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RESEARCH ARTICLE

Nest predation risk modifies nestlings' immune function depending on the level of threat

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ABSTRACT

Predation risk is thought to modify the physiology of prey mainly through the stress response. However, little is known about its potential effects on the immunity of animals, particularly in young individuals, despite the importance of overcoming wounding and pathogen aggression following a predator attack. We investigated the effect of four progressive levels of nest predation risk on several components of the immune system in common blackbird (*Turdus merula*) nestlings by presenting them with four different calls during 1 h: non-predator calls, predator calls, parental alarm calls and conspecific distress calls to induce a null, moderate, high and extreme level of risk, respectively. Nest predation risk induced an increase in ovotransferrin, immunoglobulin and the number of lymphocytes and eosinophils. Thus, the perception of a potential predator per se could stimulate the mobilization of a nestling's immune function and enable the organism to rapidly respond to the immune stimuli imposed by a predator attack. Interestingly, only high and extreme levels of risk caused immunological changes, suggesting that different immunological parameters are modulated according to the perceived level of threat. We also found a mediator role of parasites (i.e. *Leucocytozoon*) and the current health status of the individual, as only nestlings not parasitized or in good body condition were able to modify their immune system. This study highlights a previously unknown link between predation risk and immunity, emphasizing the complex relationship among different selective pressures (predation, parasitism) in developing organisms and accentuating the importance of studying predation from a physiological point of view.

KEY WORDS: Alarm calls, H/L ratio, IgY, Innate immunity, Nest predation risk, Parasites

INTRODUCTION

Predation is an agonistic biological interaction that entails the killing of a prey by a predator, thus representing an important selective pressure in many natural systems, including birds (Caro, 2005). Nestlings are generally highly exposed to predation because nest predation represents the primary cause of mortality in many birds, particularly in altricial species, whose offspring are linked to a specific place, the nest, for the development period (Martin and Briskie, 2009). Thus, nest predation pressure is used to model several avian life-history traits in nestlings (Martin, 1995). Although many studies have analyzed the role of nest predation in anti-predatory behavioural responses, our knowledge on how nest predation may affect the physiological condition of prey nestlings is still poor (Ibáñez-Álamo et al., 2015). Predation risk is thought to modify the physiology of prey mainly through the variation of hormonal levels (i.e. corticosterone) triggered by the stress response (Sapolsky et al., 2000). However, there are important knowledge gaps on how predation risk might affect other biologically relevant physiological mechanisms, such as the immune system. Physiological responses might be of extreme importance, particularly in nestlings, given that they cannot usually escape from predators and, therefore, physiological and immunological changes might be the only remaining option, particularly in recently hatched nestlings.

The immune system of vertebrates is commonly divided into an innate and an acquired component, whose functions interact in a coordinated manner in the recognition and defence of pathogens. Both innate and acquired immunity include a cellular component (i.e. leukocytes) and a humoral component (i.e. circulating proteins; Roitt et al., 2001). The immune system promotes survival by limiting the negative impacts derived from microbes, diseases or infections (Horrocks et al., 2011b), and, therefore, it can play a fundamental role in predator–prey interactions given that predator attacks often produce injuries and wounds. Nevertheless, the effects of a stressful situation (i.e. predator threat) on immunity are generally considered suppressive, as organisms could reduce the costs involved in activating and maintaining a functional immune system in order to reallocate resources towards activities that are vital for their immediate survival (Sapolsky et al., 2000). However, some studies have shown that the effect of stress on immunity varies according to the characteristics of the stimulus (e.g. duration or intensity) as well as to the sensitivity of each immune component (Dhabhar, 2009; Martin, 2009), so a particular stressor may result in the downregulation of some immune components and the upregulation of others.

Despite theoretical evidence and the critical fitness consequences resulting from nest predation, only a few studies have investigated the link between nest predation risk and immunity, most of them focusing on adult birds (reviewed in Ibáñez-Álamo et al., 2015). Parents under predation risk showed changes in their leukocyte profile (Caetano et al., 2014), circulating immunoglobulins (Thomson et al., 2010) and locally induced proliferation of T lymphocytes (Navarro et al., 2004). In contrast, knowledge on the effect of predation on the immune system of nestlings is limited (Ibáñez-Álamo et al., 2015), particularly regarding short-term effects. This is surprising, as the consequences of a nest predator attack, which is generally a punctual and short-term event, could be much more critical to offspring than to parents: nestlings would lose

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their whole fitness, whereas parents would lose only the fitness of a single reproductive event. Nevertheless, older nestlings, which have already developed a certain capacity of flying, could survive a predator's attack (Halupka, 1998; Robinson and Robinson, 2001) and, therefore, the immunological changes triggered by the perceived predation risk can adaptively improve the probability of those nestlings overcoming the negative consequences of the predator attack. To our knowledge, only one study has directly examined the effects of predation risk on immunity in nestlings. Tilgar et al. (2010) found an increase of heterophil to lymphocyte (H/L) ratio in pied flycatcher (*Ficedula hypoleuca*) nestlings that were chronically exposed to the playback of conspecific distress calls, while no effect was found in response to an acute stress. Other studies have indirectly examined the immune variation in response to predation by measuring nestling immunocompetence after the handling procedure. Goedert et al. (2014) found that nestlings of the campo flicker (*Colaptes campestris*) with a high cell-mediated immune response showed a stronger anti-predator response when captured by a potential predator (i.e. researcher), and Chin et al. (2013) found that nestlings of the ring-billed gull (*Larus delawarensis*), a semi-precocial bird, reduced their innate immunity in response to handling stress. Nevertheless, handling of nestlings by researchers did not seem to alter the immune system in other species, such as the American kestrel, *Falco sparverius*, or the European starling, *Sturnus vulgaris* (Butler and Dufty, 2007). In light of these results, it seems that predation risk may play an important role in modulating nestling immunity, even though the direction of these immunological changes is not consistent.

These studies also highlight the importance of understanding the predatory cues that induce these immune changes. Older nestlings can gather information about the presence of a potential predator both directly (i.e. sounds emitted by predators; Magrath et al., 2007) and indirectly, through the signals given by other individuals that have already detected the predator, such as parents (parental alarm calls; Magrath et al., 2007), or siblings and conspecific nestlings (distress calls; Tilgar et al., 2010). The ability of nestlings to recognize the potential predator by using different cues is crucial to evaluating the level of predation risk and selecting the best anti-predator strategy (Magrath et al., 2007), such as staying silent and hidden in the nest or leaving it suddenly (Ibáñez-Álamo et al., 2015). Furthermore, it has been proposed that variability in the source of stress, such as its intensity, novelty and duration, can affect immunity differently (Martin, 2009), also supporting the relevance of studying the effects of different predation risk cues on nestling immunity.

In this study, we investigated the effects of acute nest predation risk on the immune system of common blackbird (*Turdus merula* Linnaeus 1758; hereafter blackbird) nestlings. We simulated four progressive levels of nest predation risk (i.e. null, moderate, high and extreme) by exposing nestlings to playback calls (i.e. non-predator calls, predator calls, alarm calls and distress calls, respectively) for 1 h, and analyzed their immune response according to the intensity of the risk. We measured 11 immunological parameters to study and capture the complexity of the immune system (Matson et al., 2006). We can make two alternative predictions: based on the general assumption that predator-induced stress results in an immunological suppression (Sapolsky et al., 2000), we predict that (1a) nestlings that experience an increased nest predation risk will downregulate their immune function; however, as studies on mammals have shown some immunoenhancing effects of an acute stress event (Dhabhar, 2002; Martin, 2009), our alternative hypothesis predicts that (1b) perceived nest predation risk could stimulate the immune function of blackbird nestlings in order to limit the negative consequences of a predator attack and thus promote their survival. As the intensity of the stress-induced response can be positively correlated to the intensity of the stress experienced (Dhabhar and McEwen, 1997), in either case (1a and 1b) we predict that (2) immunity of the nestlings from the extreme predation risk group will be affected the most, by decreasing (prediction 1a) or increasing (prediction 1b) the immunological parameters. Finally, given that the particular conditions in which the altricial nestlings develop (i.e. parasite load or infection status; Navarro et al., 2004; De Coster et al., 2010) could generate additional sources of variation in immune response induced by nest predation risk (Møller et al., 1990), we evaluate the presence of blood parasites and mites in the nest. Specifically, we expect that (3) those individuals infected by endoparasites will respond more weakly to the risk of predation as the cost of immunosuppression will be higher for these individuals compared with non-parasitized nestlings (Forrester and Greiner, 2009).

MATERIALS AND METHODS

The experiment was conducted on a blackbird population in the morning and early afternoon, from 08:30 to 14:30 h, during the breeding period of 2015, between mid-April and mid-June. The study area was located in the Valley of Lecrín (36°56′N, 3°33′W), a rural area situated at 580 m above sea level, in the south east of Spain, where almost half of the nests are depredated (48.9%; Ibáñez-Álamo and Soler, 2010). We actively searched for nests from the start of the breeding season. Once the nest was located, we visited it every 2 days in order to determine its hatching date (±1 day), arbitrarily starting to count from 1 March (i.e. 1 March=1). All the nests were checked using a mirror attached to a pole to minimise the disturbance produced by nest inspection.

Experimental design and data collection

We altered nest predation risk by creating four experimental groups in which we manipulated the acoustic calls perceived by blackbird nestlings: predator calls group (PC), adult alarm calls group (AC), distress calls group (DC) and control group (CON), corresponding to a non-predator group. Call playbacks for AC, PC and CON groups were selected from a virtual platform on the web (www.xeno-canto.org). PC playbacks included calls emitted by 20 individuals corresponding to different local predators, such as Eurasian sparrowhawks, *Accipiter nisus* (Newton, 1986; Ibáñez-Álamo and Soler, 2012), and black-billed magpie, *Pica pica* (Collar, 2005), to simulate a moderate threat to the nest. This situation simulated the presence of a potential nest predator that lives and moves in the vicinity of the nest (Blumstein et al., 2008) and is based on the ability of nestlings to independently assess the current risk of nest predation by recognizing direct calls of a predator's presence (e.g. Magrath et al., 2007). For AC playbacks, we selected 15 blackbird alarm calls from both males and females, by including the several kind of alarm calls emitted by this species. For example, females emit a typical rhythmical whistle, especially when a predator is moderately near the nest, whereas a strong and sudden call is used when predators get close. Adult alarm calls usually warn the partner and the nestlings about the presence of a potential predator close to the nest and can trigger specific anti-predatory responses in nestlings of various species (e.g. Magrath et al., 2007; Suzuki, 2011). Using these calls, we wanted to simulate a higher perceived predation risk compared with that experienced by the PC group. In the DC group, nestlings were exposed to playbacks of blackbird nestling distress calls in order to simulate the direct attack of a predator at the nest. These calls are produced by nestlings in extremely threatening situations (e.g. when caught by a predator;

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Marler and Slabbekoorn, 2004) and can induce anti-predatory behaviours and physiological changes in nestlings (e.g. Tilgar et al., 2010). Because few calls of blackbird nestlings were available on the internet, we recorded distress calls of 10 nestlings from six broods of our population (Sony ICD-PX333 Digital Voice Recorder). This was done by handling them inside a car to ensure that the other nestlings in the area would not hear the calls. By doing this, we also avoided undesirable background sounds (i.e. alarm calls) and we standardized the recording parameters. Broods used for distress call recordings were successively excluded from the experimental manipulation. The CON group, corresponding to the null level of predation risk, involved exposure to the songs of other passerine species living in the study area, which do not represent any threat for blackbird nestlings. We selected 20 individuals of different species, such as the European serin (*Serinus serinus*), European goldfinch (*Carduelis carduelis*), Sardinian warbler (*Sylvia melanocephala*) and common chaffinch (*Fringilla coelebs*). We excluded alarm call vocalizations of these species, which may indirectly indicate the potential presence of a predator in the surroundings of the nest.

All calls were converted to digital audio files using Audacity software (version 2.1.0; https://www.audacityteam.org/) in order to divide calls of each individual into different sequences and keep only the high-quality ones. Each playback consisted of 3 min of call activity (20 s of calls interspersed with 40 s of silence) followed by 5 min of silence. The playbacks were joined together in a single 1 h and 15 min long audio file, in which several calls of distinct selected individuals were reproduced randomly to avoid habituation of blackbirds to the sounds. The first 15 min of each audio file consisted of silence in order to calm the nestlings in case they could perceive our presence during placement of the speakers. We created eight unique audio files for each treatment, and nestlings of each nest heard the playback only once.

The speakers were hidden under a camouflaged cloth, connected to an MP3 player and broadcasted near the nest (6 m) at 70 dB. A similar methodology has been successfully used before in our model system to manipulate acoustic calls (Ibáñez Álamo et al., 2011; Ibáñez-Álamo and Soler, 2016). When we were simultaneously broadcasting two or more playbacks, we left a buffer zone (80–100 m) between the areas to grant acoustic isolation during the experimental manipulation. We used a sequential order to assign each nest to a particular treatment (CON, PC, AC, DC), which allowed us to evenly balance the treatments throughout the season. We performed the experiment when nestlings were 10–11 days old, just before they leave the nest (Ibáñez-Álamo and Soler, 2010), in order to reduce the chances of nestlings not being able to perceive and recognize the acoustic calls.

Once we verified the presence of the nestlings in the nest, we initiated the corresponding playback. After broadcasting the playbacks, we turned off the speakers and immediately collected a blood sample (250–300 µl) from the brachial vein of the nestlings. All blood samples were collected between 10:00 and 14:00 h to standardize for the time of the day, and stored at 4°C (for a maximum 5 h after collection) until centrifugation (7571 ***g*** for 10 min). Plasma was stored at –25°C. A drop of blood was smeared on a marked glass slide and dried in open air. We also took common biometrical measures (i.e. body mass and tarsus) for each nestling and scored presence/absence of mites (subclass Acari) while we were manipulating them.

We completed the experimental manipulation for 66 nests including 161 nestlings. The number of nests (nestlings) by group was as follows: CON=15 (36), PC=16 (41), AC=17 (42) and DC 18 (42). Please note that the final sample sizes for some analyses were slightly smaller than those previously described owing to limited plasma availability for some individuals.

Immunological assays

Humoral immunity

Haemagglutination (HA)/haemolysis (HL) titres

Both HA and HL quantify levels of innate immunity. In particular, HA is indicative of the levels of circulating natural antibodies. These proteins facilitate the initial recognition of pathogens and promote the activation of adaptive immune responses (Carroll and Prodeus, 1998). HL titres estimate the action of complement and other lytic enzymes (Carroll and Prodeus, 1998). We performed HA and HL assays following Matson et al. (2005) with modifications described by Mauck et al. (2005). Scans of individual samples were randomized and scored by a single person (G.R.).

Haptoglobin (Hp)

Hp is an acute phase protein that is able to bind free haemoglobin released from erythrocytes in order to inhibit its oxidative activity. Under normal conditions, Hp is present in the blood at low concentrations, but it can rapidly increase in response to acute infection, inflammation or trauma (Matson et al., 2012). We measured Hp concentration in plasma with a commercial kit (TP801; Tridelta Development Ltd, Maynooth, Ireland), which colorimetrically quantified the haemoglobin binding capacity of the plasma.

Ovotransferrin (OVT)

OVT acts as an acute phase protein by binding free iron, which is an essential nutrient for bacterial growth. High levels of OVT are usually considered an as indicator of inflammation, infection, poor nutrition or disease (Horrocks et al., 2011a). We measured the OVT concentration following Horrocks et al. (2011a).

Nitric oxide (NOx)

Blood levels of NOx increase in response to the presence of inflammatory cytokines, microorganisms or endotoxins (Sild and Hõrak, 2009). NOx is considered a measure of innate immunity as many cell types are capable to produce it, especially macrophages, which release NOx by exocytose in order to destroy pathogens (Crippen et al., 2003). Quantification of plasmatic NOx was performed following Sild and Hõrak (2009).

Immunoglobulins (IgY)

Immunoglobulins are glycoproteins with antibody activity, produced by B lymphocytes. The antibodies neutralize pathogens, induce the activation of the complement system and promote cell migration to the sites of infection (Härtle et al., 2014). We used direct ELISA (following Martinez et al., 2003) to measure the total IgY concentrations. This assay provides information about total circulating IgY because the anti-immunoglobulins antibody we used likely binds to different isotypes of immunoglobulins (i.e. IgG, IgM), as observed in previous studies on different species (great tit *Parus major*: Kilpimaa et al., 2005; magpie: Pihlaja et al., 2006; pied flycatcher: Kilpimaa et al., 2007). Our experimental methodology did not expose the nestlings to new antigens through immunization, so we are confident that this measure reflects the general baseline levels of adaptive humoral defences and captures the abundance of circulating antigen-binding antibody molecules. These IgY levels represent the abundance in the bloodstream of the natural antibodies and the antibodies of maternal origin, as well as the specific antibodies produced by each nestling in response to recent infections associated

with developing in a natural environment; therefore, they provide important information about the health status of an organism (Gustafsson et al., 1994) and its immune capacity in a natural context (Johnsen and Zuk, 1999). Using this method, we measured both natural antibodies already present in nestlings (i.e. maternally transmitted; Grindstaff et al., 2003) and those developed by nestlings in response to naturally encountered antigens during the first days after hatching. We adapted this method to common blackbird nestlings by calculating the optimal plasma dilution (1:9000). In this assay, when an IgY molecule binds to the detection antibody, a yellow-coloured compound is produced. Thus, the sample content of total IgY measured is directly proportional to the amount of coloured product measured with a spectrophotometer. Data obtained are expressed in optical density units. For each sample, the mean absorbance value was calculated from three replicates and 'corrected' by subtracting the mean value of 'blank' absorbance to account for non-specific binding related to background activity. The cut-off value was calculated as the mean optical density of black values plus 3×s.d.

Cellular immunity

Leukocyte profile

In order to quantify white blood cells, we fixed blood smears in absolute methanol and stained them with Giemsa (GS500-500 ml, Sigma-Aldrich, St Louis, MO, USA) diluted 1:10 in PBS (pH 7.2) for 45 min. Subsequently, smears were scanned with an optical microscope (1000× magnifications with oil immersion). We counted a minimum of 100 leukocytes on each slide. Each cell was classified as heterophil, lymphocyte, eosinophil, basophil or monocyte, following Campbell and Ellis (2007). Leukocyte counts allowed us to calculate the H/L ratio. Smears were also examined to evaluate the presence of hematozoan parasites (genus *Leucocytozoon*). To estimate the presence/absence of *Leucocytozoon* infection, we inspected infected blood cells, which develop into gametocytes to complete the reproductive cycle (Forrester and Greiner, 2009). Blood smears were examined by a single investigator (E.C.).

Statistical analysis

As a general procedure, the effect of predation risk treatment on the different immunological components was first tested analyzing each immunological parameter separately. This method allowed us to inspect the individual changes in each parameter considering that parameters may not show similar patterns (Buehler et al., 2008; Pap et al., 2010). Subsequently, we investigated how all immunological parameters covaried among the four treatment groups, using discriminant analyses in order to obtain a general overview of the immune response.

Linear mixed models (LMM; lme function in the 'nlme' package; Pinheiro et al., 2016) were used to analyze each immunological parameter: HA, Hp, OVT, NOx, IgY, the number of leukocytes, lymphocytes, heterophils, eosinophils and the H/L ratio. It was not possible to fit a model for HL or for the numbers of basophils and monocytes because data on these immunological parameters were insufficient. In each model, we considered: treatment, the effect of breeding season (expressed by hatching date; Dubiec and Cichon, 2005), the effect of body condition [calculated by the body mass index (BMI), which corresponds to the residuals of the regression between body mass and tarsus length; Jakob et al., 1996], and the presence of mites and *Leucocytozoon*. When we fitted the IgY model, we also controlled for the number of lymphocytes because a specific type of lymphocyte (i.e. B cell) is responsible for the production of immunoglobulins. We also considered the interactions between treatment and each of the other predictors in order to evaluate the possible mediated effect of these factors on nest predation risk, and we included nest as a random factor in order to control for the non-independence of nestlings from the same nest. Before fitting the models, we checked for possible collinearity among predictors (Fig. S1, Table S1). We found a positive correlation between the presence of mites in the nest and hatching date (Spearman's rank correlation, r_S=0.69, P<0.001), indicating that the proportion of nests parasitized by mites significantly increased throughout the season. Therefore, we decided to drop the presence of mites from our models (Quinn and Keough, 2002) and kept the effect of breeding season (hatching date). In this way, we indirectly included the effect of mites; moreover, we were able to indirectly control for the effect of other environmental factors not directly measured (such as temperature or food availability), but that may influence the immune function (Christe et al., 2001; Serra et al., 2012). We also controlled for collinearity after fitting the models by calculating the variance inflation factor (VIF). No predictors exceeded the threshold of 3 (Zuur et al., 2010). By using a backward selection procedure, we excluded the predictors that showed the highest (non-significant) P-values, dropping firstly the non-significant interactions in order to obtain simpler alternative models (Engqvist, 2005). We did not remove treatment as it reflects the hypotheses to be tested. After checking the homogeneity of variance and the normal distribution of the residuals of our models (Zuur et al., 2010), we used logarithmic transformations for those variables that violated these assumptions (i.e. NOx, the number of leukocytes, lymphocytes, heterophils, eosinophils and H/L ratio). When appropriate, we explored significant treatment effects using Fisher's *post hoc* tests [least significant difference (LSD)]. Correlation coefficients between the immunological parameters are provided in the supplementary information (Table S2, Fig. S2).

Discriminant analysis was performed using the function lda in the 'MASS' package (Venables and Ripley, 2002). This analysis showed which immunological parameters are chosen by discriminant functions to classify nestlings into the four treatment groups. This method works by investigating the relationships among the groups' covariance to find a fixed number of linear functions that are used to discriminate between groups. Significance of a linear function indicates that the parameters with the high loadings on this function differ among the groups (Crawley, 2007).

All analyses were performed using R version 3.1.1 for Windows (https://www.r-project.org/), except those regarding the cellular component, which were analyzed with STATISTICA 10 (StatSoft Inc., Tulsa, OK, USA). Values are presented as means±s.e.

RESULTS

Perceived predation risk directly affected OVT and IgY concentrations, and the number of eosinophils, whereas the effect of predation risk on the number of lymphocyte and the H/L ratio depended on the presence of *Leucocytozoon* and body condition, respectively (Table 1).

Direct immune responses

The levels of OVT were significantly higher in the DC group compared with the AC group (Fig. 1A). Nestlings of the AC group had higher IgY levels compared with the CON group (Fig. 1B), and nestlings of the AC and DC groups had significantly more eosinophils than those of the PC and CON groups (Fig. 1C). Furthermore, IgY levels were positively correlated with the number of lymphocytes (β=0.006±0.002) and non-parasitized nestlings had more eosinophils than nestlings parasitized by *Leucocytozoon* (Table 1). NOx and HA levels were not affected by treatment.

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Table 1. Statistics of the linear mixed models for each of the immunological parameters

	β±s.e.	d.f.	*F*	*P*
Humoral innate immunity				
HA				
Treatment		3, 53	0.83	0.48
Hatching date	+0.04±0.02	1, 53	37.72	<0.001*
BMI		1, 67	0.015	0.94
Leucocytozoon		1, 69	4.82	0.03*
Treatment×Hatching date		3, 53	2.02	0.09
Treatment×BMI		3, 61	0.78	0.51
Treatment×*Leucocytozoon*		3, 64	1.33	0.27
Hp				
Treatment		3, 60	0.83	0.48
Hatching date		1, 59	0.67	0.41
BMI		1, 85	2.04	0.16
Leucocytozoon		1, 78	0.08	0.77
Treatment×Hatching date		3, 51	1.12	0.35
Treatment×BMI		3, 72	0.16	0.92
Treatment×*Leucocytozoon*		3, 75	0.42	0.074
OVT				
Treatment		3, 48	3.14	0.03*
Hatching date	+0.02±0.01	1, 48	5.07	0.03*
BMI		1, 52	1.13	0.29
Leucocytozoon		1, 49	1.30	0.26
Treatment×Hatching date		3, 43	0.66	0.58
Treatment×BMI		3, 46	1.53	0.22
Treatment×*Leucocytozoon*		3, 43	0.64	0.59
NOx				
Treatment		3, 53	0.16	0.92
Hatching date		1, 52	0.001	0.98
BMI		1, 66	2.60	0.11
Leucocytozoon		1, 68	1.99	0.16
Treatment×Hatching date		3, 49	0.52	0.67
Treatment×BMI		3, 60	1.31	0.28
Treatment×*Leucocytozoon*		3, 63	1.79	0.15
Unspecific adaptive immunity				
IgY				
Treatment		3, 38	2.85	0.04*
Hatching date	+0.005±0.002	1, 58	6.56	0.01*
BMI		1, 86	0.35	0.85
Leucocytozoon		1, 88	0.50	0.48
Lymphocytes	+0.006±0.002	1, 96	5.50	0.02*
Treatment×Hatching date		3, 55	0.40	0.76
Treatment×BMI		3, 77	0.04	0.98
Treatment×*Leucocytozoon*		3, 80	0.09	0.96
Treatment×Lymphocytes		3, 83	1.75	0.16
Cellular component immunity				
Leukocytes				
Treatment		3, 58	0.30	0.84
Hatching date	+0.007±0.002	1, 58	14.32	<0.001*
BMI		1, 87	0.81	0.37
Leucocytozoon		1, 89	0.95	0.33
Treatment×Hatching date		3, 55	1.01	0.40
Treatment×BMI		3, 81	0.70	0.55
Treatment×*Leucocytozoon*		3, 84	2.08	0.11
Heterophils				
Treatment		3, 58	0.13	0.94
Hatching date	+0.007±0.003	1, 58	4.81	0.03*
BMI		1, 87	0.01	0.91
Leucocytozoon		1, 89	0.001	0.99
Treatment×Hatching date		3, 55	0.49	0.69
Treatment×BMI		3, 81	0.93	0.43
Treatment×*Leucocytozoon*		3, 84	1.28	0.28
Lymphocytes				
Treatment		3, 58	1.36	0.25
Hatching date	+0.007±0.002	1, 58	10.08	<0.001*
BMI	+0.17±0.008	1, 84	6.21	0.01*
Leucocytozoon		1, 84	7.67	0.006*
Treatment×Hatching date		3, 55	1.46	0.24
Treatment×BMI		3, 81	2.27	0.09
Treatment×*Leucocytozoon*		3, 84	3.01	0.03*
Eosinophils				
Treatment		3, 58	5.27	0.001*
Hatching date		1, 58	0.70	0.40
BMI		1, 87	1.49	0.23
Leucocytozoon		1, 88	3.61	0.06
Treatment×Hatching date		3, 55	1.13	0.35
Treatment×BMI		3, 84	0.69	0.56
Treatment×*Leucocytozoon*		3, 81	0.52	0.67
H/L ratio				
Treatment		3, 59	1.22	0.31
Hatching date		1, 58	0.26	0.61
BMI		1, 85	0.86	0.36
Leucocytozoon		1, 85	1.69	0.20
Treatment×Hatching date		3, 55	0.90	0.44
Treatment×BMI		3, 88	3.08	0.03*
Treatment×*Leucocytozoon*		3, 82	0.39	0.76

The parameters included in the simplified model are in bold. Significant predictors are marked with an asterisk. β coefficient and s.e. are shown for significant covariates. HA, haemagglutination; Hp, haptoglobin; OVT, ovotransferrin; IgY, immunoglobulin; H/L ratio, heterophil to lymphocyte ratio; BMI, body mass index.

Indirect immune responses

Non-parasitized nestlings of the AC and DC groups had higher numbers of lymphocytes than those of the CON group (LSD *post hoc* test P=0.007 and P=0.03, respectively), whereas in the presence of *Leucocytozoon*, nestlings of the AC group had more lymphocytes than those of the DC group (LSD *post hoc* test P=0.003). Moreover, nestlings parasitized with *Leucocytozoon* had a significantly higher number of lymphocytes than non-parasitized nestlings for the PC, AC and CON groups (LSD *post hoc* test P=0.003, 0.02 and 0.05, respectively), but not for the DC group (LSD *post hoc* test P=0.44; Fig. 2). The number of lymphocytes was positively correlated with body condition (β=0.17±0.008; Table 1). The H/L ratio changed in relation to the nestlings' body condition (Table 1): nestlings in lower body condition had a higher H/L ratio compared with nestlings in good body condition, but only in the CON group (β=−0.009±0.038; P=0.02; Fig. 3).

Interaction with environmental factors

There was a positive relationship between hatching date and several immunological parameters: HA, OVT, IgY concentrations, and the number of leukocytes, heterophils and lymphocytes (Table 1).

Discriminant analysis

Discriminant analysis resulted in two highly significant discriminant functions which together explained 83% of the variance among treatment groups (Table 2). The first function indicated H/L ratio, eosinophils, lymphocytes and heterophils as the immunological parameters that contributed most to differences among groups (Table 2). According to this function, AC and DC groups were similar to each other and different from the PC and CON groups (Fig. 4). The second function, which is mainly explained by OVT and IgY levels, showed that nestlings in the DC and AC groups were different from each other (Fig. 4). The third discriminant function grouped the remaining immune indices, explaining 17% of the total variation (Table 2).

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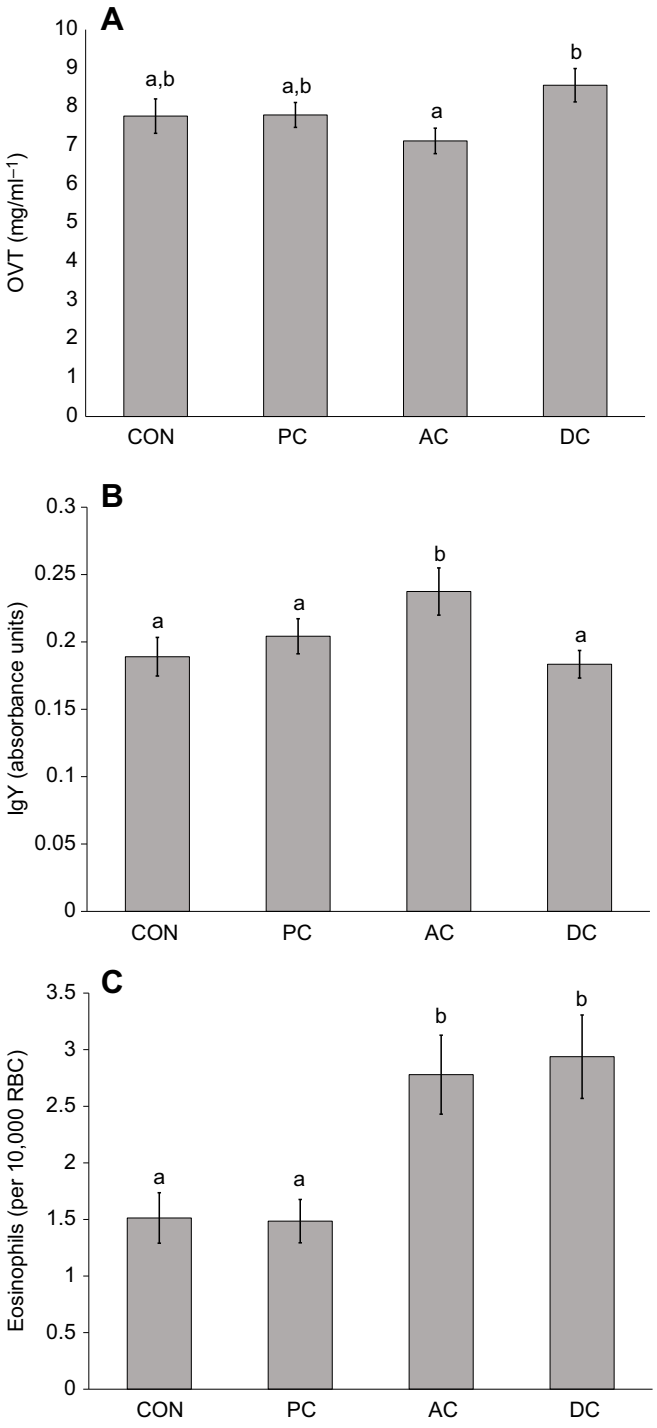

Fig. 1. Effects of short-term increase of nest predation risk on immunological parameters in blackbird nestlings in the four experimental groups: predator calls (PC), adult alarm calls (AC), distress calls (DC) and control (CON). (A) Ovotransferrin (OVT; *N*: CON=27, PC=32, AC=29, DC=27), (B) immunoglobulin (IgY; *N*: CON=36, PC=41, AC=42, DC=42) and (C) number of eosinophils (*N*: CON=33, PC=39, AC=42, DC=40). Means and standard errors are shown. Different letters indicate significant differences for the *post hoc* test at the $P<0.05$ level. RBC, red blood cells.

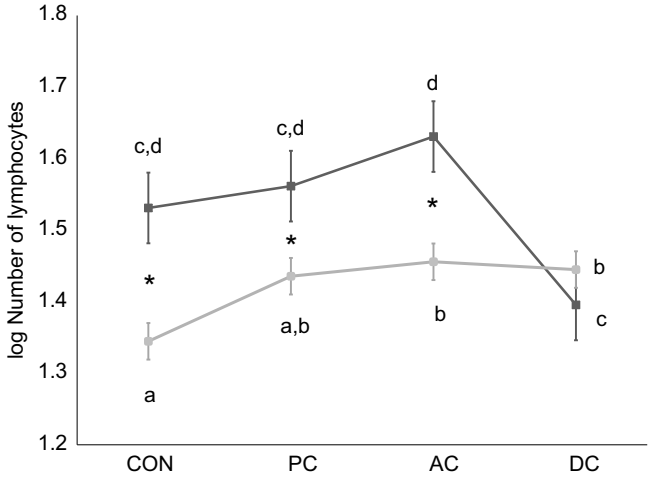

Fig. 2. Effect of treatment on the number of lymphocytes for parasitized (with *Leucocytozoon*) nestlings (dark grey line) and non-parasitized nestlings (light grey line). Sample sizes in the four treatment groups are as follows: CON (*N*=33), PC (*N*=39), AC (*N*=41) and DC (*N*=40). Different letters indicate significant differences among groups for the *post hoc* test at the $P<0.05$ level. Asterisks indicate a significant difference in the number of lymphocytes between non-parasitized and parasitized nestlings within each treatment group.

DISCUSSION

The experimental increase of nest predation risk directly affected OVT, IgY and the number of eosinophils, whereas the effect on lymphocytes and H/L ratio was mediated by the presence of *Leucoctytozoon* and body condition, respectively. We found an overall positive association between the magnitude of the effect on immunity and the intensity of nest predation risk, with the main changes linked to the most risky situations (DC and AC groups) and, surprisingly, no alterations for the moderate predation risk treatment (PC group).

Immune responses to nest predation risk

Our results showed that OVT, IgY and the number of eosinophils increased in response to a short-term increase of nest predation risk, indicating a reinforcement of the processes involved in immune defence. This finding supports the idea that a stimulation of immune activity occurs promptly during acute stress conditions (prediction 1b), whereas it contrasts with the assumption of the suppressive

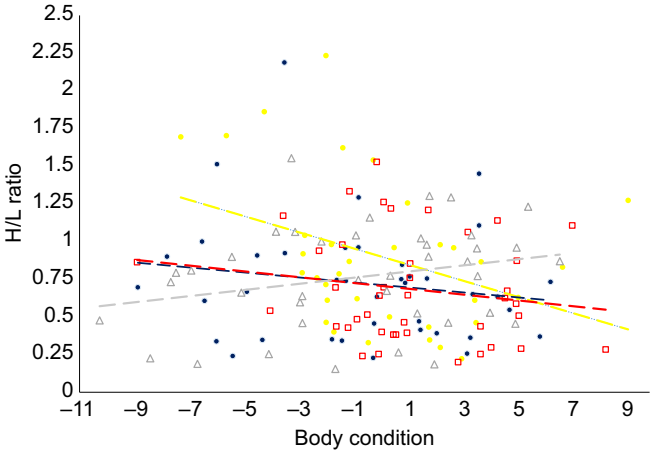

Fig. 3. Patterns of the interaction between treatment and body mass index (BMI) on the heterophil to lymphocyte (H/L) ratio. Yellow circles, blue circles, red squares and grey triangles represent CON (*N*=34), PC (*N*=39), AC (*N*=42) and DC (*N*=40) groups, respectively. Correlation was significant only in the CON group (Pearson $r=-0.37$, $P=0.03$); the H/L ratio decreased in nestlings with better BMI (yellow dashed line).

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Table 2. Results of the discriminant analysis of the immunological parameters measured among the four treatment groups

	Function 1	Function 2	Function 3
HA	−0.110	−0.174	0.184
HL	0.187	−0.192	−0.314
OVT	−0.111	−0.721	−0.231
NOx	0.270	−0.358	0.372
IgY	−0.276	0.579	0.138
Leukocytes	−0.065	−0.151	0.202
Heterophils	0.321	−0.090	0.067
Lymphocytes	−0.324	−0.133	0.416
Eosinophils	−0.414	−0.058	−0.338
H/L ratio	0.630	0.005	−0.261
Eigenvalues	0.38	0.27	0.13
Variance explained (%)	48.39	35.03	16.58
Cumulative proportion	48.39	83.42	100
P-value	<0.001	0.001	0.04

The higher the eigenvalues, the larger proportion of variance explained. This indicates the function that better differentiates among groups.

effect of stress on immunity (prediction 1a; Wingfield et al., 1997). Generally, the goal of the immune-suppression effect produced by stress is to redirect resources toward emergency biological functions (Sapolsky et al., 2000). However, in certain situations, such as during a predator attack or a territorial conflict, which may lead to physical aggression and, consequently, provoke important injuries, the suppression of the immune function could not be adaptive (Dhabhar, 2002). In these cases, the rapid activation of the immune defences may be critical for the survival of an individual. Our experiment supported this hypothesis as blackbird nestlings, by perceiving an imminent attack, displayed stimulated immunity possibly to increase survival following the attack of a nest predator. In particular, the observed increase in OVT levels for the extreme level of nest predation risk (DC group) would be suitable to contrast the spread of pathogens promptly and to limit the negative consequences of an imminent injury. OVT is a multifunctional protein – its concentration increases in the bloodstream in response to inflammation and it is involved in bactericidal, antiviral and immunomodulatory activity (Giansanti et al., 2012).

Interestingly, nestlings exposed to adult alarm calls (AC group) showed higher levels of total IgY. Unfortunately, our data do not allow us to clarify whether the resulting increase of blood immunoglobulins mirrors the stimulus of the production of a specific antigen-binding molecule (e.g. IgY, IgM, natural antibodies; Parmentier et al., 2012). Regardless of this issue, we can hypothesize that the perceived predation risk stimulated the reinforcement of circulating proteins, including natural antibodies and those developed in response to natural antigens related to the environmental rearing conditions of nestlings (e.g. mites, parasites, faeces). This finding suggests that predator stress-induced immunomodulation can rapidly activate the levels of IgY. The effect of stress on immunoglobulin production has been studied during chronic stress conditions and the results have shown an immunosuppression in nestlings (Bourgeon et al., 2011). The elevation of IgY is probably associated with general physiological changes in response to acute predator stress. These changes include variation in corticosterone levels (Sapolsky et al., 2000) and stress protein production (i.e. HSP 70), which are involved in immune function, by binding antigens and presenting them to the immune system (Tsan and Gao, 2009). These changes occurred rapidly (i.e. within 2 h after induced stress; Gu et al., 2012). Thus, we may assume that the increased IgY in the bloodstream acts as a stress protein and contributes to a general state of preparedness of the immune system to cope with incoming infections caused by a predator attack (Dhabhar and McEwen, 1997).

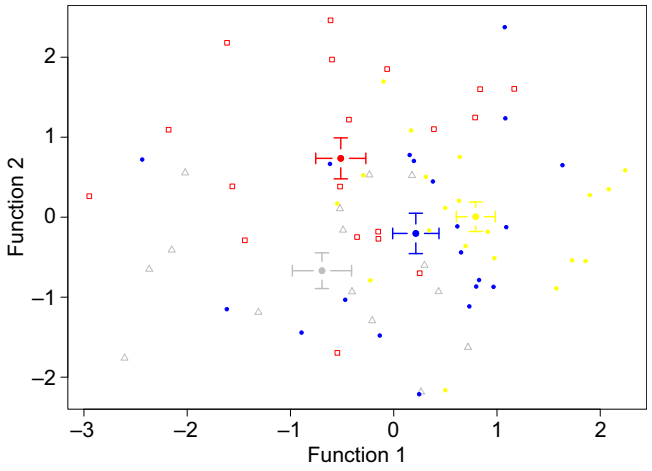

Fig. 4. Distribution of nestlings according to the first and second discriminant functions. Dots and whiskers refer to means±s.e. Yellow circles, blue circles, red squares and grey triangles represent CON, PC, AC and DC groups, respectively.

The perceived predation risk also modified part of the nestling leukocyte profile. Eosinophils increased in nestlings of the DC and AC groups, whereas lymphocytes increased only in the AC group, and their increase was associated with the presence of parasites. By contrast, the number of heterophils did not significantly change. Thus, the acquired cellular component (i.e. lymphocytes) might have a major role compared with the innate immunity (i.e. heterophils). The redistribution of lymphocytic cells enables their employment from the lymph system (i.e. bursa of Fabricius), where they receive antigens released from sites of interest in the peripheral tissue (Dhabhar et al., 1995). Therefore, under the risk of predation, lymphocytes would leave the lymph system, possibly as a consequence of the increase in blood pressure caused by fear of the perceived threat. The redistribution of lymphocytes might contribute to preparing nestlings to a possible pathogen infection in case of injuries after the predator's attack. The results also suggested that different types of leukocytes could respond at different times after the treatment started (Buehler et al., 2008; Davis, 2005). This mechanism seems to be associated with the two stages of leukocytic response distribution to stress observed in mammals: in the initial phase, leukocytes increase in blood circulation and, subsequently, in the second stage, they exit the bloodstream and migrate towards the sites of interest, such as wounds, infection sites or lymphoid tissues, where they could perform their specific functions (Dhabhar and McEwen, 1999). In the context of nest predation, the redistribution of leukocytes is evolutionarily explained as an important component of the fight-or-flight reaction during predatory attacks (Dhabhar and McEwen, 1997). Short-term stress-induced immune modulation would prepare the organism's defences by incrementally increasing immune function in the external compartments, such as skin (Dhabhar and McEwen, 1997), which are more exposed to injuries. According to these premises, it is likely that the immune suppression benefits (prediction 1a) are favourable only over a long period (chronic stress), but not during short-term stress situations, such as a predatory attack (Dhabhar and McEwen, 1999). In fact, the activation of the immune system is known to be associated with energetic and nutritional costs (Lochmiller and Deerenberg, 2000; Fargallo et al., 2002), as well as autoimmune reactions and oxidative stress

(Hasselquist and Nilsson, 2012). Our results show that the benefits of a short-term increase in immunity induced from predation risk would overcome these costs. Actually, the costs related to a short-term stress situation are generally transitory because they are associated with the redistribution of white blood cells and can therefore be compensated for after the end of the event that provoked the stress (Eggers et al., 2008).

The significant increase in the number of eosinophils that we observed indicates that these cells could have an important role in the initial stage of immunomodulation (Jacobsen et al., 2007), at least in response to nest predation risk. This offers an interesting addition to our limited knowledge of avian eosinophils, which are generally associated with parasite exposure, antibody-mediated response and the regulation of the inflammatory response (Campbell and Ellis, 2007; Davis et al., 2008).

Responses according to the intensity of nest predation risk

Our results show that the intensity of the immunological changes was positively correlated with the intensity of perceived nest predation risk, thus supporting our second prediction. Based on the first function of the discriminant analysis, nestlings' immune responses are gathered into two different groups: low (CON and PC groups) versus high risk situations (AC and DC groups; Fig. 4), showing a different level of variation than initially expected (four treatments). Although high and extreme predation risk induced an increase in several immune components, perceiving a moderate predation risk (i.e. predator calls) did not provoke significant changes. This finding is highly relevant as the use of predator calls to increase predation risk is a common manipulation procedure in nest predation studies (e.g. Mougeot and Bretagnolle, 2000; Ibáñez-Álamo et al., 2011; Caetano et al., 2014). Our results suggest that low levels of predation risk are not sufficient to induce a significant immunomodulatory effect, which is evident only when the threat to be preyed upon becomes severe (AC and DC group). Alternatively, conspecific calls (alarm or distress calls) are a better indicator of nest predation risk than predator calls, at least for common blackbird nestlings. Parental alarm calls contain detailed information for nestlings about the nature of predators (Platzen and Magrath, 2005), predator distance or behaviour (Suzuki, 2011), thus parent–offspring communication would represent the main informative process through which nestlings can monitor the current nest predation risk. Overall, our findings indicate that using even simple gradients of intensity of (nest) predation risk can help to better understand predator–prey interactions.

The important result highlighted by the second function of the discriminant analysis concerns the differences in the OVT and IgY concentrations shown by the AC and DC groups (Fig. 4). Increased OVT concentration seems to be efficient in response to an imminent predator attack (DC group), whereas IgY increased when nestlings experienced a level of predation risk just lower than the previous one (AC group). This could be due to the temporal component of a predatory event. In a natural situation, hearing parental alarm calls usually corresponds to an earlier stage in the predatory sequence (i.e. the predator is located in the surrounding of the nest) whereas distress calls start when the predator is already at the nest (Caro, 2005). In this context, distress calls could trigger different (quicker) immune responses. Another non-exclusive explanation might be that IgY levels do not need to be raised in response to distress calls because under natural conditions their values would already be high as a consequence of a previous increment in an earlier stage of a predator attack (i.e. owing to parental alarm calls, when the predator is close to the nest). In the latest stage of the predatory attack, when the predator is already at the nest, IgY levels would still be elevated and the defensive responses might be more oriented towards other types of strategies (e.g. escape from the nest).

Interaction with parasites and environmental variables

According to our third prediction, the parasitism status mediated the ability of nestlings to cope with a predatory situation, at least for some components of the immune system (Table 1). Non-parasitized nestlings showed an elevated number of blood lymphocytes when exposed to high predation risk (AC and DC group), suggesting a mobilization of the acquired immunity in response to the threat to be preyed upon, whereas nestlings parasitized with *Leucocytozoon* were not able to maintain the same levels of lymphocytes when perceiving extreme predation risk (DC group; Fig. 2). Non-parasitized nestlings of the DC group showed a similar lymphocyte level than parasitized ones, which suggests that the number of lymphocytes observed in the blood of parasitized nestlings could still be suitable to cope with future costs associated with potential injuries. However, these lymphocytes may not be enough to fight against the *Leucocytozoon* infection, suggesting a potential cost of nest predation risk on parasitized nestlings. It is possible that the mobilization of lymphocytes could be too costly for the nestlings exposed to the extreme predation risk because they are also currently parasitized: higher levels of lymphocytes could involve energetic costs and be associated with the risk of autoimmune diseases and oxidative stress (Hasselquist and Nilsson, 2012).

Another interesting line of evidence is that the effect of nest predation risk on H/L ratio was influenced by nestling body condition, thus suggesting that variations in the H/L ratio were strongly associated with the health status of nestlings (Masello et al., 2009). Here, nestlings exposed to no predation (CON group) showed a negative correlation between body condition and H/L ratio, meaning that nestlings in better body condition invested in an elevated number of lymphocytes and therefore showed a low H/L ratio, whereas those exposed to a predator threat did not show this relationship (Fig. 3). This seems to suggest that a short-term exposure to nest predation risk may alter the ability of nestlings to maintain an investment in acquired immunity. A mediator role of stress hormones, specifically corticosterone, could have determined the altered leukocyte profile (Davis et al., 2008). Perceived nest predation risk is known to modify the levels of corticosterone in blackbird nestlings (Ibáñez-Álamo et al., 2011), which in turn, affects the number of circulating leukocytes and, therefore, the H/L ratio. Our results might have important implications in those studies measuring H/L ratio if parents perceive researchers as potential predators and thus give alarm calls, making it more difficult to obtain biologically meaningful results.

Finally, we detected a strong effect of breeding season on the immune parameters (Table 1) as the nestlings hatching later in the season showed a larger investment in most of the immune parameters. Higher levels of immunity may indicate two situations: (i) higher quality nestlings (Roulin et al., 2003), or (ii) an activation of the immune system, which usually occurred under poor conditions (Lindström et al., 2004; De Coster et al., 2010). The deterioration of environmental conditions that occurs at the end of the breeding period in the study areas, when temperatures are higher (Spanish Meteorological Agency) and the number of ectoparasites increases (i.e. mites), suggests that nestlings would have to invest more in immune defence, thus supporting the latter explanation. Moreover, poor nestling condition could also be derived from parental quality because the seasonal decline in breeding success could be related to the poorer quality of late breeders (Hipfner, 1997).

Conclusions

In summary, we conclude that a short-term increase in nest predation risk may induce changes in the immune system of nestlings. This effect is complex and multifactorial and depends on the immune variable measured and the type of manipulation of nest predation risk. The different levels of nest predation risk may result in different immunological changes, suggesting that conspecific (alarm or distress) calls are important sources of information about current predation threats and can activate the immune system. Relevant natural factors, such as parasites and seasonal variability (i.e. temperature, food availability), can affect and impede the normal physiological response to nest predation, while at the same time, this selective pressure can imbalance some trade-offs between immune indexes (H/L ratio) and body condition. This study highlights the relevance of studying the physiological consequences of predator–prey interactions, not only to better understand the proximate mechanisms behind them, but also to reveal new trade-offs among several selective pressures.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: G.R., M.S., J.D.I.-A.; Methodology: G.R., E.C., M.A.V., F.R.-R., M.G.S., J.D.I.-A.; Software: G.R., E.C.; Validation: M.S., B.I.T., J.D.I.-A.; Formal analysis: G.R., E.C., M.V., M.G.S., J.D.I.-A.; Investigation: G.R., E.C., F.R.-R.; Resources: M.S.; Data curation: G.R., E.C., M.G.S.; Writing - original draft: G.R., J.D.I.-A.; Writing - review & editing: G.R., E.C., M.S., B.I.T., M.V., F.R.-R., M.G.S., J.D.I.-A.; Supervision: M.S., B.I.T., M.A.V., J.D.I.-A.; Project administration: M.S.; Funding acquisition: M.S., B.I.T.

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Supplementary information

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