation of immune challenge. Results show that an experimental increase of the reproductive investment has frequently impaired some aspect of immunity (rev. in Knowles et al., 2009).

The reverse approach, i.e. how an immune challenge can affect self maintenance and reproductive output, has been utilized in some species models by using an array of different immune challenges and vaccinations, hence affecting differentially innate and acquired, humoral, and cell-mediated immunity. The costs of activating an immunological reaction in a reproducing female is well evidenced considering her reproductive output. In oviparous species, immune-challenged females may lower the frequency of relaying (Ilmonen et al., 2000; but see Uller et al., 2006), may reduce the broods size (Råberg et al., 2000; Hanssen et al., 2004; Marzal et al., 2007), or may decrease reproductive investment in terms of egg mass (Uller et al., 2006; but see Williams et al., ’99). Aside egg mass, the composition of eggs is a key feature of egg quality that can influence the future prospect of propagule survival. Recent studies on insects showed that an immune challenge can alter egg total protein contents (Ahmed et al., 2002; but see Shoemaker and Adamo, 2007) and lysozyme content (Ahmed et al., 2002). In poultry, an immune challenge altered the yolk fatty acids composition (Burnham...
et al., 2003; Viscone et al., 2008), and in the house martin (Delichon urbica) there was a reduced egg androgen deposition in response to a challenge of their immune system (Gil et al., 2006).

In general, selection should favor plasticity in immune responses and their optimal level be modulated by environmental factors (Moret, 2006). It has been shown that environmental conditions may mold the trade-off between immunity and reproductive investment, e.g., in the tree lizard (Urosaurus ornatus), females produced smaller egg follicles than controls when food was limited, but they were able to invest both in reproduction and immune activity when food was unlimited (French et al., 2007). Similarly, with food and water provided ad libitum, adult female crickets (Grillus texensis) maintained a normal reproductive output after repeated immune challenges (Shoemaker and Adamo, 2007). In this line, not all studies aimed at investigating the occurrence of immune-related costs provide unequivocal responses (Schmid-Hempel, 2003). Overall, there are taxonomically widespread demonstrations of immune system costs, but it is quite clear that the exact nature of these costs can vary. Trade-offs and energetic costs seem to depend on the species, the immune challenge employed, and the background environmental conditions (Sadd and Schmid-Hempel, 2008). The aim of our study was to experimentally test the possible trade-off between immune response, self maintenance, and reproductive output in the Grey Partridge (Perdix perdix). According to the hypothesis of a physiological trade-off between various functions (French et al., 2009), we tested whether a stimulation of the immune system did impair female’ condition. Moreover, we tested whether the possible conflict between reproduction and immunity resulted in a decrease of number and quality of eggs.

In this study, breeding females were immune challenged with a Newcastle disease virus (NDV) vaccine. We analyzed females’ health condition, egg size, and egg quality (concentration of avidin and lysozyme, two substances involved in antibacterial innate immunity). The Grey Partridge is an interesting model species, as it has a high investment in eggs (clutch size 15–20) and is highly susceptible to experimental infection with NDV (Geral et al., ’76). Our birds, kept in outdoor aviaries, were all of the same age (first year), to avoid possible age effects (Bonneaud et al., 2003), and food was provided ad libitum to reduce environment-related variations.

METHODS
Study Area and Experimental Design
The study was conducted on grey partridges reared in 2005 at a game breeding farm in Alessandria, NW Italy (Cucco et al., 2006a,b). Twenty-four breeding pairs were housed in individual outdoor reproduction cages (4 m long x 1 m wide x 0.5 m high). The birds experienced natural light and temperature conditions throughout the year, and all of them were 1 year old. The rearing food was a powdered mixture commonly used by aviculturists to provide proper nutrition during egg laying (nutrition facts: protein 19.5%, fat 3.7%, ash 11.5%; vitamin E addition per kg of food: 50 mg). Each pair had food and water available ad libitum. From late April to early July, the hens laid a total of 856 eggs.

Two weeks before laying the first egg, breeding partridges were randomly assigned to two groups, i.e. a vaccinated group (V+) and a control group (V–). Females were immunized orally with NDV live vaccine (Bio-Vac NDV 1000 doses made by Fatto in Ozzano Emilia, Italy) in accordance with the procedure of Kiss et al. (2003). Boosters were given three times at 2-week intervals. Newcastle disease is highly contagious, prevalent worldwide and causes severe economic loss to the poultry industry (Alexander, ’97). NDV was chosen because partridges are highly susceptible to experimental infection with NDV (Geral et al., ’76). Moreover, NDV has been shown to induce both humoral and CMI responses, and the CMI response is considered important for conferring resistance to velogenic NDV (Marino and Hanson, ’87).

Measurements on Breeding Females
Five body, blood, and immune response variables were measured before the vaccination, before the breeding period began (March) and four months later at the end of the breeding period (July). Body mass was measured with an electronic balance (± 0.01 g accuracy) and tarsus length with a calliper (± 0.1 mm accuracy). Blood was drawn from the brachial vein into 75-mm heparinised capillary tubes to measure the erythrosedimentation rate (ES rate) and hematocrit value. The ES rate is diagnostic of many acute and chronic diseases, including infections and rheumatic and inflammatory diseases (Merilä and Svensson, ’95). The ES rate was measured as the ratio between the length of the capillary tube not occupied by blood cells and the total length after the capillaries stood vertically for 4 hr in a refrigerator at 4°C. Hematocrit is an easily measured serological variable, which is diagnostic of acute and chronic diseases, bacterial infections, anemia and dehydration or may reflect nutrition deficiencies of some minerals (Rupley, ’97). Blood samples were centrifuged in a portable apparatus for 4 min at 4,000 rpm, and the hematocrit was expressed as volume of the part of the capillary occupied by blood cells/blood volume in the capillary. We used the phytohaemagglutinin (PHA) test to estimate the cell-mediated immune response. Subcutaneous injection with PHA produces a local inflammation, proportional to the intensity of T-lymphocyte cell-mediated immunocompetence (Smits et al., ’99), and its relative thickness (wing-web index) is directly related to the immune condition [Merino et al., ’99]. Some authors cautioned against a straightforward interpretation of reaction to PHA injection as the classical CMI response, because this method may elicits a complex inflammatory response (Martin et al., 2006). However, recently Tell et al. (2008) justified the widespread
use of the PHA skin test as a reliable evaluator of acquired T-cell-mediated immunocompetence. We measured the thickness of the wing-web area of the breeding individuals with a spessimeter (Alpa spa, Milan, Italy, accuracy \( \pm 0.01 \) mm); the birds were then injected with 0.25 mg of PHA (Sigma L-8754, Milan, Italy) diluted in 0.05 ml of phosphate-buffered saline solution (PBS). After 24 hr, we re-measured the web thickness at the injection point. The response to PHA injection was measured both in March, i.e. before the beginning of the laying period, and in June, after the last egg was laid.

**Measurements on Eggs**

In total, 856 eggs laid by 24 grey partridge hens were collected from April to June, 2005. Clutch size did not differ between vaccinated and control groups (34.9 ± 4.9 vs. 33.6 ± 4.8 se eggs; \( t = 0.19, P = 0.85 \) ns). In this study, we limit the analysis to the first 20 eggs laid (total 469 eggs), i.e. the natural range occurring in nature (Potts, '86). Eggs were collected at the day of laying, individually marked, and weighed using an electronic balance \( (\pm 0.01 \) g precision). The eggs were also measured (length and breadth with a calliper, \( \pm 0.01 \) mm). In the lab, the eggs were separated into their constituent parts with a domestic egg separator sieve. Albumen was then stored at −20°C until analysis.

Lysozyme activity was measured by the method of Osserman and Lawlor ('66): an agar gel with a dried strain of *Micrococcus lysodeikticus* (M-3770; Sigma, Milan, Italy), which is particularly sensitive to lysozyme activity, was inoculated with 25 µL of albumen. Standard dilutions of crystalline hen egg white lysozyme (L-6876; Sigma) (25, 100, 500 and 1,000 µg/ml) were run with each group of test samples. The plates were incubated in a laboratory incubator at 25°C for 18 hr, during which bacterial growth was inhibited in the area of the gel surrounding the albumen inoculation site. The diameters of the cleared zones are proportional to the log10 of the lysozyme concentration. This area was measured using an *ad hoc* ruler, and converted on a semilogarithmic plot into hen egg lysozyme equivalents (HEL equivalents, expressed in mg/L) according to the standard curve. Each egg albumen was assayed twice (HEL was calculated from the average of the two measurements), and for each sample we measured lysozyme activity in triplicate. Intra-assay coefficient of variation was 1.53%, whereas inter-assay coefficient of variation was 1.84%.

The biotin-binding capacity (i.e. active avidin concentration) of albumen samples was measured using biotinylated insulin and biotinylated alkaline phosphatase (AP) (New England Biolabs, Celbio, Milan, Italy), according to the method reported in Groman et al. ('90). The 96-well plates were coated with biotinylated insulin (10 µg/ml) in sodium carbonate buffer (50 mM, pH 9.6) at 37°C for 2 hr, followed by washing three times with PBS–Tween (T-PBS) and blocking with 1% BSA in PBS (PBS–BSA). Albumen samples were diluted 1:6 with 0.5% BSA in PBS, vortexed briefly and incubated for 2 hr at room temperature. Duplicate samples were allowed to bind to biotinylated insulin at 37°C for 1 hr, followed by washing five times with T-PBS. Biotin-saturated samples (17 mg/L; Sigma-Aldrich, Milan, Italy) and BSA were used as negative controls and the assay was standardised with chicken avidin (Sigma-Aldrich) diluted to known concentrations. Biotinylated AP was used to probe the bound biotin-binding proteins diluted 1:3,000 in PBS–BSA (1 hr, 37°C). Para-nitrophenyl phosphate (1 mg/ml, Sigma-Aldrich) was used as a signal molecule and absorbances were measured at 405 nm with a plate reader (Sirio S, SEAC, Florence, Italy). Each egg albumen was assayed twice, and concentration values were calculated as the mean of the two measures. Intra-assay coefficient of variation was 9.51%, whereas inter-assay coefficient of variation was 10.70%, as assessed from a subsample of 12 eggs that were assayed twice.

**Statistical Analysis**

The effects of treatment on female mass, ES, hematocrit, and immune response were tested by repeated-measure ANOVAs, with vaccine treatment as an independent categorical variable. The effects of treatments on egg mass, length, breadth, lysozyme and avidin concentrations were analyzed using linear mixed models (LMM procedure in SYSTAT 12, Wilkinson, 2007), with vaccine treatment as a fixed effect. Because each female laid several eggs, egg characteristics may not have been independent. Hence, to control for the potential effects of female identity, we inserted the parent identity as a random effect in all the analyses, and we also included egg position in the laying order as covariate.

**RESULTS**

**Effect of Vaccination on Adults**

At the beginning of the breeding period, birds of the two groups did not differ in mass, hematocrit, ES rate or immune response (Table 1). At the end of the breeding period, the vaccinated group had a higher ES rate than the control group (Table 1, Fig. 1B), whereas mass, hematocrit, and immune response (Fig. 1A) did not differ between vaccinated and control females.

**Effect of Vaccination on Egg Characteristics**

Egg characteristics were unrelated to egg position in the laying order (Table 2). Eggs laid by vaccinated females were lighter (Table 2, Fig. 2A) and smaller than eggs laid by control females (egg breadth: 20.4 ± 0.1 vs. 20.9 ± 0.1 mm; egg length: 29.1 ± 0.1 vs. 29.8 ± 0.1 mm. Statistics reported in Table 2). Vaccination had no effect on the albumen lysozyme or avidin concentrations (Table 2, Fig. 2B and C).

**DISCUSSION**

Activation of an immune response is considered energetically costly (Sheldon and Verhulst, '96). Evolutionary theory predicts that the immune response should be traded-off with other vital
functions, such as growth, self-maintenance, and/or reproductive output (Norris and Evans, 2000; Hanssen et al., 2004). In this study, challenge with the NDV vaccine significantly altered the ES rate, a blood parameter related to worsened health conditions (Heylen and Matthysen, 2008). Moreover, the vaccinated females laid smaller eggs than controls. These results support the idea of a trade-off between mounting an immune response and allocating resources to other vital functions (Hanssen et al., 2004). However, the egg concentrations of lysozyme and avidin, two important albumen substances with bactericidal action, were unrelated to the hen’s immune challenge, indicating that not all aspects of egg quality were equally impaired.

Captive experiments with food ad libitum have shown both negative (Bertrand et al., 2006) or no effect of immune challenge on body mass (this study; Martin et al., 2003; Pap et al., 2008). However, studies in the wild show in general a negative effect of immune challenge on body mass (Ots et al., 2001; Hanssen, 2006). Not surprisingly, mass loss can be accompanied by an increase of basal metabolic rate, probably causing weight loss (Ots et al., 2001; Eraud et al., 2005). Our results on body mass are in line with the idea that the energetic cost of activating an immune response is low (Svensson et al., ’98; Lee et al., 2005). However, an alternative explanation is that it is difficult to detect high energetic costs in terms of mass in our experimental setup: as the females did not have food limitations, they could easily recover the energy allocated in mounting the immune response.

In immune-challenged females, we found an impaired ES rate, a blood parameter diagnostic of many diseases and

Table 1. Breeding grey partridges.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vaccine</th>
<th>Control</th>
<th>F_{1,22}</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beginning of the experimental period</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mass (g)</td>
<td>396.2±27.9</td>
<td>401.2±15.4</td>
<td>0.294</td>
<td>0.59</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>47.3±11.7</td>
<td>52.5±14.6</td>
<td>0.458</td>
<td>0.51</td>
</tr>
<tr>
<td>Erythrosedimentation rate</td>
<td>0.395±0.148</td>
<td>0.335±0.096</td>
<td>1.38</td>
<td>0.25</td>
</tr>
<tr>
<td>Immune response (mm)</td>
<td>0.402±0.357</td>
<td>0.382±0.215</td>
<td>0.03</td>
<td>0.86</td>
</tr>
<tr>
<td><strong>End of the experimental period</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mass (g)</td>
<td>383.4±40.7</td>
<td>361.7±24.1</td>
<td>3.65</td>
<td>0.09</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>50.0±16.9</td>
<td>48.3±7.3</td>
<td>3.15</td>
<td>0.13</td>
</tr>
<tr>
<td>Erythrosedimentation rate</td>
<td>0.474±0.149</td>
<td>0.332±0.105</td>
<td>4.88</td>
<td>0.038*</td>
</tr>
<tr>
<td>Immune response (mm)</td>
<td>0.350±0.162</td>
<td>0.369±0.163</td>
<td>2.26</td>
<td>0.15</td>
</tr>
</tbody>
</table>

N = 24 females, mean ± SD are reported. Comparison of mean values of mass, haematological parameters, and immune response to PHA in groups tested with different vaccine treatment.
*P<0.05, **repeated measures ANOVA.

Figure 1. Immune response to PHA injection (A) and erythrosedimentation ES rate (B) of grey partridge females with respect to the treatment with a NDV vaccine.

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infections (Heylen and Matthysen, 2008). This blood parameter has also been used to test the relationships with food scarcity (Acquarone et al., 2002), stress of migration (Merilä and Svensson, '95), egg production (Wagner et al., 2008), and more generally appears to be a good indicator of individual quality and condition (Masello and Quillfeldt, 2004). However, we did not find an influence of immune challenge on hematocrit, indicating that the challenge did not cause anemia in the breeding females. The literature on the correlation between different blood parameters and body condition in stressful situations or energetically costly activities (egg laying, fasting, parasite attack, social stress, etc.) is substantial, but the results are somewhat contradictory (Masello et al., 2009). This heterogeneity of results makes a direct interpretation of blood parameters difficult and has stimulated strong debate (Dawson and Bortolotti, '97; Salvante, 2006).

Measuring the strength of the immune response has been used as an alternative to blood parameters in investigations of the effects of an increased immune system demand in the face of a challenge (Forsman et al., 2010). However, it is often difficult to obtain a complete picture of the immune system, as its different aspects (innate and acquired, humoral, and cell-mediated immunity) can be differentially affected by the immune challenging stress, and one component of the immune system can even have antagonistic effects on the others (Arriero, 2009; Allen et al., 2009). In our study, there was no effect of NDV treatment on the reaction to PHA injection. This finding is in accordance with that recently reported in the great tit (Nilsson

\begin{table}
\centering
\begin{tabular}{|l|l|l|l|l|}
\hline
Dependent variable & Factors & F & df & P \\
\hline
\textit{Egg mass (N = 469)} & Vaccine treatment & 4.348 & 1,445 & 0.038* \\
& Laying order & 3.29 & 1,445 & 0.07 \\
\hline
\textit{Length (N = 469)} & Vaccine treatment & 4.181 & 1,444 & 0.041* \\
& Laying order & 1.208 & 1,444 & 0.27 \\
\hline
\textit{Breadth (N = 469)} & Vaccine treatment & 5.028 & 1,445 & 0.025* \\
& Laying order & 0.007 & 1,445 & 0.93 \\
\hline
\textit{Lysozyme concentration (N = 145)} & Vaccine treatment & 1.340 & 1,120 & 0.25 \\
& Laying order & 0.973 & 1,120 & 0.33 \\
\hline
\textit{Avidin concentration (N = 69)} & Vaccine treatment & 0.808 & 1,46 & 0.37 \\
& Laying order & 1.452 & 1,46 & 0.23 \\
\hline
\end{tabular}
\caption{Generalized linear mixed models of eggs laid by grey partridges in groups tested with different vaccine treatment.}
\end{table}

Figure 2. Mass (A), lysozyme (B), and avidin (C) concentrations of eggs laid by grey partridges.
et al., 2007), although further studies are needed to test whether other components of the immune system were impaired (Hőrak et al., 2006; Sarv and Hőrak, 2009). In particular, initial and final antibody titers assessment could be useful to show to what extent the experimental challenge caused the immune response (i.e. production of anti-NDV antibodies).

As found in the majority of bird species (Christians, 2002), the grey partridge egg mass is rather constant (Cucco et al., 2006a), even if external factors such as temperature can slightly influence it (Cucco et al., 2009). In our study, immune-challenged grey partridges laid smaller eggs than controls. Thus far, the only other studies reporting egg mass variation after immune challenge concern oviparous non-avian species, i.e. the Mallee dragon (Ctenophorus fordi: Uller et al., 2006), the tree lizard (French et al., 2007), and a cricket (Shoemaker and Adamo, 2007). In the last two cases, a decrease of egg mass was observed, but only in extreme circumstances. In tree lizards with an immune response after cutaneous wounds, the follicle sizes were smaller but only in females subjected to a food scarcity regimen (French et al., 2007). In food-provided crickets, females maintained reproductive output after repeated immune challenges, and only very high doses of lipopolysaccharide reduced egg weight (Shoemaker and Adamo, 2007). In our study, we found an egg mass decrease in challenged females fed ad libitum. The role of egg mass in influencing future prospects of survival is still debated because the relationship is difficult to infer from correlational studies (Krist, 2009) and a general pattern has not been agreed on due to contrasting results (Williams, 94).

Egg composition is also a key feature of egg quality that can influence future prospects of propagule survival (Williams, 94). In avian studies, the only specific data on egg quality after a maternal immune challenge concern a decrease of yolk testosterone in the house martin (Delichon urbica; Gil et al., 2006) and a change of yolk fatty acids profile in laying hens (Burnham et al., 2003; Viscione et al., 2008). Recent studies on insects showed that an immune challenge can alter the total egg protein contents (Ahmed et al., 2002; but see Shoemaker and Adamo, 2007) and the maternal lysozyme contents (Ahmed et al., 2002). In our study, the albumen concentration of two antibacterial substances (avidin and lysozyme) did not change after vaccination of the mothers. This indicates that the detrimental effect on mass was not accompanied by a variation of egg quality, at least for these two specific albumen substances. Nevertheless, it is conceivable that other yolk (i.e. egg hormones, carotenoids, and Ig) or albumen substances could be reduced by immune solicitation.

In conclusion, this study lends support to the existence of trade-offs among immune response, self-maintenance and reproductive output in the grey partridge. Future studies will provide a more complete picture of the long-term effects of immune challenge in a transgenerational perspective (Lozano and Ydenberg, 2002; Hasselquist and Nilsson, 2009).

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LITERATURE CITED


Lee KA, Martin II LB, Wikelski MC. 2005. Responding to inflammatory challenges is less costly for a successful avian invader, the house sparrow (Passer domesticus), than its less-invasive congener. Oecologia 145:244–251.


