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Effects of immune challenge and supernormal clutch production on egg quality in the red-legged partridge

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ABSTRACT

Background: Because maintenance of the immune system is thought to be resource-limited, trade-offs between immune function, body condition, and reproductive allocation are expected.

Questions: Do females confronted with the simultaneous challenges of immune stimulation and supernormal egg production face a trade-off in terms of self-maintenance (body mass and blood parameters) and/or egg quality?

Organism: Red-legged partridge, *Alectoris rufa*, a precocial bird species with a huge investment in eggs.

Methods: We challenged the immune systems of females, before egg laying, with a novel antigen (Newcastle Disease virus vaccine, NDV). We also removed eggs as they were laid, so as to induce supernormal egg production.

Conclusion: Compared with the other eggs, the last-laid eggs of hens with supernormal production were smaller, contained less yolk, had a lighter shell, and contained albumen with less lysozyme. However, the immune challenge had no effect on female condition or egg quality. Thus we found no evidence of a trade-off between immune function, body condition, and reproductive allocation.

Keywords: *Alectoris rufa*, egg quality, laying order, maternal investment,
NDV vaccine challenge, supernormal clutch.

INTRODUCTION

Life-history theory provides a theoretical evolutionary framework in which to analyse how organisms allocate their resources in relation to different priorities for survival and reproduction (Stearns, 1992). All animals have evolved optimal allocation strategies to counteract the aggression of parasites, organisms able to reduce the fitness of their hosts (Price, 1980). Adaptive defence against parasites is mainly down to the immune apparatus (Boughton *et al.*, 2011), a complex and heterogeneous system whose maintenance can be

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energetically costly and resource-limited (Loye and Zuk, 1991). In this light, life-history theory predicts the existence of trade-offs among immune function, body condition, and reproductive allocation (Sheldon and Verhulst, 1996).

There is experimental evidence to show that an investment in immunity may impair reproduction. In oviparous species, immune-challenged females may lower the frequency of relaying (Ilmonen *et al.*, 2000), reduce brood size (Råberg *et al.*, 2000; Marzal *et al.*, 2007) or decrease reproductive investment in terms of egg mass (Uller *et al.*, 2006; Cucco *et al.*, 2010). In the house martin, *Delichon urbica*, reduced egg androgen deposition has been observed in response to an immune system challenge (Gil *et al.*, 2006), while an immune challenge altered the yolk fatty acid composition in the chicken, *Gallus gallus* (Burnham *et al.*, 2003; Viscione *et al.*, 2008). In some cases, immune-challenged females showed a worsening of their health condition (Costantini and Møller, 2009), accompanied by a decrease in egg quality (Cucco *et al.*, 2010).

For oviparous animals, clutch production represents a huge investment of energy and reserves in a limited period (Nager, 2006). Females can adaptively modify egg composition through the differential deposition of substances that influence embryo growth and health [maternal effects (Mousseau and Fox, 1998)]. Since the allocation of substances in a clutch is costly (Gil *et al.*, 1999; Pilz *et al.*, 2003), a differential within-clutch allocation can be hypothesized and can be related to the expected fitness of the young (Stearns, 1992). Although variation of avian eggs is largely attributable to differences among species, eggs can also vary considerably within species (Ricklefs, 1974). Williams *et al.* (1993) suggested that females allocate nutrients to eggs within a clutch according to the fitness that each egg has by virtue of its position in the laying sequence. Indeed, detailed investigations of intra-clutch variation of egg components in different species have shown a variety of relationships between egg size or components and laying sequence (Kennamer *et al.*, 1997; Lessells *et al.*, 2002). Egg characteristics generally vary with laying order according to species-specific patterns, such as (1) increasing, (2) decreasing or (3) increasing up to the middle eggs and decreasing thereafter (Aparicio, 1999). Furthermore, many bird species ('indeterminate layers') lay additional eggs in response to egg removal (Kennedy, 1991). However, the quality (composition) of the eggs decreases with increasing egg number (Heaney *et al.*, 1998; Nager *et al.*, 2000).

Nidifugous birds are of special interest for the study of female investment in eggs, since they are specialized in a vast nutritional investment in each egg (because they do not feed the chicks after hatching). It has been observed that the eggs of nidifugous birds contain a high amount of yolk (Sotherland and Rahn, 1987; Carey, 1996). Nidifugous birds generally lay large clutches (Ricklefs, 1974), in which the high quality of the egg characteristics must be maintained so as not to decrease the fitness expectancies of propagules (Kennedy, 1991).

In this study, we analysed the relationship between female health condition and egg quality (total mass, shell, yolk, albumen, and concentration of lysozyme, a substance involved in innate antibacterial immunity) in the red-legged partridge, *Alectoris rufa*. Birds were bred in captivity to reduce environment-related variations [a discussion of the advantages and disadvantages of experiments in captive vs. wild conditions is given in Boughton *et al.* (2011)]. Breeding females were subjected to egg removal (inducing a protracted reproductive effort) and to an immune challenge (Newcastle disease virus, NDV). The red-legged partridge is an indeterminate layer that has a naturally high investment in eggs (Cramp and Simmons, 1980; Alonso *et al.*, 2008). Moreover, it is highly susceptible to immune challenge with the NDV vaccine (Geral *et al.*, 1976).

The aim of the present study was to experimentally test the hypothesis of a physiological trade-off among various functions (French *et al.*, 2009), including immune response, self-maintenance, and reproductive output. We wished to determine whether stimulation of the immune system would impair female condition or egg quality, and if females would be able to maintain egg characteristics despite a supernormal clutch production.

METHODS

We studied 45 pairs (22 in 2008, 23 in 2009) selected from a breeding farm stock in Alessandria, northwest Italy (Cucco *et al.*, 2011). All birds were one year old and in their first reproductive season. Throughout the year, the birds were maintained in natural light and temperature conditions. The rearing food was a powdered mixture commonly used by aviculturists to provide proper nutrition during egg laying. Food and water were provided *ad libitum*. In the pre-breeding season, partridges were housed in small groups in outdoor aviaries (20 m long × 3 m wide × 2 m high). Groups were exclusively composed of individuals of the same sex, and females and males had only visual or acoustic contacts during this period.

In 2009, two weeks before laying the first egg, breeding partridges were randomly assigned to one of two groups: a vaccinated group (Vaccine09) and a control group (Control09). Females were immunized orally with NDV live vaccine (Bio-Vac NDV 1000 doses made by Fatro in Ozzano Emilia, Italy) in accordance with the procedure of Kiss *et al.* (2003). Boosters were given three times at two-week intervals. Newcastle disease is highly contagious, prevalent worldwide, and causes severe economic loss to the poultry industry (Alexander, 1997). NDV was chosen because partridges are highly susceptible to experimental infection with NDV (Geral *et al.*, 1976). Moreover, NDV has been shown to induce both humoral and cell-mediated immune responses, and the cell-mediated response is considered important for conferring resistance to velogenic NDV (Marino and Hanson, 1987). In 2008, no vaccination occurred (Control08 group).

The number of days elapsed between vaccination and laying of the first egg was 24.1 ± 3.2 days (mean ± S.E.). Vaccinated and control females did not differ in laying date ($t_{19} = 0.79$, $P = 0.27$).

Measurements on breeding females

Four body, blood, and immune response variables (body mass, erythrosedimentation rate, haematocrit, and immune response to phytohaemagglutinin injection) were measured before the vaccination and before the breeding period began (March). Body mass was measured with an electronic balance (±0.01 g accuracy). Blood was drawn from the brachial vein into 75-mL heparinized capillary tubes to measure the erythrosedimentation rate and haematocrit value. The erythrosedimentation rate is diagnostic of many acute and chronic diseases, including infections and rheumatic and inflammatory diseases (Merilä and Svensson, 1995). The erythrosedimentation 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 had stood vertically for 4 h in a refrigerator at 4°C. Haematocrit is a serological variable diagnostic of acute and chronic diseases, bacterial infections, anaemia, and dehydration, or may reflect nutritional deficiencies of some minerals (Rupley, 1997). Blood samples were centrifuged in a portable apparatus for 4 min at 4000 rpm, and the haematocrit was expressed as the volume of that part of the capillary occupied by blood cells/blood volume in the capillary. We used the phytohaemagglutinin test to estimate the cell-mediated immune response. Subcutaneous injection with phytohaemagglutinin produces a local inflammation proportional to the intensity of T-lymphocyte cell-mediated immunocompetence (Smits *et al.*, 1999), and its relative thickness (wing-web index) is directly related to the immune condition (Tella *et al.*, 2008). We measured the thickness of the wing-web area of the breeding individuals with a spessimeter (Alpa spa, Milan, Italy, accuracy ±0.01 mm); the birds were then injected with 0.25 mg of phytohaemoagglutinin (Sigma L-8754) diluted in 0.05 mL phosphate-buffered saline solution. After 24 h, we re-measured the web thickness at the injection site.

Egg collection and analyses

We inspected the aviary and collected eggs ($N = 554$ in 2008, 566 in 2009) daily during the laying period. This egg removal schedule caused a supernormal deposition of eggs. Instead of a typical clutch [maximum of 10–12 eggs (Cramp and Simmons, 1980)], females laid a mean of 24.9 eggs. All eggs were weighed with an electronic balance (±0.1 g accuracy), and their length and width were measured with a caliper (±0.05 mm accuracy). Some of these eggs were taken for shell, yolk, and albumen weight measurements and for lysozyme analysis (laboratory eggs, $N = 132$ in 2008, 125 in 2009). We took the second, third, fifth, eighth, eleventh, and fourteenth eggs of all females, with the exception of five females that stopped laying before 14 eggs were laid. After separating the yolk from the albumen, we froze the eggs at −20°C.

Lysozyme activity was measured using the lyso-plate method (Osserman and Lawlor, 1966; with modifications according to D'Alba *et al.*, 2010): an agar gel with a dried strain of *Micrococcus lysodeikticus* (M-3770; Sigma), which is particularly sensitive to lysozyme activity, was inoculated with 25 mL of albumen. Standard dilutions of crystalline hen egg-white lysozyme (L-6876, Sigma) (250, 500, 1000, 2000, and 4000 $\text{mg} \cdot \text{mL}^{-1}$) were run with each group of test samples. The plates were incubated at 25°C for 18 h, 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 logarithm of the lysozyme concentration. This area was measured from a photographic image using the image processing software ImageJ (available at http://rsb.info.nih.gov/ij) and converted on a semi-logarithmic plot into hen egg lysozyme equivalents (HEL equivalents, expressed in $\text{mg} \cdot \text{mL}^{-1}$) according to the standard curve.

Statistical analysis

The effects of vaccine treatment on female mass, erythrosedimentation rate, haematocrit, and immune response were tested by repeated-measures analysis of variance (ANOVA), with vaccine treatment as an independent categorical variable.

We analysed egg characteristics using multivariate mixed models with egg mass and egg characteristics as dependent variables. The vaccine treatment was inserted as a fixed effect, and clutch was included as a random effect to control for among-female variation. The possible effect of female condition was tested by inserting mass, haematocrit, blood erythrosedimentation rate, and immune response in the models as covariates. The effect of egg mass and position in the laying order was tested by inserting these variables as covariates. Position in the laying order was inserted both as a linear and a quadratic term to allow a U-shaped pattern of variation, with intermediate eggs being different from first and last eggs laid.

Initially, we simultaneously entered all variables in the mixed models. Then, we used a stepwise backward procedure of selection of independent variables by eliminating, at each step, the variable that had the least predictive power. The stepwise backward procedure led to a final model containing only the variables reaching the 0.05 level of significance. Statistical analyses were performed using SYSTAT v.12 (SYSTAT Software Inc., San José, CA).

RESULTS

Effect of vaccination and prolonged laying on adults

At the beginning of the breeding period, birds of the two groups (Vaccine09 and Control09) did not differ significantly in mass, haematocrit, erythrosedimentation rate or immune response (Table 1). Similarly, at the end of the breeding period, there were no significant differences between vaccinated and control females.

In all groups, the values recorded at the end of the supernormal laying effort did not differ significantly from those measured at the beginning (Table 1).

Effect of vaccination on egg characteristics

In 2009, vaccinated females (Vaccine09 group females) and control females (Control09 group females) did not differ significantly in the number of eggs laid (mean ± s.d.: 25.92 ± 8.28 eggs for the Vaccine09 group, 24.20 ± 7.80 eggs for the Control09 group; $t_{21} = 0.496$; $P = 0.62$). In 2008, the females (Control08 group) laid a similar number of eggs (25.78 ± 6.95 eggs).

Vaccination had no significant effect on egg characteristics. In 2009, eggs laid by vaccinated females did not differ from eggs laid by control females (Table 2). However, there was a

Table 1. Comparison of mean values of mass, haematological parameters, and immune response to phytohaemoagglutinin in groups of breeding red-legged partridges tested with different vaccine treatment (*n* = 23 females) (mean ± s.e.)

	GROUP		ANOVA	
Parameter	Vaccine	Control	$F_{1,21}$	P
Beginning of the experimental period				
Mass (g)	446.6 ± 9.99	436.2 ± 10.54	0.510	0.49
Haematocrit	0.567 ± 0.014	0.556 ± 0.016	0.279	0.60
Erythrosedimentation rate	0.765 ± 0.033	0.743 ± 0.038	0.205	0.66
Immune response (mm)	0.566 ± 0.068	0.387 ± 0.078	3.008	0.10
*End of the experimental period**				
Mass (g)	463.7 ± 11.07	469.5 ± 12.38	0.123	0.73
Haematocrit	0.662 ± 0.030	0.614 ± 0.035	1.108	0.31
Erythrosedimentation rate	0.735 ± 0.028	0.739 ± 0.040	0.009	0.93
Immune response (mm)	0.427 ± 0.073	0.476 ± 0.085	0.177	0.68

* Repeated-measures analysis of variance (ANOVA).

Table 2. Multivariate mixed model analysis of egg mass and egg characteristics, with vaccine as factor, and female mass, haematocrit, erythrosedimentation rate, immune reaction, and position in the laying order of the focal egg as covariates

	Complete model			Stepwise model	
Variable	*t*	*P*	Coefficient (S.E.)	*t*	*P*
Egg mass (n = 1104)					
Vaccine 2009	0.165	0.87	0.067 (0.404)	—	—
Control 2008	−1.301	0.19	−0.711 (0.547)	—	—
Position laying order	6.955	**0.001**	0.116 (0.017)	6.618	**0.001**
Position × position	−5.566	**0.001**	−0.003 (0.001)	−5.135	**0.001**
Female mass	0.758	0.45	0.003 (0.004)	—	—
Haematocrit	1.346	0.18	4.988 (3.706)	—	—
Erythrosedimentation	−0.343	0.73	−0.005 (0.015)	—	—
Immune reaction	−0.252	0.80	−0.093 (0.370)	—	—
Length (n = 1081)					
Vaccine 2009	0.974	0.33	0.246 (0.253)	—	—
Control 2008	−0.817	0.41	−0.281 (0.343)	—	—
Position laying order	−5.700	**0.001**	−0.082 (0.014)	−5.650	**0.001**
Position × position	3.098	**0.002**	0.001 (0.001)	3.020	**0.003**
Female mass	0.610	0.54	0.002 (0.003)	—	—
Haematocrit	1.391	0.16	3.218 (2.313)	—	—
Erythrosedimentation	0.472	0.64	0.005 (0.010)	—	—
Immune reaction	−1.798	0.07	−0.418 (0.233)	−2.044	**0.041**
Egg mass	20.777	**0.001**	0.54 (0.026)	21.715	**0.001**
Breadth (n = 1082)					
Vaccine 2009	−0314	0.75	−0.033 (0.104)	—	—
Control 2008	−3.830	**0.001**	−0.540 (0.141)	−5.086	**0.001**
Position laying order	6.071	**0.001**	0.046 (0.008)	5.783	**0.001**
Position × position	−5.160	**0.001**	−0.001 (0.001)	−4.899	**0.001**
Female mass	−1.191	0.23	−0.001 (0.001)	—	—
Haematocrit	0.514	0.608	0.487 (0.947)	—	—
Erythrosedimentation	−0.252	0.80	−0.001 (0.004)	—	—
Immune reaction	2.148	**0.032**	0.207 (0.096)	2.086	**0.037**
Egg mass	29.972	**0.001**	0.401 (0.013)	31.603	**0.001**
Shell mass (n = 252)					
Vaccine 2009	1.270	0.21	0.124 (0.097)	—	—
Control 2008	−1.736	0.08	−0.231 (0.133)	−3.592	**0.001**
Position laying order	−2.207	**0.028**	−0.057 (0.026)	−4.385	**0.001**
Position × position	1.147	0.25	0.002 (0.002)	—	—
Female mass	0.196	0.85	0.001 (0.001)	—	—
Haematocrit	0.662	0.51	0.591 (0.892)	—	—
Erythrosedimentation	0.845	0.39	0.003 (0.004)	—	—
Immune reaction	−1.110	0.27	−0.096 (0.087)	—	—
Egg mass	4.390	**0.001**	0.094 (0.021)	4.584	**0.001**

Table 2. – *continued*

	Complete model			Stepwise model	
Variable	*t*	*P*	Coefficient (S.E.)	*t*	*P*
Yolk mass (n = 252)					
Vaccine 2009	−0.668	0.51	−0.119 (0.178)	—	—
Control 2008	−2.343	**0.020**	−0.562 (0.240)	—	—
Position laying order	2.833	**0.005**	0.101 (0.036)	3.107	**0.002**
Position × position	−2.603	**0.010**	−0.006 (0.002)	−2.985	**0.003**
Female mass	1.235	0.22	0.002 (0.002)	—	—
Haematocrit	−0.11	0.91	−0.178 (1.620)	—	—
Erythrosedimentation	−1.100	0.27	−0.007 (0.007)	—	—
Immune reaction	1.550	0.12	0.248 (0.160)	—	—
Egg mass	7.848	**0.001**	0.247 (0.032)	8.663	**0.001**
Albumen mass (n = 250)					
Vaccine 2009	0.376	0.71	0.087 (0.232)	—	—
Control 2008	−1.200	0.232	−0.376 (0.313)	—	—
Position laying order	3.822	**0.001**	0.171 (0.045)	3.750	**0.001**
Position × position	−2.717	0.007	−0.008 (0.003)	−2.637	**0.009**
Female mass	0.042	0.97	0.001 (0.02)	—	—
Haematocrit	0.762	0.45	1.613 (2.117)	—	—
Erythrosedimentation	−0.086	0.93	−0.001 (0.009)	—	—
Immune reaction	−0.358	0.72	−0.075 (0.209)	—	—
Egg mass	7.330	**0.001**	0.293 (0.040)	8.272	**0.001**
Lysozyme (n = 148)					
Vaccine 2009	1.473	0.144	184.7 (125.3)	—	—
Control 2008	3.791	**0.001**	671.7 (177.2)	4.372	**0.001**
Position laying order	−1.001	0.32	−60.3 (60.2)	−2.401	**0.018**
Position × position	0.498	0.62	2.25 (4.51)	—	—
Female mass	−1.109	0.27	−1.53 (1.38)	—	—
Haematocrit	−0.047	0.96	−56.7 (1202.6)	—	—
Erythrosedimentation	1.188	0.24	5.74 (4.83)	—	—
Immune reaction	−1.587	0.11	−123.1 (318.4)	—	—
Egg mass	1.868	0.06	58.3 (31.3)	—	—

Note: In the models, female identity was treated as a random factor.

significant difference between eggs laid by control females in 2008 and 2009: the eggs laid in 2008 had a smaller breadth, shell and yolk mass, and had more lysozyme in their albumen (Table 2).

Egg characteristics were significantly related to immunocompetence, but not to the other indices of female health condition (mass, haematocrit, and erythrosedimentation rate). Immune reaction was negatively related to egg length and positively related to egg breadth; hence females in better condition laid more spherical eggs.

Effect of supernormal laying on egg characteristics

Position in the laying order was positively related to egg mass, breadth, yolk mass, and albumen mass (Fig. 1), while there was a decrease in egg length, shell weight, and lysozyme concentration along the laying order (Fig. 1). There were significant negative quadratic coefficients for egg mass, breadth, yolk mass, and albumen mass (Table 1). This determined an inverse U-shaped pattern of variation, with higher values for intermediate egg positions, while the extreme eggs (first- and last-laid eggs) showed lower values (Fig. 1). The only positive quadratic coefficient was found for egg length; in this case, the eggs at extreme positions in the laying order were longer.

DISCUSSION

Evolutionary theory predicts that the immune response should be traded against other vital functions, such as growth, self-maintenance, and/or reproductive output (Norris and Evans, 2000; Hanssen *et al.*, 2004). In this study, to experimentally test for such trade-offs, we challenged female red-legged partridges with the NDV vaccine and induced them, via egg removal, to produce supernormal clutches. Our birds were all of the same age (first year) to avoid possible age effects (Bonneaud *et al.*, 2003; Cabezas-Diaz *et al.*, 2005), and food was provided *ad libitum* to reduce environment-related variations.

Female body condition variables (mass, haematocrit, erythrosedimentation rate, and immune response to phytohaemagglutinin injection) were not significantly affected by the vaccine challenge and none of the health parameters differed significantly between the beginning and end of the protracted egg deposition. These results indicate that conditions were not affected by the two experimental challenges. The vaccine treatment also had no effect on egg characteristics, whereas the increased effort due to laying supernumerary eggs led to a significant decrease in egg quality.

Previous experiments under *ad libitum* food conditions have shown both negative (Bertrand *et al.*, 2006) and nil effects of immune challenge on female body mass (Martin *et al.*, 2003; Pap *et al.*, 2008; Cucco *et al.*, 2010). On the other hand, studies in the wild have generally shown a negative effect of immune challenge on body mass (Ots *et al.*, 2001; Hanssen, 2006). Some studies have shown that mass loss can be accompanied by an increase in basal metabolic rate, probably resulting in weight loss (Ots *et al.*, 2001; Eraud *et al.*, 2005), and in the pigeon *Columba livia* an immune challenge induced a decrease in mass and also caused higher oxygen consumption and an increase of cloacal temperature (van de Crommenacker *et al.*, 2010). Our results for body mass are in line with the idea that the energetic cost of activating an immune response can be low (Svensson *et al.*, 1998; 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 set-up: as the females did not have food limitations, they could easily recover the energy allocated in mounting the immune response.

In the present study, there was no significant effect of vaccine challenge on immunocompetence or blood parameters. In particular, there was no effect on haematocrit, indicating that the NDV immune challenge did not cause anaemia in the breeding females (Williams *et al.*, 2004; Wagner *et al.*, 2008). This result is similar to that reported in the grey partridge *Perdix perdix* (Cucco *et al.*, 2010). In the present study, the erythrosedimentation rate and the cellular immune response to phytohaemagglutinin injection were not markedly influenced by the NDV immune challenge. In a similar experimental set-up with the grey partridge,

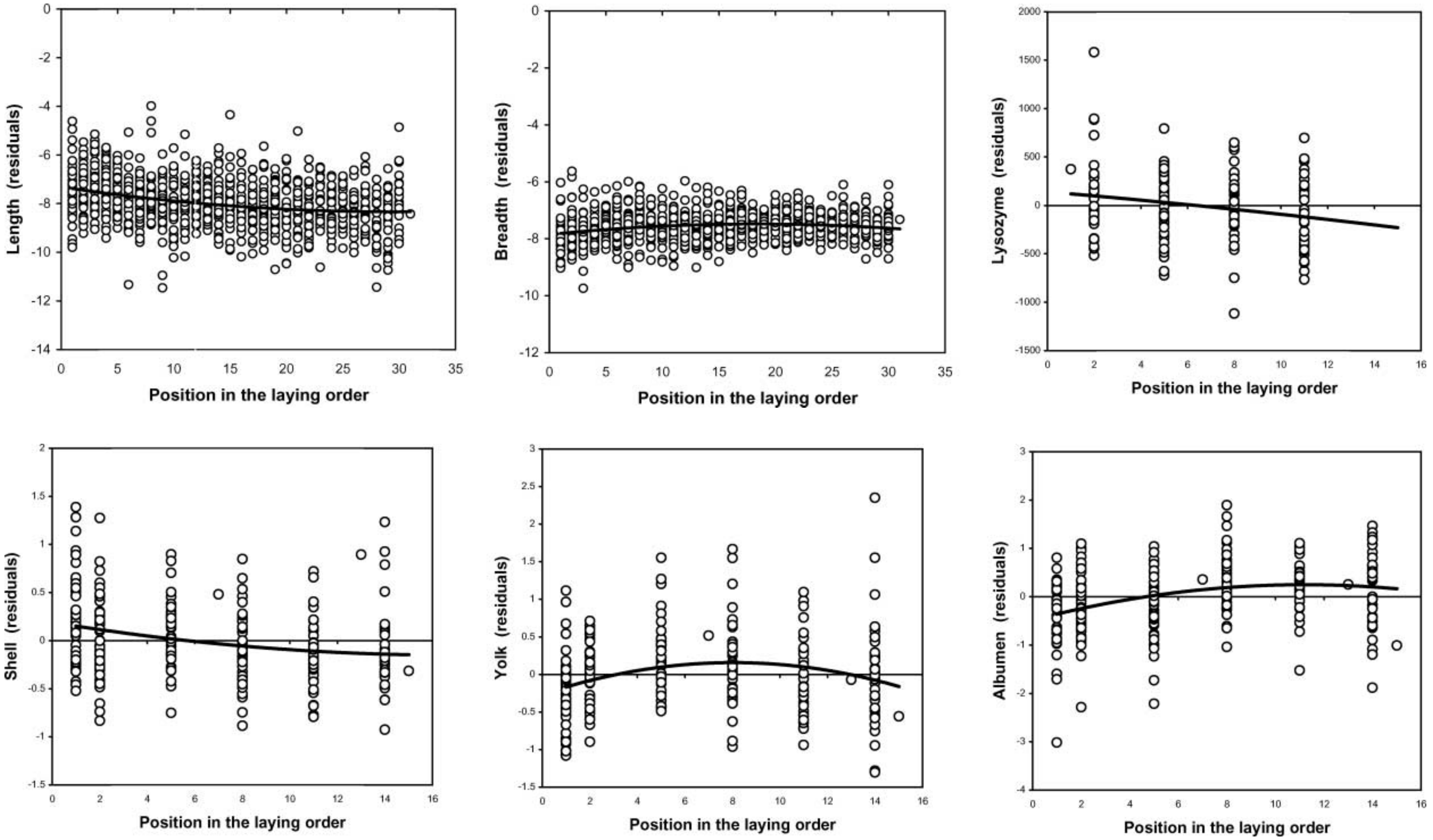

Fig. 1. Relationship between egg length, breadth, and components (residuals: deviations from within-clutch means) and laying sequence for clutches of red-legged partridges. Second-order polynomial lines are shown for each measured variable.

challenged females showed an impaired erythrosedimentation rate, a blood parameter diagnostic of many diseases and infections (Heylen and Matthysen, 2008) and a good indicator of individual condition (Masello and Quillfeldt, 2004). The difference between grey and red-legged partridges could be related to a different susceptibility to NDV of the two species (Geral *et al.*, 1976; Aldous and Alexander, 2008). Our finding on cellular immune response is similar to observations in the great tit *Parus major* (Nilsson *et al.*, 2007). In general, our data on the red-legged partridge suggest that the immune challenge does not impair female health. However, it is difficult to obtain a complete picture of state of health, as its different aspects (blood parameters, humoral and cell-mediated immunity) can be differentially affected. Further studies are needed to determine whether other components are impaired (Hõrak *et al.*, 2006; Sarv and Hõrak, 2009).

Immune-challenged partridges laid eggs similar in mass to the control eggs in the present study. This indicates that red-legged partridges are able to invest in egg production even with stimulation of the immune system. There are few data on possible detrimental effects of immune stimulation on egg mass. In birds, a slight decrease in egg mass after an immune challenge was found in the grey partridge (Cucco *et al.*, 2010). A decrease in egg mass was observed in two reptiles (Uller *et al.*, 2006; French *et al.*, 2007) and a cricket (Shoemaker and Adamo, 2007), but in two of these cases it was found only in extreme circumstances – a food scarcity regimen (French *et al.*, 2007) or a very high dose of immune-stimulating lipopolysaccharides (Shoemaker and Adamo, 2007). It should be noted that the role of egg mass in influencing future prospects of survival is still a matter of debate because the relationship is difficult to infer from correlational studies (Krist, 2011) and a general pattern has not been agreed on due to conflicting results (Williams, 1994).

In this study, albumen, yolk, egg shell, and the concentration of the antibacterial substance lysozyme did not change significantly after vaccination of the mothers. This indicates that the preservation of egg mass was accompanied by maintenance of egg quality. In avian studies, the few 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 acid profile in laying hens (Burnham *et al.*, 2003; Viscione *et al.*, 2008). Our result is similar to that reported in the grey partridge, in which an immune challenge did not alter the concentration of two antibacterial substances (lysozyme and avidin) in the albumen (Cucco *et al.*, 2010).

We measured female condition at the beginning and at the end of a prolonged period of laying, during which there was supernormal egg production. The female mass and blood parameters at the end of egg deposition were not statistically different from those at the beginning. This suggests that, after prolonged egg production, the females did not experience deterioration in health or energetic conditions. Our findings are in line with those of Willie *et al.* (2010) on the health of the zebra finch *Taeniopygia guttata* and of Christians (2000) on mass stores in the starling *Sturnus vulgaris*. Willie *et al.* (2010) investigated the effect of repeated cycles of egg production on haematological traits in female zebra finches. Females maintained haematocrit and haemoglobin concentration at some minimal functional level independently of reproductive effort, supporting the hypothesis that egg-laying females have functionally reduced haematocrit and haemoglobin concentration. Regarding energy stores, starling females experimentally induced to lay one extra egg did not have reduced protein and lipid stores (Christians, 2000). In contrast, Monaghan *et al.* (1998) found that producing one extra egg reduced the lean dry mass of the lesser black-backed gull *Larus fuscus*.

It is conceivable that laying supernormal clutches could have a detrimental effect on egg quality. Indeed, we observed a decrease in egg mass, breadth, yolk mass, albumen mass, and lysozyme content in the last-laid eggs. The relationship between laying order and egg quality was inverse U-shaped, with smaller values at the beginning and at the end of the laying sequence. The low quality of the first eggs was not unexpected: it is known that in this and other partridge species the first-laid eggs are usually of low quality and are laid scattered in an area different from the definitive nest (Cramp and Simmons, 1980).

To date, few studies have reported changes in egg quality in response to supernormal egg production. In each case, the specific egg component was different: a decrease in shell calcium was found in the common tern *Sterna hirundo* (Heaney *et al.*, 1998), a decrease of yolk proteins in the zebra finch (Williams and Miller, 2003), a lower yolk-to-albumen ratio (Verboven *et al.*, 2010) and an increased water content with a decrease of yolk lipids in the lesser black-backed gull (Nager *et al.*, 2000). Female zebra finches, lesser black-backed gulls, and common terns laying extended clutches were able to maintain egg mass in the extra eggs (Heaney *et al.*, 1998; Williams and Miller, 2003), while in the red-legged partridge (this study) and in the great tit the extra eggs where smaller than control eggs (Mänd *et al.*, 2007).

In conclusion, we examined the possible trade-offs among immune response, self-maintenance, and prolonged reproductive output in breeding red-legged partridges. Our results show that of the two challenges, immune and supernormal egg deposition, the former did not cause evident costs, while the latter did not impair female condition but affected the quality of last-laid eggs. Future studies might clarify whether egg phenotype will translate into offspring growth, survival, and recruitment.

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