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ORIGINAL RESEARCH

Experimental Implantation Trials of *Xenopsylla cunicularis* Smit (Siphonaptera: Pulicidae) in Northern France with the Objective to Use it as Vaccine Vector

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Abstract: To combat animal diseases, we propose that *Xenopsylla cunicularis* Smit (Siphonaptera: Pulicidae), a specific flea of the European wild rabbit *Oryctolagus cuniculus* L. (Lagomorpha), can be used to carry a vaccine into wild rabbit populations to protect them against lethal diseases. *Oryctolagus cuniculus* is widespread throughout Europe, but *X. cunicularis* occurs naturally only in drier areas of Morocco, Spain, and southwestern France, raising questions about the flea's general use and the subsequent risk of uncontrolled proliferation outside its natural distribution. To evaluate this risk, fleas were released in five experimental enclosures containing rabbits (four in northern France and one in southwestern France as a control). Approximately one year later, adult and immature fleas were recovered from rabbits and warrens. The climate during the experiments was recorded and warren substrate granulometry was defined. Our results showed that northern France is not suitable for persistence of *X. cunicularis* because low temperatures reduce flea development and high rainfall all over the year keeps the soil damp, which is asphyxic for fleas, even on a sandy substrate. These implantation trials suggest that uncontrolled proliferation and permanent establishment of fleas are unlikely in northern France.

Keywords: Flea, rabbit, wildlife disease, insect vector of vaccine, myxomatosis, rabbit hemorrhagic disease

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Introduction

Wild rabbit *Oryctolagus cuniculus* L. populations in France declined markedly following the arrival of myxomatosis in 1952 and rabbit hemorrhagic disease (RHD) at the end of the 1980s.[1,2] Over the same period, new agricultural practices have transformed landscapes and led to habitat modification and fragmentation of rabbit populations, reducing their number even more.[3,4] Although habitat loss is difficult to estimate, disease control is one action that can be implemented in rabbit management and conservation programs.

Since the 1980s, blood-sucking insects such as fleas have been considered potential agents for introducing and transmitting vaccines to control some wildlife diseases. In 1986, Saurat filed a patent on the use of fleas carrying myxoma virus of low pathogenicity to vaccinate rabbits against virulent field strains of myxomatosis within wild populations of rabbits that were difficult to capture and vaccinate directly.[5] Using wingless vectors rather than "flying vaccinators" such as mosquitoes[6] also minimizes escape risks. Ideally, the vector should accomplish its function (ie, transmit the virus-vaccine to the rabbit) and then disappear quickly.

In France, rabbits are parasitized by four host-specific flea species.[7,8] The rarest are *Odontopsyllus quirosi* Beaucournu and Gilot and *Caenopsylla laptevi* Beaucournu et al which are found in south of France. *Xenopsylla cunicularis* Smit occurs in south-western France, where it is relatively scarce. The most abundant is *Spilopsyllus cuniculi* Dale, which is wildly distributed throughout the country. The latter species is heavily dependent on the hormonal cycle of its host: adult fleas mature on pregnant does, then they mate and lay eggs on rabbit kittens soon after parturition.[9,10]

Continuing Saurat's work, we used the flea *X. cunicularis* because of its simple life-cycle,[11–13] easy laboratory rearing, and high specificity for rabbits (persistence is impossible with another host).[14–16] We developed efficient production methods for large-scale field release of the vector[17] (and Darries-Vallier, unpublished data on optimization) and showed that the fleas can transmit an attenuated vaccinating virus among wild rabbits (unpublished data and see also Bárcena et al[18]) as well as constructed a recombinant myxomatosis/RHD vaccine to vaccinate against both diseases.[19] In the wild, the low-pathogenic virus-vaccine we chose[19] is badly transmitted by insect vectors.[20] This is an advantage for our purpose because it limits vaccine spreading and permits targeting of the rabbit populations for vaccination. However, we had to define a field release method[17] permitting fleas to reach more than 75% of the rabbits, which is the level required for preventing the disease from becoming epizootic (Charles Nicolle's Law[21]).

To use this flea for biological control, it must be verified to have no unforeseen impacts on the ecosystems into which it is introduced. Of particular importance is the observation that *X. cunicularis* is found only in the extreme western Mediterranean Basin, and south-western France represents the northern limit of its natural distribution (Fig. 1). It has been observed

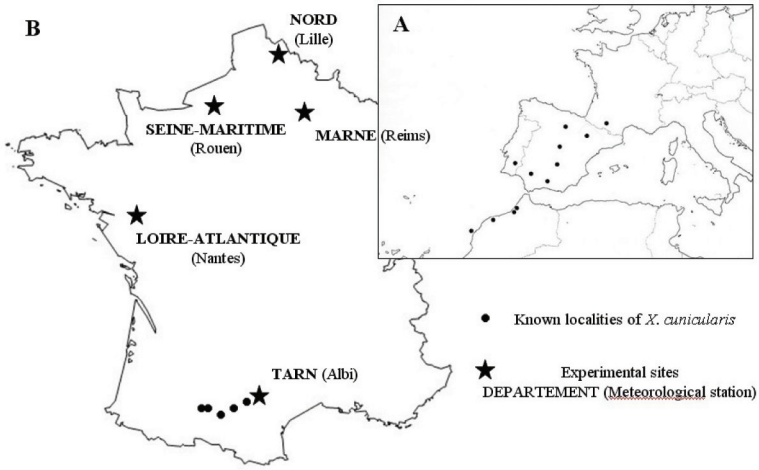

Figure 1. A. Known distribution of *Xenopsylla cunicularis*.[15] **B.** Location of experimental sites and known localities where *X. cunicularis* occurs in France.[15,21]

near Toulouse (Haute-Garonne): Fauga and Portet-sur-Garonne and more recently in 4 new sites in the departments of Gers, Haute-Garonne and Tarn.[8,15,22,23] This makes it extremely important to ascertain that any release of fleas to spread vaccines would not lead to an increase in the distribution of fleas across France with potentially deleterious effects on the transmission of naturally occurring viral diseases or interspecific competition with another flea species.

The basic biology of this flea is well-known and so provides a strong theoretical framework for further investigating this potential risk.[8,11–13,17,24] In particular, it is known that *X. cunicularis* lives in rabbit burrows and that adults jump on their hosts only to feed; eggs are laid into the soil of the warren where the detritiphagous larvae grow to maturity and pupate.[8,11] Thus, the distribution of *X. cunicularis* is linked to strict ecological requirements related to the host, substrate, and climate:[24] (i) regular warren occupation by the rabbit host is essential to ensure a food supply for egg-laying fleas, (ii) larval development is favored by loose and well-drained soils, with sandy substrate being the most suitable, and (iii) warm weather with rainfall between 280–660 mm annually as well as a spring rain regime allow optimal development of larvae. Climate is the more important parameter for flea survival. Although larvae can tolerate low humidity (RH $<$ 60%), which allows *X. cunicularis* to colonize arid areas, optimal development occurs at 80–85% RH.[12,17] Beyond this level of humidity, production of larvae decreases because a wet substrate is unsuitable for most life stages.[24] Cooke showed that when annual rainfall exceeds about 600 mm, the soil in rabbit burrows remains damp for much of the year.[25] Moreover, the seasonality of rainfall is as important as rainfall quantity, and variations of these 2 parameters are linked to important inter- and intra-annual variations of *X. cunicularis* populations.[24,12] In the area of the flea's natural distribution, monthly average temperatures vary between 5–10°C for the coldest month to 21–27°C for the hottest, while average annual temperature lies between 12.9 and 15.9°C.[24] The importance of climate was emphasized in Australian studies, in which the potential distribution of the flea in Australia was projected using a computer model,[26] which also accurately described the general distribution of *X. cunicularis* in Europe.[16,27–29] In addition, Launay showed that there was a strong decline in the productivity of *X. cunicularis* upon moving northward from Morocco into Spain and the south of France, which was attributed to decreasing temperatures and increasing rainfall.[24] Similarly, Darries-Vallier and Beaucournu confirmed the naturally low abundance and weakness of this species near the northern limits of its distribution.[23]

Because the climate is even colder and wetter in northern France,[30] *X. cunicularis* is unlikely to become permanently established outside of its current distribution if it is widely released as a temporary vaccine vector. To test this hypothesis, we carried out a first series of field trials in the north of France, which it is well outside the normal distribution of *X. cunicularis*. These trials used rabbits and fleas confined within field enclosures from which neither rabbits nor fleas could escape. They were carried out between 2007 and 2009 at 5 sites (Fig. 1B): Seine-Maritime, Marne, Nord, and Loire-Atlantique departments, and were compared with a site in the department of Tarn located at the northern limit of the natural distribution of *X. cunicularis*. The purpose of this study was to determine whether *X. cunicularis* can develop and survive in locations where it is not naturally present.

Materials

Experimental enclosures

The sites used for trials were chosen because they were characteristic of wide areas of departments described above in terms of geographic, climatic, and edaphic factors (source INRA—www.gissol.fr). High-altitude sites were ruled out because of their harsh climates.

Enclosures were either newly-built or existing and modified for the experiment. All had the following characteristics: a minimum area of 300 m^2, surrounded with a rabbit-proof fence 2 m high, and netting 40 cm below ground to prevent rabbits digging out and escaping. A 50 cm high plastic film, with the base buried, surrounded the area to prevent fleas escaping; an anti-predatory net was installed overhead to prevent inadvertent spread of fleas on rabbits by birds of prey. A sheltered manger and a water source were provided for the rabbits. Three artificial warrens were constructed to maintain both rabbits and fleas, allowing rabbits to behave normally and minimize territorial conflicts. Each warren was made of hollow cement blocks arranged to form a central chamber of 1.5 to 2 m^2 with 2 entrance tunnels 1 m long, one on each side and removable water-tight

tops. About 1.5 m^3 of soil was placed on the top. Before the burrows were closed, vegetation was removed to make later scraping easier, and soil sprinkled with a mixture of powdered dried daphnia, yeast, and sand (in a 4-1-5 part mixture) to provide a source of food for flea larvae. A volume of approximately 500 cm^3 was spread on the soil of each warren (1,500 cm^3 for each enclosure). The daphnia and yeast mixture was chosen because it is used successfully in our laboratory mass-rearing.[17] The quantity of larval food spread in the artificial burrows greatly exceed that necessary to feed fleas larvae; however, we wanted to ensure that larvae would survive before the establishment of natural food sources (flea feces, yeasts, etc) within the artificial warrens.

The characteristics of each of the 5 enclosures (Nord, Marne, Seine-Maritime, Loire-Atlantique, and Tarn) are shown in Table 1. Each enclosure was managed locally by a technician of Hunters Departmental Federation or by a hunting association member.

Rabbits

Animals were wild-caught except for Seine-Maritime rabbits, which were domesticated. Only female were used to keep populations stable and avoid complications caused by increasing rabbit numbers. Before release into the enclosures, wild-caught rabbits were held in isolation until confirmed as not pregnant. Rabbits were vaccinated against myxomatosis and RHD (eg, Dervaximyxo® and Cunical®, Merial Lab, Duluth, GA, USA) and an anticoccidial agent was added to the water supply or integrated directly into the food. To ensure that there were about 10 rabbits in each enclosure during the first stage of our experiments, 10–13 individuals were released into each site to allow for natural mortality (see Table 1).

We considered that these semi-natural conditions allowed normal rabbit behavior with little stress that could disrupt flea reproduction. Rabbit density and the number of does/warren in our experiments were equivalent to those observed in the wild (Villafuerte R, December 2012, personal communication) and sufficient to ensure regular use of the warrens, so host abundance was not a limiting factor for flea persistence. Indeed, according to Villafuerte (December 2012, unpublished data), rabbits in enclosures often exhibit less stress than animals living in the normal environment. Finally, male and female rabbits are known to form unisexual dominance hierarchies and previous studies have shown that in unisex colonies of female rabbits, a stable network of social relationships is established in 4 to 7 days.[31–34] Agonistic behavior amongst females is mainly due to reproduction[35] and its absence in our experiments minimized conflicts. The lack of reproduction in our colonies was considered unimportant because *X. cunicularis* is successfully reared on virgin rabbit females throughout the year in our laboratory.[17]

Wild rabbits were not checked for native fleas prior their release because we assumed that other flea species that may be present would not disturb our experiment. *Odontopsyllus quirosi* and *Caenopsylla laptevi* are not present in the areas tested. *Spilopsyllus cuniculi* is wildly spread throughout France but its biology and its ecological niche is very different from those of *X. cunicularis*; and its multiplication was not

Table 1. Geographical locations and ecological characteristics of the experimental enclosures.

Department (enclosure location)	Date	GPS coordinates	Surface (m^2)	Characteristics	Rabbits initial number
Nord (Beuvry-la-Forêt)	2008 2009	03°17'11"E/50°27'36"N	300	Short grass	10
Marne (Romain)	2008 2009	03°46'18"E/49°19'49"N	300	Short grass Slope ~10°	13
Seine-Maritime (Belleville-en-Caux)	2007 2008	00°59'20"E/49°42'22"N	460	Short grass Slope ~7–8°	13
Loire-Atlantique (Montbert)	2007 2008	01°29'16"W/48°03'31"N	300	Short grass under a pine forest	11
Tarn (Albi)	2007 2008	2°08'47"E/43°55'44"N	300	Tall grass	10

possible in the study sites as rabbits reproduction was not allowed in our protocol. In Tarn, wild rabbits came from an area where *X. cunicularis* was not present.[23]

Fleas

Fleas were mass-reared in our laboratory in Murviel-les-Montpellier (Hérault-France). The colony has been maintained on New Zealand White Rabbits since 1994. Breeding conditions (80–85% HR, 22–23°C for egg-laying and 21°C for development) are a trade-off between adult fitness and production output to obtain insects with high physiological quality in terms of fecundity, longevity, and capacity to withstand cold storage (8°C–85% HR). For the experiments, males and females were unfed, virgin and 2–3 days-old by the time they were released on our experimental sites. They were transported in perforated plastic tubes (8 cm high and 2 cm in diameter) with 50 fleas in each tube. For transportation, vials were placed in plastic bags with wet paper to maintain high humidity and reduce mortality during overnight express transport to distant sites. The fleas did not carry vaccine as we were only interested in their capacity to reproduce and persist in these trials.

Methods

The general protocol involved releasing fleas onto rabbits in experimental plots to follow their capacity to breed in the field and persist until the following year. Sampling was carried out approximately 3 months after the first flea release, while in the summer of the following year all rabbits were captured and combed to count the fleas they carried. The substrate within the warrens was scraped to recover all *X. cunicularis*, including those at early developmental stages.

Flea release

If fleas are used in a vaccination program in the field, several successive releases would be recommended. Thus, three separate releases were conducted at each site (in April, May, and June) when populations of *X. cunicularis* normally increase.[8] Each release consisted of 300 fleas (sex-ratio 50%) or about 30 fleas/rabbit/month, resulting in a total of 900 fleas in each enclosure. This number is much higher than recommended for the use of fleas carrying vaccine in nature (10/rabbit)[17] to give the fleas the chance to become established. The number of fleas released corresponded to the mean number of on-host fleas found in the wild,[8,13,36] without considering adults or immature individuals already present in the wild burrows and not present in our artificial warrens at the beginning of the study. Furthermore, as described above, there was sufficient larval diet in the warrens to ensure development of immature fleas. The first flea releases were conducted 1–2 weeks after the rabbits had been settled into the enclosures and had sufficient time to adapt to their new environment and behave normally. The fleas were released into each of the 6 burrow entrances late in the day to ensure rapid host-parasite contact and to reduce flea mortality, as rabbits are most active at dusk and dawn.[37]

Initial sampling to confirm flea breeding and survival

Xenopsylla cunicularis in France normally breeds throughout the spring season, so we sampled fleas in late July, about 3 months after the first flea release. At each site, we wished to verify whether the flea had established by determining the number of adult fleas carried by rabbits (flea index). We also sought evidence of breeding (number of adults and immature stages in burrow substrate samples).

As it was important not to disturb the experimental setup (minimizing rabbits stress and number of fleas collected), only three rabbits were randomly caught with ferrets, nets, or box-traps. The number of animals present at sampling time was estimated according to visual observations made during the experiment by technicians. The 3 sampled rabbits were immediately placed in separate plastic boxes with lids to avoid loss of fleas, which quickly leave stressed animals (Cooke B, personal communication and personal observations made during rabbit handling in our flea rearing), likely due to body temperature increase[38] (Bigler L, April 2012, personal communication). The rabbits were then intensively combed for 10–15 minutes and subsequently released. All fleas were collected using an aspirator. Fleas from each rabbit were placed in a labeled tube containing 70% alcohol. Fleas were later counted and observed in the laboratory. Other flea species, if present, were noted. Males of *X. cunicularis* were dissected to separate fed (digestive tract with blood) from unfed individuals. Females were dissected to determine their reproductive status based on the method of Launay as follows: unfed-females,

maturing fed-females (oocytes diameter < 330 µm), ovigerous females (oocytes $> 240 \times 330$ µm), and old-females.[8]

As *X. cunicularis* lives in the soil covering the floor of rabbit warrens, typically between 50 and 75 cm from the burrow entrance, we scraped the first meter of the soil from 2 galleries in each enclosure with a long handled spatula.[8,11] We chose to sample only 2 galleries (of 6) in order to not disturb experiment by removing too many fleas. When natural burrows were present, additional samples were taken. Each sample of soil was placed into 20-L plastic bags with a piece of wet paper to prevent desiccation and transported back to the laboratory using an air-conditioned vehicle (21°C).

In the laboratory, soil from each bag was carefully spread in 2-L aluminium trays to a depth of 3–4 cm. Any adult fleas present were collected and placed in tubes of 70% alcohol for sexing and dissection. The trays were then placed in a room maintained at 23°C and samples were observed 2 times per week for 2 months to recover newly hatched *Xenopsylla*. A ball of wet paper (regularly changed) maintained the humidity level between 75 and 85%. To collect emerging adults, each box was placed separately before opening in a large plastic container to prevent fleas from escaping. Adult flea emergence was stimulated by gently blowing on the substrate and shaking it slightly. Fleas collected with an aspirator were transferred to a referenced tube containing 70% alcohol for further sexing. These individuals were in immature stages (eggs, larvae, and nymphs mixed) at the time of sampling.

Final flea collection to confirm persistence over a year

At the end of the trials (ie, the second summer), all surviving rabbits in the enclosures were captured and thoroughly combed to collect all fleas carried by the rabbits. The fleas from each rabbit were stored in 70% alcohol and returned to the laboratory for classification as previously described.

The artificial warrens were opened to retrieve the substrate. The soil surface was gridded into small units of about 400 cm^2. The first centimeter of each unit was collected using trowels and placed in a referenced plastic bag as delicately as possible to preserve fragile flea eggs. The substrate was transported to the laboratory and spread in trays as previously described to establish the number of adults and immature stages present in burrows substrate. At some sites, rabbits also dug additional burrows and soil from these new sites was also sampled over 1 m lengths to determine whether they also contained significant populations of fleas. Approximately 90–100 trays per enclosure were collected.

For logistical reasons, Tarn substrate could not be processed using the same method as that used for other sites. Soil in each plastic bag was carefully spread into plastic boxes and any adult fleas present were collected into tubes of 70% alcohol for later examination as described above. The substrate was then covered with 70% alcohol to preserve all immature stages for later retrieval under a binocular microscope. As it was not possible to search through the entire substrate in this manner, trials were performed and 20% of the total was considered as representative. This sample was then gently washed and filtered (mesh 200 µm) to remove as much sediment as possible. The total number of immature fleas in the substrate collected at Tarn was estimated to be 5× the number of stages counted. Flea larvae and pupae were easily identifiable, but flea eggs were difficult to detect under a binocular microscope, resulting in underestimation of the total number of immature stages.

The total flea population (adults and immature individuals) at each site was related to the initial number of adults introduced (900) to calculate the multiplication rate (R). Such data do not reflect a natural evolution of a population because there was a very unbalanced population (neonate adults only) at the beginning of the study and a structured population with all age groups present at the end. Nonetheless, this parameter allows comparison between the different sites even if the values are not representative from a purely ecological perspective.

Climatic data

Throughout the experiment, temperature and humidity were recorded every week on the same day and approximately the same hour for each site. Measurements were taken within the same single warren as well as outside (under cover) using a thermo-hygrometer. As these measurements were not taken simultaneously for all enclosures, they were not strictly comparable. We therefore collected additional climatic data from nearby meteorological stations: temperature,

relative humidity, and total rainfall observed during the study period (meteofrance.com, www.infoclimat.fr, November 2010, and Fig. 1). In calculating climatic parameters at the time of our first check on fleas' survival and productivity we used (i) the average of weekly temperatures and relative humidity levels inside and outside the warrens, (ii) accumulated rainfall, and (iii) the average temperatures and relative humidity levels given by the meteorological station. For the final assessment, we used the same parameters calculated over the entire intervening year, as well as the average temperatures corresponding to the breeding season of the flea (from April to July).

Substrate analysis

Soil texture is an important element for *X. cunicularis* survival. As sandy soil is an optimal medium for flea development,[24] a granulometric analysis was performed from warrens substrate sample in each site by an independent agronomic laboratory (Eurofins Lara-Toulouse, France) using a sedimentation technique.

Statistical analysis

The mean number of fleas per rabbit (flea index), mean number of preimaginal stages, and adults per warrens in each enclosure were compared using one-way analysis of variance (ANOVA). The homogeneity of variances was tested before with Bartlett test.[39] Because variances were assumed to be equal and sample sizes were small, confidence intervals at 95% were established for all differences taken in pairs to evaluate for significantly different means. All analysis were performed with the software Nemrod W (Marseille, France).[40]

Results

Initial establishment of *Xenopsylla cunicularis*

Fleas on rabbits

Very few fleas were collected from the rabbits in northern sites compared to Tarn (Table 2): 2 in Loire-Atlantique (the three rabbits carried respectively 0, 1, and 1 flea), 3 in Nord (2-1-0), 5 in Marne (0-5-0), 6 in Seine-Maritime (3-0-0-3), and 21 in Tarn (8-5-8). Since rabbits survived well in the Seine-Maritime enclosure, 4 rabbits were sampled instead of the 3 planned. Flea index was significantly higher in Tarn (ANOVA, $F = 7.38$, $df = 4$, $P < 0.004$). Fleas were all fed and in Tarn, females were mainly ovigerous. In Seine-Maritime, the three *Xenopsylla* females recorded were old. Some *S. cuniculi* were collected on sampled rabbits, but less than 10 everywhere, except for Marne in which 51 were identified as some rabbit females had born young (and were therefore attractive to *S. cuniculi*) before their release in the enclosures.

Fleas in warren substrate

Few adult fleas were found in soil samples. In Nord, 3 female fleas (2 in maturation and 1 ovigerous) and in Marne, 1 male (neonate) and 2 females (1 in maturation and 1 ovigerous) were collected. In these two cases, the adults were found in the gallery of the sunniest warren. In Seine-Maritime, a female in ovarian maturation was recovered, but no individual in Loire-Atlantique. No adults hatched from soil samples thereafter. In contrast, in Tarn no adult fleas were present in the substrate, but 5 (2 males, 3 females) hatched later from the substrate.

Table 2. Initial sampling—Average climatic data for the period between the first release and sampling, and number and physiological states of *Xenopsylla cunicularis* caught on 3 sampled rabbits 3 months after the beginning of the study.

Department (Meteorological station)	Meteorological station data			INT		EXT		Rab	Flea index ± sd	Males			Females					Total fleas sampled
	T°	RH	Rain	T°	RH	T°	RH			neo	fed	Total M	neo	mat	ovi	Old	Total F	
Nord (Lille)	17.2	71	210	21.5	71	19.2	66	9	1.0 ± 1.0[a]		2	2		1	0	0	1	3
Marne (Reims)	17	76	179.4	16.8	72	21	63	11	1.7 ± 2.9[a]		1	1		2	2	0	4	5
S.-Maritime (Rouen)	15.5	81	285.4	16.5	87	17.9	81	13	*1.5 ± 1.7[a]		3	3		0	0	3	3	6*
L.-Atlantique (Nantes)	16.7	83	278.6	16.1	93	19.9	71	9	0.7 ± 0.6[a]		0	0		0	2	0	2	2
Tarn (Albi)	19	72	238.2	20.6	71	23	63	10	7.0 ± 1.7[b]		11	11		1	9	0	10	21

*Sampling performed with 4 rabbits (see text); Flea index not associated with the same letter differ at $P < 0.05$.
Abbreviations: T°, temperature (°C); RH, relative humidity (%); Rain, total rainfall (mm); INT-EXT, interior-exterior of warrens; Rab, estimated number of rabbits at sampling time; sd, standard deviation; neo, neonate; mat, in maturation; ovi, ovigerous; M, male; F, female.

Climate

Tarn stood out against the other sites with the highest average temperature (19°C) (Table 2). The four northern sites were colder, between 17.2 and 15.5°C. During the period between release and sampling, rainfall was highest in Seine-Maritime (285.4 mm with 167 mm falling in July), followed closely by Loire-Atlantique (278.6 mm with 124 mm in May). Rainfall was regularly distributed at the 3 other sites. Inside the warrens, humidity was generally higher and temperatures were lower compared to outside the warren.

Final sampling

Fleas on rabbits

Rabbits survived well in enclosures in Seine-Maritime (13), in Marne (8) and in Tarn (8), but only 4 and 5 survived elsewhere (Table 3). Compared with results from the first sampling, the final flea index of Nord, Seine-Maritime, and Loire-Atlantique decreased to nearly zero, while in contrast, the index at Marne and Tarn increased significantly. Flea numbers on rabbits were significantly higher in these areas (ANOVA, $F = 19.82$, $df = 4$, $P < 10^{-6}$).

Fleas in warren substrate

Adults found in the substrate (Table 4) confirm the results obtained by combing rabbits. There was a significant difference between Nord, Seine-Maritime, and Loire-Atlantique with rare adults and Marne and Tarn with 161 and 159 adults, respectively (ANOVA, $F = 19.39$, $df = 4$, $P < 10^{-4}$). The same strong difference was observed for the immature stages (Table 5) (ANOVA, $F = 3.85$, $df = 4$, $P < 0.04$), but in addition, Tarn (despite an underestimation) was significantly higher than Marne ($P < 0.05$), with 937 vs 264 immature stages. The sex ratio was 50 to 53% female. In Marne, the distribution of immature stages and adults between the 3 artificial burrows was heterogeneous. Indeed, most fleas were found in the warren in the highest part of the enclosure: 75% of adults and 83% of immature stages. In all enclosures in general, the driest and/or the highest burrows contained most individuals. These were used the most by rabbits. In Tarn, the most heavily used warrens also contained the most fleas.

Natural burrows

Three months after the beginning of the experiments, natural warrens were rare in the enclosures and no fleas were found (Table 6). At the end of the study, however, all enclosures (except Nord) had a small network of galleries. Additionally, in Marne and Tarn, some galleries were found to have fleas. In Marne, 12 adult fleas were found in 3 of 7 natural galleries, mainly in the highest point in the enclosure (attached to the highest artificial warren), but no individuals hatched from the soil samples collected. At the time of sampling, the soil was wetter than that inside the artificial warrens. In Tarn, 10 adults (ovigerous females only) and 81 immature stages (7% of the total preimaginal stages) were present in 2 galleries (those used most by rabbits).

Total fleas collected and multiplication rate

In Marne and Tarn, one-third of the fleas were carried by rabbits and two-thirds were in the warrens (Table 7). It is important to note that if the number of adult fleas was statistically the same in these 2 sites (see above), preimaginal stages were (at least) four times

Table 3. Final sampling—Numbers, sex ratio and physiological status of *Xenopsylla cunicularis* adults collected on all rabbits.

Departments	Rab	Flea index ± sd	Males			Females					Total fleas on rabbits	Sex-ratio (% females)
			neo	fed	Total	neo	mat	ovi	Old	Total		
Nord	4	0.3 ± 0.5^{a}	0	1	1	0	0	0	0	0	1	–
Marne	8	10.6 ± 6.0^{b}	0	30	30	0	10	41	4	55	85	65
Seine-Maritime	13	0.8 ± 0.7^{a}	0	0	0	1	2	7	1	11	11	100
Loire-Atlantique	5	0.6 ± 0.9^{a}	0	0	0	0	0	3	0	3	3	100
Tarn	8	12.4 ± 5.3^{b}	0	64	64	0	3	31	1	35	99	35

Flea index not associated with the same letter differ at $P < 0.05$.
Abbreviations: Rab, number of rabbits at sampling time; sd, standard deviation; neo, neonate; mat, in maturation; ovi, ovigerous; M, male; F, female.

Table 4. Final sampling—Numbers and physiological status of *Xenopsylla cunicularis* adults found in the substrate of the 3 artificial warrens (A-B-C).

Departments	Adults in artificial warrens substrate				Physiological status								Total adults	SR
					Males			Females						
	A	B	C	Mean ± sd	neo	fed	Total	neo	mat	ovi	Old	Total		
Nord	1	0	5	2.0 ± 2.6[a]	0	1	1	0	1	1	2	5	6	83
Marne	25	15	121	53.7 ± 58.5[b]	14	21	35	9	34	52	31	126	161	78
Seine-Maritime	1	0	2	1.0 ± 1.0[a]	1	0	1	0	2	0	0	2	3	67
Loire-Atlantique	3	3	3	3.0 ± 0.0[a]	0	0	0	0	6	3	0	9	9	100
Tarn	71	58	30	53.0 ± 20.9[b]	14	19	33	10	28	61	27	126	159	79

In a same column, means not associated with the same letter differ at $P < 0.05$.
Abbreviations: neo, neonate; mat, in maturation; ovi, ovigerous; sd, standard deviation; SR, sex ratio in % females.

Table 5. Final sampling—Numbers of immature instars found in the substrate of the 3 artificial warrens (A-B-C).

Departments	Artificial warrens			Mean ± sd	Total	SR
	A	B	C			
Nord	0	0	0	0[a]	0	–
Marne	15	31	218	88 ± 112.9[b]	264	53
Seine-Maritime	5	7	2	4.7 ± 2.5[a]	14	50
Loire-Atlantique	15	1	10	8.7 ± 7.1[a]	26	50
Tarn	585	151	201	312.3 ± 237.5[c]	937	no data see text

In a same column, means not associated with the same letter differ at $P < 0.05$.
Abbreviations: neo, neonate; mat, in maturation; ovi, ovigerous; sd, standard deviation; SR, sex ratio in % females.

Table 6. Final sampling—Numbers of immature instars, physiological status and numbers of adults of *Xenopsylla cunicularis* found in the substrate of natural galleries.

Departments	N	Ni	Total immature stages	Physiological status of adults								Total adults	Total fleas
				Males			Females						
				neo	fed	Total	neo	mat	ovi	Old	Total		
Nord	0	–	–			–					–	–	–
Marne	7	3	0	1	4	5	0	3	1	3	5	12 (2-3-7)*	12
Seine-Maritime	3	0	0			–					–	0	0
Loire-Atlantique	4	0	0			–					–	0	0
Tarn	3	2	81 (31–50)*	0	0	0	0	0	10	0	10	10 (0–10)*	91

(*) Details of the numbers of adults or immature stages found in the infested galleries.
Abbreviations: N, number of natural galleries in the enclosure; Ni, number of natural galleries infested with fleas; neo, neonate; mat, in maturation; ovi, ovigerous.

as numerous in Tarn. This explains the slight increase of *X. cunicularis* population (R = 1.4). Even if the Marne site seemed to be favorable for limited *X. cunicularis* development, the multiplication rate was less than 1 (R = 0.6). For Nord, Seine-Maritime, and Loire-Atlantique, values were close to zero.

Reproductive status and population structure of fleas

The sex ratio of fleas on rabbits and in the warrens showed a larger number of females (except in Tarn, in which rabbits carried more males) and most were ovigerous (Tables 3 and 4). Neonates

Table 7. Final sampling—Total number of *Xenopsylla cunicularis* (immature instars and adults) collected in each enclosure (from warrens + rabbits) at the end of the study, and multiplication rate (R).

Departments	Preimaginal stages in warrens substrate			Adults					Total fleas (enclosure)	R
				In warrens substrate			On rabbits	Total		
	Artif	Nat	Total	Artif	Nat	Total				
Nord	0	–	0	6	–	6	1	7	7	0.01
Marne	264	0	264	161	12	173	85	258	522	0.6
Seine-Maritime	14	0	14	3	0	3	11	14	28	0.03
Loire-Atlantique	26	0	26	9	0	9	3	12	38	0.04
Tarn	937	81	1018	159	10	169	99	268	1286	1.4

Abbreviations: Artif, artificial; Nat, natural.

were found exclusively in the warrens (with one exception in Seine-Maritime). The sex ratio of the preimaginal stages was 53% in Marne and 50% in Seine-Maritime and Loire-Atlantique (no data for Tarn, see protocol).

Climate

During the year following the first sampling, the average temperature was almost identical for the three northern-most enclosures (10.3–10.7°C), with Loire-Atlantique showing an average of 12.3°C (Table 8). The average temperature between April and July, corresponding to the expected breeding season of *X. cunicularis*, ranged from 14.2 to 15.7°C. In these 4 departments, winter nights were regularly below zero, with monthly average minimum temperatures between 6 and 8°C. The monthly average maximum temperatures ranged between 21.5 and 24°C. In Tarn, the southern enclosure, these parameters were higher: 13.3°C during the year, 17.4°C from April to July, average minimum 9.3°C and average maximum 28.4°C. Rainfall was highest in Seine-Maritime (881 mm) and lowest in Marne (660 mm). It was regularly distributed, except in Tarn where April and May (beginning of the flea breeding season) were very rainy (about 140 mm during each month).

Texture of substrates

The proportion of clay was similar at the 5 sites (Table 9), but the Marne substrate had a higher proportion of sand (54.9%). Its soil was silt-clay sand, while the others were sand-clay silt. When soil was sampled in summer, the substrate of these later enclosures was damp and, for Nord and Loire-Atlantique sites, it remained wet during the entire observation period in the laboratory.

Discussion

Overall, our results show that the trial sites in northern France, which are representative of the broad region considered, are less suitable for the persistence of *X. cunicularis*. Even during the first sampling, fleas showed poor survival compared to Tarn (control). Few fleas were recovered from either rabbits or burrows even though 900 insects were released at each site only 2–3 months earlier. At the end of the

Table 8. Final sampling—Average climatic data between the first control and the end of the study (12 months).

Departments	Meteorological station data				INT		EXT	
	t°	T°	Rain	RH	T°	RH	T°	RH
Nord	15.7	10.7	765.4	80	13.9	70	14.9	67
Marne	15.5	10.3	660	81	9.8	80	12.7	78
Seine-Maritime	14.2	10.5	881.1	81	11.3	86	12.6	78
Loire-Atlantique	15.7	12.3	785.2	81	13.1	86	16.2	69
Tarn	17.4	13.3	714.4	73	17.8	53	19.4	51

Abbreviations: t°, temperatures from April to July (°C); T°, temperature all over the whole year (°C); RH, relative humidity (%); Rain, total rainfall (mm); INT-EXT, interior-exterior of warrens.

Table 9. Texture of soils of the experimental sites: clay, sand and silt proportions (%).

Departments	Texture	Clays	Sand	Silt
Nord	sandy-clay silt	17.7	40	42.3
Marne	silty-clay sand	15.4	54.9	29.7
Seine-Maritime	sandy-clay silt	16.9	25.4	57.7
Loire-Atlantique	sandy-clay silt	13.3	41.1	45.5
Tarn	sandy-clay silt	15.2	41.6	43.1

experiments, in the artificial water-tight warrens the effective multiplication rate (R) was close to zero in Nord, Seine-Maritime, and Loire-Atlantique, reflecting a high mortality rate. In Marne, flea reproduction was observed but the multiplication rate was less than 1. Tarn only showed a slight increase in the flea population.

Previously, Launay described the ecological requirements for fleas based on their behavior and needs for development.[8,24] Adults only briefly feed on rabbits and live mainly within the warrens, burrowing into the loose surface soil of the burrow floor where they mate and lay eggs. Thus, a well-drained substrate is essential for both adults and developing larvae. Sandy soils generally provide an adequate environment except in areas with heavy rain and low temperatures, which reduces evaporation and keeps the soil damp for excessive periods, even in the summer when the soil is warm enough for larval development. According to the report of Launay,[24] *Xenopsylla cunicularis* is present in areas where the average annual temperature lies between 12.9 and 15.9°C and where rainfall averages between 280 and 660 mm per year, essentially in the late spring and early summer. Indeed, rainfall pattern is very important as continuous rainfall impedes the soil from drying, and even within its natural distribution, *X. cunicularis* populations fluctuate significantly from year to year depending on the amount and pattern of rainfall.[24,36] Low temperatures and inadequate substrates (due to soil texture and/or humidity) slow down egg laying and increase development time and mortality,[17] limiting population increases. Warren occupation by rabbits is the third-most important parameter for flea survival because it ensures food supply for egg-laying fleas.[8,24] The greater or lesser adequacy of these three factors (climate-substrate-host) will determine the ability of *X. cunicularis* to breed and persist.

In our study, the micro-climate (humidity and temperature) experienced by *X. cunicularis* within burrows in northern sites is not that required for larval development. First, the weather is too wet: rainfall is higher (765–881 mm), except in Marne where the annual average of 660 mm per year just meets Launay's suggested upper limit, and rainfall is also evenly spread throughout the year, even in Marne. Second, the average annual temperature is too low (between 10.3 and 12.3°C), reducing egg-laying and increasing development time. It is likely for this reason that in Seine-Maritime, females collected in the first control were old. These fleas had likely came from the last release in June due to the low average temperature observed during this first phase (15.5°C). They could neither have belonged to a first generation nor have completed their reproduction cycle. Low temperatures also explain why in Marne, immature stages in burrows soil were 4 times less numerous than in Tarn.

Warren substrate was also found to not be suitable. The proportion of silt and clay was too high to form a well-drained soil. This was particularly prominent in Nord and Loire-Atlantique, where the soil was very wet even in the summer. This observation is in accordance with Cooke's report which shows that burrows substrate remains humid throughout the year when annual average rainfall exceeds 600 mm.[25] One exception was that in Marne sand and low rainfall predominated, explaining better flea productivity. In this department, it is important to take into account the sloping configuration of the enclosure and that about 80% of adults and immature stages were in the artificial burrow at the highest, best-drained area of the enclosure. However, even this was not sufficient for the successful development of *X. cunicularis* if we consider that no fleas emerged from samples in the wetter natural holes associated with the best-drained warren, while few ovigerous fleas were present at the time of sampling. They likely came from the artificial burrow, with its water-tight top, leading to better flea survival due to better protection from rain. It is likely for the same reasons that flea development was successful in artificial warrens of Tarn. The small population found in natural galleries dug by rabbits shows that environmental conditions are not the most suitable (silty substrate, soil too wet). It seems more likely that these fleas also reinfested from artificial

burrows rather than being a population which had overwintered into the galleries.

Host availability was not a limiting factor in our protocol. However, rabbit mortality observed in Nord and Loire-Atlantique sites at the end of the experiment could have been unfavorable because of reduced use of the burrows. Nevertheless, considering climate and substrate, a larger number of hosts would likely not change the results, as in Seine-Maritime where there were 13 rabbits present throughout the study.

The reproductive status of the flea population in all experiments corresponds to the values observed in the wild for the majority of fleas.[15] The proportion of females is important, especially in the warrens. This is likely due to a more rapid mortality of males, as observed in our laboratory colonies. It is also true that they grow more slowly than females[17] and they could be as larvae or nymphs in warrens. However, the adults sex-ratio collected from the substrate is balanced (50–53%) and not in their favor. In addition to being more numerous, females are also mostly ovigerous, which is characteristic of breeding populations.[8] Additionally, one-third of the fleas were found on rabbits and two-third in the warrens: these numbers are consistent with the data of Cooke[12] and observations made in our laboratory rearing. The standard deviation of flea index observed in all experiments was probably related to the fact that rabbits living in warrens poorly infested by fleas may be less parasitized than others.

In the 4 northern enclosures, our results showed that flea populations are bound to disappear more or less rapidly. Fleas were only recovered from the artificial warrens which were better water-proofed than natural warrens; no adult *Xenopsylla* emerged from substrates collected from natural borrows. In the wild, without this added protection, we argue that their extinction would be faster. In addition, the protocol of this study deliberately favored fleas, at least in terms of their food resources (high rabbits density, powdered food for larvae) and the large number of adult fleas initially released (ie, large number compared with the quantity that would be recommended for vaccinating rabbits, not compared to the numbers occurring in the wild).[8,13,17,36]

It was not possible to repeat these experiments over several years to estimate the impact of climatic variations, but existing climatic data[30] (and meteofrance.com) confirm that climate is generally unsuitable for *X. cunicularis*. These results give a clear indication that the risk of *X. cunicularis* development in the whole northern half of France is extremely low due mainly to low temperatures and rainfall evenly distributed throughout the year.[30] In the northeastern region of France, *X. cunicularis* would experience a climate even colder and wetter than in tested sites, making flea persistence unlikely. In the central region, climatic conditions are temperate as in Marne, but we saw that even with a sandy substrate flea development was not possible in natural warrens. In the western region, temperatures are warmer due to oceanic influences, but rainfall is also heavier, as in Loire-Atlantique. Even with a sandy substrate, often encountered in coastal borders, these characteristics would not allow for flea development and successful establishment.

Natural water-tight warrens similar to Marne could be found in northern France only if local topography and substrate allowed for good soil drainage and most importantly if they were dug under structures that did not permit rain to penetrate in the ground (construction, abandoned sheet metal, etc). Such conditions are scarce but, nevertheless, what would happen if few fleas temporarily persisted in northern France? Their spreading risk seems unlikely as fleas carried away by rabbits, accidental hosts as predators, or other burrowing mammals[15] would survive and establish only if they were deposited in a suitable rabbit hole for the reasons observed in this study. Due to its high specificity and strict ecological requirements, spreading of *X. cunicularis* would be much more difficult than that observed since 2000 for the Ixodid ticks *Ixodes ricinus* and *Dermacentor reticulatus* in Europe.[41] Competition with other rabbit flea species, eg, the widely spread and well-adapted flea *Spilopsyllus cuniculi*, is unlikely as they do not share the same ecological niche. Concerning disease transmission, one could argue that released *X. cunicularis* may increase transmission of existing pathogens in rabbit populations. The major transmitted disease of veterinary importance in rabbits is myxomatosis. It is therefore important to come back to the initial reason of this work, which is the use of *X. cunicularis* as potential vaccine vector against myxoma virus (and RHDV). Transmission risk of this lethal disease

is though unlikely among vaccinated populations in which this flea would be present. According to our observations, the likely benefits linked to vaccination in rabbit management and conservation programs significantly outweigh the risks of an unlikely establishment of *X. cunicularis*.

Thus, our implantation trials suggest that this flea can be released with minimal risk of unforeseen impact in northern France. These preliminary results should be confirmed in warmer and drier conditions in southern France, where this species could find potentially favorable areas.

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Author Contributions

Conceived and designed the experiments: ADV. Analysed the data: ADV. Wrote the first draft of the manuscript: ADV. Contributed to the writing of the manuscript: AA. Agree with manuscript results and conclusions: ADV, AA, PB. All authors reviewed and approved of the final manuscript.

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As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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