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Jonas R. Etougbétché, Azra Hamidović, Henri-Joël Dossou, Maeva Coan-Grosso, Roxane Roques, et al.. Molecular prevalence, genetic characterization and patterns of *Toxoplasma gondii* infection in domestic small mammals from Cotonou, Benin. *Parasite*, 2022, 29, pp.58. 10.1051/parasite/2022058 . hal-03957367

HAL Id: hal-03957367

<https://hal.inrae.fr/hal-03957367>

Submitted on 26 Jan 2023



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Molecular prevalence, genetic characterization and patterns of *Toxoplasma gondii* infection in domestic small mammals from Cotonou, Benin

Jonas R. Etougbétché^{1,2,*} , Azra Hamidović³, Henri-Joël Dossou^{1,4}, Maeva Coan-Grosso³, Roxane Roques³, Nicolas Plault³, Gualbert Houéménou¹, Sylvestre Badou¹, Antoine A. Missihoun², Issaka Youssao Abdou Karim⁵, Lokman Galal³, Christophe Diagne⁶, Marie-Laure Dardé^{3,7}, Gauthier Dobigny^{6,8}, and Aurélien Mercier^{3,7,*} 

¹ Ecole Polytechnique d'Abomey-Calavi, Laboratoire de Recherche en Biologie Appliquée, Unité de Recherche sur les Invasions Biologiques, Université d'Abomey-Calavi, 01 BP 2009, Cotonou, Benin

² Laboratoire de Génétique Moléculaire et d'Analyse des Génomes, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, 01BP 526, Cotonou, Bénin

³ Inserm U1094, IRD U270, Univ. Limoges, CHU Limoges, EpiMaCT - Epidémiologie des maladies chroniques en zone tropicale, Institut d'Epidémiologie et de Neurologie Tropicale, Omega Health, 87000 Limoges, France

⁴ Institut du Cadre de Vie (ICaV), Université d'Abomey-Calavi, BP 2899, Abomey-Calavi, Benin

⁵ Ecole Polytechnique d'Abomey-Calavi, Laboratoire de Biotechnologie Animale et de Technologie des Viandes, Université d'Abomey-Calavi, 01 BP 2009, Cotonou, Benin

⁶ Institut de Recherche pour le Développement, UMR CBGP (IRD, INRA, Cirad, Montpellier SupAgro), Montpellier Université d'Excellence, 755 avenue du campus Agropolis, 34988 Montpellier-sur-Lez Cedex, France

⁷ Centre National de Référence (CNR) Toxoplasmose/*Toxoplasma* Biological Center (BRC), Centre Hospitalier-Universitaire Dupuytren, 87000 Limoges, France

⁸ Unité Peste, Institut Pasteur de Madagascar, BP 1274 Ambatofotsikely Avaradoha, 101 Antananarivo, Madagascar

Received 6 July 2022, Accepted 28 November 2022, Published online 21 December 2022

Abstract – Toxoplasmosis, one of the most prevalent parasitic infections in humans and animals, is caused by the intracellular protozoan parasite *Toxoplasma gondii*. Small mammals play a key role as intermediate reservoir hosts in the maintenance of the *T. gondii* life cycle. In this study, we estimated the molecular prevalence and provide genetic diversity data for *T. gondii* in 632 small mammals sampled in four areas of Cotonou city, Benin. Both the brain and heart of each individual were screened through *T. gondii*-targeting qPCR, and positive samples were then genotyped using a set of 15 *T. gondii*-specific microsatellites. Prevalence data were statistically analyzed in order to assess the relative impact of individual host characteristics, spatial distribution, composition of small mammal community, and urban landscape features. An overall *T. gondii* molecular prevalence of 15.2% was found and seven genotypes, all belonging to the *Africa 1* lineage, could be retrieved from the invasive black rat *Rattus rattus* and the native African giant shrew *Crocidura olivieri*. Statistical analyses did not suggest any significant influence of the environmental parameters used in this study. Rather, depending on the local context, *T. gondii* prevalence appeared to be associated either with black rat, shrew, or mouse abundance or with the trapping period. Overall, our results highlight the intricate relationships between biotic and abiotic factors involved in *T. gondii* epidemiology and suggest that *R. rattus* and *C. olivieri* are two competent reservoirs for the *Africa 1* lineage, a widespread lineage in tropical Africa and the predominant lineage in Benin.

Key words: *Toxoplasma gondii*, Small mammals, Parasite ecology, Infectious disease, Molecular epidemiology, Urban eco-epidemiology, Benin.

Résumé – Prévalence moléculaire, caractérisation génétique et schémas d'infection par *Toxoplasma gondii* chez les petits mammifères domestiques de Cotonou, Bénin. La toxoplasmose, l'une des infections parasitaires les plus répandues chez l'homme et les animaux, est causée par le parasite protozoaire intracellulaire *Toxoplasma gondii*. Les petits mammifères jouent un rôle clé en tant qu'hôtes réservoirs intermédiaires dans le maintien du cycle de vie de *T. gondii*. Dans cette étude, nous estimons sa prévalence moléculaire et fournissons des données sur sa diversité génétique chez 632 petits mammifères échantillonnés dans quatre localités de la ville de Cotonou. Le cerveau et le

Edited by Jean-Lou Justine.

*Corresponding authors: jonas.etougbetche@gmail.com; aurelien.mercier@unilim.fr

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cœur de chaque individu ont été analysés par qPCR ciblant *T. gondii*, et les échantillons positifs ont ensuite été génotypés à l'aide d'un ensemble de 15 microsatellites spécifiques à *T. gondii*. Les données de prévalence ont été analysées statistiquement afin d'évaluer l'impact relatif des caractéristiques individuelles de l'hôte, de la distribution spatiale, de la composition de la communauté des petits mammifères ainsi que des caractéristiques du paysage urbain. Une prévalence moléculaire globale de *T. gondii* de 15,2 % a été estimée et sept génotypes, tous appartenant à la lignée *Africa I*, ont pu être extraits du rat noir *Rattus rattus*, espèce envahissante, et de la musaraigne *Crocidura olivieri*, espèce indigène. Les analyses statistiques n'ont pas suggéré d'influence significative des paramètres environnementaux utilisés dans cette étude. Au contraire, selon le contexte local, la prévalence de *T. gondii* semble être associée à l'abondance de rats noirs, de musaraignes ou de souris ainsi qu'à la période de piégeage. Dans l'ensemble, nos résultats mettent en évidence les relations complexes entre les facteurs biotiques et abiotiques impliqués dans l'épidémiologie de *T. gondii* et suggèrent que *R. rattus* et *C. olivieri* sont deux réservoirs compétents pour la lignée *Africa I*, une lignée répandue en Afrique tropicale et prédominante au Bénin.

Introduction

Small mammals, including rodents, have an important epidemiological role in the evolutionary ecology and transmission cycle of *Toxoplasma gondii*, for which they act as natural reservoirs. They are one of the preferred prey groups of the definitive felid hosts (e.g., cats [14, 35]), and therefore play a key role in the circulation of *Toxoplasma* strains within local and novel environments [30, 42, 62, 67]. This is particularly true in the increasingly changing global context that favors the ongoing introduction and spread of invasive rodents [40, 41]. Accordingly, rodents are generally considered relevant hosts to assess environmental contamination by toxoplasmosis, and to estimate the infection risk for definitive hosts [1, 6, 67]. Nevertheless, considering rodents a meaningful indicator to predict environmental contamination by *T. gondii* requires us to differentiate between potential biological and ecological differences within and between reservoir rodent species. As an illustration, a positive relationship between rodent body mass and probability to be infected by *T. gondii* has been found both within (i.e., older individuals were more often infected [20, 67]) as well as between species (i.e., larger species had a longer life span, hence more opportunities to encounter *T. gondii* [2, 20, 46]). Species-specific ecological requirements have also been found to influence *T. gondii* prevalence, essentially due to variation in oocyst-rodent contact patterns [68, 81]. For example, fossorial species that live in burrows are always in contact with soil and likely include *T. gondii* paratenic hosts (such as earthworms, [2]) in their diet. In addition to biological and ecological species-level features, the local environment is expected to influence the persistence of oocysts within the external environment, thus greatly impacting parasite eco-epidemiology [81, 84].

Despite this burgeoning knowledge on the reservoir-based ecology of *T. gondii*, the effects of environmental, ecological, and host-individual factors on the circulation of *T. gondii* in natural populations of rodent reservoirs have been scarcely investigated in a concomitant way. Moreover, studies on the prevalence of toxoplasmosis in small mammals sampled in various parts of the world show great variability following region and habitat, host species, and detection method [30]. In Africa, most studies are based on serological detection of *T. gondii* and seroprevalence was found to be relatively low: e.g., 1.6% and 1.2% in *Mus musculus* and *Mastomys natalensis*, respectively in Niamey, Niger [58]; 2.3% and 4.2% in *Rattus rattus* from rural Gabon [57] and across Senegal [11],

respectively and 3.8% in *Rattus norvegicus* in Egypt [59]. However, such seroprevalence-based results have largely been questioned from several points of view, such as the intrinsic differences in host immune response [40] and the variable sensitivity/specificity of serological tests used [17, 30]. These limitations have therefore led to recognize qPCR-based approaches as more sensitive and reliable, since this method allows direct targeting of the parasite DNA in examined tissues [40].

Furthermore, while rodents, and specifically laboratory mice, are widely used to study toxoplasmosis in laboratory conditions (e.g., host immune response, parasite virulence and/or parasite amplification for subsequent genetic analyses), studies using the direct molecular approach for investigations in natural populations are largely lacking, especially in the African context as far as we are aware (but see e.g., [30, 40, 41]). However, investigations in natural populations are key to unravel the intricate eco-evolutionary processes underlying the circulation of *T. gondii* in various habitats. As an illustration, the first Types I, II and III isolates of this parasite were historically classified according to their virulence in the laboratory mouse [73]. However, such a virulence in laboratory mice is clearly not generalizable to all rodent species, and distinct resistance profiles have been observed in different subspecies or strains of the same rodent host species [49, 61]. These resistance profiles result from a complex evolutionary balance between the host immune response and the genetic diversity of *T. gondii* in each region of the world [22, 48, 69, 72].

The circulation of *T. gondii* in domestic animals has been demonstrated in several parts of Benin (West Africa), including Cotonou city [47, 82, 83], but no data are available for small mammals hosts. Nevertheless, this city has a major international seaport that has been shown to be responsible for the introduction of invasive rodent species and associated zoonotic pathogens into the country [13], thus questioning the genetic diversity and ecology of *T. gondii* strains that may circulate locally through these reservoir species. In this study, we explore the role of natural populations of small mammals (rodents and shrews) from Cotonou city in the epidemiology of *T. gondii*. To do so, we focus on the Cotonou small mammal community in order to (i) assess the molecular prevalence of *T. gondii* within each species-specific population, (ii) explore the impact of certain intrinsic and extrinsic factors on individual infection by *T. gondii*, and (iii) further document the genotypes circulating among small mammals within this large West African seaport city.

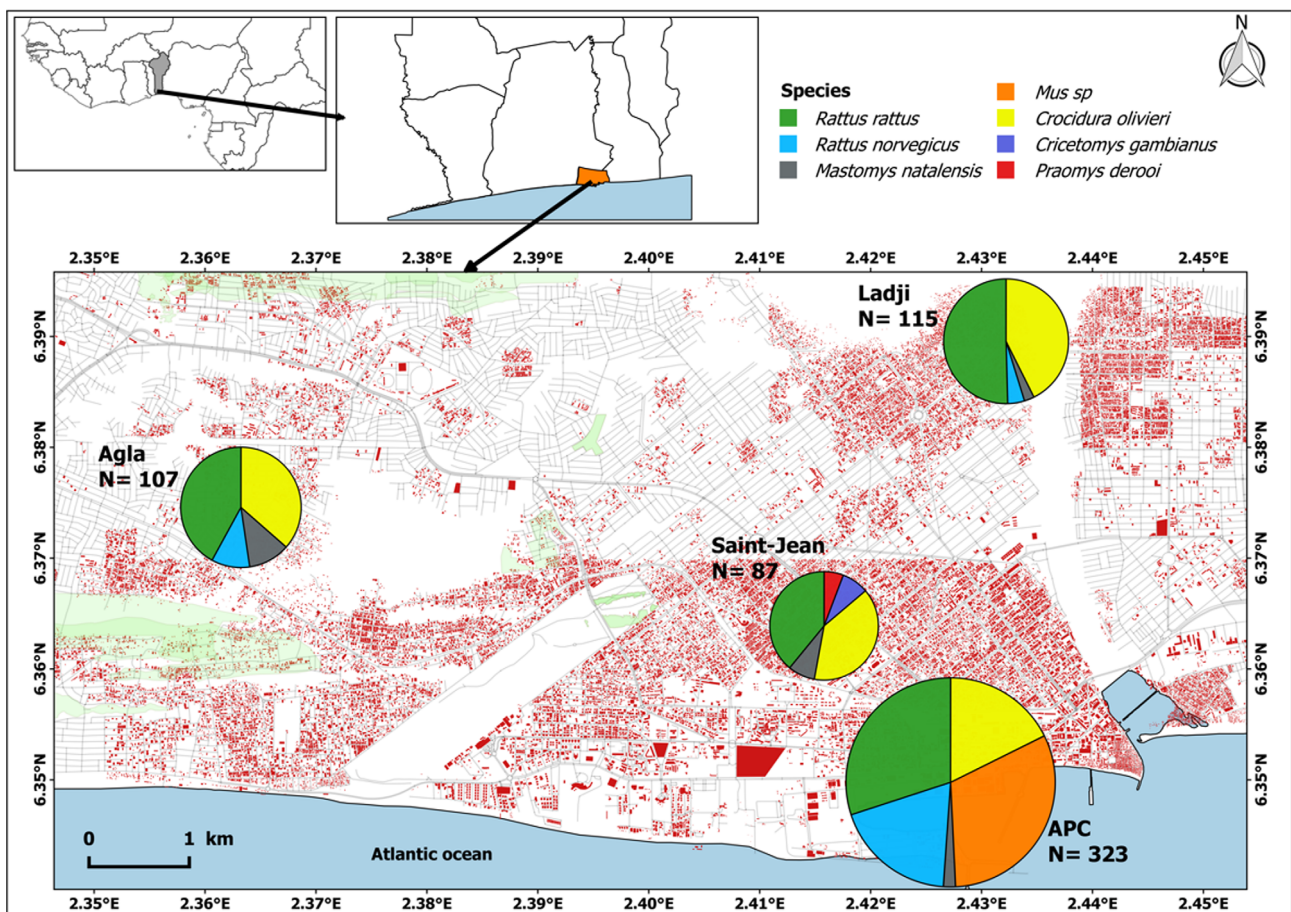


Figure 1. Distribution of sampling localities within Cotonou city, as well as micro-mammal trapping results. Circle sizes are proportional to the number of host individuals investigated for *T. gondii*.

Material and methods

Ethics statement

Sampling was conducted under the research agreement signed between the Republic of Benin and the French Research Institute for Sustainable Development (IRD) renewed on April 6, 2017, as well as the scientific collaboration agreement between IRD and Abomey-Calavi University signed on the September 30, 2010 and renewed on July 3, 2019. All trapping sessions were conducted following the explicit oral authorization of local authorities (Cotonou City Hall and local heads of urban districts) as well as the agreement of each household's owners and inhabitants. Field campaigns within the Cotonou Autonomous Seaport were implemented following official written authorization issued by its General Management Board. None of the species captured in this study have protection status (see CITES list, <https://checklist.cites.org/>). All animals were handled and processed in a humane manner, with respect to animal welfare following the guidelines of the American Society of Mammologists [74]. Our study and the uses of associated samples and genetic data were implemented following Nagoya protocol recommendations (Access and Benefit Sharing agreement number 608/DGEFC/DCPRN/PF-APA/SA).

Study sites

Our study was performed across two major types of areas: (i) three socio-environmentally contrasted urban districts of Cotonou city between 2017 and 2018, namely Ladji, Agla and Saint-Jean; and (ii) Cotonou seaport (Autonomous Port of Cotonou, or APC) (Fig. 1). Though of rather recent origin, Agla is currently one of Cotonou's most populous neighbourhoods. At the time of the present study, it was a still a partially swampy area undergoing rapid urbanization. It is located within a large shallow land and was thus characterized by wide flooded areas at the early stages of the rainy season, following accumulation of rainfall and elevation of the water table [52]. Ladji is located on the shore as well as upon Lake Nokoué (i.e., stilt-built houses). With over 10,000 inhabitants, this informal settlement is socio-environmentally very disadvantaged and suffers from insufficient basic services. It is partly flooded at the end of the rainy season following overflow of Lake Nokoué. Saint-Jean is a formal residential area of colonial origin. Even though the population is working-class, many services are formally available, and the area does not flood *per se* during the rainy season. However, wide ponds may persist in some places for days to a few weeks after heavy rains. APC is located in the central administrative highly human-made part of the city. Two traditional and seven industrial port

zones were targeted: the former gather areas where traditional fishing products are disembarked, prepared, stored and sold, while the latter correspond to the canteens (where workers take lunch) as well as areas under restricted access (e.g., administrative services, docks, storehouses, workshops, garages, and parking lots) (for details about these APC sampling sites).

Sampling

In each of the three districts, 9–11 households (hereafter designated “district sites”) were investigated (see details in [24, 26]), while nine sites were sampled in APC (hereafter designated “APC sites”). Each area was investigated twice: in October 2017 and June 2018 for Agla, Ladj, and Saint-Jean on the one hand, and in September–November 2017 and March 2018 for APC on the other. Standardized longitudinal trapping sessions were conducted for three consecutive nights using both Sherman and locally made wire mesh traps. A fish/peanut butter mixture was used as bait. Captured small mammals were brought alive into the laboratory where they were euthanized within the same day following diethyl-ether exposure and subsequent cervical dislocation, then sexed, weighed and measured. Immediately after euthanasia, presence of ectoparasitic fleas was checked by combing the animal. Particular attention was paid to unambiguous species-specific identification of all individuals through morphology and external measurements, cytochrome b DNA sequencing, PCR-RFLP analyses and microsatellite genotyping (see details in [24, 26]). The age of individuals (i.e., adult vs. juvenile stage) was assessed according to the body mass following Granjon & Duplantier [45] and signs of sexual maturity (i.e., external testicles and/or active seminal vesicles in males; developed mammae and uterus, presence of embryos and/or embryo scars in females). Among other samples, ethanol-preserved or directly frozen hearts and brains were collected on each small mammal specimen for subsequent molecular processing.

Molecular detection of *T. gondii*

Toxoplasma gondii presence was investigated on both heart and brain samples of each small mammal collected. To do so, brains were ground separately in 1 mL of physiological water (0.9% NaCl) and 30 mg of heart cut at the apex for DNA extraction and qPCR. We relied on DNA extraction and qPCR-based screening as previously described [5, 40]. Briefly, DNA was extracted using a commercial kit as recommended by the manufacturer (Qiagen QIAamp DNA Mini Kit, Courtaboeuf, France) from 200 µL of liquid brain homogenate, and from 30 mg of the heart apex. Real-time PCR targeting the non-coding 529 bp repeat region was used to assess the presence of *T. gondii* DNA as described by Ajzenberg *et al.* [5]. Each PCR contained 5 µL of extracted DNA, mixed with 15 µL of a PCR reagent mix composed of 1X LightCycler FastStart DNA Master Hybridisation Probes kit (Roche Diagnostics, Mannheim, Germany), 0.5 U of UNG (Roche Diagnostics, Mannheim, Germany), 5 mMol/L of MgCl₂, 0.5 µMol/L of each primer, and 0.1 µMol/L of TaqMan probe (Eurofins, Ebersberg, Germany) which is labeled with a fluorescent dye

(6-carboxyfluorescein, 6-FAM) at the 5' end and a dark quencher (Black Hole Quencher, BHQ1) at the 3' end. A cycling protocol was run on a Rotor-Gene 6000 thermocycler (Corbett Life Science, Sydney, NSW, Australia) as follows: initial decontamination by UNG at 50 °C/2 min and denaturation at 95 °C/10 min, followed by 50 cycles at 95 °C/20 s and 60 °C/40 s. The results obtained were expressed in cycle threshold (Ct) values. Each amplification curve was visually checked to verify its sigmoidal appearance in order to avoid the inclusion of false positives. Genomic DNA isolated from Type I (RH strain, <http://toxodb.org>) *T. gondii* isolate cultures and sterile water were used as positive and negative qPCR controls, respectively. Each heart and brain extraction sample was investigated twice by qPCR, and individuals that provided at least one positive test were considered positive.

Microsatellite genotyping

Genotyping relied on 15 microsatellite (MS) marker multiplex PCR corresponding to 15 loci located on 11 different *T. gondii* chromosomes [4]. Some of them were described as poorly polymorphic but still valuable for strain typing (TUB2, W35, TgM-A, B18, B17, M33, IV.1, and XI.1), while others are highly polymorphic and allow us to distinguish between strains of a same type (M48, M102, N60, N82, AA, N6, and N83) [4, 57]. The Beninese multi-locus genotypes (MLGs) were compared to reference strains described in previous studies (see Table 1) in order to reach strain-specific assignment. For genotyping, DNA isolated from Type II (ME49 strain, <http://toxodb.org>) *T. gondii* isolates cultures and sterile water were used as positive and negative controls, respectively.

Statistical analyses

We used Chi-square tests on the whole dataset to compare *T. gondii* prevalence between host species, sampling localities (Agla, Ladj, Saint-Jean, and APC), and screened organs. Then, analyses of APC data (hereafter referred to as “APC-based analysis”) were performed separately from the three urban districts (hereafter referred to as “District-based analysis”) since (i) they display distinct socio-economic, historical and environmental characteristics, (ii) no landscape/GIS data were available for the APC, and (iii) trapping campaigns were not performed at the same period in both types of area. After determining prevalence levels of *T. gondii* (i.e., the percentage of infected hosts calculated within a 95% confidence interval (CI) estimated with Wald’s method) for each small mammal population, we carried out a three-way analysis using specific packages in RStudio v.4.1.2 software [79].

We first used Chi-square and Fisher’s exact tests to investigate possible relationships between *T. gondii* infection and certain host individual variables (sex, age, and presence of ectoparasitic fleas), period of capture (trapping session) and organs screened for *T. gondii* detection (heart or brain).

Second, we carried out generalized linear mixed models (GLMMs) on specific datasets to test whether *T. gondii* prevalence in small mammals was significantly associated with specific intrinsic and extrinsic parameters, namely the relative abundance of each species within the small mammal

Table 1. Multilocus microsatellite (MS) genotyping of micromammal-borne *Toxoplasma gondii* from Cotonou.

Isolate ID	Host species	Origin	MS alleles															Genotype Reference	
			TUB2	W35	TgM-A	B18	B17	M33	M IV.1	M XI.1	M48	M102	N60	N82	AA	N61	N83		
#803 ^ϕ	<i>R. rattus</i>	APC	291	248	205	NA	342	165	274	354	229	166	NA	NA	269	NA	NA	<i>Africa I</i>	This study
#918 ^{ϕ; ♂}	<i>C. olivieri</i>	Agla	291	248	205	160	342	NA	274	354	227	166	147	111	269	NA	306	<i>Africa I</i>	This study
#954 [♂]	<i>C. olivieri</i>	Agla	291	248	205	160	342	NA	274	354	221	166	149	NA	275	89	306	<i>Africa I</i>	This study
#1000 [♂]	<i>C. olivieri</i>	Ladji	291	248	205	160	342	NA	274	354	227	166	147	111	269	89	306	<i>Africa I</i>	This study
#1491 ^ϕ	<i>C. olivieri</i>	Ladji	291	248	205	NA	342	NA	274	354	225	166	147	NA	267	91	306	<i>Africa I</i>	This study
#1589 ^{ϕ; ♂}	<i>C. olivieri</i>	St-Jean	291	248	205	160	342	NA	NA	354	227	NA	147	NA	267	89	NA	<i>Africa I</i>	This study
#1616 ^{ϕ; ♂}	<i>C. olivieri</i>	St-Jean	291	248	NA	160	342	NA	274	354	225	NA	147	NA	273	87	306	<i>Africa I</i>	This study
P19S1AJ6	Chicken	Benin	291	248	205	160	342	165	274	354	227	166	147	111	267	89	306	<i>Africa I</i>	[47]
TgH 13002	Human	Senegal*	289	248	205	160	336	165	274	354	225	166	145	111	273	89	306	<i>Africa 2</i>	[57]
GAB3DOM9	Chicken	Gabon	291	242	207	160	342	165	274	354	229	166	142	111	273	95	310	<i>Africa 3</i>	[57]
P676	Chicken	Benin	293	242	203	156	336	165	274	354	215	174	129	109	291	107	306	<i>Africa 4</i>	[47]
GT1	Human	USA*	291	248	209	160	342	169	274	358	209	168	145	119	265	87	308	<i>Type I</i>	[57]
PRU	Human	France*	289	242	207	158	336	169	274	356	209	176	142	117	265	127	310	<i>Type II</i>	[57]
NED	Human	France*	289	242	205	160	336	165	278	356	209	190	147	111	267	91	312	<i>Type III</i>	[57]

*: Strains obtained from Biological Resource Center for *Toxoplasma* (<http://www.toxocrb.com>). Numbers indicate the length (bp) of amplified fragments at each microsatellite locus, NA: Microsatellite allele not determined at this locus. ^ϕ and [♂] Strain isolated from brain and heart, respectively.

community (estimated by species-specific trapping success at each sampling site), host individual characteristics (sex, age, and presence/absence of fleas), period of capture (trapping session) as well as environmental features (defined by trapping site coordinates on the first four axes of PCA carried out on set landscape metrics). For the latter parameters, we took advantage of a previous study relying on the same experimental design (i.e., same sampling campaigns, hence same sampling sites) for which the dataset encompassed our own samples in the same districts of Cotonou ([27]). In this study, a wide range of landscape descriptors including landcover data, social uses associated with buildings, as well as surface water occurrences were mapped and quantified using a dedicated Geographic Information System [27]. These landscape data were integrated and explored through a principal component analysis (PCA; see [Supplementary figure](#) and Dossou *et al.* [27]). For the purpose of our study, site-specific PCA coordinates along the four first components (representing 56% of total inertia of all axes) were extracted and used as explanatory socio-environmental variables in GLMMs (only for “district-based analysis” given that this information was not collected for APC). Using the Akaike Information Criterion (AICc), we carried out a model selection procedure from a full starting model containing all predictors. *Toxoplasma gondii* prevalence was treated as a binary response (therefore assuming a binomial distribution) and either districts (for “district-based analysis”) or APC sites (for “APC-based analysis”) were considered random factors to account for possible spatial variation. Models with all possible combinations of the terms included in the starting model were generated, and the most parsimonious model (i.e., the one explaining the highest variance level with the fewest explanatory variables) was chosen among those selected within two AIC units of the best model retrieved [12]. The significance of explanatory variables and their interactions was determined by deletion testing and log-likelihood ratio tests. The final model was validated by the graphical checking of normality, independence, and variance homogeneity of residuals.

Lastly, as implemented in Mariën *et al.* [55] and Sirdar *et al.* [76], possible spatial/site clustering of *T. gondii*-carrying reservoirs was investigated in the sampling localities through Cuzick–Edwards (nearest-neighbour) tests [19, 70]. To do so, Monte-Carlo permutations ($n = 10,000$) were run to infer whether clustering of the field data was significantly higher ($p \leq 0.05$) than under random simulations [36]. After confirming that *T. gondii*-positive animals were significantly clustered, we used Kulldorff’s spatial scan statistics in order to characterize the local clusters, which make it possible to assess the number and geographical position of local clusters, as well as the number of rodents involved in the potential clusters identified [53].

All these analyses were performed using the following packages: lme4 [9] for GLMMs, MuMIN [8] for model selection, Ade4 for PCA [28], and Smacpod [36] and Spatstat [7] for spatial clustering analysis.

Results

Sampling

We analyzed a total of 632 small mammals belonging to seven species: Black rat, *Rattus rattus* ($n = 234$ individuals), African giant shrew, *Crocidura olivieri* ($n = 179$), House mouse, *Mus musculus domesticus* ($n = 102$), Brown rat, *Rattus norvegicus* ($n = 77$), Natal multimammate mouse, *Mastomys natalensis* ($n = 28$), Gambian pouched rat, *Cricetomys gambianus* ($n = 7$) and Deroo’s mouse, *Praomys derooi* ($n = 5$). These host populations comprised 481 adults and 106 juveniles (the age of 45 individuals could not be unambiguously determined) and 282 males and 350 females. Only 13.5% (81 out of 598) of individuals carried at least one flea. In our dataset, *R. rattus* (Agla: $n = 45$; Ladji: $n = 58$; Saint-Jean: $n = 34$; and APC: $n = 97$) as well as *C. olivieri* (Agla: $n = 39$; Ladji: $n = 49$; Saint-Jean: $n = 34$; and APC: $n = 57$) showed high levels of presence in all localities. *Mastomys*

natalensis was mostly trapped in the districts (97.5% of all *Mastomys* individuals captured), while *R. norvegicus* and *M. m. domesticus* were mostly trapped in APC with 79.2% and 100% of individuals captured in this locality, respectively (see Fig. 1 and Supplementary Tables 1 & 2 for details).

Molecular prevalence of *T. gondii*

Overall localities in Cotonou

Toxoplasma DNA was detected in 96 small mammals, thus representing an overall molecular prevalence of 15.2% (95% [CI = 12.39; 17.99]) (see Supplementary Tables 1 & 2 for details of prevalence). Regarding localities, the highest prevalence was found in Saint-Jean and APC, with 18.4% [10.25; 26.53] and 17.1% [12.93; 21.13], respectively, followed by Ladji with 12.2% [6.20; 18.15] and Agla with 10.3% [4.53; 16.03]. However, Chi2 tests revealed that differences were not significant between localities ($\chi^2 = 4.4$; $p = 0.23$). Regarding the host species, the highest prevalence was found in *M. m. domesticus* and *C. olivieri* with 20.6% [12.74; 28.44] and 19.6% [13.74; 25.36], respectively, followed by *M. natalensis* with 14.3% [0.0; 40.21], *R. norvegicus* with 14.3% [6.47; 22.10] and *R. rattus* with 9.9% [6.01; 13.64]. The two species *C. gambianus* and *P. derooi*, which were poorly represented in the sampling ($n = 7$ and $n = 5$), had only one and two individuals infected, respectively and were not taken into account in the subsequent analyses. Regarding the targeted organ, no significant differences were found between the brain and heart, with prevalence reaching 9.9% [7.63; 12.3] and 7.4% [5.25; 9.3], respectively. Only thirteen individuals displayed positive results in both organs (13.5% [9.49; 17.56]).

District-based analysis

For the three sampled districts, comparison of the prevalence showed a significant difference between species ($\chi^2 = 9.9$; $p = 0.02$), with pairwise comparisons indicating that shrews were significantly more infected than black rats ($\chi^2 = 8.8$; $p = 0.003$). No statistical pairwise difference was found between other species. Regarding the district sampled, no significant difference was found ($\chi^2 = 2.9$; $p = 0.23$). Regarding the trapping session, no difference was found between species nor between districts, except in Saint-Jean where prevalence was higher in June 2018 than in October 2017 ($\chi^2 = 8.2$; $p = 0.004$). Finally, regardless of the species considered, there was no significant relationship between *T. gondii* infection and respectively sex, age, or flea carriage (χ^2 , all $p > 0.2$).

APC-based analysis

Our analyses excluded *M. natalensis*, for which only six individuals were trapped and none of them were infected. We found no significant difference in *T. gondii* infection between other species ($\chi^2 = 1.8$; $p = 0.62$). *Toxoplasma gondii* prevalences in the mouse population were significantly higher in September–November 2017 than in March 2018 ($\chi^2 = 5.6$; $p = 0.02$), while no differences were found in other species

depending on the trapping period. Irrespective of the species considered, there was no sex, age, and fleas carriage-associated distribution of *T. gondii* infection (χ^2 , all $p > 0.3$) except in *R. norvegicus* where juveniles seemed to be more commonly infected than adults (Fisher's test, $p = 0.005$).

Genotyping

Most of the qPCR-positive small mammals displayed DNA concentrations of *T. gondii* in their tissues that were too low to enable genotyping (Ct > 32). Samples from seven individuals (samples #803, 918, 954, 1000, 1491, 1589 and 1616) had sufficient *T. gondii* DNA for partially successful MS-markers amplification (10/15 to 14/15 amplified markers). Despite incomplete genotyping, enough markers were amplified for lineage identification: all seven samples belonged to the *Africa 1* lineage genotypes (Table 1). These genotypes were retrieved from one black rat captured in the APC and two shrews in each of the three urban neighbourhoods.

Influence of intrinsic/extrinsic factors on *T. gondii* molecular prevalence

Several GLMMs were tested, either considering all small mammals species investigated or separately for each species with at least 50 individuals sampled, in both “district-based” and “APC-based” models (Table 2).

District-based GLMMs

We found a parsimonious model only when considering all small mammal species. A high *T. gondii* prevalence was (i) negatively associated with *R. rattus* relative abundance ($\chi^2 = 7.99$; $p = 0.005$), and (ii) positively associated with *C. olivieri* relative abundance ($\chi^2 = 5.06$; $p = 0.024$). Therefore, the socio-environmental proxies used in this study failed to show any influence on *T. gondii* prevalence in small mammal communities.

APC-based GLMMs

Considering all small mammal species, *T. gondii* infection was positively correlated with the relative abundance of *M. m. domesticus* ($\chi^2 = 7.22$; $p = 0.007$). For this rodent species, the most parsimonious model best explaining *T. gondii* infection included the trapping session ($\chi^2 = 7.45$; $p = 0.006$) only; they were significantly more commonly infected in September–November 2017 than in March 2018.

Spatial clustering of *T. gondii*-infected small mammals

Regardless of the trapping period considered, no significant clusters were observed in the four studied localities; however, when the two trapping sessions were grouped, a significant clustering of *T. gondii*-infected animals was observed in Saint-Jean district, with Monte Carlo p -values lower than 0.05 for $q = 10$ nearest neighbours (p -value = 0.03) based on the Cuzick-Edwards test (Table 3). Kulldorff's spatial statistic

Table 2. Results of Generalized Linear Mixed Models (best models) explaining *T. gondii* infection of main rodent host species in the sampled districts. Rra, Rno, Mna, Cro, Mus represent *Rattus rattus*, *Rattus norvegicus*, *Mastomys natalensis*, *Crocidura olivieri* and *Mus musculus domesticus* yield, respectively; Fleas: presence of fleas on the individual. Session: trapping session. PCA 1–4: PCA sites coordinates of axis 1–4.

Localities	Species ^a	Explanatory variables	N ^b	Predictors ^c	Estimate ± SE	p-value
Districts (Agla, Ladji & Saint-Jean)	All species	Species, Sex, Age, Fleas, Session, PCA1, PCA2, PCA3, PCA4, Rra, Rno, Mna, Cro	260	Rra Cro	−6.3668 ± 2.6852 5.9944 ± 2.6073	0.004 0.02
	<i>C. olivieri</i>	Sex, Age, Fleas, Session, PCA1, PCA2, PCA3, PCA4, Rra, Rno, Mna	91	None	–	–
	<i>R. rattus</i>	Sex, Age, Fleas, Session, PCA1, PCA2, PCA3, PCA4, Rno, Mna, Cro	125	None	–	–
APC	All	Species, Sex, Age, Fleas, Session, Rra, Rno, Mna, Cro, Mus	295	Mus	7.256 ± 2.663	0.007
	<i>C. olivieri</i>	Sex, Age, Fleas, Session, Rra, Rno, Mna, Mus	50	None	–	–
	<i>R. rattus</i>	Sex, Age, Fleas, Session, Rno, Mna, Cro, Mus	89	None	–	–
	<i>R. norvegicus</i>	Sex, Age, Fleas, Session, Rra, Mna, Cro, Mus	54	None	–	–
	<i>M. m. domesticus</i>	Sex, Age, Fleas, Session, Rra, Rno, Mna, Cro	96	Session	1.6045 ± 0.6653	0.006

^a Species included in the model.

^b Total number of individuals included in model selection.

^c Selected variables.

scan also indicated a single significant local cluster (p -value = 0.005) at one given Saint-Jean site (i.e., S6) where 5 out of 9 individuals were infected (Fig. 2).

Discussion

To our knowledge, the present study is the first to focus on interrelationships between several abiotic and rodent-based biotic factors and *T. gondii* circulation in African urban dwellings. Our results are consistent with previous findings from Europe and North America, showing that small mammals play an important role in the maintenance and circulation of *T. gondii* within the urban environment [11, 30, 40–42]. Furthermore, our qPCR-based approach led to higher prevalence values (20.6% in *M. m. domesticus*, 19.6% in *C. olivieri*, 14.3% in *M. natalensis*, 14.3% in *R. norvegicus* and 9.9% in *R. rattus*) than those previously observed elsewhere using molecular methods, regardless of the *T. gondii* targeted gene, the methodology of screening, and the geographical scale considered (e.g., in *R. rattus*: 1.8% in São Paulo, Brazil [60]; 4.5% in Gran La Plata, Argentina [23]; 4.3% in Sichuan, China [85]). In the same manner, within the African context, the prevalence exhibited here was also higher than that found in Senegal with the same qPCR-based approach (i.e., 3.8% in black rats, 13.3% in house mice, and 15.5% in shrews; [40]). These differences in prevalence could result from the nature of the tissues analyzed. Importantly, the study in Senegal (and in most studies) was based only on brain samples, whereas our study relied on both brain and heart. Although no significant differences in molecular prevalence were found between organs, only 13.5% of the 96 positive animals displayed positive results in both organs. When focusing only on brain samples, our results

show a prevalence of 6.4% in black rats, 14.7% in house mice, and 14.5% in shrews, which is much closer to the prevalence observed in Senegal [40]. This suggests that both organs (brain and heart) should be considered together when investigating molecular prevalence of *T. gondii* in future small mammal-centered surveys. Another non-exclusive hypothesis on the differences observed between the Senegalese and Beninese contexts could be the differences in socio-environmental conditions (e.g., climate, nature of soil, vegetation type) between both countries. As such, sampled localities in Senegal (Dakar, Rufisque and Joal-Fadiouth [40]) are characterized by a hot semi-arid climate [33], which could contribute to reduce the survival rate of *T. gondii* oocysts, while Cotonou is favorably more humid, with recurring floods [52, 86]. For a clearer view, we advocate for study of different geographic contexts of *T. gondii* epidemiology within commensal small mammal communities showing contrasted eco-evolutionary trajectories.

Nevertheless, our analyses provide interesting insights on the tested factors. As previously described by Galal *et al.* [40], we showed that shrews were significantly more often infected than black rats over the three Cotonou districts. This could be due to differences in life traits: black rats are omnivorous and good climbers, while shrews forage only on the ground where they show marked digging and exploratory behavior for feeding [10, 15, 16]. The latter habits may likely favor contact with *T. gondii* oocysts excreted in the environment by stray and/or domestic cats. Moreover, no gender difference was observed for *T. gondii* infection for any of the reservoir species investigated here. This was congruent with findings outlined elsewhere [20, 58, 67], suggesting that gender-based differences in rodent behavior, immunity, and/or physiology do not influence *T. gondii* infection. Within-species comparisons also showed no difference in infection with age,

Table 3. Monte Carlo p -values (10,000 simulations) to investigate significant clustering of *T. gondii*-infected small mammals within each study area based on the Cuzick–Edwards test.

Localities	Session	Total ^a	N. Pos ^b	$N^c = 2$	$N = 4$	$N = 6$	$N = 8$	$N = 10$	Kulldorf p -values
Agla	Sept/Nov 2017	50	6	0.37	0.68	0.86	0.49	0.17	0.36
	June 2018	57	5	0.4	0.46	0.19	0.16	0.08	0.14
	All	107	11	0.22	0.55	0.66	0.53	0.16	0.02
Ladji	Sept/Nov 2017	52	9	0.52	0.48	0.82	0.25	0.31	0.18
	June 2018	63	5	0.37	0.61	0.58	0.41	0.2	0.78
	All	115	14	0.16	0.10	0.07	0.24	0.33	0.02
St-Jean	Sept/Nov 2017	46	3	0.2	0.16	0.26	0.06	0.07	0.07
	June 2018	40	13	0.07	0.32	0.08	0.07	0.09	0.2
	All	86	16	0.72	0.63	0.14	0.1	0.03	0.005
PAC	Oct 2017	162	18	0.94	0.87	0.9	0.89	0.91	0.42
	March 2018	159	16	0.67	0.64	0.61	0.77	0.74	0.84
	All	321	54	0.8	0.64	0.57	0.58	0.61	0.12

^a Total number of captured rodents.

^b Total number of *T. gondii*-Infected rodents.

^c Number of nearest neighbours (q).

Bold numbers were considered to be significant.

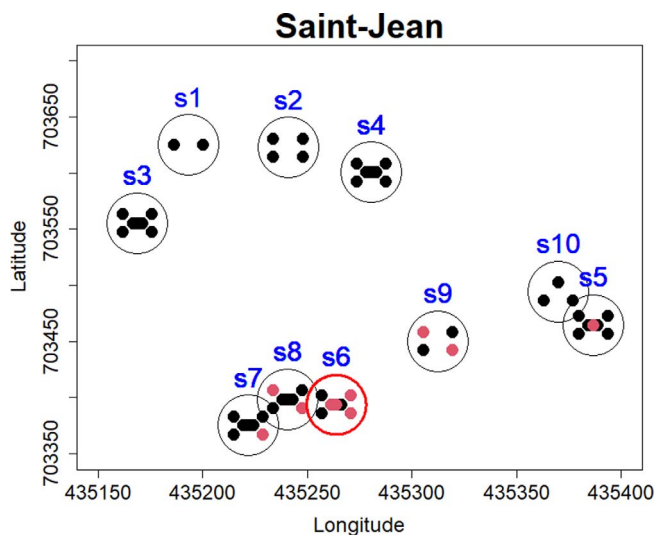


Figure 2. Spatial clustering of *T. gondii*-infected small mammals of Cotonou. Each black circle corresponds to a sampled site graphically represented by its GPS coordinates. Red and black dots represent *T. gondii*-infected and *T. gondii*-uninfected animals, respectively. Red circles represent significant clusters of cases based on the spatial statistic scan.

except in APC where juveniles were more commonly infected than adults in *R. norvegicus*. Nonetheless, we should be cautious when interpreting this outcome given the low number of juveniles trapped for certain species, including *R. norvegicus* (e.g., in APC: 7 juveniles out of 61 individuals). Regardless of the locality and the small mammal species, no relationship was found between *T. gondii* prevalence and flea carriage. Although ectoparasites have been hypothesized as paratenic hosts and vectors of *T. gondii* [66, 77, 87], our results did not allow us to identify a significant relationship between *T. gondii* infection and the presence of ectoparasitic fleas, suggesting that flea parasitism does not appear to influence *T. gondii* infection in small mammals.

Furthermore, except for Saint-Jean where prevalence was higher in June 2018 than in October 2017 (32.5% vs. 6.4%), no differences in *T. gondii* prevalence were found between trapping sessions irrespective of the district or the species considered. This observation was also made for rodent-borne *T. gondii* seroprevalence in Niamey city (Niger) [58], suggesting that infection in small mammals may consistently occur throughout the year in West African urban regions. Nevertheless, we cannot exclude that the difference observed in Saint-Jean may be because both trapping sessions correspond to the rainy season. However, our data are restricted to only two trapping sessions, thus precluding any robust and definitive conclusion about potential seasonal variations in small mammal-borne *T. gondii* prevalence. Temporal surveys during each season and over at least two years would be required to capture such possible temporal dynamics.

The “district-based GLMMs” for explaining *T. gondii* infections did not show any clear influence of the local socio-environment – at least as defined by the proxies used in this study. This finding may be interpreted in the light of the ubiquitous nature of *T. gondii*, which is adapted to diverse environmental matrices, including soil, vegetation, standing water, and the marine environment [71, 86] – and even in polar areas where felids are absent [31]. Rather than being impacted by socio-environmental determinants, *T. gondii* prevalence in Cotonou was shown to be related to the relative abundances of black rats and shrews. In fact, a high prevalence of the parasite appeared to be significantly associated with a low abundance of *R. rattus*, and a high abundance of *C. olivieri*. The social and feeding behavior of *R. rattus* could explain the low prevalence of *T. gondii* observed in this species. As explained above, the black rat is a good climber which often builds nests in roofs of houses [34], which might limit direct contact with the bare soil where *T. gondii* oocysts disseminate. Moreover, the large size of *Rattus* species [18, 64] makes them less prone to predation by domestic cats which act as final hosts [14, 35, 65, 78]. However, this interpretation, outlined elsewhere [1, 40, 67], should be taken with caution since the main

mode of *T. gondii* transmission in natural small mammal populations is still unknown [32]. Alternatively, shrews may appear to be an important host for the circulation of *T. gondii* within the urban environment. Altogether, it appears that species-specific susceptibility to *Toxoplasma* may be a key element of the ecology of this ubiquitous parasite. Therefore, we stress the need to further investigate the intricacies of host-*T. gondii* interactions at the species level.

Our “APC-based GLMMs” showed that the probability of *T. gondii* infection in the small mammal community was positively correlated with the abundance of *M. m. domesticus*. This rodent species is generally considered to be the preferred prey of domestic cats [35, 65], which could lead to increasing rates of oocysts excretion in the environment and thus higher infection opportunities for other species, including small mammals. Of note, although probably not very frequent, cats were sometimes observed wandering inside the seaport storehouses during our field trapping campaigns. Moreover, in wild-derived mice, the hypothesis of the maintenance of *T. gondii* infection over several generations by congenital transmission has been formulated [50, 56, 62, 63], and might at least partly explain the high prevalence observed in APC where all house mice were caught. Mice were found to be significantly more infected in September 2017 (rainy season) than in March 2018 (end of the dry season). Assuming environmental contamination, such a trend could be explained by the characteristics of sampled APC sites. Mice were essentially trapped in the industrial zones of APC that are characterized by large cemented surfaces and a total absence of vegetation. These conditions could be particularly unfavorable to the survival of oocysts during the dry season. Such a hypothesis is congruent with wet weather and water runoff as factors facilitating *T. gondii* oocyst survival and/or dissemination [67, 80, 84], and might explain the high prevalence observed in APC mice during the rainy season. From there, spatio-temporal surveys over different seasons in this very peculiar environment are needed to draw robust conclusions.

No significant spatial cluster of *T. gondii*-infected animals was observed in Agla, Ladjé and APC, but one significant cluster was retrieved in Saint-Jean – at site S6 where 56% of individuals were found to be infected. This site is adjacent to sites S7 and S8 where prevalence reached 27% (4/15) and 30% (3/10), respectively. This cluster can be interpreted as a local hotspot of parasite circulation. Based on our knowledge of the major routes of *T. gondii* transmission to intermediate hosts, we would expect the parasite infection to be strongly dependent on cat density [3, 29, 43, 44, 88]. Unfortunately, to our knowledge, robust data on cat abundances and activities in Cotonou are not available. During a previous survey (Houémènou *et al.*, personal information) in the exact same households of Agla, Ladjé and Saint-Jean conducted from September 2016 to June 2017 (i.e., just before the start of the sampling campaigns for the present study in October 2017 and June 2018), cats were observed or indicated by inhabitants at least once in 2/10 sites in Ladjé, 3/10 sites in Agla, and up to 5/10 sites in Saint-Jean, thus allowing a rough indication of cat abundance in our districts. The apparently frequent occurrence of domestic cats in Saint-Jean households could explain the local hotspot identified by our clustering analyses. However,

in the absence of reliable data, this hypothesis remains to be formally tested and it would therefore be critical in future studies on *Toxoplasma* eco-epidemiology in southern Benin to take cats (and other potentially impacting factors, such as climate data, co-infection, and human exposure) into account, since they should represent rarely investigated but obviously crucial factors.

The seven samples genotyped here were all classified as belonging to the *Africa 1* lineage, a *T. gondii* lineage widespread in most West and Central African countries, including Benin [38, 40, 47, 57]. They were retrieved in six shrews (two individuals in each urban district) and one black rat (originating from APC). Although the number of genotyped individuals is low, it nonetheless confirms the predominance of *Africa 1* strain in domestic and peri-domestic environments of Southern Benin, including Cotonou, as previously shown in chickens [47] and, beyond, throughout tropical Africa on different hosts [38, 57]. The *Africa 1* strain is considered lethal in laboratory and wild mice (*Mus musculus domesticus*) [40, 47, 49, 57] and the link between strain virulence and host availability in shaping genetic diversity of *Toxoplasma* worldwide is supported by an increasing number of studies [37, 54, 72]. In Senegal, the invasive house mouse *Mus musculus domesticus* is currently the most abundant rodent in seaport cities such as Dakar and Saint-Louis, as well as in several central and northern localities where most *T. gondii* isolates belong to non-mouse virulent lineages [21]. On the contrary, this invasive rodent species has not yet disseminated to certain inland regions where the *Africa 1* mouse-virulent *T. gondii* lineage was shown to be predominant [39]. In Benin, house mice invasion is probably still in its earliest phases since this species has been described almost only within APC [25, 51]. These differences in the respective history and dynamics of the house mouse invasion taking place in these two countries may explain the distinct *T. gondii* strains that circulate among local *M. musculus* populations. Testing such a hypothesis requires us to investigate the virulence of the autochthonous *Africa 1* strain in invasive house mice from Senegal and Benin. Extended genomic analyses to better capture and understand the diversity of circulating *T. gondii* strains from wild small mammal are needed since infectivity and morbidity of toxoplasmosis was shown to be related to the protozoan genotype involved [48, 72, 75].

In conclusion, our study provides a new support for the ecological ubiquity of *T. gondii* in a West-African country. Especially, we (i) showed the need to investigate both brain and heart organs in molecular surveys of *T. gondii* to draw a more accurate picture of the parasite prevalence among small mammals, (ii) confirmed the dominance of *Africa 1* lineage as the dominant strain within West-African urban areas, and (iii) highlighted the intricate interrelationships between several biotic and abiotic determinants that make the circulation of *T. gondii* a local context-dependent process in which the underlying factors seem to differ – even at a very local scale – from one socio-environmental situation to another. From all the foregoing, multidisciplinary studies are key to integrate socio-economic, biological and environmental components when studying the ecology and transmission to humans of *T. gondii* in domestic environments.

Conflicts of interest

The authors declare that there are no competing interests.

Acknowledgements. This study is part of a long-term partnership between Cotonou Autonomous Seaport, the Polytechnic School of Abomey-Calavi, the French Institute of Research for Sustainable Development, and the Tropical Neurology Institute (Inserm U1094, IRD U270 EpiMaCT, University of Limoges). We are grateful to Ladj, Agla and Saint-Jean authorities as well as inhabitants who kindly authorized us to access their households for trapping and interview purposes. We thank the Autonomous Port of Cotonou authorities and staff who facilitated our access to their infrastructures. We also thank the CDBG Small Mammal Collection (Centre de Biologie pour la Gestion des Populations, 2018, “CBGP – Small mammal Collection”, <https://doi.org/10.15454/WWNUPO>) for the conservation of samples from Benin.

Funding

This work was supported by funds from the French *Agence Nationale de la Recherche* (ANR project IntroTox 17-CE35-0004 given to A. Mercier), the Nouvelle Aquitaine region of France, and recurrent funding (given to G. Dobigny) from the French Institute of Research for Sustainable Development (IRD). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors contributions

Design of the study: A. Mercier and G. Dobigny; Sampling: J. R. Etougbétché, H.-J. Dossou, S. Badou, and G. Dobigny; Molecular biology work: A. Hamidović, N. Plault, M. Coan-Grosso, R. Roques; Data analysis and processing: J. R. Etougbétché, C. Diagne, H.-J. Dossou; Paper writing & review: J. Etougbétché, A. Hamidović, Lokman Galal, C. Diagne, G. Dobigny, A. Mercier. Supervision: A. Mercier, G. Dobigny, M-L Dardé, G. Houéménou, A. A. Missihoun and I. Youssou Abdou Karim.

Supplementary material

The Supplementary material of this article is available at <https://www.parasite-journal.org/10.1051/parasite/2022058/olm>.

Supplementary Table 1: Captures and species-specific prevalence by sampled localities and sites

Supplementary Table 2: Prevalence by sex, age, session, and flea carriage.

Supplementary Figure: Biplot PCA Cotonou, axis 1 & 2 and 3 & 4.

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Cite this article as: Etougbéché JR, Hamidović A, Dossou H-J, Coan-Grosso M, Roques R, Plault N, Houéménou G, Badou S, Missihoun AA, Youssao Abdou Karim IY, Galal L, Diagne C, Dardé M-L, Dobigny G & Mercier A. 2022. Molecular prevalence, genetic characterization and patterns of *Toxoplasma gondii* infection in domestic small mammals from Cotonou, Benin. *Parasite* 29, 58.



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