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Artificial Irrigation Impacts the Seasonal Occurrence of Pathogenic *Leptospira* in Its Wild Reservoirs in a Mediterranean Environment

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ABSTRACT

Background: Human activities play a significant role in the emergence of infectious diseases. We aimed to test whether artificial irrigation affects the occurrence of a zoonotic bacteria sensitive to desiccation, pathogenic *Leptospira* species (pathoLep), in micromammals inhabiting Mediterranean ecosystems.

Methods: A total of 361 individuals, including 217 Algerian mice (*Mus spretus*), 79 wood mice (*Apodemus sylvaticus*), and 65 greater white-toothed shrews (*Crocidura russula*), were captured during the four seasons of 2022 in six sites along a riparian forest close to a large city in north-eastern Spain and the surrounding agricultural fields, which are irrigated by flooding. A piece of kidney from each individual was analysed by means of two real-time PCR protocols targeting the *lipL32* gene, which is exclusively found in pathoLep. Generalised Linear Models were used to study the factors that may be related to the presence of pathoLep.

Results: DNA of pathoLep was detected in 28% of the individuals, a relatively high occurrence compared to similar studies. The best model for the general micromammal population included four significant factors: season, age, species, and habitat. Prevalence was significantly lower during the dry seasons; in juveniles than in adult individuals; in the wood mouse than in the Algerian mouse and the shrew; and in natural than in agricultural habitats. Prevalence was consistently higher in agricultural habitats during all the seasons, reaching over 55% prevalence in these areas during spring. For the core species, the Algerian mouse, the best model included two factors (seasons and habitat), in the same sense as the general population model.

Conclusions: This study shows that pathoLep are widespread among micromammals in the Middle Ebro Valley and that their occurrence is shaped by intrinsic and extrinsic factors. We identified a human activity (artificial irrigation) as an important driver favouring leptospiral survival in rural environments.

1 | Introduction

Human activities have played a significant role in the emergence of infectious diseases, often driving the spread of pathogens into new areas and populations. Deforestation, urbanisation, and agricultural expansion have altered ecosystems, increasing human-wildlife interactions and facilitating zoonotic disease transmission (Jones et al. 2008). These human-driven factors

Impacts

- This study investigated how artificial irrigation affects the prevalence of pathogenic *Leptospira* in micromammals from Mediterranean ecosystems in north-eastern Spain.
- Leptospiral DNA was detected in 28% of individuals, with higher prevalence in irrigated agricultural habitats than in natural environments. Prevalence was also affected by season, host species, and age.
- The findings highlight artificial irrigation as a key human-driven factor promoting *Leptospira* survival, emphasizing the impact of land-use practices on zoonotic disease emergence.

have transformed the landscape of disease emergence, highlighting the need for a more integrated approach to public health and environmental conservation.

Leptospirosis is a globally significant zoonotic disease caused by pathogenic *Leptospira* spp., characterised by a strong interrelationship between humans, animals, and the ecosystem (Caimi and Ruybal 2020). This infection has a considerable impact on both public and animal health, as it affects not only humans but also domestic and wild animals, with the latter two serving as reservoirs (Levett 2015). It is estimated that leptospirosis causes over one million severe cases and 60,000 deaths annually worldwide (Guglielmini et al. 2019; Schmidt et al. 2021). Traditionally, human cases have been associated with workers in sectors such as agriculture and livestock farming, as well as residents of rural areas with poor hygiene and sanitation conditions. The disease is particularly prevalent in wet tropical and subtropical regions with heavy rainfall, frequent floods, and stable temperatures (Haake and Levett 2015; Mazzotta et al. 2023). In developed countries, high-risk groups for leptospiral exposure include environmental workers such as wetland farmers, red crab harvesters, livestock farmers, veterinarians, and sewage workers (Shieh et al. 1999). However, in recent years, the source of infection has shifted, with an increasing number of leptospirosis cases linked to sports activities that involve exposure to water sources, such as triathlons, kayaking, open-water swimming, and canyoning (Harran et al. 2024; Mazzotta et al. 2023; Millán et al. 2019).

Additionally, leptospirosis causes economic losses in the livestock sector, as leptospiral infection can lead to abortions and reduced milk production (Pearson et al. 1980; Boey et al. 2019; Guglielmini et al. 2019). Synanthropic species, such as small and micromammals from various genera, are the most important carriers and reservoirs of pathogenic leptospires (Mazzotta et al. 2023; Millán et al. 2018). In such carriers, once the bacteria reach the lumen of the proximal renal tubules in the host, they establish themselves along the epithelial border. There, they can multiply and continue to be excreted into the environment through urine for the host's entire lifetime without causing severe pathological lesions (Ko et al. 2009; Adler and de la Peña Moctezuma 2010; but see Imlau et al. 2025). After excretion, leptospires can survive for weeks in water and on moist surfaces in temperate environments with neutral or slightly alkaline pH conditions (Fraga et al. 2015). In contrast, incidental hosts—such as humans or dogs—typically develop an acute illness after infection. They rarely develop persistent renal colonisation and are not considered major sources of environmental contamination (Adler and de la Peña Moctezuma 2010). Although numerous mammals can be infected by pathogenic leptospires, the relative contribution of a specific host to environmental contamination depends primarily on the host-pathogen interaction, as well as on the interaction between animal populations and their environment. In this regard, studies have shown that the prevalence of *Leptospira* in wildlife can vary depending on the season, geographic distribution, and annual precipitation (Mazzotta et al. 2023; Millán et al. 2018). Floods caused by heavy rainfall have often been linked to an increase in leptospirosis incidence in affected regions (Holt et al. 2006; Togami et al. 2018). Irrigation water also plays a key role as a reservoir for leptospires (Gamage et al. 2020). However, a study conducted on micromammals in peri-urban Barcelona found no significant differences in *Leptospira* prevalence between natural and residential areas, which are artificially irrigated (Millán et al. 2018).

The middle Ebro valley, located in the Autonomous Community of Aragón, is characterised by its Mediterranean continental climate, with rainfall localised in spring and autumn, and annual thermal rhythms, with hot summers and cold winters (https://idearagon.aragon.es/atlas/Aragon/info/medio-natural). A serological survey performed among students of the Veterinary Faculty of Zaragoza revealed antibodies in about 10% of the studied population (Simón et al. 1999). Since *Leptospira* cannot survive on arid surfaces (White et al. 2000), a low survival rate of the spirochetes would be expected in this valley during the dry season. However, in this region, flood irrigation is a common technique, accounting for 46% of the irrigation, according to the 2018 survey by the National Institute of Statistics on water use in the agricultural sector. Therefore, we hypothesize that, in the study area, artificial irrigation may benefit the survival of *Leptospira*, resulting in an increased prevalence in the micromammals inhabiting such areas. In consequence, the objective of the present study was to compare the occurrence of pathogenic *Leptospira* when controlling for other intrinsic and extrinsic factors potentially affecting such occurrence. We predict that occurrence will be higher in adult individuals when compared to juveniles due to a longer time of exposure; i-n males than in females, due to larger home ranges and/or the immunosuppressive effect of testosterone; and in animals captured in wet seasons and irrigated areas due to an enhanced bacterial survival.

2 | Material and Methods

2.1 | Study Area and Field Methods

Saragossa (Zaragoza) is a city of approximately 700,000 inhabitants located in north-eastern Spain. We selected six riparian forests along the Ebro River on the city's periphery (41°36′16″ N, 0°49′21″ W; Figure 1). The ecosystems of the Ebro River are highly dynamic, characterised by significant fluctuations in water levels depending on the season. These forests are characterised by ash trees (*Fraxinus* sp.), black poplars (*Populus* sp.), willow trees (*Salix* sp.), tamarisk shrubs (*Tamarix gallica*), and common reed grasses (*Phragmites australis*). We also selected six agricultural fields adjacent to the limits of these forests.

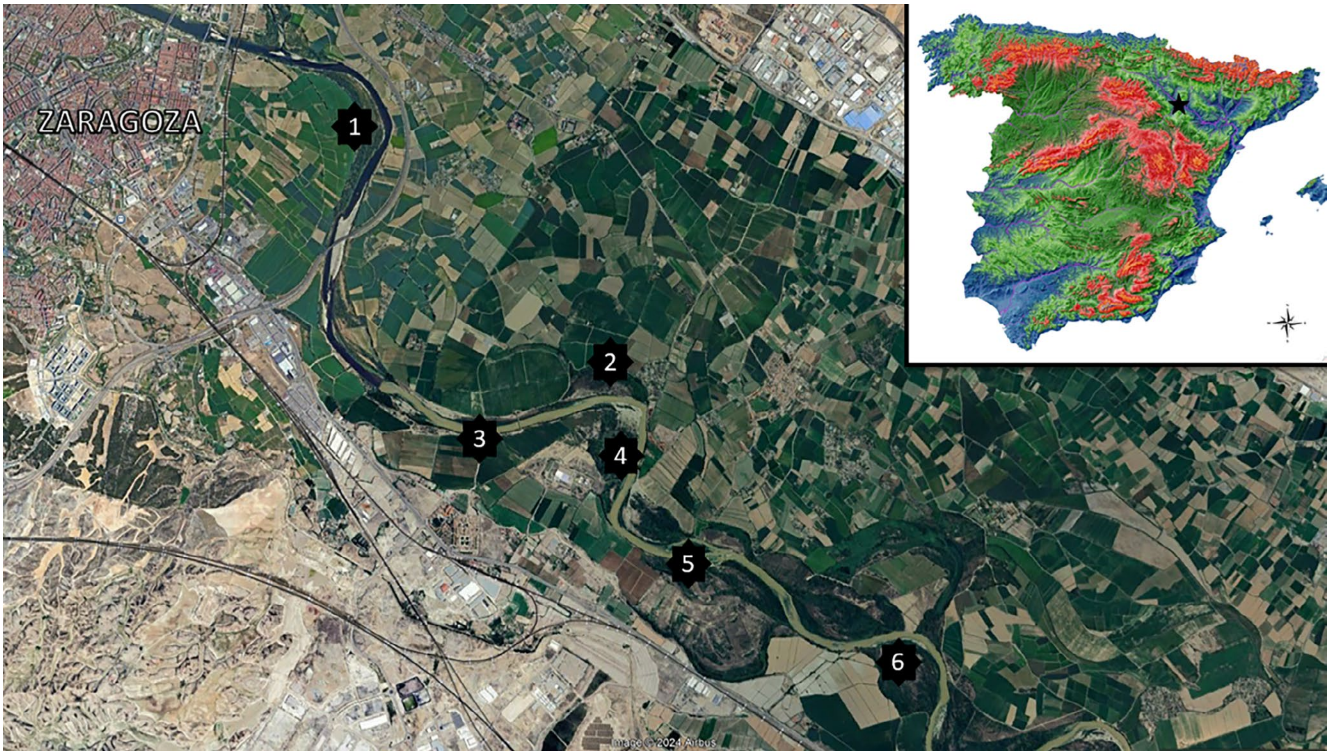

FIGURE 1 | Map of the study area, showing the six capture sites along the Ebro River.

These fields, devoted mostly to alfalfa with some scattered plantations of corn and barley, are artificially irrigated using the flooding method from April to October. Micromammals were livetrapped during 2022 using 25 Sherman traps (H.B. Sherman Traps Inc., Tallahassee, Florida) spaced every 10 m in the two habitats (natural and agricultural) per sample area. In the agrarian habitats, traps were placed in the vegetation-covered edges between fields. A mixture of wheat flour and vegetable oil was used as bait, and a piece of hydrophobic cotton as nesting material. Traps were set up in the morning and inspected after 24 h for 4 consecutive days. The total capture effort amounted to 4800 trap/nights. Animals were transferred without handling to a plastic bag and weighed using a Pesola scale to the nearest 0.5 g and anaesthetised via intraperitoneal with a combination of ketamine (Domtor, Esteve, Barcelona, Spain) and medetomidine (Imalgene, Merial, Barcelona, Spain; Chirife and Millán 2014). Animals were then euthanized by bleeding and necropsied in detail, sexed, aged, and measured. A kidney was obtained from each individual, and was stored at −21°C the same day until processing. A total of 361 individuals of three different species were captured: 217 Algerian mice (*Mus spretus*), 79 wood mice (*Apodemus sylvaticus*), and 65 greater white-toothed shrews (*Crocidura russula*) (Table 1).

TABLE 1 | Number of individuals captured in each habitat as a function of the season and the species.

			Habitat	
		Total	**Agricultural**	**Natural**
n		361	227	134
Season	Spring	42	27	15
	Summer	69	45	24
	Autumn	191	111	80
	Winter	59	44	15
Species	Algerian mouse *Mus spretus*	217	167	50
	Wood mouse *Apodemus sylvaticus*	79	28	51
	White toothed shrew *Crocidura russula*	65	32	33

2.2 | Laboratory Methods

Genomic DNA was extracted from kidney tissue samples using the Speedtools tissue DNA Extraction Kit (Speedtools DNA Extraction Kit, Biotools B&M Labs. S.A) following the manufacturer's instructions. This kit is based on the binding of DNA to the silica particles of the column membrane once cell lysis has occurred during the incubation of the sample with proteinase K/SDS. Subsequently, successive washes of the column are performed with wash buffers to remove any contaminants that may be present in the sample. Finally, the DNA is specifically eluted in a buffer with a slightly alkaline pH and low ionic strength. Two consecutive final washes were performed on each column with 50 μL of the final BB3 buffer, obtaining 100 μL elutions from each sample, which were analysed using two real-time PCR protocols. The same elution was evaluated with both molecular assays.

2.2.1 | VIASURE *Leptospira* Real-Time PCR Kit

This lyophilized kit is an assay that allows for the real-time PCR detection of the *lipL32* gene specific to pathogenic *Leptospira* species. Each reaction had a total volume of 20 μL, consisting of 15 μL of master mix and 5 μL of extracted DNA from the sample. A total of 361 DNA samples were analysed across five different PCR runs. The amplification protocol consisted of an initial step of 2 min at 95°C followed by 45 cycles (10 s at 95°C, 50 s at 60°C). Amplifications were carried out in real-time thermocyclers, CFX-96 OPUS (Bio-Rad, USA), and AriaMX (Agilent, USA). Fluorescence detection was carried out using the FAM channel for the detection of the *lipL32* gene and the HEX channel for the detection of the internal DNA control. The analysis of amplification curves was performed using the built-in software of the respective thermocyclers, and result interpretation was conducted following the VIASURE manufacturer's instructions.

2.2.2 | BactoReal Kit *Leptospira* spp.

The multiplex BactoReal Kit *Leptospira* spp. (Ingenetix GmbH) is a real-time PCR kit that allows for the detection and differentiation of the *lipL32* gene from pathogenic *Leptospira* species and the *16S* rRNA gene from both pathogenic and intermediate *Leptospira* species. Samples positive for the VIASURE *Leptospira* Real Time PCR detection kit were individually analysed with the BactoReal device. A 20 μL reaction volume was used, consisting of 11 μL of Master Mix and 9 μL of DNA from the positive samples for the VIASURE *Leptospira* kit. In the case of samples that were negative for VIASURE *Leptospira* kit, they were analysed in pools of ten samples; for this, 3 μL of eluate from each was added, loading 5 μL from each pool for PCR analysis. The amplification protocol consisted of 2 min at 50°C, followed by a 2-min program at 95°C and a third program of 45 cycles (5 s at 95°C, 60 s at 60°C). Amplifications were carried out in real-time thermocyclers, CFX-96 OPUS (Bio-Rad, USA) and AriaMX (Agilent, USA). The amplification channels for the detection of the different targets were FAM for *lipL32* detection, HEX for *16S rRNA*, and Cy5 for the detection of the internal positive DNA control.

For both kits, a positive-control sample and a negative-control sample provided by each kit were used in each run. Data analysis was performed using the Agilent AriaMx 2.0 and Bio-Rad CFX Maestro software, considering samples with Cq-values lower than 38 as positive for BactoReal kit and Cq-values lower than 40 for VIASURE *Leptospira* kit.

2.3 | Statistical Analyses

Because the dependent variable (observed prevalence) was binary (absence/presence) and fixed and random effects were incorporated, Generalised Linear Models (GLMs) were used to study the factors that may be significantly related to the response variables. The full models were fitted containing the following fixed effects: species (wood mouse/Algerian mouse/shrew), age (juvenile/adult), habitat type (natural/agricultural), and season (spring/summer/autumn/winter). For the individual GLMs of each species, the following fixed effects were established: habitat type, season, and sex. Age was excluded due to the low number of juveniles. Prevalence was analysed using the "aggregate" function from the "stats" package, which is included by default in the R software. The best model was selected using the "glm" function from the "stats" package in R, specifying a binomial distribution, generating a set of models with combinations of terms from the full model. To select the best model, the "step" function was applied, which performs an automatic variable selection based on the Akaike Information Criterion (AIC), with the best-fitting models being those with the lowest AIC. All the analyses were performed in R software (version 4.3.2; R Core Team 2023).

3 | Results

DNA of *Leptospira* sp. (*lipL32* gene) was detected in 27.9% (95% CI: 23.3%–32.6%) of the 361 samples analysed, with a prevalence of 30.4% (95% CI: 24.3%–36.5%) in the Algerian mouse, 15.2% (95% CI: 7.3%–23.1%) in the wood mice, and 35.4% (95% CI: 23.8%–47%) in the shrew. A 99.16% concordance was obtained in the real-time PCR analysis results for the detection of pathogenic leptospires with the VIASURE and BactoReal assays. One sample was invalid for BactoReal because there was no amplification in any channel, which could have been due to contamination of the DNA eluate or a failure in the preparation and dispensation of the master mix. Similarly, this error was observed in two samples analysed with VIASURE device. The Cq-values obtained in the study were low. Specifically, in 61.4% of the positive samples (62/101), the Cq was less than 30, indicating a high bacterial load in the samples.

The best model that explained the pathogen prevalence in the micromammal community ($n = 311$) included four significant factors: season, age, species, and habitat (Table 2, File S1). Prevalence in the wood mouse was significantly lower than in the Algerian mouse and the shrew (Figure 2, File S1). A lower prevalence was observed in young individuals (14.6%), being twice as high in adult individuals (34.1%) (Figure 3, File S1). Prevalence was also lower in summer (18.2%) and autumn (21.4%) than in spring and winter (43%). Furthermore, the prevalence of *Leptospira* also varied significantly depending on habitat type, with a higher prevalence in agricultural habitats (34.9%) compared to natural habitats (13.3%). Prevalence in agricultural habitats was consistently higher during all the seasons (Figure 4).

The best model for the most abundant species, the Algerian mouse ($n = 213$), included two significant factors in the presence of leptospires: habitat and season (File S1). The prevalence of *Leptospira* ranged from 23.4% to 56.2% across different seasons, with spring and winter being the seasons with the highest prevalence. A prevalence four times higher was observed in agricultural habitats (37.6%) compared to natural

TABLE 2 | Results of the Generalised Linear Model (GLM) with binomial distribution for micromammals. The influence of species, age, season and habitat on the individual probability of infection is observed. Bold values are significant factors.

Factor	Estimate	Std. Error	z-Value	p
Intercept	−0.8498	0.4701	−1.808	0.07065
Species-White toothed shrew	1.2102	0.5992	2.020	**0.04343**
Species-Algerian mouse	1.0867	0.4247	2.559	**0.01050**
Age-Juvenile	−1.2506	0.3984	−3.139	**0.00170**
Age-Subadult	−0.7642	0.6991	−1.093	0.27431
Season-Autumn	−0.5176	0.3953	−1.309	0.19042
Season-Spring	0.3996	0.4664	0.857	0.39162
Season-Summer	−1.2385	0.4635	−2.672	**0.00754**
Habitat-Natural	−0.8959	0.3633	−2.466	**0.01368**

Abbreviation: Std Error = Standard Error.

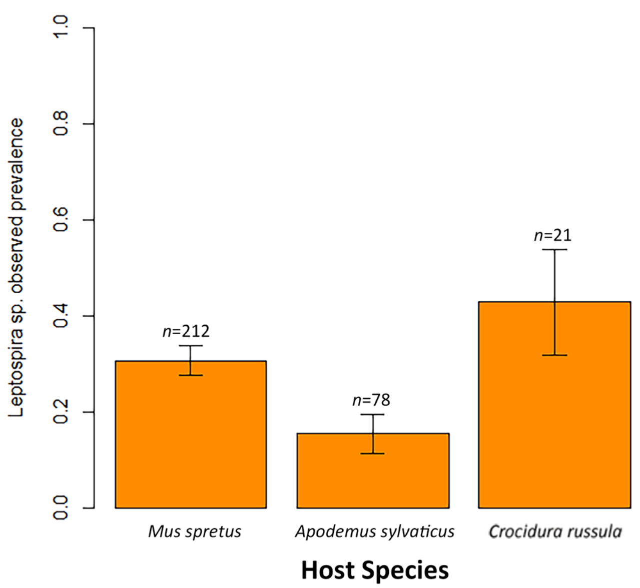

FIGURE 2 | Occurrence of pathogenic *Leptospira* depending on the species. Different letters indicate significant differences among species.

habitats (8.3%) (File S1). For the wood mouse ($n = 78$), season was the only significant factor, with spring and winter being the seasons with the highest prevalence (File S1). Although there was a tendency for higher prevalence in agricultural habitats and during spring and winter, no significant factors were identified for the prevalence in shrews, likely due to the small sample size ($n = 62$; File S1).

4 | Discussion

Considering the crucial role of micromammals in the environmental circulation of leptospires, understanding the prevalence and the factors that may impact their survival and prevalence in the hosts will help in establishing prevention and control measures for leptospirosis tailored to each specific context. Although we have not identified the *Leptosira* species or serovars infecting the studied micromammals, these are pathogenic *Leptospira* because both tests are designed to amplify the *lipL32* gene. The sequence and expression of this outer membrane protein are highly conserved in pathogenic leptospires, making it one of the most abundant proteins in this group of leptospires (Haake et al. 2000). Previous studies have shown that micromammals are most commonly infected by serovars such as Icterohemorrhagiae, Grippotyphosa, Ballum, Copellhageni, and Sejroë (Humphreys et al. 1953; Brockie 1977; Stanko et al. 1996). In fact, these serovars accounted for 95% of cases in a micromammal community composed of the same three species captured herein in another Mediterranean study site (Millán et al. 2018), and they are expected to be the most prevalent in this study area as well. Nevertheless, for the purposes of this study—specifically, to assess whether artificial irrigation influences the occurrence of pathogenic *Leptospira* in a wild reservoir—we believe that the lack of serovar identification does not undermine the epidemiological results obtained herein. It is also worth noting that the processed tissues were renal samples, confirming that the captured individuals were not only infected with leptospires but were also in a phase of bacterial shedding through urine, thereby perpetuating the presence of pathogenic leptospires in the environment.

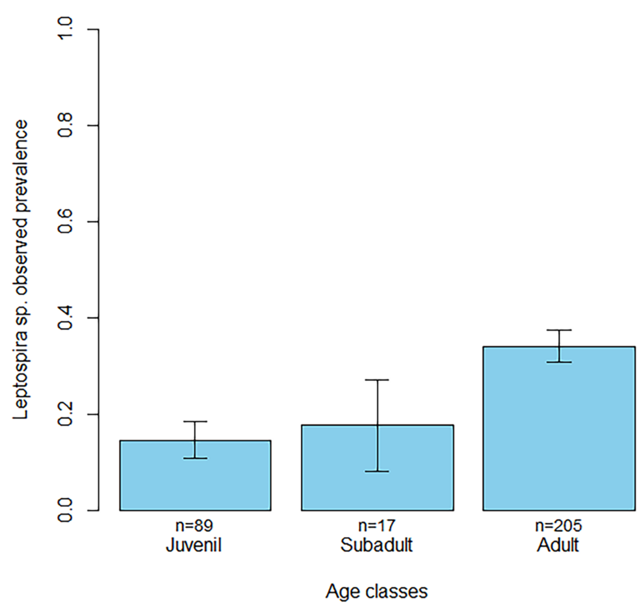

FIGURE 3 | Occurrence of pathogenic *Leptospira* depending on the age group. An asterisk denotes significant differences among age groups.

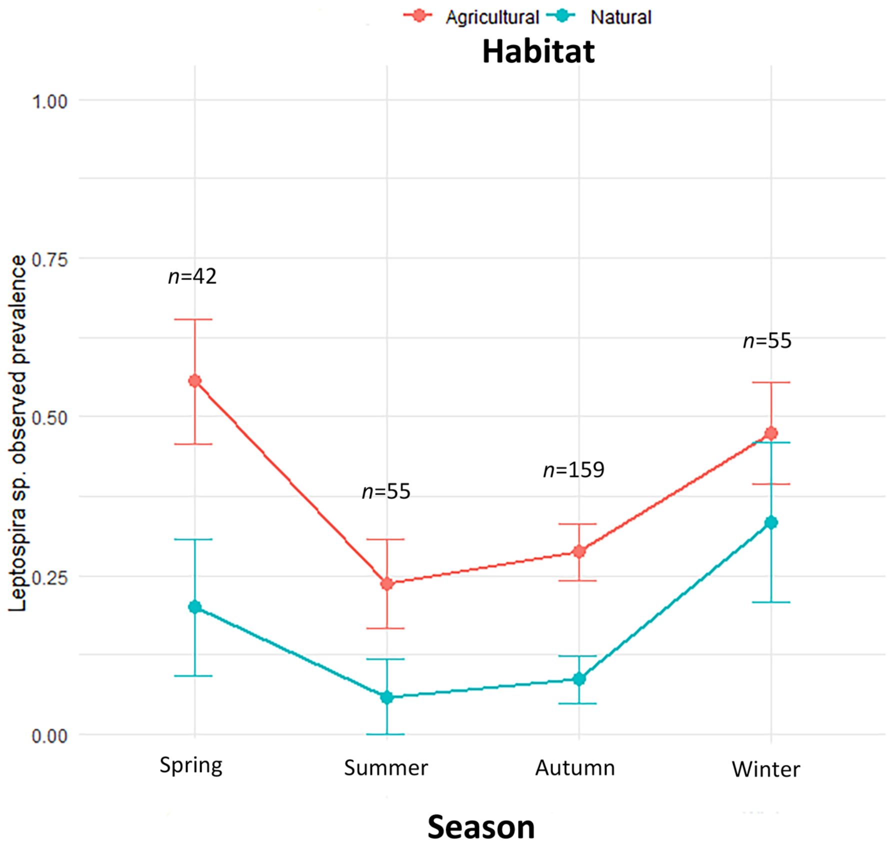

FIGURE 4 | Occurrence of pathogenic *Leptospira* depending on the season and the type of habitat (agricultural vs. natural). Statistical differences were found among seasons (lower in summer) and type of habitat (higher in agricultural).

This study found a mean prevalence of pathogenic *Leptospira* of approximately 28%, with levels exceeding 55% in agricultural areas during the spring. These rates are notably higher than those previously reported in small mammals from areas with similar climates, such as peri-urban zones of Barcelona in Spain (12%) (Millán et al. 2018), Italy (19%) (Mazzotta et al. 2023), and regions with wetter climates like France (11%) (Garcia-Lopez et al. 2024) and Germany (21%; Jeske, Emirhar, et al. 2021; Jeske, Jacob, et al. 2021). It is important to note that these differences could be attributed to variations in small mammal species across studies (Garcia-Lopez et al. 2024), differences in habitat characteristics, or seasonal sampling biases (Millán et al. 2018). Among the species studied, shrews exhibited a prevalence four times greater than that observed in urban park populations in Lyon (10%) (Garcia-Lopez et al. 2024) and in diverse European countries (12%) (Haring et al. 2023); five times higher than that reported in peri-urban areas of Barcelona (8%) (Millán et al. 2018); but very similar to the prevalence recently reported in France (35% by immunohistochemistry) (Imlau et al. 2025). Additionally, the prevalence in the Algerian mouse was nearly three times higher than that seen in Barcelona (Millán et al. 2018), while the prevalence in the wood mouse aligned with figures reported in other European populations (Garcia-Lopez et al. 2024; Jeske, Emirhar, et al. 2021; Jeske, Jacob, et al. 2021; Millán et al. 2018).

Our findings confirm previous observations regarding the strong seasonality of pathogenic leptospirosis in wildlife. Given that *Leptospira* spp. cannot survive in arid conditions (White et al. 2000), the lower prevalence observed in summer and autumn across the three species was expected. This is due to the reduced survival rate of the spirochetes during the warmer summer months, which coincide with the highest annual temperatures in Mediterranean climates. This seasonal decline in prevalence was previously noted in wild small mammals on the outskirts of Barcelona (Millán et al. 2018). Other studies have also documented significant seasonal variations, though with a higher prevalence in autumn compared to spring. This difference can be attributed to regional climatic variations, which affect soil moisture and temperature (Garcia-Lopez et al. 2024). Additionally, this seasonal pattern may be influenced by the 'juvenile dilution effect,' observed in bank vole (*Clethrionomys glareolus*) populations in Germany. This effect is linked to seasonal shifts in population composition, where infected adults that survive the winter transmit leptospires to the population in spring. As the juvenile population increases in summer, prevalence tends to decrease (Schmidt et al. 2021).

On the other hand, and in line with the proposed prediction, a significant difference was observed between natural and agricultural habitats across all four seasons. This difference is likely linked to the availability of water in irrigated agricultural areas, sustained by irrigation channels from spring to autumn (Jeske, Emirhar, et al. 2021; Jeske, Jacob, et al. 2021). It is important to note that the natural areas studied are part of a riparian ecosystem, which is inherently wet. However, the ecosystems along the Ebro River are highly dynamic, with fluctuating water levels that impact the distribution of plant and animal species, as well as other organisms like the leptospires studied here. In contrast to our findings, a previous study in peri-urban areas of Barcelona did not detect differences between natural and residential areas (Millán et al. 2018). The discrepancy between the studies may stem from the fact that artificial humidity levels in residential areas are lower than those created by flood irrigation.

Age was recognised as another risk factor for the presence of *Leptospira* spp. While there are few studies on leptospiral detection that account for the host's age, our results align with those of previous studies (Jeske, Emirhar, et al. 2021; Schmidt et al. 2021; Treml et al. 2012). This is likely due to the longer exposure time of adult individuals to the pathogen: as the host's age increases, so does the likelihood of infection (Schmidt et al. 2021). Sex might be an additional demographic factor influencing *Leptospira* prevalence. Males might be more exposed to the bacteria due to the immunosuppressive effect of androgens or by their behaviours (aggressiveness, dispersal, foraging). However, our study, in concordance with previous studies, did not find a significant effect of sex on *Leptospira* prevalence (Cortez et al. 2018; Jeske, Emirhar, et al. 2021), supporting the theory that sex does not affect the probability of being a carrier of leptospires.

In conclusion, this study shows that pathogenic *Leptospira* are widely distributed among micromammals in the Middle Ebro Valley, with their occurrence influenced by both intrinsic and extrinsic factors, including human activities. From a public health perspective, this is a crucial finding, as the *Leptospira* species detected are pathogenic. In addition to observing differences in occurrence among species, most of the initial hypotheses were confirmed, with individual infection probability being influenced by season, habitat, and host age. Healthcare workers should be aware that certain at-risk groups—such as farmers, hunters, environmental workers, as well as canoeists and fishers—may be exposed to pathogenic *Leptospira* in areas of the Middle Ebro Valley.

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Ethics Statement

Capture was authorised by Gobierno de Aragón under permit 500201/24/2022/07284. The study was granted ethical approval by the bioethics committee of Universidad de Zaragoza (PI16/22).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** zph70043-sup-0001-DataS1.docx.