University of Minho
School of Sciences

Gabriel Filipe Horta Rosa

Characterization of the diet of the European otter (*Lutra lutra*) in areas invaded and non-invaded by the signal crayfish (*Pacifastacus leniusculus*)

October 2024

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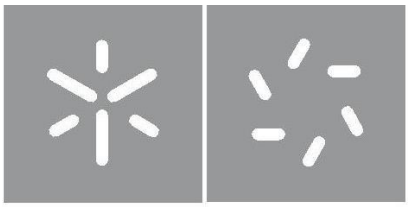

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Characterization of the diet of the European otter (*Lutra lutra*) in areas invaded and non-invaded by the signal crayfish (*Pacifastacus leniusculus*)

Master thesis in Biodiversity, Ecology and Global Change

Work made under supervision of:
Professor Doutor Ronaldo Sousa
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October 2024

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Caracterização da dieta da lontra-europeia (*Lutra Lutra*) em locais invadidos e não invadidos por lagostim-sinal (*Pacifastacus leniusculus*)

Resumo

Espécies invasoras são um grande desafio para a conservação da biodiversidade e funcionamento do ecossistema, afetando significativamente os ecossistemas de água doce. A introdução de uma espécie pode impactar significativamente o ecossistema, com efeitos tanto positivos quanto negativos, dependendo do contexto. O lagostim sinal, *Pacifastacus leniusculus*, é uma das espécies invasoras mais bem-sucedidas em ecossistemas de água doce Europeus. Este estudo teve como objetivo avaliar o impacto do lagostim sinal na distribuição, abundância e dieta da lontra-europeia, *Lutra lutra*, um predador de topo, em locais invadidos e não invadidos no Parque Natural de Montesinho e áreas adjacentes ao longo dos Rios Mente, Rabaçal, Tuela e Baceiro. Para essa finalidade foram amostrados excrementos de lontra e posteriormente analisados, também foram recolhidos dados sobre a abundância e biomassa de peixes, lagostim sinal, anfíbios e macroinvertebrados, juntamente com dados ambientais, em 30 locais distintos. Os resultados revelaram que a lontra-europeia prefere alimentar-se em locais invadidos pelo lagostim sinal, com maior abundância de excrementos de lontra correlacionada com a abundância de lagostim sinal. O aumento do consumo de lagostim sinal, especialmente nos meses de verão, quando a sua atividade é maior, sugere que as lontras estão a aproveitar a maior disponibilidade desta presa nos locais invadidos, confirmando a elevada plasticidade alimentar e comportamento oportunista da lontra-europeia. O lagostim sinal afetou negativamente as comunidades de macroinvertebrados, o que pode impactar espécies de peixes que destes se alimentam, reduzindo assim a disponibilidade de peixes para a lontra-europeia. No geral, este estudo indica que, embora o lagostim sinal afete negativamente as comunidades de macroinvertebrados, a sua abundância aumenta a disponibilidade de energia para a lontra-europeia, destacando as interações complexas entre espécies invasoras e predadores nativos.

Palavras-chave: alimentar; ecossistemas de água doce; espécies invasoras; excrementos; lagostim-sinal; lontra-europeia

Characterization of the diet of the European otter (*Lutra lutra*) in areas invaded and non-invaded by the signal crayfish (*Pacifastacus leniusculus*)

Abstract

Invasive species are a major challenge to biodiversity conservation and ecosystem functioning, significantly affecting freshwater ecosystems. Introducing an invasive species can significantly impact the ecosystem, with both positive and negative effects, depending on the context. The signal crayfish, *Pacifastacus leniusculus*, is among the most successful invasive species in European freshwater ecosystems. This study aimed to evaluate the impact of signal crayfish on the distribution and abundance of the European otter*, Lutra lutra*, a top predator, across invaded and non-invaded sites in the Montesinho Natural Park and adjacent areas along the Mente, Rabaçal, Tuela, and Baceiro Rivers. For this, European otter spraints were sampled and analysed, and data on fish, signal, crayfish, amphibian, and macroinvertebrate abundance and biomass, as well as environmental data, were recorded in 30 sampling sites. Results revealed that the European otter exhibited a foraging preference for sites invaded by the signal crayfish, with increased European otter spraints abundance correlating with signal crayfish abundance. The increased consumption of signal crayfish, especially during the summer months when their activity is higher, suggests that European otters are capitalizing on the greater availability of this prey, confirming with the European otter's high foraging plasticity and opportunistic feeding behaviour. Signal crayfish also negatively affected macroinvertebrate communities. This decline could affect fish species that primarily consume macroinvertebrates, reducing fish availability for the European otter. Overall, this study indicates that while signal crayfish negatively affect macroinvertebrate communities, their abundance increases the energy availability for the European otter, highlighting the complex interactions between invasive species and native predators.

Keywords: freshwater ecosystems; European otter; foraging; invasive species; signal crayfish; spraints

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1 – Introduction

1.1 – Importance of freshwater ecosystems

Despite covering approximately only 1% of the Earth's surface, freshwater ecosystems are crucial for supporting biodiversity, hosting almost 10% of all animal species and over a third of vertebrate species, while also playing an essential role in sustaining environmental health, economic prosperity, and human well-being (Dudgeon et al., 2006; Dudgeon, 2019; Tickner et al., 2020; Nogueira et al., 2021). However, these ecosystems are increasingly threatened by human activities, resulting in habitat loss and fragmentation, pollution, overexploitation of resources, and climate change (Dudgeon et al., 2006). These factors, combined with the introduction of invasive alien species (IAS), are jeopardizing the conservation of native species, leading to a significant decline in freshwater species populations, with recent reports showing a sharper decrease compared to marine and terrestrial species (Dudgeon et al., 2006; Almond et al., 2022; Dudgeon & Strayer, 2024). Notably, the most recent World Wide Fund for Nature report on the Living Planet Index reveals an 85% decline in the abundance of analysed vertebrate populations in freshwater ecosystems between 1970 and 2020 (WWF, 2024).

1.2 – Invasive alien species and their positive and negative impacts

The introduction of a species into a new location, either deliberately or accidentally, may have consequences for the ecosystem in question, with both positive and negative effects, depending on the context, which can lead to significant ecological, economic, and social changes (Charles & Dukes, 2008; Sax et al. 2022). Many of these species eventually become invasive, causing disruptions to the structure and functioning of ecosystems, including changes in the food chain (Simberloff et al., 2013). IAS are species with invasive potential and their introduction and spread has been facilitated by human action through activities such as aquaculture, ballast water, construction of canals, global commerce, sport fisheries, tourism, among many others (Charles & Dukes, 2008; Simberloff et al., 2013). These activities have substantially accelerated the rate at which these species are introduced, become established, and spread (Strayer, 2010), making them a severe threat to aquatic ecosystems and native biodiversity by disrupting energy flux and

nutrient cycles, altering hydrology, degrading habitat complexity, and affecting native communities through competition, hybridization, parasitism, infection, and predation (Andersen et al., 2004; Charles & Dukes, 2008; Ehrenfeld, 2010; Sousa et al., 2009; Strayer, 2012; Gallardo et al,. 2016). For instance, in mountainous streams, microbial decomposers and invertebrate detritivores drive key ecosystem processes such as leaf litter decomposition, a crucial mechanism for the flux of energy and carbon, which can be directly affected if IAS consume leaf litter or prey on the organisms responsible for decomposition (Carvalho et al., 2016).

The invasion process and its potential effects are influenced by a variety of ecological, evolutionary and social factors, which can amplify or reduce the impacts of IAS over time and space, with these impacts varying significantly based on their population size, geographic range, and position within the food web (With, 2002; Strayer et al., 2006; Gallardo et al., 2016; Jarnevich et al., 2021). IAS that occupy higher trophic levels often exert top-down control, affecting lower-level organisms and altering ecosystem structure (Pace et al., 1999). For example, invasive predators such as rainbow trout (*Oncorhynchus mykiss*), alburnus (*Alburnus alburnus*), and the spiny water flea (*Bythotrephes longimanus*) have been linked to a significant decline in fish, benthic invertebrates, and zooplankton abundance, which in turn leads to the proliferation of phytoplankton. (Gallardo et al., 2016). Conversely, IAS that affect primary production or nutrient cycles can create bottom-up effects, reducing or increasing energy availability for higher trophic levels, depending on the context (Heath et al., 2014). This is the case of some invasive aquatic plants, such as water chestnut (*Trapa natans*), that despite providing habitat or food for herbivores, can create dense shade and high respiration on riverbeds, drastically reducing dissolved oxygen levels and causing frequent and severe hypoxic or even anoxic conditions that severely impact native macroinvertebrates and fish, thus impacting upper trophic levels (Strayer, 2010; Gallardo et al., 2016). Furthermore, IAS often cause major landscape changes, as seen in the case of the North American beaver (*Castor canadensis*) in Tierra del Fuego (Argentina and Chile), which transformed ecosystems by constructing dams, altering waterway habitats and introducing large amounts of plant litter affecting riparian and submerged vegetation, macroinvertebrates and fish species (Henn et al., 2016). Another well-known example of physical ecosystem engineering in aquatic ecosystems is the presence of invasive bivalves that can

significantly affect benthic communities with positive and negative effects being described at several trophic levels, from plants to fish species, depending on the ecological context (Sousa et al., 2009). However, not all introductions lead to a negative impact. In fact, and although IAS are often linked to native species extinctions, particularly in island and freshwater ecosystems (Schlaepfer et al., 2011), they also contribute to regional biodiversity through their own immigration (Sax et al. 2022). Many IAS, such as deer, pheasants, and fish, support recreational activities such as hunting and fishing, which promote mental and emotional well-being, and provide essential food resources in many regions of the planet (Sax et al., 2022). Additionally, conservation benefits can also emerge from IAS introductions, which may offer energetic benefits (Almeida et al., 2012). This pattern is evident in Mediterranean countries like Spain, Portugal, and Italy, where recovering river European otter (*Lutra lutra*) populations have adapted to the abundance and easier capture of non-native prey such as the red swamp crayfish (*Procambarus clarkii*) (Beja, 1997). In temperate regions, however, the anticipated decline of native fish fauna, coupled with the rise of IAS, may negatively affect native fish, invertebrates and aquatic plants, but could potentially enable river otters to recolonize parts of their former native range due to the abundance and ease of capturing non-native prey (Almeida et al., 2012).

These landscape and ecosystem changes highlight the complexity of IAS impacts, which require detailed study at both the individual, population, community and ecosystem levels to fully understand their ecological consequences (Simberloff et al., 2013). The environmental context in which an invasion occurs can lead to vastly different outcomes, with some species having high impacts in certain locations while having minimal effects in others (Ehrenfeld, 2010; Strayer, 2012; Sousa et al., 2024). In fact, recent studies highlight the need to study the spread and impacts driven by invasive species at the population level, rather than solely focusing on the species level (Sousa et al., 2024).

1.3 – Signal crayfish in Europe, with particular emphasis in Portugal

Several non-native crayfish species have been introduced in Europe, with the signal crayfish (*Pacifastacus leniusculus*) being one of the most widespread and problematic IAS (Figure 1) due to its significant ecological and economic impacts (Sousa et al., 2019). Signal crayfish activity varies seasonally, with the period of greatest activity and abundance being between May and October and exhibiting reduced activity during cooler months (Johnson et al., 2013). This species is native to North America and was introduced to Sweden during the 1960s to replace the native crayfish (*Astacus astacus*), which was becoming increasingly scarce due to the crayfish plague (Anastácio, 2021). It spread across the European continent autonomously within river basins, but also frequently through human action via new illegal introductions from North America and other regional or international translocations (Anastácio, 2021). It entered Portugal, spreading from Spain where it is abundant and was introduced in 1974. The species was first detected in Portugal in 1997 in the Maçãs River, near the Quintanilha village, northeast of Portugal (Anastácio, 2021). This IAS causes changes in the trophic chain and negatively affects other invertebrates, as the increased abundance of the signal crayfish leads to a decrease in the taxonomic and functional diversity of invertebrates and other native species due to predation and competition (Meira et al., 2019; Sousa et al. 2019; Carvalho et al., 2022). In addition to impacting lower trophic levels, crayfish species can also serve as food for a large number of predators, including fish, mammals, and birds (Britton et al., 2017).



Figure 1 – The non-native signal crayfish (*Pacifastacus leniusculus*). Photo by Ronaldo Sousa.

1.4 – European otter distribution and dietary preferences

The European otter (Figure 2) is a native species and a piscivorous predator with a high dietary plasticity (Krawczyk et al., 2016). This species is distributed across Europe, Asia, and Africa and colonized various biomes, from tundra to tropical forests (Jo et al., 2017). It is associated with freshwater wetlands but can also occur in saltwater coastal areas. In Portugal, the species has a relatively wide distribution (Beja, 1997). Due to its high dietary plasticity, the otter preys on almost all species present in rivers, such as fish, amphibians, reptiles, insects and other invertebrates, birds, and even other mammals (Krawczyk et al., 2016). The significant decline in otter populations across Europe since the 1950s has greatly stimulated research into the species distribution and ecology, with diet emerging as a central focus of study (Beja, 1997; Clavero et al., 2003).



Figure 2 – The European otter (*Lutra lutra*). Photo by Bernard Landgraf.

In many habitats the otter exhibits a preference for smaller and slower-moving species, which offer higher hunting success, likely because they are more abundant and easier to catch (Kruuk, 1995). The optimal approach to foraging focuses on the balance between the benefits and costs associated with feeding (Griffiths, 1980). While the benefits, such as the weight or energy content of the food, are relatively straightforward to measure, the costs are more complex to assess (Griffiths, 1980; Kruuk 1995; Beja, 1997). These costs, including the time and energy spent searching for, pursuing, and subduing prey, are closely linked to prey size with larger prey being generally harder to catch and subdue, leading to higher handling costs and lower capture success rates as the difficulty of subduing them increases (Griffiths, 1980). Therefore, predators must weigh these foraging costs against the potential benefits of capturing a given prey. In Mediterranean regions, where red swamp crayfish are more abundant and easier to catch, otters have adapted to include this IAS in their diet (Beja, 1997; Clavero et al., 2003; Almeida et al., 2012), though less is known about the impact of the signal crayfish on otter populations.

A predator's diet can vary based on habitat type and along the latitudinal gradient. The impact of the latitudinal gradient in species diversity, characterized by increasing richness of flora and fauna from the poles to the tropics, plays a significant role in shaping predator diets (Clavero et al.,

2003). Prey abundance and diversity are crucial factors determining a predator's niche width, so predators with wide geographic ranges, like the European otter, may exhibit variations in dietary breadth in response to changes in prey availability (MacArthur & Pianka, 1966; Schoener, 1971). In Europe, this variation in otter diets can be linked to two major climatic zones: the temperate region in central and northern Europe and the Mediterranean region in the south (Clavero et al., 2003). While temperate climates are marked by cold winters and mild, rainy summers without summer droughts, Mediterranean climates are transitional between cold temperate and dry tropical zones, featuring a unique combination of hot, dry summers and cool, humid winters, conditions that influence prey community composition and structure (Clavero et al., 2003). In Mediterranean ecosystems, the limited and unpredictable availability of fish during the intense summer drought, rather than the abundance of alternative prey, plays a more significant role in expanding the dietary diversity of otters (Clavero et al., 2003). Many streams and small rivers dry up or fragment into isolated pools, drastically reducing fish availability (Prenda & Gallardo, 1996; Pires et al., 1999). Additionally, the high variability in precipitation and temperature patterns, characteristic of Mediterranean regions, greatly impacts freshwater ecosystems, leading to unpredictable fish availability (Prenda et al., 2001). Under these harsh conditions, the otter's diet becomes most diverse during the summer, compensating for the reduced fish populations with increased predation on alternative prey, including crayfish, amphibians, and aquatic invertebrates (Erlinge, 1986; Ruiz-Olmo et al., 2001; Clavero et al., 2003).

The European otter’s diet is influenced by the latitudinal gradient, yet it still varies significantly depending on habitat type. Along sea shores, fish are the most frequently consumed prey, but in lakes, rivers and streams, amphibians and crustaceans may play a larger role in the diet (Jędrzejewska et al., 2001). In lakes, rivers and streams, otters tend to behave as more generalist predators compared to coastal areas, where fish dominate their prey selection (Jędrzejewska et al., 2001). This pattern likely reflects the varying abundance and predictability of fish resources across habitats, with the highest availability along coastal areas, intermediate levels in temperate freshwater habitats, and the lowest in Mediterranean freshwater systems (Clavero et al, 2003). Such differences influence the degree to which otters rely on fish, their preferred prey (Jędrzejewska et al., 2001; Clavero et al., 2003).

1.5 – Methods to assess European otter population abundance and diet

A common approach for studying carnivore diets is through spraint analysis and identification of prey remains, as it offers insights into dietary composition and, through the number of fresh spraints, can provide an indication of abundance. The use of spraints as an indicator of habitat preference and population density has been debated for years, with various authors suggesting that fresh spraints may reflect European otter activity and abundance (Mason and Macdonald, 1986; Ruiz-Olmo et al., 2001). Some researchers have reported that spraint density correlates with population size, frequency of visits to specific areas, and habitat preferences (Hutchings & White, 2000; Hong et al., 2018). However, others have cautioned that spraint density may not always reliably indicate otter population status, highlighting the need for further research to better understand these relationships and that it should remain an indirect measure rather than a precise count of population density (Kruuk & Conroy, 1987).

The use of morphological analysis to characterize diet relies on five methods to quantify the relative importance of prey in spraint analysis: percent of biomass (Jędrzejewska et al., 2001), relative frequency of occurrence (RFO) (Clavero et al., 2004), frequency of occurrence (FO) (Clavero et al., 2004), frequency of individuals (Lanszki & Kormendi, 1996), and bulk estimate (Taastrùm & Jacobsen, 1998). Among these, biomass provides the most accurate estimate of actual diet, highlighting prey with the highest energetic significance (Krawczyk et al., 2016). FO is particularly valuable for recording rare dietary components, irrespective of their biomass (Krawczyk et al., 2016), and RFO is especially useful for comparing diets across different regions (Clavero et al., 2003). Although spraint analysis remains the most common method for studying carnivore diets, including that of the European otter, it suffers from several well-recognized caveats. These include potential misidentification due to similarities with other species spraints (Fernández et al., 1997; Harrington et al., 2010), variations in prey retention times (Carss & Parkinson, 1996), and the challenge of statistical independence when multiple samples are produced by a single individual (Carss & Parkinson, 1996). Despite these limitations, dietary studies still heavily rely on spraint analysis. However, advances in molecular techniques, such as next-generation sequencing (NGS), offer a solution (Jang-Liaw, 2021). Metabarcoding using NGS improves resolution, detects rare events, and identifies small or inconspicuous prey without

relying on the identification of remaining prey fragments (Schuster, 2008; Jang-Liaw, 2021). While high-confidence methods like metabarcoding and DNA analysis provide more accurate species identification (Martínez-Gutiérrez et al., 2015), their high cost and technical demands mean that spraint studies continue to be the primary source of dietary data for carnivores (Krawczyk et al., 2016). Understanding the diet of a given species is an important step to better understand autecological processes with eventual pay-offs towards its conservation.

1.6 - Objectives of the study

This study stems from the need to better understand the ecological effects mediated by the signal crayfish on the European otter population in the Montesinho Natural Park and adjacent areas along the Mente, Rabaçal, Tuela, and Baceiro Rivers.

In areas with a high abundance of crayfish, regardless of the species, these are important prey for the otter and represent a significant proportion of the total number and biomass of prey consumed annually by otters, especially in Mediterranean regions (Beja, 1997). Given the extensive research on otter interactions with red swamp crayfish (Beja, 1997; Almeida et al., 2012; Carvalho et al., 2016), this study aims to assess the role of the signal crayfish in the European otter's diet and examine how this IAS may influence European otter populations, and, consequently, the entire trophic chain, since the European otter is the top predator in these ecosystems. Therefore, analysing European otter spraints is essential to determine how the presence and abundance of the signal crayfish and other prey affects the European otter’s dietary patterns and distribution. In addition, we aimed to investigate the impacts of signal crayfish on the other trophic groups considered in this study, such as fish, amphibians and macroinvertebrates, as well as how various environmental factors influence the abundance of spraints of European otter and the signal crayfish.

This study was conducted under the MULTI-CRASH project: "Ecological cascades at multiple dimensions triggered by an invasive species in pristine habitats," funded by the Foundation for Science and Technology, which aims to assess trophic chains invaded and not invaded by the signal crayfish.

2 - Material and Methods

2.1 - Study area and sampling sites

This study was conducted in the Montesinho Natural Park and its adjacent downstream areas, located in northeast Portugal (Figure 1). The park, created in 1979, spans approximately 748 km² and is recognized for its high biodiversity, hosting numerous species of both animals and plants. It was originally established to protect birds, terrestrial vertebrates, and plants but also contains vital habitats for aquatic species, such as the endangered pearl mussel (*Margaritifera margaritifera*), that are now threatened by invasive species such as the signal crayfish (Cabrita, Cunha & Henriques, 2000; Sousa et al., 2019; Nogueira et al., 2021). The altitude in the Montesinho Natural Park ranges from 438 m to 1,481 m and its climate is predominantly Mediterranean with an Atlantic influence, marked by significant seasonal variability, with average annual temperature between 8.5 ºC and 12.8 ºC, and precipitation ranging from 1,000 mm to 1,600 mm, peaking in winter and nearly absent during summer (Oliveira et al., 2012; Sousa et al., 2015).

For this study, 30 sites were surveyed (Figure 3).

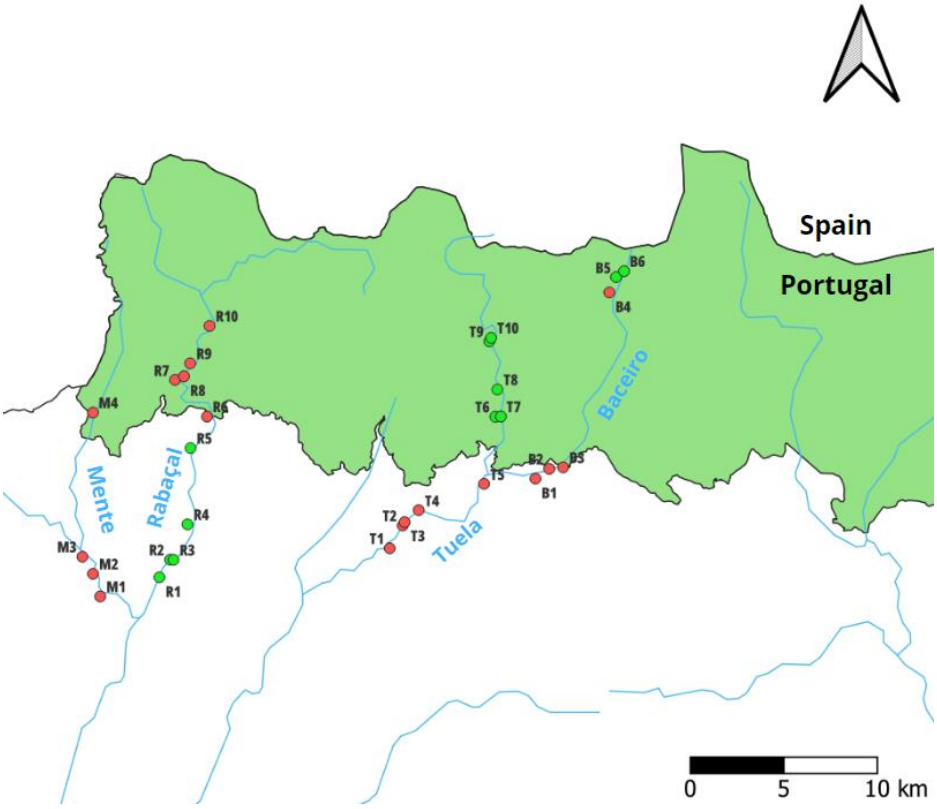

Figure 3 - Map of the surveyed area showing the location of the 30 sampling sites (18 invaded - red; 12 non-invaded - green) in Mente (M), Rabaçal (R), Tuela (T) and Baceiro (B) Rivers. Map produced using QGIS software (QGIS Development Team, 2022).

The region has a low human population density, with subsistence agriculture being the main land use, making it one of the less human disturbed areas in Portugal (Nogueira et al., 2021). The study area encompassed four rivers (Mente, Rabaçal, Tuela and Baceiro) within and around the Montesinho Natural Park. These rivers, which originate in Spain, are tributaries of the Tua River in the Douro River basin, with total lengths of 57 km, 88 km, 102 km and 60 km, respectively (Sousa et al., 2019). The rivers share similar climatic patterns and are subject to abrupt seasonal changes in river flow, with maximum values registered in winter or early spring and minimum values in late summer or early autumn (Sousa et al., 2018). The rivers feature a mix of pools and riffles, with substrates primarily composed of cobbles, boulders, gravel, and sand, while sections near weirs and reservoirs, where water tends to be more stagnant, have substrates largely

consisting of mud and with a much higher organic matter content (Sousa et al., 2018; Sousa et al., 2019; Sousa et al., 2020).

These watercourses are part of the Natura 2000 network and are considered critical habitats for many endangered species, such as the Iberian desman (*Galemys pyrenaicus*), the pearl mussel (*Margaritifera margaritifera*), the Northern Iberian spined loach (*Cobitis calderoni*), the dragonfly (*Macromia splendens*), among several other species (Sousa et al., 2015; Sousa et al., 2019). However, the biodiversity of these rivers has been impacted in recent years by droughts, habitat loss, and fragmentation, along with the introduction of invasive species, such as the red swamp crayfish and the signal crayfish. The latter has spread through all four studied rivers, first detected in these areas in 2013 and nowadays present in significant numbers (Sousa et al., 2019; Anastácio, 2021). Despite the invasive pressure, the riverbanks remain rich in vegetation, dominated by willows (*Salix spp.*), alders (*Alnus glutinosa*), ash trees (*Fraxinus angustifolia*), and poplars (*Populus nigra*) (Sousa et al., 2019), contributing to the overall ecological integrity of these habitats.

2.2 – European otter spraints sampling and analysis

The European otter spraints were sampled and analysed following the methodology outlined in Macieira (2023). In summary, the sampling of the European otter spraints was carried out by two experienced researchers along transects on the banks of four rivers (Mente, Rabaçal, Tuela and Baceiro) within the study area during July and August of 2022. The sampling covered 30 sites (Figure 3), with each site spanning a river stretch of approximately 600 meters long (or 1 hour of prospecting), with 18 located in sites invaded by the signal crayfish and 12 in non-invaded sites. GPS coordinates were recorded for all spraints (Figure 4), and only fresh samples were collected for dietary analysis. A total of 196 spraints were collected and preserved in 96% ethanol.

The number of fresh spraints found in each river stretch is going to be used as an indirect index to indicate European otter abundance in this study.



Figure 4 – European otter (*Lutra lutra*) spraints. Photo by Ronaldo Sousa.

Two spraints were discarded as they originated from another species, leaving a total of 194 European otter spraints analysed, with 22 collected from the Baceiro River, 74 from the Mente River, 60 from the Rabaçal River, and 38 from the Tuela River, with a total of 337 occurrences, with 282 in invaded sites and 55 in non-invaded sites.

The spraints analysis followed the standardized procedures described by Beja (1997). Using sterilized tweezers, samples were placed into labelled Petri dishes and dried in an oven at 45°C for 48 hours. Once dried, the samples dry weight was measured with an analytical balance (d=0.1 mg). The samples were then moistened with water and a drop of detergent in a cuvette to remove mucilaginous material and then washed over a 0.5 mm mesh sieve. Fragments were separated by taxonomic group and transferred to Petri dishes, divided into quadrants. After drying for 24 hours, morphological analysis was performed using a binocular stereoscope to identify key structures like fish scales, jaws, opercula, or crayfish uropod's (Beja, 1997). Spraints were

categorized into seven main taxonomic groups: fish, amphibians, birds, reptiles, crustaceans, other invertebrates and mammals. The 'crustaceans' group specifically referring to the invasive signal crayfish, as it was the only crustacean species identified, while the 'other invertebrates' category included several species such as aquatic insects and arachnids.

The minimum number of individuals per prey type was estimated based on these key structures, and when absent, fragments were assigned to a single individual. Identified fragments were separated, stored in labelled zip bags by taxonomic group, and weighed to determine weight per taxonomic group in each spraint. Every identified prey type was recorded as an "occurrence" and expressed in two ways: frequency of occurrence (FO), calculated by dividing the number of occurrences of a specific prey type by the total number of spraints analysed, and relative frequency of occurrence (RFO), determined by dividing the number of occurrences of a specific prey type by the total number of all prey occurrences (Macieira, 2023).

2.3 – Sampling of the fish, signal crayfish, macroinvertebrates and amphibians communities

The fish community survey was conducted using electrofishing, following the methodology outlined in Oliveira (2023). In summary, the sampling of the fish community was carried out in 30 sites (Figure 3) by three experienced researchers with portable Hans Grassl equipment equipped with a pulsed DC-300-600 V generator, during July and August of 2022. Each sampling session lasted 20 minutes, during which fish were temporarily stunned and collected for measurement. The researchers recorded the weight, length, species identification, and the number of individuals per species at each site. After measurements, the fish were safely released without harm.

To determine the distribution of signal crayfish populations, following the methodology outlined in Alves (2024), 30 sites were sampled, during July and August of 2022, across the four rivers: 4 in the Mente River, 10 in the Rabaçal River, 10 in the Tuela River, and 6 in the Baceiro River (Figure 3). At each site, a river stretch of 50-100 meters was selected, encompassing all available habitats, including pools, runs, and riffles, from the banks to the center of the river channel. The signal crayfish were captured using 8 funnel traps (Figure 5A) per site, designed specifically for decapod crustaceans. Each cylindrical trap (43 cm diameter, 22 cm height; 1.5 cm mesh) was baited with

fish remains and set for 24 hours. The captured signal crayfish (Figure 5B) were counted, weighted, measured for total length (from the rostrum tip to the rear edge of the telson), and sex determined following Sousa et al. (2019). Signal crayfish abundance at each site was recorded as the total number of individuals per catch per unit of effort (ind. CPUE).



Figure 5 – Funnel traps (A) used during signal crayfish (*Pacifastacus leniusculus*) sampling (B). Photo by Ronaldo Sousa.

The macroinvertebrate community was sampled during July and August of 2022 in 30 sites (Figure 3) using a hand net, following the methodology outlined in Nogueira (2023), across various microhabitats, including riverbanks, centre of the channel, areas with different sediment types and the presence or absence of macrophyte, with the kick sampling technique. The sampling effort was standardized by timing each collection, with abundance expressed as the total number of individuals per catch per unit of effort (ind. CPUE). The hand net used had a 25 cm diameter opening and a mesh size of 500 µm. Afterwards, the collected samples were passed through a sieve, and the captured organisms were preserved in alcohol. In the laboratory, the macroinvertebrates were separated taxonomically and weighed after drying in an oven at 60ºC for 24 hours to determine their biomass, following the method outlined by Sousa et al. (2006).

The amphibian community was sampled at the end of spring 2023 in the same 30 sites as the spraints sampling (Figure 3). At each site, a 100-meter stream reach was thoroughly surveyed for

amphibians, including larvae, juveniles, and adults. Four observers walked slowly along the banks and waded through shallow sections of the stream, using dip nets to collect aquatic larvae and adults, and they systematically searched stream banks for terrestrial adults, using torches to inspect cavities and shaded areas. All amphibians encountered were identified to species level in situ and then released. In a few cases, unidentified larvae were preserved for laboratory identification. Since individual weights could not be recorded during sampling, species-specific weights were applied based on previous studies by Clavero et al. (2004) and Blanco-Garrido et al. (2007). The following constant values were attributed to adult individuals: *Bufo spinosus* – 50g (Clavero et al., 2004); *Lissotriton boscai* – 10g (Blanco-Garrido et al., 2007); *Pelophylax perezi* – 20g (Clavero et al., 2004); *Rana iberica* – 20g (Clavero et al., 2004); and *Salamandra salamandra* – 10g (Blanco-Garrido et al., 2007). For juveniles and larvae, their weights were estimated as 30% and 5% of the adult weight, respectively.

2.4 – Sampling of environmental data

Environmental data was collected in 30 sampling sites (Figure 3), during July and August of 2022, following the methodology outlined in (Nogueira, 2023). This information was used to correlate with European otter and signal crayfish data. The parameters measured *in situ* at all sites included temperature, oxygen, water conductivity, total dissolved solids (TDS), and pH, using a YSI EXO 2 multi-parameter probe. Additionally, each site underwent an *in situ* River Habitat Survey (RHS), which provided information on altitude, Habitat Quality Assessment (HQA), Habitat Modification Score (HMS), and the Riparian Quality Index (RQI) following Raven et al. (1998), with data collected along a standard 500-meter length of river channel at each site. For a full description of the HQA, HMS and RQI scores consult Table S1, S2 and S3.

2.5 – Data analysis

Analysis of deviance for generalized linear models (GLM) (Quasi-Poisson and negative binomial) and one-way ANOVAs (Analyses of variance) tests were conducted based on assessments of data homoscedasticity and normality (Shapiro-Wilk test) to guide model selection. Logarithmic transformations were applied when needed.

European otter spraints abundance in relation to signal crayfish presence, abundance, biomass, and various environmental factors was analysed using negative binomial models (using package "MASS", version 7.3-58.4). Biomass data was subjected to a logarithmic transformation.

Due to the ecological context, a redundancy analysis (RDA) was also performed to examine how the European otter spraint abundance is related to the abundance and biomass of the different trophic groups considered in this study (fish, signal crayfish, macroinvertebrates and amphibians). This analysis result was obtained with the use of a PERMANOVA (9999 permutations) test (using package "vegan", version 2.6-6.1). All plotted data was obtained using package "ggplot2", version 3.4.2.

For comparisons between invaded and non-invaded sites in terms of FO and RFO variations, GLM (Quasi-Poisson family) models and one-way ANOVAs tests were applied as according to data homoscedasticity and normality; a Levene test (using package "CAR", version 3.1-2) was conducted alongside the one-way ANOVAs tests to verify homogeneity of variances. GLM (Quasi-Poisson family) models were also used to evaluate FOs and RFOs variations with the abundance and biomass of the different trophic groups (fish, amphibians, signal crayfish, and macroinvertebrates).

The influence of signal crayfish abundance and biomass on the abundance and biomass of different trophic groups (fish, amphibians and macroinvertebrates) was analysed using GLM (Quasi-Poisson family) and negative binomial models. Additionally, GLM models (Quasi-Poisson family) were applied to assess the relationship between signal crayfish abundance and biomass, and environmental factors.

All statistical analysis were carried out using the software RStudio Team (2022).

3 – Results

3.1 – European otter distribution across invaded and non-invaded sites

The abundance of European otter spraints and signal crayfish obtained along the sampling points in Mente, Rabaçal, Tuela, and Baceiro Rivers varied from 0 to 19, and from 5 to 458, respectively (Table 1).

Table 1 – Values for the total amount, mean, maximum, and minimum of signal crayfish, in invaded sites, and European otter (*Lutra Lutra*) spraints abundance, across all sites.

	Signal Crayfish	Otter - Invaded	Otter – Non-Invaded
Total	3431	163	31
Mean	180.6	8.2	2.6
Maximum	458	19	9
Minimum	5	0	0

There is a significant positive difference in European otter spraints abundance between invaded and non-invaded sites (Figure 6 and Table 2). There is also a significant positive relationship between European otter spraints abundance and signal crayfish abundance (Figure 7A) and biomass (Figure 7b) (Table 2).

Table 2 - Results of the analysis of deviance of GLM models for the European otter (*Lutra lutra*) spraints abundance and the presence, abundance and biomass of signal crayfish (*Pacifastacus leniusculus*). The asterisk indicates significant values ($p<0.05$).

Signal crayfish	Otter spraint abundance
Presence	z value = 2.940, p = 0.003 *
Abundance	z value = 2.082, p = 0.037 *
Biomass	z value = 3.094, p = 0.002 *

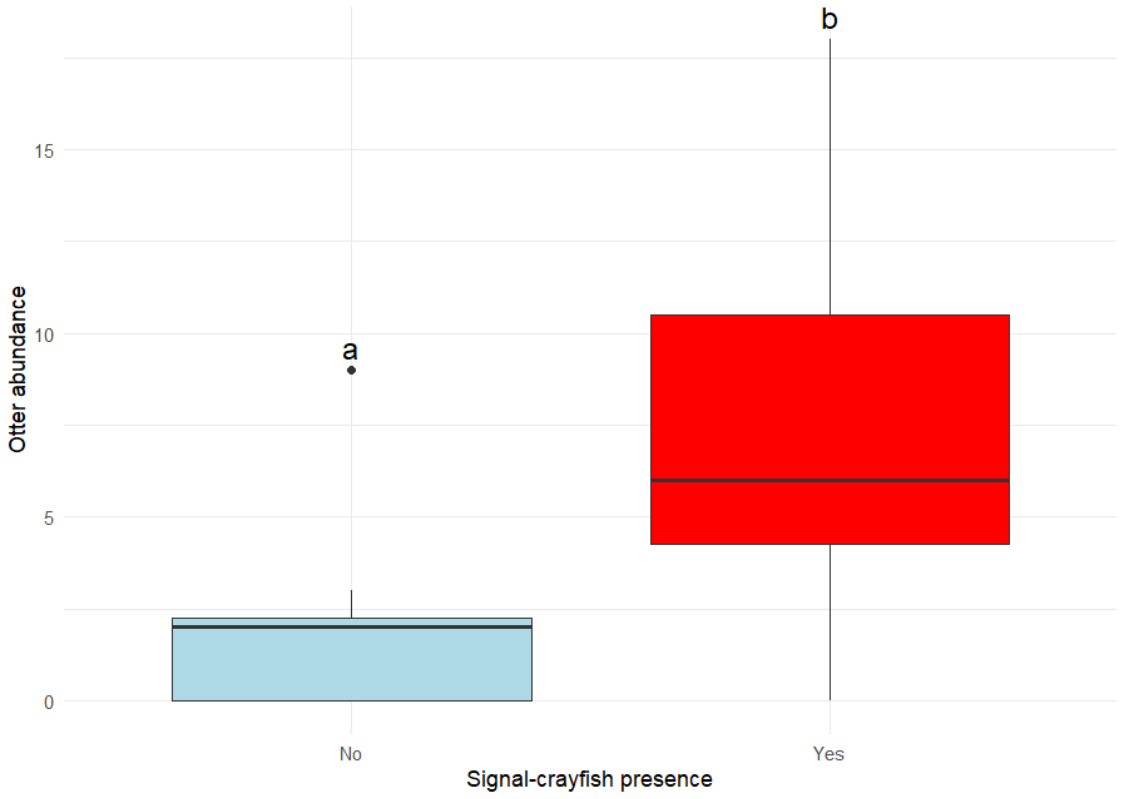

Figure 6 – European otter (*Lutra lutra*) spraint abundance between invaded (red) and non-invaded (blue) sites by signal crayfish (*Pacifastacus leniusculus*). Different letters indicate significance (a,b).

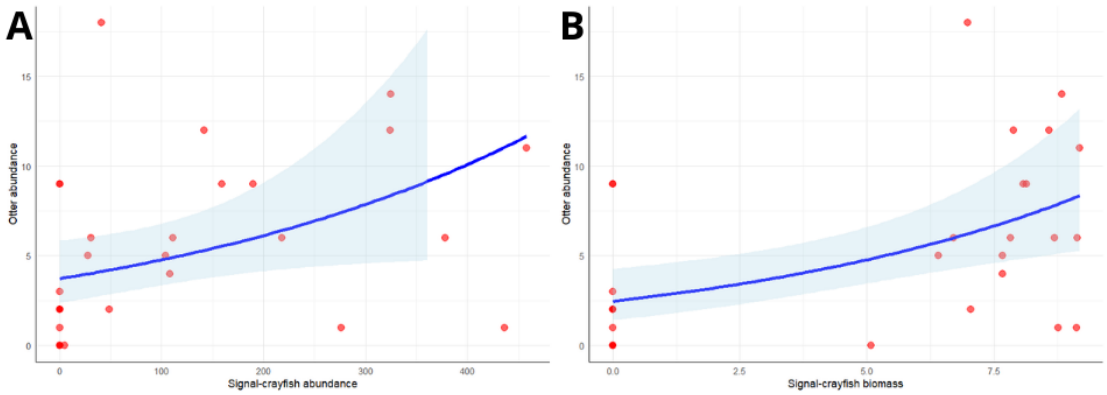

Figure 7 – Relationship of European otter (*Lutra lutra*) spraint abundance with signal crayfish (*Pacifastacus leniusculus*) abundance (A) and biomass (B).

A Redundancy Analysis (RDA) was also performed to examine how the European otter spraints abundance is related to the abundance and biomass of the other trophic groups considered in this study. Signal crayfish abundance and biomass exhibited a significant relationship with European otter spraints abundance, while the abundance and biomass of fish, amphibians and macroinvertebrates were not significant (Figure 8 and Table 3).

Table 3 – Results of the redundancy analysis (RDA) of European otter (*Lutra lutra*) spraints abundance and the signal crayfish, fish, amphibians and macroinvertebrate abundance and biomass. The asterisk indicates significant values ($p<0.05$).

Biotic data	**Otter spraint abundance**
Signal crayfish abundance	F = 4.661, p = 0.040*****
Signal crayfish biomass	F = 3.487, p = 0.045*
Fish abundance	F = 0.572, p = 0.466
Fish biomass	F = 1.156, p = 0.270
Amphibian abundance	F = 0.775, p = 0.441
Amphibian biomass	F = 0.735, p = 0.426
Macroinvertebrate abundance	F = 3.591, p = 0.070
Macroinvertebrate biomass	F = 2,694, p = 0.098

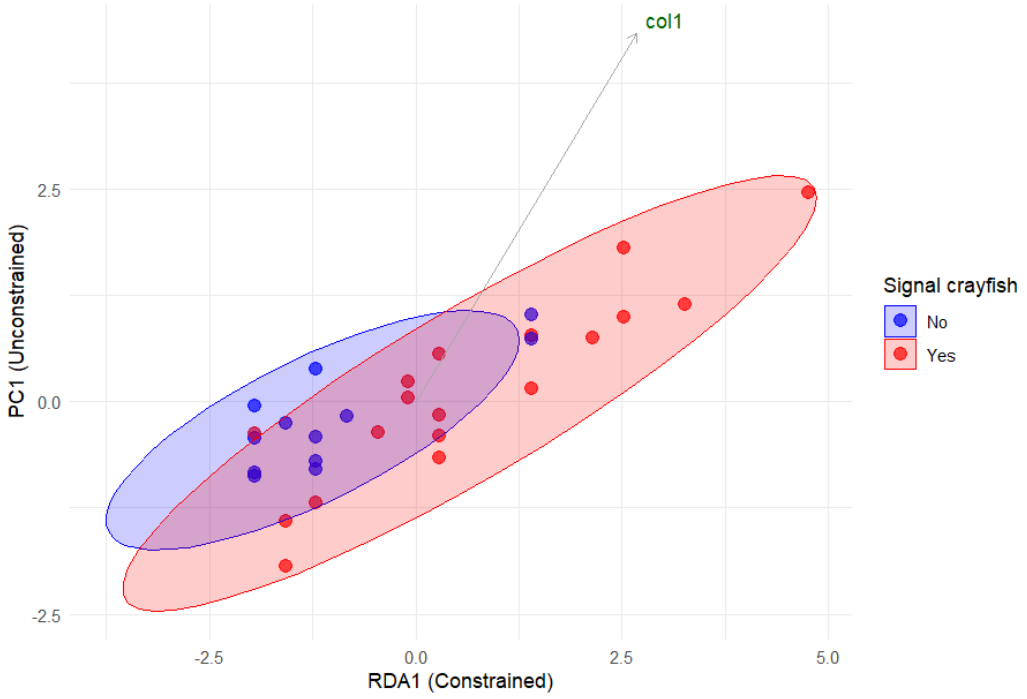

Figure 8 - Redundancy analysis (RDA) of European otter (*Lutra lutra*) spraints abundance and the signal crayfish, fish, amphibians and macroinvertebrate abundance and biomass, for invaded (red) and non-invaded sites (blue).

3.2 – European otter diet characterization

Crustaceans were the most consumed group overall by the European otter, followed by fish and other invertebrates, while mammals, reptiles, amphibians, and birds were consumed less frequently (Table 4).

Table 4 – Composition of the European otter diet, expressed through frequency of occurrence (FO) and relative frequency of occurrence (RFO), for each taxonomic group.

Taxonomic group	FO	RFO
Crustaceans	0.84	0.48
Fish	0.36	0.20
Invertebrates	0.34	0.19
Mammals	0.06	0.04
Reptiles	0.06	0.04
Amphibians	0.06	0.03
Birds	0.03	0.02

In invaded sites, crustaceans dominated the diet, with other invertebrates and fish being less frequently consumed, with the reptiles, mammals, amphibians and birds being consumed to an even smaller degree (Table 5).

Table 5 – Composition of the European otter diet in invaded sites, expressed through frequency of occurrence (FO) and relative frequency of occurrence (RFO), for each taxonomic group.

Taxonomic group	FO	RFO
Crustaceans	0.90	0.54
Fish	0.31	0.18
Invertebrates	0.32	0.20
Mammals	0.05	0.03
Reptiles	0.05	0.03
Amphibians	0.02	0.01
Birds	0.02	0.01

In non-invaded sites, fish were the most consumed group, followed by crustaceans and other invertebrates, while amphibians, reptiles, mammals, and birds were less consumed (Table 6).

Table 6 – Composition of the European otter diet in non-invaded sites, expressed through frequency of occurrence (FO) and relative frequency of occurrence (RFO), for each taxonomic group.

Taxonomic group	FO	RFO
Crustaceans	0.42	0.20
Fish	0.65	0.31
Invertebrates	0.38	0.18
Mammals	0.15	0.07
Reptiles	0.15	0.07
Amphibians	0.30	0.13
Birds	0.06	0.04

For the signal crayfish, its abundance and biomass showed a significant positive relationship with both FO and RFO (Table 7). For fish, amphibians and macroinvertebrates there was no significant relationship of their abundance or biomass on FO and RFO (Table 7).

Table 7 - Results of the analysis of deviance of GLM models between the Frequency of Occurrence (FO) and Relative Frequency of Occurrence (RFO) of key prey taxonomic groups in the European otter (*Lutra Lutra*) diet and their respective abundance and biomass (fish, amphibians, macroinvertebrates, and the signal crayfish). The asterisk indicates significant values ($p<0.05$).

Biotic data	FO	RFO
Crayfish abundance	t = 2.350, p = 0.028 *	t = 2.496, p = 0.020 *
Crayfish biomass	t = 2.356, p = 0.027 *	t = 2.500, p = 0.020 *
Fish abundance	t = 1.189, p = 0.247	t = 0.889, p = 0.383
Fish biomass	t = 1.625, p = 0.118	t = 2.030, p = 0.054
Amphibian abundance	t = -0.401, p = 0.692	t = -0.435, p = 0.668
Amphibian biomass	t = -0.412, p = 0.684	t = -0.454, p = 0.654
Macroinvertebrate abundance	t = 0.104, p = 0.918	t = -0.651, p = 0.521
Macroinvertebrate biomass	t = 1.247, p = 0.225	t = 0.580, p = 0.567

It was also analysed the relationships between the presence of signal crayfish and the FOs and RFOs of the different trophic groups. For fish and amphibians, both FO and RFO exhibited a negative significant relationship to signal crayfish presence (Table 8). However, for macroinvertebrates, no significant relationship was found in either FO or RFO (Table 8). For the signal crayfish itself, there was a positive relationship in both FO and RFO (Table 8).

Table 8 - Results of the analysis of deviance of GLM models and ANOVAs test between the presence of signal crayfish (*Pacifastacus leniusculus*) and the Frequency of Occurrence (FO) and Relative Frequency of Occurrence (RFO) of key prey taxonomic groups in the European otter (*Lutra Lutra*) diet (signal crayfish, fish, amphibians and macroinvertebrates). The asterisk indicates significant values ($p<0.05$).

Diet data	Signal crayfish
Crayfish FO	t = 2.336, p = 0.029 *
Crayfish RFO	F = 14.03, p = 0.001 *
Fish FO	t = -2.915, p = 0.008 *
Fish RFO	F = 7.428, p = 0.013 *
Amphibian FO	t = -3.869, p = 0.001 *
Amphibian RFO	t = -3.317, p = 0.003 *
Macroinvertebrate FO	F = 0.559, p = 0.462
Macroinvertebrate RFO	F = 0.052, p = 0.822

3.3 - European otter key prey groups in invaded and non-invaded sites

When analysing the relationship between fish and amphibians abundance and biomass with the signal crayfish abundance and biomass, no significant relationships were observed (Table 9). However, a significant negative relationship was found between macroinvertebrate abundance and biomass, and both crayfish abundance and biomass (Table 9).

Table 9 - Results of the analysis of deviance of GLM models between the abundance and biomass of signal crayfish (*Pacifastacus leniusculus*), and the abundance and biomass of the different trophic groups. The asterisk indicates significant values ($p<0.05$).

Biotic data	Abundance	Biomass
Fish abundance	z = 0.375, p = 0.708	z = 0.327, p = 0.743
Fish biomass	t = -0.753, p = 0.457	t = -0.821, p = 0.418
Amphibian abundance	z = -0.346, p = 0.729	z = -0.893, p = 0.372
Amphibian biomass	t = -1.420, p = 0.165	t = -1.739, p = 0.091
Macroinvertebrate abundance	z = -2.443, p = 0.015 *	z = -2.620, p = 0.009 *
Macroinvertebrate biomass	t = -2.186, p = 0.036 *	t = -2.183, p = 0.036 *

3.4 – Environmental data affecting European otter and signal crayfish populations

Environmental data (maximum and minimum) values collected along the Mente, Rabaçal, Tuela, and Baceiro Rivers are summarised in Table 10 and 11 (full environmental data in Table S4).

Table 10 – Maximum and minimum values of the collected Environmental data, according to Nogueira (2023).

Data	Maximum	Minimum	Mean
Altitude	843 m	385 m	539.1 m
Oxygen	9.47 mg/L	7.98 mg/L	8.4 mg/L
pH	7.1	6.45	6.8
Total dissolved solids	28.9 mg/L	5 mg/L	16.9 mg/L
Temperature	23 ºC	16.1 ºC	20.3 ºC
Water conductivity	57. 7 µS/cm	24.5 µS/cm	36.6 µS/cm

Table 11 – Maximum and minimum scores of HQA, HMS and RQI, with the respective quality, according to Nogueira (2023).

Data	Maximum score	Minimum score	Mean score
Habitat modification score	1635 - Severely modified	0 - Pristine	262.3 – Obviously modified
Habitat quality assessment	91 - Excellent	59 - Good	71.2 - Excelent
Riparian quality index	65 - Moderate	115 - Very high	98.9 - Very high

European otter spraints abundance and temperature exhibited a significant positive correlation, while habitat quality assessment (HQA) and altitude had a significant negative relationship (Table 12). Other factors such as oxygen, water conductivity, total dissolved solids (TDS), pH, habitat modification score (HMS) and riparian quality index (RQI) showed no significant relationship (Table 12).

Table 12 - Results of the analysis of deviance of GLM models for different environmental data and the abundance of European otter (*Lutra lutra*) spraints. The asterisk indicates significant values ($p<0.05$).

Data	Otter spraints abundance
Altitude	z = -2.989, p = 0.003 *
Habitat Modification Score	z = 0.614; p = 0.539
Habitat Quality Assessment	z = -2.086, p = 0.037 *
Oxygen	z = -1.303, p = 0.193
pH	z = 1.236, p = 0.216
Riparian Quality Index	z = -0.420, p = 0.674
Total dissolved solids	z = 1.256, p = 0.209
Temperature	z = 2.262, p = 0.024 *
Water Conductivity	z = -0.247, p = 0.805

Significant negative relationships were found with oxygen and habitat quality assessment (HQA) for signal crayfish abundance and biomass, while TDS was positively associated with both (Table 13). Additionally, pH showed a significant positive effect on signal crayfish abundance, though biomass was marginally non-significant (Table 13). Altitude and the riparian quality index (RQI) had a marginally non-significant negative effect on both signal crayfish abundance and biomass (Table 13). The habitat modification score (HMS), water conductivity and temperature had no significant effect with both signal crayfish abundance and biomass (Table 13).

Table 13 - Results of the analysis of deviance of GLM models for different environmental data and the abundance and biomass of signal crayfish (*Pacifastacus leniusculus*). The asterisk indicates significant values ($p<0.05$).

Data	Abundance	Biomass
Altitude	t = -1.953, p = 0.061	t = -1.734, p = 0.093
Habitat Modification Score	t = 0.687, p = 0.498	t = 0.889, p = 0.381
Habitat Quality Assessment	t = -2.504, p = 0.018 *	t = -2.450, p = 0.021 *
Oxygen	t = -2.279, p = 0.031 *	t = -2.475, p = 0.020 *
pH	t = 2.122, p = 0.043 *	t = 1.933, p = 0.063
Riparian Quality Index	t = -1.998, p = 0.056	t = -1.986, p = 0.057
Total Dissolved Solids	t = 2.510, p = 0.018 *	t = 2.342, p = 0.027 *
Temperature	t = 1.062, p = 0.297	t = 0.662, p = 0.514
Water Conductivity	t = 1.093, p = 0.284	t = 0.993, p = 0.329

4 – Discussion

This study showed that the European otter displayed a clear higher abundance of spraints in invaded sites over non-invaded sites. Analyses of European otter frequency of occurrence (FO) and relative frequency of occurrence (RFO) revealed reduced predation on fish and amphibians in invaded sites and showed a positive correlation with signal crayfish abundance and biomass, meaning that the more signal crayfish available, the greater the European otter's reliance on it as prey. Furthermore, the signal crayfish exhibited a negative impact on macroinvertebrate communities.

4.1 – Biotic characterization

This study hypothesized that the recent introduction of the signal crayfish is also influencing European otter's diet, ultimately increasing European otter abundance in invaded sites due to a preference for foraging on signal crayfish. The results supported this hypothesis, as European otter spraints abundance in invaded sites was significantly higher compared to non-invaded sites, demonstrating a positive relationship with signal crayfish. Therefore, this recent invasion by the signal crayfish in the study area appears to increase the energy availability for the European otter and demonstrates a possible bottom-up effect by the signal crayfish to this top predator. Additionally, the correlation between the FOs and RFOs of signal crayfish in European otter's diet was significant when compared to signal crayfish abundance and biomass in invaded sites, with signal crayfish being the most consumed prey. Also, the FO and RFO of both fish and amphibians exhibited a negative relationship with the presence of signal crayfish. This suggests that the European otter's diet is clearly influenced by the signal crayfish availability, highlighting the European otter's foraging preference for signal crayfish in the study area.

This foraging preference could be attributed to several factors. European otters primarily feed on fish, which remains their main prey whenever abundant (Clavero et al., 2003; Clavero et al., 2004). However, when fish availability decreases, they often shift to a more diverse diet (Clavero et al., 2003). Although fish are the main prey for European otters across Europe, their consumption declines notably in southern areas (Carss, 1995; Kruuk, 1995). In fact, in temperate regions, when

fish availability is constant, this taxonomic group is the most consumed prey, while in Mediterranean ecosystems, where fish availability is more unpredictable, especially during summer conditions, European otters are driven to diversify their diet, increasing predation on alternative prey, including amphibians, crayfish and other aquatic invertebrates (Erlinge, 1986; Ruiz-Olmo et al., 2001; Clavero et al., 2003; Clavero et al., 2004). The European otter's opportunistic nature and its remarkable foraging flexibility is also highlighted by the variation in prey availability across habitats, where in lakes, rivers and streams, amphibians and crustaceans play a larger role in their diet and in coastal regions, fish are the most frequently consumed prey (Jędrzejewska et al., 2001). Such differences influence the degree to which European otters rely on fish, their usually described preferred prey (Jędrzejewska et al., 2001; Clavero et al., 2003; Clavero et al., 2004). In the particular case of our study area, it should be noted that comprises oligotrophic mountain rivers with naturally low fish diversity and abundance (Oliveira, 2023), requiring European otters to diversify their diet under these conditions (Tolrà et al., 2024b).

The "crustaceans" group in European otter diet characterization refers specifically to the invasive signal crayfish. At non-invaded sites, crustaceans were the second most consumed group (FO=0.42 and RFO=20%), which may be due to a few possibilities. First, low signal crayfish abundance might have led to them not being detected during our sampling using traps, while still being present and preyed upon by European otters. Therefore, and although we considered these sites as free of signal crayfish (i.e. non-invaded), the reality is that some of them are already colonized by this invasive species, but with a very low abundance. Another possibility is European otter movement: otters may prey on signal crayfish at invaded sites and then move to adjacent non-invaded sites, where they leave spraints containing signal crayfish remains. Although both hypothesis may be correct, only future studies can test their validity.

While fish are typically the European otter's primary prey, they often display opportunistic feeding behaviour, selecting prey based on local abundance rather than strict preferences (Clavero et al., 2003; Almeida et al., 2012). One reason for this shift may be the energetic benefits provided by foraging on non-native prey species such as crayfish (Almeida et al., 2012). In Mediterranean regions, where red swamp crayfish are more abundant and easier to catch, in comparison to other taxonomic groups, European otters have adapted to include this invasive species in their diet

(Beja, 1997; Clavero et al., 2003; Almeida et al., 2012). The same may happen with the signal crayfish in our study area. This dietary shift contrasts with the European otter's foraging habits in other parts of Europe, where fish remain the primary food source, despite crayfish presence (Clavero et al., 2003; Almeida et al., 2012; Britton et al., 2017).

The increased consumption of signal crayfish, especially during the summer months when their activity is higher, suggests that European otters are capitalizing on the greater availability and detectability of this prey (Almeida et al., 2012). This shift could also reflect a decrease in freshwater fish abundance, leading to a diversification of the European otter's diet toward alternative, energetically less valuable food items such as the signal crayfish (Aparicio et al., 2000; Clavero et al., 2003; Clavero and García-Berthou, 2006; Almeida et al., 2012). As ecosystems change, the European otter's diet can serve as an indicator of shifts in prey species populations (Copp & Roche, 2003; Miranda et al., 2008; Smiroldo et al., 2009; Almeida et al., 2012). This adaptability underscores the European otter's role as both a predator and a reflection of the health and balance of freshwater ecosystems.

At invaded sites, signal crayfish was the most consumed prey (FO=0.90, RFO=0.54) and in non-invades sites, fish was the most consumed prey (FO=0.65, RFO=0.31), while macroinvertebrates did not show a significant difference for invaded (FO=0.32 and RFO=0.20) and non-invaded (FO=0.38 and RFO=0.18) sites in European otter's diet. However, the presence of signal crayfish showed a negative impact on macroinvertebrate communities, significantly reducing their abundance and biomass (Carvalho et al., 2022; Nogueira, 2023). This decline is largely driven by direct predation on macroinvertebrates by the signal crayfish, but also results from factors such as the loss of habitat complexity, trophic cascades, and changes in nutrient cycling (Jackson et al., 2014; Carvalho et al., 2022; Nogueira, 2023). While this decline on macroinvertebrate communities may not directly significantly impact the European otter's diet, it is likely that fish species like the brown trout (*Salmo trutta*), which primarily consume invertebrates, may be negatively affected (Montori et al., 2006; Rocaspana et al., 2016; Piria et al., 2022; Oliveira, 2023). Additionally, macroinvertebrates may appear in European otter's diet through indirect predation, such as gut content of other species (Almeida et al., 2012), since both fish and the signal crayfish

prey on macroinvertebrates, European otter's main prey types for non-invaded and invaded sites, respectively.

It should be noted that European otter abundance in this study is expressed by the number of fresh spraints found at each sampled site. The use of fresh spraints to estimate European otter activity and abundance is debated, with some supporting it as a useful indicator (Mason and Macdonald, 1986; Ruiz-Olmo et al., 2001), though it remains an indirect measure rather than a precise count of population density.

4.2 - Environmental data

It was also investigated the relationship between European otter abundance, measured by abundance of spraints, signal crayfish abundance, and signal crayfish biomass in relation to several environmental factors. This analysis included variables such as water temperature, dissolved oxygen, water conductivity, total dissolved solids (TDS), pH, altitude and habitat quality indicators like the Habitat Modification Score (HMS), Habitat Quality Assessment (HQA) and River Quality Index (RQI). By assessing these environmental factors, the goal was to identify the possible influence of these factors on the distribution and abundance of European otters and signal crayfish across the study area. The water temperature showed a positive relationship with the European otter spraints abundance, which could be due to higher values of habitat productivity, typically related to slow flow velocity river reaches with high water temperature, with abundant aquatic vegetation (Prenda, 1996; Tolrà et al., 2024a). Crayfish abundance and biomass were negatively related to dissolved oxygen levels, likely reflecting the effects of crayfish bioturbation, which can reduce oxygen content in water (Gherardi, 2007). However, the oxygen values in all sampling sites are relatively high and so this negative relationship may be spurious and with no ecological meaning. Additionally, a positive relationship was observed with total dissolved solids (TDS) and crayfish abundance and biomass. This aligns with previous findings that crayfish activity increases sediment disruption, negatively impacting water quality by increasing TDS and by reducing light penetration and plant productivity (Gherardi, 2007). Conversely, a positive relationship was observed with pH, which is consistent with the crayfish sensitivity to low

pH (Beaune et al. 2018). This sensitivity affects calcification, moulting, growth and reproduction, making it a limiting factor for crayfish establishment (Beaune et al., 2018). The European otter spraints abundance had a negative relationship with altitude, with the signal crayfish abundance and biomass also showing a negative tendency towards it. This pattern likely reflects the influence of altitude on temperature, with colder conditions at higher elevations (Ohmura, 2012). In addition, at these higher altitudes the studied rivers start to be even more oligotrophic and less productive (Nogueira, 2023; Oliveira 2023). Since European otters primarily feed on signal crayfish during the summer, their distribution tends to align with invaded sites where crayfish abundance and biomass are higher.

The HQA measures the rarity, richness and diversity of river habitats and with it is possible to obtain an indication of the general habitat diversity provided by the natural features in the river corridor and channel, where the higher the value, the higher the habitat quality of each site and the lower the HQA, the greater the artificial intervention and modification of the river channel at a given site, affecting the quality of natural habitats (Raven et al., 1998; Nogueira 2023). The HQA showed a negative relationship with the European otter abundance, signal crayfish abundance and biomass, this could be due crayfish-mediated bioturbation and signal crayfish consumption by the European otter. Bioturbation refers to the biological reworking of soils and sediments and is often linked to crayfish constructing burrows, as commonly observed in *P. clarkii* and *P. leniusculus*, and it can also result from crayfish movement (Gherardi, 2007), contributing to the impoverishment of water quality and structure of riverbanks in invaded sites (Gherardi, 2007). The rivers in our study area are primarily composed by clear waters and sediments dominated by cobbles, boulders, gravel, and sand, making bioturbation unlikely, demonstrating a need to study this interaction to better understand the relationship between HQA and the signal crayfish. Although the habitat quality declines as the signal crayfish populations grow, this does not impact European otter populations so far, indicating that despite suboptimal habitat conditions, otters still select these sites, highlighting their dietary preference for crayfish.

5 - Conclusion and future directions:

This study showed that the presence, abundance and biomass of the invasive signal crayfish positively affected the spraints abundance of European otter across the study area, with the signal crayfish being the most consumed prey by this top predator in invaded sites during summer conditions. This relationship illustrates a bottom-up effect mediated by the signal crayfish within the ecosystem with an increase in the energy availability for the European otter, highlighting the complex interactions between IAS and native predators.

Future research should prioritize seasonal studies, as the species present in a river are influenced by certain environmental conditions, such as temperature and river flow, resulting in their presence varying throughout the year. In the case of the signal crayfish, the period of greatest activity and abundance is between May and October (Johnson et al., 2013). During autumn and winter, low temperatures significantly reduce signal crayfish activity (Johnson et al., 2013), making it theoretically more difficult for the European otter to prey on it. Therefore, conducting a study during winter conditions, when the signal crayfish is less active could provide valuable insights into how the European otter's diet adapts in response to this seasonal shift. Another interesting line of future research would be the study of European otter's body size, as prey availability likely drives body size differences between European otters in temperate and Mediterranean regions, with temperate European otters having greater access to fish resources, while Mediterranean European otters rely more on alternative prey such as crayfish and amphibians (Erlinge, 1986; Ruiz-Olmo et al., 2001; Clavero et al., 2003; Clavero et al., 2004). Some studies have mentioned that the reduced fish consumption and corresponding expansion of the food-niche in Mediterranean European otters may be related to their smaller body size, due to their reliance in alternative prey, with European otters in this region being 11% to 35% lighter than those in temperate areas (Ruiz-Olmo et al., 1998). In Mediterranean freshwater environments, where fish availability is unpredictable and often low, smaller body size offers energetic advantages, allowing European otters to adjust their feeding habits, with their smaller size possibly linked to the higher consumption of less profitable prey (Gittleman & Purvis, 1998; Clavero et al., 2003). The same could happen in our study area as the European otter seems to heavily rely on the signal crayfish as prey. Additionally, future research using radio-tracking and

censuses (tracks and visual) could be done in invaded and non-invaded sites, as this could help to obtain a more precise measure of European otter abundance and habitat use.

It is important to note that dietary studies based on spraint analysis face notable uncertainties, such as misidentifying spraints from other species and variations in prey retention time, which may lead to repeated detection in subsequent samples (Carss & Parkinson, 1996; Fernández et al., 1997; Harrington et al., 2010; Krawczyk et al., 2016). Multiple spraints from the same individual also challenges the statistical independence of samples, and while DNA analysis can provide more accurate identification, it is often limited by cost and resource demands (Martínez-Gutiérrez et al., 2015; Krawczyk et al., 2016). Despite these issues, spraint analysis remains a primary method for understanding carnivore diets (Krawczyk et al., 2016) and this study clearly demonstrates that signal crayfish is affecting the diet and potentially the habitat use by this top predator in Montesinho Natural Park and adjacent areas.

Developing comprehensive management strategies is essential to prevent the introduction, control and mitigate the spread and impact of signal crayfish and other IAS in freshwater ecosystems. While this study showed that the signal crayfish negatively affects the macroinvertebrate communities, it also revealed a positive effect on the European otter. This creates some dilemmas as controlling or eradicating this IAS could benefit macroinvertebrate populations but may negatively impact European otters. Further research is needed to clarify the European otter's reliance on this IAS as prey and to assess how management strategies might influence European otter populations and broader ecosystem health in the Montesinho Natural Park and adjacent areas.

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Annex

Table S1 - Habitat Quality Assessment (HQA) scores. Classification according to Raven et al. (1998).

Habitat Quality Assessment Score	Category description
≥ 65	Excelent
59 - 45	Good
44 - 30	Moderate
29 - 15	Mediocre
14-0	Bad

Table S2 - Habitat Modification Score (HMS). Classification according to Raven et al. (1998).

Habitat Modification Score	Category description
0 - 16	Pristine - Semi-natural
17 - 199	Predominantly unmodified
200 - 499	Obviously modified
500 - 1399	Significantly modified
≥ 1400	Severely modified

Table S3 - Riparian Quality Index (RQI) score. Classification according to Raven et al. (1998).

Riparian Quality Index Score	Category description
120 - 97	Very high
96 - 73	High
72 - 49	Moderate
48 - 25	Low
24-0	Very low

Table S4 – Environmental data values, temperature, oxygen, water conductivity (conductivity), total dissolved solids (TDS), pH, altitude, habitat quality assessment (HQA), habitat modification score (HMS), riparian quality index (RQI), for each river and site.

River	Site	Temperature	Oxygen	Conductivity	TDS	pH	Altitude	HQA	HMS	RQI
Mente	Mente 1	19,9	8,21	29,7	16,43	7,01	385	68	0	110
Mente	Mente 2	19,6	8,46	30,9	16,28	7,07	395	71	300	104
Mente	Mente 3	20,8	7,99	31,6	16,19	6,89	398	70	430	85
Mente	Mente 4	19,7	8,17	28,9	15,15	6,78	452	69	180	102
Rabaçal	Rabaçal 1	21,4	8,44	28,3	14,18	6,78	385	67	0	111
Rabaçal	Rabaçal 2	20,6	8,39	28,1	14,45	7,04	395	76	0	115
Rabaçal	Rabaçal 3	21,5	8,28	28,2	14,18	6,95	398	62	0	110
Rabaçal	Rabaçal 4	21,4	8,27	28,1	14,41	6,53	409	66	0	112
Rabaçal	Rabaçal 5	22,4	8,27	28,1	13,93	6,48	459	73	80	114
Rabaçal	Rabaçal 6	22,5	8,24	26,1	12,75	7,1	471	59	955	93
Rabaçal	Rabaçal 7	23	8,67	26,3	12,67	7,08	487	68	320	89
Rabaçal	Rabaçal 8	23	8,45	26,3	12,67	7,08	490	69	330	101
Rabaçal	Rabaçal 9	22,4	8,5	26,5	13,3	6,66	493	68	90	111
Rabaçal	Rabaçal 10	21,1	8,48	25	12,42	6,5	520	66	10	113
Tuela	Tuela 1	22	8,05	57,7	28,8	6,92	421	67	0	94
Tuela	Tuela 2	20,7	8,06	48,6	25	6,98	427	66	685	79
Tuela	Tuela 3	20,7	8,81	48,6	25	6,98	430	63	0	84
Tuela	Tuela 4	20,2	8,1	42,6	21,5	7,02	532	74	20	102
Tuela	Tuela 5	20,1	8,06	41,3	21,5	7,01	630	73	305	87
Tuela	Tuela 6	19,9	8,75	39,1	20,78	6,93	634	73	0	99
Tuela	Tuela 7	19,5	8,89	62,4	7,14	6,96	643	69	0	100
Tuela	Tuela 8	20,3	9,47	36,7	18,7	6,79	655	76	690	90
Tuela	Tuela 9	22,2	9,33	38,9	19,29	6,72	656	78	560	87
Tuela	Tuela 10	21,4	9,34	38	19,1	6,69	684	82	20	109
Baceiro	Baceiro 1	18,9	7,98	55,2	28,9	6,75	594	74	160	104
Baceiro	Baceiro 2	18,1	8,02	51,5	27,9	6,74	608	74	100	100
Baceiro	Baceiro 3	17,2	8,05	49,6	27,3	6,83	612	72	1635	65
Baceiro	Baceiro 4	16,2	8,12	32,3	5,58	6,45	831	73	435	95
Baceiro	Baceiro 5	16,1	8,18	31,5	5,45	6,69	835	91	60	104
Baceiro	Baceiro 6	16,3	8,2	30,5	5	6,6	843	79	505	99