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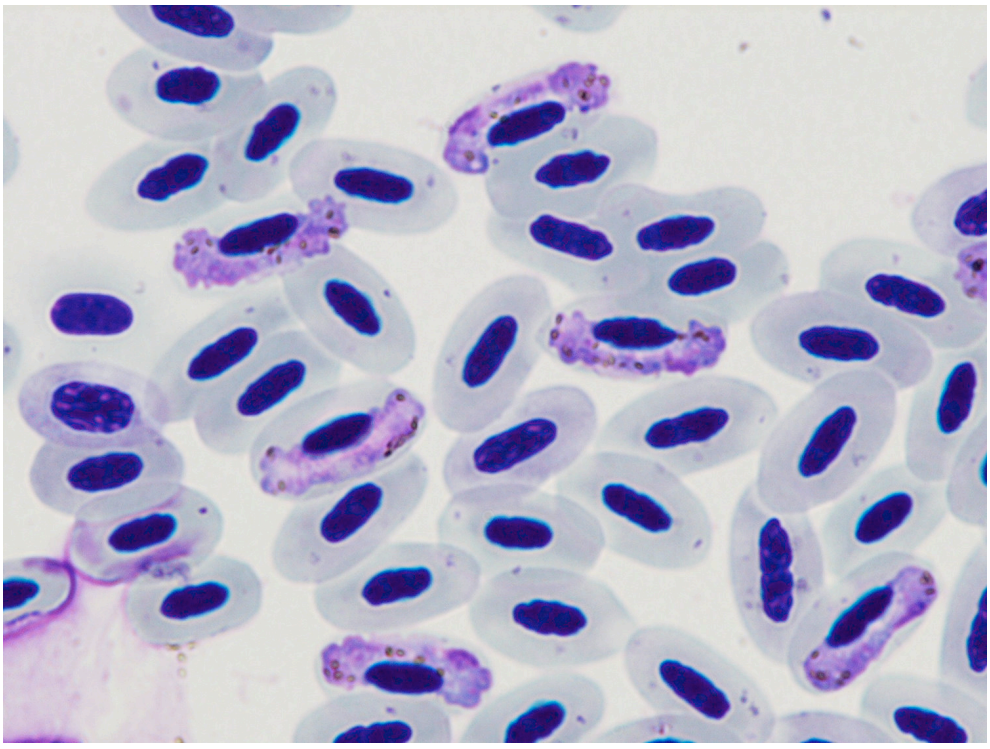
# Species divergence in parasite resistance:

## Are pied flycatchers tighter co-evolved with the shared malaria parasites than collared flycatchers?



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**ABSTRACT**

Parasites are ubiquitous. They are possibly the biggest ecological group in nature and no species is known that would not be affected by them. In my study I investigate the differences in prevalence and susceptibility to blood parasites of the group *Haemosporidia* and *Haemoproteus pallidus* of two competing species Collared (*Ficedula albicolis*) and Pied Flycatcher (*F. hypoleuca*). To reveal the prevalence of haemospiridians, I use nested PCR protocol amplifying parasite DNA. To measure the level of infection of *Haemoproteus pallidus*, I used quantitative PCR (qPCR) with a set of species-specific primers. I show that Pied Flycatchers are more frequently infected than Collared Flycatcher (44.3% and 17.4% respectively) and there is no difference in the level of infection between these flycatcher species. Moreover, Pied Flycatchered showed to be more tightly co-evolved with blood parasites than Collared Flycatchers, by showing stronger relationships between the frequency and level of infection with other condition-depended (body mass, wing length) and sexually selected traits (forehead and wing patch size, grey plumage on the back). I conclude, that experimental approach is necessary to reveal the direction of casual relationship and I also recommend to extend future studies to genotyping of generally infected samples. Our knowledge about population dynamics in species under interspecific competition is still developing but it is far from being complete. Parasites play a significant role in such systems that cannot be neglected. My study is a small contribution and step forward towards understanding it.

## INTRODUCTION

Parasitism can be described as an ecological relationship between two different organisms - the parasite and the host. Parasites are physiologically or metabolically dependent on their hosts, but their reproductive potential is much higher. Parasites can affect the hosts in different ways – influencing their behavior, mating and reproductive success, or even cause their death. This situation sets the stage for an antagoinistic co-evolutionary arms-race where the parasite evolves better ability to unitilize its host while the host evolves a higher resistance. In the host, the favourable genes coding for resitace will be specific for a particular parasitic environment. Because parasites affect fitness of hosts e.g. through their longevity (*Morand & Harvey, 2000*), fecundity (*Obrebski 1975*) or other life history traits, they may by extension influence competitive interactions between different host species. For example, the relative competitive ability of two or more host species may vary across parasitic environments.

Birds may carry many different kinds of parasites – macroscopic creatures found on the external parts of their body (ectoparasites), but most of them are microscopic: bacteria and protozoans found internally (endoparasites). In my study I focused on protozoan blood parasites, genus *Haemoproteus*, member of *Haemosporidia* family, evoking malaria in infected individuals. More specifically, the aim of this paper is to investigate influences of haemosporidians on two competing flycatcher species.

### Competition, Exclusion and Coexistence

*„Interspecific competition is one of the most fundamental phenomena in ecology, affecting not only the current distribution and success of species but also their evolution"* as Townsed and co-workers ( put it. It comes from the fact, that due to competitive effects, such as reduction in growth, fecundity or survivorship, the population dynamics is affected. It might have, in turn, a significant impact on species' distribution and their evolution.

Pied (*Ficedula hypoleuca*) and collared flycatchers (*Ficedula albicolis*) are closely related and ecologically similar species (Lundberg & Alatalo 1992) that co-occur in central and eastern Europe. They breed also on the Baltic islands of Öland and Gotland where Collared flycatchers are dominant species. Both bird species share a very similar ecological niche, therefore in these areas Collared Flycatchers tend to exclude Pied Flycatchers from some microhabitats (Gustafsson & Pärt 1991; Sætre et al. 1999a,b).

Yet, Qvarnström and co-workers showed that PF differ from CF in some life-history traits and in their response to environmental changes (Qvarnström et al. 2005). It can affect the final population dynamics of these species. According to the research, PF not only produce larger broods, breed later, produce more fledged offspring, but also their reproductive success is less sensitive to the seasonal decline in environmental conditions

(low availability of food, for instance). It is suggested that adaptation of Pied flycatchers to poor environmental conditions might reflect a higher stress of tolerance, whereas Collared flycatchers seem better to exploit good conditions. This research demonstrats that even species using similar resources may experience a reversal in relative fitness. It is interesting to ask: how these species cope with parasitic infections and how it affects their life-history strategies under circumstances of competition?

Parasites may play a significant role in dynamics of populations, having an immense impact on functioning of ecosystems (Morcogliese, 2005; Hudson et al. 2006). The effect of parasites on their hosts can be even more drastic under stressful conditions, like in shrinking ecosystems (Holmes 1996). Combe (1996) gives the example of two closely related *Drosophila* species – *D. simulans* and *D. melanogaster*. The latter overcompetes *D. simulans* under standard environmental conditions, two species coexist when parasite (parasitoid wasp, *Leptopilina boulardi*) is added to a system and finally, *D. simulans* overcompetes *D. melanogaster* in stressful conditions (lowered temperature) and presence of parasite. Therefore parasites can play a key role in the outcome of interspecific competition.

**What are malaria parasites?**

The popular definition of malaria – presented, for instance, on World Health Organization web site (http://www.who.int/en/ ) – that it is a human disease caused by four *Plasmodium* species that are transmitted by *Anopheles* mosquitoes was recently challenged (Pérez-Tris et al. 2005). Now, the view that other groups of *Haemosporidia* family that consists of *Plasmodium*, *Haemoproteus* and *Leukocytozoon* genera, should be included, prevails.

All three genera are closely related, intracellular parasitic protozoans that can be found in blood cells and other tissues, causing hemosporidiosis – or avian malaria (Friend and Franson, 2001). Avian haemosporidians are very common bird parasites, recorded in about 68% species (Loye and Zuk, 1991). As Bennett with co-workers (1994) showed, only 6% of 53 passerine families by then studied were free from Haematozoan blood parasites. *Haemoproteus* is the most common genus encountered among the family, infecting up to 67% of bird species. Additionally, it is believed to be least pathogenic but still having the biggest impact on young birds that did not achieve their resistance yet (Loye and Zuk, 1991).

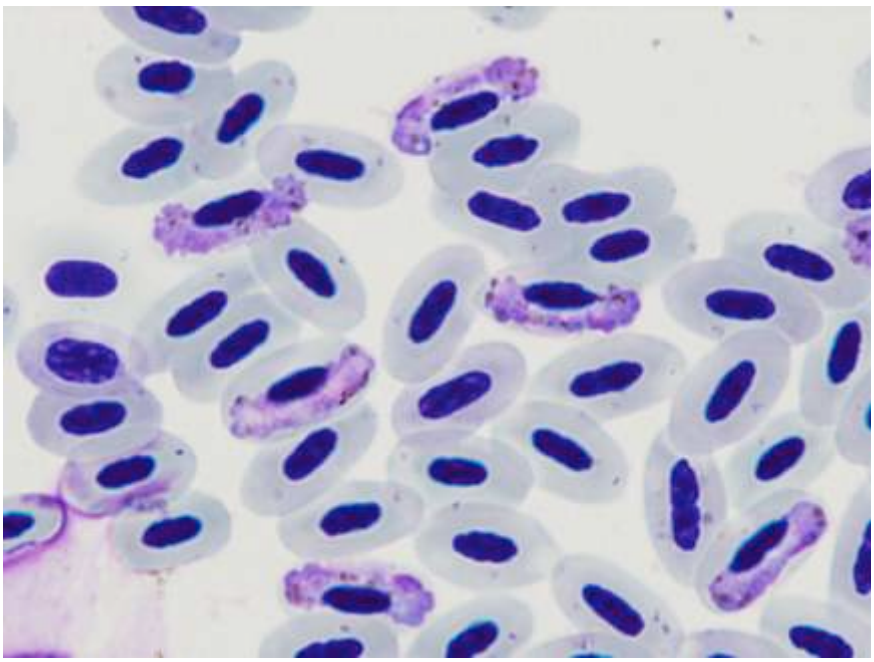


**Figure 1.** Micro- and Macrogametocytes of *Haemoproteus pallidus* (Picture kindly provided by Vaidas Varinauskas)

**Life cycle**

Haemosporidian parasites have complex life cycle involving birds as a host where the development to sexual maturity takes place and blood-sucking insects – as a stage where sexual and asexual reproduction takes place. Birds get infected by one of blood-sucking insects that serve as a vector (Table 1.) During a blood meal, infective stages of the parasite – sporozoites – are transmitted to the tissues and blood of a bird. After infection sporozoites invade the tissues and reproduce to become merozoites. The latter have an ability to penetrate red blood cells, where after maturation they become infectious gametocytes (**Figure 1.**). From now on gametocytes, as they are in circulating blood cells, can be transmitted to another blood-sucking insect. When it happens, cycle becomes completed, as parasites undergo first sexual, and second asexual reproduction to become sporozoites.

**Table 1.** Avian hemosporidia parasites and their documented vectors (*Friend and Franson, 2001*)

| Parasite | Vector | Common name |
|---|---|---|
| ***Haemoproteus*** | *Ceratopogonidae (Culicoides sp.)*<br>*Hippoboscidae (Ornithomyia sp.)* | Punkies, no-see-ums, sand flies<br>Hippoboscid or louse flies |
| ***Plasmodium*** | *Culicidae*<br>*(Culex, Aedes sp.)* | Mosquitoes |
| ***Leucocytozoon*** | *Simulidae (Simulium sp.)* | Black flies |

**Flycatcher Study System**

Flycatchers are small passerine migratory birds. They have their wintering sites in sub-Sahara Africa, from where they migrate for breeding season to Europe. Collared (*Ficedula albicolis*) and pied flycatchers (*F. hypoleuca*) co-occur throughout central and eastern Europe, as well as on the Swedish island Öland. Pied flycatcher (PF) is a less common species, representing 18% of breeding flycatchers (*Wiley et al. 2005*). Males of this species show uniformly black back and relatively small white patch on their forehead. Collared males are easily recognizable in their breeding plumage with clear black and white pattern and visible collar on their neck (see Figure 2.). Females of both species are very similar with grayish-brown plumage, but can be distinguished by subtle differences (*Svensson & Grant 1999*). Flycatchers have adapted to breed in nest boxes which had been installed on the island. Breeding season starts at the end of April and it lasts until the beginning of July.

**Figure 2**. **Left**: Adult male Collared Flycatcher (*Ficedula albicolis*), **Middle**: Adult male Hybrid, **Right**: Adult male Pied Flycatcher (*F. hypoleuca*) *Photos kindly provided by Thor Veen*

**Trade-offs, life history traits and different life history strategies**

In order to investigate how haemosporidians influence the relative fitness of the two flycatcher species I need to not only study differences in infection *per se* but I also need to investigate how infection relates to different aspects of reproductive performance in the two species.

Traits of the organism that are involved in maximizing fitness are called *life-history traits*. These include growth, reproduction, survival and immune defense (Sandland and Minchella, 2003). If the trait is costly to maintain but still adaptive, it generates trade-offs with other traits. Species, or even individuals, differ regarding strategies of resource investment into different traits, i.e. in how the trade offs between different costly traits are solved. Such strategies are called *life history strategies*.

No wonder that more and more researches provide a support for the idea, that maintaining immune defense, including resistance against parasites, is a costly trait demanding a big resource investment. They show that there are among other trade-offs between immunity and breeding success (Ilmonen et al. 2000), reproductive investment (Allander 1998; Nordling 1998), parental effort (Råberg et al. 2000) and basal metabolic rate (Ots et al. 2001)

Another trait having a potential to play an important role in birds' survival, is flight performance. It not only helps in escape from predators, but more importantly for insectivorous species foraging on flying insect (such as flycatchers) it is an important tool to feed offspring effectively. One of the indicators of flight performance is wing – tail length ratio (W/T ratio). Some studies suggest that shorter wings (lower W/T ratio) may promote higher maneuverability, whereas longer wings (higher W/T ratio) might facilitate long-

distance flight, for instance during migration (Wysocki and Kiriaka, 2007). Therefore individuals might benefit from different life history strategies depending on different selection pressure and environmental conditions (i.e. food abundance, competition, etc.). I will compare the relationships between the above listed key traits and high parasitemia between the two flycatcher species.

In conclusion, the aim of my study is to investigate susceptibility to haemosporidians of two flycatcher species. Additionally, I want to reveal if there is any association between frequency and level of infection and their life-history traits and how it affects two competing species. Also I want to find out if there is any trade-off between resistance against parasites and performance of secondary sexual traits. Finally, I want to check if there is any pattern or species specificity in infections of *Haemoproteus pallidus* and *H. balmorali*. To achieve my goal I will:

- identify general infections of *Haemosporidia* parasites using molecular method - nested PCR
- distinguish infections between two parasite species that are most frequently found in flycatchers: *Haemoproteus pallidus* and *H. balmorali*
- estimate a level of infection by quantifying parasite DNA in infected samples using qPCR method

## MATERIALS AND METHODS

### Samples collection and DNA extraction

The blood samples used in this investigation were collected from 167 Collared (*Ficedula albicolis*) and 115 Pied Flycatchers (*F. hypoleuca*) breeding on Baltic island Öland during the field season in 2007. Blood was collected from females during their incubation period and from males during the time of feeding nestlings. The collected blood samples were stored in SET buffer or absolute ethanol and later extracted in the laboratory using standard phenol-chloroform extraction followed by ethanol precipitation (Sambrook et al., 1989) DNA concentration was measured for each sample and PCR dilutions were prepared (4 – 20ng/ml). 10 random samples with genomic DNA were run on 2% agarose gel to validate DNA quality.

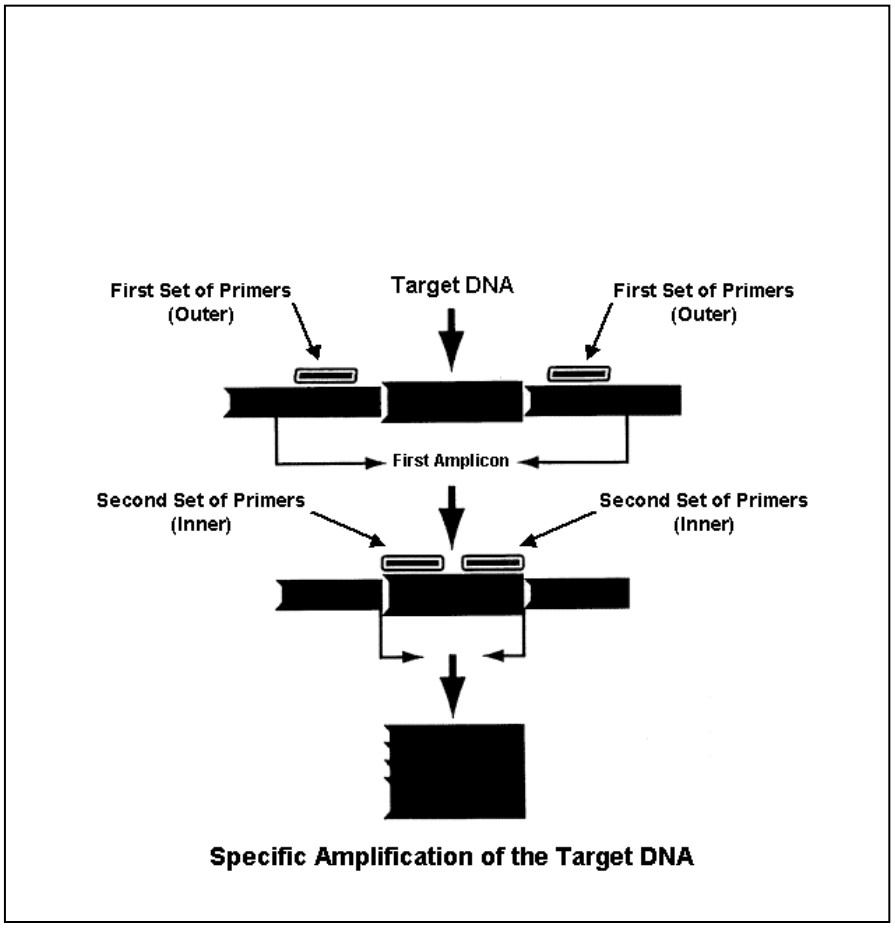
### Nested PCR – description

The aim of polymerase chain reaction (PCR) is to amplify specific DNA fragments. Nevertheless, it might happened that due to insufficient specificity of primers or simple mistakes, unwanted DNA fragments can be amplified and contaminate the PCR product. To avoid such a contamination, nested PCR is used.

Nested PCR is divided into two phases – first, a set of primers is used to amplify a longer fragment of DNA that consists of target DNA fragment and neighboring region. In second phase, PCR product from a first stage is used as template DNA. This time different set of primers is used, amplifying specifically target DNA (Figure 3.).

In this research I used a nested PCR method to amplify parasite DNA. More specifically, I focused on their mitochondrial DNA - cytochrome b fragments. As other researches have shown, nested PCR assay with cyt b fragments amplification is more accurate and sensitive compared to blood smear screening (Bentz et al. 2006) or other PCR assays based on ribosomal 18s rRNA fragments amplification (Buling et al. 2007).

**Figure 3.** A scheme of nested PCR method (http://www.wisconsinlab.com/)




**Quantitative PCR – description**

Quantitative polymerase chain reaction (qPCR) or Real time polymerase chain reaction (RT – PCR) is a modification of PCR which simultaneously quantifies and amplifies a specific part of a given template DNA. Its mechanism is based on the kinship of fluorescent dye to double – stranded DNA (dsDNA), which fluoresce once binded to it. It helps to determine whether a specific nucleic acid sequence is present in the sample and how many its copies there are after each full cycle. That is its *real time* aspect. It is a useful technique for the investigation of gene expression, viral load, pathogen detection and numerous other applications.

**Nested PCR**

All samples were run with a nested PCR as Waldenström et al. (2004) showed. It was performed in 25-ml volumes with the primers HAEM $NR_2$ (5'-AGAGGTGTAGCATATCTATCTAC-3') and HAEM NF (5'-CATATATTAAGAGAATTATGGAG-3') in the first run, amplifying 580-bp fragment. Each reaction contained approximately 5 - 20 ng of genomic DNA, 1.5 mM $MgCl_2$, 2.5 ml 103 PCR buffer II (100 mM Tris–HCl, pH 8.3, 500 mM KCl), 400 mM of each deoxynucleoside triphosphates, 0.6 mM of each primer, and 0.625 U of AmpliTaq (Applied Biosystems, Foster City, California). The thermal profile started with 3 min of denaturation at 94°C, followed by 20 cycles at 94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec, and ended with an elongation step at 72°C for 10 min.

For the final PCR, 1.0 ml of the PCR products from the initial PCR was used as template in a 25-ml-volume reaction with the primers HAEM $R_2$ (5'GCATTATCTGGATGTGATAATGGT-3') and HAEM F (5'ATGGTGCTTTCGATATATGCATG-3'), amplifying 524-bp fragment. The second run of nested PCR was performed with the same reagents and the same proportions as the first run, but with 35 cycles instead of 20. Negative control samples with $ddH_2O$ instead of genomic DNA, as well as positive control samples with genomic DNA of individual with known infection were routinely used to make sure that the PCR outcome resulted from a real infection, not a contamination.

All PCR products were visualized on 2% agarose gels stained with ethidium bromide. Positive results were interpreted as a presence of parasitic gametocytes or marozoites. All samples showing a band on the gel were re-run in order to confirm the infection. The ones that showed infection at the first time and did not confirm it at the second time, were run a third time to achieve a final result. 20% of uninfected samples were randomly re-run in nested PCR to confirm lack of infections.

**Quantitative PCR (qPCR)**

I set two standard mixes with infection of *Haemoproteus pallidus* and *H. balmorali* in 5-step dilution series. At each step the concentration of parasitic DNA was diluted 5 times in a DNA extract (1ng/µl) from a non-infected flycatcher. Flycatcher's DNA was needed to keep the same DNA concentration throughout the dilutions series. The initial infected DNA concentration was exactly 1ng/µl with infection intensity at the level of 3%. To check the parasitemia in infected birds I prepared qPCR dilutions (1ng/µl) of genomic DNA, ran each sample in qPCR with two different sets of primers specific for a parasite. Each sample was run with two repetitions and their curves development was compared with standard curves. Quantitative PCR protocol was performed 25µl volume using Invitrogen™ SYBR® Green qPCR Master Mix with ROX dye and species specific primers for *H. pallidus* HAEM FX (5'- TAACTGGTGTATTATTAGCAACTTG - 3') and PALL 1R (5'-TGCTACTGGTGCTACATTTGTA-3') and *H. balmorali* HAEM FX and BALM 1R

(5'-CACAGGTGCTACATTTGTATTT-3'). Each reaction contained 5ng of DNA, 12.5 µl of master mix and 0.6 mM of each primer. Moreover, for mixes with *H. balmorali* primers 1.5 mM $MgCl_2$ was added. The thermal profile started with 3 min of denaturation at 94°C, followed by 20 cycles at 94°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec, and ended with an elongation step at 72°C for 10 min. The final outcome of parasitemia for each individual was achieved by taking the arithmetical average from both repetition results . The outcome was interpreted as non-infected with any analyzed strain of parasite when the level of parasitemia was at least one order of magnitude lower than the level of infection of standard mix with the highest dilution. All samples where amplification results were unclear (positive and negative result in repetitions, very low amplification rate) were re-run to confirm the final outcome.

**Data analysis**

All data, after proper transformations, were statistically analyzed using JMP® 7.0.1 software. The results of qPCR were visualized using Mx-Pro – Mx3000P® software.

## RESULTS

### 1. General patterns

In total 282 samples were analyzed, 167 of Collared (*Ficedula albicolis*) and 115 of Pied Flycatchers (*F. hypoleuca*). Pied Flycatcher, PF, were more frequently infected with *Haemosporidia* parasites than Collared Flycatchers, CF, ($\chi^2$ = 23.40, p < 0.0001) with parasitemia of 44.3% and 17.4% respectively. No significant sex-specific prevalence was found neither in CF ($\chi^2$ = 0.002, p > 0.96) nor in PF ($\chi^2$ = 2.02, p > 0.16). In addition, there was no significant differences in susceptibility to parasites between different age groups of birds (in CF: $\chi^2$ = 0.27, p > 0.61; in PF: $\chi^2$ = 0.57, p > 0.45).

Among all 80 samples that showed positive result in nested PCR (general infection), 63 gave an amplified product in qPCR using species-specific primers for *Haemoproteus pallidus* and *H. balmorali*. 6 samples were excluded from analysis as they did not give a clear amplification result even after re-running the protocol.

Only 1 individual was infected with *H. balmorali* parasite and 2 individuals showed mixed infection with both parasite strains. Therefore, no species-specificity in parasitic infections was found among two species of flycatchers. For further analysis regarding parasite load only individuals infected by *H. pallidus* were taken into account, all the other ones were excluded.

Even though CF are much less frequently infected, they are no significant differences in the level of infection between two flycatcher species ($t_{1,53}$ = 1.42, p > 0.16). Adults tend to be non-significantly more heavily infected than juveniles in PF ($\chi^2$ = 2.91, p > 0.09), but not in CF ($\chi^2$ = 0.36, p > 0.55). No sex is more prone to heavy infections, neither in CF (One-way ANOVA, $F_{1,43}$ = 0.004, p > 0.95) nor in PF (One-way ANOVA, $F_{1,34}$ = 0.004, p > 0.95).

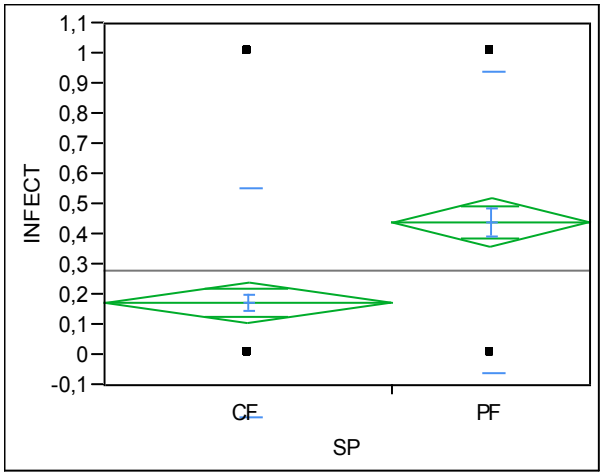



**Figure 3**. The prevalence of two flycatcher species CF- Collared Flycatcher and PF – Pied Flycatcher to Haemosporidians

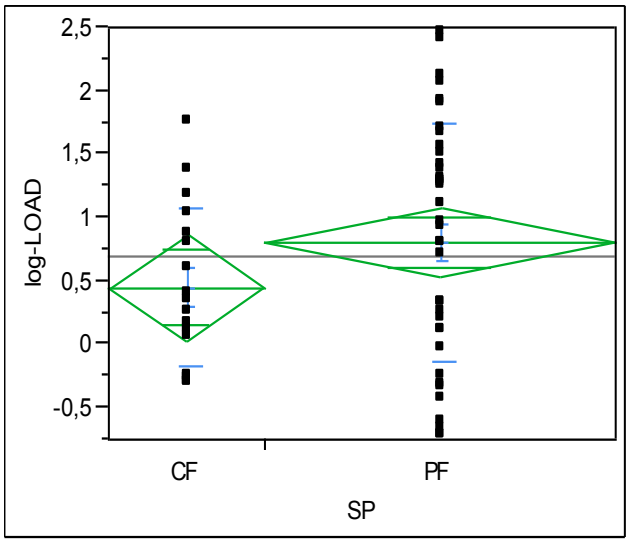



**Figure 4.** The level of infection with *H. pallidus* of two flycatcher species – CF – Collared Flycatcher and PF – Pied Flycatcher

## 2. Parasite infection, condition – dependent traits and reproductive success

Collared Flycatchers seem to be marginally affected by parasites regarding their condition and reproductive success. No significant relation was found between frequency or level of infection and laying date clutch size, number of hatchings, beak length, tarsus, tail length, wing length or mass (in all $p > 0.1$). There is a tendency, for infected birds to have more fledglings ($F_{1, 165} = 2.53$, $p > 0.11$). Moreover, CF females are significantly lighter when infected ($F_{1, 86} = 4.85$, $p > 0.03$), but no other condition-dependent traits show correlation with susceptibility to parasites (Table 2.),

**Table 2.** Traits of Collared Flycatchers related to frequency of infection with haemosporidians.

| Trait | Df | F - value | N | P > |
|---|---|---|---|---|
| Laying date | 1 | 0.006 | 154 | 0.94 |
| Clutch size | | 0.120 | 167 | 0.73 |
| Number of hatchlings | | 0.089 | 165 | 0.77 |
| Number of fledglings | | 2.530 | 165 | 0.11 |
| Beak length | | 0.790 | 167 | 0.37 |
| Tarsus | | 0.320 | 167 | 0.57 |
| Tail length | | 0.019 | 166 | 0.89 |
| Wing Length | | 0.340 | 167 | 0.56 |
| Sum of white on the wing | | 0.094 | 167 | 0.76 |
| Grey on the back | | 1.030 | 70 | 0.31 |
| Body mass | | 1.440 | 165 | 0.23 |
| Patch size | | 0.006 | 159 | 0.94 |

Pied Flycatchers do not show any significant relationship between the frequency of infection and components of reproductive success such as clutch size, number of hatchlings or fledglings (in all $p > 0.2$). Also condition-dependent traits like laying date ($F_{1,104} = 0.30$, $p > 0.58$) or tarsus ($F_{1,115} = 0.15$, $p > 0.70$) do not seem to be significantly correlated with prevalence to parasites. However, I found a significant tendency for heavier birds to be more frequently infected ($F_{1,112} = 5.73$, $p > 0.02$), conversely to Collared Flycatchers. Also among juveniles, more frequently infected individuals are heavier ($F_{1,67} = 6.36$, $p > 0.01$). This result is not affected by higher frequency of females – as they are much heavier than males during

breeding season – because sex ratio between age groups does not differ ($\chi^2$ = 0.0002, p > 0.86).

Moreover, in males we can see also significant negative correlation between frequency of infection and wing length ($F_{1,41}$ = 6.69, p > 0.01), what might affect their flight performance (see below).

### 3. Parasite infection and sexually selected traits

When both species are analyzed together, in males there is a strong negative association between the frequency of infection and wing length ($F_{1,282}$ = 18.50, p < 0.0001) and sum of white on wings ($F_{1,\ 283}$ = 9.37, p > 0.002). These results might be confounding, though, as wing length and the sum of the white are strongly and positively correlated ($F_{1,167}$ = 36.66, p < 0.0001).

In CF males' sexually selected traits such as forehead patch size ($F_{1,80}$ = 0.06, p > 0.80) or the proportion of grey on the back ($F_{1,70}$ = 1.03, p > 0. 31) show no relation to frequency of infection. Another trait – the sum of white on the wings – is also treated as sexually selected (Sheldon and Ellegren, 1999) , where individuals with a high sum of white also tend to have more fledglings ($F_{1,165}$ = 4.97, p > 0.03) and come to breeding sites earlier ($F_{1,154}$ = 12.61, p > 0.0005). Additionally, these individuals tend to be more heavily infected ($F_{1,10}$ = 7.85, p > 0.02). Neither this kind of association nor any other is found in CF juvenile individuals.

In males of PF prevalence to parasites tends to be negatively related to the proportion of grey on the back ($F_{1,33}$ = 7. 93, p > 0.008). . Interestingly, malesmore heavily infected tend to have smaller patch on their forehead ($F_{1,6}$ = 5.019, p > 0.09), but this association is not significant. Moreover, general tendency shows that birds with higher sum of white on the wings are more heavily infected ($F_{1,29}$ = 7.52, p > 0.01), what we can find specifically in females ($F_{1,21}$ = 5.63, p > 0.03), but not in males ($F_{1,8}$ = 1.75, p > 0.23).

### 4. Flight performance

W/T ratio may play an important role in flight performance in birds, influencing their maneuverability and cost of flight in general. In Pied Flycatchers there is no difference between age classes in W/T ratio ($F_{1,113}$ = 2.27, p > 0.13). However, even though females have shorter wings ($F_{1,113}$ = 11.04, p > 0.001) and tales than males ($F_{1,113}$ = 22.44,p < 0.0001), they have significantly higher W/T ratio ($F_{1,113}$ = 4.19, p > 0.04).

Collared Flycatchers' juvenile individuals tend to have lower W/T ratio than adults ($F_{1,161}$ – 7.07,p > 0.01), but there is no difference between sexes ($F_{1,166}$ = 0.73, p > 0.39).

Wing-to-tail length ratio (W/T) was associated with fitness in the two flycatchers species: Pied Flycatchers females tend to benefit from a low W/T ratio by larger clutch size ( $F_{1,73}$ =

4.28, $p > 0.04$), number of hatchlings ($F_{1,73} = 11.43$, $p > 0.001$) and fledglings ($F_{1,73} = 14.39$, $p > 0.0003$), whereas males only by bigger clutch size ( $F_{1,40} = 4.47$, $p > 0.04$). On the other hand, Collared Flycatchers with low W/T ratio come to breeding sites later ($F_{1,153} = 20.60$ $p < 0.0001$), that means when the environmental conditions and food abundance go worse, and at the same time they have smaller clutches than individuals with higher W/T ratio ( $F_{1,166} = 4.68$, $p > 0.03$). Moreover, CF females specifically show negative association between laying date and W/T ratio ( $F_{1,80} = 22.82$, $p < 0.0001$).

In PF more heavily infected birds have lower W/T ratio ($F_{1,33} = 7.56$, $p > 0.01$). It's also a case specifically for females ($F_{1,24} = 6.43$, $p > 0.02$), but not for males ($F_{1,9} = 1.06$, $p > 0.34$). Conversely, males with lower W/T ratio tend to be more frequently infected ($F_{1,40} = 7.34$, $p > 0.01$), but not females ($F_{1,73} = 0.73$, $p > 0.40$). Also among juveniles, individuals more heavily infected seem to have lower W/T ratio ($F_{1,24} = 6.44$, $p > 0.02$).

In opposition to PF, in CF W/T ratio is not affected by the frequency ($F_{1,166} = 0.16$, $p > 0.69$) or the level of infection ($F_{1,13} = 0.43$, $p > 0.84$).

## DISCUSSION

There were significant differences both in the frequency of infection by haemospirians and in the level of infection of *Haemoproteus pallidus* parasites between two competing flycatcher species . Pied flycatchers were 2.5 times more frequently infected than collared flycatchers, yet,there is no differences in the level of infection between the species. One possibility, is that pied flycatchers are more frequently infected because they are exposed to more insect vectors due to differences in their migration patterns and location of wintering grounds (Veen et al. 2007) or because of microhabitat segration during the breeding season (Adamík and Bureš, 2007).

The difference in prevalence (frequency of infection) may be explained by various factors. Pied flycatchers might be more frequently infected due to ecological niche they inhabit where are more insect vectors and probability of infection is higher. Bennett with co-workers (1995) showed that northern populations of pied flycatchers suffer more from increased prevalence and intensity of parasitism and concluded that ecological conditions may determine the composition, transmission and prevalence of blood parasites. Moreover, Qvarnström with co-workers (2005) put it, Pied Flycatchers are exposed to a more northern climate, as they breed throughout a larger part of Europe compared to Collared Flycatchers. Therefore Pied Flycatchers, because of range of their breeding sites may be subjected to parasite infection more often than Collared Flycathers.

Still, these differences may also result from disparity in immune defense. On one hand, lower susceptibility of collared flycatchers might be explained by higher resistance to specific parasites. On the other hand, it may be the effect of lower resistance that results in high mortality of infected birds, so that we can analyze only survivors with relatively low prevalence. However, if we consider that collared flycatchers seem to be much less affected by parasites in condition-depended and sexually selected traits than pied flycatchers, the last suggestion seems to be improbable. Additionally, the fact that *Haemoproteus sp.* parasites tend to have a minor effect on other life history traits in collared flycatchers with high parasitemia in infected birds may support the idea, that due to ecological differences they are less exposed to parasites, that even if infect a host, do not make much harm to it.

Given that pied flycatchers are much more often infected the parasite spend much more time co-evolving with this species. Hence, we should expect the parasite to do less harm when they infect collared flycatchers (which is in line with the observed stronger negative impact on life history traits in pied flycatchers). The difference in prevalence and susceptibility to parasites in two competing species may play an important role in the final outcome of this competition. The parasite infection may weaken (or strengthen) competitive abilities to the significant degree due to different life-history strategy forced (see Combes 1996). In this case, Pied Flycatcher is more susceptible species to blood parasites. They also seem to be more tightly co-evolved with blood parasites, what we can see in strong

correlations between the frequency and level of infection with other traits. At the same time this species is outnumbered by Collared Flycatchers and tend to be competitively excluded from better microhabitats (Qvarnström et al. 2005). It is hard to conclude that exclusion results from a higher prevalence to parasites, but without a doubt it has its contribution to it.

The outcome of the interspecific competition depends not only on competitive abilities and life-history strategies of breeding birds, but also the shape and recruitment of juvenile individuals. The latter may be to the significant extend affected by parasites and various infections. One could predict that we should observe a significant difference in frequency or level of infection between yearlings and adults. On one hand, juvenile individuals might be more susceptible to parasites due to lower acquired immunity or lack of behavioral abilities helping to avoid infection (Sol et al. 2003). On the other hand, adults might be more exposed to parasites for a longer period of time and therefore be more frequently infected than juveniles (Weatherhead and Bennett, 1999). In my study I did not find any significant differences in parasitemia between the age groups in either species. Even though I found a non-significant tendency for pied adults to be more heavily infected, it does not support an idea of considerable differences in resistance against parasites between young and older birds, or of higher risk of being infected later in life. Collared Flycatchers seem to be marginally affected by parasites regarding their condition and reproductive success. No significant relation was found between frequency or level of infection and condition-depended traits or reproductive success. One exception shows sex-specific, significant negative relation between frequency of infection and mass in CF females. Loss of mass in females during breeding season is commonly observed in passerines. It is presumed to reflect either physiological stress from activity while feeding nestlings or adaptive mass adjustment in order to reduce power required to remain aloft in flight before the most demanding period of feeding nestlings (Freed 1981; Merila and Wiggins 1997, Hillström 1995). As all blood samples from females were collected during incubation period, lower mass in infected individuals may reflect the physiological stress due to incubation and strengthened by parasite infection. This shows negative effect of parasites, by the possible trade-off between mass maintenance and a mount of immune response.

Conversely to collared flycatchers, infected pied flycatchers tend to be heavier and this tendency is even stronger for yearlings. This opposite trend might reflect a different life – history strategy in resource investment in two species. Body mass is a commonly known reliable survival predictor in fledglings (Sagar and Horning 1997; Naef-Daenzer et al. 2001) and factor influencing the success of migration (Schaub and Jenni, 2001). The energy investment into augmented body mass may be more beneficial to young birds than maintenance of elevated immune response.

On the other hand, it is possible that only superior individuals – with higher body mass or other condition – depended trait – may be able to pay the augmented price for breeding under parasitic infection. In individuals of worse quality the breeding could be too demanding and costly when they are infected. We cannot exclude that the superior individuals are selected through mate-choice, that means – by sexual selection. Hamilton and Zuk (1982) suggested that parasitism may facilitate the evolution of conspicuous secondary sexual traits which would indicate mate quality in terms of resistance against local parasites. This hypothesis was based on several assumptions: 1) host fitness decreases with increased parasite infection, 2) ornament condition decreases with increased parasite burden, 3) there is heritable variation in resistance to parasites, 4) female choice favors the most ornamented males, 5) female choice favors the least infected males. As it was explained by the authors, suitable parasites for testing their theory are those that 1) debilitate their host instead of killing it or allowing total recovery; and 2) that cause a disease, which can be acute and can result in heavy juvenile mortality, but persists in chronic form in survivors.

If we take into account the sum of white on the wings as a sexually selected trait then tendency in collared and pied flycatchers is opposite to what we could expect according to Hamilton & Zuk hypothesis. Namely, more infected birds tend to be even more conspicuous than non-infected individuals. That could suggest the existence of a strong trade – off between resource investment into performance of sexually selected traits and immunity.

Does the fact that Idid not find a negative association between a size of a sexually selected trait (wing patch) and the level of infection by hemosporidians in the flycatchers mean that I can, in this case, reject the Hamilton-Zuk hypotheses? That conclusion would be premature. This is because I cannot exclude the possibility that only individuals in high condition (indicated by a large sexually selected trait) can survive and breed with a high level of infection in their bodies. An experimental approach would be needed in order to exclude this possibility or a complete knowledge on the level of infection and ornamentation among individuals that died before breeding. The fact that the frequency of infection was found to be negatively associated with wing white among one-year old individuals also corroborate this interpretation.

Exclusively for pied flycatchers, the higher frequency of infection correlates with less grey on the back in males.

Darker plumage is more costly than white one (McGraw 2003) and it’s a good predictor of more advanced age and experience (AQ, verbal information) so that it is beneficial to invest into darker plumage and augment the probability of being chosen by females. Apparently it is more beneficial than investment into increase of resistance against parasites that might decrease chances of survival in minor manner. That supports a thesis about the existence of trade – off between sexually selected traits and immune response.

Alternatively, the high ratio of grey plumage on the back in Pied Flycatchers may reflect the alternative colour morph of the male. We distinguisch two colour morphs in these males during breeding season – a brownish, female – like colour morph, and conspicuous black – and – white morph. The brown males tend to escape from the predation (Slagsvold et al. 1995), as well as from intraspecific aggression (Järvi et al. 1987). The fact that males with more grey on the back tend to be less frequently infected than uniformely black males, may indicate that brown males can escape aggression also from Collared Flycatchers, once they breed in the same microhabitats and therefore be potentially less exposed to vectors. Moreover,in case of avoiding Collared Flycatchers' aggression, PF could invest more resources into mounting immune response and therefore be less frequently infected by blood parasites. That shows how important role may parasites play in the system of closely related, competing bird species.

The other interesting trait that shows correlative relationship with the frequency of infection, is wing-to-tail length ratio, that reflects the efficiency and type of flight performance. Again, Collared Flycatchers do not seem to be affected by frequency or the level of infection in this respect. Conversely, in Pied Flycatchers W/T ratio shows strong relation with heamosporidian infection. Firstly, it is not intuitive why pied flycatchers benefit from a low W/T ratio by bigger clutch size or number of hatchlings at all. Low W/T ratio may account for higher maneuverability (Wysocki and Kiriaka, 2007) and therefore a higher success in capturing flying insects. Therefore it is understandable that pied females with lower W/T ratio tend to have more fledglings. It is interesting they are also more heavily infected than other females, even though there is no difference in probability of being infected. Perhaps it has something to do with parental effort put into capturing more insects for hatchlings and costs put into this activity. That would make females more susceptible to deleterious effects of parasites once they are infected. On the other hand, males with lower W/T ratio tend to be more frequently infected, without suffering from infection in remarkable manner. That could suggest that performed behavior facilitates a contact with parasite vectors and make males more prone to infection. However, it remains a riddle, why increased parental effort reduce immune response in females, but not in males. Possible explanation is that, like in case of some condition - depended and sexually selected traits in Pied Flycatchers, also in this case only individuals in better condition and of better quality can afford breeding while being infected. Without a doubt more experimental and not only correlative studies need to be carried out, to show the direction of casual relationship between the frequency and level of infection and other traits.

## CONCLUSION

Parasites are ubiquitous. They are possibly the biggest ecological group in nature and no species is known that would not be affected by them (Windsor 1998). In my study I investigate the differences in prevalence and susceptibility to blood parasites of the group *Haemosporidia* and *Haemoproteus pallidus* of two competing species Collared (*Ficedula albicolis*) and Pied Flycatcher (*F. hypoleuca*). I show that Pied Flycatchers are more frequently infected than Collared Flycatcher and seem to be more tightly co-evolved with parasites, as they show more and stronger relations to condition – depended and sexually selected traits. The possible explanation for some of the results I have got is that birds of better quality and in better condition are more likely to breed under parasite infection. Nevertheless, correlative studies are not enough to draw such a strong conclusion, that is why experimental approach is necessary to have a clear image of direction of casual relationships and differences in life – history strategies in these two bird species. That would give us an invaluable input into the studies of the issues of competition over similar niches between closely related species in wild populations.

Moreover, in my study I focused on general infections of Haemosporidians, to specify the level of infection with the species of *Haemoproteus pallidus*. It would be interesting to genotype all samples generally infected to have a full image of prevalence to blood parasites in flycatcher species and to reveal the influence of specific genera or even parasite species on other condition – depended and sexually selected traits of their hosts.

Our knowledge about population dynamics in species under interspecific competition is still developing but it is far from being complete. Parasites play a significant role in such systems that cannot be neglected. My study is a small contribution and step forward towards understanding it.

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**REFERENCES**

**Adamík P. and Bureš S.** (2007) Experimental evidence for species-specific habitat preferences in two flycatcher species in their hybrid zone, *Naturwissenschaften*, **94**, 859–863

**Allander K.** (1998) Reproductive investment and parasite susceptibility in the Great Tit, *Functional Ecology*, **16**, 358-364

**Bennett G. F., Peirce M. A., Earle R. A.** (1994) An annotated checklist of the valid species of *Haemoproteus, Leucocytozoon* (*Apicomplexa*: *Haemosporida*) and *Hepatozoon* (Apicomplexa: Haemogregarinidae), *Systematic Parasitology,* **29,** 61–73

**Bennett G. F., Squires-Parsons D., Siikamaki P., Huhta E., Allander K., Hillstrom L.** (1995) A Comparison of the Blood Parasites of Three Fenno-Scandian Populations of the Pied Flycatcher *Ficedula hypoleuca, Journal of Avian Biology,* **26,** 33-38

**S. Bentz, T. Rigaud, M. Barroca, F. Martin-Laurent, D. Bru, J. Moreau, B. Faivre** (2006) Sensitive measure of prevalence and parasitaemia of haemosporidia from European blackbird (Turdus merula) populations: value of PCR-RFLP and quantitative PCR, *Parasitol.*, **133**, 685–692

**A. Buling, A. Criado-Fornelio, G. Asenzo, D. Benitez, J.C. Barba-Carretero, M. Florin-Christensen** (2007) A quantitative PCR assay for the detection and quantification of Babesia bovis and *B. bigemina*, *Vet Parasitol*, **147**, 16–25

**Combes C.** (1996) Parasites, biodiversity and ecosystems stability. *Biodiversity and Conservation,* **5**, 953-962

**Freed L. A.** (1981), Loss of Mass in Breeding Wrens: Stress or Adaptation? *Ecology*, **62**, 1179-1186

**Friend and Franson** (1999) **-** Field Manual of Wildlife Diseases: General Field Procedures and Diseases of Birds, USGS-National Wildlife Health Center

**Gustafsson, L. and Pärt, T.** (1991) Interspecific relations between collared and pied flycatchers. In Proc. XX Int. Ornithol. Congr., (ed. B.D. Bell, R.O. Cossee, J.C.E. Flux, B.D. Heather, R.A. Hitchmough, C.J.R. Robertson & R.A. Williams), pp 1425-1431, Wellington: New Zealand Ornithological Congress Trust Board

**Hillström L.** (1995) Body mass reduction during reproduction in the pied flycatcher *Ficedula hypoleuca*: physiological stress or adaptation for lowered costs of locomotion? *Functional Ecology,* **9,** 807–17.

**Holmes, J. C.** 1996. Parasites as threats to biodiversity in shrinking ecosystems. *Biodiversity and Conservation* **5**, 975-983

**Hudson P. J., Dobson A. P., Lafferty K. D.** (2006) Is a healthy ecosystem one that is rich in parasites? *Trends in Ecology & Evolution*, **21**, 381-385

**Ilmonen P.,Taarna T., Hasselquist D**. (2000) Experimentally activated immune defence in female pied flycatchers results in reduced breeding success, *Proc. R. Soc. Lond.* B, **267**, 665-670

**Loye and Zuk**, 1991

**Lundberg A. and Alatalo R. V.** (1992) The Pied Flycatcher, Poyser, London

**Marcogliese D. J.** (2005) Parasites of the superorganism: Are they indicators of ecosystem health? *International Journal for Parasitology*, **35**, 705-716

**McGraw K. J.** (2003) Melanins, metals, and mate quality, *Oikos*, **102**, 402 - 406

**Merila J. and Wiggins D. A.** (1997), Mass Loss in Breeding Blue Tits: The Role of Energetic Stress, *The Journal of Animal Ecology*, **66**, 452-460

**Morand S., Harvey P. H.** (2000) Mammalian metabolism, longevity and parasite species, *Proc. Roy. Soc*., **267**, 1999-2003

**Naef-Daenzer B., Widmer F., Nuber M.** (2001) Differential post-fledging survival of great and coal tits in relation to their condition and fledging date, *Journal of Animal Ecology*, **70**, 730 – 738

**Nordling D.** (1998) Trade-offs between life history traits and immune defense in the collared flycatcher *Ficedula albicolis*, Acta Universitatis Upsaliensis, Uppsala

**Obrebski S.** (1975) Parasite reproductive strategy and evolution of castration of hosts by parasites, *Science*, **188**, 1314-1316

**Ots I., Kerimov A. B., Ivankina E. V., Ilyina T. A., Hõrak P.** (2001) Immune challenge affects basal metabolic activity in wintering great tits, *Proc. R. Soc. Lond.* B, **268**, 1175 – 1181

**Pérez-Tris J. Hasselquist D. , Hellgren O., Krizanauskiene A., Waldenström J., Bensch S.** (2005) What are malaria parasites?, *Trends in Parasitology*, **21**, 209-211

**Qvarnström A., Svedin N., Willey C., Veen T. , Gustafsson L.** (2005) Cross-fostering reveals seasonal changes in the relative fitness of two competing species of flycatchers, *Biol. Lett*, **1**, 68–71.

**Råberg L., Nillson J-Å, Ilmonen P., Stjernman M., Hasselquist D.** (2000) The cost of an immune response: vaccination reduces parental effort, *Ecol. Let.*, **3**, 382-386

**Rintamäki P. T., Ojanen M., Pakkala H., Tynjälä M.** (1998) Blood parasites of migrating Willow Warblers (*Phylloscopus trochilus*) at a stopover site, *Can. J. Zool.*, **76**, 984–988

**Saetre, G-P., Kral, M., Bures, S. & Ims, R.A.** (1999) Dynamics of a clinal hybrid zone and a comparison with island hybrid zones of flycatchers (*Ficedula hypolecua* and *F. albicollis*). *Journal of Zoology*, **247**, 53-64.

**Sagar, P. M.; Horning, D. S.** (1997) Mass-related survival of fledgling sooty shearwaters *Puffwus griseus* at the Snares, New Zealand. *Ibis,* **140***,* 329-339

**Sambrook J., Fritsch E. F., Maniatis T.** (1989) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

**Sandland G. J. and Minchella D. J.**, (2003) Costs of immune defense: an enigma wrapped in an environmental cloak? *Trends in Parasitology*, **19**, 571-574

**Sanz J. J., Moreno J., Arriero E., Merino S.** (2001) Interactions between hemoparasite status and female age in the primary reproductive output of pied flycatchers, *Oecologia*,**126**, 339–344

**Sheldon B. C. and Ellegren H.** (1999) Sexual selection resulting from extrapair paternity in collared flycatchers, *Animal Behavior*, **57**, 185-198

**Slagsvold T., Dale S., Kruszewicz A. (**1995) Predation favours cryptic coloration in breeding male pied flycatchers, *Animal Behavior*, **50**, 1109-1121

**Sol D., Jovani R.,Torres J.** (2003) Parasite mediated mortality and host immune response explain age-related differences in blood parasitism in birds, *Oecologia*, **135**, 542–547

**Svensson L., Grant P. J.** (1999) Bird Guide, HarperCollins*Publishers*, London

**Thomas F., Poulin R., Guégan J-F., Michalakis Y.,F. Renaud** (2000), Are there Pros as well as Cons to being Parasitized?, *Parasitology Today*, **16**, 533-536

**Colin R. Townsend C. R., , Begon M., Harper J. L.** (2003) Essentials of Ecology, Malden, MA : Blackwell Publishing 2003.

**J. Waldenström, S. Bensch, D. Hasselquist, Ö. Östman** (2004) A New Nested Polymerase Chain Reaction Method Very Efficient in Detecting Plasmodium and Haemoproteus Infections From Avian Blood, *J. Parasitol.*, **90(1)**, 191–194

**Weatherhead P., Bennett, G. F.** (1991) Ecology of red-winged blackbird parasitism by haematozoa, *J. Can. Zool*. **9**, 2352-2359

**Wiley C., Bengtson J. M., Svedin N., Qvarnström A.** (2005) Hybridization cost of delayed maturation of secondary sexual traits in the collared flycatcher, *Evolution*, **12**, pp. 2711–2716

**Windsor D. A.** (1998) Most of the species on Earth are parasites, *Int. J. Parasitol.***28**, 1939-1941

**Wysocki and Kiriaka** (2007) Wing to tail length ratio in European blackbirds (*Turdus merula* L.) of different age, *Pol. J. Ecol.*, **1**, 121 - 125

**Veen T., Borge T., Griffith S. C., Sætre G., Bures S., Gustafsson L., Sheldon B. C.** (2001) Hybridization and adaptive mate choice in flycatchers, *Nature*, **411**, 45-50