DR. ANTONIO VAZQUEZ (Orcid ID : 0000-0002-0894-1708)

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Genetic diversity and relationships of the liver fluke *Fasciola hepatica* (Trematoda) with native and introduced definitive and intermediate hosts

Running tittle: F. hepatica: genetic and infective diversity

Antonio A. Vázquez[1,2,a]*, Emeline Sabourin[1,a], Pilar Alda[1,3], Clémentine Leroy[1], Carole Leray[4], Eric Carron[1], Stephen Mulero[1,5], Céline Caty[4], Sarah Hasfia[1], Michel Boisseau[1], Lucas Saugné[1], Olivier Pineau[4], Thomas Blanchon[4], Annia Alba[2,6], Dominique Faugère[1], Marion Vittecoq[1,4], Sylvie Hurtrez-Boussès[1,7]

[1] MIVEGEC, University of Montpellier, CNRS, IRD, Montpellier, France

[2] Laboratory of Malacology, Institute of Tropical Medicine "Pedro Kourí", Havana, Cuba

[3] Laboratorio de Zoología de Invertebrados I, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, Bahía Blanca, Buenos Aires, Argentina

[4] Institut de Recherche de la Tour du Valat, Arles, France

[5] IHPE UMR 5244, University of Perpignan, Perpignan, France

[6] University of Corse-Pascal-Paoli, Corte, Corsica, France

[7] Département de Biologie-Ecologie, Faculté des Sciences, Université de Montpellier, Montpellier, France

[a] equal contribution

* Corresponding author: Antonio A. Vázquez (applesnail@hotmail.fr)

Summary

Fasciolosis is a worldwide spread parasitosis mainly caused by the trematode *Fasciola hepatica*. This disease is particularly important for public health in tropical regions but it can also affect the economies of many developed countries due to large infections in domestic animals. Although several studies have tried to understand the transmission by studying the prevalence of different host species, only a few have used population genetics approaches to understand the links between domestic and wildlife infections. Here we present the results of such genetic approach combined with classical parasitological data (prevalence and intensity) by studying domestic and wild definitive hosts from Camargue (southern France) where fasciolosis is considered as a problem. We found 60% of domestic hosts (cattle) infected with *F. hepatica* but lower values in wild hosts (nutria, 19%; wild boars, 4.5%). We explored nine variable microsatellite loci for 1,148 adult flukes recovered from four different populations (non-treated cattle, treated cattle, nutria and wild boars). Populations from the four groups differed, though we found a number of migrants particularly non-treated cattle and nutria. Overall, we detected 729 different multilocus genotypes (from 783 completely genotyped individuals) and only 46 genotypes repeated across samples. Finally, we experimentally infected native and introduced intermediate snail hosts to explore their compatibility with *F. hepatica* and assess the risks of fasciolosis expansion in the region. The introduced species *G. truncatula* and *P. columella* attained the higher values of overall compatibility in relation to the European species. However, concerning the origin, sympatric combinations of *G. truncatula* were more compatible (higher prevalence, intensity and survival) than the allopatric tested. According to our results, we should note that the assessment of epidemiological risks cannot be limited to a single host-parasite system, but should focus on understanding the diversity of hosts in the heterogeneous environment through space and time.

Key words

Fasciolosis; domestic and wildlife infection; population genetics; lymnaeid snails

1. Introduction

Among the trematode species affecting domestic and wild mammals with major impacts in human populations worldwide, the liver fluke *Fasciola hepatica* displays the largest known altitudinal and latitudinal distribution (Mas-Coma, Bargues, & Valero, 2018). This parasite presents a heteroxenous life cycle including freshwater snails of the family Lymnaeidae as intermediate hosts (around 30 verified species; review in Correa et al., 2010; Vázquez et al., 2018) and a wide range of mammals as definitive hosts (Hurtrez-Boussès, Meunier, Durand, & Renaud, 2001). At a global scale, *F. hepatica* is responsible for fasciolosis, a food and waterborne disease affecting an estimate range between 35 to 72 million people annually and over 180 million at risk of infection (Nyndo & Lukambagire, 2015). Yet, it remains among the most Neglected Tropical Disease (WHO, 2007). This disease is also prevalent in temperate countries such as France where several outbreaks are usually linked to the consumption of contaminated watercress (Mailles et al., 2006)from cattle-rearing areas (Rondelaud, Dreyfuss, Bouteille, & Dardé, 2000). At veterinary level, fasciolosis infects around 600 million domestic animals and directly affects the national economies with about US $3 billion losses annually worldwide (Khan et al., 2013; Toet, Piedrafita, & Spithill, 2014). Although wild species are much less studied, some of them may maintain the circulation in non-domestic and domestic environments (e.g. Wild Red Deer and Wild Nutria: French et al., 2016; Kim, Kong, Kim, Yeon, & Hong, 2018). In any case, the risk of fasciolosis transmission usually relates to areas with some degree of anthropic incidence (*e.g.* management of hydrographic networks, husbandry practices using sensible breeds, introduction of exotic species, etc.; Sabourin, Alda, Vázquez, Hurtrez-Boussès, & Vittecoq, 2018). Such facts bring forward the need of knowing precisely the infective capacity of the circulating strains and the hosts they infect along with the circumstances that actually promote their infection.

For instance, the analysis of the population genetic structure of parasites helps to understand their circulation among their hosts (de Meeûs et al., 2007). Recent genetics studies using microsatellites markers on *F. hepatica* from different regions present most populations as highly diverse (Beesley, Williams, Paterson, & Hodgkinson, 2017; Cwiklinski, Allen, LaCourse, Williams, & Paterson, 2015). These results are usually explained through different hypothesis: (1) *F.*

hepatica is hermaphroditic but adults usually reproduce by cross-fertilization (Hurtrez-Boussès et al., 2004); (2) flukes infecting domestic animals (particularly bovines) are passively introduced into distant regions because of poor cattle managerial activities (Vázquez et al., 2016); and (3) introduction of non-native intermediate and definitive hosts in natural environments may prompt the transmission bridging the infecting wildlife genotypes with common domestic flukes (Beesley et al., 2017). Although there has been a handful of studies addressing *F. hepatica* variability within domestic animals (Beesley et al., 2017; Vázquez et al., 2016; Vilas, Vázquez-Prieto, & Paniagua, 2012), to our knowledge none has considered a detailed analysis of its diversity within wild hosts. Here we propose a study that allows to explore precisely how the dynamics of a host-parasite system may rapidly evolve from a natural complexity (heteroxenous life cycle, multiple hosts, combination of sexual and asexual reproduction according to the host) increasing through local human activities (cattle management and introduction of invasive species).

Understanding host-parasite relationships is fundamental in the attempt of preventing and controlling the transmission of infection diseases (Hawley & Altizer, 2011). However, laboratory approaches can only approximate reality to a certain point (*e.g.* revealing alleles or genotypes will rely upon the number of sampled individuals and the random probability of having higher or lower diversity). Moreover, field studies oblige to deal with some undesirable constraints (*e.g.* finding infected snails has proved to be challenging due to usually low prevalence; Sabourin, Alda, Vázquez, Hurtrez-Boussès, & Vittecoq, 2018; Vázquez, Sánchez, Alba, Pointier, & Hurtrez-Boussès, 2015). In an attempt to have a closer glance to the genetics and infection patterns in this system, we have combined field and experimental approaches in order to study different intermediate and definitive hosts susceptible to *F. hepatica.* To this end, we carried out our study within the Camargue Regional Park. In this region, fasciolosis transmission is long reported (de Rivière, 1826) and largely affected by human activities (PNRC, 2014), and thus, ideal to the study of this system. The objective is to provide a better knowledge on the epidemiology of *F. hepatica* in wild and domestic animals and its ability to infect different lymnaeid species. We characterized the population genetic structure of the parasite within each host using microsatellites markers in order to unveil the potential roles of each species as reservoirs and disseminators. Finally, we conducted experimental infections to test the compatibility of the circulating strain of *F. hepatica*

with some native and introduced lymnaeid species and to assess the risks of transmission beyond the study area.

2. Materials and Methods

2.1. Study area and F. hepatica sampling in domestic and wild definitive hosts

The study was carried out in the domain of Tour du Valat (TdV), a territory within the Regional Natural Park of Camargue (Provence-Alpes-Côte d'Azur region, France) including lands classified as Regional Natural Reserve (Figure 1). The domain covers about 2,650 ha intended for the study and protection of wetlands including the typical natural habitats of Camargue such as temporary marshes, dunes and extensive halophile meadows. Although being a protected area, the territory have some managed resources activities including cattle farming. Cattle in this place belongs to the rustic race "Raço di Biòu" (*Bos taurus*) adapted to the harsh conditions of Camargue. Because of the natural reserve regulations, the use of anthelmintic drugs definitively stopped in 2005 in all animals belonging to the TdV herd (hereafter considered as 'non-treated cattle'). However, some private cattle farms rent the surrounding lands off the limits of the Natural Reserve within TdV and use the flukicide Nitroxinil as anthelmintic drug (hereafter considered as 'treated cattle'). Treatment in this case occurs every once a year in November/December. Among wild mammals, the domain harbors the native wild boar (*Sus scrofa*) and the introduced nutria (*Myocastor coypus*). Both species are known to be susceptible to *F. hepatica* infection (Ménard et al., 2001; Mezo et al., 2013).

We explored the infection with *F. hepatica* in the domestic and the two wild species from 2013 to 2019. Cattle from TdV (non-treated, n = 210) and surrounding farms (treated, n = 15) were slaughtered for meat consumption in the nearby abattoirs of Tarascon (Bouches-du-Rhône) and Pézenas (Hérault). Veterinary authorities kindly allowed us to exhaustively dissect the livers and sample adult flukes from recently slaughtered cattle strictly following the particular hygiene and best practices guidelines of the abattoir. Liver examination was performed by cutting the whole liver in 2 cm perpendicular slices and pressing the biliary ducts. Concomitantly with liver examination, the content of the bile vesicle was examined (see exact methodology in section 2.3) and either recovering the adults from the ducts or the eggs from the bile confirmed the prevalence. Wild boar samples (n = 156) were kindly donated by private hunters during the

hunting season whereas nutrias (n = 42) were obtained through the conservation program carried out to reduce this invasive species in the area (order approving the departmental hunting management scheme for Bouches-du-Rhône, 2014 –available at: http://www.bouches-du-rhone.gouv.fr/content/download/9416/57472/file/ARRETE_SDGC.pdf). As with cattle, all received wild individuals were also thoroughly dissected and their liver examined following the same methodology as in cattle. All liver-infecting trematodes were recovered and stored in ethanol 80% for further molecular analysis. Parasite prevalence and intensities were noted following Reiczigel, Marozzi, Fábián, and Rózsa (2019). Data on sex and age were also recorded for non-treated cattle from TdV herd.

2.2. Molecular studies of F. hepatica *(DNA extraction, microsatellite amplification, genotyping and data analysis)*

We used a random sample of adult flukes from each definitive host (range 1–30 upon availability according to individual intensities) for molecular studies in an attempt to include a maximum number of infected individuals. A small piece of tissue (2 mm²) from the posterior end of each adult *F. hepatica* (n = 1,148) was used for DNA extraction using the Chelex extraction technique adapted for 96-well plates after Estoup and Martin (1996). Briefly, the tissue was dried and placed in a well (one well per individual) containing a mix of 5 µL of proteinase K (Promega) and 100 µL of 5% Chelex® (Bio-Rad Laboratories, California, USA). The plates were vortexed and incubated overnight at 56 °C followed by vortex again and incubated 10 min at 95 °C. Afterwards, the plates were centrifuged at 10,000 x *g* for 5 min and the supernatant containing the DNA was collected and stored at -20 °C until use.

Individual DNA extracts were diluted 1:10 for PCR amplification of nine microsatellite loci following the protocols of Hurtrez-Boussès et al. (2004) and Cwiklinski et al. (2015). Used markers (GenBank accession numbers) were FH15 (AJ508371), FH25 (AJ508373), FH222CBP (AJ003821), Fh_2 (LN627942), Fh_5 (LN627876), Fh_6 (LN629193), Fh_7 (LN635535), Fh_10 (LN628015) and Fh_12 (LN628360). Each locus was amplified using 1 µL of diluted DNA (1:10) in a 10 µL final reaction volume containing 2 µL buffer 5X (Promega, Wisconsin, USA), 1.2 µL 25 mM $MgCl_2$, 2 µL 2 mM deoxynucleoside triphosphates (dNTPs) (Invitrogen/Life Technology, Massachusetts, USA), 1 µL of each primer (10 pmol) and 0.2 µL of 1U Taq DNA polymerase (Promega). Thermocycling

was performed in a 96-well MJ-Research PTC 100 (MJ Research, California, USA) and consisted of an initial denaturation at 94 °C for 4 min; 30 cycles of 94 °C for 35 s, annealing temperature (FH15 = 48°C; Fh_2, Fh_5, Fh_6, Fh_7, Fh_10, FH222CBP = 55 °C; FH25 = 57 °C; Fh_12 = 60 °C) for 30 s, and 30 s at 72 °C; and a final elongation step of 72 °C for 10 min. Primers were fluorescently labelled to be used in an ABI automated sequencer (ABI-Prism 310 Genetic Analyzer, Applied Biosystems, Perkin-Elmer, California, USA). Each PCR product was diluted 1:100 and 1 µL was used to prepare a mix containing 0.5 µL of internal size standards (GENESCAN 500 LIZ, Applera France) and 15 µL of Hi-Di Formamide (20 µL qsp) for the automated electrophoreses. All allele lengths were read using GeneMapper® v. 4.0 software (Applied Biosystems, California, USA).

We estimated current parameters of population genetics such as mean number of alleles (A_r), observed (H_O) and expected (H_E) heterozygosities, departure from Hardy-Weinberg equilibrium (F_{IS}) and pairwise differentiation between populations (F_{ST}). We considered all individuals sampled within a particular host group (non-treated cattle, treated cattle, nutria and wild boar) as different *F. hepatica* populations. Estimations were computed using the software FSTAT v2.9.3.2 (Goudet, 2001) and Bonferroni corrections were applied for multiple tests (Rice, 1989). We identified identical multilocus genotypes (MLGTs) only in flukes with all nine loci amplified and tested the probability of observing *n* copies of a given MLGT as the result of random mating (P_{sex} values) using RCLone (Bailleul, Stoeckel, & Arnaud-Haond, 2016). Individuals with statistically significant P_{sex} values ($P < 0.05$) were considered the result of clonal amplification (Vilas et al., 2012). Genotypic diversity (*D*) was also calculated using the Simpson index. The average number of migrants between populations (N_m) was estimated with GENEPOP v. 4.2.1 (Rousset, 2008) using the private allele (A_{priv}) method developed by (Slatkin, 1985) for which parasites were grouped by definitive hosts. We used a Bayesian algorithm to determine the most appropriate number of genetic clusters (*K*). Twenty independent iterations of 200 000 burn-in length were followed by 100 000 Markov chain Monte Carlo repeats at each level for *K* = 1–30. Δ*K* was determined using the method proposed by Evano, Regnaut, and Goudet (2005) computed in Structure Harvester (Earl & vonHoldt, 2012). We performed a discriminant analysis of principal components (DAPC) using the package *adegent* in R (Jombart, Devillard, & Balloux, 2010) to explore the relatedness of MLGTs observed in each population *F. hepatica*. All MLGTs

were reduced to one instance for the DAPC analysis and for the construction of the genetic distance matrix.

2.3. Experimental infection of lymnaeid snails

Six populations of four lymnaeid species (two introduced and two native in Europe; see details in table 1) were exposed to the circulating *F. hepatica* isolate from TdV collected during the 2019 campaign (Camargue) and served as experimental models.

From these field populations, we reared two successive generations in the laboratory using plastic cages. Snails were fed with boiled minced lettuce *ad libitum* and maintained at constant temperature (22°C) and relative humidity (55%) conditions. Three-week old snails were used for experimental infections. A pool of *F. hepatica* eggs from non-treated cattle (n = 25) was selected to obtain miracidia (the larvae that infect the intermediate host) to expose the snails as it resulted the most genetically diverse parasite isolate (see section 3.2). Briefly, we collected the content of the bile vesicle from cattle obtained in the abattoir (see section 2.1) for mass egg retrieval. The bile was sift using pore size-decreasing set of sieves and continuous washing with saline solution (0.85%) to isolate the eggs that were stored in tubes containing saline solution at 4 °C in the dark until use (Vázquez et al., 2019). When the snails were one-week old, we changed the eggs to spring water and incubated for 15 days in darkness at 27 °C. The experimental infection methodology was followed as in Vázquez et al. (2019) individually exposing 30 snails of each population to a dose of five *F. hepatica* miracidia.

Mortality was recorded daily. All snails that died after the first week post-exposure were dissected and those individuals that showed the intramolluscan stage of *F. hepatica*, the rediae, were marked as infected. Those snails dying during the first week post-exposure were considered as non-infected since the early stages are very difficult to detect and would introduce uncontrollable bias. After 30 days post-exposure all remaining snails were dissected under a stereoscope following Caron, Rondelaud, and Losson (2008) and all living *F. hepatica* rediae (irrespective of their size or generation; Rondelaud, Belfaiza, Vignoles, Moncef, & Dreyfuss, 2009) were counted. We kept 30 individuals of each population unexposed but submitted to the same experimental manipulation as negative controls. Compatibility was evaluated using three parasitological variables according to Reiczigel et al. (2019): (1) prevalence (%) *i.e.* proportion of

infected individuals from all the exposed within the host sample; (2) mean parasite intensity as the average of intensity values –rediae/snail– calculated for a sample with 95% confidence intervals (CI); and (3) survival (%) as the percentage of alive individuals in the sample (exposed or unexposed population). To that end, a one-way ANOVA followed by a post hoc Tukey test was used to compare parasite intensities among trials after normality and homogeneity of variance were verified by the Shapiro-Wilk and Levene tests. Differences in survivorship among trials were statistically assessed through a log-rank test of Kaplan-Meier curves built from day 0 to day 30 post-exposure. All statistical tests were performed in Statistica v.12 (StatSoft. Inc., Tulsa, OK, USA 2014) and differences were considered significant at values of $P < 0.05$.

3. Results

3.1. Trematode liver infections in domestic and wild definitive hosts

The total prevalence of *F. hepatica* in cattle (domestic definitive hosts; N = 225) was 60% but with higher values in non-treated cattle (85%; mean intensity 15.7 ± 2.3 CI) than in treated cattle (67%; mean intensity 16.3 ± 7.4 CI), although non-significant (Chi-square = 3.36, $P = 0.06$) (Table 2). Intensities among all combinations of non-treated (young/adult/male/female) and treated cattle (ANOVA results: $F = 0.583$; $P = 0.67$) were not significant. In non-treated cattle, prevalence was higher in females (91.6%) than in males (81%) (Chi-square = 5.06, $P = 0.02$). Both sexes combined, prevalence in young individuals (1-3 years old) were higher than in adults (Chi-square = 4.03, $P = 0.04$). We observed a slight trend of increasing intensity with age but it was non-significant ($P = 0.24$). Prevalence was lower in wild definitive hosts with 19% in nutria (N = 42) and 4.5% in wild boars (N = 156) (Table 2). During liver dissection, we also noted the infection with the trematode *Dicrocoelium dendriticum* (Lancet liver fluke) in some individuals from all examined host species. Overall *D. dendriticum* prevalence was higher in non-treated cattle (76%) and nutria (50%) compared to treated cattle (25%) and wild boars (11%). Co-infection occurred in cattle (non-treated, 70%; treated 25%) and in nutria (38%) but it was not detected in wild boars.

3.2. Genetic diversity and population structure of F. hepatica from definitive hosts

We attempted the genetic characterization at nine microsatellite loci for 1,148 recovered adult liver flukes from non-treated cattle (949), treated cattle (100), nutria (81) and wild boar (18). The characterization of all examined loci among the four definitive host species of *F. hepatica* from

TdV is presented in Table 3. We did not find significant associations (Spearman correlation) between the number of analyzed flukes and the number of alleles per locus for all loci among the parasites found in the definitive host species.

Overall, all loci resulted polymorphic with non-significant deviations from the Hardy–Weinberg equilibrium in any of the examined groups of hosts (Table 4). Globally, *F. hepatica* populations from domestic hosts presented significantly higher values of allelic richness (5.7 alleles per locus; range: 4–25 alleles) and expected heterozygosity ($P < 0.05$) than those from wild hosts (mean: 5 alleles per locus; range: 2 – 19) ($P < 0.001$, Wilcoxon rank sum test). The populations from the four definitive host species shared 42 alleles (32%, N = 131). However, private alleles (A_{priv}) were observed only in cattle populations (Table 4). These unique alleles attained a maximum of six per locus.

3.3. Genetic differentiation in F. hepatica

All populations from the four definitive host species appeared slightly differentiated but still differed significantly (overall $F_{ST} = 0.024 \pm 0.013$ SD; $P < 0.05$). Actually, we detected a number of migrants among populations (mean 13.9 ± 7.4) particularly between non-treated cattle and nutria (Table 5).

Overall, we found 729 different MLGTs among 783 liver flukes with the nine loci amplified and 47 MLGTs repeated across the sample. However, the number of MLGTs found in each definitive host species was dependent of the sample size (Pearson correlation $r = 1$, $P < 0.001$). All identical MLGTs were the result of clonal amplification ($P_{sex} < 0.05$) with a global high genotypic diversity (Table 5). We found 41 MLGTs repeated in two flukes, five MLGTs repeated in three flukes and only 1 MLGT repeated in four flukes. Overall, 21 MLGTs were shared by different host individuals but only in non-treated cattle and five were recovered at different years (2014-2015). None MLGT was found shared by different host species.

The ΔK values were very low indicating low structuration among the analyzed flukes but still three clusters ($K = 3$) were found. To gain deeper insight into the genetic structure of *F. hepatica* populations and obtain a clearer view of the genotypic variation, we performed a DAPC that identifies clusters of genetically related individuals only for individuals with the nine loci amplified. Although most MLGTs are closely related we still are able to observe three clusters

separating treated cattle, non-treated cattle/nutrias and wild boars. MLGTs from nutrias and non-treated cattle could not be differentiated (Figure. 2), however, it should be noted that only individuals with all nine loci amplified were kept for this analysis.

3.4. Infection of different lymnaeid species with F. hepatica

All four snail species and populations were susceptible to *F. hepatica* infection (Figure 3A). Overall, the introduced species presented higher values of prevalence (*G. truncatula* from TdV, 70%; *P. columella*, 57%) than native lymnaeids. The allopatric population of *G. truncatula* from Rieu Massel attained 30% prevalence with lower values of parasite intensities and survival than the rest of analyzed populations. Overall, parasite intensities were significantly different (ANOVA results: F = 20.3; P < 0.001), particularly *G. truncatula* from Pesquier (TdV) with the highest values (mean 36 ± 6 CI) (Figure 3B). However, similar values were observed among *P. columella*, *L. stagnalis* and *G. truncatula* (Anciennes Vignes, TdV) after the *post hoc* Tukey test. The species with the lowest values of prevalence and parasite intensities was *R. balthica*. Survival was globally higher than 60% (Figure 3C) for most species and populations, reaching 100% in *G. truncatula* from TdV. Only *G. truncatula* from Rieu Massel attained low survival rate (13.3%). All control groups attained zero mortality (data not shown).

4. Discussion

4.1. High transmission of liver trematodes in domestic and wild definitive hosts in TdV

The overall prevalence of *F. hepatica* in cattle from TdV (>60%) is considerably higher than in other regions such as central France (13% to 25%; Mage, Bourgne, Toullieu, Rondelaud, & Dreyfuss, 2002) but similar to certain endemic regions elsewhere (Nepal >70%; Yadav, Ahaduzzaman, Sarker, Sayeed, & MA, 2015; Cuba >70%; Vázquez et al., 2016; Zambia >60%, Nyirenda et al., 2019). The absence of differences in prevalence or intensity between treated and non-treated cattle may suggest a failure of grazing management and/or treatment strategy by either wrong appliances or the existence of resistance to the used flukicide Nitroxinil. In this region, cattle is treated once a year in November-December (breeder's communication) and the exposure to the fluke would be potentially all-year-round. Given that flukes from the non-treated herd were recovered usually in late December, a failure of the drug can be a plausible explanation. Elsewhere, Nitroxinil has been used successfully in Triclabendazole-resistant flocks

(Romero et al., 2019) but it is known to lower its efficacy in 7- to 9-weeks old liver fluke's juveniles (Martínez-Valladares et al., 2010).

Although the probability of infection may increase with age (*i.e.* definitive hosts have more time to encounter the parasite cysts in the field; Khan et al., 2013), we found no direct link between age and parasite intensity (calculated as the burden of flukes within a single host). Our non-significant trend (analyzed individuals ranged from 1 to 18 years old) could be understood as a similar risk of infection every year associated with a natural parasite turnover during the lifespan of the host (flukes usually live for less than 2-3 years in cattle). This maintained risk of infection could be supported by a very high prevalence of field-occurring cysts of *F. hepatica* supporting reinfection. Given that females and males graze together in the studied area, the proportionally more infected females could be due to a lower immune competence by stress during pregnancy and parturition (Spithill, Smooker, & Copeman, 1999), or by a differential pattern of grazing areas because of particular management practices in this region.

The infection observed in wild hosts was not negligible and agrees with studies elsewhere. Ménard et al. (2001) found an overall 8.7% prevalence in nutria from Loire-Atlantique (western France) although it increased up to 40% when nutria were sampled from areas containing farms with infected cattle. In the case of wild boars, a study from Galicia (Spain) recorded over 11% prevalence suggesting a role as secondary reservoir of fasciolosis (Mezo et al., 2013). In addition to cattle, both wildlife species are particularly interesting in fasciolosis epidemiology. Nutrias are, however, probably more relevant in maintaining fasciolosis in a given small area due to their territoriality (Carter & Leonard, 2002) and typical habitats that usually overlaps with those of intermediate hosts snails (*e.g.* irrigation and drainage channels). On the contrary, wild boars are known to use over 100 ha of effective territory (Boitani, Mattei, Nonis, & Corsi, 1994) and thus, would be able to use different non-connected biotopes. For instance, both species would be key players in disseminating different liver fluke's genotypes among intermediate hosts that could entangle the genetic background of the overall metapopulation. This hypothesis is supported by the fact that all alleles observed in both wild hosts were shared in domestic hosts, particularly between non-treated cattle and nutrias. Whatever the case may be, we should note that transmission in the studied area is mainly due to cattle (higher prevalence and genetic diversity)

and that they are generally responsible for raising the probability of infection in wildlife or eventually humans (Vázquez et al., 2016).

On the other hand, the observed co-infection between *F. hepatica* and *D. dendriticum* (*e.g.* 70% in non-treated cattle) is particularly interesting as both trematode species target the liver of definitive hosts. In fact, this particular co-infection event is commonly reported (Ducommun & Pfister, 1991; Khanjari et al., 2014). The observed prevalence in this study reveals that *D. dendriticum* is also highly prevalent in both domestic and wild mammals. Contrary to *F. hepatica*, *D. dendriticum* uses land snails and ants as first and second intermediate hosts, respectively, and the existence of humid conditions or lasting waterbodies is not needed for transmission to occur. Indeed, alkaline and carbonate-rich soils are the most important conditions for their intermediate hosts (Manga-González, González-Lanza, Cabanas, & Campo, 2001) whilst parasite eggs can last long periods in pastures (Taylor, 2012). These facts would suggest that the infection by *D. dendriticum* might be higher than that by *F. hepatica* and that it might affect the establishment of F. hepatica. Notwithstanding, this was not the case here and a plausibly explanation could be that the studied territory is a wetland harboring lymnaeid populations highly compatible with the local strains of *F. hepatica* (see section 3.4). In a potential scenario of strong interspecific competition inside the definitive hosts, *F. hepatica* seems able to resist and survive. Future studies should model the outcome of such competition by examining the intensities of both interacting trematode species since it could overall affect the transmission epidemiology.

4.2. High polymorphism in F. hepatica populations from different hosts

The allelic sizes found in this study fall within the reported range elsewhere (Cwiklinski et al., 2015; Hurtrez-Boussès et al., 2004). However, here we found less alleles per locus for most explored loci (except Fh_7) compared to those observed by Beesley et al. (2017). In Cuba, for example, where *F. hepatica* is endemic in cattle (Alba et al., 2016), a population genetic study using FH15, FH25 and FH222CBP markers from eight different cattle isolates, showed similar values with a mean of 7.5 alleles per locus and up to 30 alleles among the three loci (Vázquez et al., 2016). Other studies that have attempted to explore *F. hepatica* using haplotype diversity also show high variability. For instance, studies using Nad1 gene found 24 haplotypes from 79

individuals in Brazil (Schwantes, Quevedo, D'Avila, Molento, & Graichen, 2019) and 37 haplotypes from 130 individuals in Iran (Bozorgomid, Rouhani, Harandi, Ichikawa-Seki, & Raeghi, 2020).

We found an absence of deviations from the Hardy-Weinberg equilibrium and high genetic diversity of *F. hepatica* that could be explained by cross-fertilization among parasites and high migration rate thanks to definitive host movements. In fact, private alleles were only found in non-treated (14%) and treated (3%) cattle. In Spain, a study showed that structuration might occur between flukes from sheep and cattle (Vilas et al., 2012). Here, after analyzing three different host species we can only think of an established flow of parasites among them, particularly in non-treated cattle and nutrias that may explain the results observed in the DAPC analysis. In the case of the more evident differentiation between treated and non-treated cattle, we should keep in mind that although farms are adjacent to one another, cattle from both herds never mix. However, we still observe some overlaps indicating that genetic distances are not very high. This raises concerns on breeding and managing practices that may promote year-round infections and the encountering of distant flukes through passive dissemination by definitive hosts. Host movements may act as 'bridging agents' playing an important role in homogenizing the metapopulation of *F. hepatica*. As a result, the random emergence of resistant genes to anthelmintic treatment may mix in the metapopulation through these migration events and complicate the eventual control of fasciolosis. For instance, clonal expansion of resistant genotypes to the flukicide Triclabendazole within the snails have been highlighted in *G. truncatula* (Hodgkinson et al., 2018). Usually, important genetic bottlenecks are expected in populations routinely treated with anthelmintic (Vázquez et al., 2016) but here we still find a high genotypic diversity. It would be nevertheless interesting to develop tests aimed at exploring the existence of resistant genes to the different used anthelminthic drugs. Such genes would explain the prevalences observed in treated cattle or expose hazardous management of grazing patterns here and elsewhere.

4.3. Field transmission and risks of fasciolosis expansion in Camargue

We should note that 40 repeated genotypes were observed in cattle. Some of these genotypes were recovered not only from different host individuals but also from different years. Two

different explanations can be given: (1) *F. hepatica* may live at least over two years inside their mammal host (Takeuchi-Storm et al., 2018); and (2) metacercariae cysts are capable of resisting for several months before being ingested by the definitive hosts (Andrews, 1999). In either case, the field contamination with metacercarie should be sufficiently large to infect different individuals with the same MLGT.

Overall, we found 687 MLGTs in cattle of which 647 were recorded once. Although snails are susceptible to be infected with more than one miracidium (see Rondelaud, Vignoles, & Dreyfuss, 2004), if we accept, theoretically, that one MLGT equals one infected lymnaeid snail (infection more commonly occur by only one miracidium that will expand asexually), there should had been up to as many infected snails as observed MLGTs in the studied period. This result suggests the existence of multiple sources of field infection for the definitive hosts allowing for such high genotypic diversity. This is particularly important if we understand that natural prevalence in the snails are usually low (Dreyfuss, Vignoles, & Rondelaud, 2003; Mekroud, Benakhla, Vignoles, Rondelaud, & Dreyfuss, 2004; Vázquez et al., 2015). Susceptible populations of lymnaeid snails should then be scattered throughout the grazing areas at TdV and managerial activities should recognize the location of intermediate hosts populations as a predictor for fasciolosis infection.

Here, we proved that the isolate of *F. hepatica* from TdV is capable of infecting several species of lymnaeid snails. Overall, we found differences in compatibility regarding their susceptibility, capacity of the parasite to expand inside the snails and survival of the latter after the exposure. The compatibility showed by sympatric *G. truncatula* is in agreement with the existence of local adaptation in this system (Vázquez et al., 2019). Inversely, the allopatric population of *G. truncatula* showed low compatibility with *F. hepatica*. This population was recovered from a non-transmission region in a commonly drought-floodable habitat (side route ditch). Genetic drift might have rendered those individuals highly incompatible through a continuous series of genetic bottlenecks that make them impossible to sustain the infection. Similar results were obtained by Dreyfuss, Vignoles, & Rondelaud (2012) when testing local adaptation in *G. truncatula* to *F. hepatica*.

Anyhow, although *G. truncatula* appears as the best suitable intermediate host (at least the sympatric populations), we should not disregard the results from the other species, even if we

did not allowed for cercarial shedding in our experiments. This is particularly the case of *P. columella*, an invasive species known for its ability of inserting itself into the epidemiological scenario of fasciolosis after recent incidental introductions worldwide (Lounnas et al., 2017). In a world-changing climate, conditions may shift to render more suitable the establishment of this species in southern Europe and consequently promote fasciolosis transmission. For instance, *P. columella* is already introduced into many artificial habitats in many European countries (Glöer, 2019) and has already been found living in the wild in France (Pointier, Coustau, Rondelaud, & Théron, 2007). Moreover, native species such as those tested here (*L. stagnalis* and *R. balthica*) would be capable of facilitating the transmission in nearby areas not yet colonized by *G. truncatula* or *P. columella*. In fact, both lymnaeids species are known to transmit *F. hepatica* in several European countries (Vázquez et al., 2018).

4.4. Concluding remarks

These results highlight the importance of studying the epidemiology of an infectious trematode disease by taking into account the diversity of occurring definitive and intermediate hosts. Indeed, this kind of approach helps in the implementation of efficient integrated management programs to prevent and/or control the transmission. For instance, in the case of fasciolosis, human activity has proved to be a key factor in promoting the transmission (Sabourin, Alda, Vázquez, Hurtrez-Boussès, & Vittecoq, 2018). Herd density and grazing management coupled with topographic features and environmental conditions that favor the occurrence of snail hosts would influence prevalence and parasite intensity of *F. hepatica*. Thus, limiting access to high-risk pastures and implementing good anthelmintic treatments are key aspects in reducing the transmission in regions such as Camargue. In addition, addressing the epidemiological risks cannot be limited to a single host-parasite system, but should focus on understanding the diversity of hosts in the heterogeneous environment through space and time. The existing polymorphism of compatibility in the snail host and *F. hepatica* has revealed that different species may play different roles and that certain combinations are particularly dangerous (Vázquez et al., 2019). Although not very well studied, something similar may occur at the definitive host level. For example, rapid evolution can take place through human activities and the introduction of new host species or the replacement of rustic breeds may facilitate the

transmission. Surveillance is, therefore, mandatory if we are to meet the challenge of tackling the emergence or re-emergence of such infectious diseases.

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Conflict of interest

The authors declare that they have no competing interests.

Ethical Statement

The study was conducted in compliance with ethical standards.

Data availability statement

All data is available upon direct request to the authors.

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Table 1. Populations of lymnaeid snail species occurring in France used in the infection trials with the circulating strain of *Fasciola hepatica* from Tour du Valat (TdV), Camargue.

Code	Species	Status	Origin	Commune	Department	Relation to the *F. hepatica* isolate
Gt.AV[S]	*Galba truncatula*	introduced	Anciennes Vignes (43.5017°N; 4.6789°E)	Le Sambuc (TdV)	Bouches-du-Rhône	sympatric
Gt.PE[S]			Pesquier (43.4573°N; 4.6640°E)	Le Sambuc (TdV)	Bouches-du-Rhône	sympatric
Gt.RM[A]			Rieu Massel (43.6556°N; 3.8122°E)	Grabels	Hérault	allopatric
Pc.PZ[A]	*Pseudosuccinea columella*	introduced	Montpellier Zoo (43.6393°N; 3.8740°E)	Montpellier	Hérault	allopatric
Ls.BL[A]	*Lymnaea stagnalis*	native	Bagne Loup (43.8623°N; 3.9632°E)	Brouzet-lès-Quissac	Gard	allopatric
Rb.SJ[A]	*Radix balthica*	native	Saint Jean stream (43.6785°N; 3.7373°E)	Combaillaux	Hérault	allopatric

Table 2. Prevalence (number of examined hosts, n) and mean parasite intensity (± 95% confidence intervals, CI) of *Fasciola hepatica* for each definitive host from 2013 to 2019 in Tour du Valat.

Host species	% prevalence (n)								Mean Intensity (± 95% CI)
	2013	2014	2015	2016	2017	2018	2019	*Overall*	
Cattle (non-treated)									
young males	-	-	0 (1)	100 (2)	-	-	-	*66.6 (3)*	13.5 (± 4.9)
adult males	100 (6)	100 (11)	91.6 (24)	100 (4)	33.3 (18)	-	69.2 (13)	*81.6 (76)*	13.4 (± 3.6)
young females	100 (3)	-	100 (11)	100 (28)	100 (1)	-	66.6 (3)	*97.8 (46)*	17.3 (± 4.5)
adult females	100 (2)	-	88.9 (9)	96.8 (32)	81.8 (22)	-	70 (20)	*88.2 (85)*	16.6 (± 4)
Overall	*100 (11)*	*100 (11)*	*91.1 (45)*	*98.5 (66)*	*60.9 (41)*	-	*69.4 (36)*	*84.8 (210)*	*15.7 (± 2.3)*
Cattle (treated)	-	-	66.6 (15)	-	-	-	-	*66.6 (15)*	16.3 (± 7.4)
Nutria	16.6 (6)	20.8 (24)	0 (1)	14.3 (7)	25 (4)	-	-	*19 (42)*	3.2 (± 1.1)
Wild boar	2.9 (35)	-	0 (15)	4.8 (42)	6 (50)	7.1 (14)	-	*4.5 (156)*	6.6 (± 0.7)

Table 3. Observed size range and number of alleles of all studied microsatellite loci of *Fasciola hepatica.*

Locus	Overall allele size range (bp)	Alleles per locus	Allele size range by population (alleles per locus)							
			Cattle (non-treated)		Cattle (treated)		Nutria		Wild boar	
FH15	230–243	8	230–243	(8)	230–243	(8)	230–241	(6)	233–237	(3)
FH25	272–282	6	276–282	(4)	272–282	(6)	278–282	(3)	280–282	(2)
FH222CBP	144–172	14	144–172	(14)	144–166	(9)	150–166	(9)	154–166	(5)
Fh_2	179–239	15	179–239	(15)	183–239	(11)	183–231	(11)	183–215	(5)
Fh_5	159–321	26	159–321	(24)	159–318	(19)	162–318	(12)	162–209	(8)
Fh_6	165–250	25	165–250	(25)	174–244	(18)	174–244	(19)	177–229	(8)
Fh_7	163–193	11	163–193	(11)	163–190	(8)	163–187	(8)	163–184	(4)
Fh_10	201–238	13	201–238	(13)	206–235	(11)	201–232	(11)	209–223	(5)
Fh_12	199–240	13	199–240	(13)	199–237	(11)	199–240	(10)	199–240	(8)

Table 4. Mean number (± standard deviation) of alleles (A_r), observed (H_O) and expected (H_E) heterozygosities, F_{IS} values (level of significance after Bonferroni correction) and private alleles (A_{priv}) of *Fasciola hepatica* populations from different domestic and wild definitive hosts.

F. hepatica population	Analyzed flukes	A_r	H_O	H_E	F_{IS}	A_{priv}
Cattle (non-treated)	949	5.78 (±2.01)	0.66 (±0.26)	0.72 (±0.23)	0.082 (NS)	18 (13.7%)
Cattle (treated)	100	5.62 (±1.55)	0.68 (±0.23)	0.73 (±0.18)	0.061 (NS)	4 (3.05%)
Nutria	81	5.53 (±1.87)	0.69 (±0.24)	0.72 (±0.22)	0.032 (NS)	0 (0.00%)
Wild boar	18	4.51 (±1.58)	0.65 (±0.38)	0.65 (±0.26)	-0.011 (NS)	0 (0.00%)

Table 5. Number of multilocus genotypes (MLGTs), repeated MLGTs (all P_{sex} values for repeated MLGTs are significant at a minimum n = 2 individuals), genotypic diversity (*D*) and mean number of migrants (N_m ± standard deviation, SD) for all the studied populations of *Fasciola hepatica* (N is the number of analyzed flukes after excluding individuals with at least one non-amplified locus).
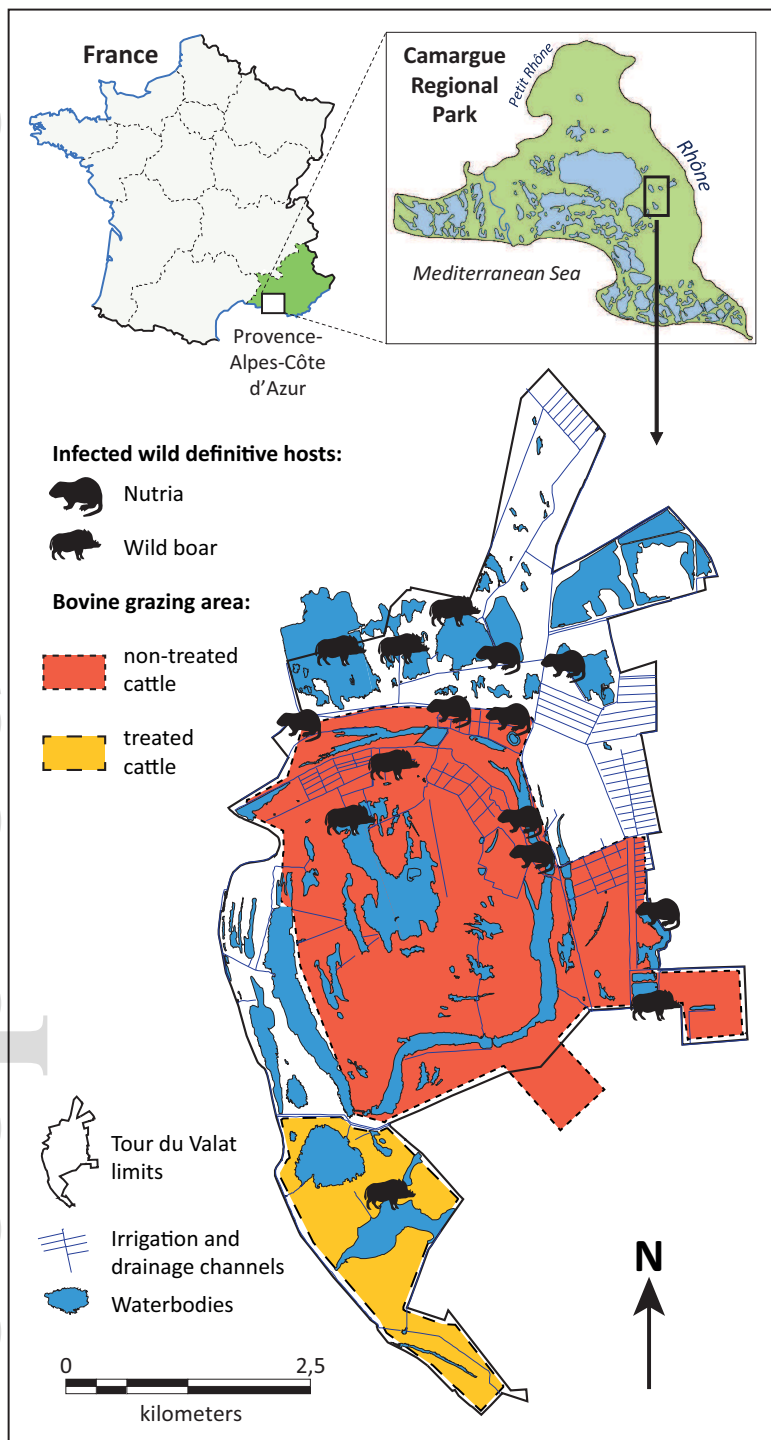
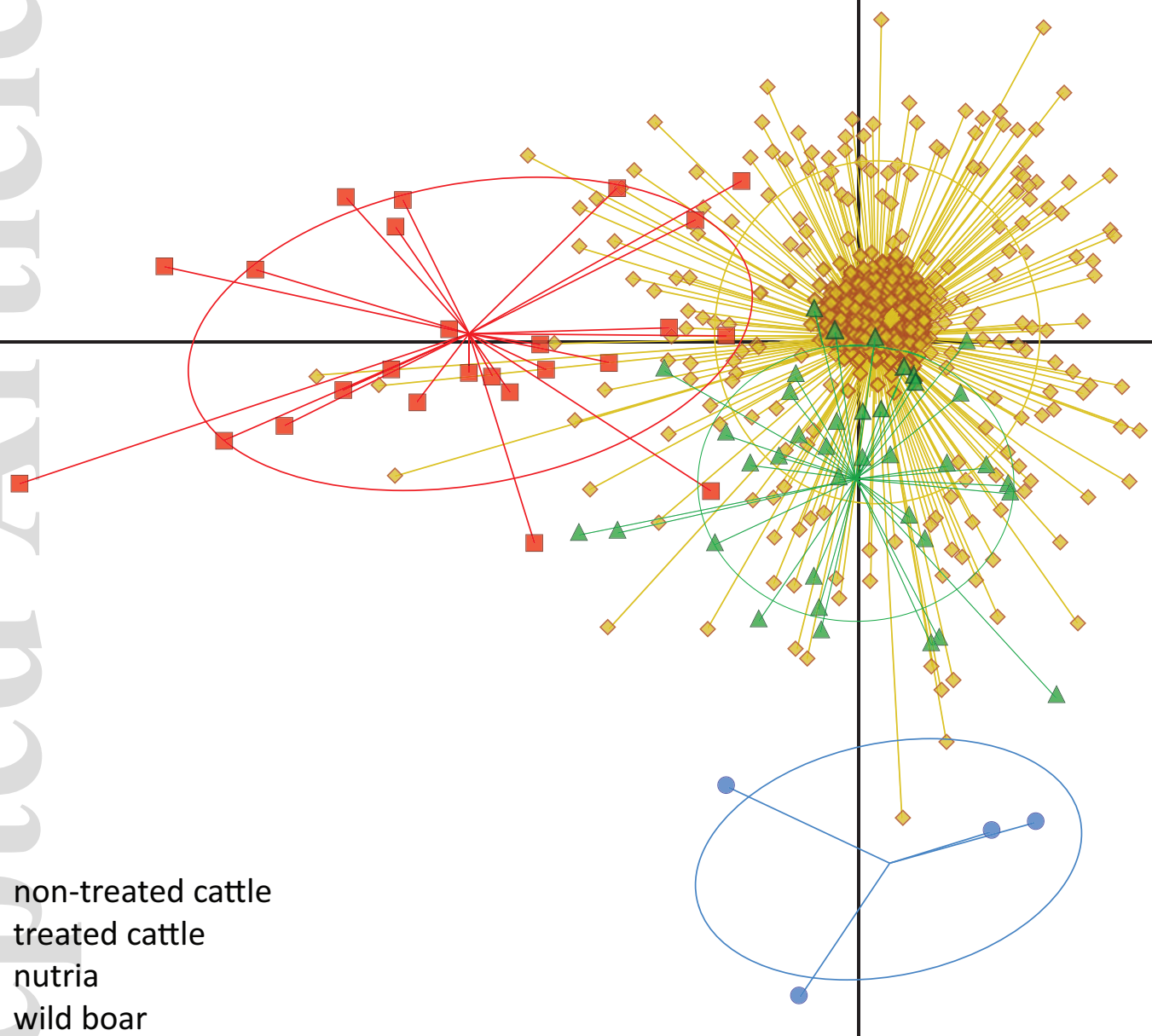| *F. hepatica* population	N	MLGTs (repeated)	*D*	N_m
Cattle (non-treated)	709	662 (40)	0.999	17.8 ± 7.1
Cattle (treated)	25	25 (0)	1.000	14.9 ± 6.7
Nutria	43	38 (5)	0.994	16.3 ± 7.2
Wild boar	6	4 (1)	0.800	6.8 ± 0.9

Figure Captions

Figure 1. Map of Tour du Valat within the Camargue Regional Park with details of cattle farms and sampled wild host infected with *Fasciola hepatica*.

Figure 2. Population genetic structure of *Fasciola hepatica* sampled from different definitive hosts groups. Scatterplot of multilocus genotypes within the first two axes of a Discriminant Analysis of Principal Components (axis 1 and axis 2 represent 37.52% and 27.3% of total variance).

Figure 3. Results of experimental infections of different lymnaeid species with *Fasciola hepatica* from non-treated cattle at Tour du Valat (Camargue). A: prevalence of infection, B: parasite intensity (different letters mean statistical differences after a one-way ANOVA and Tukey test), C: survival curves for each combination (all curves differed statistically except indicated, *NS*). Codes: *Gt=Galba truncatula*, *Pc=Pseudosuccinea columella*, *Ls=Lymnaea stagnalis*, *Rb=Radix balthica*; AV=Anciennes Vignes, PE=Pesquier, RM=Rieu Massel, PZ=Parc Zoologique, BL=Bagne Loup, SJ=Saint Jean; [A]=allopatric, [S]=sympatric.

France

Camargue Regional Park

Petit Rhône

Rhône

Mediterranean Sea

Provence-Alpes-Côte d'Azur

Infected wild definitive hosts:

Nutria

Wild boar

Bovine grazing area:

non-treated cattle

treated cattle

Tour du Valat limits

Irrigation and drainage channels

Waterbodies

0   2,5

kilometers

N

Legend:
- ◆ non-treated cattle
- ■ treated cattle
- ▲ nutria
- ● wild boar

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