UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

DIPARTIMENTO DI

MEDICINA VETERINARIA E PRODUZIONI ANIMALI

DOTTORATO DI RICERCA IN "SCIENZE VETERINARIE"

XXXV CICLO

COTUTELA UNIVERSIDAD DE LEÓN

DOCTORADO EN "CIENCIAS VETERINARIAS Y DE LOS ALIMENTOS"

ANALYSIS AND EVALUATION OF ECOSYSTEM SERVICES AND THEIR ROLE FOR THE CONSERVATION OF BIODIVERSITY AND SOCIO-ECONOMIC BENEFITS IN THE AGRO-ECOSYSTEMS OF THE MEDITERRANEAN BIOGEOGRAPHICAL REGION

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GENNAIO 2023

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ABSTRACT

The work carried out during the XXXV cycle of the PhD in Cotutela between the University of Naples Federico II (Italy) and the Universidad de León (Spain) involved Dr. Nadia Piscopo in Italian and Spanish scientific teams. In a common context such as the Mediterranean Biogeographical Region, our thesis wanted to demonstrate that the different components of the agro-ecosystem play an active and passive role in providing Ecosystem Services or Disservices. The wild boar model studied between 2019 and 2022 involved one of the sites present in the Natura 2000 network. The presence of wild boar (Sus scrofa), a species considered one of the most invasive on the planet, in a Special Conservation Area/Site of Community Interest (SCA/SCI) affects the biodiversity present, both of particular conservation interest and on that considered least concern. This situation involves the sites present in the Natura 2000 network in all 27 countries of the European Union. Almost all of the existing bibliography reports the wild boar as a demographic surplus and as an agent responsible for damage to agriculture and, lately, for road accidents and invasion of urban centres. The innovative element that distinguishes the experimental research work consists in demonstrating that, together with the ecosystem disservices produced by the wild boar to human activities, there are a series of ecosystem services that the suidae provides to man and the ecosystem. The first part of the work was carried out to determine a numerical estimate of the wild boars present in a sufficiently large area. We then proceeded to identify an area of approximately 13,000 hectares defined as the "PSR Area" and, within this, a smaller area called the "Focal Area".

The vegetation characteristics and biodiversity were described by the Standard Data Form of the SCA/SCI. Together with other operators, the areas of interest were checked with direct and indirect census methods. The results obtained made it possible to define a population of wild boars residing in the PSR Area equal to 244 specimens (average of the surveys between the years 2019 and 2022). The experience gained in the Regional and private Game Reserves of Castilla y Leon has made it possible to use the mathematical method of hunting effort (CPUE). The datum calculated thanks to field data produced a result comparable to that experienced in the field (264 specimens obtained from the average of the years observed). The negative effects consisting in damage to agriculture (413,950 Euros) and road accidents (25,000 Euros) were provided by the Campania Region and represented the items relating to the disservices caused by the wild boar. The positive effects consisting in the provisioning provided by the shot of wild boars in the PSR area (242,316 Euros) and the estimate of cultural factors (280,560 Euros) were calculated on an experimental basis and represented the Services connected to the presence of the wild boar. The resulting difference showed a weight in favor of disservices (-83,926 euros). This result could be reversed when the definition of the influence of wild boar on other living species (plants and animals) will be completed. At the moment, it has not been possible to quantify the monetary value of the biodiversity present in the habitat under study (Habitat/Biodiversity). However, direct observations have allowed us to ascertain the presence of 3/22 species indicated in the art. 4 of Directive 2009/147/EC. If we consider that there are 10 bird species described in the Natura 2000 site, the percentage increases to 30% (3/10). Another 17 species were observed including 10 birds and 7 mammals. Among the mammals, the description of the presence of Canis lupus was important. Three domesticated animal species frequented the same habitats as the animals listed above.

1. INTRODUCTION

1.1 ECOSYSTEM SERVICES, A NEW VISION FOR THE SUSTAINABILITY

Esposito, L., Piscopo, N., Aloisio, A. (2022) I sistemi agro-zootecnici e i service ecosistemici, quali strumenti di pronto uso per la transizione ecologica, le persone, il pianeta e la prosperità. Libro Verde sulla Prevenzione Sostenibili (05) Quaderni sulla Sanità Pubblica. Ed. MIDA, Pertosa, Auletta ISBN 978-88-946717-4-2

In the "Green Book on Sustainable Prevention", the authors (Esposito, Piscopo & Aloisio, 2022) address some evolutionary aspects of our planet. Starting from the present and imagining the future of the sustainability concept. Agenda 2030 proposes specific solutions using social/economic sectors and finally analyzing the effects and transformations achieved.
The Third Millennium began with invading human societies ("citizens of the world"), through an uncontrollable technology opulence, mainly linked to information technology and telecommunications. The development of new knowledge has brought about radical global changes in political and economic choices which are often identified with denominative forms that have repercussions in the common lexicon both on an intralinguistic and on an interlinguistic level. The harmonious development of the language also depends on the responsible use that is made by "authoritative people", ensuring that each citizen becomes aware of the individual behavior necessary to guarantee the survival of a correct environmental balance (the environment is a good for everyone). A living language, in good health and capable of adapting correctly to historical changes, will also allow the new generations to learn more about their past in order to be able to live the future effectively and incisively (Adamo & Della Valle, 2019). However, some definitions that are used in modern jargon are rooted in intellectual currents that have developed over the last two centuries. For example, the term "Biodiversity", which is sometimes abused, was used for the first time in 1988 by the American entomologist Edward Osborne Wilson (National Academy of Sciences, 1988) and indicates the variety of living beings on Planet Earth (biosphere): plants, animals, microorganisms, genes, complex ecosystems. Similarly, we will have to wait until 2005 and the publication of the "Millennium Ecosystem Assessment" project (MEA, 2005) to formalize the conceptual definition of "Ecosystem Services".

1.2 ECOSYSTEM SERVICES AND THE 2030 AGENDA

In general, an environment where life develops (ecosystem) is able to provide biodiversity, including man, with "direct benefits" and "indirect benefits" which are classified by the MEA into four categories: 1) provisioning; 2) regulating; 3) cultural; 4) support. This classification becomes complex when trying to quantify and economically evaluate the different ecosystem services. In 2009, the Institute for European Environmental Policy (Cooper, Hart and Baldock, 2009) indicated that services usable by all individuals and whose use by one individual does not reduce the availability for others, could be considered public and were grouped into "non-provisioning" services (regulating, cultural, supporting). The complexity of the services offered by the ecosystem and the difficulty of describing the benefits from an economic point of view leads the project "The Economics of Ecosystems and Biodiversity" (TEEB, 2010) to include the supporting services in a separate category, defined "Habitat and supporting", which includes the services of "Habitat for species" and "Maintenance of genetic diversity". Currently, unfortunately, the only services that are easily measurable and quantifiable from the point of view of economic value remain the procurement services (provisioning) which, being privatizable, respond to market systems and are easily monetised. Even the need to safeguard Planet Earth is not a recent alarm. Already in 1987 the World Commission on Environment and Development (WCED) and the United Nation Environment Program (UNEP) in the Report "Our Common Future" (WCED, 1987; Report of the World Commission on Environment and Development, 1988) introduce the concept of "sustainable development" (Brundtland Report) defining it as "development that satisfies the needs of the present generation without compromising the ability of future generations to satisfy their own".

Subsequently, during the "Earth Summit" held in Rio de Janeiro in 1992 (United Nations, 1992), the political bases were defined for the realization of sustainable development through the "Action Plan for the XXI Century" (Agenda 21).
The commitments made and the definition of the steps necessary to be able to proceed towards sustainable development were renewed in the Rio+20 Conference of 2012 (United Nations, 2012) while, in 2015, the United Nations General Assembly adopted «the Agenda 2030 for sustainable development» within which the guidelines to be adopted at a global level are clearly identified through an action program for people, the planet and prosperity (Andreoni & Miola, 2016). The 17 Sustainable Development Goals (SDGs) take into account the need to support universal peace, freedom and the eradication of poverty in a major action program which achieves, through 169 targets, the sustainable transformation of society, of the economy and environment by the year 2030 by monitoring results with 240 indicators (United Nations, 2021a; b). In this meaning it is evident that the objective proposed more and more urgently is that of inverting the paradigm of the currently dominant model of "unsustainable growth" (unbridled consumption of renewable resources, depletion of non-renewable resources, waste of materials, invasion of residues material cycles, pollution, corruption, crime) towards a "model of sustainable growth" at all levels (global, national, regional, urban) based on an integrated vision of the various dimensions of development (economic growth, protection of the environment, human and social rights) in order to preserve the planet for future generations both from an ecological point of view and from a social and civil point of view. The structure of the "Agenda 2030" action program is based on three dimensions (MiTE, 2022) which aim to obtain positive results on the "Biosphere" (SDGs 6, 13, 14, 15), on the "Society" (SDGs 1, 2, 3, 4, 5, 7, 11, 16) and on the "Economy" (SDGs 8, 9, 10, 12). The pyramid structure of the program indicates that only by achieving the objectives linked to the Biosphere will those linked to Society and, therefore, the economic ones be supported, to the benefit of the current biodiversity present on Planet Earth (fig. 1): "there cannot be a healthy economy in absence of a healthy society; society cannot be healthy if the environment is not healthy" (UN, 2021).

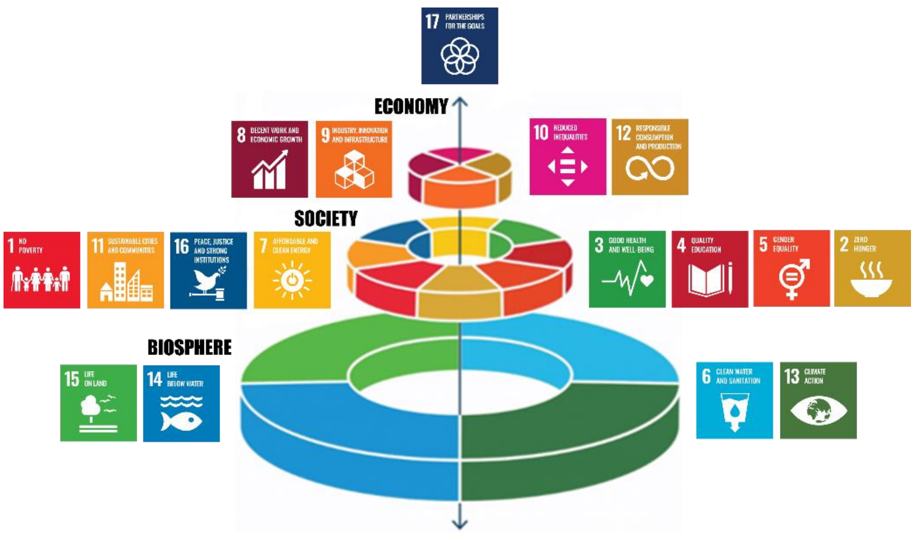

Figure 1. Agenda 2030 goals developments.
(https://www.mite.gov.it/pagina/il-contesto-internazionale-l-agenda-2030; United Nations, 2021)

1.3 The Ecosystem at the service of the planet

It appears evident that the future activities of mankind must be closely linked to the compatibility between economic development and environmental protection. It is precisely in this context that the "Ecosystem Services" enter which, however, are still struggling to find a classification that is able to economically quantify and map, in a clear way, the services that ecosystems provide to biodiversity, man in the lead. In order to standardize and hierarchically classify ecosystem services for their quantification and economic evaluation, the European Environment Agency (EEA, 2010) has decided to identify the final services that derive from support services and has reduced the categories of three-way services (provisioning, regulating and maintenance). For the foreseeable future, the European Union has based its environmental policies on the concrete integration of ecosystem services in the sustainable development actions of the Euro-Union. It is no coincidence that the "EU Biodiversity Strategy 2030", in continuity with the EU strategy on biodiversity for 2020, consists of a long-term plan which, through specific actions and commitments, is able to reverse the degradation of ecosystems to bring Europe's biodiversity on the road to recovery by 2030. With the "Mapping and Assessment of Ecosystems and their Services" (MAES) initiative, the EU has asked the Member States to map and evaluate the state of ecosystems and their services in the various national territories and, through the "Common International Classification of Ecosystem Services" (CICES) provided guidelines and indicators to identify ecosystem types (fig. 2) and assess their status (https://cices.eu).

Categoria Servizio Ecosistemico	Servizio Ecosistemico	Esempio
Approvvigionamento ***Provisioning***	Alimenti	Tutti gli ecosistemi (agro-ecosistemi) in grado di produrre risorse trofiche o Alimentari
	Materie prime	Tutti gli ecosistemi in grado di produrre materiali (legno, oli, resine, fibre, rocce, etc.)
	Acqua	Tutti gli ecosistemi regolano il ciclo globale delle acque (flussi, depurazione, disponibilità)
	Medicamenti	Tutti gli ecosistemi forniscono materia per l'industria farmaceutica
Regolazione ***Regulating***	Clima e qualità aria	Gli ecosistemi influenzano le precipitazioni localmente e globalmente e regolando la qualità dell'aria
	Stoccaggio Carbonio	Gli ecosistemi sono in grado di sequestrare il Carbonio nel suolo e nelle piante
	Mitigazione catastrofi	Gli ecosistemi strutturati mitigano gli eventi estremi (incendi, frane, valanghe, alluvioni)
	Depurazione acque reflue	Gli ecosistemi riducono la concentrazione di nutrienti e inquinanti attraverso piante e microorganismi
	Prevenzione erosione Fertilità suoli	Gli ecosistemi forniscono nutrienti ai suoli e alle piante che contengono erosione e desertificazione
	Impollinazione	Gli ecosistemi funzionanti garantiscono la presenza di insetti e organismi necessari all'impollinazione
	Controllo biologico	Gli ecosistemi regolano la diffusione di organismi nocivi e delle malattie attraverso la competizione
Habitat e Supporto ***Habitat & Supporting***	Habitat di Specie	Gli ecosistemi forniscono tutti gli elementi necessari alla vita della biodiversità
	Diversità genetica	Gli ecosistemi garantiscono il mantenimento della diversità naturale e dei relativi geni
Culturali ***Cultural***	Ricreazione e salute	Gli ecosistemi offrono possibilità per le attività ricreative a beneficio della salute mentale e fisica
	Turismo	Gli ecosistemi consentono diverse forme di turismo e indotti economici diretti e indiretti
	Estetica e ispirazione	Gli ecosistemi e i paesaggi e la biodiversità ispirano arte, cultura e ricerca scientifica
	Esperienza spirituale Senso di appartenenza	Gli ecosistemi con le foreste, le montagne, le caverne, sono considerate sacre o hanno significati spirituali. Contribuiscono al benessere mentale e al senso di appartenenza

Figure 2. Classification of Ecosystem Services according to TEEB (modified).

1.4 ECONOMIC GROWTH AND ECOSYSTEM SERVICES

The wealth and economic growth of a nation are strongly influenced by the capital it owns (stock) and by the way in which this is managed and invested in order to increase and improve the individual and collective well-being of present and future generations.

The capital of a nation is made up of different types of resources which, over time, have been recognized as assets that have contributed to the prosperity of some peoples (Natural Capital Committee, 2017). The economies of the countries and the public opinion have no difficulty in recognizing the Manufacturing Capital (machinery and real estate); Human Capital (people with their skills and knowledge); the Social Capital (set of rules, institutions and values that regulate the interactions between both public and private entities); Financial Capital (currency and all the financial instruments that allow it to be invested). Over time, all these types of capital have been identified, quantified and recognized as fundamental for increasing a country's development capacity. Not in the same way has a global economic value been given to Natural Capital (functionality of the ecosystems on which human life depends and from which natural resources and raw materials for the economy and human development are obtained) (World Bank, 2006). The UK Natural Capital Committee (2013) specifies that "Natural capital includes the entire stock of natural assets - living organisms, air, water, soil and geological resources - which help to provide goods and services of direct or indirect value to man and that they are necessary for the survival of the very environment from which they are generated". It follows that Natural Capital is a stock quantity and therefore it is identifiable with the physical or monetary value of all the elements that compose it at

a given moment. Using the ecosystem approach promoted by the Convention on Biological Diversity (U.N., 1992) it is possible to classify the different components of the planet into "biotic" and "abiotic" components. The biotic components include all terrestrial and marine ecosystems, with the flora and fauna (biodiversity) they contain (AA.VV., 1956-2015; AA.VV., 2001-2009; Minelli et al., 2002 ; AA.VV., 2003; AA.VV., 2004; AA.VV., 2005; AA.VV., 2011; Audisio, 2013; Audisio et al., 2014), while abiotic components are minerals, metals , fossil fuels, but also air, wind or solar energy (Daly and Cobb, 1989; FAO, 2016; UK Natural Capital Committee, 2013; UK Natural Capital Committee, 2014; UK Natural Capital Committee, 2015). It is also essential to underline that, while almost all biotic components are renewable, abiotic components can be both non-renewable (minerals, energy from fossil fuels) and renewable (solar energy) (Costanza and Daly, 1992).
A further classification of Natural Capital is based on the type of source from which the resources derive:

Soil	(forests, flora and fauna, soil microbes, etc.);
Subsoil	(minerals, fossil fuels)
Water	(rivers, lakes, oceans, groundwater, and marine flora and fauna)
Atmosphere	(air and climate elements)

The set of constituents of Natural Capital (stock) produces a flow of services which, finally today we identify as "Ecosystem Services" (De Groot, 1992). Since man obtains a continuous flow of benefits from the environment that are necessary for his own life, as well as for the production of goods and services, for their consumption but also for the usability of free time, the value of the flow of Ecosystem Services also defines the value of the Natural Capital stock from which they are generated. From Natural Capital we obtain, for example, air to breathe, water to drink and to grow, energy from the sun or from fossil fuels, genetic diversity for food and medical and industrial research, fish fauna for to feed us, textile fibers to produce clothes, a mountain landscape or an urban park for walking, the systems of plants and micro-nutrients in the soil that protect us from hydrogeological instability, bacteria for the natural purification of water, the biodiversity of insects necessary to pollination. All these benefits, in order to be measured and assigned in qualitative and quantitative terms to the assets that produce them, are classified in comprehensive categories. The classification of "Ecosystem Services" reported by TEEB (2010), which can be linked through CICES (2016) with the classifications originally proposed by the Millennium Ecosystem Assessment (fig. 5), indicates the following categories whose common basis is provided by the “Support Services”:

- ***Provisioning/sustenance*** of food, materials and energy obtained from ecosystems.
- ***Regulating*** of the functioning of ecosystems.
- ***Cultural***, associated with the benefit obtained from recreational uses of ecosystems or natural assets.

«Ecosystem Services» can be provided exclusively by Natural Capital (raw materials, water, food) but, in most cases, they are obtained in complementarity with other types of capital and production factors: this is an example the case of agriculture is clear. Food production depends on the type and quality of the soil and the climate but is significantly influenced by the use of agricultural machinery, the skills of those who optimize the functioning of the machinery, as well as the skills of the farmer who uses them. The agriculture example allows us to clearly see how by replacing the types of capital it is possible to obtain a result opposite to the "Service" or cause a "Disservice". In fact, substitution can be responsible for the onset of environmental sustainability problems: for example, soil fertility can be increased with the use of chemical fertilizers rather than through the regulation offered by natural nutrients and this substitution, causing an excess of chemical elements in the soil, is responsible for the pollution (excess of nutrients) of the soil and groundwater. However, as the science of ecological economics demonstrates (Comitato Capitale Naturale, 2017 Annex A), it is possible to state that without Natural Capital there is no human well-being since some assets (air, water, soil, oceans, biodiversity) are unique, not replaceable and constitute the essential basis for human life and needs (Costanza et al., 1997). Ecosystem Services therefore represent the tool that allows you to manage (use), while preserving (resilience), the set of constituents of the Natural Capital (stock) in a dynamic and evolutionary state capable of providing services in the present, ensuring them also in the future. However, if this vital and essential function in support of human activities is recognized, the importance of "Ecosystem Services" is still largely ignored today because many of these services, not being traded on the market, do not have a price that indicates their social value (reduced or no monetary valuation).

1.5 PAC and the European models for sustainability

Although there is a generalized recognition of the positive influences exerted by ecosystem services on all human activities, there is still a difficulty in implementing and inserting the conceptual and operational schemes in other community policies (Bouwma et al., 2018; Allen, 2020). However, an attentive observer of Community Agricultural Policies (CAP) will not have escaped the fact that from the initial actions of economic support tout court to the productions of the primary sector (Agriculture, Forestry and Animal Husbandry) we have moved on to the current revisions which include saving and conservation strategies such as , for example the “Farm to Fork” strategy which aims to accelerate the transition towards a sustainable food production system for humans (EC, 2020; Meredith et al., 2021). In fact, there is a trend towards a holistic system that can be integrated within the "European Green Deal" (EC, 2018). The European Commission calls for the adoption of proposals capable of transforming the EU's climate, energy, transport and taxation policies so as to reduce net greenhouse gas emissions by at least 55% by 2030 compared to the levels of the 1990. The "Farm to Fork" strategy is at the heart of the European Green Deal (EGD) with the aim of making food systems fair, healthy and environmentally friendly. It is extremely clear that we need to redesign the food production and food consumption systems that today account for a third of global greenhouse gas emissions, consume large quantities of natural resources, cause biodiversity loss and negative impacts on health (under- or over-nutrition) and do not allow fair economic returns and livelihoods for all actors, especially primary producers. The success of the "Farm to Fork" strategy depends on the ability of those who manage agro-forestry-pastoral resources, both from a political point of view (land management bodies) and from an entrepreneurial point of view (farmers, foresters, breeders) and from a technical point of view (agronomists, zoonomists, biotechnologists, veterinarians), to accelerate the transition from the current food system of Homo technologicus towards a sustainable food system thus obtaining:

- a neutral or positive environmental impact;
- action to mitigate climate change;
- an adaptation of resources to the new impacts due to climate change;
- a reversal of global biodiversity loss;
- a control system aimed at food safety for the benefit of consumers;
- a new food scheme that guarantees nutrition and public health;
- a modern vision of food production systems;
- fair governance to ensure:
    - a) the accessibility of safe, nutritious and sustainable food products to all;
    - b) adequate competitiveness with satisfactory economic returns;
- fair control of the EU supply sector;
- a convinced promotion of fair trade and the importance of sustainability.

The objectives of the "Farm to Fork" strategy and the transformative role it will have for European agri-food systems are in line with all the Sustainable Development Goals (SDGs) of the 2030 Agenda and the direct and indirect effects that its implementation will be able to generate will greatly depend on the ability to transform theoretical objectives into concrete ecosystem services.

A concrete and ready-to-use tool to evaluate the environmental impact and the deriving ecosystem services is the "animal breeding" sector.

The third millennium must consider that most of the planet's ecosystems have undergone radical transformations due to agricultural and zootechnical activities (fig. 3).

Figure 3. Agro-ecosystem and animal breeding in the province of Benevento (San Giorgio la Molara).

1.6 Livestock and Agro-Ecosystems

At best, the transformation of the original environments has given rise to mixed systems that today we define as «agro-ecosystems». Agriculture and animal breeding are the anthropic activities which, over time, have created agro-ecosystems and which, through agro-zootechnical systems and management practices, obtain supply services for the Genus Homo, i.e. food for the man and for farmed animals (Ramanzin et al., 2021). The ecosystem services linked to agro-zootechnical systems have been well described (Zhang et al., 2007) and both the services that support agricultural and animal breeding activities and the services that are produced by man's activities on the so-called Utilized Agricultural Area (SAU). The impact of land management (agricultural processing, crops, canalization and use of water, grazing, etc.) can cause negative effects on the environment and agro-zootechnical systems can produce ecosystem disservices (fig. 4).

Figure 4 gives a graphic schematization of the "Services" and "Disservices" that the agricultural and zootechnical systems are capable of producing/providing in the environments they occupy. Currently, agriculture and animal husbandry benefit from direct services which, since they are monetarily well identifiable, are defined as «Services with market». At the same time, however, the activities carried out by anthropic practices associated with agriculture and animal breeding produce effects (services) that are difficult to quantify from a monetary point of view which, although of fundamental importance for the existence of the ecosystem, are defined «Services without market».

It therefore follows that agro-zootechnical systems produce a series of components useful to man (food, fibres, workforce, fuels) which, once supplied, are identified as «Provisioning Services». As in a cascade process, in order to be produced, supply services need the presence of a whole series of conditions (soil fertility, nutrient cycle) which are identified as «Regulating Services» and which, in turn, in order for continue to provide services and do not turn into disservices, they need «Support-habitat services» identified in the contemporary presence of biological diversity (natural diversity, domestic diversity such as farmed breeds and cultivated plant varieties).

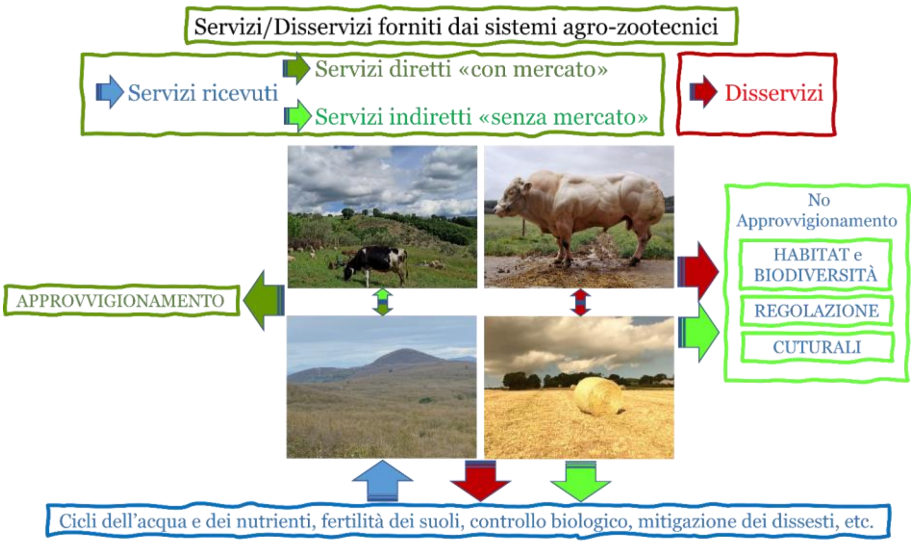

Figure 4. Agriculture, animal husbandry and ecosystem services (from Ramanzin et al., 2021 modified).

Very often the support, regulation, habitat and biodiversity, cultural services involved in the process of direct provision of services are also indirectly involved in "non-provisioning services". This results in services or disservices depending on whether positive or negative effects arise from the management methods and the type of agro-zootechnical system (Ramanzin et al., 2021). The Millenium Ecosystem Assessment (MEA, 2005) defines biodiversity as "the variability between living organisms and the ecological complexes of which they are part (terrestrial, marine and other aquatic systems); including the diversity of species, between species and of ecosystems". Making a parallel with biodiversity, we can define the agro-zootechnical system as "the variability between farmed organisms (plants and domestic animals), their compatibility with wild species and the ecological complexes of which they are part; including the diversity of species, between species and agro-ecosystems" (Esposito, Piscopo & Aloisio, 2022). Therefore, both in the case of biodiversity and in that of agro-zootechnical systems, only a multidimensional and multi-criteria evaluation of the various components of the inhabited system can provide indicative information on the structure and functioning of ecosystems or agro-ecosystems as well as the services provided in terms of sustainability (ISPRA, 2009). Biodiversity in a broad sense (living beings; the biological evolution process; the instrumental use of resources for human needs; cultural, artistic and engineering creativity; the landscape, etc.) therefore represents the operational basis of the preserved or modified ecosystems and therefore guarantees the provision of services which, precisely, are defined as ecosystem services (fig. 5):

1) Life support services (nutrient cycling, soil formation, photosynthesis);

2) Natural resource supply services (air, water);

3) Regulating services (climate, air and water quality);

4) Cultural services (recreation, aesthetic enjoyment, spiritual expressions).

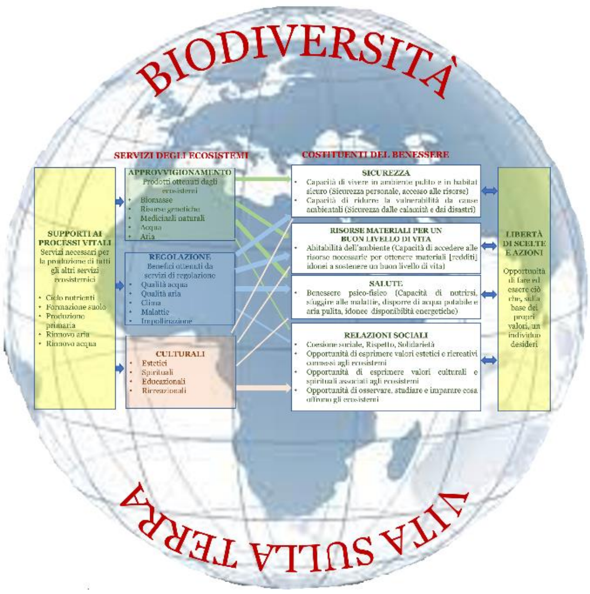

Figure 5. Relationships between biodiversity, ecosystem services and human well-being.
(MEA, 2005 modified; La Camera, 2009 modified)

1.7 THE AGRO-LIVESTOCK MODEL

The agro-livestock model, with its ability to produce primary goods, is able to describe the influence of the anthropic activities connected to it, positive or negative, on the environment and in the socio-economic field. This model is, therefore, able to describe the production of services or disservices that agricultural and animal breeding activities produce in a given environment. Ecosystems and agroecosystems provide, through their structures and processes including the work of man, services related to the ecological sphere, which, once used by society, become beneficial in the socio-economic sphere. The resulting value will determine the public and private choices of economic development and the various policies that will decide the management of the ecosystems themselves (Haines-Young and Potschin, 2010). Generally, the use of intensive practices (chemical fertilization, use of herbicides and pesticides) and an industrial management of agricultural crops (monocultures, horticulture, greenhouse crops) produce disservices on overexploited environments that outweigh the benefits obtained. Conversely, sustainable management of agricultural practices provides ecosystem services to the environments in which cultivated land is located and, even if to a lesser extent, to natural ecosystems. Objective 2 of the Agenda 2030 in terms of food security directs Governments to integrate national policies by incorporating Community policies that promote rural development towards sustainable actions in agriculture. To these actions is added the commitment undertaken at international level, to reduce greenhouse gas emissions [art. 4 of the Paris Agreement (COP21, 2015; COP26, 2021)]. In this context, knowledge of Ecosystem Services would help a lot in undertaking activities which, while continuing to guarantee the supply of human resources, would provide services useful for maintaining the ecosystem or, at worst, the agro-ecosystem with all the diversity they contain. A considerable help in the interpretation of the most important agro-ecosystem services is provided by TEEB (2010) which classifies them in the following areas:

(i)	**Provisioning/Sustenance**:	Supply of fiber and food, a key element for food security;
(ii)	**Regulating**:	Contribution to the conservation of soil fertility, pollination and water quality and for the role of containing greenhouse gas emission;
(iii)	**Habitat & Supporting**:	both agricultural and natural biodiversity.

In this regard, the Common Agricultural Policy (CAP) has recognized the importance of maintaining good environmental status in the agricultural sector, by virtue of maintaining and enhancing the supply of agro-ecosystem services in the cultivated areas.

In particular, the CAP has prepared a series of loans for "greening activities" or for all those activities that favor the formation of a link between the agricultural territory and the natural ecosystem in which it is inserted. Direct payments are intended for example:

- activities to enhance agricultural biodiversity through crop diversification;

- maintaining the quality of the soils and their fertility through the use of less invasive practices;

- the conservation of microhabitats, ecotones and natural corridors (Ecological Focus Area) within areas adjacent to crops that have been renaturalized or that have been destined for traditional agro-pastoral systems.

The role of agriculture in mitigating climate change has also been re-evaluated in the context of art. 4 par. 2 of the Paris Agreement (COP 21, 2015). Each "Party" or each State, must prepare, communicate and comply with a national contribution aimed at achieving the greenhouse gas reduction objective (Nationally Determined Contributions - NDCs). Since crops, livestock and land use changes account for more than 30% of emissions of anthropogenic origin, many of the NDCs presented by the Parties have included agriculture and its agricultural emissions among the mitigation tools for greenhouse gases greenhouse. The Report on Natural Capital, released jointly by the MATTM and the MiPAAF (Comitato Capitale Naturale, 2017), indicates that the inclusion of the agriculture, forestry and other land uses sector would allow Italy (through the related definition of policies and technical conditions that should have been completed by 2020) to contribute to the overall reduction of European greenhouse gases with a 40% reduction in national emissions by 2030 compared to 1990. The data published by ISPRA (2016) indicate that Italian agricultural emissions represent 7.2% of the national total, with a decreasing trend of 16.2% from 1990 to 2014 mainly due to the reduction in the number of reared animals and the increase in the collection of biogas from animal manure (about 10%). The National Inventory Report reports that, in 2014, the agricultural sector, on the total of national emissions, contributed by producing 42.7% of methane (CH_4); 61.7% nitrous oxide (N_2O); 0.13% carbon dioxide (CO_2). Although carbon dioxide is the best known gas for the greenhouse effect, overheating is mainly attributable to N_2O and CH_4, respectively 300 and 30 times more harmful than CO_2, for a Total Greenhouse Gas Emissions of 30.338 Gg CO_2-eq . If the agricultural sources responsible for the most significant greenhouse gas emissions are recognized in the categories agricultural soils, enteric fermentation, manure management, rice paddies and stubble combustion; the carbon sinks linked to the agro-zootechnical system are identified in multi-year woody crops (olive groves, vineyards, orchards, coppices) in which the accumulation in the biomass can be considered significantly positive unlike in annual crops where, at the end of the cycle, the biomass production and loss correspond to a zero balance. In order to tend towards a zero or negative balance, it is necessary to know the different storage capacities of greenhouse gases in soils. The margins for change in the final balance of emissions vary enormously with the variation of cultivation techniques and soil treatment (agronomic practices, trampling of grazing animals). If we evaluate, for example, the net emissions of CO_2 for the year 2014 (ISPRA, 2016) it can be seen that in grasslands these are less than 6.611 Gg while for crops the net emissions of carbon dioxide are 3.216 Gg, therefore much with the most impact (agriculture and land use sector, land use change and forestry). To obtain the containment of greenhouse gas emissions and tend towards a zero balance, studies (FAO and ITPS, 2021) report a series of indications that translate into good practices that favor the storage of carbon in soils such as, for example:

- the incorporation of crop residues into the soil rather than their combustion;

- the introduction of species with deep roots to stabilize and enrich the soils as well as to save water;

- the extension of crop rotations and crop diversification, also in consideration of the positive correlation that exists between biodiversity and carbon stocks.

Although the accounting of agricultural emissions has historically been carried out within the framework of the IPCC (Agriculture, Forestry and Other Land Use - AFOLU), there are still many gaps in the assessment of soil carbon stocks and in the ways of integrating them into climate policies. In this direction, Europe has recently introduced, with the LULUCF accounting decision (529/2013/EU), the obligation at Community level of the accounting of emissions and carbon absorption of cultivated lands and pastures (Natural Capital Committee, 2017).

1.8 AGRI-ENVIRONMENTAL SUSTAINABILITY INDICATORS

Territorial problems must be addressed through adequate planning of resource use actions as well as those of habitat and biodiversity conservation, supported by appropriate political-administrative choices. The results expected from the sustainable use of agro-ecosystems must be adequately monitored through environmental assessment (De Groot, 1992; Costanza et al., 1997).
To describe the sustainability of the agricultural system, it has been proposed to use a series of indicators (sustainability indicators of the agro-ecosystem, agri-environmental performance indicators, agri-environmental indicators) which provide statistical information useful for environmental assessment (Greco, 2002; ISPRA, 2009). According to the multifactorial and multilevel criteria already indicated in the description of "Ecosystem Services", the sustainability indicators must be able to describe and consider the relationships between the spatio-temporal, social, economic, environmental, institutional, etc. dimensions. occupying a given environment. The European Commission recommends using agri-environmental indicators to evaluate, with the statistical method, the results of the actions implemented and, if necessary, modify them with a new action plan (EC, 2003). European policies have paid close attention to the compatibility of agricultural-zootechnical production systems with natural and semi-natural ecosystems (agro-ecosystems) and with the biodiversity they contain. In this context, due importance has been given to landscape indicators such as, for example, the perimeter borders of the plots of land (length of the hedges; height of the dry stone walls; extension of the non-agricultural border areas) but also the incidence of structures of farms on their functioning and on the agro-ecosystem in which the company is included. At the same time, the indicators must translate the relationships between human activities and the environment in a quantifiable way (Conference of European Statisticians, 2001; Tellarini and Caporali, 2000) for which the O.E.C.D. (1999a, 1999b, 2001) has developed an international reference framework for the development of sustainability indicators called "Driving force - Pressure - State - Impact - Response" (Kristensen, 2004) (fig. 6).

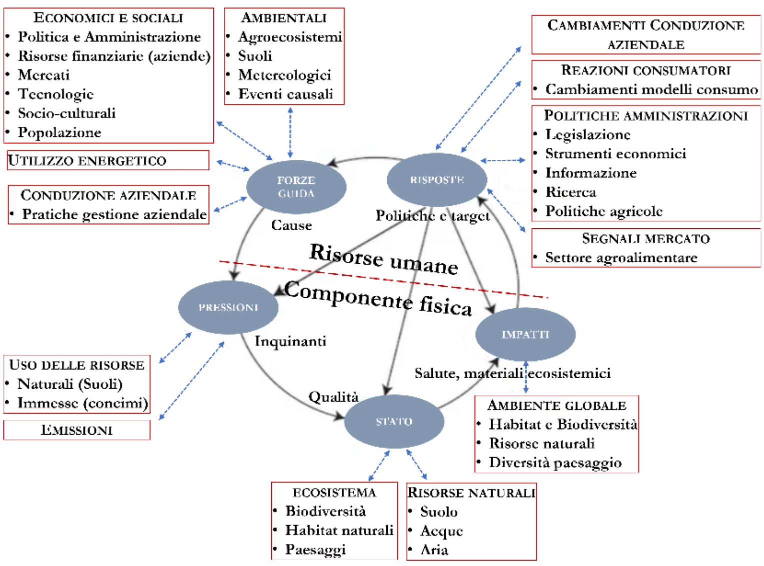

Figure 6. D.P.S.I.R scheme: identification of sustainability indicators (O.E.C.D., 1997 modified).

2. AIM OF WORK

2.1 AIMS AND PURPOSES

The analysis of the multiple variables that contribute to the determination of "Services" or "Disservices" is greatly influenced by the context in which one operates. Although the holistic approach dates back to the end of the 20th century, at present it is not yet possible to quantify all the ecosystem services provided by biodiversity. Our study therefore started from the identification of the different components of supply, regulating, habitat and biodiversity, cultural services already expressed previously. The special part, on the other hand, wanted to use a dynamic experimental approach in an attempt to apply a series of customized methodologies aimed at the possibility of quantifying, in monetary terms, the Service/Disservice associated with a species, the wild boar, which is seen only as a negative impact. Over the three years of work, the biotic communities present, in a specific territory were identified, quantified and contextualized. The updating and integration of field data has provided information that has proved to be a valid preparatory tool for solving complex wildlife and environmental management problems which, until now, have only been perceived as a disservice linked to the damage caused by the wild suidae.

The difficulty of managing the fauna heritage correctly derives from a series of critical points connected to a scarce or incomplete knowledge:

- the biology and habits of wild species that live in a habitat that no longer corresponds to the original one;
- deeply changed habitats and environmental dynamics as a function of the very high anthropic pressure and climate change.

The approximation of know-how translates, in a decisive way, into the inadequate use of this knowledge by the bodies responsible for management decisions.

A correct assessment of ecosystem Services/Disservices:

- ❖ must provide an interpretation of the phenomena of interaction between the environment, anthropic activities and animal populations (sedentary and migratory) that can occur over a suffucuously vast territory;
- ❖ it must facilitate the understanding of the general/particular status of the possible medium and long-term scenarios;
- ❖ it must show, clearly and through a complete analysis of the biocenoses, the possible actions, the ways for solving management problems that from time to time the planning bodies identify and decide to face.

The experimental work therefore proposes a model on which to draw a specific multilevel analysis which, if well applied and subsequently verified over time, becomes a tool that can be transferred to any living being to evaluate its monetary value in an ecosystem context.
In particular, starting from the data available for the wild boar, a multifactorial analysis was carried out from which, after evaluating a good number of variables, it is possible to quantify, in monetary terms, the services and inefficiencies connected to the target species.
Each item is described, updated and compared in the appropriate chapters. Some of the results obtained have been published in indexed journals and are reported at the end of the thesis.

This PhD thesis in "Veterinary Sciences" has the following aims:

Priority purpose.
“the protection, conservation and improvement of wild and domestic fauna” for
- Ensure the best conditions of natural and semi-natural habitats.
- Obtain the maximum expression of biodiversity (animal and vegetable).

- Define a monetary value to the components of the agroecosystem.
- Propose a "correct" management of all the components of the agro-ecosystem (dynamic and controlled management).

Alongside the primary purpose, secondary purposes are indicated, identified with the social role expressed by the *Sus scrofa* species:

- **Economic purpose.**

Interpreted as:
- Resilience of Ecosystem Services.
- Guarantee of ecological balances.
- Recreational, cultural and socio-economic opportunities.
- Negative pressures on human activities.

- **Educational purpose.**

Summarized in “knowing to protect”
- In the schools of the first cycle of education (primary, lower secondary).
- In the schools of the second cycle of education (secondary level).
- In universities and research institutions.
- In recreational and/or cultural associations or circles.
- In the community and public opinion.

3. MATERIAL AND METHODS

3.1 CHOICE OF THE AREA OF INVESTIGATION

Agriculture is, among the systems of human activity, the one that has most affected the change of natural ecosystems, necessarily providing for their replacement with agro-ecosystems organized and managed to obtain food and raw materials for human physiological needs (ISPRA, 2009). However, it is not possible not to consider how agriculture has settled in different ways in the different habitats present on the emerged lands of Planet Earth, modifying and transforming their balances.

The Palearctic ecozone is one of eight ecozones (Udvardy, 1975) that divide the Earth's surface (Palearctic; Nearctic; Indomalaya; Australasian; Neotropical; Afrotropical; Oceanian; Antarctic).

The steppe, for example, is an ecoregion of the Palearctic ecozone and represents a relatively dry variant of the temperate continental climate (hard winters, very hot summers); forms the transition belt between wooded areas (deciduous forest, mixed temperate forest and taiga) and desert areas of semi-arid climates.

If we consider the westernmost part of the Eurasian steppe starting from Kazakhstan, it is possible to state that the agro-zootechnical systems operated in the country condition the original ecosystems to a limited extent and, certainly, less than the fossil hydrocarbon extraction works. In fact, the agricultural land is occupied almost exclusively by cereal crops and, mainly, by pastures for livestock (bovine, ovine and caprine) with traditional and valuable products such as the astrakhan furs obtained from the Karakul sheep.

A second example is represented by the semi-desert and the Nubian-Sinaitic tropical desert of the Red Sea, also considered an ecoregion of the Palearctic ecozone, consisting mainly of immense flat expanses of sand, gravel or lava. In some areas the flat relief is broken up by isolated mountains of granite and sandstone which rise in an elevated range in the southern part of the Sinai Peninsula. If, as in the previous case, we consider the westernmost part of the desert, used by Israel, we can highlight that, even in such extreme areas, agriculture and animal husbandry have an important influence on the territories, radically modifying their ecosystem and landscape. In the Negev desert (part of the arid belt that extends from the Atlantic Ocean to India) 80,000 hectares of sand and loess (very fine wind sediment, similar to silt) with a small percentage of organic matter (<0.6%) they have been transformed into agricultural land where vegetables, cereals and citrus fruits are grown. A survey conducted by the WWF (2020) indicates that 75% of the non-frozen land has been modified by human activities. «The Living Planet Report 2020» (WWF, 2020) highlights that biodiversity includes all ecosystems on earth (deserts, forests, wetlands, mountains, lakes, seas, rivers and agricultural landscapes). In any ecosystem, living creatures, including humans, form a community, interacting with each other and with the air, water, and soil around them. The destruction of habitats is mainly due to human actions responsible for changes in land use as well as the use and trade of natural resources (animals, plants, living resources). Human predation actions added to climate change, also accelerated by man (IPCC, 2022), are accused of having caused (between 1970 and 2016) an average decline of 68% of the populations of global vertebrate species. The severe decline in wildlife populations is a clear indicator of reduced quality of life and an alarming signal for human health. Our investigation refers to one of the most envied areas of the planet which, due to the unequal distribution of organisms on the globe (latitudinal gradient of species diversity) seems to be the most inhabited by living things after the tropics. The Mediterranean Biogeographic Region is, together with the Alpine and Continental Biogeographic Regions, one of the three ecoregions of the Palearctic ecozone present in Italy (Fig. 7).

3.2 MEDITERRANEAN BIOGEOGRAPHICAL REGION

This biogeographical region includes the Mediterranean Sea and seven Member States, either partially (France, Portugal, Italy, Spain) or completely (Greece, Malta, Cyprus). It has specific regional features: a climate of hot dry summers and humid, cool winters and a generally hilly

landscape. The Mediterranean has not only a very rich biodiversity but also a large number of species that do not exist anywhere else.
To best protect the Mediterranean region, the relevant Member States and key stakeholders team up to devise nature protection measures, tailored to suit the particular needs of the entire region and to target its specific pressures.

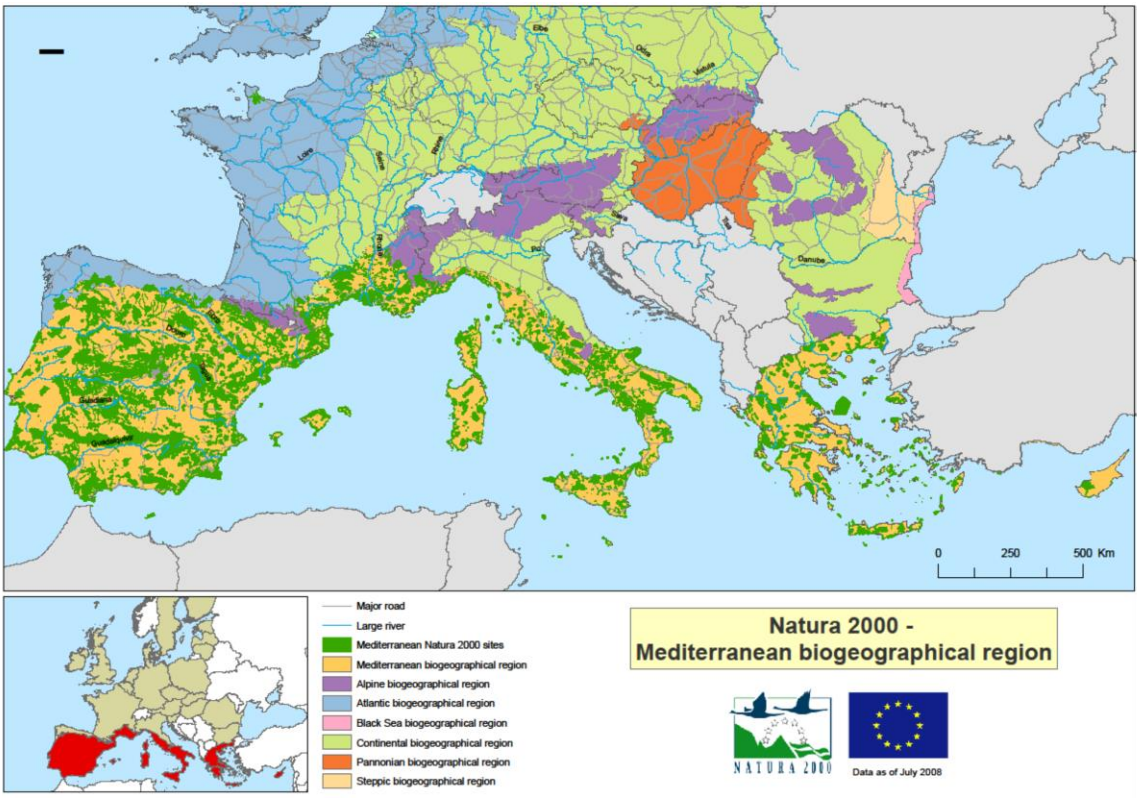

Figure 7. Map of the Biogeographical Regions of the European Union (27).
Source: https://ec.europa.eu/environment/nature/natura2000/biogeog_regions/maps/mediterranean.pdf

3.2.1 Regional features

The climate is characterised by hot dry summers and humid, cool winters. It is also very changeable with sudden heavy rain or bouts of high winds such as the Sirocco and Mistral. This climate has a profound influence on the vegetation and wildlife of the region.
For a region that takes its name from the sea it surrounds, the Mediterranean is surprisingly hilly. It includes high mountains and rocky shores, thick scrub and semi-arid steppes, coastal wetlands and sandy beaches as well as a myriad of islands dotted across the sea.
Man has left its mark across much of the landscape. The Mediterranean scrub, with its many flowers and aromatic plants, is a direct result of centuries of human activities (livestock grazing, forest fires and disforestation). This scrub has evolved into a complex and intricate mobile mosaic of habitats, home to an exceptionally rich biodiversity.

3.2.2 Biodiversity

Mediterranean wildlife and habitats are very specific as the region was not affected by the last Ice Age. The rate of endemism is exceptionally high. The Mediterranean is one of the world's top biodiversity hotspots.
Whilst the Mediterranean scrub is synonymous with the region, there are many other species-rich habitats here. Large tracts of natural, virtually pristine, forests have remained relatively untouched by man. While most central and northern European forests are now dominated by only a dozen or so tree species, the Mediterranean forests are much more diverse, harbouring up to 100 different tree species. Too dry for trees, other areas of the Mediterranean are covered in grasslands. These semi-

arid steppic areas may seem barren and lifeless but, on closer inspection, reveal an equally rich wildlife. These grasslands are prime locations for the great bustard (*Otis tarda*), the little bustard (*Tetrax tetrax*) and a whole range of ground-nesting birds such as the pin-tailed sandgrouse (*Pterocles alchata*).
Where water is more bountiful, wetlands appear at regular intervals, ranging from tiny coastal lagoons to vast deltas around the long coastline. They harbour hundreds of species of endemic fish, amphibians, and insects which, in turn, attract huge flocks of waders and ducks, especially during the migration season. Up to two billion birds migrate to, or through, the Mediterranean Region every year. Some merely stop over for a few days to refuel before crossing the Sahara, but others spend the entire winter here to escape the cold weather further north.
As for the Mediterranean Sea, its clear blue waters are famous throughout the world. It harbours a tremendous diversity of marine organisms, many of which are endemic to the region. It is estimated that the Mediterranean contains 8–9% of all the world's marine creatures. Many of the lesser-known sponges, sea squirts and crustaceans can be found hidden amongst the vast underwater meadows or Posidonia beds that grow in shallow coastal waters.

3.2.3 Pressures

The Mediterranean Region is under tremendous pressure due to human activities. It is the number one tourism destination in the world. As a result, much of the Mediterranean coastline has disappeared under concrete. There are chronic water shortages and a constant threat of forest fires. Inland, many of the ancient pastoral regimes are being abandoned because they are no longer economically viable. (https://ec.europa.eu/environment/nature/natura2000/biogeog_regions/mediterranean/index_en.htm).
The European Commission, aware of the excessive anthropic pressure on the natural environments of the 27 member countries (then 28), to ensure the conservation of natural habitats, wild fauna and flora, has chosen to protect some habitats even if these fall outside the traditional protected areas. Following the Conference of the Parties in Rio de Janeiro in 1992, it approves Directive 92/43/EEC better known as the "Habitats Directive", recently implemented[1].

[1]COMMISSION IMPLEMENTING DECISION (EU) 2022/234
of 16 February 2022
adopting the fifteenth update of the list of sites of Community importance for the Mediterranean biogeographical region
(notified under document C(2022) 862)

THE EUROPEAN COMMISSION,
Having regard to the Treaty on the Functioning of the European Union,
Having regard to Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora (1), and in particular the third subparagraph of Article 4(2) thereof,
Whereas:
(1) The Mediterranean biogeographical region referred to in Article 1(c)(iii) of Directive 92/43/EEC comprises the Union territories of Greece, Cyprus, in accordance with Article 1 of Protocol No 10 of the 2003 Act of Accession, and Malta, parts of the Union territories of Spain, France, Italy, Portugal and Croatia as specified in the biogeographical map approved on 20 April 2005 by the committee set up by Article 20 of that Directive (the 'Habitats Committee').
(2) The initial list of sites of Community importance for the Mediterranean biogeographical region, within the meaning of Directive 92/43/EEC, was adopted by Commission Decision 2006/613/EC (2). That list was last updated by Commission Implementing Decision (EU) 2021/159 (3).
(3) The sites included in the lists of Community importance for the Mediterranean biogeographical region form part of the Natura 2000 network which is an essential element of the protection of biodiversity in the Union. In order to make further progress in the actual establishment of the Natura 2000 network and in the context of a dynamic adaptation of that network, the lists of sites of Community importance are reviewed regularly.
(4) Between 10 August 2020 and 11 February 2021 Member States have proposed additional sites of Community importance for the Mediterranean biogeographical region. Member States have also submitted changes in the site-related information contained in the list of sites of Community importance for the Mediterranean biogeographical region.
(5) On the basis of the draft list drawn up by the Commission in agreement with each of the Member States concerned, which also identifies sites hosting priority natural habitat types or priority species, an updated list of sites selected as sites of Community importance for the Mediterranean biogeographical region should be adopted. Article 4(4) and Article 6 of Directive 92/43/EEC apply to the newly included sites.
(6) Knowledge of the existence and distribution of the natural habitat types and species is constantly evolving as a result of the surveillance undertaken in accordance with Article 11 of Directive 92/43/EEC. Therefore, the evaluation and selection of sites at Union level was carried out using the best available information at the time.

The work carried out during the PhD period developed on the assumption that an essential part of the EU strategy for the conservation of biodiversity was based on the assessment of forest ecosystem services and conditions. Forest is the largest terrestrial ecosystem in the European Union covering around 40% of the territory and is home to much of the continent's biodiversity. In addition, forests provide a multitude of benefits to humans in terms of climate regulation, water supply and regulation, timber, energy, habitat for biodiversity, clean air, erosion control and many others. (https://forest.jrc.ec.europa.eu/en/activities/forest-ecosystem-services/).
Knowledge of the extent and spatial distribution of Forest Available for Wood Supply (FAWS) is key for the forest bioeconomy. The importance of the presence of forest (fig. 8), requires to assessing the some main restrictions to wood availability and the related forest area and biomass stock. The Forest Not Available for Wood Supply (FNAWS) was defined as "Forests where there are environmental, social or economic restrictions that have a significant impact on the current or potential supply of wood" The economic restrictions accounted for most of the FNAWS, followed by environmental restrictions, while social restrictions had a marginal role. Copernicus HRL Forest Type map 2015 was used as a base map of forest areas. FNAWS areas were mapped using the following restrictions: high slope, high altitude, protected areas, protected species, low accessibility (distance to roads), and unproductive forests (Avitabile et al., 2018).
Forests are biologically diverse ecosystems that provide habitat for a multiplicity of plants, animals and micro-organism. Forest biodiversity can be considered at different levels, including the ecosystem, landscapes, species, populations and genetics. In high biodiversity forests this complexity allows organisms to adapt to continually changing environmental conditions and to maintain ecosystem functions. Forest ecosystems and biodiversity are strongly interlinked. On the one hand, biodiversity levels depend to a large extent on the integrity, health and vitality of forests. On the other hand, losses of forest biodiversity lead to decreased forest productivity and

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(7) Certain Member States have not proposed sufficient sites to meet the requirements of Directive 92/43/EEC for certain habitat types and species. Furthermore, knowledge of the existence and distribution of some habitat types listed in Annex I and some of the species listed in Annex II to Directive 92/43/EEC remains incomplete. For those habitat types and species it can therefore not be concluded that the Natura 2000 network is complete.
(8) In the interests of clarity and transparency, Implementing Decision (EU) 2021/159 should be repealed.
(9) The measures provided for in this Decision are in accordance with the opinion of the Habitats Committee,

HAS ADOPTED THIS DECISION:

Article 1

The fifteenth update of the list of sites of Community importance for the Mediterranean biogeographical region as set out in the Annex is adopted.

Article 2

Implementing Decision (EU) 2021/159 is repealed.

Article 3

This Decision is addressed to the Member States.

Done at Brussels, 16 February 2022.

ANNEX

PART 1

Fifteenth update of the list of sites of Community importance for the Mediterranean biogeographical region

Each site of Community importance (SCI) is identified by the information supplied in the Natura 2000 format, including the corresponding map. This information has been transmitted by the competent national authorities in accordance with the second subparagraph of Article 4(1) of Directive 92/43/EEC.
The table below gives the following information:
A : SCI code comprising nine characters, the first two being the ISO code for the Member State;
B : name of SCI;
C : * = presence on the SCI of at least one priority natural habitat type and/or species within the meaning of Article 1 of Directive 92/43/EEC;
D : area of SCI in hectares or length of SCI in km;
E : geographical coordinates of SCI (latitude and longitude) in decimal degrees.
All the information given in the Union list below is based on the data proposed, transmitted and validated by Greece, Croatia, Spain, France, Cyprus, Italy, Malta, Portugal.
http://data.europa.eu/eli/dec_impl/2022/234/oj

sustainability. The European Union indicates that sustainable forest management is geared towards supporting the provision of forest services and improving biodiversity levels.
Therefore, taking literally what has been said by the European Commission, the purpose of this thesis is to contribute in a concrete way, of asustainable forest management is oriented to support the provision of forest services and to enhance biodiversity levels.
Our work starts from a sample forest site, included in the Natura 2000 network, and from a component of biodiversity considered highly harmful to all habitat types, the wild boar. Three years of data recovery have permit to provide information and tools which are able to signal the services connected to the presence of the suidae trying to insert its presence in a balanced way in the studied habitat. Indeed, the results obtained contribute to the EU forest strategy and suggest some ways to assess and halt biodiversity loss. Finally, a calculation is proposed to attribute a monetary value to the wild boar to be used in the near future for calculating the value of the ecosystem service provided by this animal.

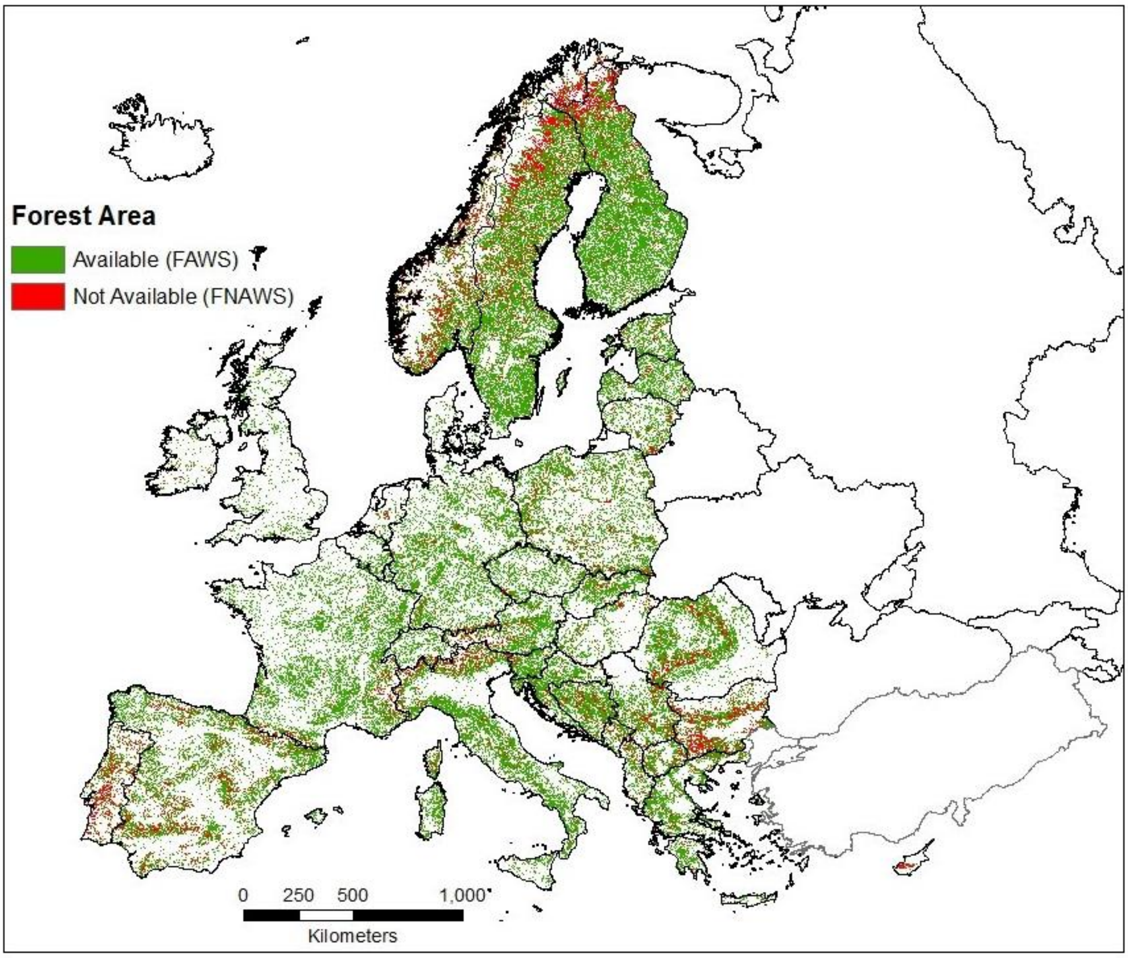


Figure 8. Preliminary map of Forest Available for Wood Supply (FAWS) and not available (FNAWS) in Europe. The sum of FAWS and FNAWS corresponds to forest area mapped by the 2015 Copernicus Forest type map.
Source: https://forest.jrc.ec.europa.eu/en/activities/forestbioeconomy/fawsmapping/

3.3 Description of the intervention context

The experimentation carried out during the research doctorate period involved the agro-forestry-pastoral territory (TASP) of the Avellino's province (fig. 9). The agro-environmental resources present in the area are under the political-administrative competencies of various public management bodies, while the economic-monetary valorisation is entrusted to the numerous private companies present. At the moment, there is no estimate of the monetary value of the ecosystem services produced by the agro-forestry-pastoral environments of the Campania Region.

Administrative Authorities
• Campania Region
• Regional Parks
• Avellino's Province
• Municipalities
• Mountain Communities
• Water Reclamation Authority
Companies
• Territorial Hunting Area
• Farms
• Farm holiday
• Meat processing laboratory

Figure 9. Geographical overview of the Avellino's Province.

Since the beginning of the experimentation, we have been aware that it would not have been possible to study the entire province of Avellino. The trial, therefore, started with the analysis of what was reported by the available official data relating to a "wide area", and subsequently through in the field collection data, into a "point area" as descripted in the subsequently subparagraphs (3.3.1 and 3.3.2). The data collected in the field were necessary to create a management model to be proposed as the beginning of the monetary evaluation of all the elements that make up a given agro-ecosystem and related to the "Ecosystem Services" connected to it.

3.3.1 Wide Area

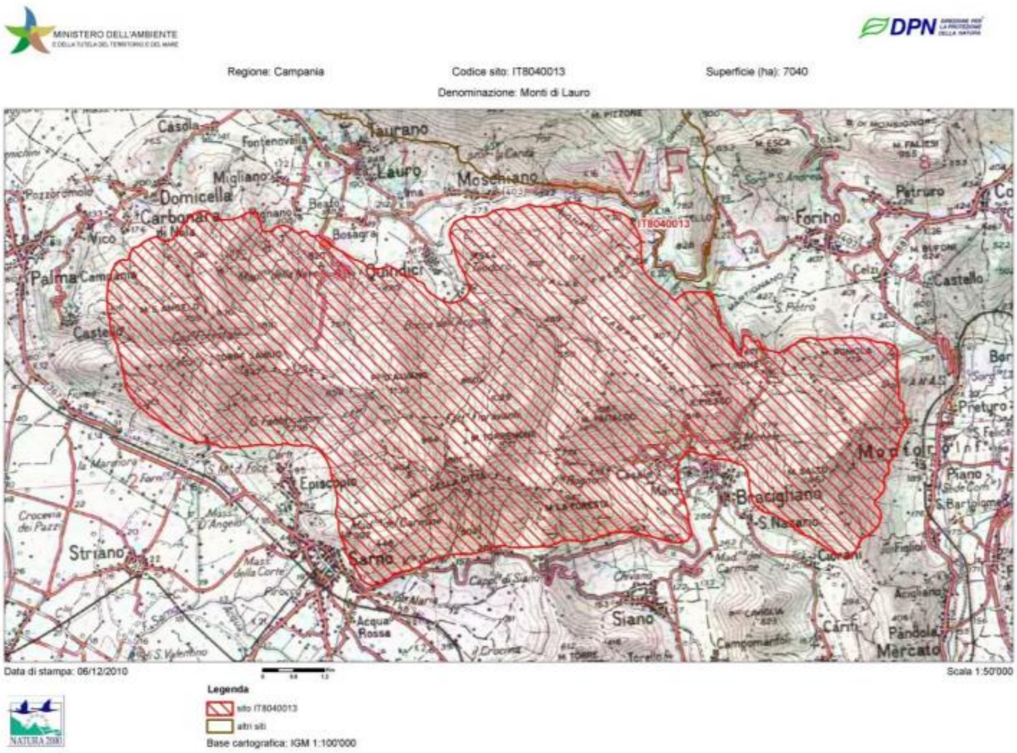
The "wide area" is divided according to the type of habitat:

- STR 18. The agro-forestry territory of the Campania Region is divided into 28 Rural Territorial Systems. The territory of the STR 18 (Monte Partenio-Monti di Avella-Pizzo d'Alvano) includes 31,800 hectares falling within 23 municipalities, 15 of which in the province of Avellino (230.2 sq km); 4 in the province of Benevento (28.1 sq km); 3 in the province of Naples (45.6 sq km); 1 in the province of Caserta (14.1 sq km).

The experimental investigation started from a representative sample area of the STR 18. The STR 18 is, in fact, made up for 68% of landscapes of the high and medium limestone mountains typical of the pre-Apennines (beech woods, grasslands of the peaks and summit karst plateaus; sub-Mediterranean hardwood forests, xerophilous grasslands on the middle and lower slopes). The total forest area amounts to about 16,955 hectares and the grazing areas have an extension of about 1,500 hectares (5%).

The foothills are mainly for agricultural use, with hazelnut and chestnut groves. The foothill plain (12%) is characterized only by hazel groves while the alluvial plain (8%) shows a landscape dominated by arable land and industrial crops, interspersed with vineyards and fruit orchards. The urban area has reached 6% of the territory.

• SCA IT8040013. For the purposes of our investigation, the presence of the Special Conservation Area/Site of Community Interest (SCA/SCI) IT8040013 “Monti di Lauro” (fig. 10) helps us a lot.



A	B	C	D	D	E	E
CI code	**Name of SCA/SCI**	**Priority**	**Area of SCA/SCI (ha)**	**Length of SCA/SCI (km)**	**Longitude**	**Latitude**
IT8040013	Monti di Lauro	*	7,040	0	14,66361	40,84083

Figure 10. SCA/SCI IT 8040013 “Monti di Lauro” (www.minambiente.it)

The SCI IT8040013 "Monti di Lauro" was established in May 1995 (D.G.R. n. 795/2017).
Although there is still no management plan, the Italian Ministry of the Environment (MiTE) recognizes it as SCA IT8040013 "Monti di Lauro", with the Ministerial Decree of 21/05/2019 - G.U. no. 129 of 04/06/2019.
The latest update made by the Campania Region is in December 2019, sent by the MiTE database to the European Commission in December 2020. The European Commission approves the proposal with decision (EU) 2022/234 of February 16, 2022 (adopting the fifteenth update of the 'List of sites of importance of the community for the Mediterranean Biogeographic Region - notified under document C (2022) 862).
(ftp://ftp.minambiente.it/PNM/ Natura2000/Transmission%20CE_December2020/)
The SCA IT8040013 “Monti di Lauro” is shared between 8 municipalities [Lauro, Quindici, Forino (province of Avellino); Carbonara di Nola, Palma Campania (Metropolitan city of Naples); Bracigliano, Sarno, Siano (province of Salerno)], and includes particular forms of plant and animal biodiversity (fig. 10a and 10b).
This biodiversity is threatened by bad anthropic management but also by the presence of animals and alien plants or that in competition with the native species create ecosystem imbalance.

Codice sito	Regione bio-geografica	Area [ha]	Latitudine	Longitudine	Altezza minima [m.s.l.m.m]	Altezza massima [m.s.l.m.m]
IT8040013	Mediterranea	7040	40 50 27	E 14 39 49	400	1133

Tipi di habitat presenti	Superficie coperta
Prati magri, steppe	40%
Brughiera, macchia, macchia mediterranea e gariga, phygrana	5%
Boschi misti	25%
Boschi di latifoglie decidue	15%
Aree non forestali coltivate con piante legnose (inclusi frutteti, oliveti,vigneti, pascoli arborati)	10%
Habitat rocciosi, detriti di falda, aree sabbiose, nevi e ghiacci perenni	5%
Copertura totale habitat	100%

Habitat di interesse comunitario (elencati nell'Allegato I della Direttiva 92/43/CEE)						
Codice	Tipo	Superficie coperta	Rappresentatività	Superficie relativa	Grado di conservazione	Valutazione globale
6220*	Percorsi substeppici di graminacee e piante annue dei Thero-Brachypodietea	30%	B	C	C	C
9260	Foreste di Castanea sativa	20%	B	C	B	B
6210	Formazioni erbose secche seminaturali e facies coperte da cespugli su substrato calcareo (Festuco-Brometalia) (*stupenda fioritura di orchidee)	10%	C	C	B	B
8210	Pareti rocciose calcaree con vegetazione casmofitica	5%	B	C	C	C
9210*	Faggeti degli Appennini con Taxus e Ilex	5%	C	B	B	C
8310	Grotte non ancora sfruttate a livello turistico	1%	A	C	A	B

*Habitat prioritari

Figure 10a. Natura 2000 – Standard Data Form Site IT 8040013 "Monti di Lauro".

A species of all is the wild boar (*Sus scrofa*) which is included in the black list of the 100 most harmful invasive species in the world. The presence of this wild suide has been demonstrated and quantified by our study within the two Point Areas.

Invertebrati (elencati nell'Allegato II della Direttiva 92/43/CEE)					
Codice	Specie	Popolazione	Conservazione	Isolamento	Valutazione globale
1062	Melanargia arge	C	B	C	B
1078	Callimorpha quadripunctaria	C	B	C	A

Altre specie importanti di flora e fauna		
Gruppo	Specie	Popolazione
Anfibi	Triturus italicus	Comune
	Hyla italica	Rara
	Rana italica	Rara
	Salamandra salamandra gigliolii	Rara
Rettili	Coluber viridiflavus	Comune
	Anguis fragilis	Rara
	Elaphe longissima	Rara
	Lacerta bilineata	Comune
	Podarcis sicula	Comune
	Podarcis muralis	Rara
	Chalcides chalcides	Rara
Mammiferi	Felis silvestris	Molto rara
Invertebrati	Lucanus tetraodon	Presenza
	Ceriagrion tenellum	Presenza
	Cordulegaster boltoni	Presenza
	Scarabaeus sacer	Presenza

Uccelli migratori abituali (non elencati nell'Allegato I della Direttiva 79/409/CEE)					
Codice	Specie	Popolazione	Conservazione	Isolamento	Valutazione globale
A247	Alauda arvensis	C	C	C	C
A155	Scolopax rusticola	C	C	C	C
A298	Acrocephalus arundinaceus	C	C	C	C
A255	Anthus campestris	C	C	C	C
A338	Lanius collurio	C	C	C	C
A283	Turdus merula	C	B	C	B
A285	Turdus philomelos	C	B	C	B
A113	Coturnix coturnix	C	C	C	C
A210	Streptopelia turtur	C	B	C	B

Mammiferi (elencati nell'Allegato II della Direttiva 92/43/CEE)					
Codice	Specie	Popolazione	Conservazione	Isolamento	Valutazione globale
1303	Rhinolophus hipposideros	C	A	C	A
1304	Rhinolophus ferrumequinum	C	A	C	A
1305	Rhinolophus euryale	C	A	C	A
1324	Myotis myotis	C	A	C	A
1316	Myotis capaccinii	C	A	C	A
1307	Myotis blythii	C	A	C	A
1310	Miniopterus schreibersii	C	A	C	A

Anfibi e rettili (elencati nell'Allegato II della Direttiva 92/43/CEE)					
Codice	Specie	Popolazione	Conservazione	Isolamento	Valutazione globale
1193	Bombina variegata	C	A	C	A
1167	Triturus carnifex	C	B	C	B
1279	Elaphe quatuorlineata	C	A	C	A

Figure 10b. Natura 2000 – Standard Data Form Site IT 8040013 "Monti di Lauro".

3.3.2 Point Area

- PSR area. Since the assessment of the wide area, as a whole, would have been too problematic, with the project "S.U.S. Campania" of the 2014-2020 Rural Development Plan of Campania, we concentrated the attention of field sampling on a smaller area but corresponding to the vegetational and ecological peculiarities of the wide area (STR 18 and SCA IT8040013).

The territory of interest in the PSR area is a total of 13,118 hectares characterized by habitat homogeneity (fig. 11) of the Municipalities of Domicella, Forino, Lauro, Monteforte Irpino, Moschiano, Pago del Vallo di Lauro, Quindici, Taurano. The results obtained from the sampling analysis will be able to provide a model for the evaluation of Ecosystem Services and their role for the conservation of biodiversity and socio-economic benefits in the agroecosystems of the Mediterranean biogeographical region.

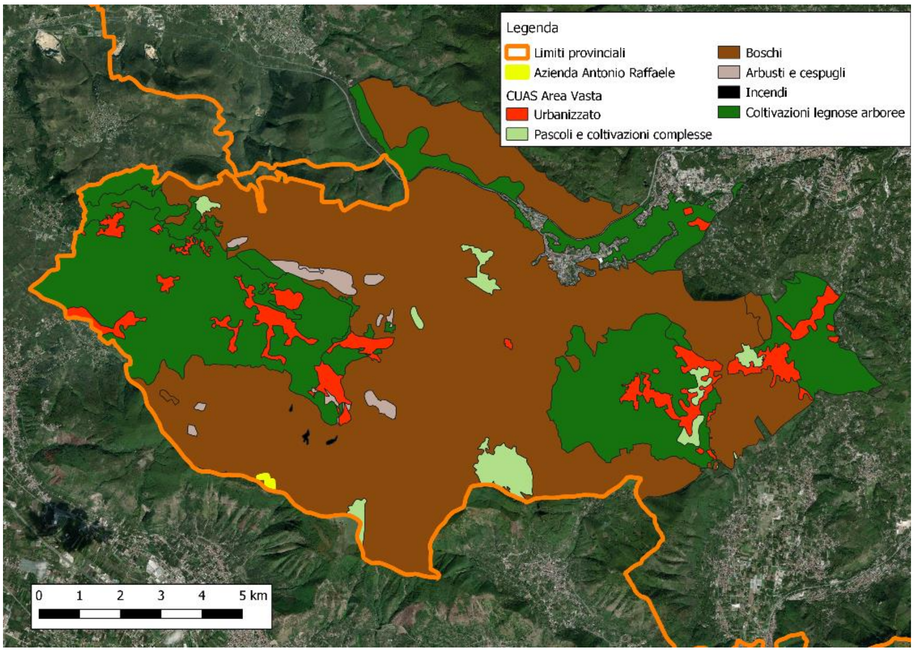


Figure 11. Land cover of the "PSR Area".

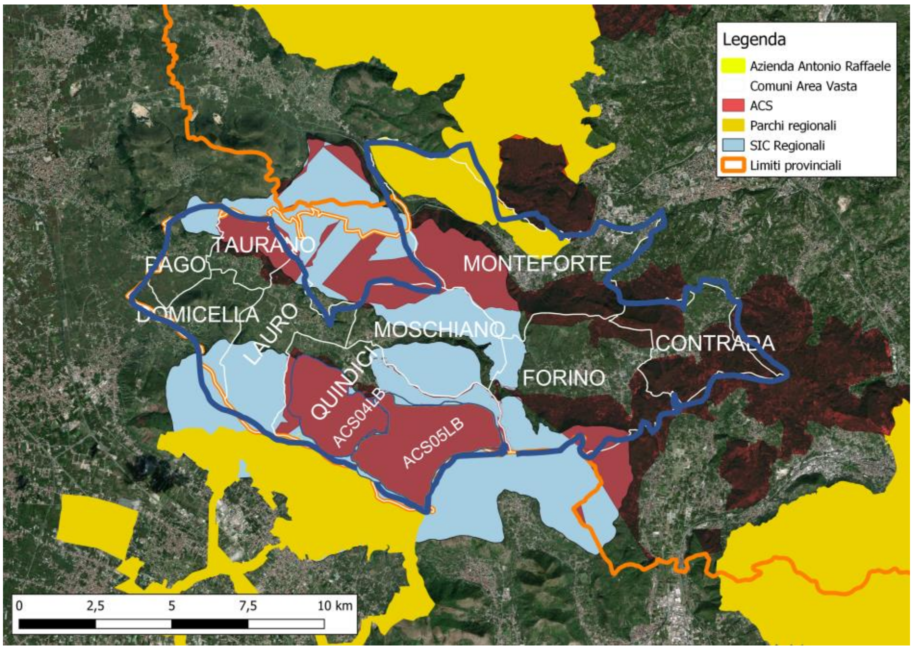


Figure 12. Subdivision of the territory of the "PSR Area" according to wildlife management.

Figure 12 shows that the PSR Area is included in the SCA/SCI IT8040013 "Monti di Lauro" close to, but not included in, the Regional Park of the hydrographic basin of the river Sarno.
The wildlife-hunting management is exercised under the control of the Territorial Hunting Area (ATC) of the Avellino's Province in compliance with the Regional Law of Campania 09 August 2012, n. 26 as amended and the Hunting Calendars approved with a Regional Council Resolution for the different hunting seasons. With reference to wild boar hunting, the focal area is close to ACS04LB (834 ha) and ACS05LB (930 ha) falling within the municipality of Quindici and included in District 10 "Vallo di Lauro".

- Focal area. The collection of samples and the evaluation of Ecosystem Services and their role for the conservation of biodiversity was carried out, during the period 2019-2022, in a farm representative of the "wide area" and "point area". The focal area is the "Raffaele Antonio farm" identified in the Campania Region (fig. 13) in the municipality of Quindici (AV). The land on which the study was carried out occupies an area of 4.70 hectareas which has as its intended use agricultural management classified as permanent crops (hazelnut), Chestnut Forest (code 9260) other than permanent meadows (code 6210). The land is identified by the coordinates 40°50'33.2999"N; 14°37'40.0001"E.

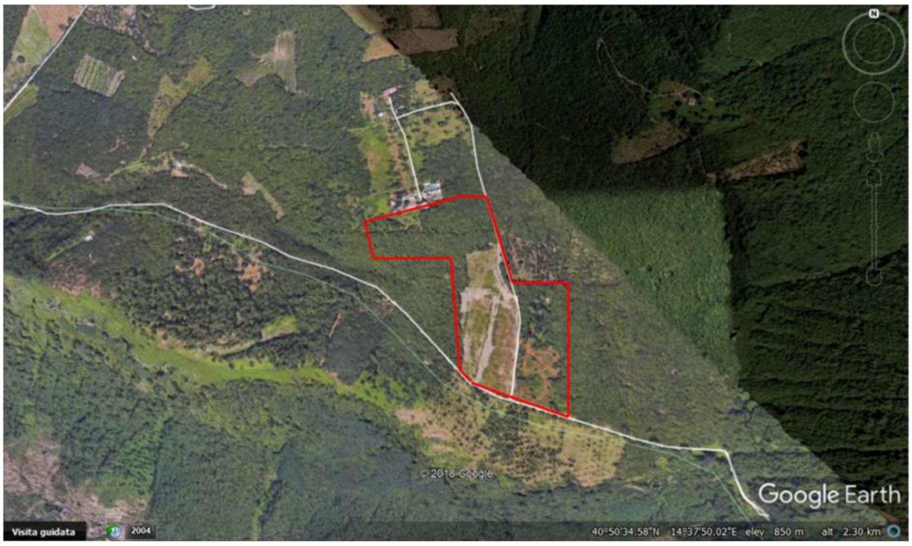


Figure 13. Farm "Antonio Raffaele" - Location of the focal area in the Campania Region.

Avellino's province, on the basis of the Provincial Territorial Coordination Plans (PTCP), is divided into 5 homogeneous districts (fig. 14) which contain 3 main classes of land use distribution. It is also characterized by two landscape typologies: the first of a mountainous type, predominantly tectonic in nature, the second typically fluvial, in which the action of erosion, transport and sedimentation of surface waterways overlaps the tectonic phenomena. The sample company falls within the District 1 Partenio and in the mountainous landscape typology even if we can frame it in a natural landscape with strong anthropic influence. During the three-year period, the agronomic and structural conditions of the "Raffaele Antonio" farm were monitored, in order to verify the starting conditions reported in the company file. The inspections carried out confirm that the Company occupies 10.48.95 hectares falling within the Municipality of Quindici (AV), of which 05.91.19 hectares of agricultural management classified as permanent crops other than permanent meadows. In particular, there are tree crops or non-rotation crops that provide repeated crops and occupy the land for at least 5 years. Among the specialized tree crops there is the hazelnut which occupies 05.17.49 hectares (with 930 plants) and mixed tree crops, including the chestnut (00.16.73 hectares) and the hazelnut (00.19.44 hectares). There is also a forest which occupies 00.33.39 hectares. Finally, an area of 00.04.13 hectares is classifying as "use other than agricultural or forestry". The company, although engaged in the production of hazelnut and chestnut trees, shares the company surfaces with the forest and its importance at an environmental level is closely associated with the SAC/SCI "Monti di Lauro" (IT8040013).

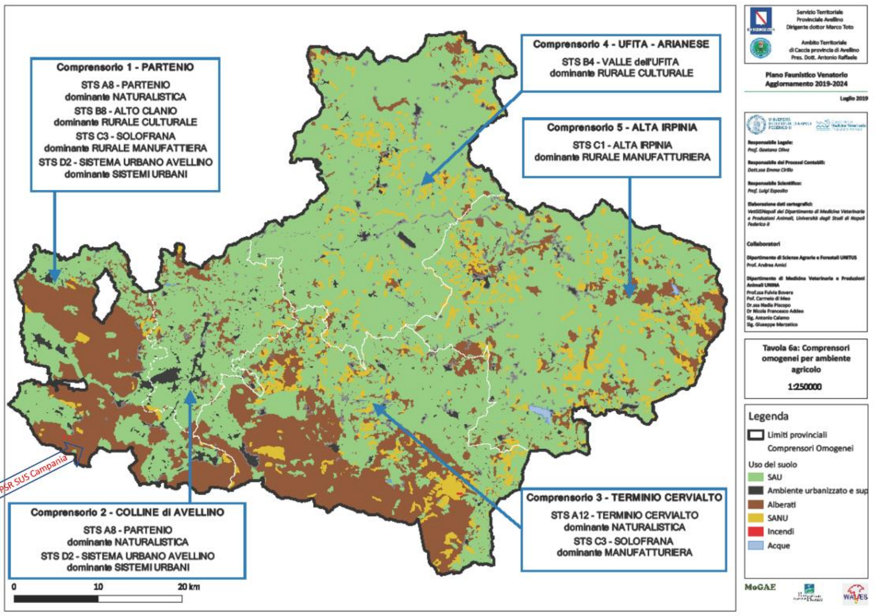


Figure 14. ATC homogeneous districts of the Province of Avellino.

Biodiversity is threatened in the event of anthropic mismanagement but also by the presence of alien animals and plants that compete with the native species. One species among all is the wild boar (*Sus scrofa*). The farmlands are subject to continuous visits by wild boars, coming both from Monte Pizzo d'Alvano and from the reliefs of Sarno, and from the mountains between Bracigliano and Siano as evidenced by the identification of transhumance routes documented by the camera traps used for censuses. The Farm's participation in the PSR project made it possible to identify the wild boars and a great number of animal biodiversity around a specific area of observation.

The PSR project suggest an innovative procedure aimed at the numerical verification of wild boars, and the possible role (Ecosystem Service) of this swine species for preserving the natural resources present and, mainly, at enhancing ecosystem services in a context of rural supply chain and sustainable development. The presence of specialized tree crops mixed with woods and bordering forest areas characterized by the presence of holm oaks, downy oaks, beeches and carob trees represents an opportunity to demonstrate that, by implementing correct management, it is possible not only to conserve the existing biodiversity in agroecosystems from balance, but also guarantee the presence of exuberant species without resorting to the eradication of harmful species or exclusively to hunting..

3.4 WILDLIFE MANAGEMENT IN THE INTERVENTION AREA

Esposito, L., Piscopo, N. et al. (2023) Piano Faunistico Venatorio Provinciale 2019-2024 - Avellino. Ed. Regione Campania, Avellino. https://campaniacaccia.it/pianofaunaav.php

To correctly interpret the investigation site, it is essential to know who and how carries out wildlife management activities in the area of interest. As in the entire province of Avellino, wildlife management is the responsibility of the Campania Region. However, two areas must be distinguished (fig. 15):

1) The agro-forestry-pastoral territory falling within the Protected Areas (managed by the Park Authorities).

2) The agro-forestry-pastoral territory in which it is possible to hunt (managed by the Hunting Territorial Area of the Avellino’s province - ATC);

.

3.4.1 Protected areas

The Protected Areas in the territory of the wide and point areas (Table 1, Fig. 15) are:
The Partenio Regional Park, with its 14,870 hectareas, occupies the North-West part of the Avellino's Province and is shared with the Metropolitan City of Naples (3,415 hectareas), the Province of Caserta (750 hectareas) and the Province of Benevento (1,660 hectareas). Of the 9,045 hectareas falling within the Avellino's Province, approximately 1,400 hectareas are included in the PSR area (only to the Municipality of Monteforte Irpino). Focal area does not fall within any protected area.

Table 1: Surfaces of the Protected Areas falling within or contiguous to the wide area.

Protected area	Surface (ha)
Partenio Regional park	14,870
Regional Park of the hydrographic basin of the river Sarno	3,436
Total	**18,306**

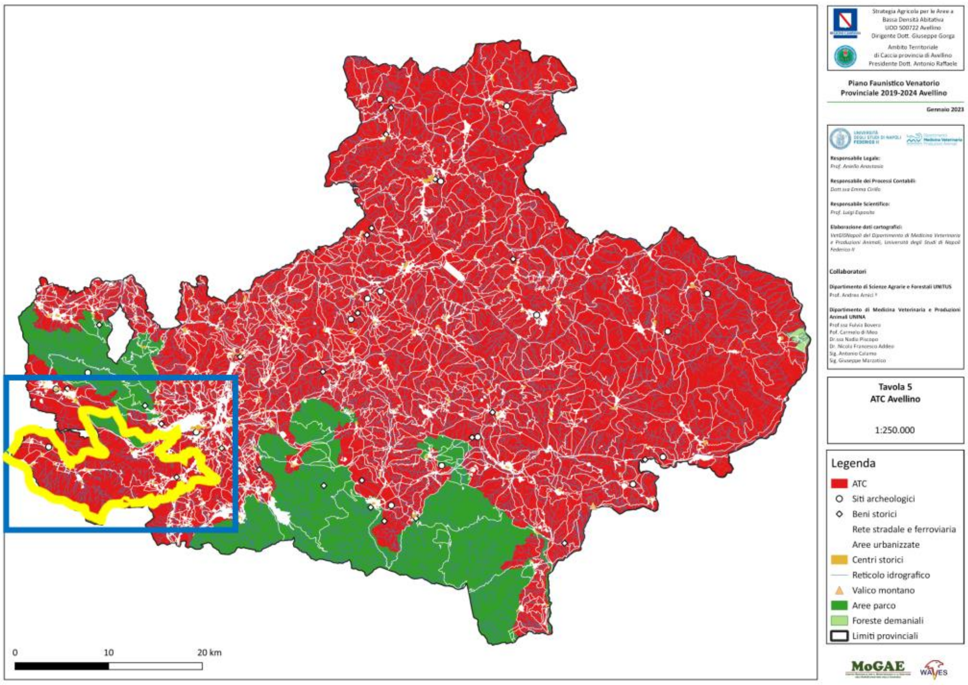


Figure 15. Protected areas and urbanized areas/infrastructures (roads and railways) in the territory of the ATC of the Avellino's Province with particular in the PSR Area.

The Regional Park of the hydrographic basin of the river Sarno (fig. 11), with its 3,436 ha, is close to the Province of Avellino but belongs to the Metropolitan City of Naples (791 ha) and the Province of Salerno (2,645 ha) and it does not concern the two study areas.

3.4.2 Wildlife institutes of the Avellino's Territorial Hunting Area

During the drafting of the Provincial Wildlife Hunting Plan 2019-2024 - Avellino it was found that the focus areas do not include any Wildlife Institutes (fig. 16 and 17). In fact, there are no Repopulation and Capture Areas (ZRC); Dog Training Zones (ZAC) with or without shooting; Wildlife Protection Oasis (OPF).

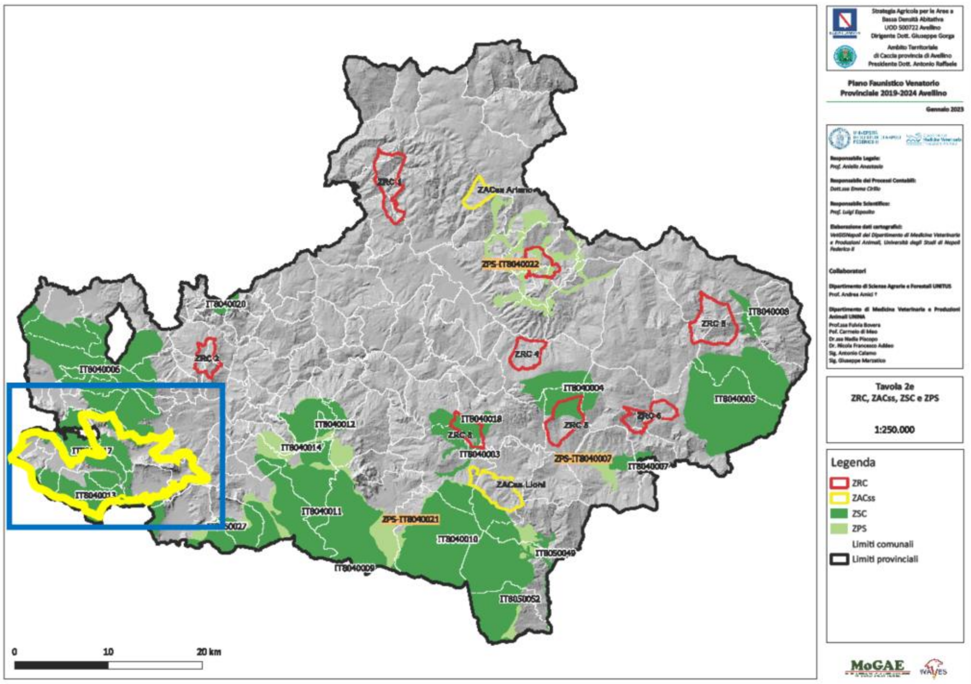


Figure 16. Repopulation and Capture Areas, Dog Training Areas, SCA, SPA in the ATC territory of the Avellino's Province (particular PSR area).

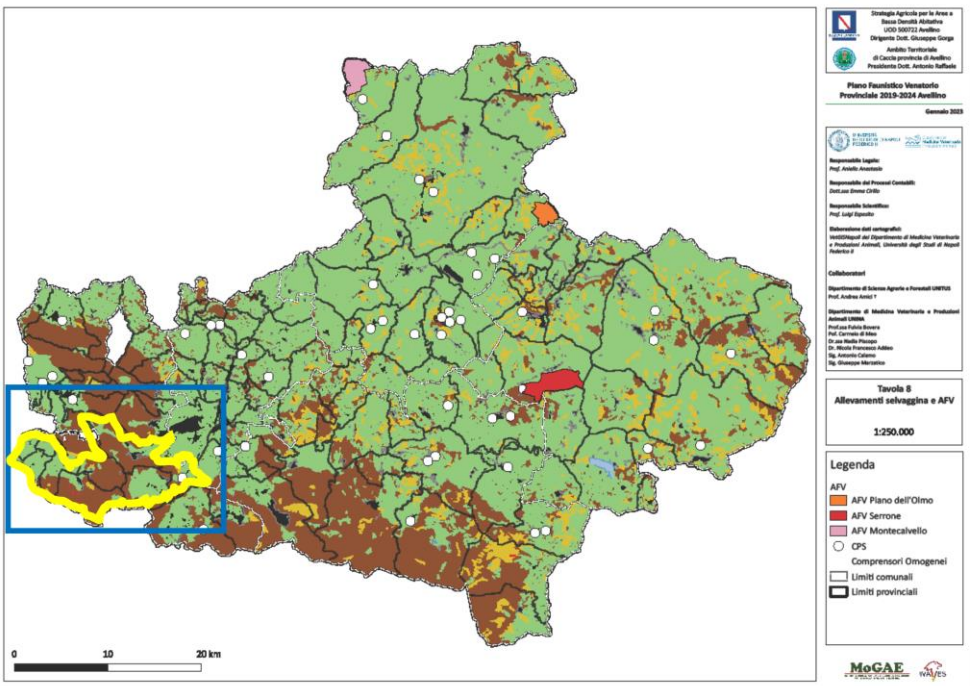


Figure 17. Wildlife Hunting Companies, Game Production Centers in the ATC territory of the Avellino's Province (particular PSR area).

Conversely, in the PSR Area and in the Focal Area, there are two "Specific Wild Boar Hunting Areas" of Hunting District no. 10 “Vallo di Lauro” and in particular ACS04LB and ACS05LB (fig. 18). The focal area is therefore included both in the ACS04LB and in the SCA IT8040013 "Monti di Lauro”.

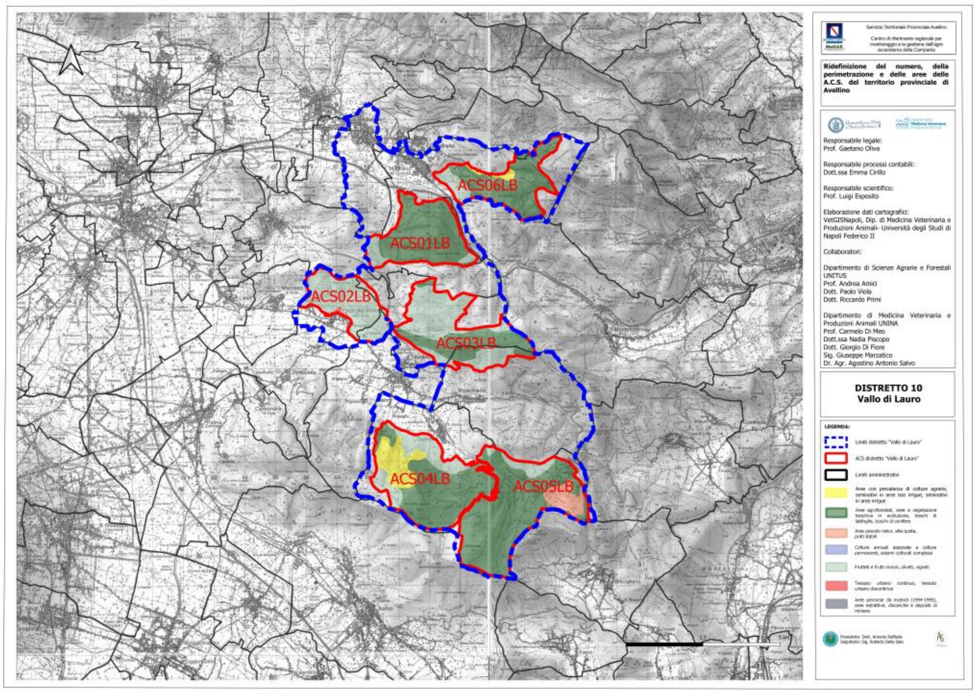


Figure 18. Specific Wild Boar Hunting Areas (ACS04LB; ACS05LB) of District 10 Vallo di Lauro in the ATC territory of the Avellino’s Province (particular in the PSR area and in the Focal Area).

3.5. METHODS FOR THE IDENTIFICATION OF ANIMAL BIODIVERSITY

Lo studio delle popolazioni selvatiche (Lovari S. & Rolando A. 2004) può essere effettuato attraverso differenti metodi: 1) conteggio diretto per osservazione; 2) conteggio indiretto; 3) analisi sperimentale; 4) conteggio per indici.
Nel periodo 30 luglio 2019 - 24 agosto 2022 nella Focal Area e nella PSR Area sono state realizzate operazioni di censimento diretto e indiretto per la identificazione delle specie animali presenti all’interno degli habitat rappresentativi della wide area.

3.5.1 – Direct count

Direct observation is a method based on the observation of animals by an operator. The systems used are of two types:
1) Total census. The total count (census in the strict sense) involves the observation and counting of each individual in the study area. Carrying out a total count of large animals can be effective, especially in very small, fenced areas; on the other hand, it is usually inaccurate and excessively expensive when it concerns large areas and/or with a rather low animal density.
2) Sample census. The sample census is a method based on the analysis of a portion of the total area (sample unit) and on the extrapolation from this of data relating to the whole. In the sampled area an absolute census is carried out to all intents and purposes, for this reason we often also speak of absolute counting for sample areas. In wildlife censuses the sample unit is generally represented by quadrants, transects or irregular portions chosen randomly from the totality.
From an operational point of view, the techniques used can be:

- Direct survey on a linear path (Line transect): the counts are carried out along pre-established paths within the area of interest, traveled by various means (on foot, by car, by plane, etc.) at constant speed. (Gatto M. & Casagrandi R. 2003).
- Direct survey in parceled out areas (block census) or strip (strip census): it is based on direct and simultaneous observation by several detectors operating within a sufficiently defined territorial unit, moving along pre-established routes or from fixed locations with the aid of suitable optical instruments (Gatto M. & Casagrandi R. 2003).

3.5.2 – Indirect counting

Indirect detection is a method based on the observation of signs and traces (beds, passage points, footprints, hair, excrement) left by animals during their movements. This area includes a set of methods that see the use of camera traps, radio transmitters, or GPS positioning systems.
The most used indirect estimation methods are:
a) “Capture - Mark - Recapture" system proposed by Petersen” (citato da Robson, 1969).
b) “Stool detection in sample areas”.
c) “Fingerprinting under suitable environmental conditions”.
d) “Measurement of hunting effort”.
e) “Use of camera traps from georeferenced points”.

a) The system “Capture - Mark - Recapture” consists in capturing a small part of the population, marking it appropriately, releasing it and subsequently recapturing new samples by counting the previously marked and recaptured animals. If it is assumed that the number of tagged animals remains constant, the ratio between the number of initially tagged animals and the number of tagged and subsequently recaptured animals will give an estimate of the total population.
In a first phase, therefore, the method provides for the capture of a sample of N1 individuals, which are marked and then released into the population. Subsequently, a second random sample of N2 individuals is collected, of which M are marked. Assuming that tagged individuals mix perfectly with untagged animals, the proportion of tagged individuals within the population of unknown size N and within the recaptured sample remains constant:

$$\frac{M}{N_2} = \frac{N_1}{N}$$

and, therefore, from this relationship it is possible to derive an estimate of N:

$$N = \frac{N_1}{M} \cdot N_2$$

Depending on the species, different objects are usually used for marking. In the case of the wild boar, plastic marks are used, generally positioned on the auricle. In medium-large sized mammals, colored collars or radio collars can be used. In some cases, it is possible to identify the animals by the presence of particular signs of a permanent nature such as mutilations or scars but also chromatic alterations of the coat.

b) The system of "Detection of faeces in sample areas" (*pellet and pellet-group census*) is a sample-type method which allows to estimate the population density by counting, in each sample area, the number of groups of "pellets fecal" found.
b1) Faecal Accumulation Rate (FAR). The FAR count data start from the subdivision of the sample area into different parcels of approximately 50x50 metres. A certain number of areas to be sampled are randomly chosen. On a central survey line about 1000 meters long and about 500 meters wide (250 meters on each side) the observers look for the droppings of the species to be studied.
The original scheme, proposed for the lagomorphs (Taylor & Williams, 1956) and adapted to the ungulates, foresees that the areas to be sampled are at time zero (T0) cleared of the pre-existing

facts after a certain period of time (Tn), normally not less than 15 days, the operators go through the sample area and count the number of faecal agglomerates (5-10 cm) present.
The calculation of the estimate of the number of subjects present in the sample area derives from the following formula:

$$N = \frac{n}{DDR \times days} \times 100$$

Where:
N= number of animals
n= number of faecal agglomerates
DDR= daily defecation rate
For wild boar the daily defecation rate is between 3.8 and 4.5 (Briedman, 1986)
days= number of days between collections
x100= 100 hectares

b2) Faecal Standin Crop (FSC).
The FSC counting data start from the subdivision of the sample area into different parcels of about 50x50 meters. A number of areas to be sampled are randomly selected. On a central survey line about 1000 meters long and about 500 meters wide (250 meters sideways) the observers look for the droppings of the species to be studied.
The original scheme (Laing et al., 2003) adapted to ungulates, foresees that the areas to be sampled are at time zero (T0) cleaned of the pre-existing faecal agglomerates after a certain period of time (Tn), normally not less than 15 days, the operators they pass through the sample area and count the number of faecal agglomerates (5-10 cm) present. Unlike FAR, FSC takes into account, for the different species, the average time of disappearance (decay) of the excreta in the different habitats. For wild boar, the decay time is considered to be 125 days in open pasture, 46 days in the oak wood and 19 days in the chestnut wood.
The calculation of the estimate of the number of subjects present in the sample area derives from the following formula:

$$N = \frac{n}{DDR \times DR}$$

Where:
N= number of animals
n= number of faecal agglomerates
DDR= daily defecation rate
For wild boar the daily defecation rate is between 3.8 and 4.5 (Briedman, 1986)
DR= number of days for the faecal agglomeration to decay

c) The system of "Taking footprints in suitable environmental conditions" (e.g. ground covered by fresh snow) is a method which overall does not always provide completely reliable data and, therefore, is not used for the numerical calculation of the individuals who make-up the group (Gatto M. & Casagrandi R., 2003).

d) The measure of hunting effort is considered as an indirect index of the relative abundance of a given species in an area, but can, in theory, also be used to obtain an estimate of the absolute abundance of the target species.

The hunting statistics provide for the analysis of the data coming from the killings and represent a further method to evaluate some structural parameters of the populations of species subject to hunting (Gatto M. & Casagrandi R., 2003).
The evaluation of the hunting effort consists in quantifying the average number of hunting days necessary for the killing of a head of a species.
The method used to estimate fish populations and to evaluate the degree of exploitation of fishing banks has also proved to be applicable to terrestrial fauna, but with varying degrees of effectiveness. It is based on the De Lury depletion model (Sanders, 1988) and rests on some critical assumptions that must be respected so that the final result gives information corresponding to reality:

- The number of animals slaughtered per unit of time dedicated to their capture must be proportional to the population density;
- The population should be closed;
- The animals' vulnerability should be constant over time;
- The skills of those who collect data should not influence their quality;
- Hunting should be done individually.

The effectiveness of the model does not seem to be influenced by the factors vulnerability, closed population, hunter's ability. The result obtained, if compared for at least three consecutive years, provides a real indication of the population in a specific area. It seems obvious that even if the factors mentioned above were standardized, the result obtained would be more precise.
The hunting effort is measured by the "Catch Per Unit Effort" (CPUE) which indicates the ratio between the individuals hunted and the number of hunting days or hours necessary to kill the individuals. The CPUE is an almost always available source of information for harvested ungulates and can therefore be used to estimate population sizes or rates of population change (Crichton, 1993., Lancia et al., 1996). Numerous studies have demonstrated its usefulness as an index of abundance (Fryxell et al., 1991, Roseberry & Woolf, 1991).
The simplest model of the relationship between CPUE and abundance is represented by a linear equation (Ricker, 1940):

$$CPUE_t = qN_t$$

Were
q = huntability coefficient
N = abundance of the population under study
t= time required for shoot

In sostitution of the previous form, the relationship between effort and abundance can be represented by a curve described by a function where, in addition to the hunting coefficient, we also find an exponent (Cooke and Beddington, 1985):

$$CPUE_t = \alpha N_t^{\beta}$$

Were
α = huntability coefficient
β = huntability exponent
N = abundance of the population under study
t= time required for shoot

In this second model there are 3 possible cases of relationship between the CPUE and the abundance depending on the value assumed by β. The three possible cases are:
$\beta < 1$
$\beta = 1$
$\beta > 1$

The first case (β <1) occurs when the collection is extremely efficient or when it is concentrated in areas where the abundance of animals is higher or when the animals, even if in decline, tend to remain concentrated.
This situation is often referred to as "*hyperstability*".
In the second case (β = 1) the CPUE is proportional to the abundance.
The third case (β >1) occurs when CPUE decreases faster than abundance, for example if hunting first focuses on a small but extremely vulnerable slice of the population and then decreases in efficiency even though the population is still abundant (assumed of constant vulnerability). This situation is referred to by the term "*Hyperdepletion*"

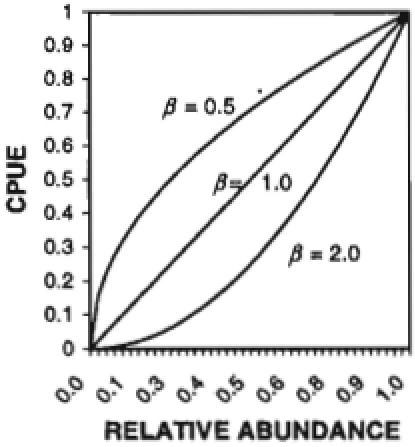


Figure 19. Three possible relationships between abundance and CPUE. Situation of "Hyperstability" (β = 0,5); proportionality (β = 1) e "Hyperdepletion" (β = 2) (Hatter, 2001).

The huntability of ungulates is influenced by several factors:

- variable weather conditions
- seasonal change of habitat
- ability to move (migrations)
- changes in the hunting calendarmbi del calendario venatorio
- evolution of hunting techniques

It can be argued that without independent estimates of absolute or relative abundance, the capture exponent cannot be estimated, and the reliability of the CPUE for determining the rate of change cannot be assessed (Hatter, 2001).

e) Use of camera traps from georeferenced points. In the preliminary investigations, the passage points of the family groups of wild boars at entry and exit points of the focal area were identified. As a result of these observations, 6 points were chosen. Each camera trap was fixed on natural supports and equipped with metal boxes to protect against atmospheric agents. In order to have as much information on the animals, each camera trap was reloaded with a new video card every week from 30 July 2019 to 24 August 2022. The camera trap model used is "Spromise"® Full HD + SMS (Digital Trail Camera S358). The weight of each camera trap is 0.38 kg while the dimensions are 15.3x12.4x9.0 cm. High resolution images (12 MP), infrared night lighting, motion sensors (PIR) set to high mode have been installed. Sound recording has been turned on to record animal sounds made during videos. Finally, the detection range and flash range of the camera traps are 25 meters and 18 meters respectively.

3.5.3 Experimental analysis

Experimental analysis is a method whose purpose is to subject a hypothesis advanced theoretically or as a result of previous descriptive studies to practical tests. For example, it is possible to take advantage of climatic changes, natural or otherwise, to evaluate the behavioral and/or biological variations of the subjects in question (Lovari S. & Rolando A., 2004).

3.5.4 – Index counting

The relative counts or by indices are aimed at defining indices of density or relative abundance when it is not possible to establish the exact size of a population, or of a part of it, in relation to the scarce permanence and the low coefficient of contactability of many species (Gatto M. & Casagrandi R. 2003).
A density index is an attribute that changes in a predictable manner with a change in population density. The indices, when repeated several times at established time intervals, provide information about the trend of a population. Instead, they can be used to estimate the absolute abundance of a population only if the relationship between an attribute that changes in a predictable way and the population under consideration is proportional, if it is then demonstrated through independent estimates of both.

Density indices can be direct and indirect.

1) Direct density indices. Among the direct density indices, the "kilometric abundance index (KAI - from Kilometric Aboundance Index)" is of particular importance, which allows you to make a direct comparison between the abundances of an animal species detected both in a single area but in periods different (population trends) and in sites with different territorial typologies. This index is expressed as the ratio between the number of sightings of a given species (or one of its subclasses) along the itinerary of a transect and the number of kilometers actually travelled. Numerous studies have in fact demonstrated the relationship between the kilometric abundance index and the absolute abundance of a given animal species, above all if the population in question exceeds a certain density (Vincent J. P. et al., 1991; Acevedo P. et al., 2008; Fernandez-de-Simon J. et al., 2011).

2) Indirect density indices. Among the commonly used indirect density indices there is the "Fecal detection in sample areas" (FAR) and the Faecal Standing Crop (FSC) which has already been mentioned above.

During the experimental period, priority was given to identifying the presence of wild boar in the focal area. At the same time, an attempt was made to identify the animal biodiversity that shared the habitat of the swine. To this end, it was decided to use the direct counting method for sample census for direct block census survey from a fixed location located in known passage points. 10 operators were assigned 5 observation posts in the four cardinal points and in the special observation point of the Focal Area. After 15 days, two hours of observation were carried out simultaneously by the observers in the twilight hours (before and after sunset) through the use of optical (binoculars, telescopes) and photographic equipment.
The accuracy of the data obtained (Fryxell J.M. et al., 2014) was assessed by the difference between the estimate made and the actual observations (accuracy) as well as by the repeatability of the actual observations (precision).
Four routes have been identified with a North-South and East-West orientation for a total length of approximately four kilometres (fig. 20).
On different dates from the direct census, the wild boar presence was indirectly determined in the Focal Area. All the signs of the presence (footprints, skin marks, hairs, roots, puddles, “insogli” and “lestre”) were detected and collected along the tracks, which were traveled every 15 days.
In specific points (georeferenced) of the Focal Area (crossing points, water points, refreshment points, feed points) 9 Camera Traps were mounted (fig. 21). Once a week, the operators replaced the memory cards from the Camera Traps.

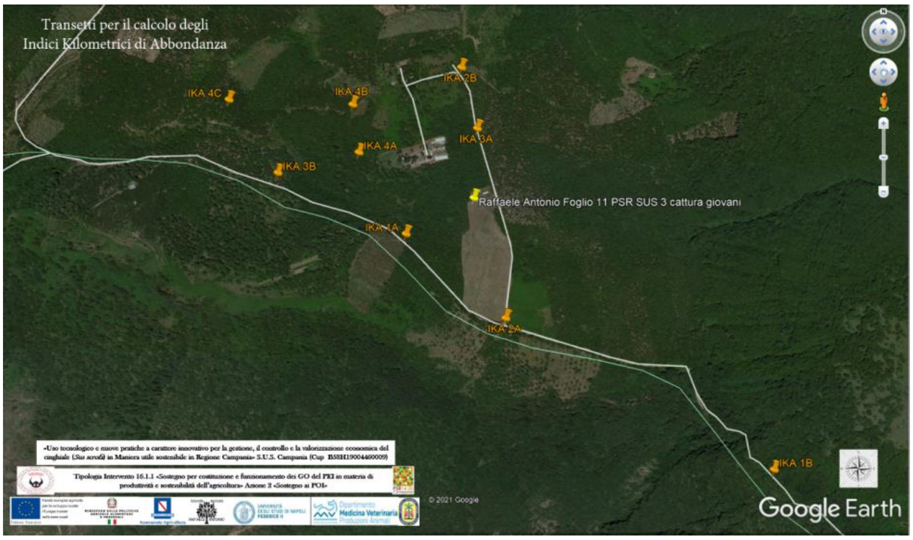


Figure 20 Paths for the indirect determination of wild boar presence into Focal Area.

The collected data was downloaded and stored in a dedicated memory to be subsequently analyzed and interpreted.

Figure 21 Camera Traps observation points around Focal Area.

Among the census systems present were used, the "Faecal Accumulation Rate"; the "Faecal Standing Crop"; the "taking of footprints in suitable environmental conditions"; the "measure of hunting effort". Despite having prepared structures for the "Capture-Marking-Recapture" due to a series of bureaucratic problems this method could not be implemented.

4. STATISTICAL ANALYSIS

4.1 ANALYSIS OF THE DENSITIES OF WILD POPULATIONS IN THE STUDY AREA

For the statistical analysis we proceeded, in the first instance, with the numerical quantification of the wild boar population present in the focal area. Subsequently, the densities of the individual animal species present were quantified. The data collection operation was repeated for three comparable periods (July 2019-July 2020; July 2020-July 2021; July 2021-July 2022). In the same period, the negative pressure exerted by wild boars on human activities (damages and road accidents) was quantified. The statistical analysis was performed using JMP® PRO14 software. For each species, the difference between the density detected over the years considered was compared using the t Test (*One sample t test*) in order to verify the significance of this difference. The results expressed as a percentage were compared using the test of independence of Chi Quadro.
It was decided to evaluate the density pairs referring to the probability of error, resulting from the test:
$p > 0,05$ not significant (NS)
$0,01 < p < 0,05$ statistically significanti (SS) ($p \leq 0,05$)
$0,001 < p < 0,01$ statistically very significant (MS) ($p \leq 0,01$)
$p < 0,001$ estremely significant (ES) ($p \leq 0,001$).

4.2 HUNTING EFFORT ANALYSIS

In the special wild boar hunting areas (ACS) present in the point area (PSR area; focal are) the hunting effort expressed as CPUE was calculated for each hunting season (2019-2020; 2020-2021; 2021-2022), as the ratio between the number of animals killed in that season and the sum of hunting days actually used to do so, in the time interval in which data are available for each ACS.
For the wild boar, a species for which hunting is authorized in the ACS, the correlation between the estimated population and the hunting effort was studied.
The Paerson correlation coefficient (r) was calculated as:

$$r = \frac{\sum_i (x_i - \overline{x})(y_i - \overline{y})}{\sqrt{\sum_i (x_i - \overline{x})^2}\sqrt{\sum_i (y_i - \overline{y})^2}}$$

Based on its r value, each correlation was placed into one of the following categories:

$0,7 < \|\mathbf{r}\| \leq 1$	Strong Correlation
$0,4 < \|\mathbf{r}\| < 0,7$	Moderate Correlation
$0,2 < \|\mathbf{r}\| < 0,4$	Weak Correlation
$0 \leq \|\mathbf{r}\| < 0,2$	Absence of Correlation

The level of significance expressed as a value is also reported together with each coefficient *p* (probability value).

Subsequently, for each pair in which a statistically significant correlation between abundance and hunting effort was demonstrated, a power regression ($f(x) = \alpha x \beta$) was performed to build a model capable of estimating the abundance of the especially starting from the CPUE values. The values of α and β were then used to predict the abundance of each species separately for each year and the data thus obtained were compared with those estimated in the field.
The difference for each "true abundance" – "CPUE predicted abundance" pair was described in percentage terms and the relationship studied.

Specie	α	β
Wilde Boar	0,335495	0,305496

In the final analysis, the densities detected in the different years of observation were compared.

4.3 CAMERA TRAPS

During the total experimental period it was possible to make 405 single evaluations on the animals captured by camera traps video, 302 examined snaps and 3.619 minutes of recorded images are studied. Time of entrance and time of exit from the observational point is measured. On the basis of a timescale of six hours each, the snaps were grouped into two different clusters: CL1 (6-11 pm); CL2 (11pm-4am). The frequencies obtained on the total number of observations per month were compared with the non-parametric Chi-square test assuming that the three age groups attended the observation point at the same times. In the same way, the presence of the other species was evaluated in the different times in which they appeared in the three years of observation.

5. RESULTS

5.1 CENSUS SPECIES REFERRED TO ART. 4 OF DIRECTIVE 2009/147/EC AND LISTED IN ANNEX II OF DIRECTIVE 92/43/EEC AND SITE EVALUATION FOR THEM

In the area subject to intervention, census operations were carried out with help of the Regional Center for Monitoring and Management of the Agro-Ecosystem of Campania (MoGAE - DG 730 of 27/11/2017). In the period 30 July 2019 – 24 August 2022 census operations were sistematically continued within the point areas (PSR Area; Focal Area).

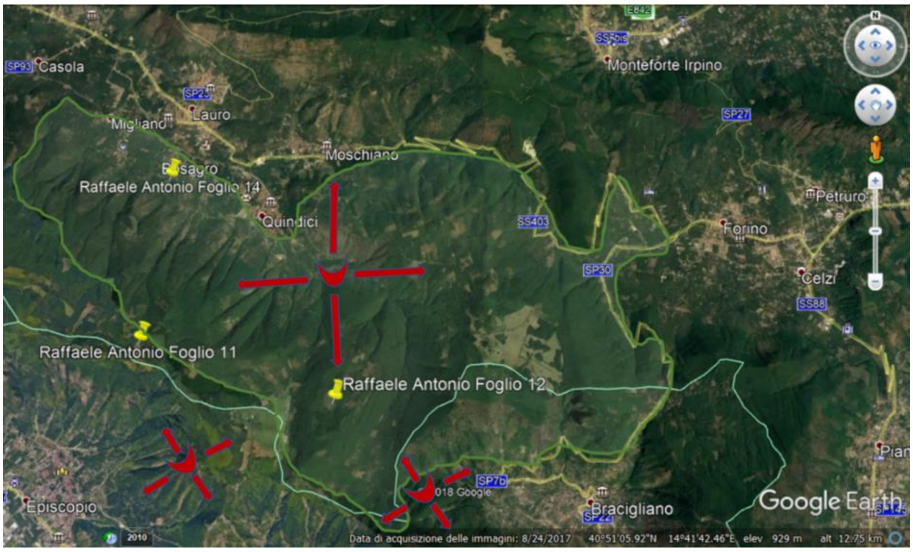


Figure 22. Area of intervention and points of dispersion of the wild boar.

In particular, the presence of some species listed in the Natura 2000 File - Standard Data Form relating to SAC/SCI IT8040013 "Monti di Lauro" - point 3 Ecological Information (2019-12 update) was found in the Focal Area (fig. 22).

The results obtained from sampling with Camera Trap allowed to identify the following species: *Streptopelia turtur (A210)*; *Turdus merula (A283)*; *Turdus philomelos (A285)*.
In addition, in the focal area we have detected the presence of wild species not reported in the SAC/SCI IT8040013: *Athene noctua*; *Buteo buteo*; *Columba palumbus*; *Corvus cornix*; *Falco tinnunculus*; *Garrulus glandarius*; *Phasianus colchicus*; *Pica pica*; *Picus viridis*; *Strix alauco; Canis lupus; Erinaceus europaeus Lepus europaeus*; *Martes foina*; *Martes martes*; *Talpa europaea*; *Vulpes vulpes*.
Finally domestic animals were signaled in focal area: *Felis catus*; *Canis lupus familiaris*; *Ovis aries*.

5.2 WILD BOAR CENSUS

Starting from 30 July 2019, in the three years of activity, the research group has dedicated itself to census activities for the definition of the number of wild boars on which to structure the multi-criteria Zootechnical Model (TBL) for the containment of wild boars in the Campania Region and quantify his ecosystem value. We started from the Focal Area (Antonio Raffaele farm) and then extrapolated the precise data obtained to the entire PSR Area. It was not possible to use the data obtained to estimate the distribution of wild boars in the entire Avellino's Province but we extrapolated the data from the Focal Area to estimate the number of wild boars in the Wide Area.

5.2.1 - Number of wild boars in the Wide Area

Wild boar populations are significantly increasing in all Italian agro-forestry-pastoral territories and represent an emerging problem. However, the Ungulate Data Bank (ISPRA) complains about a serious lack of information about the size of the wild boar populations and reports a population of one and a half million distributed over the national surface (ISPRA, 2023). In Campania, ISPRA (2009) reports a number of wild boars killed in the 2004-2005 hunting year of 2,100 heads (significantly underestimated) while in the Extraordinary Plan "Emergency Wild Boars" in Campania the samples examined by the IZSM for health checks went from 151 of 2010 to 4,508 of 2015 and the Plan provides for the increase of controls, starting from 2016, on 12,000 samples (http://burc.regione.campania.it).
The Campania region, on the https://www.campaniacaccia.it/cinghialefaq.php site, reports that in the last four hunting seasons, on average, between 9,000 and 10,000 wild boars have been killed exclusively with the "hounded course hunting" method.
In 2009, 1,360 wild boars reared in family or industrial farms were reported in the Campania Region (Fontana et al., 1999), of these 620 were bred in the province of Avellino in 124 farms (Fontana et al., 2007). Today it seems that in the province of Avellino there are 33 family farms where there would be 112 wild boars raised (PFV AV 2019-2024) even if their real location is unknown.
Our observations carried out between the first and third year of the study (2019-2022) allowed us to extrapolate the data and, therefore, estimate the number of wild boars also in the Wide Area (31,800 hectares) of the Campania Region (Rural Territorial System 18 : Monte Partenio, Monti di Avella, Pizzo di Alvano). The numerical estimate, although not precise, appears to be fundamental in the innovative process proposed for the demographic containment of wild boars which is based on standardized management processes.
On the basis of the censuses carried out in the Focal Area it is possible to estimate in the Wide Area between 7,018 and 8,214 subjects. The value is only indicative as it is calculated in proportion to what is obtained from the census operations (direct and indirect) in the Focal Area.

5.2.2 - Number of wild boars in the Focal Area

In the period 2019 - 2022, the census operations were carried out with the combined census methods described in the materials and methods (direct and indirect). The results obtained allowed to calculate the average number of animals representative of the environments present in the Focal Area. The numerical estimate is reported in the respective tables (tabs. 2, 3, 4, 5).
In the period 01 August - 31 December 2019 the census operations, in addition to the direct and indirect survey activities, were carried out using the visual control operated through camera traps made available by the MoGAE center.
The numerical estimate of the wild boars presents in the Focal Area during the year 2019 is shown in table 2. Total number of wild boars surveyed (all age classes) varied from a maximum of 240 specimens (end of the breeding season) to a minimum of 86 (end of the hunting season).

Table 2. Wild boar numerical estimate (combined census operation) during period 01 August - 31 December 2019 (in red hunting season).

	2019				
	A	S	O	N	D
Estimated number	240	240	199	131	86

The numerical estimate of the wild boars presents in the Focal Area during the year 2020 is shown in table 3. Total number of wild boars surveyed (all age classes) varied from a maximum of 239 specimens (end of breeding season) to a minimum of 123 (end of the hunting season).

Table 3. Wild boar numerical estimate (combined census operation) during period 01 January - 31 December 2020 (in red hunting season).

	2020											
	G	F	M	A	M	G	L	A	S	O	N	D
Estimated number	123	144	172	200	225	231	239	234	234	219	196	173

The numerical estimate of the wild boars presents in the Focal Area during the year 2021 is shown in table 4. Total number of wild boars surveyed (all age classes) varied from a maximum of 290 specimens (near the end of breeding season) to a minimum of 189 (end of the hunting season).

Table 4. Wild boar numerical estimate (combined census operation) during period 01 January - 31 December 2021 (in red hunting season).

	2021											
	G	F	M	A	M	G	L	A	S	O	N	D
Estimated number	189	216	232	262	284	290	284	270	278	254	216	190

The numerical estimate of the wild boars presents in the Focal Area during the year 2022 is shown in table 5. Total number of wild boars surveyed (all age classes) varied from a maximum of 219 specimens (at the start of breeding season) to a minimum of 122 (end of the hunting season).

Table 5. Wild boar numerical estimate (combined census operation) during period 01 January - 31 December 2022 (in red hunting season).

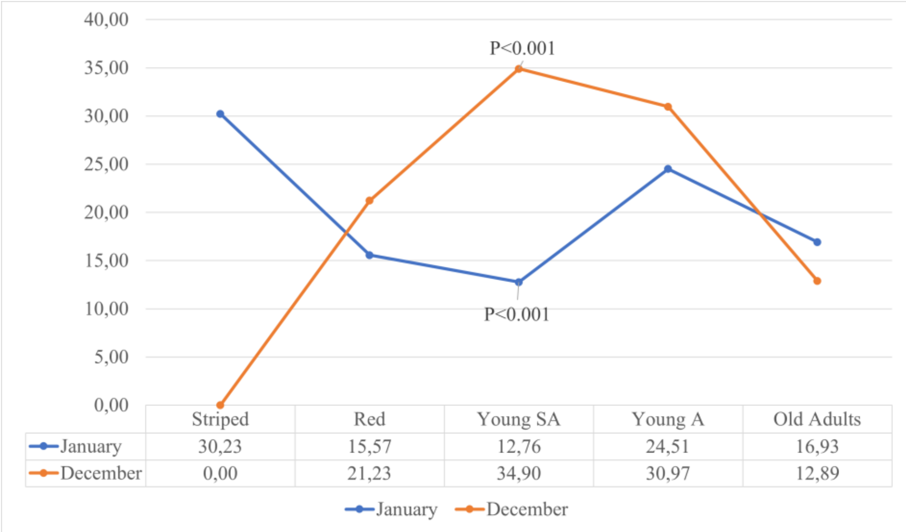
	2022											
	G	F	M	A	M	G	L	A	S	O	N	D
Estimated number	196	196	205	203	204	214	216	216	219	188	150	122

The use of camera traps and the direct census operations made it possible to identify and quantify the different age groups within the Focal Area (tab. 6).

Table 6. Estimate of the wild boars' class of age present in the Focal Area.

	2019		2020		2021		2022	
	Aug	Dec	Jan	Dec	Jan	Dec	Jan	Dec
Striped	78	0	31	0	61	0	64	0
Red	87	21	35	41	15	47	20	19
Young Subadults	0	29	0	53	4	71	71	44
Young Adults	40	19	38	62	57	49	25	38
Old Adults	35	17	19	17	52	23	16	21
Total	240	86	123	173	189	190	196	122

Table 6 shows the number of animals observed in the various years, divided by age group.



Graph 1. Distribution of class of age at the end of the hunting season.

Graph 1 shows the average presence of animals belonging to the different age groups between the years 2020-2022 in the months of January and December. The two months considered collect the animals that survived the hunting season. The comparison between the age groups shows a highly significant difference ($P<0.0001$) only for the animals of the young subadult age group (9-12 months). The number of young subadult subjects present in the month of December drastically decreases in the month of January. It can therefore be hypothesized that a certain number of animals is killed during the hunt and that a part of the survivors disperses to form new families. The absence of differences between the other age groups would mean that the structured family groups remain in the Focal Area.

If we consider the average of only adult animals (old adults 19.50 min 24.66 max), the calculation of the number per 100 hectares returns a number of animals 415 min and 525 max in 100 hectares.

5.3 Number of boars killed in ACS 04LB and 05 LB of District 10

The hunting teams operating in the observation area fall within the District 10 "Vallo di Lauro Baianese" and are the ACS04LB and the ACS05LB. The two teams are each made up of thirty hunters whose responsibility is attributed to a Huntsman. In accordance with the Hunting Calendars, teams can only go wild boar hunting from 1st October to 31st December for a defined number of 34 days overall. Each hunter, for each hunting season, pays an equal concession fee.

The authorized hunting teams killed a different number of wild boars (all age groups except striped) in the years considered. The highest number of killed animals was that relating to the year 2019 (155). In the years 2020 and 2021, as a result of the pandemic lockdown, the number of wild boars killed resulted lower (tab. 7).

Table 7. Number of wild boars killed near Focal Area (ACS) in different years. Costs related with hunting activity.

	2019	2020	2021	2022
Number of wild boars killed	155	84	88	97
Cost of hunting concession / year	451	451	451	451
Cost of hunting day / year	2.380	2.380	2.380	2.380
Cost of cartridges / year	350	350	350	350
Cost of dogs / year	1.495	1.495	1.495	1.495
una tantum costs	2.450			

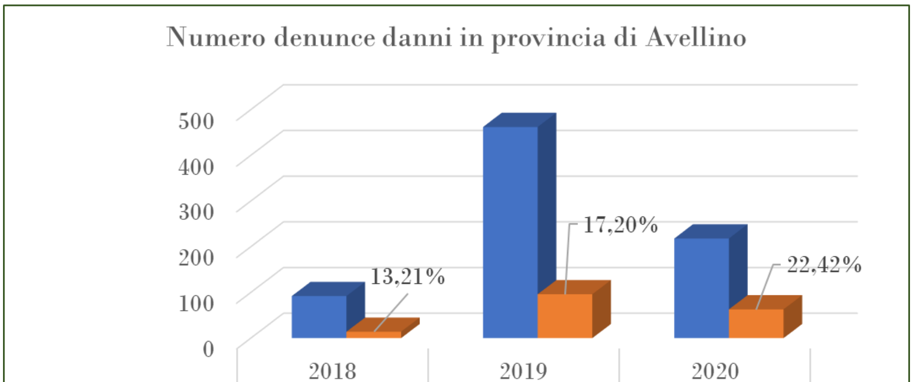
In order to evaluate the economic value linked to the wild boar, table 7 shows also the costs incurred by each hunter for a hunting season.

5.4. EVALUATION OF THE NEGATIVE IMPACTS ATTRIBUTED TO THE WILD BOAR ON ANTHROPIC ACTIVITIES

Among the wild animals, the wild boar is the one best known by the public opinion and whose presence is the most disputed due to its association with damage to agriculture (Amici et al., 2012; Geisser, Reyer & Krausman, 2004; Herrero et al., 2006; Ficetola et al., 2014; Laznik & Trdan, 2014), road accidents (Primi et al., 2009; Kruuse, Enno & Oja, 2016) and, in recent years, the invasion of cities (Castillo-Contreras et al., 2021). In the 21st century, or rather the era defined by the "social networks", in the web spaces dedicated to animals, the wild boar appears to be highly competitive with the most loved cats and dogs but, unlike these, they seem to be hated for their well quantified, population explosion (Saez-Royuela & Telleriia, 1986; Massei et al., 2014). It is currently considered one of the most invasive species on the planet after man (Lowe et al., 2018), with which it has always had a close relationship. Over the decades, the wild boar populations present in Italy (Apollonio, Randi & Toso, 1988; Gallo Orsi et al., 1995) have undergone the influence of restocking carried out, first for wildlife purposes and immediately afterwards for hunting purposes. Since the 1950s, the introduction of animals from northeastern Europe (Scillitani, Monaco & Toso, 2010; Amici et al., 2015) and uncontrolled hybridization with domestic pigs (Marsico et al., 2007; Schleimer et al., 2022) influenced the original phenotypic expression (Pedone, Mattioli & Mattioli, 1995). In the three years of the research doctorate, the objective was pursued of tackling the problems related to the conflict between natural resources, such as wild boars, with agricultural and zootechnical activities, producing ecosystem services or disservices; in this chapter the disservices connected to the presence of the wild boar will be dealt with

5.4.1 Trend of damage to the agricultural sector in the province of avellino: psr sus area and focal area

The data provided by the Campania Region STP Avellino for the three-year period 2018-2020 indicate that the trend of damage to the agricultural sector caused by wild boar shows an undulating trend due to the high number of complaints recorded in 2019 (fig. 23).



	2018	2019	2020
Provincia AV	92	462	218
n. Area Vasta	14	96	63

Figure 23. Number of wild boar damages to agriculture: Comparison between the Avellino's Province and the PSR Area.

By extrapolating the PSR Area from the provincial data, it is possible to note that the trend of wild boar damages shows an increasing trend with the highest percentage of damages reported in the year 2020. Examining the eight municipalities of the PSR Area S.U.S. (fig. 24) it is possible to report the absence of complaints in the municipalities of Domicella and Pago del Vallo di Lauro while a growing number of complaints starting from Monteforte Irpino to reach the peak in the municipality of Quindici which expresses the highest percentage of damagesThe town of Fifteen is also the town hosting the S.U.S. Campania and represents the focal area of the proposed Plan. The S.U.S. Project Campania highlights the need to identify an adequate integrated management strategy and proposes experimental capture for which an opinion is sought. The preliminary investigations conducted in 2019 and 2020 by the S.U.S. Campania indicate that the data provided by the Campania Region are largely underestimated since the interviews carried out with local farmers show a failure to present the compensation request.

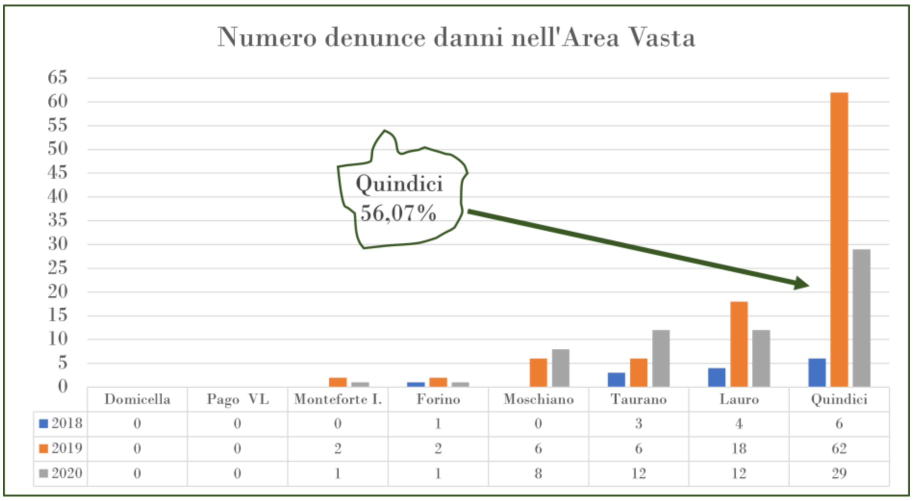


Figure 24. Number of wild boar damages to agriculture in the municipalities of the PSR Area.

The latter cause, which leads to an indefinable underestimation of the damages, is due to various factors, including:

- many companies, after years of delays and only partial acknowledgment of the damage suffered against advance investigation costs, do not report the harmful event due to the distrust they now place in the compensation system;
- many companies do not report the harmful event as they are not eligible for compensation under the "de minimis" regime which provides for the disbursement of a maximum of €15,000.00 over three years;
- many harmful events remain submerged because they are suffered by categories other than those of "Direct Farmers" and "Professional Agricultural Entrepreneurs" who, pursuant to the regional law, are the only ones entitled to compensation.

5.4.2 Damages to the agricultural sector in the psr area and in the focal area

Within the perimeter of the PSR SUS Area, the trend in the value of damage to agricultural crops underwent a sharp increase in the three-year period 2018-2020. However, from the interviews carried out in the three-year PhD period (2019-2022) it emerged that, due to the inefficiency of the damage verification systems by the Region as well as the enormous delay in paying the damages suffered in previous years, many farmers prefer not to present a claim for compensation and that, therefore, the damages are not reported causing an underestimation of the same. In this paragraph, for a detailed analysis of the characteristics of the damage, it is deemed necessary to refer to the most recent information available, i.e. that relating to the period 2018-2020. The category most affected is that of nuts (hazelnuts and chestnuts), followed by arable land, arable land and coppice (fig. 25).

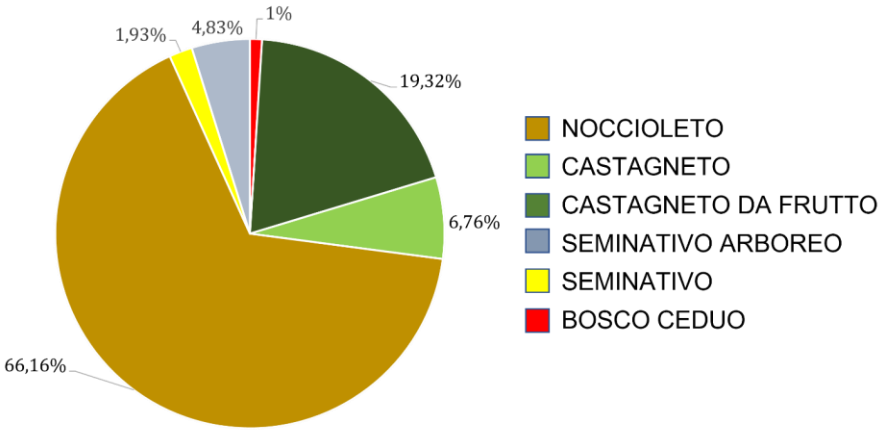


Figure 25. Damages from wild boar, by macro-category, estimated in the period 2018-2020 in the PSR area.

The breakdown by type of crop reflects the agricultural vocation of the district identified even if the damages are characterized differently in the three years considered. Damage to hazelnut and chestnut groves was reported in all three years considered, while in 2020 damage was reported to traditional arable land or trees and to coppice (fig. 26).

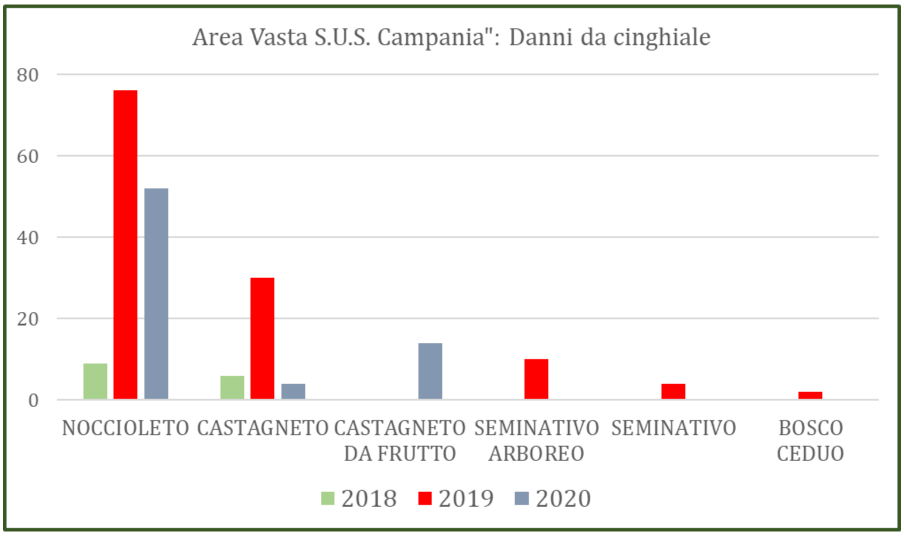


Figure 26. Damages from wild boar, by macro-category, estimated in the period 2018-2020 in the PSR Area.

The analysis of the 2018-2020 period (fig. 27) allows us to highlight that the damage caused by the wild boar to agricultural crops is directed towards the Agricultural Woody Crops and in particular towards the hazelnut groves, vineyards and chestnut groves followed by the arable land and in particular by the seed plants (wheat, oats and corn) and a discreet attention to forage crops and in particular to clover. It should also be reported that the ATC of the province of Avellino receives continuous reports of damage from agricultural associations and individual farmers, which however do not translate into requests for compensation. The estimates provided show a localization of the damage distributed over the whole area dedicated to agricultural activities in the province of Avellino, with more evident problems in the vicinity of the Protected Areas and the Oasis of Protection. In general, considering both the damages officially reported and the reports that have not resulted in requests for compensation, the situation does not seem to have substantially changed compared to previous years, even if the latter are not supported by comparable data.

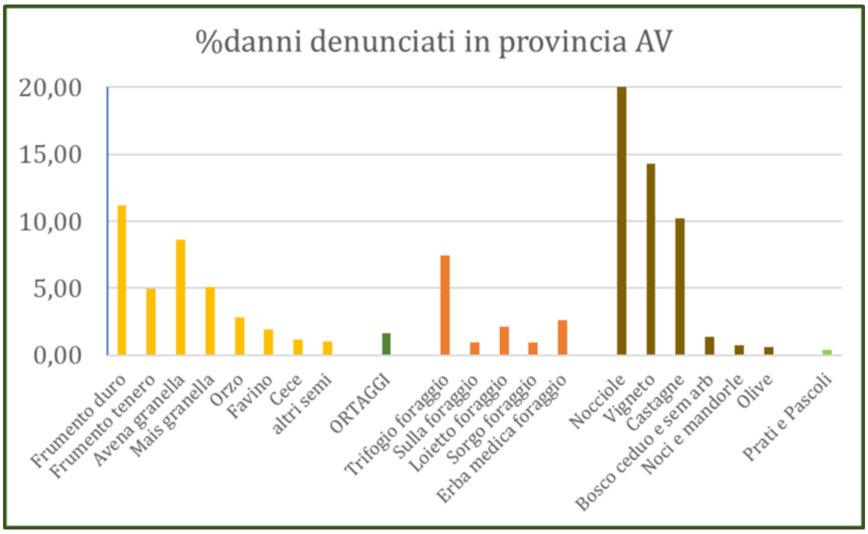


Figure 27. Number of wild boar damages to reported crops in the province of Avellino in the period 2018-2020.

5.4.3 Trend of road accidents in the province of Avellino: psr sus area and focal area

The Provincial Territorial Service of Avellino has provided the database relating to road accidents caused by wild boars on the various roads in the province of Avellino in the period 2016-2020. The trend of claims was growing (fig. 28) increasing by 3.5 times in 2020 compared to 2016. It should be noted that, at the moment, the "Selective sampling of wild boar in unsuitable areas of the Campania Region" (DD n. 33 of 12/02/2021) which was to be operational from 1 February - 31 December 2021, has not yet given results and, the measures and initiatives for the prevention and protection of road accidents implemented by the managing bodies of the road networks, they do not seem to be able to control the phenomenon.

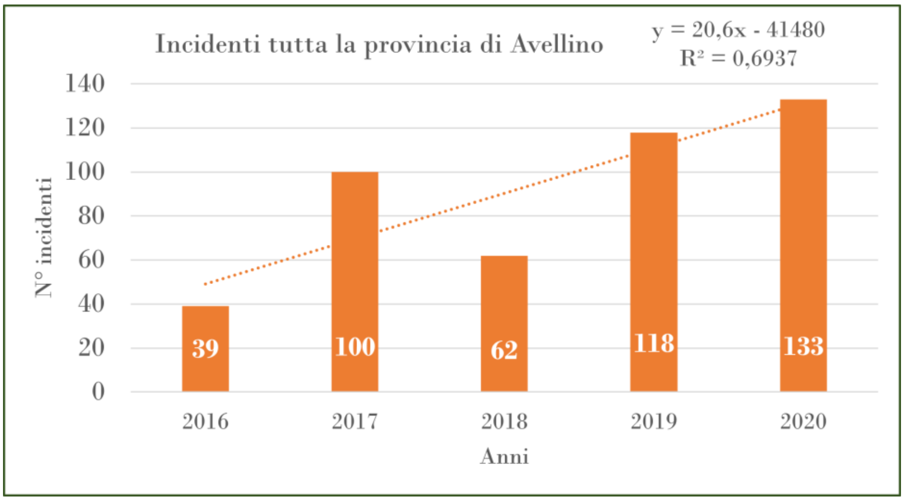


Figure 28. Number of road accidents caused by wild boar in Avellino's Province.

Up to now, therefore, it seems that only the intensification/rationalization of hunting in collective forms and the efforts in prevention and dissuasion, made by the ATC and the locally involved wild boar hunting teams, have ensured a slight decrease in the impact indicators agricultural which, however, have produced a significant numerical increase in wild boar (demonstrated in the focal area) due to the downsizing of wild boar hunting in the 2019-2020 and 2020-2021 hunting years, due to the ban on circulation following the COVID 19 Pandemic.
For the years 2019 and 2020, the results of insufficient demographic control of the wild boar can be linked to the bureaucratic slowdowns that the "Wildlife-Hunting Plan" and the "Management and control interventions of the wild boar in Campania" plan both under Evaluation procedure VAS + VI. Therefore, the elements of necessity remain for the planning of interventions to prevent damage and to reduce the number of the population, both through the strengthening of hunting and numerical control with alternative methods to shooting.

5.4.4 Toad accidents in the province of Avellino and in the PSR Area

In 2016 and 2020, 452 road accidents caused by wild boar were recorded and officially reported on the roads of the entire province of Avellino (fig. 29).
Road accidents have undergone a markedly increasing trend in recent years, currently amounting to around 100 accidents/year in the province of Avellino. This evidence is to be considered above all with respect to public safety, which is seriously endangered by the increasingly frequent impact between animals and cars along provincial and state roads.

Figure 29. Distribution of road accidents caused by wild boar in Avellino's Province in the years 2016-2020.

5.4.5 Reporting by citizens of problem events caused by wild boar in the Avellino's province

Together with what has already been highlighted in relation to damage to agricultural crops and road accidents, reports are increasingly frequent, from citizens living in the Irpinia countryside and those who live in the urban territory of the cities bordering agricultural areas and woodlands, as well as by some agricultural associations, of inconvenience due to the presence of single individuals and/or groups of wild boar.
These reports refer to encounters near homes, road crossings, incursions into public and private green areas, etc. Many of these are also reported by the local press, which also receive video-photographic footage of the events.

Here is a brief press review of some local newspapers:

15/09/2015 h ttps://www.binews.it/lauro-e-dintorni/quindici-emergenza-cinghiali-nel-vallo-la-testimonianza-di-un-anziano-contadino-e-linteressamento-di-rubinaccio/

30/08/2017 https://youtu.be/yfKBesr259w?t=20

08/07/2021 https://www.youtube.com/watch?v=go8CEwIBncE

05/08/2021 www.irpinia24.it/wp/blog/2021/08/05/ariano-irpino-continuano-le-attivita-estive/

08/09/2021 Allarme cinghiali in Irpinia, Regione e associazioni: "Stiamo studiando un modo per sopperire alla vicenda" | Irpinia24

25/11/2022
ttps://www.ottopagine.it/av/economia/275101/allarme-cinghiali-e-strategie-di-contenimento-esperti-a-confronto-ad-avellino.shtml

30/12/2022
https://www.cronachedellacampania.it/2022/12/avellino-auto-si-ribalta-per-evitare-cinghiali-in-strada-3-feriti/

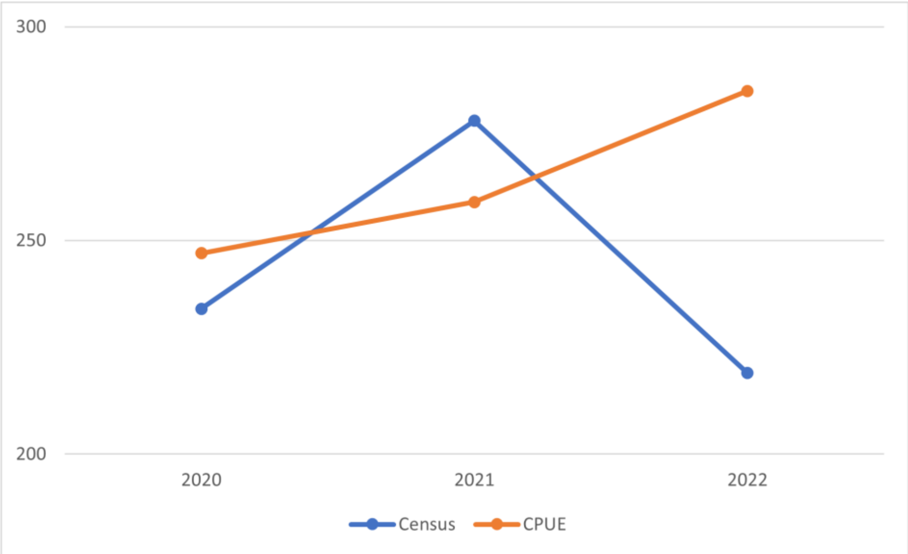
In the face of reports from citizens, the Prefectures stimulated by the flash-mobs and by the demonstrations through which they wanted to sensitize the institutions, have begun to address the wild boar problem to identify and adopt suitable measures to stem the risk of damage from wildlife.
During the last few years the problem, amplified by the media, has overwhelmed the first citizens who have found themselves forced to issue, not always in accordance with current legislation, Ordinances of Mayors for the capture of wild boars considered dangerous for public safety, as they circulate in the vicinity of homes.
It was also considered appropriate to highlight this aspect linked to citizens' concerns, for a better understanding of the ongoing phenomenon. In this regard, it is hoped that citizens and all social partners will be increasingly involved in the management of the wild boar (as well as other wild species), in order to reach a real solution to the problems.

5.4.6 Hunting effort analysis results

Hunting effort (CPUE) and wild boar numerical abundance in the two ACS involving swine populations in the Focal Area (r= - 0.44; P <0.05). Table 8 shows the results of the censuses carried out according to the two counting methods. Even if the correlation indicates a moderate association with the negative r index, the CPUE value was able to predict the population increase that occurred in the 2019-2020 and 2021-2022 hunting seasons. Even the predicted decrease in the 2020-2021 season would seem to have occurred (Graf. 2).

Table 8. Hunting season, census population, CPUE and population estimated by CPUE of the wild boar (Sus scrofa) in the ACS bordering the Focal Area.

Species	Hunting season	Census population	CPUE*100 Estimate poplation
Wild boar	2019-2020	234	247
	2020-2021	278	259
	2021-2022	219	285



Graphic 2. Comparison between the real population and the population estimated by CPUE of wild boar in the Focal Area.

6. OTHER FACTORS NECESSARY FOR THE CALCULATION OF THE GENERAL ECOSYSTEM VALUE RELATED TO THE PRESENCE OF THE WILD BOAR

6.1 CENSUS OF STRUCTURES POTENTIALLY CONNECTED TO THE PRESENCE OF WILD BOARS

In order to produce an estimate of the Ecosystem Services potentially connected to the presence of the wild boar, the research group also surveyed the factories and operators in the primary sector (farms, livestock farms, agritourism companies) and in the secondary sector (slaughterhouses, meat processing laboratories , food distribution areas) that could have been involved in the creation of the "S.U.S. Campania", in the absence of the advent of reports of African swine fever in Italy starting from 07 January 2022.

The results obtained from the study of the problems connected to agro-food production and the conflict with the wild boar are summarized below.

- ✓ **Landscape.**Within the eight Rural Territorial Systems involving the Municipalities of Irpinia, the POI S.U.S. Campania develops in an extremely articulated territory, made up of high and medium mountain landscapes (STR18: Monte Partenio Monti di Avella Pizzo d'Alvano).
- ✓ **Habitat**. The characterizing environment with which you have to work is made up of the typical altitudinal succession of the environments of the Campania Apennines (beech woods, grasslands of the peaks, summit karst plateaus, deciduous woods, xerophilous grasslands) with a total forest area of about 16,955 ha and a scarce presence of pasture.
- ✓ **Agricultre**. Agricultural activities develop on the foothills and are mainly characterized by tree crops (specialized hazelnut groves and fruit chestnut groves). The remaining portions of the territory are occupied by arable land and industrial crops, interspersed with vineyards and fruit orchards.
- ✓ **Farmhouse**. In the list of agritourism operators in the Campania Region, 125 agritourism companies are registered located in 57 Irpinia municipalities. In the first year of activity, the POI verified the existence and real vocation of 19 agritourisms present in 15 Municipalities (Avella, Avellino, Atripalda, Cesinali, Contrada, Domicella, Forino, Grottolella, Monteforte Irpino, Moschiano, Pago del Vallo di Lauro ; Pietrastornina, Salza Irpina, Santo Stefano del Sole, Summonte) which can be reached within 10 km from the intervention area in Quindici.
- ✓ **Slaughterhouses**. The slaughter of captured animals represents the critical point to be addressed with the Mayor and the competent ASL. Currently, in the province of Avellino, there are 19 slaughterhouses in which, in 10, cattle, sheep, goats and solipeds are slaughtered in addition to swine; in 6 cattle and sheep and goats; in 1 sheep and goats and in a factory only the pig species is slaughtered. Since the slaughterhouses in Irpinia do not appear to slaughter wild boars and in the whole province of Avellino there are no officially present processing centers for game killed in hunting attached to fresh meat production plants, the best proposal that can be made by the POI S.U.S. Campania consists in activating the S.U.S. Campania by regulating and controlling the slaughter of wild boar directly in the companies which will be entrusted with the captured striped. This proposal is justified by the presence in the Campania Region of a disciplinary that allows private slaughtering for self-consumption ("Regulation of private slaughtering" - DGR n. 2234 of 06/07/2002).
- ✓ **Urbanization**. Between 1970 and 2020 the urban area that occupied 2% of the total area, today occupies 6% of the Irpinia territory. The anthropic invasion has led to the fragmentation of habitats which has produced a double negative result: 1) the reduction of habitats available for wildlife; 2) the easier accessibility of fauna in urban centers through the roads. Both cases have produced a greater presence of wild animals in areas frequented by humans and, as a result, an increase in claims.

7 MONETARY EVALUATION OF ECOSYSTEM SERVICES RELATED TO WILD BOAR

7.1 ECOSYSTEM DISSERVICES

Our study starts from Liekens' citation et al. (2013) "*The monetary valuation of ecosystem services offers a promising approach to highlight the relevance of ES to society and the economy, to serve as an element in the development of cost-effective policy instruments for nature restoration and management, and to use in impact assessments in cost-benefit analysis*". In order to estimate the economic value of the Ecosystem Services linked to the presence of the wild boar in the study area, we started from the evaluation of the disservices clearly attributed to the wild swine.

7.1.1 Damage to the agricultural sector in the study areas

Table 9 shows the wild boar damage reported in the observation areas and it can be deduced that in the habitats considered natural or in any case not managed primarily by man, the areas affected by wild boar only concern the year 2019 for a very small percentage (min 0.03% max 0.58%).

Table 9. Agronomic classification of crops presents in the PSR Area with the relative percentages of area damaged by wild boar in the years made available by the Campania Region.

Classification		2018		2019		2020	
	Total surface	Damage	%	Damage	%	Damage	%
WOOD	Ha						
Avellino Province	38,704.00	0.00	0.00	0.00	0.00	0.00	0.00
PSR Area	3,173.62	0.00	0.00	2.40	0.08	0.00	0.00
Focal Area	413.07	0.00	0.00	2.40	0.58	0.00	0.00
PASTURE							
Avellino Province	15,606.48	0.00	0.00	4.00	0.03	1.00	0.01
PSR Area	118.77	0.00	0.00	0.00	0.00	0.00	0.00
Focal Area	77.10	0.00	0.00	0.00	0.00	0.00	0.00

It appears evident that the reports of damage to these two habitats cannot be considered real since it is known that woods and pastures are considered suitable areas for wild boar and their presence is proven by the plowing of the topsoil referred to the two habitats which benefit from surface rooting.
The monetary value referring to the surfaces reported, equal to 4 hectares and 80 (sum of the PSR Area and the Focal Area) of forest and 4 hectares of pasture in the province of Avellino, not being reliable, are not quantified.
More substantial are the damages attributed to the wild boar on tree crops, among which hazelnut and chestnut are the most affected crops.
The Focal Area reports the highest percentage of damage to chestnuts and hazelnuts in 2019, as do the PSR SUS area and the Provincial AV area, albeit with low percentages (tab. 10).
If the yields per hectare of each tree cultivation are calculated and the entire annual production is considered lost for the largest area damaged in the three-year period considered, it follows that the province of Avellino declares damages for chestnut equal to 97.78 ha; the core 103.60 ha; 134.01 ha for vines and 6.16 ha for olive groves, respectively equivalent to €244,450; €518,000; €804,055 and €9,856.

Table 10. Agronomic classification of crops presents in the PSR Area with the relative percentages of area damaged by wild boar in the years made available by the Campania Region.

Classification	Total surface	2018 Damage	2018 %	2019 Damage	2019 %	2020 Damage	2020 %
CHESTNUT GROVE							
Avellino Province	2,459.35	12.16	0.49	97.78	3.98	25.37	1.03
PSR Area	1,130.82	12.96	1.15	50.62	4.48	29.17	2.58
Focal Area	219.35	11.17	5.09	42.12	19.20	25.50	11.63
HAZELNUT							
Avellino Province	6,322.87	18.15	0.29	68.38	1.08	103.60	1.64
PSR Area	2,908.05	13.46	0.46	92.98	3.20	57.48	1.98
Focal Area	563.97	3.20	0.57	64.80	11.49	33.85	6.00
VINEYARD							
Avellino Province	6,916,39	8.00	0.12	134.01	1.94	111.74	1.62
PSR Area	38.78	0.00	0.00	0.00	0.00	0.00	0.00
Focal Area	0.00	0.00	0.00	0.00	0.00	0.00	0.00
OLIVE GROVE							
Avellino Province	7,294.89	0.00	0.00	6.16	0.08	1.00	0.01
PSR Area	158.32	0.00	0.00	0.00	0.00	0.00	0.00
Focal Area	1.77	0.00	0.00	0.00	0.00	0.00	0.00

7.1.2 Road accidents in the PSR Area

The data provided by the regional administration of Campania, relating to the years 2019 and 2020, indicate that, in the municipalities in which the Point Area falls, 10 car accidents were recorded due to wild boar. Table 11 shows the number of road accidents recorded in the individual municipalities of the Point Area (PSR Area and Focal Area).

The average monetary value, calculated on the basis of the compensation recognized by the Campania Region for recognized road accidents attributable to wild boar, was Euro 2,500 therefore, the total compensation would amount to 25,000 Euros.

Table 11. Road accidents recorded in the PSR Area in the period 2019-2020 and average compensation.

Municipality	n.	€ average	€ tot
DOMICELLA	0		
FORINO	2		
LAURO	0		
MONTEFORTE IRPINO	1		
MOSCHIANO	3		
PAGO DEL VALLO DI LAURO	2		
QUINDICI	2		
TAURANO	0		
Total	10	2,500.00	25,000.00

7.2 ECOSYSTEM SERVICES

In order to estimate the economic value of the Ecosystem Services linked to the presence of the wild boar in the study area, we complete our evaluation by quantifying the services clearly attributed to the wild swine.

7.2.1 Provisioning related with wild boar presence

Table 12 lists the possible revenues in Euros for the different salable parts of the wild boar.

Table 12. Wild Boar salable parts and monetary value in Euros.

	Unit	cost/unit	n. shot WB	Monetary value
Wild boar	1 animal		106	Euro
Meat	1 kg	10	5,088	50.880
Pappardelle	1 kg	40	5,088	203.520
Cured meat	1 kg	47	3,562	167.414
Teeths	1 teeth	1,5	424	636
Bristle	1 kg	989	10.6	10.483
Complete skin	1 unit	360	106	38.160
Head	1 unit	150	106	15.900
Hoof	1 unit	109	424	46.216

Table 13 shows 5 different choices to obtain from each wild boar killed during the hunting season an economic value attributable to the supply factor.
Although an indisputable monetary advantage can be identified for each choice, with maximum optimization it is evident that the most economically advantageous choice is choice 4 deriving from the combination of the sale of meat in the form of typical dishes at the restaurant; of the 4 canines; of whole skin after taxidermy treatment. However, since taxidermy is not required for every animal killed, choice 2 would seem to be the really best one (sum of the pappardelle gastronomic dish; the teeth and bristles).

Table 13. Wild Boar salable parts and monetary value in Euros.

				choise 1	choise 2	choise 3	choise 4	choise 5
Wild boar	1 animal	€/U	Quantity	1	1	1	1	1
Meat	1 kg	10	48 Kg	480.00				
Pappardelle	1 kg	40	48 Kg		1,920.00		1.920	1.920
Cured meat	1 kg	47	33 Kg			1.579		
Teeths	1 teeth	1,5	4 U	6.00	6.00	6.00	6.00	6.00
Bristle	1 kg	989	100 gr	98.90	98.90	98.90		
Complete skin	1 unit	360	1 U				360.00	
Head	1 unit	150	1 U					150.00
Hoof	1 unit	109	4 U					109.00
				584.90	2,024.90	1,683.90	2,286.00	2,185.00

Finally, table 14 shows the sum of the monetary value of the provisioning that at the end of the hunting season could be obtained from the killing of wild boars.

Table 14. Wild boars Provisioning value in Euros at the end of hunting season.

	€/choise	Quantity	€/choise/quantity
choise 1	584.90	106	61,999.40
choise 2	2,024.90	106	214,639.40
choise 3	1,683.90	106	178,493.40
choise 4	2,286.00	106	242,316.00
choise 5	2,185.00	106	231,610.00

7.2.2 Cultural related with wild boar presence

As seen in table 7, each hunter incurs fixed costs in order to exercise his activity in the period permitted by law.

Table 15 shows the costs relating to the two hunting teams authorized in the ACS bordering the Focal Area.

Table 15. Monetary value in Euros related with authorized hunters for wild boar shot.

	n. hunters	€/cultural
ACS04LB+ACS05LB	60	
€/ concession year	451.00	27,060.00
€/equipment/year	2,380.00	142,800.00
€/cartridges/year	350.00	21,000.00
€/dog/year	1,495.00	89,700.00
€ Total		280,560.00

CONCLUSIONS

Starting from an area of Campania included in the Mediterranean Biogeographical Region, a shared process with the Universidad de León has begun to express an assessment of ecosystem services by analyzing some biodiversity indicators.
The model that we wanted to use is that of the wild boar, a species to which only ecosystemic disservices are attributed (damage to agriculture, road accidents, invasions of urban centres). Our work, whose diffusion has begun through the submission of some data to the scientific community in the form of publications and reported below, intends to attribute to one of the animal species considered among the most invasive on the planet, also a positive value in the habitat it frequents.
The comparison between the disservices and the services reported in chapter 7 would demonstrate that even the wild boar would be able to produce ecosystem services. In the case of our survey (tab. 16), which was unable to fully analyze the values relating to the habitat/biodiversity, the disservices would be even greater than the services. However, the value of the outages would be significantly reduced.

Table 16. Comparison between Ecosystem Disservices/Services related with Focal Area.

	Disservices	Services	Differences
	Total amount €	Total amount €	
Damage to agricolture	413,950.00		-413,950.00
Roads accidents	25,000.00		-25,000.00
Provisioning		242,316.00	+242,316.00
Cultural		280,560.00	+280,560.00
Habitat/biodiversity	???	???	???
			-83,926

In conclusion, we can hope that a detailed multilevel analysis will be able to restore a real value of agro-zootechnical systems and related agro-ecosystems. At present, even if having to resort to the monetary parameter, it seems possible to use the tools of sustainability as indicated in detail by the 2030 Agenda. Objective 2 indicates that, through a correct quantification of Ecosystem Services, it is possible to obtain correct conservation results and proper use of natural resources.
Running sustainable food production channels while simultaneously creating new economic opportunities would considerably limit the problem of environmental pollution which will not be resolved if water, air and soil management are addressed separately. If the goals of *Homo technologicus* stop at the challenges of digitization and the application of technological knowledge, without concretely addressing the problems of man's compatibility with the planet, we will have to deal not so much with the end of the world but with the extinction of one of the species that dominated Planet Earth.
As was asked in a recent publication (Aloisio, 2021; Esposito, 2021) new technologies and scientific discoveries, combined with the growing awareness of public opinion towards the concepts of sustainability, will lead towards "electronics for life or life for electronics”?

Eur J Wildl Res (2017) 63: 62
DOI 10.1007/s10344-017-1121-6

ORIGINAL ARTICLE

Hair cortisol levels in captive brown hare (*Lepus europaeus*): potential effect of sex, age, and breeding technology

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Received: 12 December 2016 / Revised: 14 April 2017 / Accepted: 13 June 2017 / Published online: 26 June 2017

Abstract Breeding wild animals for restocking imply their ability to respond to wild conditions and to overcome the stress of transport and cage rearing. Cortisol was used in animals to measure stress levels. Aims of the study were to ascertain if a difference in hair cortisol concentration (HCC) existed between hares raised according to different breeding technologies (familiar, semi-industrial, industrial breeding) before transportation and after a pre-acclimatization period, and if a short period of adaptation could reduce the stress status. Hair samples were collected from 120 hares from an area of approximately 13.5 sp cm from the left *sulcus jugularis* by using an electric hair clipper, at day 0 (d0) and after 15 days (d15), and processed by previously established procedures. HCC significantly decreased at d15 compared to d0 in all groups. A significant difference in HCC was detected between female hares of the three groups at d0, between male and female animals belonging to the industrial group at d15, between d0 and d15 in female hares belonging to the familiar and to the industrial group, and between d0 and d15 in male hares belonging to all groups. The different breeding technologies considered resulted exerting different stress levels in hares. Fifteen days of pre-acclimatization in a low-stress environment significantly reduced HCC. Controversial results have been obtained with female subjects from the industrial group, suggesting possible complex relationships between gender and HCC.

Keywords *Lepus europaeus* · Brown hare · Stress · Hair · Cortisol · Breeding technology

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Introduction

The source of the stress, the stressor, is defined as any environmental disturbance that disrupts homeostasis (Sheriff et al. 2009; Stalder and Kirschbaum 2012). Vertebrates cope with stressors by initiating a stress response, i.e., the set of neural and endocrine responses that help to respond to the threat and then restore homeostasis (Sheriff et al. 2011). One of them is the activation of the hypothalamic–pituitary–adrenal (HPA) axis and the secretion of glucocorticoid (GC) hormones (primarily cortisol or corticosterone in mammals) (Sheriff et al. 2011; Brearley et al. 2012). HPA activation is designed to deal with acute perturbations, minimizing or shutting down non-essential functions (Sheriff et al. 2011). However, when activated chronically, the stress response is associated with a range of maladaptive effects, with a severely deleterious impact on health status, affecting long-term survival (Romero 2004; Bennett and Hayssen 2010; Stalder and Kirschbaum 2012).

Cortisol (the major GC hormone in hares) is responsible for a wide range of functions, e.g., regulation of metabolism, growth, and development, as well as physiology and endocrinology of the reproductive and immune systems (Sheriff et al. 2010; Bechshøft et al. 2012). Cortisol exerts powerful anti-inflammatory and immunosuppressive effects (Franci et al. 1996; Stalder et al. 2012). It has long been considered a reliable measure of stress in both domestic and wild mammals, and it has been measured in matrices such as blood, bird eggs, feces, saliva, whale blow, urine, feathers, liver and gonad tissue, and hair (Accorsi et al. 2008; Bennett and Hayssen 2010; Caslini et al. 2016).

Determination of blood cortisol concentrations is the standard procedure to evaluate stress conditions in farm animals (González-de-la-Vara et al. 2011). However, such concentrations vary due to different factors including circadian, ultradian, or annual rhythms; diet; environmental temperature or humidity; management; and physiological conditions (Franci et al. 1996; Szeto et al. 2004; González-de-la-Vara et al. 2011). Furthermore, restraint and handling required for blood sampling may be stressors by themselves, causing sharp increases in peripheral GC concentrations within minutes (Accorsi et al. 2008). Hence, there is an increasing attention to alternative, non-invasive techniques for monitoring adrenal function, i.e., urine, feces, and hairs (Accorsi et al. 2008; Arias et al. 2013). Hair can be collected non-invasively and is already used to extract DNA, trace metals, compounds, and drugs. Hair is readily available and easy to store and transport. Sampling does not involve pain or possible infection, and the analysis is unaffected by the momentary stress of capture (Koren et al. 2002). Hair analysis may allow monitoring hormonal changes over weeks or months by shaving off a patch of hair and resampling the newly grown hair; it offers only a long-term profile and is not suitable for monitoring hourly or daily fluctuations (Koren et al. 2002).

Hair cortisol concentration (HCC) has been measured in several species (humans, non-human primates, horse, cow, cat and dog, lynx, reindeer, grizzly and polar bear), and intraindividual stability, differences between sexes, and differences in cortisol incorporation in the hair shaft between different hair color and/or treatments have been investigated (Sauvé et al. 2007; Accorsi et al. 2008; Bennett and Hayssen 2010; Dettmer et al. 2011; Hamel et al. 2011; Manenschijn et al. 2011; Russell et al. 2011; Stalder et al. 2011; Bechshøft et al. 2012; Comin et al. 2012; Stalder et al. 2012).

Significant decrease of brown-hare (*Lepus europaeus*) population in Europe caused establishment of several farms in order to produce sufficient number of hares for hunting as well as for restocking purposes (Janicki et al. 2006). Cage breeding presumed keeping of mating couple of hare in the same cage. Handling procedures may cause additional stress with negative influence on reproduction (Janicki et al. 2006). Therefore, estimation of long-term stress status of animals in reproduction might be an important parameter pertaining management strategies, relocation or reintroduction, habitat disturbance, and population dynamics (Janicki et al. 2006; Sheriff et al. 2009).

The aim of this work was to evaluate (1) differences in cortisol levels in brown hares (*Lepus europaeus*) belonging to farms applying different breeding technologies, (2) influence of a short period of adaptation (pre-acclimatization) on HCC, and (3) if animals from different breeding technologies respond similarly to pre-acclimatization. Differences in HCC between sexes were investigated, as well.

Materials and methods

All procedures conducted in this study were carried out in accordance with the Guiding Principles in the Care and Use of Animals approved by Italian laws. The study used private client-owned animals and involved informed client/owner consent. It was performed with the best standard of veterinary care and in accordance with animal behavior.

Animals and farming technologies

European brown hares (*Lepus europaeus*) (n = 120) included in the experiment belonged to three different typologies of hare-breeding farms, and they were acquired for restocking for hunting purposes by provincial administrations of Napoli and Catanzaro. The animals were individually fitted with plastic ear tags of different color for each farm (light blue = FA; red = IN; yellow = SI). The farm typologies were categorized depending on the number of mating couples: familiar (FA) (n = 4) with about 10 couples, semi-industrial (SI) (n = 1) with about 100 couples, and industrial (IN) (n = 1) with about 250 couples.

Regardless of the breeding technology, hares older than 6 months are kept as mating couples. Animals younger than 5 months are kept in groups of eight subjects per cage in SI and IN farms, and groups of four hares in FA farms. All hares are fed with industrial feed (humidity 12.0%, crude protein 16.2%, crude fat 2.2%, crude fiber 17.8%, ash 8.2%) and alfalfa hay ad libitum and with free access to water. In FA farms, cages (150 cm length, 125 cm width,100 cm height; 1.87 mq/hare) are kept indoors and are made of zinc-coated wire gratings; in SI and IN farms, the cages (200 cm length, 100 cm width, 90 cm 127–132 height; 2.00 mq/hare) are made of waterproof material on three sides and on the ceiling, and of zinc-coated wire gratings on the remaining side and on ground; they are kept outdoor, south-north oriented and aligned in rows, with the open side toward the south. The cages' shadow was ensured by proper north-south position, as windbreak trees and canopies dark plastic, in both farms SI and IN. All cages with mesh floor welded (12.5 × 25 mm diameters, wire 2 mm) are placed on (50 cm height) stands.

The FA farms were in the province of Napoli and the SI farm was in the province of Catanzaro. The IN farm was located in Teramo province, and thus hares belonging to IN farm were transported from Teramo to Napoli (353 km, about 4 h) using wooden boxes (50 cm in length, 50 cm in width, and 50 cm in height) with 2-cm-diameter holes to allow air circulation, with two hares per box (male female ratio 1:1). Upon arrival at Napoli Provincial Administration, all the hares underwent sanitary and health controls performed by Public Health officers, before starting the pre-acclimatization period.

Acclimatization period

The period of acclimatization is carried out in areas close to those of the release in 12 different pens of 100 mq completely fenced with lightweight plastic net, supported by a stake placed every 5 m, 150 cm tall. Each pre-acclimatization area, set on the ground with two electrified wires at 15 and 30 cm height, has hosted for 15 days 10 subjects (five males and five females). Each hare could benefit from 10 mq, water, and food (hay, grass, and industrial pellets).

Sample collection

The experiment was performed between June and July 2012. The first hair sample collection determined day zero (d0); due to the high number of animals, samples were collected from 10 to 20 animals every morning, over a 10-day period, and in any case, no more than 48 h after the arrival. Before starting the collection, bodyweight was measured. Hair samples were collected from an area of approximately 4.5 cm width and 3 cm length from the left *sulcus jugularis* by using an electric hair clipper close to the skin (Oster model Turbo A5 2009, Jarden Corporation, NY, USA). After d0 sampling, all the hares included in the experiment were transported to acclimatization areas. Fifteen days after d0 (d15), another hair sample was collected from the same area with the same procedure and schedule adopted for d0. The hairs, with an approximate length of 4–7 mm were labeled, and the samples were kept in polyethylene envelope and stored at room temperature until analysis.

Sample analysis

Hair samples were processed following the procedure described by Davenport et al. (2006). Briefly, pooled hair samples were placed in glass plain tubes and mixed gently with 2 ml isopropanol for 3 min at room temperature (18–23 °C), to remove steroids present in sweat/sebum from the external surface of the hair. Then samples were allowed to dry for approximately 5 days in a clean protected hood. Once the samples were washed and dried, a portion of each one was cut at 1 cm from the proximal end and such sections were ground to a fine powder. Approximately 50 mg of powdered hair was weighed out and carefully placed into a 2-ml microcentrifuge tube. One milliliter of methanol was added to each sample, and the tubes were incubated at room temperature for 24 h with slow rotation to extract the steroids. Following extraction, samples were centrifuged for 30 s in a microcentrifuge and 0.6 ml of each extract of the methanol was aliquoted into a new tube and then dried down at 38 °C under a stream of nitrogen gas. The dried extracts were reconstituted with 0.4 ml of phosphate buffer. Duplicate samples were run for HCC using a commercially available EIA kit designed for cortisol quantification in saliva (1-3002 Cortisol Salivary Immunoassay Kit (ELISA/EIA), Salimetrics Europe, Ltd., Oaks drive, Newmarket, Suffolk, CB8 7SY, UK). Intra-assay and inter-assay coefficients of variation were 3.9 and 6.5%, respectively. Resulting values were converted from μg/dl to pg/mg for data analysis.

Statistical analysis

Data collected were imported in an electronic spreadsheet (Microsoft® Excel for MAC 2011) prior to importing them in a program for statistical analysis (JMP® 8.0, SAS Institute Inc.).

Age and weight by sex of the hares were compared between the breeding technologies and between sexes within the same farm type, by a van der Waerden analysis of variance (ANOVA). The same statistical approach was used to compare HCC at d0 and at d15 by sex between the breeding technologies. Significant differences were investigated post hoc with Tukey's HSD.

Differences between d0 and d15 in HCC in male and female hares belonging to a specific group were explored by using an unpaired Student's *t* test and Wilcoxon's rank-sum test, depending on data distribution. Normality was tested by Shapiro-Wilk's *W* test. Significance was declared at $P \leq 0.05$.

Results

All results are reported as mean ± standard deviation (range; median). One hundred twenty European brown hares (*Lepus europaeus*) were included in the analysis; 10 animals were selected from each FA farm ($n = 4$). Each group comprised the same number of hares ($n = 40$), with an equal distribution of sex (20 males and 20 females).

Age and bodyweight of hares belonging to the three groups are reported in Table 1. No significant differences were detected regarding age ($P = 0.34$) and bodyweight ($P = 0.33$). No significant differences were detected between the three groups and between female hares (age $P = 0.60$; bodyweight $P = 0.40$) or male hares (age $P = 0.72$; bodyweight $P = 0.45$). No significant differences were detected between male and female hares within the same group (FA: age $P = 0.74$, bodyweight $P = 0.45$; SI: age $P = 1$, bodyweight $P = 0.27$; IN: age $P = 0.90$, bodyweight $P = 0.82$).

HCC at d0 and at d15 are reported in Table 2. No significant difference resulted in HCC between the three groups at d0 ($P = 0.08$) or at d15 ($P = 0.58$). HCC significantly decreased at d15 compared to d0 in all groups (FA $P = 0.0002$; SI $P = 0.002$; IN $P < 0.0001$).

Table 1 Age and body weight of the hares included in the experiment, according to breeding technology and by gender within the farms. Data are reported as mean ± SD (range; median)

Farm type	Age (months)	Weight (kg)	Age (months)		Weight (kg)	
			Males	Females	Males	Females
FA	5.7 ± 2.9 (3–12; 4)	2.513 ± 0.542 (1.694–3.367; 2.453)	5.5 ± 3.0 (3–12; 4)	5.9 ± 2.9 (3–10; 5)	2.503 ± 0.523 (1.884–3.367; 2.359)	2.523 ± 0.577 (1.694–3.318; 2.599)
SI	5.6 ± 3.4 (2–15; 4)	2.457 ± 0.605 (1.408–3.613; 2.308)	5.6 ± 3.4 (2–15; 4)	5.6 ± 3.4 (2–15; 4)	2.522 ± 0.614 (1.451–3.613; 2.409)	2.391 ± 0.603 (1.408–3.250; 2.283)
IN	4.7 ± 2.1 (2–8; 4)	2.380 ± 0.596 (1.350–3.207; 2.383)	4.7 ± 2.1 (2–8; 4)	4.8 ± 2.2 (2–8; 4)	2.379 ± 0.592 (1.380–3.207; 2.350)	2.381 ± 0.614 (1.350–3.071; 2.442)

HCC at d0 and at d15 by sex are reported in Table 3. A significant difference was detected between female hares of the three groups at d0 ($P = 0.0003$); subjects belonging to the IN group had HCC significantly higher than SI hares ($P = 0.0004$) and FA hares ($P = 0.0009$). No difference was detected between female hares belonging to the SI and FA groups ($P = 0.95$). No difference was detected between male hares of the three groups at d0 ($P = 0.46$).

No difference was detected between female hares belonging to the three groups at d15 ($P = 0.10$). No difference was detected between male hares of the three groups at d15 ($P = 0.06$).

At d0, no difference was detected between male and female hares belonging to the FA group ($P = 0.09$) and SI group ($P = 0.29$). A significant difference resulted at d0 between male and female animals belonging to the IN group ($P = 0.0003$).

At d15, no difference was detected between male and female hares belonging to the FA group ($P = 0.42$) and SI group ($P = 0.56$). A significant difference resulted at d15 between male and female animals belonging to the IN group ($P = 0.003$).

A significant difference in HCC between d0 and d15 was detected in female hares belonging to the FA ($P = 0.03$) and IN ($P = 0.0007$) groups. No difference resulted in HCC between d0 and d15 in female animals belonging to the SI group ($P = 0.13$).

A significant difference in HCC between d0 and d15 was detected in male hares belonging to all groups (FA: $P = 0.02$; SI: $P = 0.004$; IN: $P = 0.009$).

Discussion

Non-invasive monitoring of stress in wild animals is becoming critical to conservation (Brearley et al. 2012). Each non-invasive technique has pros and cons, but all have the advantage of eliminating bias caused by animal capture and handling (Sheriff et al. 2010). Hormonal hair analysis offers a long-term profile and provides the resolution needed for studies of main behavioral trends and of environmental stress (Koren et al. 2002; Sheriff et al. 2010; Bechshøft et al. 2012). Hair has already been used to detect steroid hormones in cattle and anabolic steroid and corticosteroid abuse in athletes (Koren et al. 2002), as a biomonitoring tool for pollutant exposure, and to monitor HPA-axis activity in many species (Sauvé et al. 2007; Accorsi et al. 2008; Bennett and Hayssen 2010; Dettmer et al. 2011; Hamel et al. 2011; Manenschijn et al. 2011; Russell et al. 2011; Stalder et al. 2011; Bechshøft et al. 2012; Comin et al. 2012; Stalder et al. 2012).

The precise mechanisms by which substances are incorporated into hair are still incompletely understood and, only the unbound, free hormone fraction should be incorporated

Table 2 HCC in the hares included in the experiment, according to breeding technology. Data are reported as mean ± SD (range; median)

Farm type	d0 hair cortisol (pg/mg)	d15 hair cortisol (pg/mg)
FA	14.48 ± 3.51* (9.5–19.8; 13.9)	12.02 ± 2.91* (8.4–16.7; 10.9)
SI	16.43 ± 5.20* (9.8–26.0; 14.8)	12.00 ± 3.56* (7.8–17.9; 11.8)
IN	14.06 ± 3.57* (9.1–27.2; 13.7)	11.68 ± 2.79* (6.1–20.2; 12.4)

*$P \leq 0.01$

(Stalder and Kirschbaum 2012). Following the multicompartment model, different incorporation routes have been hypothesized: passive diffusion from blood capillaries, incorporation from sweat or sebum and from external sources, active synthesis and secretion of cortisol by hair follicle (Stalder and Kirschbaum 2012).

The release of GCs as a measure of the stress response is being used increasingly in studies in ecology and conservation biology to infer the "health" of individuals (Brearley et al. 2012), as well as monitoring stress status due to cage-breeding technology (Janicki et al. 2006).

Prolonged stress during capture, handling, captivity, transport, and release in the new habitat causes the poor success of translocation/restocking operations (Fischer and Tagand 2012). With acclimatization, animals no longer respond in the same robust manner to stressors. In effect, the psychological context of the stressor changes and the animal no longer perceives it to be as noxious (Romero 2004). In an experimental setting, after 2 weeks of handling several times a day, the GC response of adult rats resulted significantly reduced (Dobrakovová et al. 1993). It has been supposed that translocated wild hares need an acclimatization period of up to 2 months, and wild hares are considered to have lower mortality rates after release compared to captive bred hares (Fischer and Tagand 2012). There are no studies about pre-acclimatization during restocking procedures, and the present study did not evaluate the survival rates of relocated hares. Nonetheless, in this experiment, all hares had significantly lower HCC after 15 days of pre-acclimation; even if basal cortisol levels are unknown in these species, it seems that such pre-acclimatization period was sufficient to mitigate HPA-axis activity.

Correlation between sex and HCC has been investigated in many studies (Bennett and Hayssen 2010; Bechshøft et al. 2011; Russell et al. 2011; Bechshøft et al. 2012; Brearley et al. 2012; Comin et al. 2012; Stalder et al. 2012). The effect of gender is controversial in humans. It has been considered a potential confounding factor in an adult human, with males exhibiting higher HCC than females, an effect that, however, was not seen by other researchers (Stalder and Kirschbaum 2012). In patients with adrenal insufficiency on hydrocortisone replacement, HCC was found to be associated with perceived stress in males but not in females (Stalder and Kirschbaum 2012). Furthermore, it has been shown a significant correlation between daily dose of hydrocortisone and HCC in the female subgroup of human patients with adrenal insufficiency (Russell et al. 2011). In a first study in polar bears, Bechshøft et al. (2011) found significantly higher HCC in females; in a later study, with a fivefold sample size, such a difference was not found (Bechshøft et al. 2012). HCC did not differ between male and female in dogs, foals, and squirrel gliders (Bennett and Hayssen 2010; Stalder et al. 2012; Brearley et al. 2012; Comin et al. 2012). In the present study, HCC differed between male and female hares only for the IN group. Since results about the influence of gender over HCC are so varied, it is presumable that, if a relationship exists, it would be a very complex one.

Repeatedly shaving and resampling patches of hair from the same area can allow monitoring hormonal levels over weeks or months; furthermore, hormonal extraction from hair

Table 3 HCC in the hares included in the experiment, according to breeding technology and sex. Data are reported as mean ± SD (range; median)

Farm type	d0 hair cortisol (pg/mg)		d15 hair cortisol (pg/mg)	
	Males	Females	Males	Females
FA	14.85 ± 3.70[c] (11.3–19.8; 12.5)	14.11 ± 3.35[b, d] (9.5–18.3; 15.1)	12.20 ± 2.94[c] (9.1–16.4; 10.5)	11.83 ± 2.93[d] (8.4–16.7; 11.9)
SI	14.39 ± 2.76[c] (9.1–20.2; 14.4)	13.73 ± 4.27[b] (9.5–27.2; 12.8)	11.47 ± 2.27[c] (6.1–14.2; 12.4)	11.89 ± 3.27 (8.0–20.2; 12.4)
IN	13.62 ± 3.84[c, a] (9.8–19.8; 12.3)	19.24 ± 4.92[b, d, a] (12.0–26.0; 20.2)	10.51 ± 3.27[c, a] (7.8–16.1; 14.9)	13.49 ± 3.26[d, a] (8.5–17.9; 13.1)

[a] Statistical difference between sexes by breeding technology within the sampling day ($P \leq 0.01$)

[b] Statistical difference between breeding technologies by sex within the sampling day ($P \leq 0.01$)

[c] Statistical difference between males at d0 and at d15 ($P \leq 0.05$)

[d] Statistical difference between females at d0 and d15 ($P \leq 0.05$)

is insensitive to the immediate stress of trapping and handling wild animals (Koren et al. 2002).

Studies attempting to interpret physiological stress from HCC should validate the hair-sample-collection method. This should include determination of hair-growth rate between sexes and with age and season. Hair-growth rate was not determined in this study, and this should form a long-term study on captive brown hares to confirm results. This study focuses on the variation in hair cortisol levels; thus, the standardized technique described was appropriate to work it out.

Conclusions

To the authors' knowledge, this is the first study evaluating HCC as an indicator of management stress in brown hares (*Lepus europaeus*). The breeding technologies that characterize hare farming resulted to exert different stress in the animals, as the intensively reared group showed higher levels of HCC. Fifteen days of pre-acclimatization significantly reduced the HCC level in all the groups. Controversial results have been obtained with females belonging to the intensive rearing system group, suggesting possible complex relationships between gender and HCC.

For all mentioned, it needs to be said that HCC could be a valuable tool for the monitoring of long-term stress, but requires physiological validation to be sure that any cortisol detected will reflect the stress experienced by the animal. ACTH challenge in case of HCC was used in only few studies so far. Administered ACTH successfully increased HC levels in lynx, reindeer/caribou, and chipmunks, but not in others (Keckeis et al. 2012; Ito et al. 2005; Mastromonaco et al. 2014; Ashley et al. 2011; Terwissen et al. 2013).

Acknowledgements The authors thank Amministrazione Provinciale of Napoli, Ambiti Territoriali di Caccia of Napoli and Catanzaro1, and "Scerbo Serena" farm.

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SIMeVeP

PARCO NAZIONALE DI ABRUZZO, LAZIO E MOLISE

Cause di morte del lupo nel territorio agro-silvo-pastorale

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La distribuzione del lupo, lungo la catena appenninica, subisce una drastica riduzione alla fine degli anni '50 del novecento [1].

Da una stima del 1972, sul territorio italiano, risultavano essere presenti circa 100 lupi, suddivisi in 4 aree principali: 1. una grande area tra Abruzzo, Molise, Lazio orientale, Umbria e Marche; 2. una tra Lazio settentrionale e Toscana meridionale; 3. una tra Campania, Basilicata e Calabria settentrionale; 4. altopiano della Sila.

La situazione relativa alla presenza del *Canis lupus*, nell'ultimo censimento del 2012 riconosciuto in ambito scientifico, in Europa e Italia è di circa 12.375 individui (Forgione *et al.*, 2015). Partendo dai dati ufficiali si evince che la popolazione del lupo in Italia è stimata in un range che va da 600 a 800 esemplari suddivisi nelle popolazioni alpine (Italia, Francia, Austria, Slovenia, Svizzera) e peninsulari o appenniniche (http://ec.europa.eu/).

Nei territori del PNALM la pressione antropica è, da sempre, esercitata dalle attività agricolo-zootecniche e silvicole ma, negli ultimi decenni, è incrementata ad opera delle frequen-

Tabella 1. Numero di lupi ritrovati morti nel territorio del PNALM tra il 2000 e il 2014.					
	Morti	**Causa ignota**	**Patologia**	**Veleno**	**Trauma**
2000	6	1	1	1	3
2001	4	0	0	0	4
2002	5	0	0	3	2
2003	10	2	1	4	3
2004	5	1	0	2	2
2005	7	0	1	1	5
2006	6	0	0	0	6
2007	18	3	0	11	4
2008	3	2	0	1	0
2009	7	3	0	1	3
2010	11	1	0	2	8
2011	7	4	0	0	3
2012	12	5	2	2	3
2013	35	3	19	4	9
2014	7	0	4	1	2
143	**25**	**28**	**33**	**57**	

tazioni turistiche stagionali che si sono rese responsabili della frammentazione di molti ambienti naturali e in particolare hanno aumentato il flusso di autoveicoli di passaggio nell'area parco, sia di inverno, sia di estate [2].
Il presente lavoro mira a descrivere le cause dei morti dei lupi nel Parco Nazionale d'Abruzzo, Lazio e Molise.

Materiali e metodi

L'area di studio ha riguardato l'intero territorio dei 24 Comuni Parco Nazionale d'Abruzzo, Lazio e Molise (PNALM) costituito di antichi borghi e piccoli paesi che hanno conservato in gran parte la loro identità e il loro territorio agro-silvo-pastorale al quale si aggiunge un grande superficie costituita da ambienti naturali di alta montagna. La superficie dell'area protetta è di 49.680 ettari condivisa nelle provincie di L'Aquila, Frosinone e Isernia.
Il campione su cui si è lavorato consiste in un numero totale di 143 lupi trovati morti dagli operatori del PNALM tra gli anni 2000 e 2014. I dati sono stati inseriti in apposite schede e ordinati in funzione delle principali cause di morte diagnosticate, per la maggior parte, dall'Istituto zooprofilattico sperimentale dell'Abruzzo e del Molise.
Le cause di morte sono state raggruppate in 4 gruppi principali: patologie, avvelenamento, traumatismi e cause ignote, a loro volta comprendenti 23 sottogruppi, elencati di seguito:
- *Trichinella* spp., *Strongylus* spp. (patologie endoparassitarie)
- rogne demodettica; rogna sarcoptica (patologie ectoparassitarie);
- Aujezky; parvovirosi; cimurro (patologie virali);
- sostanze velenose non determinate, dicoumarol; arsenico; diazinon; stricnina; carbofuran; metaldeide; fosfato di zinco; Phorate; fenthion; malathion; eptaclor; endosulfan) (sostanze velenose);
- aggressione da congeneri o da cani, ferite da arma da fuoco o da tagliola, incidenti da mezzi di trasporto veicolare (traumi).

Le differenti incidenze di mortalità sono state confrontate con il test del χ^2.

Risultati

Nella tabella 1 viene riportato il numero di lupi trovati morti dal personale tecnico nel territorio di competenza del PNALM tra l'anno 2000 all'anno 2014. La figura 1 mostra una grande variabilità tra gli anni, infatti si passa da un minimo di 3 lupi trovati morti nell'anno 2008 a un massimo di 35 nel 2013. Il numero medio di lupi trovati morti nel PNALM risulta essere 17.88/anno.
Le cause di morte sono state classificate come ignota, patologia, veleno e trauma (tabella 1) la cui incidenza totale è risultata 17.48%, 19.58%, 23.08% e 39.86% rispettivamente. Un'analisi più dettagliata (figura 2) mostra che, nell'ambito delle mortalità attribuite alle patologie, sono state descritte

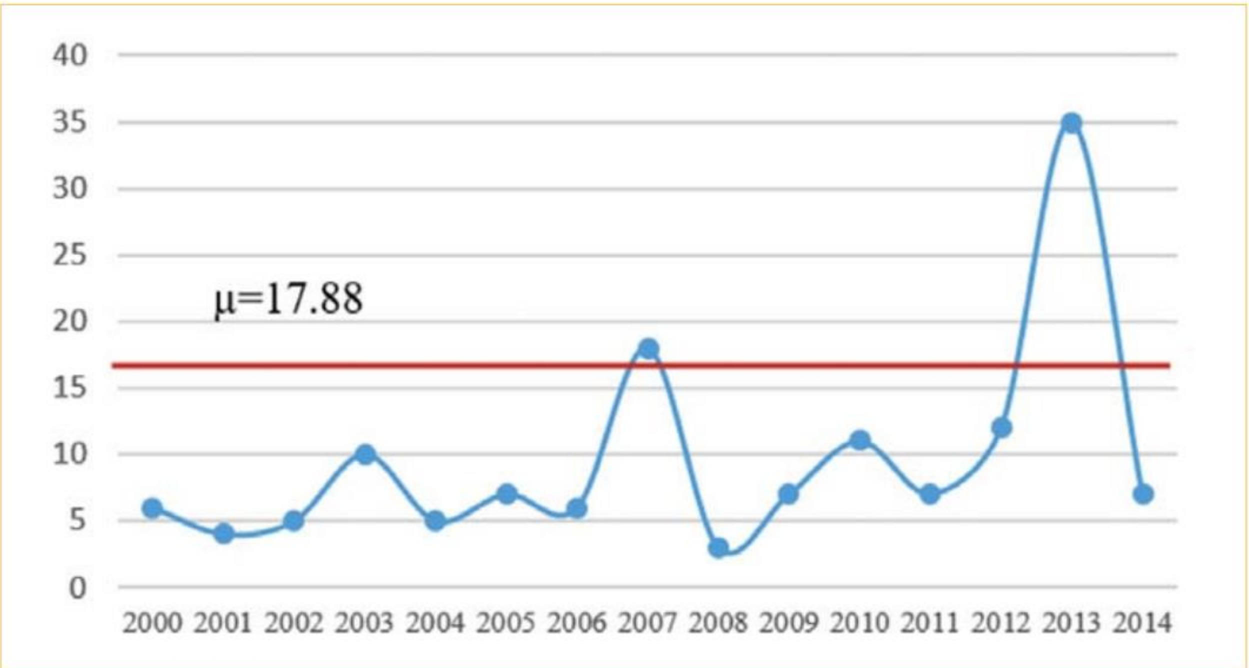

Figura 1. Andamento delle morti dei lupi negli anni e media tra gli anni; periodo 2000-2014.

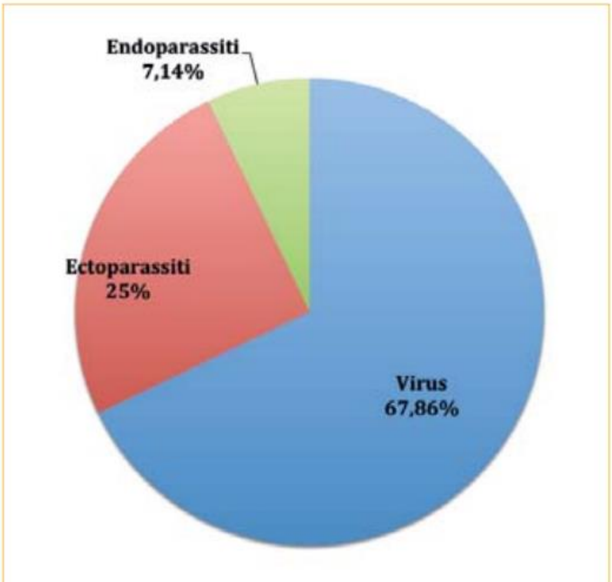

Figura 2. Incidenza percentuale del tipo di patologia riscontrata nei lupi morti nel PNALM.

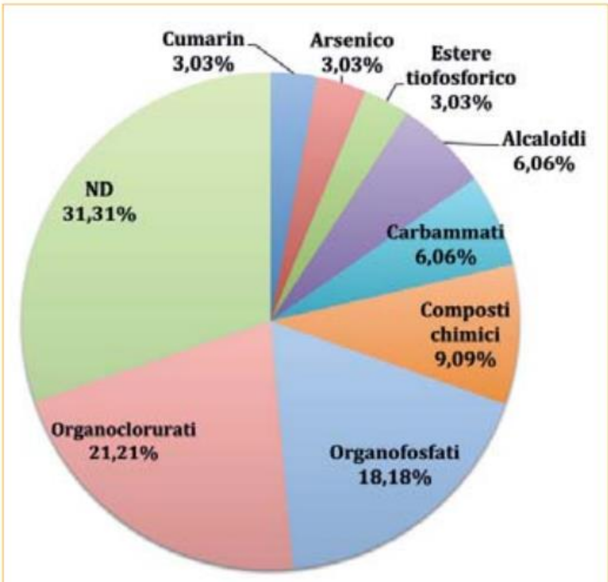

Figura 3. Incidenza percentuale del tipo di veleno riscontrato nei lupi morti nel PNALM.

in ordine crescente agenti etiologici afferenti alle endoparassitosi (*Trichinella* spp. 3,57%; *Strongylus* spp. 3,57%); agli ectoparassiti responsabili delle rogne (*demodex* spp. 10,71%, *Sarcoptes scabiei* 14,29%); ai virus responsabili delle malattie di Aujezky (Herpesvirus 3.57%); parvovirosi (Parvovirus 7,14%); Cimurro (Morbillivirus 57,14%).

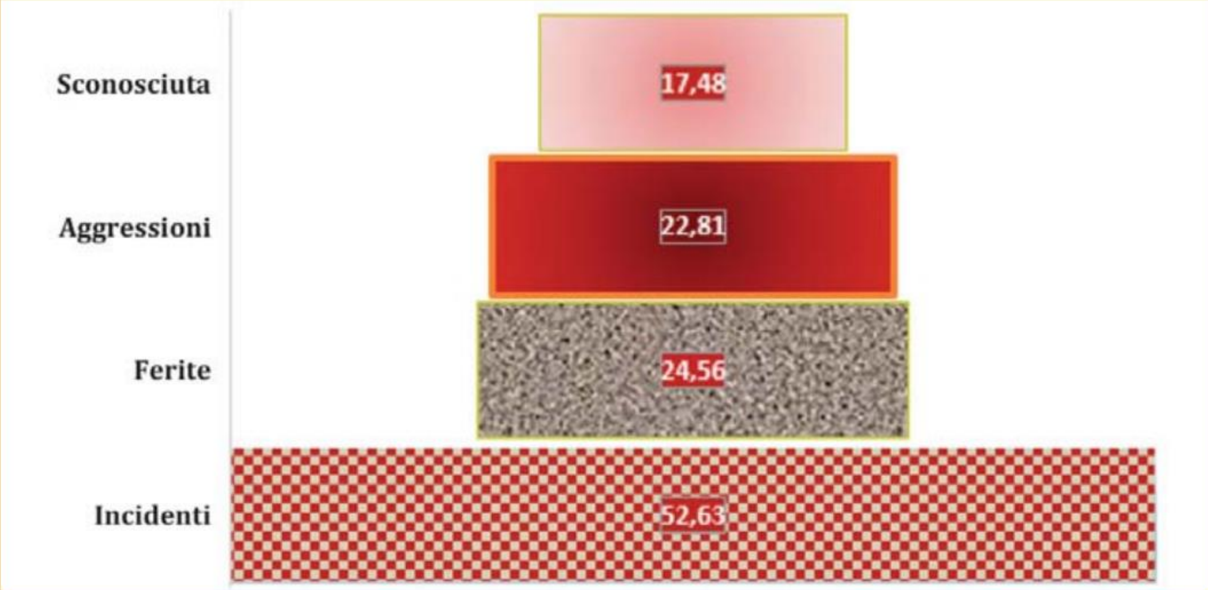

Figura 4. Incidenza percentuale del tipo di veleno riscontrato nei lupi morti nel PNALM.

All'interno delle cause di morte attribuite ai veleni (figura 3), la maggiore incidenza è rappresentata da sostanze velenose non determinate (33,31%) mentre tra le sostanze identificate si riportano, in ordine crescente, il cumarin (dicoumarol 3,03%), l'arsenico (3,03%), l'estere tiofosforico (diazinon 3,03%), gli alcaloidi (stricnina 6,06%), i carbammati (carbofuran 6,06%); i composti chimici (metaldeide 6,06%, fosfato di zinco 3,03%); gli organofosfati (fosfato 12,12%, fenthion 3,03%, malathion 3,03%), organoclorurarti 21,21% (eptaclor 3,03% e endosulfan 18,18%).
Tra le cause di morte attribuite ai traumi (figura 4) sono state registrate, in ordine crescente, l'aggressione da congeneri o da cani (22,81%), le ferite da arma da fuoco o da tagliola (24,56%), l'investimento da automobili o da mezzi di trasporto veicolare (52,63%).

Discussioni e conclusioni

L'analisi complessiva della situazione delle popolazioni di lupo presenti nel PNALM permette di stimare una certa stabilità delle popolazioni presenti nel Parco e di confermare quanto riportato dai dati EU e IUCN che considerano la popolazione di *Canis lupus* (Gray Wolf) in uno *Status* di *Least Concern* (minima preoccupazione) con un trend della popolazione stabile. Tuttavia non è da sottovalutare il dato dell'anno 2013 nel quale si è segnalata una mortalità di 35 esemplari e un'alta incidenza di malattie scarsamente segnalate in precedenza. Tale risultato potrebbe essere associato alla aumentata antropizzazione accompagnata da cani da compagnia tra i quali non è raro incontrare razze come il cane lupo cecoslovacco. Non bisogna, infatti dimenticare che il fenomeno dell'ibridazione è una variabile che, negli anni a seguire, sarà sempre maggiormente da indagare, in particolare nelle aree protette alle quali è demandata, in Italia, la funzione di protezione della biodiversità.

Per saperne di più

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Guaranteeing safety of animals under risk of fire: conceptual framework and technical issues analysis

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***Abstract*—The paper introduces the conceptual framework of a Distributed Sensor Network for the monitoring of the wooded areas under risk of fire in the so-called "Vesuvius' red zone". Main goal is the determination of the OPtimal Evacuation Route for Animals (OPERA) in case of fire, for all the animal species living in the Mount Vesuvius' surrounding areas, under the perspective of the Disaster Risk Management principles.**

***Keywords*—Fire risk, OPERA, Animal safety, Distributed Sensor Network, Disaster Risk Management**

I. Introduction

According to the 2013 Annual Report of the United Nations office for disaster risk reduction [1] "[…] *the devastating impact of forest fires on natural resources was neither quantified nor adequately took into account. Fires are harmful for a number of ecosystem services (whose loss is estimated at around 146–191 Bln dollars per year) including carbon storage, biodiversity support, protection of water sources, reduction of soil erosion, land degradation, and climate regulation*".

In this scenario, the CeRVEnE (Italian acronym for "*Regional Veterinary referral Center for non-epidemic emergencies*") was established in 2017 in the Italian Region of Campania, pursuing the objective of the Regional Government to improving and protecting people health through the timely management of both veterinary and non-veterinary epidemic emergencies related to animal health as well as to food safety.

Among the initiatives of CeRVEnE the FRAC Program (Fire Risk Assessment in Campania Region) was presented in 2018, as a project through which the Center intends to provide the Regional Government with a strategic tool able to (i) gathering and supplying in short time all the necessary information to those professionals called to handle fire-related risks, (ii) having a large (both specialized and not specialized) public informed about the operational processes to get started after the fire damages in terms of safeguard and recovery of ecosystems (and related services), wild and domestic fauna, production supply chains, and (iii) figuring out a standardized methodology for gathering data and supporting decisions consistent with the specifics of the "Disaster Management Cycle" model [2].

II. State of the Art

A. Disaster Risk Management

The last decades have seen the thriving of the disaster risk management field of study, which includes the total sum of all activities, programmes and measures that can be taken up before, during and after a disaster with the purpose to avoid it, reduce its impact or recover from its losses [3].

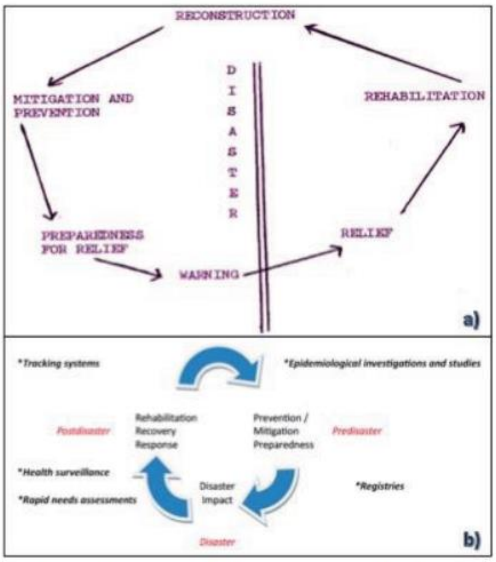

Fig. 1. Disaster Management Cycle (a). Disaster epidemiology actions and the disaster management cycle (b).

Particular interest is then assumed by the concept of "Disaster Management Cycle" (Fig. 1a) that shares isomorphically phases and associated concepts with the linear disaster phase [4–6]. Other scholars [7] also derived the key disaster-related activities employing epidemiological methods, including rapid needs assessments, health surveillance, tracking systems, epidemiology investigations and studies, and registries (Fig. 1b).

B. *Fire risk monitoring*

The problem of early detection of fires in forest areas is widely recognized at both national and international level, as witnessed by the European Forest Fire Information System (EFFIS), developed jointly by the European Commission (EC) services (Directorate General Environment and the Joint Research Centre) and the relevant fires services in the countries (forest fires and civil protection services) in response to the needs of European bodies such as the Monitoring and Information Centre of Civil Protection, the European Commission Services and the European Parliament [8].

Furthermore, For what concerns in particular the Mediterranean countries, different technologies are adopted as to the measurement of the relevant parameters for an early fire detection in risky areas: on the one side, the implementation of hierarchical Wireless Sensor Networks (WSN) means the use of a number of sensing nodes that are capable of effectively gathering information from the surrounding environment and communicating with each other to send the measured data to a base station for further processing, in order to a reduction of fire damage [8–9]; on the other side, Geographic Information Systems (GIS) can be used to combine different forest-fire causing factors for demanding the forest fire risk zone map. GIS implementation allows the evaluation of a set of parameters that effect the fire, such as topography and vegetation, with the other land use information including population, settlements, forest fire towers, fire stations, intervention places, the characteristics of the staff that will intervene, and transportation: this can make possible for example to figure out the shortest way of intervention during the disaster, and/or the areas to be emptied [11–13].

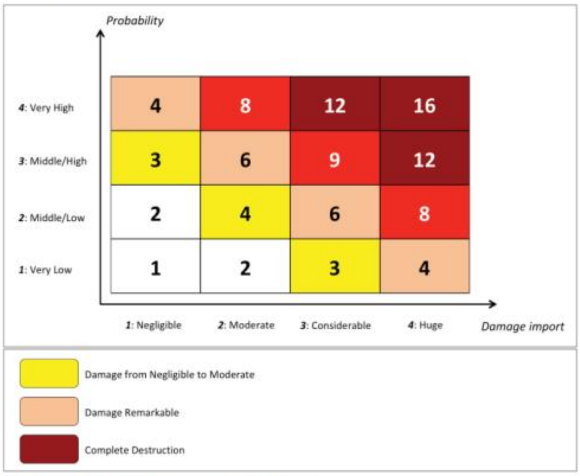

Fig. 2 Fire Risks Assessment Matrix

In line with these actions, within the FRAC program a first extended mapping of the territories surrounding the Mount Vesuvius was performed during the years 2018 and 2019 with a twofold purpose: (i) detect and count all the farming activities concerning poultry, sheep and goats, cattle, bees, swine, and equines; (ii) figure out a reliable map to classify the different fire risk areas, according to the Fire Risks Assessment Matrix (see Fig. 2) [14–16]

III. Paper Contribution

The present work intends to describe the conceptual framework and to introduce some preliminary results of an IT system to be implemented in order to support the activities pursued by the FRAC Program. In particular, the main scope of the system is twofold, that is on the one side to monitor the wooded areas under risk of fire in the so-called "Vesuvius' red zone", and on the other side to determine the OPtimal Evacuation Route for Animals (OPERA from now on) in case of fire, for each of the reported animal species living in the mentioned red zone.

Fig. 3 depicts the necessary steps to accomplish the mentioned goals; in a first moment the gathering of a set of specific information (e.g. Fuel parameters, DEM/Digital Elevation Model Map, …) is requested to create a *Fire Propagation Map*, through which characteristics and dynamics of a fire episode can be analyzed and evaluated. Starting from this, an *Evacuation Plan Model* can be figured out by also adding anthropic-layered data related to both the urbanization rate and the road system development surrounding the Vesuvius. Eventually, in order to determine the *OPERA* such model has to be enriched by means of the mapping of the animal presence (in terms of both typology and distribution) and the clear definition of type and size of vehicles requested to rescue the animal species involved in a fire episode.

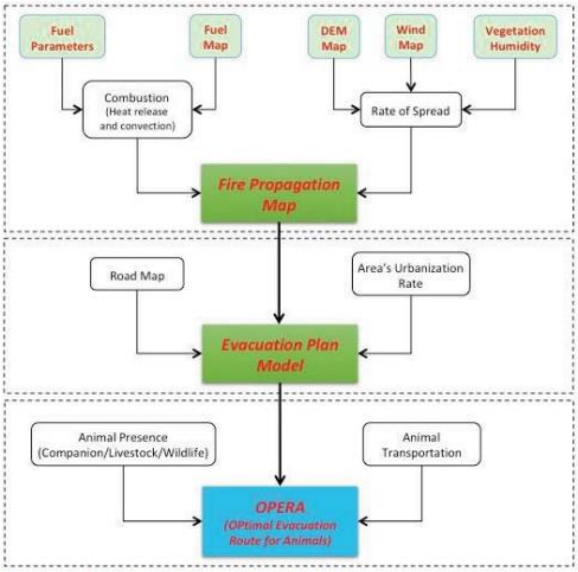

Fig. 3 Particular on the Work methodology

To that end, the software architecture is supposed to integrate a Distributed Sensor Network (DSN) to cover the red zone, an ad-hoc software to generate timely simulation models for predicting the rate of fire spreading [17], and a GIS for both the activities of web mapping and OPERA definition – in particular, the latter step will be performed as an arborescence optimization problem to be solved for instance via label setting-correcting algorithms. The Unified Modeling Language (UML) graphical notation [18] will be used to figure out the overall process model and to detail its different dynamic and static aspects.

The determination of the OPERA for beekeepers will be analysed as a first-case scenario, in order to provide some preliminary results as to the logic of the system and its working dynamics.

IV. Technical issues analysis

Technical issues are mainly related to the realization of a DSN capable of offering early warnings, and eventually monitoring the dynamics of critical events. In the following, the requirements of a network suitable to the purpose are specified and a candidate solution is discussed. The mathematical model underlying the optimization problem is also introduced and formalized.

A. *Network requirements*

The employment of distributed sensor networks (DSNs) for fire detection in forest areas has widely been investigated in the past, highlighting benefits in terms of cheapness and easy deployment [9–10]. Anyway, paying attention to the use of DSN in severe working conditions, such as those related to the presence of fire, several other requirements cannot be jeopardized. One can start enlisting flexibility requirements in terms of integration of sensor terminals, in order to have no constraints at choosing the optimal parameters for the identification of anomalies and the prevention of dangerous situations. Also, no constraints, or at least minor ones, related to the distance between sensor nodes would be of help in order to assure extensive coverage of the monitored area. Another key point is the presence of electric and/or telecommunication infrastructures and the distance of the farthest node from their coupling points. The possibility and costs of maintenance operations represent significant aspects as well. All these factors affect the topology of the network, the design of the hardware for main energy supply systems, as well as of ancillary energy harvesting systems, and sensor equipment. Nonetheless, they deeply impact the choice of the data communication solution.

B. *Network architecture*

The innovative paradigms enabling Internet of Things (IoT) applications, which are sketched in Fig. 4, seem to offer advantages in both design and implementation of a candidate DSN with respect to more traditional solutions.

The candidate network is essentially made up of two types of nodes, namely sensor and gateway nodes. Each sensor node includes a smart power module, one or more probes, a processing machine, such as the one available in a commercial *BeagleBone* board or else *Raspberry pi* board, and an interfaced wireless transmitter. The electric power module has to supply power to all terminal modules. Electricity demand should also be managed with efficient strategies. The typical sleep/deep-sleep operative modes between data refresh instances are therefore implemented, which involve that all non-critical electronic circuits are disconnected from the power supply except during their short operative slots. Probes consist of both measurement modules to collect the parameters of interest, such as: relative humidity, temperature, atmosphere pressure, mono and di-oxide carbide (CO, CO_2) percentages, solar irradiance, rain percentage, infrared/ultraviolet radiation intensity, volumetric water content and suction in soil, etc., and detectors for instance for smoke presence, or intruders. It is worth noticing for now that sensor nodes could involve troubles in the implementation of maintenance programs, needed for instance for battery replacement, since they could be distributed in difficult-to-reach places.

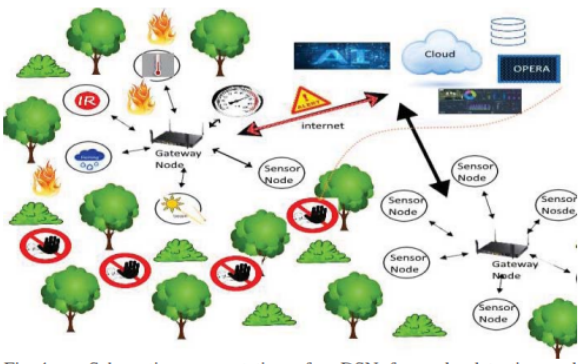

Fig. 4 Schematic representation of a DSN for early detection and monitoring of fire in forest areas, eventually integrated with several facilities made available by IoT solutions.

The processing machine has to deal with both on-site data analyses and data transmission management, as well as with synchronization tasks. It is in fact responsible of assuring reliable data routing to gateway nodes. The availability of a network clock signal is necessary for synchronization purposes: to this end the exploitation of the GPS reference clock is possible by means of a low-cost receiver. Gateways are conceptually different from sensor nodes because their chief role is interconnecting local area networks to the Internet; they require therefore more hardware in terms of processing and memory resources. For the proposed network, each gateway has to merge the data streams transmitted by the dependent sensor nodes, and, in turn, multiplex them into a single mainstream that is transmitted to a general remote collector. Gateways also provide local storage services to temporarily save critical data, thus avoiding information loss in case of occasional connection unavailability. Trading-off between performance and costs, a subset of all gateways of the proposed network is equipped with additional memory resources to assure storage capabilities comparable to those of a secondary database service. A robust implementation consists in distributing the additional memory resources between the gateways of the subset, granting them the possibility of storing a recent history of the mainstreams that should be forwarded to the general collector.

The proposed network is also complemented with a last protected gateway node, operated according to a black box model, that allows post–disaster analysis. Its protection is assured by physically placing the critical parts of its hardware underground. Data transmitted by sensor nodes should not have complex structure, so that traffic between sensor nodes and gateways can be accomplished without claiming for wide bandwidth resources. Data refresh is therefore programmed at low rate, which is sufficient for regular monitoring. The network is designed such that data refresh is automatically increased during incipient emergencies. Low data rates cope with the low power requirements of long–range wide area networks (LoRaWANs) [19]. LoRaWAN is a novel wide range distance communication protocol that is robust to noise and interference, and is capable of offering a coverage radius up to 15 km. Most important, modules compliant with LoRaWANs' specifications require ultra-low power electronic circuits. In addition, they can be complemented with solar, micro-wind, or piezoelectric energy harvesting systems that sustain the state of charge of the supply, which can be made up of both batteries or performant and reliable supercapacitors. The equipment selected for the proposed network adopts a microcontroller board, namely STM32F3DISCOVERY board, which is characterized by reduced power requirements.

Moreover, concerning some constructive details, the use of military chips can increase the resistance to heat so that even in harsh conditions, like those experienced during forest fires, the equipment has still operating time. An IP67 anti-fire package can grant to each sensor node the ability of uninterrupted operation in very hostile environments. Eventually, it has to be added that IoT paradigms generally integrate cloud–computing services, which can be activated on demand by an end customer. The exploitation of cloud resources can simplify storage and computational tasks. In particular, cloud solutions are ideal to produce more consistent, accurate, and useful information than that provided by any atomic systems. Nonetheless, they also represent efficient platforms to implement artificial intelligence (AI)–based approaches, which are becoming important backings to solve very complex problems.

C. Route Optimization

The mathematical modelling of the problem requires the creation of an oriented graph G = G(N, A), whose nodes represent the accessible junctions, while the arks represent the roads that connect two consecutive junctions. For each node the parameter $J_{r,t}$ is defined, where: $r \in [0; M]$ means one specific junction among all the possible M junctions that can be identified once figured out all the possible alternative routes between the starting point and the end point (animal safe zone); $t \in [0; +\infty[$ refers to the time moments for which the discrete simulation of the fire spreading is run. We can have: $J_{r,t} = 0$ (the junction r has already been reached from the fire at the moment t); $J_{r,t} = 1$ (the junction r hasn't been reached from the fire at the moment t yet).

A first set of constraints can be set for each node, which assures that the difference between the whole (road) flow entering the generic node v – ark (i; v) – and the whole flow leaving the same node – ark (v; j) – corresponds exactly to the requirements of such node (positive difference for the final node; negative, for the starting node; equal to zero, for a transient one). Another set of constraints can be set for each ark, which refers to their capacity, and limits the entity of the (traffic) flow that passes through the ark itself.

The problem of the determination of the OPERA can be therefore figured out as similar to a classic problem of minimum path [20], and expressed therefore in formula as follows:

$$OPERA = \min \sum_{r=0}^{M} \sum_{t=0}^{\infty} J_{r,t} \neq 0 \qquad (1)$$

V. Preliminary Results

As said, a first–case scenario is introduced, which focuses on beekeepers as the beehives transportation is less problematic in terms of general set up and execution.

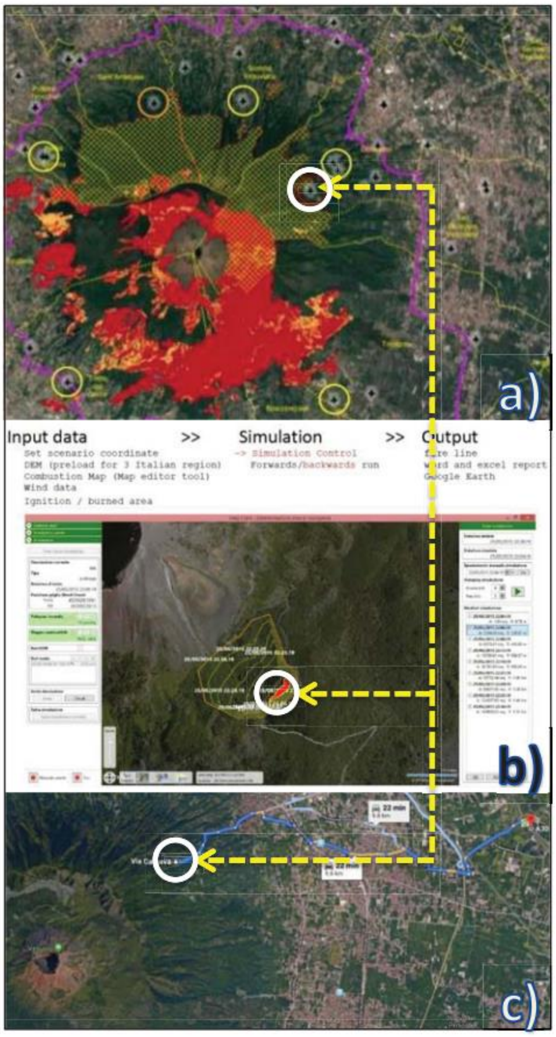

Fig. 5 First implementation of the framework

Fig. 5 reports the three main steps of the simulation, currently still in progress, performed considering a timely not much extended route to get from the starting node on the Vesuvius' slope, up to the safe zone (the A30 highway, in this case), so that a limited number of junctions could be considered. The starting node is represented in Fig. 5a within a white circle, along with the other beekeepers' sites standing on the territory under consideration, and characterized by different fire risk areas (from yellow to red, in increasing danger level). In Fig. 5b the fire spreading simulation is run, considering the beekeeper's site as the fire source, and the enlarging areas interested by the fire at specific times are calculated from the software. In Fig. 5c the OPERA is "roughly" defined by: (i) identifying in Google Maps © all the alternative routes from the starting point to the safe zone; (ii) overlapping the simulation output to the satellite map, in order to identify, time after time, the junctions not yet interested by the fire; (iii) visualizing each junction–to–junction path, and then manually combining them so as to draw the OPERA for the case study. In particular, the result of (1) was 12.

VI. Discussion and Conclusions

The overall system logic is yet to be fully disclosed, as the present paper only describes the conceptual framework of the entire software architecture; nonetheless, the development of the proposed integrated system becomes somehow critical, as the fire risk (and especially arsons risk) has considerably increased in the last years in the whole Mount Vesuvius' surrounding area, which features a unique combination of both animal and anthropic elements within a very delicate natural ecosystem.

The first choice is therefore to design and test the system on a narrow area (the mentioned "red zone") to verify its viability, as in the FRAC Program are actively involved a number of organizations (State Forestry Corps, Local Health Trusts, breeders' associations, up to the very Regional Government) that need to strictly interface with each other – and, in case of fire, in a very short time – in order to build up a solid and efficient system of surveillance planning, risk analysis, and data gathering.

The implementation of a specific integrated system to support the mandate of the FRAC Program is supposed to boost an improvement for the CeRVEnE's activities, pursuant the innovation perspectives coming with the effectual application of the Disaster Management principles.

Acknowledgment

Authors want to thank, for the help provided in the first phases of the FRAC Program: the Regional Veterinary referral Center for non-epidemic emergencies (CeRVEnE); the Department of Prevention and Public Health of Campania Region; the Civil Protection Organization; the Municipality of Somma Vesuviana (province of Naples).

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Bulgarian Journal of Veterinary Medicine, 2021, **24**, No 1, 159–163
ISSN 1311-1477; DOI: 10.15547/bjvm.2019-0054

Short communication

SIMILARITIES BETWEEN SKIN CULTURABLE BACTERIAL SPECIES OF POOL FROGS (*PELOPHYLAX LESSONAE*) AND THEIR HABITAT

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Summary

Nocera, F. P., A. De Filippis, N. Piscopo, L. Esposito & L. De Martino, 2021. Similarities between skin culturable bacterial species of pool frogs (*Pelophylax lessonae*) and their habitat. *Bulg. J. Vet. Med.*, **24**, No 1, 159–163.

The aim of the present study was to investigate the culturable microflora of pool frogs (*Pelophylax lessonae*) and their belonging aquatic environment. A total of 60 samples (56 frog cutaneous swabs, 4 water samples) were inoculated onto different selective and differential agar plates to isolate Gram-positive and Gram-negative bacteria or yeasts. Microbial investigation of the water hosting frogs was also performed. Isolates were identified by API system and their antibiotic resistance profiles were evaluated by disk diffusion method on Mueller Hinton agar plates. *Aeromonas hydrophila* and *Enterococcus durans* were detected in almost all collected samples. Many of the bacterial isolates showed multidrug-resistant profiles. Importantly, this study highlights that skin frog microbiota is correlated to the belonging environment, and, moreover, some isolated bacterial strains resulted to be of interest in animal and public health, since the park was frequented by visitors of all ages.

Key words: antibiotic resistance, frog, skin microbiota, water environment

Skin is the first line of defense against pathogenic microorganisms and the intimate contact between the epidermis and microbes has been already studied. Commonly, the skin flora is nonpathogenic, and either commensal (not harmful to the host) or mutualistic (offers a benefit). The skin microbiota can protect from transient pathogenic organism's colonisation either by competing for nutrients, secreting chemicals against them, or stimulating the skin's immune system (Cogen *et al.*, 2008).

It has been reported that, in a host species-specific manner, amphibian skin may select for microbes that are generally in low abundance in the environment (Walke *et al.*, 2014). Researchers in USA have recently identified symbiotic bacteria living on amphibians' skins that protect them from the deadly fungal diseases (Muletz-Wolz *et al*., 2017). The cutaneous microbiota of amphibians can be defined as a biological component of protection, since it can be composed of bacteria that produce antimicrobial compounds (de Assis *et al*., 2017). The rich microbial community harboured on amphibian skin, thus, represents a complex ecosystem which constantly interacts with the environment and host factors that influence colonisation (Rollins-Smith *et al*., 2011).

Here, we investigated, by bacterial growth on selective and differential media agar plates, the skin microbiota of pool frogs correlated to their aquatic environments located in Calabria Region, Italy.

The pool frog (*Pelophylax lessonae*) is a European frog protected by the Biodiversity Action Plan (BAP) which is an internationally recognised programme addressing threatened species and habitats, and it is designed to protect and restore biological systems (Glowka *et al*., 1994). Typically, the pool frog resides in small water pools and prefers places surrounded by woodlands. This research aims to build a basic ecological foundation to better understand the role of culturable microbial communities in frog and their specific habitats. Frog specimens, precisely swabs were collected from back, abdomen, gullet and throat in April 2015 from frogs living in two different habitats, a natural bath and a pool, near the Angitola lake in the Regional Natural Park of Serre (Vibo Valentia, Calabria, Italy). The capture was carried out during the project Angitola FISH2O (EFF Calabria Cod. 02/BA/12 – authorised June 28, 2013 prot. n. 756). All procedures were carried out before the actual Italian law (DgLs 4 marzo 2014, n. 26) but in total accordance with the guidelines of the current European Directive (2010/63/EU) related to care of animals used for scientific purposes. For sampling, the capture and handling of *Pelophylax lessonae* were conducted by hand using fresh latex gloves for each frog. Additionally, all animals were in good health at clinical examinations. Sterile cotton-tipped swabs were drawn across the selected anatomical sites of each frog and immediately transferred to Amies transport medium (Oxoid Ltd, UK), then maintained at 4 °C (not longer than 24 h) until processing.

A total of 60 samples (56 frog cutaneous swabs and 4 water samples) were processed at the Microbiology Laboratory of the Department of Veterinary Medicine and Animal Production, University of Naples Federico II (Italy). All samples were plated on blood agar base supplemented with 5% sheep blood, a selective medium used for the isolation of Gram-positive microorganisms, on mannitol-salt agar, a selective medium to identify staphylococci, and on MacConkey agar, a selective and differential medium for the growth of Gram-negative bacteria, which were all incubated aerobically at 37 °C for 24–48 h. Furthermore, the presence of yeasts on skin was also investigated by incubation in the dark at 30 °C for 3 days. The used agar plates were all produced by Oxoid Ltd, UK.

Bacteria were identified by macroscopic observation of the colonies, Gram staining, standard laboratory methodologies (catalase, staphylocoagulase tube test, aesculin), and miniaturised biochemical tests API system (bioMérieux SA, Marcy L'Etoile, France). The species identification by miniaturised biochemical tests was accepted when probability was > 88%.

The isolates were tested for susceptibility to 16 antibiotics by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar incubated at 37 °C, as recommended by the principles described in the Clinical and Laboratory Standards Institute standard method for veterinary pathogens (CLSI, 2015). Discs of amoxicillin/clavulanic acid (AUG 30 µg), ampicillin (AMP 10 µg), cefoxitin (FOX 30 µg), ceftiofur (EFT 30 µg), ceftriaxone (CRO 30 µg), ceftazidime (CAZ 30 µg), doxycycline (DO 30 µg), erythromycin (E 15 µg), gentamicin (CN 10 µg), kanamycin (K 30 µg), tetracycline (TE 30 µg), penicillin G (P 10 UI), enrofloxacin (ENR 5 µg), streptomycin (S 10 µg), trimethoprim/sulfamethoxazole (SXT 25 µg) and vancomycin (VA 30 µg) were tested.

By means of the culturing methods, the skin frog microbiota from tub frogs was the same independently from the different sampling sites such as back, abdomen, gullet and throat; precisely, few colonies of *Pantoea* spp., *Acinetobacter baumannii*, *Aeromonas hydrophila* and *Enterococcus durans* were isolated and identified from the different skin districts with no important change in the concentration of each bacterial population. Instead, *Aeromonas hydrophila*, *Enterococcus durans* and *Citrobacter freudii* were detected from the different sampling skin sites of frogs living in pool. The half of skin frog swabs was positive for *Penicillium* spp. yeasts with a higher and significant presence in the tub frogs than the pool frogs.

The different aquatic habitat influenced the composition of the cutaneous microbiota of the frogs, in fact dominant bacterial population on tub frog skin including, *Aeromonas hydrophila* and *Enterococcus durans*, were detected in all the collected samples including tub water samples. The most frequent bacterial isolates from water tub samples, in duplicates of 4 samples, were *Aeromonas hydrophila* (51%; 95% confidence interval [CI]: 40.86–61.06%) and *Acinetobacter baumannii* (26%; 95% CI: 17.97–35.90%). No bacterial and fungal growth was revealed from pool water.

Only from an aquatic environment, both from skin and water samples, the opportunistic pathogen *A. baumannii* was isolated. This last bacterial isolate deserves special attention, since this bacterium has been often isolated from human clinical samples. This bacterial species is generally responsible of nosocomial infections, that are difficult to treat for its documented increased resistance to antibiotics (Howard *et al.*, 2012). Indeed, recent reports indicate that *A. baumannii* has also evolved into a veterinary nosocomial pathogen and, however, it cannot be excluded that animals may occasionally play a role as reservoir for *A. baumannii* (van der Kolk *et al*., 2019).

A. hydrophila, present in all the samples examined, is an aquatic microorganism that is able to produce cytotoxins and enterotoxins associated with acute gastroenteritis and wound infections in human, and haemorrhagic septicaemia in fish, reptiles and amphibians (Fernández *et al.*, 2000). Experiments on pathogenicity of *A. hydrophila* demonstrated that frogs exposed to these bacteria showed no morbidity or mortality (Schadich & Cole, 2010).

Furthermore, both *A. hydrophila* and *A. baumannii* were present in skin samples of frogs from tub, as well as *E. durans*, a commensal member of the normal intestinal flora of humans and animals used also as indicator of hygienic quality of water (Ashbolt *et al.*, 2001), and revealed in low percentage, herein, also in tub water samples.

Additionally, *A. viridans*, was only isolated from the tub water samples, while it was not found in the skin samples of frogs living in the tub. *A. viridans* has been associated with different human infections and different animal clinical specimens, such as crustaceans, sea turtles, pigs and cow's milk (Martin *et al.*, 2007).

Penicillium spp., the only yeast identified in our samples, represents the most common fungus observed worldwide that only occasionally is agent of human and animal mycoses (Guevara-Suarez *et al.*, 2016).

It was also noticed that the resident bacteria on frogs' skin were the same present in their own aquatic environment, except for the pool water sample containing chlorine able to inhibit the bacterial growth. Chlorine is by far the most commonly used antimicrobial agent and it is known that, even in very low levels, can show high antimicrobial activity.

The present work revealed that a large proportion (80–100%) of the isolates from skin of the tub and pool frogs was resistant to amoxicillin/clavulanic acid, ampicillin, gentamicin and tetracycline, while all the isolates showed high susceptibility (100%) against trimethoprim/sulfamethoxazole and vancomycin. The isolates from tub water showed higher percentages of resistance to amoxicillin/clavulanic acid, ampicillin, cefoxitin, doxycycline and gentamicin.

Our results about microbial resistance, that is of crucial significance to public health, have important implications and deserve considerable attention for the park environment where samplings were made. It is important to continuously monitor antibiotic-resistant bacteria because resistance may be spread among bacteria presenting on aquatic animals and in the water that hosts them.

Taken together, these results suggest that frog skin microbiota is susceptible to the belonging environment, the adopted chlorine treatment works well to sanitise water pool and several isolated bacterial strains resulted to be of interest in animal and public health. The percentage of multidrug resistant strains was high, and more studies are needed to understand this dissemination. However, in order to better characterise the interaction between animal and habitat, future research is required. The implications of this research are stimulating and they suggest that monitoring the skin-associated bacteria may provide a useful way to define the health of these animals and inferences with their habitat, also in relation to health of humans as visitors and insiders.

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Paper received 15.04.2019; accepted for publication 29.06.2019

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Journal of Applied Animal Welfare Science

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/haaw20

Assessment of living conditions in wild boars by analysis of oxidative stress markers

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JOURNAL OF APPLIED ANIMAL WELFARE SCIENCE
https://doi.org/10.1080/10888705.2020.1790365

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Assessment of living conditions in wild boars by analysis of oxidative stress markers

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ABSTRACT

This study demonstrated that it is possible to differentiate wild boars living in habitats with different animal densities by the measurement of oxidative stress markers. Therefore, reactive oxygen metabolites, the antioxidant barrier, i.e., the biological antioxidant potential and the antioxidative power (OXY-Adsorbent), as well as cortisol were measured in freely ranging wild boars. In two different areas of a State Forest in the Campania Region (Italy), 42 freely ranging, managed wild boars were captured with a corral trap, and blood samples were collected. The wild boars were divided by age (>1 year old and <1 year old) and sex (male and female). Animals in one area showed significantly higher values of oxidative stress parameters than those living in the other area. The annual boar censuses of areas highlighted a higher population density in the first area than in the second one, and this would explain the results obtained. Since the health or nutritional status of animals is reflected by oxidative stress, wild boars in areas with higher animal densities may live under worse conditions than animals in areas with lower densities which becomes evident by measuring oxidative stress markers. As cortisol in blood is only reflecting a short period, the measurement of oxidative stress level may be a better indicator to evaluate the living conditions of wild boars.

KEYWORDS
Wild boar; wildlife welfare; oxidative stress markers; cortisol; free-range breeding

Background

Wild boar (*Sus scrofa*) numbers have continuously increased throughout Europe (Amici, Cifuni, Contò, Esposito, & Failla, 2015; Massei et al., 2014; Ramanzin et al., 2010), and numerous studies have demonstrated an increasing conflict between wild boar and human activity, especially in agriculture (Ficetola, Bonardi, Mairota, Leronni, & Padoa-Schioppa, 2014; Hua, Yan, Li, He, & Li, 2016; Kruuse, Enno, & Oja, 2016; Laznik & Trdan, 2014; Lebocký & Petráš, 2015). Few studies have been conducted on the effect of the population density on the welfare of wild boars in the disturbed areas. Ecological, health, and welfare issues are usually considered together in the management of wild fauna.

The health and welfare problems that wild animals are faced with are also widely considered by the European community (Esposito & Amici, 2009). The maintenance of wild animals in captivity presents the same welfare problems that are common to domestic species (Esposito et al., 2017; Ranade, Talukder, Muscatello, & Celi, 2014). Welfare monitoring represents one of the instruments

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to assess the management procedures for wild fauna. Wild boar is a possible wild animal model to evaluate the stress factors involved in animal welfare.

Vertebrates cope with different environmental stimuli by initiating a stress response. The activation of the hypothalamic–pituitary–adrenal axis and the resultant secretion of glucocorticoid hormones (primarily cortisol) are characteristics of this response (Sheriff, Krebs, & Boonstra, 2011).

Oxidation is a ubiquitous phenomenon on earth, and all organisms are thus exposed to its damaging effect on cells (Cocchia et al., 2019; Tafuri et al., 2019). The increase in the oxidation state of biomolecules mediated by free radicals produced by cellular respiration or by exogenous factors can negatively impact the function of those biomolecules and ultimately leads to cellular deterioration and even to cell death (Finkel & Holbrook, 2000; Tafuri, Ciani, Iorio, Esposito, & Cocchia, 2015). Several factors may affect the oxidative status of an individual; immune response (Costantini & Dell'Omo, 2006; Klebanoff & Clark, 1978), sexual hormones (Zhu, Huang, Li, & Shen, 1997), stress hormones (Ohtsuka, Kojima, Ohtani, & Hayashi, 1998), thermal conditions (Flanagan, Moseley, & Buettner, 1998), diet (Sohal & Weindruch, 1996), and/or growth (Costantini et al., 2006) may affect the balance between prooxidants and antioxidants, increasing oxidative stress (Finkel & Holbrook, 2000). To counteract these effects, the body can rely on an antioxidant barrier, which is composed of both external (carotenoids, ascorbate, vitamin E, etc.) and internal (Gluthatione [GSH], proteins, bilirubin, uric acid, etc.) substances. Each of these components blocks the potential damaging action of free radicals giving electrons as the free radical reactivity is linked to the deficiency of these small negatively charged particles. Any insult against this barrier allows free radicals to attack and damage cell structures, setting the stage for the occurrence of lesions typical of oxidative stress and all their dangerous consequences (premature aging, disease, etc.) (Ciani, Cocchia, d'Angelo, & Tafuri, 2015; Del Prete et al., 2018).

The present study involved two groups of wild boars maintained under semi-free condition in a State Forest in the Campania region (Italy) and aimed to assess the boars' blood levels of cortisol and some markers of oxidative stress. The prooxidant state was evaluated by measurements of reactive oxygen metabolites (ROMs), and the antioxidant barrier was evaluated by the biological antioxidant potential (BAP) test and by the Oxydant-Adsorbent test (OXY-Adsorbent test). The comparison of the investigated parameters may provide information about the living conditions of wild boars, their health and welfare.

Methods

Study area and habitat

The study area, called "Cerreta Cognole," is classified as a State Forest in the Campania region (Italy). It is located between 40°14′39.23"N and 15°39′52.53"E. In total, the area, which is part of the "Cilento, Vallo di Diano e Alburni" National Park, covers 823 hectares.

The altitude ranges between 550 and 1100 mabove sea level; the temperatures vary from −2°C to 28°C, with an annual precipitation average of 800–900 mm/year.

Approximately 90% of the forest is covered by the dominant tree species, Austrian oak (*Quercus cerris*), of which 70% is mature forest and 20% is coppice (immature or degraded forest), while the remaining percentage is accounted for by mixed coppice Mediterranean forests, namely, deciduous species (*Fraxinus ornus, Ostrya carpinifolia, Acer spp.*), coniferous species (*Pinus halepensis, Pinus domesticus, Pinus pinaster*), chestnuts (*Castanea sativa*), and scrub (3%). A very small area (0.19%) is covered by pasture. The entire perimeter of the State Forest is completely fenced (27 km), and the whole area is subdivided into four 200-ha fenced zones (A, B, C, and D), each of which has internal water sources (streams and canals).

This study was performed in two areas, area C, of 222.3 hectares, and area D, of 217.7 hectares. Data from 2011 indicated that area C contained 49 wild boars per 100 hectares, and area D contained 95 wild boars per 100 hectares (Esposito et al., 2011). In the two areas considered in our study (C and D), two capture corrals (C1, C2, D1, and D2, respectively) of approximatively 40 square meters each were present. The position guaranteed the control of the animals distributed over the entire area (C1: 40°14′16.12”N–15°38′24.28”E; C2: 40°14′9.58”N–15°37′57.15”E; D1: 40°14′38.42”N–15° 38′37.98”E; D2: 40°15′0.88”N–15°38′16.05”E). The wild boars freely feed, although the corrals, in addition to aiding in capturing the animals to sell, are used for food supplementation during a specific time of the year (winter–summer).

Animals

The study was carried out during the year 2010, with 42 freely ranging, managed wild boars that were captured during two different periods in March and in April, during the spring season of the same year, in four corrals inside the fenced areas (C and D). All captured wild boars were classified according to age (>1 year and <1 year) and sex.

The captured wild boars were maintained inside the corral for a minimum of 7 hr and a maximum of 10 hr, starting from 1 hr after the traps were set (sunset) to the time the boars were captured (sunrise).

The presence of veterinary staff ensured the appropriate handling of the wild animals and guaranteed that the investigation was carried out in accordance with the EU Directive 2010/63/EU and the guidelines of the Animal Ethics Committee of the University of Naples Federico II (Italy).

Blood sampling

From the corral, each animal entered a wooden chute that led to a containment cage. Once inside the cage, each subject was held by the throat with a dogcatcher's hook. Blood samples were drawn from the jugular vein by a Veno Jet tube (Terumo, Leuven, Belgium) with a multi-sample needle 20-G × 1.5," according to good veterinary practice. After the blood sample was collected, each wild boar was marked with an ear band, and then, the cage was weighed both with and without the animal, and the difference between the two weights established the animal's weight. The animal was released at the end of the procedure. The blood sampling was performed early in the morning. Samples were transported on icepacks to our laboratory within 4 hr of collection. After refrigeration at 4°C, sera were obtained by gentle centrifugation (800 RPM for 5 min at 4°C). Sera with a hemoglobin content <0.3 mg/ml were considered suitable for further tests and labeled, before being stored at −20°C.

Sample analysis

The serum samples were used for the determination of ROMs, the BAP test, the OXY-Adsorbent test, and cortisol levels.

Measurement of ROMs, BAP test, and OXY-Adsorbent test

The ROM test determines the level of oxidative stress by measuring the amount of organic hydroperoxide (ROOH) that oxidizes N,N-diethyl-p-phenylenediamine (Alberti, Bolognini, Macciantelli, & Caratelli, 2000; Brambilla et al., 2003; Tafuri et al., 2018; Trotti, Carratelli, & Barbieri, 2002). The phenomenon is associated with the progressive and gradual color change to a pink reaction mixture, which was initially colorless. In the d-ROM test, ROMs of a biological sample are able to generate alkoxyl and peroxyl radicals, according to Fenton's reaction. Such radicals are able to oxidize an alkyl-substituted aromatic amine that is solubilized in a chromogenic mixture, thus producing a pink-colored derivative, which is photometrically quantified at 505 nm. The intensity of the developed color is directly proportional to the concentration of ROMs, according to Lambert–Beer's law, and is expressed as Carratelli units (1 CARR U = 0.08 mg hydrogen peroxide/dl).

The antioxidant barrier was evaluated using the BAP test and the OXY-Adsorbent test (Tafuri et al., 2018). In the BAP test, the dye solution contains a mixture of ferric chloride and a derived thiocyanate in solution. The addition of a sample of plasma to this causes a discoloration. The intensity of this discoloration is determined photometrically by using a wavelength of 505 nm, and the results are proportional to the ability of plasma to reduce ferric ions. The results obtained are evaluated as µmol/L of reduced ferric ions.

OXY-Adsorbent test measures the ability of plasma to counteract the oxidation induced by a solution of hypochlorous acid (HClO), a powerful and physiological oxidant able to mimic situations that occur *in vivo*. Unreactive HClO radicals further react with the chromogen solution of N,N-diethyl-p-phenylenediamine and produce a colored complex, which is measured at 505 nm. The results were expressed as µmol HClO/L.

In the BAP test, the antioxidant potential is evaluated as ferric reducing power, while in the OXY-Adsorbent test, it is measured as the ability of the antioxidant barrier to oppose the massive oxidant action of HClO.

The oxidative stress kits were purchased from Diacron International, Grosseto, Italy.

Measurement of cortisol

Duplicate sera samples were tested for cortisol using a chemiluminescent enzyme immunoassay (Immulite 1000 Assay System, Siemens Healthcare GmbH, Erlangen, Germany) under well-controlled conditions and according to the manufacturer's instructions.

Statistical analyses

All statistical analyses were performed using a program for statistical analysis (JMP® 8.0 SAS Institute Inc.).

For each of the variables considered, normal distributions were evaluated (JMP software). Since not enough samples were available for the variable "age," it was not possible to evaluate their statistical significance. However, we considered important to report the results obtained, reserving the right use of values as soon as possible. Van der Waerden's analysis of variance (one-way ANOVA) was used to test the differences in reactive oxygen species (free radicals and non-radical species), as measured by ROMs, BAP, OXY, and serum cortisol between the areas of capture and sex of the sampled wild boars according to the following formula: $Y_{ijk} = \mu + A_i + Sex_j + (A^*Sex)_{ij} + \varepsilon_{ijk}$.

Differences among data were also tested performing the analysis of variance with the method of ordinary least squares (JMP Software), and the significant differences between the measured parameters were confirmed. Significance was set at $P \leq 0.05$.

Results

The census carried out in the two areas indicated the presence of 95 adult wild boars in area D and 49 in area C. The catch percentage was therefore 34.74% in area D and 18.37% in area C.

Table 1 shows the numbers of captured wild boars in areas D and C divided into age and sex classes. The weights of males and females older than 1 year were higher in area D with respect to area C (Table 1).

Table 2 shows that the animals captured in area D (males + females) had significantly higher values of ROMs than those in area C (535.70 vs 379.23; $P < 0.01$).

Conversely, the values of BAP test were higher in wild boars of area C than those of area D (4107.42 vs. 3736.74; $P < 0.05$), while no difference was recorded for OXY-Adsorbent test values in both areas. As shown in Table 2, males had lower BAP values than females as well as lower ROM values than females. Blood cortisol was higher in animals originating from area D than in animals

Table 1. Number of wild boars (*n*), classes of age and sex (M and F), live body weight (LBW kg), and standard deviation (SD) in two survey areas (C and D).

		Area C		Area D	
Class of age	Sex	*n*	LBW (kg ± SD)	*n*	LBW (kg ± SD)
>1 year old	M	3	69.67 ± 1.53	4	80.50 ± 3.70
>1 year old	F	2	61.50 ± 3.54	14	67.93 ± 5.43
<1 year old	M	2	39.50 ± 2.12	5	43.20 ± 2.39
<1 year old	F	2	37.50 ± 2.12	10	40.40 ± 4.50
All	M	5	57.60 ± 16.59	9	59.78 ± 19.87
All	F	4	49.50 ± 14.06	24	57.10 ± 14.63
>1 year old	M + F	5	66.40 ± 4.93	18	70.72 ± 7.34
<1 year old	M + F	4	38.50 ± 2.08	15	41.33 ± 4.06
Total		9	54.00 ± 15.16	33	57.36 ± 16.02

Table 2. Number of wild boars (M + F) in both areas of investigation (C and D) and all boars examined for different stress parameters (cortisol, ROMs, BAP, and OXY).

	n	Cortisol (µg/dl)	ROMs (UCarr[a])	BAP (µmol/L)	OXY (mmol/L)
M + F C	9	26.46 ± 2.30*	379.23 ± 127.89*	4107.42 ± 777.16	312.38 ± 62.88*
M + F D	33	28.97 ± 2.97*	535.70 ± 83.90*	3736.74 ± 829.92	356.92 ± 65.89*
Total M	14	29.98 ± 2.73*	449.33 ± 106.30*	3247.93 ± 507.57*	317.08 ± 54.41*
Total F	28	27.65 ± 2.87*	528.59 ± 109.50*	4100.29 ± 813.13*	362.53 ± 68.54*

*$P < 0.05$.
[a]1 UCarr corresponds to 0.08 mg hydroperoxide/100 ml H_2O_2.

originating from area C (28.97 vs. 26.46; $P < 0.05$) and higher in males than in females (29.98 vs. 27.65; $P < 0.01$) (Table 2).

Table 3 shows the results for all measured parameters dependent on area, age, and sex of the animals.

Discussion

In this study, stress level markers were quantified in wild boars. Therefore, ROMs and the antioxidant barrier, i.e., the BAP and the antioxidative power (OXY-Adsorbent), as well as cortisol were measured in freely ranging wild boars to get basic knowledge concerning (oxidative) stress in wild boars. The number of subjects was not consistent between the two study areas, but this is very common for studies with wild or semi-wild animals. Despite this limitation, the present study performed on wild boars revealed different stress and oxidative stress responses in the animals captured in the two survey areas, placed at "Cilento, Vallo di Diano e Alburni" National Park of Campania Region.

A state of oxidative stress is not easy to identify since it depends on the balance established between the prooxidant and antioxidant species (Esposito et al., 2017; Tafuri et al., 2018). Oxidative stress is a health risk factor involved in aging and in several diseases, in humans and/or in animals (Tafuri, Cocchia, Landolfi, Iorio, & Ciani, 2016), and if it is monitored over time, it may be a valid approach to predict the health status of an individual.

The measured stress parameters varied between animals from area C and area D and between males and females.

The results obtained suggest that the animals from area D lived under worse conditions than those from area C (higher values of cortisol, ROMs, and OXY and lower values of BAP – Table 2). It was also found that males showed higher cortisol levels and lower BAP values than females (Table 2). These results may be explained first by the different population densities in areas D and C, with 95 wild boars/100 hectares vs 49 wild boars/100 hectares, respectively, and secondly by different physiological responses typical of species that are closely linked to behavioral manifestations in

Table 3. Different stress parameters (cortisol, ROMs, BAP, and OXY) measured in animals (M and F) of different ages (>1 year old and <1 year old) in the two study areas (C and D).

		M > 1 year old		F > 1 year old		M < 1 year old		F < 1 year old		All
Cortisol										
Area	*N*	(µg/dl)	*n*	(µg/dl)	n	(µg/dl)	*n*	(µg/dl)		
C	3	29.13 ± 0.28	2	25.10 ± 0.17	2	26.51 ± 0.66	2	23.77 ± 1.44	9	26.46 ± 2.30
D	4	33.76 ± 0.68	14	28.83 ± 2.72	5	28.86 ± 1.16	10	27.29 ± 2.58	33	28.97 ± 2.97
ROMs										
	N	(UCarr)	*n*	(UCarr)	*n*	(UCarr)	*n*	(UCarr)		
C	3	383.84 ± 122.24	2	367.92 ± 196.07	2	384.50 ± 246.44	2	378.38 ± 37.56	9	379.23 ± 127.89
D	4	520.28 ± 55.28	14	607.82 ± 37.87	5	457.81 ± 36.53	10	479.85 ± 79.45	33	535.70 ± 83.90
BAP										
	N	(µmol/L)	*n*	(µmol/L)	*n*	(µmol/L)	*n*	(µmol/L)		
C	3	3817.45 ± 482.98	2	4858.23 ± 70.57	2	3081.86 ± 85.67	2	4817.14 ± 8.41	9	4107.42 ± 788.16
D	4	3301.14 ± 459.94	14	4012.74 ± 793.93	5	2930.08 ± 417.11	10	3927.90 ± 886.42	33	3736.74 ± 829.92
OXY										
	N	(mmol/L)	*n*	(mmol/L)	*n*	(mmol/L)	*n*	(mmol/L)		
C	3	293.95 ± 40.07	2	342.79 ± 57.23	2	238.81 ± 24.96	2	383.20 ± 28.31	9	312.38 ± 62.88
D	4	320.20 ± 43.91	14	350.48 ± 66.12	5	359.76 ± 38.29	10	379.20 ± 81.29	33	356.92 ± 65.89

a defined period of the year (reproductive seasonality and giving birth). The environmental characteristics are significantly conditioned by the different concentrations of the animals mentioned above. The higher concentration in area D changes the habitat characteristics and reduces the food resource availability. In the spring season, the mothers have greater nutritional requirements consequent to the birth of the newborn boars and the initiation of lactation; therefore, the presence of lower food resources and the greater intra-specific competition increase the stress condition.

Our results do not agree with another study carried out in swine and wild boars by Brambilla et al. (2003), where the absolute sera values of ROMs were lower than those described in our study.

Obviously, large variations between different populations are possible. In the two survey areas, it is possible to hypothesize that the conditions in the D area were more stressful despite the animals having a greater weight than those in area C (Table 1). This stress could be linked to the need for stronger competition to obtain food and maintain the structures of family groups.

Although the concentrations of blood cortisol are not suitable to evaluate long-term stress, our findings showed higher values in all animals sampled in area D with respect to area C, and a stronger response of whole males (Table 2), due to the capture that is a stressful factor in itself (Esposito et al., 2017).

Oxidative stress is indicative of the health status or the nutritional status of an animal, and its measurement is important for animal welfare. Therefore, pro- and antioxidative parameters may be helpful in evaluating the living conditions of wild animals, together with other welfare indicators.

Conclusions

This study demonstrated that it is possible to differentiate between two groups of wild boars living under different environmental conditions based on the measurement of oxidative stress parameters.

In a previous trial, we demonstrated that the cortisol level is a stress indicator in hares (*Lepus europaeus*) but that only one parameter is not sufficient to evaluate a response in a wild animal that is subjected to many variables (Esposito et al., 2017). The evaluation of both pro-oxidative and antioxidative states can help us to follow the metabolic responses of wild animals and improve wildlife management.

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Article

A Night at the OPERA: A Conceptual Framework for an Integrated Distributed Sensor Network-Based System to Figure out Safety Protocols for Animals under Risk of Fire †

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† The present paper is the extended and revised version of: Tamburis, O.; de Pandi, F.; Tocchi, A.; D'Arco, M.; Giannino, F.; Piscopo, N. and Esposito, L. Guaranteeing safety of animals under risk of fire: conceptual framework and technical issues analysis. In Proceedings of the 2019 IEEE International Workshop on Metrology for Agriculture and Forestry (*MetroAgriFor*), Portici, Italy, 24–26 October 2019; pp. 100–104.

Received: 3 March 2020; Accepted: 27 April 2020; Published: 29 April 2020

Abstract: Large scale wildfire events that occurred around the world involved a massive loss of animal lives, with a consequent economic impact on agricultural holdings and damages to ecosystems. Preparing animals for a wildfire evacuation requires an extra level of planning, preparedness and coordination, which is missing in the current practice. This paper describes a conceptual framework of an ICT system implemented to support the activities of the Regional Veterinary referral Center for non-epidemic emergencies (CeRVEnE) in the Campania Region for the twofold objectives. On the one hand, it realizes the monitoring of the wooded areas under risk of fire in the so-called "Mount Vesuvius' red zone". On the other hand, it determines the **OP**timal **E**vacuation **R**oute for **A**nimals (**OPERA**) in case of fire, for each of the reported animal species living in the mentioned red zone. The main innovation of the proposed system lies in its software architecture that aims at integrating a Distributed Sensor Network (DSN), an ad-hoc software to generate timely simulations for fire risk modeling, and a GIS (Geographic Information System) for both the activities of web mapping and OPERA definition. This paper shows some effective preliminary results of the system implementation. The importance of the system mainly lies in its accordance with the so-called "Foresight approach" perspective, that provides models and tools to guarantee the prevention of systematic failure in disaster risk management, and becomes moreover critical in the case of Mount Vesuvius, which hosts a unique combination of both animal and anthropic elements within a delicate natural ecosystem.

Keywords: fire risk; OPERA; animal safety; Distributed Sensor Network; simulation; disaster risk management

1. Introduction

Any world's complex system interacts with its environment, and this in turn features microsystems interacting with it: a large and delicate chain of interactions that, once broken down, not only affects the named system, but also the whole surrounding system in an irreversible manner (systematic failure) that leads to the loss of its function, thus causing a not readily recovering to a previous situation [1]. Under such a premise, providing a well-specific and shared definition for the term "disaster" is not an easy task, as it encompasses any kind of rapid-onset natural and man-made hazards, from disease spreading to avalanches, to railway accidents, just to name a few [2–4]. In particular, for what concerns fire-related disasters, the 2013 Annual Report of the United Nations office for disaster risk reduction stated that "[…] the devastating impact of forest fires on natural resources was neither quantified nor adequately took into account. Fires are harmful for a number of ecosystem services (whose loss is estimated at around 146–191 Bln dollars per year) including carbon storage, biodiversity support, protection of water sources, reduction of soil erosion, land degradation, and climate regulation" [5].

Accordingly, the whole set of actions itself to be defined, organized and eventually deployed to handle a disaster goes under many declinations, depending in this case on the specific aspects to be accounted for: the panorama spans therefore the concepts of disaster management (whose main focus is handling the events), risk management (which reviews trends with a concentration on the analysis of the risks and develops a certain response), and disaster risk management (which also has a look at trends and events, and proposes some actions) [6].

In this scenario, the CeRVEnE (Italian acronym for "Regional Veterinary referral Center for non-epidemic emergencies") [7] was established in 2017 in the Italian Region of Campania, pursuing the objective of the Regional Government to improving and protecting people's health through the timely management of both veterinary and non-veterinary epidemic emergencies related to animal health as well as to food safety. CeRVEnE's mission is to analyze the consequences of disaster events, especially in social and economic terms, on territories, agricultural supply chains, and zootechnic systems, along with a particular emphasis on the Veterinary field: a structured approach to making decisions that aims at aligning with the main principles of the so-called "disaster relief supply chain quality management" (DRSCQM) system, as introduced by [8]. In accordance to this, among the initiatives of CeRVEnE, the FRAC Program (*Fire Risk Assessment in Campania Region*) was presented in 2018, as a project through which the Center intends to provide the Regional Government with a strategic tool able to: (i) gather and supply in a short amount of time all the necessary information to those professionals called to handle fire-related risks; (ii) have a large (both specialized and not specialized) public informed about the operational processes to get started after the fire damages in terms of safeguard and recovery of ecosystems (and related services), wild and domestic fauna, production supply chains; and (iii) figure out a standardized methodology for gathering data and supporting decisions.

The present work intends to describe the conceptual framework and to show some preliminary results of an ICT system to be implemented in order to support the activities presented in the FRAC Program. The aim is to enrich the line of inquiry into fire risk monitoring innovation by addressing a number of technical exigencies all-at-once, pursuing a twofold objective, that is on the one side to monitor the wooded areas under risk of fire in the so-called "Mount Vesuvius' red zone", and on the other side to determine the **OP**timal **E**vacuation **R**oute for **A**nimals (**OPERA** from now on) in case of fire, for each of the reported animal species living in the mentioned red zone. During a wildland fire, local animal rescue organizations' and/or farms' employees work with law enforcement and fire departments to rescue as many animals as they can. Such rescue operations are not well planned ahead, and typically depend on spontaneous volunteers in disaster response, despite the availability of government regulations such as the 2006 PETS Act in the US or the Directive 2010/63/EU within the European Union. Collaborative and ICT technologies in emergency response have a great impact and role, and there is quite a vast body of literature on this. Despite being used with proficiency in human rescue activities, sensor/cellular networks and decision support systems have not found the same application for animal rescue, yet. This study aims at filling this gap by proposing one of the first attempts for devising an ICT-enforced rescue system for companion, farm and wild animals.

The paper is organized as follows: after the Introduction, Section 2 describes the related research work conducted for disaster risk management and fire risk monitoring. The proposed ICT system is presented in three core sections. Specifically, Section 3 gives a model of the proposed ICT system analyzing the issues of creating a fire propagation map and defining a compatible evacuation plan; Section 4 surveys the infrastructures supporting the implementation of the ICT system; Section 5 focuses on the route optimization problem and the design of a system to grant its effective operation. The work also presents in Section 6 some preliminary results, including the analysis of a first-case scenario. Finally, Section 7 reports some concluding remarks.

2. Related Research Work

2.1. Disaster Risk Management

Disasters have always coexisted with civilizations. With technological advancements, development initiatives resulted in the creation of a lot of infrastructure and permanent assets, as well as in the thriving of the field of study concerning the management of disaster events, which includes the total sum of all activities, programs and measures that can be taken up before, during and after a disaster with the purpose to avoid it, reduce its impact or recover from its losses. The last decade of XX century was observed by the International Community as the "International Decade for natural disaster reduction", and was dedicated to promoting solutions to reduce risks from natural hazards. The definition of fairly common vocabulary for the specific field was therefore attempted by many scholars: for instance, according to [9], hazard may be defined as "a dangerous condition or event, that threat or have the potential for causing injury to life or damage to property or the environment." Hazards can be grouped into two broad categories, namely natural (e.g., cyclones, earthquakes, volcanic eruptions) and manmade (due to human negligence, such as chemical, industrial and nuclear accidents). Landslides, floods, drought, and fires are instead introduced as socio-natural hazards, since their causes are both natural and manmade. Vulnerability may be defined as "the extent to which a community, structure, services or geographic area is likely to be damaged or disrupted by the impact of particular hazard, on account of their nature, construction and proximity to hazardous terrains or a disaster prone area". Vulnerabilities can be categorized into physical and socio-economic. Capacity can be defined as "resources, means and strengths which exist in households and communities and which enable them to cope with, withstand, prepare for, prevent, mitigate or quickly recover from a disaster". People's capacity can also be taken into account. Capacities could be classified into physical and socio-economic capacities. Risk is a "measure of the expected losses due to a hazard event occurring in a given area over a specific time period. Risk is a function of the probability of particular hazardous event and the losses it would cause." The level of risk depends upon: (i) nature of the hazard; (ii) vulnerability of the elements which are affected; (iii) economic value of those elements. A community/locality is said to be at 'risk' when it is exposed to hazards and is likely to be adversely affected by its impact.

In the wake of this, the authors in [6] pointed out some questions as to how to envision a picture of "future disasters", such as: how reliable those visions are; whether the approaches applied to the future are on the right track; and whether there are any alternatives to improve current visions. To that end, the so-called "Foresight approach" provides models and tools to guarantee the prevention of systematic failure in disaster management [10]. As implied by [11,12], 'the future' is considered as emerging from the interaction of four components, namely: (i) events (those making people doubt the efficacy of thinking about the future at all); (ii) trends (a first type includes those being continuations of both the present and the past; a second type involves the cyclical patterns not being part of our own personal experience; a third type relates to emerging issues which are completely new); (iii) images (fears, hopes, beliefs, and concerns about the future); (iv) actions (based on the forecasts).

Critical importance is then assumed by the concept of "Disaster Management Cycle/DMC" (Figure 1), which shares isomorphic phases and associated concepts with the linear disaster phase [13,14]. Despite the alternative terminologies used in the literature (e.g., [2,15]), the Cycle aims at making clear that disaster and its management is not a series of events which start and stop with each disaster occurrence, but rather a continuum of interlinked activities. Other scholars [16] also derived the key disaster-related activities employing epidemiological methods, including rapid needs assessments, health surveillance, tracking systems, epidemiology investigations and studies, and registries.

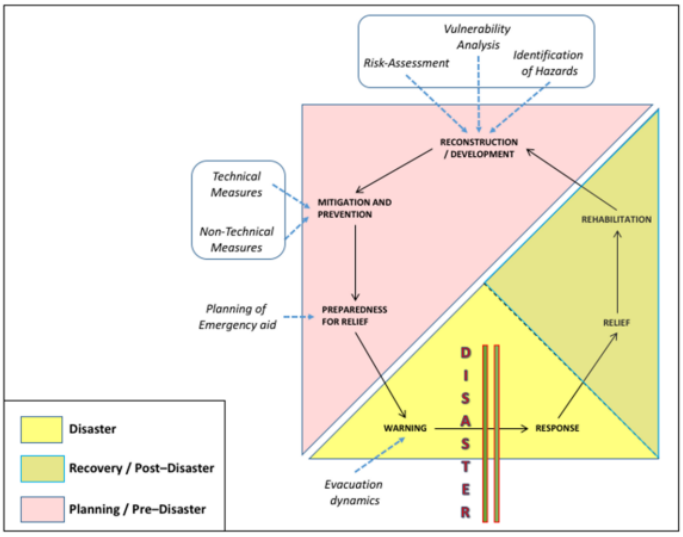

Figure 1. Disaster Management Cycle (elaborated from: Baird et al., 1975; Shorbi and Wan Hussin, 2015).

The main phases of the DMC can be briefly described as follows [17]:

- *Disaster Impact*: point (in time and space) at which a disaster occurs. However, including it serves as a reminder that—in disaster management terms—impact can vary between different types of disaster.
- *Response*: encompasses all the measures usually taken immediately prior to and following disaster impact, the latter being the most frequent application of the concept. Such measures are mainly directed toward saving life and protecting property, and to dealing with the immediate disruption, damage, and other effects caused by the disaster.
- *Recovery*: means the process by which communities and the nation are assisted in returning to their proper level of functioning following a disaster. The recovery process can be very protracted, taking 5–10 years, or even more.
- *Development*: such a segment provides the link between disaster-related activities and national development. Its inclusion is intended to ensure that the results of disaster are effectively reflected in future policies in the interest of national progress.
- *Mitigation*: specific programs intended to reduce the effects of disaster on a nation or community.
- *Prevention*: means the set of actions designed to impede the occurrence of a disaster and/or prevent such an occurrence having harmful effects on communities or key installations.
- *Preparedness*: usually regarded as comprising measures which enable governments, organizations, communities, and individuals to respond rapidly and effectively to disaster situations.

As a dynamic entity, all phases of the DMC infer and involve action. This obviously requires a range of specialist facilities and systems, usually needed to cover things such as direction and coordination of disaster-related action, emergency operations center activities, as well as warning dynamics-related information management. Among the main contributions brought to the development of the DMC, worth mentioning are: the mentioned "Foresight Approach", as its importance is being increasingly recognized by disaster managers and decision-makers for figuring out reliable long-term planning actions; the introduction of a social vulnerability index (SVI) that refers to the socioeconomic and demographic factors that affect the resilience of communities, after the original emphasis on infrastructure and technology [18]; the need to include animal issues into an overall emergency management strategy for a community [19].

2.2. Fire Risk Monitoring

The problem of early detection of fires in forest areas is widely recognized at both national and international level, as witnessed for instance by the European Forest Fire Information System (EFFIS), developed jointly by the European Commission (EC) services (Directorate General Environment and the Joint Research Centre) and the relevant fires services in the countries (forest fires and civil protection services) in response to the needs of European bodies such as the Monitoring and Information Centre of Civil Protection, the European Commission Services and the European Parliament [20]. Likewise, the Canadian wildland fire information system (CWFIS) is the national fire management information system, which elaborates daily information on fire weather, fire behavior potential and selected upper atmospheric conditions [21,22], while the Federal Land Assistance, Management and Enhancement (FLAME) Act of 2009 in the USA requires the Secretaries of Agriculture and Interior to develop a Cohesive Wildland Fire Management Strategy (CWFMS) and initiate a collaborative process between government and non-government agencies and devise solutions to wildland fire management issues [23]. A necessary look to Australia reveals a long story of forest fires (a long time before what occurred at the beginning of 2020), against which an as long tradition is standing of collection and elaboration of fire-mapping data derived from Moderate Resolution Imaging Spectroradiometer (MODIS) satellite imagery, and then, made available into the North Australia Fire Information (NAFI) website [24], along with the deployment of long-term and landscape-scale studies concerning the interactions between fire regimes and introduced livestock to pursue the conservation goal of population recovery for small mammals [25].

Forest fire is a sudden, strong and harmful natural disaster, which is why its quantitative analysis and modeling are important foci for many branches of research. Accordingly, different kinds of technologies have been developed and implemented for the measurement of the relevant parameters for early fire detection in risky areas. In particular, the use of GIS and remote sensing technology plays an important role in forest fire detection and prediction: on the one side, the implementation of hierarchical Wireless Sensor Networks (WSN) means the use of a number of sensing nodes that are capable of effectively gathering information from the surrounding environment and communicating with each other to send the measured data to a base station for further processing [26,27]; on the other side, Geographic Information Systems (GIS) can be used to combine different forest fire causing factors for attaining the forest fire risk zone map. GIS implementation allows the evaluation of a set of parameters that affect the fire, such as topography and vegetation, with other land use information including population, settlements, forest fire towers, fire stations, intervention places, the characteristics of the staff that will intervene, and transportation; this can make it possible for example to figure out the shortest way of intervention during the disaster, and/or the areas to be emptied [28,29].

With reference to EFFIS, this has been equipped with remote sensing techniques since 1998, while GPS tools are only used to determine fire perimeters. Although this methodology may be considered very precise for small fires, it introduces errors when mapping fires of large size. On the other hand, remote sensing is considered very reliable for mapping large fires, while less precise when mapping fires of small size, which may be omitted in the process of automatic classification of remote sensing imagery [30,31]. Moreover, many scholars developed general models to provide an assistant decision-making means for forest fire prevention systems, and thus, provide a scientific basis for disaster prevention, mitigation and assessment of post-disaster losses; it is e.g., the case of [32,33]. In other cases, the matter has been addressed for specific areas, such as Canada [34], India

[35,36], Europe (e.g., [37]), or Mumbai [38]; in addition, [39] have studied the use of drones to map environments and survey them for items of interest such as forest fires, landslides or wild animals, thus gaining traction in various research communities.

3. ICT System Modeling

The software architecture presented in this paper aims at integrating a Distributed Sensor Network (DSN), an ad-hoc software to generate timely simulations for fire risk modeling, and a GIS (Geographic Information System) for both the activities of web mapping and OPERA definition. In particular, the latter step will be performed as an arborescence optimization problem to be solved, for instance, via label setting-correcting algorithms [40]. Such a framework is supposed to overcome some of the limitations emerged in the description of many of the current solutions adopted worldwide and descripted in the previous sections. Specifically, an effective integration of the single aspects proposed as components for our system is actually lacking, nor solutions capable of addressing all the issues of the DMC appear to be available. Moreover, the clear inclusion of animal issues within the larger dimension of the emergency management strategies seems not to be a "trendy topic", whilst the inextricable connections between humans, animals, and surrounding environment emerges as one of the cornerstones of the One Health concept, which is inevitably called into account when dealing with the management of (not only fire-related) hazards [41,42].

Figure 2 depicts the architecture of the proposed framework [43]. At the beginning, a set of specific information is gathered to create a Fire Propagation Map, through which characteristics and dynamics of a fire episode can be analyzed and evaluated (represented in the figure in the first upper rectangle). Starting from this, an Evacuation Plan Model can be determined (the middle rectangle in the figure) by also adding anthropic-layered data related to both the urbanization rate and the road system development surrounding the area of interest, e.g., the Vesuvius in our study. For both steps, the deployment of specific sensors is required in order to collect the necessary mentioned situation- and context-aware information. Eventually, the model has to be enriched with the mapping of the animal presence (in terms of both typology and distribution) and the clear definition of type and size of vehicles requested to rescue the animal species involved in a fire episode. The determination of the OPERA for the involved rescuers (e.g., the beekeepers in our study) will be analyzed as a first-case scenario (in the lower rectangle of the figure), in order to the show the internal logic of the system as well as to provide some preliminary results as to its working dynamics.

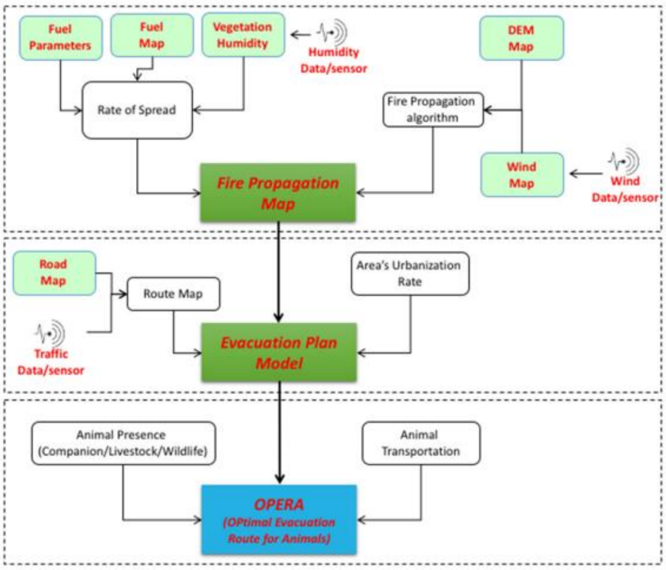

Figure 2. Architecture of the proposed conceptual framework.

3.1. Mapping of the Territories

Satellite data represent the primary source of information for mapping areas under risk of fire. Figure 3 reports the main results of a first extended mapping of the territories surrounding Mount Vesuvius performed during the years 2018 and 2019 within the FRAC program. The purpose was twofold: (i) figure out a reliable map to classify the different fire risk areas, according to the Fire Risks Assessment Matrix (Figure 4) [44–46] (left side); (ii) detect and count all the farming activities concerning poultry, sheep and goats, cattle, bees, swine, and equines (right side).

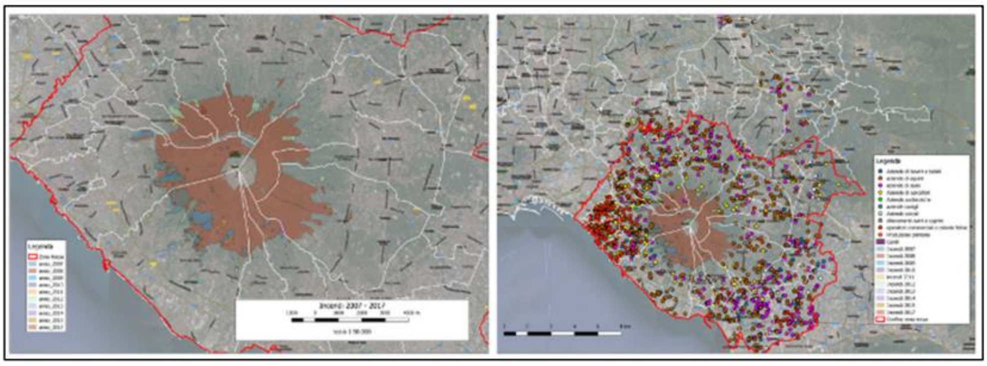

Figure 3. Map of the territories surrounding Mount Vesuvius, reporting fire risk areas (left) and farming activities classified according to the Fire Risk Assessment Matrix (right) (Source: FRAC program).

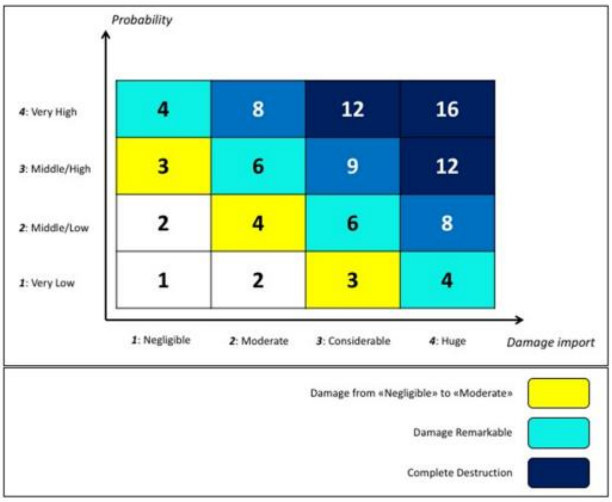

Figure 4. Fire Risk Assessment Matrix.

3.2. TIGER Simulation Tool

With reference to Figure 2, the Fire Propagation Map is the output of the so-called TIGER simulation tool [47,48]. In particular, TIGER calculates a discrete time spread of the fire perimeter in a 2D landscape using two simple modules:

- the first allows calculating the rate of spread (ROS) maximum according to fuel type/vegetation distribution and vegetation humidity data;
- the second performs a geometric algorithm to calculate the perimeter evolution of the fire according to Digital Elevation Map (DEM) and wind data.

The effective ROS, derived by the calculation of the fire intensity / speed, is determined by the Equation (1) as follows:

$$ROS\_Effective = ROS\_max \cdot Wind\_Effect \cdot Slope\_Effect \quad (1)$$

where: ROS_max (m / hour) is the maximum ROS; Wind_Effect and Slope_Effect are the limiting factors of the wind and slope, respectively.

Moreover, each area covered by fire is described by a perimeter formed by nodes and segments. In the model, in each simulation step, the nodes move outward to form a new larger perimeter according to the ROS_effective parameter. Consequently, the model requires the following input data:

- DEM (digital elevation map), with a horizontal grid resolution of 20–40 m. The projection must be metric UTM WGS84;
- a fuel classification map based on the Anderson types [49];
- vegetation humidity data;
- observations and/or wind forecasts for the areas of interest (average speed and directions).

In the proposed system, the vegetation humidity and wind data will be provided from the implementation of an integrated sensor net distributed on the landscape.

4. ICT System Infrastructures

An outline on the DSN duties are given as a foreword to a thorough analysis of the technical issues related to its terminal units, i.e., on-field devices. State-of-the-art technologies footing terminal units, network gateway, and structured data management are then reviewed, with the aim of figuring out background notes for an executive design of the DSN as a part of a much complex ICT system. Models, methods and software solutions that constitute the application level of the ICT system are discussed at the end of the section. (Anyway, although sensor networks based on wireless connections are becoming widespread because of their cheapness and easiness of installation, more general distributed sensor networks that allow coexistence of wireless and cable connections are here considered.)

4.1. DSN Duties

DSNs have widely been investigated in the past, especially after the widespread deployment of wireless technologies, which have largely simplified and made cheap network installation and maintenance operations [26,27]. At present, one can say that the design of networks for a variety of applications is easily affordable. However, for severe working conditions, such as those related to the presence of fire or other kinds of harsh environments, the work of the designer becomes sensitive. The aimed DSN is in charge of detecting critical events, monitoring their dynamics, and, eventually, signaling early warnings. Additionally, it is capable of tracking people and animals' activities in the area at risk, as well as informing about traffic jams along escape routes. The reliability of the aforementioned information is essential to the final goal of the overhead ICT system, devoted to real-time mapping risks and effectively implementing, on the need, the evacuation plan.

Due to the plurality of the targets demanded to the DSN, the network designer has to consider several technologies and arrange solutions to let them coexist; satisfying all specifications is otherwise unfeasible. Nonetheless, he has to obey the constraints for interfacing the network with the application level of the ICT system, where the algorithms for the control of high level operations, such as identification of optimal evacuation routes and most favorable flows scheduling, are executed.

4.2. DSN Technical Issues

The main technical issues at the design and deployment stage concern both the very purpose of the DSN and the requirements of it being a part of a multifaceted IT system.

Scrolling down the requirements of the DSN, scalability and upgradability stand as major ones. Scalability is intended as the possibility of integrating additional units in order to improve the basic network operation. Future upgradability is instead granted by conceiving the network as an open system with minimal constraints on interface requirements. In perspective, a relevant boost in the DSN's performance is, in fact, expected from the utilization of Internet of Things (IoT) devices now under testing. Thanks to the inherent granularity of IoT systems, as well as to their connectivity and integrated adaptation schemes, the DSN will definitely accomplish improved robustness and reliability with the integration of compatible IoT units.

The electricity demand of the network units should be managed with efficient strategies, also considering the presence of pre-existing infrastructures. The typical sleep/deep-sleep operative modes can be exploited, which involve that all non-critical modules are disconnected from the power supply except during their short operative slots. Nonetheless, smart power modules complemented with solar, micro-wind, or piezoelectric energy harvesting systems can sustain the electricity supplies made of batteries or super-capacitors.

Concerning data communications aspects, a hybrid design approach, that merges both wireless and cable connectivity, can offer superior robustness and reliability; as for the electricity infrastructure, therefore, the presence of pre-existing cabling has to be considered. Hybrid solutions also provide additional degrees of freedom, thus offering a larger set of solutions for trading-off between number of terminal units, adopted communication means, complexity and costs of the network.

The availability of a network clock signal is necessary for synchronization purposes. To this end, gateways equipped with low-cost GPS receivers are opportune. The network can be complemented with an additional protected gateway, implementing the black-box concept, such that post-disaster analysis is made possible. The protection of the black-box gateway represents a sensitive aspect and has to be assured by physically placing, or constructing for it, (at least for the critical parts of its hardware) a safe sheltered site.

In order to face issues related to harsh operating conditions, like those experienced during forest fires, the designer has to assure sufficient operating time also to other selected critical equipment, considering for them the use of superior casing structures, like IP67 anti-fire packages. The design cannot jeopardize maintenance issues after the DSN deployment. In particular, the on-field units of a DSN represent hot entries in maintenance programs, since most of them claim for on-site operations, such as battery replacement, which is troublesome if the unit is installed in a harsh environment. To this end, new prognostic and diagnostic methods, based on Artificial Intelligence (AI) approaches, can offer relevant benefits, in terms of faults reduction, improved overhauls rapidity, and maintenance costs as a whole. An AI sub-system supporting the DSN maintenance program can be included at application level in the ICT system.

Conclusively, the designer has the responsibility of finding the synthesis that best satisfies the mandatory requirements and grants compliance with standards that are applicable to network topology, specific hardware and software solutions, as well as to energy supply and ancillary energy harvesting systems.

4.3. State-of-the-Art Technologies Footing DSN

At the state-of-the-art, the innovative paradigm enabling IoT applications, sketched in Figure 5, seems to offer superior advantages in both design and implementation of a candidate DSN with respect to more traditional solutions. The DSN therefore has to be conceived as a network of smart devices, or smart things, with sensing and actuating capabilities, as sketched in Figure 6.

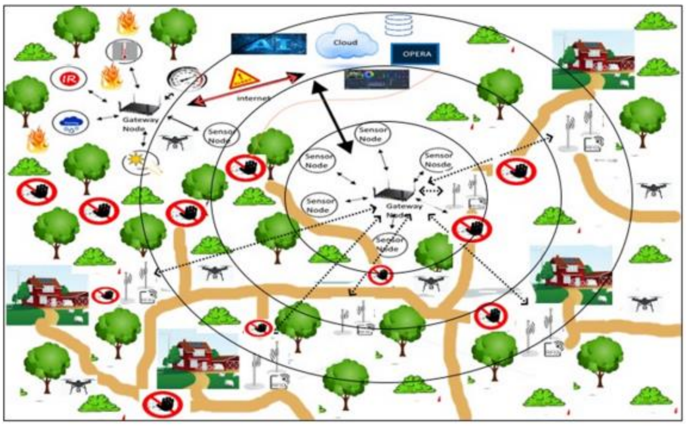

Figure 5. Schematic representation of a DSN for early detection and monitoring of fire in forest areas, eventually integrated with several facilities made available by IoT solutions.

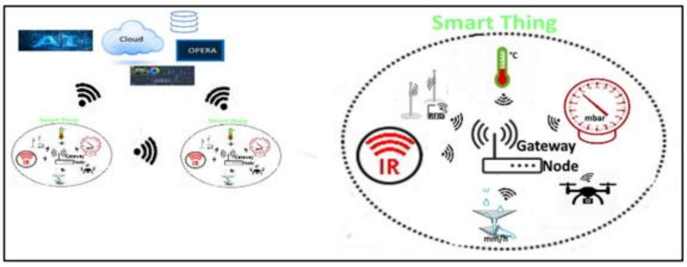

Figure 6. Smart devices with sensing and actuating capabilities and their inter-networking.

Smart devices mainly consist of sensors/actuators and gateway sections. Each sensor includes a smart power module, one or more probes, a processor, such as the one available in commercial boards hosting system-on-chips, and a communication module. The latter is often optionally included in the processor board; if not, it has to be connected as an external unit to obtain connectivity. Probes typically include an analog front-end and electronics for preprocessing the input. Most of them even provide analog to digital conversion, local data storage, and one or more standard digital interfaces. Smart detectors for smoke, relative humidity, temperature, atmosphere pressure, mono and dioxide carbide (CO, CO_2) percentages, solar irradiance, rain percentage, infrared/ultraviolet radiation intensity, volumetric water content and suction in soil, including the aforementioned characteristics, are commercially available. More recently, unmanned aerial vehicles (UAV), also known as drones, have successfully been used as sensors/actuators, thus showing the possibility of overtaking the barrier of the static concept of sensor in a DSN. Drones offer dynamic configurability and widen DSN terminal units to new kinds of operations not possible with fixed units. Furthermore, DSN units can include radio-frequency identification (RFID) options, by hosting one or more tags, and allow the intervention of personnel at the network level with the use of a handheld reader device. The tags exchange short data packets with the reader by exploiting the back-scattering principle, namely by radiating back a modulated version of the received signal, where the modulation is used to convey information. The reader equipment is made up of a power module, a processing machine and an RF transponder; it extracts the information from the on-field unit and makes it readable to the technician.

More in detail, RFID tags can be classed into active, passive and semi-passive. Active RFID transponders are self-powered, whereas passive ones are powered with an external electromagnetic signal. For passive tags, the reader equipment transmits energy, which is temporarily stored as charge in an auxiliary capacitor complementing the tag. Further classification is based on the adopted frequency band: the low-frequency (LF) tags operate in the range 125 up to 135 kHz, the high-frequency (HF) ones use the band centered at 13.56 MHz, the ultra-high-frequency (UHF) ones use the bands centered at 433, 868 and 956 MHz; the very last RFIDs on the market can use the free microwave band centered at 2.4 GHz. UHF and microwave RFIDs need shorter antennas with respect to LF ones and allow reading operations at longer distances, up to 10 m. UHF frequency range is also well suited to tracking applications, where fixed readers interrogate tags associated to animals, for instance inserted in a collar, to identify them and extract further information, which can be useful during emergencies events.

Data transmitted by terminal units should match simple structures and be concise, so that traffic between terminal units and gateways can easily be upgraded to upcoming technologies and communication protocols. Concise data and low data rates cope with the low power requirements of long-range wide-area networks and related protocols. At present, LoRaWAN, a novel wide range distance communication protocol robust to noise and interference, and capable of offering a coverage radius up to 15 km, deserves particular attention since it can be implemented using ultra-low power electronic circuits. A variety of low-cost equipment recently presented on the market supports long-range data transmission and allows rapid prototyping network solutions. For instance, there are starter packs and evaluation boards offered by major manufacturers that combine powerful 32-bit microcontrollers with several different expansion boards. These can include several micro electro-mechanical sensors (MEMS) as inertial (accelerometer and gyroscope), pressure, humidity, and temperature sensors. The use of proprietary solutions that operate as serial long-range transmitters, where data are encapsulated in key-value proper structure and transmitted to the gateway, should be considered as a secondary spare.

4.4. State-of-the-Art Technologies Footing Network Gateway and Structured Data Management

Gateways collect data from the terminal units deployed on field and forward them to the core processor of the ICT system. A network can include several gateways, each one connected to a subset of the terminal units. More specifically, gateways have to preprocess and merge the atomic data produced by the terminal units into structured information, and transmit it to the ICT platform. They require much more hardware in terms of processing and memory resources than terminal units.

Commercial boards, such as BeagleBone and Raspberry-pi, represent viable choices. They are very cheap single-board computers based on a renowned operative system (Linux), which can be equipped with additional modules for signal conditioning, analog to digital conversion, and wired/wireless standard communication interfaces. By exploiting the local storage capabilities of gateways, copies of critical data can be temporarily saved, thus avoiding information loss in case of occasional connection failure.

Gateways can communicate with the overhead ICT platform by means of either standard mobile or Ethernet protocols or message queue telemetry transport (MQTT) protocol, a publisher/subscriber application protocol fancied by IoT developers. Data refresh can be programmed at a low rate, which is sufficient for regular monitoring. The network can be designed such that data refresh is automatically increased during emergencies, eventually by complementing LoRa technology with redundant solutions. These can rely on different technologies capable of assuring wider bandwidth even at the expense of increased power requirements and shorter range coverage, and a suitable dynamic switching strategy.

Merging atomic data to produce structured information involves a distributed and hierarchical database architecture. In this scenario, each gateway plays its own role by saving data of a set of terminal units, namely those directly connected to it, and manage a portion of the whole database assembled by the overhead ICT system. Additionally, a subset of primary gateways of the network can be equipped with additional memory resources to store clones of the local databases hosted by adjacent gateways; this assures more robustness of the network in terms of capability to keep safe the collected data. Redis or MongoDB databases represent ideal candidates. They are recent solutions featuring innovative storage and data retrieval mechanisms, which are different from the tabular approaches used in relational databases. In particular, Redis is an open source in-memory data store, that grants high availability and fast responsiveness. It can cope with many data structures, from simple ones as strings to more complex ones as hash tables and lists. Nonetheless, its built-in replication mechanism, implemented according to a master/slave model, mostly simplifies the production of exact copies of database instances. It provides automatic partitioning, which is a way to automatically split and distribute data across different memory units. It offers a mechanism of self-diagnostics to constantly check if master and replica instances are synchronized.

Finally, the overhead ICT system can integrate cloud computing services that simplify storage and computational tasks, and produce more consistent, accurate, and useful information than atomic systems. Cloud services also represent efficient solutions for the implementation of artificial intelligence (AI)-based approaches, which are becoming important backings to solve very complex problems.

5. Route Optimization and Effective Implementation

5.1. Optimization Model

Defining OPERA means setting up an arborescence optimization problem, that could be tackled via the implementation of a label setting-correcting algorithm. In order to model such a problem [50], let then G = G(N, E, φ) be an oriented graph, where:

- N = *nodes*: set of all the accessible road junctions in Mount Vesuvius' red zone;
- E $\subseteq N \, x \, N$ = *edges*: roads that connect two consecutive junctions/nodes. It can be stated that: $e_k = \langle n_k, n_{k+1} \rangle$;
- $\varphi: E \rightarrow [0,1]$ = *weight function* (normalized) that assigns to each edge a value of intensity of traffic flow (defined in the continuous interval from 0 to 1).

Let moreover be:

- $t \in [0, t^*[\subset [0, +\infty[$ a discrete time interval, where t^* represents the final moment of the simulation;
- $n_{k,t_i} \in N$: junction occupied by the *k*-th token at the moment t_i.

Under such premises, some conditions can be defined. In the first moment, for any interval [t_i, t_{i+1}], it is possible to identify at least one edge, as expressed in Equation (2):

$$\forall t_i, t_{i+1} \in [0, t^*[\, , \exists \, \langle n_k, n_{k+h} \rangle \in E, with \, n_k, n_{k+h} \in N \tag{2}$$

For each moment t_i, each junction/node could be/could not be interested by the fire spreading. This can be expressed by introducing a function ρ as reported in the Equations (3) and (4):

$$\rho: N \, x \, [0, t^*[\rightarrow [0; 1] \tag{3}$$

where:

$$\rho = \begin{cases} 0, & n_k \, reached \, by \, the \, fire \, at \, t_i \\ 1, & n_k \, not \, reached \, by \, the \, fire \, at \, t_i \end{cases} \tag{4}$$

If the token is in n_k at the moment t_i, and from there many alternative routes are available, it can be assumed that there will be only one n_{k+1} junction related to the edge e_k with the least intensity of traffic flow. This is expressed in Equation (5) as:

$$\forall n_{k,t_i} \in N \, x \, [0, t^*[\, , \exists! \, n_{k+1} \in N \, with \, e_k \in E: min\varphi(e_k) \tag{5}$$

A further condition to be added to the previous one is that assigning a value of intensity of traffic flow to an edge means as well evaluating the most likely average speed a vehicle will deploy to cover the edge itself [51]. To that end, Equation (6) describes the average speed evaluated for *n* vehicles in a given time period:

$$S_t = \frac{1}{n} \cdot \sum_{i=1}^{n} S_i \tag{6}$$

where: S_i = speed of the *n*-th vehicle. As a consequence, the average speed evaluated on a given distance is expressed by Equation (7) as:

$$S_S = \frac{n}{\sum_{i=1}^{n} \frac{\Delta t_i}{L}} = \frac{1}{\frac{1}{n} \cdot \sum_{i=1}^{n} \frac{1}{S_i}} \quad (7)$$

Besides the speed average values, also necessary are: (i) the evaluation of the size and the number of the vehicles requested to transport the animals; (ii) S_{85} = the 85th percentile of the variable S, that is the speed only excessed by 15% of vehicles; and (iii) V_{mod} = most frequent cinematic interval.

The complete model is presented as follows, with which the objective is to minimize the escape route for each of the reported animal species living in the Vesuvius' red zone in case of fire, in order to the definition of effective safety protocols from CeRVEnE for the FRAC program.

$$OPERA = min \sum_{k=1}^{N} \rho(n_{k,t_i}) \neq 0 \quad (8)$$

$$s.t.\ (2)–(7)$$

OPERA poses very similar characteristics with the category of *exact problems*, for which the corresponding optimization problems can be polynomially solvable [52]. What was here presented is the attempt to find out a timely heuristic to provide an acceptable solution to the problem, even considering the peculiar context of application, as the core for the realization of a distance analysis tool for the GIS module of the integrated system at study to be endowed with [53–55].

5.2. Design of a Real-Time Routing Tasks Helping System

Section 4 has surveyed duties, technical issues, and available technologies for DSNs, with the main intent of showing the large number of possibilities at disposal of the designer. Additionally, network gateways and related system logics have been illustrated, still adopting a very general point of view, aiming at delineating a framework useful to the designer for tailoring solutions that can cope with general requirements.

Hereinafter, a specific solution for the implementation of the optimal escaping plan, according to the route model discussed in Section 5.1, is presented. The solution includes products for traffic flows, meteorology and environmental measurements, and software packages that can be exploited by means of ubiquitous facilities, like tablets or smartphones. An image list of products, available on the market and compatible between each other, to set up the proposed solution is given in Figure 7.

Figure 7. Image-list of compatible commercial products to set-up the system infrastructure. The system is capable of collecting the critical information needed to a real-time implementation of the route optimization strategy. On the top, from left to right: monitoring management and control software platform, meteorological station unit, wind speed relative humidity and atmospheric pressure sensors, solar irradiance sensor. On the bottom, from left to right: rain gauge, environmental sensor (CO, NO_2, O_3, PM10), multifunction local control unit, visibility sensing unit, microwave traffic counter and traffic classifier.

Traffic flow measurements can be carried out by means of non-intrusive and low power sensors available on the market for counting and classifying vehicles as well as measuring speed parameters, as required by the considered model. These sensors are typically installed on the center of the monitored lane, and, thanks to microwave technology and time-of-flight measurements of the electromagnetic signal, are capable of detecting the vertical profile of the vehicles (useful to the classification purpose), the direction of travel, and the corresponding speed. They are also capable of detecting headway and gap, the time the lane is occupied, and stop-and-go or stationary events, which are typical marks of a traffic jam. They are complemented by a firmware-hosting algorithm to identify accidents, overtaking, open worksites, and to perform statistical analyses in order to estimate average speed and percentiles, like those required by the model in Section 5.1. The sensors can be deployed on a selected subset of roads to directly sample the traffic flows, whereas software approaches can be exploited to infer traffic data on secondary roads.

Meteorology data are available at a marginal cost in systems like the described one by deploying low-cost and low power ancillary sensors. These offer valuable help in real-time forecasting programs by presenting weather data such as atmospheric temperature, precipitation, wind knots and direction, and visual range. It is worth noticing that temperature and visual range parameters are highly correlated to fire events and smoke, and can be used as indirect detectors or as options contributing in redundancy at monitoring. Similarly, environmental sensors can be included in the system with a marginal impact on the cost. They allow measuring air quality parameters such as carbon monoxide (CO), nitrogen dioxide (NO_2), ozone (O_3) and particulate matter (PM10), which are highly correlated to traffic and industry operation, too.

All the considered sensors can be powered by the electrical network, or alternatively by photovoltaic panels, or even battery, and are designed for low power consumption and energy savings. Nonetheless, the designer of the system can rely on customer programs by major manufacturers, according to which the aforementioned products are sold as open solutions, which can be complemented with optional functions implemented on demand.

Taking into account the specific application needs, traffic, environmental and meteorology sensors can be deployed in proximity to each other and connected to the same local control unit. The latter has to integrate standard interfaces for data transmission to remote collectors as well as on-site configuration and firmware updates. The local control units at present available on the market host one or more standard interfaces that can be selected among Ethernet, Wi-Fi, or 3G and 4G cellular networks, which are all capable of supporting even internet browsers.

The hardware of the communication infrastructure and the processing capabilities of the local units can be fully exploited when stepping from the peripheral level to the central level, where data from all local units are gathered, processed and validated. The central level system is in charge of supporting the main actors during the hazardous event. To this end, the central mainframe has to offer a service that should be accessible by the subject in an easy and effective way, for instance by means of a mobile application (app) designed for tablets and smartphones (to be further explored in the next phase).

6. Preliminary Results

The first case scenario chosen to figure out the proposed framework focuses on beekeepers as the beehives' transportation is in general less problematic in terms of general set up and execution.

The geospatial data provided from the South Italy Experimental Zoo-prophylactic Institute (Istituto Zooprofilattico Sperimentale del Mezzogiorno, IZSM) concerning the fires that occurred in the Mount Vesuvius surrounding area during the years 2007–2017, made it possible to localize all the beekeepers' farms, other than collecting information as to the negative impact of the fires on their production dynamics. Since it is known that the maximum flight range of a bee swarm is of 7 km from the source point (the beehive), two buffer zones were then considered (Figure 8):

- the first (orange line; width of the buffer radius = 1 km) was meant to localize all the beekeepers' farms directly involved in fire episodes for the period 2007–2017;

- The second (yellow line; width of the buffer radius = 7 km) was meant to figure out the areas indirectly involved by the effects of the mentioned fire episodes.

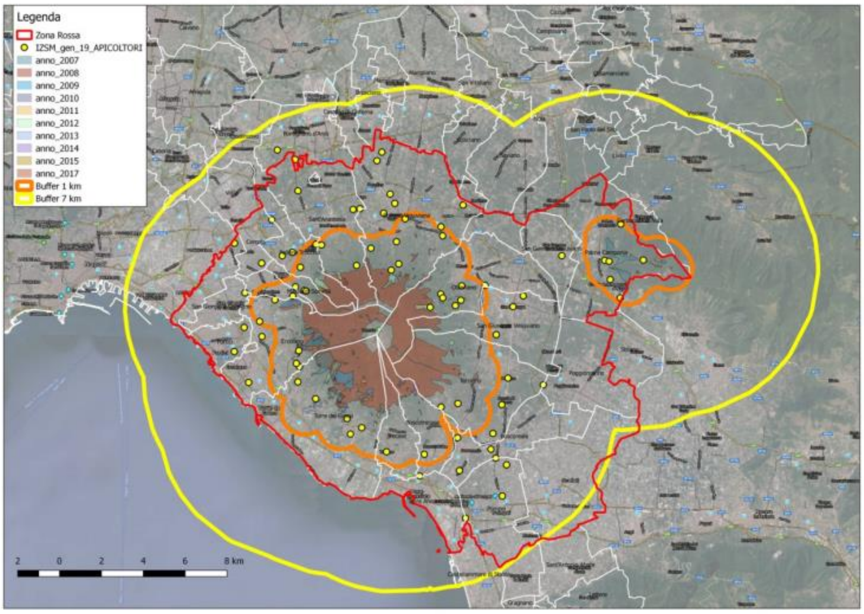

Figure 8. Buffer zones interested by direct (inner zone) and indirect (outer zone) effects of fires on beekeepers' farms for the period 2007–2017.

There are 81 mapped farms, located as follows:

- Thirty-four farms in the Vesuvian area, and set at a distance of less than 1 km from the areas directly involved in fire episodes;
- Six farms within the boundaries of the Municipality of Palma Campania, and set at a distance of less than 1 km from the areas directly involved in fire episodes;
- Forty-one farms comprised between 1 km and 7 km from the areas directly involved in fire episodes. In particular, four farms are outside the Vesuvius' red zone (two in the Municipality of Pomigliano d'Arco, and two in the Municipality of Nola).

The results of the analysis of the data highlighted that, since all the farms are set within the radius of the external buffer, it is highly likely that all the bees have been affected by the disaster episodes that occurred in the period of study.

Figure 9 reports the main working steps of the introduced ICT systems towards the definition of the OPERA for the case of a beekeeper farm set in via Carcova (starting junction/node) on Mount Vesuvius' northeast slope, up to the safe zone located at the nearest entrance of the A30 highway (ending junction/node). The map in Figure 9a was obtained by means of a GIS software for the purposes of the FRAC program. It shows the starting node within a white circle, along with the other beekeepers' sites standing on the territory under consideration, characterized by different fire risk areas (from yellow to red, in increasing danger level). In Figure 9b, the TIGER simulation tool is run, considering a fire source quite close to the beekeeper's site. In this case, the spreading of the fire front is calculated setting up a 5-min distance between two consecutive fire perimeters. In Figure 9c, both the simulation- and the route optimization-focused aspects have been combined as a two-layers representation on a Google Maps © chart: on the one side, the dashed yellow perimeters refer to the spreading of the fire front. In this case the simulation was run considering a 10-min distance between two consecutive fire perimeters. On the other side, the segmented white line was obtained by implementing the model from the Equation (8) that made it possible to identify the single junction-to-junction edges, then manually connected so as to draw the OPERA for the case study. The figure also shows the red cross which points out that that particular node, according to the simulation, would have already been reached by the fire by the time the token was supposed to get there, therefore it was necessary to calculate another path among all the available ones. The OPERA was eventually calculated for the case analyzed, and resulted to be equal to 16, i.e., the minimum path to get from the beekeeper farm in via Carcova to the A30 highway with a single vehicle to escape fire, comprising 16 nodes/junctions. Eventually, the UML sequence diagram of the ICT system is depicted in Figure 10 [56].

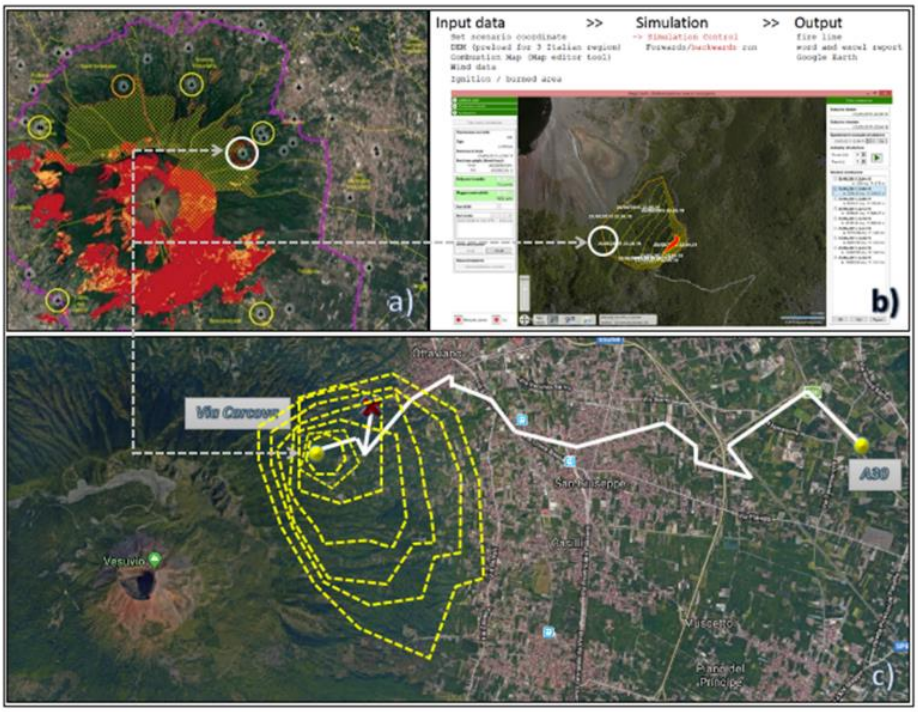

Figure 9. (**a**) mapping of the beekeepers' farms in Mount Vesuvius' red zone; (**b**) example of the TIGER simulation tool; (**c**) evaluation of the OPERA for a beekeeper farm.

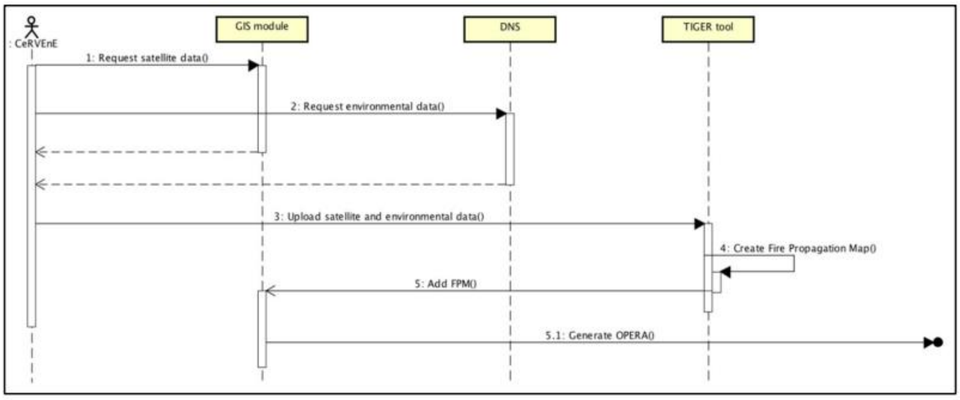

Figure 10. Sequence Diagram of the integrated ICT system.

7. Discussion and Concluding Remarks

The conceptual framework of the DSN-based system introduced in the present work represents a trustworthy data source for multi-risk models as it contributes in the different phases of a disaster management cycle, as has been described in Figure 1 [57].

With reference to the Planning/Pre-Disaster phase, its main contribution lies in helping the organization of effective actions for disaster mitigation, thanks to the possibility to previsualize escape routes so that timely and effective safety protocols can be figured out for each different animal species living in the concerned area. For what concerns the Disaster phase, the development of an advanced sensor network as early-warning system is on the other hand the key for the detection of precursory signals, which serves in short-term hazard prediction and in the activation of early warnings. It is as well called to play, in our case, a guiding role in fire-related emergency situations by offering real-time synoptic views on the affected areas, thus providing the necessary damage-related information to increase knowledge about loss distribution in the entire involved ecosystem. The Recovery/Post-Disaster phase relies eventually on the low-cost monitoring and evaluation of the recovery and reconstruction actions offered by remote sensing imagery. The monitoring is carried out through the analysis of a time series of satellite imagery that can be used e.g., to track the vegetation recovery after a wildfire.

That said, the development of such integrated system turns out as critical, since the fire risk (and especially arsons risk) has considerably increased in recent years in the whole Mount Vesuvius' surrounding area, which features a unique combination of both animal and anthropic elements within a very delicate natural ecosystem. Actually, the intent to design and test the system on a narrow area (the mentioned "red zone") to verify its viability also depends on another important feature of the FRAC Program, that is, the active involvement of a number of organizations (State Forestry Corps, Local Health Trusts, breeders' associations, up to the very Regional Government) that need to strictly interface with each other—and, in case of fire, in a very short time—in order to build up a solid and efficient system of surveillance planning, risk analysis, and data gathering. This means therefore to call into account and address disaster diplomacy-related issues as well [58]. As consequences of disasters are determined by a nuanced balance between vulnerability and resilience, figuring out (and of course, putting into practice) safety protocols for animals under risk of fire implies namely: understanding of ecosystem weaknesses (mitigation); fostering relationships between different actors (planning/preparation); engaging them in creative and cooperative ways (response); handling the transition from immediate relief toward longer-term development aid (recovery).

Under such a perspective, the implementation of a specific integrated system to support the mandate of the FRAC Program is supposed to boost an improvement for the CeRVEnE's activities, pursuant of the innovation perspectives coming with the effectual application of the disaster relief supply chain quality management principles. To such a purpose, the next steps to undertake will be particularly focused on the active involvement of the mentioned organizations, as future users of the ICT system here described, in order to funnel their vision and experience into the actual realization activities, thus enhancing robust design-based performances and routing protocols [59].

Author Contributions: Conceptualization, L.E. and O.T.; methodology, O.T., L.E., F.G., M.D. and A.T.; software, G.D.F., C.E.; validation, O.T., L.E., F.G. and M.D.; formal analysis, O.T., F.G., M.D., A.T., and C.E.; investigation, L.E., N.P., G.D.F. and O.T.; writing—original draft preparation, O.T.; writing—review and editing, O.T., F.G., M.D., N.P. and G.D.F.; supervision, O.T.; funding acquisition, L.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Regional Veterinary referral Center for non-epidemic emergencies (CeRVEnE) through the FRAC project (Good practices of the services of the Prevention Department in case of fire risks) funded by Campania Region, Office for Prevention and Veterinary Public Health.

Conflicts of Interest: The authors declare no conflict of interest.

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Management Models Applied to the Human-Wolf Conflict in Agro-Forestry-Pastoral Territories of Two Italian Protected Areas and One Spanish Game Area

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Citation: Piscopo, N.; Gentile, L.; Scioli, E.; Eguren, V.G.; Carvajal Urueña, A.M.; García, T.Y.; Alberti, J.P.; Esposito, L. Management Models Applied to the Human-Wolf Conflict in Agro-Forestry-Pastoral Territories of Two Italian Protected Areas and One Spanish Game Area. *Animals* **2021**, *11*, 1141. https://doi.org/10.3390/ani11041141

Academic Editors: Darryl Jones and Pia Lucidi

Received: 1 March 2021
Accepted: 12 April 2021
Published: 16 April 2021

Simple Summary: Conservation practices in the nature of some animal species are very difficult when they are in conflict with anthropogenic activities. In order to make possible the coexistence of a predator such as the wolf with animal breeding activities in the wild, the EU has produced solid and structured legislation through the Natura 2000 network. The application of the Habitats Directive allows the various member countries to choose biodiversity management actions as long as they maintain their resilience. Our work compares two different management methods developed in Spain and Italy with the aim of evaluating a possible difference in the conservation of wolf packs present in their respective territories. The results obtained show that both in Spain and Italy, the presence of the wolf causes damage to livestock. The economic damage is quite substantial and affects, in different ways, sheep, goat, bovine, and equine breeding. Nevertheless, wolf populations are stable in Spain, where hunting is allowed, and slightly increasing in Italy, where the species is particularly protected.

Abstract: Our work shows that, despite the persistence of persecutory actions, conservation activity has proved successful for the return of numerous wild mammals to different habitats, including the wolf. The human-wolf conflict is still described in all countries where the wolf is present. This is evidenced by the high number of damages on livestock, and the corpses of wolves found both in protected areas and in those where hunting is permitted. The diagnosis of road accidents, together with poisoning and poaching, are major causes of mortality. Although hunting records the highest percentage of kills in Spain, the demographic stability reported by the censuses suggests that this activity does not have a consistent influence on the Iberian wolf population's survival. In Italy, where wolf hunting is prohibited, wolf populations are to be increasing. In some Italian situations, wolf attacks on horses seem to cause unwanted damage to foals, but they represent a very precious source of information about the habits of carnivores. A simple management plan would be sufficient to help the coexistence between the productive parts and the ecosystem services ensured by the presence of the wolf. The presence of hybrids is a negative factor.

Keywords: wolf; conservation programs; human-wolf conflicts; wolf monitoring; protected areas; game reserve

1. Introduction

Before assessing the damage related to livestock and, even more so, the human-wolf conflict, it is necessary to describe the current state of the wolf presence in Europe.

Although the wolf present in Italy has been described by Altobello [1] as a typical subspecies *italicus*, and this classification is still given today [2], the discussions on its classification are not yet concluded. Issues such as wild, abandoned, and stray dogs, the genetic selection frequently made by humans to obtain new dog breeds, as well as disaster risks (e.g., fires) complicate the problem linked to the presence of wolf-dogs in natural environments [3–5].

The Iberian wolf (*Canis lupus signatus*) is an endemic subspecies of the Iberian Peninsula (Spain and Portugal). The scarcity of wild prey with consequent livestock predation seems to invest principally in Portugal and Spanish regions below the Duero River but does not prevent Iberian wolf survival.

1.1. Human-Wolf Conflict

General conflict at the European level generated by the presence of large carnivores into the agro-ecosystems has been underlined by the large number of damages that farmers-breeders complain about during the agro-food production. With reference to the recently published State of Nature 2020 report, it is essential to find workable solutions to mitigate the conflicts between human interests and large carnivores in line with EU law [6].

After the convention on biological diversity, numerous protected areas were established, which, thanks to specific management plans, have allowed the conservation of wolves and large carnivores in Europe. However, the presence of the wolf has also increased in unprotected areas, and, depending on the different management models, this predator has been responsible for greater or lesser damage to livestock farmers [7,8].

At the European level, the European Economic Community (EEC) Regulation implementing CITES (9 December 1996, n. 338) includes the Italian wolf population in Annex A, while the wolf populations of northern Spain, north of the Duero River, and Greece, north of the 39th parallel, have been placed in Annex B. This regulatory framework imposes specific authorizations for the movement of wolves, which can only be granted on the basis of careful assessments of destination site conditions.

The wolf has been protected in Italy since 3 July 1971, when hunting was prohibited by Decree of the Ministry of Agriculture. The Law 11 February 1992, n. 157 includes the wolf among the particularly protected species (art. 2, c. 1) and the President of the Italian Republic Decree (D.P.R.) 8 September 1997, n. 357, transposing the Habitats Directive (92/43/EEC), includes the wolf in Annex D, among the species of community interest that require rigorous protection.

The current regulatory framework for the protection of the wolf is reported on the new national plan for wolf conservation and management [9].

In Council Directive 92/43/EEC of 21 May 1992 about the "conservation of natural and semi-natural habitats and of wild flora and fauna," the Spanish populations of *Canis lupus* living south of the Duero River are covered in Annex II and IV. Both indicate for these carnivores the need to conserve them through the designation of Special Conservation Areas. Conversely, the Spanish populations of *Canis lupus* which live north of the Duero River, are among the species whose taking in the wild and exploitation may be subject to management measures (Annex V).

Both Italian and Spanish regulatory framework, in line with international guidelines [10,11], gives priority to conservation at the population level. A large part of the responsibilities regarding the monitoring, management, and requalification of wildlife, the repression of crimes, the implementation of any control plans, and the reimbursement of damages are left to the regions.

1.2. Specific Wolf Conservation Plans in EU

The wolf is present in 20/27 EU countries (74%) with an estimated population (2012 Census) of 12,375 individuals (18 countries) and 37 identified groups in Germany/Poland (36 groups) and in Spain (1 group). The comparison with the 2016 census allows us to report a growing population trend with the exception of the Karelian, Northwestern Iberian populations which seem to have increased or stabilized. The 36 groups described in Germany have become 780–1030 individuals, while the Sierra Morena group seems to be extinct (Table 1) [10].

Table 1. Distribution and presence of the wolf in Europe and in the EU member states.

Population Name	Countries	Size		Trend
		Census 2012	Census 2016	
Scandinavian	Norway, Sweden	260–330	c. 430	Increase
Karelian	Finland	150–165	c. 200	Stable to increase
Baltic	Estonia, Latvia, Lithuania, Poland	870–1400	1700–2240	Stable
Central European lowlands	Germany, Poland	36 groups	780–1030	Increase
Carpathian	Slovakia, Czech Republic, Poland, Romania, Hungary, Serbia	3000	3460–3849	Stable
Dinaric-Balkan	Slovenia, Croatia, Bosnia and Herzegovina, Montenegro, the former Yugoslav Republic of Macedonia, Albania, Serbia (incl. Kosovo), Greece, Bulgaria	3900	c. 4000	Unknown
Alps	Italy, France, Switzerland, Austria, Slovenia	280	420–550	Increase
Italian peninsula	Italy	600–800	1100–2400	Slightly increasing
		Census 2007		
NW Iberian	Spain, Portugal	2500	2500	Unknown
Sierra Morena	Spain	1 group	0	Extinct

Estimated number of wolves in Europe ~12,375 (2012)–14,590–17,199 (2016) in bold EU countries. http://ec.europa.eu/environment/nature/conservation/species/carnivores/conservation_status.htm (accessed on 22 February 2021).

Comparison between two censuses allows estimating the number of individuals from 12,411 growth to a maximum of 17,199 animals. Table 2 highlights which EU countries have used special funds (LIFE+).

Table 2. Specific conservation plans (LIFE+) for the large carnivores in EU countries (up to 2018).

	Country	*n.*		Country	*n.*
	Croatia	1		Slovenia	3
	Finland	1		Greece	4
	Germany	1		Romania	5(1)
	Hungary	1		U.K.	
	France	2		Spain	5
	Portugal	3		Italy	21

1.3. Specific Wolf Conservation Plans in Italy

The wolf population in the National Park of Abruzzo, Lazio and Molise (PNALM) would amount, according to the 1999 census data, between 30 and 38 specimens [12]. This data, compared with the latest official data produced at the national level (1100–2400 head) recognized by the European Union (census 2016), would indicate that the resident populations in the PNALM would represent 1.58–3.45% of the entire peninsular population [10].

Like other wolf conservation institutes in other states of the Union, the PNALM manages, in the town of Civitella Alfedena, a fenced structure of about 4 hectares (Area Faunistica del Lupo) within which there is a nucleus of 13 wolves.

1.4. Specific Wolf Conservation Plans in Spain (Castilla y Leòn)

Spanish populations of *Canis lupus* living south of the Duero River are considered protected. Conversely, the Spanish populations of *Canis lupus* present north of Duero River are managed with specific action plans of the different regions.

In the Autonomous Community of Castilla y León (CyL), all actions relating to the Iberian wolf (*Canis lupus signatus*) are coordinated by the Wolf Conservation and Management Plan implemented with the Decree 14/2016, 19 de mayo (currently judicially annulled). The plan for wolf conservation in Castilla y León is structured to keep the wolf population in a favorable and constant state. The state of conservation of the predator is possible if his presence is compatible with the traditional use of domestic livestock that together balances natural ecosystems. *Canis lupus signatus* is considered a dynamic element of rural development [13]. The wolf population in Castilla y León (94,223 km^2) would amount, according to the 2001 census between 1000 and 1500 specimens [14], but according to the latest census (2012–2014) [15], the region would have 60% of the Iberian wolf populations present throughout the peninsula corresponding to 179 packs and 1600 wolves during the summertime with an increase of 20% [16,17]. Like other wolf conservation institutes in other states of the Union, the Castilla y León manages, in the town of Robledo de Sanabria, a fenced structure of about 23 hectares (Félix Rodrìguez de la Fuente) [18] within which there is a nucleus of 14 wolves (7 adults and 7 sub-adults).

This work aims to describe different types of wolf management in two EU member states in which there is the same conservation law. Data were analyzed in order to evaluate a possible no-conflictual presence of the wolf and breeding activities.

2. Materials and Methods

2.1. Animals

The damages reported by farmers in two Italian areas (protected areas) and in one Spanish area (game reserve) were considered, within which, as required by law, agricultural activities are allowed.

In particular, as regards PNALM, damages were described on 5064 livestock in the period 2004–2016; however, to allow comparison with the other two realities, work was carried out on 2948 heads killed by the wolf in the period 2010–2016 (Table 3).

Table 3. Number of livestock heads attacked by wolves in the three study areas (National Park Abruzzo, Lazio, and Molise = PNALM; Regional Park of Aurunci Mountain = Aurunci; Coto de Caza Valle de Vidriales = Vidriales).

PNALM	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**
Sheep	172	235	174	146	115	160	176
Horse	48	65	48	40	32	44	49
Goat	129	176	130	109	87	121	132
Cow	82	112	82	69	55	76	84
Total	431	588	434	364	289	401	441
Aurunci	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**
Sheep	45	54	47	43	38	45	49
Horse	36	48	40	47	32	27	31
Goat	30	49	43	34	33	41	42
Cow	0	3	5	7	6	8	9
Total	111	154	135	131	109	121	131
Vidriales	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**
Sheep	0	0	0	0	0	0	0
Horse	0	0	0	0	0	0	0
Goat	0	0	0	0	0	0	0
Cow	0	0	0	0	0	0	0
Total	0	0	0	0	0	0	0

During the 2010–2016 period, Aurunci Regional Natural Park (Aurunci), damages were reported on 892 heads (Table 3). In a specific area of the park, there is a particular situation that consists of a herd in the natural state of Esperia's ponies. These were used to describe the dynamics of the wolf's predatory activity. In the period 2002–2020, 2094 foals born from a number of mares varied; between 110 and 240 were studied. In the same period (2010–2016), damages were examined in the Coto de Caza Valle de Vidriales (Vidriales) and in the whole of Castilla y León (CyL) [19].

Table 1 shows the official data relating to the number of wolves surveyed in the years 2012 and 2016 and published by the environment commission of the European Union [10].

2.2. Study Areas

2.2.1. PNALM

The study area is a national protected area in which hunting activities are prohibited but not agricultural and breeding. It is geographically localized on latitude 41°48′30.2″ N and longitude 13°47′11.3″ E and, consist of a territory of 49,680 hectares shared by three regions (Abruzzo, Lazio, and Molise) and including 25 municipalities (12 Abruzzo; 8 Lazio; 5 Molise).

PNALM extends mainly in the mountainous and pastoral territory of the Alto Sangro basin, surrounded by the Marsicani mountains to the south, while to the northeast, it is divided by the Majella, the Abruzzo plateaus, the Gizio and Tasso-Saggitario valleys. Habitat is characterized by the presence of rivers and lakes and has formed by beech woods for about 2/3 of the surface (33,286 hectares), meadows, and pastures (11,426 hectares) conditioned by the seasons that leave little room for agricultural crops (4968 hectares).

Most of the centuries-old pasture is still used today for livestock breeding. In the period of transhumance, shepherds, and their flocks, use large spaces of the protected area. In recent times, the open-air breeding of cows (meat and milk) has also developed. At the same time, it is possible to find, in the open spaces, a representative number of horses used mainly for equestrian tourism.

2.2.2. Aurunci

The study area is a regional protected area in which hunting activities are prohibited but not agricultural and breeding. It is geographically localized on latitude 41°24′32.94″ N and longitude 13°29′51.66″ E and consist of a territory of 19,374 hectares included on Lazio region formed by 10 municipalities.

A specific area of 300 hectares, shared between the Municipalities of Lenola and Campodimele, has been studied for the presence of a large population of Esperia ponies which have been associated with a series of wolf attacks

Descending from the summit of Appiolo Mountain (901 m asl) toward the valley floor, you will encounter a natural landscape that develops on a carbonate relief dominated by rock outcrops, with little presence of soil and therefore of specific vegetation (chasmophytic community typical of southern Italy (*Dianthion rupicolae*, *Saxifragion australis*) and Festuco-Brometalia (xerophilous to semimesophilic-perennial polispecific grasslands dominated by hemicryptophytic grasses, generally secondary, from arid to semimesophilic)).

The agricultural areas count 1350 hectares, mostly represented by the cultivation of olives and grapevines. They are limited to the flanks and to the foothills at an altitude, normally below 400 m asl. The secular pasture is now reduced to 1162 hectares in which the last shepherds exercised, on the two sides of Lenola and Campodimele, residual breeding of native breeds endangered such as the white Monticellana goat, the Sopravvissana sheep, and the Esperia pony.

2.2.3. Vidriales

The Spanish studied area is a private game reserve (Coto de Caza Valle de Vidriales) currently recognized by the Wildlife Estates agency in Brussels as a Nature Reserve, responsible not only for hunting but also for the management and conservation of wildlife in the area. It was founded in 1992 from the reorganization of land [11]. It is geographically localized on latitude 42°6′16.38″ N and longitude 5°55′56.05″ W and consists of a territory of 5493 hectares included on Castilla y León region formed by six municipalities.

The territory is divided into an agricultural area of 1350 hectares (where dry farming is the most frequently used cultivation technique, there are also large grapevine areas), and a remainder consisting of a forest area of 2840 hectares (Mediterranean woods and *Quercus ilex*); a scrub area of 1153 hectares (dominated by shrubs, small woody plants, and *Lavandula stoechas);* and an area of meadows and pastures of 150 hectares (grasses, legumes, *piperaceae*, and other herbaceous plants both natural and artificial).

The game reserve is equipped with a hunting plan that provides for the maximum killing of 380 red-legged partridge, 600 wild rabbits, 550 hares, 3 deers (2 males and 1 female), 9 roe deers (6 males and 3 females), 1 wolf and an unlimited number of wild boars in 5 hunts in a year (*monteria*).

The game reserve also provided to actions of management as: (1) corrective measures aimed at reducing limiting factors for small hunting animals; (2) choose, at regular intervals, four areas of the reserve (approximately 300–400 hectares) to be allocated to hunting activities for a period of 3–4 consecutive years. At the end of the aforementioned period, four other areas will be choosing to rotate the land subjected to hunting pressure and allow an adequate demographic restoration of the game species populations, including the wolf; (3) choose hunting type aimed primarily at hunting large animal species with particular reference to wild boar (*Sus scrofa*) and wolf (*Canis lupus signatus*); (4) improvement of habitats and protection of the entire ecosystem with particular actions of: (a) food supplement (blocks of salt, hunting products not included in the game bag, hay, straw and waste from local crops); (b) recovery of natural water sources and creation of artificial pools; (c) control of predators (huntable mammals) through hunting; (d) implement health programs aimed mainly at two species present on the reserve: rabbit (*Oryctolagus cuniculus*) and wild boar (*Sus scrofa*); (e) restocking of small game.

2.3. Models Applied to the Human-Wolf Conflict

In order to describe the current conflicts between anthropogenic activities and the presence of the wolf, two factors that indicate the conflict in the sampled territories were considered.

- Livestock damages

When on one or more animals a predatory attack by canids occurs, inspections are organized by the competent authorities to obtain a picture of the event as complete as possible.

The operations carried out are aimed at validating the most credible hypothesis on the identification of the responsible predator for the attack based [9] on five categories of judgment to assign responsibility to the canid involved (wolf, dog, or others) and, when possible, to confirm using molecular biology tests.

- Finding of dead wolves

When the presence of a dead canid is reported in the sample areas, an inspection is organized by authorized personnel who, after having filled out an identification form and taken photographs, collect the corpses and send them to the specialized centers for necropsy diagnosis and laboratory analysis.

2.4. Statistical Analysis

The processed data were collected in Italy (Abruzzo, Lazio and Molise regions) within protected areas where wolf hunting is prohibited and in Spain (Castilla y León region) within an area where it is possible to hunt the wolf. The comparison between two completely different realities, but both included in the Mediterranean biogeographical region, allows evaluating the human-wolf conflict, with a different but fundamental variable (hunting) on the existing wolf populations.

All the in-field collected data have been inserted in Excel tables so that they can be used with the available statistical programs (JMP®).

For the quantification and for the statistical analysis, the cases in which the final judgment falls into the categories "Certain canid responsibility" were considered and, of these, the cases judged in the "Probable wolf responsibility" category were then attributed to the wolf, eliminating those belonging to the category "Wolf liability excluded".

For the statistical analysis, contingency tables were used for the comparison between the years and the attacks attributed to the wolf.

Quantitative data relating to mortality and damage to animal husbandry caused by the examined carnivores, as well as the differences between the averages of the victims, were tested with the Student's t-test, producing significant results for the compared values ($p < 0.01$). As for the comparison of the qualitative data, the Chi-square test was used, which returned as well significant results ($p < 0.05$ and $p < 0.01$).

The Pearson correlation moment coefficient was calculated to evaluate the relationships between mortality and damage.

3. Results

3.1. General Level of Conflict in Europe

The European Parliament has recognized the importance of the role played by rural actors and the socio-economic importance of countryside activities for the conservation of biodiversity in the European biogeographic regions (ecosystem service).

The problem of large carnivores was discussed, and the related challenges and solutions to improve coexistence with anthropogenic activities. With reference to the recently published State of Nature 2020 report, it is essential to find workable solutions to mitigate the conflicts between human interests and large carnivores in line with EU law [6].

Many stakeholders are awaiting the commission's revised guidance on strict protection to better understand how conservation and management priorities can be correctly applied toward achieving long-term coexistence with large carnivores in Europe's densely populated and multifunctional landscapes.

The critical points to be addressed in order to identify the best practices to ensure the coexistence of large carnivores-human activities involve the answer to the following questions [20]:

- Which large carnivore species and populations are in need of greater conservation efforts based on the latest Member State reports?
- What is the appropriate scale to achieve favorable conservation status?
- How to assess a large carnivore's "favorable conservation status in their natural range"?
- How can derogations be used in populations with unfavorable conservation status: What does recent case law say?
- How to deal with individual "bold" large carnivores?
- Favorable reference values (FRVs) and conservation status assessments at the population level: A way forward?

3.2. *Specific Level of Human-Wolf Conflict into PNALM*

Since the 1920s, the presence of wolves has been described in the areas of the National Park of Abruzzo [1]. Subsequently, between the mid-1950s and the early 1970s, it is possible to hypothesize a disappearance of the wolf in these environments. With the first conservation policies of the species [21], the census operations also began, reporting about 100 specimens throughout the Italian Peninsula [22].

Ever since in the current territory of the PNALM, the wolf has always been the object of protection, management, and conservation. Proof of this are the interventions themselves to reintroduce prey species such as deer and roe deer (carried out since the early 1970s), or the first experiences of compensation for affected farmers. Such these interventions resulted in the recovery of the wolf not only under a demographic perspective but, above all, as to ecological aspects in the territory of the park and in the neighboring Apennine areas [23].

During the 1980s and 1990s, the wolf was the subject of various monitoring programs. Since 2005 a new research project involved on large carnivores in the park aims at defining the numerical entity, the genetic identity, the social and territorial organization of the populations, and the food ecology through the analysis of predations operated on wild and domestic species [24].

- Analysis of the samples studied (PNALM)

The open conflict between the wolf and the breeders is confirmed by the analysis of the causes of death of the predator reported within the National Park from 1999–2017 (Table 4). In order to evaluate the differences between the three realities considered, the data relating to the mortality of wolves and the predations carried out by them in the period 1999–2017 are taken. However, where data are available outside this period, it was decided to describe them to corroborate what was reported in a different period.

In Table 4, during the period 1999–2017, the causes of mortality of wolves found dead (corpses or remains, n = 139) in the PNALM are reported [25,26].

Figure 1 reports the trend of the percentages of dead wolves and damage recorded in the PNALM, comparing them among years.

Table 4. Number of wolves founded dead in the samples studied areas of Italy (National Park Abruzzo, Lazio, and Molise = PNALM; Regional Park of Aurunci Mountain = Aurunci).

Period 1999–2017	PNALM	Aurunci	Total
Accidents	50 (36%)	1 (20%)	51
Hunting	0	0	0
Control	0	0	0
Unknown	43 (31%)	2 (40%)	45
Poisoning	31 (22%)	0	31
Poaching	15 (11%)	2 (40%)	17
Total dead wolves	139	5	144
Damages to livestock (in heads dead 2010–2016)	2948	892	3840

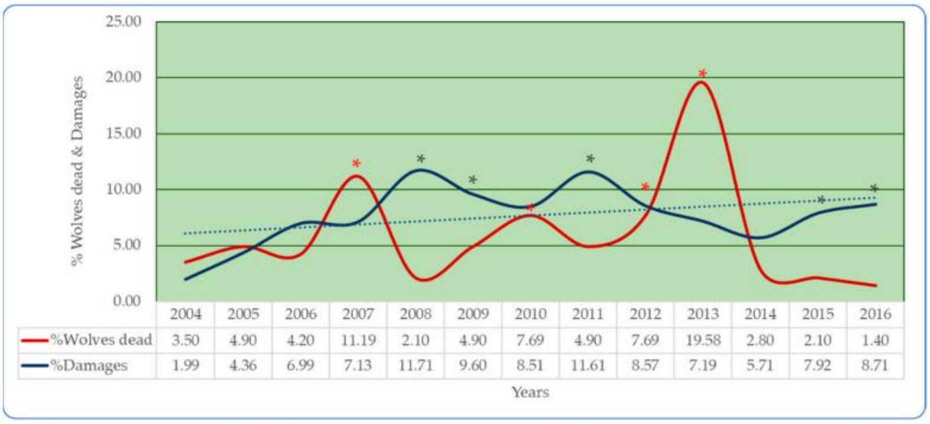

Figure 1. Relationship between percentages of dead wolves and damage caused by the canid. The asterisk indicates significant differences ($p < 0.01$) between the years compared.

In Figure 1, it is also possible to see a negative correlation: as the damage caused by the wolf decreases, the mortality rate increases ($r = -0.0567$).

The analysis of Figure 1 allows us to consider relatively uniform the mortality of wolves and the damages percentage within the Italian National Park. There are no statistical differences between the years, with the exception of the years 2007, 2010, 2012, and 2013 ($p < 0.01$), when the number of dead wolves is higher than 10, up to a maximum of 28 in 2013.

The damages are repeated with the same frequency, and significant differences ($p < 0.01$) are reported only for the years 2008, 2009, 2011, 2015, and 2016 compared to the other years considered.

Finally, Figure 2 shows the correlation ($R^2 = 0.506$) between the percentage of dead wolves and the reported damage.

The major cause of mortality of wolves (males 48%; females 38%; indeterminate 14%) is represented by injuries (accidents and traumas 47%).

Among the injuries and trauma, the highest percentage is attributed to road accidents (53%), while 24% is caused by gunshot wounds or traps and 23% by aggressions between congeners or from other canids [25].

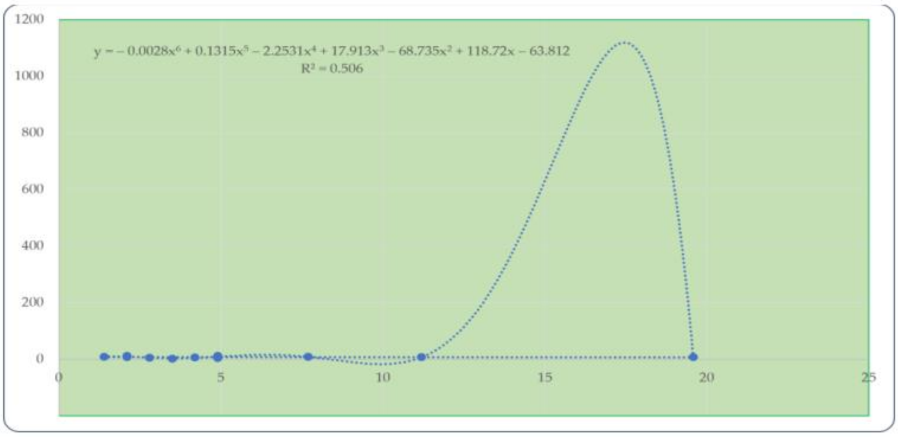

Figure 2. Correlation between dead wolves and damage caused by the canid.

Apart from the undetermined causes of death, which represent the highest share (31%), poisoning from organo-chlorine (21%) and organophosphates (18%) are the most represented features in percentage. Following, in descending order there are: chemical compounds (9%); alkaloids, and carbamates (6%); coumarins, thiophosphoric ester, and arsenic (3%).

Among the pathogens identified at necropsies and laboratory analysis, the presence of viruses and endo and ectoparasites are described. To these etiological agents is not always possible to ascribe the cause of death.

The analysis of the overall situation of the wolf presence in the PNALM allows to estimate certain stability of the National Park population and confirm what is reported by the EU and International Union for Conservation of Nature (IUCN) data which consider the *Canis lupus* population in a status of least concern (LC), with a tendency to stability [27].

In the three study areas, in order to homogeneously compare the dynamics and wolf predatory preferences on domestic livestock, the data were sorted for the same period, 2010–2016. The complaints received by breeders amounted to 2948 head of livestock. The attacks were certainly attributed to the wolf for 62%. The type of predation is slightly different between the years considered.

The prey of choice appears to be the sheep, followed by the goat, the bovine, and the horse (Table 5).

Table 5. Percentage of domestic animals preyed by wolves in Italian studied areas. (National Park Abruzzo, Lazio, and Molise = PNALM; Regional Park of Aurunci Mountain = Aurunci).

Year	Protected Area	Sheep	Goat	Cow	Horse
2010	PNALM	39.91	29.93	19.03	11.14
	Aurunci	40.38	26.92	32.69	0.00
2011	PNALM	39.97	29.93	19.05	11.05
	Aurunci	35.34	31.90	31.03	1.72
2012	PNALM	40.09	29.95	18.89	11.06
	Aurunci	34.42	31.82	29.87	3.90
2013	PNALM	40.11	29.95	18.96	10.99
	Aurunci	33.17	25.63	35.68	5.53
2014	PNALM	39.79	30.10	19.03	11.07
	Aurunci	34.84	30.32	29.03	5.81

Table 5. *Cont.*

Year	Protected Area	Sheep	Goat	Cow	Horse
2015	PNALM	39.85	30.08	19.05	11.03
	Aurunci	37.50	33.93	22.32	6.25
2016	PNALM	39.95	30.02	18.96	11.06
	Aurunci	36.50	31.73	24.04	6.73

3.3. Specific Level of Human-Wolf Conflict into Aurunci

As already described for the PNALM, in the 1920s, the wolf was present in all the Italian mountains [28,29], so it can be said that it was also present on the extreme offshoot of the Lazio Antiapennines of the Ausoni Mountains and the Aurunci Mountains.

By the same principle, it is possible to hypothesize a disappearance of the wolf in these environments between the mid-1950s and the early 1970s [22].

Surveys carried out in 2000–2001 exclude the presence of stable wolf nuclei into the Aurunci. However, the research carried out between 2004 and 2005 [30,31] indicated the presence in the entire range of the Regional Natural Park of the Aurunci Mountains of 3–4 individuals of the *Canis lupus* species.

These reports seem to be the first after those of 1985 and refer to a spontaneous return of the canid without having resorted to reintroduction. In the same year, 2005, the Regional Natural Park of the Aurunci Mountains Authority began an indemnity campaign for damage caused by predation to breeders in the protected area.

From that moment on, the reports of predation and sighting of wolves have not stopped throughout the park area, with particular reference to Monte Petrella (1500 m asl) [32] and Monte Appiolo (901 m asl) [33].

- Analysis of the samples studied (Aurunci)

Also, for Aurunci Park, we have analyzed the data relating to the mortality of wolves and the predations carried out by them in the period 1999–2017 and evaluated the differences between the other two realities. However, as in the case of PNALM, it seemed useful to describe a particular human-wolf conflict that we have been able to study for a long period of time. During the period 1999–2017 (Table 4), five dead wolves were found in the Regional Natural Park of the Aurunci Mountains (one for accident, 2005; two for unknown causes, 2006, 2007). In the same period, in-field checks and expert investigations indicated the simultaneous presence of stray dogs. Stray dogs are one of the main issues involved in the conservation of endangered and protected species such as wolves. Therefore, in 2008, the Park Authority launched the census of stray dogs [34]. In the years 2014 and 2016, two wolves dead for poaching were found.

The reasons that lead to the realization of the specific work, as below reported, have their roots in the framework law on protected areas 394/1991. The human presence in the agro-forestry-pastoral habitat guarantees therefore, a timely application of One Health-related environment management dynamics.

The Sustainable Development Agenda [35] reiterates the need for restoration methods suitable for achieving integration between man and the natural environment, including by safeguarding anthropological, archaeological, historical, and architectural values and traditional activities.

In the area of the park, there is a consistent representation of an endangered equine breed: Esperia's pony. These are animals whose original breeding area includes the Aurunci Mountains and the Ausoni Mountains and in which the result of a very rigid natural selection is evident, which has shaped their contained forms (height at the withers 132–138 cm) and the extreme rusticity. The survival of this breed, able to use fodder resources that are difficult to reach and, in any case otherwise lost, appears as the only condition to be able to maintain the human presence in territories that seem increasingly destined for abandonment and degradation [36].

In the study area, data relating to predatory attacks by wolves on domestic livestock in the period 2010–2016 were collected. The complaints received by breeders amounted to 892 head of livestock. The attacks were certainly attributed to the wolf for 70%. The type of predation is slightly different between the years considered. The prey of choice appears to be the sheep, followed by the goat, the horse, and the bovine (Table 5).

- The case of the reproductive tendency of the Esperia's ponies explains the trend over time of the predatory attitude of the wolf (Aurunci)

The sample on which we worked consists of a group of 150 Esperia's pony (15% of the Italian population estimated at about 1000 mares) reared in the wild on an area of 300 hectares of Monte Appiolo. The mares' pregnancies were followed from 2002 to 2020.

The births take place outdoors and, normally, without human help, between February and October of each year. During the same period, neonatal and foal mortality rates at different ages (within the year) were noted. Attacks by canids on foals were analyzed, reported by the shepherd.

Table 6 shows the number of births registered from 2002 to 2020 and the relative mortality of Esperia's foals reported by the breeder. The results indicate that from the year 2002 to 2006, the number of mares increased, stabilizing on 150 breeding mares until the year 2016. A new increase from 200 to 240 mares is recorded in the period 2017–2020.

Table 6. Vivinatality of newborn foals; mortality numbers and percentage of Esperia's ponies due to canid attacks in the period 2002–2020.

Year	Mares	Vivinatality Birth		Mortality Total		Neonatal		Preyed	
	n	*n*	%	*n*	%	*n*	%	*n*	%
^ 2020	240	97	40.42	62	63.92 ***	9	9.28	53	54.64
2019	240	103	42.92	48	46.60 **	10	9.71	38	36.89
2018	240	108	45.00	48	44.44 **	6	5.56	42	38.89
2017	200	90	45.00	30	33.33 **	5	5.56	25	27.78
2016	150	106	70.67 *	25	23.58 *	4	3.77	21	19.81
2015	150	108	72.00 *	25	23.15 *	4	3.70	21	19.44
2014	150	113	75.33 *	45	39.82 **	7	6.19	38	33.63
^ 2013	150	112	74.67 *	71	63.39 ***	11	9.82	60	53.57
2012	150	109	72.67 *	46	42.20 **	8	7.34	38	34.86
2011	150	125	83.33 *	36	28.80 **	12	9.60	24	19.20
§ 2010	150	121	80.67 *	17	14.05 *	10	8.26	7	5.79
2009	150	110	73.33 *	12	10.91 *	12	10.91	0	0.00
2008	150	115	76.67 *	15	13.04 *	15	13.04	0	0.00
2007	150	120	80.00 *	14	11.67 *	14	11.67	0	0.00
2006	150	120	80.00 *	12	10.00 *	12	10.00	0	0.00
2005	130	112	86.15 **	10	8.93	10	8.93	0	0.00
2004	120	108	90.00 ***	6	5.56	6	5.56	0	0.00
2003	120	110	91.67 ***	1	0.91	1	0.91	0	0.00
2002	110	107	97.27 ***	0	0.00	0	0.00	0	0.00

Asterisks indicate significant differences among the years (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$); the differences among the percentages were calculated using the chi-square test; § year in which the breeder begins to report canids attacks; ^ years in which the highest mortality is recorded since the beginning of canid attacks.

Obviously, during the same period, the number of births increased, which, in the entire period considered, was 110.21 ± 8.31 per year. The neonatal mortality of foals ranges from zero to 15 in the period 2002–2009 (7.63 ± 4.95) and 7.16 ± 2.34 in the period 2010–2020, where mortality of foals was also ascribed to predation by canids.

Predation begins to be reported in 2010 and becomes significant in 2011 (5.79 vs. 19.20% $p < 0.01$). The increase in mortality is significantly high in 2012 (19.20% vs. 34.86%; $p < 0.05$) and 2013 (19.20% vs. 53.57%; $p < 0.01$).

This last year is the first in which was described the highest number of foals dead by an attack of wolves. The mortality rate will decrease compared to 2013 but will remain constant (24.29 ± 8.08%) and, in any case, significantly high compared to 2010. In the last four years, predatory attacks have again increased the mortality rates (39.55% ± 11.16%), and they peak (54.64%) comparable to that recorded in 2013 (Table 6).

Foals birth alive (vivinatality) was highest in 2002 (97.27%), maintained an average of 82.42% between 2003 and 2011 and, then collapsed between 2012 and 2016 to 73.07%. In the last four years, vivinatality was the lowest in the entire study period (43.33%).

The analysis of the above data, combined with the information provided by Table 5, allows us to hypothesize a significant influence of predations on the reproductive results of the Esperia's ponies.

An important observation (Figure 3) about predatory attacks consists of a significant seasonal pattern. All attacks on horses from 2002 to 2020 in the various months in which they occurred were classified. The attacks begin, in fact, in the month of April, during which the attention of predators would be directed toward foals born in February (that is, about two months old). The monthly mortality continues with an ascending curve until the month of June and then gradually decreases until it reaches zero in the month of December. The total percentage of foals' deaths caused by predation, compared to the number of births, is 24.93%. The mortality rates begin to increase from April (11.30%) to become more consistent and descendant in May (31.03%), June (28.16%), July (13.60%), and August (11.69%). Percentages that are reduced in residuals in September (2.30%), October (0.96%), and November (0.96%).

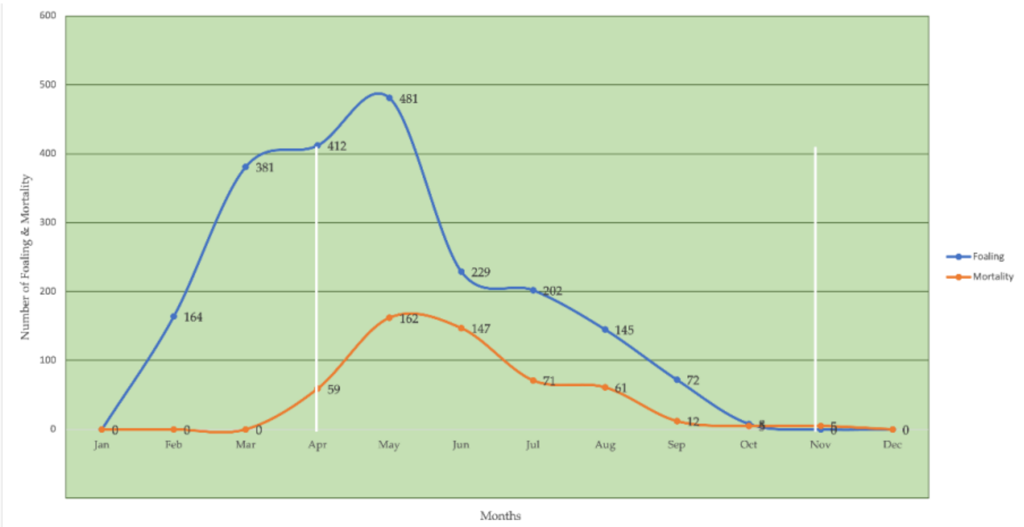

Figure 3. Foaling (vivinatality) and monthly mortality in Esperia's ponies among the months.

The analysis of the data also allows us to observe that in the months of December, January, February, and March, there are no predatory attacks, which would indicate a different trophic choice by the predators, probably justified by the absence of foals older than two months but less than six months of age, in the predation area.

Evaluating the sum of the number of dead foals, divided into the different months of the year, it is clear that, while the births are concentrated between February and October, predations are concentrated between April and November.

The advanced considerations would indicate a seasonal presence of wolves or groups of wolves in the area under study, which would begin to arrive in March–April and leave at the end of November.

Unfortunately, due to the lack of the authorizations, it was not possible, in this work, to indicate how many wolves or how many groups have occurred in the area and whether there have been litters, all elements that would explain the dynamics of the recorded predations and would make it possible to identify fundamental management actions to preserve a breed of a horse in danger of extinction and in the same way a wild species, the wolf, that does not deserve human inattention, especially in an area under protection.

3.4. Specific Level of Human-Wolf Conflict into Vidriales

Table 7 shows the causes of mortality of wolves found dead between 1999 and 2004 in the Castilla y León [18] and into Vidriales between 1999–2017.

Table 7. Number of wolves found dead in the areas of Spain.

Period 1999–2017	Castilla y León *	Vidriales
Accidents	56 (7.98%)	0
Hunting—Legal control	300 (42.73%)	0
Unknown	15 (2.14%)	1
Poisoning	6 (0.85%)	0
Poaching—Illegal control	325 (46.30%)	0
Total dead wolves	702	1
Damages to livestock (Attacks paid for dead livestock 2010–2016)	6813	0

* from the year 1999 to 2004.

The biggest difference that is immediately noticed is the high percentage of wolves killed for hunting activities (42.73%), which in the Spanish region is allowed in derogation from the Habitats Directive. In addition, the samplings that are carried out by the park rangers for population control operations (3.14%).

Conversely, like the two Italian study areas, the other causes of death are represented by road accidents (7.98%), unknown (2.14%), and poisoning (0.85%) causes.

Although hunting activities are allowed in Vidriales, in the reference period, no wolf hunts were organized. However, in the study area, the presence of the wolf is evidenced by the discovery of one dead subject for unknown causes in 2015.

The management of the hunting reserve is based on European legislation (Directive 92/43/EEC) and on the law of Castilla y León, number 4 of 12 July 1996, which exercises exclusive jurisdiction as an autonomous region.

Title II, in Article 7 defined as game species those listed in Annex I. Among these, the wolf (*Canis lupus*) is mentioned as the big game species north of the Duero River.

Annex II indicates that wolf hunting can be carried out from the fourth Sunday in September until the fourth Sunday in February of the following year.

The owner of the agricultural land that can be used for hunting purposes (Title IV) can request the consideration of €2500–3000 for a wolf killed.

However, the use of this practice would not negatively affect the size of the populations residing in the region. In fact, Castilla y León harbors around 60% of the total Iberian wolves (14). At least 179 packs were found in the last census 2012–2014 [16,17]. Due to its close presence in rural environments, persecutory acts against the wolf have also increased in Spain. In fact, in large areas of the Iberian territory, it is common to find dead animals from poaching or poisoning [19].

The reports received from Vidriales count no attack by wolves on livestock.

This result is due to two orders of factors:

(a) the type of farming.
(b) the availability of natural prey.

In Vidriales, there is the intensive breeding of the dairy sheep organized in modern stables that house from 250 to 500 sheep. The animals go out to pasture for about 4 h in the morning, strictly controlled by the shepherd accompanied by at least 3 Spanish mastiff dogs and 2–3 shepherd dogs (perro carea) to return indoors for milking and the rest of the day. Other livestock (goats, cows, and horses) are scarce.

In all environments that make up the coto de caza, there are wild boars and roe deers, which represent the major trophic resource of the wolves present in Vidriales.

4. Discussion

The human-wolf conflict was measured by estimating the number of domestic livestock preyed and the number of wolves found dead in three experimental areas. The effects of predations have significantly affected the mortality of some animal species. As a consequence, it is possible to hypothesize a significant influence of predations on the reproductive results, for example, of the Esperia's Pony.

The greater number of available and analyzed data relating to PNALM allows us to hypothesize a close relationship between the number of predated animals and the number of wolves found dead.

Conversely, in the other two realities, in contrast to predations, there are no significant reports of dead wolves.

The analysis of the overall data of the entire region of Castilla y León allows us to describe the causes of mortality of the recovered wolves. The results show that these causes of mortality are comparable in the two EU countries.

The experience gained in about 20 years in the two Italian parks indicates that the promotion of scientific studies in protected areas, through results, is able to explain in a consistent manner the behavior of predators and their presence compatible in agro-forest-pastoral territories. The acquisition of this knowledge is able to provide the management entities with the appropriate tools for the coexistence between sustainable anthropic activities and the protection of the wolf. All of this in a healthy and balanced environment, as recommended by the United Nations in the context of Agenda 2030, in accordance with the directives inherent to the One (Digital) Health paradigm [37].

5. Conclusions

The increase in damage which, although unable to prove it, could be ascribed to a demographic increase of the wolf, has caused two emerging problems linked to each other: (1) the onset of new conflicts with humans (especially in territories, as in Italy, where the absence of the carnivore for a long time has meant that the memory of living with the predator was lost); (2) the doubt of the genetic purity of domestic fauna predators.

Both problems exacerbate the anthropic conflict and contribute to what was seen in the second half of the last century or to the progressive abandonment of the territories with the consequent deterioration of the trophic qualities available.

Our work highlights that the actions to protect farms, the knowledge and containment of the persecution, as well as the actions aimed at individual species, included action of well-being assessment [38], have been able to bring back many species of mammals, as also demonstrated by European Rewilding Network [7]. It also demonstrates that veterinary assistance and wildlife technicians represent one element that, along with the support of operators in the agro-forestry-pastoral sector and the multidisciplinary work, favors the increase of already existing packs and allows the correct settlement of new populations. It is critical (at both national and international level) the presence of a good collaboration among the scientific world, the animal breeding, and the administration entities.

Author Contributions: Conceptualization, L.E.; methodology, N.P. and V.G.E.; investigation, N.P., L.G., E.S., T.Y.G. and J.P.A.; resources and funding acquisition, L.E.; writing—original draft preparation, L.E. and A.M.C.U.; writing—review and editing, L.E. and V.G.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Publicly available datasets were analyzed in this study. This data can be found here: https://wolf.fandom.com/wiki/Wolf-dog_hybrid (accessed on 22 February 2021); https://medioambiente.jcyl.es/web/es/medio-natural/plan-conservacion-gestion-lobo.html (accessed on 22 February 2021); https://www.minambiente.it/comunicati/lupo-il-nuovo-piano-di-conservazione-e-gestione-prevede-la-prevenzione-attiva-e (accessed on 22 February 2021); https://ec.europa.eu/easme/sites/easme-site/files/italy_update_it_june20.pdf (accessed on 22 February 2021); https://ec.europa.eu/environment/nature/conservation/species/carnivores/conservation_status.htm (accessed on 22 February 2021); https://www.minambiente.it/pagina/elenco-ufficiale-delle-aree-naturali-protette-0 (accessed on 22 February 2021). In-field data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors acknowledge the National Park of Abruzzo, Lazio, and Molise, and the Junta of Castilla y León.

Conflicts of Interest: The authors declare no conflict of interest.

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pathogens

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Communication

Histopathological Features of Symptomatic and Asymptomatic Honeybees Naturally Infected by Deformed Wing Virus

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Abstract: Deformed wing virus (DWV) is capable of infecting honeybees at every stage of development causing symptomatic and asymptomatic infections. To date, very little is known about the histopathological lesions caused by the virus. Therefore, 40 honeybee samples were randomly collected from a naturally DWV infected hive and subjected to anatomopathological examination to discriminate between symptomatic (29) and asymptomatic (11) honeybees. Subsequently, 15 honeybee samples were frozen at -80° and analyzed by PCR and RTqPCR to determine the presence/absence of the virus and the relative viral load, while 25 honeybee samples were analyzed by histopathological techniques. Biomolecular results showed a fragment of the expected size (69bp) of DWV in all samples and the viral load was higher in symptomatic honeybees compared to the asymptomatic group. Histopathological results showed degenerative alterations of the hypopharyngeal glands (19/25) and flight muscles (6/25) in symptomatic samples while 4/25 asymptomatic samples showed an inflammatory response in the midgut and the hemocele. Results suggest a possible pathogenic action of DWV in both symptomatic and asymptomatic honeybees, and a role of the immune response in keeping under control the virus in asymptomatic individuals.

Keywords: deformed wing virus; hypopharyngeal glands; flight muscles; honeybee immunity; honeybee pathology

Citation: Power, K.; Martano, M.; Altamura, G.; Piscopo, N.; Maiolino, P. Histopathological Features of Symptomatic and Asymptomatic Honeybees Naturally Infected by Deformed Wing Virus. *Pathogens* **2021**, *10*, 874. https://doi.org/10.3390/pathogens10070874

Academic Editor: Giovanni Cilia

Received: 28 May 2021
Accepted: 8 July 2021
Published: 10 July 2021

1. Introduction

Among the factors that threaten the health and wellbeing of honeybees, a noteworthy variety of pathogens such as bacteria, viruses, fungi and parasites are mentioned. In recent decades, honeybee viruses have been studied for their potential impact on beekeeping productions, acquiring more and more importance in the research world. Viruses in honeybees were first described in 1913 [1] when an American researcher attributed to a virus the "sac" appearance showed by some diseased larvae, although the causative agent (Sacbrood virus) was not characterized until 1964 [2]. To date, at least 22 viruses that can infect honeybees have been described. Most investigated hives are found to be infected by at least one virus, but often multiple viruses are detected in one hive [3–5].

In addition to causing high economic losses, viruses negatively affect the morphology, physiology, and behavior of honeybees and, although individuals do not always show clinical signs, they are frequently associated with weakening and colony collapse [6]. Depending on the different pathways of infection and on the health status of the colonies, viruses can cause symptomatic infections, i.e., overt or clinical, and asymptomatic infections, i.e., covert or subclinical [7]. Symptomatic infections are characterized by clinical signs and by high levels of viral particle production, to which the insect either succumbs or survives according to the status of the immune system.

These symptomatic infections can be further divided into acute and chronic: the acute involve the active replication of the virus, with a high titer of viral particles in a short time and cause rapid death of the host with evident clinical signs. In extreme cases, when the production of high viral titers occurs during a short time, sudden death can occur without previous clinical signs (hyperacute infections) [8]. Chronic infections, on the other hand, imply a slow but constant production of viral particles during the life of the host, or during the duration of the infected life stage, with subsequent appearance of clinical signs. On the contrary, asymptomatic infections are characterized by persistence of the virus beyond life stage, vertical transmission and the absence of obvious symptoms, although there could still be a hidden cost for the host [8–10]. Asymptomatic infections can be latent and persistent [6]. In the first case the viral genome may be present as an extrachromosomal episome or may be integrated into the host genome with incomplete replication or no replication at all. In the second case, there is a constant but low production of viral particles in the host cells, and either the infected cell survives, or the limited number of dead cells is counterbalanced by the production of new cells. Persistent infections, therefore, represent a balance between host and persistent viral replication, where despite the infection, the host does not die. Moreover, asymptomatic infections can become symptomatic when the host homeostasis is unbalanced by stressors such as other pathologies, food deficiencies, and other environmental factors [9].

Deformed wing virus (DWV) is positive single-stranded RNA virus belonging to the genus Iflavirus, family Iflaviridae of the order Picornavirales [11] and is the most prevalent virus in honeybees, with a minimum average of 55% of apiaries infected across 32 countries [12]. The virus was first isolated in the 1982 in the UK by Bill Baley and Brenda Ball from dead Japanese honeybees showing particular deformity of wings [13]. Soon after, honeybees from the UK, Belize and South Africa died showing DWV symptoms. Ten years after, in the UK the virus was found in Varroa destructor-infested colonies, and it was then found in every location where *V. destructor* was well established [12]. Due to the link with *V. destructor* and following the huge spread of it around the world between 1970 and 1980, DWV altered its epidemiology and has currently a global distribution [14]. Except for Australia, Uganda and the Canadian island of Newfoundland, where the *V. destructor* mite has not been found, the presence of this particular virus has been reported in Africa, Asia, Europe, North America and South America [15]. DWV appears in three master variants DWV-A, DWV-B and DWV-C, plus numerous recombinations, often more virulent than the masters [16]. DWV-A was the first variant to be detected and it is closely associated to colony collapse [17]; however, DWV-B, previously termed Varroa Destructor virus-1, was found to be equally or more virulent than DWV-A when injected in high viral loads [18]. DWV-C was first described in U.K. honeybee samples from 2007 and linked in combination with DWV-A to the death of overwintering colonies [19].

Recent studies have shown that DWV is present in more than 64 species of insects and highly prevalent not only in honeybees, but also in more than 29 arthropod species associated with honeybee hives [12,20,21]. Within insects, DWV was found in bumblebee species *Bombus terrestris* and *Bombus pascuorum*, wasp species *Vespula vulgaris* and *Vespa crabro* and *Lasius* spp. ants [12,22,23], *besides A. mellifera*. DWV is a major pathogen of honeybees and its prevalence, strongly connected with the ectoparasite *V. destructor*, strongly increases honeybee colony mortality [24]. DWV is a low pathogenic virus that is capable of infecting all stages of development of honeybees, from eggs to adults, although it shows a higher replication in pupae [25,26]. It takes its name from the characteristic symptom that manifests itself in newly hatched honeybees with deformed or underdeveloped wings; these honeybees, unable to fly, can die shortly after emerging from the cell. Initially, the deformity of the wings had been attributed to the action of the *V. destructor* mite, as the symptom was more evident in conjunction with a strong infestation by the parasite [27]. Subsequently, DWV was identified as the etiological agent of wing deformity, emphasizing the association between the viral titers and the symptom [25,28]. However, although DWV is one of the few honeybee viruses to have its own characteristic clinical manifestation, it is known also to be present in apparently healthy colonies [10].

Although the pathology and virulence of DWV remain linked to horizontal vectored transmission by *V. destructor*, the presence of DWV has been demonstrated also in the absence of *V. destructor* [29]. Varroa-mediated virus transmission from adult honeybees to developing pupae is responsible for the display of the symptoms [24,30] such as early pupal death, deformed wings, shortened and swollen abdomen and discoloration of the cuticle in adult bees, and learning deficiencies [14]. Symptomatic DWV infection occurs primarily during autumn and in highly mite-infested colonies, where it constitutes predictive marker for winter colony losses [30,31]. According to the epidemiological model proposed by Chen et al. [9] two distinct moments of viral presence and infection can be recognized: in healthy and viable colonies, the virus remains latent / persistent without determining evident symptoms. Vice versa, in weak "stressed" colonies, the virus can abandon the state of latency, considerably replicate, increasing its virulence and causing the death of single individuals and depopulation of the colony. Among the main stressors identified in DWV infection, temperature decline could increase severity of viral infection in newly emerged honeybees (probably explaining the high levels of winter losses), while pesticides and poor nutrition could trigger the honeybee immune system making them more susceptible to viral infection, leading to colony collapse [32,33]. However, the main trigger for DWV symptomatic infection remains the uncontrolled Varroa infestation.

There is no doubt that the impact of viral diseases, especially DWV, in apiaries is a global threat to beekeeping and it is associated to honeybee colony loss [34]. Possible treatments against viral infections in honeybees are not known and legally recognized to date. Currently, a suitable treatment against Varroa is the best approach to fight DWV, since, after treatment, there is a gradual reduction of viral titers in colonies [35,36].

A deep knowledge of the crucial aspects of the viral pathogenicity, is important for realizing an effective control program, therefore, the aim of this preliminary study was to analyze any anatomo-histopathological findings in symptomatic and asymptomatic honeybees collected from a hive infected from DWV to try to better understand the pathological events underlying the infection.

2. Results

2.1. Anatomopathological Results

Anatomopathological examination confirmed the presence of alterations in 29/40 (72.5%) honeybees, namely deformed and crippled wings, discolored and shortened abdomens while 11/40 (27.5%) honeybees showed no lesions. Samples displaying alterations were classified as symptomatic (S) honeybees, while samples not showing anatomopathological alterations were classified as asymptomatic (A).

2.2. Biomolecular Results

A fragment of the expected size (69bp) of DWV was successfully amplified from 10/10 (100%) S samples and 5/5 (100%) A samples by RT-PCR but not in negative control (NTC) (data not shown). Act β amplification (151bp product) confirmed the integrity of all analyzed cDNAs. To gain insights on the possible difference of viral load between S honeybees and A group, samples were further investigated by qPCR. A successful and reproducible Cq of reference and target genes was obtained in 10/10 (100%) S and 5/5 (100%) A samples. RQ analysis according to $2^{-\Delta\Delta Cq}$ method revealed that the viral load was higher in 9/10 S samples (90%) compared to the A group (Figure 1). Further variant specific PCR analysis for identification of the DWV variant has revealed the presence of DWV-A but not DWV-B in all the 15/40 analyzed samples (data not shown).

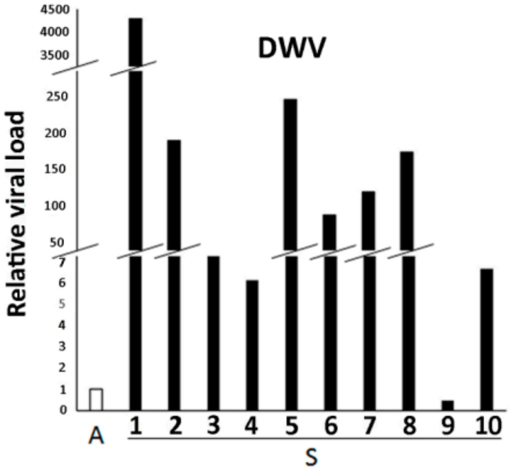

Figure 1. Analysis of relative viral load in honeybee samples showing clinical signs (S 1–10) compared with apparently healthy honeybee samples (A). Relative quantization data obtained by Real-time qPCR are expressed as fold change of each S sample with respect to the A samples considered as group (n = 5), which were set equal to 1, according to the $2^{-\Delta\Delta Cq}$ method.

Moreover, multiplex PCR for six honeybee viruses (Acute Bee Paralysis Virus-ABPV, Chronic Bee Paralysis Virus-CBPV, Sacbrood Virus-SBV, Black Queen Cell Virus-BQCV, Kashmir Bee Virus-KBV, Israeli Acute Paralysis Virus-IAPV) revealed the presence in all the 15/40 previously analyzed samples (symptomatic and asymptomatic honeybees) of ABPV (data not shown).

2.3. Histopathological Results

The histopathological analysis of symptomatic honeybees (19/25; 76%) revealed alterations of the hypopharyngeal glands in 19/19 (100%) honeybees and of flight muscles in 6/19 (31%) honeybees. The hypopharyngeal glands were characterized by small irregularly shaped acini, consisting of cells showing hyperchromic often fragmented nuclei and more or less abundant cytoplasm filled with few small vacuoles and numerous eosinophilic granules. Moreover, in the gland lumen and in the hemocele, it was noticed the presence of small cells with strongly basophilic nuclei and eosinophilic cytoplasm (Figure 2a).

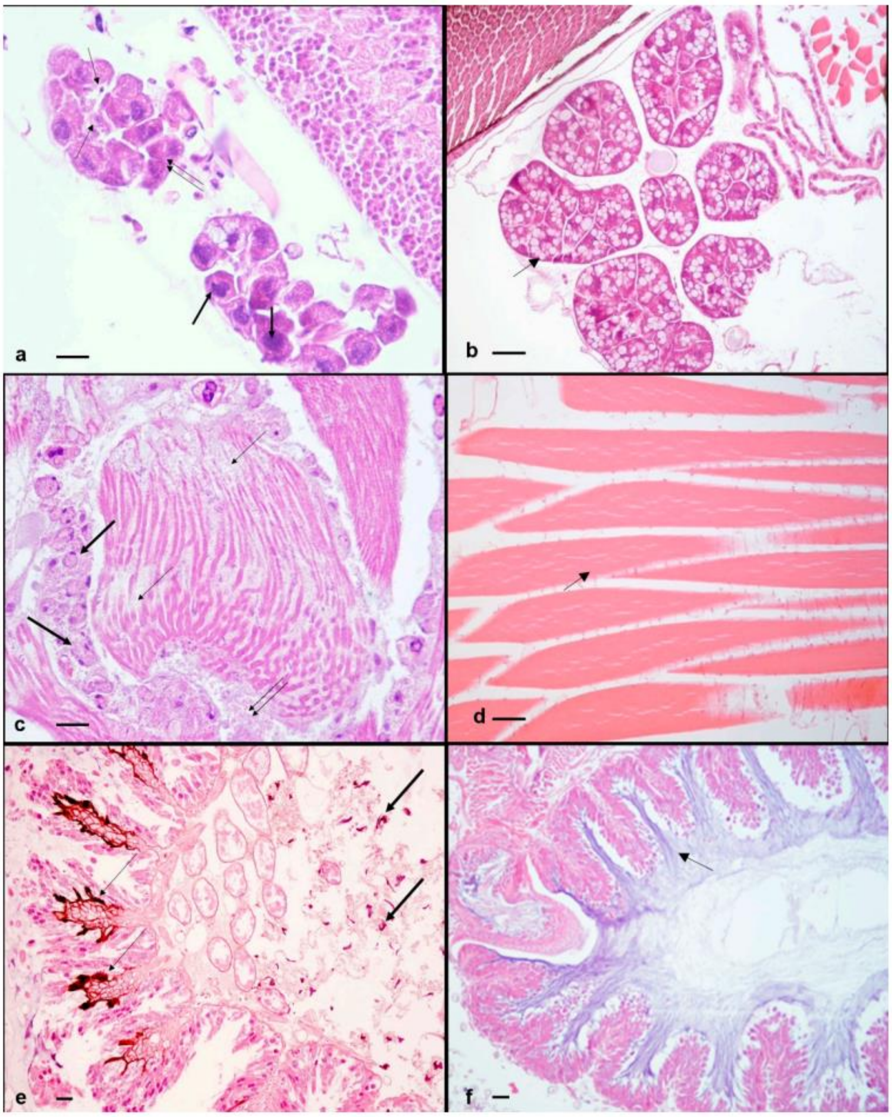

Figure 2. *A. mellifera* with symptomatic and asymptomatic infection of DWV and confirmed presence of ABPV. (**a**) Symptomatic honeybee. Hypopharyngeal glands. Small irregular acini showing cells with hyperchromic nuclei (thick arrows), cytoplasm filled with few small vacuoles and eosinophilic granules (double arrow), plasmatocytes in the gland lumen (thin arrows). H-E. 400× (40× objective and 10× ocular). (**b**) Asymptomatic honeybee. Hypopharyngeal glands. Large acini showing cells with cytoplasm filled with numerous large vacuoles with clear foamy material (thin arrow). H-E. 400×. (40× objective and 10× ocular). (**c**) Symptomatic honeybee. Flight muscles. Fibers with few not completely formed myofibrils (thin arrows), numerous muscle-forming nuclei (thick arrows), trophocytes with nuclear fragmentation and eosinophilic material between the muscle fibers (double arrow). H-E. 400× (40× objective and 10× ocular). (**d**) Asymptomatic honeybee. Flight muscles. Numerous fibers with many well-formed myofibrils (thin arrow). No trophocytes are present. H-E. 400× (40× objective and 10×ocular). (**e**) Asymptomatic honeybee. Midgut. Melanin accumulation between the fold of the villi (thin arrows) and in the hemocele (thick arrows). H-E. 200× (20× objective and 10× ocular). (**f**) Symptomatic honeybee. Midgut. Absence of melanization and hemocytes. The midgut epithelium appears intact and the peritrophic membrane appears well lined (thin arrow). H-E. 200× (20× objective and 10× ocular). Scale bar: 50 µm.

In contrast, hypopharyngeal glands of asymptomatic honeybees appeared composed of larger acini, consisting of cells showing numerous large vacuoles with clear foamy material in the cytoplasm (Figure 2b). The flight muscles showed absence of tonofibrils, few myofibrils often not completely formed and many new muscle-forming nuclei indicative of an ongoing myogenesis and incomplete maturation. Moreover, trophocytes with nuclear fragmentation or absence of nuclei, intermingled with eosinophilic material were evident between the muscle fibers. (Figure 2c). In contrast, the flight muscles of asymptomatic honeybees consisted of numerous fibers showing many well-formed myofibrils. No trophocytes were observed in asymptomatic samples (Figure 2d). The histopathological evaluation of asymptomatic honeybees (6/25) highlighted the presence in 4/6 (66%) honeybee samples of a great amount of melanin between the folds of the villi and in the lumen of the midgut, and scattered in the hemocele (Figure 2e). Moreover, the presence of two cell populations (hemocytes) was observed: one population characterized by small cells, showing small and hyperchromic nuclei, often localized at the periphery, and clear, bright eosinophilic cytoplasm, identified as plasmatocytes; the second population characterized by bigger cells with dark nuclei and granular light eosinophilic cytoplasm, identified as granulocytes. Plasmatocytes were localized in the epithelium of the midgut and in the hemocele; granulocytes were mainly present near the abdominal fat body (Figure 3).

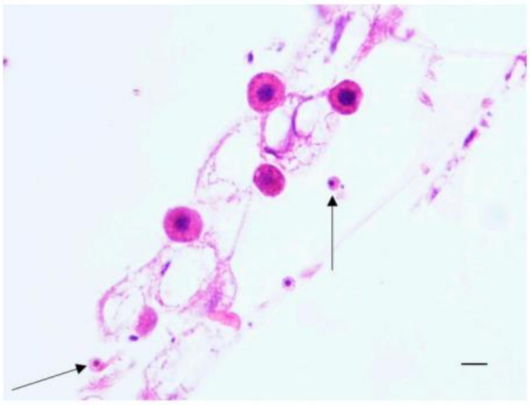

Figure 3. *A. mellifera* with asymptomatic infection of DWV and confirmed presence of ABPV. Fat Body. Granulocytes near the abdominal fat body (thin arrows). H-E. 400× (40× objective and 10× ocular). Scale bar: 40 µm.

Where melanin deposition occurred at the basal lamina level of the midgut villi and high infiltration of plasmatocytes was present at this level, high level of midgut epithelial cell exfoliation and only few regenerative cell nests were observed; in the severest cases, epithelial cells showed pyknotic nuclei, and disruption of whole villi was noticed (Figure 4). On the contrary, in symptomatic honeybees melanization was not present and hemocytes were not observed in the midgut neither in the hemocele. The midgut epithelium appeared intact and the peritrophic membrane appeared well lined (Figure 2f).

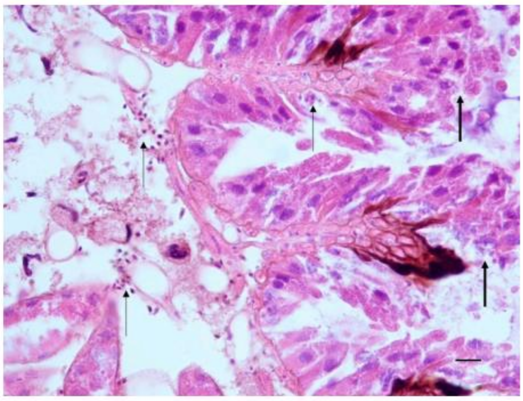

Figure 4. *A. mellifera* with asymptomatic infection of DWV and confirmed presence of ABPV. Midgut. Plasmatocytes in the epithelium and in the hemocele (thin arrows), epithelium exfoliation and disruption of the villi (thick arrows). H-E. 400× (40× objective and 10× ocular). Scale bar: 40 µm.

Moreover, no spores of Nosema spp. were observed in any of the 25/40 analyzed samples.

3. Discussion

DWV is recognized, in association with *V. destructor*, as one of the main causes of colony collapse.

Unlike many other viruses, it is characterized by typical symptomatic infections showing high pupal mortality, wing deformities, shortened, bloated and discolored abdomens; however, the virus is also capable of infecting the entire colony silently [10,37]. Therefore, the scarce presence or absence of clinical signs may not reflect the actual state of health of the colony.

In this study, symptomatic and asymptomatic honeybee samples were collected and subjected to biomolecular analysis to highlight the presence of viral genome and to anatomo-histopathological analysis to evaluate the presence of any alterations of organs and tissues. Biomolecular results showed elevated DWV viral titers in S samples compared to A samples. Despite the limited number of samples analyzed, the results obtained agree with previous studies [10,35]. We can therefore imply that also the samples used for histopathological had high viral titers in symptomatic honeybees and lower viral titers in asymptomatic honeybees.

Honeybees exhibiting anatomopathological alterations showed also histopathological alterations of the hypopharyngeal glands and of the flight muscles.

In *A. mellifera* the hypopharyngeal glands are part of the digestive system and, according to the role played in the colony, they are responsible for the production of royal jelly, storage of glycogen for the flight muscles, synthesis of enzymes important for the transformation of nectar into honey and for social immunity [38,39]. Moreover, the presence of vitellogenin, a glycoprotein necessary to produce immune system components and for longevity, has been demonstrated in the hypopharyngeal glands [40]. In this study the hypopharyngeal glands of symptomatic honeybees appeared hypotrophic, containing few small vacuoles (mucous origin) and numerous eosinophilic granules (serous origin). This seems to suggest an alteration of the secretory activity, particularly a shift towards an increase production of serous secretion, typical of foragers [41,42]. A possible early passage of honeybees to their role as foragers could be responsible for an unbalance in the castes and premature aging of the colony. Considering the role of hypopharyngeal glands in producing components of worker and royal jelly, essential for the efficient development of larvae [43,44], a modification in secretion, could lead to an altered production of the components of this substance and a consequent altered development of the larvae, which could be weaker and more susceptible to the action of the virus and of other pathogens [45,46]. Moreover, it can be hypothesized that alterations of the hypopharyngeal glands could also lead to a reduced secretion of vitellogenin, and a consequent, at least partial, impairment of the immune system [47,48]. These effects, in the long run could compromise colony fitness and survival. Alterations of hypopharyngeal and mandibular glands of honeybees infected with DWV, have already been described by Koziy et al. [49] and our observations match what previously found, corroborating the theory of an action of the virus on these organs.

At the thoracic level, symptomatic honeybees showed incomplete development of the flight muscles. In healthy honeybees, the mature muscles begin to form during pupal development by replacement of the larval muscles with mature muscles, starting from new muscle nuclei with an end-to-end trend. At the same time there is a gradual reduction of the fat body due to the degeneration of the trophocytes. The myogenesis process ends 70 h after cell capping with the attachment of the muscles to the epidermis of the cuticle using tonofibrils [50]. The histopathological study of the flight muscles of symptomatic honeybees has highlighted the presence of eosinophilic material and trophocytic nuclear debris between the muscle fibers, most of which appeared immature and detached from the cuticle, consequent to the absence of tonofibrils. These aspects, found in adult honeybees, could be indicative of incomplete myogenesis and could be responsible of an altered development of honeybees and of a reduction of their size and inability to hatch and fly. Localization of DWV in the flight muscles of symptomatic honeybees was described by Lamp et al. [51], using immunohistochemical techniques, and we here describe for the first time the presence of lesions at this level. Additionally, in this study not all samples showed incomplete myogenesis, and the reason could be found in the different developmental moment in which the virus infects the honeybee or in the titer of the virus.

Interestingly, honeybees showing no anatomopathological alterations, despite being infected by the virus, did not show the same tissue alterations as the symptomatic ones, but revealed the presence of a high number of inflammatory cells (plasmatocytes and granulocytes) and melanin accumulation between the midgut villi and in the hemocele. These findings suggest a strong activation of the immune system, particularly of the cellular response. Honeybees can try to keep the virus under control thanks to an efficient individual immune system, which is mainly composed by a first line defense and a second line defense. Honeybee venom is present on the cuticle of adult honeybee and can be considered as a chemical barrier and a first line defense against pathogens in the individual [52]. The exoskeleton cuticle and the peritrophic membranes of the digestive tract, also are considered as a first line defense as they prevent pathogens from entering the body and have access to the cells [53]. If unfortunately, a pathogen manages to surpass these physical barrier, cellular and humoral immune responses will be activated as a second line of defense [54]. The cellular response consists in activation of hemocytes function including phagocytosis, nodulation, encapsulation of the pathogen, what in pathology is defined as "granulomatosis reaction", and melanization [55]. The humoral response involves secretion of antimicrobial peptides (AMP), and other effectors, melanization, and the enzymatic degradation of pathogens by different pathways [54]. Richardson et al. [56] have identified and described the presence of two predominant cell types involved in the cellular response: granulocytes and plasmatocytes. Granulocytes exhibit a strong propensity for phagocytosis while plasmatocytes are involved in the encapsulation activity [57]. A strong and efficient immune system is the key for honeybee health and colony fitness.

In our study, the midgut epithelium of asymptomatic honeybees showed slugged epithelial cells, and as only few regenerative cell nests were present the adequate turnover that could restore the non-functional epithelium was not guaranteed. It is intuitive that a midgut showing these alterations cannot be functional both in absorption of nutrients and secretion of substances useful for the wellbeing of the peritrophic membrane, and consequently of the honeybees. It seems evident that, although no symptoms are evident, the virus is still acting on cells and tissues and that the activation of the immune response comes with a cost for the host.

This study has highlighted the presence of significant morphological alterations in symptomatic and asymptomatic honeybees infected with DWV and the results could suggest a possible pathological action of the virus in both groups of honeybees, and a possible role of the immune system, particularly of the cellular response, in keeping under control the virus in asymptomatic infections. It could be discussed that the alterations found could be linked to the action of other pathogens such other viruses or Nosema spp. However, histopathological examination of the midgut has been proven to be an efficient diagnostic tool for identifying the parasite in honeybees [58,59] and, as no spores have been observed in our samples, we can exclude the role of the parasite in generating the lesions observed. Regarding the possible action of other viruses, in this study we have screened for the presence of six different viruses and ABPV was found in all samples. ABPV is often associated to DWV in honeybee colonies [60], yet ABPV alone does not trigger humoral or cellular immune response in honeybees and therefore should not be considered as directly responsible for generating the immune response and melanization observed in the midgut and in the hemocele [61]. However, we cannot exclude a co-participation of ABPV to the generation of the alterations here found.

Therefore, further studies using other techniques such as FISH and immunohistochemistry, are necessary to deepen this preliminary study and better understand the etiopathogenesis of the findings here described.

4. Materials and Methods

During a regular visit to a beehive at an apiary located in Naples, Campania Region, it was possible to observe the presence of numerous small honeybees with deformed wings and shortened and discolored abdomens, suggesting the presence of a DWV infection.

The infected hive was clinically inspected, and the levels of *V. destructor* infestation were evaluated using the icing sugar technique [62] and assessed at 6% (18 mites/300 honeybees). A total of 40 adult honeybee samples were randomly captured from the frames and transported in 50 mL tubes to the laboratory of Veterinary General Pathology and Anatomical Pathology of the Department of Veterinary Medicine and Animal Productions, University of Naples "Federico II".

4.1. Anatomopathological Analysis

After immobilization with chilling for 3 min at −20 °C [63], all collected samples (40) were observed at the stereo microscope (Microscope Axioskop HBO50, Zeiss, Milan, Italy) to better identify any anatomopathological lesions and classify individuals in symptomatic and asymptomatic according to the presence/absence of typical clinical signs of the disease.

4.2. RNA Extraction, Reverse Transcription (RT) and PCR

A total of 15/40 honeybees were subjected to biomolecular investigation to verify and, in case of positive results, quantify the presence of viral RNA.

Samples were individually chopped up with a sterile blade to facilitate subsequent homogenization with the TissueLyser mechanical homogenizer (Qiagen, Hilden, Germany). Each sample was put in 2 mL tubes along with a grinding metal bead and subjected to lysis by two steps of five minutes at 50 Hz, interspersed with a cycle of ice cooling of 2 min to avoid overheating and preserve the integrity of the biological molecules.

RNA was extracted and purified from genomic DNA using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), according to the protocol provided by the manufacturer, and RNA concentration was measured by spectrophotometric reading.

For each sample, 250 ng of RNA were subjected to RT using the commercial iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's recommendations.

Subsequently, 12.5 ng of cDNA for each sample were subjected to PCR to amplify a segment of DWV genetic material and verify the presence/absence of the virus in the samples using the AmpliTaq Gold DNA Polymerase kit (Applied Biosystems, ThermoFisher

Scientific, Waltham, MA, USA) according to manufacturer's instructions. The housekeeping gene β-actin (Act β) of A. mellifera was also amplified to ensure the presence of amplifiable cDNA in each sample. One no template control (NTC) was included in each PCR reaction as negative control.

Subsequently, a new PCR was performed on the same samples to discriminate between the two different variants DWV-A and DWV-B according to the protocols found in the literature [64,65].

Moreover, a multiplex PCR was executed on the previous samples (15/40) to screen for the presence of six other relevant honeybee viruses (ABPV, CBPV, SBV, BQCV, KBV, IAPV) according to the protocol proposed and validated by Cagirgan and Yazici [66]. The set of primers used for amplification of the genetic material of viruses and Act β used in this study were found in literature and a complete list, together with the product size, annealing temperature and application is reported in Supplementary Materials.

Amplification products were migrated by electrophoresis on 2.5% agarose gel in TBE buffer (Tris-Borate-EDTA) along with a 50 bp molecular marker (Bioline), stained with ethidium bromide and observed under UV with the ChemiDoc gel scanner (Bio-Rad).

4.3. Real-Time PCR (qPCR) for Detection of Relative Viral Load

In order to determine a relative quantization (RQ) of viral load of the samples, a Real-Time PCR (qPCR) was carried out using the primers described above.

For each sample tested positive for DWV in PCR, 12.5 ng of cDNA were subjected to qPCR using iTaq Universal SYBR Green Supermix kit (Bio-Rad), according to the manufacturer's instructions.

Amplification of honeybee Act β as reference gene was also performed in parallel to allow normalization of the results and an NTC was included in the reaction as negative control.

Relative quantization of DWV viral load was calculated by using the $2^{-\Delta\Delta Cq}$ method as previously described [67,68]. Briefly, fold change in viral load was estimated for each individual S sample against A samples considered as control group.

4.4. Histopathological Analysis

Samples were processed as previously described [69]. Briefly, honeybees were individually injected with 10 µL of 10% buffered formalin and then stored for 24 h in 50 mL tubes containing the same fixative.

Subsequently, each sample was placed in an embedding cassette and processed. 3 µm sections were cut, stained with hematoxylin and eosin, and observed by light microscopy (Microscope Nikon Eclipse E-600, Tokyo, Japan). All tissues were observed to identify possible alterations and analyzed for the presence of visible pathogens, i.e., Nosema spp.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10070874/s1, File S1: Oligonucleotides used for amplification of viruses and Act β in this study. Sequences, products size, annealing temperature and applications are indicated.

Author Contributions: Conceptualization, K.P. and P.M.; Methodology, K.P. and G.A.; Validation, M.M., N.P. and P.M.; Formal Analysis, N.P.; Investigation, K.P. and G.A.; Writing—Original Draft Preparation, K.P.; Writing—Review and Editing, M.M. and P.M.; Supervision, P.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by PSR 14/20 Campania. Tipologia intervento 16.1.1 "sostegno per costituzione e funzionamento dei GO del PEI in materia di produttività e sostenibilità dell'agricoltura". Azione 2 "Sostegno ai POI". "Uso tecnologico e muove pratiche a carattere innovativo per la gestione, il controllo e la valorizzazione economica del cinghiale (Sus scrofa) in maniera sostenibile in Regione Campania". S.U.S Campania (CUP B58H19004460009).

Institutional Review Board Statement: Ethical review and approval were waived for this study, as according to the D.L. 4 March 2014 n.26, and national implementing decree following the European regulation 2010/63/UE, ethical approval is not necessary for insects with the except of cephalopoda.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available on reasonable request to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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Influence of Days after Calving and Thermal Stress on the Efficacy of a Progesterone-Based Treatment in Acyclic Italian Mediterranean Buffalo

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Simple Summary: "Mozzarella di Bufala Campana DOP" (mozzarella cheese) is mainly produced and marketed during the spring and summer months. The buffalo is a seasonal species that increases its reproductive activity when daylight hours decrease. Therefore, to increase milk production in the favourable period, the so-called "Out of Breeding Season Mating" technique is applied. It consists of the interruption of sexual promiscuity during the naturally occurring breeding season and concentrating calving and milk production during periods of increasing daylight length. However, the application of this technique increases the incidence of anoestrus, as animals are forced to breed outside of their natural and favoured period of the year, although other factors can also increase the incidence of anoestrus, such as climate. A reduction of seasonal anoestrus can be achieved by using some hormonal treatments. In this study, primiparous acyclic buffaloes were selected and divided into three classes according to their days in milk. Animals were synchronized using P_4 vaginal implants, and artificial insemination (AI) was performed according to protocol. The temperature–humidity index (THI) was recorded to evaluate its influence on anoestrus. Statistical analysis showed that the implemented P_4-based treatments were highly effective in removing the anoestrus condition in buffaloes. On the contrary, no influence of the THI on the efficacy of the P_4 synchronization treatment was observed.

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Citation: Matera, R.; Cotticelli, A.; Salzano, A.; Piscopo, N.; Balestrieri, A.; Campanile, G.; Neglia, G. Influence of Days after Calving and Thermal Stress on the Efficacy of a Progesterone-Based Treatment in Acyclic Italian Mediterranean Buffalo. *Animals* **2021**, *11*, 3166. https://doi.org/10.3390/ani11113166

Academic Editor: Roslyn Bathgate

Received: 13 September 2021
Accepted: 2 November 2021
Published: 5 November 2021

Abstract: The aim of this study was to evaluate the efficacy of a progesterone-based treatment on anoestrus in buffaloes. Primiparous acyclic buffaloes (n = 276), were divided into three classes according to their days in milk (DIM): from 50 to 90 (Class I; n = 86), from 91 to 150 (Class II; n = 102) and from 150 to 200 (Class III; n = 88). Animals were synchronized using P_4 vaginal implants, followed by timed artificial insemination (TAI). They were then allowed to enter into a larger group of buffaloes for natural mating 15 days after AI was performed, and pregnancy status was monitored from then on at 15-day intervals. Finally, the temperature–humidity index (THI) was calculated. Statistical analysis was performed by ANOVA by means and both multiple and linear regression. The total pregnancy rate (PR) was 87.7%, with no differences among DIM classes (88.0, 92.4, and 80.0% in Classes I, II, and III, respectively). However, the PR at TAI tended to be higher (p = 0.07) in buffaloes in Class II. The follicle (FL) area in Class II buffaloes was larger ($p < 0.01$) than that of the other classes. No influence of the THI on the total PR was recorded. The pregnancy outcome at TAI was affected by the FL area (odds ratio = 2.237; $p < 0.05$) and body condition score (BCS) (odds ratio = 1.256; $p < 0.05$). In conclusion, treatment with vaginal P_4 optimizes pregnancy rates in anoestrus buffaloes, particularly when the animals are in mid-lactation and show an optimal BCS. Furthermore, the THI does not seem to affect the efficiency of the progesterone treatment.

Keywords: Mediterranean buffalo; progesterone; anoestrus

1. Introduction

The buffalo species is an important livestock resource in both developing and developed countries [1,2], as demonstrated by the worldwide increase in the population in the last decades [3]. Although it is used in many countries for draught power and meat production, in developed countries, milk production is the main reason for buffalo farming. In Italy, the breeding strategies applied on buffalo farms have reached a high grade of innovation and mechanization, similarly to modern dairy cattle farms. In fact, according to the statistics of the Italian Association of Buffalo Breeders (ANASB), in the last several years, the average reported yield in Italian Mediterranean buffalo was 2368 kg of milk/270 days of lactation, and the fat and protein percentages were 7.77% and 4.64%, respectively [4].

In order to guarantee optimal milk production and ensure economic farm profitability, particular attention should be paid to the optimization of reproductive efficiency [3,5]. In Italy, buffalo milk is entirely used for the production of mozzarella cheese, which, in the late 90s, received important recognition by being included in the list of national DOP (Protected Denomination of Origin) products, and it is marketed mainly during the spring–summer period. Therefore, in order to achieve most of the mozzarella milk production during the desired marketing period, the "Out of Breeding Season Mating" (OBSM) technique [6] must be applied. It consists in the interruption of sexual promiscuity (or performed AI) during the breeding season (when daylight length decreases), with the goal of concentrating calving and milk production during the remaining periods of increasing daylight length [7]. In fact, although heats and ovarian activity can be observed and monitored in some animals throughout the year, the buffalo at our latitudes are tendentially "short day" breeders [2], resulting in higher reproductive activity when daylight hours decrease. Such tendential seasonality may be derived from its origin as animal species in the Indo Valley region, where forage availability is recorded during periods of decreasing daylight length [5,6]. Indeed, as observed in other seasonal species, anoestrus and embryonic mortality in buffaloes are the main reproductive problems reported during the period of the year characterized by increasing daylight hours [8].

The application of OBSM increases the incidence of anoestrus, as animals are forced to breed outside of their natural period. In recent studies, prolonged post-partum anoestrus in buffaloes calving during periods of increasing daylight hours was reported [9], probably due to low pulsatile secretion of Luteinizing Hormone (LH) that cannot guarantee appropriate follicle development [10]. Although it is mainly due to increasing daylight length, other factors can also increase the incidence of anoestrus, such as climate, parity, and farm management [9]. Several studies have been carried out to elucidate the hormonal basis of anoestrus [5,9], although little and contrasting information is actually available on the influence of other variables, such as days in milk (DIM) and the temperature-humidity index (THI), on productive and reproductive performance in buffalo [11,12]. In particular, buffaloes are able to adapt to different environmental conditions, from tropical to temperate environments, and the response to thermal stress is quite variable (for review, see [12]). According to several authors, buffalo are considered more tolerant to heat stress than cattle [13], although high relative humidity and direct solar radiations may severely affect their performance. A mean THI value of 73 for 20 days before breeding was demonstrated to have a deleterious effect on the conception rate in dairy cattle [14], while no information is available on the impact of heat stress on reproduction, particularly during synchronization protocols, in buffalo.

A reduction of seasonal anoestrus can be achieved using some hormonal treatments [15–17]. Significant results have been recorded using either intravaginal progesterone (P_4) devices [15,18] or norgestomet ear implants [19], which are associated with prostaglandin and equine chorionic gonadotrophin (eCG). However, little information is available on the efficacy of progesterone devices in buffaloes with different days open and maintained in the same conditions with the subsequent resumption of the ovarian cycle and pregnancy rate (PR). Therefore, the aim of this study was to assess the influence of thermal stress and days after calving on progesterone-based treatment in acyclic buffaloes undergoing TAI and natural mating.

2. Materials and Methods

2.1. Farm

The Ethical Animal Care and Use Committee of the Federico II University of Naples approved the experimental design of the study (protocol no. 996072017). The trial was carried out on a commercial buffalo farm located in the South of Italy between 39.0°N and 41.5°N, where about 900 buffaloes were bred. Lactating cows received a total mixed ration administered twice daily. The detailed composition of the diet and the chemical nutritional value are reported in Table 1. The ration was formulated to meet the nutritional requirements for maintenance and production according to previous studies [20]. A total of 276 primiparous adult buffaloes, weighing about 550 kg and showing an optimal body condition score (between 7.0 and 8.5 on a 1 to 9 scale with 0.25 increments [21]) were included in the study. The average BCS was 7.8 ± 0.02, and the average milk yield was 12.1 ± 0.2 kg.

Table 1. Composition (kg), chemical characterization (% of dry matter, DM), and energy density (Milk Forage Unit—MFU) of the diet utilized during the trial.

Feed	Kg
Maize silage	20.0
Brewers grains	8.0
Alfalfa Hay	4.4
Straw	1.0
Corn flakes	3.1
Soybean meal	2.1
Hydrogenated fat	0.25
Calcium carbonate	0.08
Sodium bicarbonate	0.06
Chemical Composition	
DM (kg)	16.82
CP (%/DM)	14.80
EE (%/DM)	4.85
CF (%/DM)	19.51
NDF (%/DM)	41.09
ADF (%/DM)	23.68
Ashes (%/DM)	5.62
NSC (%/DM)	32.03
Starch (%/DM)	21.66
Calcium (%/DM)	0.84
Phosphorus (%/DM)	0.45
MFU (%/DM)	0.92

CP = crude protein; EE = ether extract; CF = Crude fibre; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin; NSC = non-structural carbohydrates.

2.2. Reproductive Management

Reproductive management was carried out by means of both artificial insemination (AI) and natural mating. Adult buffalo cows were maintained in groups of 60 animals in cement open yards that allowed 15 m^2/head. The OBSM technique was implemented on the farm; thus, mating was allowed from February to September (8 months) in order to centre most of the deliveries and, consequently, the maximum milk production from January to August. Immediately (within 3–5 days) after calving and within 20 days post-partum, the buffaloes underwent gynaecological examinations and assessments of the reproductive tract (Control Genital Apparatus—CGA) to assess the conditions of the apparatus. A voluntary waiting period (VWP) of 40 days was maintained. Following the VWP, natural mating was allowed and guaranteed by the presence of four bulls in each group (1 bull for every 15 cows). Gynaecological examinations of the animals were carried out 7 days apart by means of ultrasound to assess the ovarian and uterine status. Animals found to be affected by other conditions (mastitis, lameness, etc.) were excluded from the study. Furthermore, if the animals did not show corpora lutea and/or follicles larger than 0.7 cm following three consecutive examinations, they were considered to be in anoestrus and were subjected to oestrus synchronization [17] and AI (see below). On the contrary, if a pregnancy was recorded, the diagnosis was confirmed at least two times 30 days apart, and the animals were excluded from further investigations.

2.3. Experimental Design and Selection of the Animals

Only non-cyclic primiparous lactating buffaloes were selected from a larger number of animals during a period of increasing daylight length (from April to July). Following VWP, animals underwent gynaecological examinations 7 days apart; if no corpora lutea or large follicles were detected, two further examinations were carried out to confirm the anoestrus condition. Therefore, the selection criteria were: (i) absence of corpus luteum and/or follicles larger than 0.7 cm in at least three consecutive clinical examinations by ultrasound 7 days apart, (ii) no clinically evident inflammatory condition of the uterus, and (iii) a BCS between 7 and 8.5. If these characteristics were met, the animals were entered into a synchronization treatment based on a progesterone intravaginal device and AI (see below). A paillette from the same lot to be used for AI was analysed under a light microscope to assess sperm progressive motility and the live spermatozoa percentage. Fifteen days after AI, inseminated buffaloes were included in a larger group for natural mating. Pregnancy diagnosis was carried out 27 days after AI and confirmed on days 45 and 90 after AI; if the buffaloes were not pregnant, they underwent clinical examinations for pregnancy diagnosis every 15 days. According to pregnancy, animals were classified as pregnant at TAI or pregnant at the 1°, 2°, and 3° oestrous cycle post-insemination. Finally, data on temperature and humidity were collected on the first day of the synchronization treatment and the day of TAI to calculate the THI and the differences between the initial and final THI (ΔTHI).

2.4. Synchronization Treatment and TAI

Acyclic buffaloes were synchronized by means of a P_4-based synchronization treatment (Figure 1). Briefly, selected buffaloes received a GnRH agonist (buserelin acetate, 12 µg; Receptal®; Intervet, Milan, Italy) on day 0 together with the insertion of a 1.38 g progesterone intravaginal device (CIDR®; Zoetis SRL, Rome, Italy). After 10 days, the device was removed, and the animals received a luteolytic dose of cloprostenol (500 mg of Estrumate®; MSD Animal Health, Milan, Italy) together with 750 IU of PMSG (Folligon; Intervet MSD, Milan, Italy). Forty-eight hours later, a further GnRH agonist was administered to ensure the synchronization of ovulation. Timed artificial insemination (TAI) was performed 60 h after prostaglandin administration and 16 h after the last GnRH administration. On the day of AI, the animals underwent ultrasound examination (MyLab 30Gold®; Esaote, Genova, Italy), and the size of the preovulatory follicle was recorded. The area of the preovulatory follicle was calculated as previously described [22]:

$$\text{FL area} = \frac{a}{2} \times \frac{b}{2} \times \pi \quad (1)$$

where:

- ✓ FL = follicle
- ✓ a = major axis of the follicle
- ✓ b = minor axis of the follicle
- ✓ $\pi = 3.14$

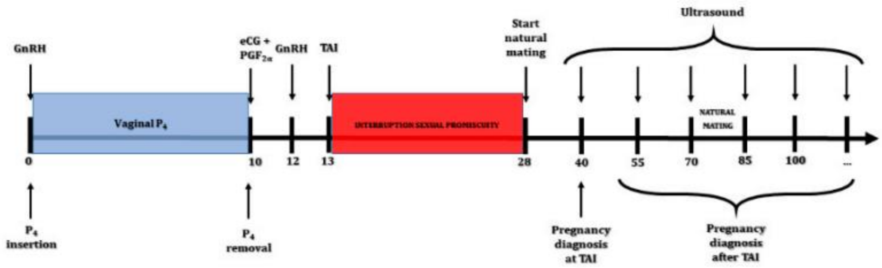

Figure 1. Synchronization treatment and experimental design during the study.

TAI was carried out by the same experienced veterinarian using frozen and thawed semen of one buffalo bull.

2.5. Meteorological Information

The environmental temperature and relative humidity (both on an hourly basis) were recorded at an official weather station (Davis Vantage Pro 2 plus wireless, Hayward, CA, USA) located 1 km from the farm. Data were collected daily and used to calculate the average temperature–humidity index (THI) according to the following formula [23]:

$$THI = (0.8 \times T) + (RH/100) \times (T - 14.4) + 46.4 \quad (2)$$

where:

- ✓ T is the environmental temperature in °C
- ✓ The relative humidity (RH) in %. The daily average RH value was calculated using the arithmetic mean and included in the equation

Data on environmental minimum temperature, maximum temperature, thermal excursion, and relative humidity were recorded. THI values recorded at the start of the synchronization protocol (THI_{start}) and the end of the synchronization protocol (THI_{end}) were used to calculate the average during the period of synchronization (THI_{mean}) and the excursion throughout the period of synchronization (ΔTHI).

2.6. Statistical Analysis

Statistical analyses were performed using SPSS (28.0) for Windows 10 (SPSS Inc., Chicago, IL, USA). Buffaloes were assigned to three classes according to their days in milk (DIM): from 50 to 90 DIM (catabolic phase of lactation; n = 86), from 91 to 150 DIM (first phase of the anabolic phase of lactation; n = 102), and from 150 to 200 DIM (second phase of the anabolic phase of lactation; n = 88). Moreover, according to the THI_{mean} values, buffaloes were divided into four classes: Class A (THI < 63), Class B (THI between 63.1 and 72), Class C (THI between 72.1 and 75), and Class D (THI > 75.1). Pregnancy rates among post-synchronization oestrus cycles (I, II, III, IV, and +IV), among DIM classes (I, II, and III), and among different months were compared using nonparametric tests (Pearson and X-square). Differences between groups were evaluated by Analysis of Variance (ANOVA). A General Linear Model (GLM) procedure was utilized to assess the influence of THI_{start}, THI_{end}, THI_{mean}, and ΔTHI on the follicle area at TAI by multivariate ANOVA. BCS, DIM, month of calving, and their interaction were included in the model. All data are reported as means ± standard error. A difference of $p < 0.05$ was accepted as statistically significant.

The relationship between the DIM, BCS, THI, follicular area, and treatment–conception interval was studied using multiple linear regression (stepwise procedure). Finally, the odds ratio for the pregnancy outcome at TAI was assessed by multiple logistic regression (stepwise procedure). Days in milk, milk yield (MY), BCS, follicle area, month of calving, THI_{start}, THI_{end}, THI_{mean}, and ΔTHI were included in the model as continuous variables.

3. Results

At the end of the mating period, an overall pregnancy rate of 87.7% was recorded (Table 2). In particular, more than 50% and 35% of the animals were pregnant after AI and within 3 oestrus cycles after AI, respectively (Table 2).

Table 2. Pregnancy rate recorded throughout the study in buffaloes that underwent TAI and natural mating.

ANIMALS	TAI	Cycles Post-Insemination			TOTAL
		1°	2°	3°	
Buffaloes inseminated (*n*)	276	128	74	50	276
Number pregnant (*n*)	148	54	24	16	242
Percentage pregnant (%)	53.6 [a]	42.2 [b]	32.4 [b]	32.0 [b]	87.7
Percentage of total pregnant (%)	61.2	22.3	9.9	6.6	100

Values in the same rows with different superscripts are significantly different ([a,b], $p < 0.05$).

No differences were recorded in the total pregnancy rate when the buffaloes were classified according to their days in milk at the start of the treatment (88.0, 92.4, and 80.0% PR in Classes I, II and III, respectively). However, the number of pregnant buffaloes at TAI in Class II (91–150 DIM) tended to be higher ($p = 0.07$) compared with those in Class I (60.4 and 48.0 in Classes II and I, respectively). Furthermore, a significantly ($p < 0.01$) higher pregnancy rate was observed in Class II in the first cycle post-insemination compared with the others (Figure 2). The BCS was significantly higher ($p < 0.01$) in buffaloes in Classes II and III compared with those in Class I (7.60 ± 0.1, 7.97 ± 0.1, and 8.07 ± 0.1 in Classes I, II, and III, respectively).

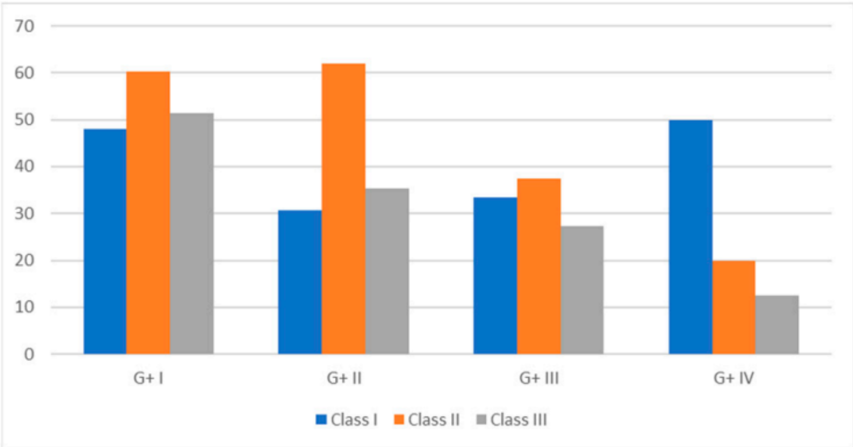

Figure 2. Pregnancy rate recorded throughout the study in buffaloes with different days in milk (Class I: <90 DIM; Class II: 91–150 DIM; Class III: >151 DIM).

Excluding buffaloes pregnant at TAI, the mean interval between the end of treatment and conception was significantly ($p < 0.05$) higher for animals in Class I compared with those in Classes II and III (48.2 ± 4.1 days in Class I vs. 27.5 ± 2.4 and 34.6 ± 4.7 days in Classes II and III, respectively).

The follicle area recorded at TAI was significantly ($p < 0.01$) larger in buffaloes in Class II compared with those in other classes (Table 3). Although no differences were recorded between pregnant and non-pregnant buffaloes within each class, buffaloes pregnant at TAI had significantly larger follicles ($p < 0.01$) than their non-pregnant counterparts (Table 3).

Table 3. Area of the dominant follicle recorded on the day of TAI in pregnant and non-pregnant buffaloes with different days in milk (Class I: <90 DIM; Class II: 91-150 DIM; Class III: >151 DIM).

CLASS	PR TAI	NP TAI	TOTAL
Class I	1.24 ± 0.0^{X}	1.14 ± 0.0^{X}	1.19 ± 0.0^{X}
Class II	1.59 ± 0.0^{Y}	1.49 ± 0.0^{Y}	1.55 ± 0.0^{Y}
Class III	1.23 ± 0.0^{X}	1.16 ± 0.0^{X}	1.22 ± 0.0^{X}
TOTAL	1.39 ± 0.0^{A}	1.25 ± 0.0^{B}	1.34 ± 0.0

Values are expressed as mean ± standard error. ^{X,Y}, values within the same column differ significantly at $p < 0.01$. ^{A,B}, values within each row are significantly different at $p < 0.01$.

A further analysis was carried out to evaluate the pregnancy rate (PR) in animals treated throughout the year (Figure 3). Few differences were observed throughout the months. However, a higher ($p < 0.05$) PR was recorded in buffaloes treated in June compared with those treated in July. Similarly, the PR at TAI and in the first cycle post-insemination was significantly ($p < 0.05$) higher in June than in May (Figure 3).

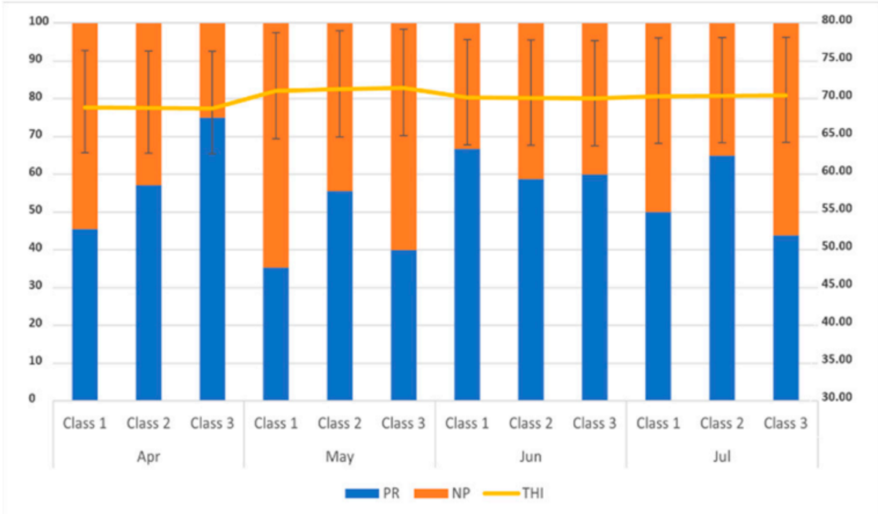

Figure 3. Pregnancy risk recorded in different months in buffaloes with different days in milk (Class I: <90 DIM; Class II: 91–150 DIM; Class III: >151 DIM) and THI values recorded throughout the study.

The average THI throughout the study was 70.2 ± 0.42. No influence of THI on the total pregnancy rate was recorded. In particular, when the animals were divided into the four classes of THI, pregnancy rates of 43.3, 52.2, 59.7, and 57.5 were recorded in Classes A, B, C, and D, respectively. Furthermore, the multiple linear regression analysis only showed a significant ($p < 0.01$) influence of BCS at TAI on the follicular area ($r^2 = 0.242$), according to the following equation:

$$FL_{area} = -0.299 + (0.255 \times BCS) \quad (3)$$

Additionally, in this case, no influence of THI was recorded. Finally, the multiple logistic regression analysis (Table 4) showed that the pregnancy outcome at TAI was affected by the FL area (odds ratio = 2.237; $p < 0.05$) and BCS at TAI (odds ratio = 1.256; $p < 0.05$), while no influences of other variables were recorded.

Table 4. Results (odds ratios and 95% confidence intervals) of the logistic regression analysis for the risk of pregnancy at timed artificial insemination (TAI), considering several factors: days in milk (DIM); body condition score (BCS); area of the preovulatory follicle (FL area); month of calving ($month_{calv}$); milk yield (MY); and the THI recorded at the start (THI_{start}), at the end (THI_{end}), and during (THI_{mean}) the trial. Data on THI excursion during the synchronization period were also considered (ΔTHI).

Variable	Coefficient	Odds Ratio	95% Conf. Int.	*p* Value
Constant	−2.427	0.0883	64.346	0.470
DIM	0.149	1.161	1.808	0.509
BCS	0.101	1.215	2.312	0.031
FL area	0.698	2.010	3.794	0.014
$Month_{calv}$	0.189	1.208	1.804	0.354
MY	0.0424	1.043	1.142	0.291
THI_{start},	−0.1000	0.905	0.974	0.215
THI_{end},	0.0601	1.062	1.158	0.394
THI_{mean}	0.140	1.150	1.370	0.156
ΔTHI	−0.0304	0.970	1.026	0.284

4. Discussion

Anoestrus is a naturally occurring event in buffaloes during the out-of-breeding season, which coincides with the time of year characterized by increasing day length. In Italy, its incidence is particularly high when the OBSM technique is implemented, thus affecting the reproductive efficiency of the herd. In this study, we evaluated the influence of DIM and THI on exogenous progesterone administration and synchronization in buffaloes in intensive conditions after TAI and subsequent natural mating. In our study, P_4-based treatment was able to guarantee both an optimal conception rate and the resumption of ovarian activity, leading to a pregnancy rate of more than 87% at the end of the breeding season. These results are in agreement with previous trials carried out in the buffalo species in which treatment with exogenous progesterone guaranteed the removal of seasonal anoestrus, achieving a satisfactory pregnancy rate [15,18]. According to several studies (for review, see [9]), the anoestrus condition in the buffalo species is probably due to the low secretion of LH. It is largely known that LH RNA levels are increased in rat pituitary cells in vitro following P_4 action, probably via GnRH receptor regulation [24]. In fact, in vivo treatment by exogenous progesterone reduces the pulsatile secretion of GnRH [25] and, consequently, the synthesis of GnRH receptors [26]. This causes a reduced sensitivity of the pituitary gland to GnRH. Furthermore, an increase in LH and FSH was observed during P_4 treatment in ovariectomized and hypothalamic–pituitary-disconnected ewes [27] due to augmented synthesis and inhibition of secretion. Similarly, P_4-based treatments have been demonstrated to increase the pulsatile secretion of LH during and after treatment in cattle [28], favouring follicular growth and oestradiol concentration; this in turn leads to a higher number of LH receptors of granulosa and theca cells and, consequently, ovulation [29]. It is likely that this process is also the basis of the removal of seasonal anoestrus in buffalo species.

It is also worth pointing out that a PR of more than 94% was recorded within the second cycle after TAI. In a previous trial carried out on buffalo heifers, the efficiencies of two re-synchronization treatments throughout the year were compared [18]. Although in this case, buffaloes were mated through TAI, a pregnancy rate of about 90% was achieved within the third cycle when synchronization was performed with a P_4-vaginal implant. This interesting result confirms our finding identifying a high fertile condition in buffalo in the first cycles after synchronization.

According to several studies, a low pregnancy rate (from 7 to 30%) has been recorded out of the breeding season in buffalo (for review, see [9]) due to the high incidence of anoestrus. This is the first study evaluating the influence of the days in milk on the efficacy of synchronization programs with P_4 vaginal implants in buffaloes. However, the PR after TAI and in the first subsequent cycle in Class II buffaloes tended to be higher than that recorded in Class I. Furthermore, it is worth pointing out that buffaloes in Class II showed larger preovulatory follicles at TAI compared with their Class I counterparts. It is known that buffaloes in Class I experienced a typical post-partum anoestrus, previously described in cattle [30] and buffalo [31]. This is not influenced by the calving season but probably by the negative energy balance that the animals encounter during early lactation. This condition significantly affects both the resumption of ovarian activity and preovulatory follicle growth, impairing reproductive efficiency. This interesting finding is further confirmed by the lower BCS recorded in buffaloes in Class I compared with those in Class II. Several authors have demonstrated that low BCS is associated with a low pregnancy rate in both cattle [32] and buffalo [31]. This is probably due to the utilization of nutrients for metabolism maintenance rather than for reproduction [33]. On the contrary, a typical seasonal (or summer) anoestrus has been recorded in buffaloes in Class II [6,12]. In a natural environment, this is a physiological condition that avoids mating and, consequently, calving in periods in which the environmental conditions and availability of forage would not be sufficient to ensure the survival of the offspring and the conservation of the species. Usually, in these cases, a great proportion of buffaloes resume their cyclic ovarian activity only in coincidence with the following breeding season when the animals are exposed to periods of decreasing daylight length [9].

Pregnant buffaloes also showed a larger preovulatory follicle compared with their non-pregnant counterparts. It is known that follicular growth is stimulated by the pulsatile secretion of gonadotrophin [34], but in anoestrus animals, it is guaranteed by eCG administration [35], which has both FSH and LH activity. Monteiro et al. [36] also demonstrated a high correlation between the size of the dominant follicle at TAI and both ovulation and pregnancy rate at 45 days post-AI. Furthermore, larger follicles were also associated with larger corpora lutea and augmented progesterone secretion [22], reducing the incidence of embryonic mortality [8].

Little information is available on the influence of the THI on productive and reproductive performance in buffaloes [37]. Several authors agree on the high thermo-tolerance of buffaloes to hot and humid climates, which is due to several factors, such as the presence of melanin pigments in the epidermis to prevent the penetration of the dermis by ultraviolet rays and a high number of sebaceous glands [38]. However, according to some recent studies [37], it has been demonstrated that THI values higher than 82 cause a decline in milk yield of about 1%. Similarly, a reduced conception rate after AI from 4 to 21% has been recorded in three buffalo genotypes in Egypt together with an increase in THI values. In our study, no influence of THI was recorded on follicular growth or BCS. Furthermore, according to the multiple regression analysis, the THI also did not influence the PR at TAI, while strong effects of the FL area and BCS were recorded. It is worth pointing out that the THI values recorded in this trial rarely reached 80, and no differences were recorded between the THI at the start of the synchronization protocol and that at the end. It is known that a deleterious effect of the THI is observed from a THI higher than 72 in dairy cows (for review, see [39]) and higher than 75 in buffalo [37]. In our study, a THI_{mean} higher than 75 was recorded in only 29.7% of cases, and it never exceeded 80; it cannot be ruled out that the low number of animals affected this result, as demonstrated by the evidence that buffaloes that encounter a THI higher than 75 showed a similar PR to the others.

5. Conclusions

In conclusion, this study demonstrated that a P_4-based treatment is highly effective in removing the anoestrus condition in buffaloes, allowing a PR higher than 87% within the third oestrus cycle after treatment. According to our results, DIM does not seem to influence PR results, with the exception of a trend for a higher PR recorded in Class II, which represents animals in a positive energy balance (after 91 days of lactation). This is probably due to both the larger size of the preovulatory follicle and the optimal BCS. On the contrary, no influence of THI on the efficacy of the synchronization treatment was observed.

Author Contributions: Conceptualization, G.N. and A.B.; methodology, R.M. and G.C.; software, A.C.; validation, A.C., A.S. and R.M.; formal analysis, A.C., R.M. and N.P.; investigation, R.M.; resources, G.N.; data curation, R.M., N.P. and A.C.; writing—original draft preparation, R.M. and A.S.; writing—review and editing, G.C. and A.B.; supervision, G.N. and G.C.; project administration, A.B.; funding acquisition, G.N. and G.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Italian Ministry of Agriculture (MIPAAF—DISR 07), Programma di Sviluppo Rurale Nazionale 2014/2020. Caratterizzazione delle risorse genetiche animali di interesse zootecnico e salvaguardia della biodiversità. Sottomisura: 10.2, Sostegno per la conservazione, l'uso e lo sviluppo sostenibili delle risorse genetiche in agricoltura. Project: "Bufala Mediterranea Italiana—tecnologie innovative per il miglioramento Genetico—BIG" Prot. N. 0215513 11/05/2021. CUP: 2015.99.42203.5966.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki; the Ethical Animal Care and Use Committee of Federico II University of Naples approved the experimental design of the study (protocol no. 996072017) in July 2017.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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Zearalenone (ZEN) and Its Metabolite Levels in Tissues of Wild Boar (*Sus scrofa*) from Southern Italy: A Pilot Study

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Abstract: Zearalenone (ZEN) is a non-steroidal estrogenic mycotoxin produced by the fungi of the *Fusarium* genera, and is a contaminant of cereals and plant products. ZEN and its metabolites are considered endocrine disruptors, and could have various toxic effects on animals and humans. In recent years, there has been a significant demographic increase in wild boar (*Sus scrofa*) in many mountainous and hilly areas of Italy, including the Campania region, mainly due to global climate change. The wild boar can be defined as a generalist and omnivorous species capable of varying its diet; therefore, it can play a role as an environmental bioindicator towards contaminants such as mycotoxins. This study was conducted to evaluate, for the first time, the concentrations of ZEN and its metabolites in the liver, kidney, and muscle of 82 wild boars shot in their habitat by hunters with hunting permits in different localities of Avellino province (Campania region, Southern Italy) from 2021 to 2022. The samples were collected and analyzed with an SPE clean-up and high-pressure liquid chromatography method with fluorescence detection. The results indicated that ZEN and α-Zearalenol were present in most of the samples, suggesting that a plan to monitor these mycoestrogens is essential to achieve the goals of "One Health".

Keywords: Zearalenone; α-Zearalenol; β-Zearalenol; wild boar; HPLC-FLD

Key Contribution: This study suggests that wild boar may represent a source of ZEN, potentially causing a risk to human health, especially for consumers of game meat. Consuming excessive amounts of mycotoxins can result in so-called "mycotoxicosis" poisoning, posing a considerable threat to animals and humans.

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Citation: Longobardi, C.; Damiano, S.; Ferrara, G.; Montagnaro, S.; Meucci, V.; Intorre, L.; Bacci, S.; Esposito, L.; Piscopo, N.; Rubino, A.; et al. Zearalenone (ZEN) and Its Metabolite Levels in Tissues of Wild Boar (*Sus scrofa*) from Southern Italy: A Pilot Study. *Toxins* **2023**, *15*, 56. https://doi.org/10.3390/toxins15010056

Received: 5 December 2022
Revised: 23 December 2022
Accepted: 4 January 2023
Published: 9 January 2023

1. Introduction

The wild boar (*Sus scrofa*) is an opportunistic omnivore whose diet is highly variable and strongly influenced by environmental changes. In Europe, the crops consumed by wild boar mainly include energy-rich plant foods such as acorns, chestnuts, pine seeds, and olives, in addition to other agricultural crops, such as maize, wheat, barley, rye, oats, rice, sorghum, potatoes, and sugar beet [1]. In recent years, we have witnessed an increase in the population and habitat of wild boars in many mountainous and hilly areas of Italy, including the Campania region as a result of global climate change. Global climate change has led to an increase in temperature and humidity, which favors the development of fungi on various food crops, especially cereals [2]. Zearalenone (ZEN) is a toxic secondary metabolite produced by several *Fusarium* species that grow on crops [3]. ZEN is a mycoestrogen classified as an endocrine disruptor and, since its chemical structure is similar to the endogenous estrogen 17-estradiol, it can bind estrogen receptors [4]. This mycotoxin contaminates cereals such as wheat, barley, corn, sorghum, rye [5,6], rice [5], corn silage [7], sesame seeds, hay [8], flour, malt, soybeans, beer [9], corn oil [10], dried fruits, spices, and milk [11], and its thermostability allows it to withstand storage, milling, processing, and cooking [12]. ZEN is also known to be immunotoxic, hepatotoxic [13], nephrotoxic, hematotoxic [14], and genotoxic [15]. The International Agency for Research on Cancer (IARC) has classified ZEN as a Group 3 substance (not carcinogenic to humans) [16]. Its toxicity depends on the immune status of the organism and the state of the reproductive system (juvenile or pregnant) [17]. The liver is the major distribution organ of ZEN [18] and it is metabolized mainly by hepatic hydroxysteroid dehydrogenase into α-Zearalenol (α-ZEL) and β-Zearalenol (β-ZEL) [19]. ZEN and its metabolites exhibit estrogenic [20] and anabolic effects in several animal species, resulting in infertility, hormonal dysfunction, and hyperplasia of the reproductive tract [21,22]. Animal species that are particularly sensitive to the effects of ZEN exposure include pigs and ruminants [12]. In humans, ZEN causes premature puberty [23]. In pregnant women, long-term exposure to ZEN can result in decreased embryo survival and fetal weight, as well as decreased milk production. In men, ZEN reduces sperm count and viability, impairing spermatogenesis [24]. The European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain has established a tolerable daily intake (TDI) of 0.25 μg/kg body weight of ZEN for human consumption and published a no-observed-effect level (NOEL) of 10 μg/kg body weight of ZEN per day for pigs [25]. Since most scientific information about ZEN and its metabolites in wild boars is sourced from Poland [26–28], we considered it necessary to investigate the concentrations of this mycotoxin and the levels of its metabolites in the muscle, liver, and kidney of wild boar in the Campania region for the first time. The animals were hunted in different locations of the province of Avellino in the Campania region, Southern Italy, where wild boar meat is traditionally used to make typical products such as "coppa" and "salami". It follows that monitoring the mycotoxin content of wild boar meat could be important for the protection of consumer health, since contaminated products can lead to enormous economic losses and pose risks to humans and animals.

2. Results

During the study period, a total of 82 hunted wild boar samples from eight hunting areas in the province of Avellino were examined (Figure 1).

For each animal, information on gender, age, body weight, and hunting areas was recorded. For the risk factor analysis, the animals in which ZEN or one of its metabolites were detected in at least one organ were considered positive, as shown in Table 1. The samples were divided as follows: 46.3% males and 53.7% females. Young animals (0–12 months) were the most represented category (53.7%; $n = 44$), followed by older animals (>36 months) (24.4%; $n = 20$) and finally adult animals (13–36 months) (21.9%; $n = 18$). A total of 40 out of 82 wild boar samples tested (48.7%; 95% confidence interval (CI) 37.9–59.6) were positive for ZEN and/or α-ZEL. No β-ZEL was detected by HPLC in the liver, muscle, or kidney of the studied animals (Table 1). No statistical significance was demonstrated between the presence of this mycotoxin and gender, age, or body weight, although males (50.0%, 95% CI 34.1–65.9), adults (13–36 months) (61.1%, 95% CI 38.6–83.6), and those belonging to the 70–89 kg body weight class (64.3%, 95% CI 39.2–89.3) had higher percentages of ZEN and/or α-ZEL.

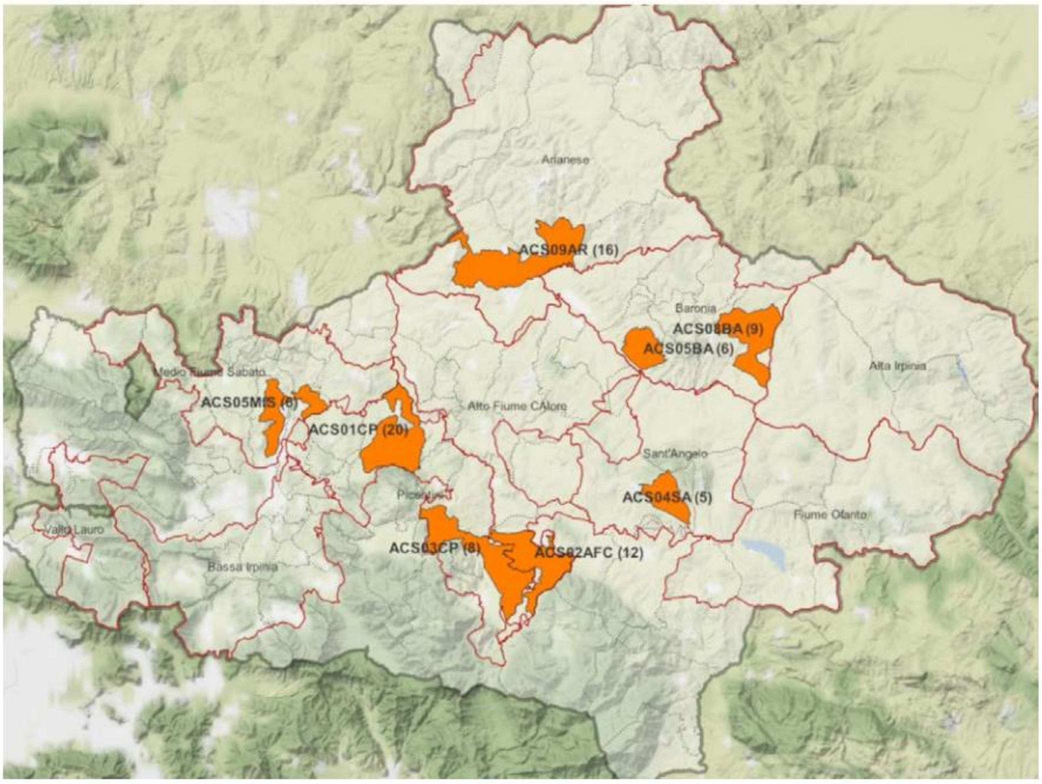


Figure 1. Cartographic image of the province of Avellino (Campania region, Southern Italy) with hunting areas (in orange) involved in wild boar (*Sus scrofa*) sampling.

The results of the validation study are reported in Table S1 of the Supplementary Materials. The average recoveries were between 63% and 75% with satisfactory RSD, thus completely fulfilling the performance criteria fixed by the European Commission Regulation (2006) [29], i.e., recovery in the range of 50–120% and 70–110% for levels < 1 and between 1 and 10 ng/g, respectively. Figure 2 shows chromatograms of the liver sample of one wild boar naturally contaminated with ZEA and α-ZEL.

The mean concentrations of ZEN in the liver, muscle, and kidney samples were 1.71 ng/g, 1.49 ng/g, and 0.65 ng/g, respectively; data analysis revealed statistical significance between ZEN concentrations in the liver samples ($p < 0.0003$) (Table 2; Figure 3, Panel A). The mean α-ZEL values in the liver, muscle, and kidney samples were 0.65 ng/g, 0.66 ng/g, and 0.77 ng/g, respectively (Table 2; Figure 3, panel B). The Kruskal–Wallis test showed no statistical significance between the α-ZEL values in the liver, muscle, and kidney, although the mean α-ZEL values were higher in the kidney samples (Table 2; Figure 3, Panel B).

A weak correlation was observed between the body weight of the wild boars and the concentrations of ZEN and α-ZEL in the liver, muscle, and kidney. Similar results were found between the age of the wild boars and the concentrations of ZEN and α-ZEL in the same organs. The results are presented in Table 3.

Table 1. ZEN and α-ZEL prevalence and risk factor analysis in wild boars from Avellino Province (Campania region of Southern Italy, 2021–2022).

Wild Boars	*n*	Positive	%	SE%	95% CI	X^2	*p*	OR	95% CI
Total	82	40	48.7	10.8	37.9–59.6	-	-	-	-
Gender									
Female	44	21	47.7	14.7	32.9–62.4	0.04	0.9871	0.9130	0.38–2.17
Male	38	19	50.0	15.9	34.1–65.9				
Age									
0–12 months	44	21	47.7	14.7	32.9–62.5	1.732	0.4206	Ref.	
13–36 months	18	11	61.1	22.5	38.6–83.6			0.581	0.19–1.77
>36 months	20	8	40.0	21.5	18.5–61.5			1.36	0.46–4.00
Body Weight									
30–49 kg	34	17	50.0	16.8	33.2–66.8	2.181	0.5357	Ref	
50–69 kg	14	6	42.8	25.9	16.9–68.7			0.75	0.21–2.62
70–89 kg	14	9	64.3	25.1	39.2–89.3			1.8	0.49–6.49
≥90 kg	20	8	40.0	21.5	18.5–61.5			0.66	0.21–2.04
Hunting areas									
ACS01CP	20	9	45.0	21.8	23.2–66.8	3.103	0.875	Ref.	
ACS03CP	8	3	37.5	33.5	3.95–71.1			0.73	0.13–3.93
ACS02AFC	12	5	41.6	27.8	13.7–69.5			0.87	0.20–3.70
ACS04SA	5	3	60.0	42.9	17.1–100.0			1.83	0.24–13.46
ACS05MFS	6	2	33.3	37.7	0.0–71.1			0.61	0.09–4.13
ACS09AR	16	9	56.2	24.3	31.9–80.6			1.57	0.41–5.9
ACS05BA	6	3	50.0	40.0	9.9–90.0			1.22	0.19–7.59
ACS08BA	9	6	66.7	30.8	35.8–97.4			2.44	0.47–12.6

SE: standard error; 95% CI: 95% confidence interval; OR: odds ratio; Ref: reference category; *p*: $p < 0.05$ was considered statistically significant.

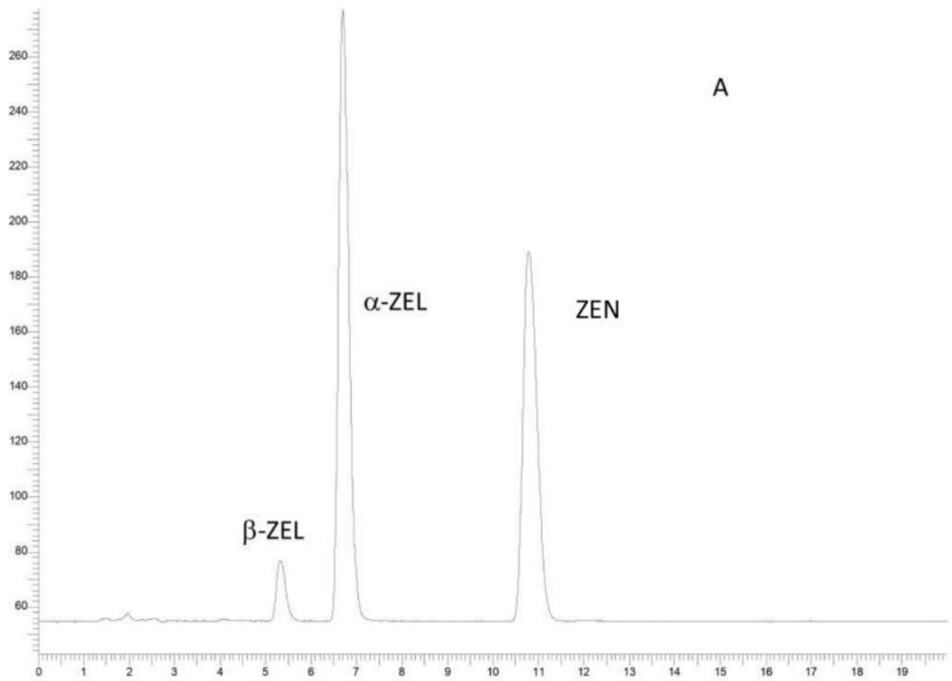
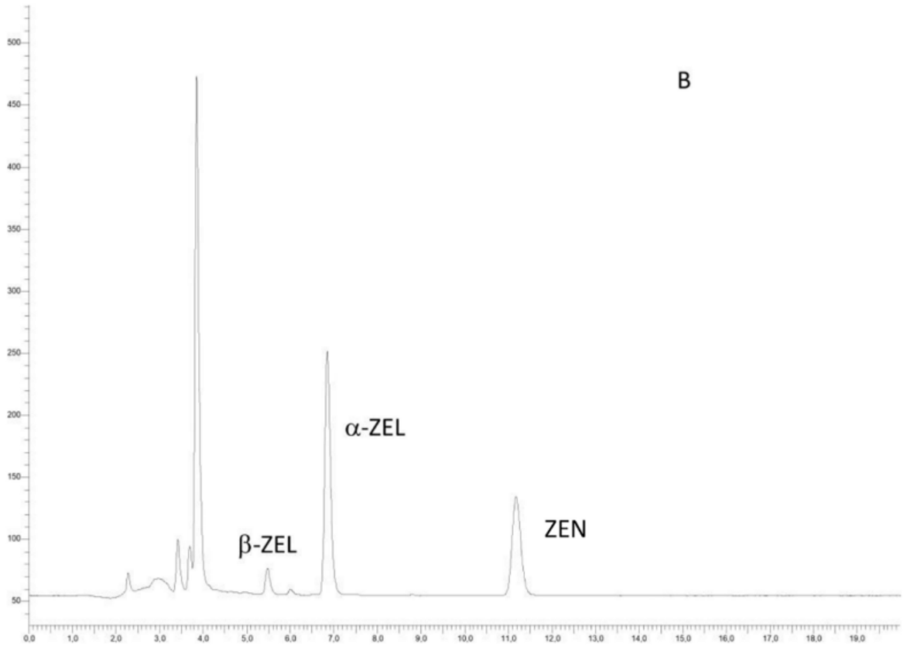


Figure 2. *Cont.*



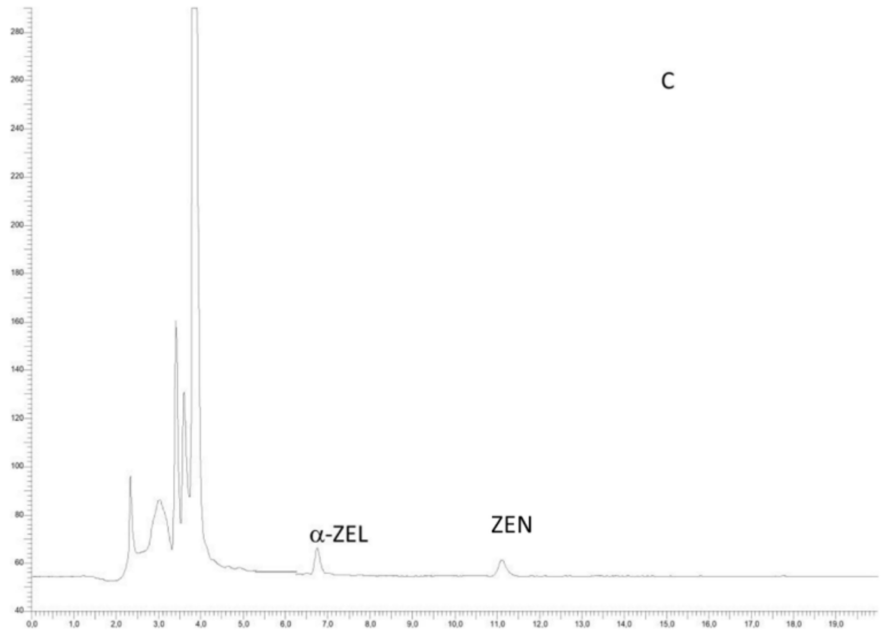


Figure 2. *Cont*.

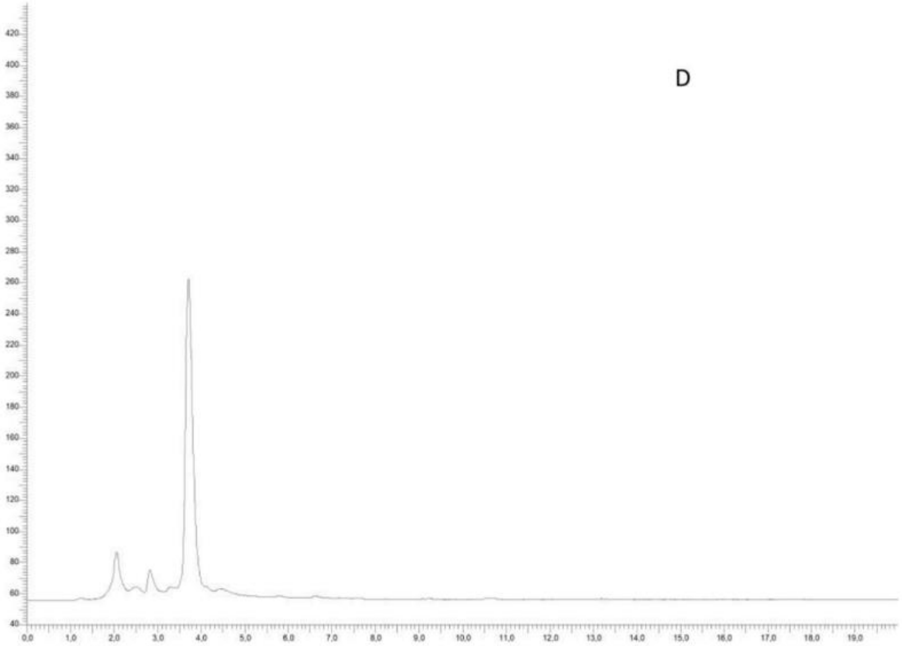


Figure 2. HPLC-FLD chromatograms of: (**A**) ZEN, α-ZEL and β-ZEL standard solutions (at 100 ng/mL); (**B**) a liver sample spiked with ZEN, α-ZEL and β-ZEL standard solutions (at 100 ng/mL); (**C**) a liver sample naturally contaminated with ZEN and α-ZEL; (**D**) a blank liver sample.

Table 2. Mean of ZEN and α-ZEL concentrations (ng/g) in liver, muscle, and kidney of wild boars from Avellino Province (Campania region of Southern Italy, 2021–2022).

Mycotoxin	Sample	Positive	%	SE%	95% CI	Chi-Square	*p*	Mean Concentration (ng/g)	SE	Kruskal–Wallis Statistic	*p*
	Liver	34	41.5	10.6	30.8–52.1			1.71	0.339		
ZEN	Muscle	21	25.6	9.5	16.2–35.2	15.056	0.0005	1.49	0.493	16.46 *	0.0003 ***
	Kidney	12	14.6	7.7	6.9–22-3			0.65	0.26		
	Liver	16	19.5	8.5	10.9–20.1			0.65	0.2409		
α-ZEL	Muscle	11	13.4	7.4	6.0–20.8	1.972	0.3730	0.66	0.1706	1.218	0.5439
	Kidney	10	12.2	7.1	5.1–19.3			0.77	0.311		

SE: standard error; asterisk: indicates the level of significance; * $p < 0.05$ was considered statistically significant; *** $p < 0.001$.

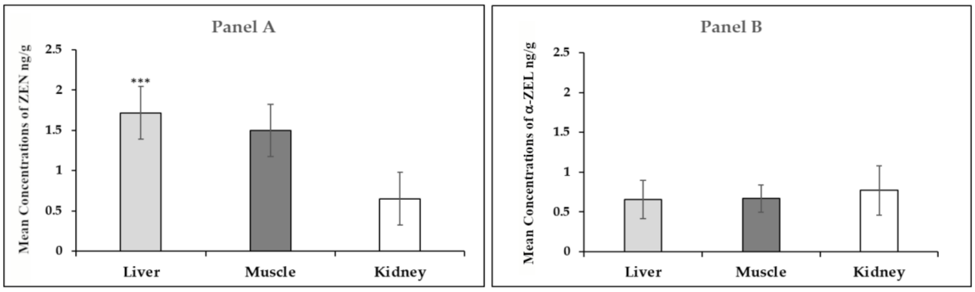


Figure 3. Mean of ZEN (**Panel A**) and α-ZEL (**Panel B**) concentrations (ng/g) in liver, muscle, and kidney of wild boars from Avellino Province (Campania region of Southern Italy, 2021–2022); *p*: a value of $p < 0.05$ was considered statistically significant; *** $p < 0.001$.

Table 3. Spearman correlation coefficient analysis shows the correlation between the concentrations of ZEN and α-ZEL in liver, muscle, and kidney in relation to the body weight and age of the sampled wild boars.

Variable	Mycotoxin	Organ	Mean Concentration (ng/g)	SD	r	95% CI	*p*
Body Weight							
	ZEN	Liver	1.716	3.065	0.004	−0.213–0.221	0.9727
		Muscle	1.495	4.467	−0.023	−0.239–0.195	0.8338
		Kidney	0.652	2.352	0.020	−0.198–0.236	0.8604
	α-ZEL	Liver	0.657	2.174	−0.078	−0.029–0.141	0.4813
		Muscle	0.666	1.540	−0.072	-0.284–0.148	0.5193
		Kidney	0.770	2.807	−0.077	−0.289–0.143	0.4908
Age							
	ZEN	Liver	1.716	3.065	0.006	−0.211–0.223	0.9539
		Muscle	1.495	4.467	−0.104	−0.314–0.115	0.3477
		Kidney	0.652	2.352	−0.073	−0.286–0.146	0.5099
	α-ZEL	Liver	0.657	2.174	−0.017	−0.233–0.201	0.8804
		Muscle	0.666	1.540	−0.059	−0.273–0.160	0.5951
		Kidney	0.770	2.807	−0.066	−0.279–0.153	0.5505

SE: standard error; $p < 0.05$ was considered statistically significant.

3. Discussion

The problem of residue of substances with potentially toxic effects, such as mycotoxins, in foods has taken on considerable importance in terms of food safety [30]. Clearly, knowledge of the epidemiological behavior of this toxic agent is one of the key elements for planning a monitoring or management plan for this mycotoxin, which is important for public health, domestic animal health, livestock production, and wildlife conservation. To plan a management strategy, bioindicators must be identified, i.e., species that can be used to monitor exposure to toxic substances. Wild boar is an excellent species for use as a biological indicator for the detection of mycotoxins in wild meat, both because of its eating habits and because of its wide distribution. Moreover, although it is a wild species, it can be easily sampled. In the Campania region, wild boar meat is traditionally used to produce niche products, especially "coppa" and "salami", and its liver is also highly appreciated and frequently used in local cooking recipes. Therefore, our data suggest that health surveillance of this species is needed to protect these niche products and reduce the introduction of ZEN into the human diet. As indicated in the European Community guidelines (Commission Regulation (EC) No. 401/2006) [29], control of the quality of meat intended for processing is a priority to reduce the possibility of toxin transmission to humans. In this study, ZEN and α-ZEL were detected in 48.7% (40/82; 95% CI 37.9–59.6) of the wild boars tested. No statistical significance was demonstrated between the presence of ZEN and gender, age, body weight or hunting areas, although males (50.0%, 95% CI 34.1–65.9), adults (13–36 months) (61.1%, 95% CI 38.6–83.6), and animals in the 70–89 kg body weight class (64.3%, 95% CI 39.2–89.3) had higher percentages of ZEN and α-ZEL. Thus, it seems that young animals are more sensitive than adult and heavier animals. This result may be due to the fact that wild boars with a higher body mass have greater degradation capacity [31] and access to less contaminated food sources in relation to social behavior and feeding hierarchy [32]. The primary route by which ZEN enters organisms is through the consumption of contaminated food. Our results show that ZEN and α-ZEL are mainly found in liver and muscle, and less frequently found in the kidneys. The highest concentration of ZEN was found in liver (mean = 1.71 ng/g) and this value was statistically significant ($p = 0.0003$) compared with the concentrations of ZEN in muscles (mean = 1.49 ng/g) and kidneys (mean = 0.65 ng/g). This may be related to the fact that liver and muscle are the most widely distributed organs and also to the fact that they are produced by liver microsomes, as is the case in pigs [7]. The influence of ZEN on wild boars is poorly studied. In the published data, considerably more information can be found on ZEN mycotoxicosis in domestic pigs, which are highly sensitive to ZEN [33,34]. No statistical significance was demonstrated for the concentration of the metabolite α-ZEL in the studied samples (liver, muscle, and kidney), although the highest concentrations were found in the kidney (mean = 0.770 ng/g). A possible explanation for the higher α-ZEL concentrations in the kidney than ZEN could be related to the metabolic processes of the organism. In this respect, further molecular and biochemical studies are necessary to clarify the mechanisms of ZEN toxicity in wild boar. Finally, the data found in this study showed no correlation between the concentrations of ZEN and α-ZEL in different tissues in relation to the body weight and age of the studied wild boar. Although the concentrations of ZEN and α-ZEL were not statistically significant, wild boar has proven to be an excellent biological indicator for the presence of mycotoxin in food, which leads us to infer that this mycotoxin is widely distributed in the environment. This is a global problem that leads to livestock disease, serious economic loss, and a negative impact on human health.

4. Conclusions

This work supports the concept that animal-derived products, particularly wild boar meat, are a potential source of ZEN and its metabolites, and that wild boars serve as sentinels for the presence of mycotoxins in the environment and crops. We detected ZEN and metabolites in almost 50% of the tested animals, with no variations based on age, gender, or region, demonstrating the extensive contamination of the collected animals. Our research improves our knowledge of mycotoxin contamination in wild boar meat and offal, demonstrating how mycotoxins, such as ZEN, can enter the human food chain in a variety of ways, posing a significant public health risk. To achieve this goal, a health monitoring plan that includes identifying mycotoxins in wild boars should be implemented.

5. Materials and Methods

5.1. Ethical Approval

No ethics committee approval was required for because the wild boars were harvested by hunters, and thus not culled for research purposes. These animals were legally killed in their own habitat by licensed hunters under the 2021–2022 annual hunting plan, approved by the Province of Avellino in the Campania region of Southern Italy.

5.2. Sampling Area

The Campania region is in the southern Italian peninsula, with a total area of 13,595 km^2 and a coastline of 350 km (217 mi) bordering the Tyrrhenian Sea. The region has a temperate Mediterranean climate with cold winters and dry summers [35]. Wild boars occur in 40% of the regional territory (except for Cilento National Park, Vallo di Diano, and Alburni), and Avellino is the province with the highest percentage, followed by Salerno, Caserta, and Benevento (Figure 3). The study was carried out in eight hunting areas distributed throughout the province of Avellino (Table 1).

5.3. Reagents

ZEN, α-ZEL, and β-ZEL reference standards were purchased from Merck (Milan, Italy). Working solutions were prepared by diluting the stock solution with a mobile phase consisting of water/acetonitrile/methanol 50/46/4% *v*/*v*. HPLC-grade water, methanol (CH_3OH), and acetonitrile (CH_3CN) were purchased from VWR (Milan, Italy).

5.4. Chromatographic Method

The chromatographic system consisted of a PerkinElmer 200 series variable flow pump (PerkinElmer, Waltham, MA, USA) connected to a Jasco 1521 fluorescence detector (Jasco, Tokyo, Japan). The excitation wavelength (λex) and emission wavelength (λem) were set to 274 and 440 nm, respectively. The system was controlled via a PerkinElmer interface module (NCI 900 Network Chromatography Interface), and chromatograms were processed using PerkinElmer TotalChrom Navigator software. An X-Bridge C18 5 µm 250 × 4.6 mm chromatography column (Waters, Milford, MA, USA) was used. Analyses were performed at room temperature with a flow rate of 1 mL/min and an injection volume of 100 µL was used. The mobile phase consisted of water/acetonitrile/methanol 50/46/4% *v*/*v*.

5.5. Sample Preparation

Samples of muscle, liver, and kidney (2 g) were collected from each wild boar and homogenized for several minutes with 10 M CH_3CN in an Ultra Turrax T25 homogenizer. The homogenizate was shaken for 10 min, the extracts were centrifuged at 4000 rpm at 4 °C for 10 min, and the supernatant was collected. The supernatant was concentrated to 2 mL by evaporation at 50 °C under a stream of nitrogen. The concentrate was mixed with 8 mL of water and the solution was applied to an Oasis HLB cartridge (60 mg, 3 cm^3, Waters, USA) at a flow rate of 0.5 mL/min. The cartridge was previously conditioned with 2 mL CH_3OH and 2 mL water. After washing with 2 mL of water, mycotoxins were eluted with 4 mL of CH_3OH, and the eluate was evaporated to dryness at 50 °C. The residue was redissolved in 500 µL of HPLC mobile phase, and 100 µL of the final extract was injected into the HPLC system. Samples spiked before extraction were used to verify the performance of the extraction and purification procedure and to obtain validation parameters. Spiking solutions of ZEN and metabolites were prepared daily by dilution with the HPLC mobile phase. For the muscle, kidney, and liver samples, the spiked homogenate was left at room temperature for at least 2 h after thorough mixing for 30 min to allow equilibration, and was used to check the purification procedure before HPLC analysis.

5.6. Method Validation

The HPLC-FLD method was validated according to the European Commission (2002) by evaluating specificity, recovery, linearity, LOD and LOQ, repeatability and reproducibility. Several wild boar meat samples were analyzed to verify the absence of the target analyte and potential interfering compounds; then, 30 blank samples were pooled and used for the validation study. The linearity was evaluated by spiking muscle, liver, and kidney samples with ZEN, α-ZEL, and β-ZEL at 0.25, 0.5, 1, 2.5, and 5 ng/g and analyzing them using the extraction and HPLC-FLD method. The experiment was repeated three times. The repeatability was tested by analyzing the muscle, liver, and kidney samples spiked with ZEN, α-ZEL, and β-ZEL at the levels of 0.1 ng/g, 1 ng/g, and 5 ng/g. All samples were measured in triplicate on the same day. For the within-laboratory reproducibility test, each of the contamination levels was tested in triplicate over a period of five days. Repeatability and reproducibility were given as the mean of the concentrations for three and six, respectively, fortification levels were determined at three different times, and the relative standard deviation was computed as %RSD = (standard deviation/mean concentration) × 100. The results of these experiments were also used for the determination of the recovery. The LOD and LOQ were determined by the signal-to-noise approach, defined at levels resulting in signal-to-noise ratios of 3 and 10, respectively. The analytical response and chromatographic noise were measured from the chromatogram of a blank sample extract (1 mL) to which ZEN, α-ZEL, and β-ZEL solutions were added.

5.7. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 7 software (GraphPad Software Inc, La Jolla, CA, USA). All data were tested for normality using the Kolmogorov–Smirnov test. Since the data were not normally distributed, the Kruskal–Wallis test was used to evaluate the significance of differences between mycotoxin concentrations in different tissues. Spearman correlation coefficient analysis was used to evaluate the correlation between the concentrations of ZEN and α-ZEL in muscle, liver, and kidney, as well as the body weight of the wild boar (Med Calc). The ranges of correlation strength were $r \geq 0.8$, $0.6 \leq r < 0.8$, $0.3 \leq r \leq 0.5$, and $r \leq 0.2$ for strong, moderately strong, moderate, and weak correlation, respectively. A value of $p < 0.05$ was considered statistically significant. MedCalc Statistical Software version 20.118 (MedCalc Software (free trial), Ostend, Belgium; www.medcalc.org; 2022) was used to compare proportions of positivity in relation to categorical dependent variables and to determine statistical significance within each class (gender, body weight, age, and location). Chi-square tests were used to compare the proportions of positivity related to categorically dependent variables and to determine the statistical significance within each class (gender, body weight, age, and location). Variables associated with the presence of ZEN and its metabolites were entered into binary logistic models using JMP Pro version 15.0.0 (SAS Institute Inc., Campus Drive, Cary, NC, USA). For the risk factor analysis, wild boar in which ZEN or one of its metabolites were detected in at least one organ sample were considered positive. $p < 0.05$ was considered significant. Significant differences between categories were quantified by calculating odds ratios (OR) and 95% confidence intervals (CIs).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxins15010056/s1, Table S1: Validation parameters of HPLC method; LOD = limit of detection, LOQ = limit of quantification, r2 = coefficient of correlation, SD = standard deviation, RSD = relative standard deviation.

Author Contributions: Conceptualization, C.L., S.D. and R.C.; methodology, C.L., S.D., G.F., S.M., V.M. and S.B.; investigation, C.L. and G.F.; resources, S.D., L.E. and N.P.; data curation, R.C. and S.F.; writing—original draft preparation C.L., S.D. and S.M.; visualization, A.R. (Antonio Rubino), A.R. (Antonio Raffaele) and R.C.; supervision, L.I., S.F. and R.C.; project administration, R.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data sets used and/or analyzed during the current study are available from the corresponding author.

Acknowledgments: The authors would like to thank Giuseppe Marzatico for the technical support.

Conflicts of Interest: The authors declare no conflict of interest.

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