Mixed haemosporidian infections are associated with higher fearfulness in Yellow Wagtail, *Motacilla flava*

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Mixed haemosporidian infections are associated with higher fearfulness in Yellow Wagtail, *Motacilla flava***M. P. Marinov**

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Mixed haemosporidian infections are associated with higher fearfulness in Yellow Wagtail, *Motacilla flava***Martin P. Marinov, Chiara Marchetti, Dimitar Dimitrov, Mihaela Ilieva and Pavel Zehindjiev****Abstract**

Blood parasite infections have been shown to influence behavioural traits of their avian hosts, in particular activity level and boldness. Following the hypothesis that a mixed infection by different parasite species should have higher effects than single-species infections, we analysed activity and boldness in wild-caught Yellow Wagtails (*Motacilla flava* L., 1758), a trans-Saharan migrant, during the energetically demanding spring migration. Eighty-five percent of the birds were naturally infected with *Haemoproteus* or *Plasmodium* (Sporozoa, Haemosporida) and 27% of individuals had parasites from both genera. No differences in activity were found among uninfected, single and mixed infection groups. Birds with infections from both genera appeared to be more fearful when first introduced to a cage. These birds also tended to be less likely to approach a novel object compared to uninfected birds and birds infected by a single genus only.

Key-words: avian haemosporidians, co-infections, fearfulness, host-parasite interactions, migration, *Motacilla flava*, Yellow Wagtail

Introduction

Parasites can alter their host's behaviour (Moore 2002) and the changes can result from a pathological side-effect, an adaptive response of the host to reduce parasite virulence, or an adaptive manipulation by the parasite beneficial for its own transmission (Poulin 2010). Behavioural manipulation occurs mostly in parasites whose infective stages are habitat-specific, in parasitoid insects, in trophically-transmitted parasites, and in vector-borne parasites (Thomas et al. 2005; Poulin 2010). Vector-borne blood parasites, such as the group related to bird malaria, manipulate their invertebrate vectors' behaviour into repeated probing and shorter duration of individual blood meals, thus causing them to feed on multiple hosts (Hamilton and Hurd 2002). In contrast, their effect on vertebrate hosts' behaviour is still poorly explored.

Avian haemosporidians of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* (Sporozoa, Haemosporida) are parasites with a broad geographical distribution and are known to affect mating behaviour, exploration, boldness, problem solving and startle response of their vertebrate hosts (see Marinov et al. 2017, and references therein). While haemosporidian infections are often lethal to naïve host populations in the first, acute phase of infection (Warner 1968; Atkinson and Van Riper III 1991; Valkiūnas 2005), long coexistence ensures co-adaptation and the ability of hosts to survive and reproduce while being chronically infected (Davidar and Morton 1993; Woodworth et al. 2005). However, subtle parasite effects during the chronic phase, while theoretically plausible (Asghar et al. 2011), are difficult to detect. Some studies suggested that avian haemosporidians reduce host survival (Martínez-de la Puente et al. 2010; Krams et al. 2013; Asghar et al. 2015; Krama et al. 2015) and affect host life-history traits such as spring arrival (DeGroot and Rodewald 2010; Santiago-Alarcon et al. 2013), reproductive success (Merino et al. 2000; Marzal et al. 2005; Knowles et al. 2010; Asghar et al. 2011; Marzal et al. 2013b), and offspring quality (Asghar et al. 2015). However, no such effects were detected in other studies (Weatherhead and Bennett 1992; Bensch et al. 2007; Grillo et al. 2012; Knutie et al. 2012; Dias et al. 2016). Several studies found a connection between blood parasites and susceptibility to stress during the reproductive season (Norris et al. 1994; Richner et al. 1995; Allander 1997; van de Crommenacker et al. 2012). Chronically infected Great Reed Warblers (*Acrocephalus arundinaceus* L., 1758) showed a higher rate of telomere shortening than uninfected individuals, and infected females produced offspring with shorter telomere length (Asghar et al. 2015).

Although mixed haemosporidian infections are frequent among bird populations (Valkiūnas et al. 2003; Grillo et al. 2012; van Rooyen et al. 2013) only few studies on their possible impact on avian behaviour have

been made. An enhanced negative effect of mixed infections with more than one parasite genus in a single host is equally difficult to prove. Some studies showed negative effects of mixed infections on body condition and positive on reproductive output (Marzal et al. 2008; Marzal et al. 2013a) while others did not find any relationship (Sanz et al. 2001; van Rooyen et al. 2013, 2014). Mixed *Leucocytozoon* and *Plasmodium* infections affected the ability of Mountain White-crowned Sparrows (*Zonotrichia leucophrys oriantha* Oberholser, 1932) to produce consistent songs (Gilman et al. 2007). Also, sex-related differences between uninfected and infected Great Tits (*Parus major* L., 1758) in exploration, problem solving and startle response were most obvious in birds co-infected with *Leucocytozoon* and *Plasmodium* (Dunn et al. 2011). Further studies are needed about parasite-avian host relationship.

The aim of this study was to assess if mixed chronic infections have a higher effect on behaviour than single or no infections. We caught Yellow Wagtails (*Motacilla flava* L., 1758) during spring migration, a delicate phase where birds are subjected to a high energetic demand, faced with the challenge of replenishing their fat reserves at stopover sites (Klaassen 1996) while minimizing the cost of exposure to predators (Lima and Dill 1990). Behavioural traits such as activity, fear and boldness play a key role in these challenging circumstances (Groothuis and Carere 2005; Blumstein 2006). We might expect clear-cut behavioural differences in these traits between infected and non-infected birds, under the assumption that chronically infected individuals might be more sensitive to stressful conditions. The Yellow Wagtail is a long-distance migrant wintering in sub-Saharan Africa (Curry-Lindahl 1965), therefore potentially exposed to a range of parasites due to its different wintering and breeding habitats (Figuerola and Green 2000; Jenkins et al. 2012), and indeed it is host to a large number of blood parasites (Valkiūnas and Iezhova 2001). A study in Bulgaria showed high prevalence of the genus *Haemoproteus*, followed by the genus *Plasmodium*, with frequent mixed infections (Shurulinkov et al. 2012). The prevalence and number of mixed infections in spring were much higher than in autumn, probably because seasonal haemosporidian relapses (increase of parasitaemia in previously infected hosts) occur usually during spring (Valkiūnas 2005). This allows more precise parasite identification and more exact assessment of parasite prevalence (Norris et al. 1994; Valkiūnas et al. 2003).

In this study, we address the following questions: (i) is there an effect of single and mixed heterogeneric infections on behavioural traits such as activity and boldness of the Yellow Wagtail during spring migration and, assuming that such effect is present, (ii) is this effect sex-dependent. We also controlled for differences in body condition and checked for the relative distribution of the parasite genera in the study sample.

Materials and methods

Study site and data collection

The study was carried out at the Biological Experimental Station “Kalimok” (Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences) situated in a wetland area near the Danube riverbank, NE Bulgaria (44°00'N 26°26'E). Seventy-four Yellow Wagtails (*Motacilla flava*) were caught with mist nets during spring migration, on 9-16 April 2009. Bird species, age and sex were identified according to Svensson (1992). The birds were caught mostly in the evening hours (19:00-20:00 h), ringed and measured following Bairlein (1995), kept in cotton bags overnight and put in keeping cages as soon as it was light (about 7:00 h).

Keeping cages and control of onset of eating

Each bird was kept in an individual standard wire cage, 18cm × 9.5cm × 13.5cm. The birds could hear but not see each other. They had water *ad libitum* and at first each received 6 mealworms (*Tenebrio molitor* L., 1758) and 6 dipteran larvae (Calliphoridae). Every 2 hours we counted the number of larvae left by the bird and in case some had disappeared the original number was restored, until the birds ate all the food. This normally happened within a few hours, with some between-individual variation. At this point, the diet was enriched with egg food and both larvae *ad libitum*. Some individuals started eating by the next morning. Birds which did not eat were released within a few hours or on the next day if their condition declined.

Parasite diagnostics

A detailed description of all procedures involved in the haemosporidian sampling and identification was published elsewhere (Dimitrov et al. 2013). Briefly, blood-samples obtained by venipuncture of the brachial vein of each bird were scanned both by microscopic examination and PCR-based molecular diagnostic method for the presence and identification of haemosporidians. Blood smears were examined using a light microscope for 10–15 min at low magnification (400×) and then at least 100 fields were studied at high magnification (1000×). Birds identified as bearing haemosporidians by either microscope or molecular method were recognised as infected. Parasite identification was performed according to the morphological and morphometric characters described by Valkiūnas (2005). The intensity of infection was estimated as a percentage by counting the number of parasites per 1000 red blood cells or per 10,000 red blood cells if infections were light (i.e. < 0.1%). A standard ammonium acetate method was used for total DNA extraction (Richardson et al. 2001). DNA was used

as a template in PCR assays for detection of parasites. Primers and temperature profiles described in Hellgren et al. (2004) were used. The samples were not screened for *Leucocytozoon* spp. since parasites of this genus were not recorded in the blood films. We used mostly microscopic examination to count for mixed infections of the genera *Haemoproteus* and *Plasmodium*. Such infections were largely underestimated by the PCR-based method. We defined four groups of birds according to their infection status: uninfected birds, *Haemoproteus* infections, *Plasmodium* infections, and mixed infections.

Behavioural tests protocol

As soon as each bird was put in the keeping cage, it was filmed for 5 min to assess its reaction to the new situation (First Cage Test). It was kept in the cage until the next morning and then tested in the presence of a novel object ($n = 64$). At first it was filmed for 3 min (control), then a bright-coloured plastic ball, 4 cm in diameter, was hung on one side of the cage (Novel Object Test). The bird was then filmed for 15 min and its latency to approach the object was recorded. These tests took place between 7:00 and 12:00 h. After the last test a blood sample was taken to check for parasites, and the bird was released.

Behavioural assessment

The individual response to a new situation or object in laboratory conditions could be a relevant assessment of a bird's ability to adapt to novel environments during migration, e.g. stopovers and wintering sites (Nilsson et al. 2010). The first reaction to the keeping cage (First Cage Test) was used to assess the extent of individual variation in coping with an unfamiliar situation. In the First Cage Test, we measured the following variables: number of moves, percentage of hops and turns, frequency of flying, duration of looking around, duration of looking out of the cage, duration of staying still and frequency of escape attempts. We applied principal component analysis (PCA) to summarize these variables to identify behavioural dimensions. Two axes with eigenvalues >1 were extracted (the others were not significant). The first component (PC1) was positively correlated with activity and explained 48.65% of the total variance, while the second component (PC2) identified a curiosity/fearfulness axis (24.14% of the total variance explained), with higher values corresponding to more fearful behaviour (Table 1). Within non-active birds, which had lower scores on PC1, some were still and frozen, moving very little (higher scores on PC2), while others showed a considerable amount of head-turning and looking around (lower scores on PC2). Also, within the more active birds, scoring higher on PC1, a distinction could be made between individuals that frantically moved up and down and tried to escape from the cage (higher

scores on PC2), and others (with lower scores on PC2) which moved but performed head-turning and hops (movements without changing position, like turning around or little jumps) rather than moves (displacement from one position to the other). So both fearful and curious birds showed variation in activity level.

In the Novel Object Test we measured the latency to approach a new object in order to assess neophilia. According to Mettke-Hofmann (2012), in this setup the object has to be placed at a neutral location in the cage and the bird should be free to approach or stay away from the object. We coded the latency to approach the object as a binary variable since, out of 64 trials, approximately 69% of the focal individuals approached the object within 14 min (coded “yes”) while approximately 31% did not approach within 15 min at all (coded “no”).

Statistical analysis

We checked the correlation between the latency to approach a new object and each of the two PCs (activity and curiosity/fearfulness, respectively) using Spearman's rank test. A two-way analysis of variance (Factorial ANOVA) was performed on both principal components separately to investigate the association with infection status (uninfected, *Haemoproteus* infection, *Plasmodium* infection, and mixed infections) and sex as explanatory variables. Bonferroni test was used in post-hoc analysis when significant differences were detected. A logistic regression was performed to ascertain the effects of infection status on the likelihood of birds to approach a novel object. Additionally, we tested for correlations between infection status (no infection, single or mixed infection) and the intensity of each haemosporidian genus. We calculated body condition index (the relative size of energy reserves) as suggested by Jakob et al. (1996) using the standardised residuals from a linear regression of body mass against wing length (both variables were log-transformed prior to calculation) and compared for the effects of sex, infection status and sex $\times$ infection status using two-way ANOVA. McNemar's test for paired-sample nominal scale data (Zar 2010) was performed to determine whether there was a significant difference in the prevalence of the two haemosporidian genera in the studied population.

Statistical analyses were performed using STATISTICA, Version 8.0 (Statsoft Inc., Tulsa, OK, USA) and an evaluation version of MedCalc Statistical Software, Version 16.2.1 (MedCalc Software bvba, Ostend, Belgium).

Results

Prevalence and intensity of infection

In total, 85% of the birds were infected with blood parasites (Table 2) of two haemosporidian genera: 52 out of 74 individuals (70.3%) were infected with *Haemoproteus* spp. and 31 individuals (41.9%) with *Plasmodium* spp.; 20 individuals (27.0%) were infected with both genera. The frequency of *Haemoproteus* infections was significantly higher than *Plasmodium* (McNemar Test: $n = 74$, $\chi^2 = 9.3$, $P = 0.002$).

Infection status was significantly correlated with intensity of infection for each haemosporidian genus: for *Plasmodium* $r_s = 0.93$, $P < 0.01$, $n = 74$; for *Haemoproteus* $r_s = 0.7$, $P < 0.01$, $n = 74$.

The two sexes differed significantly in body condition; however the effect of infection status on body condition index was not significant, nor was it significant when interaction with sex was included (Table 3).

Behavioural tests

In the First Cage Test, there was no difference in activity (PC1) in relation to infection status ($F_{3,66} = 1.51$, $P = 0.22$, Fig. 1a, Table 3). However, individuals with mixed infections had significantly higher values of PC2 (i.e., were more fearful) than uninfected birds ($F_{3,66} = 3.06$, P (Bonferroni corrected) = 0.025, Fig. 1b). There was an interaction between sex and infection status in PC2 ($F_{3,66} = 3.25$, $P = 0.027$, Table 3). Females with mixed infections showed higher fearfulness than uninfected females (Bonferroni test, $P = 0.05$), while there were no differences in males (Table 4).

The logistic regression model for the Novel Object Test showed a tendency for the chance to approach a new object to be eight times larger in uninfected birds than in birds with mixed infections (Odds ratio = 8, $P = 0.07$, 95% CI = 0.84/76.37, Fig. 2).

Correlation between behavioural test measurements

The latency to approach a new object was positively correlated both to PC1 ($r_s = 0.3$, $P = 0.017$, $n = 64$) and PC2 ($r_s = 0.3$, $P = 0.016$, $n = 64$). Highly active and fearful birds took longer to approach a novel object in the cage.

Discussion

In this study, we found that migrating Yellow Wagtails with mixed infections were more fearful than uninfected individuals, with intermediate effects in single infections, and a stronger tendency of this in females. No parasite

effect was found on activity or on body condition. The frequency of *Haemoproteus* was significantly higher than *Plasmodium* in the study sample.

The principal components describing the birds' behaviour at their first experience in a new cage had a logical structure. PC1 was related to activity and PC2 to curiosity/fearfulness. The two variables are, by definition, not correlated, in fact fearful birds could be either active and trying to escape, or still, almost freezing. At the same time, exploring birds could either move around or sit still, but in both cases they would look around, appear relatively at ease and interested in their environment. Activity *per se* is thus not enough to describe a bird's motivational state. Further research should focus on the presence of stress hormones to connect internal status with behavioural patterns.

We found no effect of parasites on PC1, related to activity, and no evidence of an acute effect of parasites due to a relapse in the energetically demanding migrating season. Hawaiian Apapane (*Himatione sanguinea* Gmelin, 1788) and Eurasian Siskins (*Spinus spinus* L., 1758) experimentally infected with *Plasmodium relictum* Grassi and Feletti, 1891 showed an acute infection with a clear drop in activity (Yorinks and Atkinson 2000; Mukhin et al. 2016). Birds caught during active migration are obviously able to endure a chronic parasitic infection; those which were unable to cope would have not made it to our study site. Other variables than activity levels are more likely to be influenced in the long run, such as survival or reproductive output (Marzal et al. 2008).

In contrast, the second principal component of the First Cage Test, connected to fearfulness, showed an effect of parasites: birds with mixed infections were more fearful than uninfected. These results were similar in the Novel Object Test, and the two tests were correlated. This result was reinforced by the fact that, again in both tests, single infections showed intermediate values, and the intensities of the single infections were lower than in the mixed infections. Larger sample size might yield clearer differences among the groups.

The shift between curiosity and fearfulness, expressed as approach to, or avoidance of novelty has been explained as an outcome due to the discrepancy between perceived and expected events: a moderate discrepancy provokes curiosity, a large discrepancy - the tendency to flee (Van Kampen 2015). The shift in motivational state underlying the response to novelty implies between-individual variation (Berlyne 1950). Parasite infections may change the threshold to different motivational states, thus increasing fearfulness. Mixed infections with *Plasmodium* and *Leucocytozoon* in Great Tits were associated to higher probability of startle response (an alternative measure of fearfulness, Cockrem (2007)) in males and faster exploration in females, as sex-specific hormonal effects have been supposed (Dunn et al. 2011). Also, an interaction between *Leucocytozoon* and

Plasmodium infections on the ability of male Mountain White-crowned Sparrows to produce consistent songs was detected (Gilman et al. 2007). Our study found a tendency to be more fearful in females. While this could be a selective advantage in view of protecting the nest by more cautious behaviour, a question remains about the extent of the influence of high testosterone levels in males on their behaviour. Such study may require to be repeated in non-breeding conditions, to ascertain if sex related differences persist outside the pre- and breeding season.

In theory, a bird with a chronic infection could increase its own survival probability by being wary of its environment. Previous studies showed the opposite, a higher tendency of infected birds to take risks, where the mechanisms proposed involved a negative selection of sick birds to counteract parasite pressure (Marinov et al. 2017; see also Horváth et al. 2016). However, an experimental study in House Sparrows (*Passer domesticus* L., 1758) showed higher escape rates in infected birds, possibly increasing survival chances against predators and securing future parasite transmission (García-Longoria et al. 2015). More detailed studies in different experimental situations could answer this question.

Yellow Wagtails caught in Bulgaria during spring showed a high prevalence of single and mixed infections with *Haemoproteus* and *Plasmodium*, in accordance with the results reported by Shurulinkov et al. (2012). Earlier studies have suggested that haemosporidian prevalence depends on host susceptibility to infection and environmental conditions determining dipteran vectors abundance (Ricklefs and Sheldon 2007), and may vary spatially and temporally across populations of a single host (Merilä et al. 1995; Bensch and Åkesson 2003; Gibb et al. 2005; Shurulinkov and Ilieva 2009). The high occurrence of mixed infections in our study suggests that transmission of *Haemoproteus* and *Plasmodium* in the Yellow Wagtails is not spatially isolated and probably happens in breeding sites, because juvenile birds caught during autumn migration also showed single and mixed infections with the two genera (Shurulinkov and Chakarov, pers. comm.). The lack of correlation between the presence of *Haemoproteus* and *Plasmodium*, and the lack of negative effect on host's body condition in our results suggests the absence of strong competition (see Hatchwell et al. 2000) due to the long co-evolution between both parasites in the same host species (Atkinson and Van Riper 1991). More research is needed on the chance of being infected with a particular haemosporidian genus.

In conclusion, Yellow Wagtails with a mixed chronic infection by *Haemoproteus* and *Plasmodium* blood parasites show higher fearful reactions than non- and single-infected birds, a phenomenon that has been demonstrated by two tests. Further studies on various host species are needed to assess the importance of multiple parasite infections, which frequently occur in natural populations, on the host fitness and survival.

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Figure captions

Fig. 1. Comparisons of levels of activity (PC1, a) and curiosity/fearfulness (PC2, b) in four groups of Yellow Wagtails (*Motacilla flava*) with different parasite infection status (PC1: negative values – low activity; positive values – high activity; PC2: negative values – high curiosity; positive values – high fearfulness). Vertical bars denote 0.95 confidence intervals.

Fig. 2. Percentage of Yellow Wagtails (*Motacilla flava*) with higher neophilia (approaching a novel object), for each of four groups with different parasite infection status. Yes – approach a novel object; No - not approach.

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Fig.1

143x48mm (300 x 300 DPI)

Fig. 2. Percentage of Yellow Wagtails (*Motacilla flava*) with higher neophilia (approaching a novel object), for each of four groups with different parasite infection status. Yes – approach a novel object; No - not approach.

Fig. 2
63x40mm (300 x 300 DPI)

Table 1. Behavioural variables comprising the two principal components (PCs). The significant correlation coefficients between variables and PCs are marked in bold.

Principal components	PC1	PC2
Number of moves	0.8653	-0.2039
% hops and turns	-0.5938	-0.4513
Frequency of flying	0.8910	-0.1751
Duration of look around	-0.5853	-0.7255
Duration of look out of the cage	0.7912	-0.1428
Duration of staying still	-0.2137	0.9287
Frequency of escape attempts	0.7041	-0.0691
Expl.Var	3.4052	1.6900
Prp.Totl	0.4865	0.2414

Table 2. Summarized data of haemosporidian prevalence in the Yellow Wagtail (*Motacilla flava*). The percentage of individuals in each group is shown in brackets.

Sample size (<i>n</i>)		Uninfected (<i>n</i>)		Single infections (<i>Plasmodium</i>) (<i>n</i>)		Single infections (<i>Haemoproteus</i>) (<i>n</i>)		Mixed infections (<i>Plasmodium</i> and <i>Haemoproteus</i>) (<i>n</i>)		Total prevalence	
♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
47	27	5	6	8	3	19	13	15	5	42 (89.4)	21 (77.8)
Total:	74	11 (14.9)		11 (14.9)		32 (43.2)		20 (27.0)		63 (85.1)	

Table 3. Two-way (factorial) ANOVA for the effects of sex, infection status and sex $\times$ infection status on the Yellow Wagtails' (*Motacilla flava*) activity (PC1), curiosity/fearfulness (PC2) and body condition index (BCI). The significant *P*-values are marked in bold.

Effect	PC1					PC2					BCI				
	SS	DF	MS	F	P	SS	DF	MS	F	P	SS	DF	MS	F	P
Sex	0.02	1	0.02	0.02	0.89	1.72	1	1.72	1.96	0.17	0.006	1	0.006	13.00	0.001
Infection status	4.35	3	1.45	1.51	0.22	8.07	3	2.69	3.06	0.03	0.000	3	0.000	0.34	0.80
Sex*Infection status	3.06	3	1.02	1.06	0.37	8.59	3	2.86	3.25	0.03	0.003	3	0.001	2.15	0.10
Residual	63.39	66	0.96			58.11	66	0.88			0.031	66	0.000		

Table 4. Estimated marginal means of PC2 scores for the male and female Yellow Wagtails (*Motacilla flava*) in relation to their infection status.

Sex	Status	<i>n</i>	Mean	SE	95% CI
Males	Uninfected	5	-0.3	0.42	-1.14 to 0.53
	<i>Haemoproteus</i>	19	0.18	0.22	-0.25 to 0.61
	<i>Plasmodium</i>	8	-0.47	0.33	-1.13 to 0.19
	Mixed infections	15	0.07	0.24	-0.41 to 0.56
Females	Uninfected	6	-0.83	0.38	-1.6 to -0.07
	<i>Haemoproteus</i>	13	-0.15	0.26	-0.67 to 0.37
	<i>Plasmodium</i>	3	0.9	0.54	-0.18 to 1.98
	Mixed infections	5	1.01	0.42	0.17 to 1.85