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Interpreting immunological indices: The importance of taking parasite community into account. An example in blackbirds *Turdus merula*

Clotilde Biard[1], Karine Monceau[2], Sébastien Motreuil[2] and Jérôme Moreau[2]*

[1]*Sorbonne, Université, UPMC Univ Paris 06, UPEC, Paris 7, CNRS, INRA, IRD, Institut d'Écologie et des Sciences de l'Environnement de Paris, F-75005 Paris, France; and* [2]*UMR CNRS 6282 Biogéosciences, Equipe Ecologie-Evolutive, Université de Bourgogne, 6 Bd Gabriel, F-21000 Dijon, France*

Summary

1. Despite the intensive use of immune indices in immunoecology, whether to interpret the results of immune indices in terms of actual immune competence (i.e. ability to control and clear parasite infections as indicated by high values of immune indices associated with low parasite loads) or current immune activation (pathogenic infection being associated with high parasite load and high values of immune indices) is still an open question. Most studies to date have produced contrasting results focused on the effect of a single parasite species despite the fact that hosts usually harbour a community of parasites that influences one another's impact on host immune response.

2. We simultaneously assessed blood parasites, intestinal parasites and ectoparasite loads in male blackbirds and compared these measures to several immune indices to investigate how parasites explain the variation around the mean of these immune indices.

3. Parasite loads covaried within hosts. Immune indices better reflected the interacting effects of these parasites than their independent effect. Immune indices may therefore be better indicators of ongoing pathogenic infections than immunocompetence. Furthermore, intestinal parasites explained a significant part of the variance in most immune indices through their interactions with other parasites, suggesting that they have a strong influence in modulating immune function.

4. Taking the parasite community into account in immunoecology studies will certainly help increase our understanding of immune indices.

Key-words: birds, immune assay, immunocompetence, immunoecology, intestinal parasites, ongoing infection, parasite community

Introduction

Immunocompetence is a general term referring to the capacity of an individual to mount an efficient immune response when challenged by pathogens (Sheldon & Verhulst 1996; but see Vinkler & Albrecht 2011). The immune system is complex, and therefore, immune function can be quantified in many ways (Viney, Riley & Buchanan 2005; reviewed in Boughton, Joop & Armitage 2011). In vertebrates, two different types of indicators are commonly employed by ecologists to assess immunocompetence (reviewed in Demas *et al.* 2011). First, monitoring measures such as white blood cell counts, haematocrit or sedimentation rate are relatively simple to obtain and provide integrative measures of the individual's health status (e.g. Ots & Hõrak 1996; Zuk & Johnsen 1998; Norris & Evans 2000; Lamková *et al.* 2007). Secondly, measures of the host cellular or humoral immune responses to non-replicative antigens such as phytohemagglutinin (PHA) or sheep red blood cells (SRBC), respectively, test an individual's ability to mount an immune response while measuring the magnitude of that response (e.g. Zuk & Johnsen 1998; Norris & Evans 2000; Faivre *et al.* 2003; Goüy de Bellocq *et al.* 2006; Tella *et al.* 2008; Owen, Nelson & Clayton 2010).

Despite the fact that these tools are frequently used in ecological studies, a central question remains unresolved: Do measures of immune indices reflect an individual's current status of pathogenic infection or its actual immunocompetence (Adamo 2004)? For example, an elevated lymphocyte count can be interpreted as a good immunocompetence (i.e. an indicator of healthy individuals who highly invest in immune function to maintain low parasite levels, Read & Allen 2000) or as a sign of an ongoing infectious disease (i.e. a physiological response to infection in unhealthy individuals with high parasite levels; Siva-Jothy 1995; Ots & Hõrak 1998; Nunn, Gittleman & Antonovics 2000). To solve this issue, most studies used a correlative approach, measuring immune indices and relating these measures to the prevalence of parasites or parasite load (Dawson & Bortolotti 1997; Johnsen & Zuk 1998, 1999; Ots &

*Correspondence author. E-mail: jerome.moreau@u-bourgogne.fr

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Hõrak 1998; Ots, Murumägi & Hõrak 1998; Poiani, Goldsmith & Evans 2000; Esparza *et al.* 2004; Goüy de Bellocq *et al.* 2006; Valera, Herbert & Kristin 2006; reviewed in Owen, Nelson & Clayton 2010 for ectoparasites). If immune indices reflect the level of immunocompetence, individuals with higher values should harbour a lower parasite load. In contrast, if immune indices reflect ongoing infections and current levels of activation of the immune system, individuals with higher values should be more heavily parasitised.

Studies aimed at investigating these relationships produced controversial results, and the actual significance of immune indices remains a subject of debate (e.g. Owen & Clayton 2007). For example, the ability to mount a cell-mediated immune and the magnitude of the response are often assessed using injections of PHA and were shown to be either positively (Christe *et al.* 2000; Gwinner *et al.* 2000; Saks *et al.* 2006; quadratic: Huyghe *et al.* 2010) or negatively (Johnsen & Zuk 1999; Navarro *et al.* 2003; Esparza *et al.* 2004; Goüy de Bellocq *et al.* 2006; Mougeot *et al.* 2009; Martínez-De La Puente *et al.* 2013) related to parasite load. These discrepancies between results could first arise because the majority of studies focused on a single parasite species (ectoparasites: Christe *et al.* 2000; Gwinner *et al.* 2000; Goüy de Bellocq *et al.* 2006; Huyghe *et al.* 2010; intestinal parasites: Saks *et al.* 2006; Mougeot *et al.* 2009; blood parasites: Navarro *et al.* 2003; Esparza *et al.* 2004; Martínez-De La Puente *et al.* 2013; nematodes: Johnsen & Zuk 1999). However, in nature, hosts usually harbour a community of parasite species (Petney & Andrews 1998; Cox 2001). Coinfections by multiple parasite species are likely to be the rule rather the exception (Cox 2001). In addition, more recent studies have shown that the intensities of infection by two parasite species could be negatively correlated (Hatchwell *et al.* 2000; Frontera *et al.* 2007; Goüy de Bellocq *et al.* 2007). This finding could therefore influence the interpretation of results and, consequently, the conclusions. The relationship between infection and immune indices strongly depends on the parasite species in question. Indeed, different parasites may trigger different immune responses and potentially lead to different immune trade-offs (Bush *et al.* 2001; Schmid-Hempel 2011). Ectoparasites typically elicit inflammatory responses (e.g. Owen, Nelson & Clayton 2010), intracellular parasites such as protozoan stimulate T-helper 1 (Th1) and Th17 immune responses, while extracellular parasites such as helminths mainly induce Th2 immune response (Moreau & Chauvin 2010; Hayward 2013; Moreno *et al.* 2013). Immune responses may also vary according to the tissue or organ where parasites are localised, as well as to the life stage and life cycle of the infecting parasite (Roulin *et al.* 2003; Moreau & Chauvin 2010; Auld & Tinsley 2015). Different predictions regarding the relationships between immune indices and parasite abundance may therefore arise, depending on the parasite species considered. For example, Goüy de Bellocq *et al.* (2007) found that the magnitude of the immune response to PHA was negatively related to the intensity of cestode infection but positively related to that of nematodes in white-toothed shrew *Crocidura russula*. In this case, immune response to PHA could have been interpreted as either reflecting strong immunocompetence or an ongoing infection, if the authors had exclusively considered cestodes or nematodes, respectively. This study not only highlighted the difficulties in drawing conclusions based on these data but also that ecoimmunologists must consider different species of parasites infecting the same host to understand the significance of a particular immune index. Such studies simultaneously investigating variation in multiple immune indices and relating them to the parasite community are still very rare (but see Lamková *et al.* 2007; Roulin *et al.* 2007; Dittmer *et al.* 2011). However, interactions among different parasite species play a crucial role in determining the parasite community composition and relative abundance of each parasite species, as well as the host's immune responses. Parasites may interact directly, or indirectly through competition for shared resources ('bottom-up') or through the host's immune response ('top-down'), when the immune response towards a particular parasite either enhances or suppresses the response towards other parasites, and may consequently either prevent or facilitate infection by these other parasites, respectively (Pedersen & Fenton 2007; Graham 2008; Moreno *et al.* 2013; Nunn *et al.* 2014). Relationship between immune indices and parasite loads may thus differ depending on the composition of the parasite community.

On the one hand, studies of the ecology and evolution of parasite communities are urged to use immune indices in order to dissect interactions among parasites and understand how the dynamics and composition of host–parasite assemblages might be mediated by trade-offs within the immune system (Bradley & Jackson 2008). On the other hand, studies in ecoimmunology are increasingly using multiple immune indices in order to investigate immunocompetence and its implications in evolutionary ecology and disease ecology (Owens & Wilson 1999; Demas *et al.* 2011; Brock, Murdock & Martin 2014; Downs, Adelman & Demas 2014). However, in both cases, a deeper knowledge of the relationships between immune indices and the infection with single and multiple parasites is needed, in order to be able to interpret variation in immune indices (Boughton, Joop & Armitage 2011).

Here, we took a correlative approach similar to previous studies that investigated the covariations of immune indices and parasite loads for a twofold purpose. We investigated (i) whether and to what extent the abundance of different parasite species (blood parasites, intestinal parasites and ectoparasites) may covary within individual avian hosts and (ii) the relationship between the immune indices most commonly used in immunoecology (haematocrit, sedimentation rate, white blood cell counts, PHA skin test) and ecto- and endoparasite loads. We considered the relationships of each immune index first with each parasite species independently and then with the parasite community as a whole. We used the common blackbird, *Turdus merula*, as a model species because it is very common and abundant in urban parks and naturally harbours numerous identifiable parasite species (Biard *et al.* 2010). We were therefore able to test whether the interpretation drawn from the relationships of immune indices and parasite loads (i.e. strong immunocompetence or ongoing infectious disease) may

vary according to the particular parasite species considered or may depend on interactions between parasite species.

Materials and methods

BIRD TRAPPING AND GENERAL MAINTENANCE

Fifty adult male blackbirds (at least 2 years old) were caught using mist nets in four major urban parks in Dijon, France (47°19′N, 5°02′E), over a 1-month period in February 2006. Only adult males were captured to minimise the potentially confounding influences of age and sex. After capture, birds were immediately weighed (electronic balance Scout Pro, Ohaus, precision: ±0·1 g) and were kept in outdoor aviaries (220 × 150 × 250 cm) for 2 days. Water and food for large turdids (COFNA) were provided *ad libitum* and replaced daily. After 2 days in these large outdoor aviaries, the blackbirds were isolated in individual outdoor cages (69·5 × 44·5 × 82·5 cm) and fed using the same regime (day 1) (Fig. 1). The birds were then allowed a 1-week acclimatisation period (days 1–7, Fig. 1), which was necessary for the birds to recover from the stress of capture and captivity. Birds entered the experiment in batches of individuals captured on the same day, having all experienced the same 9-day delay between capture and the start of the experiment. Although birds were kept in a relatively mild environment as compared to their natural habitat with regard to food availability and variation in quality, the aviaries and the single cages were placed outdoor. Birds were thus still exposed to the natural weather, daylight and potential arthropod vectors, as well as to their own intestinal parasites between two cage-cleaning sessions.

STUDY DESIGN

To account for possible daily variations in the measures of parasite load and immune function, we performed at least two measures for each test over the course of the study (with the exception of the PHA assay). In the morning of day 7, ectoparasite load was assessed, and a blood sample was collected from the brachial vein in a heparinised micro-haematocrit tube to measure haematocrit and sedimentation rate and to make a thin blood smear for leucocyte and blood parasite counts. Then, the birds were weighed (as previously done) and returned to their individual cages (Fig. 1). From day 8 to day 13, faeces produced over 24 h were collected daily to assess intestinal parasite load over a 6-day period to account for the strong daily variability in the release of eggs of these parasites (Hõrak *et al.* 2004; Filipiak, Mathieu & Moreau 2009) (Fig. 1). On day 14, the birds were weighed, blood sampled and assessed for ectoparasite load using the same procedure as on day 7. On days 21 and 22, the capacity to mount a cell-mediated immune response and its magnitude were measured using PHA injection (Fig. 1). At the end of the study (on day 22), the birds were released in large outdoor aviaries (220 × 150 × 250 cm).

The repeatability of each type of measure was high and significant (always >0·52 for parasite loads and 0·76 for immune indices, see Table S1). In addition, mean value for each parasite load and immune index generally did not significantly change over the course of the experiment (see Table S2). Similarly, bird body mass did not significantly change between the capture and the end of the experiment, indicating that captivity and access to food *ad libitum* did not change host body condition (see Table S2). Therefore, for each variable, the means of the different measures were used in statistical analyses. Details on mean values and repeatability of the measures of all parasite loads and immune indices, as well as tests of their variation with time, are given in Table S1 and Table S2, respectively.

ASSESSMENT OF PARASITE LOAD

Ectoparasites (day 7 and day 14)

Each bird was carefully inspected for 5 min over the whole body surface to detect all visible ectoparasites (Clayton & Walther 1997; Grégoire *et al.* 2002). In a previous study (Biard *et al.* 2010), only one type of ectoparasite (the feather-chewing lice, *Philopterus* sp., Phthiraptera: Philopteridae) was found in this blackbird population, and this ectoparasite was found to infect half of all birds examined. Urban blackbirds have very low tick infestation rates (2% of birds, Grégoire *et al.* 2002), which agree with our observations as no tick was found on the birds in our study. Therefore, we focused our counts on chewing lice. Chewing lice were counted by carefully examining the bird's head, throat, belly and rump. Ectoparasites were not removed during inspection, and two counts were conducted for each bird to obtain a mean number of *Philopterus* sp.

Blood parasites (day 7 and day 14)

Thin blood smears were made from a droplet of blood from each bird sampled. Slides were air-dried and immediately fixed in absolute methanol for 5 min and stained with a Giemsa solution for 40 minutes. Blood smears were examined under 1000× magnification and oil immersion. The number of parasitised cells was determined on the basis of an examination of 10 000 erythrocytes per smear (Hatchwell *et al.* 2000). Blood parasites were identified using the description of

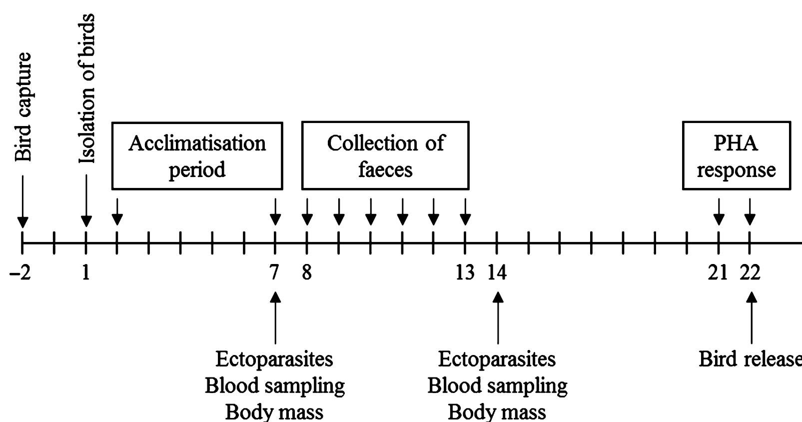

Fig. 1. Summary of experimental schedule, indicating the timing of the different measures performed and samples collected on birds.

Valkiūnas (2005). As it is difficult to identify *Plasmodium* to the species level using only the blood stage, we identified this blood parasite to the genus level (Bennett, Bishop & Pierce 1993). Two counts were conducted for each blood smear, and the mean number of *Plasmodium* was used in subsequent analyses. Other parasites such as *Haemoproteus*, *Leucocytozoon* or *Trypanosoma* were found only rarely (<3% of blackbirds in our urban area). Due to this low prevalence preventing robust statistical analyses, birds infected with these parasites were excluded from our analyses (see Biard *et al.* 2010) and are not presented among the 50 birds of this data set. This is consistent with previous studies that report urban blackbird populations are most frequently parasitised by the haematozoan parasite *Plasmodium* (Barroca 2005). Although haematozoan parasite counts from blood smears underestimate the actual parasite prevalence, they are nevertheless significantly and positively correlated with parasitaemia (Bentz *et al.* 2006) and are unlikely to lead to false positives (Valkiūnas *et al.* 2008). In the context of our study, haematozoan parasite counts therefore provided a biologically meaningful measure of infection (Bentz *et al.* 2006).

Intestinal parasites (day 8 to day 13)

To estimate intestinal parasite load, an aluminium sheet was placed on the bottom of cages for a 24-h collected faeces. Faecal samples were weighed to the nearest 0·01 g (electronic balance Scout® Pro) and placed in a 50-mL Falcon tube. Samples were gently homogenised with 14 mL of Sheater solution (45% sugar solution in tap water). Parasites were counted in a McMaster chamber in which 600 μL of the sample solution was placed undisturbed for 10 min to allow oocysts and eggs to float. In a previous study, most blackbirds were infected with one species of cestode *Hymenolepis* sp. and oocysts of one species of coccidia *Isospora turdi* (Biard *et al.* 2010). Very few nematodes (*Capillaria* sp., Capillariidae) and (*Trichostrongylus* sp., Trichostrongylidae) were found (Biard *et al.* 2010). Therefore, counts were restricted to coccidia and cestodes and two counts were conducted for each faecal sample, and the mean number of oocysts was used in subsequent analyses (Filipiak, Mathieu & Moreau 2009). Coccidia and cestode concentrations were expressed as the number of parasites per gram of faecal sample.

IMMUNE INDICES

Sedimentation rate and haematocrit (day 7 and 14)

Capillary tubes containing blood were vertically stored at 4°C for 4 h, and sedimentation rate (proportion of blood sedimented in 4 h) was calculated as the height of plasma divided by the total height of blood sample (plasma and cells) (Svensson & Merilä 1998). Capillaries were centrifuged at 5000 rpm for 5 min immediately afterwards to determine haematocrit or packed cell volume (i.e. proportion of the tube filled with red blood cells divided by the total sample volume in the capillary tube, Svensson & Merilä 1998). Lengths were measured with an electronic calliper to the nearest 0·01 mm. High blood sedimentation rate is indicative of acute infections and inflammatory diseases, leading to elevated levels of immunoglobulins and fibrinogen in the blood (e.g. Sturkie 1986; Svensson & Merilä 1998). Haematocrit reflects metabolic activity and nutritional state, and low values (anaemia) may indicate bacterial infection and/or impairment of intestinal absorption of nutrients (Ots, Murumägi & Hõrak 1998). Measures obtained on days 7 and 14 did not significantly differ, thus mean values were subsequently used.

White blood cells count and H/L ratio (days 7 and 14)

After blood parasite counts (see previous section), blood smears were also used to estimate the total numbers and proportions of different types of leucocytes under 1000× magnification and oil immersion. The total proportion of white blood cells (WBC) was estimated by counting the number of leucocytes found for every 10,000 erythrocytes. All leucocyte types were identified and counted following Campbell & Dein (1984). The numbers of heterophils, lymphocytes, eosinophils, monocytes and basophils were estimated for every 100 leucocytes. Basophils were identified in very few blood smears and were not included in further analyses. The ratio of the relative numbers of heterophils to lymphocytes (H/L ratio) was used as an index of physiological stress and current infection (e.g. Ots, Murumägi & Hõrak 1998). Heterophils are phagocytosing cells involved in non-specific inflammatory responses, during which their lysis may induce oxidative damages to a host's tissues (Ots, Murumägi & Hõrak 1998). Heterophilia has been experimentally shown to occur as a result of coccidian infection (Hõrak *et al.* 2004). Monocytes, eosinophils and basophils are also part of the non-specific immune response and are involved in responses to chronic bacterial or parasitic infections, delayed hypersensitivity reactions and early acute inflammatory responses, respectively (Coles 1997; Clark, Boardman & Raidal 2009). Lymphocytes are effectors of the highly specific immune responses; T cells are responsible for the regulation of the immune response and clearance of antigens, and B cells are involved in immune memory and the production of immunoglobulins (Ots, Murumägi & Hõrak 1998). Leucocyte number increases in cases of stress and infection (e.g. Sturkie 1986; Ots, Murumägi & Hõrak 1998). Leucopenia, in particular heteropenia, arises from severe toxaemia, with or without concurrent overwhelming viraemia or septicaemia (Coles 1997). Stress responses mediated by glucocorticoids increase the relative number of heterophils and decrease that of lymphocytes, leading to an increased H/L ratio (Davis, Maney & Maerz 2008). Lymphopenia may also occur as a result of acute viral infection (Coles 1997) and has been found to indicate immunosuppression resulting from increased reproductive effort (e.g. Hõrak, Ots & Murumägi 1998). There were no significant differences between leucocyte counts on days 7 and 14, thus mean values were subsequently used.

PHA assay (day 21 to day 22)

The ability to mount a cell-mediated immune response was measured using the simplified protocol of phytohaemagglutinin (PHA) skin test (Smits, Bortolotti & Tella 1999; Biard *et al.* 2009). The PHA-induced immune response provides a direct measure of the potential proliferative response of circulating T lymphocytes to the injection of a mitogen combined with that of cytokines and inflammatory cells (Davison, Morris & Payne 1996) and thus involves both innate and adaptive components of the immune system (Martin *et al.* 2006; Tella *et al.* 2008). Blackbirds were injected with 100 μL of 10 mg/mL phytohemagglutinin (PHA-P, Sigma-Aldrich, Lyon, France) dissolved in phosphate-buffered saline (PBS) in the centre of the right wing web (patagium). All individuals were injected in the morning between 9 and 12 am (day 21, Fig. 1). Patagium thickness at the injection site was measured just before and exactly 24 h after injection (day 22, Fig. 1) using a pressure-sensitive spessimeter with an accuracy of 0·01 mm (Teclock SM-112, Alpa SpA, Milano, Italy). An assistant restrained the bird while the micrometer was placed over the injection site by the same observer (JM). Each individual was measured twice. Two individuals are missing from PHA data due to failed injection.

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STATISTICAL ANALYSIS

Haematocrit values followed a normal distribution. The proportions of different leucocytes (heterophils, monocytes, eosinophils, lymphocytes) were square-root-arcsine-transformed, and the results of PHA, sedimentation rate, total leucocyte counts and the H/L ratio were natural-log-transformed to reach normality. Immune indices were moderately correlated and generally did not hold after correction for multiple tests (Table S3). One individual had a very high *Plasmodium* sp. intensity in comparison with the others. As the overall results were not qualitatively altered without it and because there was no obvious biological reason to exclude this individual, we presented the results including this individual in the analyses. Pathogen distributions did not significantly differ among parks (results not shown here).

Associations between different species of parasites were tested with a matrix of Kendall's τ correlations because intensities of infection with most parasite species were not normally distributed. The potential relationships between all immune indices and parasite loads were tested independently for each parasite species using Kendall's τ correlations associated with Benjamini–Yekutieli's (BY's) correction in order to reduce the incidence of false positives (Benjamini & Yekutieli 2001).

Multiple regressions were used to determine which parasite species best explained variance in the different immune indices. As our aim was to test whether the most popular immune indices used in the field of immunoecology may depend on interactions between parasite species, we ran separate analyses for each index instead of a global analysis. The original full models included intensities of infection with the four parasites, body mass, the park of origin of the birds and all interactions. A stepwise analysis (backward procedure) was used to remove non-significant effects and interactions (at $P > 0{\cdot}05$). The residuals of all models followed a normal distribution. In all models, date of capture, body mass and the park origin of the birds were non-significant and thus were not retained in the final models presented here.

All statistical tests were performed using JMP software (Version 3.2.2, SAS Institute Inc., Cary, NC, USA).

Results

INFECTION SCREENING AND RELATIONSHIP BETWEEN PARASITE SPECIES

Prevalence of, and the means and ranges of intensity of infection with, different parasite species in blackbirds are detailed in Table 1. All birds were infected with *Hymenolepis* sp. and *Isospora turdi*, whereas only approximately three quarters of birds were infected with *Plasmodium* sp. and the feather-chewing lice *Philopterus* sp. The means and ranges of intensity of infection with different parasites were highly variable, with some birds being highly infected and others showing low infections (Table 1).

Of all possible correlations between the intensities of infection by the four parasite species, only the intensity of infection with mallophaga *Philopterus* sp. was significantly negatively correlated with that of *Plasmodium* sp. and positively correlated with that of *Hymenolepis* sp. (Table 2). No other correlations were significant.

RELATIONSHIPS BETWEEN PARASITE SPECIES AND HAEMATOCRIT AND SEDIMENTATION RATE

When each parasite species was considered separately, haematocrit was significantly and positively correlated with *Philopterus* sp. infection intensity and negatively correlated with that of *Plasmodium* sp. (Fig. 2, Table 3a). After adjusting *P*-values with BY's correction, no correlation remained significant. In a multiple regression model, intensity of infection with *Plasmodium* sp. explained most of the variation in haematocrit (stepwise regression, global model and effect of *Plasmodium* sp.: $F_{1,49} = 9{\cdot}79$, $P = 0{\cdot}003$, $R^2 = 0{\cdot}17$). *Philopterus* sp. intensity was excluded from the best model at $P = 0{\cdot}09$. Sedimentation rate was not significantly correlated with any of the parasite species in simple two-by-two correlations (Table 3b) or in a multiple regression model (global model including infection intensities of the four parasite species: $F_{4,49} = 0{\cdot}81$, $P = 0{\cdot}53$).

RELATIONSHIPS BETWEEN PARASITE SPECIES AND WHITE BLOOD CELLS

Analyses of the relationships between white blood cell indices (WBC, proportion of lymphocytes, heterophils, monocytes, eosinophils and H/L ratio) and parasite infection intensity in simple two-by-two correlations highlighted only one significant correlation (Table 3c–h), which remained so after adjusting *P*-values with BY's correction. The proportion of monocytes and the intensity of infection with *Plasmodium* sp. were significantly negatively correlated (Table 3f). Multiple regression models confirmed the absence of relationship between parasites and WBC (global model on natural-log-transformed WBC: $F_{4,49} = 0{\cdot}39$, $P = 0{\cdot}81$) and between parasites and the proportion of eosinophils (global model on square-root-arcsine-transformed proportion of eosinophils: $F_{4,49} = 0{\cdot}34$, $P = 0{\cdot}85$). However, the interaction between *Hymenolepis* sp. and *Plasmodium* sp. infection intensities significantly and strongly explained the proportions of lymphocytes and heterophils and the H/L ratio (Table 4a–c). In males harbouring a high load of *Hymenolepis* sp., no relationship was found between the *Plasmodium* sp, infection intensity and proportions of lymphocytes and heterophils and the H/L ratio (Fig. 3a–c). However, in males with a low load of *Hymenolepis* sp., the *Plasmodium* sp. infection intensity was negatively correlated with the proportion of lymphocytes (Fig. 3a) and positively correlated with that of heterophils and the H/L ratio (Fig. 3b,c). The proportion of monocytes was significantly explained by the interaction between the intensities of infection with *Plasmodium* sp. and *Philopterus* sp. (Table 4d). There was a negative relationship between the intensity of infection with *Plasmodium* sp. and the proportion of monocytes in males heavily parasitised by *Philopterus* sp., but no relationship was found when considering males with a low load of *Philopterus* sp. (Fig. 4).

Table 1. Prevalence (percentage of infected hosts), mean intensities (mean numbers of parasites per infected host) ± SEM and ranges of intensity of infection with different parasite species recorded in blackbirds for all individuals (n = 50) or for infected individuals only

	Prevalence	Mean intensity for all individuals	Range for all individuals	Mean intensity for infected individuals	Range for infected individuals
Philopterus sp.	72	15·8 ± 1·94[1]	0–50[1]	21·94 ± 1·87[1]	2·5–50[1]
Plasmodium sp.	84	11·34 ± 3·67[2]	0–174[2]	13·5 ± 4·30[2]	1–174[2]
Hymenolepis sp.	100	198·29 ± 22·30[3]	7·78–560[3]	198·29 ± 22·30[3]	7·78–560[3]
Isospora turdi	100	2060 ± 383·41[4]	31–13794[4]	2060 ± 383·41[4]	31–13794[4]

[1]Number of mallophaga *Philopterus* sp. found during a 5-min feather inspection.
[2]Number of parasitised cells by *Plasmodium* sp. per 10 000 erythrocytes.
[3]Number of cestode eggs *Hymenolepis* sp. per gram of faecal sample.
[4]Number of oocysts of *Isospora turdi* per gram of faecal sample.

Table 2. Kendall's τ rank correlation coefficients between the intensities of mallophaga *Philopterus* sp., one blood parasite *Plasmodium* sp. and two intestinal parasites (*Hymenolepis* sp. and *Isospora turdi*) in blackbird

	Philopterus sp.	*Plasmodium* sp.	*Hymenolepis* sp.
Plasmodium sp.	**−0·30*** **		
Hymenolepis sp.	**0·22***	−0·08	
Isospora turdi	−0·13	0·16	0·03

Values in bold indicate a significant correlation. *indicates a P-value < 0·05, ***indicates a P-value < 0·001. When P-values were adjusted for multiple comparisons with BY's correction, the two correlations remain significant.

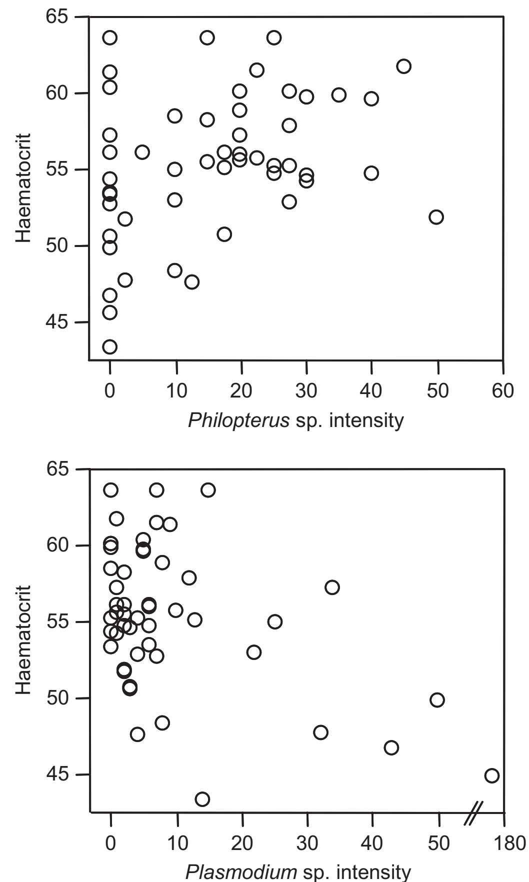

Fig. 2. Correlation between haematocrit and the intensities of infection with *Philopterus* sp. and *Plasmodium* sp. (top and bottom figure, respectively).

RELATIONSHIPS BETWEEN PARASITE SPECIES AND PHA ASSAY

Looking at all possible associations between wing web swelling and the intensities of infection with each parasite, no significant relationship emerged (Table 3i). Variation in the magnitude of the immune response to PHA was best explained in a multiple regression model including the intensities of infection with *Hymenolepis* sp. and *Isospora turdi*. There was a highly significant interaction between these two intestinal parasites (Table 4e). PHA response was significantly positively related to the intensity of infection with *Hymenolepis* sp. when blackbirds were more heavily coinfected with *Isospora turdi*. However, when male blackbirds were less infected by *Isospora turdi*, this relationship was significantly negative. For an intermediate load of *Isospora turdi*, there was no relationship between the PHA response and the *Hymenolepis* sp. intensity (Fig. 5).

Discussion

The results of this study (summarised in Table 5) further illustrate that the relationships between parasite infection and immunological indices are complex and vary according to the parasite considered. One cause of this complexity, among others not assessed in this study (age, sex or environment effects), is that hosts harbour an assemblage of parasites and the intensities of infection with different parasite species covary. These results therefore highlight the difficulty in using a single immune parameter in studies of ecological immunology, as already noted (Norris & Evans 2000; Adamo 2004; Salvante 2006). Further investigations, especially those employing experimental infections and selectively cleaning the host of one or more of its parasites, would help confirm these relationships between immunological indices and parasite infections (Viney & Graham 2013; Fenton *et al.* 2014). Our results remain, however, correlative and need to be cautiously interpreted, especially since field birds were kept in outdoor aviaries during

Table 3. Summary of the results from Kendall's τ correlations between intensities of infection with different parasite species (*Philopterus* sp., *Plasmodium* sp., *Hymenolepis* sp. and *Isospora turdi*) and immune indices in blackbirds. The total white blood cell (WBC) count was estimated by counting the number of leucocytes for 10 000 erythrocytes. The numbers of lymphocytes, heterophils, monocytes and eosinophils was estimated by examining 100 leucocytes and is expressed as a proportion

			Parasites							
	Descriptive statistics		*Philopterus* sp.		*Plasmodium* sp.		*Hymenolepis* sp.		*Isospora turdi*	
Immune indices	Mean ± SEM	Range	τ	p	τ	p	τ	p	τ	p
(a) Haematocrit	55·42 ± 0·67	43·38–63·64	**0·22**	**0·03**	**−0·20**	**0·04**	0·02	0·82	0·10	0·31
(b) Sedimentation rate	0·39 ± 0·04	0·05–1·35	−0·11	0·29	0·10	0·34	0·05	0·62	0·02	0·85
(c) WBC	54·54 ± 3·64	12–119	0·09	0·36	0·04	0·70	0·008	0·93	−0·01	0·89
(d) % lymphocyte	0·79 ± 0·01	0·51–0·92	0·02	0·83	0·02	0·83	−0·03	0·76	0·03	0·73
(e) % heterophil	0·09 ± 0·01	0·00–0·41	−0·13	0·22	0·18	0·07	−0·03	0·78	0·09	0·37
(f) % monocyte	0·04 ± 0·005	0·00–0·16	0·13	0·21	**−0·28**	**0·007**	0·03	0·80	0·04	0·67
(g) % eosinophil	0·06 ± 0·006	0·00–0·23	−0·03	0·77	0·06	0·58	0·06	0·53	0·08	0·39
(h) H/L ratio	0·13 ± 0·02	0·00–0·82	−0·13	0·21	0·17	0·08	−0·02	0·83	0·09	0·37
(i) PHA	1·18 ± 0·08	0·34–2·64	−0·06	0·54	-0·01	0·91	−0·04	0·70	0·08	0·43

Values in bold indicate a significant correlation. When *P*-values were adjusted for multiple comparisons with BY's correction, only the correlation between *Plasmodium* sp. and percentage of monocyte remained significant.

Table 4. Multiple regression models investigating variation in (a) H/L ratio (ln-transformed), (b) proportion of lymphocytes (arcsine-square-root-transformed), (c) proportion of heterophils (arcsine-square-root-transformed), (d) proportion of monocytes (arcsine-square-root-transformed) and (e) magnitude of the immune response to PHA (ln-transformed) as a function of different parasite species intensity and their one-way interactions

Source of variation	Factor d.f.	Error d.f.	R^2	Slope Estimate ± s.e.	F	P
(a) H/L ratio						
Global model	3	49	0·46		12·97	**< 0·0001**
Hymenolepis sp.	1	49		−0·0001 ± 0·001	1·24	0·27
Plasmodium sp.	1	49		0·006 ± 0·001	32·60	**< 0·0001**
Hymenolepis sp. × *Plasmodium* sp.	1	49		$-0.0003 \pm 4.5 \times 10^{-6}$	33·17	**< 0·0001**
(b) Proportion of lymphocytes						
Global model	3	49	0·31		6·84	**0·0007**
Hymenolepis sp.	1	49		$-3.7 \times 10^{-7} \pm 7.0 \times 10^{-5}$	0·0001	0·99
Plasmodium sp.	1	49		−0·003 ± 0·0007	14·29	**0·0005**
Hymenolepis sp. × *Plasmodium* sp.	1	49		$1.29 \times 10^{-5} \pm 2.9 \times 10^{-6}$	20·20	**< 0·0001**
(c) Proportion of heterophils						
Global model	3	49	0·36		8·82	**< 0·0001**
Hymenolepis sp.	1	49		$6.2 \times 10^{-5} \pm 6.8 \times 10^{-5}$	0·84	0·36
Plasmodium sp.	1	49		0·003 ± 0·0007	21·25	**< 0·0001**
Hymenolepis sp. × *Plasmodium* sp.	1	49		$1.3 \times 10^{-5} \pm 2.8 \times 10^{-6}$	23·13	**< 0·0001**
(d) Proportion of monocytes						
Global model	3	49	0·16		2·89	**0·04**
Philopterus sp.	1	49		−0·0006 ± 0·0007	0·92	0·34
Plasmodium sp.	1	49		−0·002 ± 0·001	5·14	**0·03**
Philopterus sp. × *Plasmodium* sp.	1	49		$-0.0001 \pm 6.5 \times 10^{-5}$	4·34	**0·04**
(e) Immune response to PHA						
Global model	3	47	0·29		5·92	**0·02**
Hymenolepis sp.	1	47		−0·0002 ± 0·0004	0·15	0·70
Isospora turdi	1	47		$3.8 \times 10^{-5} \pm 2.6 \times 10^{-5}$	2·23	0·14
Hymenolepis sp.x*Isospora turdi*	1	47		$8.6 \times 10^{-7} \pm 2.1 \times 10^{-7}$	16·34	**0·0002**

Values of $P < 0{\cdot}05$ are given in bold.

3 weeks with food *ad libitum*. Releasing food constraint might have altered the body condition of birds and their ability to cope with parasites. Nevertheless, bird body mass, measures of parasite loads and of immune indices did not vary within the two-first weeks after capture, and their values were significantly and highly repeatable, suggesting the results obtained in the aviary should still reflect natural variation. Such an absence of change in immune indices and parasite burdens has been previously, reported, showing that a period of 3 weeks is not sufficient to alter immune indices or host body condition for birds transferred from the field to aviaries (see Hõrak *et al.* 2002 or Sepp, Sild & Hõrak 2010).

The blackbirds were naturally infected by different parasites, and significant correlations between parasite loads were found

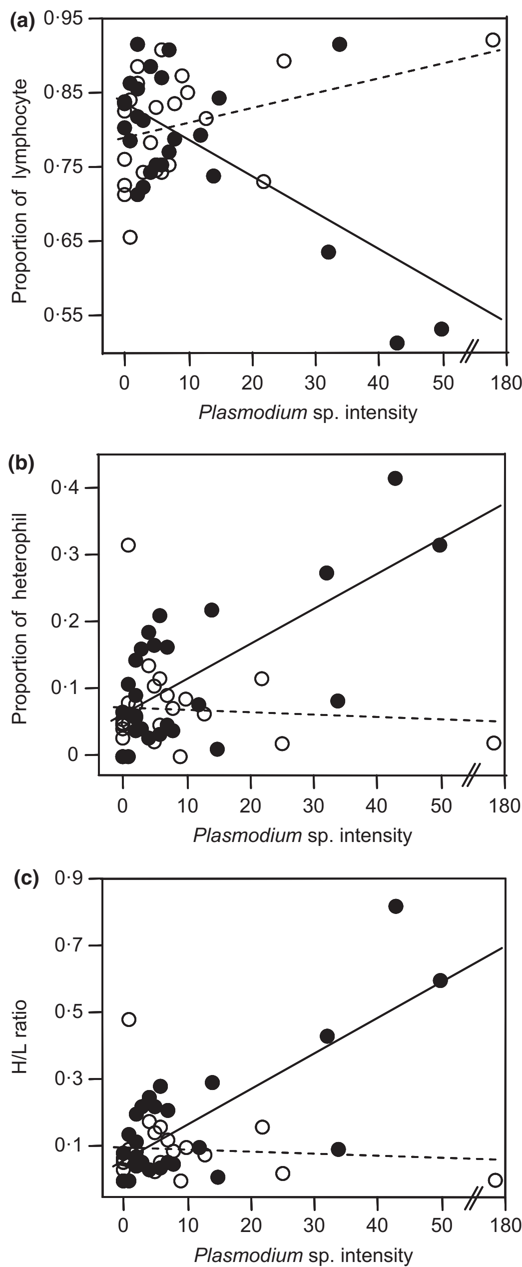

Fig. 3. Relationships between the intensity of infection with *Plasmodium* sp. and (a) proportion of lymphocytes, (b) proportion of heterophils and (c) H/L ratio, as a function of the intensity of infection with *Hymenolepis sp*. For illustrative purposes, birds were divided into two groups according to their intensity of infection with *Hymenolepis sp*. (above or below median values: high parasite load is indicated by open circles and dashed lines, and low parasite load is indicated by black circles and solid lines).

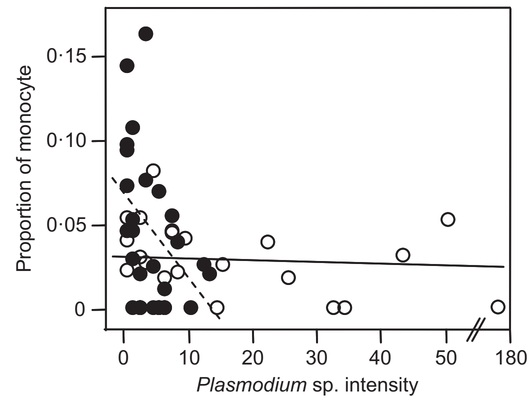

Fig. 4. Relationship between the intensity of infection with *Plasmodium* sp., the intensity of infection with *Philopterus* sp. and the proportion of monocytes. For illustrative purposes, birds were divided into two groups according to the intensity of infection with *Philopterus sp*. (above or below median values: high parasite load is indicated by black circles and dashed lines, and low parasite load is indicated by open circles and solid lines).

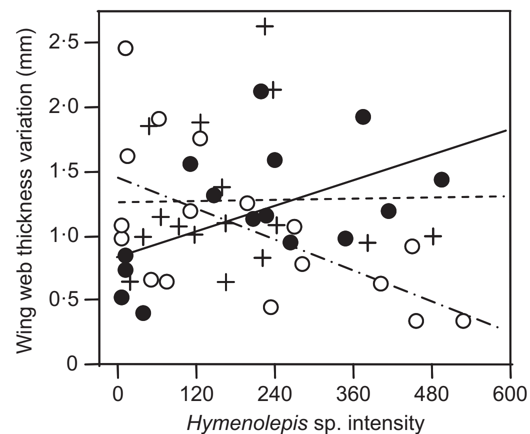

Fig. 5. Relationship between the magnitude of the immune response to PHA injection (variation in wing web thickness in mm after 24 h) and intensity of infection with *Hymenolepis* sp. and *Isospora turdi*. For illustrative purposes, birds were divided into 3 equal groups according to the intensity of infection with *Isospora turdi* (high parasite load is indicated by black circles and solid lines, medium parasite load is indicated by black crosses and dashed lines, and low parasite load is indicated by open circles and dashed lines).

within hosts. These covariations between different parasite species confirm previous results (Biard *et al.* 2010), although another study failed to find clear aggregation patterns between blood and intestinal parasites in a different population of the same host species (López, Soriguer & Figuerola 2011). In addition, the covariations between parasites were not the same as those observed previously in the same population (Biard *et al.* 2010). Altogether, these results suggest that associations between parasites likely exhibit temporal and spatial variability due to biotic and abiotic factors of both the host and parasite environment (see also Moreno *et al.* 2013 for an example in mammals), or an effect of other parasites that were not assessed, and might differentially affect covariations within the parasite community in this study and in the previous one (Biard *et al.* 2010). Coinfecting parasite species interact within the host and may have null, antagonistic or synergistic effects on each other. This will, in turn, affect their respective consequences on their host (Viney & Graham 2013), with previous infections leading to sequential effects affecting interspecific parasite covariations (Karvonen, Seppala & Valtonen 2009).

Table 5. Summary table of the relationships found between measured immunological indices and (a) individual parasites (+: significantly positive correlation, −: significantly negative correlation or 0: not significant; see Table 3 for more details) or with (b) multiple parasites (parasites main effects and in interaction, see Table 4 for more details)

	(a) Individual parasite				
Immune indices	Ectoparasite	Blood parasite	Cestode	Coccidia	(b) Parasites in interaction
Haematocrit	+	–	0	0	Blood parasite
Sedimentation rate	0	0	0	0	n.s.
WBC	0	0	0	0	n.s.
Proportion of lymphocytes	0	0	0	0	Blood parasite and Cestode × Blood parasite
Proportion of heterophils	0	0	0	0	Blood parasite and Cestode × Blood parasite
Proportion of monocytes	0	–	0	0	Blood parasite and Ectoparasite × Blood parasite
Proportion of eosinophils	0	0	0	0	n.s.
H/L ratio	0	0	0	0	Cestode × Blood parasite
PHA	0	0	0	0	Cestode × Coccidia

Ectoparasite, *Philopterus* sp.; Blood parasite, *Plasmodium* sp.; Cestode, *Hymenolepis* sp.; Coccidia, *Isospora turdi*.

Parasites may interact directly within their host, or indirectly, through its immune system and through shared resources (Viney & Graham 2013; Griffiths *et al.* 2015). In this study, hosts heavily parasitised by mallophaga *Philopterus* sp. were also heavily infected by cestodes *Hymenolepis* sp. but harboured few *Plasmodium* sp. parasites. Unfortunately, there are too few studies exploring the relationships between different parasite groups to understand how the intensity of infection with a parasite may influence that of other parasites in the same host (but see Holmstad & Skorping 1998; Holmstad, Jensen & Skorping 2008; Boag, Hernandez & Cattadori 2013; Knowles *et al.* 2013; Pedersen & Antonovics 2013; for experimental studies). Previous studies of male satin bowerbirds *Ptilonorhynchus violaceus* have already shown a positive correlation between the intensity of the protozoan *Haemoproteus* and the amblyceran lice (Borgia *et al.* 2004). To our knowledge, our study provides the first evidence of a negative relationship between the abundance of an ectoparasite such as chewing lice and that of a blood parasite. It would be beyond the scope of this paper to propose different hypotheses for these relationships between different parasite species, but some potential explanations for the positive relationship between infections with mallophaga and cestodes can be found in Biard *et al.* (2010).

Whatever the mechanisms behind the covariations among parasites, the results presented here highlight the difficulty in interpreting results of immune indices and show that the interpretation of immune indices (as evidence for ongoing infection vs. immunocompetence) is parasite dependent. This is particularly well illustrated by the relationship between haematocrit and parasite loads. Haematocrit is generally negatively influenced by bacterial or parasite infections and is considered to be an indicator of nutritional condition (Fair, Whitaker & Pearson 2007 for a review). Here, our study reports expected relationships, such as that between the protozoan *Plasmodium* sp. and anaemia, while also revealing less intuitive interactions between parasite species that underlie the disparity between individual correlations and results of multiple regressions. For example, *Philopterus* sp. was highly positively correlated with haematocrit, but was excluded as an explanatory variable in a global model investigating variation in haematocrit, which strongly suggests that this parasite has no influence on haematocrit values when taking simultaneous infection with other parasites into account. This example illustrates that a measure of one particular immune index could be misinterpreted when investigating the effects of only one parasite species.

Our study also raises important issues regarding the use and interpretation of two of the most popular tools in ecological immunology: white blood cells counts and PHA skin test. Most commonly, it is considered that (i) the number of WBC and/or the number of one particular white blood cell type (such as lymphocytes and monocytes) increases with stress and infection (Johnsen & Zuk 1998; Ots & Hõrak 1998; Ots, Murumägi & Hõrak 1998), (ii) the heterophil/lymphocyte (H/L) ratio increases with physiological stress (Gross & Siegel 1983; Ots & Hõrak 1996; Moreno *et al.* 2002) and (iii) a larger wing web swelling after an injection of PHA reflects the general efficiency of cell-mediated immunity and is a general measure of immunocompetence and immune responsiveness (e.g. Johnsen & Zuk 1999; Smits, Bortolotti & Tella 1999; Tella, Scheuerlein & Ricklefs 2002; Navarro *et al.* 2003; Martin *et al.* 2006; Biard *et al.* 2009). A large number of studies confirmed these findings (references above), but some of them have failed to find any relationship or even found the reverse (Dufva & Allander 1995; Hõrak, Ots & Murumägi 1998; Christe *et al.* 2000; Gwinner *et al.* 2000; Ricklefs & Sheldon 2007). In our study, when correlations were tested separately for each parasite species, there was no significant relationship between the total number of WBC, the proportions of each type of WBC, H/L ratio or the immune response to PHA and parasite infection (with the exception one relationship between the proportion of monocytes and *Plasmodium* sp.). However, the proportions of some WBC types, H/L ratio and immune response to PHA were significantly explained by the interaction between two different parasite species (mostly *Hymenolepis* sp. and *Plasmodium* sp. for WBC and *Hymenolepis* sp. and *Isospora* sp. for PHA). These results suggest a differential modulating effect on WBC or cell-mediated response to PHA of parasite species interacting within the same host. To our knowledge, only few

studies examined immune indices in relation to multiple parasite species (Figuerola *et al.* 2005; Valera, Herbert & Kristin 2006; Goüy de Bellocq *et al.* 2007; Roulin *et al.* 2007). The differential influence of two intestinal parasites on the immune response to PHA supports the results found in white-toothed shrews, where immune response to PHA was negatively related to cestode intensity and positively related to nematode intensity (Goüy de Bellocq *et al.* 2007). In addition, variation in WBC proportions in relation to parasites found in our study also suggests complex interactions between different species of parasites and host immune system. Blood parasites *Plasmodium* sp. and gut parasites *Hymenolepis* sp. are very different pathogens and their presence stimulates different arms of the host immune system: helminths induce a Th2 response and protozoans induce a Th1 response (Pedersen & Fenton 2007). Coinfection thus results in a trade-off between the simultaneous immune responses towards the two parasite species. This competitive interaction influences the outcome of the host's response in terms of infection control and pathogenesis that would in turn differentially influence the reaction of the immune system. In addition, helminths are known to evade the host's immune system by exploiting immunoregulatory pathways to suppress immune responses to intra- and extracellular parasites. They stimulate regulatory T cells to produce cytokines that downregulate both Th1 and Th2 responses, and suppress Th17 responses (Knowles 2011; Nunn *et al.* 2014). The trade-off between different arms of the immune system and/or the multiple suppressive effects of helminths might explain that cestodes are involved in strong interactions terms with other parasites explaining variation in WBC (see Table 4) and, in particular, the absence of a detectable relationship between the intensity of infection with *Plasmodium* and the H/L ratio in blackbirds heavily infected with *Hymenolepis* sp. We might thus have concluded that the H/L ratio does not reflect immunocompetence or a current immune response to an infection with *Plasmodium* sp. On the other hand, when *Hymenolepis* sp. load was low, there was a positive relationship between the intensity of infection with *Plasmodium* sp. and the H/L ratio, suggesting that a high H/L ratio reflects a reaction to infection against *Plasmodium* sp. These results should draw attention to the fact that caution should be used when interpreting results of very popular tools such as WBC proportions or PHA skin test as indicators of parasite resistance.

Whatever the mechanisms underlying the relationships between immune indices and different parasite species simultaneously infecting a host, our results also highlight the importance of extracellular gut endoparasites such as tapeworms. The degree of infection with *Hymenolepis* sp. strongly and significantly explained variation in most immune indices (WBC, H/L ratio, PHA) in interaction with infection levels with other parasite species. The impact of cestodes on host physiology (including immune responses and behaviour) as well as cestode prevalence remains largely underexplored in natural bird populations (Piersma *et al.* 2001; Righi & Gauthier 2002; Figuerola *et al.* 2005; Mazur, Pronin & Tolochko 2007) as compared to mammals (Bordes & Morand 2009a,b and references therein). These studies suggest that tapeworm infections are frequent in natural bird populations and are often associated with significant inflammatory and cellular immune responses. Relationships between tapeworm infection and the different immune indices found in this study suggest a strong influence of this parasite on modulating immune function. Our findings are in agreement with those by Bordes & Morand (2009a), who showed that an increase in the number of helminth parasite species is positively correlated with an increase in white blood cells counts across a wide range of mammalian species. Therefore, we recommend monitoring helminth community in future studies of birds to obtain a more comprehensive overview of the effects of this parasite on host physiology.

CONCLUSIONS & RECOMMENDATIONS

This study shows that different parasite species covary within hosts, confirming the findings of previous studies (Holmstad & Skorping 1998; Cox 2001; Holmstad, Jensen & Skorping 2008; Bordes & Morand 2009a,b; Biard *et al.* 2010; Krasnov, Shenbrot & Khokhlova 2011) and raising the question whether these covariations are more widespread than previously accepted including instances where parasites share the same compartment inside a host (i.e. Hatchwell *et al.* 2000 for covariation between two blood parasites). In addition, most immune indices assessed in this study were significantly related to the parasite load of at least one parasite species. Therefore, the interpretation of a particular immune index will certainly depend on which parasite species are monitored in the field. Furthermore, the value of immune indices likely reflects the combined effects of an assemblage of parasite species rather than that of a single main parasite species. Consequently, studies relating the variation in immune indices to a single parasite species are at risk of overly simplistic interpretations. An absence of significant relationships between immune indices and parasite infection may indeed indicate that another parasite species not assessed may interact with either parts of the relationship and consequently bias its interpretation. It has been suggested to use immune indices in order to explore patterns of coinfection and processes ruling parasite community dynamics within their host (Bradley & Jackson 2008), and to assess multiple immune indices, as well as their temporal and spatial variation, in order to more deeply investigate immunocompetence (Boughton, Joop & Armitage 2011; Pigeon *et al.* 2013; Brock, Murdock & Martin 2014). Conversely, and as already noted by Bordes & Morand (2009b), we encourage future investigators to assess different parasite species when relating their intensity to variation in immune indices to gain a better understanding of this complexity. Taking the host–parasite community into account will certainly help to prevent misinterpretations.

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Data accessibility

Data will be available from the Dryad Digital Repository (Biard *et al*. 2015).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Mean values (± SEM) for all measurements (see Fig. 1 for the experimental schedule and for the timing of the different measures performed) for parasite intensities, immune indices, body mass, and repeatabilities assorted with their 95% confidence interval (95% CI).

Table S2. Differences among paired measures (see Table S1) for parasite intensities, immune indices and body mass.

Table S3. Matrice of Spearman's ρ coefficient of correlation between pairs of immune indices (above diagonal) and associated *P*-values (below diagonal).