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Host resistance and parasite virulence in greenfinch coccidiosis

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Abstract

The question why different host individuals within a population differ with respect to infection resistance is of fundamental importance for understanding the mechanisms of parasite-mediated selection. We addressed this question by infecting wild-caught captive male greenfinches with intestinal coccidian parasites originating either from single or multiple hosts. Birds with naturally low pre-experimental infection retained their low infection status also after reinfection with multiple strains, indicating that natural infection intensities confer information about the phenotypic ability of individuals to resist novel strains. Exposure to novel strains did not result in protective immunity against the subsequent infection with the same strains. Infection with multiple strains resulted in greater virulence than single-strain infection, indicating that parasites originating from different host individuals are genetically diverse. Our experiment thus demonstrates the validity of important but rarely tested assumptions of many models of parasite-mediated selection in a wild bird species and its common parasite.

Introduction

Host-parasite relationships have been in the focus of research in evolutionary ecology because parasite-mediated selection has a potential to explain the origin and/or maintenance of sexual reproduction, ornamental traits, and MHC diversity (reviewed in Clayton & Moore, 1997; Little, 2002; Summers *et al.*, 2003). Significant amounts of relevant theory, such as the hypotheses of parasite-mediated sexual selection (Hamilton & Zuk, 1982) and dispersal (Møller & Erritzøe, 2001), and the immunocompetence-handicap hypothesis of Folstad & Karter (1992) have been stimulated and explored in the research of wild animals (particularly birds) and their parasites in natural environments. A crucial assumption of such models is that within a population, the hosts should vary either genetically or phenotypically in resistance to infections while the parasites should vary in virulence. A few experimental tests of this assumption in nondomestic vertebrates originate from studies of fish (e.g. López, 1998; Wegner *et al.*, 2003; Kurtz *et al.*, 2004) and lizards (Oppliger *et al.*, 1999). As regards the birds, two studies of barn swallows *Hirundo rustica* (Møller, 1990; Møller *et al.*, 2004) and a study of kittiwakes *Rissa tridactyla* (Boulinier *et al.*, 1997) have detected significant heritability of ectoparasite resistance. On the other hand, to our knowledge the assumption that parasite strains inhabiting different host individuals may appear genetically diverse has never been experimentally studied in a wild bird species. Assuming that avian models are most likely to remain in the scope of active research of parasite-mediated selection, it would therefore be important to determine the sources of variation in host resistance and parasite virulence in species available for traditional field studies, such as passerine birds.

Among such possible model systems, the association between coccidian intestinal parasites and their avian hosts seems especially promising. Coccidians from the genus *Isospora* (Protozoa, Apicomplexa) infect a number of passerine species (reviewed by Giacomo *et al.*, 1997; Duszynski *et al.*, 2000; McGraw & Hill, 2000). Related coccidians from the genus *Eimeria* are common parasites of poultry where they directly inhibit the uptake of essential dietary components, including carotenoids and other fat-soluble antioxidants, in the gastrointestinal tract of chickens (e.g. Allen & Fetterer, 2002a), and consequently depress carotenoid-based pigmentation ('pale bird syndrome'; Tyczkowski *et al.*, 1991). Thus, in

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the context of the vivid interest of animal ecologists in carotenoid-based ornaments as potential signals of phenotypic quality (e.g. Lozano, 1994; Olson & Owens, 1998; von Schantz *et al.*, 1999; Møller *et al.*, 2000), such parasites should be especially suitable for the detection of mechanisms ensuring the honesty of signals. Indeed, the effect of experimental coccidian infection upon the carotenoid-based ornaments has been detected in three cardueline finch species (Brawner *et al.*, 2000; McGraw & Hill, 2000; Hõrak *et al.*, 2004). Importantly, coccidian infection intensity, measured as concentration of parasite oocysts in faeces, directly indicates parasite reproductive success (e.g. Chapman, 1998). Thus, unlike in many other parasite models, proportional relationships between host resistance, parasite virulence and parasite fitness can be assumed. Another appealing aspect of coccidiosis is that reproduction of parasites can be stopped with coccidiostatic drugs, which enables standardizing the infection status of hosts and later reinfection with parasite strains isolated from different donor individuals.

We address the issues of variation in host resistance and parasite virulence in the study of wild-caught greenfinches and their coccidian parasites. Greenfinches (*Carduelis chloris* L.) are medium-sized (ca. 28 g), sexually dichromatic gregarious seed-eating passerines native to the western Palearctic region. The colour of carotenoid-based feathers has been shown to be a sexually selected trait, as more brightly coloured male greenfinches are favoured by females as mates (Eley, 1991). Our previous study (Hõrak *et al.*, 2004) has demonstrated severe effects of infection with Isosporan coccidians on the physiology and expression of carotenoid-based plumage coloration in greenfinches. This study compared birds inoculated with the mixed parasite strains with those continuously medicated during the experiment. Infection resulted in drastic but transient decreases in serum carotenoid, vitamin E, triglyceride and albumin concentrations, and reduced body mass, indicating serious pathology and probable nutrient malabsorption due to damaged intestinal epithelium. This model system thus proved useful for experimental manipulations of host infection status. In the current study, we use experimental infections and reinfections with homologous and heterologous parasite stocks in order to address the general issue about why individual hosts differ in their parasite loads. Specifically, we ask the following questions:

1. Is the natural variation in parasite loads caused by different resistance of individuals? Animal parasites generally exhibit an aggregated or overdispersed distribution within their host populations (see e.g. Boag *et al.*, 2001 for a review). Such heterogeneities can be generated either by variation between individuals in their exposure to parasites or by differences in their susceptibility to infection (Wilson *et al.*, 2002). These options can be distinguished by infecting hosts with initially low or high infection levels with the same parasite strains. If the differences between natural infection levels are caused by different resistance of those host categories, then the differences in infection intensities between different bird categories should remain prominent also after experimental reinfection with the same parasite strains. Alternatively, if the birds with initially low infection have low parasite loads just because they have not encountered truly virulent pathogens yet, then the new infection should be similarly virulent among the individuals with initially low and high parasite loads.
2. Does encounter with novel parasites confer protective immunity against subsequent infection with the same strains? For the parasite-mediated selection to occur, at least some hosts in the population should remain susceptible to at least some parasite strains present in that population. This means that hosts should not be able to build up effective immunity against any novel parasite strains. We thus predicted that if the same individuals were infected twice with the same parasites, then infection intensity would not decrease after the second infection. Alternatively, if the birds are able to acquire resistance subsequent to each new encounter with a novel strain, then the second infection with the same parasites should result in lower virulence than the first infection.
3. Do heterologous infections (parasites originating from multiple hosts) yield more severe parasitemias than homologous infections with parasites originating from a single host? For the host-parasite coevolution to occur, the parasite strains present in the population must be genetically variable. This assumption can be indirectly tested by comparing infection intensities resulting from heterologous and homologous infections. Genetic variation in parasites inhabiting different host individuals will be manifested if infection with multiple novel strains results in greater virulence than infection with a single novel strain or the host's own parasites. It should be noted, however, that such a result by itself would not be sufficient proof that different parasite strains vary specifically in their virulence. Theoretically, it is also possible that due to competitive host exploitation, multiple infections with strains of similar virulence also leads to higher pathology than infection with the same strains separately (e.g. Wedekind & Rüetschi, 2000; but see Brown *et al.*, 2002). However, in such a case also, those parasite strains cannot be genetically identical.

Materials and methods

A total of 52 male greenfinches were caught in mist-nets in the Sõrve Bird Observatory in Island Saaremaa (57°55′N; 22°03′E) during 2 (day 0) and 3 January 2004. Birds were transported to Tartu and housed in individual indoor cages (27 × 51 × 55 cm) with sand bedding. The birds were fed *ad libitum* with sunflower

seeds and tap water. During the study, birds were kept on the natural day-length cycle. All procedures in the aviary were done in the dark before illumination (hereafter 'morning') or after the lights were turned off (hereafter 'evening'). During the setting of the paper bedding (see *Parasites* section) the lights were turned off. Birds were released on 8 March (day 98). The study was conducted under a license from the Estonian Ministry of the Environment.

Research protocol

The course of the experiment is described in Fig. 1. After transportation to Tartu birds were allowed a 3-day acclimatisation period (days 2–4) in the aviary. After day 5 (days 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35 and 38–41) we started the monitoring of individual parasite loads to determine the individual average pre-experimental infection level (hereafter 'pre-exp. oocyst count'). Concurrently (during days 6–26) the oocysts were collected for the experimental inoculations. In the morning of day 39, all the birds were blood sampled (time point 'pre-exp.' in the Fig, 1 and 3–5). In the evening, 2 days later (day 41) all birds were administered a coccidiostatic cure by adding Vetacox PLV (Sanofy-Synthelabo Inc., Paris, France) to their drinking water (1 g of Vetacox dissolved in 2 L of water) for 5 days (days 41–45). During the subsequent 5 days (46–50), the effects of the coccidiostatic treatment waned and a relapse in oocyst counts was detected from the day 48 onward. At the end of this relapse period (day 47) birds were blood sampled for the second time (time point '1. rel.' in the Fig, 1 and 3–5). In the evening of day 51, all birds were inoculated orally with 2000 sporulated oocysts of *Isospora lacazei* diluted in 2×100 μL of water. Birds were allocated to four equal (13 birds) treatment groups, which received different inoculates (however, our sample sizes vary slightly in different analyses due to our inability to measure all variables in all individuals during all the sampling episodes). The first group (hereafter 'own') was inoculated with the oocysts collected from their own faeces while the second (hereafter 'mixture') group was inoculated with a mixture of oocysts collected from six birds with higher than average pre-experimental oocyst counts. The third group (hereafter 'single strain') received oocysts collected from a single bird with the highest oocyst output, and the fourth group (hereafter 'initially low') was inoculated with the same mixture of oocysts as the 'mixture' group. The oocyst mixtures did not contain parasites from the donor of a single strain. The groups did not differ by age ($\chi^2_3 = 0.3$, $P = 0.959$) or body mass ($F_{3,48} = 0.45$, $P = 0.717$). The groups 'own', 'mixture' and 'single strain' did not differ in their pre-experimental oocyst counts ($F_{2,36} = 1.41$, $P = 0.256$), while the 'initially low' group consisted of birds with significantly lower than average pre-experimental oocyst counts ($F_{3,48} = 16.01$, $P < 0.001$).

On the ninth (day 60) morning after the first inoculation, the third set of blood samples was collected (time point '1. inf.' in the Fig, 1 and 3–5) and the same evening, birds entered the second coccidiostatic cure with Vetacox (days 60–64). After the second relapse period (days 65–69), birds were assigned to the second experimental inoculation (day 70). During the second inoculation, birds from the group 'single strain' received oocysts collected from their own faeces and the group 'initially low' received pure tap water (due to shortage of infection material). The birds from groups 'own' and 'mixture' received the same treatment as during the first inoculation. The fourth blood sampling (time point '2. inf.' in the Fig, 1 and 3–5) was performed on the ninth morning after the second inoculation (day 79). On the 86th day of the experiment, all the birds were injected intradermally in the wing web with 0.2 mg of phytohaemagglutinin (PHA) in 0.04 mL of isotonic saline in order to measure cell-mediated immunity. On day 88, all the birds were injected with a 50 μL suspension of sheep red blood cells (SRBC) diluted in sterile isotonic saline to induce the humoral immune response. Results of these experiments will be reported elsewhere.

Plasma triglyceride concentrations from each blood sampling were determined by enzymatic colorimetric test

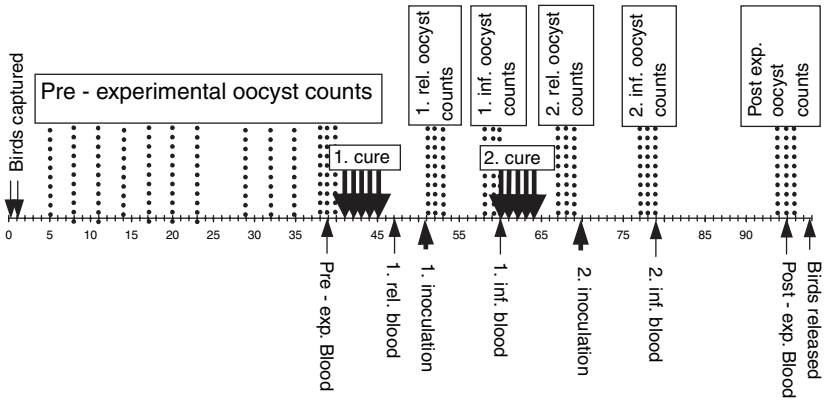

Fig. 1 Course of the experiment. Day 0 = 2nd January. Boxes 1. cure and 2. cure indicate the days of administration of the coccidiostatic treatment. Boxes describing oocyst counts indicate the periods over which the daily oocyst counts (dotted lines) were averaged. 1. and 2. inf. stand for the first and second experimental infection, respectively. 1. and 2. rel. denote measurements of infection intensities during the periods of natural relapses of infection, subsequent to the periods of medication with a coccidiostatic drug.

as described in Hõrak *et al.* (2004). High blood triglyceride levels are indicative of a resorptive state during which fat is deposited to adipose tissues. Hence triglyceride concentrations reflect the individual's state of fattening by indicating the amount of food absorbed during the few hours before blood sampling (Jenni-Eiermann & Jenni, 1998). To assess the intensity of coccidian infections, faecal samples were collected during 3 days around the blood samplings and the averages of parasite counts for these 3 days were used in statistical analyses (days 51–53 for point '1. rel.', days 58–60 for paint '1. inf.', days 67–69 for point '2. rel.', days 77–79 for point '2. inf.' and days 94–96 for points 'post-exp.' in the Fig, 1 and 3–5).

Parasites

The coccidian species present in the faeces of migrating greenfinches in Estonia has been previously identified as *I. lacazei* (see Hõrak *et al.*, 2004 for details). Since coccidian parasites are known to be highly host specific (e.g. Lillehoj & Trout, 1993) it was assumed that birds used in the current data set were infected with the same species of *Isospora*.

Because of diel periodicity in oocyst shedding (e.g. Brown *et al.*, 2001), two sheets of paper (paper bedding) were placed upon the sand bedding in the individual birdcages 2 h before turning off the lights. After the lights were turned off in the evening, the faeces were collected from the papers. Faecal samples were weighed to the nearest 0.01 g with an electronic balance (Mettler Toledo AB-S), suspended in 1 mL of water and held at room temperature for 30 min. Then, the solution was drained through gauze into individual tubes and centrifuged at 1500 r.p.m. (179 ***g***) for 7 min. The supernatant was removed and 0.5 mL of saturated NaCl water solution was added to the 0.5 mL of residue. The number of oocysts was counted using the McMaster chamber (volume = 0.15 mL) and their concentration was expressed as number of oocysts per gram of faecal sample. Repeatability of infection intensity, measured from two faecal samples collected at the same time, was 0.91 ($F = 20.34$; $P < 0.0001$; $n = 20$). During the pre-experimental period, coccidiosis was diagnosed for all the birds with an average intensity of 105918 ± 354320 (SD) oocysts per g. Difference in individual infection intensities was very high, ranging from 266 ± 552 to 2502444 ± 1415898 oocysts per g (however, the second highest infection intensity was already considerably lower than the maximal, with an average pre-experimental oocyst count of 487638 ± 804029 oocysts per g). The distribution of the pre-experimental parasite loads was highly aggregated (Fig. 2).

Oocysts to be used for oral inoculations were collected during the 20-day period before the first blood sampling (days 6–26). Faecal samples of each bird were pooled to individual cell culture flasks with 75 cm^2 culture area and filter caps for continuous venting, and preserved in 2% potassium dichromate ($K_2Cr_2O_7$) solution at room temperature and aerated daily. Sporulation of oocysts was registered 15 days after collecting the last sample (day 41) by microscopic observation. To prepare the inoculates, the mixture was drained through gauze and the resulting potassium dichromate solution containing oocysts centrifuged at 2500 r.p.m. (496 ***g***) for 10 min. After centrifugation, the supernatant was removed and 0.2 mL of residue was resuspended in 1 mL of water. This mixture was centrifuged again at 2500 r.p.m. (496 ***g***) for 10 min and the supernatant removed leaving 0.2 mL of residue. This washing procedure was

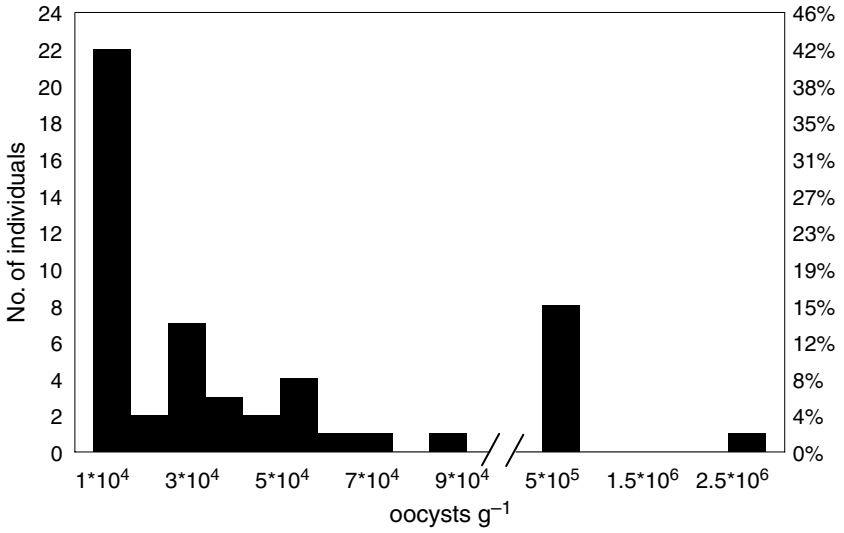

Fig. 2 Frequency distribution of average pre-experimental infection intensities.

repeated 3–4 times until the potassium dichromate was removed from the solution.

Results

Infection dynamics: group averages

During the 13 sampling days of the pre-experimental period, infection intensities of individual birds were moderately but significantly repeatable ($r = 0.43$, $F_{51,727} = 12.11$, $P < 0.00001$). After the first experimental infection, birds inoculated with the multiple strains developed higher infection intensity than birds inoculated with their own strain (Fig. 3a; $F_{5,120} = 3.91$, $P < 0.01$ for time × group interaction term in repeated measures ANOVA with main effects of group ($F_{1,24} = 0.04$, $P = 0.837$) and time ($F_{5,120} = 2.39$, $P < 0.05$)). Average infection intensity in the former group also remained higher than that of the birds inoculated with their own strain during the periods subsequent to the second medication and second infection. Birds infected with the single external strain developed infection

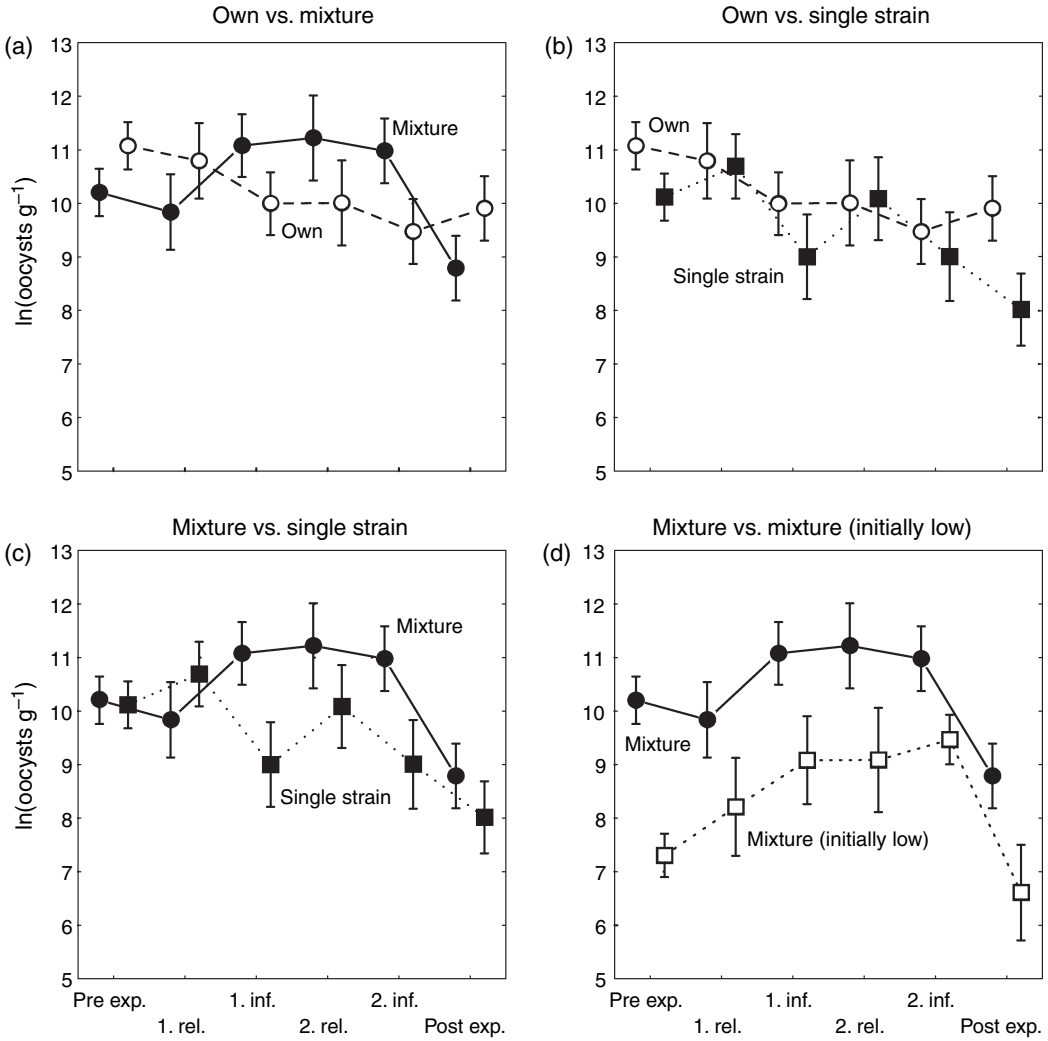

Fig. 3 Effect of experimental infections upon the coccidian oocyst shedding (per gram of feces) in different treatment groups. 'Own' stands for double infection with own strain; 'mixture' denotes double infection with mixture of strains in 'susceptible' hosts; 'single strain' is for infection with a single external strain (second time infected with own strain) and 'mixture (initially low)' denotes infection with a mixture of strains in 'initially low' hosts (second time treated with water). Exact time intervals for sampling are shown in Fig. 1. Coccidian reproduction was completely arrested both before the first and second infection (not shown in the figure), $n = 12$–13 birds per group. Vertical bars are SE. In repeated measures ANOVA, including all time points depicted on the figure, time × group interaction term is statistically significant ($F_{15,230} = 2.44$, $P = 0.003$) when all groups are included in a single model with main effects of group ($F_{3,46} = 2.83$, $P = 0.049$) and time ($F_{5,230} = 6.17$, $P < 0.001$).

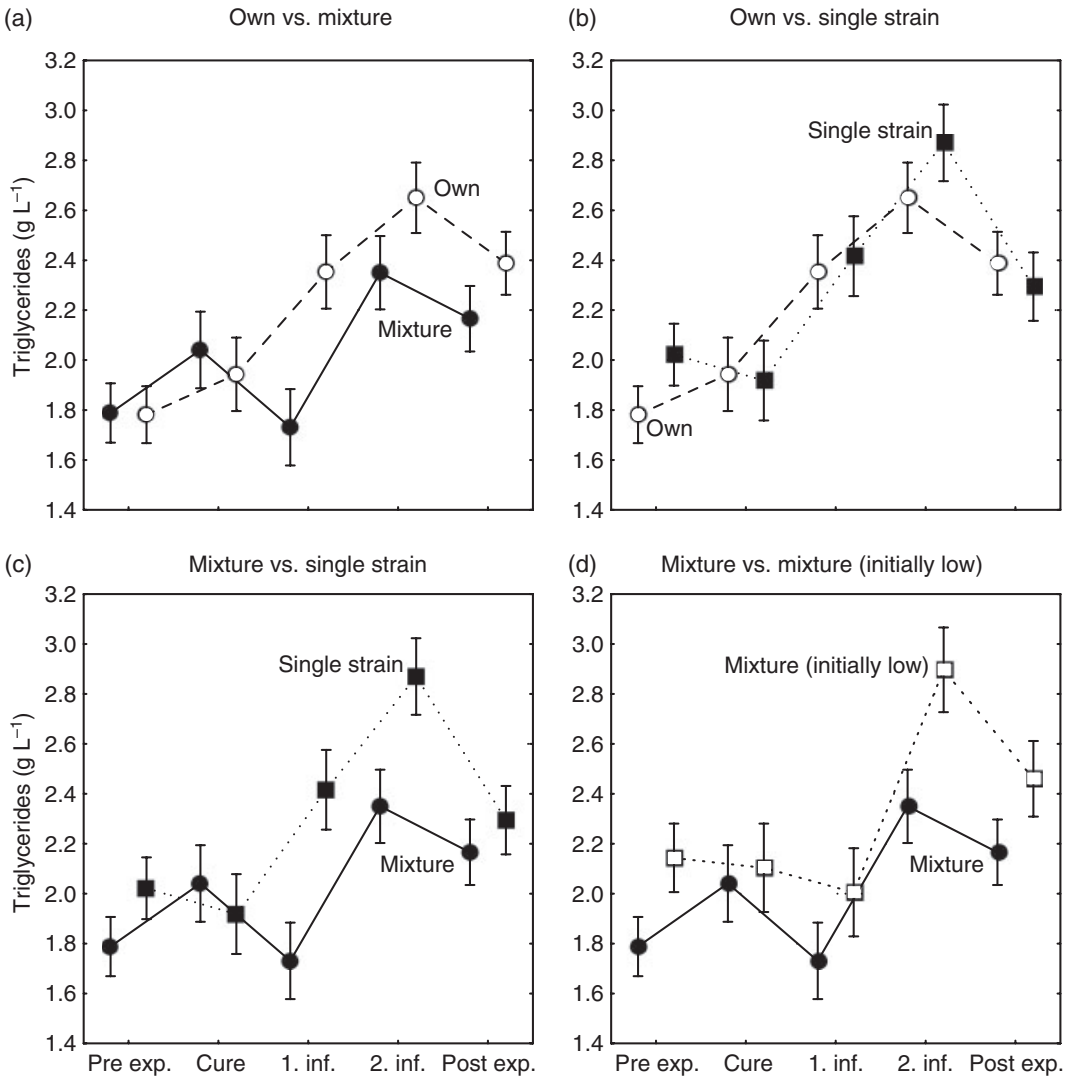

Fig. 4 Effect of experimental infections upon the plasma triglyceride concentration in different treatment groups. See the legend of Fig. 3 for the details. Time × group interaction term is statistically significant ($F_{12,164} = 2.78$, $P = 0.002$) when all groups are included in a single model with main effects of group ($F_{3,41} = 1.60$, $P = 0.204$) and time ($F_{4,164} = 32.83$, $P < 0.001$).

dynamics, indistinguishable from the birds infected with their own parasites (Fig. 3b; $F_{5,115} = 1.46$, $P = 0.209$ for time × group interaction in the model with main effects of group ($F_{1,23} = 0.81$, $P = 0.376$) and time ($F_{5,115} = 6.06$, $P < 0.001$)). Subsequent to the first infection, birds infected with the single external strain also developed weaker infection than birds infected with multiple strains (Fig. 3c; $F_{5,115} = 3.28$, $P < 0.01$ for time × group interaction term in the model with main effects of group ($F_{1,23} = 0.04$, $P = 0.837$) and time ($F_{5,115} = 2.39$, $P < 0.05$)). Comparison of birds with relatively low and high average pre-experimental infection intensity (but infected with the same multiple strains) revealed that infection dynamics was parallel in time in both groups (Fig. 3d; $F_{5,115} = 0.36$, $P = 0.877$ for time × group interaction term in the model with main effects of group ($F_{1,23} = 7.09$, $P < 0.05$) and time ($F_{5,115} = 6.00$, $P < 0.001$)). The significant main effect for the group factor indicates that both groups remained different in their average infection intensities throughout the experiment.

Plasma triglyceride concentrations followed similar pattern as infection dynamics. Triglyceride levels of birds inoculated with the multiple strains dropped sharply after the first infection and remained generally lower than those of birds infected with their own strain (Fig. 4a; $F_{4,92} = 4.36$, $P < 0.01$ for time × group interaction term in the model with main effects of group ($F_{1,23} = 2.66$, $P = 0.117$) and time ($F_{4,92} = 16.57$, $P < 0.001$)). The same holds for the comparison of birds

inoculated with mixture vs. single external strain (Fig. 4c; $F_{4,84} = 4.63$, $P < 0.01$ for time × group interaction term in the model with main effects of group ($F_{1,21} = 2.95$, $P = 0.101$) and time ($F_{4,84} = 14.17$, $P < 0.001$)). Again, the birds infected with the single strain and their own parasites remained indistinguishable in their triglyceride levels (Fig. 4b; $F_{4,88} = 0.94$, $P = 0.419$ for time × group interaction term in the model with main effects of group ($F_{1,22} = 0.25$, $P = 0.626$) and time ($F_{4,88} = 22.2$, $P < 0.001$)). With regard to the comparison of two bird categories infected with the mixed strains, birds with initially low infection intensity had relatively higher plasma triglyceride levels before the experiment and after the second infection. However, the group factor in the model was only marginally significant ($F_{1,19} = 4.21$, $P = 0.054$) in a model with effects of time ($F_{4,76} = 16.89$, $P < 0.001$) and time × group interaction term ($F_{4,76} = 1.38$, $P = 0.259$; Fig. 4d).

Body mass dynamics during the experiment were generally parallel to that of triglycerides (Fig. 5). However, in this case no significant interactions were found when comparing a group with multi-strain infection with those infected with their own strain (Fig. 5a; $F_{4,96} = 1.08$, $P = 0.354$ for time × group interaction term in the model with main effects of group ($F_{1,24} = 1.38$, $P = 0.252$) and time ($F_{4,96} = 26.5$,

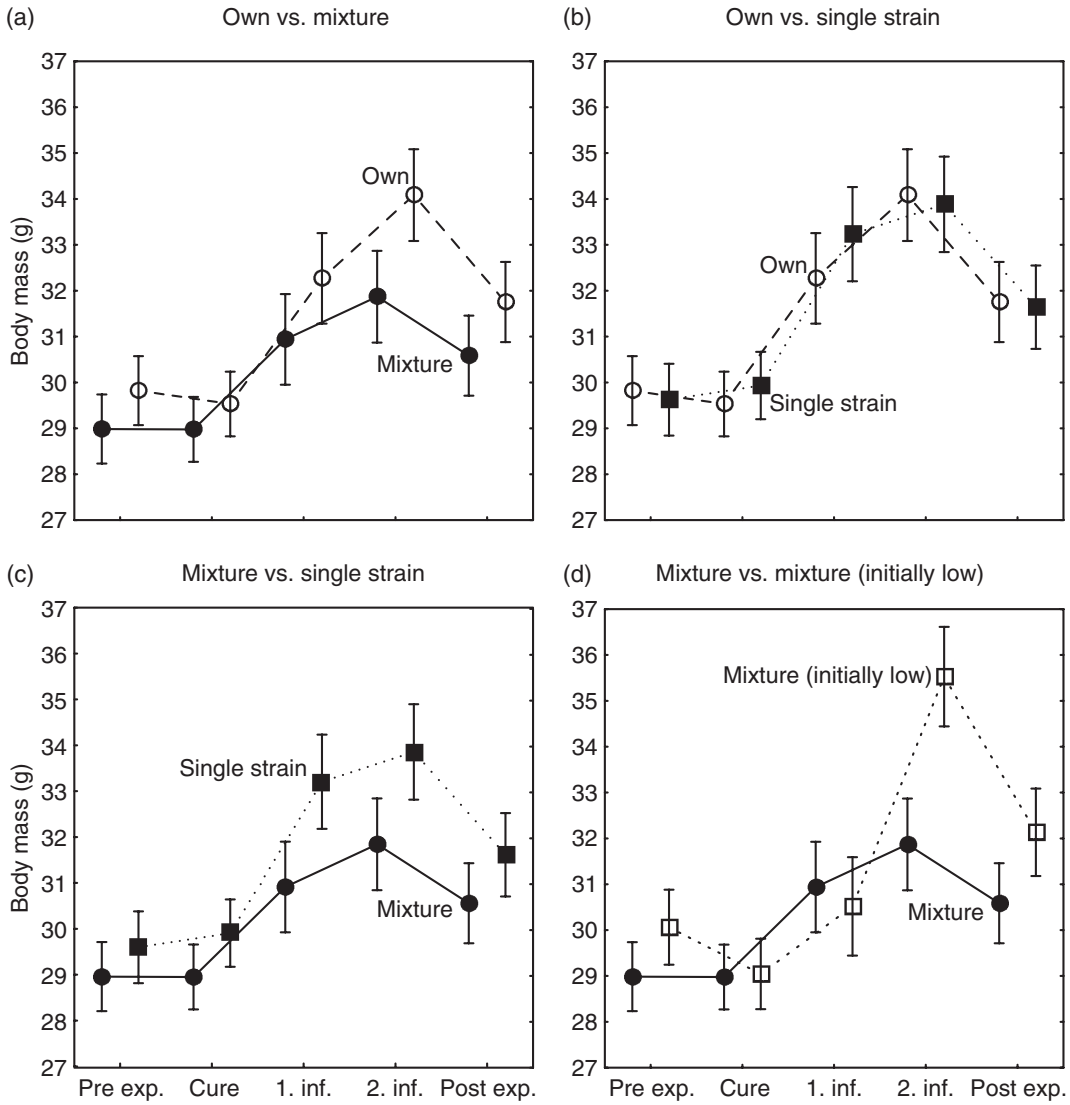

Fig. 5 Effect of experimental infections upon the body mass dynamics in different treatment groups. See the legend of Fig. 3 for the details. Time × group interaction term is statistically significant ($F_{12,180} = 2.36$, $P = 0.008$) when all groups are included in a single model with main effects of group ($F_{3,45} = 0.71$, $P = 0.549$) and time ($F_{4,180} = 50.09$, $P < 0.001$).

$P < 0.001$)). The same holds for the comparison of mixture vs. single external strain (Fig. 5c; $F_{4,92} = 0.98$, $P = 0.385$ for time × group interaction term in the model with main effects of group ($F_{1,24} = 1.54$, $P = 0.227$) and time ($F_{4,92} = 18.86$, $P < 0.001$)). Mass dynamics of birds inoculated with own parasites and single strain were virtually identical (Fig. 5b; $F_{4,92} = 0.42$, $P = 0.655$ for time × group interaction term in the model with main effects of group ($F_{1,23} = 0.03$, $P = 0.860$) and time ($F_{4,92} = 22.48$, $P < 0.001$)). However, this time a different pattern emerged in the comparison of groups with initially high and low infection intensities and infected with the same mixture strain. Subsequent to the second infection (when the former group received second time the same mixture and the latter group received water), body mass of the water-treated 'initially low' group rose significantly higher than that of the infected group. This was also reflected in significant time × group interaction term in the model ($F_{4,88} = 6.01$, $P < 0.01$) with main effects of group ($F_{1,22} = 0.94$, $P = 0.344$) and time ($F_{4,88} = 32.22$, $P < 0.001$; Fig. 5d).

Infection dynamics: individual patterns

To describe the individual patterns of susceptibility to infection, we introduce the term 'response to infection' to denote a difference in individual infection intensities measured during the peak phase of the first infection (data point 3 in Fig. 3) and during the whole pre-experimental period. In 50% of birds, infection intensity increased while in the other half of the birds, infection intensity declined subsequent to the first infection. Birds with different responses to infection were not equally distributed between treatment categories ($\chi^2_3 = 8.3$, $P < 0.05$). Among both groups inoculated with mixed parasite strains, 9 birds of 13 (69%) increased their infection intensities, while among the birds inoculated with their own strain, only 3 of 13 (23%) increased in their infection intensities. This difference between groups was significant ($P < 0.05$, Fisher exact test). Response to infection was intermediate among the birds inoculated with a single external strain (5 birds of 13, i.e. 38% increased in infection intensities). This proportion did not differ significantly from that observed among birds infected with their own strain or among birds infected with multiple strains ($P > 0.2$).

Birds whose infection intensity increased after the first infection evidently suffered deterioration of their physiological condition as the response to infection correlated negatively with the change in plasma triglyceride concentration between first infection and pre-experimental period ($r = -0.34$, $P < 0.05$, $n = 50$). Change in plasma triglyceride levels, in turn, correlated strongly with the corresponding change in body mass during the same period ($r = 0.79$, $P < 0.0001$, $n = 50$).

Discussion

Typical for most animal parasites, the distribution of infection intensities among greenfinches was highly aggregated (Fig. 2). Our experiment succeeded in generating different patterns in infection dynamics among greenfinches infected with coccidian oocysts originating from different hosts. The study also confirmed our assumption that host resistance varies proportionally with parasite virulence (i.e. damage caused to the host) and parasite fitness (i.e. its reproductive rate). This was indicated by the patterns in plasma triglyceride levels and body mass dynamics in different treatment groups, which were generally inversely proportional to the patterns of oocyst output in the same time periods. Furthermore, individual changes in plasma triglyceride levels; body mass and infection intensities were significantly intercorrelated. These results mean that our experimental inoculations caused significant changes in physiology of treated birds. Application of these premises implies that our model system is suitable for explorations of the sources of variation in host resistance and parasite virulence. However, it should also be noticed that not all the changes in host physiology that occurred during our study were due to experimental infections. For instance, transient increases of body mass and plasma triglyceride levels after first infection might have occurred due to habituation of birds to captivity and/or handling stress or changes in hormonal profiles due to increase in day length. Similarly, the decline in body mass and triglyceride levels at the end of the experiment could probably be related to stress and/or extra energetic expenditures associated with immune responses to PHA and SRBC. It is therefore important to rely on between-group differences in infection dynamics (i.e. time × treatment interaction terms in repeated measures ANOVA models) when interpreting the results of our experiment. Next, we will discuss our main findings in the light of questions concerning sources of variation in parasite virulence and host resistance as posed in the Introduction.

First we asked whether the natural variation in parasite loads is caused by differences in resistance of birds to standard infection. This hypothesis was most clearly supported by the result that infection intensities of birds with initially low parasitemia remained low throughout the experiment, although they received exactly the same heterologous inoculum as the birds with average pre-experimental infection intensity. The infection dynamics of these two groups remained parallel in time, although consistently lower among 'initially low-infection' birds (Fig. 3d). This result implies that natural infection intensities confer information about the ability of individuals to also resist novel strains. This is an important finding in the context of immunoecological research where the relative importance of different sources of variation in natural infection levels has been

under continuous debate (e.g. Clayton, 1991; McLennan & Brooks, 1991; Poulin & Vickery, 1993; John, 1997). Furthermore, intra-populational variation in host resistance, especially when occurring simultaneously with variation in parasite virulence, is an important assumption of models of parasite-mediated selection listed in the Introduction. As regards the coccidian infection, various breeds of chickens have been shown to differ remarkably in their resistance to challenge with standard Eimerian strains (e.g. Pinard-van der Laan *et al.*, 1998; Smith *et al.*, 2002). However, to our knowledge, the results of the present study appear to be the first experimental demonstration of individual variation in resistance to coccidiosis in a wild animal population.

At present, we cannot distinguish whether between-individual differences in susceptibility to coccidiosis were primarily caused by genetic or ontogenetic differences in immune function of individual greenfinches. Maternal effects (e.g. Grindstaff *et al.*, 2003) and environmental conditions experienced during ontogeny (e.g. Blount *et al.*, 2003) have been shown to exert considerable effects on individuals' capability to respond to immune challenges later in life. On the other hand, in domestic chickens the outcome of coccidian infection has been shown to depend on the interactions between the genes of both host and parasite. A recent study of *E. maxima* infection in chickens (Smith *et al.*, 2002) has demonstrated full protective immunity against the reinfection with the same strain of the parasite, while cross-protection against heterologous parasite strain varied from zero to almost 100%, depending on host genetics. Yet it is at present unknown whether genetic variation in parasite resistance is also responsible for the differential susceptibility to coccidian infections in wild birds. The similarity of the natural situation to the above-mentioned one is not necessarily obvious because of the vastly different selection pressures in the wild as compared to those imposed by the past and current poultry industry (e.g. Knap & Bishop, 2000). However, as regards greenfinches, any mechanism leading to consistent between-individual differences in parasite resistance would be sufficient for triggering parasite-mediated sexual selection. This is because in a species where females can gain direct benefits by mating with resistant males, it does not matter whether individual differences in disease susceptibility are of genetic or environmental origin. In greenfinches the resistance to coccidiosis is likely to affect the quality of the parental care provided by males, as suggested by the serious health impact of infection, detected in this study and by Hõrak *et al.* (2004). Second, birds whose general condition is weakened by coccidiosis might be more susceptible to infections transmitted via physical contact and thus more likely to infect their breeding partners. Third, infected males with a weakened condition might be more vulnerable to predation (e.g. Møller & Erritzøe, 2000) during breeding, which would again put a premium on females to mate with more resistant individuals. Coccidian infection has been shown to depress the expression of carotenoid-based plumage coloration in greenfinches (Hõrak *et al.*, 2004) and those ornaments are targets of female choice (Eley, 1991). It is thus likely that proceeding from the indicators of resistance to coccidiosis in their mate choice would enable greenfinch females to obtain at least direct benefits from their mates.

Our second question was whether the encounter with novel parasites confers protective immunity against subsequent infection with the same strains. This is an important issue because parasite-mediated selection could not work if hosts were able to build up effective immunity against any novel parasite strains. In our study, average infection intensity did not decrease after secondary infection among birds that were repeatedly infected with the same heterologous parasites. This result indicates that encounters with a mixture of parasite strains did not help the birds to suppress efficiently the subsequent infection with the same strains. This finding also supports our previous contention that individuals really differed in their general capability to resist coccidiosis. Because we could not observe the development of protective immunity at reinfection with the heterologous inoculum, we can eliminate the possibility that birds with initially low parasite loads retained their low infection status during the experiment just because they were already familiar with the strains contained in that mixture. Although, to our knowledge, the development of immunity against avian *Isospora* has never been described, we believe that the time interval between subsequent infections (19 days) was sufficient to enable birds to develop the immunological memory. According to previous work on chicken coccidiosis, birds can develop immunity against homologous strains during 2–4 weeks after initial inoculation (e.g. Vermulen *et al.*, 2001) and parasite reactive serum antibodies reach maximum levels at 8–14 days after oral infections (Lillehoj & Ruff, 1987). We thus consider it likely that the lack of protective immunity due to previous exposure to the same parasite strains reflects the genuine inability of greenfinches to become coadapted with just any coccidian strains encountered during their lifetime. On the other hand, the result that reinfection of hosts with their own parasite fauna resulted in lower infection intensities than infection with mixed strains (Fig. 3a) indicates that hosts can tolerate their 'own', previously acquired parasites better than novel ones. Such a situation can occur, for instance, if each observed individual represents a viable and unique host-parasite assemblage, retained after selective elimination of such host-parasite combinations, which resulted in deadly overexploitation of hosts. Under such a scenario, the outcome of infection would depend on the genetic variation between both hosts and parasites as predicted by the Red Queen models of host-parasite coevolution (e.g. Frank, 1994). Such a scenario seems plausible, given

that chickens can acquire immunity against Eimerian infections on the basis of MHC-mediated responses, although, notably, the innate immune responses also play an important protective role (Allen & Fetterer, 2002b).

The question whether coccidian strains inhabiting greenfinches are genetically heterogeneous was addressed by comparing the infection success of parasite inocula originating from single or multiple hosts. We expected that genetic variation among parasite strains infecting different hosts would be revealed by the higher virulence of parasites originating from multiple hosts as compared to infection with parasites originating from a single host. Consistent with our expectations, birds inoculated with multiple strains developed higher infection intensities than birds infected with parasites from a single host (Fig. 3c). This also means that our assumption about different individuals harbouring different *Isosporan* strains was justified. This was expected on the basis of previous research on chicken coccidiosis where different strains of coccidia are known to interbreed within a host (e.g. Williams, 2002) which means that infection material originating from a single individual can be considered relatively homologous. Thus, although we do not know how many different founder strains participate in the forming of the coccidian fauna of individual greenfinches, our results suggest that between individual differences resulting in individual faunas are sufficient to lead to differential pathogeneicity. However, the patterns of multiple infections resulting in greater virulence than infection with a single novel strain or a host's own parasites can have at least four possible explanations.

First, and probably the most parsimonious explanation would be that because parasite strains differ in their virulence, hosts are more likely to encounter virulent parasites from multiple rather than from single infections. Such an outcome would be compatible with previous findings about immunological variability of different Eimerian strains in chicken coccidiosis (e.g. Martin *et al.*, 1997; Williams, 1998, 2002; Smith *et al.*, 2002) and microparasite infections in general (reviewed by Read & Taylor, 2001). It is noteworthy in this context that infection with a single novel strain could even be considered avirulent in our study because such birds had infection dynamics (Fig. 3b), as well as patterns in plasma triglyceride levels (Fig. 4b) and body mass (Fig. 5b) which were virtually indistinguishable from the birds of the control group infected with their own parasites. Given that coccidians invading previously infected host are expected to face fierce competition by preceding strains (e.g. Williams, 1998), and more virulent parasite strains are generally supposed to outcompete milder strains (reviewed in Wedekind, 1999), it seems plausible that coccidians from a single host were less likely to compete with pre-existing strains than coccidians originating from multiple hosts.

Second, a slightly modified version of the first explanation would be that multiple infections increase the likelihood of encounter between such host and parasite genotypes that result in the most virulent infections. Such an outcome would be expected under many models of host-parasite evolutionary dynamics (e.g. Frank, 1994) and it would also compare favourably with findings of genotypic interactions between host and parasites in chicken coccidiosis described above.

The third possibility is that multiple infections lead to higher parasite replication for mechanistic reasons because simultaneously fighting antigenic variants of different parasite strains might be more difficult for the host's immune system than handling a single-strain infection. Such an explanation would be compatible, for instance, with the findings of de Roode *et al.* (2003) who showed that mixed clone infections were harder to clear than single clone infections in the murine malaria.

Finally, at least theoretically, we cannot exclude the scenario where parasite strains inhabiting different hosts possess similar virulence but different degrees of relatedness to each other. This explanation would be based on the reasoning that the optimal rate of host exploitation, and hence virulence, is higher in genetically diverse infections because in-host relatedness is reduced (e.g. Wedekind & Rüetschi, 2000; Read & Taylor, 2001; but see Brown *et al.*, 2002). However, even such a scenario would require that parasite strains inhabiting different host individuals are genetically diverse.

To summarize, the results of our experiment indicate that the outcome of coccidian infection in greenfinches depends on concurrent variation in host resistance, parasite virulence and their interaction. Importantly, we showed that natural infection intensities reflect individuals' ability to resist novel strains and that greenfinches do not develop protective immunity against arbitrary parasite strains. This study also showed for the first time in a wild bird species that coccidian parasites inhabiting different host individuals are genetically diverse, which demonstrates the validity of important but rarely tested assumption of many models of parasite-mediated selection. In line with our findings, Bensch & Åkesson (2003) have demonstrated spatial and temporal variation in different lineages of *Haemoproteus* sp. blood parasites in Swedish willow warblers (*Phylloscopus trochilus*). Similarly, temporal variation in different lineages of an avian malarial parasite community in Puerto Rico has been detected by Fallon *et al.* (2004). However, not much is known about the fitness consequences of avian malaria infections, and therefore it is unclear whether they will contribute to the maintenance of variation in the host's resistance genes. We consider it much more likely that Isosporan coccidians can play such a role in passerine birds, because their pathogeneicity (which can ultimately lead to the host's death) has been well documented (Box, 1977; Sironi, 1994; Giacomo *et al.*, 1997; Hõrak *et al.*, 2004). This suggests that avian coccidiosis offers a great

potential for microevolutionary research, especially in the context of current advances in molecular characterisation of different parasite strains and host immune system diversity.

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