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Within-brood size differences, sex and parasites determine blood stress protein levels in Eurasian Kestrel nestlings

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Summary

1. Brood hierarchies established through hatching asynchrony are supposed to be costly for small chicks because of impaired growth and survival. An additional cost that has remained unexplored is the stress imposed by competition for resources in the nest.
2. In the present study of broods of Eurasian kestrels, we have used the level of heat shock proteins such as Hsp60 and Hsp70 in peripheral blood as well as the heterophile/lymphocyte ratio to detect stress in nestlings. The sex of nestlings and their *Caryospora* sp. oocyst excretion were included in analyses.
3. Nestlings showing a large size difference with respect to their largest sibling had higher levels of both stress proteins, and this effect was stronger for female chicks as indicated by a significant interaction sex × size difference, presumably because of their higher food requirements for growth. Nestlings for which the largest sibling was a female had higher levels of Hsp60 than when it was a male. The heterophile/lymphocyte index was a much poorer predictor of competitive stress.
4. Stress proteins are effective estimators of competitive, nutritional and parasite-mediated stress of nestlings in the wild. The cost of sustained stress has to be included in future analyses of the fitness repercussions of dominance hierarchies in avian broods.

Key-words: *Caryospora* sp., *Falco tinnunculus*, hatching asynchrony, Hsp60, sibling competition

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Introduction

Hatching asynchrony is a common trait in birds although its functional basis is still poorly understood (Stoleson & Beisinger 1995). However, there is no doubt that the first consequence of hatching asynchrony for broods is the creation of a hierarchy in age and size at hatching. Thus, a competitive disadvantage is imposed by hatching asynchrony on the last hatched chicks. This handicap could be modulated through a more competitive disposition in last hatched chicks, enabling them to catch up with their larger siblings (Saino *et al*. 2001). Competition in the nest may be mediated hormonally through a higher hormone activity in small siblings (Nuñez-de la Mora, Drummond & Wingfield 1996). It has been suggested that females may use differential allocation of testosterone in last laid eggs in order to improve their capacity for sibling competition at hatching (Schwabl 1993; but see Ellis, Borst & Thompson 2001). However, little attention has yet been paid to the costs of maintaining high levels of competition towards siblings, although size hierarchies imposed by hatching asynchrony have been proposed to be, at least in some species, including raptors, a mechanism to reduce sibling competition (Hahn 1981; Wiebe & Bortolotti 1994; Viñuela 1999).

A bird's sex is one of the factors that may affect competitive disposition in nestlings (Bortolotti 1986; Drummond *et al*. 1991). Additionally, differential mortality between male and female nestlings during the period of parental care has been found to favour survival of the smaller sex (Clutton-Brock 1991). In reversed sexually dimorphic birds such as raptors, males may suffer in conditions of food shortage, owing to size-dependent capacity to compete for resources in the nest (Bortolotti 1986; Anderson *et al*. 1993; Arroyo 2002; Fargallo *et al*. 2002; Fargallo *et al*. 2003). However, in another species with reversed sex dimorphism, last hatched females suffered a greater increase in mortality than last hatched males, owing to their greater food demands (Torres & Drummond 1997). Thus, it is not obvious which sex should suffer more from stress given the implications of size both for competitive capacity and for food

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requirements. In any case, the relationship between within-brood size differences and nestling sex may be reflected in stress conditions. Another consequence of sexual size dimorphism in the nest may be that dominance hierarchies could depend on the sex of the larger siblings, with situations when the oldest sibling belongs to the larger sex being more stressful for its smaller broodmates.

Previous studies have found that competition within broods may result in higher levels of circulating corticosterone (Nuñez-de la Mora *et al*. 1996; Tarlow, Wikelski & Anderson 2001; although see Sockman & Schwabl 2001). However, corticosterone levels may indicate short-term physiological stress rather than chronic stress effected by a sustained competitive situation. Chronic stress may be better expressed by the heterophile/lymphocyte ratio (H/L) and heat shock protein levels. The H/L has been suggested as an indicator of stress in domestic birds (Maxwell 1993; Maxwell & Robertson 1998) and early studies suggest its relevance for studies of nutritional stress in wild avian populations (Hõrak *et al*. 1999; Moreno *et al*. 2002a,b). Stress proteins, traditionally called heat shock proteins or Hsps, are evolutionarily highly conserved molecules that help cells in recovering from stress situations by correcting misconfigurations in protein structures (Morimoto 1991). Hsp levels do not fluctuate as rapidly as hormone levels (see Burel *et al*. 1992), and may thus be independent of capture and handling stress. Moreno *et al*. (2002a) have observed that Hsp levels are related to H/L and to nestling growth in wild bird populations. They detected a positive association between mean Hsp level in broods and the within-brood variance in size traits, and suggested that Hsp levels reflected the stress imposed by asymmetric sibling competition. To our knowledge, competitive stress in nestling birds has not been previously measured with Hsp levels.

In addition, parasitism has been revealed as an important source of stress (Møller 1997), as shown by recent studies of Hsp levels in relation to ectoparasite load and blood parasites (Merino *et al*. 1998; Merino *et al*. 2002). Little is known about the impact of intestinal parasites on different aspects of the ecology of wild birds. The effects of parasites should be controlled in any analysis of the stress of sibling competition given their potential as significant stressors for nestling birds.

Here we have tried to explore four related questions in a wild population of Eurasian Kestrels. The first issue is the influence of size differences among siblings on stress because of competition for food. The second question is whether there are sexual differences in stress in a species where females are larger than males, and whether this difference interacts with the size differences imposed by hatching asynchrony. Merino, Møller & de Lope (2002) have suggested that there are sexual differences in adult Barn Swallow Hsp levels, while nothing is known about differences in nestlings. In addition, the third issue is the influence of sex of the largest sibling in the brood on stress imposed by its larger size or its stronger competitive disposition (Anderson *et al*. 1993; Fargallo *et al*. 2003). Finally, the fourth issue was related to the stressful effects of the different types of parasites of nestling kestrels in our study population.

Materials and methods

FIELD PROCEDURES

We monitored breeding in Eurasian Kestrels, a small and reversed sexually dimorphic falcon (Village 1990). The study was conducted during the breeding season of 2002 in the Campo Azálvaro region, a montane (1300 m above sea level) grassland area in central Spain (for more details see Fargallo *et al*. 2001). All licences that allowed us to conduct the field work and to manipulate nestlings were issued by Junta de Comunidades de Castilla y León. All broods used in our study were located in nestboxes that were monitored every 2 days during laying and eggs were marked according to laying order (one egg is laid every 2 days; see Aparicio 1994; Wiebe, Wiehn & Korpimäki 1998). To record hatching order, we visited nests every day from 25 days after the end of laying, and twice every day from the day when we detected the first evidence of hatching until all eggs of the clutch hatched. We estimated hatching asynchrony as the number of hours elapsed between the estimated hatching time for the first and last hatched chicks in a brood (Viñuela 2000). Hatchlings were identified by marking them with indelible and harmless ink on the hatching tooth during the hatching process, and later with ink on the head until banding. Chicks were weighed with a Pesola spring balance to the nearest 0·25 g and wing length was measured with callipers to the nearest 0·01 mm just after hatching. When they were 24 days old chicks were weighed to the nearest gram with a Pesola spring balance and their wing length measured with callipers to the nearest mm. Additionally 250 µl of blood were extracted by brachial venipuncture. A drop of blood was used to determine nestling sex by molecular procedures (Fridolfsson & Ellegren 1999), and another drop for leucocyte counts and blood parasite screening in blood smears.

HAEMATOLOGY AND PARASITES

Blood smears were immediately air dried and later fixed with absolute ethanol and stained with Giemsa (1/10 v/v) for 45 min. Half of the symmetrical smear was scanned at ×200 magnification in search of large blood parasites, whereas small intraerythrocytic parasites were searched at ×1000 magnification (Merino & Potti 1995a; Merino, Potti & Fargallo 1997). Smears were scanned at ×1000 magnification and at least 100 white blood cells were counted to obtain the differential leucocyte count. We counted leucocytes in a part of the smear where cells are separated in a monolayer and

we scanned the smear along its short axis to minimize differences in the thickness of the blood layer. Leucocyte counts were related to 10 000 erythrocytes by counting red blood cells in the field scanned. This method has been reported to be highly repeatable (Saino, Møller & Bolzern 1995; Moreno *et al*. 1998). Heterophils, eosinophils, basophiles, lymphocytes and monocytes were differentiated and counted following recommendations by Dein (1986) and Hawkey & Dennet (1989).

When nestlings were 24 days old we collected faecal samples during manipulation just after defecation. Every chick was kept on a separate clean one-use paper towel, so we know the origin of each faecal sample. Chicks were maintained on individual towels while their siblings were measured and weighed. This method allowed the collection of only 44 samples, which were analysed by zinc sulphate flotation and counting in a MacMaster chamber (Teddington, UK). Both quantitative and qualitative analyses were carried out. Propagules were identified following Melhorn, Düwell & Raether (1992). The parasitological terms used are based on Bush *et al*. (1997). Although the quantitative faecal examination is only an approximate indication of the actual intensity of infection, this is the only non-invasive technique suitable for the study of intestinal parasites in wild animals (Watve & Sukumar 1995). In addition, the abundance of *Carnus* spp. flies was estimated when nestlings were 24 days old. To estimate *Carnus* infestation for other studies in the study area, wes found that the best method to estimate them was to take the chicks individually from the nest and immediately place them in a bag while blowing on them, counting all flies that could be collected in the bag and returning the nestlings to the nest when all siblings in the brood had been explored. We estimated *Carnus* with this method because these flies are extremely mobile and do not adhere to nestlings, so a more thorough search would not have rendered more accurate estimates. However, no *Carnus* flies were found on nestlings at 24 days of age.

MEASUREMENT OF HEAT SHOCK PROTEINS (HSPS)

All procedures to obtain soluble proteins were carried out at 4 °C to prevent denaturation. Blood cells were homogenized by sonication in approximately 0·4 ml of distilled water to release Hsps. The homogenate was centrifuged (14 000 *g*, 20 min) and the supernatant collected. The total protein concentration was determined using the Bio-Rad Protein Assay (Hercules, CA, USA). Samples of soluble proteins obtained from the blood cells (35 μg well^{-1}) were separated by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). The total protein value was within the linear rank of the antibody–antigen response for the species and antibodies studied. Electrophoresis was performed in a discontinuous buffer system. Polyacrylamide gels consisted of a stacking gel (4%, pH = 6·8) and a separating gel (10%, pH = 8·8). Protein separation was performed at a constant voltage of 200 V, following the manufacturer's (Bio-Rad) guidelines. Electroblot transfer from the polyacrylamide gels was performed at 150 V for 1·5 h. The polyvinylidene fluoride (PVDF) blots were washed in PBS containing 0·05% Tween-20 (PBS-Tw), and incubated with 5% non-fat powdered milk in PBS-Tw for 1 h to block all additional binding capacity of the PVDF membranes. After incubation, blots were tested with antiserum. The primary monoclonal antibodies (Sigma, St Louis, MO, USA) were anti-Hsp60 (clone LK2) and anti-Hsp70 (clone BRM22) diluted 1/1500 and 1/5000 in PBS-Tw. These antibodies react specifically with Hsp60 and Hsp70, respectively, and have been used to recognize these Hsps in a large variety of vertebrates, including birds.

A peroxidase-conjugated secondary antibody (Sigma) was used at 1/6000 dilution. This dilution was chosen because it allows clear detection of Hsps without unspecific binding. Positive bands were detected using 50 mM Tris buffer containing 0·03% diaminobenzidine and 0·001% hydrogen peroxide. Primary and secondary antibodies were incubated overnight at 4 °C and for 2 h at room temperature, respectively. Three washes with PBS-Tw were performed after each step. Finally, protein bands were quantified using image analysis software for Windows (Scion Corporation). Immunoreactivities (arbitrary units) were obtained using the following formula:

$$\text{Immunoreactivity} = \text{area} \times \text{mean intensity of the band.}$$

STATISTICAL PROCEDURES

Since eggs, hatchlings and nestlings from the same nest share parents and environment we used the chick as the unit and the nest as a random factor in general linear mixed models (GLMM) using a normal distribution of errors and an identity link function (Littell *et al*. 1996) in SAS statistical software (SAS 1989–96 Institute Inc., Cary, NC). The nest was included as a random term in the manner of a randomized complete block design to avoid pseudoreplication (Hurlbert 1984). In addition, by introducing this random term we ensured that the effects of the fixed variables were not influenced by characteristics of the parents or the nest. Statistical tests associated with random terms denote significant nest variation in the variables examined ($Z = 2{\cdot}59$, $P = 0{\cdot}0048$). Therefore, this design allowed us to use a chick as sample unit and avoided pseudoreplication because we controlled for the significant random variance of each nest. In addition, Hsp levels were analysed on three different days where random variability in measurements were found. Thus, 'analysis day' was introduced into the model as a random factor in mixed models ($Z = 6{\cdot}03$, $P < 0{\cdot}0001$), allowing us to control this variation. Residuals of the mixed model with Hsp60 as dependent variable controlling by random effects of 'nest' and 'analysis day' were employed as the *y*-axis to

give graphical results. In order to explore the relationships between the different cell types and growth or size measurements, mixed models were performed with nest as random variable.

We performed a mixed model to evaluate the effect of the nest when we explored variables related to each different nestling in each nest. The effect of position in the within-brood size hierarchy was expressed as size differences of each nestling with respect to its largest sibling. These differences were included as a covariate in the mixed model, like laying date and clutch size. The mass and wing length differences of each individual chick with their largest sibling at 24 days of age were considered as mass differences and size differences, respectively. Other variables such as laying order and hatching order were also considered as fixed factors. Laying order and hatching order were encoded as first, middle or last hatched/laid eggs, allowing us to compare laying and hatching sequences among different clutch sizes. *Caryospora* infection as found in our kestrel population was treated as a factor (0, uninfected nestling; 1, infected nestling) and as a covariate (abundance of infection). Some of the explanatory variables could covary, so we fitted their effects to the observed data following a forward stepwise procedure, testing the significance of each variable and adding only the variable that resulted in a better fit of the model. The significance of the remaining variables was tested again until no additional variable or interaction reached significance. All tests are two-tailed.

Results

HATCHING ASYNCHRONY

Nestling size hierarchy was strongly related to hatching asynchrony (logistic regression Wald statistic = 18·97, $P < 0{\cdot}0001$). Hatching asynchrony increased with laying order ($F_{2,106} = 11{\cdot}91$, $P < 0{\cdot}0001$), indicating that last hatched chicks hatched from last laid eggs and consequently showed larger size differences in relation to their siblings ($F_{2,109} = 6{\cdot}33$, $P = 0{\cdot}003$).

HSP LEVELS IN RELATION TO SIZE HIERARCHIES WITHIN BROODS AND SEX

The mean levels of Hsp60 and Hsp70, expressed as immunoreactivities, of 112 nestlings were 188·0 ± 22·3 and 118·9 ± 26·0, respectively. Of these, 61 were females and 51 were males. The Hsp60 level was related negatively to nestling wing length ($F_{1,78} = 26{\cdot}09$, $P < 0{\cdot}0001$) and nestling mass ($F_{1,78} = 9{\cdot}33$, $P = 0{\cdot}0031$) and positively to size difference ($F_{1,78} = 30{\cdot}65$, $P < 0{\cdot}0001$) and mass difference ($F_{1,78} = 14{\cdot}01$, $P = 0{\cdot}0003$). GLIMMIX only found the positive relationship between Hsp60 level and size differences, measured as wing length differences with the largest sibling (see methods) when all variables were considered in the model (Table 1), suggesting that size differences was the most adequate variable related to Hsp60 levels. This indicates that undersized nestlings with respect to the largest sibling in the brood showed higher levels of stress (Fig. 1). In

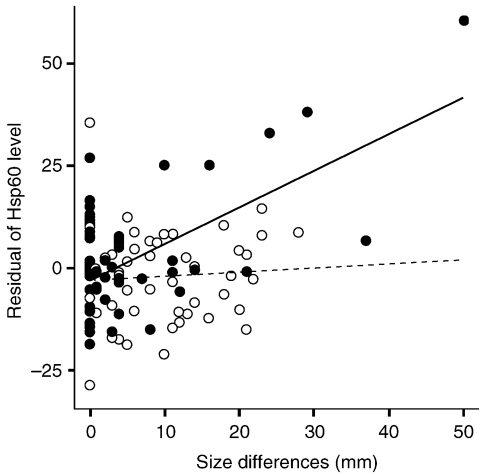

Fig. 1. Effects of the interaction between sex and size difference with respect to the largest sibling on the levels of Hsp60 from peripheral blood of Eurasian Kestrel nestlings. The continuous line represents females and the broken line represents males. The *y*-axis represents the residuals from the GLIMMIX model controlling the random effects of 'nest' and 'analysis day' (see Materials and methods for more details).

Table 1. GLMM with normal error and identity link function on intensity of Hsp60 levels of Eurasian Kestrel nestlings. The model retained the variance of nest introduced into the model as a random term ($Z = 2{\cdot}59$, $P = 0{\cdot}0048$). Other potentially influencing variables and the remaining interactions between the variables were not significant ($P > 0{\cdot}07$). In the final model 'nestling sex' was maintained to explore interactions. Statistically significant *P*-values in bold type

Response term	Explanatory term	Rejected term	*F*	df	*P*
Hsp60	Size difference		14·66	1,69	**0·0003**
	Nestling sex		0·51	1,69	0·4768
	Sex * Size difference		8·73	1,69	**0·0043**
	Sex of largest sibling		6·23	1,69	**0·0150**
	Laying date		4·29	1,69	**0·0420**
		Nestling mass	1·84	1,68	0·2612
		Wing length	1·11	1,68	0·2953
		Laying order	1·19	2,67	0·3096
		Clutch size	0·77	1,67	0·3844
		Hatching order	0·63	2,62	0·5351
		Mass difference	0·01	1,60	0·9105

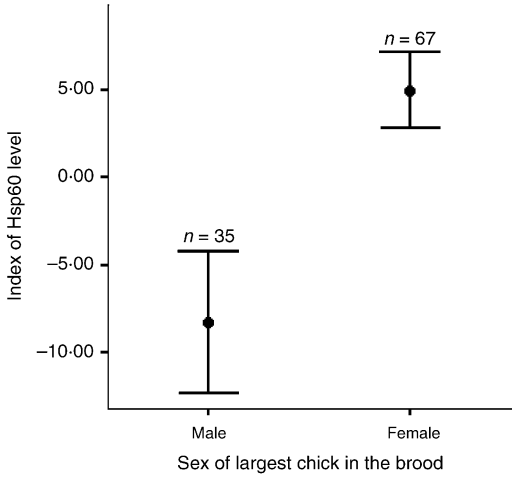

Fig. 2. Mean (± SE) Hsp60 level according to sex of the largest sibling in a brood. Numbers above bars are sample sizes. The *y*-axis represents the residuals from the GLIMMIX model between random effect of the nest and Hsp60 level.

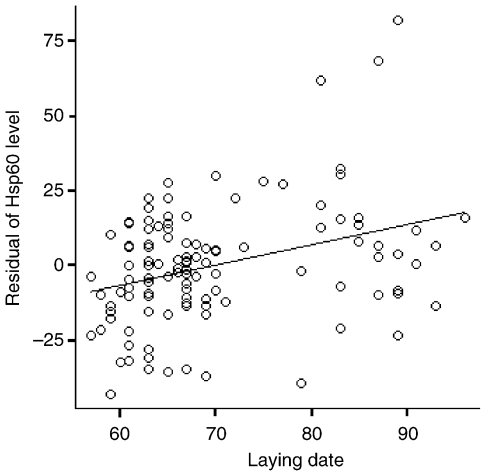

Fig. 3. Correlation between laying date (0 is March 1) and Hsp60 level (see Table 1 for statistics). The *y*-axis represents the residuals from the GLIMMIX model of the association between random effect of the nest and Hsp60 level.

addition, nestling sex was related to Hsp60 level ($F_{1,78}$ = 12·39, P = 0·0007), although this relationship disappeared when the interaction between sex and size hierarchy was considered (Table 1). The significant interaction indicates that females are more stressed than males for a similar level of size differences with the largest sibling in the brood (Fig. 1).

In addition, sex of the largest sibling was related to the Hsp60 level of its broodmates (Table 1) and with mean Hsp60 level in broods, with those broods where the largest chick was a female showing a higher Hsp60 level (Fig. 2). Laying date was also positively related to Hsp60 levels (Table 1), suggesting that stress increases during the breeding season (Fig. 3). Hsp70 levels were related to size differences with the largest sibling ($F_{1,77}$ = 20·55, P < 0·0001) and nestling sex ($F_{1,77}$ = 12·42, P = 0·0007), where undersized nestlings were more stressed and females were more stressed than males (193·6 ± 22·8 and 183·3 ± 20·9, respectively) without any significant interaction (all P > 0·3491).

LEUCOCYTE COUNTS IN RELATION TO SIZE DIFFERENCES WITHIN BROODS AND SEX

We collected 88 blood samples from nestlings in order to explore leucocyte counts and blood parasites. No relationships were found between counts of different types of white blood cells (see Materials and methods) and wing length (all P > 0·301) or nestling mass (all P > 0·054). No relationships were found between numbers of different types of leucocytes and Hsp60 (all P > 0·139) or Hsp70 levels (all P > 0·217). Mean H/L of nestlings was 0·63 ± 0·45 (n = 88, range 0·12–2·64). There was also no relationship between H/L and Hsp60 and Hsp70 levels ($F_{1,54}$ = 1·76, P = 0·1899; $F_{1,55}$ = 0·02, P = 0·8856, respectively). However, H/L was positively correlated to nestling size differences ($F_{1,59}$ = 4·19, P = 0·0451) and wing length ($F_{1,59}$ = 5·00, P = 0·0291) in independent analyses, but only the second association remained significant when all variables were included in a GLIMMIX analysis (Table 2). Neither sex nor nestling size differences were related to H/L, and no significant interactions were found (all P > 0·53).

PARASITES AND STRESS

The screen of blood smears of 86 nestlings for haematozoa was also negative. We collected 44 faecal samples from nestlings for which we could measure Hsp levels, where the coprological analyses of the faeces revealed the presence of *Caryospora* sp. in 29 (65·9%) of the samples analysed. Mean oocyst excretion was 4379·4 ±

Table 2. GLMM with normal error and identity link function on H/L of Eurasian Kestrel nestlings. The model retained the variance of nest introduced in the model as a random term (Z = 1·73, P = 0·0420). Other potentially influencing variables and the remaining interactions between the variables were not significant (P > 0·21)

Response term	Explanatory term	Rejected term	F	df	P
H/L index	Wing length		5·00	1,59	**0·0291**
		Sex	1·35	1,58	0·2494
		Laying date	0·42	1,57	0·5209
		Hatching order	0·94	2,52	0·3961
		Size difference	0·10	1,52	0·7547
		Laying order	0·21	2,50	0·8129
		Nestling mass	0·02	1,49	0·8998

Table 3. GLMM with normal error and identity link function on Hsp60 levels of Eurasian Kestrel nestlings. The model retained the variance of nest, introduced in the model as a random term ($Z = 2{\cdot}51$, $P = 0{\cdot}0060$). Other potentially influencing variables and the remaining interactions between variables were not significant ($P > 0{\cdot}35$). In the final model 'nestling sex' was maintained to explore interactions

Response term	Explanatory term	Rejected term	*F*	df	*P*
Hsp60	Size difference		9·439	1,15	**0·008**
	Nestling sex		1·690	1,15	0·210
	Sex * Size difference		6·647	1,15	**0·021**
	Caryospora infection		11·419	1,15	**0·004**
		Laying date	1·390	1,14	0·259
		Sex of largest sib	0·100	1,14	0·762

6851·8 oocysts/g of faeces. No other parasite propagules were detected. No relationships were found between infection and laying date, nestling mass gain, nestling growth, wing length or size differences (Mann–Whitney *U*-tests, all $P > 0{\cdot}333$). In addition, H/L index was not correlated to parasite infection (Mann–Whitney *U*-tests, all $P > 0{\cdot}511$). However, we introduced *Caryospora* infection status as a factor in the model explaining Hsp60 or Hsp70 levels, finding that *Caryospora* infection increased Hsp60 levels (Table 3), but was not related to Hsp70 levels ($F_{1,15} = 0{\cdot}03$, $P = 0{\cdot}8584$). The intensity of infection was not related to Hsp60 or Hsp70 levels (both $P > 0{\cdot}3101$).

Discussion

THE EFFECT OF POSITION IN THE SIZE HIERARCHY

The relationship found between Hsps and size differences clearly shows that levels of these proteins in kestrel nestlings increase in relation to size differences with respect to their largest sibling and are thus dependent on position in the within-brood size hierarchy. This hierarchy is imposed by the within-brood hatching pattern. Both stress proteins show a similar trend, reinforcing the evidence for hierarchy-related stress. Competition-related stress could be mediated by hormonal levels, given that smaller chicks increase corticosterone levels under food deprivation (Nuñez-de la Mora *et al*. 1996) and that testosterone levels increase with laying order in order to compensate for age- and size-related disadvantages in posthatching development (Schwabl 1993). Earlier studies have shown that Hsp60 levels were related to within-brood size variation (Merino *et al*. 1998; Moreno *et al*. 2002a). These authors suggested that Hsp60 could be expressing the greater stress induced through competition in broods with large size differences. However, these studies did not report that the smallest siblings had higher levels of stress proteins, as they worked with brood means. Our results clearly show that the smaller nestlings in each brood are more stressed than their larger siblings, as has been shown in other species under food shortage using stress hormones (Nuñez-de la Mora *et al*. 1996; Kitaysky, Wingfield & Piatt 2001). We propose that Hsp levels, especially Hsp60, may be a good index of sustained competition-related stress in avian broods. It is possible that begging signals and adopting strategic positions in the nest during parental visits could be stressful (Brzek & Konarzewski 2001), especially in species such as the kestrel, where nest position is an important factor determining success in obtaining food under food shortage (Fargallo *et al*. 2003).

Stress protein levels and H/L have shown a positive association in a recent study (Moreno *et al*. 2002a), a result that could not be confirmed for nestling kestrels. Also, H/L did not relate to size hierarchies within broods, confirming the suggestion by Moreno *et al*. (2002a) that it is a poorer index of the stress due to competition in the nest. There was a positive association of H/L with wing length, a variable that has been shown as a good estimate of nutritional condition in nestling birds (Nowicki *et al*. 2000). This indicates that as found for broilers, food restriction does not affect H/L (Maxwell 1993), which thus seems not to be a good index of nutritional stress in kestrels.

We also found that Hsp60 levels increase during the course of the breeding season. This trend could be related to a seasonal decline in food abundance or to thermal stress imposed by an increase in ambient temperature. Parasites of nestlings increase in prevalence as the breeding season progresses in some studies (Merino & Potti 1995b; although see Dawson & Bortolotti 1997), suggesting costs in terms of growth and immune response (Merino *et al*. 2000; although see Roulin *et al*. 2003 for Eurasian Kestrels). However, our results suggest that laying date is not a relevant factor that increases infection in kestrel chicks. It is widely assumed that resources decrease with date in most temperate areas (Price 1996). In our study population there is a seasonal decline in nestling size and in reproductive success (Fargallo *et al*. 2001), which can be due to low food allocation by poor quality parents to their nestlings. It is thus reasonable to suppose that competition for food will increase with date and lead to more competition-related stress. Poor nutrition itself could also be driving the seasonal trend in stress (Moreno

et al. 2002a). However, the expected seasonal increase in ambient temperature could be reflected in a thermal increase in nestboxes, so Hsp60 levels could also reflect a seasonal increment in thermal stress (Krebs & Feder 1995). These two possibilities are not mutually exclusive, so future research should explore their relative importance.

THE EFFECT OF NESTLING SEX

Our results suggest that stress protein levels of small siblings depend on sex when size hierarchies are extreme. Thus, subordinate female nestlings were more stressed than males when the size differences with respect to the largest sibling increased. In raptors, female nestlings are larger than males, suggesting that males are in a competitive disadvantage under conditions of food scarcity (Anderson *et al*. 1993; Fargallo *et al*. 2003), meaning a higher male mortality (Arroyo 2002) or lower capacity of mounting an adequate immune response under food shortage (Fargallo *et al*. 2002). Our results are partially in disagreement because nestling sex is excluded in our model, while our results suggest additionally that female nestling kestrels are more stressed than males when size differences increase in relation to larger siblings. Different studies have shown that the larger sex has higher energetic requirements for growth, suggesting an adaptive disadvantage in relation to the smaller sex (Bortolotti 1986; Wiebe & Bortolotti 1992; Torres & Drummond 1997). This implies that they would have to compete more strongly for food when in a subordinate position in the brood hierarchy. This could lead to a higher stress for small female siblings. Our results suggest that female kestrel chicks are more stressed than males when they lose their competitive size advantage because of the higher food requirements in this sex, suggesting additionally that males may better confront a size-disadvantage situation. The association of competition-related stress with sex-dependent hormone levels in nestlings remains to be studied to clarify the role of testosterone and corticosterone in the determination of stress levels.

Our results show that when the largest chick in a brood is a female, average stress protein levels in broods are higher than if it is a male. This suggests that females in a dominant position impose a more competitive situation for their siblings just before they fledge, which is expressed in increased Hsp levels. Alternatively, subordinates with a large female dominant could experience higher stress because they cannot compete effectively, rather than because they are trying to compete more. In our population, nestling mass decreased with hatching order in clutches initiated with male eggs, while fledging mass of chicks hatched in different orders did not differ in nests initiated with female eggs (Blanco *et al*. 2003). This result suggests that when females are in a dominant position, subordinate siblings may compensate for their size handicap by competing more effectively for food. This compensation may not be without the costs imposed by higher stress levels. In general, reviews of the costs of growth compensation (Metcalfe & Monaghan 2001) should consider the effects of stress induced by increased sibling competition.

STRESS AND PARASITES

Our results suggest that infection by *Caryospora* sp. increases individual stress, at least as measured by stress proteins. The effect of parasites is independent of position in the hierarchy and sex, contrary to findings by Christe, Møller & de Lope (1998). In this context, chicks could be infected by contact with faeces of their siblings or directly by eating infected prey. Therefore, patterns of nestling *Caryospora* infection could be related to random transmission between brood mates. Very little is known about coccidian infection in wild populations of raptors, although severe coccidiosis may cause death in free-living kestrels (Krone 2002). Protozoan parasites are large enough to avoid phagocytic uptake, although inflammatory cells could act directly by adhering to them and releasing a variety of potent mediators creating a hostile microenvironment (Wakelin & Apanius 1997). In kestrel nestlings, *Caryospora* infection could induce fever and an inflammatory response, which in turn could raise stress protein levels (Macario 1995). However, *in vitro* studies have shown that stress proteins could be stimulated by the parasite itself (Garbe 1992), especially during the first stage of infection.

To conclude, we have shown the usefulness of stress proteins from peripheral blood for detecting the effects of different types of stressors for nestling birds. A low position in the brood hierarchy increases stress, and this effect is more marked for female chicks in species where females are larger and require more food in the nest. Parasites and breeding date also affect stress. When the dominant chicks belong to the larger sex, their siblings are more stressed. Trying to compensate for a bad start in life may be stressful, and this stress may have long-term consequences for fitness. The link between stress protein levels and future survival and reproduction in the wild remains to be elucidated to confirm their utility for ecological studies.

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References

Anderson, D.J., Budde, C., Apanius, V., Martínez-Gómez, J.E., Bird, D.M. & Weathers, W.W. (1993) Prey size influences competitive dominance in nestling American kestrels (*Falco sparverius*). *Ecology* **74**, 367–376.

Aparicio, J.M. (1994) The effect of variation in the laying interval on proximate determination of clutch size in the European kestrel. *Journal of Avian Biology* **25**, 275–280.

Arroyo, B. (2002) Sex-biased nestling mortality in the Montagu's harrier *Circus pygargus*. *Journal of Avian Biology* **33**, 455–460.

Blanco, G., Martínez-Padilla, J., Serrano, D., Dávila, J.A. & Viñuela, J. (2003) Mass provisioning to different-sex eggs within the laying sequence: consequences for adjustment of reproductive effort in a sexually dimorphic bird. *Journal of Animal Ecology* **72**, 831–838.

Bortolotti, G.R. (1986) Influence of sibling competition on nestling sex ratios of sexually dimorphic birds. *American Naturalist* **127**, 495–597.

Brzek, P. & Konarzewski, M. (2001) Effect of food shortage on the physiology and competitive abilities of sand martin (*Riparia riparia*) nestlings. *Journal of Experimental Biology* **204**, 3065–3074.

Burel, C., Mezger, V., Pinto, M., Rallu, M., Trigon, S. & Morange, M. (1992) Mammalian heat shock protein families. Expression and functions. *Experientia* **48**, 629–634.

Bush, A.O., Lafferty, K.D., Lotz, J.M., & Shostak, A.W. (1997) Parasitology meets ecology on its own terms: Margolis *et al.* revisited. *Journal of Parasitology* **83**, 575–583.

Christe, P., Møller, A.P. & de Lope, F. (1998) Immunocompetence and nestling survival in the house martin: the tasty chick hypothesis. *Oikos* **83**, 175–179.

Clutton-Brock, T.H. (1991) *The Evolution of Parental Care*. Princeton University Press, Princeton, NJ.

Dawson, R. & Bortolotti, G.R. (1997) Ecology of parasitism of nestling American kestrels by *Carnus hemapterus* (Diptera: Carnidae). *Canadian Journal of Zoology* **75**, 2021–2026.

Dein, F.J. (1986) Hematology. *Clinical Avian Medicine and Surgery* (eds B. G. H. Harrison & L. R. Harrison), pp. 174–191. W.B. Sanders, Philadelphia.

Drummond, H., Osorno, J.L., Torres, R., García-Chavelas, C. & Merchant-Larios, H. (1991) Sexual size dimorphism and sibling competition: implications for avian sex ratios. *American Naturalist* **138**, 623–641.

Ellis, L.A., Borst, D.W. & Thompson, C.F. (2001) Hatching asynchrony and maternal androgens in egg yolks of house wrens. *Journal of Avian Biology* **32**, 26–30.

Fargallo, J.A., Blanco, G., Potti, J. & Viñuela, J. (2001) Nestbox provisioning in a rural population of Eurasian kestrels: breeding performance, nest predation and parasitism. *Bird Study* **48**, 236–244.

Fargallo, J.A., Laaksonen, T., Poyri, V. & Korpimäki, E. (2002) Inter-sexual differences in the immune response of Eurasian kestrel nestlings under food shortage. *Ecology Letters* **5**, 95–101.

Fargallo, J.A., Laaksonen, T., Korpimäki, E., Pöyri, V., Griffith, S.C. & Valkama, J. (2003) Size-mediated dominance and begging behaviour in Eurasian kestrel broods. *Evolutionary Ecology Research* **5**, 549–558.

Fridolfsson, A.K. & Ellegren, H. (1999) A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology* **30**, 116–121.

Garbe, T.R. (1992) Heat shock proteins and infection: interactions of pathogen and host. *Experientia* **48**, 635–639.

Hahn, D.C. (1981) Asynchronous hatching in the laughing gull: cutting losses and reducing rivalry. *Animal Behaviour* **29**, 421–427.

Hawkey, C.M. & Dennet, T.B. (1989) *Atlas de Hematología Veterinaria Comparada*. Grass Editions, London.

Hõrak, P., Tegelman, L., Ots, I. & Møller, A.P. (1999) Immune function and survival of great tit nestlings in relation to growth conditions. *Oecologia* **121**, 316–322.

Hurlbert, S.H. (1984) Pseudoreplication and the design of ecological field experiments. *Ecological Monographs* **54**, 187–211.

Kitaysky, A.S., Wingfield, J.C. & Piatt, J.F. (2001) Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. *Behavioral Ecology* **12**, 619–625.

Krebs, R.A. & Feder, M.E. (1995) Natural variation in the expression of the heat-shock protein HSP70 in a population of *Drosophila melanogaster* and its correlation with tolerance of ecologically relevant thermal stress. *Evolution* **51**, 173–179.

Krone, O. (2002) Fatal *Caryospora* infection in a free-living juvenile Eurasian kestrel (*Falco tinnunculus*). *Journal of Raptor Research* **36**, 84–86.

Littell, R.C., Milliken, G.A., Stroup, W.W. & Wolfinger, R.D. (1996) *SAS System for Mixed Models*. SAS Institute, Cary, NC.

Macario, A.J.L. (1995) Heat-shock proteins and molecular chaperones: Implications for pathogenesis, diagnostics, and therapeutics. *International Journal of Clinical and Laboratory Research* **25**, 59–70.

Maxwell, M.H. (1993) Avian blood leukocyte responses to stress. *World Poultry Science Journal* **49**, 34–43.

Maxwell, M.H. & Robertson, G.W. (1998) The avian heterophil leukocyte: a review. *World Poultry Science Journal* **54**, 155–178.

Melhorn, H., Düwell, D. & Raether, W. (1992) *Atlas de Parasitología Veterinaria*. GRASS Editions, Barcelona, Spain.

Merino, S. & Potti, J. (1995a) High prevalence of hematozoa in nestlings of a passerine species, the pied flycatcher (*Ficedula hypoleuca*). *Auk* **112**, 1041–1043.

Merino, S. & Potti, J. (1995b) Mites and blowflies decrease growth and survival in nestling pied flycatcher. *Oikos* **73**, 95–103.

Merino, S., Potti, J. & Fargallo, J.A. (1997) Blood parasites of some passerine birds from central Spain. *Journal of Wildlife Diseases* **33**, 638–641.

Merino, S., Martínez, J., Barbosa, A., Møller, A.P., de Lope, F., Pérez, J. & Rodríguez-Caabeiro, F. (1998) Increase in a heat-shock protein from blood cells in response of nestling house martins (*Delichon urbica*) to parasitism: an experimental approach. *Oecologia* **116**, 343–347.

Merino, S., Møller, A.P. & de Lope, F. (2000) Seasonal changes in cell-mediated immunocompetence and mass gain in nestling barn swallows: a parasite-mediated effect? *Oikos* **90**, 327–332.

Merino, S., Martínez, J., Møller, A.P., Barbosa, A., de Lope, F. & Rodríguez-Caabeiro, F. (2002) Blood stress protein levels in relation to sex and parasitism of barn swallows (*Hirundo rustica*). *Ecoscience* **9**, 300–305.

Metcalfe, N.B. & Monaghan, P. (2001) Compensation for a bad start: grow now, pay later? *Trends in Ecology and Evolution* **16**, 254–260.

Møller, A.P. (1997) Parasitism and the evolution of host life history. *Host–Parasite Evolution: General Principles and Avian Models* (eds D.H. Clayton & J. Moore), pp. 105–127. Oxford University Press, Oxford.

Moreno, J., de León, A., Fargallo, J.A. & Moreno, E. (1998) Breeding time, health and immune response in the chinstrap penguin *Pygoscelis antarctica*. *Oecologia* **115**, 312–315.

Moreno, J., Merino, S., Martínez, J., Sanz, J.J. & Arriero, E. (2002a) Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. *Ecoscience* **9**, 434–439.

Moreno, J., Merino, S., Sanz, J.J. & Arriero, E. (2002b) An indicator of maternal stress is correlated with nestling growth in pied flycatchers *Ficedula hypoleuca*. *Avian Science* **2**, 175–182.

Morimoto, R.I. (1991) Heat shock: the role of transient inducible responses in cell damage, transformation, and differentiation. *Cancer Cells* **3**, 295–268.

Nowicki, S. Hasselquist, D. Bensch, S. & Peters, S. (2000) Nestling growth and song repertoire size in great reed warblers: evidence for song learning as an indicator mechanism in mate choice. *Proceedings of the Royal Society of London Series B* **267**, 2419–2424.

Nuñez-de la Mora, A., Drummond, H. & Wingfield, J.C. (1996) Hormonal correlates of dominance and starvation-induced aggression in chicks of the Blue-footed booby. *Ethology* **102**, 748–761.

Price, T. (1996) Maternal and paternal effects in birds. Effects on offspring fitness. *Maternal Effects as Adaptation* (eds A.M. Timothy & W. Charles), pp. 202–226. Oxford University Press, Oxford.

Roulin, A., Brinkhof, M.W.G., Bize, P., Richner, H., Jungi, T.W., Bavoux, C., Boileau, N. & Burneleau, G. (2003) Which chick is tasty to parasites? The importance of host immunology vs. parasite life history. *Journal of Animal Ecology* **72**, 75–81.

Saino, N., Møller, A.P. & Bolzern, A.M. (1995) Testosterone effects on the immune system and parasite infestations in the barn swallow (*Hirundo rustica*): an experimental test of the immunocompetence hypothesis. *Behavioral Ecology* **6**, 397–404.

Saino, N., Incagli, M., Martinelli, R., Ambrosini, R. & Møller, A.P. (2001) Immunity, growth and begging behaviour of nestling barn swallows *Hirundo rustica* in relation to hatching order. *Journal of Avian Biology* **32**, 263–270.

Schwabl, H. (1993) Yolk is a source of maternal testosterone for developing birds. *Proceedings of the National Academy of Sciences of the USA* **90**, 11446–11450.

Sockman, K.W. & Schwabl, H. (2001) Plasma corticosterone in nestling American kestrels: effects of age, handling stress, yolk androgens, and body condition. *General and Comparative Endocrinology* **122**, 205–212.

Stoleson, S.H. & Beisinger, S.R. (1995) Hatching asynchrony and the onset of incubation in birds, revisted. When is the critical period? *Current Ornithology* **12**, 191–270.

Tarlow, E., Wikelski, M. & Anderson, D.J. (2001) Hormonal correlates of siblicide in Galapagos Nazca boobies. *Hormones and Behaviour* **40**, 14–20.

Torres, R. & Drummond, H. (1997) Female-biased mortality in nestling of a bird with size dimorphism. *Journal of Animal Ecology* **66**, 859–865.

Village, A. (1990) *The Kestrel*. T. & A. D. Poyser, London.

Viñuela, J. (1999) Sibling aggression, hatching asynchrony and nestling mortality in the black kite (*Milvus migrans*). *Behavioral Ecology and Sociobiology* **45**, 33–45.

Viñuela, J. (2000) Opposing selective pressures on hatching asynchrony: egg viability, brood reduction and nestling growth. *Behavioral Ecology and Sociobiology* **48**, 333–343.

Wakelin, D. & Apanius, V. (1997) Immune defence: genetic control. *Host–Parasite Evolution: General Principles and Avian Models* (eds D.H. Clayton & J. Moore). Oxford University Press, Oxford.

Watve, M.G. & Sukumar, R. (1995) Parasite abundance and diversity in mammals. Correlates with host ecology. *Proceedings of the National Academy of Sciences USA* **92**, 8945–8949.

Wiebe, K.L. & Bortolotti, G.R. (1992) Facultative sex ratio manipulation in American kestrels. *Behavioral Ecology and Sociobiology* **30**, 379–386.

Wiebe, K.L. & Bortolotti, G.R. (1994) Energetic efficiency of reproduction: the benefits of asynchronous hatching for American kestrels. *Journal of Animal Ecology* **63**, 551–560.

Wiebe, K.L., Wiehn, J. & Korpimäki, E. (1998) The onset of incubation in birds: can females control hatching patterns? *Animal Behaviour* **55**, 1043–1052.

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