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Lack of resistance against the tick *Ixodes ricinus* in two related passerine bird species

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ABSTRACT

Although many wild bird species may act as reservoir hosts for tick-transmitted diseases and/or support long-distance dispersal of infected ticks, to date no research has been done on the extent to which songbirds may acquire resistance to ixodid ticks. Here we investigate whether two passerine species belonging to the family Paridae, the blue tit (*Cyanistes caeruleus*) and the great tit (*Parus major*), are able to acquire resistance after repeated infestations with *Ixodes ricinus* nymphs. As blue tits are less frequently exposed to *I. ricinus* in the wild than great tits, we expected *I. ricinus* to be less adapted towards the blue tit's resistance mechanisms. Over the three infestation sessions we observed consistently high tick attachment rates and yields, high engorgement weights, and short engorgement and moulting durations, indicating that neither of the two songbird species is able to mount effective immune responses against *I. ricinus* nymphs after repeated infestations. As a consequence of the lack of resistance, birds were unable to prevent the direct harm (acute blood depletion) caused by tick feeding. Birds compensated the erythrocyte loss without reduction in general body condition (body mass corrected for tarsus length). The lack of resistance suggests that *I. ricinus* has a long co-evolutionary history with both avian hosts, which enables the tick to avoid or suppress the host's resistance responses.

1. Introduction

Few studies have focussed on the interactions between ixodid ticks and avian hosts. Although many wild bird species may act as reservoir hosts for tick-transmitted diseases and support long-distance dispersal of ticks (Anderson et al., 1986; Olsen et al., 1993, 1995; Comstedt et al., 2006; Larsson et al., 2007), to date little information is available on the extent to which wild birds may acquire resistance to ticks. Population dynamics of hosts, vectors and their pathogens strongly depend on the ultimate outcomes of vector–host interactions (Anderson and May, 1978, 1979; Randolph et al., 2002). Therefore, in order to gain insights into the epidemiology of tick-transmitted diseases in the wild, it is essential to obtain knowledge of the hosts' innate and acquired resistance to the ticks to which they are naturally exposed (Randolph, 1979; Randolph and Nuttall, 1994; Nunn et al., 2006).

On a global scale, ticks and tick-transmitted diseases cause huge annual economic losses due to their negative impact on public health and livestock (Lehmann, 1993; Wikel, 1996; Zhang et al., 2006). During the last few decades there has been an increasing interest in the immunology of the host–tick interface and host resistance in particular. Since the work of Trager (1939), who reported that Guinea pigs were able to mount a very efficient immune response to the tick *Dermacentor variabilis*, many studies have reported on acquired resistance to ticks, especially in domestic mammals. Resistance to an ectoparasite reflects the ability of the host to recognise the antigens of the ectoparasite and to protect itself against harm from the parasite (Wakelin, 1996; Wikel, 1996). If the tick's counter-measures to the host immune system are not effective, tick feeding and salivation will be hindered. Consequently premature detachments and hence reductions in engorgement weights occur, which result in reduced tick survival or offspring numbers. Ticks that remain attached to resistant hosts feed very slowly or not at all and many die in situ (Rechav, 1992; Hillyard, 1996; Wikel, 1996).

The ability to acquire immunity in response to tick bites varies strongly amongst host species. It is assumed that in longer-established parasitic relationships, ticks are more likely to successfully evade or suppress the host's rejection mechanisms (Ribeiro, 1989; Sonenshine, 1993). Few studies have investigated the physiological responses developed by natural hosts against ixodid ticks (e.g. Randolph, 1979; Fielden et al., 1992; Dutoit et al., 1994; Hughes and Randolph, 2001). This is surprising, since processes at the tick–host interface may influence the biology of ticks, as well as pathogen transmission (Randolph, 1979; Wikel, 1980; Fivaz et al., 1989; Randolph and Nuttall, 1994; Wikel et al., 1997). To our knowledge, the occurrence of resistance in natural tick-songbird systems has not been examined to date.

The aim of the work described in this paper was to investigate whether two passerine birds belonging to the family Paridae – the blue tit (*Cyanistes caeruleus*) and the great tit (*Parus major*) – are able

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to acquire resistance after repeated infestations in controlled conditions with *Ixodes ricinus* nymphs. *Ixodes ricinus* is a widespread tick in Europe, where it has been recognised as a key vector of numerous human and/or animal pathogens, including bacteria (e.g. *Borrelia burgdorferi* sensu lato, *Rickettsia* spp.), viruses (e.g. tick-borne encephalitis virus) and protozoans (e.g. *Babesia* sp.) (Gray, 1991; Jongejan and Uilenberg, 2004). It is a non-nidicolous tick with a broad range of vertebrate hosts and has a limited vertical distribution inside understory vegetation (Lees, 1948; Mejlon and Jaenson, 1997; Gray, 1998). During questing, the subadult developmental stages (larvae and nymphs) of *I. ricinus* frequently infest *C. caeruleus* and *P. major* individuals of different European populations (prevalence: 6–58%) (Humair et al., 1993; Olsen et al., 1995; Hubalek et al., 1996; Comstedt et al., 2006; Kipp et al., 2006). *Cyanistes caeruleus* (mean prevalence ± standard error (SE): 7.8 ± 2.2%) is less often infested than *P. major* (33.1 ± 9.4%) (Humair et al., 1993; Olsen et al., 1995; Hubalek et al., 1996; Comstedt et al., 2006; Kipp et al., 2006), which may be because *C. caeruleus* is less inclined than *P. major* to forage at low heights, inside the habitat of *I. ricinus* (Hartley, 1953; Betts, 1955; Gosler, 1987). However, interspecific differences in tick prevalence may not only be explained by differences in foraging behaviour (Comstedt et al., 2006) but also by interspecific variation in the birds' ability to acquire resistance.

If resistance is acquired we expected reduced feeding success by the ticks over successive infestations, as well as a reduction in blood depletion of the host. Furthermore, we wanted to investigate to what extent other health parameters of the host (erythrocyte sedimentation rate and general body condition) were negatively affected by successive tick infestations. Due to the natural differences in tick exposure, we hypothesised that the co-evolution had been less intense between *I. ricinus and C. caeruleus* than *P. major*. Consequently, we predicted poorer adaptations in the *C. caeruleus–I. ricinus* association, characterized by a stronger reduction in feeding success of ticks with successive infestations, and therefore more reduced impacts on *C. caeruleus*' health parameters.

2. Materials and methods

2.1. Birds

Cyanistes caeruleus and *P. major* are small passerine birds (body mass 7–15 and 15–20 g, respectively) which commonly breed in woodlands and gardens of the western and eastern Palearctic. Nest boxes are readily accepted as surrogates for tree-holes, in which they naturally breed and roost (Cramp and Perrins, 1993; Gosler, 1993). *Parus major* may act as a competent reservoir for the tick-borne pathogen *B. burgdorferi* sensu lato (Humair et al., 1993; Olsen et al., 1995; Comstedt et al., 2006). Because resistance outcomes can be influenced by cross-reactivity of humoral and cell-mediated immunity as a result of infestations with other blood sucking ectoparasite species (Denhollander and Allen, 1986; HellerHaupt et al., 1996; Krasnov et al., 2005; Gallizzi et al., 2008), we worked with ectoparasite-naïve birds. These were obtained by collecting nestlings from parasite-free boxes in the wild and introducing them with their parents into ectoparasite-free aviaries. Nestlings were collected from woodlots in a long-term study population in northern Belgium (see Matthysen et al., 2001 for details). Nests in this population are frequently infested with other ectoparasites such as *Ceratophyllus gallinae*, *Protocalliphora* sp. larvae, *Dermanyssus gallinae* and *Ixodes arboricola* (personal observations). During the month preceding the breeding season (March), selected nest boxes were sprayed twice with permethrin (2.2 g L^{-1}). Permethrin has a low avian and mammalian toxicity (Elliott, 1977). In addition, after hatching of the chicks, nests were microwaved three times at 4-day intervals. In order to increase genetic variation within the families transferred to the aviaries, two groups of four nestlings per *C. caeruleus* nest, and three to four nestlings per nest in *P. major* were switched with two to three other nests that were also kept free of ectoparasites, at 8 days of nestling age. A few days before fledging, nestlings and parents of four selected nests of *C. caerulues*, and five nests of *P. major* were transferred into outdoor aviaries located at the University of Antwerp (Belgium), where parents continued to feed the nestlings until independence. In total 31 *C. caeruleus* individuals and 28 naïve *P. major* individuals from 10 different broods of each bird species participated in the experiment. Birds received food and water ad libitum during their stay in captivity, and birds had the opportunity to take a bath in fresh water. After experiments were finished, birds were re-introduced into the wild. During the experiment, one and five individuals died, respectively, in the *C. caeruleus* and *P. major* groups. Ringing procedures and experiments were carried out in accordance with national environmental legislation and university regulations. Wild birds were introduced from the wild into aviaries with a licence from the Agency for Nature and Forests (Flemish Government, Belgium). The infestation procedure (see below) was approved by the Ethics Committee for Animal Experiments of the University of Antwerp (Belgium).

2.2. Study design

When birds were 9 weeks old, *C. caeruleus* and *P. major* individuals were infested with *I. ricinus* nymphs three times in succession. Each infestation lasted 4–5 days, and birds were kept free of ticks for a duration of 5–6 days between the consecutive infestations. To be able to control for a time and/or age effect, unrelated to the effect of previous infestations, we divided the birds into three treatment groups whereby the infestations of the second and third treatment group started, respectively, 10 days and 20 days later than the first treatment group (Fig. 1). Each treatment group was stratified according to nests of origin.

In one of the woodlots of the study population, *I. ricinus* nymphs were caught by dragging a white flannel flag over suitable vegetation. After capture and identification (Hillyard, 1996) *I. ricinus* nymphs were kept under sterile conditions in a climate room at >90% relative humidity and 16:8 h (light:dark photoperiod; 25:15 °C temperature cycle) until infestation. As the degree of acquired resistance has been reported to increase with high tick challenges in laboratory and natural hosts (Randolph, 1979, 1994; Hughes and Randolph, 2001), we infested the birds with tick loads around the maximum level found under natural conditions in our study population. Using moistened tweezers, 12 nymphs for *C. caeruleus* and 17 nymphs for *P. major* were put underneath the feathers on the head of each bird in each infestation session. Immediately afterwards birds were kept for 2 h in an air-permeable cotton bag (sized: 20 × 15 cm) inside a darkened cage which kept them inactive (Heylen and Matthysen, 2008). In order to check for escaped ticks, bags were suspended over water by a rope covered with adhesive tape. No ticks escaped from the bags. Inspection of the bags revealed that some of the questing nymphs were bitten by the birds and consequently had died. Other nymphs became stuck in bird excretions, while a number did not attach for unknown reasons. After tick exposure, birds were placed in individual cages with a wire-mesh floor (40 × 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 at 7 a.m. and 7 p.m. each day with minimal disturbance to the host. Some nymphs were presumably lost because they could not be found amongst the faeces or food remains beneath the wire-mesh or they may have been eaten by the hosts before dropping out of reach. Each nymph was rinsed with purified water, blotted on dry, clean filter paper and weighed individually on an electronic microbalance to the nearest 0.01 mg.

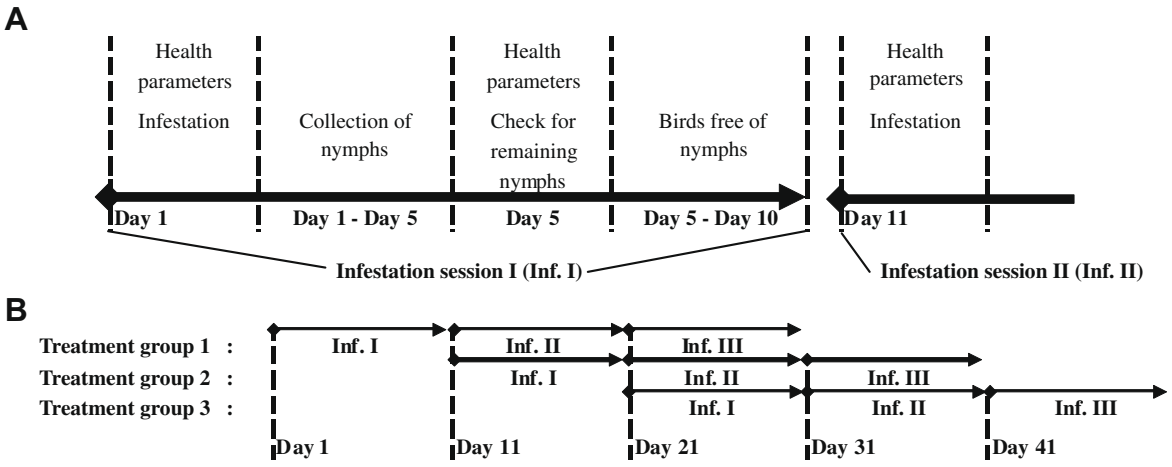

Fig. 1. Schematic overview of the study design. (A) Actions taken during an infestation session. (B) Schedule of successive infestation sessions for each treatment group.

For *P. major*, engorged nymphs were kept in individual tubes at 25 °C and >90% relative humidity (Christe et al., 1998) until moult to the adult stage was completed. Moulted nymphs were sexed and the duration of the pre-moult period was determined (regular checks every 2 days). Successfully engorged nymphs that did not complete their moult were sexed by executing a discriminant analysis based on their weight (see below). For *C. caeruleus* only some (approximately one-half, due to losses from an accidental chemical contamination) of the collected ticks were kept in individual climatised conditions as described above and sexed after moulting. No pre-moult period was determined.

To investigate the influence of repeated infestations on feeding success of *I. ricinus*, we estimated the following parameters: (i) the proportion of attached ticks, (ii) the proportion of the administered ticks that successfully engorged (tick yield), (iii) the weights of engorged male and female nymphs and (iv) their feeding duration (to the nearest 12 h). For *P. major* we additionally registered (v) the proportion of ticks that failed to moult to the adult stage, and (vi) the duration of the pre-moult period. 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 failure of ticks to complete their development. However, the durations of feeding may either increase or decrease (see Section 1) when resistance is acquired. From these criteria, engorgement weight is considered to be one of the most consistent indicators of resistance (Varma et al., 1990; Rechav, 1992).

In addition we measured $\Delta_{after-before}$ (the differences of measurements after infestation minus measurements before infestation, see Fig. 1A) in three parameters reflecting the hosts' health: haematocrit level (Hct), erythrocyte sedimentation rate (ESR) and body condition (mass/tarsus ratio). Immediately before and 5 days after infestation, body mass was measured to the nearest 0.1 g using a digital balance and blood samples were taken (maximum 65 μL) from the ulnar vein into 75 μL heparinized capillary tubes. We used the ratio between body mass and a skeletal measurement (tarsus length, measured with a digital caliper to the nearest 0.01 mm) as a measure of body condition (e.g. Gorney et al., 1999; Yom-Tov, 2001). The ESR is a diagnostic method based on the fact that the movement of erythrocytes through plasma is enhanced by increased levels of major acute-phase proteins and immunoglobulins. High values signify an impaired health status (Sharma et al., 1984; Saadeh, 1998). To measure ESR, heparinized capillary tubes containing blood samples were placed vertically for 4 h at 4 °C. The level of ESR was measured by calculating the ratio between the volume of the part of a capillary tube not occupied by red blood cells and the total volume of blood in the capillary tube. After measuring the ESR, blood samples were centrifuged at 14,000*g* for 10 min to measure the Hct. Acute or chronic anaemia, as indicated by low Hct, results in a reduced oxygen-carrying capacity of the blood and consequently restricts oxygen-demanding processes (Dein, 1986). ESR and Hct were measured with a digital calliper to the nearest 0.01 mm under optimal light conditions.

2.3. Statistical analysis

General linear mixed effects models (GLMM) and generalised estimation equations (GEE) were fitted to test hypotheses on response variables, taking into account the correlation structure of the repeated measurements from the same individual (for methodological details see: Verbeke and Molenberghs, 2001; Molenberghs and Verbeke, 2005).

For each resistance measure we modelled a linear trend with tick infestation order. In each model we controlled for potential differences in the initial response values at the start of treatment and we tested – besides the overall change – whether the changes in responses differed amongst the three treatment groups. Pairwise differences in initial response values were tested by means of post hoc tests. If responses showed a monotonic trend both in initial treatment-group values and within-treatment group changes, this could be due to a time effect rather than an effect of resistance. For response variables showing such patterns we did a second analysis in which we modelled a linear date effect and tested whether the residual variation could still be explained by the successive infestations. A formal method to detect multicollinearity between independent variables (sequence of infestations and date) indicated minor problems for the interpretation and inference of parameter estimates in these models (variance inflation factor = 1.9) (Neter et al., 1996). In the analysis of health parameters of the birds, an additional time factor (two levels: 'before' and 'after' infestation) was integrated into the models.

To control for sex differences of nymphs, successfully engorged nymphs of unknown gender (animals that failed to moult or that were followed up only until drop-off for *C. caeruleus*) were assigned to the most likely gender by the use of cut-off values for engorgement weights based on Fisher's linear discriminant functions (Sharma, 1996). For *C. caeruleus* and *P. major*, the cut-off value above which ticks were considered as females, respectively, equalled 4.34 and 4.33 mg with a misclassification error of 1.5% for *C. caeruleus* (based on 444 ticks with known gender) and 1.7% for *P. major* (based on 857 ticks with known gender). Analogous low error counts have been observed in field-collected nymphs engorged on BALB/c

Table 1
Feeding parameters of *Ixodes ricinus* nymphs fed on *Cyanistes caeruleus* individuals with successive infestations.

	Infestation I 12 Ticks/bird (n = 31)	Infestation II 12 Ticks/bird (n = 31)	Infestation III 12 Ticks/bird (n = 31)[a]
% of attachment	93 ± 1 (346/372)	94 ± 1 (351/372)	92 ± 2 (344/372)
Feeding period (days)			
Female	3.63 ± 0.05 (187)	3.62 ± 0.06 (152)	3.72 ± 0.07 (173)
Male	3.31 ± 0.07 (137)	3.38 ± 0.07 (138)	3.46 ± 0.08 (118)
% Tick yield	86 ± 2 (324/372)	75 ± 4 (290/372)	78 ± 3 (291/372)[b]
Engorged weight (mg)			
Female	5.21 ± 0.04 (187)	5.34 ± 0.06 (152)	5.27 ± 0.05 (173)
Male	3.19 ± 0.04 (137)	3.31 ± 0.04 (138)	3.28 ± 0.03 (118)

n, number of birds.
Data are presented as means ± standard error. The total numbers of ticks are shown in parentheses.
[a] One bird died after ticks had dropped off.
[b] Significance levels after Bonferroni adjustment of pair-wise comparisons with Wilcoxon signed-rank test: $P = 0.52$ between infestations I and III.

mice (Dusbabek, 1996). A similar approach in which successfully engorged nymphs of unknown sex are dichotomized into 'males' and 'females' to go on with further analyses has been used in other studies (e.g. Dusbabek et al., 1994; Ogden et al., 2002).

Since a decrease in infestation and development success of ticks is expected when resistance is acquired, null-hypotheses were tested one-sided ($\alpha = 0.05$). However, feeding durations of the nymphs could either increase or decrease (see Section 1), hence the null-hypotheses for this parameter were tested two-sided. With regard to the health parameters, the alternative hypothesis assumes ticks are harmful (Heylen and Matthysen, 2008), so here null-hypotheses were tested one-sided ($\alpha = 0.05$) as well. Since multiple hypotheses were tested on the same individuals, all P-values were adjusted by applying Bonferroni correction, in order to avoid statistical significance that might occur by chance. All data manipulations and statistical analyses were performed using SAS v9.1 (SAS Institute, Cary, North Carolina, USA).

3. Results

3.1. Resistance measures

Marginal means (collapsed over the individual birds of the three treatment groups) with standard errors of the feeding and development parameters with infestation order are presented for *C. caeruleus* (Table 1) and *P. major* (Table 2). Marginal frequency distributions of the engorgement weights and marginal views of the profiles of the health parameters are presented in Figs. 2 and 3. Parameter estimates of the models that analyse the change in feeding and development parameters with infestation order in relation to treatment groups are presented in Table 3.

Attachment success on *C. caeruleus* did not change with infestation order, and no significant differences were found between treatment groups. On the other hand, for *P. major* the attachment success significantly decreased with infestation order ($F_{1,53} = 13.26$; $P = 0.002$). In addition, success rates in the first infestations decreased monotonically over treatment groups ($F_{2,24.3} = 6.84$; $P = 0.004$; Table 3). An additional analysis in which we controlled for the date effect (estimate: $-4.6 \pm 1.3\%$/10 days; $F_{1,26.4} = 14.15$; $P < 0.0001$) showed no residual trend in success rate with infestation order ($-0.6 \pm 2.0\%$/session; $F_{1,70.7} = 0.17$; $P = 0.68$). Thus, the decrease in success rate can be attributed to a date effect rather than resistance.

The overall tick feeding duration on *C. caeruleus* did not increase with infestation order ($F_{1,875} = 3.75$; $P = 0.16$), but we found significant differences amongst treatment groups: feeding durations in the first infestations increased monotonically over treatment groups, and in the second treatment group there was a significant increase with infestation order ($F_{1,873} = 3.96$; $P < 0.0001$; Table 3).

Table 2
Feeding and development parameters of *Ixodes ricinus* nymphs fed on *Parus major* individuals with successive infestations.

	Infestation I 17 Ticks/bird (n = 28)[a]	Infestation II 17 Ticks/bird (n = 26)[b]	Infestation III 17 Ticks/bird (n = 25)[c]
% of attachment	93 ± 2 (444/476)	87 ± 2 (384/442)	82 ± 3 (349/425)[e]
Feeding period (days)			
Female	3.47 ± 0.04 (194)	3.66 ± 0.05 (175)	3.75 ± 0.05 (158)[e]
Male	3.20 ± 0.06 (167)	3.27 ± 0.04 (144)	3.47 ± 0.07 (104)[d]
% Tick yield	78 ± 4 (361/459)	72 ± 5 (319/442)	67 ± 4 (262/391)
Engorged weight (mg)			
Female	5.24 ± 0.05 (194)	5.29 ± 0.04 (175)	5.30 ± 0.05 (158)
Male	3.17 ± 0.03 (167)	3.19 ± 0.03 (144)	3.31 ± 0.03 (104)
Time to moult (days)			
Female	32.5 ± 0.3 (181)	32.0 ± 0.3 (151)	32.3 ± 0.3 (130)
Male	29.9 ± 0.4 (148)	29.4 ± 0.3 (130)	31.7 ± 0.6 (88)[f]
% of succeful moult	91 ± 2 (329/361)	86 ± 2 (281/319)	82 ± 4 (218/262)

n, number of birds.
Data are presented as mean ± standard error. The total numbers of ticks are shown in parentheses.
[a] One bird died while infested, one bird died after ticks had dropped off.
[b] One bird died after ticks had dropped off.
[c] Two birds died while infested.
Significance levels after Bonferroni adjustment of pair-wise comparisons with Wilcoxon signed-rank test:
[d] $P < 0.05$ between infestations I and III.
[e] $P < 0.01$ between infestations I and III.
[f] $P < 0.05$ between infestations II and III.

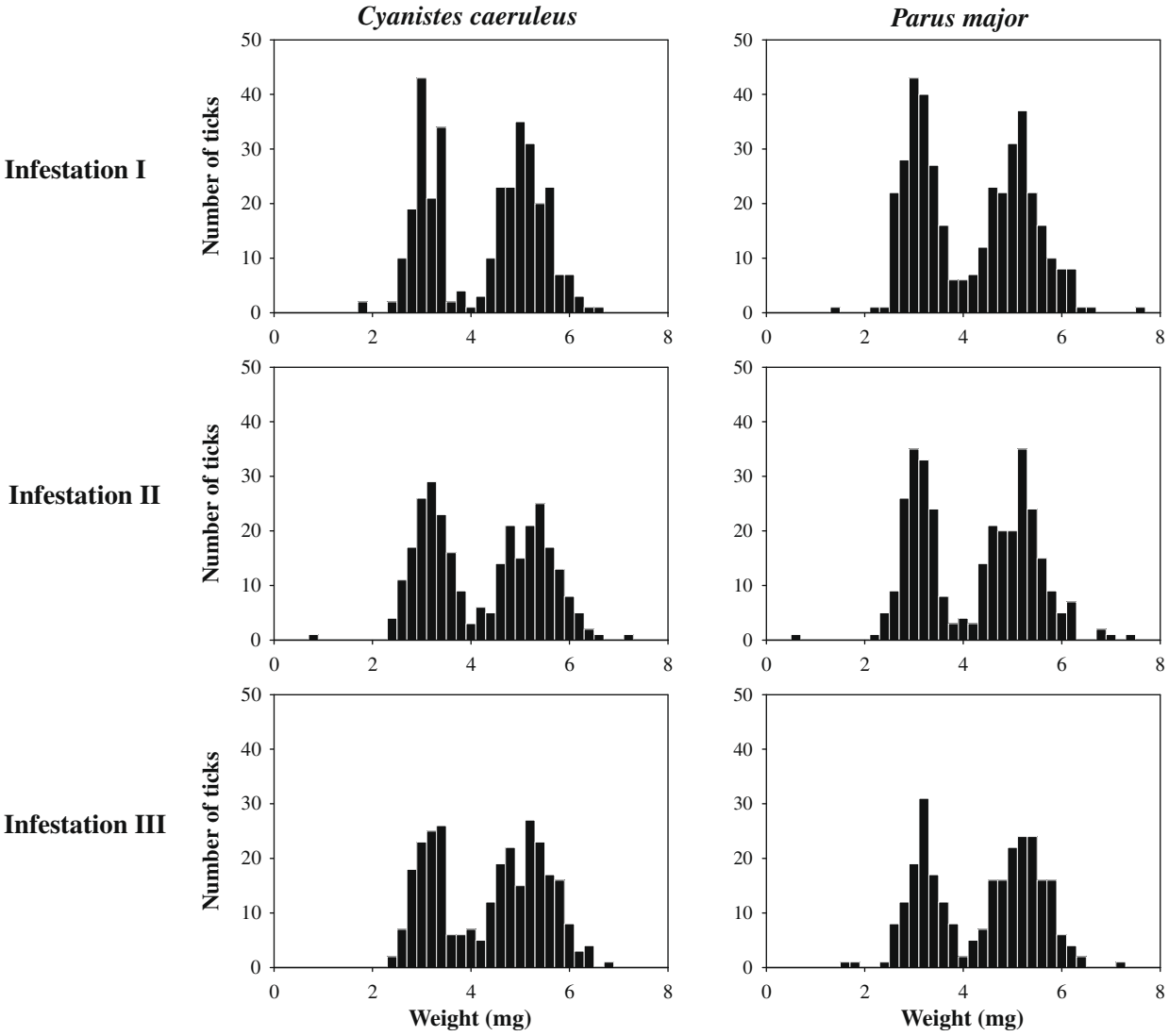

Fig. 2. Frequency distribution of *Ixodes ricinus* nymph engorgement weights during each of three successive feed applications on *Cyanistes caeruleus* and *Parus major*.

For *P. major* we found a highly significant overall increase in feeding duration with infestation order ($F_{1,935} = 39.98$; $P < 0.001$). When testing for the interaction between change in feeding duration and treatment group, we found that in the first treatment group the increase was higher ($T_{933} = 3.18$; $P = 0.0015$) than the other two treatment groups. A significant monotonic increase was also present at the start of the first infestation (Table 3). After correction for a linear date effect, no evidence remained for an increase in feeding duration with infestation order ($F_{1,61.3} = 2.34$; $P = 0.13$). Feeding durations in pre-male nymphs were significantly shorter than in pre-female nymphs (difference for *C. caeruleus* and for *P. major*: 0.29 ± 0.04 days; all $P < 0.0001$). There was no significant interaction between the gender of the tick and the change in feeding duration with infestation order. For both *C. caeruleus* and *P. major*, less than 1% of the observed ticks did not drop off within 5 days after attachment.

Tick yield decreased with infestation order for *C. caeruleus* ($F_{1,61} = 5.59$; $P = 0.043$), but remained high throughout the experiment (Table 1). In this model, there was also a suggestion of a possible time effect, since the yields in the first two treatment groups were higher compared to the third treatment group ($T_{28.5} > 3.08$; $P < 0.012$; Table 3). When we controlled for the date effect (estimate: −6.3 ± 2.3%/10 days; $F_{1,29.4} = 7.64$; $P = 0.009$), no significant association with infestation order remained (1.8 ± 2.8%/session; $F_{1,60.6} = 0.43$; $P = 0.52$) hence this should again be interpreted with care. For *P. major* tick yield did not change with infestation order. Tick yields in the first infestations also decreased monotonically over treatment groups (Table 3). For *C. caeruleus* as well as *P. major*, all recovered nymphs were the normal grey/dark grey colour.

Engorgement weights did not decrease with infestation order (Fig. 2). There was even a slight increase for ticks fed on both *C. caeruleus* ($F_{1,875} = 3.81$; $P = 0.05$) and *P. major* ($F_{1,930} = 4.25$; $P = 0.04$). Engorged pre-male nymphs had a lower body mass than pre-female nymphs (estimated difference in *C. caeruleus*: 2.02 ± 0.03 mg; in *P. major*: 2.05 ± 0.03; all $P < 0.0001$, see bimodal distributions shown in Fig. 2). There was no difference amongst treatment groups in either the initial engorgement weights or the trend with infestation order. No significant interaction between the change in engorgement weight with infestation order and tick gender was found. For *C. caeruleus* as well as *P. major* very few under-weighted and partially engorged ticks were recovered.

The time to moult (data available for *P. major* only) did not change with infestation order, and slight differences amongst the treatment groups were found in the estimates of the first infestations (see Table 3). Males moulted faster to the imago stage than females (estimated difference: 0.29 ± 0.04 days; $T_{805} = 4.32$; $P < 0.0001$). The proportion of ticks that successfully developed to the imago stage (data available for *P. major* only) almost significantly decreased

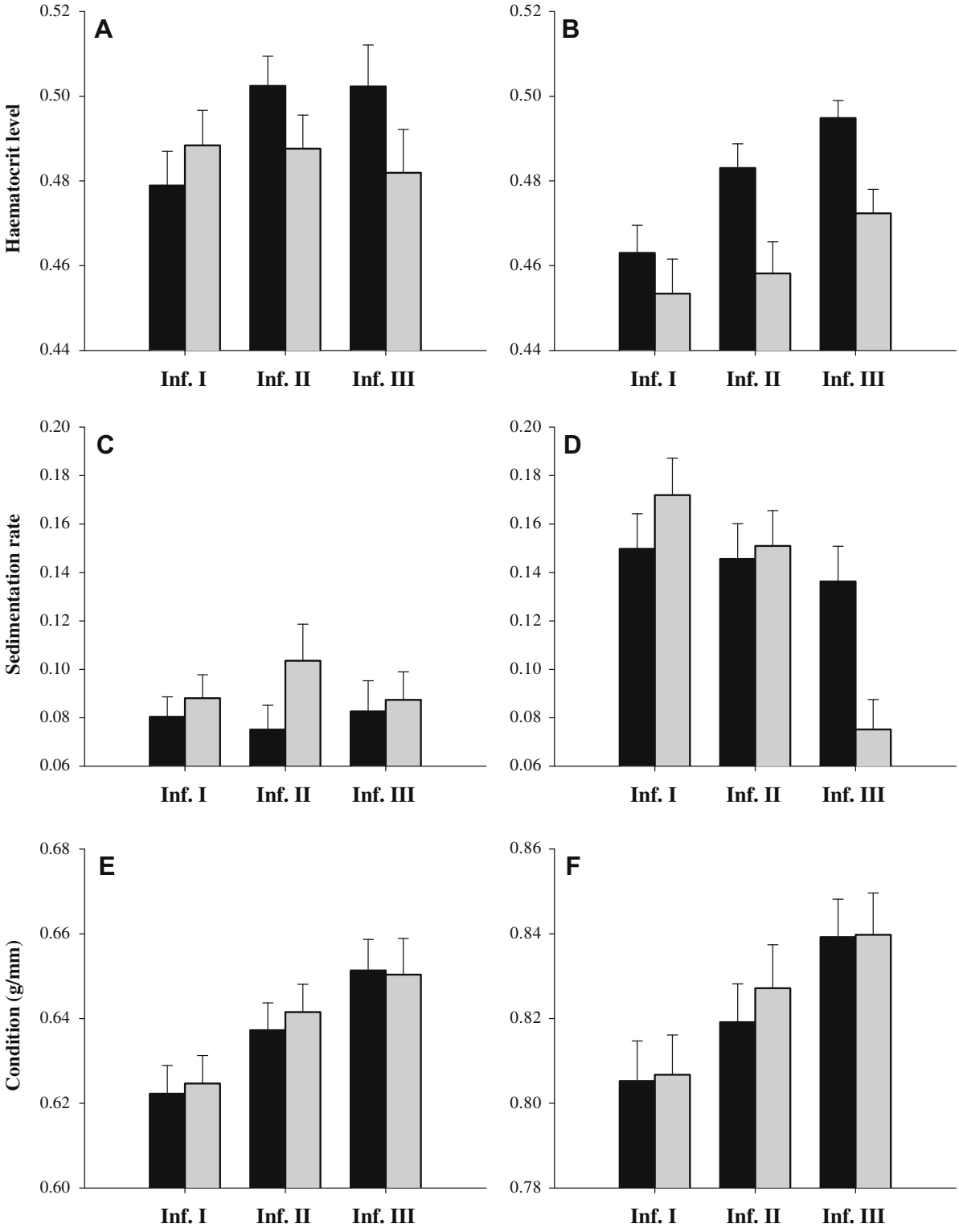

Fig. 3. Mean haematocrit level, relative sedimentation rate and body condition (±1 standard error) of *Cyanistes caeruleus* (A,C,E) and *Parus major* (B,D,F) immediately before (black bars) and after (grey bars) each of three successive feed applications (Infestation (Inf.) I, II and III) of *Ixodes ricinus* nymphs.

with infestation order ($F_{1,44.6} = 6.01$; $P = 0.054$), and did not differ between treatment groups (see Table 3).

3.2. Health measures

For *C. caeruleus* the decrease in Hct levels after tick infestations became greater with consecutive infestations (Fig. 3A; main effect of $\Delta_{after-before}$: $-2.3 \pm 1.5\%$; $F_{1,145} = 2.41$; $P = 0.18$; interaction $\Delta_{after-before}$ with infestation order: $-1.7 \pm 7\%$/session; $F_{1,146} = 5.37$; $P = 0.03$). Note however, that during the first infestation there was no significant decrease in Hct levels for *C. caeruleus* ($\Delta_{after-before}$: $0.9 \pm 1.0\%$; $T_{143} = 0.97$; $P = 0.33$, Fig. 3A), although all feeding parameters of the ticks show that infestation was successful (see above). Mean Hct increased significantly with infestation

Table 3
Change in feeding and development parameters of *Ixodes ricinus* nymphs fed on *Cyanistes caeruleus* and *Parus major* with successive infestations in relation to three treatment groups whereby the infestations of the second and third treatment groups started, respectively, 10 days and 20 days later than the first treatment group. If no differences between treatment groups were found, only the overall estimate is given.

	Cyanistes caeruleus		*Parus major*	
	Infestation I	Change	Infestation I	Change
% Of attachment				
Overall	93.4 ± 1.1	−0.3 ± 0.8[f]		−5.2 ± 1.5[d,g]
TG 1	N.D.	N.D.	97.5 ± 2.6[b]	N.D.
2	N.D.	N.D.	94.5 ± 2.7[b]	N.D.
3	N.D.	N.D.	87.8 ± 2.3[a]	N.D.
Feeding period (days)				
Overall		0.05 ± 0.02[f]		0.14 ± 0.02[e,g]
TG 1	3.37 ± 0.07[a]	0.03 ± 0.06[a,f]	3.25 ± 0.05[a]	0.23 ± 0.04[b,c]
2	3.38 ± 0.07[a]	0.18 ± 0.06[b,e]	3.33 ± 0.04[b]	0.08 ± 0.04[a,c]
3	3.66 ± 0.07[b]	−0.02 ± 0.04[a,f]	3.42 ± 0.05[b]	0.11 ± 0.04[a,c]
% Tick yield				
Overall		−4.4 ± 1.7[c,g]		−4.4 ± 4.7[f]
TG 1	90.6 ± 4.4[b]	N.D.	85.7 ± 5.8[b]	N.D.
2	90.8 ± 4.4[b]	N.D.	78.3 ± 6.1[a]	N.D.
3	78.2 ± 3.5[a]	N.D.	71.8 ± 4.7[a]	N.D.
Engorged weight (mg)				
Overall	4.23 ± 0.03	0.04 ± 0.02[f]	4.22 ± 0.03	0.05 ± 0.02[f]
Time to moult (days)				
Overall	–	–		0.23 ± 0.17[f]
TG 1	–	–	31.27 ± 0.25[b]	N.D.
2	–	–	30.41 ± 0.32[a]	N.D.
3	–	–	31.35 ± 0.53[b]	N.D.
% Of succeful moult				
Overall	–	–	91.2 ± 2.2	−5.8 ± 2.1[f]

TG, treatment group; N.D., no statistically significant differences amongst treatment groups.
[a,b] Values with the same postscript letter do not differ significantly ($P > 0.05$). Significance levels for testing whether 'change' differs from zero.
[c] $P < 0.05$.
[d] $P < 0.01$.
[e] $P < 0.001$.
[f] Not significant.
[g] Not significant after correction for date (see main text for details). Significance levels of overall change are Bonferroni adjusted.

order (main effect: 1.3 ± 0.5%/session; $F_{1,146} = 6.1$; $P = 0.015$), and the treatment groups for *C. caeruleus* did not differ in their mean Hct level at the first infestation. For *P. major* Hct levels significantly decreased during each infestation ($\Delta_{after-before}$: −1.9 ± 0.4%; $F_{1,121} = 26.49$; $P < 0.0001$; see Fig. 3B). The average Hct increased with infestation order (1.0 ± 0.2%/session; $F_{1,122} = 19.79$; $P < 0.0001$). However, changes in Hct within sessions were independent of infestation order. On average the values of Hct levels in the first treatment group (45.1 ± 0.7%) were significantly lower than the mean of the pooled values of the last two treatment groups which did not differ from each other (estimated difference: 2.9 ± 0.9%; $T_{22.3} = -3.02$; $P = 0.006$).

For *C. caeruleus* ESR tended to increase during each infestation (main effect of $\Delta_{after-before}$: 1.4 ± 0.8%; $F_{1,146} = 3.61$; $P = 0.062$). There was no interaction with infestation order ($F_{1,146} = 0.29$; $P = 0.59$) and there were no differences found amongst the treatment groups. For *P. major* the increase in ESR due to tick infestations became weaker and in the last infestation session ESR tended to decrease (interaction $\Delta_{after-before}$ ESR with infestation order: −4.1 ± 1.4%/session; $F_{1,116} = 8.13$; $P = 0.005$; Fig. 3D). The estimated mean difference ($\Delta_{after-before}$) during each of the infestation sessions equalled 7.1 ± 3.1% ($F_{1,116} = 5.46$; $P = 0.03$) and the overall change with infestation order equalled −0.7 ± 1.0%/session ($F_{1,119} = 0.44$; $P = 0.51$). Furthermore, there was a monotonic decrease across the treatment groups, in which only the ESR of the final treatment group (11.8 ± 0.8%), was significantly lower than the mean of the pooled values of the other two treatment groups which did not differ from each other (estimated difference: 3.3 ± 1.1%; $T_{17.5} = -3.11$; $P = 0.006$).

The general body condition of both bird species did not change during tick infestations, but there was a highly significant improvement in body condition with infestation order (0.019 ± 0.002 g/mm/session; for *C. caeruleus*: $F_{1,149} = 81.48$; $P < 0.0001$; for *P. major*: $F_{1,123} = 95.07$; $P < 0.0001$; Fig. 3E and F). For both species the general body condition in the first infestation increased monotonically over the treatment groups.

4. Discussion

The present study demonstrates that two closely related passerine birds do not acquire resistance to *I. ricinus* nymphs after repeated infestations. High tick attachment rates, yields and engorgement weights, and short engorgement and moulting durations indicate that neither of the two songbird species is able to mount effective immune responses against attached *I. ricinus* nymphs after repeated infestations. Compared with BALB/c-mice, which are known to be incapable of developing resistance (Dusbabek et al., 1994, 1995; Dusbabek, 1996; Mbow et al., 1994; Christe et al., 1998), average feeding durations were consistently lower (range BALB/c mice: females 3.81–5.75 days; males 3.56–5.14 days), and average engorgement weights were higher (females: 4.08–5.07 mg; males: 2.53–3.13 mg). Tick yields lay within the BALB/c mice range (61–98%), but care should be taken in interpretation, since in the mouse studies ticks were released and followed up inside plastic capsules glued to the skin. As a consequence of the lack of resistance, the birds in our study were unable to overcome the direct harm (acute blood depletion) caused by tick feeding (Heylen and Matthysen, 2008) after repeated infestations. It should be emphasised that in our experiment, birds were infested with relatively high numbers of ticks, but within the range of natural conditions. From fledging onwards (mid-May until mid-June) birds may become exposed to high levels of the subadult developmental stages of *I. ricinus* (Gray, 1991), which makes the experimental data representative for situations in the wild.

However, in line with the prediction of acquisition of resistance, attachment rates and feeding durations of ticks, respectively, decreased and increased for *P. major*, and the engorgement success for *C. caeruleus* decreased, although the overall effect was weak. Here, the presence of time-dependent processes that are not related to the tick infestations may have influenced our observations. For *P. major* individuals, the quality of body and crown feathers became visibly impaired during their stay in the cages (personal observations), which could have reduced the attachment rate of the ticks. On the other hand, the increase in feeding duration could be the consequence of age-dependent changes in physiological parameters of the host, such as the observed increase in Hct levels, which has also been observed in *P. major* individuals in the wild (unpublished data) and in other songbirds (Fair et al., 2007). Increased Hct levels raise blood viscosity (Birchard, 1997), and hence could have decreased the ticks' feeding efficiency. In support of this we found a significant association between feeding durations and the Hct levels immediately before each infestation session for *P. major* (Pearson's $\rho = 0.3$ in both male and female nymphs, $P < 0.03$) but not for *C. caeruleus*. Whatever the reason may be for the increase in feeding durations, visual inspection of birds at the end of each infestation session confirmed that virtually all ticks dropped off within 5 days after attachment. Since ticks are expected to remain attached and feed very slowly or die in situ on tick-resistant hosts (Hillyard, 1996; Wakelin, 1996; Wikel, 1996), our observations do not correspond to the acquisition of resistance. Furthermore, tick engorgement weights even tend to increase with

infestation order in both bird species, clearly indicating that no resistance was acquired (Varma et al., 1990; Rechav, 1992). Similar outcomes that indicate an enhancement of tick feeding were observed in experiments with other incompetent hosts (Fielden et al., 1992; Mbow et al., 1994; Dusbabek et al., 1995).

Little knowledge is available on resistance in natural bird – ixodid tick systems. Experimental infestations of Guinea fowl with immature *Amblyomma marmoreum* and *Amblyomma hebraeum* (Fielden et al., 1992) – common natural non-nidicolous ticks of Guinea fowl in Africa (Horak et al., 1991) – similarly failed to elicit acquired resistance. In guillemots (*Uria aalge*) observational data suggests that birds do not become immune to the nidicolous tick *Ixodes uriae*, since more nymphs were found on old than young guillemots, and female tick weight was not related to host age during the nymphal stage (Nunn et al., 2006). In another colonial seabird, the black-legged kittiwake (*Rissa tridactyla*), there is some evidence that *I. uriae*'s feeding success may differ according to the genetic origin of the gulls (McCoy et al., 2002), since ticks had shorter engorgement times on sympatric nestlings than allopatric nestlings (although infestation levels were similar between the two types of nestlings). An earlier report on this seabird suggests that susceptibility to *I. uriae* is heritable, since tick loads in the nestling stage are positively correlated between parents and offspring, while controlling for various environmental factors (Boulinier et al., 1997). Although the latter two reports provide evidence for genetic variation in the degree of resistance against *I. uriae*, no experimental data was obtained that could distinguish the innate component from the acquired (adaptive) component in the measures of resistance. To our knowledge, there is no information on resistance in passerines against ticks. However, there are some reports that songbirds may have the capacity to acquire resistance against other types of ectoparasites, as shown e.g. in *P. major* nestlings exposed to hen fleas (*Cerathophyllus gallinae*) (Walker et al., 2003) and barn swallow nestlings (*Hirunda rustica*) exposed to tropical fowl mites (*Ornithonyssus bursa*) (Moller, 2000).

Acquired resistance against ticks has frequently been demonstrated in laboratory rather than in natural hosts (Randolph, 1979; Fielden et al., 1992; Dutoit et al., 1994). Therefore, it has been suggested that tick resistance is confined to artificial host–tick associations (Ribeiro, 1989) 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). However, the discovery that voles (*Clethrionomys glareolus*), but not mice (*Apodemus* spp.) have the capacity to develop resistance to larvae of *I. ricinus* and *Ixodes trianguliceps* (Randolph, 1979, 1994; Dizij and Kurtenbach, 1995) revealed that some natural hosts may develop efficient resistance against ticks. Interestingly, the degree of acquired resistance in voles depends on the level of exposure to *I. trianguliceps* larvae (Randolph, 1994) since tick feeding success and survival is higher when infestation intensity is low (Randolph, 1994), as if a critical threshold level should be exceeded to elicit an effective immunological response (Dineen, 1963). Similar findings were observed in laboratory mice that were able to acquire resistance (Randolph, 1979). Although we kept tick loads in the present study high throughout the experiment, we did not find evidence for acquired resistance in the two natural avian hosts. Therefore we assume our data represents the outcome of a presumed long-established relationship with *I. ricinus*, in which ticks have evolved mechanisms to suppress or evade immune reactions of both bird species. As a consequence of the lack of resistance, birds were unable to overcome the direct harm (acute blood depletion) caused by tick feeding. Still, this harm was limited and birds quickly compensated for the erythrocyte loss without reduction in their general body condition (body mass corrected for tarsus length). The levels of inflammation at the end of each infestation session (ESR) stayed low in *C. caeruleus* or decreased in *P. major* with successive infestations. Those results can be viewed as mechanisms of tolerance rather than resistance (Sprent, 1962; Randolph, 1979), with tolerance being defined as mechanisms reducing the impact of the parasite on the host rather than affecting parasite growth or survival (Raberg et al., 2009).

The health impact of natural tick intensities for *C. caeruleus* (mean intensity: 1.2 ± 0.3 ticks per infested bird) and *P. major* (2.2 ± 0.4 ticks per infested bird) (Humair et al., 1993; Olsen et al., 1995; Hubalek et al., 1996; Comstedt et al., 2006; Kipp et al., 2006) is presumably very low compared with the impact of the high tick burdens in the present study (Heylen and Matthysen, 2008). Therefore we hypothesise that natural *I. ricinus* infestations may have limited fitness consequences in nature, and hence there may be low selective pressure for the birds to evolve effective resistance mechanisms. In addition, a more economical defence strategy against ticks in songbirds may be to develop behavioural avoidance mechanisms (Hart, 1990, 1997; Moore, 2002) as observed in seabirds (King et al., 1977; Duffy, 1983; Mangin et al., 2003) which may take precedence over selection towards physiological resistance mechanisms.

The present results contribute to our understanding of the occurrence of non-nidicolous ticks on songbirds in the wild. Since *C. caeruleus* and *P. major* showed a similar lack of resistance to *I. ricinus* nymphs, the observed difference in infestation in nature is presumably the result of different contact rates with ticks due to differences in foraging behaviour (Comstedt et al., 2006), rather than physiological resistance. *Cyanistes caeruleus* and *P. major* are model organisms in evolutionary ecology and population biology research, and further studies on their interactions with ticks may offer opportunities to understand the role of common resident birds in the population dynamics of the ticks and their role in the eco-epidemiology of tick-borne diseases.

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