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Avian malaria in Collared flycatchers

Fitness consequences and a relation to a secondary sexual character

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Abstract

All organisms have limited amounts of energy, time and nutrients to spend during their lifetime and this gives rise to trade-offs in life histories. It has been shown that increased reproductive effort can reduce parasite resistance and specific immune response in birds. This suggest that there is a trade-off between spending energy on immune system and reproductive effort. Within birds there are several suitable blood parasites that can be used as model organisms for the study of parasite - host interactions, of which 3 genera of protozoan haemosporidians which one could call malaria parasites. Since a few years back it is possible to detect and investigate with high accuracy and speed whether individual organisms are infected with blood parasites or not. Still, there is not sufficient knowledge about how avian malaria parasites affect their host's fitness. In this study I investigated how reproductive success of collared flycatchers is affected by the presence of malaria and if a certain secondary sexual character is correlated to infection. I also used old biometrical data to see if there is a correlation between size as nestling and malaria infection as old.

I found that females infected with avian malaria have a lower reproductive success, and that adult males infected with malaria have on average less white on their 3rd tertial feather than non-infected ones. I also show that infected individuals were smaller as 12 day nestlings than non-infected individuals.

Introduction

Life history theory predicts that there is a cost of reproduction and that there is trade-offs between traits such as parental care, numbers of offspring, and future reproduction (Williams 1966). All organisms have limited amounts of energy, time and nutrients to spend during their lifetime and this gives rise to these and other different trade-offs in life histories (Ricklefs 2007). It has been shown that increased reproductive effort can reduce parasite resistance and specific immune response in birds (Nordling 1998). This could suggest that there is a trade-off between spending energy on immune system and reproductive effort. In the year 1982, Hamilton and Zuk proposed that blood parasites limit the amount of which secondary sexual characters are expressed due to trade-offs, and how that may be connected to sexual selection when the secondary sexual characters works as an honest signal of individuals quality to resist parasites (Hamilton & Zuk 1982). Before this proposal, behavioural ecologists did not focus very much on parasite-host interactions, but this changed dramatically as a result of this article (Clayton 1991). Now, about 30 years later, a vast number of publications have been produced on the subject and parasite-host interactions are well studied.

Within birds, there are several suitable blood parasites to use as model organisms for the study of these interactions, of which 3 genera of protozoan haemosporidians which one could call malaria parasites, *Haemoproteus, Plasmodium and Leucocytozoon*. There are several lineages of each genera and they are all found within erythrocytes and cell tissue in their hosts, in which they acts as parasites (Pérez-Tris *et al.* 2005).
Haemosporidians infects their hosts through the vector of blood sucking insects such as mosquitoes and black flies, and the different parasites have been found in the majority of examined wild bird species (Friend & Franson 1999).

To detect and investigate whether individual organisms are infected with blood parasites can be done since the last years with high accuracy and speed. This due to the new polymerase chain reaction (PCR) techniques that have been developed. Especially since the development of the nested PCR technique described by Waldenstrom *et al.* (2004), the genuses *Haemoproteus* and *Plasmodium* can easily and quickly be detected. This made it possible to analyse host - blood parasite interactions in a better way since the new method is more accurate, sensitive and reliable than former methods (Waldenstrom *et al.* 2004). Still, there is not sufficient knowledge about how avian malaria parasites affect their host's fitness, (Marzal *et al.* 2004). One experimental study in a House martin (*Delichon urbica*) population has showed that birds treated with primaquine, which is an anti-malarial chemical, showed a significantly lower amount of the blood parasite (*Haemoproteus prognei*), increased clutch size, higher proportion of hatched eggs and higher fledgling success (Marzal et al. 2004). Higher fledgling success is also reported in another experiment with Blue tits (Parus caeruleus) where parents where medicated with the anti-malarial chemical primaquine (Merino *et al.* 2000). Moreover, infections of *Haemoproteus* have been shown to reduce survival among Blue tits (*Parus caeruleus*) in a population in Spain (Martinez-de la Puente *et al.* 2010). Another anti-malarial drug is Malarone, which has been tested on Blue tits (*Parus caeruleus*). It was found that medicated infected females got higher fledging success, they provided more food and hatched more eggs (Knowles *et al*. 2009a). That parental care can possibly be negatively affected by presence of blood parasites is also presented by Buchanan *et al.* (1999), in a population of Sedge warblers (*Acrocephalus schoenobaenus*). Male Pied flycatchers (*Fidedula hypoleuca*) infected with *Trypanosoma* blood parasites have been

shown to arrive later to breeding places in Finland after migration compared to non infected individuals. Thus, it seemed that *Trypanosoma,* which is not a malaria parasite, had higher cost for the host than *Haemoproteus* in this trait, since no difference in arrival time could be found among those individuals (Rätti *et al*. 1993). Early arrival to breeding places is an important factor in fitness, since arrival time affects breeding success (Both & Visser 2001). A meta-analysis of elevated reproductive effort and how that affects malarial infection intensity and immune function has been conducted by Knowles *et al.* in (2009b) and it showed that there is good support for causative relationships.

Secondary sexual characters are well studied in Collared flycatchers (*Ficedula albicollis*). For example, females have been shown to choose males with respect to wing patch size, tarsus length and song versatility (Sirkiä & Laaksonen 2009). In the Swedish population, the white forehead patch predicts outcome in competition for territories between males (Pärt & Qvarnström 1997) and also that males with bigger white forehead patches suffer less from *Haemoproteus* parasites (Andersson 2001). This suggests that the forehead patch signals male quality and the ability to resist malaria infections. A female mating with a male with large forehead patch could possibly then increase her fitness by gaining direct benefits. For example, the chicks would be provided more food and/or indirect benefits, such as the chicks would get better genes to avoid or suppress malaria infections. A study made on House finches (*Carpodacus mexicanus*) showed that more brightly colored males were preferred by females and that those males provided more parental care than less colored males. Also, brighter males had a higher survival rate (Hill 1991). Another study on flycatchers made by Szöllösi *et al.* (2009) showed no correlation between neither wing patch size nor forehead patch size on the presence of malaria infection. This study was on a population in Hungary, so it seems that secondary sexual characters and their functions might differ between populations. In other species of birds, correlations between brightness in plumage and malaria infection have been found (Figureola *et al.* 1999). Therefore, it seems that malaria parasites might be costly for their hosts and affect their fitness in both the long and short term.

Aims

This study aims at investigating if presence of malaria infection affects reproductive success among female and male Collared flycatchers in a population on Gotland. I was testing if the amount of white on the males and females 3rd tertial is a possible signal of parasite resistance, as it has been shown in males with the white forehead patch. Also, the amount of white was tested against the reproductive success, lay date, age and condition as chick.
In addition, data from the previous years was used to investigate if there is a difference between infected and non infected Collared flycatchers with respect to their size/condition as 12 day old nestlings. If so, condition already early in life could affect the individuals future susceptibility to parasites. I was investigating whether proportions of infected individuals vary among age groups.

About the Collared flycatcher

The collared flycatcher, *Ficedula albicollis,* is a small insectivorous migratory passerine bird in the family *Muscicapidae*. The males are chromatically colored in black and white with some prominent white patterns such as a white collar, fore head patch, white wing bars and white patterns on the tertials. Females are more indistinctively colored, with most of upper parts in brownish grey. The clutch normally consists of 4-8 pale blue eggs. They lay one egg per day and they start to incubate them on the same day as they laid their last egg. Incubation then lasts for 14 days, and the chick leaves the nest around 2 weeks after they hatched. Breeding occurs in natural holes and nest boxes in trees in deciduous and mixed forests, gardens and parks in central and eastern Europe (Svensson *et al.* 2009). The population in Sweden is restricted to the islands Öland and Gotland in the Baltic sea, even though there have been a few records of breeding pairs on the main land of Sweden.

Methods

Field methods

The study was performed in the southern part of Gotland, an island in the Baltic sea, during mid April to early July in 2010. This population of collared flycatchers has been studied since 1980 (Gustafsson *et al*. 1994) and long term data is available from 14 different woods/sites in the area. The different sites are mainly covered by mixed or deciduous forest and a high density of lower trees and bushes. Every nestbox was checked every fourth day, so if we found eggs we would know the exact laying day of the first egg (from now on referred to as lay date). Once we found eggs, we checked that box every day until the number of eggs in the clutch was not increasing anymore and with that information, we could calculate the expected hatching date. The nests were then visited on the expected hatching date to confirm, and were they not hatched then, we visited them every day until they had hatched. The female was caught during incubation and the male after the chicks were at least 6 days old during feeding, to avoid disturbance during their first days. To catch the adults, we used a special trap that was placed inside the nest box, that would close the entrance hole once the bird went inside. All birds that were not previously ringed were ringed with an aluminum ring with a specific serial number engraved. At the same time, we collected all blood samples and feathers et cetera. After the field season we had all necessary data, including lay dates of first eggs, hatching dates, and number of fledglings for each breeding pair.

Lab methods

The blood samples were collected in the field in EDTA tubes and then centrifuged to separate corpuscles from blood plasma directly after one got in from the field every day. The protocol for centrifuging was 4 minutes at a speed of 6000r/min. The plasma was taken away with a pipette and stored separately. Samples were then kept in a -18°C freezer until we got back to the lab in Uppsala were they were kept in a -80°C freezer until they were to be analysed. The DNA of the bloodsamples was extracted through high salt purification, and to get a suitable concentration of DNA, we measured the concentration in a spectrophotometer and then diluted with ddH_2O to the right concentration (20-50µl/ml) if necessary. A DNA quality check of the extractions was also performed, through electrophoresis on agarose gel (1.5%

agar), 1.5µl Blue dye in a 5µl extraction sample. After this, the gel could be put over UV light to make the marked DNA visible. To investigate whether the individual bird was infected with *Haemoproteus* or *Plasmodium* parasites, the successful extractions were analysed through a nested polymerase chain reaction protocol developed by Waldenstrom *et al*. (2004). The final PCR products where then handled in the same way through electrophoresis as the DNA quality check of the extractions. If a band showed up in the gel, the bird was infected with either *Haemoproteus* or *Plasmodium*. Each extraction sample was processed in the same way 3 times, to be sure of the results. Negative and positive controls were used after every 15th sample to control for possible procedure mistakes. The negative control consisted of ddH_2O and the positive of a known positive sample from an infected bird.

Scanning methods

The amount of white on the birds left 3rd tertial was recorded in the following way:
First the feather was scanned with a black paper background with a Canon MX310 combined scanner/printer. It was scanned in black and white, 1200dpi, contrast "50", paper size "letter", and the output file was saved as a .tif file. The contrast was made quite high to make only the white on the feather visible towards the black background.

The files were opened in Adobe Photoshop CS version 8-0.1 and copy-pasted into a new empty .jpg picture with the size 80x80mm at 1200dpi and 8 bits grey scale.
Since the new picture frame was smaller than the original scanning, I had to fit the feather into the new picture so that the whole feather was visible and then saved in jpg format at maximum quality. The reason to make the images smaller was that they were then not that heavy for the computer to handle and analyse. The amount of white on the feather was measured against the black background in the picture, not against the amount of black on the feather itself. This was because feathers were not completely black and white, sometimes grayish and had rather indistinctive patterns. Also, the pattern on the feather varies a lot and it would be hard to measure it in another way.

Measurements of the amounts of white were performed with the software ImageJ 1.23u (National Institutes of Health 2010). The image was opened and a histogram was made with the corresponding list of the amount of pixels in each grayscale value in the images. Since the image was black and white with a high contrast, the only values in the list was 0 (black) and 255 (white). Total amount of pixels in each image was 14288400, and the percentage of white pixels was calculated with this number.

Statistical analyses

A test of reproductive success among flycatchers with respect to malaria infection (1/0), lay date, and age (1 year / >2 year) and clutch size was performed using a generalized linear model, with Poisson distribution and the link function Log. The test was made both for females and males separately. It has been previously shown that lay date and age may affect reproductive success (Gustafsson *et al.* 1994). Consequently, these factors were included as well. We also tested possible effect of lay date on reproduction success alone, with a generalized linear model with Poisson distribution (link function Log). Age effect (1 year / >2 year) was tested in the same way. The standard 95% CI was used.

To test whether there is a difference in the amount of white on the 3rd tertial between infected and non infected males and females a 2 sample t-test was performed. As nominal factors I used malaria (1/0) and the amount of white as the response variable. The test was performed 2 times, one time for 1 year olds and one time for >2 year olds for each sex, since it has been shown before in collared flycatchers that secondary sexual characters might correlate with, or signal different things at different age (Pärt & Qvarnström 1997).

The amount of white was also tested against definite age (Oneway Anova), lay date (standard least square regression) and reproductive success (Oneway Anova) separately. Also, tarsus length, weight and the residuals between weight and tarsus length were tested against amount of white with a standard least squares regression. To check that residuals were normally distributed, a Shapiro-Wilk W test was performed. The standard 95% CI was used.

Presence of malaria was tested against definite age with a generalized linear model with binominal distribution with the link function Logit.

To test if growing up circumstances might affect malaria presence, I made generalized linear model (binominal errors and link function Logit) with presence of malaria as response factor and as factor variable I birds condition when they were 12 days old (thus, data was used from previous years) and also I used definite age as a control factor. Weight, tarsus length and the residuals between weight and tarsus length was tested. The residuals between weight and tarsus length are a common way of estimating body condition in collared flycatchers (Lars Gustafsson pers. com.). Tarsus length and weight among flycatcher chicks is positively correlated with food provisioning from rearing parents (Moreno *et al*. 2008), and thus can it be used as a measurement of size and/or condition. All the generalized linear models were checked for overdispersion and the standard 95% CI was used.

Results

The breeding season 2010

The breeding season in 2010 was generally late, the median lay day was 4 days later compared to the overall median the last decade (2009 excluded) (Lars Gustafsson, unpublished data). In total 366 pairs of Collared flycatchers started to breed during the season in 2010. Of all chicks, 59.9% survived from egg stage until they fledged two weeks old, which is almost exactly the same as usual (60.0%) when compared to the mean over the last decade (2009 excluded) (Lars Gustafsson, unpublished data). As for the mean number of fledglings per nest, this season produced 3.85 fledglings, a little bit higher than the last decade's overall mean number of 3.74 fledglings per nest (Lars Gustafsson, unpublished data).

Malaria infection and its effect on number of fledglings

Within females and males respectively, 29.9% and 34.8% out of 394 successfully analysed samples showed infection with malaria (*Haemoproteus/Plasmodium*). Presence of malaria had a significant effect on the amount of fledglings among females when controlling for lay date, and age (n= 100, p= 0.0149) (Table 1). Overall test statistics for this test were as follows: (n= 100, df= 6, chi-square= 74.1298, p= <0.0001). Lay date and clutch size also turned out to be significant in the model but not age (Table 1). Among males, no significant effect of malaria could be found (p= >0.05) when running the same test with the same factors. No other factors except clutch size turned out significant either. Overall test statistics for this test: (n=73, df= 7, chi-square= 22.5825, p= 0.020)

Table 1. The effect of the factors; lay date, malaria infection, age and clutch size on females reproductive success.

Factor	ChiSquare	Df	P
Lay date	6.02253081	1	0.0141
Malaria infection	5.9302372	1	0.0149
Age	1.2571928	1	0.2622
Clutch size	43.865581	3	< 0.001

There was a difference between 1 year old versus >2 years old females when testing for fledgling success alone (n= 109, df= 1, chi-square= 4.4474, p= 0.0350). Mean number of fledglings for 1 year old females was 3.79 ± 2.06 (SD) and 4.70 ± 2.05 (SD) for >2 year olds. Lay date also had a significant effect on females number of fledglings (n= 286, df= 1, chi-square= 34.6283, p= <0.0001). Reproductive success decreased with time (Figure 1).
As for males, no significance could be detected when comparing fledgling success with age (n= 76, df= 1, chi-square= 0.5383, p= >0.05) but when comparing fledgling success with lay date a significant effect was found (n= 215, df =1, chi-square= 19.8503, p= <0.0001).

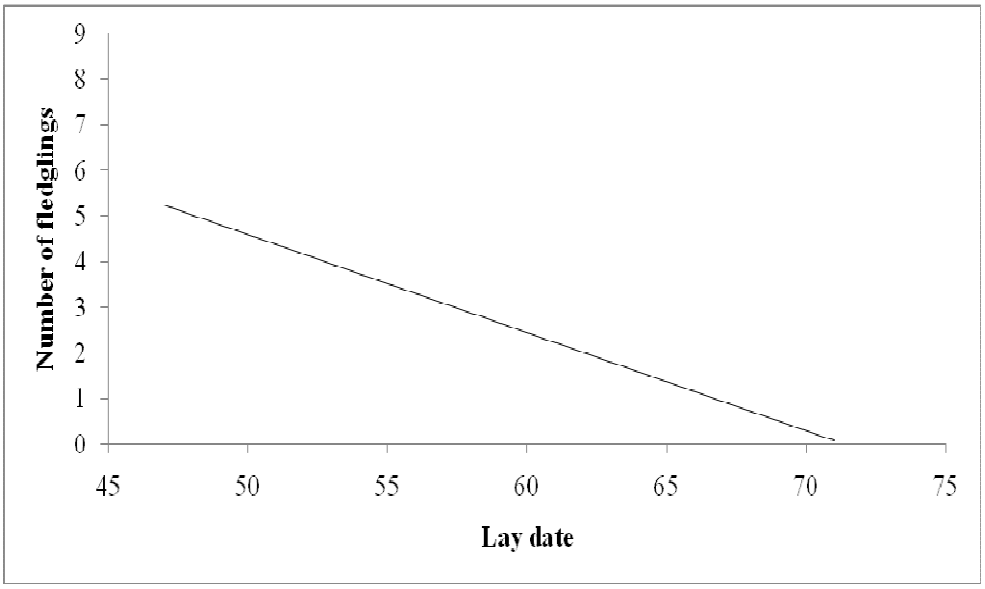

Figure 1. The correlation (fitted line) among females, between lay date and number of fledged chicks during the 2010 season. 1st of April is day 0.

Amount of white on 3rd tertial

A sexual dimorphism was found with respect to the amount of white on their third tertial (t-test; n= 287, t= 22.36934, p= <0.05). The mean among males and females was 2.2% ± 0.41 (SD) and 1.25% ± 0.03 (SD) respectively. Within males, there was no significant correlation between amount of white and lay date (n= 148, f= 2.2792, p= 0.1333). Also, no difference in white with respect to definite age (n= 65, f= 1.6128, p= 0.1504) or reproductive success (n= 147, f= 1.0685, p= >0.3870) could be detected.
In females the results looked about the same. No significant correlation was found between amount of white versus; lay date (n= 135, f= -0.0498, p= 0.8238), age (n= 75, f= 0.6202, p= 0.7134) and reproductive success (n= 127, f= 0.8788, p= 0.5255). In 1 year old males, no significant difference could be found on the amount of white on the 3rd tertial when comparing infected and non infected individuals (n= 6, t= 1.720455, p= 0.9193). However, for the >2 year old males a significant difference could be found (n= 58, t= -1.77392, p= 0.0413). The ones infected had on average less white (mean 2.13% ± 0.32 (SD)) than the non infected (2.29% ± 0.34 (SD)). Among females, the opposite was observed. 1 year old females

infected with malaria had significantly less white on their 3rd tertial than non-infected (n= 16, t= -1.93925, p= 0.0365). Infected females had on average less white (mean 1.10% ± 0.15 (SD)) than non infected (mean 1.34% ± Std Dev: 0.34(SD)). The >2 year old females showed no significant difference in the amount of white with respect to malaria infection (n= 54, t= -0.4239, p= 0.3377). Mean values for non-infected and infected females were 1.22% ± 0.31 (SD) and 1.18% ± 0.36 (SD) respectively. No significant correlation among males was found when testing amount of white with their conditions (weight, tarsus length and residuals between weight and tarsus length) as chicks (in all; n= 34, p= >0.05). The same was found among females (in all; n= 42, p= >0.05). All Shapiro-Wilk tests for normal distribution turned out non significant (p= >0.05)

Age and infection

I found a significant difference between ages with respect to presence of malaria (n= 186, df= 6, chi-square= 15.8392, p= 0.0146) (Table 2).

Table 2. Numbers of birds with known age, numbers infected and the percent of infected individuals

Age (year)	Nr	Infected	% Infected
1	43	11	25.6%
2	61	15	24.6%
3	30	10	33.3%
4	30	18	60.0%
5	15	6	40.0%
6	4	0	0%
7	3	1	33.3%
Sum	186	61	32.8%

Growing up conditions

When testing residuals of weight by tarsus length as 12 days old chick with presence of malaria (with definite age as control), no significance was found. Whole model test gave; n= 104, chi-square= 11.7921, p= 0.0378. The effect of the factor residuals weight by tarsus length was non significant (p= 0.4337). But, when testing only for weight and tarsus length separately there was significance in tarsus length (whole model; n= 104, chi-square= 19.9470, p= 0.0013, effect test for tarsus length; p= 0.0031) but not in weight (whole model; n=104, chi-square= 14.4258, p= 0.0131, effect test for weight; p=0.0716). Mean tarsus length for

infected was 19.59mm ± 0.56 (SD), and for non infected 19.82mm ± 0.49 (SD). Regarding weight, mean for infected individuals was 14.00grams ± 0.69 (SD), and non infected 14.79 grams ± 1.13 (SD).

Discussion

The proportion of avian malaria infected individuals is about the same as has been shown before on the island (Nordling 1998). On another Swedish island, Öland, also in the Baltic sea, similar numbers are observed (Katarzyna Kulma, pers. com.) and this is not surprising since the populations are so close to each other. This could support the fact that the detection through the developed nested PCR is consistent, since the same technique is used on Öland.

This study supports the findings that infection of malaria parasites can reduce fledgling success among birds, even though it was a purely observational study, compared to most of other studies with similar results. The study suggests that presence of malaria is costly and can reduce reproductive success among females, and that infection together with lay date may be important factors in reproductive success among female collared flycatchers on Gotland. No similar significant results could be found among males, but this could possibly be explained by the fact that when the number of fledglings was 0, the nest was abandoned/dead early in the breeding attempt before the male was caught (and thereby known) or missing. This study only detected the blood parasites as present or absent, and it would be of great interest to investigate whether there is a gradient of how the amount of parasites can influence the reproductive success among collared flycatchers on Gotland. Also, it would be useful to catch the males earlier, so that they are known before nests possibly die. This would generate start data (0 data) also for males. An experiment with treatment of malaria would also be beneficial, because otherwise it is difficult to identify definite causative effects.

The amount of white on their 3rd tertial is a sexually dimorphic character, and in males individuals more than one year old showed a difference in the amount of white on their third tertial with respect to presence of malaria. Young males did not show this correlation and this suggests that amount of white could act as a signal of male quality among adult birds but not among young individuals. Perhaps infection does not have enough impact on the bird early in infection stage and that then the trait is used later in life. There are uncertainties regarding when in life malaria infects birds. Cosgrove *et al*. (2006) found no evidence of infected chicks in blue tits and they suggest that either the chicks were not bitten by malaria carrying vectors, or the disease had not yet developed. Also the prepatent period of avian malaria can last for several months. *Haemoproteus* has indeed been found in 12 day old collared flycatcher chicks on Gotland, even though it only was in 7 out of 303 samples (Nordling 1998). My test with young males only consisted of 6 samples and the result should be interpreted with great caution considering the probable low power of the test. No significant correlation could be found between amount of white and lay date, age or reproductive success. Since lay date was negatively correlated with reproductive success and reproductive success was difficult to investigate because of regular missing data for males when number of fledglings in the nest is low or even 0, this result may be misleading. One important issue is also that if the amount of white acts as a signal of parasite resistance, it does not necessary reflect the current situation (i.e. the situation when the bird was caught) It would rather tell if the bird was infected, and possibly how much parasites it had when it moulted the last time and not when we caught it and took the blood sample. Adult collared flycatchers moult their tertials both after the

breeding season and during their time in their wintering areas (Svensson1992). It also yet remains to examine whether the amount of white in the tertial is a sexually selected trait. Regarding the white forehead patch in male collared flycatchers, it has been shown that females gain indirect benefits when mating with a male with a large badge (Sheldon *et al*. 1997) and that the size of the badge predicts male parental care (Qvarnström 1997). This would be interesting to investigate with respect to the amount of white on the tertial. Among females, the opposite was observed, young infected individuals showed significantly less white in their 3rd tertial than non-infected and this was not found among old females. I found no reasonable arguments for this result. The test of young females included only 16 birds, the standard deviations differed somewhat and the p-value was not very low (even though < 0.05) so maybe this result also should be treated with caution.

Presence of malaria was significantly connected to age and it seems like the proportion increases with age and then decreases again (table 2). This could suggest that it takes some time before these birds get exposed to the parasite and possibly that malaria is correlated negatively with survival. However, it is hard to point out causative effects with this data and analysis, and low amount of data in the older ages causes some doubts. More data on this is would be needed to establish and possibly strengthen the conclusions.

As for the test comparing body condition as 12 days old chick and the presence of malaria later in life, I found significant results pointing out that among larger birds (longer tarsus) malaria is less prevalent when older, than among smaller ones. This results suggests that the growing up circumstances could affect the future susceptibility of the individuals to parasites and thereby also their reproductive success, at least among females, as shown in this report. Brood size manipulations (*cf*. Nordling 1998) would be a useful tool to investigate this phenomenon in more depth, since manipulated brood size can lower and heighten the condition of the chicks.

Conclusions

This study found some important correlations; that presence of malaria is connected to fledgling success among females, and that the amount of white on males 3rd tertial is correlated to presence of malaria. It was also found that small chicks seem to be more susceptible to malaria parasites. As with many other studies, this one gives rise to more questions, mentioned earlier in the discussion, and I think that experimental methods are needed to find more causative effects.

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