



**NICHE MODELLING, CURRENT DISTRIBUTION  
AND FUTURE SCENARIOS FOR THE OTTERS IN  
HIMALAYAS**

A dissertation presented by

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# Exposure during doctoral research 2017-20

## List of courses followed in third year:

Course on Academic English 29/11/2019-31/1/2020, 32 ore,]. Dott.ssa Laura Tommaso

Course on “Research Article or Proposal Writing Techniques — Concepts & Practices”, seven days / 21 ore Dr. Sudeep Shukla

Course on “Biostatistics-Concepts, Tools & Applications” seven days / 21 ore Dr. Damini Singh

List of presentations, seminars and workshops in 2020-21:

Three days’ workshop on “Statistical Analysis of Ecological Parameters” organized by G.B. Pant National Institute of Himalayan Environment (14-16 September 2020) Dr. Suresh K Rana

Seven days’ Workshop on National Wildlife Week 2020 celebrated in collaboration with Ladakh Science Foundation (2-8 October 2020). (A) “Plants of Ladakh” (B) “Migratory birds in the Himalayas”, (C) Mammals of Ladakh (D) Birds of Ladakh (E) Quiz competition (F) Debate on Wildlife of Ladakh by Pankaj Raina

Webinar: “Impact of Covid-19 on Biodiversity Conservation”: International day for Biological Diversity. 22 May, 2020. WWF- India

26th to 28th February 2021 (Oral Presentation) “Monitoring Otters Using Water Samples from Water Bodies: A Practical guide for eDNA sample collection”. First Eurasian Otter Workshop

21st to 23rd February 2020 (Oral presentation, invited speaker) “Environmental DNA as a promising tool to survey elusive species in harsh environments “. 3rd Himalayan Otter Network Meeting — Kathmandu, Nepal, February 2020.

## List of courses followed in second year:

Legal, economic, ethical and social aspects of the intellectual property of scientific research results Prof. ssa Barbara Troncarelli

Workshop “Effective communication Dott. Umberto Maria Di Giacomo and Dott. Gabriella Sferra

Basic course for the use of software “R” (5 cfu) Dott. Mirko Di Febbraro

## List of presentations, seminars and workshops in 2019-20:

13.12.2018. Conferenza dei dottorati di ricerca. (Poster presentation) Potential Distribution and Niche Overlap Analysis in Three Otter Species: *Lutra lutra*, *Aonyx cinereus* and *Lutrogale Perspicillata*, Campobasso.

26.03.2019. Giornata della ricerca. Scientifica del Department of biosciences and territory. (Poster presentation) Distribution and Niche Overlap Analysis in Three Otter Species: *Lutra lutra*, *Aonyx cinereus* and *Lutrogale perspicillata* in Day of Scientific Research.

08.03.2019. (Oral Presentation). Predicting current and future potential distribution of *Aonyx cinereus* *Lutra lutra* and *Lutrogale perspicillata* in Himalaya under different climate change scenarios in: 14th International Otter Congress in Tangjiahe National Nature Reserve, China.

## List of courses followed in first year

“Basic course for the use of the software ‘R’” — Dr. Mirko Di Febbraro

e-DNA training in Department of Biotechnology and Bioscience, University of Milano-Bicocca.

## List of presentations, seminars and workshops in 2017-18:

14/12/2017: “Employment Opportunities of the Biologist in Production Systems: Roles and Tasks” — Prof. Luca Martirani (1 CFU)

09/04/2018: “Research funding” — Prof. Fabio Pilla, Dott.ssa Silvia Reale (1 CFU).

11/04/2018: “Research funding (construction of the financial economic plan)” — Prof. Francesco Capalbo (1 CFU).

29/05/2018: Career Day “What will I do when I grow up? Job opportunities for a biologist “.

6.18/06/2018: “The evaluation of the research” —

Prof. Giuseppe Vanoli (2 CFU).

03/07/2018 and 05/07/2018: “Information technology for scientific research” — Prof. Rocco Oliveto (2 CFU).

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#### **Awards**

Best Poster presentation “Potential Distribution and Niche Overlap Analysis in Three Otter Species: *Lutra lutra*, *Aonyx cinereus* and *Lutrogale Perspicillata* (2018) in “Conferenza dei dottorati di ricerca, Campobasso.



## An Otter by Ted Hughes

Underwater eyes, an eel's  
Oil of water body, neither fish nor beast is the otter:  
Four-legged yet water-gifted, to outfish fish;  
With webbed feet and long ruddering tail  
And a round head like an old tomcat.

Brings the legend of himself  
From before wars or burials, in spite of hounds and vermin-poles;  
Does not take root like the badger. Wanders, cries;  
Gallops along land he no longer belongs to;  
Re-enters the water by melting.

Of neither water nor land. Seeking  
Some world lost when first he dived, that he cannot come at since,  
Takes his changed body into the holes of lakes;  
As if blind, cleaves the stream's push till he licks  
The pebbles of the source; from sea

To sea crosses in three nights  
Like a king in hiding. Crying to the old shape of the starlit land,  
Over sunken farms where the bats go round,  
Without answer. Till light and birdsong come  
Walloping up roads with the milk wagon.

The hunt's lost him. Pads on mud,  
Among sedges, nostrils a surface bead,  
The otter remains, hours. The air,  
Circling the globe, tainted and necessary,

Mingling tobacco-smoke, hounds and parsley,  
Comes carefully to the sunk lungs.  
So the self under the eye lies,  
Attendant and withdrawn. The otter belongs

In double robbery and concealment --  
From water that nourishes and drowns, and from land  
That gave him his length and the mouth of the hound.  
He keeps fat in the limpid integument

Reflections live on. The heart beats thick,  
Big trout muscle out of the dead cold;  
Blood is the belly of logic; he will lick  
The fishbone bare. And can take stolen hold

On a bitch otter in a field full  
Of nervous horses, but linger nowhere.  
Yanked above hounds, reverts to nothing at all,  
To this long pelt over the back of a chair.

By Ted Hughes

# Summary

Otters (Family Mustelidae; Subfamily Lutrinae) are regarded as bioindicators of the wetland habitats which they inhabit. They are involved in a variety of processes that govern ecosystems and hence any disturbance to these ecosystems caused by hunting or climate change is initially reflected in the otter populations of the area. As such, ensuring the protection of these tiny carnivores is critical on a worldwide scale. A fundamental understanding of species ecology is a necessary precondition for species conservation and management. Aspects such as feeding and habitat ecology are critical for species survival because they enable educated choices on the protection and management of the species' preferred habitats. Additionally, the Himalayan mountain ranges are anticipated to warm faster than the worldwide average. As a result, any animal found in this region, such as the otter, is of special significance. Future climatic changes are expected to have a disastrous effect on both the ecosystem of this area and the animals that occupy the environment.

When compared to other regions of the globe, otter research in the Himalayan area has received less academic attention, resulting in a scarcity of scientific knowledge on the species and its ecosystem. The conservation and scientific value of otters in the Himalayas has expanded significantly in recent years. However, several unknown aspects of this species, such as population size, habitat use, and a variety of other ecological questions related to adaptation to climate change and adoption to threats like human wildlife conflict, poaching and hunting, and the influence of exotic competitors are not well studied. Hence, the proposed research attempts to fill a knowledge gap regarding the vulnerability of the Himalayan otters. We also examine ecological niche overlap, similarity and potential distribution, in three otter species: *Lutra lutra*, *Aonyx cinereus* and *Lutrogale perspicillata* across the ranges.

It is a well-known fact that determining the presence of rare and endangered species in freshwater environments is critical for ecological research and conservation, though it can be time-consuming and difficult under extreme conditions. Detecting DNA traces in the environment

(Environmental DNA, eDNA) allows more innovative and reliable monitoring and conservation of rare and elusive species such as the Eurasian otter (*Lutra lutra*). We devolved this state of art technology and tested it in Italian and Trans-Himalayan river system to detect the presence of otters with an aim of updating the current knowledge of the distribution of otters and exploring new places which were not known to be otter positive before.

This thesis is organized into six chapters:

## **CHAPTER 1.**

### **Introduction to the research project**

Introduction to the three species of otters in the Himalaya: *Lutra lutra*, *Aonyx cinereus*, and *Lutrogale perspicillata*, and how global warming, species distribution modeling, and environmental DNA can contribute to fill knowledges gaps and support species conservation planning.

## **CHAPTER 2.**

### **Investigating the effects of interaction of land-use and climate change on the vulnerability of Himalayan otters**

Climate Change Vulnerability Assessment (CCVA) prescribes the quantification of species vulnerability based on three components: sensitivity, adaptive capacity and exposure. Such assessments should be performed through combined approaches that integrate trait-based elements (e.g., measures of species sensitivity such as niche width) with correlative tools quantifying exposure (magnitude of changes in climate within species habitat). Furthermore, as land-use alterations may increase climate impacts on biodiversity, CCVAs should focus on both climate and land-use change effects. Unfortunately, most such assessments have so far focused exclusively on exposure to climate change. We evaluated the vulnerability of three otter species occurring in the Himalayan region, i.e., *Aonyx cinereus*, *Lutra lutra* and *Lutrogale perspicillata*, to 2050 climate and land-use through the recently proposed Climate Niche Factor Analysis (CNFA). Our results underline how coupling climate and land-use change components in CCVAs can generate diverging predictions of species vulnerability

compared to approaches relying on climate change only.

### **CHAPTER 3.**

#### **Global assessment of ecological niche overlap, similarity and potential distribution, in three otter species: *Lutra lutra*, *Aonyx cinereus* and *Lutrogale perspicillata* across the ranges**

Understanding the areas where suitable environmental conditions support a species' survival is critical for ecological, evolutionary, and conservation studies of that species. As a result, estimating niche overlap has become a critical tool for determining a species' ecological requirements, relative abundance, geographic distributions, coexistence of species, evolutionary diversification, and conservation strategies. Our study's objective was to determine the most recent global distribution ranges of *L. lutra*, *A. cinereus*, and *L. perspicillata*. To accomplish this, we conducted a distribution range survey and used Species Distribution Modelling (SDM) to determine the three species' possible distributions. Additionally, we attempted to quantify the extent of niche overlap and similarity between each pair of species. Finally, we looked for discrepancies between each species' actual and potential distribution ranges that might indicate competitive exclusion.

### **CHAPTER 4.**

#### **Assessing the feasibility of an environmental DNA (eDNA)-based detection approach to monitor the presence of Eurasian otters: first accounts from southern Italy**

Determining the presence of endangered species in freshwater settings is necessary for ecological research and conservation, but it can be difficult and time consuming. Detection of DNA traces in the environment (Environmental DNA, eDNA) enables the monitoring and conservation of rare and elusive species such as the Eurasian otter (*Lutra lutra*) to be more innovative and dependable. We evaluated an experimental approach based on a target qPCR assay for detecting *L. lutra* eDNA in water samples in the southern Italy river system as a rapid monitoring tool to be used in conjunction with traditional field surveys on a finer scale. This is the first time that a technique based on eDNA was used

to monitor the occurrence of *L. lutra* in Italy. We compared the eDNA-based findings to traditional survey observations and established the validity of this innovative strategy for monitoring such aquatic elusive species on a large scale.

### **CHAPTER 5.**

#### **Test an environmental DNA approach to detect *Lutra lutra* in the Himalayan rivers**

Little is known about the habitat preferences of the Eurasian otter across the Himalaya and, in particular, across the Trans-Himalayan region. This reflects a significant gap in the knowledge of poorly-known species-biology and conservation in the region. Moreover, the Himalayan mountain ranges are expected to experience higher rates of warming than the global average. Therefore, future changes in climate are likely to have catastrophic impacts on the ecology of this region as well as on the species inhabiting the landscape. eDNA monitoring is a cost-efficient, non-invasive, and easy-to-standardize method of sampling. It entails the collection of genetic material directly from environmental samples (water, soil, snow, and ice) that show no evidence of biological activity. DNA sequencing technology that is cost-effective, sensitive, and constantly improving is becoming the best contender for species and macro-organism community monitoring projects. The results of eDNA analyses have been shown to be effective in monitoring present biodiversity in terrestrial and aquatic habitats. In the present study, to overcome the deficiencies of monitoring method, we used eDNA to detect the rare and elusive otters in Trans-Himalayas. We tested an experimental workflow based on a target qPCR assay to detect DNA traces of *L. lutra* in water samples as a large-scale monitoring tool, which could be supplemented by fine-scale surveys once the species was identified. In the present study, we used the eDNA technique to determine the distribution of otters in Trans-Himalayas ranging from 2600 m to 3810 m asl.

### **CHAPTER 6.**

#### **Concluding remarks**



## The Otter

When you plunged  
The light of Tuscany wavered  
And swung through the pool  
From top to bottom.

I loved your wet head and smashing crawl,  
Your fine swimmer's back and shoulders  
Surfacing and surfacing again  
This year and every year since.

I sat dry-throated on the warm stones.  
You were beyond me.  
The mellowed clarities, the grape-deep air  
Thinned and disappointed.

Thank God for the slow loadening,  
When I hold you now  
We are close and deep  
As the atmosphere on water.

My two hands are plumbed water.  
You are my palpable, lithe  
Otter of memory  
In the pool of the moment,

Turning to swim on your back,  
Each silent, thigh-shaking kick  
Re-tilting the light,  
Heaving the cool at your neck.

And suddenly you're out,  
Back again, intent as ever,  
Heavy and frisky in your freshened pelt,  
Printing the stones.

*By Seamus Heaney*

## Introduction to the research project

### Global Warming

The human population has had a major influence on the environment in the last few thousand years (Ojima et al., 1994; Sanderson et al., 2002; Vitousek et al., 1997). In the previous century alone, the human population rose from less than 2 billion in the early 1900s to over 6 billion in the year 2000 (Loh & Wackernagel, 2004). An exponential increase in the human population and consumerism in the 20th century have caused problems that were earlier unknown to humans and the planet (Sanderson et al., 2002).

Strong evidence demonstrates that atmospheric concentration of greenhouse gases (especially carbon dioxide) has been growing. For example, it was reported in 2019 that the average CO<sub>2</sub> content in the atmosphere (414.7ppm) was 45 percent greater than it was between 1980 and 1990 (Apadula et al., 2019). Climatic changes caused by rising CO<sub>2</sub> levels are accepted as the leading contributor to global warming. Climatic changes projected for the 21st century can be compared to the largest global changes in the last 65 million years; and global warming is the most significant climatic change. Over the next several decades, global warming is predicted to increase. Climate predictions and carbon scenarios predict an increase from 1.8 to 4.0 °C global average temperatures by the year 2100 (Serreze, 2010).

Regardless of agreements to cut carbon emissions, the climate will continue to change for at least a few centuries due to the persistence of the oceanic and atmospheric circulation systems, to which species will respond in an unanticipated manner (Castiaux, 2012). Warming climatic impacts have already been demonstrated to alter species phenology and physiology, as well as cause shifts in species ranges, which might lead to higher extinction rates (Parmesan, 2005; Root et al., 2003; Walther et al., 2005).

### Climate and Land-use Change

Effects of climate change on the three otter species, Smooth-coated otter (*Lutrogale perspicillata*), Oriental small-clawed (*Aonyx cinereus*) and the Eurasian (*Lutra lutra*). have been modelled at a large scale by (Cianfrani et al., 2018) combining species sensitivity, habitat exposure and other metrics to calculate a global vulnerability index. Climate change can severely alter habitats and food sources for wildlife (Stenseth et al., 2002; C. D. Thomas et al., 2004) as well as the structure and function of ecosystems (Parmesan & Yohe, 2003). There is viable proof that climate change will become one of the principal drivers of species extinction (Chen et al., 2009; Salick et al., 2009) by affecting the distribution as well as the biological traits of a species. Also, land use and anthropogenic disturbance were considered as static factors by (Cianfrani et al., 2011) to evaluate the effects of climate change on the future distribution of the Eurasian otter in Europe. Indeed, land-use change is among the most critical drivers of global change and biodiversity loss (Lambin, 1999) since it influences many aspects of geo-environmental systems (Guzha et al., 2018; Kiruki et al., 2017; Rogora et al., 2018), hampering a species' capacity to move to suitable habitats (Oliver et al., 2015; Pereira et al., 2012). Since land-use change is expected to increase climate change impacts on biodiversity, it is critical to integrate these two components to understand likely consequences of such pressures on biodiversity (Sirami et al., 2017; Titeux et al., 2017). Investigating how these species could respond to future climate and land-use change in a highly vulnerable area such as the Himalaya represents a crucial task for delineating effective conservation strategies against global change impacts (Foden et al., 2019).

Climate and land-use changes have been ranked as the biggest threats to global biodiversity (Sala

et al., 2000) but the majority of studies exploring species' responses to climate change have largely disregarded the role of land-use characteristics, assuming that species are limited only by the shift of climatic conditions (Pacifi et al., 2015; Sirami et al., 2017), also see (Di Febbraro et al., 2019). Our current knowledge of the implications of climate and land-use change interactions on ecological systems is limited and mainly founded on broad assumptions rather than on observed interactions (Brook et al., 2008; Oliver & Morecroft, 2014). Forecasting species responses to climate and land use change is becoming more crucial for biodiversity conservation. Planning and implementing sustainable biodiversity protection is impeded due to our lack of understanding on how species will adapt to these coupled environmental changes (Davies et al., 2012; Lioubimtseva et al., 2005).

When modelling future changes in species distributions, a number of studies have underlined the need for integrating land use change with climate change (Sala et al., 2000; Sirami et al., 2017). As a result, for biodiversity conservation, forecasting species responses to climate and land use change is becoming more crucial. The ability of a species to persist in a landscape that is experiencing a certain extent of habitat loss in conjunction with changing climatic conditions, or the ability of a species to migrate to new areas as they become climatically suitable, are important questions for the management and conservation of sustainable landscapes (Travis, 2003).

### **Species Distribution Modeling**

Determining populations of rare, endangered, and poorly known species is a vital consideration in biodiversity conservation for evaluating species' conservation status, and for generating effective strategies for in situ and ex situ conservation. Often, field surveys are constrained by low population sizes with widely dispersed individuals, rugged terrain, political unrest, inadequately understood distributional ecology. Ecological niche modelling (ENM) gives a robust predictive framework for targeting areas for field surveys to locate additional populations. ENM fuses known occurrence records with appropriate environmental layers to predict species' ecological demands and potential geographic distributions (Soberón & Peterson, 2005).

Species distribution or habitat suitability are required for many aspects of environmental research, resource management, and conservation planning (Franklin, 2010). Over the last 20 years, the importance of modelling species distributions has increased, with examples ranging from meta-population ecology (Hanski, 1998), climate change research (Huntley et al., 2007) and niche theory (Pearman et al., 2008) to the optimization of

survey design for rare species (Guisan et al., 2006).

A wide range of theoretical and applied analyses in animal ecology, biogeography, and conservation biology involve the production or use of maps of species' distribution (Boitani & Powell, 2012). Though the use of traditional methods of distribution modelling using generalised linear models (GLMs; (McCullagh & Nelder, 1989) or generalised additive models (GAMs; (Hastie, T. & Tibshirani, R. J., 1990) are still on trend, recent developments are becoming popular. Ecological Niche Factor Analysis (ENFA) (Hirzel et al., 2002), Markov Chain Monte Carlo (MCMC) image restoration (Heikkinen & Högmander, 1994) and Occupancy based distribution (Kery and Schmidt, 2008) are other popular approaches of modeling distribution, occurrence and potential use of habitat by species.

Overlap of current and future species distribution was used to identify areas where habitat suitability was lost or gained following climate change, their fragmentation levels and connectivity needed to maintain dispersal of a species to deal with isolation and habitat-specific stochasticity. Outputs allowed us to identify critical factors affecting species distribution and evaluate species vulnerability regarding exposure and sensitivity to climate changes, its potential to adapt to changes.

Information on the presence and presence-absence of species was modelled using habitat information, climate data, human existence and land-use land cover variables. Climate change projections were derived from the Intergovernmental Panel on Climate Change (IPCC). The species' distributions were modelled using an ensemble forecasting approach. Maps of the probability of occurrence were transformed into presence/absence binary maps based on specific thresholds. To evaluate potential species co-occurrence, binary maps were overlapped, and binary maps were overlaid on the human-wildlife conflict map to identify areas in need of specific mitigation measures.

### **Predictive Distribution Modelling**

Recent changes in climate change have already had an impact on biological systems around the world, resulting in shifts in species distribution. Upward movement of alpine flora has been documented in the Himalaya. It has been said that Himalayan mountain ranges are expected to experience higher rates of warming than the global average. Therefore, future changes in climate are likely to have catastrophic impacts on the ecology of this region as well as on the species inhabiting the landscape. Species distribution models (SDMs) are currently the most widely used of scientific approaches to examine potential climate-change impacts on biodiversity.

### Species Monitoring Using Environmental DNA

Monitoring the presence of rare and/or endangered species is essential for ecology and conservation purposes, but often can be conducted only by adopting non-invasive methods, or actively searching for indirect signs (e.g. roadkills, footprints, scats, spraints). In the past, efforts have been made to explore mammal species diversity by using various techniques, such as transect sampling (L. Thomas et al., 2010), camera trapping (Jamwal et al., 2016) and sign surveys (Sadlier et al., 2004). Camera trapping has largely been used to study ecology worldwide (Rovero et al., 2014). This approach has an advantage over other types of surveillance since cameras can operate in the field for more extended periods and do not require the same level of attention as human observers (Meek et al., 2014). However, the issue of theft and the ongoing costs of using camera traps are significant drawbacks. Sign surveys using trails and transects are not only effective for wildlife monitoring, but they are also relatively inexpensive in terms of dedicated facilities and analytics requirements and are straightforward to conduct (Jathanna et al., 2003). Nonetheless, sign survey is often incapable of identifying congeners species or to quantify their relative abundance (Harrington et al., 2010). On the other hand, scat survey is a widely used strategy in mammal monitoring studies to assess distribution through space and time of a certain species (Lee et al., 2019; Prat-Mairet et al., 2017), such as the Eurasian otter (*Lutra lutra*) (Reuther, 2000). However, scat detection is time-consuming and often difficult to be applied in harsh environments due to logistic impediments (Lerone et al., 2015). Within the last decade, we faced a revolution in species detection, due to the implementation of molecular techniques able to detect DNA traces from environmental matrices (e.g. water) (Taberlet et al., 2018). Environmental DNA (eDNA) is released by organisms into the environment from faeces, mucus, skin cells, or extracellular DNA (Taberlet et al., 2012) and its detection soon became an invaluable ally in wildlife conservation studies (Cristescu & Hebert, 2018; Valsecchi et al., 2021). This is the case of many top predator carnivores, including the Eurasian otter.

eDNA monitoring is an efficient, non-invasive and easy-to-standardise sampling approach. It consists of collecting genetic material directly from environmental samples (water, soil, snow, ice). Cost-efficient, sensitive, and ever-advancing DNA sequencing technology is becoming more and more the best candidate for species and macro-organismal community monitoring programs. Results from eDNA approaches have proven

useful for monitoring contemporary biodiversity in terrestrial and aquatic ecosystems (Thomsen & Willerslev, 2015). DNA left in the environment from higher organism such as mammals and fish may come from excreted cells or tissue such as urine (Valiere & Taberlet, 2000), faeces (Poinar et al., 1998), hairs and skin (Bunce et al., 2005; Lydolph et al., 2005), and apparently from dead individuals leaking genetic material. Preservation of DNA left in the environment is much higher in cold and dry climate, up to hundreds of thousands of years, and thus its application in the Himalaya is particularly promising (Willerslev et al., 2003). The eDNA approach can be applied to monitor the current distribution of the otter and its prey, especially fish.

### Himalayas

In mountain ecosystems, global climate change can exert its strongest effects on freshwater habitats, e.g. frequent and increased extreme flooding and erosion. These systems are already heavily affected by anthropogenic factors, such as pollution, dam construction, and habitat disturbance. An effective assessment of global change threats to biodiversity relies on identifying highly vulnerable species, together with the drivers of such vulnerability. Vulnerability assessments are particularly crucial in mountain environments, which are amongst the most sensitive ecosystems to global change (Beniston, 2003). Temperature in the Himalaya, for instance, is increasing at twice the average warming rate of the northern hemisphere (Chen et al., 2009). Increasing climate change impacts, followed by rising resource demands of growing populations, cause serious concerns about the future of Himalayan biodiversity (Chakraborty et al., 2018). As part of its rich biodiversity, the Himalaya hosts three otter species, i.e., Smooth-coated (*Lutrogale perspicillata*), Oriental small-clawed (*Aonyx cinereus*) (Gupta et al., 2020; Hussain et al., 2008) and the Eurasian (*Lutra lutra*). *Lutra lutra* was documented in the Indian Trans-Himalayan rivers, Indus, Suru and Dras in Ladakh (Jamwal et al., 2016).

### Otters

Otters are highly vulnerable species, sensitive to habitat loss and water pollution. As keystone aquatic predators, otters are considered an indicator species of healthy freshwater ecosystems (Kruuk, 2006; Ruiz-Olmo et al., 1998).

### The Smooth-Coated Otter (*Lutrogale Perspicillata*)

Although the smooth-coated otter, *Lutrogale perspicillata*, is now restricted to a few protected

areas, they were once common in wetlands and low-lying areas throughout South Asia and Southeast Asia, where fishermen, particularly in eastern India and Bangladesh, trained them to drive fish into their nets. Based on an estimated population loss of more than 30% in the last 30 years, this species is currently classified as Vulnerable (Pacifi et al., 2013) Appendix II of the Convention on International Trade in Endangered Species. It has been found in Nepal, India, Bangladesh, Bhutan, Southwest China, Myanmar, Singapore, Thailand, Singapore, Vietnam, Malaysia, Sumatra, Java, Borneo, Indonesia (Hussain, 1993) and southern Iraq (Al-Sheikhly & Nader, 2013).

Smooth-coated otters are identified by their long, flattened tails, deeply webbed front paws, and a smooth top rhinarium border, thus the name. They have non-spotted muzzles and a naked, dark rhinarium with a peaked top margin-inverted V shaped rhinarium (Hussain, 1999; Prater, 1965). The size of the species ranges from 1067 to 1300 mm, and they may weigh up to 11.4 kg (Foster-Turley, 1992). Their litter size ranges from 2 to 5 puppies. A smooth-coated otter family typically includes a male, female, and up to five young. (Prater, 1965) identified two smooth-coated otter subspecies: *L. p. perspicillata* in northeast and southern India, Myanmar, and Sumatra, and *L. p. sindica* in north and northwestern India and Pakistan (Sindh province). In addition to these, another subspecies, *L. p. maxwelli*, has been discovered in the marshes of southern Iraq (Mason & Macdonald, 2009). Large rivers, lakes, peat swamp forests, coastal mangroves, estuaries, and rice fields are preferred by smooth-coated otters (Foster-Turley, 1992), as long as there is enough riverside vegetation for protection and escape, as well as rocky regions or deep soil for excavating natal dens. These otters have been spotted swimming out to the seas, but they need enough fresh water to remove the salt off their coats.

The expanding human population in Asia is the greatest danger to Asian otters, resulting in the loss of wetland habitats, a decrease in prey biomass, dam building, pollution, and poaching. The Smooth-coated otter is directly threatened by the illicit wildlife trade. Between 1980 and 2015, 5,881 otter pelts were collected in 15 Asian nations, with India accounting for about half of the pelts and the Smooth-coated and Eurasian otters being the most common species (Gomez et al., 2016).

Increased aquaculture operations in South and Southeast Asia are also resulting in the indiscriminate slaughter of otters. While small-scale fishermen tolerate otters, commercial fishers consider and kill them as pests. Climate change will also have a significant impact on otter populations

across the globe as smooth-coated otters rely on rivers, lakes, and streams, which will change dramatically as a result of global warming, lowering water levels in long-term droughts and changing prey concentrations.

#### **Small-clawed Otter (*Aonyx cinereus*)**

Small-clawed Otter *Aonyx cinereus* is the smallest otter, it is ubiquitous in Southeast Asia but is quickly diminishing owing to wetland habitat loss, poaching, and, more lately, the online pet trade. Due to an estimated historic population loss of more than 30% over the previous 30 years, or three generations, the Asian small-clawed otter is classed as Vulnerable (Pacifi et al., 2013). Appendix II of the Convention on International Trade in Endangered Species.

The range of the small-clawed otter is extensive, stretching from India in South Asia eastward to Southeast Asia, including Lao People's Democratic Republic, Malaysia, Myanmar, Cambodia, Bangladesh, and Indonesia, to Palawan in the Philippines and southern China (Hussain et al., 2011; Wozencraft, 1993). As its name indicates, the small-clawed otter has very small claws and webbed feet. Their pelage ranges from dark brown to light brown, with a pale, occasionally almost white chest, neck, cheeks, and chin. Small-clawed otters range in length from 652-939 mm and weigh between 2.7 and 5.4 kg. (Hussain et al., 2008) describe front paws as being similar to hands with decreased nails for grabbing small prey in shallow, filthy water. Their behaviour and ecology are mostly unknown, with just a few captive studies are conducted. Females begin reproducing at around 15 months of age and have a gestation period of roughly 60 days (Foster-Turley, 1992). Their lifetime in captivity is around 11 years (Crandall, 1964). Asian small-clawed otters inhabit a diverse range of natural and man-made environments, preferring slower-moving water sources such as meandering rivers, streams, peat swamps, mangrove forests, tidal pools, rice fields, irrigation ditches, and fish ponds. The food of the small-clawed otter is mostly comprised of crabs and other invertebrates, as opposed to the fish that other otter species enjoy. The Small-clawed otter is endangered throughout its habitat by human development and activity. Aquaculture, swamp reclamation, siltation caused by deforestation, pesticide contamination, mining, quarrying, slash-and-burn agriculture, and habitat loss owing to agricultural conversions such as coffee, tea, and palm oil plantations, as well as rice fields, all have a negative impact on otter habitat. The illicit pet trade is a significant danger to small-clawed otters. Small-clawed otters are endearing and popular attractions in zoos and, more recently, in Asian pet stores, pet

festivals, and even coffee cafes (Aadrean, 2013; Gomez & Bouhuys, 2018; Gonzalez, 2010). A recent modelling study indicates that climate change would have a severe influence on Small-clawed otter habitats, with up to 40% of viable regions disappearing by 2070. This scenario is exacerbated by the Small-clawed otter due to its climatic niche's marginality (Cianfrani et al., 2018). This scenario is exacerbated by the Small-clawed otter due to its climatic niche's marginality (Cianfrani et al., 2018).

### **The Eurasian Otter (*Lutra lutra*)**

*Lutra lutra*, the Eurasian otter, is the most widespread otter species. It may be found in a wide range of environments, from Western Europe to the Palearctic, India, Southeast Asia, and North Africa. The Eurasian otter has been reclassified from Vulnerable to Near Threatened owing to a sustained population loss of no more than 30% during the last three generations (23 years) (Pacifci et al., 2013) Appendix I of the Convention on International Trade in Endangered Species. The Eurasian otter has one of the most extensive ranges of any Palearctic animal, including Europe, North Africa, and Asia (Hung & Law, 2016). With isolated populations of *L. l. monticola* in the Himalayas and *L. l. kutab* in Jammu and Kashmir, where they are scarce in the rivers Indus, Suru, and Dras in Ladakh, India has a small number of Eurasian otters (Jamwal et al., 2016).

The Eurasian otter is dark brown in colour, with a paler underbelly and pale spots on the neck (Kruuk, 2006) Total length is 920-1200 mm, head to body is 550-570 mm, and tail length is 35-375 mm (Foster-Turley, 1992). They range in weight from 4

to 12 kg (Reuther, 1991). Furthermore, each of the four feet has five toes, each with powerful claws and webs between them (Pocock, R. I., Seymour Sewell, R. B., & Shebbeare, E. O., 1939). Male and female Eurasian otters attain sexual maturity at roughly 18 and 24 months, respectively, in the wild, but it takes them considerably longer in captivity, generally 3 to 4 years (Reuther, 1991). After a 65-day gestation period, they give birth to a brood of 1 to 5 puppies (Acharjyo & Mishra, 1983). The Eurasian otter may be found in a broad range of aquatic environments, including lakes, reservoirs, rivers, marshes, swamp woods, and coastal regions, and may be found from sea level to 3,500 metres in the Himalayas (Jamwal et al., 2016). The Eurasian otter is normally territorial, solitary, and nocturnal, but it may be seen during the day in regions where human activity is minimal. The aquatic environments of otters are particularly susceptible to human alteration.

River canalization, destruction of bankside vegetation, dam building, oil spills, wetlands draining, and aquaculture are all detrimental to otter habitat (Reuther & Hilton-Taylor, 2004). Acidification of rivers and lakes, as well as organic pollution from nitrate fertilisers, untreated sewage, and agricultural slurry, cause declines in fish biomass. In western and central Europe, pollution is a severe danger to otters. Demand in East Asia, notably China, fuels a considerable illicit traffic in otter pelts, which are obtained in South and Southeast Asia. Isolation of scattered populations of subspecies (*L. l. monticola*, *L. l. Kutab*, and *L. l. nair*) might be a major danger in India, according to (Hung & Law, 2016)

**This work addressed the gap of information on the ecology and conservation status of the otter in the Trans-Himalayan region of India with the following primary objectives:**

**Investigating how the interaction of land-use and climate change affects the vulnerability of otters in the Himalaya**

- To model the current potential distribution using topographic, climatic and land-use variables
- To predict their potential distribution in 2050 under climate and land-use change scenarios
- To assess the degree of range net change and shift experienced
- To assess species vulnerability to these global change scenarios by coupling SDMs and the “CNFA” framework (Climate Niche Factor Analysis)
- To quantify the relative contribution of species sensitivity and habitat exposure in shaping vulnerability

**Global assessment of ecological niche overlap, similarity and potential distribution, in three otter species: *Lutra lutra*, *Aonyx cinereus* and *Lutrogale perspicillata* across the ranges**

- To define the most up-to-date potential distribution of *Lutra lutra*, *Aonyx cinereus* and *Lutrogale perspicillata* worldwide through species distribution modelling

- To detect any discrepancy between actual and potential distribution range of each species that could be interpreted as evidence of competitive exclusion
- Quantification of degree niche overlap and similarity between each combination of species pair

**Assessing the feasibility of an environmental DNA (eDNA) based detection approach to monitor the presence of Eurasian otters: first accounts from southern Italy**

- To detect the presence of *Lutra lutra* using water samples from southern Italy
- To compare the efficiency of eDNA sampling with traditional methods

**Test an environmental DNA approach to detect *Lutra lutra* in the Himalayan rivers**

- To detect the presence of *Lutra lutra* using water samples from Indian Trans-Himalaya
- To compare the efficiency of eDNA sampling with traditional methods
- To identify key ecological variables influencing otter presence in Indian Trans-Himalaya

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# Global change on the Roof of the World: vulnerability of Himalayan otter species to land-use and climate alterations

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## ABSTRACT

**Aim.** Climate Change Vulnerability Assessment (CCVA) prescribes the quantification of species vulnerability based on three components: sensitivity, adaptive capacity and exposure. Such assessments should be performed through combined approaches that integrate trait-based elements (e.g., measures of species sensitivity such as niche width) with correlative tools quantifying exposure (magnitude of changes in climate within species habitat). Furthermore, as land-use alterations may increase climate impacts on biodiversity, CCVAs should focus on both climate and land-use change effects. Unfortunately, most of such assessments have so far focused exclusively on exposure to climate change.

**Location.** Himalaya

**Methods.** We evaluated the vulnerability of three otter species occurring in the Himalayan region, i.e. *Aonyx cinereus*, *Lutra lutra* and *Lutrogale perspicillata* to 2050 climate and land-use through the recently-proposed Climate Niche Factor Analysis (CNFA) framework combined with Species Distribution Models.

**Results.** Future climate and land-use change will reduce (6 – 15%) and shift (10 – 18%) the geographic range of the three species in the Himalaya, with land-use alterations exerting far more severe effects than climate change. Among vulnerability components, sensitivity played a greater role than exposure in determining the vulnerability of the otters. Specifically, the most specialist species, *L. perspicillata* showed the highest vulnerability in comparison with the most generalist, *L. lutra*.

**Main conclusions.** Our results underline how coupling climate and land-use change components in CCVAs can generate diverging predictions of species vulnerability compared to approaches relying on climate change only. Moreover, intrinsic components, such as species sensitivity, proved significantly more important in determining vulnerability than extrinsic metrics such as habitat exposure.

## KEYWORDS:

Climate change, land-use change, climate change vulnerability assessment, species distribution models, SDMs, climate niche factor analysis, niche width, habitat exposure

## INTRODUCTION

Climate change has recently emerged as a key threat to biodiversity and ecosystems (Chen et al., 2011; Pacifici et al., 2015). It is expected to surpass habitat loss as the prime threat to global biodiversity in the future (Leadley, 2010). The majority of studies exploring species' responses to climate change have largely disregarded the role of land-use characteristics, assuming that species are limited only by the shift of climatic conditions (Pacifici et al., 2015; Sirami et al., 2017; but see Di Febbraro et al., 2019). Indeed, land-use change is among the most critical drivers of global change and biodiversity loss (Lambin, 1999), since it influences many aspects of geo-environmental systems (Guzha et al., 2018; Kiruki et al., 2017; Rogora et al., 2018), hampering the capacity of species to move to suitable habitats (Oliver et al., 2015; Pereira et al., 2012). Our current knowledge of the implications of climate and land-use change interactions on ecological systems is limited and mainly founded on broad assumptions rather than on observed interactions (Brook et al., 2008; Oliver & Morecroft, 2014). Since land-use change is expected to increase climate change impacts on biodiversity, it is critical to integrate these two components to understand likely consequences of such pressures on biodiversity (Sirami et al., 2017; Titeux et al., 2017).

From this perspective, an effective assessment of global change threats to biodiversity relies on identifying highly vulnerable species, together with the drivers of such vulnerability. One of the most comprehensive frameworks developed to assess species vulnerability to climate change is the "Climate Change Vulnerability Assessment" (CCVA; Foden et al., 2019). This analytical framework uses an array of different approaches (i.e. biological traits, correlative and mechanistic models, etc.) to quantify species vulnerability based on three main components: i) sensitivity (i.e. degree to which a species is affected by climate change as a consequence of intrinsic attributes such as niche specialization, environmental tolerance, etc.); ii) adaptive capacity (i.e. ability to adjust to climate change by virtue of high levels of phenotypic plasticity, dispersal ability or genetic diversity) and; iii) exposure (i.e. rate and magnitude of climate changes within species habitat; Foden et al., 2019). Despite CCVAs having been widely used in recent years, most of these assessments have focused solely on the exposure component (L. M. Thompson et al., 2015), usually quantified through correlative approaches (i.e. Species Distribution Models, "SDMs" hereafter; Pacifici et al., 2015). On the other hand, assessments involving a strictly trait-based approach (i.e. using species' biological characteristics, such as niche width or dispersal capabilities, as predictors of climate-driven

extinction risk), mostly quantified species vulnerability according to direct observations, published literature or expert judgment (Borges et al., 2019), though precise vulnerability thresholds associated with each trait are often unknown (Pacifici et al., 2015). Several authors underlined the advantages of using combined approaches able to assess vulnerability by integrating e.g., correlative and trait-based elements (Foden et al., 2019; Pacifici et al., 2015). Yet, such compound frameworks have thus far only been used in a limited number of studies (but see e.g., Garcia et al., 2014; Warren et al., 2018), especially if compared to those based solely on correlative models (Pacifici et al., 2015). Along with this general recommendation towards the use of combined approaches, a deeper understanding of how climate change and other pressures, such as land-use alterations, could interact to affect the future survival of species was highlighted as another major challenge, as well as a better integration of this interplay in CCVA techniques (Foden et al., 2019).

Vulnerability assessments are particularly crucial in mountain environments, which are amongst the most sensitive ecosystems to global change (Beniston, 2003). Temperatures in the Himalaya, for instance, are increasing at twice the average warming rate of the northern hemisphere (F. Chen et al., 2009). Increasing climate change impacts, followed by rising resource demands of growing populations, cause serious concerns about the future of Himalayan biodiversity (Chakraborty et al., 2018). Under the current combination of accelerating global warming and habitat degradation and loss, many Himalayan species may not be able to adapt to such overwhelming pressures (Xu et al., 2009).

In mountain ecosystems, global climate change can exert its strongest effects on freshwater habitats (e.g., increased extreme flooding and erosion; Cianfrani et al., 2018). These systems are already heavily affected by anthropogenic factors, such as pollution, dam construction, and habitat disturbance. Otters are highly vulnerable species, sensitive to habitat loss and water pollution (Kruuk, 2006; Ruiz-Olmo et al., 1998). As keystone aquatic predators, otters are considered an indicator species of healthy freshwater ecosystems (Kruuk, 2006; Ruiz-Olmo et al., 1998). The Himalaya encompasses the altitudinal range edge of three otter species (Figure S1): the Small-clawed otter (*Aonyx cinereus*), the Eurasian otter (*Lutra lutra*) and the Smooth-coated otter (*Lutrogale perspicillata*; Duplaix & Savage, 2018). The IUCN Red List classifies *L. lutra* as Near Threatened (NT), and *A. cinereus* and *L. perspicillata* as Vulnerable (VU; Duplaix & Savage, 2018; see also Appendix S1). Effects of climate change on the three species have been modelled at large scale by Cianfrani et al. (2018), combining species sensitivity, habitat exposure and other metrics to calculate a global

vulnerability index. Also, land use and anthropogenic disturbance were considered as static factors by Cianfrani et al. (2011) to evaluate the effects of climate change on the future distribution of the Eurasian otter in Europe. Investigating how these species could respond to future climate and land-use change in a highly vulnerable area such as the Himalaya represents a crucial task for delineating effective conservation strategies against global change impacts. In addition, this particular context represents a unique opportunity to test innovative, combined approaches to assess species vulnerability to multiple global change drivers (i.e. climate and land-use), in keeping with the prevailing future perspectives delineated for CCVAs (Foden et al., 2019).

In this scenario, the present study aims to explore determinants of otter species vulnerability to future climate and land-use change in the Himalaya in terms of species sensitivity (i.e. a measure of niche specialization) and habitat exposure (dissimilarity between current and future environmental conditions within species habitat). Specifically, we formulated two hypotheses: i) including land-use change in vulnerability assessments yields higher vulnerability values than assessments relying only on climate change; ii) species sensitivity exerts a significantly higher contribution than habitat exposure in determining vulnerability values. To test these hypotheses, we have adopted the recently-proposed “CNFA” (Climate Niche Factor Analysis) framework (Rinnan & Lawler, 2019) combined with state-of-the-art SDMs. In particular, we explicitly integrated SDMs into the “CNFA” framework by calculating species sensitivity and habitat exposure within the presence/absence maps predicted by SDMs.

Specific objectives of this study were: (a) to model the current potential distribution of the three otter species in the Himalaya, considering different combinations of topographic, climatic and land-use variables, (b) to predict their potential distribution in 2050 under climate and land-use change scenarios, (c) to assess the percentage of gain/loss in range area and the degree of shift experienced by the three species under such scenarios, (d) to assess species vulnerability to these global change scenarios by coupling SDMs and the “CNFA” framework, and (e) to quantify the relative contribution of species sensitivity and habitat exposure in shaping vulnerability.

## METHODS

### 2.1 Analytical framework

To evaluate the effect of climate and land-use change on the three species of interest, we modelled their distribution considering present-day environmental conditions under two alternative setups: i) including

only topographic and climate predictors (climate SDMs), and ii) also adding land-use variables (full SDMs). Subsequently, we projected climate SDMs under two 2050 climate change scenarios (IPCC, 2013), and full SDMs by including two additional 2050 land-use change scenarios (Li et al., 2017). Predictions from both climate and full SDMs were then used alternatively to quantify range net change and shift, as well as included into the “CNFA” framework to calculate species sensitivity, habitat exposure and vulnerability.

### 2.2 Study area

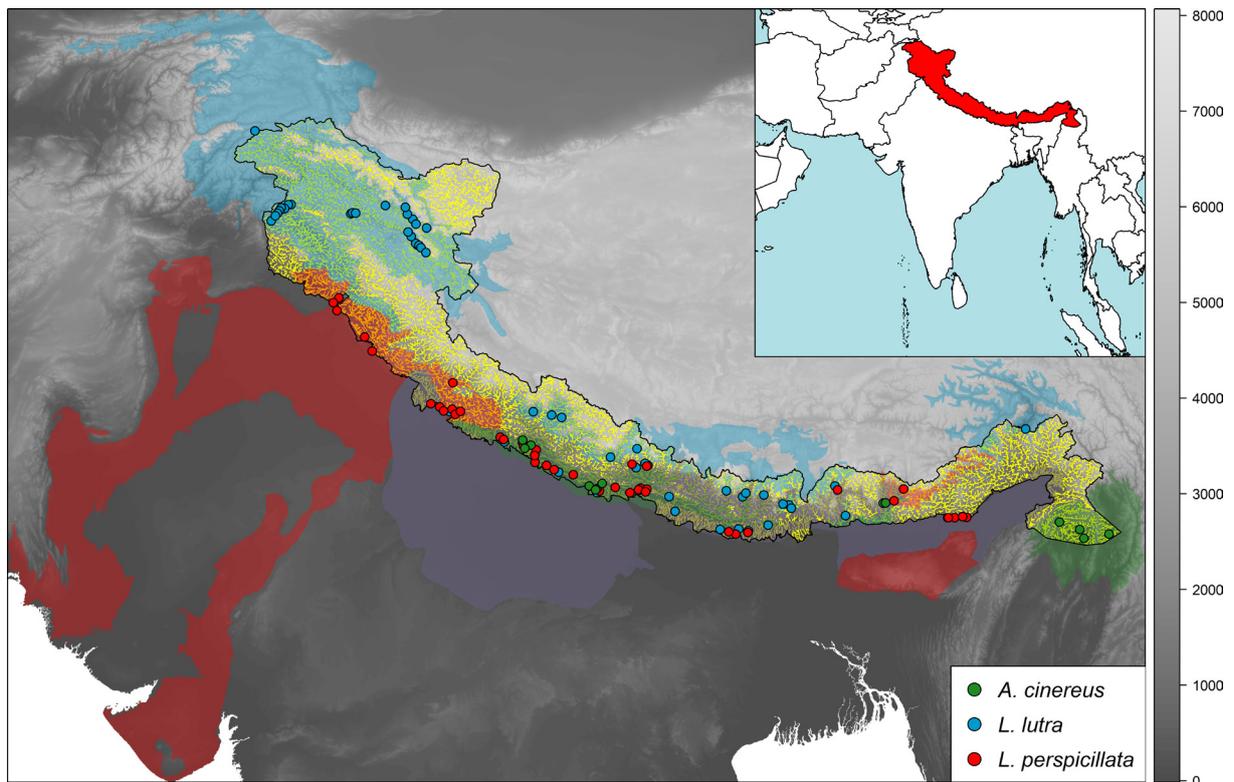
The Himalaya is a vast mountain region located between the Indian Subcontinent in the south and the Tibetan Highland in the north. The region extends from Afghanistan in the northwest (36° N and 70° E) to China in the southeast (26° N and 100° E), covering an area of more than 1.000.000 km<sup>2</sup> with a length of 3000 km and a maximum width of 400 km. The climate ranges from a tropical zone in the lowlands of India and Nepal, to permanent ice and snow at the highest elevations, and from a more continental regime in the NW to a more oceanic regime in the SE. Its abruptly rising mountains result in a high diversity of ecosystems and species, characterizing this area as a unique biodiversity hotspot (Xu et al., 2019).

### 2.3 Species occurrence data

Occurrence records were gathered from the literature and from online databases, as well as published technical reports (full data source list is provided in Appendix S2). We cross-checked all the occurrences excluding those with a minimum geographical precision coarser than 1 km and collected before 1970, as well as those referring to captive specimens (Cianfrani et al., 2018). Moreover, we drop any occurrence falling farther than 1 km from the hydrographical network. Although opportunistic records may yield accurate predictions of species distribution (Tiago et al., 2017), they are often spatially autocorrelated and/or discontinuous due to a usually unknown and unbalanced sampling effort that can vary noticeably across space (van Strien, Swaay, & Termaat, 2013). Hence, the occurrence dataset for SDMs calibration, which originally included 229 records among the three species, underwent a further filtering procedure to remove clustered data (Appendix S3). After this step, we obtained a final dataset of 12 records for *A. cinereus*, 59 records for *L. lutra*, and 43 records for *L. perspicillata* (Figure 1).

### 2.4 Environmental predictors

We considered the 19 Worldclim predictors, two



**Figure 1.** Study area, species occurrence data and ecoregions considered for SDMs calibration (green: *A. cinereus*; blue: *L. lutra*; red: *L. perspicillata*; purple polygons indicate overlapping ecoregions by *A. cinereus* and *L. lutra*). The Digital Elevation Model (grey shadowed) and the hydrographic network (yellow) are reported as background layers

topographical and seven land-use variables that could represent proxies of ecological requirements at medium-large scale (Table S1). All parameters were rasterized at a resolution of 1 km. Land use variables were derived from the GeoSOS global database (Li et al., 2017; <http://geosimulation.cn/GlobalLUCCProduct.html>), which describes remotely sensed land-use categories as detected by MODIS in 2010 (product MCD12Q1; <https://lpdaac.usgs.gov/>). In particular, for each pixel we calculated the Euclidean distance from each of these land-use categories. In addition, we calculated the alpha diversity (i.e. count) of land-use categories by applying a moving window approach. Specifically, the count of each category was calculated within a 10 km-radius circular window moved throughout each pixel of the land-use map. Such radius length was coherent with the average home range size of the three species (Kruuk, 2006). Topographical variables included elevation (gathered from Jarvis et al., 2008) and slope (derived from the elevation map). Predictors were further subselected by checking for multicollinearity ( $VIF \leq 5$ ), retaining the following 13 covariates: elevation, mean diurnal temperature range, temperature seasonality, precipitation of the driest month, precipitation

of the warmest quarter, precipitation of the coldest quarter, richness of land-use categories and Euclidean distance from farmlands, forests, grasslands, urban areas, barren lands and water bodies (Table S1). As specified above, elevation and climate variables were used to calibrate climate SDMs, while the entire predictors set was used for full SDMs.

### 2.5 Species Distribution Models

To calibrate climate and full SDMs, we applied the so-called “ensemble of small models” approach to avoid model overfitting (Breiner et al., 2015), an issue arising when few records are available with respect to the number of predictors (Guisan & Zimmermann, 2000). Accordingly, we trained a set of bivariate models for each species, i.e. considering all possible combinations of the environmental covariates taken two at a time (10 combinations per species in climate SDMs and 78 combinations per species in full SDMs), and then averaging each model’s results. Bivariate models were calibrated by using two algorithms: generalized linear models (GLMs) and maximum entropy models (Maxent; Phillips et al., 2006). We chose these two algorithms as they reported the highest predictive accuracies within an “ensemble

of small models” context (Breiner et al., 2015, 2018). For each species, we randomly placed a set of 10,000 background points over a region identified by all the WWF Terrestrial Ecoregions (Olson et al., 2001) intersecting the species records gathered in the Himalayan study area (Figure 1; Barve et al., 2011). To evaluate predictive performance of the models, we performed a “block” cross-validation (Muscarella et al., 2014) by splitting data into four geographically non-overlapping bins of equal numbers of occurrences, corresponding to each corner of the entire geographical space. Occurrences falling into three out of four bins were used in turn for model calibration, while the data from the held-out bin were used for evaluation purposes. This method proved useful in assessing model transferability, i.e. the ability to extrapolate predictions into new areas/times (Roberts et al., 2017), as well as to penalize models based on biologically meaningless predictors (Fourcade et al., 2018). The predictive performance of each model was assessed by measuring the area under the receiver operating characteristic curve (AUC; Hanley & McNeil, 1982) and the true skill statistic (TSS; Allouche et al., 2006). In particular, models achieving  $AUC \geq 0.80$  and  $TSS \geq 0.40$  are considered “good” ones *sensu* Swets (1988) and Landis & Koch (1977), respectively. Models were optimally tuned through an intensive procedure as implemented by (Breiner et al., 2018). Specifically, for each bivariate model we varied parameters and complexity as generated by alternative settings, then choosing the configuration that yielded the highest AUC under the block cross-validation scheme (Breiner et al., 2018). For GLMs, we tested the shape of the relationship (i.e. linear, quadratic or cubic) and the interaction level (absent or present). For Maxent, we evaluated regularization values between 0.5 and 4, with 0.5 steps, and tested the following feature classes: linear, linear + quadratic, hinge, linear + quadratic + hinge, linear + quadratic + hinge + product and linear + quadratic + hinge + product + threshold (Fourcade et al., 2018). The settings configurations that emerged as the optimal ones for most of the tested bivariate models were then used to calibrate final GLMs and Maxent bivariate models (Breiner et al., 2018). Among these, we dropped poorly calibrated models (i.e. achieving  $AUC < 0.7$ ; Di Febbraro et al., 2019) from the subsequent analyses. Ensemble models were obtained by averaging individual GLMs and Maxent models projections weighted by their respective AUC scores (Marmion et al., 2009). For each species, we calculated the relative importance of predictors from the ensemble model using the functionality provided in the “biomod2” package (Thuiller et al., 2009). Lastly, we calculated the spatial autocorrelation in ensemble models

residuals through Moran’s *I* correlograms (Appendix S4). Differences in predictive performance between climate and full SDMs were assessed through *t*-test.

### 2.6 Future scenarios

Climate SDMs were projected on two climate change scenarios (i.e. RCP.26 and RCP85) from the fifth IPCC assessment (IPCC, 2013). Full SDMs were instead projected on four climate and land-use change scenarios obtained by coupling the two abovementioned IPCC scenarios with two land-use change scenarios (LUC.B1 and LUC.A2) elaborated by Li et al. (2017; Table S2). Since different global circulation models may lead SDMs to predict diverging climate change effects (Buisson et al., 2010), we considered three alternative versions for the RCPs scenarios, yielded respectively by the CCSM4, CNRM-CM5 and MIROC-ESM global circulation models. The scenarios predict mild (RCP.26 and LUC.B1) to severe (RCP85 and LUC.A2) alterations in climate and land-use in 2050. The corresponding mean temporal trends of the covariates used in the models under all the scenarios are shown in Figure S2. We calculated a multivariate environmental similarity surface (MESS; Elith et al., 2011) to evaluate the effect of model extrapolation on values of predictors lying outside the calibration range (Appendix S5). Current and future SDMs projections were binarized to obtain range maps according to four threshold approaches (i.e. “equalize sensitivity and specificity”, “maximize TSS”, “mean occurrence probability”, and “minimize receiver operating characteristic plot distance”; Liu et al., 2013), as to account for the effect of using different binarization schemes (Di Febbraro et al., 2019).

### 2.7 Quantification of the effects of climate and land-use change on otter distribution and vulnerability

For each species, model and scenario, we calculated two metrics of climate and land-use change effect on binary range maps: net change (in terms of gain/loss percentage) and geographical shift (expressed as 100 – percentage overlap between current and future range maps; Franklin et al., 2013). We also assessed the direction of geographical shift by measuring changes in the position of range centroids (Aguirre-Gutiérrez et al., 2016) and margins (Taheri et al., 2021) along longitude, latitude and elevation between current and 2050 range maps (further details are provided in Appendix S6). Species vulnerability to climate and land-use change was calculated by following the so-called “CNFA” (i.e., “Climate Niche Factor Analysis”) approach, which was recently implemented by Rinnan & Lawler (2019). Similarly, to what done by Cianfrani et al. (2018) to evaluate effect of climate change on freshwater otters at global scale, this approach

relies on ecological niche factor analysis (ENFA, a technique that calculates the environmental niche of a species using presence-only data; Hirzel et al., 2002), to derive two metrics: i) species sensitivity and ii) habitat exposure to global change. In particular, sensitivity is calculated as the array of environmental conditions a species tolerates (the so-called “specialization vector” within the ENFA framework) with respect to a given environmental variable. The rationale behind this metric is that the smaller the range of environmental conditions a species tolerates for a given variable, the smaller its niche width and, consequently, the more sensitive the species will be to strong deviations from these optimal conditions (e.g., under global change; Rinnan & Lawler, 2019). Habitat exposure is calculated as a dissimilarity measure between present and future environmental conditions within a species current habitat: the higher such dissimilarity, the larger the amount of environmental change a species might experience if it remains in place. Species sensitivity and habitat exposure are then combined to calculate vulnerability through the following equation (adapted from (Rinnan & Lawler, 2019):

$$v_j = \sqrt{(1 + e_j)s_j}$$

where  $s$  and  $e$  respectively indicate species sensitivity and habitat exposure to the  $j$ -th environmental variable. According to the CNFA framework, both sensitivity and exposure were measured within the current distribution of the species (Rinnan & Lawler, 2019). Hence, we alternatively used binary predictions by climate and full SDMs to delineate spatially such current habitat. Specifically, we calculated a different vulnerability value for each binary map deriving from each scenario, global circulation model and binarization threshold. All the vulnerability analyses were carried out using the “CENFA” R package (Rinnan & Lawler, 2019). Differences in vulnerability values between each species pair were assessed through analysis of variance and Tukey post-hoc test. To evaluate the relative contribution of species sensitivity and habitat exposure in determining species vulnerability to climate and land-use future scenarios, we regressed vulnerability scores against sensitivity and exposure, respectively, considering each environmental variable. For this purpose, we used linear mixed effect models (LMM), setting sensitivity and exposure in turn as fixed terms, and global circulation model, global change scenario and binarization threshold as random intercepts. LMMs were fitted using the “lme4” R package (Bates et al., 2015).

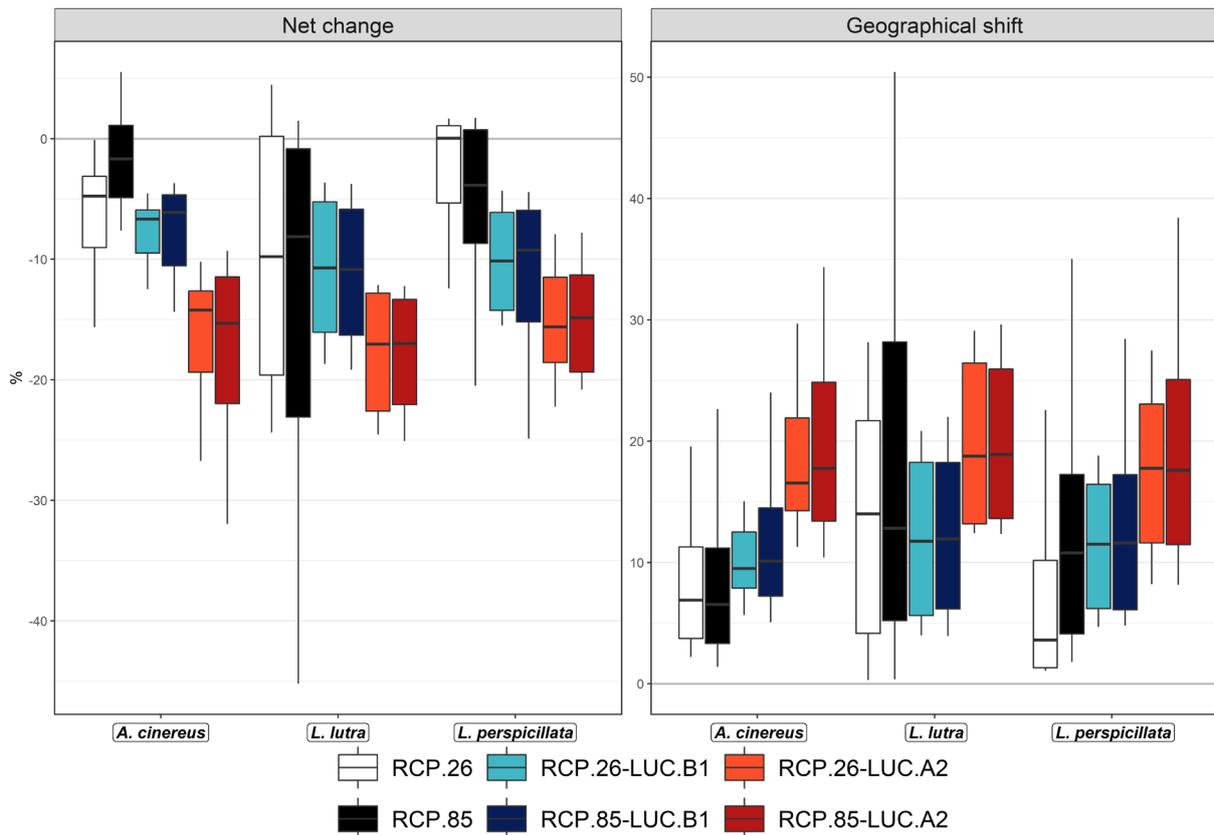
## RESULTS

### 3.1 Species Distribution Models

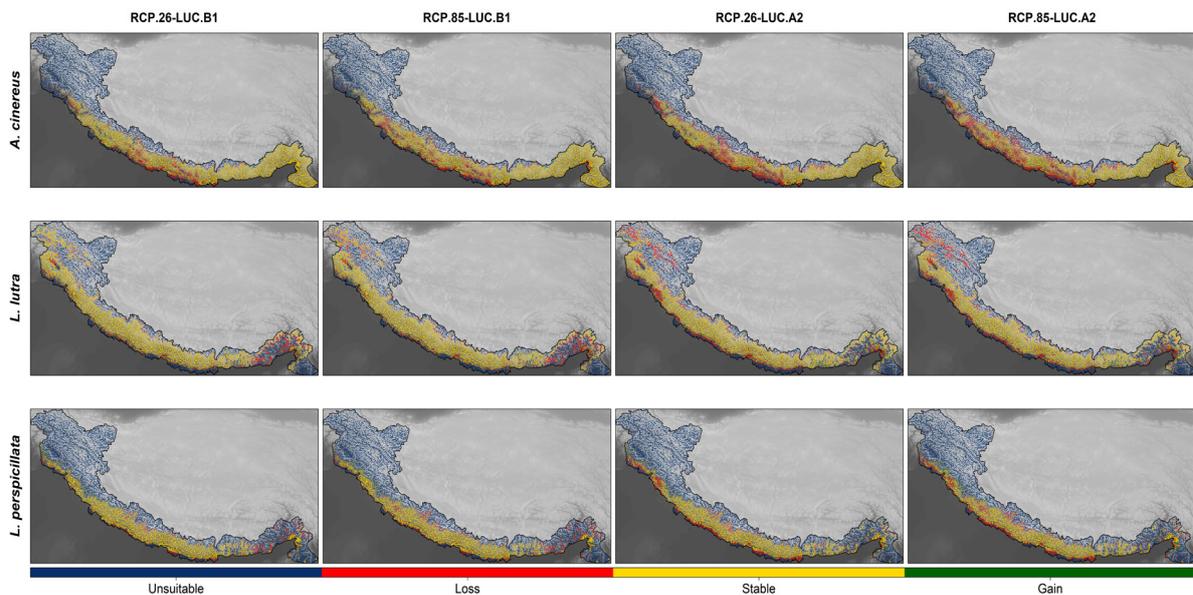
Overall, full SDMs significantly outperformed climate SDMs in terms of both AUC ( $t = 4.85, p < 0.001$ ) and TSS ( $t = 4.14, p < 0.001$ ). Specifically, while full SDMs achieved good predictive performances *sensu* Swets (1988) and Landis & Koch (1977), with AUC values  $> 0.80$  and TSS values  $> 0.40$  for all species (Table S3), climate SDMs were less accurate, with just one species (i.e. *L. perspicillata*) reporting an AUC  $> 0.80$  and two (i.e. *A. cinereus* and *L. perspicillata*) achieving TSS values  $> 0.40$ . In light of that, we hereafter showed and discussed response curves and spatially explicit predictions only for full SDMs (Figure S3 – S10). According to these models, both climate and land-use variables were listed amongst the most important predictors shaping the distribution of the three species (Figure S4). For example, distance from forests was among the three most contributing predictors for all the species (along with distance from grasslands in *L. lutra*), showing an inverse relationship with their suitability. Moreover, precipitation during the warmest-driest periods was maximally important in *L. perspicillata*, and within the four most important predictors for *A. cinereus* and *L. lutra*, overall indicating moderate precipitation conditions as the most preferred (Figure S4). Elevation was highly important for *L. lutra* (i.e. highest suitability at 1000-3500 m asl) and, to a lesser extent, for *A. cinereus* (i.e. suitability peak around 1000 m asl), while predictors describing temperature fluctuations (i.e. mean diurnal range and temperature seasonality) were among the five most contributing predictors for all the three species, showing the otters to prefer a rather high thermal stability (Figure S4). Full SDMs for all the three species exhibited a negligible spatial autocorrelation in their residuals (Appendix S4), as well as a minimal extrapolation effect in models predictions (see Appendix S5 and Figures S7 – S9).

### 3.2 Quantification of the effects of climate and land-use change on otter distribution and vulnerability

Climate SDMs, by accounting for climate change scenarios only, predicted far less severe alterations of otter distribution than full SDMs, which also involve land-use change scenarios (Figure 2). Specifically, net change in future distribution as predicted by climate SDMs is clearly negative only for *L. lutra* (median values between  $-9\%$  and  $-10\%$ ), while it shows far higher values in the other two species, even achieving positive scores (i.e. a net gain in distribution) for *A. cinereus* under RCP85 scenario, and *L. perspicillata* under RCP26 scenario (Figure 2).



**Figure 2.** Percentage of net area change (on the left) and geographical shift (on the right) in otter distribution under 2050 climate and land-use change scenarios. The box plots depict climate change only (i.e. RCP26 and RCP85) and combined climate/land-use change scenarios, which range from mild (RCP26-LUC.B1) to severe (RCP85-LUC.A2) alterations in climate and land-use. Models projections are generated by three global circulation models and four binarization thresholds. Black/white colours indicate climate change only scenarios, while blue (red) colour tones refer to low- (high-) severity land-use change scenarios. In the box plots, the central line represents the median, while the box comprises the interquartile range (IQR). The upper whisker represents the 3rd quartile + 1.5 IQR while the lower whisker is 1st quartile - 1.5 IQR

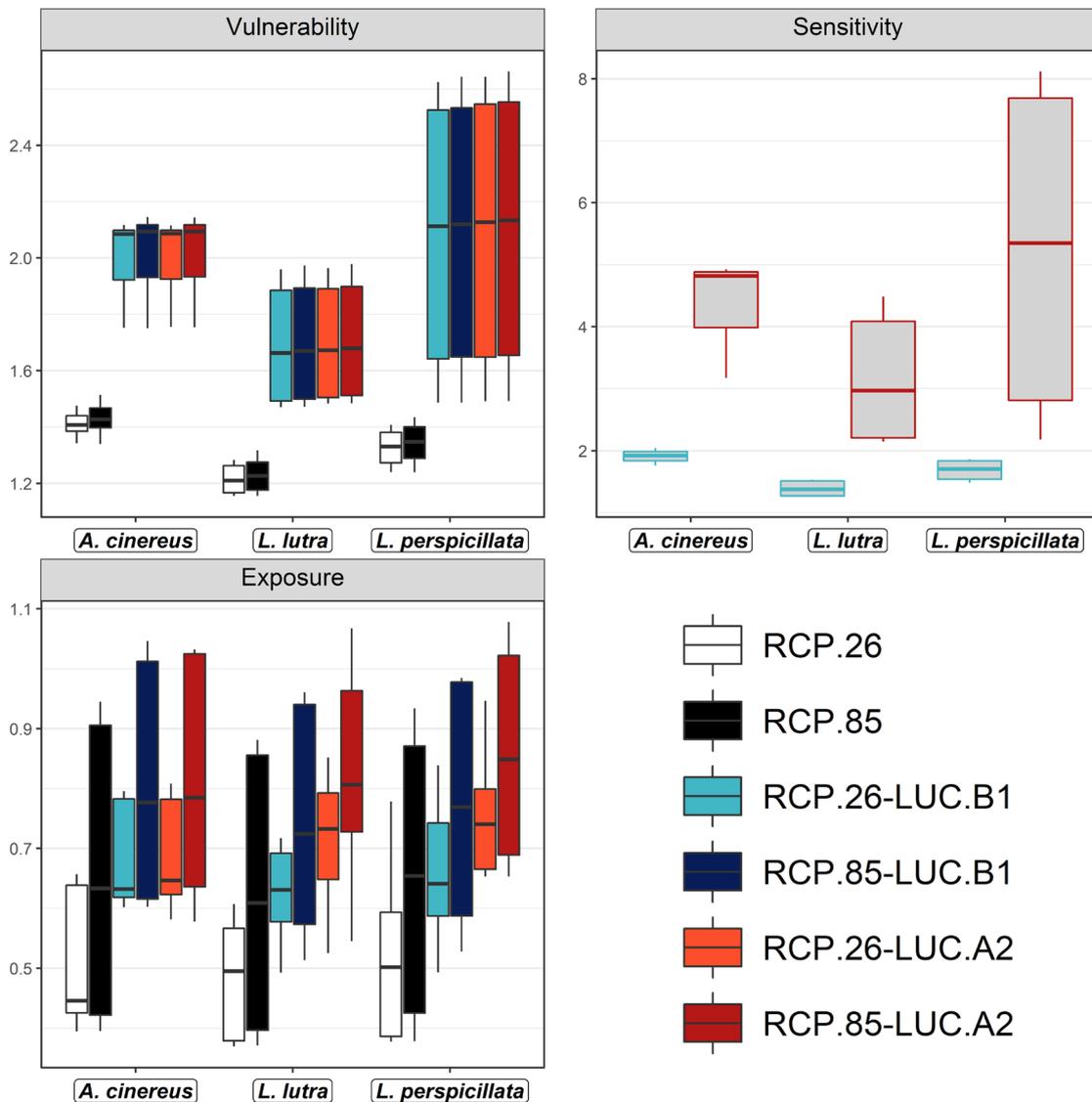


**Figure 3.** Spatially-explicit predictions of otter range modifications under 2050 climate and land-use change scenarios, as generated by the MIROC-ESM global circulation model and the “maximize TSS” binarization threshold. Blue: stable unsuitable habitat, red: habitat loss, yellow: stable suitable habitat, green: habitat gain.

On the contrary, all the three species showed a negative net change in their future distribution under full SDMs, ranging from ca. -6% for *A. cinereus* under RCP.85-LUC.B1 scenario to ca. -15% for *L. lutra* under RCP.85-LUC.A2 (Figure 2 and 3).

Similarly, the three species reported lower range shifts under climate SDMs (from ca. 6% for *L. perspicillata* to 14% for *L. lutra*, both under the RCP.26 scenario; Figure 2) than under full SDMs (from ca. 10% for *A. cinereus* under RCP.26-LUC.B1 to 18% for *L. lutra* under RCP.85-LUC.A2; Figure 2 and 3). Overall, the three species exhibited slight to moderate shifts in range centroids towards south – east and lower elevations under most of the scenarios

(Appendix S6). Moreover, range margins severely shrank for *A. cinereus* along longitude and latitude, while extending mostly northward for the other two species. Elevation range margins shrank for all the species under almost all scenarios (Appendix S6). In general, scenarios including the most severe land-use alterations (i.e. LUC.A2) produced the strongest impact on species distribution, irrespectively of the associated climate change scenario (Figure 2). Such a pattern is particularly evident for *A. cinereus*, where the differences in net change and geographical shift are highest between LUC.B1 and LUC.A2 scenarios and lowest between RCP.26 and RCP.85 scenarios. These differences are less pronounced in the other



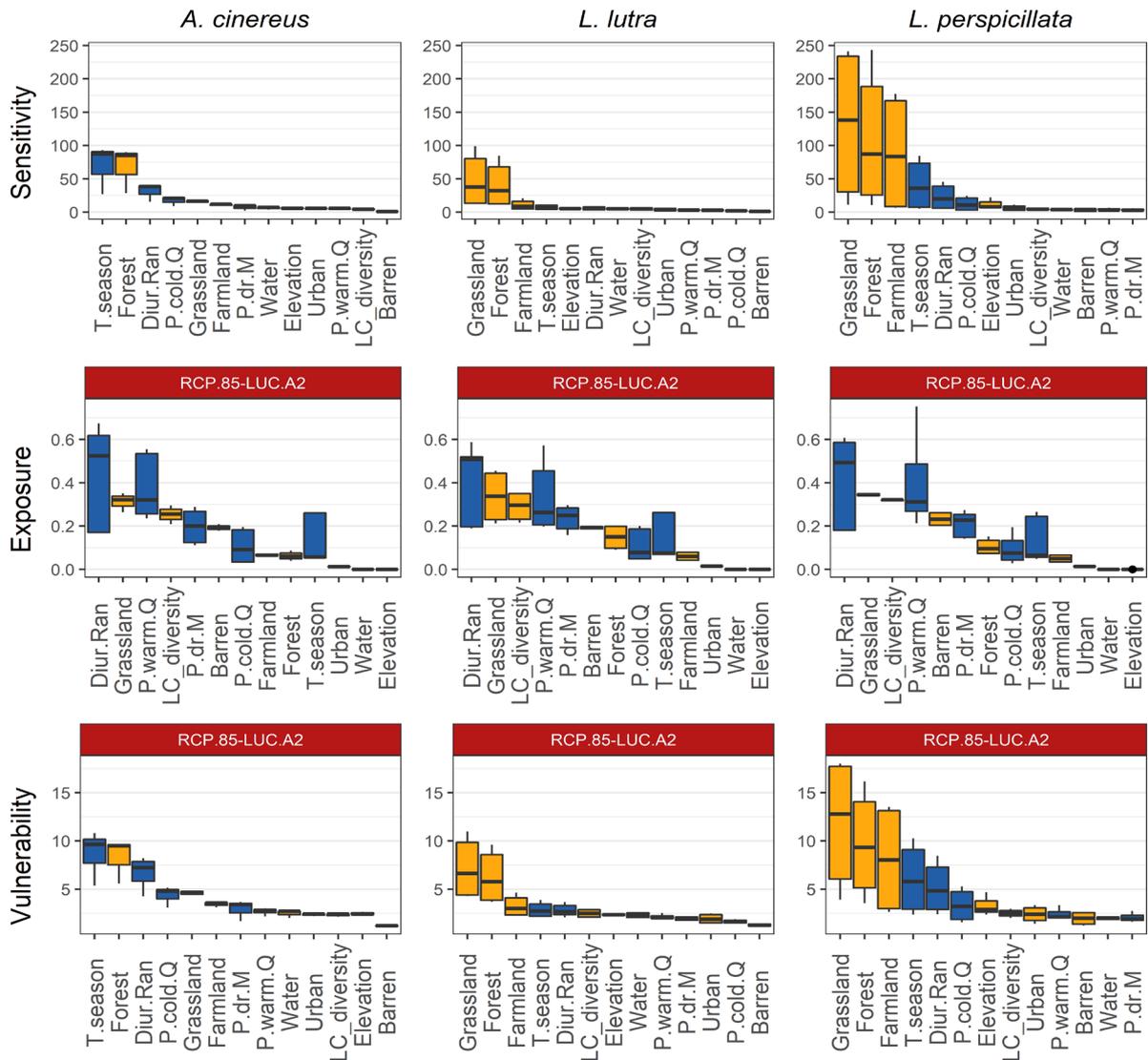
**Figure 4.** Otter total vulnerability, along with species sensitivity and habitat exposure to the six 2050 climate and land-use change scenarios, as generated by three global circulation models and four binarization thresholds. Box plots colours for vulnerability and exposure panels refer to climate change only (i.e. black and white) and combined climate and land-use change scenarios (from blue to red tones), while border colours in box plots for sensitivity refer to such metric calculated on climate and topographic variables only (cyan; as done under climate SDMs) and on the entire predictors set (dark red; as done under full SDMs).

two species, although LUC.A2 scenarios still hold as the most severe ones (Figure 2).

For all the three species, vulnerability values calculated under climate SDMs were largely lower than those computed under full SDMs (Figure 4).

Under climate SDMs, *L. lutra* and *A. cinereus* resulted significantly the least and the most vulnerable species to 2050 climate change, whereas *L. perspicillata* showed intermediate vulnerability scores, though always significantly different from those of the other two species (Figure 4; Table S4). Under full SDMs, *L. lutra* achieved significantly lower vulnerability values than both the other two species, while *L. perspicillata* overcame *A. cinereus* as the most vulnerable species, though their vulnerabilities are not significantly different (Figure 4; Table S4). Exactly the same figures apply to species sensitivity

scores under the two alternative modelling setups, whereas habitat exposure values are rather similar among the three species (Figure 4). The three other species also exhibited different patterns in their vulnerability, sensitivity and exposure as partitioned among each climate and land-use predictor. Under the worst scenario (i.e. RCP85–LUC.A2), both *L. lutra* and *L. perspicillata* showed the highest vulnerability to distance from grasslands and forests, though the magnitude of such an effect was far smaller for the first species. *Anonyx cinereus* was moderately vulnerable to temperature seasonality, distance from forests and mean diurnal range (Figure 5, S10). This pattern follows very closely the sensitivity scores exhibited by the three species. In particular, *L. lutra* exhibited a low sensitivity (i.e. wide tolerance) to several variables (Figure 5, S10), emerging as

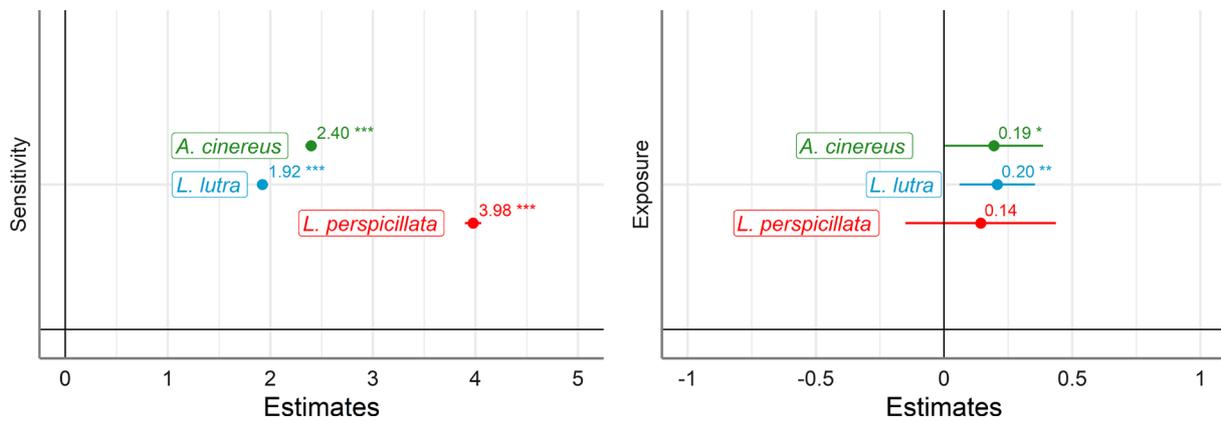


**Figure 5.** Species sensitivity, habitat exposure and vulnerability to 2050 worst scenario (i.e. RCP.85-LUCA2) calculated for each climate and land-use predictor. Box plots include results generated by three global circulation models and four binarization thresholds. Land-use variables are depicted in gold, while climate variables are shown in blue. Variables' complete names and descriptions are provided in Table S1.

the most generalist species. On the other hand, *L. perspicillata* showed a high sensitivity to distance from grasslands and forests, while *A. cinereus* showed a moderate sensitivity to temperature seasonality and distance from forests (Figure 5).

In contrast to sensitivity, the three species exhibited habitat exposure scores of comparable magnitude among each other. In fact, their current habitat is similarly exposed to alterations in

mean diurnal range, precipitation of the warmest quarter and distance from grasslands (Figure 5). Taking together the differential contribution of sensitivity and exposure among global circulation models, global change scenarios and binarization thresholds, LMMs outcomes highlighted sensitivity to exert a higher influence than habitat exposure on vulnerability scores. Although statistically significant for all the species, the preponderant



**Figure 6.** Estimates and statistical significance of the coefficients in the linear mixed effect models fitted between otter vulnerability and sensitivity or exposure, in turn (p value legend: \*= $<0.05$ ; \*\*= $<0.01$ ; \*\*\*= $<0.001$ )

role of sensitivity emerged most clearly for *L. perspicillata* (Figure 6). On the other hand, the statistical relationship between habitat exposure and vulnerability is overall weaker, and not even significant for *L. perspicillata* (Figure 6).

## DISCUSSION

According to our results, future climate and land-use change will exert an overall detrimental effect on the geographic range of the three otter species in the Himalaya, contributing to high vulnerability values. In particular, all three species will undergo moderate to severe shrinking and shift in their distribution under 2050 global change scenarios, especially as predicted by models accounting for both climate and land-use future alterations. In fact, amongst the global change scenarios considered in our assessment, land-use modifications are predicted to exert the most severe effects on all the three species, irrespectively of the associated climate change scenario. Our results allowed us to confirm both the initial hypotheses, reporting far higher vulnerability scores to 2050 global change when including both climate and land-use drivers (hypothesis i), as well as a significantly preponderant effect of niche width rather than habitat exposure (which is overall high and similar

among the species) in determining vulnerability values (hypothesis ii). In keeping with that, the most specialist species, *L. perspicillata*, showed the highest vulnerability as opposed to the most generalist, least vulnerable species, *L. lutra*.

The potential distribution of the three otter species is overall shaped by a mixture of climate and land-use variables. For instance, *A. cinereus* and *L. perspicillata* showed similar environmental preferences, reporting the highest suitability in forest habitats with moderate precipitation during the warmest season. Such outcomes are in keeping with the well-known preference of the two species for moist forests (e.g., mangrove or peat swamp forests; Duplaix & Savage, 2018; Foster-Turley, 1992; Gupta et al., 2020). Moreover, *L. perspicillata* and *A. cinereus* respectively feed on fish and invertebrates (i.e. crustaceans and shellfish; Anoop & Hussain, 2005; Duplaix & Savage, 2018; Foster-Turley & Markowitz, 1982; Kanchanasaka & Duplaix, 2011), whose abundance in freshwater streams can be altered by scarce (i.e. drought) or extreme (i.e. floods) rainfall regimes (Chea et al., 2020; Gratwicke, 2004; Milner et al., 2013). This evidence further confirms the importance of precipitation-related variables in shaping the distribution of these two species. Particularly

for *A. cinereus*, we also predicted a marked effect of temperature fluctuations, as indicated by the high importance scores of seasonality. Although with a lower magnitude, this outcome is apparent also for *L. perspicillata*, where mean diurnal range and temperature seasonality emerged among the most important predictors. In fact, the two species shows the highest suitability in overall thermally stable habitats. This pattern partly recalls the evidence provided by Cianfrani et al. (2018), which showed *A. cinereus*, and to a lesser extent also *L. perspicillata*, to be affected by temperature fluctuations, although mostly in terms of thermal diurnal range. Grassland and forest habitats with moderate precipitation during the driest month emerged as the most preferred environments by *L. lutra*. These outcomes are largely consistent with previous investigations of this species. For instance, the role of herbaceous and forested habitats as resting and reproduction sites for *L. lutra* also emerged in Cianfrani et al. (2010), in keeping with previous similar evidence (e.g., Beja, 1996; Prenda et al., 2001; Prenda & Granado-Lorencio, 1996). Moreover, Cianfrani et al. (2011) and Cianfrani et al. (2018) carried out modelling studies reporting precipitation during the driest month among the most important drivers affecting *L. lutra* geographic distribution. The relevance of this predictor is likely linked to its effects on hydrological regimes and river characteristics, which in turn affect *L. lutra* habitat availability, especially during the drought season (Kruuk, 2006; Scorpio et al., 2016). Overall, elevation also contributed to shape otter distribution, especially for *L. lutra*. While this species exhibited moderate to high suitability values along a wide altitudinal gradient, confirming its environmental flexibility (e.g., Kruuk, 2006; Remonti et al., 2009), for *A. cinereus* suitability sharply decreased toward high elevations (Duplaix & Savage, 2018), although we cannot entirely exclude such pattern to be altered by a lower sampling effort at higher elevation, or by competitive exclusion possibly exerted by *L. lutra*. Raha & Hussain (2016), exploring the habitat use of the three species in Western Ghats (i.e. a tropical plain and mountain area in Southwestern India), found *L. perspicillata* and *L. lutra* to prefer dense forested areas at low to intermediate elevations, while *A. cinereus* tends to occur in waterbodies at higher elevations surrounded by dense grassland. In fact, such evidence is only partly coherent with our findings, though it is important to remark that our study context (i.e. the Himalayan chain) is really different than the area investigated by Raha & Hussain (2016). In addition, such partial

inconsistencies may depend upon the great variability of habitat types the three species are known to use throughout their range (Duplaix & Savage, 2018).

Interestingly, our predictions showed land-use change to remarkably override the future role played by climate change. In fact, A2 land-use change scenario was systematically associated with the severest range contraction and shift in all the three species, whether in combination with low- or high-intensity climate change scenarios. Under A2 scenarios, Himalayan landscape will increase farmlands and more urbanized areas in lieu of forests and grasslands (Figure S2), therefore impacting two of the most important land-use categories driving otter distribution (Figure S4). Despite a noticeable scarcity of studies designed to forecast both climate and land-use change impacts on species distribution (Titeux et al., 2017), a pattern where land-use change effects overtake those by climate change has been observed several times (Sirami et al., 2017). For instance, Eglington & Pearce-Higgins (2012) pointed out how long-term trends in UK bird populations have been predominantly affected by alterations in land-use intensity rather than by climate change. An even stronger divergence between the effects of these two global change drivers was showed by Di Febbraro et al. (2019), which predicted future climate change to ease invasion risk by alien squirrels as opposed to the strongly detrimental effect played by land-use alterations. The importance of considering both climate and land-use in predictive studies on biodiversity has been repeatedly underlined (Sirami et al., 2017; Titeux et al., 2017), though it started to be adequately accounted for only in rather recent times (e.g., Newbold, 2018; Peters et al., 2019). It is well-established that modelling species response to climate change only may provide substantially different predictions compared to those including land-use change as well, thus hampering adequate conservation recommendations (Di Febbraro et al., 2019; Sirami et al., 2017). This evidence substantially explains why the outcomes from full SDMs are largely divergent (and overall, more accurate) from those yielded by climate SDMs, as well as by other studies that analyzed otter future distribution exclusively under climate change scenarios. For instance, Cianfrani et al. (2018) predicted a loss in global suitable area of -5% to -20% for *L. perspicillata*. While such prediction largely coincides with our results under climate SDMs, we reported a severer loss (-7% to -25%) under high-intensity land-use

change scenarios. Even, Cianfrani et al. (2018) showed an overall increase in suitable area under climate change for *L. lutra* (+10% to +11%), whereas we predicted a systematical loss for this species, especially under the high-intensity land-use change scenarios. That said, we cannot exclude that such inconsistencies may partly depend on the different geographical extent adopted in Cianfrani et al. (2018; i.e. at a global scale) with respect to our study setup. It is also noteworthy that we focused on the Himalayan region, which encompasses the margins of the altitudinal ranges of the three species. Accordingly, resident otters might present local adaptations to mountain environments, thus likely being more susceptible to climate and land-use alterations if compared to lowland populations. This pattern seems particularly plausible for *L. lutra*, whose several subspecies are distributed in discrete spatial enclaves, especially along the Himalaya (Hung & Law, 2016). In fact, a solid body of literature has shown that there can be important geographical intraspecific variation in the sensitivity and response to changing environmental conditions (e.g., Nice et al., 2019; Peterson et al., 2019). Such intraspecific variation in environmental requirements substantially derives from local adaptation or adaptive plasticity along the gradient of environmental conditions (Macdonald et al., 2018). Consequently, predictions of species response to global change that neglect to account for such intraspecific differences may strongly diverge from those generated from local partitions of species distributions (Martin et al., 2020). This evidence could further explain the difference between our predictions of otter future distribution and those provided by Cianfrani et al. (2018).

The evaluation of otter vulnerability determinants showed species sensitivity (i.e., a measure of niche width) to play the most important contribution, whereas habitat exposure effect was mostly negligible for all the three species. This pattern emerges even more clearly by looking at how the three species are ranked according to sensitivity, exposure and vulnerability. Despite reporting an intermediate to high exposure values, *L. lutra* was ranked as the least vulnerable species, by virtue of its lowest sensitivity. Analogously, *A. cinereus* scored a median vulnerability ca. 30% higher than *L. lutra*, though these two species showed largely similar exposure values. Such outcomes confirm well-documented evidence that habitat exposure, while being the most used metric in vulnerability assessments (Foden et al., 2019),

is not necessarily consistent with niche measures of species intrinsic sensitivity (Dickinson et al., 2014). This tendency was also highlighted for otters within the global scale vulnerability assessment performed by Cianfrani et al. (2018). As for the three species we focused on, such assessment reported *L. lutra* and *L. perspicillata* as the least and the most vulnerable species, respectively. Interestingly, although Cianfrani et al. (2018) showed a higher exposure for *A. cinereus* than for *L. perspicillata* (in contrast to our results), the latter was consistently more vulnerable by virtue of a far higher sensitivity. This body of evidence substantially emphasizes the importance of assessing vulnerability by including trait-based elements, e.g., measures of niche width, into “classical” correlative approaches usually adopted to predict range dynamics (Foden et al., 2019; Pacifici et al., 2015).

As for the main limitations of this study, we did not consider the third component usually included in the CCVA theoretical framework, i.e. the adaptive capacity (Foden et al., 2019). Such ability to adjust to environmental change should be inferred by traits as e.g., phenotypic plasticity or dispersal abilities, which are often unavailable for many species. This lack of information makes adaptive capacity the most challenging component to be quantified in CCVAs, which often simply neglect it (Cianfrani et al., 2018; Foden et al., 2019). It is worth mentioning that species sensitivity and habitat exposure can be given different weights as to tune their relative contribution to the final vulnerability score (Rinnan & Lawler, 2019). Although the way such weights are defined should bias, at least in theory, the vulnerability score toward sensitivity or exposure, Cianfrani et al. (2018) designed a specific analysis showing that different weighting schemes on the single vulnerability components altered the final score only marginally (especially for the three species analyzed here). Furthermore, we were interested in quantifying the relative contribution of sensitivity and exposure on vulnerability accounting for several variability sources (e.g., global circulation models, global change scenario and binarization thresholds). Accordingly, we found the most obvious choice was to assign the same weight (i.e., 1) to both vulnerability components, instead of artificially introducing any weighting scheme that lacked of a strong ecological rationale. Lastly, we did not include any projection of climatic extreme events (e.g., droughts or floods) in our vulnerability assessment. Despite a well-established evidence that such events will likely have stronger effects on ecological systems than simple shifts in mean

values (R. M. Thompson et al., 2013), modelling their frequency and occurrence is particularly difficult due to their rarity and stochastic nature. As a result, such phenomena have usually been neglected in conservation studies until very recently (e.g., Palmer et al., 2017).

### CONCLUSIONS

Notwithstanding these drawbacks, our study underlines how coupling climate and land-use change in CCVAs can generate profoundly diverging results compared to approaches relying on solely climate change. Such ever-mounting evidence appears particularly relevant both to reassess more cautiously the species ranked as insensitive or favored by climate change (e.g., *L. lutra*), and, even more, to increase attention on those species (e.g., African otters *A. congicus* and *A. capensis*) that were already classified as highly vulnerable under climate change scenarios only (Cianfrani et al., 2018). The outcomes of this study also highlight

the potentialities of performing CCVAs through a “combined” framework, thus emphasizing the importance of including trait-based elements (e.g., species sensitivity) into purely correlative approaches (Foden et al., 2019; Pacifici et al., 2015). In fact, such intrinsic elements proved significantly more important in determining species vulnerability than extrinsic metrics such as habitat exposure. Our analyses show that such a pattern still holds when accounting for several variability sources usually linked to correlative models (e.g., alternative global circulation models and binarization schemes). Future research efforts should focus on developing tools to quantify adaptive capacity in a more reliable and generalizable way than currently available approaches (Williams et al., 2008). Including this “great absent” in CCVAs, along with a deeper focus on modelling climate variability and extreme events, will surely bring this conservation tool toward a more advanced level.

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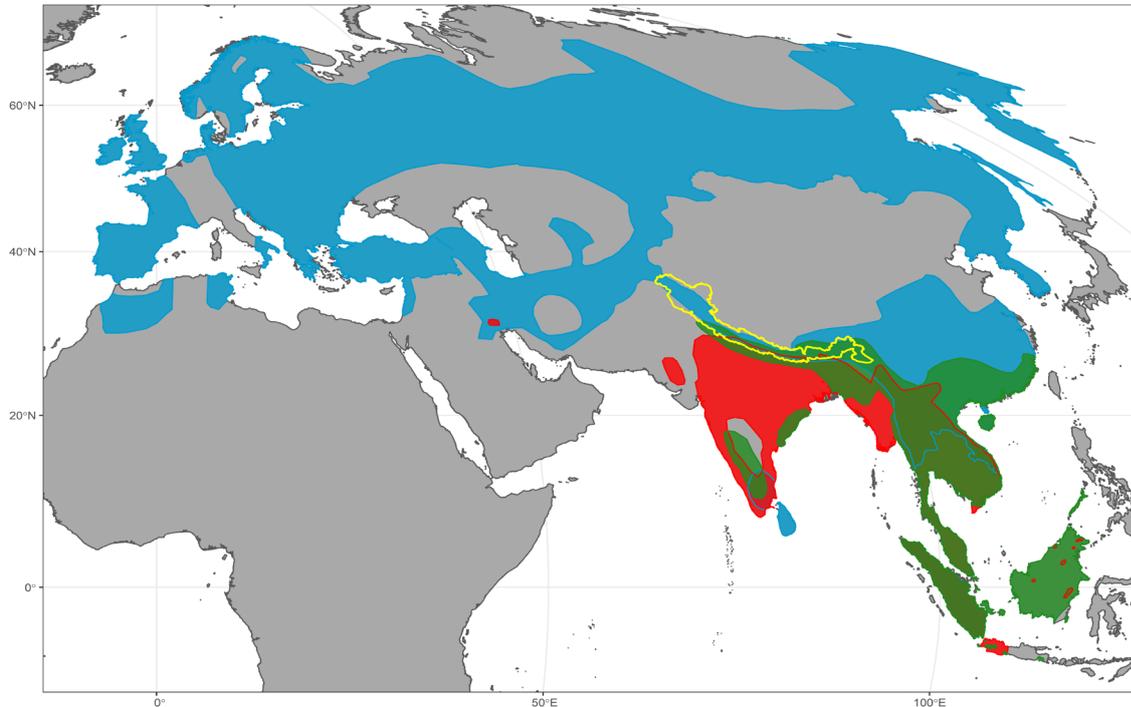
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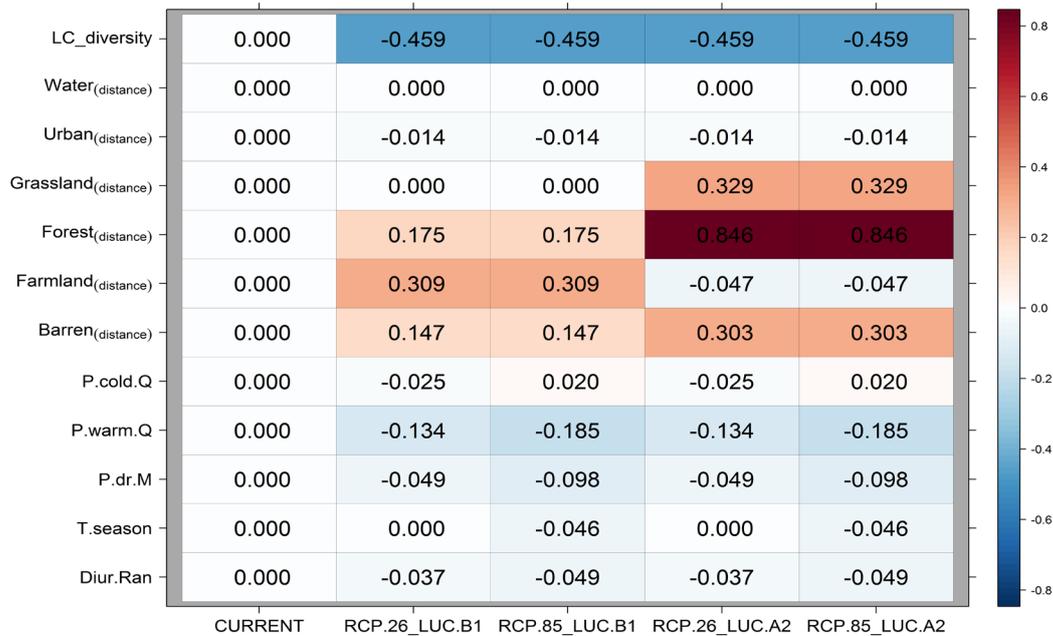
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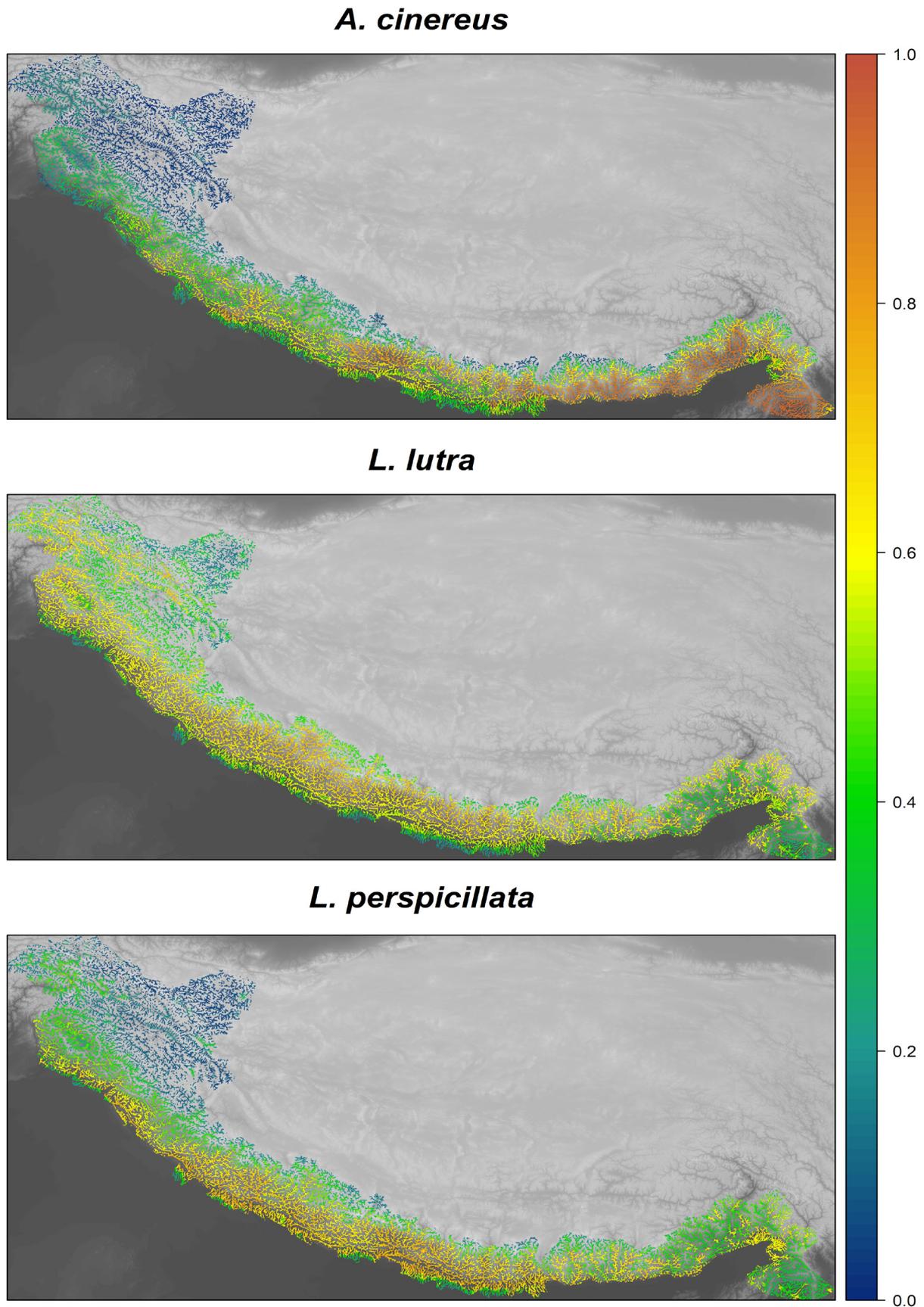
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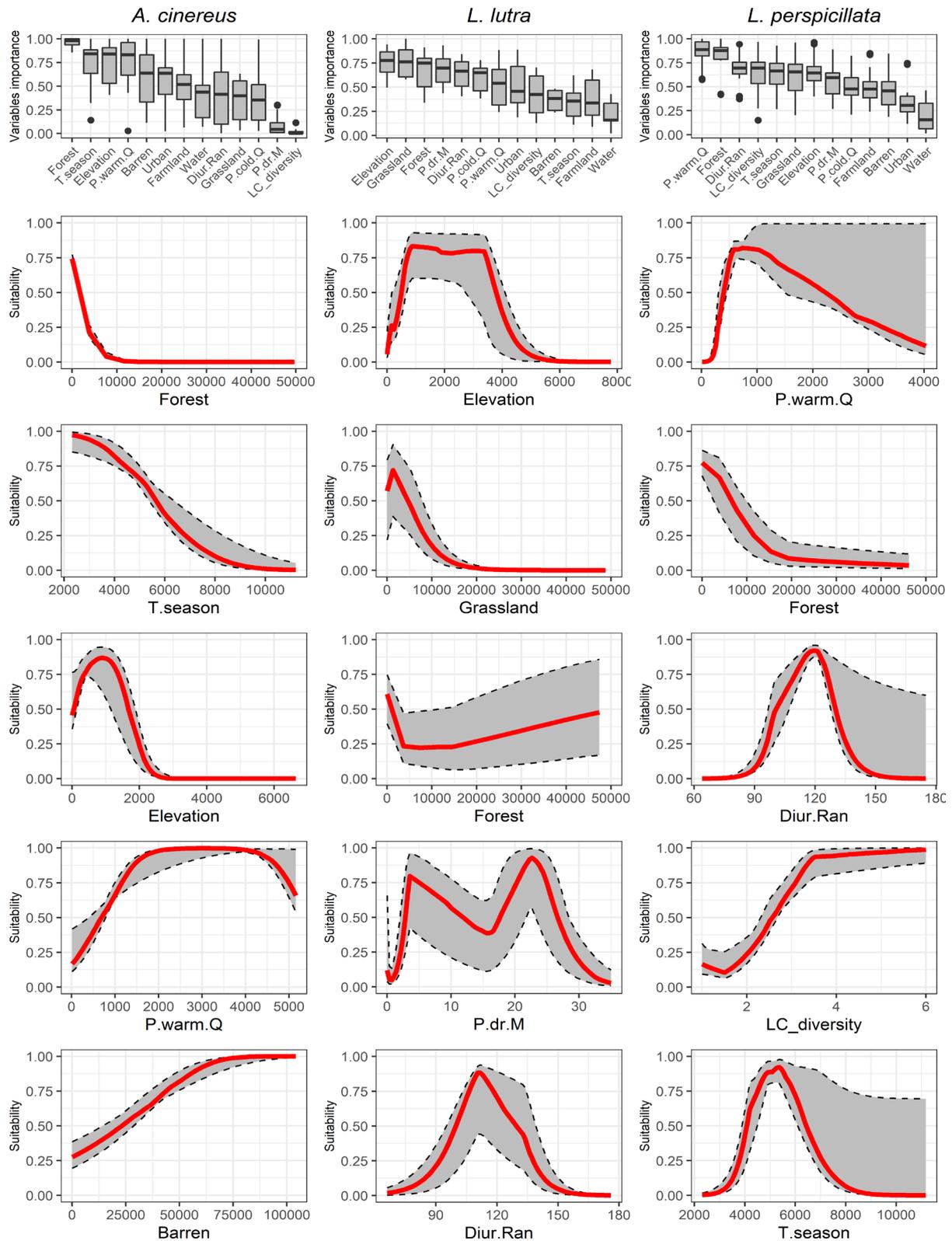
**Figure S1.** IUCN range maps of the three analyzed otter species: *Aonyx cinereus* (green polygons), *Lutra lutra* (blue polygons) and *Lutrogale perspicillata* (red polygons). Yellow line delimits the boundaries of the study area



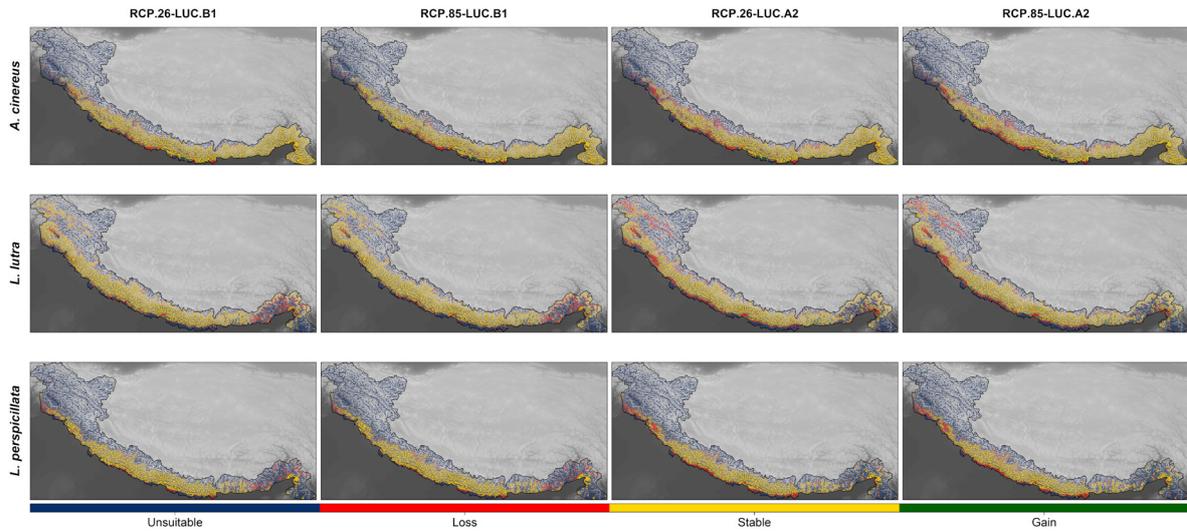
**Figure S2.** Temporal trends of the covariates (rows) used in SDMs under the four combined climate and land-use change scenarios (columns). The trends are quantified as the standardized difference between the medians of the pixel values of each predictor in future scenarios and current time. A negative value (blue tones) for a given predictor indicates a future reduction of the median pixel value for that variable, while a positive value (red tones) indicates a future increase. Importantly, since land-use variables are quantified as Euclidean distances from land-use categories, a negative value for one of these predictors (except LC\_diversity) indicates a future decrease in such distance, i.e. an increase in the cover of the corresponding land-use category



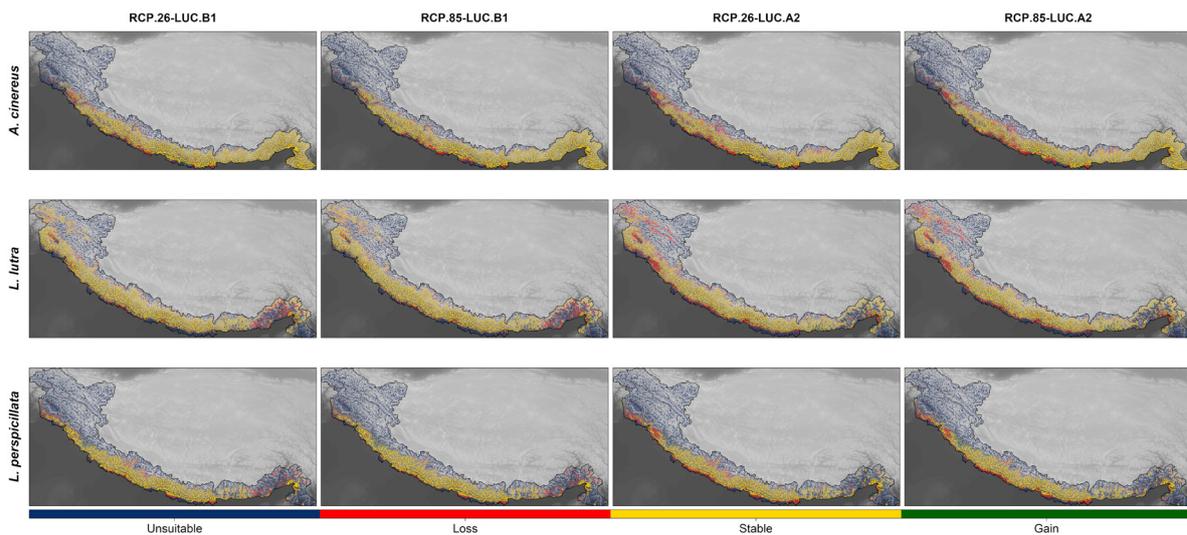
**Figure S3.** Present-day occurrence probability of the three otter species as predicted by full SDMs



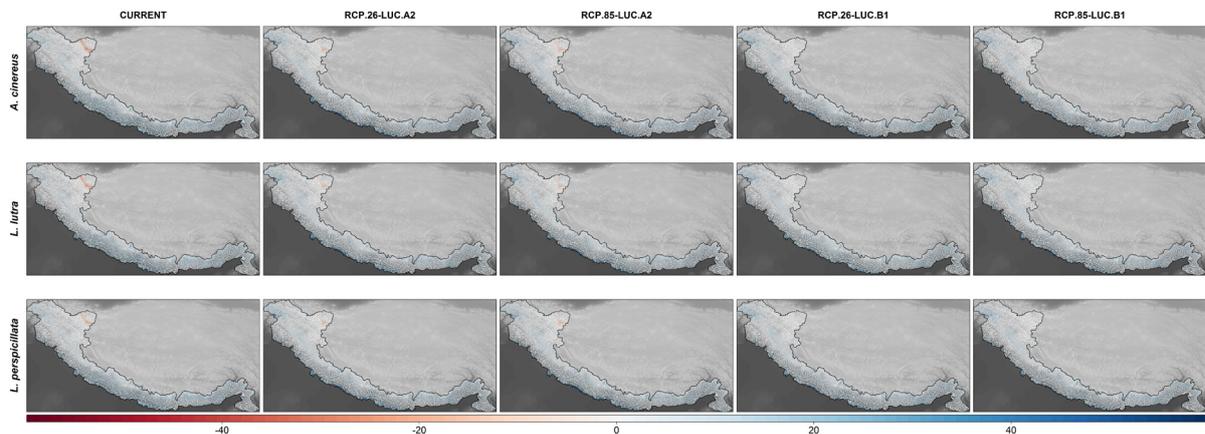
**Figure S4.** Relative contribution and related response curves of each predictor used in the SDMs calibration. Variables in the box plots are listed in order of importance from left (high importance) to right (low importance). Similarly, the predictors' response curves, which depict the variation of the occurrence probability versus the first five most important variables, are sorted from top to bottom according to their relative importance to the models. The grey region around the response curves refer to the 95% confidence interval of the estimate.



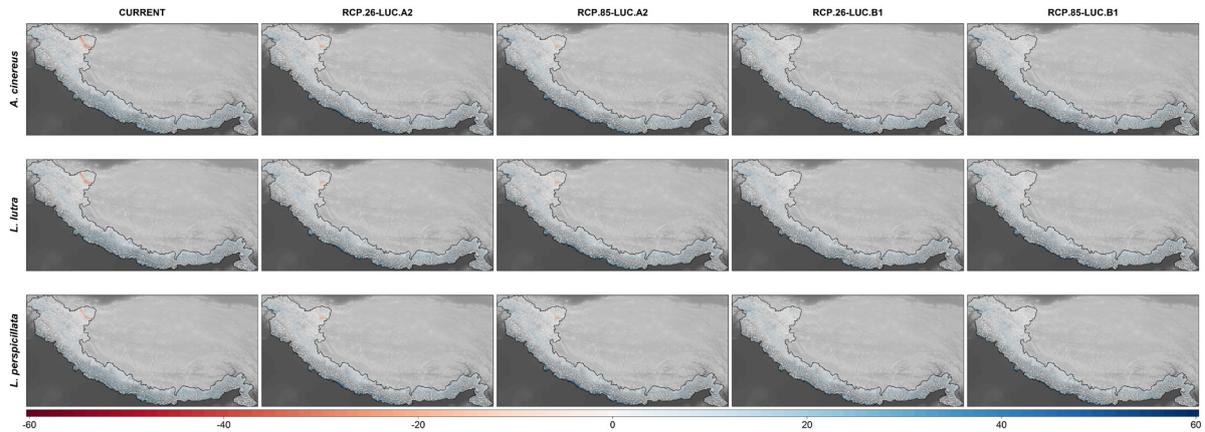
**Figure S5.** Spatially-explicit predictions of otter range modifications under 2050 climate and land-use change scenarios, as generated by the CCSM4 global circulation model and the “maximize TSS” binarization threshold. Blue: stable unsuitable habitat, red: habitat loss, yellow: stable suitable habitat, green: habitat gain.



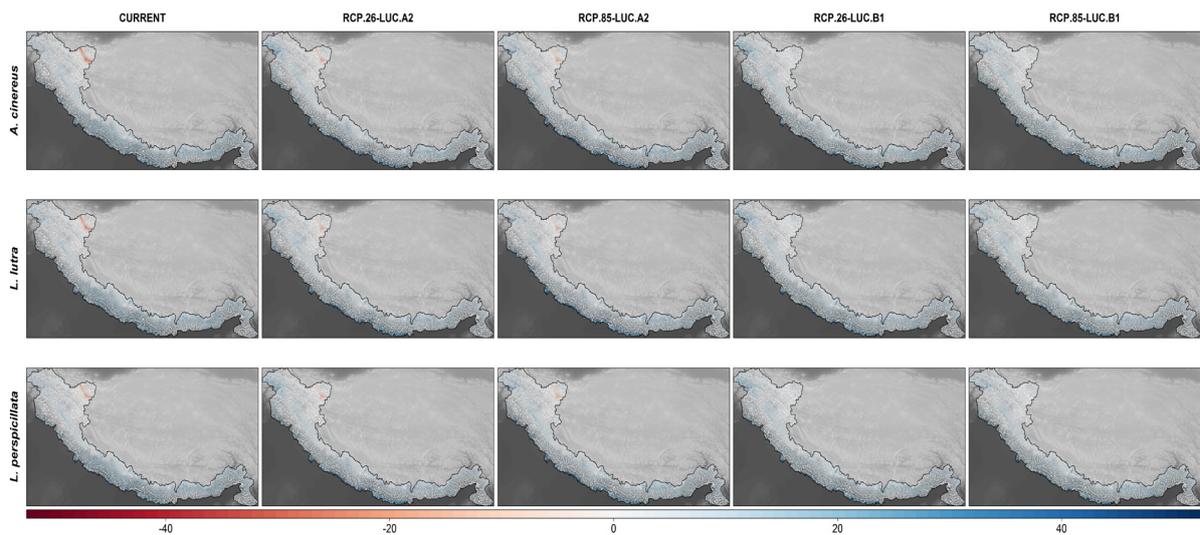
**Figure S6.** Spatially-explicit predictions of otter range modifications under 2050 climate and land-use change scenarios, as generated by the CNRM-CM5 global circulation model and the “maximize TSS” binarization threshold. Blue: stable unsuitable habitat, red: habitat loss, yellow: stable suitable habitat, green: habitat gain.



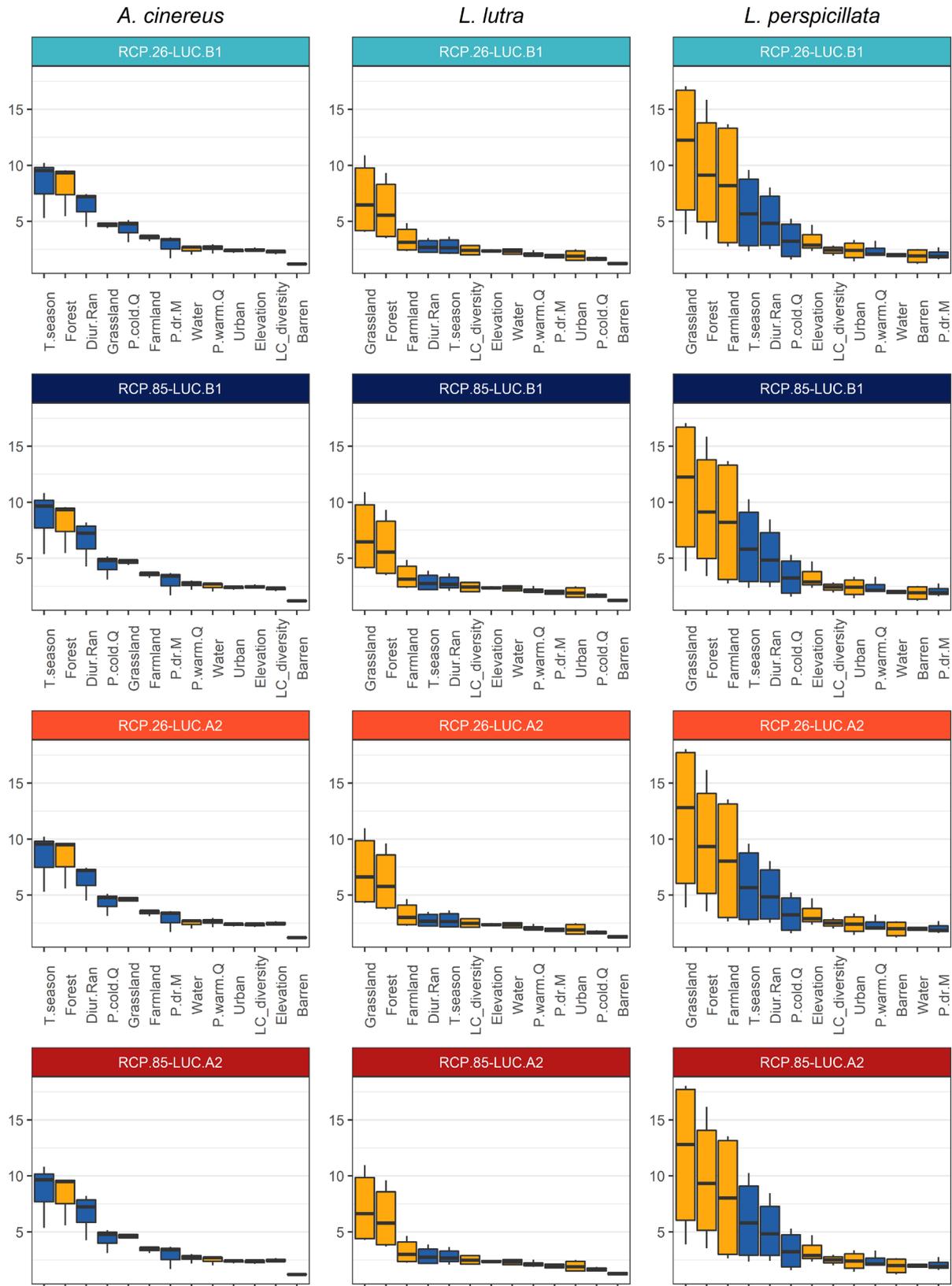
**Figure S7.** MESS results for SDM predictions under the CCSM4 global circulation model. Blue colors indicate no extrapolation, while red colors indicate strong extrapolation



**Figure S8.** MESS results for SDMs predictions under the CNRM-CM5 global circulation model. Blue colors indicate no extrapolation, while red colors indicate strong extrapolation



**Figure S9.** MESS results for SDMs predictions under the MIROC-ESM global circulation model. Blue colors indicate no extrapolation, while red colors indicate strong extrapolation



**Figure S10.** Species vulnerability to the four 2050 climate and land-use change scenarios calculated for each predictor. Box plots include results generated by three global circulation models and four binarization thresholds. Land-use variables are depicted in gold, while climate variables are shown in blue. Variables complete names and descriptions are provided in Table S1

**SUPPLEMENTARY MATERIAL**  
**SUPPLEMENTARY FIGURES CAPTIONS**

**Table S1.** List of variables used in SDMs calibration, along with their acronyms as depicted in figures and tables, units, sources and type.

Variable	Acronym	Unit	Source	Type
Elevation	<i>Elevation</i>	m a.s.l.	(Jarvis, Reuter, Nelson, & Guevara, 2008)	Topography
Mean diurnal range	<i>Diur.Ran</i>	°C * 10	(Hijmans, Cameron, Parra, Jones, & Jarvis, 2005)	Climate
Temperature seasonality	<i>T.season</i>	°C * 10	(Hijmans et al., 2005)	Climate
Precipitation of driest month	<i>P.dr.M</i>	mm	(Hijmans et al., 2005)	Climate
Precipitation of warmest quarter	<i>P.warm.Q</i>	mm	(Hijmans et al., 2005)	Climate
Precipitation of coldest quarter	<i>P.cold.Q</i>	mm	(Hijmans et al., 2005)	Climate
Alpha diversity of land-use categories within a 10 km-radius window	<i>LC_diversity</i>	count	(Li et al., 2017)	Land-use
Euclidean distance from farmlands	<i>Farmlands</i>	m	(Li et al., 2017)	Land-use
Euclidean distance from forests	<i>Forests</i>	m	(Li et al., 2017)	Land-use
Euclidean distance from grasslands	<i>Grasslands</i>	m	(Li et al., 2017)	Land-use
Euclidean distance from urban areas	<i>Urban</i>	m	(Li et al., 2017)	Land-use
Euclidean distance from barren lands	<i>Barren</i>	m	(Li et al., 2017)	Land-use
Euclidean distance from water bodies	<i>Water</i>	m	(Li et al., 2017)	Land-use

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**Table S2.** Characteristics of 2050 climate and land-use scenarios used in SDMs projections.

Scenario	Main characteristics
RCP.26	The scenario assumes that global annual CO <sub>2</sub> emissions will peak between 2010–2020, declining substantially thereafter. A +1 °C global warming is forecasted (IPCC, 2013).
RCP.85	The scenario assumes CO <sub>2</sub> emissions keep rising throughout the 21st century. A +2 °C global warming is forecasted (IPCC, 2013).
LUC.A2	The scenario assumes a high population growth, a sprawling urban expansion and a medium economic growth. As a result, the scenario predicts an increase in farmlands and a decrease in grasslands and forests (Li et al., 2017).
LUC.B1	The scenario assumes a low population growth, a compact urban expansion and a high economic growth. As a result, the scenario predicts a decrease in farmlands and an increase in grasslands and forests (Li et al., 2017).

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**Table S3:** Predictive performance of climate and full SDMs assessed through “block” cross-validation.

Species	Climate Species Distribution Models		Full Species Distribution Models	
	AUC	TSS	AUC	TSS
<i>A. cinereus</i>	0.779 ± 0.029	0.554 ± 0.092	0.894 ± 0.029	0.762 ± 0.077
<i>L. lutra</i>	0.696 ± 0.032	0.363 ± 0.029	0.833 ± 0.057	0.566 ± 0.120
<i>L. perspicillata</i>	0.823 ± 0.026	0.537 ± 0.057	0.893 ± 0.021	0.709 ± 0.082

**Table S4.** Results of ANOVA and Tukey post-hoc test comparing mean vulnerability values among species for each climate and land-use change scenario (p value legend: \*=<0.05; \*\*=<0.01; \*\*\*=<0.001).

Scenario	ANOVA sum of squares	Tukey pairwise comparison	Difference in mean vulnerability
RCP26	0.22***	<i>L. lutra</i> – <i>A. cinereus</i>	-0.19***
		<i>L. perspicillata</i> – <i>A. cinereus</i>	-0.08**
		<i>L. perspicillata</i> – <i>L. lutra</i>	0.11***
RCP85	0.23***	<i>L. lutra</i> – <i>A. cinereus</i>	-0.20***
		<i>L. perspicillata</i> – <i>A. cinereus</i>	-0.08**
		<i>L. perspicillata</i> – <i>L. lutra</i>	0.11***
RCP26-LUC.B1	1.06**	<i>L. lutra</i> – <i>A. cinereus</i>	-0.32*
		<i>L. perspicillata</i> – <i>A. cinereus</i>	0.07
		<i>L. perspicillata</i> – <i>L. lutra</i>	0.40*
RCP85-LUC.B1	1.07**	<i>L. lutra</i> – <i>A. cinereus</i>	-0.33*
		<i>L. perspicillata</i> – <i>A. cinereus</i>	0.07
		<i>L. perspicillata</i> – <i>L. lutra</i>	0.40*
RCP26-LUC.A2	1.06*	<i>L. lutra</i> – <i>A. cinereus</i>	-0.31*
		<i>L. perspicillata</i> – <i>A. cinereus</i>	0.09
		<i>L. perspicillata</i> – <i>L. lutra</i>	0.40*



# Global assessment of ecological niche overlap, similarity and potential distribution, in three otter species: *Lutra lutra*, *Aonyx cinereus* and *Lutrogale perspicillata* across the ranges

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## INTRODUCTION

The study of species niche and geographical distribution has held an important place in biology (Brown, 1984; Grinnell, 1917; Hutchinson GE, 1957). It improves our understanding of natural history for taxa, and helps in ecological, biogeographical, and evolutionary studies (Sagarin et al., 2006). Also, the capacity to estimate species distributions has proven critical to species conservation and land management (Ferrier, 2002; Scott & Csuti, 1997). Resource selection for a species is a hierarchical process that includes behavioural responses to certain environmental features. (Horne et al., 2008; Vanak & Gompper, 2010). Habitat selection at the distribution range level is the first order habitat selection (Milleret et al., 2019). With increased access to statistical and digital mapping tools, niche dimensions are increasingly fundamental to ecological biogeography, focusing on ecological communities' spatial designs (Devictor et al., 2010; Lomolino et al., 2006). The crucial factor that determines the potential distribution of a species is its fundamental niche (Hutchinson GE, 1957), which also plays the main role in its evolutionary changes (Voje et al., 2015). In the last two decades, the area of distribution modelling has grown significantly,

creating a plethora of literature and techniques for field overviews (Guisan & Zimmermann, 2000), (Guisan & Thuiller, 2005). Understanding areas where appropriate environmental circumstances sustain the survival of a species is crucial for ecological, evolutionary, and conservation studies of a species (Evans et al., 2009). Also, estimation of niche overlap has become an important tool for investigating species coexistence (Gregory & Macdonald, 2009), evolutionary diversification (Araya et al., 2011), and conservation strategies (Russo et al., 2015a). In fact, species' potential distribution based on fundamental niche is seldom realized, as biological interactions constrain it to the portions of the niche that can be utilized, namely the realized niche, resulting in a geographically constrained distribution (Anderson et al., 2002; Jiménez-Valverde et al., 2008). According to the competitive exclusion principle (Hardin, 1960), coexisting species must vary in some element of resource utilization. The "ghost of competition past" forecasts the coexistence of species with a relatively minimal overlap in the use of resources (Connell, 1980). Alternatively, if there is competition for limited resources, there should be significant overlap in the niches of two species.

Ecological Niche Modelling (ENM, (Anderson, 2012) is a powerful method for characterizing broad-scale environmental conditions under which species populations occur (Guisan et al., 2017). ENM techniques integrate species occurrence records with digital environmental data to predict Grinnellian niches and corresponding geographic distributions (Guisan & Zimmermann, 2000; Peterson, 2011).

Otters are considered bio-indicators of the wetland ecosystems they live in (Ruiz-Olmo et al., 1998). They play essential roles in many processes that regulate ecosystems (Ben David et al. 1998), and thus, any perturbation by hunting, climate change, in these ecosystems is first reflected in the distribution and status of the otter populations of the area (Monson et al., 2000) (Ben-David et al., 2001).

In the present study, we quantified the ecological niche overlap of three Eurasian species of otters, i.e. the Eurasian otter (*L. lutra*), the Smooth-coated otter (*L. perspicillata*), and the Small-clawed otter (*Aonyx cinereus*). Their geographic ranges overlap in Asia (Duplaix & Savage, 2018), and the three species are known to be sympatric in some parts of India (Raha & Hussain, 2016) and Southeast Asia (Kruuk et al., 1994), (Foster-turley et al., 1990). The IUCN Red List of Threatened Species includes the Near Threatened Eurasian otter (*L. lutra*), the Vulnerable Smooth-coated otter (*L. perspicillata*), and the Vulnerable Small-clawed otter (*Aonyx cinereus*). *L. perspicillata*

and *A. cinereus* decline is caused by habitat loss and exploitation (de Silva et al., 2015;Wright et al., 2015), whereas the Asian population of *Lutra lutra* attributed to the species' sensitivity to recent intensification of human-induced threats (Roos et al., 2015).

Understanding the environmental conditions that promote their survival and coexistence will be critical to conservation success, especially in human-dominated settings (Karanth et al., 2010).

Our study's goal was to model the most recent global distribution of *L. lutra*, *A. cinereus*, and *L. perspicillata*, and measure the extent of niche overlap and similarity among each species pair combination. Specific objectives were: i) to update the actual range of occurrence of three species ii) to quantify the niche of each species based on environmental predictors related to otter's ecological requirements iii) to quantify the niche overlap and similarity between each combination of species pairs, iv) to predict the potential distribution of each species, v) to detect any discrepancy between the actual and potential distribution range of each species that could be interpreted as evidence of competitive exclusion. Outcomes will likely assist conservation biologists and land managers in understanding the critical features for both population persistence, connectivity, as well as understanding whether interacting species may be able to coexist in the same geographical areas, i.e. the distribution and persistence of species hotspots (Morelli et al., 2016).

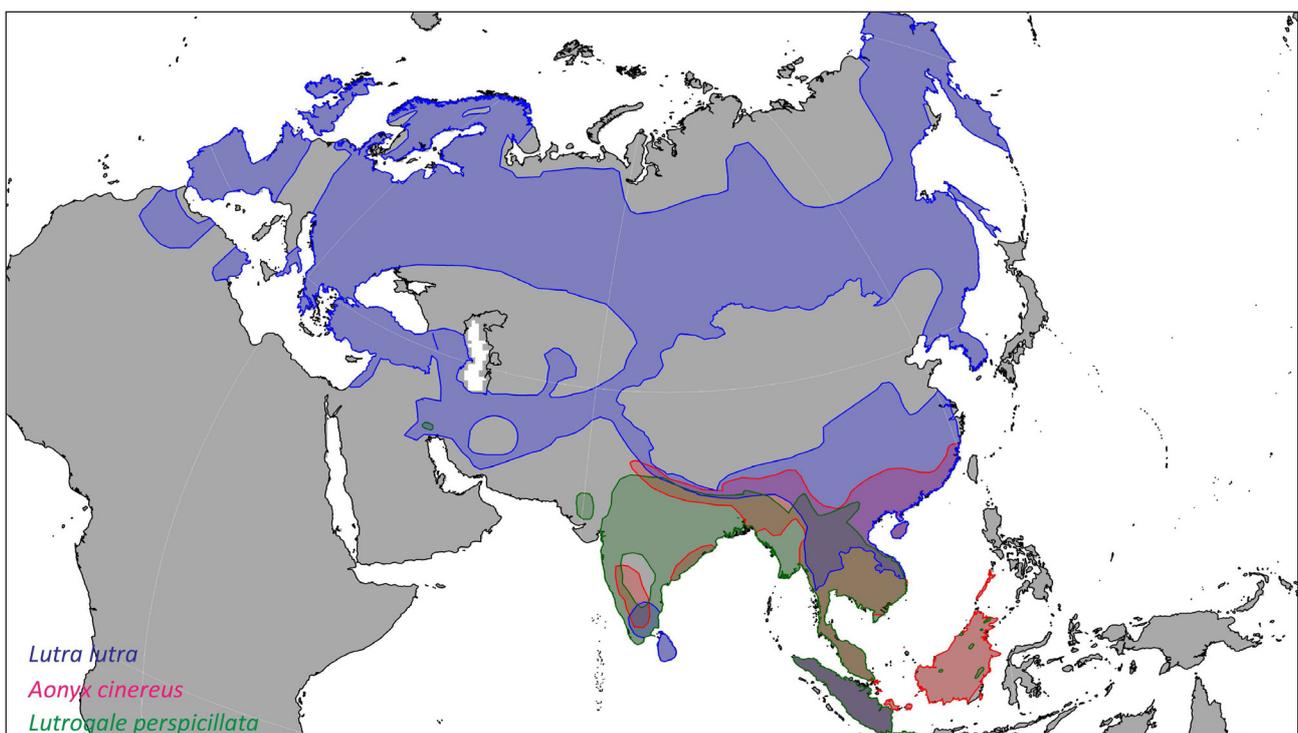


Fig 1. Study area showing the distribution range of the three otter species

## METHODS

### 2.1. Occurrence data

To gather species occurrences across their whole range, we first searched through journals and online sources, such as the Global Biodiversity Information Facility (GBIF; <https://www.gbif.org>) and iNaturalist (<https://www.inaturalist.org>). We removed occurrences older than 2000 and those likely related to captive specimens. Following (Yannic et al., 2014), we expanded the dataset by adding 10000 occurrences selected randomly within the polygons of the IUCN range map of each species. To minimize the impact of sampling bias, we used the spatial thinning algorithm implemented in the spThin R package (Aiello-Lammens et al., 2015) to filter the dataset used for analysis (Table 1), finally obtaining 1743, 114, 108 occurrence data for *L. lutra*, *L. perspicillata*, and *A. cinereus*, respectively.

### 2.2. Environmental variables

As environmental variables, we considered 19 bioclimatic predictors derived from the WORLDCLIM database, along with Normalized Difference Vegetation Index (NDVI) and Net Primary Productivity (NPP) gathered from MODIS covering the 2000-2017 period, flow accumulation (Lehner et al., 2008) and elevation (Jarvis et al., 2008). All the predictors were rasterized at a spatial resolution of 20 km. We checked for predictors multicollinearity by setting a variance inflation factor  $\leq 7$  (Zuur et al., 2010).

### 2.3 Species Distribution Models

The distribution of the three species were modelled using an ensemble forecasting approach within the biomod2 R package (Thuiller et al., 2009), which averaged the predictions of five commonly used modelling techniques: generalised linear model (GLM), generalised additive model (GAM), gradient boosting machine (GBM), random forest (RF), and maximum entropy (MAXENT) models. We randomly generated sets of 70% of the starting occurrences used for calibration, while the remaining 30% of random selected occurrences were used to assess the model predicting abilities. Models were evaluated using the receiver operating characteristic curve (AUC) (Hanley & McNeil, 1982) and the true skill statistic (TSS) (Allouche et al., 2006).

### 2.4. Niche overlap analysis

The niche analysis was performed independently of the SDMs that were used to define the potential distributions of the species (Stiels et al., 2015). We explored species niches using the analytical framework proposed by (Broennimann et al., 2012) that has been adopted in several other

studies (e.g. Di Febbraro et al., 2013; Loy et al., 2017; Russo et al., 2015). Within this framework, the same environmental variables used to build the SDMs and describing three dimensions of the otter's niche (climate, hydrography and vegetation landscape) were recorded on species occurrence locations and on a set of random points covering all environmental conditions occurring within the range of each species. All environmental data were entered a principal components analysis (PCA). Environmental variables recorded at species locations were projected into this PCA space, following the procedure used by (Loy et al., 2017). Subsequently, the density of the background environments and species records across the first two PCs were calculated by a kernel density smoother and, respectively, divided by the maximum number of occurrences in any one cell of the environmental space and by the number of sites with the most common environment (Broennimann et al., 2012). Biplots were then produced to evaluate the relative influence of the environmental parameters on the species' niche (Loy et al., 2017).

The density grids of  $r \times r$  cells in the PCA environmental space were used to compute niche overlap between each species pair. A niche similarity test (Warren et al., 2008) was then used to evaluate if two species were more similar/different in their niche space than expected by chance. Schoener's D values of niche overlap between species were compared to a null distribution of 100 overlap values, yielding a significant outcome if the observed value was higher (significant similarity) or lower (significant dissimilarity) than the 95% of the null distribution ( $P < 0.05$ , (Loy et al., 2017). The similarity between niches was tested in both directions by measuring the amount of one species niche included in the other niche, and vice versa (Broennimann et al., 2012). All procedures were performed using the R package 'ecospat' (Broennimann et al., 2012). We also produced ten replicated occurrence datasets, averaged the relative values of Schoener's D, and evaluated the percentage of tests yielding significant outcomes.

## RESULTS

### 3.1. Model accuracy and potential distribution

The SDMs for the three species achieved excellent predictive performances (Swets, 1988)(sensu Swets 1988; Fig1) with mean AUC values 0.93 (SD=0.07) and TSS values 0.73 (SD = 0.020) for *L. lutra*, 0.95 (SD=0.02) and 0.87 (SD=0.05) for *A. cinereus*, and 0.97 (SD=0.04) and 0.89 (SD=0.01) for *L. perspicillata* (Table 1).

	<i>Lutra Lutra</i>		<i>Aonyx cinereus</i>		<i>Lutrogale perspicillata</i>	
	Mean	SD	Mean	SD	Mean	SD
AUC	0.93	0.006	0.95	0.01	0.96	0.00
TSS	0.73	0.020	0.87	0.04	0.89	0.013

Table 1. ROC and TSS model evaluation scores for all species

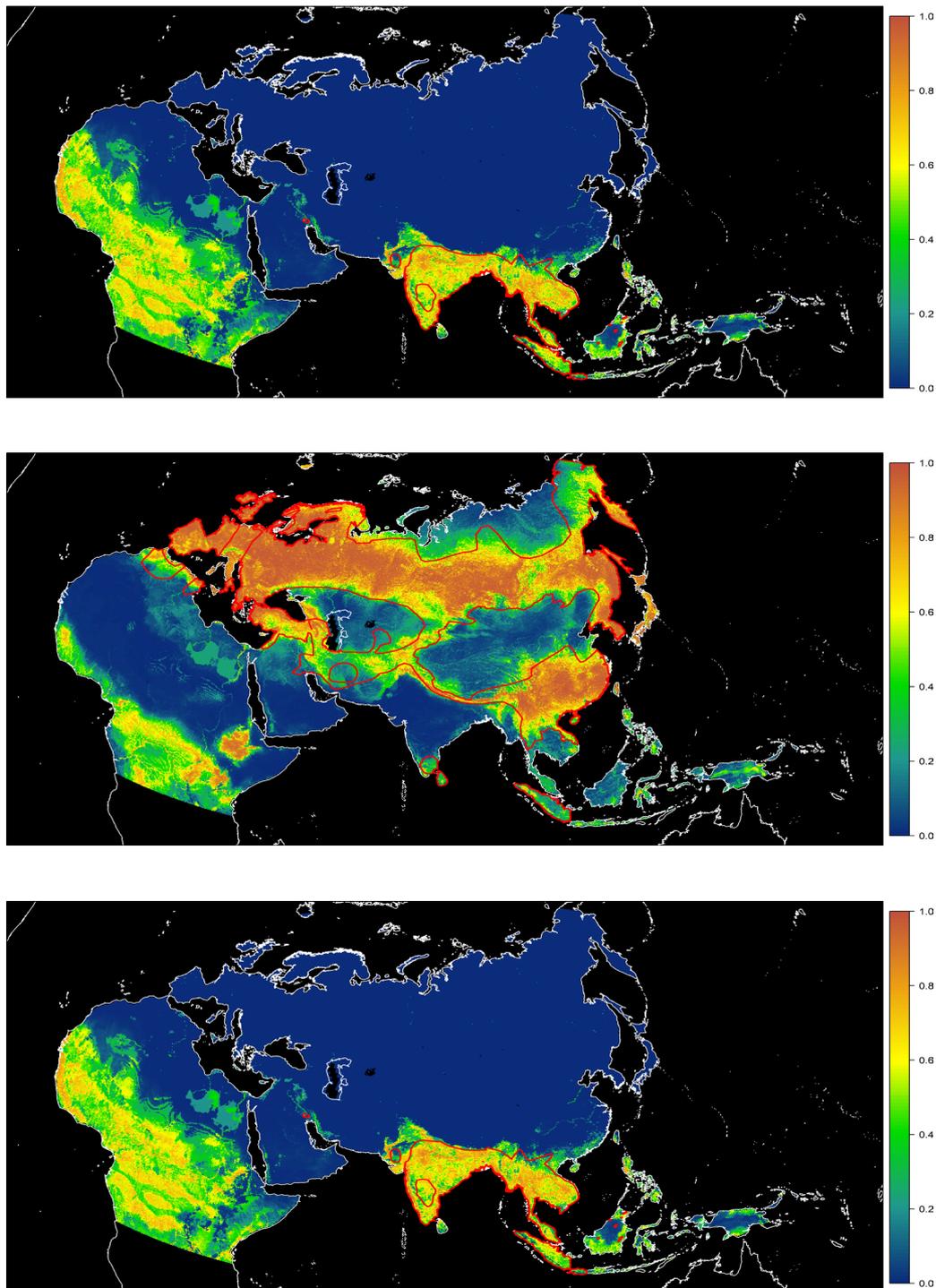


Fig 1. Potential distribution of *A. cinereus* (top), *L. lutra* (centre), and *L. perspicillata* (bottom) derived from SDMs within each species range

Results showed that 77.48 % (SD=10.19), 77.32 % (SD=9.31) and 75.26 % (SD=10.68) of the respective IUCN ranges is suitable for *L. lutra*, *L. perspicillata* and *A. cinereus* respectively (Fig 1).

3.2. Niche overlap

The first two PCs explained ca. 60% of the total variance in the data. Within the PCA space, the environmental niche of *L. lutra* was the widest among the three species, showing a core placed toward high values of July NDVI, NPP and Precipitation of Driest Quarter (bio17, Fig. 2). The other two species niches

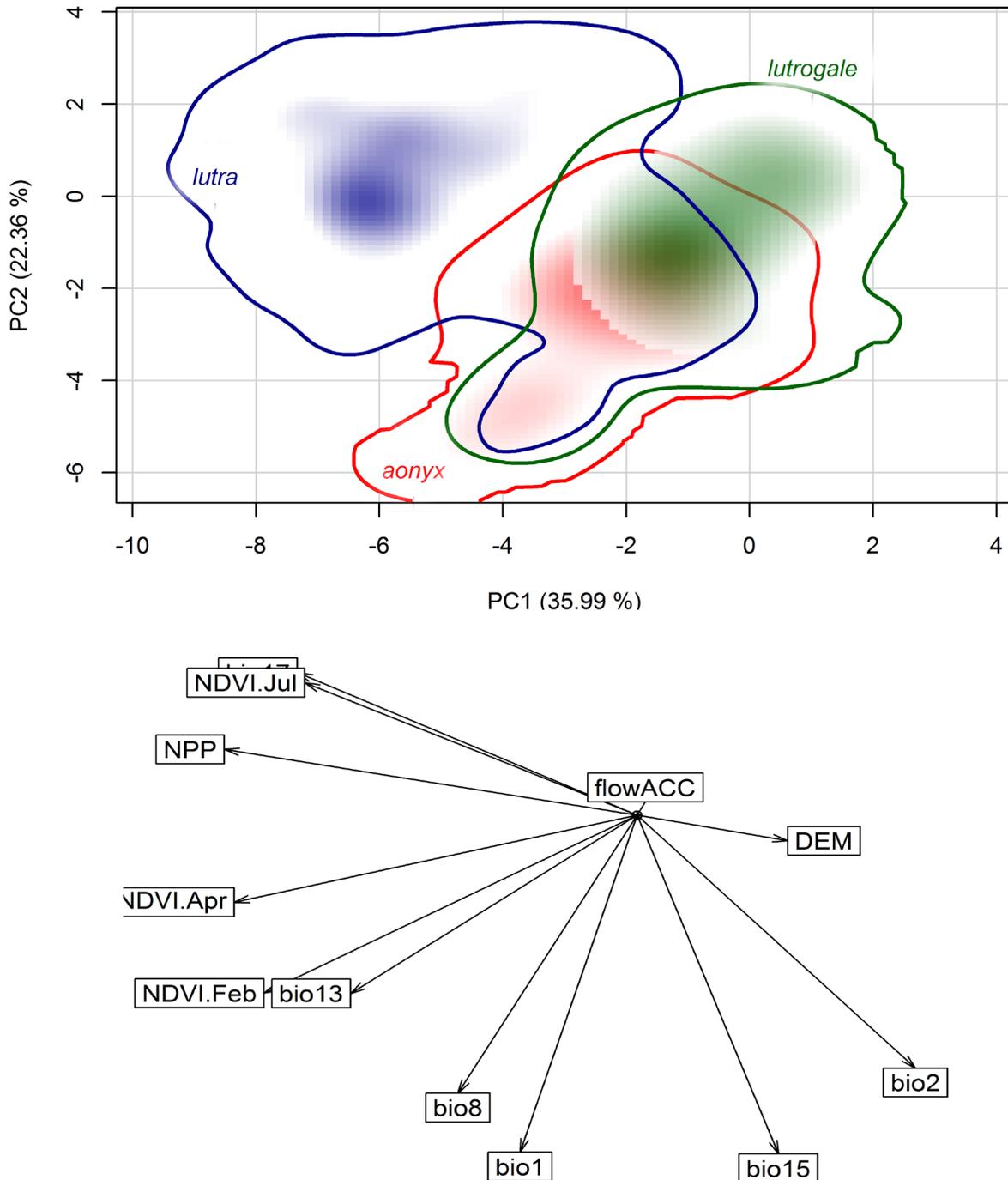


Fig 2. Species niches along the first two PCA axes (PC1 and PC2) of environmental variables recorded at presence sites and in the background environment within the species ranges (top), and the biplot of original variables (bottom). Red: *Aonyx cinereus*; green: *Lutrogale perspicillata*; blue: *Lutra lutra*. Colour shading shows the density of the species occurrence by cell, with the solid and dashed contour lines indicating 80 and 50% isopleths, respectively.

are placed along a gradient influenced by February NDVI, Annual Mean Temperature (bio1), Mean Temperature of Wettest Quarter (bio8), and Precipitation of Wettest Month (bio13), with the niche of *A. cinereus* laying toward higher values than *L. perspicillata*. (Fig 2).

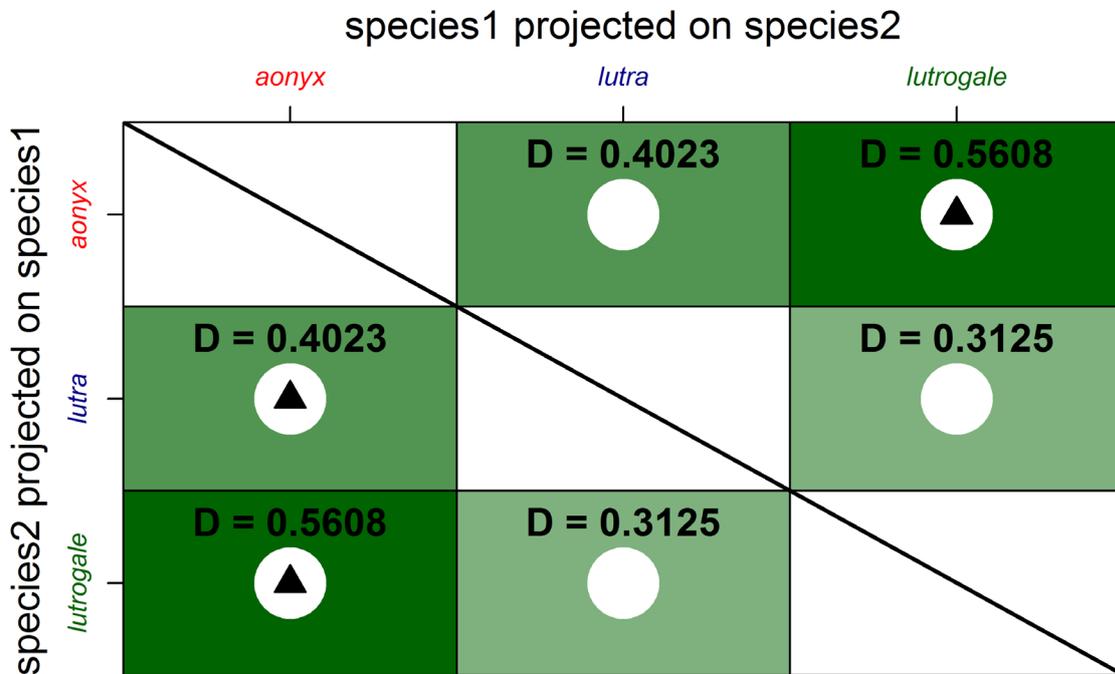


Fig 3 Niche similarities between species pairs. Color intensity is proportional to the Schoener's D values, triangles refer to significant similarity tests.

Niche similarity tests revealed significant niche similarities between *A. cinereus* and *L. lutra* ( $D=0.40$ ,  $p<0.05$ ), and between *L. perspicillata* and *A. cinereus* ( $D=0.56$ ,  $p<0.05$ ), whereas the comparison between *L. perspicillata* and *L. lutra* was not significant ( $D=0.31$ ,  $p\geq0.05$ ) (Fig 3).

Other than examining suitable area of extent of occurrence for each species we also examined the percentage of suitable area of each species falling within other's species' range. We discovered that 8.51% ( $SD = 11.08$ ) of *L. lutra* suitable areas and 13.89% ( $SD = 8.67$ ) of *L. perspicillata* fall within the IUCN range of *A. cinereus* whereas 12.66% ( $SD = 9.28$ ) of suitable areas for *A. cinereus* and 6.60% ( $SD = 7.27$ ) of suitable areas for *L. lutra* were included in the range of *L. perspicillata*. *L. lutra* showed the least overlap, with only 2.1% ( $SD = 1.77$ ) and 2.39% ( $SD = 1.81$ ) of its range included suitable areas for *A. cinereus* and *L. perspicillata* respectively.

## DISCUSSION

SDMs produced reliable potential distribution maps for the three otter species occurring in Asia. The spatial and climatic niche of *L. lutra* was mainly affected by Precipitation of Driest Quarter, confirming previous findings (Cianfrani et al., 2011,

2018). The niche of *A. cinereus* and *L. perspicillata* were instead shaped by almost similar variables, i.e. February NDVI, Temperature, and Precipitation. Both species' well-known affinity for forests (e.g., mangrove or peat swamp forests; Foster-Turley, 1992; Gupta et al., 2020). Furthermore, *A. cinereus* and *L. perspicillata* rely heavily on fish, shrimp, crayfish, and crabs, (Foster-Turley, 1992; Hussain & Choudhury, 1998) and quantity of which in freshwater streams can be affected by various rainfall regimes (Gratwicke, 2004). Our study also coinciding the trend which is reminiscent of the evidence provided by (Cianfrani et al., 2018), which showed that *A. cinereus* is impacted by temperature changes.

The extent of suitable habitat for each species was between 75 and ca 78% of their IUCN range. Two of the species are listed as Vulnerable in the IUCN red list, due to an inferred population decline of more than 30% over the past 30 years, whereas the range of *L. lutra* in East Asia is highly fragmented and the species is considered rare (Loy, 2018). Despite being well adapted to human impacted landscapes, all species are threatened by the loss of wetlands, poaching, pollution, and decline in prey biomass (Hussain et al 2018, Aadrean et 2018,

Loy 2018). As many other mammals in South Asia (Schipper et al., 2008), otters are threatened by human development and activities, similar to many terrestrial mammals (Duplaix & Savage, 2018). Our results suggest that a strict protection of the three species and their habitat could likely promote an increase of their current areas of occupancy (AOO, *sensu* IUCN) and the expansion of their extent of occurrence (EOO, *sensu* IUCN) in East Asia.

The future survival of these might also be hampered by competitive interactions among species with similar ecological requirements like otters. Our results showed significant similarities between the spatial and climate niche of *A. cinereus* with both *L. perspicillata* and *L. Lutra*, whereas niche similarity between *L. perspicillata* and *L. lutra* was not significant. Despite the similarity in their spatial niche, *A. cinereus* is known to coexist with both *L. perspicillata* and *L. Lutra* in many parts of its range (Kruuk et al., 1994, Raha & Hussain (2016). *L. perspicillata* may compete for resources with *A. cinereus* (Sabrina 1985), as *A. cinereus* and *L. perspicillata* primarily prey on shrimp, crayfish, and crabs, among other prey (Anoop & Hussain, 2005; Foster-Turley, 1992). Remains were identified by the comparison of body parts with a reference collection of appropriate prey species. Two methods were used to estimate the proportions of different prey consumed: frequency of occurrence and score-bulk estimate. The number of prey types in a spraint varied between one and seven. More prey species were found during low water levels (March-April). According to our findings, *A. cinereus* is the species of main concern because it has the least suitable IUCN range compared to *L. perspicillata* and *L. Lutra*, it is the smallest specie, and it shares both its spatial and feeding niche with *L. perspicillata*, likely leading to add in competition for resources and

space. These concerns are expected to increase in the light of climate change, that is expected to have a significant impact on *A. cinereus* habitats, with up to 40% of suitable areas disappearing by 2070 (Cianfrani et al., 2018).

### CONCLUSIONS

Human activities, both in the past and present (Millennium Ecosystem Assessment, 2005), have influenced and are influencing freshwater ecosystems. Freshwater ecosystems, which may be the most vulnerable to climate change, have been influenced and are influencing (Milly et al., 2005; Dudgeon et al., 2006; Vorosmarty et al., 2010). Freshwater habitats are currently under threat as a result of crop irrigation, contamination, land-use activities, and infrastructure development, among other factors (Vorosmarty et al., 2010). The Lutrinae subfamily contains thirteen different otter species that can be found all over the world. In both marine and freshwater environments, they are able to thrive. Similarly, (Duplaix & Savage, 2018) over the last century, the eleven remaining freshwater otter populations around the world have seen significant declines as a result of habitat destruction, mistreatment, and bioaccumulation of toxins. Otters, as the top freshwater predators, are among the most vulnerable species on the planet, making them one of the most endangered. Due to the fact that otter populations require large expanses of adequate freshwater and riverine habitat, and because riverine habitats are considered to be particularly vulnerable to anthropogenic pressures, this is the case (Kruuk, 2006). Many species are still endangered or extinct, many of them are found in Asia and Africa. Furthermore, new challenges such as climate change may put recovered ecosystems at risk of extinction in the long term (Kruuk, 2006).

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# First assessment of Environmental DNA based detection approach to monitor the presence of Eurasian otter in southern Italy

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## ABSTRACT

The assessment of the occurrence of rare and endangered species in freshwater environments is crucial for ecological studies and conservation issues, but it can be time-consuming and challenging in harsh environments. Detecting DNA traces from the environment (environmental DNA, eDNA) can provide innovative and reliable solutions for the monitoring and conservation of rare and elusive species, such as the Eurasian otter *Lutra lutra*. We tested an experimental workflow based on target qPCR assay to detect *L. lutra* eDNA from water samples as a speditive monitoring tool at large scale to be coupled with fine-scale traditional field surveys. This is the first application of an eDNA-based approach to monitor the presence of *L. lutra* in Italy. We compared the eDNA-based results with traditional survey observations and confirmed the reliability of this innovative approach for the large-scale monitoring of such aquatic elusive species.

## INTRODUCTION

Monitoring the presence of rare and/or endangered species is essential for ecology and conservation purposes, but often can be conducted only adopting non-invasive methods, or actively searching for indirect signs (e.g. roadkills, footprints, scats, spraints). In the past, efforts have been made to explore mammal species diversity by using various techniques, such as transect sampling (Thomas et al., 2010), camera trapping (Jamwal et al., 2016), and sign surveys (Sadlier et al., 2004). Camera trapping has largely been used to study ecology worldwide (Rovero et al., 2014). This approach has an advantage over other types of surveillance since cameras can operate in the field for more extended periods and do not require the same level of attention as human observers (Meek et al., 2014). However, the issue of theft and the ongoing costs of using camera traps are significant drawbacks. Sign surveys using trails and transects are not only

effective for wildlife monitoring, but they are also relatively inexpensive in terms of dedicated facilities and analytics requirements and straightforward to conduct (Jathanna et al., 2003). Nonetheless, sign survey is often incapable of identifying congeneric species or to quantify their relative abundance (Harrington et al., 2010).

On the other hand, scat survey is a widely used strategy in mammal monitoring studies to assess distribution through space and time of a certain species (Lee et al., 2019; Prat-Mairet et al., 2017), such as the Eurasian otter (*Lutra lutra*) (Reuther et al., 2000). However, when dealing with elusive species and field surveys, conducting a capillary and long-term monitoring can be challenging: reaching sampling sites difficult to access and following signs of the target species in areas with extreme topography and ruggedness require manpower, investment, and is time-consuming. Especially when sampling campaigns are over a wide spatial scale and extended in time these issues appear to be determinant. (Lerone et al., 2015). Within the last decade, we faced a revolution in species detection, due to the implementation of molecular techniques able to detect DNA traces from environmental matrices (e.g. water) (Taberlet et al., 2018). Environmental DNA (eDNA) is released by organisms into the environment from faeces, mucus, skin cells, or extracellular DNA (Taberlet et al., 2012) and its detection soon became an invaluable ally in wildlife conservation studies (Valsecchi et al., 2021; Cristescu and Hebert, 2018). This is the case of many top predator carnivores (Harper et al., 2019; Lyet et al., 2021; Wilcox et al., 2021), including the Eurasian otter.

*L. lutra* population showed a decline in the last century, due to habitat fragmentation and destruction across its range, especially in Western and Central Europe (Pigneur et al., 2019; Marcelli et al., 2012; Mucci et al., 2010; Koelewijn et al., 2010; Janssens et al., 2008; Ferrando et al., 2004; Dallas et al., 2002). Law enforcement and banding of harmful chemical compounds used in agriculture allowed the species to recover in many European countries, and in 2007 *L. lutra* moved from Vulnerable (VU) to Near Threatened (NT) category in the IUCN Red List. However, the status of *L. lutra* is still perilous in Italy, and the species is listed as Endangered (EN) in the national red list (Rondinini et al., 2013). Remnant viable populations only occurred in southern Italy (Loy et al., 2004). The return of otters in the river Sangro in 2007 (De Castro and Loy, 2007) marked the onset of a still ongoing recovery in south-central regions (Giovacchini et al., 2018; Loy et al., 2015;). In this context, traditional field monitoring of otters can be highly demanding in terms of time and costs. eDNA analysis could be a valuable alternative, especially to assess the species

occurrence in peripheral areas of the species range (Sales et al., 2020). In the present study, we tested for the very first time in Italy an eDNA-based experimental workflow to detect *L. lutra* DNA from water samples by a target qPCR assay. As a speditive monitoring tool for aquatic elusive species, *L. lutra* eDNA-based assay can be proficiently coupled with traditional field surveys to facilitate rapid, large-scale biomonitoring, representing a natural progression in bioassessment and ecosystem surveillance.

## METHODS

### *Study area and sampling sites*

The study area is located at the northern boundary of the Eurasian otter range in Italy. Six sampling sites (S1-S6) were selected along the rivers Sangro, Volturno and Biferno, including areas (S1-S4) where otters have been monitored and detected by sign surveys (spraints and tracks) since 2000 (Loy et al., 2015; De Castro et al., 2013; Loy et al., 2004), and an area (S5-S6) upstream of a steep dam located along the river Sangro in the National Park of Abruzzo, Lazio and Molise (PNALM). Here, otter signs were never recorded (Marcelli, 2006; De Castro, 2007; Imperi, 2011; Lerone, 2013), with the exception of S6, where spraints were first found in September 2018 (Caldarella, pers. comm.; Lerone, pers. comm.; Giovacchini, pers. comm.) (see Table 1 for detailed information).

During our survey, at each sampling site, indirect signs of otter presence (spraints and footprints) were actively searched and recorded along 600 m of riverbank. According to the standard protocol for otter survey in Europe, this is the searching distance that allows to decide whether a site is positive or negative for otter occurrence (Reuther et al., 2000).

Field surveys for four sampling sites (S1, S2, S3 and S4) were carried out in June 2018, and for two sites (S5 and S6) in February 2019.

eDNA survey and field survey were carried out at the same time, in order to ensure the most up-to-date information on otter presence.

For eDNA analyses, three litres of water for sites S1-S4 and two liters of water in the case of sites S5-S6 were aseptically collected using sterile 1 L bottles. Samples from S1-S4 sites were transported (within 24 h, kept at 4 °C) in a laboratory equipped for eDNA analyses for water filtration; orthogonal water filtration was performed under a laminar flow cabinet and vacuum was generated by a vacuum pump (Vacuubrand™) connected to a filtering apparatus. Samples from S5-S6 sites were tested for on-site filtration by a portable hand vacuum pump connected to a polypropylene flask (1 L, Nalgene®) using sterile disposable filter units (Sartorius). Due to the heterogeneity of water turbidity among samples, we developed an experimental

Site name	River name	Altitude (m asl)	Sampling date	References for past field surveys	Otter signs searched in past field surveys
S1	Volturno	186	June 2018	Loy et al., 2004; De Castro et al., 2013; Loy et al., 2015	Spraint and tracks
S2	Volturno	217	June 2018	Loy et al., 2004; De Castro et al., 2013; Loy et al., 2015	Spraint and tracks
S3	Biferno	134	June 2018	Loy et al., 2004; De Castro et al., 2013; Loy et al., 2015	Spraint and tracks
S4	Volturno	184	June 2018	Loy et al., 2004; De Castro et al., 2013; Loy et al., 2015	Spraint and tracks
S5	Sangro (PNALM)	1118	February 2019	Marcelli, 2006; Imperi, 2011; Lerone, 2013  - all negative -	Spraint and tracks
S6	Sangro (PNALM)	975	February 2019	Marcelli, 2006; De Castro, 2007; Imperi, 2011; Lerone, 2013  - Always negative - First spraint found in September 2018 (Caldarella, pers. comm.; Lerone, pers. comm.; Giovacchini, pers. comm.).	Spraint and tracks

**Table 1. Sampling sites details and references of past surveys.**

scheme designed as follows. Briefly, where water was evaluated as transparent at naked eye, it was filtered directly on a nitrocellulose membrane filter with a pore size = 0.2  $\mu\text{m}$  (sites S1, S3, S5, S6). Moreover, in the case of S1 and S3 sites, each 1 L bottle of the three liters of water collected was filtered in parallel, obtaining three replicas, whereas in the case of on-site filtration (sites S5 and S6) the entire volume of water (2 L) was directly filtered on the disposable filter unit, to limit sample handling. In the case of high turbid waters (sites S2 and S4), the water of each bottle was mixed thoroughly and preliminary filtered (for a total of 3 L) with 8  $\mu\text{m}$ -based nitrocellulose membranes; then, the water recovered was further filtered by 0.2  $\mu\text{m}$  membrane

filters. In this case, no filtration replicates were accounted for, but to increase eDNA concentration the entire volume (3 L) was directly filtered on the filter.

All tubes and containers were sterilised with sodium hypochlorite between samples.

#### **DNA extraction**

All the instrumentations used, if not disposable, were sterilized with sodium hypochlorite or autoclaved prior to each experiment. Pre and post amplification phases were carried out in separate laboratory rooms with dedicated equipment and every step was conducted in the laminar flow cabinet, in order to avoid any possible contamination

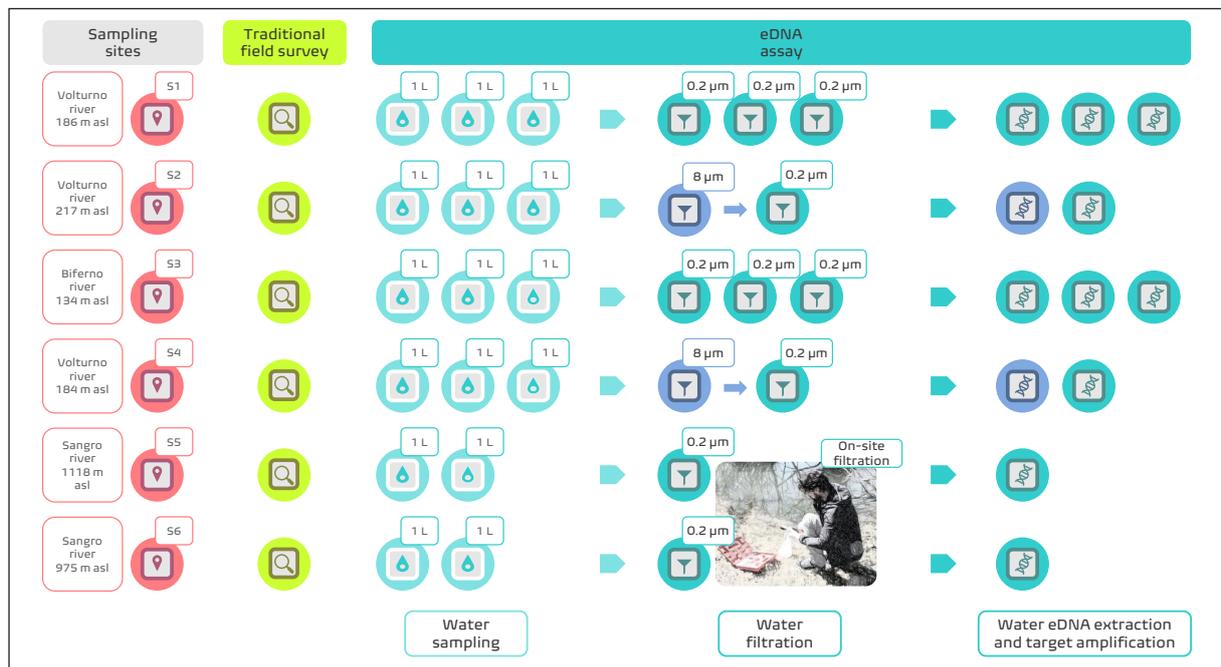


Figure 1. Experimental workflow. For each sampling site, samples collected, filtered and processed for eDNA extraction and target amplification are reported. The volume of water collected for each sample is indicated. The membrane filter pore size used is reported and highlighted with different colours (0.2 µm pore size in cyan and 8 µm pore size in blue).

with exogenous eDNA.

eDNA was extracted from each filter using the DNeasy® PowerWater® Kit (Qiagen), following the Manufacturer's protocol with minor modifications; briefly, eDNA was eluted in 75 µL of warmed (40 °C) elution buffer, to increase the final eDNA concentration. If not processed immediately, the filter samples were stored at -80 °C (Figure 1).

Positive controls for eDNA assay were obtained from two *L. lutra* tissue samples stored in ethanol at the University of Molise. Genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) following the Manufacturer's protocol.

#### qPCR assay set up

First, we tested with end-point PCR the otter positive controls obtained from the reference *L. lutra* tissue samples. To do that, we used otter specific primers reported by Park et al., 2011, LutCyt-F and LutCyt-R, which were designed to amplify a 227 bp long region of the mitochondrial cytochrome b of *L. lutra* (positions 32-259). Amplification conditions consisted in an initial denaturation for 5 min at 94 °C, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min (Park et al, 2011). The reaction mix was composed as follows: 0.25 µL of Wonder Taq Thermostable DNA polymerase© (EuroClone S.p.A.), 4 µL of Wonder Taq Reaction Buffer (EuroClone S.p.A.), 0.2 µL each primer [100 µM], 2 µL of DNA sample

and Milli-Q water to reach the volume of 20 µL. Amplicons were purified from agarose using EuroGOLD Gel Extraction Kit® (EuroClone S.p.A.) following Manufacturer's protocol.

Cyt b amplicons obtained by end-point PCR were bidirectionally Sanger-sequenced at Eurofins Genomics (<https://www.eurofinsgenomics.eu/>). A GenBank-NCBI BLAST analysis of the obtained consensus sequences confirmed that the two reference tissue samples corresponded to the expected Cyt b fragment of *L. lutra* (i.e. maximum identity > 99%; query coverage 100%).

Quantitative Real Time PCR (qPCR) assays were performed with an AB 7500 (Applied Biosystem) instrument. To set up the qPCR assay, first a standard curve was generated. Briefly, we amplified the DNA of positive controls using *L. lutra* specific primer pairs (LutCyt-F, LutCyt-R). qPCR conditions included 10' initial denaturation at 95 °C and 40 cycles of denaturation at 95 °C for 15 s and annealing-elongation at 55 °C for 1 min. Amplification reaction consisted of 5.0 µL SsoFast EvaGreen Supermix with Low ROX (Bio-Rad), 0.1 µL each [10 µM] primer solution, 2 µL of extracted DNA. Ten-fold serial dilutions of positive control were tested in triplicate and the assay was repeated in triplicate as well, in order to calculate qPCR amplification efficiency and limit of detection (LoD). The LoD is defined as the lowest concentration (and as a consequence the highest Ct) at which 95% of the positive samples are

detected (Bustin et al., 2009).

All the amplification data were collected and analyzed with the SDS 7500 Real-Time PCR System Software (Applied Biosystems).

**otter eDNA qPCR assay**

Environmental water samples, together with positive controls and negative controls (no template), were amplified in the same run-in triplicate. We further included, in the same assay, otter negative controls, constituted by eDNA extracts of water samples (AQU01-05) from a sampling site characterized by the ascertained absence of otter eDNA and the likely occurrence of heterogeneous DNA and inhibitors. These samples were collected from the river Lambro. They were processed previously, according to the protocol tested for the other environmental samples, i.e., 1 L of water was filtered on a membrane filter (0.2 µm pore-sized) and eDNA was extracted using the DNeasy® PowerWater® Kit (Qiagen). For all the filtered water samples (both those from S1-S6 sites and AQU01-05), 5 µL of eDNA template was used in the reaction to take into account low target eDNA concentration. To ensure that no otter detection in environmental samples was due to the absence of *L. lutra* eDNA and not to a failure of eDNA extraction, the same samples were also tested with qPCR using the MiFish primer pairs, a set of universal 12SrRNA

oligonucleotides for the untarget DNA metabarcoding characterization of fishes (Miya et al., 2015). The amplification reaction conditions were the same tested for LutCyt assay. Amplification profile included an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing-elongation for 1 min at 58 °C. All the amplification data were collected and analyzed with the SDS 7500 Real-Time PCR System Software (Applied Biosystems).

Ct (Threshold Cycles) values were converted into counts (DNA copies) using the formula  $Counts = E^{(40-Ct)}$  where E is the efficiency of amplification (Bruno et al., 2017).

To avoid any possible contamination with exogenous DNA, pre and post amplification phases were carried out in separate rooms, and every step was conducted in a laminar flow cabinet

**Results and Discussion**

Amplification efficiency of qPCR assay, estimated by means of serial dilutions of positive controls, approached 100%. The limit of detection (LoD) approximated at Ct=35.4. According to the calculated LoD, the tested environmental samples were considered negative (no detection) if Ct was greater than 35.4 in at least 2/3 of amplification replicates. If at least one sampling replicate resulted in positive detection, the site was considered otter-positive for

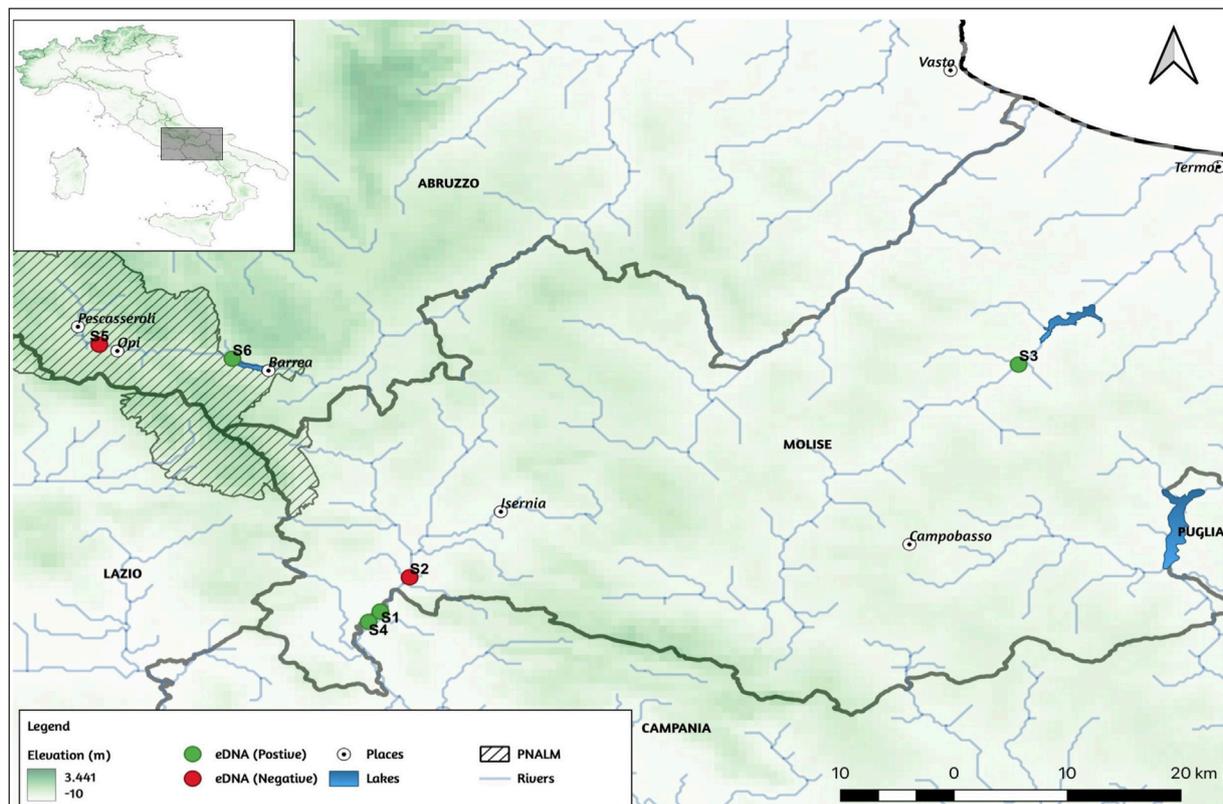


Figure 2. Map of eDNA results for each sampling site

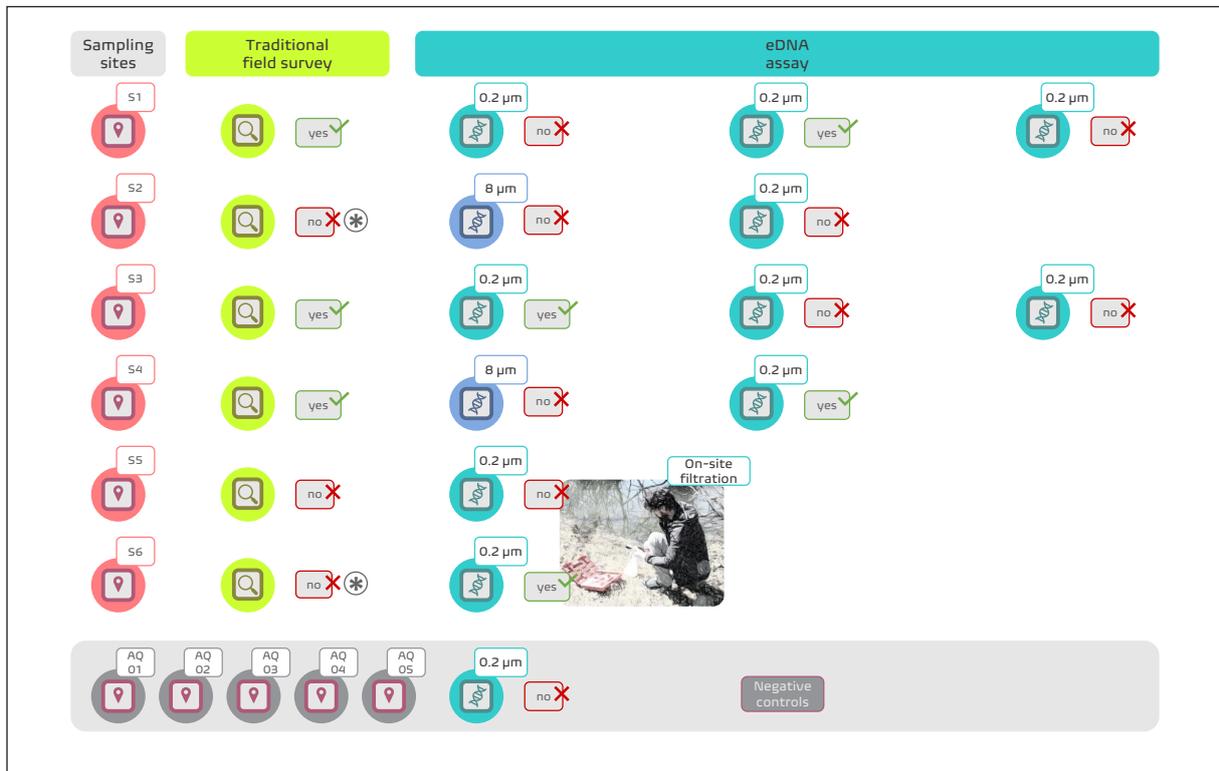


Figure 3. Otter detection from field survey and eDNA assay at each sampling site (S1-S6), compared to negative controls (AQ01-05). The membrane filter pore size used is reported and highlighted with different colours (0.2 µm pore size in cyan and 8 µm pore size in blue). \*otter detection in previous field surveys only.

eDNA assay. Water eDNA of *L. lutra* measured by qPCR ranged from 0 to 178 DNA counts/µL. Negative controls (no otter DNA: AQU01-05 and no DNA) provided no amplification signal. Considering otter-positive environmental samples (n=4), we reported an average of 22 DNA counts/µL (s.d.: 29 DNA counts/µL), indicating low DNA concentration of the target species. However, this value matches with other studies on rainbow trout eDNA detection from water samples (Wilcox et al., 2018). MiFish qPCR assay, used for the untarget DNA metabarcoding characterization of fishes (Miya et al., 2015), confirmed the successful DNA extraction for each tested sample, with the exception of positive and no template controls, as expected.

Figure 2 shows the results of eDNA assay for each sampling site, whereas otter presence estimation through both visual observation and eDNA assay is reported in Figure 3. eDNA samples collected at S1 and S3 sites (positive for otter visual records) produced positive results in 1/3 of sampling replicates. Previous studies (Kasai et al., 2020; van Bochove et al., 2020; Bylemans, 2018; Tsuji et al., 2017) suggest that low target DNA concentration together with DNA degradation can have a role in false negative results and recommend replicates to detect eDNA traces of target species. Samples collected at site S2 (otter records only in the past) resulted negative for eDNA otter detection. For site S4 (positive for otter signs), eDNA

amplification occurred for the 0.22 µm fraction. The 8 µm pore size filter, used to remove water turbidity, showed no amplification of target DNA. In the case of S5 (on-site filtration), we obtained no detection with both visual observation and eDNA assay. Conversely, site S6 (on-site filtration) (otter presence reported only by previous studies) resulted positive for eDNA assay. In this study, considering sampling sites, and taking into account the eDNA and traditional survey contextually carried out, we obtained 5 out of 6 sampling sites concordant in results. S6 only was not congruent in the results obtained, with positive otter eDNA signal, but no otter signs recorded at the time of our survey. If we include the information gained from past field surveys, S2 represents an “otter-positive” site for data collected in past surveys, but negative in otter eDNA detection; conversely, S6 reported otter records from the past (but not for the present survey) and an otter eDNA signal in our assay. Considering this last evidence regarding S6, eDNA could reveal otter presence when no visual signs are reported, increasing the positive location for otter presence from 50% (3 out of 6 sites) to 67% (4 out of 6 sites).

On the whole, our data revealed that an eDNA approach based on the amplification of a specific genetic marker from water samples could be a reliable tool to monitor the occurrence of the elusive Eurasian otter in a non-invasive way. We tested

different filtration strategies (i.e., preliminary filtration to remove water turbidity vs direct filtration) and different experimental scenarios (i.e., laboratory vs on-site filtration), in order to account different fieldwork requirements and conditions. Pre-filtration with a wide-mesh filter can be useful to avoid filter clogging in case of high-water turbidity, enabling the recovery of eDNA in the downstream fine-mesh filter. Further, on-site filtration could be useful in harsh environments, to avoid issues related to transport and storage of bulky water samples. Despite the low number of samples, we obtained a good congruence (5 out of 6 sampling sites) of results, suggesting the protocol we adopted could be used in areas where field survey is unfeasible or unaffordable and in large scale monitoring. However, the accuracy of site detection still needs to be tested thoroughly, taking into consideration specific environmental variables such as water temperature, water flow, water chemical composition, and all the other factors (including minimum water volume and sampling replicates) that could affect DNA detection (Furlan et al., 2016; Ficetola et al., 2015). One step forward the use of eDNA as a well validated approach for monitoring can be achieved by modelling eDNA detection considering effects of intrinsic sample characteristics, environmental conditions in the field and sample treatment in the molecular laboratory (Song et al., 2017; Harrison et al., 2019; Jo & Minamoto, 2021). Increasing efforts in this direction can help in correctly defining a threshold accounting for positive or negative sites for a stable otter presence.

In this study, several measures were taken in the field and in the lab to circumvent false positive and false negative results. To obtain a reliable test of the method for a target species we strongly recommend to 1) select sampling localities where the target taxon is surely absent (as a negative control) or present (integrating data from well validated traditional methods and recording indirect signs of the presence of the target species); 2) evaluate and record environmental variables, such as water turbidity, which could affect experimental procedures; 3) in case of turbid water, a pre-filtration step with a large mesh filter can be useful to avoid filter clogging and it could improve detectability of eDNA reducing particulate matters and inhibitors; 4) use disposable instruments and carefully clean non disposable ones among samples. Considering that a key factor that strongly influences the reliability of eDNA

results is the laboratory set-up, we also recommend to 5) employ a pre-amplification room specific for low quantity DNA samples, and a post amplification room, with a unidirectional workflow through the different rooms of the laboratory; 6) use dedicated equipment for each phase; 7) include quality controls for each experimental step (i.e. DNA extraction positive and negative controls, as well as DNA amplification positive and negative controls); 8) include technical (repeated measures of the same sample) and biological (repeated measures of biologically distinct samples that capture random biological variation) replicates (Blainey et al., 2014) to avoid false negative results.

## CONCLUSIONS

Under a reliable experimental design and laboratory conditions/procedures, eDNA investigations offer several advantages compared to traditional field surveys of rare and elusive species. Among these, we can mention the null disturbance to the ecosystem or to the target species, fast analytical time, possibility of being applied in harsh field conditions, and the possible scalability to multiple species detection from one environmental sample, that is especially useful for required monitoring obligations under European regulation. Considering the pros and cons of both field survey and eDNA assays, we believe that to make sound wildlife management decisions (e.g.: evaluation of the effectiveness of conservation measures or regulating plans) it is critical to adopt an integrated approach, exploiting the advantages of sensitivity of molecular assays and the robustness of traditional well-validated methods. In addition, comparing the distribution of the target species through both target eDNA assays and field surveys will broaden the understanding of limits and caveat of these monitoring strategies. Finally, environmental water samples collected for target species detection can be proficiently exploited to unlock the huge amount of information hidden in the nucleic acids extracted: a DNA metabarcoding approach, relying on the high-throughput analyses of DNA sequences, can be usefully applied for species occupancy modelling, population genetics, biodiversity estimation, and food web and trophic niche exploration (Compton et al., 2020), deepening our knowledge about the state of an ecosystem on the whole.

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# Environmental DNA to survey elusive species in harsh environments: *Lutra lutra* detection in Himalayan rivers

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## INTRODUCTION

A precise framework of species occurrence and distribution trends is crucial to identifying their conservation status (IUCN red list criteria), and identify critical areas to prioritize conservation measures. Studying otter species may be challenging, since they are rare, elusive, can be nocturnal, seldom live in difficult-to-access environments, and have relatively large regions and home ranges (Kruuk, 2011).

Several traditional methods, including transect sampling (Thomas et al., 2010), camera trapping (Silveira et al., 2003) and sign survey (Sadler et al., 2004) have widely been used to study several mammals with differing outcomes. In particular, scat surveys have become a standard monitoring method and an accepted tool for monitoring the distribution of otters over time (Reuther, 2000). These traditional methods are often labour-intensive and time-consuming, and can be ineffective in detecting species presence, especially when they are rare and scattered over large areas (van Bochove et al., 2020; Zhang et al., 2020). However, in the case of rare and elusive species,

the acquisition of presence information is not only challenging and time-consuming, but also deceptive (Chadès et al., 2008; Goldberg et al., 2018; Lahoz-Monfort et al., 2016).

Environmental DNA (eDNA) is new tool that could play a vital role in overcoming the limits of traditional methods in the species detection, especially when surveying rare, elusive and endangered species at large geographic scale (Rees et al., 2014). eDNA investigations rely on DNA fragments to infer the presence of organisms (Harrison et al., 2019). eDNA is released by organisms into the environment from faeces, mucus, skin cells, or extracellular DNA (Prié et al., 2020; Taberlet et al., 2012). The presence of a target organism can be inferred by collecting, for instance, water, soil, or even air samples. For rare and endangered species the identification by eDNA has immense potential for understanding their ecology and distribution trends, as it enables the detection of DNA traces rather than the direct sampling of the organism (Bohmann et al., 2014; Goldberg et al., 2015). Particular attributes of eDNA sampling can be addressed to multiple species, thus providing

a cost-effective tool to monitor biodiversity in critical areas. The application of molecular based techniques targeting environmental DNA (eDNA) has been proved to be particularly useful to detect rare and elusive species in aquatic environments. However, an eDNA-based approach for the detection of rare semi-aquatic mammals has not been tested yet, especially in extreme conditions like the Himalayan rivers.

Otter populations are declining throughout Asia and the Himalayan region, threatened by human pressure on water resources, habitat fragmentation, and illegal trade (de Silva, 2011; Duplaix & Savage, 2018; Foster-turley et al., 1990; Kafle, 2009). Himalayan region hosts three otter species, i.e., the Smooth-coated otter (*Lutrogale perspicillata*), the Asian small-clawed otter (*Aonyx cinereus*) (Duplaix & Savage, 2018), and the Eurasian otter (*Lutra lutra*) (Jamwal et al., 2016). Specifically, only Eurasian otter is known to rarely occur in two Trans-Himalayan rivers: Indus, Drass (Jamwal et al., 2016).

Himalayan range represents the upper altitudinal limit of the distribution of the species. However, despite the crucial role of otters as top predator in these freshwater ecosystems, and their value as flagship species for conservation (Kruuk, 2006, 2011; Pardini, 1998; Stevens et al., 2011), main conservation and survey efforts in trans-Himalaya have been traditionally focused on other charismatic fauna, such as the Snow Leopard (*Panthera uncia*) (Jamwal et al., 2016). This lack of interest produced a large gap of knowledge on the occurrence, distribution, and ecological requirements of otters in the Trans-Himalayan region. The only extensive survey using camera trap and indirect sign surveys was recently conducted by (Jamwal et al., 2016). Additionally, otter monitoring in Trans-Himalayas is complicated and requires a high logistic investment due to topographically challenging and extreme landscapes (Joshi et al., 2020). In this context, eDNA could offer a valuable alternative, especially to assess the species occurrence at a large scale.

In the present study we tested the efficiency of the eDNA approach to detect the rare and elusive otters in six rivers in the Trans-Himalaya, and compared the results to those obtained from a traditional field survey. Our specific aims were: i) evaluate any discrepancy between eDNA and traditional field otter survey ii) identify environmental factors that could influence the efficacy of otter detection both through eDNA survey.

## METHODS

### Study area

The research was conducted in six rivers of Ladakh (32°15'–36° N; 75°15'–80°15'E) covering about 200 km of river stretches at altitudes ranging from 3,500 to 4,400 m asl. Ladakh is a union territory in

northern India with elevations ranging from 2900 to 7600 m asl. Ladakh mountains are one of the Trans-Himalayan biogeographic provinces, which includes also the Tibetan Plateau, and the Himalaya Sikkim (WII 2009). The Trans-Himalaya is a cold desert with an altitude range of 2,200 metres to over 7,000 metres and winter temperatures that can drop below - 40 degrees Celsius. The Trans-Himalaya gets 100-1000 mm of annual precipitation, which decreases to the north and east (Fox et al., 1994). The majority of the Trans-Himalayan range is located in India's Ladakh Union region, between 32°15 and 36° N and 75°15 and 80°15 E. Ladakh's primary drainage is formed by the Indus River and its two major tributaries, the Zaskar and Shyok rivers. There are several small and large brackish and freshwater lakes in the region. The territory of Ladakh is split into two districts—Kargil and Leh (Fig. 1)—and shares boundaries with Pakistan-controlled Kashmir, China-controlled Aksai Chin, Tibet, and Lahaul Spiti (Himachal Pradesh, India). The Zaskar, Ladakh, and Karakoram Mountain ranges run parallel in the area. Ladakh has an arid to semi-arid atmosphere (Lone et al., 2017) with an average annual rainfall of 115 mm (Lone et al., 2017, 2019). The average annual monthly precipitation is 13.4 millimetres in January, and the average minimum monthly precipitation is 6.9 millimetres in August (Lone et al., 2019). Rivers, especially the Indus and its tributaries such as the Zaskar, Markha, Shyok, Nubra, and Suru, scar the barren mountain landscape. Siachen, the region's largest glacier, is situated in Ladakh's far northwest corner. Eastern Ladakh is a vast plateau with a plethora of huge brackish water reservoirs.

### Traditional monitoring method

Between the 10th of July and the 30th of October 2018, survey was conducted in Ladakh, at 15 locations (Indus 2, Indus 2, Indus 3, Agam, Shyok 1, Turtuk 1, Turtuk 2, Panamik 1, Panamik 2, Drass 3, Drass 2, Drass 1, Suru 1, Zaskar Up and Zaskar low) (Fig. 1) along six rivers: Indus, Zaskar, Shyok, Panamik, Dras, and Suru rivers (Fig. 1). Using a sample technique similar to that employed in the Trans-Himalayas and Himalayas, indirect otter signs like scats were checked and recorded at 15 locations along the rivers. At each site otter signs were searched along 100 metres and were spaced 900 metres apart along one bank of each river (Chettri & Savage, 2014; Jamwal et al., 2016).

### eDNA monitoring, sample collection and processing

Water samples were collected at the aforementioned 15 locations while conducting the traditional surveys. We collected water samples along six rivers: Indus (n=3), Zaskar (n= 2), Shyok (n= 4), Panamik (n= 2), Dras (n= 3), and Suru (n=1) rivers. (Fig. 1). All equipment, tubing,

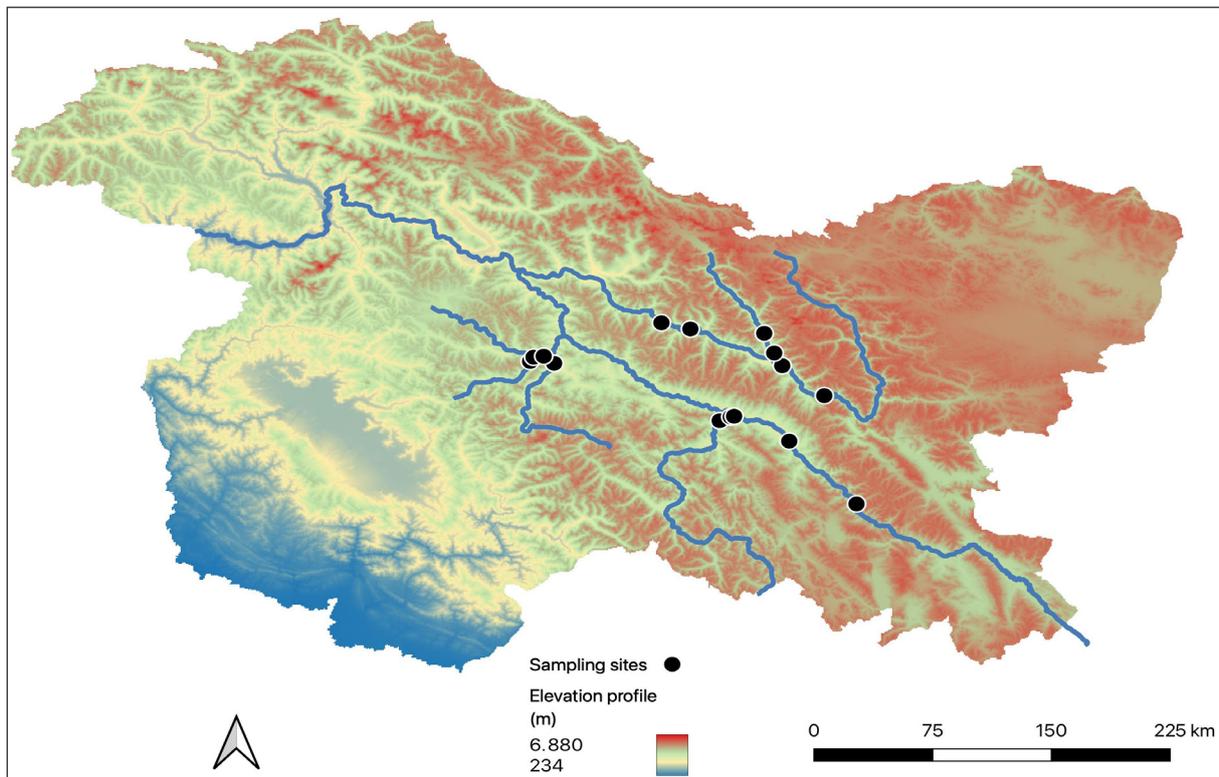


Fig 1. Map of study area

tubing connections, and containers were sterilized. Two liters of water from each site ( $n=15$ ) were collected and filtered on site. In order to allow field operations, vacuum was generated by a portable hand vacuum pump, connected to a polypropylene filtering flask (capacity 1 L, Nalgene®) with Tygon® tubing. All tubing, tubing connections, and containers were sterilized. Vertical (orthogonal) filtration using sterile disposable filter units (Sartorius), characterized by membrane filters with a pore size =  $0.2 \mu\text{m}$  (47 mm diameter, nitrocellulose membrane filter) and then stored in refrigerator.

#### eDNA extraction

eDNA extraction and all the subsequent experimental steps were performed in a laboratory equipped for environmental DNA analyses. All the instrumentations used, if not disposable, were sterilized with sodium hypochlorite or autoclaved prior to each experiment. Pre and post amplification phases were carried out in separate rooms and every step was conducted in the laminar flow cabinet, in order to avoid any possible contamination with exogenous DNA.

Then, total DNA was extracted from the filters obtained by water filtration using DNeasy® PowerSoil® Kit (Qiagen) following the protocol. DNA was eluted in  $75 \mu\text{L}$  of warmed ( $40^\circ\text{C}$ ) elution buffer, to increase DNA concentration. If not processed

immediately, samples were stored at  $-80^\circ\text{C}$ .

#### DNA extraction of positive controls (*Lutra lutra*)

*L. lutra* scat was collected from Turtuk 2, Indus 2 and Drass 2. DNA from otter scat samples dried at room temperature was obtained using DNeasy® PowerSoil® Kit (Qiagen) following the protocol, with few modifications: the lysis step was carried out at  $45^\circ\text{C}$  for 15' and extended time of incubations for the other steps was adopted. DNA was eluted in  $75 \mu\text{L}$  of warmed ( $40^\circ\text{C}$ ) elution buffer, to increase DNA concentration. If not processed immediately, samples were stored at  $-20^\circ\text{C}$ .

#### qPCR assay set up

First, we tested with end-point PCR the otter positive controls obtained from reference *L. lutra* scat samples. To do that, we used otter specific primers reported by (Park et al., 2011), LutCyt-F and LutCyt-R, which were designed to amplify a 227 bp long region of the mitochondrial cytochrome b of *L. lutra* (positions 32-259). Amplification conditions consisted in an initial denaturation for 5 min at  $94^\circ\text{C}$ , then 35 cycles of  $94^\circ\text{C}$  for 30 s,  $55^\circ\text{C}$  for 30 s, and  $72^\circ\text{C}$  for 30 s; and a final extension at  $72^\circ\text{C}$  for 7 min (Park et al., 2011). The reaction mix was composed as follows:  $0.25 \mu\text{L}$  of Wonder Taq Thermostable DNA polymerase® (EuroClone S.p.A.),  $4 \mu\text{L}$  of Wonder Taq Reaction Buffer (EuroClone S.p.A.),

0.2 µL each primer [100 µM], 2 µL of DNA sample and Milli-Q water to reach the volume of 20 µL. Amplicons were purified from agarose using EuroGOLD Gel Extraction Kit © (EuroClone S.p.A.) following Manufacturer's protocol.

Cyt b amplicons obtained by end-point PCR were bidirectionally Sanger-sequenced at Eurofins Genomics (<https://www.eurofinsgenomics.eu/>). A GenBank-NCBI BLAST analysis of the obtained consensus sequences confirmed that the two reference scat samples corresponded to the expected Cyt b fragment of *L. lutra* (i.e., maximum identity > 99%; query coverage 100%).

Quantitative Real Time PCR (qPCR) assays were performed with an AB 7500 (Applied Biosystem) instrument. To set up the qPCR assay, a standard curve was first generated according to the qPCR assay developed for *L. lutra* in Italy (Molise) (Jamwal et al., submitted). All the amplification data were collected and analyzed with the SDS 7500 Real-Time PCR System Software (Applied Biosystems).

#### **otter eDNA qPCR assay**

Environmental water samples were run in triplicate with positive and negative controls (no template) at the same amplification conditions. The same assay included a negative control constituted by eDNA extracts of water samples (AQU01-05) from a site of ascertained absence of otter eDNA, and the likely occurrence of heterogeneous DNA and inhibitors. These samples were collected in the river Lambro, located more than 300 km far from the current otter range. Following the standard protocol for environmental samples (ref), 1L of water was filtered on 0.2 µm pore-sized membrane filters. eDNA was then extracted from the membranes using the DNeasy® PowerWater® Kit (Qiagen). For all the filtered water samples, 5 µL of eDNA template was used in the reaction to take into account low target eDNA concentration. To ensure that absence of *L. lutra* eDNA in water samples was not due to a failure of eDNA extraction, the same samples were also tested with qPCR

using the MiFish primer pairs, a set of universal 12SrRNA oligonucleotides for the untarget DNA metabarcoding characterization of fishes (Park et al., 2011). The amplification reaction conditions were the same set for LutCyt assay. Amplification profile included an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing-elongation for 1 min at 58 °C. All the amplification data were collected and analyzed with the SDS 7500 Real-Time PCR System Software (Applied Biosystems). Ct (Threshold Cycles) values were converted into counts (DNA copies) using the formula  $Counts = E^{(40-Ct)}$  where E is the efficiency of amplification calculated in Jamwal et al. (submitted).

Considering the limit of detection (LoD) reported by Jamwal et al. (submitted), we considered the sample negative (no otter detection) for DNACounts/microL < 5. For each triplicate, we considered a reliable result when at least 2/3 replicas were in concordance. To avoid any possible contamination with exogenous DNA, pre and post amplification phases were carried out in separate rooms, and every step was conducted in a laminar flow cabinet.

#### **Environmental variables**

A total of 15 environmental parameters were recorded at each sampling sites (Table 1). Altitude was measured with an Etrex 10 GPS, while river width, bank height, and channel width were measured using an ATN Laser ballistics 1000 range finder. Deeper Smart Sonar Pro+ was used to collect maximum and average water depth. A Kestrel® 4500 Pocket Weather Tracker was used to measure the relative humidity at each site. Surface velocity, temperature, percent of dissolved oxygen, dissolved oxygen (mg/l), total dissolved solid, salinity, acidity, and nitrate levels were all recorded using a YSI Pro20 meter outfitted with a galvanic dissolved oxygen sensor (YSI Inc). Prior to performing Principal component analysis, we standardized the variables.

Site n.	Parameters	Min	Max	Units
1	Altitude	2660	3819	meters
2	River Width	14	74	meters
3	Max Depth	0.6	7.3	meters
4	Avg Depth	0.85	4.80	meters
5	Surface velocity	0.46	1,24	m/s
6	Temperature	5.5	12.7	°C
7	Dissolved Oxygen	64.7	77.7	%
8	DO mg/L	7.29	9.34	mg/L
9	Total Dissolved Solid	73	257	mg/L
10	Salinity	0.05	0.19	SAL-ppt
11	Acidity	8.06	8.53	pH
12	Nitrate	0.19	0.69	NO3-N mg/L
13	Relative Humidity (RH %)	19.5	50.3	RH %
14	Bank heigth	0.5	3	Ft
15	Channel width	40	1100	meters

**Table 1:** Details of environmental parameters recorded at each sampling site

### Statistical Analysis

A principal component analysis (PCA) was run to investigate the relationship between environmental factors and presence/absence of otter at each site. Parameters having a factor loading higher than 0.7 were considered significantly crucial in contributing to a given principal component. A biplot was produced to explore the influence of each parameter on presence and absence sites. Significant combinations of environmental variables influencing the presence of otters at sites were then explored through a generalized linear model (GLM) with binomial family and a “logit” function, considering otter presence as the response variable (values 0 and 1). Prior to GLM a correlation matrix was produced to check for variables multicollinearity among 15 variables (Table 1). Variables with a correlation coefficient over 0.8 were then removed (Dissolved Oxygen (%), Dissolved Oxygen (mg/L) DO<sub>2</sub>, Maximum Depth (m) and Salinity (SAL-ppt)). The best model was selected by calculating the Akaike information criterion (AIC). Using  $\Delta$ AIC and AIC weights ( $\omega_i$ ), we computed AIC support values as well. Multiple variable combinations were prepared and run using

R package glmulti 1.0.8. (Calcagno & de Mazancourt, 2010). Model averaging for the best performing models ( $<2 \Delta$ AIC) was carried out in R package MuMin 1.43.1. (Bartón, 2020).

### RESULTS

#### *Traditional monitoring method & eNDA monitoring method*

Traditional monitoring method results showed that 53 % of the 15 sites were positive for otters, whereas 47% sites were negative for otters. For eNDA monitoring method qPCR was used to determine the amount of *L. lutra* eDNA in each sample. In terms of *L. lutra* eDNA presence, 11 out of 15 Himalaya samples gave positive findings which is 73% compare to 53 % traditional monitoring method.

There was no amplification signal in the negative controls (no otter DNA: AQU01-05 and no DNA). The MiFish qPCR technique, which was used to characterise untarget DNA metabarcoding in fishes (Miya et al., 2015), demonstrated effective DNA extraction for all examined samples, with the exception of positive and no template controls, as predicted.

Sampling site	eNDA monitoring method	Traditional monitoring method	Match
Shyok 1	Yes	No	No
Turtuk 1	Yes	No	No
Suru 1	Yes	No	No
Indus 1	Yes	Yes	Yes
Indus 2	Yes	Yes	Yes
Indus 3	Yes	Yes	Yes
Agam	Yes	Yes	Yes
Turtuk 2	Yes	Yes	Yes
Drass 3	Yes	Yes	Yes
Drass 2	Yes	Yes	Yes
Drass 1	Yes	Yes	Yes
Panamik 2	No	No	Yes
Panamik 1	No	No	Yes
Zanskar Up	No	No	Yes
Zanskar low	No	No	Yes
AQU01	No	No	Yes
AQU02	No	No	Yes
AQU03	No	No	Yes
AQU04	No	No	Yes
AQU05	No	No	Yes

**Table 2.** Comparison between eDNA and traditional survey results at each sampled site

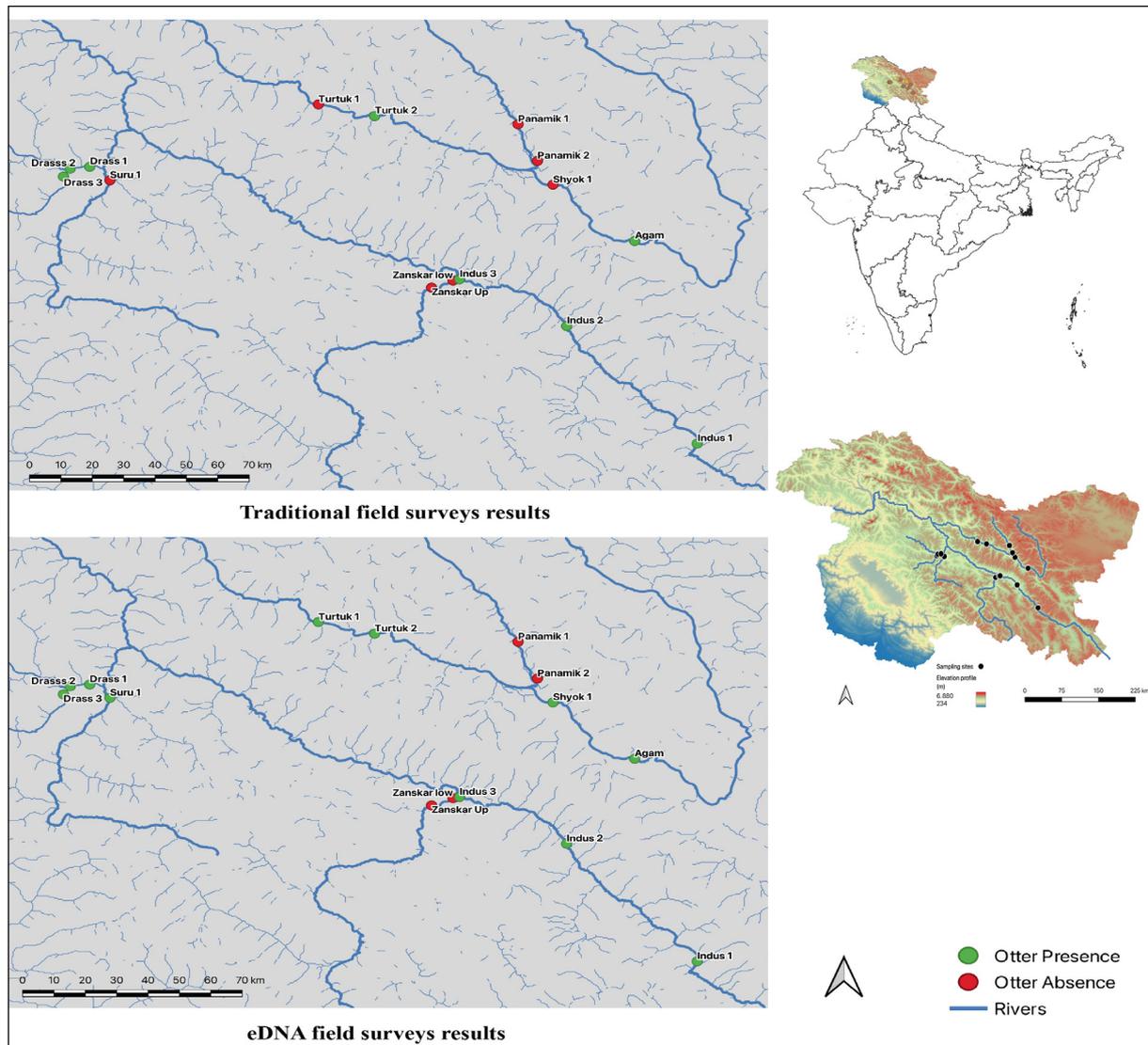


Fig. 2 Results from traditional (above) and eDNA (below) surveys at each site.

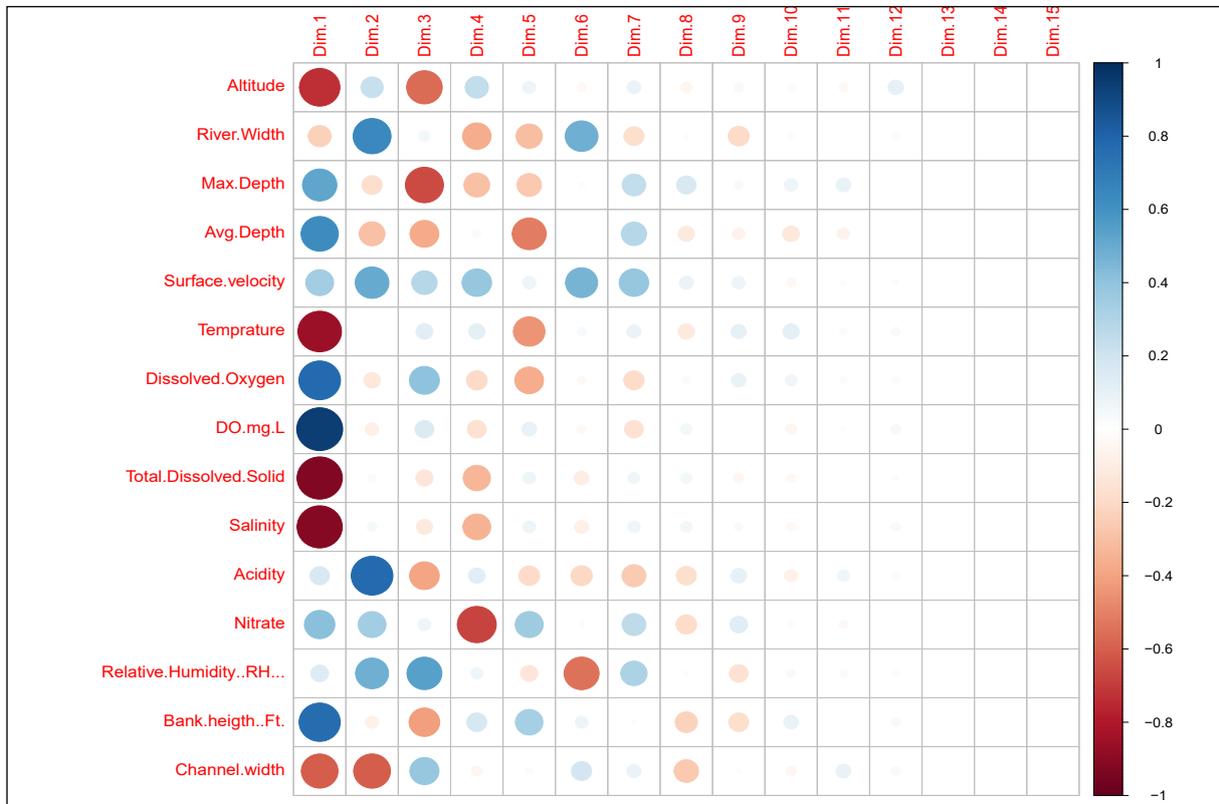
While using traditional monitoring method we found otter presence signs at 8 sites and at 7 sites we did not found any sign. Indus 2, Indus 3, Agam, Turtuk 2, Drass 2, Drass 1, Suru 1, were found to be otter positive and no otter presence signs were recorded at Shyok 1, Turtuk 1, Panamik 1, Panamik 2, Suru 1, Zanskar Up, and Zanskar. (Fig. 2)

When compared to eDNA monitoring method, Panamik 1, Panamik 2, Zanskar Up, and Zanskar Down were found to be otter negative and Indus 2, Indus 2, Indus 3, Agam, Turtuk 2, 3, Drass 2, Drass 1, Suru 1, and Shyok 1, Turtuk 1, Suru 1 were otter

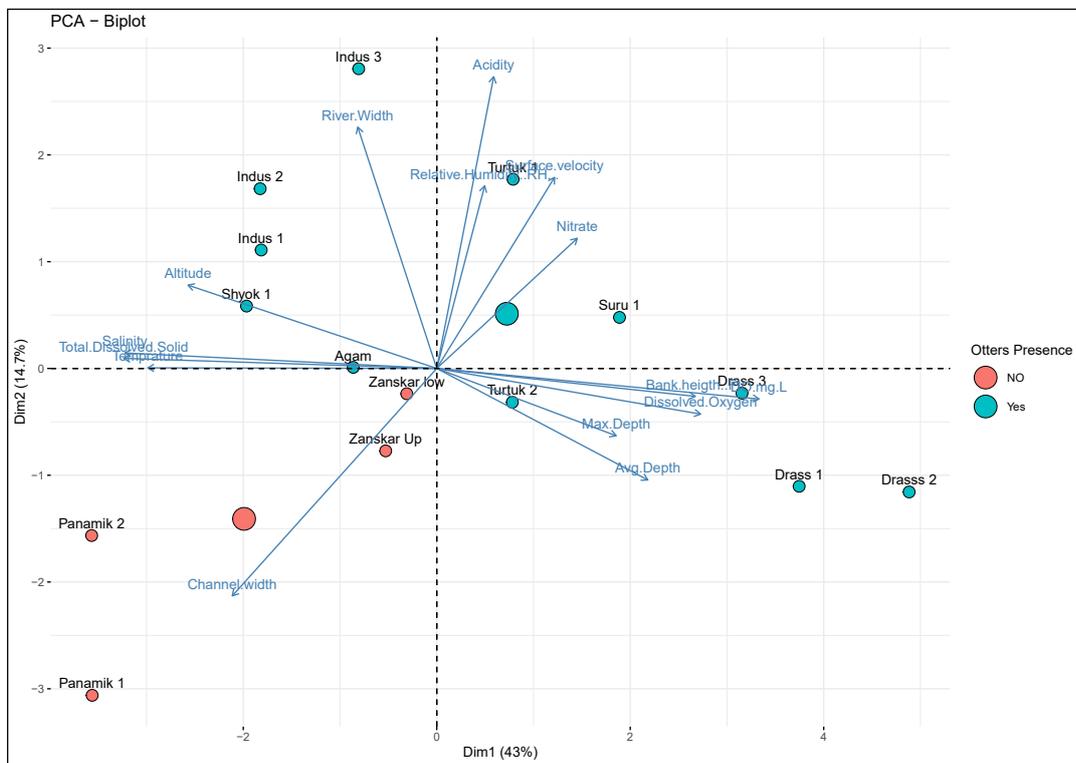
positive (Fig 2). We discovered three more otter positive sites, Shyok 1, Turtuk 1, and Suru 1, that had previously been recorded otter negative in traditional monitoring method. (Fig. 2)

**Environmental variables**

Fig. 3 shows the magnitude and sign of coefficients for each PC eigenvector of PCA run on environmental variables recorded at each site. Sites avoided of otter eDNA clearly segregated when both PC1 and PC2 axes were negative, explaining 57% of the variance (Fig. 4).



**Fig 3-** Relative contribution of each environmental variable to PC1-PC15. The colour and size of circles respectively indicate the sign (blue = positive, red = negative) and magnitude of coefficient in each eigenvector



**Fig. 4.** Biplot on the first two PCs of environmental parameters collected at each site.

	Model	AIC	dAIC	df	wi
1	eDNA ~ 1 + Acidity	10.46	0.00	2	0.55
2	eDNA ~ 1 + Acidity + Channel width	11.51	1.05	3	0.32
3	eDNA ~ 1 + Surface velocity + Channel width	15.08	4.62	3	0.05
4	eDNA ~ 1 + Channel width	15.46	5.00	2	0.04
5	(Null model) eDNA ~ .	24	13.54	12	<0.001

**Table 3** Models showing the most significant combination of environmental variables affecting the presence/absence of Edna. Only the top 5 models with a ΔAIC are shown

Parameters	Estimate	Std. Error	Adjusted SE	z value	p-value	parameter weigh	containing models
(Intercept)	-183.716	111.419	1.227.978	1.496	0.135		
Acidity	2.222.624	1.350.127	1.487.798	1.494	0.135	1	2
Channel.width	-0.0177	0.01975	0.02196	0.806	0.42	0.25	1

**Table 4.** Models averaged coefficients of the best performing models (<2 ΔAIC).

The biplot superimposed to the scatter plot evidenced that absence sites were characterized by high Channel width and low values of Nitrate, Acidity, Surface velocity, and Relative Humidity. Positive sites were instead widespread in the scatter plot and characterized by different parameters depending of the rivers. Instead, results from traditional monitoring method failed to identify any clear pattern relating environmental variables to presence/absence of otters.

**GLM**

We used glmulti to autorun different combinations of the variables, which tested 2100 models. Acidity, channel width, and surface velocity were the variables that contributed to the best performing top five GLM models explaining the occurrence of eDNA at the 15 sampling sites (Table 4). Specifically, the concentration of acidity (pH) was the most critical factor for the presence of otters (AIC = 10.45).

**DISCUSSION**

We first assessed the efficiency of eDNA target assay in detecting the elusive Eurasian otter in the Himalayan region. In the present study, traditional

monitoring method revealed 53% otter positive sites and 47% otter negative sites, while eDNA monitoring method revealed 73% otter positive sites and 27% otter negative sites. Our preliminary results suggest that an eDNA monitoring method based on the amplification of specific genetic markers from water samples can be a reliable tool for non-invasively monitoring the presence of elusive semi-aquatic species. For Eurasian otters, eDNA monitoring approach is a valuable supplement to monitoring method (track identification and scat count), which is both time consuming and prone to error (Davison et al., 2002; Hansen & Jacobsen, 1999). Additionally, when compared to traditional monitoring methods, the eDNA method discovered more otter positive sites (20%) which clearly indicates that the eDNA sampling method is more effective.

Detection of otters at an altitude of 3810 m using eDNA monitoring method showed that eDNA monitoring method is very robust even in extreme environments.

We discovered a strong correlation between otter presence in the field and molecular evidence along the Himalayan rivers. The scat count provided field evidence, while the molecular results

came from the analysis of biological material in the water, which was detected using real-time quantitative PCR (qPCR). We discovered an 80% match for positive and negative sites for traditional monitoring method and eDNA monitoring method, and other positive sites discovered using the eDNA method were exactly the same (100%) as those discovered using traditional monitoring method. Our research revealed that Panamik and Zanskar rivers were otter negative for both the traditional monitoring and the eDNA monitoring methods, but Suru was otter positive with the eDNA monitoring method while it was negative with the traditional monitoring method, indicating that the eDNA survey method was better when compared with the traditional method. When these results were compared with a previous study conducted in this region (Jamwal et al., 2016) Zanskar has always been otter negative, but Suru has become otter positively in contrast.

Despite the fact that many assays, particularly those published in previous years, used endpoint PCR, qPCR is known to perform best in case of sensitivity and quantitative data. In our case study, high sensitivity has proved to be crucial, since the low number of DNA copies detected from water samples. Despite the small number of samples and sites studied, we obtained 100% congruence of results for positive-to-positive site, implying that the protocol we used could be used with high reliability in areas where field surveys are impractical or prohibitively expensive.

The accuracy of site detection still needs to be evaluated thoroughly, taking into consideration specific environmental variables such as water temperature, water flow, water chemical composition, and all the other factors that could affect DNA detection. Moreover, a quantitative estimation of otter presence and/or the distance of the target organisms from the sampling site could be deepened with further analyses.

eDNA approach encompasses the entire workflow, from field sampling to results interpretation. One of the main challenges is the risk of contamination: molecular assays relying on DNA amplification are both highly sensitive, allowing the detection of DNA traces, and highly prone to cross-contamination, because of the exponential increase of DNA copies during the amplification step (Ficetola et al., 2015). Another difficulty stems from the inherent properties of environmental samples: eDNA degrades very quickly in certain conditions (e.g., high temperatures) (Bylemans et al., 2018; Kasai et al., 2020; Tsuji et al., 2017; van Bochove et al., 2020). Furthermore, the degradation rate is

difficult to estimate and is still being debated in the scientific community. Finally, even if the target DNA is present, the presence of DNA polymerase inhibitors common in environmental samples (e.g., humic acid) can impede DNA amplification (Bylemans et al., 2018; Kasai et al., 2020; Tsuji et al., 2017; van Bochove et al., 2020).

Several precautions were taken in this study, both in the field and in the lab, to avoid false positive and false negative results. To obtain a reliable test of the method for a target species, we strongly recommend 1) selecting sampling localities where the target taxon is surely absent or present; 2) using disposable instruments and carefully cleaning non disposable ones from one sample to the other. Given that the laboratory setup is a key factor that strongly influences the reliability of eDNA results, we also recommend that you: 3) use a pre-amplification room specific for low quantity DNA samples, as well as a post-amplification room with a unidirectional flow 4) use dedicated equipment for each phase; and 5) include a quality control for each experimental step (i.e. DNA extraction control).

The biplot superimposed to the scatter plot evidenced that absence sites were characterized by high Channel width and low values of Nitrate, Acidity, Surface velocity, and Relative Humidity and these are the key variables especially in identifying in the negative sites. Positive sites were instead widespread in the scatter plot and characterized by different parameters depending of the rivers.

Generalized linear model clearly stating that acidity, channel width (Table 4) is among the main variable that are affecting the occurrence of otter in Indian Trans-Himalayas.

Previous research on *L. lutra* established a correlation between pH and otter sprainting sites (Mason & Macdonald, 1987, 1989). Additionally, some studies demonstrated how DO reduced otter prey items and how nitrates impacted otter prey items (Acharya & Rajbhandari, 2014; Bedford, 2009). Studies also showed how temperature of the water has an effect on the metabolic rate of *L. lutra* (Kruuk et al., 1994).

However, in the Indian Trans-Himalayas we require extensive in-depth research to determine these relationship between otter occurrence and how they affecting the ecology of otters.

## CONCLUSION

We first assess the efficiency of eDNA target assay in detecting elusive mammals in natural aquatic ecosystems in the Himalayan region. Concordance between eDNA detection and traditional survey findings was seen in 80% of samples. On comparing

Traditional and eDNA survey method in Indian Trans-Himalayas we found eDNA increased detection of positive sites by 20% and same time eDNA detected 20% more positive sites which were not known to be otter positive sites in traditional surveys. Overall, our findings suggest that an eDNA approach based on the amplification of a specific genetic marker from water samples could be a reliable tool for monitoring the presence of otters in a non-invasive manner. Water samples can be beneficial. One step forward is the use of eDNA as a well validated approach for monitoring, which can be accomplished by modelling eDNA detection while taking into account the effects of intrinsic sample characteristics, field environmental conditions, and sample treatment in the molecular laboratory. Increasing efforts in this area can aid in correctly defining a threshold that accounts for positive and negative sites for otter presence. eDNA assays can be performed at peak abundance, at carefully selected sampling points along multiple river courses, to provide a reliable snapshot of the species present in

a catchment area. To fully capture solitary, rare, and invasive species, we currently recommend the use of eDNA metabarcoding in conjunction with other non-invasive surveying methods (such as camera traps) to maximize monitoring efforts (Sala et al., 2000).

We are confident that, under these strict conditions, eDNA investigations offer several advantages over direct or indirect field surveys of rare and elusive species that are worth using: no disturbance to the ecosystem or to the target species, a top concern regarding conservation or restoration measures and for social and ethical valuation of scientific surveys of biodiversity; less time-consuming; decisive when sampling is required in harsh environments; opportunity to detect multiple species from one environmental sample. To make sound wildlife management decisions (e.g., evaluating the effectiveness of conservation measures or regulating plans), an integrated approach that takes advantage of the sensitivity of molecular assays and the robustness of well-validated methods is required.

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## Concluding remarks

### Advances in otter conservation

Anthropogenic activities in the past and present had and continue to have a significant impact on freshwater ecosystems, making them potentially the most vulnerable to climate change (Dudgeon et al., 2006; Milly et al., 2005).

Freshwater habitats are under threat also as a result of crop irrigation, contamination, land-use activities, and infrastructure (Vörösmarty et al., 2010). The Lutrinae subfamily includes thirteen otter species found all over the world. As top predators they thrive in both marine and freshwater environments (Duplaix & Savage, 2018). Eleven surviving freshwater otter populations worldwide have declined significantly over the last century as a result of habitat destruction, maltreatment, and bioaccumulation of toxins (Sergio et al., 2006).

The IUCN Red List of Threatened Species includes the Near Threatened Eurasian otter (*L. lutra*), the Vulnerable Smooth-coated otter (*L. perspicillata*), and the Vulnerable Small-clawed otter (*Aonyx cinereus*). *L. perspicillata* and *A. cinereus* decline is caused by habitat loss and exploitation (De Silva et al., 2015; Wright et al., 2015) whereas the Asian population of *Lutra lutra* attributed to the species' sensitivity to recent intensification of human-induced threats (Roos et al., 2015). Investigating the effects of interaction of land-use and climate change on the vulnerability of Himalayan otters, our study showed how combining climate and land-use change in CCVAs can produce vastly different results than approaches focusing solely on climate change. Such mounting evidence appears particularly relevant both to reassess the species ranked as insensitive or favoured by climate change (e.g., *L. lutra*), and, more importantly, to focus more attention on those species (e.g., African otters *A. congicus* and *A. capensis*) that were already classified as highly vulnerable under climate change scenarios only (Cianfrani et al., 2018). The findings of this study also highlighted the advantages of conducting CCVAs using a “combined” framework, emphasizing the importance of incorporating

trait-based elements (e.g., species sensitivity) into purely correlative approaches (Foden et al., 2019; Pacifici et al., 2015). In fact, intrinsic factors were found to be far more important than extrinsic factors like habitat exposure in determining species vulnerability. Our findings showed that this pattern persists even after accounting for several sources of variability commonly associated with correlative models (e.g., alternative global circulation models and binarization schemes). Future research should concentrate on developing tools to measure adaptive capacity in a more reliable and generalizable manner than currently available methods (Williams et al., 2008). Including this “great absent” in CCVAs, as well as a stronger focus on modelling climate variability and extreme events, will undoubtedly advance this conservation tool.

Understanding the environmental conditions that promote species survival and coexistence will also be critical to conservation success, particularly in human-dominated environments (Karanth et al., 2010). By using SDM we produced reliable potential distribution maps for three over four Asian otter species (*Lutra lutra*, *Aonyx cinereus*, and *Lutrogale perspicillata*), as well as their spatial and climatic niche overlap and similarity. Two of the species are currently listed as Vulnerable on the IUCN red list, owing to an inferred population decline of more than 30% over the last 30 years, whereas *L. lutra* is highly fragmented and rare in its East Asian range (Loy, 2018) as is the case with many terrestrial mammals in South Asia (Schipper et al., 2008). Despite being well adapted to human-threatened landscapes, the extent of suitable habitat for each species ranged around 75% of their current range; all species face threats from wetlands loss, poaching, pollution, and a decline in prey biomass (Hussain et al 2018, Aadrean et 2018, Loy 2018). Our findings suggest that a strict protection and freshwater habitat restoration could likely promote the expansion of their current areas of occupancy and extent of occurrence in their Asian range.

### Advances in otter monitoring

Various monitoring methods have previously been used to study multiple mammals with different results. These includes transect sampling (Thomas et al., 2010), camera trapping (Silveira et al., 2003) and indirect sign survey (Sadler et al., 2004). Scat surveys have especially become a standard monitoring method and an accepted tool for monitoring otter distribution over time (Reuther, 2000). These monitoring methods usually involve labour and time and can be ineffective in the identification of the real biodiversity.

In this context environmental DNA (eDNA) survey can play a key role in overcoming the costs and limitations in species detection of the traditional monitoring methods (Rees et al., 2014).

Our findings proved for the first time that non-

invasive monitoring strategies based on molecular techniques could be successfully used to detect the presence of *Lutra lutra* in freshwater bodies, especially at medium-large scale.

However, it should be underlined that the use of eDNA approach to detect otter presence in water samples should be used with caution, especially when assessing an area using only a few sampling points or a small number of replicates. Future studies should be addressed to test eDNA surveys in different seasons and under different environmental conditions, as eDNA detection can be influenced by temperature and other environmental variables that can contribute to DNA degradation (Harrison et al., 2019).

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