Malaria parasites, immune challenge, MHC variability and predator avoidance in a passerine bird

László Zsolt Garamszegi[1,*], Magdalena Zagalska-Neubauer[2], David Canal[1], Gábor Markó[3,4,5], Eszter Szász[3], Sándor Zsebők[3,6], Eszter Szöllősi[3,5], Gábor Herczeg[3] and János Török[3]

[1]*Department of Evolutionary Ecology, Estación Biológica de Doñana-CSIC, Seville, Spain;*

[2]*Polish Academy of Sciences, Museum and Institute of Zoology, Ornithological Station, Gdansk, Poland;*

[3]*Behavioral Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Budapest, Hungary;*

[4]*Department of Plant Pathology, Corvinus University of Budapest, Budapest, Hungary;*

[5]*MTA-ELTE-MTM Ecology Research Group, Biological Institute, Eötvös Loránd University, Budapest, Hungary;*

[6]*Université Paris-Sud, Centre de Neurosciences Paris-Sud, UMR 8195, Orsay, France;*

[*] address for correspondence:

Department of Evolutionary Ecology
Estación Biológica de Doñana-CSIC
c/ Americo Vespucio, s/n, 41092, Seville, Spain;
Tel: (+34) 954 232 340 (ext. 1111)
Fax: (+34) 954 621 125
E-mail: laszlo.garamszegi@ebd.csic.es

Abstract

Several hypotheses predict a relationship between parasite burden and risk-taking behavior, but the underlying causal mechanisms are poorly understood due to the scarcity of experimental studies and the neglected focus on immune defense. Here, in three sets of field studies on the collared flycatcher, *Ficedula albicollis*, we investigated how among-male variation in flight initiation distance (FID, the distance at which an individual flee a potential predator) is linked to among-male variation in health status. First, we correlatively assessed the relationship between FID and the prevalence of haemosporidian blood parasites. We found no difference in risk-taking behavior between parasitized and non-parasitized individuals rejecting a hypothesis that predicts that malaria infection status affects the costs of predator avoidance. Second, we performed an immune challenge experiment, in which randomly chosen birds were injected with a novel antigen (sheep red blood cell) and their change in FID was compared with birds that received a placebo treatment. This experiment revealed no evidence for the immunological treatment affecting risk-taking behavior, thus we failed to obtain support for the hypothesis that posits that immediate health status mediates decisions about when to flee a predator. Finally, we detected a negative relationship between the number of alleles of the major histocompatibility complex (MHC) and FID. This result, in concordance with the above negative results, supports the "avoidance" hypothesis that states that only individuals with efficient immune defense machinery are able to bear the costs of risk-taking that can emerge through the increased infection rates of risk-taker individuals.

Keywords: boldness, flight initiation distance, immunogenetics, parasite-mediated selection, personality, phenotypic correlation, temperament

Introduction

Risk-taking decisions of animals (i.e. when to postpone current activity and flee from a predator) are determined by several extrinsic and intrinsic factors, all of which interact with the cost-benefit balance of different actions of the potential prey (Lima and Dill 1990; Cooper and Frederick 2007). Given that pathogens may profoundly influence the outcome of predator-prey encounters, infection status has been suggested as one of the key properties of individuals that can affect their behavioral strategies for predator avoidance and determine if they end up as a survivor or prey (Moore 2002; Barber and Dingemanse 2010; Kortet et al. 2010). The relationship between parasitism and host risk-taking behavior can arise through a variety of mechanisms that incorporate different causal links to connect host and parasite traits and that are briefly reviewed below and also summarized in Table 1.

From the parasite's perspective, the altered host behavior hypothesis emphasizes the parasite's ability to actively manipulate risk-taking in the host (Table 1). A list of trophically transmitted parasites can interact with the nervous system of their intermediate hosts in a way that leads to maladaptive, active and/or bold behaviors in infected individuals and causes higher predation rate in their population, thus enhancing the efficiency of transmission (Sanchez et al. 2008; Kaushik et al. 2012; Goblirsch et al. 2013; Poulin 2013; Jacquin et al. 2014; Kekäläinen et al. 2014). Malaria parasites can also be considered as manipulative parasites, as they are known for the cyclically occurring immobilization of their hosts by high fewer (Coatney et al. 1971; Valkiūnas 2005). Such an ability of parasites can enhance their transmission success, as immobilized hosts cannot freely exhibit behaviors that prevent or interrupt mosquito bites (Waage and Nondo 1982; Hodgson et al. 2001). Accordingly, host immobilization by malaria parasite can decrease general activity, which can lead to inefficient escape behaviors in the presence of predators (Møller and Nielsen 2007; Møller 2008).

From the host's perspective, the economic hypothesis (Ydenberg and Dill 1986) posits that prey should opt for investing in predator avoidance behaviors once the risk of being caught by the predator exceeds the cost of fleeing. The presence of pathogens may mediate this optimization and shift decisions towards apparently higher risk-taking (Godin and Sproul 1988; Møller 2008). Given that individuals harboring debilitating pathogens should be in generally worse condition than parasite-free individuals, the formers can be expected to interrupt their current activity (e.g. feeding) later and will be less inclined to engage in costly runaway behaviors in the presence of threat than healthy individuals.

The asset protection hypothesis (Clark 1994) operates through the cost-benefit balance due the residual reproductive value of individuals (Cooper and Frederick 2007; Wolf et al. 2007). Organisms that are in prime condition and have a high probability to survive until the next breeding season and produce offspring in the future have more to lose if becoming predated than organisms that have no such prospects in subsequent reproductive bouts. Therefore, for injured, parasitized, or diseased individuals, the lower residual reproductive value turns the current reproductive event relatively more valuable (Binning et al. 2014), thus they will develop and maintain elevated levels of risk-taking behavior than healthy individuals (Table 1).

The above-mentioned hypotheses rely on the general causal scenario, in which parasitism mediates risk-taking behavior, but there are other hypotheses that postulate an opposite causality by assuming that risk-taking behavior has consequences for parasite burden (Table 1). For example, one can formulate the side-effect of risk-taking hypothesis (Table 1), which predicts a positive relationship between risk-taking and parasitism as a result of higher general behavioral activity that make individuals more susceptible to parasite infection (Wilson et al. 1993; Garamszegi et al. 2007; Boyer et al. 2010; Kortet et al. 2010; Dizney and Dearing 2013). Aggressive, explorative and risk taking individuals may have higher chances

100 to get in physical contact with numerous parasites and vectors (thus malaria parasites are also
101 involved) through their higher interaction rate with different components of the socio-
102 ecological environment. Bold animals may often suffer injuries from fights with conspecifics
103 and/or predators, which also make them particularly susceptible to infections (Semple et al.
104 2002; Johnson et al. 2006; Ondrackova et al. 2012). Another side effect may operate through
105 immuno-suppression, as risk-taking lifestyle may negatively alter the efficiency of the
106 immune system that can lead to elevated parasite levels (e.g. Navarro et al. 2004).
107 Finally, the avoidance hypothesis postulates that the increased hazard of parasitism
108 through the higher encounter rate or suppressed immune function in risk-taking individuals
109 may favor certain avoidance mechanisms via physiological or behavioral means that prevent
110 the negative side effects of their behavior (Hart 1990). Accordingly, only those individuals
111 will be able to engage in high-risk behaviors under predatory threat or in other challenging
112 situations that are armored with a preventive machinery against increased susceptibility to
113 parasites (Table 1). For example, the major histocompatibility complex (MHC), which is the
114 most important genetic region coding the vast majority of proteins that participate in immune
115 defense in vertebrates (Apanius et al. 1997; Hedrick 1999; Piertney and Oliver 2006) is likely
116 involved in this mechanisms (Kortet et al. 2010). Furthermore, individuals may also balance
117 how much they invest in immune function depending on the experienced risk of exposure to
118 pathogens and predators: animals that generally display behaviors that incur lower levels of
119 risk in different ecological and social contexts may be expected to allocate less resources to
120 immune defense than other animals that rely on riskier behavioral strategies (Zylberberg et al.
121 2014).
122 To test the above, not mutually exclusive hypotheses in a wild bird, we investigated
123 how malaria prevalence, immediate health status and genetic resistance to parasites can shape
124 risk-taking behavior, or *vice versa*, in the collared flycatcher, *Ficedula albicollis*. We

performed series of field studies, in which we assessed the relationship among these traits by combining both experimental and correlative approaches. We focused on the risk-taking behavior of males during the courtship period that can be characterized without the capture of individuals (Garamszegi et al. 2008; 2009; 2012; 2014). We estimated flight initiation distance (FID), which is the distance at which an individual flee an approaching predator. FID is a widely used measure of risk-taking in a variety of bird and other animal taxa (Stankowich and Blumstein 2005; Blumstein 2006; Cooper and Frederick 2007; Møller 2008), and in the studied flycatcher population, it can be approximated with relatively high repeatability ($R > 0.6$) within the same breeding season (Garamszegi et al. 2012). Here, we rely on our long-term data collected since 2007 and present results from three different studies investigating different aspects of the relationship in focus. First, we show how FID correlates with the prevalence of haemosporidian blood parasites (mostly caused by a *Haemoproteus* strain), which are abundant malaria agents that birds bring from the African wintering sites (Szöllősi et al. 2009). Second, we show results from an experimental study, in which we manipulated the health status of males by injecting them with an antigen and followed changes in their FID. Third, we present the output from a survey, in which, we assessed genetic resistance against parasite by counting the number of MHC alleles that individuals possess and related this estimate to FID. The relevance and predictions of these three studies for the considered hypotheses linking parasitism to risk-taking are given in Table 1.

Methods

Behavioral measurements and ringing protocols

Our fieldwork was carried out in a Hungarian population of the collared flycatcher in the Pilis Mountains in Hungary (47°43´N, 19°01´E). Our breeding plots involve more than 800 nest-boxes that have been established for the long-term study of hole-nesting passerines in the

150 early eighties (Török and Tóth 1988). From the expected arrival date from the wintering sites
151 (middle of April), we regularly visit the field site for territorial but unpaired flycatcher males
152 showing the typical courtship behavior on their territory during the most active morning
153 period (usually between 6.00 to 12.00h). Then, we apply non-invasive methods to
154 characterize three behavioral traits in males during the courtship period. Among these traits,
155 in accordance with our hypothetical framework investigated here, we focus on our assessment
156 of risk-taking based on flight initiation distance (FID as described by Blumstein 2003)
157 collected from 2007 to 2013 (albeit different components of the study rely on different
158 subsamples within this frame, see below).

159 To score FID, we first stimulated aggressive responses from territory owners by
160 exposing them to a caged stimulus male positioned 2-3 meters away from their nest-box.
161 When the focal male showed an aggressive response, whereby it was observed on the top or at
162 the side of the cage showing clear intention to fight, we initiated measuring FID starting from
163 our hiding position (about 20-30 meters from the nest box). To take these measurements, the
164 observer walked toward the focal bird, at a normal walking speed, until it interrupted its
165 aggression displays and fled as a consequence of the approach of a potential predator. The
166 observer continued walking if the resident returned to the decoy's cage to fight within at least
167 one minute. This sequence was repeated until the bird did not return to the reference position
168 anymore (each individual returned at least once). At this point, we measured the distance
169 between the observer and the decoy's cage (i.e. the closest distance at which the focal male
170 could be approached by a human) as the number of steps of approximately one meter. By
171 applying this framework to estimate of FID, we could eliminate the confounding effect of
172 individual differences in the ability of noticing a predator. For example, very aggressive
173 males may not be aware of the presence of a human, thus by allowing the birds to flee and
174 return at least once, we ascertained that it had the chance to perceive the risky situation and

make a decision. In addition, we previously found that FID measured during the context of territorial aggression strongly correlates with the distance from the human observer when the bird is engaged in singing (Garamszegi et al. 2008), thus we can reasonably assume that motivation to fight may not be a confounding factor. Therefore, we assumed that FID is inversely related to risk-taking, as birds with short FID can be considered to take high risk by allowing potential predators to approach until close proximity. Our earlier studies based on the repeated measurement of the same individuals in the same breeding season also indicated a considerable amount of within-individual consistency in FID, as the repeatability estimate of the trait revealed a value ($R > 0.6$, Garamszegi et al. 2012) that was noticeably higher than the mean seen in a meta-analysis of a large number of behavioural traits (Bell et al. 2009).

For each observation, we recorded the calendar date and exact time to statistically control for date and temporal effects. The date when the first behavioural test for a particular male had been realized was used to reflect arrival date. We assumed that the date of first observation is a good surrogate of real arrival date, because we monitor our breeding plots for newly arrived, displaying birds in a standard way with high intensity. It is therefore likely that males used in this study were recorded just upon their arrival. Arrival order of males translates to differences in territory quality, as early arrival is generally known to lead to the occupation of prime breeding sites (Kokko 1999). Therefore, we used arrival date as a proxy variable for territory quality. We used this variable to control for biases stemming from the non-standard environment during our behavioural essays (flycatchers were assessed at the place they chose), as risk-taking might be affected by territory quality (Garamszegi et al. 2009).

After the behavioral assays, we immediately captured males with a conventional nest-box trap to perform standardized ringing protocols and to take measurements and blood samples. Males were classified as yearlings or older based on the typical sub-adult plumage

coloration (brown remiges) of yearling males (Svensson 1984). The size of the forehead patch (FPS, the product of its maximum height and width) and wing patch (WPS, the sum of the lengths of white bars on the outer vanes of the 4th-8th primaries), which are important sexually selected traits that may reflect male quality (Hegyi et al. 2002; Török et al. 2003), were measured with a digital caliper (to the nearest 0.1 mm). WPS was standardized across age categories by bringing the age classes to a common mean of 0 and standard deviation of 1. To assess body size and body condition, we also took measurements of tarsus length with calipers, and body mass with a Pesola spring balance (to the nearest 0.1 g).

We took blood samples from the brachial vein for subsequent molecular analyses (see parasite and MHC screens below). Blood samples in the field were stored in absolute alcohol. After all procedures birds were released thus allowed to continue their breeding performance.

Immune challenge experiment

In 2009, after the assessment of FID, as soon as we successfully captured the birds we performed an immune challenge experiment, in which we elicited an antibody-mediated immune response by injecting sheep red blood cell suspension (SRBC, with approximately 5x107 SRBCs in 100µl sterile PBS) intraperitoneally into birds that were randomly assigned to an experimental group (N = 21). This challenge stimulates the production of large numbers of B-cell-dependent antibodies resulting in a health status similar to that during a real infection (Hay and Hudson 1989). Previous studies in birds have demonstrated that the activation of the immune system by SRBC alters metabolic activity, leukocyte profile and plasma protein content, thus increasing heat production and protein turnover, and generating fever (Siegel et al. 1982; Klasing and Austic 1984; Fair et al. 1999; Ots et al. 2001). In a previous study of the same flycatcher population, we found that the administration of SRBC

to males during the courtship period reduced singing activity in a few days (Garamszegi et al. 2004).

We also randomly selected males (N = 14) to receive a placebo treatment (100 µl sterile phosphate-buffered saline). Following the treatments and after applying individual markings on the belly by water resistant color pens, we released the experimental birds. On the subsequent days (1-5 days after the first behavioral test), we searched SRBC- or placebo-treated males displaying in the same or in another territory, and if a previously injected bird was found (N = 23), we measured FID again. The probability of finding a bird in a courtship activity few days after treatment was not related significantly to the original FID that was estimated upon the first observation ($t = 1.658$, $df = 16.24$, $P = 0.117$). Therefore, samples in the immune challenge experiment can be considered as being unbiased. During the course of the experiment, information on FID may have become unavailable for some males in some pre- or post-treatment days due to various constraints. Therefore, not every individual could be followed throughout the entire sequence of their re-sights.

Molecular methods

Parasite screening

Blood samples were collected throughout the entire 7-year observation period, of which we could successfully screen 159 samples for parasite prevalence for individuals with information on FID. DNA from blood samples was extracted by either ammonium-acetate (Nicholls et al. 2000), after which the concentration of genomic DNA was adjusted to 25-35 ng/µl. Polymerase chain reactions (PCRs) were performed to amplify a region of cytochrome *b* gene in the mitochondrial DNA (mtDNA) of *Haemoproteus* and *Plasmodium* parasites using the protocol described by Waldenström et al. (2004). In all PCRs both negative (ddH2O) and positive controls (samples from birds which were previously confirmed to be

infected) were included among the samples to control for possible contaminations and failures during PCRs, respectively. To ensure that none of the samples went through degradation between sample collection and analysis, all samples were previously sexed (as a control for DNA quality) by amplifying the CHD (chromo-helicase-DNA-binding) genes of the host DNA (Ellegren 1996), and screened twice in independent PCRs. PCR results were visualized in agarose gels stained with ethidium bromide.

All samples with positive amplification were sequenced (by both sides) directly using the BigDye® Terminator v3.1 cycle sequencing kit and products from the sequencing reactions were run on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems) or by Macrogen Inc. Sequences were edited and aligned using the program BioEdit (Hall 1999) and Geneious v. 5.6.5 (Biomatters 2012) and identified to genus level (and classified them to be *Haemoproteus* or *Plasmodium* by comparing sequence data with those of previously identified parasites (Bensch et al. 2009). Parasites with sequences differing by one nucleotide substitution were considered to represent evolutionary independent lineages (Bensch et al. 2004). Sequences (N = 6) showing double peaks in the electropherograms due to multiple infections were treated as evidence for infection by the most common parasite lineage COLL2, as the corresponding sequence always appeared on the double peaks. However, the results do not change qualitatively if we remove these samples from the analyses below (data not shown). The overall prevalence of malaria parasites was 54.09% (86 out of 159), which was caused by 11 lineages of *Plasmodium* spp. and *Haemoproteus* spp (see Figure 1 for names and sample sizes).

MHC genotyping

Blood samples for MHC screening for this study in relation to behavior originated from three years (2007-2009). Altogether, we screened 123 males for MHC, of which we had data on FID for 52 individuals.

Amplification and 454 sequencing. We used 454 sequencing platform to amplified 197-bp fragment of the collared flycatcher MHC class IIB 2nd exon, related to the peptide binding region. Fusion primers used in the PCR composed of (1) 454 Roche universal tail (forward - 454 amplicon adaptor A, reverse - 454 amplicon adaptor B), (2) 6-bp forward MID which allow distinguish between individuals, and (3) primer specific for MHC flycatcher (forward - FicL1938, and reverse - FicR1938) (Zagalska-Neubauer et al. 2010). PCR was performed in 20 μl volumes and contained approximately 100 ng of genomic DNA, 0.2 mM of each dNTPs, 1 μM of each primer, 25 mM $MgCl_2$, 1 U of Taq polymerase (Fermentas) and 2 uL of 10× PCR buffer with $(NH_4)_2SO_4$. The thermal profile consisted of 3 min. denaturation at 94°C, followed by 33 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, ended with final elongation at 72°C for 3 min. PCR product concentration was estimated on the agarose gel and measured on NanoDrop. The samples were pooled into approximately equimolar quantities and then purified with use of the MiniElute PCR Purification Kit (Qiagen). Obtained pools were sequenced as a part of a single 454 Titanum technology run (the run contained also MHC amplicons from other species) at the Functional Genomics Center, Uni /ETH Zurich. The run was divided into seven sections that allowed using the same MID several times. Data were extracted and analysed with jMHC software (Stuglik et al. 2011). The output was further analyzed with use of Excel, Bioedit (Hall 1999), MEGA 5.1 and Geneious v. 5.6.5 (Biomatters 2012).

MHC genotyping and distinguishing true alleles from artifacts. Given that a high number of artifacts are often generated when using Next-Generation Sequencing (NGS) technologies (Babik et al. 2009) at different stages of the analysis (e.g. PCR point mutation or small

297 indels), we followed several complementary criterions to distinguish real alleles from
298 artifacts.
299 First, to preliminary filter out artifacts we applied a procedure (Zagalska-Neubauer et al.
300 2010), which aimed to exclude variants present in data set in just one and two copies or
301 variants which contain indels. Then, we calculated the maximum per-amplicon frequency
302 (MAPF) of each sequence variant and sorted the variants according to MAPF criterion.
303 Almost all variants from 0.5% could be regarded as artifacts, whereas variants with and above
304 2.5% have satisfied coverage and at the first site meet the criterion of putative true alleles.
305 Variants with MAPFs from 1.5% to 2.4% were examined especially careful as could contain
306 high number of artifacts.
307 An additional sequence extraction to examine the existence of chimeras originated by *in*
308 *vitro* recombination between true alleles (Lenz and Becker 2008; Galan et al. 2010) was
309 performed by grouping the sequences within individuals by similarity using Geneious
310 (Biomatters 2012). After that, chimeras could be easily detected because they typically appear
311 in a low number between the two clusters of sequences corresponding to the putative alleles
312 from they are originated. As an additional control, sequences were translated to confirm that
313 they are apparently functional (e.g. without stop codons or frameshifts) and inspected by eye
314 to verify that the variants found in the polymorphic sites were also presented in other alleles
315 from the population. As a result, we identified 172 true alleles in the processed sample.
316 Samples from 28 individuals were amplified in independent PCRs and sequenced (i.e.
317 with different barcodes and genotyped blindly) as a control for genotyping error. No
318 discrepancy was found between the genotypes, confirming that our genotyping approach is
319 reliable. It is possible that, even after following all the steps outlined above, artifacts were not
320 completely excluded from the data set. In such case, it is reasonable to assume that the
321 variables under study and the number of artifacts are unrelated (e.g. individuals taking high

risk were not more probably of generating artifacts during NGS) and thus that our conclusions are unbiased. Individuals with a low coverage (< 200) were discarded from further analyses because a low number of sequence could lead to underestimate the individual MHC diversity. After that, coverage and number of alleles found in an individual were unrelated (Pearson correlation: r = 0.107; P = 0.267).

Statistical analyses

The distribution of continuous variables was investigated graphically, before entering them into any analyses (histograms and Q-Q plots). If these figures indicated strong deviance from normality, we applied an appropriate transformation to obtain more symmetrical distribution As a result, FID and the number of MHC alleles was $\log_{10}$-transformed, while the rest of variables was left untransformed. The date of the behavioral observation was standardized among years by defining day 1 in each season based on the date when the first males were seen on the field site. For the categorical predictors, such as for age, the prevalence of blood parasites and treatment categories, we drew frequency diagrams to see the number of cases within each group. Based on this diagnostics it was evident that analyzing parasite prevalence at the lineage level would incur categories with extremely rare cases (see sample sizes in Figure 1) that would be disproportionally influential on the model outputs. Therefore, we created a bivariate variable for overall prevalence ("yes" or "no") that indicated if an individual was parasitized by any of the screened haemosporidian lineages. Before performing any analyses, we verified that each individual was represented with a single measurement (i.e. if data for the same male was available from different years we used the first measurements available).

To analyze the relationship between FID and the prevalence of blood parasites, we built a general linear mixed model with the following structure. FID was treated as response

variable, and was modeled by using a Gaussian distribution. The focal predictor in this model was general parasite prevalence, while the model also included standardized date, time, age and other male traits (FPS, WPS and body condition) as covariates (due to modest sample sizes, we avoided modeling their interactions). Year effects were handled by entering this variable as a random factor in the model. Initially, we considered both random intercepts and slopes to capture potential between-year differences in the focal association, but our model comparison exercises revealed that the inclusion of random-slopes into the model did not generally offer higher fit to the data (all $P > 0.7$). Hence, we proceeded with the simpler models without random-slope structure.

To investigate the effect of the immune challenge treatments on risk-taking behavior in male flycatchers, we analyzed repeated measure data on FID in a mixed model design considering the hierarchical structure of data as caused by the within- and between-individual levels of observations. During the course of the experiment, we scored behavior at least once prior the treatment (pre-treatment state) and one to three times following the different injections (SRBC or placebo) depending on how many times we succeeded to relocate males and assess their FID following the treatment they had received (see Figure 2 for more details about successful re-sights). Therefore, we built a mixed model with repeated FID estimates as a response variable, which included individual identity as a random effect and treatment group (pre-treatment state, SRBC and placebo) as a fixed effect (model A). In this model we also entered the considered covariates (time, date, age, WPS and FPS) as predictors (since the experiment was performed in a single year, we did not control for year effects). We also created a similar model (model B), in which FID estimates obtained in pre-treatment state were entered as a predictor, and in which bivariate treatment effects (SRBC and placebo) were tested for, while the other random and fixed effects were defined as above. In this

model, we hence achieved a control for initial differences in FID and assessed the effect of treatment on the change in FID independent of the pre-treatment states.

Finally, we constructed a model to evaluate how between-individual differences in genetic resistance against pathogens are associated with between-individual differences in risk-taking phenotypes. In this exercise, we used FID as a response variable, while the number of MHC alleles was treated as a predictor in parallel to the other variables (date, time, age and other male traits).

Before interpreting the model outcomes, we systematically performed numerous model diagnostics statistics to avoid misleading results based on statistical artifacts. We first checked assumptions about the distribution of residuals i.e. whether they were normally and homogeneously distributed. The visual inspection of the corresponding diagnostics plots (e.g. Q-Q plot and residuals plotted against fitted values) indicated no obvious deviations from these assumptions. Second, we examined issues about multicollinearity that might potentially lead to instable results and unreliable parameter estimates (Freckleton 2011). For this purpose, we calculated variance inflation factors (VIF, O'Brien 2007) to the standard linear model analogue of each mixed model that was obtained after excluding the random effect (as this method is not available for mixed models). These analyses showed that collinearity among predictors is not a serious issue to consider further (VIFs < 2). Finally, we verified the absence of influential data points by excluding each of them one by one from the data and then contrasting the derived parameter estimates and fitted values against those that correspond to the model based on the full data. This jackknife procedure revealed no evidence for influential cases strongly affecting the interpretations.

Parameter estimates from the fitted models were obtained by fitting models using Maximum Likelihood rather than Restricted Maximum Likelihood (Bolker et al. 2009). To determine the strength of the focal relationship between FID and categorical predictors

(parasite prevalence, experimental treatment), we performed likelihood ratio tests, in which we compared full models that included the focal predictor with their restricted counterparts without the same predictor (for this comparisons we relied on Maximum Likelihood estimations). The statistical significance of the focal predictor is then described by the probability function of the chi-square distribution at the degrees of freedom reflecting the difference between models in the number of parameters estimated (df = 1). Repeatability estimates from mixed models were extracted *sensu* Nakagawa and Schielzeth (2010). Their 95% confidence intervals (95% CI) were estimated based on parametric bootstrap procedures.

The statistical analyses were carried out in the statistical environment of R (R Development Core Team 2013). For the mixed modeling, we used the package lme4 (Bates et al. 2011). For a part of the model diagnostics, we relied on the VIF function available in package car (Fox and Weisberg 2011). For some verification, we also exploited some functions from packages languageR (Baayen 2007), bkrtest (Halekoh and Højsgaard 2013) and influence.ME (Nieuwenhuis et al. 2012).

Results

The relationship between risk-taking and prevalence by haemosporidian blood parasites

When we compared FID between individuals that were infected with any haemosporidian lineage (N = 86) with parasite-free individuals (while statistically controlling for the effect of potentially confounding variables), we found no significant relationship between overall prevalence of parasites and our estimate of risk-taking (Table 2, likelihood ratio test comparing the full model with a model that lacks parasite prevalence as predictor: $\chi^2 = 0.746$, P = 0.388). We repeated this analysis with the most common parasite lineage in our sample, COLL2, which was detected in 68 out of 159 (42.77%) individuals with data on FID (Figure

1). The mean FID of individuals parasitized with this lineage was 10.52 m that was very similar to the mean FID (10.70 m) of individuals that were not parasitized by any of the lineages (73 (45.91%) out of 159 males, Figure 1).

Immune challenge experiment

When we analyzed repeated estimates of FID altogether by including assessments at the first observation as a pre-treatment state and by controlling for the considered potentially confounding variables (Table 3, model A), we found a marginally significant treatment effect (likelihood ratio test comparing the full model with a model that lacks the focal categorical predictor: $\chi^2 = 5.236$, P = 0.073). However, any difference in FID among the three experimental categories is likely to appear due to the fact that FID estimates obtained before capturing and injecting birds tended to be lower than during subsequent observations following the experimental manipulation (group-specific LS means ± SE of FID from model A: pre-treatment: 12.15 ± 1.86 m, SRBC: 15.05 ± 2.12 m, placebo: 15.76 ± 2.21 m). Repeatability of FID relying on the entire observation history of individuals was 0.603 (95% CI = 0.335 - 0.790; since none of the other variables appeared as an important confounder of FID, repeatability was estimated from a model that only included individual identity as a random factor). When we focused on the subset of individuals that received a SRBC injection, the repeatability estimate of the trait including was 0.705 (95% CI = 0.327 - 0.888). Similar calculations for individuals in the placebo group gave 0.525 (95% CI = 0.00 - 0.801).

We also constructed a model, in which FID estimates corresponding to the pre-treatment state were entered as an additional predictor (Table 3, model B). This model revealed a significant effect for FID upon the first assessment confirming that the trait is repeatable within a season (Table 3, model B), but not for the experimental treatment (likelihood ratio test comparing the full model with a model that lacks the focal categorical

predictor: $\chi^2 = 0.820$, P = 0.365). LS means from the model indicated that FID is very similar in the two groups (group-specific LS means ± SE of FID from model B: SRBC: 12.10 ± 2.90 m, placebo: 12.93 ± 2.03 m). Overall, we failed to provide any statistical evidence that immune challenge caused by SRBC injection would alter the within-individual variation of FID over the observation period in 2009 (Figure 2).

The number of MHC alleles and risk-taking

The maximum number of MHC alleles per individual was 15 (mean ± SE = 8.514 ± 0.164, as estimated from the entire validation sample based on 123 males). The number of MHC alleles that an individual harbors was negatively correlated with FID (r = -0.322, N = 52, P = 0.020, Figure 3), suggesting that males that take higher risk are armored with a higher diversity in their MHC. This relationship was also observed when we controlled for the effect of the potentially confounding variables (Table 4).

Discussion

The major findings emerging from this field study of the collared flycatcher were that i) males harboring lineages of haemosporidian parasites in their blood do not express higher or lower levels of risk-taking when a potential predator approaches them during their territorial defense activity than parasite free males; that ii) the administration of an antigenetic agent via an SRBC injection does not alter the temporal variation of risk-taking behavior compared to the control birds that received a placebo; and that iii) individuals that flee a predator at a shorter distance have a higher number of MHC alleles than individuals that apply a less risky predator avoidance strategy by higher FID. In addition, corroborating previous studies (Garamszegi et al. 2012), we could confirm that FID as triggered by an approaching human is

highly repeatable across different days of courtship period, a pattern that is independent of the health status of individuals. Altogether, these results lend the most support to the avoidance hypothesis (Hart 1990) that posits that only individuals with efficient immune defense will be able to tolerate any parasite-mediated costs that high risk-taking can have (Table 1).

If the screened haemosporidian parasites evoked any debilitating physiological costs or/and had considerable consequences for host survival, we should have observed different FID estimates between the group of parasitized and non-parasitized males. Particularly, given that infection by these pathogens can decrease host mobility and enhance freezing as opposed to fleeing (Holmstad et al. 2006), it would be reasonable to detect shorter FID in males with non-zero prevalence in accordance with the economic and the altered host behavior hypotheses. A similar relationship could be expected on the basis of the fitness costs of avian malaria (Marzal et al. 2008; Krams et al. 2013) and in line with the asset protection hypothesis that assumes that the prevalence of harmful parasitic agents reduces residual reproductive values thus makes malaria infected individuals take higher risk. Unfortunately, we do not know anything about the pathology and survival effects of malaria parasitism in our flycatcher population, but we can at least infer that experimental infection and medication studies on other passerines revealed considerable fitness consequences of chronic malaria infection (Atkinson et al. 1995; Merino et al. 2000; Marzal et al. 2005; Zehtindjiev et al. 2008; Cellier-Holzem et al. 2010; Knowles et al. 2010). Furthermore, other correlative studies were able to demonstrate a relationship between the prevalence of blood parasites and risk-taking behavior both at the intra- and interspecific levels (Møller and Nielsen 2007; Møller 2008; Dunn et al. 2011; García-Longoria et al. 2014). Therefore, we can infer on one hand that such parasite-mediated costs are so low in the collared flycatcher that these are unlikely to drive between-individual differences in risk-taking. On the other hand, relying on the correlative study of surviving individuals, we cannot exclude the possibility that we

disproportionally sampled fewer males that seriously suffered from the consequences of infection, realized lower FID and were more likely to become the victim of predation. Furthermore, it remains possible that other, more harmful parasites impose higher costs on the host individuals, thus can mediate risk-taking more obviously. Note that we did not expect the risk taking > parasitism scenario (e.g. side effect or avoidance mechanisms) to be relevant in the current context. This is because malaria parasites originate from the wintering sites and have a long-lasting prevalence through the whole breeding season (Szöllősi et al. 2009), thus it is impossible that variance in our FID estimates caused variance in the prevalence of these pathogens.

The result of our study unraveling no effect of experimental immunization on FID also suggests that the causal scenario in which parasites mediate risk-taking is very unlikely. From the perspective of an immunologically naïve bird, SRBC is a novel, non-pathogenic and non-replicating antigen, against which the immune system mounts a B-cell-specific response (Hay and Hudson 1989). An injection with SRBC alters basal metabolic rate, albumin and leucocyte profiles in the blood, suppresses cell-mediated immune response, generates fever, decreases behavioral activity and causes mass loss in relatively short period of time (Siegel et al. 1982; Klasing and Austic 1984; Fair et al. 1999; Ots et al. 2001; Garamszegi et al. 2004; Horak et al. 2006), but can also have long-term fitness consequences (Hanssen et al. 2004; Hanssen 2006). Therefore, the experimental administration of SRBC to males is likely to solicit a health status that is similar to what is experienced under a parasite attack, thus the immune challenge should have led to a shift in the cost-benefit balance of different behavioral responses towards an approaching predator, a pattern that we were unable to observe. Based on our previous experience in the studied species and with the effect of SRBC on behavioral (singing) activity over a similar time window (Garamszegi et al. 2004), we can reasonably assume that the treatment was effective and caused a decline in body condition. However,

sample size and the power of the associated analyses might be an issue. In any case, the current study failed to provide any support for the hypotheses that state that parasites mediate risk-taking behavior in the hosts due to the cost of mounting an immune defense. Our negative result emerging in the immune challenge experiment is not an exceptional case, other studies also reached similar conclusion (Kekäläinen et al. 2014; DiRienzo et al. 2015; but see Butler et al. 2012; Binning et al. 2014 for positive evidence).

The negative relationship between FID and the number of MHC alleles that individuals possess in their genomes implies that males that take higher risk in the presence of a potential predator may have a more efficient immune system. It is generally thought that MHC diversity in terms of the number of different alleles provides genetic means for the individuals to efficiently combat against a broader spectrum of antigens and a more diverse parasite fauna (Apanius et al. 1997; Hedrick 1999; Piertney and Oliver 2006). However, other hypotheses emphasize that having too many alleles may have some costs (e.g. due to negative selection limiting the lymphocyte repertoire or outbreeding depression), and there might be a selection for an optimal number of MHC alleles. Currently, we do not have empirical evidence about how higher MHC diversity translates into parasite resistance in the studied population (albeit research is in progress), but a study on a Swedish population detected a linear and negative relationship between the probability of malarial infection and functional MHC diversity (Radwan et al. 2012) Therefore, it makes an intuitive sense to interpret the relationship between the number of MHC alleles and FID as an indication of a relationship between parasite resistance and risk-taking decision. Along this interpretation our results would suggest that only those individuals can bear the parasite-mediated costs of risk-taking that are armored with an appropriate, genetically based, immune defense machinery corroborating the predictions of the avoidance hypothesis (Table 1).

We do not have data to demonstrate the parasite-mediated costs are associated with FID, but it may be that our study just has a too narrow focus in terms of parasites, and other than malaria pathogens may be relevant. In theory, several costs due to frequent contact with parasites and vectors or immunosuppression may be in effect (see Introduction). Actually, the avoidance hypothesis does not require observed parasite prevalence to be related to risk-taking, because individuals adjust their behavioral responses based on their capacity to defend against parasites and not by the immediate physiological condition evoked by parasites. That is, individuals with inefficient immune system that apply inappropriate bold behavioral strategies will not be able to tolerate the cost of increased infection thus will be more likely to die. Accordingly, boldness should not necessarily correlate with observed parasite prevalence *per se*, because only those bold individuals can survive that are better in resisting against the higher parasite pressure (thus risk-avoiders and risk-takers will have modest parasite levels). Similarly, given that the avoidance hypothesis predicts a causal mechanism, in which risk-taking affects parasitism and not *vice versa*, it is not surprising that the immune challenge treatment appeared ineffective on behavior. We did not specify immunological response to the injection, but it might be straightforward to test in the future if individual-specific FID estimates predict antibody titers to SRBC. Such a relationship could be predicted if risk-taking strategies are adjusted based on the individual general capacity to resist against parasites.

An important prediction of the avoidance hypothesis is that both risk-taking and immunocompetence are stable, individual-specific attributes. A selection for the development of the avoidance machinery is only required if the observed FID responses to an approaching human reflects how individuals generally cope behaviorally to various life-threating situations and not just an immediate reaction that can only be observed in the given experimental set-up. Unfortunately, based on the low probability of finding and assaying males in two different

570 years, currently, sample size limitations do not permit us to reliably estimate repeatability of
571 FID for a longer time window, and thus to make inferences for the stability of the trait over
572 lifetime. However, it is notable that the between-day repeatability of FID found in this and an
573 earlier study (Garamszegi et al. 2012) is generally higher ($r > 0.5$) than for other behavioral
574 traits (Bell et al. 2009). Furthermore, it also correlates with other behaviors that can be
575 considered as the manifestations of the same characteristics along the shy-bold continuum
576 indicating that general individual-specific risk-taking strategies may exist (Garamszegi et al.
577 2008; 2009; 2012). Regarding the immunological trait we measured, it remains without doubt
578 that the number of MHC alleles is genetically determined and should therefore be consistent
579 during the entire life of an individual.

580 Beside the avoidance hypothesis, it is also partially plausible that individuals with high
581 MHC allele diversity are generally in good condition and can therefore afford high risk-
582 taking. However, MHC polymorphism is not directly related to immediate body condition,
583 but it provides information about a genetic potential, and the link with body condition cannot
584 be interpreted without considering parasite exposure.

585 Kortet et al. (2010) have suggested the co-evolutionary dynamics between hosts and
586 parasites favor between-individual differences in immune function within the host population,
587 in which MHC genes may play a crucial role. One step forward, such genetic variation within
588 a group of animals can generate intrinsic differences in individual states resulting in
589 phenotypic variation in behavior via positive feedback loop mechanisms (Luttbeg and Sih
590 2010). Accordingly, pre-existing, small genetic differences among individuals in parasite
591 resistance can be regarded as generators of disparities in initial assets that are consequently
592 translated into phenotypic variation in condition-dependent behaviors. Such innate differences
593 will be reinforced and stabilized through positive feedback loops, in which high resource-
594 intake rates in risk-taker individuals lead to better immunocompetence. Furthermore,

individuals that effectively defend against parasites will pay a smaller cost under parasite pressure than individuals that have an immune system of inferior performance, thus the former individuals can be expected to generally apply bold and active behavioral strategies than the individuals of the latter type. Our results with regard to the relationship between the number of MHC alleles and FID provide the first empirical support for this model.

In summary, in this field study of the collared flycatcher, we found that there was no relationship between the prevalence of haemosporidian blood (malaria) parasites and FID, an estimate of risk-taking. Similarly, using an experimental approach we failed to demonstrate that an immune challenge alter the risk-taking behavior of males during the onset of their courtship. However, we observed a significant association between the number of MHC alleles and FID indicating that individuals with better resistance against parasites take higher risk. These results concordantly lend support for avoidance hypothesis that posits that only individuals with efficient immune defense can bear the parasite-mediated costs of risky behaviors.

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844 **Tables**

845 **Table 1**. Hypotheses that predict a relationship between parasitism and host risk-taking
846 behavior against predators, consider different causal scenarios and that have different
847 predictions. See main text for explanations.

Hypothesis	Causality	Parasite prevalence	Experimental infection	Genetic resistance to parasites
Altered host behaviour hypothesis	Parasites > Risk taking	Higher prevalence in risk-takers (but only for parasites that are able to manipulate host behaviour)	Immune challenge is ineffective if it does not mimic infection by host-manipulating parasites	Resistant individuals against host-manipulating parasites will take lower risk
Economic hypothesis	Parasites > Risk taking	Higher prevalence in risk-takers	Immune challenge mediates risk-taking	Resistant individuals will be risk-avoiders
Asset-protection hypothesis	Parasites > Risk taking	Higher prevalence in risk-takers	Immune challenge mediates risk-taking	Resistant individuals will be risk-avoiders
Side effect (due to higher exposure to parasites or immune-suppression) of risk-taking hypothesis	Risk-taking > Parasites	Higher prevalence in risk-takers	Immune challenge does not directly affect behaviour	No difference between resistant and non-resistant individuals
Avoidance hypothesis	Risk taking > Avoidance mechanisms > Parasites	No difference between risk-takers and risk-avoiders	Immune challenge does not affect behaviour	Only resistant individuals can take high risk

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Table 2. The relationship between the prevalence of blood parasites and flight initiation distance (FID) while controlling for other potentially confounding variables in male collared flycatchers. Output from a mixed model with overall prevalence that combined prevalence across all parasite lineages (for lineage specific effects, see Figure 1) in a bivariate-state categorical predictor. Year was used as random effect (variances: year = 0.007, residual = 0.070). df and P values were calculated based on Satterthwaite's approximations. For the significance for the effect of parasite prevalence (based on likelihood ratio test), see main text.

Predictor variables	**Estimate (SE)**	***df***	***t***	***P***
Intercept	1.424 (0.571)	141.95	2.495	0.014
Parasite prevalence (yes)	0.041 (0.045)	140.89	0.920	0.359
Age (juvenile)	-0.105 (0.055)	133.58	-1.913	0.058
Date	0.004 (0.005)	141.26	0.856	0.393
Time	0.016 (0.015)	140.26	1.043	0.299
FPS[A]	<0.001 (<0.001)	141.80	0.809	0.420
WPS[B]	0.008 (0.030)	139.11	0.283	0.778
Condition	-1.032 (0.735)	141.49	-1.404	0.162

[A] Forehead patch size

[B] Wing patch size

Table 3. The effect of immune challenge on flight initiation distance (FID) while controlling for other potentially confounding variables in male collared flycatchers, as revealed by two differentially constructed mixed model that include individual identity as random term (variances, Model A: individual = 0.058, residual = 0.025; Model B: individual < 0.001, residual = 0.022). Model A considers a three-state categorical variable (pre-treatment state, SRBC and placebo) as treatment effect, while model B involves the pre-treatment states in FID as a continuous predictor and a two-state binary variable for the SRBC and placebo treatments as a categorical predictor. df and P values for the coefficients were calculated based on Satterthwaite's approximations. For the significance of treatment effects (based on likelihood ratio test), see main text.

Predictor variables	**Estimate (SE)**	***df***	***t***	***P***
Model A)				
Intercept	1.540 (0.449)	37.81	3.428	0.001
Treatment (pre-treatment)	-0.123 (0.065)	44.41	-1.891	0.065
Treatment (placebo)	0.001 (0.087)	41.80	0.009	0.993
Age (juvenile)	-0.071 (0.135)	30.17	-0.523	0.605
Date	-0.023 (0.015)	36.73	-1.489	0.145
Time	-0.019 (0.017)	39.16	-1.104	0.276
FPS	<0.001 (<0.001)	28.52	0.498	0.623
WPS	0.018 (0.054)	26.58	0.326	0.747
Condition	-0.006 (0.011)	27.95	-0.524	0.605

Model B)				
Intercept	0.402 (0.390)	22.99	1.031	0.313
pre-treatment FID (day 0)	0.487 (0.123)	22.99	3.945	0.001
Treatment (placebo)	0.058 (0.075)	22.99	0.772	0.448
Age (juvenile)	-0.165 (0.212)	22.99	-0.776	0.445
Date	-0.016 (0.010)	22.99	-1.541	0.137
Time	-0.016 (0.017)	22.99	-0.945	0.355
FPS	<0.001 (<0.001)	22.99	1.553	0.134
WPS	-0.041 (0.049)	22.99	-0.844	0.407
Condition	0.001 (0.008)	22.99	0.142	0.888

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Table 4. The relationship between the number of MHC alleles and flight initiation distance (FID) while controlling for other potentially confounding variables in male collared flycatchers. Output from a mixed model, in which year was used as random effect (variances: year = 0.023, residual = 0.085). df and P values were calculated based on Satterthwaite's approximations.

Predictor variables	**Estimate (SE)**	***df***	***t***	***P***
Intercept	2.096 (1.093)	38.03	1.917	0.063
number of MHC alleles	-0.847 (0.417)	36.08	-2.029	0.049
Age (juvenile)	-0.092 (0.108)	40.63	-0.846	0.403
Date	0.003 (0.011)	33.63	0.245	0.808
Time	0.008 (0.033)	39.18	0.255	0.800
FPS	<0.001 (<0.001)	40.85	-0.281	0.780
WPS	0.032 (0.074)	38.49	0.436	0.665
Condition	-0.547 (1.354)	33.76	-0.404	0.689

885 **Figure legends**

886

887 **Figure 1**. The relationship between the prevalence of different lineages of blood parasites
888 (abbreviations are taken from the MalAvi database (Bensch et al. 2009)) and flight initiation
889 distance (FID assessed in meters). Group-specific means (grey circles) and standard errors
890 (bars) are given. Group-specific sample sizes are provided on the top.

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892 **Figure 2**. The effect of immune challenge (an injection with sheep red blood cells, SRBC)
893 and control treatment (physiological water, placebo) on flight initiation distance (FID
894 assessed in meters) in a field study of the collared flycatcher males in 2009. The figure on the
895 left shows group-specific statistics for the pre-treatment state, while figure on the right are for
896 the post-treatment state. Boxplots show the extreme of the lower whisker, the lower hinge, the
897 median, the upper hinge and the extreme of the upper whisker.

898

899 **Figure 3**. The linear relationship between the number of MHC alleles and flight initiation
900 distance (FID, assessed in meters) in male collared flycatchers. The line shows the regression
901 line that can be fitted to the untransformed data (FID = 22.57 – 1.26 * Number of MHC
902 alleles).

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