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Are incubation costs in female pied flycatchers expressed in humoral immune responsiveness or breeding success?

Abstract Although clutch size variation has been a key target for studies of avian life history theory, most empirical work has only focused on the ability of parents to raise their altricial young. In this study, we test the hypothesis that costs incurred during incubation may be an additional factor constraining clutch size in altricial birds. In the pied flycatcher (*Ficedula hypoleuca)*, we manipulated the incubation effort of the female by enlarging and reducing clutch sizes. To manipulate incubation effort only, the original clutch sizes were restored shortly after hatching. We found that fledging success was lower among broods whose clutches were enlarged during incubation. There was, however, no effect of manipulation on female body condition or on their ability to mount a humoral immune response to diphtheria or tetanus toxoid during the incubation or nestling provisioning period. Instead, we found that the original clutch size was related to the immune response so that females with seven eggs had significantly lower primary antibody responses against tetanus compared to those with six eggs. Our results suggest that incubating females are not willing to jeopardise their own condition and immune function, but instead pay the costs of incubating a larger clutch by lower offspring production. The results support the view that costs of producing and incubating eggs may be substantial and hence that these costs are likely to contribute to shaping the optimal clutch size in altricial birds.

Keywords Clutch size · Cost of reproduction · *Ficedula hypoleuca* · Immunocompetence · Life history trade-offs

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Introduction

Clutch size variation continues to attract considerable interest in studies of life history evolution. According to optimality models, the optimal clutch size should be determined by the cost and benefit ratio that maximises lifetime reproductive success (Godfray et al. 1991). Like any other life history trait, it is thought to be subject to allocation trade-offs that, in iteroparous organisms, can operate both within and between reproductive attempts (e.g. Sibly and Calow 1984; Lessells 1991).

Clutch size theory has largely been tested in altricial birds with considerable parental care. Most of the experimental work has concentrated on investigating trade-offs between consecutive breeding attempts (cost of reproduction; Williams 1966). Such studies have shown considerable costs for parents feeding experimentally enlarged broods (reviewed in Lindén and Møller 1989; Roff 1992). In contrast, potential costs arising between different phases of the same breeding attempt have gained less attention (e.g. Lessells 1991; Heaney and Monaghan 1996; Jönsson et al. 1998). For example, costs associated with incubation have traditionally been overlooked (e.g. King 1973; Walsberg and King 1978), and experimental tests of costs for later success of a brood arising through incubating a larger clutch are still rare (see Monaghan and Nager 1997 for a review). Such costs might be expected, not least because energy expenditure increases with incubation effort (see Thomson et al. 1998 for a review). The few studies addressing this question have indeed found that an experimentally enlarged clutch has adverse effect on female condition (Jones 1987; Moreno and Carlson 1989), hatching success (Moreno et al. 1991; Siikamäki 1995), prolongs the incubation period (Moreno and Carlsson 1989; Siikamäki 1995) and increases hatching asynchrony (Moreno and Carlsson 1989). Hence, incubation costs apparently play a role in shaping the optimal clutch size.

In this study, we investigated the costs of incubation that a female pied flycatcher (*Ficedula hypoleuca* Pallas) might be facing within a breeding attempt. To do this, we

manipulated clutch sizes after clutch completion, and after hatching, restored the original clutch sizes. Hence, only the incubation effort was altered, while brood sizes (number of nestlings) were kept similar to those which would have resulted under unmanipulated conditions. This experimental design avoids the problem that costs associated with incubation may be confounded by costs of brood rearing. We investigated effects of increased incubation effort on female humoral immune responsiveness, parent feeding rates and breeding success. By restoring the original brood sizes, we were able to test whether manipulated incubation effort affects subsequent parental feeding effort and fledging success, parameters rarely measured in this context. Humoral immune responsiveness during the incubation and brood-rearing phases was measured by immunising females with non-pathogenic antigens (diphtheria-tetanus vaccine). The trade-off between immune defence and parental effort has recently attracted much interest in avian life history studies (see Gustafsson et al. 1994; Sheldon and Verhulst 1996; Møller 1997). Immune defences have been shown to be costly (Ilmonen et al. 2000; Moret and Schmid-Hempel 2000; Råberg et al. 2000), and studies on birds show that an experimentally increased work load during offspring feeding can reduce immune responses (Deerenberg et al. 1997; Nordling et al. 1998; Moreno et al. 1999) and increase blood parasite infections (e.g. Richner et al. 1995; Nordling et al. 1998; Wiehn and Korpimäki 1998). In the current study, we examine whether female flycatchers compromise immune function when incubating enlarged clutches.

Material and methods

Study area and species

The study was carried out in a nest box breeding population (in total ca 320 nest boxes) of pied flycatchers in the surroundings of the town of Harjavalta ($61°20'$ N, $22°10'$ E), south-west Finland during 1998. The pied flycatcher is a small (ca 12–13 g) insectivorous passerine bird that breeds throughout northern and eastern Europe (Cramp and Perrins 1993). It winters in West Africa, and males arrive in our study area in May, about 1 week earlier than females. In our study population, egg laying starts at the end of May, and females lay a clutch of three to nine eggs, with a mode of six. The female incubates alone for approximately 2 weeks. During this period, the male provides some food to the female. Both sexes feed the nestlings until they fledge at the age of 14–16 days. Each female rears only a single brood during a breeding season.

The breeding season of 1998 was exceptionally rainy with the highest monthly rainfall recorded for June during the last 10 years (data from Peipohja Meteorological station, $61°16'$ N, $22°15'$ E). These harsh environmental conditions provided a good opportunity to study within-breeding season trade-offs, because trade-offs will become more obvious when resources are limited (Tuomi et al. 1983; Bell and Koufopanou 1986). Moreover, harsh conditions have been shown to increase the allocation trade-off between parental effort and defence against parasites in birds (Wiehn and Korpimäki 1998; Ilmonen et al. 1999; Wiehn et al. 1999).

Manipulation of incubation effort

We altered the incubation effort of female pied flycatchers by manipulating the number of eggs in their nests. Only females with an original clutch size of six or seven eggs were included in the experiment. Treatments were randomly assigned to females with the same laying date and clutch size. On the second or third day after clutch completion, two eggs were transferred from their natal nest to a foster nest. In control nests, two eggs were exchanged between nests without changing the original clutch size. As a result, three matched groups of nests were formed, with reduced, control and enlarged clutch sizes, all within the limits of natural variation in clutch size. On the second day after hatching, the original (unmanipulated) brood sizes were restored by transferring back two nestlings from enlarged to reduced nests. In the control nests, two nestlings were exchanged between nests without changing the brood size. Unfortunately, a number of nest boxes were exposed to predation by a cat during the incubation period, which resulted in uneven sample sizes among treatment groups. This did not, however, result in among-group differences in laying date ($F_{2,39}=0.44$, $P=0.65$) or original clutch size ($\chi^2=0.63$, $P=0.73$).

General methods

Nest boxes were visited as needed (every 1–7 days) to determine the clutch initiation date, clutch size, hatching date, and number of hatched and fledged young. At the age of 2 days, nestlings were weighed to the nearest 0.1 g with a Pesola 30-g spring balance, and their wing length was measured to the nearest 0.5 mm with a steel ruler. Females were captured for the first time 2–3 days after clutch completion, the second time 2–3 days after hatching, and the third time when their chicks were 13–14 days old and about to fledge. Parent birds and their 13- to 14-day-old chicks were ringed, weighed to the nearest 0.1 g, and had tarsus length measured to the nearest 0.1 mm with sliding callipers, and wing length to the nearest 0.5 mm by the maximum method (Svensson 1992). As a measure of female body condition, we used the residuals from a linear regression of body mass on tarsus length.

Immunisation protocol

Females were immunised for the first time 2–3 days after clutch completion by injecting 100 µl of diphtheria-tetanus vaccine into the pectoral muscle (Finnish National Public Health Institute; diphtheria 38 Lf and tetanus 10 Lf, mixed with the adjuvant aluminium phosphate 1.0 mg/ml). Just prior to injection, we collected a pre-injection blood sample from each female (120–150 µl in heparinised capillary tubes by puncturing the brachial vein with a needle). After 13–14 days (i.e. 2–3 days after chicks hatched), we collected a second blood sample to measure the primary humoral immune response. The females were then immunised for a second time, and a third blood sample was collected 11–12 days later (when chicks were about to fledge) to measure the secondary immune response. The blood was transferred into Eppendorf tubes containing 3 µl of heparin. The tubes were immediately stored in ice-boxes and within 3 h centrifuged at 3,000 rpm for 8 min. The plasma was extracted and stored at –20°C until later ELISA analysis.

ELISA assay

We measured female humoral immune responsiveness as the antigen-specific antibody levels in the females' sera using an ELISA previously developed for red-winged blackbirds (for details of methods, see Hasselquist et al. 1999; Ilmonen et al. 2000). This assay has proved to work for other passerines as well, as shown by much higher post- than pre-injection antibody titres against the injected antigens (D. Hasselquist, unpublished data), and this was also true in our study population of pied flycatchers (Ilmonen et al. 2000). This ELISA method provides sensitive measures of the

amount of passerine antibodies that specifically bind to a certain antigen (here diphtheria or tetanus toxoid). We used the magnitude of the antibody titre as a standardised measure of an individual's ability to mount an antibody response; a higher antibody titre indicated superior humoral immune responsiveness.

We used a diluent of 1% powdered milk in 0.01 phosphate-buffered saline (pH 7.2) to produce 1:1,600 dilutions of each pre- and post-immunisation serum sample. To avoid between-plate variation, we ran serum samples from all studied females on three 96-well ELISA plates for each of the two antigens, and analysed all plates on the same day. A pre-immunisation serum sample from each female was run to investigate each individual's background level of antigen-specific antibodies. For each individual, post-immunisation serum samples were added to the plate in duplicate and the average of these was our measure of antibody titre. On each plate, we ran at least two wells with blank samples (these wells were treated in the same way as test sample wells except that no bird serum was added). As our measure of pre- and post-immunisation antibody titres of individual females, we subtracted the mean value of these blanks from the measured antibody concentration. On each plate, we ran three standard samples covering the range of antibody titres for the immunised females. We used the differences between standard curves and adjusted pre- and post-immunisation antibody titers to control for between-plate variation.

Monitoring feeding rates

Parental feeding rates (feedings per hour) were monitored using video cameras. When nestlings were 10 days old, we placed a video camera about 10 m from the nest box between 9.00 a.m. and 5.00 p.m. For both parents, the feeding rate was not related to the time of the day ($P > 0.5$). Each nest was monitored for a period of 80 min and only during periods with no rain. The first 15 min and last 5 min of recordings were excluded from the analyses to prevent effects of disturbance at the nest. A monitoring period of 1 h has previously been used to successfully estimate parental feeding rates in this population of pied flycatchers (Ilmonen et al. 2000).

Statistical analyses

We ran ANOVA and ANCOVA models, in which we first tested for effects of the original clutch size, the treatment, and their interactions on the dependent variables. If the effect of clutch size and the interaction were non-significant, they were subsequently dropped from the model. We used initial female body condition as a covariate in the analysis of post-hatching body condition. Female post-hatching body condition was used as a covariate in the analysis of body condition at the end of the nestling period. In the analyses of secondary antibody responses, we used primary responses as covariates. The ratio of eggs hatched and young fledged followed Poisson distributions. Hence hatching success (number of hatched chicks/number of incubated eggs) and fledging success (number of fledged young/number of chicks after resuming the original brood size) were analysed using Poisson distribution and log link functions in the GENMOD procedure of SAS statistical software version 6.12 (SAS 1995). Pairwise comparisons in the GENMOD were ran by using the CONTRAST statement (SAS 1997). All results are reported with two-tailed probability values.

Results

Hatching success and the length of the incubation period

The clutch size manipulation had no significant effect on hatching success (number of hatchlings/incubated eggs) or the duration of the incubation period (Table 1). The same was true for the mean body mass and wing length of chicks on the day when we returned the chicks restoring the original brood size (chicks 2 days old; Table 1).

Effects of incubation effort on breeding success

Our clutch size manipulation had a significant effect on fledgling success (number of fledglings/number of

Table 1 Effects of manipulation of incubation effort on female hatching success (percentage of hatchlings/incubated eggs), duration of incubation in days, mass and wing length of 2-day-old nestlings, mass and wing length of 13- to 14-day-old fledglings, female body condition (residuals from the linear regression of body mass on tarsus length) 2–3 days after hatching and at the time their chicks were about to fledge, and female primary and secondary antibody titres to diphtheria and tetanus. Presented are either F-values from ANOVA or ANCOVA, or log-likelihood ratio χ^2 (*) from GENMOD in SAS (SAS 1995)

	Reduced		Control		Enlarged		F/χ^2	P-value
	n	Mean±SE	n	Mean±SE	n	Mean±SE		
Hatching success (%)	18	93.80±3.30	11	95.50±4.20	13	91.50±3.80	0.03*	0.99
Duration of incubation	18	13.67±0.24	11	14.18±0.31	13	13.77±0.28	0.92	0.41
Mean nestling mass	18	3.95±0.11	10	3.69±0.14	13	3.87±0.12	0.98	0.39
Mean nestling wing length	18	8.76±0.26	10	8.90±0.31	13	8.88±0.26	0.09	0.92
Mean fledgling mass	17	14.11±0.25	9	14.45±0.35	10	14.60±0.33	0.78	0.47
Mean fledgling wing length	17	50.84±0.58	9	48.39±0.79	10	49.65±0.75	3.22	0.05
Female post-incubation condition[a]	18	0.96±1.61	11	−1.79±2.06	13	0.19±1.89	0.56	0.58
Female condition at fledgling[b]	17	−0.96±1.49	8	0.46±2.19	10	1.26±1.93	0.44	0.65
Primary response to diphtheria	18	13.01±3.25	11	9.27±4.16	13	15.01±3.83	0.53	0.59
Primary response to tetanus[c]	18	30.57±4.24	11	27.11±5.41	13	24.73±5.10	0.40	0.67
Secondary response to diphtheria[d]	17	37.98±2.75	8	42.92±4.03	10	39.93±3.60	0.52	0.60
Secondary response to tetanus[e]	17	55.01±1.90	8	61.08±2.76	10	55.39±2.47	1.79	0.18

Note: covariates and fixed factor:
[a] Female initial condition $F=6.38$, $df=38$, $P<0.016$
[b] Female post-incubation condition $F=12.54$, $df=31$, $P<0.001$
[c] Original clutch size $F=4.06$, $df=1$, $P=0.05$; manipulation×original clutch size interaction $F=0.69$, $df=2$, $P=0.51$

[d] Primary response to diphtheria $F=32.32$, $df=31$, $P<0.001$
[e] Primary response to tetanus $F=29.65$, $df=31$, $P<0.001$
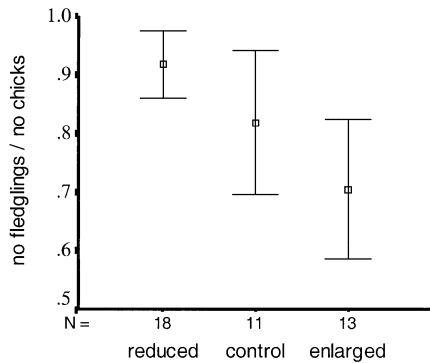
Fig. 1 The mean±SE fledgling success (number of fledglings/ brood size after return) of experimental female pied flycatchers

chicks after restoring the original brood size; GENMOD: treatment $\chi^2=13.71$, $df=2$, $P=0.001$). Hence, females in nests with enlarged clutch sizes had lower fledging success compared to the reduced group ($\chi^2=13.71$, $df=1$, $P<0.001$), whereas there was only a tendency for a difference between the enlarged and control group ($\chi^2=2.90$, $df=1$, $P=0.09$) and between the control and reduced group ($\chi^2=3.12$, $df=1$, $P=0.08$; Fig. 1; pairwise contrasts in GENMOD). We did not find any significant among-treatment-group differences in body mass of fledglings (Table 1). However, the mean wing length of fledglings seemed to be affected by the treatment; females incubating reduced clutches produced fledglings with longer wings compared to control females (Tukey pairwise test, $P=0.04$; Table 1), whereas other pairwise comparisons were non-significant ($P>0.1$).

Effects of incubation effort on feeding rates

We did not find any significant among-treatment-group differences in female feeding rates (one-way ANOVA: treatment $F_{2,31}=0.50$, $P=0.61$; means±SE of feeding rates per hour: reduced 16.69±1.97, $n=16$; control 13.67±2.63, $n=9$; enlarged 14.44±2.63, $n=9$). Similarly, male feeding rates did not differ significantly among the treatment categories (ANOVA: treatment $F_{2,31}=1.37$, $P=0.27$; reduced 15.31±1.57, $n=16$; control 17.78±2.09, $n=9$; enlarged 12.89±2.09, $n=9$). To control for possible variance in parental feeding rates caused by among-treatment-group differences in brood sizes, we ran ANCOVAs where brood size during video recordings was used as a covariate. However, this did not essentially change the results.

Effects of incubation effort on female body condition and immunocompetence

Treatment did not have any significant effect on female post-hatching body condition or body condition measured at the time their chicks were about to fledge (Table 1). Nor did it have significant effects on either female primary humoral responses (measured shortly after hatching) or secondary humoral responses (measured when feeding large nestlings) against either of the two antigens (Table 1). Interestingly, females with an original clutch size of seven eggs had lower primary responses against tetanus compared to those with six eggs (22.49±4.27 and 34.01±3.41, respectively, $P=0.05$; Table 1). The original clutch size did not, however, have any significant effect on the primary responses against diphtheria or secondary responses against either of the two antigens (results not shown).

Discussion

In this study, we investigated within-breeding-attempt costs of incubation in the pied flycatcher by manipulating only the clutch size and measuring its consequences on humoral immune responsiveness and body condition of females, as well as brood-rearing investment and breeding success of the pairs. In some other studies, experimentally increased clutch sizes have resulted in reduced incubation efficiency (Moreno and Carlsson 1989; Moreno et al. 1991; Siikamäki 1995). In this study, however, we did not find any significant among-treatment-group differences in duration of incubation or hatching success. This indicates that females with enlarged clutch sizes accepted the extra eggs and did not reduce incubation efficiency.

Despite the fact that the females apparently adjusted incubation effort according to the manipulated clutch size, increase in effort did not lower humoral immune responsiveness or body condition. Instead, the costs of incubation seemed to have been paid by their young later during the nestling rearing period. This was seen as lower fledging success, measured as the proportion of fledglings per nestlings, in nests with enlarged compared to reduced clutch sizes. In addition, females that had experienced reduced incubation effort produced fledglings with longer wings compared with controls. This is interesting, since wing length has been found to be a good estimate of nutritional condition in nestling birds (reviewed in Nowicki et al. 2000). As there were no among-treatment-group differences in incubation efficiency or body mass of chicks 2 days post-hatching, the lower fledging success is unlikely to have been a result of poor-quality chicks in nests with enlarged clutches, due, for example, to a slow development rate of embryos (see Webb 1987; Doblhammer and Vaupel 2001). Hence, nests with enlarged clutches apparently did less well due to events taking place later during the nestling period. However, we could not find any significant among-treatment-group differences in parental feeding frequencies. Note, however, that feeding frequencies were only recorded when chicks were about 10 days old. Females with enlarged clutch sizes may have already reduced brooding investment in small nestlings during the first half of the nestling period. Brooding investment has been shown to be energetically demanding for females (e.g. Moreno 1989; Moreno and Hillström 1992) and essential for nestling growth and survival (Sanz 1997). Another possibility for lower success in nests with enlarged clutches is that parents provided their nestlings

with smaller prey items or lower-quality food, which may be easier to catch (e.g. Lifjeld 1988; Wright and Cuthill 1989, 1990). Unfortunately, the video recordings do not allow us to test these possible explanations.

Irrespective of the actual causal mechanism, our finding of reduced fledging success as a consequence of increased female incubation effort is in agreement with the results of three other studies where incubation, but not brood-rearing, effort was manipulated (Heaney and Monaghan 1996; Cichoń 2000; Reid et al. 2000; but see Moreno et al. 1991), and contradicts the traditional assumption of incubation as a non-significant effort in avian reproductive costs (see Monaghan and Nager 1997). Heaney and Monaghan (1996) in a study on common terns (*Sterna hirundo*) and Cichoń (2000) in a study on collared flycatchers (*F. albicollis*) found that increased incubation effort resulted in lowered fledgling quality. Reid et al. (2000) in a study with starlings (*Sturnus vulgaris*) found that experimental reduction in the energy demands of incubation led to improved fledging success during the same breeding attempt and better hatching success of second clutches. Our results indicate that within-breeding-attempt costs can have a role in shaping the optimal clutch size in birds. Even if trade-offs between different phases within a single breeding event may be important in understanding adaptive life history decisions (Jönsson et al. 1995, 1998), earlier experimental studies have almost solely focused on between-breeding-attempt costs. Our results also suggest that a common practice to solely manipulate chick rearing effort to measure avian reproductive costs may give an incomplete picture of the costs of producing extra young, because potential costs of parental effort during the early breeding phase are neglected.

Recent studies have shown that an experimental increase in avian nestling provisioning effort can lead to immunosuppression (Deerenberg et al. 1997; Nordling et al. 1998; Moreno et al. 1999), and such physiological trade-offs between parental effort and immune function have been offered as a mechanism behind costs of reproduction (Gustafsson et al. 1994; Sheldon and Verhulst 1996). In the present study, we did not find any detrimental effects of experimentally increased incubation effort on female body condition or antibody production against tetanus or diphtheria toxoid. Similarly, female collared flycatchers with enlarged clutch sizes did not show any reduction in their body condition or antibody responses against sheep blood red cells, but instead paid the costs of incubation with lower-quality fledglings (Cichoń 2000). One possible explanation is that even if incubation is costly (Heaney and Monaghan 1996; Cichoń 2000; Reid et al. 2000; this study), it is likely to be less energy demanding than nestling provisioning. Thus females may be able to increase incubation effort without immediately jeopardising immune defence.

Moreover, a female's lifetime reproductive success may be favoured by investing in self-maintenance and immunity during the early breeding phase, and paying the costs of incubation later, by reducing parental effort during the brood-rearing phase, when parental duties (and costs) can be shared with the male. This might especially be the case if egg production was also demanding. We found that females with an original clutch size of seven eggs showed lower primary antibody responses against another antigen, tetanus, compared to those with six eggs. This result, although correlative, suggests that there may be a resource allocation trade-off between egg production and immune defence. As egg production can be nutritionally and energetically costly in birds (e.g. Ankney 1980; Alisauskas and Ankney 1985; Asthaimer and Grau 1985; Monaghan et al. 1998 and references therein), females might not be willing to pay, in addition, the cost of incubation directly in the currency of reduced body condition or immune function. The combined costs of producing and incubating eggs could have severe fitness effects jeopardising both current and future reproductive success. While there are no earlier studies that have examined the relationship between clutch size and immune function, high investment in egg production has been shown to reduce females' resistance to blood parasites (see Chernin 1952; Oppliger et al. 1996, 1997; Ilmonen et al. 1999).

In conclusion, our finding of lowered reproductive success due to increased incubation effort is in agreement with the results of the three previous experimental studies of fitness consequences of incubation effort (Heaney and Monaghan 1996; Cichoń 2000; Reid et al. 2000). These findings suggest that incubation effort can create within-breeding-attempt costs which reduce the female's capacity to invest in subsequent brood rearing. Furthermore, the correlative finding that females with large original clutch sizes showed low primary antibody responses against tetanus indicates that females may have to trade egg production against their ability to fight parasites and pathogens. Our results suggest that egg production and incubation costs may play a role in determining avian clutch size, and thus that these costs can at least partly explain why the most common clutch size has often found to be less than the calculated optimum.

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