

## Daytime variation in T-cell-mediated immunity of Eurasian kestrel *Falco tinnunculus* nestlings

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Host-parasite interactions are central in evolutionary and behavioural ecology. In the last few years, skin injections of the mitogen Phytohaemagglutinin (PHA) have become one of the most important and widely used in-vivo assays of immune function in birds. However, there are no studies of the circadian variation suggesting that care should be taken interpreting results when using this technique. This 3-year study assessed PHA responses as a function of daylight time in 310 Eurasian kestrel *Falco tinnunculus* nestlings at 24 days of age in Central Spain. I found that T-cell-mediated immunity was positively related to nestling mass and varied among years. Controlling for these variables, I also found that T-cell-mediated immunity decreased with the hour of sampling, and that this pattern was consistent between years. In addition, I found that at the end of the day only, T-cell-mediated immunity decreased with brood size. Parasites seem not to be behind this pattern, but I suggest that the cumulative effect of sibling competition during the day might explain the decrease of cellular immunity with the hour of sampling. Thus, I strongly recommend that future studies of cellular immunity should control for this potential source of variation when nestling self-maintenance is evaluated by the PHA-induced skin-swelling response.

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Host-parasite interactions are central in evolutionary and behavioural ecology (Møller et al. 1999). Studies about what, how and how much hosts invest in their immune defences against parasites are thus critical within those fields. Measuring the ability of an individual to produce an efficient immune response is crucial for ecological studies and for a better comprehension of host-parasite interactions (Møller et al. 1999), host life history (Martin et al. 2001, Tella et al. 2002), host sexual selection (Hamilton and Zuk 1982) or parent-offspring conflict (Saino et al. 2000). In birds, evaluations of immune function have been carried out in poultry within areas of pathology and physiology (Goto et al. 1978, van der Zijpp 1983, Chu et al. 1995). Additionally, in the last few years, a high number of ecological studies have used a skin injection of the mitogen Phytohaemagglutinin (PHA), which provides a measure of T-cell mediated recruitment of circulating leukocytes (Stadecker et al.

1977, McCorkle et al. 1980), one of the components of the immune system (Apanius 1998).

T-cell-mediated immune responses have provided interesting results into behaviour or ecology in wild bird species (Moreno et al. 1998, Fargallo et al. 2003, Møller et al. 2003, Müller et al. 2003, Saino et al. 2003a) and they are currently recognized as an informative proxy for body condition and performance (Hörak et al. 1999, Tella et al. 2000a, Soler et al. 2003, Haussmann et al. 2005), which may ultimately linked to individual fitness. However, care should be taken interpreting results when using this measurement, because previous studies have shown response variation with age (Haussmann et al. 2005), season (Merino et al. 2000a), sex (Fargallo et al. 2002, Müller et al. 2003), nestling hierarchy (Christe et al. 1998), or that its relationship with individual traits vary between years (Jovani et al. 2004). In addition, differences in cellular immunity

between day and night have been described in adult captive sparrows *Passer domesticus* (Navarro et al. 2003). However, the effect of daytime in cellular immune responses in wild birds remains widely unknown, despite the fact that almost all studies evaluate cellular immunity during the day (but see, Mougeot et al. 2004). Thus, it is crucial (1) to know how cellular immunity varies throughout the day (rather than between day and night) in a non-captive bird, controlling for other variables, and (2) to evaluate whether this pattern is consistent between years. In this paper, I describe the diurnal variation of T-cell-mediated immunity in a wild population of Eurasian kestrel *Falco tinnunculus* nestlings through three years.

## Methods

The study was conducted in 2000, 2001 and 2002 in an Eurasian kestrel (kestrel hereafter) population breeding in nest-boxes in Campo Azávaro region (40°40'N, 4°20'W, 1,300 m a.s.l.), a homogenous mountain grassland area in central Spain (for more details see Fargallo et al. 2001). I monitored nest boxes every 1–2 days during the prelaying and laying periods to estimate laying date, laying order and clutch size. Eggs were marked with felt pens as they were laid in order to know the laying sequence. To record hatching order, I started visiting nests every day from 25 d after the end of laying, and two to three times every day from when I detected the first evidence of hatching until all eggs of the clutch hatched. I marked the chicks with indelible ink on the hatching tooth, and later every five days on the head until they were ringed at 17–20 d. This allowed me to determine from which egg each chick had hatched. When nestlings were 24-days old I measured body mass to the nearest 0.25 g and cellular immunity according the PHA test (see below). Since all nestlings were sampled at the same age, body mass was used as a measure of body condition. In addition, a blood sample was taken to sex nestlings by molecular procedures (Fridolfsson and Ellegren 1999). For both body mass, size and T-cell-mediated immunity measurements, I recorded the time of day in which the birds were sampled.

## Cellular immunity

Twenty-four days after hatching, kestrel chicks were injected intradermally in the left wing web with 0.1 ml of a solution of Phytohaematogglutinin PHA (3 mg of PHA in 1ml of PBS, PHA Sigma L8754-50MG) after measuring its thickness at the point of injection. Twenty-four hours after injection wing web thickness was measured again and I considered the difference

between initial and final measurement as the cell-mediated immune response (Smits et al. 1999). In both cases (before and 24 h after injection), three measures were taken with a digital spessimeter (Mitutoyo Absolute ID-112B) to the nearest 0.01 mm. Both the initial (intra-class correlation coefficient,  $r=0.95$ ,  $F_{338,678}=130.3$ ,  $P<0.001$ ) and final ( $r=0.97$ ,  $F_{338,678}=205.4$ ,  $P<0.001$ ) measurements were repeatable. I used the mean values of the three measurements in the analyses.

## Statistical analyses

Statistical analyses were performed using SAS software (SAS 1989–96 Institute Inc., Cary, NC, USA). Since nestlings from the same nest had probably more similar characteristics than those from different nests due to the influence of sharing common parents and rearing environments, they were not considered as independent samples. Thus, I performed General Linear Mixed Models (GLMM), with “nest” as a random factor. By doing so, I avoided pseudoreplication (Hurlbert 1984). Statistical tests associated with the random term denoted significant nest variation in the variables examined (in all analyses  $Z>2.87$ ,  $P<0.002$ ). Cellular immunity was considered as the dependent variable, using a normal distribution of errors and an identity link function. Initial models included year, clutch size, brood size, nestling sex, laying and hatching order as fixed factors, day of assessment, nestling mass and daytime as covariates. For analyses, daytime (local time) was transformed to minutes where 00:00 was 0, 09:00 was 540, 12:00 to 720 and so on. In order to test for between-year differences in diurnal variation in immune response, I also included the interaction between “year” and daytime in the model. I coded laying and hatching order as initial, middle or last laid/hatched eggs, which allows comparisons of nestling parameters between clutches or broods of different sizes. To check for potential daytime biases in nestling sex-ratio, nestling mass, seasonality (date of assessment), hatching and laying order, I used GLMMs (with nest as a random variable) with those as dependent variables, and daytime as a covariate.

In order to explore whether the daytime variation of cellular immunity varied in relation to reproductive parameters (brood size, clutch size, laying date), I included the interactions between those variables and hour of sampling in a GLM model where year was considered as a fixed effect and cellular immunity as the dependent variable. For these analyses, “hour of sampling” was coded into three equal periods according to the overall range of variation of the three years (1 or morning: from 8:30 to 12:30; 2 or Midday: from 12:30 to 17:00; 3 or evening: from 17:00 to 20:55).

Some of the explanatory variables could covary, so I fitted their effects to the observed data following a forward stepwise procedure, testing the significance of each variable and adding only variables 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 were two-tailed.

## Results

The average immune response of nestlings was  $3.49 \pm 1.28$  mm (2000:  $4.13 \pm 0.14$  mm,  $n = 82$ ; 2001:  $3.08 \pm 0.12$  mm  $n = 108$ ; 2002:  $3.34 \pm 0.10$  mm,  $n = 120$ ). I did not find any daytime bias in assessment date, sex-ratio, hatching or laying order (GLMM, all  $P > 0.25$ ). Nestling mass was also not affected by sample time (GLMM,  $F_{2,223} = 1.40$ ,  $P = 0.248$ ).

Nestling immune response decreased with the hour of sampling, once controlling for nestling mass and year (Table 1, Fig. 1). Other potentially influencing variables, such as day of assessment, clutch size or brood size or their interactions, were not statistically significant (all  $P > 0.3$ ). Similarly, the interaction between year and daytime was not significant (GLMM,  $F_{2,224} = 2.12$ ,  $P = 0.123$ ). As expected, the relationship was also negative considering each year separately (GLMM's, 2000: estimate =  $-0.004$ ,  $F_{1,59} = 5.95$ ,  $P = 0.017$ ; 2001: estimate =  $-0.002$ ,  $F_{1,78} = 8.63$ ,  $P = 0.004$ ; 2002: estimate =  $-0.001$ ,  $F_{1,85} = 7.66$ ,  $P = 0.006$ ).

When considering three categories of daytime sampling (see methods), I found a significant interaction between brood size and daytime variation in cellular immunity (Table 2). Cellular immunity decreased with brood size at the end of the day (GLMM, estimate =  $-0.344$ ,  $F_{1,53} = 5.45$ ,  $P = 0.023$ , Fig. 2; year and nestling mass as covariates, both  $P < 0.023$ ), whereas no significant relationship was found in the other two categories (both  $P > 0.420$ , Fig. 2). No such relationship was found with either clutch size or laying date ( $P > 0.222$ ).

Table 1. Results of a GLMM (with normal error and identity link function) of T-cell-mediated immunity in Eurasian kestrel nestlings. The random term ( $Z = 5.02$ ,  $P < 0.001$ ) explained 63.7% of the variance, and 37.3% of the variance was due to the fixed effects. The variance explained by nestling mass, year, and daytime were 21.1%, 4.5% and 7.9% respectively. All interactions between variables were not significant (all  $P > 0.33$ ).

Source of variation	Estimate $\pm$ SE	F	df	P
Nestling mass	$0.0143 \pm 0.0021$	44.15	1,226	$< 0.001$
Year	$0.7642 \pm 0.1730$	13.32	2,226	$< 0.001$
Daytime	$-0.0018 \pm 0.0004$	15.84	2,226	$< 0.001$

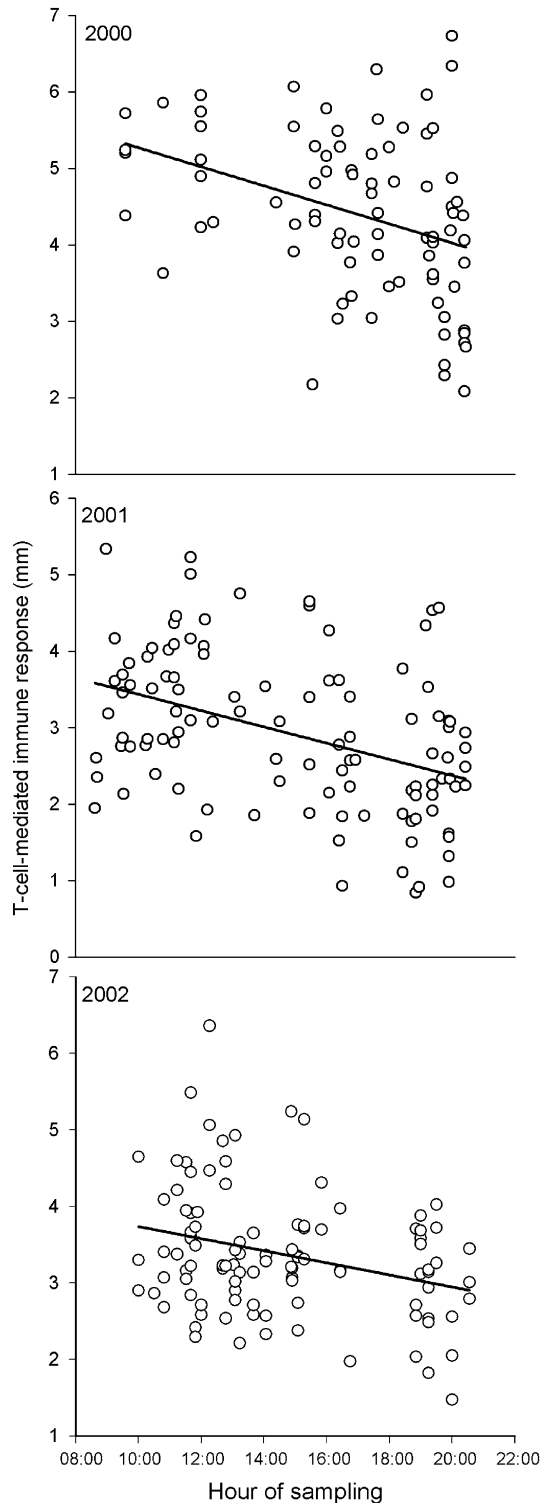


Fig. 1. Linear regressions between T-cell-mediated immune response and hour of sampling during the day of Eurasian kestrel nestlings in three different years. Note that although hour of sampling was transformed into minutes for the statistical analyses, I show the hour of sampling as military time for an easier interpretation. See results for statistics.

Table 2. Results of a GLMM (with normal error and identity link function) of T-cell-mediated immunity in Eurasian kestrel nestlings in relation to brood size. The model explained 37.2% of the original variance without considering the random term and the fixed effects as follow: nestling mass (20.07%), year (5.7%) and interaction brood size  $\times$  daytime (3.8%). The random term ( $Z=2.87$ ,  $P=0.002$ ) explained 62.8% of the variance. All other interactions between variables were not significant (see Methods and Results for more details).

Source of variation	Estimate $\pm$ SE	F	df	P
Nestling mass	0.0148 $\pm$ 0.0021	46.53	1,223	< 0.001
Year	0.7639 $\pm$ 0.1670	12.74	2,223	< 0.001
Daytime	-0.9312 $\pm$ 0.8689	2.15	2,223	0.118
Brood size	-0.4060 $\pm$ 0.1702	1.66	1,223	0.199
Brood size $\times$ daytime	0.4420 $\pm$ 0.2006	3.47	2,223	0.032

## Discussion

In this study I found that T-cell-mediated immune response significantly varied with the time of day in which nestlings were challenged and this variation was consistent between years (i.e., the slope of the relationship was similar each year). This is the first time that such daytime variation is described in birds although a previous study with white storks *Ciconia ciconia* found no relationship (Jovani et al. 2004). My results also show that cellular immune response is highly and positively related to nestling mass, as it has been widely suggested in birds (Tella et al. 2000b, Alonso-Álvarez and Tella 2001, Tella et al. 2001). However, I did not find variation in nestling mass through the day, possibly because nestling mass variance within nests was higher than the overall variance according to time of the day.

Parasites can have an important effect on T-cell-mediated immunity (Saino et al. 1998, Merino et al. 2000b). However, a study in 2002 showed that kestrel nestlings do not have blood parasites at the age they were sampled (Martínez-Padilla et al. 2004) and intestinal parasites do not have a significant effect on cellular immunity (Martínez-Padilla and Millán unpubl. data). So other factors could influence the results at least in kestrels. The relationship between cellular immunity and daytime might be linked to daytime variations in energy available to the nestlings, especially through sibling competition, an energy-demanding activity (Massemin et al. 2002) that affects cellular immunity in kestrels (Fargallo et al. 2002). Sibling competition is especially high in kestrel nestlings close to fledging, because at that age parents leave the prey in the nest and nestlings fight among them to take the prey (Village 1990). Parent kestrels deliver prey continuously throughout the day (Village 1990), and the continued physical exercise due to sibling competition, which involves increased oxygen consumption and production of free radicals (Rose 1991), may cause a negative effect on immune system (Svensson et al. 1998). This daily negative physiological effect could be improved through the night by sleeping, restoring physiological functions (McFarland 1981, Amlaner and Ball 1983) and turning back to maximum conditions at the beginning of the day. The idea that sibling competition causes a negative effect on cellular immunity is supported by the observation that cellular immunity decreased with brood size, but only at the end of the day. Similarly, in other studies, experimentally increased brood size decreased nestling cellular immunity (Naguib et al. 2004). However, little is known about the mechanism behind the negative relationship between

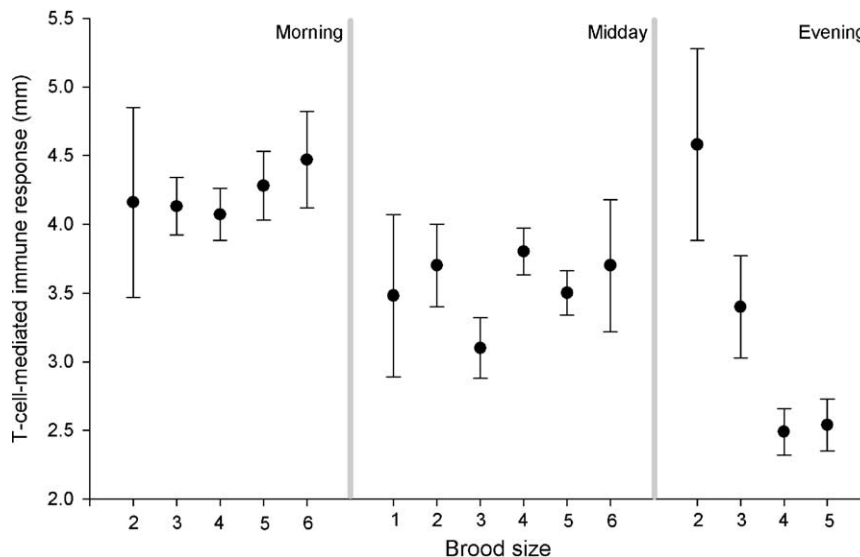


Fig. 2. Means  $\pm$  SD of T-cell-mediated immune response in relation to brood size according to the time of the day (see Methods).

T-cell-mediated immune response and the time of day. It has been described that corticosterone (Saino et al. 2003b) and testosterone (Naguib et al. 2004, Müller et al. 2005) depress cellular immunity. Furthermore, it has been suggested that corticosterone facilitates begging and resource allocation in nestling birds (Kitaysky et al. 2001) and testosterone levels increase with brood size (Naguib et al. 2004). Therefore, if the effects of sibling competition are more intense at the end of the day and in bigger brood sizes, I cannot rule out glucocorticoids or testosterone as proximal mechanisms of my results. However, more studies are needed to clarify this association. Although controversial and thus hard to generalize to other bird species, it has also been suggested that levels of corticosterone and testosterone show circadian variations. Morning peaks of corticosterone levels have been shown (de Souza et al. 2001, Tarlow et al. 2003, but see Vleck and Van Hook 2002), and testosterone is also elevated around night time or early morning (Schanbac et al. 1974, Balthazart 1976, Balthazart et al. 1981, Bachman et al. 1987, Aschoff 1989). The potential enhancement of these endogenous rhythms by sibling competition and its implications on cellular immunity remains to be explored. I suggest that kestrel nestlings may face a trade-off cyclically every day between two different energy demanding functions, such as T-cell-mediated immunity (Alonso-Álvarez and Tella 2001) and sibling competition. Different dynamics of sibling competition through the day may explain the lack of effect of time of the day in cellular immunity in white storks *Ciconia ciconia* (Jovani et al. 2004).

Future studies of bird cellular immunity should take into account this potential source of variation, either statistically (considering daytime as a covariate) or by homogenising the challenge time. Thus, I strongly recommend in future controlling by time of the day when nestling self-maintenance is measured as T-cell-mediated immunity. I also recommend to control by this variable in comparative studies when possible.

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