Food availability and competition do not modulate the costs of *Plasmodium* infection in dominant male canaries

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Keywords:
Avian malaria, competition, infection, Plasmodium relictum, SGS1, group living, social rank, virulence, social stress

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Abstract

Understanding the different factors that may influence parasite virulence is of fundamental interest to ecologists and evolutionary biologists. It has recently been demonstrated that parasite virulence may occur partly through manipulation of host competitive ability. Differences in competitive ability associated with the social status (dominant or subordinate) of a host may determine the extent of this competition-mediated parasite virulence. We proposed that differences between subordinate and dominant birds in the physiological costs of infection may change depending on the level of competition in social groups. We observed flocks of domestic canaries to determine dominant or subordinate birds, and modified competition by providing restricted (high competition) or *ad libitum* food (low competition). Entire flocks were then infected with either the avian malaria parasite, *Plasmodium relictum* or a control. Contrary to our predictions we found that food availability had no effect on the outcome of infection for dominant or subordinate birds, though we found evidence that our food availability manipulations did alter competition and behaviour within our experimental cages. We found that dominant birds appeared to suffer greater infection mediated morbidity in both dietary treatments, with a higher and more sustained reduction in haematocrit, and higher parasitaemia, than subordinates. Our results show that dominance status in birds can certainly alter parasite virulence, though the links between food availability, competition, nutrition and virulence are likely to be complex and multifaceted.

1. Introduction

The ability to resist and recover from pathogenic infection is one of the major fitness-determining traits shared by all animals. However, often parasites will differ in their virulence, the degree of morbidity and mortality they inflict upon hosts. Understanding the factors that drive these differences in virulence is of fundamental interest. For a given host, extrinsic factors such as parasite genotype and environment may modulate parasite virulence. For example, it has been shown that parasite virulence may be altered when host environment differs in factors such as temperature (Blanford et al., 2003), host density (Steinhaus, 1958), and food availability (Bedhomme et al., 2004). Similarly, intrinsic factors such as host genotype (Lefevre et al., 2007), sex (de Roode et al., 2007) or age (Gardner and Remmington, 1977) may affect parasite virulence. A further difference between hosts that may potentially shape the outcome of parasitic infection is the social status of the host, especially in vertebrate species with social hierarchies (Larcombe et al. in press). There is growing interest into how some animals, including birds, develop stable and profound differences between individuals in their behavioural profiles (Sih and Bell, 2008). How such differences in behaviour or social status translate into differences in parasite virulence following infection remains unclear.

Dominance is associated with a number of benefits in wild birds, for example access to the best feeding opportunities (Parisot et al., 2004), predator free foraging sites (Schneider, 1984), roosting positions (Weatherhead and Hoysak, 1984), or mating success (Post, 1992). Despite these benefits there is increasing understanding of the costs of dominance. Social stress, the physiological stress associated with attaining or maintaining a dominant social position, has received attention as a cost of dominance (Creel et al., 1996). Several studies have demonstrated chronic elevated levels of potentially damaging hormones in dominant birds, compared with subordinates (e.g. Goymann et al., 2004; Goymann and Wingfield, 2004). In addition, some evidence suggests that dominant individuals may have reduced immune function compared to subordinates (Li et al., 2007), although in other cases the reverse is true (Ungerfeld and Correa, 2007). In a recent experiment, we showed that parasite-mediated morbidity and mortality in canaries was dependent on the social status of the host, when receiving a reduced diet (Larcombe et al. in press). Throughout that study, all birds received a reduced quantity of seeds in a single feeder, a way to experimentally increase competition between individuals in their groups. This food manipulation may have altered the patterns of parasite virulence we observed in subordinate and dominant birds. Firstly, the energetic costs of obtaining and protecting food resources are likely to be higher for dominants than subordinates, especially since there is some evidence that dominant birds may have higher metabolic rates (Hogstad, 1987). These costs of food gathering and food site protection will be increased when less food is available. Competition-mediated differences in parasite virulence may therefore be more severe for dominants than subordinates, when food is scarce, compared to food rich environments. For socially tolerant subordinates, the influence of food availability on competition and parasite virulence, is likely to be less severe. Secondly, when flocks of birds are provided only one feeder there may be unnaturally high levels of competition compared to a more natural environment where secondary feeding sites may be available, reducing the requirement for all birds to feed simultaneously. In this study, we tested whether mortality or morbidity of canaries infected with *Plasmodium relictum* differed between dominants and subordinates, receiving either a reduced or *ad libitum* diet. Importantly, in *ad lib* groups, several feeders were available in each cage meaning that dominant birds could not monopolise and protect the food resource, and more birds could feed simultaneously without encountering aggression from other birds.

The goal of this study was to assess the interactive effects between social status, infection and food availability on parasite virulence using domestic canaries as hosts and

Plasmodium relictum (lineage SGS1), an avian malarial parasite. By keeping canaries in flocks of 5 birds, and scoring for consistent feeding behaviours, we divided birds into 2 categories: dominant (D) and subordinate (S) within each flock. Half of the flocks received an *ad lib* diet, and the other half received a limited though adequate diet. Whole flocks were then either infected with Plasmodium, or given a control inoculation. Following infection, we measured morbidity (change in mass and haematocrit) and mortality of hosts, in addition to parasitaemia. We predicted that infected dominant birds would have higher morbidity/mortality than infected subordinates in reduced food groups and infected dominant birds receiving an *ad lib* diet would have lower morbidity/mortality, than infected dominants receiving a reduced diet.

2. Materials and methods

We used 60 adult male canaries during the experiment, and prior to commencement each bird was molecularly sexed following a standard PCR technique (Fridolffson and Ellegren, 1999). We only used male canaries in the experiment as we did not wish to confound the experiment with differences between sexes, or by interactions in- and between pairs of birds. After confirming the sex of each bird, we divided the birds between 6 aviaries (2.5 * 1.5 * 2.2 m), 5 birds per aviary. Each bird was weighed, and had its tarsus length measured prior to re-housing in a new flock.

2.1 Husbandry and Diet Manipulation

Before commencing the diet manipulation, all cages were provided with *ad libitum* food (a commercial seed mix, lettuce, apple and hard-boiled egg) for 7 days. Since we were interested in determining costs of dominance and infection under different environmental conditions, we divided the flocks between two different feeding regimes. Following the 7 days of acclimation, the birds were provided with either *ad libitum* food or reduced food. *Ad lib* diet consisted of 3 large round feeding dishes, each full of seeds. The feeders were deliberately interspersed throughout the cage with large gaps between to reduce contact between birds while feeding, and to allow several birds to feed at once. Reduced diet consisted of just one dish per cage, with 12g of seeds per bird per day. We had previously found that 12g of seeds is the maximum amount a single bird would eat per day (Larcombe et al. in press). This amount of seed was thus sufficient to nourish each bird, though allowed competition between birds (pers. obs). During the course of the experiment, the cages were monitored daily, and if a bird died the amount of seed was reduced accordingly in reduced food flocks.

2.2 Behavioural observation

We performed behavioural observations to assess the social status and dominance related behaviours of each the birds in each flock. The procedure was similar to that outlined in Larcombe et al. (in press), but with some modifications. The first phase of observations was carried out 3 days before the start of the experimental diets, when all birds received an identical diet. The second phase of observations took place 11 days after being placed in their flocks. We performed behavioural observations for 3 consecutive days in both phases. Each morning at 09.00 we removed the remaining seed from the day before, and left cages for 30 minutes without seeds. Following the 30-minute food deprivation, we placed a seed feeder in each cage that allowed only a single bird to feed at a time. We also placed a video camera in each cage and filmed the interactions between birds at the feeder for 20 minutes, starting when the feeder was first entered. Birds were marked with non-toxic coloured pen on the back of the head or wings for identification on the video tapes.

In order to score the bird's behaviour, when the video was re-watched the 20 minute time period was divided into 10 two minute blocks. Birds were scored for the frequency of certain behaviours in each block: Primary Access (PA) to the feeder, where a bird successfully fed directly from the hole in the feeder. Secondary Access (SA), when a bird was motivated to feed, and appeared at the feeder, either attempting to feed, or pecking at discarded seeds, but did not achieve Primary Access. Antagonistic encounters (ANT), where a bird aggressively postured towards another, typically by lowering its head and fanning and trembling its wings, or by pecking out at the other bird, sometimes escalating into a physical fight, or when a bird received these physical cues from another individual. We previously found that these behaviours are repeatable across days for canaries (Larcombe et al., in press). We summed the counts of PA, SA and ANT in pre-experiment trials, and again for the observations taken during the experimental phase in order to analyse the change in behaviour following the experimental procedures.

In this experiment we were interested in associating costs of infection and competition with differences in social behaviour. Rather than categorizing birds based on an assumption of linear hierarchies in each cage, here we scored birds as dominant or subordinate depending on the ratio of primary to secondary access to the feeder. Both these scores indicate a motivation to feed and so comparing the occasions spent as the primary bird, to a secondary bird (waiting near the feeder), offers a good approximation of the relative dominance status. We calculated this dominance ratio based on data from the second phase of observations as (PA day 9 + PA day 10 + PA day 11 +1) / (SA day 9 + SA day 10 + SA day 11 +1). Where the ratio was ≥ 1 a bird was categorized as dominant, where it was <1 the bird was classified as subordinate. We did not use the data from the first phase of observations, since at that time the seed diet was augmented with other food items (see above), and overall the birds were less motivated to feed. However, it is important to note that even allowing for this, the dominance ratio pre experiment (phase 1) was significantly positively correlated with the dominance ratio during the experiment (phase 2) (spearman's $\rho = 0.787$, $p < 0.0001$).

2.3 Experimental Infection

We used the avian malaria parasite *Plasmodium relictum* (lineage SGS1) originally obtained from a natural population of house sparrows, and cross-transferred to naive canaries. Infected blood was cryopreserved and stored at -80°C (see details in Bichet et al., 2012). For the purpose of the present experiment, cryopreserved blood was thawed (Bichet et al., 2012) and transferred intraperitoneally to 5 domestic canaries. Eleven days post-infection (dpi), parasitaemia was evaluated from thin blood smears (absolute methanol fixation, 10% Giemsa staining, observation of 10,000 erythrocytes). Blood was collected from donors to prepare a stock suspension diluted in PBS containing the desired number of parasites per inoculum ($5x10^5$asexual parasites) that served to infect birds.

On the day of infection, we captured all birds within a flock. Each bird was weighed, and a small volume of blood was taken in a capillary tube for subsequent haematocrit assessment. Finally, the bird was either injected with *Plasmodium*-infected canary blood, or with control non-infected canary blood. Infected and non-infected flocks were distributed randomly throughout the aviary.

2.4 Post-infection monitoring

Following the experimental infection (day 0), birds were left in their flocks, and were monitored at regular intervals. We re-caught all birds on days 5, 9, 12, 15 and 19 post-infection. On each of these sampling days, we took a small blood sample for haematocrit measurement and qPCR, and weighed each bird. The measurement of haematocrit can be directly representative of damage caused by malarial parasites in canaries (Spencer et al., 2005, Cellier-Holzem et al., 2010).

2.5 Assessing parasite intensity

Parasite intensity was assessed using the quantitative PCR assay (Cellier-Holzem et al. 2010). For each individual we conducted two qPCR reactions in the same run: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7 (5'-AGC CTG AGA AAT AGC TAC CAC ATC TA-3'), 18sPlasm8 (5'-TGT TAT TTC TTG TCA CTA CCT CTC TTC TTT-3'), and fluorescent probe Plasm Hyb2 (5'-6FAM-CAG CAG GCG CGT AAA TTA CCC AAT TC-BHQ1-3')) and the other targeting the 18s rDNA gene of bird (Primers

18sAv7 (5’-GAA ACT CGC AAT GGC TCA TTA AAT C-3’), 18sAv8 (5’-TAT TAG CTC TAG AAT TAC CAC AGT TAT CCA-3’) and fluorescent probe 18sAv Hyb (5’-VIC-TAT GGT TCC TTT GGT CGC TC-BHQ1-3’)).

Parasite intensities were calculated as relative quantification values (RQ) as $2^{-(Ct\ 18s\ \mathit{Plasmodium}\ -\ Ct\ 18s\ Bird)}$ using the software SDS 2.2 (Applied Biosystem). Ct represents the number of PCR cycles at which fluorescence is first detected as statistically significant above the baseline and RQ can be interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (host 18s rDNA). All qPCR reactions were carried out in an ABI Prism 7900 cycler (Applied Biosystem).

2.6 Statistical analyses

For body mass, haematocrit, and parasitaemia we constructed an identical GLMM using SAS (9.1.3). This approach allows for missing values caused by mortality and/or sampling problems. RQ values of parasitaemia were log-transformed before analysis, and thereafter body mass, haematocrit and parasitaemia were modelled with a normal distribution. The models were fully factorial and included fixed factors dominance status (dominant/subordinate), infection (infected/non-infected) and diet (reduced/*ad lib*), in addition to time and time^2 as continuous fixed effects to examine mean changes over time. We also included all possible two and three way interactions between these terms. The interaction among diet, dominance and infection were designed to test our predictions that differences in virulence between dominant and subordinate birds would depend on food availability. For parasitaemia infection and its interactions were removed from the model, since only infected birds have parasites. Additionally we had three random factors in each model. Bird identity nested within cage (bird(cage)) was added, as this allows the model to control for non-independence of birds housed in the same cage over the course of the experiment, and permitted the variance between birds to be estimated. We added cage as a random factor to estimate the variance between cages. We also used time as a random factor with bird(cage) as a subject, using an autoregressive type 1 covariance matrix to estimate within-individual variation, controlling for correlations between observations taken closer together in time. Baseline measures prior to the experiment were included for models of haematocrit and body mass. For our models explaining parasitaemia we did not have a baseline, since parasitaemia is always zero pre-infection. We also analyzed mortality using a simpler model. We tested the probability of mortality using a binary distribution, with infection, dominance, prevalence, and their interactions as fixed factors, and including cage as a random factor to control for the non-independence of birds grouped together. This model did not assess time, since very few birds died during the experiment. To analyze the change in behaviour for the birds, we used the summed frequency of each behaviour during pre-experiment and mid-experiment trials. A model was constructed that included diet, infection and time (pre- or mid-experiment) as fixed factors, and cage and bird(cage) as random factors to account for non-independence of data from the same birds housed in the same cages as before. Dominance was not included in these models as this behavioural data was used to classify subordinate and dominant birds to begin with. These counts were analyzed with a Poisson distribution. Non-significant terms were dropped from the models starting with higher-order interactions, until only significant terms remained. Throughout the results relevant statistics are reported from the final model, though statistics for non-significant terms of interest are reported from the point they were dropped from models. Degrees of freedom were corrected using the satterthwaite method.

2.7 Ethical note

This experiment was carried out in 2009 under the permit # 21-CAE-085 approved by departmental veterinary services.

3. Results

There were no significant differences in mass (means: dominants =23.49 +/- 0.54, subordinates = 25.05 +/- 0.89, F=2.19, p =0.15) or haematocrit (means: dominants =0.427 +/- 0.009, subordinates = 0.444 +/- 0.015, F=0.93, p =0.34) prior to the experiment.

Food availability had no effect on change in haematocrit (Table 1). There was a significant impact of infection on haematocrit: plotting changes in haematocrit (Figure 1) shows that haematocrit reduced sooner, and the reduction was more sustained, in infected than in non-infected birds. The reduction in haematocrit in non-infected birds probably reflects anaemia caused by our experimental procedures i.e. repeated capturing, handling and regular blood sampling. Overall, dominant birds also had a greater reduction in haematocrit than subordinates during the experiment, and this reduction was sustained for longer. Dominant birds reached peak anaemia on day 15 compared to day 12 in subordinates, and by day 18 dominant birds had not recovered in terms of haematocrit (Table 1, Figure 2)

We found a marginally non-significant interaction between dominance and time^2 on parasitaemia (Table 2), and again, diet had no effect. As for haematocrit, our data show that dominant birds had a greater peak in parasitaemia than subordinate birds, though the birds appeared to recover (Figure 3).

We found a significant interaction between dominance status, infection and time on post-treatment body mass (Table 3). This difference appears to be driven by differences in non-infected birds, where non infected dominant birds suffered a great loss of body mass throughout the experiment than non-infected subordinates (Figure 4). Surprisingly, we found no evidence that our food availability manipulation had a significant effect on body mass.

We found no evidence that mortality was affected by either dominant status ($p>0.9$), infection ($p=0.14$) or diet ($p=0.12$).

In order to assess the success of our food-availability treatments on competition, we tested for changes in two measures of behaviour. Total frequency of feeding behaviour was analyzed to test for differences in motivation to feed. This included both primary and secondary feeding, to assess the overall motivation to feed for every bird. Total frequency of antagonistic encounters (each time a bird was aggressive towards another bird, or encountered aggression from another bird) was analyzed to test for differences in competition. We found a significant effect of food-availability on the change in feeding (time*diet $F_{1, 112} = 5.1$, $p=0.026$). There was no effect of either infection or its interactions ($p > 0.2$ in all cases). All birds were more motivated to feed during the experiment than in pre-experimental trials, but birds fed an *ad lib* diet were less motivated to feed during the experiment than birds receiving a reduced diet (Figure 5). We also found a significant interaction between infection and food-availability on antagonistic encounters (time*infection*diet $F_{1, 108} = 5.1$, $p=0.02$). The results were broadly similar to those for feeding behaviour: all birds were involved in more antagonistic encounters during the experiment than before, but during the experiment birds receiving the reduced diet were involved in more antagonistic encounters than those receiving an *ad lib* diet (Figure 6). These results strongly indicate that our dietary treatments were successful in modifying competition in the cages. The effect of infection is less clear, and appears to be driven by the low frequency of antagonistic encounters in pre-manipulation non-infected birds receiving the *ad lib* diet.

4. Discussion

Our aim in this experiment was to assess whether differences between subordinate and dominant canaries in the virulence of avian malaria infection were dependent on host food availability. We found that dominant birds had higher apparent costs of infection; however, we found no evidence that this was altered by the food treatment the birds received. This is a surprising result which we discuss in terms of host behaviour and physiology.

Firstly, we found an effect of dominance on the change in both haematocrit and parasitaemia. Dominant birds had a significantly greater decrease in haematocrit than subordinate birds. There was also a trend for dominant birds to have a sharper (and more variable) increase in parasitaemia than subordinate birds. These results are broadly similar to a previous experiment (Larcombe et al., in press), and our initial prediction that dominant birds would show greater post-infection morbidity and mortality than subordinates in reduced-food groups. However, we expected that this difference between social groups would be ameliorated in *ad libitum* groups, where the costs of protecting or monopolising a scarce food resource would not exist. In fact, there was no effect of food availability on either haematocrit or parasitaemia.

In this study, the loss of haematocrit we observed in dominant birds was apparent in both infected and non-infected birds. Haematocrit readings can be used as an effective measure of the destruction of red blood cells by malaria parasites in canaries (Cellier-Holzem et al., 2010; Spencer et al., 2005), though is subject to modification by many other factors in birds (reviewed in Fair et al., 2007). Fasting and nutritional deficiencies can sometimes result in decrease in haematocrit in birds (e.g. Merino and Potti, 1998; Piersma et al., 2000), however, if this were responsible for the patterns we observed, we would expect that increased food availability would prevent reduction in haematocrit, or reduction in haematocrit would be associated with a concomitant decrease in body mass. We found that non-infected dominant birds suffered a greater reduction in body mass than non-infected dominants (though no difference between infected dominants and subordinates). Why then do dominant birds fare worse than we expected, even when non-infected and provided with *ad lib* food? We are confident that our food-availability treatments had the desired effect on competition: we found that diet significantly impacted both the propensity to feed, and the number of aggressive encounters (indicative of overall competition) between birds. Birds receiving the restricted diet were involved in significantly more antagonistic encounters, and were more motivated to feed during the experiment, that those receiving an *ad lib* diet. Perhaps, rather than competition, fundamental differences in physiology between dominants and subordinates determine the outcome of infection. It has been noted elsewhere that subordinate birds in captivity cannot escape their dominant competitors, leading to unnaturally increased stress levels (Katrschal et al., 1998). Our feeding treatments were designed to ameliorate the competition associated with having a shared food resource: in *ad lib* cages there were three feeders full of seeds, arranged such that they could not be monopolised. Despite this, it is possible that dominant birds were still motivated to exclude other birds from the feeding territory, as they might in the wild, even though they were unable to achieve this. Chronic elevation of hormones associated with this unnatural conflict (Goymann and Wingfield, 2004) may explain why dominant birds generally decreased haematocrit compared to subordinates, or why non-infected dominants suffered greater loss in body mass than non-infected subordinates. Nonetheless, our results for haematocrit and parasitaemia show that the ability of hosts to monopolise food resources may be associated with higher parasite virulence.

In this experiment, our predictions were based on the simple premise that increased food availability would ameliorate the energetic costs of infection and competition. However, interactions between diet and malaria virulence may be more complicated than initially expected. Indeed, the assumption that generally better nutritional state in hosts will benefit resistance to parasites is far from clear cut. In humans, for example, evidence that Protein Energy Malnutrition (PEM) can actually result in *decreased* malaria virulence is widespread, though disputed (reviewed in Shankar, 2000). Additionally, there are some pathogens for which an over-reacting immune system is responsible for greater post-infection damage than direct parasite exploitation (Sorci and Faivre, 2009; Long and Graham, 2011), and these circumstances may favour malnourished individuals, with weaker immune responses. Despite this, we found no evidence that our reduced diet actually helped reduce malaria virulence.

A further consideration is that in this experiment we only modified one dimension of food availability: the quantity of seed available. Perhaps, the quality of food available, rather than simply the quantity, is more important in determining the outcome of parasitism. Key nutrients in the diet such as antioxidant vitamins, minerals and carotenoids can alter immune function, and several studies have shown that dietary availability of these nutrients can have immunomodulatory effects (Bendich, 2001; McGraw and Ardia, 2003; Cha et al., 2010). Indeed, a recent study showed that canaries fed a diet supplemented with egg, lettuce and apples had markedly different responses to *Plasmodium relictum* infection than birds fed a control diet (Cornet et al., in press). However, although parasites achieved larger population sizes and produced more sexual stages in control host than in supplemented hosts, for a given parasitaemia supplemented birds had lower haematocrit than control birds. This shows that the links between food availability, competition, nutrition and immunity are likely to be complex and multifaceted.

In this study we set out to investigate whether the virulence of malaria infection in canaries was modified by social status and/or food availability. As expected, we showed that dominant birds appeared to suffer greater infection-mediated morbidity in reduced food flocks, however, contrary to our expectations this difference was not ameliorated by diet. Indeed, we found little evidence that greater food availability had any effect on traits specifically related to parasite virulence, despite finding that competition was increased by reducing the seed available. Our results show that dominance status in birds can certainly alter parasite virulence, though differences between individual hosts are likely to be multifaceted. Further experiments are required to disentangle the different effects of environment, host behaviour and physiology on the costs of parasitic infection.

Acknowledgements

Financial support was provided by the Région Bourgogne, the CNRS (Programme MIE) and the ANR (IRMAL).

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Figure Legends

Figure 1: Haematocrit change during experiment for infected and non-infected birds. The graph shows reduction in haematocrit value from pre-experiment haematocrit for each sampling point during the experiment (higher values represent more anaemic birds).

Figure 2: Haematocrit change during the experiment in dominant and subordinate birds. Data plotted shows reduction in haematocrit value from pre-experiment haematocrit for each sampling point during the experiment (higher values represent more anaemic birds).

Figure 3: Change in parasite intensity for infected birds. The legend describes the dominance status of individuals

Figure 4: Reduction in body mass for birds during the experiment. The legend describes the dominance status and infection status of individuals

Figure 5: Frequency of feeding behaviours in birds receiving an *ad libitum* or reduced diet, prior to- or during the experiment

Figure 6: Frequency of antagonistic encounters in birds receiving an *ad libitum* or reduced diet, prior to- or during the experiment