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Ectoparasites help to maintain variation in cell-mediated immunity in the blue tit–hen flea system

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ABSTRACT

Hypothesis: Host–parasite interactions have the potential to maintain genetic variation, especially in traits related to defence against parasites.

Organism: Blue tit (*Cyanistes caeruleus*) nestlings.

Methods: Rear nestlings in artificially created conditions of low and high abundance of ectoparasitic hen fleas (*Ceratophyllus gallinae*). Cross-foster siblings between these environments to estimate parasite-induced genotype–environment interactions. Measure components of phenotypic variance in morphology (tarsus length and body mass) and in immune defence (plasma immunoglobulins and cell-mediated immunocompetence).

Results: Hen flea infestation lowered growth, cell-mediated immunocompetence, and haematocrit. Genotype–environment interactions were observed in cell-mediated immunocompetence, body mass, and haematocrit, with significant crossing reaction norms for cell-mediated immunocompetence and haematocrit. Immunoglobulin concentrations were unaffected by fleas. Thus, host–parasite interactions constrain the expression and evolvability of some traits, but also create genotype–environment interactions with the potential to maintain genetic variation in immune defence.

Keywords: ecological immunology (immunoecology), ectoparasites, evolutionary quantitative genetics, reciprocal cross-fostering, restricted maximum likelihood linear mixed model.

INTRODUCTION

Host–parasite interactions are thought to be a major force in maintaining genetic variation in traits, because the co-evolutionary dynamics between host and parasite is a continuous process whereby hosts adapt for defence against parasites and parasites adapt for overcoming host defences (Hamilton, 1980; Hamilton and Zuk, 1982). From the host's point of view, parasites

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create different environments, which interact with genetic effects to give rise to phenotypic plasticity, where a single genotype presents different phenotypes as a function of the environment (Pigliucci, 2005). The functions mapping genotypes to environment are termed 'reaction norms' (Via *et al.*, 1995; Lynch and Walsh, 1998) and these may cross, resulting in changes in the ranking of genotypes when parasite loads differ – that is, there is a low genetic correlation across parasite environments (Lynch and Walsh, 1998). Under environmental heterogeneity, this type of genotype–environment interaction has the possibility to maintain genetic variation in traits subjected to directional selection, because – depending on what environment is experienced – a different genotype will be selected for (Lynch and Walsh, 1998). On the other hand, the amount of additive genetic variance may vary between environments (Hoffmann and Parsons, 1991; Hoffmann and Merilä, 1999), which can cause differences in heritability (the proportion of phenotypic variance due to additive genetic effects). This latter form, however, is not by itself expected to contribute to the maintenance of genetic variation, as the ranking of genotypes does not change between environments.

The study of immune defence in an ecological context has become one of the major topics in behavioural ecology (Hamilton, 1980; Hamilton and Zuk, 1982; Sheldon and Verhulst, 1996; Norris and Evans, 2000; Ricklefs and Wikelski, 2002). In general, studies in natural populations (reviewed in Hoffmann and Merilä, 1999; Merilä and Sheldon, 2001; Charmantier and Garant, 2005) have mostly considered trait heritability as a function of nutritional or weather conditions, and have typically found lower heritabilities in unfavourable conditions, suggesting that adaptation to parasite-rich conditions may be constrained. In this context, immunological traits are especially interesting, because they may be involved in the host's response to parasites and are thus selectively important (e.g. Møller and Saino, 2004). Aspects of the immune defence have been shown to be under genetic control in laboratory conditions (Wakelin and Apanius, 1997) and in free-living vertebrate populations (Coltman *et al.*, 2001; Svensson *et al.*, 2001; Råberg *et al.*, 2003). Hence, unfavourable conditions created by parasites may suppress the evolutionary potential to adapt an efficient immunological defence against them, which may have profound implications for trait evolution in a host–parasite system. On the other hand, while parasites may constrain trait variation, they potentially also create a mechanism for maintaining variation in host traits if the trait's reaction norms cross between parasite environments. For example, in blue tits *Cyanistes caeruleus*, blowfly (*Protocalliphora* sp.) ectoparasites suppress the additive genetic variance of the host's tarsus length, but also cause crossing reaction norms between clean and infested nests. The latter aspect provides a mechanism for maintaining genetic variation for size in a heterogeneous environment (Charmantier *et al.*, 2004b). Whereas we have a reasonable understanding of genotype–environment interactions in size traits, our current knowledge of the sources of observed variation and the evolutionary potential of immunity traits in natural environments is still relatively poor.

Here, we use a host–parasite system consisting of the blue tit and its ectoparasite, the hen flea (*Ceratophyllus gallinae*), to investigate causes of variation in immune parameters and growth traits of nestlings. Hen fleas are common in nests of passerines, and they may exert a strong influence on hosts' development and fitness (e.g. Clayton and Moore, 1997). Here, we experimentally alter the intensity of hen flea parasitism in nests, and cross-foster nestlings between these environments. Within this set-up we explore the effects of hen fleas on two 'traditional' measures of growth (tarsus length and body mass) and three indices of immunity and physiological condition: cell-mediated immunocompetence, measured as the hypersensitivity response to phytohaemagglutinin; plasma total concentration of immunoglobulins; and haematocrit. We quantify the broad-level genetic parameters as 'nest

of origin'-related (which includes genetic, but also maternal and part of the genetic dominance variation) versus environmental components of phenotypic variance in these traits. In particular, by adopting a reaction norm approach, we examine the possibility that genotype–environment interactions either constrain (by negatively affecting heritability) or maintain (by generating crossing reaction norms) the evolvability of various traits.

MATERIALS AND METHODS

Study system

Blue tits (*Cyanistes caeruleus*) are small hole-nesting passerines that readily accept nest-boxes. They have a relatively large clutch size (average = 10–12 eggs) and a short period of development (young hatch after 2 weeks of incubation, reach their final size 2 weeks post-hatching, and fledge at the age of 18–22 days). Nests of tits are often infested with haematophagous hen fleas (*Ceratophyllus gallinae*) (Harper *et al.*, 1992; Tripet and Richner, 1999). Hen flea larvae feed on detritus from the nest; adult fleas suck blood from their hosts, but otherwise live in the nest material.

The study was conducted in spring 2005 in two populations breeding in nest-boxes: on the Swedish island of Gotland (57°10′N, 18°20′E) in the Baltic and in southern Finland (Ekenäs: 60°01′N, 23°31′E). Before the onset of breeding, nest-boxes were cleaned and old nests collected to obtain fleas for subsequent infestations. Boxes were regularly monitored to determine laying date of the first egg, clutch size, and hatching date. Hatching date was defined as the day when the first chick hatched (= day 0).

Manipulation of ectoparasite load

Broods were paired according to hatching date and number of hatchlings, allowing a maximal difference of two hatchlings (mean difference = 0.59, S.D. = 0.78). The pair of broods is referred to as a 'dyad' hereafter. On day 2 post-hatching, original nest material was replaced with another blue tit nest that had been heat-treated for several minutes in a microwave oven. Heat treatment kills all nest-based ectoparasites (e.g. Richner *et al.*, 1993). Special care was taken to clean the box well and remove all of the original nest-material. Then, one randomly chosen nest within a dyad was re-infested with 200 adult fleas ('flea-infested' nest) while the other remained uninfested ('parasite-free' nest). As immigration rates of hen fleas are low (Heeb *et al.*, 1996), the parasite-free nest should remain fairly free from parasites until the end of the nestling period. The reproductive cycle of hen fleas takes at least 3 weeks (Harper *et al.*, 1992), so the development of nestlings should therefore only have been affected by the number of adult fleas we added to the nests. The number of fleas added falls within the natural range observed in non-manipulated nests (own data). On Gotland all infestations were made using fleas from the local population, but in Finland some nests were infested with fleas from Gotland.

Parental response to ectoparasite manipulation

In the Gotland population, the feeding behaviour of parents was video-recorded for one hour, when their offspring were 9 days old. Recordings were made between 07.00 and 12.00 h local time, at the same time for both nests in a dyad. Feeding rates were expressed as the total number of visits to the nest (by male and female) in the hour.

Cross-fostering

Simultaneously with ectoparasite treatment, we performed a partial cross-fostering procedure. We swapped half of the nestlings from each brood between nests within a dyad, so that siblings from one family were raised in two different ('parasite-free' and 'flea-infested') environments. All nestlings were individually identifiable throughout the protocol. Before cross-fostering, each nestling was marked by clipping a specific combination of its nails and it was ringed on day 9. Before cross-fostering, nestlings were weighed with an electronic balance (to the nearest 0.1 g) and the treatment ('stay' or 'swap') was alternated within this weight hierarchy. The treatment of the heaviest nestling was decided at random. The mean difference in initial mass between means of sibling groups reared together was 0.27 ± 0.23 g. The differences in mass on day 2 between sibling group means were not correlated with differences in mass on day 16 ($r = 0.13$, $P > 0.4$). Therefore, a significant component of origin would not arise simply because of initial size differences and the advantage of one of the sibling groups in within-nest competition (Merilä, 1996). Nestlings were weighed again on day 5. For other research purposes, half of the nestlings were fed a supplement on days 5–9 containing amino-acid methionine, which has been shown to directly affect blue tit cell-mediated immunocompetence and growth (see Brommer, 2004). However, in the present experiment, methionine supplementation had no effect on any of the traits we measured (methionine supplementation as a fixed-effect term and its interaction with other fixed-effect terms in analyses following the mixed model structure outlined below, $P > 0.6$ in all cases). To simplify the presentation of results, we did not include this factor in the final statistical models.

Assessment of immunocompetence

At the age of 13 days, nestlings' cell-mediated immunocompetence was assessed as a response to phytohaemagglutinin (PHA; Sigma, code L8754) injection. This assay is widely used in avian studies to measure cell-mediated immunity *in vivo*. We used the simplified protocol described by Smits *et al.* (1999) and injected intradermally 0.04 ml of 5 $mg \cdot ml^{-1}$ PHA solution in saline into the right wing web. The thickness of the wing web at the site of injection was measured to the nearest 0.01 mm with a thickness gauge (Mitutoyo 700-117SU, with spring removed) twice before injection (repeatability: 94.0%, $P < 0.0001$) and three times 24 h ($\pm$ 1 h) after injection (repeatability: 94.2%, $P < 0.0001$). The difference between mean post-injection and mean pre-injection measurements (wing web index) was used as a measure of cell-mediated immunocompetence. All nestlings in each population were injected and measured by one observer. This response reflects acquired T-cell-mediated immunocompetence (Tella *et al.*, 2008).

As further physiological variables, we measured haematocrit and the concentration of immunoglobulins in the blood plasma. On day 16, a blood sample was collected into a haematocrit capillary, after which the capillary was sealed and stored in the cold until it was further processed later the same day. Samples were centrifuged at 5000 $rev \cdot min^{-1}$ for 5 min. Haematocrit (cell fraction in the total sample volume) was measured using sliding digital callipers, after which plasma and blood cells were separated. Plasma was stored at $-20°C$ until immunoglobulin analysis.

Nestlings' immunoglobulin concentration was determined with an indirect enzyme-linked immunosorbent assay (ELISA) using commercial anti-chicken immunoglobulin-G

(IgG) antibody (10 $\mu l \cdot ml^{-1}$, C6409, Sigma Chemicals Co., St. Louis, MO, USA). This method has been validated for several wild bird species, including *Parus* species and blue tit (for details, see Müller *et al.*, 2004; Kilpimaa *et al.*, 2005; Pihlaja *et al.*, 2006). In the immunoglobulin analysis, 96-well microplates (ImmunoPlate Maxisorp, Nunc Co., Nunc A/S, Roskilde, Denmark) were first coated overnight at 4°C with IgG antibody. After emptying, the wells were saturated for 1 h with 1% bovine serum albumin (BSA, Roche Diagnostics GmbH, Manheim, Germany) prepared in phosphate-buffered saline (PBS, pH 7.4), and then washed three times with PBS-Tween 20 (0.25%). Samples were diluted with 1% BSA/PBS and each sample incubated in duplicate (50 µl per well, sample dilutions 1 : 100 and 1 : 2000) for 3 h at room temperature. Pooled plasma samples of nestlings were used as calibrators and they were prepared as serial dilutions for generating the standard curve. The immunoglobulin concentrations in the samples are presented relative to this standard. An arbitrary value of 10^6 units equals the mean concentration of the individuals of the pooled sample. After washing, alkaline phosphatase conjugated anti-chicken IgG antibody (A-9171, Sigma Chemical Co.) was added and the plates were incubated overnight at 4°C (dilution of 1:10,000). Finally, after a final washing, *P*-nitrophenyl phosphate (1 $mg \cdot ml^{-1}$, Sigma Chemical 104 Phosphatase Substrate) in a diethanol amine buffer (1 $mol \cdot l^{-1}$, pH 9.8) was applied. The optical density was read at 405 nm with a plate reader (Multiskan Ascent, Therma Oy, Finland).

Measurements of growth

When nestlings were 16 days old, we measured their final size. Body mass was measured with a Pesola spring balance to the nearest 0.1 g. Tarsus length was measured twice using sliding digital callipers to the nearest 0.1 mm (repeatability: 97.9%, $P < 0.0001$).

Statistical procedures

Data were analysed using restricted maximum likelihood (REML) linear mixed models, with the program S-PLUS 6.1 (Insightful Corporation) following the modelling approach detailed by Pinheiro and Bates (2000). This approach hinges on introducing random effects within a series of hierarchical models of increasing complexity. First, we built a mixed model with fixed effects including 'fleas' (experimental manipulation of ectoparasites), 'methionine treatment', 'population', and their interactions as explanatory variables, and brood identity as a random effect. These models were simplified by removing all non-significant interactions and factors using maximum likelihood to solve the mixed model, until minimal adequate models were obtained (e.g. Crawley, 2002).

The same fixed-effects structure was retained in analysing the effects of the cross-fostering experiment. The significance of inclusion of each additional random effect could then be tested with a likelihood ratio test in a comparison with the hierarchically simpler model. We considered random effects in the following order: (1) 'Dyad', which accounted for differences between dyads in brood size, timing, and other properties. (2) 'Origin (dyad)', which described the effect of common origin. In a partial cross-fostering design, the origin variance component estimates the sum of half the additive genetic variance, a quarter of dominance variance, and all pre cross-fostering effects. (3) Random slopes across the flea treatment, which allowed for the interactions 'fleas × dyad' and 'fleas × origin (dyad)', where the former effect allows for variation in the rearing environment across flea treatment and

the latter expresses the interaction between origin-specific effects and flea treatment [for structure and interpretation of these random effects, see Merilä (1996, 1997)]. This approach allows for rigorous testing of the random effects. The full model provided REML estimates of all variance–covariance components and the correlations, including their 95% confidence intervals. Significant crossing of the origin-specific reaction norms across flea environments was inferred using the full model's REML-estimated 95% confidence interval of the correlation between elevation ('origin (dyad)') and slope ('fleas × origin (dyad)'). Because we consider two environments ('parasite-free' and 'flea-infested'), reaction norms between the environments cross in the case where this correlation differs from unity (Lynch and Walsh, 1998). To visualize the reaction norms, we generated Best Linear Unbiased Predictor (BLUP) values for each trait for each family (i.e. origin) in both parasite-free and flea-infested environments. By connecting the flea environment-specific BLUPs, origin-specific reaction norms can be displayed graphically for each trait.

The study is based on 389 nestlings, from 44 families, that survived to day 13. Three of these nestlings died before day 16 and for some nestlings not all traits were measured, thus sample sizes differ between analyses. Because of logistic constraints, we could not measure immunoglobulin concentrations of nestlings from two dyads, and the sample size for this variable was therefore lower.

RESULTS

Parental response to ectoparasite

Parental feeding rate did not differ between parasite-free and flea-infested nests (ANOVA accounting for brood size: $F_{1,25} = 0.603$, $P = 0.45$), indicating that any observed effects of parasite treatment on nestlings were directly caused by fleas and not by altered parental behaviour in providing resources to their offspring.

Nestling traits in response to ectoparasite infestation

Experimental manipulation of ectoparasite load significantly reduced nestlings' growth: nestlings reared in infested nests had shorter tarsi and weighed less than nestlings reared in parasite-free nests (Fig. 1). The differences in body mass were already visible on the third day after manipulation [weight on day 5: parasite-free 5.00 ± 0.07 (S.E.); flea-infested 4.51 ± 0.07; REML mixed model $F_{1,344} = 15.83$, $P = 0.0001$). Haematocrit values were significantly lower in flea-infested nests (Fig. 1). Ectoparasites also tended to reduce measures of immunocompetence: Nestlings from flea-infested nests had lower cell-mediated immunocompetence than nestlings from parasite-free nests, although the difference was only marginally significant in the full mixed model (Fig. 1). However, plasma immunoglobulin concentration on day 16 was not significantly affected by experimental treatment (Fig. 1).

Origin effects and their interaction with the flea environment

Origin-specific effects accounted for a significant part of variation in tarsus length, cell-mediated immunocompetence, plasma immunoglobulin, and haematocrit (Table 1, Fig. 2). However, origin-specific effects on body mass were weak (Fig. 2) and non-significant

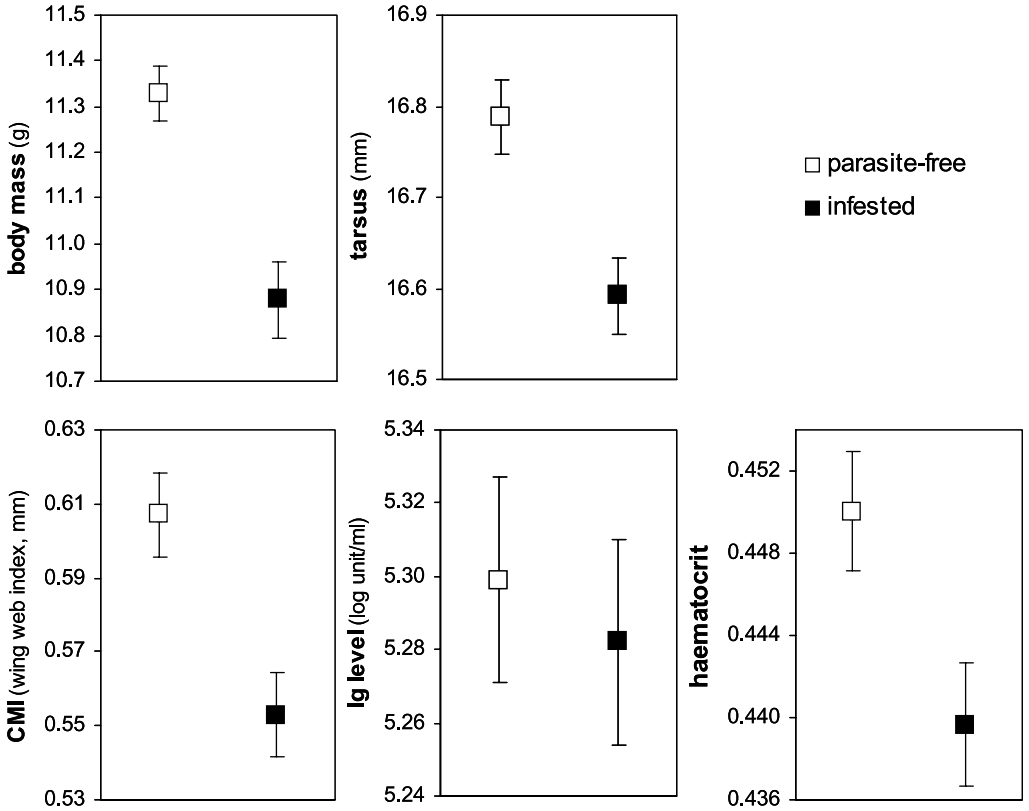

Fig. 1. Effect of flea infestation on body mass, tarsus length, cell-mediated immunocompetence (CMI), immunoglobulin (Ig) concentration, and haematocrit of nestlings (mean ± S.E., raw data). Results of tests for the effect of flea treatment in REML mixed model: body mass, $F_{1,341} = 4.5$, $P = 0.03$; tarsus length, $F_{1,340} = 4.3$, $P = 0.04$; CMI, $F_{1,344} = 3.2$, $P = 0.08$; Ig concentration, $F_{1,299} = 0.02$, $P = 0.88$; haematocrit, $F_{1,334} = 4.9$, $P = 0.03$.

(Table 1). Rearing effects, expressed by the 'fleas × dyad' interaction, were significant for all studied traits (Table 1) and were most pronounced for body mass, for which they explained 25% of the variation (Fig. 2). We found indications of an interaction between the flea environment and origin-specific effects for cell-mediated immunocompetence, body mass, and haematocrit, whereas this interaction did not explain any variation in tarsus length or plasma immunoglobulin concentration (Table 1, Fig. 2).

The reaction norms of different families across ectoparasite environments showed various patterns for different traits. Cell-mediated immunocompetence showed a low cross-environment origin-specific correlation (REML-estimated $r = -0.409$; 95% CI: −0.873, 0.442), indicating significant crossing reaction norms (see Fig. 3). We found a similar though less clear pattern for haematocrit (REML-estimated $r = 0.703$; 95% CI: −0.959, 0.999). For body mass, ranking of families was similar in the two environments (REML-estimated $r = 1.000$; 95% CI: −1.000, 1.000), although origin-specific variance was higher in flea-infested nests than in parasite-free nests, resulting in different slopes of the reaction norms (Fig. 3). Reaction norms for tarsus length and immunoglobulin

Table 1. Comparison of linear mixed-effects models fitted for body mass, tarsus length, cell-mediated immunity (CMI), immunoglobulin (Ig) concentration, and haematocrit of nestlings

Model	Random effect structure	d.f.	AIC	logLik	Models compared	L.Ratio	*P*
Body mass							
1		4	1116.4	−554.2			
2	D	5	1066.1	−528.0	2 vs. 1	52.33	<0.0001
3	D, O(D)	6	1068.0	−528.0	3 vs. 2	0.12	0.730
4	**D, O(D), F × D, F × O(D)**	**10**	**959.5**	**−469.7**	**4 vs. 3**	**116.51**	**< 0.0001**
5	D, F × D	7	963.0	−474.5	4 vs. 5	9.57	0.023
Tarsus length							
1		3	685.9	−340.0			
2	D	4	651.1	−321.5	2 vs. 1	36.87	<0.0001
3	D, O(D)	5	621.6	−305.8	3 vs. 2	31.47	<0.0001
4	**D, O(D), F × D, F × O(D)**	**9**	**605.0**	**−293.5**	**4 vs. 3**	**24.61**	**0.0001**
5	D, F × D	6	636.6	−312.3	4 vs. 5	37.63	<0.0001
CMI							
1		4	−300.3	154.2			
2	D	5	−315.0	162.5	2 vs. 1	16.69	<0.0001
3	D, O(D)	6	−323.9	168.0	3 vs. 2	10.91	0.001
4	**D, O(D), F × D, F × O(D)**	**10**	**−369.9**	**195.0**	**4 vs. 3**	**54.03**	**< 0.0001**
5	D, F × D	7	−353.9	184.0	4 vs. 5	21.98	0.0001
Ig concentration							
1		4	296.4	−144.2			
2	D	5	279.4	−134.7	2 vs. 1	18.93	<0.0001
3	D, O(D)	6	275.1	−131.6	3 vs. 2	6.36	0.012
4	**D, O(D), F × D, F × O(D)**	**10**	**250.9**	**−115.5**	**4 vs. 3**	**32.15**	**< 0.0001**
5	D, F × D	7	255.2	−120.6	4 vs. 5	10.22	0.017
Haematocrit							
1		4	−1387.4	697.6			
2	D	5	−1444.5	727.2	2 vs. 1	59.38	<0.0001
3	D, O(D)	6	−1444.6	729.5	3 vs. 2	4.45	0.035
4	**D, O(D), F × D, F × O(D)**	**10**	**−1453.4**	**736.7**	**4 vs. 3**	**14.46**	**0.006**
5	D, F × D	7	−1452.4	733.2	4 vs. 5	7.02	0.071

Random effects: dyad (D), nest of origin nested in dyad (O(D)), interaction between fleas (experimental treatment) and dyad (F × D), and interaction between fleas and origin nested in dyad (F × O(D)). Random effects were sequentially added and their significance assessed with the likelihood ratio test in a comparison with the simpler model. The structure of the models requires that two interactions (F × D and F × O(D)) are added in the same step. To provide more information on the significance of F × O(D), the fit of the model containing dyad and its interaction with fleas, but without origin and its interaction with fleas, is also given. Model #1 includes fixed effects only. The Akaike information criterion (AIC) and log likelihood (logLik) and likelihood ratio (L.Ratio) are given. The best models are indicated in **bold** font.

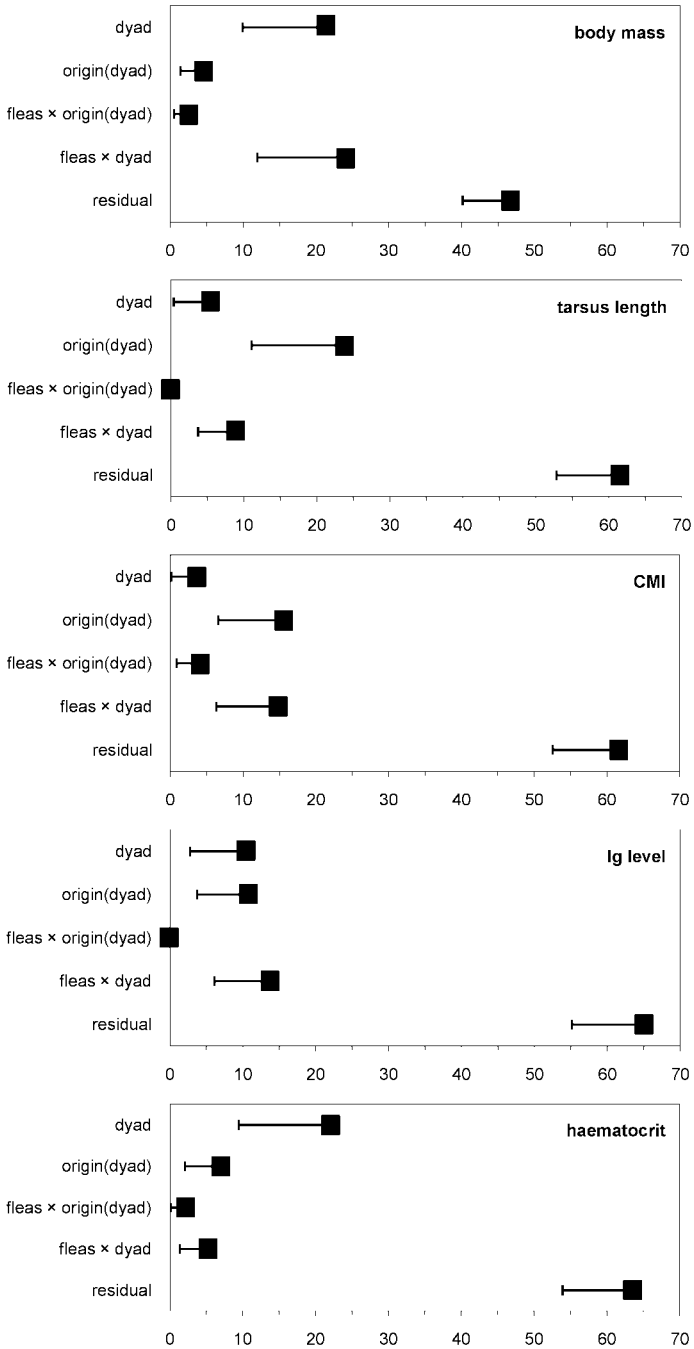

Fig. 2. Components of variance (percentage) for body mass, tarsus length, cell-mediated immunocompetence (CMI), immunoglobulin (Ig) concentration, and haematocrit of nestlings. Lower 95% confidence interval is indicated. 'Fleas × origin(dyad)' expresses a genotype–environment interaction and 'fleas × dyad' is an effect of nest of rearing.

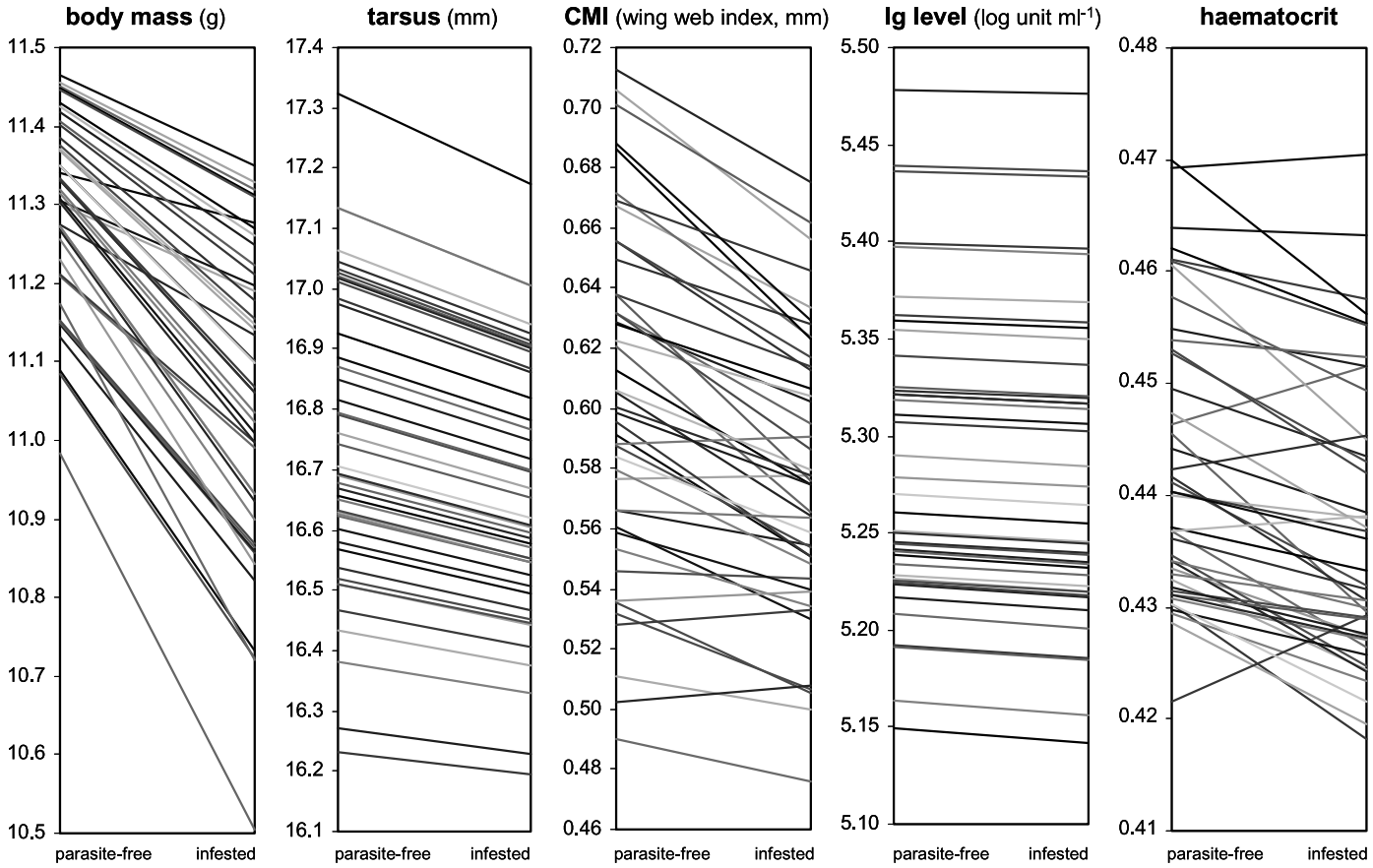

Fig. 3. Origin-specific values of body mass, tarsus length, cell-mediated immunity (CMI), immunoglobulin (Ig) concentration, and haematocrit. Values are the BLUP (Best Linear Unbiased Predictor) for 'origin' derived from the best-fitting REML linear mixed-effect models in Table 1. Lines connect BLUPs for siblings raised in different ectoparasite environments. BLUPs are used here as an illustration only, and the correlation across flea environments presented in the text is based on the full REML mixed model of Table 1.

concentration were parallel (Fig. 3), as illustrated by the lack of contribution of the 'fleas × origin (dyad)' term to phenotypic variance in these traits (Fig. 2). Estimated maximal heritability (twice the proportion of phenotypic variance explained by nest-of-origin effects) of tarsus length was 0.47 and of immunoglobulin concentration 0.21.

DISCUSSION

Blue tit nestlings raised in flea-infested nests suffered impaired growth, shown by a lower body mass and tarsus length at the end of the nestling period. Moreover, the general physiological condition of nestlings, as quantified by their haematocrit, was reduced in ectoparasite-infested nests. In general, negative effects of hen fleas are expected and are well documented in the closely related great tit (e.g. Richner *et al.*, 1993; Richner and Tripet, 1999; Fitze *et al.*, 2004). We measured two aspects of immune defence, cell-mediated immunocompetence and a measure of the humoral response (immunoglobulin concentration). Immunoglobulin concentration remained largely unchanged when exposed to fleas. In line with previous work on great tits (Tschirren *et al.*, 2003), we found lowered cell-mediated immunocompetence in hen flea-infested nests. Mounting an immune response is expected to be costly (Klasing and Leshchinsky, 1999), and a trade-off between cell-mediated immunocompetence and growth has been demonstrated experimentally in blue tits (Brommer, 2004). However, growth and cell-mediated immunocompetence were both reduced by hen fleas, and immunoglobulin concentration also tended to be lower. Hence, a trade-off does not appear to be involved. It is possible that hen fleas are so effective in reducing the amount of available resources that both allocation to immune defence and growth are reduced. Pitala and colleagues (N. Pitala, H. Siitari, L. Gustafsson and J.E. Brommer, submitted) found that supplementing food to blue tit nestlings that are exposed to hen fleas strongly increases their cell-mediated immunocompetence, suggesting that hen fleas cause this component of the immune system to be resource limited. In general, arthropod ectoparasites also have the capacity to down-regulate host defences (Schoeler and Wikel, 2001), suggesting that a flea-induced reduction in growth and immunological measures may be unrelated events.

We observed a significant origin-related component of variation in immune parameters. First, origin effects explained approximately 10% of variance in plasma immunoglobulin concentrations. High heritabilities of antigen-specific humoral responses have been reported in great tits (Kilpimaa *et al.*, 2005) and blue tits (Råberg *et al.*, 2003). Second, we found significant effects of origin on cell-mediated immunocompetence. The results of earlier work, mostly based on cross-fostering, are equivocal, as some found no heritable variation in cell-mediated immunocompetence (Christe *et al.*, 2000; Soler *et al.*, 2003; de Neve *et al.*, 2004; Kilpimaa *et al.*, 2005; Cucco *et al.*, 2006) whereas others did find evidence of a significant 'nest of origin'-related influence similar to that found in the present study (e.g. Saino *et al.*, 1997; Brinkhof *et al.*, 1999; Tella *et al.*, 2000; Ardia and Rice, 2006; Cichoń *et al.*, 2006). Importantly, however, Pitala *et al.* (2007) found that relatively strong nest-of-origin effects on collared flycatcher cell-mediated immunocompetence were not due to additive genetic variance. In particular, cell-mediated immunocompetence is known to correlate with heterozygosity in some species (Hawley *et al.*, 2005; Reid *et al.*, 2005), and estimates of the origin effect on cell-mediated immunocompetence may thus contain genetic dominance effects. Furthermore, maternal effects on nestling immunocompetence are known to occur, at least for nestlings' immunoglobulin concentrations (Grindstaff *et al.*, 2003, 2006; Kilpimaa *et al.*, 2007).

Genotype–environment interactions

We found little evidence that traits that are more closely related to fitness show reduced environment-dependent heritability, as suggested by a meta-analysis (Charmantier and Garant, 2005). On the one hand, traits that capture nestling condition and are presumably related to fitness, such as body mass (Tinbergen and Boerlijst, 1990) and heamatocrit, do appear to have higher heritability under favourable conditions. On the other hand, the effect of nest of origin on nestling cell-mediated immunocompetence, which is a known correlate of offspring recruitment in this population (Cichoń and Dubiec, 2005), is lowered by the presence of fleas in the nest (unfavourable environment). Nevertheless, these three traits all show evidence of a genotype–environment interaction, because their reaction norms either fan out (body mass) or cross (cell-mediated immunocompetence, haematocrit). Our findings thus illustrate the importance of not only quantifying changes in estimates of additive genetic variance and heritability across an environment, but also of quantifying the correlation between environments.

We found no interaction between origin-specific effects and the flea environment for tarsus length, which is strong evidence for an absence of a genotype–environment interaction across hen flea environments. This is unlikely to be due to power limitations of our cross-fostering design, as our estimated heritability of tarsus length (0.47) is very close to the narrow-sense heritability estimated for a Corsican population [0.47–0.49 (Charmantier *et al.*, 2004a)]. This lack of a genotype–environment interaction on tarsus length is surprising, because an earlier cross-fostering study in this population showed crossing reaction norms of tarsus length between control and unfavourable conditions [created by increasing brood size (Merilä and Fry, 1998)]. Furthermore, Charmantier *et al.* (2004b) observed a marked increase in heritability of tarsus length in Corsican blue tit nests where all blowfly larvae were experimentally removed, as well as a low narrow-sense cross-environment genetic correlation. Apparently, hen fleas create an unfavourable environment that is different from increased sibling competition or blowfly parasitism, implying that conclusions about the consequences of environmental heterogeneity for a genotype–environment interaction in a particular system are not easily generalized to others.

Three traits showed the potential for a genotype–environment interaction across hen flea environments. Because the ranking of families in body mass does not change across flea environments, selection is expected to favour the same genotypes. Selective consequences of nestling haematocrit are unknown and the consequences of a genotype–environment interaction in this component cannot therefore be evaluated. Given that nestling cell-mediated immunocompetence correlates with recruitment in this population (Cichoń and Dubiec, 2005), the clear and highly significant change in ranking of families in their cell-mediated immunocompetence across hen flea environments has an important implication: heterogeneity in the exposure to fleas has the potential to maintain variation in cell-mediated immunocompetence in the face of selection. In addition, maternal effects may contribute to these effects, because the ranking of the families is based on their nest-of-origin effects and thus contain maternal effects. Maternal indirect effects on offspring cell-mediated immunocompetence are possible (e.g. Koutsos *et al.*, 2007), but additional work is required to address the issue of how much of the origin-specific variance in cell-mediated immunocompetence is due to maternal effects, whether there is any genetic component to such a maternal effect, and whether such effects are related to maternal exposure to hen fleas. While it remains to be shown that cell-mediated immunocompetence is causally involved in lowering hen flea

fecundity, Tschirren and colleagues (2007) recently suggested that variation in cell-mediated immunocompetence may contribute to controlling hen fleas. Certain families (whether due to additive genetic or maternal effects) may be much better than others in suppressing hen flea ectoparasites and this genotype–environment interaction may play an important role in host–parasite co-evolutionary dynamics between blue tits and hen fleas.

Selection is expected to erode genetic variance for a trait, but – in a heterogeneous environment – a genotype–environment interaction has the potential to maintain variation (Lynch and Walsh, 1998). We found evidence that the ranking of trait values for families was maintained across parasite environments for the morphological traits, although it showed an increase in variance (reaction norms fanning out) for body mass. For cell-mediated immunocompetence and haematocrit, we detected a significant change in the ranking of trait values across families between the parasite environments (crossing reaction norms). Although more detailed work is needed to evaluate the additive genetic components in these patterns, our results suggest that ectoparasitism is a potentially powerful factor in maintaining variation in measures of immunocompetence in the wild.

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