UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

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La Nutria eurasiática (*Lutra lutra*) como bioindicador ambiental en relación con las especies exóticas invasoras: Caña común (*Arundo donax*), Cangrejo rojo americano (*Procambarus clarkii*) y Visón americano (*Neovison vison*).

D. Ettore Emanuele Dettori

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LA NUTRIA EURASIÁTICA (*Lutra lutra*) COMO BIOINDICADOR AMBIENTAL EN RELACIÓN CON LAS ESPECIES EXÓTICAS INVASORAS: CAÑA COMÚN (*Arundo donax*), CANGREJO ROJO AMERICANO (*Procambarus clarkii*) Y VISÓN AMERICANO (*Neovison vison*).

Memoria presentada para optar al Grado de Doctor por:
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Resumen:

La nutria euroasiática (Lutra lutra, Lineaeus 1758), atravesó en la cuenca del río Segura (SE España) un periodo crítico desde la década de 1960 hasta principios de los 80, seguido de un periodo de estabilidad durante los 90 tras el cual ha experimentado un ligero incremento durante el siglo XXI. Así, dependiendo del buen estado y conservación de los ecosistemas acuáticos, este mamífero semiacuático es uno de los principales depredadores apicales del río Segura y sus afluentes, y un potencial bioindicador de la situación ambiental de la red hidrográfica. En este trabajo se ha relacionado la presencia y distribución, uso del hábitat y dieta de la nutria euroasiática con las condiciones ambientales del río Segura y sus afluentes en la Región de Murcia ambientales, a partir de datos, obtenidos fundamentalmente dentro del proyecto LIFE13BIO/ES/001407 RIPISILVANATURA, con el fin de poder aplicar una política de conservación efectiva para las poblaciones de este carnívoro amenazado. Se ha intentado proporcionar directrices para una gestión que tenga en cuenta la actividad humana y la calidad del hábitat en el entorno de sus zonas de presencia, con el objetivo mejorar la base de información disponible sobre la distribución, uso del hábitat, respuesta a la urbanización, a la presencia de especies exóticas invasoras (EEIs) y a la restauración ambiental, mediante la implementación de programas de investigación y seguimiento adecuados.

En la primera parte del trabajo se han recopilado datos cualitativos y cuantitativos sobre la distribución de la nutria euroasiática en la Región de Murcia, con el fin de comprender mejor su ocupación de la cuenca hidrográfica y sus relaciones con la gestión y restauración ambiental. Para ello, dentro del proyecto RIPISILVANATURA (2014-2019) se ha evaluado la distribución y uso del hábitat en el tramo medio del río Segura y sus afluentes. Entre abril de 2016 y junio de 2018, se han muestreado (1 a 10 veces) 42 secciones (longitud media ± SD = 0,5 ± 0,2 km) en busca de excrementos de la especie, cubriendo 109,8 km lineales de río. El período de estudio ha coincidido con las principales actividades de restauración ambiental del proyecto RIPISILVANATURA, consistentes en la erradicación de la caña común (Arundo donax) que ha invadido espacios anteriormente ocupados por la vegetación de ribera autóctona. La preferencia de hábitat de la nutria euroasiática se ha evaluado en relación con diez parámetros ambientales que podrían haber influido en su presencia, mediante un análisis de regresión logística (LRA) y el modelo no paramétrico de regresión Random Forest. Se ha observado que la nutria euroasiática evitaba las aguas contaminadas y su presencia respondía de forma inversa a la anchura de la banda de vegetación riparia, compuesta principalmente por manchas densas de Arundo. Además, en los tramos bajo el control

del proyecto, se ha encontrado que su intensidad de marcaje aumentaba después de la eliminación de la caña. Los valores medios han sido mayores en los transectos restaurados del proyecto LIFE RIPISILVANATURA (1,18 marcas/100m) respecto al tramo intermedio aguas abajo y no sometido a ninguna gestión de restauración (0,76 spraints/100m). Además de su influencia directa sobre la nutria euroasiática, los efectos combinados de la contaminación del agua y la propagación de la caña común podrían también alterar la distribución y abundancia de peces, reduciendo la disponibilidad de presas para esta especie. Por lo tanto, mejorar la calidad del hábitat y las comunidades de peces nativos es clave para favorecer la recuperación de la nutria euroasiática en un área tan humanizada como la cuenca del río Segura.

La segunda parte del trabajo se ha centrado principalmente en la composción de la dieta de la nutria euroasiática en la cuenca del río Segura. En climas semiáridos, la escasez e impredecibilidad de las precipitaciones, junto con la excesiva regulación del caudal (para destinar más agua al regadío agrícola) y la conexión entre distintas cuencas hidrográficas (Trasvase Tajo-Segura), ha favorecido la expansión de peces no nativos en las cuencas receptoras, en sustitución de las comunidades nativas. Desde la década de 1980, esto ha llevado a que el número de especies de peces no autóctonos se incremente progresivamente en el río Segura precisamente como resultado de la construcción de la gran infraestructura de trasvase de agua dulce que conecta el río Tajo con el Segura desde 1979. Con el objetivo de resaltar cómo los cambios impulsados por el ser humano han afectado a la diversidad de las comunidades de peces, se ha estudiado el cambio en la dieta de un depredador apical de peces comparando la dieta de la nutria euroasiática (Lutra lutra) entre dos periodos separados entre sí 20 años (1997-98 y 2016-19). También se ha comparado el tamaño del barbo gitano (Luciobarbus sclateri) depredado, como especie preferida de pez autóctono en el río Segura, y se ha observado como la calidad del hábitat ha afectado a su condición durante este período de tiempo. En general, los peces y el cangrejo rojo americano (Procambarus clarkii), una especie exótica invasora, han formado la mayor parte de la dieta de la nutria euroasiática durante los dos períodos considerados en nuestro estudio. En el período 2016-19 la contribución de las especies exóticas a la dieta de la nutria euroasiática ha aumentado significativamente, hasta incluir diez especies no nativas. Los hábitos alimentarios de la nutria eurasiática han reflejado fielmente la variación en la composición de la comunidad de peces y han confirmado la importancia del cangrejo rojo como presa alternativa a los peces en la Península Ibérica. Finalmente, la longitud promedio de los barbos gitanos depredados ha disminuido en el segundo período de estudio, con una consistente disminución en la rentabilidad trófica del barbo gitano para la nutria euroasiática.

A continuación del estudio de su dieta en la cuenca del río Segura, se ha relacionado la recuperación de la nutria euroasiática (Lutra lutra) con la expansión concurrente de asentamientos humanos, que están propiciando el aumento de registros en tramos urbanos y otras zonas fuertemente antrópicas. Se ha analizado la dieta de la especie a lo largo de un gradiente natural-urbano, con el objetivo principal de evaluar en qué medida la variación en la disponibilidad de alimento determinada por el hombre, afecta a sus hábitos alimenticios y cómo puede prosperar este esquivo mustélido en hábitats aparentemente inadecuados. Desde 2016 hasta 2019 se han recolectado 521 excrementos, monitoreando 37 estaciones de muestreo (longitud media ± SD = 0.5 ± 0.3 km). En las áreas naturales, el porcentaje de cobertura de ribera no alterada fue mayor, coincidiendo con el menor número de extracciones de agua, mientras que la contaminación del agua fue mayor en las áreas urbanas. El cangrejo rojo americano (Procambarus clarkii) y los peces constituyeron la mayor parte de la dieta de la nutria euroasiática. Los dos peces más depredados fueron el barbo gitano (Luciobarbus sclateri) y la boga del tajo (Pseudochondrostoma polylepis), que parecen estar entre las especies más abundantes en el río, lo que confirma la regla general de alimentación de la nutria euroasiática en la Península Ibérica. La contribución de los peces no nativos ha sido en promedio baja, mientras que el cangrejo rojo americano (especie exótica invasora), ha superado en importancia a los peces en todos los hábitats excepto en las áreas urbanas. Sin embargo, los datos de dieta no permiten identificar al alimento como un recurso limitante para las nutrias eurasiáticas en áreas urbanas. Por el contrario, la intensidad de marcaje varía consistentemente con el gradiente natural-urbano (0.65 a 0.31 marcas /100 m entre los dos extremos del gradiente, respectivamente), lo que sugiere que las perturbaciones humanas (modificación del hábitat y contaminación del agua) deben desempeñar también un papel importante en la distribución de las nutrias euroasiáticas.

La última parte del trabajo se ha realizado atendiendo a la simpatría de los mustélidos semiacuáticos nativos y exóticos, y su potencial interacción en función de su situación en la Península Ibérica, tratando de desarrollar un plan de prevención frente a la invasión por mustélidos exóticos para la Región de Murcia. Por eso, se ha introducido el estudio del visón americano (Neovison vison), un mustélido semiacuático liberado accidentalmente al medio natural en toda Europa por su uso en la industria peletera y que se ha comportado como una especie exótica invasora. Esta especie se ha extendido por casi toda Europa continental, por lo que se han realizado varios programas orientados a su erradicación masiva. En la Península Ibérica, existe un programa de este tipo desde 2001 como parte del plan de acción nacional para la conservación del visón europeo, Mustela lutreola (cuya principal y más fructífera actuación ha sido el proyecto LIFE LUTREOLA SPAIN / LIFE13 NAT/ES/001171 "Nuevos enfoques en la conservación del visón europeo en España"). Efectivamente un correcto plan de acción es fundamental tanto para el manejo, como para monitorear su distribución y abundancia, con el fin de prevenir la recolonización de las áreas de las cuáles ha sido eliminada esta EEI. Generalmente, los excrementos de visón americano se pueden confundir con los de otros mustélidos del mismo tamaño, pero también con los de la nutria euroasiática (Lutra lutra), ya que tienen la costumbre de depositarlos a lo largo de los cursos de agua. Una ayuda válida para superar estas dificultades puede ser el uso de métodos genéticos no invasivos, por lo que, con el objetivo de proporcionar una técnica fiable y conveniente para diferenciar animales simpátricos y morfológicamente similares, se ha probado la efectividad de un método de PCR-RFLP basado en ADN mitocondrial procedente de heces para distinguir el visón americano de la nutria euroasiática. La reacción de PCR-RFLP ha diferenciado claramente los excrementos de visón americano de los excrementos de nutria euroasiática, ya que las enzimas de restricción han producido patrones genéticos distintos para los dos mustélidos, y además bien definidos. El resultado ha sido que el 85% de las 94 muestras fecales analizadas se han atribuido a una especie de mustélido, y que solamente tres de las siete inicialmente atribuidas visualmente al visón americano, pertenecían realmente a ese mustélido. Esto confirma que la precisión de la identificación visual de los excrementos es cuestionable y, por lo tanto, que los métodos genéticos no invasivos son apropiados para una evaluación más fiable en los programas de erradicación.

A partir de todo lo anterior, se concluye que las estrategias de conservación para la nutria euroasiática en el área estudiada deberían centrarse en la restauración de los hábitats de agua dulce y estar dirigidas principalmente a mejorar la ictiofauna nativa. Estas acciones no pueden ser efectivas sin un diseño de caudales que imite los patrones del régimen de flujo natural y mejore la conectividad longitudinal de los cauces. En cuanto a las especies exóticas invasoras, si bien los cangrejos rojos americanos son difíciles de erradicar, se ha demostrado que el manejo puede controlar la expansión de la caña común. No obstante, para el control de esta última especie se requiere un mantenimiento y monitoreo a largo plazo para confirmar los efectos positivos de la restauración ambiental tanto en la calidad del hábitat como en la expansión de la nutria euroasiática en la cuenca hidrográfica del Segura. Por estas razones, se considera que la investigación y el seguimiento la nutria euroasiática debería realizarse con una mayor regularidad, dada su utilidad como indicador del éxito de las acciones de mejora y restauración del hábitat. Al mismo tiempo, en áreas donde se espera que la expansión de nutrias y visones conduzca a la superposición entre ellos, la aplicación de métodos genéticos no invasivos PCR-RFLP permitiría la identificación confiable de las especies objetivo y la implementación inmediata de acciones de gestión. Las conclusiones del trabajo están en línea con algunas de las principales medidas del Plan de Recuperación de la Nutria en la Región de Murcia, que incluyen proteger y mejorar su hábitat, fomentar la cooperación institucional y social para desarrollar un plan de acción integrado de restauración ribereña (que debería abordar específicamente las EEIs), reducir la fragmentación fluvial, y establecer vedas y regulaciones de pesca. También, gestionar los recursos hídricos de manera compatible con la especie, para mejorar la calidad del agua y la disponibilidad de los recursos tróficos, y por último asegurar el seguimiento biológico e investigación de sus poblaciones y de los factores que afectan a su conservación. Finalmente, la recuperación en curso de una especie carismática como la nutria euroasiática puede contribuir a una percepción positiva de las acciones de gestión tanto por parte de los organismos gubernamentales como del público, mejorando así la imagen de futuros proyectos de restauración del río Segura. En este contexto, la nutria puede ser la especie emblema en proyectos de restauración de ríos destinados a mejorar la calidad del agua y la vegetación ribereña en áreas urbanas y, en última instancia, de fomento de la sostenibilidad urbana y la recuperación para el público de las riberas de los ríos.

Abstract

The Eurasian otter (*Lutra lutra*, Linnaeus 1758) declined throughout the basin of the River Segura (Murcia Region, SE Spain) from the 1960s to the early 1980s. After a decade of stability during the 1990s, it has started to recover since the beginning of the 21st century. This semi-aquatic mammal is a top-predator of the River Segura and its tributaries, and a potential bioindicator of the status of hydrographic networks. In this work, the distribution, habitat use and diet of the Eurasian otter have been related to the environmental conditions of the River Segura and its tributaries. Data were mainly collected within the project LIFE13BIO/ES/001407 RIPISILVANATURA, with the aim of providing guidelines for the management of freshwater habitats that take into account increasing urbanization and spread of invasive alien species (IAS) in the Region of Murcia.

In the first part of the work, we collected data on the distribution and habitat preferences of the Eurasian otter. The study period coincided with the main environmental restoration activities carried out within the framework of the project RIPISILVANATURA, consisting in the eradication of the common giant reed (*Arundo donax*), a non-native species which has invaded riparian habitats to the detriment of native species. To assess the effects of habitat management on otter distribution, between April 2016 and June 2018, 42 river stretches (mean length ± SD = 0.5 ± 0.2 km) were sampled for otter spraints, covering a 109.8 km long stretch of the river and including both managed and unmanaged riverine sections. To assess otter habitat preferences ten environmental parameters potentially affecting its occurrence were recorded and analysed using logistic regression analysis (LRA) and non-parametric Random Forest regression models. Otters tended to avoid polluted waters and stretches covered by thick *Arundo* patches. Otter occurrence increased after the removal of giant reed, average marking intensity being 1.18 spraints/100 m in restored sampling stations and 0.76 spraints/100m on unmanaged stretches. The combined effects of water pollution and spread of giant reed may alter the distribution and abundance of fish, reducing prey availability for otters. Therefore, improving habitat quality and native fish communities are key factors for enhancing the recovery of the Eurasian otter in as heavily altered areas as the catchment of the River Segura.

The second part of the work focused on the diet of the Eurasian otter in the River Segura basin. In semi-arid climates the combination of several factors, including the scarcity and unpredictability of rainfall, excessive regulation of water flow (to allocate more water to agricultural irrigation) and connection between different hydrographic basins (Trasvase Tajo-Segura), since the 1980s has led to the progressive increase in the number of non-native fish species in the river Segura. In order to highlight how human-driven changes have affected the diversity of fish communities, variation in the diet of otters has been assessed by comparing two study periods (1997-98 and 2016-19), separated from each other 20 years. The size of preyed Andalusian barbel (*Luciobarbus sclateri*), the most widespread native fish of the river, was also compared, to assess how habitat quality affected its condition during this period of time. In general, fish and invasive red swamp crayfish (*Procambarus clarkii*), formed the bulk of otter diet during both periods. In 2016-19 the contribution of exotic species to otter diet increased significantly, to include ten non-native species. Its feeding habits faithfully reflected the variation in the composition of the fish community and confirmed the importance of the invasive red crayfish as an alternative-to-fish prey in the Iberian Peninsula. Finally, the average length of preyed Andalusian barbel, and thus its profitability for the otter, decreased in the second study period.

The ongoing recovery of the Eurasian otter in Murcia region is leading to a progressive increase in otter records both in urban and peri-urban river stretches. With the main goal of evaluating to what extent man-driven variation in food availability affects its feeding habits and how this elusive mustelid can thrive in apparently unsuitable habitats, otter diet has been analysed along a natural-to-urban gradient. From 2016 to 2019, 521 spraints were collected by monitoring 37 sampling stations (mean length ± SD = 0.5 ± 0.3 km). In natural areas, the percent cover of undisturbed banks was the highest, with the lowest number of water abstraction systems, whilst water pollution was the highest in urban areas. As expected, introduced red swamp crayfish and fish formed the bulk of otter diet. The two most preyed fish, *Luciobarbus sclateri* and *Pseudochondrostoma polylepis*, were among the most abundant species in the river, confirming the generalist feeding behaviour of the Eurasian otter. The contribution of non-native fish was on average low, while invasive crayfish exceeded the overall importance of fish in all habitats except urban areas. Overall, diet data did not allow attesting food as a limiting resource for otters in urban areas. In contrast, marking intensity varied consistently along the natural-to-urban gradient (from 0.65 to 0.31 spraints/100m), suggesting that human disturbance (i.e. habitat modification and water pollution) may play a major role in shaping otter distribution.

Concomitant otter recovery and spread of non-native species in the Iberian Peninsula has led to overlapping otter and American mink (*Neovison vison*) ranges. This semi-aquatic, invasive mustelid has been introduced in Spain due to its use in fur industry. Successively, it has spread throughout almost all continental Europe, which is why several programs have been carried out aimed at its eradication. In the Iberian Peninsula the American mink is controlled since 2001 as part of the national action plan for the conservation of the European mink, *Mustela lutreola* (see the project: LIFE LUTREOLA SPAIN/LIFE13 NAT/ES/001171 "New approaches in the conservation of the European mink in Spain"). In order to prevent the recolonization of areas from which non-native mink has been eradicated and highlight its occurrence in new areas it is of pivotal importance to dispose of an effective monitoring method. Generally, American mink scats can be confused with those of other mustelids of the same size, but also with those of the Eurasian otter, particularly when containing undigested remains of aquatic prey. To overcome such difficulties cost-effective genetic methods are needed. With the aim of providing a reliable technique to differentiate sympatric, morphologically similar animals, we tested the effectiveness of a faecal mtDNA-based PCR-RFLP method. Our method clearly differentiated American mink scats from those of Eurasian otters, since restriction enzymes produced distinct and well-defined genetic patterns for the two mustelids. Overall, 85% of 94 faecal samples were attributed to a mustelid species, and only three out of the seven samples initially attributed to the American mink based on their morphology (shape and size), were confirmed. As the accuracy of field-identification of scats is questionable, non-invasive genetic methods should be used for assessing the results of any eradication program.

Based on the results, we argue that conservation strategies for the Eurasian otter in the study area should focus on the restoration of freshwater habitats and improvement of native ichthyofauna. These actions cannot be effective without a flow design that mimics the patterns of the natural flow regime and improves the longitudinal connectivity of watercourses. Regarding invasive exotic species, while American red crayfish are difficult to eradicate, it has been shown that management can control the spread of giant reed. However, long-term management and monitoring are required to control this invasive species and confirm the positive effects of environmental restoration on both habitat quality and otters in the Segura watershed. For the latter aim, otter surveys should be carried out regularly. At the same time, in areas where the expansion of otters and mink is expected to lead to range overlap, non-invasive PCR-RFLP methods may allow the reliable and cost-effective identification of the target species and the immediate implementation of management actions.

The action plan for otter recovery in Murcia Region includes the conservation and improvement of freshwater habitats, involvement of public cooperation, control of alien species, enhancement of river connectivity and regulamentation of sport fishing. The ongoing recovery of a charismatic species such as the Eurasian otter can contribute to a positive perception of management actions by both government agencies and the public, supporting future restoration projects of the River Segura.

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Chapter I

General Introduction

Introduction

1 Aims of the study

The “Plan de Recuperación de la Nutria en la Región de Murcia” (Otter Recovery Plan in Murcia region), approved in 2016 by the regional government, aims at improving the quality and suitability to Eurasian otters (*Lutra lutra*, Linnaeus 1758) of freshwater ecosystems, monitoring its population trend and stimulating the involvement of the whole society in the conservation of the species. It also stresses the lack of information on otter status and current threats to its conservation.

Within this framework, the main goals of this work were the updating of the distribution and ecology of the otter in the catchment of the River Segura under different habitat restoration regimes (Chapter 1), the analysis of diet in relation to historic changes in native and exotic fish resources (Chapter 2), and natural-urban gradients (Chapter 3), also by setting up a cost-effective non-invasive genetic method for the prompt and reliable identification of faecal samples (Chapter 4), which, in areas of sympatry, can be confused with those of non-native American mink (*Neovison vison*, Schreber 1777). Monitoring was carried out in relation to ecological restoration projects, with the aim of highlighting the otter’s potential role as bioindicator of environmental quality in semi-arid Mediterranean rivers. Particular attention was devoted to otter feeding habits, with the aim of assessing the suitability of freshwater habitats along a natural-to-urban gradient and highlight the changes occurred in the composition and diversity of fish communities in the last decades, as a consequence of flood regulation and spread of non-native species.

The Eurasian otter (*Lutra lutra*) is a semi-aquatic carnivore, and it is particularly sensitive to the environmental variations which affect freshwater ecosystems, particularly in Mediterranean climates (Magalhaes et al. 2002). As in many European countries, in Spain the Eurasian otter declined throughout the 1970s and 1980s (Delibes 1990) and started to recover in the last decade of the 20th century (Ruiz-Olmo & Delibes 1998). The third national otter survey showed that the species occurred in 70% of the country (López-Martín & Jiménez 2008). This positive trend has induced to classify the otter as “Least Concern” according to the IUCN Red List criteria (Ruiz-Olmo 2014). In Murcia, the Eurasian otter (*Lutra lutra*) suffered a large reduction in its distribution since the 1960s and 1970s, when it occurred on the whole River Segura and five tributaries (370 km), until the early 1990s, when it was only located in the upper third of the main river (50-60 km; Palazón and Carmona 1998; Pastor et al. 2008). In the last 30 years, the otter has recovered and currently is reported on a ca. 230 km long stretch of the river (Dirección General de Medio Ambiente, 2019 and Dettori et al. 2019a).

Rivers have experienced intense and long-standing human pressures. These impacts favor the spreading of opportunistic and exotic species, with a detrimental effect on native communities. In particular, the giant reed (*Arundo donax*, L. 1753), a species of Asian origin historically used by man, which has progressively colonized the Mediterranean basin and large areas of the five continents, becoming one the 100 most dangerous invasive species worldwide (Lansdown 2013). In Spain, and especially in the River Segura basin, the giant reed is thoroughly spread disturbing the native riparian vegetation. With LIFE13BIO / ES / 001407 RIPISILVANATURA (2014-2019) the aim was, through soft engineering techniques, to control invasive alien species and to improve riparian habitats by reducing the coverage of the invasive giant reed *Arundo donax*. Among invasive animal species, one with the greatest effects on the structure and functioning of the river biological communities and habitats is the red swamp crayfish (*Procambarus clarkii* Girard, 1852), whose role as prey for the Eurasian otter is another relevant aspect of research interest in semiarid Mediterranean rivers (Ruiz-Olmo & Palazón 1998; Clavero et al. 2003). The invasion by red swamp crayfish is only one of the many symptoms of the strong alteration suffered by the biota of these rivers, in which important changes are also observed in the ichthyofauna, for which the otter can represent an interesting indicator (Castejón-Bueno et al. 2011; Loureiro et al. 2015; Souty-Grosset et al. 2016).

The American mink (*Neovison vison*) is an invasive semi-aquatic mustelid that it is considered a threat to endemic and threatened native species (e.g. Palazón and Ruiz-Olmo 1997; Ferreras and MacDonald 1999; Jefferies 2003; Macdonald and Harrington 2003; Fisher et al. 2009), and also competes with other mesocarnivores (Melero et al. 2012; Palazón and Melero 2014), in particular the endangered European mink (*Mustela lutreola* Linnaeus, 1761), and European polecat (*Mustela putorius*, Linnaeus, 1758). The American mink has been introduced throughout Europe for its fur. In the Iberian Peninsula, the first farms date back to the late 1950s in central and north-western Spain. Since then, recurrent escapes have given rise to various nucleus scattered in 12 autonomous communities (Lecis et al. 2008; Melero and Palazón 2017), reaching as far as north-western Portugal (Vidal-Figueroa and Delibes 1987). The American mink is found mainly along river courses and in wetlands (Palazón et al. 2016), where it affects the distribution of Miller's water shrew (*Neomys anomalus* Cabrera, 1907) and southwestern water vole (*Arvicola sapidus* Miller, 1908) (García-Díaz et al. 2013).

Where sympatric, the faeces of otters and mink can be confused when the latter preys on aquatic prey, or, otherwise, with those of other mustelids in the case of the American mink. Therefore, cost-effective techniques are needed to discriminate the presence of both species, particularly in areas of potential overlap resulting from the ongoing expansion of both species' ranges. Such techniques may serve as surveillance tools to prevent the spread of American mink into territories not yet occupied.

To introduce the research work, we review the role of molecular techniques in the study and management of otters and mink, to conclude with a brief presentation of the target species and study area.

Figure 1. Eurasian otter (*Lutra lutra*). Photo taken during a sampling carried out in 2017 in the Teselas 37-38 "La Mulata", station of the LIFE Ripisilva project.

1.1 Molecular Ecology and Conservation Genetics

In the last three decades, the use of molecular markers has acquired a leading role in fields such as: taxonomy, ecology, evolutionary biology and conservation biology. Disciplines such as molecular ecology and conservation genetics result from the integration of genetics with ecology (Frankham et al. 2002). Progress in these disciplines has brought to highlight a number of topics in relation to evolutionary genetic changes in populations exposed to anthropogenic disturbance (e.g., habitat fragmentation, direct persecution, introductions, etc.). The rapid development of non-invasive molecular techniques *(non-invasive genetic sampling*) occurred in the last three decades has made it possible to combine the analysis of molecular markers with the extraction of DNA from biological material, such as hair or feces (Waits and Paetkau 2005).

The spread of these techniques has led to a real revolution in the study of wildlife and in particular of elusive, rare or threatened species, making it possible to gather information that would have been difficult to obtain through traditional invasive techniques (Taberlet and Luikart 1999; Piggott and Taylor 2003).

Non-invasive molecular techniques have been applied to wildlife studies since the early 1990s (Höss et al. 1992; Taberlet and Bouvet 1992), particularly to carnivorous mammals (Piggott and Taylor, 2003; Waits and Paetkau, 2005; Beja-Pereira et al. 2009), such as: ursids (Taberlet et al. 1997; Wasser et al. 1997; Murphy et al. 2003; Bellemain and Taberlet 2004), canids (Lucchini et al. 2002; Adams et al. al. 2003; Creel et al. 2003; Marucco et al. 2009), felids (Ernest et al. 2000; Palomares et al. 2002; Alda et al. 2008) and mustelids (Frantz et al. 2004; Gómez-Moliner et al. 2004; Hedmark et al. 2006; Ferrando et al. 2008; Ruiz-Gonzalez et al. 2008). Several species-specific protocols of analysis have been proposed (Taberlet and Luikart 1999).

The application of non-invasive techniques allows the assignment of biological samples to three systematic levels: species, population and individual. This allows to obtain information of primary importance for management and conservation purposes.

At the species level, it is possible to reliably assign those signs of presence that would be difficult or even impossible to distinguish on the sole basis of morphological characteristics, thus representing an effective method for estimating the distribution of elusive or otherwise difficult to detect species (Alda et al. 2008; Gómez-Moliner et al. 2004; Palomares et al. 2002). In addition, using markers linked to sex chromosomes, it is possible to assess the sex ratio of the target population (Dallas et al. 2000; Dallas et al. 2002). Finally, by using hypervariable markers (microsatellite markers), individual genotypes can be achieved, allowing to study ranging behaviour and habitat preferences, population dynamics (density, dispersion, immigration rates, etc.) and composition of social groups (Waits and Paetkau 2005).

2 Non-invasive genetic monitoring

2.1 General considerations

Before embarking on any study focused on non-invasive sampling methods, we must keep in mind a number of general considerations: first, we must be aware that non-invasively collected DNA is generally of low quantity and is partially degraded (Broquet and Petit 2004); secondly some contamination problems may occur due to the presence of PCR inhibitors (Taberlet and Luikart 1999). Thus, identification or genotyping success depends on several related factors, including: the type and age of the sample, sampling conditions, storage, and the protocol used for DNA extraction and amplification. Optimizing each phase and coordinating field work and laboratory analysis is essential to obtain a result that is overall satisfactory (Beja-Pereira et al. 2009).

2.2 Preservation of samples

Two main storage methods are commonly used:

silicates (SiO_2), in powder or granules, are used to quickly dry the sample so that it can be stored at room temperature for a long time. It is a simple and convenient method also to be used in the field. The method is used for both feces and hair (Randi 2006).

Ethanol> 90% is very efficient in preserving DNA and can be used directly in the field to stop DNA degradation at the moment of sample collection. The advantage of this method lies in the possibility of storing the sample at room temperature even for a few days. It is used for both feces and hair. The ratio of faecal sample to solution should be 1:2 or 1:4 (Randi 2006).

Tubes can be temporarily stored in a cool place at + 4ºC, but if the extraction is not carried out in a short time it is advisable to freeze them at–20°C. Each sample must be collected using sterile instruments, to avoid both contamination between samples and contamination by human DNA, and each sample must report the date, location, sample code, and notes (Randi 2006).

2.3 Most used molecular markers in non-invasive genetic samples

The choice of molecular markers depends on the aims of the analysis. Mitochondrial DNA (mtDNA) is usually chosen for species-level work, while microsatellite loci are better suited for identifying individuals. The mtDNA is replicated in the mitochondria, and each cell contains 5,000 to 10,000 mitochondria, each of which contains 10 or more mtDNA molecules. Each mtDNA sequence, defined as a "haplotype", is transmitted intact over the generations, and therefore can be used to reconstruct genealogies in populations and to identify samples belonging to different populations, subspecies and species. The most used sequences of mDNA are the *Cytochrome b,* which can be easily aligned between different species and the *D-Loop* control region, provided that the amplified fragment includes one hypervariable domain, which in some cases makes it possible to distinguish also the subspecies or population of origin (Randi 2006; Ruiz-Gonzalez et al. 2008). PCR products are then sequenced and compared to the sequences filed in international data banks, such as GenBank, using specific softwares (e.g. BLAST). Sequences with the highest percentage of similarity are generally the most suitable for identifying the species.

For mammals, a rapid, unexpensive and alternative method to sequencing is the analysis of restriction sites (Restriction Fragment Length Polymorphisms, RFLP). This technique consists in digesting by restriction enzymes a fragment of mtDNA (usually Cytochrome b or D-loop region), which has diagnostic restriction sites for the different species, and thus yield fragments of species-specific length (Ruiz-Gonzalez et al. 2008; Dettori et al. 2020b).

Microsatellites consist of sequences shorter than 8 nucleotides that are repeated a few times and produce blocks of up to a few hundred nucleotides at each locus. So far, they have been identified in the genome of all analysed organisms and are randomly distributed in the chromosomes. They are included within the non-coding sequences of genes (introns).

Sex markers are unique sequences of DNA of sex chromosomes. This assay consists in amplifying a specific fragment present only on the Y chromosome. As not-amplified fragments can be erroneously attributed to females, usually the DNA fragment used for sex determination is amplified whatever the sex of the sample, but its length varies between sexes.

Sex can be determined by a simple electrophoretic run on agarose gel with separation of the fragments based on their molecular weight, although errors can be generated due to deletion or insertion of alleles (Seddon 2005).

2.3.1 DNA extraction from non-invasive samples

The aim of this process is to obtain the greatest possible quantity and quality of DNA, while avoiding the possible presence of contaminating PCR inhibitors. For this reason, DNA extraction must be carried out paying attention to its handling and using standard protocols that ensure the best capture and purification of the small available amount of DNA. Currently, commercial kits are the simplest and quickest method to extract DNA, being easily adaptable to any type of sample (Hausknecht et al. 2007, Luikart et al. 2008, Vallet et al. 2008). The extraction of faecal DNA is not selective (all the DNA present in the faeces is extracted) and this may affect the next phase of the protocol. Next step is PCR (Polymerase Chain Reaction) using molecular markers in order to amplify the target region of the species under study. DNA amplification is subsequently verified by an agarose gel electrophoresis, under standard conditions.

2.3.2 PCR amplification

The molecular markers usually used for non-invasive genetic analyzes are: mitochondrial DNA (mtDNA), microsatellites (also called Simple Tandem Repeats = STR) and DNA sequences present only on sex chromosomes. The choice of the molecular marker characteristic should be specific for the species to be studied. The markers must be easily amplified even if the DNA is degraded, therefore the amplified fragment should not exceed 200-300 bp for nuclear DNA and 300-400 bp for mitochondrial DNA, the optimal size being 100-150 bp for both DNAs. Another feature of the markers should be high variability, in order to provide more detailed information.

2.3.3 PCR errors

When we design a non-invasive genetic study, we must consider a series of recommendations to recover as much DNA as possible.

Presence of PCR inhibitors, that is those substances that frustrate the normal amplification of DNA, may affect the sensitivity of the assay or even lead to false-negative results. PCR inhibitors are a heterogeneous group of substances with different properties and mechanisms of action, but some of them are predominantly found in specific types of samples, which therefore require matrix-specific protocols for the preparation of nucleic acids prior to PCR (Schrader et al. 2012).

Regardless of the effort to best process a non-invasive sample, non-invasive DNA will always be partially degraded. For this reason, it is recommended to select shorter loci which will grant greater reliability when obtaining a genotype (Broquet and Petit 2004; Buchan et al. 2005). Problems related to low DNA availability (Bellemain & Taberlet; 2004) can be mitigated choosing commercial kits that allow much more specific and reliable DNA amplifications by combining high-throughput PCR buffers and thermoreactive polymerases (Randi 2006).

Exogenous and contaminated DNA. Contamination by human DNA is a major problem in non-invasive studies during laboratory processing. For this reason, in order to avoid the possible interference of alien DNA, the solution is to use species specific markers. It is recommended that no person in the laboratory comes into direct contact with products to be amplified (Gilbert et al. 2005).

3. The target species

The Eurasian otter (*Lutra lutra*) and American mink (*Neovison vison*) are eutherian mammals belonging to the order Carnivora and family Mustelidae. This includes 56 species, grouped into 22 genera and 5 subfamilies: *Mustelinae*, *Melinae*, *Mellivorinae*, *Taxidiinae*, *Lutrinae*. Common characteristics to both species are slender-elongated bodies and a prevalent fish-based diet.

3.1 Eurasian otter (*Lutra lutra*, Linnaeus 1758)

The genus *Lutra* belongs to the subfamily *Lutrinae* and includes three semi-aquatic species - Eurasian otter (*L. lutra* Linnaeus, 1758), Japanese otter (*L. nippon* Imaizumi and Yoshiyuki, 1989), Sumatran otter (*L. sumatrana* Gray, 1865) - and 12 subspecies.

3.1.1 Distribution

Lutra lutra is the most widely distributed otter species (Pocock 1941; Ellerman & Morrison-Scott 1966; Romanowski et al. 2010). Its historical range extended from Japan in the East to Portugal in the West, and from the Arctic regions of Asia and Europe to as far as Indonesia in the South (Foster-Turley et al. 1990). The Eurasian otter declined during the 20th century especially in central and western Europe (Ruiz-Olmo et al. 2008). Currently otter European range includes Denmark, western parts of Germany, the Netherlands, Belgium, Luxembourg, eastern and central regions of France, Switzerland, western Austria, and fragmented populations in southern Italy and Spain (Roos et al. 2015).

Otter home range covers between 1 and 40 km of watercourses, depending on food availability. Faeces and gel, collectively known as “spraints”, are used to mark the territory and provide information about the individual's status to other group members (Kruuk 1992). The home range of each male overlaps that of one or more females, nonetheless otters are usually solitary, and the male does not contribute to cub rearing (Erlinge 1968). Females become sexually mature at 18-24 months of age and the first reproduction occurs, on average, when they are 30 months old. After a gestation of 60-70 days, females give birth to 2-3 puppies which are lactated for 3 months and live with the mother for more than a year (Hauer 2002).

In the Iberian Peninsula Eurasian otters are smaller than in central and northern Europe. The most relevant population occurs on the Atlantic side, ranging from Galicia in the north to Extremadura in the south and including Portugal. In the eastern part of the peninsula the otter is more dispersed tending to be more abundant in the north-east quadrant than in the south-east. It is almost absent in most of ephemeral Mediterranean rivers and in intensively cultivated or urbanized areas (López-Martín & Jiménez 2008; Ruiz-Olmo and Delibes 1998; Morales et al. 1998; Barrientos et al. 2003; Pedroso et al. 2004; 2007, Ruiz-Olmo et al. 2005b), where habitat loss, alterations in water chemistry (Gutleb and Kranz 1998; Kruuk 1995), poaching and road traffic are the main threats to otter. Although population trend is stable, the species conservation is still threatened by environmental pollution, habitat fragmentation, illegal hunting and increasing water consumption.

In Murcia, the Eurasian otter (*Lutra lutra*) suffered a large reduction in its distribution since the 1960s and 1970s, when it occurred on the whole River Segura and five tributaries (370 km), until the early 1990s, when it was only located in the upper third of the main river (50-60 km; Palazón and Carmona 1998; Pastor et al. 2008). In the last 30 years, the otter has recovered and currently is reported on a ca. 244 km long stretch of the river (Dirección General de Medio Natural 2019). Flowing in the semi-arid southeastern Iberian Peninsula, the River Segura (Murcia, Spain) is one of the most regulated European catchments. Reservoirs and water withdrawal and transfer, deeply alter natural flow regimes, reversing the seasonal pattern of flows, and exacerbating both floods and droughts (Vidal-Abarca et al. 2002; Belmar et al. 2010). Since the second half of the 20th century, outstanding spread of irrigated agriculture and urban growth in plain areas have deeply transformed the landscape, increasing water demand with an unsustainable intensity, to the point of giving rise to a structural water deficit (Martinez-Fernandez et al. 2000; Grindlay et al. 2011).
Water pollution, due to both urban and industrial discharges, exacerbates eutrophication and reduces fish availability to otters (Pastor et al. 2008).

Figure 2. Geographic distribution of *Lutra lutra*. Map redrawn from IUCN Otter Specialist Group 2020. The Eurasian otter is an elusive, solitary otter that has one of the widest distributions of all palearctic mammals, from Ireland to China and down to Southeast Asia.

3.1.2 Food habits

Otter food habits are primarily studied by scat (“spraint”) analysis, with the aim of outlining the local composition of its diet, comparing the availability and use by otters of trophic resources, and assessing predator–prey relationships. It is a prerequisite for investigating interspecific competition with other semi-aquatic predators, such as the American mink, and for designing conservation management strategies (Kruuk 2006).

Although fish usually forms the bulk of otter diet, variation in diet composition has been shown to occur among habitat types. Jedrzejewska et al. (2001) reported that the fish component of the diet declined from 94% on seashores, to 71% on lakes and ponds, and 64% on rivers or streams, while the importance of amphibians and crayfish increased inversely. In the sea, otters mainly prey in the littoral zone, and less in benthic and pelagic zones (Erlinge 1968; Mason & Macdonald 1986; Kruuk 2006).

In general, the frequency of use of fish species corresponds to their availability, i.e. the diversity of local fish assemblage (Erlinge 1968; Lanszki et al. 2001; Lanszki & Sallai 2006; Bauer-Haáz et al. 2014). As an exception, usually, otters prey on non-native fish less than expected based on their relative abundance (Balestrieri et al. 2013)., alien fish being mostly preyed upon in the presence of fish farms, which provide artificially high biomass and density of prey (Lanszki 2016). Eurasian otters should prefer large fish (Lanszki et al. 2001; Lanszki et al. 2007) but mainly prey on less than 10 cm long fish (Erlinge 1969; Wise, Linn & Kennedy 1981; Kruuk & Moorhouse 1991; Kloskowski 1999; Taastrom and Jacobsen 1999; Copp & Roche 2003; Remonti et al. 2010). Small watercourses that have a high risk of drying out are of particular interest (Lanszki et al., 2009a), as this may affect prey use of otters (Lanszki et al. 2006; Lanszki & Széles 2006; Lanszki et al. 2009b).

Diet varies as a function of the biogeographical region, fish consumption decreasing from the Atlantic and Boreal regions to the Continental and Pannonian regions and being the lowest in the Alpine and Mediterranean regions (Lanzski et al. 2016). Clavero, et al. (2003) reported a latitudinal gradient in trophic niche breadth, otter diet being more diverse in southern Europe. Amphibian consumption is the highest (9-22%) in the Alpine region, indicating that altitude is also an important factor (Remonti, Balestrieri & Prigioni 2009). Crustaceans are often recorded as a major secondary food resource in the Mediterranean region, while their contribution is the lowest in the Boreal and Alpine regions (Lanzski et al 2016).The highest reptile consumption has been recorded in the Mediterranean and Pannonian regions while bird consumption was the highest in the Continental region. The contribution of mammals is usually negligible (1-3%).

All thirteen otter species show to be remarkably conservative in their diet. Summing up, the Eurasian otter is primarily a fish-predator eater, with crabs and frogs as major secondary foods in southern Europe. It is a generalist predator, the diet of which faithfully reflects the local availability of aquatic prey (Kruuk 2006).

In the Iberian Peninsula, in autumn and winter Eurasian otters mainly rely on fish, probably because of the large availability of juveniles and reduced activity, due to low water temperature. On the contrary, crayfish are more active during summer, increasing their availability to otters during this season (Amboage Domínguez 2016). The spread of introduced red swamp crayfish (*Procambarus* *clarkii*) may have had a positive impact on otter ongoing recovery, despite its strong impact on freshwater habitats (Mendes et al. 2011).

3.1.3 Genetic sampling of otters

The Eurasian otter has an intense marking activity, mainly carried out through spraints and the jelly-like, brown and sticky secretions of anal glands. When fresh they have a black-greenish color which slowly turns to whitish in old spraints, which anyway retain their characteristic odour, which Bouchardy (1986) compared to a mixture of fish and linseed oil. Upon visual analysis, food remains, such as fish scales and vertebrae, bone fragments of amphibians and remains of crustacean exoskeleton, are generally evident in the faeces. Sprainting sites, i.e. sraints or gels deposited within a radius of about 1 meter (Kruuk et al. 1986), can be marked by several individuals.

The so-called “standard method “for otter surveys (Macdonald 1983) contemplates the identification of a proper number of survey stations, consisting of stretches of river between 600 and 1000 m in length, to be visited periodically in search of signs of presence of the species. Marking intensity is expressed as the number of sites or signs (spraints and gels) per 100 m of watercourse and is considered as an rough index of otter abundance (Mason and Macdonald 1987)., Data on marking intensity combined with the percentage of positive stations allow to define the distribution pattern of the species (Mason and Macdonald 2004). Geo-referencing of collected spraints by Territorial Information Systems (GIS) may allow to assess the movements of individuals and estimate population size (Kohn et al. 1999; Pearse et al. 2001; Wilson et al. 2003; Eggert et al. 2003).

For genetic analysis, "fresh", i.e. deposited within the past 12 hours, spraints should be selected (Randi 2006). For still not fully understood reasons, genotyping success of otter faecal DNA is usually lower with respect to other mustelids, usually ranging between 20% and 30%. In a study conducted in the Pollino National Park (Southern Italy), out of a total of 187 fresh faeces collected in the field, 77 (41.2%) were successfully genotyped (Prigioni et al. 2006). Notwithstanding, in Mediterranean areas, riparian vegetation cover is usually scarce along most river stretches, and insulation and high air temperatures are expected to make DNA degradation more rapid than in northern Europe.

Despite these difficulties, given the high elusiveness of the species, non-invasive genetic monitoring is currently the most reliable method for gathering information on otter distribution and abundance.

Genetic studies have highlighted low levels of mtDNA variation (Ferrando et al. 2004; Mucci et al. 2010), which may be due to genetic drift in postglacial founder populations with long-term low densities, and overhunting. In Eastern Germany the mitochondrial genome of 81 individuals has been investigated to assess the actual genetic variability and identify population subsets for conservation management (Effenberger & Suchentrunk, 1999). Mucci and Randi (2007) developed a PCR/RFLP system targeted to amplify and cut a segment of the ZFX/ZFY gene in non-invasive otter (*Lutra lutra*) samples. This assay produces one sex-specific fragment in females (XX genotypes) and two fragments in males (XY genotypes), allowing sex identification in 72/91 (79%) of individuals.

In the northern Iberian Peninsula (Basque Country), non-invasive genetic sampling effort was carried out to assess the distribution and population size of an endangered otter population (Vergara et al., 2014). Samples were identified to species level by sequencing a 226 bp mtDNA fragment prior to genotyping and all samples corresponding to otter (127 out of 134, while two samples were identified as mink) were subsequently individually genotyped using a multiplex panel of 11 microsatellite markers and sexed by the sex-chromosome-related gene ZFX/ZFY. Genotyping success was 43%, corresponding to 20 different individuals (11 females, 6 males, and 3 individuals of unknown gender) and a mean otter density of be 0.09 (0.06–0.12) ind./km of watercourse (Vergara et al., 2014).

In the River Segura basin, for his doctoral thesis (University of Murcia) Lucas-Canovas (2019) used 13 microsatellite loci according to the Dallas & Piertney (1998) and Dallas (1999) criteria and designed a PCR-Multiplex system to optimize all reactions. From his study, 17 consensus genotypes were obtained, and population size was assessed at 26 individuals.

Figure 3. Photo taken during a sampling carried out in 2017 in the Teselas 42 and 42, stations of the Ripisilva LIFE project.

3.1.4 Status and conservation

In Spain, the Eurasian otter was back in the spotlight when the "Ministerio de Medio Ambiente" published the work entitled "La Nutria (*Lutra lutra*) en España", edited by M. Delibes (1990), a review of current knowledge on otter distribution and population trend in the previous decades. In the Iberian Peninsula, the species suffered a strong regression from the 1950s to the 1980s, mainly due to direct persecution for fur, a situation that went on until 1973, when legal protection was finally granted (Decreto 2573/73) (Galián et al., 2011). Other causes of its decline were the alteration of riparian systems (Adrian et al., 1985), and water pollution, particularly by Persistent Organic Pollutant, such as DDT and PCBs (Ruiz-Olmo et al. 2000). In 1981 a national-scale survey was carried out (Elliot, 1983), which, together with the work of Delibes and Callejo (1983), allowed to draw a picture of otter current distribution.

At the European level, the Eurasian otter is listed in Annexes II and IV of the Habitats Directive (Directive 92/43/EEC), which stipulate that otter conservation requires strict protection and the designation of special protection areas. In Spain, the Eurasian otter is included in the List of Species in the Special Protection Regime (RD 139/2011). In the Murcia region, the otter is included in the Catalogue of threatened species (Art. 16) with the classification "Endangered" (EN) (Ley 7/1995 de la Fauna Silvestre, “Law for Wild Life”); as such, an Action Plan for the removal of major threats is required to be drafted. Finally, RD 59/2016 approved the Eurasian otter Recovery Plan in the Region (“Comunidad Autónoma”) of Murcia (BORM, 2016).

3.2 American mink (*Neovison vison* Schreber, 1777)

Neovison vison is the only extant species of the genus *Neovison*, belonging to the Mustelinae subfamily. It occurs throughout the United States, except Arizona, and in most of Canada, including an introduced population in Newfoundland.

Its size is about 30-40 cm, excluding the tail. This is just over a third of the total length of the head-body. A certain degree of sexual dimorphism can be recorded: males are slightly longer (head-body length: 34-44 cm vs. 30-37 cm) and heavier (1.2-1.5 kg vs. 0.7-0.9 kg) than females. Its water repellent fur is usually dark brown to black. American minks have white markings on their chin, lower lip, abdomen, and groin.

The fingerprints of the American mink are very characteristic, despite practically indistinguishable from those left by European mink (Ceña and Ceña 1999). They are between 2.5 and 4 cm long and 2.5 cm wide, almost half that of the otter (Bravo 1999).

Scat can be deposited on riverbanks, on the top of stones or other prominent objects, and generally are blackish. Their shape is that typical of the mustelids from 6 to 9 cm in length. The scent is strong and very distinctive.

The breeding period starts in March and lasts four weeks. As in other Mustelids, delayed implantation occurs while, gestation lasts between 40 and 75 days, on average 58. The female suckles 3-6 young for about 5-6 weeks. The young come out of the den when they are 6-7 weeks old (Bravo 1999).

3.2.1 Distribution area

Native to North America (Dunstone 1993), the American mink has been introduced in most of Europe, from Iceland and Norway in the north to the Iberian Peninsula in the south (Bonesi and Palazón 2007). It became widespread since the 1920s (Gerell 1971; Bonesi and Palazón 2007), mainly due to animals escaping from fur farms. Large established populations occur in northern and central-eastern Europe, while more localized populations are spread in Mediterranean countries, namely Italy and the Iberian Peninsula (Bonesi and Palazon 2007).

This invasive semi-aquatic mustelid is considered to threaten endemic and native crustaceans, fish, amphibians, voles and ground-nesting birds as a predator (e.g. Palazón and Ruiz-Olmo 1997; Ferreras and MacDonald 1999; Jefferies, 2003; Macdonald and Harrington 2003; Fisher et al. 2009) and it also competes with other mesocarnivores (Melero et al. 2012; Palazón and Melero 2014), particularly the endangered European mink *Mustela lutreola* and polecat *Mustela putorius*.

In Spain, fur farms are distributed in the northern area of Segovia and Pontevedra, which were the first regions to start this type of industry, Catalonia, Cantabria, Madrid, Castilla y León, Aragon, Valencian Community, and the Basque Country. In 1992, in Spain there were 214 registered farms, which then dropped to 37 in 2011. Currently, there are farms in Cantabria, Soria (Lubia), Vizcaya, Guipúzcoa (Motrico), Álava (various locations), Teruel (Carrión and Mora de Rubielos), Zaragoza (Albalate del Arzobispo), Girona (Ullastret and Serra de Daró). There is also a farm in Valença do Minho (Portugal).

In 1997, the Ministerio de Medio Ambiente published a monograph on the European and American mink coordinated by Jordi Ruiz-Olmo and Santiago Palazón, reporting the status of the species in 1992-93. According to this monograph, in 1992-93 the American mink range covered a total of 18,300 km^2, about 3.6% of Spain.

In 2020, due to the SARS-Cov2 Pandemic (COVID-19), farming activities have been suspended indefinitely throughout Europe and massive culls have become widespread (Lesté-Lasserre, 2020).

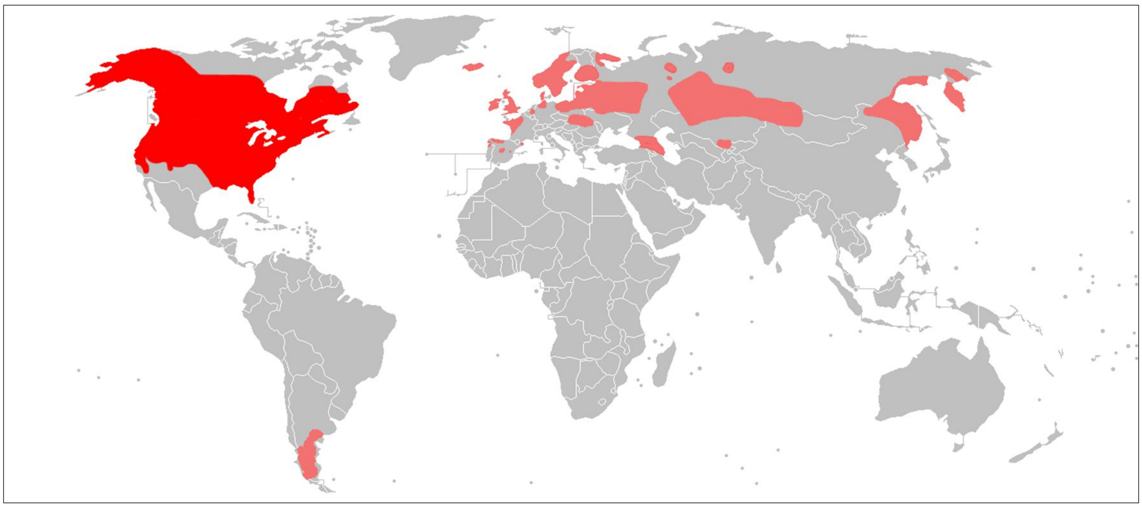

Figure 4. (IUCN and Juan Carlos Blanco, Mamíferos de España. ISBN 8408027492) The species natural range encompasses most of North America. Introduced in South America, Europe, and the old Soviet Union.

3.2.2 Diet

Information on alien predator diets is essential for assessing their impact on native prey and understanding competitive inter-specific interactions with native predators (Jędrzejewski et al. 1995; Bartoszewicz et al. 2008; du Preez et al. 2017).

The American mink is considered a generalist and opportunistic species that feeds on both aquatic and terrestrial prey (McDonald 2002; Hammershj et al. 2004); its diet depends on the local and seasonal availability of food resources.

In the Iberian Peninsula, as described by Bravo and Bueno (1999), the American mink relies on a wide range of prey, with fish and mammals being primary resources and birds secondary prey (Vidal-Figueroa and Delibes 1987; Bueno 1994). Among fish, it prefers cyprinids as they are slower and easier to catch than salmonids, but eels (*Anguilla 39larkia39*) are also a favorite prey, while, among mammals, rabbits (*Oryctolagus cuniculus*) are a preferred prey, as well as a large variety of small mammals (Bravo & Bueno 1999). Furthermore, it can feed on amphibians, particularly during their breeding period.

Usually, the consumption of mammals increases in summer and autumn, when they are more abundant, while fish are more frequent in winter, due to their reduced mobility (Bravo & Bueno 1999). The consumption of birds is also more abundant in the warm season when pullets are abundant. In areas where colonial birds breed on mainland, they may be the main resource (Vidal-Figueroa and Delibes 1987). The American crayfish *Procambarus 40larkia* is a key prey for the American mink in the Iberian Peninsula (Bueno 1996).

3.2.3 Genetic sampling of American mink

Several genetic methods have been developed for identifying American mink based on non-invasive sampling. These methods include the amplification and sequencing of either the mitochondrial cytochrome b gene (Harrington et al. 2010), or microsatellite locus Mel08 (López-Giráldez et al. 2005), and polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) methods. For the latter, widely applied, indirect approach, a variety of PCR primers and restriction enzymes have been designed to recognize the American mink among a range of closely related species, targeting either the mitochondrial cytochrome b gene (Hansen and Jacobsen 1999; Harrington et al. 2010) or D-loop region (Gómez-Moliner et al. 2004; Statham et al. 2005; Ruiz-González et al. 2008).

Figure 5. Laboratory extraction of DNA from American mink and Eurasian otter feces.

3.2.4 Status and control

This invasive species is regulated by the Real Decreto 630/2013, 2 August 2013, and is included in the Catálogo Español de Especies Exóticas Invasoras that provides a strategy for management, control and eradication of the species. In Spain the American mink cannot be possessed or sold, nor released in nature.

In those countries where the American mink constitutes a pest and a threat to indigenous biodiversity, a great variety of studies and control programs are being carried out (Bonesi and Palazón 2007). In Spain, since 2001 there has been an Eradication Plan for the American mink, coordinated within the European mink Working Group (Ministry of the Environment and Rural and Marine Affairs) and framed in the National Strategy for the Conservation of the European mink. Since the start of this century, a systematic control of the populations of Álava, Burgos, Teruel and Castellón has been applied, and then extended to those of Vizcaya, Guipúzcoa, La Rioja, Navarra, Palencia, Soria, Zaragoza and Catalonia. So far, the only methodology for controlling the American mink is selective trapping using trap boxes. It is a method that requires a lot of effort and adequate personnel, as well as a high and constant budget over several years. The effort and economic costs of trapping different proportions of the whole population per unit area have been estimated, as well as the possibility of eradication in continental areas (Zuberogoitia et al. 2010). Results suggest that while eradication may be possible in some areas with a moderate cost, control is the most sustainable option (Zabala et al. 2010).

4. Competition between Eurasian otters and American mink

Connell (1980) defined "the ghost of past competition" referring to species coexisting despite resources overlap, and which are supposed to have adapted as to avoid the deleterious effects of competition. This does not apply to native mustelids and American mink, the latter occurring in Europe only since the second half of the 20th century. Studies on competition between American mink and Eurasian otter are manifold (Erlinge 1972; Jenkins and Harper 1980; Chanin 1981; Clode and Macdonald 1995; Bueno 1996; Strachan and Jefferies 1996; Previtali et al. 1998; Ruiz-Olmo et al. 2001; Bonesi et al. 2004; Bonesi et al. 2006). Most of these studies attempted to establish dietary overlap between the two species. Wickens (1991) observed that Eurasian otters interfered with the American mink by over-marking their latrines. In the United Kingdom the American mink established in the 1950s and 1960s following escapes from fur farms and by the 1970s occurred throughout England and Wales (Dunstone 1993; Halliwell & Macdonald 1996). American mink settled in the wild at a time when otters were declining due to water pollution (Chanin and Jefferies 1978), and it was suggested that competition with the non-native predator may had played a role (Lever 1985). On the contrary, by comparing diets and densities of mink and otter in the mid 1970s and late 1990s in a same river, it has been observed that mink shifts its diet as otter density increases, suggesting that mink habitat use may be affected by competition with the larger otter (Strachan et al. 1990; Jefferies et al. 2000; Bonesi et al. 2004). Among other factors, food availability is a major variable that can determine the coexistence of Eurasian otters and American mink. Therefore, it is important to know the composition of their diet.

In Spain, the existence of important populations of American mink did not prevent the Eurasian otter from recovering in large areas of the central peninsula (Ruiz-Olmo et al. 1997). A study by Bueno (1996) showed that there is a clear segregation in the size of the fish consumed by the two species. The American mink tends to capture fish smaller than 15 cm, whereas the otter avoids the very small ones (< 5 cm) and prey on the other size classes depending on their availability. Moreover, when sympatric, mink tend to rely more on terrestrial prey to lower competition.

In conclusion, there is a clear diet overlap and the American mink is capable of occupying the otter's niche in those locations where the latter is absent (Bueno et al. 1996). On the opposite, in the best-preserved aquatic habitats, Eurasian otter presence may hinder the expansion of the non-native mustelid (Bravo & Bueno 1999). The assessment of the distribution of the two species in potential areas of sympatry is partially hindered by the actual possibility of misidentifying their scats (e.g. Lampa et al. 2015). For this reason, cost-effective genetic methods are required to allow the prompt and reliable identification of faecal samples.

5. Study area

The two species' distribution and diet were monitored in area of both sympatry (rivers Tagus, Turia and Ebro) and allopatry (rivers Segura, Tirso and Flumendosa).

5.1 Segura Hydrographic Basin

The catchment of the River Segura is located in the southeast of the Iberian Peninsula, with an approximate area of 18,879 km^2. Data were collected on the river stretch flowing from the Comarca del Noreste (municipality of Calasparra) through the Comarca de la Vega Alta del Segura (municipalities of Cieza, Abarán) and del Ricote (municipalities of Ojós and Ulea) downstream ward to Comarca Huerta of Murcia, which includes the municipalities of Alcantarilla and Murcia. The area belongs to the geological-structural unit of the Betic Mountains. It is one of the driest regions in Europe, characterized by mild winters, hot summers, scarce and variable rainfall, less than 350 mm per year, and a moderate thermal amplitude. Because of prolonged droughts the catchment area is mostly semi-arid, with a moderate increase in rainfall in mountainous areas (Sanchez & Carmona 2006).

5.2 Tagus Hydrographic Basin

The River Tagus (Tajo) has been connected to the River Segura through the "Transvase Tajo-Segura" (Tajo-Segura inter-basin water transfer), a 286 km long water transfer facility connecting, since 1978, the Entrepeñas and Buendia dams in the upper River Tajo, to the Talave Dam in the catchment of the River Segura (Pittock et al., 2009). Monitoring areas were located in: Sacedón municipality, province of Guadalajara, and in the protected area "Reserva Natural Fluvial del arroyo Ompolveda", which takes its name from a pluvial-Mediterranean stream (Ompolveda) that springs on limestone mountains.

The River Manzanares flows from the Sierra de Guadarrama through Madrid and into a Tagus's tributary, the River Jarama. Its upper basin, from the source to "monte de el Pardo" is included in the Regional Park "de la Cuenca Alta del Manzanares" (52,796 ha). The lower course is included within the protected area "Parque regional del Sureste".

The River Jarama springs at the feet of the Peña Cebollera and crosses the Spanish provinces of Guadalajara and Madrid. Its main tributaries are, on the right bank, the rivers Lozoya, Guadalix and Manzanares; and, on the left-side, the rivers Jaramilla, Henares and Tajuña. Being the only biological corridor that crosses the Community of Madrid from north to south, it plays a major role in the spread of both Eurasian otters and American mink. The river Tajuña crosses the provinces of Guadalajara and Madrid. It feeds a 409 ha reservoir in the valleys of Torrecuadrada de los Valles and El Sotillo. River Henares, a 160 km long tributary originates in the Sierra Ministra (1220 m a.s.l.).

5.3 Turia and Ebro Hydrographic Basin

In Teruel province of the autonomous community of Aragon, sampling was conducted on the River Jiloca, that it is part of the watershed of the Ebro basin. its course runs through the provinces of Teruel and Zaragoza. This 126 km long river flows in a generally north easterly direction from its source near Monreal del Campo. The watershed covers an area of 2,957 km^2.

The River Alfambra is a tributary of the River Turia, which springs on the Sierra de la Moratilla (2000 m a.s.l.), in the province of Teruel. It is a low-discharge, irregular watercourse with strong floods in autumn and late winter.

5.4 Tirso and Flumendosa Hydrographic Basins

Surveys for non-native American mink were carried out also in the autonomous region of Sardinia, in Italy (Oristano, Núoro and Ogliastra provinces). Two major watercourses of the island were sampled, namely the River Tirso and River Flumendosa, belonging to two distinct hydrographic basins.

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Chapter II

The recovery of the Eurasian otter in the framework of restoration and control of Invasive Alien Species (IAS) in the Segura River Water Basin (SE Spain).

PART 1: Eurasian otter distribution and habitat use in a Mediterranean catchment managed for the control of invasive giant reed *Arundo donax*.

Abstract

We assessed the distribution and habitat use of the Eurasian otter (*Lutra lutra*) on the middle reaches of the River Segura and its tributaries (Murcia, SE Spain) within the framework of the project LIFE13BIO/ES/001407 RIPISILVANATURA (2014-2019). Between April 2016 and June 2018, we surveyed (1-10 times) 42 sampling stretches for otter spraints (mean length ± SD = 0.5 ± 0.2 km), covering a 109.8 km long section of the river. The study time frame coincided with the main restoration activities of the LIFE project, consisting in the eradication of widespread, invasive giant reed Arundo donax and plantation of autochthonous riparian vegetation. Otter habitat preferences were assessed by measuring ten environmental parameters potentially affecting its occurrence. The relationship between the presence/absence of the otter and habitat variables was evaluated by both Logistic Regression Analysis (LRA) and non-parametric Random Forest regression model. Otters avoided polluted waters and their occurrence was inversely related to the width of the riparian buffer, mostly consisting of Arundo reed beds. Moreover, in managed stretches, otter marking intensity increased after the removal of alien reeds and was higher in RIPISILVANATURA restored transects (1.18 spraints/100 m) than in the downstream, unmanaged and invaded half of the watercourse (0.76 spraints/100 m). The combined effects of water pollution and giant reed spread may alter the distribution and abundance of fish, reducing prey availability to otters. The improvement of habitat quality and native fish communities is thus pivotal to enhance otter recovery in the heavily modified catchment of the River Segura.

Key words: invasive alien species, freshwater habitat, habitat restoration, riparian vegetation, Lutra lutra. environmental management.

Introduction

The current scale and intensity of human-induced environmental change is unprecedented, and the rate of species extinctions is still accelerating, representing a major threat to biodiversity (Brooks et al. 2002; Hanski 2011), and humankind (United Nations 2019). Economic activities affect species abundance and distribution through landscape modifications which alter ecosystem properties and functions (Foley et al. 2005), including productivity (Haberl et al. 2007) and food availability (Muhly et al. 2013). The main causes of environmental change are variation in land use, direct exploitation of organisms, climate change, pollution and invasive alien species (United Nations 2019).

Environmental degradation is especially worrying in inland aquatic ecosystems, as they have been recognized as one of the most threatened habitats in the world (Saunders et al., 2002). Rivers have experienced intense and long-standing human pressures, especially made by socioeconomic drivers (agriculture, water storage and diversion, urban and industrial sewage discharge), which have caused the impairment of aquatic and riparian habitats worldwide (Dudgeon et al., 2006; Pletterbauer et al., 2018; Schumtz & Sendzimir, 2018) mainly through water overexploitation, flow regulation, agricultural and urban pollution, habitat modification and riparian deforestation, among others (e.g. Dudgeon et al., 2006).

Climate change is expected to synergistically affect aquatic ecosystems (Moss et al., 2009) rising global temperatures and increasing the frequency and intensity of droughts and extreme flow events in some regions (Milly et al., 2005), such as Mediterranean catchments where altered precipitation seasonality and an increase in aridity and flow intermittence is predicted (Giorgi & Lionello, 2008). In addition, Mediterranean landscapes have been subjected to intense human influence for centuries, which has led to the progressive loss of riverine species and habitats (Tierno de Figueroa et al., 2012). Therefore, the combination of historical anthropogenic pressure and recent climate change has resulted in a critical situation for Mediterranean biodiversity.

Anthropogenic impacts and global change enhance the spreading of opportunistic and exotic species, with a detrimental effect on native communities.

In particular, the giant reed (Arundo donax), one the 100 most dangerous Invasive Alien species (IAs) worldwide (Lansdown, 2013), has progressively colonized the Mediterranean region. Arundo donax is a perennial, rhizomatous grass that was actively introduced from Asia several decades and centuries ago for hedgerows, bioconstruction (e.g., traditional roofs), agricultural stakes and erosion control (Elorza et al., 2004). More recently, European projects enhanced its cultivation for biomass production (El Bassam and Dalianis, 1998). Its large rhizomes enhance tolerance to environmental stress and enable quick resprouting after mowing, removal and other disturbances (Quinn & Holt, 2008; Lambert et al., 2010; Mann et al., 2013). For these reasons, Arundo donax flourishes in the riparian habitats of urban and agricultural areas, where it outcompetes native reeds and woody plants, increasing flooding, siltation (Lambert et al., 2010), and the frequency and intensity of fire (Giessow et al., 2011). Moreover, it not only affects native flora, but also soil arthropods (Maceda-Veiga et al., 2016) and can have a dramatic impact on threatened fish, amphibians and riparian birds (Giessow et al., 2011; see also Bruno et al., 2019, and references therein).

Flowing in the semi-arid southeastern Iberian Peninsula, the River Segura (Murcia, Spain) is one of the most regulated European catchments. Reservoirs and water withdrawal and transfer, deeply alter natural flow regimes, reversing the seasonal pattern of flows, and exacerbating both floods and droughts (Vidal-Abarca et al., 2002; Belmar et al., 2010). Since the second half of the 20th century, outstanding spread of irrigated agriculture and urban growth in plain areas have deeply transformed the landscape, increasing water demand with an unsustainable intensity, to the point of given rise to a structural water deficit (Martinez-Fernandez et al., 2000; Grindlay et al. 2011). Favoured by these man-driven environmental alterations on the main river and its tributaries, the giant reed has widely spread in the midlands and lowlands of the catchment, covering up to 40% of riverbanks in these areas, where it has displaced native riparian vegetation, originally constituted by a peculiar mixture of European and north-African flora (Bruno et al. 2019).

With the aim of controlling this invasive reed and enhancing the recovery of native riparian habitats, soft-engineering techniques were applied between 2014 and 2019 in 37 selected river stretches of the middle segments of the Segura river, in order to decrease the density and coverage of A. donax within the framework of the LIFE+ project RIPISILVANATURA (LIFE13BIO/ES/001407). Given that A. donax coexists with remnants of native riparian vegetation including species with conservation interest, the removal of above-ground stems was mainly carried out manually (portable electric lawnmower) in winter to minimize disturbance, followed by either monthly (i.e. extensive) or quarterly (i.e. intensive) maintenance; to enhance the effects of competition (Curt et al., 2017). In addition, site-specific combinations of native riparian species have been planted since winter 2016 in mowed patches, depending on the local environmental conditions and the potential native vegetation of the reach. Till now, the effects of restoration actions have been tested using ecological indicators such as riparian vegetation, aquatic macroinvertebrates and birds. A significant reduction of A. donax height and density was observed, together with an improvement in riparian quality and increase in the richness of riparian plant species (Bruno et al., 2019). While the diversity and richness of the first indicator were recorded to have increased, bird abundance indices did not show significant short-term responses in a preliminary assessment (Bruno et al., 2018). In a more recent evaluation, however, Bruno et al. (2019) recorded differential effects between extensive (positive-neutral effect) and intensive treatments (neutral-negative effect) for bird richness, density and abundance.

Evaluations of restoration success based on a few better-known taxonomic groups may be inadequate to represent the biodiversity response of other groups and properly assess the influence of riparian restoration in associated biological communities (Golet et al., 2011). Given that the response of biological communities to restoration can differ between taxonomic groups and rivers, assessments at the ecosystem scale should include several levels of biological organization (Pedersen et al., 2007; Pander and Geist, 2013). The Eurasian otter (*Lutra lutra*) is a semi-aquatic carnivore which, being at the top of the trophic chain, is particularly sensitive to the environmental variations affecting freshwater ecosystems, particularly in Mediterranean climates (Gasith & Resh 1999, Magalläes et al. 2002). Furthermore, it has been previously but marginally used in the evaluation of river restoration success (Pedersen et al., 2007).

In Murcia, the otter suffered a large reduction in its distribution since the 1960s and 1970s, when it occurred on the whole River Segura and five tributaries (370 km), until the early 1990s, when it was only located in the upper third of the main river (50-60 km; Palazón and Carmona, 1998; Pastor et al., 2008). As in other Mediterranean areas, the contraction of otter distribution range was mainly due to water pollution, human disturbance, habitat destruction and hunting (Cortés et al., 1998). In the last 30 years, the species has progressively recovered and currently is reported on a ca. 244 km long stretch of the river (Dirección General de Medio Natural, 2019), which could be associated to improvements in habitat quality as wells as a greater food availability due to the spread of red swamp crayfish (Clavero et al., 2010) but also to increased flow regulation (Bueno-Enciso et al., 2014). As otter recovery can be affected by both water discharge and habitat quality (Prenda et al., 2001; Ruiz-Olmo et al., 2004; Remonti et al., 2008), we assessed its distribution and habitat use through surveys for indirect signs of presence through gradients of water discharge and habitat quality. By comparing otter marking intensity in managed vs. unmanaged river stretches, we also evaluated the effects of restoration activities within RIPISILVANATURA, focusing on the removal of giant reed beds and the plantation of native riparian species. We hypothesized that the use of restored/managed stretches will be used by otter in a greater extent than those completely invaded by *A. donax*.

Study area

Located in one of the driest European regions (SE Spain), the River Segura (Fig. 1) is a semi-arid (mean annual rainfall: 382 mm; mean annual temperature: 17 ºC) catchment (14,432 km2), where both natural and anthropogenic gradients contribute to shape a very diverse landscape. Despite the remarkable cover of forested or semi-natural areas (45.2%), the expansion of agricultural land (52.1%) and increasing flow regulation have caused the progressive reduction of natural areas and spread of IAs. Agriculture is the main source of diffuse pollution - mainly phosphates and nitrates -, discharged into the river (Pellicer-Martínez and Martínez-Paz, 2018).

The study area coincided with the middle sector of the river (Fig. 1), showing a mixture of Eurasian and Ibero-African flora (Salix spp., Fraxinus spp., Populus spp., Tamarix spp., Nerium oleander L.; Bruno et al., 2014). Notwithstanding native habitats have been progressively invaded by the giant reed, which can cover up to 90% of riverbanks (mean ± DS =54 ± 27%; authors' unpublished data). To control the spread of this invasive species, restoration activities were carried out on a 57 km long stretch of the river (Fig. 1).

Methods

From April 2016 to July 2018, 42 sampling stations (mean length ± SD = 0.5 ± 0.2 km) were monitored on the River Segura and its tributaries, covering a 109.8 km long stretch of the river (Fig. 1). Each station was surveyed 1-10 times (mean ± SD = 6 ± 2.5), the interval between two consecutive visits varying from 3 to 18 days. Surveys were carried out using the widely used "standard method" (Reuther et al., 2000). Typical otter marking sites (e.g. large stones, bridges, pool borders, confluences; Macdonald and Mason, 1983; Prigioni, 1997) were searched for otter spraints (i.e. faeces and anal secretions) on both river banks and around small islands. To gather additional information about the number of spraints per unit of length of sampling stretches, differently from the standard method, surveys were not halted as soon as signs of the species occurrence were found (Balestrieri et al., 2011). Otter marking activity was expressed as the percentage of surveys positive for otters [P% = (number of positive surveys / total number of surveys) × 100)], and as the mean number of spraints per 100 m (MI).

For each transect, ten habitat variables potentially affecting otter distribution were recorded (Table 1): 1. Discharge (0: dried, 1: low, 2: medium, 3: high); 2. Water speed (m/s); 3. Water turbidity (0: none, 1: very low, 2: low, 3: average 4: high); 4. Mean width of the wet riverbed (m); 5. Mean width of the riverbed (m); 6. Mean river depth (m); 7. Pollution (0: none, 1: low, 2: medium, 3: high); 8. Mean width of riparian vegetation on both river banks (m); 9. Mean percent cover of (semi-)natural vegetation in a 30 m large belt on both banks; 10. Mean percent cover of aquatic vegetation. By (semi-) natural vegetation we refer to the percent native cover within the riparian vegetated belt.

Discharge was assessed visually, based on the distribution and cover of hydromorphologic units (HMU; Parasiewicz, 2007), which broadly reflect the progressive increase in water speed and sur-face turbulence (1: runs and large isolated pools caused by summer drought; 2: ruffles and slow rif-fles; 3: riffles and rapids). Water turbidity and vegetation cover were assessed by eye, while pollu-tion was scored based on the recording of a total of six signs of presence (foam, oil, wastewater, garbage, algal blooms, anoxic sediment). The remaining variables were measured in 7-10 sections per site and averaged. Considering that, in Mediterranean rivers, otters tend to mark suitable feeding sites, such as pools (Remonti et al., 2011), the first six variables aimed to assess the role played by discharge variation and river morphology on the distribution of marking sites. Variables 7-10 were measured to assess the environmental quality of aquatic (7, 10) and riparian (8-9) habitats.

The relationship between each environmental variable and MI was first tested by non-parametric correlation (Spearman's test). To avoid collinearity, those variables clearly representing redundant information (1, 4, 10 in Table 1) were omitted from subsequent analysis.
The influence of the measured variables on MI was then tested by a backward stepwise linear multiple regression, using Fisher's F test to check the level of significance of the model and to enter or remove the variables (SPSS 12.0.1; SPSS, Chicago, IL, USA). Before the analysis, whenever the habitat variables were not normally distributed, the best transformation to improve the distribution of the data was identified using Box-Cox's method (Box and Cox, 1964).

As the use of transformations is not avoid of limitations and misinterpretations (Robert and Casella, 2004; Tang et al., 2012), data were also analysed using the non-parametric, machine learning method, Random Forest, which is highly suitable for analysing both compositional data and longitudinal settings with the aim of identifying non-linear relationships amongst both continuous and categorical variables without processing (Vincenzi et al. 2011; Brűckner and Heethoff 2017). A random forest model is made up of hundreds of unpruned classification and regression trees, each trained by selecting a random bootstrap subset (Xi) and a random set of predictor variables. Predictor variables are evaluated by how much they decrease node impurity or how often they make successful predictions in the forest of classification and regression trees (Breiman 2001). We assessed variable importance by both, the percent Gini increase of mean square error in nodes (%Inc MSE) and the increase in node purity (Inc Node Purity). The RF regression model was applied 1000 times, assessing the significance level of each individual variable by the 95 percentile of the ordered distribution of node impurity values (Balestrieri et al. 2013).

Variation in otter MI was compared by Mann-Whitney U test between the 52 km domain of the RIPISILVANATURA project and the unmanaged, downstream river stretch (58 km); and, within the former, between reference areas (sections of well-preserved riparian forest; N = 23) and ecological monitoring stations (EMEs; N = 30), i.e. the stretches where eradication treatments were conducted. Between 2016 and 2018, the relationship between MI and variation in A. donax cover was tested for nine EMEs using Spearman non-parametric correlation (rho).

Results

The otter was detected along the whole study area. The percentage of positive surveys kept constant (ca 80%) throughout the study period (2016-2018), while marking intensity increased, on average, from 0.81 spraints/100 m in 2016 to 0.88 and 1.25 in 2017 and 2018, respectively, for a total of 585 spraints recorded in the study period.

Multiple linear regressions indicated that otters preferred stretches surrounded by more natural vegetation (p=0.001), while tended to avoid polluted waters (p=0.025, Table 2, Figure 2). In contrast, marking intensity decreased with increasing width of the riparian vegetation belt on riverbanks (p=0.002, Table 2). The Random Forest model (Supplementary material S1) displayed similar results since it included as most important variables the width of riparian vegetation and riverbed, followed by pollution and percent cover of (semi-) natural vegetation (Fig. 3).

Mean marking intensity was higher in RIPISILVANATURA transects (Calasparra and Cieza municipalities, 1.18 spraints/100 m) than in the downstream half of the watercourse (Cieza, Ulea and Murcia, 0.76 spraints/100 m) during the whole period. Within the domain of the LIFE project, we found no differences in MI between reference areas and EMEs, except for 2016 (Table 3), when MI was markedly higher in the latter. Percent variation in A. donax cover between 2016 and 2018 was negatively correlated with MI (rho = -0.7, P = 0.036; Fig. 4).

Discussion

Our results provide an overview of the variables explaining otter distribution in Murcia Region, a relevant contribution in the framework of its Regional Recovery Plan, drawn up in consideration of its classification as a species in risk of extinction (http://www.murcianatural.carm.es/web/guest/41). Although initially our sampling design did not focus specifically on restoration projects, its aim being instead to assess the current distribution of the Eurasian otter in the catchment of the River Segura and to point out the environmental variables which may affect its habitat use, the coincidence with a restoration initiative provided an unique opportunity to incorporate such assessment into the set of biological indicators of recovery. RIPISILVANATURA surveys included several treatment areas allowing to relate the variation in otter marking intensity with the removal of Arundo donax and habitat quality. Two years after the beginning of habitat management, otter marking intensity slightly increased, otter occurrence being higher in the river stretch where restoration actions were carried out. Moreover, at site level, marking intensity decreased in the areas experiencing a lower reduction in giant reed density as a result of restoration interventions (Figure 4).

The use of spraints in the assessment of otter habitat preferences and abundance has been long debated (Kruuk et al. 1986; Jefferies 1986; Mason and Macdonald 1987; Kruuk and Conroy 1987). Although there is no direct relationship between numbers of spraints and numbers of otters, sprainting activity is believed to reflect changes in the distribution of otters (Chanin 2003), MI increasing with both habitat use (Clavero et al. 2006) and otter numbers (Mason and Macdonald 1993; Strachan and Jefferies 1996; Lanszki et al. 2008).

If we assume that MI is an index of habitat use, our results support the hypothesis that habitat quality is a major factor determining the recovery of otters on the River Segura and that the further implementation of giant reed control may enhance otter occurrence in the catchment. In general, the effects of invading giant reed or riparian vertebrates of semiarid Mediterranean river corridors have been poorly assessed. Usually, the effects on vertebrates of Arundo invasion are inferred through the examination of invertebrate based-food chains (e.g. Herrera & Dudley, 3003: Maceda-Veiga et al., 2016). The effects on bird communities have been dealt with in a few studies (Bruno et al, 2018, 2019, and references therein), but rarely on other vertebrates. Although the wild boar (*Sus scrofa*) may contribute to its biological control through rhizome digging (Quinn & Holt, 2008), giant reed is usually not attractive to herbivores due to its low palatability and accumulation of noxious compounds (Deltoro-Torró et al., 2012). Large and medium-sized mammals find difficult to penetrate densely infested areas (Coffman et al., 2004). In areas invaded by this species in California, the detectability of carnivore species was significantly lower, suggesting that Arundo-dominated habitats may function as refuge areas for small mammals (rodents, squirrels) that serve as prey for these species, altering predator-prey dynamics (Hardesty-Moore et al., 2020). This is consistent with previous studies carried out in Mediterranean riparian habitats (Matos et al., 2009; Santos et al., 2011, Grilo et al., 2016), where carnivore diversity and abundance were positively related to riparian quality.

In the case of aquatic or semi-aquatic species, the presence of *Arundo* is frequently assumed as a normal component of riparian vegetation. In the Iberian Peninsula, the typical habitat of Eurasian otters and American mink on Mediterranean rivers is commonly described as a “riparian vegetation composed of […] and reeds, *Phragmites, Arundo*” (e.g. Melero et al., 2008, 2011). Nevertheless, otter breeding sites are commonly associated to the occurrence of dead trunks and cavities among tree roots (Arizpe et al., 2008), which are not common in invaded areas. Hypothetically, dense giant reed beds may offer additional cover and protection from external disturbance, but it is not known whether the encroachment of riparian areas by this invasive species is positive or detrimental to otters. The apparently counter-intuitive negative relation between marking intensity and the width of riparian vegetation buffer may indicate that otters tend to avoid dense reeds, as on unmanaged river stretches the giant reed is widespread and dominates on its preferred native riparian vegetation. Giant reeds alter the morphology of the river, reducing the width of the water channel and affecting the distribution of fish species (Giessow et al., 2011). The decreased availability of free waters where searching for fish prey may explain the selection by otters for large river sections, where reeds can spread on the rivered without obstructing the water corridor.

As recorded for birds (Bruno et al., 2019), the otter showed short-term responses to giant reed mowing, which may mainly depend on the sharp reduction in reed cover rather than on gradual improvement in riparian habitat quality. Nonetheless, pollution, although roughly assessed by eye, was included in both regression models, indicating that the overall quality of freshwater habitats may affect otter occurrence. Water pollution can alter the abundance and distribution of fish (Vila-Gispert et al., 2002), suggesting that the combined effect of giant reed spread and pollution may reduce the availability of the main prey of otters. Accordingly, on river stretches covered with giant reeds, otter diet has been reported to mostly consist of alien red swamp crayfish Procambarus clarkii (Rubio et al., 2019). In Mediterranean rivers, the use by otters of alternative-to-fish prey has been related to fish shortage, (Karamanlidis et al., 2013; Smiroldo et al., 2019) and is consistent with the impact of man-driven habitat modifications on native fish communities.

Long-term monitoring is required to confirm the positive effects of giant reed management on both the overall quality of freshwater habitats and otter expansion in the catchment of the River Segura. Notwithstanding, the ongoing recovery of as a charismatic species as the Eurasian otter may contribute to a positive perception of management actions by both government agencies and public audience, enhancing the application of further river restoration projects. To be effective, these should include the improvement on native fish populations.

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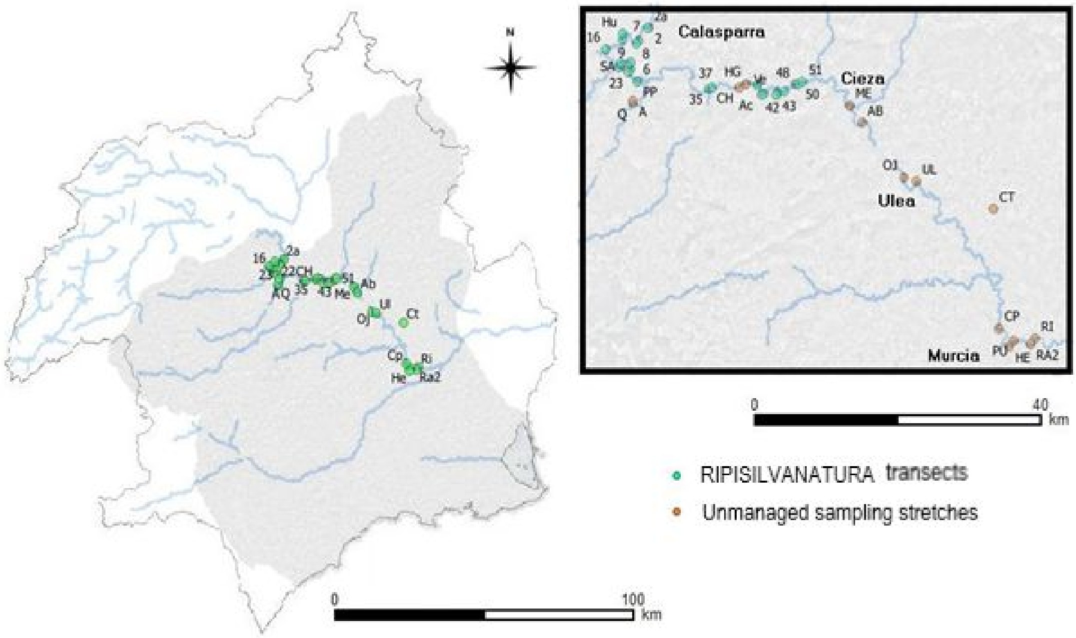

Figure 1. Study area: surveys for otter were carried out in both the upstream half of the River Segura (Calasparra), included in the Life project RIPISILVANATURA (giant reed removal) and in the downstream, unmanaged river stretch (Cieza, Ulea and Murcia).

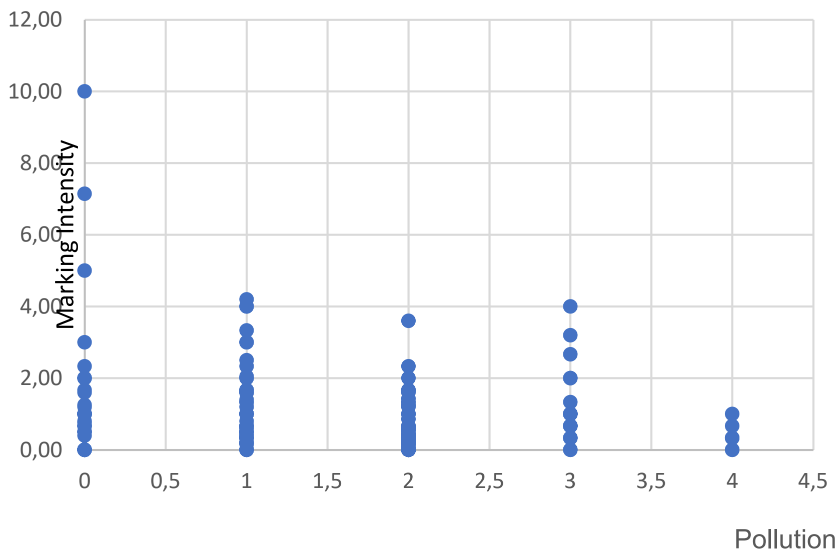

Figure 2. Relationship between otter marking intensity and water pollution (rho = -0.183, P = 0.016, N = 172).

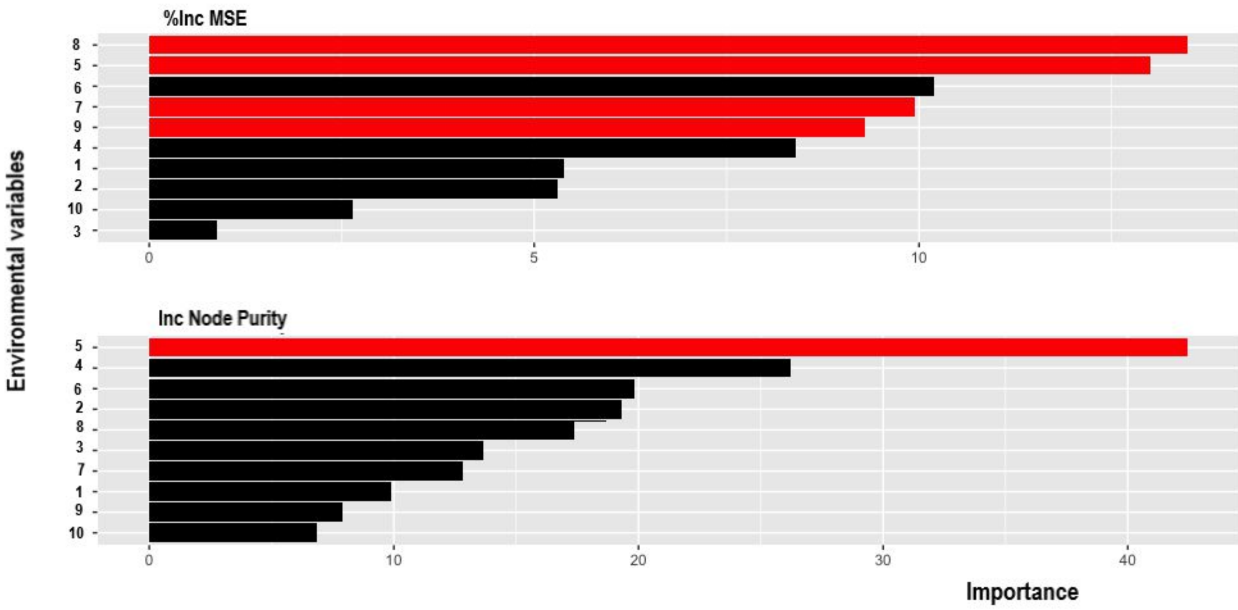

Figure 3. Variable importance, as assessed by both the percent Increase of Mean Square Error and Increase in Node Purity, in the Random Forest Regression model; significant variables (permutation test) are shown by red bars (1. Discharge; 2. Water speed; 3. Water turbidity; 4. Mean width of the wet riverbed; 5. Mean width of the riverbed; 6. Mean river depth; 7. Pollution; 8. Mean riparian vegetation width; 9. Mean % cover of (semi-)natural vegetation; 10. Mean % cover of aquatic vegetation).

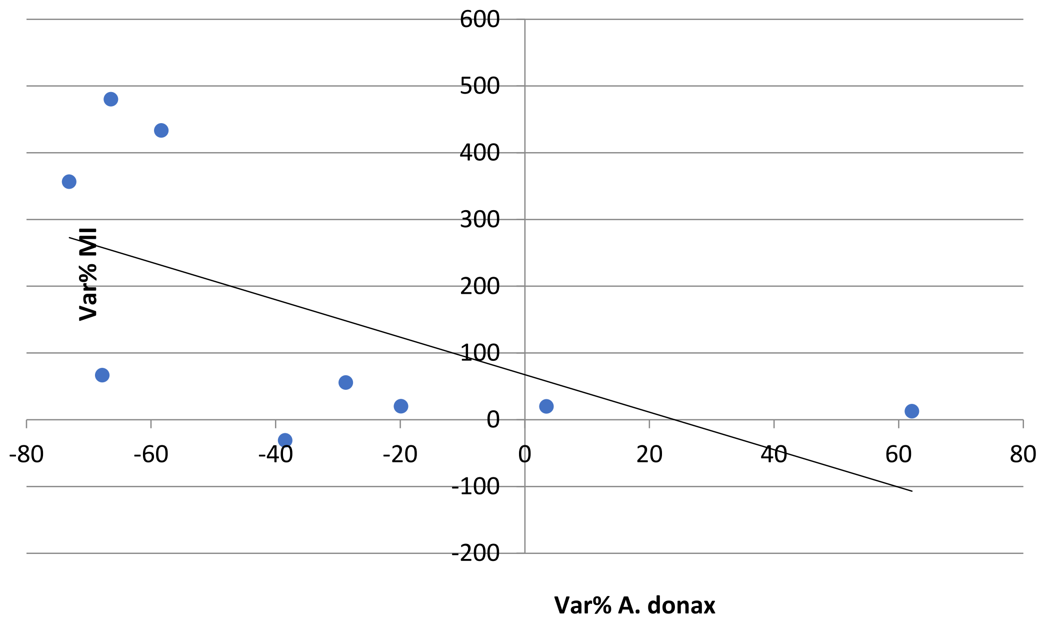

Figure 4. Relationship between the percent variation in *Arundo donax* cover and otter Marking Intensity, as assessed between 2016 and 2018 for nine sampling stretches where the removal of giant reed was carried out.

N	Acronym	Variable	Measure
1*	**Dis**	Discharge	Classes by eye (0-3)
2	**Vel**	Water speed	m/s
3	**Tur**	Water turbidity	Classes by eye (0-5)
4*	**WW**	Width of wet riverbed	(m)
5	**RW**	Width of the whole riverbed	(m)
6	**RD**	Water depth	(m)
7	**Pol**	Pollution	Classes by eye (0-4)
8	**VeW**	Riparian vegetation width (in both margins)	(m)
9	**RiV**	Surface occupied by riparian vegetation	% cover on a 30 m tape on both margins
10*	**WaV**	Aquatic vegetation cover	% cover

Table 1. Habitat variables potentially affecting otter distribution recorded for each transect (*excluded after Spearman correlation analysis).

Variables	B	SE	t	P
Constant	-0.615	0.146	-4.200	<0.001
Pollution	-0.175	0.077	-2.267	0.025
Riparian vegetation width	-0.149	0.048	-3.140	0.002
% cover of riparian vegetation	0.136	0.041	3.285	0.001

Table 2. Parameters of the multiple regression, with otter marking intensity as dependent variable.

	Marking Intensity		
	2016	**2017**	**2018**
Reference sites	0.07	2.34	0.89
EMEs	0.87	2.18	1.19
Mann-Whitney U	128.5	264	318.5
P	**0.036**	0.96	0.62

Table 3. Differences in Marking Intensity (MI) between different sections of the RIPISILVANATURA Project: reference sites (N = 23) vs Ecological Monitoring Stations (EMEs, N = 30).

Supplementary material S1

	%IncMSE	%IncMSE.pval	IncNodePurity	IncNodePurity.pval
Discharge (1 –Dis)	53.798.149	0.24752475	9.903.257	0.17821782
Water speed (2 – Vel)	52.993.009	0.32673267	19.333.338	0.19801980
Turbitity (3 – Tur)	0.8803082	0.69306931	13.680.966	0.55445545
Width werb (4 – WW)	130.098.552	0.01980198	42.449.149	0.00990099
Width whrb (5 – RW)	84.051.922	0.31683168	26.219.887	0.79207921
Water depth (6 – RD)	0.1921560	0.06930693	19.842.121	0.96039604
Pollution (7 – Pol)	99.437.418	0 .01980198	12.835.190	0.35643564
Rip veg w (8 – VeW)	134.898.019	0.00990099	17.385.752	0.43564356
Rip veg w (8, rigth m)	112.802.182	0.03960396	18.685.499	0.42574257
Rip veg s (9 – RiV)	92.914.709	0.02970297	7.888.199	0.71287129
Aquat veg (10 – WaV)	26.456.891	0.40594059	6.852.687	0.24752475

%IncMSE is the most robust and informative measure. It is the increase in mse of predictions (estimated with out-of-bag-CV) as a result of variable j being permuted (values randomly shuffled).

grow regression forest. Compute OOB-mse, name this mse0.
for 1 to j var: permute values of column j, then predict and compute OOB-mse(j)
%IncMSE of j'th is (mse(j)-mse0)/mse0 * 100%

the higher number, the more important

IncNodePurity relates to the loss function which by best splits are chosen. The loss function is mse for regression and gini-impurity for classification. More useful variables achieve higher increases in node purities, that is to find a split which has a high inter node 'variance' and a small intra node 'variance'. IncNodePurity is biased and should only be used if the extra computation time of calculating %IncMSE is unacceptable. Since it only takes ~5-25% extra time to calculate %IncMSE, this would almost never happen.

Chapter III

Diet of the Eurasian otter during its recent recovery in the Segura River Water Basin (SE Spain).

PART 2: Eurasian otter *Lutra lutra* diet mirrors the decline of native fish assemblages in a semi-arid catchment (River Segura, SE Spain).

Abstract

In semi-arid environments, the effects of irregularly distributed rainfall, flow regulation and water inter-basin transfer enhance the spread of non-native fish to the detriment of native communities. In the River Segura, since the 1980s the number of non-native fish species has progressively increased, also as a consequence of the building of water transfer facility connecting the rivers Segura and Tajo. With the aim of highlighting how man-driven changes in the diversity of fish communities affect the diet of top-predators, we compared Eurasian otter Lutra lutra diet over a 20-year period. As habitat quality affects the condition of Andalusian barbel Luciobarbus sclateri, the most widespread native fish, we also compared the size of preyed barbels. Fish and introduced red swamp crayfish Procambarus clarkii formed the bulk of otter diet in both study periods. In 2016-19 the contribution of non-native species to otter diet increased significantly, both for crayfishand fish, which included ten non-native species. Otter feeding habits faithfully mirrored the variation in the composition of the fish community and confirmed the importance of crayfish as alternative-to-fish prey in the Iberian Peninsula. The average length of preyed barbels was significantly lower in the second study period, consistently with a decline in barbel profitability for otters.

Key words: environmental stress, non-native fish, Procambarus clarkii, inter-basin water transfer, invasive species.

Introduction

Throughout arid lands, water demand is increasing with human population density and climate warming, resulting in the alteration of flow regimes and freshwater communities (Miller et al., 1989; Kingsford, 2000; Olden & Poff, 2005). Since the second half of the 19th century, inter-basin water transfer (IBT) projects have been considered as an effective solution to solve water resource deficits and management problems (Shiklomanov, 1999), and, currently, there are over 170 active IBTs (Schmidt et al., 2019). Connecting different biogeographic regions, water diversions can act as invasion corridors, facilitating the spread of non-native invasive species (Galil et al., 2007; Gallardo & Aldridge, 2018), as demonstrated in all continents (e.g.: River Rhine, NW Europe, Leuven et al., 2009; River Huai, China, Qin et al., 2019; Tasmania, rivers Gordon and Pedder, Sanger, 2001; rivers Great Fish and Sundays, South Africa, Kadye & Booth, 2013; coastal drainages of southern California, Swift et al., 2015). Chronic colonisation by non-native fishes, coupled with the buffering of seasonal variability due to damming and flow regulation can drive the decline of native fish assemblages, especially in water-limited catchments (Propst et al., 2008; Clavero et al., 2015).

Interactions between introduced and native species are highly complex, being exacerbated by environmental factors (Mack & D'Antonio, 1998). Native semi-aquatic predators usually prey on non-native fish less than expected based on their relative abundance (e.g. Eurasian otter *Lutra lutra*, Balestrieri et al., 2013; kingfisher *Alcedo atthis*, Nessi et al., in press). Nonetheless, few studies have been carried out in arid and semi-arid freshwater habitats, where anthropogenic impacts add to strong natural environmental stress (Ormerod et al., 2010). In arid drivers of Morocco, Eurasian otters mainly prey on widespread, native Andalusian barbel *Luciobarbus sclateri* (Libois et al., 2015; Riesco et al., 2020), despite about 60% of recorded species are non-natives (Clavero et al., 2015, 2017).

Flowing in south-eastern Spain, the River Segura is one of the driest and most regulated (24 dams higher than 10 m and 121 weirs > 2 m; CHS, 2007; Grindlay et al., 2011) European watersheds. Annual rainfall is ca. 400 mm, with large fluctuations in both seasonal and yearly values and pronounced summer droughts (Machado et al., 2011; Belmar et al., 2013). As in arid and semiarid areas of Australia and South Africa, intermittent and ephemeral streams are the predominant watercourse classes in the river catchment (Martinez-Capel et al., 2011). Water availability, on average $318 \cdot 10^6$ m^3/year, is insufficient to meet water demand, totalling $1483 \cdot 10^6$ m^3/year, of which 75% for irrigation-dependent agriculture (Uche et al., 2015). To solve this large deficit, water is mainly supplied from saltwater desalination plants and through the Tajo-Segura IBT, a 286 km long water transfer facility connecting, since 1978, the Entrepeñas and Buendia dams in the upper River Tajo, to the Talave Dam in the catchment of the River Segura (Pittock et al., 2009). Fish dispersion through the Tajo-Segura IBT system has been imputed as the cause of the spread of several non-native fish species in the recipient basin (e.g. Andreu-Soler et al., 2004; Oliva-Paterna et al., 2005). Currently, the fish community of the River Segura includes 18 freshwater species, of which 13 (72%) are non-natives (Oliva-Paterna et al., 2014).

Fish availability is known to affect the diet of the Eurasian otter, a semi-aquatic top-predator of freshwater habitats (Kruuk, 2006). As in many European countries, in Spain otter populations declined throughout the 1970s and 1980s due to water pollution, man-driven alteration of freshwaters habitats and hunting (Delibes, 1990), and started to recover in the last decade of the 20th century (Ruiz-Olmo & Delibes, 1998), following improvement in water quality (mainly through water treatment plants) and habitat restoration (Jiménez et al., 2008). The third national otter survey showed that the species occurred in 70% of the country (López-Martín & Jiménez, 2008). This positive trend has induced to classify the otter as “Least Concern” according to the IUCN Red List criteria (Ruiz-Olmo, 2014).

In SE Spain, the distribution of the otter suffered a large reduction since the 1970s, when it occurred on the whole River Segura and five tributaries (370 km), until the early 1990s, when it was recorded only in the upstream stretch of the main river (50-60 km; Yelo & Calvo, 2004). In the last three decades, the species has progressively recovered and is currently reported on a ca. 230 km long stretch (Dettori et al., 2019a).

With the aim of highlighting if otter feeding behaviour may reflect man-driven changes in the diversity and richness of the fish community and the ongoing recovery of this semi-aquatic mustelid may be hindered by food availability, we compared its diet, as assessed by the analysis of faecal samples, over a 20-year period (in 1997-98 and 2016-19).

Andalusian barbel was the most abundant and widespread fish in both study periods (Miñano et al., 2003; Oliva-Paterna et al., 2014). As habitat quality (Vila-Gispert & Moreno-Amich, 2001; Oliva-Paterna et al., 2003) and the spread of introduced species (Castejón Bueno, 2010) may affect barbel condition, we expected otters to prey on smaller fishes in the second study period. To test for this hypothesis, the length of preyed barbels was assessed using diagnostic bones and available regression equations.

Study area

The catchment of the River Segura (352 km) covers 14,432 km^2 and hosts a stable population of about 1,850,000 inhabitants (Uche et al., 2015). The climate ranges from sub-humid to semi-arid Mediterranean, with mean annual rainfall of 382 mm and mean annual temperature of 17 ºC.

Land use includes shrubland and woodland (45%), crops (52%), urban areas (2%), pastures and sparsely vegetated areas (Bruno et al., 2014a). Riparian vegetation consists of both European and Ibero-African species (*Salix* spp., *Fraxinus angustifolia*, *Populus* spp., *Tamarix* spp., *Nerium oleander*) (Bruno et al., 2014b). From the first half of the 1980s traditional rain-fed crops have been progressively replaced by irrigated ones, and urban areas have grown (Alonso-Sarría et al., 2009). River longitudinal connectivity has been recently improved through restoration projects aimed at eliminating or permeabilizing barriers (e.g. LIFE12 ENV / ES / 1140 SEGURA RIVERLINK; Sanz-Ronda et al., 2019), and controlling the invasive giant reed *Arundo donax* using soft engineering techniques (e.g. LIFE13 BIO / ES / 001407 RIPISILVANATURA; Bruno et al., 2019).

Methods

In 1997-1998 and 2016-2019, undisputable spraints were searched for on a 110 km long stretch of the River Segura (Fig. 1), by surveying typical otter marking sites (e.g. large stones, bridges, pool banks, confluences) on both banks.

The oldest sample consisted of 951 scats collected between February 1997 and March 1998. All faecal samples collected in a same sampling station/date were pooled together.

Between April 2016 and June 2019, 600 faecal samples were collected along 37 sampling stations, consisting of a 0.5 ± 0.05 km long stretch of the river, stored in test tubes containing ethanol, labelled and frozen at -20ºC.

For the analysis, each spraint was soaked for 24 hours in a solution of Hydrogen Peroxide 30% w/v (100vol) stabilized pure. Each spraint was then washed by a strong water jet into a sieve with 0.5 mm wide meshes. Undigested remains were carefully examined using a microscope. Fish remains were identified from their vertebrae, pharyngeal teeth and scales, using personal collections and the keys of different authors (Prigioni, 1997; Oliva-Paterna et al., 2014, 2019). Feathers and chelae and thoracopods were the main diagnostic features for birds and crustaceans, respectively. The diagnostic remains of amphibians and reptiles were identified by the keys of Prigioni (1997) and Smiroldo et al. (2019a).

For both study periods, data were split into two seasons (warm: IV-IX; cold: X-III) and, to allow comparison, results were expressed as percent relative frequency of occurrence [FR% = (number of occurrences of an item / total number of items) × 100]. Raw frequency data were compared by the chi-squared (χ^2) test, using Bonferroni's sequential method as a conservative correction for multiple testing (Rice, 1989). Trophic niche breadth was estimated by standardized Levins' index $B = 1/(R\ \Sigma p_i^2)$ (Feinsinger et al., 1981), with p_i = RF and R = 20.

The length of preyed barbels was assessed based on key diagnostic bones (maximum length/width of pharyngeal teeth, length of cephalic, thoracic and caudal vertebrae), using the methods and equations proposed by Ruiz-Olmo (1995). Average total body lengths were compared between the two study periods by Mann-Whitney's U test.

Results

Fish and introduced red swamp crayfish *Procambarus clarkii* formed the bulk of otter diet in both study periods (Tab. 1), with the minor contribution of birds, frogs and small mammals (< 5% each). In 1998-99 fish (%RF=76.8), particularly native Andalusian barbel (59.9), was the main otter prey, while in the second study period crayfish dominated (47.9). In the late 1990's, preyed fish included only four species, of which two (Eastern mosquitofish *Gambusia holbrooki* and Iberian nase *Pseudochondrostoma polylepis*) were non-natives. In 2016-19, otter diet was more diverse (B = 0.2 vs. 0.1), including twelve fish species, of which ten were non-natives (Tab. 1). In the second study period the contribution of introduced species to otter diet increased significantly, both for crayfish (47.9 vs. 17.5, χ^2 = 255.6, P < 0.001) and fish, particularly Iberian nase (3.4 vs. 0.1, χ^2 = 93.3, P < 0.001) and mosquitofish (2.3 vs. 0.3, χ^2 = 65.6, P < 0.001), while the relative frequency of Andalusian barbel declined (15.5 vs. 59.9, χ^2 = 234.4, P < 0.001). In 2016-19 also the frequency of non-fish prey, frogs (2.9 vs. 0.3, χ^2 = 72.6, P < 0.001) and small mammals (4.5 vs. 0.9, χ^2 = 76.4, P < 0.001) tended to increase.

In both study periods, crayfish were preyed on more frequently in the warm season (1998-99: 20.9 vs. 15.5, χ^2 = 7.2, P < 0.05; 2016-19: 64.8 vs. 40.2, χ^2 = 18.7, P < 0.01), while barbels prevailed in autumn-winter (1998-99: 63.0 vs. 54.8, χ^2 = 9.7, P < 0.05; 2016-19: 20.3 vs. 11.5, χ^2 = 8.9, P < 0.05) (Fig. 2). In 1998-99, insects were used only in the warm season, while birds were preyed on in the cold season of 2016-19.

The average (± SD) length of preyed barbels was significantly lower in the second study period (18.9 ± 5.6 cm vs. 16.1 ± 5.1 cm; U = 2936, P < 0.001, N = 200 and 46, respectively; Fig. 3).

Discussion

The catchment of the River Segura is characterised by low and irregularly distributed rainfall leading to either drought or flooding events. Human activities, including reservoirs, water withdrawal and transfer, deeply alter natural flow regimes, blunting the magnitude of flows and reversing their seasonal pattern, and exacerbating droughts (Vidal-Abarca et al., 2002; Belmar et al., 2010).

Life history traits of native species enhance their tolerance to droughts and environmental disturbance (Matthews & Marsh-Matthews, 2003; Ferreira et al., 2007), but may make them vulnerable to human-altered flow regimes (Lytle & Poff, 2004). Moreover, flow regulation affects the composition and structure of fish assemblages and may facilitate the establishment and spread of non-native species (Bunn & Arthington, 2002; Clavero et al., 2013; Almeida & Grossman, 2014).

Accordingly, since the 1980s the number of non-native fish species in the River Segura has progressively increased, with some species – bleak *Alburnus alburnus*, Iberian gudgeon *Gobio lozanoi*, pumpkinseed *Lepomis gibbosus* and Iberian nase -, currently occurring as frequently as native barbels (Oliva-Paterna et al., 2014). The initial spread of non-native species coincided with the completion of the Tajo-Segura IBT, which probably caused the invasion of Iberian gudgeon (Mas, 1986), golden carp *Carassius auratus* (García de Jalon et al., 1992), Iberian nase (Torralva & Oliva-Paterna 1997), and zander *Sander lucioperca* (Miñano et al., 2002), but also with a phase of exponential increase in the cumulative number of alien fish acclimatized in Spain, suggesting that the “improvement” of fish resources for sport fishing and aquaculture also played a major role in fish introductions (Elvira & Almodóvar, 2001).

Although the frequency of occurrence of non-native fish in otter diet in 2016-19 was rather low with respect to their relative availability, particularly for bleak (see Remonti et al., 2010 about prey selection by otters), otter feeding habits faithfully mirrored the variation in the composition of Segura’s fish assemblage in the last two decades.

While non-native fish usually represent a minor component of otter diet (Balestrieri et al., 2013), throughout the Iberian Peninsula red swamp crayfish are preyed by a wide array of native predators (Correia, 2001; Tablado et al., 2010), including otters (Spain: Adrian & Delibes, 1987; Portugal: Beja, 1996). North

American crayfish were introduced in the marshes of the River Guadalquivir, southwestern Spain, in 1973, and by the end of the decade spread throughout eastern Spain (Gutiérrez-Yurrita et al., 1999; Oficialdegui et al., 2019). Being highly resistant to adverse conditions, red swamp crayfish are capable to spread through dry land and temporary habitats, coping with large seasonal fluctuations in water levels (Barbaresi & Gherardi, 2000), as those occurring in our study area.

In a study carried out between October 2004 and February 2005 by the analysis of 943 spraints (Pastor González, 2011) the relative frequencies of occurrence of fish and crayfish in otter diet were intermediate (48% and 38.2%, respectively) to those recorded in 1998-99 and 2016-19, suggesting that throughout the study period the importance of crayfish as prey has progressively increased. Although otters can rely on a relatively wide range of aquatic food resources, including crabs, crayfish, amphibians and invertebrates, fish are their preferred prey (Ruiz-Olmo & Palazón, 1997; Clavero et al., 2003; Remonti et al., 2008; Smiroldo et al., 2019a). As the use by otters of alternative-to-fish prey mainly depends on fish shortage (Remonti et al., 2010; Krawczyk et al. 2010; Smiroldo et al. 2019b), the recorded trend may indicate the progressive decline of native fish resources, mainly Andalusian barbels. Consistently, in the catchment of the River Segura, waterflow regulation has been demonstrated to affect the growth, age structure and body condition of barbel populations (Torralva et al., 1997; Oliva-Paterna et al., 2003), and, recently, a decrease in barbel abundance has been reported (Oliva-Paterna et al., 2019). While the general improvement of water quality and recent restoration projects may have partially offset the negative effects of pollution and barriers, the expansion of red swamp crayfish may still affect barbel populations through habitat and food web alterations and even direct predation on fingerlings (Geiger et al., 2005; Loureiro et al., 2015; Souty-Grosset et al., 2016).

The lower prey size recorded in 2016-19 with respect to the late 1990s is consistent with a decline in barbel profitability for otters, which may have forced the mustelid to switch to alien crayfish, particularly in the warm season, when large crayfish are most abundant (Beja, 1996).

Although the relationship between prey abundance and carnivores' population regulation is not straightforward, prey availability has been reported to affect otter breeding success and mortality (Kruuk, 2006). Otters need to eat the equivalent of 15–20% of their bodyweight every day (Heggberget, 1995) and variation in the availability of their preferred prey items may affect their survival. In man-altered freshwater ecosystems, non-native crayfish may play an important role as alternative prey and have enhanced the ongoing recolonisation of Iberian catchments (see also Beja, 1996), particularly in peri-urban and agricultural land, where native fish are often replaced by non-native species (Dettori et al., 2019b).

As climate warming is expected to increase water deficit, with direct and indirect consequences for cyprinid populations (Beja 1995; Ilheu et al., 2007), information on the ability of semi-aquatic species to face the expansion of arid areas is pivotal for implementing conservation actions (Harms et al. 2008). The spread of invasive aquatic species, interacting with climate change (Burgiel & Muir, 2010; Capdevila-Arguelles et al., 2011), will pose additional threats on the whole trophic web of semiarid rivers. In the Iberian Peninsula, strategies aimed at otter conservation should focus on the restoration of freshwater habitats, which have been demonstrated to improve both habitat quality and otter abundance (Bruno et al., 2019; Dettori et al., 2019a). Specific actions should be directed at improving native fish species through site-specific measures (Santos et al., 2018), whereas invasive crayfish are difficult to eradicate. In addition, the design of flows that mimic natural flow regime patterns may provide more suitable environmental conditions for native fish species (Sánchez-Perez et al., 2020). The Eurasian otter should be regarded as a useful indicator of the effectiveness of such actions, as already demonstrated with respect to both the recovery of fish in depleted rivers (Narváez et al., 2020) and contaminant accumulation by red swamp crayfish (Rodríguez-Estival et al., 2020).

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Table 1. Percent relative frequency %RF of food items in the diet of Eurasian otter *Lutra lutra* on the River Segura (SE Spain) in 2016-19 (N = 600) and 1998-99 (N = 951). Raw frequency data were compared by the χ^2 test with sequential Bonferroni's correction (n.s.: non-significant).

Food items	**2016-19**	**1998-99**	**P**	**Status**
	%RF			
Procambarus clarkii	47.9	17.5	<0.001	introduced
Fish	37.0	76.8	<0.001	
Cyprinus carpio	1.7	0.4	<0.05	introduced
Luciobarbus sclateri	15.5	59.9	<0.001	native
Pseudochondrostoma polylepi	3.4	0.1	<0.001	introduced
Alburnus alburnus	0.1	-	n.s.	introduced
Gobio lozanoi	0.6	-	n.s.	introduced
Tinca tinca	0.2	-	n.s.	introduced
Unidentified Cyprinids	3.1	2.5	n.s.	
Sander lucioperca	1.1	-	n.s.	introduced
Micropterus salmoides	0.4	-	n.s.	introduced
Lepomis gibbosus	0.5	-	n.s.	introduced
Esox lucius	0.1	-	n.s.	introduced
Gambusia holbrooki	2.3	0.3	<0.001	introduced
Salmo trutta	-	0.3	n.s.	native
Unidentified fish	7.9	13.4	<0.01	
Birds	1.7	2.8	n.s.	
Turdus merula	-	0.1	n.s.	
Gallinula chloropus	1.0	-	n.s.	
Anas platyrhynchos	0.1	-	n.s.	
Unidentified birds	0.6	2.7	n.s.	
Ranidae	2.9	0.3	<0.001	
Natrix maura	0.5	1.2	n.s.	
Muridae	4.5	0.9	<0.001	
Coleoptera	0.6	0.4	n.s.	
Orthoptera	0.2	0.1	n.s.	
Gastropoda	0.6	-	n.s.	
Vegetal Matter	3.7	-	n.s.	
Garbage	0.2	-	n.s.	

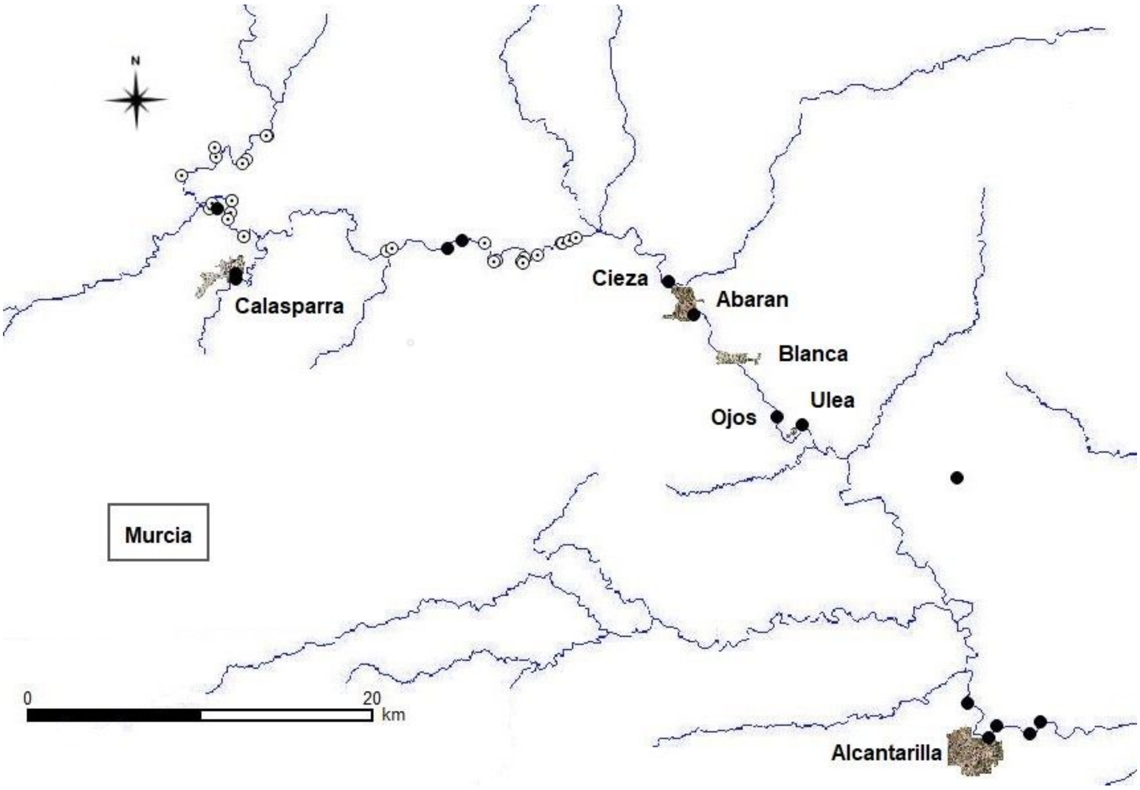

Figure 1. Sampling sites surveyed for otter spraints on the River Segura (SE Spain) in 1998-99 and 2016-19.

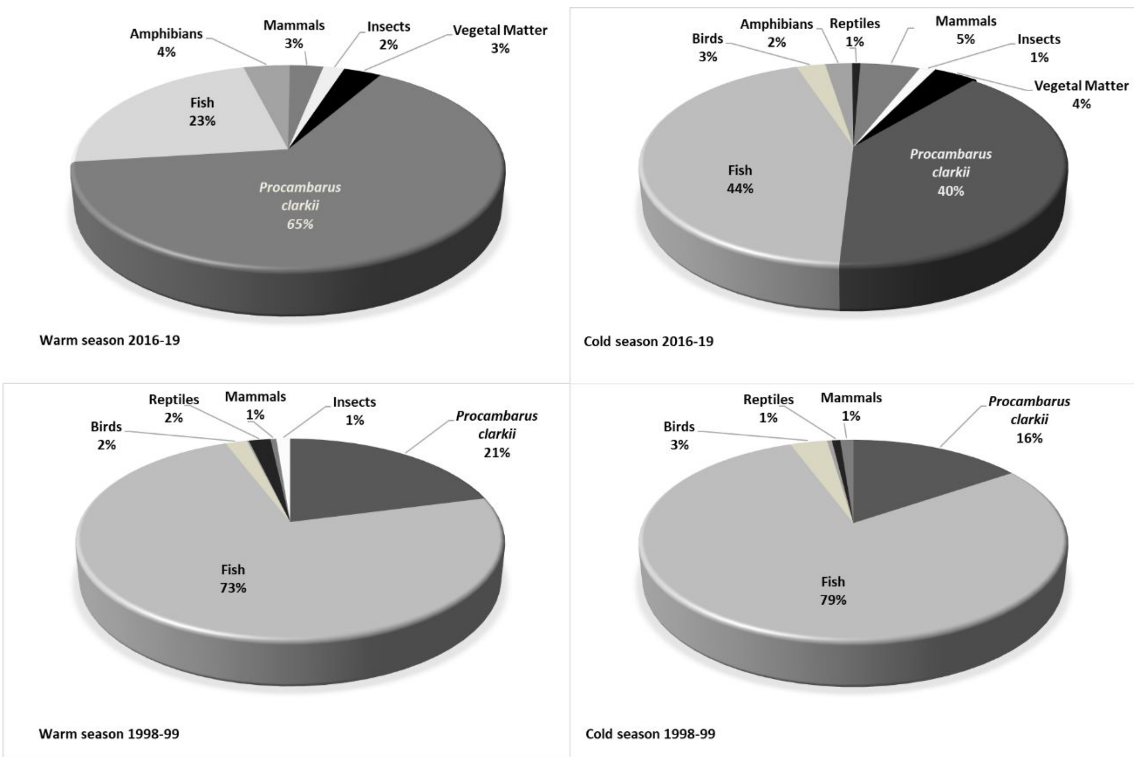

Figure 2. Seasonal variation in otter diet on the River Segura, as assessed by the analysis of faecal samples collected in 1998-99 and 2016-19.

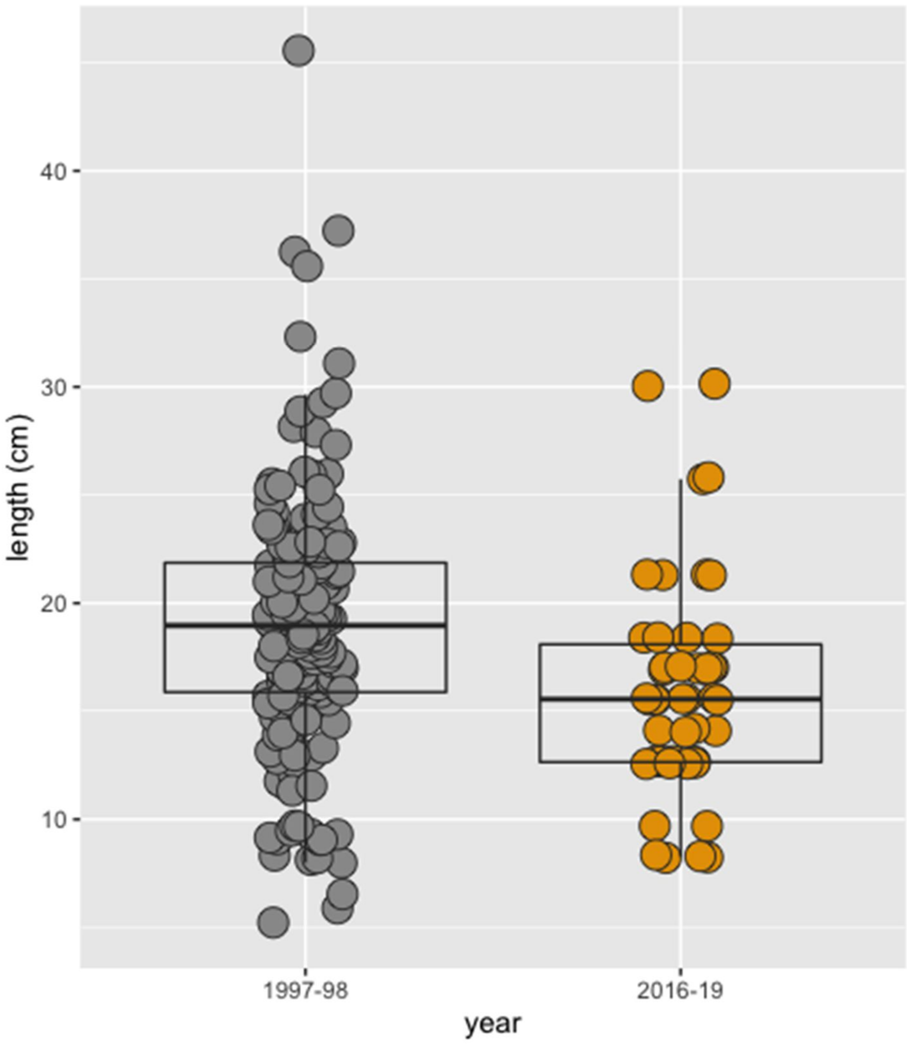

Figure 3. Box-plot diagram showing variation in the mean length of barbels preyed on by otters in 1998-99 and 2016-19.

Chapter III

Diet of the Eurasian otter during its recent recovery in the Segura River Water Basin (SE Spain).

PART 3: Distribution and diet of recovering Eurasian otter (*Lutra lutra*) along the natural-to-urban habitat gradient (River Segura, SE Spain).

Abstract

In the catchment of the River Segura (SE Spain), ongoing Eurasian otter (Lutra lutra) recovery and concurrent spread of human settlements are resulting in increasing otter records into urban areas. We analysed otter diet along a natural-to-urban gradient, with the main aim of assessing to which extent man-driven variation in food availability affects its feeding habits and how this secretive mustelid can thrive in apparently unsuitable habitats. From 2016 to 2019, we collected 521 spraints, monitoring 37 sampling stations (mean length ± SD = 0.5 ± 0.3 km). In natural areas, the percent cover of undisturbed banks was the highest, with the lowest number of water abstraction systems, whilst water pollution was the highest in urban areas. Introduced red swamp crayfish (Procambarus clarkii) and fish formed the bulk of otter diet. The two most preyed fish, Luciobarbus sclateri and Pseudochondrostoma polylepis, were among the most abundant species in the river, confirming the generalist feeding behaviour of the otter. The contribution of non-native fish was on average low, while invasive crayfish exceeded the overall importance of fish in all habitats except urban areas. Overall, diet data did not allow attesting food as a limiting resource for otters in urban areas. In contrast, marking intensity varied consistently with the natural-to-urban gradient (from 0.31 to 0.65 spraints/100 m in urban- and natural areas, respectively), suggesting that human disturbance (i.e. habitat modification and water pollution) may play a major role in shaping otter distribution.

Key words: feeding habits, urbanization, urban adapters, non-native fish, red swamp crayfish

Introduction

Urbanization is one of the main drivers of biodiversity decline (Soulé 1991; Crooks 2002; McKinney 2002) and, as urban areas will grow and intertwine with natural land further (Eurostat 2016), urban development is expected to be the main cause of land-use change, habitat loss and fragmentation in the next decades (Theobald 2001; Kasanko et al. 2006).

Along natural-to-urban gradients, increasing human population and building density affect several abiotic factors - including micro-climatic conditions, pollution, night lighting and soil properties -, and resources availability, with indirect effects on the composition and structure of plant and animal communities (McDonnell et al. 1997; Pickett et al. 2001; Hansen et al. 2005; Brown 2012).

Species vary widely in their ability to adapt to these changes and can be grouped in three broad categories of increasing tolerance towards urbanization: avoiders, adapters and exploiters; while species belonging to the last group achieve their better performance in urban areas, adapters generally tolerate low-to-moderate levels of urbanization (Crooks 2004; McKinney 2006, 2008), varying their behavioural patterns to cope with novel conditions (Brown 2012).

Among mammals, generalist predators are the most capable to exploit novel habitats and the related array of anthropogenic food resources, and often attain higher densities in urban than in rural habitats (Bateman and Fleming 2012). Several carnivore species currently occur in highly urbanised areas from red foxes (*Vulpes vulpes)* badgers (*Meles meles*) and stone martens (*Martes foina*) in European cities (Huck et al. 2008; Herr et al. 2009; Iossa et al. 2010) to coyotes (*Canis latrans*), striped skunks (*Mephitis mephitis*), black bears (*Ursus americanus*) and raccoons (*Procyon lotor*) in North America (Beckmann and Berger 2004; Gross et al. 2012; Santana and Armstrong 2017; Schneider et al. 2019).

The Eurasian otter (*Lutra lutra*) generally avoids human settlements and does not use anthropogenic resources. Introduced fish species usually form a negligible fraction of their diet (Balestrieri et al. 2013), although the otter has shown to be capable of adapting to man-driven changes in food availability in heavily altered freshwater ecosystems (Beja 1996; Smiroldo et al. 2019).

Negative relationships between otter occurrence and human disturbance have been recorded throughout the whole species' range (Cortés et al. 1998; Robitaille and Laurence 2002; Romanowski et al. 2013; Jo et al. 2017). Water pollution levels have been shown to affect otter occurrence, suggesting that it may be considered a bioindicator of the quality of freshwater ecosystems (Ruiz-Olmo et al. 1998a; Prenda et al. 2001). Human-related landscape modifications have been reported as the main factor limiting otter expansion, otters tending to avoid highly cultivated and urban areas (Clavero et al. 2010).

Nonetheless, ongoing otter recovery and concurrent spread of urban areas are resulting in increasing otter presence close to or into urban areas in several countries, from Ireland (White et al. 2013) to South Korea (Jo et al. 2018). The colonisation of these suboptimal areas is likely driven by the increase in otter range and numbers rather than habitat improvement, suggesting a certain degree of behavioural plasticity (Remonti et al. 2008a; Romanowski et al. 2013).
As throughout central and western Europe (Macdonald and Mason, 1994), in the Region of Murcia (SE Spain), the otter suffered a large reduction in its distribution since the 1960s and 1970s, when it occurred on the whole River Segura and five of its major tributaries (370 km), until the early 1990s, when it was recorded only in the upstream third of the main river (50-60 km; Palazón and Carmona 1998; Pastor et al. 2008).

In the past, the river was heavily polluted by industrial and urban discharges, but in the last two decades it has been the object of a restoration projects which have led to the progressive improvement of water quality and recovery of aquatic flora and fauna (Ródenas and Albacete 2014; Ródenas 2016). Currently, the otter is reported for a 230 km long stretch of the river and steadily occurs in the metropolitan area of Murcia (the largest city in the river catchment), where records of otters in urban habitats are increasing (Table 1).

Flowing through one of the driest European areas, the River Segura crosses a diversity of habitats, including Mediterranean shrubland and forests, agricultural land (mainly rice fields, apricot and peach orchards), and urban areas (Bruno et al. 2015). Along this natural-to-urban gradient, morphological and physicochemical alterations can alter stream water quality, reducing habitat quality and thus affecting the richness and diversity of fish communities (Walters et al. 2003; Helms et al. 2005). Urbanization may also enhance the invasion of exotic species while negatively affecting native fish and amphibian species (Davidson et al. 2001; Riley et al. 2004).

The catchment of the River Segura is one of the most regulated in Europe (Sabater et al. 2009) and few studies have been carried out in freshwater habitats where anthropogenic impacts combine with strong natural environmental stress (Ormerod et al. 2010). Moreover, as climate warming is expected to increase water deficit in Europe, information on the ability of semi-aquatic species to face the expansion of arid areas is urgently needed (Harms et al. 2008).

By spraint analysis, we analysed the diet of otters with the main aim of pointing out to which extent man-driven variation in food availability affects their feeding habits and how they thrive in apparently unsuitable habitats. In more disturbed habitats, we expected otters to rely mostly on non-native species and other-than-fish prey.

Study area

The River Segura (352 km) is the main watercourse of a semi-arid Mediterranean catchment (14,432 km^2; max elevation >2000 m a.s.l.) in the southeastern Iberian Peninsula. The climate ranges from sub-humid to semi-arid Mediterranean, with mean annual rainfall of 382 mm and mean annual temperature of 17 ºC. Land use is mainly characterized by shrubland and woodland (45%), crops (52%), urban areas (2%), pastures and sparsely vegetated areas (Bruno et al. 2014a), ranging from more natural habitats in the headwaters to widespread agricultural areas in the lowlands. The river is intensively regulated for agricultural and industrial purposes, with five large water reservoirs, for a total storage capacity of 704 million m^3. Despite the strong flow regulation, water demand exceeds by 224% water availability and only 4% of runoff reaches its mouth (Zimmer 2010).

Native riparian vegetation consists of both European and Ibero-African flora (*Salix* spp., *Fraxinus angustifolia*, *Populus* spp., *Tamarix* spp., *Nerium oleander*) (Bruno et al. 2014b). Invasive giant reed *Arundo donax*, is widespread on riverbanks in midlands and lowlands, but currently controlled by applying soft-engineering techniques in the framework of LIFE13BIO/ES/001407 RIPISILVANATURA project (Bruno et al. 2019).

Methods

To assess otter responses to increasing levels of urbanization, we used four broad categories of land use: natural areas, agricultural land, rice fields, and urban areas. Natural habitats consisted of Aleppo pine forests (*Pinus halepensis*), Mediterranean shrubland (mastic *Pistacia lentiscus* or cade juniper *Juniperus oxycedrus*), and perennial grassland (*Brachypodium retusum Stipa tenacissima* and *Lygeum spartum*). In these areas, anthropogenic activities are rare and disturbance negligible. Agricultural land (mainly orchards and poplar plantations) and rice fields were characterized by low to moderate human population densities, while urban areas included land mainly covered by buildings and with the highest human densities.

Surveys for otter spraints

From April 2016 to June 2019, we monitored 37 river stretches (mean length ± SD = 0.5 ± 0.3 km) on the River Segura and its tributaries, covering, in total, a 110 km long river (between 41 and 428 m a.s.l.). Based on land cover maps and direct surveys, sampling stations were chosen as to be representative of the different types of habitats (natural areas: 14; agricultural land: 9; rice fields: 5; urban areas: 9; Fig. 1). Each station was visited on average 9 ± 6.5 times (min-max: 1-22), searching for typical otter sprainting sites (e.g. large stones, bridges, pool banks, confluences) by walking, whenever possible, on both riversides and around small islands (Prigioni 1997; Balestrieri et al. 2011).

Otter marking activity was expressed as percentage of surveys positive for otter spraints [P% = (number of positive surveys / total number of surveys) × 100)], and as mean number of spraints per 100 m (marking intensity, MI).

To assess how habitat quality changes along the habitat gradient, i.e. what environmental parameters other than land-use may vary and affect habitat suitability to otters, for each transect we recorded by eye 13 habitat variables once a year, in winter, from 2016 to 2019 (Table 2). As qualitative assessment methods depend on the surveyor's fieldwork experience (Patton 2002), variables were recorded always by the same surveyor. Contamination of freshwater habitats by organochlorine compounds (PCBs and DDTs) has been recognized as the main cause of otter decline in the second half of the 20th century (Ruiz-Olmo et al. 2000) and otters have been demonstrated to avoid polluted water (Ruiz-Olmo et al. 1998b); moreover, pollution affects its main food sources (Mason and Macdonald 1986). Human disturbance on riverbanks is a major factor for resting site selection by otters (Weinberger et al. 2019), while riparian vegetation provides cover and exerts a strong influence on fish biomass (Albertson et al. 2018).

To compare the four habitats we applied nonparametric multivariate analysis of variance (NPMANOVA) with 10000 permutations and post-hoc pairwise comparisons using Bonferroni's correction for multiple comparisons (Anderson 2001; Hammer et al. 2001). Kruskal-Wallis' test was used to compare single variables and also mean MI among the four habitats. Raw P% data were compared by the chi-squared test (χ^2) for contingency tables.

Diet analysis

Firstly, each sample was soaked for 24 h in a solution of distilled water and hydrogen peroxide, then washed through a sieve with a 0.5-mm-wide mesh, dried in oven, and observed under a binocular microscope (Leica Wild M3Z 6.5-40x, UK) (Smiroldo et al. 2019). Fish remains were identified from their vertebrae, jawbones and scales, using personal collections and the keys of different authors

(Wise 1980; Camby et al. 1984; Prigioni 1997). Amphibians were identified by the keys of Di Palma and Massa (1981), whilst telson, chelae and thoracopods were the main diagnostic features for crustaceans.

Following Prigioni et al. (1991), results were then expressed as percent frequency of occurrence (F% = (number of spraints containing a specific food item/total number of examined spraints) × 100), percent relative frequency of occurrence (RF% = (number of occurrences of an item/total number of items) × 100), estimated percent volume (V%= total estimated volume of each food item as ingested/number of spraints containing that item) and percent mean volume (mV%= total estimated volume of each food item as ingested/total number of examined spraints), which outlines the proportional contribution of each food item to the overall diet (Kruuk and Parish 1981). For each sample, the minimum number of individuals of each kind of prey was estimated by the number and position (left–right) of diagnostic hard parts (as mouth bones for fish, ilia for amphibians). When no diagnostic part was found, the remains of a prey were considered to belong to a single individual. The weight of preyed fish was assessed by comparing the size of the jawbones found in otter spraints to a personal reference collection of jawbones collected from fish of known size. A constant weight was assigned to the other prey items: insects 1 g, amphibians 30 g, reptiles and crustaceans 50 g, birds 100 g (Kruuk and Parish 1981).

Trophic niche breadth was estimated by standardized Levins' index $B = 1/(R\ \Sigma p_i^2)$ (Feinsinger et al. 1981), using mV% (p_i) and 25 food categories (R). As a first step, raw frequency data were compared among habitats using the chi-squared test (χ^2) for contingency tables, while volumes were compared by Kruskal-Wallis' test. A Principal Components Analysis (PCA) was used to describe the main sources of habitat-related variation in otter diet. PCA was performed on an arcsine transformed 4 x N matrix, where N was the number of items scoring mV% > 1 for at least one out of the four habitats. The relationship between the mV% of fish and crayfish was tested by Spearman's correlation coefficient.

Results

By surveying a total of 153.5 km of transects (natural areas: 37.8 km; agricultural land: 46.6 km; rice fields: 30.0 km; urban areas: 39.1 km), we collected 521 spraints (126, 165, 104 and 126, respectively). Marking intensity ranged between 0.31 spraints/100 m in urban areas and 0.65 spraints/100 m in natural areas (agricultural land: 0.34; rice fields: 0.41). The percentage of surveys positive for otters varied significantly (χ^2 = 26.8, 3 d.f., $P < 0.001$), being the lowest in urban and agricultural areas (P% = 23.9 and 32.5, respectively) and the highest in natural areas and rice fields (P% = 51.1 and 62.8).

Overall, the four habitats were significantly different ($F = 4.98$, $P < 0.001$). Post hoc comparisons showed that urban areas differed from both natural areas ($P < 0.001$) and agricultural land ($P = 0.006$). In natural areas the percent cover of riparian vegetation was, on average, the highest (χ^2 = 13.1, $P = 0.004$), as so as the occurrence of undisturbed banks (χ^2 = 11.4, $P = 0.01$), with the lowest number of water abstraction systems (χ^2 = 9.6, $P = 0.02$); water pollution, as expressed by variables “garbage” (χ^2 = 7.6, $P = 0.05$) and “sewage” (χ^2 = 8.4, $P = 0.04$), was the highest in urban areas.

Introduced red swamp crayfish *Procambarus clarkii* and fish formed the bulk of otter diet (mV% = 56.5 and 38.4, respectively; Table 3, Fig. 2).

Most frequently preyed fish were Andalusian barbel *Luciobarbus sclateri* (F% = 21.0) and Iberian nase *Pseudochondrostoma polylepis* (F% = 4.7), followed by introduced Eastern mosquitofish *Gambusia holbrooki* (F% = 3.2) and pike-perch *Sander lucioperca* (F% = 1.5). Food items other than fish accounted for 1–2% of the overall diet each (Fig. 2).

Crayfish and fish were otter’s main prey in rice fields (χ^2 = 32.4, 3 d.f., $P < 0.001$) and urban areas (χ^2 = 26.5, 3 d.f., $P < 0.001$), respectively. Common carp *Cyprinus carpio* was preyed by otters more often than expected in urban areas (χ^2 = 17.0, 3 d.f., $P < 0.001$), while the Iberian nase prevailed in natural areas (χ^2 = 11.6, 3 d.f., $P = 0.01$) and was not recorded in rice fields (Online Resource 1).

Volumes of crayfish and fish were inversely correlated in the diets of all four habitats (Spearman's rho = -0.72 − -0.95; $P < 0.001$ for all; Fig. 3).

On the whole, Andalusian barbel and Iberian nase characterized otter diet in both urban and natural areas, while in rice fields the otter was less piscivorous (Fig. 4), with only four fish species preyed on (vs. 9 in both urban areas and agricultural land and 7 in natural areas). Non-native fish were preyed more frequently in urban areas (RF% = 56.6; $\chi^2 = 7.6$, 3 d.f., $P = 0.05$) than in the other habitats ($RF\%_{RF}$ = 26.9; $RF\%_{AL}$ = 37.3; $RF\%_{NA}$ = 40.9). Niche breadth was the highest in urban areas (B = 0.2) and the lowest in rice fields (B = 0.08).

Discussion

In the last four decades, agricultural intensification and urbanization, and associated increasing water demand, have deeply transformed the catchment of the River Segura (Grindlay et al. 2011; Belmar et al. 2013; Bruno et al. 2015). Inter-basin water transfer, allowed by 300 km of canals which connect the water-rich River Tajo to the River Segura has further worsened the alteration of the fish community, which is currently dominated by non-native species (ca. 70%; Oliva-Paterna et al. 2005, 2017), while yielding questionable benefits from the water supply standpoint (Gupta et al. 2008).

Eurasian otter diet included 11 out of the 17-18 fish species known to occur in the sampled river stretch (Oliva-Paterna et al. 2014, 2019). Both the two most preyed fish, the Andalusian barbel and Iberian nase, are widespread and among the most abundant species of the river (Oliva-Paterna et al. 2014), confirming the generalist feeding behaviour of the otter (Clavero et al. 2003; Remonti et al. 2008b). Only the first is native to the River Segura catchment, while the Iberian nase is endemic to the central Iberian Peninsula and most likely has colonized the river through the Tajo-Segura aqueduct (Torralva and Oliva-Paterna 1997). Although some introduced species are currently widespread on the river, such as bleak *Alburnus alburnus*, pumpkinseed *Lepomis gibbosus* and Eastern mosquitofish *Gambusia holbrooki* (Oliva-Paterna et al. 2014), their contribution to otter diet was negligible with respect to the Andalusian barbel's, consistently with the generally minor importance of non-native fish to otters recorded throughout its European range (Balestrieri et al. 2013). Habitat-related variation in the relative importance of common carp and Iberian nase probably depends on their distribution, the first being widespread in the lower course of the sampled river stretch, where urbanization is the highest, and the latter forming more abundant populations in the upstream half of the watercourse. Consistently, a rather tight relationship between the content of spraints and food availability at sprainting sites has been reported (Clavero et al. 2006; Remonti et al. 2009), despite the home range of each individual might cover several kilometers of watercourses (Prigioni et al. 2006).

In contrast, as previously reported for both SW Spain (Adrian and Delibes 1987) and Portugal (Beja 1996; Correia et al. 2001), invasive red swamp crayfish was a major, alternative-to-fish resource, exceeding the overall importance of fish in all habitats except urban areas and, particularly, in rice fields. It has been suggested that both crayfish abundance (Beja 1996) and fish availability (Remonti et al. 2008) are the major factors affecting the use by otters of this alternative food resource. Although the lack of biomass or density data hinder the sound relationship between otter feeding habits and prey availability, the network of irrigation ditches which feeds rice fields with water is likely to provide suitable habitats for an abundant crayfish population (Gherardi and Barbaresi 2000), supporting their large consumption by otters. Red swamp crayfish represents a conservation dilemma, since its relevant contribution as otter prey (Ruiz-Olmo and Clavero 2008) has to be contrasted with the disruption it causes to several river processes and functions (Tablado et al. 2010), claiming the better understanding of the role played by this invasive species in aquatic food webs (Mendes et al. 2011).

Urban areas alter local environments producing novel, man-made “niche opportunities” (Shea and Chesson 2002) or simply promoting the invasion of non-native species by putting native species at a competitive disadvantage along a natural-to-urban disturbance gradient (McKinney 2006). Otter diet was consistent with this pattern, supporting the hypothesis that the alteration of the physical and chemical environment (see McDonnell et al. 1997) may promote the spread of non-native fish.

Contrary to our expectations, otter diet in urban areas was mainly piscivorous. This result prevents from outlining a variation in its feeding habits clearly consistent with the worsening of habitat suitability to otters along the habitat quality gradient (natural areas-agricultural land-rice fields-urban areas) suggested by our estimates of levels of pollution, human disturbance and vegetation cover in the four habitats. Although human disturbance, namely pollution and introductions, can alter fish assemblages (Vila Gispert et al. 2002), fish diversity and biomass generally increase from headwaters to downstream (Remonti et al. 2009). As most urban areas border the downstream half of the monitored river stretch, probably otter diet was simply governed by fish availability, suggesting that feeding plasticity may allow this mustelid to endure also in man-altered habitats and that it may be a poorer bioindicator for habitat quality than previously reported (Reid et al. 2013).

While diet data did not allow attesting food as a limiting resource for otters in urban areas, both the percentage of visits positive to otter signs and marking intensity were, on the whole, consistent with the natural-to-urban gradient, supporting the hypothesis that, as for other mustelids (Balestrieri et al. 2019), human disturbance may play a major role in shaping otter distribution.

Lack of disturbance is an important feature at both resting- and, particularly, breeding sites. Apart from people, dogs can be a major threat, especially for cubs or wherever dens are in above-ground cover (Liles 2003; Weinberger et al. 2019). Moreover, urban otters likely suffer a greater risk of being exposed to threats such as roadkilling (Fabrizio et al. 2019), high levels of chemical contaminants and pollutants (Smit et al. 1998; Peterson and Schulte 2016), the still under-investigated effects of microplastics (Smiroldo et al. 2019), and parasites and diseases associated to domestic animals (Barros et al. 2018), as well as the synergistic interaction between these disturbance agents (Jessup et al. 2007). Taking all this into account, the decrease of anthropogenic pressure on urban river stretches may enhance otter occurrence and increase river connectivity for semi-aquatic species. The naturalization of urban river stretches, such as that undertaken for the River Manzanares in Madrid (Magdaleno 2017), is currently regarded as an advisable management goal by both municipal and river authorities.

Ongoing otter recovery in the catchment of the River Segura may contribute to a positive perception of recent restoration actions by funders and government agencies (Dettori et al. 2019). Furthermore, the increasing detection of otters in urban areas provides opportunities to boost the interest of a wider public audience in the conservation of otters and freshwater habitats. In these terms, the otter may be the flagship species of further river restoration projects aiming at improving water quality and riparian vegetation in urban areas, and, ultimately, urban sustainability and public use of riverbanks for recreation and amenity.

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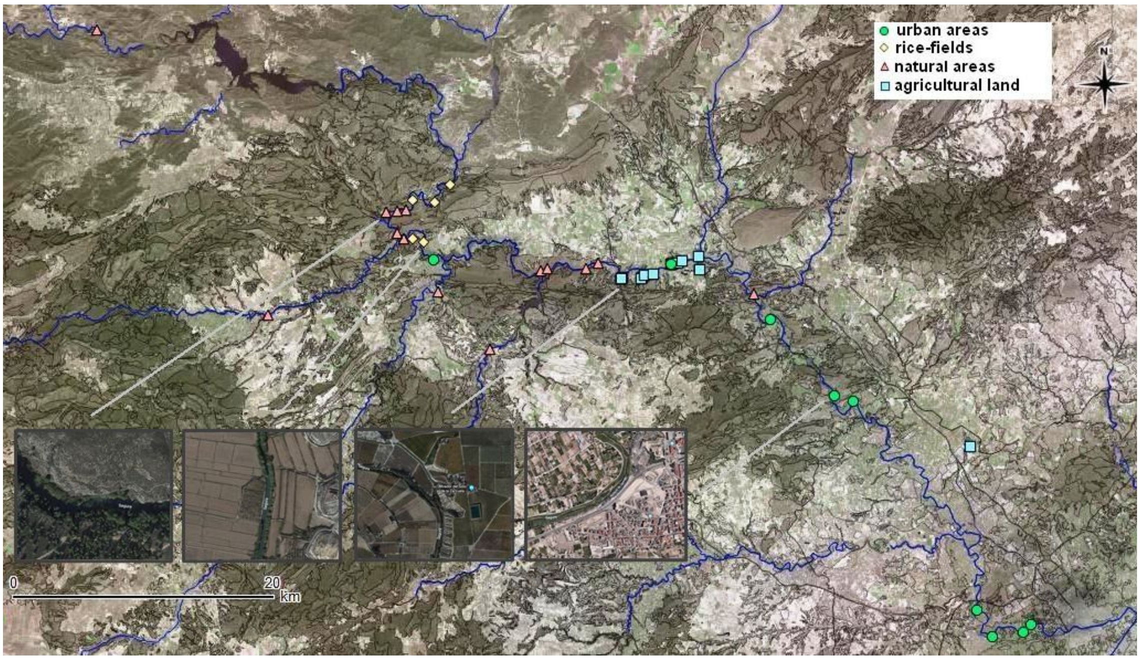

Figure 1. River stretches (N = 37, length ± SD = 0.5 ± 0.3 km) searched for otter spraints between April 2016 and April 2019 on the River Segura (SE Spain).

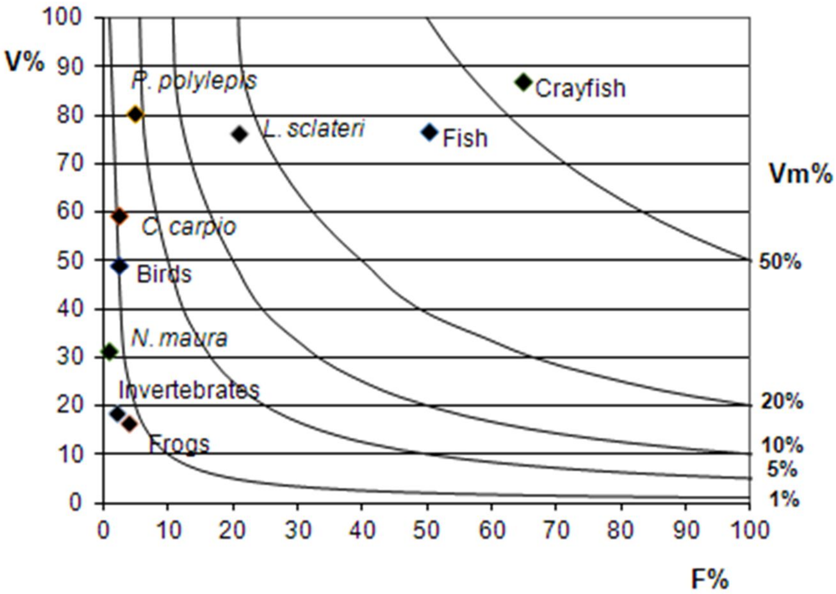

Figure 2. Estimated percent volume (V%) of main food categories, whenever eaten, vs. their frequency of occurrence (F%) for the overall diet (N = 521) of the otter on the River Segura. Isopleths connect points of equal percent mean volume in the diet (mV%).

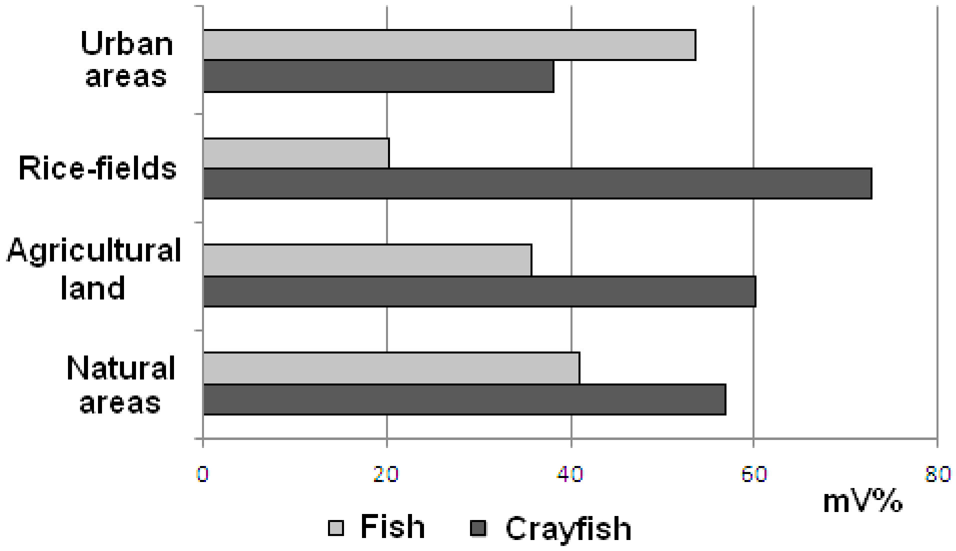

Figure 3. Percent mean volume (mV%) in otter diet of fish and red swamp crayfish for the four main habitats along a 110 km long stretch of the River Segura.

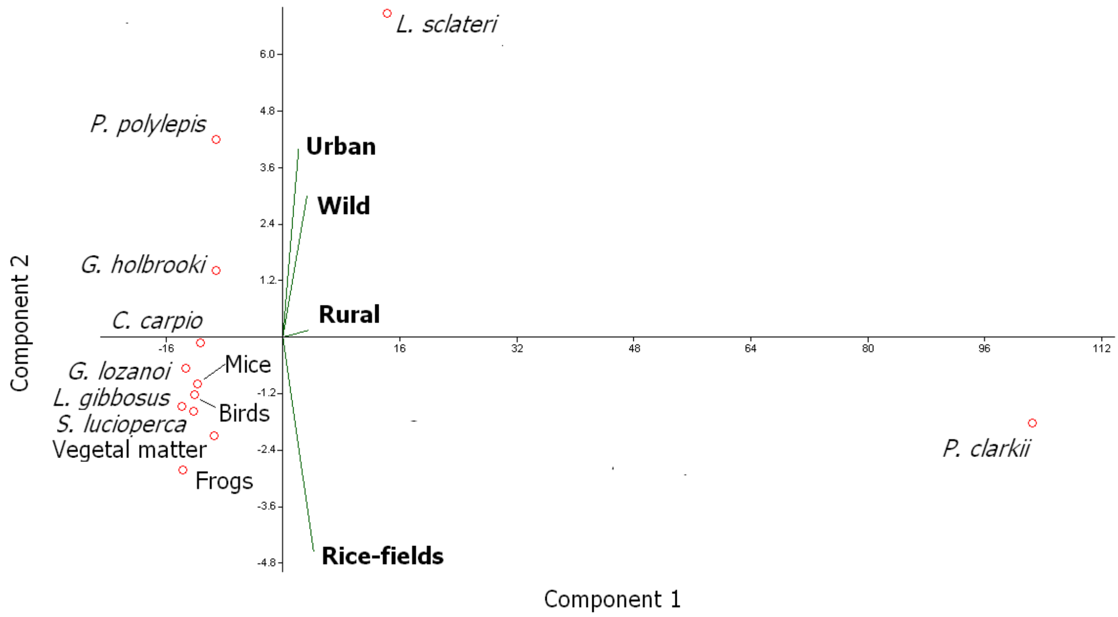

Figure 4. Plot of otter habitat-related diets in relation to the first two Principal Components extracted from the mean percent volumes (mV%) of 12 major food categories. The first component explained 97% of the total variance.

Table 1. Recent otter records in urban areas of Murcia County.

Date	**Locality**	**Signs**
02/06/2013	Barriomar (Murcia City)	spraints
23/01/2017	Molina del Segura	roadkill
29/12/2017	Javalí (Murcia City)	videoclip
03/04/2018	Cieza	videoclip
23/11/2018	Benejuzar (Alicante)	videoclip
20/05/2019	Murcia City Centre	videoclip

Table 2 Habitat variables as assessed by eye (presence/absence except for vegetation cover) for each of the 37 sampling stations for otter spraints on the River Segura.

Main effect	Variable	Measure
Alteration of water quality	Water pollution	Observation of foam, oil or other non-natural substances, unpleasant or chemical odours
	Garbage	Unregulated dump on the banks or into the riverbed
	Algal cover	Unnatural levels of (bacterial) alga growth
	Sewage	Observation of sewage pipes
	Water abstraction	Occurrence of active pipes, ditches or other abstraction systems
Human disturbance	Boating	Canoeing or motorboat navigation
	Hunting-Fishing	Observation of sport hunters or fishers
	Sheep-grazing	Occurrence of herds on the riverbanks
	Country roads	Presence of roads bordering the riverbanks (up to 30 m from the riverbed)
	Paths	Presence of hiking paths on the riverbanks (up to 30 m from the riverbed)
	Undisturbed banks	No recordable sign of access to the river by man
Vegetation cover	Riparian vegetation	Mean percent cover on the riverbanks
	Aquatic vegetation	Mean percent cover into the riverbed

Table 3. Diet of the otter on the River Segura as assessed by the analysis of 521 spraints collected between April 2016 and April 2019 (F%: percent frequency of occurrence; RF%: percent relative frequency of occurrence; V%: percent volume; mV%: mean percent volume; see methods).

Food items	F%	RF%	V%	Vm%
Procambarus clarkii	65.0	47.9	86.8	56.5
Fish	50.2	37.0	76.6	38.4
Undetermined fish	10.7	7.9	75.7	8.1
Cyprinus carpio	2.3	1.7	59.6	1.4
Luciobarbus sclateri	21.0	15.5	76.5	16.1
Pseudochondrostoma polylepis	4.7	3.4	80.4	3.8
Alburnus alburnus	0.2	0.1	50.0	0.1
Gobio lozanoi	0.8	0.6	73.0	0.6
Tinca tinca	0.3	0.2	52.5	0.2
Undetermined Cyprinids	4.2	3.1	82.6	3.4
Sander lucioperca	1.5	1.1	71.7	1.1
Micropterus salmoides	0.5	0.4	55.0	0.3
Lepomis gibbosus	0.7	0.5	87.5	0.6
Esox lucius	0.2	0.1	5.0	-
Gambusia holbrooki	3.2	2.3	90.8	2.9
Ranidae	4.0	2.9	16.5	0.7
Natrix maura	0.7	0.5	31.3	0.2
Birds	2.3	1.7	49.3	1.2
Undetermined birds	0.8	0.6	65.0	0.5
Gallinula chloropus	1.3	1.0	39.4	0.5
Anas platyrhynchos	0.2	0.1	50.0	0.1
Muridae	6.2	4.5	25.1	1.6
Insects	2.0	1.5	18.8	0.4
Coleoptera	0.8	0.6	10.0	0.1
Orthoptera	0.3	0.2	42.5	0.1
Gasteropoda	0.8	0.6	18.0	0.2
Vegetal matter	5.0	3.7	23.7	1.2
Garbage	0.3	0.2	5.0	-

Chapter IV

Tools for the detection of invasive mustelids in areas of sympatry with Eurasian otter (*Lutra lutra*).

PART 4: A cost-effective PCR-RFLP method for monitoring invasive American mink: preliminary field test in control areas of Spain

Abstract

American mink Neovison vison is a semi-aquatic mustelid that has been introduced throughout Europe. Several attempts to eradicate this invasive species have been carried out since its spread. In the Iberian Peninsula, an eradication program has started since 2001, as part of the national action plan for the conservation of European mink Mustela lutreola. Proper detection is crucial for management, both for monitoring variation in mink abundance and prevent the recolonization of cleared areas. Mink scats can be confounded with those of several same-sized mustelids, and with the spraints of Eurasian otter Lutra lutra, which also occur along watercourses. However, these difficulties can be overcome by non-invasive genetic methods. With the aim of providing a cost-effective and reliable technique for differentiating mink scats from those of the largest possible number of species with morphologically similar scats, we tested the effectiveness of an already available faecal mDNA-based PCR-RFLP method for distinguishing mink- and otter faeces. We then applied the method for analysing scats collected and identified in the field by trained surveyors for monitoring the success of mink control operations. The PCR-RFLP method clearly differentiated mink scats from those of otters, as restriction enzymes produced well-defined different patterns. Eighty-five percent of 94 faecal samples could be assigned to a mustelid species. Only three out of seven samples were assigned to mink, confirming that the accuracy of the visual identification of scats is questionable and genetic methods are needed for the reliable assessment of the success of eradication programs.

Key words: species identification, non-invasive methods, faecal DNA, *Neovison vison*, *Lutra lutra*

Introduction

To reduce the costs of management and impact on ecosystems, strategies for monitoring invasive alien species require the reliable identification of the target species at low densities, either in the first phases of colonisation, to ensure early detection and prevent it from spreading, or, after invasion, to assess the success of eradication operations (Mehta et al. 2007).

For many elusive species, surveys rely on indirect signs of presence, mostly hairs or faeces (MacKay et al. 2008), which, however, often cannot be differentiated morphologically, particularly for related species or species with similar diets (Davison et al. 2002). These limitations in monitoring efficacy have been overcome by DNA-based methods of identification, which potentially address a wide variety of applications specifically relevant to invasive species detection and management (Darling and Blum 2007). To be effective, genetic tools must be precise, cheap, technically accessible, and allow the identification of the target species across the widest possible range of similar species (Darling and Blum 2007).

The family Mustelidae is the largest of the order Carnivora and, throughout Europe, forms the bulk of the mesocarnivore guild. A further species, American mink *Neovison vison*, native to North America (Dunstone 1993), has been introduced in most of Europe, from Iceland and Norway in the north to the Iberian Peninsula in the south (Bonesi and Palazón 2007). It became widespread since the 1920s (Gerell 1971; Bonesi and Palazón 2007), mainly due to animals escaping from fur farms.

Large established populations occur in northern and central-eastern Europe, while more localized populations are spread in Mediterranean countries, namely Italy and the Iberian Peninsula (Bonesi and Palazon 2007).

This invasive semi-aquatic mustelid is considered to threaten endemic and native crustaceans, fish, amphibians, voles and ground-nesting birds as a predator (e.g. Palazón and Ruiz-Olmo 1997; Ferreras and MacDonald 1999; Jefferies, 2003; Macdonald and Harrington 2003; Fisher et al. 2009) and it also competes with other mesocarnivores (Melero et al. 2012; Palazón and Melero 2014), particularly the endangered European mink *Mustela lutreola* and polecat *Mustela putorius*.

In the Iberian Peninsula, the first farms were established at the end of the 1950s in Central and North-western Spain. Since then, recurrent escapes have given rise to 8-10 nuclei spread across 12 autonomous communities (Lecis et al. 2008; Melero and Palazón 2017) and allowed the colonization of North-western Portugal (Vidal-Figueroa and Delibes 1987). American mink mainly occur along river courses and in wetlands (Palazón et al. 2016), where they affect the distribution of Mediterranean water shrew *Neomys anomalus* and southern water vole *Arvicola sapidus* (García-Díaz et al. 2013).
As part of the national action plan for the conservation of the European mink, an eradication program has started since 2001, and currently involves ten provinces (divisions within autonomous communities; Melero and Palazón 2017). Mink are captured using live-cages on floating platforms, a method with negligible impacts on other species and which has proven to be very effective for controlling American mink populations both in the UK and Spain (Palazón et al. 2016). Mink monitoring at trapping sites is usually carried out by surveying floating platforms for tracks and scats and camera-trapping (LIFE Lutreola, 2019), while the “standard method” for Eurasian otter *Lutra lutra*, consisting in the search for signs of presence, mainly scats, along 600 m of riverbanks, is considered suitable for assessing mink distribution (Melero et al. 2013; Garcia et al. 2020).

Proper detection is crucial for its management, both for monitoring variation in mink range and abundance and because a few escaped mink may nullify the success of control campaigns (Oliver et al. 2016). However, signs cannot always be visually assigned with certainty (Davidson et al. 2002; MacKay et al. 2008). Mink scats can be confounded with those of other mustelids, such as polecat, otter, stoat *Mustela erminea,* pine marten *Martes martes* and stone marten *Martes foina*, and common genet *Genetta genetta* (Harrington et al. 2010). Moreover, otters and mink occur syntopically in freshwater habitats and their scats can be found on the same marking site (Lampa et al. 2015), making the assignment of scats particularly difficult when containing fish remains (Harrington et al. 2010; Grimm-Seyfarth et al. 2019).

Several genetic methods have been developed for identifying American mink based on non-invasive sampling. These methods include the amplification and sequencing of either the mitochondrial cytochrome b gene (Harrington et al. 2010), or microsatellite locus Mel08 (López-Giráldez et al. 2005), and polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) methods. For the latter, widely applied, indirect approach, a variety of PCR primers and restriction enzymes have been designed to recognize the American mink among a range of closely related species, targeting either the mitochondrial cytochrome b gene (Hansen and Jacobsen 1999; Harrington et al. 2010) or D-loop region (Gómez-Moliner et al. 2004; Statham et al. 2005; Ruiz-González et al. 2008).

The method proposed by Ruiz-González et al. (2008) was specifically designed for faecal DNA and allows identifying the largest number of mustelids, the American mink and six native European species (*Martes martes*, *M. foina*, *M. zibellina*, *Mustela erminea*, *M. putorius* and *M. lutreola*), through their different RFLP haplotypes. Having been developed to distinguish *Martes* species, the method had not been tested on otter spraints, which, in turn, can be easily confused with mink scats.

Thus, with the aim of checking the effectiveness of this PCR-RFLP method for assessing the distribution of American mink, we amplified the mDNA extracted from indisputable otter scats and compared the restriction patterns to those obtained from mink tissues. We then applied the method to scats collected and identified in the field by trained surveyors for monitoring the success of routine control operations.

Materials and methods

Sample collection and DNA extraction

Eurasian otter spraints were collected on the River Segura (Murcia region, SE Spain), as part of the monitoring of the success of a river restoration project (LIFE+ RIPISILVANATURA; Dettori et al., 2019). American mink DNA was isolated from fresh tissues of trapped individuals, euthanized during the eradication campaign carried out in Guadalajara province (Castilla-La Mancha autonomous community, central Spain).

DNA was isolated from both faecal samples (N = 94) and tissues (N = 9) using, respectively, the FavorPrep Stool DNA Isolation Mini Kit, following the manufacturer's instructions, and Sambrook protocol (1989).

PCR-RFLP analysis

We used the forward primer Mm_L1 (5′- CCCAAAGCTGACATTCTAAC-3′) and reverse primer Mm_H1 (5′-ATGGGCCCGGAGCGAGAAGAGGTACAC-3′), both designed for *Martes* species by Ruiz-González et al. (2008). To maximize the probability of amplifying faecal mDNA, the primers were designed to amplify a short fragment of 276 bp of the D-loop region.

A 5-μL volume of the DNA extraction mixture was added to 20 μL of the PCR mixture, containing 2 μL of forward primer Mm_L1 and 2 μL of reverse primer Mm_H1 (5 pmol/ μl), 12.5 μL 2× Xpert Taq Mastermix with dye, and 3.5 μL of sterile water. After incubation for 5 min at 94°C, the samples were subjected to 35 amplification cycles using an Applied Biosystems Accrt ABI9700 96 Well Pcr thermal cycler, consisting of denaturation for 1 min at 94°C, annealing for 1 min at 63.5°C and a final extension stage of 1 min at 72°C. Six microlitres of the final amplified product was analysed by electrophoresis on 2,5% agarose gel. Negative controls were used to check for contamination.

To confirm the obtained haplotypes, PCR products were sequenced using a HITACHI 3500 Genetic Analyzer (Applied Biosystems). The new nucleotide sequences were deposited in GenBank under accession numbers MT227875 (*Lutra lutra*), and MT227876 (*Neovison vison*).

MEGA X 10.1 (Kumar et al. 2018) was used to check and to align the mitochondrial D-loop sequences and their different haplotypes (Tab. 1). The restriction enzymes RsaI and BsuRI (HaeIII) were confirmed as those generating RFLP patterns for the otter different from those of the other mustelids listed in Table 2.

DNA digestions by endonucleases were run in 14-μL volumes, consisting of 1.4 μL of the appropriate 10×reaction buffer (supplied by the manufacturer with the respective enzyme), 0.2–0.4 μL of restriction enzyme solution (10 U/μL), 5 μL of the PCR product and the remaining volume of pure water. Incubations were performed for 6 h at 37°C, followed by a 3% agarose gel electrophoresis of 8 μL of the digestion products. RedSafe DNA Stain bands were visualized and photographed using a Biobee Tech UV Transilluminators.

Field test

The PCR-RFLP method was applied for identifying the faecal samples collected between May 2018 and January 2019 to monitor the success of eradication campaigns performed in two areas of the current sympatric distribution range of American mink and Eurasian otter, namely the autonomous community of Madrid (central Spain) and Teruel province (Aragon autonomous community, NE Spain). Sampling was conducted along riverbanks, where both species occur syntopically. Coordinates were recorded for all the samples collected using a global positioning system (Garmin).

Out of 94 faecal samples collected (80 in Madrid and 14 in Teruel), based on their external morphology 84 were initially identified as *Lutra lutra* and seven as *Neovison vison*, while three were filed as “uncertain” (two as “otter/mink” and one as “mink/rat”). Faecal samples were stored in autoclaved tubes containing ethanol 96% and frozen at −20°C until processed.

As a further check, six samples, four suspected to belong to American mink and two uncertain scats, were also sequenced.

Results

After digestion with BsuRI, *Lutra lutra* displayed a thick electrophoretic band corresponding to a 273-bp fragment, while the remaining 5-bp-long band was invisible on agarose gel (restriction pattern B). RsaI digestion produced only one restriction pattern (D), consisting of two well-defined electrophoretic bands corresponding to 161 and 94-bp-long fragments, and a third 23-bp-long invisible band (Tab. 2).

Neovison vison showed a slightly longer (282-bp) fragment, corresponding to the restriction pattern B of BsuRI, and the so-called restriction pattern "G" (Ruiz-González et al. 2008) after RsaI digestion: 129-30, 117, 40-1-bp (Tab. 2).

Thus, the simultaneous use of the two restriction enzymes allowed to obtain species-specific restriction patterns (otter: BD; American mink: BG) and unequivocally discriminate the two species.

Out of 94 faecal samples analysed, 80 (85%) could be assigned to one of the mustelid species for which diagnostic restriction patterns were available (75 *Lutra lutra*, 3 *Neovison vison* and 2 *Martes foina*), while 14 DNA samples (15%) were not amplified by the primers used.

All samples classified as "otter" in the field were confirmed by our PCR-RFLP analysis. Out of the seven samples assigned to the American mink, three were confirmed, two gave no amplicons, and two, both collected in Teruel, showed the restriction pattern of the stone marten (BC). Finally, all uncertain samples belonged to the otter. PCR-RFLP responses were confirmed for all the six sequenced samples (i.e.: three mink, two otters and one stone marten).

Overall, only 30% of the faecal samples suspected to belong to the American mink (3/8 in Madrid and 0/2 in Teruel) were genetically assigned to the same species.

Discussion

Regular monitoring is currently needed for both American mink and Eurasian otter, to assess the success of control operations and maintain a high level of surveillance in the areas cleared of the first, or track and possibly assist the ongoing recovery of the latter by informing conservation actions (Bryce et al. 2011; Gariano and Balestrieri 2018; Garcia et al. 2020).

As previously suggested, the search for signs on riverbanks may be a suitable and cost-effective method to assess the occurrence of both species (Melero et al. 2013), provided that faecal samples can be assigned to each species with certainty in areas of sympatry. Genetic sampling is increasingly necessary for improving our knowledge on the distribution, density and behavior of semi-aquatic mustelids, allowing to compare scat-based estimates of abundance to actual species numbers (Sittenthaler et al. 2020).

The tested PCR-RFLP method clearly differentiated American mink scats from those of Eurasian otters, as the position of cleavage site for the restriction enzyme RsaI produced well-defined different patterns. In addition, the use of two endonucleases allows the reliable identification of up to eight different mustelid species producing morphologically similar scats.

Although fresh mink scats have been claimed to be easily distinguishable from those of otters because of their characteristic foetid odour (Dunstone 1993), the field study confirmed that the accuracy of the visual identification of scats is questionable, as American mink faeces can be confused with both otter spraints and stone marten scats. Keeping in mind, that even experts can fail to distinguish the scats of several same-sized mustelids (Harrington et al. 2010; Grimm-Seyfarth et al. 2019), particularly when the target species occurs at low density (Davison et al. 2002), PCR-RFLP analysis, accounting for a broad range of morphologically and ecologically similar species, can reliably overcome this difficulty.

Moreover, as both otters and mink are currently expanding their ranges in several countries - e.g. Scotland (Fraser et al. 2015), Germany (Lampa et al. 2015), Portugal (Pedroso et al. 2014) and NE Italy (Iordan et al. 2012; Balestrieri et al. 2016) -, overlap in their ranges is expected to increase in the next future, as does the need for effective survey methods.

While genetic analysis confirmed the presence of American mink on the monitored rivers of Madrid autonomous community, albeit the proportion of marking sites attributed to this species was halved, in Teruel (rivers Alfambra and Jiloca) no mink scat could be confirmed, suggesting that the eradication campaign is succeeding in controlling this invasive species. Accordingly, throughout autumn 2018 trapping yielded no captures, although one mink track was possibly recorded on floating platforms (Latizal 2018).
The PCR-RFLP species identification rate was high (85%) and close to those previously obtained for *Martes* spp. (88%, Ruiz-González et al. 2008; 81.3%, Balestrieri et al. 2015). However, the method had been designed for faecal DNA and both the higher copy number of mithocondrial DNA compared to nuclear DNA sequences and the small fragment (276 bp) that was amplified were expected to improve the success rate of the analyses (Frantzen et al. 1998). Although we had no useful information on the sampling protocol of the two field surveys, based on our experience the success of PCR-RFLP methods is poorly affected by scat age, making it particularly suitable for low density populations (see also Palomares et al. 2002).

In addition to being less sensitive to low-quality and low-quantity DNA, with respect to the sequencing of amplified products, PCR-RFLP is also cheaper (for materials, we estimated a current cost of € 2.5-3 per sample) and can be carried out into “basic” laboratories.

Finally, the method allows the prompt and quick identification of large numbers of samples and electrophoretic results can be immediately interpreted by eye, enabling field surveys and laboratory analyses to be handled in parallel. This ability is pivotal to assess the effects of eradication programs on distribution patterns and the in-play, cost-effective tuning of control activities. Moreover, the method may be useful for the routine monitoring of potential areas of colonization and expansion of the American mink. In the catchment of the River Segura, mink expansion may be facilitated by its artificial connection with the River Tagus, which has already caused dramatic changes in the composition of fish communities (Oliva-Paterna et al. 2014), and which we therefore propose as priority surveillance area.

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Table 1. Aligned sequences of the amplified mitochondrial D-loop fragment in *Lutra lutra* and *Neovison vison* (restriction pattern BG). The primer sequences are underlined, while restriction sites for RsaI and BsuRI (HaeIII) are shown in bold and bold/italic, respectively.

Lutra lutra	CCCAAA	GCTGAC	ATTCTA	ACTAAA	CTATTC	CCTGAT	TCTCTC	ACCCCA	CATTTC	AATTCA	60
Neovison vison	CCCAAA	GCTGAC	ATTCTA	ATTAAA	CTATTC	CCTGAT	TTCCTC	ACCACC	ATTTTT	TCAATT	60
Lutra lutra	TATATT	CAACGA	CATTTA	CTGTGC	CTGCCC	AGTATG	TATTCC	GCGCAC	CGCCCC	CTATGT	120
Neovison vison	CATATA	TTTAAC	AACATT	CAATGT	GCTTCC	CCAGTA	TGTATT	CTTCTT	ATTTTT	TTTCCT	120
Lutra lutra	ATATCG	TGCATT	AATGGT	TTGCCC	CATGCA	TATAAG	CAT**GTA**	**C**ATACT	ATGGTT	GATTTT	180
Neovison vison	CCCCCT	AT**GTAC**	GTCGTG	CATTAA	TGGCTT	GCCCCA	TGCATA	TAAGCA	**GTAC**AT	ATTATG	180
Lutra lutra	ACATGT	ATCCAC	CTCACC	TAGATC	ACGAGC	TTGATC	ACCATG	CCTCGA	GAAACC	ATCAAT	240
Neovison vison	ATTAAT	CTTGCA	TGCATG	CATTTC	ACTTAG	ATCACG	AGCTTT	ATCACC	ATGCCT	CGAGAA	240
Lutra lutra	CCTTGC	GCGACG	T**GTAC**C	TCTTCT	CGCTCC	G***GGCC***C	AT				278
Neovison vison	ACCATC	AACCCT	TGCCTG	AAGTAT	ACCTCT	TCTCGC	TCCG***GG***	***CC***CAT			287

Table 2. Restriction patterns generated for *Lutra lutra* (this study) and *Neovison vison* (confirmed by this study) compared to those reported by Ruiz-González et al. (2008) for other mustelids. Capital letters used to designate the patterns correspond to those previously chosen by Ruiz-González et al. (2008). Fragment sizes (in base pairs, bp) that could be visually identified on agarose gel for each enzyme are shown in italics. Those in bold cannot always be identified

Species	Amplicon length (bp)	BsuRI (HaeIII) fragment size (bp)	BsuRI (HaeIII) restriction pattern	RsaI fragment size (bp)	RsaI restriction pattern	Combined restriction pattern
Lutra lutra	278	*273*, 5	B	*161, 94*, 23	D	BD
Neovison vison	286-287	*281-2*, 5	B	*130, 117, **40***	G	BG
				*117, 99, **41***, 30	E	BE
Mustela lutreola	280-281	*275-6*, 5	B	*134, 121-122,* 25	G	BG
				134, 82, 39-40, 25	H	BH
Mustela putorius	280-287	*275-282*, 5	B	*121-8, 93, **41***, 25	E	BE
				93, 82, 40, 41, 25	F	BF
Mustela erminea	276-277	*135-6, 136*, 5	C	*118-19, 94, **41***, 23	E	CE
Martes martes	276	*220, 51, 5*	A	*97, 94, 62*, 23	A	AA
				97, 94, ***41***, 21, 23	B	AB
Martes foina	276	*271*, 5	B	*94, 82, 62*, 23, 15	C	BC
Martes zibellina	276	*271*, 5	B	*97, 94*, ***41***, 21, 23	B	BB
		220, 51, 5	A	*158, 94,* 24	D	AD

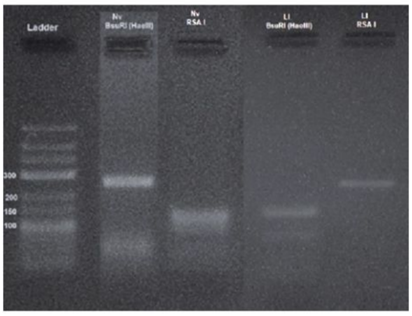

Figure 1. Image of a 3% agarose gel showing RedSafe™ Nucleic Acid Staining Solution bands and diagnostic restriction enzyme patterns (BD; BG) generated using BsuRI (HaeIII) and RsaI for *Neovison vison (Nv)* and *Lutra lutra (Ll)*.

Chapter V

Conclusions and Management recommendations.

Conclusions

The following conclusions can be drawn from the studies carried out in this work:

I. Our results provide an overview of the variables that explain the distribution of the Eurasian otter in the Murcia region. Within the Life RIPISILVANATURA project it was possible to include different treatment areas which allowed to relate the variation in otter marking intensity with the removal of *Arundo donax* and habitat quality. Two years after the start of habitat management, the intensity of otter marking had already slightly increased, suggesting the greater use of river stretches where restoration actions had been carried out. Although there is no direct relationship between the number of droppings and population abundance, marking activity is believed to faithfully reflect changes in the distribution of otters. If we assume that marking intensity is an index of habitat use, our results support the hypothesis that habitat quality is a major factor determining the recovery of otters and giant reed control may enhance otter occurrence.

In the Mediterranean region, *Arundo donax* is frequently assumed as a normal component of riparian vegetation and, in general, the effects of giant reed spread in semi-arid river corridors have been poorly evaluated with regard to large and medium-sized mammals and particularly in the case of aquatic or semi-aquatic species. Despite dense giant reed beds may offer to otters additional cover and protection from external disturbance, they alter the morphology of the river, potentially affecting the distribution of fish species. Our results suggest that the decreased availability of free waters where searching for fish prey may explain otter tendency to avoid reed beds.

II. The River Segura is one of the driest and most regulated European watersheds. Water withdrawal has deeply altered its natural flow regimes, affecting the composition and structure of fish communities and enhancing the spread of non-native species through the Tajo-Segura inter-basin transfer.

By comparing otter diet, as assessed by the analysis of faecal samples, over a 20-year period, we showed that the feeding habits of otters were deeply affected by variation in the composition of Segura's fish assemblage and that, in the last two decades, the condition of the most widespread native fish, the Andalusian barbel, significantly worsened, probably because of interspecific competition with alien species.

Between the late 1990s and nowadays, the importance of crayfish as otter prey has progressively increased. As the use by otters of alternative-to-fish prey has been demonstrated to depend on fish shortage and non-native fish usually represent a minor component of their diet, the recorded trend further points at the decline of native fish resources.

Red swamp crayfish represents a conservation dilemma, since its relevant contribution as prey for a wide array of native predators, otters included, must be contrasted with the disruption it causes to several river processes and functions. As reported by previous studies, in the Iberian Peninsula non-native crayfish may have played a major role in the recolonization by Eurasian otters of fragmented freshwater habitats, where native fish are often replaced by non-native species.

III. In the last four decades, urbanization and agriculture intensification have deeply transformed the catchment of the River Segura. Differently from several other meso-carnivores, the Eurasian otter generally avoids human settlements and does not rely on anthropogenic resources. Moreover, as also demonstrated by our analysis of otter's habitat preferences in the study area, water pollution levels have been shown to affect otter distribution and habitat use. Notwithstanding, records of otters in urban habitats of the region are increasing and otters seem to steadily occur in the metropolitan area of Murcia. On these bases, with the aim of pointing out how otters may thrive in such apparently unsuitable habitats and anthropogenic disturbance affects their feeding habits and distribution, we analysed the variation in otter diet along a natural-to-urban gradient.

Contrary to our expectations, the diet of otters in urban areas was mainly piscivorous, suggesting that fish availability is not the main reason for otters to avoid urbanized areas. Thus, as both the percentage of visits positive to otter signs and marking intensity were, consistent with the natural-to-urban gradient, we concluded that human disturbance may play a major role in shaping otter distribution. On one side, otters need undisturbed wood patches for both resting and breeding sites, while the risk associated to roadkills, pollution and free-ranging dogs is higher in high-density residential and urban areas.

IV. The use of the PCR-RFLP method used to distinguish sympatric semiaquatic species clearly differentiated American mink droppings from those of Eurasian otters, as the location of the cleavage site for the restriction enzyme RsaI yielded well-defined different patterns. Despite it has been claimed that American mink droppings are easily distinguishable from otter droppings, the field study confirmed that their identification is uncertain whenever fish and crayfish form the bulk of undigested remains. In contrast, when mink eat terrestrial prey, their scats can be confused with those of other similar-sized mustelids, such as the stone marten. The use of this PCR-RFLP method, being effective for the identification of a wide range of ecologically similar meso-carnivores, can reliably overcome these difficulties. Moreover, this method allows the rapid and timely and cost-effective identification of several samples, allowing to simultaneously manage field investigations and laboratory analyses. This ability makes the method a useful tool to assess the effects of mink eradication programs, tune control activities and also for the routine monitoring of potential areas of expansion of the American mink.

Genetic analyses confirmed the presence of American mink on the monitored rivers of the autonomous community of Madrid, but not in those of the province of Teruel (Aragon), where no mink droppings could be confirmed, suggesting that there the eradication campaign is successfully managing to control this invasive species.

Management recommendations

Climate warming is expected to increase water deficit, with direct and indirect consequences for native cyprinid populations and the spread of invasive aquatic species.

These interacting factors are expected to have deep impacts on semi-arid and arid catchments, where increasing water demand and flow regulation exacerbate seasonal droughts.

Strategies aimed at otter conservation should focus on the restoration of freshwater habitats and be predominantly directed at improving native fish assemblages through site-specific measures. These actions cannot be effective without the prior design of flows that mimic natural flow regime patterns, which, in their turn, may provide more suitable environmental conditions for native fish species. Corrective measures like barrier removal or permeation (fish passages) can also help improve native fish populations, as has been tried through projects such as LIFE RIVERLINK, focused on improving the longitudinal connectivity of the Segura River.

While invasive crayfish are difficult to eradicate, management has been demonstrated to be able to control the expansion of invasive giant reed, with positive effect on the overall quality of freshwater habitats. Despite long-term monitoring is required to confirm the positive effects of giant reed management on both habitat quality and otter expansion in the catchment of the River Segura, our results suggest that the Eurasian otter may be considered a useful indicator of the effectiveness of such actions. For these reasons, we argue that otter surveys should be carried out on a regular basis. In areas where the expansion of otters and invasive mink is expected to lead to overlapping ranges, the application of the PCR-RFLP method successfully tested during this research would allow to the reliable identification of the target species and prompt implementation of management actions.

The ongoing recovery of a charismatic species as the Eurasian otter may contribute to a positive perception of management actions by both government agencies and public audience, enhancing the application of further river restoration projects. This is particularly true for urban and residential areas, where the decrease in anthropogenic pressure on river stretches can increase the presence of otters.

The improvement of the status, integrity and resilience of urban rivers is requested by both the Water Framework Directive (2000/60/EC) and the Directive on the assessment and management of flood risks (2007/60/EC), which highlight integrated sustainable urban development as one of the major milestones of EU policies. In this context, the otter may be the flagship species of river restoration projects aiming at improving water quality and riparian vegetation in urban areas, and, ultimately, urban sustainability and public use of riverbanks for recreation and amenity. In the lower sections of the Segura River, where it crosses and irrigates large areas of traditional orchards affected by intense urban sprawl, this role of flagship species can be extended to the management and restoration of these agricultural landscapes and their historic irrigation systems, enhancing their contribution to green infrastructure of urban and peri-urban areas.

As demonstrated by projects carried out on urban rivers in Spain (e.g. Manzanares in Madrid), the improvement of environmental flow regime, restoration of riparian vegetation, removal of exotic species and enhancement of native fish assemblages are the main restoration measures which need to be planned to optimize ecosystem services and counteract ecological alterations.

The Otter Recovery Plan in the Region of Murcia

, approved by Decree No. 59/2016, of June 22 (Supplement 4 of Boletín Oficial de la Región de Murcia number 155, of 07/06/2016), establishes five blocks of measures with their corresponding indicators. Of these, the following three can be considered in line with the conclusions of this thesis, highlighting those to which its results and future research on the subject could contribute:

- Protect and improve the riparian habitat for the species, including the study of the quality of the habitat in the hydrographic basin and the cooperation between the regional environmental authorities and the state water basin managers to develop an integrated action plan for riparian restoration (that should specifically address Exotic Invasive Species and be built as a continuity of projects such as RIPISILVA) and barrier adaptation to reduce fragmentation (as an extension of projects like RIVERLINK), and the establishment of fishing closures and regulations in breeding areas, as well as stewardship and collaboration agreements with owners to promote habitat conservation measures.

- Manage water resources in a manner compatible with the species, improving water quality and the availability of trophic resources, including collaboration agreements with the water managers, local authorities and social agents to identify the target areas and measures that guarantee the good ecological status of the river systems; projects to improve fish and other trophic resources of the species; and to coordinate the recovery measures in the Segura River hydrographic basin with the neighboring autonomous communities (the latter could include preventive measures against Exotic Invasive Species like American Mink, for which a protocol has been proposed in this thesis).

- Ensure population monitoring, and determine the factors involved in their conservation, including a program of biological monitoring of the species, its diet and habitat (to which this thesis has made a non-negligible contribution); monitoring the different threat factors, develop lines of research in collaboration with scientific institutions and universities; and capacity building through seminars on the recovery of the species, directed at technicians and environmental wardens.

Annexes

Annex I. Riassunto

La lontra eurasiatica (*Lutra lutra*, Lineaeus 1758), ha attraversato un periodo critico nel bacino del fiume Segura (Regione di Murcia, SE Spagna) dagli anni '60 all'inizio degli anni '80, seguito da un periodo di stabilità durante gli anni '90 con un leggero incremento durante il 21° secolo. Questo mammifero semi-acquatico è uno dei principali predatori del fiume Segura e dei suoi affluenti e risulta essere un potenziale bioindicatore delle condizioni ambientale della rete idrografica. In questo lavoro, la distribuzione, l'uso del habitat e la dieta della lontra sono stati analizzati in relazione alle variazioni dell'uso del suolo e della composizione delle comunità ittiche occorse negli ultimi due decenni, principalmente sulla base dei dati ottenuti nell'ambito del progetto LIFE13BIO / ES. / 001407 RIPISILVANATURA. Si è cercato di fornire delle linee guida per la conservazione della lontra e per una gestione efficace degli habitat acquatici in relazione alla crescente urbanizzazione e alla diffusione di specie esotiche invasive.

Nella prima parte del lavoro sono stati raccolti dati sulla presenza della lontra nella Regione di Murcia, al fine di stimarne la distribuzione e le preferenze ambientali nel bacino idrografico. Il periodo di studio è coinciso con le principali attività di ripristino ambientale del progetto RIPISILVANATURA (2014-2019), consistente nell'eradicazione della canna comune (*Arundo donax*), specie alloctona che ha invaso gli ambienti ripariali precedentemente occupati dalla vegetazione fluviale autoctona. Per questo, l'attività di marcamento della lontra è stata monitorata sia in tratti di fiume oggetto di interventi, sia in tratti non gestiti. Tra aprile 2016 e giugno 2018, 42 sezioni (lunghezza media ± DS = 0,5 ± 0,2 km) sono state campionate (da 1 a 10 volte) alla ricerca degli escrementi della specie, coprendo un tratto di 109,8 km di lunghezza. La preferenza di habitat della lontra eurasiatica è stata valutata in relazione a dieci parametri ambientali che avrebbero potuto influenzarne la presenza, utilizzando l'analisi di regressione logistica (LRA) e il modello di regressione non parametrico Random Forest. È stato osservato che la lontra eurasiatica evita le acque inquinate e le fitte macchie di *Arundo*. L'intensità di marcamento è aumentata dopo la rimozione della canna (1,18 spraints/100 m contro 0,76 spraints/100 m). Gli effetti combinati dell'inquinamento dell'acqua e della diffusione della canna comune potrebbero alterare la distribuzione e l'abbondanza delle specie ittiche, riducendo la disponibilità di prede per la lontra. Pertanto, il miglioramento della qualità del habitat e il ripristino di comunità ittiche autoctone sono fondamentali per favorire il recupero della lontra eurasiatica in un'area antropizzata come il bacino del fiume Segura.

La seconda parte del lavoro si è concentrata principalmente sulla composizione della dieta della lontra nel bacino del fiume Segura. Nei climi semi-aridi, la scarsità e imprevedibilità delle piogge ,l'eccessiva regolazione delle portate (a causa dei prelievi per l'irrigazione) e il collegamento tra diversi bacini idrografici (TrasvaseTajo-Segura), hanno favorito il progressivo aumento del numero di specie ittiche alloctone nel fiume Segura. Al fine di evidenziare come i cambiamenti causati dalle attività antropiche abbiano influenzato la composizione e struttura delle comunità ittiche, la dieta della lontra è stata confrontata in due periodi separati tra loro di 20 anni (1997-98 e 2016-19). Sono state anche confrontate le dimensioni dei barbi gitani (*Luciobarbus sclateri*) predati nei due periodi. In generale, pesci e gambero rosso della Louisiana (*Procambarus clarkii*), una specie aliena e invasiva, hanno costituito la maggior parte della dieta della lontra durante entrambi i periodi considerati. Nel 2016-19 il contributo delle specie esotiche nella dieta della lontra eurasiatica è risultata notevolmente aumentata, includendo dieci specie non autoctone. Le abitudini alimentari della lontra eurasiatica hanno rispecchiato fedelmente la variazione nella composizione della comunità ittica e hanno confermato l'importanza del gambero rosso come preda alternativa ai pesci nella penisola iberica. Infine, la lunghezza media dei barbi predati è diminuita nel secondo periodo di studio, suggerendo che le alterazioni occorse negli ultimi due decenni possano aver influito negativamente anche sulla struttura dellle popolazioni autoctone.

In continuazione dello studio sulla dieta nel bacino del fiume Segura, si è messo in relazione il recupero della lontra eurasiatica (*Lutra lutra*) con la contemporanea espansione degli insediamenti umani, che stanno portando ad un progressivo incremento di avvistamenti in aree urbane e peri-urbane. La dieta della specie è stata considerata lungo un gradiente da naturale- a urbano, con l'obiettivo principale di valutare in che misura la variazione della disponibilità di cibo determinata dalle attività antropiche influenzi le sue abitudini alimentari e come questo mustelide possa prosperare in habitat apparentemente inadatti. Dal 2016 al 2019 sono stati raccolti 521 escrementi, monitorando 37 stazioni di campionamento (lunghezza media ± SD = 0,5 ± 0,3 km) distribuite in quattro macro-habitat: aree naturali, coltivi (principalmente frutteti e pioppeti), risaie e aree urbanizzate. Nelle aree naturali, la percentuale di copertura della vegetazione ripariale è risultata più elevata, in coincidenza di un minor numero di prelievi d’acqua, mentre l'inquinamento idrico è risultato maggiore nelle aree urbane. Come atteso, la dieta è risultata costituita prevalentemente da gambero rosso americano (*Procambarus clarkii*) e pesci. Le due specie ittiche più predate, il barbo gitano (*Luciobarbus sclateri*) e la lasca del Tago (*Pseudochondrostoma polylepis*), sono le specie più abbondanti nel fiume, confermando il carattere generalista della dieta della lontra eurasiatica. Il contributo dei pesci non autoctoni è stato in media abbastanza basso, mentre il gambero rosso della Louisiana (una specie aliena ed invasiva) è risultato la preda principale in tutti gli habitat ad eccezione delle aree urbane. Nel complesso, i dati sulla dieta non consentono di identificare il cibo come una risorsa limitante per la lontra nelle aree urbane. Al contrario, l'intensità di marcamento varia in modo coerente con il gradiente naturale-urbano (da 0,65 a 0,31 spraints/100m), suggerendo che il disturbo antropico (modificazione del habitat e contaminazione dell'acqua) svolgano un ruolo importante nella distribuzione delle lontre eurasiatiche.

L'ultima parte del lavoro ha avuto come obbiettivo la messa a punto di un metodo efficace di monitoraggio della lontra e del visone americano (*Neovison vison*), un mustelide semiacquatico alloctono, introdotto nella Penisola Iberica per scopi commerciali. Questa specie si è diffusa in quasi tutta l'Europa continentale, motivo per il cui sono stati realizzati diversi programmi volti alla sua eradicazione. Nella penisola iberica esiste un programma di questo tipo dal 2001 nell'ambito del piano d'azione nazionale per la conservazione del visone europeo, *Mustela lutreola* (la cui azione principale e più fruttuosa è stata il progetto LIFE LUTREOLA SPAIN/LIFE13 NAT/ES/001171 "Nuovi approcci nella conservazione del visone europeo in Spagna"). Per valutare gli effetti dei piani di controllo e individuare la presenza della specie in aree di espansione è necessario disporre di un metodo di campionamento efficace e sicuro. Le feci del visone americano possono essere confuse con quelle di altri mustelidi della stessa dimensione, ma anche con quelli della lontra, in particolare quando contengono resti di prede acquatiche. Un valido aiuto per superare queste difficoltà può essere l'utilizzo di metodi genetici non invasivi È dunque stato sviluppato un metodo PCR-RFLP basato sul DNA mitocondriale estratto da campioni fecali. Il metodo ha chiaramente distinto le feci di visone americano da quelli di lontra eurasiatica, poiché gli enzimi di restrizione hanno prodotto pattern ben differenziati per le due specie. L'85% dei 94 campioni fecali analizzati è stato attribuito a una specie di mustelide, e solo tre dei sette inizialmente attribuiti al visone americano su basi morfologiche (forma e dimensione degli escrementi), sono stati confermati appartenere a quel mustelide. Ciò dimostra che l'accuratezza dell'identificazione visiva degli escrementi è discutibile e, quindi, che i metodi genetici non invasivi sono appropriati per una valutazione più affidabile nei programmi di eradicazione.

Sulla base dei risultati ottenuti, si conclude che le strategie di conservazione per la lontra eurasiatica nell'area studiata dovrebbero concentrarsi sul ripristino degli habitat di acqua dolce ed essere principalmente finalizzate al miglioramento dell'ittiofauna nativa. Queste azioni non possono essere efficaci senza una regolamentazione delle portate che tenga conto delle variazioni naturali del flusso idrico e il ripristino della connettività longitudinale dei corpi idrici. Per quanto riguarda le specie esotiche invasive, mentre i gamberi rossi americani sono difficili da estirpare, è stato dimostrato che la gestione può controllare la diffusione della canna comune. Tuttavia, è necessario proseguire sia le azioni di ripristino ambientale sia il monitoraggio della lontra, al fine di confermare gli effetti positivi degli interventi effettuati. Per questo motivo si ritiene che il censimento della lontra debba essere svolto con maggiore regolarità. Allo stesso tempo, nelle aree in cui si prevede che l'espansione di lontre e visone comporti la sovrapposizione dei rispettivi areali, l'applicazione di metodi genetici non invasivi può consentire l'identificazione affidabile delle specie bersaglio e l'immediata implementazione delle azioni di gestione. Le conclusioni del lavoro sono in linea con alcune delle principali misure del Piano di recupero della lontra nella Regione di Murcia, che includono la protezione e il miglioramento del suo habitat, la promozione della cooperazione istituzionale e sociale per lo sviluppo di un piano d'azione integrato di ripristino degli ambienti ripariali (che dovrebbe includere specificamente le specie esotiche invasive), l'incremento della connettività fluviale e una maggiore regolamentazione della pesca. L'espansione di una specie carismatica come la lontra eurasiatica può contribuire a una percezione positiva delle azioni di gestione da parte sia delle agenzie governative, sia dell’opinione pubblica, favorendo lo sviluppo di ulteriori progetti di ripristino del fiume Segura. In questo contesto, la lontra può svolgere il ruolo di specie “bandiera” di progetti volti a migliorare la qualità dell'acqua e della vegetazione ripariale nelle aree urbane e, in ultima analisi, promuovere la sostenibilità urbana e il recupero per il pubblico degli ambienti ripariali.

Annex II. FIELD FORM

Locality **Date**

Water body or river

Length of the sampling station (*in meters*.)

Water regime dry mínimum average máximum

Flow rate in the sampling station *m/sec.*

Use of the shore roads close to the shore trails with fishing spots
paths next to the river banks not reachable
shores intact

Water speed (*1-slow / 5-extremely fast*) 1 2 3 4 5

Cloudy water (*1-extremely cloudy / 5-very clean*) 1 2 3 4 5

Visible traces of pollution foam bad smells oils
strange colors urban solid residues
algal cover bacterial coverage
discharges other

Uses of the body of water water capture motorboats b. sailing
rowing boats canoeing fishing
hunting bath sheep-farming
sand quarry other

Fishing pressure number of fishermen detected in the investigation transect

Sprainting sites

n° site substrate n° anal secretions n° excrements
n° site substrate n° anal secretions n° excrements
n° site substrate n° anal secretions n° excrements
n° site substrate n° anal secretions n° excrements

River bank vegetation cover

< 5% 5-25% 26-50% 51-75% 76-100%

Amplitude of the riparian vegetation (*in meters*)

Dominant vegetation

River measure width at the station point (*in meters*)

River bed width (*in meters*)

Depth of the watercourse

Ground

rock pebbles gravel
sand mud o other

Aquatic vegetation cover

< 5% 5-25% 26-50% 51-75% 76-100%

Dominant aquatic plants

Notes

Annex III. DISSECTION PROTOCOL

FISH:

1. If frozen, the fish should be allowed to thaw.

2. Measure the size of the fish, including tail and head length, and scale size during thawing.

3. Remove some scale samples.

4. Scales are kept well separated from each other, in a Petri dish sealed with film.

5. Boil the fish.

6. Once boiled, remove the fish from the water and let it cool.

7. Dissect the fish using a scalpel, carefully removing the tissues until the spines.

8. Carefully remove the spinal column, trying not to fracture the rasps.

9. Split the tail from the body.

10. The skull must be carefully disassembled, separating pharyngeal bones.

11. Soak all hard parts in hydrogen peroxide (H_2O_2).

* *The amount of peroxide varies according to the size of the fish*.

12. Hydrogen peroxide is left to act for 24 hours, as to remove all the mucosa and soft tissues.

13. The remains are poured into a strainer with a 2 mm mesh and washed with tap water.

14. Allow the bones to dry completely and store them in a film-sealed Petri dish.

15. Mark the Petri dish with the scientific name of the species.

OTHER ANIMALS (SOFT TISSUES AND HARD PIECES):

1. If the animal is frozen, let it thaw.

2. Measure the size of the animal (or its diagnostic parts) during thawing.

3. Prepare tubes filled with 96º alcohol for storing diagnostic remains.

* *The size of the tubes and the amount of alcohol used will vary depending on the size of the animal.*

4. Whenever necessary, the animal is dissected with a scalpel, removing with care the tissues until chitinous or keratinous parts are released.

5. Remove all hard parts very carefully.

6. Separate diagnostic parts from the rest of the body, carefully disassembling them.

7. Soak all hard parts in hydrogen peroxide (H_2O_2).

* *The amount of peroxide varies according to the size of the animal*.

8. Hydrogen peroxide must act for 24 hours to remove all mucous membranes and soft tissues.

9. The content is poured into a strainer with a 2mm mesh screen and washed with tap water.

10. Diagnostic elements are left to dry completely, and then stored in the tubes (step 3), sealed with film.

11. Finally, each tube is marked with the scientific name of the species.

Annex IV. GENETIC ANALYSIS PROTOCOLS

FavorPrep Stool DNA Isolation Mini Kit (Favorgen)

General Protocol [FASTI 001-1 (100 preps)]:

1. Add up to 200 mg of stool sample to a bead tube and place the tube on ice.

-- If the sample is dry, reduce the sample size to ≤ 50 mg.

-- If the sample is liquid, add 200 μl of sample into a bead tube.

2. Add 300 μl of SDE1 Buffer and 20 μl of proteinase K (10 mg/ml) to the sample. Vortex at maximum speed for 5 minutes. Incubate the sample mixture at 60 °C for 20 minutes and vortex the sample every 5 minutes during the incubation.

-- Make sure stool sample is homogenized completely.

-- For isolation of DNA from gram positive bacteria, do a further incubation at 95 °C for 5 minutes after proteinase K lysis.

3. Briefly spin the tube to remove drops from the inside of the lid.

4. Cool down the sample mixture and add 100 μl of SDE2 Buffer. Mix well by vortexing and incubate the sample mixture on ice for 5 minutes.

5. Centrifuge at full speed (~18,000 x g) for 5 minutes.

6. Carefully transfer the supernatant to a 1.5 ml microcentrifuge tube (not provied) and discard the stool pellet.

--Avoid pipetting any debris and pellet.

7. Add 200 μl of SDE3 Buffer. Mix well by vortexing and incubate the sample mixture at room temperature for 2 minutes.

-- SDE3 Buffer must be suspended completely by vigorously vortexing before every using.

-- Cut off the end of 1 ml tip to make it easier for pipetting the SDE3 Buffer.

8. Centrifuge at full speed for 2 minutes.

9. Carefully transfer 250 μl of supernatant to a 1.5 ml microcentrifuge tube (not provied).

--Avoid pipetting any debris and pellet.

10. (Optional) If RNA-free DNA is required, add 1 μl of 100 mg/ml RNase A (not provided). Mix well and incubate the sample mixture at room temperature for 2 min.

11. Briefly spin the tube to remove drops from the inside of the lid.

12. Add 250 μl of SDE4 Buffer and 250 μl of ethanol (96~100%). Mix thoroughly by pulse-vortexing.

13. Place a SED Column into a Collection. Transfer all of the sample mixture to the SDE Column. Centrifuge at full speed for 1 min and discard the flow-through then place the SDE Column into a new Collection Tube.

14. Add 750 μl of Wash Buffer (ethanol added) to the SDE Column. Centrifuge at full speed for 1 min then discard the flow - through. Return the SDE Column back to the Collection Tube.

--Make sure that ethanol (96~100%) has been added into Wash Buffer when first use.

15. Repeat step 15.

16. Centrifuge at full speed for an additional 3 min to dry the SDE column.

--Important step! This step will avoid the residual liquid to inhibit subsequent enzymatic reactions.

17. Place the SDE Column into a 1.5 ml microcentrifuge tube (not provided). Add 50 ~ 200 μl of preheated Elution Buffer or ddH2O to the membrane center of the SDE Column. Stand the SDE Column for 2 min at room temperature.

--Important step! For effective elution, make sure that the Elution Buffer or ddH2O is dispensed onto the membrane center and is absorbed completely.

18. Centrifuge at full speed for 1 min to elute DNA.

PCR amplification of the D-loop region

Basic Protocol Xpert Taq Mastermix (2X)

Xpert Taq Mastermix (2X)	12.5 µl	1X
Forward primer (5 pmol/ µl)	2 µl	0.4 µM
Reverse primer (5 pmol/ µl)	2 µl	0.4 µM
Template DNA	3.5 µl	1-250 ng*
PCR –grade water	5 µl	
Total	25 µl	

Forward primer Mm_L1 (5′- CCCAAAGCTGACATTCTAAC-3′ adapted for *Martes* genus from the L1607 primer of Davison et al. 1999) and a reverse primer, Mm_H1 (5′-ATGGGCCCGGAGCGAGAAGAGGTACAC-3′ designed bu Ruiz-Gonzalez et al. (2008) for *Martes* genus).

Temperature	Time	
94ºC	5’	
94ºC	1’	35 cycles
63,5ºC	1’	
72ºC	1’	
72ºC	10’	
4ºC	*Inf.*	

Agarose D1 Low EEO	1,5%	DNA extraction	100/400/30
Agarose D1 Low EEO	2,0%	PCR product	100/400/40
Agarose D1 Low EEO	3,0%	PCR-RFLP	100/400/50

Ladder used: **Thermo Scientific™ O'GeneRuler Low Range DNA Ladder, Ready-to-Use 25-700 bp**.

Nucleic acid staining solution: **RedSafe Nucleic Acid Staining**.

Analyses were conducted using the: **Horizontal electrophoresis chamber**.

Electrophoresis check: **Industrial Automatic UV Transilluminator**.

Annex V. Further presentations in corollary of this work were presented in chronological order:

Poster, III Congresso Nazionale Fauna Problematica. *Recent spread of invasive American mink* Neovison vison *in Sardinia*. Cesena, 24 – 26 novembre 2016.

Poster, III Jornadas Doctorales Universidad de Murcia. *A non-invasive genetic survey of sympatric carnivorous mammals in a riparian corridor along the intensively cultivated valley of the Segura River (SE Spain)*. Murcia, 30 de mayo – 1 de Junio, 2017.

Poster, XIII Congreso SECEM. *Uso de areas suburbanas por la nutria paleártica (*Lutra lutra*)*. Guadalajara, 6 – 9 de diciembre, 2017.

Poster VI Jorandas Doctorales Universidad de Murcia. *Distribution and diet of the Eurasian otter (*Lutra lutra*) on the river Segura (SE Spain)*. Universidad de Murcia, 29 – 31 de mayo, 2018.

Comunicación Oral, III Congreso de Biodiversidad y Conservacion de la Naturaleza. *Diet of the Eurasian otter (*Lutra lutra*) on the river Segura (SE Spain) and its comparison with the Iberian Peninsula*. Murcia, 27 – 29 de Septiembre de 2018.

Oral Comunication, Oral Comunication, 4th International Conference Integrative sciences and sustainable development of rivers. *LIFE+ RIPISILVANATURA: Biomonitoring and shortterm assessment of restoration measures to control*. Lyon, 21-25 June, 2018.

Poster, 1st Iberian Ecological Society & XIV AEET Meeting. *Improving the ecological knowledge of Eurasian otter in the Segura river basin (SE Iberian Peninsula)*. 4th – 7th February 2019, Barcelona, Spain.

Oral Comunication. *LIFE+ RIPISILVANATURA: short-term evaluation of riparian restoration actions across multiple taxa*. 4th – 7th February 2019, Barcelona, Spain.

Comunicación Oral, III Congreso Ibérico restauración fluvial, RESTAURARIOS 2019. *LIFE+ RIPISILVANATURA: preliminary assessment of the effect of riparian restoration actions on aquatic and terrestrial biodiversity*. Murcia, 12 – 13 – 14 de junio 2019

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