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Effects of avian malaria and *Eimeria* (Euccocidiorida: Emeriidae) on body condition in Eurasian Blue Tit (*Cyanistes caeruleus*)

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CONTENTS

Abstract

Recently, there has been an interest in studying host-parasite predator-prey interactions due to the importance parasites have on host community structure and function. The Eurasian Blue Tit (*Cyanistes caeruleus*) is a well-known passerine bird that serves as host for different parasites, among them avian malaria blood parasites. Effects on plumage coloration due to infection have been demonstrated previously but the effect of avian malaria on body condition has not been well studied. In this project, the blue tits were tested for avian malaria by using nested-polymerase chain reaction (PCR) and were also evaluated for a body condition index, with the aim of finding out whether or not parasite presence is associated with lower values of body condition. Our results showed no relationship between body condition and infection, as similar body condition values in both infected and uninfected birds were observed. Also, fecal samples from Blue Tits were tested using standard endpoint PCR for the presence of parasites of the genus *Eimeria*, a gastrointestinal coccidian parasite. Some birds, which also tested positive for malaria, showed evidence of possible infection by an *Eimeria* species. This supports the occurrence of parasitic co-infection in the study birds.

Laburpena

Azkenaldian jakin-min handia dago bizkarroi-ostaraliaren harrapari-harrapakin elkarrekintzetan, bizkarroiek ostalari komunitatearen egitura eta funtzioan duten garrantzia dela eta. Amilotx urdina (*Cyanistes caeruleus*), Passeriformes ordeneko txori ezaguna da, zeinak bizkarroi desberdinentzako ostalari modura jarduten duen, horien artean, txoriengan eragiten duten malaria odol parasitoa. Bizkarroi hauen infekzioak lumen kolorean eragina duela frogatu da jada, baina gorputzaren egoeran duen eragina ez da behar bezala ikertu oraindik. Proiektu honetan, amilotx urdinak malariaz gaixoturik dauden aztertuko da nested-PCR bidez eta gorputzaren egoeraren ebaluazioa egingo da ere, indize bat lortzeko asmoz. Horrela, kondizio balio baxuak infekzioarekin bat datozen egiaztatuko da. Lortutako emaitzek kondizio indize eta infekzioaren artean erlaziorik ez dagoela adierazten dute, gaixotu eta gaixotugabeen artean kondizio balio antzekoak lortu baitira. Horrez gain, amilotx urdin hauetan *Eimeria* generoko parasito intestinalen presentziarik dagoen aztertu da PCR bidez, kasu honetan gorotzetatik lortutako material genetikoa erabiliz. Azken honetarako aztertutako txori batzuk, zeintzuek malaria gaixotasunerako positibo eman zuten, *Eimeria* generoko espezieren batez infektatuta daudelaren ebidentziak azaldu dute. Emaitza hauek bat datoz txori hauetan egindako ikerketetan lortutako ko-infekzio gertaerekin.

1. INTRODUCTION

There is continuing interest in understanding host-parasite interactions because they can shape community structure through their effects on trophic interactions, competition, and biodiversity, while also serving sometimes as keystone species (Preston & Johnson, 2010). Parasites function as micro-predators on their host, and these interactions are ubiquitous in ecological communities, producing fitness (Powell & Prior, 2016) and behavioral (Preston & Johnson, 2010) consequences for the host. Parasites have negative impacts at various organizational levels including impacts on individuals, populations and species, altering both host community structure and function (Smith *et al.*, 2009), and leading to epidemics, which may reduce host diversity (Valkiūnas, 2004). 'Multi-host' parasites are capable of infecting more than one host and impact the evolution and dynamics of infectious diseases, while complicating disease control effectiveness (Gandon, 2004). Thus, having a better understanding of parasite-host dynamics can help decrease parasite transmission, reduce infection, and predict epidemic cycles.

Multi-host parasites represent at least 60% of human zoonotic pathogen species (Taylor *et al.*, 2001), constituting a public health problem due to our close relationship with animals (CDC, 2017). In addition, a single host can be co-infected, which means the host is simultaneously infected by more than one parasite (Clark *et al.*, 2016). Since host-parasite interactions are ubiquitous and can vary among different hosts and parasites, altering parasite communities could lead to an alteration of disease dynamics, thus, altering wildlife populations (Rogalski *et al.*, 2017). These stressful situations could reveal a negative body condition-infection relationship in hosts, even if resources are not limited and conditions are favorable (Sánchez *et al.*, 2018). Infection can also affect condition-dependent preference during mate choice (Cotton *et al.*, 2006). Birds, as many other living beings, serve as hosts for different type of parasites including those of the blood, gastrointestinal system, and external surfaces (Bensch *et al.*, 2000; Hellgren *et al.*, 2004; Schwarz *et al.*, 2009), but unlike most animals, birds are able to migrate long distances, potentially helping to spread the parasite and to transmit it to other animals. Parasitism can reduce the reproductive success and post-breeding condition of birds (Merino *et al.*, 2000), and parasites such as avian malaria also affect plumage coloration (del Cerro *et al.*, 2010). However, it has barely been studied how parasite infection affects body condition, creating a knowledge gap that should be covered since body condition can influence individual fitness and mate choice (Merino *et al.*, 2000).

Avian haemosporidian multi-host parasites are a diverse and prevalent group of blood protozoans in the phylum Apicomplexa, and cause avian malaria (Bensch *et al.*, 2000; Bensch *et al.*, 2004). The order Haemosporida includes the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, that are transmitted by mosquitoes acting as vectors (Kimura *et al.*, 2010). Analysis of mtDNA indicates that avian malaria presents over 10,000 species and is widely distributed (Bensch *et al.*, 2004). Malaria causes anemia, reduces mobility (Allander & Bennett, 1995) and has a role in plumage coloration, especially,

carotenoid-based colors (del Cerro *et al.*, 2010). In addition, infection caused by haemosporidian parasites can stabilize at low levels, relapsing upon stressful and demanding situations such as breeding season, food shortage, and migration (Cornet *et al.*, 2014). Another multi-host parasite infecting birds, are species in the genus *Eimeria*, which are found in the gastrointestinal system (Badparva *et al.*, 2015). *Eimeria* is a one-celled protozoan in the phylum Apicomplexa and is mostly homoxenous, meaning it only has one host (Perkins, 2000). Birds can get infected by *Eimeria* through contaminated food, soil, and water, or even from exposure to contaminated insects and other animals. These parasites are very important in the poultry industry, and result in substantial economic losses. Birds within the order Passeriformes also become infected with *Eimeria* as has been demonstrated in previous studies (Dolnik *et al.*, 2010; Berto & Lopes, 2013; Oliveira *et al.*, 2017).

Body condition has long been used to deduce impacts of parasitism or animal fitness (Sanchez *et al.*, 2018) but currently, little is known about how avian malaria affects body condition index. Body mass is considered a body condition indicator since it helps in estimating the ability of birds to store energy, and thus, is used as index of individual fitness (Cresswell, 2009). The correlation between body mass and fitness can be negative or positive (Beldomenico *et al.*, 2008), even if in nature is usually positive, and additional factors like environmental conditions (Macleod *et al.*, 2008), habitat quality (Weimerskirch & Lys, 2000) and host-parasite interactions (Beldomenico *et al.*, 2008) should be accounted for. Birds with low mass in cold weather could be in the process of starving to death (positive correlation between mass and fitness), while in other situations, lower body mass than other individuals could indicate a better survival probability, meeting all foraging requirements without having to store fat in excess (negative correlation) or requiring less energy to reach sexual maturity (Cresswell, 2009). Another body condition indicator is tarsus length, which refers to the structural size of the bird, and is taken into account with the aim of obtaining a better body condition index (Brown, 1998). The effects that parasites may have on birds and individual parasite status could supplement information on body condition of the bird and also indicate how communication is affected by infection. Infected birds are expected to have lower body condition than uninfected birds, since infection acts negatively on the host, but no relation at all or/and limited correlation within a range of body condition values is also a possibility.

A good way to prove the effect of avian malaria in bird body condition is by studying species within the order Passeriformes, since they the most diverse, largest, ubiquitous avian order that represents 60% of all living birds (Ericson *et al.*, 2014) and also a susceptible order, possibly due to their dominance in diversity (Valkiūnas, 2011). The specie we chose for our experiment is the Eurasian Blue Tit (*Cyanistes caeruleus*), a non-migratory passerine with a wide European distribution and which is a known host for various parasites, including those causing avian-malaria. These birds are noteworthy because of their plumage coloration such as the dark blue-black eye-stripe, blue crown, yellow chest and white bright cheeks (Hill, 2006b) and avian malaria infection in these birds is known to cause less saturated

carotenoid-based coloration production (Hill, 2006a; Brawner *et al.*, 2000; Merino *et al.*, 2000), reducing reproductive success and post-breeding condition (Merino *et al.*, 2000). However, the correlation between avian malaria and body condition has barely been studied. Considering that body condition is important for birds, this project would be a good opportunity to acquire knowledge about the effect of avian malaria on body condition, at both specie (*Cyanistes caeruleus*) and order level (Passeriformes).

To approach this, a captive Blue Tit population will be tested for avian malaria and *Eimeria*, and also evaluated for body condition index. The detection test will be done by using the PCR method, nested-polymerase chain reaction (nested-PCR) for the avian malaria and a standard endpoint polymerase chain reaction (PCR) for the *Eimeria*, which currently is the predominant method for parasite detection and has been previously used in different studies, using both blood and fecal DNA (Xiao *et al.*, 1999; Bench *et al.*, 2000; Hellgren *et al.*, 2004; Waldenström *et al.*, 2004; Duval *et al.*, 2007; Sehgal, 2015). This method allows us to detect parasitic infections using very small DNA and is also sensitive and specific (Wedrychowicz, 2000). Through this, I will test our hypothesis, which is that avian malaria infection lowers body condition, and will see if the results obtained support any of the models I presented. In addition, the birds will be tested for intestinal parasites of the genus *Eimeria*, supporting the occurrence that the Blue Tit can be co-infected.

2. MATERIAL AND METHODS

2.1 Sampling

The birds used in this study are from a captive population in Montpellier, southern France. Each outdoor aviary includes 1 Blue Tit pair, one or two oak trees, water and food ad libitum and soil and natural substrates-covered ground. For the birds to fill up their nest chambers, moss and lining material are provided (Lambrechts & Caro, 2018). It is important to state that these birds do not breed on the aviaries. For this study, forty birds were captured using mist nests and then placed in paper bags. This was done between 9:30 AM and 16:00 PM of May 25th of 2020, during the breeding season. Some of those birds were born in 2015, while the others were born in 2019, meaning there is a four year difference in age between some of them. Sixteen birds were from 2015 while the other 24 were from 2019. Later, their tarsus length, wing and body mass was measured, and the bag was also checked to see if they defecated. If so, those feces were collected in Eppendorf tubes and preserved with 95% ethanol. These tubes were later held at 4ºC. After the measurement, blood samples were drawn from the birds. Researchers drew approximately 85µl of blood with a syringe from the jugular vein and separated it in aliquots, from which 5µl were put in a 1.5ml Eppendorf tube with 0.5ml Queen's lysis buffer as a perservative. This was also held at 2ºC (David Lopez-Idiáquez, unpublished data).

2.2 DNA extraction

Both blood and fecal DNA were extracted using DNeasy Blood and Tissue Kit (QUIAGEN, Hilden, Germany) but for each DNA source the first few steps differed. For blood DNA parasite extraction, I took 100 µl of preserved blood, and placed it a 1.5ml Eppendorf, where then I added 100µl PBS and 20µl proteinase K. For the next step, I added 200µl of kit buffer AL, vortexed and incubated the samples at 56ºC for 10 minutes. Once incubated, 200µl ethanol (96-100%) was added to the tube and vortexed again. After that, the mixture was pipetted into a spin column placed in a 2ml collection tube and centrifuged at 8,000 rpm for 1 minute, and the flow through was discarded. The spin column was placed into another collection tube, then 500µl kit buffer AW1 was added, and the column centrifuged again at 8,000 rpm for 1 minute. This step was repeated but the buffer added was AW2 and centrifuged at 13,300 rpm for 4 minutes. In the final step, the DNA in the membrane of the spin column was eluted with 200µl buffer AE, incubated at room temperature (15-25ºC) for 1 minute and then centrifuged at 8,000 rpm for 1 minute. The spin column was then discarded. For the fecal DNA, the samples were centrifugated at 13,300 rpm for 5 min to pellet the sample and the supernatant ethanol was removed with a pipette without touching the pellet. Later, the samples were dried at 37ºC for 10 minutes, resuspended with 200µl of H_2O and centrifugated at 13,300 rpm for 10 minutes. Without disturbing the pellet, all the H_2O was extracted and 180µl of buffer ATL was added to the tube in order to resuspend the pellet. Then 20µl of proteinase K was added, vortexed to mix. The tubes were placed in an Eppendorf thermomixer set to 56ºC and 500 rpm to incubate overnight until the tissue was completely lysed. After incubation, the samples were softly vortexed and 200µl of kit buffer AL was added. From there, the same procedure for column loading and elution as in blood DNA extraction was followed. At the final step, only 50µl of buffer AE was added to ensure maximal DNA concentration.

2.3 Avian malaria detection

The DNA obtained from the blood extraction was tested for *Plasmodium* and *Haemoproteus* presence using the nested-PCR. The primers I used were those of Waldenström *et al.* (2004). The first pair, used in the first PCR, HAEMNF1/HAEMNR2, were developed by Waldenström *et al.* (2004) while the second pair, HAEMF/HAEMR, were developed by Bensch *et al*. (2000). These primers amplify a ~580 bp long fragment of the mitochondrial cytochrome *b* gene, commonly used to study the order Haemosporida in avian blood (Palinauskas *et al.*, 2007). All the information about the primers is given in table 1.

Table 1: Primer ID with its direction, the target gene, primer sequences and the expected amplicon size.

Target	Primer ID	Direction	Primer sequence (5'-3')	Size (bp)
Cyt b	HAEMNF1	Forward	CATATATTAAGAGAATTATGGAG	580
Cyt b	HAEMNR2	Reverse	AGAGGTGTAGCATATCTATCTAC	
Cyt b	HAEMF	Forward	ATGGTGCTTTCGATATATGCATG	524
Cyt b	HAEMR2	Reverse	GCATTATCTGGATGTGATAATGGT	

Both PCR tests were performed using Multiplex Qiagen PCR Kit with a final volume of 10µl. A single positive control (*Plasmodium relictum* DNA from canary samples) and a negative control (ddH_2O) were included per PCR run to detect potential contamination and confirm amplification. The reaction content and PCR conditions were provided by colleagues in France (David Lopez-Idiáquez, unpublished data). Each reaction contained 1µl of DNA, equivalent to 25ng, 2µM each primer (0.2µM final), 5µl Multiplex PCR Master mix® 2x (QUIAGEN, Hilden, Germany) and 2µl RNAse-free H_2O. The procedure consisted of 10 min at 94ºC for denaturalization, followed by 20 cycles of 30s at 94ºC, 40s at 51ºC (T_{ann}: ºC), 50s at 72ºC and ending with 5 min of elongation at 72ºC. For the second PCR, the composition was identical, except the 1µl DNA used was the amplicon obtained in the first PCR. The PCR conditions in this case were 10min at 94ºC for denaturalization, 35 cycles of 30s at 94ºC, 40s at 52ºC (T_{ann}: ºC), 50s at 72ºC and ending with 10 min of elongation at 72ºC. The results were visualized in a 2% agarose gel with Green Safe stain (NZYTech, Lisboa, Portugal) and the ladder used to determine amplicon size was NZYDNA ladder VI (NZYTech, Lisboa, Portugal).

Body condition

Body condition was calculated separately for the 2015 and 2019 birds. For the calculation, each individual's body mass and tarsus length were measured at capture, and values were then used to calculate a body condition index. In both cases, the calculation was done the same way; total mass of the bird divided by its tarsus length. The index was analyzed taking into consideration the infections detected by the nested-PCR to evaluate the hypotheses, and detect any visible trend (Figure 1). In addition, two statistical analyses were performed. The Mann-Whitney-U test statistical analysis was done to determine whether there are significant differences in condition between birds of different infection status, while the z-test analysis was done with the aim of testing the difference in the proportion of infected birds between Blue Tits born in 2015 and 2019.

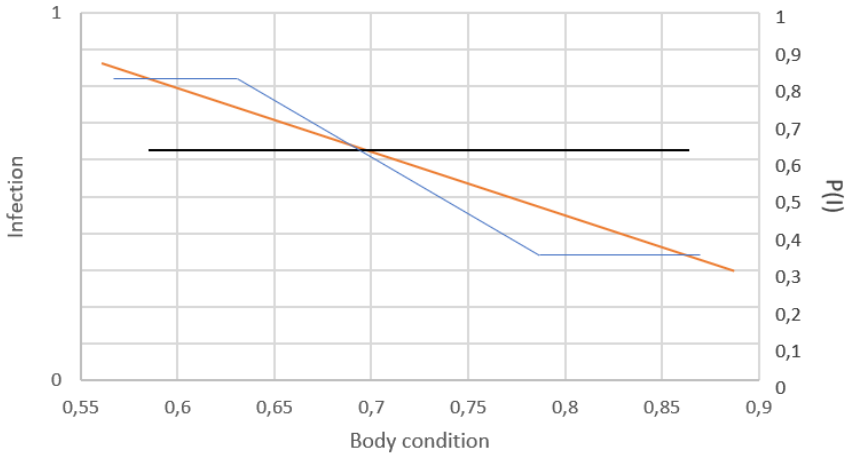

Figure 1: Three possible (theoretical) correlative relationships between body condition and probability of infection, P(I). Orange represents a linear negative correlation between body condition and infection status, while black represents no relationship at all. The blue line represents a limited correlation within a range of condition values. Within the Y axis, 1 represents infection, while 0 represents no sign of infection

Eimeria detection

A standard endpoint PCR was performed with 16 samples and a negative control (ddH_2O). Those samples belong to three different groups: Positive control samples, blood samples and fecal samples. Five positive *Eimeria spp*. samples were obtained from chickens and provided by colleagues from the Animal Health and Diagnostic Center, Cornell University (New York, USA). Another four samples were DNA extracted from Blue Tit blood and controlled for bird DNA. The last 7 samples were DNA extracted from Blue Tit feces. The primer pair used in the PCR was ERIB1/ERIB10, designed by Schwarz *et al.* (2009). These primers amplify a fragment of the 18S rRNA gene, a conserved gene previously used for identification and phylogenetic analysis of *Eimeria* by Stenzel *et al.* (2019). The expected amplicon size was approximately 1790bp long (Table 2).

Table 2: Primer ID with its direction, the target gene, specificity, primer sequences and the expected amplicon size.

Target	Specifity	Primer ID	Direction	Primer sequence (5'-3')	Size (bp)
18S rRNA	Genus	ERIB1	Forward	ACCTGGTTGATCCTGCCAG	~1,790
18S rRNA	Genus	ERIB10	Reverse	CTTCCGCAGGTTCACCTACGG	

The reaction content and PCR conditions I chose were based on the DNA polymerase used in the PCR and also the annealing temperature calculated based on the melting temperature of the primer sequences. The PCR tests had a final volume of 50µl. Each reaction contained 1µl of genomic DNA, equivalent to

9,33ng, 0,25 µM each primer, 25 µl 5U of NZYTaqII® Colourless Master mix 2x (NZYTech, Lisboa, Portugal) and 21,5µl of H_2O. The procedure consisted of 3 min at 95ºC for denaturalization, 35 cycles of 30s at 94ºC, 30s at 57ºC (T_{ann}: ºC), 1 min at 72ºC and ending with 5 min of elongation at 72ºC. The results were visualized in a 1% agarose gel using Green Safe (NZYTech, Lisboa, Portugal). The ladder used was GeneRuler 1Kb Plus DNA ladder (Thermo Scientific, Waltham, United States).

3. RESULTS

In the first nested-PCRs that I performed, out of 40 individuals, 24 birds tested positive, while the other 16, tested negative, as seen in figure 2, meaning malaria prevalence was 60% (Figure 2). The presence of a band indicates the sample was positive for avian malaria, while no band indicates absence of *Plasmodium* or/and *Haemoproteus*. In the samples, single bands were obtained, which were 520bp long. However, in the positive control extra bands showed up, especially an amplicon around 1200bp long. To make an approximation of the size of the bands, we looked at the ladder we used.

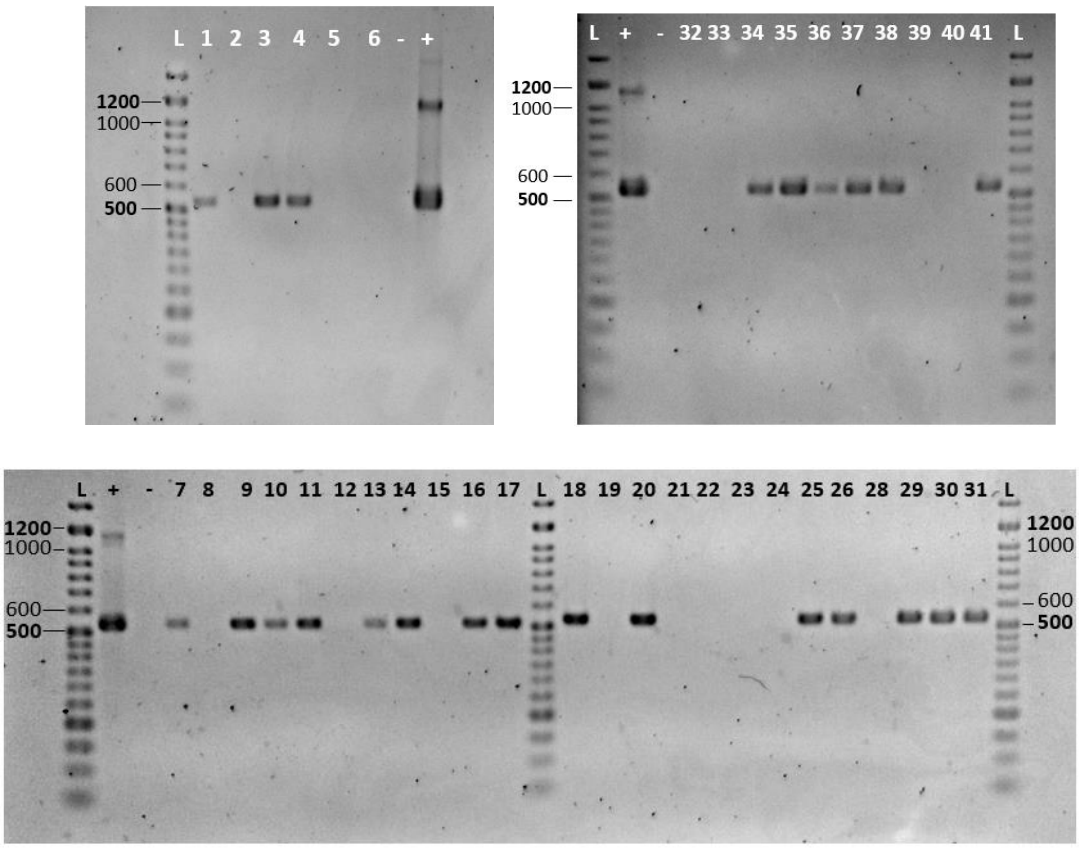

Figure 2: 2% agarose gel showing the results of the nested-PCRs for 40 samples and controls using HAEMNF1/HAEMNR2 and HAEMF/HAEMR primer pairs on the blood DNA extracted from the Blue Tits. Components of the figure: NZYDNA VI ladder (L), positive control (+), negative control (-), and enumerated samples from the capture.

When comparing these results with the nested-PCRs performed by our colleagues in France (David Lopez-Idiáquez, unpublished data), no false positives were found but I detected eleven false negatives. To determine whether the samples were actually positive or negative, a new nested-PCR was performed with the putatively false negative samples. In this second nested-PCR, the positive control was the same one we used for previous nested-PCRs. Two additional positives were found out of eleven samples that I tested (Figure 3). This means that taking into account the new positives, 26 individuals tested positive for avian-malaria, while 14 samples tested negative, increasing malaria prevalence to 65%.

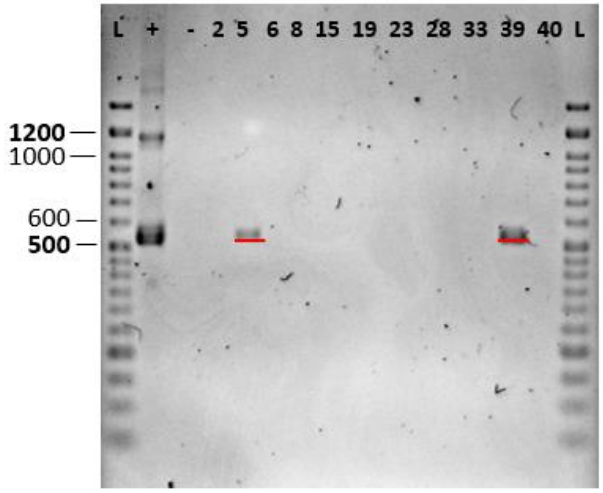

Figure 3: Nested-PCR performed with the false negatives. The primer pairs used were also HAEMNF/HAEMNR2 and HAEMF/HAEMR. Components of the figure: NZYDNA VI ladder (L), positive control (+), negative control (-) and enumerated samples.

At the beginning of the study three different hypothesis were presented for the possible correlation between the infection of avian-malaria and the body condition of individuals (Figure 1). In the analysis done for this potential correlation, both 2015 (5-year old) and 2019 (1-year old) bird body condition values were similar in infected and uninfected birds, even if two of the infected birds of 2015 showed slightly higher body condition than uninfected birds (Figure 4). The body condition index values of the infected Blue Tits born in 2015 (5-year old) went from 0,617 (minimum) to 0,883 (maximum), with an average value of 0,709 and standard deviation of 0,021, while the values of the uninfected Blue Tits went from 0,621 to 0,713, with an average value of 0,657 and standard deviation of 0,016. The body condition index values obtained from the infected Blue Tits born in 2019 (1-year old) went from 0,580 to 0,729, with an average value of 0,643 and standard deviation of 0,021, while the values obtained from uninfected Blue Tits went from 0,602 to 0,748, with an average value of 0,647 and standard deviation of 0,016. The body condition value of 0,883 in one infected individual from the 2015 birds (5-year old) was considered a notable outlier. The condition values of the uninfected birds fall within the range of

values of the infected birds and no trend in values is observed. The Mann-Whitney-U test (Online calculator: https://www.socscistatistics.com/tests/mannwhitney/) revealed non-significant differences in the body condition index of infected (Median=0,699, n=11) and uninfected (Median=0,653, n=5) birds born in 2015, U=97, z=0,5082, p=0,610, r=0,127. In the birds that were born in 2019, the Mann-Whitney-U test also revealed non-significant differences in the body condition index of infected (Median=0,636, n=15) and uninfected (Median=0,634, n=9) birds, U=268, z=0,032, p=0,976 r=0,0065. Furthermore, the z-test (Online calculator: https://www.socscistatistics.com/tests/ztest/) revealed a non-significant difference in the proportion of infected 1-year old (2019) (M=0,709, SD=0,021) and 5-year old (2015) (M=0,643, SD=0,009) birds, with a 95% level of confidence, z=0,406, p=0,6818.

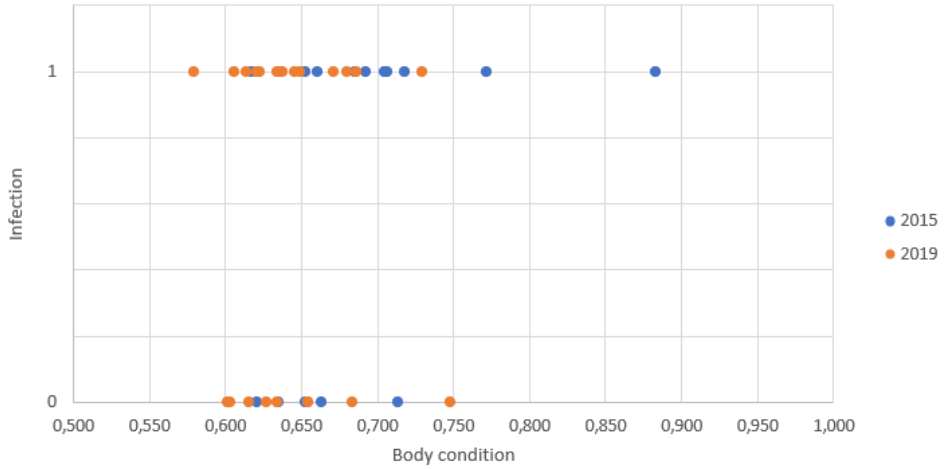

Figure 4: A Graph showing the analysis done between infection and body condition obtained in the nested-PCRs performed. In the X axis body condition values are shown for birds born in 2015 (blue) and 2019 (orange). In the Y axis birds were separated in two groups: Infected (1) and uninfected (0).

In the PCR performed for the *Eimeria* detection at genus level, no band was obtained in the negative control but variability in amplicon size was found in the rest of the samples (Figure 5). The tested samples belong to three different groups. First, in the known-positive controls (chicken), three different bands showed up. The biggest one is around 2000bp long and only appeared in number 5. The smallest one, around 1500bp long, appeared in sample number 2, 3 and 4. The intermediate band, which is closer to 2000bp than it is to 1500bp, was obtained in samples 1, 3 and 4. Second, two bands appeared in all the blood control samples. The size of one band was the similar than the intermediate band from the positive samples and the other band was even smaller, slightly less than 1000bp. Last, in the fecal samples, all samples produced a single band except sample number 7, which produced two. The band obtained in samples 9, 26, 34 and one of the bands in sample 7 (all red in Figure 5) have the same size, a little over 1500bp, but do not match with any of the bands obtained in the known-positive or blood

control groups. The second band obtained in sample 7 matches with the larger amplicons obtained in the blood samples and in the positive samples numbered 1, 3 and 4. The band obtained in sample 35 matches with the band that *Eimeria spp.* positive sample number 5 obtained, which is around 2000bp long. Sample 28 also obtained a band, but this one was much larger, close to 4000bp. Sample 41 showed no band at all.

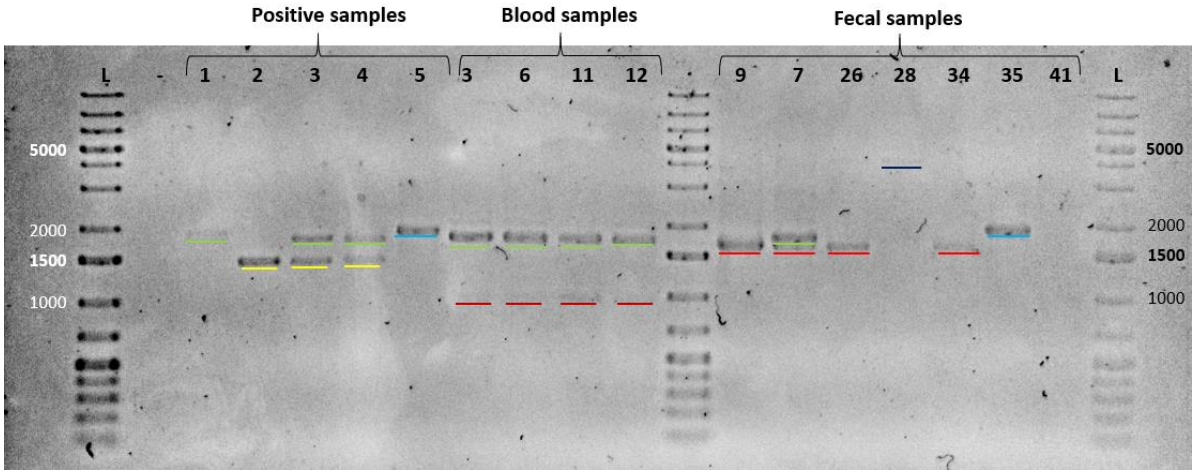

Figure 5: 1% agarose gel showing the results of the PCR using ERIB1/ERIB10 primer pair on the positive *Eimeria spp*. samples, blood samples and the fecal samples from the Blue Tits. Components of the figure: GeneRuler 1Kb ladder (L), negative control with ddH_2O (-), 5 positive samples, 4 blood samples and 7 fecal samples. Amplicon size approximations are indicated with the ladder fragment enumeration on the right and the colors refer to different amplicon sizes obtained in the PCR. Same color means same band size.

4. DISCUSSION

Avian malaria is a common Blue Tit parasite known to affect condition-dependent traits such as carotenoid-based pigmentation (Brown, 1998; Merino *et al.*, 2000; del Cerro *at al.*, 2010), but the effect on other morphological indicators of body condition such as body mass and tarsus length has not been well studied. Since the condition-infection relationship could have consequences for the host, it is important to understand this relationship. The results obtained in this study showed that even if 65% of the birds were infected (Figure 2), there is no statistical evidence for difference in condition between infected and uninfected birds in neither 5-year old (2015) nor 1-year old (2019) Blue Tits, since both the figure 4 and the performed statistical analysis indicated the differences were insignificant. However, the number of infected birds was different between the two groups, with 4 more infected individuals in the group of birds born in 2019 (1-year old). Also, comparing with the results obtained by our colleagues

in France (David Lopez-Idiáquez, unpublished data) no false positives were found, meaning likely that I had no contamination in the nested-PCR tests, but false negatives did show up (Figure 3).

When comparing previous results (Wood *et al.*, 2007; Cosgrove *et al.*, 2008; Szöllősi *et al.*, 2011; Dubiec *et al.*, 2015) with the ones obtained in this study, both similarities and differences were found. In this study, malaria prevalence was 65%, very close to the 65,2% prevalence obtained by Podmokła *et al.* (2014) in their study. However, in the studies done by Cosgrove *et al.* (2008) and Szöllősi *et al.* (2011) completely different results were obtained; one got 25,6% of overall prevalence while the other one got 87,9%. In addition, it is important to emphasize that the false negatives obtained in the nested-PCRs performed in this experiment, had impact on the general prevalence. This variability could be due to different factors such as sample size and DNA extraction. Sample size is a very important factor since the bigger the study population, the smaller the error will be. In this project, the number of individuals tested for malaria were 40 (n=40) while Cosgrove *et al.* (2008) had 816 individuals tested. Such a big difference of sample size could be the reason for the variability in prevalence. Another important procedure affecting prevalence could be the DNA extraction. If just part of the sampled blood is extracted without mixing it first, the infected cells would be left at the bottom. Thus, the quantity of infected cells in the extracted DNA would be too low to detect the infection, not reflecting the real infection of the bird, and therefore, getting false negatives. This could explain my false negatives, since I extracted the DNA just once. A good way to avoid this would be to make sure the blood sample is mixed before extracting anything or/and to extract blood DNA every time a nested-PCR is performed. Additionally, the population status could have affected the malaria prevalence. Cosgrove *et al.* (2008), Szöllősi *et al.* (2011) and Dubiec *et al.* (2015) studied wild Blue Tit populations, while in the present study a captive Blue Tit population was studied. It is important to state that these captive Blue Tits do not breed in the aviaries (David Lopez-Idiáquez, unpublished data), still being exposed to mosquito vectors.

Moreover, birds have some vulnerable times such as breeding season where stress levels are high and new infection and relapses occur. Valkiūnas *et al.* (2004) found higher parasite prevalence on spring, prior to breeding season, which coincides with the date these birds were sampled. This could also be a reason for the high prevalence found in this experiment. Furthermore, studies done by Wood *et al.* (2007), Dubiec *et al.* (2016) and Cosgrove *et al.* (2008) found prevalence variation with age. As explained in our results, more infected birds were found in the 2019 group, the young birds, although this was not significantly different from older birds. This is the opposite of what it was found in the studies just mentioned, since they found a higher probability of infection and also prevalence in older birds. These might have higher probability of infection since they have been exposed longer and also had more opportunity to have the parasites enter the dormant form and relapse upon stress conditions. Even when I took the false negatives into account (Figure 3), my results would not support the ones obtained in the previous studies. However, the z-test analysis I performed revealed a non-significant

difference in the proportion of infected birds born both in 2015 and 2019. In order to know if our results do not support the prevalence variation with age observed in other studies or the lack of significance was just a coincidence, the sample size tested for avian malaria should be larger, since the capture I tested had only 40 individuals. The infected 2019 Blue Tits from our capture would be expected to be newly infected, while infection in the 2015 cohort would mostly be due to the relapses or persistent infection. Perhaps, the old birds developed parasite resistance that the young ones had not yet, removing the infection from the blood and therefore, testing negative in the nested-PCR.

Body condition index was also evaluated in this experiment, providing interesting results. No difference on body condition values was found between infected and uninfected birds (Figure 4) and this is in accordance with one of the three possible correlative models I provided at the beginning for the hypothesis in figure 1, which is presented by the horizontal line in black. This line, which is located at P(I)=0,6 is the most appropriate model for my results since it indicates that infection does not have any impact body condition, getting same results on the body condition values of infected and uninfected birds. Bennett *et al.* (1998) obtained the same results when they compared body mass of infected and uninfected different passerines. They found some interaction among the year and also the area of capture but did not find any effect of parasitemia in body mass. One of the conclusions they got was that parasitism does not affect body condition. The results Granthon & Williams (2017) obtained in their study of the effect of avian malaria on the body condition of songbirds, type of passerines, are consistent with the lack of correlation I found here. Since parasitism can impact bird fitness negatively (Powell & Prior, 2016), the lack of correlation could be due to the parasite resistance developed by the birds, which would potentially have ameliorated the effect on body condition, or also due to the removal of less resistant birds from the population by mortality. It has been observed that Heat Shock Protein 60 (HSP60) expression is induced with the presence of parasites (Merino *et al.*, 1998), giving the host an effective immune and stress response system against avian malaria (Martinez *et al.*, 1999; Tomas *et al.*, 2005). Garcia-Longoria *et al.* (2019) studied the host-parasite interaction to explain prevalence and parasitemia effect on passerines birds, suggesting host immune defenses have an important role on the relationship. However, Milenkaya *et al.* (2015) proposed that body condition indexes are not always meaningful indicators of fitness and Lailvaux & Kasumovic (2011) suggested that the body condition indicators are context-dependent, since some strategies may be adaptive in some situations and non-adaptive in others. Furthermore, Harrison *et al.* (2013) and Cotton *et al.* (2004) in their studies, concluded that not all indexes are relevant to all species. Therefore, another potential explanation for the lack of relationship obtained in our results is that the body condition index studied in this experiment was not either relevant for the Blue Tit or context-dependent.

In the detection of *Eimeria* parasites, the results obtained varied between samples. Even if this parasite has mostly been studied in poultry and farm animals, Dolnik *et al.* (2010) in their study demonstrated that these parasites are widely distributed in wild passerines, including the Blue Tit. In addition to the

negative control with H_2O, I also tested 4 blood samples to make sure the bands I obtained meant parasite presence and not unwanted amplification of bird DNA. Since *Eimeria* is a coccidian found in the gastrointestinal system (Badparva *et al.*, 2015), no amplification was expected in the blood samples but as can be seen in figure 5, two bands showed up in all the blood samples; 3,6,11 and 12. This could mean blood DNA amplification, likely because the primers I used were complementary to some sequences of the extracted blood DNA, resulting in off-target amplification. The positive samples, which were obtained from dead infected chickens, (Cornell University, New York, USA) were supposed to be positive for *Eimeria spp*. In the PCR I performed (Figure 5), a 1500bp band was obtained in sample 2,3 and 4, which could indicate the presence of an *Eimeria* specie, as well as the 2000bp band obtained in number 5. It is important to remember the primers I used amplify at genus level (Schwarz *et al.*, 2009), being able to detect different species and, thus, potentially producing different sized amplicons. The intermediate band obtained in samples 1,3 and 4 match the ones obtained in the blood samples. There is a chance that the feces that were collected from those chickens had blood in them, which is an *Eimeria* infection symptom (Li & Ooi, 2009), so when extracting the DNA, both fecal and blood DNA got extracted, and since these primers seem to be able to attach and hybridize certain blood sequences, amplification occurred. This could also be the case of fecal sample number 7 since one of the two bands obtained matches the blood sample amplicon size. Furthermore, the amplicon of the fecal sample number 35 matches the one obtained in the positive sample number 5, meaning this too could had been infected by the same *Eimeria* specie. However, the amplicons obtained in fecal samples 9, 7, 26 and 34, do not match any of the other amplicons but were of a consistent size. This could belong to a third *Eimeria* specie. In sample 41, an amplicon of about 1500bp was expected, since in all the other PCRs performed in our lab, a band showed up, likely meaning PCR failure. Last, in sample number 28 a 5000bp band was obtained, doubling the size of the other ones. This could be the result of other non-target amplification. Taking all this into consideration, and in order to make sure what each band belongs to, the bands should be extracted and sent for sequencing. If what I suggest to be blood DNA amplification is correct, the primer specificity should be checked and if needed, its PCR conditions optimized.

Finally, all the fecal samples that showed evidence of possible infection by an *Eimeria* species, tested positive for avian malaria, which means these Blue Tits are likely co-infected. These results support the ones obtained by Clark *et al.* (2016), in which they demonstrated that wild birds could be simultaneously infected by other parasites besides *Plasmodium* or/and *Haemoproteus*. Out of all the co-infected Blue Tits, only sample number 9 was from 2019, which could be expected, since older birds have been exposed to different parasites for longer time. This sample also had the lowest body condition value. The body condition index of sample 9, born in 2019 (1-year old) was 0,671, while the average body condition index of the rest of the birds, who were born in 2015 (5-year old), was of 0,746 with a standard deviation of 0,030. Even if the z-test revealed the difference in proportion of infected birds of both ages

was non-significant, by analyzing a larger sample size, the potential compensation of the effect of parasitemia in body condition by older birds could be studied more precisely, obtaining more accurate results.

Conclusions

Body condition is considered a fitness indicator and also demonstrated to have effect on reproductive success and output (Powell & Prior, 2016). However, no correlation between avian malaria and body condition index was found in the present study. This could be due to a resistance developed by the host, the lack of meaningfulness of these indicators in the Blue Tit or the small sample size. It has been observed that HSP60 expression increase is related with parasite resistance (Merino et al., 1998). Thus, measuring its expression on this captive population would supplement information from these results. Also, a potential co-infection in the Blue Tits was observed. Sequencing could determine if the amplicons I obtained belong to *Eimeria* species. In addition, a possible variation in age was also observed between body condition and co-infection. To investigate this, a larger study size should be implemented, more PCR, with more samples and more body condition evaluations should be done. In summary, no significant relationship was found between body condition and avian malaria infection, and age of the bird showed not to have any effect on this correlation, suggesting further studies should be done to explain this. Also, these Blue Tits are probably infected by different parasites simultaneously, suggesting that co-infections should be taken into consideration when studying parasite-bird interactions and dynamics.

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