UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

THE SYLVATIC AND SYNANTHROPIC CYCLES OF *ECHINOCOCCUS* SPP., *TAENIA* SPP. AND *TOXOCARA* SPP. IN PORTUGAL: COPROLOGIC AND MOLECULAR DIAGNOSIS IN CANIDS

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I dedicate this work to my parents for making everything possible…

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Abstract

THE SYLVATIC AND SYNANTHROPIC CYCLES OF *ECHINOCOCCUS* SPP., *TAENIA* SPP. AND *TOXOCARA* SPP. IN PORTUGAL: COPROLOGIC AND MOLECULAR DIAGNOSIS IN CANIDS

Echinococcus spp., *Taenia* spp. and *Toxocara* spp. are important parasites of domestic and wild canids and neglected zoonotic helminths. Despite their relevance in Public Health, little is known about their prevalence in Portugal. An epidemiological study was conducted to clarify the role of canids in the sylvatic and synanthropic cycles of these pathogens in our country.
Fecal samples from dog (n = 51), red fox (n = 62) and Iberian wolf (n = 68) were collected from two regions. *Toxocara* spp. and taeniid eggs were isolated through a Sieving-flotation technique. Species identification in taeniids was made using Multiplex-PCR followed by sequencing of amplified material; in *Toxocara* spp. by measuring the eggs.
Taenia hydatigena, T. serialis, T. pisiformis, T. polyacantha, *Echinococcus canadensis* (G7) and *Toxocara canis* were detected in wolves. This was the first time taeniid species were studied in Portuguese Iberian wolf populations, with the first records of *T. polyacantha* and *E. canadensis* in Iberian wolves. *T. hydatigena* and *Toxocara canis* were found in both dogs and foxes and *T. polyacantha* in foxes.
Dogs were considered the most important link between domestic and synanthropic cycles but wolves and foxes can be regarded as the most relevant hosts in maintaining the sylvatic and synanthropic cycles of taeniids and *Toxocara* spp., respectively. Control programs should consider these species as part of their measures, and dogs, since they are more easily reached, should be dewormed more frequently.

Key words: canids, *Echinococcus*, epidemiology, Portugal, *Taenia, Toxocara*.

Resumo

OS CICLOS SILVÁTICOS E SINANTRÓPICOS DE *ECHINOCOCCUS* SPP., *TAENIA* SPP. E *TOXOCARA* SPP. EM PORTUGAL: DIAGNÓSTICO COPROLÓGICO E MOLECULAR EM CANÍDEOS

Echinococcus spp., *Taenia* spp. e *Toxocara* spp. são parasitas importantes de canídeos domésticos e silvestres e agentes de zoonoses negligenciadas. Apesar da sua relevância em Saúde Pública, pouca informação existe acerca da prevalência em Portugal. Foi realizado um estudo epidemiológico para compreender o papel que espécies de canídeos poderiam desempenhar nos ciclos silvático e sinantrópico destes parasitas no nosso país.
Foram recolhidas amostras fecais de cão (n =51), raposa (n = 62) e lobo ibérico (n = 68) de duas regiões. Os ovos de *Toxocara* spp. e de tenídeos foram isolados por uma técnica de Filtração-Flutuação. As espécies de tenídeos foram identificadas por Multiplex-PCR e sequenciação do material amplificado; para *Toxocara* spp. foram medidos os ovos.
Em lobos foram detectadas as espécies *Taenia hydatigena, T. serialis, T. pisiformis, T. polyacantha*, *Echinococcus canadensis* (G7) e *Toxocara canis,* sendo este o primeiro estudo das espécies de tenídeos na população portuguesa de lobo ibérico*. T. polyacantha* e *E. canadensis* (G7) foram detectados pela primeira vez em lobo ibérico. *T. hydatigena* e *Toxocara canis* foram encontrados em raposas e cães e *T. polyacantha* apenas nas raposas.
Considerou-se que os cães serão o principal elo de ligação entre os ciclos doméstico e sinantrópico, enquanto os lobos e as raposas o serão para os ciclos silvático e sinantrópico de tenideos e *Toxocara* spp., respectivamente. As medidas dos programas de controlo deverão, por isso, focar-se também nestas espécies, e os cães, por serem mais facilmente manipulados, devem ser desparasitados mais frequentemente.

Palavras-chave: canídeos, *Echinococcus,* epidemiologia, Portugal, *Taenia*, *Toxocara*.

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bp – base pairs
bw – body weight
DNA – Deoxyribonucleic Acid
e.g. – *exempli gratia*
ELISA – Enzyme-linked Immunosorbent Assay
EPG – Eggs per gram
g – gram
i.e. – *id est*
kg – kilogram
mg – milligram
ml – milliliter
N – normal
n – sample size
No. – number
OIE – World Organization for Animal Heath
PCR – Polymerase Chain Reaction
pi – post infection
WHO – World Health Organization
x *g* – gravity
µL - microliter

1. Introduction

In September 2005, a meeting of the World Health Organization (WHO) in Geneva, Switzerland, developed the concept of Neglected Zoonotic Diseases to address the increasing concern towards a group of pathogens with specific characteristics. All these were ancient diseases, largely known by the human populations, with severe clinical signs and deep economic impact. Most importantly, they presented an endemicity that was strongly supported by the interaction between humans, domestic animals and wildlife. Underdiagnosis led to underestimation of their real importance which resulted in few control measures being taken nowadays (World Health Organization [WHO], 2010).

Since its creation, the list of neglected zoonotic diseases increased every year. Two diseases included in this list are Cystic Echinococcosis/Hydatidosis and Cysticercosis/Taeniasis, both present in Portugal. Although not a part of this list, Toxocarosis still fulfills the majority of the criteria and some recent works consider it an important neglected zoonosis in some countries (Hotez & Wilkins, 2009).

Base-line epidemiological studies are needed for these diseases, about which little is known, especially regarding their wildlife reservoirs.

Five years of Veterinary studies in Lisbon and the great interest developed by the author in the fields of Parasitology, Wildlife and Public Health led to the choice of the research project described. In the first part of this document will be given a brief description of the training period undertaken by the author, followed by a thorough literature review of the main topics, i.e. Echinococcosis, Taeniosis and Toxocarosis. The second part addresses the project developed, including its main goals, materials and methods, results, discussion and conclusion of all the research performed.

1.1. Training Period Activities

In the last year of his Integrated Masters in Veterinary Medicine, the author took a training period of approximately six months in two different locations - at the Parasitic Diseases Routine Laboratory, Faculty of Veterinary Medicine, Technical University of Lisbon, Portugal (FVM-TUL) from September 5th to September 28th 2011; and at the Institute of Parasitology, Vetsuisse-Faculty, University of Zurich, Switzerland (IPZ-UZH), the latter under the LLP/Erasmus Program, between October 3rd 2011 and February 29th 2012.

The training period was supervised by Prof. Doctor Peter Deplazes (IPZ-UZH) and co-supervised by Prof. Doctor Luís Manuel Madeira de Carvalho (FVM-TUL).

During the first part of the training period, held in Lisbon, the author followed the routine activities of the Laboratory, gathering knowledge and practice in sample processing and diagnosis, mainly in Hemoparasites and Gastrointestinal helminths of carnivores. In January 2012, the student also spent two weeks at the FVM – TUL analyzing fecal samples from stray dogs that were eventually used in this study.

In the Institute of Parasitology, Zurich, the student developed the majority of the work presented in this document. In the first month, the student was introduced to the general techniques and projects done in the Institute. It was possible to follow and also to practice techniques of egg isolation in feces and from adult worms, Single and Multiplex PCR for taeniids, ELISA to detect coproantigens of *Echinococcus multilocularis* in fecal samples. During the remaining period, the student started working on his project, supervised by Prof. Doctor Peter Deplazes and Doctor Teresa Armua.

During the training period in Zurich it was also possible for the student to follow several steps of other ongoing projects about *Echinococcus multilocularis, Fasciola hepatica, Toxoplasma gondii* and *Angyostrongylus vasorum*. He could learn and help at fox and rodents necropsies, snail infection and molecular techniques such as Western-blot.

Each Tuesday there were internal seminars hosted by a member of the Institute or a guest researcher. The student was able to attend to the following sessions:

11.10.11. Christoph Lippuner (IPZ-UZH): "Transcriptomics of intestinal stages of *Cryptosporidium*";

18.10.11. Stefanie Wagner (IPZ-UZH): "Mosquitoes in Central Europe: ecological and physiological investigations";

25.10.11. Michael Grigg (Laboratory of Parasitic Diseases, National Institute of Health, Bethesda, USA): "Biology of *Toxoplasma* Pathogenesis: Strain Type and Polyparasitism as Predictors of Disease";

08.11.11. Catherine Eichwald (Institute of Virology, University of Zürich): "*Bacillus subtilis* spores, a safe alternative for enteric antigen delivery";

15.11.11. Relja Beck (Head of Laboratory for Parasitology, Department for Bacteriology and Parasitology, Croatian Veterinary Institute, Zagreb, Croatia): "Vector borne diseases in Croatia";

22.11.11. Hubertus Hertzberg (IPZ-UZH): "Vakzination gegen *Haemonchus contortus*";

29.11.11. Nick Smith (Queensland Tropical Health Alliance, James Cook University, Cairns Australia): "The Molecular Biology of Oocyst Wall Formation in Coccidian Parasites";

06.12.11. Petra Wampfler (IPZ-UZH): "Proteomics of secretory organelles in *Giardia*";

12.12.11. Matthias Rottmann (Swiss Tropical and Public Health Institute, Basel, Switzerland) "Malaria Drug Discovery projects at the Swiss TPH";

24.01.12. Sofia Gersão (FVM-TUL) "Control of cyathostominosis: A comprehensive approach".

2. Literature Review

2.1. *Echinococcus* spp.

Echinococcus spp. are hermaphrodite heteroxenous tapeworms, whose life cycle develops in different mammal species. The adult parasite infects the small intestine of the definitive host, whereas the larval stage occurs in different tissues of the intermediate host.

2.1.1. Taxonomy

Echinococcus genus belongs to the Phylum Platyhelmynthes, Class Cestoda, Subclass Eucestoda, Order Cyclophyllidea, Family Taeniidae (Kassai, 1999; Roberts, Janovy & Schmidt, 2009a).

The taxonomy within the genus *Echinococcus* is subject of a great debate. After some initial classifications, taxonomic revision recognized four species – *E. granulosus*, *E. multilocularis*, *E. vogeli* and *E. oligarthrus* (Thompson & McManus, 2001; Thompson, 2008). However, some authors were concerned with the oversimplified approach to *E. granulosus* as one single species, since there were phenotypic variations between isolates from distinct regions (Thompson & McManus, 2002). New classifications have been proposed with the outcome of molecular techniques. Genetic analysis, especially of mitochondrial DNA, confirmed a great variety within *E. granulosus*, which reflected not only different phenotypic characteristics, but also distinct transmission patterns, host specificity, pathogenicity and even sensibility to chemical control. Therefore, *E. granulosus* was divided in several strains/genotypes, named after the main intermediate host. Nowadays, according to some authors, further genetic analysis revealed sufficient variability to enable the creation of distinct species instead of strains (Thompson, 2008; Tappe, Kern, Frosch & Kern, 2010). The proposed taxonomy, according to the latest data, is described in Table 1.

Further attention should be paid to the ongoing debate regarding the creation of an additional species, *E. intermedius*, which would include genotypes G6 and G7 (Thompson, 2008; Nakao et al., 2010).

Recently, two species have been discovered. *E. shiquicus* was described in the Tibetan fox and the Plateau pika from Tibet, China (Xiao et al., 2005). *E. felidis* was isolated from African lions in Uganda by Huttner et al. (2008) and this was actually a rediscovery since this species had already been described in 1937 by Ortlepp, but was lately included into *E. granulosus* sensu lato.

E. granulosus sensu lato is the only species documented in Portugal (Eckert & Deplazes, 2004; Beato, 2008). Therefore, from hereafter this cluster of species will be discussed with greater detail and will be mentioned simply as *E. granulosus*. When referring to genotypes G1, G2 and G3, *E. granulosus* sensu stricto will be used. Additional information concerning the other species shall be presented whenever scientific relevant for this work.

Table 1 – Taxonomy of genus *Echinococcus* and main characteristics of the different species.

Species [a]	Genotype classification [a]	Definitive hosts [b]	Intermediate hosts [b]	Geographical distribution [b]
E. granulosus sensu stricto *	G1 – sheep strain	Dog, fox, dingo, jackal, hyena	Sheep, cattle, pig, camel, goat, buffalo, macropods, wild boar, cat	Worldwide
	G2 – Tasmanian sheep strain	Dog, fox	Sheep, buffalo, cattle, camel	Tasmania, Argentina, Italy, Algeria
	G3 – Oriental buffalo strain	Dog, fox	Buffalo, camel, cattle	Argentina, Brazil, Portugal, Italy, Greece, Morocco, Turkey, Iran, India,
E. equinus (Williams and Sweatman, 1963)	G4 – horse strain	Dog	Horse, donkey	Europe
E. ortleppi * (López-Neyra and Soler Planas, 1943)	G5 – cattle strain	Dog	Cattle, sheep, goat, buffalo, pig	Europe, Russia, South Africa, India, Nepal, Sri Lanka, Central and South America
E. canadensis * (Webster and Cameron, 1961)	G6 – camel strain	Dog	Camel, sheep, cattle, goat, pig	Africa, Middle East, Iran, China, Nepal, Argentina, Peru
	G7 – pig strain	Dog	Pig, wild boar, goat	Spain, Portugal, Austria, Eastern and South-eastern Europe, Turkey, Argentina, Peru
	G8 – cervid strain	Wolf, dog	Moose, reindeer	North America, Estonia
	G9 [1]	?	?	Poland
	G10 [2]	Wolf, dog, fox?	Moose, reindeer	North America, Finland, Sweden, Estonia
E. felidis (Ortlepp, 1937)	-	Lion, hyena?	Warthog	Uganda
*E. multilocularis** (Leuckart, 1863)	-	Red fox, Arctic fox, dog, cat, wolf, raccoon dog, coyote	Rodents, domestic and wild pig, dog, monkey, horse	North America, North and Central Eurasia
E. oligarthrus [3] (Diesing, 1863)	-	Wild felids	Rodents	Central and South America
E. shiquicus (Xiao et al., 2005)	-	Tibetan fox	Plateau pika	Tibetan Plateau (China)
*E. vogeli** (Rausch and Bernstein, 1972)	-	Bush dog, dog	Rodents	Central and South America

* Humans as intermediate hosts

[1] Genotype only found in humans (Scott, Stafaniak, Pawlowski & McManus, 1997)

[2] Zoonotic potential still unclear (Romig, Dinkel & Mackenstedt, 2006)

[3] Only three confirmed cases in humans (D'Alessandro & Rausch, 2008)

[a] adapted from Thompson (2009), Nakao et al. (2010), Tappe et al. (2010) and Knapp et al. (2011).

[b] data collected from Scott et al. (1997); Lavikainen, Lehtinen, Meri, Hirvelä-Koski & Meri (2003); McManus & Thompson (2003); Dinkel et al. (2004); Eckert & Deplazes (2004); Mwambete, Ponce-Gordo & Cuesta-Bandera (2004); Xiao et al. (2005); Lavikainen et al. (2006); Romig et al. (2006); Beato (2008); D'Alessandro & Rausch (2008); Huttner et al. (2008); Hüttner, Siefert, Mackenstedt & Romig (2009); Omer et al. (2010); Kamal, Romig, Kern & Rihab (2011); Konyaev et al. (2011); Rojo-Vazquez et al. (2011); Sharifiyazdi, Oryan, Ahmadnia & Valinezhad (2011).

2.1.2. Morphology

The adult stage of *Echinococcus granulosus* sensu lato is a small tapeworm whose length ranges from 2 to 7 mm. Body is composed by a scolex in the anterior end, followed by a neck and the strobila (see Figure 1-a). The scolex contains two rows of hooks (rostellum) and four muscular suckers (acetabula) that help parasite fixation to the intestine wall. The strobila consists of three to six proglottids. Proglottids mature progressively towards the posterior end and the last one is usually a gravid proglottid carrying eggs in uterus (Thompson & McManus, 2001; Eckert & Deplazes, 2004).

Echinococcus species lack digestive and respiratory systems, so the metabolic exchanges take place through the tegument. This structure covers the body of the parasite and also has a protective role against host enzymes and immune responses (McManus, 2009).

Echinococcus sp. eggs are morphologically indistinguishable from other taeniids eggs. They are ovoid, with approximately 30-36 µm in diameter, including inside the first larval stage (oncosphere or hexacant embryo harboring three pairs of hooks) (Roberts et al., 2009a). A layered wall surrounds the egg (see Figure 1-b). It consists of a thin outer capsule that is rapidly lost when eggs are released in the environment, a thick embryophore with a striated appearance and finally the oncosphere membrane which cannot be detected by light microscopy (Thienpont, Rochette & Vanparijs, 1986; Roberts et al., 2009a).

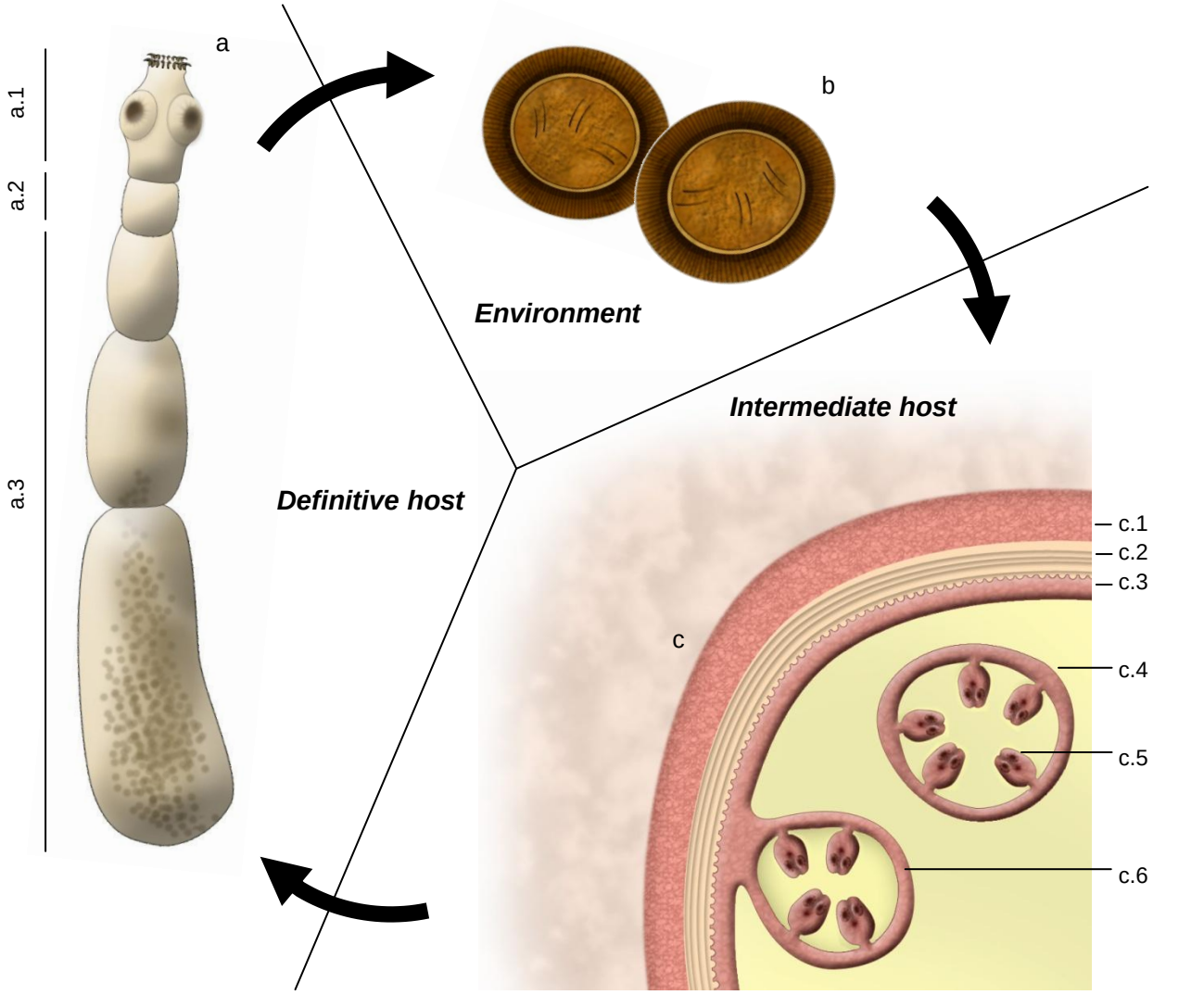
The eggs eventually develop into the next stage, the metacestode or hydatid cyst (see Figure 1-c). It is a fluid-filled bladder surrounded by an inner cellular layer (germinal layer) from where brood capsules arise through inward budding. In the inner surface of these brood capsules, the protoscoleces develop by asexual reproduction reaching numbers as high as hundreds of thousands in one single hydatid cyst (Thompson & McManus, 2001; Diaz et al., 2011a). Protoscoleces are only present in fertile cysts and each has the potential to evolve into a sexual mature adult parasite. They are invaginated immature forms of the adult worm and already present hooks and four suckers. Released protoscoleces and ruptured brood capsules precipitate in the hydatid fluid creating hydatid sand (David de Morais, 1998).

The structure of metacestodes is a key element to distinguish between different species of *Echinococcus*. In *E. granulosus*, the hydatid cyst is a single chamber which grows concentrically. Although some incomplete septa may develop, separating the primary cyst from secondary chambers, the metacestode is unilocular and asexual reproduction occurs internally (Thompson & McManus, 2001). If brood capsules rupture, daughter cysts develop, structurally similar to the primary cyst (Siracusano, Delunardo, Teggi & Ortona, 2012).

The outer surface of the germinal layer is covered with microtriches which are microvillus that increase the contact area, enhancing the nutrient uptake and other metabolic exchanges between the parasite and the intermediate host. This layer is also responsible for the synthesis of an external, acellular layer that separates the hydatid cyst from the host tissue. This laminated layer

has about 3 mm thickness in *E. granulosus* and protects metacestode against host immune system (Diaz et al., 2011a). Usually, as a result of this immune response, an adventitial layer is produced by the host surrounding the cyst (see Figure 1-c).

Figure 1 – Morphology of different stages of *Echinococcus granulosus.* (Original illustrations)



(a – adult stage; a.1 – scolex; a.2 – neck; a.3 – strobila; b – eggs in feces; c – metacestode; c.1 – adventitial layer; c.2. – laminated layer; c.3 – germinal layer; c.4 – daughter cyst; c.5 – protoscolex; c.6 – brood capsule)

2.1.3. Life Cycle

The life cycle includes a definitive mammalian host, usually a canid, carrying the adult egg-shedding stage, with low pathogenicity. The intermediate mammalian host, generally an ungulate, is infected by the eggs that develop into a larval stage (Eckert & Deplazes, 2004) (see Table 1).

Transmission to the definitive host occurs after ingestion of organs infected with the metacestode. Pepsin action in stomach, as well as increased temperature and bile secretions in duodenum,

stimulate evagination of protoscoleces. The adult parasite develops, reaching maturity in four to six weeks in the anterior quarter of the small intestine, where it actively migrates to the crypts of Lieberkühn and attaches to the intestinal mucosa (Eckert et al., 2001a). Thousands of adult worms may be found in only one dog (David de Morais, 1998).

Gravid proglottids detach from strobila and are released with feces, contaminating the environment. Rupture of proglotiids can occur still in the intestine or eggs may be actively excreted out of the proglottids. Each proglotiid may contain 200-800 eggs (David de Morais, 1998). Prepatent period in dogs is about 45 days (European Scientific Counsel Companion Animal Parasites [ESCCAP], 2010).

After being ingested by a suitable intermediate host, eggs suffer the action of gastrointestinal enzymes. The oncosphere then releases itself from the embryophore before crossing the intestinal wall. This step is triggered by bile action, hooks movement and own lytic secretions (Eckert et al., 2001a, Siracusano et al., 2012). Through blood or lymph vessels, the oncosphere reaches internal organs. Lungs and liver are the most common location of hydatid cysts (Diaz et al., 2011a), although there is a broad spectrum of other possibilities, including spleen, kidneys, brain, muscles and heart (Eckert et al., 2001a; Infante Gil, 2005). Secondary Echinococcosis occurs when new cysts develop in new organs after rupture of an initial one (Eckert et al., 2001a).

If ingested by unsuitable intermediate hosts, some metacestodes lack production of protoscoleces, thus being called sterile/infertile metacestodes, in contrast with fertile ones. This intermediate hosts are considered aberrant or accidental hosts. They may be infected by the metacestodes, however these stages do not become fertile or the hosts do not interact with other animals from the life cycle. In both the former and the latter situation, there is no further transmission of the parasite and the cycle is not completed. Human beings are considered aberrant hosts (Eckert et al., 2001a).

2.1.4. Pathology and Immunity

Definitive hosts are infected with the adult stage of *Echinococcus* spp. This is called the intestinal form of Echinococcosis. Some histological changes may occur in response to parasite attachment (e.g. thickening of intestinal mucosa due to cellular infiltration or higher mucus production) but generally infection is asymptomatic in definitive hosts, although some degree of pruritus may develop if gravid proglotiids penetrate the anal glands (Eckert et al., 2001a).

Excretory/secretory (E/S) products are released from the parasite which stimulates an immune response by the definitive host. In experimentally infected dogs (n=3), Jenkins & Rickard (1986) could detect the presence of antibodies against protoscolex antigens at day 5 post-infection (pi) and against E/S scolex antigens at 10 days pi. Moreno et al. (2004) also detected a consistent increase in IgG against protoscolex antigens at least 14 days after experimental infection of six dogs. IgA and IgE were also detected but the results varied greatly among dogs. Although an immune response is mounted against parasites, studies failed to correlate IgG titers with worm

burden (Gasser, Lightowlers, Obendorf, Jenkins & Rickard, 1988; Moreno et al., 2004). Peyer patches are involved in immunity against infection, producing specific IgA and IgG (Deplazes, Thompson, Constantine & Penhale, 1994).

Overall, evidences exist of both humoral and cellular immune responses against *E. granulosus* infection in canid hosts. As reviewed by Torgerson (2006), data suggest some degree of protection against reinfection. Recently, Rossi et al. (2012) demonstrated that after a primary infection, dogs developed a markedly humoral immune response. In further trials, parasite burden was lower and the immune response was both cellular and humoral mediated.

Cystic Echinococcosis is the form of Echinococcosis in intermediate and aberrant hosts, caused by *E. granulosus*. Growth of the metacestode is slow and several years are required to develop clinical signs (10-12 months in pigs; 2-4 years in sheep) (Eckert et al., 2001a). Since the majority of farm animals are slaughtered in earlier ages, meat inspection in the abattoirs reveals a high percentage of subclinical infections. Clinical signs are rare and strongly depend on location, dimension and number of cysts and their relation with adjacent structures. In ruminants, the majority of cysts are found in lungs and liver (Eckert et al., 2001a; Correia, Beato, Parreira & Grácio, 2010). In Portugal, sheep harbor a higher percentage of cysts in liver, whereas in cattle the majority is in lungs (Correia et al., 2010). In pigs and horses, liver is the most commonly affected organ. Changes in liver function is the most common biochemical finding in these animals (Eckert et al., 2001a). Clinical signs have been found in horses with heavy infections (Eckert & Deplazes, 2004).

After infection of intermediate hosts, egg and oncosphere antigens are recognized and both humoral and T-cell mediated responses are mounted (Siracusano et al., 2012) which allow some control of the parasite as well as immunity to reinfection (Zhang, Ross & McManus, 2008). In vitro experiments demonstrated the antibody-mediated lytic activity of complement in sheep serum against oncospheres. Lower concentrations of serum from immune sheep were needed to destroy the oncospheres (Heath, Holcman & Shaw, 1994). Although cellular immunity is also mentioned, the mechanism is not yet fully understood (Zhang et al., 2008). As reviewed by Eckert & Deplazes (2004), the majority of experimental studies regarding immunity in intermediate hosts use parasitic burdens higher than those that would occur naturally. Hence, although some degree of immunity was demonstrated experimentally, the same should not happen in natural conditions, since high percentage of older animals is infected.

The laminated layer of the hydatid cyst induces a regulatory response, which attenuates host's innate immune system. Ergo, involving the metacestode, host tissues produce an advential layer made from collagen fibers and few inflammatory cells, making a chronic evolution of the disease possible (Diaz et al., 2011b).

Fertility of cysts depends on the infective strain and species of intermediate host. In Portugal, in infections by *E. granulosus* sensu stricto, higher percentage of fertile cysts is found in sheep while

in cattle the majority are sterile (David de Morais, 1998; Beato, 2008; Correia et al., 2010). Paredes et al. (2011) found high concentration of bovine IgGs in the germinal layer of infertile cysts. According to the authors, fertility of cysts may be related in some degree with host's immune response. Biding of antibodies to the germinal layer would prevent cellular division or differentiation and development of brood capsules, thus being responsible for the cyst infertility.

2.1.5. Diagnosis

There is a wide range of diagnostic tools available for *Echinococcus granulosus*. The choice for one method or the other depends on (i) the purpose of the study; (ii) the sample origin (definitive/intermediate host; animal/human; domestic animal/wildlife); (iii) whether it is *in vitam* or *post mortem* diagnosis; (iv) the equipment and financial resources available.

Regardless of the chosen technique, for safety reasons, infective materials (intestines or fecal samples) should be previously frozen at -70º C for at least 4 days or at -80º C for 2 days (Eckert, Gottstein, Heath & Liu, 2001). Heating to 70 ºC for 12 hours is also a viable option. Personal protective equipment and laboratory decontamination must not be forgotten.

2.1.5.1. Diagnosis in Definitive hosts

Echinococcus spp. eggs can be found in fecal samples in both *in vitam* or *post mortem* diagnosis. A concentration method with a saturated solution of zinc sulphate or sucrose can be used followed by microscopic examination. Mathis, Deplazes & Eckert (1996) developed a flotation and sieving method that allows higher sensitivity in recovering eggs from feces. As an example, a study in Lithuania with 240 dogs found prevalences of 14.2% with this technique against only 5.0% using a modified McMaster method (Bružinskaitė, Šarkūnas, Torgerson, Mathis & Deplazes, 2009).

Despite being quick, cheap and ideal for routine diagnosis, this method alone lacks sensibility as it can only detect patent infections and false negative results can appear due to low worm burden, irregular shedding of eggs or intact gravid proglotiids released in feces. Taking samples in different days, as well as analyzing higher amounts of feces may increase sensitivity (Torres, Pérez, Segovia & Miquel, 2001; Torgerson & Deplazes, 2009). In rare cases, proglotiids can be found in feces which may allow identification (Eckert et al., 2001a).

Purgation with arecoline hydrobromide was a common technique in the past (Kamiya, 2008; Deplazes, Knapen, Schweiger & Overgaauw, 2011). The drug, administered orally, acted as a parasympathomimetic agent that paralyzed the worms and stimulated intestinal smooth muscle motility. This eventually led to the elimination of the adult parasite in feces, which could later be identified (Eckert et al., 2001a). It had nearly 100% specificity but sensitivity could be lower than 65%. It was also a time-consuming method, with potential safety risks to animals, but also to humans since animals would be shedding adult worms and eggs (reviewed by Torgerson & Deplazes, 2009).

The gold standard technique for diagnosing both *E. granulosus* and *E. multilocularis* in carnivores is the Sedimentation and Counting Technique (SCT). Briefly, small intestine should be divided into five sections and incubated in physiological saline solution. After removal of intestine, the remaining liquid is left for successive sedimentation and decantation steps. Worms may be visualized and quantified using a stereomicroscope (Kamiya, 2008). Although this method does not need too much advanced equipment, is considered cost-effective, it is time-consuming and not suitable for a high number of samples. Concerning this, Umhang, Woronoff-Rhen, Combes & Boué (2011) recently described an adaption from this technique, the Segmental Sedimentation and Counting Technique (SSCT), where only two segments from the small intestine were screened. They found a sensitivity of 98.3% against 100% of SCT for *E. multilocularis*, just by screening the fourth segment combined with the first or second ones. Since *E. granulosus* has different location patterns in the gut, the screened segments have to be accessed for this species.

The Intestinal Scraping Technique (IST) is another necropsy-based method, in which scrapings from the intestinal mucosa in different locations are examined microscopically (Kamiya, 2008). Although it takes less time, a sensitivity of 78% was found for *E. multilocularis*, comparing with the SCT (Hofer et al., 2000). In the "Shaking in a Vessel" Technique (SVT), described by Duscher, Prosl & Joachim (2005), the small intestine is opened longitudinally and its content placed in a vessel filled with water, followed by shaking and decantation steps until the content is clear. The lid contains a mesh and the sediment here attached is screened with a stereomicroscope in several Petri dishes. Duscher et al. (2005) found a sensitivity of 96.2% for this method when searching for *E. multilocularis* in foxes.

Worms of *E. granulosus* can be easily morphologically differentiated from *E. multilocularis*. However if immature stages or just scoleces are found, staining may be necessary for differential diagnosis. Rostellar hooks are often lost during freezing so they cannot be used to differentiate species (Eckert et al., 2001a).

More advanced techniques have been developed for diagnosis of *Echinococcus* species. Diverse coproantigen-ELISAs are available for detection of antigens in fecal samples from canids (Deplazes et al., 1992; Allan et al., 1992; Deplazes, Alther, Tanner, Thompson, Eckert, 1999). Some are specific for one species, whereas others detect both *E. granulosus* and *E. multilocularis.* They can be used both in live or dead hosts and allow the screening of high number of samples at the same time. Furthermore, prepatent infections can be detected. Deplazes et al. (1992) were able to detect positive samples 10-20 days pi. Nevertheless, false negative results may occur in areas with low prevalence, and also due to cross-reactivity with *Taenia* antigens (Christofi et al., 2002; Torgerson & Deplazes, 2009). For example, validation of a commercial ELISA kit revealed specificities of 98% in a group of helminth-free dogs but this value decreased to 80% in a group of

the same area, infected with *Taenia* species (Christofi et al., 2002). Another limitation is the impossibility to distinguish between current or past infections (Kamiya, 2008).

Serology methods to detect antibodies in serum from infected dogs are available, capable of giving positive results in just less than two weeks after infection (Moreno et al., 2004). However, as previously mentioned, no correlation was found between antibody titers and worm burden. Moreover, for epidemiological studies, especially in wild populations, this method presents serious limitations.

PCR-based techniques are highly specific and allow species and sometimes strain identification. They can be used in eggs recovered from feces. Since they are time-consuming and expensive, usefulness in large scale studies is questionable. When analysis is made in material collected from feces, this method will suffer from the same low sensitivity and limitations as the ones mentioned earlier for coprological techniques, namely detection of patent infections only. Furthermore, fecal inhibitors may interfere with PCR results (Torgerson & Deplazes, 2009). One way of overcoming this is if eggs are isolated through a sieving-flotation technique (Mathis et al., 1996) which, combined with PCR, presents sensitivity as high as 78% and specificity of 93% (Ziadinov et al., 2008). Several PCR-based techniques are now available for diagnosis in fecal samples (copro-PCR). Examples include methods for G1 (sheep) strain (Abbasi et al., 2003; Štefanić et al., 2004), multiplex-PCR to differentiate between *E. granulosus, E. multilocularis* and *Taenia* sp. with a specificity of 100% (Trachsel, Deplazes & Mathis, 2007), and a PCR/dot blot assay developed for different *Taenia* and *Echinococcus* species, including *E. granulosus* genotype 1 (Armua-Fernandez et al., 2011). Sequencing of amplified materials in multiplex-PCR can be used in species and strain differentiation for *E. granulosus*.

2.1.5.2. Diagnosis in Intermediate hosts

Since majority of infections are subclinical, *post-mortem* diagnosis is the best diagnostic tool for intermediate hosts. This is mainly done in slaughterhouses by visual inspection of organs, especially liver and lungs. However, prevalences collected from abattoirs may be underestimated since the majority of the individuals, namely sheep, are slaughtered in very young ages when hydatid cysts are still too small to be detected (Eckert & Deplazes, 2004; Kamiya, 2008).

Some studies carried out ultrasound screenings in lungs and liver from intermediate hosts. In Kenya, this method revealed a sensitivity of just 54.36% and a specificity of 97.64% against *post-mortem* examination of 300 small ruminants (Sage et al., 1998).

Serology to detect antigens collected from hydatid fluid may be useful, especially in younger animals where cysts could be overlooked in *post-mortem* diagnosis. (Eckert et al., 2001a) However, since this methods lacks sensitivity, they are usually more suitable to identify the parasite in the overall herd, rather than for an individual diagnosis (Eckert & Deplazes, 2004).

Molecular techniques may be used for species and strain identification. A random amplified polymorphic DNA-PCR (RAPD-PCR) was developed to distinguish between species of *Echinococcus* and between strains of *E. granulosus* (Scott & McManus, 1994). Also, restriction fragment length polymorphism associated with PCR (RFLP-PCR) has been used successfully in distinguishing *E. granulosus* strains in cysts isolated from intermediate hosts (Beato, 2008).

2.1.6. Epidemiology

E. granulosus is the most widespread species in the genus, with a variety of life cycle patterns that translates geographic, epidemiologic and cultural diversity. *Echinococcus* sp. evolved from an ancient population located in Northern Europe, where a predator-prey cycle between wolf and cervids was established (David de Morais, 1998). The growth of human civilization, colonization of different regions and domestication of ungulates and canids eventually led to the creation of multiple other life cycles. Presence of this parasite can now be found worldwide, except in some remote places as Greenland and in confined areas, mainly islands, where intensive control programs were mounted (e.g. Iceland, New Zealand, Tasmania) (Eckert & Deplazes, 2004).

The Mediterranean region is considered endemic for *E. granulosus*. Different factors are responsible for maintaining infection: (i) high tradition in extensive farming of sheep; (ii) close contact between human, dog and sheep in rural areas; (iii) abundance of stray dogs; (iv) home slaughter and feeding dogs with potentially infected organs; (v) lack of hygiene education and control programs (David de Morais, 1998; Eckert & Deplazes, 2004).

In the last decades, few studies accessed the prevalence and the transmission patterns of Echinococcosis in Portugal. In this section, a comprehensive review is presented about the epidemiological studies available. Since data are scarce, information from Spain is also presented whenever comparable conditions may exist.

2.1.6.1. Definitive Hosts

According to the current knowledge, domestic dog is the main definitive host for *E. granulosus* in Portugal, while sheep is the main intermediate host (David de Morais, 1998; Beato, 2008).

The prevalence of infection in dogs from Portugal, mentioned in the latest edition of the Manual on Echinococcosis in Humans and Animals by the WHO, refers to a study published by in 1970 (Eckert et al., 2001c). On that time, a prevalence of 10.44% was found in necropsies of 450 dogs from several locations in Portugal. Santarém revealed the highest prevalence (29.4% in just 17 animals). No significant differences were found between prevalences from the main geographic regions (North, Centre, Lisbon Metropolitan Area and South) (reviewed by David de Morais, 1998). David de Morais (1998) compiled almost all the studies done in Portugal about canine Echinococcis since 1938 and less than 10 are cited. Although Alentejo is the region were most human cases are detected (David de Morais, 2010), prevalences found in dogs were lower than

5%. In this study, compiled by David de Morais (1998), however, authors admit not using sheep dogs, and since sample included animals from both rural and urban areas, real prevalence in key endemic areas may have been underestimated. Still, in another survey in Alentejo, only 1 case was found in 153 necropsied dogs (David de Morais, 1998). Further studies are needed, especially in regions where most human cases are reported.

Stray, sheep and hunting dogs are important in epidemiology, since they cover large areas and have contact with possibly infected intermediate hosts. In Spain, Garrudo Arias et al. (1999) found a prevalence of 2.12% of *Echinococcus* spp. in 754 stray dogs from the province of Extremadura, through necropsy. During a control program in La Rioja, Spain, Jiménez et al. (2002) considered stray dogs the most important reservoirs for *Echinococcosus* sp.. Likewise, in the province of Alava, Northern Spain, a higher prevalence of 14% (n = 726) was found in sheep dogs through coproantigen ELISA (Benito, Carmena, Joseph, Martínez & Guisantes, 2006) while in a previous study using necropsy just 0.5% of 1040 dogs from urban environments were positive (Benito et al., 2003, cited by Benito et al., 2006).

Previous studies failed in detecting infected foxes in Portugal or Spain (Carvalho-Varela & Marcos, 1993; Carvalho-Varela, Marcos & Grácio-Moura, 1993; Rodríguez & Carbonell, 1998; Criado-Fornelio et al., 2000; Eira, Vingada, Torres & Miquel, 2006; Martínez-Carrasco et al., 2007). Foxes do not seem to play an important role as definitive hosts for *E. granulosus*, except in specific ecosystems, e.g. Australia (Thompson & McManus, 2001).

E. granulosus sensu stricto (G1 strain) was already diagnosed through necropsy in the Iberian Wolf (four positive cases in a group of 27 cadavers; three of them from provinces near the Spain-Portugal northern border) (Sobrino et al., 2006). No records exist about the Portuguese populations of the Iberian wolf.

2.1.6.2. Intermediate Hosts

Sheep, goats, cattle and pigs are known intermediate hosts for *E. granulosus* in Portugal. David de Morais (1998) compiled national statistical data about meat rejection in abattoirs during a period of over 20 years, between 1944 and 1968. The majority of infected sheep originated from the southern provinces of Portugal, with a prevalence of 1.7% in more than 4 million animals. Still, this number is thought to be an underestimation, since during those years several faults were pointed to meat inspection conditions and accuracy. The most recent publications do not provide clear statistical information regarding the infection of sheep. In the Zoonoses Report of the European Food Safety Authority (EFSA) from 2007, a prevalence of 9.4% is referred in a sample of just 32 animals. Prevalences of sheep infection are probably underestimated, since the majority is slaughtered with less than one year old, which makes diagnosis very difficult (Rojo-Vazquez et al., 2011).

Regardless the prevalences, sheep are considered important intermediate hosts for multiple reasons: (i) they are the most common ungulate in Portugal; (ii) extensive farming is common, with close contact between sheep and dog; (iii) this species feeds close to the soil, making infection easier; (iv) fertility of cysts seems to be higher in sheep than in cow (David de Morais, 1998; Beato, 2010; Correia et al., 2010).

During 1944-1968, pigs were the species most affected by hydatidosis with an overall prevalence of 4.6% (more than twice the value found for sheep). The majority of reported cases were from the Northern and Center regions, where prevalences of rejection were 8.9% and 6.3%, respectively (in a population of 11 487 595 animals) (David de Morais, 1998). In the following years, drastic reduction of pig population occurred in result of African Swine Fever foci in Portugal. Also intensive production of pigs largely replaced the previous systems. Overall, it resulted in a reduction of cases diagnosed, at least until 1983 (David de Morais, 1998). Recent studies in the Northern region, confirm that hydatidosis is still present among pigs in the North of Portugal. Prevalence of 8.1% was found in the District of Bragança, in 333 pigs from Bísaro breed (Freire et al., 2005). Also in Cantanhede preliminary data of a monitoring program found 5 animals harboring fertile cysts in a sample of 66 animals (Conceição, 2005). Nowadays, increasing popularity of extensive farming of pigs may give rise to new concerns and therefore this situation should be carefully monitored (Hernández Mira et al., 2008).

Bovine cases of hydatidosis were also mostly detected in the Northern region, with an overall prevalence of 4.3% in the Portuguese territory during 1944-1968 that decreased during 1968-1983 (David de Morais, 1998). The report from EFSA (2009) reveals a prevalence of less than 0.1% in a total of 174 834 animals, which is not a representative number of the total cattle slaughtered in Portugal. However, since majority of cysts found in cattle are sterile, this species does not seem to play an important role in the dissemination of the parasite (Beato, 2010).

According to the EFSA report published in 2011, 159 infected deer and 264 wild boars were reported in 2009 in Spain but these are only results from random inspections and no spatial distribution of infected cases is presented (Rojo-Vazquez et al., 2011). A recent work found a wild boar highly infected with fertile cysts from *E. granulosus* G1, the most common genotype infecting humans (Martín-Hernando, González, Ruiz-Fons, Garate & Gortazar, 2008). Since population of wild boar in the Iberian Peninsula is increasing, several recent works emphasize the importance that this species may play because they are important preys for Iberian wolf and also are available to dogs during hunting season (Martín-Hernando et al., 2008; Rojo-Vazquez et al., 2011).

2.1.6.3. Genetic Characterization

The few genetic characterization studies in Portugal revealed the presence of *E. granulosus* sensu stricto G1 and G3 (Beato, 2008) in cysts collected from sheep and cattle and also G7 (pig strain) in pigs (Castro et al., 2005). In Central Spain, Mwambete, Ponce-Gordo & Cuesta-Bandera (2004)

isolated G1 (sheep strain) hydatid cysts from sheep, cattle, humans, goats, pigs and wild boars, G4 (horse strain) from horses, and G7 from goats, pigs and wildboars. This study confirmed that G1 is the most important cause of hydatidosis in humans in this region and that the fertility of cysts in sheep was higher than in the other species, similarly to what happens in Portugal (Correia et al., 2010). Still, human infection with G7 was already recorded in several European countries (Rojo-Vazquez et al., 2011) and in a study in Austria the majority of Cystic Echinococcosis patients were infected with G7 genotype (Schneider, Gollackner, Schindl, Tucek & Auer, 2010).

In the Southern Portugal, the most important strain is the sheep strain, with higher number of human cases, whereas in the North, the pig strain seems more prevalent, with apparently low infectivity to humans (David de Morais, 2010).

2.1.6.4. Environmental Contamination

According to Gemmell, Roberts, Beard & Lawson (2001), biotic potential is "[…] the potential number of viable cysts which can be established in an intermediate host by an individual definitive host per day." For *Echinococcus* sp. this is assumed to be relatively low compared to other taeniid species. Still, egg contamination in the environment is possible. Eggs are already infective when excreted in feces (Deplazes et al., 2011) and more than 8000 eggs can be shed, per day, in an average infected dog (Gemmell et al., 2001). Taeniid eggs are very resistant in the environment and they can still be infective until 1 year, sometimes longer, if kept in adequate temperatures (4 ºC to 15º C) and humidity levels (Gemmell et al., 2001; Thevenet et al., 2005). Blowflies can act as vectors, transporting the eggs through long distances (Deplazes et al., 2011).

Eggs are very sensitive to desiccation, being destroyed after 4 days at 25% humidity and in 1 day after 0%. Extreme temperatures can also destroy them (death occurs in 5 min at 60-80º C) (Eckert et al., 2001b).

Taeniid egg contamination in public places was revealed in several studies worldwide. Prevalences may be low, especially due to the fact that coprology has low sensitivity for *Echinococcus* sp., as previously mentioned. More details will be given in the next chapter (*Taenia* spp.) about fecal contamination with taeniid eggs.

2.1.6.5. Hydatidosis in Humans

E. granulosus is an important zoonotic agent. Human infection is associated with poor hygiene conditions. Theoretically, egg ingestion can occur after contact with dogs and their feces, contaminated water and food, but not much is known of which transmission pathway plays a more important role. Dog fur was found contaminated with eggs (Eckert & Deplazes, 2004) and in Portugal, similarly to other countries, possession of dogs was significantly higher in human patients with Cystic Echinococcosis (David de Morais, 2010). As reviewed by Deplazes et al. (2011), there

is a possibility of subcutaneous infection by taeniid eggs occurring through a solution of continuity in skin.

Human hydatidosis is usually asymptomatic in early stages. Clinical signs develop in a variable degree, depending on the same factors previously stated for intermediate hosts. More than half of primary human hydatidosis is located in liver (Eckert & Deplazes, 2004). David de Morais (2010) found that 85% of human cases in Portugal had hepatic cysts. Clinical signs are often liver-associated, e.g., cholestasis, hepatomegaly, portal hypertension and ascitis. Pulmonary and neurological signs have been described after cyst infection of respiratory system and brain, respectively. After rupture of cysts and release of hydatid fluid, strong anaphylactic reactions may occur (Thompson & McManus, 2001).
Diagnosis is usually made in older ages. David de Morais (2010) reviewed all the newly diagnosed human cases between 1979 and 2008 in Portugal, and approximately 70% were people older than 40 years old.

Recently, throughout the world several cases of emergence and reemergence of Echinococcosis are documented. In former Soviet Union countries, the decreased quality of meat inspection and veterinary activities may have resulted in increasing prevalences in humans and animals (Eckert & Deplazes, 2004). In Australia, recent prevalences found in dogs were unexpectedly high, and wildlife is supposed to play an important role in maintaining the infection pressure (Jenkins, Romig & Thompson, 2005). Also in China, high prevalences are found among humans (Eckert & Deplazes, 2004).
In 2009, 790 cases were notified in the European Union (EU), 11.3% less than in 2008. Seventy percent of these cases were from Bulgaria, Germany, Spain and Romania. Some countries have much higher incidences than Portugal, as will be presented further: incidence of 4.25/100 000 in Bulgaria (323 cases), 1.07/100 000 in Lithuania (36). Spain had 86 confirmed cases in 2009, with an incidence of 0.19/100 000 (EFSA, 2011). In Europe, however, some of the results shown include also *E. multilocularis*. As reviewed by Rojo-Vazquez et al. (2011), data from EFSA has several limitations, especially since notification does not always occur.
In the latest WHO/OIE Manual on Echinococcosis in Humans and Animals, the incidence of this disease in humans is Portugal is 2.2/100000 inhabitants but again this is an old reference, dating from 1944-1968. Recent data compiled by David de Morais (2010) and available at Direcção Geral de Saúde (DGS) (2010) were analyzed. During 1987-2007, 467 hydatid patients were officially notified, 76.9% from Alentejo, in the South of Portugal. David de Morais (2010) showed an increasing number of diagnosed cases in 1980s and 1990s. The highest values were found in the late 1980s / early 1990s and then a sudden decrease in the last decade with 112 cases diagnosed

between 2000 and 2008 (DGS, 2005; DGS, 2010). When confirmed, *E. granulosus* was the only species found (EFSA, 2009; EFSA, 2010).

As reviewed by David de Morais (2010), data shows that both in the 1980s (incidence 2.2 cases/100 000 inhabitants/year) and during 2003-2005 (0.1 cases/100 000 inhabitants/year) prevalences were too low to consider Portugal a hiperendemic country in its whole. According to the EFSA report published in 2011, the incidence of Echinococcosis confirmed cases in Portugal was 0.04/100 000 inhab. in 2009. These results are similar to the ones found in the previous four years: 9 cases (2005), 9 (2006), 10 (2007), 4 (2008). Although there seems to be a reduction in notified cases, is important to refer that many cases may not be notified and some, because they are asymptomatic, are never diagnosed.

The district of Évora, in Alentejo is the one with the highest rates of human hydatidosis, with an average incidence of 3.2/100 000 inhabitants/year between 2004 and 2008. The county of Alandroal has the highest in all country – 18.2 incidence /100 000 inhab./year (2004-2008) (David de Morais, 2010).

There are several environmental and socio-ecological factors that explain the higher prevalence of Human Hydatidosis in Alentejo. It is an area mainly inhabited by older people, with low educational levels, that strongly depend upon agriculture and livestock production and where hunting is an important economic activity (Hernández Mira et al., 2008). Sheep farming in extensive and semi-extensive systems is abundant and the contact between humans and dogs is high. Moreover, home slaughter still occurs and dogs are still fed with animal viscera.

2.1.7. Treatment and Control

Treatment of dogs should be made in areas of high prevalence at least each 6 weeks with praziquantel or epsiprantel (ESCCAP, 2010). For praziquantel, single treatment in the adequate dose of 5.0 mg/kg/bw (*per os*) or 5.7 mg/kg/bw (IM) revealed effective (Eckert et al., 2001a). Epsiprantel should be given at 5.5 mg/kg/bw (*per os*). Several experimental studies regarding immunity in dogs have been made but so far no vaccine is available (Kamiya, 2008)

Treatment of intermediate hosts is not common. Diagnosis is usually made *post-mortem* and when performed *in vitam*, it is not cost-effective (Kamiya, 2008). Treatment with mebendazole was studied in sheep and pigs, but long periods of daily treatment were needed (Eckert et al., 2001a). A recombinant vaccine (Eg95) was developed with almost 100% of protection achieved in sheep. It is also effective in goats and cattle. Two doses of the vaccine are injected subcutaneously in the neck with a one-month interval and immunity can last as long as 12 months (Heath, Jensen & Lightowlers, 2003). Vaccination, however, does not act upon already established cysts and so other control measures need to be taken in animals already infected. Passive immunity in colostrum was also achieved after vaccination (Eckert et al., 2001a).

As stated by Eckert & Deplazes (2004), baseline studies are important before implementing control programs, so that achievement of long-term results can be evaluated (e.g. prevalence and incidence in humans, dogs and livestock). Control measures are essential for managing dissemination. They should include public education and improvement of meat inspection and other veterinary-related activities, and focus should be made in individual protection of people working with infective material. Appropriate control of dog population is also important, namely correct deworming, dog registration and control of stray populations. Furthermore, attention should be paid to wildlife populations, since they can work as reservoirs.

Several control programs were held worldwide, e.g. New Zealand, Cyprus and South America (Gemmell et al., 2001b). Due to the epidemiological proximity, a successful control program held in La Rioja, Spain during 14 years is mentioned (Jiménez et al., 2002). Control measures targeted dogs, sheep and human populations. Dogs were treated regularly with praziquantel, covering all dog population but increasingly focusing on sheep dogs in later phases of the program. Some stray dogs were also killed to access the prevalence throughout the program. In local abattoirs, correct disposal of rejected organs was guaranteed. Furthermore, public health education was made on risk groups. After this program, human incidence was reduced from 19 to 4 newly diagnosed cases/ 100 000 inhabitants and prevalences in dogs and adult sheep decreased from 7.0 to 0.2% and 82.3 to 20.3%, respectively. A net benefit of more than 2 million USD was estimated after 14 years of this program.

Removing dead ungulates from fields is another important measure, especially since older animals harbor higher number of cysts, having a higher potential to spread the parasite (Torgerson, Ziadinov, Aknazarov, Nurgaziev & Deplazes, 2009).

Rough estimates of economic impact of Cystic Echinococcosis were determined by Budke, Deplazes & Torgerson (2006). Annual economic losses due to human cases were estimated over 300 million USD for Western Europe, USA, Canada, Australia and New Zealand and more than 700 million USD globally. Worldwide, livestock-associated economic losses accounted for more than 2 billion USD annually, with more than 100 billion just for liver condemnation alone. Although these are just estimates, still they translate high economic losses. In practice, effects on livestock also account for decreasing in milk production and animal body condition, which altogether has a huge impact, especially in poor communities and regions that strongly depend upon farming (Eckert & Deplazes). As stated by Budke et al. (2006) control measures are necessary and cost-effectiveness may support all the efforts.

After implementation of effective programs, permanent surveillance and other control measures must be maintained (Gemmell et al., 2001b). Furthermore, treatment of imported dogs should be mandatory; otherwise, infection levels will increase again.

Efforts in Portugal to control this disease may have begun in the late 1970s in the Southern region of the country (Borges Ferreira, 1979), where some campaigns against Echinococcosis began. These were based in public education and dog treatments. Dogs presented by their owners were treated with arecoline hydrobromide in the beginning and praziquantel in later years. Feces released from dogs were either taken for analysis or burnt. In 1991, after the end of these campaigns, the Fighting Group against Echinococcosis-Hydatidosis in the Counties of Elvas and Alandroal started its work. This is a group made by volunteers, mainly health care professionals that performed several screenings and sanitary education in Alentejo (Hernández Mira et al., 2008).

In 1996, a Control and Monitoring Program of Echinococcosis/Hydatidosis was approved and co-funded by the European Union in Portugal. Despite cessation of European funding, the program continued in Alentejo and later on in Algarve and Beira Interior. The activities include the notification of positive cases in meat inspection, followed by appropriate epidemiological inquiries and sanitary education, especially in schools. This disease is still part of the list of mandatory notifiable diseases in Portugal and OIE (Direção-Geral de Alimentação e Veterinária [DGAV], 2012). Also free deworming of dogs with praziquantel is performed during the annual Rabies vaccination campaigns in specific approved regions. Few results are available of this program, with no base-line studies done, since some years ago, which makes it difficult to understand the impact of these measures.

2.2. *Taenia* spp.

Taenia species are long hermaphrodite tapeworms, with relevance in Human and Veterinary Medicine. They are heteroxenous parasites and in their life cycle, similarly to *Echinococcus* sp., predation is the basic transmission mechanism between intermediate and definitive hosts.

2.2.1. Taxonomy

The genus *Taenia* belongs to the same taxonomic family as genus *Echinococcus*. Taxonomy is the same given previously, i.e., Phylum Platyhelmynthes, Class Cestoda, Subclass Eucestoda, Order Cyclophyllidea, Family Taeniidae (Kassai, 1999; Roberts et al., 2009a).

According to Loos-Frank (2000) and Hoberg (2006), there were 42 valid *Taenia* species, but recent studies created new taxa and clarified others (see Table 2). Still, genetic and morphological analysis is required to correctly validate and characterize all the species presented.

For instance, there is evidence that *T. mustelae* may represent a different genus (Lavikainen et al., 2008) and debate has arisen over the status of *T. krabbei*. Some authors defend it should stay as an individual species and others as a subspecies of *T. ovis*, *T. o. krabbei* (Hoberg, 2006; Lavikainen et al., 2008).

T. serialis has 2 subspecies: *T. s. serialis*, the cosmopolitan subspecies, and *T. s. brauni*, a subspecies from Africa (Loos-Frank, 2000). *T. polyacantha* is considered to also have 2 subspecies: *T. p. polyacantha* from the south of the tundra zone in Eurasia and *T. p. arctica* in the northern area (Loos-Frank, 2000; Lavikainen et al., 2008).

Table 2 – Species within the genus *Taenia*.

Species	Definitive Host	Intermediate Host	Geographic distribution
T. arctos (Haukisalmi et al., 2011)	Brown bear	Elk, Moose	Finland, Alaska (USA)
T. acinonyxi (Ortlepp, 1938) *	Leopard, cheetah	African ruminants, warthog	Sub-Saharan and sub-Sahelian regions
T. asiatica (Eom & Rim, 1993)	Human	Pig	Southeastern Asia
T. crassiceps (Zeder, 1800) *	Canids, felids	Rodents, lagomorphs, European mole	North America, Europe, former USSR
T. crocutae (Mettrick & Beverly-Burton, 1961)	Spotted and Brown Hyena	African ruminants, baboon	Africa
T. endothoracicus (Kirschenblatt, 1948)	Red fox	Rodents	Northern Africa, Asia, Russia
T. gonyamai (Ortlepp, 1938)	Cheetah, lion	African ruminants	Central and Eastern Africa
T. hyaenae (Baer, 1926)	Spotted and Brown Hyena, African wild dog	African ruminants, Zebu cattle, Dromedary	Africa
T. hydatigena (Pallas, 1766)	Canids and felids	Domestic and wild ungulates	Cosmopolitan
T. macrocystis (Diesing, 1850)	Wild felids	Lagomorphs	North America
T. madoquae (Pellegrini, 1950) Jones et al., 1988	Black-backed jackal	Dik-dik	Kenya

Table 2 – continuation.

Species	Definitive Host	Intermediate Host	Geographic distribution
T. martis (Zeder, 1803)	Mustelids	Rodents	Europe, North America
T. multiceps (Leske, 1780) *	Domestic and wild canids, felids	Domestic and wild ruminants and pigs, lagomorphs	Cosmopolitan
T. mustelae (Gmelin, 1790)	Mustelids	Rodents	Europe, North America
T. olnojinei (Dinnik and Sachs, 1969)	Spotted and Brown Hyena	Antelopes	Central Africa
T. omissa (Lühe, 1910)	Wild felids	Cervids	North and South America
T. ovis (Cobbold, 1869)	Canids	Ruminants	Cosmopolitan
T. parenchymatosa (Pushmenkov, 1945)	Arctic fox, dog	Reindeer, red deer	Russia
T. parva (Baer, 1926)	Wild felids, viverrids, herpestids	Rodents	Europe, Africa
T. pencei (Rausch, 2003)	Ringtail	Deer mouse	Oregon (USA)
T. pisiformis (Bloch, 1780)	Domestic and wild canids, felids	Rodents, lagomorphs	Cosmopolitan
T. polyacantha (Leuckart, 1856)	Arctic fox, red fox, wolf, dog, Iberian lynx	Rodents	Europe, North America
T. regis (Baer, 1923)	Lion, leopard	Wild ruminants	Africa
T. rileyi (Loewen, 1929)	Lynx, bobcat	Rodents	North America
T. saginata (Goeze, 1782)	Human	Cattle, wild ruminants	Cosmopolitan
T. selousi (Mettrick, 1962)	Wild cat	Rodents	Southern Africa
T. serialis (Gervais, 1847) *	Dog, wolf, jackal, red fox	Rodents, lagomorphs	Cosmopolitan
T. simbae (Dinnik and Sachs, 1972)	Lion	Antelope	Tanzania
T. solium (Linnaeus, 1758) *	Human	Pigs, primates, lagomorphs, rodents, camel, bear, canids	Cosmopolitan
T. taeniaeformis (Batsch, 1786) *	Domestic cat, wild cat, bobcat, red fox, viverrids,	Rodents, lagomorphs	North America, Europe, Egypt
T. talicei (Dollfus, 1960)	Dog?	Tuco-tuco	Argentina
T. taxidiensis (Skinker, 1935)	Badger, coyote	Ground squirrel	North America
T. twitchelli (Schwartz, 1927)	Wolverine	Porcupine	North America

* Zoonotic – human may serve as intermediate hosts (Loos-Frank, 2000; Hoberg, 2002)

Data adapted from Loos-Frank (2000), Jones & Pybus (2001), Rausch (2003), Hoberg (2006), Nakao et al. (2010), Rossin, Timi & Hoberg (2010) and Haukisalmi, Lavikainen, Laaksonen & Meri (2011). Ten species have been left out of the most recent phylogenetic analysis due to incomplete life cycle or relevant morphological data lacking for accurate comparisons (Hoberg, Alkire, de Queiroz & Jones, 2001; Lavikainen et al., 2008; Nakao et al., 2010). These were not included in this table.

Given the huge variety of species, an overall review will be made. Since *Taenia crassiceps, T. hydatigena, T. multiceps, T. pisiformis, T. polyacantha*, *T. serialis* and *T. taeniaeformis* have all been reported in the Iberian Peninsula in canids (Carvalho-Varela & Marcos, 1993; Carvalho-Varela et al., 1993; Rodríguez & Carbonell, 1998; Balmori, Rico, Naves & Llamazares, 2000; Criado-Fornelio et al., 2000; Torres et al., 2000; Segovia, Torres, Miquel, Llaneza & Feliu, 2001; Segovia, Guerrero, Torres, Miquel & Feliu, 2003; Eira et al., 2006; Martínez-Carrasco et al., 2007) these ones will be discussed in greater detail whenever relevant.

2.2.2. Morphology

Sexually mature *Taenia* spp. worms inhabit the intestine of mammals. The scolex contains four muscle suckers (acetabula) and a protrusible portion in its apex, the rostellum, sometimes armed with rings of hooks (Figure 2). These are important criteria for species identification. Number of hooks ranges from 24 to 76, distributed in one or two rings and some species, e.g. *T. saginata*, lack a rostellum (Loos-Frank, 2000; Murrell, 2005; Roberts et al., 2009a).

The strobila is flat and segmented, formed by the all proglottids. Lengths of an adult tapeworm can be as high as 12 m (Murrell, 2005). After strobilation, these segments mature and become longer than wider as they progress towards the posterior end, where both reproductive systems are fully developed. Data from reproductive systems is also another important criterion for species identification (Loos-Frank, 2000).

After fertilization, gravid proglotiids, containing the eggs, detach from the strobila and are released intact in feces (Roberts et al., 2009a). *Taenia* sp. eggs are morphological identical to the ones described previously for genus *Echinococcus* (see 2.1. *Echinococcus* spp.).

In intermediate hosts, the oncosphere matures into the metacestode. Morphology and location of metacestodes varies within the species (see Table 3 and Figure 2-c). The cysticercus is a fluid-filled bladder with approximately 10 mm in diameter, involved by a germinative membrane with an invaginated scolex (protoscolex). The coenurus is similar to cysticercus, but the germinative layer of a single bladder may develop multiple invaginated scolices. Strobilocercus is also similar to cysticercus but some degree of strobilation occurs (Loos-Frank, 2000; Roberts, Janovy & Schmidt, 2009b).

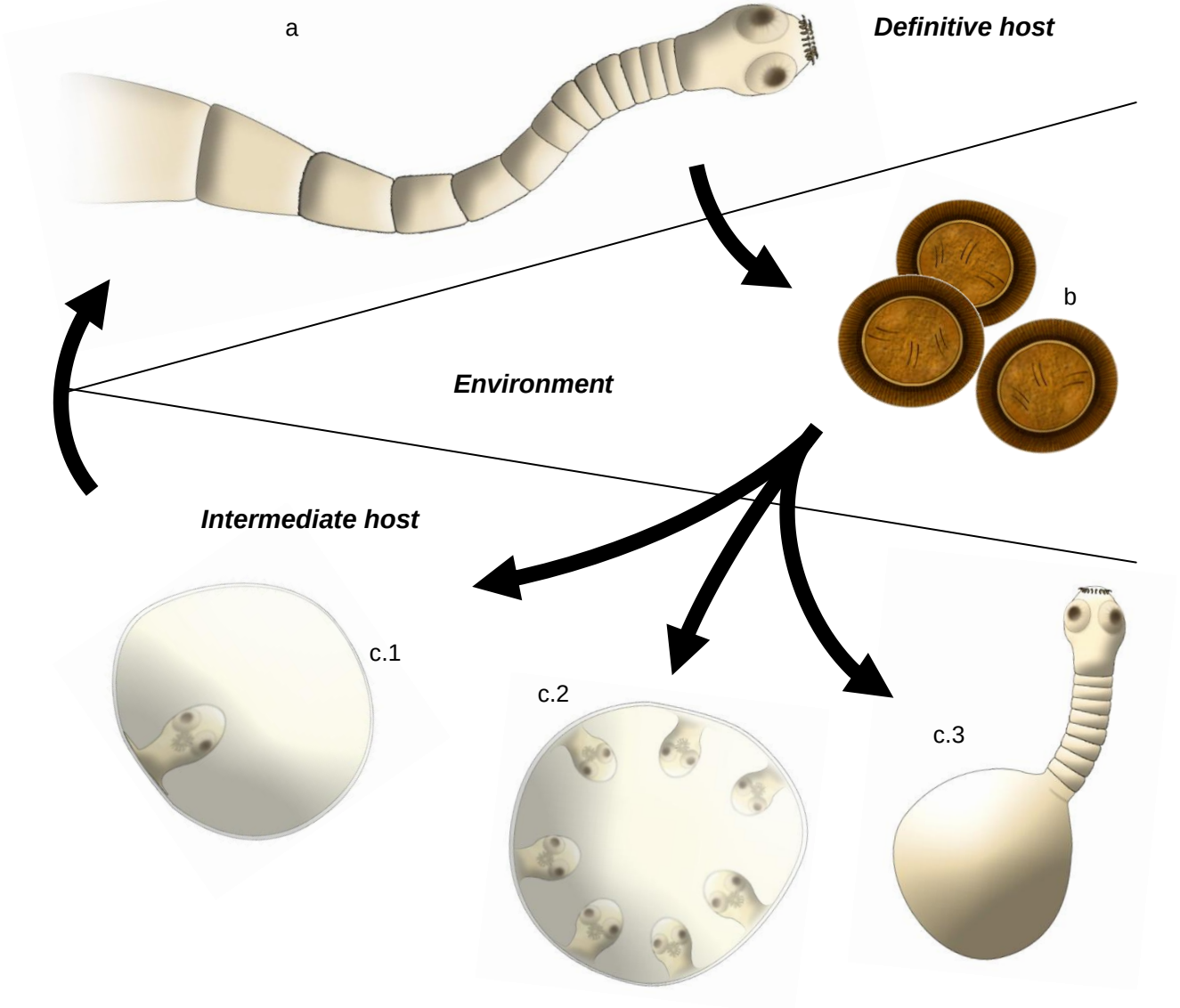
Table 3 – Main features of metacestodes from *Taenia* spp.

Species	Metacestode morphology	Location in the intermediate host
T. crassiceps	Multiplying Cysticercus*	Peritoneal cavity, subcuteanous tissue, intramuscular, lungs, eye, brain
T. hydatigena	Cysticercus	Mesentery, liver surface, lungs
T. multiceps	Coenurus	Brain
T. pisiformis	Cysticercus	Mesentery, liver surface, pelvic cavity
T. polyacantha	Multiplying Cysticercus*	Peritoneal cavity
T. serialis	Coenurus	Muscles in subcutaneous tissue, heart and serous membranes
T. taeniaeformis	Strobilocercus	Liver surface

* These cysticerci are capable of asexual reproduction through budding;

Data adapted from Loos-Frank (2000), Jones & Pybus (2001), Kyngdon et al. (2006) and Roberts et al. (2009b).

Figure 2 – Morphology of different stages of *Taenia* spp. (Original illustrations)



a – adult stage; b – eggs in feces; c – metacestodes; c.1 – Cysticercus; c.2 – Coenurus; c.3 – Strobilocercus

2.2.3. Life Cycle

After infection of definitive host, bile salts stimulate protoscolex to evaginate, while the vesicle is either digested by host's enzymes or absorbed by the protoscolex (Roberts et al., 2009a). Strobilation and worm growth then starts in the small intestine and the parasite attaches to the intestinal mucosa (Beveridge & Rickard, 1975). After more than 6 weeks pi, fully developed adults of *T. pisiformis* were found in experimental infection in dogs. Between 9000 and 28000 eggs were found per gravid proglottid of *T. pisiformis*, and an average of 13000 for *T. crassiceps*. (Beveridge & Rickard, 1975; Miyaji et al., 1990)

Different experiments reveal that patent period is at least 4-6 weeks for *T. pisiformis* and *T. crassiceps* in dogs (Beveridge & Rickard, 1975; Miyaji et al., 1990; Zurabian, Aguilar, Jiménez, Robert & Willms, 2008; ESCCAP, 2010). Prepatent period is of 8-10 weeks for *T. hydatigena* with an average number of 28000 eggs per proglottid (Deplazes, Gottstein, Stingelin & Eckert, 1990). A similar prepatent period was found for *T. multiceps* (Gauci et al., 2008).

Taenia species are hermaphrodites and autogamy seems the most frequent reproductive behavior (Knapp et al., 2011). Mature tapeworms release gravid proglottids in feces but these segments can also migrate out of the anus. Drying and rupture of the gravid proglottids releases the eggs in the environment and they are already infective for the intermediate hosts (Roberts et al., 2009b). Sánchez Thevenet, Basualdo & Álvarez (2010) demonstrated that eggs from *T. hydatigena* exhibited metabolic activity in the environment, and the usage of endogenous lipids could be an important factor in maintaining the viability of the embryo outside the definitive host.

Similarly to *Echinococcus* sp., after ingestion of *Taenia* spp. eggs by intermediate hosts, digestive secretions in duodenum destroy the embryophore and the oncosphere hatches. The hexacant embryo then crosses the intestinal wall, migrating through the bloodstream and reaching diverse locations in the body (see Table 3) (Murrell, 2005; Roberts et al., 2009b).

2.2.4. Pathology and Immunity

In definitive hosts, a humoral immune response is triggered after infection, although it seems weak and not really effective against reinfections (Cordero del Campilho & Rojo-Vázquez, 1999). Jenkins & Rickard (1985) found positive titers of antibodies against *T. hydatigena* and *T. pisiformis* E/S scolex antigens 3 weeks after infection. Antibodies against oncosphere antigens were only found later. Cross-reactivity was found between sera of animals infected with one species and the other. Antibody titers (IgG) against *T. hydatigena* E/S scolex antigens did not correlate with worm burden, and persisted in uninfected dogs (Jenkins, Gasser, Romig, Zeyhle & 1991). Taeniasis in dogs is usually asymptomatic, although some kind of pruritus may develop and dogs tend to rub their bottom against the ground (ESCCAP, 2010).

Immunity develops in several intermediate hosts against different *Taenia species*. Lightowlers (2010) reviewed the existence of a concomitant immunity in intermediate hosts against reinfection, i.e., an immune response develops against early stages of the metacestode, protecting from further infection, without affecting previously installed cysts.

In sheep infected with *T. hydatigena*, a cellular response developed around the cyst, with lymphocytes and eosinophils (Meeusen, Gorrell, Rickard & Brandon, 1989) and a similar capsule is formed in other species (Lloyd, 2008). Metacestodes seem to be capable of avoiding hosts immune response, which translates as a thinner capsule later in infection (Lloyd, 2008).

Cysts survive in the intermediate hosts for variable periods of time, depending on *Taenia* species and host tissue (Lloyd, 2008). Cyst degeneration is accompanied by thickening of capsule; liquid within converts into a caseous material.

Immunity against *T. hydatigena* develops in 7-14 days and can persist for a long time, but only in regions where sheep are continuously exposed to infective eggs (Cabrera et al., 1995; Murrell, 2005). Also, immunity to reinfection was achieved in rabbits (reviewed by Lightowlers, 2010). Humoral response with IgG developed after experimental infection with eggs. *T. pisiformis* oncospheres were lysed after 9-10 days of incubation with immune serum (Kyngdon et al., 2006).

T. hydatigena migration in liver of sheep creates hemorrhagic traces that evolve into white fibrotic lesions. Cysticerci are usually located superficially in liver parenchyma, while hydatid cysts infect deeper regions. Same lesions are found in rabbits affected by *T. pisiformis* cysticercus or in sheep brain with *T. muliceps* coenurosis (Harcourt-Brown, 2002; Lloyd, 2008).

Pathogeny of metacestode depends largely on its location and dimension, and also on host immune status. Mice infected with *T. crassiceps* cysticercus may exhibit skin lumps if cysts are located subcutaneously. Behavior changes, mostly impaired coordination, were recorded in brain infections (Jones & Pybus, 2001). Furthermore, experimentally infected male mice revealed a decrease of testosterone levels and a more submissive behavior if dominance was not yet established (Gourbal, Lacroix & Gabrion, 2002).

T. multiceps metacestodes infect the central nervous system of sheep, creating a disease called gid. Clinical signs include blindness, muscle tremors, with ataxia and ophistotonus if cerebellum is affected (Lloyd, 2008; Roberts et al., 2009b). If coenurus is located in the spinal cord, paresis may occur. In acute onsets of the disease, animals may die but a greater percentage survives and gradually develops neuromuscular and ocular signs (Radostitis, Gay, Blood & Hinchcliff, 2006). Infection with *T. hydatigena* cysticercus is usually asymptomatic in small ruminants. Clinical signs such as weakness and depression may develop in acute cases, with high liver infections (Pugh & Baird, 2011).

Coenurus from *T. serialis* can be located in the retrobulbar space, axilae and cheek of rabbits but usually do not create severe clinical signs; skin tumefactions or exophtalmus were already recorded (O'Reilly, Mccowan, Hardman & Stanley, 2002; Harcourt-Brown, 2002). Rabbits heavily

infected with *T. pisiformis* cysticerci may present abdominal pain and intestinal obstruction (Harcourt-Brown, 2002) however infections are usually chronic with growth retardation and sometimes paralisis and nervous alterations (Cordero del Campilho & Rojo-Vázquez, 1999). Hepatomegaly and emaciation were recorded in rabbits infected with *T. polyacantha* cysticerci (Jones & Pybus, 2001). Also in deer mice, infection with *T. taeniaeformis* did not reveal severe clinical signs except hepatomegaly (Jones & Pybus, 2001).

2.2.5. Diagnosis

Diagnosis in definitive hosts closely follows the coprologic and necropsy techniques already described in the *Echinococcus* spp. chapter. Loos-Frank (2000) extensively reviewed morphologic criteria to identify the different *Taenia* species. In the WHO/FAO/OIE Guidelines for the Surveillance, Prevention and Control of Taeniosis/Cysticercosis a method of Graham is referred, in which adhesive tape is used to collect eggs sticking to skin/fur of the perianal region (Murrell, 2005).

Techniques to detect copro-antigens also have been created. Deplazes et al. (1990) developed a copro-ELISA for *T. hydatigena* which was able to detect prepatent infections as soon as 18 days pi. Cross-reactivity was found with other *Taenia* species but high specificity was found, namely with *Echinococcus* spp. Allan et al. (1992) also developed a copro-ELISA test based on *T. pisiformis* antigens.

A multiplex-PCR to identify *Taenia* sp. and *Echinococcus* spp. eggs was already discussed (Trachsel et al., 2007), as well as a PCR/dot blot assay that identifies different species of *Echinococcus* and *Taenia* (namely, *T. crassiceps*, *T. hydatigena*, *T. multiceps*, *T. ovis* and *T. taeniaeformis*) (Armua-Fernandez et al., 2011). Al-Sabi & Kapel (2011) created a multiplex-PCR to differentiate *Taenia* species (both adults and larval stages). Final result is based on different band patterns created by different species in the electrophoresis gel. Some patterns were very similar and could be misleadingly interpreted, so authors suggest a combination of the result with data from morphology, host and location of the parasite.

In livestock, meat inspection is the most important diagnostic tool. Only 20-50% of cases are assumed to be detected (Lloyd, 2008), and this also includes other medical important taeniasis such as *T. solium* and *T. saginata*. Light infestations are easily missed. Serology tests would reduce economic losses due to carcass damage after meat inspection.

Similar molecular techniques described for *Echinococcus* spp. diagnosis apply here.

2.2.6. Epidemiology

Taenia species are distributed worldwide and in each one of them is evident the diversity of life cycle patterns adapted to each geographic, cultural and ecological regions (see Table 2). Evolution involved an increasing degree of host-switching, which was higher among definitive hosts. An

example is reviewed by Hoberg (2006). Diet shift of humans from herbivore to omnivore created the opportunity for acquisition of *T. saginata*, *T. asiatica* and *T. solium* ancestors in Africa. Later migration of human populations to Eurasia, allowed the distribution of these species. In addition, extensive domestication of ungulates is assumed to have played an important role in defining domestic pigs and cattle as intermediate hosts for *T. solium* / *T. asiatica* and *T. saginata*, respectively.

In the Iberian Peninsula, several *Taenia* species have been found in canid hosts, and they strongly translate food habits in different ecosystems (Carvalho-Varela & Marcos, 1993). Studies on the red fox and Iberian wolf are summarized in Table 4 and Figure 3, respectively. No data is available for the *Taenia* species infecting Portuguese populations of Iberian wolves, thus studies from nearby regions in Spain are cited.

Figure 3 – *Taenia* species infecting Iberian wolf in Northwestern Iberian Peninsula (Original)

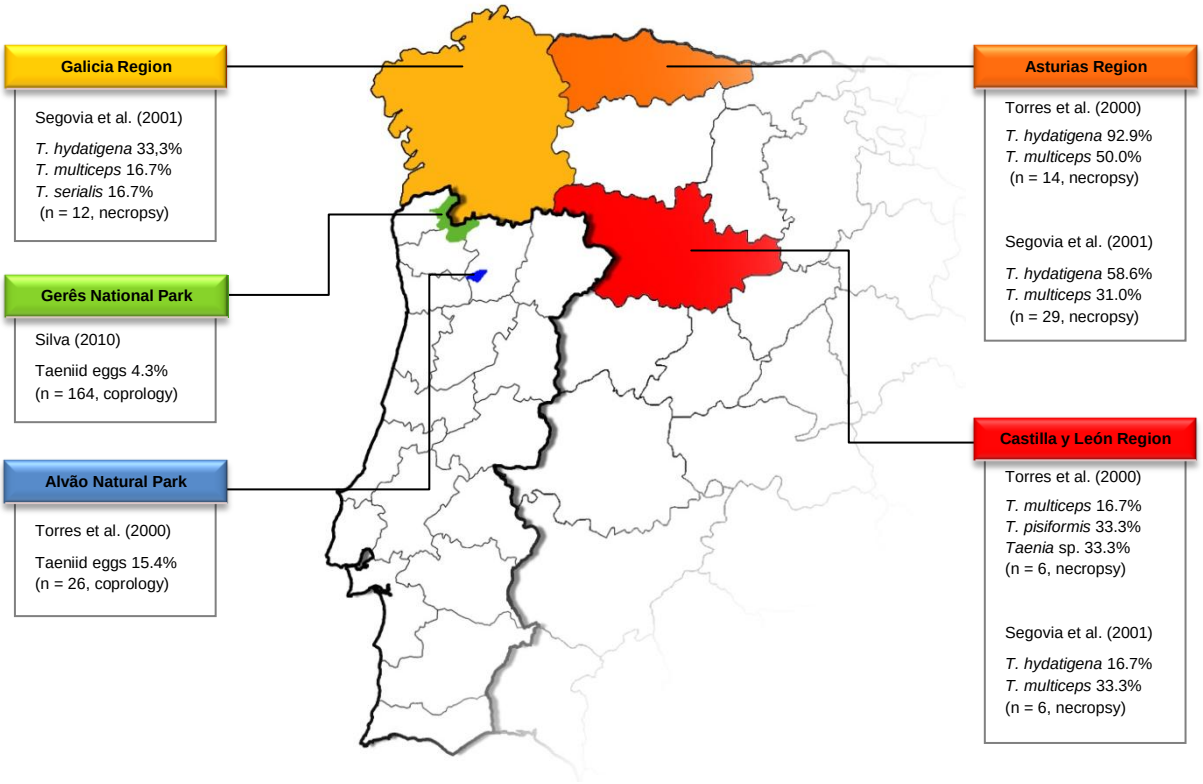


Table 4 – Prevalence of different Taenia species in Red fox populations from Portugal.

Location	Sample size	Prevalence of *Taenia* sp. (%)						Diagnostic tool	Reference
		T. c.	***T. h***	***T. pi***	***T. po***	***T. s***	***T. t.***		
All territory	306	1.3	-	3.3	2.0	3.0	-	necropsy	Carvalho-Varela & Marcos, 1993
Serra da Malcata	20	-	25.0	-	35.0	-	-	necropsy	Segovia et al., 1997
Dunas de Mira	62	-	-	1.6	-	-	3.2	necropsy	Eira et al., 2006

T. c. – Taenia crassiceps; *T. h. - T. hydatigena*; *T. pi - T. pisiformis*; *T. po. - T. polyacantha*; *T. s. - T. serialis; T.t. - T. taeniaeformis*

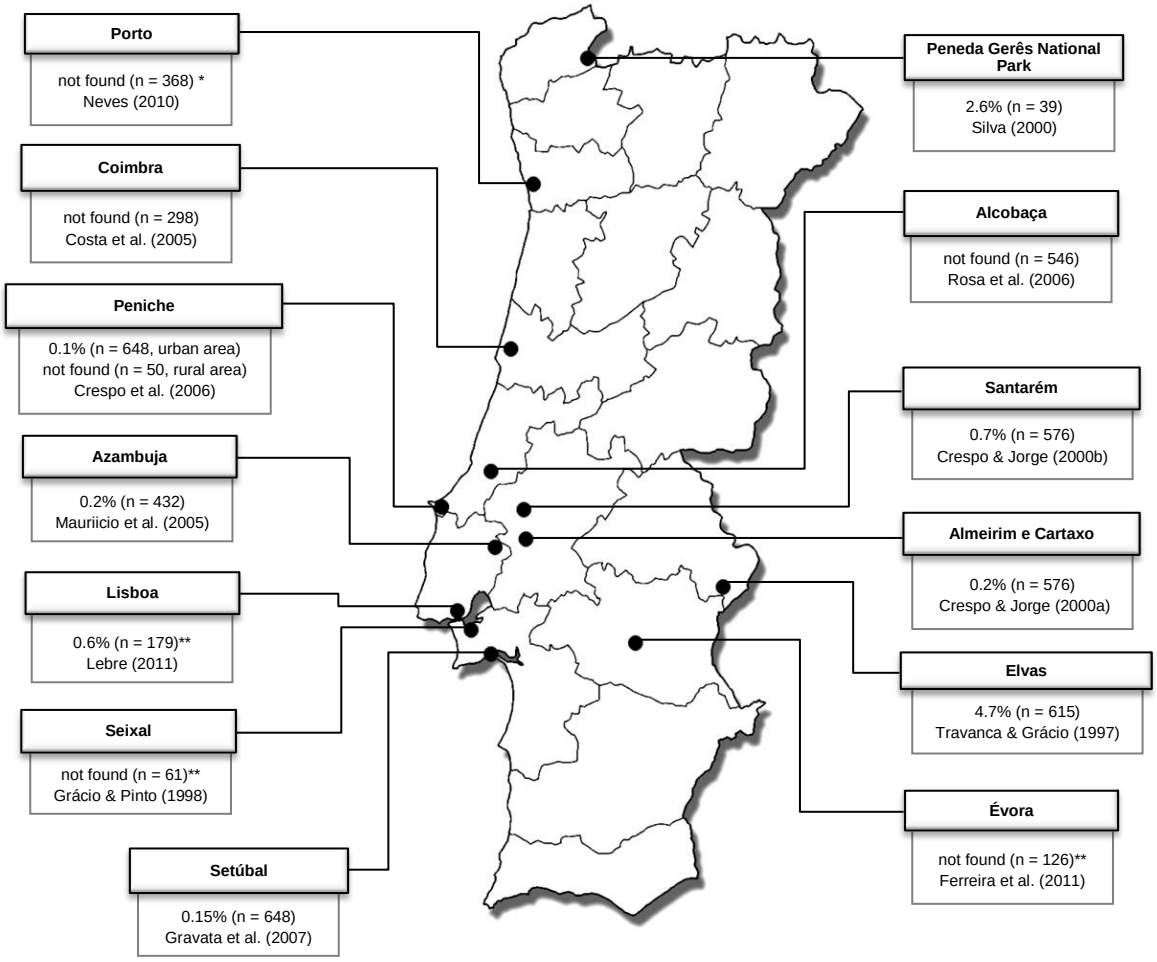
Few data is available regarding dogs as definitive hosts, although they are known hosts for the majority of these *Taenia* species. The majority of data comes from epidemiological studies accessing environmental contamination with dog feces. *Taenia* species have a high biotic potential, with thousands of infective eggs per gravid proglottid as previously stated. Asexual budding and dissemination of parasites by arthropod vectors increase the probability of infection (Cordero del Campilho & Rojo-Vázquez, 1999). Figure 4 summarizes several studies about prevalence in fecal samples collected from public places in Portuguese cities.

Prevalence of Taeniid eggs in dog fecal samples is low in urban areas. The highest levels were found in Elvas, followed by a rural environment in Peneda Gerês National Park. Most of these *Taenia* species circulate in a sylvatic cycle, with rodents and lagomorphs as intermediate hosts. In some cases, domestic ungulates, namely sheep, can act as intermediate hosts and synanthropic, or even rural domestic, cycles develop. *T. hydatigena* is known to infect sheep in Alentejo and Ribatejo, the Southern-Center regions of Portugal. Prevalence of 8.74% was found in a sample of 103 animals from these regions (Crespo & Jorge, 1999). Cysticerci from *T. hydatigena* were found in the chorion-allantoic membrane of a goat's foetus in the Northern Portugal but the significante for vertical transmission is not yet known (Payan-Carreira, Silva, Rodrigues & dos Anjos Pires, 2008). Goats and cows are as well referred as intermediate hosts in the Iberian Peninsula (Cordero del Campillo, 1994, cited by Segovia 2001). In wildlife, this *Taenia* species was found in roe deer (Navarrete et al., 1990), red and fallow deer (Maia, 2011) and in wild boar (De-Le-Muela, Hernandez-De-Lujan & Ferre, 2001).

T. multiceps coenurus is known to infect goats, cows, horses and donkeys (Cordero del Campillo, 1994, cited by Segovia 2001). Reports from lagomorphs infected with *T. pisiformis* occur through all country, with high infestations being found lately in the Center regions and Alentejo (Madeira de Carvalho & Valverde, personal communication, May, 2011). Reports were also made in Viseu district (Coelho, 2010). The Iberian hare is another possible intermediate host (Alzaga et al., 2008). *T. taeniaeformis* strobilocerci was detected in rodents from Setubal region (Behnke et al., 1993)

and *T. serialis* coenuri in rodents from Galicia, Spain (Jones & Pybus, 2001) and in wild rabbits from Spain (Cordero del Campilho & Rojo-Vázquez, 1999). No data was found about *T. polyacantha* and *T. crassiceps* intermediate hosts, although in France, the rodent *Microtus arvalis* is a known one (Jones & Pybus, 2001).

Figure 4 – Prevalence of Taeniid eggs in dog's fecal samples collected from public places. (Original map)



Saples were collected from public places or collected from dogs presented at a veterinary hospital (*) or from kennel dogs (**). Samples were analysed through different coprologic tests.

Taenia crassiceps, T. multiceps, T. serialis and *T. taeniaeformis* are all able to infected humans as intermediate hosts. Human infection follows similar pathways as the ones described for *Echinococcus*, namely ingestion of uncooked meat or contaminated water or food items. *T. crassiceps* is rare in humans and frequently occurs in immunosupressed patients, locating in the subcutaneous and intermuscular tissues (Heldwein et al., 2006). There are some cases described in Europe, namely in Germany. *T. multiceps* is a rare condition already detected in Europe (Italy

and France). Coenurus locates in the brain and clinical signs are pressure-related, e.g., cephalea, motor impairment and vomiting (El-On, Shelef, Cagnano & Benifla, 2008). Sporadic cases of *T. taenaeiformis* were found in human and this species is not considered a zoonotic risk (Jones & Pybus, 2001).

2.2.7. Treatment and Control

Treatment regimens in dogs and control measures described for *Echinococcus* also apply for *Taenia* species. In infected pigs and cattle, treatment with albendazole and oxfendazole resulted in smaller viable cysts of *T. sollium* and *T. saginata*, respectively (Lloyd, 2008).

Recombinant DNA vaccines have been developed for *T. ovis* in sheep, *T. saginata* in cattle and *T. solium* in pigs (Lloyd, 2008). Recently, recombinant oncosphere antigens were obtained for *T. multiceps* (Gauci et al., 2008). The authors refer the possibility of a joint vaccine against *T. multiceps* and *Echinococcus granulosus* for sheep.

2.3. *Toxocara* spp.

Toxocara species are monoxenous roundworms of human and veterinary medical importance. The adult stages inhabit the small intestine of different mammalian hosts.

2.3.1. Taxonomy

Toxocara genus belongs to the Phylum Nemathelminthes, Class Nematoda, Order Ascaridida, Superfamily Ascaridoidea, Family Toxocaridae (Kassai, 1999; Despommier, 2003). A great number of species is cited in the literature but references are often old and data seldom reliable, with some species being reported only once. A thorough analysis, using morphological and genetic criteria, would be useful to clarify all these information gaps. Some of the species with most reliable background information are listed in Table 5.

Table 5 – Some species within the genus *Toxocara*.

Species	Definitive hosts	Geographical distribution
T. alienata (Rudolphi, 1819)	Procyonids	South America
T. apodemi (Olsen, 1957)	Rodents	Eastern Asia
T. canis (Werner, 1782) *	Domestic and wild canids	Cosmopolitan
T. cati (Schrank, 1788) *	Domestic and wild felids	Cosmopolitan
T. genettae (Warren, 1972)	Viverrids	Spain, Africa
T. mackerrasae (Sprent, 1957)	Rodents	Australia, Papua New Guinea
T. malaysiensis (Gibbons et al., 2001)	Cat	Malaysia
T. pteropodis (Baylis, 1936)	Fruit bats	Australia, USA
T. sprenti (Warren, 1972)	Viverrids	Thailand
T. tanuki (Yamaguti, 1940)	Raccoon dog	Japan
T. vajrasthirae (Sprent, 1972)	Hog-badger	Thailand
T. vitulorum (Goeze, 1782) [?]	Cattle, buffalo	Tropical and subtropical regions

* Zoonotic potential
[?] Zoonotic potential not confirmed

Data collected from SanMartín et al. (1992), Zhu et al. (1998), Sato, Inaba, Ihama & Kamiya (1999), Gibbons, Jacobs & Sani (2001), Fisher (2003), Gasser, Zhu, Hu, Jacobs & Chilton (2006) and Roberts, Janovy & Schmidt (2009c).

As *T. canis* and *T. cati* are the relevant species for this study, the following review sections will focus on these ones only.

2.3.2. Morphology

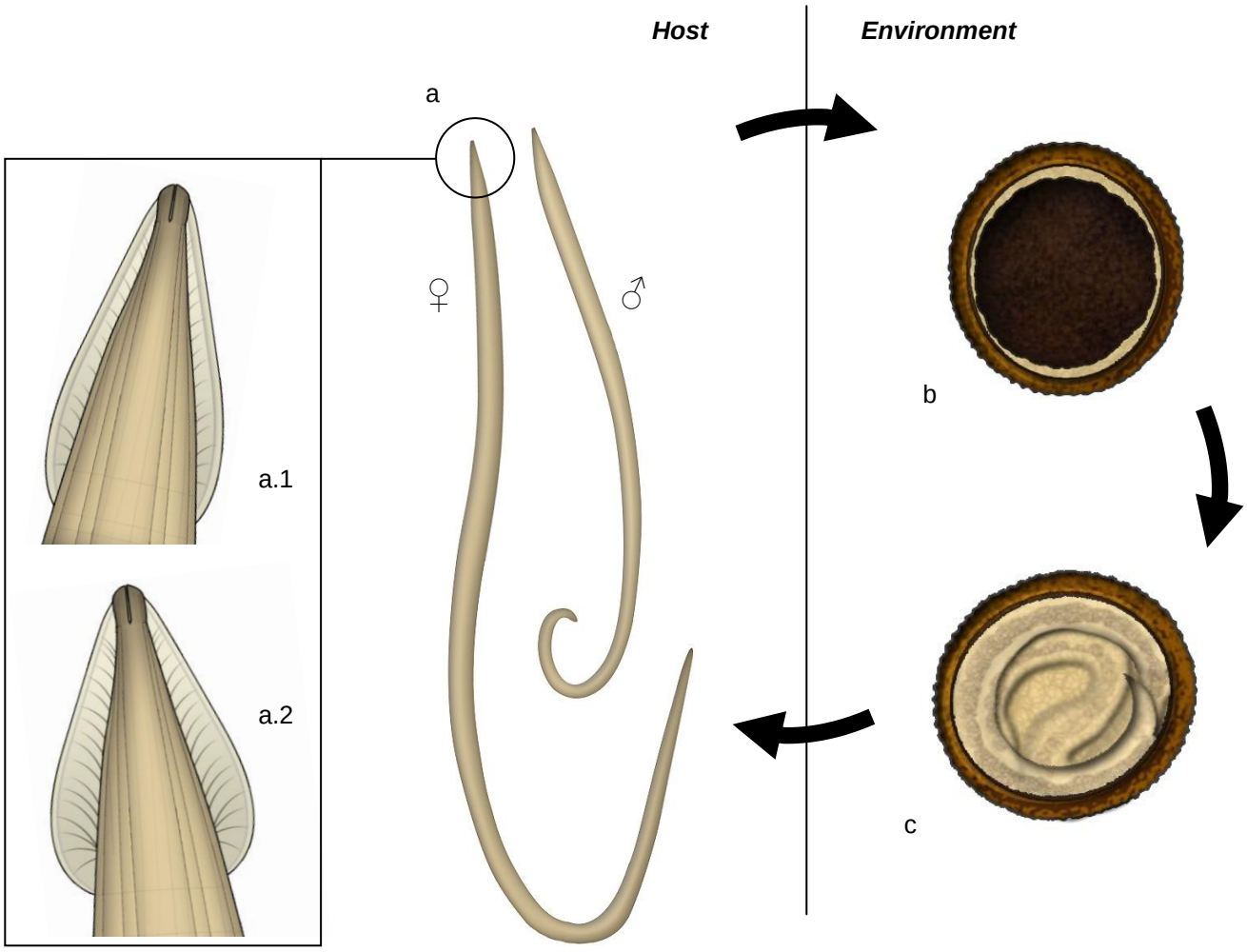
Toxocara canis and *T. cati* adults are large intestinal roundworms. Length ranges from 4 to 6 cm (males) and 6.5 to 15 cm (females) in *T. canis* and from 3 to 6 cm (males) and 4 to 10 cm (females) in *T. cati* (Cordero del Campilho & Rojo-Vázquez, 1999; Roberts et al., 2009c). The anterior end features three well-developed lips that surround the bucal cavity and communicate with the digestive system. The entire roundworm body is covered by a cuticle from where arise

cervical alae. These structures differ between species, being spear-shaped in *T. canis* and broader, arrow-shaped, in *T. cati* (Smith & Noordin, 2006) (see Figure 5). The body wall also features a powerful musculature that allows larvae movement.

After copulation, females lay unembryonated eggs (Roberts et al., 2009c). These are round to ovoid, with a thick alveolar capsule and a dark brown granulated content (Thienpont et al., 1986) (see Figure 5). The capsule has three different layers – an outer vitelline layer, a chitinous intermediate one and an innermost lipid layer – that confer resistance in the environment.

Eggs from those two species were often regarded as morphologically indistinguishable. However, Fahrion, Schnyder, Wichert & Deplazes (2011) observed significant differences between egg dimensions of *T. canis* [74.8 (74.0–75.7) x 86.0 (85.1–86.8) µm] and *T. cati* [62.3 (61.8–62.7) x 72.7 (72.2–73.3) µm]. As soon as maturation begins in the environment, egg content divides into a morula and eventually an infective larva, which, if ingested by a suitable host, can develop again into the adult stage.

Figure 5 – Morphology of different stages of *T. canis* and *T. cati*. (Original illustrations)



(a – male and female adult forms of *Toxocara* sp.; a.1 – spear-shaped anterior end of *T. canis*; a.2 – arrow-shaped anterior end of *T. cati*; b – non-embryonated egg; c – larvated egg)

2.3.3. Life Cycle

Life cycles of *T. canis* and *T. cati* are both complex and very alike. Ergo, a description for *T. canis* will be given with additional remarks in the end about its differences with *T. cati* life cycle.

Infection of a dog, or other suitable host, may occur through four pathways: (i) ingestion of eggs from feces; (ii) ingestion of infected paratenic hosts; (iii) intrauterine transmission; or (iv) through transmammary transmission.

Unembryonated eggs shed in feces maturate in the environment. This involves two molting steps where larval cuticle undergoes structural modifications until the third and infective larval stage (L_3) develops (Brunaska, Dubinsky & Reiterova, 1995). Hatching of infective eggs takes place in the small intestine 2-4 hours after ingestion by a dog or another suitable host. Larvae then penetrate the intestinal wall, through enzymatic and mechanic mechanisms. After entering bloodstream, they migrate via portal circulation and liver to the lungs.

From this step forward, parasite migration seems to depend on some degree on the hosts age and infectious dose (Fahrion, Staebler & Deplazes, 2008; Schnieder, Laabs & Welz, 2011). In puppies younger than 3 months, tracheal migration is the most common pathway. After crossing alveolar capillaries, L_3 reach pulmonary alveoli and then bronchioles, bronchi, trachea and pharynx until being swallowed and reaching stomach as a fourth stage larvae (L_4). Fifth stage larvae develop in the small intestine where they further mature into adults 7 to 15 days pi. After sexual reproduction, embryonated eggs are excreted in feces by female worms (Despommier, 2003; Schnieder et al., 2011). Prepatent period lasts for 31-35 days pi (in puppies) and up to 40-56 days pi (in adults) (Fahrion et al., 2008; Schnieder et al., 2011).

In older puppies and adults, the percentage of larvae undertaking tracheal migration is lower. Instead, larvae undergo somatic migration after being trapped in lungs capillaries and reentering bloodstream. Then they may reach a status of arrested development in kidneys, skeletal muscles, myocardium, brain or liver where they can stay alive for several months (Saeed, Taira & Kapel, 2005; Schnieder et al., 2011).

The dormant larvae in somatic tissues may be reactivated. Such example is pregnant bitches previously infected. Hormone levels in the final third of gestation period or periparturient imunosuppression may be responsible for larvae reactivation. Transplacental migration can occur with infection of the fetus liver. After birth, tracheal migration occurs in puppies and they shed eggs in feces after less than 3 weeks (Lloyd, Amerasinghe & Soulsby, 1983; Overgaauw, 1997; Schnieder et al., 2011). As reviewed by Overgaauw (1997), transplacental transmission may occur in consecutive pregnancies even in the absence of reinfection between them.

Other infectious route is the transmammary pathway, after reactivated larvae enter bloodstream and reach mammary glands acini, colostrum and milk 38 days after parturition (Overgaauw, 1997). In mice, prolactin seems to be an important stimulus for larvae migration (Jin, Akao & Ohta, 2008).

The transmammary transmission is however of less importance, since a smaller percentage of larvae is recovered from milk, comparatively to prenatal transmission (Burke & Roberson, 1985). Bitches infected after partum can also shed larvae in milk. In post-natal infected puppies, larvae do not need tracheal migration; instead, they mature directly in the intestine.
Lactating bitches can also be infected by their offspring in grooming activities, by accidentally ingesting eggs shed in feces or L_4 released in vomit. Recently, eggs were also found in dog fur; however it is still unclear if in a sufficient amount to create infection (Aydenizöz-Ozkayhan, Yagci & Erat, 2008; Deplazes et al., 2011). Presumably non-infected bitches may develop patency postpartum. Two hypotheses might explain this. The first one is just a simple intestinal passage of ingested eggs. Secondly, since immunity is acquired against L_3 only, L_4 are able to complete life cycle and patency develops (Lloyd et al., 1983; Schnieder et al., 2011). These infections tend to disappear spontaneously after lactation (Overgaauw, 1997).

If eggs are ingested by accidental hosts, larvae hatched in the intestine undergo somatic migration but do not reach sexual maturity. These paratenic hosts play an important role in epidemiology, since they maintain infection pressure to canid hosts, and transmission may occur through predation. Birds, rodents, pigs and sheep, and also humans, can act as paratenic hosts. Somatic migration in paratenic hosts resembles the one described in adult definitive hosts (Taira, Saeed, Permin & Kapel, 2004; Deplazes et al., 2011; Kolbeková, Kolářová, Větvička & Syrůček, 2011).
It seems that the outcome of ingested larvae from paratenic hosts depends on their stage. If they had already mature in paratenic hosts tissues, intestinal development can occur directly and patency is present. However, if in early stages, larvae would undergo tracheal or somatic migration (Schnieder et al., 2011).

Life cycle of *Toxocara cati* is very similar to the one previously described for *T. canis*. Third-stage larvae are also the infective stages (Brunaska et al., 1985), reaching intestine as adult worms at day 28 pi, with patency developing 56 days pi (Overgaauw, 1997). One major difference is that tracheal migration is also frequent in adult cats. Also, as queens harbor few somatic larvae, transplacental transmission is uncommon. Transmammary transmission is the main vertical transmission and adult worms develop directly in kittens after infection (Overgaauw, 1997; Coati, Schnieder & Epe, 2004).

2.3.4. Immunity and Pathology

A humoral response is triggered after *Toxocara* infection. In natural infected puppies, IgM titers rise and may still be elevated after 3 months (Maizels, Schabussova, Callister & Nicoll, 2006). In experimentally infected dogs, aged 8-10 months, Fahrion et al. (2008) found increasing positive IgG specific reactions against *T. canis* excretory/secretory (E/S) antigens 10 days pi. Larval

migration was shown to trigger a T-cell mediated response in mice responsible for white granulomatous lesions, and that also stimulated peripheral eosinophilia, similarly to what happens in dogs (Kayes & Oaks, 1980).
Despite the existence of an immune response against *Toxocara* larvae, they still manage to evade it, remaining alive in tissues for a long time. As reviewed by Schnieder et al. (2011), two mechanisms may explain this fact. The first one proposes that an antigenic coat of E/S products is rapidly shed by the larvae. This quick turnover makes larvae inaccessible to the host's immune system, because adherent antibodies, complement and immune cells are released with the remaining products. A second approach says that E/S antigens may structurally resemble some hosts antigens, which prevents activation of immune system (Schnieder et al., 2011).

The concept of age resistance was created to explain the lower degree of patent infections in adult dogs. This was mainly due to acquisition of immune competence, as well as immunity in lungs and gastrointestinal tract that would prevent from further infections. Past studies about *T. canis* in adult dogs sustained this concept by using high doses of infective eggs which failed to create patent infections (Overgaauw, 1997). However, patent infections can develop in adults. For example, in Belgium, Claerebout et al. (2009) found a prevalence of 4.4% *Toxocara* spp. eggs in fecal samples of 301 household dogs older than 3 months. Gates & Nolan (2009) also found patent infections in adult dogs and Richards & Lewis (2001) found high EPG of *Toxocara* eggs in feces from adult red foxes, sometimes even higher than cubs. Although some cases could be due to intestinal passage of eggs, experimental trials were able to create patency in naïve and non-naïve adult dogs infected with low burden of infective larvae 40-56 days before (Fahrion et al., 2008). These doses are a more approximate value of the ones occurring in the natural infection. Saeed et al. (2005) also detected adult worms in experimentally infected foxes with low egg burden. Hence, as reviewed by Schnieder et al., (2011) and Deplazes et al. (2011), the role of adult hosts in *T. canis* epidemiology might be underestimated, and their susceptibility to infection may be higher than previously assumed. Despite this, Fahrion et al. (2008) suggest that previous contact with the parasite, as well as deworming practice, may increase resistance to infection.

Toxocarosis is usually subclinical in adult dogs and in foxes (Overgaauw, 1997; Saeed et al., 2005). In puppies, however, clinical signs include gastrointestinal manifestations such as diarrhea, vomiting, flatulence, pain and a dilated belly. Fatal cases occur in intense infections, after obstruction and rupture of the intestine, colonization of bile ducts and eventually peritonitis. Also, lung migration can cause severe respiratory signs, including coughing and dyspnea, due to pneumonia (Overgaauw, 1997; Schnieder et al., 2011). Growth reduction may occur as a result of digestion and nutrient uptake inefficiency. Since transplacental transmission does not occur in

kittens, infection takes place in an older age than puppies which makes clinical signs less frequent in cat (Overgaauw, 1997).

As a result of larval migration, necropsy may reveal white *milky* spots in liver surface and renal cortex, and also petechiae and white and red lesions in lungs (Schnieder et al., 2011). Similar white lesions were found in experimentally infected foxes (Saeed et al., 2005).

Eosinophilia is the main laboratorial alteration and develops 10 days after infection (Fahrion et al.. 2008), which may be less clear in pregnant bitches (Lloyd et al., 1983). In puppies infected prenatally eosinophilia started to decrease in the beginning of patent period. Red blood cell count may be reduced in puppies due to internal bleeding after parasite migration. Liver enzyme levels, special alanine transaminase (ALT), increase until the 14th day pi (Schnieder et al., 2011).

2.3.5. Diagnosis

Presumptive diagnosis of Toxocarosis in pets can be achieved conjugating the clinical signs (sometimes very suggestive as a distended belly or vomiting of larvae) with background information of the pet (age, deworming status, previous cases in family). Fecal examination can confirm this suspicion through a sedimentation-flotation or a sieving technique (Mizgajska-Wiktor & Uga, 2006). Fecal examination is also the best option to diagnose patent infections in samples from wild hosts and stray pets. These techniques have however lower sensibility due to non-patent infections or low egg shedding. If possible necropsy is a more reliable technique. Saeed et al. (2005) found significant differences between prevalences of *T. canis* revealed by necropsy (76.0%) and fecal examination using a concentration McMaster method (41.0%) in 381 foxes from Denmark. Also, Torres et al. (2001) obtained higher combined prevalences for *Toxocara* sp. and *Toxascaris leonina* in necropsy – 42.2% against only just 9.4% in coprology with flotation in Zinc Sulphate preceded by a concentration step. Martínez-Carrasco et al. (2007), however, did not find significant differences between necropsy and fecal examination with centrifugation-flotation in saccharose in 49 dogs.

To access soil contamination, Ruiz de Ybáñez, Garijo, Goyena & Alonso (2000) found that washing and sieving techniques were the ones that could retrieve the majority of eggs. The former involves washing and centrifugation in distilled water, followed by flotation in sacharose solution. In the latter soil samples are passed through sequential sieves and finally mixed with a saccharose flotation solution.

As reported by Fahrion et al. (2011), egg measurement may be useful for species discrimination since *T. cati* eggs were significantly smaller than *T. canis*. This should be a more practical and economic technique than molecular analysis.

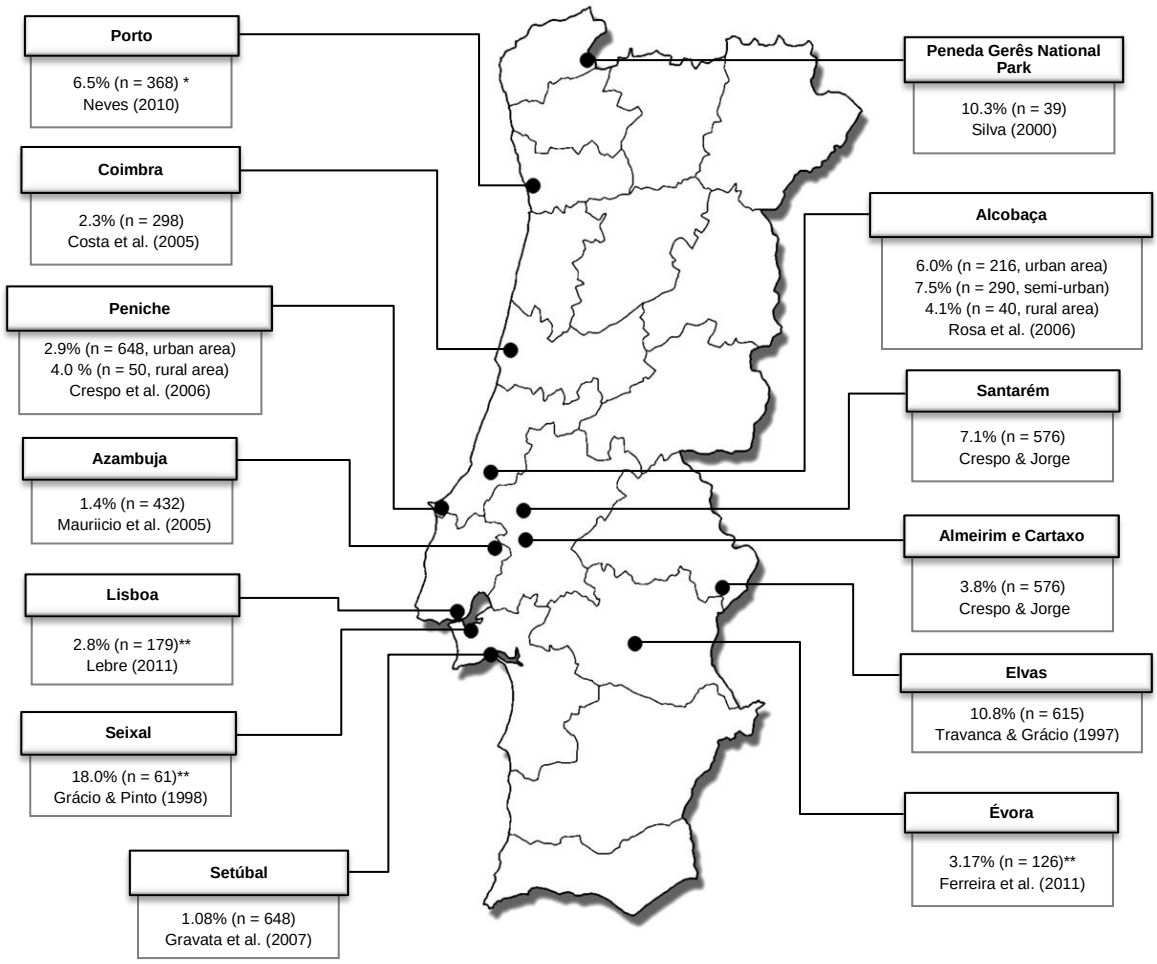
Although maybe not suitable for routine diagnosis, molecular and serological methods were also developed. Both a multiplex-PCR and a PCR-linked Restriction Fragment Length Polymorphism (RFLP) were developed by Jacobs, Zhu, Gasser & Chilton (1997) to distinguish between *T. canis,*

T. cati and *Toxascaris leonina* from eggs isolated from feces and larvae in host tissue. ELISA to detect T. canis larvae E/S antigens have been used successfully in monitoring *Toxocara* infections in adult dogs (Fahrion et al., 2008).

2.3.6. Epidemiology

Toxocara canis and *T. cati* have been found in a lot of domestic and wild hosts. As reviewed by Okulewicz, Perec-Matysiak, Buńkowska & Hildebrand (2012), definitive hosts for *T. canis* include dogs, coyotes, dingoes, jackals, wolves, fennecs and several fox species, including the red fox. In Portugal, it has been found in the main canid hosts, including dogs, red foxes and Iberian wolves (Carvalho-Varela & Marcos, 1993; Crespo & Jorge, 2000b; Eira et al., 2006; Silva, 2010). Prevalences of *Toxocara* sp. in dogs from Portugal are described in Figure 6. Prevalence in wild hosts in the Iberian Peninsula is listed in Table 6.

Figure 6 – Prevalence of *Toxocara* sp. eggs in dog's fecal samples collected from public places. (Original map)



Samples were collected from public places or collected from dogs presented at a veterinary hospital (*) or from kennel dogs (**). Samples were analysed through different coprologic tests.

Sexual differences in *Toxocara* infections vary within host species. In dogs, since data from different studies are contradictory, it is still discussable whether sex may influence parasite migration (Overgaauw, 1997; Fahrion et al., 2008; Deplazes et al., 2010; Schnieder et al., 2011). Contrarily, a higher degree of infection was consistently found in male foxes (Eira et al., 2006; Deplazes et al., 2010; Okulewicz et al., 2012). Saeed & Kapel (2006) proposes that different food habits as well as broader territory occupation contribute to this fact.

Table 6 – Prevalences of *Toxcara* eggs and *T. canis* worms in wild hosts from Portugal and Spain.

Host species	Location [1]	Prevalence (sample size)	Diagnostic test	References
Fox	Múrcia (SP)	45.5% (55)	Necropsy	Martínez-Carrasco et al. (2007)
	Dunas de Mira (PT)	37.1% (62)	Necropsy	Eira et al. (2001)
	Serra da Malcata (PT)	35.0% (20)	Necropsy	Segovia et al. (1997)
	Galicia (SP)	23.0% (201)	Necropsy	Alvarez et al. (1995)
	Overall Portugal	11.1% (306)	Necropsy	Carvalho-Varela & Marcos (1993
	Guadalajara (SP)	4.4% (67)	Necropsy	Criado-Fornelio et al. (2000)
	Gerês (PT)	24.7% (81)	Coprology	Silva (2010)
	Elvas (PT)	15.0% (55)	Coprology	Valverde (2011)
Wolf	Gerês (PT)	7.3% (164)	Coprology	Silva (2010)
	Northwestern Spain	6.4% (47)	Necropsy	Segovia et al. (2001)
	Astúrias and Castilla y León (SP)	5.0% (20)	Necropsy	Torres et al. (2000)

[1] PT – Portugal; SP – Spain

Toxocara cati infects cats as well as wild cats, cheetahs, foxes, American leopard, lions, lynxes, ocelots and tigers (Rodríguez & Carbonell, 1998; Okulewicz et al., 2012). Recently, Fahrion et al. (2011) detected *T. cati* eggs in dog feces, which could be attributed to intestinal passage due to coprophagy and not to true parasitism. Nevertheless, this may be important in disseminating the parasite and further studies are needed in this area. Concerning the potential hosts existing in Portugal, besides domestic cats, *T. cati* was also diagnosed in lynxes in Portugal (Castro, 1992) and nearby regions from Spain (Torres, Garcia-Perea, Gisbert & Feliu, 1998; Millán & Casanova, 2007; Duarte et al., 2010; Ferreira et al., 2011) and also in red foxes (Rodríguez & Carbonell, 1998). In foxes, however, *T. cati* is a satellite species. Also in Iberian lynxes, the prevalence of *T. cati* was low: 2.1% against 8.7% of *Toxascaris leonina*, another common ascarid of carnivores Madeira de Carvalho, Pereira da Fonseca & Carvalho-Varela, 1994).

Data on prevalence of *T. cati* in Portugal are scarce. Duarte et al. (2010) reported a prevalence of 10.8% in 74 fecal samples from stray cats in Lisbon Metropolitan area. A study in the Southern city of Évora, failed to detect *T. cati* patent infections in a small sample of 22 household and kennel cats (Ferreira et al., 2011). A compilation of studies in the Iberian Peninsula are presented in Table 7.

Table 7 – Prevalences of *T. cati* in cats from Portugal and Spain.

Location *	Prevalence (sample size)	Type of cat	Diagnostic test	References
Lisbon (PT)	10.4% (74)	Stray	Coprology	Duarte et al. (2010)
Évora (PT)	0 (22)	Kennel and Household	Coprology	Ferreira et al. (2011)
Zaragoza region (SP)	55.2% (58)	Stray	Necropsy	Calvete et al. (1998)
Sierra Morena and Doñana area (SP)	31% (19)	Stray	Necropsy	Millán & Casanova (2007)
Barcelona (SP)	22.0% (50)	Stray and kennel	Coprology	Gracenea et al. (2009)
La Rioja region (SP)	20.3% (231)	Stray	Coprology	Miró et al. (2004)
	25.0% (48)	Farm		
	10.7% (103)	Household		

* PT – Portugal; SP – Spain

Prevalence revealed by necropsy was high. The results of fecal examination were mild, in some cases higher that 20%, which could mean that the real prevalences are also relatively high.
The finding of *T. cati* eggs shed in dog feces makes us rethink epidemiological studies (Fahrion et al., 2011). Moreover, Fisher (2003) stresses that studies in urban areas should focus more on sandpits, since these are the places preferred by cats for defecation. Using the same locations to compare *T. canis* and *T. cati* contamination may have led to the assumption that the former is more important.

Environmental contamination with *Toxocara* eggs has been widely studied. Different environmental conditions interfere with egg maturation period which lasts from two weeks up to several months or years (Brunaska et al., 1985; Keegan & Holland, 2012). Temperature and humidity strongly affect egg development. At 20ºC, Keegan & Holland (2012) found the majority of eggs embryonated in two weeks. Under experimental conditions, Gamboa (2005) found it was at 30º C with 30% of humidity that most percentage of eggs reached full development. Also, temperature of 21º C at 15% or 30% had high results. Although egg development was higher at 30ºC, egg degradation also increased at this temperature. Low humidity was responsible for a higher percentage of non-viable eggs (Gamboa, 2005; Keegan & Holland, 2012). However, even in extreme temperatures during summer or winter, eggs manage to survive in soil, especially if it has enough depth to attenuate the environment conditions. *T. cati* eggs resistance is assumed to be similar to *T. canis*, although some experiments show they are more resistance to low temperatures (Mizgajska-Wiktor & Uga, 2006).
Distribution of eggs in soil profile is affected by several factors. Earthworms may ingest the eggs, transporting them to soil surface. Some soil fungi revealed ovicidal effects, which could reduce the the risk of transmission in public areas (Ciarmela, Minvielle, Lori & Basualdo, 2002).

Public places contamination with infected feces was relatively high in some cities of Portugal, namely Alcobaça, Santarém or Elvas (see Figure 6). Curiously several studies in Portugal reported higher prevalences in the city centers, comparatively to the city borders and rural areas nearby (Mauricio et al., 2005; Rosa et al., 2006). Same results were found in other studies elsewhere (Deplazes et al., 2011), and this may be a result of the considerable density of stray pets in urban areas.

Toxocara eggs are found in hair coat from both dogs and cat (Overgaauw et al., 2009). In puppies and kittens, where the higher EPG were found, this may be a result of self-contamination or acquisition in litter, whereas in adults contamination from the environment is most probable (Keegan & Holland, 2012). It was initially assumed that fur contamination played an important role in transmission to humans (Wolfe & Wright, 2003). Although maturation of eggs in fur was demonstrated experimentally (Keegan & Holland, 2012), percentage of embryonated eggs in natural populations was low, thus reducing the probability of transmission (Aydenizöz-Ozkayhan et al., 2008; Roddie, Stafford, Holland & Wolfe, 2008; Keegan & Holland, 2010). As stated by Overgaauw et al. (2009), high doses of hair have to be ingested to create an infection. Also, in well cared pets, fur contamination is presumably less probable (Keegan & Holland, 2012).

Paratenic hosts also play a relevant role in epidemiology. Behavior changes were documented in mice due to larval infection of central nervous system. Hamilton, Stafford, Pinelli & Holland (2006) detected memory impairment and in other studies, as reviewed by Schnieder et al. (2011), less activity was found, as well as a higher probabiblity of open areas occupation. This altogether enhances the possibility of predation and maintenance of parasitic life cycle.

Few studies exist on the prevalence of infected paratenic hosts. Antolová, Reiterová, Miterpáková, Stanko & Dubinsky (2004) found a seroprevalence of 7.7% for *Toxocara* sp. in rodents and other small mammals in Slovak Republic. Although not statistically significant, prevalences were higher in suburban areas, comparatively to rural areas. Reperant, Hegglin, Tanner, Fischer & Deplazes (2011) also had higher seroprevalences for *Toxocara* sp. in rodents in urban areas. This, combined with the contamination of urban areas with fecal samples, might translate as a synanthropic life cycle developing between stray dogs, foxes and small mammals which maintain infections near cities. Importance of paratenic hosts was also verified when high seroprevalences of *Toxocara* spp. were found in animals kept in zoological collections, where deworming treatments are performed (Okulewicz et al., 2012).

Toxocara canis and *T. cati* are zoonotic roundworms, and Humans may serve as paratenic hosts. It was generally assumed that *T. canis* was the most important source for human cases and *T. cati* was often neglected. However, recent reviews have stated that the zoonotic importance of the latter may be underestimated (Fisher, 2003; Lee, Schantz, Kazacos, Montgomery & Bowman,

2010; Deplazes et al., 2011). Several facts are given to support this theory: (i) incapacity to distinguish serologically *T. canis* and *T. cati* infections; (ii) findings of higher prevalences of environmental contamination by *T. cati* than *T. canis* in some places; (iii) presence of Toxocarosis in Muslim countries where contact with dogs is avoided, although paratenic hosts might have an important role here.

Infection pathways for humans include ingestion of undercooked meat infected with *Toxocara* larvae and poor hygiene which eventually allows ingestion of eggs from contaminated soil, water or vegetables (Lee et al., 2010; Deplazes et al., 2011). Contamination of public places, such as gardens and playgrounds, presents a high risk for users in general, but children in particular, especially the ones exhibiting geophagia. The highest number cases of Human Toxocarosis are found in children (Deplazes et al., 2011) and some studies suggest a direct relationship between soil contamination and human cases (Mizgajska, 2001). Although soil contamination seems higher in cities, human cases are often common in rural areas, where more contact exists with soil and animals, and also a lower degree of hygiene condition as well as veterinary care (Lee et al., 2010).
Infection of humans by larvae of *T. canis* can cause several syndromes, some with severe clinical signs. Visceral Larva Migrans (VLM) occurs mostly in young children. Spleen, liver, lungs and also central nervous system are affected, resulting in hepatitis, pneumonia and meningoencephalitis, eventually followed by seizures. In Ocular Larva Migrans, larvae infect the eye and optic nerve, with variable degree of ocular tissues inflammation that can lead to vision impairment or strabismus. This syndrome affects especially older children and teenagers. Covert Toxocarosis is considered a non-classic syndrome of Toxocarosis and patients exhibit some of the signs presented in VLM, including coughing, abdominal pain, sleep and behavior changes. At some point, a relationship was attempted between Asthma and Toxocarosis, however recent data does not seem to support this (Despommier, 2003; Smith & Noordin, 2006; Hotez & Wilkins, 2009).
Clinical signs are not common and many epidemiological studies detected seroposivity in healthy individuals. In Europe, low values of prevalence were found in Switzerland (2.7 – 5.1%), in Italy (1.6%), and in the Netherlands (6-11%). Higher seroprevalences were detected in Salamanca, Spain (29.4 – 33.1 %) and in Slovak Republic (27.4%). Worldwide highest prevalences were found in South America and Southeastern Asia (data reviewed by Smith & Noordin, 2006).
In Portugal, Grácio, Trinca & David de Morais (2005) studied 276 human sera from Évora Distric, Alentejo, and found seroprevalences of 11.6% and 13.4% using ELISA tests to detect somatic and E/S antigens, respectively. Two previous studies cited by these authors reveal prevalences of 11% and 30%. Martins et al (2005) analysed by ELISA 457 suspected cases in a period of 10 years, and found 100 (21.9%) positive cases. This confirms that humans have contact with this parasites in Portugal.

2.3.7. Treatment and Control

According to the guidelines provided by the ESCCAP Association, treatment in adult dogs and cats should occur in a monthly basis, especially in populations at risk. Since this may be difficult to achieve in practice, it could be replaced by coprological examination each 1-3 months. Also, kittens and puppies at risk should start treatment three or two weeks after birth, respectively, with consecutive repetitions each 15 days until two weeks after weaning. Also, due to the ability of vertical transmission, bitches and queens should be dewormed beginning at the same time as their offspring (ESCCAP, 2010).

Major anthelminthic drugs may be used for treatment of Toxocarosis in dogs and cats, including Macrocyclic lactones, Benzimidazoles, Nitroscanate, Pyrantel or Emodepside, with especially regards for the first two classes which are effective against both adult and larval stages (Epe, 2006). More than the immediate effect of parasite control, Fahrion et al. (2008) suggested that deworming could enhance the immunologic responses since higher antigens of destroyed larvae are released after treatment.

Besides appropriate deworming strategies, control of *T. cati* and *T. cati* involves basilar rules that apply for a wide range of other intestinal parasites – including hygiene, correct disposal of samples, correct meat preparation, control of stray animals and public education. Quarantine and treatments before the entrance of animals in kennels or houses are important. Public places, especially playgrounds or gardens should be carefully protected to prevent the entrance of stray cats and dogs (Epe, 2006; Alho, Seixas, Rafael & Madeira de Carvalho, 2010; ESCCAP, 2010). Also sand and soil in these areas should be regularly cleaned and changed, or even replaced by artificial soft pavements as has happened lately in a lot of public parks in Portugal.

Since wild populations, namely foxes and rodents, can help maintaining the life cycle, attempt of control could be thought. Rodents control is performed regularly by other reasons. Deworming of wild populations as a control strategy would be a very complicated process, and the cost-benefit may not be high enough. It is difficult, however, to access economic indicators for Toxocarosis since information available in humans and paratenic hosts relates to seroprevalence and not to the disease itself (Overgaauw, personal communication, March 2012).

Some efforts have been made in characterizing antigenic candidates for future vaccines (Epe, 2006) that could confer long-lasting immunity against infection. However, given the complexity of this process no vaccines have been developed so far.

3. Main Objectives of the Study

From the previous review section, one can clearly see that Portugal harbors specific conditions for maintenance of *Echinococcosis* and *Taeniosis* in sylvatic and domestic cycles. Despite the clinical and economic importance of these diseases, base-line studies focusing on definitive hosts, core elements in control programs, are lacking in Portugal. Therefore the aims of the first part of this study were:

(i) For the first time in studies like this in Portugal, use a sieving-flotation technique to diagnose patent taeniid infections in canid hosts;

(ii) Through molecular analysis characterize the *Taenia* species, as well as the genotypes of *E. granulosus*;

(iii) Understand the possible role of different definitive hosts in the epidemiology of the sylvatic and synanthropic cycles of these cestodes, especially for *E. granulosus,* and also for *T. pisiformis* that has been increasingly found in wild rabbits hunted in the Center-South Portugal;

(iv) Characterize for the first time the taeniid species infecting the threatened Iberian wolf in its Portuguese distribution;

(v) Compare the results of these techniques with methods currently used in the Parasitic Diseases Routine Laboratory (FVM-TUL).

Toxocara canis is an important zoonotic agent and a synanthropic cycle develops near cities. The recent findings about *T. cati* as an underestimated zoonosis, as well as the ability to morphologically distinguish its eggs from the *T. canis* ones, allows further analysis in this field. Hence, the main goals of the second part of this study were:

(i) Use a sieving-flotation technique to diagnose patent infections for *Toxocara* spp. in susceptible hosts, in two different geographic and epidemiological regions from Portugal;

(ii) Understand the possible role of different definitive hosts in the epidemiology of the sylvatic and synanthropic cycles of *Toxocara*;

(iii) Use morphologic criteria to assess the possibility of *T. cati* contamination in fecal samples from canid hosts;

(iv) Compare the results with the ones obtained previously by other methods used in the Parasitic Diseases Routine Laboratory (FVM-TUL).

4. Materials and Methods

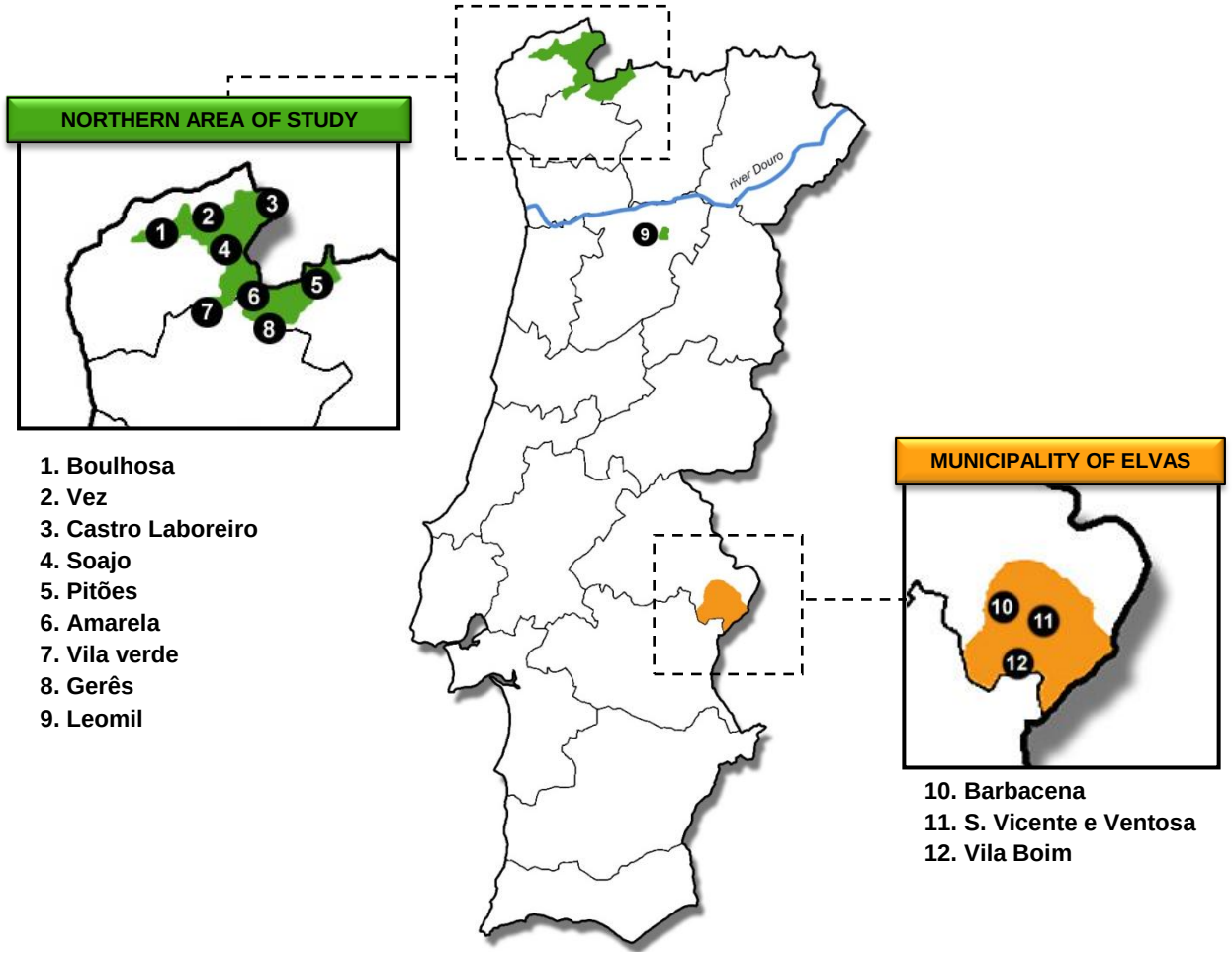
4.1. Sample Population and Study Areas

This epidemiological study was conducted in two different regions from Portugal. One area in the North corresponded to the distribution of the Portuguese populations of the Iberian Wolf (*Canis lupus signatus* Cabrera, 1907). The second area corresponded to the Municipality of Elvas, in the Southern-Centre Portugal. These are regions where the sylvatic and domestic cycles of the parasites in study are probable to overlap. One hundred and eighty one (n = 181) fecal samples from three different canid species were collected from these two areas. Details regarding their distribution and the species included are listed in Table 8 and Figure 7.

Table 8 – Overall number of species collected by host species and area of study.

Species	Northern area of study	Elvas Municipality
Iberian wolf	68	-
Red fox	33	29
Dog	16	35
Sub-Total	117	64
Total	181	

Figure 7 – Areas of study and geographical distribution of fecal samples. (Original map)



Northern Area of Study

Wolf, fox and dog fecal samples from this area had been previously collected between October 15th 2008 and January 30th 2009 and analyzed by coprological techniques such as McMaster, Willis and Sedimentation, as part of the Veterinary Master Thesis of student Marta Silva (Silva, 2010). Samples were collected from the environment and identified by several variables, e.g. morphology, location, smell, content, known distribution and transects of hosts. They originated from nine areas, each corresponding to the territory of a specific wolf pack; eight were located North of the river Douro and one (Leomil) in the South, where a subpopulation from the main population of Iberian wolf in Portugal exists which is isolated from the other by human and natural borders (Alexandre, Cândido & Petrucci-Fonseca, 2000) (see Table 9). More specific data regarding the location of samples can be seen in Figure 7. For each sample, species, date and location were recorded.

Since then, samples were stored at 4º C at the Parasitic Diseases Routine Laboratory of FVM-TUL.

Table 9 – Distribution of samples, according to each wolf pack territory in the Northern area of study.

Wolf pack territory *	Species		
	Wolf	Fox	Dog
1. Boulhosa	1	-	-
2. Vez	20	14	2
3. Castro Laboreiro	1	-	-
4. Soajo	7	4	3
5. Pitões	4	2	4
6. Amarela	10	-	1
7. Vila verde	1	11	4
8. Gerês	13	2	2
9. Leomil	11	-	-

* Numbers refer to the locations in Figure 7.

This region extends through the Districts of Viana do Castelo, Braga and Chaves and next to the Spanish border on the North. It is mainly a mountain area, crossed by a dense hydrographic net, and a great part occupied by the Peneda Gerês National Park. Pluviosity is high and average temperature is 13º C, with increasing thermal amplitudes in the more continental areas. Human populations are aged, mostly rural, strongly depending on agriculture. Extensive production of cattle, goats and sheep is an important income source (Instituto de Conservação da Natureza e Biodiversidade [ICNB], 2012).

Municipality of Elvas

Between December 27th 2010 and February 7th 2011, 29 fecal samples were collected from foxes (15 females; 14 males) killed during the hunting season of 2010-2011. The cadavers had been gathered nearby the village of Barbacena, for skinning purpose, and samples were taken from the last 10 cm of the large intestine. These samples were also previously analyzed by coprological techniques (McMaster, Willis and Sedimentation), at the Parasitic Diseases Routine Laboratory of FVM-TUL as a part of the Veterinary Nurse Thesis of student António Valverde (Valverde, 2011). Samples were then stored at 4º C.

Thirty five fecal samples from hunting (n = 28) and sheep dogs (n = 7) were also collected in October 2011 from hunting association areas in the Municipality of Elvas. Dogs, 21 females and 14 males, aged between one and 11 years old, were dewormed once a year with Praziquantel, at the same time as the Rabies vaccination. Distribution of samples per area is in Table 10.

Samples were firstly analyzed by the author through the same coprological methods as the previous ones and then stored at 4º C at the Parasitic Diseases Routine Laboratory of FVM-TUL.

Table 10 – Distribution of samples, according to each hunting association area in the Municipality of Elvas.

Hunting Associations area	No. of dogs sampled
11. S. Vicente e Ventosa	6
12. Vila Boim	29

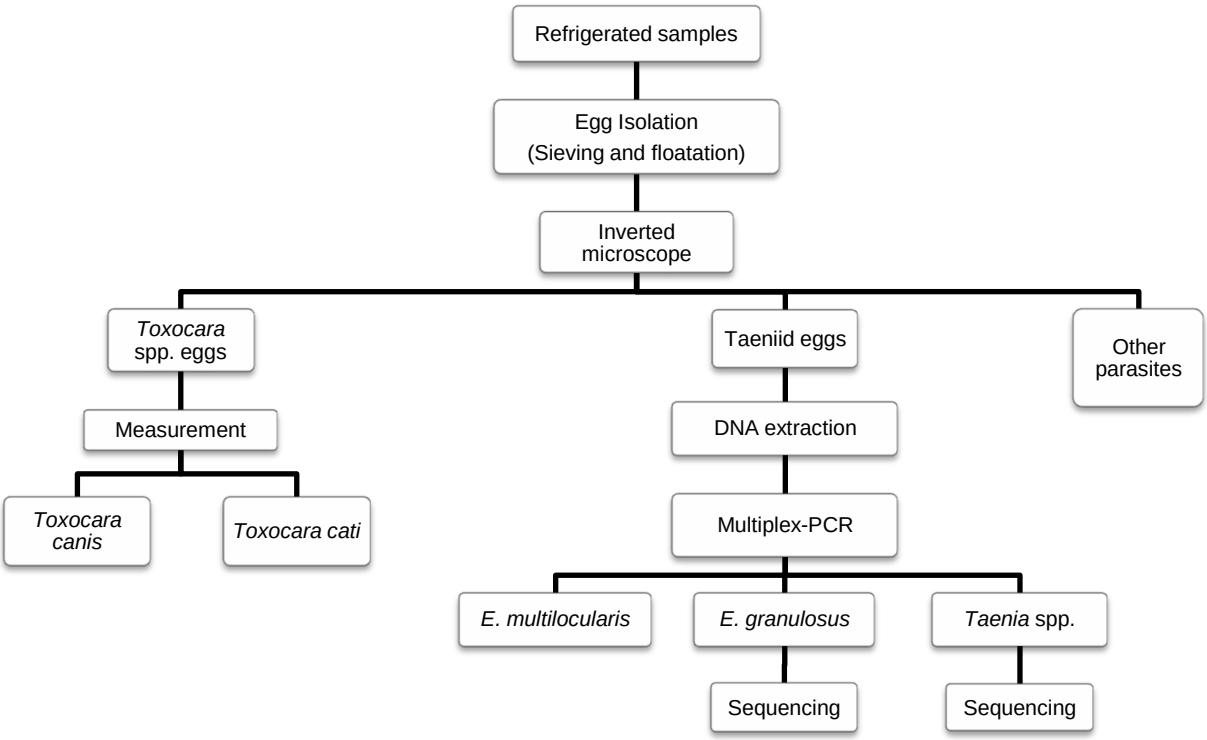
Note: numbers refer to the locations in Figure 7.

The Municipality of Elvas is located in the Portalegre District, Alentejo. It is limited by Évora District and Spain. The climate is dry, mainly Mediterranean. Annual thermal amplitudes range 20º C, reaching 45º C in summer. Landscape is composed mainly of cork oak woodland with some agriculture patches and small villages. Population has aged progressively in the last years and in each area of study human population is less than 1200 people. Agriculture is an important economic activity and cattle and sheep are the main livestock produced (Câmara Municipal de Elvas, 2009; Câmara Municipal de Elvas, 2012). Hunting is also a relevant occupation activity and birds, foxes, wild boars and lagomorphs are the main hunting trophies (Associação de Caçadores da Freguesia de São Vicente e Ventosa, 2012).

4.2. Decision Tree for the samples analysis

According to the main goals of this study, samples were submitted to different analytical pathways, according to the parasite identified (see Figure 8).

Figure 8 – Decision Tree for samples analysis



4.3. Note concerning safety procedures

As previously stated, when handling fecal samples potentially infected with *Echinococcus* eggs, deep freezing at – 80º C is advisable to kill the eggs. In this case, however, deep freezing was shown to severely alter *T. canis* eggs morphology (data not shown) making identification hard. In this instance, we opted not to freeze the samples, but remaining safety measures were followed, namely working in a proper confined environment, with protective clothing. After work, bench and materials were carefully clean with Sodium Hypochlorite 2% solution and then washed with water.

4.4. Taeniid and *Toxocara* spp. eggs isolation through sieving and flotation procedure

This technique is a modification of the one described by Mathis et al. (1996) for the detection of eggs of *Echinococcus multilocularis* in feces from foxes.

Each fecal sample was firstly homogenized and approximately 2 g of it diluted 1:4 in PBS containing 0.3% Tween 20. Samples were double analyzed to increase the sensibility. Data regarding humidity (dry, average, liquid) and content (i.e., fur, stones) of samples were recorded.

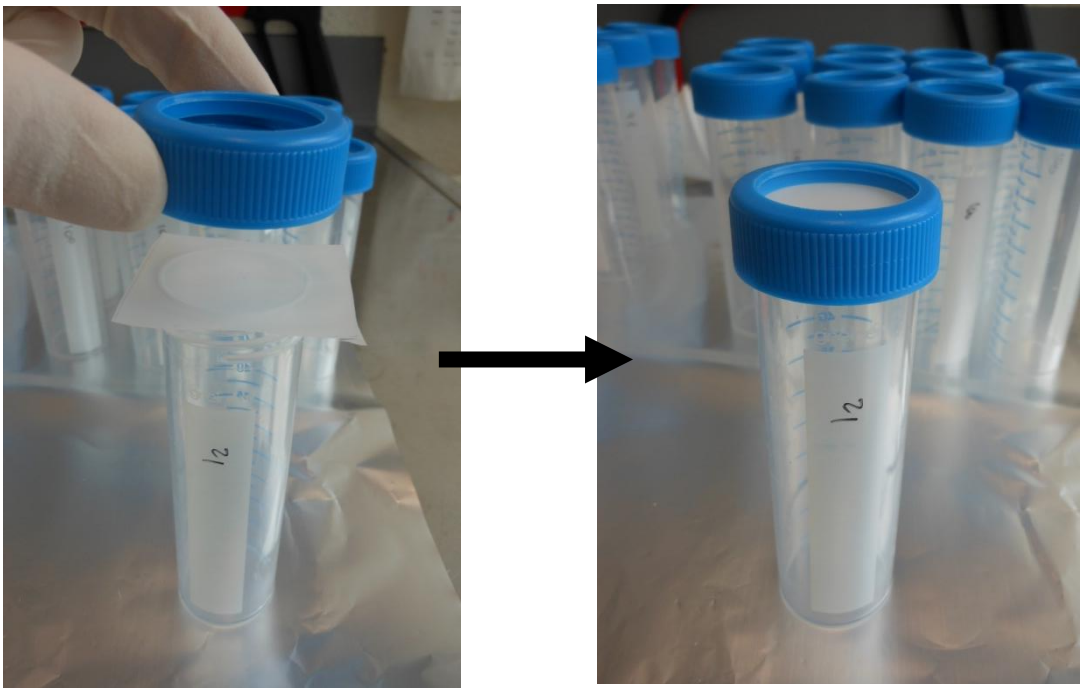
After being stored at 4º C overnight (~ 12h), samples were centrifuged for 10 min at 1600 x *g* (Allegra X-15R centrifuge, Beckman Coulter Inc, Switzerland). The supernatant was discarded and the pellet (approx. 2 mL) resuspended in 8 mL of zinc chloride solution (density 1,45 g/mL), followed by a new centrifugation step (30 min at 1000 x *g*). Supernatant was then passed through sequential funnels and sieves with different mesh sizes: 100 µm, 40 µm and 21 µm, respectively

(See Figure 9). The 40 µm and 21 µm sieves were put upside down, washed thoroughly with water and washed material collected in different flattened side tubes (Nunclon, Nunc, Denmark) for the identification of *Toxocara* sp. and taeniid eggs, respectively. Eggs were identified in a bifocal, inverted microscope at 100x and 400x magnifications.

Taeniid and *Toxocara* sp. eggs were counted up to 40 per sample. Each *Toxocara* sp. positive sample was also classified in one or more categories according to egg morphology. Classification was adapted from Overgaauw et al. (2009): non-viable eggs (empty with disrupted wall); unembryonated (intact egg wall with non-divided content), embryonated (with morula), larvated (with larvae).

Figure 9 – A 21-µm sieve for egg isolation.

[Sieves were house made with a Nylon filter mesh size 21 or 40 µm (Scrynel NY 21 or 40 HC) adapted to a Blue Max falcon tube (Becton and Dickinsen, New Jersey) with a modified lid].



4.5. Measurement of *Toxocara* spp. eggs

Positive sample for *Toxocara* spp. in flattened side tubes were centrifuged for 1 min at 500 g (Allegra X-15R centrifuge, Beckman Coulter Inc, Switzerland). The pellet was transferred to a glass slide and the available eggs, up to 10, were measured (maximum and minimum diameter) under a microscope at 400 x magnification with software Leica Application Suite version 2.8.1. (Leica Microsystems, Switzerland).

4.6. Multiplex-PCR for identification of taeniid eggs in feces from carnivores

DNA extraction

DNA from taeniid eggs was extracted based on the method described by Štefanić et al. (2004). Material was centrifuged for 10 min at 200 x *g* (Allegra X-15R centrifuge, Beckman Coulter Inc, Switzerland) and the pellet transferred to a 1.5 mL Eppendorf tube. After centrifuging at 800 rpm for 1 min (Biofuge pico, Heraeus, UK), supernatant was discarded and pellet resuspendend in 200 µL of distilled water. Then 25 µL 1M KOH and 7 µL of 1M DTT (Dithiothreitol) were added and the samples homogenized in vortex and incubated at 65º C for 15 min. This was followed by spinning down the samples and neutralizing the pH with 60 µL of 2M Tris-HCl (pH 8.4) and 2 µL of concentrated HCl (12.4 N / ≥ 37%). Material was vortexed and spinned down again and 20 µL of Proteinase K and 200 µL of Buffer AL (QIAmp DNA mini kit; Qiagen, Hilden, Germany) were added. After another vortexing step, samples were incubated at 56º C for 10 min, followed by a spin down step and the addition of 50 µL of Chelex beads (50% w/v in distilled water; Bio-Rad Laboratories, Hercules, California). Samples were mixed for 2 hours on a rotator (Heto Rotamix, Heto, Denmark) at room temperature, centrifuged (1 min, 13000 rpm) and the supernatant (approximately 400 µL) transferred to a new 1.5 mL Eppendorf tube. Two hundred microliters (200 µL) of ethanol (100%) were added before vortexing and spinning down the samples. The final mixture (less than 800 µL) was transferred to a Qiagen spin column from the same kit and centrifuged (1 min, 8000 rpm). New collection tubes were taken and samples washed in several steps: twice with 300 µL Buffer AW1 and subsequent centrifugation (1 min, 8000 rpm), once with 300 µL Buffer AW2 and centrifugation (1 min, 8000 rpm) and finally once with 300 µL Buffer AW2 and centrifugation (3 min, 13000 rpm). Before adding Buffer AW2, collection tubes were discarded and new ones taken. The washing step was followed by an additional centrifugation (1 min, 8000 rpm) and the spin column transferred to a new Eppendorf tube, to where 100 µL of Buffer AE were added. Finally, samples were incubated for 1 min at room temperature, centrifuged 1 min at 8000 rpm and the flow through fraction containing the DNA stored at -20º C until used for PCR analysis.

Multiplex-PCR

The Multiplex-PCR for identification of taeniid eggs was done according to Trachsel et al. (2007), using the Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany). For each sample (2 µL of DNA template), the amplification reaction mixture included 25 µL of Mastermix, 18 µL of distilled water from the kit and 5 µL of primer mix. Primers used are listed in Table 11. Primer mix was done with 80 µL of UnivSr, 10 µL of each other primer and 380 µL of distilled water.

For negative control was added 2 µL of distilled water. For positive controls were added 1 µL of distilled water and 1 µL of control DNA (*E. multilocularis*, *Taenia* spp. and *E. granulosus*). In all tubes were added 2 drops of mineral oil and finally they were vortexed and spinned down. Cycling

conditions were: 15 min/95 ºC; 40 cycles of (30 sec / 94 ºC; 90 sec / 58 ºC; 10 sec / 72 ºC); 10 min / 7 ºC.

Amplicons were detected by electrophoresis in 2% agarose gel stained with GelRed and revealed by ultraviolet radiation.

Table 11 – Primers targets and sequences amplified for Multiplex-PCR (adapted from Trachsel et al.; 2007).

Target species	Target Gene	Primer designation	Sequences (5' – 3')	Amplicon size (bp)
E. multilocularis	*nad1*	Cest1	TGCTGATTTGTTAAAGTTAGTGATC	395
		Cest2	CATAAATCAATGGAAACAACAACAAG	
E. granulosus	*rrnS*	Cest4	GTTTTTGTGTGTTACATTAATAAGGGTG	117
		Cest5	GCGGTGTGTACMTGAGCTAAAC	
Taenia sp.	*rrnS*	Cest3	YGAYTCTTTTTAGGGGAAGGTGTG	267
		Cest5	GCGGTGTGTACMTGAGCTAAAC	

4.7. *Taenia* spp. and *Echinococcus granulosus* amplicons sequencing

Taenia spp. and *E. granulosus* amplicons obtained in the Multiplex-PCR were sequenced. For DNA purification a MinElute PCR Purification Kit (Qiagen, Hilden, Germany) was used. For one volume of the Mutliplex-PCR material, were added five times the volume of Buffer PB. Mixture was transferred to a MinElute column and centrifuged for 1 min at 13,000 rpm (Biofuge pico, Heraeus, UK. Flow-through fraction was discarded and 750 µL of Buffer PE were added to the column, followed by centrifugation for 1 min at 13,000 rpm. After discarding flow-through, samples were additionally centrifuged in the same conditions. The MinElute column was then placed in a 1.5 mL Eppendorf and 12 µL of Buffer EB were added. The flow-through fraction was collected after centrifugation (1 min; 13,000 rpm).

Samples were further analyzed to confirm if DNA concentration was within the range of 2 and 10 ng/µL, using a NanoDrop 1000 Spectrophotometer and a NanoDrop 3.1.0 computer software (Thermo Fisher Scientific). Further dilutions were made using Buffer EB. Finally, 1 µL of sequencing primer $Cest5_{seq}$ (5' GATTCTTTTTAGGGGAAGG 3') or Cest4 (Trachsel et al., 2007) was added to each final solution of *Taenia* spp. or *E. granulosus*, respectively.

DNA sequencing was performed by Synergene Biotech GmbH, Biotech Center Zurich, Switzerland (http://www.synergene-biotech.com). Results were analyzed using free software FinchTV by Geospiza, Inc., Seattle (http://www.geospiza.com) and compared with GenBank nucleotide collection with BLAST (Basic Local Alignment Search Tool) with high similar sequences (megablast) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.8. Statistical Analysis

For Statistical Analysis the free software Quantitative Parasitology 3.0 was used. It was developed by Reiczigel & Rózsa (2005), Budapest, and available online (http://www.zoologia.hu/qp/qp.html).

For Taeniid and *Toxocara* spp. eggs positive samples prevalences were determined, as well as the confidence interval (95%) of the prevalence.

According to the Portuguese Red Book of Vetertebrates, the Portuguese population of Iberian wolf is estimated in 200-400 individuals (Cabral et al., 2008). The sample size (n = 68) was good, accounting for approximately 20% of the number of wolves in Portugal. Since sample size, though good, was small in number, to compare prevalence rates between wolf subpopulations (two independent groups), the non-parametric Fisher's Exact Test was used, with a critic value of p = 0.05. For this test only, the online free software GraphPad was used (available at: http://www.graphpad.com).<

The non-parametric McNemar's test was used to compare results obtained by the two methods of egg isolation (dependent groups) – Sedimentation-Flotation with saturated sacharose solution (done at the FVM-TUL) and the Sieving-flotation technique (performed at the IPZ-UZH).

A statistical analysis comparing parasite infections in the Northern area of study with Elvas Municipality was not performed because methods used to collect the samples were different which could give bias results. In addition, number of samples from dogs and foxes was low, both in the Northern and Southern area, not being a significant sample, which makes difficult to infer conclusions with these results. Unfortunately, for logistical questions, it was not possible to collect a higher amount of feces and so no attempt was made for statistical analysis in these samples.

5. Results

5.1. *Taenia* spp. and *Echinococcus* spp.

Of the 181 samples, 19 were considered positive for taeniid eggs (Figure 10) and taken to Multiplex-PCR analysis (Figure 11). Results of Microscopy, Multiplex-PCR and Sequencing are listed in Table 12. Detailed results of sequencing and GenBank analysis can be seen in Annex 1.

One wolf fecal sample, positive for taeniid eggs but negative in the Multiplex-PCR, did not have fecal material left to repeat the DNA isolation, so species identification was not possible.

Sequencing revealed *Echinococcus canadensis* and four *Taenia* species.

Figure 10 – Taeniid eggs in a wolf fecal sample positive for *T. hydatigena.* (Original pictures)

(Scale bar: 50 µm)

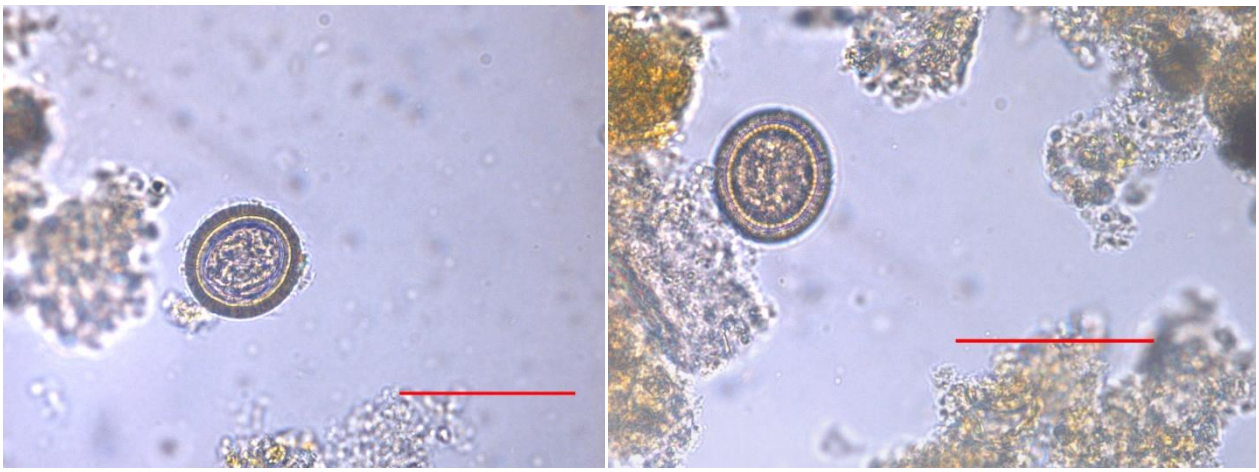


Figure 11 – Example of Multiplex-PCR results of two samples (Original picture).

[1 – 100 bp DNA ladder; 2 – positive sample for *Taenia* sp.; 3 – positive sample for *Taenia* sp. and *E. granulosus*; 5 – negative control; 7 – positive control for *E. granulosus* (117 bp); 8 – positive control for *Taenia* sp. (267 bp); 9 – positive control for *E. multilocularis* (395 bp); lanes 4 and 6 were intentionally left empty].

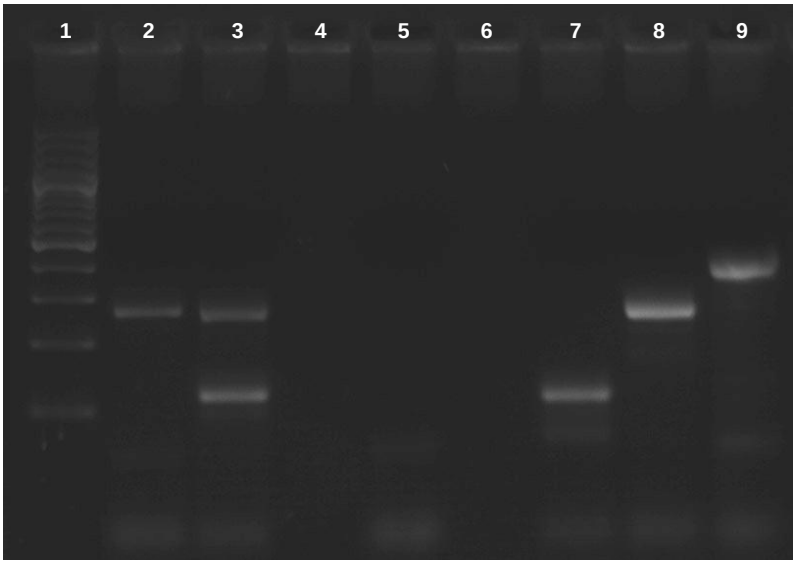


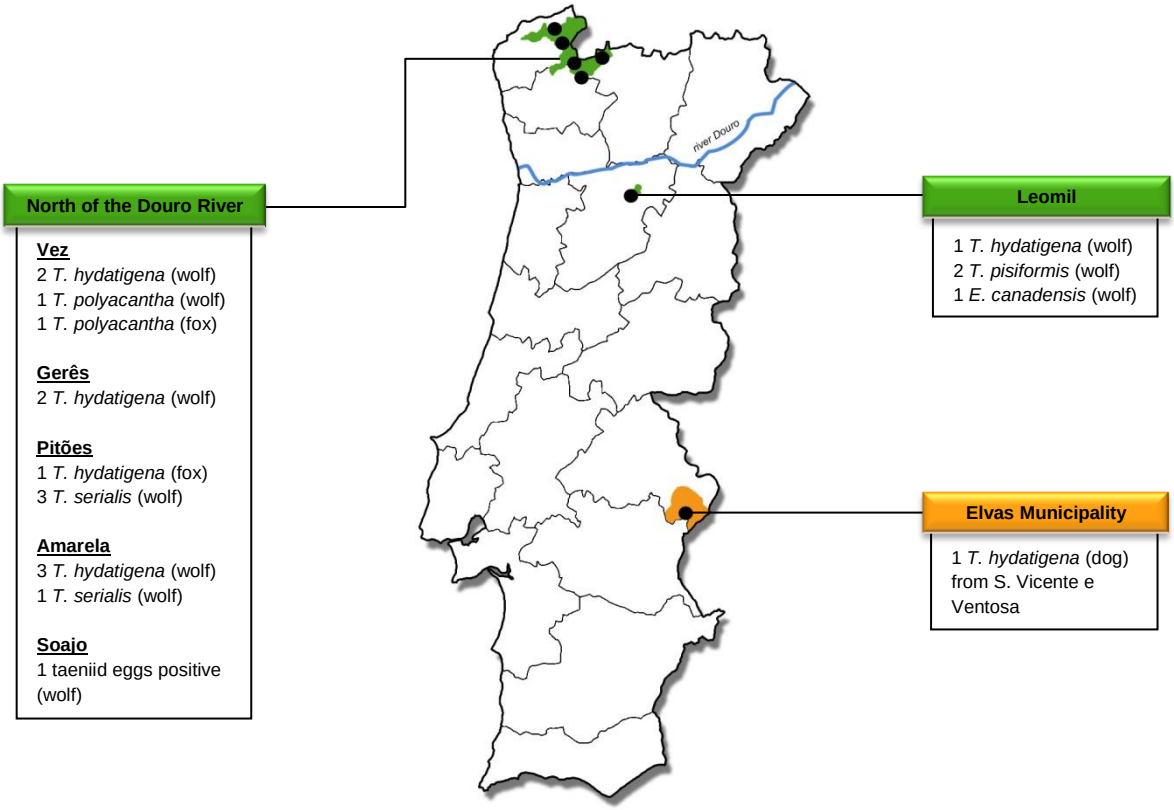
Table 12 – Positive fecal samples for Taeniid eggs and results of Multiplex PCR and Sequencing.

[Results are presented as the absolute frequency, followed by prevalence % (CI 95%)]

	Northern area of study			Elvas Municipality	
	Wolf (n = 68)	**Fox (n = 33)**	**Dog (n = 16)**	**Fox (n = 29)**	**Dog (n = 35)**
Microscopy result					
Taeniid eggs positive	16 23.5 (14.5 – 35.2)	2 6.1 (1.1 – 19.4)	-	-	1 2.9 (0.1 – 15.2)
Muliplex-PCR results					
Taenia sp.	15 22.1 (13.6 – 33.7)	2 6.1 (1.1 – 19.4)	-	-	1 2.9 (0.1 – 15.2)
Taenia sp. + E. granulosus	1 1.5 (0.8 – 7.8)	-	-	-	-
Sequencing results					
E. canadensis	1 1.5 (0.8 – 7.8)	-	-	-	-
T. hydatigena	8 11.8 (5.5 – 21.9)	1 3.0 (0.1 – 16.1)	-	-	1 2.9 (0.1 – 15.2)
T. polyacantha	1 1.5 (0.1 – 7.8)	1 3.0 (0.1 – 16.1)	-	-	-
T. pisiformis	2 2.9 (0.5 – 10.1)	-	-	-	-
T. serialis	4 5.9 (2.0 – 14.5)	-	-	-	-

Overall distribution of taeniid species and its geographic location can be seen in Figure 12.

Figure 12 – Approximate geographic location of samples positive for taeniid species.



Note: In the boxes are indicated: number of positive samples followed by the taeniid species (and host species)

Comparisons of Taeniid species between the two subpopulations of Iberian wolf are listed in Table 13.

Table 13 – Prevalence of Taeniid species per subpopulation of Iberian wolf.

	Northern subpopulation (n = 57)	**Southern subpopulation (n = 11)**	**Fisher's exact test p value (2-sided)**
Taeniid eggs	13 22.8 (13.5 – 35.9)	3 27.3 (7.9 – 60.0)	0.712
E. canadensis	0 (0 – 6.6)	1 9.1 (0.5 – 40.4)	0.162
T. hydatigena	7 12.3 (5.9 – 23.5)	1 9.1 (0.5 – 40.4)	1.000
T. serialis	4 7.0 (2.4 – 17.3)	0 (0 – 26.5)	1.000
T. polyacantha	1 1.8 (0.0 – 9.3)	0 (0 – 26.5)	1.000
T. pisiformis	0 (0 – 6.6)	2 18.2 (3.3 – 50.0)	0.024*

For each sample is given absolute frequency followed by prevalence % (confidence interval 95%)
* significant value ($p < 0.05$)

Number of taeniid eggs per gram (EPG) for each sample can be seen in Table 14. Results were compared with the ones previously obtained in a flotation technique with saturated saccharose solution [data from Silva (personal communication, September 2011) and Valverde (personal communication, May 2011)].

Table 14 – Epg of Taeniid samples per host species.

Host	**Sample[1]**	**Species**	**Epg**	**Previous results***
Wolf	G061	*T. hydatigena*	1.15	-
	G104	*Taenia* sp.	0.47	-
	G118	*T. hydatigena*	2.45	-
	G135	*T. polyacantha*	1.36	-
	G145	*T. hydatigena*	1.82	-
	G182	*T. serialis*	1.60	**+**
	G184	*T. serialis*	> 10.15	-
	G188	*T. serialis*	2.26	-
	G224	*T. hydatigena*	0.25	-
	G254	*T. hydatigena*	6.90	-
	G255	*T. serialis*	2.60	**+**
	G261	*T. hydatigena*	0.25	-
	G262	*T. hydatigena*	0.43	-
	G275	*T. pisiformis*	> 9.66	**+**
	G278	*T. pisiformis*	0.47	-
	G301	*T. hydatigena* *E. canadensis*	> 9.66	**+**
Fox	G136	*T. polyacantha*	3.14	-
	G179	*T. hydatigena*	2.47	-
Dog	E026	*T. hydatigena*	> 9.71	**+**

[1] Samples beginning with and "G"or "E" belonged to the Northern or Southern areas of study, respectively.
* Positivity for Taeniid eggs in flotation. + positive result; - negative result.

Comparison was made between results obtained with Sieving-Flotation technique (18 taeniid eggs positive in 117 samples) with the ones obtained previously by Silva (2010) (4 positive samples in 117) (Table 15). McNemar's test p value was 0.0005 (< 0.05). No differences were obtained in the results from the Elvas Municipality, so no statiscal analysis was performed.

Table 15 – Frequency Table comparing two techniques to isolate taeniid eggs from fecal samples of the Northern Area of study.

		Flotation Technique [1]		
		(+)	(-)	total
Sieving-Flotation Technique	(+)	4	14	18
	(-)	0	99	99
	total	4	113	117

[1] Data from Silva (personal communication, September 2011)
(+) positive result; (-) negative result

Multiple parasitism was found in 6 out of the 15 wolf sample with taeniids. Four samples were also positive for hookworms, 1 for *Toxocara* sp. and 1 for *Trichuris vulpis.* The two positive fox samples also revelead positive results for hookworms (n = 2) and *Toxocara* sp. (n = 1). The dog sample did not contain other parasites.

5.2. *Toxocara* spp.

Toxocara spp. eggs were found in 16 samples with the following distribution (see Table 16). Examples of isolated *Toxocara* sp. eggs are in Figure 13.

Table 16 – Positive fecal samples for *Toxocara* sp. eggs per area of study.

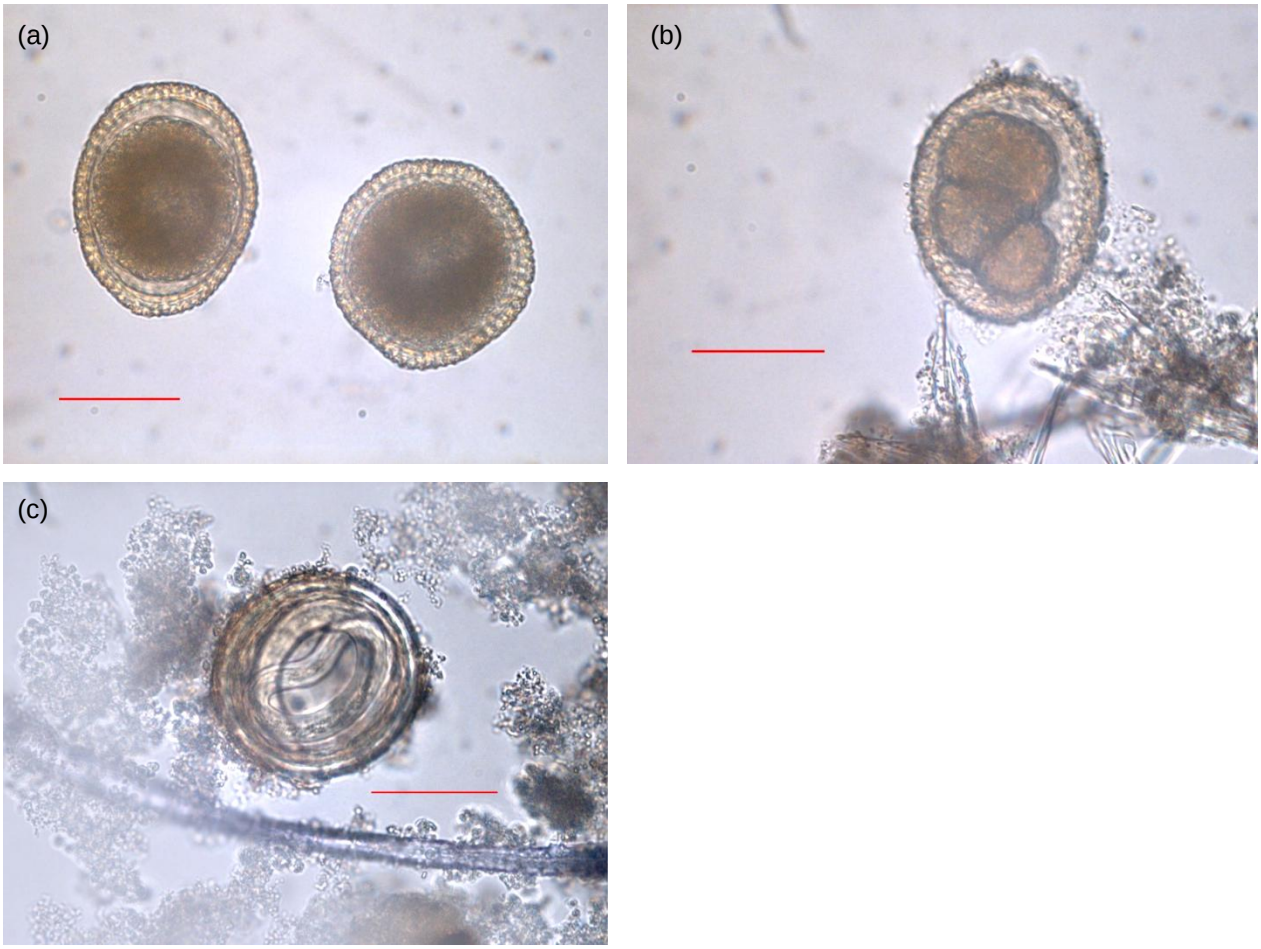
Parasite	**Northern area of study**			**Elvas Municipality**	
	Wolf (n = 68)	**Fox (n = 33)**	**Dog (n = 16)**	**Fox (n = 29)**	**Dog (n = 35)**
Toxocara spp.	8 11.8 (5.5 – 21.9)	4 12.1 (4.3 – 28.5)	0 (0.0 – 20.8)	3 10.3 (2.9 – 27.2)	1 2.9 (0.2 – 15.2)

Note: Results are presented as the absolute frequency, followed by prevalence % (CI 95%)

In wolves, no *Toxocara* sp. eggs were found in the southern subpopulation. Fisher's exact test p value was 0.337 comparing the two prevalences (0.0% in the southern subpopulation versus 14.0% in the northern one).

Figure 13 – (a) unembryonated (b) embryonated and (c) larvated *Toxocara* spp. eggs recovered from fecal samples (Original pictures).

(Scale bar: 50 µm)



Eggs classification per sample, as well as egg measurements and respective comparsion with *T. cati* and *T. canis* reference values (Fahrion et al., 2011) are listed in Table 17.

Table 17 – EPG, egg classification and maximum and minimum length of *Toxocara* spp. eggs.

Host	Sample[1]	Egg classification[2]				No. of eggs measured	Average (µm)	*Toxocara* sp.
		NV	UE	E	L			
Dog	E008	-	+	-	-	10	80,1 – 91,3	*T. canis*
Fox	E035	-	+	-	-	3	70,5 – 89,2	*T. canis*
	E036	-	+	+	-	2	79,9 – 91,4	*T. canis*
	E038	-	+	+	-	5	75,8 – 88,3	*T. canis*
	G008*	-	+	-	-	0	(-)	*Toxocara sp.*
	G108	-	+	+	-	6	70,9 – 83,3	*T. canis*
	G124	-	+	+	-	10	72,2 – 82,9	*T. canis*
	G136	-	+	+	-	10	72,7 – 79,0	*T. canis*

[1] Samples beginning with and "G" and "E" belong to the Northern or Southern areas of study, respectively.

[2] For each sample are indicated qualitative cathegories, according to the morphology of eggs present: NV – non-viable; UE – unembryonated; E – embryonated (morula); L - larvated

* EPG was to small and it was not possible to collect and mesure eggs.

- negative; + positive; (-) not done

Table 17 – continuation.

Host	Sample[1]	Egg classification[2]				No. of eggs measured	Average (µm)	*Toxocara* sp.
		NV	UE	E	L			
Wolf	G002*	-	+	-	-	0	(-)	*Toxocara sp.*
	G084*	-	+	-	-	0	(-)	*Toxocara sp.*
	G097	-	+	+	-	1	81,7 - 90,4	*T. canis*
	G114*	-	+	-	-	0	(-)	*Toxocara sp.*
	G117	-	+	+	+	9	74,3 – 87,5	*T. canis*
	G135	-	+	+	-	10	71,4 – 81,6	*T. canis*
	G183*	-	+	-	-	0	(-)	*Toxocara sp.*
	G258	-	+	+	+	4	77,6 – 83,3	*T. canis*

[1] Samples beginning with and "G" and "E" belong to the Northern or Southern areas of study, respectively.
[2] For each sample are indicated qualitative cathegories, according to the morphology of eggs present: NV – non-viable; UE – unembryonated; E – embryonated (morula); L - larvated
* EPG was to small and it was not possible to collect and mesure eggs.
- negative; + positive; (-) not done
Some eggs in G117 samples exhibited living larvae inside, moving.

From the total samples, 9 were positive in both techniques, and eight just in this study (Table 18). Unlike taeniid eggs, some species had been previously positive for *Toxocara* eggs but were negative in this study (see Table 19).

Table 18 – EPG and comparison of positive results of Sieving-flotationg technique with the ones obtained previously.

Host	Sample	Epg	Result this study	Previous results*
Dog	E008	35.7	+	-
Fox	E035	5.7	+	-
	E036	5.3	+	+
	E038	9.9	+	+
	G008	1.1	+	+
	G108	7.7	+	+
	G124	> 17.7	+	+
	G136	> 25.2	+	+
Wolf	G002	0.6	+	-
	G084	1.5	+	-
	G097	0.5	+	-
	G114	0.5	+	-
	G117	16.8	+	+
	G135	> 18.2	+	+
	G183	0.5	+	-
	G258	0.8	+	+

[1] Samples beginning with and "G"or "E" belonged to the Northern or Southern areas of study, respectively.
*Data obtained from Silva (personal communication), Valverde (personal communication) and data not shown.
+ positive result
- negative result

Table 19 – Frequency Table comparing two techniques to isolate *Toxocara* spp. eggs from fecal samples.

		Flotation Technique								
		Northern Area of Study[1]			**Elvas Municipality (fox)[2]**			**Elvas Municipality (dog)[3]**		
		(+)	(-)	total	(+)	(-)	total	(+)	(-)	total
Sieving-Flotation Technique	(+)	7	5	12	2	1	3	0	1	1
	(-)	8	93	105	0	26	26	0	32	32
	total	15	98	117	2	27	29	0	33	33
P value in McNemar's test		0.5791			1.0000			1.0000		

[1] Data from Silva (person. communication), [2] Valverde (person. communication) and [3] data not shown.
(+) positive result; (-) negative result

From the eight wolf samples positive for *Toxocara* eggs, four were also positive for other parasites: *Toxascaris leonina* (n = 2), *Trichuris vulpis* (n = 1), and *T. polyacantha* (n = 1). Also, the four foxes from the Northern study regions all had other parasite infections, namely hookworms (n = 2), *T. leonina* (n = 1) and *T. polyacantha* (n = 1).

In the Municipality of Elvas, the three positive foxes also had hookworms (n = 3), trematodes (n = 2) and *Spirocerca lupi* (n = 1) and the dog also shed eggs of tremadotes and *Capillaria* sp.

5.3 Other parasites

With the Sieving and Flotation procedure were found other species of parasites. The prevalences found in each species are listed in Table 20.

Table 20 – Other parasites identified in the sieving procedure.

Parasite	North			Alentejo	
	Wolf (n = 68)	**Fox (n = 33)**	**Dog (n = 16)**	**Fox (n = 29)**	**Dog (n = 35)**
Acanthocephala					
Macracanthorhinchus sp.	-	-	-	2 6.9 (1.2 – 22.1)	-
Cestodes					
Mesocestoides spp. and Hymenolepis spp.	-	1 3.0 (0.2 – 16.1)	-	-	2 5.7 (1.0 – 19.5)
Nematodes					
Capillaria sp.	-	1 3.0 (0.2 – 16.1)	3 18.8 (5.3 – 43.6)	3 10.3 (2.9 – 27.2)	4 11.4 (4.0 – 26.8)
Hookworms	12 17.6 (10.1 – 28-6)	8 24.2 (11.7 – 42.3)	5 31.3 (13.2 – 56.4)	19 65.5 (46.5 – 81.6)	10 28.6 (15.2 – 45.7)
Physaloptera sp.	-	-	-	3 10.3 (2.9 – 27.2)	1 2.9 (0.2 – 15.2)
Spirocerca sp.	-	-	-	1 3.4 (0.2 – 16.9)	-
Strongyloides sp.	1 1.5 (0.1 – 7.8)	3 9.1 (2.5 – 23.9)	1 6.3 (0.3 – 30.5)	-	1 2.9 (0.2 – 15.2)
Toxascaris leonina	5 7.4 (3.0 – 16.0)	2 6.1 (1.1 – 19.4)	-	-	-
Trichuris spp.*	1 1.5 (0.1 – 7.8)	-	-	-	-
Trichuris vulpis	4 5.9 (2.0 – 14.5)	2 6.1 (1.1 – 19.4)	8 50.0 (27.2 – 72.8)	-	5 14.3 (5.8 – 29.8)
Trematodes					
Trematodes	-	1 3.0 (0.2 – 16.1)	-	12 41.4 (24.7 – 60.5)	5 14.3 (5.8 – 29.8)

Note: Results are presented as the absolute frequency, followed by prevalence % (CI 95%)

* One dog had *Trichuris* spp. positive; eggs dimensions were too small to be *Trichuris vulpis* and most compatible with *T. muris* or *T. leporis*.

6. Discussion

6.1. *Taenia* spp. and *Echinococcus* spp.

Iberian Wolf

Overall prevalence rates for taeniid eggs in wolves were 23.5%. Since this is the first study employing these techniques in the Iberian Peninsula is difficult to compare values. However, one can see that prevalences fall between the results of coprology and necropsy obtained in nearby regions from Portugal and Spain (Figure 3).

This was the first study to address the species of taeniids infecting wolves in Portugal and since size was good, conclusions can be withdrawn with relatively good certainties. This is also the first time genetic characterization was performed in isolates from wild definitive hosts in Portugal.

The most common taeniid species found in wolves was *T. hydatigena* with an overall prevalence of 11.8% and with no significant differences between the two subpopulations. Similar results were found by Torres et al. (2000) and Segovia et al. (2001). A review by Craig & Craig (2005) on helminthfauna of wolves also revealed that *T. hydatigena* was the most common cestode in all the 25 studies cited. An ecological analysis of the intestinal helminth community of Iberian wolves found *T. hydatigena* as a core species, not only one of the most prevalent, but also one with the highest mean abundance of worms per wolf (Segovia et al., 2003). Likewise, in a study in Italy, *T. hydatigena* was considered a dominant species, i.e., characteristic of the populations and well adapted to the host (Guberti, Bolognini, Lanfranchi & Battelli, 2004). This association would contribute to the maintenance of the parasite's life cycle. Similar assumptions can be made here. Because wild ungulates are scarce, wolf populations in Portugal, strongly depend on human activity and their distribution follows the areas where livestock is farmed (Carreira & Petrucci-Fonseca, 2000). The main preys of wolf in this region are domestic goats (in both subpopulations) and horses and cattle (especially in the northern subpopulation) (Álvares, Pereira & Petrucci-Fonseca, 2000). All these three ungulates are known intermediate hosts of this species in the Iberian Peninsula. This explains the high prevalences found for this tapeworm and it is likely that a synanthropic cycle between wolves-domestic ungulates, or even a sylvatic between wolves-wild ungulates, exists in the North of Portugal.

T. serialis was the second most common taeniid species with a prevalence of 5.9%. In Galicia it was already found in wolves and rodents, and wild rabbit is also a known intermediate host. Lagomorphs and micromammals represent only 1.5% and 1.6%, respectively, of the wolf diet in Peneda Gerês National Park (Álvares et al., 2000) and 3% in the south, in Alvão Natural Park (Carreira & Petrucci-Fonseca, 2000). *T. pisiformis*, which has similar intermediate hosts as *T. serialis*, was only found in the southern subpopulation, with significant differences arising, relatively to the northern subpopulation. In other studies, *T. pisiformis* also was not found in the

more Northern areas of Spain, but positive results were obtained in Castilla y Léon (see Figure 3). Cysterci from this species heavily infect lagomorphs in the Center and Southern parts of Portugal. Inversely, *T. serialis* was only found in Galicia, but not in Asturias or Castilla y Léon. Since small mammals are of low importance in wolf diet it is understandable the lower results, comparatively to *T. hydatigena*. Although assumptions need to be made carefully in this point, the apparent geographic differences may reflect distinct availability of preys and parasite distribution. Wild rabbits are more common in the South of Portugal than in North (Carvalho & Gomes, 2004), but still *T. serialis* was found in the northern latitudes. Studies in intermediate hosts in these areas are needed to clarify the presence/absence of these parasites. Prevalence of *T. pisiformis* was also lower than *T. serialis* in other studies in Spain (Segovia et al., 2003).

This is the first time *T. polyacantha* has been found in Iberian wolves. Rodents are the main intermediate hosts and it is possible that infection occurs after predation. In Eastern Europe it is known to infect wolves (Craig & Craig, 2005). Although no information was found about intermediate hosts, this species was already found on foxes in Portugal (Carvalho-Varela & Marcos, 1993).

T. multiceps was not found in this study. It was surprising, since it has the same intermediate hosts as *T. hydatigena*, and in other studies in Spain it was the second most common tapeworm, with prevalences of 16.7-50.0% in necropsied wolves (Torres et al., 2000; Segovia et al., 2001). Explanations to this fact are difficult. Different parasite distributions in the Northern part of the Iberian Peninsula may explain it. Sheep, because of their feeding behavior, are usually the most susceptible to be infected by taeniid eggs. If wolves seldom feed on sheep in the study areas, maybe this could explain the absence of this parasite.

E. canadensis was found for the first time in Iberian wolf and, as far as we know, also for the first time in wolves in Europe. Although the amplified materials was small (75 bp), GenBank analysis revealed 100% similarity only with G6/G7 isolates, namely a G7 isolated from a pig in Slovakia (Annex 1). This two genotypes are very similar, but background information supports that the genotype here is G7 (pig strain), which is known to occur in domestic pigs in the Northern part of the country. The wolf fecal sample positive for *Echinococcus* sp., was from the southern subpopulation, where wild boars are more available and the second most important prey in their diet, after goats (Carreira & Petrucci-Fonseca, 2000). This wolf was also shedding eggs from *T. hydatigena*.

Sample is too small to comment on prevalence, although it confirms that wolf can also participate in *E. canadensis* (G7) life cycle. In the Iberian Peninsula, this genotype infects not only domestic pigs, but also wild boars and goats. Since in the last host there is a higher percentage of infertile cysts (Mwambete et al., 2004), transmission from wild boars should be more important.

No genetic studies are available addressing the genotype mostly infecting humans in Portugal. In Spain, the few analyzed isolates from human patients all belonged to the G1 genotype (Mwambete et al., 2004). In Austria, a higher prevalence was found for G7 (Schneider et al., 2010).

Iberian wolf was already a known definitive host for *E. granulosus* sensu strico G1 (Sobrino et al., 2006) and now infection with *E. canadensis* (G6) was found. *E. equinus* (G4) is also present in horses from nearby regions, and horses are very important preys in wolf diet, especially in the North of Portugal, where they are preferable if available (Carreira & Petrucci-Fonseca, 2000). If wolves are found infected with *E. equinus* (G4), this canid species can be truly considered the most important definitive host for *E. granulosus* in Iberian Peninsula. Still, the fact that it is included in a great variety of life cycles with ungulates (*E. granulosus* G1) and domestic or wild pigs (*E. canadensis* G7) already supports this hypothesis. More importantly, since wild boar and wild ungulates can be infected with fertile cysts of *E. granulosus* sensu lato, one can conclude that at least two genuine sylvatic cycles can develop, independently of human activity. The synanthropic cycle between wolf and domestic ungulates is also present, which maintains the infection. Wolves feed from trash sites too, near urban areas (Carreira & Petrucci-Fonseca, 2000), and this can be another contact point with human populations. Altogether, the data is very relevant if serious control programs are attempted, since focus should be made not only on the domestic cycle, but also in the sylvatic one, which helps maintaining infection pressure to ungulates an humans.

Red Fox

Both *T. hydatigena* and *T. polyacantha* were found in foxes from the Northern area of study. As previously stated, fox sample was too small to infer strong conclusions.

In other studies in Portugal, prevalences of tapeworms on foxes did not reveal a dominant species (see Table 4), which reflects the generalist feeding behavior of this canid. These results also support that. Foxes diet strongly depends upon their ecosystem. In the North of Portugal, rodents are the most common species (which explains the *T. polyacantha* infections), although rabbits and insects can also be used in a lower degree (Carvalho & Gomes, 2004). Carcasses of sheep and other ungulates left on the field after predation or disease and viscera from home slaughters are available to foxes that can then be infected with *T. hydatigena* or *T. multiceps.* Still, wolves, and may be dogs, are the most important definitive hosts. All the others species of *Taenia* addressed in this study use lagomorphs and rodents, and so foxes are potential hosts.

No *E. granulosus* was found, which is in concordance with previous studies. Foxes are important hosts for *E. multilocularis* in Center-eastern Europe, but they do not seem very adapted hosts for *E. granulosus* and when found, prevalences of infection were low (Eckert et al, 2001a; Jones & Pybus, 2001).

The negative results found in foxes from Elvas Municipality were surprising. Either sample size or prevalence of infection was too small, or maybe both. The most important question in study was whether foxes from the South of Portugal played an important role in the epidemiology of *T. pisiformis,* which infects large numbers of wild rabbit in this area. Wild rabbit, and probably hare, are the most important prey of foxes in Southern Portugal. Still, background information supports that foxes are not very good hosts for *T. pisiformis*. Beveridge & Rickard (1975) succeeded in experimentally infecting foxes with *T. pisiformis*. However in this species, adults were significantly smaller than the ones retrieved from dogs, and no gravid proglottids were found at the time when that had already happened in dogs. Prevalence of *T. pisiformis* in foxes is low in the majority of studies consulted (Carvalho-Varela & Marcos, 1993; Jones & Pybus, 2001; Eira et al., 2006). Further statements will be made about this topic when discussing dogs.

Dog

Just 16 samples were collected from dogs in the North of Portugal and none was positive for taeniid eggs. Again, this was a very small sample because 2.3% of dogs were found positive for taeniid eggs in this same area by Silva (2010) using a larger sample (n = 39). Dogs, most probably stray or sheep dogs, can have similar role as wolf in the epidemiology of all these tapeworms, although wolf, being a top predator and hunting large ungulates may have a higher importance. However, transmission of tapeworms to humans is indeed mostly done by dogs. In further studies, larger samples should be taken to study these subjects.

In the dogs from Elvas Municipality, only one was found positive for *T. hydatigena*. It was a one-year-old female sheep dog, which was under control with praziquantel and had concurrent coccidiosis (data not shown). It is known that home slaughter of pigs and sheep still occurs in rural areas where dogs are fed potential infected viscera and veterinary care is often neglected.

According to Cordero del Campilho & Rojo-Vázquez (1999), in sheep dogs the most probable taeniids would be *T. hydatigena, T. multiceps* and *E. granulosos*, whereas in hunting dogs, with higher contact with smaller mammals, *T. pisiformis, T. taeniaeformis* and *T. serialis* would be more frequent.

If the assumption is correct that foxes are not very suitable hosts for *T. pisiformis*, then dogs, especially hunting dogs would play the crucial role in maintaining its life cycle in this area. In this study, we could see that deworming of dogs in this region rarely occurs. Usually it is done once a year in the time of rabies vaccination but this does not cover at all the small pre-patent period for taeniasis. This is shown by the positive dog found with *T. hydatigena*.

To correctly address the problem of *T. pisiformis*, and to test the hypothesis that hunting dog may be part of the source problem, it would be interesting to study dog infections before and after hunting season, and see if prevalence of infection with taeniid species increased after.

Concerning *E. granulosus*, dog seems the only suitable definitive host in this area where the incidence of human cases is the highest in the country. A rural domestic cycle between sheep and dog (especially sheep dog) is the most probable.

Comparison of Techniques for Taeniid eggs Isolation

One sample did not give a positive result in PCR. In this sample only one egg had been found. Since this Multiplex-PCR is capable of amplifying DNA from one single egg (Traschel et al., 2007), maybe previous steps, such as DNA extraction, failed to correctly do that. Since no fecal samples remained, it was impossible to identify the species.

A high significant value was found with McNemar's test which proves the Sieving-flotation technique is much more sensitive to isolate taeniid eggs from fecal samples, finding more than four times positive samples. In the studies of the Southern area, with foxes and dogs, no differences were found. The positive results in both studies occurred when high EPG were found (> 1.6; average 6.1). Likewise, in Bružinskaitėa, Šarkūnasa, Torgerson, Mathis & Deplazes (2009), *Echinococcus* samples with low infections were not detected in the McMaster method but were identified with the Sieving-flotation technique.

Increasing the amount of fecal samples analyzed (4 g instead of 2 g) may have resulted in higher sensitivity of the method. One important fact is that 10 out of the 14 (71.4%) samples with false negative results were mainly fur, nine of them from wolves (data not shown). Eggs easily stick to fur and so the incubation done with PBS-Tween may have helped in detachment of eggs, allowing a higher recovery. Overall, this technique presents a very good alternative to the classic coprologic methods, if sample size is not very big, and especially in wolf fecal samples which frequently have high amounts of fur.

6.2. *Toxocara* spp.

Iberian wolf

Although in some cases *Toxocara* species couldn't be identified, all the remaining revealed *T. canis* infections. The problem with the first samples was that EPG was too low, often with only one egg found, which was difficult to recover after centrifugation.

The prevalence of this parasite found in wolves was 11.8% with no significant differences between the two subpopulations. This value was a little higher than the 5.0% and 6.4% obtained in Northern Spain by Torres et al. (2000) and Segovia et al. (2001), respectively. Although no data could be collected about the average age of the animals, the sampling in the Northern area of this study took place between October and January. Since births occur before Fall (Álvares et al., 2000), and postparturient bitches may also shed eggs, we could assume that there is a higher possibility of patent infections being sampled at least in the samples collected in the middle of Autumn.

Curiously, in the study from Segovia et al. (2001), all the worms were found in adult animals. In Italy, Guberti et al. (2004) found higher prevalence of *Toxocara canis* in puppies, whereas in adults *E. granulosus* and *Trichuris vulpis* were most frequent.

Since wolves are gregarian species, transmission of *Toxocara* sp. is enhanced in wolf packs. Moreover, they can also feed on paratenic hosts such as rodents. An interesting fact is that wolves in Portugal can feed on carnivores as well (Álvares et al., 2000; Carreira & Petrucci-Fonseca, 2000). Foxes, and especially dogs, are sometimes preyed. If these animals are parasitized with larvae or adults, there is the possibility of transmission.

Most of the EPG found in wolves was low, although more than reflecting low parasite burden, this could be related to an advanced time in the patent period.

Red fox

Similar prevalences were found in foxes from both study areas and similar to the results found by Carvalho-Varela & Marcos (1993) in all the Portuguese territory (11.1%). Some authors already addressed the link between *T. canis* and reproductive cycle of foxes, finding higher prevalences in Spring and Summer, after births (Saeed et al., 2006). On this study samples were collected after this period – October-January (North) and December-February (Elvas). We can conclude that the majority of sampled animals were adults. Interestingly, this suggests that adults are as well susceptible to infection and to tracheal migration, being able to shed eggs in feces.

From the studies consulted, prevalence in foxes seems to be higher than in wolves. Silva (2010) found significant differences between the prevalences of *Toxocara* sp. in wolves (7.3%) and foxes (24.7%) in Peneda Gerês National Park. Since rodents and lagomorphs, as well as birds and insects are important in fox diet, is it understandable why they present higher prevalences. Furthermore, foxes are also known definitive hosts for *T. cati*, although low prevalences are usually found.

As previously stated, periurban areas are probably the places where the link between the domestic and sylvatic cycle occurs. Red fox populations are widespread in Portugal and it is considered a species of Least Concern (LC) according to the Red Book of Portuguese Vertebrates. Associating this to the probably higher infections rates and the possibility of infection by *T. cati*, red fox may be the most important definitive host in the sylvatic cycle, also participating in a synanthropic cycle in rural and periurban areas.

Dog

A negative result was again obtained in dogs from the Northern area of study, which reflects the low number of animals sampled, since Silva (2010) obtained a prevalence of 10.3% in dogs from the same area. In the Municipality of Elvas, only one positive case was diagnosed in a male

hunting dog. Again the sample was very small. Travanca & Grácio (1993) found a prevalence of 10.3% in dogs from the same district.

The finding of this positive case makes sense since hunting dogs have higher contact with paratenic hosts and also are kept together with other dogs, which facilitates contact transmission.

Toxocara infects both stray, kennel and domestic dogs in all Portuguese territory and the presence of *T. canis* and *T. cati* in urban areas is evident. Dogs are again the most important link with humans, transmitting the parasite between the domestic and synanthropic cycles.

Comparison of Techniques for *Toxocara* sp. eggs isolation

The different techniques exhibited mismatched results. In Elvas Municipality a higher sensibility was obtained with Sieving-flotation technique with no false negatives, although no significant difference was found. In the Northern area of study, from the 12 positive samples detected with the Sieving-flotation technique, only 7 were also positive in the common Flotation technique. All seven samples positive in both tests with exception of one (EPG = 35.7), had EPG lower than 5.7 and the majority lower than 1.5. Nevertheless, eight samples were false negatives with the Sieving-flotation technique. From these, 5 were considered dry in the preliminary evaluation of fecal material, but when they were first analyzed by Flotation technique they were classified as fresh. *Toxocara* sp. eggs are very sensitive to low humidity, and it is most probable that the false results obtained are a result of a destruction or deformation of eggs rather than a low sensitivity of the technique. It must be reminded that some of these samples had been stored for almost three years. Other explanation may be the heterogeneous distribution of eggs in feces. Especially in dry samples or fur, homogenization of samples is difficult.

The Sieving-flotation technique seems also a relatively good technique to isolate *Toxocara* eggs; however, samples should be preferable fresh.

One important result was that nine of the 16 positive samples had embryonated eggs, one with living larvae. The majority of these samples had a mild humidity level. Gaspard, Ambolet & Schwartzbrod (1997) also found living eggs after two years at 4ºC. In this study, some eggs survived for a period of three years at 4º C. This confirms that *Toxocara* eggs are very resistant, especially if humidity and temperature are not too extreme. Contamination of public places is known in Portuguese cities and since a correlation was made between this risk factor and human infections, further attention should be paid as well as more monitoring of human infections to access the real problem.

6.3. Other Parasites

Other parasites were found in this study. However, one should remember that the eggs isolated are between 21 and 99 µm, and so this is not a true reflection of the parasite community of canids

studied. The majority of protozoa were lost. Another example is the hookworms. They were still the most prevalent parasite found in almost all hosts. Nevertheless, especially in the Northern area of study infection rates were low, comparatively with previous results that revealed prevalences of 50% or higher (Silva, 2010).

The most interesting results were obtained in the trematodes and *Trichuris* sp. The trematode eggs found were most probably *Alaria* sp. a species known to infect foxes and dogs in Portugal (Eira et al., 2006). It has a complex life cycle, using amphibians, reptiles and rodents as intermediate hosts (Roberts, Janovy & Schmidt, 2009d). This trematode was mostly found in Elvas, with a high prevalence of 41.4% in foxes, which reflects the generalist feeding behavior of this carnivore. Moreover, it was also found in five hunting dogs, which is dangerous since it has zoonotic potential.

Trichuris vulpis was one of the most prevalent parasite found in dogs. This parasite is relevant in Veterinary Medicine since it can cause severe intestinal inflammation in dogs (Roberts et al., 2009d).

McNemar's test revealed significant differences between the two techniques (data not shown) for both Trematodes and *T. vulpis* which confirm that the Sieving-Flotation technique can be also used with very good results to detect this species. For *Alaria* sp., since eggs are larger than 100 µm, to increase the recovery rate, the first sieve used should be bigger than 120 µm.

Finally, the results obtained from the domestic dogs in Elvas, confirm that the treatment schedules and deworming strategy are not enough to control helminths in these dogs, especially the zoonotic ones, which can pose real health issues.

7. Conclusions and Further Studies

Toxocara and taeniid species can be considered neglected zoonoses and many reasons explain this: (i) these are not vector-borne diseases; (ii) they often occur in rural areas with poor hygiene and education levels, and so data available may be scarce; (iii) incubation periods are long both in human and animals and, since livestock is usually asymptomatic, few attention is paid by farmers; (iv) generally, they are not lethal diseases, but still they are resilient pathogens, with a chronic evolution that can cause impairment of some functions in the long-term; (v) control is difficult due to the life cycle of the parasites and lack of understanding towards the need of a frequent fecal analysis/deworming scheme.

These parasites are confirmed in Portugal where they affect wild and domestic animals and also human populations. Therefore, domestic, sylvatic and synanthropic life cycles are possible.

From the results of this study and the data from the literature review, one can conclude that dogs are the most important canids in the domestic cycle and eventually in transmitting these diseases to humans. Wolves and foxes are important hosts for maintaining the sylvatic and synanthropic cycles of taeniids and *Toxocara* sp, respectively. The existence of a sylvatic cycle, independent from human activity is an important fact. If control programs are attempted, focus should be made not only on dogs but also in wild canids.

For the first time *T. polyacantha* and *E. canadensis* (G7/pig strain) were detected in wolves from the Iberian Peninsula. Studies like this are important, particularly in an endangered species such as the Iberian wolf, as they help understanding ecological and biological aspects of these populations.

The Sieving-Flotation technique revealed a very good alternative to isolate *Toxocara*, and more importantly, taeniid eggs from fecal samples, especially in wolves. Measurement of eggs is a cheap way of identifying some parasite species and should always be a part of routine diagnosis.

All this work fits in the One Health concept; although the *Taenia* species addressed here are of lower zoonotic importance, the same does not happen with *Echinococcus* spp. and *Toxocara* spp. It is evident that controlling these helminths is a hard task and many barriers exist that postpone this final goal. Nevertheless, one should remind that education, especially of children, is a cheap and powerful tool to prevent dissemination at some degree and must not be forgotten.

The main goals of this study seem to be in generally fulfilled. Still this was a preliminary study, which poses several new questions to be addressed. Here are some of them:

(*Taeniids*)

a) New studies with larger samples should be made on definitive hosts. Besides, using copro-ELISA technique may be very useful in wild populations;

b) There is a lack of information regarding infection in intermediate hosts. For *Echinococcus* sp. it would be important to see the prevalence of infection in wild boars and other ungulates, identify the infecting genotype and cyst fertility. A curious study would be analyzing the horses from Portugal, particularly the wild breed of Garrano and search for *E. equinus.* Equally, this parasite should be searched in canid hosts;

c) No studies seem to identify the genotype mostly infecting humans in Portugal. It would be important to characterize it and understand which life cycle patterns are the most important;

d) The hypothesis that hunting dogs may be partially responsible for the high infestations of *T. pisiformis* in wild rabbit/hare population should be tested. It would be interesting to compare the infection rates in hunting dogs before and after the hunting season.

(*Toxocara*)

a) More studies should be made focusing on feline populations, not only to understand their role as reservoirs for *T. cati*, but also to check the overall parasite community in this species;

b) Human seroprevalences of *Toxocara* sp. should be evaluated, especially in veterinarians, veterinary students, hunters, farmers and other risk groups.

c) Snails are referred as paratenic hosts of *Toxocara* sp. Portugal has a very long tradition in dishes with snails and these can be tried everywhere, even in restaurants with low quality standards. Detecting the presence o *Toxocara* sp. in snails would be important to access the risk of transmission to humans by consumption of this species.

8. References

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Annex 1. Results of GenBank analysis

Study Area	Host	Sample	Species	Acession number	Query coverage	E value	Max ident
Northern Area of Study	**Wolf**	61	*T. hydatigena*	GQ228819	98%	3e-108	100% (216/216)
		118	*T. hydatigena*	GQ228819	100%	2e-94	100% (191/191)
		135	*T. polyacantha*	DQ408419	100%	1e-86	97,4% (182/191)
		145	*T. hydatigena*	GQ228819	99%	4e-107	100% (214/214)
		182	*T. serialis*	EU219546	98%	9e-93	99,5% (190/191)
		184	*T. serialis*	EU219546	100%	8e-109	99,1% (222/224)
		188	*T. serialis*	EU219546	99%	2e-105	99,5% (214/215)
		224	*T. hydatigena*	GQ228819	100%	2e-89	98% (187/190)
		254	*T. hydatigena*	GQ228819	100%	6e-110	100%(219/219)
		255	*T. serialis*	EU219546	100%	6e-110	98,6% (221/224)
		261	*T. hydatigena*	GQ228819	97%	2e-79	100% (164/164)
		262	*T. hydatigena*	GQ228819	74%	3e-109	100% (218/218)
		275	*T. pisiformis*	GU569096	100%	3e-108	99,5% (218/219)
		278	*T. pisiformis*	GU569096	100%	2e-105	98,6% (218/221)
		301	*T. hydatigena*	GQ228819	100%	6e-105	100% (210/210)
			E. canadensis	AY462128	100%	2e-30	100% (75/75)
	Fox	136	*T. polyacantha*	EU219542	82%	3e-73	97% (164/169)
		179	*T. hydatigena*	GQ228819	99%	1e-107	99,5% (217/218)
Elvas Municipality	**Dog**	26	*T. hydatigena*	GQ228819	99%	1e-107	100% (215/215)