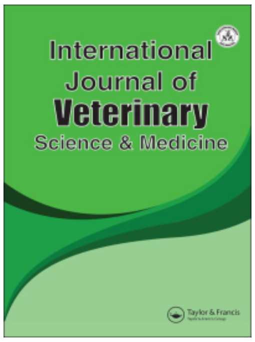

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Distribution, identification and ecology of *Phortica* genus (Diptera: Drosophilidae) in Spain

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ABSTRACT

The genus *Phortica* (Diptera: Drosophilidae) includes five species of small flies in Europe. *Phortica variegata*, the zoophilic fruit fly, is the main vector of *Thelazia callipaeda*, a zoonotic parasite that is rapidly spreading througout Europe. Despite extensive studies on thelaziosis in animals and humans, there is limited knowledge about the geographical distribution and hovering activity of these vector flies. In 2023, 1,462 *Phortica* flies were sampled across 12 Spanish provinces, providing new records of *Phortica variegata* and *Phortica oldenbergi*. Surprisingly, *P. oldenbergi*, previously considered a rare Afrotropical species, was prevalent in most regions sampled in Spain. However, *Phortica semivirgo* was not collected. The abundance of *Phortica* spp. correlated positively with altitude and certain tree species. Rural oak-wooded areas in central and northern Spain showed the highest densities of *P. variegata*. Both drosophilid species were analysed morphologically and molecularly, providing new morphological descriptors and sequence barcodes for species identification. Phylogenetic analysis based on COI sequences, showed *P. oldenbergi* grouped with Asian origin *Phortica* species, while *P. variegata* in America was closer to Spanish sequences than those from other European countries. The hovering activity of *P. variegata* causes significant discomfort to humans during outdoor activities. This paper also reviews the historic records of *P. variegata, P. semivirgo* and *P. oldenbergi* in Spain over the last 90 years. This study enhances the understanding of the distribution, identification, ecology, and behaviour of these zoophilic flies in Europe.

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1. Introduction

The Drosophilidae family is a diverse and cosmopolitan group of flies, which includes some species commonly known as fruit flies, vinegar flies or pomace flies. Notably, male adults within the Steganinae subfamily (including *Amiota* spp., *Apsiphortica* spp., and *Phortica* spp.) not only feed on fermenting substrates (such as sup runs), but also exhibit a unique zoophilic behaviour known as "lacryphagy". This involves feeding on the lachrymal secretions (tears and eye fluids) and occasionally sucking perspiration from mammals [1,2].

In Europe, only five species of the genus *Phortica* have been recorded. While *Phortica variegata* and *Phortica semivirgo* are widely reported in many European countries, *Phortica erinacea* [3], *Phortica goetzi* [4], and *Phortica oldenbergi* are rarely captured and have a more restricted distribution [4–6]. In Spain, three species, *P. variegata, P. semivirgo,* and *P. oldenbergi,* have been identified to date [7]. However, most of the records of the genus *Phortica* in the country are primarily published in local journals, digital platforms or remain as grey

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literature difficult to access. Therefore, there is an urgent need to update this information for accurate predictions.

Phortica flies, particularly *P. variegata*, are recognized as a nuisance to both animals and humans [2,5,8]. *Phortica variegata* is the most widely distributed species and is native to the Palearctic region. Males primarily (females rarely) feed on the lacrimal secretions and cutaneous transpiration (sweat) of domestic dogs, cats, several wild carnivores, hares, and humans [2,3,9]. During this feeding process, they can transmit the parasitic nematode *Thelazia callipaeda* , 1910 (Spirurida: Thelaziidae), commonly referred to as the "oriental eye worm", which can cause eye diseases of varying severity and symptoms known as thelaziosis [6]. The infection cycle begins when *P. variegata* becomes infected with the L1 stage of *T. callipaeda* while feeding on the ocular secretions of hosts, acting as an intermediate host and biological vector [10]. Thelaziosis is an emerging disease in Europe, with the first reported case in 2001, indicating active circulation between countries and an expanding distribution and increasing incidence [11]. In the Iberian Peninsula, thelaziosis has been documented in dogs, cats, red foxes, wild rabbits and humans, with a rise in the number of canine thelaziosis detected in some areas of mainland Spain [12–14], and across other countries [11].

The hovering activity around the head and eyes can be a significant nuisance for hosts, particularly humans [8], but no previous research has focused on the discomfort caused when large populations of *P. variegata* flies in a specific area. To date, information on the biology, ecology, and zoophilic habits of the *Phortica* genus in Europe is scarce. Therefore, this study aims to i) update the distribution of *Phortica* species by compiling historic data and the results of a field sampling; ii) describe regions where *Phortica* spp. pose a nuisance to humans in Spain; iii) provide a more detailed morphological and molecular characterization of *Phortica* flies.

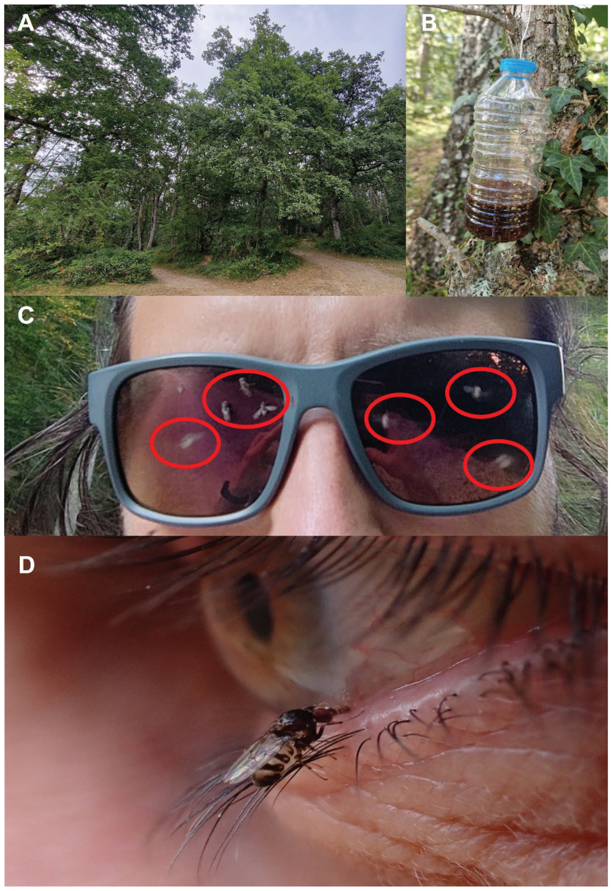

Figure 1. (A) Typical deciduous forest habitat of *P. variegata*. (B) Suspended vertical bottle traps used on trees for this study. (C) Singular hovering flight activity of *P. variegata* on human face (red circles denote flies). (D) Male of *P. variegata* feeding on human ocular tears.

2. Material and methods

2.1. Entomological trapping

Twenty-five sampling points at forested areas (Figure 1A) distributed across 12 different provinces with distinct ecological ecosystems and altitudes were selected throughout Spain (Table 1). Trapping was made using handmade plastic bottle traps baited with a mixture of apple cider vinegar and wine (Figure 1B), a valid and standard approach for collecting *Phortica* flies [8]. Three to four traps were deployed for seven consecutive days at each sampling point, giving a total of 84 traps along 25 sampling points in August of 2023. The peak activity for these flies occurs at this time of the year [13,15]. Bottle traps were positioned 10–50 m from each other and vertically suspended from trees in shaded areas at a height of 1.30–1.80 m above the ground. After seven days, the bottle traps were retrieved and transported to the laboratory. Dominant tree and shrub species were recorded for each sampling point.

2.2. Morphological identification

The bottle contents were first filtered through a mesh and then transferred to Petri dishes containing ethanol (70%), where trapped individuals were identified, counted and sexed. The number of *Phortica* spp. individuals was calculated as the number of captured individuals/trap/day/speciesper sampling point. The identification of the genus *Phortica* is complex, and unequivocal determination relies on the genitalia of males and vaginal sclerites of females. Thus, identification to the species level should be confirmed by clarification of the terminalia. Flies were first grouped in different taxa based on leg and abdominal terga features. Then, a subsample of *Phortica* flies (2–5 specimens/site) from the investigated territories was digested by incubating the tissue in 15% potassium

Table 1. Summary of the Spanish provinces sampled for *Phortica* spp. flies in summer 2023.

Province	Mean/trap/day (Min-Max)		Municipality	Altitude (m.a.s.l.)	Plan community composition
	P. variegata	*P. oldenbergi*			
Álava	2.08 (0.75–3.00)	0.00	Legutiano	580	*Quercus robur, Crataegus monogina, Ilex aquifolium*
Álava	39.58 (29.00–47.25)	0.00	Izki	710	*Quercus pyrenaica, Crataegus monogina, Ilex aquifolium*
Álava	0.21 (0.00–0.36)	0.00	Kripan	830	*Quercus ilex, Fagus sativa*
La Rioja	1.21 (1.09–1.27)	0.12 (0.00–0.27)	Sojuela	725	*Quercus pyrenaica*, shrubs, *Pinus* sp.
Valencia	0.00	0.00	La Eliana	85	*Quercus coccifera, Pistacia lentiscus, Pinus halepensis*
Valencia	0.00	0.00	Paterna	100	*Pinus halepensis, Pistacia lentiscus, Olea europea, Ceratonia siliqua*
Valencia	0.00	0.00	Rocafort	70	*Pinus halepensis*, fruit crops of *Citrus sinensis*
Cáceres	1.79 (0.29–5.86)	0.11 (0.00–0.43)	Jarandilla de la Vera	550	*Quercus pyrenaica*
Cáceres	0.32 (0.14–1.00)	0.46 (0.14–0.86)	Losar de la Vera	520	*Quercus pyrenaica*
Cáceres	0.00	0.07 (0.00–0.24)	Sierra de Fuentes	485	*Quercus ilex*
Cáceres	0.14 (0.00–0.63)	0.06 (0.00–0.19)	Cáceres	470	*Quercus ilex, Quercus suber, Olea europaea, Pyrus, Rhus*
Huesca	1.75 (1.38–2.00)	0.04 (0.00–0.13)	La Peña (water dam)	560	*Quercus* sp.,*Buxus sempervirens, Amelanchier ovalis, Crataegus monogyna, Rosa canina, Populus nigra, Rubus ulmifolius*
Huesca	27.17 (18.75–33.25)	0.17 (0.13–0.25)	La Peña (Ways)	560	
Pontevedra	0.00	0.00	Vigo	90	*Quercus robur, Castanea sativa*
Pontevedra	0.00	0.00	Vigo	405	*Quercus robur, Pinus pinaster, Eucalyptus globulus, Castanea sativa*
Gerona	0.00	0.07 (0.00–0.14)	Blanes	30	*Pinus halepensis, Quercus ilex, Quercus suber, Rhamnus alaternus, Quercus cerrioides*
Gerona	0.00	0.00	Tordera	25	*Quercus petraea, Quercus robur, Quercus canariensis, Sorbus torminalis, Alnus glutinosa*
Mallorca	0.09 (0.00–0.18)	0.00	Palma	130	*Olea europaea var. sylvestris (Quercetum ilicis)*
Mallorca	0.00	0.00	Palma	155	*Quercus ilex (Cneoro-Ceratonietum)*
Córdoba	0.00	0.00	Córdoba Arroyo Palomera	160	*Cistus albidus, Pistacea lentiscus, Olea* sp., *Luma apiculata*
Córdoba	0.00	0.03 (0.00–0.15)	Córdoba Villares Park	570	*Quercus ilex, Pinus pinea, Arbutus unedo, Pistacea lentiscus, aromatic plants*
Madrid	0.00	0.04 (0.00–0.13)	Madrid	695	*Quercus ilex, Pinus halepensis*
Madrid	0.04 (0.00–0.13)	0.42 (0.13–0.63)	El Berruego	925	*Quercus ilex, Quercus pyrenaica*
Sevilla	0.50 (0.00–1.00)	0.28 (0.22–0.33)	El Castillo de las Guardas	340	*Acer negundo, Nerium oleander, Eucaliptus* sp., *Quercus rotundifolia, Quercus ilex*
Huelva	0.26 (0.00–0.44)	0.37 (0.00–1.00)	Arroyomolinos de Léon	620	*Quercus ilex, Quercus rotundifolia, Olea europaea, Pistacea lentiscus, Ficus carica*

m.a.s.l.: metres above sea level.

hydroxide (KOH) at 45°C for 24 h. After clearance, genitalia were extracted and slide-mounted with Hoyer's medium. Specimens were identified to the species level under a binocular microscope by observing key morphological characteristics [3,5,16].

2.3. Molecular identification and phylogenetic analysis

Genomic DNA was extracted from the legs (either 2 or 3 legs) in *P. variegata* ($n = 8$) and *P. oldenbergi* ($n = 4$) from the different sampling points (Table 1) using Qiagen DNA kit following the manufacturer's protocol. A 658 bp fragment of the mitochondrial cytochrome c oxidase subunit 1 (Cox1 or COI) gene was amplified following the protocol conditions outlined by Folmer *et al.* [17]. The amplified products were sequenced on both directions using the Capillary Electrophoresis Sequencing Service of the UCM (Madrid, Spain), and a consensus sequence was generated by Geneious v.2020.0.3 [18]. Similarity with GenBank sequences was determined using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Detailed specimen records and sequence information of *Phortica* species have been deposited in the DNA Data Bank of Japan (DDBJ: https://www.ddbj.nig.ac.jp/index-e.html). The accession numbers are LC820476 to LC820483 (*P. variegata*) and LC822030 to LC822033 (*P. oldenbergi*). Twelve *Phortica* sequences from our study were included along with other *Phortica* species worldwide ($n = 27$) in phylogenetic analysis, while *Drosophila melanogaster* (MT807020) was set as an outgroup. Phylogenetic reconstructions were conducted using the Maximum Likelihood (ML) optimization criterion, employing the GTR+I+G4+FO model as defined by IQ-TREE [19] and model selection was based on Akaike information criterion. The robustness of the resulting ML trees was tested by a SH-aLRT (Shimodaira – Hasegawa-like approximate Likelihood Ratio Test) with 5000 replicates. The tree was visualized using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Finally, the average evolutionary divergence was estimated for all sequence pairs within the species *P. variegata* and *P. oldenbergi*. Additionally, the evolutionary divergence between the two resulting clusters of *P. variegata* was assessed. These analyses were conducted using the Kimura 2-parameter model with MEGA11 [20,21].

2.4. Literature records

For the bibliographic review, we consulted the "taxonomy of Drosophilidae" database, which compiles all published works on this family (http://taxodros.unizh.ch). The search criteria used were global region – Europe, and specific country – mainland Spain, resulting in a total of 2,395 drosophilid localities, of which 48 corresponded to "*variegata*", "*oldenbergi*" and "*semivirgo*". Each of these records was checked to verify species identity. Inaccurate or inaccessible records were excluded. Additionally, records were gathered from biodiversity platforms and digital networks such as Virtual Biodiversity, the Global Biodiversity Information Facility (GBIF), and iNaturalist, as long as they included high-quality pictures showcasing diagnostic morphological features for unequivocal identification of the targeted drosophilid species. We categorized entries as *Phortica* spp. when identification to species level was not possible. The location of all records was geographically represented using QGIS software version 3.34 LTR.

2.5. Nuisance records

We assessed the direct nuisance inflicted by *Phortica* flies on humans through different approaches. First, sampling sites with captures of *Phortica* flies were revisited immediately afterwards to evaluate the level of nuisance (low, medium, and high infestation) based on the number of flies hovering around humans' heads in a 2-min interval (low numbers = 1–5, moderate numbers = 5–15, and high numbers ≥ 15) (Figure 1C). Second, we documented complaints from people over the last 4 years who reported discomfort caused by flies. These complaints arrived usually through informal communications during fieldwork. To confirm that these issues were indeed caused by *Phortica* flies, we visited these locations to verify the species and assess the level of nuisance. To limit unnecessary visits, we only considered potential report cases describing flies that exhibited characteristic constant and incipient fluttering around the eyes, settling on the tear ducts or areas with sweat (ears, nape, forehead), making them an easily recognizable genus [2]. If a visit was not feasible, we requested high-quality pictures, fresh specimens, and/or video recordings for accurate species identification. Location, habitat and altitude were recorded for each of these nuisance observations.

2.6. Statistical analysis

The effect of vegetation type on the mean captures per day and trap of *Phortica* spp. was evaluated through a series of non-parametric variance tests, as abundance data were not normally distributed. Mann-Whitney non-parametric tests were used to compare the abundance of *P. variegata* and *P. oldenbergi* in areas with either the presence or absence of both *Pinus* spp. and *Quercus* spp. We considered it as present when at least 25% of the habitat was dominated by these trees. Four independent tests were done, one for each tree/*Phortica* species combination.

The relationships between sampling site altitude (metres above sea level, m.a.s.l.) on the capture rates of either *P. variegata* or *P. oldenbergi* were tested with Spearman correlation tests. All the analyses were run in JMP v9, and graphs were built using the package *ggplot2* in R version 4.1.1.

3. Results

Passive trapping of *Phortica* flies was conducted in 12 provinces of Spain, resulting in the capture of 1,462 *Phortica* spp. flies. *Phortica variegata* (n = 1,395; 95.4%) was the most abundant species, while *P. oldenbergi* (n = 67; 4.6%) was present in low numbers. *Phortica semivirgo* was not recorded in any of the sampled habitats. Regarding the sex ratio, *P. variegata* and *P. oldenbergi* consisted of 96.1% and 74.0% males, respectively.

3.1. *Phortica* flies distribution in mainland Spain and the Balearic Islands

Phortica flies were recorded in 10 of the 12 sampled provinces: Huesca (n = 699), Álava (n = 507), Cáceres (n = 149), La Rioja (n = 44), Huelva (n = 27), Sevilla (n = 17), Madrid (n = 12), Mallorca (n = 4), Córdoba (n = 2) and Gerona (n = 1) (Table 1). No *Phortica* flies were recorded in the Valencia (Eastern mainland Spain) and Pontevedra (Northwestern mainland Spain) provinces (Table 1; Figures 2 and 3). *Phortica variegata* and *P. oldenbergi* were present in 13 and 12 sites (out of the 25), respectively, indicating that both species are widely distributed nationwide. Higher densities of *P. variegata* flies were observed in Northern and Central Spain. The general density of *Phortica* flies (*P. variegata* + *P. oldenbergi*) was generally low (1.32 specimen/trap/day), although higher numbers were recorded in Izki and La Peña (39.6 and 27.2 specimens/trap/day, respectively) (Table 1; Figure 3).

3.2. Altitude and vegetation effects on *Phortica* spp. abundance

Phortica spp. were detected between 30 and 925 m.a.s.l. Altitude was positively related to *P. variegata* (Rho = 0.59,

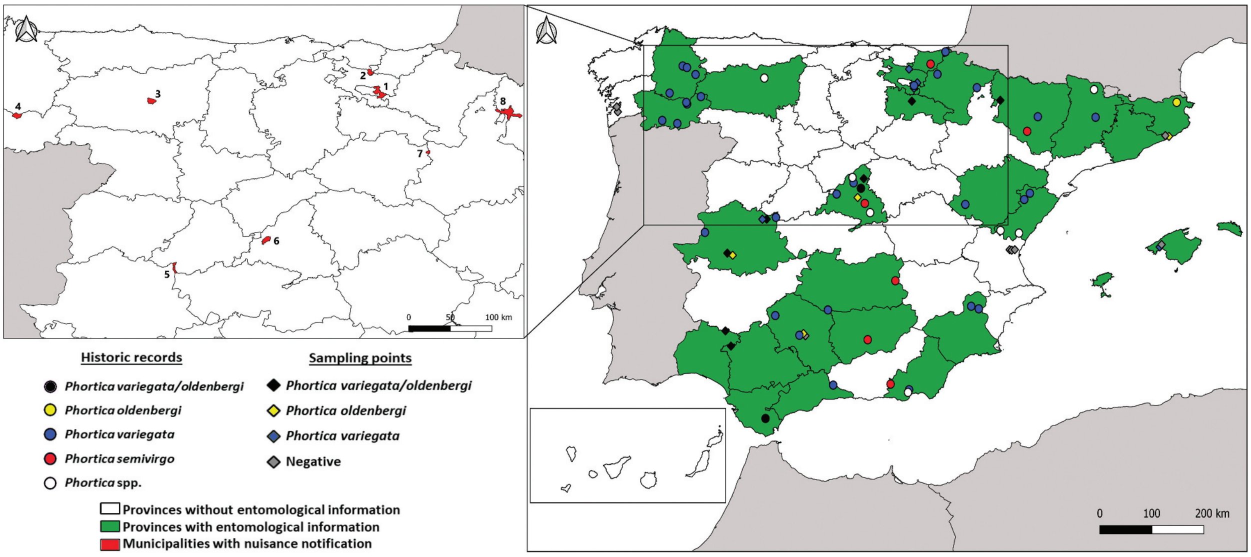

Figure 2. Left map: municipalities with reports of disturbances to humans caused by *P. variegata* flies. Right map: distribution of *Phortica* species in Spain based on entomological trapping collections during the summer of 2023 (with abundances by region) and historic records (1935–2023) based on the references included in table 1 and table S1, respectively. Numbers (1–8) in the municipalities with reports of disturbances refer to table S1.

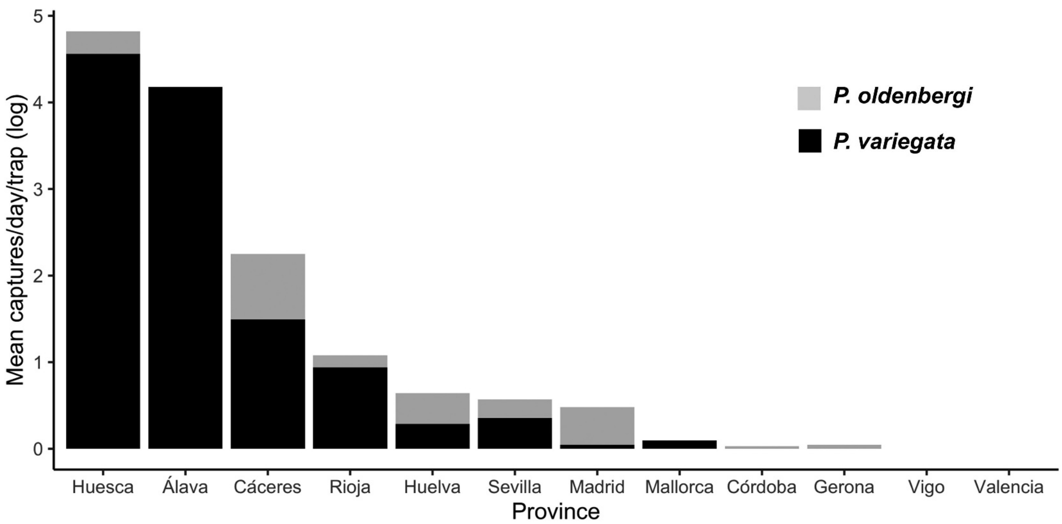

Figure 3. Number of *P. variegata* (black) and *P. oldenbergi* (grey) captured per trap day in the sampling sites in the 12 different provinces studied in Spain.

$p = 0.002$) and *P. oldenbergi* (Rho = 0.44, $p = 0.032$). *Phortica variegata* showed a marked increase in abundance after 350 m.a.s.l., peaking at around 600 m.a.s.l., before declining at higher altitudes. In contrast, *P. oldenbergi* capture rates increased linearly with altitude, with a pronounced increase over 700 m.a.s.l. (Figure S1).

Quercus was the most dominant plant genus at selected sites ($n = 19/25$; 76%), with other main genera reported including *Acer* sp., *Olea* sp., and *Pinus* spp. Vegetation in the trap environments appeared to affect *Phortica* spp. abundance. The presence of *Pinus* spp. was associated with lower capture rates of *P. variegata* (Mann-Whitney non-parametric test, $z = 2.29$, $p = 0.021$), but no differences in the abundance of *P. oldenbergi* ($z = 0.96$, $p = 0.340$). Higher numbers of *P. oldenbergi* were captured at sampling sites with *Quercus* ($z = 2.01$, $p = 0.040$) while no differences were found for *P. variegata* ($z = 1.41$, $p = 0.160$) (Figure 4).

Habitats with *Phortica* spp. presence included wild areas, rural areas in natural green environments, golf courses and surroundings, rural houses, green areas, and footpaths, among others. The highest density of *P. variegata* flies was recorded in an oak woodland located within a golf course (Figure 1A) and in an environment in the pre-pyrenean mountains, next to a reservoir that collects water from the river Gállego, surrounded by holm oaks, poplars, brambles, and boxwoods. New flora communities with populations of *Phortica* flies were recorded, including mixed-species plant communities' dominance by one of each species:

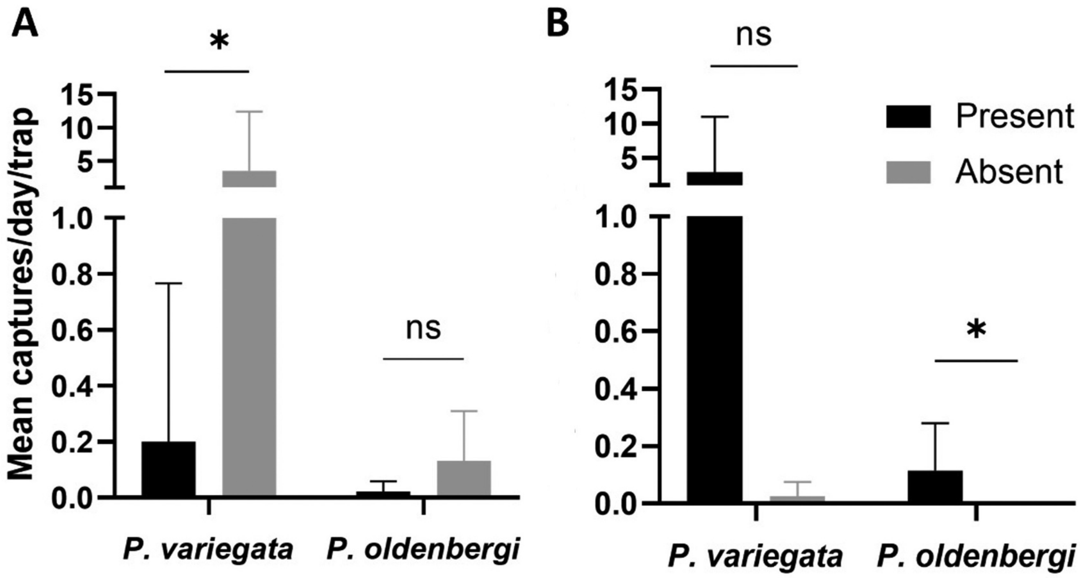

Figure 4. Effect of environmental variables on the mean captures/day/trap (capture rates) of *Phortica* spp. (A) Effect of either presence or absence of *pinus* spp. On capture rates of both *P. variegata* (black) and *P. oldenbergi* (gray). (B) Effect of either presence or absence of *Quercus* spp. on capture rates of both *P. variegata* (black) and *P. oldenbergi* (grey).

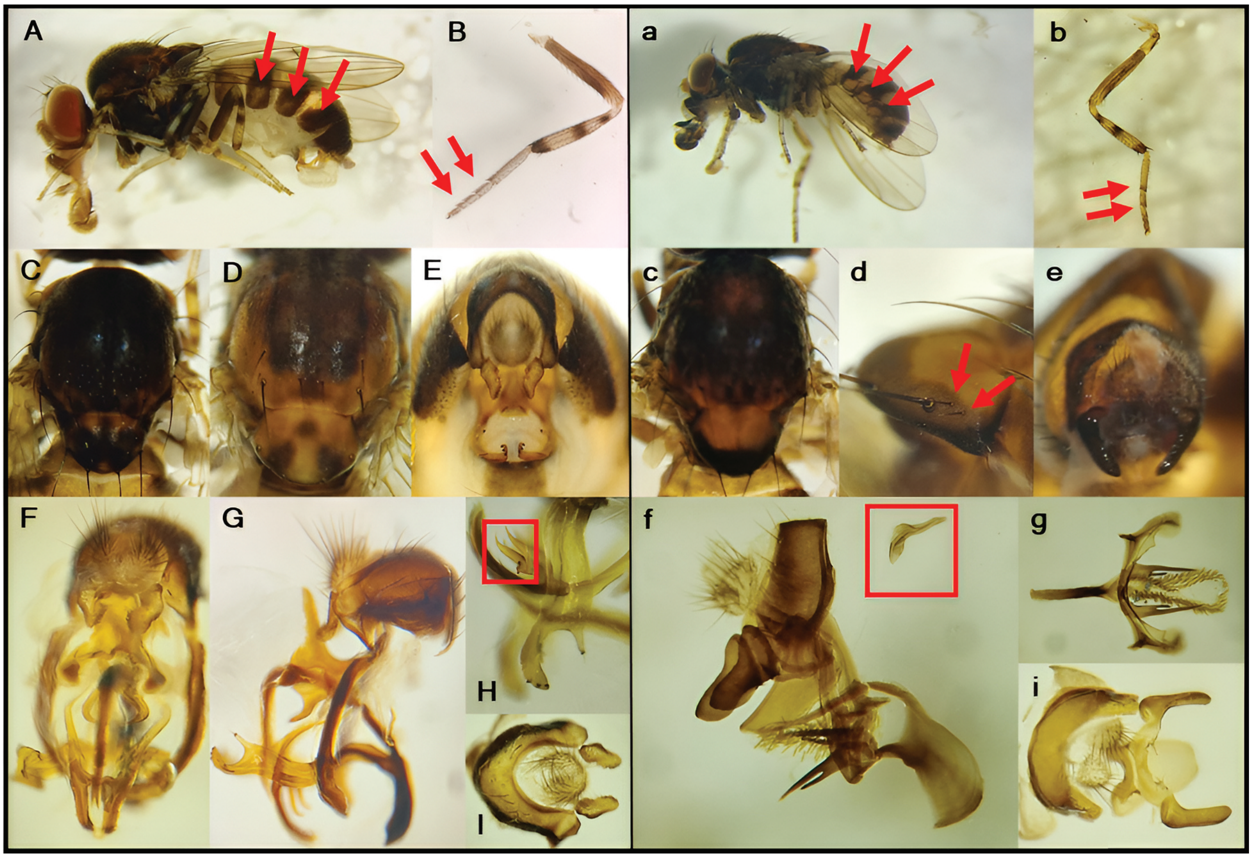

Figure 5. Morphological features used for the identification of adult *Phortica* specimens. Male of *Phortica variegata* on the left (capital letters) and male of *Phortica oldenbergi* on the right (lowercase letters). (A,a) General aspect: arrows showdetails of lateral spots on abdominal tergites. (B,b) Legs: tibia bears three conspicuous dark transverse bands on a light background. Arrows highlight the presence or absence of a dark spot on tarsomeres. (C,c and D,d) Scutellum: different colour patterns. Arrows denote the unique supplementary bristles proximal to each of the basal scutellar setae in *P. oldenbergi*. (E,e) External aspect of the abdominal terminalia before clarification. (F,f and G,g) Details of genitalia after clarification (digestion), showing the hypandrium, gonopods, *aedeagus,* postgonites and phallic organs in different views. Red rectangle shows ejaculatory apodeme. (H) Red rectangle shows the well-developed lateral rods in the inner paraphysis, which is a distinctive and singular feature of *P. variegata* species. (I,i) View of periphalic organs (epandrium, surtyli, and cercus).

Pinus halepensis, *Quercus ilex*, *Olea europaea*, and *Acer negundo*.

3.3. Morphological identification remarks

Our study has identified various straightforward descriptors for the accurate separation of males and females of the two species collected (Figure 5). The genus *Phortica* typically measures 3.5–4.0 mm in length and has a distinctive appearance characterized by yellowish tibiae on the hindlegs, usually with three distinct dark spots. The thorax has small, multiple confluent spots and numerous partially confluent grey spots, as well as eight irregular rows of setae. The abdomen displays a pattern of variable orange spots on a dark or light background (Figure 5). While males of both species are well-defined and are easier to identify, females, particularly of the *P. oldenbergi* species, have often gone unnoticed.

Males. Abdomen: abdominal tergites are dark in both *P. variegata* and *P. oldenbergi*, but light spots on lateral margins of tergites 3–5 are absent or indistinct in the former(Figure 5A) and more delimitated and circular in the latter (Figure 5a). Legs: Segments of tarsus in *P. variegata* are mostly yellowish, with at most, tarsomere 5 slightly darkened (Figure 5B). At least half of the tarsomere 5, and often also other tarsomeres, are apically darkened in *P. oldenbergi* (similar to *P. semivirgo*) (Figure 5b). *Scutellum*: The colour pattern in *P. variegata* is usually entirely dark brown (or greyish) with a variable pattern of undefined light spots (same ground colour as *scutum*) (Figure 5C,D). In contrast, *P. oldenbergi* usually has a conspicuous well-defined amber-coloured spot occupying most of the anterior surface of the dark brown *scutellum* (Figure 5c). The reliable diagnostic feature in *P. oldenbergi* is the presence of one or two supplementary bristles proximal to each of the basal scutella setae, whereas in *P. variegata* this region is bare of bristles (Figure 5d). Genitalia: The separation of both species can be done under the stereomicroscope by observing the visible external terminalia of the males (Figure 5E,e). For further confirmation, the internal genitalia of *P. oldenbergi* is strikingly different from other species of the subgenus, with the *aedeagus* covered by numerous nipples and parameters apically bifid (Figure 5f,g). Possible confusion can occur with *P. semivirgo*, but among other features, in *P. variegata* the inner paraphysis (lateral rods) are strongly developed, long, extended beyond the tip of medial rod, curved (Figure 5H), and pointed in contrast to *P. semivirgo*.

Females. They are usually bigger and lighter compared to males. However, abdomen pattern in females of both species is similar, with well-defined circular light spots in lateral margins. Therefore, the observation of the black spots on leg tarsus, the colour pattern of the *scutellum*, and bristles in the *scutellar* region is necessary for species determination.

3.4. Molecular and phylogenetic analysis

All *P. variegata* sequences (LC820476–83) achieved an identity between 98.30–100% with other GenBank sequences of the same species (e.g. OQ359821 or OQ359798). However, no *P. oldenbergi* sequences were available in GenBank. The highest similarities of the *P. oldenbergi* sequences of this study (LC822030–33) were 94.53–94.68% with *Phortica floccipes* sequences (EU431953) and 94.38% with *Phortica hani* (NC081079).

The set of *P. variegata* sequences showed low average evolutionary divergence ($d = 0.007$; S.E. $= 0.002$). However, they form two distinct branches: one that includes Spanish sequences along with specimens from Portugal and North America, and another that comprises other European sequences (see Figure 6). The genetic distance between both groups was 1.3%. For *P. oldenbergi*, according to the phylogenetic tree, formed a single monophyletic group ($d = 0.000$; S.E. <0.001), and the closest related species come from Asia (Figure 6).

3.5. Literature records

A total of 51 historic records of the three species of *Phortica* genus in Spain between 1934 and 2024 were compiled (Figure 2; Table S1). *Phortica variegata* was widely distributed throughout most of Spain, but without any previous reports in the Balearic and Canary Islands. It has been most frequently cited in northern regions, although there have been occasional records in Extremadura, Andalucía, and Murcia. According to the authors, flies were collected across areas ranging from 50 to 1,500 m.a.s.l., with captures being more prevalent between 700 and 800 m.a.s.l. (Table S1). The most common trapping method were fermented baits traps (bottles lured with either vinegar or beer), flight interception traps (drop traps) and entomological sweeping (Table S1). On the other hand, *P. semivirgo* exhibited a patchy distribution, with occasional collections primarily occurring in the southern half of the Iberian Peninsula. Interestingly, this species seemed to be associated with more diverse habitats other than those used by its congener *P. variegata*.

3.5.1. Description of nuisance cases

Phortica variegata flies pose a significant problem in certain regions during the summer months (Figure 1C), severely impacting the enjoyment of outdoor and recreational activities. In addition to humans (Figure 1D), domestic pets such as dogs, are also affected by these flies, which frequently settle in their eyes. No similar cases have been reported for the other

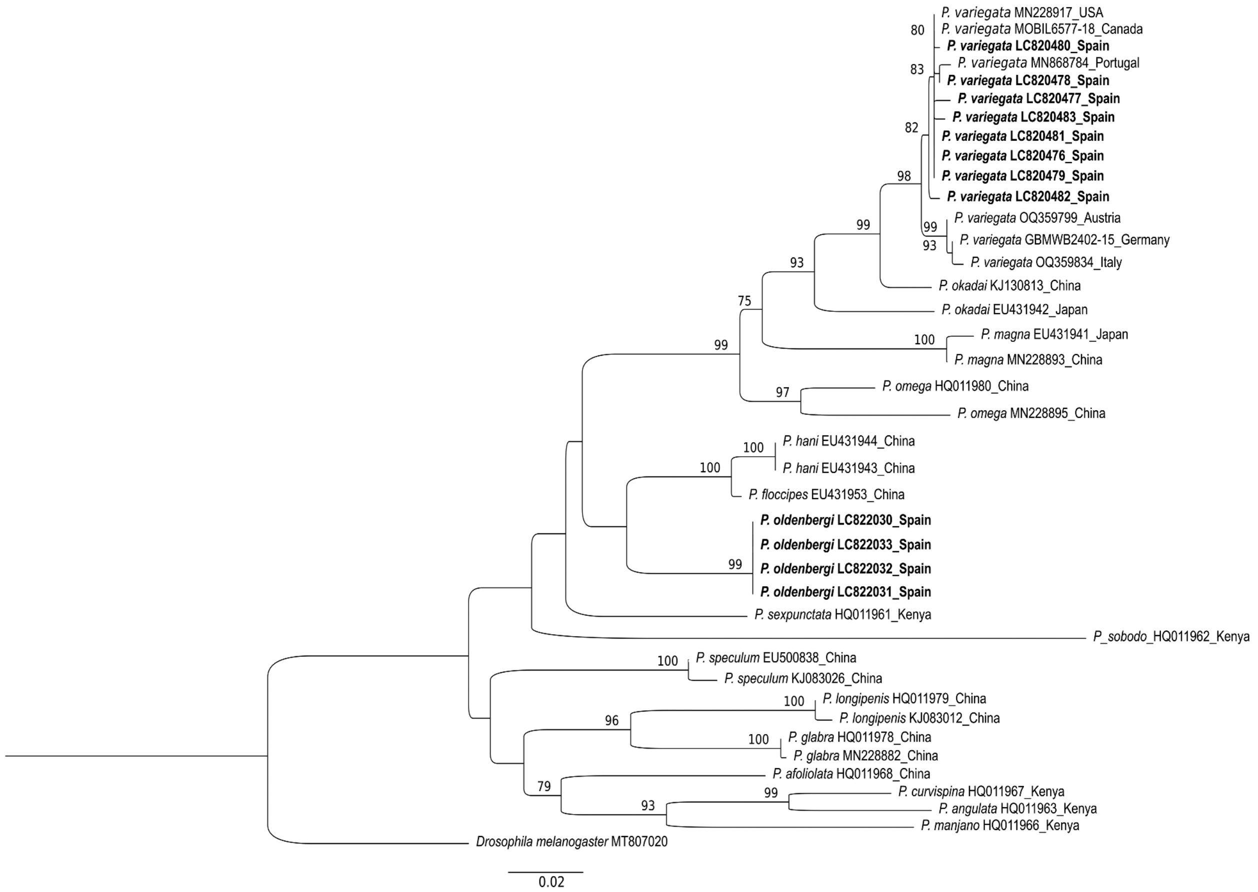

Figure 6. Phylogenetic tree of *Phortica* species based on 26 sequences from known species deposited in GenBank and 12 new sequences generated in this study (in bold). Evolutionary history was inferred using the ML method based on the general time reversible model. The bar indicates the average number of substitutions per site. Bootstrap values > 75% in 5000 repetitions are indicated at specific branch nodes. *Drosophila melanogaster* (MT807020) was used as an outgroup.

two *Phortica* species. Table S2 provides an overview of the specific cases where *P. variegata* became problematic due to its persistent flight patterns during host seeking. This table represents only a fraction of the reported cases. Our data show that nuisance becomes apparent at densities higher than 5–10 flies.

4. Discussion

This paper reviews literature records on the distribution of flies in the genus *Phortica* and provides updated, novel, and significant data on their distribution across mainland Spain and the Balearic Islands. Our study reveals the widespread presence of *P. variegata*, including territories with high density and *P. oldenbergi*. In contrast, *P. semivirgo*, a species commonly recorded in many European countries (particularly in the East European Plain) [22–24], was not found in our 2023 sampling. This is surprising given its historic records in six Spanish provinces between 1996 and 2015. Further studies are necessary to clarify whether the absence of *P. semivirgo* in our study could be due to habitat-related factors, sampling design or other unknown variables. Historic records indicate that *P. semivirgo* inhabits a range of habitats not included in our study, such as arid areas with characteristic *Juniperus* vegetation and humid areas with diverse flora such as riparian vegetation, *Tamarix* sp., *Salsola* sp. and other flora [25], although in other northern countries it has also been found in deciduous forests [23,26]. The other two European *Phortica* species (*P. erinacea* and *P. goetzi*) were not found in the present study.

Based on captures presented in this work and historic records, *P. variegata* is more abundant in the northern half of Spain. However, specimens are also present in moderate numbers in suitable woodlands in the southern regions, aligning with results of habitat suitability models [2,27]. Notably, *P. variegata* has been recorded in the Balearic Islands for the first time. Since cases of thelaziosis are diagnosed in dogs and cats throughout most of Spain [14], it is expect *Phortica* vectors to be present in all autonomous communities. However, there is a significant bias, as domestic animals potentially carrying thelaziosis might have been infected at other endemic areas, for example, during the holidays of their owners. Our findings indicate a risk of thelaziosis circulation across most regions of Spain, excluding the Canary Islands, if *T. callipaeda*-positive animals are introduced into areas already inhabited by *Phortica* species with vectorial competence. This underscores the potential public health threat in regions with high fly populations. *Phortica variegata*, native to the Palearctic region, has been recently reported in Canada [28], which may be considered a novel risk of *T. callipaeda* infection to humans and other animals in this country. The phylogenetic analyses of the sequences of the present study suggest a closer relationship of the *P. variegata* from North America with sequences from the Iberian Peninsula than with those from the rest of Europe, which could suggest a possible introduction from this part of Europe towards America.

Surprisingly, *P. oldenbergi*, a fly recorded as a rare Afrotropical species in Europe, present in areas of Germany [29], Spain [25] and Italy [30], showed a greater distribution in Spain than expected, albeit with lower abundances than its congener *P. variegata*. *Phortica oldenbergi* is presumed to belong to the subgenus *Allophortica*, along with other African species [31]. However, our phylogenetic analysis revealed closer relationships with Asian species than with African ones. The potential role of *P. oldenbergi* as vector has recently been evaluated under experimental conditions, suggesting it may act as an intermediate host for *T. callipaeda* [32].

Various research groups have investigated the environmental factors (landscape, temperature, humidity, barometric pressure, altitude, and relative humidity) that influence the phenology and behaviour of *P. variegata* [2,8,15]. By combining historic and recent records, we found that *P. variegata* flies are more abundant at latitudes over 500 m.a.s.l. and less common in areas with *Pinus halepensis*, while *P. oldenbergi* are more abundant in areas with *Quercus* spp. These associations are most commonly observed in rural woodland ecosystems, including deciduous oak forests such as *Q. pyrenaica, Q. ilex, Q. robur, Quercus rubra,* and *Quercus rotundifolia*, as well as beech (*Fagus sylvatica*), holly (*Ilex aquifofium*), chestnut (*Castanea sativa*), and fruit trees. This pattern is consistent with previous research conducted in Europe [2,3,13,15,27].

Most cases of thelaziosis occur in the supra-Mediterranean or meso-mediterranean bioclimates, with mean altitudes of 960 and 560 m.a.s.l., respectively. *Phortica variegata* specimens have been captured in bioclimatic zones at altitudes ranges ranging from 20 m.a.s.l. (United Kingdom) to 1,147 (Miraflores de la Sierra, Spain) [13]. These findings align with those of the present study, which also considered altitudes above 1,500 m.a.s.l. The new data from the present study show that *P. oldenbergi* is present at altitudes ranging from 30 to 925 m.a.s.l, with higher abundances over 600 m.a.s.l.

Regarding trapping methods for *Phortica* flies, exist but may not be standardized across scientific or entomological communities. It appears that the three *Phortica* fly species are captured using different traditional trapping methods. However, the two most frequently employed techniques are active sweep netting on hosts and fruit-baited traps due to their simplicity and effectiveness [13]. Recently, González *et al.* [8] suggested using a binary blend of cider vinegar (75%) and red wine (25%) in homemade plastic bottle traps to capture *P. variegata*, noting this mixture was more effective than fermented fruit blends.

Despite the availability of identification keys, the existence of intermediate forms, intraspecific variation, and teneral specimens makes the identification of *Phortica* spp. flies challenging [3,5,16]. Additionally, taxonomic descriptions are often difficult to follow, and the diagnostic features used to distinguish species have evolved over the last years. Females are rarely trapped, possess fewer morphological diagnostic features, and are rarely lachryphagous, diminishing their veterinary importance and explaining why the morphology of females of most drosophilid species remain poorly described [1]. We have noted some discrepancies in the descriptors compared to previous authors [3,5]. For instance, tarsomere 5 in *P. oldenbergi* was darker or slightly darker in most specimens, contrary to the yellowed colouration described previously [3,5]. Additionally, previous descriptions did not mention the distinctive amber yellow spot on the anterior part of the *scutellum* in *P. oldenbergi*, also observed in specimens collected in Italy (Dr. Otranto, personal communication). The morphology of *P. oldenbergi* differs from other African species [29], and it appears strikingly different from other Palearctic and Oriental *Phortica* species, with its occurrence in central Europe still considered enigmatic. Considering its greater phylogenetic similarity to Asian species, studies should be directed at corroborating the origin of this species.

Significant efforts have been dedicated to study the prevalence and expansion of thelaziosis, as well as the phenology and vectorial role of *P. variegata* to *T. callipaeda* [11]. However, no studies have addressed the direct consequences of the hovering activity of *Phortica* flies on hosts. Our study is the first to identify regions in Spain where the high presence of *Phortica* flies (*P. variegata*) represents a genuine nuisance to humans and domestic animals. Moderate-to-high population densities of *P. variegata* flies can significantly disrupt human well-being by interfering with leisure, sporting and recreational activities like golfing, adventure facilities, camping, hiking and hunting. This annoyance and discomfort results from the singular, curious and distinctive behaviour of these zoophilic fruit flies. They patrol in groups along forest clearings at the height of branches in wooded areas and, upon detecting a host, fly slowly towards them, positioning themselves at the upper torso level in front of the face to reach eyes and areas of perspiration [1,8,28]. They repeatedly approach hosts of interest from various angles before landing on his eyes [1]. A similar behaviour is observed in animals like dogs, where dozens of flies may hover over their eyes without being deterred. According to our observations, these flies can persistently follow individuals for tens of metres, even during high-speed movement, making them difficult to evade.

A higher fly density is linked with altitude and *Quercus* spp. forests, which we identified as areas with substantial *Phortica* spp. densities. Unfortunately, little can be done to mitigate the presence of these diurnal flies since they typically inhabit protected locations such as natural parks or rustic green spaces, where treatments are impractical both due to the large areas involved and the environmental consequences of biocides application in high biodiversity areas. Effective control methods for *P. variegata* flies remain largely undocumented and undeveloped. Recently, González *et al.* [8] proposed using traps baited with specific attractants for both vector detection (entomological surveillance) and population reduction (mass capture). However, their effectiveness in reducing nuisance levels remains untested. While complementary methods, such as insecticide applications or toxic baits, may help mitigate nuisance levels, their use in natural environments like forests poses significant risks to non-target species and local biodiversity. Limited effectiveness has been observed with insecticide treatments applied to lower oak branches in golf courses during summer; however, these methods lack selectivity and can disrupt delicate ecosystems. Furthermore, no repellents or substances have been identified as reliably effective in preventing these flies from approaching or landing on hosts. It is crucial to emphasize that any control strategy must balance the goal of reducing nuisance levels with the imperative of preserving environmental integrity, recognizing that indiscriminate eradication is neither ethical nor sustainable.

5. Conclusion

This study provides key insights into the distribution, behaviour, and identification of *Phortica* flies in Spain. *Phortica variegata* was the most abundant species, while *P. oldenbergi*, previously considered rare, showed widespread distribution. The absence of *P. semivirgo* suggests differences in habitat preferences or population dynamics, revealing a gap in current knowledge. Molecular and morphological analyses introduced new descriptors and COI sequences for *P. variegata* and *P. oldenbergi*, aiding identification and revealing potential phylogenetic relationships. The hovering behaviour of *P. variegata* and its attraction to eye secretions raise concerns for animal and human health, especially during outdoor activities.

This study highlights the relevance of *Phortica* spp. as vectors of thelaziosis, emphasizing their impact on Spanish ecosystems and the need for further research, particularly for veterinarians and other stakeholders, given their role as vectors of thelaziosis in animals and humans.

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