# Parasites suppress immune-enhancing effect of methionine in nestling great tits

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**Abstract** After birth, an organism needs to invest both in somatic growth and in the development of efficient immune functions to counter the effects of pathogens, and hence an investment trade-off is predicted. To explore this trade-off, we simultaneously exposed nestling great tits (*Parus major*) to a common ectoparasite, while stimulating immune function. Using a $2 \times 2$ experimental design, we first infested half of the nests with hen fleas (*Ceratophyllus gallinae*) on day 3 post-hatch and later, on day 9–13 post-hatch, and then supplemented half of the nestlings within each nest with an immuno-enhancing amino acid (methionine). We then assessed the non-specific immune response by measuring both the inflammatory response to a lipopolysaccharide (LPS) and assessing the levels of acute phase proteins (APP). In parasite-infested nestlings, methionine had a negative effect on body mass close to fledging. Methionine had an immune-enhancing effect in the absence of ectoparasites only. The inflammatory response to LPS was significantly lower in nestlings infested with fleas and was also lower in nestlings supplemented with methionine. These patterns of immune responses suggest an immuno-suppressive effect of ectoparasites that could neutralise the immune-enhancing effect of methionine. Our study thus suggests that the trade-off between investment in life history traits and immune function is only partly dependent on available resources, but shows that parasites may influence this trade-off in a more complex way, by also inhibiting important physiological functions.

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## Introduction

The immune system is an important mediator of life history trade-offs and may limit investment of resources in other life history traits such as growth and development, or in secondary sexual traits (Norris and Evans 2000). Parasites impose strong selection on their hosts and thus play a pivotal role in the evolution of immune defence (Clayton and Moore 1997). Parasites are known to affect clutch size, brood size and thus lower the reproductive output of hosts (e.g. Oppliger et al. 1994; Richner et al. 1993). Nestlings of altricial species are particularly prone to ectoparasites, first because they are nest-bound, and second because of their naïve or underdeveloped immune system (Sol et al. 2003; Wakelin and Apanius 1997). The maternally derived antibodies deposited in the egg may then provide the primary form of immune defence. Their deposition depends on the presence of ectoparasites before egg formation (Buechler et al. 2002; Gasparini et al. 2001). The transition from relying on maternal antibodies to endogenous immune defence is species specific and most likely restricted to a short period in the early life of birds, yet knowledge is still scarce (Hasselquist et al. 2011; Killpack and Karasov 2012).

In experimental studies that provided methionine to boost immune function (Tsiagbe et al. 1987), nestling magpies and blue tits showed stronger response to an injection of phytohaemagglutinin (PHA), a measure of the T cell-mediated immune response, but lower growth rate and higher mortality (Brommer 2004; Soler et al. 2003). In mountain bluebirds, female nestlings only showed

compensated mass gain after methionine supplementation (O'Brien and Dawson 2013). In a great tit study, methionine led to reduced growth during supplementation and augmented growth after the treatment in the presence of haematophagous ectoparasites only, suggesting a parasite-mediated allocation trade-off between growth and immune function (Tschirren and Richner 2006). This was partially also confirmed in methionine-supplemented blue tits, which showed suppressed growth during the supplementation and showed higher mass gain in parasitized but not in deparasitized nestlings shortly after methionine treatment (Pitala et al. 2010). However, nestlings of all treatments compensated initial growth reduction and reached equal body size prior to fledging.

Parasite fitness may be limited by host condition and availability of specific nutrients (Bize et al. 2008; Tschirren et al. 2007), host body temperature and skin thickness (Elliot et al. 2002; Owen et al. 2009). The host defence is pathogen or parasite dependent, and integrates physiological, behavioural and morphological strategies. Inflammation is a major component of the innate immune response and an important defence mechanism of birds against blood-sucking ectoparasites, and has been shown to limit parasite access to blood that may lead to lower parasite survival (Owen et al. 2009, 2010). Biting induces tissue damage, followed by release of foreign molecules in the saliva (Owen et al. 2010). In turn, the host releases cytokines, which trigger the acute phase response (Petersen et al. 2004).

The objectives of this study were twofold: we experimentally tested whether parasite load and an immune-enhancing methionine supplementation influence the trade-off between investment in immune defence and growth, using two parameters of the innate immune response; we then tested whether previous host exposure to parasites had an effect on parasite survival.

In a $2 \times 2$ experimental design, half of the nests in a great tit (*Parus major*) population were infested with naturally occurring hen fleas (*Ceratophyllus gallinae*) after hatching, and on day 9 post-hatch when growth enters the asymptotic phase, half of the nestlings within each nest were supplemented with methionine for 4 subsequent days. Supplementation was done at this phase because we were interested in an early methionine-independent immune response towards fleas on day 9, and thereby measured the concentration of haptoglobin, an acute phase protein involved in an early non-specific immune response, and in flea survival. We measured nestling immune response by injecting a novel antigen [lipopolysaccharide (LPS) from the cell wall of *Escherichia coli*] that mimics a bacterial infection and triggers a non-specific immune response (Dunn and Wang 1995; Parmentier et al. 1998b). The response to a novel antigen was measured on two levels:

first, the skin swelling as a response to the inflammation process, which corresponds to the recruitment of cytokines at the infection site; second, we assessed the acute phase response, which is involved in the progression of inflammation and tissue repair (Barnes et al. 2002). Acute phase proteins are supposed to be elevated following an LPS challenge or an inflammation (Millet et al. 2007), and reduced under anaemic conditions (Gabay and Kushner 1999; Yee et al. 2008).

We predicted nestlings with methionine supplementation to show stronger immune response following an LPS challenge as shown in previous studies towards PHA (e.g. Brommer 2004; Soler et al. 2003; Tschirren and Richner 2006), and nestlings that were infested with fleas to show a weaker immune response. Therefore, methionine-supplemented nestlings growing up in parasite-infested nests were expected to show an intermediate immune response. Furthermore, flea survival may depend on whether the nestlings they were feeding from were in the parasite-infested or parasite-free group, provided that nestlings have a functioning immune system on day 9 post-hatch.

## Materials and methods

The study was conducted in spring 2010 in a natural population of great tits (*P. major*) breeding in nest boxes in Spilwald, a forest near Bern, Switzerland (47°56′N, 7°18′E). The great tit is one of the main hosts of the ectoparasitic hen flea *Ceratophyllus gallinae* (Tripet and Richner 1997). Hen fleas live in the nest material of hole-nesting birds and suck blood from nestlings, but also from adults that visit the nest (Tschirren et al. 2007). We regularly visited nest boxes to determine the start of incubation and hatching date (day 1). Three days after hatching of the first nestling, we cross-fostered nestlings by exchanging whole broods having the same hatching date (±1 day) and number of nestlings (±1). During the transfer, nestlings were kept warm with commercial hand-warmer bags. After brood exchange, each pair of nests was then randomly assigned to the flea treatment, i.e. flea-infested ($n = 52$) or control group ($n = 46$). All the nests were first heat treated in a microwave to eliminate all nest parasites (Richner et al. 1993). Half of the nests were then infested with 100 hen fleas (*C. gallinae*) collected from old nest material of the previous year. Three breeding pairs of the infested group versus eight in the control group abandoned their nests before day 12 post-hatching. After catching the adults 12 days post-hatch, 14 pairs of the control group and 11 pairs of the infested group abandoned their nests, potentially due to the unusually rainy and cold conditions in spring 2010 in this relevant time frame for breeding (NABEL weather station, http://www.meteosuisse.admin.ch/web/

de/klima/klima_heute/monatsflash/flash201005.html) in combination with the LPS injection of the parents. Another study in the same population but in a different year showed no desertion events after LPS injection (Losdat et al. 2011), but see (Bonneaud et al. 2003) for opposite results in house sparrows.

Nestlings were ringed on day 9 and body mass measured on the day of hatching, and days 9, 12 and 16 post-hatch using an electronic balance (Sartorius, Germany) with a precision of 0.01 g. To assess whether methionine supplementation influenced body mass gain, we compared body mass gain (1) during supplementation between day 9 and 12, and (2) after the supplementation between day 12 and 16 post-hatch. On day 16, measurements of tarsus length (±0.01 mm) and body mass were taken and blood extracted from either the foot (day 9) or the brachial vein (day 16) for haptoglobin analysis (see below). We recorded mortality of nestlings throughout the breeding period and date of fledging. The majority of the adults was captured on day 11 and injected with LPS in the wing web for the purpose of another experiment. LPS injection of the adults did not affect nestling growth between day 12 and 16 ($F_{1,62} = 2.24$, $p = 0.14$).

### Methionine treatment

Within each nest, every second nestling along the weight hierarchy was randomly assigned (by throwing a dice) to receive a methionine supplementation ($n = 297$) or tap water ($n = 283$). Methionine is a sulphur amino acid that is required during immune defence (Grimble and Grimble 1998). Methionine (DL-methionine; Sigma Chemicals, Germany) was suspended in tap water (0.1 g/ml) and supplied orally using a syringe. Experimental nestlings were provided with 200 µl dissolved methionine on days 9, 10, 11, 12 and 13 and control nestlings with an equal amount of tap water.

### Immune response of nestlings

LPS induces an inflammatory response and activates B cells regardless of their antigenic specificity. The response is independent of the presence of T cells (Kuby et al. 2007). LPS (Sigma-Aldrich) was dissolved in phosphate buffered saline with a concentration of 0.5 mg/ml. On day 15 post-hatch, all nestlings were injected with 0.02 ml dissolved LPS in the patagium (wing web), thus receiving 0.01 mg of LPS (Bonneaud et al. 2003; Coslovsky and Richner 2012). The thickness of the patagium was measured with a constant-tension dial micrometer (Mitotuyo, type 2046S) before injection and 24 h later to measure the skin swelling due to inflammation (Berthouly et al. 2008). Each measurement was taken 3 times and the strength of the swelling response was calculated as the difference between the mean

value before and after injection. The swelling difference was then corrected for the time difference between the two measurements using a regression model and the resulting residuals were used as response variable for further analyses. All nestlings in a brood were measured by the same person on both days.

### Acute phase protein concentration

The acute phase response provides an early non-specific defence mechanism against tissue destruction, infection or bacterial products (Suffredini et al. 1999). It is induced by cytokines acting as messengers between the local site of injury and the hepatocytes synthesising the acute phase proteins and is detectable for several days after the application of a stimulus (Petersen et al. 2004). Specifically, haptoglobin offers protection against harmful end products of the immune response, namely haem from damaged host cells and free radicals from phagocytes (Matson et al. 2006), and constitutively circulates in the blood at low concentrations. Concentrations can increase significantly in response to an infection or inflammation (Matson et al. 2006). We analysed the concentrations of Hp (mg/ml) from the nestling plasma (7.5 µl) with a colorimetric assay, following the manufacturer's protocol (TP801; Tridelta Development, Ireland). The absorbance was measured at 630 nm with a microplate reader (PowerWave XS reader; Witec, Switzerland). Haptoglobin concentrations were generally not normally distributed, therefore a Box-Cox transformation was applied ($\lambda = 0.1$ on day 9, $\lambda = 0.2$ on day 16) (Box and Cox 1964).

### Flea survival

The flea survival was assessed following Coslovsky and Richner (2011), to test the survival time of parasites that fed on flea-treated nestlings or controls, and to evaluate the correlation with nestling body mass. On day 9, we used groups of five male hen fleas, kept in a climatic chamber at 4 °C until the day of exposure to nestlings of rank 1 and 4 within each nest (based on body mass the second day after hatching). When leaving for the field on the mornings of the feeding trials, we placed each group of fleas in a plastic tube kept on ice until shortly before the beginning of the feeding trial. After allowing the fleas to warm up for a few minutes, we placed each group of fleas on a nestling inside a plastic bag with air holes and allowed them to feed for 20 min. Fleas were then recollected with a Falcon tube aspirator and put back in a plastic tube placed on ice. Back in the lab, fleas were separated into small individual plastic tubes containing a small amount of plaster on the bottom. We added two drops of water to the plaster to maintain humidity levels, and placed all the tubes in a climatic

chamber at 25 °C. Survival of fleas was checked daily, and mean survival time (days) calculated for each group of five fleas. In the analysis we included only fleas that survived at least 1 day after the feeding trial. We included nestling rank and body mass at the day of trial in the analysis.

## Statistical analyses

All statistical analyses were performed using R 3.0.2 (R Core Team 2013). We used linear mixed effect (LME) models [nlme package with restricted maximum likelihood estimation (Pinheiro et al. 2012)] to analyse the effect of parasite infestation and methionine supplementation on nestling body mass, growth, LPS response and levels of haptoglobin. Parasite infestation, methionine supplementation and their interaction, and sex were included as fixed factors in the models, and brood size and hatching date (brood size and hatching date were centred on the mean) as covariates. We performed post hoc tests by running the same models on the single levels. Nestling mortality was analysed with a generalised linear mixed model (glmer) using a binomial error distribution [package lme4 (Bates 2010)], excluding nests that were abandoned after catching the adults. Age at fledging was analysed with glmer and poisson error distribution. In all models we included the nest of origin as a random factor to account for the non-independence of nestlings originating from the same family or sharing the same rearing environment (since we exchanged whole broods, nest of origin accounts for the rearing environment as well). Furthermore, we removed interactions when non-significant ($p > 0.1$) in order to allow the interpretation of main effects (Engqvist 2005). Flea survival was analysed using an LME including flea infestation as fixed factor and rank, sex, body mass of the nestling and day of trial as covariates. Model validation was applied to check for normality and heteroscedasticity. We report $F$-values and $df$ from ANOVA tables for main effects and interactions and $z$-values for generalised mixed model with binomial distribution.

## Results

Nestling body mass on day 9 post-hatch, i.e. before methionine treatment started, was not affected by parasite infestation ($F_{1,88} = 0.006$, $p = 0.94$). Nestling body mass increased significantly with hatching date ($F_{1,498} = 7.99$, $p = 0.005$). However, nestling body mass on day 16 post-hatch was not influenced by parasite treatment or the methionine supplementation alone (no significant main effect), but showed a significant interaction effect between methionine supplementation and parasite infestation (Table 1; Fig. 1). To interpret the interaction effect, we analysed the model for parasite-free and parasite-infested

**Table 1** Nestling body mass on day 16 analysed with linear mixed effects models

| Variable | Estimate (±SE) | $F_{df}$ | $p$ |
|---|---|---|---|
| Intercept | 13.46 (±0.28) | | |
| Flea treat[a] | 0.54 (±0.35) | $0.18_{1,61}$ | 0.68 |
| Methionine[b] | 0.20 (±0.24) | $0.69_{1,293}$ | 0.41 |
| Sex[c] | 0.70 (±0.16) | $19.44_{1,293}$ | **<0.001** |
| Brood size | −0.41 (±0.12) | $12.68_{1,61}$ | **<0.001** |
| Hatching date | 0.04 (±0.32) | $1.44_{1,293}$ | 0.23 |
| Flea treat[a] × methionine[b] | −0.61 (±0.31) | $3.82_{1,293}$ | **0.05** |

Significant terms ($\alpha > 0.5$) are in bold

[a] Parasite-infested relative to parasite-free nests

[b] Methionine-supplemented relative to control nestlings
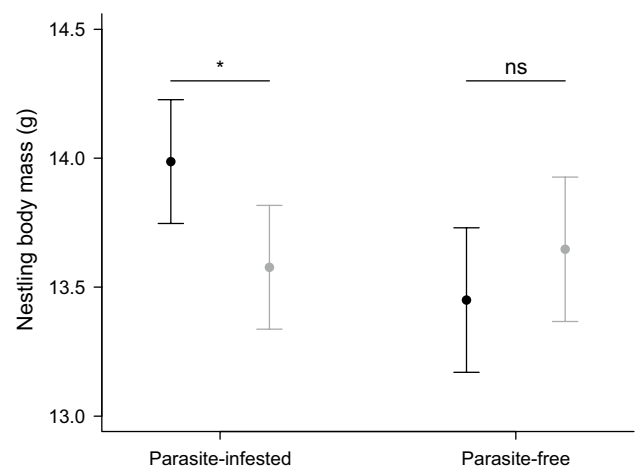
[c] Male relative to female siblings



**Fig. 1** Nestling body mass on day 16 post-hatch (model coefficients ± SE) differed significantly between methionine-supplemented (*grey*) and control nestlings (*black*) when growing up in parasite-infested nests, but did not differ in parasite-free nests (significant interaction effect of the parasite and methionine treatment, $p = 0.05$). Post hoc tests showed that the effect of methionine treatment differed in the parasite-infested broods only (indicated by an *asterisk*), and not in the parasite-free broods [not significant (*ns*)]. There is no significant difference between parasite-infested and parasite-free nests

nests separately. In parasite-free nests, methionine-supplemented nestlings ($F_{1,119} = 1.19$, $p = 0.28$) were not significantly different from control nestlings, whereas in parasite-infested nests, body mass estimates of methionine-supplemented nestlings were lower ($F_{1,172} = 4.07$, $p = 0.05$) compared to control nestlings (Fig. 1).

Nestling growth during methionine supplementation, from day 9 to 12, was not influenced by parasite infestation, methionine treatment or their interaction, and did not depend on brood size or hatching date. Males gained significantly more body weight than females (Table 2). Nestling growth after methionine supplementation, from day 12 to 16, was not influenced by parasite infestation, methionine

**Table 2** Growth rate during methionine supplementation (*Day 9–12*), after methionine supplementation (*Day 12–14*) and from before supplementation until fledging (*Day 9–16*), analysed with linear mixed effect models

| Variable | Day 9–12 | | | Day 12–16 | | | Day 9–16 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Estimate (±SE) | $F_{df}$ | *p*-value | Estimate (±SE) | $F_{df}$ | *p*-value | Estimate (±SE) | $F_{df}$ | *p*-value |
| Intercept | 2.38 (±0.66) | | | 2.61 (±0.79) | | | 4.87 (±1.15) | | |
| Flea treatment[a] | 0.09 (±0.22) | $0.16_{1,85}$ | 0.69 | 0.14 (±0.27) | $0.13_{1,61}$ | 0.72 | 0.35 (±0.39) | $0.61_{1,61}$ | 0.44 |
| Methionine[b] | 0.05 (±0.06) | $1.01_{1,459}$ | 0.32 | −0.14 (±0.08) | $2.44_{1,294}$ | 0.12 | −0.08 (±0.11) | $0.32_{1,294}$ | 0.57 |
| Sex[c] | 0.21 (±0.07) | $9.68_{1,459}$ | **<0.01** | 0.10 (±0.09) | $1.13_{1,294}$ | 0.29 | 0.31 (±0.12) | $6.92_{1,294}$ | **0.01** |
| Brood size | −0.01 (±0.08) | $0.03_{1,85}$ | 0.87 | −0.21 (±0.10) | $4.31_{1,61}$ | **0.04** | −0.20 (±0.15) | $1.93_{1,61}$ | 0.17 |
| Hatching date | 0.02 (±0.02) | $0.83_{1,459}$ | 0.36 | −0.04 (±0.03) | $2.17_{1,294}$ | 0.14 | −0.01 (±0.04) | $0.10_{1,294}$ | 0.75 |
| Flea treatment[a] × methionine[b] | | $0.05_{1,458}$ | *0.83* | | $0.21_{1,293}$ | *0.64* | | $0.46_{1,294}$ | *0.50* |

Significant terms (α > 5) are in bold and terms eliminated from the final model are in italics

[a] Parasite-infested relative to parasite-free nests

[b] Methionine-supplemented relative to control nestlings

[c] Male relative to female siblings

**Table 3** Skin-swelling response to lipopolysaccharide challenge in nestlings

| Variable | Estimate (±SE) | $F_{df}$ | *p*-value |
|---|---|---|---|
| Intercept | 1.71 (±1.64) | | |
| Flea treatment[a] | −4.42 (±2.01) | $4.87_{1,37}$ | **0.03** |
| Methionine[b] | −2.20 (±1.08) | $4.26_{1,166}$ | **0.04** |
| Sex[c] | 0.11 (±1.11) | $0.04_{1,166}$ | 0.84 |
| Brood size | −0.98 (±0.74) | $1.53_{1,37}$ | 0.22 |
| Hatching date | 0.66 (±0.22) | $9.01_{1,166}$ | **0.003** |
| Flea treatment[a] × methionine[b] | *1.35 (±2.16)* | $0.39_{1,165}$ | *0.53* |

Significant terms (α > 5) are in bold and terms eliminated from the final model are in italics

[a] Parasite-infested relative to parasite-free nests

[b] Methionine-supplemented relative to control nestlings

[c] Male relative to female siblings



**Fig. 2** Skin-swelling response towards lipopolysaccharide (*LPS*) (model coefficients ± SE) was lower in nestlings (**a**) growing up in parasite-infested nests (*Fleas*) compared to non-infested nests (*No fleas*) and (**b**) lower in methionine-supplemented nestlings (*methionine*) compared to non-supplemented nestlings (*control*)

treatment or their interaction, and also not by sex and hatching date, but compensatory growth was significantly higher in smaller broods (Table 2). Mass gain from day 9 to 16 was neither influenced by parasite infestation, methionine treatment nor their interaction. Males showed larger mass gain than females and mass gain was unaffected by brood size and hatching date (Table 2c).

The skin swelling in response to the LPS challenge was significantly lower in nestlings in flea-infested nests and lower in methionine-supplemented nestlings (Table 3; Fig. 2). There was no significant interaction between treatments. Nestlings that hatched later in the season showed a significantly higher response to LPS (Table 3).

Haptoglobin concentrations on day 9 did not differ between nestlings in parasite-free and parasite-infested nests before methionine supplementation (Table 4). However, on day 16 post-hatch, haptoglobin concentrations showed a significant interaction effect between methionine
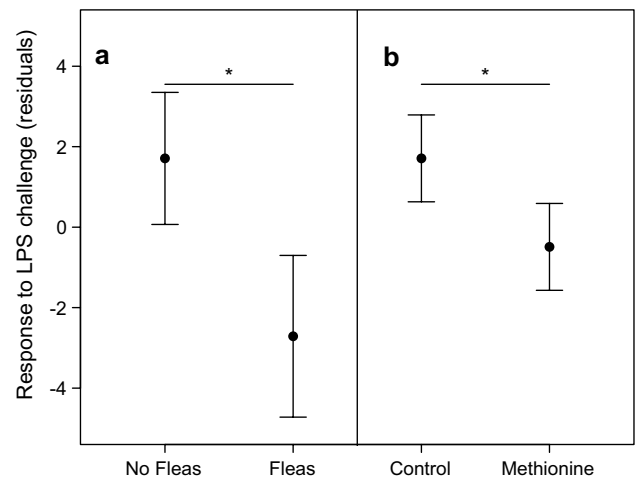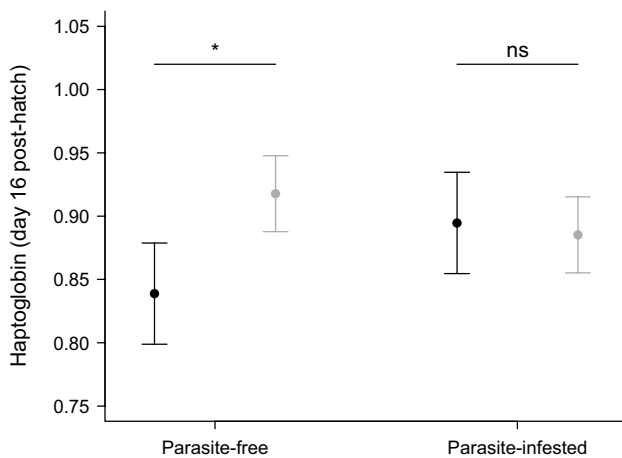
supplementation and parasite infestation (Table 4; Fig. 3). Methionine-supplemented nestlings growing up in parasite-free nests showed higher levels of haptoglobin ($F_{1,84} = 7.15$, $p = 0.01$) than controls (Fig. 3). In parasite-infested nests, however, the two groups did not differ.

Nestling mortality between the day of cross-fostering and a day before fledging was not significantly affected by parasite infestation ($z = 0.25$, $p = 0.80$), or methionine supplementation ($z = 0.50$, $p = 0.62$). Age at fledging was not influenced by parasite infestation ($z = 0.67$, $p = 0.51$), or methionine supplementation ($z = −0.12$, $p = 0.91$).

Mean flea survival was significantly higher when feeding on heavier nestlings, or on nestlings hatched later in the breeding season, but was unrelated to nestling sex, rank, or prior parasite treatment (Table 5).

**Table 4** Summary of the generalised linear mixed models testing for the effect of haptoglobin concentrations (Box-Cox transformed) in response to parasite infestation before (*Day 9*) and after (*Day 16*) methionine supplementation

| Variable | Day 9 | | | Day 16 | | |
|---|---|---|---|---|---|---|
| | Estimate (±SE) | $F_{df}$ | *p*-value | Estimate (±SE) | $F_{df}$ | *p*-value |
| Intercept | 0.85 (±0.02) | | | 0.84 (±0.04) | | |
| Flea treatment[a] | 0.03 (±0.02) | $2.92_{1,64}$ | 0.09 | 0.06 (±0.05) | $0.04_{1,56}$ | 0.84 |
| Methionine[b] | | | | 0.08 (±0.03) | $2.51_{1,192}$ | 0.11 |
| Sex[c] | −0.01 (±0.01) | $0.39_{1,198}$ | 0.54 | 0.01 (±0.02) | $0.22_{1,192}$ | 0.64 |
| Brood size | −0.01 (±0.01) | $1.05_{1,64}$ | 0.31 | −0.01 (±0.02) | $0.13_{1,56}$ | 0.72 |
| Hatching date | −0.002 (±0.002) | $1.20_{1,198}$ | 0.27 | −0.01 (±0.005) | $1.31_{1,192}$ | 0.25 |
| Flea treatment[a] × methionine[b] | | | | −0.09 (±0.04) | $4.97_{1,192}$ | **0.03** |

Significant terms (α > 0.5) are in bold

[a] Parasite-infested relative parasite-free nests

[b] Methionine-supplemented relative to control nestlings

[c] Male relative to female siblings



**Fig. 3** Nestling haptoglobin concentrations on day 16 post-hatch (model coefficients ± SE) showed a significant interaction between the methionine supplementation and parasite treatment. Post hoc tests showed that in parasite-free nestlings, methionine-supplemented nestlings (*grey*) showed significantly higher haptoglobin concentrations than control nestlings (*black*) as indicated with an *asterisk* ($F_{1,84} = 7.15$, $p = 0.01$); however, this effect was not apparent in parasite-infested nests (*ns*, $F_{1,106} = 0.09$, $p = 0.77$). There is no difference in haptoglobin levels between parasite-infested and parasite-free nests

**Table 5** ANOVA table and coefficients of linear mixed effect model for flea survival

| Variable | Estimate (±SE) | $F_{df}$ | *p*-value |
|---|---|---|---|
| Intercept | −2.67 (±1.84) | | |
| Flea treatment[a] | 0.34 (±0.41) | $0.27_{1,80}$ | 0.61 |
| Rank | 0.01 (±0.13) | $0.14_{1,56}$ | 0.71 |
| Nestling body mass | 0.16 (±0.16) | $14.31_{1,56}$ | **<0.01** |
| Sex | 0.51 (±0.43) | $2.33_{1,56}$ | 0.13 |
| Date of experiment | 0.18 (±0.05) | $14.54_{1,56}$ | **<0.01** |

Significant terms (α > 0.5) are in bold

[a] Whether nestlings were exposed to parasites before or not

## Discussion

Nestling immune defence mainly relies on maternal antibodies; however, the transition from reliance on these maternal antibodies to the development of their own immune system is only little understood (Hasselquist et al. 2011; Killpack and Karasov 2012). Parasites may trigger the development of the nestlings' own immune defence (Tschirren and Richner 2006). Hence, we infested nestlings with blood-sucking ectoparasites, the hen flea, and simultaneously supplemented half the nestlings with the immune-enhancing amino acid methionine to test the predicted trade-off between investments in immune function versus growth. The immunocompetence was measured by the skin swelling and the concentration of circulating haptoglobin after an LPS challenge.

Parasite infestation after hatching had no effect on nestling body mass on day 9 or 16, and no overall effect on haptoglobin concentrations on day 9 and 16. However, on day 16 post-hatch, the skin-swelling response to LPS was lower in nestlings infested with fleas. This would be predicted if a specific response against fleas limited a further response towards pathogens. Furthermore, it is likely that saliva of fleas contains immunosuppressive molecules that activate an anti-inflammatory TH2 response, as has been found in sand flies and ticks, which is often associated with the presence of extracellular pathogens (reviewed in Andrade et al. 2005; Boughton et al. 2011). Ectoparasite infestation has been shown to bias the host immune response towards the TH2 response favouring the parasite's survival (Andrade et al. 2005; Harrington et al. 2010; Wikel and Alarcon-Chaidez 2001), thus, parasites may indeed have an immunosuppressive effect.

Methionine supplementation did not result in an altered growth rate, which is in contrast to previous studies where

nestlings showed impaired growth under methionine supplementation (Brommer 2004; Soler et al. 2003; Tschirren and Richner 2006). In these studies nestlings were supplemented at the beginning of growth from day 3 to 6, whereas we supplemented them from day 9 to 13, hence rather towards the end of the main growth phase. Methionine was expected to boost the immune responses. Interestingly, the skin-swelling response and the acute phase response showed an opposite pattern in the presence of methionine. We found that methionine-supplemented nestlings showed a lower skin-swelling response towards LPS, but higher haptoglobin concentrations in the absence of parasites. A possible explanation for a lower swelling response in methionine-supplemented nestlings may be due to altered protein metabolism during an acute phase response (Barnes et al. 2002) or IL-1/TNF-α activity (Parmentier et al. 1998a), and may therefore advance the peak of the maximum LPS response, and thus remain undetected if tested 24 h later. Former studies found evidence for a higher immune response towards PHA, a cell-mediated immune response, which however may be different from a response to LPS that is eliciting a more specific response (Bize et al. 2010). Alternatively, methionine helps synthesise glutathione, an important antioxidant that may suppress inflammatory components, but enhances components related to cell-mediated immunity (Grimble 2006). Although inflammation is an important part of the early immune response, there is also a risk of immunopathology (Sorci and Faivre 2009). However, this is speculative and more studies are needed to provide insight into the different arms of the immune response, and specifically the inflammation process with different feedback loops to avoid immunopathology or autoimmune responses.

Yet, parasite and methionine effects are interlinked. We found that nestling immune response towards a novel antigen was mediated by parasite infestation since flea infestation in combination with methionine had an interacting effect on nestling immune response. This suggests that fleas induce an immunological stress, for example by enhancing the anaemic condition of nestlings (Boughton et al. 2006; e.g. Richner et al. 1993). Haptoglobin is known to be downregulated under anaemic conditions (Brus and Lewis 1959; Gabay 1999; Rogerson 2006), and it may thus explain the interaction effect between parasite infestation and methionine supplementation. A further indication for an interaction between parasites and methionine was the negative effect on nestling body mass on day 16 post-hatch in the presence of ectoparasites in methionine-supplemented nestlings. Besides the immune-enhancing effect, methionine is the amino acid most susceptible to oxidation by free radicals (Stadtman et al. 2003) and may lead to a potential increase in the production of reactive oxygen species (Pamplona and Barja 2007). Thus, our results show that the trade-off between investment in life history traits (e.g. growth, survival) and immune functions is not only dependent on resources, but that parasites can mediate this trade-off in a more complex way by also inhibiting important other functions.

Flea survival after feeding on a nestling on day 9 mainly depended on nestling body mass, as also shown in other studies (Tschirren et al. 2007). It was also influenced by trial date, indicating that fleas profited more from nestlings born later in the season. Previous exposure of nestlings to fleas did not affect flea survival. This indicates that flea survival on nestlings still depends on maternal effects (Coslovsky and Richner 2011; Gallizzi et al. 2008).

The findings suggest that the immune system of nestlings is not fully developed at the age of 9 days post-hatch. Haptoglobin levels were detectable on day 9 and increased after LPS challenge on day 16, and were influenced by the combined parasite infestation and methionine supplementation. Thus, it seems that nestlings were still relying on maternal antibodies by the age of 9 days, but developed their own immune response around fledging age. This is in agreement with a previous study in nestling pied flycatchers, where total immunoglobulin levels were low at the age of 5 days and increased until the age of 14 days (Grindstaff et al. 2006), and also with a study in great tits where natural antibody expression was fully developed by the end of the nestling stage (De Coster et al. 2010). Other studies on magpies (Pihlaja et al. 2006) and house sparrows (King et al. 2010) suggest an earlier loss of maternal antibodies in the neonates' system, yet provide no information on the endogenous immune function of these nestlings. Further studies are needed to investigate this transition in a more systematic manner. The interpretation of the functioning of the immune system is complex (Schmid-Hempel 2009), and different arms of the immune system cover different facets (Gonzalez-Braojos et al. 2013), and may thus allow for different interpretations of our results.

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