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Article

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Predation is one of the most relevant selective forces in nature. However, the physiological mechanisms behind anti-predator strategies have been overlooked, despite their importance to understand predator–prey interactions. In this context, the immune system could be especially revealing due to its relationship with other critical functions and its ability to enhance prey's probabilities of survival to a predator's attack. Developing organisms (e.g. nestlings) are excellent models to study this topic because they suffer a high predation pressure while undergoing the majority of their development, which maximizes potential trade-offs between immunity and other biological functions. Using common blackbirds *Turdus merula* as model species, we experimentally investigated whether an elevated nest predation risk during the nestling period affects nestlings' immunity and its possible interactions with developmental conditions (i.e. body condition and growth). Experimental nestlings modified some components of their immunity, but only when considering body condition and growth rate, indicating a multifaceted immunological response to predation risk and an important mediator role of nestlings' developmental conditions. Predation risk induced a suppression of IgY but an increase in lymphocytes in nestlings with poor body condition. In addition, experimental but not control nestlings showed a negative correlation between growth and heterophils, demonstrating that nest predation risk can affect the interaction between growth and immunity. This study highlights the importance of immunity in anti-predator response in nestlings and shows the relevance of including physiological components to the study of predation risk.

Keywords: developing organisms, heterophils, immunoglobulins, long-term predation risk, nestling period, trade-off

Introduction

Predation is one of the most important selective pressures in nature (Caro 2005). Beyond the direct impact on fitness caused by the killing of prey (Preisser et al. 2005), the consequences related to the prey's perception of predation risk (i.e. visual, auditory

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and olfactory) can be decisive (Lima 1998, Cresswell 2008, Zanette et al. 2011). Predation risk induces prey to respond with a wide variety of anti-predator defenses (Lima and Dill 1990, Caro 2005, Creel et al. 2005, Hawlena and Schmitz 2010, Díaz et al. 2013, Ibáñez-Álamo et al. 2015). The scientific community has been traditionally focused on behavioral responses (Sheriff and Thaler 2014, Zanette et al. 2014, Ibáñez-Álamo et al. 2015) while little attention has been paid to the physiological anti-predator responses, despite their benefits to fully comprehend the costs associated with predation risk (Clinchy et al. 2004, 2013, Zanette et al. 2011, 2014). The single exception is the hormonal response to predation, which is studied within the more general framework of stress-induced factors (reviewed by Sapolsky et al. 2000, Hawlena and Schmitz 2010). Other physiological components, like the immune system, have been less explored (Hawlena and Schmitz 2010) and mainly focused on invertebrates (Rigby and Jokela 2000, Stoks et al. 2006, Duong and McCauley 2016). These studies found contrasting results, some of them showing a down-regulation of immune values like phenoloxidase or the number of immune cells while others found the opposite pattern.

In vertebrates, the immune system protects the organism from pathogens, diseases and infections (Roitt et al. 2001). Therefore, it is expected to play an important and direct role in prey survival (Dhabhar and McEwen 1997, Dhabhar 2002). Thus, immunological changes in response to predation risk variations might also be relevant among vertebrates. Nevertheless, only few studies have been conducted on these taxa, particularly on amphibians. An immunosuppressive effect in response to an increased predation risk was found in tadpoles (Seiter 2011, Groner et al. 2013). Authors suggested that this change could be a direct response for allocating resources to behavioural or morphological anti-predator strategies (Seiter 2011). Birds, and particularly young individuals (i.e. nestlings), are an ideal model to study effect of predation risk on the immune system. Predation is the first cause of mortality for nestlings and is able to exert an important selective pressure in several avian life-history traits (Martin and Briskie 2009), including their physiology (reviewed by Ibáñez-Álamo et al. 2015). Therefore, an involvement of the immune system in response to predation risk may exist by its participation in limiting the negative consequences associated with the possible injuries imposed by predators (Viswanathan and Dhabhar 2005, Martin et al. 2006). Despite this possibility, very little is known about the mechanisms that regulate the relationship between immunity and predation risk in birds. Two studies conducted with adult birds linked a significant elevation in some immunological components (i.e. white blood cells and immunoglobulins) after an increase in perceived predation risk (Clinchy et al. 2004, Thomson et al. 2010). On the other hand, studies on developing organisms (e.g. nestlings) showed responses that are more complex. There is evidence indicating that a high cell-mediated immune response is associated with higher probabilities of escaping a predator in campo flicker *Colaptes campestris* nestlings, as their better overall condition favors the emission of more efficient distress calls that attract more adults to mob the predator (Goedert et al. 2014). Tilgar et al. (2010) found variations in the leucocyte profile (i.e. H/L ratio) in nestlings that were exposed to higher nest predation risk during one week. These immunological changes were associated with an increase of stress-hormone levels (Tilgar et al. 2010), confirming the mediator role of the endocrine system. A recent study in common blackbird *Turdus merula* nestlings has shown a more complex immune response to predators as only some of the parameters analyzed (e.g. ovotransferrin, immunoglobulins and part of their white blood cells) changed due to a short-term increased predation risk (Roncalli et al. 2018). These alterations are suggested to be used for preparing the organism to cope with the potential deleterious consequences imposed by the imminent predator attack (Roncalli et al. 2018). These changes may therefore be adaptive and not a by-product of corticosterone variations, enabling nestlings to improve their probability of surviving a predator's attack. Partial nest predation is in fact relative common in birds (Lyons et al. 2015, Ellis et al. 2018) and it is particularly relevant in older nestlings, for which the probability of survival after a predator attack can be up to 60% (Halupka 1998).

Consequences due to a long-term nest predation exposure could be even more complex. In fact, short- and long-term risks can produce different anti-predator responses (Lima 2009), which can be modulated by prey to avoid physiological-induced costs. For example, the ratio of heterophils to lymphocytes in blood (H/L ratio), a typical measure of stress in birds, usually increases in response to a short-term stress exposure (Maxwell 1993), whereas it declines in prolonged conditions (Maxwell 1993, Caetano et al. 2014). Indeed, prey are known to experience chronic stress under long-term exposure to predators (Hik et al. 2001, Sheriff et al. 2011, but see Creel et al. 2009), which generally provoke a general drop of the immune defenses (Sapolsky et al. 2000). The consequences related to the immunosuppression induced by predation risk may be severe also in term of fitness costs, for example by rendering an organism more vulnerable to infections or inflammation processes (Dhabhar 2002). The immune effects of long-term manipulations in nest predation (i.e. whole nestling period) typically refer to the non-lethal impacts of predation (Lima 1998, Zanette et al. 2014), whose effects can have crucial consequences for developing organisms given the fact that the immune system is involved in critical trade-offs with other fitness-related functions. Energetic or nutrient elements needed for a good immunological maintenance (Klasing 2004) typically generate trade-offs with other biological components in an organism (Hasselquist and Nilsson 2012) because of the competition in employing limited resources (Zera and Harshman 2001). Therefore, the resources available for nestlings during their developmental stages and, consequently, their body condition, might interact with the strength of the immune response. Developing individuals with a good

body condition could have more opportunities for the maintenance of a strong immune system (Christe et al. 1998, Martin-Vivaldi et al. 2006) and, thus, conditioning their possibilities to survive a predator attack. Moreover, several studies have shown a negative relationship between immunity and growth in nestlings (Saino et al. 1998, Soler et al. 2003, Brommer 2004). Growth is probably the most important vital process for developing birds, especially for altricial species (Lack 1968, Ricklefs 1983), since the high energetic and nutritional investment required by nestlings occurs in short time (Starck and Ricklefs 1998). Post-natal development of altricial nestlings is considered the phase of a bird's life cycle where they suffer the highest predation pressure (Martin 1995); so, it could be predicted that long-term predation risk can induce changes on the trade-offs between immunity and growth during this early stage of their life.

In this study, we have two objectives. First, we investigate whether a long-term (i.e. whole nestling period) increase in nest predation risk induces an immune response in nestlings and, second, whether nest predation can unbalance trade-offs between immunity and important biological functions in developing organisms. We experimentally increased the risk of perceived nest predation during the whole nestling period in common blackbird nestlings and analyzed 12 immunological variables to obtain a complete overview of the potential changes associated with our long-term experiment. Regarding the first objective and based on the results of a short-term manipulation of nest predation risk in the same blackbird population (Roncalli et al. 2018), we predict that chicks exposed to our treatment will activate (at least) part of their immune system to face a potential attack (prediction 1a). However, given that an activation of the immune system seems to be initially induced by a threat, whereas an immunosuppression is a more common response to long-term stressors, when stress hormonal levels are high (Dhabhar 2002, Martin 2009), it could be also predicted a down-regulation of the immune system when facing a long-term increase in predation risk (prediction 1b). With respect to the second objective and based on the information mentioned above, we predict that nest predation risk will unbalance the trade-offs between immunity and body condition (prediction 2), and between immunity and growth (prediction 3).

Material and methods

Study model

We conducted our study during the spring of 2014 on a common blackbird population located in the Valley of Lecrín (36°56′N, 3°33′W; 580 m a.s.l.), an agricultural area in southeast Spain. In this population blackbird nestlings stay in the nest for 11–13 days (Ibáñez-Álamo and Soler 2010). Predators occurring in the study area consist of avian species (e.g. Eurasian sparrowhawks *Accipiter nisus*), mammals (e.g. genets *Viverra genetta*) and snakes, with an overall nest predation rate of 48.9% (Ibáñez-Álamo and Soler 2010).

We actively searched the nests from the start of the breeding period (early March). Once a nest was located, we checked the content every two days to know the exact hatching date. We used a mirror attached to a pole to reduce our disturbance when approaching to the nests.

Experimental design

In order to manipulate nest predation risk at a long-term level, we followed a similar procedure of that previously used in other studies (Zanette et al. 2011, Coslovsky and Richner 2012, Hua et al. 2014). Basically, we created two experimental groups in which we played predator acoustic cues (experimental group) or non-predator sounds (control group) during the entire duration of the nestling period (Fig. 1).

We selected calls of nest predators, such as Eurasian sparrowhawks *Accipiter nisus* (Newton 1986, Ibáñez-Álamo and Soler 2012) or black-billed magpie *Pica pica* (Collar 2005) for the experimental group, whereas we used non-predator species, like European serin *Serinus serinus*, European goldfinch *Carduelis carduelis* or Sardinian warbler *Sylvia melanocephala*, for the playbacks of the control group. We avoided using alarm call vocalizations for non-predator species as they may indirectly indicate the presence of a predator (Haff and Magrath 2012, 2013). All the species are known to be present in the study area (unpubl.). We chose avian calls from a virtual platform on the web (<www.xeno-canto.org>) carefully selecting only high-quality records. We created specific playbacks for our experiments with the Audacity software. Each playback consisted of 6 min and 40 s of call activity (40 s of calls interspersed with 1 min of silence for four times) followed by 7 min of silence. We joined nine different playbacks to compose a single 1 h and 45 min long audio file, which was then broadcasted continuously. Eight different unique audio files were created for each group.

We broadcasted sounds in plots, which consisted in circles of 100 m of radius including 1–3 synchronous blackbird nests. Blackbirds' territories have approximately 30–35 m of radius from the nest (Ibáñez-Álamo and Soler 2010). We placed each speakers at 7–10 m from the focal nest and we left a buffer zone (150 m) between plots, calculated from the point where the speakers were located (center to center), to grant acoustic isolation regarding our manipulation (Krams et al. 2009). In addition, we avoided to perform two experimental manipulations close to each other (< 300 m) simultaneously. So, we are confident that the effect of our manipulation is due to the broadcasting of playbacks in each plot and not from neighboring plots. The speakers were hidden under a camouflage cloth and placed in the center of each plot, pointing to a different (randomly assigned) direction every other day to ensure a similar influence of our acoustic manipulation on the plot. Speakers operated during the day (from 8:00 am to 8:00 pm) at 70 dB. We tried to avoid the habituation of blackbirds to the speakers by: 1) carefully selecting calls of different bird species and from different individuals for each species (i.e. approximately 15 distinct calls were reproduced in each audio file); 2) randomising the order of playbacks

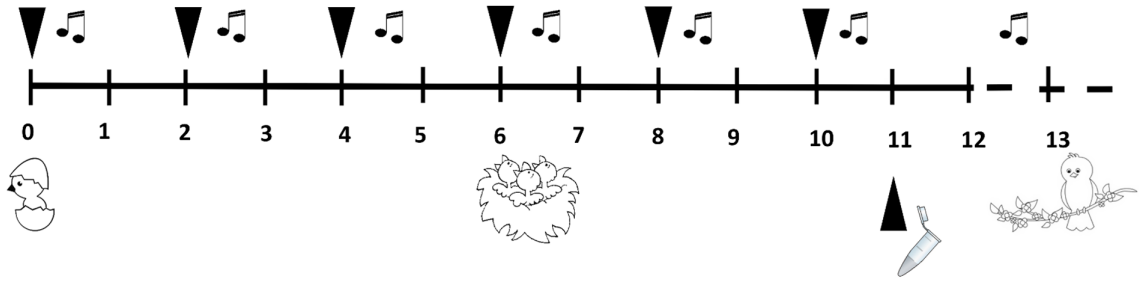

Figure 1. Experimental design. Speaker played every other day during nestling period (from day 0 ± 1 to day 11 ± 1; triangles above the line). Tarsus length were measured every other day just before playing the speakers. At day 11 ± 1, we collected blood sample (triangle below the line), measure body mass and tarsus length for calculating body condition. The speakers continued playing according the two-days procedure until the chicks fledged naturally.

within the audio file; 3) changing the direction of speakers within the plot; and 4) operating the speakers every other day (Fig. 1). We visited nests only when speakers were playing. In order to avoid potential confounding effects due to maternal effects (Morosinotto et al. 2013), we started our manipulation the day of hatching and randomly assigned each plot to the experimental or control group.

We completed the experimental manipulation for 20 nests (43 nestlings) and 17 nests (39 nestlings) for the control and the experimental group respectively. The final sample sizes for some analyses was slightly smaller than those previously described owing to limited plasma availability for some nestlings.

Immunological assays

When the second hatched nestling was 11 days old (Fig. 1), we collected a blood sample (250–300 µl) from the brachial vein of all chicks of that nest. We always collected the blood sample in a day in which the speakers did not play to ensure that we quantify the long-term effect of increased predation rather than the short-term increase in nest predation risk (Roncalli et al. 2018). All blood samples were collected between 10 am and 2 pm and kept refrigerated (4°C, maximum 5 h) until centrifugation (13 000 rpm for 10 min). Plasma was separated and stored at −25°C until their analyses. We also collected a drop of blood that was smeared on a marked glass slide and dried in open air.

We performed several immunological assays to quantify 12 parameters belonging to both the humoral and cellular component of the immune system as well as to innate and acquired immunity (Janeway et al. 1997). The objective of this multiple measurements was to capture the complexity of the immune system (Matson et al. 2006), since we previously found that not all components of blackbirds' immune system respond similarly to a (punctual) increase of nest predation risk (Roncalli et al. 2018).

Innate humoral immunity

1) *Haemolysis/Haemagglutinationtitres (HL-HA)*

Haemagglutination (HA) and haemolysis (HL) assays were used to quantify the levels of non-specific natural antibodies and titers of complement-like lytic enzymes respectively. These molecules are part of the innate immunity facilitating the initial recognition of pathogens and promoting the activation of adaptive immunity (Carroll and Prodeus 1998). We followed the procedure described by Matson et al. (2005); scan of individual samples was randomized among all plates and scored by a single person (GR; see Matson et al. 2005 for more details).

2) *Haptoglobin (HP)*

Haptoglobin (HP) is an acute phase protein found in several species, including birds, which is able to inhibit the oxidative activity of erythrocytes, by binding the free hemoglobin released (Galicia and Ceuppens 2011). In response to acute infection or inflammation, HP results in a high blood concentration (Matson et al. 2012). A commercial colorimetric assay kit (TP801; Tridelta Development Ltd., Maynooth, Ireland) was used to quantify its concentration following Matson et al. (2006).

3) *Ovotransferrin (OVT)*

OVT is another acute phase protein, which can bind free iron, an essential nutrient for bacterial growth. Ovotransferrin is a protein with antibacterial, antiviral and antifungal activities (Giansanti et al. 2012) and therefore, it is usually considered as an indicator of inflammation and infection, poor nutritional state or diseases (Horrocks et al. 2011). OVT concentration was quantified following Horrocks et al. (2011).

Acquired humoral immunity

4) *Immunoglobulins (IgY)*

Immunoglobulins are important serum proteins produced by B lymphocytes that identify and counteract pathogens and promote leukocytes migration to the sites of infection (Härtle et al. 2014). Total immunoglobulin concentrations (IgY) were assessed using a sensitive enzyme-linked immune absorbent assay (ELISA) method. Antichicken antibodies were used following the procedure developed by Martinez et al. (2003). We adapted this method for common blackbird nestlings following Roncalli et al. (2018).

Cellular immunity

5) *Leukocyte profile*
In order to quantify white blood cells, blood smears were fixed in absolute methanol the day of collection and stained for 45 min with Giemsa (GS500-500 ml SIGMA-ALDRICH Giemsa stain). The smears were scanned with an optical microscope (1000× magnification with oil immersion) in order to count a minimum of 100 leukocytes in each slide. The number of leukocytes per 10 000 erythrocytes was calculated by counting the number of erythrocytes per field and multiplying by the number of field scanned to count 100 leukocytes. Each cell was classified as heterophils, lymphocytes, eosinophils, basophils or monocytes following the description of (Campbell and Ellis 2007) in order to estimate the relative proportion of each cell type and to calculate H/L ratio.

Body condition and growth rate

All chicks of the brood were individually marked with non-toxic markers (FaberCastel Multimark) to allow individual recognition. Body condition were calculated as the residuals of the regression between body mass (0.01 g) and tarsus length (0.01 mm), both measured at the 11 ± 1 days (Jakob et al. 1996). Growth rates were estimated by using the logistic function of growth described by Starck and Ricklefs (1998). We obtained the growth rate constant (k) of each nestling based on tarsus length, which were measured every other day until the day of blood sampling. We decided to use tarsus length as predictor of structural growth because body mass is more susceptible to variation due to other causes rather than growth such as parental food provisioning or fat accumulation (Rising and Somers 1989).

Statistical analyses

To examine the effect of the perceived predation risk on the immune system we tested each immunological parameter separately. This is a common procedure, which allowed us to consider the possibly opposing responses of the individual immunological components (Norris and Evans 2000, Pap et al. 2010, Hegemann et al. 2012, Roncalli et al. 2018). We fitted a linear mixed model (LMM; lme function in the 'nlme' package; Pinheiro et al. 2016) for each immunological parameter measured (HA, HP, OVT, IgY levels, number of leukocytes, heterophils, lymphocytes, eosinophils, basophils and H/L ratio). It was not possible to fit any model for the lysis activity (HL) or monocytes because the low number of individuals showing values above zero. We included treatment (high versus low predation risk), body condition, growth rate (k) and hatching date as predictors; moreover, we consider the interaction between treatment and each of the other predictors. The inclusion of the interaction between treatment and body condition/growth rate allowed us to test the second and third predictions respectively. The hatching date (the day on which the first nestling of each brood hatched; day 1 = 1 March) were added to control for the temporal variation in environmental factors that can affect the immune system (Dubiec and Cichon 2005, Hegemann et al. 2012, Roncalli et al. 2018). We fitted a nested random structure in which nest identity was nested within plot identity in order to control for the non-independence of nestlings from the same nest, and nests from the same plot. Following a backward selection procedure, we successively excluded the factors that showed the highest (non-significant) p-values to obtain simpler alternative models, dropping firstly the non-significant interactions (Engqvist 2005). We did not remove treatment as it reflects the hypotheses to be tested. To control whether body condition differed between nestlings of control and treatment groups we run a LMM including the treatment as predictor and fitting the same nested random structure used in the other models. Models were validated by the visual inspection of the residuals to verify the homogeneity of the variances and the normality (Zuur et al. 2010) and we used the logarithmic transformation for those models that violated the assumptions of linearity (i.e. IgY, the number of eosinophils and H/L ratio). All data were analyzed using R ver. 3.3.2 (<www.r-project.org>).

Results

Our nest predation risk manipulation did not affect blackbird immune parameters directly but indirectly. Only when considering the interaction with nestlings' body condition and growth rate we found an effect on the acquired immunity (IgY and lymphocytes) of blackbird nestlings and some aspects of their innate component (i.e. heterophils) (Table 1).

We found a positive association between body condition and the number of lymphocytes in control nestlings, but this relationship disappeared in nestlings exposed to nest predation risk since the experimental nestlings in poor body condition had a higher number of lymphocytic cells compare with control ones (Fig. 2A). Additionally, we found a positive relationship between the number of eosinophils and body condition ($p = 0.02$; Table 1). Nestlings exposed to higher nest predation risk showed lower IgY levels compared with those of the control group, but again, this relationship occurred only for the nestlings in poor body condition (Fig. 2B). However, body condition between control and experimental nestlings did not differ ($F_{1,35} = 0.37$, $p = 0.54$).

Our results showed a significant effect on the number of heterophils of the interaction between the experimental increase in predation risk and growth ($p = 0.03$; Table 1). The slopes of experimental and control nestlings are different; specifically, experimental nestling with lower values of growth rate had a higher number of heterophils compared with control nestlings (Fig. 2C). In addition, we found a negative significant effect of growth on acute phase proteins, showing that nestlings investing more in growth had lower levels of HP and OVT ($p = 0.04$; Table 1).

Finally, it is worth mentioning that we found a strong significant positive effect of hatching date for most white blood cells, as well as for IgY, regardless of our predation risk treatment (Table 1).

Table 1. Statistics of the linear mixed model LMM for each of the immunological parameters. Predictors included in the final model are underlined and significant predictors are marked with an asterisk. Numbers in parenthesis correspond to the sample size.

	β ± SE	df	F	p
Humoral innate immunity				
HA (69)				
Treatment		1,34	0.069	0.79
Body condition		1,32	1.245	0.27
Growth		1,25	0.029	0.86
Hatching date		1,33	0.576	0.45
Treatment × body condition		1,24	1.077	0.31
Treatment × growth		1,23	0.091	0.76
Treatment × hatching date		1,24	0.467	0.50
HP (84)				
Treatment		1,26	0.016	0.90
Body condition		1,35	0.249	0.62
Growth	−0.254 ± 0.125	1,33	4.311	0.04*
Hatching date		1,26	1.805	0.19
Treatment × body condition		1,33	0.772	0.38
Treatment × growth		1,34	1.079	0.30
Treatment × hatching date		1,25	0.027	0.87
OVT (80)				
Treatment		1,21	0.355	0.55
Body condition		1,18	0.232	0.63
Growth	−15.111 ± 6.961	1,19	4.712	0.04*
Hatching date		1,20	0.035	0.85
Treatment × body condition		1,16	0.004	0.94
Treatment × growth		1,17	1.864	0.19
Treatment × hatching date		1,19	0.309	0.58
Humoral acquired immunity				
IgY (84)				
Treatment		1,34	0.271	0.60
Body condition		1,43	0.016	0.90
Growth		1,33	0.005	0.94
Hatching date	+0.012 ± 0.004	1,34	9.004	0.01*
Treatment × body condition		1,43	5.884	0.02*
Treatment × growth		1,32	0.341	0.56
Treatment × hatching date		1,25	0.001	0.98
Cellular component immunity				
Leukocytes (74)				
Treatment		1,33	1.782	0.19
Body condition		1,36	3.366	0.07
Growth		1,26	0.915	0.34
Hatching date	+0.689 ± 0.261	1,33	6.341	0.01*
Treatment × body condition		1,36	2.923	0.09
Treatment × growth		1,25	0.477	0.49
Treatment × hatching date		1,24	0.096	0.76
Heterophils (74)				
Treatment		1,25	0.703	0.41
Body condition		1,25	1.224	0.28
Growth		1,25	0.301	0.59
Hatching date	+0.473 ± 0.159	1,25	8.363	0.01*
Treatment × body condition		1,25	3.334	0.08
Treatment × growth		1,25	5.340	0.03*
Treatment × hatching date		1,24	0.006	0.94
Lymphocytes (74)				
Treatment		1,26	2.412	0.13
Body condition		1,26	0.935	0.34
Growth		1,26	0.413	0.52
Hatching date		1,25	0.069	0.79
Treatment × body condition		1,26	5.061	0.03*
Treatment × growth		1,25	0.006	0.94
Treatment × hatching date		1,24	0.165	0.68

(*Continued*)

Table 1. (Continued)

	β ± SE	df	F	p
Eosinophils (73)				
Treatment		1,26	2.793	0.10
Body condition	+0.064+0.027	1,27	5.720	0.02*
Growth		1,27	0.83	0.37
Hatching date		1,25	0.07	0.79
Treatment × body condition		1,26	1.233	0.27
Treatment × growth		1,25	0.028	0.86
Treatment × hatching date		1,24	2.855	0.10
Basophils (72)				
Treatment		1,33	1.058	0.31
Body condition		1,27	0.437	0.51
Growth		1,28	2.861	0.10
Hatching date	+0.082 + 0.034	1,33	6.718	0.01*
Treatment × body condition		1,26	0.638	0.43
Treatment × growth		1,25	0.001	0.98
Treatment × hatching date		1,24	0.166	0.68
H/L ratio (74)				
Treatment		1,25	0.577	0.45
Body condition		1,27	0.185	0.67
(Growth)		1,28	0.473	0.49
Hatching date		1,25	3.682	0.07
Treatment × body condition		1,26	0.331	0.57
Treatment × growth		1,25	3.090	0.09
Treatment × hatching date		1,24	0.005	0.94

Discussion

Our study shows that the changes in the immune system of developing nestlings caused by perceived predation risk were significant only when mediated by nestling condition (i.e. body condition and growth rate), supporting partially prediction 1. Our experimental manipulation entailed both an immunoenhancement (lymphocytes and heterophils) and immunosupression (IgY levels) effect, fitting with prediction 1a and 1b, respectively. The mediator role of body condition and growth supports prediction 2 and 3 and suggests that anti-predator strategies in developing organisms are condition-dependent during this critical stage (i.e. nestling period).

Immunoenhancement effect of predation risk

According to Caro (2005), anti-predator responses can be divided into three groups: 1) to avoid the encounter with the predator, 2) to escape the potential predator if the encounter happens and 3) to overcome the negative consequences of that encounter (i.e. injuries). The activation of the nestlings' cellular immunity due to our experimental manipulation seems to suggest that the immune system is involved in the third type of anti-predator responses. Generally, chronic stress conditions tend to reduce cellular immunity (Dhabhar 2002) because of the immunosuppressive effect of glucocorticoids during the stress response (Sapolsky et al. 2000). However, previous studies show conflicting results at this respect (Boonstra et al. 1998, Clinchy et al. 2004, Navarro et al. 2004). Our results also challenge this assumption and match with the alternative possibility (prediction 1a): a stimulation of the immune system in response to the increased predation risk that could be helpful for prey as it favors an efficient healing of wounds, monitors potential infections and promotes tissue repairing (Dhabhar and McEwen 1997, Dhabhar 2009). As a matter of fact, heterophils and lymphocytes represent the predominant white blood cells in birds (Janeway et al. 1997). Heterophils mainly contribute to inflammatory responses and to the control of antimicrobial activity (Harmon 1998), whereas lymphocytes are crucial to fight infections (Janeway et al. 1997). However, the immunoenhancement effect in lymphocytic and heterophilic cells was significant only when considering the interaction with developmental conditions, in particular for those nestlings showing poor body conditions and reduced growth rate, respectively. In the first situation, the positive association between the number of lymphocytes and the body condition found in control nestlings confirms previous findings for both adults (Navarro et al. 2003) and nestling passerines (Christe et al. 1998). This association is lost under a situation of high predation risk (experimental treatment; Fig. 2A) creating thus, a trade-off between immunity and body condition, probably due to the competition for resources and a new cost-benefit balance (Brommer 2004). On the other hand, we found that predation risk modified the association between the number of heterophils and growth rate (Table 1, Fig. 2C), providing another piece of evidence that predation risk can alter established trade-offs in developing organisms. The trade-off between immunity and growth in young organisms has been described for several species (De Neve et al. 2007, Romano et al. 2011, Van der Most et al. 2011), and it is also supported by our results on the negative effects of growth on acute phase proteins, but to

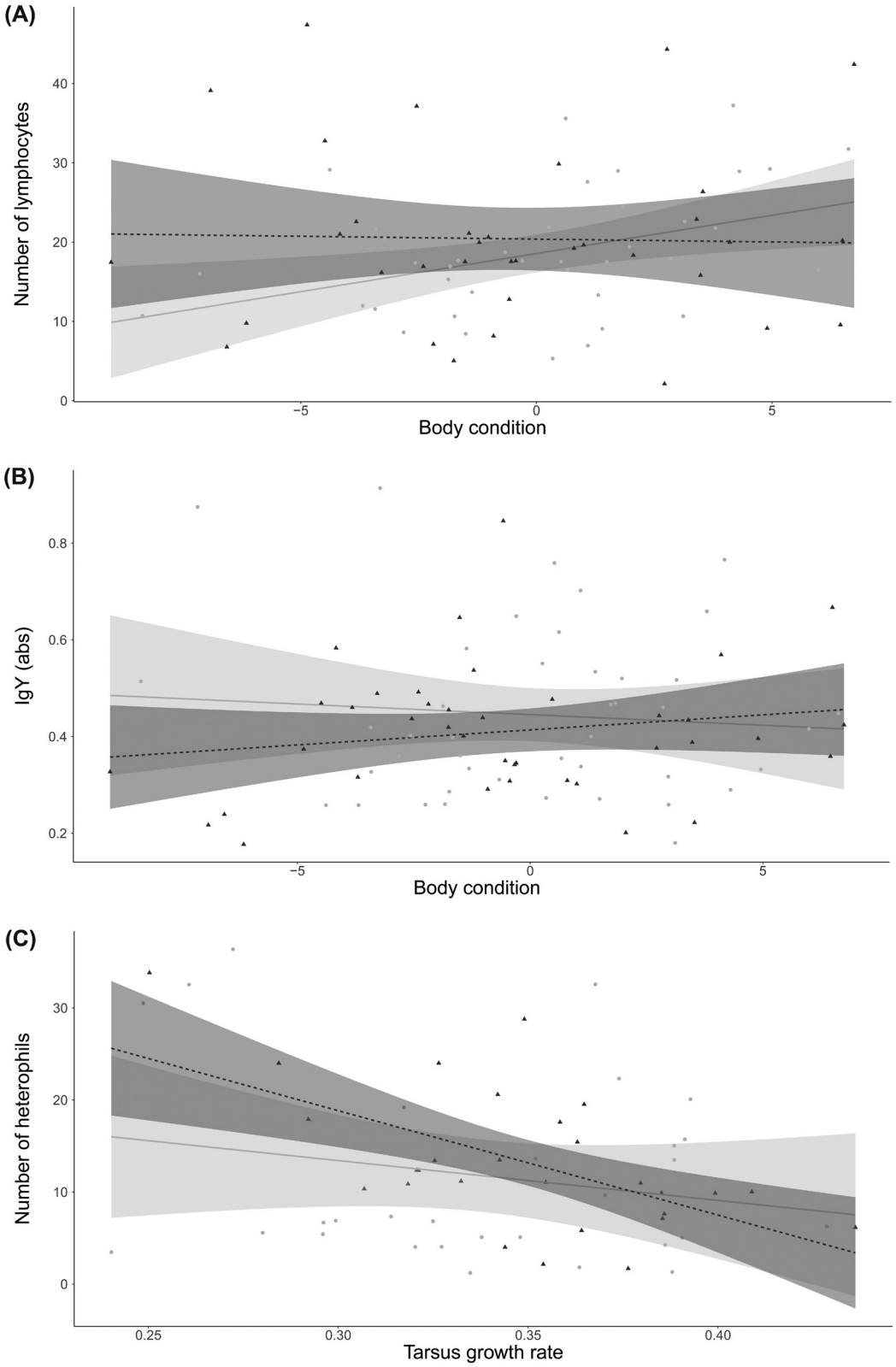

Figure 2. Effects of the experimental predation risk on the immunological parameters. Dark grey triangles and dotted lines indicate experimental (high risk) nestlings, whereas light grey circles and solid lines indicate control (low risk) nestlings. Effects on the trade-off between (A) body condition and the number of lymphocytes; (B) body condition and IgY levels; (C) growth (kt) on the number of heterophils.

our knowledge, this is the first demonstration that predation risk can impact its balance. Our results indicate differences in the number of heterophils between control and treatment nestlings when they experienced lower growth rates (i.e. difference in the slopes; Fig. 2C). This suggest that experimental nestlings might employ their resources to increase their innate immune response under an elevated nest predation risk at the cost of their growth. This mechanism is lost when nestlings grow faster (Fig. 2C). This effect could be at least part of the mechanistic response behind the common trade-off between predation and growth in birds ('Lack's hypothesis'; Lack 1948, Bosque and Bosque 1995, Martin 1995, Remeŝ and Martin 2002).

Immunosuppressive effect of predation risk

In contrast with the immunoenhancement effect of long-term nest predation risk on the cellular component, we found a down-regulation of IgY levels (Fig. 2B), which is a crucial component of acquired immunity. This finding fits with the immunosuppressive effect of chronic stress (Martin 2009). Nevertheless, also in this case, the predator-induced changes were significant only considering the effect of nestlings' body condition, specifically in nestlings with the lowest levels of body condition. Our results suggest therefore, that these nestlings had a limited availability of resources to respond immunologically to the perceived predation risk, whereas nestlings in better condition were able to keep their normal functions, thus maintaining their general wellness, and modify their immunoglobulin levels simultaneously (prediction 2). An alternative mechanism may be that the immunosuppressive effect reflect a protective role of the immune system in preserving developing organisms from potential autoimmune diseases, which are known to be related to stress-induced immunoenhancement under certain conditions (Dhabhar 2009). Accordingly, nestlings in poorly body condition, which showed the lower levels of IgY, could be those more sensitive to autoimmune disorders.

The immunosuppressive effect found for the IgY levels contrasts with the immunoenhancement found in blackbirds of the same populations a consequence of an acute increase in predation risk (Roncalli et al. 2018). This confirm that the duration of the exposure to predator acoustic calls would be determinant in the immune adjustment of prey (Martin 2009), at least regarding the humoral acquired immunity (i.e. immunoglobulins). Supporting previous studies, it seems that growing under a constant nest predation risk would provoke more subtle but continuous changes in young individuals (Zanette et al. 2011, Clinchy et al. 2013).

The possible role of hormones and parental behavior

The activation of the HPA axis in response to a source of stress entails the release of corticosteroid hormones (Sapolsky et al. 2000), which usually cause a down-regulation of different immunological components (Nazar and Marin 2011, Gao et al. 2017). However, the interplay between the immune system and hormones is intricate and can vary according to the immunological component involved (Stier et al. 2009). For example, Chin et al. (2013) found that ring-billed gull *Larus delawarensis* nestlings exposed to handling stress increased their corticosterone levels and down-regulated some components of their immune system (natural antibodies and complement-mediated lysis) but not others (IgY levels). In addition, the link between corticosteroids and nest predation is not totally clear yet and seems to depend on different factors such as age (Tilgar et al. 2010) or the type of cue perceived by nestlings (Ibáñez-Álamo et al. 2015). For instance, although there is no information on endocrine effects of long-term predation risk in blackbird nestlings, a short-term elevation decreases corticosterone levels instead of increasing them (Ibáñez-Álamo et al. 2011). In our study, we did not measure hormonal levels and therefore, we cannot discard a mediator effect of this endocrine regulator in the observed predator-induced immune modifications. Nevertheless, we have demonstrated an effect of predation risk on the immunity of developing organism that interplayed with other important physiological conditions occurring during this delicate period. Further studies on predation risk, analysing both hormonal and immunological components simultaneously, would help to understand the connection between the two physiological parts and the potential adaptive role of the immune system in prey protection.

Finally, it is worth to consider that the immunological variations found in our manipulation could be the consequences of changes in parental care behaviour, which can be altered (i.e. reduced) by predation risk (Ghalambor et al. 2013, Hua et al. 2014). Actually, body condition of altricial nestlings depends mainly on the amount of food supplied by their parents and it was found that the immune system of nestlings can be related to food availability and parental effort (Saino et al. 1997, Hoi-Leitner et al. 2001). Nevertheless, our control and experimental nestlings did not vary in their body conditions. Further, it is known that parents are able to compensate for the reduced provisional rates under predation risk by deliver a larger load of food in each visit to the nest (Martin et al. 2000, Eggers et al. 2008). Given all these reasons, it seems unlikely that changes in parental feeding behavior could be indirectly the responsible for the nestlings' immune variations observed in this study.

Nest predation risk and breeding season

According to the results shown in a recent study on the short-term predator-induced immune changes (Roncalli et al. 2018), we found that the effect of nest predation on the nestling immunological parameters was constant throughout the whole breeding season (i.e. no significant interaction between our experiment and hatching date; Table 1), suggesting that the immunological alterations due to predation pressure are not affected by time (at least within the breeding period). However, and in agreement with intra- (Roncalli et al. 2018)

and interspecific studies (Sorci et al. 1997, Dubiec and Cichon 2005, Hegemann et al. 2012), we found a temporal increase in some leukocytes and IgY levels (Table 1), which would be associated with a deterioration of the environment at the end of the breeding season (Roncalli et al. 2018 for details).

Conclusions

Our experimental study provides novel findings that advance our understanding on the physiological mechanisms behind anti-predator responses, particularly of developing organisms. According to these outcomes, the immune system could play a crucial role as part of the anti-predator responses. Whether these changes are adaptive (i.e. helping organisms to overcome the negative consequences following the encounter with a predator) or a consequences of other physiological changes (e.g. hormonal modifications) requires additional studies, but overall supports the idea that growing under a long-term nest predation risk can cause relevant physiological changes (Dhabhar 2009, Zanette et al. 2011). Furthermore, our results combined with those testing the immune effect of short-term nest predation risk (Chin et al. 2013, Goedert et al. 2014, Roncalli et al. 2018) strongly suggest that developing organisms modulate their anti-predator immune responses depending on the duration of the risk. Importantly, we found that the developmental conditions of nestlings are crucial in modulating the effect of nest predation risk on the immunity, which at the same time suggest the capacity of predation risk to alter several trade-offs in developing organisms.

Data availability statement

Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.8kprr4xjs> (Roncalli et al. 2020).

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Author contributions – Conceptualization: GR, MS, JDI-A; Methodology: GR, EC, MAV, FR-R, MGS; Software: GR; Immunological assays: GR, EC, MAV, MGS; Resources: MS; Writing – original draft: GR, JDI-A; Writing – review and editing: GR, MS, JDI-A, EC, MAV, FR-R, MGS, BIY; Funding acquisition: MS, BIT.

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