Fluctuating pathogen-mediated selection drives the maintenance of innate immune gene polymorphism in a widespread wild ungulate

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Abstract (246 words)

Toll-like Receptors (TLR) play a central role in recognition and host frontline defence against a wide range of pathogens. A number of recent studies have shown that *Tlrs* often exhibit a large polymorphism in natural populations. Yet, there is little knowledge on how this polymorphism is maintained and how it influences disease susceptibility in the wild. We investigated the selective mechanisms by which multiple-pathogens drive the genetic diversity of *Tlr* genes, as well as the commonly-used *Mhc -Drb* in European roe deer (*Capreolus capreolus*), an abundant and widespread ungulate in Europe. In a previous work, we showed that some *Tlrs* exhibit contemporary signature of balancing selection in roe deer. To assess the relative contribution of "heterozygous advantage", "negative-frequency dependence" and "fluctuating selection", we tested associations between *Mhc-Drb* or *Tlr* variants and infection or co-infection with *Toxoplasma* and *Chlamydia*, two intracellular pathogens known to cause reproductive failure in ungulates. We showed that *Toxoplasma* and *Chlamydia* exposures vary significantly across space and time with few co-infection events and that the two pathogens act antagonistically on *Tlr* polymorphism leading to fluctuating selection. By contrast, we only observed *Tlr* heterozygous advantage for a single pathogen and we did not find evidence of negative-frequency dependence. We did not detect any association with *Mhc-Drb.* Our study confirmed the importance of looking beyond *Mhc* genes in wildlife immunogenetic studies. It also emphasized the necessity to consider multiple pathogen challenges and their spatio-temporal variations to improve our understanding of vertebrate defence evolution against pathogens.

Keywords: balancing selection, habitat heterogeneity, Toll-like genes, antagonistic effects, roe deer

Running title: Fluctuating selection on TLR genes in deer

38 **1. INTRODUCTION**

39 While evolutionary theory predicts that selection and random drift should deplete genetic diversity, a
40 number of genes and genomic regions have maintained abundant polymorphism in natural
41 populations with exceptionally high ratio of non-synonymous (protein-altering) to synonymous
42 substitutions (Hedrick, 2006; Trowsdale & Parham, 2004). Balancing selection is a collective term for
43 evolutionary processes that adaptively maintain variation in populations. It is a widespread process
44 driving the diversity of genes involved in numerous biological functions including pathogen resistance
45 (Sommer, 2005), color polymorphism (Wellenreuther, 2017) or mate choice/male competiveness
46 (Johnston et al., 2013; Moore & Moore, 1999). However, identifying the mechanisms driving
47 balancing selection has been a major challenge. At least three not-mutually exclusive processes
48 (putting aside sexual selection) can act in synergy: the "heterozygote advantage" or
49 "overdominance" hypothesis (Doherty & Zinkernagel, 1975; Lewontin & Hubby, 1966) proposes that
50 heterozygous individuals have a higher relative fitness, on average, than homozygous individuals. The
51 "negative frequency-dependent selection" (also called rare-allele advantage) hypothesis suggests
52 that the fitness-advantage of alleles is inversely proportional to their frequency in the population as a
53 result of the host-pathogen (Takahata & Nei, 1990) or prey-predator co-evolutionary dynamics
54 (Cook, 2003). Lastly, the "fluctuating selection hypothesis" (Hill, 1991; Levene, 1953) proposes that
55 high genetic diversity may be maintained when spatially and/or temporally varying directional
56 selection occurs with sometime antagonistic effects on fitness (Wittmann, Bergland, Feldman,
57 Schmidt, & Petrov, 2017).

58 Immunity-related genes are ideal candidates for studying how adaptive genetic diversity is
59 maintained in wild populations because of their direct effect on survival (Møller & Saino, 2004) or
60 reproductive success (Pedersen & Greives, 2008). The genes of the major histocompatibility complex
61 (MHC) are often used as a model since they show an exceptional polymorphism in vertebrates
62 (Spurgin & Richardson, 2010). Their structure and mechanisms of interaction with antigens are now
63 well described (J. Klein & Figueroa, 1986). Furthermore, their ability to trigger a rapid adaptive
64 response to varying pathogen-mediated selective pressures (PMS) has been demonstrated
65 experimentally (Eizaguirre, Lenz, Kalbe, & Milinski, 2012; Phillips et al., 2018). Over the last few years,
66 a growing body of literature identified PMS at *Mhc* genes in a large range of taxa (see Spurgin &
67 Richardson, 2010 for a reviews) but few of them convincingly elucidated the relative influence of the
68 three mechanisms mentioned above (but see Oliver et al., 2009; Philips, 2018). One likely reason is
69 that most studies have an unrealistic view of the functioning of immune system and of the ecological
70 context in which it evolves, by modelling associations between a single gene (most often a *Mhc* gene)
71 and only one pathogen.

72 *Mhc*, although important, corresponds to a small fraction of the genetic variation in pathogen
73 resistance. Before the adaptive immunity has an opportunity to intervene in the host response,
74 invading pathogens interact with different effectors of innate immunity that recognize specific
75 structures (Acevedo-Whitehouse & Cunningham, 2006). Therefore many genes encoding proteins
76 involved in pathogen recognition or immune regulation may be associated with the resistance
77 against the same pathogen (Turner, Begon, Jackson, Bradley, & Paterson, 2011). In particular, genes
78 encoding Toll-like receptors (TLR) play a key role in the host frontline defence against a wide range of
79 microparasites such as fungi, bacteria, protozoa. This family of genes conserved in both invertebrate
80 and vertebrate lineages are critical for the stimulation of many immune pathways such as
81 inflammation, innate or acquired immunity (Akira, Takeda, & Kaisho, 2001). A multitude of studies
82 have revealed associations between *Tlr* gene polymorphism and infectious diseases in humans and
83 domestic species (Mun et al., 2003; Yang & Joyee, 2008). However, very few (most focusing on birds
84 or rodents) have investigated their role in host-pathogen interactions in natural environment and
85 their contemporary mechanisms of evolution at a population level (but see Turner et al., 2011;
86 Tschirren et al., 2013; Grueber et al., 2013).

87 Moreover, co-infection with multiple pathogens is a real world rule (Maizels & Nussey, 2013) and
88 some selective mechanisms such as heterozygous advantage or fluctuating selection may better be
89 detected when accounting for the multi-pathogen context (Sin et al., 2014; Wegner, Kalbe, Kurtz,
90 Reusch, & Milinski, 2003). Most immunity-related genes have pleiotropic effects on resistance
91 against several pathogens (Hedrick, 1999) and the maintenance of 'susceptible' (deleterious) alleles
92 in a wild population can be due to antagonist effects of a given immunity-related gene on several
93 pathogens (*i.e.* antagonistic pleiotropy). To date, only few studies have examined the selective
94 effects of multiple pathogens on host immunogenetic patterns and they have only focused on *Mhc*
95 genes (Tollenaere et al. 2008 on voles, Loiseau et al. 2008 on house sparrows, Kamath et al. 2014 in
96 plain zebras, Froeschke & Sommer 2012 on striped mouse).

97 Here, we investigated the selective mechanisms by which multiple pathogens could drive the
98 genetic diversity of immunity-related genes in European roe deer (*Capreolus capreolus*), the most
99 abundant and widespread ungulate in Europe. We studied a free-ranging population of roe deer
100 inhabiting an agricultural landscape in Southwestern France. This population is heavily exposed to a
101 range of pathogens that also infect livestock, including the abortive *Toxoplasma gondii* or *Chlamydia*
102 *abortus* (Candela et al., 2014). These two pathogens are known to have negative influence on the
103 survival and reproductive performances of ungulates (Dubey, 2009; Pioz et al., 2008). They may
104 therefore exert selective pressures on immunity-related genes. We focused on four genes encoding
105 pattern-recognition receptors (PRRs) that detect specific microbes or pathogen-associated molecular

106 patterns: the widely-used *Mhc class II-Drb* gene and three non-*Mhc* genes encoding Toll-like
107 receptors genes (*Tlr2, Tlr4, Tlr5*). *Mhc* and *Tlr* genes may have pleiotropic effects on the
108 resistance/susceptibility to pathogens harboring similar pathogen-associated molecular patterns
109 (Kaisho & Akira, 2004; Loiseau et al., 2008). Moreover, *Tlr* genes have been shown to play a major
110 role in host defence against multiple pathogens, including *Toxoplasma* (Miller, Boulter, Ikin, & Smith,
111 2009) and *Chlamydia* (Beckett et al., 2012). In a recent work, we showed that these genes exhibit
112 high levels of amino acid diversity within French roe deer populations including the population
113 studied here, hence suggesting an important functional repertoire (Quéméré et al., 2015). *Tlr2* and
114 *Tlr4* also exhibited contemporary signatures of balancing selection: several haplotypes were
115 observed at moderate frequency and showed very low levels of differentiation among roe deer
116 populations compared to the patterns obtained with neutral markers. These findings support the
117 hypothesis that the polymorphism of these genes is shaped by multiple pathogen-mediated
118 selection.

119 More specifically, we assessed the role of "heterozygous advantage", "negative-frequency
120 dependence" and "fluctuating" selection in shaping the signatures of balancing selection observed in
121 the European roe deer. We analyzed whether the prevalence of *Toxoplasma* and *Chlamydia* or a
122 combination of these pathogens (*i.e.* co-infection status) was associated with specific patterns of
123 genetic diversity observed at the four immunity-related genes (e.g. heterozygosity/allelic divergence,
124 presence of rare or common alleles), while accounting for ecological predictors (age, sex, spatial and
125 temporal variation in infection level). The "heterozygote advantage" hypothesis has received limited
126 support when authors focused on a single pathogen species (but see Olivier et al., 2009) but a
127 growing number of case studies has revealed *Mhc* heterozygous superiority in animals co-infected
128 with multi-pathogens (Hughes & Nei, 1992; McClelland et al., 2003; Froeschke & Sommer, 2012 but
129 see Sin et al., 2014). In agreement, we expected associations between immunity-related gene
130 heterozygosity and the co-infection status of deer, in particular because *Toxoplasma* and *Chlamydia*
131 employ similar strategies to invade host cells (Romano & Coppens, 2013). Negative-frequency
132 dependence, through host-pathogen "Red Queen" co-evolution, may confer selective advantage to
133 novel alleles as recently demonstrated experimentally in wild populations (Phillips et al., 2018).
134 Therefore, under this hypothesis, we expected negative relationships between rare *Mhc* or *Tlr* alleles
135 and pathogen infection. Lastly, given the high temporal and spatial heterogeneity in pathogen
136 exposure in the study area (Candela et al., 2014; Sevila et al., 2014), balancing selection was also
137 predicted to act on *Mhc-Drb* and *Tlr* genes through spatial and/or temporal fluctuations in pathogen-
138 mediated selection. We thus expected to find various associations between particular *Mhc-Drb* or *Tlr*
139 alleles and pathogen prevalence.

140 **2. MATERIAL AND METHODS**

141 **2.1 Study population and data collection**

142 The study focused on a roe deer population inhabiting a heterogeneous agricultural landscape in
143 Southern France (43°13'N, 0°52'E). This area is covered by a mix of open fields (including 38%
144 meadows and pastures for cattle and sheep, and 32% crops), hedges (16%), small woodlands patches
145 (14%) and two larger forest blocks (5%). Individuals were caught by drive-netting during winter (from
146 January to March) between 2008 and 2016 in seven sampling sites with varying vegetation cover
147 (Figure S1). Individual sex, age class (juveniles: ≤ 1 year of age versus adults: > 1 year of age) and
148 body mass were recorded. Blood samples were collected for pathogen screening (see below) and ear
149 punches for genetic analyses. In total, we gathered samples from 433 annual captures corresponding
150 to 328 different individuals (190 females and 138 males, 164 juveniles and 164 adults). All applicable
151 institutional and European guidelines for the care and use of animals were followed. The study was
152 permitted by the land manager. All the procedures involving animals were approved by the Ethical
153 Committee 115 of Toulouse and authorized by the French Ministry in charge of ethical evaluation (n°
154 APAFIS#7880-2016120209523619v5).

155 **2.2 *Tlr* and *Mhc-Drb* genotyping**

156 DNA was extracted from alcohol-preserved tissues using the DNeasy Blood and Tissue kit (QIAGEN).
157 The second exon of the *Mhc-Drb* class II gene encoding the ligand-binding domain of the protein was
158 amplified and sequenced for 293 deer samples using Illumina MiSeq system following the procedure
159 detailed in Quéméré et al. (2015). Haplotypes and individual genotypes were identified using the
160 SESAME barcode software (Piry, Guivier, Realini, & Martin, 2012). *Tlr* genes (*Tlr2*, *Tlr4* and *Tlr5*) were
161 genotyped using the two-step procedure described in Quéméré et al. (2015). Previously, a pilot study
162 on 32 individuals was performed to identify polymorphic sites (SNPs) and Linkage-disequilibrium (LD)
163 groups. We screened almost the entire coding region of the three *Tlr* genes (82% in average)
164 including the leucine-rich extracellular region of receptors involved in antigen-binding. Detailed on
165 primer sequences, SNP positions and codon changes are provided in Quéméré et al. (2015). We then
166 selected one SNP per LD group (primarily targeting non-synonymous sites) that was genotyped for all
167 individuals (N=328) using the KASPar allele-specific genotyping system provided by KBiosciences
168 (Hoddesdon, UK). A total of 13 SNPs were typed including 5, 3 and 5 SNPs for *Tlr2*, *Tlr4* and *Tlr5*
169 respectively. Details on SNP position and codon change can be found in Table S2. Haplotypes were
170 then reconstructed from the phased SNPs using the procedure implemented in DNASP v5 (Librado &
171 Rozas, 2008). All sequences have been submitted to NCBI Genbank (Accession nos. are in Table S1,
172 Supporting information).

173 **2.3 Pathogen screening**

174 We determined infection by *Toxoplasma gondii* and/or *Chlamydia abortus* by analysing the
175 serological status of roe deer with regard to these pathogens using classical enzyme-linked
176 immunosorbent assays (ELISA) with specific commercial kits (Sevila et al., 2014). According to the
177 manufacturer's instructions, blood samples with antibody recognition level (ARL) > 30% (respectively
178 40%) were considered positive for *T. gondii* (respectively *C. abortus*) (see Sevila et al., 2014 for
179 further details). In total, we obtained the *Toxoplasma* and *Chlamydia* serological status of 277 (with
180 74 repeated measures) and 196 (36 repeated measures) roe deer respectively (caught between 2008
181 and 2016). The proportion of co-infected individuals was examined in the years where both
182 pathogens were screened (between 2008 and 2013). The importance of interactions between
183 *Toxoplasma* and *Chlamydia* in roe deer was analyzed using a screening association method
184 (Vaumourin et al., 2014). This method tests whether some pathogen associations are significantly
185 more or less detected than expected under the null hypothesis that these associations are random.

186 **2.4 Statistical analyses**

187 We analyzed the relationships between the prevalence of *T. gondii* or *C. abortus* infection (binary
188 response variable – presence/absence) and immunity-related gene variation using generalized linear
189 mixed-effect models (GLMM) with a binomial family (using a logit link function) and the *glmer*
190 function implemented in the lme4 package (Bates, Maechler, & Bolker, 2012). We also tested
191 whether immunity-related gene variation affected the number of pathogens co-infecting roe deer (0,
192 1 or 2) using a GLMM with a Poisson family (using a log link function).

193 In a first step, we built for each pathogen and for the co-infection status a reference "non-
194 genetic" model containing biological and environmental factors that possibly affect pathogen
195 exposure. We tested the influence of sex (male *versus* female) and age-class (juvenile *versus* adult)
196 because both behaviour and host susceptibility may vary between sexes and across an individual's
197 lifetime (Klein, 2000) and because infection persistence is expected to result in a positive age-
198 prevalence relationship (Gotteland et al., 2014). We also considered the spatial and inter-annual
199 variation in environmental conditions that may affect pathogen survival and thus encounter
200 probability (e.g. climate, food resources, population density, etc.). For each individual and year, we
201 calculated an annual probability of infection (API) corresponding to the number of infected
202 individuals in a given year (except the focal individual) divided by the number of animals sampled this
203 year (except the focal individual). Similarly, we calculated a spatial probability of infection (SPI) as the
204 number of infected individuals in a given sampling site (except the focal individual) divided by the
205 number of animals sampled within this site (except the focal individual). In the co-infection models,

206 we tested the effect of sex, age, API and SPI of both pathogens. In all models, we included individual
207 identity as a random effect to account for correlation amongst different observations of the same
208 individual.

209 In step two, using the best 'non-genetic' model as reference, we tested, independently for each
210 pathogen/co-infection and gene, the three mechanisms potentially underlying balancing selection.
211 The "heterozygote advantage" hypothesis was tested either by i) adding heterozygosity as a binary
212 fixed effect (heterozygote vs homozygote) or ii) fitting the number of SNPs (allelic distance) among
213 the two alleles with an individual's genotype. The "negative frequency dependence" hypothesis was
214 investigated by including the presence or absence of rare haplotype (<10%) as explanatory variable.
215 Finally, the "fluctuating selection" hypothesis was examined by considering associations between
216 infection status and the presence/absence (0/1) or the number of copies (0, 1 or 2) of the most or
217 the second most frequent haplotype. Each genetic effect was tested either as a simple additive effect
218 or in interaction with API or SPI. These latter enabled to test the hypotheses that the genetic effect
219 on pathogen prevalence varies across time or space, according to the intensity of exposition.

220 In total, 28 models were tested for each combination of gene and pathogen or gene and co-
221 infection status (Table S3). We used a model selection procedure using Akaike information criterion
222 with a correction for small sample sizes (AIC_C) to determine which model best explained variation in
223 pathogen infection (models with lowest AIC) . We only examined models with ΔAIC< 2 relative to the
224 best model. Statistical analyses were performed using the software R version 3.3.3 (R Development
225 Core Team, 2017).

226 **3. RESULTS**

227 **3.1 Immunogenetic variation**

228 Among the 293 tested individuals, we found nine functional alleles for *Mhc* class II-*Drb* gene, coding
229 for different amino acid sequences. One haplotype (CacaDRB*0302) was common (60%), two other
230 (CacaDRB*0201 and CacaDRB*0102) had intermediate frequency (18% and 10% respectively) while
231 the six others were rare (<5%). Observed heterozygosity (H_O) reached 0.55. We isolated six different
232 haplotypes for *Tlr2* in 327 genotyped individuals, all corresponding to different amino acids
233 sequences. Two haplotypes ("CATCG" and "TGCCG") showed particularly high frequency (35% and
234 51% respectively) while the four others were rare (< 5%). The two most common haplotypes
235 belonged to two highly divergent genetic clusters and were separated by seven substitutions
236 (including four non-synonymous ones) (Quéméré et al., 2015). The *Tlr4* gene exhibited four
237 functional haplotypes in 327 individuals including two haplotypes ("CGG" and "GAG") with moderate
238 to high frequency (26% and 55% respectively) and two haplotypes with weak frequencies (10% and

239 7%). Lastly, we isolated six haplotypes (in 324 individuals) for the *Tlr5* among which two ("GCTCG",
240 "ACCTG") had high frequencies (38% and 31% respectively), one ("ATCCG") had intermediate
241 frequency (18%) and the three others were rare (<5%). Observed heterozygosity was relatively high
242 for the three *Tlr* genes (H_O = 0.62, 0.64 and 0.69, *Tlr2*, *Tlr4* and *Tlr5* respectively).

243 **3.2 Ecological patterns of parasitism**

244 Seroprevalence of *Toxoplasma* and *Chlamydia* reached 35% and 16% respectively (Table 1). Among
245 the 232 annual captures for which both pathogens were screened (196 different deer), only 18
246 individuals (7%) were co-infected by two pathogens simultaneously while 93 (40%) and 36 (15%)
247 animals were infected solely by *Toxoplasma* and *Chlamydia* respectively. We did not reveal any
248 significant pathogen association using the screening analysis (*P* = 0.39) (Figure S2).

249 For the two studied pathogens, we found a significant positive effect of annual and spatial
250 variation in environmental conditions (modeled as a linear effect of API and SPI) on the prevalence of
251 the two pathogens: API ranged between 0.16 (in 2014) and 0.83 (in 2009) for *Toxoplasma*; between 0
252 (in 2012 and 2013) and 0.37 (in 2010) for *Chlamydia*. SPI ranged between 0.15 (in site 9) and 0.55 (in
253 site 1) for *Toxoplasma;* between 0.11 (in site 9) and 0.22 (in site 7) for *Chlamydia.* Additionally, the
254 *Toxoplasma* reference (non-genetic) model revealed a significant effect of age class, with lower
255 antibody prevalence in juvenile individuals compared to adults (OR = 0.38 [CI 0.15-0.91]). Lastly, we
256 found a significant positive effect of $API_{TOXO,}$ API_{CHLA} and negative effect of SPI_{CHLA} on co-infection
257 status. Most co-infections (16 out of 18) occurred in 2009 and 2010 (Table 1).

258 **3.3 Genetic effects on pathogen infection**

259 *Toxoplasma gondii*

260 The inclusion of *Tlr2* genotype significantly improved the *Toxoplasma* infection non-genetic model.
261 The best-fitted model (delta AICc = -4.68 with non-genetic model) (Table S3) included an effect of
262 individual age-class, the yearly prevalence (API) and the number of copies of the "TGCCG" haplotype
263 of *Tlr2*. Roe deer carrying two copies of this frequent haplotype (51%) showed a decreased
264 *Toxoplasma* seroprevalence (odd ratio OR = 0.24 [0.075-0.76]) compared to individuals without this
265 haplotype (Figure 1a). The model with the same haplotype fitted as a binary effect
266 (presence/absence) received a slightly lower support (delta AICc = -2.86). We also observed a strong
267 relationship between *Toxoplasma* prevalence and *Tlr4* heterozygosity (delta AICc = -3.86) or *Tlr4*
268 allelic distance (delta AICc = -4.19), each time in interaction with the annual probability of infection
269 (Figure 2). Roe deer carrying two different or distant copies of *Tlr4* had a significantly lower
270 probability of being infected with *Toxoplasma* than other animals, in years when population

271 exposure was high. The opposite pattern was observed when population exposure was low. Other
272 models testing rare allele advantage received a weaker support than the reference non-genetic
273 model. We did not detect any association between *Toxoplasma* infection and *Tlr5* or *Mhc-Drb*
274 genetic variation.

275 *Chlamydia abortus*

276 We revealed a strong relationship between *Tlr2* genotype and *Chlamydia* prevalence. The best-
277 fitted model (delta AIC = -6.15 with reference model) included a positive effect of API, SPI and the
278 number of copies of the most-common haplotype ("TGCCG"). Homozygous individuals for this allele
279 had an increased probability to be infected (OR = 9 [1.11-80]) (Table S3, Figure 1). Alternatively, the
280 second best-fitted model (delta AIC = -5.48) indicated that deer carrying one or two copies of the
281 second most-frequent haplotype ("CATCG") were less infected with *Chlamydia* (OR = 0.46[0.13-1.68]
282 for deer carrying a single copy ; OR = 0.019 [0.004-0. 95] for deer carrying two copies). We also found
283 a significant association between *Tlr5* genotype and *Chlamydia* infection (delta AIC = -4 with
284 reference model). Individuals carrying rare alleles (<5%) were more likely to be infected (OR= 5.44
285 [1.02-28.9]) (Table S3). All models testing directional selection or heterozygosity advantage received
286 a weaker support than the minimal non-genetic model. No association was observed when
287 considering *Mhc-Drb* and *Tlr4* genes.

288 *Co-infections*

289 We did not find any relationship between the co-infection status of individuals and their
290 immunogenetic profiles (Table S3).

291 **4. DISCUSSION**

292 So far, very few studies, focusing on a limited number of taxa (most exclusively birds or rodents),
293 examined the mechanisms behind pathogen-mediated selective processes contemporarily acting on
294 immunity-related genes within natural populations (Grueber et al., 2013; Barbara Tschirren, Råberg,
295 & Westerdahl, 2011; Turner, Begon, Jackson, & Paterson, 2012). Being evolutionary ancient and
296 under strong functional constraints, genes involved in innate immune responses are expected to
297 evolve primarily under purifying selection and to exhibit limited polymorphism (Parham, 2003). Yet, it
298 is now quite clear that some of these genes, including *Tlr* genes encoding surface recognition
299 receptors, may have relaxed selective constraints and show large nucleotide diversity, sometimes
300 comparable to *Mhc* genes (Seabury et al., 2011). In a previous work, we have shown that the high
301 sequence and allelic diversity observed at roe deer *Tlr* genes could be maintained through balancing
302 selection (Quéméré et al., 2015). In this study, we developed a multi-gene/multi-pathogen

303 association approach to bring new insights into the mechanisms underlying such balancing selection.
304 Most of the pathogen-gene associations found here suggested a key role of fluctuating directional
305 selection and, to a lesser extent, of heterozygous advantage. We only found a limited influence of
306 negative-frequency dependence process. We showed that different *Tlr* genes may be associated with
307 resistance to the same microparasite (here *Tlr2* and *Tlr4* with *Toxoplasma* or *Tlr2* and *Tlr5* with
308 *Chlamydia*) in agreement with the general idea that pathogens interact with many immune
309 recognition receptors. We also revealed that different pathogens may act antagonistically on the
310 polymorphism of a given gene (here *Tlr2* with *Toxoplasma* and *Chlamydia*).

311 **4.1 Predominant role of directional and fluctuating selection on *Tlr* gene variation**

312 We revealed that the polymorphism of *Tlr2* is predominantly shaped by directional selection exerted
313 by both *Toxoplasma* and *Chlamydia*. Roe deer carrying the most or second most frequent haplotype
314 were less likely to be infected either by *Toxoplasma* or *Chlamydia*. We know that these two abortive
315 pathogens may have significant impact on the annual reproductive success of ungulates (Pioz et al.,
316 2008). TLRs have been shown to activate the complement system, a component of innate immunity
317 known to be important for resistance to microparasites during early infection (Raby et al., 2011). A
318 higher affinity of a *Tlr* haplotype to *Toxoplasma* or *Chlamydia* ligands may result in a stronger
319 complement response and thus in an increased resistance to these pathogens (Tschirren et al., 2013).

320 Our results also revealed a high temporal and spatial heterogeneity in both pathogen prevalence
321 probably owing to variation in exposure. Annual variation in pathogen prevalence may be related to
322 both climate and host factors. Climatic conditions such as temperature or humidity are known to
323 affect pathogen survival in the environment and may influence host transmission *via* the quantity of
324 infected aerosols for *Chlamydia* (Tang, 2009) or oocysts for *Toxoplasma* (Gotteland et al., 2014). For
325 example, Sevila et al. (2014) showed that *Toxoplasma* prevalence in roe deer was higher in mild and
326 wet years. The high turnover of individuals in the population in relation with a strong harvest
327 pressure may also partly explain why we observed a so high inter-annual variability (Candela et al.,
328 2014). Moreover, habitat heterogeneity and land use within roe deer's home range may explain the
329 observed spatial variation in pathogen exposure. For example, *Toxoplasma* prevalence increased
330 with the proportion of human dwellings (a proxy of the cat presence) within home range.

331 Spatial and temporal variation in pathogen-mediated directional selection means that *Tlr* haplotypes
332 inheritance may vary among years and according to where females established their home range.
333 This supported the hypothesis that fluctuating selection is a key process in the maintenance of
334 balanced polymorphism in *Tlr* genes in this population. This finding is fully consistent with the
335 Simpsonian 'dynamic' Adaptive Landscape concept (Simpson, 1944) that states that genetic variance

336 and evolution of fitness characters in the wild is mainly shaped by spatial and temporal fluctuations
337 in selection, specially variation in biotic interactions (Calsbeek, Gosden, Kuchta, & Svensson, 2012;
338 Siepielski, DiBattista, & Carlson, 2009). This general concept can be easily applied to host-pathogen
339 interactions and immunity-related gene evolution. Different habitats in terms of biotic and abiotic
340 factors are likely to support distinct pathogen communities and so distinct pathogen-mediated-
341 selection regime (Eizaguirre & Lenz, 2010). Using simulation-based models, Hedrick (2002)
342 demonstrated that variation in space and time in pathogen resistance can in itself be a strong
343 mechanism for the maintenance of polymorphism at immunity-related genes. In agreement, several
344 empirical studies found that *Mhc* genetic diversity varies across space in a mosaic of habitats likely
345 due to differences in pathogen communities (Alcaide, Edwards, Negro, Serrano, & Tella, 2008;
346 Eizaguirre & Lenz, 2010; Landry & Bernatchez, 2001). We noted that few studies have convincingly
347 elucidated the ecological drivers of this immunogenetic variation and that all focused on *Mhc* genes.

348 **4.2 Antagonistic pleiotropy and potential immunogenetic trade-off**

349 Interestingly, we observed that different haplotypes of a same *Tlr* gene (here *Tlr2*) may be associated
350 with the infection status of the two pathogens. Pleiotropic effects in *Tlr2* were expected since this
351 receptor is known to be implicated in the recognition of many bacterial, fungal, protozoa (de Oliviera
352 Nascimento, Massari, & Wetzler, 2012) including *Toxoplasma* (Yarovinsky, 2008) and *Chlamydia*
353 (Beckett et al., 2012; Darville et al., 2003). Here, the most common *Tlr2* haplotype ("TGCCG") (51%)
354 was associated with decreased *Toxoplasma* infection and increased *Chlamydia* infection while the
355 concurrent haplotype ("CATCG") (35%) showed opposite trends. This result provides an example of
356 antagonistic pleiotropy, which has been proposed as a widespread mechanism by which genetic
357 variation is maintained within populations (Roff & Fairbairn, 2007; Rose, 1982), in particular at
358 immunity-related genes (Aderem & Ulevitch, 2000; Carter & Nguyen, 2011). Antagonistic pleiotropy
359 has been rarely evidenced in natural populations (Turner et al., 2011) and, to our knowledge, this
360 study is the first demonstration with regard to a *Tlr* gene. Two non-exclusive mechanisms may be
361 invoked to explain this antagonistic effect: decrease of intracellular pathogen competition (Loiseau et
362 al., 2008) and host immunogenetic trade-off (Kamath et al., 2014). *Toxoplasma* and *Chlamydia* use
363 similar strategies to invade cells route and compete for the same nutrient pools in co-infected cells
364 (Romano & Coppens, 2013). Therefore, antagonistic effects might arise because *Tlr2* gene alters the
365 competitive interactions between the two pathogens. In other words, because individuals carrying
366 the most frequent *Tlr2* haplotype are less infected by *Toxoplasma*, this may indirectly promote
367 infection by *Chlamydia* without a direct role of the second most frequent haplotype (and *vice versa*).
368 In a study on house sparrows, Loiseau et al. (2008) found antagonistic effects of a *Mhc* class I gene on
369 multiple malarial parasite strains. In this particular case, deleterious 'susceptibility alleles could be

370 maintained in the population due to within-host competition between malaria parasites. Here, we
371 did not observe fewer co-infections than expected given the prevalence of the two pathogens.
372 Moreover *Tlr2* genotype had no effect on individual co-infection status. So it is unlikely that
373 antagonistic effect may result from the competitive interactions between *Toxoplasma* and
374 *Chlamydia*.

375 The most likely hypothesis is the presence of a host immunogenetic trade-off (Kamath et al.,
376 2014). In this case, pathogens do not directly compete but *TLr2* genotype has a direct effect on the
377 resistance/susceptibility to both pathogens. The allele inferred to be beneficial for decreasing
378 *Toxoplasma* infection is also associated with increased susceptibility to *Chlamydia* (and *vice versa*).
379 This scenario is reinforced by the fact that the two concurrent *Tlr2* haplotypes exhibit very distinct
380 DNA and amino-acid sequences, what suggests marked functional differences. Similar patterns and
381 processes have previously been described for ticks and nematodes infections (Kamath et al., 2014;
382 Turner et al., 2011). To our knowledge, our study is the second one showing evidence of antagonistic
383 selection at non-*Mhc* gene in a wild population (see for the other one Turner et al., 2011).

384 **4.3 Marginal role of heterozygous advantage**

385 A negative relationship between gene heterozygosity and deer co-infection status was expected
386 under the heterozygosity advantage hypothesis (McClelland et al., 2003). Instead, we found only an
387 association between *Tlr4* heterozygosity or allelic divergence and the infection to a single pathogen
388 (*Toxoplasma*). This finding must be interpreted with caution since we analyzed two pathogens only.
389 However, Sin et al. (2014) also revealed several single gene-single pathogen associations in European
390 badger (*Meles meles*) and did not find any support for *Mhc* heterozygosity being associated with
391 fewer co-infecting pathogens despite examining 13 pathogenic species from a wide variety of taxa. In
392 our study, the absence of relationship between heterozygosity at immunity-related genes and co-
393 infection could be due to the low individual's probability of being exposed to both pathogens
394 *Toxoplasma* and *Chlamydia* across their life-time, explaining the low fitness benefit of heterozygous
395 genotypes. The lack of association between Mhc-Drb and the two pathogens supported the general
396 idea that MHC class II molecules principally bind exogenous antigens and are primarily involved in
397 the immune response to extracellular pathogens (Hughes & Yeager, 1998)

398 The strong association between *Tlr4* allelic divergence and *Toxoplasma* infection supported the
399 divergent allele advantage hypothesis (Wakeland et al., 1990). Individuals carrying highly distant
400 copies of *Tlr4* appeared less likely to be infected by *Toxoplasma* than those carrying one haplotype or
401 two genetically closer haplotypes, but in years of high *Toxoplasma* exposure only. This genetic effect
402 in interaction with inter-annual variation in pathogen exposure again emphasizes the importance of

403 fluctuating selection on immunogenetic polymorphism. We also found an advantage for
404 homozygotes or individuals carrying two genetically close haplotypes when *Toxoplasma* exposure
405 was low. However, further information on the genetic diversity of *Toxoplasma* in this deer population
406 and on the co-evolutionary dynamics with *Tlr4* is needed to fully understand this complex pattern.

407 **5. Conclusion**

408 In conclusion, our study illustrates the importance of looking beyond *Mhc* genes (Acevedo-
409 Whitehouse & Cunningham, 2006) and single snapshot gene/pathogen association (Maizels &
410 Nussey, 2013) in wildlife immunogenetic studies. In the present case, we did not find any influence of
411 *Mhc* diversity on the resistance to two microparasites. Moreover, we highlighted the importance of
412 antagonistic effects and fluctuating selection on the maintenance of innate immunogenetic diversity,
413 what could not have been possible using a classical single gene-single pathogen approach.

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595

596 **Data Accessibility:**

597 Haplotype DNA sequences: Primers and genBank accessions are in Table S1. SNP positions and
598 characteristics are in Table S2. Genotypes of all individuals at each *Tlr* and *Mhc-Drb* locus and
599 environmental/parasitological data can be found on Dryad: Dryad entry doi:10.5061/dryad.qt0079b.

600 **Authors' contributions**
601 EQ, EGM and NC designed the study and interpreted the data; AJMH, HV and YC led the data
602 collection process on the field; MG, JM and EQ carried out the *Tlr* and *Mhc-Drb* genotyping; EQ, PH,
603 NM and MF performed the statistical analyses; EQ led the writing, all authors contributed to the
604 manuscript and gave final approval for publication.

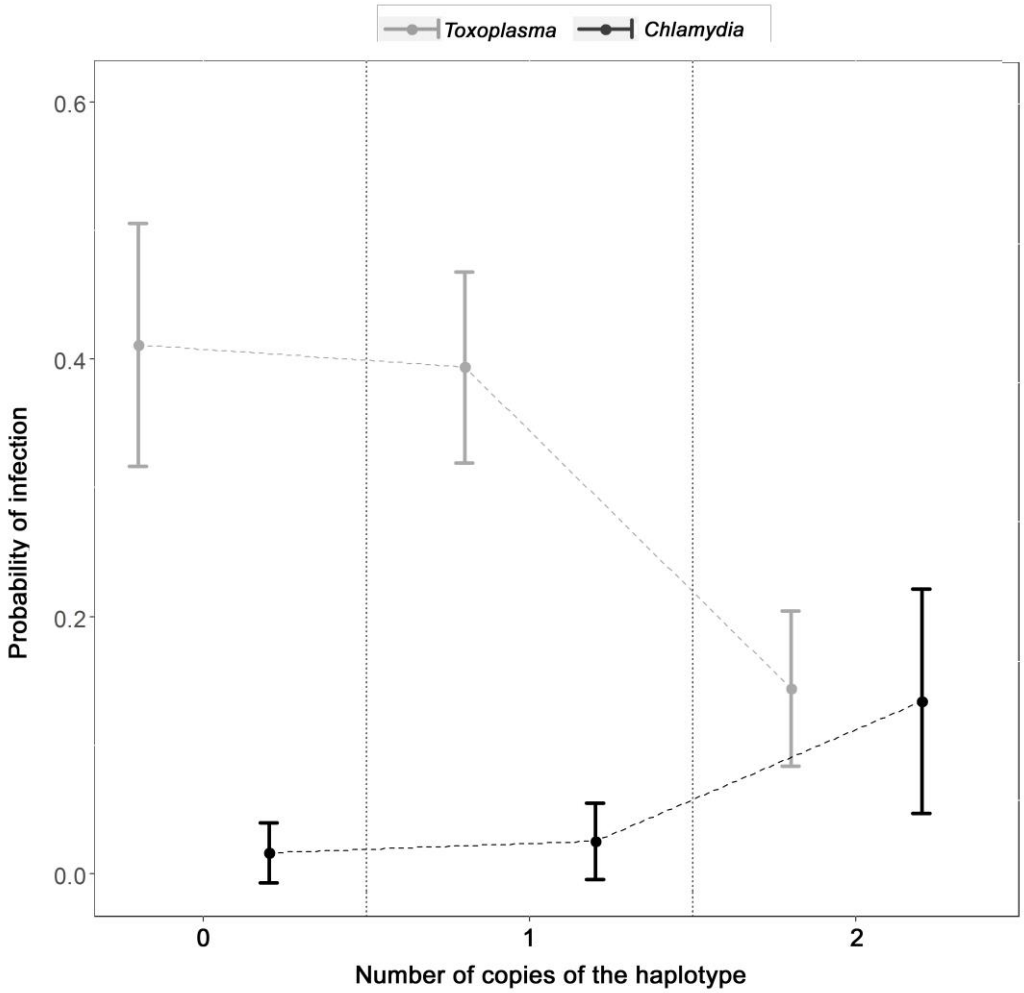
605 **Tables and Figures.**

606 **Table 1 - Summary of the pathogen** data (number of samples per year in brackets). NA indicates the years for
607 which no data were available.

Pathogen	Number of samples	Number of individuals	Overall seroprevalence	Seroprevalence evaluated per year								
				2008	2009	2010	2011	2012	2013	2014	2015	2016
Toxoplasma	351	277	0.35	0.23 (40)	0.83 (42)	0.56 (43)	0.24 (50)	0.21 (42)	0.24 (38)	0.16 (31)	0.3 (49)	0.43 (16)
Chlamydia	232	196	0.16	0.00 (40)	0.14 (41)	0.37 (43)	0.28 (50)	0.05 (42)	0.00 (16)	NA	NA	NA
coïnfection	232	196	0.08	0 (40)	5 (41)	11 (43)	2 (50)	0 (42)	0 (16)	NA	NA	NA

608

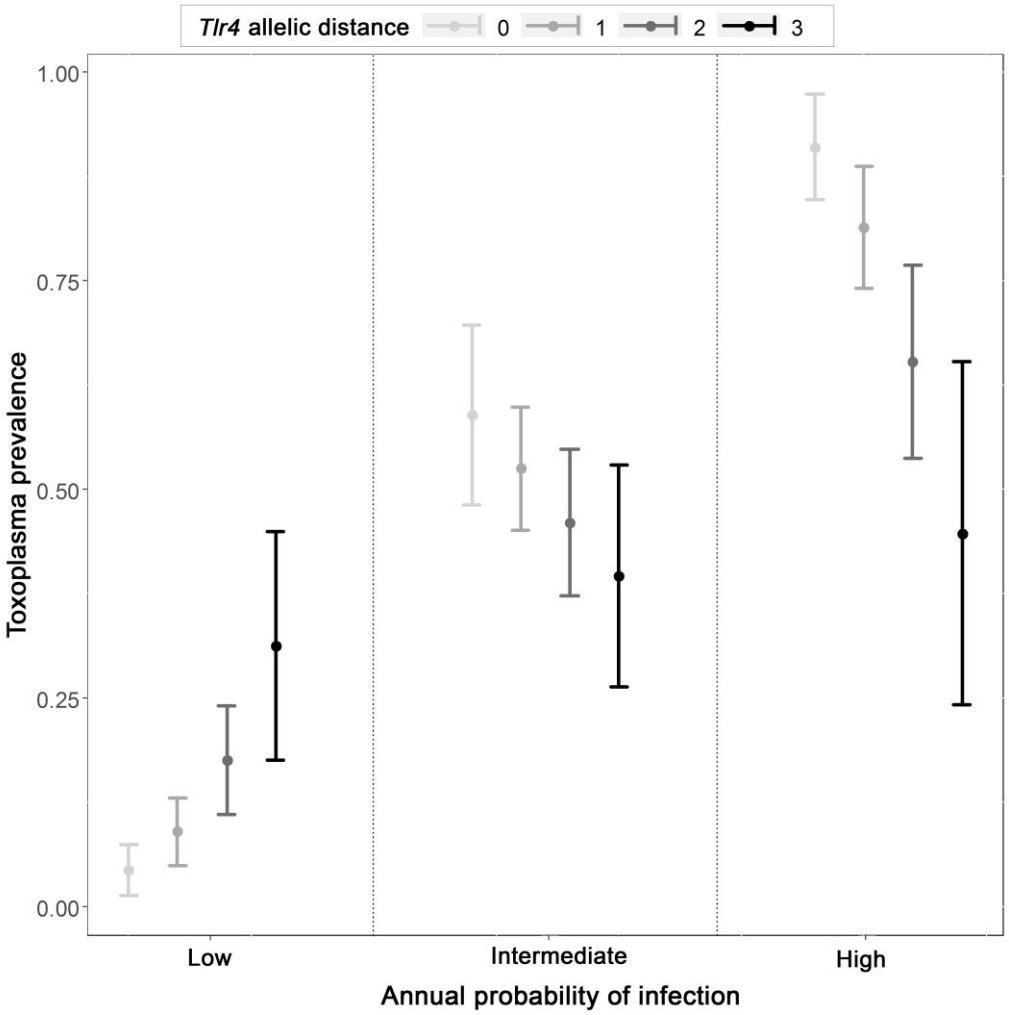
609 **Figure 1.** Genetic polymorphisms at *Tlr2* are associated with both *Chlamydia* and *Toxoplasma*
610 serological status. Predicted prevalence of *Toxoplasma* and *Chlamydia* infection in roe deer with the
611 number of copies (0/1/2) of the most common haplotype ("TGCCG", 51%).



612

613

614 **Figure 2.** Infection to *Toxoplasma* is associated with *Tlr4* allelic distance. Predicted prevalence of
615 *Toxoplasma* infection in roe deer in function of the distance (in base pairs) between the two alleles of
616 the *Tlr4* gene with a positive interaction with the annual probability of infection (Low API = 0.3,
617 Intermediate = 0.5 and high = 0.7)



618

619 **Supporting information**

620 **Table S1.** Details of primer sequences, product size and genbank accessions.

621 **Table S2.** Summary of the 13 *Tlr* SNPs genotyped using the KASPar SNP genotyping system.

622 **Table S3.** Details of the mixed linear models of *Toxoplasma*, *Chlamydia* infection or co-infection for
623 each gene (*Trl2*, *Tlr4*, *Tlr5* and *MHC-Drb*).

624 **Figure S1.** Map of the study area.

625 **Figure S2.** Outputs of the screening association analysis.

626