A Study of Pathological Lesions in European badgers (*Meles meles*) with and without mycobacteriosis in England (northern Edge area)

Davide Corbetta DVM MRCVS AFHEA

Senior Clinical Training Scholar (Resident) in Veterinary Anatomic Pathology

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University of Nottingham, United Kingdom

Student ID: 4293396

Supervisors: Malcolm Bennett (year 1-2-3), Llorenç Grau Roma (year 1-2)

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Abbreviations

ACVP = American College of Veterinary Pathologists

AFHEA = Associate Fellow of Higher Education Academy

AIH = Autoimmune lymphocytic hepatitis

AMDV = Aleutian Mink Disease Virus

ATP = Associate Teaching Program

BBSRC = Biotechnology and Biological Sciences Research Council

BCS = Body Condition Score

BCG = Bacillus Calmette–Guérin

BSVP = British Society of Veterinary Pathology

bTB = Bovine Tuberculosis

BW = Body Weight

CD = Cluster of Differentiation

CDV = Canine Distemper Virus

CIN = Chronic Interstitial Nephritis

CL = Containment Level

CMI = Cell-Mediated Immunity

CPD = Continuous Professional Development

CTP = Clinical Training Program

DEFRA = Department of Environment, Food and Rural Affairs

DNA = Deoxyribonucleic Acid

DVM = Doctor of Veterinary Medicine

DVPS = Diagnostic Veterinary Pathology Service

ECVP = European College of Veterinary Pathologists

ECVCP = European College of Veterinary Clinical Pathologists

ESVP = European Society of Veterinary Pathologists

ESVCP = European Society of Veterinary Clinical Pathologists

ESAT-6 = Early Secreted Antigenic Target 6 kilodaltons

ESRC = Economic and Social Research Council

HRA = High Risk Area

HE = Haematoxylin and Eosin

HEV = Hepatitis E Virus

HPF = High Power Field

IFN-γ = Interferon gamma

IL = Interleukin

iNOS = Inducible Isoform of Nitric Oxide Synthase

IS6110 = Insertion Sequence 6110

IUCN = International Union for Conservation of Nature

ID = Identification

LRA = Low Risk Area

M. = *Mycobacterium*

M = Mycobacterial

MAC = *Mycobacterium avium* Complex

MAT = Microscopic Agglutination Test

MTB = *Mycobacterium tuberculosis*

MTC = *Mycobacterium tuberculosis* complex

MRCVS = Member of Royal College of Veterinary Surgeons

mRNA = messenger Ribonucleic Acid

MVM = Master of Veterinary Medicine

MZN = Modified Ziehl Neelsen

NO = Nitric Oxide

NOD2 = Nucleotide-binding Oligomerization Domain-containing protein 2

NTM = Non-Tuberculous Mycobacteria

PAS = Periodic Acid Schiff

PCR = Polymerase Chain Reaction

PhD = Doctor of Philosophy

PME = Post-mortem Examination

RSPCA = Royal Society for the Prevention of Cruelty to Animals

RVC = Royal Veterinary College

SD = Standard Deviation

SLMs = Silica-Laden Macrophages

SOPs = Standard Operating Procedures

SVMS = School of Veterinary Medicine and Science

TB = Tuberculosis

TLRs = Toll-Like Receptors

TNFα = Tumour necrosis factor-α

Th = lymphocyte T Helper

UNISVET = Unione Italiana Società Veterinarie

UoN = University of Nottingham

UK = United Kingdom

VBNC = Viable But Non-Culturable

WSC = Wednesday Slide Conference

ZN = Ziehl Neelsen

µm = Micrometre

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Abstract

European badgers (*Meles meles*) are omnivores members of the family Mustelidae and are widely present in all Europe and the United Kingdom. They are believed to play an important role in the epidemiology of bovine tuberculosis (bTB) in England and several other European countries. Previous studies focused on badgers from cattle bTB endemic areas. European badgers (*Meles meles*) are also known to be infected by a wide range of other bacterial, viral, protozoal and parasitic diseases.

The aim of this study was to evaluate non-traffic-injury-related macroscopic and histological lesions in road-killed badgers with and without mycobacteriosis from the northern edge of the bTB epidemic, which is considered an area of lower bTB prevalence.

Overall, 607 badger carcasses were collected from six counties in England (northern edge) and necropsied. During post-mortem examination, gross lesions were recorded, and several samples were collected for histopathology, culture and molecular analysis. A subset of eighty eight (n=88) badgers, 44 positive (M+) microbiologically confirmed mycobacteriosis (whether or not *Mycobacterium bovis*), and 44 negative (M-), were selected for histopathological examination.

Macroscopically, bite wounds show a trend towards significance in the M+ badgers. Histologically, in decreasing order of frequency, the most common findings were silica-laden macrophages (85%), granulomas (53%), sarcocystosis (47%), nephritis (31%), portal/periportal hepatitis (26%), and ulcerative dermatitis (18%). M+ badgers had a significantly higher prevalence of granulomas ($p < 0.001$) and lower prevalence of hepatitis ($p = 0.003$) than M- badgers. Of the 44 M+ badgers, 31 had *M. bovis* infection and 13 other mycobacterial infections. Sequencing of rRNA PCR amplicons identified *M. nonchromogenicum* and *M. malmoense* in two badgers. *M. nonchromogenicum*

was associated with granulomatous pneumonia and few acid-fast bacilli. *M. malmoense* was associated with pyogranulomatous dermatitis and abundant acid-fast bacilli.

In conclusion, this study provided a pathological analysis of the most common lesions in road-killed badgers and might set the foundation for further studies of the complex relationship between mycobacteriosis and other diseases in badgers. It also introduced two environmental mycobacteria capable of inducing histological lesions in badgers similar to those caused by *M. bovis*, a finding relevant to prevalence studies using histology, especially in non-endemic populations, and possibly also to the efficacy of vaccines.

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1 Introduction and background

Badgers are omnivores and members of the family Mustelidae and subfamilies Melinae, Helictidinae, Mellivorinae and Taxidiinae (Delahay et al., 2008; Lariviere and Jennings, 2009). The classification of the badgers is complex and several species and genera are recognised within the subfamilies (Delahay et al., 2008; Lariviere and Jennings, 2009). The badgers belonging to the subfamily Melinae are the Hog badgers (*Arctonyx collaris),* Japanese badgers (*Meles anakuma*), Asian badgers (*Meles leucurus*), European badgers (*Meles meles*) (Delahay et al., 2008; Lariviere and Jennings, 2009). Ferret badgers (*Melogale* spp.) belong to the subfamily Helictidinae (Delahay et al., 2008; Lariviere and Jennings, 2009) and honey badgers (*Mellivora capensis*) are the only species and genus of the subfamily Mellivorinae (Delahay et al., 2008; Lariviere and Jennings, 2009). American badgers (*Taxidea taxus*) are the most common badgers belonging to the subfamily Taxidiinae (Delahay et al., 2008; Lariviere and Jennings, 2009).

1.1 European Badger (*Meles meles*)

1.1.1. Geographical distribution

European badgers (*Meles meles*) are present in all Europe and some parts of the West Asia (Kranz et al., 2016). European badgers are occasionally referred as Eurasian badgers given their distribution in both Europe and Asia (Kranz et al., 2016). They localise in Europe west of the River Volga up to the Middle Volga, the southern mountains of Middle Asia, Iran, Iraq, Israel, and the islands of the UK, Ireland, Rhodes, Crete (Abramov and Puzachenko, 2013; Proulx et al., 2016).

1.1.2. Subspecies

Several subspecies of European badgers are recognised. The subspecies of the European badgers (*Meles meles*) are the Common badgers (*Meles meles meles*), Norwegian badgers (*Meles meles milleri*), Continental badgers (*Meles meles taxus*), Transcaucasian badgers (*Meles meles canescens*), Cretan badgers (*Meles meles arcalus*), Rhodes badgers (*Meles meles rhodius*) (Abramov and Puzachenko, 2013). The subspecies *Meles meles meles* predominates over most of Europe (Kranz et al., 2016). In the UK, the most common subspecies found is *Meles meles meles* (Kranz et al., 2016). The international Union for Conservation of Nature (IUCN) classified the subspecies *Meles meles meles* as being of least concern and is characterised by a wide population size, which is steady and even expanding in some areas (Kranz et al., 2016).

1.1.3. Habitats

The preferential habitats of the European badgers (*Meles meles*) are deciduous woods and open pastureland (Abramov and Puzachenko, 2013). They occasionally inhabit mixed and coniferous woodlands and have adapted their lives to urban parks and suburban areas (Abramov and Puzachenko, 2013). Badgers are mostly nocturnal and reside in clusters of mixed sexes and ages in subterranean burrow systems described as setts (Rogers et al., 2003; Abramov and Puzachenko, 2013). European badgers hibernate during winter in their setts and prepare for winter dormancy in the late summer by accumulating adipose tissue, which reaches a climax in October (Rogers et al., 2003). Both males and females are heaviest in November prior to the winter sleep, and thinner in May, due to the utilisation of the subcutaneous and visceral fat deposit accumulated during the autumn (Rogers et al., 2003).

1.1.4. Diet

Unlike other mustelids, European badgers feed on a large selection of plants and animals and are considered opportunistic foragers with an omnivorous diet, that includes nuts, fruit, tubers, bulbs, acorns and cereal crops (Cleary et al., 2009). They consume a variety of earthworms (*Lumbricidae*), the contents of bee nests and wasps, birds' eggs, carrion, and also prey on live vertebrates such as European hedgehogs (*Erinaceus europaeus*), moles (*Talpa europaea*), and rabbits (*Oryctolagus cuniculus*) (Kauhala and Auttila, 2010). In England, it appears that *Lumbricidae* make up a large proportion of their diet in all seasons except summer (many worms aestivate), during when they increased their consumption of cereals (Cleary et al., 2009).

1.1.5. Anatomy

Anatomically, the skull of European badgers (*Meles meles*) possess a well-developed sagittal crest and short, triangular paraoccipital processes (Delahay et al., 2008). They have characteristic short limbs with pronounced palmar and plantar surfaces and the feet possess five digits each and varies from plantigrade to semidigitigrade (Delahay et al., 2008). The claws are strong, not retractable, obtuse and wear with age, in particular the hind claws, which are sometimes worn away in old badgers (Delahay et al., 2008). The pelage is soft and form a dense undercoat (Delahay et al., 2008).

The haircoat in badgers presents few peculiarities. According to the pigmentation of the hair, European badgers (*Meles meles*) are divided in black/white, erythristic, yellow and albino (Delahay et al., 2008; Lariviere and Jennings, 2009). Black/white badgers are known for their distinguishing parallel and symmetrical black stripes that run laterally to the nose, and through the eyes they reach the frontal and parietal region of the head (Delahay et al., 2008; Lariviere and Jennings, 2009). These two dark stripes are separated by a white medial stripe (Delahay et al., 2008). The dorsal pelage is grizzled grey and the ventral pelage tends to be dark grey or black (Delahay et al., 2008). Erythristic badgers are characterised by having a sandy pale red discoloration on the parts of the body usually black (Delahay et al., 2008). Yellow badgers are also described but extremely rare (Delahay et al., 2008). Albino badgers are characterised by a generalised lack of pigmentation and can be white or slightly yellow with pale pink eyes due to reduced pigmentation of the iris (Delahay et al., 2008).

European badgers (*Meles meles*) are characterised by a distinct sexual dimorphism. Boars typically have thicker necks, wider skulls, and more tapered tails

than sows, which show shorter and narrower heads and softer and fuzzier tails (Delahay et al., 2008). Both males and females have three pairs of mammary glands which tend to be more developed in females (Delahay et al., 2008). Male badgers are generally heavier than females. Male badgers usually weigh between 9.1 and 16.7kg and female badgers weigh between 6.6 and 13.9kg (Delahay et al., 2008). Their weights vary depending of food availability and seasons. They grow until autumn where they reach a peak before the hibernation which occurs in winter (Delahay et al., 2008). European badgers can weigh up to 24kg in autumn (Delahay et al., 2008). In both sexes, the body length measured from the occipital bone to the base of the tail ranges from 56cm to 90cm (Delahay et al., 2008). The length of the tail varies from 11cm to 20cm (Delahay et al., 2008). Both males and females possess specialised apocrine and sebaceous (scent) glands, which are localised at the base of the tail (subcaudal gland) and around the anus (anal gland) (Delahay et al., 2008; Lariviere and Jennings, 2009). Both glandular structures work as scent-marking tools (Delahay et al., 2008; Lariviere and Jennings, 2009).

1.2. Tuberculosis in cattle, European badgers and other wildlife species in the United Kingdom

1.2.1. Tuberculosis in cattle (*Bos taurus taurus*) and bovine tuberculosis density areas

Mycobacterium bovis is the causative agent of bTB in cattle and TB in wild, domestic animals and humans (De Lisle et al., 2001). It is a member of the *M. tuberculosis* complex that includes seven species: *M. africanum, M. caprae, M. microti, M. tuberculosis, M. canettii, , M. pinnipedii,* and *M. bovis* (Smith et al., 2006). Morphologically, the mycobacteria are small bacilli, non-motile, 1 to 4 μm in length, characterised by a mycolic acid cell wall that shields the cell from phagocytosis within phagocytic cells and is responsible for their acid-fast feature (Smith et al., 2006). TB in animals keeps on posing a significant risk to livelihoods in non-developing and developing countries through the high threat of zoonotic transmission and loss of production (Smith et al., 2006).

In Great Britain, bTB was first isolated in 1979 where only 0.01% of the British cattle were identified as infected (Department for Environment Food & Rural Affairs, 2014). Since then, bTB in cattle increased dramatically, with breakdowns magnifying every nine years and causing a significant animal loss (up to 300,000 animals slaughtered in order to control the disease) and leading to exorbitant costs and misery to national farmers (Department for Environment Food & Rural Affairs, 2014). In the last forty years, bTB has been becoming one of the most critical and urgent animal health problem in the UK and is considered a priority for the national government DEFRA policy (Department for Environment Food & Rural Affairs, 2014). bTB in cattle is concentrated in South Wales and Southwest England and has been slowly

expanding northwards (Silk et al., 2017; McDonald et al., 2018; Delahay et al., 2007; Department for Environment Food & Rural Affairs, 2018).

DEFRA set up three geographical regions in the UK according to the incidence and risk of TB in cattle: the Low Risk Area (LRA), the Edge Area and the High Risk Area (HRA) (Department for Environment Food & Rural Affairs, 2018) (see figure 1). The Edge area is defined as the boundary of the HRA and LRA where bTB in cattle is thought to be expanding (Department for Environment Food & Rural Affairs, 2018). The northern part of the DEFRA bTB Edge area in cattle is believed to be the point where the bTB in cattle is spreading out and includes six counties: Cheshire, Derbyshire, Nottinghamshire, Leicestershire, Warwickshire and Northamptonshire (Department for Environment Food & Rural Affairs, 2018). The northern Edge area is characterised by variable herd bTB density in cattle, with higher density identified in the county of Cheshire (up to 1.64 herds affected by bTB per square kilometre) compared with the other counties (Department for Environment Food & Rural Affairs, 2018) (see figure 1). The lowest bTB density in the northern Edge area is registered in the East Northamptonshire and most of Nottinghamshire (between 0 and 0.27 herds per square kilometre) (Department for Environment Food & Rural Affairs, 2018) (see figure 1).

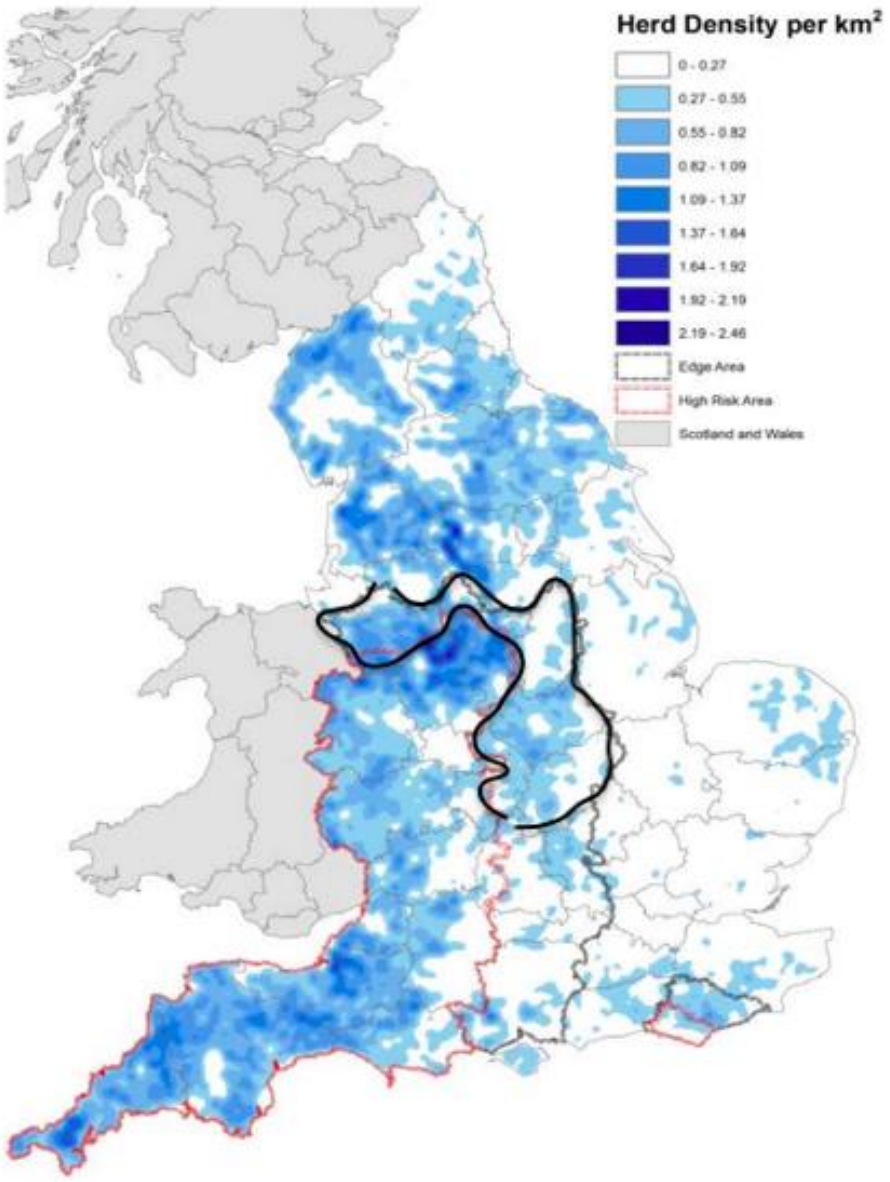

Figure 1. Northern Edge area and bTB cattle herd density in England. The map published on the 2018 DEFRA report shows the herd density of bTB in England in the 2017, measured as the number of herds per square kilometre (Department for Environment Food & Rural Affairs, 2018). The herd density is highlighted with a different gradation of colour (white – low density, dark blue – high density) according to the scale on the right side of the map. The area highlighted with the pale red line is the HRA and the one highlighted with the pale green line is the Edge area, according to the bTB risk assessment in cattle of DEFRA (Department for Environment Food & Rural Affairs, 2018). The Northern Edge area (Cheshire, Derbyshire, Nottinghamshire, Leicestershire, Warwickshire and Northamptonshire) is highlighted with a black line and shows a variable bTB density in cattle with the highest being in Cheshire (up to 1.64 herds per square kilometre) (Department for Environment Food & Rural Affairs, 2018). The lowest bTB density is registered in the East Northamptonshire and most of Nottinghamshire (coloured in white in the map – between 0 and 0.27 herds per square kilometre) (Department for Environment Food & Rural Affairs, 2018).

1.2.2. Tuberculosis in European badgers (*Meles meles*) and badger population density areas

Tuberculosis in badgers was first reported in the south-west of England in 1971 (Gallagher and Clifton-Hadley, 2000). Since then, badgers have been regarded as the principal wildlife source of *M*. *bovis* to cattle (Dalley et al., 2019; Infantes-Lorenzo et al., 2019).

Whether or not badgers are true reservoirs, i.e. can maintain the infection in the absence of reintroduction as well as being a source of infection to the 'target' population (in this case cattle), is highly debated as the basic reproductive number (R_0) is not known but thought to be close to 1 (Department for Environment Food & Rural Affairs, 2018; Haydon et al., 2002). While the term reservoir is often used in the UK and Ireland, in other European countries, such as Poland, badgers are not considered a reservoir of the disease, although in some, such as France, some cattle outbreaks are thought to be due to the transmission via badgers (Bouchez-Zacria et al., 2018; Lipiec et al., 2018).

In a study conducted in 2014 in Cheshire, on the northern-most edge of the British cattle epidemic TB area, there was a strong evidence that both cattle and badgers were part of the same expanding epidemic with evidence of co-localisation of *M. bovis* SB0129 (genotype 25) infection in both cattle and badgers, based on epidemiological investigations of the outbreak and the genetic analysis (spoligotyping) (Sandoval Barron et al., 2018). However a more recent and geographically wider study found different rates of infection and different genotypes between cattle and badgers in other northern 'Edge' counties (Swift B. and Bennett M., per. comm).

The prevalence of TB in badgers in the UK is poorly understood in the current literature. Within the northern Edge Area, the prevalence of TB in badgers was

previously unknown and found recently to vary between counties from around 4% to 15% (Bennett, 2017). In Cheshire, the estimated prevalence of bTB infection (*M. Bovis*) in badgers was 21.3% (CI 95% 14.2 to 30.6%) (Sandoval Barron et al., 2018).

The population density of the badgers in the northern Edge is also poorly understood and considered by some epidemiological studies an area of 'moderate' badger density, which varies from 1 badger per sq-km to 1 badger per three sq-km (Baldwin, 2017; Delahay et al., 2008) (see figure 2).

Several studies of bTB in badgers are available in the current literature. Most previous researches of TB in badgers have concentrated on areas with known, longstanding and high prevalence of bTB and often on TB disease investigation and pathology, combining microbiological culture and PM (Post mortem) examination or combined PM examination, histology and culture without necessarily characterising the mycobacteria detected (Silk et al., 2017; McDonald et al., 2018; Murphy et al., 2010; Goodchild et al., 2012).

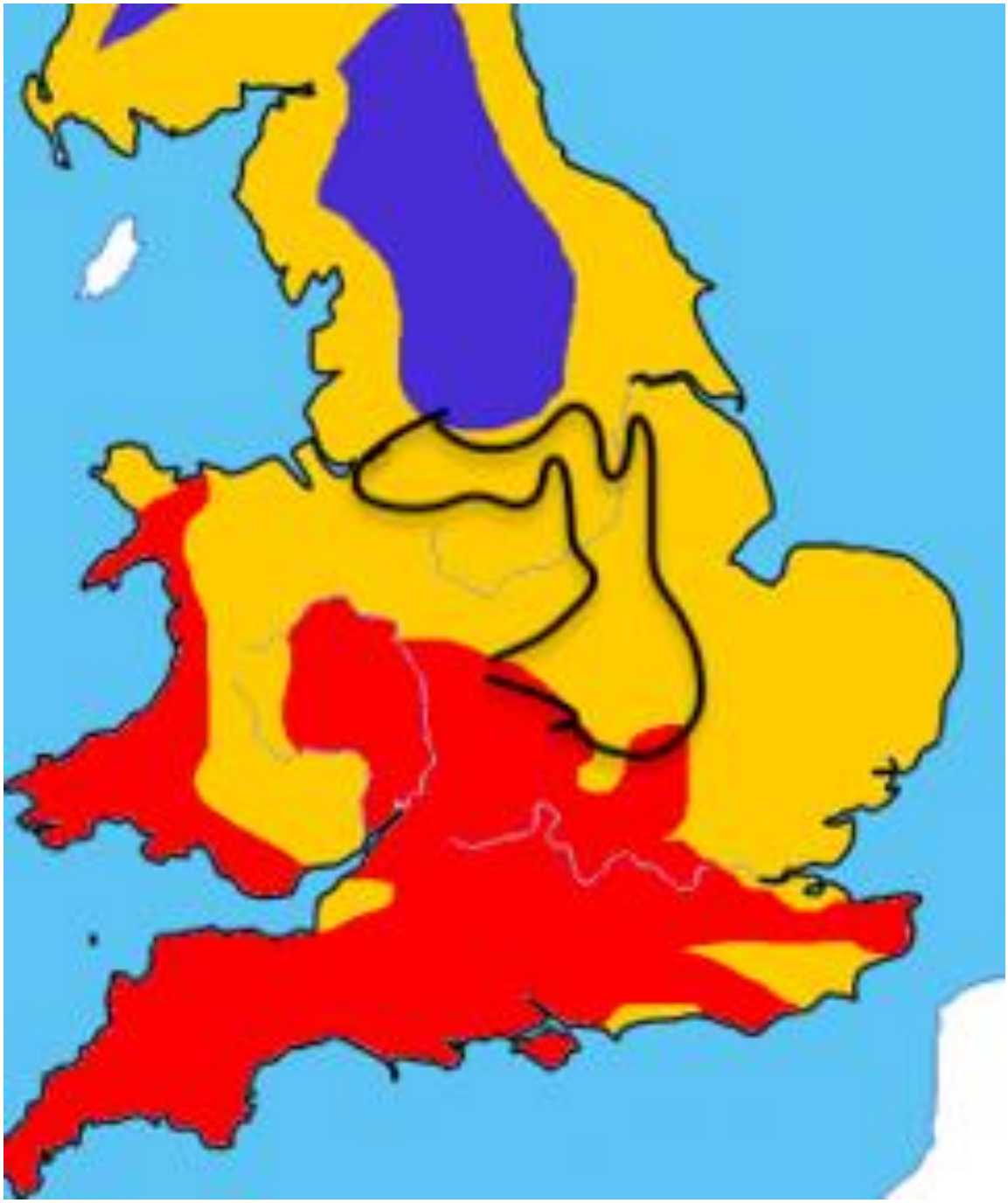

Figure 2. Study area (northern Edge area) and badger density in England. The map illustrates the approximate distribution of the badgers in England (Baldwin, 2017). The different colours in the map highlight the different density of badger population. Blue = Low density (< 1 badger per 3 sq-km); Yellow = Moderate density (from 1 badger per sq-km to 1 badger per 3 sq-km); Red = High density (>1 badger per sq-km). The map is based on previously published data (Delahay et al., 2008; Baldwin, 2017). The northern Edge area is included in the map and is highlighted with a thick black line and includes six counties, such as Cheshire, Derbyshire, Nottinghamshire, Leicestershire, Warwickshire and Northamptonshire. The Northern edge area is considered as a 'moderate' badger density (Baldwin, 2017; Delahay et al., 2008). Highest densities of badgers are identified in the Southern England and West Wales (red colour).

1.2.3. Tuberculosis in other wildlife species

Badgers are not the only wildlife species infected with TB in the UK. In a survey in south-west England, muntjac (*Muntiacus reevesi*), field vole (*Microtus agrestis*), stoat (*Mustela erminea*), yellow-necked mouse (*Apodemus flavicollis*), fallow deer (*Dama dama*), grey squirrel (*Sciurus carolinensis*), polecat (*Mustela putorius*), fox (*Vulpes vulpes*), common shrew (*Sorex araneus*), wood mouse (*Apodemus sylvaticus*), roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) were also found to be infected (Delahay et al., 2007, 2008). The highest prevalence of *M. bovis* infections was found in muntjac (*Muntiacus reevesi*) (5%), fallow deer (*Dama dama*) (4%), polecat (*Mustela putorius*) (4%), foxes (*Vulpes vulpes*) (3%) and stoats (*Mustela erminea*) (3%) (Delahay et al., 2007).

1.2.4. Pathogenesis of tuberculosis in European badgers (*Meles meles*)

The pathogenesis of TB in badgers is slightly different from that in cattle. Although considered a respiratory disease following from the inhalation of infectious aerosol particles, bite wound infections in badgers also represent a significant route of transmission and may result in a more rapid and progressive disease (Corner et al., 2011; Gormley and Corner, 2018). The role of the respiratory route in the badger epidemic is controversial in the current literature (Corner et al., 2011; Gormley and Corner, 2018). The oral route of transmission and gastrointestinal infection of TB in badgers are not completely understood (Gormley and Corner, 2018).

Tuberculosis is considered an immunopathological disease and the complexity of the microbial-host interaction has been extensively investigated (McAdam et al., 2015). Researches in animal models attempted to clarify what really drives the disease progression and to understand whether the bacteria or the host really benefits from the development of the granuloma (Martinot, 2018). These interactions may influence not only the formation of the mycobacterial granuloma but also the development and persistency of non-tuberculous mycobacterial infections (NTM) and interactions with other infectious agents (Martinot, 2018). It is postulated that during TB infection, an organised granuloma is a hallmark of an adequate host immune response (Egen et al., 2008; Orme and Basaraba, 2014). However, the formation of a granuloma may also allow a niche for the development and progression of other infectious agents (Martinot, 2018). This might mean that concomitant non-TB infections can predispose to a clinical disease and re-activation of TB or even TB itself can predispose or perhaps protect from secondary infections (Martinot, 2018). The intimate relationship between TB and concomitant infectious and non-infectious diseases is a large topic in human medicine and the subject of experimental studies, reviews, case reports and large

epidemiological studies (Aksoy et al., 2003; Li and Zhou, 2013; Cadena et al., 2018; Huaman et al., 2015). However, this relationship still needs to be understood for many non-human animals and particularly badgers. Badgers are exposed continuously to other infectious agents, which could potentially influence their immune system and so the progression of TB (McAdam et al., 2015; Martinot, 2018).

The kinetics of TB progression and the immunological responses of badgers to TB have been studied experimentally (Gormley and Corner, 2018). The early specific immune response following *M. bovis* infection is T cell mediated (McAdam et al., 2015). Dendritic cells and macrophages initially recognise *M. bovis* via pathogen-recognition receptors such as nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and Toll-like receptors (TLRs) (McAdam et al., 2015). The TLRs involved in recognition of *M. bovis* are TLR2, TLR4, TLR8 and TLR9 (McAdam et al., 2015). Other receptors expressed by phagocytes, such as mannose binding lectin and CR3, can mediate recognition and entry of *M. bovis* (McAdam et al., 2015). This results in the production of cellular mediators such as interleukin (IL)-1, IL-6, IL-2 and IL-12 that in turn activate CD4+ and CD8+ T lymphocytes and secretion and release of pro-inflammatory cytokines, including interferon-γ (IFN-γ), which is involved in mycobacterial killing (McAdam et al., 2015; Gormley and Corner, 2018). The degree of the initial IFN-y responses correlates with the progression of mycobacterial infection in badgers (Buzdugan et al., 2017; Gormley and Corner, 2018). Activated and infected macrophages produce TNF-α involved in monocyte recruitment and granuloma formation (McAdam et al., 2015). After IFN-y secretion, activated macrophages respond differently in badgers than in other animal species and do not produce either nitric oxide (NO) or the inducible isoform of nitric oxide synthase (iNOS), which are bactericidal against mycobacterium species (Bilham et al., 2017).

1.2.5. Pathology of tuberculosis in European badgers (*Meles meles)*

The pathological macroscopic and microscopic changes in badger tissues with TB are well documented. Grossly, visible TB morphological changes are rare in badgers (Corner et al., 2011; Sandoval Barron et al., 2018). If noted, the lesions are reported mainly in the thoracic cavity, mostly lung (Corner et al., 2011; Sandoval Barron et al., 2018). Clinically, the severity of the disease in badgers varies from subclinical infection (associated with non-visible lesions macroscopically) to clinically evident as mild disease (associated with extra-pulmonary and minimal pulmonary lesions grossly) to severe disease (associated with more systemic involvement, cachexia, debilitation and death) (Corner et al., 2011). Histologically, discrete granulomas are seen, composed of small nests of epithelioid macrophages (the predominant cell type) surrounded by a rim of plasma cells and small lymphocytes (Corner et al., 2011). Mineralisation is uncommon (Corner et al., 2011). Histology of TB lesions in badgers is described in the lung, trachea, bronchi, serosae, liver, spleen, lymph nodes, intestine, skin, kidney (Corner et al., 2011). Some of the histological features of TB described in cattle, such as thick encapsulation, abscesses, cavitation, large areas of caseation or the layered organisation of the granulomas with multinucleated (Langhans) giant cells, are not observed in badgers (Corner et al., 2011). The presence of granulomas and acid fast bacilli (detected with Ziehl-Neelsen staining) is characteristic of TB in badgers (Gavier-Widen et al., 2001). As in other species, the combination of histopathology, culture and molecular testing (Polymerase Chain Reaction (PCR) amplification) is considered the most accurate approach in diagnosing TB in badgers (Corner et al., 2011). Spacer oligonucleotide typing (spoligotyping) can be implemented for genotyping strains of the *M. tuberculosis* complex (MTC) (Driscoll, 2009).

1.2.6. Atypical mycobacteriosis

The genus *Mycobacterium* comprises other species than just members of the MTC, often referred to as ‘atypical’ or ‘nontuberculous’ mycobacteria (NTM) (Porvaznik et al., 2016; Koh, 2017). These are usually environmental mycobacteria, which do not cause typical bovine or human TB or leprosy (Porvaznik et al., 2016; Koh, 2017). They are classified according to the Runyon classification based on the bacterial growth, the production of yellow pigment and whether this pigment was produced after exposure to light or only in the dark (Porvaznik et al., 2016; Koh, 2017).

The slow growing mycobacteria belonging to the Runyon group III include the most prevalent opportunistic pathogens in people, which are the mycobacteria of the *M. avium* complex (MAC), *M*. *xenopi, M*. *terrae* complex, *M*. *genavense, M*. *nonchromogenicum, M*. *haemophilum, M*. *malmoense* and *M*. *ulcerans* (Porvaznik et al., 2016; Koh, 2017).

The interest in infection with NTM in humans has been consistently increasing. NTM are associated with a panoply of disease in humans, including soft-tissue, pulmonary and systemic infections (van Ingen et al., 2018; Ratnatunga et al., 2020).

The treatment of NTM disease is challenging due to their long time span, to the variability in bacterial profiles, different antimicrobial susceptibility patterns and to the lack of guidelines, not to mention diagnosis, as many NTM are unculturable (van Ingen et al., 2018; Ratnatunga et al., 2020). Some NTM, such as *M. genavense, M. marinum, M. ulcerans, M. haemophilum*, *M*. *xenopi*, are considered unculturable or fastidious as culture requires specific nutrients or temperatures (Simon et al., 2019). *M. leprae*, although not classified as NTM, also remains unculturable *in vitro* (Simon et al., 2019). Other NTM, such as *M. smegmatis*, although normally considered culturable, can

reach a viable but nonculturable (VBNC) state due to a temporarily low metabolic activity (Oliver, 2010).

Although the epidemiology, pathogenesis, gross and histopathological appearance of *M. bovis* infection are widely described in the current literature, to our knowledge little information on infection and disease associated with NTM in badgers is to be found in the literature; indeed the only pathological description of NTM lesions in badgers appears to be of histological lesions secondary to MAC in badgers in Spain (Balseiro et al., 2011).

The role of NTM infections in badgers appears to have been poorly studied, yet badgers – through burrowing underground and eating earthworms, which in turn ingest and are covered in soil - might be expected to be exposed to a range of NTM. NTM infections could interfere with diagnostic tests and affect the efficacy of vaccines, as is likely the case in humans and could even influence the pathogenesis and epidemiology of bTB (von Reyn, 2017).

1.3. Other common infectious and parasitic diseases of European badgers (*Meles meles)* and other species of badgers

Badgers can be affected by a wide range of viral, bacterial, parasitic and fungal agents (Ethier et al., 2017). The species *Meles meles* in the UK is known to be susceptible to babesiosis, sarcocystosis, gamma herpesvirus, polyomavirus, trypanosomiasis and salmonellosis (Bartley et al., 2017; Guardone et al., 2020; Lepore et al., 2017; Banks et al., 2002; Banks et al., 2002; Ideozu et al., 2015; Wilson et al., 2003; Swift B.,Bennett M., personal communication). Another documented infectious disease in European badgers (*Meles meles*) in the UK is adiaspiromycosis, a fungal infection caused by the saprotrophic fungus *Emmonsia crescens or E. parva* (formerly *Chrysosporium parvum*) (Borman et al., 2009, 2018). It is a respiratory infection in badgers and is potentially zoonotic (Schwartz et al., 2015; Borman et al., 2009, 2018). Other infectious agents include the lungworm *Perostrongylus falciformis* (formerly known as *Aelurostrongylus falciformis*), *Angiostrongylus daskalovi*, *canine distemper viru*s, borreliosis, histoplasmosis, cryptosporidiosis, *Troglotrema acutum* and leptospiral infection (Davidson et al., 2006; Byrne et al., 2019; Deak et al., 2017; Di Sabatino et al., 2016; Gern and Sell, 2009; Wodecka et al., 2016; Wohlsein et al., 2001; Mateo et al., 2017; Heddergott et al., 2015; Millán et al., 2009; Ayral et al., 2016) (see table 1).

Ferret badgers (*Melogale spp.)* are known to be infected with *rabies virus,* babesiosis, *Toxoplasma gondii* (Chang et al., 2015; Chiou et al., 2016; Guardone et al., 2020; Chen et al., 2017). American badgers (*Taxidea taxus*) are reported to be infected with leptospirosis, *canine distemper virus* and *Alaria alata* mesocercariae (Takeuchi-Storm et al., 2015; Ethier et al., 2017).

Bacteria	
Salmonella spp. *(S. enterica, S. dublin, S. agama)* *Leptospira spp.* *(L. Icterohemorragiae)* *Borrelia spp.* *(B. burgdorferi, B. garinii)*	Italy (Chiari et al., 2014), United Kingdom (Wray et al., 1977; Wilson et al., 2003; Swift B. and Bennett M., personal communication) Spain (Millán et al., 2009), France (Ayral et al., 2016) Switzerland (Gern and Sell, 2009), Poland (Wodecka et al., 2016)
Virus	
Gamma herpesvirus *Canine distemper virus* *Aleutian mink disease virus* *Polyomavirus*	United Kingdom (Banks et al., 2002) Italy (Di Sabatino et al., 2016), Finland (Knuuttila et al., 2015) United Kingdom (Banks et al., 2002),
Protozoa	
Babesia spp. *(B. annae, B. microti)* *Trypanosoma pestanai* *Cryptosporidium hominis* *Sarcocystis lutrae*	United Kingdom (Bartley et al., 2017; Guardone et al., 2020); Italy, Bosnia and Herzegovina, Hungary and Spain (Guardone et al., 2020) United Kingdom (Ideozu et al., 2015) Spain (Mateo et al., 2017) United Kingdom (Lepore et al., 2017)
Trematodes	
Troglotrema acutum	Germany (Heddergott et al., 2015)
Fungi	
Histoplasma spp. *Emmonsia spp.* *(E.crescens, E. parva)*	Germany (Wohlsein et al., 2001) United Kingdom (Borman et al., 2009, 2018)
Nematodes	
Perostrongylus falciformis *Angiostrongylus daskalovi*	Ireland (Byrne et al., 2019), Norway (Davidson et al., 2006) Romania (Deak et al., 2017)

Table 1. Infectious agents in European badgers (*Meles meles*). Listed in the first column (left) are the genera and, occasionally, the species of bacteria, virus, protozoa, trematodes, fungi and nematodes identified in European badgers (*Meles meles)* in the current literature. For each infectious agent, the references of the studies and the countries where the researches were performed are specified in the second column (right).

1.4. Aims and objectives

The aims of this research project are to describe the pathological changes associated with *M. tuberculosis* complex (MTC+) and other mycobacterial infections (OM+) seen in European badgers (*Meles meles*), compare other non-traumatic macroscopic and microscopic changes with and without mycobacterial infections, and to perform a survey of lesions not related to road-traffic injury, other than just tuberculosis, found in road killed badgers. This study focuses on European badgers (*Meles meles*) in areas with a lower prevalence of bTB in England (northern Edge area) and attempts to differentiate *M. bovis* from other mycobacteria as it was hypothesised that the prevalence of non-tuberculous or atypical mycobacterial (NTM) infections might be relatively more significant in low bTB prevalence populations.

The study aims to achieve the desired outcome by performing a complete post-mortem macroscopic and microscopic (histopathology) examinations of found dead (road-killed) badger carcasses found on the roadside and by comparing the pathological findings with microbiological (culture) and molecular (PCR) results of pooled tissue samples collected during post-mortem.

2 Material and methods

2.1 Animals (European badgers)

During the period July 2016-November 2017, 658 found dead (road-killed) European badger (*Meles meles*) carcasses were collected by using a local network of farmers (recruited through the National Farmers' Union of England and Wales and local bTB groups such as the Cheshire TB Eradication Board), conservation wildlife groups, university project staff, veterinarians and local authorities and businesses. I was directly involved in examining the carcasses for this project during the period January 2017 to November 2017. Overall, 607 carcasses were suitable for necropsy and sampling. The remaining 51 carcasses were rejected due to advanced state of putrefaction.

2.2 Study area (northern Edge Area)

The sampled area of study was the northern half of DEFRA's Edge Area of the TB cattle epidemic in England, which is the boundary between the cattle HRA and LRA (Department for Environment Food & Rural Affairs, 2018).

The carcasses of the badgers were collected in six English counties. Specifically, as part of this study, 103 out of the 607 animals (17.0%) originated from Cheshire, 99 (16.3%) from Derbyshire, 101 (16.6%) from Nottinghamshire, 105 (17.3%) from Leicestershire, 101 (16.6%) from Warwickshire and 98 (16.2%) from Northamptonshire. Within the period of collection, the overall peak carcass collection was recorded in February 2017. The peaks of collection were later in Northamptonshire which occurred in March and July 2017 owing to a later start in that county with up to 20 animals registered. The highest peak was reached for the county of Leicestershire which showed more than 50 animals collected during February. This was followed by the county of Warwickshire with 48 animals, Derbyshire with a peak of 35 animals and, finally, Nottinghamshire and Cheshire with similar collection peaks of 28 animals each (see figure 3). County-level collections stopped once roughly 100 suitable badger carcasses per county had been sampled.

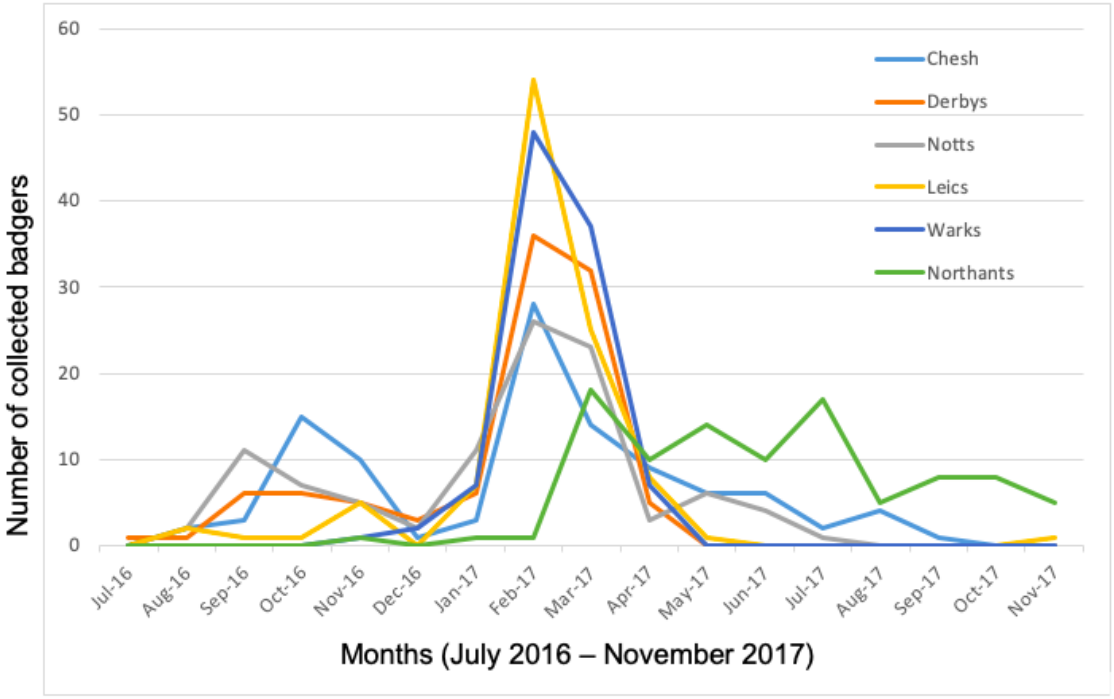

Figure 3. Number of badger carcasses collected by month and by county (Bennett, 2017). As illustrated in the graph, the collection reached a peak in February 2017 for most of the counties, with the exception of Northamptonshire (green line) in which the collection peak was recorded in March 2017. Northamptonshire also showed a second peak in July 2017. In March and July, Northamptonshire registered a peak of 18 to 20 animals, respectively. The highest peak was reached for the county of Leicestershire (yellow line) which showed more than 50 animals collected during February. This was followed by the county of Warwickshire (blue line) with 48 animals. Derbyshire (red line) registered a peak of 35 animals, whereas Nottinghamshire (grey line) and Cheshire (turquoise line) have a similar collection peak in February with approximately 28 animals each.

2.3 Carcass delivery, handling and recording

Most of the carcasses (n=504) for this study were delivered to the veterinary necropsy and laboratory facilities at Sutton Bonington Campus, University of Nottingham (UoN), in double thickness plastic bags supplied as part of collection kits (Bennett, 2017). The remaining badgers (n=103) covering the county of Cheshire were delivered to the veterinary pathology department at the University of Liverpool. The collection kits included safety advice, gloves, submission forms, individual pre-labelled tags and documents for recording the coordinates, time and date of the carcass being found and its collection (Bennett, 2017). Each carcass was given a unique ID supplied with the collection kits and already entered onto the tags and submission form. In addition to hard copy records kept in each laboratory and by individual researchers (enabling auditing of records), all submissions and results were entered into a shared spreadsheet (Excel ®), which was checked regularly to ensure data were both up-to-date and correct. I was involved daily in creating records and keeping the spreadsheet of Excel ® file up to date. In addition, submission and necropsy forms were scanned and stored by technicians on a shared folder on the UoN server, along with photographs taken during necropsy. Carcass collection and delivery to the UoN were performed by stakeholder groups and researchers who are included in the acknowledgments as reference.

2.4 Post mortem examination and sample collection

Necropsies were undertaken within 24 hours of arrival in a negative pressure cabinet at enhanced Containment Level (CL) 2, according to established published guidelines (Advisory Committee on Dangerous Pathogens, 2018). Animals were necropsied and samples were collected using standard operating procedures (SOPs) based on previous studies (Byrne et al., 2015; Sandoval Barron et al., 2018).

I undertook over half of the necropsies and tissue collections delivered to UoN personally, under the supervision of my supervisors (I performed the post-mortem of approximately 350 badgers). 103 animals originating from Cheshire were necropsied by a boarded-certified veterinary anatomic pathologist at the University of Liverpool who is included in the acknowledgments as reference. The remaining post-mortems were performed by a boarded-certified veterinary anatomic pathologist at the UoN.

Identification number, body mass, photographs, date and time of the post-mortem examination, location and name of the collectors were recorded (Bennett, 2017). The gender of the badgers was recorded. The age was estimated as juvenile, adult and old based on the dentition and degree of tooth wear, the degree of wear of the claws and the pelage, according to criteria reported in the current literature (Hancox, 1988; Abramov and Puzachenko, 2013). The state of preservation of the carcass was categorised as “fresh”, “mild”, “moderate”, or “advanced” autolysis, following published criteria (Brooks, 2016). Well-defined criteria for assessment of the Body Condition Score (BCS) in badgers and other mustelids are lacking in the current literature, so BCS was based on the canine and feline criteria (5-point score system) defined by the World Small Animal Veterinary Association (German, 2016).

The tissue sampling and culture protocols were similar to those used in previous studies (Byrne et al., 2015; Sandoval Barron et al., 2018). Lymph nodes, lung

lobes and any lesions consistent with TB were collected for culture of *M. bovis*, and positive cultures after 8-12 weeks were tested by IS6110 PCR to determine whether or not they were MTC. Five pools of tissue were sampled: a 'head and neck pool' of parotid, mandibular, retropharyngeal lymph nodes; a 'carcass pool' of prescapular, axillary, popliteal, superficial inguinal lymph nodes; an 'abdominal pool' comprising hepatic and mesenteric lymph nodes; a 'thoracic pool' of bronchial, mediastinal lymph nodes; a 'lung pool' of lung lobe samples. Tissue samples (including lung, spleen, tongue, left gonad, stomach, left kidney, masseter skeletal muscle, heart, large intestine, small intestine, urinary bladder, diaphragm and liver) were also preserved in buffered 10% formalin for histopathology (see table 2)**.** A sample of hair pluck, whole blood, kidney and liver were also collected and stored frozen (-20°C) for further testing.

Tissue samples **for *Mycobacterium* spp. culture, PCR**	Tissue samples **for histology** (10% buffered formalin fixed)
Parotid LNs (right, left)	Spleen
Mandibular LNs (right, left)	Tongue
Retropharyngeal LNs (right, left)	Left gonad
Mediastinal LNs (anterior, posterior)	Stomach
Bronchial LNs (right, left)	Liver
Hepatic LNs (right, left)	Left kidney
Mesenteric LNs (right, left)	Lung (left apical and caudal lobes)
Prescapular LNs (right, left)	Masseter muscle
Axillary LNs (right, left)	Heart (left ventricle free wall)
Inguinal LNs (right, left)	Colon
Popliteal LNs (right, left)	Jejunum
Lung (right and left apical and caudal lobes)	Urinary bladder
Any TB-compatible lesions*	Diaphragm
	Any TB-compatible lesions*

Table 2. List of the collected tissues. The table shows the list of the organ tissues collected during post-mortem examination. The samples were divided according to storage and use. The left column shows a list of the tissues (lymph nodes LNs and lung lobes) that were collected in individual plain pots and pooled for culture and PCR. The right column shows the organ tissues immersed / fixed in 10% buffered formalin for histopathological examination. In both, right and left column, any lesion compatible grossly with TB (TB-compatible lesions*) was also collected in formalin for histology and in plain pots for culture and PCR. The TB-compatible lesions comprise nodules, areas of thickenings and consolidation, and skin wounds present in any organ.

2.5 Microbiology

Tissue processing of *Mycobacterium spp.* was undertaken in the UoN CL3 laboratory using a SOP (Ruettger et al., 2012; Jagielski et al., 2014; Sandoval Barron et al., 2018). Microbiology was performed by PhD students, posdoctoral research fellows and lecturers who are included in the acknowledgments as reference. Pooled samples were inoculated on to Stonebrink Selective agar and Middlebrook 7H11 agar and incubated at 37°C for a minimum of 12 weeks (Sandoval Barron et al., 2018; Goodchild et al., 2012). Cultures were examined every week for the appearance of colonies characteristic of *Mycobacterium spp.* (Sandoval Barron et al., 2018).

2.6 Polymerase Chain Reaction

Tissue processing for IS6110 PCR of MTC was undertaken in the UoN CL3 laboratory using a SOP (Ruettger et al., 2012; Jagielski et al., 2014; Sandoval Barron et al., 2018). PCR was performed by PhD students, posdoctoral research fellows and lecturers who are included in the acknowledgments as reference. DNA was extracted from slow-growing mycobacteria colonies and confirmed as MTC by amplification of the IS6110 genetic elements. Mycobacterium-like colonies that tested negative by PCR for MTC, were further characterised by ribosomal RNA (rRNA) PCR and sequencing (Sandoval Barron et al., 2018). The M+ group comprises all the *Mycobacterium* positive badgers including those with colonies at culture confirmed by PCR as MTC (MTC+) and those with colonies at culture negative by PCR for MTC and characterised as other mycobacteria (OM+). The M- group comprises all the culture-negative badgers, and those producing *Mycobacterium*-like colonies which were further identified as *Mycobacterium*-negative by PCR/sequencing.

2.7 Histopathologic examination

Eighty eight animals (n=88) were selected for histopathological examination based on the results of the culture, PCR amplification and degree of autolysis. The tissue samples were fixed in 10% buffered formalin, pH 7.2 before being embedded in paraffin wax, sectioned at 5µm, stained with haematoxylin and eosin (HE) and examined by light microscopy. Special stains for mycobacteriosis (Ziehl–Neelsen ZN and Wade-Fite modified Ziehl-Neelsen MZN) were used in all cases with histological lesions compatible with TB. Additional special stains (Periodic Acid Schiff PAS, Gram stains and Prussian Blue) were also occasionally used in order to investigate specific aetiologies and/or pathological changes.

The histopathological examination of the tissue slides stained with HE and special stains was undertaken by myself, under supervision. The tissue processing, embedding and staining were performed by qualified laboratory technicians through the routine SVMS Diagnostic Pathology service, who are included in the acknowledgments as reference.

2.8 Statistical analysis

Analyses were performed using an online statistical package software called VassarStats website calculator (Lowry, 1998).

Data in a 2 x 2 contingency table format (two categorical variables and two levels within categories) were analysed by Fisher's Exact test and data with a 2 x *n* (two categorical variables and greater than two levels within categories) format were analysed by chi-square test (Quinn and Keough, 2002). For the latter, the Yates' correction for continuity was applied because it allows for improved test precision when data frequencies are low or have zero cells (Quinn and Keough, 2002).

The lower and upper limits of the 95% confidence interval for a proportion were obtained by using the Wilson procedure with a correction for continuity (Newcombe, 1998).

A p value < 0.05 was accepted as statistically significant (Senthilkumar, 2006).

2.9 Ethics

The research project was approved by the relevant Research Convenor and the Ethical Review Panel of the SVMS, UoN (Animal Welfare and Ethical Review Body, 2020). The project described below complies with published codes of conduct, ethical principles and guidelines of professional bodies associated with my research discipline.

3 Results

3.1 Gross post mortem macroscopic examination

3.1.1 Gender and age evaluation

Overall, 318 male (53%) badgers were recorded compared with 276 (45%) female animals. The proportion of male badgers was higher than the female and this was statistically significant (p = 0.02). Most of the counties also showed a trend towards the male population which represented 49% (n=54) for the county of Cheshire, 55% (n=54) for the county of Nottinghamshire, 57% (n=59) for the county of Leicestershire, 53% (n=51) for the county of Warwickshire and 55% (n=53) for the county of Northamptonshire. The only exception was the county of Derbyshire which registered a slightly increment of the female population (53%, n=52) compared with the male one (47%, n=47), although the difference was not significant (see table 3).

The proportion of adults was consistently higher in all the counties with a total of 440 animals (72%), compared with the young (24%, n=143) and old (2%, n=13) badgers, by a ratio of approximately 2:1. The same ratio in favour to the adult population was identified when considering the individual counties with a proportion of 68% (n=73) for Cheshire, 72% (n=74) for Derbyshire, 71% (n=71) for Nottinghamshire, 72% (n=73) for Leicestershire, 72% (n=71) for Warwickshire and 81% (n=78) for Northamptonshire. The highest percentage of old animals was registered in the county of Cheshire (4%, n=4). Five male badgers were macroscopically identified as cryptorchid. Gender and age were not recorded for a small percentage of animals (n=13, 2% and n=11, 2% respectively) due to post analytic errors (lack of records) (n=4) or advanced autolytic changes or severe traumatic lesions which hampered external examination assessment, included age and sex evaluation (n=20) (see table 3).

	Total	Cheshire	Derbys	Notts	Leics	Warks	Northants
GENDER							
Male number % (95% CI)	318 53% (48.3-56.4)	54 49% (39.1-58.2)	47 47% (37.4-57.7)	54 55% (44.2-64.4)	59 57% (46.6-66.3)	51 53% (42.2-62.7)	53 55% (44.2-64.6)
Female number % (95% CI)	276 45% (41.4-49.5)	44 40% (30.6-49.3)	52 53% (42.2-62.5)	45 45% (35.5-55.7)	45 43% (33.7-53.3)	46 47% (37.2-57.7)	44 45% (35.3-55.7)
No record number % (95% CI)	13 2% (1.1-3.7)	13 11% (6.6-19.5)	0 0% (0.0-0.0)	0 0% (0.0-0.0)	0 0% (0.0-0.0)	0 0% (0.0-0.0)	0 0% (0.0-0.0)
Total	607	111	99	99	104	97	97
AGE							
Adult number % (95% CI)	440 72% (68.7-75.9)	73 68% (58.4-76.7)	74 72% (61.9-80.0)	71 71% (60.9-79.4)	73 72% (61.6-79.8)	71 72% (61.6-80.0)	78 81% (71.7-88.2)
Old number % (95% CI)	13 2% (1.1-3.7)	4 4% (1.2-9.8)	2 2% (0.3-7.5)	1 1% (0.1-6.2)	2 2% (0.3-7.5)	2 2% (0.3-7.8)	2 2% (0.3-8.0)
Juvenile number % (95% CI)	143 24% (20.2-27.1	25 23% (15.9-32.7)	22 21% (14.1-30.7)	27 27% (18.8-36.9)	27 26% (18.4-36.2)	26 26% (18.1-36.2)	16 17% (10.1-25.9)
No record number % (95% CI)	11 2% (0.9-3.3)	5 5% (1.7-11.9)	5 5% (1.8-11.5)	1 1% (0.1-6.2)	0 0% (0.0-0.0)	0 0% (0.0-0.0)	0 0% (0.0-0.0)
Total	607	107	103	100	102	99	96

Table 3. **Gender and age of the badger carcasses collected.** The table illustrates the proportion of badgers divided by gender (male, female) and estimated age (adult, old, juvenile). Gender and age were not recorded for a small percentage of animals (identified as “no record”). For each category, it is shown the number of animals, the percentage and 95% CI (Confidence Interval). The predominance of male badgers is evident for all the counties with the exception of Derbyshire where the number of females is slightly higher than males. The predominance of adult animals is striking and roughly double compared to the remaining aging groups (old, juvenile).

3.1.2 Degree of autolysis

The majority of badgers had advanced (240/607, 40%) to mild (190/607, 31%) autolysis (see figure 4). Only 12 animals (2% of the received carcasses) were considered fresh, following previously described criteria (Brooks, 2016) (see figure 4). Despite the initial exclusion of 51 carcasses as considered in an advanced state of putrefaction, within the remaining 607 badgers, still 40% of the animals were considered as in a state of advanced autolysis (not putrefaction) and considered still suitable for further assessment and included in this study.

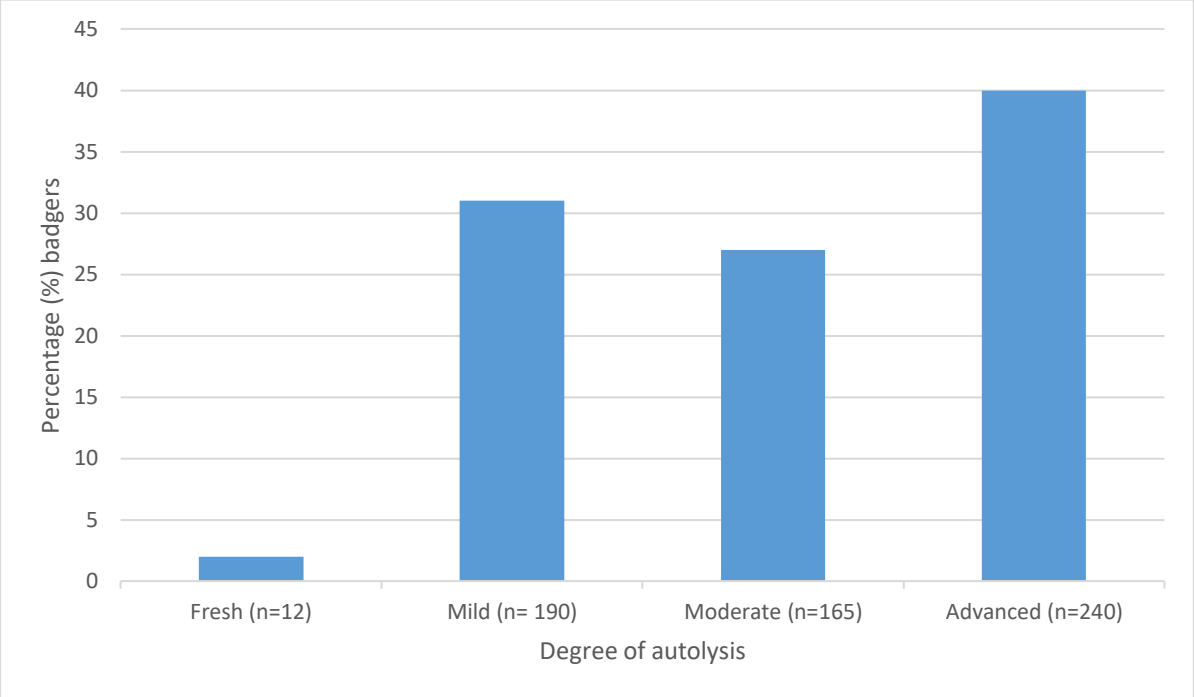

Figure 4. Degree of autolysis of the badger carcasses collected. The bar chart gives information about the proportion of badger carcasses (% of badgers, Y axis) presented mild, moderate, advanced autolysis or were fresh (degree of autolysis, X axis). The numbers of badgers are written under each blue bar (n). There were upward trends in both categories of badgers showing mild and advanced autolysis, with a more rapid increase for the advanced ones. Overall, the trends of advanced autolytic carcasses were the most predominant (n=240, 40%).

3.1.3 Macroscopic gross post mortem lesions

All the studied animals showed evidence of trauma compatible with road traffic accidents. Road traffic accident lesions included fractures (affecting mostly the skull and the ribs), subcutaneous and cutaneous contusions, haemothorax, hemoperitoneum, pneumothorax, laceration of parenchymal organs (such as lung, spleen and liver) and haemorrhages or pelvic fractures. Of the 607 badgers examined, 227 (37%) had macroscopic lesions unrelated to road-traffic accident (see table 4). No other significant non-traffic-injury-related gross abnormalities were observed in the remaining 380/607 (63%) badgers examined. The following chapters will detail the macroscopic findings identified in the 227 badgers.

Animal (n=227)	Granuloma (n=10 4%)	Lymph-adenomegaly (n=26 11%)	Bite wounds (n=49 22%)	Lice (n=18 8%)	Tail ulcers (n=26 11%)	Mixed Lesions (n=98 44%)
Young (n=58)	2/58 *(3%)*	6/58 *(10%)*	11/58 *(19%)*	5/58 *(9%)*	9/58 (16%)	25/58 *(43%)*
Adult (n=160)	8/160 *(5%)*	20/160 *(13%)*	34/160 (*21%)*	13/160 *(8%)*	15/160 *(9%)*	70/160 *(44%)*
Old (n=5)	0/5 *(0%)*	0/5 *(0%)*	4/5 *(80%)*	0/5 *(0%)*	0/5 *(0%)*	1/5 *(20%)*
No record (n=4)	0/4 *(0%)*	0/4 *(0%)*	0/4 *(0%)*	0/4 *(0%)*	2/4 *(50%)*	2/4 *(50%)*

Table 4. Macroscopic gross post-mortem lesions. The table illustrates the animals with non-traffic-related lesions (n=227) which include badgers with single gross lesions (granulomas, lymphadenomegaly, bite wounds on the neck, lice or tail ulcers) or combined lesions (mixed lesions) and divided by age groups (young, adult, old). In few animals (n=4), the age was not recorded. Road-traffic accident injuries are excluded. The majority of the young and adults presented mixed lesions (respectively 43% and 44%). The least numbers of young and adult badgers showed only granulomas (respectively 3% and 5%). The old badgers showed a higher proportion of bite wounds, seen as only identifiable lesion (80%). Only one old badger showed a combination of multiple lesions which comprised pulmonary granulomas associated with lymphadenomegaly of the mediastinal and submandibular lymph nodes, lice infestation, bite wounds and ulcers at the base of the tail. The differences within the age groups and lesion groups were not statistically significant.

3.1.3.1 Multiple gross lesions

A high proportion of animals (n=98, 44%) presented multiple mixed macroscopic lesions which were identified in 43% of young animals (n=25), 44% of adults (n=70) and 20% of old badgers (n=1). The age was not recorded in the remaining two animals (n=2) with multiple lesions (see table 4).

The most common combination of gross lesions seen in badgers was the presence of bite wounds and the ulcers on the tail which were identified in six young badgers (n=6, 10%), twenty adults (n=20, 12%) and one old animal (n=1, 20%), however this change was not statistically significant. Lice infestation was also frequently identified in combination with other gross changes. The combination lice infestation / bite wound was registered in four young badgers (n=4, 7%) and thirteen adults (n=13, 8%), whereas the combination lice infestation / tail ulcer was seen in six young animals (n=6, 10%) and sixteen adults (n=16, 10%).

Lymphadenomegaly (involving a single lymph node or multiple lymph nodes) was frequently seen with other macroscopic lesions, such as with granulomas (3% of young animals, n=2; 3% of adult animals, n=4; 20% of old animals, n=1), with bite wounds (2% of young animals, n=1; 5% of adult animals, n=8; 20% of the old animals, n=1), with lice infestation (3% of young animals, n=2; 3% of adult animals, n=5; 20% of the old animals, n=1), and with tail ulcers (4% of adult animals, n=6; 20% of the old animals, n=1 and no young animals recorded).

In addition to lymphadenomegaly, the granulomas were seen in combination with bite wounds (5% of young animals, n=5; 1% of adult animals, n=2; 20% of the old animals, n=1), with lice infestation (1% of adult animals, n=2; 20% of the old animals, n=1 and no young animals recorded) and with tail ulcers (2% of young animals, n=1; 2% of adult animals, n=3; 20% of the old animals, n=1).

3.1.3.2 Granulomas

A small proportion of badgers (n=10, 4%) presented only macroscopically granulomatous lesions and comprises two young badgers showing granulomatous nephritis (n=1, 10%) and granulomatous pneumonia (n=1, 10%) and eight adult badgers with granulomatous hepatitis (n=1, 10%) and granulomatous pneumonia (n=7, 70%) (see table 4). Granulomas were present predominantly in adult badgers, however the difference was not statistically significant.

Granulomas were also seen in other eleven badgers combined with other macroscopic lesions (‘mixed lesions’) and include granulomatous pneumonia associated with bite wounds and tracheobronchial lymphadenomegaly (n=8, 72%), subcutaneous granulomas associated with bite wounds on the neck and lymphadenomegaly of the right and left prescapular lymph nodes (n=1, 9%) and granulomatous pneumonia associated with tracheobronchial lymphadenomegaly and tail ulcers (n=2, 18%) (see figure 5). One of the badgers affected by granulomatous pneumonia (compatible with miliary TB) was also severely cachectic (see figure 6).

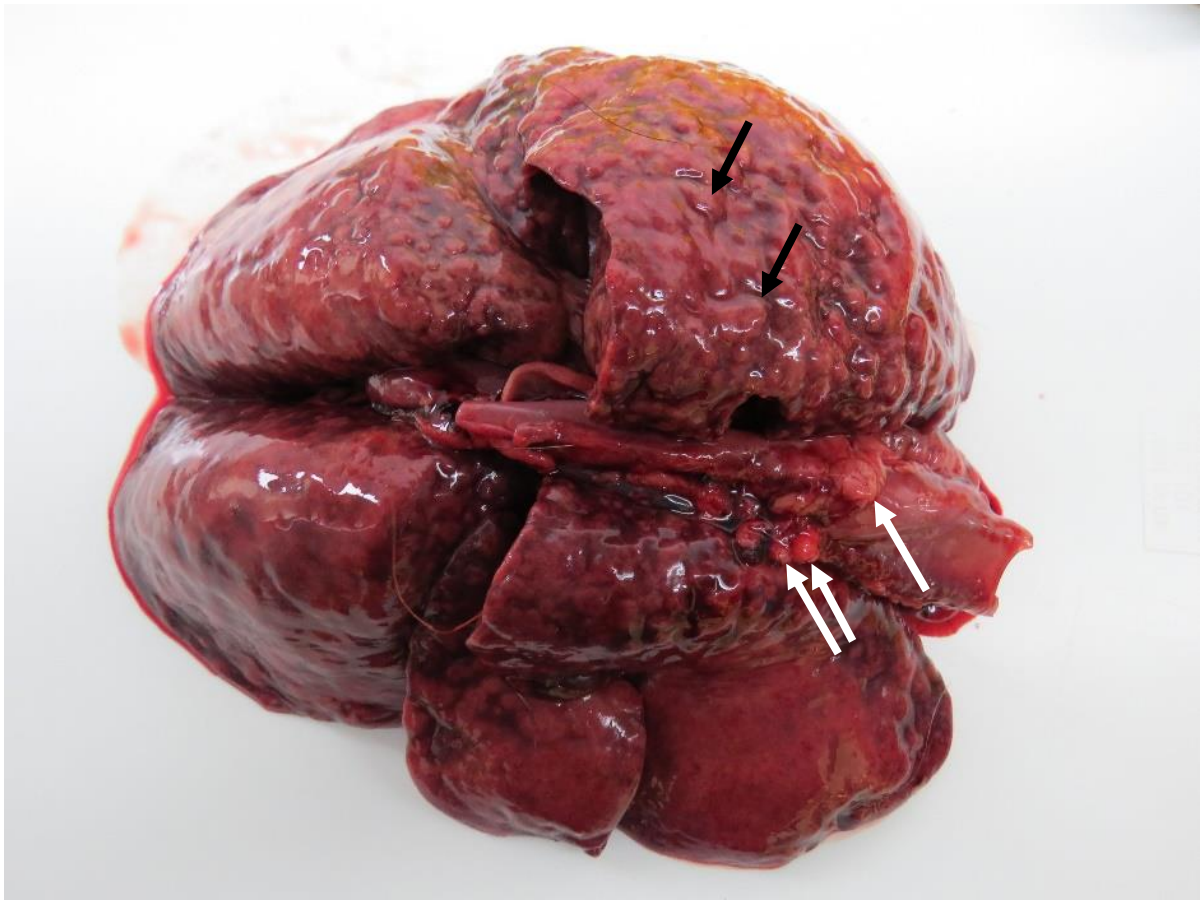

Figure 5. Granulomatous pneumonia. Lung; Badger number 1414. Affecting the lung lobes, the pulmonary parenchyma shows abundant multifocal to coalescing, raised, approximately 1.0 x 0.5cm in diameter, well demarcated, solid and firm nodules (black arrow). The tracheobronchial lymph nodes are moderately enlarged, solid and firm (lymphadenomegaly) (white arrow). The lung lobes are diffusely swollen, heavy and with a red to dark discoloration of the pulmonary parenchyma.

Figure 6. Cachexia and granulomatous pneumonia (compatible with miliary TB). Badger number 350. Whole body (A). The badger shows a diffuse and marked muscle atrophy with prominent vertebral spinous processes and lack of subcutaneous adipose tissue (cachexia). Thorax (B). The lung lobes contain abundant, multifocal, up to 0.3 x 0.3 x 0.3cm, solid, well demarcated, slightly raised, white nodules (arrowheads). The lung lobes show a diffuse red to dark discoloration. Within the heart, the epicardial adipose tissue is depleted (white arrow).

3.1.3.3 Fibrinous suppurative pleurobronchopneumonia: a case report

One badger presented with cachexia and severe fibrinous and suppurative bronchopneumonia, pleuritis and marked pyothorax which were not considered characteristic of TB (see figures 7 and 8). This badger (number 1150) was confirmed as negative for *Mycobacterium* species, after pooling of the tissue samples. Microbiology swab of the lesion in the lung was not taken and culture not attempted due to road-traffic injury-related external contamination.

3.1.3.4 Lymphadenomegaly

Twenty six animals (11%) presented a regional lymphadenomegaly without any other concomitant macroscopic change unrelated to road-traffic accident. Within this group, six animals (10%) were young and the remaining twenty animals (13%) were adults (see table 4).

Figure 7. Pyothorax and fibrinosuppurative pleuritis. Thorax; Badger number 1150. The thoracic cavity is filled with abundant friable white to yellow strands (fibrin) admixed with abundant yellow purulent exudate (asterisk). The parietal and visceral pleura are markedly thickened and expanded by similar purulent and fibrinous exudate (white arrow).

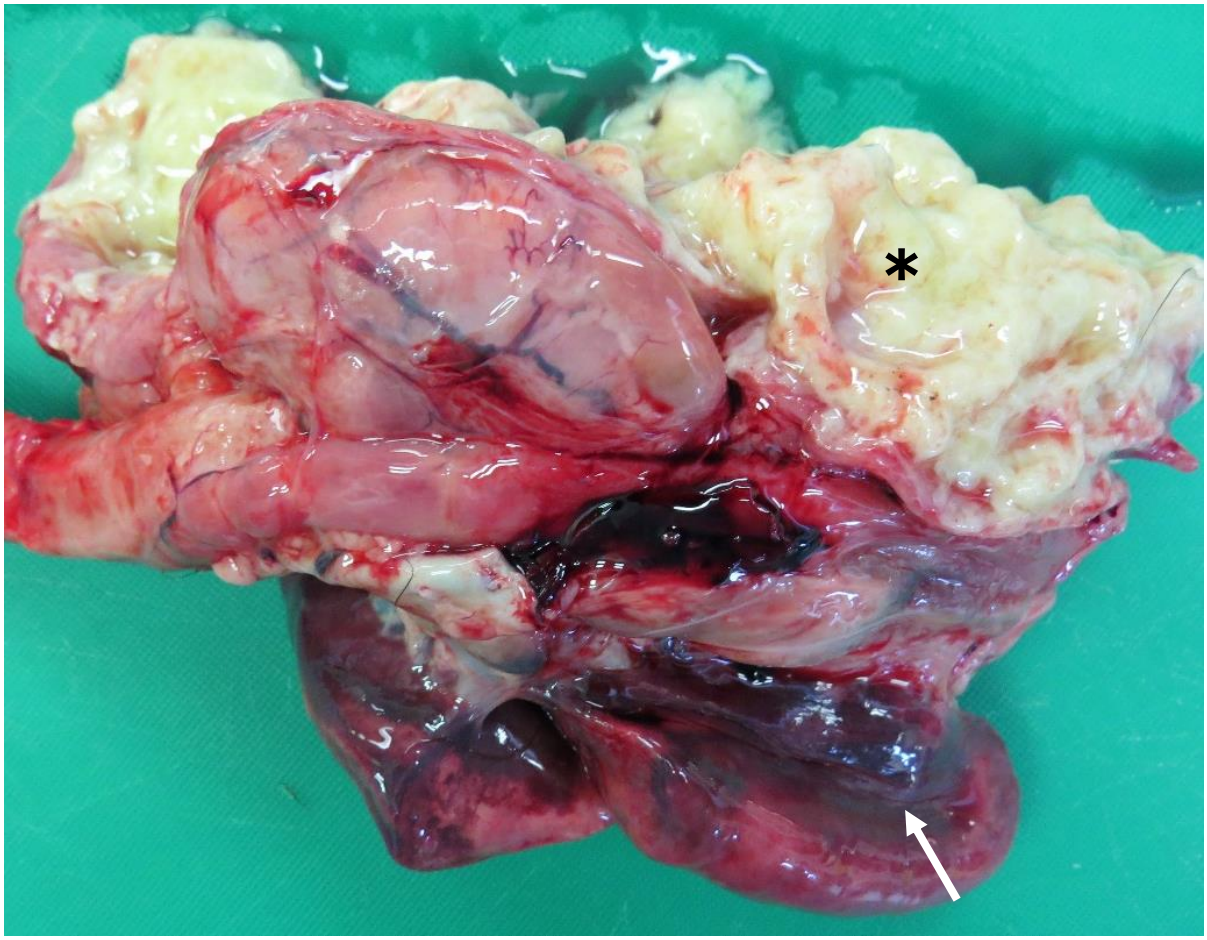

Figure 8. Fibrinosuppurative bronchopneumonia with marked fibrinosuppurative pleuritis. Lungs; Badger number 1150. The pleura and the pulmonary parenchyma of the right cranial, medial and caudal lung lobes are diffusely covered by fibrin and purulent exudate (asterisk). The left cranial, medial and caudal lung lobes are moderately swollen, dark to red and heavy (white arrow).

3.1.3.5 Integumentary macroscopic changes (bite wounds, tail ulcers, lice)

Upon examination of the integument, 49 (22%) out the 227 badgers showed multifocal, well demarcated, up to 10 x 10 x 0.2cm in diameter, depressed, red to dark, cutaneous ulcers on the neck compatible with bite wounds (see figure 9). A higher proportion of bite wounds was identified in the old badgers (4/5, 80%) compared with the young (11/58, 19%) and adult (34/160, 21%) ones (see table 4).

Twenty-six (11%) of the 227 badgers showed multifocal to coalescing, up to 10 x 9 x 0.2 cm, irregularly shaped, well-demarcated ulcerative lesions on the back at the base of the tail (see figure 10). The majority of the ulcers on the tail was detected in young animals (9/58, 16%) compared with the adults (15/160, 9%). Old badgers did not present any ulcer on the tail (see table 4). These cutaneous ulcers differ from those on the neck as they consistently included a large, up to 15cm, circumferential peripheral areas of hair loss, which were absent in the bite wounds (see figure 10).

Eighteen badgers (8%) presented low to moderate numbers of lice (*Trichodectes melis*) randomly distributed on the surface of the haired skin (see table 4). Direct identification and classification of the lice were performed by a parasitologist at the UoN who is included in the acknowledgments as reference. No other ectoparasites (mites nor ticks) were identified. There was a minimal increased trend of lice infestation in the young badgers (5/58, 9%) compared with the adults (13/160, 8%) (see table 4). No significant differences were noted between the age groups of badgers in relation to the lesions detected on the skin.

Figure 9. Ulcerative dermatitis at the neck (compatible with bite wound). Skin, lateral right neck; Badger number 458. The right ventrolateral area of the neck shows a focal, well demarcated, irregularly shaped, depressed, pink, 2x2cm, cutaneous ulceration compatible with bite wound (white arrow).

Figure 10. Ulcerative dermatitis at the base of the tail. Skin, base of the tail; Badger number 479. The skin at the base of the tail shows abundant multifocal, irregularly shaped, depressed, red to pink, 5x6cm, cutaneous ulcerations (black arrows) which are surrounded by thickened (hyperplastic) skin and an extensive area of hair loss (asterisks).

3.2 Mycobacteriosis and gross post mortem macroscopic examination

The body mass was the first parameter measured before starting a post-mortem examination.

Body mass values in badgers ranged between 5.1kg (the lightest) and 16.5kg (the heaviest) with a mean value of 10.3 kg (median 10.2kg, SD 1.96) (see figure 11). The body mass was also evaluated and compared within the sub-categories of badgers according to age, gender and mycobacterial infectious status and no significant differences were identified. A slight decrease in trend was noted in the category of the young badger (mean value 8.8kg) compared with the adults (mean value 10.7kg) and old badgers (mean value 11.1kg) (see figure 11). The population of males showed a slightly increased body mass (mean value 10.6kg) compared with the females (mean value 9.9kg). According to the mycobacterial infectious status, the body mass of the M+ animal (mean value 10.2kg) was approximately the same as the M- badgers (mean value 10.3kg) (see figure 11).

A comparison between the body mass and food availability and/or season was not possible.

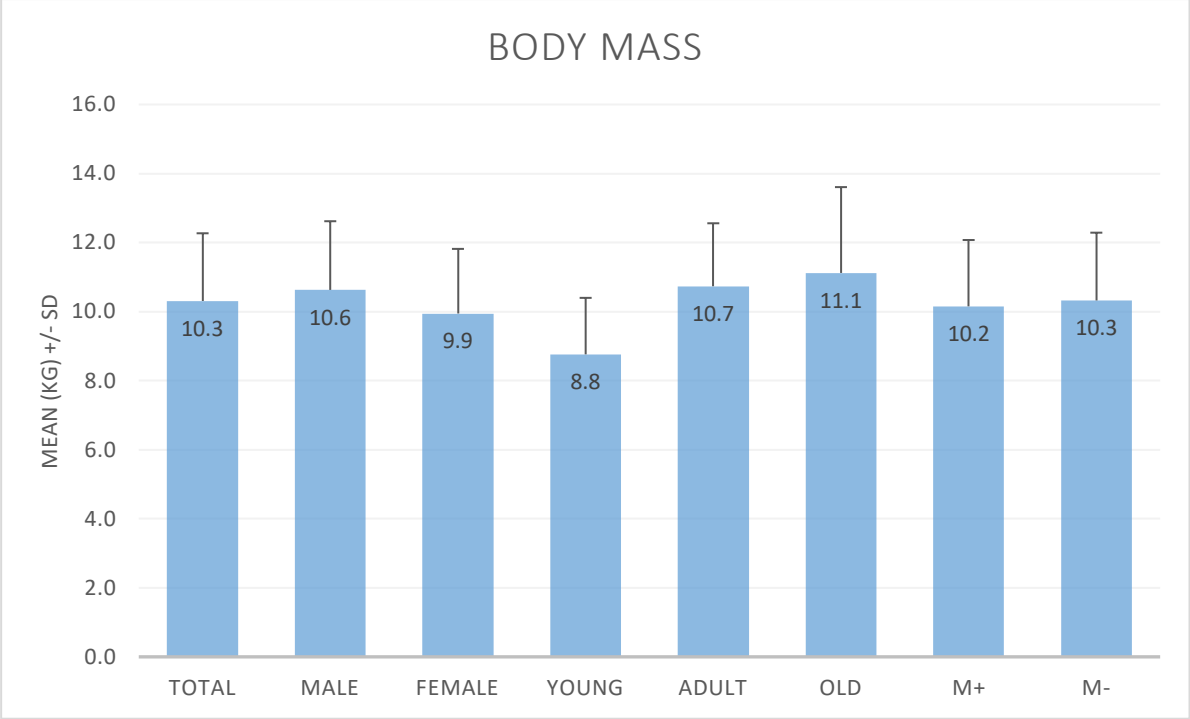

Figure 11. Body mass mean values (Kg) and gender, age, mycobacteriosis. The bar charts are demonstrating the mean body mass measurements in the total badger population (n=607) and subdivided in the different sub-categories, such as gender (male, female), estimated age (young, adult, old) and infection status (M+, M-). The bars indicate the mean value ± SD in the total number of badgers and sub-categories. Overall, the total mean value of the badger population is 10.31kg with a median of 10.20kg and SD of 1.96. The values were similar in the different categories with an exception for the young badgers which show a slightly reduced mean value (8.8kg). No statistically significant differences in body masses were noted in the different categories, M+ and M- included. SD=Standard Deviation, M+= positive to mycobacterial infection, M-= negative to mycobacterial infection.

The comparison between gross lesions and mycobacterial infectious status showed that the MTC+ animals presented more commonly a combination of multiple macroscopic lesions (12/25, 48%) rather than a single change (see table 5). The binomial of granulomas / lymphadenomegaly, lymphadenomegaly / bite wounds and lymphadenomegaly / tail ulcers were the most common changes in the MTC+ animals (respectively being each 2/25, 8%) but not statistically significant. The proportion of M+ animals (16/37) and M- animals (82/190) showing multiple lesions was the same (both 43%). The concomitance of bite wounds and tail ulcers were frequently observed in both M+ (n=5, 13%) and M- animals (n=23, 12%).

The bite wounds showed a trend towards significancy in the M+ group (11/37, 30%) and OM+ group (5/12, 42%) compared with the M- group (38/190, 20%), although the difference was not statistically significant (see table 5).

Within the 10 animals showing only granulomas, only one (3%) was M+ (specifically OM+) and the remaining nine badgers (5%) were M-. Granulomas as single lesion were only identified in OM+ animals (1/12, 8%) and none of the MTC+ ones. The granulomas in the MTC+ badgers were identified only in combination with other changes, such as lymphadenomegaly. On the other hand, lymphadenomegaly as single lesion was identified only in the MTC+ animals (3/25, 12%) and none of the OM+ ones (see table 5).

The proportion of lice infestation was slightly higher in the M+ animals (11%, n=4) compared with the M- badgers (7%, n=14), but not statistically significant. On the other hand, the ulcers on the tail were predominantly seen in the M- animals (13%, n=24) compared with the M+ badgers (5%, n=2), although not significant (see table 5).

Animal (n=227)	Granuloma (n=10, 4%)	Lymph-adenomegaly (n=26, 11%)	Bite wounds (n=49, 22%)	Lice (n=18, 8%)	Tail ulcers (n=26, 11%)	Mixed lesions (n=98, 44%)
M+ (n=37, 16%)	1/37 *(3%)*	3/37 *(8%)*	11/37 *(30%)*	4/37 *(11%)*	2/37 *(5%)*	16/37 *(43%)*
MTC+ (n=25, 11%)	0/25 *(0%)*	3/25 *(12%)*	6/25 *(24%)*	3/25 *(12%)*	1/25 *(4%)*	12/25 *(48%)*
OM+ (n=12, 5%	1/12 *(8%)*	0/12 *(0%)*	5/12 *(42%)*	1/12 *(8%)*	1/12 *(8%)*	4/12 *(34%)*
M- (n=190, 84%)	9/190 *(5%)*	23/190 *(12%)*	38/190 *(20%)*	14/190 *(7%)*	24/190 *(13%)*	82/190 *(43%)*

Table 5. Macroscopic gross post-mortem lesions and mycobacteriosis. The table illustrates the number of animals (number/total and percentage) with one single lesion (granulomas, lymphadenomegaly, bite wounds, lice ot tail ulcers) or with multiple changes (mixed lesions) subdivided within the M+, MTC+, OM+ and M- groups according to *Mycobacterium* culture and PCR results. M+ includes both MTC+ and OM+. The only M+ badger showing granulomatous pneumonia as single lesion macroscopically was OM+. Lymphadenomegaly as only change was only detected in MTC+ animals. A higher proportion of M+ animals (30%) and OM+ badgers (42%) showed bite wounds when compared with the M- animals (20 %), although the difference was not statistically significant. The highest proportion of MTC+ badgers presented multiple lesions (48%). No statistical differences were found between any of the categories. M+=badgers positive to mycobacterial infection, MTC+= badgers positive to M. tuberculosis complex, OM+= badgers positive to other mycobacteria, M-= badgers negative to mycobacterial infection.

3.3 Histopathologic microscopic examination

A total of 88 animals were examined for histopathology. Following culture and IS6110 PCR results, two age and sex-balanced subgroups within the M+ and M- groups were selected for histopathological examination. Fresh carcasses and animals with the least autolytic changes (mild and mild to moderate) were selected. Thus, 44 out of the 607 badgers from each group (M+ and M-) were included. Both groups included fresh animals (n=1), with mild autolysis (n=21), with moderate autolysis (n=22).

The observed histological changes were the presence of silica-laden macrophages (SLMs) in the lung and tracheobronchial lymph nodes, granulomas in multiple organs, sarcocystosis in the skeletal muscles, lymphoid follicular hyperplasia in the lymph nodes and spleen, chronic interstitial nephritis in the kidney, portal to periportal hepatitis or hepatic necrosis in the liver, ulcerative dermatitis affecting the skin of the neck or base of the tail, nematodiasis and adiaspiromycosis in the lung. SLMs was the most common histological change (n=75, 85%), followed by granulomatous inflammation (n=47, 53%), muscular sarcocystosis (n=41, 47%), lymphoid follicular hyperplasia (n=34, 38%), chronic interstitial nephritis (n=27, 30%), portal to periportal hepatitis (n=23, 26%), ulcerative dermatitis (n=16, 18%) and pulmonary nematodiasis (n=10, 11%). The least common histopathological changes were both hepatic necrosis and pulmonary adiaspiromycosis (both n=8, 9%) (see figure 12).

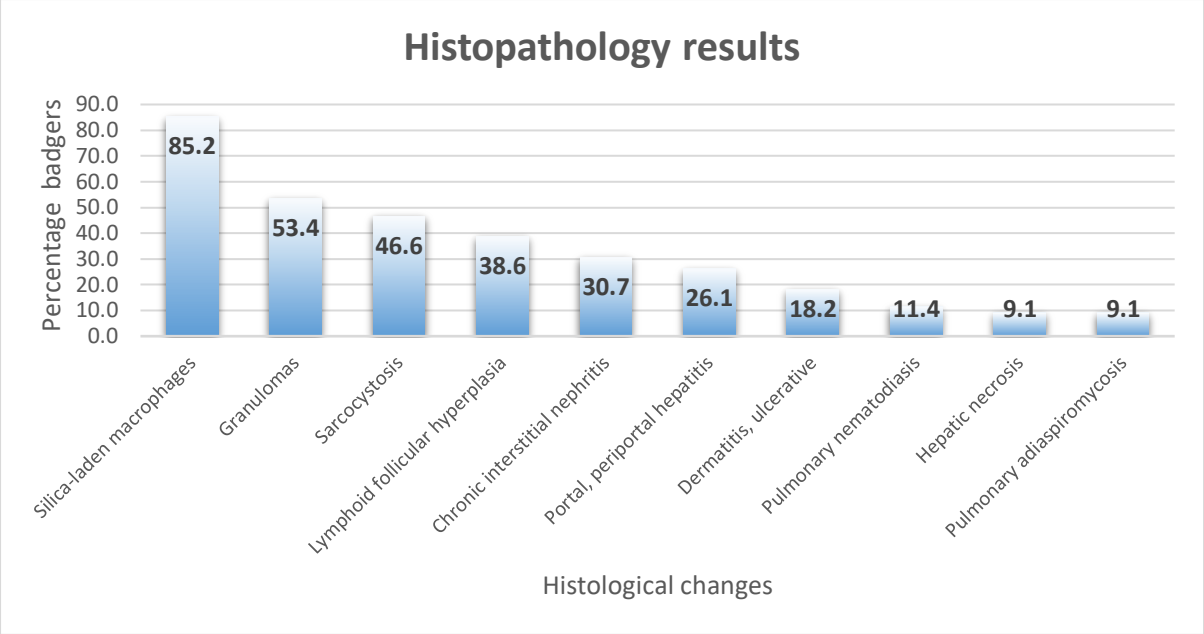

Figure 12: Histopathology results. The bar charts illustrate the percentage of animals (Y axis) affected by the identified histological changes (X axis) in decreased order of frequency (left to right). Only a group of animals (n=88) were selected for histopathological examination. The most common histological change identified in the badgers was the presence of SLMs (n=75, 85%) which was noted predominantly in the lung, followed by granulomatous inflammation (n=47, 53%) and muscular sarcocystosis (n=41, 47%). Thirty four badgers (n=34, 38%) showed lymphoid follicular hyperplasia in the peripheral lymph nodes and spleen. Chronic interstitial nephritis was identified in twenty seven animals (n=27, 30%), portal to periportal hepatitis in twenty three badgers (n=23, 26%), ulcerative dermatitis in sixteen animals (n=16, 18%) and pulmonary nematodiasis was seen in ten badgers (n=10, 11%). The least common histopathological changes were both hepatic necrosis and pulmonary adiaspiromycosis (both n=8, 9%).

3.3.1 Silica laden macrophages

SLMs were present in the lung and in the tracheobronchial lymph nodes and appeared histologically as aggregates of macrophages containing moderate amounts of intracytoplasmic dark to light brown, granular to specular material. The clusters of macrophages were identified within the alveolar septa and peri-bronchial and perivascular interstitium (see figure 13).

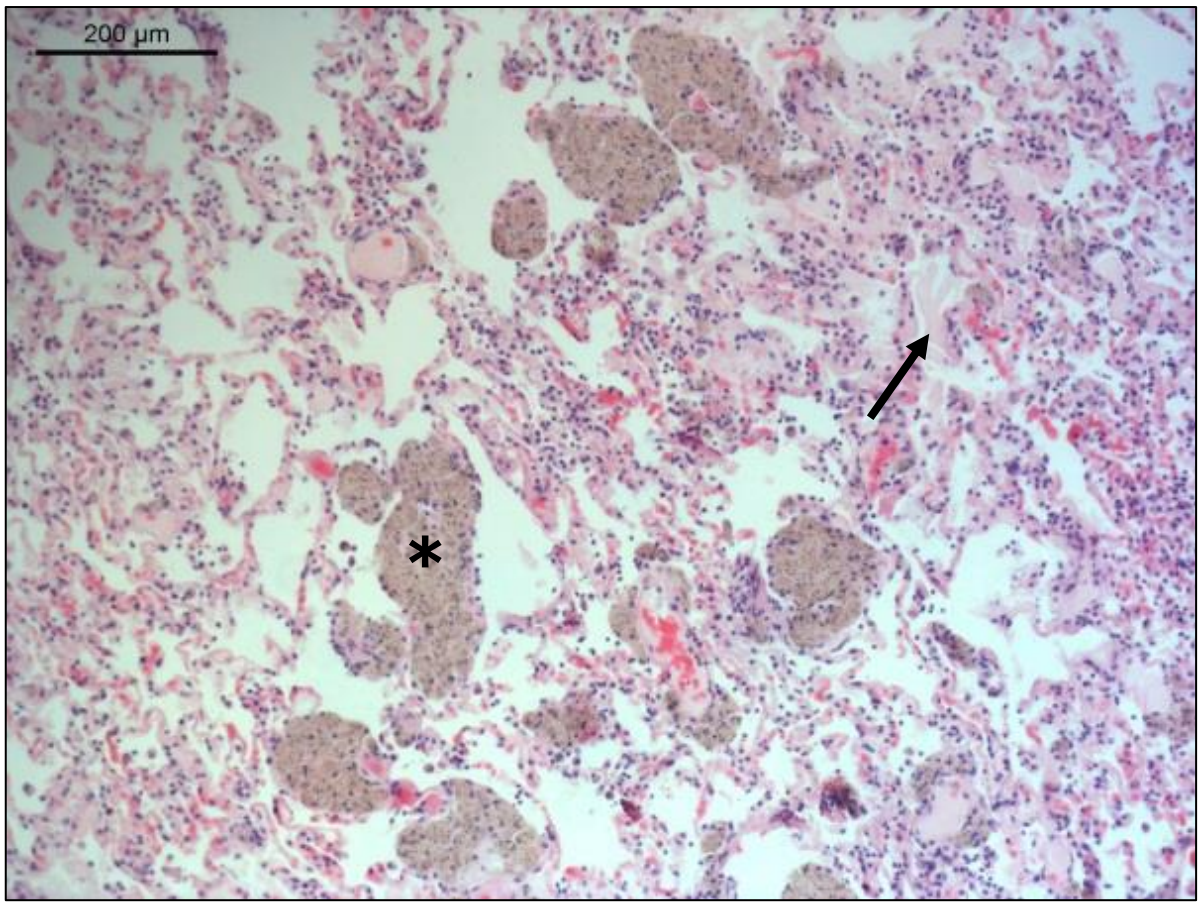

Figure 13. Silica-laden macrophages (SLMs); Lung, HE, 10X HPF. Badger number 350. Multifocally within the interalveolar septa, there are aggregates of macrophages containing abundant brown and finely granular intracytoplasmic material (interpreted as silica) (asterisk). Adjacent alveolar spaces occasionally contain minimal amount of eosinophilic proteinaceous fluid (oedema) (black arrow). HE = haematoxylin and eosin. HPF = High Power Field.

3.3.2 Granulomas

The histomorphology of the granulomas varied from small nests of epithelioid macrophages surrounded by a rim of lymphocytes and plasma cells. Multinucleated cells were absent, and areas of lytic necrosis were rarely seen. Areas of mineralisation were not identified. Granulomas were noted histologically in several organs, such as spleen, kidney, lymph node, haired skin, testicle, stomach, lung and liver (see figures 14 and 15).

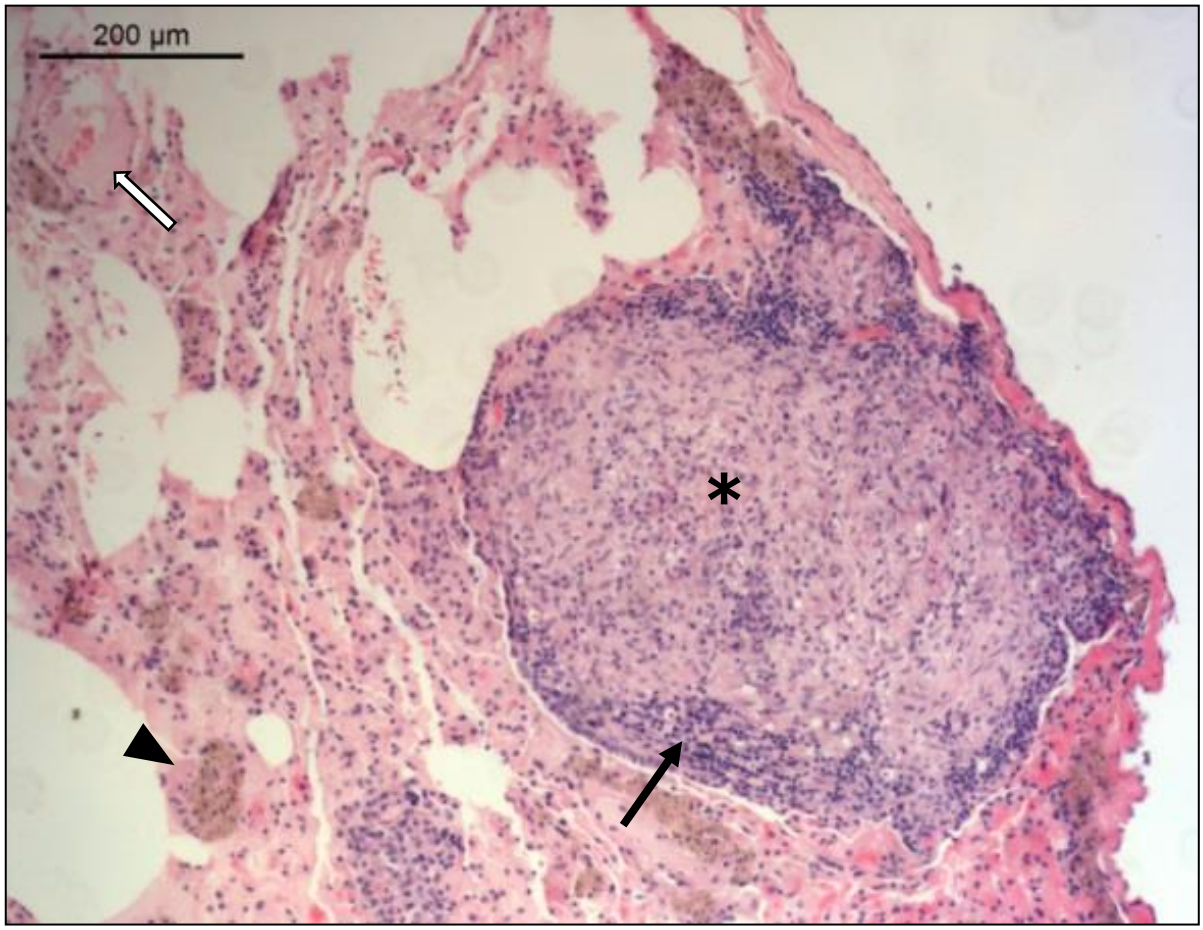

Figure 14. Pulmonary granuloma; Lung, HE, 10X HPF. Badger 1414. Focally, there is a discrete granuloma (asterisk) composed of abundant large, up to 10µm, macrophages rimmed by mature lymphocytes and plasma cells (black arrow). Adjacent alveolar spaces contain moderate amount of eosinophilic and finely granular proteinaceous fluid (oedema) (white arrow). Few aggregates of SLMs (arrowhead) are also multifocally present. HE = haematoxylin and eosin. HPF = High Power Field.

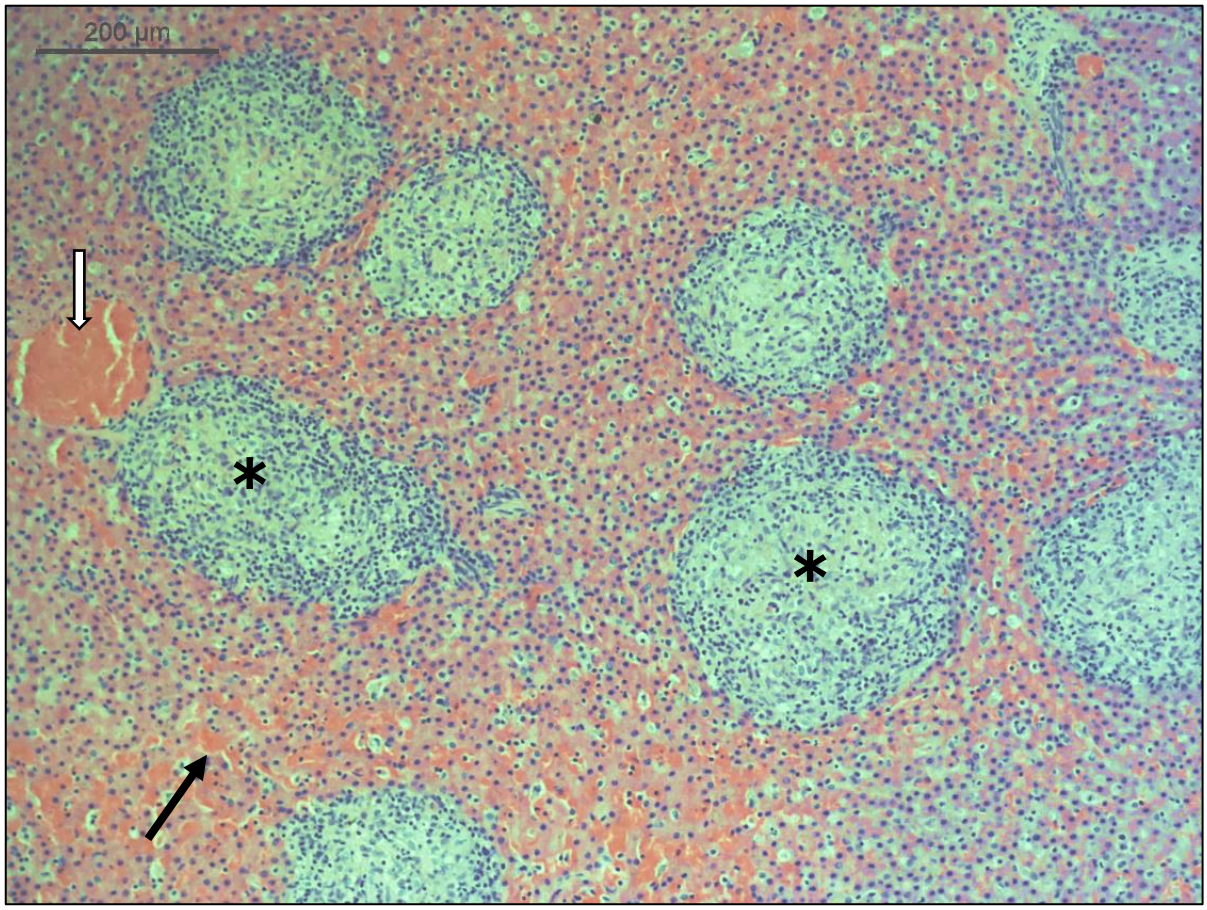

Figure 15. Hepatic granulomas; Liver, HE, 10X, HPF. Badger number 1414. The hepatic parenchyma contains numerous multifocal granulomas (asterisks) composed of macrophages rimmed by mature lymphocytes and plasma cells. There is moderate congestion of the adjacent sinusoids (black arrow) and central veins (white arrow). HE = haematoxylin and eosin. HPF = High Power Field.

3.3.3 Portal hepatitis and hepatic necrosis

Within the liver, in addition to the granulomas, the most common histological change (n=23, 26%) was the presence of portal to periportal, lymphocytic and plasmacytic hepatitis, characterised by moderate numbers of small mature lymphocytes and fewer plasma cells in the portal triads and surrounding portal tracts admixed with dilated lymphatics and minimal increased collagen (fibrosis) (see figure 16). A further eight animals (9%) had a random, lytic hepatic necrosis characterised by karyorrhectic and hypereosinophilic hepatocytes admixed with fibrin, haemorrhage and variable numbers of viable and non-viable neutrophils. No infectious agents were observed within these lesions.

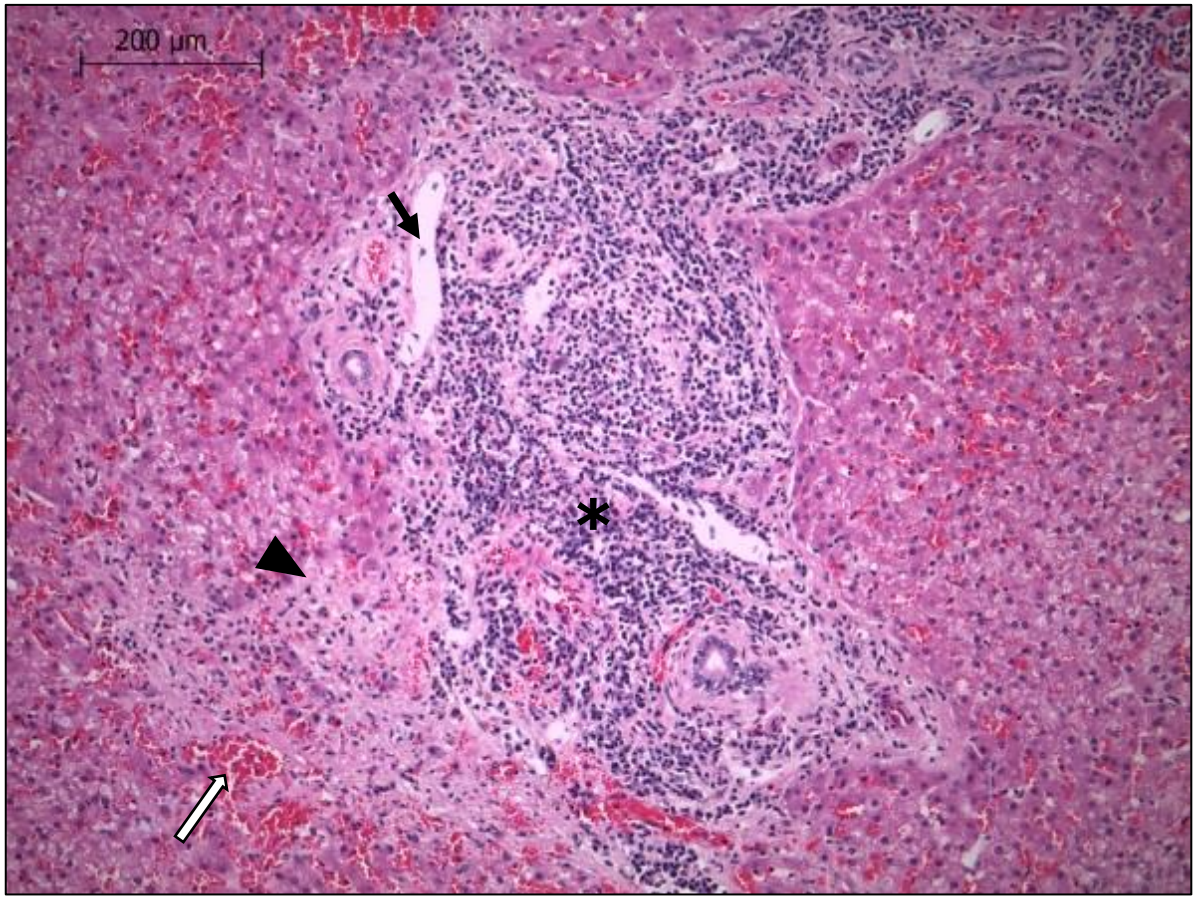

Figure 16. Portal to periportal hepatitis; Liver, HE, 10X, HPF. Badger number 662. Multifocally expanding the portal tracts, there are moderate numbers of mature lymphocytes with fewer plasma cells (asterisk). Portal tracts also show dilated lymphatic vessels (black arrow). There is minimal periportal increased amount of pale collagenous material (mild fibrosis) (arrowhead). The sinusoids are moderately congested (white arrow). HE = haematoxylin and eosin. HPF = High Power Field.

3.3.4 Interstitial nephritis

Chronic interstitial nephritis was seen mostly in the renal cortex and the corticomedullary junction and was characterised by a variable degree of lymphocytic or lymphoplasmacytic infiltration within the interstitium and compressing adjacent renal tubules. There was a variable degree of tubular degeneration and regeneration and tubular ectasia (see figure 17).

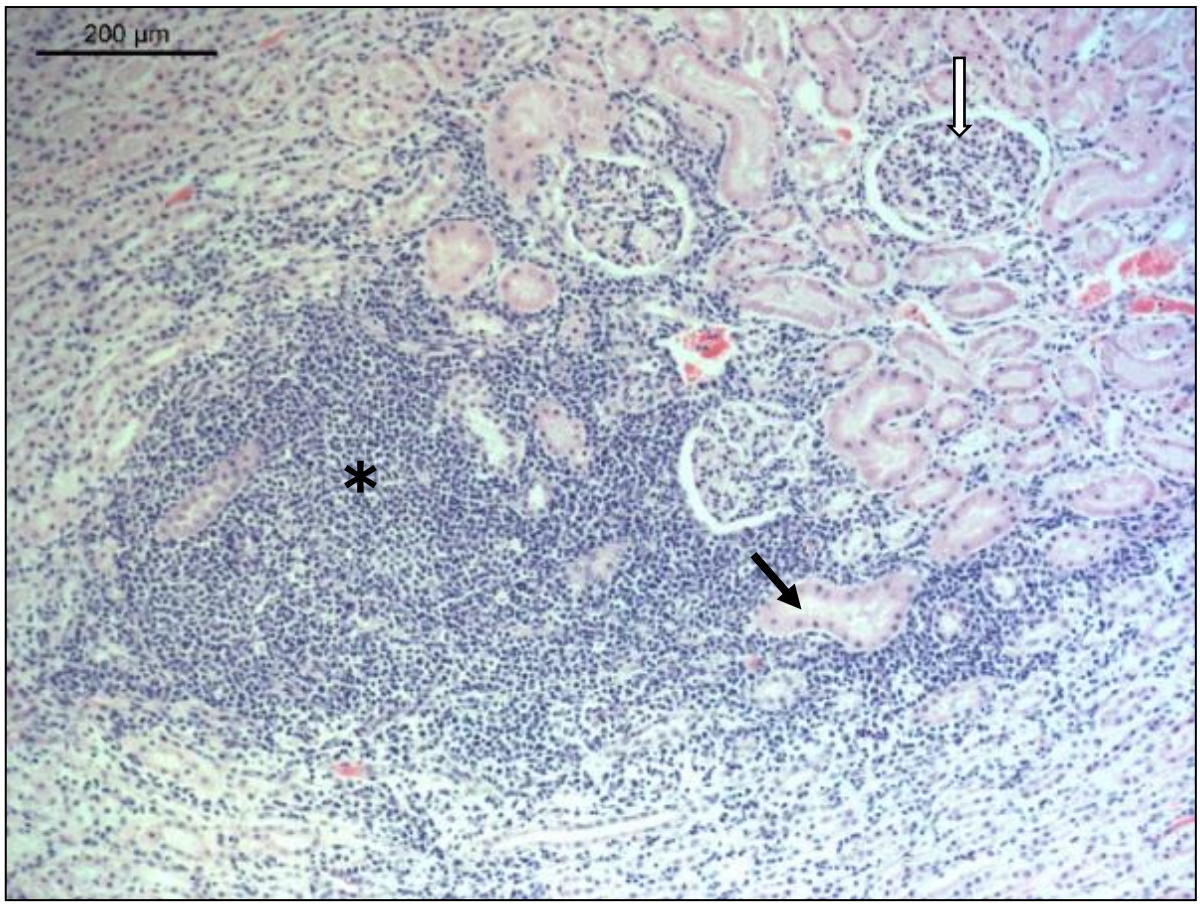

Figure 17. Chronic Interstitial Nephritis (CIN); Kidney, HE, 10X, HPF. Badger number 74. Multifocally within the renal corticomedullary junction and renal cortex, the interstitium is expanded by abundant small mature lymphocytes and plasma cells (asterisk). Adjacent proximal renal tubules are mildly ectatic (black arrow) and the epithelium is either swollen and vacuolated (degeneration) or show a prominent vesicular nucleus, basophilic cytoplasm and rare mitotic figures (regeneration). The renal glomeruli (white arrow) are unremarkable. HE = haematoxylin and eosin. HPF = High Power Field.

3.3.5 Pulmonary adiaspiromycosis

Pulmonary adiaspiromycosis consisted on few multifocal granulomatous foci composed of epithelioid macrophages and fewer lymphocytes, which surrounded large, variably sized (up to 300µm diameter), uninucleate, round, fungal adiaspores with a 5-µm thick hypereosinophilic wall (consistent in morphology with *Emmonsia spp*.) (see figure 18). The fungal wall occasionally was confirmed with PAS special stain.

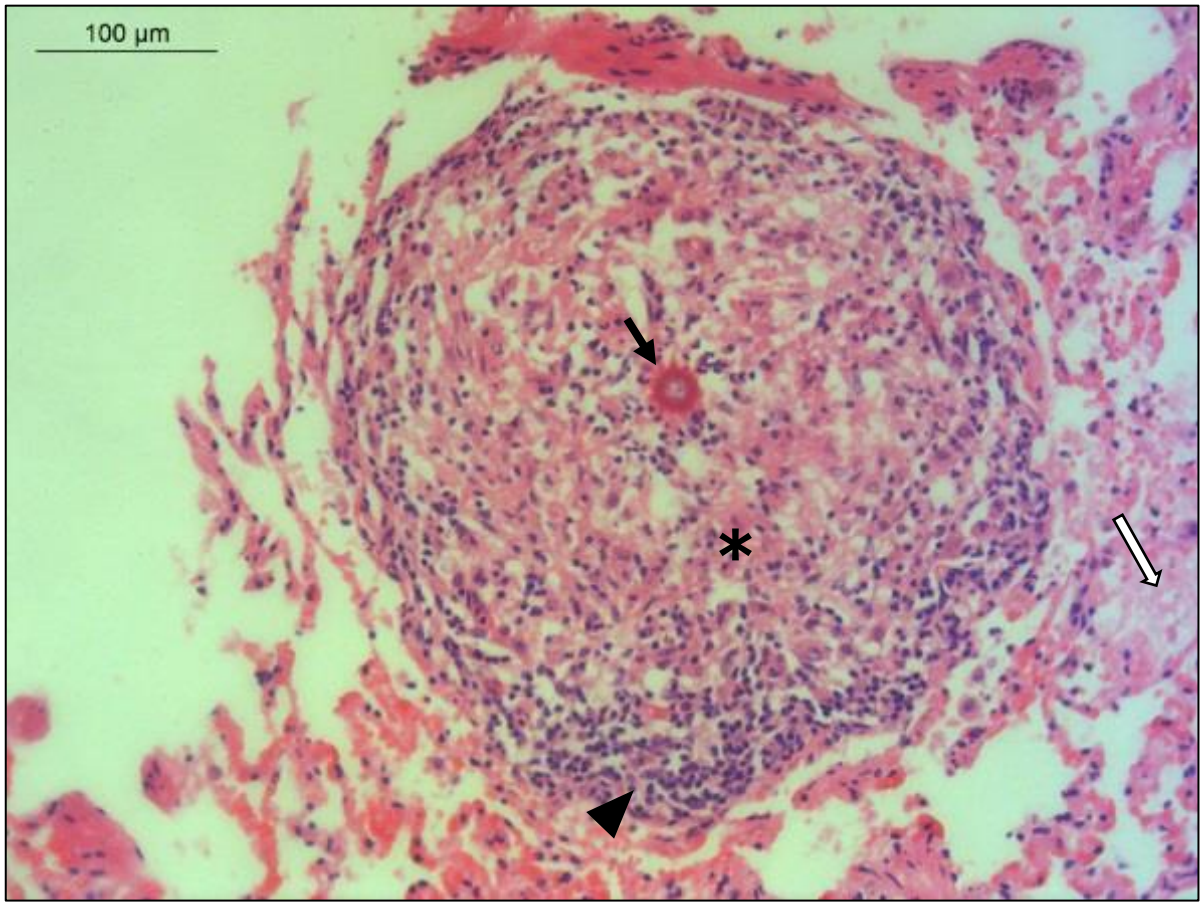

Figure 18. Pulmonary adiaspiromycosis; Lung, HE, 10X, HPF. Badger number 1283. There is a focal granuloma composed of abundant epithelioid macrophages (asterisk) rimmed by moderate lymphocytes and plasma cells (arrowhead). At the centre of the granuloma, there is a single, round, approximately 30µm in diameter, purple, adiaspore consistent in morphology with the fungus *Emmonsia spp.* (black arrow). The adjacent alveolar spaces are mildly compressed by the granuloma and contain minimal amount of pale proteinaceous eosinophilic fluid (oedema) (white arrow). HE = haematoxylin and eosin. HPF = High Power Field.

3.3.6 Sarcocystosis

Sarcocystosis was detected in the skeletal muscle layer of the tongue and in the masseter skeletal striated muscle. Protozoal cysts were up to 250µm, contained numerous 7µm, crescent-shaped bradyzoites and were surrounded by a thin hyalinised wall. Cysts were observed within the sarcoplasm of the myocytes. The parasites were consistent in morphology with *Sarcocystis spp.* and no to minimal lymphocytic myositis were associated with the cysts (see figure 19).

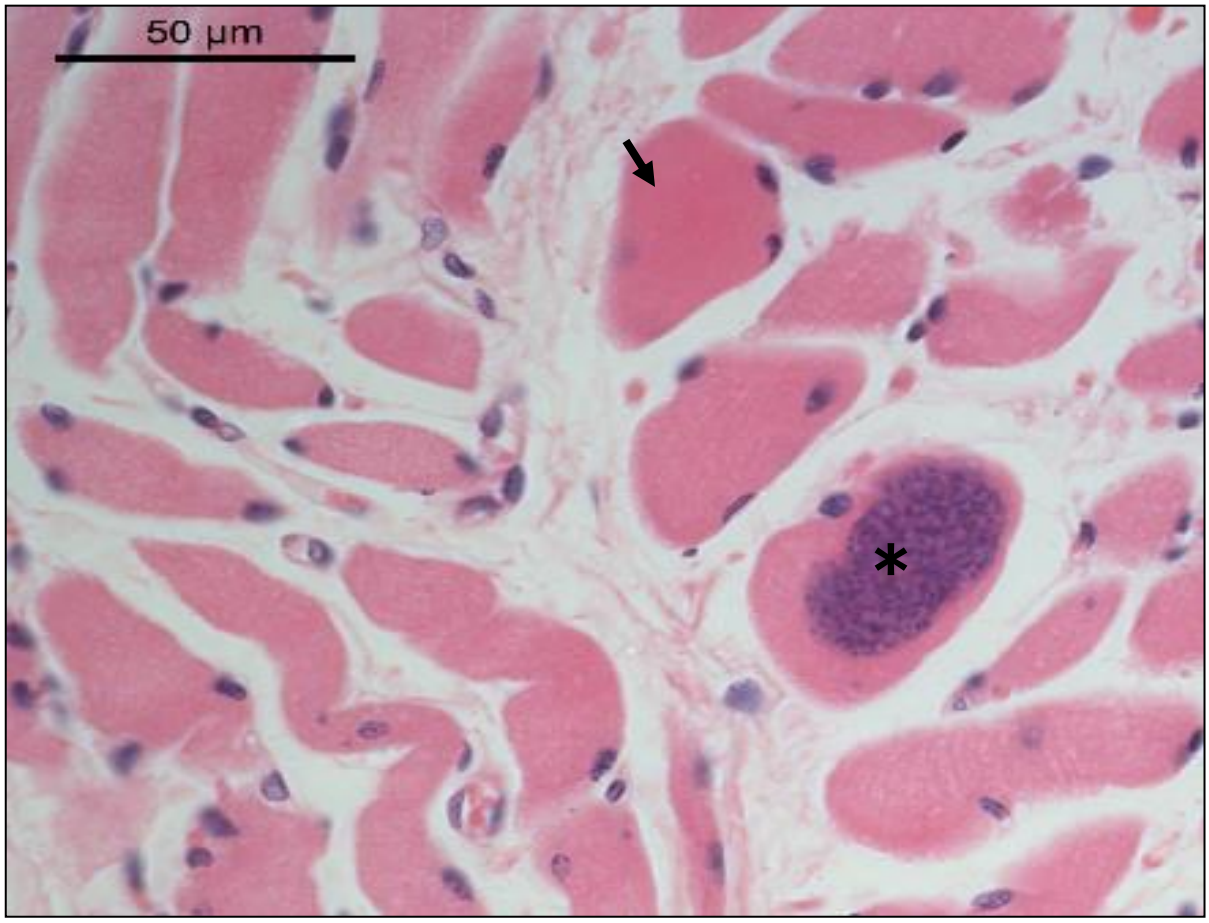

Figure 19. Sarcocystosis, tongue (Skeletal muscle layer), HE, 40X, HPF. Badger number 883. The sarcoplasm of the myocyte contains a protozoal cyst with abundant basophilic crescent-shaped structures (bradyzoites, asterisk). The cyst measures approximately 60µm in diameter. There is no evidence of inflammation and the adjacent myofibers (black arrow) are unremarkable. HE = haematoxylin and eosin. HPF = High Power Field.

3.3.7 Pulmonary nematodiasis

In the lung, 10 animals (11%) had histological evidence of nematodes compatible in morphology with *Perostrongylus falciformis.* Alveolar lumina were markedly expanded by aggregates of intra-alveolar nematode adults containing abundant embryonated and non-embryonated nematode eggs and larvae in various stages of development, which were surrounded by minimal numbers of lymphocytes, plasma cells and occasionally histiocytes and more frequently no inflammation (see figure 20).

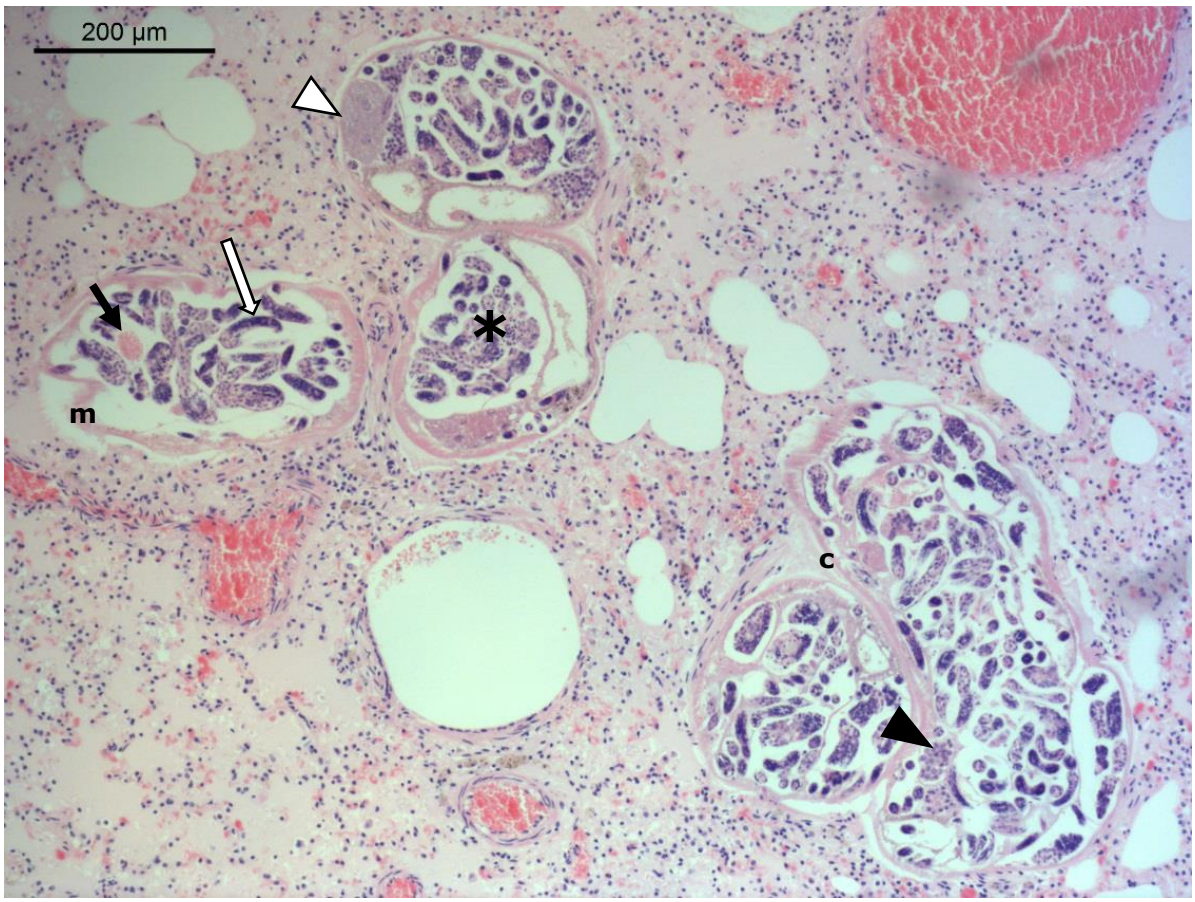

Figure 20. Pulmonary nematodiasis, Lung, HE, 10X HPF. Badger number 1409. Multifocally, alveolar lumina are markedly expanded by abundant adult nematodes (asterisks) with no associated inflammation. The parasites have a thin smooth cuticle (c), coelomyarian-polymyarian musculature (m), a pseudocoelom which contains intestine (white arrowhead) and a large uterus filled with larvae and embryonated and non-embryonated nematode eggs. Larvae (white arrow) are approximately 70µm, with 4-6µm diameter, basophilic nuclei and 1µm, amphophilic cuticle. Non-embryonated eggs (black arrow) are oval, approximately 30µm and eosinophilic. Embryonated eggs (black arrowhead) are 50µm and multinucleated. HE = haematoxylin and eosin. HPF = High Power Field.

3.4 Mycobacteriosis and histopathologic microscopic examination

The histopathological changes in badgers are assessed with and without confirmed mycobacteriosis (M+, MTC+, OM+ and M-). Overall, 77% M+ group badgers (34/44) had granulomatous lesions in at least one organ compared to 29% M- badgers (13/34) ($p < 0.001$) (see table 6). The prevalence of 84% in MTC+ badgers (26/31) was also significantly larger than in M- badgers (13/34) ($p < 0.001$). Granulomas were detected histologically in twenty-six MTC+ badgers (26/31, 84%), eight OM+ badgers (8/13, 62%) and thirteen M- badgers (13/44, 29%) (see table 6). There were not enough 'other mycobacteriosis' (OM+) badgers to compare statistically the prevalence of granulomas in them with that in MTC+ badgers, but no obvious difference in prevalence was observed.

In contrast, there was a significantly smaller prevalence of portal to periportal hepatitis in M+ badgers (5/44, 11%) and also in the MTC+ badgers (3/31, 10%) ($p = 0.0030$) compared to the M- badgers (18/44, 41%) ($p = 0.0036$) (see table 6).

Regarding the other histological lesions, no significant changes were identified. SLMs showed a similar prevalence in all the groups and varied from 84% (37/44) in the M+ to 87% (38/44) in the M- badgers. Hepatic necrosis registered the same prevalence in both M+ and M- animals (both 9%). The only M+ animals showing hepatic necrosis were MTC+. A slight decrease in trend of M+ animals showing nephritis (10/44, 23%) was identified when compared with the M- group (17/44, 39%), although not significant. A similar decreased trend in the M+ group was noted for the animals presenting muscular sarcocystosis (18/44, 40%) and pulmonary adiaspiromycosis (2/44, 4%) compared with the M- animals (respectively 23/44, 52% and 6/44, 11%). The only M+ animals with concomitant adiaspiromycosis were OM+.

In contrast, pulmonary nematodiasis showed an increased trend in the M+ animals (6/44, 14%) compared to the M- badgers (4/44, 9%) (see table 6).

Among the M- animals with granulomatous lesions (13/44, 29%) other causes than *Mycobacterium* spp. were histologically identified in some animals, which included pulmonary adiaspiromycosis (6/13, 46%) and spermatic granuloma in the testicle (1/13, 8%). In the remaining M- granulomas localised in the lung (5/13, 38%) and stomach (1/13, 8%), a clear cause could not be determined histologically but parasitic (nematode) migration tracts were suspected.

Two M+ badgers (4%) had granulomatous pneumonia and concomitant lung infections with *Emmonsia* spp. and *Mycobacterium* spp. Six M+ badgers (14%), had interstitial lymphocytic and histiocytic pneumonia with concomitant pulmonary nematode larvae (compatible in morphology with *Perostrongylus falciformis*) and *Mycobacterium spp.* infection.

Animal (n=88)	M+ (n=44)	MTC+ (n=31)	OM+ (n=13)	M- (n=44)
Granuloma	34/44* *(77%)*	26/31* *(84%)*	8/13 *(62%)*	13/44 *(29%)*
SLMs	37/44 *(84%)*	25/31 *(81%)*	12/13 *(93%)*	38/44 *(87%)*
Periportal Hepatitis	5/44** *(11%)*	3/31** *(10%)*	2/13 *(15%)*	18/44 *(41%)*
Hepatic necrosis	4/44 *(9%)*	4/31 *(13%)*	0/13 *(0%)*	4/44 *(9%)*
Chronic interstitial Nephritis	10/44 *(23%)*	7/31 *(23%)*	3/13 *(23%)*	17/44 *(39%)*
Sarcocystosis	18/44 *(40%)*	13/31 *(42%)*	5/13 *(38%)*	23/44 *(52%)*
Pulmonary adiaspiro-mycosis	2/44 *(4%)*	0/31 *(0%)*	2/13 *(15%)*	6/44 *(11%)*
Pulmonary nematodiasis	6/44 *(14%)*	4/31 *(13%)*	2/13 *(15%)*	4/44 *(9%)*

Table 6. Histopathology and mycobacteriosis. The table illustrates the most common histopathological changes noted in the selected badger population (n=88) which comprises 44 M+ animals (31 MTC+ and 13 OM+) and 44 M- badgers. For each category, there is the number of the animal showing the histological lesions and percentage (frequency) of the affected badgers. There is a significant trend for the M+ animals showing histological granulomas compared with the M- badgers (*$p < 0.001$). The same significant trend was also noted in MTC+ badgers showing granulomas (*$p < 0.001$). On the other hand, both MTC+ and M+ badgers show a significant lower frequency of hepatitis than M- animals (**$p = 0.003$). No significant differences were identified in the other subgroups. M+=badgers positive to mycobacterial infection, MTC+= badgers positive to *M. tuberculosis* complex, OM+= badgers positive to other mycobacteria, M-= badgers negative to mycobacterial infection.

3.5 Mycobacteriosis and granulomas: location and distribution in the body

The distribution of the granulomas in the organs was also assessed histologically. Lungs and lymph nodes were the organs with more frequently observed granulomas (respectively 34/47, 79% and 5/47, 11%). They were followed by liver and haired skin (both 4/47, 8%) and kidneys and spleen (both 2/47, 4%). The least frequent locations were stomach and testicle (both 1/47, 2% of prevalence) (see figure 21).

The most common location of granulomas in both M+ and M- badgers was the lung (37/47, 78%) compared with all the other organs combined (19/47, 40%), and this was statistically significant (χ^2=12.77, df=1, p = 0.0004). There was, however, no obvious difference in the prevalence of lung granulomas between M+ (26/34, 77%) and M- animals (11/13, 84%) (see table 7). There were very few granulomas in the testicle (1/13, 8%) or stomach (1/13, 8%) (both M- badgers) and the difference in prevalence of granulomas between M+ and M- badgers was driven by 6-15% M+ badgers having microscopic granulomas in lymph nodes, liver, skin, spleen and kidney. Specifically, 6% of M+ granulomas were identified in the spleen (2/34) and kidney (2/34), followed by a 12% identified in the liver (4/34) and skin (4/34) and, finally, 15% identified in the lymph nodes (5/34). There were no M- granulomas in the liver, spleen, kidney, lymph nodes and skin (see table 7).

All the tissues with granulomas were stained with ZN and MZN. Acid fast bacilli were detected in all the selected MTC+ and OM+ badgers (34/34, 100%). No acid-fast bacilli were detected in the M- granulomas.

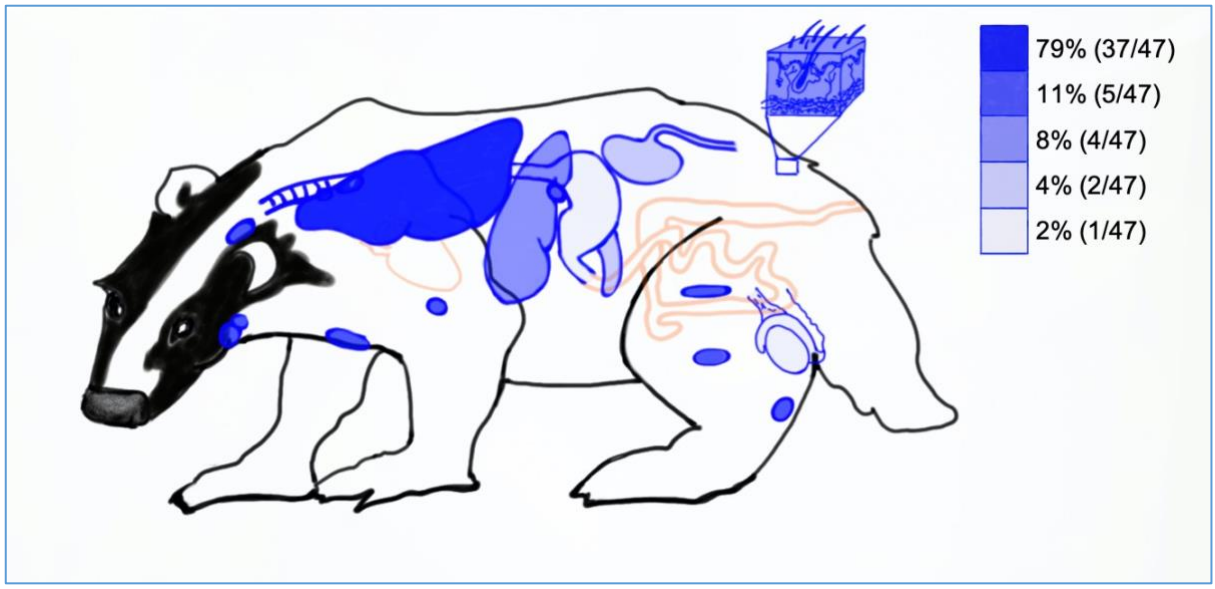

Figure 21. Location of granulomas in badger tissues. The picture shows the frequency (%) with the number of animals affected by granulomatous lesions (n / total of badgers with granuloma) within the organs of badgers as shown; blue colour intensity indicates dark=high to light=low granuloma frequencies. Note: lungs and lymph nodes were the most common locations of the granulomas on histological examination, respectively 79% and 11% of frequency. They are followed by liver and haired skin (both 8%), kidneys and spleen (both 4%) and stomach and testicle (both 2%).

Organs	Granuloma M+ badgers (n=34)	Granuloma M- badgers (n=13)
Lung	26/34 *(77%)**	11/13 *(84%)**
Liver	4/34 *(12%)*	0/13 *(0%)*
Spleen	2/34 *(6%)*	0/13 *(0%)*
Kidney	2/34 *(6%)*	0/13 *(0%)*
Lymph nodes	5/34 *(15%)*	0/13 *(0%)*
Haired Skin	4/34 *(12%)*	0/13 *(0%)*
Testicle	0/34 *(0%)*	1/13 *(8%)*
Stomach	0/34 *(0%)*	1/13 *(8%)*

Table 7. Distribution of M+ and M- granulomas in badger tissues. The table illustrates the distribution of the M+ and M- granulomas in the different organs. For each category, the number of badgers (n/total) and the percentage (frequency %) are listed. The lung was the most common organ affected by granulomas in both M+ (77%) and M- (84%) compared with the other organs (*p = 0.0004). There were no M+ granulomas in stomach and testicle. The only few granulomas identified in the testicle (n=1, 8%) and stomach (n =1, 8%) were M-. The liver, spleen, kidney, lymph nodes and haired skin presented only M+ granulomas. No trends of significance were identified between the M+ and M- pulmonary granulomas. M+=badgers positive to mycobacterial infection, M-= badgers negative to mycobacterial infection.

3.6 Atypical mycobacteria

3.6.1 *Mycobacterium nonchromogenicum*

Among the OM+ group, one badger was identified as *M. nonchromogenicum* (Swift B. and O'Cathail C., per. comm). Histologically, there was evidence of pulmonary granulomas identical in morphology to the ones identified in the MTC+ animals (see figure 22). Sparse intra-histiocytic and extracellular MZN acid fast bacilli were detected (see figure 23).

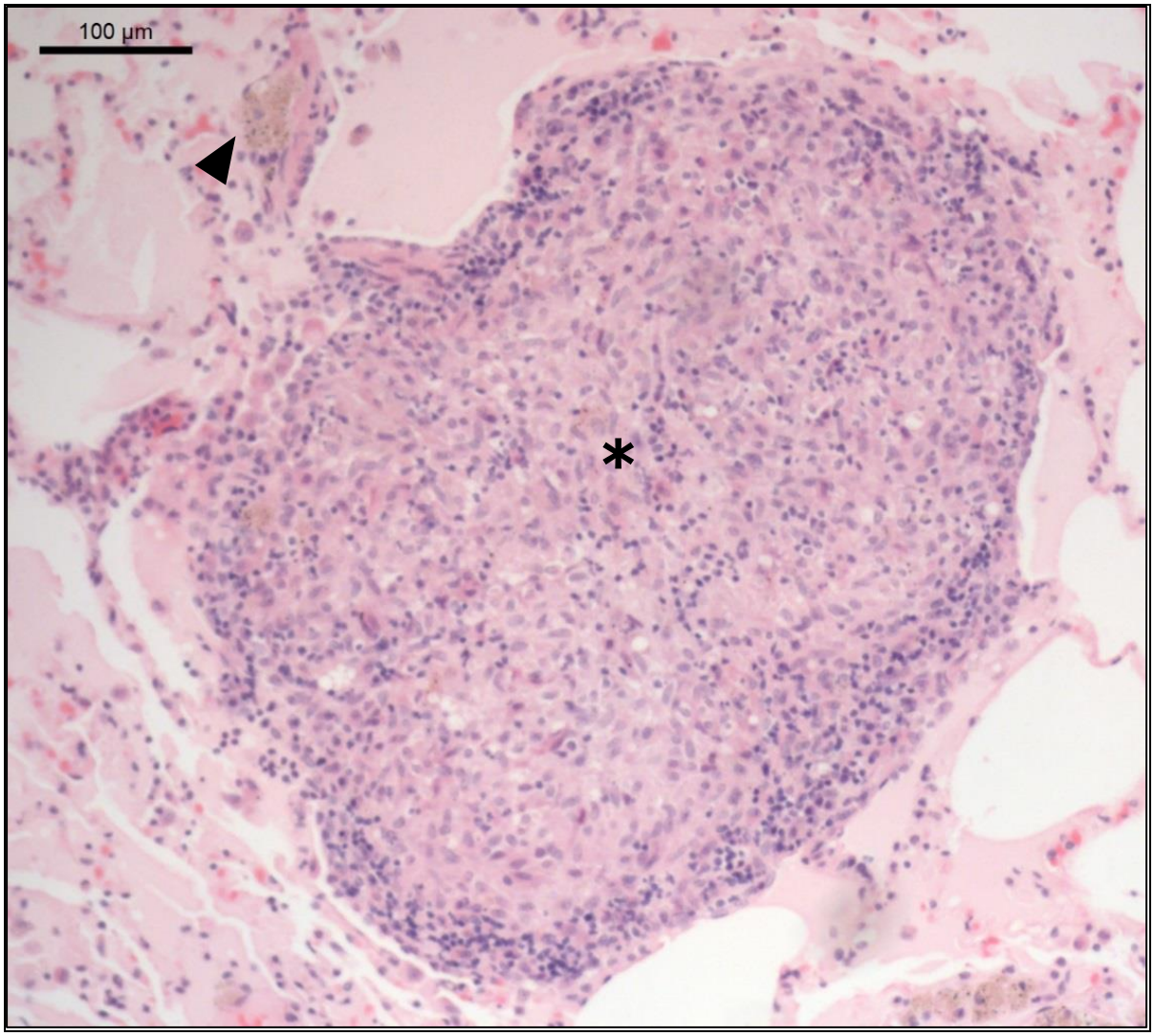

Figure 22. 'Atypical' mycobacteriosis (*Mycobacterium nonchromogenicum*); Lung, HE, 10X HPF. Badger number 516. Compressing the alveolar spaces, there is a focal discrete granuloma (asterisk), with a centre of epithelioid macrophages rimmed by moderate lymphocytes and plasma cells. Note few SLMs (arrowhead) surrounding the granulomas. HE = haematoxylin and eosin. HPF = High Power Field.

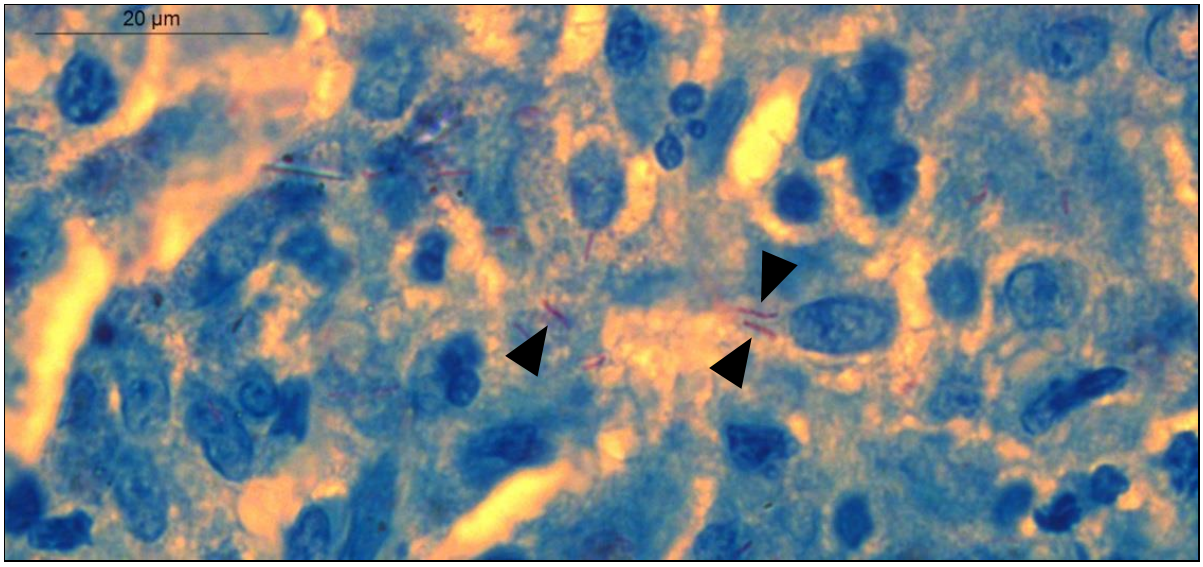

Figure 23. 'Atypical' mycobacteriosis (*Mycobacterium nonchromogenicum*); Lung, MZN, 40X HPF. Badger number 516. Within the cytoplasm of the epithelioid macrophages and occasionally extracellularly, there are few, approximately 4µm, pale, red ("acid fast"), bacilli (arrowheads) consistent in morphology with *Mycobacterium* species. MZN = Modified Ziehl Neelsen. HPF = High Power Field.

3.6.2 *Mycobacterium malmoense*

Among the OM+ group, one badger was found to be infected with *M. malmoense* (Swift B. and O'Cathail C., per. comm). There was histological evidence of pyogranulomatous and ulcerative dermatitis and panniculitis with abundant intra-histiocytic and extracellular MZN acid-fast positive bacteria (see figures 24 and 25).

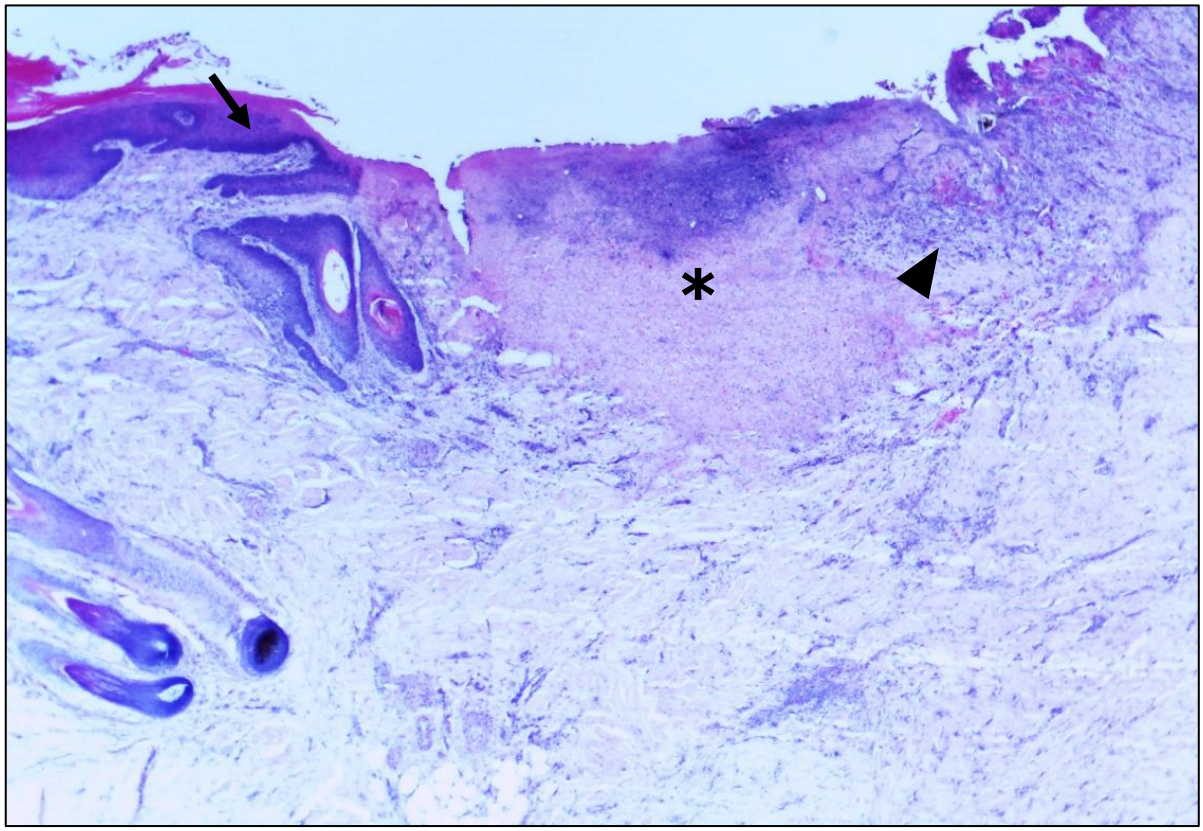

Figure 24. 'Atypical' mycobacteriosis (*Mycobacterium malmoense*); Haired skin, dorsum, HE, 10X HPF. Badger number 458. There is a focal and extensive ulceration of the skin (asterisk) composed of karyorrhectic and necrotic eosinophilic and basophilic debris and fibrin. The underlying tissue within the dermis contains a focally extensive pyogranulomatous inflammation (arrowhead) composed of histiocytes and viable and degenerate neutrophils admixed with minimal areas of haemorrhages. Adjacent epidermis shows moderate orthokeratotic hyperkeratosis and acanthosis (black arrow). HE = haematoxylin and eosin. HPF = High Power Field.

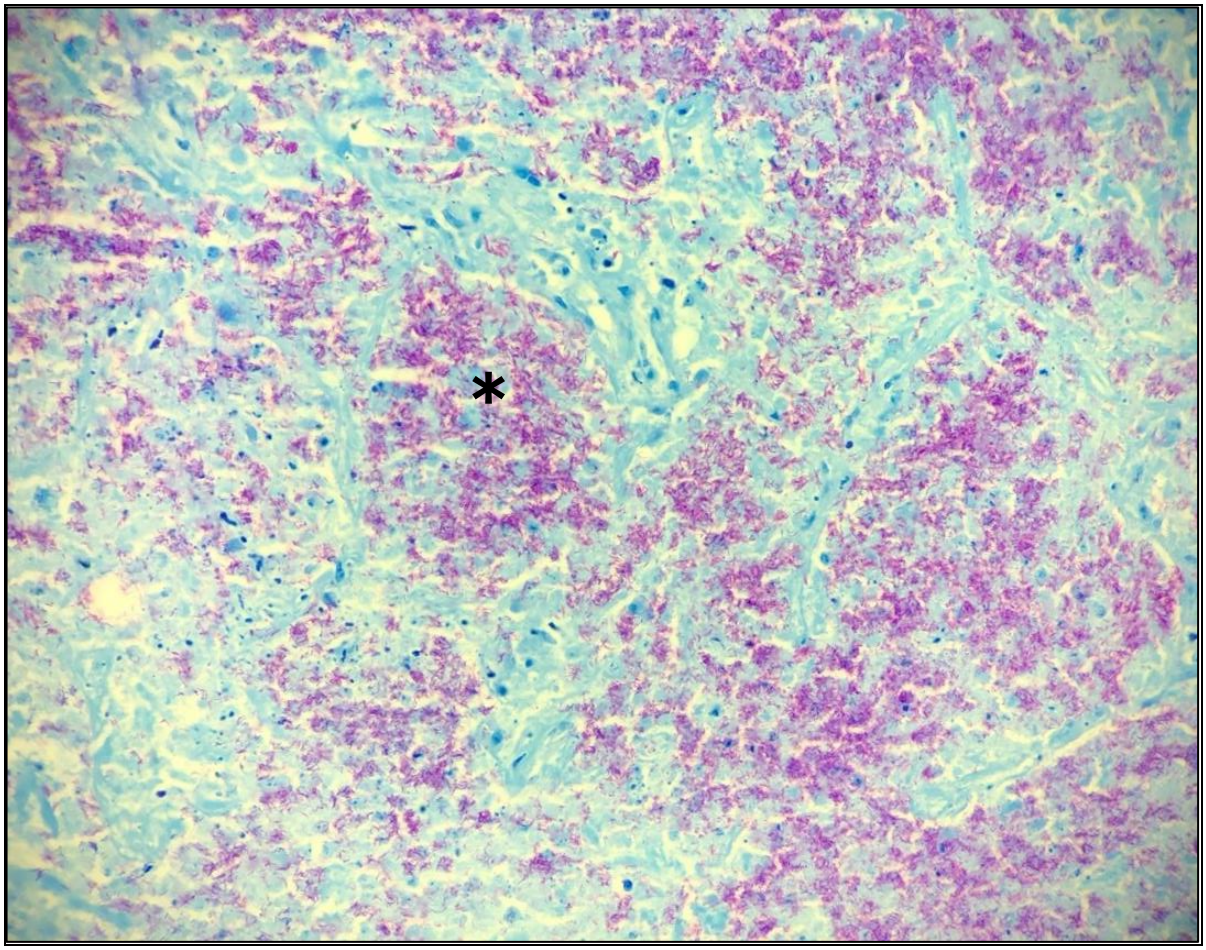

Figure 25. Atypical mycobacteriosis (*Mycobacterium malmoense*); Haired skin, dorsum, MZN, 10X HPF. Badger number 458. Within the deep dermis and subcutis, there are abundant, up to 4µm, intra-histiocytic and extracellular, red ("acid fast") bacterial bacilli consistent with *Mycobacterium* species (asterisk). MZN = Modified Ziehl Neelsen. HPF = High Power Field.

4 Discussion

Bovine TB caused by *M. bovis* is an important health and economic problem for the British cattle industry, and badgers are believed to be an important source, and possibly reservoir, of the infection in some areas of the UK (Dalley et al., 2019; Infantes-Lorenzo et al., 2019).

As part of the government's strategy for achieving the official bTB-free status, England has different surveillance systems to *M. bovis* and the control measures were set up according to the incidence and risk of bTB (Department for Environment Food & Rural Affairs, 2018).

This study focused on road-killed badgers in six counties within the northern Edge Area, where the prevalence of TB in badgers was previously unknown and found to vary between counties from around 4% to 15% (Bennett, 2017). Although poorly understood, the northern Edge is considered by some researchers an area with moderate badger density population, which varies from 1 badger per sq-km to 1 badger per 3 sq-km, variable bTB density and low TB prevalence in cattle (Baldwin, 2017; Delahay et al., 2008; Department for Environment Food & Rural Affairs, 2018).

This study used road-killed badgers collected by volunteers, as previously performed (Sandoval Barron et al., 2018).

4.1 Gross Post mortem examination

4.1.1 Gender, age, body mass and autolysis

For the 607 badgers for which gross post mortem examinations were performed in the study, the gender ratio was 53 male : 45 female and the age ratio was 72 adult : 24 juvenile. There are no other recent studies in the northern Edge Area, with which to compare the results of gender and age of the current survey, however these ratios are similar to those reported in previous studies of found dead badgers in other areas of the UK (Goodchild et al., 2012; Delahay et al., 2008, 2009).

The total body mass varied between 4.7kg and 15.7kg, with a mean value for adults of 10.3kg, which is consistent with the known body mass reference intervals established in European badgers (Delahay et al., 2008). It is important to point out that seasonal fluctuation should also be taken into account when assessing body masses in badgers, however given the unknown post-mortem interval of the collected carcasses, it is not possible to reliably correlate the body mass with the season in this study.

There was a high rate of badgers showing advanced autolysis (40%). However, as the study made use of road killed badgers, there was an inevitable delay between time of death and sampling, as well as how long carcasses had been on the road and the conditions of storage. The carcasses were collected within 48 hours of notification, and if possible the same day and necropsies and processing occurred within 24 hours following storage at 4°C (Bennett, 2017; Department for Environment Food & Rural Affairs, 2018). Although carcasses affected grossly by severe putrefaction were rejected and not included in the study (including demographics data), it was however interesting to record the degree of post-mortem autolysis, something that previous studies appear not to have addressed (Gallagher and Clifton-Hadley, 2000; Gavier-

Widen et al., 2001; Corner et al., 2011; Goodchild et al., 2012; Bhuachalla et al., 2015; Gormley and Corner, 2018). Anecdotal accounts suggest that autolysis, particularly in tandem with freeze-thawing, has had a significant effect on microbiological studies of TB prevalence in badgers. Autolysis is different from putrefaction. Autolysis is defined as a chain reaction of enzymatic self-digestion that results from the degradation of tissues by intrinsic (lysosomal) enzymes, and which normally precedes putrefaction which is defined as the process of decomposition caused by tissue digestion of the anaerobic bacteria (Brooks, 2016). Both autolysis and putrefaction can severely hamper both macroscopic and histological examination of the tissues (Dettmeyer, 2018). Most of the carcasses in our study showed advanced autolytic changes following the criteria described by Brooks (Brooks, 2016). These data must be interpreted with caution however, because the distinction between moderate and marked autolysis is subjective and variation between pathologists might have affected the evaluation.

4.1.2 Macroscopic lesions

The carcasses had injuries consistent with road traffic collisions, including subcutaneous and cutaneous contusions, haemothorax, hemoperitoneum, pneumothorax, parenchymal organs (such as lung, spleen and liver) lacerations and haemorrhages or pelvic fractures consistent with a previous literature review (Ressel et al., 2016).

At gross post-mortem examination, only two badgers showed signs of severe cachexia. The first one was affected by granulomatous pneumonia (miliary tuberculosis), which was confirmed as MTC+. The second case in this study, which had no detectable mycobacterial infection, presented a severe fibrinous and suppurative bronchopneumonia and suppurative pleuritis with marked pyothorax, compatible histologically with bacterial pneumonia. The latter also presented traumatic injuries due to traffic collisions and a culture swab from the affected lung was not attempted given the likely environmental contamination.

Grossly, the majority of the badgers (44%) presented multiple combined macroscopic changes, rather than a single gross change, the most common being the concomitant presence of ulcerative cutaneous lesions on the neck and the base of the tail, with no statistical differences between the age groups. The comparison with the mycobacterial infectious status demonstrated that the majority of the MTC+ animals (48%) showed a combination of multiple gross lesions with the most common being lymphadenomegaly in combination with pulmonary granulomas and/or bite wounds, similar to the ones described by previous authors (Corner et al., 2011; Gormley and Corner, 2018); these changes suggest that systemic/multiorgan involvement (in particular lymph nodes) is a key factor in the pathogenesis of TB in badgers and

highlight two most important transmission routes, such as respiratory and bite wounds (Corner et al., 2011).

Overall, macroscopic granulomas as only detectable change were identified only in 3% of the M+ badgers, in the lung, and similar prevalence was noted in other studies of TB in badgers (Gavier-Widen et al., 2001; Gormley and Corner, 2018). The macroscopic appearance of the granulomas in the lung, whether in M+ or M- badgers, was similar to that previously described for tuberculous lesions, suggesting that granulomas are not pathognomic of TB (Gavier-Widen et al., 2001; Gormley and Corner, 2018). No statistically significant differences in prevalence of grossly visible pulmonary granulomatous lesions were established between M+ and M- badgers.

Macroscopically, 8% of the M+ badgers showed only enlarged lymph nodes (lymphadenomegaly) with no other concomitant gross pathological changes, which was interpreted as secondary non-specific antigenic stimulation due to traffic injury-related trauma. Lymphadenomegaly was the most common macroscopic change identified in combination with other gross changes in MTC+ badgers, although no statistically significant. Previous studies of TB in badgers identified the thoracic lymph nodes (in particular tracheobronchial and mediastinal lymph nodes) as more affected than abdominal lymph nodes (Gavier-Widen et al., 2001; Gormley and Corner, 2018). In this study, there were no significant differences between the prevalence of thoracic, abdominal or peripheral lymphadenomegaly in M+ and M- badgers. This probably reflects the constant exposure of badgers to a wide variety of antigenic stimulation (not just mycobacteriosis) which can induce a reactive enlargement of the peripheral lymph nodes. Interestingly, in the subset of badgers selected for histopathology, thirty four badgers showed granulomatous inflammation and, within them, 15% presented

granulomatous lymphadenitis which were all identified as enlarged on macroscopic examination.

Although not statistically significant, M+ and OM+ badgers had an increased prevalence of ulcerative dermatitis on the neck, compatible macroscopically with bite wounds. These resembled the ‘degloving’ wounds caused by blunt canine teeth, described by a previous study as typical of badger bites (Bailey et al., 2012). This trend toward a relationship between bite wounds and mycobacterial positivity has been described in previous studies and has been taken as showing the importance of bite wounds as a transmission route for TB in badgers (Corner et al., 2011; Gormley and Corner, 2018).

In addition to bite wounds on the neck, 11% of the badgers had a focal ulcerative dermatitis at the base of the tail for which a clear cause was not found. Few studies have linked the ulcers on the tail to bite wounds, however the gross morphology of the ulceration on the tail was not typical of bites as previously described and unlike the neck lesions, the presence of the tail lesions was not correlated with M+ (Gavier-Widen et al., 2001; Bailey et al., 2012). One hypothesis was that these lesions might be associated with ectoparasites, but significant correlation was not established between ulcers at the base of the tail and the presence of lice (*Trichodectes melis),* consistent with previous findings who also found no cutaneous pathological changes associated with lice in badgers (Kozina et al., 2014). Self-induced trauma secondary to mutual allomarking is also a possibility. Mutual allomarking in European badgers (*Meles meles*) is defined as behavioural mechanism which involves two badgers pressing the subcaudal apocrine scent glands against each other and is used to keep strong cohesion between groups and share group odours (Buesching et al., 2003). Finally, any self-induced trauma secondary to pruritus

could have led to the ulcers at the tail. Although the causes of spontaneously occurring pruritus in wild badgers and other mustelids are not investigated in the current literature, in other carnivores the most common causes of pruritus include ectoparasites (particularly mite infestation or flea allergy/infestation), fungal infection, viral infection, bacterial pyoderma, immuno-mediated dermatosis, seborrhoea, food allergy, atopic dermatitis or any underlying neoplasm (Dunbar Gram, 2011). Histologically, a system of pattern analysis is normally applied to inflammatory, neoplastic and non-neoplastic skin lesions which can help generate a list of possible differential diagnoses and reach a more precise final diagnosis (Mauldin and Peters-Kennedy, 2016). In this study, in most of the cases of cutaneous ulcers at the base of the tail, the ulceration was associated with a pattern of lymphocytic and neutrophilic perivascular dermatitis which is considered the least specific pattern of inflammation (Mauldin and Peters-Kennedy, 2016). In a case report of a badger (*Meles meles*) from south-west England, superficial perivascular dermatitis, hyperkeratosis and ulceration were associated with sarcoptic mange and histological evidence of intracorneal mites compatible with *S. scabiei* (Collins et al., 2010). It has been hypothesised that red foxes (*Vulpes vulpes*) might have been the source of the infection in the badgers as result of spillover event (Collins et al., 2010). In contrast to this report however, intralesional mites were not identified in any of the ulcerated lesions. The lack of detection of sarcoptic mange in the study is unknown, however it was reported that badgers with a severe sarcoptic mange infestation tend to hide in the underground setts which can limit the identification and diagnosis of the lesions (Soulsbury et al., 2007). This aspect might have been contributory to an underestimation of sarcoptic mange in the badgers of this study.

The only ectoparasite identified on macroscopic examination was the lice *Trichodectes melis.* Interestingly, other ectoparasites (ticks, fleas or mites) were not detected. A previous study conducted in England found a higher prevalence of lice (80%) which is consistent with this study (Hancox, 1980). In contrast to previous researches, however, ticks were not identified (Hancox, 1980; San E. Do Linh, 2007). Low loads of ticks, mites and fleas remain unexplained. Ticks and fleas had likely abandoned the carcasses before post-mortem examination. Other studies discussed the possibility of underlying reasons that are linked to reduced ticks and flea infestation in badgers, such as the ones linked to social behaviours (auto- and allogrooming and the replacement and removal of the bedding material) or due to increased immunocompetence or resistance to certain parasites (Hancox, 1980; San E. Do Linh, 2007).

4.2 Histopathological examination

4.2.1 Silica-laden macrophages

The most common histological change was the presence of SLMs (85%) in the lungs and tracheobronchial lymph nodes. SLMs are common in burrowing animals having a subterranean social lifestyle, such as badgers, which are constantly exposed to environmental dust (Schoening et al., 2018). The histological changes due to SLMs were considered incidental in this study. Haemosiderin can cause a similar brown pigmentation in the cytoplasm of the alveolar macrophages to the silica seen in this study, but haemosiderin stains with Perls Prussian Blue special stain (Caswell and Williams, 2016). None of the SLMs stained positively with Prussian Blue. There was no significant association between age, gender or M+ animals and the presence of SLMs, consistent with previous studies (Schoening et al., 2018). Although incidental, some concerns were raised regarding considering SLMs as an early stage of pneumoconiosis. Histopathological criteria of the chronic stage of dust pneumoconiosis (such as interstitial lung disease, hyaline nodules and septal fibrosis) as occurs in domestic animals and people were not seen in the study (Caswell and Williams, 2016; Kambouchner and Bernaudin, 2015). Although a definitive diagnosis of chronic pneumoconiosis is not possible given the absence of the mentioned histological criteria, the presence of SLMs in the study are similar to the macules described by previous researches in people (Kambouchner and Bernaudin, 2015). “Macules”, as defined, are the consequence of the accumulation of silica dust-laden macrophages which surround small respiratory bronchioles, large bronchi and along interlobular septa (Kambouchner and Bernaudin, 2015). They are caused by relatively ‘inert’ dusts, such as the silica dusts found in the ground and could suggest an early phase of dust accumulation (Kambouchner and Bernaudin, 2015).

4.2.2 Mycobacterial histological lesions: granulomas and atypical mycobacteriosis

In this study, granulomas were identified histologically in 53% of the animals and were significantly more common in the lung compared with other tissues ($p = 0.0004$). As expected, granulomas were significantly increased in M+ and MTC+ animals ($p < 0.001$) compared with M- population, however this difference was driven by a higher prevalence of granulomas in extrapulmonary sites as there was no difference in the prevalence of granulomas in lung between M+ and M- animals. The morphology of the granulomas in badgers with TB is also consistent with previous studies where predominance of epithelioid macrophages and lack of multinucleated cells were both consistent features (Gavier-Widen et al., 2001; Corner et al., 2011; Gormley and Corner, 2018). However, the histomorphology of the granulomas in the M+ badgers did not differ from the morphology of the granulomas in M- badgers. This is an important point in diagnosing TB, because the use of routine histopathology (HE) alone could lead to over-diagnosis of TB, unless combined with special chemical stains for mycobacteria (such as ZN and MZN). The lack of multinucleated cells is thought to be correlated with decreased ESAT-6 secretion in M+ infected badgers and possibly associated with reduced pathogenicity of TB (Lesellier, 2018). ESAT-6 is a virulence factor released by many species of pathogenic and environmental mycobacteria and for an unknown reason, the secretion is reduced in experimentally TB-infected badgers (Lesellier, 2018). The lack of multinucleated cells was also a consistent feature in M- granulomas in the study, however the reason of their lack in non-mycobacterial infected badgers has not been reported. In a recent case series of captive badgers, multinucleated giant cells were identified in the spleen secondary to idiopathic systemic amyloidosis which displayed a peculiar star-like morphology

(Bianco et al., 2020). The role of multinucleated cells is this condition is controversial and unknown (Bianco et al., 2020). No case of systemic amyloidosis was identified in this study. Areas of lytic necrosis within the granulomas were extremely rare in both M+ and M- badgers, consistent with previous researches (Gavier-Widen et al., 2001; Corner et al., 2011; Gormley and Corner, 2018). In contrast to previous findings, however, no evidence of mineralisation within the mycobacterial granulomas was detected (Corner et al., 2011). There were other differences found between this and other studies of TB in badgers (Gavier-Widen et al., 2001). Firstly, granulomas in the intestine were absent. This discrepancy needs to be interpreted with caution as the intestine often showed autolytic changes so advanced as to hamper a detailed evaluation. Secondly, acid fast bacilli free in the lumina of bronchioles with necrotic debris were never identified. The number of acid fast bacilli was variable and mostly within the cytoplasm of the epithelioid macrophages or rarely extracellularly and, when present, in the areas of necrosis, consistent with previous studies (Gavier-Widen et al., 2001; Corner et al., 2011; Gormley and Corner, 2018). Interestingly, acid fast bacilli were consistently found in all the M+ positive animals and we found a 100% agreement ZN and MZN acid fast stain, culture and molecular testing.

Of the 44 M+ badgers in this study, thirteen of the *Mycobacterium* cultures were negative for MTC PCR, and so classified as other mycobacteria (OM+). The rRNA PCR products from two badger cultures were sequenced and identified as *M. nonchromogenicum* and *M. malmoense*. Interestingly, both these badgers had histologically confirmed lesions. Lesions associated with other NTM (MAC) have been previously described in badgers (Balseiro et al., 2011). This appears to be the first time however, that histologically confirmed lesions have been identified in badgers with *M. nonchromogenicum* and *M. malmoense* infections. For the remaining eleven

badgers, although histological changes compatible with tuberculosis were found, further characterisation of the mycobacteria is still pending.

M. nonchromogenicum is a slow growing nonpigmented *Mycobacterium* species in the *M. terrae* complex. While often considered non-pathogenic, it can cause human pulmonary disease, but has not been reported in any other species (Sawai et al., 2006). The infected badger in this study had granulomatous pneumonia with rare intrahistiocytic acid fast bacilli, similar to that described in humans (Sawai et al., 2006).

M. malmoense is a slow-growing non-photochromogenic mycobacterium normally present in the environment, such as in waters, wet soil, dust, fur of domestic and wild animals and food waste. *M. malmoense* infections can cause pulmonary diseases and subcutaneous infections in humans (Cowan et al., 2009; Lapierre et al., 2017; Zweijpfenning et al., 2018). In domestic animals, *M. malmoense* infection has been described in cats, causing disseminated disease or subcutaneous granulomatous inflammation with abundant acid-fast mycobacteria (Karkamo et al., 2016; Hetzel et al., 2012). The histological changes in the badger of this study are similar to a previous case report from a cat (*Felis catus)* which presented a similar pyogranulomatous dermatitis at the base of the tail (Hetzel et al., 2012). The badger case, additionally, showed evidence of ulceration and, in contrast to the case report, the pyogranulomatous inflammation was not accompanied by multinucleated giant cells (Hetzel et al., 2012). This discrepancy, however, is not surprising as the granulomatous inflammations (caused by mycobacterial infection or other diseases) are commonly seen without multinucleated giant cells (Gavier-Widen et al., 2001; Lesellier, 2018). Some exceptions are also reported (Bianco et al., 2020). Consistent with the findings in the cat, there were abundant numbers of acid fast mycobacteria in the cutaneous lesion of the badger (Hetzel et al., 2012).

Thus, NTM can cause similar histological granulomatous lesions in badgers to those caused by *M. bovis*. Although from the pathological point of view this change does not represent a novelty we believe that this finding is relevant in prevalence studies where histology is used as a sole diagnostic criterion, especially in TB non-endemic populations.

The presence of atypical mycobacteria could also raise concerns in terms of efficacy of the vaccine. Vaccination of badgers has been part of the control plans for the disease in the UK, and in-vitro researches have proved that vaccination with live attenuated bacillus Calmette-Guérin (BCG) vaccine is protective in badgers, and also in wild boars (Balseiro et al., 2020). In humans, it is known that the efficacy of the BCG vaccination in protection against pulmonary TB shows profound variations between individuals (Colditz et al., 1994). According to some researchers, a possible reason for this variability is the increased exposure of certain populations to NTM (Poyntz et al., 2014). Previous published studies are limited to local surveys or experimental models and still need to explain with certainty the role of NTM and BCG vaccination interaction (Poyntz et al., 2014). Large scale studies in badgers with higher numbers of histologically detectable lesions are needed in order to confirm the presence of NTM active disease and infections and to assess the prevalence of NTM infection in badgers.

4.2.3 Parasitic granulomatous pneumonia (pulmonary nematodiasis)

Parasitic granulomatous pneumonia was identified in 11% of the badgers examined and was histologically compatible with *Perostrongylus falciformis* infection (Byrne et al., 2019). *P. falciformis* (formerly known as *Aelurostrongylus falciformis*) has an indirect life cycle with terrestrial gastropod molluscs, such as snails (*Cornu aspersum*) and slugs (*Arion ater)* serving as intermediate hosts (Deak et al., 2018). Stage 1 (L1) larvae are excreted in the faeces of infected badgers and infiltrate the foot of the gastropod, where they undergo development into stage 3 (L3) larvae (Deak et al., 2018; Byrne et al., 2019). Following ingestion by the badgers (the definitive host) of the L3 larva, the nematode matures to the adult stage in the terminal bronchioles and alveolar spaces (Deak et al., 2018; Byrne et al., 2019). The European badger appears to be the only definitive host of the species *P. falciformis* and American minks are known to be the definitive hosts of a different species of the same genus (*P. pridhami*) (Deak et al., 2018). *P. falciformis* has a strong similarity histologically to *Angiostrongylus* species (Deak et al., 2018). PCR amplification is considered a useful diagnostic tool to confirm *P. falciformis* and rule out angiostrongyliasis (Deak et al., 2018). As part of their life cycle, *Angiostrongylus* species normally reach the mature adult larval (L3) stage in the pulmonary arteries (Caswell et al., 2016). In this study, adult parasites were not identified in the pulmonary arteries during gross examination, so the possibility of angiostrongyliasis was considered unlikely.

4.2.4 Pulmonary adiaspiromycosis

Adiaspiromycosis was detected histologically in 9% of the animals. This disease is caused by the dimorphic fungus *Emmonsia* species and is a common respiratory infection of wild rodents and mustelids, badgers included (Borman et al., 2018). The diagnosis was based on assessment of the morphology of the non-replicating adiaspores on routine histological staining (HE) for most of the cases with, occasionally, evidence of PAS positive fungal walls, as applied by previous studies (Borman et al., 2018). Definite diagnosis of pulmonary adiaspiromycosis would require microdissection of adiaspores, PCR amplification and fungal culture, which are however frequently considered challenging and unsuccessful (Borman et al., 2009). No additional confirmatory tests have been performed in this study. Given the size of the spores on histopathology (up to 500μm), it is likely that *E. crescens* was the species seen, as *E. parva* is much smaller (20–40μm) (Borman et al., 2018). Infection in badgers likely occurs through inhalation of dust-borne fungal aleuriospores. The pathological effects of adiaspiromycosis in humans depend on host immunocompetence and fungal (adiaspore) load and gross pathological changes secondary to adiaspiromycosis are well documented in both people and badgers (Schwartz et al., 2015; Borman et al., 2018). The badgers showing pulmonary adiaspiromycosis were all in a good body condition, suggesting a subclinical infection or mild clinical signs antemortem. In all the cases, only a single adiaspore was commonly seen in the centre of a granuloma of the examined section of lung. Adiaspore do not replicate in the host, so the few numbers of conidia detected reflect the numbers of the inhaled ones (Schwartz et al., 2015; Borman et al., 2018). No gross changes were identified, and no significant correlation was found between M+ positive animals and adiaspiromycosis.

4.2.5 Portal to periportal lymphocytic and plasmacytic hepatitis

A portal to periportal lymphocytic to lympho-plasmacytic hepatitis was seen in 26% of badgers. Portal hepatitis is an unspecific finding in domestic animals (occasionally referred as “non-specific reactive hepatitis”) most commonly secondary to a variety of extrahepatic disease processes, especially febrile illnesses, septicaemic bacterial disease, gastro-intestinal disease or it represents the residual lesion of a previous intrahepatic inflammation (M. Van den Ingh et al., 2006; Cullen and Stalker, 2016). The inflammation as seen in this study is usually mainly mononuclear with plasma cells and lymphocytes in the portal areas (M. Van den Ingh et al., 2006; Cullen and Stalker, 2016). All the badgers with portal hepatitis presented concomitant histological lesions elsewhere in the body, with the most common being granulomatous pneumonia, adiaspiromycosis, lymphoid follicular hyperplasia or interstitial nephritis. Although portal hepatitis does not seem to have been described previously in badgers, there are reports in other mustelids such as ferrets and minks (Li et al., 2016; LaDouceur et al., 2015). Ferrets are experimentally susceptible to Hepatitis E Virus (HEV), which can induce chronic lymphocytic hepatitis (Li et al., 2016). Portal hepatitis is also a well-documented pathological feature of Aleutian mink disease virus (AMDV) infection and Aleutian Disease in minks (LaDouceur et al., 2015). It has been shown that badgers also can be infected with AMDV (Knuuttila et al., 2015). AMDV is a member of the family *Parvoviridae* and genus *Amdoparvovirus* and is one of the most important viruses causing significant welfare problems in farmed and feral American minks (*Neovison vison*), European minks (*Mustela lutreola*), ferrets (*Mustela putorius furo*), polecats (*Mustela putorius*), stoats (*Mustela erminea*), martens (*Martes foina, Martes martes*) (Knuuttila et al., 2015). Otters (*Lutra lutra, Lontra canadensis*) can also be infected with detectable pathological lesions

(Wells et al., 1989). AMDV infection induces high antibody titres in mink, plasmacytosis and immune complex disease, autoimmune hepatitis (AIH), the clinical signs of which range from subclinical to severe and fatal (Virtanen et al., 2019). The histological feature of AIH is a portal mononuclear inflammation that expands the parenchyma and comprises B and T lymphocytes, plasma cells and macrophages (Vergani and Mieli-Vergani, 2008).

Interestingly, the percentage of badgers with portal to periportal hepatitis was significantly lower in M+ ($p = 0.003$) than in M- badgers. It is not immediately obvious why this might be, but two possible hypotheses were raised. The first hypothesis is that concomitant mycobacterial infection might have induced stronger IFN-γ expression, capable of clearing the viral infection without chronic lymphocytic inflammation. Strong IFN-γ expression is consistent with progression of tuberculosis in badgers (Buzdugan et al., 2017; Gormley and Corner, 2018). In humans, those who clear the hepatitis virus (mostly B and C) have better IFN-γ production than those who develop chronic infection (Whittaker et al., 2019). Inhibition of IFN-y can also predispose to reactivation of viral hepatitis virus in people (Chen et al., 2018). This hypothesis could be tested by assays for IFN-γ mRNA transcription in the badger tissue samples, alongside PCR analysis for suspected viral infections. A second hypothesis could be that the mycobacterial immunomodulation might have suppressed the autoimmune response and chronic inflammation associated with, for example, AMDV infection. Mycobacteria can induce suppression of autoimmunity through several mechanisms involving altered T-cell trafficking, B cell regulation, and suppression of the Th17 response, by inducing apoptosis of activated cells or by inducing regulatory T cells (Lee et al., 2010).

4.2.6 Hepatic necrosis

Random hepatic necrosis was identified histologically in 9% of the badgers. The cause of it was not identified. The main likely differential diagnoses would include parasitic migration and bacterial infection. Hepatotoxicity (for example strychnine, organophosphate, organochlorine pesticides or anticoagulant rodenticides toxicity) was also taken into account, however the random distribution of the necrosis made this unlikely (Cullen and Stalker, 2016). No parasites were identified in the liver and Gram stain failed to identify bacteria in the lesions. Hepatic necrosis is a frequent finding with salmonellosis in both domestic animals and experimental studies (Giuliano et al., 2015; Wray et al., 1977). Badgers are known to be infected with a range of *Salmonella enterica* serovars (Wilson et al., 2003). Salmonellas were isolated from around 20% of the badgers in this study (B. Swift, personal communication).

4.2.7 Interstitial lymphocytic nephritis

Interstitial nephritis was identified in 31% of the badgers. Lymphocytic infiltration in the renal interstitium of badgers was also briefly described in a previous study, with however no information about prevalence (Gavier-Widen et al., 2001). Previous studies linked interstitial nephritis in American badgers (*Taxidea taxus*) with *Leptospira* infection (Ethier et al., 2017). In a study conducted in Andalusia (Spain), 50% prevalence of *Leptospira* serovar Icterohemorragiae detected by serology was found in European badgers (*Meles meles*) with microscopic lesions in the kidneys, however it is not clear if the seroprevalence was assessed in wild or captive badgers (Millán et al., 2009). In another survey conducted in France, European badgers (*Meles meles*), stone martens (*Martes foina*), pine martens (*Martes martes*) and foxes (*Vulpes vulpes*) were infected by various *Leptospira* genotypes and the prevalence of leptospiral infection in badgers was 8.2% based on PCR analysis of kidney samples (Ayral et al., 2016). Leptospiral infections have been found in badgers in the south west of England but did not appear to be associated with overt disease (Gallagher and Nelson, 1979). Nephritis in badgers was also described associated with cardiovascular calcification (Karstad and Lankester, 1975). Areas of cardiovascular mineralisation were not noted histologically in this study.

4.2.8 Sarcocystosis

Sarcocystosis was found in almost half the badgers, most likely caused by *S. lutrae* (Lepore et al., 2017). The tongue was the most common location for these lesions; myocytes containing bradyzoites were surrounded by minimal to no lymphocytic inflammation which is consistent with the current literature (Lepore et al., 2017). The life cycle of sarcocystosis occurs within two hosts: intermediate host, where the asexual reproduction occurs, and definitive host, where the sexual reproduction takes place (Lepore et al., 2017). The morphology of *Sarcocystis spp.* in this study suggests that is an intermediate host stage of the parasite. The life cycle stages of *Sarcocystis* species in badgers are poorly understood (Lepore et al., 2017). It is hypothesised that the transmission to badgers could occur via the ingestion of sporocysts from faeces of the definitive hosts or via contaminated food or water. (Lepore et al., 2017). The definitive hosts in the life cycle of sarcocystosis in badgers are not clearly identified. It is speculated that some species of birds belonging to the family *Corvidae*, or the white-tailed sea eagles (*Haliaeetus albicilla*), as well as foxes (*Vulpes vulpes*) and even other badgers may behave as definitive hosts of *Sarcocystis lutrae* (Lepore et al., 2017).

4.3 Conclusion and summary

In conclusion, the study of road-killed badgers attempted to provide a survey and analysis of prevalence of diseases in European badgers (*Meles meles*) in an area in the UK considered as with a lower prevalence of bTB in cattle and poorly understood TB prevalence and density population in badgers.

A broad list of pathological changes was demonstrated both macroscopically and histologically. The most striking and relevant pathological lesions were the ones directly linked to infectious agents which include sarcocystosis, adiaspiromycosis, pulmonary nematodiasis and mycobacteriosis. For some histological lesions (hepatic necrosis, nephritis and hepatitis), however a cause could not be identified phatomorphologically.

In regard to sarcocystosis and adiaspiromycosis, histopathological identification allowed a diagnosis of the infections. In regard to pulmonary nematodiasis, histopathology showed its limitations in differentiating the nematode species, in particular within the same family of Metastrongylidae in order to differentiate *P. falciformis* and *Angiostrongylus* species.

In regard to mycobacteriosis, a full diagnostic work-up which included gross post mortem examination, histopathology, culture and PCR was performed. In this study, mycobacteriosis in badgers was associated histologically with granulomas (in several organs) and ulcerative pyogranulomatous dermatitis. An overall 77% prevalence of mycobacteriosis (M+) showing histological lesions was demonstrated. In all these cases, there was the presence of acid-fast bacillary bacteria visible with ZN and MZN. As expected, a statistically significant higher proportion of granulomas was identified in M+ badgers compared to the M- badgers, but, interestingly, without differences when considering only the granulomas present in the lung, suggesting that

extrapulmonary granulomas were driving the prevalence of M+ granulomas compared with the M-. This study also identified a 62% prevalence of OM+ for most of which the sequencing and mycobacterial identification are still pending. For the first time, two atypical mycobacteria (*M. nonchromogenicum* and *M. malmoense*) were identified in badgers both causing histological detectable lesions. Given the immunological implication that mycobacterial infection can cause on badgers in relation to other concomitant disease, this study also focused on comparing the macroscopic and histological lesions with M+ and M- animals. An apparent decreased prevalence of portal hepatitis in M+ badgers compared to the M- badgers was identified.

4.4 Future plans and implications

Future plans will include the engagement of additional molecular techniques to confirm the aetiologies identified histologically and to identify the species and genera of the infectious agents involved, particularly for sarcocystosis, adiaspiromycosis, pulmonary nematodiasis, as performed in previous studies (Deak et al., 2018; Lepore et al., 2017; Schwartz et al., 2015; Borman et al., 2018).

Mycobacteriosis was confirmed in 77% of the badgers and extrapulmonary granulomas were driving the prevalence of M+ granulomas compared with the M- ones. This brought up some considerations and important implications in regard to the pathogenesis and the way of transmission of TB in European badgers and pointed out the importance of other transmission routes rather than the respiratory one. Additionally, this study identifies an important target for future studies, such as investigation of prevalence of NTM within the badger population. In this study, the granulomatous lesions in badgers with NTM infections are similar on histology to those described with bTB. This aspect has obvious consequences for the diagnosis of bTB in badgers based only on HE and ZN alone, and potential consequences for the efficacy of BCG vaccination and for the pathogenesis and epidemiology of bTB, as it is the case in humans. Future works should focus on identifications of NTM species to fully understand the role they play in prevalence, diagnosis and vaccine efficacy. This study allowed identification of few NTM species, however more pending rRNA PCR products from OM+ badgers need to be sequenced in order to identify further species. The latter represents a short term future plan.

In this study, portal hepatitis presented a lower prevalence in M+ badgers compared to the M- badgers. Further action could be planned in order to try to confirm this association and, if so, to understand its possible causes, which may potentially be

linked to immunomodulation triggered by the mycobacteria and concomitant infections. Two hypotheses were raised, and future plans would be needed to prove such hypotheses and explain the correlation. Assays for IFN-γ mRNA transcription in badger liver samples, alongside with PCR analysis for suspected infections causing hepatitis (such as HEV and AMDV) can be implemented. Induction of regulatory T cells (Tregs) is hypothesised as cause of the mycobacterial-induced autoimmunity. For this reason, alongside with IFN-γ mRNA transcription, future plans could focus on demonstrating Tregs-expressing transcription factor Foxp3 in badger liver samples.

This study also identified histological lesions in which their causes could not be identified, which are hepatic necrosis, nephritis and hepatitis. Further research is required to identify the underlying cause of them as well as their significance to individuals and populations.

Overall, considering the diseases internationally recognised in European badgers (*Meles meles*), their zoonotic risk and their lack of information of prevalence in naturally infected badgers in England, future plans should be prioritised and focussed on detection of leptospirosis, babesiosis and trypanosomiasis.

Leptospirosis is a zoonotic infectious disease and the lesions in the kidneys identified histologically together with the lack of significant large prevalence studies and the potential high risk of exposure of badgers to contaminated soils and waters should justify a further investigation by molecular techniques (PCR) on frozen kidneys, the use of immunohistochemistry to identify leptospiral antigens in formalin fixed tissue, or immunofluorescence methods on urine sample, as demonstrated by previous studies (Millán et al., 2009).

At the moment, there is currently little information available concerning the prevalence of haemoparasites in badgers in England (in particular *Babesia* species).

Although no ticks were identified in this study, the underestimation of tick-borne diseases is of concern. Trypanosomiasis is considered a zoonotic disease caused by a hemoflagellate protozoan and, at the moment, the prevalence of naturally infected infection in a large badger population in England is not fully understood in the current literature (Ideozu et al., 2015). Nested PCR on blood for both *Babesia* and *Trypanosoma* species are considered effective tools for diagnosing these infectious diseases and further plans should consider the use of them as screening tools of infection (Bartley et al., 2017; Guardone et al., 2020; Ideozu et al., 2015).

Based on the pathomorphology of the observed lesions, other pathogens of potential interest for future research may include *Salmonella spp*. in badgers affected by hepatic necrosis, *AMDV* in the ones affected by hepatitis and *Borrelia burgorferi* (agent of Lyme disease) detection in individuals affected by nephritis.

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