Causes and consequences of variation in nestling immune function

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List of original articles

This thesis is based on the following articles (chapters), which are referred to in the text by their Roman numerals:

I *N. Pitala, H. Siitari, L. Gustafsson, J.E. Brommer*
Costs and benefits of increased investment in immune function under differential exposure to ectoparasites
manuscript

II *N. Pitala, H. Siitari, L. Gustafsson, J.E. Brommer*
Benefits of increased food availability depend on exposure to parasites: an experiment with blue tit nestlings and haematophagous fleas
manuscript

III *N. Pitala, H. Siitari, L. Gustafsson, J.E. Brommer*
Exploring the role of genotype-environment interactions in maintaining/constraining evolvability in an avian host-ectoparasite system
manuscript

IV *N. Pitala, L. Gustafsson, J. Sendecka, J.E. Brommer*
Nestling immune response to phytohaemagglutinin is not heritable in collared flycatchers
Biology Letters (2007) 3: 418–421

V *N. Pitala, S. Ruuskanen, T. Laaksonen, L. Gustafsson*
The effects of experimentally manipulated yolk androgens on growth and immune function of male and female nestling collared flycatchers
manuscript

VI *N. Pitala, L. Gustafsson, J. Sendecka, J.E. Brommer*
Against the rules: nestling immunocompetence index and the probability of local recruitment in a population of collared flycatchers
manuscript

Contributions

	I	*II*	*III*	*IV*	*V*	*VI*
Initial idea	JB	NP	JB, NP	JB	NP, TL	NP
Study design and field methods	NP, JB	NP, JB	NP, JB	—	NP, TL, SR	—
Field data	NP, JB	NP	NP, JB	NP, JS, LG, JB	NP	NP, JS, LG, JB
Logistic support	LG	LG	LG	LG	TL, LG	LG
Laboratory methods	HS	HS	HS	—	—	—
Data analysis	NP	NP	NP, JB	NP	NP	NP, JB
Manuscript preparation	NP, JB	NP	NP, JB	NP, JB	NP	NP

All co-authors have commented on the manuscripts.

Key:

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JB Jon E. Brommer

LG Lars Gustafsson[1]

HS Heli Siitari[2]

JS Joanna Sendecka[1]

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Kochani, ta książka jest dla Was! (a szczególnie ostatnie strony ;-)

Abstract

Defence against pathogens is a vital need of all living organisms that has led to the evolution of complex immune mechanisms. However, although immunocompetence – the ability to resist pathogens and control infection – has in recent decades become a focus for research in evolutionary ecology, the variation in immune function observed in natural populations is relatively little understood. This thesis examines sources of this variation (environmental, genetic and maternal effects) during the nestling stage and its fitness consequences in wild populations of passerines: the blue tit (*Cyanistes caeruleus*) and the collared flycatcher (*Ficedula albicollis*).

A developing organism may face a dilemma as to whether to allocate limited resources to growth or to immune defences. The optimal level of investment in immunity is shaped inherently by specific requirements of the environment. If the probability of contracting infection is low, maintaining high growth rates even at the expense of immune function may be advantageous for nestlings, as body mass is usually a good predictor of post-fledging survival. In experiments with blue tits and haematophagous hen fleas (*Ceratophyllus gallinae*) using two methods, methionine supplementation (to manipulate nestlings' resource allocation to cellular immune function) and food supplementation (to increase resource availability), I confirmed that there is a trade-off between growth and immunity and that the abundance of ectoparasites is an environmental factor affecting allocation of resources to immune function. A cross-fostering experiment also revealed that environmental heterogeneity in terms of abundance of ectoparasites may contribute to maintaining additive genetic variation in immunity and other traits.

Animal model analysis of extensive data collected from the population of collared flycatchers on Gotland (Sweden) allowed examination of the narrow-sense heritability of PHA-response – the most commonly used index of cellular immunocompetence in avian studies. PHA-response is not heritable in this population, but is subject to a non-heritable origin (presumably maternal) effect. However, experimental manipulation of yolk androgen levels indicates that the mechanism of the maternal effect in PHA-response is not *in ovo* deposition of androgens.

The relationship between PHA-response and recruitment was studied for over 1300 collared flycatcher nestlings. Multivariate selection analysis shows that it is body mass, not PHA-response, that is under direct selection. PHA-response appears to be related to recruitment because of its positive relationship with body mass. These results imply that either PHA-response fails to capture the immune mechanisms that are relevant for defence against pathogens encountered by fledglings or that the selection pressure from parasites is not as strong as commonly assumed.

Summary

1. Introduction

The immune system evolved because protection against micro- and macroparasites is a prerequisite for survival. The usefulness of immune defence does not raise doubts. The vertebrate immune system is a most complex machinery enabling defence against pathogens, but the need for antiparasite defence is common to all living organisms, and all – vertebrates, invertebrates and plants – have developed immune mechanisms. However, the variation in immune function observed in natural populations is relatively little understood. What are the sources of this variation, how is this variation maintained, and what are its consequences? How does variation in the level of immune defence translate into variation in fitness? The answer to this question may seem trivial, as it is intuitive that immunity contributes to survival. But this is obvious only for immuno-incompetent individuals, unable to survive in non-sterile environments. An example is severe combined immunodeficiency disease (SCID) in humans. Infants born with SCID due to genetic defects lack both cellular and humoral immune defences and, unless immunity is reconstituted by medical intervention, die of infections in the first year of life (Chan and Puck 2005). Immunocompetence is, however, a quantitative trait, and the fitness implications of variation in the level of immune defence depend on the strength of the selective pressure of pathogens in relation to other sources of selection. This implies that the optimal investment in immune defence is shaped by environmental conditions.

In my thesis I have focused on immune function during development. Life history theory predicts trade-offs between fitness components which arise from allocation of limited resources to different traits (Stearns 1992). Conditions experienced during ontogeny have a major influence on performance in adulthood (Lindström 1999; Metcalfe and Monaghan 2001). The trade-offs during development may be especially important, since they may shape an individual's phenotype in an irreversible way. Patterns of growth may affect many physiological processes, entailing consequences that may be expressed much later in life (Metcalfe and Monaghan 2003). A trade-off that is likely to occur in developing organisms between growth and immune function (Soler *et al.* 2003; Brommer 2004) is of particular interest, as both body size and immunocompetence may be important components of fitness. Nonetheless, little is currently known about the ecological interactions that underlie the outcome of this important trade-off.

In my studies I used nestlings of two passerine species and investigated what forces shape their investment in immune function and how immune function relates to fitness. Fig. 1 presents a framework of my thesis. A central theme in my research has been the trade-off between growth and immune function in developing individuals. The outcome of this trade-off is governed by genes, maternal effects and environmental conditions, and their interactions. In particular, environmental factors especially relevant for growth and immune function are parasites and food resources. The nestling phenotype (here, growth and immunocompetence expressed during the nestling stage) determines an individual's survival and adult phenotype.

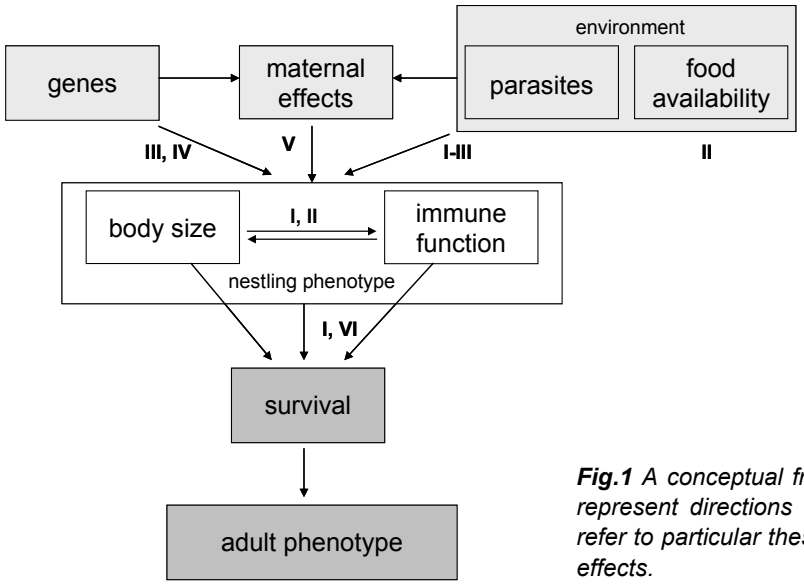

Fig.1 *A conceptual framework of the thesis. Arrows represent directions of influence. Roman numerals refer to particular thesis chapters investigating these effects.*

2. Empirical approaches of the thesis – methods and goals

Studies performed under controlled laboratory conditions and observational studies investigating natural variation in focal traits are of obvious value, and in some cases they may be the only feasible empirical approaches. Nevertheless, inference of the causality from correlative data is generally not possible. The main advantage of laboratory studies, i.e. eliminating background variation coming from multiple unidentified and possibly confounding sources, is also a limitation: a simplified and standardized laboratory environment does not allow establishment of the real-life importance of phenomena under investigation. My credo as an empiricist is as follows: (1) studying organisms in their natural environment, using (2) carefully designed experimental manipulations, and (3) adopting a long-term perspective. Integrating these three elements offers the possibility of determining the ecological and evolutionary significance of studied traits and processes in the most unambiguous way. This is rarely possible to achieve, but in research included in this thesis I tried to approach this methodological ideal.

The following sections give an overview of the study systems and methods I used in my research on the evolutionary ecology of nestling immune function, as well as the objectives of applying these methods (see Chapters **I**-**VI** for technical details).

2.1. Populations under study

2.1.1. Study area

The studies were conducted in the southern part of the Swedish island of Gotland in the Baltic Sea (ca. 57˚10`N, 18˚20`E). The study area consists of separate woodland patches equipped with nest-boxes, surrounded by agricultural land (Fig. 2a). Nest-box

plots are mostly deciduous forests, dominated by oak (*Quercus robur*) and ash (*Fraxinus excelsior*), with an understory of hazel (*Corylus avellana*), and include also some "änge" (forest hay-meadows) – remnants of ancient rural landscape (Fig. 2b) – and coniferous forest, with pine (*Pinus silvestris*) and birch (*Betula pubescens*) (Fig. 2c). Nest-boxes are occupied by collared flycatchers (*Ficedula albicollis*), great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*), some coal tits (*Parus ater*), and occasionally nuthatches (*Sitta europea*).

In the central part of the study area, nest-box plots make up most of the land suitable for hole-breeding birds. Thus, inspection of nest-boxes provides a good control of the local populations.

Data for Chapter **III** were also collected in southern Finland, north-east of the city of Tammisaari (60˚01`N, 23˚31`E). The study area of 6 km^2 consists of a mixed forest (spruce *Picea abies*, pine and birches) interspersed with agricultural land and lakes, a landscape typical for southern Finland.

2.1.2. The blue tit and its parasite

The blue tit (*Cyanistes caeruleus*) is a small hole-nesting passerine, readily breeding in nest-boxes and common throughout the western Palearctic. In the Gotland population females lay a single clutch per season, consisting on average of 10-12 eggs, which they incubate for approximately two weeks. Young fledge at the age of 18-22 days and reach independence about two weeks post-fledging. Both parents participate in provisioning offspring with food; nestling diets consist mainly of caterpillars. In years of adverse weather conditions nestling mortality can be substantial (Chapter **I**), but the large clutch size enables applying within-brood treatments (Chapters **I**-**III**, see § 2.4.2., 2.4.3.). Recruitment of nestlings to the local population is low (1-3 %), hence the possibility of follow-up studies on recruits in this species was limited.

For the purposes of this thesis, the blue tit is particularly interesting as a host of ectoparasites. Blue tit nests are commonly infested with haematophagous hen fleas (*Ceratophyllus gallinae*) (Harper *et al.* 1992). Hen fleas and their main hosts, blue and great tits (*Parus major*), are well studied systems: hen fleas were found to negatively affect development of nestlings and increase their mortality, lower the condition and survival of parents attending infested nests, and influence future reproduction of nestlings exposed to fleas (e.g. Richner *et al.* 1993; Richner and Tripet 1999; Fitze *et al.* 2004). There is some evidence for induced immune defence against hen fleas (Walker *et al.* 2003; Tschiren *et al.* 2007), although its mechanism is largely unknown.

Hen fleas time their reproduction cycle to the breeding of their hosts and may produce 2-3 generations per season (Harper *et al.* 1992). Adult fleas that overwinter in bird nest-sites start to reproduce when hosts become permanently present in nests, as female fleas need a blood meal to produce eggs (Harper *et al.* 1992). Larvae feed on organic debris from the nest material. Fleas develop from eggs to imagos in approximately three weeks (Harper *et al.* 1992), and imagos of the first offspring generation emerge around the hatching time of nestlings. After the nestlings have fledged, some fleas spin cocoons and remain dormant until new hosts appear the next spring, while the others gather around the entrance to the nest-site and disperse.

Fig. 2 *Habitats of the Gotland study area in early spring. Photo by N. Pitala*

(a) *Study area consists of separate forest patches; here ecoton between deciduous forest and cultivated field.*

(b) *forest hay-meadow*

(c) *mixed coniferous forest*

2.1.3. The collared flycatcher

The collared flycatcher (*Ficedula albicollis*) is a small, migrant passerine, wintering in central Africa and breeding in central and eastern Europe. The Gotland population (as well as the population of another Baltic island, Öland) is isolated from the main species range and is characterized by high returning rates of adults and high recruitment of juveniles (Pärt and Gustafsson 1989; Pärt 1991). Flycatchers arrive from the wintering grounds at the end of April to mid-May, and produce one brood per season. They willingly occupy nest-boxes. Females lay 4-8 eggs and incubate the clutch alone for approximately two weeks. Nestlings are fed by both parents, largely with flying insects (Sendecka and Bolund 2007), and leave the nest 13-18 days after hatching. After fledging, the young are fed by the parents for another two weeks. Most males are monogamous, but some take up another, separate territory, and ca. 9% of males attract a second female there (Gustafsson and Qvarnström 2006). Extra-pair copulations are common, and approximately 15% of all nestlings are sired by extra-pair mates (Sheldon and Ellegren 1999).

2.2. Growth, morphology and condition

The recurrent theme in the thesis is the investment in immunocompetence versus somatic growth. I weighed young at intervals throughout the nestling period to monitor their development and recorded their final morphology shortly before fledging, using several standard measures in ornithology. Tarsus length is an index of skeletal size; since skeletal growth ceases in birds after the nestling period, tarsus measurements indicate nestlings' adult body size (e.g. Merilä and Fry 1998). Body mass reflects, to a large extent, body fat reserves, i.e. an individual's nutritional status (condition). Wing and tail length are good indicators of the pace and stage of development, since altricial birds can leave the nest only after they have grown long enough feathers.

In addition to morphological and immunological (§ 2.3.) traits, I measured haematocrit (packed cell volume in blood samples) as an index of physiological condition. Low haematocrit values are indicative of anaemia and poor nutrition (e.g. Potti *et al.* 1999).

2.3. How to measure immunocompetence?

Immunocompetence can be defined as an ability to resist parasites or control disease. Generally, there are two different approaches to measuring immunocompetence: monitoring and challenge methods (reviewed in Norris and Evans 2000). Monitoring methods quantify the levels of elements involved in immune responses, such as different types of leukocytes, immunoglobulins, or other immunologically active components (e.g. Gustafsson *et al.* 1994; Ots and Hõrak 1996; Zuk and Johnsen 1998), the assumption being that these levels correlate with the effectiveness of an individual's immune system. For instance, higher immunoglobulin levels would indicate better protection

against pathogens. Another form of monitoring is assessment of parasite loads (e.g. blood parasites or ectoparasites). Here, lower parasite load would be interpreted as an indication of higher immunocompetence, since healthier individuals are probably the ones that are more efficient in fighting-off pathogens. The interpretation is, however, complicated by the fact that under natural conditions the exposure to parasites will differ between individuals in a manner that is usually uncontrollable for researchers. Low parasite load may indicate high parasite resistance, or simply living in a parasite-free environment. Similarly, elevated levels of immunoglobulins or leucocytes may indicate ongoing infection, and not superior immunity (Norris and Evans 2000).

These problems with the interpretation of monitoring results have prompted ecologists to assess immunocompetence as a response to a standard immunological challenge (Norris and Evans 2000). These methods involve introducing a standard amount of non-pathogenic, novel antigen into an organism (e.g. Nordling *et al.* 1998; Ilmonen *et al.* 2000; Råberg *et al.* 2003), which simulates infection and forces the immune system to respond, but eliminates the harmful effects of pathogen multiplication in host tissue. The subsequent immune response is then quantified, and its strength is assumed to positively correlate with an individual's immunocompetence, i.e. that individuals with strong response to artificial antigen would also respond effectively to any antigen occurring in their environment.

In my studies I used both a challenge and a monitoring method, commonly applied in avian ecological studies. I quantified hypersensitivity responses to phytohaemagglutinin (PHA) (challenge), and concentrations of circulating antibodies (Ig level) (monitoring). PHA is a plant lectin that stimulates the proliferation of circulating T-lymphocytes (but is also mitogenic to other cell types). Response to PHA is not specific, as PHA stimulates many T-cell lineages. PHA injected under the skin (in small birds in the wing web) produces inflammation and a local swelling, the size of which reflects the strength of the immune response. PHA-response consists of an array of immune mechanisms, involving also macrophages, basophils, heterophils (Martin *et al.* 2006b), and therefore is a composite measure of cellular activity of the immune system.

The concentration of plasma immunoglobulins (IgY and IgM) was quantified in 16 day old blue tit nestlings as an index of the humoral immune function. This measure reflects the general efficiency of B-lymphocytes (Apanius 1998). A newly hatched bird is totally reliant on maternally derived antibodies and its own immunoglobulin production starts several days after hatching (Apanius 1998). Specific antibody responses arise following a time lag, it is therefore not feasible to measure a specific humoral response to a standard challenge prior to fledging.

2.4. Experimental manipulations

2.4.1. Manipulation of ectoparasite load

The basis of the blue tit studies included in this thesis (Chapters **I**-**III**) was manipulation of the intensity of ectoparasitism by hen fleas. It is possible to control the infestation by sterilizing nests in a microwave oven (e.g. Richner *et al.* 1993), since hen fleas

are nest-based parasites, dwelling in the nest material. By sterilizing all nests and re-infesting some of them with a fixed number of adult fleas (within the range observed in non-manipulated nests) standard differences in the intensity of flea parasitism can be introduced. The parasite treatment was performed shortly after the nestlings had hatched. This treatment created two types of environmental conditions for nestling development (deparasitized and flea-infested) differing in nestling food demand, as fleas drain resources from the hosts, but also in stimulation of the immune system, as ectoparasitism constitutes an immunological challenge for the host (see Results and discussion).

2.4.2. Methionine treatment

A direct manipulation of investment in immunocompetence is needed to demonstrate its fitness importance (Sheldon and Verhulst 1996; Norris and Evans 2000). Such a manipulation was carried out in three recent studies on wild passerine birds (Soler *et al.* 2003; Brommer 2004; Tschirren and Richner 2006), by supplementing nestlings with methionine, a sulphur amino acid, showing immunoenhancing properties.

Methionine is involved in the synthesis of glutathione (Grimble 2006), intracellular concentrations of which affect functions of T-cells, such as cytotoxicity and proliferation (Liang *et al.* 1989; Gmünder and Dröge 1991; Grimble and Grimble 1998; Grimble 2006). In birds, methionine supplementation increases *in vivo* response to PHA (Tsiagbe *et al.* 1987; Soler *et al.* 2003; Brommer 2004; Tschirren and Richner 2006). Magpie (*Pica pica*) nestlings supplemented with methionine also had lower blood parasite loads (Soler *et al.* 2003), which indicates that a methionine-induced increase in PHA-response may reflect a biologically significant improvement in immune defences. An addition of methionine to natural nestling diets, applied in wild bird studies (Soler *et al.* 2003; Brommer 2004; Tschirren and Richner 2006), does not change the amount of resources (proteins or energy) available to nestlings for growth or development of physiological functions, but should alter their resource allocation (see Soler *et al.* 2003) by stimulating them to increase investment in their immune system.

In Chapter **I**, methionine supplementation was applied in combination with the parasite treatment (§ 2.4.1) in order to study whether and how the costs and benefits of increased investment in immune defences depend on environmental conditions determined by ectoparasitism. Methionine treatment (supplementation or control) was applied within nests and alternated between nestlings along the weight hierarchy, to control for size/age related effects.

2.4.3. Food supplementation

In Chapter **II**, I continued to investigate how optimal allocation decisions (growth vs. immune defence) depend on environmental conditions. I reversed the experimental set-up from the previous study and instead of manipulating the allocation (Chapter **I**) of naturally available (presumably limited) resources, I provided some nestlings with additional food, this way manipulating resource availability. Also in this study, I applied

a supplementation treatment within nests and in combination with the parasite treatment to study whether nestlings would differentially allocate extra resources depending on the presence of parasites.

Nestlings were fed once a day with either Nutri-plus gel (Virbac), a high-energy nutritional supplement, or with NutriBird A21 (Versele-laga), a complete hand-rearing formula for birds, with a dosage corresponding to 10-15% of their daily energy expenditure. Control nestlings did not receive any supplements, but were otherwise handled similarly to experimental nestlings (i.e. taken out and kept outside of the nest for the duration of feeding).

2.4.4. Yolk androgen manipulation

In Chapter **V** I studied maternal effects mediated by yolk androgens in the collared flycatcher. Birds provide a good system to study hormone-mediated maternal effects, because an embryo develops in the closed environment of an egg, outside the mother's body. This enables the experimental manipulation of prenatal exposure to hormones, without interference with the mother (Groothuis *et al.* 2005b). Androgen hormones deposited by avian females in the egg yolk have been shown to affect a variety of offspring traits, including immune function (Groothuis *et al.* 2005b). I manipulated androgen levels by injecting physiological doses (within the natural range of variation) of testosterone and androstenedione dissolved in sesame oil into the yolk of eggs at the start of incubation. Control eggs received injections with oil only. I assigned whole clutches to the same treatment, to study between-nest effects of elevated yolk concentrations of androgens.

2.4.5. Cross-fostering

Partial cross-fostering is an experimental technique allowing for the separation of origin-related effects (genetic and maternal) from the effects of post-natal environments. Nestlings are exchanged between nests, and sibling groups are reared apart, together with unrelated nest-mates. Cross-fostering is often used in avian quantitative genetic studies (Merilä and Sheldon 2001), though heritability estimates based on cross-fostering may be inflated by maternal and other early environmental effects, and by non-additive genetic effects (Lynch and Walsh 1998).

In Chapter **III**, nestlings were cross-fostered between deparasitized and flea-infested nests, thus nestlings originating from one family were raised under different exposures to ectoparasites. This allowed me to study the changes in broad-sense heritability in relation to environmental conditions and genotype-environment interactions.

In Chapter **IV**, I combined cross-fostering with an animal model statistical technique (Kruuk 2004). Animal models are efficient in separating additive genetic from other origin-related effects and this approach allowed estimation of the narrow-sense heritability of PHA-response in the collared flycatcher.

3. Results and discussion

3.1. Of fleas and birds…

3.1.1. Ectoparasites and host immune responses

Clearly, hen fleas have detrimental effects on their blue tit hosts. Nestlings from parasitized nests have reduced growth: body mass, tarsus length, feather growth, and lowered haematocrit (Chapter **I**, **II**, **III**; Fig.3). However, the strength of this effect can vary between years (cf. Chapters **II** and **III**). Haematophagous ectoparasites exert a pressure on their hosts by resource drainage, acting as vectors for other pathogens (e.g. Schoeler and Wikel 2001), and creating wounds that may further increase the risk of secondary infections. Infestation with ectoparasites thus constitutes an immunological challenge. Vertebrate immune systems respond to this challenge and both innate and adaptive cell-mediated responses, as well as humoral responses, are invoked by arthropod ectoparasites (e.g. Brossard and Wikel 2004). Host immune responses can directly counteract ectoparasites (Wikel *et al.* 1996; Walker *et al.* 2003; Tschirren *et al.* 2007). However, the relationship between ectoparasites and host immune responses is not well understood in ecological literature.

PHA-response involves both innate and acquired cell-mediated immunological mechanisms (Martin *et al.* 2006b). If those mechanisms are used in defence against ectoparasites (or infections associated with ectoparasites), one might expect elevated PHA-response in individuals subjected to ectoparasites. However, manipulation of ectoparasite load typically either has no effect on PHA-response (Merino *et al.* 2001; Brinkhof *et al.* 1999; Saino *et al.* 1998; Tschirren *et al.* 2005; Tschirren *et al.* 2007, Chapter **I**) or lowers PHA-response (Christe *et al.* 2000; Berthouly *et al.* 2007; Chapter **III**; Fig. 3). Interpretation of the relationship between response to PHA and ectoparasites is complicated by two factors. Firstly, ectoparasites can downregulate immune responses of their hosts (Schoeler and Wikel 2001). Immunomodulatory effects of ectoparasites often involve a shift from T helper 1 (cell-mediated) immune responses to T helper 2 (antibody) responses (Schoeler and Wikel 2001). Thus, an increased investment in immune function in response to parasitism may be masked by the immunomodulating components of flea saliva. Secondly, comparing the swelling induced by injecting PHA between parasitized and non-parasitized nests does not appear to accurately describe the relevant immunological responses involved. In great tit nestlings previously exposed to hen fleas, a higher PHA-response was associated with lowered hen flea fecundity, whereas PHA-response of naïve hosts was not related to the fitness of fleas (Tschirren *et al.* 2007). This suggests that the mechanism of PHA-response as such is not directly involved in the defence against ectoparasites, but PHA-response may rather be an index of the ability of an organism to develop adequate immune defences.

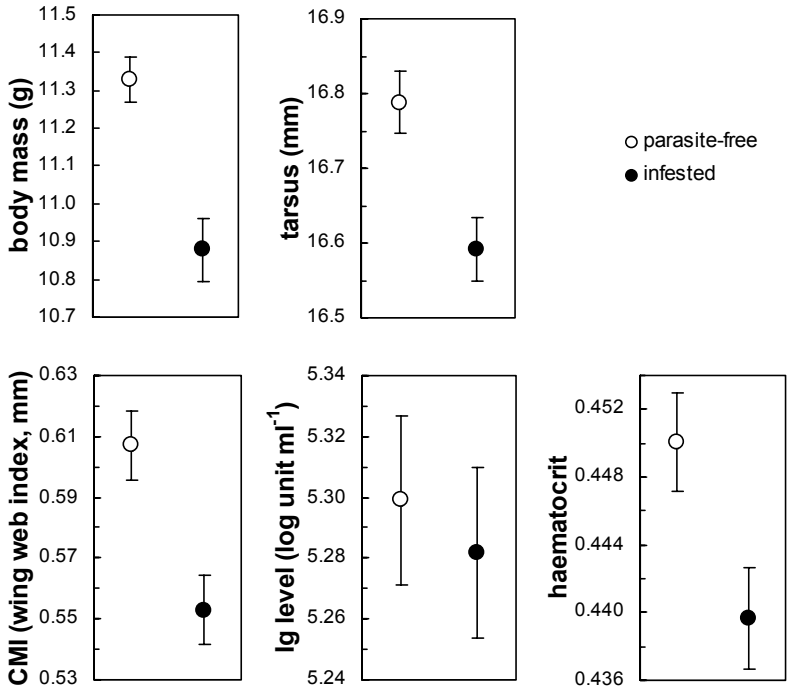

Fig. 3 *Hen fleas have a clear negative effect on blue tit hosts. Nestlings raised in flea-infested nests are smaller and in worse condition than nestlings from ectoparasite-free nests. From: Chapter **III**.*

Total Ig levels – reflecting humoral immune defence – were, in my studies, in general not affected by hen flea ectoparasitism (Chapter **I**, **III**; Fig.3) although the production of immunoglobulins is known as a response to components of ectoparasitic arthropod saliva (Wikel *et al.* 1996), and was shown to be an effective host response to hen fleas in the closely related great tit (Buechler *et al.* 2002). I did not measure the concentration of immunoglobulins specific to antigens from hen flea saliva, hence it is possible that a generally high level of exposure to other antigens overwhelmed any specific humoral response to hen fleas. However, I found that providing nestlings with additional food increases their Ig levels in flea-infested nests, but not in parasite-free nests (Chapter **II**). This result may be interpreted as adaptive allocation of resources, in agreement with the findings that antibody responses may be efficient in defence against ectoparasitic arthropods.

3.1.2. Growth-immunity trade-off and optimal investment in immune function

Understanding the significance of immune defence and its role in life histories requires assessment of the costs and benefits associated with immune defence. Evolutionary ecology has focused on the costs (Schmid-Hempel and Ebert 2003) which can be attributed to building up the immune machinery and maintaining a competent immune system, and to using it, i.e. responding to an immunological challenge (Lochmiller and Deerenberg 2000; Schmid-Hempel 2003). The costs of immune defence can be expressed as trade-offs between immune function and other aspects of an individual's performance (e.g. Ilmonen *et al.* 2000; Råberg *et al.* 2000; Bonneaud *et al.* 2003; Kilpimaa *et al.* 2004) and as the risk of autoimmune pathology (Råberg *et al.* 1998). To demonstrate the benefits of immunocompetence, i.e. a causal relationship between a fitness

component (e.g. survival) and immune function, the latter needs to be manipulated to break up its relationship with condition (Sheldon and Verhulst 1996; Norris and Evans 2000). This is because a higher amount of resources at an individual's disposal is likely to increase its immune defence as well as survival, irrespective of whether there is a causal link between them.

The optimal level of investment in immunity is shaped inherently by specific requirements of the environment. If the probability of contracting infection is low, maintaining high growth rates even at the expense of immune function may be advantageous for nestlings, as body mass is usually a good predictor of post-fledging survival (Nur 1984; Tinbergen and Boerlijst 1990). Also, shortening the nestling period may decrease

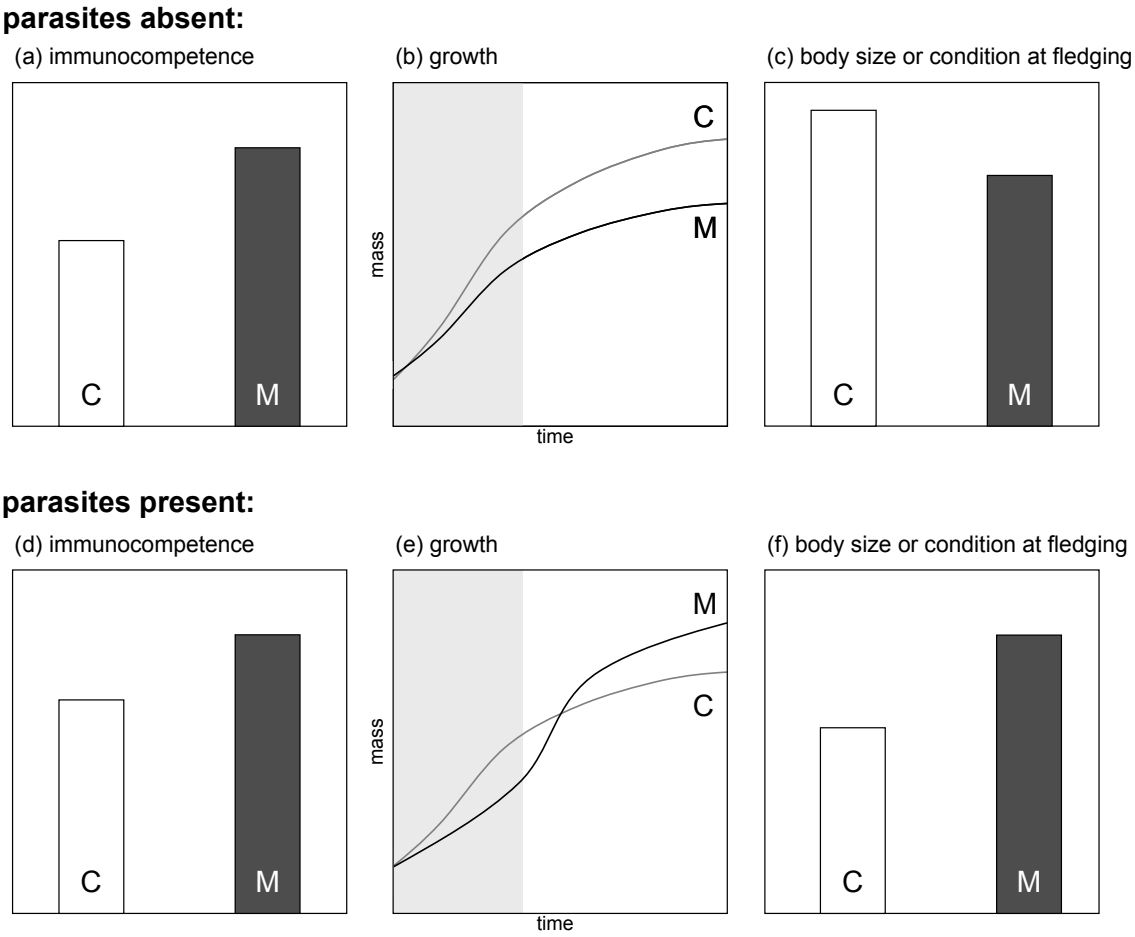

Fig. 4 *Hypothetical effects of methionine supplementation on nestlings in deparasitized environment (upper panels, a-c) and when parasites are present (lower panels, d-f); C – control nestlings, M – methionine-supplemented nestlings. Methionine stimulates nestlings to increase their investment in immunity, and in consequence enhances immunocompetence; this effect is independent of environment (a, d). During the supplementation period (shaded area in panels b and e) methionine-treated nestlings in both environments suffer reduction in growth due to the allocation trade-off between growth and immunocompetence. In the absence of parasites, investment in immunity is not advantageous and after methionine treatment stops, control and supplemented nestlings grow at similar rates (b); as a consequence nestlings with boosted immunocompetence are smaller at fledging (c) – this is the anticipated cost of increased investment in immunocompetence. Increased immunity is beneficial when parasites are present; enhanced immunity allows methionine-treated nestlings to grow better than control nestlings after supplementation stops (e). Nestlings with boosted immunocompetence fledge bigger and in better condition (f) – this is the anticipated net benefit of increased investment in immunity. From: Chapter* ***I****.*

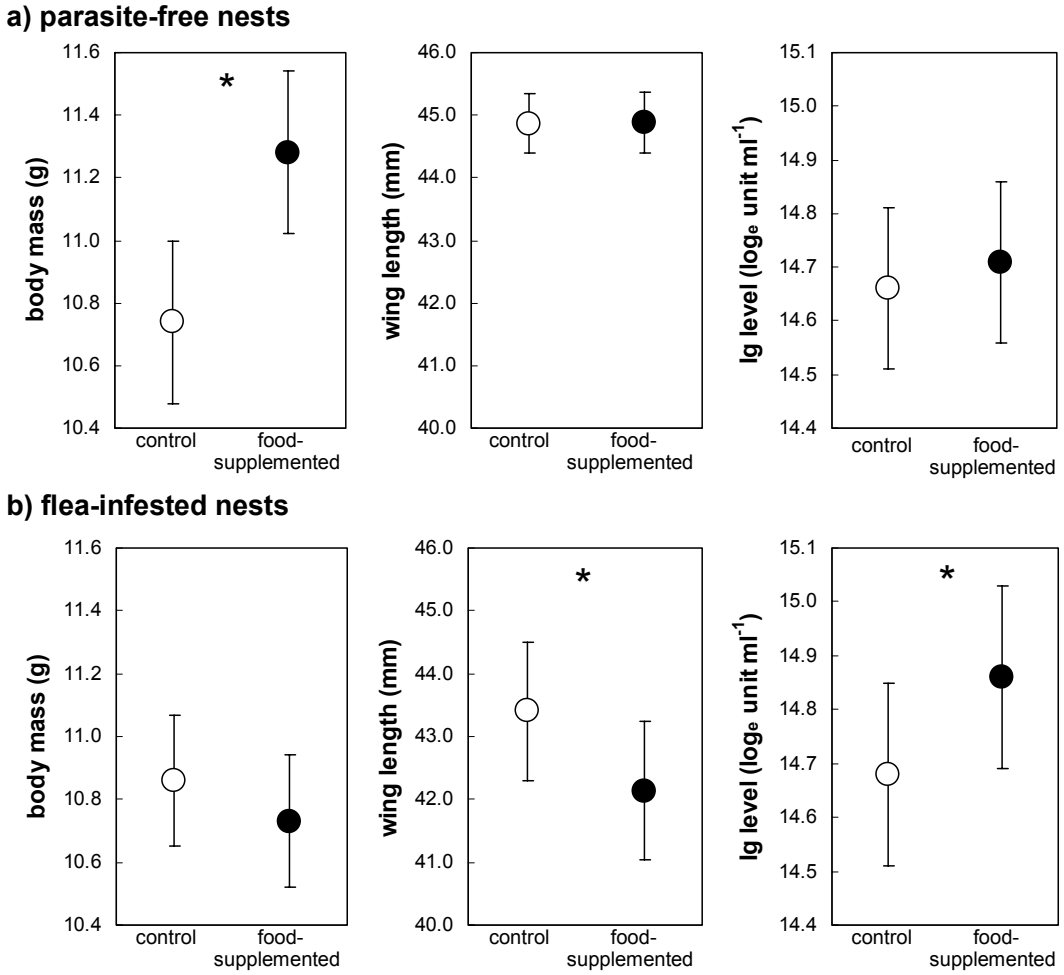

Fig. 5 *The effects of food supplementation on components of growth and development of the immune system in blue tit nestlings depend on the presence of ectoparasites. Significant differences (*p < *0.05) marked with asterisk. From: Chapter* ***II****.*

a risk of predation (Martin 1995; Remeš and Martin 2002). Manipulation of ectoparasite abundance in combination with methionine supplementation (Tschirren and Richner 2006) may be a useful method to study both the costs and benefits of immunocompetence, as ectoparasites themselves or pathogens they transfer may be counteracted by the host's immune system. I studied the costs and benefits associated with methionine-induced increased investment in immune function, in a blue tit-hen flea system in Chapter **I**. The logic of such an experiment, based on results and ideas formulated by Soler *et al.* (2003) and Brommer (2004), is detailed in Fig. 4.

Nestlings in parasitized nests reacted to methionine treatment with increased immune PHA-response and suppressed growth, but afterwards compensated for the initial growth reduction and reached equal body size at fledging as the controls. Nestling growth was not affected by methionine treatment in deparasitized nests and the effect of methionine on PHA-response in this group was not statistically significant. These results suggest that nestling responsiveness to methionine supplementation depends on parasite load and immune defence is enhanced specifically in the presence of ectoparasites, which may be beneficial. The strong evidence for fitness effects of en-

hanced immune function is still missing, as methionine-supplemented nestlings did not attain higher body mass or size at fledging than controls. Methionine supplementation itself and/or redirecting resource allocation from growth to immune function may also incur mortality costs.

Nevertheless, Chapter **I** does show the existence of a trade-off between immune function and growth. In Chapter **II** I further confirmed that growth and immune function compete for the same resources and that ectoparasites shape allocation of resources to these traits. In the reversed experimental approach, where the level of available resources (and not their allocation) was manipulated, I showed that in the presence of ectoparasites investment in immune function is favoured over investment in growth, whereas in relatively ectoparasite-free environments growth is prioritized (Fig. 5). The effects of additional food provided to nestlings depended on whether nestlings were exposed to parasites or not. My studies show that environmental factors, such as abundance of ectoparasites and food availability, play a role in the resolution of an allocation dilemma – growth vs. immune function (Chapter **I**, **II**).

3.2. Quantitative genetic basis of immunological traits

The amount of additive genetic variance underlying a trait determines its evolvability (Lynch and Walsh 1998), and hence is of obvious interest in evolutionary ecology. I investigated the additive genetic basis of immune function in Chapters **III** and **IV**. Immunological traits have been shown to be under genetic control in laboratory conditions (Wakelin and Apanius 1997) and also in free-living vertebrate populations (Coltman *et al.* 2001; Svensson *et al.* 2001; Råberg *et al.* 2003). Nevertheless, our knowledge of the sources of observed variation and the evolutionary potential of immunity traits in the natural environment is still relatively poor.

The cross-fostering experiment I conducted in the blue tit population (Chapter **III**) revealed significant origin-related components of variation in nestling immune parameters. Origin effects account for 10.6% of variance in nestling plasma Ig levels and 15.7% of phenotypic variance in PHA-response, which gives estimates of broad-sense heritability (i.e. including maternal effects and dominance variance) of 0.21 and 0.31, respectively.

Total Ig level provides a general index of humoral immune function. High heritabilities of antigen-specific humoral responses were previously found in great tits (Kilpimaa *et al.* 2005) and in blue tits (Råberg *et al.* 2003). In domestic chickens, selection for a specific antibody response also raised general antibody responsiveness (Cheng and Lamont 1988; Heller *et al.* 1992). The origin influence on plasma Ig level found in Chapter **III** may, however, also reflect maternal transfer of immunity (see § 3.3). Results of earlier work concerning heritability of cellular immune function (measured as PHA-response) are equivocal, suggesting heritable PHA-response in 6 out of 10 species (see Table 1 in Chapter **IV**).

Cross-fostering is a method frequently used in avian quantitative genetics, allowing, to some extent, partitioning of phenotypic variance into its additive genetic and environmental components (Merilä and Sheldon 2001). However, estimates of heritability

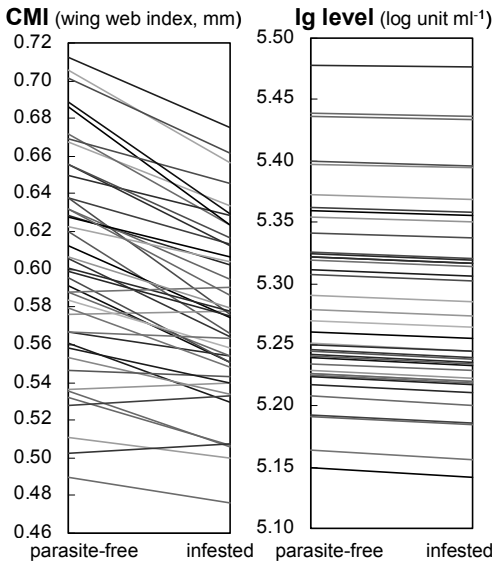

Fig. 6 *Visualization of genotype-environment interactions on blue tit nestlings' immunological traits (cell mediated immunocompetence, measured as PHA-response, CMI, and total Ig levels in plasma) in relation to ectoparasites (hen fleas). Lines connect genotypic values of traits for siblings raised in parasite-free and infested nests, and present reaction norms of families. Genotype-environment interactions do not contribute to variation in Ig levels (parallel reaction norms). The reaction norms for CMI significantly cross, hence the ranking of genotypes is different in flea-infested and parasite-free environment. From: Chapter* ***III****.*

based on full-sib comparisons may be inflated by dominance variance and early environmental (maternal) effects (Lynch and Walsh 1998). In Chapter **IV**, I estimated narrow-sense heritability of PHA-response using an animal model statistical technique efficient in separating additive genetic effects from non-heritable origin effects (Kruuk 2004). This analysis revealed that in the Gotland population of collared flycatchers, PHA-response does not have a significant additive genetic basis, although it appeared so in an earlier cross-fostering study (Cichoń *et al.* 2006), but is influenced by non-heritable origin effects accounting for 10.4% of variance. Chapter **IV** is the first study that quantified narrow-sense heritability of PHA-response. However, other recent studies suggest that the finding of significant non-heritable origin effects may be general. PHA-response is related to heterozygosity (Reid *et al.* 2005; Hawley *et al.* 2005), i.e. non-additive genetic effects. Additionally, the finding that extra-pair nestlings mount higher responses to PHA than within-pair young (both their maternal and paternal half-sibs) (Johnsen *et al.* 2000; Garvin *et al.* 2006), indicates the role of genetic compatibility rather than additive genetic effects. PHA-response may also be affected by pre-hatching maternal effects (see § 3.3).

Assuming, however, that observed origin effects on immunological traits in blue tit nestlings (Chapter **III**) reflect heritable variation, Chapter **III** suggests a mechanism by which high additive genetic variation for these traits (which might be subject to strong selection) may be maintained.

Interactions between genetic and environmental effects give rise to phenotypic plasticity, where a genotype presents different phenotypes as a function of the environment (Pigliucci 2005). This function forms a reaction norm (Via *et al.* 1995; Lynch and Walsh 1998). There are two (not mutually exclusive) forms of genotype-environment interaction: reaction norms may cross, resulting in genotypes having different rankings in different environments or the amount of additive genetic variance may vary between environments (Hoffman and Merilä 1999). The former occurs when a trait is not determined by the same set of genes in different environments, i.e. the ge-

netic correlation for that trait between environments is low (Lynch and Walsh 1998). This type of genotype-environment interaction has the possibility to maintain genetic variation in traits subjected to strong directional selection (Lynch and Walsh 1998).

I found that variation in blue tit nestling PHA-response has a significant genotype-environment interaction component in relation to hen flea parasitism (Chapter **III**). PHA-response showed little origin-specific (genetic) correlation across deparasitized and flea-infested environments, indicating significant crossing reaction norms (see Fig. 6), i.e. a significant change in the ranking of trait values of the families across environments. Although further work is needed to evaluate the additive genetic components in this pattern, this result suggests that environmental heterogeneity in parasite abundance is a potentially powerful factor in maintaining variation in certain measures of immunocompetence in nature.

3.3. Maternal effects and immune function

Parents shape the phenotype of their offspring not only by their genetic input, but also through phenotypic parental effects. Paternal and maternal effects are flexible mechanisms allowing adjustment of offspring phenotype to particular requirements of the environment (Mousseau and Fox 1998). Variation in immune function in vertebrates is commonly subject to maternal effects (Roitt *et al.* 1998; Grindstaff *et al.* 2003; Groothuis *et al.* 2005b) and there is also evidence for maternal effects on immunity in invertebrates (Huang and Song 1999; Moret and Schmid-Hempel 2001). Passive immunity (antibodies) can be directly transferred to offspring via yolk in oviparous vertebrates, and across the placenta and through the colostrum and milk in mammals (reviewed by Grindstaff *et al.* 2003). Maternal effects also modify the development of active immunity in offspring (Grindstaff *et al.* 2003).

In the collared flycatchers early (pre-hatching) maternal effects contribute to variation in cellular immune function, measured by PHA-assay (Chapter **IV**). Compounds transferred by avian females into eggs include e.g. antioxidants (Royle *et al.* 2001; 2003) and hormones (e.g. Hayward and Wingfield 2004; Groothuis *et al.* 2005b), which may modify offspring immune function. Steroid hormones, androgens and estrogens, have a role in regulating adult immunity and the response of immune cells to gonadal steroids in adulthood may depend on the pattern of hormonal stimulation during early development (Martin 2000). The yolk of all bird species studied so far contains significant amounts of androgens, in concentrations far exceeding those detected in maternal circulation (Groothuis *et al.* 2005b). Maternally transferred yolk androgens adaptively modify a variety of offspring phenotypic traits (see Groothuis *et al.* 2005b for a review), but elevated levels of yolk androgens may also result in suppressed cellular and humoral immune function in nestlings (Groothuis *et al.* 2005a; Müller *et al.* 2005b; Navara *et al.* 2005). Offspring immunosuppression mediated by yolk androgens is usually regarded as a cost of, otherwise beneficial, deposition of androgens into eggs. This immunosuppression has also been postulated as an adaptive effect: yolk androgens may provide a mechanism for directing resource allocation into a more important trait (growth) in a situation where the resources are limited, this way enhancing survival

(Groothuis *et al.* 2005b; 2006). When resources are abundant, yolk androgens may enhance both growth and immunocompetence (Navara *et al.* 2006).

Nevertheless, in the collared flycatcher the maternal effect in PHA-response does not result from differences in androgen transfer to eggs by females (Chapter **V**). Manipulation of yolk androstenedione and testosterone levels did not affect nestling PHA-response, although it modified their growth and haematocrit (Chapter **V**). Our results add to studies that have not found yolk androgen-related effects on immune function (Andersson *et al.* 2004; Tschirren *et al.* 2005; Rubolini *et al.* 2006; Müller *et al.* 2007). Thus, a decrease in immune function of nestlings exposed to high concentrations of androgens during the embryonic stage does not seem to be a general pattern.

3.4. Immunocompetence and fitness

Despite the fact that immunocompetence has become a focus for research in evolutionary ecology, few studies have examined how variation in immune function observed among individuals in natural populations is translated into variation in fitness. An improved immune defence is generally thought to increase lifespan and, in consequence, fitness (e.g. Lochmiller and Deerenberg 2000). But the question of a relationship between immunocompetence and fitness is not a trivial one. If all individuals in a population attain a basic level of immunocompetence that provides sufficient (though not perfect) protection against pathogens, an increase in the level of immune defence may have little or no implications for fitness if other selection pressures, e.g. predation, are stronger than parasitism. Alternatively, if immune defence is related to fitness, this relationship may take the form of positive directional or stabilising selection (Råberg *et al.* 2003), or even negative selection, if strong immune responses involving high energetic (Martin *et al.* 2003) or autoimmunity costs (Råberg *et al.* 1998) would be elicited by pathogens of low virulence.

In the population of collared flycatchers from Gotland PHA-response appears to be under selection (Chapter **VI**). Nestlings found breeding in the population in subsequent years had higher PHA-response (although only marginally significantly) than nestlings that failed to recruit (Fig. 7). However, collared flycatcher nestlings that have high body mass (i.e. are in good condition) also exhibit high PHA-response. Using multivariate selection analysis I have shown that the latter trait experiences no selection independent from selection on body mass itself.

Only three studies to date analysed post-fledging survival in relation to the strength of immune response (Christe *et al.* 2001; Cichoń and Dubiec 2005; Moreno *et al.* 2005). Immunoglobulin levels correlate positively with returning rates of house martin (*Delichon urbica*) nestlings (Christe *et al.* 2001). In a Spanish population of pied flycatchers (*Ficedula hypoleuca*) PHA-response turned out to be the best predictor of recruitment among several studied traits (Moreno *et al.* 2005). In the Gotland blue tit population PHA-response was found to be related to recruitment, however, only in interaction with rearing environment (Cichoń and Dubiec 2005).

One possible interpretation of the pattern found in Chapter **VI** is that other selection pressures, related e.g. to predation, migration or food availability, operate with

greater strength and override any effects of PHA-response on survival. Alternatively, immunocompetence is important for survival, but PHA-response fails to capture variation in those immune components that are relevant to defence against the parasites encountered by juvenile collared flycatchers.

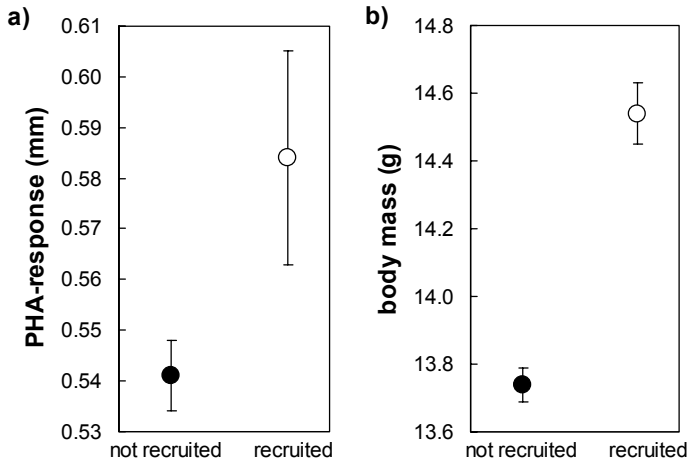

Fig. 7 *Body mass and PHA-response of collared flycatcher nestlings that recruited (*N = *132) or did not recruit (*N = *1196) to the local population in subsequent years. From: Chapter* ***VI****.*

4. Conclusions

In this thesis I have shown that developing individuals experience a trade-off between growth and immune defence, which is mediated by the abundance of ectoparasites (Chapters **I**, **II**). This trade-off is likely to have serious implications for individual life histories, considering that conditions experienced during development may affect the phenotype in an irreversible way. I have shown that excessive investment in immune function may have little or no benefits (Chapter **I**), and may even incur survival costs for individuals (Chapter **I**). I used birds and their insect ectoparasites as a study system, nevertheless my results have a general importance, as the defence against pathogens is a necessity for all living organisms.

Heritability is crucial for a consideration of any trait in an adaptive scenario, as well as the knowledge of the strength of selection acting upon the trait. At present such information for immunity-related traits in wild populations is scarce. In this perspective, the extensive data from the Gotland collared flycatcher population I present in my thesis (Chapters **IV** and **VI**) are unique. I was able to reliably estimate additive genetic variance and narrow-sense heritability of a commonly used index of cellular immune function, the PHA-response (Chapter **IV**) and quantify selection gradients acting on PHA-response after fledging (Chapter **VI**). However, my results raise the question of what the information content of the PHA assay is. PHA-response reflects condition and is lowered when the environment imposes resource limitation (Chapter **I**, **II**, **III**, **IV**, **V**, **VI**). Some evidence suggests that PHA-response may reflect the ability to mount relevant immune responses, detrimental to ectoparasites (Tschirren *et al.* 2007). Nevertheless, Chapter **VI** reveals that in collared flycatchers the strength of response to

a PHA challenge does not predict post-fledging survival. Recent studies show that PHA response is an indicator of non-additive genetic variation (Reid *et al.* 2005; Hale and Briskie 2007). Yet, the PHA assay may not be a proper tool to address research questions assuming heritable immunity, as PHA-response does not have a significant *additive* genetic component (Chapter **IV**) in collared flycatchers.

The field of evolutionary and ecological immunology is relatively young and it is now undergoing a debate, in which approaches and methods used commonly to date are questioned (e.g. Viney *et al.* 2005; Martin *et al.* 2006a; Martin *et al.* 2006b; Kennedy and Nager 2006). My results may contribute to this debate. Specifically, my thesis demonstrates that an *a priori* assumption that a high PHA-response indicates fitness benefits is not justified (Chapter **I**, **VI**). Further progress in the field of ecological immunology requires that a greater effort now be undertaken to develop alternative methods of assessing immune function in the field, especially for nestling birds.

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