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

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Abstract (233 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 high levels of genetic diversity and contemporary signatures of balancing selection in roe deer. To assess the relative contribution of "heterozygous advantage", and "fluctuating selection", we evaluated associations between *Mhc-Drb* or *Tlr* variants and 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 detected, and that the two pathogens act antagonistically on *Tlr* polymorphism leading to fluctuating selection. By contrast, we found no evidence of association with *Mhc-Drb* and *Tlr* heterozygosity. 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

1. INTRODUCTION

While evolutionary theory predicts that selection and random drift should deplete genetic diversity, a number of genes and genomic regions have maintained abundant polymorphism in natural populations with exceptionally high ratio of non-synonymous (protein-altering) to synonymous substitutions (Hedrick, 2006; Trowsdale & Parham, 2004). Balancing selection is a collective term for evolutionary processes that adaptively maintain variation in populations. It is a widespread process driving the diversity of genes involved in numerous biological functions including pathogen resistance (Sommer, 2005), color polymorphism (Wellenreuther, 2017) or mate choice/male competiveness (Johnston et al., 2013; Moore & Moore, 1999). However, identifying the mechanisms driving balancing selection has been a major challenge. At least three not-mutually exclusive processes (putting aside sexual selection) can act in synergy: the "heterozygote advantage" or "overdominance" hypothesis (Doherty & Zinkernagel, 1975; Lewontin & Hubby, 1966) proposes that heterozygous individuals have a higher relative fitness, on average, than homozygous individuals. The "fluctuating selection hypothesis" (Hill, 1991; Levene, 1953) proposes that high genetic diversity may be maintained when spatially and/or temporally varying directional selection occurs with sometime antagonistic effects on fitness (Wittmann, Bergland, Feldman, Schmidt, & Petrov, 2017). Third, the "negative frequency-dependent selection" (also called rare-allele advantage) hypothesis suggests that the fitness-advantage of alleles is inversely proportional to their frequency in the population as a result of the host-pathogen (Phillips et al., 2018; Takahata & Nei, 1990) or prey-predator co-evolutionary dynamics (Cook, 2003). This third mechanism is particularly challenging to evidence in natural systems as it requires long time series of species prevalence and allele frequencies of genes involved in "Red Queen" co-evolution (Spurgin & Richardson, 2010, but see Charbonnel & Pemberton, 2005).

Immunity-related genes are ideal candidates for studying how adaptive genetic diversity is maintained in wild populations because of their direct effect on survival (Møller & Saino, 2004) or reproductive success (Pedersen & Greives, 2008). The genes of the major histocompatibility complex (MHC) are often used as a model since they show an exceptional polymorphism in vertebrates (Spurgin & Richardson, 2010). Their structure and mechanisms of interaction with antigens are well described (Klein & Figueroa, 1986). Furthermore, their ability to trigger a rapid adaptive response to varying pathogen-mediated selective pressures (PMS) has been demonstrated experimentally (Eizaguirre, Lenz, Kalbe, & Milinski, 2012; Phillips et al., 2018). Over the last few years, a growing body of literature identified PMS at *Mhc* genes in a large range of taxa (see Spurgin & Richardson, 2010 for a review) but few of them convincingly elucidated the relative influence of the three mechanisms mentioned above (but see Oliver et al., 2009; Philips et al., 2018). One likely reason is

71 that most studies modelling associations between a single gene (most often a *Mhc* gene) and only
72 one pathogen. However, decreasing sequencing costs now offer new opportunities to examine
73 multiple genes and diseases in a single study, leading to a more realistic view of the functioning of
74 the immune system and of the ecological context in which it evolves.

75 *Mhc*, although important, only corresponds to a fraction of the genetic variation in pathogen
76 resistance (Acevedo-Whitehouse & Cunningham, 2006). Before the adaptive immunity has an
77 opportunity to intervene in the host response, invading pathogens interact with different effectors of
78 innate immunity that recognize specific structures (Jepson et al., 1997; Lam-Yuk-Tseung & Gros,
79 2003). Therefore many genes encoding proteins involved in pathogen recognition or immune
80 regulation may be associated with the resistance against the same pathogen (Turner, Begon, Jackson,
81 Bradley, & Paterson, 2011). In particular, genes encoding Toll-like receptors (TLR) play a key role in
82 the host frontline defence against a wide range of microparasites such as fungi, bacteria, protozoa.
83 This family of genes conserved in both invertebrate and vertebrate lineages are critical for the
84 stimulation of many immune pathways such as inflammation, innate or acquired immunity (Akira,
85 Takeda, & Kaisho, 2001). A multitude of studies have revealed associations between *Tlr* gene
86 polymorphism and infectious diseases in humans and domestic species (Mun et al., 2003; Yang &
87 Joyee, 2008). However, very few (most focusing on birds or rodents) have investigated their role in
88 host-pathogen interactions in natural environment and their contemporary mechanisms of evolution
89 at a population level (but see Kloch et al., 2018; Tschirren et al., 2013).

90 Moreover, infection with multiple pathogens is a real world rule (Maizels & Nussey, 2013) and
91 some selective mechanisms such as heterozygous advantage or fluctuating selection may better be
92 detected when accounting for the multi-pathogen context (Sin et al., 2014b; Wegner, Kalbe, Kurtz,
93 Reusch, & Milinski, 2003). Most immunity-related genes have pleiotropic effects on resistance
94 against several pathogens (Hedrick, 1999) and the maintenance of 'susceptible' (deleterious) alleles
95 in a wild population can be due to antagonist effects of a given immunity-related gene on several
96 pathogens (*i.e.* antagonistic pleiotropy). To date, only few studies have examined the selective
97 effects of multiple pathogens on host immunogenetic patterns and the great majority focused on
98 *Mhc* genes (Tollenaere et al., 2008 on voles; Loiseau et al., 2008 on house sparrows ; Kamath et al.,
99 2014 in plain zebras; Froeschke & Sommer, 2012 on striped mouse but see Antonides et al., 2019).

100 Here, we investigated the selective mechanisms by which multiple pathogens could drive the
101 genetic diversity of immunity-related genes in European roe deer (*Capreolus capreolus*), the most
102 abundant and widespread ungulate in Europe. We studied a free-ranging population of roe deer
103 inhabiting an agricultural landscape in Southwestern France. This population is heavily exposed to a

104 range of pathogens that also infect livestock, including the abortive *Toxoplasma gondii* or *Chlamydia*
105 *abortus* (Candela et al., 2014). These two pathogens are known to have negative influence on the
106 survival and reproductive performances of ungulates (Dubey, 2009; Pioz et al., 2008). They may
107 therefore exert selective pressures on immunity-related genes. We focused on four genes encoding
108 pattern-recognition receptors (PRRs) that detect specific microbes or pathogen-associated molecular
109 patterns: the widely-used *Mhc class II-Drb* gene and three non-*Mhc* genes encoding Toll-like
110 receptors genes (*Tlr2, Tlr4, Tlr5*). *Mhc* and *Tlr* genes may have pleiotropic effects on the
111 resistance/susceptibility to pathogens harboring similar pathogen-associated molecular patterns
112 (Kaisho & Akira, 2004; Loiseau et al., 2008). Moreover, *Tlr* genes have been shown to play a major
113 role in host defence against multiple pathogens, including protozoans and gram-negative bacteria
114 (Beckett et al., 2012; Gazzinelli & Denkers, 2006; Miller, Boulter, Ikin, & Smith, 2009; Netea,
115 Wijmenga, & O'Neill, 2012; Yarovinsky, 2008). In a recent work, we showed that these genes exhibit
116 high levels of amino acid diversity within French roe deer populations including the population
117 studied here, hence suggesting an important functional repertoire (Quéméré et al., 2015). In
118 particular, *Tlr2* and *Tlr4* exhibited several haplotypes at moderate frequency and very low levels of
119 differentiation among roe deer populations compared to the patterns obtained with neutral markers.
120 These findings support the hypothesis that the polymorphism of these genes is shaped by balancing
121 selection although the underlying mechanism(s) remains to be investigated.

122 In this work, we assessed the role of "heterozygous advantage" and "fluctuating" selection in
123 shaping the signature of balancing selection observed in the European roe deer. We analyzed
124 whether the prevalence of *Toxoplasma* and *Chlamydia* was associated with specific patterns of
125 genetic diversity observed at the four immunity-related genes (e.g. heterozygosity/allelic divergence,
126 presence of dominant haplotypes), while accounting for ecological predictors (age, sex, year,
127 sampling site). Support for the *Mhc* heterozygote advantage hypothesis has been found in many
128 studies of mammals (Hedrick, 2012; Oliver et al., 2009; Sin et al., 2014b), including wild ungulates
129 (Brambilla, Keller, Bassano, & Grossen, 2018; Paterson, Wilson, & Pemberton, 1998). However, its
130 importance for innate immunity-related genes has rarely been assessed (but see Antonides et al.,
131 2019; Grueber et al., 2013). A recent study in birds (Grueber, Wallis, & Jamieson, 2013) has found
132 evidence for survival advantage associated with *Tlr4* heterozygote genotypes. Here, we expected
133 associations between *Tlr* and/or *Mhc-Drb* heterozygosity and infection with *Toxoplasma* and
134 *Chlamydia*¸ two pathogens employing similar strategies to invade host cells (Romano & Coppens,
135 2013). Moreover, there is a high temporal and spatial heterogeneity in pathogen exposure in the
136 study area (Candela et al., 2014; Sevila et al., 2014), and TLR and MHC receptors have pleiotropic
137 functions (Akira et al., 2001; Hedrick, 1999). As such we expected to find fluctuations in pathogen-

138 mediated directional selection acting on different *Mhc-Drb* or *Tlr* alleles. Such fluctuations could
139 underlie the signatures of balancing selection previously detected on these genes (Quéméré et al.,
140 2015).

141 **2. MATERIAL AND METHODS**

142 **2.1 Study population and data collection**

143 The study focused on a roe deer population inhabiting a heterogeneous agricultural landscape in
144 Southern France (43°13′N, 0°52′E). This area called "Vallons et Côteaux de Gascogne" (VCG) is part
145 of the Aterlier Pyrénées Garonne(https://pygar.omp.eu/). The area is covered by a mix of open fields
146 (including 38% meadows and pastures for cattle and sheep, and 32% crops), hedges (16%), small
147 woodlands patches (14%) and two larger forest blocks (5%). Individuals were caught by drive-netting
148 during winter (from January to March) between 2008 and 2016 in seven neighboring sites located a
149 few kilometers apart, with varying vegetation cover (Figure S1). Deer from these sites have been
150 shown to belong to the same panmictic population (Coulon et al., 2006; Gervais et al., 2019).
151 Individual sex, age class (juveniles: ≤ 1 year of age versus adults: > 1 year of age) and body mass were
152 recorded. Blood samples were collected for pathogen screening (see below) and ear punches for
153 genetic analyses. In total, we gathered samples from 433 annual captures corresponding to 328
154 different individuals (190 females and 138 males, 164 juveniles and 164 adults) among which 157
155 deer had been included in a previous immunogenetic studies (Quéméré et al., 2015). All applicable
156 institutional and European guidelines for the care and use of animals were followed. The study was
157 permitted by the land manager. All the procedures involving animals were approved by the Ethical
158 Committee 115 of Toulouse and authorized by the French Ministry in charge of ethical evaluation (n°
159 APAFIS#7880-2016120209523619v5).

160 **2.2 *Tlr* and *Mhc-Drb* genotyping**

161 The *Mhc-Drb* class II and *Tlr* (*Tlr2*, *Tlr4* and *Tlr5*) genes of 157 roe deer from VCG had been genotyped
162 in a previous study (Quéméré et al., 2015). We completed this dataset by genotyping 171 new
163 individuals using exactly the same procedure. DNA was extracted from alcohol-preserved tissues
164 using the DNeasy Blood and Tissue kit (QIAGEN). *Tlr* genes were genotyped using a two-step
165 approach: a pilot study on 32 individuals was first performed to identify polymorphic sites (SNPs) and
166 linkage-disequilibrium (LD) groups. We screened almost the entire coding region of the three *Tlr*
167 genes (82% in average) including the leucine-rich extracellular region of receptors involved in
168 antigen-binding. Detailed on primer sequences, SNP positions and codon changes are provided in
169 Quéméré et al. (2015). We then selected one SNP per LD group (primarily targeting non-synonymous

170 sites) that was genotyped for all individuals using the KASPar allele-specific genotyping system
171 provided by KBiosciences (Hoddesdon, UK). A total of 13 SNPs were typed including 5, 3 and 5 SNPs
172 for *Tlr2*, *Tlr4* and *Tlr5* respectively. Details on SNP position and codon change can be found in Table
173 S2. Lastly, haplotypes were reconstructed from the phased SNPs using the procedure implemented in
174 DNASP v5 (Librado & Rozas, 2008). The second exon of the *Mhc-Drb* class II gene encoding the ligand-
175 binding domain of the protein was amplified and sequenced using Illumina MiSeq system as detailed
176 in Quéméré et al. (2015). Haplotypes and individual genotypes were identified using the SESAME
177 barcode software (Piry, Guivier, Realini, & Martin, 2012). All sequences have been submitted to NCBI
178 Genbank (Accession nos. are in Table S1, Supporting information).

179 **2.3 Pathogen screening**

180 The serological status of roe deer for *Toxoplasma gondii* and/or *Chlamydia abortus* was analyzed
181 using classical enzyme-linked immunosorbent assays (ELISA) with specific commercial kits (Sevila et
182 al., 2014). The specificity and sensitivity of these kits were respectively 97.4 and 99.4 % for *T. gondii*
183 and 92.2 and 89 % for *C. abortus*. Although the kits were developed for domestic ruminants, they
184 were shown to be reliable and efficient in wild ungulates (Gotteland et al., 2014) with a good
185 concordance with classical test (e.g. high concordance (0.8) between ELISA and the Modified
186 Agglutination Test, a reference test for *Toxoplasma* in roe deer). According to the manufacturer's
187 instructions, blood samples with antibody recognition level (ARL) > 30% (respectively 40%) were
188 considered positive for *T. gondii* (respectively *C. abortus*) (see Sevila et al., 2014 for further details).
189 In total, we obtained the *Toxoplasma* and *Chlamydia* serological status of 277 (with 74 repeated
190 measures) and 196 (36 repeated measures) roe deer respectively (caught between 2008 and 2016,
191 Table S3). The individual repeatability (R) and its standard error (SE) for *Toxoplasma* and *Chlamydia*
192 seroprevalence were calculated using the R package 'rptR' (Stoffel, Nakagawa, & Schielzeth, 2017).
193 The proportion of individuals seropositive for both *Toxoplasma* and *Chlamydia* was examined in the
194 years where both pathogens were screened (between 2008 and 2013).

195 **2.4 Statistical analyses**

196 We analyzed the influence of immunity-related gene variation, environmental and life-history factors
197 on the seroprevalence of *T. gondii* or *C. abortus* (binary response variable – presence/absence) using
198 generalized linear mixed-effect models (GLMM) with a binomial family (using a logit link function).
199 We used multimodel inference to establish which explanatory variables had an effect, averaged over
200 plausible models (Burhnam & Anderson 2002; Grueber et al., 2011). Analyses were performed using
201 the *glmer* function implemented in the 'lme4' package (Bates, Maechler, & Bolker, 2012), 'MuMin'
202 v1.7.7 (Barton, 2009), and 'AICcmodavg' v1.25 (Mazerolle, 2013) in R version 3.3.3 (R Development

203 Core Team, 2017). Multimodel inference was conducted in two steps to reduce the number of
204 factors in a single model and the total number of models evaluated (Sin et al., 2014b).

205 In the first step, we investigated the effects of non-genetic biological and environmental factors
206 that possibly affect pathogen exposure. The starting model included roe deer sex (male *versus*
207 female) and age-class (juvenile *versus* adult) because both behaviour and host susceptibility may vary
208 between sexes and across an individual's lifetime (Klein, 2000) and because infection persistence is
209 expected to result in a positive age-prevalence relationship (Gotteland et al., 2014). We also included
210 the capture year (9 levels) and site (7 levels) as fixed effects to account for the spatial and inter-
211 annual variation in environmental conditions that may affect pathogen survival and thus encounter
212 probability (e.g. climate, food resources, population density). Roe deer classically inhabit a single
213 home range across the entire adult life that is relatively small (mean 0.76 km^2) (Morellet et al., 2013)
214 and generally included in only one capture site. As such, we considered capture sites as the spatial
215 units shared by individuals captured at the same location. In all models, we included individual
216 identity as a random effect to account for correlation amongst different observations of the same
217 individual. We conducted an initial exploration of our data to ascertain their distribution and identify
218 potential outliers (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). Model averaged parameter estimates
219 are provided with their unconditional standard errors (SE), 95% confidence intervals and relative
220 importance, after model averaging (ΔAIC< 5 relative to the best model) (Burnham, Anderson, &
221 Huyvaert, 2011). Significant variables were retained in a reduced model for use in step 2.

222 In the second step, we investigated the "heterozygote advantage hypothesis" by fitting a binary
223 fixed effect (heterozygote *vs* homozygote) and the "haplotype advantage" hypothesis by considering
224 associations between the infection status and the number of copies (0, 1 or 2) of the most or second
225 most frequent haplotype of *Tlr2*, *Tlr4*, *Tlr5* and *MHC-Drb*. We used variance inflation factors (VIF) to
226 measure collinearity among predictors and retained variables with VIF values <3 (Zuur et al., 2009).
227 To minimize multicollinearity, the effects of the two most frequent haplotypes of a gene and its
228 heterozygosity were evaluated in separate models. The identity of the site and/or the year were
229 included as random effects in all models because (i) individuals at a site/year all experience similar
230 conditions of exposure (see the results of step 1) and (ii) to account for spatial/temporal
231 autocorrelation in allele frequencies that may lead to spurious associations with pathogen
232 prevalence. In total, 256 models were evaluated for both *Toxoplasma* and *Chlamydia* (see Table S4
233 for the full list of models).

234 **3. RESULTS**

235 **3.1 Immunogenetic variation**

236 Among the 293 tested individuals, we found nine functional alleles for *Mhc* class II-*Drb* gene, coding
237 for different amino acid sequences. One haplotype (CacaDRB*0302) was common (60%), two other
238 (CacaDRB*0201 and CacaDRB*0102) had intermediate frequency (18% and 10% respectively) while
239 the six others were rare (<5%). Observed heterozygosity (H_O) reached 0.55. We isolated six different
240 haplotypes for *Tlr2* in 327 genotyped individuals, all corresponding to different amino acids
241 sequences. Two haplotypes ("CATCG" and "TGCCG") showed particularly high frequency (35% and
242 51% respectively) while the four others were rare (< 5%). The two most common haplotypes
243 belonged to two highly divergent genetic clusters and were separated by seven substitutions
244 (including four non-synonymous ones) (Quéméré et al., 2015). The *Tlr4* gene exhibited four
245 functional haplotypes in 327 individuals including two haplotypes ("CGG" and "GAG") with moderate
246 to high frequency (26% and 55% respectively) and two haplotypes with low frequencies (10% and
247 7%). Lastly, we isolated six haplotypes (in 324 individuals) for the *Tlr5* among which two ("GCTCG",
248 "ACCTG") had high frequencies (38% and 31% respectively), one ("ATCCG") had intermediate
249 frequency (18%) and the three others were rare (<5%). Observed heterozygosity was relatively high
250 for the three *Tlr* genes (H_O = 0.62, 0.64 and 0.69, *Tlr2*, *Tlr4* and *Tlr5* respectively).

251 **3.2 Ecological patterns of parasitism**

252 Seroprevalence of *Toxoplasma* reached 35% and was moderately repeatable among years at the
253 individual level (R=0.21, SE=0.06). By comparison, seroprevalence of *Chlamydia* was lower (16%) but
254 showed a higher individual consistency among capture events (R=0.70, SE=0.05). Among the 232
255 annual captures for which both pathogens were screened (196 different deer), only 18 individuals
256 (7%) had anti-*Toxoplasma* and anti-*Chlamydia* antibodies simultaneously while 93 (40%) and 36
257 (15%) animals were seropositive solely for *Toxoplasma* and *Chlamydia* respectively (Table 1). We
258 found a strong effect of "the year of capture" for the two pathogens with a significant increased
259 probability of being seropositive for *Toxoplasma* in 2009 and 2010 (Seroprevalence = 83% and 56%
260 respectively) and a decreased seroprevalence in 2009 for *Chlamydia* (16%) (Table 1). Additionally, we
261 revealed a significant relationship between *Toxoplasma* infection status, roe deer age and capture
262 site: antibody prevalence was lower in site 9 and in juveniles compared to adults (Table S5, Figure
263 S2).

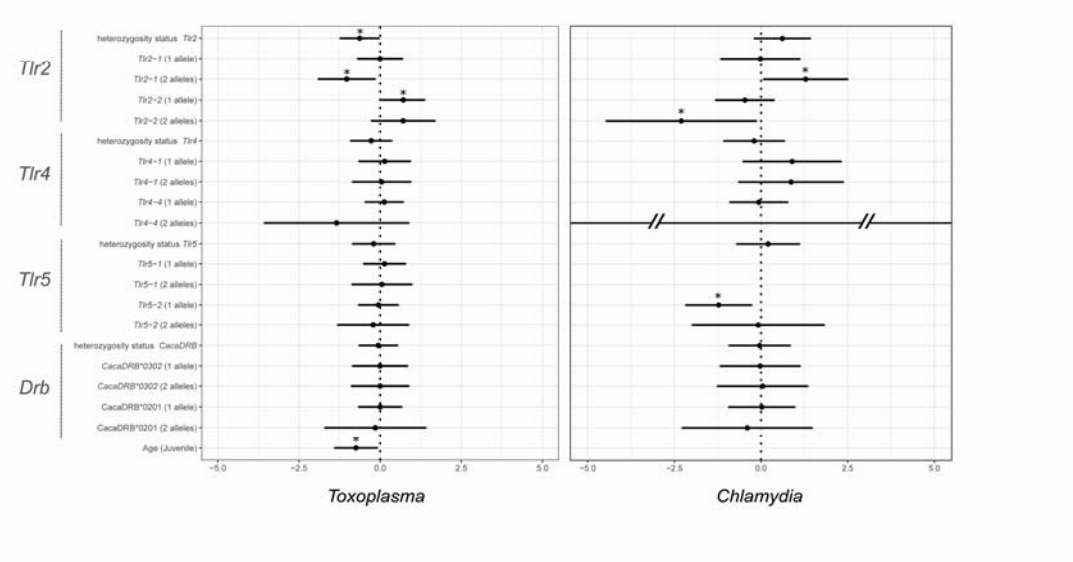
264 **3.3 Genetic effects on pathogen infection**

265 We revealed a strong relationship between *Toxoplasma* seroprevalence and the number of copies of
266 specific *Tlr2* haplotypes. Roe deer with two copies of the most frequent "TGCCG" haplotype (*Tlr2-1*)
267 showed a decreased *Toxoplasma* seroprevalence (model-averaged parameter estimates (AES) = -1.03
268 [95% CI -1.91, -0.14]) while individuals carrying the second most frequent haplotype ("CATCG", *Tlr2-*

269 *2*) were more likely to be *Toxoplasma* seropositive (Figure 1) (AES = 0.71 [0.04, 1.38]). We also found
270 a negative relationship between *Toxoplasma* seroprevalence and individual heterozygosity at *Tlr2*
271 gene (AES = -0.63 [-1.24, -0.02]). *Toxoplasma* seroprevalence was not associated with *Tlr4*, *Tlr5* or
272 *Mhc-Drb* genetic variation (neither heterozygosity status nor specific alleles).

273 Similarly to *Toxoplasma*, we found a strong relationship between *Tlr2* genotype and *Chlamydia*
274 seroprevalence. However, we observed the opposite pattern of association: homozygous individuals
275 for the most-common *Tlr2* haplotype (*Tlr2-1*) had an increased probability to be seropositive (AES =
276 1.28 [0.06, 2.51]) while individuals carrying *Tlr2-2* were less likely to be seropositive for *Chlamydia*
277 (AES = -2.30 [-4.48, -0.12] for "CATCG" homozygous deer). We also revealed an association between
278 *Tlr5* genotype and *Chlamydia* seroprevalence: roe deer with the "ACCTG" haplotype were less likely
279 to be Chlamydia seropositive (AES = -1.22 [-2.18, -0.25]). No association was observed when
280 considering *Mhc-Drb* and *Tlr4* genotypes (neither number of alleles nor heterozygosity status).

281 **Figure 1. Model averaged parameter estimates and their 95% confidence intervals for genetic (*Tlr2, Tlr4, Tlr5 and Mhc-*
282 ***DRB* genotype)** predictors associated with *Toxoplasma* and *Chlamydia* seroprevalence (predictors included in the top model
283 sets with ΔAic < 5). * indicate a parameter with a significant effect. //* indicates extremely wide 95% confidence intervals.



284

285 **4. DISCUSSION**

286 So far, very few studies, focusing on a limited number of taxa (most exclusively birds or rodents),
287 examined the mechanisms behind pathogen-mediated selective processes contemporarily acting on
288 innate immunity-related genes within natural populations (Grueber et al., 2013; Barbara Tschirren,
289 Råberg, & Westerdahl, 2011; Turner, Begon, Jackson, & Paterson, 2012). Being evolutionary ancient
290 and under strong functional constraints, genes involved in innate immune responses are expected to

291 evolve primarily under purifying selection and to exhibit limited polymorphism (Parham, 2003). Yet, it
292 is now quite clear that some of these genes, including *Tlr* genes encoding surface recognition
293 receptors, may have relaxed selective constraints and show large nucleotide diversity, sometimes
294 comparable to *Mhc* genes (Seabury et al., 2011). In a previous work, we have shown that the high
295 sequence and allelic diversity observed at roe deer *Tlr* genes could be maintained through balancing
296 selection (Quéméré et al., 2015). In this study, we developed a multi-gene/multi-pathogen
297 association approach to bring new insights into the mechanisms underlying such balancing selection.
298 Our results suggested a key role of directional selection on specific *Tlr* haplotypes rather than
299 heterozygote advantage. We showed that different *Tlr* genes may be associated with resistance to
300 the same microparasite (here *Tlr2* and *Tlr5* with *Chlamydia*) in agreement with the general idea that
301 pathogens interact with many immune recognition receptors. We also revealed that different
302 pathogens may act antagonistically on the polymorphism of a given gene (here *Tlr2* with *Toxoplasma*
303 and *Chlamydia*). Given the high temporal and spatial heterogeneity in pathogen exposure in the
304 study area, this supports the hypothesis that fluctuations in pathogen-mediated directional selection
305 may promote the maintenance of *Tlr* genetic diversity.

306 **4.1. High temporal and spatial variation in pathogen exposure**

307 Our results revealed a high temporal heterogeneity in both pathogen seroprevalence at individual
308 and population levels. In contrast with the traditional view of lifelong persistence of *Toxoplasma*
309 infection (Tenter, Heckeroth, & Weiss, 2000), we observed low individual repeatability of
310 *Toxoplasma* seroprevalence at the within-individual level (R=0.21). This is in line with a previous
311 study on the same population that reported frequent seroconversion with initially seropositive
312 individuals becoming seronegative within 1 to 3 years (Sevila et al., 2014). There is no clear
313 consensus on the persistence rate of antibodies against *T. gondii* in domestic and wild ungulates.
314 *Toxoplasma* seroprevalence is known to increase with age in wild ungulate populations, but it is most
315 likely due to repeated exposure from the same environment rather than antibody persistence
316 (Gotteland et al., 2014; Opsteegh, Swart, Fonville, Dekkers, & Van Der Giessen, 2011). Overall, this
317 suggests that the presence of antibodies in the study population would reflect relatively recent
318 rather than long-term infection. The observed annual variation in pathogen prevalence at population
319 level may be related to both climate and host factors. Climatic conditions such as temperature or
320 humidity are known to affect pathogen survival in the environment and may influence host
321 transmission *via* the quantity of infected aerosols for *Chlamydia* (Tang, 2009) or oocysts for
322 *Toxoplasma* (Gotteland et al., 2014). For example, Sevila et al. (2014) showed that *Toxoplasma*
323 prevalence in roe deer was higher in mild and wet years and that *Chlamydia* prevalence increased in
324 cold years. The high turnover of individuals in the population in relation with a strong harvest

325 pressure may also partly explain why we observed a so high inter-annual variability in multi-cohort
326 samples (Candela et al., 2014). Moreover, we also observed a strong variation in *Toxoplasma*
327 exposure across sampling sites that most likely results from habitat heterogeneity and land use
328 within roe deer's small home range (e.g. proportion of human dwellings within home range as a
329 proxy of the cat presence, Sevila et al., 2014).

330 **4.2 Predominant role of directional and fluctuating selection on *Tlr* gene variation**

331 We revealed that *Tlr2* gene polymorphism is predominantly shaped by directional selection exerted
332 by both *Toxoplasma* and *Chlamydia*. Roe deer carrying the most frequent *Tlr2* haplotype were less
333 likely to be infected by *Toxoplasma* but more likely infected by *Chlamydia*. The opposite pattern was
334 revealed for the second most frequent haplotype. These two abortive pathogens may have
335 significant impact on the annual reproductive success of ungulates (Pioz et al., 2008). TLRs have been
336 shown to activate the complement system, a component of innate immunity known to be important
337 for resistance to microparasites during early infection (Raby et al., 2011). A higher affinity of a *Tlr*
338 haplotype to *Toxoplasma* or *Chlamydia* ligands may result in a stronger complement response and
339 thus in an increased resistance to these pathogens (Tschirren et al., 2013).

340 Spatial and temporal variations in pathogen-mediated directional selection suggest that *Tlr*
341 haplotypes inheritance may vary among years and according to sites where females established their
342 home range. This supported the hypothesis that fluctuating selection is a key process in the
343 maintenance of *Tlr* genes' balanced polymorphism in this population. This finding is fully consistent
344 with the Simpsonian 'dynamic' Adaptive Landscape concept (Simpson, 1944) stating that genetic
345 variance and evolution of fitness characters in the wild are mainly shaped by spatial and temporal
346 fluctuations in selection, specially variation in biotic interactions (Calsbeek, Gosden, Kuchta, &
347 Svensson, 2012; Siepielski, DiBattista, & Carlson, 2009). This general concept can be easily applied to
348 host-pathogen interactions and immunity-related gene evolution. Different habitats in terms of biotic
349 and abiotic factors are likely to support distinct pathogen communities and so distinct pathogen-
350 mediated-selection regime (Eizaguirre & Lenz, 2010). Using simulation-based models, Hedrick (2002)
351 demonstrated that variation in space and time in pathogen resistance can in itself be a strong
352 mechanism for the maintenance of polymorphism at immunity-related genes. In agreement, several
353 empirical studies found that *Mhc* genetic diversity varies across space in a mosaic of habitats likely
354 due to differences in pathogen communities (Alcaide, Edwards, Negro, Serrano, & Tella, 2008;
355 Eizaguirre & Lenz, 2010; Landry & Bernatchez, 2001). We noted that few studies have convincingly
356 elucidated the ecological drivers of this immunogenetic variation and that all of them focused on
357 *Mhc* genes.

358 **4.2 Antagonistic pleiotropy and potential immunogenetic trade-off**

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

384 The most likely hypothesis is the presence of a host immunogenetic trade-off (Kamath et al.,
385 2014). In this case, pathogens do not directly compete but *TLr2* genotype has a direct effect on the
386 resistance/susceptibility to both pathogens. The allele inferred to be beneficial for decreasing
387 *Toxoplasma* infection is also associated with increased susceptibility to *Chlamydia* (and *vice versa*).
388 This scenario is reinforced by the fact that the two concurrent *Tlr2* haplotypes exhibit very distinct
389 DNA and amino-acid sequences, what suggests marked functional differences. Similar patterns and
390 processes have previously been described for ticks and nematodes infections (Kamath et al., 2014;

391 Turner et al., 2011). To our knowledge, our study is the second one showing evidence of antagonistic
392 selection at non-*Mhc* gene in a wild population (see for the other one Turner et al., 2011).

393 **4.3 Limited role of other genes and selective mechanisms**

394 While many empirical studies in a variety of organisms revealed signatures of pathogen-mediated
395 directional or balancing in *Mhc* loci (see Bernatchez & Landry, 2003; Sommer, 2005; Spurgin &
396 Richardson, 2010 for reviews), we did not find any association between *Mhc-Drb* and *Toxoplasma* or
397 *Chlamydia*. This is congruent with the findings of Quéméré et *al.* (2015), who showed clear evidence
398 of historical positive selection on *Mhc-Drb* in roe deer at the sequence level but no signature of
399 contemporary selection (in contrast to *Tlr* genes). This study suggests that genetic drift and migration
400 would the predominant contemporary forces shaping *Mhc* variation in roe deer. Moreover, the few
401 studies showing associations between MHC class II genetic diversity and pathogen infection in other
402 wild ungulates focused on strongyle parasites (Charbonnel & Pemberton, 2005; Kamath et al., 2014;
403 Paterson et al., 1998) while we looked at interactions with micro-pathogens. This supports the
404 general idea that MHC class II molecules principally bind exogenous antigens and are primarily
405 involved in the immune response to extracellular pathogens (Hughes & Yeager, 1998).

406 Our data provided only weak support for the "heterozygous advantage hypothesis". *Tlr2* is the only
407 gene that exhibited a relationship between heterozygosity status and *Toxoplasma* seroprevalence.
408 However, this pattern most likely resulted from the correlation between *Tlr2* heterozygosity and the
409 number of copies of *Tlr2-2* haplotype (Pearson's r = 0.3; P-value <0.001), this later being strongly
410 associated with *Toxoplasma* infection status. This illustrates the complexity of disentangling different
411 mechanisms underlying pathogen-mediated selection (Sin et al., 2014a; Spurgin & Richardson, 2010).
412 We noted that the "heterozygous advantage hypothesis" only received a limited support when
413 authors focused on a single pathogen species (but see Oliver et al., 2009), while a growing number of
414 case studies has revealed *Mhc* heterozygous advantage in animals co-infected with multiple
415 pathogens (Hughes & Nei, 1992; McClelland et al., 2003; Froeschke & Sommer, 2012). In our case,
416 the lack of association with roe deer heterozygosity status could be due to the low individual's
417 probability of being exposed to both pathogens *Toxoplasma* and *Chlamydia* across their life-time,
418 explaining the low fitness benefit of heterozygous genotypes. A limitation of this work is that we
419 could not investigate the importance of other mechanisms such as negative frequency-dependent
420 selection, because of insufficient statistical power to test the role of rare variants and the lack of long
421 time series (see Charbonnel et al., 2005 for an example).

422 **5. Conclusion**

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

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Data Accessibility:

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

Authors' contributions

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

Tables

Table 1 - Summary of the pathogen data (number of samples per year in brackets). NA indicates the years for 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

Supporting information

Table S1. Details of primer sequences, product size and Genbank accessions.

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

691 **Table S3**. Sample size per year and site for *Toxoplasma* and *Chlamydia*.

692 **Table S4**. Full list of models tested for genetic association with *Chlamydia* and *Toxoplasma*.

693 **Table S5.** Prevalence of Toxoplasma and Chlamydia in the seven sizes.

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

695 **Figure S2.** Model averaged parameter estimates and their 95% confidence intervals for biological and
696 environmental factors