

Ectoparasitism during an avian disease outbreak: An experiment with *Mycoplasma*-infected house finches and ticks

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ABSTRACT

Hosts are typically co-parasitized by multiple species. Parasites can benefit or suffer from the presence of other parasites, which can reduce or increase the overall virulence due to competition or facilitation. Outcomes of new multi-parasite systems are seldom predictable. In 1994 the bacterium *Mycoplasma gallisepticum* jumped from poultry to songbirds in which it caused an epidemic throughout North America. Songbirds are often parasitized by hard ticks, and can act as reservoirs for tick-borne pathogens. We tested the hypothesis that *Mycoplasma* infection in house finches influences North America's most important tick vector *Ixodes scapularis*, by affecting the tick's feeding success, detachment behaviour and survival to the next stage. Most ticks detached during the daylight hours irrespective of the bird's disease status and time since infestation. Birds incrementally invested in anti-tick resistance mechanisms over the course of the experiment; this investment was made earlier in the *Mycoplasma*-infected birds. At higher tick densities, the feeding success on birds with more severe conjunctivitis was lower than in the uninfected birds. Throughout the experiment we found positive density dependent effects on the tick's feeding success. More diseased hosts suffered more from the tick infestations, as shown by reduced haematocrits. Three *Mycoplasma*-infected birds died during the weeks following the experiment, although all birds were kept in optimal housing conditions. *Mycoplasma* made the bird a less accessible and valuable host for ticks, which is an example of ecological interference. Therefore, *Mycoplasma* has the potential to ultimately reduce transmission outcomes of tick-borne pathogens via songbird hosts.

1. Introduction

Significant numbers of parasitic organisms that cause disease in humans have an origin in wildlife, including bird hosts. Because birds can move over large distances they can act as long-range carriers for parasites, including vector-borne diseases to humans, thereby establishing new pathogen transmission foci far away from existing ones (Chen et al., 2005; Hubálek, 2004; Lanciotti et al., 1999; Ogden et al., 2008). Emergent infectious diseases can increase in prevalence over vast ranges in short periods of time, thereby having detrimental effects on their previously unaffected host populations (Chen et al., 2005; Lühken et al., 2017). How these emergent diseases affect the transmission dynamics of established diseases that have been thriving in

wildlife populations for centuries remains poorly studied. Invasive parasites can alter the ecology of native pathogens across different scales (i.e. from host individual to population) ultimately affecting R_0 (the basic reproductive number) of established micro-parasites. Effects of co-infections on co-occurring parasites varies: infectious agents can benefit or suffer from the presence of another parasite in the same host while some co-infections seem not to affect any of the parasites present (Schmid-Hempel, 2011).

Around 1994 a host shift of the poultry bacterium *Mycoplasma gallisepticum* (further: '*Mycoplasma*') to wild American songbirds (Fischer et al., 1997) caused a major epidemic of mycoplasmal conjunctivitis, particularly in the house finch *Haemorrhous mexicanus* (P. L. Statius Müller, 1776) (Dhondt et al., 1998; Ley et al., 1997). In eastern

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USA the disease caused a decrease of house finch abundance by more than half (Hochachka and Dhondt, 2000). The epidemic has now spread across most of the USA (Dhondt et al., 2006) and is found in a variety of songbird species (Dhondt et al., 2013, 2014, 2015) that suffer in different degrees depending on the host species (Dhondt et al., 2008, 2014) and *Mycoplasma* strain involved (Hawley et al., 2013).

Birds are often infested by ixodid ticks: non-permanent ectoparasites, that are characterized by their low intrinsic mobility, by several days of non-stop feeding on the avian hosts, and by long time-spans off-host. Ticks are vectors for multiple micro-parasites that include the bacterium complex *Borrelia burgdorferi* s.l. causing Lyme disease in humans (Ginsberg et al., 2005). Several ixodid tick species can be found on the same bird species (Heylen et al., 2014, 2017a; Loss et al., 2016; Norte et al., 2015). Birds actively disperse attached ticks and the pathogens they carry. Even when birds are not infected, they can function as vehicles for ticks that have been infected before by other hosts (Heylen et al., 2014, Heylen et al., 2017). While *Mycoplasma* and ticks share songbird species as hosts, the bacterium is not tick-borne.

Most of the research on pathogen–ectoparasite interactions within the same host has focused on micro-parasites and their vectors. Many vector-borne parasites have been shown to alter phenotypic traits of their arthropod vectors and hosts in a way that increases the probability of parasite transmission, including vector feeding behaviour, survival and immune system, as well as the host's attractiveness, defensive behaviour, blood characteristics and immune system (Lefèvre and Thomas, 2008). Experimental studies on co-occurring pathogens and their non-vector ectoparasites are rare, but important for our understanding of parasite community associations and transmission dynamics. Best-known examples originate from fish, where bacterial infections together with a skin ectoparasite increase host mortality by an increased susceptibility to other harmful bacteria (Bandilla et al., 2006; Kotob et al., 2016). Co-occurrence of heterologous parasites in individuals are the rule rather than the exception, and fitness outcomes for parasites and hosts are not necessarily additive. Only experiments can disentangle the complex interactions involved in multi-species parasitism interactions.

In this study we experimentally investigated whether, following its host shift to wild birds, *Mycoplasma* has the potential to affect the population dynamics of ticks that parasitize songbird hosts, by addressing the following two question: **(1) Does *Mycoplasma* infection influence a tick's timing of detachment?** This timing of detachment, that could be influenced by the bird's activity, is important for the tick's survival, spatial distribution, and hence its exposure to a subsequent host. We therefore infested bird hosts that were or were not infected with *Mycoplasma* in the morning or in the evening to determine if ticks detached after a fixed time interval or preferentially during the day or the night; **(2) Does a *Mycoplasma* infection have an effect on tick feeding success?** Hosts can either resist or tolerate the ticks. In house finches *Mycoplasma* causes conjunctivitis and thus causes disease in the body regions preferred by ticks (i.e. eye region and head) (Fracasso et al., 2019) where both parasites can interact. Combined acute effects of ticks and *Mycoplasma* could lead to the bird's death, reducing future tick feeding chances. Furthermore, parasite competition and facilitation could influence tick attachment, engorgement and moulting success (Heylen et al., 2010). In addition, transmission rates of potential tick-borne pathogens could be affected by the presence of *Mycoplasma*, and eventually affects the ecological outcomes for tick and host.

2. Materials and Methods

2.1. Host, pathogen and ectoparasite

In our experiments we compared the interaction between house finch host (exposed or not to the pathogen *Mycoplasma*) and the parasitic black-legged tick *Ixodes scapularis*. This generalist multi-host non-nidicolous ectoparasite is North America's most abundant and

important vector for tick-borne diseases in humans and domestic animals (Gray, 1998). The two most common tick species found on American songbirds - including house finches - are *Ixodes scapularis* and *Haemaphysalis leporispalustris* (Packard, 1869) (Brinkerhoff et al., 2018; Loss et al., 2016; Peters, 1999). Millions of tick individuals feed every year on North American songbirds, underscoring the birds' importance in maintenance of the tick population and their pathogen cycles (Brinkerhoff et al., 2018; Ginsberg et al., 2005; Loss et al., 2016; Richter et al., 2000). Typically, immature *I. scapularis* developmental stages (larvae and nymphs) are found on birds that frequently forage on the ground, and on small to medium-sized mammals. As larvae will develop into nymphs (i.e. the developmental stage to which humans are most often exposed) factors that affect outcomes of this stage's survival and behaviour are of utmost relevance for human epidemiology of tick-borne diseases (Gray, 1998).

2.2. Experimental design for the study of the timing of tick detachment behaviour (Exp. a and Exp. b)

Timing of detachment was studied by performing an experiment according to a randomized, 2 × 2 factorial design, with factors 'Mycoplasma infection' (Yes, No) and 'moment of infestation' ('dawn': 7:30 a.m., or 'dusk': 7:30 p.m.). The combination of the two factors led to four experimental groups: birds were infested either at dawn or at dusk, and inoculated either with *Mycoplasma* or with buffer only. Three hypotheses will be tested: **(H1)** duration until detachment is equal for morning- and evening infested birds. This means that the time to tick detachment is pre-programmed and independent of the birds' circadian activity or disease status. We therefore expect that equal numbers of ticks detach during the day (between 7:30 a.m. and 7:30 p.m.) and the night (from 7:30 p.m. to 7:30 a.m.) and independently of bird disease status. **(H2)** all ticks, no matter when they are placed on a bird, detach during the daylight hours, and disease status has no effect on this robust pattern. We expect that detachments is triggered by minimal changes in bird activity levels between night and day; therefore tick detachment behaviour from diseased birds and uninfected birds does not differ. **(H3)** ticks in morning- and evening-infested birds all detach during the daylight hours in healthy birds only, while in the diseased birds the detachments are spread over day and night. If tick detachment is triggered by the amount of bird activity which would represent daylight hours, and given that diseased birds become much less active, soon after inoculation during both day and night (Dhondt et al., 2007; Kollias et al., 2004), detachment is predicted to be more variable and spread over day and night, especially during the later stage of the disease (Exp. b; see Fig. 1).

To test these hypotheses, birds were infested with pathogen-free ticks at two separate occasions after the *Mycoplasma* inoculation: early in the disease cycle (Day 0–6 post-inoculation 'PI'; Exp. a) when birds develop fever and start to decrease in activity, and late (Day 11–17 PI; Exp. b) when high conjunctivitis scores and minimal activity scores are measured (Hawley et al., 2012). Each bird was infested with 25 larvae and 10 nymphs, numbers within the range of natural conditions in North American songbirds, but likely outside the natural range for House Finches (Brinkerhoff et al., 2018; Loss et al., 2016). Especially ground foragers in forested areas are disproportionately likely to have high tick intensities (Loss et al., 2016). While house finches are often found foraging on the ground, they prefer more open and urban areas, places that are less preferred by *I. scapularis*.

2.3. Experimental design to study effects of tick density (Exp. c)

In order to investigate effects of the late disease phase (Day 20–26 PI) on feeding success of larvae - i.e. the instar most sensitive to immune reactions of the host - in a final infestation session (Exp. c) birds were exposed again. This time, tick loads varied between 25 and 250 larvae, to investigate whether high levels of co-feeding would increase

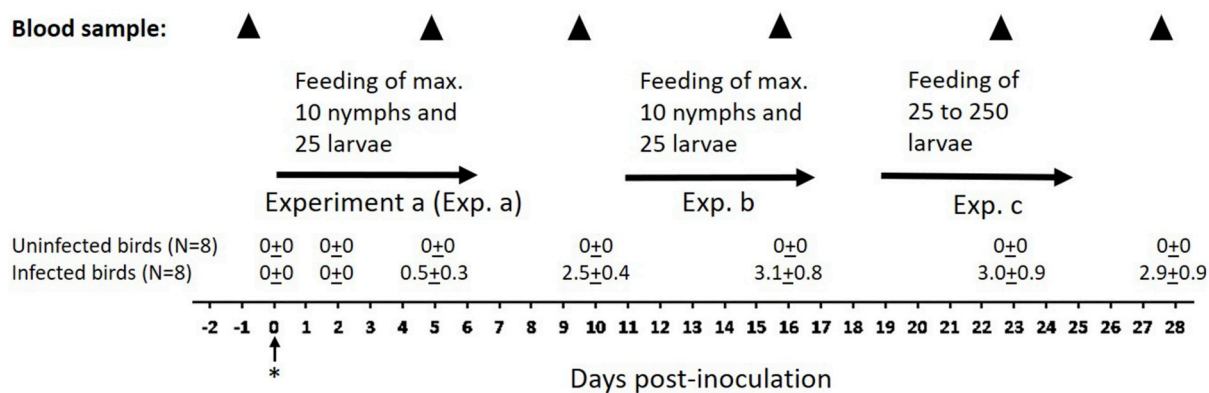


Fig. 1. Schematic overview of the study design, with actions performed during the tick infestation sessions. At day 0 (*) the birds of the experimental group were inoculated with *Mycoplasma gallisepticum*, while the uninfected birds received a sham-treatment (buffer only). At each time point, average eyescores \pm standard errors are presented for both uninfected and infected birds. The start of a horizontal arrow indicates the day of tick exposure, the end indicates the day when the last ticks were collected from the plastic boxes underneath the cages.

or decrease tick success under the influence of the co-parasite *Mycoplasma*. For both infected and uninfected birds, two individuals each received 25, 50, 100, or 250 larvae. Tick loads higher than 50 are considered to be rare in North American songbirds (Loss et al., 2016), and have – as far as we know – never been observed in House finches. Due to a human mistake (tubes were not perforated, such that air flow was hindered and humidity too low for the larvae that were in it) one bird received ticks of which at least half were of poor condition and/or dead. Tick outcomes from this bird were excluded from the analyses of Exp. c.

2.4. Experimental *Mycoplasma* infection and tick infestations (Exp. a – c)

All experiments were approved by Cornell University's IACUC protocol 2009-0034. In early Spring of 2019, 16 house finches were captured in Ithaca (Tompkins County, New York (42° 46' N, 76° 45' W)) with mist nets under permit (New York State Fish and Wildlife License 39, Albany, NY; United States Geological Survey, Department of the Interior, Laurel, MD, permit 22669). Birds were kept in individual wire mesh cages (Height: 45, Width: 45, Length: 75 cm) until April 2019 when the experiment started. This is the period of the year when *I. scapularis* become active and start questing (Ogden et al., 2018). The cages were placed inside a BSL2 lab facility. In all cages, the arrangement of perches, water and food containers was identical. Water and food (Roudybush, Inc. Cameron Park, CA) were offered *ad libitum*. Before starting the experiment, all birds were negative for *M. gallisepticum* by two methods: visual inspection for eye lesions (Sydenstricker et al., 2006) and Realtime Polymerase Chain Reaction (qPCR) designed to test for the presence of the bacteria using the DNA from conjunctival swabs (Grodio et al., 2008). During the entire duration of the experiments, birds were maintained with a photophase beginning at 7:30 a.m. and a scotophase beginning at 7:30 p.m.

After a habituation period of at least 4 days, eight randomly selected birds were inoculated with *Mycoplasma* using a micropipette (further: 'infected birds') while the others were inoculated with Frey's medium using a micropipette (further: 'uninfected birds'). Each bird in the infected group was inoculated on 4 April 2019 (Day 0) with 50 μ L of the *M. gallisepticum* isolate CA2015.022–3(2P) at 2.8×10^7 CFU/ml in each eye. The birds in the uninfected group were similarly inoculated with Frey's medium only.

Twelve hours later, birds were artificially infested with *I. scapularis* at either 7:30 a.m. or 7:30 p.m. (see 'moment of infestation'). Using a paintbrush, ticks were put underneath the feathers on the head of the bird. Immediately afterwards each bird was kept for 1 h in an air-permeable darkened 20×15 cm cotton bag which kept them inactive in the darkened inside of bag (Heylen and Matthyssen, 2008), after

which they were replaced in their cage. All ticks that did not attach and remained in the bag were counted, to obtain the attachment success (i.e. % of ticks that stayed on the bird after 1 h in the cotton bag calculated as: $100 \times [\text{number of ticks placed on birds} - \text{ticks in bag}] / \text{number of ticks placed on birds}$).

2.5. Monitoring of tick detachments and feeding success (Exp. a – c)

A removable deep plastic box with damp (water) filter paper and whose edges were covered with vaseline was placed underneath each cage to prevent ticks that had released from the host from escaping. The engorged ticks of Exp. a and Exp. b that dropped into the plastic box through the mesh floor were collected twice a day for seven days: the morning check started at 6:45 a.m., the evening check around 6:45 p.m. Boxes were removed, and checked in good light conditions. The boxes that needed to be checked were temporarily replaced by a second set of boxes, and replaced 45 min later. Checking the 16 plastic boxes took on average 45 min. The action of changing the boxes upon entering the room took only 5 min so that the birds that were kept in the dark experienced almost no disturbance. Larvae of Exp. c were sampled on 2 occasions (4 and 6 days after tick exposure) and no further attention was given to their timing of release. Some immature ticks were presumably lost because they could not be found amongst the faeces and food remains beneath the wire-mesh or because the host ate them before they were able to detach. Each collected tick was rinsed with purified water, blotted on dry filter paper, and weighed individually on an electronic microbalance to the nearest 0.001 mg. Engorged individuals were kept individually in tubes at 95% relative humidity at (16 h:8 h light:dark photoperiod, 25 °C:15 °C temperature cycle) until moult to the next development stage was completed.

2.6. Monitoring disease signs and physiology following infection (Exp. a – c)

To measure the host response to *Mycoplasma* infection and tick infestation, eye lesions of both eyes were scored in all birds on a scale for each eye of 0 (no lesions) to 3 (severe lesions) following Sydenstricker et al. (2006) on days PI given in Fig. 1. In seven out of eight birds, *Mycoplasma* inoculation led to conjunctivitis (range eyescores of both eyes combined: 1–6) and all uninfected birds had eyescores equal to zero. Blood samples were taken (maximum 65 μ L) from the ulnar vein into 75 μ L heparinized capillary tubes (see Fig. 1 for sampling days) in order to measure haematocrit levels (Hct). They were centrifuged for 5 min at 11,000 g to calculate the ratio between the volume of the part of a capillary tube occupied by red blood cells and the total volume of blood in the tube. These volumes were measured with digital callipers to the nearest 0.01 mm under optimal light conditions. 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). For comparison, we added Hct values of twenty-four birds that were not infested with ticks, but were exposed to poultry *M. gallicpticum* strains concurrently with our infected birds. No formal statistical tests were executed on these additional birds, as they were not sham-treated.

2.7. Statistical analysis

Generalized estimation equations (GEE) were fitted (logit-link, and binomial distributed residuals) when modelling the proportion of ticks that detached, taking into account the statistical dependence of measurements on the same bird. For normal distributed variables (Hct, tick weights) the identity-link was used. The proportion of engorged ticks that diurnally detached was modelled against the moment of infestation, the tick stage and their interaction. We used methods of survival analysis (time-to-event data) for modelling the duration until tick detachment (Cox and Oakes, 1984). The duration until detachment (in days) was modelled by a marginal cox proportional hazards model for clustered data (Shu and Klein, 1999) with tick stage and the moment of infestation added into the model. Data on feeding duration is represented by Kaplan-Meier curves. All data manipulations and statistical analyses were done in SAS v 9.3 (SAS Institute, Cary, North Carolina, USA).

3. Results

3.1. Tick detachment behaviour in relation to *Mycoplasma* infection (Exp. a and Exp. b)

The vast majority of larvae (range over treatment groups: $89 \pm 4\%$ - $100 \pm 0\%$) and nymphs ($72 \pm 24\%$ - $100 \pm 0\%$) detached during the day, and infected and uninfected birds did not differ in the extent of diurnal detachment (for all experiments and stages: P 's > 0.05 ; Fig. 2). This conclusion did not change when using eye scores as a proxy for disease status. Additional observations: Larvae placed at dusk were on average significantly less likely to detach during the day, although the effect size was small ($\text{logit}_{\text{dusk-dawn}} = -1.51 \pm 0.37$; $Z = -4.04$; $P < 0.001$). For the nymphs, no significant contrasts were observed in relation to the moment of infestation. Nymphs were slightly less likely to detach during the day than the larvae placed at dawn ($\text{logit}_{\text{larva-nymph}} = 1.60 \pm 0.43$; $Z = 3.75$; $P < 0.001$).

Following inferences could be made from the Kaplan-Meier curves (Fig. 3): In larvae, in the early stage of the *Mycoplasma* infection (Exp. a), there was no difference between infected and uninfected birds as regards time to detachment; in the late stage of the infection (Exp. b) duration until detachment in uninfected birds (3.46 ± 0.03 days) was significantly shorter than in the infected birds (3.66 ± 0.05 days; hazard ratio: 2.29; $\chi^2 = 13.76$; $df = 1$; $P < 0.0002$), although the difference was small.

Additional observations: In Exp. a (although not in Exp. b) ticks placed at dawn (4.54 ± 0.02 days) detached sooner than those at dusk (4.81 ± 0.03 days; hazard ratio: 8.8; 95%-CI: 5.3–14.7; $\chi^2 = 71.62$; $df = 1$; $P < 0.0001$; Fig. 3A). Unexpectedly, the larvae of Exp. b (3.56 ± 0.03 days) released on average sooner than those of Exp. a (4.68 ± 0.02 days; hazard ratio: 89.9; 95%-CI: 49.9–161.9; $\chi^2 = 224.6$; $df = 1$; $P < 0.0001$) and the between-bird individual variation in feeding durations doubled (variance in Exp. a: 0.09 ± 0.02 ; Exp. b: 0.18 ± 0.03 ; Wilcoxon test: $P = 0.019$). Furthermore, this variation in durations was considerably lower in the uninfected birds exposed at dawn (variance Exp. a: 0; Exp. b: 0.023 days) compared to the other birds (range variances Exp. a: 0.07–0.15 days; Exp. b: 0.19–0.25 days; Wilcoxon test: P 's < 0.008).

In nymphs, as regards duration until detachment, we did not find differences between infected and uninfected birds in either Exp. a or b

(All P 's > 0.05). Additional observations: nymphs fed a shorter time in Exp. b (4.39 ± 0.09 days) than Exp. a (5.11 ± 0.05 days; hazard ratio: 5.8; 95%-CI: 3.7–9.2; $\chi^2 = 55.5$; $df = 1$; $P < 0.0001$). In this comparison the within-bird individual variation did not differ between the experiments. Conclusions of the above models did not change when including the eye scores as a proxy for *Mycoplasma* infection and/or adding the gender of the nymphs to the survival analyses.

Taken together: We can reject H1 and H3 (see Materials and Methods): ticks strive for diurnal detachments and likely base their strategy on minimal changes between night and day (H2). The negative effects of *Mycoplasma* on bird activity levels are not sufficient to disturb this robust behaviour. Although almost all larvae detached during the daylight hours, infection and moment of exposure had an unexpected effect on the variation in larval detachments.

3.2. Infestation success and development to next stage (Exp. a - c)

In Exp. a and b, there was no difference between infected and uninfected birds on overall infestation success (i.e. the percentage of ticks placed on the birds that successfully reached the next developmental stage after feeding) (all P 's > 0.05 ; Table 1). After decomposing the infestation process into the separate events until moult (i.e. attachment, engorgement, detachment and moult), similarly no effect of *Mycoplasma* was found in any of those steps (all P 's > 0.05). Using eye scores as a proxy for disease status did not change the conclusions.

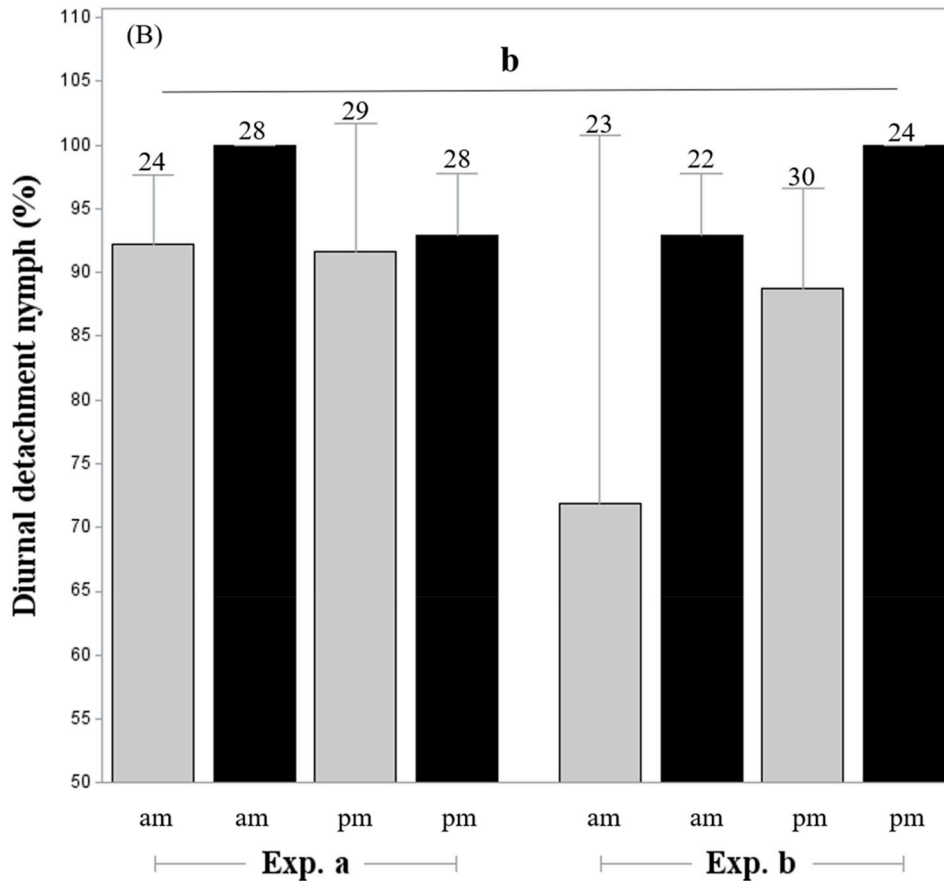
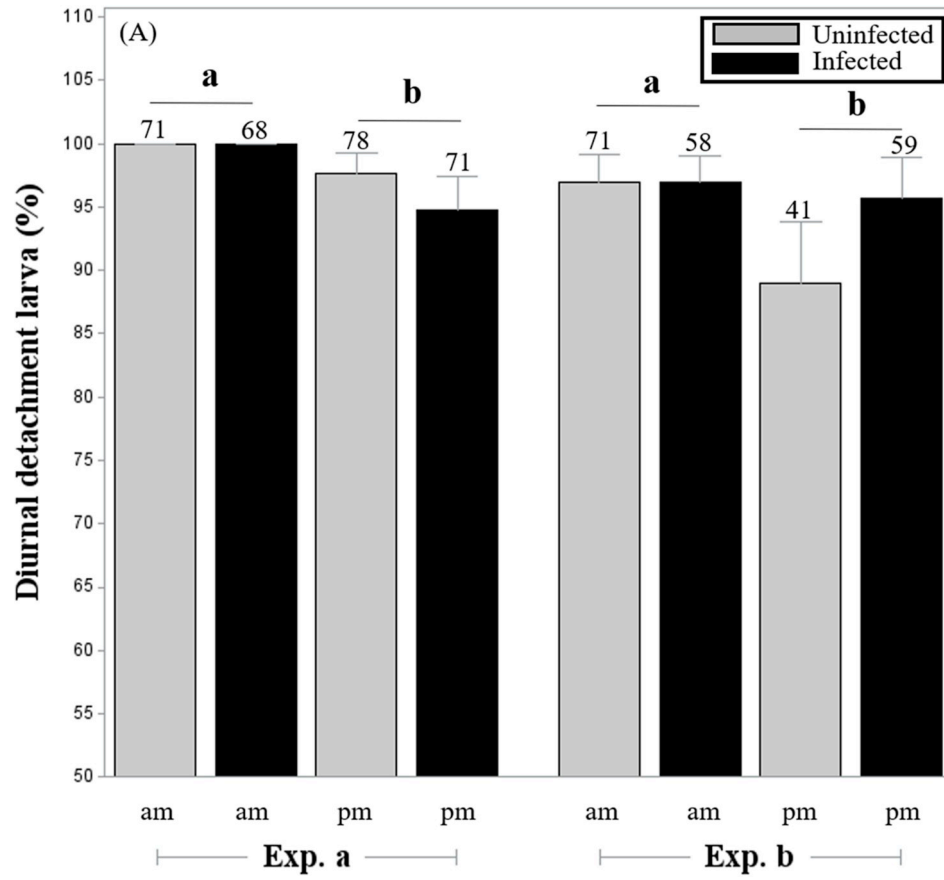
Additional observations: In larvae, the attachment success (i.e. the percentage of ticks that stayed on the birds after 1 h in the cotton bag) and moulting success (i.e. the percentage of detached ticks that did not die during moult) were high (Table 1). This contrasts with the significantly lower engorgement success (i.e. the percentage of ticks that initially stayed on birds that engorged and detached) ($\text{logit}_{\text{engorge-attach}} = -2.48 \pm 0.36$; $Z = -6.94$; $P < 0.0001$), especially for larvae of Exp. b, where engorgement success was significantly lower than in Exp. a ($\text{logit}_{\text{Exp a-Exp b}} = 0.66 \pm 0.22$; $Z = 5.79$; $P = 0.016$).

In nymphs, engorgement success tended to be lower than attachment success ($\text{logit}_{\text{engorge-attach}} = -0.50 \pm 0.27$; $Z = -1.83$; $P = 0.07$). Attachment success in turn was significantly lower than moulting success ($\text{logit}_{\text{attach-moult}} = -0.70 \pm 0.26$; $Z = -2.7$; $P = 0.007$).

Comparing larvae and nymphs for each of the successes, we found that in all comparisons attachment success of larvae was much higher than that of nymphs ($\text{logit}_{\text{larva-nymph}} = 1.47 \pm 0.41$; $Z = 3.60$; $df = 1$; $P = 0.0003$); while in Exp. a there was no significant difference between engorgement success of larvae and nymphs, engorgement success in Exp. b was lower for larvae than for nymphs ($\text{logit}_{\text{larva-nymph}} = -0.74 \pm 0.29$; $Z = -2.56$; $df = 1$; $P = 0.011$); finally moulting success did not differ between larvae and nymphs in Exp. a, but was slightly lower in nymphs of Exp. b ($\text{logit}_{\text{nymph-larva}} = -2.77 \pm 1.17$; $Z = 2.36$; $df = 1$; $P = 0.018$).

Larval engorgement weights differed between treatment groups only in Exp. b, where ticks were lighter than the uninfected birds ($\Delta \text{mean}_{\text{uninfected-infected}} = 0.016 \pm 0.010$ mg; $\chi^2 = 9.15$; $df = 1$; $P = 0.0025$; Fig. 4 and Table 1). When investigating the change in weights over the first two experiments, we found a decrease by 0.035 ± 0.012 mg in the infected group, and a tendency to increase (0.016 ± 0.010 mg) in the uninfected birds (interaction 'Exp. x treatment': $\chi^2 = 9.15$; $df = 1$; $P = 0.0025$). Nymphal engorgement weights were not affected by *Mycoplasma* exposure or infestation session.

In Exp. c, in which we varied the larval tick loads (25–250 larvae per bird) and left the nymphs out, we found that the overall infestation success was positively affected by tick density ($\text{logit}: 3.8 \pm 0.6 \cdot 10^{-3}/\text{tick}$; $\chi^2 = 36.93$; $df = 1$; $P < 0.001$) but did not differ between infected and uninfected birds. However, within the infected group, infestation success decreased with eye lesion severity ($\text{logit} = -0.10 \pm 0.02/\text{score}$; $\chi^2 = 18.43$; $df = 1$; $P < 0.0001$, Fig. 5). Note that eight days earlier in Exp. b, we did not observe such an association ($\text{logit} = 0.06 \pm 0.05/\text{score}$;



(caption on next page)

Fig. 2. Means and standard errors of the percentage of diurnally detached larvae (A) and nymphs (B) fed on Uninfected or Infected birds that were exposed at dawn ('am') or dusk ('pm'). The same letter above bars indicates no significant difference. Diurnal detachment happened significantly more often in larvae that were exposed at dawn ('a') compared to dusk ('b'). In nymphs the diurnal detachments did not significantly differ among the treatment groups, and overall they were similar to the larvae exposed at dusk ('b'). The number above the bar refers to the sample size.

$\chi^2 = 1.57$; $df = 1$; $P = 0.21$).

Additional observations: After correcting for tick density, the overall infestation success in Exp. c was shown to be considerably lower than in the previous infestation sessions ($\text{logit}_{\text{Exp. a-Exp. c}} = 1.54 \pm 0.18$; $\text{logit}_{\text{Exp. b-Exp. c}} = 0.91 \pm 0.28$; $\chi^2 = 12.43$; $df = 2$; $P = 0.002$). Also the average engorgement weights were lower than those in the two previous infestations ($\Delta_{\text{Exp. a-Exp. c}} = 0.05 \pm 0.01$; $\Delta_{\text{Exp. b-Exp. c}} = 0.04 \pm 0.01$; $\chi^2 = 6.99$; $df = 2$; $P = 0.03$). So overall, there was a decrease in larval engorgement weight from Exp. a onwards (-0.026 ± 0.007 mg/session; $\chi^2 = 6.99$; $df = 1$; $P = 0.008$; Fig. 4).

3.3. Health measures (Exp. a - c)

We measured haematocrits (Hct) values both in the birds involved with our tick experiment, and in another 24 house finches that were not infested with ticks, but were experimentally exposed to poultry *Mycoplasma gallisepticum* strains as part of another concurrent experiment (Fig. 6). None of the latter developed conjunctivitis. At the onset of the experiment (day 0) the HCT values did not differ between the

groups ($\chi^2 = 11.05$; $df = 2$; $P = 0.65$). For the total duration of the experiment, the average Hct-values of the birds without ticks remained above those of the tick infested birds. Hct decreased in the tick-infested birds; this decrease was significantly stronger during Exp. a than Exp. b (Exp. a $\Delta_{\text{after-before}} = -3.56 \pm 1.68\%$; Exp. b: $-2.63 \pm 1.19\%$; $\chi^2 = 11.05$; $df = 1$; $P < 0.0001$; interaction 'Exp. x $\Delta_{\text{after-before}}$ ' $\chi^2 = 4.54$; $df = 1$; $P = 0.03$) but there was no effect of *Mycoplasma* infection.

Over the course of the experiment (from Day -1 to Day 28), average haematocrits remained below the initial values in tick infested birds. Surprisingly, the extent of Hct decrease was not explained by the total weight of fed ticks (i.e. the sum of all engorged larvae and nymphs per bird) nor the total number of ticks that successfully fed. When including the eye scores as a proxy for *Mycoplasma* infection, we found a strong negative association with this overall Hct decrease ('eyescore x $\Delta_{\text{Day 28-Day -1}}$ ', -0.68 ± 0.13 ; $Z = -5.13$; $P < 0.0001$; Fig. 7), although this effect was not found in the comparison 'infected vs. uninfected'. During the following 2 weeks, after all the ticks had already detached, three *Mycoplasma* infected birds (all with low Hct levels on day 28 PI)

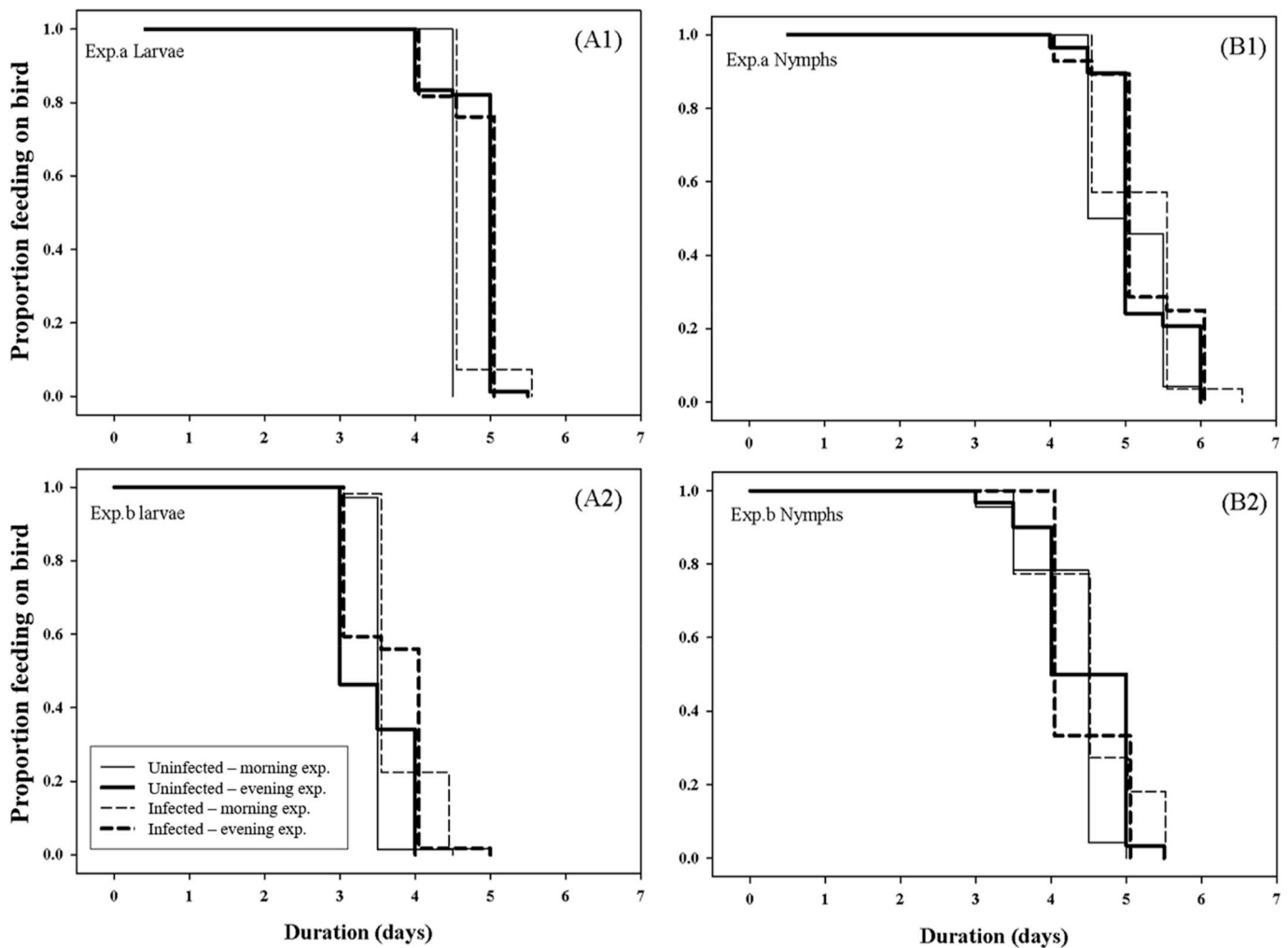


Fig. 3. Kaplan-Meier curves of time to detachment of *I. scapularis* larvae (A) and nymphs (B) placed on house finches. Lines represent the distribution functions of detached ticks that have been placed on the host respectively at 7:30 a.m. (morning) and at 7:30 p.m. (evening) to *Mycoplasma*-infected and uninfected birds, early (Exp. a) and later (Exp. b) in the disease development resulting from *Mycoplasma* infection (see main text for details).

Table 1

Feeding and development parameters of *Ixodes scapularis* larvae and nymphs placed on house finch individuals in relation to treatment groups (Uninfected vs. Infected) and early (Exp. a) and later (Exp. b) in *Mycoplasma gallicepcticum*'s disease development.

	Larvae (25 ticks/bird; N = 16)		Nymphs (10 ticks/bird; N = 16)			
	Uninfected	Infected	Uninfected	Infected		
Overall infestation success %						
Exp. a	65.5 ± 5.3	59.7 ± 6.0	N	63.8 ± 5.0	63.8 ± 3.8	N (N)
Exp. b	44.8 ± 9.0 L, .03	47.1 ± 5.0 L, .05	N	61.3 ± 9.9 N	50.8 ± 5.0 N	N (N)
(a) Attachment success %						
Exp. a	97.5 ± 1.5	94.5 ± 2.1	N	87.5 ± 3.7	86.3 ± 3.8	N (L, < .001)
Exp. b	93.0 ± 3.8 N	99 ± 1 N	N	88.8 ± 3.5 N	76.3 ± 5.0 N	N (L, < .01)
(b) Engorgement success %						
Exp. a	76.3 ± 3.6	73.9 ± 4.7	N	75.9 ± 4.5	81.8 ± 3.4	N (N)
Exp. b	58.8 ± 6.8 L, .04	59.2 ± 4.6 L, .002	N	73.0 ± 10.1 N	76.1 ± 5.2 N	N (H, .01)
(c) Moulting success %						
Exp. a	97.9 ± 1.4	97.2 ± 2.8	N	96.1 ± 2.5	91.0 ± 3.8	N (N)
Exp. b	96.9 ± 3.1 N	100 ± 0 N	N	93.3 ± 3.5 N	88.4 ± 4.8 N	N (L, .02)
Engorged weight (mg)						
Exp. a	0.47 ± 0.01	0.49 ± 0.01	N	♀-5.00 ± 0.14 ♂-2.84 ± 0.07	5.17 ± 0.16 2.99 ± 0.09	N
Exp. b	0.49 ± 0.01 N	0.46 ± 0.01 L, < .001	L, < .01	♀-5.35 ± 0.12 ♂-2.93 ± 0.06 N	5.44 ± 0.18 2.84 ± 0.09 N	N

Overall infestation success: % of all ticks placed on the birds that successfully reached the next developmental stage; which is the outcome of a-c.

(a) Attachment success: % of all ticks that stayed on the birds after 1 h in the cotton bag.

(b) Engorgement success: % of ticks that initially stayed on birds that successfully engorged.

(c) Moulting success: % of engorged ticks (see b) that did not die during moult.

Left-to-right comparisons (i.e. Infected vs Uninfected): Infected significantly lower ('L', P-value) or higher ('H', P-value) than Uninfected; Up-to-down comparisons (i.e. Exp. b vs. Exp. a): Exp. b significantly lower ('L', P-value) or higher ('H', P-value) than Exp. a.

In brackets: P-values for tests that compare the success between nymph and larva (left-to-right comparisons).

'N': no difference.

unexpectedly died, while none of the uninfected birds did.

4. Discussion

Mycoplasma had no strong effects on tick detachment behaviour. Both in the early and late stage of the infection, ticks mostly detached during the day, and this behaviour was very similar to that of the ticks in the uninoculated birds. In an evolutionary perspective, we can interpret the diurnal detachment as a strong adaptation to *I. scapularis*' ecology. Being an ectoparasite with low intrinsic mobility, it obtains its hosts by a passive sit-and-wait strategy ('questing') in the understory vegetation, which is a habitat that satisfies two requirements for tick survival and persistence: sufficiently high humidity to maintain the water balance (Stafford, 1994) and a variety of hosts for each parasitic stage, including large mammals on which females feed and copulate (Gray, 1998). Detachments (i.e. preference for the day) from the diseased birds during the second infestation session – when birds are least active (Hawley et al., 2012) – were not different from the uninfected birds. Therefore, we conclude that the light/dark signals and associated changes in host activity are likely sufficient for the development of this robust behaviour. Bird physiology seems to play a role in the level of detachment synchronicity, as we found that uninfected birds that were infested in the morning – i.e. the birds with presumably the highest movement activity upon tick exposure – showed lower variation in larval feeding durations (Fig. 3) (Dhondt et al., 2007).

Feeding duration of the ticks was shorter in the second than in the first infestation session. This is opposite of what was observed in the resistance experiment involving two European songbirds (*Parus major* and *Cyanistes caeruleus*) that were repeatedly exposed to *I. ricinus* nymphs (Heylen et al., 2010). Engorgement weights even slightly increased, leading the authors to conclude that these European birds did not acquire resistance when being exposed to ticks.

At higher larval exposures (more than 25; Exp. c), conjunctivitis had a negative effect on the tick's feeding success three weeks post inoculation (Fig. 5). The histological alterations around the bird's eyes that coincide with the preferred tick feeding spots, reduced tick infestation success, including the tick's willingness to attach and to feed. Another explanation is that more diseased birds have changed their behaviour: birds with severe conjunctivitis more often shake their head, and rub it against the bars of the cage or perch (pers. obs.) thereby reducing the infestation success. In addition to the *Mycoplasma* effect, there was a positive density-dependent effect, in that more ticks successfully fed when birds were exposed to higher loads. The observation that ticks perform better when they aggregate on host individuals has been shown before in nidicolous (Van Oosten et al., 2016) and non-nidicolous ticks (Ogden et al., 2002).

Birds that faced *Mycoplasma* – and brought in a physiologically challenging disease state – are investing earlier and more in resisting the ticks. We can interpret several of our outcomes as a shift from tolerance (Exp. a) towards (incomplete) resistance (Exp. b and c) under the progressing pathogenic effects of the *Mycoplasma* infection, 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). Acquired resistance was shown by significantly lower attachment and engorgement success after the consecutive exposures. Moreover, larval engorgement weights significantly decreased; reduced engorgement weights are the most reliable signs of anti-tick responses (Rechav, 1992; Varma et al., 1990). *Mycoplasma* caused an acceleration of this effect, as shown by an earlier decrease in engorgement weights in the infected birds, starting already from Exp. b (see Fig. 4). These outcomes contrast with the observed lack of acquired anti-tick resistance in the two European songbirds (Heylen et al., 2010) caught in a region where *Mycoplasma* does not occur in wild birds. Acquired resistance against ticks has frequently been demonstrated in laboratory

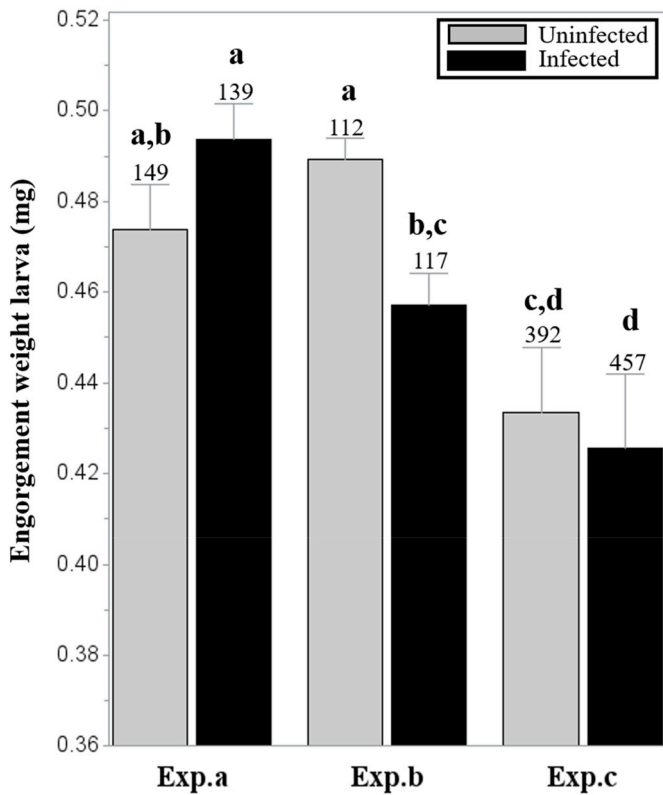


Fig. 4. Engorgement weights (means and standard errors) of larvae that fed on *Mycoplasma*-infected or uninfected birds. Only in the second infestation (Exp. b, exposure: 25 larvae and 10 nymphs) infection had a negative effect on the weights. In the first infestation (Exp. a, 25 larvae and 10 nymphs) as well as in the third infestation (Exp. c, 25–250 larvae) no effect of *Mycoplasma* was observed. The engorgement weights in Exp. c were significantly lower than Exp. a, both for uninfected and infected birds. The number above the bar refers to the sample size.

rather than in natural hosts (Fielden et al., 1992). 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. Although we found several reports of ixodid infestations in house finches (Brinkerhoff et al., 2018; Castro and Wright, 2007; Luttrell et al., 1996; Peters, 1999) - though most without any mention of *I. scapularis* infestation intensities - we assume that the experimental loads are beyond natural levels. Possibly a critical threshold load has been exceeded to elicit an immunological response to which larvae are most vulnerable (Dineen, 1963), resulting in the observed signs of resistance. Although in our study nymphs were less successful in attaching on the heads compared to larvae (Exp. a and b), they seem to feed more vigorously and are efficient in suppressing local immune responses to which they are less sensitive, as shown by their higher engorgement successes. As a consequence of the incomplete resistance in the house finches, birds were unable to overcome the direct harm (acute blood depletion) caused by tick feeding (Heylen and Matthysen, 2008, 2011). Possibly *Mycoplasma* made birds more vulnerable to the effects of co-parasites (in our case the ticks), as was shown by mortality in the experimental birds - which has never been observed in previous *Mycoplasma*-house finch experiments in captivity.

While *Mycoplasma* infection had no strong effects on tick detachment behaviour, it can act as a selective pressure to develop effective anti-tick resistance mechanisms, when it pushes songbirds towards their outer physiological limits. The seemingly accelerated mortality of co-parasitized individuals, making hosts unavailable to competing parasites, can be considered as ecological interference (Rohani et al., 2003). This phenomenon has so far - to the best of our knowledge - never been shown in a system involving ecto- and micro-parasite simultaneously parasitizing their natural host.

The results of this experiment generates a suite of questions. We may thus wonder whether avian hosts more preferred by *I. scapularis* (Loss et al., 2016) would show similar patterns; how interspecies variation in susceptibility to *Mycoplasma* strains that differ in virulence (Dhondt et al., 2008, 2014; Hawley et al., 2013) would affect bird and

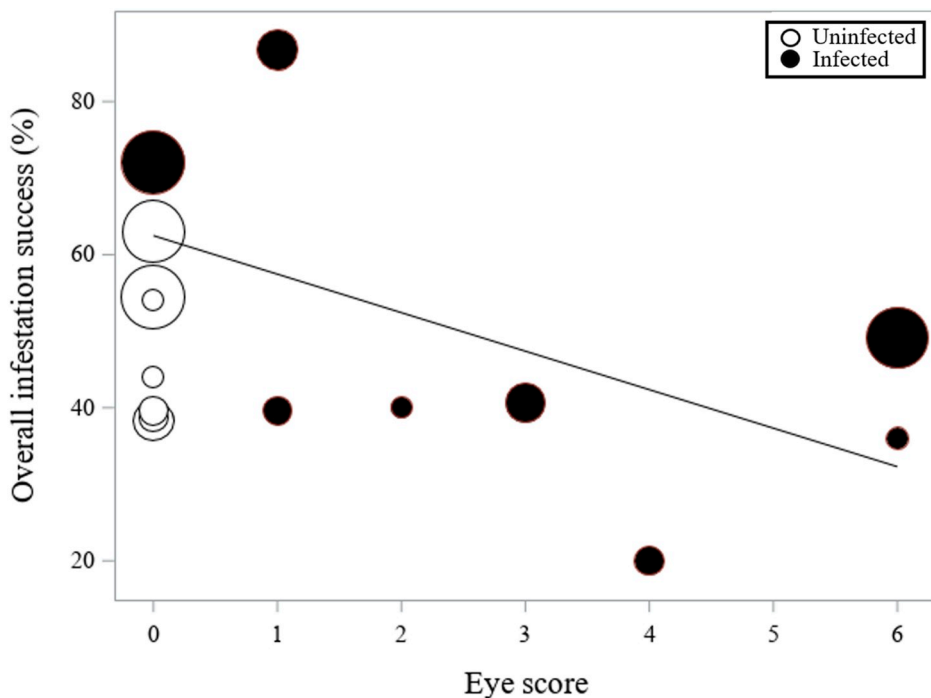


Fig. 5. Overall infestation success in relation to the level of conjunctivitis (expressed in eye scores) in *Mycoplasma*-exposed birds of Exp. c. Size of bubbles is proportional to the total density of larval ticks placed on the bird (range: 25–250). In the infected birds, the success decreased with disease severity, as illustrated by the linear regression line.

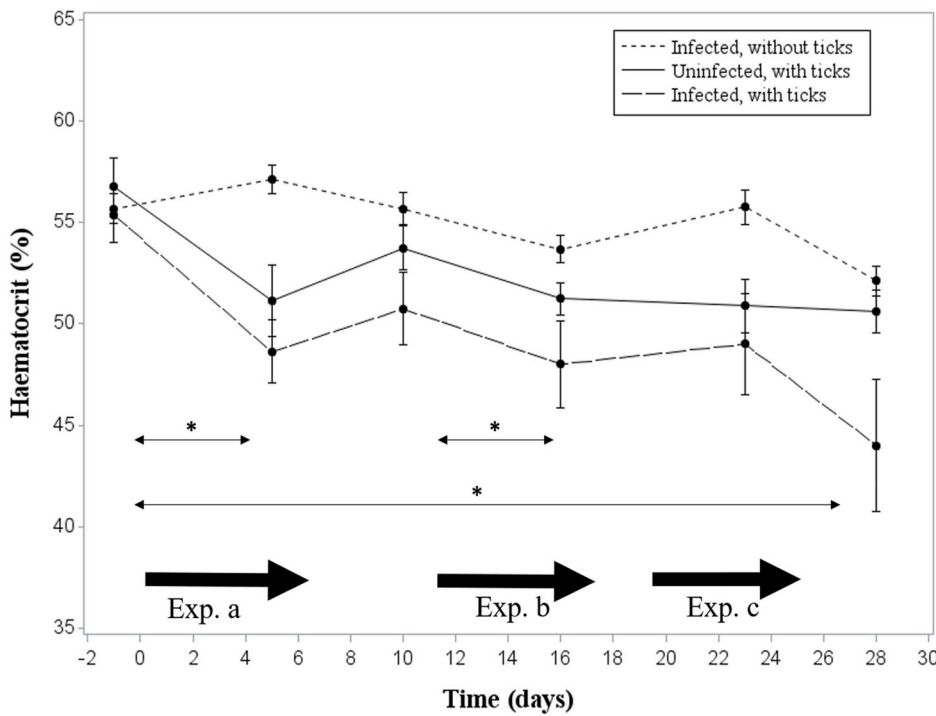


Fig. 6. The haematocrit values (Means and standard errors) over the three infestation sessions (see Fig. 1 for explanation study design). An extra set of control birds (short-dashes) that were infected with a variety of *Mycoplasma* strains - but not with ticks - was added for comparison. While there was no difference among groups at time-point zero, the haematocrit of birds without ticks remained higher than the infested birds. The significant decreases are indicated with an asterisk.

tick communities; and whether cross-resistance (Heller-Haupt et al., 1996) against other tick species (Loss et al., 2016) would develop. In addition, the question remains whether the outcomes observed here would hold under less extreme infestation loads, i.e. that resemble natural conditions (Loss et al., 2016). Furthermore, to find out whether *Mycoplasma* eventually affects tick-borne micro-parasites' basic reproductive numbers in known host reservoirs (Ginsberg et al., 2005;

Loss et al., 2016), information on the natural prevalence of co-occurring *Mycoplasma* and ticks are needed. In addition to the monitoring of spatio-temporal variation of co-parasitism in wild songbird, experiments - as presented here - are heavily required.

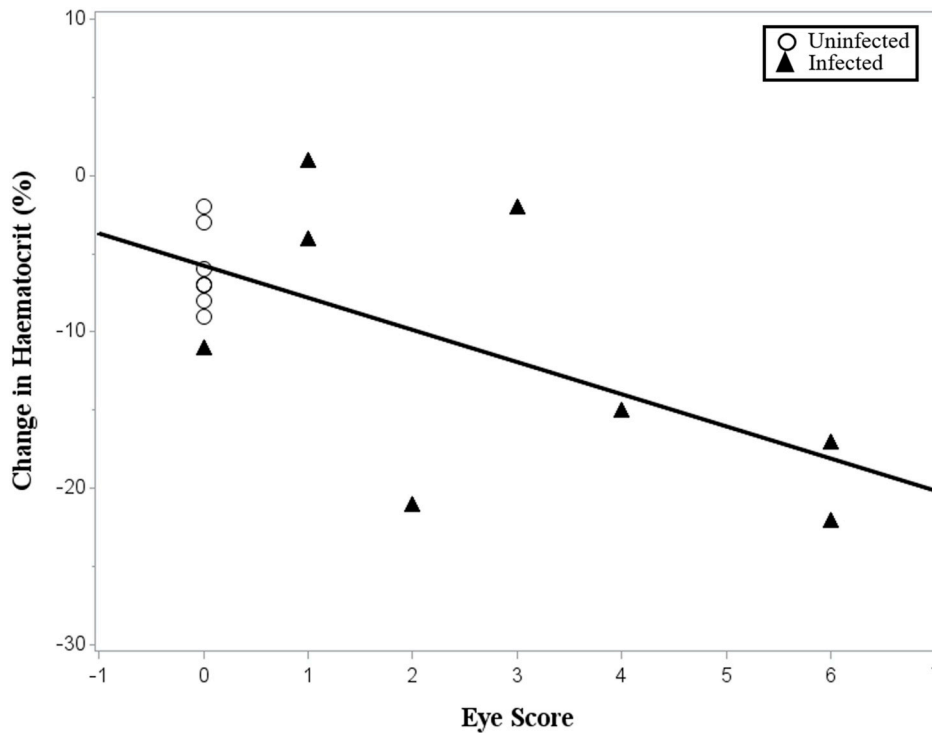


Fig. 7. Change in haematocrit levels (Day 28 minus Day -1) in relation to the eye score. All birds were infested with ticks. The haematocrit decreased more strongly with disease severity (eye scores).

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Authors' contributions

D.H. and A.D. initiated the study and designed the experiment. M.R. and K.D. executed the infections and did the physiological screenings. L.G. did the molecular analyses. D.H. and A.D. drafted the manuscript with notable input from K.D. All authors edited and approved the final manuscript.

Declarations of conflicts of interest

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

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The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

There is no conflict of interest to mention.

Data availability

All data is given in the text.

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