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Adaptive and non-adaptive explanations for hatching failure in eggs of the Red-legged Partridge *Alectoris rufa*

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Hatching success has been considered one of the main factors associated with fitness in birds. Hatching failure could be the result of two independent events, infertility and death of embryos, which have not been independently considered in most studies. Our study analyses hatching failure pattern in a precocial bird, the Red-legged Partridge *Alectoris rufa*. In trials with captive birds, we tested effects of egg size, laying order and clutch size on infertility and embryo death controlling for maternal condition and age. On the basis of previous studies we predicted that lighter eggs show higher rates of infertility and/or embryo mortality. We also predicted that infertility prevails in first and last-laid eggs.

Infertility represented the most important component of hatching failure in Red-legged Partridge. The importance of egg size in determining hatching success was supported because infertile eggs were lighter than fertile ones. Infertility prevailed in first seven eggs of the sequence of laying supporting previous findings. Hatched and unhatched fertile eggs showed similar weights, but embryo mortality increased with the sequence of laying. Clutch size and maternal body condition did not account for differences in infertility or embryo mortality. We suggest that infertility and embryo death might be caused by physiological constraints on both parents.

Key words: body condition, egg size, embryo mortality, infertility, precocial bird, laying sequence, physiological constraints

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INTRODUCTION

Hatching success has been considered one of the main factors associated with fitness in birds (Koenig 1982, Ojanen 1983, Cooke *et al.* 1985). Several factors have been related to hatching failure in birds: egg size or quality (Parsons 1970, Magrath 1992, Simmons 1997), clutch size (Blackburn 1991, Clutton-Brock 1991), laying

order (Robertson & Cooke 1993, Williams *et al.* 1993, Potti & Merino 1996), parental age or quality (Reid & Boersma 1990, Bolton 1991, Meathrel *et al.* 1993), infections (Bruce & Drysdale 1991, Cook *et al.* 2003, Cook *et al.* 2005) and environmental temperature (Webb 1987, Stoleson 1999).

It has been repeatedly found that egg size is positively related to hatching success by the mediating influence of parental quality and/or laying

order on egg size (Reid & Boersma 1990, Meathrel *et al.* 1993, Williams *et al.* 1993, Clifford & Anderson 2002, but see Potti & Merino 1996). It has also been reported that the interaction between clutch size and laying order is associated to hatching success, with last eggs in large clutches showing a low hatching success (Williams *et al.* 1993, Potti & Merino 1996). In general, the pattern of hatching failure more commonly reported follows a trend along the laying sequence with relatively high rates of failure in both first and last laid eggs of the clutch (Robertson & Cooke 1993, Williams *et al.* 1993, Potti & Merino 1996).

Hatching failure in birds could be the result of two independent events: infertility and death of the embryo (Koenig 1982, Ojanen 1983, Potti & Merino 1996). However, these events have not been independently considered in most studies (but see Potti & Merino 1996). Potti & Merino (1996) stated that hatching failure of first and last-laid eggs in Pied Flycatcher *Ficedula hypoleuca* was mainly due to infertility, although mortality rates of the embryo tended to be higher in last-laid eggs. Infertility has also been reported as the main cause of hatching failure of first-laid eggs in House Sparrows *Passer domesticus* (Cordero *et al.* 1999). Hence, there exists some evidence that infertility is the key factor determining hatching failure of first and last-laid eggs, while embryo mortality along the laying sequence remains poorly explored.

We analyse hatching failure in the Red-legged Partridge *Alectoris rufa* reared in captivity. We discriminate between failures due to infertility and those due to death of the embryo, whereas potentially confounding factors such as parental age, food abundance or disease vectors are controlled. We tested if egg size is related to infertility and embryo survival, hypothesizing that lighter eggs show higher rates of infertility and/or embryo mortality. We also tested if larger clutches show higher rates of infertility and/or embryo mortality (Williams *et al.* 1993, Potti & Merino 1996). Finally, we analysed to what extent hatching success in first and last-laid eggs is related to infertility (Potti & Merino 1996, Cordero *et al.* 1999).

METHODS

The Red-legged Partridge is a precocial bird characterized by large clutches (mean 16–18 eggs, Nadal *et al.* 1996) and double nests (Zwickel 1982, Green 1984). Females start incubation after all eggs have been produced (Nadal 1998). Laying interval between eggs may be variable, but based on our observations the mean is 2.21 (SE 0.07) days. Despite double nesting behaviour, Red-legged Partridge is considered to have only one laying attempt per year (Green 1984). We did not observe laying intervals significantly longer than the mean laying interval. We are, therefore, confident about our assumption that the partridges produced only one clutch per year.

We conducted the study in the 2001 breeding season at Lugar Nuevo farm in Jaén (Spain). In order to control parental age effects we used 32 randomly selected pairs of two-years old reared partridges (second breeding year for all individuals). All partridges were kept as pairs in outdoor pens. We maintained individuals on a high-protein and high-energy (19% and 2750 kcal kg⁻¹, respectively) commercial diet (Purina®) and water supply *ad libitum*. Before and after the breeding period, all females were weighed with a spring balance (Pesola) to the nearest 0.1 g and their tarsus lengths were measured using a digital calliper to the nearest 0.01 mm. Female body condition (as a measure for maternal quality) was estimated as the residual from the linear regression between body mass and (tarsus length)³ (Piersma & Davidson 1991).

All pens were inspected daily to collect eggs. A total of 776 eggs were collected and individually marked with mother number and laying date with a permanent marker pen. Immediately after collection we weighed eggs to the nearest 0.1 g using a dial balance. Although variation in egg quality has not been explicitly considered, fresh mass of the egg is usually correlated with dry weight of the yolk and albumen (Clair 1996, Kennamer *et al.* 1997, Flint & Grand 1999). Egg mass is therefore a good measure of egg quality in terms of macronutrient composition (Williams 1994, Hill 1995).

Eggs were stored in incubator trays located in a room where temperature and relative humidity was controlled (12–13°C and 75%, respectively) in order to arrest embryo development. The trays were turned daily, ensuring that they were tilted in the opposite direction to what they were previously to avoid adherence of embryos to one side. Most eggs were stored less than 40 days (mean 28.6, SE 0.61), which is similar to the pre-incubation period in the field (estimated from mean clutch size \times mean laying interval as 37.6 days). To control for storage time we recorded it for all eggs (see statistical analyses). Eggs were in the incubator for 20 days, and subsequently in the hatcher for the last 3 days. Once into the incubator, trays containing eggs were also turned. The temperature and relative humidity of incubation and hatching rooms were kept at optimum levels (24–28°C and 60%). Incubation temperatures were 37.5 and 37°C for the setting and hatching stages, respectively, while humidity was 47% up to the pipping stage, followed by 75% during hatching.

When eggs were transferred to the hatcher all of them were examined by visual inspection with a light source (candling) to check whether the embryo was developing properly. If not, the eggs were considered non-viable and were removed to avoid the risk of possible bacterial infections (Thear 1987). Dead embryos were detected by evidence of aberrant tissue during candling; infertile eggs were detected because the egg contents remained clear. Candling is a reliable method to detect embryos and to distinguish between viable and non-viable embryos. Eggs that could not be assigned with certainty to one of the categories were treated as viable eggs. Viable eggs were transferred to the hatcher to aid the control of carbon dioxide, as well as to simplify hygiene (Thear 1987). Once the whole process was finished we collected non-viable eggs from hatcher. Non-viable eggs were opened and scored as infertile or fertile (when containing a clearly visible embryo which died before hatching). Eggs considered as infertile contained only yellow yolk. Additionally, eggs coded as infertile were inspected under a 40x binocular microscope for the

presence of the embryonic disc to ensure that eggs were correctly classified as infertile. Nevertheless, embryos that failed at a very early stage of development might have remained undetected, potentially leading to an overestimate of infertility (see Birkhead *et al.* 1995).

Statistical analyses

We analysed factors influencing infertility and death of embryos fitting a generalised linear mixed model (GLMM) with binomial error and a logit link (MacCullagh & Nelder 1989). In this analysis the mother identity was included as a random factor to avoid pseudoreplication (Krackow and Tkadlec 2001) and either infertility of the egg or death of embryo was included as the dichotomic response variable. Several variables of interest were included as covariates: laying order, egg mass, clutch size and female body condition. We also included a quadratic term for laying order to account for the typical concave-up trajectory of hatching failure along the laying sequence recorded elsewhere (Robertson & Cooke 1993, Williams *et al.* 1993, Potti & Merino 1996). Because eggs were stored before the introduction in incubators and the time of storage might have affected the probability of death of embryos, we used time of storage (in days) as a covariate in all GLMM models. We initially fitted a maximal model containing all explanatory variables. Following the principle of parsimony we removed terms from the maximal model to reach the minimal adequate model, that is, the model with the minimal number of terms, in which all the parameters were significant (Crawley 1993). The removal process was performed by progressively deleting non-significant main effects ($P > 0.05$) (Crawley 1993). A reduced model was retained when the change in deviance (which approximates a chi-square distribution) after removing a term was not significant with respect to the ulterior model that included the term of interest.

We ensured that our data followed a binomial distribution by checking over-dispersion (over-dispersion was approximately 1 in all cases) of the statistical models. GLMM analyses were performed

using Glimmix macro in the SAS 9.0 package. Standard statistical analyses were performed using the STATISTICA 99 package. In all cases statistical significance was set at $P < 0.05$.

Table 1. Mean \pm SE for egg mass (g), clutch size (number of eggs laid per female), infertility (% of infertile eggs) and death of embryo (% of fertile eggs that did not hatched) for each female Red-legged Partridge.

Mother	Egg mass	Clutch size	Infertility	Death embryo
1	17.80 \pm 0.31	23	50	0
2	15.51 \pm 0.41	13	93	100
3	16.63 \pm 0.28	28	60	25
4	17.71 \pm 0.28	27	59	20
5	16.63 \pm 0.23	42	39	54
6	17.26 \pm 0.32	22	67	0
7	17.65 \pm 0.25	34	70	38
8	16.70 \pm 0.35	18	37	0
9	17.19 \pm 0.24	37	52	0
10	16.56 \pm 0.40	14	59	20
11	16.48 \pm 0.53	8	88	0
12	18.97 \pm 0.25	34	13	31
13	18.40 \pm 0.32	22	5	15
14	18.13 \pm 0.28	28	17	58
15	14.78 \pm 0.53	8	67	50
16	16.78 \pm 0.38	15	54	0
17	11.95 \pm 0. 75	4	100	-
18	16.58 \pm 0.45	11	50	40
19	17.71 \pm 0.23	40	79	43
20	17.31 \pm 0.30	25	57	0
21	17.05 \pm 0.24	37	49	20
22	17.12 \pm 0.25	36	10	0
23	19.07 \pm 0.22	46	25	18
24	18.28 \pm 0.35	18	50	25
25	18.08 \pm 0.35	18	40	67
26	17.48 \pm 0.23	41	52	45
27	18.98 \pm 0.32	21	44	11
28	18.46 \pm 0.30	25	32	20
29	15.33 \pm 0.33	20	85	0
30	16.57 \pm 0. 25	34	36	11
31	17.92 \pm 0.34	19	6	6
32	15.50	1	100	-

RESULTS

Reproductive parameters of each female, including fertility and hatchability of the eggs, are listed in Table 1. Including fertile eggs only, hatching failure due to death of embryo was 22.8%. Infertility rate reached the 38.3% of the total egg sample.

Correlations between the covariates used in GLMM analyses are listed in Table 2. Although, egg mass was correlated to laying order and clutch size, we used all variables in GLMM models because the effect size (r-value) was small. In addition, we used type III sums of squares in GLMM analyses to test effects of each covariate on the response variable while accounting for the presence of other correlated predictors in the model (Underwood 1997). We did not show correlations between clutch size and laying order because they have no biological sense, and then both were not included in GLMM analyses.

Egg Infertility

The minimal adequate model from the GLMM analysis indicated that period of storage significantly affected the infertility of eggs in a positive way ($F_{1,627} = 133.09$, $P < 0.001$). Additionally, we detected significant effects of laying order (quadratic term, Fig. 1) and egg mass ($F_{1,627} = 3.98$, $P = 0.04$; $F_{1,627} = 7.01$, $P = 0.008$, respectively) while controlling for effects of period of storage. Infertile eggs were lighter than fertile ones (mean 17 g, SE 0.08, vs. 17.98 g, SE 0.07, respectively). Infertile rate was higher than 60% until the 7th

Table 2. Pearson correlations between covariates used in GLMM analyses. Analyses were performed at the egg level ($n = 769$). Correlations between clutch size and laying order were not included because the lack of biological meaning of this relationship.

	Egg mass	Female body condition
Clutch size	0.18 $P < 0.001$	-0.01 $P = 0.68$
Laying order	0.21 $P < 0.001$	-0.009 $P = 0.79$
Egg mass		0.13 $P < 0.001$

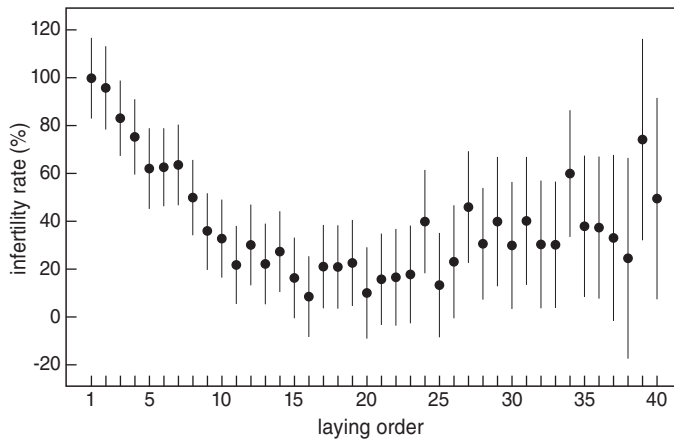


Figure 1. Infertility rate along the sequence of laying.

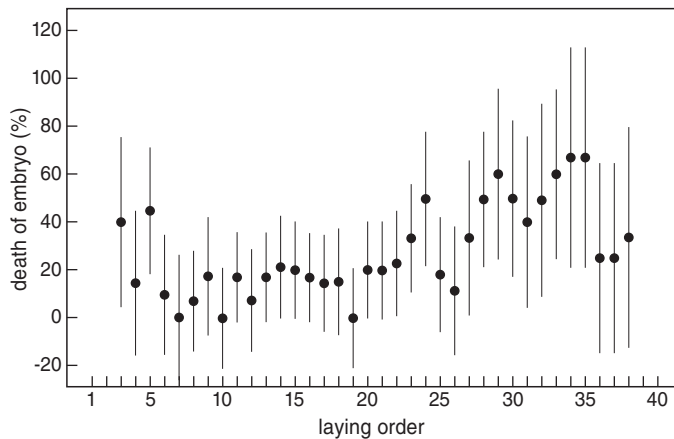


Figure 2. Rate of embryo death along the sequence of laying.

egg, showed the lower values (around 20%) between the 10th and 26th egg, and increased slightly from this position (Fig. 1). Mother identity also affected the infertility probability of eggs ($Z = 2.8$, $P = 0.003$).

Embryo mortality

Death of the embryo depended on laying order ($F_{1,334} = 42.52$, $P < 0.001$), with values around or higher than 50% from the 28th egg (Fig. 2). The percentage of embryo death showed values lower than 20 % in most of the remaining positions of the sequence of laying (Fig. 2). Similarly, time of storage had a positive effect on embryo mortality ($F_{1,334} = 24.01$, $P < 0.001$). Embryo mortality varied among birds ($Z = 2.41$, $P = 0.008$).

Egg mass, clutch size and maternal body condition did not affect death of embryos, and these parameters were not included in the minimal adequate models.

DISCUSSION

Our study showed that infertility represented the most important component of hatching failure in Red-legged Partridge. Because some of the eggs remained long periods in storage, we acknowledge that the infertility rate may have been overestimated at the cost of embryo mortality.

We observed that infertile eggs were lighter than fertile eggs. This result supports that egg

mass positively affected hatching success (Parsons 1970, Magrath 1992, Simmons 1997). Additionally, infertile eggs were most prevalent amongst first-laid eggs. This result supports previous findings (Potti & Merino 1996, Cordero *et al.* 1999). The higher prevalence of infertility in first-laid eggs allowed us to reject inbreeding depression as in that case all eggs along the laying sequence would have been affected similarly. We suggest alternative explanations for the infertility of first laid eggs. Firstly, the most obvious possibility is a lack of sperm in the utero-vaginal junction when females started laying. Hormonal and physiological changes related to seasonal variation of photoperiod, temperature and humidity could incite adult females to start laying despite the absence of sperm. Indeed, solitary females may lay complete clutches (pers. obs.). Tentatively, we suggest that egg laying by unfertilized females could be selectively favoured as a signal to encourage males to copulate, although such mechanism would be expensive for females. Unfortunately, we have no data about mismatches between male and female reproductive activity to test this hypothesis. Secondly, at the beginning of the breeding attempt, the utero-vaginal junction might contain low sperm quantity or/and quality. Both in poultry and wild birds a positive correlation has been shown between egg infertility and low numbers of spermatozoa near the site of fertilization (Small *et al.* 2000, Torok *et al.* 2003). We propose that females should mobilize sperm according to a different reproductive value of eggs along the laying sequence. First-laid eggs may suffer higher pre-incubation abandonment and predation rates (Robertson & Cooke 1993, Flint *et al.* 1994), such that females should optimize the allocation of scarce sperm avoiding the fertilization of first-laid eggs. In relation to low quality sperm, abnormal spermatozoa are not able to cross all barriers to reach the ova (Birkhead *et al.* 1995, Gomendio 2002), which would lead to egg infertility as well.

However, what could be the ultimate reason that some of the eggs in the remaining laying sequence were infertile? Based on the low weight of infertile eggs, we suggest that maternal invest-

ment in infertile eggs was lower than in fertile ones. In this context, we hypothesize that a certain 'pool' of infertile and cheap eggs along all the laying sequence favour survival of fertile eggs by spreading predation risk. We also need to consider that time of storage may influence the probability of premature death of the embryo, but the storage time in the middle of the sequence of laying was always below (mean 22 d, SE 0.56) the 37 days elapsed between first-laid egg and incubation start in the field.

Another common cause of infertility is poor parental condition (Wiggins *et al.* 1994, Potti & Merino 1996). In our study, female body condition was not related to egg infertility, and neither we found any relationship between clutch size and egg infertility.

In relation to embryo mortality, egg weight has usually been considered as a key factor (Simmons 1997 for species with small clutches). In contrast, our study showed that unhatched fertile eggs were not lighter than hatched eggs. In most bird species egg mass is a relatively good estimator of egg quality in terms of energetic contents of yolk and albumen (Ojanen 1983, Williams 1994). Hence, hatchability differences of eggs seemed not to be caused by differences in egg quality. Similarly, Potti & Merino (1996) found high rates of embryo mortality in first and last-laid eggs even though the latter were clearly larger. Consequently, they suggested that intra-clutch egg size would be a poor predictor of energetic contents (see also Bryant 1978). Although several works supported this result, it is important to take into account that most of those did not discriminate infertility from death of the embryo (Moss *et al.* 1981, Williams *et al.* 1993, Clifford & Anderson 2002). Our results indicate that the effect of egg mass on infertility and embryo death was very different, which emphasizes the importance of analyzing the two components of hatching failure independently.

Another factor that has been advocated to explain hatching failure is the position in the laying sequence (Robertson & Cooke 1993, Williams *et al.* 1993). Most studies found higher rates of embryo mortality in the first and last-laid eggs

(Potti & Merino 1996, Williams *et al.* 1993). Our results indicated that death of embryos occurred more frequently in last-laid eggs, while infertility was the main cause of hatching failure in first-laid eggs. Both in precocial and altricial species hatching failure of last-laid eggs has usually been related to the end of the incubation period ('terminal egg neglect hypothesis'; Evans & Lee 1991). However, in our study eggs were artificially incubated, which allowed us to investigate alternative underlying mechanisms. We suggest that subtle changes in egg contents (in terms of hormones and oligoelements, for example) associated with female physiological performance at the end of the breeding attempt could be responsible for the higher embryo mortality in late eggs. In agreement with this hypothesis, it has been shown in Barn Swallows *Hirundo rustica* that the first eggs in a clutch contain more lysozyme than last-laid eggs (Saino *et al.* 2002). Lysozyme is a key component of maternal antibacterial immunity which is transferred to eggs (Sato & Watanabe 1976, Tizard 1991, Braun & Fehlhaber 1996). Hence, embryos from last-laid eggs might suffer from lower immune response to bacterial agents, but further research on intra-clutch variation in egg composition and infection status of dead embryos would be necessary to test this hypothesis.

Finally, our work did not support previous findings about the positive relationship between clutch size and mortality rate of embryos (e.g. Williams *et al.* 1993, Potti & Merino 1996). Our result indicates that there needs not be a trade-off between clutch size and egg quality.

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SAMENVATTING

Het voortplantingssucces van vogels wordt onder meer door het uitkomstpercentage van de eieren bepaald. Het feit dat een ei niet uitkomt, kan het gevolg zijn van zowel onvruchtbaarheid van het ei als van embryonale sterfte. Bij het bepalen van het broedsucces wordt tussen deze twee mogelijkheden zelden onderscheid gemaakt. Dat gebeurde wel in de onderhavige studie aan de Rode Patrijs *Alectoris rufa*, een nestvlinder die de eieren relatief

lang bebroedt. In dit onderzoek aan twee jaar oude vogels in gevangenschap werd het uitkomen van de eieren in verband gebracht met eigrootte, legvolgorde en legselgrootte. Op grond van eerder onderzoek werd verwacht dat lichtere eieren een grotere kans op onvruchtbaarheid en/of embryosterfte zouden hebben, en bovendien dat embryosterfte vooral plaats zou vinden in eieren die als eerste en als laatste gelegd zijn. Het niet-uitkomen bij de Rode Patrijs had vooral met onvruchtbaarheid te maken. Hoe lichter het ei, hoe groter de kans dat het ei onvruchtbaar was. Bovendien was – in overeenstemming met eerder onderzoek – de onvruchtbaarheid het hoogst onder de eerste zeven eieren van een legsel. De embryosterfte was onafhankelijk van het gewicht van het ei, maar nam toe met de legvolgorde. Legselgrootte en lichaamsconditie van de vrouwtjes hadden geen aantoonbaar effect op vruchtbaarheid of embryosterfte. Er wordt verondersteld dat onvruchtbaarheid en embryosterfte beide veroorzaakt worden door fysiologische beperkingen bij beide ouders. (DH)

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