

A flea-induced pre-hatching maternal effect modulates tick feeding behaviour on great tit nestlings

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Summary

1. A host's defence reaction against one parasite species can modulate the habitat quality for other parasites in two ways: it can provide cross-resistance against closely related species due to antigenic similarity, or it can reduce resistance to other cohabiting species, since the mounting of multiple defence reactions is more costly.

2. Here we test whether two completely unrelated parasite species can influence each other across host generations, that is, whether a hen flea-induced maternal effect known to protect great tit (*Parus major*) nestlings against flea infestations will also alter tick (*Ixodes ricinus*) feeding behaviour on nestlings.

3. We infested experimental great tit nests with hen fleas (*Ceratophyllus gallinae*) prior to egg-laying to induce the maternal effect, while all parasites were removed in control nests. Nestlings from the two types of nests were then cross-fostered into flea-free foster nests to produce broods that contained both, nestlings with and without the flea-induced maternal effect. Five days after hatching, we put five larval ticks on each nestling and assessed tick feeding behaviour.

4. We found that ticks feeding on nestlings with the flea-induced maternal effect detached significantly earlier than ticks feeding on controls. The result is compatible with the hypothesis of a trans-generational parasite–parasite interaction, that is, it suggests that the flea-induced maternal effect alters tick feeding behaviour and that it may protect nestlings against tick-borne diseases by reducing tick attachment times. In addition, we found that more ticks attached on male than on female nestlings, suggesting that males are more susceptible to parasites than females as shown in other vertebrates.

Key-words: *Ceratophyllus gallinae*, cross-protection, *Ixodes ricinus*, parasite–parasite interaction, *Parus major*

Introduction

Parasites feeding on the same host can be viewed as a community of animals exploiting a common resource (Krasnov, Stanko & Morand 2006). They can either directly interact with each other, for example, by competing for resources, or they can indirectly influence each other by provoking defence reactions in the host such as immune responses (Zuk & Stoehr 2002) and grooming (Mooring, Blumstein & Stoner 2004) that will in turn affect other organisms parasitizing the same host (Krasnov *et al.* 2005). For example, resistance to one ectoparasite taxon might reduce resistance to other taxa. The mounting of multiple defence reactions is costly (Krasnov *et al.* 2005), and therefore challenges by several parasites can lead to an immune depression in the host (Krasnov *et al.* 2006). Also, it is known that parasites can actively suppress the host's immune system. For example, tick feeding reduces

the host's T-cell mediated immunity and antibody response for several days after infestation (Wikel 1996). Moreover, immunoglobulin-binding proteins secreted by adult male ticks, increase the engorgement weights of co-feeding females, nymphs and larvae (Rechav & Nuttall 2000), indicating that the presence of male ticks facilitates feeding for other individuals.

In contrast to this, resistance to one ectoparasite species can also be accompanied by resistance to an unfamiliar closely related species; a phenomenon known as cross-resistance or cross-protection that has been demonstrated for the hosts of several blood sucking arthropods (Brown, Graziano & Askenase 1982; Heller Haupt, Kagaruki & Varma 1996; Kumar & Kumar 1996; Khokhlova *et al.* 2004). Some studies even suggest that cross-resistance occurs in unrelated taxa of haematophagous ectoparasites, because of the similarity of salivary chemicals used to facilitate blood sucking (den Hollander & Allen 1986; Krasnov *et al.* 2005). A cross-protection effect might also occur because a parasite can activate components of the non-specific immune system, such

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as granulocytes and mast cells that are known to be involved in resistance against ticks (Wikel 1996). The activated non-specific immune system can then also induce protection against other unrelated parasites (Huang & Matsumoto 2000).

Closely related parasite species have even been shown to influence each other across host generations (Smith *et al.* 1994) by means of egg-mediated maternal effects (Mousseau & Fox 1998). Parasite infested mothers belonging to many different animal taxa transfer antibodies (or other substances) via the eggs, the placenta or the milk to induce a passive immunisation in their offspring (Heeb *et al.* 1998; Gasparini *et al.* 2001; Buechler *et al.* 2002; Grindstaff, Brodie & Ketterson 2003). In some cases the maternal antibodies can react with closely related parasite species, and therefore increase the resistance of the offspring not only to the parasite that induced the maternal effect but also to the related parasite species (Smith *et al.* 1994, but see also Khokhlova *et al.* 2004). However, it is so far unknown whether two completely unrelated parasite species can influence each other across host generations. In this study, we explored whether a hen flea-induced (*Ceratophyllus gallinae* Schrank, Siphonaptera, Ceratophyllidae) maternal effect in the great tit (*Parus major* L.) can influence the feeding behaviour of another, completely unrelated ectoparasite species, the European castor bean tick (*Ixodes ricinus* L., Acari, Ixodidae).

The great tit is a small hole-nesting passerine that is frequently infested with hen fleas (Tripet & Richner 1997). The presence of fleas has severe effects on nestlings by reducing their condition, survival and fledging success (Richner, Oppliger & Christe 1993; Oppliger, Richner & Christe 1994; Fitze, Clobert & Richner 2004). However, these effects are partly countered by a flea-induced maternal response that reduces parasite virulence for nestlings (Heeb *et al.* 1998). The exact mechanisms involved in the response are still unknown, yet it has been shown that mothers exposed to fleas during egg-laying transfer an increased amount of IgG immunoglobulins (Buechler *et al.* 2002) and a decreased amount of testosterone (Tschirren, Richner & Schwabl 2004) to their eggs. *Ixodes ricinus* is a three-host tick very common in Europe (Piesman & Gern 2004) that feeds on a large array of hosts such as mammals, reptiles and birds including the great tit (Roulin *et al.* 2003). Several hosts have been shown to acquire resistance against ticks resulting in reduced tick engorgement weight, decreased blood-meal volume, prolonged feeding duration, reduced fecundity, inhibition of moult or even the death of the tick (Wikel 1996). In addition, host quality can influence the number of ticks that attach to the host (Rechav & Fielden 1997; Harnnoi *et al.* 2006). Hence, we expect the flea-induced maternal effect to influence one or more of the factors listed above. Also, nestling gender is expected to affect these factors, because it has been shown that host sex or sex hormones (e.g. testosterone) can influence tick feeding performance (Hughes & Randolph 2001). In the present study, we experimentally induced a maternal effect in a part of the great tit nests and cross-fostered nestlings to create broods with a mix of nestlings with and without a maternal effect. The nests were then

artificially infested with ticks and we tested whether the number of ticks attached per nestling and the feeding duration of the ticks was affected by the flea-induced maternal effect, the nestling gender, or by a combination of both.

Material and methods

The experiments were performed in spring 2006 in the Forst and the Spilwald, two forests near Bern, Switzerland. In February, that is, before the birds started to build their nests, we removed the old nests from the nests boxes and brushed them out in order to remove the previous year's parasites. Old nests were collected and stored in a climate chamber at 5 °C, so that fleas present in the nest material could later be used for experimental infestations.

INDUCTION OF THE MATERNAL EFFECT

Starting at the end of March, we regularly visited all nest boxes in order to determine the beginning of nest construction. Great tits use moss to build their nests and as soon as the moss covered the floor of the nest box with a layer *c.* 2 cm thick, we removed all parasites from the nest using a microwave appliance (Richner *et al.* 1993). One-third of the nests ($n = 32$) was then randomly assigned to the 'infested'-treatment, that is, they were infested with 40 fleas collected from the previous year's nesting material. The other two-thirds of the nests ($n = 64$) were assigned to the 'uninfested' treatment, that is, they were left parasite-free. Except for the addition of the fleas the nests used in both treatments were manipulated in the same way. Territorial boundaries, and thus location of the nest site are defined by the end of March (Gosler 1993), that is, before the application of the flea treatment. Also, nest building had already started at the moment of manipulation, and it is therefore unlikely that the birds changed their nest site as a consequence of the treatment. Regular nest checks were used to determine the onset of egg-laying and the start of incubation. On the fourth day of incubation, we heat-treated all nests again to remove all fleas from the nests. Thus, in summary one-third of the parents had been experimentally infested with fleas from the beginning of nest building until the end of the egg-laying period and could thus have induced a maternal effect (Heeb *et al.* 1998; Buechler *et al.* 2002). Nestlings, however, grew up in uninfested nests. Because the strength of the maternal effect could vary with the time interval between nest infestation and egg-laying (Buechler *et al.* 2002), this interval was added as a covariate to all analyses.

CROSS-FOSTERING

In the next step we cross-fostered nestlings in order to create broods with a mix of nestlings with and without the flea-induced maternal effect. Nests were visited daily to determine the hatching day of the first nestling (defined as day 1). If all the nestlings had not hatched on the first day, nests were revisited in the afternoon of day 2. All freshly hatched nestlings were weighed with an electronic portable scale to the nearest 0.01 g and individually marked by removing some of their down feathers. In the morning of day 3, when all eggs had hatched we cross-fostered the nestlings. We used a split brood design including one 'uninfested' receiver nest ($n = 28$) and two donor nests, one 'infested' and one 'uninfested'. Half of the brood of each donor nest, that is, every second nestling in the mass-ranked hierarchy, was transferred to the receiver nest. We randomly decided which of the two donor nests would donate the nestlings with the

even ranks, and which one would donate the nestlings with the odd ranks. The donor nests were used to adopt the nestlings of the receiver nest and were then not further considered. Clutch size before and after the cross-fostering remained unchanged. Five days after hatching, that is, on the day when the experiments started (see below), nestling mass did not significantly differ between the two treatments (mixed effect model with the random effects Nest of Origin nested within the Nest of Rearing: $F_{1,24} = 2.76$, $P = 0.11$, mean 'infested' = 5.74 g, mean 'uninfested' = 5.99 g). Also the numbers of male and female nestlings were balanced between treatments ($\chi^2_1 = 0.96$, $P = 0.33$, 'infested': males = 64, females = 55; 'uninfested': males = 53, females = 59).

TICK INFESTATION

On day 5, that is, 2 days after the cross-fostering, we placed five *I. ricinus* larvae onto each nestling with the help of tweezers. The ticks did not always stay on the nestling on which they had been initially placed, but moved around before attaching onto a nestling. Because nestlings touch each other inside the nest cup, the ticks could freely move from one nestling to the other and choose the best host. The ticks used for the infestation were from a laboratory colony free of pathogens, maintained at the Institute of Biology, University of Neuchâtel, Switzerland. Since it is known from a survey in Switzerland that only larvae and nymphs of *I. ricinus* infest birds (Papadopoulos *et al.* 2001), we decided to use larvae for this experiment given the small size of the nestlings. In a natural great tit population, infestation densities of 2.45 ticks per nestlings were found (Roulin *et al.* 2003), and thus our experimental infestation was within the natural range.

On day 7, we visited the nests again and counted the number of ticks that had attached onto each nestling in order to determine the host preference of the ticks. The exact position of each tick on the nestlings was mapped in a schematic drawing. Thereafter, nests were visited daily and the ticks still remaining on the nestlings were compared with the drawings of day 7 in order to determine when each tick had detached from the nestling. We did not have problems identifying the individual ticks using this method, because at the time of the experiment the nestlings had not grown their feathers, and because the ticks rarely aggregated on a particular location and remained on the same spot when attached. We then calculated an average tick attachment time per nestling as a measure of tick feeding duration. Nest checks were stopped as soon as all ticks had detached from each nestling.

Nestlings were weighed upon each visit and were ringed on day 9 with a small numbered aluminium ring. At this age the individual marking of nestlings by the removal of down feathers at hatching is still visible, hence we always knew the identity, the nest of origin, and the flea-treatment of the parents of each nestling. At the age of 5 days, we took a small blood sample from each nestling for molecular sexing (Tschirren, Fitze & Richner 2003). Some nests were lost before the start of the experiment and thus our final sample contained 231 nestlings from 53 Nest of Origin that were raised in 28 different Nests of Rearing. In the 'infested' treatment there were 64 males and 55 females and in the 'uninfested' treatment 53 males and 59 females. Due to the loss of some nestlings in the course of the experiment, the sample size varies slightly between the different variables.

Out of the 231 nestlings in our experiment, 37 nestlings (half of them with and half without maternal effect) from 19 nests were also used for another study that tested the effect of the maternal response on flea survival. We let five fleas take one blood meal for 20 min on

these nestlings immediately before the ticks were applied. To avoid secondary infestations of the nests, the flea-feeding experiments, as well as the application of ticks, were performed inside a bus with the experimental nestling taken off the nest. After the manipulation, each single flea used was collected from the experimental nestling, and thereafter the nestling placed back into the nest. Thus all other nestlings did not come in contact with fleas. In order to test whether this treatment had an effect on the results of our tick-feeding experiment, we performed all analyses with both the entire and the reduced data set where the 37 flea-exposed nestlings were excluded.

STATISTICAL METHODS

Data were analysed with linear mixed effect models in the nlme package (Pinheiro *et al.* 2006) in R (R Development Core Team 2007). The models had two random effects: the Nest of Origin nested within the Nest of Rearing. Model assumptions were tested as recommended by Pinheiro & Bates (2004), and if necessary the response variables were transformed (log-transformation for the feeding duration and square-root-transformation for the number of attached ticks).

The variance structure of the within-treatment error in the analysis of the number of ticks attached on each nestling was modelled with varIdent, that is, with different variances for each level of stratification (Pinheiro *et al.* 2006). The initial models contained the following fixed effects: Maternal-Effect-Treatment, Nestling Mass Day 5, Nestling Sex, Brood Size, Hatching Date, Time between Induction of the Maternal-Effect-Treatment and First Egg, the interaction between the Maternal-Effect-Treatment and Nestling Sex, and the interaction between the Maternal-Effect-Treatment and the Time between Induction of the Maternal-Effect-Treatment and First Egg. The models were reduced with a stepwise backward procedure and only significant terms were kept in the model.

Results

The flea-induced maternal effect had no influence on the number of ticks that initially attached to each nestling ($F_{1,24} = 0.136$, $P = 0.715$). However, significantly more ticks attached on male (mean = 3.53, SE = 0.28) than on female (mean = 2.51, SE = 0.27) nestlings ($F_{1,176} = 12.8$, $P = 0.0005$, Fig. 1; n [nestlings] = 231, n [nest origin] = 53, n [nest rearing] = 28). In addition, the number of ticks per nestlings was positively correlated with nestling mass ($F_{1,176} = 7.10$, $P = 0.0084$). All other factors, that is, Brood Size, Hatching Date, Time between Induction of the Maternal-Effect-Treatment and first egg, and all interaction terms were non-significant (all P -values > 0.13). The same results were obtained if the 37 flea-exposed nestlings (see Material and methods section) were removed from the analyses (influence of nestling sex: $F_{1,139} = 7.76$, $P = 0.0061$, influence of nestling mass $F_{1,139} = 5.31$, $P = 0.0227$, all other factors were not significant).

The proportion of nestlings infested with at least one tick was 82%, and only the infested nestlings were used for the analysis of tick attachment times. Mean tick attachment time per nestling was significantly reduced on nestlings with the flea-induced maternal effect (mean = 2.90 days, SE = 0.0525 days) compared to control nestlings without the maternal effect (mean = 3.12 days, SE = 0.0672 days, $F_{1,22} = 4.644$,

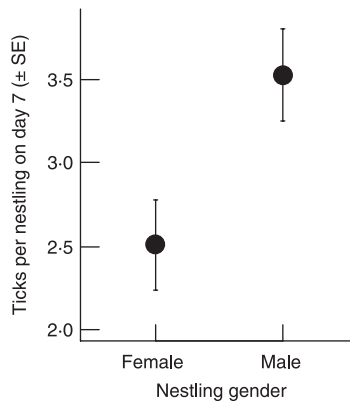


Fig. 1. Average number of ticks attached on each nestling on day 7, that is, 2 days after five larval ticks had been applied on each nestling, in relation to nestling sex (n [males] = 117, n [females] = 114). The error bars indicate ± 1 SEM.

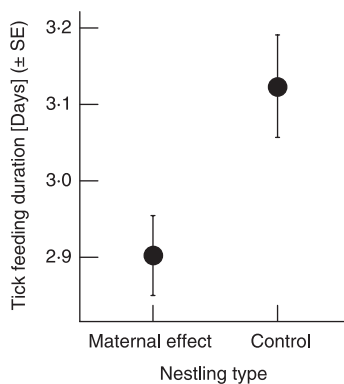


Fig. 2. Average attachment duration of ticks feeding on either nestlings with maternal effect ($n = 91$) or on control nestlings ($n = 87$). The error bars indicate ± 1 SEM.

$P = 0.0424$, Fig. 2; n [nestlings] = 178, n [nest origin] = 51, n [nest rearing] = 28). All other factors, that is, Nestling Mass Day 5, Nestling Sex, Brood Size, Hatching Date, Time between Induction of the Maternal-Effect-Treatment and First Egg, and all interaction terms did not significantly influence tick attachment times and were thus removed from the final model (all P -values > 0.28). The exclusion of the flea-exposed nestlings (see Material and methods section) did not change these results (influence of the maternal effect: $F_{1,22} = 6.399$, $P = 0.0191$, all other factors were not significant).

Discussion

CONSEQUENCES OF THE MATERNAL EFFECT

The flea-induced maternal effect did not alter tick attachment preferences but significantly reduced the ticks' feeding duration by 7%. It suggests that the flea infestation of great tit parents from the beginning of nest construction until the end of egg-laying has a significant influence on the quality of nestlings as tick hosts. To our knowledge this shows for the first time that

a parasite-induced pre-hatching maternal effect can affect parasites of a completely unrelated taxon, and that parasites can modulate the habitat (i.e. the host) quality for other parasites across host generations.

The reduced feeding duration of ticks feeding on nestlings with an induced maternal effect could be attributed to either a decreased or an increased resistance against ticks in these nestlings. For the case of a decreased resistance, many studies have shown that a resistance to ticks increases tick feeding duration until full engorgement (Fivaz, Nurton & Petney 1991; Amoo & Dipeolu 1992; Wikel 1996). Hence, assuming that most of the ticks in our experiment had reached full engorgement, the differences in tick feeding duration between maternal effect and control nestlings would indicate a reduced resistance against ticks in nestlings with the maternal effect. This would be the case if the flea-induced maternal effect would induce a defence reaction directed specifically against fleas, but not other parasites. Such a specific defence mechanism is expected to use up some of the nestlings limited resources that would then be lacking in the defence against other parasites such as ticks (Krasnov *et al.* 2005). In contrast for the case of an increased resistance, it has been shown that resistance against ticks reduces the proportion of ticks that fully engorge (Brown *et al.* 1982; Craig *et al.* 1996; Hughes & Randolph 2001). Therefore host resistance can lead to premature detachment of ticks, resulting in very short feeding times (Hughes & Randolph 2001). In this case, the decreased tick feeding duration on nestlings equipped with the maternal effect would indicate that they have an increased resistance against ticks compared to the controls. Increased resistance against ticks could be triggered by at least three non-mutually exclusive mechanisms. First, the increased resistance could be mediated via the higher amount of antibodies transferred to the nestlings by flea infested mothers (Buechler *et al.* 2002). These antibodies could either be quite unspecific and thus effective against many kinds of ectoparasites, or they could be specifically directed against flea saliva components. In the latter case they may confer resistance against ticks if they would recognize similar salivary chemicals that facilitate blood sucking in both, the tick and the flea (den Hollander *et al.* 1986; Mans, Louw & Neitz 2002; Krasnov *et al.* 2005). Second, the substances transferred to the nestlings by flea-infested mothers could activate the nestlings' innate immune system that would in turn also increase the resistance against ticks (Wikel 1996). Third, flea-infested mothers have been shown to reduce the amount of androgens deposited into their eggs (Tschirren *et al.* 2004), and it is known that increased quantities of testosterone can depress the resistance against ticks (Hughes & Randolph 2001). Therefore the reduced concentration of testosterone transmitted to nestlings by flea-infested mothers could ease their struggle against the ticks.

To assess whether the flea-induced maternal effect leads to an increased or a reduced resistance against ticks in nestlings, we would at least have to know the number of ticks that had reached full engorgement. Yet, because we used nests that contained nestlings with maternal effect and control nestlings together, the detached ticks found in the nest could not

unequivocally be attributed to one treatment group. Hence our experimental design did not allow us to check the engorgement status of detached ticks with respect to the maternal effect treatment. Other studies have assessed tick feeding success based on the theoretical, minimal time required for successful engorgement (McCoy *et al.* 2002). However, nothing is known about the minimal time required to reach full engorgement of *I. ricinus* larvae feeding on great tits, and therefore this method could not be applied in our case. Hence, further experiments are needed to settle this issue. Regardless of the mechanism, the key significance of our results is that the flea-induced maternal effect reduced tick feeding time. Because reduced tick feeding duration also reduces the risk of the transmission of tick-borne diseases, such as Lyme borreliosis (Crippa, Rais & Gern 2002), the flea-induced maternal effect is expected to positively affect nestling performance in the presence of ticks.

Thirty-seven nestlings (20 and 17 in the respective experimental groups) had been exposed to five fleas for 20 min just before the ticks were applied. Although this could theoretically influence tick feeding, if these nestlings had developed a defence reaction against the fleas and/or if the intensity of this reaction was influenced by the maternal effect (Anderson 1995; Mondal & Naqi 2001; Lemke, Hansen & Lange 2003), inclusion or exclusion of these 37 nestlings into the statistical models gave the same results. It is also rather unlikely that five flea bites lead to very strong defence reactions in 5-day-old nestlings, especially because studies on other birds indicate that the nestlings' immune system is not yet fully developed at this age (Grindstaff *et al.* 2003).

EFFECT OF HOST SEX

We found that 29% more ticks attached to male than to female nestlings. This result is not surprising, because differential tick infestation of male and female hosts is known for many species (Mooring, McKenzie & Hart 1996; Sorensen & Moses 1998; Aubret *et al.* 2005; Sinski *et al.* 2006). Yet, in most studies it is not possible to distinguish whether this is due to physiological differences between the sexes or due to different behaviours of males and females, that is, larger home ranges of the males (Randolph 2004) or stronger behavioural defences such as grooming in females (Mooring *et al.* 1996). In the present study, we infested all animals with the same number of ticks, that is, the experimental tick encounter rate was the same for both sexes. Also, we infested nestlings while they were still in the nest and therefore the environment was the same for males and females. In addition, an earlier experiment in our study area that involved filming of 6-day-old nestlings showed that at this age nestlings do not yet engage in self-grooming (Helfenstein, unpublished data). Thus, the different infestation rates of males and females in our experiment are probably not due to behavioural, but rather to physiological differences between the sexes. These differences might include a reduced immunocompetence in male nestlings (Tschorren *et al.* 2003) and/or increased androgen levels, that are known to affect tick feeding (Hughes & Randolph 2001). It could also

be argued, that ticks aggregate on the males, simply because they are larger, and therefore present a larger surface. However, this is rather unlikely since in our statistical model we corrected for nestling mass and therefore a factor other than mass must be responsible for the difference in tick aggregation between males and females. Nestling mass had a positive influence on the number of ticks attached. Again, this relationship could be based on the larger surface of heavier nestlings, but it is also conceivable that ticks attach on larger nestlings because they are in better condition and might therefore be better hosts, for example, due to a higher protein content of the blood.

In conclusion, our results show that host quality of great tit nestlings for ticks is influenced by several factors. First, ticks preferentially aggregate on large nestlings and on males; and second, the flea-induced maternal effect reduces tick feeding time, indicating that the presence of one parasite species can alter the host quality for another unrelated parasite species even across host generations.

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