RESEARCH ARTICLE

REVISED Ineffective humoral anti-tick IgY-response in birds: reaction against pathogen constituents? [version 2; peer review: 3 approved, 1 approved with reservations]

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Abstract

Background: Variation in parasite burdens among hosts is typically related to differences in adaptive immunity. Comprehension of underlying mechanisms is hence necessary to gain better insights into endemic transmission cycles. Here we investigate whether wild songbirds that have never been exposed to ticks develop adaptive humoral immunity against endemic *Ixodes ricinus* ticks.
Methods: Blue tits were exposed three times in succession to wild *Ixodes ricinus* ticks. For each infestation, serum samples were obtained. An enzyme-linked immunosorbent assay was developed, using tick salivary antigens, in order to quantify the bird's IgY response against ticks. In addition, at every sampling occasion the birds' body weight (corrected for body size) and haematocrit level was determined.
Results: Individual IgY levels against the ticks' salivary proteins increased over three consecutive tick infestations, and large among-individual variation was observed. The responses were specifically directed against *I. ricinus*; cross-reactivity against the congeneric tree-hole tick *Ixodes arboricola* was negligibly low. IgY responses did not impinge on tick feeding success (engorgement weight and attachment success). Yet, those birds with the highest immune responses were more capable to reduce the acute harm (blood depletions) by compensating erythrocyte loss. Furthermore, at the end of the experiment, these birds had gained more body weight than birds with lower IgY levels.
Conclusions: Latter observations can be considered as an effect of host quality and/or tolerance mechanisms. Birds anticipate the (future) costs of the activation of the immune system by ticks and/or ongoing tick-borne pathogen infections. Furthermore, although

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unsuccessful against tick feeding, the IgY responses may indirectly protect birds against tick-borne disease by acting against salivary protein secretions on which pathogens rely for transmission.

Keywords
Tick, Ixodes ricinus, bird, Borrelia burgdorferi s.l., IgY, antibody, constituent, immunity

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REVISED **Amendments from Version 1**

The study design has been explained in a better way. Figures have been updated, and in the discussion, we elaborated on 'maternal antibodies' and 'resistance in natural vs. unnatural tick hosts'. In addition, we put forward several unanswered questions with regard to the ecology of host-tick interactions.

Any further responses from the reviewers can be found at the end of the article

Plain language summary

Songbirds are central elements in the ecological networks of ticks, but are heavily overlooked when it comes to elementary biological mechanisms like immune responses against ticks and tick-borne diseases. No studies so far have related individual variation in wild songbird's adaptive immune responses to components of ectoparasite fitness. Although the immune response was specifically targeted against the tick *Ixodes ricinus*, tick feeding success was not reduced and thus birds clearly did not acquire immunological resistance against the ticks after being exposed for a long time. Interestingly, birds with the highest immune responses were more capable to reduce the acute harm and had gained more body weight than birds with lower IgY levels. Latter observations can be considered as an effect of host quality and/or tolerance mechanisms: birds anticipate the (future) costs of the activation of the immune system by ticks and/or ongoing tick-borne pathogen infections. Although unsuccessful against tick feeding, the immune responses may indirectly protect birds against tick-borne disease by acting against salivary protein secretions on which pathogens rely for transmission. Our study can be considered as a primer for future work exploring tick epitopes that can be targeted by bird immune components.

Introduction

All parasites show a certain degree of host specialization, partly defining the variation in burdens among host species, but also within a single host species, large variation among individuals in levels of parasitism has been proven to be the rule rather than the exception. This individual variation is - at least for parasites that live for longer periods of time in the off-host environment - determined by the following factors: (1) encounter rates, (2) mechanistic specializations in parasites for host finding and resource exploitation, and (3) host behavioural resistance, immunological resistance and susceptibility (Hudson *et al.*, 2002; Poulin, 2007; Schmid-Hempel, 2011). The latter two especially show extensive individual variation and are known to have a genetic basis, and to be heritable (Mazé-Guilmo *et al.*, 2014 and references herein). They are therefore considered to profoundly influence the co-evolutionary dynamics between parasite and host, and hence the (genetic) diversity found in natural host and parasite populations. Yet empirical evidence on how and to what extent host resistance - both behavioural and immunological – actually drives the observed variation in infestation levels in the wild, and thus the (co-)evolutionary processes, remains poorly studied in the majority of macro-parasite-wildlife systems.

This also applies to macro-parasites feeding on birds, such as ticks and mosquitos; the by far most important ectoparasites to human health vectoring micro-organisms that cause disease (e.g. Lyme disease, West-Nile virus) (Hubálek, 2004). In addition to grooming and preening (Clayton *et al.*, 2010), birds can reduce infestations by avoidance of ectoparasite-rich habitats (Christe *et al.*, 1994; Moore, 2002). But those first line behavioural defences are far from effective, particularly during the breeding season when birds face high time and energy demands while rearing their offspring, and must exploit parasite-rich habitats (Heylen *et al.*, 2013; Richner *et al.*, 1993).

However, the second line of defence, the host's immunological reaction against natural ectoparasites, has received very little attention in birds, especially with regard to ticks (Davison *et al.*, 2008; De la Fuente *et al.*, 2015; Heylen *et al.*, 2010). To investigate such immune responses, lab experiments are required in which host individuals are repeatedly exposed in order to allow immunity to develop. Intriguingly, in a previous experimental study we show that naïve songbirds did not acquire resistance against *Ixodes ricinus* (L., 1758) during the first months after fledging: tick engorgement weights and cellular immune responses remained unchanged after repeated exposures (Heylen *et al.*, 2010; Heylen *et al.*, 2015). Despite this apparent lack of immunological resistance, a substantial amount of among-bird variation was observed in the tick's feeding success and virulence, which still could be shaped by humoral immune responses.

This study focuses on the avian adaptive humoral immune response against ixodid ticks as a potential driver of the among-bird variation in feeding success. For this, we follow-up ticks that have been placed on the birds' skin, giving them the maximum opportunity to feed. As the development of an effective immunological response needs time, we exposed birds three times in succession over a time period of 25 days. Throughout the experiment, the birds' physiological health and IgY-antibody response were monitored (see 'Methods' section). For the main part of the study we used wild blue tits (*Cyanistes caeruleus*, L. 1758) and their endemic ticks in order to reproduce the natural songbird-tick interaction. Birds fledged in tick-free aviaries, ensuring they were tick-naïve before entering the experiments and got habituated to humans to reduce stress responses. In this study we put forward three questions: (1) do birds develop an IgY-antibody response specifically against *Ixodes ricinus* salivary antigens, and how strong is the inter- and intra-individual variation in this response? (2) do individual IgY-levels negatively correlate with tick feeding success (i.e. anti-tick resistance) and (3) how do they correlate with changes in bird physiology due to tick feeding?

Methods

Ethical statement

All procedures, including the tick infestation (for more details see below), were carried out in accordance with national environmental legislation and regulations, and were approved by the Ethical Committee for Animal Experiments of the University of Antwerp (Licenses N° 2009-32 and 2016-88). Wild birds were captured under licences N° S8/VERG/07-U5R26 and

ANB/BL/FF-V17-00029 of the Agency for Nature and Forests, Flemish Government, Belgium. Bird individuals were kept in optimal conditions at the University of Antwerp, with food and water *ad libitum* in large cages (surface floor 40 cm x 80 cm; height: 40 cm) and had the opportunity to take a bath in fresh water. Birds were monitored daily. Wild birds were released after a minimum time period in captivity. Manipulations of a bird (infestation, blood sampling, weighing, measurement of tarsus length) occurred in a separate section of the lab room, outside the view of the other birds. As manipulations (see below) cause mild distress or harm, the use of analgesics was not necessary.

Bird serum samples

Sera from tick-exposed birds were obtained from 16 blue tits that were exposed three times in succession with 12 nymphs over a time span of 30 days in the summer of 2008 (see Figure 1 for schematic overview of study design). All of them made part of a previous ethically approved experiment and were in good condition (Heylen *et al.*, 2010). Birds were kept in tick-free aviaries since hatching, thus naïve to ticks at the start of the experimental exposure. A blood sample (maximum 65 µL) was taken from the ulnar vein collected into 75 µL heparinized capillary tubes and subsequently centrifuged for 10 min at 14,000 g, after which the serum was separated from the blood clot and stored in Eppendorf tubes at -80°C until further analysis. To this end, the vein was superficially punctured with a needle (27G). Due to the small body size of the songbirds under study, the sampled serum volumes were kept to a minimal. As the minimum requirement for biochemical analyses was approximately 30 µL serum/bird per sampling occasion, only a limited number of birds (16) of the original experiment (31, see Heylen *et al.*, 2010) could enter the longitudinal analyses (i.e. enough volume in three consecutive infestation sessions).

Monitoring of repeatedly exposed blue tits for seroconversion and physiological changes

When blue tits were nine weeks old, individuals were infested with *I. ricinus* nymphs three times in succession (Infestation 1–3) (see Figure 1 for schematic overview of study design). Each infestation lasted 4–5 days, and the birds were kept free of ticks for a duration of 5–6 days between the consecutive infestations. We infested birds with tick loads corresponding to the maximum level found under natural conditions in our study population (Heylen *et al.*, 2013). To this end, 12 randomly sampled *I. ricinus* nymphs were put underneath the feathers on the head of each bird in each infestation session using moistened tweezers (Heylen & Matthysen, 2008). To this end, for each bird, Eppendorf tubes containing a nymph each, were randomly picked out of a box containing the remaining tubes with ticks. Birds were then kept for 2 h in an air-permeable cotton bag (size: 20 cm × 15 cm) inside a darkened cage, which kept them inactive. After tick exposure, birds were placed in individual cages with a wire-mesh floor (40 cm × 80 cm). Below the wire-mesh was a plastic tray containing damp filter paper and edges were streaked with vaseline to prevent nymphs from escaping. The engorged nymphs that dropped through the mesh cage were collected each day with minimal disturbance to the host (Heylen *et al.*, 2010).

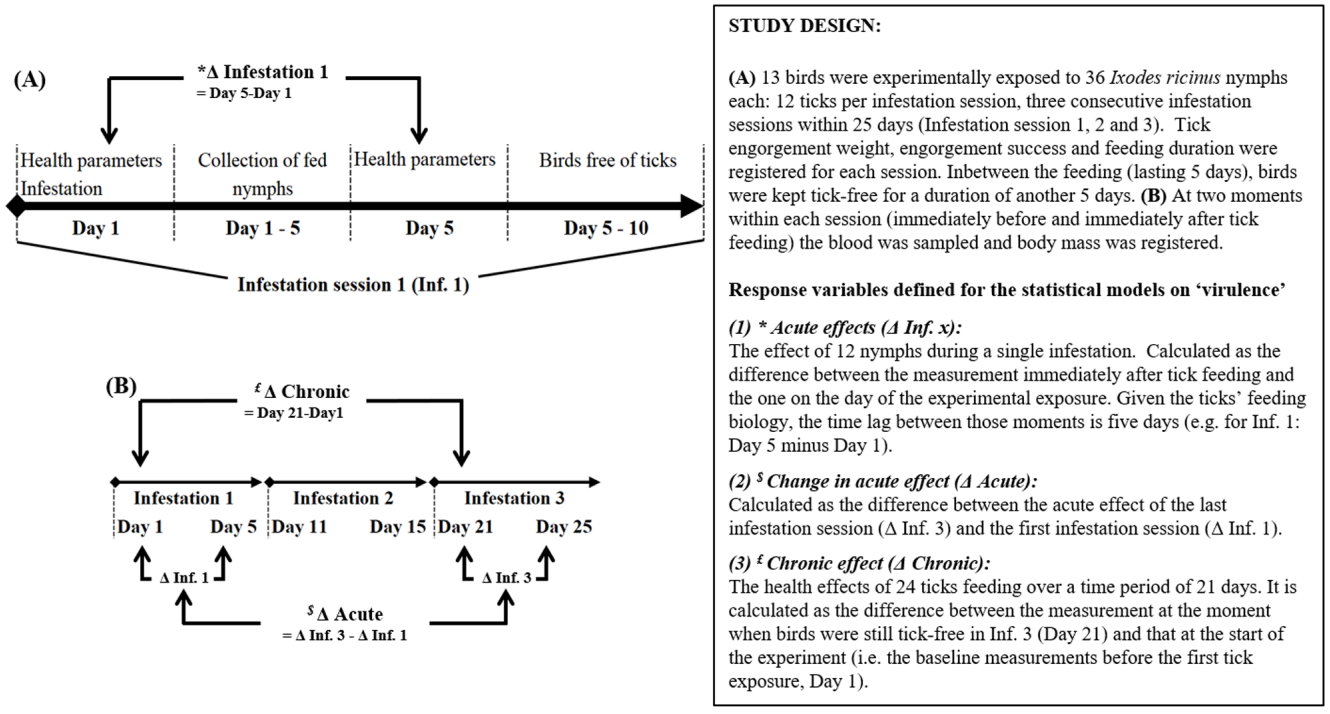

Figure 1. Schematic overview of the study design.

We estimated the effects of the IgY-response (ELISA described below) on tick measures as the change between exposure 1 and exposure 3. We measured the effect of IgY-response on the health measures as the change in health status (described below) between the moments immediately before tick exposure 1 and immediately before tick exposure 3 (i.e. 5–6 days after Inf. 2). Furthermore, we studied the change in the acute responses (i.e. the change in health status immediately before and just after tick exposure; Figure 1A) between infestation 1 and infestation 3 (Figure 1B) in response to the cumulative IgY response (i.e. the summed OD's; see further).

We measured two parameters reflecting the hosts' health status immediately before and after the infestation (Figure 1A). (1) Haematocrit (Hct) level: anaemia, as indicated by low Hct (the volume percentage of erythrocytes in the blood), results in a reduced oxygen-carrying capacity of the blood and restricts oxygen-demanding processes (Dein, 1986). Reticulocytes (i.e. immature erythrocytes) are stored in bird bone marrow, and can be instantly released in the blood stream (Martinho, 2012) upon sudden erythrocyte reduction (e.g. injury). Heparinized capillary tubes containing blood samples were centrifuged for 10 min at 14,000 g, and the ratio of packed red blood cells to the total volume was measured with a digital calliper to the nearest 0.01 mm under optimal light conditions. (2) Body condition (mass/tarsus ratio): body mass was measured to the nearest 0.1 g using a digital balance. To this end, the bird was immobilised by gently placing it in a tube (6 cm length, diameter 2.5 cm).

We subsequently calculated the ratio between body mass and a skeletal measurement (tarsus length, measured with a digital calliper to the nearest 0.01 mm) as a measure of body condition (Yom-Tov, 2001). Metabolic processes, e.g. for the compensation of parasite harm and mounting immune responses are known to be energy demanding and may lead to a reduced body condition when anabolic processes are hindered (e.g. restricted food conditions, constrained metabolic pathways, etc.) (Martin *et al.*, 2003).

Ixodes ricinus collection and feeding success

Ixodes ricinus nymphs were caught by dragging a white flannel flag over suitable vegetation. The ticks were subsequently kept under sterile conditions in a climate room at >90% relative humidity, a 16 h:8 h (light:dark) photoperiod, and a 25°C:15°C temperature cycle until infestation. After feeding on the blue tits, the engorged nymphs were weighed to the nearest 0.01 mg. To investigate the influence of repeated infestations on feeding success, we estimated the following parameters: (1) the proportion of the administered ticks that successfully engorged (tick yield), (2) the total weight of engorged nymphs and (3) their feeding duration. If hosts acquire resistance, this is expected to result in the following observations compared to naïve hosts (Rechav, 1992): lower numbers of engorged ticks, smaller blood meals (lower weight of engorged ticks), and increased feeding durations. From these criteria, engorgement weight is considered as to be one of the most consistent indicators of resistance (Varma *et al.*, 1990).

ELISA-based detection of immunoglobulins

Tick's salivary gland extraction (SGE). Salivary glands were dissected from 32 semi-fed colony-reared infection-free adult female *I. ricinus* obtained from IS insect Services GmbH in Berlin, Germany. Before dissection, all ticks had fed on sheep for 5–7 days; feeding is known to significantly increase SGE concentrations (Mateos-Hernández *et al.*, 2017; Ogden *et al.*, 2002). The glands were washed four times in PBS to remove tick debris, pooled and homogenized. To test IgY-cross-reactivity with antigens of a congeneric tick species, we used material of 12 engorged adults *I. arboricola* ticks (Heylen *et al.*, 2014) that had fed on great tit nestlings and that were dissected in the same way. A pool, containing the glands of 6–8 ticks in 60 µl PBS, was manually disrupted with a sterile pestle and the following steps were performed before storage at -80°C: sonication three times for five seconds with a treatment in ice (BRANSON 150), centrifugation at the maximum speed (10,000 rpm) for 10 minutes at 4°C, and filtering through a 0.2 µm filter (Chromafil AO-20/3 Macherey-Nagel GmbH, Düren, Germany) to remove contaminating bacteria. Total protein concentrations were estimated by Nanodrop (Cafiso *et al.*, 2019) and equilibrated to 1 mg/mL prior to use in the assays.

IgY ELISA. Basing ourselves on Ogden *et al.* (2002), after optimization of the ELISA-protocol - including the binding capacities of the anti-chicken antibodies for passerine raised antibodies (sandwich ELISA in which plates are coated with bird sera) – the following volumes and concentrations yielded the most reliable and repeatable results for the indirect ELISA tests. To coat the 96-well microtiter plates (Nunc Maxisorp flat bottom, Thermo Fisher Scientific, Geel, Belgium), 150 µL PBS per well was used, containing a concentration of 1.8 µg/mL SGE. Negative controls were coated with 150 µL PBS only. Plates were incubated for 12 hours at 4°C. After coating, the plates were washed three times with 200 mL PBS to remove the unbound material prior to blocking, using 200 µL of 0.5% Bovine Serum Albumin in PBS per well, and incubation for 1h at 37°C. Subsequently, the solution was removed and the wells were rinsed with PBS. Primary antibodies were obtained from bird sera diluted 140 times in PBS. 150 µL of the diluted bird sera were added to appropriate wells and the plates were incubated for 1 hour at 37°C. Afterwards, the wells were washed four times with 200 mL of PBS and 150 µL of the labelled secondary antibody (Rabbit anti-Chicken IgG, FC specific-alkaline phosphatase antibody, Sigma-Aldrich, code SAB3700239, Overijse, Belgium -15000 times diluted in PBS) was added. After one hour incubation at 37°C, the plates were washed three times with 200 mL of PBS and pre-washed once with 200 mL of alkaline phosphatase (AP) buffer (100 mM Tris, 2 mM MgCl2, pH 9.6 with HCl). The amount of secondary antibody bound to the primary antibodies is visualized through AP reaction after adding 150 µL of a 1 mg/mL 4-*p*-nitrofenylfosfaat dilution in AP reaction buffer and one hour incubation at 37°C. Plates are read with a plate reader (Biotek Synergy MX, BioTek, Winooski, VT, USA) measuring OD at 405nm.

Repeatability and qualitative discrimination infested vs. non-infested birds. Negative control serum samples were

obtained from three adult (sex unknown) domesticated canaries (*Serinus canaria*, L. 1758), belonging to a captive population maintained for multiple generations (15 years) at the University of Antwerp. In addition, three 1st calendar year blue tits (*Cyanistes caeruleus*) and three 1st calendar year great tits (*Parus major*, L. 1758) that were kept in tick-free aviaries since hatching (sex unknown; see Heylen *et al.*, 2010 for further details on origin and housing). Sera from tick-exposed birds were obtained from three free-living great tits (1st calendar male and female, one 2nd calendar year male) that showed to be *Ixodes ricinus* tick-infested upon capture with mist nets (early Autumn 2019, Antwerp, Belgium), three 1st calendar year great tits (sex unknown) that were three times experimentally exposed to 17 *Ixodes ricinus* nymphs over a time span of 30 days (Heylen *et al.*, 2010), and three of the abovementioned blue tits.

The IgY levels in serum samples belonging to the same individuals showed to be highly repeatable within an ELISA-plate (Pearson's Rho: 0.93; N= 17) as depicted by the scatterplot (Figure 2). One measurement was excluded (in the non-infested Sc 3) as a pipetting error had occurred. Considerable variation in IgY levels was observed among tick-exposed individuals of the same species (variance/mean in great tits: 10%; blue tits: 19%), but also in the naïve blue tits (10%). Both naturally infested (caught in the wild and blood sampled once) birds and repeatedly infested birds (following scheme depicted in Figure 1) showed noticeably higher OD values than non-infested individuals.

Statistical analysis

For the qualitative comparisons between infested and non-infested birds (only three individuals per bird species, over the two groups) no statistical tests were performed (Figure 2), neither for the description of the IgY-profiles of three blue tits - for which sufficient amounts of serum and tick antigens allowed a quantification at each of the six time points (Figure 1 and Figure 3). Data of IgY levels at Day 1, 11 and 21 (Figure 1) of the latter three birds were combined with that of ten additional blue tits, followed by the parametric statistical analyses as described below:

generalized linear mixed effect models (GLMM's) were fitted on health measures to model the acute infestation effects (Δ Inf. 1, 2 and 3; Figure 1) as a function of the bird's IgY levels (OD value) within each infestation. To avoid collinearity problems and to adjust for differences in variation between infestations, the IgY levels were standardized ($OD_{Inf.x}$ - $mean_{Inf.x}$/Standard $deviation_{Inf.x}$). By adding a random bird individual effect, and using Kenward-Roger approximation for the denominator degrees of freedom, we took into account the correlation of observations within the same individuals.

In a second statistical analysis, we tested whether the changes in acute effects ('Δ Acute', Figure 1) were related to the summed IgY levels over the three infestations (SOD), which we consider as a proxy for the bird's overall anti-tick IgY production over the course of the experiment. In a final analysis we modelled the change in initial values (Δ Inf. 3–1) (= chronic response) as a function of SOD. Effects of IgY levels on tick feeding parameters were modelled in a similar way, except for the fact that we only have one value per bird/infestation session (and not a difference). Before entering the analysis, the average tick measures (weight, feeding duration) were calculated for each bird/infestation. In all models, a stepwise

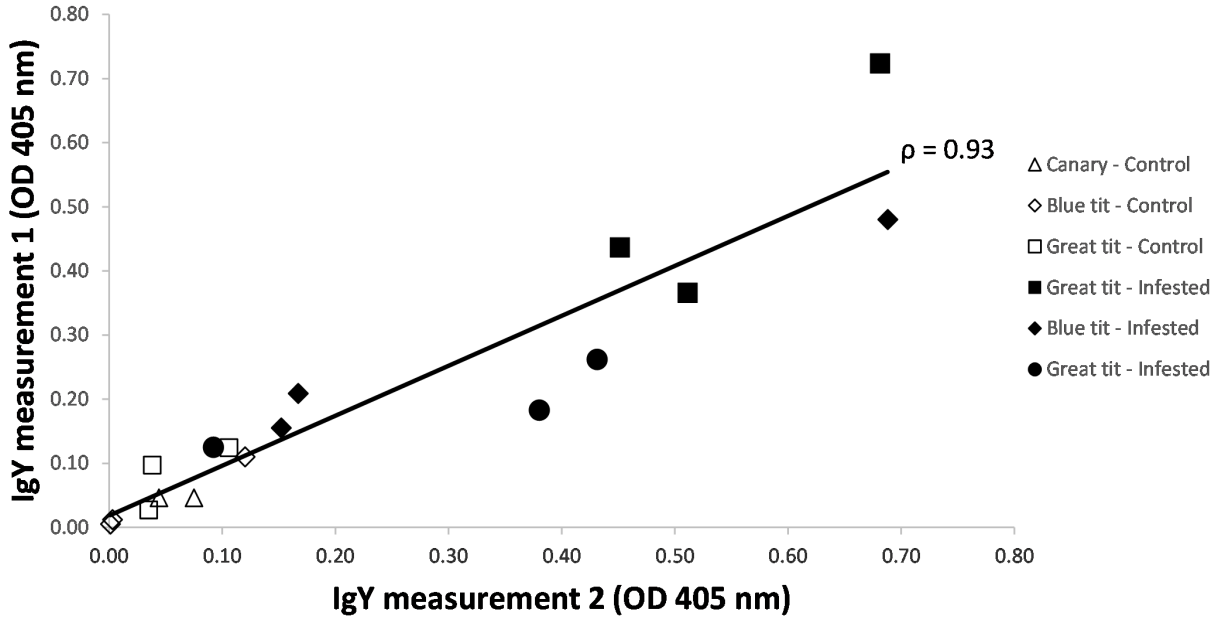

Figure 2. ELISA's optical density measures of two IgY-measurements on the same bird. IgY levels were obtained from non-infested and *Ixodes ricinus*-infested birds (great tit, blue tit, canary). Among the infested birds, three great tits were very recently infested when caught in the wild (filled squares). All other samples were obtained from birds that fledged in tick-free aviaries or cages (control), and which were three times experimentally infested with ticks (including bird 1–3 of the 16 birds in the blue tit experiment).

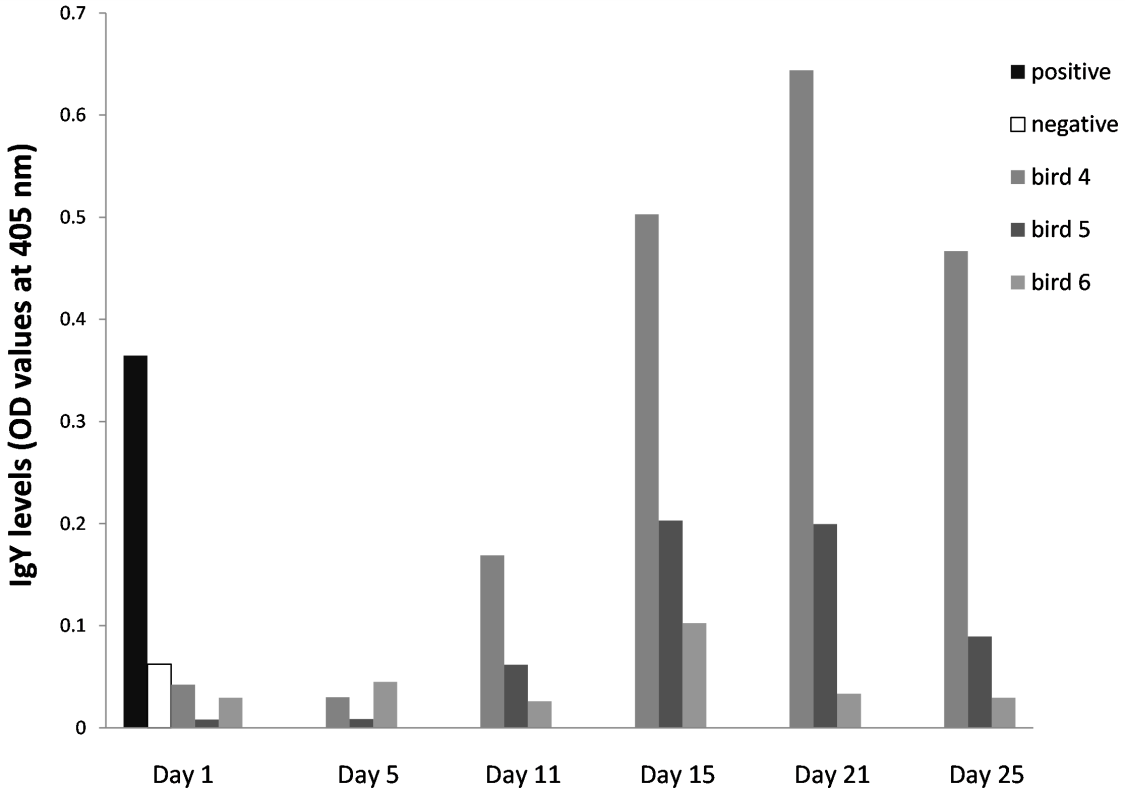

Figure 3. IgY levels of sera sampled from *Ixodes ricinus*-infested blue tit individuals (bird 4–6) that were repeatedly exposed following the study design in Figure 1 (pre-infestation: Day1, 11, 21; post-infestation: Day 5, 15 and 25). Positive and negative control birds: repeatedly exposed great tit and naïve canary, respectively.

selection procedure was used in which the model was iteratively refitted after exclusion of the least significant effect, until only significant factors and their lower order interactions terms were left. All data manipulations and statistical analyses were performed using SAS v 9.2 (SAS Institute, Cary, North Carolina, USA). Estimates are reported as mean ± standard error.

Results

Sera *I. ricinus*-exposed birds and cross-reactivity with *I. arboricola* antigens

In order to evaluate the entire temporal patterns in IgY levels over the course of the experiment, we monitored the three blue tit individuals for which sufficient serum was available at all possible sample occasions (i.e. six moments in total, see Figure 1 for study design). We observed an OD-curve that tended to be bell-shaped, with the highest IgY levels around Day 15–21 (Figure 3). The same increase in temporal pattern (Day 1, Day 11 and Day 21) was observed in the additional ten blue tits (0.014 ± 0.004 OD unit/Day unit, T-value = 3.49, df = 10.8, P = 0.0052; Figure 4). Large individual variation was observed on each of the sampling days (variance/mean Day 1: 5%, Day 11: 17%, Day 21: 16%), and variation in slopes differed from zero (estimate: 0.14 ± 0.1 10 -3; Likelihood ratio test: Z = 1.70; P = 0.044). We repeat that the reasons why the ten birds are not entirely covered are: (1) at several sample occasions there was not enough serum available, (2) the Day 15 sample has been used to investigate cross-reactivity with *I. arboricola* antigens. With regard to the latter, the antibodies against *I. ricinus*-antigens in those samples taken at Day15 showed almost no cross-reactivity (Figure 4). We observed that in two birds (bird 2 and 4) the OD's in the *I. arboricola* wells were slightly higher than the baseline values on Day 1 (i.e. when all birds were still naïve.

IgY correlations with anti-tick resistance

In order to investigate whether IgY influenced the tick's feeding performance, we utilised a GLMM for repeated measurements with feeding performance (engorgement weights, feeding durations or engorgement success) as response variable and the IgY levels as explanatory variable.

Test statistics for the cross-sectional (Inf. 1, 2 and 3) and longitudinal analyses (Δ Inf. 3–Inf. 1) in relation to the IgY levels are presented in Table 1. Neither the average engorgement weight (Figure 5) nor the feeding duration showed significant associations with IgY level in any of the analyses (Table 1), despite the strong IgY-increase and large among-individual

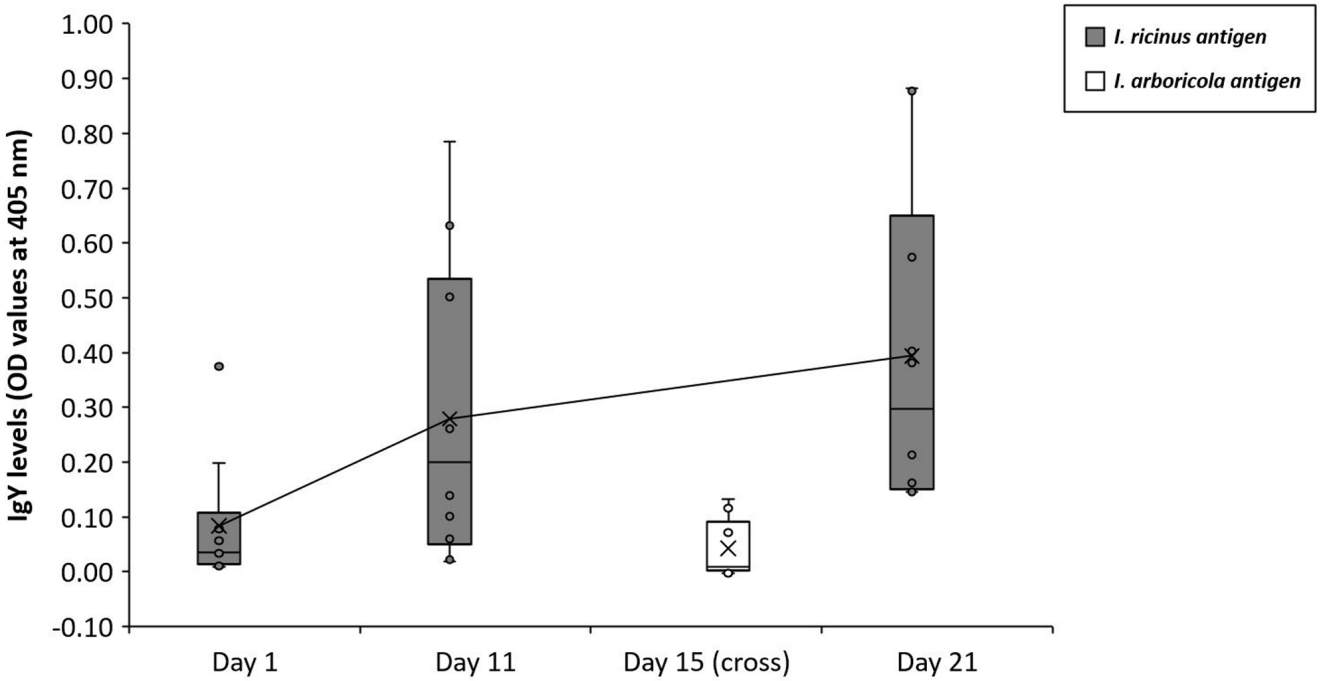

Figure 4. Box-plots of the IgY levels of sera from ten blue tits (bird 7–16) that were infested three times in succession with *I. ricinus* (for design see Figure 1). Values of a second ELISA are included, in which the cross-reactivity of anti-*I. ricinus* IgY's was tested (Day 15 samples only) against *I. arboricola* salivary antigens.

variation (Figure 3 and Figure 4). Furthermore, the proportion of ticks that successfully engorged did not covary with IgY levels. For absolute values of feeding parameters, we refer to Heylen *et al.* (2010). Conclusions did not change when restricting the analyses to the subset of 10 individuals that were simultaneously analysed on a single plate (Figure 4). In sum, none of the feeding performance factors were influenced by IgY levels across either each infestation, or across all three infections.

IgY correlations with tick virulence

In order the assess the influence of IgY on the acute health effects by the feeding ticks (i.e. the birds' changes in Hct and body condition), we used a GLMM with as response variable the acute change in the health parameter within an infestation session (see Figure 1), and as explanatory variable the IgY levels from the blood sample taken immediately before an infestation (Table 2). While on average the Hct levels did not significantly change during the first two infestations ('Acute' Inf. 1: 1.97 ± 1.29; Inf. 2: -1.14 ± 1.08%), they decreased in the third infestation (Inf. 3: -6.24 ± 1.63%, T-value = -3.82, P = 0.0028). Birds with higher IgY levels prior to an infestation showed a less severe Hct decrease (2.23 ± 0.80%/OD unit, T-value = 2.92, df = 31, P = 0.0065; Figure 6). In this analysis, two statistical outliers belonging to the same bird were removed (see Figure 6). The same analysis, but with body condition as response variable, did not show any significant associations with IgY levels.

When investigating the change in acute effects on Hct ('Δ Acute') caused by the ticks (i.e. the difference between the acute effect in infestation 1 and the one in infestation 3) we did not find an association with the total amount of IgY produced by the bird during the experiment (i.e. the summed IgY levels as a proxy). The same analysis, but with body condition as response variable, did not reveal any significant association with the summed IgY levels (Table 2).

In order the assess the influence of IgY on the chronic health effects by the ticks, we modelled the change in health (i.e. Day 21 minus the baseline values on Day 1, see Figure 1: Δ Chronic) against the summed IgY levels. We found that the increase in body condition over the infestation sessions (Δ Chronic: 0.027 ± 0.006 g/mm; T-value: 4.38, df = 11; P < 0.001) was higher in birds that had higher summed IgY levels (SOD) (0.033 ± 0.006 g/mm; T-value = 5.33, df = 11, P = 0.0002; Figure 7), however Hct levels did not show any association with SOD (Table 2).

Discussion

This is the first study that investigates the interplay between the songbirds' humoral immune response and natural blood-sucking ectoparasites (ticks), combining observational and experimental approaches. The acquisition of tick immunity has been an important topic in the field of tick biology, as patterns of immunity can be used to develop transmission blocking vaccines for humans or reservoir animals against tickborne pathogens.

Table 1. Type 3-outcomes of generalized linear mixed effect models (GLMM's) investigating the association between IgY levels and proxies for anti-tick resistance. IgY levels were measured in the serum samples taken at the beginning of each infestation session. In the analyses of the cross-sectional correlations ('per infestation') IgY levels have been standardized. For the longitudinal analysis (i.e. the cumulative response, 'Inf. 3 minus Inf. 1'), the summed IgY levels over the three infestation sessions was calculated (See Figure 1). Test statistics before exclusion from the model are given, as well as the parameter estimates and statistics for the terms that remained in the model (P-value <0.05).

Per infestation	***Infestation*** $_{(ndf,ddf)}F$	***IgY (SD)*** $_{(ndf,ddf)}F$	***IGY x Infestation*** $_{(ndf,ddf)}F$
Engorgement weight	$_{(1,35)}0.15^{NS}$	$_{(1,37)}0.21^{NS}$	$_{(2,33)}0.56^{NS}$
Feeding duration	$_{(2,35)}0.74^{NS}$	$_{(1,37)}2.74^{NS}$	$_{(2,33)}1.20^{NS}$
Engorgement success	$_{(2,31)}0.63^{NS}$	$_{(1,36)}0.64^{NS}$	$_{(2,32)}0.62^{NS}$
Inf. 3 minus Inf. 1		***Summed IgY's*** $_{(ndf,ddf)}F$	
Engorgement weight		$_{(1,11)}1.81^{NS}$	
Feeding duration		$_{(1,11)}0.55^{NS}$	
Engorgement success		$_{(1,11)}0.70^{NS}$	

Infestation: early (Inf. 1), during sero-conversion (Inf. 2) and at maximum IgY levels (Inf. 3); NS: P-value >0.05.

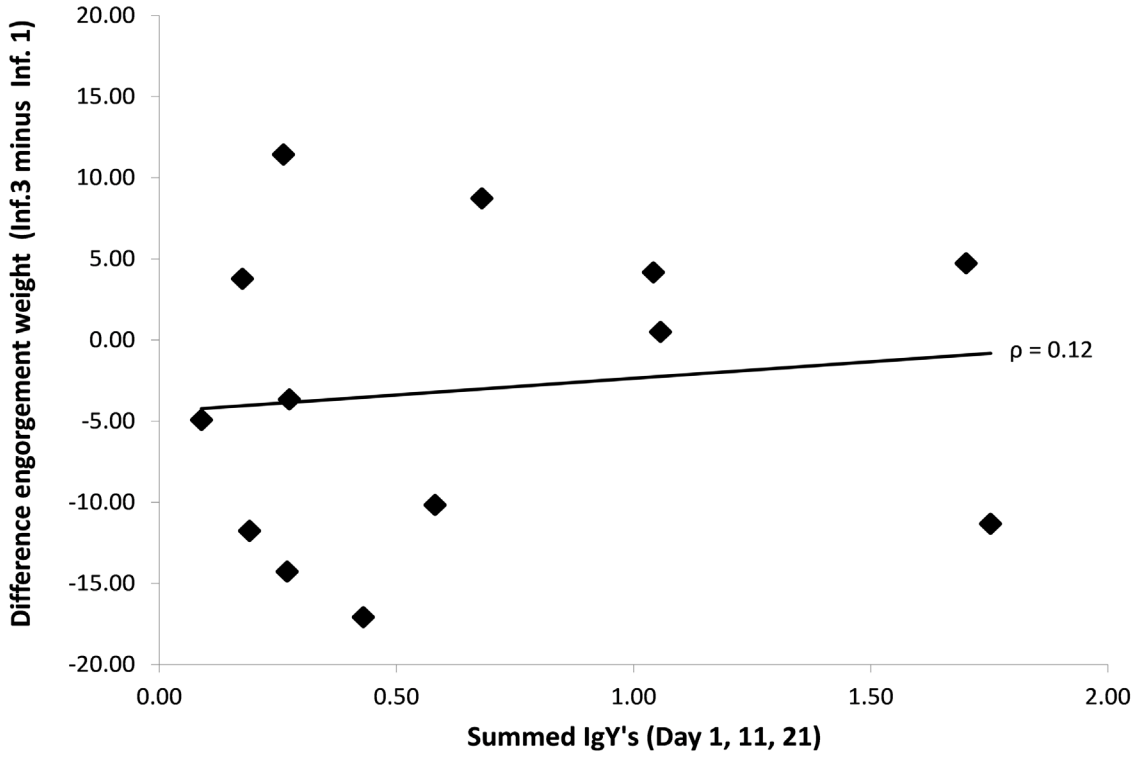

Figure 5. Difference in the summed engorgement weights between the beginning of the experiment (Inf. 1, naïve bird) and the end (Inf. 3, previously exposed to 24 ticks) as function of the summed IgY levels in blue tit sera (bird 4–16). Total engorgement weight is a function of the average engorgement weight and the proportion of successfully fed ticks, none of which showed a significant association with IgY levels (Table 1).

Table 2. Outcomes of GLMMs investigating the association between IgY levels and the health parameters in 13 birds repeatedly infested with *Ixodes ricinus* nymphs. See Figure 1 for the experimental design, and further explanation of 'Acute effect' ('after' minus 'before' exposure), Δ Acute (Change in acute effects Inf. 3 vs. Inf. 1) and Δ Chronic (values on Day 21 minus the ones on Day 1).

* Acute effect (Δ Inf.x)	Infestation	IgY (SD)	IgY x Infestation	Average Slope over the 3 infestations
Haematocrit	$_{(2,31)}$**12.06** $^{<0.001}$	$_{(1,31)}$**8.76** $^{0.006}$	$_{(2,29)}$1.70 ^{NS}	**0.024±0.008**
Condition	$_{(2,36)}$0.98 ^{NS}	$_{(1,35)}$0.06 ^{NS}	$_{(2,33)}$0.69 ^{NS}	
$^{\$}$ Change in acute effect (Δ Acute)		Summed IgY's $_{(ndf,ddf)}F$		
Haematocrit		$_{(1,11)}$0.16 ^{NS}		
Condition		$_{(1,11)}$4.42 $^{0.064}$		
$^{£}$ Chronic effect (Δ Chronic)		Summed IgY's $_{(ndf,ddf)}F$		Slope
Haematocrit		$_{(1,11)}$0.65 ^{NS}		
Condition		$_{(1,11)}$**28.41** $^{0.0002}$		**0.033±0.006**

Infestation: early (Inf. 1), during sero-conversion (Inf. 2) and at maximum IgY levels (Inf. 3).

* Outcome without the bird giving outlying residuals. This bird led to violation of the normality assumption (Shapiro-Wilk W: 0.96); removing it improved the model fit (Shapiro-Wilk W: 0.98).

NS: P-value >0.05.

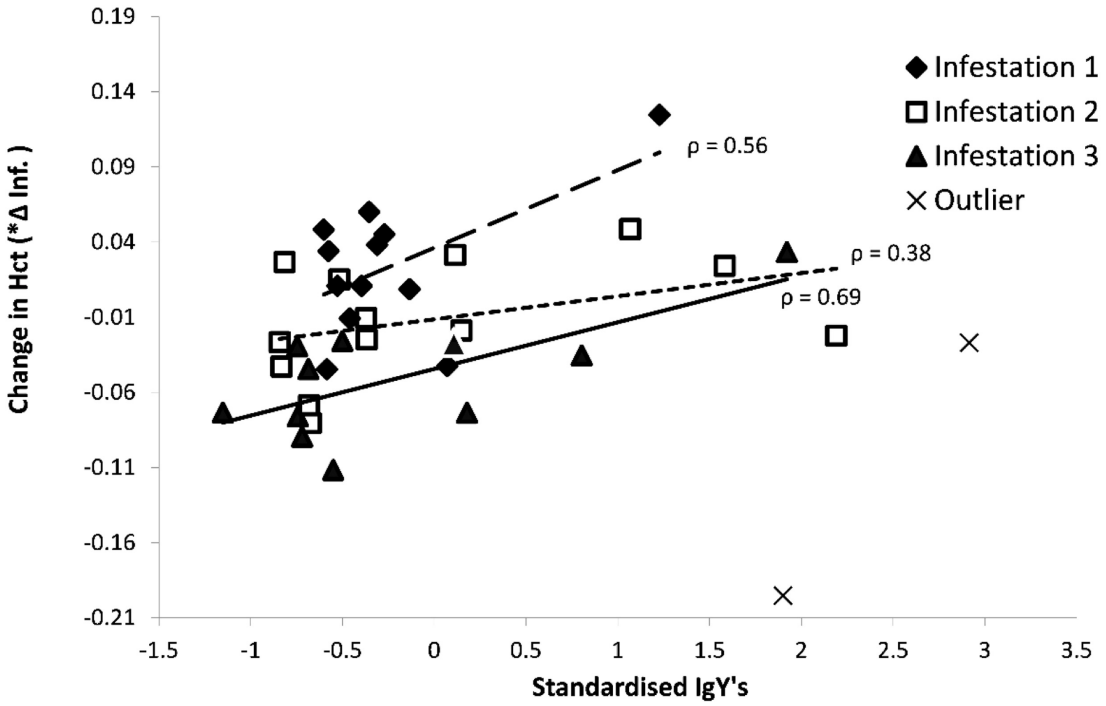

Figure 6. Acute effects of ticks on Hct levels in response to the standardized IgY levels of sera samples in 13 blue tits (bird 4-16) presented in Figure 3 and Figure 4. Two outliers from the same individual were excluded from the statistical analyses.

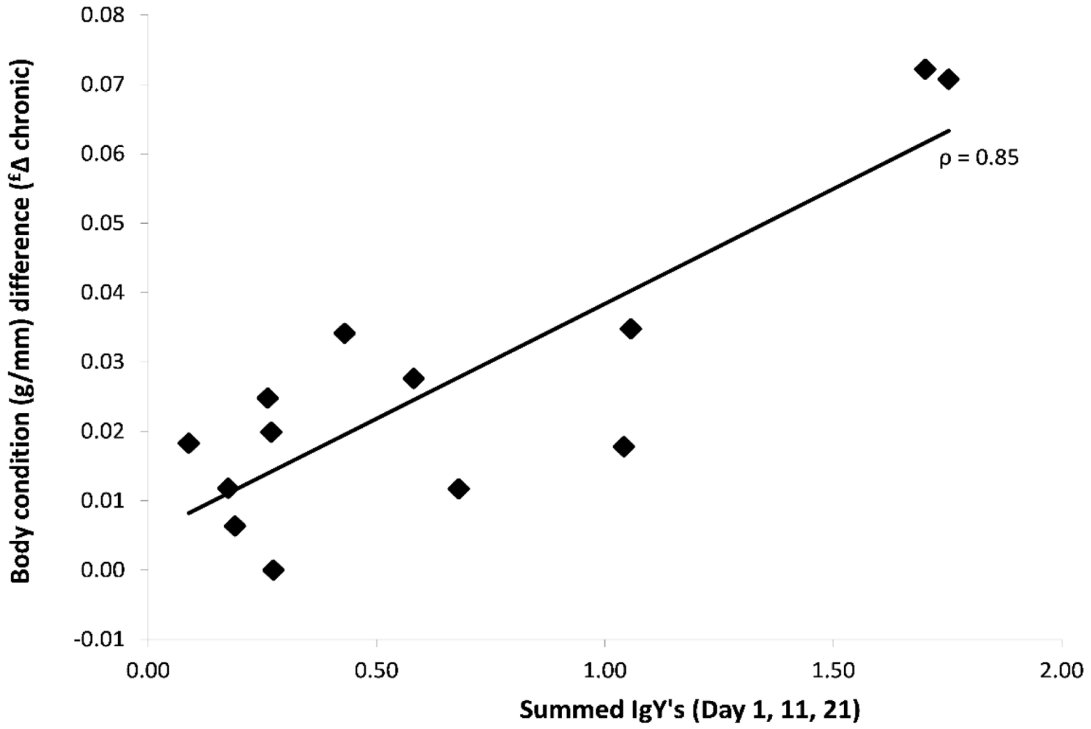

Figure 7. Chronic effects of ticks on the body condition (weight/tarsus) of 13 blue tits (bird 4-16) in response to the summed IgY levels (summed OD's over three time points prior to an infestation).

Immunity of mammals and non-mammalian hosts (such as birds) may differ in response to the same antigens (e.g. tick salivary proteins), which emphasizes the need of the current study. Furthermore, as wild birds can maintain and spread tick-borne pathogens, the relevance of the use of wild phenotype birds for endemic transmission cycles likely outweighs the limitation linked to the maintenance of wild animals in lab conditions. We put forward three questions (1) do birds develop a specific antibody response against *Ixodes ricinus* salivary antigens and how strong is the variation among birds? (2) Does the humoral immune response reduce tick feeding success (3) and virulence?

In order to address the first question, we developed an indirect ELISA, and succeeded to quantify the bird's immunoglobulins (IgY) that bind to *I. ricinus* salivary gland antigens. We then could show that the level of tick-specific IgY was low at the beginning of the experiment, when birds were naïve, then steeply increased to peak at 15–20 days - the moment of sero-conversion – and tended to decrease afterwards (Figure 3). This sero-conversion pattern is comparable to IgG-kinetics in mammals (Barriga *et al.*, 1991). IgG is the mammalian analogue to the avian IgY, present in the chronic phase of parasite exposure, and is involved in the development of long-term resistance (Davison *et al.*, 2008) against ticks (Ogden *et al.*, 2002). In our study, the IgY-response turned out to be specifically targeted against *I. ricinus* salivary antigens, as the cross-reactivity against *I. arboricola*-antigens was shown to be negligibly low (Figure 4). Thus, the observed IgY-response gives evidence for an adaptive response, rather than an acute inflammatory reaction. We found significant individual variation among birds in immune profiles, as well as in initial values before being exposed for the first time. An explanation for the latter finding might be that maternal antibodies of mothers with anti-tick IgY-concentrations in their blood have been transferred via egg yolk to nestlings, as observed in gulls (Müller *et al.*, 2004). This additional source of antibodies complicates the interpretation of IgY levels as signals of the individual's previous tick exposure, especially in juveniles (Gasparini *et al.*, 2001). However, the immunological processes in the early development of altricial birds (among which blue and great tits) differ from those of semi-precocial birds (to which gulls belong). In an experimental study by King *et al.* (2010) where House sparrows (*Passer domesticus* L.) are experimentally exposed to novel antigens, the authors observed short half-life of maternal antibodies (less than 3 days after hatching), low transfer from mother to nestlings, and a rapid production of endogenous antibodies by nestlings (8–10 days after hatching). They concluded that altricial developing birds achieve immunologic independence much earlier than precocial birds, implying that the variation in (initial) IgY levels observed in our study is synthesized by the juvenile bird itself.

For our second question, we looked at pairwise relationships between the IgY levels and either tick feeding parameters or bird health measures. As observed in other natural hosts

(Fielden *et al.*, 1992; Ribeiro, 1989), the opportunistic *I. ricinus* turns out to be extremely efficient in circumventing the bird's antibody response. In host types where anti-tick resistance is acquired, strong decreases in engorgement weights are observed in subsequent tick exposures. Acquired resistance against ticks has frequently been demonstrated in laboratory rather than in natural hosts, therefore it has been suggested that tick resistance is confined to artificial host–tick associations (e.g. guinea pigs infested with Dermacentor variabilis, rabbits infested with *I. ricinus*) (Bowessidjaou *et al.*, 1977; Ribeiro, 1989; Wikel, 1996) and that successful parasitism in natural host-tick associations is the result of an intense co-evolution, in which ticks developed adaptations to evade the host's immune system (Fielden *et al.*, 1992; Ribeiro, 1989). Here we found that, despite the high among bird-individual variation in IgY-responses and tick feeding success, there was no significant association between them. We conclude that naïve juveniles do not acquire immunological anti-tick resistance. The outcomes could be viewed as a mechanism of tolerance instead of resistance. The latter is the capacity to limit the parasite burden, while the first refers to the ability to limit the harm caused by a given burden by compensatory mechanisms (Råberg *et al.*, 2009). The concept of tolerance is notoriously difficult to measure in animals, when measuring the slope of how host fitness decreases with parasite burden. Here, high burdens did not cause direct fitness effects (bird mortality), or gave rise to indirect fitness effects via physiological measure that link up with bird fitness; both findings are in favour of tolerance.

While on average the ticks performed equally well throughout the experiment (i.e. no significant difference between infestation 3 and 1), the harm (i.e. blood depletion) seemed to be better compensated for when birds had higher IgY levels. Massive amounts of reticulocytes (i.e. immature erythrocytes) are stored in bird bone marrow, and can be instantly released in the blood stream (Martinho, 2012). We point out that the net Hct difference in each infestation (Figure 1 – Figure 6) is the result of two processes: the immediate erythrocyte compensation (i.e. addition of erythrocytes in the blood stream) and acute erythrocyte depletion due to tick feeding. Although tick feeding did not decrease with IgY, the net effect of the abovementioned processes showed a correlation with IgY. We do not know of any role of IgY in these processes, but since both immune responses and harm compensation are energetically demanding, general health could simply be driving the observed correlation. Additionally, birds with a higher overall IgY-response gained more body weight (first 21 days). Metabolic processes for the compensation of the blood depletion by the ticks, the repair of skin lesions and blood vessels, and mounting immune responses are all energy demanding (Martin *et al.*, 2003), and may lead to a reduced body condition. However, under lab conditions, with food *ad libitum*, those birds with the strongest IgY-response showed to be more successful in gaining body mass. The observed increase may relate to the gain in body mass for the regeneration of feathers (i.e. the post-juvenile moult) (Bojarinova *et al.*, 1999) or other undefined seasonal physiological changes. Also, by gaining body mass, birds possibly anticipated the costs of (chronic) activation of the immune system due to tick infestations and/or ongoing tick-borne pathogen infections (as birds were exposed to ticks from the wild) (Heylen *et al.*, 2015). In the end, this may benefit the fitness of both the ticks and micro-organism: future ticks could feed more successfully in those birds with a stronger body mass increase and it is conceivable that they may even induce such processes. We mention that our results are correlational, and do not prove causation; anti-tick IgY-response are not necessarily the cause of better health outcomes, but could be a correlated by-product of variation in quality among the birds. This quality (condition, health, vigour) could be affected by several factors, including good genetic constitution, higher quality maternal care, lower stress experience, fewer co-parasites, etc. In the ecological immunology literature, many studies have shown that variation in condition will drive associations between immunological traits (Sadd & Schmid-Hempel, 2009) but from our study it is clear that the measured IgY's did not correlate with the tick's feeding success, despite they targeted tick salivary antigens.

Birds are central elements in the ecological networks of ticks, but are heavily overlooked when it comes to elementary biological mechanisms like immune responses (De la Fuente *et al.*, 2015). In fact, surprisingly few studies have related individual variation in host immunity to components of ectoparasite or even tick- fitness. Despite being unsuccessful in reducing tick feeding success via IgY's, birds may benefit from the observed IgY-responses: by indirectly acting against vector-borne pathogen constituents (i.e. tick proteins functioning as carrier vehicles) tick-to-host transmission may become mitigated. As the transmission of pathogenic tick-borne agents heavily relies on salivary proteins (De la Fuente *et al.*, 2015), it is worth studying this hypothesis for a variety of tick-borne pathogens in (in)competent natural reservoir songbird hosts. Are anti-tick immune responses, host physiology and tick feeding performance affected by the pathogens themselves and/or other tick associated micro-organisms (including viruses)? Are these responses comparable in different tick-pathogen-host systems? Does also the bird's long-term fitness remain unaffected by high tick loads, providing further indirect evidence for tolerance (as defined by Råberg & colleagues (2009))? All these questions are heavily unexplored in birds, but are crucial for understanding local transmission and life cycles. Some of the answers could even inspire vaccine development, when mapping the tick epitopes that are effectively used by pathogens and could be targeted by host immune components.

Data availability

Zenodo: data Ineffective humoral anti-tick IgY-response in birds reaction against pathogen constituents, https://doi.org/10.5281/zenodo.4527196 (Heylen, 2021).

Acknowledgements

We thank Frans Fierens and Joris Elst for their technical assistance.

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Open Peer Review

Current Peer Review Status: ? ✓ ✓ ✓

Version 2

Reviewer Report 07 February 2022

https://doi.org/10.21956/openreseurope.15184.r28390

✓ **Courtney Waugh**
Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway

This article provides new and important information about the humoral immune response of blue tits and their ticks.

The work is clearly presented in adequate detail and cites relevant sources. The figures are clear and the statistics are sound. The study is well designed to answer the research questions and provides new data about the subject. The conclusions support the results.

A very nice paper.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Wildlife immunology and infectious diseases.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 03 February 2022

https://doi.org/10.21956/openreseurope.15184.r28391

Veerle Jaspers
Department of Biology, Norwegian University of Science and Technology (NTNU), Trondheim, Norway

The authors present an original contribution regarding the immunological responses of birds to ectoparasite infections and the potential coping mechanisms of the birds. The results are very interesting and the discussion is sound and highlights areas for further research.

After reading the revised manuscript and the responses the authors have made regarding the comments of earlier reviewers, I am of the opinion that the manuscript is suitable to pass peer review but would suggest a few minor revisions.

I have some minor comments in addition to the revisions already made. All are regarding clarification of the methods:

- Please add the number of blue tits exposed for this experiment in the methods section of the abstract.
- In my opinion, it would be clearer for the reader to move the section on Ixodes ricinus collection up, just after the ethical statement.
- One clarification is needed: I got confused about the number of birds for each specific result: 16 blue tits in total were used, but for only 3 enough blood was available at 6 times. Then again the authors talk about 10 additional birds. So these two together account for 13 birds. What happened to the other three? I got confused here and it would be helpful to list specifically in the statistical part of the methods and also clearly in the results, how the 16 birds were included/divided in the different parts of the analysis.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Avian toxicology, ecotoxicology, avian physiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 20 August 2021

https://doi.org/10.21956/openreseurope.14274.r26998

Maxime Madder
The Tamarin Commercial Hub, Clinglobal, Tamarin, Mauritius

Ineffective humoral anti-tick IgY-response in birds: reaction against pathogen constituents. Heyler *et al*.
The authors present an interesting study trying to define the effect of a humoral response in blue tits that are exposed to multiple infestations with wild caught I*xodes ricinus* ticks.

General comments
The paper offers an interesting insight the way blue ticks react to multiple infestations of ticks, but some general information is missing as pointed out below.

Introduction
It might be interesting for the reader to explain what type of host immune responses are triggered by ticks (including birds), as other studies were referred to (Heylen et al., 2010, 2015), and this to explain why IgY was selected. Also why IgY was looked at and not IgA or M.

Methods

Having used wild caught ticks, and not knowing their infection status with viral, bacterial or protozoal disease agents, could the authors clarify if the use of infected ticks might have influenced the results and conclusions? Many articles describe the influence of infected ticks on the ticks' behaviour, but also on the host immune response.
Could the authors specify where the ticks were collected, what the known pathogens are in this region and their prevalence, and where the birds originate from. Also the date the study was conducted is not specified.

Discussion
The authors state the the birds tolerated the ticks, and did not show anti-tick resistance. Could this be dose-dependent? Could higher infestation loads result in anti-tick resistance? It might be hypothesised that tolerance is seen as long as birds are not negatively impacted by infestation.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Parasitology, acarology, ticks and tick-borne diseases, vector ecology, animal health

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 10 August 2021

https://doi.org/10.21956/openreseurope.14274.r27346

?

Michelle Wille
University of Sydney, Sydney, NSW, Australia

Heylen: Ineffective humoral anti-tick IgY-response in birds: reaction against pathogen constituents

The authors aimed to understand the immune response of Blue tits to tick infestation using an experimental approach. Overall, this is an interesting paper, but currently some shortcomings in data presentation (both text and writing) make it hard to understand the details and interpret of the findings.

Some general questions:

I think a bit more careful explanation is needed for the reader to understand the idea of "tick resistance". Specifically, that it's not the prevention of ticks infesting birds, but rather a decrease in the impact of ticks on birds (eg, lower tick engorgements and perhaps a more effective immune response).

Second, humoral immunity develops following the acute response (inflammation). How do these findings link into studies assessing inflammation following tick infestation? Would this variation in IgY perhaps be due to a variation in acute responses? I recognise that the authors did not measure this, but I wonder if there is data from previous studies?

Third, as these were field collected ticks, how do the authors control for things like viral infections which may affect Hct or body condition. These viral infections may also affect Engorgement weights and feeding durations of ticks (particularly for viruses infecting the ticks, not necessarily arboviruses which are transmitted to the birds). Theoretically they shouldn't affect the IgY as this is specific. I recognise that the authors did not measure this, but it is worth point out in the discussion.

Specific comments:

Methods

- I would defer to the expertise of other reviewers to appropriately assess the methods of this paper.

Results

"Sera I. Ricinus-exposed birds and…":

- I would encourage the authors to open the first sentence with the purpose of this experiment. For example, "In order to address XXXX, we three tits were monitored at six time points (as opposed to 3). In these birds we found…."

- Similarly for the next paragraph. "Following this, 10 blue tits (including or excluding the 3 before?) were monitored at three time points. These time points corresponded to direction prior to infestation, and thus reflecting the IgY levels following the previous infestation event. In these birds, OD profiles were…."

"IgY correlations with anti tick resistance":

- The opening of the second paragraph could improve. Something like: "In order to

investigate whether XX influenced YY, we utilised a GLMM. Neither age etc…"

- Also, please help the reader a bit more. I'm not sure why "Inf 1" is cross-sectional and "delta Inf3-Inf 1" is longitudinal. Can you explain here what these values mean (especially given Figure 1 is not at all intuitive). So, based on this analysis, what did influence IgY levels? Perhaps add, "In sum, none of the factors we tested influenced IgY levels across either each infestation, or across all three infections".

"*IgY correlations with tick virulence":*

- This section is not well written – its rather written like a list of bullet points rather than a "story" whereby the reader is walked through the analysis with care. For example, adding leader statements like "In order the assess the acute effects, i.e., the variation in Hct levels, XXX YY" are useful.

- Why were these data points deemed outliers – was this a technical problem?

- Why were the IgY levels summed?

RE Body condition:

- Perhaps rephrase to say something like "Body condition…for all birds, with the exception of infestation sessions wherein we found an acute effect (here explain which sessions those were)." I also don't understand why there is reference to chronic effect here?

Paragraph 4:

- Does this mean that you did the analysis twice – once including and once excluding the Birds in Figure 3 (i.e., the birds that were measured 6 times and therefore did not have enough sera remaining to be on all plates)?

Paragraph 5:

- Again, more common language interpretations would be useful here. Why did you use summed values? Why does Hct and Body condition refer to chronic? See comments above about including a leading sentence.

Discussion

Paragraph 2 RE maternal antibodies:

- Are there studies you can reference that have quantified how long maternal antibodies persist in birds. For example, this has been done in ducks for antibodies against influenza A, but I wonder if this has been done for any passerines for any virus/bacteria? This data is useful for future studies such that you may start experiments when birds are older, and you are sure that the effect of maternal antibodies has waned.

Paragraph 3 RE tick resistance:

- Can the authors please provide an example of hosts types that have been shown to acquire resistance to ticks?

- Also, you have a statement: "We conclude that naïve juveniles do not acquire anti-tick resistance". Do adult passerines acquire this resistance? Are you sure you are using the correct measurements and experimental design for this as it is a strong statement to make?

Paragraph 3 RE tolerance.

- Given fitness wasn't really measured and the experimental design limited and controlled for tick infestations, I am not sure how convincing this is. Are there are examples that the authors could reference for animals that tolerate ticks?

My suggestions for Figure improvement:

I would think that a number of the figures that are related could be panelled into a larger figure? For example, Fig 3 and 4 together, perhaps Fig 5, 6, 7 together?

Figure 1:

- Concept figures should be immediately understandable without needing to read the legend, but unfortunately Figure 1 is very confusing. There is plenty of space so I suggestion you write out “Inf. 1” and “D1”. The legend isn’t very clear either. The figure doesn’t really explain well the different between “Acute” and “Chronic”. Overall, I suggest reconsidering this figure – there are lots of great concept figures out there in papers that rely on infection experiments for inspiration.

Figure 2:

- Based on the Figure and the legend, it is unclear to me the purpose of this figure. Perhaps a more descriptive title such as: Correlation between first and second IgY measurement of the same sample/bird”. Can you please add the Statistic onto the figure, and also the line. I expect that you could leverage more colours to make it easier for the reader.

Figure 3:

- Again, I would change the figure legend so that this plot is easier to appreciate. For example: Change in IgY levels of 3 Blue Tits that were sampled on 6 occasions. Given Figure 1 isn't intuitive, I still don’t really understand this plot. Perhaps add some extra metadata below the bars – lines or square brackets to indicate infestation periods, or different colours or arrows? Were the positive and negatives only for Day 1?

Figure 4.:

- I would suggest that this plot be a boxplot or point graph, with day on the x-axis, and the different birds in different colours.

- You could, if you wanted, connect the individuals with lines. You could also then add a line or similar for the mean? Even better, you could model the change of IgY over time? An example of a suggestion: https://stackoverflow.com/questions/59693411/r-boxplot-draw-lines-between-each-subject-in-case-of-repeated-measurements

Figure 5:

- As with Figure 2 – can you add the test statistic and regression or correlation line to this graph? Also, please add the statistic to Figure 7.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Host-pathogen interactions, virology, virus ecology, virus evolution, eco-immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 10 Sep 2021

Dieter Heylen, Institute of Tropical Medicine, Antwerp, Belgium

Dear editors and reviewer, on behalf of all authors, I am glad to re-submit the manuscript 'Ineffective humoral anti-tick IgY-response in birds: reaction against pathogen constituents?'. Songbirds are central elements in the ecological networks of ticks, but are heavily overlooked when it comes to elementary biological mechanisms like immune responses against ticks and tick-borne diseases. No studies so far have related individual variation in songbird adaptive immune responses to components of ectoparasite fitness. Our study therefore can be considered as a primer for future work exploring tick epitopes that can be targeted by bird immune components. In the following sections, we state point-by-point how we have dealt with the comments of the reviewer. Adjustments can be traced by 'track changes' formatting and alterations.

Comments to the reviewer

The authors aimed to understand the immune response of Blue tits to tick infestation using an experimental approach. Overall, this is an interesting paper, but currently some shortcomings in data presentation (both text and writing) make it hard to understand the details and interpret of the findings.

I think a bit more careful explanation is needed for the reader to understand the idea of "tick resistance". Specifically, that it's not the prevention of ticks infesting birds, but rather a decrease in the impact of ticks on birds (eg, lower tick engorgements and perhaps a more effective immune response).
*** We agree with the reviewer we should emphasize that we investigated the processes after the ticks have been placed on the bird's skin. Furthermore, we realize we could have done a better job in introducing the general idea of the study and its design. Therefore, we added more information in the first sentences of the Introduction's last paragraph, where the purpose of the study has been made clear, which reads as follows: "This study focuses on the avian adaptive humoral immune response against ixodid ticks as a potential driver of the among-bird variation in feeding success. Hereto, we follow-up ticks that have been placed on the birds' skin, giving them the maximum opportunity to feed. As the

development of an effective immunological response needs time, we exposed birds three times in succession over a time period of 25 days. Throughout the experiment the birds' physiological health and IgY-antibody response were monitored (see 'Methods' section)".
*** In addition, we screened the text for 'resistance' and found that it has been used once without 'anti-', and a couple of times where it would have been better to place 'immunological' in front of it.

Second, humoral immunity develops following the acute response (inflammation). How do these findings link into studies assessing inflammation following tick infestation? Would this variation in IgY perhaps be due to a variation in acute responses? I recognise that the authors did not measure this, but I wonder if there is data from previous studies?
*** The reviewer put forward an interesting hypothetical explanation that may explain the individual variation among birds in IgY responses. We do not have the required data to test this hypothesis, in that the more reactive birds (in terms of inflammatory response) also elicited the highest IgY responses. We mention that inflammatory reactions by the host can be expected after rupture of the integument (the skin) and exposure to novel epitopes. Remarkably, ticks are one of those ectoparasites that are specialized in maximally reducing those inflammatory responses, making the interpretation of inflammatory reactions a difficult task. Ticks evade the host repertoire of immunopharmacological agonists by counter-producing a number of salivary antagonists (Ribeiro, 1989). We also want to emphasize that ELISA's are designed to detect antibodies that specifically bind to tick-antigens. Given the birds have never been exposed to ticks before, it is expected that proteins belonging to the inflammatory reaction/acute response are driving the observed IgY-patterns. Most of the elements given above are already in the manuscript, but we added a line in the 'Discussion' section: "the observed IgY-response gives evidence for an adaptive response, rather than an acute inflammatory reaction."

Third, as these were field collected ticks, how do the authors control for things like viral infections which may affect Hct or body condition. These viral infections may also affect Engorgement weights and feeding durations of ticks (particularly for viruses infecting the ticks, not necessarily arboviruses which are transmitted to the birds). Theoretically they shouldn't affect the IgY as this is specific. I recognise that the authors did not measure this, but it is worth point out in the discussion.
*** This is an important point, indeed. Therefore, in the last paragraph of the discussion, we added the following sentence "Are anti-tick immune responses, host physiology and tick feeding performance affected by the pathogens themselves and/or other tick associated micro-organisms (including viruses)? Are these responses comparable in different tick-pathogen-host systems? … All these questions are heavily unexplored in birds."

Specific comments:

Results

"Sera I. Ricinus-exposed birds and…": I would encourage the authors to open the first sentence with the purpose of this experiment. For example, "In order to address XXXX, we three tits were monitored at six time points (as opposed to 3). In these birds we found…." Similarly for the next paragraph. "Following this, 10 blue tits (including or excluding the 3 before?) were monitored at

three time points. These time points corresponded to direction prior to infestation, and thus reflecting the IgY levels following the previous infestation event. In these birds, OD profiles were…."
*** We now have re-organized this entire section.

"IgY correlations with anti tick resistance": The opening of the second paragraph could improve. Something like: "In order to investigate whether XX influenced YY, we utilised a GLMM. Neither age etc…" Also, please help the reader a bit more. I'm not sure why "Inf 1" is cross-sectional and "delta Inf3-Inf 1" is longitudinal. Can you explain here what these values mean (especially given Figure 1 is not at all intuitive).
*** The problem with the interpretation of Figure 1 should be alleviated, given the improvements made. (1) Figure 1 provides an extensive explanation of the all response variables. (2) Inf (infestation) and D(ay) are now more often written in full (3) with symbols we refer in the explanatory section to the design figure (response variables). In addition, these symbols are used in 2 other figures (Figure 3 and Figure 6) for a better understanding.

So, based on this analysis, what did influence IgY levels? Perhaps add, "In sum, none of the factors we tested influenced IgY levels across either each infestation, or across all three infections".
*** We added the suggested sentence.

IgY correlations with tick virulence": This section is not well written – its rather written like a list of bullet points rather than a "story" whereby the reader is walked through the analysis with care. For example, adding leader statements like "In order the assess the acute effects, i.e., the variation in Hct levels, XXX YY" are useful.
*** We have rewritten the paragraphs and agree that without repeating important elements described in the 'Methods' section, this part of the 'Results' section is tough to follow.

Why were these data points deemed outliers – was this a technical problem?
*** Normality assumptions were violated. We mention that both outliers belonged to the same bird individuals.

Why were the IgY levels summed?
*** The cumulative IgY response has been defined as a proxy for the total investment in specific IgY-antibodies by the bird throughout the course of the experiment.

RE Body condition: Perhaps rephrase to say something like "Body condition…for all birds, with the exception of infestation sessions wherein we found an acute effect (here explain which sessions those were)." I also don't understand why there is reference to chronic effect here?
*** We integrated the section on body condition with the section in which the model on Hct is described. We thus removed the section the reviewer is referring to. We also removed the reference to chronic.

Paragraph 4: Does this mean that you did the analysis twice – once including and once excluding the Birds in Figure 3 (i.e., the birds that were measured 6 times and therefore did not have

enough sera remaining to be on all plates)?
*** Yes, that is correct. We removed the sentence, to make the paragraph less complicated. The IgY measurements of the 3 birds were well situated within the measurements of the other 10 birds, and the birds have been treated in exactly the same way throughout the experiment (the reason why putting them together with the other 10 birds in a single analysis makes sense).

Paragraph 5: Again, more common language interpretations would be useful here. Why did you use summed values? Why does Hct and Body condition refer to chronic? See comments above about including a leading sentence.
*** We rewrote the sentences for 'Chronic effects'.
*** Now, better explained in Figure 1: "Chronic effects: The health effects of 24 ticks feeding over a time period of 21 days. It is calculated as the difference between the measurements at the moment when birds were still tick-free in Inf. 3 (Day 21) and those at the start of the experiment (i.e. the baseline measurements before the first tick exposure, Day 1)."

Discussion

Paragraph 2 RE maternal antibodies: Are there studies you can reference that have quantified how long maternal antibodies persist in birds. For example, this has been done in ducks for antibodies against influenza A, but I wonder if this has been done for any passerines for any virus/bacteria? This data is useful for future studies such that you may start experiments when birds are older, and you are sure that the effect of maternal antibodies has waned.
*** After delving deeper into the literature, we found an important study by King et al (2010) entitled "Are Maternal Antibodies Really That Important? Patterns in the Immunologic Development of Altricial Passerine House Sparrows (Passer domesticus)", stating that "Based on the short half-life of maternal antibodies, the rapid production of endogenous antibodies by nestlings and the relatively low transfer of maternal antibodies to nestlings, our findings suggest that altricial developing sparrows achieve immunologic independence much earlier than precocial birds". The authors estimated the half-time of maternal anti-bodies in nestling plasma as 2.2 +/-0.25 days; they also estimated the immunologic independence as 8–10 days after hatch. If the blue tit – also an altricial passerine – shows similar physiological patterns, then the IgY levels observed in our study should have been synthesized by the bird itself, as all maternal antibodies should have been cleared from the plasma. We added some extra sentences in the first paragraph of the 'Discussion' section, referring to this study: "However, the immunological processes in the early development of altricial birds (among which blue and great tits) differ from those of semi-precocial birds (to which gulls belong). In an experimental study by King et al. (2010) where House sparrows (Passer domesticus L.) are experimentally exposed to novel antigens, the authors observed short half-life of maternal antibodies (less than 3 days after hatching), low transfer from mother to nestlings, and a rapid production of endogenous antibodies by nestlings (8-10 days after hatching). They concluded that altricial developing birds achieve immunologic independence much earlier than precocial birds, implying that the variation in (initial) IgY levels observed in our study is synthesized by the juvenile bird itself."

Paragraph 3 RE tick resistance: Can the authors please provide an example of hosts types that

have been shown to acquire resistance to ticks?
*** Typically, development of resistance occurs in artificial tick-host interactions, i.e. that are never observed in nature. We added: "Acquired resistance against ticks has frequently been demonstrated in laboratory rather than in natural hosts, therefore it has been suggested that tick resistance is confined to artificial host–tick associations (e.g. guinea pigs infested with *Dermacentor variabilis*, rabbits infested with *I. ricinus*) (Bowessidjaou et al., 1977; Ribeiro, 1989; Wikel 1996) and that successful parasitism in natural host-tick associations is the result of an intense co-evolution, in which ticks developed adaptations to evade the host's immune system (Ribeiro, 1989; Fielden et al., 1992)".

Also, you have a statement: "We conclude that naïve juveniles do not acquire anti-tick resistance". Do adult passerines acquire this resistance? Are you sure you are using the correct measurements and experimental design for this as it is a strong statement to make?
*** We are sure about the use of the correct measurements: tick engorgement weight is considered to be one of the most consistent indicators of the host's immunological anti-tick resistance. More than likely adults do not acquire immunological anti-tick resistance: the many experiments we have executed in our lab with a diverse set of natural host x tick interactions, all resulted in high feeding success, especially with regard to engorgement weights (great and blue tits, as well as blackbird, infested with *I. ricinus*, *I. arboricola* and *I.frontalis*). We mention that most of these experiments took place in different seasons (autumn, winter, early spring) than the current study.
*** We added 'immunonlogical' to the sentence, which reads as follows "We conclude that naïve juveniles do not acquire immunological anti-tick resistance.". We have some indications that adult passerines – given their past experience with ectoparasite exposures – became better in grooming than young birds, lowering the proportion of successfully attached ticks (not the engorgement weights).

Paragraph 3 RE tolerance. Given fitness wasn't really measured and the experimental design limited and controlled for tick infestations, I am not sure how convincing this is. Are there are examples that the authors could reference for animals that tolerate ticks?
*** The concept of tolerance is notoriously difficult to measure in animals. As shown by Raberg and colleagues (cited in the paper), the demonstration of tolerance requires experimentally infecting replicates of different host genotypes with different loads of pathogens and measuring host fitness. Tolerance for each host genotype is then estimated as the slope of how host fitness decreases with pathogen burden. Host genotypes that have a flat slope between host fitness and pathogen load are considered tolerant. All this, is very hard to measure in a single experiment (and to the best of our knowledge, this has never been tested in ticks x vertebrate systems). However, controlled experiments have shown that adult birds with and without tick infestations – with tick burdens close to the upper limit observed in nature – did not differ in breeding success (Heylen et al. 2009). Moreover, birds infested in captivity showed no decrease in body condition compared to control birds, despite the measurable drop in haematocrit. We also note that in the experiment reported here, tick burden did not affect mortality (Heylen et al. 2010). Although additional experiments with varying tick burdens and including long-term survival would be required to fully support the concept of tolerance as it was defined by Raberg and colleagues, we want to leave the following sentence in the manuscript: "Here, high burdens did not cause direct fitness effects (bird mortality) or gave rise to indirect fitness effects via physiological

measure that link up with bird fitness; both findings are in favour of tolerance". But in order to tone down our previous (hypothetical) conclusion, we added a sentence in the last paragraph of the 'Discussion' section: "Does also the bird's long-term fitness remain unaffected by high tick loads, providing further indirect evidence for tolerance (as defined by Råberg and colleagues (2009))?"

My suggestions for Figure improvement:

Figure 1: Concept figures should be immediately understandable without needing to read the legend, but unfortunately Figure 1 is very confusing. There is plenty of space so I suggestion you write out "Inf. 1" and "D1". The legend isn't very clear either. The figure doesn't really explain well the different between "Acute" and "Chronic". Overall, I suggest reconsidering this figure – there are lots of great concept figures out there in papers that rely on infection experiments for inspiration.
*** We have the opinion that without text, it will be very hard to understand the different contrasts (acute, chronic, change in acute effects) that were calculated in the different statistical models. Instead of re-drawing the time-line of the experiment, we now more extensively explained how the contrasts have been defined.

Figure 2: Based on the Figure and the legend, it is unclear to me the purpose of this figure. Perhaps a more descriptive title such as: Correlation between first and second IgY measurement of the same sample/bird". Can you please add the Statistic onto the figure, and also the line. I expect that you could leverage more colours to make it easier for the reader.
*** The purpose of the figure is to show that our assay is characterized by a relatively high repeatability. As far as we know, outcomes from this assay - with salivary gland extracts and songbird serum - have not been published before, the reason why we wanted to have the figure in the manuscript. The correlation coefficient, as a measure of repeatability, is given in the text and now in the figure as well. Symbols and shapes identify each of the groups: control birds, and experimentally infested (both in which several bird species were included). These have been described in detail in the 'Methods' section.
*** We prefer not to add P-values and test statistics in the figures, as several of the statistical analyses were far more advanced than a least-squares regression. Instead, to address the comment of the reviewer, we added a Pearson-correlation coefficient: a descriptive measure of association.

Figure 3: Again, I would change the figure legend so that this plot is easier to appreciate. For example: Change in IgY levels of 3 Blue Tits that were sampled on 6 occasions. Given Figure 1 isn't intuitive, I still don't really understand this plot. Perhaps add some extra metadata below the bars – lines or square brackets to indicate infestation periods, or different colours or arrows? Were the positive and negatives only for Day 1?
*** We extended the legend with "Positive and negative control birds: repeatedly exposed great tit and naïve canary, respectively.", making clear that those additional samples have been included, needed to show the response in completely naïve birds (among which the Canary finches which have never been in the wild, thus never been exposed to ticks) and heavily infested birds (great tits, see 'Methods' section).

Figure 4.: I would suggest that this plot be a boxplot or point graph, with day on the x-axis, and

the different birds in different colours.
*** A boxplot has been generated, including a line that connects the means of the IgY-measurements based on the ELISA's with *I. ricinus* antigens. The sera used for cross-reaction (read: IgY-measurements based on the ELISA's with *I. arboricola* antigens) has been placed in its chronological position, though not connected with the other box plots, given it depicts a different reaction from the others. *** We mention that in the 'Discussion' section (as well as in the 'Results' section), we referred to initial values of certain bird individuals for which the IgY levels were relatively high. In order to provide the reader with a graphical overview of the raw data, we kept the previous Figure 4, but placed it in the 'Supplementary materials.'

Figure 5: As with Figure 2 – can you add the test statistic and regression or correlation line to this graph? Also, please add the statistic to Figure 7.
*** Correlation coefficients have now been added, as well as a least squares regression line.

On behalf of all authors, Sincerely Yours, Dieter Heylen

Competing Interests: No competing interests were disclosed.