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Parasites as an animal model for the study of lead contamination in domestic and wild ungulates

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Preface

This thesis is divided into two parts: the first part includes a general introduction and a chapter on exploring the "suitability of parasites as biological indicators". The second part is a 'thesis by publication' format, which includes two published articles in international peer-reviewed journals.

Chapter 1. includes an overview of the suitability of parasites as biological indicators in polluted environments. Parasites can serve as effective sentinels for metal pollution, as they often accumulate heavy metals at higher concentrations than their hosts. Their bioaccumulation patterns provide insights into environmental contamination and ecosystem health, making them valuable indicators in ecotoxicological studies.

Chapter 2. Consists of "First molecular description of *Macracanthorhynchus hirudinaceus* in wild boars from Italy with pathomorphological and epidemiological insights" published in Parasitology Research. This chapter is about *Macracanthorhynchus hirudinaceus* which is a zoonotic parasite affecting suids worldwide which are the definitive hosts for this helminth species. *Macracanthorhynchus hirudinaceus* is of significant economic and management concern due to its pathogenicity, causing intestinal obstruction and perforation in the definitive hosts. The current study is the preliminary investigation from Sardinia, Italy, reporting the pathomorphological findings and molecular characterization of *M. hirudinaceus* in the wild boars.

Chapter 3. Covers "Lead concentrations in wild boar from Sardinia: analysis of food safety concerns" published in Environmental Sciences Europe. The chapter explains the risk assessment of lead (Pb) in the hunters who frequently consume game meals. It was ascertained that the margin of exposure for hunters was very low. In comparison with the European Food Safety Authority (EFSA) guidelines, none of the samples was fully fit for human consumption which calls for the inclusion of game meat in the EU regulation.

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General Introduction

Ecological communities constitute a diverse array of organisms that interact with each other in multiple ways to maintain the ecosystem's natural functioning (Poisot et al., 2013). One of the integral components of the ecosystems are the parasites which have the potential to influence ecological structure and flow of energy through the food webs (Thompson et al., 2005). It is critical to understand how parasites may affect the function, structure, and dynamics of the food webs (Lafferty et al., 2008). The major route of ensuring survival for the parasitic species has been diversification through tracking the food web interactions (Marcogliese and Cone, 1997; Marcogliese, 2002). Woven into intricate relationships with the hosts, the parasitic interactions can potentially affect the ecosystem, and any change in the free-living components of the ecosystems may also affect them. A few notable examples of parasitism in ecosystems include trophic cascades, changes in productivity, alterations in the strength of interactions, and an increase in energy flow (Lafferty et al., 2008). Additionally, parasitism can induce a suite of impacts in the host species including behavioural and physiological changes, changes in nutritional status, altered population dynamics through the reduction in fecundity and increased mortality, modification of intraspecific and interspecific competitions, reduced growth, influence on mate choice, altered sex ratios and increased susceptibility to predation (Minchella and Scott, 1991). Thus being important determinants of community structure, the parasites can provide important clues on the functioning of an ecosystem (Marcogliese and Cone, 1997).

With the beginning of the industrialization era, many new compounds have found their way into the ecosystems resulting in different ecological stresses for the organisms disrupting the function and structure of ecosystems. Increased anthropogenic activities have led to a rise in the concentrations of different inorganic and organic pollutants like heavy metals, pesticides,

persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Erasmus et al., 2022; Reshu et al., 2022). Ecosystems of all kinds, aquatic, terrestrial, and air, are contaminated by agricultural, industrial, and domestic wastes contributing to significant environmental pollution; the interfaces of these ecosystems are equally vulnerable and exposed to toxicants. The impacted ecosystems, depending upon the intensity and duration of stress, may undergo energetic imbalances, changes in species composition and abundance, altered biotic ecological strategies, and disrupted nutrient cycles making the ecosystem less stable and non-sustainable (Freedman, 2013). Hence, there is a dire and urgent need for the recognition of environmental issues to devise suitable and sustainable approaches for environmental monitoring and management.

The integrity and health of ecosystems whereby the systems maintain their organization and balance between different components are the key attributes for normal functioning and maximizing productivity (Jørgensen et al., 2010). The assessment of ecosystem health can be done by employing physico-chemical assays and/or biological sentinels (biological indicators) which are the standardized tools for the measurement of contamination levels. The bioindicators manifest not only the current environmental conditions but also provide information on temporal changes over longer periods (Zargar et al., 2022) as opposed to chemical analysis which may provide inaccurate results due to multiple antagonistic and synergistic interactions between compounds (Kerambrun et al., 2011). These species indicate the status and extent of deterioration in the environment providing early warning signals to make remedial efforts for the affected ecosystems (Adams, 2002). The biological indicator concept is merely not about exhibiting the presence or absence of species rather it extends into reflecting the prevailing environmental conditions and the level of exposure of organisms to these contaminants along with their quantitative measurements (Hellawell, 2012). The biological sentinel species must qualify certain criteria to be considered good environmental

indicators, the basic merits are: i) the species must be easy to sample and handle, ii) they should have a cosmopolitan distribution having applicability in wide geographical areas in the greatest number of ecological communities and environments, iii) they should be sensitive to small variations in environmental stress and readily accumulate pollutants providing key indications on their distributional status in relation to contaminant levels, iv) they should also be able to provide quantitative measurement of environmental impacts and have the large body for sufficient tissue analysis and v) they should be easily cultured in laboratories facilitating the understanding of their responses to pollutants in the experimental studies and field observations (Hellawell, 2012; Jørgensen et al., 2010). Besides these characteristics, the sentinel species should also have well-studied physiology and ecology to understand their responses to stress; however, in reality, it is very rare to find all these characteristics in a single organism (Sures, 2004). The most common species utilized for bioindication are free-living organisms like fish, bivalves, crustaceans, red foxes, and rats however much attention is also being given to the interrelation between contaminants and different taxa of parasites in the ecotoxicological context (Mijošek et al., 2022; Sures, 2004; Sures et al., 2017; Vidal-Martínez and Wunderlich, 2017). There are quite a number of studies that have demonstrated the usefulness of parasites, particularly cestodes, acanthocephalans, and nematodes, as bioindicators of environmental impact. Their effectiveness as accumulation and effect indicators has been emphasized in many studies (Sures et al., 2017).

Besides the devouring role that parasites are normally associated with, their implication in environmental studies is now widely accepted. Endohelminths (acanthocephalans and cestodes) of fish have proved to be the promising sentinel organisms for indicating heavy metal pollution in the aquatic environments (Sures et al., 2017), however, lesser number of studies exist in literature about the parasites of terrestrial vertebrates. Considering high metal pollution loads in the terrestrial habitats, there is an emphasized need on conducting similar studies on

the mammalian species and their endohelminthic parasitic fauna. Considering the effectiveness of cestodes and acanthocephalans in biomonitoring studies, there is a dire need to define ideal parasitic sentinel species to take into account the small scale changes in the heavy metal concentrations in the terrestrial biotopes.

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Chapter 1

Suitability of parasites as biological sentinels

The environmental issues require effective control and proper management for the disposal of contaminants and pollutants. The mitigation strategies involve understanding and prevention of the consequences of harmful degradation processes and toxic environmental exposures of animals and humans. These alterations in the environment are studied through bioindicators which respond to the environmental impact through biological responses that are more reliable and ecologically relevant (Mijošek et al., 2022; Zargar et al., 2022). Parasitism is one of the most common lifestyles on the earth (Marcogliese, 2005) and parasites are usually found in every ecosystem and on every free-living organism. The impacts of parasitism on the host species and their biological responses must be investigated, apart from simply focusing on the negative consequences of parasites on the hosts, to unravel the impacts of anthropogenic changes in the environment (Timi and Poulin, 2020). The environmental implications of parasites have led to the development of a discipline, 'environmental parasitology' which addresses the impacts of anthropogenic activities on the abundance and occurrence of parasites thereby relating changes in natural parasitic distribution with environmental quality (Erasmus et al., 2022; Sures et al., 2017). The ways in which the effects and exposure to pollutants are analyzed are effect indication and accumulation indication, respectively (Sures, 2004). Information about the chemical state of the environment through physiological, behavioural and molecular changes resulting in differential distribution, composition, and abundance of parasitic communities is the manifestation of effect indication (Nachev and Sures, 2016; Sures, 2004; Sures et al., 2017). On the other hand, parasites act as accumulation indicators by amassing quantifiable and biologically available amounts of chemical substances in the environment and reflect the ecological presence of substances of environmental concern. The parasites are able to concentrate heavy metals within their tissues to a level higher than the

ambient concentration in the host or the environment (Sures, 2003). A summary of parasite and host-level responses after exposure to metal pollutants can be seen in Figure 1.

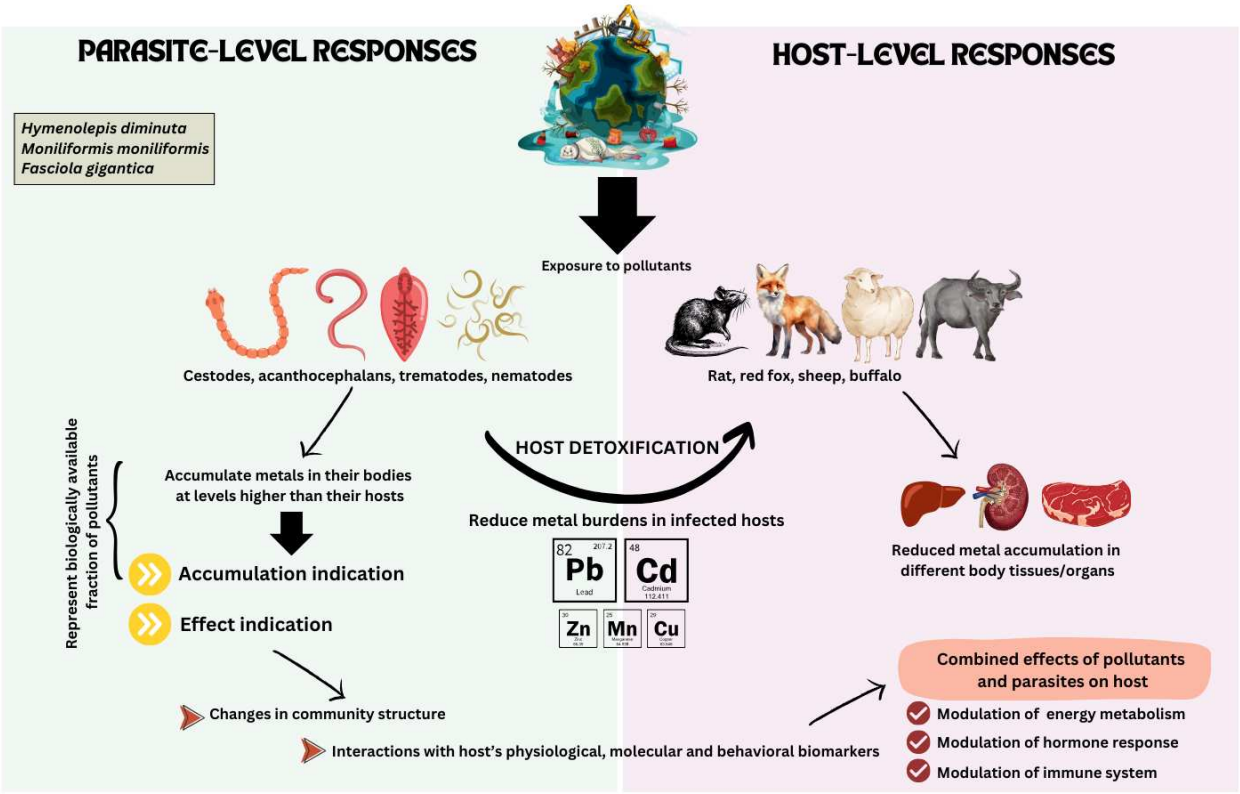

Figure 1 Parasites as accumulation and effect indicators for the metallic pollutants. Parasites reduce metal burdens, mainly Pb and Cd, for their hosts. Other metals like Zn, Mn and Cu are also known to be absorbed by the parasites but the number of studies supporting this evidence is low.

a. Parasites as accumulation indicators

The total pollutant load in the environment doesn't equal the amount available for uptake by organisms. Environmental factors affect pollutant availability and toxicity, making only the biologically available fraction relevant for biomonitoring (Nachev and Sures, 2016). Parasites are particularly suitable as accumulation indicators as they are able to bioconcentrate pollutants that have very low environmental concentrations. Thus these contaminants can easily be

detected and quantified using conventional analytical techniques (Sures et al., 2017). Bioconcentration of pollutants leads to host detoxification and has positive effects on the overall physiology of the host in pollutant environments (Perrot-Minnot et al., 2023). Moreover, parasites offer valuable insights into the biological availability of the pollutants serving as potential diagnostic tools to assess the behavior of chemicals in the environment and the extent to which they can be taken up by the organisms (Sures et al., 2017).

Certain parasites like acanthocephalans and cestodes exhibit a remarkable ability to tolerate very high pollutant burdens particularly heavy metals which indicates their applicability as sentinels for polluted environments (Sures et al., 2017). These parasite groups lack a digestive system (gutless) and virtually all substances have to pass through their tegument and membranes revealing the presence of contaminants in the host as well as the environment (Perrot-Minnot et al., 2023; Sures et al., 2004). These endohelminth taxa of parasites live inside the host intestine and any accumulated concentration of pollutants represents the biologically available fraction (Sures et al., 2017). These two taxa have shown the highest accumulation potential for different elements, both essential and non-essential, at very high levels compared to their hosts (Sures, 2004) followed by nematodes and trematodes, particularly digeneans (Zargar et al., 2022). However, the nematodes have more propensity towards accumulation of essential elements (Amini et al., 2013). Upon exposure to metals, organometallic complexes form in the liver of the host species and are typically excreted via the bile duct into the intestine, where they may be reabsorbed or eliminated through feces (Sures and Siddall, 1999). This normal hepatic-intestinal cycling is disrupted if the organisms are infected by the helminth parasites as they absorb bile acids and intercept metal-rich complexes in the intestine, potentially leading to metal accumulation (Starling, 1985). Parasitic uptake represents an efflux pathway for the host, similar to elimination processes, thereby reducing the metal levels in different tissues of the host (Le et al., 2016). Metal buildup triggers physiological responses in

the host that can counteract harmful effects at low tissue concentrations. Consequently, when parasitism reduces the toxin levels, the host is likely to experience less severe toxicity compared to uninfected individuals (Sures et al., 2017). Metal accumulation within the parasites may, however, be affected by some factors like the site of localization of the parasite within the host and its developmental stage (Nachev et al., 2013). Moreover, there is a substantial difference in the metal accumulation capacity of the larval and adult parasitic stages, with larvae showing reduced accumulation potential (Sures and Taraschewski, 1995).

In both aquatic and terrestrial ecosystems, endoparasites like acanthocephalans, cestodes, digeneans, and nematodes accumulate metals primarily through their hosts rather than through direct contact with the environment (Sures et al., 2017). While metal uptake in terrestrial ecosystems mostly happens through dietary intake, parasites in freshwater and marine environments obtain metals from their host's gills, circulatory systems, and diet (Nachev and Sures, 2016). Although the exposure pathways vary between different ecosystems, the parasites collect elevated metal concentrations in both settings. This suggests that regardless of different environmental conditions, the process of metal accumulation in parasites is comparable across ecosystems (Lotfy et al., 2013; Torres et al., 2011). The majority of the requirements for sentinel species are met by acanthocephalans, cestodes, and certain nematodes. They are very resistant to metal contamination and exhibit a remarkable ability to acquire metals. These parasites are large, abundant, widespread, and easy to sample, with pollutant levels in their bodies typically reflecting environmental concentrations (Sures et al., 2017). Additionally, acanthocephalans and cestodes could be useful for studying the availability of nanoparticles and other pollutants, as their accumulation involves crossing multiple biological membranes, providing insights into the biological availability of pollutants in ecosystems (Perrot-Minnot et al., 2023; Sures et al., 2005). However, in addition to the parasite-related factors, the differential metal accumulation in intermediate and definitive hosts

also warrants attention as distinctive mechanisms operate in each type of host (Perrot-Minnot et al., 2023) and their respective trophic levels. There are limited studies on the effects of pollutants and parasites on the definitive hosts compared to intermediate hosts which opens an important avenue for future studies.

b. Parasites as effect indicators

Effect indication within the context of environmental parasitology involves complex biological interactions and responses (Sures et al., 2017). For effect indication *via* parasites, two key approaches are mainly focused on like assessment of the direct impacts of pollutants on the survival and longevity of free-living stages and the determination of changes in parasitic population and communal structure. Parasites are integral components of ecosystems as part of food webs and environmental changes can be detected through parasites if any of their developmental stages or hosts are negatively affected. Adverse effects on parasites trigger changes in their abundance, causing shifts in biodiversity patterns and ecological indices, such as species diversity and the proportion of monoxenic and heteroxenic species (Sures, 2008a; Sures et al., 2017).

Traditional ecotoxicological approaches typically focus on behavioral, physiological, or molecular responses to environmental stressors, such as pollution or habitat disruption (Amiard-Triquet et al., 2015). Exposure to contaminants can elicit a wide range of responses, from high stress levels and protective mechanisms to complete physiological disruption and organismal death. Ecotoxicologists utilize biochemical and molecular responses (biomarkers) as early indicators of contaminant presence and anticipate potential adverse consequences (Forbes et al., 2006). The most common biomarkers employed to study the effects of contaminant exposure are changes in hormone regulation, energy metabolism, oxidative stress, and altered expression of genes and proteins related to pollutant biotransformation and

elimination (Amiard-Triquet et al., 2015; Foster et al., 2018; Monserrat et al., 2007). In natural environments, organisms are exposed to a multitude of factors, including pollutants and other intrinsic and extrinsic stressors which raise concerns about the reliability and ecological relevance of biomarkers in indicating exposure to or effects of toxicants (Forbes et al., 2006).

Indication through changes in behavioral, physiological, and molecular responses of the hosts

The parasites strongly interact with the biomarker responses induced by the pollutants within their hosts by affecting host physiology in diverse ways. The effects of parasites and pollutants on hosts are context-dependent, influenced by the specific parasitic species, host, and type of pollutant involved. These interactions can modulate biomarker levels, with significant implications like affecting biomarker reliability for pollution monitoring and potential adverse physiological effects on host organisms (Marcogliese and Pietrock, 2011; Sures, 2006). Generally, larval parasites in intermediate hosts that rely on trophic interactions for transmission tend to be more infectious, resulting in greater pathological manifestations when coupled with environmental stressors (Sures et al., 2017). Similar kinds of responses are elicited by parasites and pollutants in the hosts but only a few responses are parasite-specific (Sures, 2008b). The way biomarkers respond in organisms infected with parasites and exposed to pollutants is not well understood and needs more research.

The biomarkers of oxidative stress, for instance, may show an increasing or decreasing trend as a result of the combined effect of parasitism and pollutants, making interpretation challenging. Using multiple enzymes and substrates related to oxidative stress can help clarify the stress response in hosts (Martínez-Álvarez et al., 2005). Moreover, parasites also modulate energy metabolism markers (total lipid and glycogen levels) differently thereby necessitating a more comprehensive understanding of their impact on host physiology (Sures et al., 2017).

Conversely, decreased responses are elucidated by the heat-shock proteins (stress response) and metallothioneins in the hosts infected with acanthocephalan and digenean parasites respectively (Baudrimont and de Montaudouin, 2007; Frank et al., 2013). In this way, some effects attributed to environmental pollution might actually be linked to parasitic infections as biomarker responses are dependent on the infection status of the host (Frank et al., 2013). Exposure to the pollutants makes the host more vulnerable to parasitic infection, and both of these stressors may modulate the immune responses of the host synergistically or antagonistically (Marcogliese et al., 2010; Sures, 2006). Such concurrent exposure may either have severe or mitigated impacts on organismal health (Marcogliese and Pietrock, 2011). From an evolutionary perspective, it can be concluded that the parasites reduce the toxic burdens of their hosts to enhance their own survival as their own existence would be in peril if the host dies (Sures et al., 2017). In fact, this argument is supported by evidence as cestode parasites reduce oxidative stress in hosts making them less sensitive to metallic exposure (Sánchez et al., 2016). However, more evidence is required to highlight the positive impact of parasitosis to reduce pollutant load as parasites are not neutral with respect to their interaction with the host organisms.

Changes in the numerical patterns of the parasite population and effects on free-living stages

The trajectories of population abundance can reflect the health of an ecosystem and its functionality. The environmental conditions of the host are crucial for parasites, albeit the impacts may vary based on the life cycle of the parasite. Environmental factors must be favorable for all host levels (intermediate and final hosts) for the heteroxenous parasites, whereas, only the ecological requirements of the parasite and host must be met for the monoxenous parasites (Sures, 2008a). Hence, the environmental conditions have a less pronounced effect on the populations of monoxenous parasites in comparison to heteroxenous

parasites (Mackenzie, 1999), however, in other instances, a higher susceptibility of monoxenous parasites has been observed (Blanar et al., 2009). A close relationship between environmental factors and parasitism has been documented for heteroxenous parasites which suffer reduced abundance due to the harmful effects of pollution on intermediate hosts (Williams and Mckenzie, 2003). Alternatively, the immunosuppressive effects of pollutants may also promote parasitic infection (Sures, 2008b). Increasing levels of pollution, therefore, may result in an increase or decrease in parasite abundance depending on the susceptibility of a parasitic species to a particular contaminant (Sures, 2005). In populations exposed to environmental pollution, parasitism may exhibit a threshold effect, where low-level infections confer benefits, but higher parasite loads become harmful (Goutte and Molbert, 2022).

The ability of parasites to survive is dependent on a variety of circumstances, one of which is the effective transfer of infectious stages from one host to the next. In addition to using predator-prey relationships and vectors to infect new hosts, several helminth parasites have free-living phases of transmission that leave their hosts and enter the surrounding environment (Pietrock and Marcogliese, 2003). The effect of contaminants on the survival and infection potential of free-swimming stages has been studied more extensively in aquatic ecosystems compared to terrestrial ecosystems. The effect of metals on cecariae and miracidia of digenean parasites has been studied to evaluate if these developmental stages could be used as bioindicators (Sures et al., 2017). However, little has been accomplished in this field of study because of the lesser number of studies and inconsistent results mostly suggesting reduced survival and infectivity of cercariae (Pietrock and Marcogliese, 2003; Sures et al., 2017). Furthermore, the brief lifespan of the free-living parasite stages may make monitoring more difficult, potentially rendering the observed effects inconsequential.

Effects of environmental stressors on the parasites

Stressors in the environment can have a variety of effects on the parasites. Knowing the parasitic characteristics unique to their life cycles is necessary to comprehend and evaluate how these stressors affect parasite diversity (Llopis-Belenguer et al., 2019). Major characteristics of the host-parasite interaction that environmental stressors might take advantage of include life cycle complexity, host specificity, transmission mechanism, distribution range, and localization inside the hosts. An increase or decrease in stressors may directly or indirectly affect the parasites (Sures et al., 2023). It has been established recently that the monoxenous parasites are at an advantage in both aquatic and terrestrial habitats due to simple life cycles and the involvement of a single host (Sures et al., 2017). A multi-host parasite is at higher risk as a decrease in the abundance of one host along the chain may lead to the local extinction of the parasitic species. Additionally, a reduction in the number of multiple hosts, followed by a disturbance (habitat fragmentation/alteration), makes parasites highly vulnerable and significantly affects their prevalence (Bitters et al., 2022).

Changes in temperature and global warming driven by climate change also have considerable effects on parasitic populations. Given the intrinsic complexity of host-parasite systems, predicting the outcomes of temperature-driven alterations in parasitic life cycles and their potential interactions with other environmental and biological factors is quite challenging, despite their profound impacts on parasites and host species (Marcogliese, 2001). Digenean trematodes are among the most complicated metazoan parasites involving several vertebrate and invertebrate hosts as well as direct and indirect transmission paths. The free-living stages (miracidia and cercariae) are particularly vulnerable to environmental factors. There is a strong positive correlation between temperature and the asexual cercariae generation and appearance in the first intermediate mollusk host as well as the infectivity in the second intermediate host (Díaz-Morales et al., 2022). Nevertheless, while the rise in temperature positively impacts

cercarial transmission, these effects can differ significantly amongst sympatric trematode species, and warming temperatures may change the parasite community structures in ecosystems rather than favouring certain species over others (Selbach and Poulin, 2020). Temperature-mediated increases in parasite productivity and infectiousness can, however, be restrained by predation of free-living stages offsetting an increase in disease risk influenced by climate change (Goedknegt et al., 2015). Additionally, temperature not only modulates transmission dynamics of free-living stages but also alters the impact of parasitic infections on host organisms and their role within food webs (Wang et al., 2019). Predicting population and pathological dynamics in warming ecosystems relies on understanding different and diverging temperature optima of hosts and parasites. There are significant variations among different host-parasite associations, and understanding the mechanisms governing these interactions is crucial to determining how temperature influences them. Furthermore, host genotypes from various geographic locations might exhibit potentially variable temperature responses even within the same host-parasite system, complicating the infection dynamics even for a single parasitic species (Leicht et al., 2017).

A broad spectrum of environmental contaminants and their metabolites have been impacting ecosystems since the beginning of the industrialization era, with many new chemicals being introduced every year. The effects of pollutants on parasites and their hosts are highly concentration-dependent, with varying levels of exposure influencing the severity of stress (Sures and Nachev, 2022). The effects of pollution on parasites have been reviewed extensively (Blanar et al., 2009; Marcogliese, 2005; Sures et al., 2017; Sures and Nachev, 2022) and parasites respond to contaminants with changes in the community structure. Excessive pollution levels in the environment can also exert selective pressure driving evolutionary changes within the affected organisms (Weis and Weis, 1989) leading to speculation that different parasite taxa might have developed tolerance to pollutants but evidence has been

lacking so far (Sures et al., 2023). There was a direct relationship between acanthocephalans, cestodes, and high element levels (Erasmus et al., 2022) indicating that if host-parasite systems were able to evolve adaption mechanisms, high contaminant loads could be tolerated. There is a direct effect of pollution on adult ectoparasites and larval stages which are in contact with the environment, inducing responses that reduce transmission efficiency and change the population dynamics of the parasites (Sures et al., 2017; Thieltges et al., 2008). Similarly, endoparasites with free-living larval stages, such as cercariae and miracidia, are also sensitive to pollution (Gilbert and Avenant-Oldewage, 2021; Thieltges et al., 2008). Heavy metals can impact trematode transmission by reducing the longevity, viability, and infectiousness of cercariae (Morley et al., 2006). Similar outcomes for organic contaminants, including different pesticides, have been documented (Hua et al., 2016). The free-living stages of parasites are not only susceptible to pollutants but also capable of influencing environmental pollutant levels; large quantities of cercariae released by molluscan intermediate hosts (Díaz-Morales et al., 2022) can act as pollutant sinks at lower spatial scales, affecting chemical distribution and bioavailability (Sures and Nachev, 2022). Apart from considering the individual effects of pollutants, the interactive effects of environmental stressors must be taken into account to fully understand the behavior of parasitic species and assess how host-parasite systems are impacted.

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Chapter 2.

Research article

First molecular description of *Macracanthorhynchus hirudinaceus* in wild boars from Italy with pathomorphological and epidemiological insights

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1. Abstract

Macracanthorhynchus hirudinaceus is a zoonotic parasite affecting suids worldwide which are the definitive hosts for this helminth species. *Macracanthorhynchus hirudinaceus* is of significant economic and management concern due to its pathogenicity, causing intestinal obstruction and perforation in the definitive hosts. Current study is the preliminary investigation from Sardinia, Italy, reporting the pathomorphological findings and molecular characterization of *M. hirudinaceus* in the wild boars (*Sus scrofa meridionalis*). A total of 59 wild boars were examined showing acanthocephalan infection in 8 (13.6%) animals. In total, 49 parasites were collected with a mean intensity of 6.1. Comparatively higher infection levels were observed for males (16.7%) and young boars (14.3%); however, these epidemiological differences were statistically nonsignificant. Histopathological examination revealed the presence of a variable number of nodules (~5 mm) in the intestine of *M. hirudinaceus* infested animals surrounded by a hyperemic-hemorrhagic halo. Several parasites were recovered from the intestinal lumen attached by the means of characteristic hooks showing necrosis in muscle layers. A moderate number of plump reactive fibroblasts and lesser numbers of fibrocytes were embedded with and at the borders of the inflammatory nodules in a moderate amount of homogeneous intensely eosinophilic fibrillary material rupturing the cell membrane. For molecular characterization, six isolated worms were amplified for the partial mitochondrial *cox1* gene showing distinct interindividual variations. This first pathological and molecular description from southern Europe provided new knowledge about the diffusion of *M. hirudinaceus* in wild boars, furthering the research into the origin and transmission status of *M. hirudinaceus* in endemic localities.

Key words: Acanthocephala, *Macracanthorhynchus hirudinaceus,* Italy, mtDNA, *cox1*

Introduction

2. Introduction

The wild boars (*Sus scrofa*) are distributed throughout the Europe, Asia and North Africa (Wilson 2005). In the last decade their population has increased remarkably in most areas of Europe, including Italy (Pittiglio et al. 2018). To date, the wild boar population in Europe counts nearly four million individuals with abundance of these ungulates approximately doubled from 500,000 in 2010 to 1 million in 2020 in Italy (Iacolina et al. 2018; ISPRA, 2020). The endemic subspecies, *Sus scrofa meridionalis*, is spread throughout the Sardinian Island, occupying various habitats including low Mediterranean maquis, woodlands, both uncultivated and cultivated lands and pastures (Iacolina et al. 2016; Lombardini et al. 2017).

The wild boars are classified as one of the most invasive animal species in the world according to the International Union for the Conservation of Nature: Species Survival Commission (IUCN SSC), not only because of the environmental concern, but also due to significant damage to agricultural and natural habitats, and key infectious disease reservoirs transferring diseases to animals and humans (ISSG 2013, Fredriksson-Ahomaa 2018). Being omnivorous with high adaptation capacity, wild boars feed on small mammals, invertebrates, carrions and tubers and could therefore host a high diversity of gastro-intestinal helminths that have great public health importance (Solaymani-Mohammadi et al. 2003).

Among the helminths of wild boars is the zoonotic acanthocephalan *Macracanthorhynchus hirudinaceus* (Leng et al 1983; Schmidt 1971; Radomyos et al. 1989). The life cycle is indirect, the adult stage infects the small intestine of wild boars, the definitive hosts and can be accidentally transmitted to humans by ingestion of the intermediate host, arthropods, usually coleoptera from Scarabaeidae family (*Melolontha vulgaris*, *Cetonia aurata, Polyphilla fullo*, *Anomalia vitis*). The most important intermediate hosts in the United States are the june beetles (*Cotinis nitida*), New World scarab beetles (genus *Phyllophaga*), and other beetle species that

feed primarily on dung (Pavlović et al. 2010). Adult worms possess a retractable proboscis armed with hooks and granulomatous lesions are often found at the site of attachment. Since acanthocephalans lackdigestive tract, nutrients uptake occurs directly through the tegument (Bowman et al. 2003). Female worms measure up to 40 cm in length, while males are smaller and measure up to 10 cm (Mowlavi et al. 2006). Mature eggs released through the female's uterine are passed in the feces of the host. Deep brown in color and spindle shaped, eggs measure 110 μm by 65 μm having three layered shell and well developed acanthors. The eggs are capable of surviving under extreme climatic conditions. After being ingested by dung beetles, the acanthor larva develops to an infective cystacanth stage in around 3 months. Wild boars become infected by ingesting beetle grubs with the cystacanths developing into adults (Urquhart et al. 1996, Bowman et al. 2003, Marchiondo 2019).

Despite the large population and wide distribution of wild boars in Sardinia, very few studies on parasites of these animals have been carried out on the island (Bandino et al. 2015; Bonelli et al. 2020; Setsuda et al. 2020). Regarding *M. hirudinaceus*, the only study in wild boars in Sardinia was published in conference proceedings and refers to a case report (Pilo et al. 2012). Therefore, no data on the epidemiology and molecular characterization of this parasite in Sardinian wild boars are currently available. To fill this knowledge gap, the current study aimed to assess the prevalence of *M. hirudinaceus* in wild boars in Sardinia, as well as to report the pathomorphological findings and the molecular genetic characteristics of this parasite for the first time from Southern Europe to have a better understanding of genetic diversity of its population in Sardinia, Italy.

3. Materials and methods

Sample collection

From 2009 to 2020, a total of 59 wild boars were examined at Istituto Zooprofilattico

Sperimentale della Sardegna, Tortolì, Sardinia. Animals considered in this study were culled during the hunting seasons. At necropsy, data about age, sex and hunting site were recorded. Based on tooth development, animals were classified as juveniles (<18 months of age) and adults (≥18 months of age) (Massei and Toso 1993; Papini et al. 2018). The gastrointestinal tract was opened and macroscopically examined for the presence of parasites and pathological lesions. All acanthocephalans were collected and stored in 70% alcohol until morphological identification and biomolecular analysis at Parasitology Laboratory of the Veterinary Teaching Hospital of the University of Sassari, Italy. Morphological identification of *M. hirudinaceus* was performed using the morphological keys available in literature (Petrochenko, 1958; Richardson 2005).

Histological examination

Selected tissue samples of the intestinal tracts showing acanthocephalan lesions were fixed in 10% buffered formalin, dehydrated with a series of increasing alcohol concentration and cleared in xylene and were subsequently embedded in paraffin. Multiple sections of 3 μm thickness were stained with hematoxylin and eosin (H.E.) and evaluated at light microscopy. Tissues were imaged using Nikon Eclipse 80i and digital computer images were recorded with a Nikon Ds-fi1 camera.

Biomolecular analysis

DNA from six collected worms was extracted using G-spin™ Total DNA Extraction kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. PCR amplification of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) was performed using one set of primers, Acanth_Cox1-F1 (5'-TTTGGAGGGGTTTGATGGGT-3') and Acanth_Cox1-R4 (5'-CCCCAGACATTCTTCTCTCCA-3'), and the same conditions previously described (Kamimura et al. 2018). PCR products were purified using Nucleospin

Gel and PCR Clean Up (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and sent to an external sequencing service (Eurofins Genomics, Ebersberg, Germany). The sequences were obtained using primers Acanth_cox1-F4 (5'-TTGGGTATTTGGGCATGGTG-3') and Acanth_cox1-R2 (5'-CACCATGCCCAAATACCCAA-3') employed exclusively for sequencing (Kamimura et al. 2018) of the partial *cox1* gene and compared with the reference sequences for *M. hirudinaceus* (FR856886, Weber et al. 2013; LC350021, Kamimura et al. 2018) available on the NCBI database using BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Multiple alignment of the obtained nucleotide sequences for the *cox1* gene was done using Clustal W program. The aligned sequences, trimmed to median length (961 bp), were then exported to DnaSP program for the computation of polymorphism in the obtained sequences (Rozas et al. 2017). A maximum-likelihood tree was generated for the *cox1* sequences along with the reference sequences (FR856886, LC350021, AF416997) keeping *Monoliformis monoliformis* (AF416998) as an outgroup employing MEGA-X software (Kumar et al. 2018). The obtained nucleotide sequences for the *M. hirudinaceus* isolates were submitted in Genbank database under the accession numbers: MZ683370-MZ683375.

4. Results

In total, 59 wild boars were examined among which 38 were categorized as adults whereas 21 were juvenile and subadult. Among the 59 wild boars examined, 23 were females and 36 males. An overall prevalence of 13.6% was found among the culled wild boars; males had higher infectivity level (16.7%) than the females (8.7%) with non-significant difference in infection levels ($\chi^2 = 0.211$; $P = 0.629$). Among age cohorts, there was slightly higher proportion of infection in the young animals (14.3%) compared to the adults (13.2%) alluding to non-significant difference between the age groups in terms of infection ($\chi 2 = 0.01$; $P = 0.904$).

A total number of 49 *M. hirudinaceus* specimens were collected, with a mean abundance (mA) of 0.8 and a mean intensity (mI) of 6.1. The highest intensity of infection of *M. hirudinaceus* per wild boar was 24 worms. Males constituted 44.9% (22 out of 49) and females 55.1% (27 out of 49) of the recovered *M. hirudinaceus* specimens. Total body length ranged from 5.4 in males to 14.1 cm and 6.2 to 32.5 cm in female worms. Stereomicroscopical observation of *M. hirudinaceus* showed the cephalic region characterized by the retractile proboscis with typical hooks and epidermal elevation at their insertion point (Fig. 1). The gross examination of the infected wild boars showed a variable dilation of the intestinal lumen and the presence of several parasites at dissection (Fig. 2a and 2b) attached by means of its apical portion entirely embedded in the wall. The mesenteric lymph nodes were enlarged, and the serosa of the small intestine showed a variable number of roundish small nodules (~5mm) surrounded by a hyperemic-hemorrhagic halo (Fig. 2c). In the mucosal surface of the small intestine, the nodules after the parasites removal, showed a slight relief, centrally umbilicated (Fig. 2d).

Histological examination

The histological examination showed a moderate, focal, 0.2 × 0.2 mm, irregular nodular inflammatory chronic granulomatous reaction oriented around a longitudinal section of an adult acanthocephalan characterized by focally disrupting and ulcerating the intestinal mucosa, extending to the muscular layer and to less extent to the serosa. The parasite was characterized by a thin outer cuticle and a thicker hypodermis (up to 250 um) with both felted and cross fibers containing lacunar channels, outer circular and inner longitudinal somatic muscle layers, and lemnisci with compressor muscles within a pseudocoelom (Fig. 3). The overlying mucosa was replaced by a moderate number of karyorrhectic neutrophils and less number of eosinophils, admixed with a moderate amount of eosinophilic granular material with cellular debris (lytic necrosis), in turn surrounded by a moderate number of lymphocytes and plasma cells and few epithelioid macrophages. Admixed with and at the borders of the inflammatory

nodule were a moderate number of plump reactive fibroblasts and lesser numbers of fibrocytes embedded in a moderate amount of homogeneous intensely eosinophilic fibrillary material (collagen) interpreted as fibroplasia (pyogranuloma). Peripheral myofibers presented increased eosinophilia and loss of the sarcoplasmic cross striation (degeneration) or fragmentation of sarcoplasm with rupture of the cell membrane.

Biomolecular analysis

The partial *cox1* nucleotide sequences obtained for the six *M. hirudinaceus* isolates were (>99%) similar to a sequence from Hungary (FR856886) but showed 89-90% similarity to an isolate identified from Japan, LC350021. There were 16 nucleotide polymorphic sites within the analyzed fragment of the *cox1* gene yielding 12 singleton variable sites and 4 parsimony informative sites. There was a single non-synonymous substitution (valine to isoleucine) resulting from a point mutation at first position of the codon at nucleotide number 181 with respect to the reference sequence. It was further revealed that all six nucleotide sequences had interindividual nucleotide variations yielding six different haplotypes having 6.267 average number of nucleotide differences (*k*) among them. Phylogenetic assessment of the obtained sequences indicated a robust clustering with the reference sequence from Hungary with strong bootstrap values at the relevant tree nodes. The Japanese *M. hirudinaceus* isolate clustered outside the European population indicating the difference between these parasitic populations. Evolutionary linkages among the sequences are determined by the genetic distance between the populations depicted by the horizontal branch lengths on a phylogenetic tree (Fig. 4).

5. Discussion

The present survey provides new insights into the epidemiology and molecular characterization of *M. hirudinaceus* in wild boars for the first time in Sardinia, Italy. In addition, pathomorphological alterations due to this parasite in the gastrointestinal tract are described.

As far as we know, no previous epidemiological surveys have been carried out on *M. hirudinaceus* in wild boars in Sardinia. Furthermore, there are only a few studies on the prevalence of the acanthocephalan in these ungulates in Italy (Magi et al. 2005, Papini et al. 2018) as well as in Europe. Low prevalence observed in the current study (12.1%) was quite similar to what was ascertained from central Italy (9.4%; Papini et al. 2018), southern Italy (Sicily) (11.1% in feral pigs; Migliore et al. 2021) and Spain (12.1%, Gassó et al. 2015). Apart from this, *M. hirudinaceus* has more frequent occurrence (range: 33.0-81.8%) in other endemic areas like Morocco (Amayour et al. 2017), Argentina (Ciocco et al. 2019), Iran (Mowlavi et al. 2006; Sarkari et al. 2016; Dodangeh et al. 2018) and Tunisia (Lahmar et al. 2019). The worldwide variations in recorded prevalence could be related to the different climatic conditions and soil composition that may influence the distribution of the dung beetles (*Scarabaeidae*) which act as intermediate hosts of *M. hirudinaceus* (Foata et al. 2005; Lizana et al. 2021). Regarding reptiles, little is known about them as potential paratenic hosts of *M. hirudinaceus*, but their varied worldwide distribution may likely contribute to prevalence of *M. hirudinaceus* in wild boars. Indeed, reptiles act as paratenic hosts of different parasitic zoonoses including acanthocephalans such as *Macracanthorhynchus ingens* (Hartnett et al., 2018; Mendoza-Roldan et al., 2020). The acanthocephalan *M. hirudinaceus* can infect not only wild boars but also jackals, red foxes, domestic pigs and quite rarely dogs, where a case of pseudoparasitism or misidentification with *Macrachanthorhynchus catulinus* may likely have occurred (Dalimi et al. 2006; Lahmar et al. 2014; Barbosa et al. 2017; Gherman and Mihalca, 2017; Kouam et al. 2018). Incidental hosts include muskrats, squirrels, and humans (Baker, 2007). However, it was established that this parasitosis is most common in pigs raised in free-range and extensive production systems compared to those raised in industrial farms (Barbosa et al. 2017). In Sardinia, the free-ranging pigs are forbidden by the African Swine Fever eradication programme (PE-ASF15-18), but semi-extensive pig breeding is still present on the

island (Anagrafe Nazionale Zootecnica 2020). Furthermore, the intermediate hosts of *M. hirudinaceus* are well distributed in Sardinia (Carpaneto et al. 2011).

The prevalence found in wild boars in current study, may also represent a sanitary concern for the health status of domestic pigs, particularly in areas where semi-extensive farming allows uncontrolled contacts with wild species that favours the transmission of viral diseases. The ingestion of the intermediate host harboring cystacanth larvae by the pigs could lead to economic losses for farmers primarily due to slow growth and weight loss typical of *M. hirudinaceus* severe infection (Barbosa et al. 2017). *Macracanthorhynchus hirudinaceus* has been reported in humans from China (Leng et al. 1983; Zhong et al. 1983), Thailand (Hemsrichart et al. 1983; Radomyos et al. 1989) and Russia (Schmidt 1971) to date. There is no description of *M. hirudinaceus* in humans in Italy thus far, however, the possibility of indirect transmission of this parasite to humans could not be excluded. Indeed, the proximity of wild animals to humans has thrived recently, especially during the lockdown for the COVID-19 pandemic, as documented also in Sardinia (Bressan 2020).

The prevalence of neglected and zoonotic parasites such as *M. hirudinaceus* recorded in wild boars in Sardinia, suggests the need to carry out further investigations throughout the island on wild boars, which are known to harbor other parasitic zoonoses such as *Trichinella* spp. (Bandino et al. 2015), *Echinococcus granulosus* (Paoletti et al. 2019; Sgroi et al. 2019) and *Toxoplasma gondii* (Sgroi et al. 2020).

Based on present study the age of wild boars seemed not to represent a risk factor for infection, since no differences were found between the prevalence in young and adults, unlike to what was reported in Sicily (Migliore et al. 2021), where *M. hirudinaceus* was identified only in young feral pigs (<1-yr-old), and in Iran (Mowlawi et al. 2006) where adult wild boars were comparatively more infected than young ones.

Pathological examination showed severe damage of the small intestine wall, with the anterior

part of the thorn-headed worm penetrated into the sub mucosal layer of boar intestine and caused severe inflammation. The findings of this study are in line with those reported by previous studies (Mowlavi et al. 2006; Sarkari et al. 2016; Migliore et al. 2021).

Identification of epidemiological and pathogenic status of a parasite is of paramount importance to understand its biology and infectivity. There is accumulating evidence regarding the reliability of the morphological studies alone as they may result in misidentification of the species (Huston et al. 2020). Our study combined both morphological and molecular approaches to characterize the *M. hirudinaceus* isolates retrieved from the wild boars in Sardinia. For molecular analysis, the study involved sequencing for the *cox1* gene which is the most common and reliable marker in population genetics and phylogenetic studies. So far, molecular data on *M. hirudinaceus* are very limited; there are only two sequences in the NCBI database, one of which is a complete genome from Hungary (FR856886) whereas, the other is partial *cox1* sequence from Japan (LC350021). It is very interesting to mention that all six nucleotide sequences for the partial *cox1* gene had high genetic variability which shows an expanding *M. hirudinaceus* population within this locality. The European sequences were more similar to each other compared to the Japanese sequence displaying heterogeneity among the different geographical populations. Only two reports of *M. hirudinaceus* occurrence are reported in Japan and is yet to be established whether this species is accidentally introduced or has endemic status (Kamimura et al. 2018). But the clustering of Japanese isolate separately from the European population shows that independent factors are operative at different geographical areas affecting the genetic structure of the species. Due to scarcity of investigations and lack of molecular data, no studies on gene flow among the *M. hirudinaceus* populations could be carried out which limits our understanding of its geographical spread and population structure.

Conclusion

This preliminary investigation involving pathomorphological and molecular approaches to characterise acanthocephalan species, *M. hirudinaceus*, from Italy has provided an insight into level of infection and extent of endemism of this parasitic species in southern part of Europe. Amid lack of epidemiological and genetic information on this zoonotic parasite from European side, continued efforts are required to generate molecular data to find relationships among different regional and endemic populations. On the other side, it is equally important to find out the transmission routes of this parasite to limit its spread to humans and other host species and devise suitable strategies for its control.

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Declarations

Conflicts of interest

The authors declare no conflict of interest.

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Fig. 1 Stereomicroscopical observation of anterior end of *Macracanthorhynchus hirudinaceus* with its characteristic spiny head.

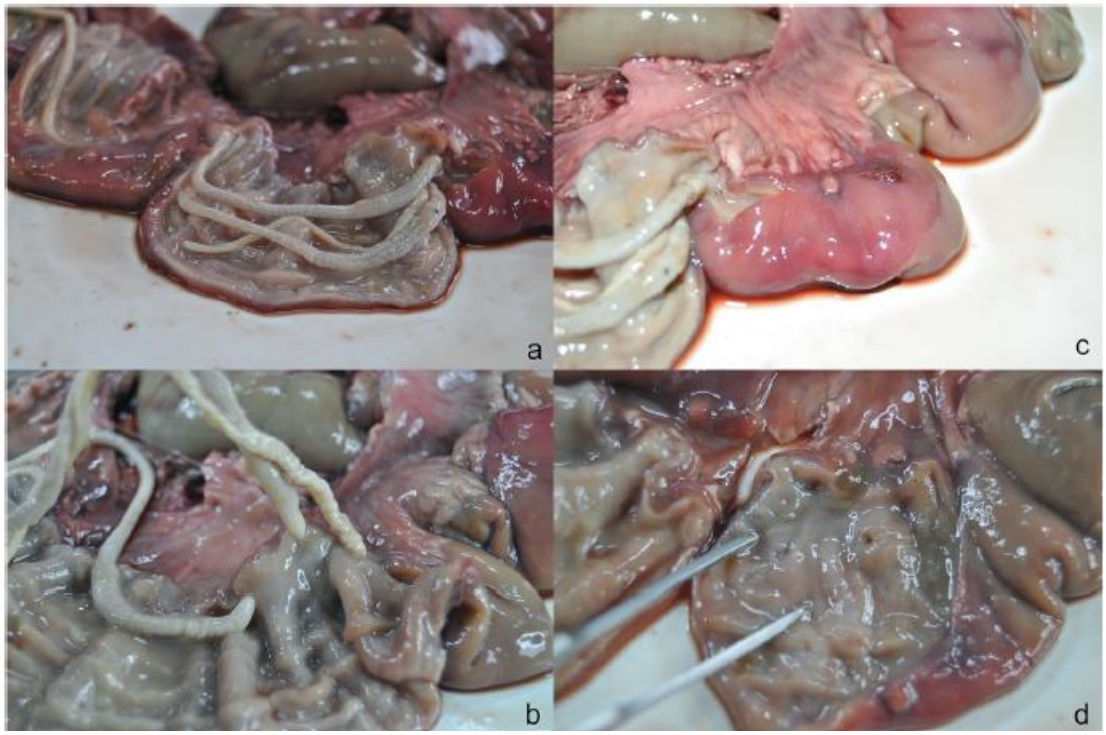

Fig. 2 Macroscopical examination of the small intestine showing the presence of several worms in the open gut (a) with the spiny-head penetrating the intestinal wall (b), and nodular lesions on the serosa (c) appearing umbilicated in the mucosal surface after removal parasites (d).

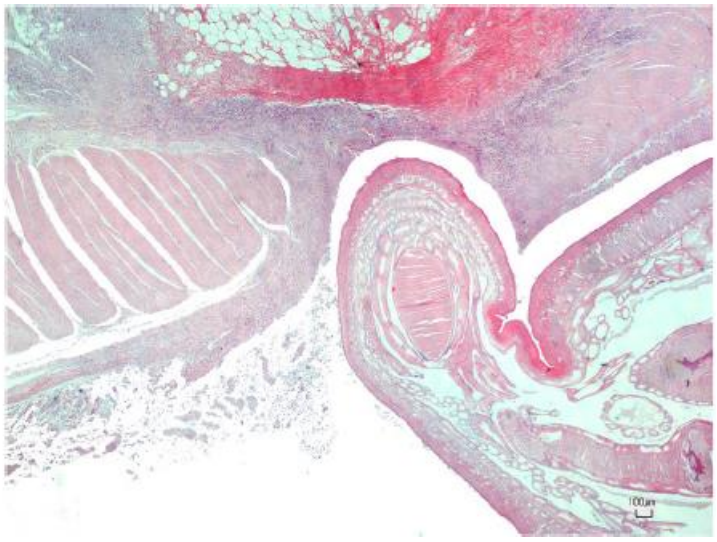

Fig. 3 Histological view of the small intestine H.E.-stained showing a moderate, focal chronic pyogranulomatous and ulcerative enteritis around a longitudinal section of *Macracanthorhynchus hirudinaceus*.

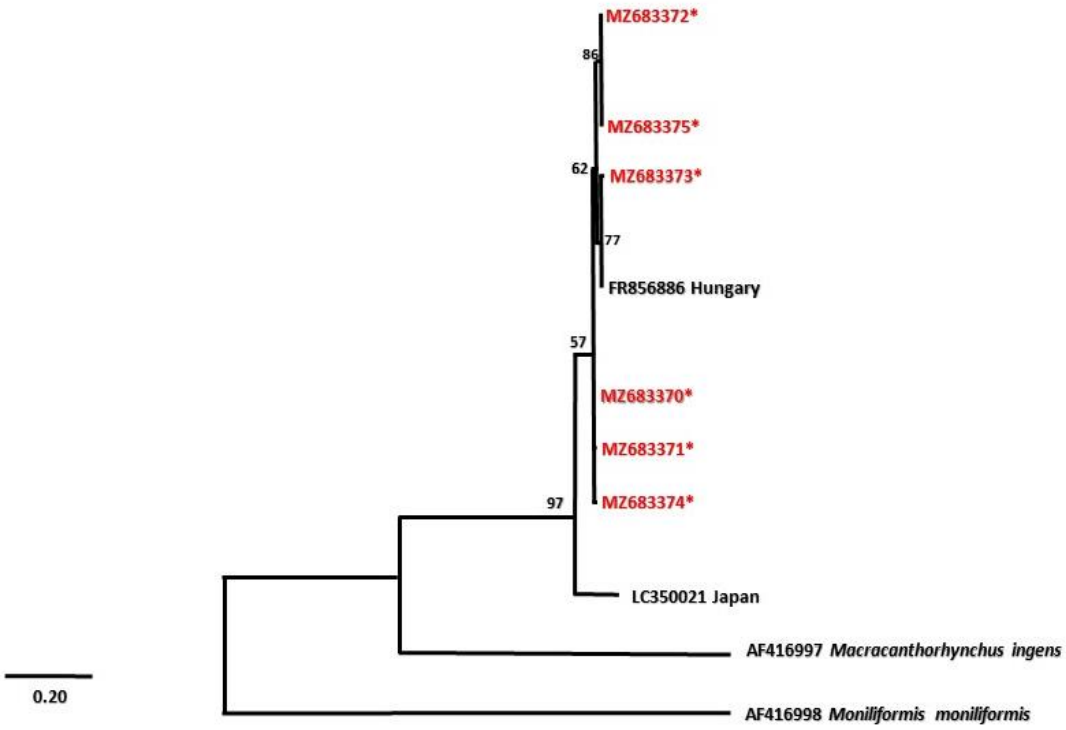

Fig. 4 Maximum-likelihood phylogenetic tree of the *cox1* mtDNA sequences for *M. hirudinaceus* specimens (written in red with asterisk) indicating positioning with Hungarian isolate. Bootstrap values are represented as number on the tree nodes.

Chapter 3.

Original article

Lead concentrations in wild boar from Sardinia: analysis of food safety concerns

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1. Abstract

A multitude of pathways within human food chains have been affected by the presence of lead (Pb) which has highly negative consequences on animal and human health. Numerous sources contribute to Pb exposure, and the use of Pb ammunition is considered one of the main unregulated means of releasing this hazardous metal into the game meat and environment. In addition to this, geogenic sources or anthropogenic activities could also result in higher contamination of game meat. Considering these aspects, the present study was carried out in wild boar (n = 53) from different hunting grounds in northern part of Sardinia (Italy) for evaluation of mean residual levels in liver, spleen, and thigh muscle through employing inductively coupled plasma mass spectrometry (ICP-MS). The highest mean concentration of Pb was estimated in liver (6.70±34.87 mg/kg dw) followed by spleen (5.11±22.36 mg/kg dw) and thigh muscle (2.67±11.74 mg/kg dw). Concerning biological sexes, females (n = 27) had higher mean levels for all tissues, however, no differences were ascertained in the bioaccumulation of Pb. Among the age groups, adults had higher accumulation of Pb in all tissues compared to juveniles. Nevertheless, the level of Pb in both groups did not yield any accumulation pattern. Additionally, a moderate correlation was calculated for liver and spleen (0.643, $p < 0.01$) for Pb accumulation. The risk assessment revealed a very high risk to the hunters who frequently consume the game meals as margin of exposure for this group was very low. In comparison with EFSA guidelines, none of the samples was fully fit for human consumption which calls for inclusion of game meat in the EU regulation.

Key words: *Sus scrofa meridionalis*; risk assessment; game meat; bioaccumulation; liver; Pb; Italy

2. Introduction

Lead occurs naturally in the earth's crust but certain human activities like mining and smelting, industrial processes, agricultural practices, hunting, and sport shooting have resulted in increased concentrations of this metal in the environment [1] ranking it as the second most significant threat to human health by the Agency for Toxic Substances and Disease Registry [2] and a chemical with long term effects accounting for 900,000 deaths worldwide [3-4]. It is one of the most hazardous and cumulative environmental pollutants which affects all biological systems with no known function in the cellular processes [5-6]. Main routes of Pb exposure are inhalation or ingestion via air, dust, soil, food and drinking water [7]. A number of traditional applications where Pb is used (for example, addition in gasoline as anti-knocking agent) are now being curtailed, however, it is still a public health priority because of its potential toxic effects [6]. Even at very low exposure levels, Pb causes significant disruption to the normal physiological processes [8]. In animal tissues, Pb causes irreversible health effects interfering with a number of body functions and primarily affecting reproductive, renal, hematopoietic, cardiovascular, hepatic, excretory and central nervous systems [2, 9]. The European Food Safety Authority (EFSA) has identified developmental neurotoxicity as the most sensitive toxicological endpoint for Pb and in the case of adult population, the most sensitive endpoints are nephrotoxicity and impacts on the cardiovascular system [10]. Because of high toxicity of Pb, no safe alimentary levels can be defined so efforts should be made to reduce and minimize Pb-intake [10]. The Commission Regulation (EC) No 1881/2006 had set the maximum level (ML) of Pb in meat of bovine animals, sheep, pig and poultry to be 0.37 mg/kg dw (0.10 mg/kg ww). This regulation was later repealed and the new regulation CR 2023/915 set the maximum limits for pig meat to be 0.55 mg/kg dw (0.15 mg/kg ww).

Amid growing concerns regarding the toxicity of Pb and its detrimental impacts on human

health, it is imperative to study Pb residues in the food and environment as the mammalian body burdens are realistic indicators of exposure to contaminants [11]. In terrestrial environments, wild mammals are often studied for indication of pollutant levels because of their significant position in the ecological pathways [12]. Certain wild species like wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) are consumed in Europe as game meat and are employed in ecotoxicological studies to assess the level of contaminants [13-15]. Game meat has historically been a crucial component of human nutrition, and game hunting continues to hold significant importance in various parts of the world, particularly Europe. Over time, hunting has evolved beyond its primary function of providing sustenance and is playing a pivotal role in the game meat production sector [16]. The consumption of wild game meat by humans is increasing, driven by a preference for "non-farmed" meat and the endorsement of wild game as a wholesome substitute for other types of meats [17]. Nonetheless, there exist notable variations in hunting and game processing regulations across different European countries, leading to distinct national game meat supply chains, game market, meat quality and hygiene standards which pose several challenges to this industry. The challenges faced in game meat production include establishing effective disease surveillance and control, implementing population control measures and managing the introduction of contaminants *via* the use of free bullets. These challenges collectively contribute to a complex landscape that requires careful consideration and management to ensure sustainable game meat production and consumption [18].

Furthermore, hunting practices result in various commercial game meat products that are harvested through the Pb based ammunition. Ingestion of Pb residues from hunted game has recently been identified as a pathway of lead exposure to humans who routinely consume this meat [19]. Wild-shot game animals often carry small fragments of Pb lodged in their meat due to the use of Pb-based ammunition, including both gunshot and bullets [20]. These fragments

are often too tiny and abundant to be detected and removed during food processing [21-22]. Despite removal of meat close to wound channel, Pb remains in the meat as its fragments penetrate the tissue like a 'splinter cloud' and there is high possibility that those tiny pieces disperse within the whole tissue and carcass [23]. Environmental sources and hunting practices are contributing to elevated levels of Pb in game meat which need to be monitored [24]. Hunting-derived meat does not usually undergo inspection which can result in uncertainties in determining foodborne risks associated with consumption and handling of this meat [25-26]. For these reasons, this study was carried out to quantify the levels of Pb in different tissues of wild boar hunted in different hunting areas of northern part of Sardinia (Italy) and appraise the Pb-associated risks to human population from consumption of game meat. It was hypothesized that due to absence of mining activities in northern Sardinia, very low levels of Pb would be found in the game meat.

3. Materials and Methods

Study area

Sardinia is the second-largest island in the Mediterranean Sea and is located in the center of western Mediterranean basin. It spans around 24,000 km² with a coastline of approximately 1,900 km. The island features a diverse orographic landscape, including plains, hills, and mountains with a rich array of biotopes. Agriculture dominates land use in the plains and hilly areas, accounting for about half of the territory [27]. The southern part of Sardinia has historically been a mining haven where silver, lead and zinc deposits were greatly excavated up until 20th century [28].Whereas the northern part of Sardinia has no metal mining operations and is characterized by Mediterranean scrub and scattered woodlands (https://www.sardegnaturismo.it/). The Sardinian wild boar, *Sus scrofa meridionalis*, is abundant throughout the island inhabiting different habitats like woodlands, pastures and

agricultural lands whose hunting is a popular sport [29]. The hunting season starts from November 1st and continues until January 31st [30]. Sardinia is an endemic region for African swine fever (ASF) and the according to the Legislative Decree n. 54/2004 pertaining to the control of ASF, hunters routinely provide blood and muscle samples from each hunted wild boar to the veterinary authorities.

Sampling methods

A total of 53 wild boar sourced from the hunting season of 2022-2023 in three different localities (Bortigiadas, Trinità D'Agultu, Sassari) of northern Sardinia were examined in the current study for evaluation of their Pb content. The information with regards to each hunted animal was collected and written on a pre-designed information sheet. All animals were classified according to sex, age class and area. Among the 53 wild boar, 25 were collected from Bortigiadas, 18 from Trinità D'Agultu and 10 from Sassari. With regards to sex, 26 wild boars were classified as males and 27 as females. The age estimation of each animal was based on patterns of tooth eruption and replacement, following the method described by Massei and Toso [31]. Adults were defined as animals older than 12 months, while juveniles as younger than 12 months. Based on these estimations, there were nine juveniles and 44 adults. The liver (n =53), spleen (n = 53) and thigh muscle (n = 44) specimens were taken from these hunted carcasses of the wild boar. Hide was removed from the carcasses and the samples were taken by the veterinary specialists with knife. For the muscle tissue, thorough examination was done to check the bullet entry and exit wound and care was taken to collect the sample away from the wound so as to avoid contamination which resulted in lesser number of muscle specimens. Liver, spleen, and thigh muscle were removed from each carcass and delivered to the Department of Veterinary Medicine, University of Sassari, Italy.

Determination of Pb

An aliquot was collected from each matrix, placed in plastic tube, and sent to the Istituto Superiore di Sanità in Rome, Italy, for the Pb analysis. Approximately 500 mg of liver, spleen and thigh muscle of each wild boar were dried at 105 °C overnight (the mean water content was 73%). The samples were then weighed in 15 mL polystyrene tubes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA). For digestion process, 3 mL of ultrapure concentrated nitric acid (HNO_3) (VWR, Leuven, Belgium) was added and the samples were digested on a heat block at 80 °C until complete dissolution (ModBlock, CPI International, Santa Rosa, CA, USA) and subsequently diluted with ultrapure deionized water. Reagent blanks were processed in parallel as a control to determine the Pb contamination from plastic tubes and nitric acid. As quality control, the Pb accuracy and precision were evaluated by the use of a reference sample (i.e., pig kidney-based ERM-BB186) from the Institute for Reference Materials and Measurements, Geel, Belgium. The accuracy resulted to be 96.5% while the precision, calculated on replicated measurements of the pig kidney ERM-BB186, less than 6%. Quantification of Pb (in dry weight) was performed by sector field inductively coupled plasma mass spectrometry (SF-ICP-MS), using a Thermo Scientific Element 2 model (Bremen, Germany). The low-resolution mode (LR, $m/\Delta m = 300$) was used for the determination of ^{208}Pb. An additional calibration method was used to quantify the metal and, as internal standards, the concentration of 1 ng/mL in the analytical solutions of ^{115}In was used. The operating conditions were: RF power, 1250 kW; plasma flow rate, 15 L/min; auxiliary and sample gas flow rates ranging from 0.85to 1.0 L/min; sample intake flow rate, 1.0 mL/min; and temperature of the spray chamber, ca. 10°C. The limit of detection and quantification for Pb were calculated as 3- and 10-times the standard deviation of the reagent blank and resulted to be 0.025 ng/ml and 0.08 ng/ml, respectively.

Statistical analysis

The statistical analysis involved expression of data in terms of arithmetic mean ± standard deviation (SD), range (minimum-maximum) and median. Before conducting analyses on the obtained data, a normality test was performed using the Shapiro-Wilk *W* test. The concentrations of Pb in all studied tissues did not follow a normal distribution. Moreover, log-transformation was performed which also displayed skewness in the data. Consequently, the non-parametric Mann-Whitney *U* test followed by Bonferroni correction was employed to assess the significance of differences among sex, age groups and locations for Pb accumulation in the liver, spleen and thigh muscle. Moreover, to understand the pattern of accumulation in the studied tissues, Spearman's correlation coefficient was computed with significance levels set at $p \leq 0.05$ and $p \leq 0.01$. The statistical analysis was performed on SPSS (version 25).

Risk assessment

The European Commission's Scientific Committee for Food (SCF), in 1992, adopted the provisional tolerable weekly intake (PTWI) for Pb (0.025 mg/kg of the body weight) proposed by the Joint FAO/WHO Expert Committee on Food Additives. However, by 2010, the EFSA CONTAM Panel determined that this PTWI was no longer suitable as there was no evidence of a safe threshold for Pb-induced effects. Based on its evaluation of risk assessment to humans, EFSA derived the benchmark dose (BMD) for Pb for the developmental neurotoxicity in children as well as cardiovascular and nephrotoxic effects in adults [10].

Based on the published literature of consumption of 200 g of game meat per day by the hunters [32], daily exposure was calculated using the following formula:

$$Estimated\ daily\ intake = \frac{CxIR}{BW}$$

Where C is the concentration of Pb in game meat, IR is the ingestion rate of game meat (g/day) and BW is body weight which is taken as 60kg. Estimated exposure is expressed as µg/kgbw/day.

A risk assessment metric proposed by EFSA, termed as margin of exposure (MOE) represents the ratio between a toxicological reference point and the estimated human exposure quantifying how far the current exposure is from levels known to cause adverse effects. The MOE is calculated as:

$$MOE = \frac{POD\ (BMDL)}{Estimated\ daily\ intake}$$

Where POD is point of departure which referes to the exposure level identified in toxicological studies that serves as the starting point for risk assessment. A smaller MOE (<1) implies that the exposure is closer to the level associated with adverse effects. The BMDLs (benchmark dose lower confidence interval) for alimentary Pb exposure for cardiovascular effects is 1.50 µg/kg bw/day and for nephrotoxic effects is 0.63 µg/kg bw/day in adults [10].

4. Results

A total of 53 hunted wild boar were sampled for the evaluation of Pb in liver, spleen and thigh muscles across hunting localities in northern Sardinia, Italy (Fig. 1). For each matrix, the Pb concentration as mean ± SD, range (min-max) and median for the pooled data, as well as for gender and age, is reported in Table 1. Overall, the highest mean concentration of Pb was determined in the liver samples (6.70±34.87 mg/kg dw) whereas the lowest mean value was obtained for thigh muscle (2.67±11.74 mg/kg dw; Fig. 2).

The males and females had varying concentrations of Pb in their tissues. The concentration of Pb in females was higher than in males, particularly in liver samples (11.92±48.73 mg/kg dw

versus 1.29±1.85 mg/kg dw, p = 0.21) followed by spleen (8.43±31.13 mg/kg dw versus 1.66±2.78 mg/kg dw, p = 0.54) and thigh muscle (4.58±16.40 mg/kg dw versus 0.76±1.25 mg/kg dw, p = 0.84). The Mann-Whitney U test did not indicate any significant difference among the metal levels in both sexes for liver, spleen and thigh muscle (Table 1, Fig. 2). Among the age groups, juveniles (<1 years) exhibited lower mean residual level of Pb in all tissues (liver: 1.33±0.69 mg/kg dw; spleen: 1.76±3.96 mg/kg dw; muscle: 0.27±0.30 mg/kg dw) compared to the adults where liver had highest mean Pb level (7.83±38.25 mg/kg dw), followed by spleen (5.79±24.50 mg/kg dw) and thigh muscle (3.05±12.86 mg/kg dw). Despite considerable variations in the mean accumulation levels of Pb, Mann-Whitney U statistic revealed non-significant differences among all tissues ($p > 0.05$) (Table 1, Fig. 2).

Some samples from Bortigiadas and two samples from Trinità D'Agultu had extremely high Pb concentrations, much higher than the mean values for the examined tissues. In Bortigiadas, two liver samples had 48.36 mg/kg dw and 251.19 mg/kg dw, while two spleen samples had 161.85 mg/kg dw and 22.28 mg/kg dw Pb concentrattion. In Trinità D'Agultu, one spleen sample had 18.68 mg/kg dw, and a thigh muscle sample contained 85.67 mg/kg dw Pb level. These samples were analyzed twice to minimize any chances of error, but same values were obtained which were considered valid to be included in the analysis. The values of Pb with respect to locations in each organ is presented in figure 3.

Spearman's rank correlation revealed a positive but moderate association (rs: 0.643, $p < 0.01$) between liver and spleen, whereas a weak but significant association between liver - muscle (rs: 0.381, $p < 0.05$) and spleen - muscle tissues (rs: 0.342, $p < 0.05$) was found.

In the risk assessment, daily intake was assessed and it was estimated that exposure through consumption of liver was 1340 µg/day which resulted in very low MOE values for both cardiovascular and nephrotoxic effects. Likewise, the exposure through consumption of

muscle was 534 µg/day which also yielded very low MOE values for both cardiovascular and nephrotoxic endpoints. MOE was also calculated for people with different consumption habits of the game meat, i.e., rare consumers (2 meals a year), high consumers (10 meals a year) and extreme consumers (90 meals a year). The frequent consumers were at high risk due to very low MOE values (Table 2).

5. Discussion

The current study tried to estimate the levels of Pb in liver, spleen and thigh muscle of wild boar hunted in Sardinia (Italy) to understand Pb-bioaccumulation and its outcomes on human health. The results are alarming as high concentrations of Pb are reported in the wild boar of Sardinia. Game meat is among those food types which have comparatively higher content of Pb, possibly because of Pb ammunition and other factors [33]. In addition to environmental sources, Pb-based ammunition can result in higher carcass contamination, especially around bullet entry and exit wounds, which may result in unavoidable contamination of game meat [34]. Wild boar is one of the most hunted and abundant game species in Europe [35] and its population has experienced significant growth both in terms of range and absolute numbers recently [36]. Wild boar have been a subject of focus in biomonitoring studies [37]. Being omnivores and holding a high position in the food chain, wild boar can potentially accumulate a variety of substances. Moreover, it is easy to acquire as a wild game and exhibits measurable responses to the toxicants [38]. Some studies suggest that wild boar may not be particularly suitable for biomonitoring studies due to their wide home range and movement across different regions [39]. However, a few studies on Pb bioaccumulation have indicated greater accumulation capacity of Pb by wild boar compared to red deer [15, 40].

So far, most of the studies on elemental concentrations in wild game animals are focused on kidneys and liver, the organs with key detoxification roles in the body [41], while limited

studies on toxicant levels in muscles are available [11]. Contrary to detoxification role of liver and kidneys, muscles are only the sites of accumulation [42]. The data in this paper report residual levels of Pb in spleen and thigh muscle in addition to liver of hunted wild boar in Sardinia. Due to main site of detoxification, mean Pb concentration was highest in liver (6.70 mg/kg dw) compared to other tissues. In comparison to current study, mean Pb concentration in wild boar liver (0.46 mg/kg dw) sampled from 11 hunting grounds over the period of seven years in North Macedonia were considerably low [43]. The mean level of Pb in liver of red deer (5.466 mg/kg dw) from Idanha-a-Nova, Spain (located near mining area) [13] was quite close to the value estimated for Pb in liver of wild boar in current study. However, the mean concentration of Pb in wild boar from industrial and agricultural areas of Spain yielded considerably low values (0.299 mg/kg dw) [14]. The concentrations of trace elements found in the liver, muscles and other tissues are influenced by the predisposition of the species and the capacities of each individual organism [44]. Moreover, the exposure to chemical elements varies among carnivores, omnivores and herbivores due to the distinct pathways through which contaminants traverse food webs. The risk of exposure is additionally managed through an intricate interplay between characteristics of contaminants that influence their fate and transport within the environment, alongside the natural history traits of animals inhabiting a particular site [45].

The wild boar samples collected from the hunting counties in Sardinia were also checked for any sex-specific association. Females exhibited considerably higher mean concentrations of Pb in all organs, however, these differences were non-significant. Similar studies on wild boar livers conducted in Northwestern and Southern Spain also failed to establish any gender-based association for Pb accumulation [46-47]. Several individual characteristics like size, nutritional status, genetics and hormonal profiles differ between sexes, affecting the niche differentiation and resulting in disparities in pollutant exposure and vulnerability. Moreover, sex related

differences can potentially affect the uptake mechanism, toxicokinetics and response to contaminants [48]. The sex related patterns for metals in mammalian species are also complicated by reproductive status and time of the year [49].

This study also indicated that mean residual levels of Pb were comparatively higher in all tissue specimens of adults compared to juveniles. These findings were in agreement with a study on wild boar in Italy (Brescia city) where no significant difference in mean elemental level was found among the adults and juveniles for muscle tissue [50]. Lead being nonessential element is resistant to biodegradation and maintains toxicity for longer time periods [51]. The degree of exposure of animals may increase with age due to persistence and long retention time of Pb in the environment. Older age animals also forage at a larger scale compared to young animals, thereby coming in contact with metals from broader geographic areas encompassing a more diverse array of vegetation types [52]. Nevertheless, interpretation of results in ecotoxicological studies could be difficult and is complicated due to multiple factors, hence it is advisable to find local sources of contamination [11].

It is usually expected that the metals initially accumulate in liver as it is the primary detoxification site [53]. Different organs and tissues like kidneys, spleen, bones and muscles which are involved in metal absorption, transport pathways, utilization and discharge, sequester immobilized form of metals [54]. Our study indicated an efficient Pb uptake by wild boar tissues with significant but moderate correlation between liver and spleen tissues and weak correlation between liver – muscle and spleen – muscle tissues. Spleen is the major site where blood gets filtered and is involved in erythrocyte turnover and storage; mammalian erythrocytes are also known to readily take up Pb [55] which might explain higher accumulation of Pb in spleen of wild boar. The metal accumulation in liver and kidney may also reflect acute exposure, whereas, the concentrations in muscle, bone or hair indicate chronic

exposure which is why the mean levels in these tissues are usually lower than liver and kidney [15, 56].

Contamination of game meat is a One Health concern as it indicates presence of toxicants in the environment posing risks to environmental, animal and public health [13]. For Pb, the advisable way to measure effects on humans is through MOE which indicates a level of concern. Wild boar are frequently consumed by the Italian hunters with the highest intakes of 3000 grams per month [32], such high intakes represent a significant risk to the consumers which could potentially manifest as changes in systolic blood pressure and cause chronic kidney disease (the endpoints described by EFSA). Current study estimated very low MOE among the high consumers of game meat which is a point of concern. This part of Sardinia, from where the samples were collected, does not have geogenic sources which could contribute to the presence of Pb in the game meat. However, the presence of Pb can possibly be explained through the mushrooming/expanding abilities of the hunting bullets which disperse across the tissues thereby leading to contamination [57].

Regulations are underway from the health and environmental authorities to define safe limits for different contaminants in food sources [17]. The game meat still remains a poorly regulated commodity even though game meat consumers are considered among the vulnerable group concerning Pb exposure (1.98 to 2.44 μg Pb/kg of body weight per day) according to EFSA [10] as they could consume up to 50-91 game meat meals per year [58]. The European regulation pertaining to Pb is solely confined to the established MLs for farmed species and neglects the game meat. According to The Italian National Research Plan of Residues [59], a surveillance plan for the breeding animals process and first transformation of animal-origin products, in the case of hunted wild boar, the only substance monitored is Pb in muscle. No specific maximum level has been established for Pb, but only the presence of the metal at

concentrations above the limit of detection (LOD) of the adopted analytical instrumentation represents the action limit. In this context, all muscle tissues examined showed Pb concentration at or above the action limit. In general, this plan describes the animal species and categories to be sampled, specific residues or substances to be investigated along with their maximum residue limits or maximum levels, sampling strategies, levels and frequencies of sampling, according to national and European standards. Any level of exposure for Pb is hazardous so there is an imminent need to minimize its entry into the environment and foodstuff thereof in accordance with ALARA principle (as low as reasonably achievable) [10].

Even though our study is preliminary, it could be speculated that accumulation of Pb in food sources of wild boar is resulting in such increased Pb levels in wild boar muscles rather than Pb-based ammunition solely. A study in Bavaria, Germany reported very high cesium concentrations in wild boar muscles which was mainly attributed to ^{137}Cs deposition in soil indicating that this radioactive element was finding its way into the wild boar through tubers (fungi) which accumulate ^{137}Cs through soil [60]. A similar mechanism might be operative for entry of Pb in wild boar's food chain thereby resulting in high bioaccumulation. Indeed, numerous plant species are recognized for their ability to adsorb Pb through roots (primary Pb accumulation sites) and tissues with high cellulose content [61]. Lead exhibits variable effects depending on the fates and distributions of its different isotopes in the environment. An understanding of partitioning behavior and biogeochemical pathways of Pb isotopes in terrestrial environments is necessary to understand shifts in Pb isotopic compositions throughout weathering processes and atmospheric-biological sub-cycles [62]. Since elevated levels of Pb are present in game meat, suitable hygienic measures should be taken in combination with replacement of Pb based ammunition with non-toxic alternatives for shooting wild game. Along with this, the ecological context in which game animals are raised must also be considered.

There are certain limitations of the study which need to be mentioned. Small sample size, sampling from a single hunting season and only including northern Sardinia for sample collection are the major concerns related to this study. Future studies should include a bigger sample size from the whole Sardinian island with longer duration to fully understand the metal contamination in the region along with a special emphasis on finding the sources of contamination.

Conclusion

Natural habitats are under ever increasing pressure from anthropogenic activities, hence the food sources once considered safe for consumption are becoming a serious source of contamination. Moreover, continuous monitoring of wildlife living in/near contaminated areas must be done with special focus on game species. Areas where the underlying geology contains elevated levels of potentially harmful elements or in regions with anthropogenic contamination, additive risks might emerge if pollutants are transferred through the food chain, leading to their accumulation in game animals that are then consumed by humans (as previously observed in the case of livestock meat attaining unacceptable levels of Pb). Likewise, it is equally important to develop regulations for identifying toxic limits for the hazardous metals in game meat. Regional management plans should also be devised for reducing Pb exposure in high end consumers of the game meat (hunters and their families). Eventually, it is crucial to understand the potential reasons behind Pb bioaccumulation, and this involves monitoring the concentrations of other metals, such as Zn, which serve as important environmental pollution marker, thereby to clarify the role of ammunition on this bioaccumulation. The heavy metal pollution in the ecosystems necessitates the use of One Health approach to achieve sustainable environmental management.

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Data Availability

All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

The study involved hunted wild boar which routinely undergo post mortem veterinary examination. The tissuespecimens were collected from these animals. The study did not involve human subjects.

Consent for publication

The study did not involve human subjects.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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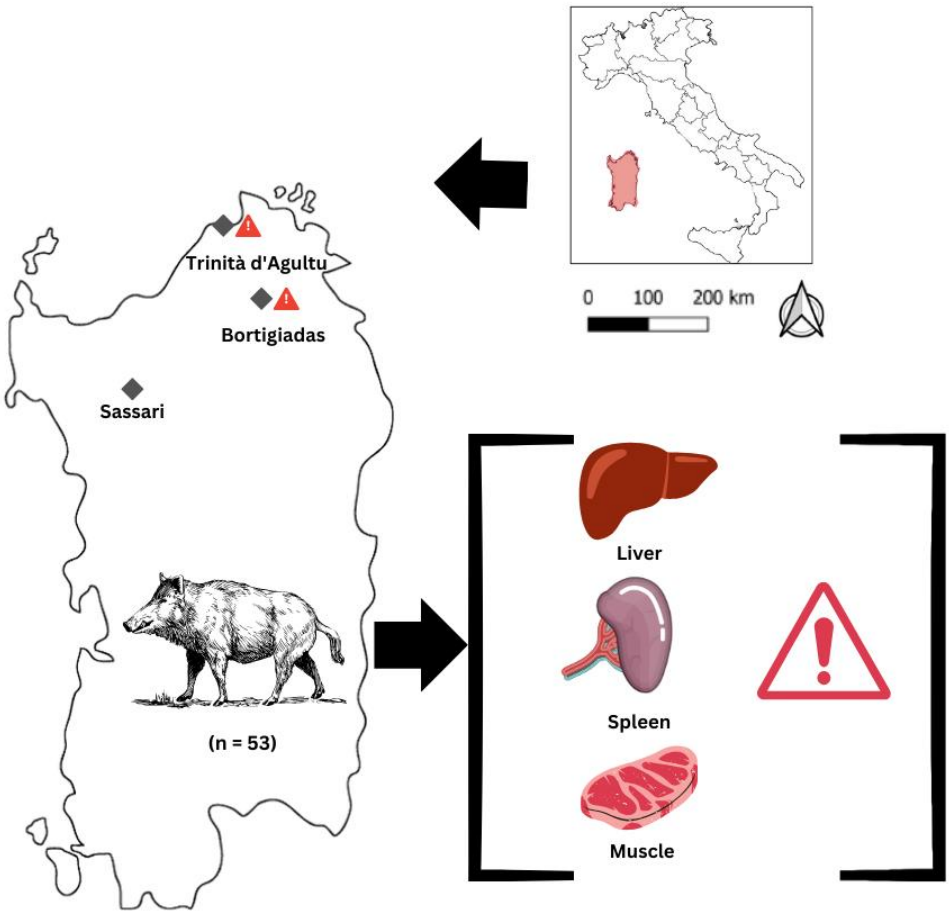

Figure 1: Map of Sardinia showing the location for the hunting grounds from where wild boar sampling was done. Hazard sign indicates unusually high levels of Pb in few of the wild boar samples from Bortigiadas and Trinità D'Agultu. It was also seen that the liver, spleen and muscle tissues were unsafe for consumption in more than 60% of the samples.

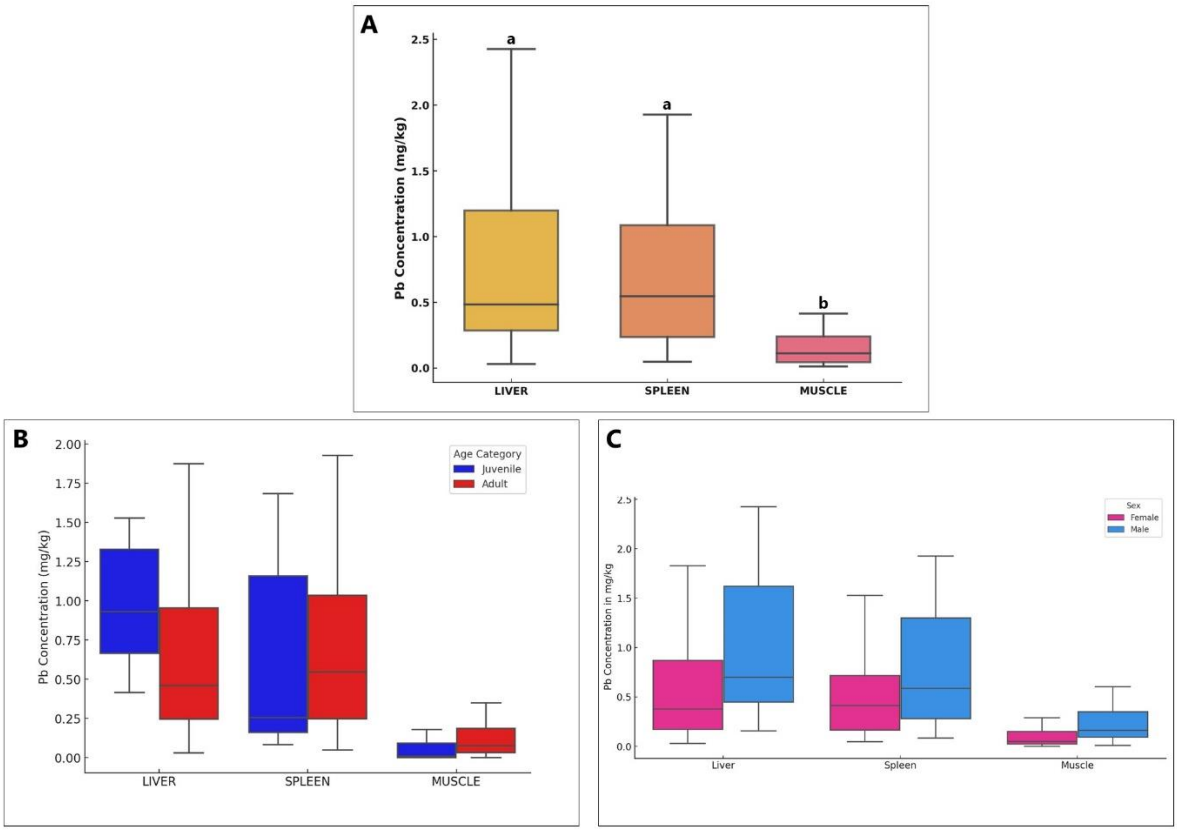

Figure 2: Mean concentration of lead (Pb) in the studied tissue samples for: pooled data (**A**), sex (**B**) and age groups (**C**). The boxplots are presented for non-outlier observations. The letters indicate statistically significant differences. No differences were observed among sex and age groups.

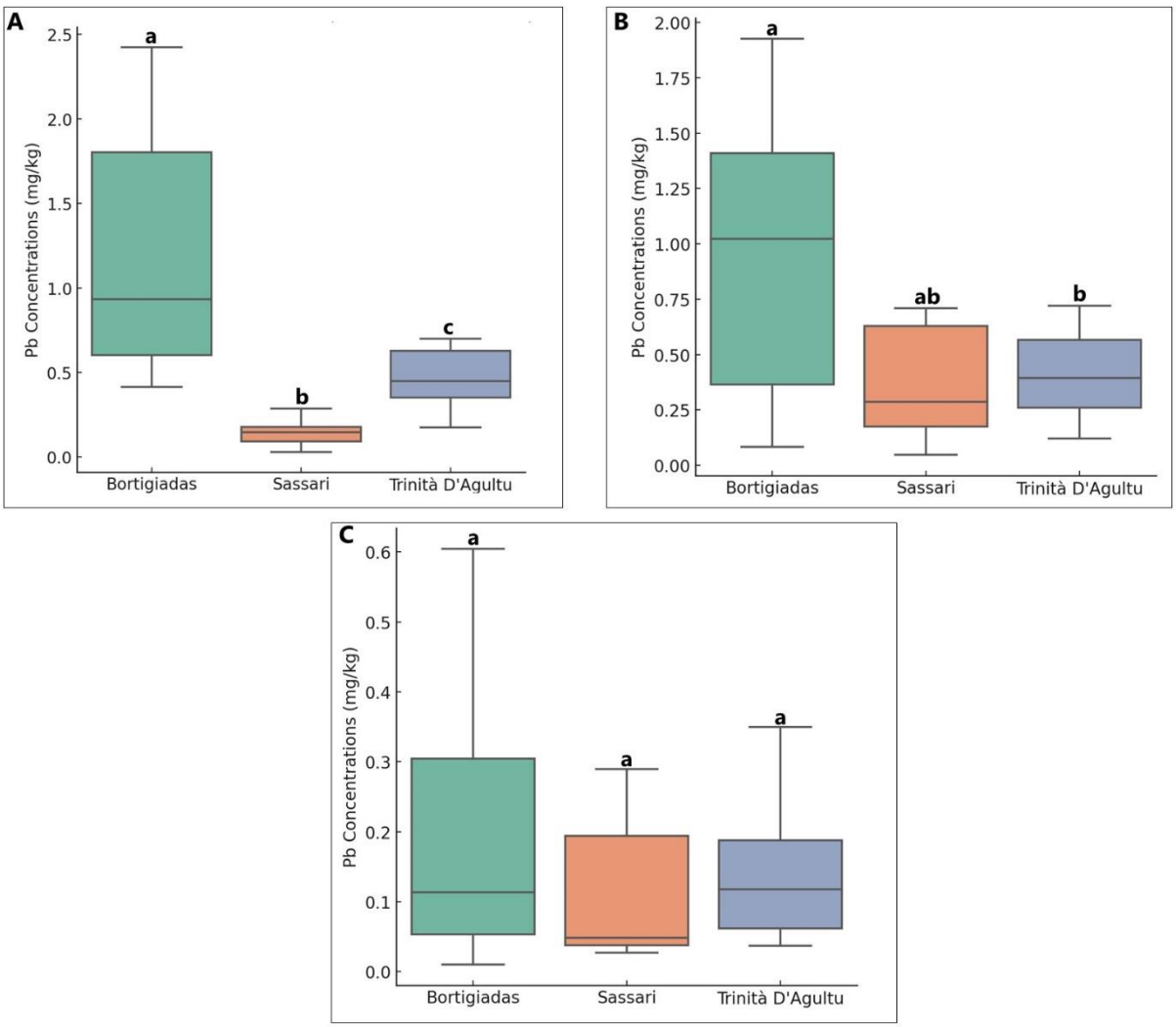

Figure 3: Mean concentration of lead (Pb) in the studied tissue samples across the sampling locations of northern Sardinia (Bortigiadas, Sassari and Trinità D'Agultu) in the liver (**A**), spleen (**B**) and muscle (**C**). The boxplots are presented for non-outlier observations. The letters indicate statistically significant differences.

Table 1. Concentration of Pb (mg/kg dw) in liver, spleen and thigh muscle of wild boar expressed as mean and standard deviation (M±SD), minimum and maximum (min-max), and median.

			Liver	**Spleen**	**Thigh muscle**
Sex	**Male**	Samples	26	26	22
		M±SD	1.29 ± 1.85	1.66 ± 2.78	0.76 ± 1.25
		Min-Max	0.157 - 9.63	0.083 - 11.8	0.011 - 4.27
		Median	0.664	0.736	0.179
	Female	Samples	27	27	22
		M±SD	11.92 ± 48.73	8.43 ± 31.13	4.58 ± 16.40
		Min-Max	0.030 – 251.19	0.048 – 161.85	0.019 - 85.7
		Median	0.452	0.546	0.173
Age group	**<1 year**	Samples	9	9	6
		M±SD	1.33 ± 0.69	1.76 ± 3.96	0.27 ± 0.30
		Min-Max	0.387 - 2.414	0.083 – 9.69	0.011 – 0.969
		Median	0.933	0.725	0.147
	>1 year	Samples	44	44	38
		M±SD	7.83 ± 38.25	5.79 ± 24.50	3.05 ± 12.86
		Min-Max	0.030 – 251.19	0.048 – 161.85	0.019 - 85.67
		Median	0.484	0.604	0.183
Overall statistics		Samples	53	53	44
		M±SD	6.70 ± 34.87	5.11 ± 22.36	2.67 ± 11.74
		Min-Max	0.030 – 251.19	0.048 – 161.85	0.011 - 85.76
		Median	0.569	0.623	0.179

Table 2. Estimated exposure and margin of exposure (MOE) for the cardiovascular and renal endpoints evaluated for Pb concentrations in liver and muscle.

	Estimated daily intake (µg/day)	**Endpoints**	**Margin of exposure (based on 200g game meat consumption per day)**	**MOE-rare consumption (2 game meals/year)**	**MOE-high consumption (10 game meals/year)**	**MOE-extreme consumption (90 game meals/year)**
Liver	1340	Cardiovascular effects	0.067[a]	12.3	2.45	0.27[a]
		Nephrotoxic effects	0.028[a]	5.15	1.03	0.11[a]
Muscle	534	Cardiovascular effects	0.17[a]	30.7	6.15	0.68[a]
		Nephrotoxic effects	0.07[a]	12.93	2.58	0.29[a]

[a] MOE<1.

General discussion and conclusions

There is a great ecological and global public health risk concern related to the contamination in the ecosystems due to the toxicity of heavy metals. The mobilization and transport of these metals in the environment has greatly accelerated since the beginning of the industrialization era (Merian, 1983). Lead (Pb) is one of the most hazardous and cumulative environmental pollutants that affect all biological systems with no known function in the cellular processes (White et al., 2007; Healey, 2009). It is one of the most studied toxicants due to its well-known toxicosis in animals and humans (Healey, 2009). Known sources of Pb exposure are paints, plumbing materials, golf balls, fishing tackle, bullets (ammunition), mining waste, batteries, and other electronic components (Humphreys, 1991; White et al., 2007; Pokras & Kneeland, 2008). Diffuse environmental contamination can occur from multiple point sources qualifying it as a multimedia and global environmental pollutant (White et al., 2007).

Even at very low levels, Pb causes significant disruption to the normal physiological processes of animal bodies (Buekers et al., 2009). Accumulating in different links of the trophic chain, Pb and its compounds are highly persistent and non-biodegradable (Dobrowolska & Melosik, 2008; Flora et al., 2012). Within the context of human health, the World Health Organization (WHO) states that "there is no level of exposure to lead that is known to be without harmful effects" especially in children and women of reproductive age (WHO, 2021). In animal bodies, it causes irreversible health effects interfering with a number of body functions and primarily affecting reproductive, renal, hematopoietic, hepatic, excretory, and central nervous systems (ASTDR, 2017; Flora et al., 2012). Other effects include hearing defects, cardiovascular issues, teratological impairments, behavioral abnormalities, immunotoxicity, impaired cognition, and neuromuscular weakness (Healey, 2009; Flora et al., 2012). Pb has also been implicated in carcinogenicity by inducing direct DNA damage, oxidative damage to DNA, inhibition of DNA synthesis/repair, or epigenetic involvement in gene expression by substituting zinc (Zn)

(Silbergeld et al., 2000).

There are a variety of sources where Pb ends up within the food webs. One of the major sources of contamination is the unregulated use of ammunition for killing the big game species in European countries. Estimates from the European Chemical Agency state that nearly 21,000 tons of Pb are annually released into the environment which is associated with the Pb-based rifle bullets (ECHA, 2018). There is an abundant amount of evidence that provides striking cases of Pb poisoning in game animals and birds due to hunting and shooting activities worldwide (Buekers et al., 2009). Many avian and mammalian predatory and scavenging species ingest Pb bullets or gunshots along with their food including viscera discarded by hunters or prey upon animals that have ingested gunshots in their digestive tracts. Predatory species may also take up Pb from an injured animal sustaining a gunshot carrying the pellets in its flesh (Pain et al., 2010). Such poisoned animals are an additional interface where human exposure occurs to Pb. Radiographs have revealed widespread metal fragment dispersion around the wound channels in hunted game which is consumed by the hunters and their families (Dobrowolska & Melosik, 2008; Hunt et al., 2009). Several tens or in some cases, several hundred folds higher Pb levels have been found in large game carcasses in comparison to admissible levels in meat (Santiago et al., 1998; Lazarus et al., 2005; Dobrowolska & Melosik, 2008). Livers, kidneys, and muscles of most mammalian game animals have shown Pb contamination (Dip et al., 2001; Kramárová et al., 2005) indicating bullet-derived Pb exposure. Lead (Pb) poisoning in the birds, identified from decades of research, has paved the way for a ban on Pb shots in waterfowl hunting, however, similar recognition has not been given to rifle-based game hunting because of lack and incoherence of data for these species. This calls for upscale studies from multiple areas to identify risks associated with rifle hunting. Additionally, risk estimates done via domestic food chain modeling have revealed a sufficient amount of risk to humans from the livestock ingesting Pb-contaminated forage. Soil-plant

continuum has been shown to accumulate a high amount of Pb leading to its bioavailability for the animals at higher trophic levels (Kim et al., 2016).

Amid growing concerns due to Pb toxicity and its adverse effects on human health, a variety of sentinel organisms have been proposed for monitoring the bioavailability and routes of contamination in the ecosystems. Pb bioaccumulates in animals as its rate of absorption is greater than the rate of elimination. In this scenario, parasites, particularly cestodes, and acanthocephalans, can act as ideal bioindicators for environmental contamination by Pb (Sures et al., 2000; Sures et al., 2003; Torres et al., 2004; Teimoori et al., 2014). There have been studies on the ways in which various forms of pollution affect the distribution and abundance of parasites and the synergistic effects of pollution and parasitism on the health of the host. However, in an alternative approach, parasites are used as accumulation indicators of contamination in the environment. An ideal sentinel species is ubiquitous and abundant, widespread, and has the capability to accumulate pollutants at a magnitude higher than their hosts (Sures et al., 2003; Sures, 2004; Torres et al., 2004; Teimoori et al., 2014). Criteria for a good accumulation indicator of metallic pollution require the sentinel organisms to have well-studied physiology and ecology in relation to the integration of environmental pollutants at different developmental stages, seasonal responses to metal toxicity, and assimilation of pollutants over long periods (Sures, 2004). Cestodes and acanthocephalans are ideally suited to elucidate and evaluate the extent of contamination in aquatic and terrestrial habitats because of their resistance to the highest possible metal levels in the environment and sufficient body size for tissue analysis (Sures et al., 2017). Ingestion of contaminated food by the host has a direct influence on the intestinal parasites which can manifest a higher pollution load than the host (Sures & Siddall, 2003) providing valuable information about the chemical state of the environment. Due to varying levels of input, bioavailability of the toxins at different times of the year cannot be determined and the parasites serve as ideal indicators of this contamination

(Sures et al., 1999). Changes in environmental metal exposure can rapidly be manifested by cestodes and acanthocephalans which are common and widespread parasites of fish (Siddall & Sures, 1998) and mammals. Duration of environmental exposure can be calculated by comparing the metal concentrations in the parasites and host tissues as metals are taken up readily by the parasites. A low ratio (high metal levels in parasite and host tissues) indicates a longer exposure duration than the time when toxicant levels are low in the host tissues and higher in the parasite (Siddall & Sures, 1998; Sures et al., 1999).

Most of the studies involving biomonitoring of metals employing parasitic species have been done on fish and their cestode and acanthocephalan parasites, however, understanding metal toxicokinetics in terrestrial host-parasite ecosystems has not been given much attention. Environmental Parasitology is relatively a new avenue in ecotoxicological studies where parasites are being employed as pollution indicators to understand the metal toxicokinetics and future research must be directed to explore the integration of environmental pollutants in the mammalian species, along with their parasites, which are specifically consumed by the humans. Mammalian helminth parasites can serve as model organisms for the risk assessment of heavy metals like Pb in terrestrial environments. Studies can be conducted on the cestode and acanthocephalan parasites of mammals to determine the experimental ratio between the equilibrium metal level in the parasites and the exposure concentration (bioconcentration factor, BF). Once such estimates are done, a similar host-parasite system could be used in multiple areas and sites to evaluate the extent of metal contamination.

There are multiple fronts where our lack of understanding hinders the employment of parasites in ecotoxicological studies. Therefore, future research should focus on exploring the mechanisms underlying parasite-pollutant interactions, including the role of parasite physiology, behavior, and ecology in accumulating and responding to pollutants (Marcogliese, 2005; Poulin, 2010). Investigating the response of parasites towards different contaminants

across diverse ecosystems and host-parasite systems can help in understanding the bioindication potential of the parasites. Additionally, basic research is needed to better understand how pollutants are taken up, stored, and detoxified by parasites. While there has been some research on metal accumulation, studies on micropollutants and nanoparticles remain limited, especially in marine host-parasite systems and in bird and mammalian hosts. Moreover, few studies have explored how parasites can act as sinks for organic pollutants, with early research suggesting they may reduce pollutant concentrations in infected hosts and influence host physiology. To deepen this understanding, host transcriptome studies can identify target genes and metabolic pathways affected by both stressors and parasites. Future research should also aim to sequence more genomes of relevant hosts and parasites to improve transcript interpretation, while next-generation sequencing could help assess parasite biodiversity through environmental DNA (eDNA) and metabarcoding techniques.

The complex relationships between parasites, hosts, and environmental stressors can only be unraveled when climate change and its effects are studied in detail with regard to parasitic communities in different ecological settings. So future applications should contextualize monitoring habitat quality, tracing pollutant dispersal, and assessing ecosystem resilience (Kutz et al., 2014; Lafferty et al., 2015). To date, there are only a few studies that have addressed the effects of stressors, such as temperature, in multiparasite and multihost settings. These studies show that host responses are complex and sometimes contradictory due to the interactions between species and the presence of parasites, which can significantly affect free-living communities. Studying a single host species is insufficient to completely comprehend the consequences of parasites at the ecosystem level thus requiring more research into this field.

The impact that parasites have on their hosts is influenced by their diverse life strategies. For example, parasites that passively absorb nutrients, such as adult cestodes and acanthocephalans, differ significantly from those that actively feed on host tissues, such as nematodes and

monogeneans. These feeding strategies also reflect the parasites' trophic positions. Additionally, the impact on hosts often depends on the developmental stage of the parasite, with larval parasites in intermediate hosts generally causing more harm than adult parasites in definitive hosts. These differences not only exist among major parasite groups but can also occur within closely related species. Therefore, studies should use parasites with varying life strategies under similar conditions, with precise descriptions of their life cycles. Additionally, there is a need to develop standardized protocols for parasite sampling, identification, and analysis to facilitate large-scale monitoring efforts to strengthen comparisons across ecosystems. Practically, informed decisions and effective management strategies can only be devised if parasitologists, ecologists, and environmental scientists come together to realize the potential of parasites as bioindicators. Testing all these assumptions in real-world conditions, particularly in areas undergoing ecological restoration, will help verify their accuracy. Integrating parasite bioindication with traditional monitoring approaches will provide a more comprehensive understanding of ecosystem health and pollution impacts.

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