Inter-sexual differences in the immune response of Eurasian kestrel nestlings under food shortage

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Abstract
When resources are limited, parents should decide the optimal number, size, and sex of progeny, and offspring should decide the optimal allocation of resources to different costly functions, such as growth and immunity. We manipulated clutch sizes of Eurasian kestrels by one egg to estimate possible cumulative effects of incubation and chick rearing costs on parental body condition, feeding effort, and offspring viability. No obvious effects of clutch size manipulations on feeding effort were found while feeding effort was adjusted to the original clutch size. Enlarged and control nests suffered from higher nestling mortality than reduced nests, and chicks of the enlarged group were in poorer body condition than chicks of the reduced group. Controlling for body mass, male chicks exhibited lower cell-mediated immunity assessed by a cutaneous hypersensitivity response than females, but only in treatments suffering from food restrictions, as indicated by chick starvation. These novel results reveal inter-sexual differences in physiological strategies in early life, suggesting sex-related differences in susceptibility to disease and consequently in survival prospects of offspring.

Keywords
Clutch size, parental effort, immunity, sex allocation, quality vs. quantity of offspring.

INTRODUCTION
The conflict between quantity and quality of offspring is a classical trade off playing a central role in current theories of life-history evolution (Stearns 1992). Most studies assessing this conflict come from experiments with avian clutch size. A frequent result arising from these investigations is the compromise found between brood size and the mass of the young at independence (Lessells 1991; Korpimäki & Rita 1996). Food shortage may promote a resource reallocation of nestlings, which may constrain growth and other costly physiological functions. Recent studies indicate that the maintenance of immune function is energetically and nutritionally costly (Lochmiller & Deerenberg 2000). Therefore, when food becomes scarce, as in experimentally enlarged broods, the immune function may compete with other physiological functions, such as growth (Saino et al. 1997a; Hörak et al. 1999).

The development of the immune function can also be associated with sex (Grossman 1985). Inter-sexual differences in immunity are a well-known phenomenon in domesticated animals and humans, males usually exhibiting greater susceptibility to diseases and weaker immune response than females (Grossman 1985; Olsen & Kovacs 1996). However, the current knowledge about sex-specific differences in immunity in the wild is scarce (Møller et al. 1998), despite its obvious importance to many aspects of parental care and sexual selection (Hamilton & Zuk 1982; Folstad & Karter 1992; Møller & Saino 1994). There is some evidence for a higher susceptibility to parasite infection (Folstad et al. 1989; Zuk 1990; Møller & Saino 1994) and weaker cell-mediated immunity (Moreno et al. 2001) in adult males compared to adult females, but only one such case has been reported from growing individuals. Male nestlings of the pied flycatcher Ficedula hypoleuca were more prone to suffer from blood-sucking mites than females (Potti & Merino 1996). If immunity is a function competing with growth, inter-sexual difference in immunity is expected to be higher in sexually size-dimorphic species, in which male and female nestlings can exhibit different patterns of growth and mortality (Slagsvold 1982; Teather 1987). In addition, if sexual advertisement is costly to produce or maintain and thus competes for limited resources (Zuk et al. 1995; Moller et al. 1996), it is expected that sexually dichromatic species will exhibit different patterns in the allocation of resources while growing.
METHODS

Study area and experimental design

The study was conducted during the breeding season of 1999 in the Kauhava region, western Finland (63°N, 23°E), where kestrels breed in nest boxes mounted on the gables of barns (Korpimäki & Wiehn 1998). Three to four days after clutch completion, three nests with approximately the same laying date (± 2 days) of the last egg were chosen and randomly assigned into reduced, control and enlarged groups. From the reduced nests, one randomly selected egg was transferred to a nest of the enlarged group. In control nests, one randomly selected egg was removed in a warm box and returned to the nest after 20 min. Ten triplets (30 nests) were finally obtained for the experiment. Nests were visited on average three times (3.2 ± 1.1) during the hatching period to estimate hatching asynchrony. If necessary, we measured wing length of hatchlings to retrospectively determine hatching date by using growth curves obtained from our kestrel population (E. Korpimäki, unpublished work). Two nests from the enlarged group and one from the reduced group were abandoned by the parents two weeks after hatching. These three nests were excluded from analyses. There was no bias in sample size reduction between different groups (\( \chi^2 = 0.30, P = 0.81 \)). There were no obvious between-treatment differences in the original clutch size (ANOVA, \( F_{2,24} = 0.51, P = 0.60 \), mean ± SD: enlarged 5.9 ± 0.4, control 5.8 ± 0.8 and reduced 6.1 ± 0.8) and laying date (ANOVA, \( F_{2,24} = 0.16, P = 0.85 \), enlarged 35.6 ± 6.1, control 33.8 ± 8.4 and reduced 33.8 ± 8.2; 1 = 1st April). There were no between-treatment differences in hatching date, defined as the date of the first egg hatched (ANOVA, \( F_{2,24} = 0.38, P = 0.68 \), enlarged 68.6 ± 6.3, control 65.0 ± 10.7 and reduced 67.1 ± 8.4) or hatching asynchrony (Kruskal–Wallis ANOVA, \( H_{2,24} = 1.41, P = 0.49 \), enlarged 1.9 ± 0.9 days, control 1.8 ± 1.0 days and reduced 1.5 ± 0.8 days).

Prey delivery rate of parents 12 days after hatching was used as an estimate of parental effort. It was measured by 24-h video recordings (Panasonic WV-CP222 camera with WV-LA210C3 lens, Panasonic AG 6124 video recorder) at each nest and defined as the number of prey items delivered to nest (see Wiehn et al. 2000). Male and female parents were captured with a swing-door trap attached to the nest box 15 days after hatching, and their body mass and structural size (culmen, wing, tail and tarsus length) were measured. Mass, tarsus and wing lengths of chicks were measured 24–27 days after hatching.

Assessment of immunocompetence

A phytohaemagglutinin-P (PHA) injection assay (Cheng & Lamon 1988) was used to evaluate in vivo T-cell-mediated immunity (CMI), one of the three major components of the immune system. PHA is a mitogen commonly used in studies of birds because it is considered a benign (Merino et al. 1999) and useful method to evaluate thymus-dependent function (Goto et al. 1978). Twenty-four to twenty-seven days after hatching kestrel chicks were injected intradermally in the wing web with 0.3 mg of PHA dissolved in 0.1 mL of PBS. The thickness of wing web was measured three times with a sliding calliper (to the nearest 0.05 mm) at the injection site before and 24 h after injection. The repeatabilities of both initial (\( r = 0.98, F_{138,278} = 282.3, P < 0.001 \)) and final (\( r = 0.98, F_{138,278} = 149.5, P < 0.001 \)) measurements were high, and we used mean values of these three measurements. The difference between initial wing web thickness and swelling was used as a response estimate (Smits et al. 1999).

Sex identification

A drop of blood (20–50 μL) was collected from hatchlings by means of brachial vein puncture and stored in ethanol for later sexing in the laboratory. Kestrel chicks were sexed by using a polymerase chain reaction (PCR) amplification based on the technique used by Fridolfsson & Ellegren (1999). DNA was isolated from the red blood cells using the Qiagen QIAamp kit and the manufacturer’s protocol. PCR amplification was run using a particular set of primers (2550F and 2718R), as proposed by Fridolfsson & Ellegren (1999). The method was tested with 16 male and 26 female parent kestrels. All of them were correctly sexed.

Statistical procedure

Body mass of parents was correlated with wing length (\( r = 0.62, F_{1,24} = 15.02, P = 0.001 \) and \( r = 0.41, F_{1,26} = 5.52, P = 0.027 \) for males and females, respectively). To explore the possible between-group variation in body mass...
of parents we controlled for the individual size including wing length as a covariate in the models instead of using residuals from both linear regressions (García-Berthou 2001). In the case of chicks, wing length at the end of the nestling period was linearly correlated with their age ($r = 0.76$, $F_{1,137} = 189.5$, $P < 0.0001$). Body size of chicks was estimated by combining wing length (corrected by age) and tarsus length in a principal component analysis. Both variables showed a normal distribution. PC1 explained 65.3% (eigenvalue = 1.31) of the variation in these two measurements. Because kestrels are sexually dimorphic in body size and weight (Village 1990; Massemin et al. 2000), we controlled for the effect of sex. Body condition was determined as the residuals from an ANCOVA, in which the cube root of body mass was the response variable, sex a factor and PC1 a covariate (ANCOVA, sex $F_{1,136} = 29.0$, $P < 0.0001$, PC1 $F_{1,136} = 19.2$, $P < 0.0001$). When using mean values of the chicks in the analyses, we removed the effect of sex on body mass by using the residuals from an ANOVA, in which body mass was entered as response variable and sex as factor (ANOVA, $F_{1,137} = 48.8$, $P < 0.0001$). Values of individual nestlings were only used when we explored the effect of sex on body condition and CMI response. Within-nest sexual differences in body condition and CMI-response of chicks were analysed using generalized linear models (GLM) in SAS software. To avoid pseudo-replication, a three-factor nested ANOVA was performed in which sex was nested within treatment and treatment nested within triplets. The interaction between sex and treatment was explored in the same model.

Analyses of sex ratio and mortality variation were performed by GENMOD procedure in SAS statistical software (SAS 1989–96 Institute Inc., Cary, NC, USA). For brood sex ratio, the number of sons was entered as the dependent variable and number of hatched chicks in the brood as the binomial denominator (PROC GENMOD, logit link function, binomial distribution). In the analysis of mortality we used the number of dead chicks per brood as the response variable (PROC GENMOD log link function, poisson distribution). Statistical tests are two-tailed and mean ± SE are given.

**RESULTS**

**Parental effort and offspring viability**

Our manipulation induced significant differences in the number of hatchlings between enlarged and reduced groups (Table 1) but there was no obvious difference in the number of fledglings. Thus, the number of dead nestlings was higher in both enlarged (0.63 ± 1.0) and control (0.30 ± 0.7) nests than in the reduced (0.0 ± 0.0) nests (GENMOD $\chi^2 = 7.54$, $P = 0.003$; between-group contrasts: reduced-control $\chi^2 = 3.85$, $P = 0.049$, reduced-enlarged $\chi^2 = 7.54$, $P = 0.006$, control-enlarged $\chi^2 = 1.05$, $P = 0.31$). However, only the difference between reduced and enlarged nests was still significant after the sequential Bonferroni correction for multiple probability estimation (Rice 1989). Hatching sex ratio was 48 ± 21.2% and did not differ significantly between treatment groups (GENMOD $\chi^2 = 2.85$, $P = 0.24$). We did not find sex bias in the nestling mortality, as the fledgling sex ratio did not differ from hatching sex ratio in either enlarged ($\chi^2 = 0.01$, $P = 0.95$) or control ($\chi^2 = 0.16$, $P = 0.69$) nests.

Prey delivery rate of parents was similar in all the experimental groups (Table 1), and it was positively correlated with original clutch size ($r = 0.64$, $n = 16$, $P = 0.007$) but not with experimental clutch size ($r = 0.38$, $n = 16$, $P = 0.14$). These correlation coefficients were not significantly different ($Z = 0.91$, $P > 0.1$). When controlling for original clutch size in a multiple regression, prey delivery rate did not vary with fledging sex ratio ($r = -0.30$, $F_{1,13} = 1.71$, $P = 0.24$). The body condition of male parents was not affected by the treatment, whereas body condition of female parents tended to be poorer in the enlarged group than in the two other groups (Table 1). Neither male nor female parent body condition was

Table 1 Effects of clutch size manipulation on reproductive traits, parental body condition and parental provisioning rates of Eurasian kestrels. Means ± SD followed by different letters (a, b or c) are significantly different according to Tukey’s *posteriori* test. Body mass of parents was corrected by wing length as a covariate. CMI response of chicks was corrected by body mass index as a covariate. Adjusted means are shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enlarged</th>
<th>Control</th>
<th>Reduced</th>
<th>d.f.</th>
<th>$F$</th>
<th>$P$</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental clutch size</td>
<td>6.9 ± 0.4a</td>
<td>5.8 ± 0.8b</td>
<td>5.1 ± 0.8c</td>
<td>9</td>
<td>2.24</td>
<td>14.0</td>
<td>0.000</td>
</tr>
<tr>
<td>No. hatching</td>
<td>6.1 ± 0.8a</td>
<td>5.5 ± 0.9ab</td>
<td>4.8 ± 0.9b</td>
<td>9</td>
<td>2.24</td>
<td>4.43</td>
<td>0.022</td>
</tr>
<tr>
<td>No. fledglings</td>
<td>5.6 ± 1.0</td>
<td>5.1 ± 0.9</td>
<td>48 ± 0.9</td>
<td>9</td>
<td>2.24</td>
<td>1.26</td>
<td>0.301</td>
</tr>
<tr>
<td>Male body mass</td>
<td>176.0 ± 18.4</td>
<td>178.6 ± 16.2</td>
<td>173.5 ± 16.4</td>
<td>7</td>
<td>2.21</td>
<td>0.33</td>
<td>0.763</td>
</tr>
<tr>
<td>Female body mass</td>
<td>210.4 ± 22.0</td>
<td>229.1 ± 18.7</td>
<td>226.5 ± 18.7</td>
<td>9</td>
<td>2.23</td>
<td>3.02</td>
<td>0.068</td>
</tr>
<tr>
<td>No. prey items 24 h$^{-1}$</td>
<td>21.0 ± 2.9</td>
<td>19.6 ± 3.2</td>
<td>21.6 ± 7.5</td>
<td>5</td>
<td>2.14</td>
<td>0.22</td>
<td>0.801</td>
</tr>
<tr>
<td>Fledgling body condition</td>
<td>12.1 ± 16.4a</td>
<td>3.7 ± 13.8ab</td>
<td>7.7 ± 16.1b</td>
<td>9</td>
<td>2.24</td>
<td>3.86</td>
<td>0.035</td>
</tr>
<tr>
<td>CMI response</td>
<td>2.6 ± 0.5</td>
<td>3.3 ± 0.6</td>
<td>3.5 ± 1.0</td>
<td>9</td>
<td>2.23</td>
<td>2.29</td>
<td>0.124</td>
</tr>
</tbody>
</table>

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correlated with fledgling sex ratio ($r = 0.06$, $F_{1,24} = 0.1$, $P = 0.74$ and $r = 0.26$, $F_{1,25} = 1.32$, $P = 0.31$, respectively).

Chicks from enlarged clutches were in poorer body condition than those from reduced clutches, whereas no difference was found between control and both reduced and enlarged groups (Table 1). Similarly, body mass index tended to be lower in enlarged than in reduced groups (ANOVA, $F_{2,24} = 3.03$, $P = 0.054$). Body mass explained 28% of the variation of the T-cell mediated immune response of chicks ($r = 0.53$, $F_{1,137} = 53.11$, $P < 0.0001$). CMI response was also positively associated with body condition index ($r = 0.39$, $F_{1,137} = 25.0$, $P < 0.0001$), but it explained a lower percentage of the variance (15%). These linear regressions are not based on independent observation units because we used all the nestlings for calculations (see Methods). But, these regressions were only performed to explore the variables for which CMI response should be controlled for. CMI response of chicks differed between treatments (ANOVA, $F_{2,24} = 3.92$, $P = 0.033$), but when corrected by body mass this difference was no more detectable (Table 1). Immune response corrected by body mass index was not significantly associated with laying date, original clutch size, prey delivery rate, or body condition of parents (all $P > 0.13$). We corrected by body mass, because it explained a higher proportion of the variance of CMI response than the body condition index.

After removing the effect of sexual size dimorphism, nested ANOVA showed that male chicks were in poorer body condition than female chicks (Model, $F_{51,87} = 9.65$, $P < 0.001$, sex, $F_{46,86} = 8.69$, $P < 0.001$, interaction sex × treatment $F_{5,86} = 11.96$, $P < 0.001$). Bonferroni $a posteriori$ tests showed that this inter-sexual difference was only observed in enlarged nests, whereas no similar differences were found in control and reduced nests. We looked for possible inter-sexual differences of CMI response in a new nested ANOVA, in which all the treatment groups were pooled. Results showed that, in general, females were prone to mount a stronger immune response than males (Model $F_{51,87} = 7.10$, $P < 0.001$). This difference was maintained after including body mass as a covariate (Model $F_{52,86} = 8.18$, $P < 0.001$, sex $F_{46,86} = 5.01$, $P < 0.001$, interaction sex × treatment $F_{5,86} = 9.21$, $P < 0.01$, body mass $F_{1,86} = 13.13$, $P < 0.001$). Bonferroni $a posteriori$ tests showed significant inter-sexual differences in CMI response in the enlarged and control nests, but not in the reduced nests (Fig. 1).

**DISCUSSION**

**Parental effort and body condition**

Our experiment induced significant between-treatment differences in the clutch size between all experimental groups and in the number of hatchlings between reduced and enlarged groups. Differences in incubation effort by parents (Moreno et al. 1991; Moreno & Sanz 1994) should be assumed as female kestrels of the enlarged nests incubated successfully more eggs than females of the reduced ones. Females of enlarged nests tended to be in poorer body condition than females of control and reduced nests, but neither the incubation effort nor brood size, nor the cumulative effort of both, resulted in any obvious variation in body condition of males. Similarly, the experiment seemed not to affect feeding effort of parents, which is known to be positively correlated with the proportion of the time the male parents use for hunting (Tolonen & Korpimäki 1994). The sample size for feeding rates was relatively small, and consequently the statistical power of the test (see Table 1) was not high enough to establish a convincing conclusion on its own. However, the association between feeding rate and original clutch size leads us to suspect that parents did not markedly respond to our experimental manipulation by varying their feeding effort. This is consistent with the proposition that kestrel males in the variable food conditions of our study area are reluctant to increase foraging effort above the level set for the original clutch size (Korpimäki & Rita 1996; Tolonen & Korpimäki 1996; Wiehn et al. 1999).

The weak trend found in female body condition according to the manipulation might indicate that females of the enlarged group are paying a cumulative cost of increased incubation and brood rearing efforts. A similar weak trend in female body condition was also found in previous studies in our kestrel population, when only brood-size manipulations were done (Korpimäki & Rita 1996). This may suggest that incubation effort is not particularly costly, at least in our study conditions, probably because the
food is supplied by males during the incubation (Village 1990; Tolonen & Korpimäki 1994).

**Nestling body condition and immunity**

If we assume that the number of prey items delivered by parents did not vary according to the experimental treatments, an increased mortality and signs of food restrictions on chicks in experimentally enlarged clutches compared to reduced clutches would be expected. Chicks of enlarged broods suffered from higher mortality and were in poorer body condition than chicks of the reduced group.

Immune response was associated with fledgling body mass and condition, a common finding in studies of birds (Alonso-Alvarez & Tella 2001). This suggests that immunocompetence is a food-dependent function when chicks are growing.

**Effect of sex on body condition and immunity**

The present study on a wild kestrel population is consistent with previous laboratory studies where sex-related differences in immunocompetence were detected (Grossman 1985; Olsen & Kovacs 1996). This difference emerged in early life and only in treatments suffering from food restrictions, as denoted by chick starvation (although after using Bonferroni correction mortality in control group was not significantly different from reduced group). The heritability of phenotypic characters, such as tarsus length and body mass, has been often reported (see Falconer 1989; Stearns 1992). Similarly, heritable components of the immune system have been identified in studies on wild birds and poultry (e.g. Cheng & Lamont 1988; Saino et al. 1997a; Brinkhof et al. 1999; but see Tella et al. 2000a). In the enlarged nests there were nestlings from two different families, inflating the variance within the enlarged group. But even so, we found inter-sexual differences in the body condition and immune response of nestlings of the enlarged group, and therefore our results are conservative in this respect. In addition, sex differences in immunity was similarly found in the control group where chick mortality was also observed. We do not have data that allow us to establish a plausible cause of the sex difference in immunity. Parallel to immune depression, we found that male (the smaller sex) chicks also had poorer body condition than female chicks in nests with food shortage. This difference could be due to a differential food intake of male vs. female nestlings. Size-mediated female dominance may be more apparent in raptor broods where siblings compete for monopolizable prey, as Anderson et al. (1993) have reported in the moderately size-dimorphic American kestrels Falco sparverius (females being 9% heavier than males).

However, sex-related differences in T-cell mediated immunity were not found in this kestrel species (Tella et al. 2000b), although food conditions were not explored in that study. In our kestrel population, we have found similar results in relation to the amount of food consumed.

Female nestlings had competitive superiority to access food over their male nest-mates when the prey delivered by parents was small enough to be handled by the nestlings (Fargallo, Laaksonen, Korpimäki, Pöyri, Griffith and Valkama, unpublished work). This is supposed to be the situation at the nest during the last 2 weeks before fledging, when parents do not dismember the prey items. These results indicate that female nestlings were able to get more food and to reach better body condition than males at fledging time in those nests suffering from food shortage. However, controlling for body condition or body mass, female nestlings still showed a stronger cell-mediated immune response, suggesting that physiological functions other than growth are in competition with the immune function in male chicks.

Sexual dichromatism has previously been observed in nestlings; male nestlings showing grey feathers on the rump and tail. If secondary sexual traits are costly to produce and/or maintain (Zahavi 1975), sexual immune dimorphism might result from an energetic trade-off between immune function and production of sexual characters (Wedekind & Fostad 1994).

Our study reveals, to our knowledge for the first time in the wild, inter-sexual differences in the performance of one component of the immune system, T-cell mediated immunity, before emancipation. The interest of these results in an evolutionary context arises from the fact that sexual differences in immunocompetence can be observed in early life and, in addition, they are only patent (or at least more patent) when food becomes scarce. Assuming that immune response can be an indicator of survival (Saino et al. 1997b; Christie et al. 1998; Hörak et al. 1999; Soler et al. 1999; Tella et al. 2000b), these findings indicate that under food shortage, male chicks can suffer from higher susceptibility to parasite infections than female chicks. This may promote inter-sexual differences in survival prospects. Our study is only applicable to a short time period during growth. It is important to know whether male nestlings are able to compensate for poorer body condition and immunity after fledging. The ability to compensate for poor juvenile nutrition is particularly well developed in birds (Ricklefs 1983). But recent research suggest that, although compensatory growth can bring quick benefits, it is also associated with a variety of costs that are often not evident until much later in adult life (Metcalfe & Monaghan 2001). On the other hand, cell-mediated immune response measured at the fledging stage seems to be a good estimator of future survival (Hörak et al. 1999; Soler et al. 1999; Tella et al. 2000b). Hormonal differences between the sexes, with their modulating effects on behaviour and immune responses,
may also induce sex differences in physiological strategies (Grossman 1985; Folstad & Karter 1992; Owens & Short 1995; Olsen & Kovacs 1996).

Inter-sexual difference in immunity in early life might be an agent mediating the operational sex ratio of populations, and potentially promoting parental decisions on the determination of offspring sex ratios associated with natural food availability (Trivers & Willard 1973). Unfortunately, despite the Eurasian kestrel being a well-studied species, precise studies about sex-related offspring survival that eliminate sex-related variation in natal dispersal as a possible interfering factor are lacking. Future work should be focused on exploration of the physiological mechanisms mediating the relationships between endocrine and immune functions, and the possible costs of producing secondary sexual characters during growth.

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REFERENCES


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