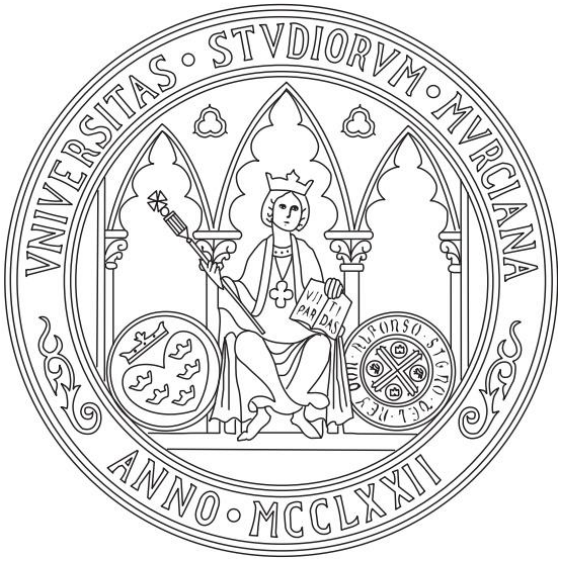

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Interaction between the red fox (*Vulpes vulpes*), helminths and environmental characteristics in semi-arid areas of southeastern Iberian Peninsula

Interacción entre el zorro rojo (*Vulpes vulpes*), los helmintos y las características ambientales en áreas semiáridas del sureste de la Península Ibérica

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Memoria presentada por la Licenciada en Ciencias Ambientales

Irene Arcenillas Hernández

Para optar al grado de Doctor en Ciencias Veterinarias

DIRECTORES

Carlos Martínez-Carrasco Pleite

María del Rocío Ruiz de Ybáñez Carnero

“Las especies que sobreviven no son las más fuertes, ni las más inteligentes;

sino aquellas que mejor se adaptan al cambio”

Charles Darwin.

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SUMMARY

Over the last decades, natural ecosystems have suffered a significant transformation due to human activity, being the fragmentation of the territory one of the main consequences. These transformations generate an impact on wildlife populations, causing the movement of species towards more suitable habitats where they can acquire the trophic resources and shelter necessary for their survival. In addition, in semi-arid Mediterranean areas, these wildlife movements may also be associated with the climatic characteristics of the environment, since scarce and irregular annual precipitation, high temperatures and high levels of evapotranspiration frequently lead to periods of scarcity of water resources. In this sense, similarly to wildlife, parasites are affected by the climatic conditions of the ecosystem in which their life cycle develops. Environmental temperature and humidity are two of the main factors for the development of a parasite's life cycle, and any alteration affecting these factors can influence both the free-living stages and the intermediate or paratenic hosts that participate in it, and can lead to changes in the transmission of the parasite itself or even affect its survival.

Currently, anthropized areas have become to interaction between wild animals, domestic animals and humans, which may pose an epidemiological risk derived from the transmission of shared pathogens, including numerous species of parasites. The red fox (*Vulpes vulpes*) is one of the most abundant and common synanthropic species in this wild-domestic-human interface, and it hosts a wide variety of parasites, many of them shared with domestic canids or even humans. Therefore, their proximity to rural or urban areas can be considered a risk of transmission in both directions, generating the spill-over effect, where the fox can also be a victim of this exchange of pathogens. In this sense, epidemiological studies should take into account these new situations where these shared areas can become important sites of parasite transmission.

The diagnostic method used in this type of epidemiological studies should be as sensitive and accurate as possible to minimize the number of false negatives and to obtain the closest value to the real parasite intensity. Therefore, the use of a suitable diagnostic method is essential to assess with the greater rigor the possible impact of parasites on the health of the individual and, on a larger scale, on the host population. Furthermore, specifically in the case of foxes, it is necessary to know the epidemiological risk associated with the presence of this canid in the wild-domestic-human interface.

The main objective of this Thesis was to describe the nematode communities that parasitize the cardiopulmonary, gastrointestinal and urinary systems of the red fox population in the Region of Murcia (SE Spain), as well as to know how the biotic and environmental variables of this semi-arid Mediterranean region influence the abundance of these parasites.

For this purpose, the first objective of this Thesis (Chapter 1) was to evaluate the sensitivity and accuracy of a post-mortem diagnostic method for the detection of cardiopulmonary nematodes in foxes. A total of 51 foxes were necropsied during 2015-2018, and both the heart and lungs of each fox were examined for nematode detection. Specifically, three consecutive methodological steps were employed: first, the opening of the tracheobronchial tree, pulmonary arteries and heart cavities (OT), followed by the manual squeezing of the lung parenchyma (WS), and finally the artificial digestion of the lung parenchyma (AD) with a solution of pepsin and hydrochloric acid. Four species of cardiopulmonary nematodes were isolated: *Angiostrongylus vasorum, Crenosoma vulpis, Eucoleus aerophilus* and *Metathelazia capsulata*. The total number of nematodes obtained in each of the methodological steps applied was 454 (OT), 285 (WS) and 141 (AD). The consecutive use of OT and WS improved the quantification of the parasitic intensity present in the host, and reduced the number of false negatives. Sensitivity was 96.1% when using OT followed by WS. When the methodological step AD was not used, the accuracy of the parasite intensity decreased to 84%, failing to detect 16% of the cardiopulmonary nematodes present. On the other hand, the use of AD increased the sensitivity of the prevalence and the intensity of parasitization of *A. vasorum* and *M. capsulata*, both species located in small capillaries and bronchioles of the lung, respectively, complicating its detection. The results obtained allow us to recommend the method we have employed when designing epidemiological studies based on the detection of cardiopulmonary nematodes in wild carnivores, since it will be possible to obtain results of prevalence and parasite intensity in accordance with the real situation of the carnivore population under study.

On the other hand, it is important to know the epidemiological factors that condition the presence of parasites in fox populations inhabiting semi-arid areas, since water stress is an environmental determinant that can lead to an approach of animals to

anthropized areas and, therefore, to higher contact rates at the wild-domestic-human interface. Therefore, the second and third objectives of this Thesis were to describe the gastrointestinal and cardiopulmonary nematode communities (Chapter 2) and that of the urinary system (Chapter 3) present in the fox population of the Region of Murcia. In addition, the influence of biotic and abiotic variables characteristic of this semi-arid Mediterranean area on five nematode species selected for their sanitary importance, either for their pathogenicity in dogs or for causing zoonosis (*Angiostrongylus vasorum, Crenosoma vulpis, Uncinaria stenocephala, Toxocara canis* and *Toxascaris leonina*) was evaluated (Chapter 2). The same analysis was carried out for *Pearsonema plica* (Chapter 3), a urinary bladder parasite little studied in domestic and wild canids and whose epidemiological situation in this type of environment is unknown. During the period 2015-2021, a total of 167 carcasses of foxes were collected from recovery centres, authorized hunting activity and road-killed individuals. Necropsy was performed, the cardiorespiratory system was initially extracted, and the methodology described in Chapter 1 was applied. In addition, the gastrointestinal system (oesophagus, stomach, small and large intestine) was examined and, finally, the kidneys, ureters and bladder were opened. Generalized linear models (GLM) were used to analyse the influence of biotic and abiotic variables on parasite abundance. Chapter 2 described a total richness of eleven species of nematodes, seven gastrointestinal (*Pterygodermatites affinis, Uncinaria stenocephala, Toxocara canis, Toxascaris leonina, Spirocerca vulpis, Oxynema crassispiculum* and *Trichuris vulpis*) and four bronchopulmonary (*Angiostrongylus vasorum, Crenosoma vulpis, Eucoleus aerophilus* and *Metathelazia capsulata*). The results obtained in the models indicated that, in this semi-arid Mediterranean region, temperature and humidity are the variables with the greatest influence on the abundance of the selected parasites, as well as forestry and agricultural areas. This cover vegetation areas provide places of refuge that, indirectly, favour optimal temperature and humidity conditions for the establishment and development of parasite eggs and free-living larvae (*U. stenocephala, T. canis* and *T. leonina*), and the gastropods involved in the life cycle of *A. vasorum* and *C. vulpis*. On the other hand, it was found that the presence of foxes in anthropized areas may pose an epidemiological risk, since a higher abundance of *U. stenocephala*, *T. canis* and *T. leonina* was detected in areas close to humanized

environments, implying a higher risk of transmission between synanthropic foxes and dogs sharing the same zone.

In Chapter 3 of this Thesis, the presence of *P. plica* was detected in the urinary bladder of 2.4% of the foxes examined (4/167). The analysis of environmental variables confirmed that humidity is the limiting factor for the development of the biological cycle of this parasite. The low prevalence of *P. plica* found could be due to the fact that the climatic conditions of the semi-arid Mediterranean regions, in general terms, are not sufficiently favourable for the development and survival of the earthworm, which is the necessary intermediate host for this parasite species to complete its life cycle. However, the confirmation that this nematode is present in the fox population of the Region of Murcia is the evidence that, even in these semi-arid Mediterranean areas, there are places where the conditions of humidity are appropriate for the presence of the parasite. This result suggests that there are specific areas in which the potential risk of transmission of *P. plica* exists, being able to maintain the sylvatic cycle due to the fox and, consequently, posing a risk to dogs that share these areas. Therefore, shared environments by domestic and wild species should be taken into account when designing health monitoring plans for those pathogens that, as in the case of *P. plica*, can affect domestic and wild canids.

During the development of the objectives previously mentioned, specimens of a spirurid nematode species were isolated from the bronchi and bronchioles of the examined foxes, whose description did not coincide with any of the species of bronchopulmonary nematodes described in this wild canid to date. However, based on the morphometric characteristics of the specimens studied, it was possible to confirm that it was a nematode species belonging to the genus *Metathelazia*, and more specifically to the species *Metathelazia capsulata*. Therefore, the fourth and final objective of this Thesis was to make a detailed morphometric description of this lung nematode species, as well as its molecular markers COI and rDNA. Measurements of the main structures were made using both light microscopy and scanning electron microscopy, which allowed comparisons with previously morphometric descriptions of *M. capsulata* made in host species different than the red fox. The majority of the measures were in agreement with those previously described by other authors, although some differences were found. Specifically, the main difference was in the length of the individuals, both males and

females (6.6 mm and 7.4 mm, respectively), but there were also slight variations in the mean values of the depth of the buccal cavity and the distance between the excretory pore and the anterior end, both of which were lower than those described previously. On the other hand, DNA extraction and PCR amplification of the mitochondrial marker COI and five rDNA markers (18S, 28S, 5.8S, ITS1 and ITS2) were carried out, and then sequenced and phylogenetically analysed. Based on the data available in GenBank, results obtained concluded that the sequences of *M. capsulata* are closely related to the family Rhabdochonidae, but also suggest distant relationships with the family Thelaziidae, both belonging to the superfamily Thelazioidea. This is the first finding of *M. capsulata* in foxes in Europe, and therefore in the Iberian Peninsula, as well as the first time that molecular markers for this species are described. This analysis provides very useful information for future phylogenetic studies on the nematodes of the genus *Metathelazia* spp. and, generally, on the species of the family Pneumospiruridae.

The results obtained throughout the development of this Thesis provide valuable information in different aspects. First, it shows the importance of using an appropriate diagnostic method for the objective desired, and a protocol is described for the diagnosis of cardiopulmonary nematodes of carnivores that allows obtaining accurate results. On the other hand, the analysis of the environmental variables in semi-arid Mediterranean areas highlights that the availability of humidity, temperature and, indirectly, cover vegetation areas, are key epidemiological factors in the maintenance, dispersion and abundance of the nematode species that affect foxes and dogs. Considering that the fox is the most abundant wild carnivore species in this semi-arid region, it should be emphasized that its presence in the wild-domestic-human interface may represent an added risk factor to parasite transmission, so prevention and control measures should be applied. Thus, these data can be useful when designing epidemiological monitoring programs, and management and conservation plans for other carnivore species or endangered species that share habitat with the fox.

RESUMEN

A lo largo de las últimas décadas los ecosistemas naturales han sufrido una notable transformación debido a la actividad humana, siendo la fragmentación del territorio una de las principales consecuencias. Estas transformaciones generan un impacto en las poblaciones de fauna silvestre, provocando el movimiento de las especies hacia hábitats más adecuados donde pueden obtener los recursos tróficos y refugio necesarios para su supervivencia. Además, en las zonas mediterráneas semiáridas, estos movimientos de fauna silvestre también pueden estar asociados a las características climáticas del entorno, debido a que las precipitaciones anuales escasas e irregulares, las temperaturas elevadas y el alto nivel de evapotranspiración originan en muchas ocasiones periodos de escasez de recursos hídricos. En este sentido, al igual que la fauna silvestre, los parásitos se ven afectados por las condiciones climáticas del ecosistema en el que se desarrolla su ciclo biológico. La temperatura y la humedad ambientales son dos de los factores principales para el desarrollo del ciclo de vida de un parásito, y cualquier cambio en dichos factores puede afectar tanto a las formas de vida libre como a los hospedadores intermediarios o paraténicos que participan en él, pudiendo dar lugar a cambios en la transmisión del propio parásito o incluso afectar a su supervivencia.

Actualmente, las áreas antropizadas se han convertido en zonas de interacción entre animales silvestres, animales domésticos y el ser humano, lo cual puede suponer un riesgo epidemiológico derivado de la transmisión de agentes patógenos compartidos, entre los que se encuentran numerosas especies de parásitos. El zorro rojo (*Vulpes vulpes*) es una de las especies sinantrópicas más abundantes y comunes en esta interfaz silvestre-doméstico-humano, y es hospedador de una amplia variedad de parásitos, muchos de ellos compartidos con cánidos domésticos, o incluso con el ser humano. Por ello, su cercanía a las áreas rurales o urbanas puede considerarse un riesgo de transmisión en ambos sentidos, dando lugar al efecto spill-over, donde el zorro también puede ser víctima de ese intercambio de patógenos. En este sentido, los estudios epidemiológicos deben tener en cuenta estas nuevas situaciones donde estos territorios compartidos pueden convertirse en importantes lugares de transmisión de parásitos.

El método de diagnóstico utilizado en este tipo de estudios epidemiológicos debe ser lo más sensible y exacto posible para minimizar el número de falsos negativos y obtener el valor más próximo al real de la intensidad parasitaria. Por ello, el uso de un

método de diagnóstico adecuado es esencial para valorar con mayor rigor cuál es el posible impacto de los parásitos sobre la salud del individuo y, a mayor escala, sobre la población de hospedadores estudiada. Además, en el caso concreto del zorro, es necesario para conocer el riesgo epidemiológico asociado a la presencia de dicho cánido en la interfaz silvestre-doméstico-humano.

Por todo lo mencionado anteriormente, el objetivo principal de esta Tesis Doctoral fue describir las comunidades de nematodos presentes en el sistema cardiopulmonar, gastrointestinal y urinario que parasitan la población de zorro rojo de la Región de Murcia (SE de España), así como conocer cómo influyen las variables bióticas y las variables ambientales características de esta región semiárida mediterránea en la abundancia de dichos parásitos.

Para ello, el primer objetivo de esta Tesis Doctoral (Capítulo 1) fue evaluar la sensibilidad y exactitud de un método de diagnóstico *post-mortem* para la detección de nematodos cardiopulmonares en el zorro. Durante los años 2015-2018 se realizó la necropsia de un total de 51 zorros, y tanto el corazón como los pulmones de cada uno de ellos fueron examinados para la detección de nematodos. En concreto, se emplearon tres pasos metodológicos consecutivos: en primer lugar, la apertura del árbol traqueobronquial, arterias pulmonares y cavidades cardiacas (OT), seguido del estrujado manual del parénquima pulmonar (WS), y finalmente la digestión artificial del mismo (AD) empleando una solución de pepsina y ácido clorhídrico. Se aislaron cuatro especies de nematodos cardiopulmonares: *Angiostrongylus vasorum, Crenosoma vulpis, Eucoleus aerophilus* y *Metathelazia capsulata*. El total de nematodos obtenidos en cada uno de los pasos metodológicos aplicados fue de 454 (OT), 285 (WS) y 141 (AD). El uso de OT y WS de forma consecutiva permitió mejorar la cuantificación de la intensidad parasitaria presente en el hospedador, a la vez que se redujo el número de falsos negativos. La sensibilidad fue del 96.1% al utilizar OT seguido de WS. Cuando el paso metodológico AD no se empleó, la exactitud del cálculo de la intensidad parasitaria disminuyó hasta el 84%, dejándose de detectar el 16% de los nematodos cardiopulmonares presentes. Por otro lado, el uso de AD incrementó la sensibilidad en la cuantificación de la prevalencia e intensidad de parasitación de *A. vasorum* y *M. capsulata*, ambas especies situadas en pequeños capilares y bronquiolos del pulmón, respectivamente, lo que dificulta en

ocasiones su detección. Los resultados obtenidos nos permiten recomendar el método que hemos empleado cuando se diseñen estudios epidemiológicos basados en la detección de nematodos cardiorrespiratorios en carnívoros silvestres, ya que se podrán obtener resultados de prevalencia e intensidad de parasitación acordes con los valores reales que tiene la población de carnívoros que sea objeto de estudio.

Por otra parte, es importante conocer los factores epidemiológicos que condicionan la presencia de parásitos en las poblaciones de zorros que habitan en zonas semiáridas, puesto que el estrés hídrico es un determinante ambiental que puede dar lugar a un acercamiento de los animales a áreas antropizadas y, por tanto, a mayores tasas de contacto en la interfaz silvestre-doméstico-humano. Por ello, el segundo y tercer objetivos de la presente Tesis Doctoral fueron describir las comunidades de nematodos gastrointestinales y cardiopulmonares (Capítulo 2) y las del sistema urinario (Capítulo 3) presentes en la población de zorros de la Región de Murcia. Además, se evaluó la influencia de las variables bióticas y abióticas características de esta zona semiárida mediterránea en cinco especies de nematodos seleccionadas por su importancia sanitaria, ya sea por su patogenicidad en perros o por ser causantes de zoonosis (*Angiostrongylus vasorum*, *Crenosoma vulpis*, *Uncinaria stenocephala*, *Toxocara canis* y *Toxascaris leonina*) (Capítulo 2). Este mismo análisis se realizó para la especie *Pearsonema plica* (Capítulo 3), un parásito de la vejiga urinaria poco estudiado en cánidos domésticos y silvestres y del que se desconoce su situación epidemiológica en este tipo de ambientes. Durante el período 2015-2021 se recogieron un total de 167 cadáveres de zorros procedentes de centros de recuperación, individuos atropellados o abatidos en cacerías autorizadas. Se realizó la necropsia y se extrajo, inicialmente, el sistema cardiorrespiratorio, sobre el que se aplicó la metodología descrita en el Capítulo 1. Además, se examinó el sistema gastrointestinal (esófago, estómago, intestino delgado e intestino grueso) y, finalmente, se procedió a la apertura de los riñones, uréteres y vejiga. Para el análisis de la influencia de las variables bióticas y abióticas sobre la abundancia de los parásitos se utilizaron modelos lineales generalizados (GLM). En el Capítulo 2 se describió una riqueza total de once especies de nematodos, siete de ellos gastrointestinales (*Pterygodermatites affinis*, *Uncinaria stenocephala, Toxocara canis, Toxascaris leonina, Spirocerca vulpis, Oxynema crassispiculum* y *Trichuris vulpis*) y cuatro broncopulmonares (*Angiostrongylus vasorum,*

Crenosoma vulpis, Eucoleus aerophilus y *Metathelazia capsulata*). Los resultados obtenidos en los modelos indicaron que, en esta región semiárida mediterránea, la temperatura y la humedad son las variables con mayor influencia sobre la abundancia de los parásitos seleccionados, así como las zonas con terrenos forestales o agrícolas. Este tipo de áreas, cubiertas por vegetación, aportan lugares de refugio que, de manera indirecta, favorecen que las condiciones de temperatura y humedad sean óptimas para el establecimiento y desarrollo de los huevos y larvas de vida libre de los parásitos (*U. stenocephala, T. canis* y *T. leonina*), y los gasterópodos implicados en el ciclo biológico de *A. vasorum* y *C. vulpis*. Por otra parte, se comprobó que la presencia del zorro en áreas antropizadas puede suponer un riesgo epidemiológico, ya que se detectó una mayor abundancia de *U. stenocephala*, *T. canis* y *T. leonina* en zonas cercanas a ambientes humanizados, lo que supone un mayor riesgo de transmisión entre los zorros sinantrópicos y los perros que estén compartiendo la misma ubicación.

En el Capítulo 3 de la presente Tesis Doctoral se detectó la presencia de *P. plica* en la vejiga urinaria del 2,4% de los zorros examinados (4/167). El análisis de las variables ambientales confirmó que la humedad es el factor limitante para el desarrollo del ciclo biológico de este parásito. La baja prevalencia de *P. plica* encontrada podría deberse a que las condiciones climáticas de las regiones semiáridas mediterráneas, en términos generales, no son lo suficientemente favorables para el desarrollo y supervivencia de la lombriz de tierra, que es el hospedador intermediario necesario para que esta especie de parásito pueda completar su ciclo biológico. Sin embargo, la constatación de que este nematodo está presente en la población de zorros de la Región de Murcia es la evidencia de que, incluso en estas zonas áridas mediterráneas, existen lugares donde las condiciones de humedad son apropiadas para la presencia del parásito. Este resultado sugiere que hay áreas concretas en las que el riesgo potencial de transmisión de *P. plica* existe, pudiendo mantenerse el ciclo selvático a expensas del zorro y, en consecuencia, suponiendo un riesgo para los perros que comparten dichas zonas. Por ello, estos ambientes compartidos por especies domésticas y silvestres deberían tenerse en cuenta a la hora de diseñar los planes de vigilancia sanitaria de aquellos patógenos que, como es el caso de *P. plica*, pueden afectar a cánidos domésticos y silvestres.

Durante el desarrollo de los objetivos de la Tesis Doctoral antes mencionados, se aislaron de los bronquios y bronquiolos de los zorros examinados ejemplares de una especie de nematodo espirúrido cuya descripción no coincidía con ninguna de las especies de nematodos broncopulmonares descritas en este cánido silvestre hasta la fecha. No obstante, en base a las características morfométricas de los especímenes estudiados, se pudo confirmar que se trataba de una especie de nematodo perteneciente al género *Metathelazia,* y más concretamente a la especie *Metathelazia capsulata*. Por ello, el cuarto y último objetivo de esta Tesis Doctoral fue hacer una descripción morfométrica detallada de esta especie de nematodo pulmonar, así como de sus marcadores moleculares COI y rDNA. La medición de las principales estructuras de este nematodo se hizo empleando tanto microscopía óptica como microscopía electrónica de barrido, lo cual permitió hacer comparaciones con las descripciones morfométricas anteriormente realizadas de *M. capsulata* en especies de hospedadores diferentes al zorro rojo. La gran mayoría de las medidas realizadas concordaron con las descritas previamente por otros autores, aunque se encontraron algunas diferencias. En concreto, la principal fue la referida a la longitud del nematodo, tanto de machos como de hembras (6,6 mm y 7,4 mm, respectivamente), pero también hubo ligeras variaciones en los valores medios de profundidad de la cavidad bucal y en la distancia entre el poro excretor y el extremo anterior, que fueron inferiores a los descritos hasta el momento. Por otro lado, se llevó a cabo la extracción del ADN y su amplificación a través de la técnica PCR del marcador mitocondrial COI y de cinco marcadores rDNA (18S, 28S, 5.8S, ITS1 y ITS2), para después proceder a su secuenciación y análisis filogenético. Basándose en los datos disponibles en el GenBank, los resultados obtenidos concluyeron que las secuencias de *M. capsulata* están estrechamente relacionadas con la familia Rhabdochonidae, pero también sugieren relaciones distantes con la familia Thelaziidae, ambas pertenecientes a la superfamilia Thelazioidea. Este es el primer hallazgo de *M. capsulata* en zorros de Europa, y por tanto en la península ibérica, al igual que es la primera vez que se describen los marcadores moleculares para esta especie. Este análisis proporciona información muy útil para futuros estudios filogenéticos sobre los nematodos del género *Metathelazia* spp. y, en general, sobre las especies de la familia Pneumospiruridae.

Los resultados obtenidos a lo largo del desarrollo de esta Tesis Doctoral aportan una valiosa información en diferentes aspectos. En primer lugar, ponen de manifiesto la importancia de emplear un método de diagnóstico adecuado para el objetivo perseguido, y se describe un protocolo para el diagnóstico de nematodos cardiopulmonares de carnívoros que permite la obtención de resultados fiables. Por otra parte, el análisis de los condicionantes ambientales en zonas semiáridas mediterráneas resalta que la disponibilidad de humedad, la temperatura y, de manera indirecta, las zonas cubiertas por vegetación, son factores epidemiológicos clave en el mantenimiento, dispersión y abundancia de las especies de nematodos que afectan al zorro y al perro. Teniendo en cuenta que el zorro es la especie de carnívoro silvestre más abundante en esta región semiárida, se debe destacar que su presencia en la interfaz silvestre-doméstico-humano puede suponer un factor de riesgo añadido a la transmisión de parásitos, por lo que se deben aplicar medidas de prevención y control teniendo en cuenta este hecho. Así, estos datos pueden ser útiles a la hora de elaborar programas de vigilancia epidemiológica y planes de gestión y conservación de otras especies de carnívoros o especies en peligro que compartan hábitat con el zorro.

GENERAL INTRODUCTION

ENVIRONMENTAL DISTURBANCES AND WILDLIFE

Throughout the last few decades human pressure on natural ecosystems has increased, leading to the destruction of habitats, being one of the major consequences of these anthropogenic activities the fragmentation of the natural areas. Fragmentation constitutes the modification of natural habitats into smaller and more isolated parcels which has significant effects on the functionality of ecosystems, including the viability of wildlife populations (Jaeger et al., 2016; Amaya-Castaño and Palomares, 2018). These modifications have favoured the emergence of new ecotones in the landscape, leading to changes in ecological interactions and, frequently, causing wild populations movement which may lead to an out of balance of certain wild species populations (Mestre et al., 2021). All these changes result in a progressive movement of these animals closer to urbanized or rural areas, where they find trophic resources or refuge. In this sense, the preference of wildlife species for a particular habitat depends on the type of vegetation cover, food availability, predation risk or environmental barriers of human origin (Amaya-Castaño and Palomares, 2018).

Mammalian carnivores are considered one of the most sensitive species to habitat loss because of their spatial and diet requirements, as well as their reduced reproductive rate (Crooks, 2002; Luck, 2007). Adaptation to changes in the landscape owed to human disturbance varies depending on the ecological and behavioural plasticity of a particular carnivore species (Bateman and Fleming, 2012). Heterogeneous environments are attractive to some wild carnivores because they provide a greater diversity of ecological niches compared to homogeneous ones (Červinka et al., 2014; Mackenstedt et al., 2015). In this sense, urban and peri-urban areas offer facilities in the availability of trophic resources or permanent water sources (Plumer et al., 2014), making these areas attractive for wildlife. In fact, mesocarnivores such as the stone marten (*Martes foina*), Eurasian badger (*Meles meles*), but also wild canids as Iberian wolf (*Canis lupus signatus*) and red fox (*Vulpes vulpes*) have matched well to this kind of environments in the Iberian Peninsula (Šálek et al., 2015). Another adaptative pattern was denominated as "Pax Romana" by Martínez-Abraín et al. (2019), and refers to the evolution of wild fauna from an evasive behaviour to a gradual approach to human settlements. In this sense, one of the carnivore species better adapted to anthropized areas is the red fox, the most widely

distributed wild carnivore in the Iberian Peninsula (Gortázar et al., 2007) whose populations are currently approaching to urban settlements, sharing territories with other domestic carnivores and human populations (Díaz-Ruiz et al., 2016; Barrera et al., 2020).

THE RED FOX

The red fox is a mammal species of the order Carnivora with a worldwide distribution (Macdonald and Reynolds, 2004) (Figure 1). Along the Iberian Peninsula it is mainly present in heterogeneous ecosystems (López-Martín, 2017), although it can occupy a wide variety of habitats (Virgós, 2001). According to the International Union for Conservation of Nature (IUCN), the fox is a species of Least Concern (LC) (Macdonald and Reynolds, 2010), with the same classification in Spain (Blanco, 2007) since it does not have any natural threat to their conservation (Sillero-Zubiri et al., 2004). Nevertheless, in the Iberian Peninsula, as well as in many other Mediterranean regions, the fox is currently a hunting species under population control, and nowadays road-kill is one of the most common causes of death (Grilo et al., 2008).

Figure 1. Worldwide distribution of the red fox (*Vulpes vulpes*). Purple areas correspond to zones where this carnivore has been introduced, and yellow ones are the original distribution area of the species. From: IUCN (International Union for Conservation of Nature) (2016).

Thanks to its ecological plasticity, the red fox is capable to successfully adapt to anthropized areas, such as rural, peri-urban and urban settlement (Díaz-Ruiz et al., 2013; Plumer et al., 2014; Jahren et al., 2020). High scavenging resources, as garbage or road-

kills individuals' carrion make these zones highly suitable for foxes feeding (Martínez-Carrasco et al., 2007; Rosalino et al., 2010; Selàs et al., 2010). Besides, agricultural or forestry activities increase the edge effect between these anthropized areas and natural habitats, which favours the abundance of prey populations and, therefore, the predation success of this carnivore. In this sense, the distribution and availability of trophic resources in the Iberian Peninsula are conditioned by a latitudinal gradient, creating biogeographical differences in the diet of the fox (Dell'Arte et al., 2007; Díaz-Ruíz et al., 2013). Accordingly, lagomorphs are more abundant and the preferred prey in southern areas, while in northern regions, small mammals such as rodents, fruits or seeds are the alternative choice for foxes (Soriguer et al., 2003; Díaz-Ruíz et al., 2013). Reptiles, insects and invertebrates complement the available resources, mostly depending on the type of habitat and the period of the year (López-Martín, 2017). Moreover, the red fox is the main predator of game species with an important hunting value, such as the red-legged partridge (*Alectoris rufa*) or the rabbit (*Oryctolagus cuniculus*) (Delibes-Mateos et al., 2008; Villanúa et al., 2008).

Its generalist and opportunistic diet also make foxes susceptible to becoming infected with a wide range of pathogens (Duscher et al., 2006), since many of their prey could act as intermediate or paratenic hosts species (Senior et al., 1980; Reperant et al., 2009; Bowman, 2014; Deak et al., 2020). Parasite infection in hosts can prompt an impact on the fitness or even the survival of individuals. In this sense, parasite species could affect host population densities, impacting on their body condition and suppressing their immune system when high parasite loads occur (Lindenfors et al., 2007; Oliver-Guimerà et al., 2017; Ferreira et al., 2019), even leading to the death of the host (Traversa et al., 2010). It may also lead to modifications in their social behaviour, foraging, or changing their movements within their home range (Moore, 2002). Depending on their own specific requirements, each parasite may occupy different biological niches in the host, provoking a variety of health consequences such as gastrointestinal or respiratory disorders (Morgan and Shaw, 2010; Traversa, 2011).

Besides the opportunity of hunting a wide range of prey, and thus become infected with the parasites they may harbour, the approach of foxes to urban or peri-urban areas could lead to new opportunities to spread these pathogens (Chase et al., 2020; González-

Ávila et al., 2020; Jahren et al., 2020; McClure et al., 2020). Thus, foxes become highly relevant from an epidemiological point of view (Dáttilo et al., 2020; Tayyrov et al., 2021) due to the health risk for domestic carnivores and humans they represent (Deplazes et al., 2004; Reperant et al., 2007; Di Cerbo et al., 2008; Karamon et al., 2018; Otranto and Deplazes).

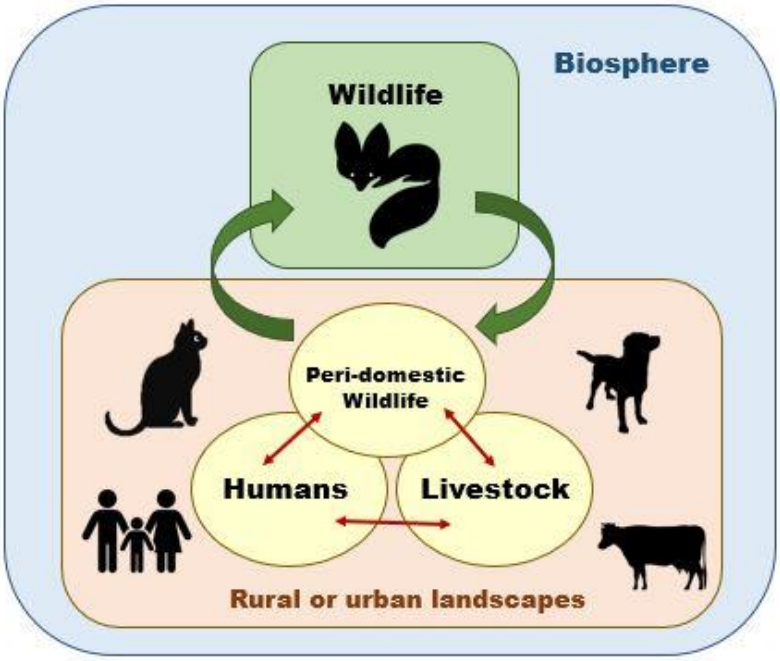

Figure 2. Pathogen spill-over at wildlife-domestic-human interface. Arrows represent the direction of pathogens transmitted among areas of interaction. This spread can lead to the appearance of new reservoirs and even generate a new transmission cycle. Figure based on Jones et al. (2013).

In fact, dogs are phylogenetically very close to foxes (Huang et al., 2014), and share many species of parasites, so their health status may become affected as a consequence of their coexistence in the same area. *Angiostrongylus vasorum* and *Dirofilaria immitis* are two of the most relevant examples of these shared parasite species both located on the right side of the heart and the pulmonary arteries (Alho et al., 2018; Carretón et al., 2020), and they cause severe signs in infected individuals (Traversa et al., 2010). Moreover, *D. immitis* is also a zoonotic parasite. Likewise, other nematode species with potential human health implications are *Ancylostoma caninum,* which causes *cutaneous larva migrans* (Schwartz et al., 2022), or *Toxocara canis*, which causes *visceral larva migrans, ocular larva migrans*, etc., where humans could become parasitized after accidentally

ingesting infective eggs present in the environment (Otranto and Deplazes, 2019; Wu and Bowman, 2020). Hence, red fox can act as a source of parasites and may originate a spill-over in the wildlife-domestic-human interface (Figure 2) (Thompson et al., 2009; 2010). In this epidemiological scenario, wild and domestic canids, and even livestock, might serve as a link between the sylvatic and domestic cycles of parasites, where also human could be concerned.

In this sense, epidemiological studies should be implemented from a One Health perspective (Garcês and Pires, 2021), a multidisciplinary view where animal, human and natural ecosystem health are considered (Figure 3). Through the adoption of this wider perspective of pathogen transmission occurring at the wildlife-domestic-human interface, new disease control plans will become significantly more effective (Mackenzie and Jeggo, 2019).

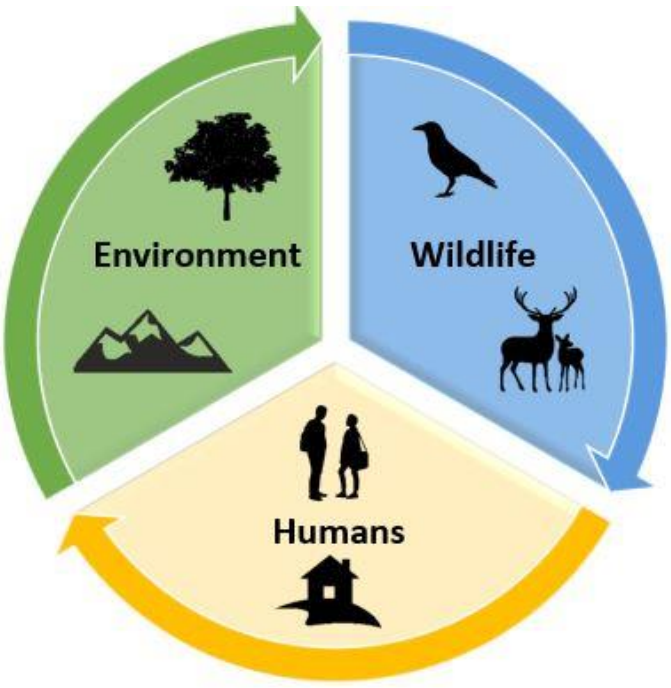

Figure 3. One Health approach, where human, animal (including both domestic and wild species) and ecosystem health are intimately connected. Figure based on Thompson et al. (2013).

PARASITES, ENVIRONMENTAL CONDITIONS AND EPIDEMIOLOGICAL IMPLICATIONS

Similarly to wildlife populations, habitat changes also affect parasite occurrence by altering parasite-host relationships (Harris and Dunn, 2010). Pathogens that proliferate in anthropized environments are those whose transmission depends on vectors or reservoirs that are well adapted to these areas (Cable et al., 2017). From a geographical point of view, parasites must be distributed in the areas where their hosts are also present, thus the relationship between host and parasite abundance can be used to identify epidemiological hotspots where the risk of occurrence of a specific parasite is higher (Poulin, 2014). Likewise, since parasite infective forms and their intermediate or paratenic hosts are present in the environment, it is essential to know as much as possible their biology in order to better understand the epidemiology of the parasite transmission (Morand, 2015; Aleuy and Kutz, 2020). In this sense, parasites occurrence is affected by the climate (Poulin et al., 2011), and many of them show a distribution conditioned by a latitudinal gradient, as a proxy for environmental variables (Morand, 2015). More precisely, climatic factors have an effect on helminths, both on those having an indirect life cycle involving intermediate and/or paratenic hosts, and on those transmitted by free infective stages present in the environment (Poulin et al., 2007, 2011; Altizer et al., 2013; Mkandawire et al., 2022). Parasites are heterothermic animals that can suffer alterations in their phenology as a consequence of changes in abiotic conditions (Mouritsen and Poulin, 2002; Ogden et al., 2005; Poulin and Mouritsen, 2005). However, those which develop within a homeotherms host will be less influenced by external conditions (Møller, 2010). Thus, the environmental temperature has a significant influence on the parasite life cycle, since any disturbance can also affect, for example, the dynamics of the intermediate hosts or their survival (Shapiro et al., 2017). Besides, the presence of humidity in the environment is essential to balance the desiccation effect and favour the survival of the free-living stages, apart from favouring the motility of the larvae in the environment (Tariq, 2015). Probably, combined interaction of these two factors, temperature and humidity, might generate ideal environmental conditions for the development of parasites. A case in point is cover vegetation areas as a proxy of moisture and mild temperatures, which influences the success of the life cycle of parasites, as well

as intermediate and paratenic host population dynamics (Martín and Sommer, 2004; Martínez-Valladares et al., 2013).

For all these reasons, the design of epidemiological studies that consider environmental variables is necessary to know the distribution of parasites and, consequently, to know which are the areas with the highest risk of occurrence of these pathogens (Sanchis-Monsonís et al., 2019; Deak et al., 2020). This type of epidemiological analysis is especially necessary in areas affected by environmental changes, either due to human action or climate change (Jenkins et al., 2011; Jones et al., 2013; McMahon et al., 2018; Aleuy and Kutz, 2020). This is the case of the semi-arid Mediterranean basin, that according to future scenarios due to the progress of climate change, is one of the regions with the greatest vulnerability, showing a high risk of suffering water stress and desertification (IPCC, 2007; Vila-Traver et al., 2021). In general terms, arid or semi-arid Mediterranean regions suffer low rainfall patterns, unequally distributed over the year, and high evapotranspiration values, which lead to a deficit of water severely impacting to biodiversity (Zereini et al., 2004). As regards Iberian Peninsula, floods or prolonged periods of drought will be increasingly frequent scenarios (García-Ruiz et al., 2011; Bangash et al., 2013; Andrade et al., 2021). In particular, southeastern Spain, including the Region of Murcia, is one of the most arid regions in Europe (Gil-Guirado and Pérez-Morales, 2019) and, based on the mean annual precipitation and temperature values, the Köppen-Geiger classification (Peel et al., 2007) categorizes the southeastern Mediterranean basin of the Iberian Peninsula as an arid desert climate (BSk and BSh) (Figure 4).

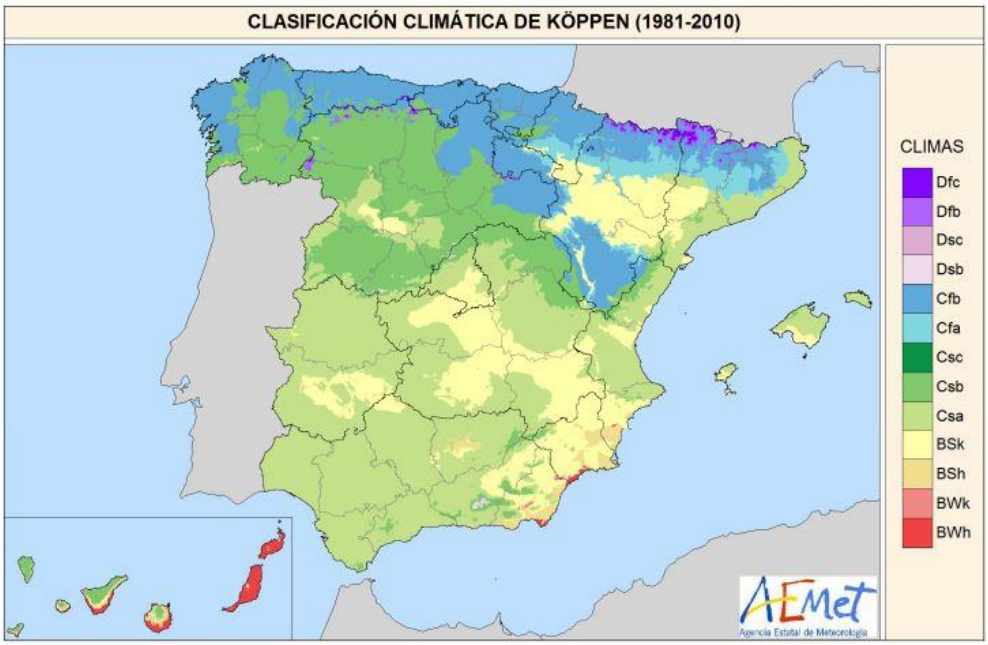

Figure 4. Köppen-Geiger Climate Classification of the Iberian Peninsula, Balearic and Canary Islands (1981-2010). Figure from Chazarra et al. (2018).

The scarcity of hydric resources predicted by these climate scenarios will increase water competition, both in urban, rural and natural environments (Vila-Traver et al., 2021). This trend towards a more intense environmental aridity drives the movement of host species to areas with better resources for their survival, and waterpoints turn to be attractive areas of confluence for domestic animals, human and wildlife (Martínez-Guijosa et al., 2021), leading to an interaction increase among synanthropic species, as the red fox. This tendency can also disturb the dynamics of disease transmission, once directly affecting the reproduction or mortality rates of intermediate and/or paratenic hosts or free-living parasite stages, or changes in the behaviour or population dynamics of hosts, their contact rates or physiology (Dobson et al., 2015).

Considering these predictions in the Mediterranean areas of the Iberian Peninsula, it is important to carry out epidemiological studies that evaluate both the biotic variables of hosts and the abiotic variables of the territory to understand how the parasites are distributed and what is the epidemiological risk of each region. Thus, we could predict in which areas a confluence between fox and domestic canids populations, or humans, is expected to exist, as well as locations where suitable conditions for the parasite life cycle

are occurring. This information is essential to elaborate wildlife management plans and prevention programs for the control of parasite transmission at wildlife-domestic-human interface.

Finally, these epidemiological studies must be based on a precise diagnostic tool, as it is necessary to provide the most accurate data for a correct analysis of the health status of the individual and of the host population studied. In this sense, it is necessary to use a method that provides the greatest possible sensitivity with the lowest false negative rates, as well as the highest accuracy to obtain the most exact values of parasite intensity. The evaluation of the effectiveness of the method used for an epidemiological study is essential, since, apart from selecting the most appropriate to obtain the best results, the costs and execution time of the study must be taken into account (Roeber et al., 2013; Llewellyn et al., 2016; Buonfrate et al., 2018), as depending on the final purpose, the functional performance or technical efficiency involved may differ (Nicolay et al., 2014).

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OBJECTIVES

The red fox (*Vulpes vulpes*, Linnaeus, 1758) can be a host of parasites shared with domestic animals and even humans, thus their epidemiological role in the interaction between the sylvatic and anthropic cycles of these pathogens may be critical. Hence, the main objective of this Thesis is to describe the communities of the nematode species that currently parasitize the red fox population from the Region of Murcia (SE Spain). In this context, the use of an accurate diagnostic method is essential to confirm the presence and intensity of parasites in this host species, which allows a more precise estimation of the potential effect exerted by the pathogen on the host and, moreover, to evaluate the epidemiological risk associated with foxes at the wildlife-domestic-human interface. This leads us to the first objective of this Thesis:

1. To evaluate the sensitivity and exactness of a *post-mortem* diagnostic method for the detection of cardiopulmonary nematodes of the red fox. Traditionally, the opening of the tracheobronchial tree, heart chambers, the lungs and their branches is the diagnostic method carried out to detect parasitized foxes and to calculate the parasite intensity. Our purpose is to analysed if the sensitivity and exactness increase by the additional application of two consecutive steps: squeezing the lung parenchyma and the artificial digestion of this tissue with hydrochloric acid and pepsin solution. This objective will be addressed in **Chapter 1.**

As mentioned above, it is well known that foxes can potentially spread pathogens to domestic animal populations, and even to humans. Therefore, understanding the epidemiological factors that determine the presence of nematodes parasitizing these carnivores in semi-arid areas is necessary, given that water stress is an environmental determinant influencing higher rates of fox contact with anthropized areas. The evaluation of the abiotic characteristics of the area of study could provide valuable information to explain the parasite occurrence in foxes, the epidemiological role of intermediate and paratenic hosts in the maintenance of the life cycle of some of these parasites, as well as the potential significance that transmission through infective stages present in the environment could have. For this purpose, the second and third objectives of this Thesis were proposed:

2. To describe the gastrointestinal and cardiorespiratory nematode communities of the red fox population in the Region of Murcia (SE Spain). The prevalence and intensity of nematode species was calculated, discussing what could be the epidemiological role of the fox in the maintenance and dispersal of some of these parasites, especially in areas shared with other domestic carnivores. In the case of nematode species that are of major health importance, either because they are pathogenic parasites shared with dogs and cats or because they cause zoonoses (i.e., *Angiostrongylus vasorum*, *Crenosoma vulpis*, *Uncinaria stenocephala*, *Toxocara canis* and *Toxascaris leonina*), a detailed study of the environmental factors that could have an effect on their parasite abundance was carried out. This objective will be addressed in **Chapter 2**.

3. To evaluate the occurrence of *Pearsonema plica* in the red fox population of the Region of Murcia (SE). This is a urinary bladder nematode with an indirect life cycle in which earthworms participate as obligate intermediate hosts. For this reason, *P. plica* has been scarcely studied in foxes and other canids from semi-arid areas, as is the case in the southeast of the Iberian Peninsula. However, it is necessary to know if, in these dry areas, there are potential zones where environmental factors allow the parasite to be present. The analysis of the abiotic variables will make it possible to know if *P. plica*, until now neglected in semi-arid Mediterranean regions, should be considered as a potential pathogen for domestic and wild carnivores in these areas. For this reason, it was analysed whether environmental factors influence the presence and abundance of *P. plica*, identifying the presence of transmission hotspots. This objective will be addressed in **Chapter 3**.

Over the course of the objectives described above, bronchial spirurid nematode specimens were isolated, but their identification to the species level did not fully agree with the morphometric descriptions published to date. These nematodes apparently belong to the family Pneumospiruridae, genus *Metathelazia.* A detailed morphometric description was carried out based on images taken with both light and electron microscopy. The main structures of the nematode were measured and described, critically comparing these results with those of similar species described in foxes and

other wild carnivores. The identification was completed by molecular techniques. This objective will be addressed in **Chapter 4**.

CHAPTER ONE:

What is the sensitivity and exactness of post-mortem diagnostic method for cardiopulmonary nematodes in wild carnivores? Towards the gold standard

ABSTRACT

Cardiopulmonary nematodes can cause health and fitness disorders in wild and domestic carnivores. The red fox (*Vulpes vulpes*) may be involved in the spread of these shared parasites at the domestic-wildlife interface. This study aims to evaluate the post-mortem diagnostic method to assess its sensitivity in the detection of cardiopulmonary nematodes in the red fox, as well as their exactness to estimate the parasite intensity of each nematode species. For this purpose, fifty-one cardiorespiratory systems of foxes were analysed. The heart and lungs of each fox were examined through three consecutively methodological steps: first, the tracheobronchial tree and the pulmonary arteries and their branches were opened (OT); next, the lung parenchyma was immersed in water and squeezed (WS); finally, the lung parenchyma was artificially digested in a solution of pepsin and chlorhydric acid (AD). Four nematode species were identified: *Eucoleus aerophilus*, *Angiostrongylus vasorum*, *Crenosoma vulpis* and *Metathelazia capsulata*. Of the total number of nematodes found, most of them were recovered using OT (n=454), followed by WS (n=285) and AD (n=141). The use of these two additional and consecutive methodological steps helped to improve parasite intensity results and decreased false negative case detection. More specifically, the results obtained using OT and WS indicate that, when used together, the sensitivity in the detection of parasitized foxes was 96.1%, while the exactness of the parasite intensity found was 84%. When the AD step is added, although it does not increase the sensitivity in the diagnosis of cardiopulmonary nematodes, results are more exact with respect to parasite intensity, increasing the total number of parasites by 16%. In addition, AD improves the sensitivity in the detection of *A. vasorum* and *M. capsulata*, as well as quantifying more exactly the parasite intensity of these two nematode species (92.5% and 92.3% of exactness without AD, respectively). Our study provides valuable information that should be taken into account when planning epidemiological studies based on cardiorespiratory nematode detection by necropsy in foxes.

INTRODUCTION

Cardiopulmonary nematodes are common parasites of wild and domestic carnivores that inhabit mainly in the tracheobronchial tree (trachea, bronchi and bronchioles). It is widely recognized that lungworms cause respiratory diseases with

serious health implications for the host (Traversa et al., 2010) and, consequently, may also influence wild population dynamics (Martínez-Rondán et al., 2019).

Environmental degradation of natural areas is one of the main factors why a large variety of wild species, including mammals, have expanded their home range into urban and peri-urban areas, attracted by the trophic resources available in anthropized environments. This leads to increased interaction among domestic animals, wildlife and humans (Plumer et al., 2014; Mackenstedt et al., 2015; Deplazes et al., 2019), promoting the spread of parasites to non-endemic areas and their establishment in domestic animals and, when possible, even in humans (Deplazes et al., 2004; Saeed et al., 2006; Traversa et al., 2010; Veronesi et al., 2014; Otranto et al., 2015).

Red fox (*Vulpes vulpes*) is one of the most widely distributed wild carnivore worldwide. This canid has great ecological plasticity, being capable of adapting to a wide range of habitats, including anthropized areas (Díaz-Ruiz et al., 2013; Hoffmann and Sillero-Zubiri, 2016). Indeed, fox prefers fragmented and heterogeneous areas (Gloor et al., 2001). This great adaptability, together with the fact that fox shares a large number of pathogens with dogs and cats, makes it a relevant species from an epidemiological point of view at the domestic-wild interface (Dáttilo et al., 2020; Tayyrov et al., 2021).

Eucoleus aerophilus, *Crenosoma vulpis* and *Angiostrongylus vasorum* are lungworms previously described in wild and domestic canids worldwide (Morgan et al., 2005; Traversa et al., 2010; Tolnai et al., 2015; Deak et al., 2020). Specifically, *E. aerophilus* (syn. *Capillaria aerophila*) is located on the tracheal and bronchial mucosa of a broad range of canids, felids, mustelids and even humans (Anderson, 2000; Lalošević et al., 2008; Traversa et al., 2009; Di Cesare et al., 2014; Tolnai et al., 2015), whereas *C. vulpis* infects the bronchioles, bronchi and trachea of canids and mustelids (Anderson, 2000; Schug et al., 2018). Both nematodes can cause mucosal oedema and lung disorders (Nevárez et al., 2005). On the other hand, *A. vasorum* is a nematode of the pulmonary arteries and the right side of the heart that can produce progressive deterioration of the respiratory and cardiac functions or even neurological disorders (Alho et al., 2018; Carretón et al., 2020). Nowadays this nematode species is considered an emerging lungworm in Europe both in domestic and in wild canids (Helm et al., 2010; Deak et al., 2017; Tayyrov et al., 2021; Tieri et al., 2021). All these nematodes have been previously described in foxes from Iberian Peninsula (Martínez-Carrasco et al., 2007; Garrido-Castañé et al., 2015; Martínez-Rondán et al.,

2019; Carretón et al., 2020). Other cardiopulmonary species described in red fox are the heartworm *Dirofilaria immitis* (Gortázar et al., 1998; Segovia et al., 2004), *Filaroides hirti* (Martínez-Rondán et al., 2019) and *Metathelazia capsulata*, a poorly described nematode found in the bronchi of Nile foxes and mustelids (Geritcher, 1948; Pence and Dowler, 1979; Jiménez et al., 2013).

Red fox is thought to be a key reservoir of cardiopulmonary nematodes worldwide, being one of the main drivers of the increase of cases in dogs (Traversa et al., 2010; Veronesi et al., 2014). The emergence of cardiopulmonary parasites in pets has led to a growing interest to understand the causes of their occurrence (Deak et al., 2020; Fuehrer et al., 2021). Therefore, it is necessary to carry out epidemiological studies based on the diagnosis of cardiopulmonary nematodes in pets and also in foxes, especially in those areas where the interaction between these host species is more intense (De Zan et al., 2021).

Many of the studies published to date aimed at detecting the presence and parasite intensity of cardiopulmonary nematodes in carnivores are based on the isolation of these parasites by necropsy. However, to the authors' knowledge, none of them have evaluated the sensitivity of this diagnostic method. Assessing the effectiveness of the method applied for epidemiological studies is fundamental, since the choice of the most appropriate method guarantees the most accurate results and can also reduce the costs and/or the execution time of the study (Llewellyn et al., 2016; Buonfrate et al., 2018). The most frequently used diagnostic method for detecting cardiopulmonary nematodes in carnivores is the opening of the tracheobronchial tree, heart chambers and the pulmonary arteries and their branches. We propose the evaluation of the sensitivity of this method in the detection of parasitized red foxes and the precision in quantifying the parasite intensity of each species of cardiopulmonary nematode. Moreover, we evaluated the accuracy of the subsequent application of two consecutive steps: first, the immersed in water and squeezing of the lung parenchyma and, second, the artificial digestion of lung parenchyma in a solution of hydrochloric acid and pepsin. The improvement of this diagnostic method could be particularly significant to address studies in areas where wild and domestic carnivores share the same habitat, with high risk of transmission of lungworms.

MATERIAL AND METHODS

Study samples

Between 2015 and 2018, 51 red foxes (23 females and 28 males; 17 juveniles and 34 adults) shot in authorized hunts or found dead by road-kill in Murcia and Alicante provinces (SE Spain) were necropsied. The sex and age of the animals were recorded, as well as their weight divided in three categories: low (< 5kg), medium (5-7 kg) and high (>7kg). The age category was determined by tooth growth (Harris, 1978). Heart and lungs, including the trachea, were extracted, individually refrigerated in plastic labelled bags and submitted to the laboratory, where they were frozen at -20ºC until their analysis.

Laboratory procedures

Analysis of cardiorespiratory system included three consecutive steps: first, the heart chambers, the tracheobronchial tree and the pulmonary arteries and their branches were opened (OT); next, the lung parenchyma was immersed in water and squeezed (WS); and finally, the lung tissue was artificially digested in a solution of pepsin and chlorhydric acid (AD) (Figure 1). Briefly, after defrosting the samples at laboratory temperature, the tracheobronchial tree and the large pulmonary vessel were longitudinally opened and inspected under the stereoscope to isolate the nematodes. Also, the heart was separated from the lungs as close as possible to the main vessels, and chambers were opened and inspected. To conclude the first step of the analysis (OT), trachea and cardiac chambers were washed and filtered through a 63 µm of mesh sieve, and the retained material was examined under the stereomicroscope.

For washing and squeezing the respiratory tracts (WS), lung pieces (3x3 cm) were immersed in a bucket with water and manually squeezed for one minute. Subsequently, the water was filtered as previously described, and the retained material was deposited in a Petri dish and examined under a stereomicroscope to remove all nematodes located in small vessels and bronchi which were not detected with the previous step (OT).

Finally, all the pieces of lung parenchyma were artificially digested (AD) as described in Martínez-Rondán et al. (2019) with some modifications. Specifically, the tissue was digested in a solution with pepsin and chlorhydric acid (1.5%) in distilled water, under a constant and slight shaking, at 40ºC for 15 min. After this time, the digestion sediment

was recovered and examined under the stereomicroscope. When parts of the parenchyma remained undigested, this procedure was repeated as described above.

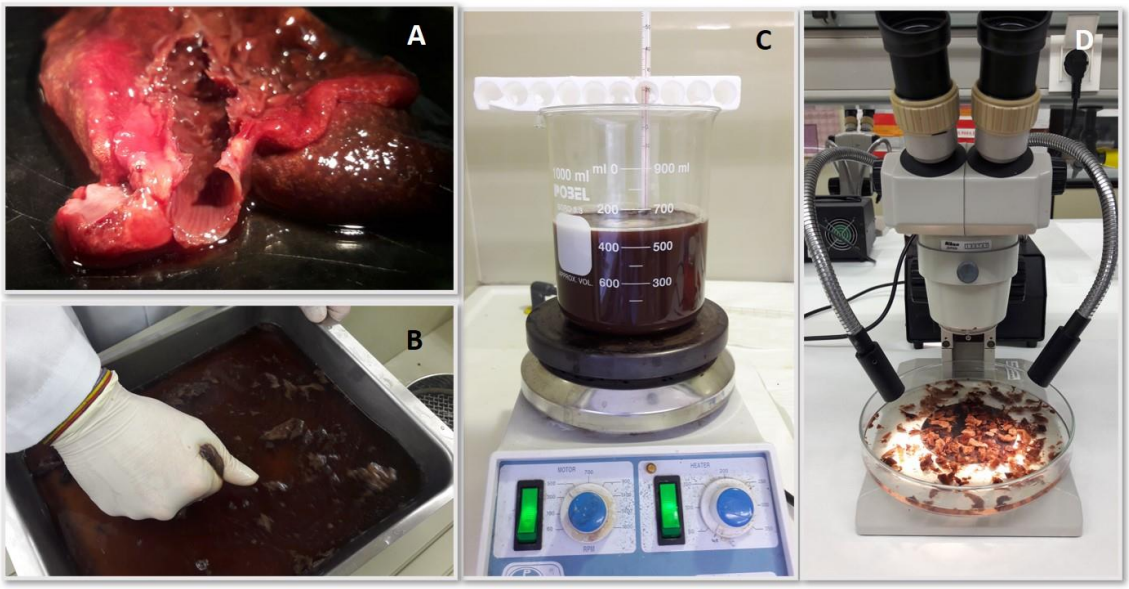

Figure 1. Analysis of cardiorespiratory system included three consecutive steps. (A) Opening of the tracheobronchial tree. (B) Squeezing of lung parenchyma. (C-D) Artificial digestion of the lung parenchyma and remains observation under the stereomicroscope.

The nematodes isolated in each methodological step (OT, WS or AD) were cleaned in distilled water and separately preserved in 70% ethanol until their morphometric identification based on Gerichter (1948), Anderson et al. (2009), Costa et al. (2003), Jiménez et al. (2013) and Latrofa et al. (2015).

Statistical analysis

Prevalence (P) and median intensity (MI) were calculated following Margolis et al. (1982) and Bush et al. (1997). According to quartiles, four categories was used for evaluate overall parasite intensity (very low: 1-2, low: 3-5, medium: 6-20 and high: >20 nematodes), and three categories (low, medium and high) for each nematode species identified (*E. aerophilus*: 1-2, 3-4, >4 specimens; *A. vasorum*: 1-5, 6-20, >20 specimens; *C. vulpis*: 1, 2, >2 specimens; and *M. capsulata*: 1-3, 4-8, >8 specimens). The distribution of data was analysed with Shapiro-Wilks test. Non-parametric Kruskal-Wallis test and Yate's-corrected chi-squared test were used to compare medians and proportions, respectively. R software

4.1.2 (R Core Team, 2021) software was used and significance threshold was established for the value $p<0.05$.

The sensitivity of a diagnostic method is defined by the formula: TP/(TP+FN), where TP is a true positive result and FN is a false negative result. In other words, it is the ability of the method to accurately classify infected animals (Rutjes et al., 2007; Carrau et al., 2021). In our study, TP occurred when cardiopulmonary nematodes were isolated in the studied fox, while FN corresponded to animals in which these parasites were not detected despite being present in the cardiorespiratory system of the host. Specificity is defined by the formula: TN/(FP+TN), where TN is a true negative result, and it is referring to the capacity of the method to detect not parasitized individuals (Coughlin et al., 1992). In this study, the specificity of the method was not taken into account, since the use of AD ensures the identification of 100% of negative foxes (i.e., not parasitized by cardiopulmonary nematodes). Moreover, the identification of the four nematode species found is certain on the basis of the taxonomic keys used, with no possibility of confusion with other nematode species.

RESULTS

Overall, 880 cardiopulmonary nematodes were isolated from the samples, and four species were identified: *Eucoleus aerophilus*, *Angiostrongylus vasorum*, *Crenosoma vulpis* and *Metathelazia capsulata*. The highest number of nematodes were detected by OT (454/880, 51.6%, MI 4 specimens, range 1-70). In the next methodological step (WS), another 285 nematodes were obtained (285/880, 32.4%; MI 4.5; range 1-35), while in the last step (AD), 16.0% of the total number of isolated nematodes were detected (141/880, MI 2, range 1-20) (Table 1, Figure 2). Attending to nematode species, 100% of *E. aerophilus* and 98.4% of *C. vulpis* were collected when the first two steps (OT and WS) were applied. In the case of *A. vasorum* and *M. capsulata*, the percentage of recovery by the first two steps was lower (84.4% and 76.6%, respectively), being necessary to digest the lung parenchyma (AD) to detect the remaining specimens of each species (15.6% and 23.4%, respectively) (Table 1).

Table 1. Distribution of the number (N) and percentage of specimens (P), median intensity (MI) and range of each nematode species isolated from red foxes by opening (OT), squeezing (WS) and artificial digestion (AD).

	OT		WS		AD		
Nematodes	**N (%)**	**MI (range)**	**N (%)**	**MI (range)**	**N (%)**	**MI (range)**	**Total specimens**
E. aerophilus	81 (97.6)	2 (1-15)	2 (2.4)	1 (1-1)	0	0	83
A. vasorum	200 (48.6)	3.5 (1-65)	147 (35.8)	6 (1-35)	64 (15.6)	3 (1-14)	411
C. vulpis	48 (78.7)	1 (1-21)	12 (19.7)	4 (1-7)	1 (1.6)	1	61
M. capsulata	125 (38.5)	2 (1-70)	124 (38.1)	2 (1-27)	76 (23.4)	2 (1-20)	325
All species	454 (51.6)	4 (1-70)	285 (32.4)	4.5 (1-35)	141 (16)	2 (1-20)	880

Overall, the sensitivity of detection of cardiopulmonary nematodes was 80.4% (41/51) when OT was the only procedure used, while it increased to 96.1% (49/51) when OT and subsequent WS were applied. Finally, when the three steps (OT+WS+AD) were used, sensitivity increased by 3.9% (2/51).

According to each of the nematode species found, a sensitivity of 100% was obtained when using OT and subsequently WS in the case of *E. aerophilus* and *C. vulpis* (Table 2). When only OT was used, the number of false negatives was high for *A. vasorum* (30.8%) and *M. capsulata* (51.9%), increasing the sensitivity to 92.3% and 92.6%, respectively, when the second methodological step (WS) was performed. It is relevant to note that up to 7.7% of foxes infected with *A. vasorum* and 7.4% of those harbouring *M. capsulata* would not have been detected as carriers of these nematode species if the last step of the diagnostic method (AD) had not been used.

Table 2. Sensitivity of the detection of the four cardiopulmonary nematode species described in the study using the three-step diagnostic method: opening (OT), squeezing (WS) and artificial digestion (AD).

	OT				OT + WS			
Nematodes	**TP[(a)]**	**FN[(b)]**	**Sensitivity (%)**	**%FN**	**TP[(a)]**	**FN[(b)]**	**Sensitivity (%)**	**%FN**
E. aerophilus	26	1	96.3%	3.7%	27	0	100%	0%
A. vasorum	18	8	69.2%	30.8%	24	2	92.3%	7.7%
C. vulpis	8	1	88.8%	11.2%	9	0	100%	0%
M. capsulata	13	14	48.1%	51.9%	25	2	92.6%	7.4%

(a) True positive foxes; (b) False negative foxes.

The evaluation of biotic factors (age, sex and weight of the foxes) did not show significant relationship with neither prevalence nor parasite intensity in spite of the use of each methodological step or even the whole procedure (p-value>0.05) (Table 3).

Table 3. Number of parasitized foxes (P: prevalence) and intensity of nematodes (MI: median intensity) detected by the three-step diagnostic method (opening: OT; squeezing: WS; artificial digestion: AD) attending to the sex, age and weight of the host.

			Diagnostic procedure					
			OT		OT+WS		OT+WS+AD	
Variable	**Level**	**Number of foxes**	**P (%)**	**Intensity (MI)**	**P (%)**	**Intensity (MI)**	**P (%)**	**Intensity (MI)**
Sex	Male	28	85.7%	299 (5)	100%	475 (7)	100%	566 (9.5)
	Female	23	73.9%	155 (2)	91.3%	264 (3)	100%	314 (3)
Age	Juvenile	17	88.2%	150 (4)	94.1%	251 (7.5)	100%	295 (8)
	Adult	34	76.5%	304 (3)	97%	488 (4)	100%	582 (5)
Weight	Low	13	92.3%	110 (3.5)	100%	187 (4)	100%	208 (4)
	Medium	25	80%	207 (3.5)	92%	336 (7)	100%	427 (9)
	High	13	69.2%	137 (4)	100%	216 (3)	100%	245 (3)

The use of OT as the unique methodological step resulted in the detection of only 56.2% of the positive animals with an overall very low parasite intensity (1-2 cardiopulmonary nematodes), showing statistically significant differences (p-value <0.05) with the results of foxes with any of the remaining ranges of parasite intensity: 90.9% (low parasite intensity), 83.3% (medium parasite intensity) and 100% (high parasite intensity) (Table 4). In addition, in the case of foxes with an overall very low parasite intensity, 12.5% were false negatives when OT and WS were applied consecutively, being only confirmed as true positives when AD was used. Although differences were not statistically significant, the number of nematodes isolated increased when AD was included in the diagnostic procedure, improving the exactness. Specifically, 20% (4 nematodes), 15% (6 nematodes), 22.3% (32 nematodes) and 14.7% (99 nematodes) of the total number of nematodes isolated from foxes with very low, low, medium and high parasite intensity, respectively, were detected when using AD. In this sense, the exactness improves when, at least, OT and WS are used as a two-step method of parasite detection as is shown in Table 4.

Table 4. Positive foxes (P (%)) and number of nematode specimens (ns) detected by the three-step diagnostic method (opening: OT; squeezing: WS; artificial digestion: AD) attending to the global parasite intensity ranges.

		Diagnostic procedure						
		OT		OT+WS		OT+WS+AD		
Intensity range	**Number of foxes**	**P (%)**	**ns**	**P (%)**	**ns**	**P (%)**	**ns**	**Exactness (OT +WS)**
Very low (1-2)	16	56.2%(*)	10	87.5%	16	100%	20	80%
Low (3-5)	11	90.9%	22	100%	34	100%	40	85%
Medium (6-20)	12	83.3%	51	100%	112	100%	144	77.7%
High (>20)	12	100%	371	100%	577	100%	676	85.3%
Total specimens			454		739		880	83.9%

(*) Statistically significant differences (p-value < 0.05).

The lowest sensitivity of detection by OT of *M. capsulata* and *A. vasorum* was recorded in the groups of foxes with low parasite intensity, showing 68.8% and 42.6% of false negatives (Table 5), although differences were only statistically significant for *M. capsulata* ($p<0.05$). This percentage remains remarkable for both nematode species (12.5% and 14.3 % of false negatives, respectively) when the diagnostic procedure included two steps (OT followed by WS). Interestingly, the use of the OT step detected 100% of the foxes with a high parasite intensity of each of the four species of cardiopulmonary nematodes found (Table 5).

Finally, the implementation of AD as the last step allowed the detection of specimens of *A. vasorum* and *M. capsulata* that were not found with the two previously applied steps (OT and WS). Specifically, in the case of *A. vasorum* it was possible to detect 25% of the total nematodes (7/28 nematodes) in the category of foxes with low parasite intensity, 24.3% (18/74) in foxes with medium parasite intensity, and 12.6% (39/309) in animals with high parasite intensity. In the case of *M. capsulata*, these same parasite intensity categories were 24% (6/25), 23.4% (7/30) and 23.4% (63/270), respectively (Table 5).

Table 5. Number of parasitized foxes (P: prevalence) and number of nematode specimens (ns) detected by the three-step diagnostic method (opening: OT; squeezing: WS; artificial digestion: AD) attending to the parasite intensity ranges of each cardiopulmonary nematode species.

			Diagnostic procedure		
			OT	OT+WS	OT+WS+AD
Species	**Intensity range**	**Number of foxes**	**P (%) (ns)**		
E. aerophilus	1-2	17	94.1% (21)	100% (22)	100% (22)
	3-4	5	100% (17)	100% (17)	100% (17)
	>4	5	100% (43)	100% (44)	100% (44)
A. vasorum	1-5	14	57.4% (11)	85.7% (21)	100% (28)
	6-20	6	66.6% (22)	100% (56)	100% (74)
	>20	6	100% (167)	100% (270)	100% (309)
C. vulpis	1	6	83.3% (5)	100% (6)	100% (6)
	2	1	100% (2)	100% (2)	100% (2)
	>2	2	100% (41)	100% (52)	100% (53)
M. capsulata	1-3	16	31.2% (*) (6)	87.5% (9)	100% (25)
	4-8	5	40% (3)	100% (23)	100% (30)
	>8	6	100% (116)	100% (207)	100% (270)

(*) Statistically significant differences (p-value < 0.05).

DISCUSSION

To the authors' knowledge, to date no study evaluated the sensitivity and exactness of the commonly used diagnostic method for the detection and quantification of cardiopulmonary nematodes in carnivores. This method consists on opening the tracheobronchial tree, the heart chambers, and the pulmonary arteries and their branches. To know the sensitivity of this method, it is necessary to have a gold standard procedure that allows knowing exactly the number of nematodes in the cardiorespiratory system. In our study, we have applied a procedure based on three consecutive steps (OT, WS, AD) that, together, allow all nematodes to be detected and, therefore, should be considered the gold standard method. Three of the cardiopulmonary nematode species detected (*E. aerophilus*, *C. vulpis* and *A. vasorum*), had been previously described parasitizing foxes in the Iberian Peninsula (Martínez-Carrasco et al., 2007; Garrido-Castañé et al., 2015; Fanelli et al., 2019; Martínez-Rondán et al., 2019). However, the fourth recovered species, *M. capsulata*, has not been recently recorded worldwide. The

last reported description of this species in foxes was in Israel in the middle of the last century (Gerichter, 1948), and later the nematode was only cited in badgers (*Meles meles*) (Pence and Dowler, 1979). Recently, a new species of this same genus, *M. mexicana*, has been described in nine-banded armadillo (*Dasypus novemcinctus*) from Central Mexico (Jiménez et al., 2013). To the author's knowledge, this is the first report of this nematode species in red foxes from Iberian Peninsula.

Since the application of the three diagnostic steps was performed consecutively, the first one (OT) was the step with the highest recovery of nematodes as expected, followed by WS and, finally, AD. The use of OT and subsequently WS allowed the detection of a high percentage of parasitized animals (96.1%). In terms of overall prevalence of cardiopulmonary nematodes, our results show that if AD is not applied as the last step in lung processing, the method will have a 3.9% false negative rate. These results, although represent a small percentage, should be taken into account when conducting epidemiological studies based on OT and WS exclusively. Regarding the parasite intensity estimation, our study shows that the exactness of the method is reduced when AD is not used (84% when OT and WS were applied), especially in the quantification of *A. vasorum* and *M. capsulata* (84.4% and 76.6%, respectively). In the case of *E. aerophilus* and *C. vulpis*, exactness values were higher although AD were not used (100% and 98.3%, respectively). Globally, when AD was used, 141 more nematodes were detected (16%). In this regard, our results indicate that better records were obtained when two consecutive 15 minutes-AD steps were performed instead of only one lasting 30 minutes. This difference helped to prevent nematodes to be digested.

Most of studies focused on the estimation of lungworms in foxes only use opening and/or flushing (Jeffery et al., 2004; Santoro et al., 2015; Houpin et al., 2016; Deak et al., 2020; Gillis-Germitsch et al., 2020; Lemming et al., 2020). However, results are not always comparable since the method used in these studies is not uniform in all cases (Lemming et al., 2020). Moreover, the aim of many studies is to determinate the presence/absence of cardiorespiratory nematodes, without evaluating in many cases the parasite intensity (Schug et al*.,* 2018). This highlights the need to standardize the diagnostic method for parasites in carnivores based on the necropsy of the cardiorespiratory system.

There are no similar studies evaluating the sensitivity and exactness of cardiopulmonary nematode detection using different methods. Only Houpin et al. (2016)

compared the results of three methods for the detection of *A. vasorum*: dissection and visual examination (comparable to OT), PCR and a canine antigen detection ELISA test. The sensitivity of detection of *A. vasorum* using the OT method described by these authors reached 84.1%, while in our study it was 69.2%. However, the application of the second step (WS) in our study reached a sensitivity of 92.3%.

Sensitivity of detection and exactness of the parasite intensity quantification found by our three-step method varied attending to the lungworm species. Specifically, the sensitivity was 100% for detecting *E. aerophilus* and *C. vulpis* infected foxes, and the exactness of the parasite intensity was 100% and 98.4%, respectively, when OT and subsequently WS were used. These results are consistent with the fact that *E. aerophilus* is usually detected in the upper parts of the respiratory tract (trachea and large bronchi) and *C. vulpis* is localized in bronchi and bronchioles (Nevárez et al., 2005), so the application of AD does not represent a significant step to extract these nematode species from their usual location.

On the other hand, sensitivity of detection of positive foxes to *A. vasorum* and *M. capsulata* was below 93% when AD step was not used, meaning that 7.7% and 7.4% of parasitized foxes by these nematode species were false negative, respectively. In terms of intensity, 15.6% of *A. vasorum* specimens parasitizing foxes (range: 1-14) were found using AD. This is justified by the location of these nematodes in areas of the lung that make their detection by OT and WS difficult. In this sense, adults of *A. vasorum* are usually located in the right ventricle and major pulmonary arteries, but also in capillary vessels (Carretón et al., 2020; Morchón et al., 2021), showing a great difficulty to be opened, even using fine-tipped scissors. So, artificial digestion represents a necessary step to extract the nematodes from these parts of the lung parenchyma. *A. vasorum* is highly pathogen to canids (Poli et al., 1991; Morgan et al., 2008). In fact, high-loaded *A. vasorum* lungs can be easily recognized as they are much heavier and denser than healthy ones, showing yellow calcareous deposits that denote the existence of damaged areas (Jeffery et al., 2004). Therefore, when a high intensity of *A. vasorum* can be perceived visually, the use of the whole procedure could be recommended to provide more exact parasite intensity results.

Likewise, *M. capsulata* collected by AD represented 23.4% (range: 1-20) of the total number of these nematodes isolated in this study. This species is found mainly in bronchi and bronchioles (Gerichter, 1948; Pence and Dowler, 1979) and, as previously mentioned,

artificial digestion allows a better isolation of specimens located in bronchioles of smaller diameter.

As noted above, the small capillaries or bronchioles are not often examined in detail when using only OT, either with or without the later use of WS, so it is not possible to recover all the nematodes present in the host nor to detect all the positive animals. Consequently, prevalence, abundance or parasite intensity rates could be underestimated (Morgan et al., 2008). Particularly, our results showed that up to 25% and 24% of the specimens of *A. vasorum* and *M. capsulata* would not be detected if AD step was not included in the diagnostic evaluation of foxes with low parasite intensities. Therefore, AD is necessary to improve recovery of nematodes as well as to decrease false negative cases (Martínez-Rondán et al., 2019).

The use of the WS step in addition to the classical OT step is decisive in estimating the prevalence of parasitized foxes with a low intensity of *M. captulata*, since the number of positive animals detected with these two phases is significantly higher than that described in the case of exclusively using OT.

Sex, age, and weight of foxes in no case influenced the results of the diagnosis of cardiopulmonary nematodes, which shows that the three-step method we describe can be applied in a general way in carnivores, regardless of the category to which the host belongs.

According to the prevalence of parasitized foxes registered by using the first two steps of the proposed method (OT and WS), the use a costly and time-consuming technique such as AD should be carefully evaluated since it will only increase the number of positive animals by 3.9%. On the other hand, AD should be of great interest if the objective of the study is to quantify the parasite intensity on the host, obtaining the exact number of specimens.

The results achieved in the present study allow us to assure that the classic method used so far to detect the presence of cardiopulmonary nematodes in carnivores, only based on the opening of the airways and the heart together with washing and squeezing of these tissues, is not sufficient to precisely analyse small respiratory organs as well as to detect the presence of very small nematodes. In these circumstances, the addition of a supplementary diagnostic step, the artificial digestion of lung tissue, should be convenient. Furthermore, this three-step method has proved to be of particular interest

in heavily parasitized animals. The fox is a synanthropic carnivore species that shares habitat with other domestic carnivores (mainly dogs and cats) and even humans. Their faeces, which may contain infective eggs or larvae, could be a source of infection for stray and domestic dogs (Traversa et al., 2014), and even humans could be affected with several bronchopulmonary zoonotic nematodes such as *E. aerophilus* (Otranto and Deplazes, 2019). The implementation of this method could provide precise information on the distribution of cardiopulmonary nematodes on wild carnivores, nowadays submitted to deep investigation given the possibility that domestic animals acquire these infections by contact with wild carnivores, even more in the present circumstances, in which the approach of populations of wild carnivores to anthropized areas has been widely verified. This knowledge would allow to significantly improve the design of the wildlife management plans currently in force.

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CHAPTER TWO:

Environmental determinants in the occurrence of gastrointestinal and cardiopulmonary nematodes in the red fox in semi-arid Mediterranean areas

ABSTRACT

Increasing fragmentation of natural ecosystems due to human activity is leading to movement of wildlife from their natural habitats to new areas, including urban settlements. Red fox (*Vulpes vulpes*) is the most abundant wild canid in semi-arid Mediterranean areas of the southeaster Iberian Peninsula, and its approach to anthropized areas could pose a risk of disease transmission to domestic carnivores and even to humans. Such transmission of pathogens may also be conditioned by the climatic factors of the region, affecting their survival or that of intermediate and/or paratenic hosts involved in the life cycle. Climatic conditions of semi-arid regions are characterized by high temperatures and low precipitation, so it is important to understand how this may influence on the distribution and abundance of parasites in the territory. On the other hand, Mediterranean basin, which includes the semi-arid Mediterranean regions of the Iberian Peninsula, is considered a vulnerable area owing to meteorological variations derived from climate change, with a predictive future with more dry periods and greater fluctuations in the rainfall dynamics. Due to the arid conditions of this region, the scarcity of water availability could result in areas shared by synanthropic fox populations and domestic animals that become hotspots for parasite transmission. Hence the main objective of this study was to describe the gastrointestinal and cardiopulmonary nematode species affecting fox population in these semi-arid areas and also the influence of environmental variables on the parasite abundance. For this purpose, the respiratory and gastrointestinal tracts of 167 foxes collected over the years 2015-2021 from the Region of Murcia (SE Spain) were necropsied. Environmental variables, composed by three datasets (climatic variables, spectral index and land cover), as well as host characteristics (sex, age and body condition using KFI index) were evaluated using a Generalised Linear Model. To determine the parasite spatial aggregation Moran index was used, as well as a continuous spatial distribution based on statistical models obtained. The total richness found was eleven species, seven were gastrointestinal nematodes (*Pterygodermatites affinis*, *Uncinaria stenocephala, Toxocara canis, Toxascaris leonina, Spirocerca vulpis, Oxynema crassispiculum* and *Trichuris vulpis*) and four cardiopulmonary ones (*Angiostrongylus vasorum, Crenosoma vulpis, Eucoleus aerophilus and Metathelazia capsulata*). The influence of these biotic and abiotic variables was performed for *A.*

vasorum, C. vulpis, U. stenocephala, T. canis and *T. leonina*, considered to be the nematode species of greater importance for domestic canid health, and in some cases, even for human health. Temperature and humidity were found to be the most important variables influencing the abundance of the parasites, as well as areas with forestry or agricultural lands. Cover vegetation areas in this semi-arid zone, provide refuge sites with mild temperatures and moisture in the soil, which help the optimal development of *U. stenocephala, T. canis* and *T. leonina* eggs, as well as the establishment of gastropod populations that participate in the life cycle of *A. vasorum* and *C. vulpis*. Although the analysis of spatial parasite distribution abundance indicates a random pattern, maps about absolute abundance distribution values showed a geographically defined location for *C. vulpis,* and *T. leonina,* whereas this distribution where homogeneous for the rest of nematode species selected. This geographical distribution is especially important in the case of *U. stenocephala, T. canis* and *T. leonina* species, since they showed points with high values of abundance near to anthropized areas. These findings have to be carefully evaluated since the presence of these parasites may result in a risk of transmission between domestic and wild canids, and even to human health, so it should be considered in epidemiological vigilance and management plans.

INTRODUCTION

The increase of the environmental pressure and the fragmentation of natural habitats are two of the consequences of the anthropogenic activities, which cause a severe impact on wildlife. These changes in ecological interactions have caused, in addition to the loss of biodiversity, that many wild species have had to adopt new strategies to find resources, often being forced to adapt to anthropized areas (Pimm et al., 1995; Ellis, 2011; Brearley et al., 2013). One of the consequences of this change in wild animal behaviour has been the increase in their contact with domestic animals and humans (Di Cerbo et al., 2008), which means an enhanced transmission risk of pathogens (Smith et al., 2009; Thompson et al., 2009; Duscher et al., 2015; Hassell et al., 2017). In this sense, anthropogenic trophic sources are a predictable and attractive resource for wildlife, leading to changes in the ecology of wild species and, indirectly, potential shifts in host-parasite interactions (Becker et al., 2018).

The maintenance and transmission of parasites is conditioned by the presence of their host species (Harris and Dunn, 2010). Nevertheless, this parasite occurrence is also affected by abiotic factors (Dobson et al., 2015; Morand, 2015; Blasco-Costa and Poulin, 2017; Turner et al., 2021). In this sense, environmental conditions could influence on the survival and embryogenesis of the parasites' free-living stages, and also invertebrate species that participate as intermediate or paratenic hosts are sensitive to these conditions (O´Connor et al., 2006; Poulin et al., 2007, 2011; Altizer et al., 2013; Mkandawire et al., 2022). For instance, very high temperatures could induce the desiccation of parasite eggs present in the environment (Okulewicz et al., 2012; Aleuy and Kutz, 2020), whereas moisture could be a factor for the successful development of embryonated eggs or infective larvae (O´Connor et al., 2006; Tarbiat et al., 2015). Likewise, molluscs and earthworms, which are common intermediate or paratenic hosts of several helminth species, are influenced by these climatic factors (Morgan et al., 2009; Singh et al., 2019; Aleuy and Kutz, 2020). Besides, areas provided with natural refuges such as woodlands or cultivated areas also favour the presence of beneficial microclimates for the development of these invertebrates (Martín y Sommer, 2004). Hence, it is important to identify locations which can become microclimates with favourable conditions for the establishment of the sylvatic cycle of parasites in order to detect potential foci of infection.

On the other hand, climate change is leading to variations in meteorological dynamics, increasing global mean temperature or decreasing precipitation. As a result of predictive climatic models, Mediterranean basin is one of the most affected areas by these changes (IPCC, 2007; Vicente-Serrano, 2007). In particular, the Mediterranean semi-arid regions of the Iberian Peninsula will be one of the most vulnerable areas due to increased drought periods or floods (Vicente-Serrano, 2007; Bangash et al., 2013; Andrade et al., 2021). Considering the importance of humidity and temperature on parasite life cycles, epidemiological studies must consider all these environmental determinants, especially in semi-arid Mediterranean areas, since estimates indicate that they will suffer greater water stress in the coming decades (Tomaszkiewicz et al., 2016; Vila-Traver et al., 2021).

The red fox (*Vulpes vulpes* Linnaeus, 1758) is an abundant carnivore with a worldwide distribution. It is an opportunistic predator, with a varied diet that includes

small and medium-size mammals, birds, reptiles, insects, fruit or rubbish (Morandi et al., 2019; Waindok et al., 2021). This wild canid is a resilient species with an ecological plasticity that allows it to adapt to different types of habitats, such as forests, agricultural lands, or even peri-urban and urban areas, where high fox density could occur due to the availability of anthropogenic trophic resources (Martínez-Carrasco et al., 2007; Sándor et al., 2017; Karamon et al., 2018). The red fox is a territorial species, with a relatively small home range (Trewhella et al., 1988; Devenish-Nelson et al., 2013) determined by several factors, such as age, sex, presence of predators or food availability (Castañeda et al., 2019; Main et al., 2020). On average, the size of their home range is between 50-250 ha, although it can sometimes reach 500-650 ha when larger territories are available (Dekker et al., 2001; Henry et al., 2005). On the other hand, the fox can act as a host for a large number of parasites. For this reason, many studies highlight the prominent role that the fox can play in the transmission of these parasites, especially in human settlements (Deplazes et al., 2004; Reperant et al., 2007; Di Cerbo et al., 2008; Otranto and Deplazes, 2019). In fact, the occurrence of foxes in anthropized areas of semi-arid Mediterranean climate in southeastern Iberian Peninsula is increasingly frequent. In addition, considering that this wild canid can be a host of parasites shared with domestic animals and even humans, its epidemiological role in the interaction between the sylvatic and anthropic cycle of these pathogens may be critical (Traversa et al., 2010). Among these parasites shared with domestic canids are cardiopulmonary nematodes such as *Angiostrongylus vasorum* and *Crenosoma vulpis* (Alho et al., 2018, Schug et al., 2018), both presenting an indirect life cycle with gastropod molluscs as intermediate hosts (Anderson, 2000; Carretón et al., 2020). Regarding the gastrointestinal nematodes of wild and domestic canids, *Uncinaria stenocephala*, *Toxocara canis* and *Toxascaris leonina* are particularly relevant because they are broadly distributed and may cause zoonoses (Okulewicz et al., 2012; Traversa et al., 2014).

Due to the epidemiological importance arising from this changing situation, the main objective of this study was to describe the cardiopulmonary and gastrointestinal nematode species affecting the fox populations in semi-arid areas of southeastern Iberian Peninsula, analysing whether there are environmental determinants that influence the abundance and spatial distribution of these parasites. Moreover, it was discussed whether

the presence of foxes in semi-arid areas represents a health risk in the transmission of these parasites to dogs and humans present at the wildlife-domestic-human interface.

MATERIAL AND METHODS

Study area and sample collection

The study was carried out in the Region of Murcia (SE Spain), an area with a predominantly Mediterranean climate and considered as one of the driest regions of the Iberian Peninsula. The annual rainfall is scarce (less than 400 mm/year), with high interannual variability and very high potential evapotranspiration values (Miró et al., 2018).

This study involved a total of 167 foxes collected between 2015 and 2021 from hunting programmes, wildlife recovery centres or road-killed individuals, covering the whole of the Region of Murcia. The site where each fox was recovered was identified by using geographic coordinates as represented in Figure 1.

Animals were categorized according to sex (96 males and 71 females), and age (51 juveniles and 116 adults) following Harris (1978). During necropsy, the respiratory and gastrointestinal tracts of foxes were extracted, refrigerated in plastic labelled bags, and frozen at -20ºC until analysis. In the case of one road-kill fox, only the small intestine could be obtained, because most of the large intestine had been lost. Moreover, the Kidney Fat Index (KFI) was used to estimate body condition of foxes. This index was calculated according to Riney (1955), applying the formula: KFI = (FW/KW) *100 (Riney, 1955), where Fat Weight (FW) is the weight of the perirenal fat and Kidney Weight (KW) the weight of the kidney after fat removal.

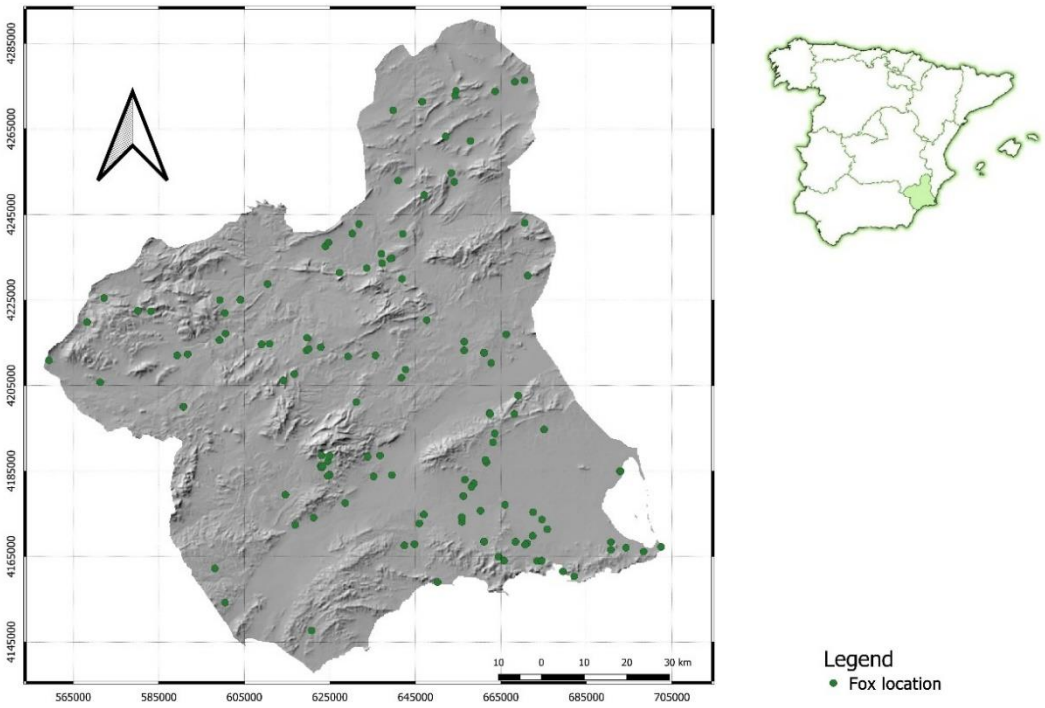

Figure 1. Location of foxes (n=167) in the study area (Region of Murcia, SE Spain).

Laboratory procedures

After defrosting, the tracheobronchial tree and the large pulmonary vessels were longitudinally opened and examined under the stereomicroscope. Besides, heart was separated from lungs and chambers were opened and inspected. Then, trachea and the cardiac chambers were washed using a sieve (mesh diameter 63 µm), and the withheld material was observed under the stereomicroscope. Subsequently, lung parenchyma was cut in small pieces (3x3 cm) and manually squeezed immersed in a bucket with water. This water was filter as described above and the content transferred to a Petri dish and examined under the stereomicroscope. In this way, parasites that would have remained in the minor blood vessels or bronchi can be flushed out. Finally, according to Martínez-Rondán et al. (2019), artificial digestion of lung parenchyma was performed. Briefly, tissue was digested in a solution with pepsin and chlorohydric acid (1.5%), constantly shacked, at 40ºC, for 15 min. The sediment was checked under the stereomicroscope.

Each section of the digestive tract (oesophagus, stomach, small and large intestine) was separated and then opened longitudinally. The mucosa of each of these portions was

scraped and washed using a sieve (mesh diameter 300 µm), and both the content and the mucosa were examined under the stereomicroscope (Figure 2). All recovered nematodes were preserved in 70% ethanol until identification.

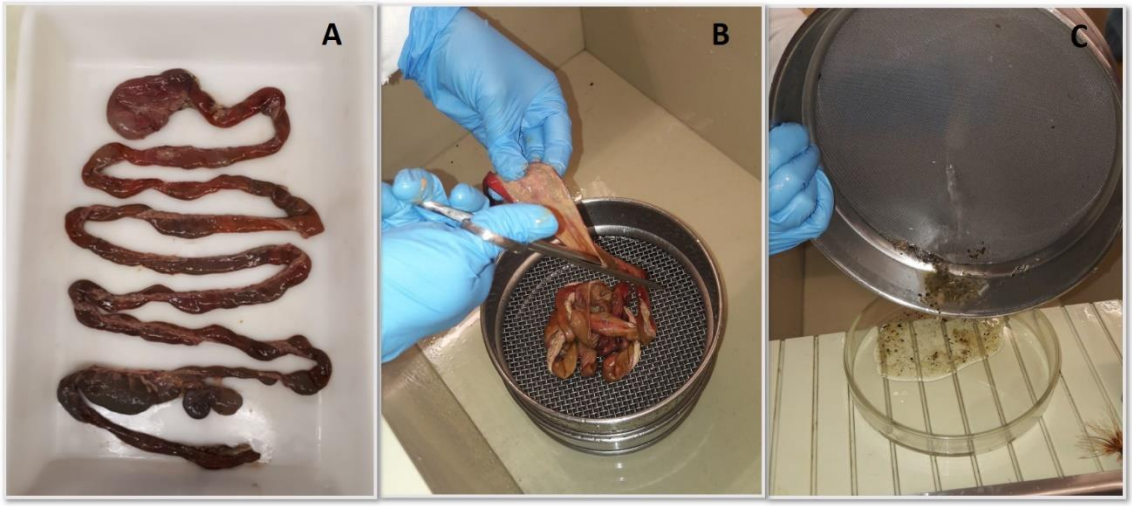

Figure 2. Digestive tract analysis procedure (A). Digestive tract extracted after necropsy. (B) Intestine opened longitudinally and the posterior washing and sieving of the contents (C).

Lactophenol was used to clear adult nematodes before their morphometrical identification according to Gerichter (1948), Yamaguti (1961), Anderson (2000), Costa et al. (2003), Popiolek et al. (2009) and Rojas et al. (2018).

Statistical analysis

Prevalence (positive animals/total animals; P) with 95% confidence intervals (95%CI), median abundance (number of parasites/total animals; MA) with the range, and median parasite intensity (number of parasites/positive animals; MI) with the range were calculated according to Margolis et al. (1982) and Bush et al. (1997) for each nematode species isolated in this study.

R software 4.1.2 (R Core Team, 2021) software was used for carrying out statistical analysis, and significance threshold was established for p value <0.05. Shapiro Wilks test was used to determine the normality of data.

Once the species were identified, the analysis of environmental and host-dependent factors that could be influencing the abundance of these species was limited to *A.*

vasorum, *C. vulpis*, *T. canis*, *T. leonina* and *U. stenocephala*. These parasites were selected because of their epidemiological, pathogenic and health relevance, since they are frequent in both domestic and wild canids and because, sometimes, they can cause infection in humans (Smith et al., 2003; Macpherson, 2013; Taylor et al., 2015; Hodžić et al., 2016; Otranto and Deplazes, 2019; Lemming et al., 2020; Tylkowska et al., 2021; Pohly et al., 2022). A Generalised Linear Model (GLM), with either a Poisson or Binomial negative distribution was used to evaluate host and environmental factors influencing parasite abundance of *C. vulpis* and *T. canis*, *A. vasorum*, *T. leonina* and *U. stenocephala*.

Three packages of environmental variables were used (Table 1). The first dataset is comprised of annual and monthly average data series of climatic variables such as precipitation, temperature, radiation and reference evapotranspiration (ET0) (Ninyerola et al., 2005). In the case of ET0, these values were calculated in accordance with Hargreaves and Samani (1985) using monthly and annual average values of temperature and radiation. The second dataset was obtained from the reflectance values of the land surface from the OLI and TIRS sensors of the Landsat 8 satellite (https://earthexplorer.usgs.gov/). Images from winter and summer seasons were considered (05/08/2019 and 12/01/2020, respectively). The Normalized Vegetation Index (NDVI), which is an index related to plants photosynthesis, is calculated from the ratio of the wavelengths of the visible spectrum in the red range ρ_r (0.64-0.67 µm) and in the near infrared ρ_{NIR} (0.85-0.88 µm). Normalized Moisture Index (NDMI) is related to the vegetation water stress, which is determined from the wavelengths of the near infrared, and the Short-Wave Infrared ρ_{SWIR1} (1.57-1.65 µm). Bare Soil Index (BSI) reports the difference in spectral behaviour between bare soil and sparsely vegetated areas. For that, algorithm uses the wavelengths mentioned above in addition to the blue range ρ_b (0.45-0.51 µm) of the visible spectrum. Moreover, the thermal infrared band ρ_{TIR1} (10.60-11.19 µm), that estimates soil moisture and thermal mapping, was applied from the TIRS sensor. Finally, Land Cover dataset was divided into five habitat categories defined in CORINE Land Cover (CLC): artificialized territory, agricultural land, forestry areas, wetlands and water surfaces (https://land.copernicus.eu/pan-european/corine-land-cover/clc2018). All these dataset groups were calculated considering a 1 km radius buffer (400 ha) from the geographic location of origin of each individual, considered as average fox home range (Deak et al., 2020), using QGIS (3.16.11) software.

The collinearity of the data was assessed by the Variance Inflations Factors (VIF), reducing the number of factors included in the model. Specifically, when VIF values were greater than 5, the collinearity was considered to be high, and these values were discarded. Package “usdm” was used to calculate VIF values (Naimi et al., 2014). Akaike's Information Criterion (AIC) (Akaike, 1974) was used for model selection.

Based on fox locations, the spatial autocorrelation of each of the selected nematode species was also analysed. For this purpose, the Moran index was used, where values close to 0 denote a random distribution, while the positive values indicate a tendency to clustering and negative values would suggest a tendency of the data to be dispersed. The result is a z-score and a p-value that will give us information on whether there is statistical significance.

Finally, the continuous spatial distribution of the abundance of the analysed nematode species was calculated using the inverse distance weighted interpolation (IDW) process. The method was performed using ArcGIS through the Geostatistical Analyst plug-in. Independent parameters were fitted for each of the variables containing parasite abundance, according to the resulting models. To predict a value at locations without records, IDW takes the measured values surrounding the prediction location. Recorded values closer to the prediction site will have a greater influence on the predicted value than those further away, assuming that each measured point has a local influence that decreases with distance (Watson and Philip, 1985).

Table 1. Host and environmental variables that predict nematode species occurrence in red foxes from the Region of Murcia (SE Spain).

		Description	Source
Host variables	Sex	Male and female levels	-
	Age	Juvenile and adult levels	Harris (1978)
	KFI (body condition)	Indicator of physiological and nutritional status of the host	Riney (1955)
Climatic variables	Evapotranspiration	Daily potential evapotranspiration (mm/day)	Ninyerola et al. (2005)
	Radiation	Extra-terrestrial solar radiation (mm/day)	
	Precipitation	Average of precipitation during the summer and winter seasons (mm)	
	Temperate	Average of temperate during the summer and winter seasons (ºC)	
Spectral index	NDVI	Vegetation quantification calculating the difference between near-infrared (vegetation reflects) and red light (vegetation absorbs).	Elaborated by the authors based on Landsat images from https://earthexplorer.usgs.gov/
	NDMI	Combine near-infrared and short-wave infrared to measure the water content of the vegetation.	
	BSI	Indicator of soil variations combining short-wave spectral bands and blue, red, near-infrared.	
Land cover	CLC category 1	Artificial surfaces	CORINE Land Cover
	CLC category 2	Agricultural areas	
	CLC category 3	Forest and semi-natural areas	
	CLC category 4	Wetlands	
	CLC category 5	Water bodies	
	Urbanization distance	The distance of the fox location point from an urban settlement.	Elaborated by the authors based on CORINE Land Cover (CLC2018)

RESULTS

A total of eleven nematode species were identified, including four cardiopulmonary species (1217 specimens collected) and seven gastrointestinal species (5779 nematodes in total). The overall prevalence of foxes parasitized by at least one nematode species was 89.2% (84.5-93.9 95%CI; 149/167). Prevalence, mean abundance and median intensity of each nematode species are presented in Table 2.

Table 2. Nematodes collected from the gastrointestinal and respiratory tracts of foxes (n=167) from Region of Murcia (SE Spain).

Species	Positive[1]	P (CI95%)[2]	MA (range)[3]	MI (range)[4]
All species (n=6996)	149	89.2 (84.5-93.9)	13 (0-389)	20 (1-389)
E. aerophilus (n=200)	45	26.9 (20.2-33.6)	0 (0-44)	2 (1-44)
A. vasorum (n=545)	46	27.5 (20.7-34.3)	0 (0-107)	2.5 (1-107)
C. vulpis (n=65)	12	7.2 (3.2-11.1)	0 (0-28)	1 (1-28)
M. capsulata (n=407)	55	32.9 (25.8-40.0)	0 (0-117)	2 (1-117)
P. affinis (n=1995)	121	73.7 (67.0-80.5)	4 (0-326)	7 (1-326)
U. stenocephala (n=290)	47	28.6 (21.1-34.9)	0 (0-53)	3 (1-53)
T. leonina (n=212)	29	17.7 (11.8-23.5)	0 (0-69)	2 (1-69)
T. canis (n=32)	16	9.7 (5.2-14.3)	0 (0-7)	1 (1-7)
S. vulpis (n=78)	25	15.2 (9.7-20.7)	0 (0-13)	1 (1-13)
O. crassispiculum (n=3149)	89	54.6 (46.9-62.2)	1 (0-222)	10 (1-222)
T. vulpis (n=23)	6	3.7 (0.8-6.5)	0 (0-7)	3.5 (1-7)

[1] Positive: number of positive foxes; [2]Prevalence of nematode species (%) with 95% confidence intervals (CI95%); [3]Median Abundance with range (minimum and maximum number of nematodes collected); [4]Median Intensity with range (minimum and maximum number of nematodes collected).

The highest parasite richness recorded was seven nematode species (4%, 0.9-7.2 95%CI; 6/149). 18.1% (11.9-24.3; 27/149) of foxes harboured only one species of nematode, 20% (14.3-27.3; 31/149) two species, and coinfections with three, four, five and six nematode species were 14% (8.5-19.7; 21/149), 20.1% (13.7-26.6; 30/149), 16.1% (10.2-22; 24/149) and 6.7% (2.7-10.7; 10/149), respectively. The prevalence and median intensity of each parasite species according to the sex and age categories of the host are shown in Table 3.

Table 3. Percentage of positive foxes (n=167) and median intensity of each nematode species according to host characteristics (sex and age).

			E. aerophilus		***A. vasorum***		***C. vulpis***		***M. capsulata***	
Variable	**Category**	**N[1]**	P (IC95)[2]	MI (range)[3]	P (IC95)[2]	MI (range)[3]	P (IC95)[2]	MI (range)[3]	P (IC95)[2]	MI (range)[3]
Sex	Male	96	25 (16.3-33.6)	2 (1-15)	26 (17.2-34.8)	2 (1-107)	7.3 (2.1-12.5)	1 (1-28)	32.3 (22.9-41.6)	2 (1-54)
	Female	71	29.6 (18.9-40.2)	2 (1-44)	29.6 (18.9-40.2)	3 (1-67)	7 (1.1-12.3)	1 (1-2)	33.8 (22.8-44.8)	1.5 (1-117)
Age	Juvenile	51	15.7 (5.7-25.6)	2 (1-8)	21.6 (10.3-32.8)	2 (1-107)	7.8 (0.4-15.2)	1 (1-1)	25.5 (13.5-37.4)	4 (1-54)
	Adult	116	31.9 (23.4-40.3)	2 (1-44)	30.2 (21.8-38.5)	4 (1-67)	6.9 (2.3-11.5)	1.5 (1-28)	36.2 (27.4-44.9)	2 (1-117)
			P. affinis		***U. stenocephala***		***T. leonina***		***T. canis***	
Variable	**Category**	**N[1]**	P (IC95)[2]	MI (range)[3]	P (IC95)[2]	MI (range)[3]	P (IC95)[2]	MI (range)[3]	P (IC95)[2]	MI (range)[3]
Sex	Male	95	73.7 (64.8-82.5)	7.5 (1-53)	29.4 (20.3-38.6)	3 (1-53)	20 (11.9-28.0)	3 (1-69)	13.7 (6.7-20.8)	1 (1-7)
	Female	69	73.9 (63.5-84.2)	7 (1-326)	26.1 (15.7-36.4)	5 (1-31)	14.5 (6.2-22.8)	2 (1-16)	4.3 (-0.4-9.1)	1 (1-2)
Age	Juvenile	50	68 (55.7-80.9)	6.5 (1-44)	18 (7.3-28.6)	3 (1-10)	14 (4.4-23.6)	5 (1-69)	14 (4.4-23.6)	2 (1-7)
	Adult	114	76.3 (68.5-84.1)	8 (1-326)	32.4 (23.8-41.0)	3 (1-53)	19.3 (12.0-26.5)	2 (1-16)	7.9 (2.9-12.8)	1 (1-3)
			S. vulpis				***O. crassispiculum***		***T. vulpis***	
Variable	**Category**	**N[1]**	P (IC95)[2]	MI (range)[3]	**Category**	**N[1]**	P (IC95)[2]	MI (range)[3]	P (IC95)[2]	MI (range)[3]
Sex	Male	95	15.8 (8.4-23.1)	2 (1-13)	Male	95	52.6 (42.6-62.6)	9.5 (1-222)	3.1 (-0.3-6.6)	6 (1-7)
	Female	69	14.5 (6.2-22.8)	1 (1-9)	Female	68	57.3 (45.6-69.1)	11 (1-163)	4.4 (-0.5-9.3)	3 (2-4)
Age	Juvenile	50	16 (5.8-26.1)	1 (1-13)	Juvenile	50	50 (36.1-63.8)	4 (1-163)	2 (-1.9-5.9)	4 (4-4)
	Adult	114	14.9 (8.3-21.4)	2 (1-9)	Adult	113	56.6 (47.5-65.7)	14 (1-222)	4.4 (0.6-8.2)	3 (1-7)

[1]N: number of foxes collected for each category; [2]P: prevalence of each nematode species by fox sex and age (%) with 95% confidence intervals (IC95%); [3]MI (range): median intensity (minimum and maximum number of nematodes collected).

The significant environmental and host-dependent variables included in the prediction models for each nematode species are shown in Table 4, including the AIC values and the explained variance obtained in each model.

Table 4. Significant predictive environmental and fox variables correlated positively (+) or negatively (-) with nematode species abundance.

Variables	Levels	Nematode species correlation				
		A. vasorum	***C. vulpis***	***T. canis***	***T. leonina***	***U. stenocephala***
Sex	Male		+	+	+	
	Female					
Age	Juvenile		-	+		
	Adult					
KFI		+				
Season	Winter					
	Summer					
	Transition					
Year		-	-			
NDMI	19/08/20		+	+		
	20/01/20	-	-	-	+	
Urban distance			-		-	
Precipitation	Winter				-	+
	Summer		-	-	+	
Radiation	Summer					
Mean maximum summer temperate				-	-	+
Land uses (CLC)	CLC 1	+				+
	CLC 2	+	-		+	+
	CLC 3	+				+
	CLC 4	+				
	CLC 5	+				+
AIC		**453.2**	**155.2**	**158**	**263.1**	**389**
Explained variance		**23.4 %**	**79.1%**	**41%**	**48.7%**	**30.6%**

Concerning to statistically significant host variables in the predictive models (Table 4, Tables S1-S5), male gender of the host was positively correlated with the lungworm *C. vulpis* and the ascarids *T. canis* and *T. leonina* abundances. Juvenile age category was positively related to *T. canis* abundance and negatively related to *C. vulpis* abundance. On the other hand, *A. vasorum* abundance was positively correlated with KFI of foxes. Land use categories showed positive relation with *A. vasorum* and *U. stenocephala* abundances (except for the land use CLC 4 in the latter nematode species); in the case of *C. vulpis*, its

abundance decreases as the surface of agricultural areas increases. Regarding the distance to urban areas, abundances of *C. vulpis* and *T. leonina* were negatively correlated to this variable. Summer NDMI was positively correlated with *C. vulpis* and *T. canis* abundances, whereas abundances of *A. vasorum*, *C. vulpis* and *T. canis* were negatively associated with winter NDMI, except for *T. leonina*. The environmental variable maximum summer temperature had a negative effect on the abundance of ascarids, while it was positive for the hookworm *U. stenocephala*. The abundances of *C. vulpis* and *T. canis* decreased when values of high mean summer rainfall raised, being positive the correlation with *T. leonina* abundance. Finally, the mean precipitation in winter were positively and negatively correlated with the abundances of *U. stenocephala* and *T. leonina*, respectively.

Spatial distribution regarding nematode abundance of the five nematode species was analysed showing a random pattern (results of Moran's index are shown in Table 5). To evaluate geographical incidence of analysed species, a continuous spatial distribution was calculated based on statistical models obtained (Figure 3). Location comparison showed a higher abundance in *A. vasorum* among other parasites. Regarding spatial mapping of absolute values, *A. vasorum, T. canis* and *U. stenocephala* provided a spatially homogeneous distribution. Instead, *C. vulpis,* and *T. leonina*, evidenced a geographically defined location.

Table 5. Spatial pattern distribution of nematodes from foxes attending to Moran index results.

Nematode species	Moran Index value	z-score	p-value
A. vasorum	0.012488	0.179918	0.857217
C. vulpis	0.014894	0.242118	0.808689
T. canis	0.013635	0.193298	0.846725
T. leonina	0.02291	0.372817	0.709284
U. stenocephala	0.037318	0.427374	0.669107

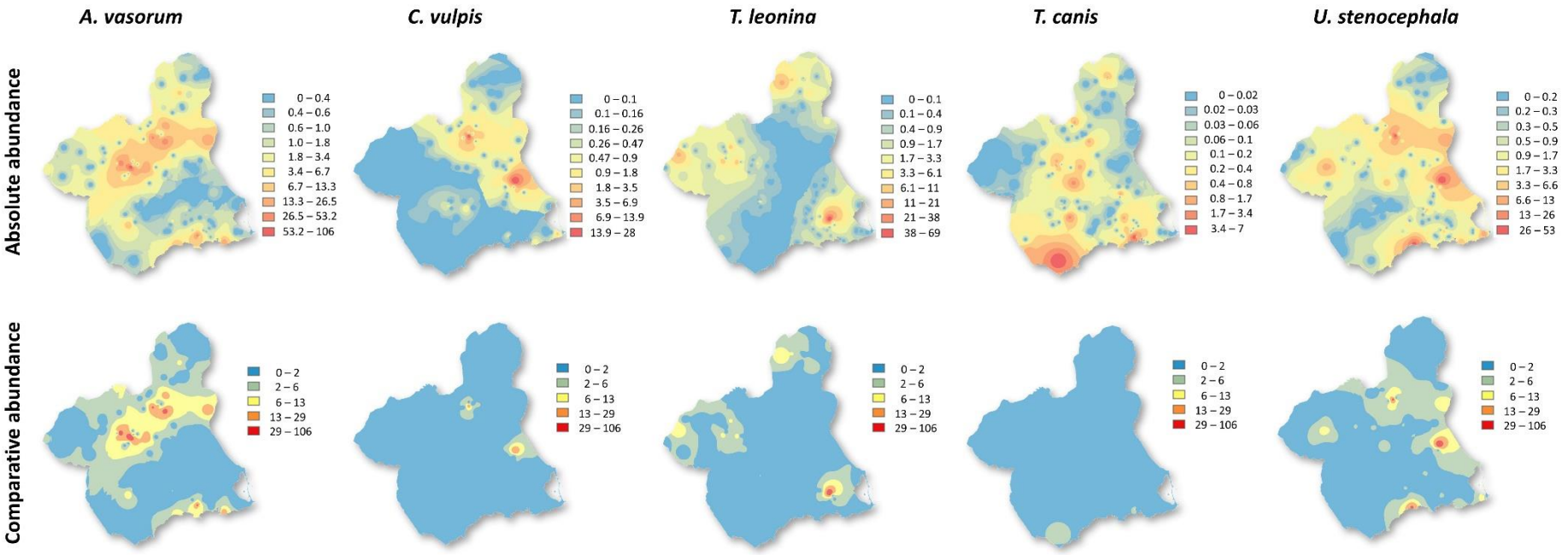

Figure 3. The top row shows the spatial distribution of the absolute abundance of each of the parasites analysed. The bottom row shows the comparative abundance between the parasites analysed. A geometric interval classification scheme has been used, which create class breaks based on class intervals that have a geometric series. This ensures that each class range has approximately the same number of values in each class and that the change between intervals is consistent. Thus, a balance is achieved between highlighting changes in the middle values and the extreme values.

DISCUSSION

Red foxes have been widely investigated as carriers of a great variety of pathogens in natural environments (Otranto and Deplazes, 2019). Previous studies have highlighted that changes in the land use of the territory, together with the fragmentation of natural ecosystems, facilitate the contact between wild and domestic animals, enhancing the transmission risk of parasites, some of them of pathogenic relevance to canids (Morgan et al., 2008). On the other hand, among other conditions, the occurrence of parasites depends on environmental factors and on the presence of the intermediate and paratenic hosts involved in the life cycle (Poulin and Morand, 2000; Bozick and Real, 2015).

All the nematode species reported in this study, except for *Metathelazia capsulata*, have been previously described in the Iberian Peninsula (Gortázar et al., 1998; Criado-Fornelio et al., 2000; Eira et al., 2006; Martínez-Carrasco et al., 2007; Garrido-Castañé et al., 2015; Figueireido et al., 2016; Fanelli et al., 2019; Martínez-Rondán et al., 2019) and other European areas (Saeed et al., 2006; Magi et al., 2015, 2016; Tolnai et al., 2015; Fiocchi et al., 2016; Schug et al., 2018; Gavrilovic et al., 2019; Deak et al., 2020; Tylkowska et al., 2021). This wide species richness highlights, among other aspects, the diverse diet of foxes in semi-arid Mediterranean areas, where invertebrates, reptiles or small mammals are the main prey of this wild carnivore (Díaz-Ruiz et al., 2013; Soe et al., 2017; Castañeda et al., 2022). These prey act as intermediate and/or paratenic host for the nematode species found in our study (Martínez-Carrasco et al., 2007; Schug et al., 2018; Mørk et al., 2019; Deak et al., 2020).

To the author´s knowledge, this is the first time that the genus *Metathelazia* has been found in foxes in Europe and, specifically, the first record of *M. capsulata* in red foxes worldwide. This lung parasite has been previously cited in marbled polecat (*Vormela peregusna*), European badger (*Meles meles*) Nile fox (*Vulpes vulpes nilotica*) and American badger (*Taxidea taxus*) (Gerichter, 1948; Pence and Dowler, 1979). The life cycle of this nematode is not completely known (Anderson, 2000), although an intermediate host is thought to be involved in transmission (Gerichter, 1948). It is noteworthy that *M. capsulata* is the most prevalent bronchopulmonary species in this study. This parasite is found mainly in small bronchi and bronchioles (Gerichter, 1948; Pence and Dowler, 1979), so its location by conventional parasitological techniques (opening of the bronchial tree)

may go undetected and, consequently, the prevalence of *M. capsulata* in the fox may be underestimated. Therefore, in addition to a thorough examination of the lung parenchyma, we recommend artificial digestion of this tissue as a final step to confirm the presence of *M. capsulata*, thus avoiding false-negative foxes. Our results highlight the need to address epidemiological studies to know the occurrence of this nematode in other European fox populations. It would also be advisable to investigate whether there is a life cycle associated with dogs or wild canids, and if the pathogenic effect of *M. capsulata* should be taken into account as a threat to the health of canids.

A. vasorum and *C. vulpis* are not considered to cause zoonosis, although their pathogenic effects on wild and domestic canids are well known (Morchón et al., 2021; Pohly et al., 2022). Their occurrence in the ecosystems depends on the density of their intermediate hosts (Lemming et al., 2020), but also on environmental conditions. In this sense, explanatory models of the abundances of these nematodes indicated that summer NDMI values are positively associated with both lung nematodes, while the opposite occurs with winter NDMI. It is known that temperature and humidity determinate the development and survival of lungworm larval stages (Ferdushy and Hasan, 2010; Robbins et al., 2021), as well as influence the activity and dynamics of their intermediate hosts, snails and slugs (Morgan et al., 2009; Alho et al., 2018), or amphibians, described as paratenic host of *A. vasorum* (Bolt et al., 1994). Recently, the survival of up to 8 weeks of *A. vasorum* L3 and *C. vulpis* infective stage in the environment once excreted by slugs or snails have been demonstrated in laboratory conditions (Conboy et al., 2017; Robbins et al., 2021). Therefore, the transmission risk for wild and domestic canids increases where environmental conditions favour the presence of intermediate and/or paratenic hosts, or the survival of free infective larvae (Maksimov et al., 2017). The study area is characterized by a semi-arid Mediterranean climate, with very dry and warm summers. Therefore, habitats with greater vegetation cover are possibly the most suitable for the occurrence of *A. vasorum* and *C. vulpis*. In our study, all land cover categories were positively correlated with *A. vasorum* abundance. Agricultural, wooded or wetland areas have suitable microclimatic conditions for the presence of snails, slugs and amphibians, with optimum humidity, temperature and vegetation cover for the survival of these intermediate and paratenic hosts, and also for the infective larvae. Although the spatial distribution of the occurrence of these two nematodes followed a random pattern,

without significance in the clustered distribution, the continuous spatial distribution map indicated the existence of specific areas where the parasite abundance was higher and, therefore, we can assume that the risk of infection is higher in these locations (Figure 4). In the study carried out by Čabanová et al. (2018) in foxes from Slovakia, it was found a clustered *A. vasorum* distribution with infection foci. This clustered pattern has also been described in other European countries (Helm et al., 2010; Tolnai et al., 2015; Maksimov et al., 2017), showing a higher infection risk in areas with environmental characteristics that do not always coincide among the studies carried out, which demonstrates that the distribution of *A. vasorum* is conditioned by specific bioclimatic determinants of each study area.

In semi-arid areas of southeastern Iberian Peninsula, the abundance of *C. vulpis* decreased when percentage of agricultural lands increased. A similar result was described by Maksimov et al. (2017), who found that *C. vulpis* infection risk in foxes was lower in agricultural areas, while urban areas had a higher risk of occurrence. In contrast, other authors have described a wide distribution pattern of *C. vulpis* occurrence, probably due to the resistance of L1 larvae to various climatic factors, including freeze temperatures (Čabanová et al., 2018).

Several mollusc species involved in the life cycle of *A. vasorum* and *C. vulpis* have been described, such as *Arion lusitanicum, Arion vulgaris, Deroceras reticulatum, Helix aspersa* (sin. *Cornu aspersum*) and *Limax maximus* (Colella et al., 2016; Maksimov et al., 2017; Fuerher et al., 2020), some of them being present in the southeast of the Iberian Peninsula (Borredà and Martínez-Ortí, 2014). Specifically, *H. aspersa* is a common snail in Mediterranean regions (Colella et al., 2016), also frequent in urban gardens. *D. reticulatum* is commonly found in cultivated fields, and *A. lusitanicus* is usually more abundant in wooded areas (Maksimov et al., 2017).

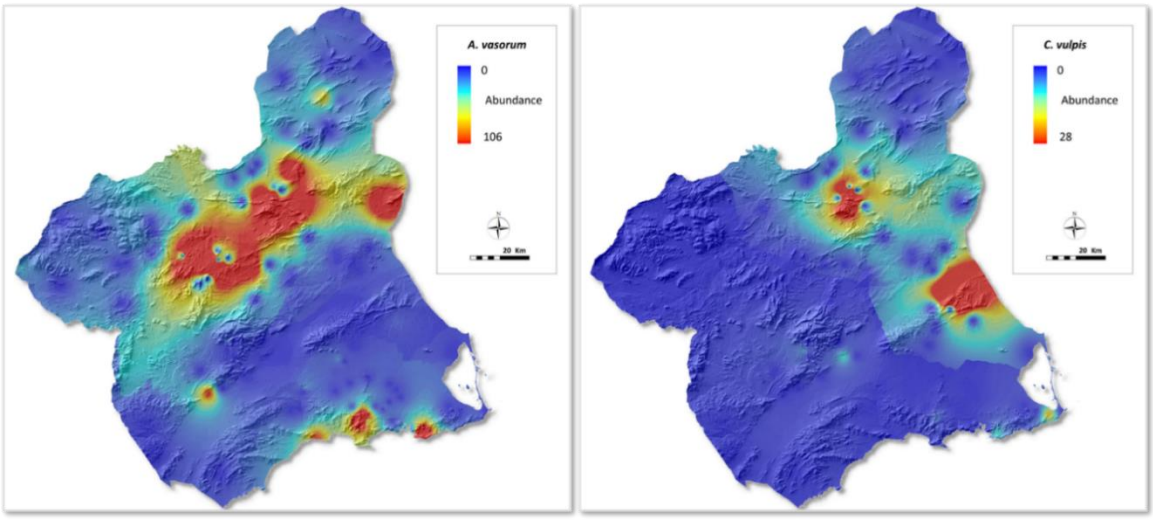

Figure 4. Abundance distribution maps of *Angiostrongylus vasorum* and *Crenosoma vulpis*.

According to previous studies, *A. vasorum* and *C. vulpis* can use different intermediate host in their life cycle, so their occurrence in different types of habitats is possible, ultimately impacting on the infection rates of these nematodes in domestic and wild canids in these areas (Garrido-Castañé et al., 2015; Deak et al., 2017; Deak et al., 2020). Further investigations on the intermediate host species involved in the life cycle of these emerging parasites are necessary to better understand the factors associated with their distribution and maintenance in sylvatic host (Gavrilović et al., 2019; Gillis-Germitsch et al., 2020; Morgan et al., 2021).

U. stenocephala is a hookworm with a direct life cycle where eggs are excreted through the host's faeces, from which the infective stage (L3) will develop, being conditioned by environmental characteristics (Anderson, 2000; Richard et al., 1995). This nematode is capable to infect domestic and wild canids, and less frequently humans (Seguel and Gottdenker, 2017; Miljević et al., 2019; Štrkolcová et al., 2022). It is one of the most prevalent nematodes in European foxes (Saeed et al., 2006; Reperant et al., 2007; Di Cerbo et al., 2008; Vergles Rataj et al., 2013; Fiocchi et al., 2016; Waindok et al., 2021), although in Spain its prevalence is lower (Gortázar et al., 1998; Criado-Fornelio et al., 2000; Martínez-Carrasco et al., 2007) as shown in our study. Transmission occurs when the definitive host ingest L3, but larvae can also penetrate through the skin (Gibbs, 1961). Moisture, mild temperature and vegetation cover are favourable conditions for the protection and viability of the free larvae and, consequently, for carnivore infection to

occur (Fiocchi et al., 2016). In this sense, attending to our results, all the variables with a positive influence on the abundance of *U. stenocephala*, are associated with these optimal habitat conditions: land uses (forestry, agricultural and even urban areas), mean winter rainfall and mean maximum summer temperate. The high temperatures frequently reached during the summer in the Region of Murcia are possibly the main limiting factor that reduces the survival of *U. stenocephala* free-living stages. Therefore, areas covered with vegetation, such as wooded or agricultural areas, protect the larvae from exposure to the sun and help to maintain a lower temperature and higher humidity. In fact, the continuous spatial distribution map revealed very specific areas in which the abundance of *U. stenocephala* is higher than in the rest of the study area (Figure 5). Specifically, the peri-urban area of Murcia city, the capital of the province, is one of these zones with the highest abundance. These findings should be of concern to public health authorities, since previous studies have shown that the presence of geohelminth eggs and infective larvae in urban and peri-urban areas carries a high risk of transmission between dogs and foxes, as well as a potential hazard to humans (Reperant et al., 2007; Traversa et al., 2014; Karamon et al., 2018).

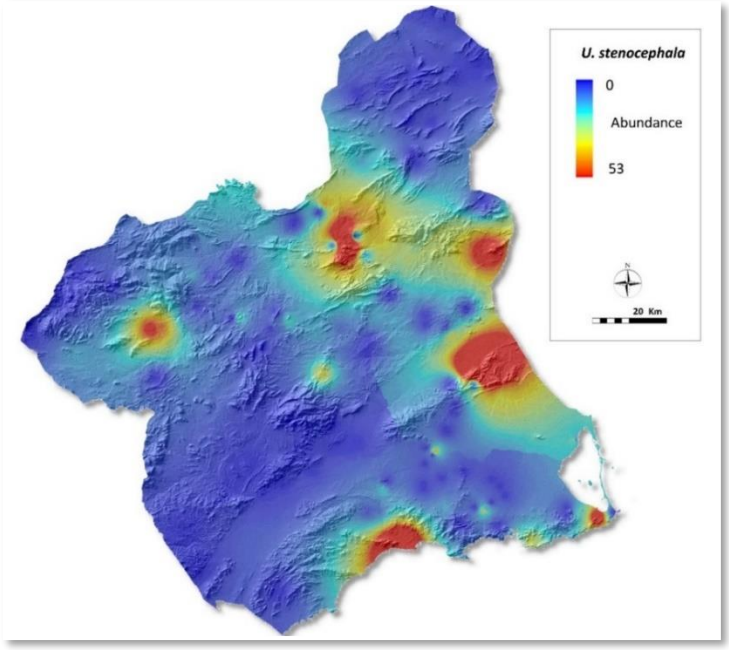

Figure 5. Abundance distribution map of *Uncinaria stenocephala*.

T. canis and *T. leonina* are two relevant parasites of wild and domestic canids that, also, cause zoonoses (Otranto and Deplazes, 2019). Despite of most of the studies in wildlife reported higher prevalences of *T. canis* than of *T. leonina* (Saeed et al., 2006; Reperant et al., 2007; Figueiredo et al., 2016; Magi et al., 2016), here there was a higher prevalence of *T. leonina* than of *T. canis*, in agreement with the results obtained by Gortázar et al. (1998) and Criado-Fornelio et al. (2000). This higher prevalence may be due to the fact that *T. leonina* eggs can survive in a wider variety of environmental conditions, including the capacity to embryonate in darkness or to resist freezing temperatures for a longer time than *T. canis* egg (Okulewicz et al., 2012). Despite being parasites with a similar life cycle, divergent results have been obtained. This is the case of the negative and positive influence of NDMI winter on the abundance, or the negative and positive effect of summer rainfall in the case of *T. canis* and *T. leonina*, respectively. All these results suggest that there are possibly other environmental factors that could be contributing to the occurrence of these parasites. The high abundance of *T. leonina* detected (212 specimens) differs notably from that of *T. canis* (32 specimens) and may cause these divergent results. However, to know more precisely the influence of these bioclimatic determinants, it would be necessary to analyse a larger number of foxes. On the other hand, *T. leonina* abundance increased in agricultural areas and when distance to urban sites decreased. Remarkably, these factors did not influence the abundance of *T. canis*. This contrast could be due to differences in the survival of infective eggs in the environment (Okulewicz et al., 2012), but this could not be justified on the diet of foxes, since both *T. canis* and *T. leonina* present life cycles with the same paratenic hosts (rodents). In this sense, Mørk et al. (2019) observed that the presence of *T. leonina* in foxes was conditioned by the abundance of rodent populations that, according to these authors, are more accessible and abundant in rural or peri-urban areas. On the other hand, Antolová et al. (2004) found that *T. canis* seropositivity in rodents from suburban areas of Slovakia was higher compared to from rural areas, and Brochier et al. (2007) detected foxes parasitized by this ascarid in urban areas from Belgium. Therefore, in agreement with other authors (Reperant et al., 2007; Traversa, 2012; Mørk et al., 2019), further studies are needed to know in more detail the environmental factors that determine the occurrence of these two ascarid nematodes, especially in urban and peri-urban areas where foxes are present and, consequently, may contribute to the

maintenance of *T. canis* and *T. leonina* by environmental contamination with ascarid eggs (Traversa et al., 2014; Nijsse et al., 2015).

One of the most remarkable results was that, as shown in the continuous spatial distribution map (Figure 6), two of the areas with the greatest *T. canis* abundance were in the southwest and the southeast of the region, being areas with one of the highest human population densities. Likewise, the spatial distribution of *T. leonina* (Figure 6) indicates points with higher abundances in the northwest and north of the region, which are also highly anthropized areas. These results suggest that foxes in these peri-urban areas should be considered as a health risk factor, so veterinarians and public health management authorities in these areas should pay special attention to this issue.

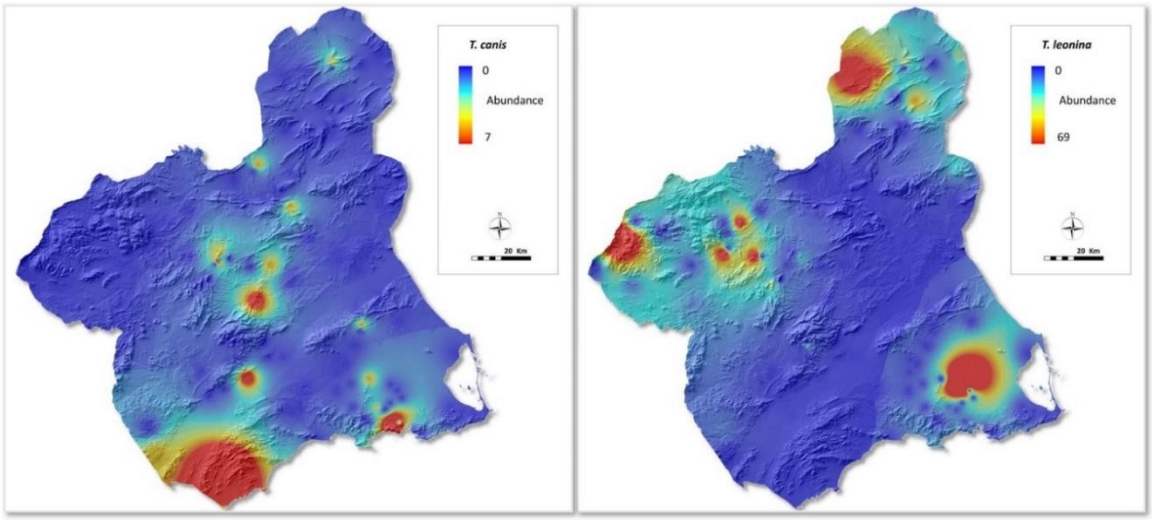

Figure 6. Abundance distribution maps of *Toxocara canis* and *Toxascaris leonina*.

In our study, male foxes had higher risk of infection by *C. vulpis*, *T. canis* and *T. leonina*. Previous studies have found that male foxes are more likely to be parasitized by *T. canis* and *T. leonina* (Richards et al., 1993; Segovia et al., 2004; Saeed and Kapel, 2006), possibly because they usually have a larger home range and, therefore, a wider choice of areas potentially contaminated with eggs of these ascarids or with the presence of paratenic hosts (Torres et al., 2006; Tylkowska et al., 2021). In contrast, other authors have found no significant differences in the presence of *T. canis* (Wolfe et al., 2001; Roddie et al., 2008) and *C. vulpis* (Magi et al., 2015; Morandi et al., 2019) between sexes. It has been suggested that differences in the prevalence of pathogens may be conditioned by

host sex, because testosterone reduces the immune response and, consequently, males are more prone to infection (Klein, 2004; Guerra-Silveira and Abad-Franch, 2013). In our study, significant differences between sexes were not detected in all parasites, so we consider that this hypothesis does not fully explain our results.

Juvenile foxes in the semi-arid area under study showed a positive correlation with the abundance of *T. canis*, while it was negative in the case of *C. vulpis*. The different transmission ways of *T. canis*, including transplacental and galactogenic transmission (Anderson, 2000; Roberts et al., 2013), could explain this result. In this sense, previous studies have found that juvenile foxes were more prone to be parasitized by *T. canis* (Richards et al., 1993; Saeed and Kapel, 2006), and, young foxes tend to have higher prevalences of *C. vulpis* than adults (Jeffery et al., 2004; Davidson et al., 2006; Hodžić et al., 2016). In our study, the significantly negative correlation detected in juveniles could be due to the less time they have had throughout their lives to become infected with molluscs or free infective larvae, so that the cumulative effect has not had time to occur and, consequently, the abundance is lower in this age category.

This study confirms that fox populations inhabiting semi-arid Mediterranean areas are a reservoir for a diversity of nematode species, many with severe implications for domestic carnivores and even humans. In spite of the arid conditions in the Region of Murcia, natural forestry together with agricultural areas favour the appearance of parasites, since one of the most important environmental features is humidity, which has proved indispensable for the development of the free-living stages and the intermediate hosts. High temperatures registered in this semi-arid region combined with the lack of rainfall and elevate levels of evapotranspiration, limit the availability of water and humidity. Therefore, those areas shared by synanthropic foxes and domestic carnivores can become a hotspot for parasite transmission among these hosts, turning proximity to urban populations in an epidemiological risk factor. Finally, it is important to note that it would be desirable a deeper study of intermediate (for *A. vasorum* and *C. vulpis*) or paratenic (for *T. canis* and *T. leonina*) hosts. A better knowing of involved species and variables determining their abundance, could help to anticipate potential fox infection hotspots.

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SUPPLEMENTARY FILES

Table S1. *Angiostrongylus vasorum* results using GLM with Negative Binomial distribution.

Coefficients	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	222.63584	328.23099	0.678	0.497588
Year	-0.45258	0.15666	-2.889	0.003865 **
KFI	0.02385	0.01005	2.374	0.017577 *
Summer NDMI	13.00436	7.19607	1.807	0.070739 .
Winter NDMI	-19.25155	5.72538	-3.362	0.000772 ***
CLC 1[(1)]	0.19884	0.06653	2.989	0.002799 **
CLC 2	0.19812	0.06646	2.981	0.002874 **
CLC 3	0.19890	0.06652	2.990	0.002788 **
CLC 4	0.43830	0.14421	3.039	0.002371 **
CLC 5	5.79711	2.14449	2.703	0.006866 **
MMS Temperature [(2)]	0.01519	0.02338	0.650	0.515939

[(1)] CLC1 = Artificial surfaces; CLC2 = Agricultural areas; CLC3 = Forestry and semi-natural areas; CLC4 = Wetlands; CLC5 = Waterbodies. [(2)] Mean Maximum Summer Temperature.

Table S2. *Crenosoma vulpis* results using GLM with Family Poisson distribution.

Coefficients	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	1.566e+03	2.620e+02	5.978	2.26e-09 ***
Winter	3.071e+00	1.704e+00	1.802	0.071593 .
Year	-7.680e-01	1.294e-01	-5.934	2.95e-09 ***
Age-Juvenile	-1.556e+00	5.411e-01	-2.875	0.004040 **
Sex-Male	2.253e+00	5.272e-01	4.275	1.92e-05 ***
Summer NDMI	4.337e+01	4.640e+00	9.347	< 2e-16 ***
Winter NDMI	-4.297e+01	7.422e+00	-5.789	7.06e-09 ***
Urbanization distance	-3.303e-04	1.020e-04	-3.240	0.001196 **
CLC 2[(1)]	-9.490e-04	3.321e-04	-2.858	0.004268 **
CLC 5	-2.695e+00	2.031e+02	-0.013	0.989413
Mean summer precipitation	-3.574e-02	1.057e-02	-3.380	0.000724 ***

[(1)] CLC2 = Agricultural areas; CLC5 = Waterbodies

Table S3. *Uncinaria stenocephala* results using GLM with Negative Binomial distribution.

Coefficients	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-3.367e+02	1.490e+02	-2.260	0.02384 *
Age-Juvenile	-8.806e-01	4.790e-01	-1.839	0.06599 .
KFI	-5.166e-03	9.710e-03	-0.532	0.59473
Winter NDMI	-7.559e+00	4.396e+00	-1.720	0.08551 .
Urbanization distance	-1.525e-04	8.914e-05	-1.711	0.08701 .
CLC 1[(1)]	8.555e-02	4.292e-02	1.993	0.04626 *
CLC 2	8.593e-02	4.287e-02	2.005	0.04499 *
CLC 3	8.626e-02	4.290e-02	2.011	0.04432 *
CLC 4	1.092e-01	8.247e-02	1.324	0.18559
CLC 5	3.818e+00	1.494e+00	2.556	0.01059 *
Mean winter precipitation	1.591e-02	4.966e-03	3.203	0.00136 **
MMS Temperature [(2)]	1.130e-01	2.786e-02	4.056	4.99e-05 ***

[(1)] CLC1 = Artificial surfaces; CLC2 = Agricultural areas; CLC3 = Forestry and semi-natural areas; CLC4 = Wetlands; CLC5 = Waterbodies. [(2)] Mean Maximum Summer Temperature.

Table S4. *Toxocara canis* results using GLM with Family Poisson distribution.

Coefficients	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	2.277e+01	6.036e+00	3.772	0.000162 ***
Age-Juvenile	1.023e+00	3.996e-01	2.561	0.010448 *
Sex-Male	1.900e+00	5.565e-01	3.414	0.000641 ***
Summer NDMI	1.658e+01	5.959e+00	2.783	0.005384 **
Winter NDMI	-7.898e+00	3.616e+00	-2.184	0.028953 *
CLC 2[(1)]	-4.051e-04	2.963e-04	-1.367	0.171500
CLC 3	-4.520e-04	3.127e-04	-1.445	0.148371
CLC 5	-2.715e+00	2.704e+02	-0.010	0.991987
Mean summer precipitation	-2.205e-02	6.607e-03	-3.337	0.000846 ***
MMS Temperature [(2)]	-6.283e-02	1.896e-02	-3.315	0.000917 ***

[(1)] CLC2 = Agricultural areas; CLC3 = Forestry and semi-natural areas; CLC5 = Waterbodies. [(2)] Mean Maximum Summer Temperature.

Table S5. *Toxascaris leonina* results using GLM with Negative Binomial distribution.

Coefficients	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	81.2249626	16.5607669	4.905	9.36e-07 ***
KFI	-0.0203092	0.0123046	-1.651	0.09883 .
Sex-Male	2.1127046	0.6452360	3.274	0.00106 **
Winter NDMI	20.3136725	6.3930281	3.177	0.00149 **
Urbanization distance	-0.0004118	0.0001323	-3.111	0.00186 **
CLC 2[(1)]	0.0014942	0.0003597	4.154	3.27e-05 ***
Mean winter precipitation	-0.0407718	0.0087913	-4.638	3.52e-06 ***
Mean summer precipitation	0.0446445	0.0089706	4.977	6.47e-07 ***
MMS Temperature [(2)]	-0.2824611	0.0511225	-5.525	3.29e-08 ***

[(1)] CLC2 = Agricultural areas. [(2)] Mean Maximum Summer Temperature.

CHAPTER THREE:

Distribution of *Pearsonema plica* in red foxes (*Vulpes vulpes*) from Iberian semi-arid areas

SUMMARY

Pearsonema plica is a nematode that parasitizes the bladder of domestic and wild canids, with an indirect biological cycle with earthworm as intermediate hosts. In most cases, this nematode causes minor signs of disease to the hosts, although severe cases have also been described. The red fox (*Vulpes vulpes*) can be definitive hosts of *P. plica* showing a high variable prevalence in Europe, including Iberian Peninsula, ranging from 1% to more than 90%.

The red fox is a wild species with synanthropic behaviour and a high ecological plasticity that allows it to inhabit different kind of habitats. So, it can be considered as a reservoir for this parasite since foxes can share territories with domestic canids. Semi-arid Mediterranean areas include a wide range of landscapes, where anthropized areas are one of the most common territories, resulting to the movement of wildlife to these zones. This approach can increase even more the contact between wildlife, domestic animals and human and, consequently, the transmission of diseases among them.

Several epidemiological studies at the domestic animals-wildlife-human interface in these semi-arid areas focus on the environmental particularities that can lead to specific contact areas between foxes and canids, where biotic and abiotic conditions can favour the presence of shared pathogens. Although the environmental conditions in these dry regions are not *a priori* favourable for the cycle of *P. plica*, the understanding of the specific conditions that allows this parasite to successfully complete its biological cycle and, therefore, to infect host species inhabiting these areas, are highly convenient. Thus, the aim of this study was to evaluate the presence of *P. plica* in red foxes from the Region of Murcia (SE Spain), a semi-arid Mediterranean region, and to elucidate the biotic and environmental characteristics which determine the abundance of this parasite.

In order to achieve these objectives, between 2015 and 2021, the urinary system (bladder, kidneys and ureters) of 167 foxes was analysed. Nematodes found after opening and examination of these organs were morphometrically identified. Sex, age and body condition (KFI) were the host variables analysed. Environmental variables were categorized into three groups for their analysis: climatic variables, spectral index and land use; these data were calculated based on foxes' home range. On the other hand, prevalence, median abundance and median intensity were calculated, and the influence

of the previously mentioned biotic and abiotic variables in relation on *P. plica* abundance was evaluated using a Generalized Linear Model (GLM). Finally, Moran's index was calculated to estimate the spatial autocorrelation of this parasite.

Four foxes (P= 2.4%, 4/167) were parasitized with *P. plica*, recovering a total of 35 specimens. Contrary to the host variables, the environmental factors showed significant results, being the winter normalized differential humidity index (NDMI), CORINE Land Cover categories (anthropogenic surfaces, agricultural land and forestry areas), distance to urban areas and mean summer precipitation, the variables included in the model with a positive influence on the abundance of *P. plica*. Moreover, Moran's index indicated a random distribution of the parasite throughout the territory ($p>0.05$).

As expected, attending to the arid environmental characteristics of the study area, the prevalence of this nematode was low. Although this result contrast with the higher values obtained in other European regions, it agrees with those found in studies carried out in areas with similar characteristics than those in the area under study. Our results revealed the importance of those environmental variables related to humidity, an essential factor for the development of the earthworm and, therefore, for the maintenance of this parasite's cycle. In addition, the positive influence of forestry and agricultural areas is indirectly related to this same parameter, since it favours soil moisture.

The present study confirms the presence of *P. plica* in red foxes from the Region of Murcia, a semi-arid area in the southeast of the Iberian Peninsula. Despite the low prevalence found, there are locations where the abiotic conditions are optimal for the development of earthworms, allowing the parasite to complete its biological cycle. Considering the synanthropic behaviour of this wild canid and its approach to urban, peri-urban or rural areas owing to the trophic and water resources that these zones provide, it is important to take these results into account when epidemiological studies or health vigilance programs are developed, especially in the case of areas shared by wild and domestic animals.

Detailed information on this chapter has been published in the *International Journal for Parasitology: Parasites and Wildlife*, which DOI is provided below to consult: https://doi.org/10.1016/j.ijppaw.2022.08.005

CHAPTER FOUR:

First report of *Metathelazia capsulata* in red foxes (*Vulpes vulpes*) in Europe and new contributions to its identification

ABSTRACT

Metathelazia capsulata is a lungworm that inhabit in the bronchi and bronchioles from mammal carnivore species, which life cycle its unknown. Spirurid nematodes were isolated at necropsy from the respiratory tract of red foxes (*Vulpes vulpes*) from the Region of Murcia (SE Spain). The main objective of this study was to describe in detail the morphometric features of these nematodes, as well as to report some molecular markers. The images obtained by light microscopy and field-emission scanning electron microscopy were used to describe and measure the principal structures, comparing these characteristics with previous descriptions performed on this nematode species. The principal morphometric difference compared to previous *M. capsulata* descriptions was the shorter total length for both males and females (6.6 mm and 7.4 mm, respectively). In addition, the mean values of buccal cavity depth and distance between the excretory pore and the anterior end of the nematode were also lower than those previously reported, while rest of features were in accordance with the ranges described to date of this species. On the other hand, sequence data of the mitochondrial (COI) and nuclear (rDNA) genes of *M. capsulata* are described, being the first time that molecular markers are reported for the genus *Metathelazia* and also for the entire family Pneumospiruridae. These results indicate, based on data available from GenBank, that sequences obtained of *M. capsulata* are closely related to the family Rhabdochonidae, which is assumed to belong to superfamily Thelazioidea, a superfamily including the family Pneumospiruridae, but also suggest the distant relations with the family Thelaziidae. This is the first time that *M. capsulata* is reported in red fox from Europe. This study provides valuable information for future phylogenetic studies on *Metathelazia* spp. nematodes and, in general, on species of the family Pneumospiruridae.

INTRODUCTION

The superfamily Thelazioidea comprises three families: Pneumospiruridae, Rhabdochonidae and Thelaziidae. This last family includes nematodes whose primarily host are birds and mammals, while those in Rabdochonidae parasitize marine and freshwater fishes (Chabaud and Bain, 1994). The Pneumospiruridae family includes nematodes parasitizing the respiratory system of carnivores, having little information about their transmission and life cycle (Anderson, 2000), although Gerichter (1948)

suggested that an intermediate host could be involved, as occurs in other Thelazioidea nematodes.

Along with *Pneumospirura* and *Vogeloides*, *Metathelazia* is the main genus included in Pneumospiruridae (Chabaud, 1975) with a large number of species described worldwide (Chabaud and Bain, 1994) affecting lung parenchyma or bronchi of their host.

The identification of nematodes belonging to Pneumospiruridae family is frequently based on the morphological characteristics of the cephalic region. In this sense, along the years, different authors have taken into account this criterion to classify Pneumospiruridae nematodes (Dougherty, 1943; Wertheim and Chabaud, 1977); in particular, the genus *Vogeloides* presents six developed lips, the genus *Pneumospirura* is small-lipped, and in the genus *Metathelazia* lips are absent (Pence and Stone, 1977).

M. californica (Skinker, 1931), *M. bassarisci* (Pence and Stone, 1977) and *M. servalis* (Wertheim and Giladi, 1977) are some of the species that have been previously reported in carnivores such bobcat (*Felis rufus*), cougar (*Puma concolor hippolestes*) or serval (*Felis servalis*). Moreover, the northern white-breasted hedgehog (*Erinaceus roumanicus sacer*) and the Egyptian mongoose (*Herpestes ichneumon*) have been described to host *M. multipapillata* and *M. oesophagea*, respectively (Gerichter, 1948).

Nile fox (*Vulpes vulpes nilotica*), European badge (*Meles meles*), American badger (*Taxidea taxus*) and marbled polecat (*Vormela peregusna*) are the three carnivore species in which *M. capsulata* has been previously reported (Gerichter, 1948; Pence and Dowler, 1979). Additionally, *M. mexicana* is a new species that has been recently described from a Nine-banded armadillo (*Dasypus novemcinctus*) in Central Mexico (Jiménez et al., 2013).

To the author´s knowledge, since Gerichter (1948) descriptions, no other species of the genus *Metathelazia* has been described until the moment in Europe. This lack of reports regarding nematodes of the genus *Metathelazia* contrasts with studies published in recent years concerning the genus *Vogeloides*. In particular, Blanco et al. (1993) isolated *Vogeloides oesophagea* in Egyptian mongooses (*Herpestes ichneumon*) from Spain, and a new species named *Vogeloides morowaliensis* has been recently described in Pallas's tube-nosed bats (*Nyctimene cephalotes*) from Indonesia (Purwaningsih et al., 2021).

In this study, a spirurid nematode detected in the bronchi of red foxes coming from Region of Murcia (SE Spain) was morphologically described. Also, six molecular markers were reported: one mitochondrial (cytochrome oxidase subunit I (COI) gene) and five

nuclear markers (18S gene, 28S gene, 5.8S, and internal transcribed spacers (ITSs) ITS1 and ITS2) of the ribosomal DNA (rDNA). Therefore, the aim of this study was to enhance the knowledge of Pneumospiruridae family, providing morphological and molecular data of this spirurid found in red foxes.

MATERIAL AND METHODS

A total of 167 cardiorespiratory tracts were obtained between 2015 and 2021 from foxes coming from hunting programmes, wildlife recovery centres or road-killed in the Region of Murcia (SE Spain). Each respiratory tract was longitudinally opened through the tracheobronchial tree and the large pulmonary vessels. Subsequently, the lung parenchyma was cut (3x3 cm pieces), immersed in water and manually squeezed. The content was filtered through a 63 µm of mesh sieve, and the remaining material was inspected under the stereomicroscope. Finally, lung tissue was artificially digested with a solution of pepsin and chlorhydric acid (Martínez-Rondán et al., 2019), in order to identify worms located in bronchioles of smaller diameter. All isolated helminths specimens were preserved in absolute ethanol until morphological identification or DNA extraction to molecular analysis.

Morphometrical identification

A total of 29 nematodes (14 males and 15 females), were measured and cleared with lactophenol to carry out their description. The visualization of internal structures was performed using a Leica DM6000B microscope connected to Leica DFC280, while for length and width of the parasite Leica Z6 APO stereomicroscope connected to Leica DFC550 digital camera was used. Both system equipment presented Leica Application Suite V 2.5.0 software. All measurements were taking using ImageJ software and expressed in micrometers, with the exception of the total length of the nematodes, that was measured in millimeters.

Sample preparation and field emission scanning electron microscope imaging

For the study of lips details, field emission scanning electron microscopy (FE-SEM) was used. As previously mentioned, samples were fixed in absolute alcohol at laboratory temperature until processing. Subsequently, postfixation was performed with 1% osmium tetroxide during 2 hours and the samples were washed in 0.1M sodium cacodylate buffer with sucrose. Specimens were dehydrated with acetone series and dried at critical point on 0.2 µm Isopore filters (Merck Millipore Ltd). Lastly, the samples were subsequently submitted to platinum sputtering with a 5.0 nm thin film (Leica EM ACE 600) and were examined employing an FE-SEM, (ApreoS Lovac IML Thermofisher), with a selected voltage of 5 kV, 0.2 nA and working distance of 10 mm.

DNA extraction, PCR amplification, cloning and sequencing

The total DNA of six female nematodes was extracted from ethanol-fixed specimens with the standard phenol-chloroform procedures using the whole specimen for each DNA extraction. The different molecular markers (COI, 18S, 28S, 5.8S, ITS1 and ITS2) were amplified by PCR (Polymerase Chain Reaction) with specifics primer pairs (Table 1). Specific primer pair were used to amplify COI, 18S, and 28S sequences, while for the ISTs and 5.8S three primer pairs combinations were used (Table 1).

The combination of the primer pair ITS1-F and nema28_R68 allowed the amplification of the ITS1, 5.8S and ITS2 in a long fragment of about 2kb (Itagaki et al., 2005; Xiang et al., 2013). As this fragment was difficult to amplify, to facilitate the amplification of these markers, two new primer ITS1-int-R and ITS2-int-F located on the 5.8S sequence were designed as internal primers to combine to the ITS1-F and nema28_R68 primers. Thus, the combination of ITS1-F and ITS1-int-R allowed the amplification of ITS1 and part of the 5.8S, and the combination of ITS2-int-F and nema28_R68 allowed the amplification of part of the 5.8S and the ITS2. PCRs were performed in 50µl of reaction, containing 30-100 ng of template DNA. The PCR conditions were as follows: initial denaturation at 95°C for 4 minutes; 35 cycles with denaturation 30s at 95°C, annealing temperature 90s and polymerase extension 120s at 72°C; and a final extension of 5 minutes at 72°C. The annealing temperatures were 50°C for COI, 18S, 28S and ITS1-5.8S-ITS2, 48°C for ITS1, and 54°C for ITS2. In order to get an accurate

sequencing, the amplicons were resolved in 1% ethidium bromide-stained agarose gels, and they were isolated from the gel with a QIAquick gel extraction kit (Qiagen) and cloned in Z-Competent JM109 *E. coli* (Zymo Research) using the PGEMTeasy vector (Promega). Positive clones were Sanger sequenced with T7 and SP6 primers.

Table 1. List of amplified markers, name, sequence and references of the primers.

Markers	Primers Pairs	Primers sequences	Amplification size (bp)	Reference
COI	COIintF	5'-TGATTGGTGGTTTTGGTAA-3'	689	Casiraghi et al. (2001)
	COIintR	5'-ATAAGTACGAGTATCAATATC-3'		
18S	nema18s_F01	5'-CCATGCAWGTCTAWGTTCAAA-3'	1775	Xiang et al. (2013)
	nema18s_R01	5'-GGAAACCTTGTTACGACTTTTG-3'		
28S	C1'	5'-ACCCGCTGAATTTAAGCAT-3'	972	De Bellock et al. (2001)
	D2	5'-TCCGTGTTTCAAGACGG-3'		
ITS1-5.8S-ITS2	ITS1-F	5'-TTGCGCTGATTACGTCCCTG-3'	2018-2027	Itagaki et al. (2005)
	nema28_R68	5'-TTAGTTTCTTTTCCTCCGCTTA-3'		Xiang et al. (2013)
ITS1	ITS1-F	5'-TTGCGCTGATTACGTCCCTG-3'	1060-1070	Itagaki et al. (2005)
	ITS1-int-R	5'-TACCAACGGGAATGAACCCG-3		This work
ITS2	ITS2-int-F	5'-CGTGGATCGATGAAGAACG-3'	1030-1062	This work
	nema28_R68	5'-TTAGTTTCTTTTCCTCCGCTTA-3'		Xiang et al. (2013)

Sequence and phylogenetic analysis

The sequences were analysed and aligned using Bioedit program (version 7.0.9.0) (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and Clustal Omega (Sievers and Higgins, 2021; Madeira et al., 2022). The obtained sequences were aligned and compared with those belonging to other nematode species using the Blast tool (Altschul et al., 1990). The phylogenetic relationships were reconstructed with Maximum-Likelihood (ML) (Nei and Kumar, 2000) using MEGA X (Kumar et al., 2018). Node supports in the analysis were assessed with 2000 bootstrap replicates. For this analysis, the COI sequences from different nematode species available in GenBank considering a 100% of coverage and the highest percentages of identity with our sequences were used: *Chandlerella quiscali*, HM773029; *Cylicospirura subaequalis*, GQ342968; *Cylicospirura felineus*, GQ342967; *Dirofilaria sp. Hongkongensis*, KX265050; *Dirofilaria repens*, KF692102; *Gongylonema*

pulchrum, AP017685; *Gongylonema nepalensis*, LC388892; *Onchocerca ochengi*, NC_031891; *Rhabdochona xiphophori*, MH778493; *Rhabdochona salgadoi*, MH778492; *Setaria labiatopapillosa*, NC_044071; *Spinitectus osorioi*, MN592671; *Spirocerca sp*, KJ605489; *Spirocerca lupi*, MT522373; *Thelazia rhodesi*, MT511659; *Thelazia callipaeda*, AM042556. The best-fit nucleotide substitution model with the lowest BIC (Bayesian Information Criterion) value was chosen using MEGA X, and for these sequences was GTR+G.

RESULTS

Measurements from the studied nematodes are shown in Table 2, as well as standard deviation, range and variation coefficient.

Species description

Nematodes have a tegumental sheath covering the body with thick and smooth cuticle with a whitish appearance. The buccal cavity has two pseudolabia and four papillae pairs in two circles, a pair each located laterally at dorsal and ventral margins of buccal capsule. Also, there are two big lateral amphids (Figure 1). Excretory pore presents a small gland and the oesophagus is club-shaped with a short muscular anterior part and a longer glandular posterior part, with a constriction coinciding with the nerve ring (Figure 2).

These parasites showed a clear sexual dimorphism. The width of males is uniform throughout the body. The tail is disposed in a spirally shaped, with conical shape ending and absent caudal alae. There are six pairs of lateral papillae with the following disposition: two preanal pairs, one adanal pair (Figure 3) and three postanal pairs (Figure 3). The spicules are falciform and slightly different in length (Table 2) with pointed end (Figure 4) and a double tear-shaped gubernaculum is present (Figure 4).

The body of females becomes gradually wider until the tail, which is rounded and presents an ending dorsal bending mucron. Vulva and anus are close to the posterior end, coinciding with a strong muscular system at the terminal portion of the vagina (Figure 5). Eggs are numerous, oval-shaped and thick-shelled. They are completely embryonated at the terminal part of the uterus (Figure. 6). Results of measurements are shown in Table 2.

Table 2. Mean, standard deviation (sd), range and coefficient of variation (CV) of the measures of male and female specimens (expressed in micrometers, except the total length of the nematode, which is expressed in millimeters).

	Male (n=14)		Female (n=15)	
	Mean ± sd (range)	**CV(%)**	**Mean ± sd (range)**	**CV(%)**
Length (mm)	6.6 ± 0.74 (5.2 – 8.4)	11.3	7.4 ± 0.75 (5.6 – 8.5)	10.0
Width	386 ± 0.04 (311 – 492)	11.0	477 ± 0.05 (318 – 592)	11.7
Oral cavity width	21 ± 4.6 (10.9 – 28.9)	21.7	20 ± 4.9 (10 – 30)	24.5
Oral cavity depth	11 ± 1.6 (7.2 – 14.2)	15.1	11 ± 2.3 (6.6 – 16.5)	21.0
Nerve ring – head	57 ± 8.2 (42.6 – 74.8)	14.4	60 ± 7.9 (43.4 – 74.1)	13.3
Excretory pore – head	99 ± 9.9 (87.6 – 125.7)	10.0	113 ± 9.3 (95.1 – 127.5)	8.2
Length oesophagus	416 ± 32.9 (355.2 – 477.6)	7.9	439 ± 28.8 (381.7 – 493.7)	6.5
Width oesophagus	64 ± 7.5 (50.8 – 78.5)	11.7	66 ± 8.7 (50.5 – 84.8)	13.2
Spicule right length	311 ± 20.7 (268.8 – 353.6)	6.6		
Spicule right width	17 ± 2.7 (10.8 – 22.8)	16.2		
Spicule left length	297 ± 19.9 (258.5 – 326.8)	6.7		
Spicule left width	16 ± 2.5 (11.3 – 20.7)	15.3		
Gubernaculum length	36 ± 4.9 (25.7 – 49.0)	13.5		
Gubernaculum width	10 ± 2.2 (6.4 – 14.5)	23.0		
Vulva – anus			122 (± 17.6) 100.3 – 149.9	14.4
Anus – tail			67 (± 10.2) 51.3 – 88.7	15.1
Eggs – length			45 (± 6.9) 29.9 – 57.3	15.3
Eggs – width			34 (± 6.5) 17.2 – 43.7	19.4

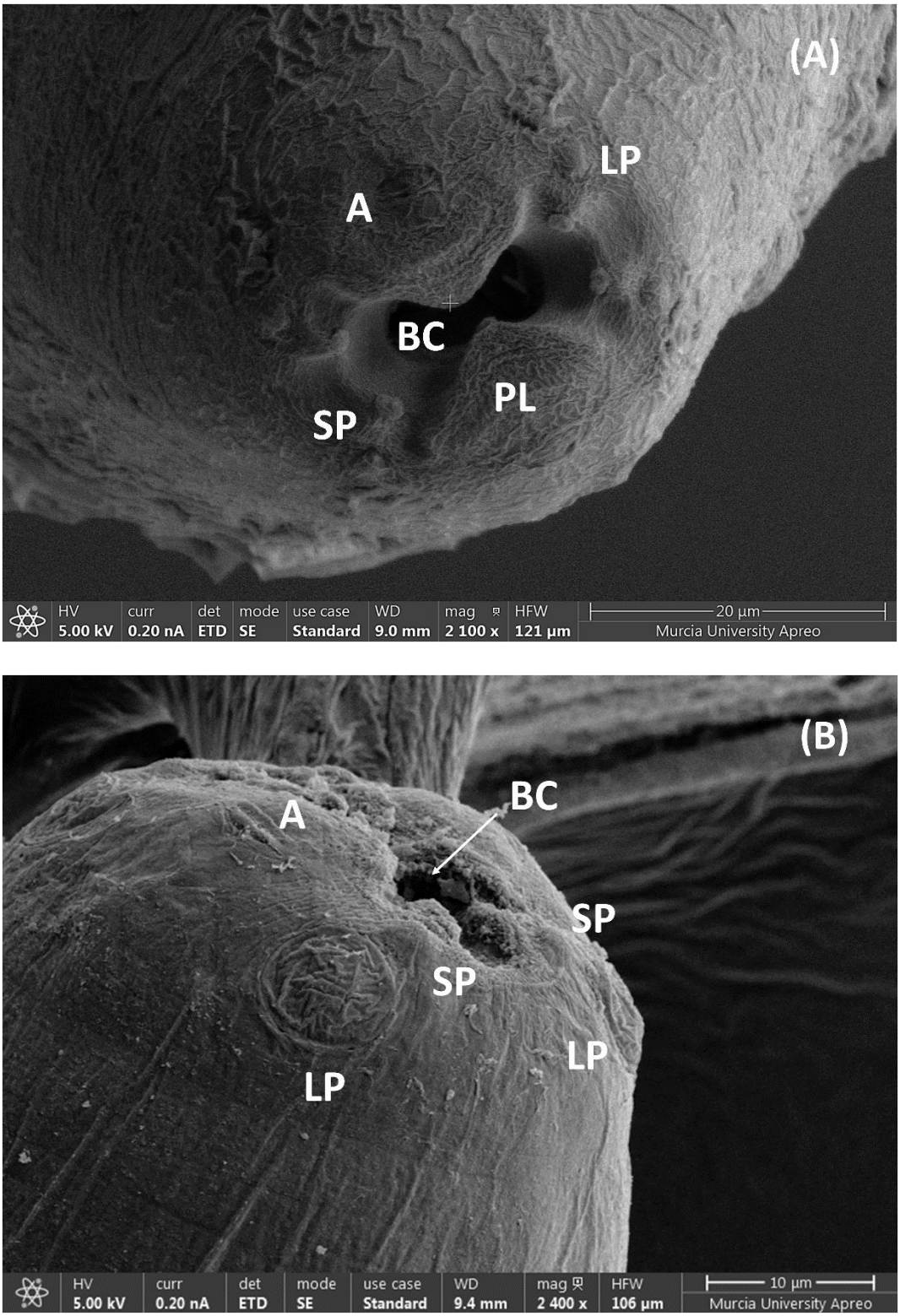


Figure 1. Scanning electron micrograph with a dorsal view (A) and lateral view (B) of the buccal cavity (BC) of a *M. capsulata* female. Four papillae pairs: two small pairs (SP) and two large pairs (LP) can be distinguished. The amphids (A) are at the bottom of the two pseudolabials (PL).

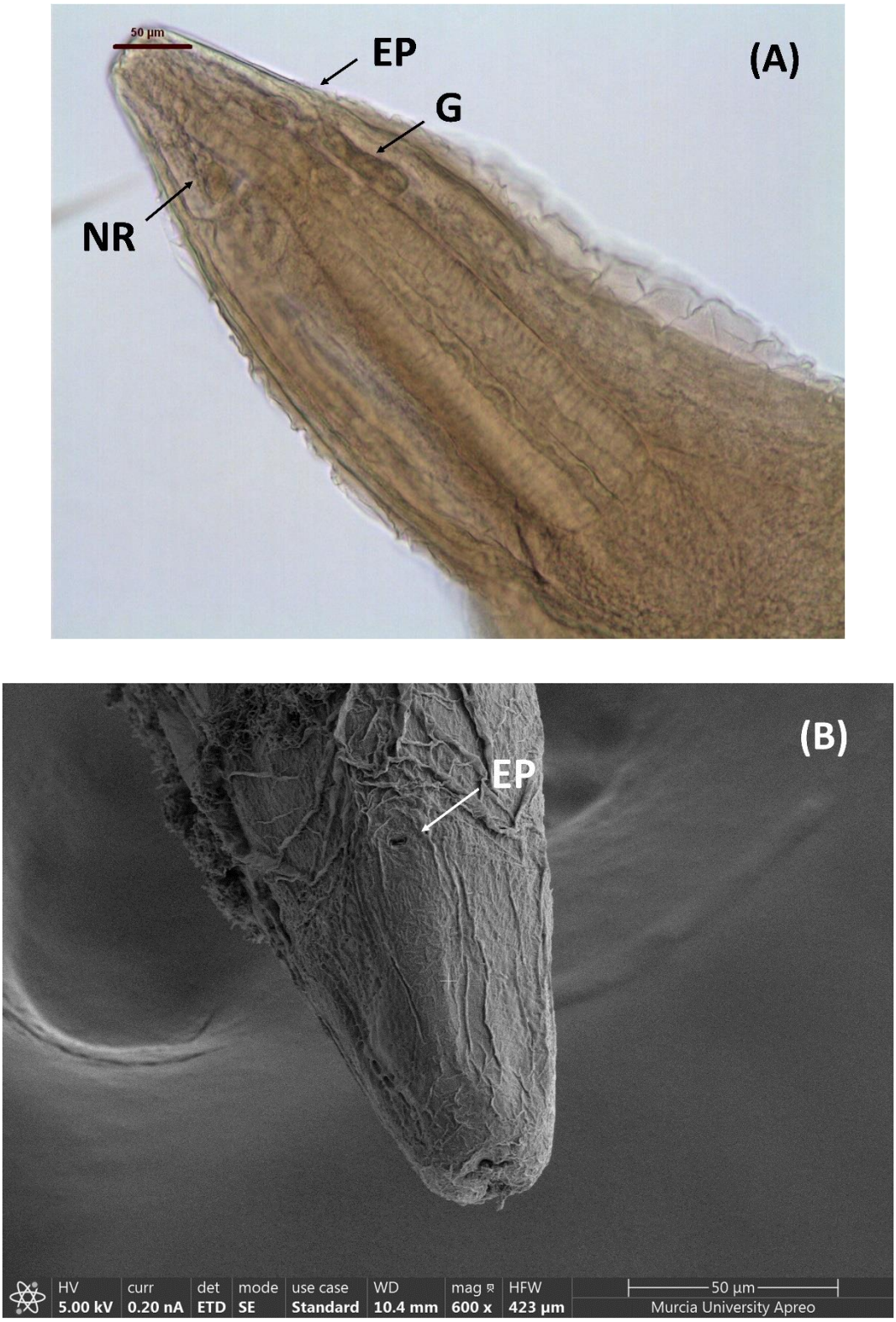
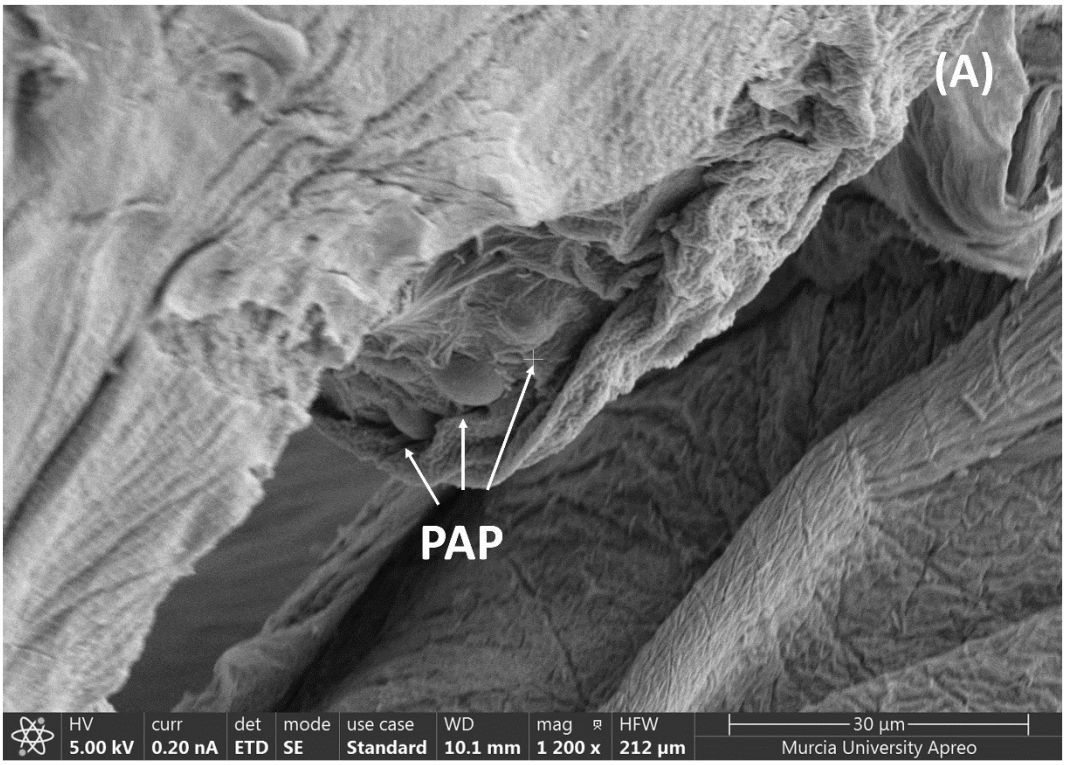


Figure 2. (A) Optical microscope image of the anterior part of a female specimen of *M. capsulata*, where the excretory pore (EP) can be distinguished together with the gland (G), as well as the constriction at the level of the nerve ring (NR). (B) Scanning electron micrograph showing the opening of the excretory pore (EP).



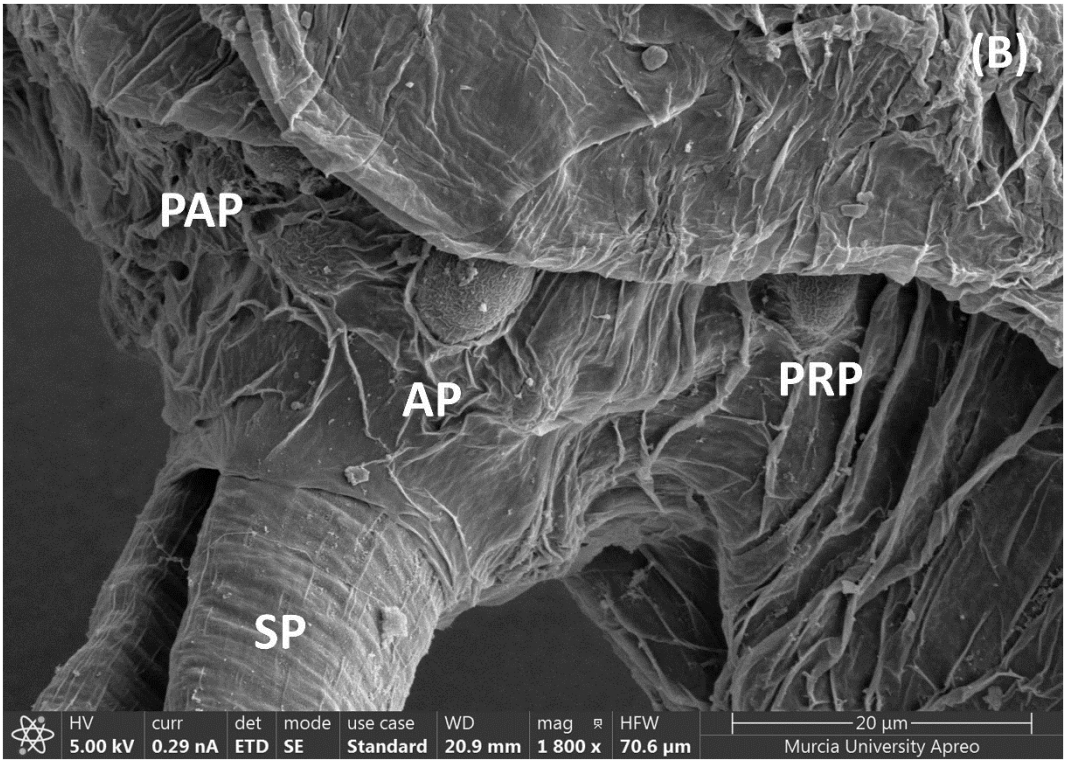
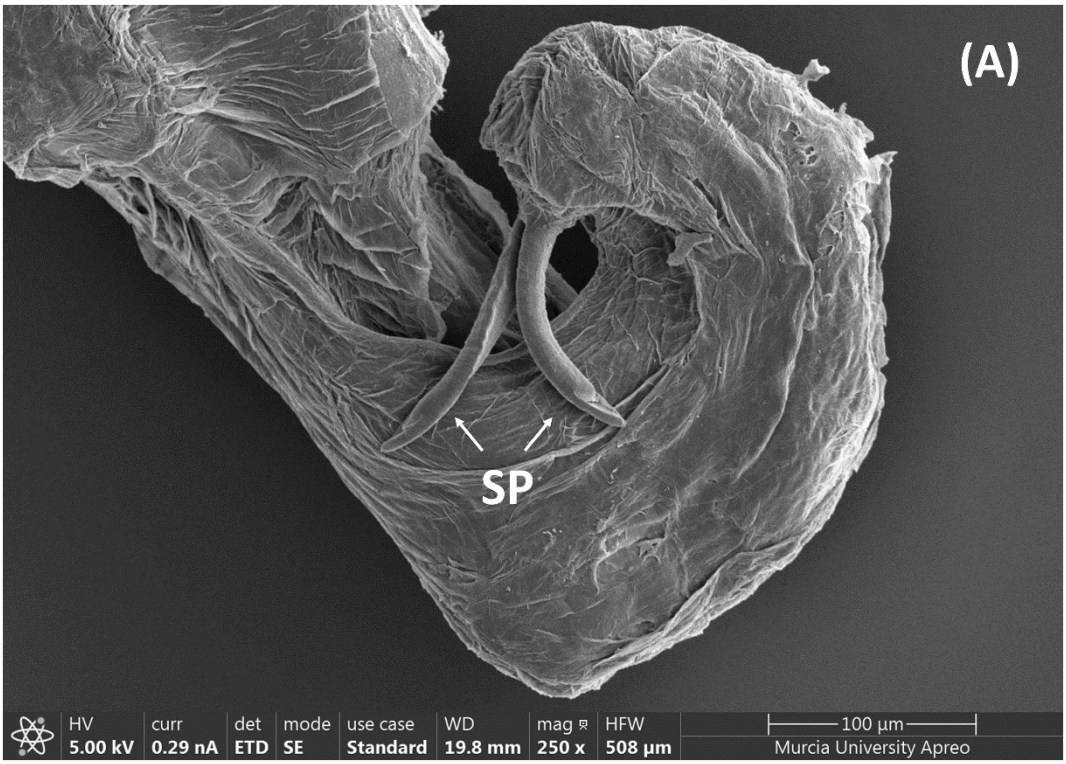


Figure 3. Scanning electron micrograph of the caudal end of a male specimen of *M. capsulata*. (A) Postanal papillae (PAP). (B) Preanal papillae (PRP), adanal papillae (AP) and postanal papillae (PAP), and spicules (SP).



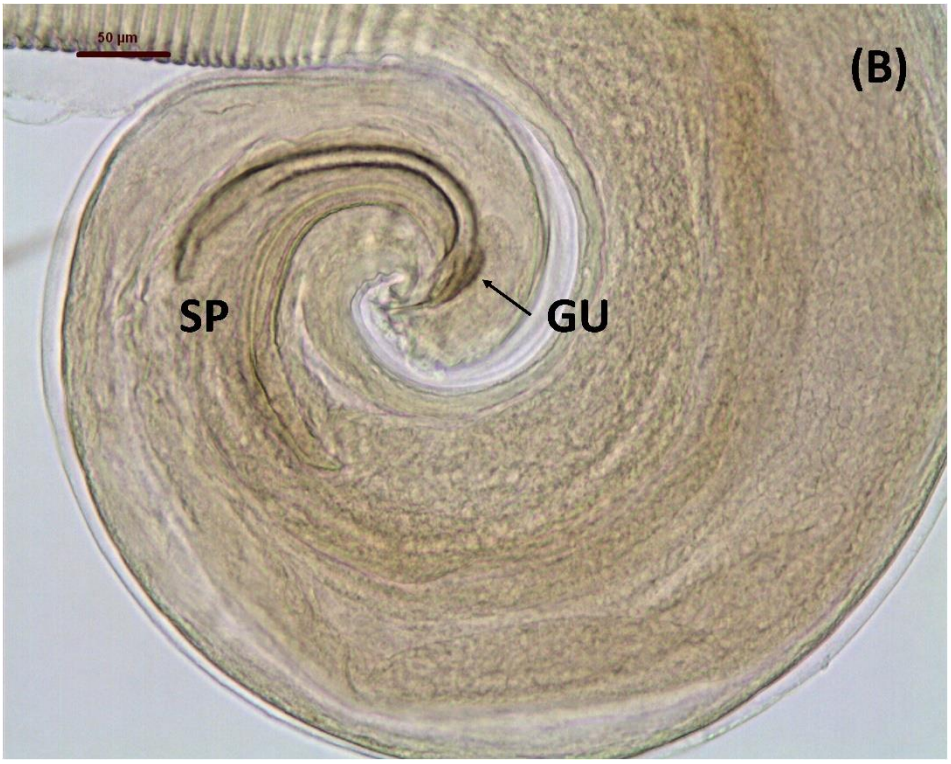
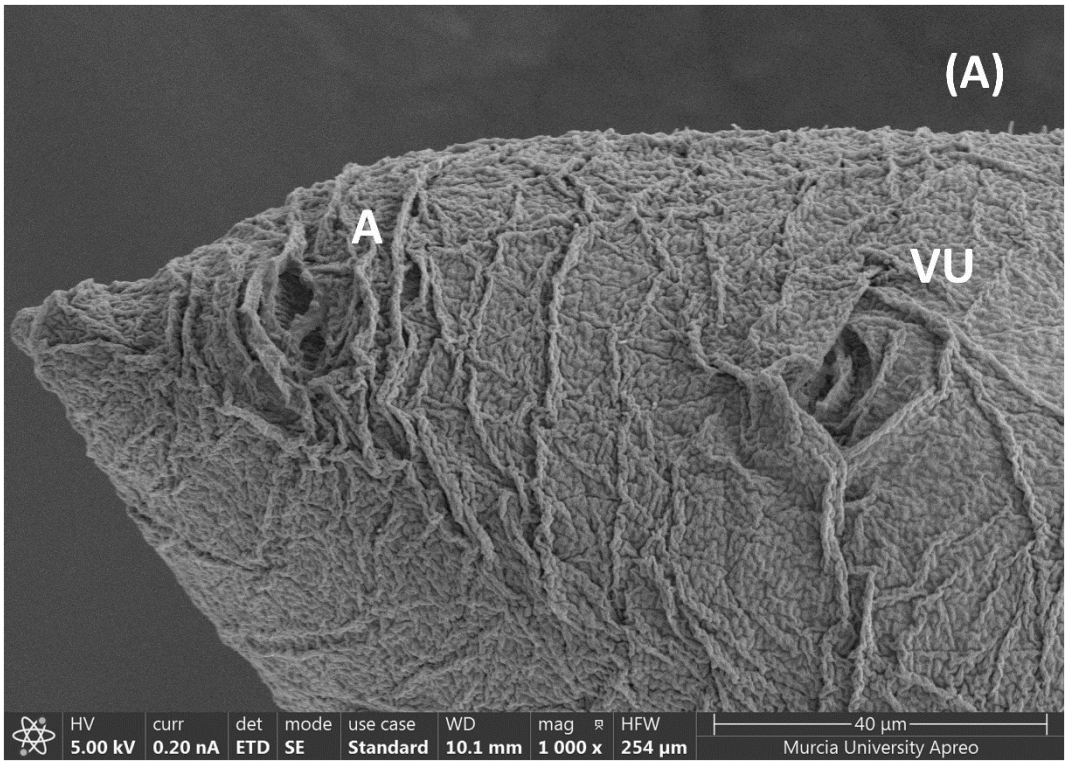


Figure 4. (A) Scanning electron micrograph of the caudal end of a male specimen of *M. capsulata*, showing falciform spicules (SP) with pointed end. (B) Optical microscope image in which the presence of double gubernaculum (GU) can be appreciated.



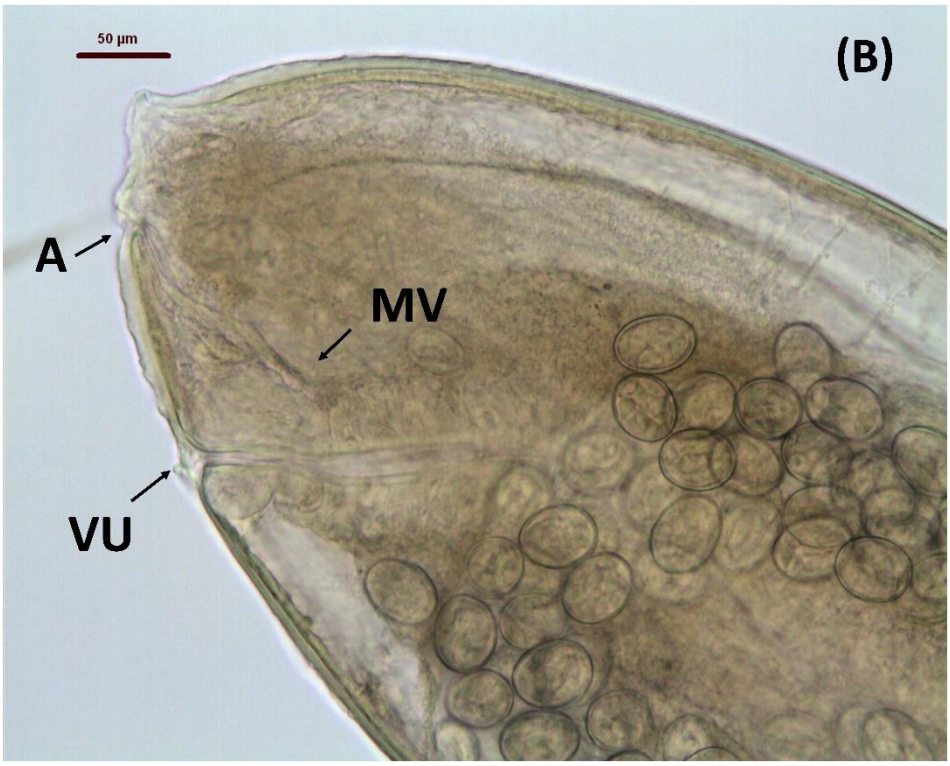


Figure 5. (A) Scanning electron micrograph of a female specimen of *M. capsulata* showing the vulva (VU) and anus (A) close to the posterior end. (B) Optical microscope image showing the musculature of the vagina (MV) and the vulva (VU) and anus (A) position.

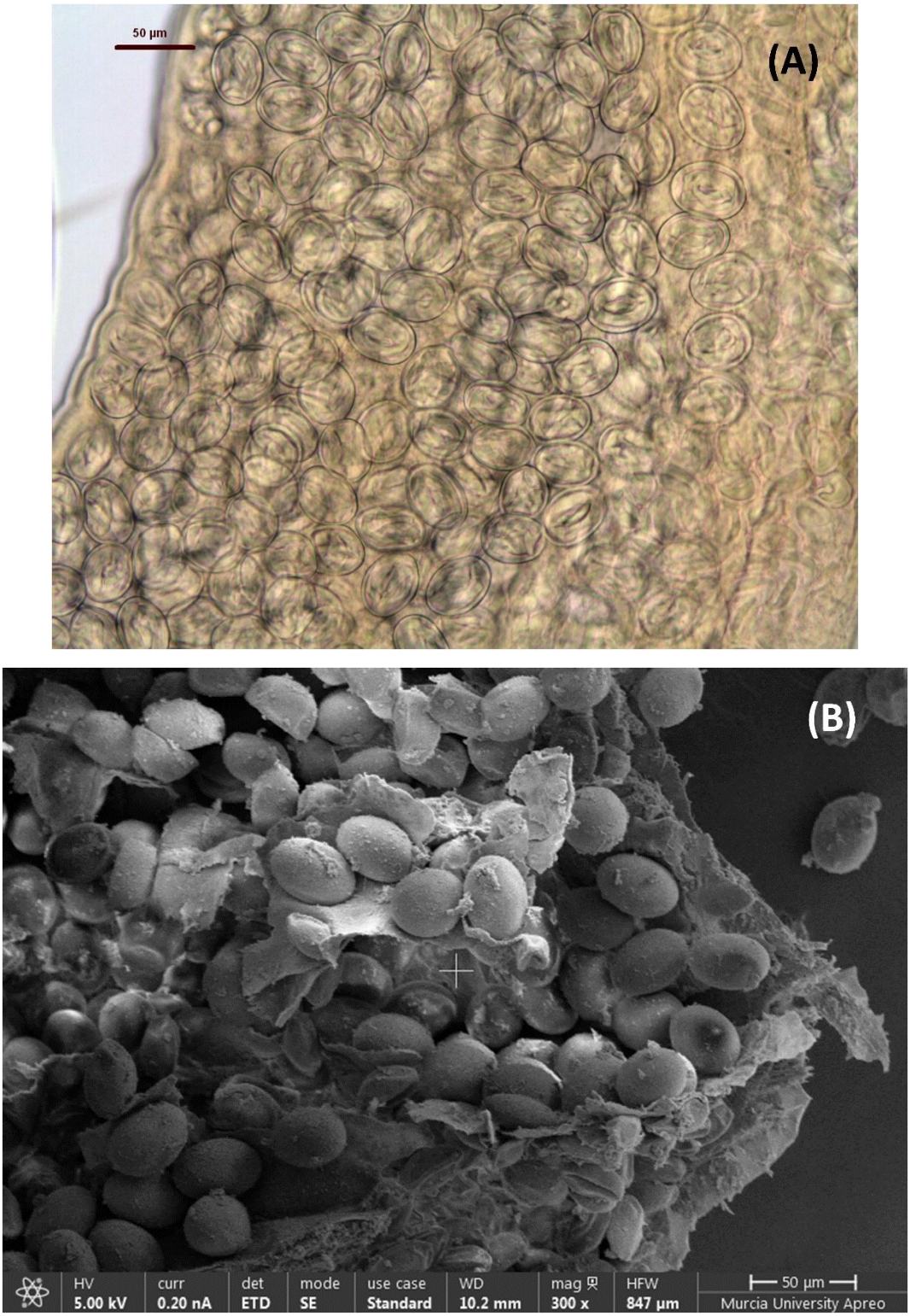


Figure 6. Oval-shaped and thick-shelled eggs viewed through the optical (A) and the scanning electron micrograph (B).

Sequences analysis

Six COI sequences (ON995618-ON995623) were obtained from three nematodes. All these sequences had the expected size of 689 base pairs (bp) and an A+T content between 69.28% to 69.81%. The alignment of the sequences showed very little intraindividual variation (0.0-0.29%); the interindividual variation was between 0.29 to 1.45%, both due to several substitutions but not to deletion or insertion. In fact, all of them could be translated to the corresponding protein fragment without stop codon. Nucleotide variation in COI sequences and the A+T content were similar to that of other nematode species (Iorio et al., 2009; Otranto et al., 2005; Bai et al., 2020). As no sequences for this genus or family are previously described we aligned the COI sequences with some that covered the same region and are available in GenBanK belonging from different nematode genera (*Chandlerella*, *Cylicospirura*, *Dirofilaria*, *Gongylonema*, *Onchocerca*, *Rhabdochona*, *Setaria*, *Spinitectus*, *Spirocerca* and *Thelazia*). The result of the alignment demonstrated that the highest identity percentage is observed with the COI sequence of the species *Rhabdochona salgadoi* (84.29%) (family Rhabdochonidae) and the lowest with the species *Thelazia callipaeda* (79.97%) (family Thelaziidae).

Four 18S rDNA sequences (OP004062-OP004065) were obtained from two nematodes. All these sequences had 1775 bp, and an A+T content between 51.04 to 51.21%. The alignment of the sequences showed very little intraindividual variation (0.06-0.39%) and the interindividual variation was between 0.17 to 0.23%. As for COI sequences, we aligned the 18S sequences with sequences of the same region that are available in GenBanK belonging from different nematode genera (*Gongylonema*, *Ichtyobronema*, *Oxyspirura*, *Physaloptera, Rhabdochona*, *Spinitectus*, *Spirocerca*, *Streptopharagus* and *Thelazia*). The result of the alignment demonstrated that the highest identity percentage is observed with the 18S sequence of *Physaloptera turgida* (94.99%) (Physalopteridae) and the lowest with *Thelazia lacrymalis* (88.67%) (Thelaziidae).

Six 28S rDNA sequences (OP021864-OP021869) were obtained from three nematodes. All these sequences had 972 bp, and an A+T content between 54.32 to 54.53%. The alignment of the sequences showed very little intraindividual variation (0.0-0.41%), and the interindividual variation was between 0.0 to 0.31%. The Blast search performed with our sequences in GenBank reported sequences from other distant

families of nematodes. However, the coverage was low (around 50%), and the highest identity (86.94%) for this coverage corresponded to the species *Gongylonema neoplasticum* belonging to the family Gongylonematidae.

With the different combinations of the ITSs primer pairs (Table 1), six ITS1, ITS2 and 5.8S sequences were obtained (OP059074-OP059083) from three nematodes. The ITS1 presented an A+T content variation ranging between 57.90% and 58.17%, and a length of 734 bp, but one of the sequences had 724 bp due to a deletion. The alignment of the sequences obtained showed a variation intraindividual and interindividual between 0.54% and 0.69%, and they are due to random distributed substitutions.

The ITS2 sequences had an A+T content ranged between 58.68% and 60.48%, the length of the sequences varied from 835 bp to 873 bp mainly due to variation in a microsatellite (TA)n. The alignment of the sequences obtained showed a very little variation due to base changes, the intraindividual and interindividual variations ranged between 1.1%-2.3% and 0.4%-4.5%, respectively. The A+T content of the ITS2 of *Habronema microstoma* and *H. muscaethe* was similar being 70.5% and 64.2%, respectively (Traversa et al., 2004).

The interspecific variation of the ITSs sequences was very high even between closely related sequences (Blouin, 2002). As no sequences for closely related species were available in GenBank, the alignment of the ITSs sequences with those of other nematode species belonging from other families was very bad. In fact, Blast search in GenBank resulted in coverages inferior to 12% of the length of the analysed sequences.

The 5.8S rDNA sequences analysed had an A+T content of 51.63% and 153 bp in length, being five of them identical, while one presented a substitution. The Blast search in GenBank demonstrated that the sequences from species of the genera *Protospirura*, *Spirurina*, *Gongylonema* and *Mastophorus* have the highest identity percentages (96.73%, 96.08%, 96.03% and 95.39%, respectively).

Phylogenetic analysis

In this work, the partial sequence of the COI was used for the phylogenetic analysis. Sixteen COI sequences from different nematode species were selected from GenBank considering a 100% coverture and the highest identity percentages with the analysed fragment (see Material and Methods section). The obtained Maximum-likelihood tree

(Figure 7) grouped all the sequences of *Metathelazia capsulata* showing a close relationship with the clade that included the species of the genera *Rhabdochona* and *Spinitectus* (family Rhabdochonidae) as expected for a species from the Pneumospiruridae family. However, the species of the family Thelaziidae (genera *Spirocerca* and *Thelazia*) are situated on clades more distant.

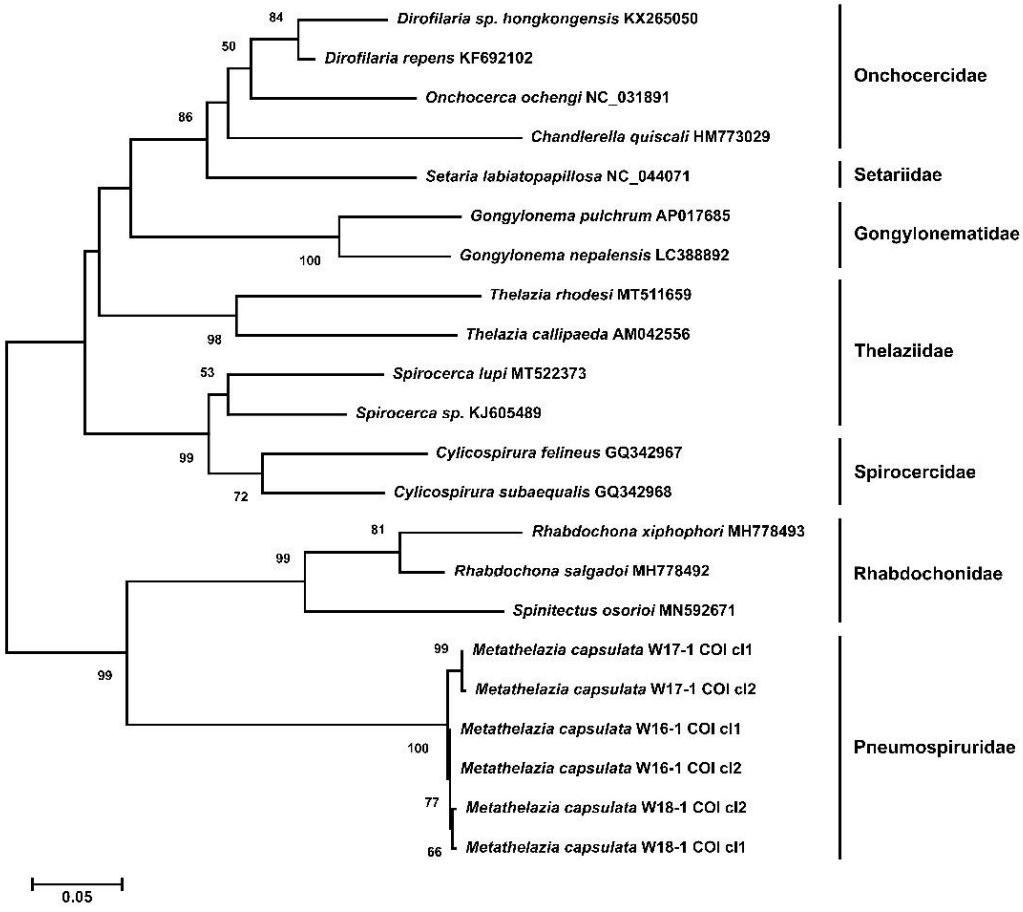


Figure 7. Maximum-likelihood tree using of COI sequences of *Metathelazia capsulata* and sixteen different nematode species (GTR+G model). Bootstrap supports of 2000 replicates greater than 50 are shown at each node. The scale bar represents the number of nucleotide substitutions per site.

DISCUSSION

To the authors' knowledge, this is the first record of the *Metathelazia* genus in red foxes in Europe, and, specifically, the first time that *M. capsulata* is found in the bronchial

tree of this wild canid. This species was reported previously in other wild carnivore species in America and Palestine (Gerichter, 1948; and Pence and Dowler, 1979). Most of the morphological characteristics found in the studied specimens were in accordance with those described by Skinker (1931): poorly developed lips and buccal capsule, similar male spicules and, in case of females, long, pointed and conical tail, and vulva distant from the anus in the posterior end.

As proposed by Wertheim and Chabaud (1977), the nematode species described in this study should be classified as *M. capsulata*, since the buccal capsule had two major lobes (pseudolabials) and four smaller submedian. Moreover, these characteristics of the cephalic region agrees with other species of the genus as *M. mexicana* (Jiménez et al., 2013).

As noted above, several species of the genus *Metathelazia* have been previously described (Table 3). Concerning *M. capsulata*, our results reveal some differences compared to previously descriptions. When comparing the measures with those described by Gerichter (1948) and Pence and Dowler (1979), one of the main differences is the individual size. Specifically, the mean length size of the nematodes found from red foxes in this study was 6.6 and 7.4 mm for males and females, respectively. However, previous authors reported higher values of the same measure (10 mm and 18 mm for males and females, respectively). The same happened to other measures as buccal cavity depth or distance between the excretory pore and the anterior end of the nematode; in both cases, our measures were lower than those previous described (Gerichter, 1948; Pence and Dowler, 1979). As for the remaining data, although the mean values were not similar, the ranges can be considered in agreement with the description of *M. capsulata*, as well as the general description of the parasite. On the other hand, the number and arrangement of the observed papillae coincides with those described by Pence and Dowler (1979).

Specimens from this study provided evidence of the wide metric variability that can occur in *M. capsulata*. In fact, Pence and Dowler (1979) discussed similar phenomenon when they compared their results with those of Gerichter (1948). In this sense, Jiménez et al. (2013) suggested that differences observed in nematodes recovered from badgers in America (New World), and those from badgers, foxes and polecats in Palestine (Old World), could in fact be different species. However, they did not dispose of the original

samples to be able to carry out comparisons and verify his theory. As recommended this author, based on the variability in the measures found in relation with previous *M. capsulata* descriptions, if the original specimens were available, more detailed comparative studies could be carried out to determine whether this is, in fact, a new species of *Metathelazia*.

On the other hand, this work is the first time that molecular markers are reported for the genus *Metathelazia* and also for the entire family Pneumospiruridae. In fact, sequence data of the mitochondrial (COI) and nuclear (rDNA) genes of *M. capsulata* are described. The marker analysed presented the usual characteristic in relation to the sequence length, A+T content and variation described for the same marker in other nematode species (Pereira et al., 2016; Bai et al., 2020).

The analysed markers are commonly used for nematode species identification, diagnostic and phylogenetic analysis (Iorio et al., 2009; Pereira et al., 2016; Choudhury and Nadler, 2018; Bai et al., 2020). As no information has been reported about molecular markers of this genus or even of this family, we have not been able to compare our data with those from closely related species. In fact, for analysed sequences, the Blast searches in GenBank reported sequences from other nematode families.

Mitochondrial molecular markers are currently being used for species identification in several organism groups including nematode species (Blouin, 2002; Otranto et al., 2005, 2007). Especially the COI gene is a widely used gene as important barcoding for species identifications and for phylogenetic analysis. Our phylogenetic analysis results with this gene agree with the consideration that the genera *Rhabdochona* and *Spinitectus* are closely related (Choudhury and Nadler, 2018). Our results indicate that sequences of *M. capsulata* are closely related to the family Rhabdochonidae, which is assumed to belong to superfamily Thelazioidea (Černotíková et al., 2011), a superfamily that includes species of the family Pneumospiruridae, but also suggest the distant relations with the family Thelaziidae.

The morphological and molecular data provided by our study will be useful in future research for the identification of *M. capsulata*, offering valuable information that will serve as a basis for phylogenetic studies of nematodes belonging to the genus *Metathelazia* and, in general, of species of the family Pneumospiruridae.

Table 3. Measurements of some nematode species described from the family Pneumospiruridae.

	Pneumospirura bassarisci **(Pence and Stone, 1977)**		***Vogeloides felis*** **(Vogel, 1928) (Pence and Stone, 1977)**		***Metathelazia californica*** **(Skinker, 1931) (Pence and Stone, 1977)**		***Metathelazia multipapillata*** **(Gerichter, 1948)**	
	Male n=4	**Female n=9**	**Male n=30**	**Female n=30**	**Male n=30**	**Female n=30**	**Male**	**Female**
Lenght	6.34 mm (5.30-6.42; 6.02)	10.69 mm (7.47-11.20; 9.49)	5.73-7.62 (6.63) mm	15.7-20.9 (18.9) mm	11.8-16.5 (13.4) mm	37.5-56.4 (43.1) mm	14-16 mm	30-40 mm
Width	227 (222-258; 235)	404 (216-408; 327)	162-346 (275)	442-589 (502)	104-125 (112)	184-236 (204)	130-140	190-260
Lenght esophagus							340-390	340-420
Width esophagus							40-50	70-80
Muscular esophagus	105 (110-117; 111)	117 (116-140; 125)	125-249 (167)	140-234 (177)	152-199 (171)	216-275 (236)		
Glandular esophagus	246 (237-257; 247)	242 (234-281; 250)	193-310 (251)	287-386 (332)	316-392 (358)	298-503 (404)		
Bucal deep	24 (22-25; 20)	26 (14-28; 19)	16-35 (22)	25-35 (28)	5-7 (6)	6-12 (10)		
Bucal lenght								
Nerve ring - head	80 (65-80; 75)	82 (76-88; 81)	61-113 (82)	64-99 (84)	99-135 (119)	117-140 (209)		
Excretory pore - head	82 (80-84; 82)	105 (90-108; 97)	70-146 (93)	59-94 (76)	140-216 (180)			
Spicule	181 (140-199; 173)		199-322 (244)		187-234 (210)		170-190	
Spicule width								
Gubernaculum	41 (41-47; 43)		32-53 (39)		29-37 (31)		27-30	
Gubernaculum width								
Cloacal opening - tail	59		47-70 (56)		40-70 (61)			
Vulva - anus		152 (106-164; 142)		118-220 (158)		74-182 (117)		100-160
Anus - tail		47 (47-59; 53)		37-88 (65)		258-353 (308)		60-70
Eggs - lenght		47 (47-53; 50)		41-49 (46)		40-48 (44)		47-49
Eggs - width		37 (36-40; 38)		26-36 (34)		28-34 (32)		33-35
Vagina								2400-3000

Table 3 (continue). Measurements of some nematode species described from the family Pneumospiruridae.

	Metathelazia oesophagea (Gerichter, 1948)		*Metathelazia capsulata* (Pence and Dowler, 1979)		*Metathelazia mexicana* (Jiménez et al., 2013)		*Metathelazia capsulata* (Gerichter, 1948)	
	Male	**Female**	**Male**	**Female**	**Male n=4**	**Female n=11**	**Male**	**Female**
Lenght	6.6-6.8 mm	7,8-8,2 mm	8.63-12.05 (10.07) mm	11.56-21.83 (17.13) mm	6.55-8.34 (7.23) mm	7.97-1.04 (1.04) mm	10-12 mm	18-20 mm
Width	260-280	340-370	131-346 (273)	294-603 (442)	238-273 (256)	261-461 (346)	290-360	440-470
Lenght esophagus	1000-1200	1230-1270			444-485 (474)	407-525 (466)	420-470	520-550
Width esophagus	190-210	230-270					70-90	70-100
Muscular esophagus			205-345 (269)	222-345 (293)				
Glandular esophagus			51-179 (117)	96-164 (137)				
Bucal deep			12-54 (30)	10-50 (27)	13-23 (17)	13-28 (21)		
Bucal lenght			12-44 (28)	12-47 (34)				
Nerve ring - head			49-140 (89)	82-132 (110)	59-79 (68)	58-81 (70)		
Excretory pore - head			72-130 (100)	62-204 (152)	69-109 (92)	90-121 (107)		
Spicule length	210-230		183-315 (260)		190-231 (214)		310-340	
Spicule width			12-15 (18)					
Gubernaculum length	38-39		25-53 (41)		40-79 (53)		45-54	
Gubernaculum width			8-18 (12)					
Cloacal opening - tail			36-76 (54)		74-83 (78)			
Vulva - anus		80-120		125-249 (187)		183-259 (204)		140-160
Anus - tail		100		35-94 (71)		66-87 (78)		100
Eggs - lenght		40-46		42-50 (44)		40-52 (44)		47
Eggs - width		30-35		29-37 (33)		24-39 (31)		38
Vagina		1000-1100						1900-2600

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GLOBAL DISCUSSION

Ecosystem modification produced by human activities are causing variations in the behaviour and distribution ranges of wild species. Wildlife can serve as a major reservoir for pathogens, either parasites, viruses or bacteria, and changes in their distribution ranges may result in a new geographic distribution of diseases (Otranto and Deplazes, 2019). Therefore, the approach of wild species to rural or urban environments can lead to new interaction with humans and domestic animals, and hence, to a potential higher risk of pathogen transmission (Smith et al., 2009; Thompson et al., 2009; Duscher et al., 2015; Hassell et al., 2017). In arid or semi-arid regions, characterized by high temperatures and low rainfall, the areas with availability of water, humidity or cover vegetation can become hotspots of interaction between domestic animals, wild animals and humans. Along this Thesis, an epidemiological study of fox population in semi-arid areas of the Iberian Peninsula, specifically in the Region of Murcia (SE Spain), was carried out.

The diagnostic method used during sample analysis must be as sensitive and accurate as possible to provide the most reliable results. Thus, to achieve the first objective of this Thesis (**Chapter 1**), the sensitivity and diagnostic accuracy of the traditional procedure used for the isolation of cardiopulmonary nematodes was compared with a new adaptation of this diagnostic method, which includes two additional and consecutive steps: squeezing of the lung parenchyma and artificial digestion in a solution of hydrochloric acid and pepsin was assessed.

From an epidemiological point of view this evaluation is important, since the choice of an appropriate method can guarantee much more accurate results and reduce costs or execution time (Llewellyn et al., 2016; Buonfrate et al., 2018). To date, most published studies dealing with the presence and parasite intensity of cardiopulmonary nematodes are based on their isolation through necropsy (Jeffery et al., 2004; Santoro et al., 2015; Houpin et al., 2016; Deak et al., 2020; Gillis-Germitsch et al., 2020; Lemming et al., 2020). Considering the importance of certain cardiopulmonary parasites for wild and domestic carnivore populations (Alho et al., 2018; Morchón et al., 2021; Pohly et al., 2022), it is necessary to have a gold standard procedure that allow to precisely know the health status of the studied populations and identify infection hotspots in order to implement effective management measures. Overall analysis showed that the consecutive use of the three methodological steps improves finding results, especially in terms of intensity.

Different results when squeezing (WS) and artificial digestion (AD) were provided according to the distribution of nematodes along the respiratory tract. Since *E. aerophilus* and *C. vulpis* are found in upper parts of the respiratory tract (Nevárez et al., 2005), the use of AD did not represent a significant improvement. However, its use significantly facilitated the isolation of *A. vasorum* and *M. capsulata* from those bronchioles or vessels with a smaller diameter. Owing to their small size, capillaries or bronchioles are usually not carefully examined when the traditional diagnostic method is employed, so it is possible that false negative results may occur. In agreement with Morgan et al. (2008) and Martínez-Rondán et al. (2019), AD is necessary to improve nematode recovery as well as to decrease false negative cases, since parasite prevalence, abundance or intensity rates could be underestimated. On the other hand, the use of AD implies an economic cost and time-consumption that must be considered. According to our results, its use could be of interest if the purpose of the study is to quantify the parasite intensity in the host, while the traditional method based on the opening with the addition of WS of the parenchyma would be enough to determine the prevalence.

The epidemiological study described in **Chapters 2** and **3** led to fulfil objectives 2 and 3 of this Thesis, respectively. Specifically, species of nematodes in red foxes and their relationship with the biotic and, especially, abiotic characteristics of the environment were evaluated. For this purpose, nematode communities present in the cardiorespiratory, gastrointestinal and urinary tracts were analysed. Likewise, considering that this study is carried out in a semi-arid area of the Iberian Peninsula, characterized by high temperatures and scarce precipitation (Miró et al., 2018), the influence of environmental variables on the abundance and distribution of parasite species throughout the study area was evaluated. As a consequence of climate change, it is estimated that this semi-arid region will suffer episodes of water stress (Tomaszkiewicz et al., 2016; Vila-Traver et al., 2021) and, possibly, changes in ecological interactions and distribution of wildlife species, thus this environmental analysis are particularly necessary in these regions, in order to detect if areas at risk of parasite transmission at the wild-domestic-urban interface could exist.

A large richness of nematode species has been described in fox populations, including a bronchopulmonary spirurid belonging to the genus *Metathelazia* that had

never been found in Europe and, hence, not even in the Iberian Peninsula (Chapter 4). This great parasite richness implies a wide variety of intermediate or paratenic hosts, such as gastropods (intermediate host of *A. vasorum* and *C. vulpis*), earthworms (intermediate host of *P. plica*), or rodents (paratenic hosts of *T. canis* and *T. leonina*). These results reflect the broad diet of the fox, thus showing that it is a generalist species capably to adapt to several types of habitats (Dell'Arte et al., 2007; Díaz-Ruíz et al., 2013; Martínez-Carrasco et al., 2007).

Bioclimatic factors were determinant on the abundance of these nematode species since they might directly influence on free-living larvae or parasite eggs in the environment, as in the case of *U. stenocephala*, *T. canis* or *T. leonina* (Chapter 2). Several environmental variables were associated with the abundance of *T. canis* and *T. leonina*. Temperature is an important component for the development of both ascarids infective stages, though *T. leonina* eggs have the ability to adapt to a wide variety of environmental conditions, better supporting extreme temperatures, even freezing, or being able to embryonate under dark conditions (Okulewicz et al., 2012). This could explain that its prevalence was higher than the one of *T. canis*. Despite the similar life cycle of both species, results revealed unequal responses, particularly in the case of NDMI values, precipitation or land cover uses. In addition, the paratenic hosts for these ascarids are common (rodents), so diet is not a key factor in these discrepancies. It is possible that other environmental conditions not analysed could be affecting both species or even their paratenic host distribution. Hence, it is necessary to continue studies to clarify in detail these differences (Reperant et al., 2007; Traversa, 2012; Mørk et al., 2019). Moreover, although the statistical results do not evidence urban areas as a risk factor, the continuous spatial distribution maps showed specific points where the highest abundances of these ascarids are near to urban settlements. Therefore, in urban and peri-urban areas foxes could be maintaining *T. canis* and *T. leonina* life cycles by soil contamination with eggs (Brochier et al., 2007; Traversa et al., 2014; Nijsse et al., 2015).

U. stenocephala also has a direct life cycle, whose infective larvae (L3) will develop from eggs excreted by the infected host (Anderson, 2000). Humidity, mild temperatures and vegetation cover are essential factors to enhance the maintenance and development of these free-stage larvae in the environment (Fiocchi et al., 2016) and infection finally

occurs in the host. In our study, abundance of *U. stenocephala* was related with the same abiotic conditions mentioned above, as it is highlighted in Chapter 2. The areas covered with vegetation (forestry and agricultural areas) provide a refuge from the effect of the high temperatures in this semi-arid region, helping to preserve a greater humidity and decreasing the effect of the temperature. However, the abundance of this parasite in urban areas should also be taken into consideration in terms of sanitary aspects, since infective larvae of this nematode are in the contaminated soil, posing a risk of transmission to dogs, foxes and even humans (Reperant et al., 2007; Traversa et al., 2014; Karamon et al., 2018).

Lands with permanent watercourses, soil moisture, or refuges providing soft temperatures, favour also the activity, dynamics and development of intermediate host populations such as gastropods and earthworms (Morgan et al., 2009; Alho et al., 2018; Sankar and Patnaik, 2018; Singh et al., 2019). Gastropods are intermediate host of *A. vasorum* and *C. vulpis*, cardiopulmonary nematode species described in Chapter 2. The abundance of both species was positively related to summer NDMI values, in contrast to winter NDMI. As occurs with the previous mentioned species, moisture and temperature are necessary for the survival of infective larvae (Ferdushy and Hasan, 2010; Robbins et al., 2021), as well as for the activity and dynamics of molluscs' populations (Morgan et al., 2009; Alho et al., 2018), or amphibians, paratenic host of *A. vasorum* (Bolt et al., 1994). In the case of *A. vasorum*, all land use categories were identified as a risk factor. Habitat with vegetation cover or water availability are more suitable for the occurrence of *A. vasorum* and *C. vulpis*. Although, it has been shown in several studies that the distribution of *A. varosum* is often observed in clusters, leading to think that this parasite is able to adapt to a wide variety of microclimates (Helm et al., 2010; Tolnai et al., 2015; Maksimov et al., 2017; Čabanová et al., 2018), in our study it was observed a random pattern. Contrary, in this semi-arid Mediterranean area of Iberian Peninsula, agricultural lands served as protective zones for the transmission of *C. vulpis*. Several mollusc species involved in the life cycle of *A. vasorum* and *C. vulpis* have been described (Colella et al., 2016; Maksimov et al., 2017; Fueher et al., 2020), and some of them are present in the semi-arid areas of southeastern Iberian Peninsula (Borredà and Martínez-Ortí, 2014). It is possible that different habitat requirements for snail and slug exist (Garrido-Castañé et al., 2015; Deak

et al., 2020), but also the host species required for each one of the lungworm species could be different (Maksimov et al., 2017). In this sense, it would be convenient to conduct further studies to evaluate all potential factors associated with the maintenance of the life cycle of these species in the environment (Gavrilović et al., 2019; Gillis-Germitsch et al., 2020; Morgan et al., 2021).

Along Chapter 3, where the prevalence and the abundance of the urinary bladder nematode *P. plica* were evaluated, results were similar to those described in previous studies. Foxes with *P. plica* came from irrigated agricultural and forest areas, both variables associated with a higher availability of moist environments, which are necessary for the presence of the intermediate host of this parasite (Sankar and Patnaik, 2018; Singh et al., 2019). However, climatic conditions in semi-arid Mediterranean areas are not favourable for the presence of earthworms, and thus the prevalence was very low. In this regard, evidence that this nematode is present in the Region of Murcia shows that, in spite of the unfavourable environmental conditions of this semi-arid area, suitable locations with humidity are available for the presence of this parasite. Therefore, the maintenance of the sylvatic cycle of *P. plica* is possible, and the existence of shared territories between foxes and dogs may pose a risk to this parasite transmission.

Adaptation to ecosystem changes depends on the ecological or behavioural plasticity of each carnivore species (Bateman and Fleming, 2012). In the case of foxes, their great ecological plasticity allows them to adapt to anthropized areas. Moreover, based on their wide feeding range, results from Chapter 2 and 3 demonstrate that foxes play a significant epidemiological role as they contribute to the dispersal of these parasites throughout the territory, as well as helping to maintain the parasite's sylvatic cycle in the natural environment; thus, foxes can potentially serve as reservoirs of a great diversity of nematode species, including those shared with domestic carnivores and humans (Duscher et al., 2015; Mackenstedt et al., 2015; Figueiredo et al., 2016; Otranto and Deplazes, 2019). In this regard, it should be noted that domestic animals can also play an important role in the transmission of pathogens, being a link between wild and domestic-urban niches, which may result in a spill-over from wildlife to domestic animals, or vice versa (spill-back) (Thompson, 2013). Besides, humans can be accidentally infected by the ingestion of infective eggs of parasite species such as *T. canis*, reported in Chapter 2. Thus,

it is important to consider this when establishing management and control measures in areas where this wild-domestic-human interface is present.

Finally, in order to achieve the objective 4, a morphometric and molecular description of a nematode recovered from bronchi and bronchioles of red foxes were performed (**Chapter 4**). The parasite was identified as *Metathelazia capsulata,* a spirurid nematode previously described in the bronchi and bronchioles of wild carnivores such as the European badger (*Meles meles*), American badger (*Taxidea taxus*), Nile fox (*Vulpes vulpes nilotica*) or marbled polecat (*Vormela peregusna*) (Gerichter, 1948; Pence and Dowler, 1979). This is the first finding of *M. capsulata* in Iberian Peninsula, and to our knowledge, also in Europe. Most of the morphological characteristics coincided with previous author´s descriptions, even though some differences were found, mainly the nematode length. Nowadays, molecular biology techniques are very useful as diagnostic tools in parasitology, and frequently employed when parasites are difficult to identify by classical morphometric techniques or when it is necessary to confirm the species on a genetic basis. In this sense, mitochondrial molecular markers such as COI gene are currently used for identification of diverse organisms, including nematode species (Blouin, 2002; Otranto et al., 2005, 2007). According to our study, sequences of the nematode identified as *M. capsulata* are closely related to the family Rhabdochonidae, included in the superfamily Thelazioidea (Černotíková et al., 2011). This superfamily is composed by three families: Rhabdochonidae, Thelaziidae and Pneumospiruridae, and the genus *Metathelazia* belongs to the last one (Chabaud and Bain, 1994). These results provided the sequence data description of the mitochondrial (COI) and nuclear (rDNA) genes of *M. capsulata*, being the first molecular markers reported for the genus *Metathelazia* and hence for the family Pneumospiruridae.

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CONCLUSIONS

FIRST: The abundance and species richness of gastrointestinal, cardiorespiratory and urinary system nematodes detected in the red fox (*Vulpes vulpes*) population in the southeast of the Iberian Peninsula evidence the important epidemiological role that this canid plays in the maintenance and dispersal of these helminths in semi-arid Mediterranean areas. Since most of these parasites can affect domestic carnivores and some cause zoonoses, the presence of foxes at the domestic-wild-human interface should be taken into account as an epidemiological risk factor for public and animal health.

SECOND: The application of squeezing and subsequent artificial digestion of the lung parenchyma to the diagnostic technique for the detection of cardiopulmonary nematodes in carnivores, decreases the overall percentage of false negatives, and provides a more accurate parasite intensity than when only the tracheobronchial tree and pulmonary vessels are opened. Therefore, the technique based on these three consecutive methodological steps is recommended to obtain more reliable results and, consequently, to make a more solid epidemiological interpretation when studying these parasites in domestic and wild canids.

THIRD: The use of artificial digestion of lung parenchyma is especially recommended to obtain more accurate results in the quantification of specimens of *Angiostrongylus vasorum* and *Metathelazia capsulata*. In addition, artificial digestion increases the sensitivity of the diagnostic technique for these two nematodes. However, in the case of nematode species located in the trachea and bronchi (*Eucoleus aerophilus* and *Crenosoma vulpis*), it is sufficient to open these tracts and squeeze the parenchyma to get a high sensitivity in the diagnosis.

FOURTH: The survival of the infective stages of cardiopulmonary and digestive fox nematodes in the environment, as well as the presence of their intermediate and paratenic hosts, are conditioned by the high temperatures and lack of humidity characteristic of semi-arid Mediterranean areas. Hence, forested or agricultural areas are those with the highest abundance of these nematodes due to the microclimatic conditions that allow the development of their biological cycles. These microhabitats can become hotspots of intraspecific and, also, interspecific transmission of nematodes in areas shared by foxes and dogs.

FIFTH: The presence of *Uncinaria stenocephala, Toxocara canis* and *Toxascaris leonina* in foxes in semi-arid areas of the southeastern peninsular highlights the epidemiological risk posed by the presence of this wild carnivore in anthropized areas, especially in rural and peri-urban areas, considering the potential zoonotic of these nematodes.

SIXTH: The prevalence of *Pearsonema plica* in foxes in the southeast of the Iberian Peninsula is very low because environmental factors are not favourable for the development of its life cycle. However, the detection of parasitized foxes shows that there are specific areas in which the environmental conditions, both natural or due to human activity, provide the necessary humidity for earthworms to be present and, in this way, for *P. plica* to survive. Consequently, even in semi-arid Mediterranean environments, it is advisable to consider the risk of transmission of this nematode, which is common to domestic and wild canids.

SEVENTH: This is the first description of the bronchopulmonary nematode *Metathelazia capsulata* in foxes in Europe. This finding highlights the need for future studies to understand the life cycle of this spirurid species and whether other domestic or wild hosts, are also involved in it.

EIGHTH: Sequences of the COI and rDNA molecular markers of *M. capsulata* have been identified for the first time. This valuable information, together with the detailed morphometric description provided, will be useful for future studies on the phylogeny of *Metathelazia* genus nematodes and, more generally, those belonging to the family Pneumospiruridae, where this genus is included.

CONCLUSIONES

PRIMERA: La abundancia y la riqueza de especies de nematodos gastrointestinales, cardiorrespiratorios y del sistema urinario detectadas en la población de zorros (*Vulpes vulpes*) del sureste de la península ibérica, demuestra el destacado papel epidemiológico que este cánido tiene en el mantenimiento y dispersión de estos helmintos en áreas semiáridas mediterráneas. Dado que la mayoría de los parásitos del zorro pueden afectar a los carnívoros domésticos y que algunos son causa de zoonosis, su presencia en la interfaz doméstico-silvestre-humano debe ser tenida en cuenta como un factor de riesgo epidemiológico en el ámbito de la salud pública y la sanidad animal.

SEGUNDA: La aplicación del estrujado y posterior digestión artificial del parénquima pulmonar a la técnica de diagnóstico de nematodos cardiopulmonares habitualmente empleada en carnívoros, disminuye el porcentaje global de falsos negativos y se obtiene una intensidad de parasitación más exacta que cuando solo se realiza la apertura del árbol tráqueobronquial y de los vasos pulmonares. Por tanto, es recomendable la aplicación de la técnica basada en estos tres pasos metodológicos consecutivos para obtener resultados más fiables y, en consecuencia, para hacer una interpretación epidemiológica más sólida cuando se estudian estos parásitos en cánidos domésticos y silvestres.

TERCERA: El uso de la digestión artificial del parénquima pulmonar es especialmente aconsejable para obtener resultados más precisos en la cuantificación de los especímenes de *Angiostrongylus vasorum* y *Metathelazia capsulata*. Además, la digestión artificial incrementa la sensibilidad de la técnica de diagnóstico referida a estos dos nematodos. Sin embargo, en el caso de las especies de nematodos localizadas en la tráquea y bronquios (*Eucoleus aerophilus* y *Crenosoma vulpis*), es suficiente la apertura de estas vías y el estrujado del parénquima para obtener una elevada sensibilidad en el diagnóstico.

CUARTA: La supervivencia en el medio ambiente de los estadios infectivos de los nematodos cardiopulmonares y digestivos del zorro, así como la presencia de sus hospedadores intermediarios y paraténicos, están condicionadas por las altas temperaturas y la falta de humedad características de las áreas semiáridas mediterráneas. Por ello, las zonas provistas de vegetación forestal o áreas de uso agrícola son las de mayor abundancia de estos nematodos, debido a las condiciones microclimáticas que permiten el desarrollo de sus respectivos ciclos biológicos. Estos microhábitats pueden convertirse

en puntos calientes de transmisión intraespecífica y, además, interespecífica en áreas compartidas entre el zorro y el perro.

QUINTA: La presencia de *Uncinaria stenocephala*, *Toxocara canis* y *Toxascaris leonina* en los zorros de zonas semiáridas del sureste peninsular pone de manifiesto el riesgo epidemiológico que supone la presencia de este carnívoro silvestre en áreas antropizadas, sobre todo en zonas rurales y periurbanas, debido al carácter zoonósico de estos nematodos.

SEXTA: La prevalencia de *Pearsonema plica* en los zorros del sureste de la península ibérica es muy baja debido a que los factores ambientales no favorecen el desarrollo de su ciclo biológico. No obstante, la detección de zorros parasitados demuestra que hay zonas concretas en las que las condiciones ambientales, ya sean naturales o debidas a la actividad humana, propician la humedad necesaria para que haya lombrices de tierra y, de esta forma, *P. plica* pueda sobrevivir. Por tanto, incluso en ambientes semiáridos mediterráneos es recomendable tener en consideración el riesgo de transmisión que tiene este nematodo, común a cánidos domésticos y silvestres.

SÉPTIMA: Es la primera vez que se describe el nematodo broncopulmonar *Metathelazia capsulata* en zorros de Europa. Este descubrimiento pone de manifiesto la necesidad de realizar futuros estudios para conocer cuál es el ciclo biológico de esta especie de espirúrido, averiguando si otros hospedadores, ya sean domésticos o silvestres, también están involucrados en el mismo.

OCTAVA: Se han identificado por primera vez las secuencias de los marcadores moleculares COI y rDNA de *M. capsulata*. Esta valiosa información, junto a la descripción morfométrica detallada que se ha realizado, servirán para futuros estudios de filogenia de nematodos del género *Metathelazia* y, en general, de aquellos pertenecientes a la familia Pneumospiruridae, en la que se incluye dicho género.