UNIVERSIDADE DE LISBOA
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HOW HISTORICAL AND PRESENT CLIMATE CONDITIONS AFFECTED THE DISTRIBUTION OF THE MEDITERRANEAN WATER SHREW? A PHYLOGEOGRAPHICAL AND ECOLOGICAL APPROACH

JOAQUIM SEVERINO TORRES TAPISSO

DOUTORAMENTO EM BIOLOGIA

(BIOLOGIA EVOLUTIVA)

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Teses orientada pela Professora Doutora Maria da Luz Mathias e pelo Professor Leszek Rychlik,
especialmente elaborada para a obtenção do grau de doutor em BIOLOGIA (BIOLOGIA EVOLUTIVA)

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Na elaboração desta dissertação foram usados artigos já publicados, ou submetidos para publicação, em revistas científicas indexadas ou em livros. De acordo com o previsto no nº 1 do artigo 45º do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no Diário da República, 2.ª série, n.º 65, de 30 de Março de 2012, o candidato esclarece que participou na concepção, obtenção dos dados, análise e discussão dos resultados de todos os trabalhos, bem como na redacção dos respectivos manuscritos.

A dissertação, por ser uma compilação de publicações internacionais, está redigida em Inglês.

Apesar de alguns dos artigos científicos integrados na dissertação já terem sido publicados a sua formatação foi alterada para uniformizar o texto.

Lisboa, Junho de 2014
Joaquim T. Tapisso

PRELIMINARY NOTE

According to Article 45.nr.1 of the Post-graduate Studies Regulation (Diário da República, 2ª série, nº 265, 30 March 2012) this dissertation includes papers published or submitted for publication and the candidate, as co-author, was involved in the scientific planning, sampling design, data collection, statistical analyses and writing of all manuscripts.

Papers format was made uniform to improve text flow.

The dissertation, being composed of a series of international publications, is written in English.

Lisboa, Junho de 2014
Joaquim T. Tapisso

DEDICATÓRIA

PARA OS MEUS PAIS E PARA A MINHA FAMÍLIA A 4

Vocês são a razão e o

coração da minha existência!

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Era impossível realizar esta tese sem ajuda. Foram muitas as pessoas que me ajudaram no trabalho de campo, no laboratório e na obtenção de amostras. Mas não menos importante foi a ajuda “invisível”. O apoio daquelas pessoas que não dão um contributo científico mas que são fundamentais por acreditarem no meu sonho de ser cientista. Esta tese é mais uma etapa realizada desse sonho. Um sonho que quero continuar a viver.

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Water shrews in Europe have a name: Professor Leszek Rychlik. I have learned so much with you not only about these amazing animals but also about field work, scientific thinking, polish way of life… You were a real mentor for me. A role model about to be a scientist and great father! You and your family received me in the best way possible almost without knowing me. I will never forget how well you take care of me. Be sure that one day I will go again to Poland that is now my second country.

A Doutora Graça Ramalhinho representa para mim um ponto de viragem no meu percurso académico. A si lhe devo a descoberta destes fascinantes animais que são os musaranhos. Mas resumir a minha relação com a Drª Graça aos musaranhos é pouco. É muito, muito pouco. Os seus ensinamentos aliados à minha teimosia levaram ao desenvolvimento de uma relação muito especial. Muito obrigado por acreditar em mim.

Antes do doutoramento assustava-me mais com um PCR do que com uma noite gelada na Serra da Estrela. Mas com um jeitinho muito especial, só ao alcance de quem fala com açúcar na voz, a Doutora Cristiane Bastos-Silveira lá me foi mostrando que os meus receios tinham pouca razão de ser. Muito obrigado Cristiane por todo o apoio.

O nosso grupo de micromamíferos é muito especial. Uma das razões para isso é o facto de três de nós já cá andarmos desde os tempos idos da licenciatura. Sofia, foi com orgulho que te vi chegar a Doutora Sofia Gabriel. Tens feito tudo ao teu alcance para que eu também possa acrescentar esse título ao meu nome. Que fique aqui escrito que tiveste azar na saída de campo. Foi talvez a saída mais dura que tive na Serra da Estrela. Muito obrigado por todo o apoio e amizade. E já agora agradecer também ao Doutor Pedro Martins que também sobreviveu aquelas verdadeiras noites loucas do Covão da Ponte.

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RESUMO

O estudo da distribuição das espécies é fundamental para a conservação da biodiversidade. O conhecimento preciso da distribuição geográfica de uma espécie bem como dos factores que mais a influenciam são essenciais na avaliação do seu risco de ameaça. Entre os factores que mais influenciam a distribuição das espécies incluem-se: i) a história evolutiva, nomeadamente o local de origem e posteriores rotas de dispersão; ii) as condições abióticas, que podem determinar limites fisiológicos de sobrevivência; iii) as interacções bióticas, tanto positivas como negativas e; iv) a capacidade de dispersão e/ou de adaptação a novas condições. Cada um destes factores pode influenciar diferencial e/ou cumulativamente o padrão de distribuição de uma espécie a nível local, regional ou global, sendo a influência exercida por cada um geralmente maior nas áreas limítrofes de distribuição específica.

A região Mediterrânica constitui o limite sul para muitas espécies que apresentam uma vasta distribuição extensível ao centro e norte da Europa. Adicionalmente, esta região alberga também inúmeras espécies endémicas, estando listada como um “hotspot” de biodiversidade mundial. A congregação de espécies na bacia do Mediterrânico deve-se em grande parte ao papel fundamental que esta região desempenhou na história evolutiva da maioria das espécies europeias, nomeadamente como área de refúgio durante as glaciações do Quaternário. No entanto, é nesta mesma área que agora se estima virem a ocorrer as maiores modificações na distribuição de muitas espécies, resultantes de alterações climáticas, nomeadamente de um aumento acentuada da temperatura.

O Musaranho-de-água, *Neomys anomalus*, é uma espécie semiaquática distribuída essencialmente pela região Mediterrânica, desde Portugal até ao Irão, com o

limite norte recentemente alterado para a Lituânia. Apesar da vasta área ocupada, a distribuição desta espécie é altamente fragmentada. O musaranho-de-água ocorre geralmente em densidades muito baixas, o que se traduz num reduzido conhecimento da sua biologia e ecologia. A União Internacional para a Conservação da Natureza e dos Recursos Naturais (UICN) refere uma tendência para o decréscimo das populações e classifica a espécie como "Least Concern". Em Portugal, a falta de informação justificou a atribuição de estatuto do "Informação Insuficiente".

O Musaranho-de-água é considerado uma espécie de elevados requisitos ecológicos devido aos seus hábitos semiaquáticos. Os habitats preferenciais desta espécie são geralmente zonas com um elevado grau de humidade, como rios, ribeiros, lagos ou charcos. A sua dieta é essencialmente constituída por macroinvertebrados terrestres, incluindo anelídeos e pequenos coleópteros, mas também presas aquáticas como larvas de tricópteros. O musaranho-de-água ocorre geralmente em sintopia com outras espécies de musaranhos sendo, no entanto, relativamente rara a coexistência com, o Musaranho-de-água-do-Norte, *N. fodiens*, uma espécie com preferências ecológicas muito similares ao Musaranho-de-água. A dieta do Musaranho-de-água-do-Norte é também ela muito parecida com a dieta do Musaranho-de-água, embora inclua geralmente uma percentagem mais alta de presas aquáticas. As duas espécies são morfologicamente muito semelhantes, ainda que o Musaranho-de-água-do-Norte apresente adaptações mais pronunciadas ao ambiente aquático. Assim, ambas as espécies apresentam uma pelagem densa e impermeável, franjas de pêlos longos e duros nas patas e uma dupla fila de pêlos na parte ventral da cauda que funciona como quilha direccional durante a natação. No entanto, a densidade de pêlos nas patas é bastante superior no Musaranho-de-água-do-Norte relativamente ao Musaranho-de-água. A quilha caudal também distingue as duas espécies, sendo extensível a toda a cauda no

Musaranho-de-água-do-Norte, ocupando apenas o último terço da cauda no Musaranho-de-água. O Musaranho-de-água-do-Norte ocorre geralmente em maiores densidades que o Musaranho-de-água, distribuindo-se continuamente desde o norte de Espanha até à Rússia e China, ocorrendo também em latitudes mais elevadas como na Grã-Bretanha e Península Escandinava. Assim, o Musaranho-de-água-do-Norte ocorre em simpatria com o Musaranho-de-água sobretudo na região Mediterrânica. A ausência de sintopia entre as duas espécies nesta região tem sido explicada pela competição interespecífica não sendo, no entanto, ainda claro a escala a que essa interacção biótica influencia o seu padrão de distribuição.

Esta tese teve como principal objectivo avaliar a influência de diferentes factores (evolutivos e ecológicos) na determinação da distribuição do Musaranho-de-água. Foi utilizada uma abordagem multidisciplinar a diferentes escalas temporais. Assim, foi avaliada a influência da história evolutiva da espécie na sua distribuição actual através do estudo da estrutura filogeográfica em toda sua área de distribuição geográfica; foram identificados os principais factores ecológicos que determinam a actual presença da espécie; foi estimada uma potencial alteração nos padrões comportamentais do Musaranho-de-água em função do contexto biogeográfico, nomeadamente a ocorrência em simpatria ou alopatria com o Musaranho-de-água-do-Norte; por último, foi estimada a influência de factores bióticos e abióticos na determinação do limite Sul de distribuição das duas espécies de forma a inferir as consequências de futuras alterações climáticas.

O estudo filogeográfico do Musaranho-de-água implicou a recolha de amostras de tecido ao longo de toda a área de distribuição da espécie. Assim, foram efectuadas campanhas de amostragem em vários pontos da Europa, com especial incidência em Portugal e Polónia. Foram também obtidas amostras de outras localizações como

Alemanha, Eslováquia, Ucrânia e Rússia através do estabelecimento de colaborações internacionais. Foram ainda utilizadas amostras museológicas obtidas em diferentes museus europeus. Para além desta amostragem, a análise filogeográfica beneficiou também da obtenção de sequências de ADN depositadas no GenBank, muito em particular das sequências utilizadas num estudo preliminar sobre a história evolutiva dos musaranhos-de-água na Europa, particularmente em Itália. Tal como neste estudo, as análises moleculares incidiram na comparação de um fragmento do ADN mitocondrial, o gene do citocromo *b*. Foram também recolhidas e analisadas amostras do Musaranho-de-água-do-Norte de forma a comparar a história evolutiva das duas espécies.

A determinação dos factores ecológicos que regulam a presença ou ausência do Musaranho-de-água foi realizada no Parque Natural da Serra da Estrela, Portugal. Foi monotorizada uma população durante um período de aproximadamente ano e meio (Outubro 2007 até Janeiro 2009), tendo sido efectuadas cinco saídas de campo, cada uma delas implicando cinco noites de armadilhagem. Os animais foram capturados em armadilhas de madeira tipo Sherman, distribuídas numa grelha semi-fixa ocupando diferentes tipos de habitat, a diferente distância de um curso de água (Rio Mondego). Todos os animais capturados foram identificados, pesados, marcados e posteriormente libertados.

De uma outra área também localizada nas proximidades do Rio Mondego e ainda dentro do perímetro do Parque Natural da Serra da Estrela, foram capturados nove Musaranhos-de-água que foram transportados para o Museu Nacional de História Natural e da Ciência da Universidade de Lisboa. O comportamento de natação e mergulho destes animais foi analisado através de testes de curta duração e a estratégia de procura de comida foi investigada também experimentalmente através de testes de longa duração. Nos testes de curta duração os animais foram induzidos a nadar num aquário durante um período máximo de 5 minutos, tendo sido filmados em três posições diferentes. Nos testes de longa duração os animais foram filmados durante 24 horas num terrário onde se simulou um habitat aquático e um habitat terrestre. Os testes realizados implicaram o fornecimento de uma quantidade fixa de alimento em ambos ou apenas num dos habitats. No final de cada teste foi registada tanto a quantidade como a localização de cada uma das porções de comida não utilizada. Todos os animais foram devolvidos ao seu habitat natural depois de concluídos os ensaios laboratoriais.

As localizações de todos os pontos de captura do Musaranho-de-água foram georreferenciadas para posterior análise da distribuição da espécie a nível regional, mais concretamente na Península Ibérica. Aos dados de campo foram acrescentados pontos de ocorrência da espécie descritos na bibliografia e no portal do Sistema Global de Informação sobre a Biodiversidade (GBIF). Procedeu-se depois à modelação espacial da distribuição das duas espécies de musaranhos-de-água de forma a estimar a sua presente distribuição na Península Ibérica, bem como prever potenciais alterações em função das mais recentes estimativas de alterações climáticas, tanto num cenário a curto prazo (2050) assim como num cenário a longo prazo (2070).

O estudo filogeográfico permitiu aumentar de forma significativa o número de amostras de musaranhos-de-água até agora utilizadas em análises moleculares. Este estudo permitiu também obter a melhor cobertura geográfica de amostras até ao momento. A análise do gene citocromo *b* permitiu identificar três linhagens genéticas, uma linhagem de ocorrência exclusiva na Península Ibérica, uma linhagem Italiana, incluindo na sua maioria amostras provenientes de Itália mas também dos Alpes Suiços, e uma terceira linhagem de distribuição mais alargada, incluindo toda a Europa Central, o Leste Europeu, a Península Balcânica e a região da Anatolia (Turquia). As distâncias genéticas encontradas entre linhagens foram, no entanto, muito diferentes. A distância encontrada entre a linhagem Ibérica e as outras duas linhagens foi muito superior à distância encontrada entre a linhagem Italiana e a linhagem do centro e leste europeu. Quando comparado com outros estudos filogenéticos, o grau de diferenciação genética da linhagem Ibérica é superior ao encontrado entre diferentes espécies de musaranhos. Não foi possível identificar qualquer linhagem genética no Musaranho-de-água-do-Norte, apesar de também se ter aumentado consideravelmente o número de amostras analisadas bem como a cobertura geográfica das amostras específicas.

A monitorização da população do Musaranho-de-água na Serra da Estrela permitiu identificar o habitat ripícola como o preferido para a ocorrência da espécie. Todas as capturas ocorreram junto à linha de água, independentemente da altura do ano. A espécie foi capturada em abundância no Verão, estando praticamente ausente da área de estudo no Inverno. Foi possível determinar que os factores que mais influenciam a presença do Musaranho-de-água são as condições abióticas do curso de água, nomeadamente a largura, profundidade e corrente do rio. A presença de outras espécies de musaranhos como o Musaranho-de-dentes-vermelhos, *Sorex granarius* ou o Musaranho-de-dentes-brancos, *Crocidura russula* não parecem influenciar a presença do Musaranho-de-água. Foi também possível observar que, apesar da ocorrência pontual destas duas espécies de musaranhos terrestres no habitat ripícola, apenas os movimentos dos Musaranhos-de-água indiciaram que vários indivíduos conseguem atravessar o rio, e utilizar simultaneamente as duas margens. Estes resultados parecem sugerir uma alteração na estratégia de procura de presas, que pode assim depender de invertebrados aquáticos.

Os resultados laboratoriais do comportamento de natação e mergulho foram consistentes com a utilização do habitat aquático observado na Natureza. Foi possível verificar que a eficiência de natação e mergulho dos Musaranhos-de-água provenientes da Serra da Estrela é maior do que a previamente observada em Musaranhos-de-água de outras zonas da Europa onde ocorrem em simpatria com o Musaranho-de-água-do_Norte. O Musaranho-de-água em Portugal consegue gerar uma maior capacidade de propulsão com as patas traseiras mergulhando também com uma configuração hidrodinâmica mais eficiente. A análise individual dos mergulhos demonstrou também que em Portugal o Musaranho-de-água apresenta uma grande diversidade no perfil desses mergulhos, alguns deles considerados de máxima dificuldade por implicarem uma longa duração e a exploração activa do fundo do aquário. A avaliação da estratégia de procura de presas veio também a demonstrar que estes animais podem alimentar-se tanto em ambiente terrestre como aquático, o que até aqui só tinha sido observado no Musaranho-de-água-do-Norte. A ausência desta última espécie parece claramente influenciar a estratégia de procura de presas do Musaranho-de-água e com isso alterar o padrão de distribuição da espécie a nível local, levando assim à selecção de habitats exclusivamente aquáticos.

Numa análise a nível regional foi também possível demonstrar que a distribuição do Musaranho-de-água está dependente da presença ou ausência do Musaranho-de-água-do-Norte. A modelação espacial da distribuição dos musaranhos-de-água na Península Ibérica permitiu observar que a área de ocorrência estimada para o Musaranho-de-água é maior que a área estimada para o Musaranho-de-água-do-Norte. Enquanto o Musaranho-de-água dispõe de áreas climaticamente adequadas tanto a Norte como a Sul da Península, o Musaranho-de-água-do-Norte está confinado a uma faixa situada a Norte da Península que se estende das Astúrias aos Pirenéus. Esta é também a faixa de simpatria das duas espécies, sendo que no resto da Península apenas se encontram populações alopátricas do Musaranho-de-água. No entanto, a distribuição potencial desta espécie é muito fragmentada, estando significativamente correlacionada com os sistemas montanhosos Ibéricos. A competição interespecífica influencia sobretudo a altitude a que as espécies ocorrem. Na zona de simpatria o Musaranho-de-água-do-Norte ocorre em altitudes mais elevadas do que o Musaranho-de-água. Mas, na ausência do seu competidor mais directo, o Musaranho-de-água ocupa áreas montanhosas a altitudes mais elevadas, sendo expectável que seleccione habitats mais aquáticos tal como se verificou na Serra da Estrela.

Estima-se que a área de distribuição de ambas as espécies sofra uma redução na Península Ibérica perante diferentes cenários de alterações climáticas. Essa redução implicará uma contracção da área de distribuição dos musaranhos-de-água. A área de distribuição do Musaranho-de-água poderá sofrer reduções de 10 a 30% enquanto no Musaranho-de-água-do-Norte os valores de redução podem variar entre 25 a 60% da sua área de distribuição actual. Os valores de redução de áreas favoráveis à ocorrência destas espécies na Península tenderão a agravar-se ao longo do tempo. Justifica-se assim assinalar que, mesmo no cenário menos grave de alterações climáticas, as populações de *N. anomalus* mais afectadas serão aquelas que actualmente ocorrem nas montanhas do Sistema Central da Península Ibérica, incluindo as populações da Serra da Estrela investigadas no decorrer desta tese. As contracções da área de distribuição potencial de musaranhos-de-água na Península Ibérica face a diferentes cenários de alterações climáticas poderão também provocar alterações na extensão das áreas de simpatria e alopatria das duas espécies. Assim, as alterações potenciais na distribuição de *N. anomalus* implicarão não só uma alteração dos limites de distribuição da espécie na Península Ibérica mas também uma alteração no tipo de habitat em que a espécie ocorre, em resultado do aumento da área de alopatria. Pelo contrário, o Musaranho-de-água-do-Norte ocorrerá quase em exclusivo em simpatria com o Musaranho-de-água o que poderá significar um aumento da competição por habitats favoráveis nestas áreas.

A análise do ADN mitocondrial do Musaranho-de-água permitiu a identificação clara de uma linhagem genética altamente divergente localizada na Península Ibérica. É no entanto prematuro atribuir um estatuto taxonómico distinto ás populações Ibéricas sem antes aprofundar o estudo molecular da espécie, nomeadamente através da análise de outro tipo de marcadores como genes nucleares. O isolamento genético do Musaranho-de-água na Península Ibérica vem também requerer que se investigue a estrutura filogeográfica da espécie nesta região, sendo que para isso será necessário aumentar ainda mais o número de amostras analisadas. Na Península Ibérica, o Musaranho-de-água ocorre num contexto biogeográfico distinto de quase toda a restante área de distribuição da espécie. As populações de Musaranho-de-água da Península Ibérica ocorrem na sua maioria em alopatria com o Musaranho-de-água-do-Norte. A ausência deste competidor directo conduz a uma alteração na selecção do habitat preferencial do Musaranho-de-água, no sentido da ocupação de áreas mais dependentes do meio aquático como as margens de rios. O estudo do uso do habitat de um população alopátrica de Musaranhos-de-água da Serra da Estrela demonstrou precisamente a alteração no nicho ecológico da espécie. As capturas sucessivas dos mesmos indivíduos nas duas margens do rio Mondego reflectiram também uma alteração no comportamento de mergulho e natação dos indivíduos, e possivelmente a modificação na estratégia de procura de presas. A demonstração experimental de alterações no comportamento predatório do *N. anomalus* confirma também a expansão do nicho ecológico da espécie, o que na Península Ibérica poderá ser potenciado pela diferenciação genética destas populações. A variação da altitude média de ocorrência do Musaranho-de-água a nível regional também parece reflectir o efeito de competição interespecífica.

A abordagem multidisciplinar no estudo dos padrões de distribuição do *N. anomalus* permitiu demonstrar a importância que a história evolutiva teve na actual distribuição da espécie, bem como aferir o papel que a competição interespecífica desempenha a diferentes escalas. Serão no entnato necessários mais estudos para clarificar o estatuto taxonómico da espécie na Península Ibérica. É também evidente que o estatuto de ameaça terá que ser revisto, não só pelo facto da identificação de uma linhagem genética indicar a existência de um novo endemismo ibérico mas também devido à previsível acentuada redução na área de distribuição do Musaranho-de-água, caso se confirmem os mais recentes cenários de alterações climáticas. A redução estimada da área de distribuição do Musaranho-de-água na Península Ibérica terá implicações significativas na ocorrência da espécie em Portugal, a qual poderá no futuro ficar confinada a pequenos núcleos populacionais no Norte do país.

Palavras-Chave: *Neomys anomalus*, filogeografia, competição interespecífica, alterações climáticas

ABSTRACT

The Mediterranean water shrew (*Neomys anomalus*) is a semi-aquatic species with a wide but fragmented distribution across southern and center Europe and Asia Minor. This species is one of the least studied small mammal species not only of the Portuguese but also of the whole European fauna. As such, it is still unclear what are the major factors determining the fragmentation of the Mediterranean water shrew populations.

The present work intended to better understand the influence of climate and other ecological factors on the distribution of the Mediterranean water shrew by following a time-scaled multidisciplinary approach, that included: i) the study of the evolutionary history of the species; ii) the analysis of ecological and behavioural aspects determining its current niche space; and iii) the determination of the effects of future climate changes on the species' distribution.

Two factors were identified as significantly contributing to the current distribution pattern of the Mediterranean water shrew: severe climatic alterations, that were extremely important in the evolutionary history of the species and that are also projected to have a significant impact in its future distribution, and the interspecific competition with a congeneric species, which is one of the main factors regulating the current habitat preferences of the Mediterranean water shrew.

The followed multidisciplinary and time-scaled approach provided relevant insights on the ecological factors determining the presence of the Mediterranean water shrew, ultimately defining its geographical distribution. It also highlighted the need to clarify the taxonomic status of the Iberian populations of this species. The detection of a unique genetic lineage in this region makes the reported findings highly relevant from an evolutionary perspective, and extremely significant for its conservation.

Keywords: *Neomys anomalus*, phylogeography, interspecific competition; climate changes

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1 Problem overview

Species distributions should be analysed under a spatial-temporal framework as the occurrence area of living organisms depends on their evolutionary history and ecology (Brown *et al*., 1996; Gaston, 2009). As such, the study of a species' historical origins and subsequent movements of its individuals is as important as the identification of the ecological factors that determine the current distribution of a species namely, abiotic conditions, biotic interactions and the current ability of individuals to disperse. Climate is among the abiotic factors most highly correlated with species distributions (Gaston, 2003; Araújo, 2005), with ambient temperature frequently being a key factor for the occurrence of a species in a given area (Jeffree & Jeffree, 1994; Gaston, 2003). Past climatic oscillations characterised by marked changes in ambient temperatures have led to significant alterations in species' geographical distributions, some with evolutionary consequences as the speciation and extinction of species (Dynesius & Jansson, 2000). Recent alterations in the distribution of many species have also been linked with human-induced climate changes, consistent with an increase in ambient temperatures (e.g. Hickling *et al*., 2006; Parmesan, 2006; Chen *et al*., 2011). It is still difficult to infer the evolutionary capacity of species to cope with the recent climate changes but it is clear that the projected extinction rates have a magnitude similar to the mass extinction events that occurred along the history of the Earth (Bellard *et al.*, 2012). Thus, increasing the knowledge on factors determining the geographical distribution of species is therefore essential to mitigate the effects of future climate changes on biodiversity.

Mammals are distributed across almost all climatic regions of the globe (Wilson & Reeder, 2005). One of the main reasons for such success is mammals' well known ability to maintain a constant body temperature, i.e., endothermy (Grigg *et al.*, 2004). Although severe climatic changes as the oscillations in Quaternary temperatures have induced significant alterations in the distribution of many mammals (Davies *et al.*, 2009), it is expected that mammals should be less affected by recent climate changes than other groups of species less effective in regulating body temperature (Thuiller *et al.*, 2011). However, a particular group of mammals seems to be especially vulnerable to changes in temperature. Given their small body size, shrews are under high physiological constraints when it comes to maintaining a constant body temperature, making them particularly susceptible in a global change scenario (Thuiller *et al.*, 2011).

Shrews (Order Soricomorpha, Family Soricidae) are among the most ancient mammals in the world. The fossil record indicates that the first shrews evolved about 45 million years ago (Wójcik & Wolsan, 1998). These species have retained several primitive features, including a small and unspecialised body plan that only suffered minor changes along their evolution (Hutterer, 1985). However, shrews are a highly diverse group, including 385 recently recognised species in 26 genera (Wilson & Reeder, 2011) distributed across almost all regions of the globe (Churchfield, 1990). The family Soricidae includes three subfamilies - Myosoricinae, Soricinae and Crocidurinae - differing in the number of species and distributional range (Wilson & Reader, 2011). The subfamily Myosoricinae includes about 20 African shrew species distributed south of the Sahara desert (Hutterer, 2005). The remaining subfamilies have a similar number of species between them and a wide distribution. Soricinae shrews, the red-toothed shrews, are mainly distributed in the Northern hemisphere, occurring in cold and humid climates, while Crocidurinae shrews, the white-toothed shrews, occur in Africa and in the southern part of Eurasia, inhabiting warmer and arid areas (Churchfield, 1990). As such, underlined by an evolutionary history in separate geographic regions, Soricinae and Crocidurinae shrews have developed different strategies to overcome the physiological constrain of being small. Red-toothed shrews are known for their extremely high metabolic rate (much higher than predicted by allometry) and by their ability to decrease body size during winter (both in mass and length), a phenomenon known as the Dehnel's effect (Weiner, 1992; Taylor, 1998). In contrast, Crocidurinae shrews, have a metabolic rate lower than red-toothed shrews (but still higher that other small mammals), a lower body temperature, and the ability to enter in daily torpor to save energy and reduce water loss (Taylor, 1998). Members of both subfamilies occur sympatrically and sometimes even syntopically in large areas over Europe, including Portugal (Hutterer, 2005; Fig. 1). However, according to the ecological, behavioural and physiological adaptations to different climate conditions that characterize each subfamily, the species projected to be most affected by climate warming are the members of the Soricinae (Thuiller *et al.*, 2011).

Most Soricinae shrews occurring in Europe belong to the genus *Sorex*, but there are also two species belonging to the genus *Neomys*, the water shrews. Water shrews differ from *Sorex* species because they have the ability to forage in water. In fact, the morphological adaptations to a semi-aquatic life-style are among the most evident specialisations found in shrews (Hutterer, 1985). Shrews are considered good models to investigate the effects of interspecific competition because of their lack of morphological, behavioural and physiological differentiation (Kirkland, 1991). Habitat and trophic niche segregation have been frequently described as the main mechanisms by which *Sorex* shrews avoid competition (e.g. Neet & Hausser, 1990; Rychlik, 2000), being spatial segregation more evident between *Sorex* and water shrews (Rychlik, 2000). By exploiting aquatic resources, water shrews avoid competition with *Sorex* shrews that frequently co-occur in communities of multiple species (e.g. Churchfield, 1991; Churchfield, 1998; Churchfield & Rychlik, 2006). However, in several parts of Europe two of the three water shrews species, the Mediterranean water shrew (*Neomys anomalus* Cabrera 1907) and the Eurasian water shrew (*Neomys fodiens* (Pennant, 1771)) overlap in their distribution area (Hutterer, 2005), which may thus induce competition between these congeneric species.

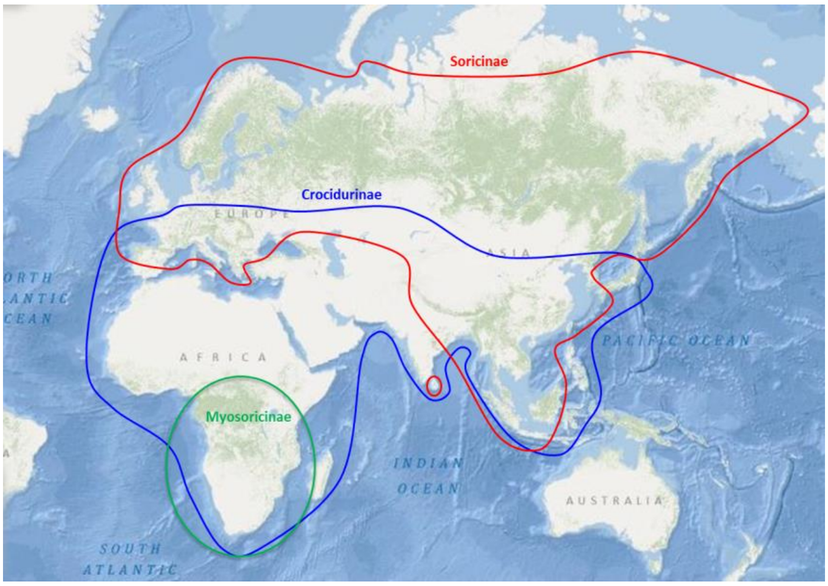

Figure 1. Distribution of three subfamiles of shrews: Myosoricinae, Soricinae and Crocidurinae; Americas are excluded (adapted from Churchfield, 1990).

This thesis proposes to uncover the main factors influencing the distributional pattern of the Mediterranean water shrew by following a time-scaled multidisciplinary approach, namely, i) the study of the evolutionary history of the species; ii) the analysis of ecological and behavioural aspects determining its current niche space and iii) the determination of the effects of future climate changes on the species' distribution.

1.2 The study species

The Mediterranean water shrew, *Neomys anomalus* Cabrera 1907, although only weighing between 7 to 20 g is one of the largest shrew species in Europe (Fig. 2). This species is usually bicoloured, having dark fur on the dorsal side, and white on the ventral side. The most remarkable morphological features of this species are related with its semi-aquatic life-style. Its fur is composed by a dense layer of woolly hairs and by guard hairs with a pronounced H-shape core, which improve its waterproof proprieties and floating capacity (Ivanter, 1994). The hind-feet of the Mediterranean water shrew are larger than those of similar sized terrestrial shrews, being covered by fringes of stiff hairs that increase the propellant power during swimming (Spitzenberger, 1990; Churchfield, 1998). The ventral side of the tail is also partially covered by two rows of stiff hairs, forming a keel that promotes stability in water. The brain case of the Mediterranean water shrew is also larger that of its terrestrial counterparts as a consequence of the reduction in the olfactory lobes and increase in trigeminal nerves, allowing the species to detect prey by contact (using the vibrissae) rather than by smell (Kryštufek *et al.*, 2000). All these morphological adaptations to a semi-aquatic life-style are shared by the other species of the same genus, being, however, more evident in the Eurasian water shrew, *N. fodiens* (Pennant, 1711) (Spitzenberger, 1990; Churchfield, 1998; Fig. 2). This species is slightly bigger than the Mediterranean water shrew (body mass ranging from 7 to 23 g) and it has larger hind-feet (16 to 21 mm compared to 13 to 18 mm in *N. anomalus*; Spitzenberger, 1990). The keel covers the entire ventral side of the tail and its feet are covered, even more densely, by fringes of stiff hairs (Spitzenberger, 1990; Churchfield, 1998). Due to these attributes, the Eurasian water shrew is considered to be better adapted to aquatic environments than the Mediterranean water shrew (Spitzenberger, 1990; Churchfield, 1998; Vogel, 1998; Mendes-Soares & Rychlik, 2009).

Figure 2. Mediterranean water shrew, *Neomys anomalus*, caught in Serra da Estrela, Portugal (left side). Eurasian water shrew, *Neomys fodiens*, caught in Białowieża Forest, Poland (right side).

As most shrews occurring in the Palearctic region, the Mediterranean water shrew breeds between spring and late summer. Although only a few studies concerning the reproduction of this species exist, these suggest that this species has a similar litter size to other Soricinae shrews, i.e., ranging from 2 to 7 young (e.g. Michalak, 1982). Their lifespan should also fall within the average range of other Soricinae species, varying between one to two years. Behavioural studies on the Mediterranean water shrew have found a high tolerance between conspecific individuals, which is indicative of a gregarious social system (Krushinska & Pucek, 1989; Krushinska *et al.*, 1994). Such social organisation is rare among Soricinae shrews (Rychlik, 1998), most species being territorial and displaying high levels of intraspecific aggressiveness (Rychlik, 1998; Rychlik & Zwolak 2006). One of the most aggressive species is the Eurasian water shrew, which is not only highly aggressive intraspecifically but also towards individuals of other species, including the Mediterranean water shrew (Krushinska & Pucek, 1989; Krushinska & Rychlik 1993; Krushinska *et al*., 1994; Rychlik & Zwolak, 2006). The smaller sized Mediterranean water shrew, actively avoids contact with the bigger sized Eurasian water shrew, a behaviour that has been considered a strategy to reduce aggression and competition between the two species (Krushinska & Rychlik, 1993; Krushinska *et al.*, 1994). This behavioural mechanism of aggressive avoidance has important implications in habitat selection by the Mediterranean water shrew. This species inhabits wet areas, such as banks of small rivers, streams, lakes, ponds, marshes and swamps (Spitzenberger, 1990; Rychlik, 2000). However, these are also the preferential habitats of the Eurasian water shrew (Spitzenberger, 1990; Rychlik, 2000), and as expected, habitat segregation has been described in the wet areas where both species co-exist (Rychlik, 2000). The microhabitats closer to water bodies are exclusively occupied by the Eurasian water shrew, being the Mediterranean water shrew only found in adjacent microhabitats already at a considerable distant from the water (Rychlik, 2000). Studies on the diet of the two species are also congruent with microhabitat segregation. In sympatric areas, the diet of the Mediterranean water shrew is mainly composed by terrestrial prey, including larvae of different insects, earthworms and spiders (Spitzenberger, 1990, Churchfield & Rychlik, 2006). Although the Eurasian water shrew also feeds on terrestrial prey, its diet includes a high percentage of aquatic invertebrates (Spitzenberger, 1990; DuPasquier & Cantoni, 1992; Churchfield & Rychlik, 2006). There is a general perception that in areas where the Eurasian water shrew is absent, the Mediterranean water shrew occurs closer to water, probably including aquatic prey in its diet (Kryštufek & Quadracci, 2008). However, field data supporting such hypothesis is still scarce and frequently contradictory. The lack of data on the ecology of allopatric populations of the Mediterranean water shrew assumes a special relevance because these populations represent almost half of the geographic distribution of the species.

The Mediterranean water shrew has a wide but discontinuous distribution through the mountains of west and central Europe, including Portugal, Spain, France, Belgium, Italy, Germany and Poland (Hutterer, 2005), with the northernmost limit of the species' distribution having been recently extended to Lithuania (Balčiauskas & Balčiauskienė, 2012; Fig. 3). This species also occurs in the lowlands of the Balkan region, Anatolia and Eastern Europe (Ukraine, Belarus and Western Russia), the eastern limit of the species being located in Iran. More than half of the Mediterranean water shrew range is also occupied by the Eurasian water shrew, which is continuously distributed throughout most of Europe, Russia, west China and northwest Mongolia (Hutterer, 2005; Fig. 4).

The International Union for Conservation of Nature reports a decreasing population trend of the Mediterranean water shrew but classifies the species as "Least Concern" (IUCN, 2014). In Portugal, the lack of information about this species justified the status of "Data Deficient" (Cabral *et al.*, 2005).

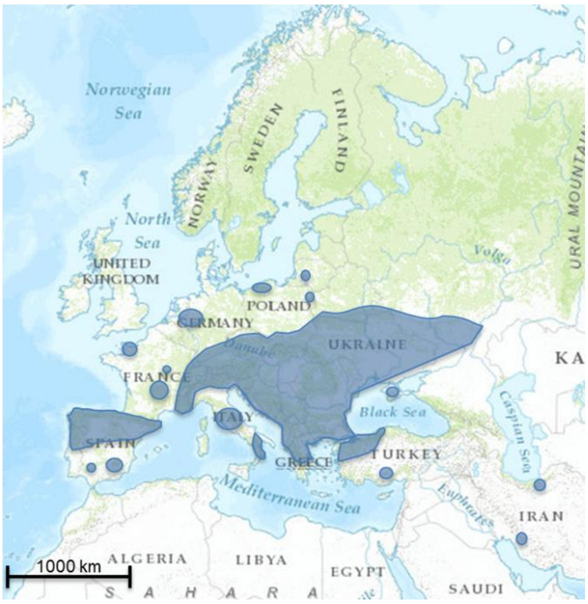

Figure 3. Geographical distribution of the Mediterranean water shrew, *Neomys anomalus* (adopted from Hutterer, 2005).

Figure 4. Geographical distribution of the Eurasian water shrew, *Neomys fodiens* (adopted from Hutterer, 2005).

1.3 Evolutionary history inferred from phylogeography

Paleobiogeographic studies have been given important contributions to clarify the ancient distribution of species and their evolutionary history (e.g. Kryštufek *et al.*, 2000). However, the fossil record is usually limited to a small number of species and geographic locations, both in space and time. With the advances in the technical ability to obtain DNA sequences from individuals across a species range, it became possible to infer the evolutionary history of a larger number of species through the identification of their phylogeographic patterns, i.e. by determining the geographical distribution of genealogical lineages (Avise *et al.*, 1987). Phylogeographic studies typically allow the determination of the exact location where a species has originated, the refuge areas during adverse climatic periods and the subsequent re-colonisation routes.

Most phylogeographic studies are based on the analysis of mitochondrial DNA (mtDNA). This molecule has specific characteristics that are particularly suitable for phylogeographic surveys. Firstly, mtDNA is highly abundant in the cell and is easily extracted. Secondly, it is typically inherited in a uniparental (matrilineal) way, thus having haploid transmission which facilitates its analyses. Thirdly, it has a rate of divergence that is fast enough to show population differences across a species range but slow enough to not saturate with recurrent mutations over a few million years (Avise, 2000). Phylogeographic studies based on the analysis of this molecule typically involve the determination of mtDNA haplotypes, which are used to infer a phylogeny, or a gene tree. Since closely similar or identical mtDNA haplotypes are geographically localized, gene trees can be used to infer the geographical distribution of major gene lineages (Templeton, 2001).

One of the major findings obtained by the accumulation of empirical data generated by the analysis of mtDNA is that closely related species often tend to be genealogically structured in similar ways, i.e., species with a similar ecology are more likely to have been originated in the same areas and to have responded similarly to climate changes over time. As such, it is expected that these species have used the same refuge areas during inhospitable climatic periods and adopted similar re-colonisation routes when environmental conditions were more favourable. Avise and colleagues (2000) reported the existence of similar phylogeographic patterns in southeastern America between different taxa, including invertebrates, freshwater and marine fish, turtles and birds. Concordant genealogical patterns were also observed in other regions, such as Central and South America, in several species of rodents (Riddle *et al.*, 2000), bats (Hoffmann & Baker, 2003) and reptiles (Victoriano *et al.*, 2008; Castoe *et al.*, 2009). The same was observed in other biogeographic regions like Australia, in which similar phylogeographic patterns have also been found in reptiles (Schneider *et al.*, 1998), snails (Hugall *et al.*, 2002) and mammals (Potter *et al.*, 2012). In Europe, congruent phylogeographic patterns have also emerged for different taxa. In fact, the number of European species showing similar phylogeographic structures is even more remarkable than in other regions of the globe. The majority of the phylogeographic studies identify three European regions - the Iberian Peninsula, the Italian Peninsula and the Balkan region – as the primary refugia for most species during the Pleistocene ice ages (Hewitt, 1996; 1999; 2000; 2004; Weiss & Ferrand, 2006), with only a few exceptions having been detected to this general trend (e.g. Deffontaine *et al.*, 2005; Kotlik *et al.*, 2006; Ruiz-González *et al.*, 2013). Thus, current geographical distributions of European fauna are strongly influenced by the location of species in one or multiple refugia and by the subsequent re-colonisation routes adopted.

Several phylogeographic studies indicate the Iberian Peninsula as the major refuge from which numerous currently wide distributed species have recolonized Europe (e.g. Michaux *et al.*, 2003). In addition, molecular surveys have also identified several cryptic species in the Iberian Peninsula (Ibañez *et al.*, 2006; Martínez-Solano, 2004; Mateus *et al.*, 2011; Paupério *et al.*, 2012), which, considering the already unusual number of endemic species here found (Baquero & Telleria, 2001), reinforce the importance of this region as a hotspot of biodiversity. Several particular characteristics of the Iberian Peninsula may have promoted the survival and diversification of such high number of species. This region is relatively isolated on the westernmost point of Europe and shows a high complex system of mountain ranges, orientated primarily east to west. Such mountain range orientation has two main consequences: i) prevent movements along a north-south axis, thus constituting gene-flow barriers between populations and ii) promotes strong climatic gradients within and between mountains due the differentiated influence of the North Atlantic and the Mediterranean Sea. Thus, in order to better infer the evolutionary history of widely distributed European species it is critical to ensure an accurate representation of Iberian populations in phylogeographic analysis.

Few molecular studies have been conducted in order to infer the phylogeographic patterns of water shrews in Europe (Kryštufek *et al.*, 2000; Castigila *et al.*, 2007). Moreover, these few studies were based on a small number of samples with a poor geographic cover. So far, only one point-located sample of the Mediterranean water shrew from the Iberian Peninsula was analysed (Castigila *et al.*, 2007).

1.4 Factors influencing species geographical distributions

Among the most important factors determining the geographic distribution of a species are abiotic conditions that impose physiological limits to species persistence and biotic interactions that can be either positive or negative (Soberon & Peterson, 2005). Species dispersal abilities, which are particularly relevant in the context of evolutionary history, may also influence the area where a species is found (e.g. Schloss *et al.*, 2012). However, this factor is usually considered less important than, for example, climatic conditions determining the presence or the absence of individuals in a given area.

1.4.1 Abiotic conditions

Every species has climatic and physical tolerances that determine where they can live, survive and reproduce, i.e. their fundamental niche (Hutchinson, 1957). At a geographical scale, environmental temperatures are considered to play a pivotal role in the determination of species distribution patterns (e.g. Jeffree & Jeffree, 1994). In fact, extensive studies have been performed in order to evaluate the effects of potential changes in environmental temperatures on the niche space of many species (e.g. Parmesan *et al.*, 1999; Parmesan & Yohe, 2003; Chen *et al.*, 2011; VanDerWal *et al.*, 2013). However, at a local scale, other abiotic factors can be as important as climatic variables influencing the occurrence of a species. For instance, there are species strongly dependent of particular edaphic conditions or water course regimes (e.g. Morueta-Holme *et al.*, 2010).

Energetic constraints are commonly referred to explain the influence of abiotic conditions on the distribution of species. In temperate regions it has been found that the ability of species to occupy a given niche is strongly dependent from seasonal energetic bottlenecks within the annual cycle (Weiner, 1992). The linear link between survival and energetic strategies is particularly relevant in small sized-mammals (e.g. Jackson *et al.,* 2001). Survivorship and reproduction of such small endotherms depend on the efficiency of their energetic strategy to cope with seasonal changes. In general, seasonal acclimatisation is acquired not only through physiological but also behavioural adjustments. The typical response of small mammal species to variations in ambient temperature involves alterations in the body mass, basal metabolic rate and daily activity (Lovegrove, 2005). Shrews, however, are so small that such adjustments are critical for their survival (e.g. Genoud, 1988; Taylor, 1998; Churchfield, 2002), which contributes to their vulnerability to alterations in ambient temperatures. Shrews have almost no fat reserves and their high metabolic rate requires a high daily food intake, frequently exceeding its body weight (Taylor, 1998). Soricinae shrews, for example, do not survive more than 3 to 4 hours without food. The energetic strategy of water shrews is even more dependent of abiotic conditions because of their use of both terrestrial and aquatic environments (Genoud, 1988; Vogel, 1998), which impose additional physiological constrains (Williams, 1998).

1.4.2 Biotic interactions

The potential occurrence area of a species based on the physiological tolerance to abiotic conditions (the fundamental niche), is usually significantly different from the area where the species effectively occurs, i.e., the realized niche (Hutchinson, 1957). The realized niche is only composed by areas with suitable abiotic conditions where the species has positive population growth rates, given the constraining effects of biological interactions, such as competition (Pearman *et al.*, 2008). One of the most effective ways to analyse the importance of competition in the occurrence of a species is through the study of space use and habitat selection (Morris, 2003). According to niche theory, the coexistence between two competitor species is mainly obtained by ecological segregation (Levins, 1968), which in a large number of small mammals involves microhabitat segregation (e.g. Eccard & Ylonen, 2003; Pita *et al.*, 2010). Several mechanisms of ecological segregation have been described in shrews, including microhabitat and trophic partitioning (e.g. Churchfield & Sheftel, 1994; Rychlik, 2000; Churchfield & Rychlik, 2006). As such, the effect of interspecific competition on the distributional patterns of shrews is usually only detected at a local scale. However, in the case of species like water shrews, in which optimal wet habitats are less available than terrestrial habitats, the effect of interspecific competition may affect their distributional patterns at larger scales. Moreover, the spatial effect of biotic interactions as interspecific competition may vary significantly across the distribution area of a species (Case & Taper, 2000). In the limits of the geographical distribution where species may occur near their physiological tolerances, the effect of interspecific competition may be magnified and ultimately determinant in defining species limits (e.g. Hersteinsson & Macdonald, 1992; Price & Kirkpatrick, 2009).

1.5 Species distribution models

Technical advances in geographic information systems, as well as the accumulation in digital databases of information about environmental variables and species occurrences, has allowed the development of computational species distribution models (Guisan & Thuiller, 2005; Elith & Leathwick, 2009; Kearney & Porter, 2009; Elith *et al.*, 2010). Most distribution models are based on the correlation between point-locations of a species and associated environmental characteristics, i.e., they only consider the fundamental niche of species (Soberón & Peterson, 2005). One of the main reasons to use correlative models instead of models involving the realized niche of a species is that biological interactions are considered to only affect the spatial patterns of a species at a local scale (Pearson & Dawson, 2003). However, depending on species and biological interaction there are cases in which the difference between the potential distribution area based on the fundamental niche is significantly different than the area estimated by models considering the realized niche (e.g. Araújo & Luoto, 2007; Gilman *et al.*, 2010; Van der Putten *et al.*, 2010; Wisz *et al.*, 2013).

Distribution models have been extensively used to predict the consequences of climatic variations on the occurrence area of a species. Usually these models are used to forecast potential alterations in the suitable climatic areas for the species considering different scenarios of climate changes at several time scales (e.g. Araújo *et al.*, 2005; Hijmans & Graham, 2006; Levinski *et al.*, 2007). The estimations given by these models are considered an important management tool in conservation of biodiversity (e.g. Araújo *et al.*, 2011), especially considering the accumulation of empirical data on recent human induced climate changes (IPCC, 2013). The increasing importance of the use of distribution models in conservation biology requires maximum accuracy when estimating the potential area of occurrence of a species, which can only be achieved by increasing the knowledge on biotic interactions and incorporating such information on realized niche based models (Urban *et al.*, 2013).

The spatial distribution of species may, however, not vary significantly even in periods of sudden climate change if they show the ability to adapt to new conditions, i.e. to expand or alter their realized niche (Parmesan, 2006; Hoffman & Sgrò, 2011). Adaptation to new environmental conditions may be achieved by an evolutionary response involving genetic alterations (e.g. Bradshaw & Holzapel, 2006; Skelly *et al.*, 2007; Visser, 2008) or by phenotypic plasticity (e.g, Charmantier *et al.*, 2008; Canale & Henry, 2010), i.e. the ability of an organism to express different phenotypes in different environments (Agrawal, 2001). Both responses may induce an alteration of the realized niche of a species, which may involve, for example, adaptation in different life-traits of the species (Réale *et al.*, 2003; Hill *et al.*, 2011).

1.6 Aims, hypotheses and thesis structure

The present work framed by different time and geographical scales intends to better understand the influence of climate and other environmental factors on the distribution of the Mediterranean water shrew (*Neomys anomalus*) both considering the post glacial routes of dispersion from Quaternary refuges and the ecological requirements regulating its presence.

The thesis specific aims are to:

1. Identify main phylogeographic patterns in the Mediterranean water shrew across its distributional range, namely the role of Mediterranean peninsulas as refugia during the Pleistocene ice ages;

2. Compare the phylogeographic structure between the Mediterranean water shrew and the Eurasian water shrew (*Neomys fodiens*), since both species are taxonomically related, morphologically similar and sympatric over a large area across Europe;

3. Identify ecological factors that may regulate the occurrence of the Mediterranean water shrew at the range borders of its geographical distribution, since these outermost populations usually occur under the absence of the Eurasian water shrew;

4. Investigate swimming and diving skills of allopatric Mediterranean water shrews as well as their foraging strategy;

5. Determine the potential distribution area of both Mediterranean and Eurasian water shrews in the Iberian Peninsula;

6. Evaluate the role of interspecific competition on the distribution of both species at a regional scale;

7. Estimate potential changes on the distribution of water shrews under different climatic scenarios and time scales.

The following **hypotheses** were tested:

H1) Glacial refugia for water shrews were located in Mediterranean Peninsulas, from where they expanded northwards;

Mediterranean Peninsulas acted as refuge areas during Pleistocene glaciations for many European species (Weiss & Ferrand, 2007). Re-colonisation routes have been identified based on present day contact zones between different genetic lineages (Hewitt, 1999; Hewitt, 2004). A previous study assessing the phylogeographical structure of water shrews in Europe clearly identified the Italian Peninsula as an important glacial refugium for *N. anomalus* (Castiglia *et al.*, 2007). However, a considerably higher number of samples from the Italian Peninsula relatively to the remaining species distribution range were used in this study. As such, the importance of the remaining Mediterranean Peninsulas as glacial refugia and the post-glacial re-colonisation routes adopted by the species remain unclear. The preliminary analysis conducted by Castiglia *et al.* (2007) indicated differences between the phylogeographic structure of *N. anomalus* and *N. fodiens*. However, similarly to *N. anomalus*, the number and distribution of *N. fodiens* samples were insufficient to clarify the role of Pleistocene glaciations in the current geographic distribution of the species.

By increasing the number and range (covering the vast majority of both water shrew’ distributions) of samples, it was expected to identify main mtDNA lineages in all putative glacial refugia and clarify the potential re-colonisation routes of northwards expansion. Samples were obtained from field trips, through the collaboration with other European research groups and from museum collections. Haplotype diversity was assessed using a mtDNA gene (cytochrome *b*).

The results of this analysis are addressed in **Chapter 2**.

H2) Water shrews from the Iberian Peninsula constitute cryptic forms congruent with the taxonomical classification of two Iberian endemic subspecies *N. anomalus milleri* and *N. fodiens niethammeri*;

Southern Peninsulas have a rate of endemic species higher than central and northern Europe (Baquero & Telleria, 2001) presumably because of their role as refuge areas during glaciations and subsequent allopatric speciation. Some of these endemic species have been only recently identified using molecular techniques, since they are morphologically similar to their northern and central European counterparts. Many of these so called cryptic species have been often identified in the Iberian Peninsula (e.g. Ibañez *et al.*, 2006; Martínez-Solano, 2004; Mateus *et al.*, 2011; Paupério *et al.*, 2012), probably due to the difficulty to disperse over the Pyrenees.

Castiglia *et al.* (2007) have found a significant genetic difference between Iberian *N. anomalus* and the species' remaining populations, including the Italian lineage. However, they suggested that further studies should be done in order to evaluate the taxonomic status of Iberian *N. anomalus*, as they were only able to analyse one sample from Spain. To test the hypothesis of cryptic speciation of Iberian water shrews, we increased the number of samples of *N. anomalus* from this region and analysed, for the first time, the mtDNA of Iberian *N. fodiens*. It was expected to obtain a more accurate genetic distance between the Iberian *N. anomalus* and the remaining populations, as well as to infer whether Iberian *N. fodiens* were also genetically divergent from the remaining European populations.

This hypothesis is addressed in **Chapter 2**.

H3) *N. anomalus* undergoes competitive release in the absence of the dominant competitor *N. fodiens*

H3a) Competitive release of *N. anomalus* involves, at a local level, the selection of habitats closer to water courses;

According to niche theory, the coexistence of competitor species induces niche segregation (Levins, 1968). In the case of water shrews, spatial segregation has been described; *N. fodiens* usually inhabits banks of streams or small rivers, while sympatric *N. anomalus* occurs in wet habitats but at some distance from watercourses (Rychlik, 2000). Character displacement as a response to competition has also been reported in water shrews, *N. fodiens* usually being bigger than sympatric *N. anomalus*. On the contrary, allopatric *N. anomalus* undergoes a character release converging in size towards *N. fodiens* (Spitzenberger, 1990; Rácz & Demeter, 1998; Kryštufek & Quadracci, 2008). As such, a habitat shift can be expected to occur in allopatric *N. anomalus,* since competition in aquatic habitats is potentially smaller than in terrestrial habitats. To test this hypothesis a study plot was set in an area outside the distribution range of *N. fodiens*. The habitat preferences, habitat use and habitat overlap between *N. anomalus* and other syntopic shrew species were determined during five trapping sessions covering different seasons. The influence of environmental conditions on the occurrence of *N. anomalus* was also investigated.

This hypothesis is addressed in **Chapter 3**.

H3b) Competitive release induces the use of a diving foraging mode by allopatric *N. anomalus*;

The microhabitat segregation between sympatric water shrews involves the adoption of different foraging strategies: diving in deep water for *N. fodiens* and wading in shallow water for *N. anomalus* (Churchfield, 1998; Vogel, 1998; Rychlik, 2000). Accordingly, experimental assays have demonstrated that *N. fodiens* is significantly more proficient at swimming and diving than sympatric *N. anomalus* (Mendes-Soares & Rychlik, 2009). Here it is hypothesised that allopatric *N. anomalus* have swimming and diving abilities similar to *N. fodiens*. If so, allopatric populations should also use the diving foraging mode. To test these hypotheses two laboratorial experiments were carried out using *N. anomalus* from an allopatric population: short-term swimming and diving assays and long-term foraging assays. The behavioural response obtained was compared with the previously published data on sympatric populations of *N. anomalus* and *N. fodiens* (Rychlik, 1997; Mendes-Soares, 2009).

These hypotheses are addressed in **Chapter 4**.

H3c) Competitive release allows, at a regional level, the occurrence of *N. anomalus* in abiotic conditions more typical of *N. fodiens*;

A shift in the preferential habitat of a species has the potential to influence its distribution pattern at different levels. When interspecific competition is the main drive for the habitat shift, it can be expected that the effects of such biotic interaction may also be detectable at different spatial scales. The effect of interspecific competition regulating the preferential habitats of a species may be particularly evident at its range borders, since these populations often have to cope with less suitable abiotic conditions (Sexton *et al.*, 2009). As such, it is expected that allopatric populations of *N. anomalus* occurring at the species' range borders tend to be characterised by abiotic conditions more similar to that of *N. fodiens* than to the typical environmental conditions of sympatric *N. anomalus*. To test this hypothesis, the potential distribution of both water shrew species was modelled for the Iberian Peninsula using a set of several bioclimatic variables obtained from the WorldClim database. Occurrence data for water shrews in this region was mainly obtained from the Global Biodiversity Information Facility. The influence of competition was inferred by testing intra- and interspecific differences on the environmental variables showing the highest contribution for each species' model.

This hypothesis is addressed in **Chapter 5**.

H4) Future climate changes will alter the southern range limit of water shrews;

Climate changes have a significant impact on the distribution of species, since they can induce the expansion, regression, dislocation or ultimately the extinction of a species from a given area (Parmesan & Yohe, 2003; Thomas *et al.*, 2004). The most up-to-date climatic models forecast significant increases in ambient temperatures for the next decades (IPCC, 2013). Soricinae shrews are the European mammals estimated to suffer the most severe reductions on their suitable climatic areas as a consequence of climate warming (Thuiller *et al.*, 2011). As such, we hypothesise that the southern limit of water shrews will change, *N. fodiens* probably being the most severely affected species, given its stronger dependence on colder climates compared to *N. anomalus*. If so, the area of competitive release of *N. anomalus* is expected to increase. To test this hypothesis the distribution of suitable climatic areas for the occurrence of water shrews in the Iberian Peninsula was modelled into the future decades (2050 and 2070), having as a basis global climate data of two concentration pathways (RCP 2.6 and RCP 8.5) representing mild and severe climate warming scenarios.

This hypothesis is addressed in **Chapter 5**.

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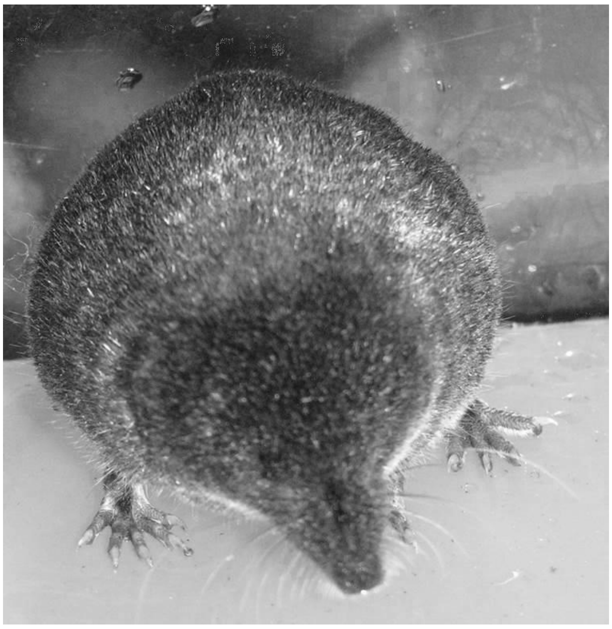
CHAPTER 2

PHYLOGEOGRAPHY OF WATER SHREWS



Contrasting post-glacial colonisation of European water shrews (*Neomys spp.*) inferred from mtDNA analyses reveals a highly divergent lineage in Iberian *N. anomalus* populations

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Abstract

We have analysed the phylogeographic patterns of two species of water shrews, *Neomys anomalus* and *N. fodiens* using mitochondrial DNA sequences (*cyt-b*). We have largely extended the geographical coverage of previous phylogeographic studies, mostly across the species' European ranges, which resulted in a better understanding of their genetic diversity and demographic histories. Our genetic analyses revealed very distinct evolutionary histories of both water shrew species, with very different genetic structures. From our results emerged three major mitochondrial lineages among *N. anomalus*, including a very divergent lineage confined to the Iberian Peninsula. In comparison, *N. fodiens* showed no phylogeographic structure even after the considerable increase in sample size and range coverage. The observed high levels of haplotype diversity, coupled with low levels of nucleotide diversity, suggest a rapid population expansion of *N. fodiens* throughout its Eurasian range following the last Ice Age. Ecological differences between both species that may help to explain these distinct patterns are discussed.

Keywords

Comparative phylogeography; cryptic species; sympatry; allopatry; *Neomys anomalus*; *Neomys fodiens*; cytochrome b

Introduction

The current genetic structure of species has been strongly influenced by historical climatic events (Hewitt, 2000). During long and inhospitable periods, like Quaternary ice ages, many species were restricted to one or a few refugia (Taberlet & Cheddadi, 2002). The geographic isolation of these refugial areas enabled species to genetically diversify, but postglacial re-colonisation led to a loss of genetic diversity in newly colonised areas, probably because of repeated founder events (Hewitt, 2000; 2004).

Phylogeographic studies conducted over the past three decades have revealed that species reacted differently to Quaternary ice ages, depending on the persistence in one or multiple refugia, colonisation routes and migration rates (see Avise, 2009 and references therein). In Europe, although three main regions were identified as refugia (Iberian Peninsula, Italian Peninsula and Balkan region), concordant phylogeographic patterns between species are rare (Hewitt, 1999; 2001). This lack of congruence is probably related with different dispersal abilities of species and capability to overcome biogeographic barriers such as the Pyrenees, the Alps or the Carpathian Mountains. Discrepancies in the geographical distribution among genetic lineages have been detected even between closely related species (e.g. Seddon *et al.*, 2001; Michaux *et al.*, 2005).

Recent molecular surveys have also revealed instances of cryptic variation, i.e., genetically divergent but morphologically indistinguishable lineages within species (Pfenninger & Schwenk, 2007). The identification and description of cryptic lineages have important implications for both biogeographic and evolutionary studies as well as for the conservation of biodiversity (Bickford *et al.*, 2007). In Europe, cryptic variation has been predominantly identified in the Iberian Peninsula, in organisms as different as lampreys (Mateus *et al.*, 2011), amphibians (Martínez-Solano, 2004), bats (Ibañez *et al.*, 2006) and rodents (Paupério *et al.*, 2012). In fact, the detection of cryptic variation has given a great contribution to the already high level of biological diversity currently found in the Iberian Peninsula, both in plant and animal species (Baquero & Telleria, 2001; Garcia-Barros *et al.*, 2002).

The Mediterranean water shrew, *Neomys anomalus* Cabrera, 1907, and the Eurasian water shrew, *N. fodiens* (Pennant, 1771) are two closely related species with a sympatric distribution through most of Europe. *Neomys anomalus* has a more southern and patchy distribution, ranging from the south of the Iberian Peninsula, to Crimea, Turkey and western Russia whereas *N. fodiens* is continuously distributed from the north of the Iberian Peninsula to central Europe and Scandinavia (Hutterer, 2005). The two species have only slight differences in morphology (Spitzenberger, 1990; Churchfield, 1998) behaviour (Mendes-Soares, 2009) and habitat preferences (Rychlik, 2000). Such differences are much more evident in locations where both species are sympatric, coexisting in the same habitats, such as banks of small rivers, streams, lakes, ponds, marshes and swamps (Rychlik, 2000). In sympatry, *N. fodiens*, the dominant species, is usually larger than *N. anomalus* (Spitzenberger, 1990), more proficient at swimming (Mendes-Soares & Rychlik, 2009) and selects microhabitats with direct access to watercourses (Rychlik, 2000). However, in allopatry, *N. anomalus* converges towards *N. fodiens* in terms of size (Rácz & Demeter, 1998; Kryštufek & Quadracci, 2008), displaying a more efficient swimming and foraging behaviour in water (Tapisso *et al.*, 2013), and having similar microhabitat preferences (Rychlik & Ramalhinho, 2005).

Despite their broad distribution across Europe, phylogeographic data on these two species is scarce and mostly limited to a study published by Castiglia and colleagues (2007). The authors suggested that both *N. anomalus* and *N. fodiens* probably shared the Iberian and Italian refugia during the Quaternary glaciations but currently show significant differences in their genetic structure. These conclusions were drawn based on small sample set and short DNA fragment. In fact, their results are not in accordance with a previous enzymatic study conducted by Catzeflis (1984) that found a higher intraspecific differentiation in *N. fodiens* than in *N. anomalus*. As such, the phylogeographic patterns of these two species remain somewhat unclear and would benefit from a broader analysis. Moreover, Castiglia *et al*. (2007) found a highly differentiated *N. anomalus* haplotype in a sample from Spain, with a genetic divergence of 9% with respect to the remainder *N. anomalus* haplotypes. Clearly, improving the sampling scheme within the Iberian range of the species is critical to clarify the taxonomic status of the extant populations of *N. anomalus* inhabiting the Iberian Peninsula. In the same way, it is also relevant to analyse samples of *N. fodiens* from this region in order to evaluate if the populations here found are also differentiated from the remaining populations of the species.

Under the assumptions that glacial refugia for water shrews were located in Mediterranean Peninsulas and that populations of the Iberian Peninsula may be highly genetic differentiated from other populations, the aims of this study were: i) to confirm if the Iberian and Italian Peninsulas were the only refugia for the two species of water shrews; ii) to check if the highly divergent haplotype already found for *N. anomalus* in Spain constitutes a case of cryptic variation and iii) to determine if this potential cryptic variation is exclusive of *N. anomalus* or, instead, also found in *N. fodiens*.

Material and Methods

Samples and DNA extraction

We analysed a total of 131 samples across the Eurasian distribution range of *N. anomalus* and *N. fodiens* (Table 1 and 2; Fig. 1). One hundred fresh samples (tail tips) were obtained by live-trapping, using baited wooden box traps. All animals were released after the tissue collection. Fresh samples were stored in absolute ethanol at 4ºC and total genomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). Additionally, 31 dry tissue samples (mostly skin) were obtained from museum collections (Museu Nacional de História Natural e da Ciência, Lisbon, Portugal; Doñana Biological Station, Seville, Spain; Museo Nacional de Ciencias Naturales, Madrid, Spain; Museum für Naturkunde, Berlin, Germany; Zoological collection of the Mammal Research Institute, Białowieża, Poland). In this case, genomic DNA was extracted using standard protocols involving incubation with SDS and digestion with proteinase K, followed by a phenol–chloroform DNA extraction (Sambrook *et al.*, 1989). To prevent contamination, DNA extractions involving museum-curated specimens were carried out in a closed environment with dedicated sterile material.

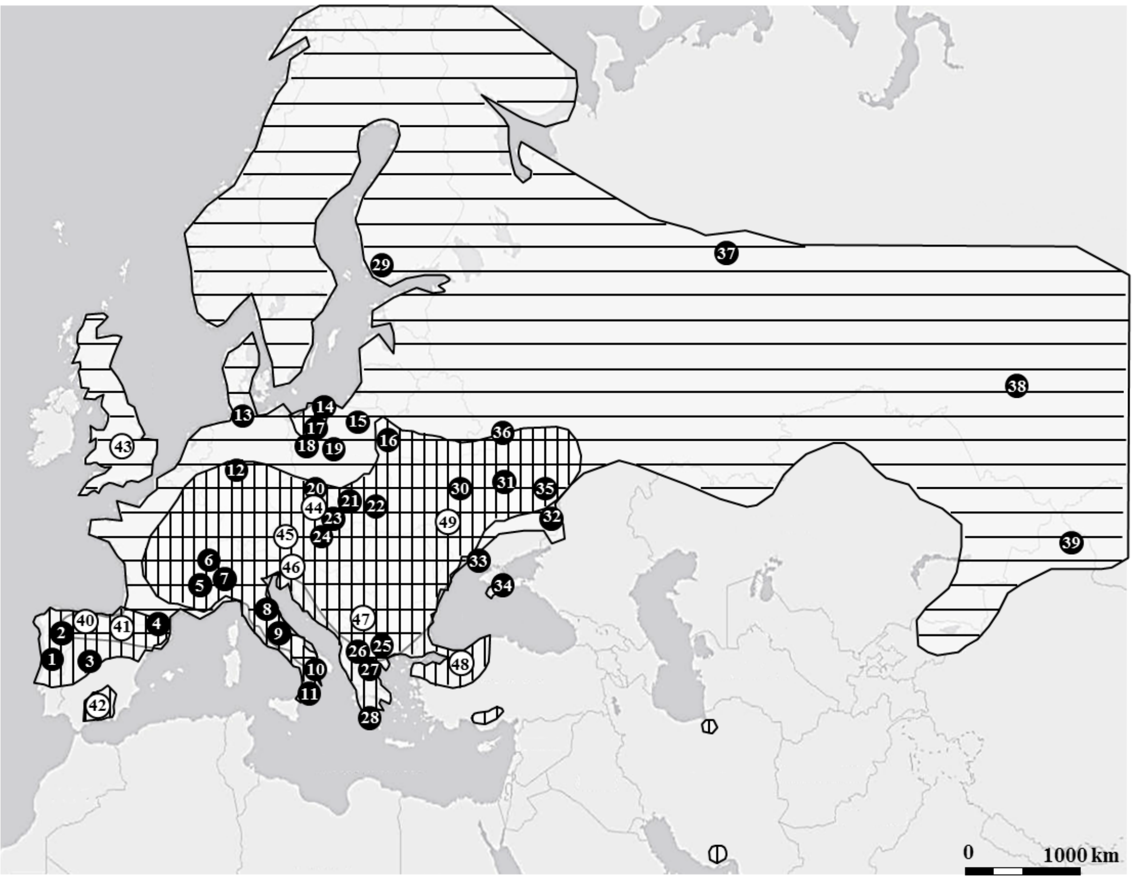

Figure 1. Map showing the Eurasian geographical range of both studied species of water shrews, *Neomys anomalus* (vertical lines) and *N. fodiens* (horizontal lines), according to Hutterer, 2005. All new sampling localities are represented by numbered solid circles and previously surveyed locations are represented by numbered open circles. Locality numbers refer to those in Tables 1 and 2.

Table 1. Locations, number of specimens and haplotype name of *Neomys anomalus* samples.

Location	Nº of Specimens	Haplotype	bp	Map number
Covão da Ponte, Serra da Estrela, Portugal	1	Nano1	1113	1
Covão da Ponte, Serra da Estrela, Portugal	1	Nano2	1113	1
Covão da Ponte, Serra da Estrela, Portugal	1	Nano3	1113	1
Covão da Ponte, Serra da Estrela, Portugal	4	Nano4	1113	1
Covão da Ponte, Serra da Estrela, Portugal	9	Nano5	1113	1
Covão da Ponte, Serra da Estrela, Portugal	1	Nano6	1113	1
Vale Glaciar, Serra da Estrela, Portugal	1	Nano7	1113	1
Vale Glaciar, Serra da Estrela, Portugal	1	Nano8	1113	1
Rio Sabor, Montesinho, Portugal	1	Nano9	1113	2
Rio Sabor, Montesinho, Portugal	1	Nano10	1113	2
Madrid, Spain	1	Nano11	1113	3
Sierra de Gredos, Spain	1	DQ991055	1113	3
Sion, Switzerland	1	AB175099	1113	6
Bassins, Switzerland	1	AB175100	1113	6
Meod vecchio, Italy	2	DQ991051	1113	8
Verrecchie, Italy	1	DQ991053	1113	9
Pescasseroli, Italy	1	DQ991054	1113	10
Sila, Calabria, Italy	1	DQ991049	1113	11
Aspromonte, Calabria, Italy	1	DQ991050	1113	12
Słowiński, Poland	2	Nano12	1113	15
Słowiński, Poland	1	Nano13	1113	15
Kletno, Poland	1	Nano14	1113	16
Kletno, Poland	3	Nano15	1113	16
Kletno, Poland	1	Nano16	1113	16
Bieszczady, Poland	1	Nano17	1113	17
Bieszczady, Poland	3	Nano18	1113	17
Bieszczady, Poland	2	Nano19	1113	17
Białowieża, Poland	2	Nano20	1113	18
Białowieża, Poland	1	Nano21	1113	18
Białowieża, Poland	1	Nano22	1113	18
Nitra, Slovakia	1	Nano23	1113	25
Nitra, Slovakia	1	Nano24	1113	25
Kerkini, Greece	1	Nano25	1113	27
Agios Germanos, Greece	1	Nano26	1113	28
Agios Germanos, Greece	1	Nano27	1113	28
Litochoro, Greece	1	Nano28	1113	29
Kiev, Ukraine	2	Nano29	1113	21
Kherson, Ukraine	1	Nano30	1113	25

Table 1. (continued).

Location	Nº of Specimens	Haplotype	bp	Map number
Kherson, Ukraine	1	Nano30	1113	25
Crimea, Ukraine	1	Nano31	1113	26
Crimea, Ukraine	1	Nano32	1113	26
Crimea, Ukraine; Bryansk, Russia	2	Nano33	1113	26; 27
Belgorod, Russia	1	Nano34	1113	28
Granada, Spain	1	Nano35	315	42
Granada, Spain	1	Nano36	315	42
La Rioja, Spain	1	Nano37	315	41
Slovenia	1	AF182184	315	46
Slovenia	1	AF182185	315	46
Macedonia	1	DQ630409	315	47
Turkey	1	AF182182	315	48
Turkey	1	AF182183	315	48

Table 2. Locations, number of specimens and haplotype name of *Neomys fodiens* samples.

Location	Nº of specimens	Haplotype	bp	Map number
Mosset, Pyrenees, France	1	DQ991063	1113	4
Alps, France	3	Nfod1	1113	5
Alps, France	2	Nfod2	1113	5
Alps, France	1	Nfod3	1113	5
Alps, France	4	Nfod4	1113	5
Bassins, Switzerland	1	AB175098 (Nfod4)	1113	6
Carlogne, Italy	1	DQ991061 (Nfod4)	1113	7
Pescorocchiano, Italy	1	DQ991056	1113	9
Pescorocchiano, Italy	1	DQ991058	1113	9
Pescasseroli, Italy	1	DQ991057	1113	10
Pescasseroli, Italy	1	DQ991059	1113	10
Opi, Italy	1	DQ991060	1113	10
Sila Calabria, Italy	1	DQ991062	1113	11
Hochsauerlandkreis, Germany	1	GU981295	1113	13
Plön, Germany	2	Nfod5	1113	14
Plön, Germany	1	Nfod6	1113	14
Słowiński, Poland	2	Nfod7	1113	15
Kletno, Poland	2	Nfod8	1113	16

Table 2. (continued).

Location	Nº of specimens	Haplotype	bp	Map number
Kletno, Poland	1	Nfod9	1113	16
Kletno, Poland	1	Nfod10	1113	16
Bieszczady, Poland	1	Nfod11	1113	17
Bieszczady, Poland	1	Nfod12	1113	17
Białowieża, Poland	1	Nfod13	1113	18
Białowieża, Poland	1	Nfod14	1113	18
Bieszczady, Białowieża, Mazury, Poland	3	Nfod15	1113	17; 18; 19
Mazury, Poland	1	Nfod16	1113	19
Ujście, Poland	1	Nfod17	1113	20
Poznań, Poland	2	Nfod18	1113	21
Poznań, Poland	1	Nfod19	1113	21
Koło, Poland	3	Nfod20	1113	22
Koło, Poland	2	Nfod21	1113	22
Koło, Poland	3	Nfod22	1113	22
Koło, Poland	1	Nfod23	1113	22
Konin, Poland	1	Nfod24	1113	23
Konin, Poland	1	Nfod25	1113	23
Konin, Poland	1	Nfod26	1113	23
Konin, Poland	2	Nfod27	1113	23
Tatra, Poland	3	Nfod28	1113	24
Tatra, Poland	2	Nfod29	1113	24
Lipót, Hungary	2	Nfod30	1113	26
Akritochori, Greece	2	Nfod31	1113	30
Bieszczady, Białowieża, Poland; Kiev, Ukraine	3	Nfod32	1113	17; 18; 21
Kiev, Ukraine	1	Nfod33	1113	21
Kiev, Ukraine	1	Nfod34	1113	21
Sunny, Ukraine	1	Nfod35	1113	22
Sunny, Ukraine	1	Nfod36	1113	22
Zhytomyr, Ukraine	1	Nfod37	1113	23
Luhansk, Ukraine	1	Nfod38	1113	24
Bryansk, Russia	1	Nfod39	1113	27
Novosibirsk, Russia	1	AB175096	1113	29
Komi, Russia	1	Nfod40	1113	30
Koverhar, Finland	1	AB175097	315	31
Altay, China	1	AB175071	315	32
Transcarpathians, Ukraine	2	Nfod41	315	49
Transcarpathians, Ukraine	1	Nfod42	315	49
Hirschegg, Austria	2	Nfod43	315	45

Table 2. (continued).

Location	Nº of specimens	Haplotype	bp	Map number
Czech Republic	1	Nfod44	315	44
Asturias, Spain	1	Nfod45	315	40
Asturias, Spain	1	Nfod46	315	40
Somogy, Hungary; Transcarpathians, Ukraine	2	Nfod47	315	26; 49
England	1	Nfod48	315	43
Macedonia	1	DQ6630421	315	47
Macedonia	1	AF182180	315	47
Macedonia	1	AF182181	315	47

DNA amplification and sequencing

A 1140-bp fragment of the mitochondrial DNA encompassing the complete cytochrome-b gene (*cyt-b*) was amplified by the polymerase chain reaction (PCR) using the primer pair L14723 (5'– ACC AAT GAC ATG AAA AAT CAT CGT T –3') and H15915 (5'– TCT CCA TTT CTG GTT TAC AAG AC –3') (Castiglia *et al.*, 2007). A shorter fragment of the *cyt-b*, with 368-bp, was amplified by using the primer pair L14841 (5'– AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA–3') and H15149 (5'– AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A–3').

All reactions contained around 100 ng of template DNA, 0.3 mM of each primer, 1U of Taq Polymerase (Promega), 2.5 mM $MgCl_2$ and 0.2 mM of each dNTP to a final volume of 25 µl. DNA amplifications were performed using the following cycling conditions: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. A final extension step at 72°C for 10 min was added at the end. All PCR products resulting from successful amplifications were purified using the FastAP

Thermosensitive Alkaline Phosphatase kit. Sequencing was performed commercially using the same primers from PCR amplification.

Sequence data analyses

The *cyt-b* sequences were aligned and manually corrected using Sequencher TM 4.8. (Gene Codes Corp.). To ensure the mitochondrial origin of the sequences, they were checked for the presence of stop codons and compared against GenBank entries of *N. anomalus* and *N. fodiens.* For the broadest possible analysis, all newly obtained sequences were truncated to a standard length of 1113 base pairs. All new (non-redundant) *cyt-b* haplotypes were submitted to GenBank (accession numbers will be available upon acceptance of the manuscript). Twenty two additional *cyt-b* sequences were included in our dataset to complement the range-wide coverage of our analyses (see Fig. 1).

jModelTest 0.1.1 (Posada & Crandall, 1998) was used to determine the best fitting model of DNA evolution for the *cyt-b* dataset (without outgroup). TVM+Γ was selected among 88 available models under the Akaike information criterion (AIC) (Posada & Buckley, 2004). The *cyt-b* dataset was then analysed for Bayesian MCMC inference on MrBayes version 3.2 (Ronquist *et al.*, 2012) with two simultaneous runs of five chains each, during 5 million generations sampled every 500 steps. After a 25% burn-in both runs were used to generate a 50% majority rule consensus tree. Due to the lack of representation of complete *cyt-b* sequences for other *Neomys* species on GenBank, the common shrew *Sorex araneus* was used as outgroup (accession number GQ374433.1). Phylogenetic networks were computed under the median-joining algorithm as implemented in Network version 4.6.1.1. (Bandelt *et al.*, 1999).

Genetic diversity parameters (nucleotide diversity, π and haplotype diversity, h) as well as neutrality tests, D (Tajima, 1989), F_S (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002), were estimated in DnaSP v.5.10.01 (Librado & Rozas, 2009). Statistical significance of observed values of D, F_S and R_2 was obtained by 10,000 coalescent simulations in the same software (Librado & Rozas, 2009). The net divergence of mitochondrial sequences between species and lineages (as determined by the phylogenetic analysis) was computed in MEGA v.5.2 (Tamura *et al.*, 2011). Statistical significance was estimated using a 10,000 bootstrap replicates.

Results

Genetic diversity, demographic history and phylogenetic analysis

In this study, we have largely extended the sample size and geographical scope of previous studies regarding the phylogeography of *Neomys anomalus* and *N. fodiens*. We have significantly increased the sampling coverage of the Iberian Peninsula, Central and Eastern Europe, including the Balkan region. A 1113 bp fragment of the mitochondrial *cyt-b* was successfully amplified for a total of 117 specimens of *N. anomalus* (n=54) and *N. fodiens* (n=63), which collapsed into 74 haplotypes, *N. anomalus* (n=34) and *N. fodiens* (n=40), most of them identified for the first time. In order to achieve the broadest possible analysis, the resulting *cyt-b* datasets of both species were joined with all the previously published sequences available in the literature (haplotype frequencies were taken into consideration for the network analysis), resulting in a dataset of 94 haplotypes (Table 3).

Sequences were highly variable, mostly for *N. anomalus*, with 173 polymorphic sites, 129 of which were parsimony informative. In *N. fodiens*, 121 polymorphic sites were detected, 66 of which were parsimony informative. Levels of genetic diversity among both species of water shrews (haplotype and nucleotide diversity) are shown in Table 3. Overall, levels of haplotype diversity were high for both species, although *N. fodiens* presented much lower values of nucleotide diversity than *N. anomalus*, suggesting a recent population expansion of the *N. fodiens* populations (Table 3). This result is well supported by the three neutrality tests, Tajima's D, Fu's F_S and Ramos-Onsins R_2 performed (Table 3). Such departures from neutrality were also confirmed by the R_2 test.

Table 3. Genetic diversity indices and neutrality tests applied to *N. anomalus* and *N. fodiens* based on *cyt-b* sequences (overall and by lineage).

		Genetic diversity				**Neutrality tests**		
		N	**N_H**	***H***	**π**	**D**	**F_S**	**R_2**
N. anomalus (Overall)		61	43	0.973 ± 0.010	0.04364 ± 0.00173	1,178	-0,042	0,1362
N. anomalus Lineages	Iberian	23	12	0.834 ± 0.069	0.00463 ± 0.00096	**-1,553**	-1,863	**0,071**
	Italian	8	6	0.893 ± 0.111	0.00610 ± 0.00132	-0,384	0,142	0,132
	Eastern	30	25	0.978 ± 0.011	0.01349 ± 0.00209	-0,959	-4,009	0,080
N. fodiens (Overall)		76	51	0.987 ± 0.004	0.00941 ± 0.00068	**-2,008**	**-27,582**	**0,039**

N – sample size, N_H – number of haplotypes, H – haplotype diversity ± SD, π – nucleotide diversity ± SD. D – after Tajima (1989), F_S – after Fu (1997), R_2 – after Ramos-Onsins and Rozas (2002). Statistically significant neutrality test result shown in bold ($p < 0.05$).

We have confirmed the low levels of genetic structure across the range of *N. fodiens* in contrast with a clear phylogeographic structure among the populations of *N. anomalus* (Fig. 2). Three well-supported lineages were found for *N. anomalus,* namely the Iberian, Italian and Eastern clades. The Iberian lineage was composed exclusively by samples from Portugal and Spain, from which 12 distinct haplotypes were identified with 31 variable positions and 13 parsimony informative sites. The *N. anomalus* sequences from Italy (Castiglia *et al.*, 2007) partially clustered together. We have confirmed the subdivision of these haplotypes according to their geographical origin, but only the sequences from northern and central Italy formed a well-supported clade. Two additional haplotypes from Switzerland were included in this lineage, clustering with the two northern Italian haplotypes. These two haplotypes were previously described by Ohdachi *et al*. 2006 but were not analysed by Castiglia *et al*. 2007. Overall, the Italian lineage comprised six haplotypes with 19 variable positions and 10 parsimony informative sites. Southern Italian haplotypes, originating from the Calabria region, did not cluster together with the remaining Italian sequences, creating a small distinctive sub-cluster, associated with a well-supported clade that comprised all other *N. anomalus* specimens, named as Eastern lineage. This extensive lineage showed the widest geographic range, including samples from Central and Eastern Europe (e.g. Poland, Ukraine and Russia) but also samples from the Balkan region (Greece). We found 25 haplotypes in this lineage with 84 variable positions from which 52 were parsimony informative.

Within the three *N. anomalus* lineages, the highest haplotype diversity was found in the Eastern lineage which also displayed the highest nucleotide diversity (Table 3). The Italian lineage presented a haplotype and nucleotide diversity higher than the Iberian lineage (Table 3). A population expansion signal was observed only for the Iberian lineage, revealed by a negative Tajima’s D and a significant R_2.

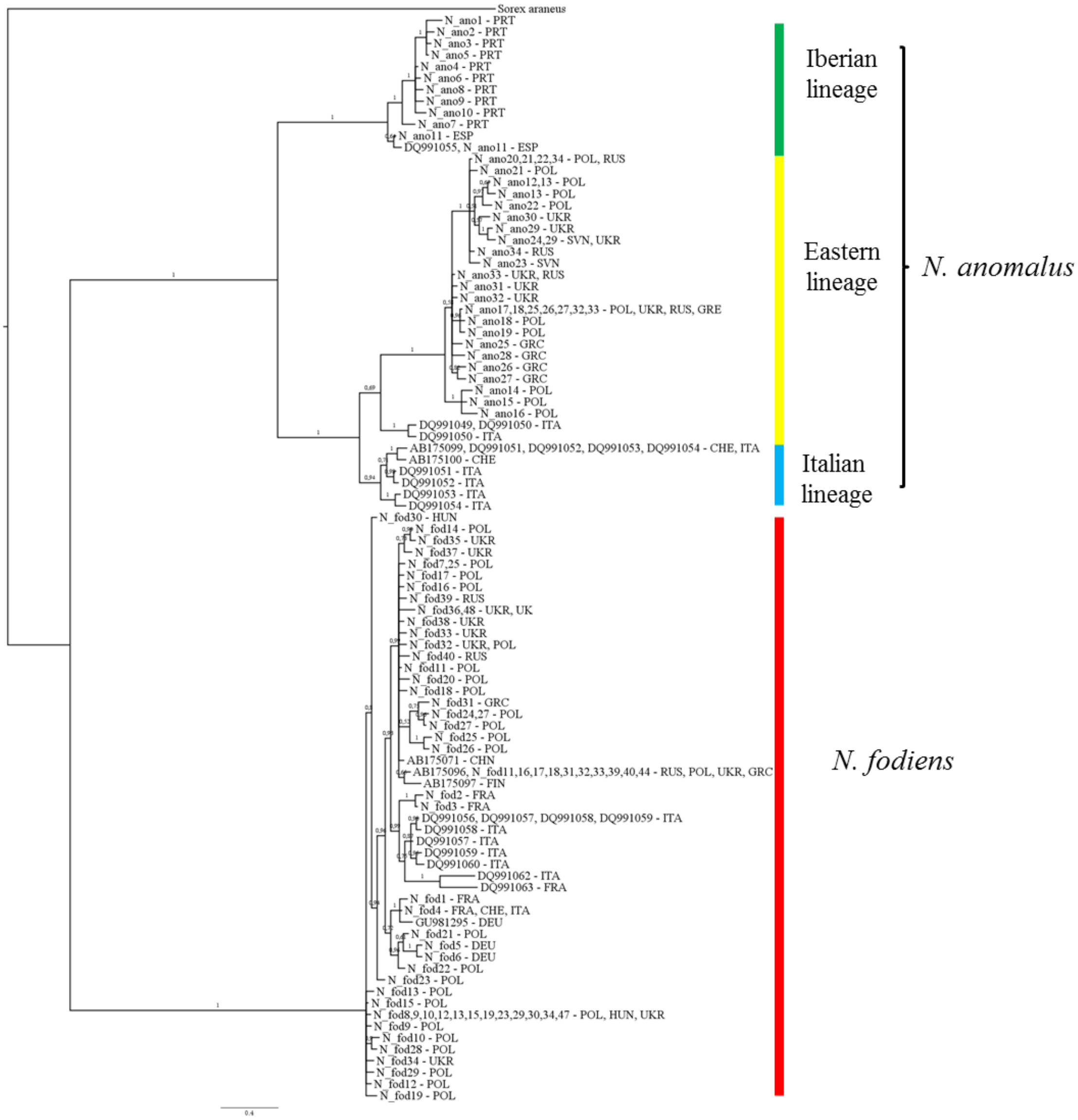

Figure 2. Bayesian 50% majority-rule phylogenetic tree of previously published *cyt-b* haplotypes (1113 bp long) of water shrews, *Neomys anomalus* and *N. fodiens*, in addition to our new sequences from across Eurasia. Three letter codes following haplotype labels correspond to all countries where certain haplotype was identified.

The Median-Joining network of *N. anomalus* (Fig. 3) showed a high differentiation between the Iberian and the Italian lineage (65 mutational steps). Within the Italian lineage, the northern and central populations appeared well separated from southern populations (12 mutational steps). Twenty four additional mutations separated these haplotypes from the remaining haplotypes belonging to the Eastern lineage. The Median-Joining network of *N. fodiens* confirmed the weak geographic structure already found in the phylogenetic tree (Fig. 2 and 4). No apparent geographical subdivisions were found, with most haplotypes being from different regions separated by only one mutational step. Overall, net divergence levels between *N. fodiens* and all the lineages of *N. anomalus* were high (15.8% – 16.5%, see Table 4). At the intraspecific level, the different lineages of *N. anomalus* showed a wider range of divergence among them (2.8% – 7.3%), with the Iberian lineage showing the highest divergence from all other lineages (see Table 4).

Table 4. Estimates of net divergence between *cyt-b* sequences (1113bp) from *N. anomalus* and *N. fodiens* using 10.000 bootstrap replicates (below diagonal). Standard error estimates are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980).

	N. fodiens	**Iberian *N. anomalus***	**Eastern *N. anomalus***	**Italian *N. anomalus***
N. fodiens	-	0.013	0.013	0.013
Iberian *N. anomalus*	0.162	-	0.008	0.008
Eastern *N. anomalus*	0.165	0.073	-	0.005
Italian *N. anomalus*	0.158	0.069	0.028	-

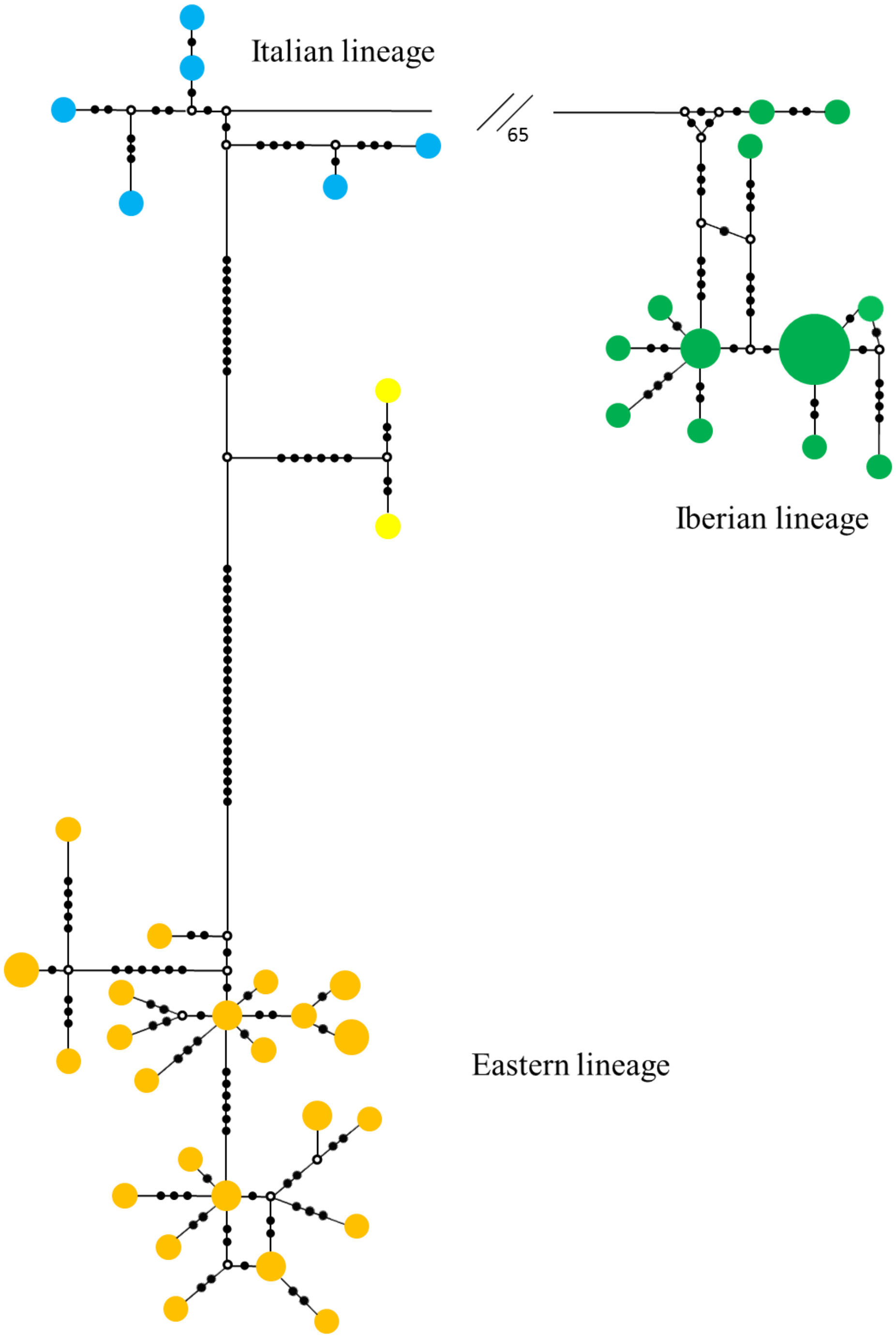

Figure 3. Median-joining network of the mitochondrial gene *Cyt-b* obtained for *Neomys anomalus* specimens across Eurasia. Circles represent unique haplotypes, with colours according to the mitochondrial lineages as determined by the phylogenetic analysis based on *cyt-b* sequences presented in Fig. 2. Circle area is proportional to the frequency of occurrence and solid dotted lines connecting haplotypes represent nucleotide differences between them. Open black circles represent hypothetical haplotypes.

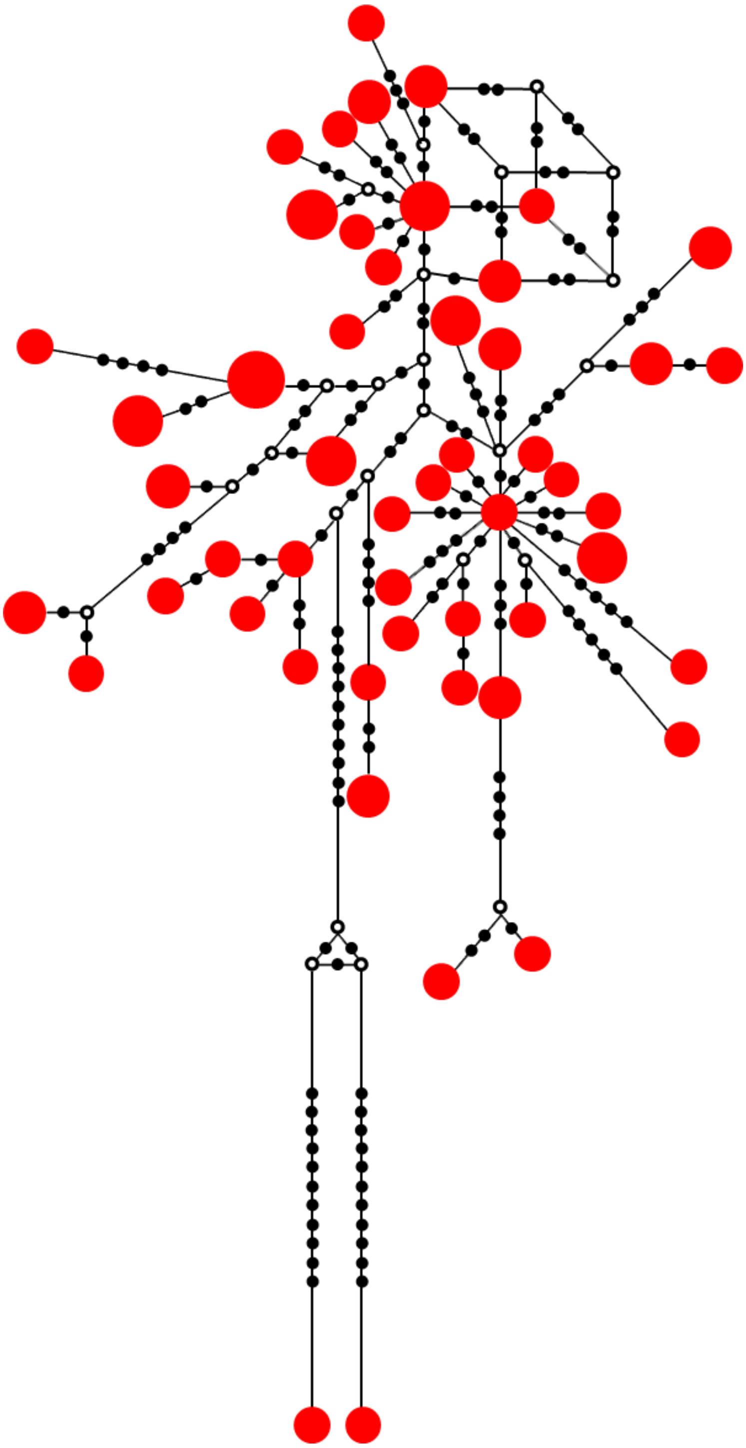

Figure 4. Median-joining network of the mitochondrial gene *Cyt-b* obtained for *Neomys fodiens* specimens across Eurasia. Circles represent unique haplotypes, with colors according to the mitochondrial lineages as determined by the phylogenetic analysis based on *cyt-b* sequences presented in Fig. 2. Circle area is proportional to the frequency of occurrence and solid dotted lines connecting haplotypes represent nucleotide differences between them. Open black circles represent hypothetical haplotypes.

Partial cyt-b (315 bp) analysis

The analysis of a shorter fragment of the mitochondrial *cyt-b* (315 bp) obtained from 14 museum specimens allowed us to increase the number of sequences of both species as well as to survey a wider geographic area (Fig. 1). In particular, it allowed important additional samples to be included in the analysis of *N. anomalus*, with an increment of sequences from northern and southern Spain (see Fig. 1). These clustered with all remaining samples from the Iberian Peninsula (Fig. 5), confirming the previously obtained well-supported Iberian clade based on longer *cyt-b* sequences (Fig. 2). The additional sequences from Slovenia, Macedonia and Turkey (Kryštufek *et al.*, 2000) clustered within the Eastern lineage (Fig. 5). The additional short fragment sequences obtained for *N. fodiens* have not rendered a higher phylogeographic differentiation among the populations of this species (Fig. 5).

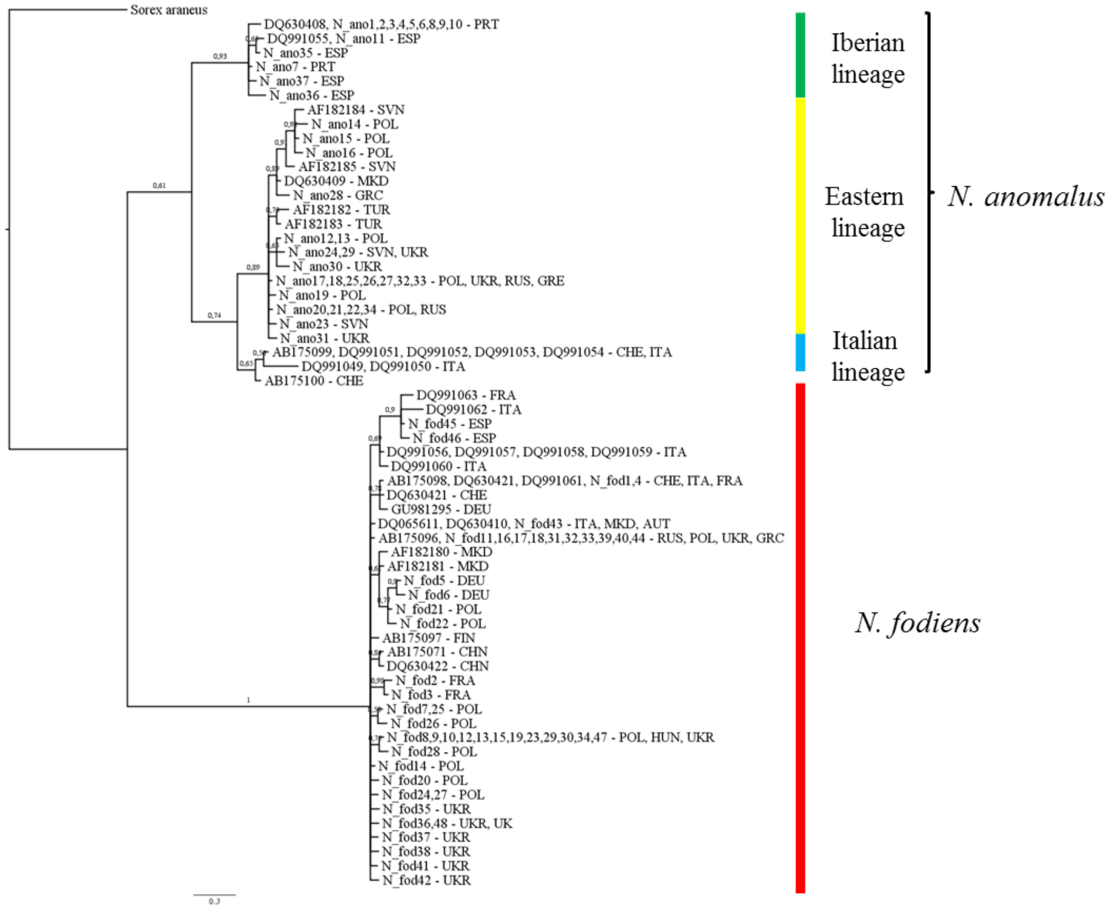

Figure 5. Bayesian 50% majority-rule phylogenetic tree of previously published *cyt-b* haplotypes (315 bp long) of water shrews, *Neomys anomalus* and *N. fodiens* in addition to our new sequences from across Eurasia. Three letter codes following haplotype labels correspond to all countries where certain haplotype was identified.

Discussion

Genetic structure of N. anomalus and N. fodiens

Our analyses clearly show that the two *Neomys* species have distinct genetic structures based on their mitochondrial DNA phylogeny. We have confirmed the findings of Castiglia *et al.* (2007) reporting that *N. anomalus* shows a greater genetic diversity than *N. fodiens*. The more extended coverage of our sample allowed an accurate identification of three main mitochondrial lineages in *N. anomalus*, in contrast with the phylogeographic pattern observed in *N. fodiens*, where no distinct lineages were detected. In *N. anomalus*, one lineage was exclusively constituted by Iberian sequences (from Portugal and Spain), including the highly divergent Spanish haplotype, A7, as highlighted by Castiglia *et al.* (2007). These results clearly indicate that the Iberian Peninsula have constituted an important glacial refugia for *N. anomalus*, although not contributing to the spread of the species through Europe and Asia. Instead, the Pyrenees seems to have acted as a geographic barrier, isolating the Iberian *N. anomalus* from the remaining lineages. Segregated Iberian genetic lineages have already been reported for other small mammal species such as the pigmy shrew, *Sorex minutus* (Mascheretti *et al*., 2003; McDevitt *et al.*, 2010) or the field vole, *Microtus agrestis* (Paupério *et al.*, 2012). The isolation of *S. minutus* in the Iberian Peninsula presents similar pattern to the one detected in our study for *N. anomalus*. The occurrence of such isolated genetic lineages supports the claim that the Iberian Peninsula is an area of endemism rather than a repository for northwards expansions after glacial periods (Bilton *et al.*, 1998; Baquero & Tellería, 2001). The case of *M. agrestis* is, however, an example in which the Iberian Peninsula can act in both ways. Initially it was postulated that central Europe had been recolonised by a southern lineage of field voles, which persisted during last glaciations in an Iberian refugium (Jaarola & Tegelstrom, 1995;

Jaarola & Searle, 2002). Recently, however, an extensive sampling in the Iberian Peninsula allowed the identification of two Iberian lineages, one of them strictly confined to Portugal, and the previously known Southern lineage in Spain (Paupério *et al.*, 2012).

Our results also seem to indicate an incipient subdivision between Portuguese and Spanish *N. anomalus* haplotypes (Fig. 2). However, such subdivision was only evident when using the long *cyt- b* fragment and is based on a more limited number of haplotypes. A pattern of refugia within refugia has already been found in the endemic Pyrenean desman, *Galemys pyrenaicus* (Igea *et al.*, 2013). A similar scenario is also possible for *N. anomalus* within the Iberian Peninsula and further efforts should be developed in order to clarify whether a phylogeographic structure within the Peninsula exists.

Similarly to the Pyrenees, the Alps could also have acted as a barrier between Italian and the Eastern *N. anomalus* lineage. However, the genetic differentiation between the Italian and the Eastern lineage is much smaller than between the Iberian lineage and the two other lineages. In fact, the subdivision of the Italian lineage is only statistically well supported for northern and central Italian haplotypes (Fig. 2). Contrary to Castiglia *et al*. (2007) findings, in our study southern Italian *N. anomalus* have clustered with the Eastern lineage (Fig. 2). A well supported mtDNA lineage including animals from Italy, Balkans and western Turkey has already been found for other small mammal species, such as the wood mouse, *Apodemus sylvaticus* (Michaux *et al.*, 2003). In fact, the widespread Eastern lineage included haplotypes from the Balkan region (Greece and Macedonia), Anatolia (Turkey), central Europe (Slovenia, Slovakia and Poland) and Eastern Europe (Ukraine and Russia). The geographic distribution of haplotypes composing this lineage is concordant with a northwards expansion from the

Anatolia region, as first hypothesized by Kryštufek *et al*. (2000). According to these authors, *N. anomalus* had presumably persisted through the Quaternary climatic oscillations in Anatolia where, by allopatric speciation, diverged into a third species of this genus, the Transcaucasian water shrew, *N. teres*. The northwards expansion of *N. anomalus* would have occurred later, during the Late Pleistocene, when a marine drawdown presumably created a land bridge allowing movements between Asia Minor and Europe (Kryštufek *et al.,* 2000).

Contrary to other red toothed shrews that were able to re-colonise all Europe from southern regions (e.g. Taberlet, 1998; McDevitt *et al*., 2010), the current northern limit of *N. anomalus* was only recently extended to the Baltic region with the identification of three specimens in Lithuania (Balčiauskas & Balčiauskienė, 2012). Such expansion of *N. anomalus* to northern latitudes was associated with the recent climate warming (Balčiauskas & Balčiauskienė, 2012). Intolerance to cold but also the competition with *N. fodiens* may have restricted the northern expansion of *N. anomalus*. In the case of the 'Iberian' lineage, the absence of *N. anomalus* from habitats at higher altitudes in the Pyrenees by the presence of *N. fodiens* (Torre & Tella, 1994) is probably the main factor isolating Iberian populations of *N. anomalus* from the rest of Europe. *Neomys fodiens* is not only more tolerant to lower temperatures but also dominant over *N. anomalus*, which is in turn excluded from optimal wet habitats (Rychlik, 2000). These two factors have probably contributed to the differences observed in the evolutionary history of the two species and the resulting current distribution patterns.

Neomys fodiens exhibited a markedly lower genetic structure than *N. anomalus* based on the mitochondrial DNA sequences, with individuals from different locations as far as Greece and Poland clustering together and individuals from the same locations (e.g. Ukraine) scattered through the *N. fodiens* branch of the tree (Fig. 2 and 5).

Moreover, the resulting haplotype network showed a star-like phylogeny, characteristic of a rapid and widespread postglacial expansion (Fig. 4). The same result was obtained from all neutrality tests, which also detect such population growth (Ramos-Onsins & Rozas, 2002).

Despite the significant increase in the number and geographic spread of the analysed specimens in comparison with Castiglia *et al.* (2007), the most divergent haplotypes found in *N. fodiens* were still the two individuals from Calabria (Italy) and the Pyrenees (France) already described by Castiglia *et al.* (2007). However, we were able to extend the distribution of these divergent haplotypes to the Iberian Peninsula since two northwestern Spanish individuals from Asturias (Spain) have clustered together with these Italian and French haplotypes in the phylogenetic tree obtained with the short fragment (315 bp) *cyt-b* sequences (Fig. 5). According to Castiglia *et al.* (2007) the divergence time between these haplotypes is 1.0 MY, which suggests a first colonisation of Europe by *N. fodiens* in the early Pleistocene. However, the lack of a clear phylogeographic structure and the star-like phylogeny found with samples ranging from the Iberian Peninsula to China, suggests that after Quaternary glaciations *N. fodiens* rapidly colonised most of Eurasia and likely from multiple northern and central refugia, as postulated for other small mammal species (Deffontaine *et al.*, 2005; Kotlík *et al.* 2006; Vega *et al.* 2010). Also, the distinctive sub-group of two haplotypes comprising *N. anomalus* specimens from Calabria (South Italy) was generated within the *anomalus* branch of the phylogenetic tree. Despite poorly supported, this branching is coherent with the phylogeographic patterns observed for other previously studied taxonomic groups, possibly indicating the existence of different refugia within the Italian Peninsula during the Last Glacial Maximum (Vega *et al.*, 2010 and references therein).

The ecological similarities between *N. anomalus* and *N. fodiens* could preclude a congruent phylogeographic structure between the two species. In fact, among the twenty shrew species occurring in Europe, shrews belonging to the genus *Neomys* are the only ones showing a semi-aquatic life-style. However, based on the molecular data here presented it seems clear that *N. anomalus* and *N. fodiens* have a distinct evolutionary history. *N. anomalus* appears to have been confined to southern refugia during the last glaciations, while *N. fodiens* probably persisted in multiple refugia in central and northern Europe. Several hypotheses have been suggested to explain the phylogeographic discrepancies found between closely related species, including i) evolutionary aspects such as the geographical origin of the species; ii) demographic causes, changes in population size of the remained population after glacial periods; or iii) physiological factors, such as thermal tolerance (e.g. Sipe & Browne, 2004; Berggren *et al.*, 2005; Michaux *et al.,* 2005). In the case of water shrews, Castiglia *et al*. (2007), based on the current distribution of both species, emphasized the ability of *N. fodiens* to inhabit colder areas than *N. anomalus,* particularly at high-altitudes. Recent findings showed that the interspecific competition between these two species could also be an important factor determining their distribution pattern (Tapisso *et al.* submitted). In fact, under the absence of *N. fodiens*, *N. anomalus* can occur at higher altitudes and in colder areas (Tapisso *et al.* submitted). We can thus assume that the faster re-colonisation of high altitude habitats by *N. fodiens,* particularly at the Pyrenees and the Alps, was probably one of the main factors contributing to the isolation of *N. anomalus* in the Iberian and Italian Peninsulas, rather than cold intolerance by the later species.

Taxonomic status of N. anomalus in the Iberian Peninsula

One of the most striking results of the investigation performed by Castiglia *et al*. (2007) on the phylogeography of water shrews in Europe was the uncover of a single and highly divergent *N. anomalus* haplotype in Spain, corresponding to a genetic distance of 9% between this sequence and the remaining *anomalus* lineages. This level of genetic differentiation was so high that the authors questioned the taxonomic status of the *N. anomalus* in the Iberian Peninsula, even based on a single haplotype originated from a single specimen. By increasing the number and geographic coverage of *N. anomalus* samples from both Portugal and Spain, we were able to confirm the presence of the highly divergent haplotype found by Castiglia *et al*. (2007). The genetic distance between the lineage constituted by Iberian samples and the remaining *N. anomalus* is around 7%, a value close to the findings of Castiglia *et al.* (2007) but much higher than the average intraspecific distance found by Fumagalli *et al.* (1999) within European Soricidae shrews (0 to 5.21 ± 0.75%).

Only few studies have investigated intraspecific genetic distances among shrews. One of those studies was carried out by O'Neill *et al*. (2005) on the phylogenetic relationship between North American water shrews, *Sorex palustris* and *S. bendirii*. The authors found a *cyt- b* lineage within *S. palustris* with a higher genetic distance than the interspecific distance between *S. palustris* and *S. bendirii*. As such, based on a 6.9% divergence, the authors proposed that *S. palustris* might in fact comprehend two species, *S. palustris* and *S. navigator*. In Europe, data on genetic distances at the intraspecific level have been published for *Crocidura russula* (Vogel *et al*., 2003) and for *C. suaveolens* (Dubey *et al.*, 2006). In the first case, a mean genetic divergence of 8.5% was found between North African western and eastern lineages, supporting a similar taxonomical subdivision (Vogel *et al.*, 2003; Cosson *et al.*, 2005). Genetic distances ranging from 4.7 to 10.8% were found within the *C. suaveolens* group, leading to the revision of the taxonomical status of the species and its recognition as a different subspecies (e.g. *C. s. caspica*).

In the light of the results from studies of Soricinae and Crocidurinae shrews, the high genetic divergence of Iberian *N. anomalus* here reported, at the mitochondrial *cyt-b*, supports the need for a deeper understanding of these populations and potentially a taxonomic revision. In fact, the taxonomic status of *N. anomalus* in the Iberian Peninsula has already been questioned due to morphological discrepancies, in particular craniometrical size differences. Corbet (1978) described two subspecies, the bigger sized *N. anomalus anomalus* Cabrera, 1907, occurring exclusively in the Iberian Peninsula and the smaller sized *N. a. milleri* Mottaz, 1907, occurring in the rest of the distribution range. However, such infra-specific categories were later questioned by Ramalhinho (1988), since the craniometrical differences between *N. a. anomalus* and *N. a. milleri* were highly dependent on the geographical location of the central and northern populations. Later studies have demonstrated that significant craniometrical differences can also be found between different *N. anomalus* populations in areas outside the Iberian Peninsula. These morphological variations seem to be highly dependent on ecological factors, namely the presence of *N. fodiens*. The size of *N. anomalus* increases in areas where the dominant and bigger sized *N. fodiens* is absent. Such character release has been found in several locations of central and eastern Europe (Rácz & Demeter, 1998; Popov & Zidarova, 2008; Kryštufek & Quadracci 2008) but also within the Iberian Peninsula (López-Fuster *et al.*, 1992). Further efforts need to be done in order to discriminate between morphological differences induced by phenotypic plasticity, from ecological changes including morphological, physiological and behavioural changes that can be enhanced by a significant genetic differentiation highly indicative of speciation. Additionally, besides the multilocus genetic component, future studies incorporating different aspects of these species’ biology namely, a geometric morphometric approach for the skull morphology, behavioural and reproductive tests involving different *N. anomalus* lineages, would surely help to unravel the taxonomic status of the Iberian populations.

Conclusions

The results generated by this study represent a key advance in the knowledge of the phylogeographic patterns of both *N. anomalus* and *N. fodiens*. At the same time, the high level of genetic divergence detected in the Iberian populations of *N. anomalus* at the mitochondrial level reflect the need of conducting further studies to elucidate their taxonomic status and to better understand their evolutionary history. Future studies would largely benefit from a multilocus approach (sex-linked and autosomal loci), expanding from the marker already used in this survey. Also, extending the sampling within the Iberian Peninsula seems mandatory in order to clarify the geographic limits of this highly divergent lineage. Finally, a geometric morphometric approach, associated with the study of skeletal structures, could also clarify whether the observed mitochondrial divergence among *N. anomalus* translates into morphologic divergence of the distinct lineages.

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CHAPTER 3

HABITAT USE OF THE MEDITERRANEAN WATER SHREW

Determinants of habitat use by the Mediterranean water shrew (*Neomys anomalus*) under the absence of the European water shrew (*N. fodiens*)

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Abstract

The Mediterranean water shrew, *Neomys anomalus* Cabrera, 1907, can use both terrestrial and aquatic environments, although the use of aquatic habitats is usually limited by the presence of a dominant competitor, the Eurasian water shrew, *Neomys fodiens* (Pennant, 1771). Information regarding ecological factors that regulate the access to water by *N. anomalus* under the absence of *N. fodiens* is still scarce. Here we investigated the abundance, habitat preferences and spatial movements of a *N. anomalus* population in central Portugal, an area outside the range of *N. fodiens*. We expected a competitive release, with *N. anomalus* occurring in aquatic habitats typically selected by *N. fodiens*. Five trapping sessions were performed between October 2007 and January 2009. Shrews were monitored in a semi-fixed trapping grid covering habitats differing in the wetness degree (river bank; meadow; ecotone and pine forest). *N. anomalus* occurred exclusively in the river bank whereas other syntopic shrew species occurred mainly in drier habitats. The abundance and movements of *N. anomalus* were determined exclusively by abiotic conditions related with the morphology of the river. These results confirm the expansion of the ecological niche by allopatric *N. anomalus* towards aquatic habitats more typical of *N. fodiens*.

Keywords

Mediterranean water shrew; *Neomys anomalus*; Eurasian water shrew; *Neomys fodiens*; habitat use; competition.

Introduction

Many mammals live in close association with water being categorized as semiaquatic (Fish, 2000) in comparison with only a few that are strictly aquatic (Veron, Patterson and Reeves, 2008). The degree of aquatic use by semiaquatic species depends on specific anatomical and physiological adaptations (Fish & Baudinette, 1999) and is mainly regulated by seasonal changes that alter the thermal maintenance cost and predation risk in water (e.g. Mclachlan-Troup *et al.*, 2010). Intersexual competition may also induce significant differences in the aquatic activity, with males and females displaying a distinct use of aquatic habitats (e.g. Zabala *et al.*, 2007). Differences between populations have also been detected as a result of interspecific competition. In the presence of dominant species, sub-ordinate competitors are frequently driven to terrestrial habitats (e.g. Ulevičius & Balčiauskas, 2007).

The Mediterranean water shrew, *Neomys anomalus* Cabrera, 1907, is a semiaquatic species with a patchy but wide distribution along central and southern Europe (IUCN, 1995; Fig. 1). Its morphological adaptations to a semi-aquatic life-style include a dense waterproof pelage, fringes of hairs on the hind feet and a keel of stiff hairs on the last third of the tail (Pucek, 1981; Churchfield, 1998). Altogether these adaptations increase swimming and diving efficiency by providing insulation, expanding the surface area of the feet and promoting stability in water (Pucek, 1981; Churchfield, 1998). Such morphological features are more prominent in a close-related species, the Eurasian water shrew, *Neomys fodiens* (Pennant, 1771) (Pucek, 1981; Churchfield, 1998). This species, known to display a higher swimming efficiency and underwater foraging ability (Rychlik, 1997; Mendes-Soares & Rychlik, 2009), has a continuous distribution throughout most of Europe and in many locations is syntopic with *N. anomalus* (IUCN, 1995; Fig. 1).

In central Europe the use of aquatic environments by *N. anomalus* is limited by the presence of *N. fodiens*. For instance, Spitzenberger (1990) verified in Austria that the dominant *N. fodiens* colonize a larger range of limnic habitats segregating *N. anomalus* to terrestrial areas. More recently, microhabitat analyses in Białowieża Forest (Poland) confirmed such pattern, with *N. fodiens* occurring strictly near watercourses and *N. anomalus* occurring mainly at some distance from streams (Rychlik, 2000). However, in southern Europe the influence of *N. fodiens* upon the use of aquatic habitats by *N. anomalus* is not as clear as in central Europe, particularly in the Iberian Peninsula. Investigations in the Pyrenees confirmed that *N. anomalus* occurs in meadows far from streams or rivers occupied by *N. fodiens* (Torre & Tella, 1994). But in other northern regions of the Iberian Peninsula, *N. anomalus* was found almost exclusively in riverbanks even when *N. fodiens* was present (Agirre-Mendi, 2004 a; b). Contradictory findings on the use of aquatic habitats by *N. anomalus* have also been reported for central and southern regions of the Iberian Peninsula, where *N. fodiens* is absent. In southwest Spain (Amores, 1975), the species was found inhabiting dry and dense pine forests while in central Portugal (Ramalhinho, 1988; 1995) *N. anomalus* has been found occupying wet habitats, but at considerable distance from watercourses. However, in a recent study in northern and central Portugal, the species was only detected at the edge of shallow and fast flowing waters (Rychlik & Ramalhinho, 2005).

Considering the above apparently disparate findings, the purpose of the present study was to identify ecological factors that may regulate the use of aquatic habitats by *N. anomalus* from southern populations, i.e. under the absence of *N. fodiens*. We hypothesize that in such populations a competitive release may occur. As such, the habitat preferences and the spatial movements of *N. anomalus* can be influenced only by seasonal-based variation of biophysical properties of watercourses, and/or by biotic interactions as intersexual or interspecific competition with shrew species other than *N. fodiens*. We selected a population inhabiting central Portugal firstly because this is an area outside the range of *N. fodiens*, and secondly because previous studies in this country achieved the most contradictory results (Ramalhinho, 1988; 1995; Rychlik & Ramalhinho, 2005). In addition, the study of *N. anomalus* populations in the Iberian Peninsula is utmost relevant because these populations are genetically divergent from other European populations (Castiglia *et al.*, 2007). As such, insights from ecological data highlighting the factors involved in the occurrence of these populations, besides improving the knowledge on the semiaquatic behaviour of *N. anomalus*, may also bear relevant implications for the conservation of a distinct evolutionary unit.

Material and Methods

Study area

The study was performed at Serra da Estrela Natural Park, Portugal (Fig. 1). Serra da Estrela is located in the western side of the Iberian Central Cordillera, a mountain range that crosses the center of the Iberian Peninsula with a maximum elevation of 1993 meters above sea level. The study plot (approximately 70 x 45 m) was set at Covão da Ponte, Manteigas (40°26'34.68''N; 7°30'43.86''W, elevation 950 m), along a straight section of the Mondego river. In this section, both slopes of the riverbank had a smooth inclination, and the riverbed was composed by medium size gravel, sand and few emerged stones.

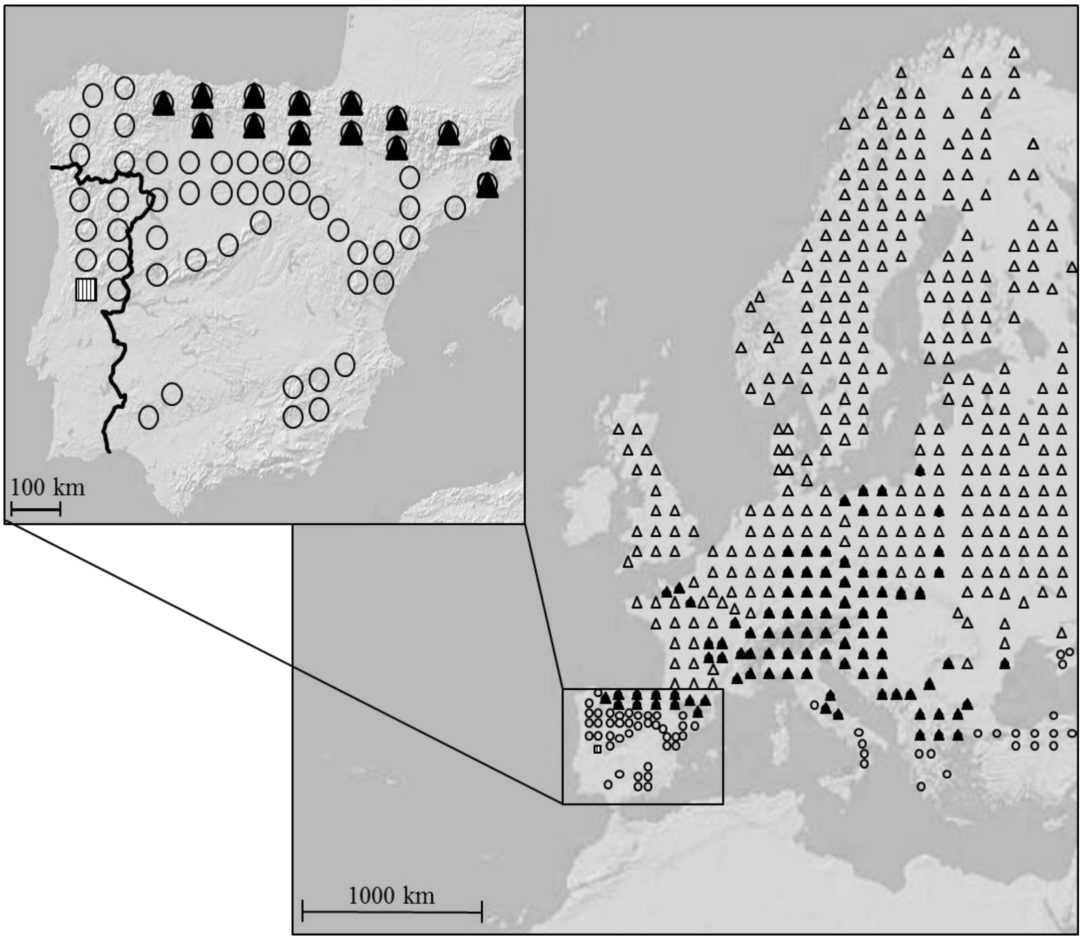

Figure 1. European distribution of the Mediterranean water shrew, *Neomys anomalus* and the Eurasian water shrew, *Neomys fodiens* (according to IUCN, 1995). Empty circle = allopatric populations of *N. anomalus*; empty triangle = allopatric populations of *N. fodiens*; black overlapping triangle and circle = sympatric populations of *N. anomalus* and *N. fodiens*. Outer box highlights the Iberian Peninsula showing the exact location of the study area (crossed bar square).

Trapping design

A total of five trapping sessions were performed seasonally between October 2007 and January 2009 (October 2007; January, May and September 2008; January 2009). In each trapping session, 75 traps were distributed in five parallel lines ca. 75 m long (15 traps per line were set at a distance of ca. 5 m from each other). Two lines were located on the left side of the river and three lines on the right side. The location of the trapping lines attempted to cover the main habitats in the study plot. As such, in the left side of the river, the first line of traps was located on the river bank, mainly characterized by sedges of *Carex gracilis*, bushes of *Erica arborea* and *Cytisus sp*. and the second line was set in a meadow area dominated by several *Juncus* species. In the right side of the river the first line of traps was placed on the river bank, with a vegetation cover similar to the left bank. The second line was set in an ecotone area composed by a narrow strip of pteridophytes. A third line of traps were set on the undergrowth cover of the pine forest, dominated by *Rubus sp*. Trapping points were kept constant in all but the two lines set along river banks. In these two lines, trapping points varied between sessions according to the river’s width as traps in these lines were always set at less than one meter from the water. Thus, distance between outer lines and river bank lines varied from 15 to 30 m; distance between river bank lines corresponded to the river width in each trapping session. In each session, wooden box live-traps (dziekanówka-trap, PPUH A. Marcinkiewicz, Poland) provided with cotton as bedding material and fly pupae as bait were open late afternoon, just before dusk, and were checked four times, every three hours. Trapping effort was kept constant at 4500 trap-hours in each session. Animals were released in the place of capture immediately after species, sex and body mass were recorded. All animals were marked by fur clipping.

Permission to capture and manipulate the animals was obtained from the Institute of Nature Conservation and Forests, Lisbon, Portugal (license number ICNB 132/2008/CAPT).

Environmental analysis

Air temperature and humidity were recorded in every trapping check using a portable weather station positioned on the ground near the first trap of the ecotone line (Oregon Scientific BAR638HG, Oregon, USA). River width was measured at three transects spaced along the river by 15 m. Depth and water flow were measured at each transect (FP101 Global Water Flow Probe, Gold River, CA, USA).

Data analysis

Habitat preferences were evaluated using the Ivlev's electivity index (Ivlev, 1961) which was derived by the formula IV_i = (use of habitat *i* – availability of habitat *i*)/(use of habitat *i* + availability of habitat *i*), where IV_i is the preference for the habitat *i*. Values of IV range from -1 (complete avoidance) to +1 (complete preference), with 0 indicating no active selection. Habitat overlaps were calculated using the Pianka index in the statistical software EcoSim 7.72 (Pianka, 1973; Gotelli & Entsminger, 2009). Pianka’s index ranges from 0 to 1, with 0 indicating the use of a habitat by a single species and 1 indicating complete habitat overlap between two or more species. Distances covered between successive recaptures were calculated for all individuals recaptured in each trapping session, considering the minimum distance between trapping points. Pearson correlation coefficients were calculated to test the dependence of the number of captures between species and environmental parameters. One-way ANOVA followed by Tukey’s test was used to compare significant levels of preference for the different habitats. Non-parametric statistics (Kruskall-Wallis followed by Mann-Whitney test) were used to test for differences in individual movements, since distances were not normally distributed. All statistical tests were performed using IBM SPSS Statistics 19 (SPSS, 2010), considering a minimum *P* value of 5%.

Results

Habitat use of Neomys anomalus

We have obtained a total of 42 captures of *Neomys anomalus*. Most of the water shrews were caught during the trapping sessions in October 2007 and September 2008 (Table 1). In the remaining periods this species was rare or even absent from the study area. There were no significant differences in the mean body mass of water shrews captured in different trapping periods (Table 1). All water shrews were caught along the river banks. *Neomys anomalus* showed an exclusive preference for this kind of habitat regardless the abundance of the species, season or sex (Table 2). All remaining habitats were significantly avoided by this species (F = 2.77, $P = 0.000$; Table 2). As such, the habitat overlap between males and females was usually high (Table 3). Water shrews' movements were characterized by long distances along the same side of the river bank (Table 4) but some individuals were also successively recaptured on both sides of the river (Fig. 2). Neither season nor sex-dependent differences were found in the direction and distance of water shrews' movements.

Table 1. Number of animals captured and mean body mass (g) of three species of shrews trapped in consecutive trapping sessions at Covão da Ponte study site (S.D. – standard deviation; i.d. – insufficient data).

Species		**October 2007**	**January 2008**	**May 2008**	**September 2008**	**January 2009**
Neomys anomalus	Nº of individuals (males × females)	2 × 4	0	1 × 0	4 × 5	0 × 2
	Nº of recaptures	6	0	0	17	1
	Mean body mass ± S.D.	10.78 ± 0.71	i.d.	10	9.05 ± 1.33	11.33 ± 0.62
Sorex granarius	Nº of captures (males × females)	1 × 1	1 × 0	0	1 × 1	3 × 1
	Nº of recaptures	0	1	0	0	7
	Mean body mass ± S.D.	7.25 ± 1.25	6.45 ± 0.05	i.d.	6.00 ± 0.50	6.05 ± 0.42
Crocidura russula	Nº of captures (males × females)	3 × 5	6 × 6	2 × 2	2 × 6	1 × 7
	Nº of recaptures	13	25	12	15	9
	Mean body mass ± S.D.	7.30 ± 0.65	8.44 ± 0.71	7.77 ± 0.48	7.80 ± 1.06	8.47 ± 1.61

Table 2. Habitat preferences (expressed by the Ivlev's electivity index) of three species of shrews in consecutive trapping sessions at Covão da Ponte study site (i.d. – insufficient data).

Species		**October 2007**	**January 2008**	**May 2008**	**September 2008**	**January 2009**
Neomys anomalus	Meadow	-1	i.d.	-1	-1	-1
	Left river bank	0.43	i.d.	0.67	0.49	0.54
	Right river bank	0.43	i.d.	-1	0.36	0.25
	Ecotone	-1	i.d.	-1	-1	-1
	Pine forest	-1	i.d.	-1	-1	-1
Sorex granarius	Meadow	-1	-1	i.d.	-1	-1
	Left river bank	-1	-1	i.d.	-1	-0.05
	Right river bank	0.43	0.67	i.d.	0.43	0.15
	Ecotone	-1	-1	i.d.	0.43	0.29
	Pine forest	0.43	-1	i.d.	-1	-0.05
Crocidura russula	Meadow	-0.17	0.2	-1	-0.64	-1
	Left river bank	-1	0.04	-1	-1	-1
	Right river bank	-0.17	-0.03	0.65	0.32	0.4
	Ecotone	0.45	-0.03	-0.52	0.48	0.35
	Pine forest	-0.02	-0.3	-1	-1	0.26

Table 3. Interspecific and intersexual habitat overlaps in capture space distribution of the three species of shrews in consecutive trapping sessions at Covão da Ponte study site (i.d. – insufficient data).

Species			**October 2007**	**January 2008**	**May 2008**	**September 2008**	**January 2009**
Neomys anomalus	×	*Sorex granarius*	0.50	i.d.	i.d.	0.34	0.55
Neomys anomalus	×	*Crocidura russula*	0.17	i.d.	0	0.34	0.33
Sorex granarius	×	*Crocidura russula*	0.40	0.40	i.d.	0.80	0.90
	Neomys anomalus						
Male	×	Female	0.89	i.d.	i.d.	0.96	i.d.
	Sorex granarius						
Male	×	Female	0	i.d.	i.d.	0	0.10
	Crocidura russula						
Male	×	Female	0.36	0.74	0.98	0.99	0.69

Table 4. Mean (± S.D.) distance of movements (MDM; in m) between successive recaptures of the three species of shrews.

	Total number of recaptures	**Total MDM**	**N**	**MDM within habitat**	**N**	**MDM between habitats**
Neomys anomalus	22	30.67 ± 5.25	14	28.00 ± 8.76	8	34.00 ± 4.96*
Sorex granarius	7	19.71 ± 3.73	4	18.00 ± 3.91	3	27.67 ± 2.33
Crocidura russula	76	12.23 ± 4.69	63	5.84 ± 2.11	13	49.37 ± 25.04

*considering both sides of the river as independent habitats

Biotic interactions between N. anomalus and other shrews

Two species of shrews co-occurred with *N. anomalus*, the Iberian shrew, *Sorex granarius* (17 captures) and the Greater white-toothed shrew, *Crocidura russula* (114 captures; Table 1). A negative correlation was found between the number of captures of *N. anomalus* and *S. granarius* (r = -0.283, P = 0.038). Since *S. granarius* was always relatively rare in the study area (Table 1), such negative correlation is mainly induced by the occurrence of *N. anomalus* affecting the trapping success of *S. granarius* rather than the reverse. The influence of *N. anomalus* in the trapping success of *S. granarius* was enhanced by the similarities in the habitat preferences of these two species. Although not occurring exclusively in river banks, *S. granarius* also showed a consistent preference for this habitat (Table 2), resulting in a relatively high habitat overlap with *N. anomalus* (Table 3). Similarly to *N. anomalus*, significant differences were also found in the habitat preferences of *S. granarius* (F = 3.404, P = 0.029), with pairwise comparisons revealing differences between the preference for river banks and the avoidance of the meadow area (Tukey test: P = 0.016). The movements of *S. granarius* were about 10 m shorter than the movements of *N. anomalus* (Table 4), though this difference was statistically insignificant. *Sorex granarius* was only successive recaptured along the same side of the river bank or recaptured in adjacent habitats such as right side of the river bank and ecotone or ecotone and pine forest (Fig. 2).

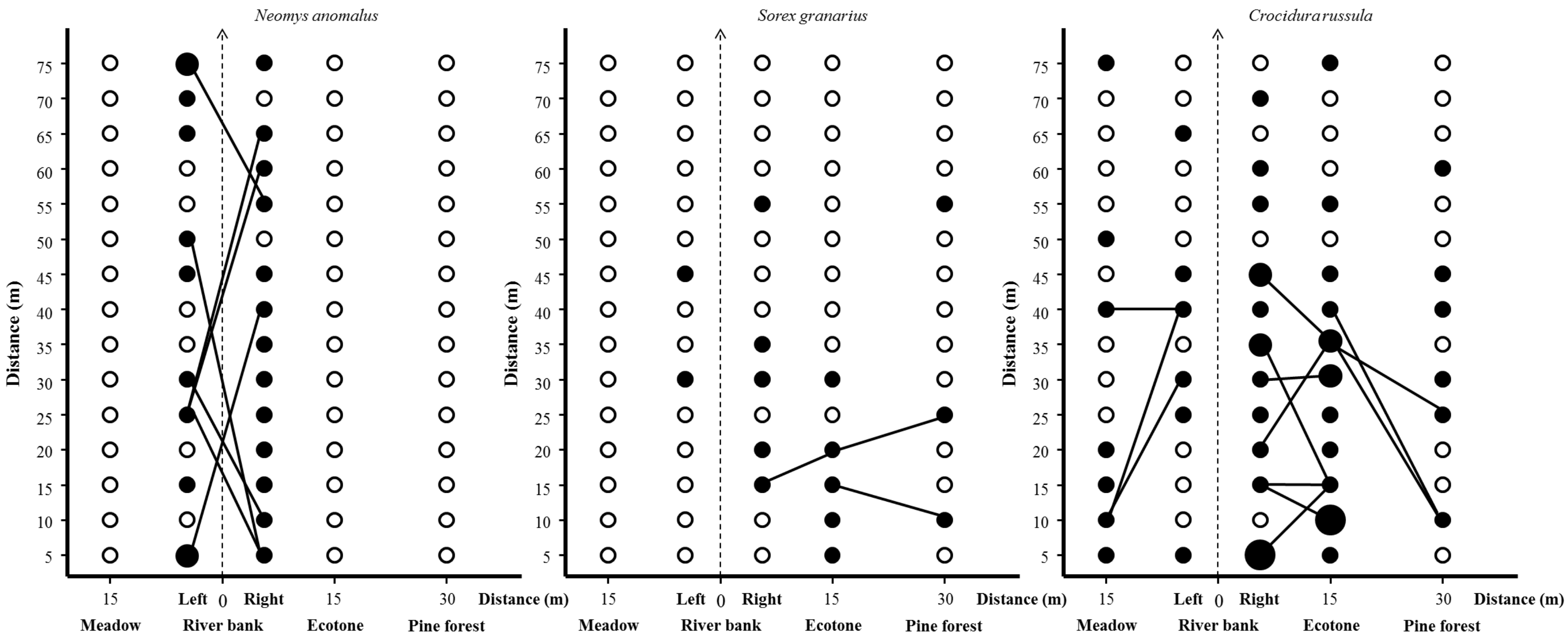

Figure 2. Distribution of captures along five trapping lines at Covão da Ponte study site. Trapping points are represented by circles. Empty circle = 0 captures; small black circles = 1 to 4 captures; medium black circles = 5 to 8 captures; big black circles = 9 to 12 captures. Successive recaptures are represented by solid lines connecting the two trapping points. Dashed line represents the center of the riverbed.

Crocidura russula was frequently captured in the study area but such high number of captures was not correlated with the trapping success of *N. anomalus* (Table 1). Although also selecting river banks as one of the preferred habitats, *C. russula* occurred in all available habitats. The habitat preferences of this species were variable but, in general, riverbanks and the ecotone area were the preferred habitats while peripheral habitats such as meadow and pine forest were avoided (Table 2). The variations in the habitat preferences of *C. russula* were statistical significant (F = 4.08, *P* = 0.008), with pairwise comparisons revealing a significant difference between the avoidance of the meadow and the preference for the ecotone (Tukey test: *P* = 0.024). The habitat overlap between *N. anomalus* and *C. russula* was smaller in most trapping periods than the overlap observed between *N. anomalus* and *S. granarius* (Table 3). However, considering the habitat overlap between males and females of each shrew species, more similarities were found between *N. anomalus* and *C. russula* than considering *N. anomalus* and *S. granarius*, even taking into account the small number of captures of this later species (Table 3). Despite the dispersion of *C. russula* in the study area, the mean distance moved between successive recaptures was significantly shorter than that observed for both *N. anomalus* and *S. granarius* (Z = -3.50, *P* = 0.000 and Z = -2.33, *P* = 0.020, respectively; Table 4). *C. russula* moved most frequently short distances within the same habitat (82.89% of the movements), with the remaining movements occurring mostly between the river bank and the ecotone (Fig. 2).

Abiotic parameters

The environmental parameters analysed at the study site varied significantly between trapping periods (Table 5). *N. anomalus* was clearly the species most influenced by such environmental variations. The number of captures of *N. anomalus* was positively correlated with air temperature (r = 0.725, P = 0.0001), but negatively correlated with all parameters related with river morphology: river width (r = -0.690, P = 0.003), river depth (r = -641, P = 0.003), and water flow (r = -0.605, P = 0.017). Temperature and humidity affected negatively the number of captures of *S. granarius* (temperature r = -0.751, P = 0.0001; humidity r = -0.335, P = 0.013), whereas the trapping success of *C. russula* was not influenced by changes in any of the environmental parameters considered.

Table 5. Environmental measurements (mean ± SD) during consecutive trapping sessions at Covão da Ponte study site.

	October 2007	**January 2008**	**May 2008**	**September 2008**	**January 2009**
Air temperature (ºC)	8.95 ± 2.44	2.54 ± 3.99	6.31 ± 1.52	13.66 ± 2.18	-4.65 ± 2.83
Humidity (%)	85.5 ± 8.70	93.82 ± 4.84	95.29 ± 2.60	87.08 ± 13.33	83.20 ± 8.64
River width (m)	2.58 ± 0.21	5.43 ± 1.57	7.40 ± 0.22	2.97 ± 0.58	6.66 ± 0.49
River depth (cm)	18.50 ± 9.30	51.75 ± 12.34	45.25 ± 10.35	17.13 ± 3.64	27.33 ± 8.22
Water flow (m/s)	0.29 ± 0.01	1.32 ± 0.17	3.20 ± 0.22	0.16 ± 0.01	0.65 ± 0.06

Discussion

In the present study the occurrence of *N. anomalus* was restricted to the riverbanks and its abundance was influenced by seasonal changes affecting this microhabitat rather than by the occurrence of other shrew species. No signs of intersexual competition were detected and the spatial distribution of *N. anomalus* supports the earlier predictions about its gregarious social system (Krushinska & Rychlik, 1993; Rychlik, 1998; 1999).

Habitat use of shrews

Neomys anomalus used exclusively riverbanks, but the riverbanks were not exclusively used by this species. *S. granarius* and *C. russula* also occurred in this habitat but showed different degrees of habitat overlap with *N. anomalus*. The overlap between *N. anomalus* and *C. russula* was relatively small because *C. russula* occurred mainly in the riverbank during periods of low abundance of *N. anomalus*. When both species were relatively abundant, *C. russula* occurred mainly in drier habitats (meadow and pine forest). Such habitat segregation between *N. anomalus* and *C. russula* had already been described in other syntopic populations of these two species (Rychlik & Ramalhinho, 2005). High levels of habitat overlap were observed between *N. anomalus* and *S. granarius*, due the preference of the later species for transitional wet to dry habitats. However, these two species differ in the way they use wet habitats since, unlike *N. anomalus*, *S. granarius* has no morphological adaptation to a semiaquatic life style (Madureira & Ramalhinho, 1981; Mathias, 1999).

High levels of habitat overlap have been described in different shrew communities (e.g. Feldhamer *et al.*, 1993; Rychlik, 2000), indicating that other mechanisms besides habitat segregation may also be important in reducing interspecific competition. For example, trophic segregation is also considered an important mechanism allowing the coexistence of multiple shrew species (e.g. Churchfield, 1984a; Churchfield & Sheftel, 1994; Churchfield *et al.*, 1999). Niche partitioning involving trophic resources is particularly evident between semiaquatic and terrestrial shrews, since only species like water shrews include aquatic prey in their diet (Churchfield, 1984a; Cástien & Gosálbez, 1999; Churchfield & Rychlik, 2006). In turn, both habitat and trophic segregation have been reported between water shrew species. In the presence of the dominant *N. fodiens*, *N. anomalus* occurs at some distance from watercourses and includes a comparatively lower proportion of aquatic prey in their diet (Niethammer, 1978; Churchfield, 1984a; DuPasquier & Cantoni, 1992; Churchfield & Rychlik, 2006). However, as here observed, *N. anomalus* can occur at small distances from water under the absence of *N. fodiens*, which may also lead to higher predation on aquatic invertebrates. Such hypothesis has already been proposed to explain the increase in body size of allopatric *N. anomalus* towards the size of *N. fodiens* (Kryštufek & Quadracci, 2008). Recent behavioral experiments also suggested a higher use of aquatic foraging by allopatric *N. anomalus*, based on their efficiency in underwater foraging as well as a more effective swimming and diving performance than sympatric *N. anomalus* (Tapisso *et al.*, 2013).

Intersexual competition does not seem to influence the habitat use of any shrew species occurring in the study area. However, the spatial distribution of captures as well as the direction and the mean distance of individual movements are indicative of different social systems. The habitat overlap and the intercrossed movements (movement of an individual intersecting a previous movement of other individuals) between males and females of *N. anomalus* indicate a gregarious social system. These results are in accordance with laboratory experiments that showed high levels of intraspecific tolerance in this species congruent with a gregarious life (Krushinska & Rychlik, 1993; Rychlik, 1998; 1999). We have also observed high levels of intersexual overlap in *C. russula*, which is in accordance with the monogamous social system already described for this species (Cantoni & Vogel, 1989). The social organization of *S. granarius* seems to be different from *N. anomalus* and *C. russula*. The patchy distributional patterns of captures as well as the absence of intercrossed movements indicate that this species is solitary and territorial, following the social organization typical for *Sorex* species (e.g. Churchfield, 1990; Cantoni, 1993; Rychlik, 1998).

Seasonal changes in the abundance of N. anomalus

The abundance of *N. anomalus* varied according to seasonal changes that induced significant alterations in the configuration of the preferred habitat of the species, particularly in the morphology of the watercourse. This species was more frequently captured in the driest periods of the year (summer and autumn), during which the river reached its minimum water depth and water flow. Such high abundance of water shrews in drier periods may be related to changes in density since less wet areas are available and because late summer and autumn represent the end of the breeding season for most of shrews, including *N. anomalus* (Churchfield, 1984b; Lardet, 1988). The trapping success of *N. anomalus* observed in summer (0.4 captures per 100 trap-hours) was, however, much higher than previously reported in *N. anomalus* populations co-occurring with *N. fodiens* (0.035 captures per 100 trap-hours; Rychlik, 2000) in the same annual period. Similar results had already been observed in other *N. anomalus* populations in Portugal (0.35 captures per 100 trap-hours; Rychlik & Ramalhinho, 2005). These results suggest that the absence of the dominant *N. fodiens* influences the abundance of *N. anomalus* via competitive release.

In winter, *N. anomalus* was either absent or only occasionally captured. Considering previous studies reporting a similar reduction in the number of captures of *N. fodiens* during cold periods (Churchfield, 1984b; Lardet, 1988; DuPasquier & Cantoni, 1992), the apparent low abundance of *N. anomalus* in winter may comparatively result from an increase in the cost of foraging in cold waters or from an increase in underground activity, including the use of burrows with direct access to water (Churchfield, 1984a; Lardet, 1988). Our results seem to better adjust to this second explanation because the few captures of *N. anomalus* in winter were still located at the riverbank. However, the occurrence of a seasonal habitat shift in other *N. anomalus* populations in Portugal cannot be excluded since this species was previously captured at considerable distances from water, especially during cold periods (Ramalhinho, 1988; 1995). Fluctuations on the abundance of *N. anomalus* may also be related to alterations in the risk of predation or winter food shortage. Laboratory studies have demonstrated a significant reduction in the foraging activity of shrews in response to the presence of predators (Saarikko, 1992). Seasonal variations in the risk of predation may be particularly relevant in southern populations of *N. anomalus* because in winter the riparian vegetation cover is significantly reduced and is not replaced by a constant snow cover, which constitutes the main protection against predators of small mammals in northern regions (e.g. Hansson & Henttonen, 1985). The abundance of *N. anomalus* in winter may have also been dependent from a low availability of aquatic prey caused by the increase in the water depth and the rapid water flow of the river.

Shift of habitat niche of Neomys anomalus

In central Europe, *N. anomalus* occurs near watercourses only when *N. fodiens* is absent (Šebek, 1970; Laar & Daan, 1976; Niethammer, 1977; 1978). But even in such allopatric populations, the habitats known to be selected by *N. anomalus* are small shallow water streams with slow water flow (Šebek, 1970; Laar & Daan, 1976; Niethammer, 1977; 1978). This has been explained by lower swimming and diving abilities of *N. anomalus* compared with *N. fodiens* (Mendes-Soares & Rychlik, 2009). So far only *N. fodiens* was found in river banks of deep and fast flowing waters (Niethammer, 1977; 1978; Lardet, 1988; Cantoni, 1993). The present study demonstrates for the first time that *N. anomalus* is able to inhabit such habitats. The occurrence of *N. anomalus* on riverbanks allows to consider that, at least some populations, are more ecologically specialized as supported by previous experimental findings (Tapisso *et al.*, 2013). However, as here also demonstrated, these *N. anomalus* populations are strongly influenced by seasonal variations that can alter the risk of predation and the foraging cost in streams or small rivers, which may explain the occurrence of some populations at longer distances from watercourses, particularly during winter (Amores, 1975; Ramalhinho, 1988; 1995). But even considering the role of seasonal variations, the absence of *N. fodiens* can determine a habitat shift of *N. anomalus* in the Iberian Peninsula. Our results clearly indicate an ecological release, with *N. anomalus* selecting habitats that are used by *N. fodiens* in other parts of Europe. The extension of the habitat shift in the Iberian Peninsula may also be magnified by the genetic differences found between Iberian and European populations of *N. anomalus* (Castiglia *et al.*, 2007).

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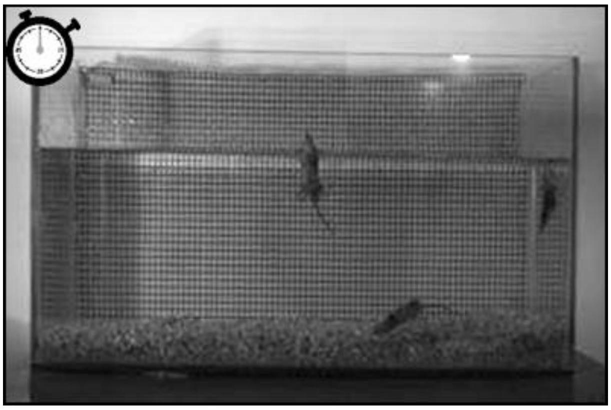
CHAPTER 4

SWIMMING AND DIVING BEHAVIOUR OF THE MEDITERRANEAN WATER SHREW



Ecological release: swimming and diving behaviour of an allopatric population of the Mediterranean water shrew

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Abstract

In regions where the 2 species are sympatric, the Eurasian water shrew, *Neomys fodiens* (Pennant, 1771), dominates behaviourally and numerically over the Mediterranean water shrew, *Neomys anomalus* Cabrera (1907), and tends to exclude it from direct access to aquatic habitats. In Portugal, ecological release can be expected in behavioural traits of *N. anomalus* due to the absence of the dominant *N. fodiens*. We investigated swimming and diving skills and efficiency in underwater foraging in 9 individuals of *N. anomalus* from Serra da Estrela, central Portugal. Efficiency in swimming and diving was analysed in short duration tests. Foraging behaviour was assessed through video-records of shrews foraging in a terrarium that simulated terrestrial and aquatic habitats. Our results were compared with data previously obtained for *N. anomalus* and *N. fodiens* co-existing in sympatry in Białowieża Forest, eastern Poland. In contrast to sympatric Polish *N. anomalus,* allopatric Portuguese *N. anomalus* were more efficient in some swimming parameters (such as stroking frequency) and showed greater variation in diving profiles. Allopatric *N. anomalus* also were able to take food portions from deep water, a behaviour that previously had been observed only in *N. fodiens*. Foraging success underwater, however, was much lower in allopatric *N. anomalus* than in *N. fodiens*. Our results suggest that in the absence of the dominant *N. fodiens*, allopatric *N. anomalus* can use 2 foraging modes: wading in shallow water (its primary foraging mode) and diving in deep water, thus extending its ecological niche.

Keywords

allopatric population, ecological release, Eurasian water shrew, interspecific competition, Mediterranean water shrew, *Neomys anomalus*, *Neomys fodiens*

Introduction

Ecological release may occur when interspecific competition is reduced, allowing species to expand their ecological niches. Several cases of competitive release have been described in which a competing species is removed from its environment (e.g., Bodey *et al.*, 2009; *Trewby et al.*, 2008). Competitive release may happen when species arrive in new environments, such as in island colonization (e.g., Diamond, 1970). Reduced interspecific competition is also known to occur in allopatric populations of closely related species. Sympatric populations with similar ecological requirements tend to accentuate differences that reduce interspecific competition, a phenomenon known as ecological character displacement (Brown & Wilson, 1956). On the contrary, allopatric populations of closely related species may converge as a result of the absence of a main competitor, becoming nearly identical in some characters, a phenomenon termed by Grant (1972) as ecological character release. Although character shifts occur in morphological, ecological, behavioural, or physiological traits (Brown & Wilson, 1956), most studies have focused on morphological traits such as size differences (Dayan & Simberloff, 1998; 2005) or ecological traits such as variations in trophic niches (Azuma, 1992; Bolnick *et al.*, 2010). Changes in morphological and ecological traits may also imply a significant change in behaviour of the species. However, behavioural traits have been used mainly to investigate how coexisting populations of similar species avoid hybridization (reproductive character displacement – Höbel & Gerhardt, 2003; Russo *et al.*, 2007), without considering the influence of ecological changes on resource use (Pfennig & Pfennig, 2009).

Shrews are insectivorous mammals that exhibit great similarity in size, morphology, and ecology. Despite this similarity, communities of multiple coexisting species are frequently found in the same habitats (Churchfield, 1990). In Europe, 2 semi-aquatic species occur, the Mediterranean water shrew, *Neomys anomalus* Cabrera, 1907, and the more widespread Eurasian water shrew, *N. fodiens* (Pennant, 1771). *N. fodiens* has a continuous distribution throughout most of Europe whereas *N. anomalus* has a wide but patchy distribution ranging from the Iberian Peninsula to Crimea, Turkey and including isolated populations in western Russia and Iran (IUCN/SSC, 1995; Hutterer, 2005). The distributional ranges of both species overlap to a large extent. However, in southern regions only *N. anomalus* occurs. In many locations the 2 species are not only sympatric but also syntopic, coexisting in the same habitats, such as banks of small rivers, streams, lakes, ponds, marshes and swamps (Rychlik, 2000; Spitzenberger, 1990).

In sympatric populations, niche segregation between *N. anomalus* and *N. fodiens* seems to be based mainly on the differentiation of microhabitats and trophic niches, both related to their aquatic dependence (Churchfield & Rychlik, 2006; Rychlik, 2000, 2004). Recent comparative laboratory experiments involving sympatric populations reveal that *N. fodiens* is a more proficient swimmer and diver, foraging in both shallow and deep water. In contrast, the swimming and diving skills of *N. anomalus* only allow the species to forage successfully in shallow water (Rychlik, 1997; Rychlik & Jancewicz, 2002; Mendes-Soares & Rychlik, 2009). These observations are in accordance with habitat selection by the 2 species: *N. fodiens* occurs strictly near water courses with direct access to deep water, whereas *N. anomalus* prefers wet ground flooded by shallow water, about 10-15 m from deep streams (Rychlik, 2000). Morphological adaptations to semi-aquatic life are more evident in *N. fodiens*. It has larger hind feet with longer hairs that form fringes and a longer keel of stiff hairs on the ventral side of its laterally compressed tail (Churchfield, 1998; Pucek, 1981). In contrast, *N. anomalus* has smaller hind feet, shorter fringes and a shorter or absent keel. The fringes of hairs increase the surface area of the feet which adds propellant power to swim, while the keel provides balance and prevents pitching and yawing (Churchfield, 1998). Interspecific diet comparisons support both microhabitat segregation and differences in underwater foraging in sympatric populations (Rychlik, 1997). The percentage of aquatic prey in the diet of *N. fodiens* can reach up to 95% (DuPasquier & Cantoni, 1992; Niethammer, 1978). In contrast, aquatic prey comprised only 11% of the diet of *N. anomalus* coexisting with *N. fodiens* in Białowieża Forest (Churchfield & Rychlik, 2006). These results, as well as the aforementioned laboratory experiments conducted by Rychlik (1997) and Rychlik & Jancewicz (2002), suggest that, in sympatry, these 2 species display distinct modes of aquatic foraging: diving in deep water by *N. fodiens* and wading in shallow water by *N. anomalus*.

In allopatric populations, however, morphological studies reveal that *N. anomalus* converges in body size and hind foot size with *N. fodiens* (Kryštufek & Quadracci, 2008; Rácz & Demeter, 1998; Spitzenberger, 1990). It is thus possible that, without the competitive pressure from *N. fodiens*, *N. anomalus* occupies aquatic habitats and uses aquatic resources more intensively than when in sympatry with *N. fodiens.* This hypothesis is supported by Rychlik & Ramalhinho (2005), who found that allopatric populations of *N. anomalus* in northern and central Portugal selected microhabitats directly adjacent to streams or rivers. Therefore, ecological release can be expected in behavioural traits, namely in swimming and diving skills, as well in foraging mode, which should be more similar to those of *N. fodiens*.

Water shrews swim by paddling, generating thrust by alternating strokes of the front and hind limbs. This mode of swimming is considered a modification of the terrestrial gait, being more common in semi-aquatic species which must compromise form and function to successfully operate both on land and in water (Stein, 1989; Williams, 1998). Differences in swimming efficiency may thus reflect the level of dependence on the aquatic environment of each species. The aims of this study were to investigate swimming skills and foraging strategy of an allopatric population of *N. anomalus* and to compare these results with data obtained from sympatric populations of *N. anomalus* and *N. fodiens* tested in similar experiments (Rychlik, 1997; Mendes-Soares & Rychlik, 2009). We expected that allopatric *N. anomalus* would display better swimming and diving skills than sympatric *N. anomalus* and have higher success in underwater foraging. Consequently, the foraging strategy of allopatric *N. anomalus* should be more similar to *N. fodiens* than what was previously described for sympatric *N. anomalus*.

Material and Methods

Experimental animals

A total of 9 subadult individuals of *N. anomalus* were trapped between October and November 2008 along Mondego River in Serra da Estrela Natural Park (2926328E; 4478212N), central Portugal, an area outside the distribution of *N. fodiens*. Traps were set along the river bank at distances < 2 m from the water course and located under or between patches of sedges (*Carex*). The vegetation of the river bank also included bushes of *Erica* and *Cytisus* and *Salix* trees. Shrews were transported to the Natural History Museum of Lisbon where they were kept for ≤ 3 weeks under an artificial photoperiod (10L:14D, with lights on at 0800 h) at a temperature of about 15-20ºC and humidity about 80%. Each shrew was maintained in an individual cage (40 × 20 × 30 cm) equipped with a shelter (inverted flower pot filled with moss) and litter (a mixture of sand, sawdust, peat, and moss). Water and food (minced meat, fly pupae, and mealworms) were given ad lib. Shrews were released at sites of capture after the experiments. All the field and lab procedures were in agreement with the American Society of Mammalogists (Sikes *et al.*, 2011) and were authorized by the Institute of Nature Conservation and Biodiversity, Lisbon, Portugal (license number ICNB 132/2008/CAPT).

Water shrews are rare and difficult to catch. They are also protected species, which limits the number of animals that can be tested in laboratory experiments. The sample size we used in this study was similar to those used in previous studies on aquatic behaviour of water shrews (Köhler, 1991, 2000; Vogel, 1990; Vogel *et al.*, 1998), namely those assessing differences in the swimming, diving and foraging behaviour between sympatric populations of *N. anomalus* and *N. fodiens* inhabiting Białowieża Forest, Poland (Rychlik, 1997; Mendes-Soares & Rychlik, 2009) with which our results were compared. Mendes-Soares & Rychlik (2009) studied swimming and diving behaviour of 10 *N. anomalus* and 12 *N. fodiens*, and Rychlik (1997) analyzed foraging behaviour of 6 *N. anomalus* and 5 *N. fodiens*. Data of both studies were here repeated for comparison. All shrews were trapped and maintained in the same way.

Experiment 1 - Swimming and diving behaviour

Experiments were performed according to the methods described by Mendes-Soares & Rychlik (2009). Briefly, swimming and diving behaviours were video-recorded between 1600 h and 1800 h in 2 aquaria. A larger aquarium (80 × 30 × 40 cm; water depth 25 cm) was used for recordings of distant and close lateral views, and a smaller one (60 × 30 × 30 cm; water depth 20 cm) for dorsal view recordings. During filming, a 1×1 cm grid was placed on the background wall of both aquaria, and two 150-watt lamps were placed at each side about 1 m apart. Each experiment consisted of three 5-min tests corresponding to recordings of the 3 types of views (distant lateral, close lateral, and dorsal) performed in a random order. At the beginning of each test animals were placed on a plastic platform which was submerged after 2-3 min, inducing the animals to swim. Between recordings of distant and close lateral views each animal rested 2 min on the platform, and some minced meat was given (no food was available under water). Recording of dorsal views were performed ≥ 30 min before or after the distant and close lateral recordings. Each animal was tested once a day for 3-5 consecutive days. Each day the water was partially replaced and walls of aquaria cleaned.

Videos were recorded with a high definition (HD) digital video camera Sony HDR-HC7E (Japan) at a shutter speed of 1/1,250 s. All recordings were converted to digital format (.jpg and .mpg) using the software AVG Video Processor 7.7. The files obtained were analysed frame-by-frame (25 frames/s) using Corel Draw 9® and Corel Photo-Paint 9®.

Three main categories of behaviour were distinguished: swimming – when animals used paws to actively move at the water surface; diving – when animals moved while completely submerged; and floating – when animals remained at the water surface using their paws only to maintain balance. The following 7 parameters were analysed within main behaviours: swimming velocity – mean distance per s in all swimming bouts in a test; stroke frequency – mean number of strokes per s in all swimming bouts in a test; body inclination – angle maintained between the central long axis of the animal’s body and the water surface – i.e., inclination from the horizontal; ratio of body width to lateral tail undulations – relation between maximum body width measured from a dorsal perspective to maximum amplitude of tail undulations at the same moment; ratio of body width to body length – relation between maximum body width measured from a dorsal perspective to maximum head and body length, also measured from a dorsal perspective, from the tip of nose to base of tail; fineness ratio while swimming, floating and diving – measure of streamlining (Fish, 1993a) calculated as the relation between maximum head and body length to the maximum body height (greatest dorsal-ventral distance); duration of bout – duration of a single floating or diving bout.

Additionally, 16 diving profiles were distinguished (Fig. 1). They were classified as easy, medium or difficult, based on the energy it was assumed shrews expended for each dive and taking into account 4 factors: descent angle measured between central long axis of the shrew's body and the water line; depth of dive; distance swum underwater after reaching the deepest point; and ascent angle measured between the central long axis of the animal's body and the bottom of the aquarium. Each action was scored with 1 for a lower energetic cost or 2 for a higher energetic cost (Fig. 1).

All behavioural parameters, including diving profiles, were identical to those defined by Mendes-Soares & Rychlik (2009) and were calculated in exactly the same way. Temporal accuracy was 0.04 s (1 frame) and spatial accuracies were 0.1 cm for distance and 0.1º for angle. For more details about the procedure, data organization and analysis, see Mendes-Soares & Rychlik (2009).

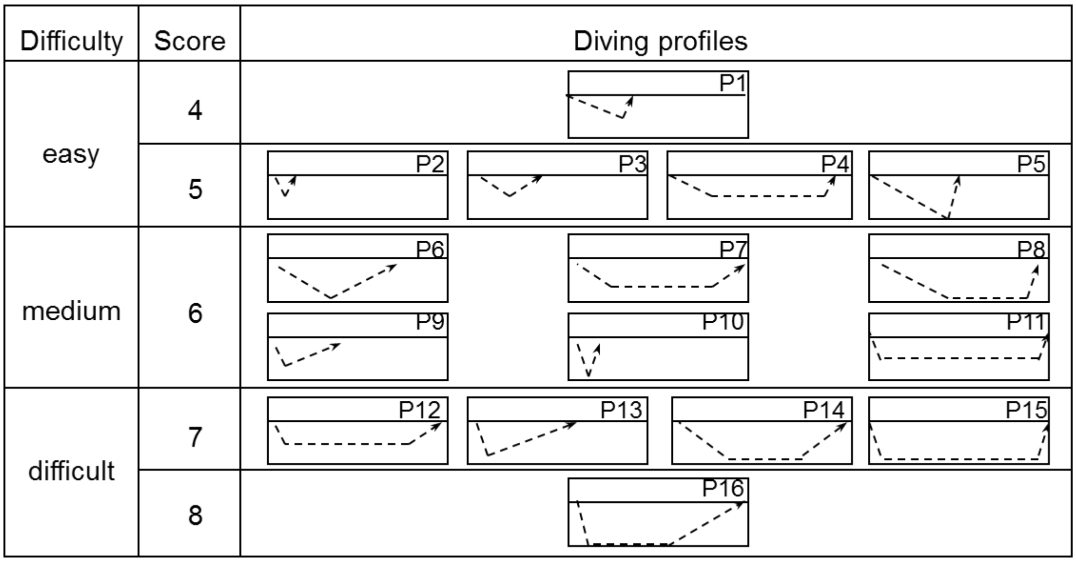

Figure 1. Diagrammatic illustration of 16 diving profiles (P) distinguished by Mendes-Soares & Rychlik (2009) and applied in this study, in order of difficulty. Difficulty classification was obtained by adding the scores of each profile based on the descent angle (score = 1 for an obtuse descent angle or score = 2 for an acute descent angle); depth of the dive (score = 1 for dives up to the half the water depth or score = 2 for more than half of the water depth); distance swum underwater (score = 1 for dives ≤ 25 cm or score = 2 for dives > 25 cm); and ascent angle (score = 1 for an obtuse ascent angle or score = 2 for an acute ascent angle). After Mendes-Soares & Rychlik (2009), with permission of the *Journal of Ethology*.

Experiment 2 - Foraging behaviour

Experiments were performed following the methods of Rychlik (1997). Briefly, experiments were carried out in an open top terrarium (150 × 50 × 50 cm) made of glass and Plexiglas, divided into terrestrial and aquatic areas, each 25 cm wide and 150 cm long. The terrestrial area was composed of a mixture of sand, soil and turf approximately 15 cm thick. The bottom of the aquatic area was covered with a mixture of gravel and pebbles (0.5–1 cm diameter) to create a slope 20 cm thick at 1 edge of the terrarium and 2 cm at the opposite side, with water depth varying from 5 to 23 cm. The bank consisted of a vertical plastic wall to which some irregularities (mainly embedded stones) were added, allowing animals to exit from water onto the land. Four structures protruded from the water: 2 'islands' made of big stones (10-12 cm diameter) completely surrounded by water and 2 platforms (8 × 8 cm) made of plastic plates located at the bank. Two 'hiding-places' constructed of wood and bark were located in the terrestrial area.

Seven of the 9 water shrews used in experiment 1 were tested in 2 variants that differed in the distribution of food and available natural structures: variant 1 - food and natural structures (stones, sticks and tufts of grass) present in both terrestrial and aquatic areas of the terrarium; variant 2 – food and natural structures only in the aquatic area of the terrarium. Variant 1 and 2 replicated the conditions described by Rychlik (1997) as variant D and W, respectively. Food portions were either small cubes (about 0.15 cm^3 and 0.2 g) or large cubes (about 1 cm^3 and 1.3 g) of pig heart muscle that were replaced in every test. Quantity and distribution of food portions were also selected according to the procedures of Rychlik (1997). In our experiments, 15 small and 4 large portions of food were distributed directly on the ground of the terrestrial area, and, 30 small and 4 large portions were distributed at the bottom of the aquatic area. Food portions were always prepared 1.5 hrs before each test and the position of each food portion in the terrarium was maintained throughout all experiments.

For 4 consecutive days each animal was tested twice in each variant. Each test lasted 3 h and was performed between 1900 h and 2300 h. Non-test-food was removed from the animal cage 30 min before the test. Animals were transported in their individual cages to the experimental terrarium and were released in the terrestrial area, near one of the hiding places. Shrews were filmed in dark (no visible light) with a high definition (HD) digital video camera Sony HDR-HC7E (Japan), using the infrared sensitive night mode. The video camera was positioned over the terrarium covering the entire test area. An infrared light lamp of 175 watts was hung 3 m above the terrarium. Shrews were returned to their home cages after each test and food portions remaining in the terrarium were counted. The water was partially replaced and the ground of the terrestrial area was dug up before testing each shrew. Structures like stones, sticks, bark, wood and the nesting material of the hiding places were washed or replaced. Video records were analyzed with Samsung Power DVD software using the digital zoom option (4x or 8x) when more detailed observations were required.

Nine behavioural categories were distinguished: hiding – animals stayed inside one of the hiding-places (if >30 s); land foraging – foraging on the terrestrial area (>1 s); food success – food portion found and sniffed, manipulated, eaten or taken; before water – short periods spent on “islands” and “shelves” or running along the bank during water foraging (see below; 10-30 s); swimming – swimming on the water surface (>1 s); diving – diving completely under water (>1 s); water foraging – the sum of time in behaviours before water, swimming and diving; rooting soil – rooting ground on the terrestrial area (>20 s); and rooting grass – rooting in grass turfs (>20 s). Other behaviours not listed above were also noted, e.g., grooming and resting outside of hiding-places (>10 s). All behavioural categories were identical to those defined by Rychlik (1997) and were calculated in exactly the same way. Time spent in each behaviour was measured with an accuracy of 0.1 s and is reported as mean percentage of total test time.

Statistical analysis

All statistical tests were performed using IBM SPSS Statistics 19 (SPSS, Inc. 2010). Because all populations were tested under identical conditions, data of allopatric *N. anomalus* were statistically compared with data of sympatric *N. anomalus* and *N. fodiens*. Differences between populations in swimming and diving behaviours (except for diving profiles) were assessed using Student's *t*-test after confirmation that data followed a normal distribution. The non-parametric Kolmogorov-Smirnov test was used to compare distribution of frequencies of the various diving profiles. The difference between observed and expected frequencies was obtained using a goodness-of-fit test. Differences in foraging behaviours between the 2 variants were analyzed with a Wilcoxon test, and interspecific differences were assessed using a Mann-Whitney test. Differences were considered significant for P value $\leq$ 5%, for all statistical tests performed.

Results

Swimming and diving behaviour

In experiment 1, allopatric *N. anomalus* spent most of the time swimming (on average 93.1%). Floating and diving behaviours were much less frequent (averaging 5.6% and 1.3%, respectively). Animals adopted a horizontal position while swimming, keeping the head slightly above the water surface and moving their hind feet in alternating strokes, synchronized with rapid undulatory movements of the tail. Average swimming velocity of allopatric *N. anomalus* (32.1 cm/s) was similar to the velocity of *N. anomalus* living in sympatry with *N. fodiens* (33.4 cm/s) but it was significantly lower than the velocity of *N. fodiens* (36.4 cm/s: Student's *t*-test: $t = 4.04$, $d.f. = 16$, $P = 0.0009$; Table 1). However, the stroking frequency was lower in allopatric than sympatric *N. anomalus* (7.5 strokes/s and 8.2 strokes/s, respectively; Student's *t*-test: $t = 3.36$, $d.f. = 18$, $P = 0.0035$; Table 1). In fact, the stroking frequency of allopatric *N. anomalus* was more similar to the stroking frequency of *N. fodiens* (7.6 strokes/s). No differences were found between allopatric *N. anomalus* and sympatric *Neomys* populations regarding the position of the body (inclination and ratio between body width and body length; Table 1). However, allopatric *N. anomalus* swam with narrower undulations of the tail, as shown by the significant difference in the ratio between body width and width of undulations of tail (Student's *t*-test: $t = 6.764$, $d.f. =19$, $P < 0.0001$ for allopatric vs. sympatric *N. anomalus*; $t = 5.559$, $d.f. =18$, $P < 0.0001$ for allopatric *N. anomalus* vs. *N. fodiens*) and with a lower fineness ratio (Student's *t*-test: $t = 3.402$, $d.f. = 19$, $P = 0.003$ for allopatric vs. sympatric *N. anomalus*; $t = 3.987$, $d.f =18$, $P = 0.0009$ for allopatric *N. anomalus* vs. *N. fodiens*; Table 1).

One animal was constantly moving and did not float, but the other 8 spent some time floating. Animals were almost completely immobile while floating, using their feet only to redirect their body. Floating bouts were characterized by several short duration periods rather than few longer bouts. Mean duration of floating bouts of allopatric animals was significantly longer than in sympatric *N. anomalus* (Student's *t*-test: t = 2.65, *d.f.* =15, P = 0.0181) but very similar to the duration of floating bouts in *N. fodiens* (Table 1). The fineness ratio while floating was lower than while swimming and, in this case, a significant difference was found between allopatric *N. anomalus* and *N. fodiens* (Student's *t*-test: t = 4.101, *d.f.* = 14, P = 0.0011; Table 1).

Table 1. Comparison of parameters observed in swimming, floating, and diving in allopatric *Neomys anomalus* from Portugal (this study) with sympatric populations of *N. anomalus* and *N. fodiens* from Poland (data from Mendes-Soares & Rychlik, 2009). Asterisks indicate statistically significant differences (Sig.) in percentages between allopatric *N. anomalus* and each population of sympatric *N. anomalus* and *N. fodiens*, based on *t*-tests: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, no significant difference. Sample sizes vary because some behaviours were not shown by or were not measureable in all shrews tested.

	Allopatric *N. anomalus*		**Sympatric *N. anomalus***			**Sympatric *N. fodiens***		
Behavioural parameter	***n***	**Mean ± *SD***	***n***	**Mean ± *SD***	**Sig.**	***n***	**Mean ± *SD***	**Sig.**
Swimming								
Velocity (cm/s)	9	32.1 ± 2.2	9	33.4 ± 1.4	ns	9	36.4 ± 2.4	**
Stroking (strokes/s)	9	7.5 ± 0.5	11	8.2 ± 0.5	**	11	7.6 ± 0.6	ns
Inclination from horizontal (°)	9	13.2 ± 1.9	11	12.0 ± 2.3	ns	9	12.6 ± 1.1	ns
Ratio, body width: body length	9	0.4 ± 0.03	8	0.4 ± 0.1	ns	7	0.4 ± 0.01	ns
Ratio, body width: tail undulation	9	1.5 ± 0.2	12	1.1 ± 0.04	***	11	1.1 ± 0.1	***
Fineness ratio (streamlining)	9	2.7 ± 0.3	12	3.2 ± 0.3	**	11	3.3 ± 0.3	**
Floating								
Duration of 1 floating bout (s)	8	2.4 ± 1.2	9	1.3 ± 0.3	*	8	2.0 ± 0.7	ns
Fineness ratio (streamlining)	8	1.7 ± 0.2	5	2.0 ± 0.2	ns	8	2.0 ± 0.2	*
Diving								
Duration of 1 diving bout	9	2.1 ± 0.8	10	2.1 ± 0.6	ns	9	3.1 ± 0.6	*
Fineness ratio (streamlining)	8	4.3 ± 0.7	10	3.9 ± 0.2	*	8	3.6 ± 0.2	**

A total of 139 dives was analyzed for allopatric *N. anomalus*. The mean duration of each dive was similar to that previously found for sympatric *N. anomalus* and significantly lower than in *N. fodiens* (Student's *t*-test: *t* = 3.03, *d.f.* = 16, *P* = 0.0081; Table 1). However, the maximum diving duration in allopatric animals reached 5.6 s, while the longest dives were only 2.3 s for sympatric *N. anomalus* and 4.0 s for *N. fodiens*. Fineness ratio while diving revealed that allopatric animals adopt a narrower shape than sympatric *N. anomalus* (Student's *t*-test: *t* = 2.19, *d.f.* = 16, *P* = 0.0433) and *N. fodiens* (Student's *t*-test: *t* = 3.918, *d.f.* = 14, *P* = 0.0010; Table 1). The distribution of diving profile frequencies did not differ between allopatric and sympatric *N. anomalus* nor between allopatric *N. anomalus* and *N. fodiens* (Kolmogorov-Smirnov test: Z = 0.707, *P* = 0.699 and Z = 1.061, *P* = 0.211, respectively). However, significant differences were found when we compared the observed frequency of diving profiles of allopatric *N. anomalus* with an expected uniform distribution (6.25% for 16 profiles; Fig. 2). Allopatric *N. anomalus* displayed 7 of 16 diving profiles more frequently than expected. Three were easy diving profiles (profiles 1, 3, and 5), 2 medium (profiles 8 and 10) and 2 difficult (profiles 13 and 15). Only 4 diving profiles (profiles 4, 7, 11, and 12) were significantly avoided by allopatric animals, all of them corresponding to shallow and long dives. The diversity of diving profiles displayed by allopatric *N. anomalus* is higher than was previously found for sympatric *N. anomalus* and *N. fodiens*. Sympatric *N. anomalus* showed a significant preference for only 3 of the 16 diving profiles (profiles 8, 10, and 15), avoiding 7 of them (profiles 4, 7, 9, 11, 12, 13, and 16), whereas *N. fodiens* showed a significant preference for only 2 diving profiles (profiles 10 and 15), avoiding 6 of them (profiles 1, 4, 7, 9, 11, and 12; Fig. 2—see also Mendes-Soares & Rychlik, 2009).

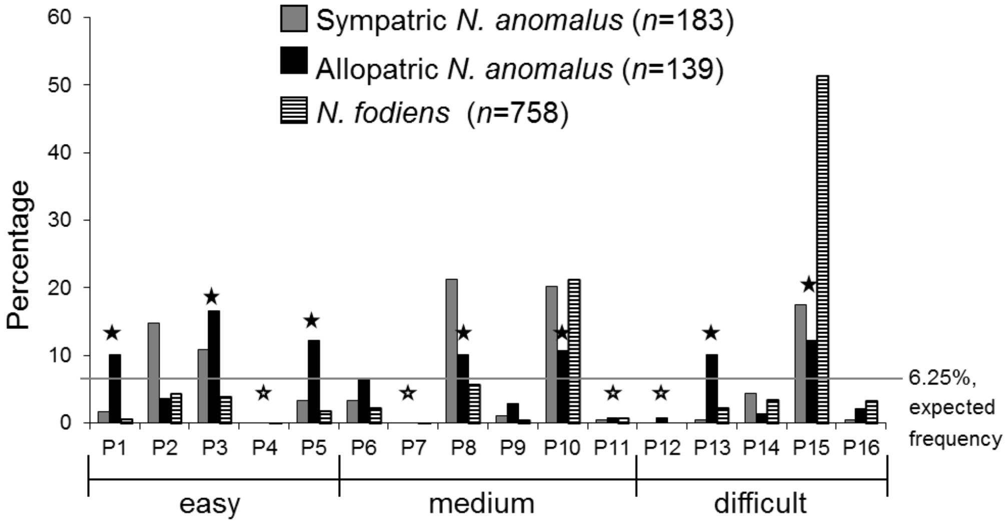

Figure 2. Differences in distributions of diving profiles (P1 to P16) displayed by allopatric *N. anomalus* (this study) and sympatric *N. anomalus* and *N. fodiens* (Mendes-Soares & Rychlik, 2009). Gray line at 6.25% indicates expected uniform distribution. Differences between expected and observed values (revealed by goodness-of-fit *G*-test): black star, preferred profile (significantly more frequent than expected; $P \leq 0.05$); white star, avoided profile (significantly less frequent than expected; $P \leq 0.05$).

Foraging behaviour

In experiment 2, allopatric *N. anomalus* spent most of the time hiding (94% of 3-h tests in both experimental variants). As previously observed for sympatric *N. anomalus* and *N. fodiens*, allopatric *N. anomalus* also spent more time foraging on land than in water. The number and duration of dives was low, and the lack of food in the terrestrial area (variant 2) did not induce a significant increase in the aquatic behaviour of allopatric *N. anomalus*. Consequently, the success rate in finding food on the terrestrial area was higher than in the aquatic area (Wilcoxon test: $Z = 3.262$, $P = 0.001$; Table 2).

No significant differences were found between allopatric and sympatric *N. anomalus* in mean percentage of time foraging on land or in water in either experimental variant (Table 2). Allopatric *N. anomalus* spent significantly less time than *N. fodiens* in foraging on land in variant 1 (Wilcoxon test: $Z = 2.646$, $P = 0.008$) and significantly less time foraging in water in both variants (Wilcoxon test: $Z = 2.535$, $P = 0.011$; Table 2).

Allopatric *N. anomalus* also dived for significantly less time than *N. fodiens* (Wilcoxon test: Z = 2.378 $P = 0.017$; Table 2). Additionally, *N. fodiens* dived in both variants while sympatric *N. anomalus* only dived in variant 2 (i.e. when there was no food on land) and then only for short periods. Consequently, although the success rate in finding food in water by allopatric *N. anomalus* was lower than that of *N. fodiens* (Wilcoxon test: $Z = 2.885$, $P = 0.006$ in variant 1; Wilcoxon test: $Z = 2.699$, $P = 0.005$ in variant 2), 4 allopatric *N. anomalus* were able to take food portions underwater in both variants, something that sympatric *N. anomalus* never did. These 4 found food underwater on both sides of the slope that composed the bottom of the aquatic part — 2 shrews were only able to find food on the shallow end (5 cm deep) but the other 2 also found food in the deepest area (23 cm), reaching an average depth of 14.6 cm.

Rooting behaviour was characterized by large variation among animals. The only significant difference was the shorter duration of rooting grass in allopatric *N. anomalus* compared to sympatric *N. anomalus* (Wilcoxon test: $Z = 2.289$, $P = 0.022$: Table 2).

Table 2.—Comparison of foraging behaviours and foraging success of allopatric *Neomys anomalus* from Portugal ((*n* = 7; this study) with sympatric populations of *N. anomalus* (*n* = 6) and *N. fodiens* (*n* = 5) from Poland (data from Rychlik, 1997). Shrews were tested in 2 experimental variants: 1 – with food and natural structures in both terrestrial and aquatic parts of terrarium, and 2 – with food and natural structures only in aquatic part. Mean duration of behaviours and mean number of items successfully taken in water and on land are shown for allopatric *N. anomalus*, and percentage of total time or of total number of food items available are shown for all 3 populations. Asterisks indicate statistically significant differences (Sig.) in percentages between allopatric *N. anomalus* and each population of sympatric *N. anomalus* and *N. fodiens*, based on Mann Whitney *U*-test: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, no significant difference.

	Allopatric *N. anomalus*		**Sympatric *N. anomalus***		**Sympatric *N. fodiens***	
	Variable as measured	**% of total**	**% of total**		**% of total**	
Behavioural parameter	**Mean ± *SD***	**Mean ± *SD***	**Mean ± *SD***	**Sig.**	**Mean ± *SD***	**Sig.**
Variant 1 (food on land and in water)						
Time foraging on land (s)	338.6 ± 197.9	3.1 ±1.8	5.1 ± 2.8	ns	8.8 ± 3.3	**
Time foraging in water (s)	40.4 ± 56.2	0.4 ± 0.5	0.4 ± 0.3	ns	3.0 ± 1.3	**
Time diving (s)	3.1 ± 1.8	0.03 ± 0.06	0	ns	0.4 ± 0.4	*
Time rooting soil (s)	60.6 ± 100.9	0.6 ± 0.9	0.1 ± 0.2	ns	0.5 ± 0.9	Ns
Time rooting grass (s)	45.9 ± 48.3	0.4 ± 0.5	8.1 ± 12.3	**	2.0 ± 3.0	Ns
Food success on land (*n* pieces)	8.1 ± 5.2	42.5 ± 27.5	79.7 ± 34.1	ns	66.3 ± 14.8	Ns
Food success in water (*n* pieces)	0.1 ± 0.4	0.4 ± 1.1	0	…	17.9 ± 13.7	**
Variant 2 (food in water only)						
Time foraging on land (s)	379.9 ± 238.4	3.5 ± 2.2	7.9 ± 7.7	ns	5.1 ± 1.8	Ns
Time foraging in water (s)	37.9 ± 66.1	0.4 ± 0.6	0.3 ± 0.2	ns	2.3 ± 1.3	*
Time diving (s)	3.3 ± 0.4	0.1 ± 0.1	0.01 ± 0.01	ns	0.6 ± 0.5	*
Time rooting soil (s)	52.9 ± 65.1	0.5 ± 0.6	0.8 ± 1.2	ns	0.7 ± 1.0	Ns
Food success in water (*n* pieces)	0.5 ± 1.1	1.5 ± 3.3	0	ns	19.4 ± 13.6	**

Discussion

Allopatric *N. anomalus* from Portugal did not display all the behaviours previously observed for *N. anomalus* sympatric with *N. fodiens*. In fact, allopatric *N. anomalus* resembled *N. fodiens* in several swimming and diving parameters. Under similar experimental conditions, Mendes-Soares & Rychlik (2009) found that the swimming velocity of *N. fodiens* is significantly higher than the velocity of sympatric *N. anomalus* (36.4 cm/s and 33.4 cm/s, respectively). In the present study the swimming velocity recorded for allopatric *N. anomalus* was similar to the velocity already observed for sympatric members of the species (32.1 cm/s). Such a result supports predictions that take into account morphological differences between *N. fodiens* and *N. anomalus*. According to Pucek (1981) and Churchfield (1998), *N. fodiens* can reach higher swimming speeds because of their larger hind-feet with longer fringes, which allow strokes with more propulsive power. When tested in larger aquaria, *N. fodiens* velocities were even higher than those observed by Mendes-Soares & Rychlik (2009). Ruthardt & Schröpfer (1985) recorded an average swimming speed of about 40 cm/s in an aquarium 300 cm long, whereas similar velocities (36.7–44.1 cm/s) were obtained by Köhler (1991) who used a 145 cm long-aquarium. High swimming speeds allow animals to increase foraging efficiency and active pursuit, but they are also energetically demanding because thrust power is directly related to the cube of velocity (Fish, 1993a). The energy expenditure for paddling is so high that some semi-aquatic species (e.g. muskrats) swim at lower mean velocities than the expected most efficient speed (Fish, 1982a).

Mendes-Soares & Rychlik (2009) also found that sympatric *N. anomalus* had to increase their stroking frequency to reach swimming speeds similar to those of *N. fodiens*. Allopatric *N. anomalus* reached similar velocities to sympatric *N. anomalus* with significantly fewer strokes per second. The stroking frequency of allopatric *N. anomalus* was, in fact, very similar to what was recorded previously for *N. fodiens* (7.5 and 7.6 strokes/s, respectively). Each paddle stroke only generates thrust power in half of the cycle and involves an energy loss in the recovery phase of the limb (Fish, 1993a). By using fewer stroke cycles to achieve the same velocity as *N. fodiens,* allopatric *N. anomalus* show greater paddling efficiency. Such an increased efficiency can result from an increase of the paddle surface. On the other hand, optimization of the body position while swimming (which could minimize the resistive forces) was not found. Therefore, allopatric *N. anomalus* seems to maximize their propulsive force by either increasing body size or the hind feet surface, rather than to reduce drag force. A convergence in body size (represented by cranio-dental measurements) of *N. anomalus* towards *N. fodiens* was reported previously in allopatric populations in Hungary and the Balkans (Kryštufek & Quadracci, 2008; Popov & Zidarova, 2008; Rácz & Demeter, 1998). In those studies, the larger size of allopatric *N. anomalus* was explained by the lack of competition with *N. fodiens* for food resources of higher quality, which is consistent with the ecological release hypothesis. The same kind of character convergence was also found in populations of *N. anomalus* inhabiting the southern Iberian Peninsula, where *N. fodiens* is absent (López-Fuster *et al.*, 1992).

The use of the tail by small mammals while swimming has been recognized as an indicator of species with aquatic abilities (Cook *et al.*, 2001; Esher *et al.*, 1978; Nicolas & Colyn, 2006). In general, skilled paddling swimmers show an undulating movement of the tail. However, the proportion of thrust generated by this movement is much smaller than the thrust power generated by the hind feet (e.g., Fish, 1982b; Fish & Baudinette, 1999; Santori *et al.*, 2008; Williams, 1983). Although alternated strokes of the hind feet are highly energetic, they also generate yaw, and, as Fish (1982b) demonstrated for muskrats, the main function of the tail of paddle swimmers is to reduce drag caused by yawing motions. Like muskrats, water shrews have a laterally compressed tail with a keel of stiff hairs on the ventral side (Pucek, 1981). The keel is more prominent in *N. fodiens* than in *N. anomalus*, allowing a higher swimming stability and lower amplitude of tail movements (given by the ratio between body and width of tail undulations). As is typical for *N. anomalus*, the animals tested in our study had a keel only on the terminal third of the tail. Nevertheless, the undulatory movements of their tail were narrower than previously observed in *N. fodiens* (Mendes-Soares & Rychlik, 2009). Allopatric animals presented a higher swimming stability, probably as a result of a more efficient relationship between stroking frequency (similar to *N. fodiens*) and swimming velocity (lower than *N. fodiens*).

Floating behaviour has also been successfully used to discriminate between swimming abilities of closely related terrestrial and semi-aquatic mammals (e.g., Fish, 1993b; Santori *et al.*, 2008). Semi-aquatic forms are able to float effortlessly and in a more horizontal position as a result of the development of a waterproof fur (Fish, 2000; Fish *et al.*, 2002) not present in terrestrial species. The water-repellent properties of the fur are mainly given by a high hair density that retains large amounts of air, providing insulation and buoyancy (Fish *et al.*, 2002). Studies of *N. fodiens* confirmed the insulating effect of the fur (Vogel, 1990). Its high buoyancy results from the retention of air in a very dense layer of woolly hairs and is enhanced by the more pronounced H-shaped core of guard hairs and 1.5-2.0 times more irregularities on the hair surface than in shrews of the genus *Sorex* (Ivanter, 1994). Floating bouts of *N. fodiens* have longer mean duration than those of sympatric *N. anomalus* (Mendes-Soares & Rychlik, 2009), but shorter duration than those of allopatric *N. anomalus* (the present study). Further studies are required to determine whether differences exist in density and structure of the fur between the 2 species of water shrews. Additional allopatric populations of *N. anomalus* should also be studied, as Portuguese animals appear to be more similar to *N. fodiens* in terms of their floating capacity, suggesting that allopatric populations may be better adapted to the aquatic environment.

The long floating periods observed for the allopatric *N. anomalus* allowed them to rest after swimming and frequently were followed by diving bouts. These diving bouts were usually very short in duration, but some animals were able to perform longer dives. Longer diving bouts, such as those observed in the present study (5.6 s), were obtained by Köhler (2000) only after training *N. anomalus* in bigger tanks and with live prey available underwater (mean dive duration, 4.7 s, maximum dive duration, 6.7 s). In such experimental conditions, the duration of single dives for sympatric *N. fodiens* averaged 11.5 s, reaching a maximum of 15.6 s (Köhler, 1991). Vogel *et al.* (1998) obtained a similar average (12 s) while testing the maximal diving depth of *N. fodiens*. However, without training but still with prey available underwater, the duration of single dives of *N. fodiens* never exceeded 4 s (Churchfield, 1985), a value which is probably closer to what would be observed under more natural conditions (Ruthard & Schröpfer, 1985). Under laboratory conditions, the 2 water shrew species not only showed differences in duration of each dive but also in diving profiles (Mendes-Soares & Rychlik, 2009). *N. fodiens* showed a clear preference for exploring the entire bottom of the tank while diving, whereas *N. anomalus* more frequently displayed diving profiles that only allowed them to explore part of the bottom. The allopatric *N. anomalus* we tested did not show a clear preference for specific diving profiles. Instead, they displayed a wide diversity of diving profiles, including shallow dives, bottom-up dives and dives in which animals explored the entire bottom of the tank, touching the gravel with their snout several times (with 2 dives lasting 4.7 s and 5.2 s).

In sympatric populations, *N. fodiens* forages mainly by diving in deep water, while *N. anomalus* forages by wading in shallow water. Such findings were first described from laboratory experiments (Rychlik, 1997) and later confirmed under natural conditions (Churchfield & Rychlik 2006; Rychlik, 2000). In the present study (experiment 2), allopatric *N. anomalus* also showed a clear preference to forage on the terrestrial part of the aquarium and only some of the animals were able to dive and find food in deep water. However, this was the 1st time that diving foraging behaviour was observed for the species. In similar experimental conditions, sympatric *N. anomalus* were unable to find any food in deep water (Rychlik, 1997). Nevertheless, underwater success rate of the allopatric population of *N. anomalus* never reached the success rate observed in *N. fodiens*, which indicates that wading is still its preferred foraging mode. Such a result can rely on morphological or physiological constraints on diving in *N. anomalus*, but it can also be related to ecological factors like interspecific competition. In the Iberian Peninsula, several populations of *N. anomalus* co-exist with the semi-aquatic Pyrenean desman *Galemys pyrenaicus*, which occurs in the same habitats but forages almost exclusively in deep water (e.g., Carvalho, 1996; Santamarina, 1993). However, no data are available on competition between this pair of species.

The present study shows that allopatric *N. anomalus* from Portugal are better adapted to the aquatic environment than conspecifics coexisting with *N. fodiens* in Poland. These allopatric shrews are more efficient while swimming, capable of resting in water, have a wider diversity of diving profiles and, at least to some extent, are able to forage in deep water. Wading still seems to be the main foraging mode but is probably enhanced by the diving foraging mode. These results are consistent with findings on habitat preferences of the species in Portugal. In the Iberian Peninsula there are populations of *N. anomalus* occurring distant from watercourses (e.g., Amores, 1975; Spitzenberger, 1990), but also populations, like the ones found in Portugal and tested in the present study, that live directly at the edge of streams and small rivers (Rychlik & Ramalhinho, 2005). Iberian *N. anomalus* populations also show significant genetic differences from other European populations, as pointed out by Castiglia *et al.* (2007), who suggested the need for a revision of the taxonomic status of the Iberian populations of *N. anomalus*. Given their findings, the results of the present study are even more relevant, because they describe behaviour of a potential new taxonomic entity. However, the ecological release hypothesis still applies because, in the Iberian Peninsula, both species of water shrews co-occur in some areas and, in those areas, *N. anomalus* seems to be constrained by the presence of *N. fodiens* as a result of the altitudinal and habitat segregation (Castién & Gosálbez, 1992; Gosálbez & López-Fuster, 1985; Torre & Tella, 1994). For example, in the Pyrenees Mountains where both species co-exist, *N. anomalus* seems to occur in low densities, confined to alpine meadows far from streams (Torre & Tella, 1994). Moreover, convergence in the aquatic behaviour of Portuguese allopatric *N. anomalus* towards *N. fodiens* is consistent with the convergence in body size already observed in other allopatric populations of *N. anomalus* outside the Iberian Peninsula (Kryštufek & Quadracci, 2008; Popov & Zidarova, 2008). Thus, the absence of the main competitor probably allows *N. anomalus* to extend their ecological niche to more aquatic environments, as here indicated by the improvement in the swimming and diving performance.

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CHAPTER 5

COMPETITION AND MODELLING DISTRIBUTION OF WATER SHREWS

Competition effects on the distribution of water shrews (*Neomys* spp., Soricidae) in the Iberian Peninsula under present and future climate variability

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Abstract

This study aimed to investigate the influence of interspecific competition on the geographic distribution of two water shrew species, *Neomys anomalus* and *N. fodiens*, in the Iberian Peninsula. It also aimed to predict how such biotic interaction may influence their future distribution under different climate change scenarios. Potential current distributions were modelled using the MaxEnt algorithm, based on occurrence data mainly obtained from the Global Biodiversity Information Facility. Bioclimatic variables were obtained from the WorldClim database. The influence of competition on the current distribution of the two species was analysed by testing intra- and interspecific differences on the environmental variables showing the highest contribution for each species' model. Model projections into the future (2050 and 2070) were also obtained using MaxEnt, based on global climate data of two representative concentration pathways (RCP 2.6 and RCP 8.5). The current distribution of water shrews in the Iberian Peninsula is mainly determined by the waterfall regime. Altitude also strongly influences the distribution of *N. anomalus* while *N. fodiens* is more affected by high temperatures. The resulting distributions showed a more extensive but fragmented pattern for *N. anomalus* and a continuous but localized distribution of *N. fodiens* in northern regions. The two species overlap in large extant in this area. Comparisons between allopatric and sympatric populations revealed that *N. anomalus* tends to occur at higher altitudes in the absence of *N. fodiens*, which is consistent with a potential competitive release. Predictions based on future climate changes showed a contraction in the distribution of both species increasing the area of potential competitive exclusion. Interspecific competition influences the distribution of water shrews in the Iberian Peninsula. Future climate changes may cause changes in the distribution of both species that will magnify negative impacts by an increase of their sympatry.

Keywords

Competition, modelling, climate changes, sympatry, allopatry, *Neomys anomalus*, *Neomys fodiens*

Introduction

Interspecific competition is known to influence the spatial distribution of species (Connell, 1983; Connor & Bowers, 1987; Durrett & Levin, 1998). Dominant competitors often restrict the presence of subordinate species to only part of their potential occurrence area (Hirzel & Le Lay, 2008). The spatial effects caused by interspecific competition can be observed at different scales. For instance, competition between closely related species frequently induces microhabitat segregation (e.g. Křivan & Sirot, 2002), affecting species distributional patterns at a local scale but allowing their coexistence in the same geographical areas. In other cases, competitive interactions can have broader effects, playing an important role in the determination of range limits (Sexton *et al*., 2009). Species with similar ecological requirements often have allopatric and/or parapatric distributions as a result of interspecific interactions driven by competitive exclusion (e.g. Anderson *et al*., 2002; Case *et al*., 2005; Price & Kirkpatrick, 2009).

The influence of interspecific competition on species' spatial patterns is regulated by abiotic conditions which constitute *per se* major determinants of species distributions (Pulliam, 2000; Soberón & Peterson, 2005). In fact, every species has climatic and physical tolerances that determine where they can live, survive and reproduce, i.e., their niche space (Ricklefs, 2010). Following G.E. Hutchison's definition of niche, the physiological tolerance to abiotic conditions determine the "fundamental niche" of a species (Hutchinson, 1957). However, the areas which are actually occupied by a species, "the realized niche", are only determined when biotic interactions are included in the calculation of the niche space (Hutchinson, 1957). In recent years, the advance of geographic information systems allowed the development of species distribution models that relate niches with distribution areas (Elith & Leathwick, 2009). These models estimate potential areas of distribution by correlating occurrence data with environmental and/or spatial characteristics of given locations (Austin, 2007; Elith & Leathwick, 2009). One of the major applications of such models has been the prediction of future changes on species distributions, namely in consequence of climatic alterations (e.g. Parmesan & Yohe, 2003; Araújo *et al.*, 2005; Austin & Niel, 2011).

According to the most recent annual report of the Intergovernmental Panel on Climate Change, abiotic factors such as surface temperatures are predicted to increase from 1.5 to 4.8º C till the end of the century (IPCC, 2013). As such, most of the models developed to estimate impacts of climate changes only consider the influence of abiotic conditions on the potential area of occurrence of single species, i.e., on their fundamental niche (e.g. Jiménez-Valverde *et al.*, 2008; Elith & Graham, 2009). However, depending on the species, geographical location and type of interaction, the effects of future changes on the distribution of species can also be strongly influenced by biotic interactions (e.g. Araújo & Luoto, 2007; Gilman *et al.*, 2010). For example, interspecific competition may be particularly relevant at the limit of distribution of many species since populations at range borders have often to cope with less suitable abiotic conditions (Hoffman & Blows, 1994; Guo *et al.*, 2005). As such, predictions of future climatic changes can be more accurate if following a previous clarification of the influence of biotic interactions on the niche space of species.

The present study was intended to investigate the interaction effects between biotic (competition) and abiotic factors (environmental conditions) in determining the distribution of two water shrew species, the Mediterranean water shrew, *Neomys* *anomalus* Cabrera, 1907, and the Eurasian water shrew, *Neomys fodiens* (Pennant, 1771), at their south-western range limit. Because these two species are morphologically similar and have similar ecological requirements, a working hypothesis considered that their space niches could be influenced by competitive interactions. Studies on microhabitat analysis and antagonistic behaviour showed that *N. fodiens* dominates over *N. anomalus* (Krushinska & Rychlik, 1993; Rychlik, 2000; Rychlik & Zwolak, 2006). Thus, in regions where both species overlap the competitive exclusion of *N. anomalus* from areas occupied by *N. fodiens* could be expected (on the contrary, in allopatric areas where only *N. anomalus* occurs, a competitive release effect could be revealed by the occurrence of *N. anomalus* in environmental conditions usually occupied by *N. fodiens*). The distribution of these two species was also forecast under different climate scenarios in order to investigate changes of the potential areas of competitive release and competitive exclusion.

Material and Methods

Species and study area

N. anomalus and *N. fodiens* are close related species, both adopting a semiaquatic lifestyle. They occur in wet areas such as banks of small rivers, lakes and swamps (Spitzenberger, 1990; Rychlik, 2000). The two species share morphological adaptations to aquatic habitats. They have a dense waterproof pelage, fringes of hairs on the hind feet and a keel of stiff hairs along the tail (Churchfield, 1998). However, these adaptations are more prominent in *N. fodiens*, which is also slightly larger than *N. anomalus* (*N. fodiens* – body mass 16-21 g, *N. anomalus* - 10-18 g; Spitzenberger, 1990). Both species have a wide distribution across Eurasia (Fig. 1). However, *N. anomalus* is patchily distributed along central and southern Europe and Asia Minor, whereas *N. fodiens* is continuously distributed throughout most of Europe, Russia, west China and northwest Mongolia (Hutterer, 2005). Thus, allopatric populations of *N. fodiens* are more frequently found in northern regions and cover approximately 89.6% of its extensive range whereas allopatric populations of *N. anomalus* are mainly located in southern regions and cover only ca. 43% of its range (Kryštufek *et al*., 2000; Fig. 1). The overlap area is mainly located in central and southern Europe (Fig. 1). This study is focused on the Iberian Peninsula because this region represents the south-western limit for both species.

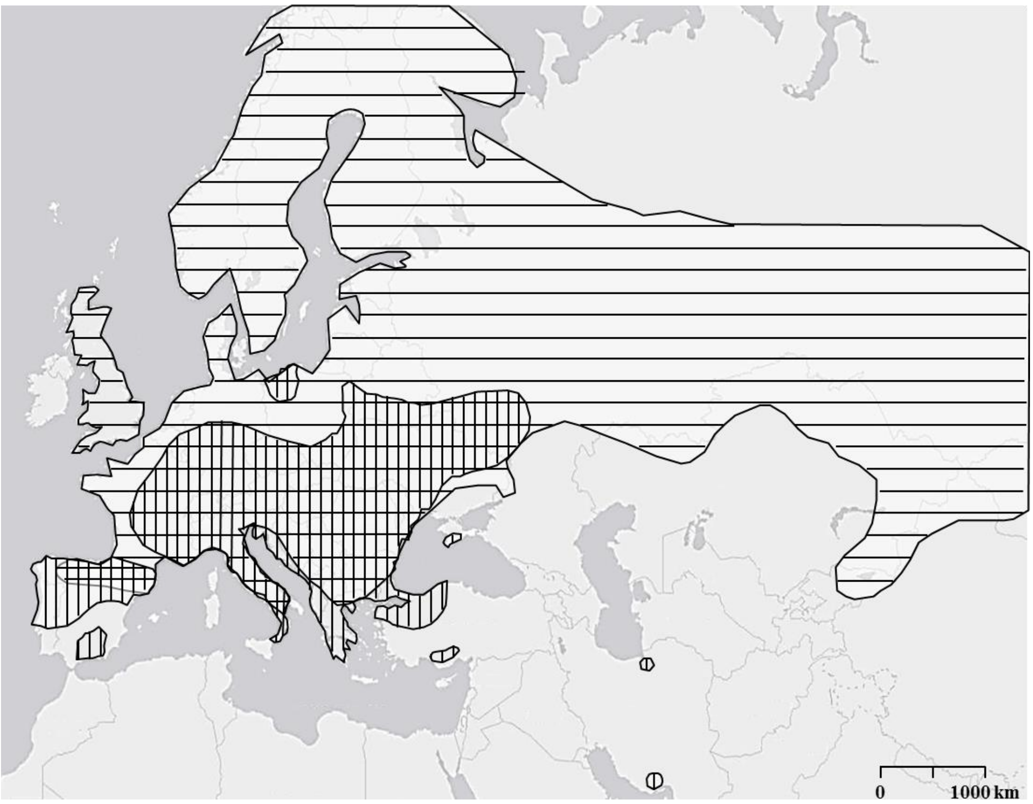

Figure 1. Geographical distribution of *Neomys anomalus* (vertical hatching) and *N. fodiens* (horizontal hatching).

The Iberian Peninsula is characterized by two main phytogeographic and climatic regions, the Eurosiberian and the Mediterranean regions (Moreno *et al*., 1990). The Eurosiberian region extends through northern Portugal, the Galician Massif, Cantabrian Mountains and the western and central Pyrenees. This region is characterized by a vegetation mainly composed of deciduous oak forests (UNESCO, 2003), an oceanic climate lacking a dry season and a constant precipitation regime (Rodriguez-Puebla *et al*., 1998). The Mediterranean region corresponds to the remaining part of the Peninsula. The geomorphology of this region also includes several mountain chains such as the “Sistema Ibérico” (central/eastern Iberia), the “Sistema Central” (central Iberia) and the “Sistema Bético” (southern Iberia). In general, the Mediterranean vegetation is composed by evergreen trees such as oak and cork oak forests. The climate is characterized by mild wet winters and dry summers and the potential occurrence of drought periods (Rodriguez-Puebla *et al*., 1998).

Species occurrence data

Occurrence data for the study species was obtained from the online portal Global Biodiversity Information Facility (GBIF), from field-trips and museum collections (see Appendix S1 in Supporting Information). Although the study area is the Iberian Peninsula, to avoid an underestimation of species space niches (Thuiller *et al*., 2004) the dataset was completed with records from peripheral countries: France, north-western Italy, south-western Switzerland and Andorra collected from GBIF (Table 1). Records that had no decimal latitude and longitude information were geo-referenced using the following geographical tools: Geolocate, Geonames and Google maps.

Table 1. Number of occurrence records of *Neomys anomalus* and *N. fodiens* by country of collection gathered from GBIF and other sources for the modelling procedure.

	N. anomalus	***N. fodiens***
Portugal	52	0
Spain	863	693
Andorra	0	7
France	57	714
Switerzland	7	22
Italy	1	1
Total	**980**	**1437**

Base environmental variables

The base environmental variables used were the available 19 bioclimatic variables of the WorldClim database (Hijmans *et al.*, 2005) at the resolution of 0.083333º (Table 2). The coverage was cut to the extent of the “enlarged” study area (35º to 50º latitude; -10º to 10ºlongitude), using R 3.0.1 software (R-Core-Team, 2013). Model projections for the next decades were based on global climate data of two representative concentration pathways (RCPs; RCP 2.6 and RCP 8.5) that were considered in the Fifth Assessment IPCC (IPCC, 2013). Global-mean surface temperature increases 1.5º C by 2100 for the lowest of the four RCPs and around 4.5ºC for highest concentration pathway, relative to pre-industrial levels. As such, RCP 2.6 was considered as the mild climate change scenario and the RCP 8.5 as the severe climate change scenario. The bioclimatic variables based on these pathways for the years 2050 and 2070 were download from the WorldClim database (GISS-E2-R), with the same resolution as for variables from current conditions.

Table 2. Base environmental variables downloaded from Worldclim with 0.083333º of resolution.

Code	Bioclimatic layer
Alt	Altitude (elevation above sea level)
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (max temp - min temp)
BIO3	Isothermality (BIO2/BIO7) (* 100)
BIO4	Temperature Seasonality (standard deviation *100)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO7	Temperature Annual Range (BIO5-BIO6)
BIO8	Mean Temperature of Wettest Quarter
BIO9	Mean Temperature of Driest Quarter
BIO10	Mean Temperature of Warmest Quarter
BIO11	Mean Temperature of Coldest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter

Modelling

Each species' potential distribution was modelled using the MaxEnt algorithm (version 3.3.3k; Phillips *et al*., 2006) across the "enlarged" area of study. Occurrence points that felt within the same grid cell (same resolution of the environmental variables) were counted as single points. The MaxEnt model was run with all of the presence records (100% training), the regularization multiplier was set to 1 and the maximum number of iterations was set to 500. To avoid an over-fitting of the relationship between the presence records and the environmental variables, instead of using all available variables, predictor variables should be selected. Therefore, a preliminary model for each species was computed using the available 19 bioclimatic variables in order to analyse the contribution of the variables in the produced MaxEnt predictions, using percent contribution values and alternated estimated of variable importance with jackknife tests. Furthermore, the ecological requirements of the species modelled were also taken into account. Finally, a model for each species was computed using the most meaningful and uncorrelated variables as predictors. The produced maps of probability of suitable conditions were converted into binary presence/absence maps using an equal training sensitivity and specificity. The area under the Receiver Operating Characteristic curve (AUC) values were used to determine the accuracy of the model (Fielding & Bell, 1997). AUC values higher than 0.7 are usually seen as indicating useful predictions (Swets, 1988). The sympatric area between *N. anomalus* and *N. fodiens* in the Iberian Peninsula was obtained by overlapping the two species' potential distributions using the overlay function of the raster package in R (Hijmans, 2012).

In order to estimate potential distribution changes for the two species given climate change in this century, MaxEnt models were projected onto the future climate scenarios (mild-RCP 2.6 and severe-RCP 8.5) for the years 2050 and 2070. Differences in suitable areas between current conditions and future conditions were calculated, for the two species and the area of overlap in species potential distributions.

Environmental analysis

The influence of competitive interactions on the current distribution of the two species were analysed by testing intra- and interspecific differences on the environmental variables showing the highest contribution for each species' model. Mean annual temperature and mean annual precipitation, were also considered since these two variables had already been used with suitable results in similar studies (e.g. Anderson *et al*., 2002). For each species, occurrence points that were located inside the predicted distributions and inside the overlap area were selected. For each of these subsets of occurrence data the corresponding values of the variables of interest, mentioned above, were extracted using extract function of the raster package in R.

Non-parametric statistics (two-tailed Mann-Whitney U-test) were used to test for interspecific differences and differences between allopatric or sympatric populations of the same species. All statistical tests were performed using IBM SPSS Statistics 19 (SPSS Inc., 2010), considering a minimum *P* value of 5%.

Results

Species distribution models

The dataset compiled a total of 980 presence records for *N. anomalus* and 1437 presence records for *N. fodiens* (Table 1). In Fig. 2, the geographical distribution of collected presence records from the Iberian Peninsula is shown. The grey area in the maps shows the predicted current distribution for both species in the Iberian Peninsula. Both models produced had AUC values greater than 0.7, thus validating the predictions (Swets, 1988). Information on the evaluation and validation of models can be found in Appendix S2.

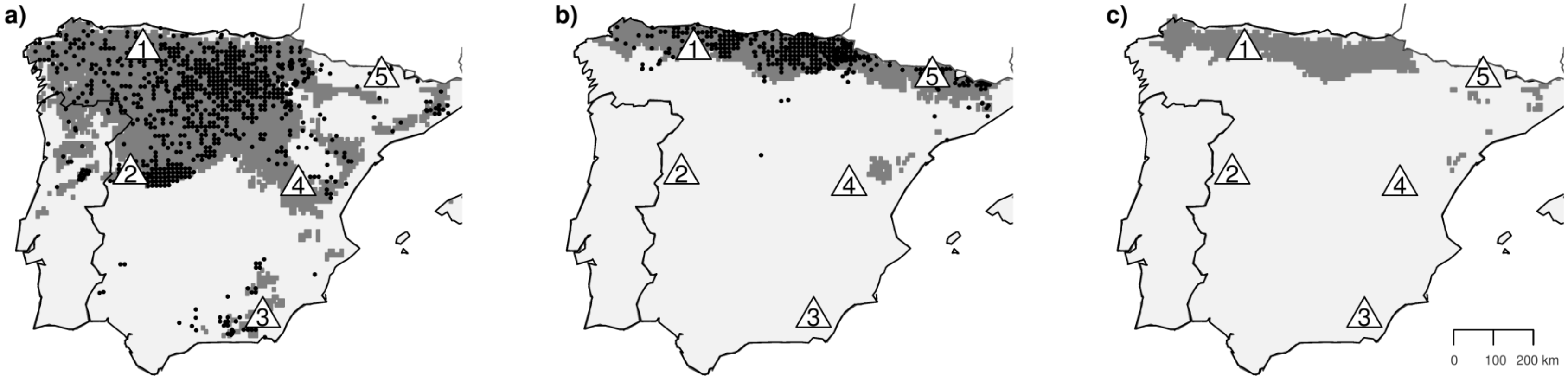

Figure 2. Occurrence localities (black points) and predicted potential distribution (grey area) for (a) *Neomys anomalus* (MaxEnt model with AUC value of 0.887) and (b) *N. fodiens* (MaxEnt model with AUC value of 0.854) in the Iberian Peninsula. Predicted potential area of sympatry between *N. anomalus* and *N. fodiens* in the Iberian Peninsula (c), indicated by the overlap between the produced models of potential distribution of the two species. Numbers inside triangles indicate the main mountain systems in the Iberian Peninsula: 1 - Cantabrian Mountains; 2 - “Sistema Central”; 3 - “Sistema Bético”; 4 - “Sistema Ibérico”; 5 - Pyrenees. Maps are in the geographic coordinate system (on the WGS84 ellipsoid).

The variables selected to model each species are presented in Table 2. The three most significant environmental variables contributing to model the distribution of *N. anomalus* were: altitude (38.4%), the mean temperature of the driest quarter (38.0%) and precipitation seasonality (13.5%). The predicted current area of occurrence of *N. anomalus* comprises 29.7% of the Iberian Peninsula. The model predicted a continuous distribution from the north of the Iberian Peninsula (northern Portugal, Cantabrian Mountains) to the mountain range of the “Sistema Central” (Fig. 2). The model also predicted several isolated areas of occurrence, the most significant being located in the Pyrenees and in the “Sistema Bético”.

The distribution model of *N. fodiens* was mainly determined by the mean temperature of the warmest quarter (44.9%), the mean precipitation on the warmest quarter (18.8%) and the mean precipitation on the driest quarter (16.2%). The model estimated an area covering 12.5% of the total area of Iberian Peninsula. The occurrence area predicted for *N. fodiens* is restricted to the north of the Iberian Peninsula, including the Cantabrian Mountains and the Pyrenees. The model also predicts the occurrence of the species in an isolated region located on the “Sistema Ibérico”. In fact, the species was already captured on that mountain system but slightly southern from the predicted area (Fig. 2).

The two species’ distribution models revealed a potential overlapping area mainly restricted to the Cantabrian Mountains and few isolated areas in the Pyrenees, comprising 5.9% of the Iberian Peninsula. The sympatric area represents 19.9% of the total predicted area of *N. anomalus* and 47.1% of the estimated distribution of *N. fodiens*. As such, allopatric areas where a potential competitive release may occur reach

80.1% of the predicted area for *N. anomalus* and 52.9% of the predicted area for *N. fodiens*.

Environmental analysis

Significant differences were found between all environmental variables characterizing the occurrence locations of *N. anomalus* and *N. fodiens* (*P* values ranging from 0 to 0.039). The results showed that *N. anomalus* occurs in warmer and drier regions than *N. fodiens*. However, the two species still show a significant overlap in their tolerances to temperature and precipitation parameters. Average elevation of occurrence locations showed the highest levels of inter- and intraspecific variability (Table 3). The environmental conditions characterizing the locations of allopatric records of *N. anomalus* were significantly different from those characterizing areas of potential sympatry with *N. fodiens* (*P* values ranging from 0 to 0.001). However, the only parameter differing in the direction predicted by competitive release was elevation (*P* = 0.001). In allopatry *N. anomalus* was found on average at higher altitudes than when in sympatry with *N. fodiens*.

Neomys fodiens is the species inhabiting the highest altitudes in the Iberian Peninsula as revealed by the results obtained in regions where only this species occurs. Allopatric regions of *N. fodiens* are also characterized by lower temperatures than sympatric regions (mean annual temperature, *P* = 0.007; temperature driest quarter, *P* = 0.0001) and by higher rates of precipitation, particularly when considering the warmest and the driest periods of the year (precipitation warmest quarter, *P* = 0.0001; precipitation driest quarter, *P* = 0.013). Overall, the environmental conditions where *N. fodiens* occurs do not match the predictions of competitive release nor competitive exclusion, since the environmental characteristics of allopatric populations are all significantly different from those obtained for *N. anomalus* (*P* values = 0.000).

Table 3. Mean ± standard error for selected environmental variables at known collection localities of *Neomys anomalus* and *N. fodiens* used in this study. Mann-Whitney U test values are given for intraspecific and interspecific comparisons (* $P < 0.05$; ** $P < 0.001$; n.s., not significant).

Environmental variables	***N. anomalus*** **allopatry**	**U value**	***N. anomalus*** **sympatry**	***N. anomalus*** **all records**	**U value**	***N. fodiens*** **all records**	***N. fodiens*** **allopatry**	**U value**	***N. fodiens*** **Sympatry**
Mean annual temperature (Cº)	11.29 ± 1.86	50.43**	10.75 ± 1.45	11.17 ± 1.79	148.81*	10.57 ± 2.54	9.52 ± 3.77	19.09*	11.07 ± 1.40
Temperature warmest quarter (Cº)	19.00 ± 1.88	25.34**	17.26 ± 1.31	18.62 ± 1.91	94.22**	17.03 ± 2.14	16.22 ± 3.22	18.12 n.s.	17.43 ± 11.59
Temperature driest quarter (Cº)	17.73 ± 4.35	30.45**	16.67 ± 2.45	17.50 ± 4.04	99.91**	15.07 ± 5.51	11.02 ± 7.79	20.61**	17.02 ± 2.06
Mean annual Precipitation (mm)	649.60 ± 261.05	100.37**	909.49 ± 149.33	707.08 ± 263.85	265.41**	1006.22 ± 172.66	1016.10 ± 207.90	15.08. n.s	1001.48 ± 153.18
Precipitation Seasonality (mm)	31.55 ± 10.25	30.24**	23.85 ± 5.36	29.85 ± 9.92	84.39**	22.29 ± 5.39	21.05 ± 7.38	19.42*	22.89 ± 3.99
Precipitation warmest quarter (mm)	97.92 ± 37.81	111.79**	163.96 ± 24.82	112.52 ± 44.73	291.92**	195.54 ± 42.96	215.11 ± 56.82	10.54**	186.16 ± 30.33
Precipitation driest quarter (mm)	91.25 ± 34.11	113.20**	157.19 ± 23.85	105.83 ± 42.2	291.41**	184.30 ± 42.33	195.59 ± 58.24	13.76*	178.89 ± 30.74
Elevation (m)	893.59 ± 342.58	43.93**	763.86 ± 294.93	864.90 ± 336.80	125.24**	772.07 ± 484.56	975.36 ± 704.14	13.05*	674.71 ± 286.06

Climate change scenarios

Considering both climate change scenarios, losses in climatically suitable area are expected to occur for both species, being aggravated from 2050 to 2070 (Fig. 3). In 2050, *N. anomalus* is predicted to lose 11.3% of its suitable area under the mild scenario and 19.3 % under more severe conditions. In both scenarios, the estimated alterations in the suitable area for *N. anomalus* follow a northward range contraction (Fig. 3). The area predicted to be more significantly affected is the central mountain system of the Iberian Peninsula ("Sistema Central"), with reductions in suitable area occurring both in central Portugal and central Spain (Fig. 3). A dislocation of the suitable area of *N. anomalus* to the Pyrenees is also estimated under both climate change scenarios. In 2070, the losses of the suitable area for the occurrence of *N. anomalus* increase to 16.5% and 29.1% under the mild and the severe climate change scenarios, respectively. Such reductions follow the trend described to 2050 but with a slightly recovery of suitable areas in the central system, namely in Portugal (Fig. 3).

The decline in the suitable climatically area of *N. fodiens* in the Iberian Peninsula due to future climate changes will imply a northeaster contraction. In 2050 the model estimates a reduction of the suitable area of *N. fodiens* of 25.3% under the mild scenario and a reduction of 44.4% under the severe scenario. The region expected to lose more significantly suitable areas for the occurrence of *N. fodiens* is the Cantabrian Mountains (Fig. 3). In 2070, the losses of climatically suitable area of *N. fodiens* in the Iberian Peninsula are quite similar to the 2050 predictions in the mild scenario (25.6%) but increase significantly in the severe scenario (60.2%). Again, the contraction of the climatically suitable area of *N. fodiens* is expected to occur mainly in the Cantabrian Mountains but also in the Western and Central Pyrenees (Fig. 3).

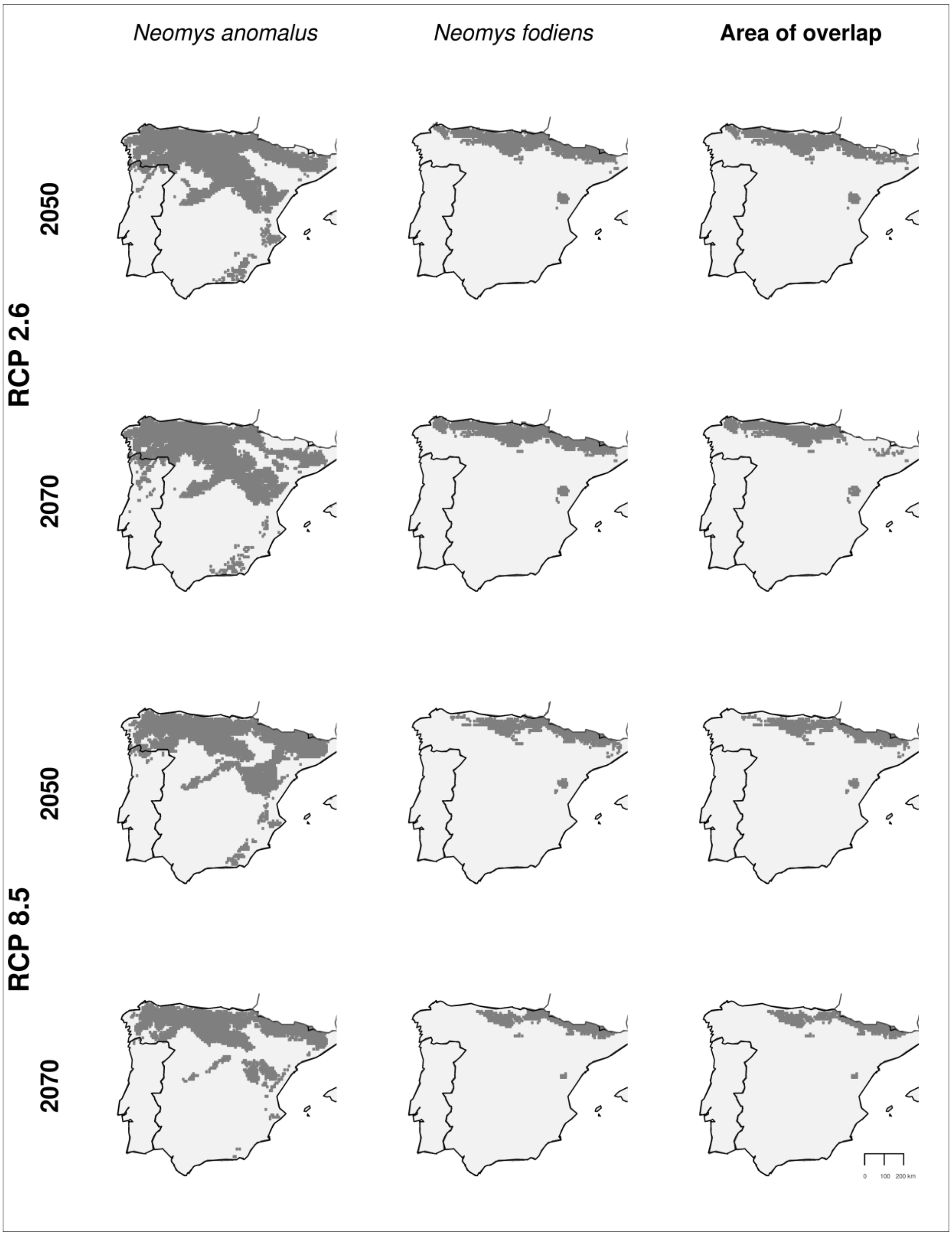

Figure 3. Future potential distribution (grey area) for (a) *Neomys anomalus* and (b) *N. fodiens* in the Iberian Peninsula under different climate change scenarios (RCP 2.6 – mild and RCP 8.5 – severe) for years 2050 and 2070. (c) Future potential area of sympatry between *N. anomalus* and *N. fodiens* in the Iberian Peninsula considering the same climate change scenarios for 2050 and 2070. Maps are in the geographic coordinate system (on the WGS84 ellipsoid).

Considering the two periods analysed, the contractions of the climatically suitable area of *N. anomalus* and *N. fodiens* in the Iberian Peninsula may also promote changes in the overlapping area occupied by both species (Fig. 3). Under the mild scenario the overlapping area between the two species is estimated to increase from the current 5.9% to 7.1%. Under the more severe scenario, the increase in the overlapping area is slightly smaller (6.1%) probably due the more significant reductions in the climatically suitable areas of both species. With the reduction of suitable climatically areas for both species projected for 2070, the overlapping area is expected to decrease, reaching 5.5% under the mild scenario and 4.7% under the severe scenario.

The expected changes in the extension and location of climatically suitable areas for *N. anomalus* and *N. fodiens* in the Iberian Peninsula under different climate change scenarios will also imply alterations in the areas of potential competitive release and competitive exclusion. However, those changes are estimated to be more significant in *N. fodiens* than in *N. anomalus*. In 2050, the competitive release area of *N. anomalus* is estimated to comprise 73.3% of the total suitable climatically area under both climate change scenarios. In 2070, the competitive release area of *N. anomalus* is expected to increase under the mild scenario (78.1%) but to remain similar to 2050 under the severe scenario (73.2%). All estimations of suitable climatically areas imply a reduction in the allopatric area of *N. fodiens*, with a consequent increase of the sympatric area with *N. anomalus*. In 2050, only 24.8% of the climatically area is expected to be suitable exclusively for *N. fodiens* under the mild scenario. The reduction of the allopatric area of *N. fodiens* is expected to be even more evident under the severe scenario (8%). Consequently, under such environmental conditions almost all climatically suitable area for the occurrence of *N. fodiens* is expected to be shared with *N. anomalus*. In 2070, the proportion of allopatric and sympatric areas of *N. fodiens* is estimated to be relatively similar (41.6 and 58.4%, respectively) under mild conditions but under more severe conditions a significant reduction of the allopatric area is again projected (5.4% allopatry versus 94.6 % sympatry).

Discussion

The influence of abiotic conditions and biotic interactions on the distribution of water shrews in the Iberian Peninsula differs between *N. anomalus* and *N. fodiens*. The two species overlap in their environmental tolerances but *N. anomalus* occurs in a wider range of climatic conditions. Moreover, interspecific competition influences the distribution of *N. anomalus* but it does not affect the distributional pattern of *N. fodiens*. As such, the hypothesis under which the realized niche is influenced by competitive interactions is supported only for *N. anomalus*. A contraction of the potential distribution of both species in the Iberian Peninsula is expected under future climate changes, although being more evident for *N. fodiens*.

The overall geographic distribution of the two water shrew species suggest that they have different tolerances to ambient temperatures and precipitation regimes since *N. fodiens* occurs farther north than *N. anomalus* (Hutterer, 2005). The predicted distribution of water shrews in the Iberian Peninsula is in accordance with the general distributional pattern of both species, i.e., *N. fodiens* is confined to north of the Peninsula while *N. anomalus* also occurs in central and southern areas. Such distributional patterns are strongly influenced by climatic conditions. Iberian *N. anomalus* occurs on average in warmer and drier places than *N. fodiens*. However, the two species still show some overlap in their tolerance to ambient temperatures, being mainly discriminated by the precipitation regime. *N. anomalus* occurs in places with a lower rainfall rate than *N. fodiens*, not only considering the average annual precipitation but also during the driest periods of the year. The occurrence of *N. fodiens* in areas with a higher annual precipitation is probably related with a stronger dependence of this species for aquatic resources. Similar relationships had already been found in other semiaquatic species such as the Iberian Desman (*Galemys pyrenaicus*; Morueta-Holme *et al*., 2010)

The climatic variables of the dry season have been considered the most important parameters discriminating the two climatic and phytogeographic areas in the Iberian Peninsula: the Eurosiberian region and the Mediterranean region (Moreno *et al*., 1990). It can thus be concluded that the precipitation regime plays a crucial role determining the distribution of water shrews in the Iberian Peninsula. The ability of *N. anomalus* to cope with unstable precipitation regimes allows this species to occur in both climatic regions, whereas *N. fodiens* is restricted to the Eurosiberian zone. In fact, the exclusive presence of *N. fodiens* in the Eurosiberian zone of the Iberian Peninsula had already been observed by Sans-Fuentes and Ventura (2000) in Catalonia (north-eastern Spain). These authors found that the main factor affecting the distribution of *N. fodiens* in that region was the mean annual precipitation, with the occurrence of the species being dependent on relatively high precipitations. The single confirmed presence of *N. fodiens* in a typical Mediterranean climatic area was reported by Aloise and colleagues (2005) in southern Italy. This finding seems to be an exception in the distribution of *N. fodiens* since it extends the southern limit of the species in Europe by 2º in latitude.

The distribution model adopted predicts the occurrence of *N. anomalus* in a large and continuous area, ranging from northern Portugal to northern and central Spain. But it also predicts the occurrence of this species in several small areas located in central Portugal, and southern and south-eastern Spain. Such fragmented pattern of distribution contrasts with the single and continuous estimated area for *N. fodiens* along northern Spain. The divergence found here between the patchy distribution pattern of *N. anomalus* and the continuous distribution of *N. fodiens* can also be found along the remaining distribution range of both species (Hutterer, 2005). Regional distribution studies have been more frequently performed for *N. fodiens* than for *N. anomalus*. Those studies have shown that in northern Europe *N. fodiens* is a relatively common species, evenly distributed over large areas (e.g. Hanski & Kaikusalo, 1989; Greenwood *et al*., 2002). On the contrary, the occurrence of *N. anomalus* is relatively rare and discontinuous even in the most southern areas (e.g. Sofianidou & Vohralik, 1991; Bego *et al*., 2008; Anděra & Zbytkovský, 2009). Two main reasons can explain the divergence found in the spatial patterns of the two species. First, optimal wet habitats for water shrews are more likely to be found in the Eurosiberian region than in the Mediterranean zone, due to their significant difference in terms of precipitation rate (UNESCO, 2003). But because *N. anomalus* also occurs partly in the Eurosiberian region overlapping with *N. fodiens,* its access to wet areas is probably restricted by interspecific competition.

In the overlapping zone of the Iberian Peninsula, the access of *N. anomalus* to habitats at higher altitudes is conditioned by the presence of *N. fodiens*, rather than by environmental tolerance to ambient temperature or precipitation. Such competitive exclusion of *N. anomalus* from high altitude habitats, due to the presence of *N. fodiens*, has already been observed in field investigations performed by Torre and Tella (1994) in the Pyrenees. These authors observed that *N. fodiens* excludes *N. anomalus* from locations close to streams or other water courses at altitudes above 1000 m. Such habitat segregation influenced by altitude was later confirmed in other southern regions where *N. fodiens* and *N. anomalus* are also broadly sympatric (Slovenia, Bosnia, Macedonia; Kryštufek *et al*., 2000). In these regions, *N. fodiens* occurs in vertical habitats whereas *N. anomalus* nearly always inhabits horizontal habitats (Kryštufek *et al*., 2000). The exception to this pattern of habitat selection was detected only for allopatric *N. anomalus* also occurring in vertical habitats (Kryštufek *et al*., 2000). In fact, the two species seem to occur at different altitudes along their geographic range, with *N. fodiens* reaching elevations of 2500 m, whereas *N. anomalus* do not occur above 1850 m (Spitzenberger, 1990; Kryštufek *et al*., 2000).

The increase in the average elevation of allopatric *N. anomalus* in the Iberian Peninsula is indicative of competitive release which may also allow the occurrence of the species in optimal wet habitats. Recent studies on habitat preferences of different allopatric populations of *N. anomalus* in Portugal have consistently demonstrated the preference of these shrews for microhabitats near water courses (Rychlik & Ramalhinho, 2005; J.T. Tapisso, L. Rychlik, M.G. Ramalhinho and M.L. Mathias, submitted). A behavioural study testing Portuguese allopatric *N. anomalus* revealed an increase in swimming and diving efficiency when compared with *N. anomalus* occurring in sympatry with *N. fodiens* (Tapisso *et al*., 2013). Such a result is also congruent with a competitive release effect considering the absence of *N. fodiens.* Since the presence of *N. anomalus* in southern Spain has been mainly confirmed by skull identifications from barn owl pellets (López-Fuster *et al*., 1990), further ecological investigations will be important to determine the influence of competition (or lack of it) on the distribution of this species in southern regions. The competitive release of allopatric *N. anomalus* has been identified in other European regions by morphological studies demonstrating an increase in its body size towards *N. fodiens* (Spitzenberger, 1990; Rácz & Demeter, 1998; Kryštufek & Quadracci, 2008).

The distribution of water shrews in the Iberian Peninsula is predicted to contract under climate change scenarios. Such result is congruent with other studies reporting that among mammals, shrews are the species more vulnerable to future climate changes (Thuiller *et al*., 2011). Nevertheless, the reduction of the ranges of *N. anomalus* and *N. fodiens* in the Iberian

Peninsula under the most updated climate change scenarios is now less pronounced than previous estimations (Araújo *et al*., 2012). Araújo and colleagues (2012) described variations in the distributional area of *N. anomalus* in the Iberian Peninsula of 50 to 90% in the period from 2020 to 2050 and 83 to 90% from 2050 to 2080. Similarly to the predictions showed in this study, they also estimated variations in the potential area of distribution of *N. fodiens* higher than in *N. anomalus,* varying between 28 to 96% in the period from 2020 to 2080 and varying between 74 to 99% from 2050 to 2080. The higher vulnerability to climate changes of species more adapted to the Eurosiberian region, like *N. fodiens,* than species adapted to the Mediterranean region, such as *N. anomalus*, is congruent with what has been already observed in other groups, namely amphibians and reptiles (Carvalho *et al*., 2010). In such ectothermic animals, species with Atlantic affinities occurring in northern regions of the Iberian Peninsula are predicted to be more vulnerable than Mediterranean species (Carvalho *et al*., 2010).

The difference in the vulnerability to future climate changes between *N. anomalus* and *N. fodiens* will alter the proportion between areas of allopatry and sympatry of the two species in the Iberian Peninsula and, consequently, the areas of potential competitive release and competitive exclusion. The area of potential competitive release of *N. anomalus* is expected to decrease under expected climate changes. As such, the potential effects of future climate changes in the distribution of *N. anomalus* in the Iberian Peninsula may be even higher due to an increase in the area of competitive exclusion with *N. fodiens*. The changes in the proportion between allopatric and sympatric areas are, however, much more evident for *N. fodiens*. In this case, most of the potential occurrence area of *N. fodiens* in the Iberian Peninsula is predicted to be sympatric with *N. anomalus*, which may increase the competition between the two species in the most northern regions of the Iberian Peninsula. Since *N. fodiens* dominates the aquatic habitats in the presence of *N. anomalus*, it may be expected that the potential increase of competition for optimal aquatic habitats, due climate changes, will have a higher impact on *N. anomalus* than in *N. fodiens*. However, both water shrew species may also be affected by the competition with other semiaquatic species like *G. pyrenaicus*. A recent study evaluating the potential impacts of future climate changes in the distribution of *G. pyrenaicus* in the Iberian Peninsula have demonstrated that the distribution of this species will also suffer a northward contraction being restricted to small areas in the north of the Iberian Peninsula (Morueta-Holme *et al.*, 2010). Such areas overlap in large extent with the future distribution of *N. fodiens* in the Iberian Peninsula, which suggests that *N. fodiens* may be more affected by future competitive interactions with *G. pyrenaicus* than *N. anomalus*.

In general, biotic interactions are considered to play a minor role determining species distributions at a regional and continental scale (Pearson & Dawson, 2003). However, depending on the species, the type of interaction, and the method considered, purely climate-based modelling may not be sufficient to explain current or future species distributions (Araújo & Luoto, 2007). The application of a niche-based modelling approach to competitor species such as water shrews allows better understanding of their distribution in the Iberian Peninsula and considerable increases the accuracy of potential impacts of future climate changes.

Acknowledgements

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Supporting Information

Appendix S1 Sources of occurrence data.

Appendix S2 Variable selection and model validation.

Appendix S1 – Sources of occurrence data used in the modeling procedures

Neomys anomalus

- **Data accessed through GBIF data portal**

Banc de dades de biodiversitat de Catalunya: Banc de dades de biodiversitat de Catalunya-VerteCat, http://data.gbif.org/datasets/resource/2473 (2013-02-22)

Banco de Datos de Biodiversidad de la Comunitat Valenciana (http://bdb.cma.gva.es), http://data.gbif.org/datasets/resource/8004 (2013-02-22)

Biological Station of Doñana (CSIC): Estacion Biologica Donana - CSIC, Mammal Collection, http://data.gbif.org/datasets/resource/1759 (2013-02-22)

Division of Mammals, Research and Collections Information System, NMNH, Smithsonian Institution: NMNH Vertebrate Zoology Mammals Collections, http://data.gbif.org/datasets/resource/1837 (2013-02-22, 2013-03-06)

European Distributed Institute of Taxonomy (EDIT) 2007-2010 - All Taxa Biodiversity Inventory + Monitoring (ATBI+M) in "Parc National du Mercantour" (France) and "Parco Naturale delle Alpi Marittime" (Italy), http://data.gbif.org/datasets/resource/7949 (2013-02-22, 2013-03-06)

Field Museum: Field Museum of Natural History (Zoology) Mammal Collection , http://data.gbif.org/datasets/resource/14349 (2013-02-22, 2013-03-06)

Field Study Group of the Dutch Mammal Society: Field Study Group of the Dutch Mammal Society (NL) - 2003 - Mammal Survey Alvao Natural Park, Portugal, http://data.gbif.org/datasets/resource/14143 (2013-02-22)

Field Study Group of the Dutch Mammal Society: Field Study Group of the Dutch Mammal Society (NL) - 2009 - Mammal Survey Serra da Estrela, Portugal, http://data.gbif.org/datasets/resource/14144 (2013-02-22)

Mammal Collection of the Spanish Museo Nacional de Ciencias Naturales, online database (www.gbif.es), http://data.gbif.org/datasets/resource/302 (2013-02-22)

Mammal Research Institute, Polish Academy of Sciences: Mammal Collection, http://data.gbif.org/datasets/resource/10971 (2013-02-22, 2013-03-06)

Netherlands Biodiversity Information Facility (NLBIF): Zoological Museum Amsterdam, University of Amsterdam (NL) – Mammalia, http://data.gbif.org/datasets/resource/12489 (2013-02-22, 2013-03-06)

Palomi, J. L, Gisbert, J. y Blanco J. C. (eds.) 2007. Atlas y Libro Rojo de los Mamíferos Terrestres de España. Dirección General para la Biodiversidad. SECEM-SECEMU. Madrid, 588 pp., http://data.gbif.org/datasets/resource/3369 (2013-02-22)

Senckenberg: Collection Mammalia SMF, http://data.gbif.org/datasets/resource/8313 (2013-03-06)

SPN - Service du Patrimoine naturel, Muséum national d'Histoire naturelle, Paris: Inventaire National du Patrimoine Naturel (I002): Mammifères de France, http://data.gbif.org/datasets/resource/14281 (2013-02-22)

ZOOTRON - 5 database at Museum of Zoology, University of Navarra (MZNA – UNAV.ES; A.H. Ariño), http://data.gbif.org/datasets/resource/791 (2013-02-22)

- **Other sources**

Museu Nacional de História Natural e da Ciência (MNHN), Lisbon, Portugal (Specimens' Catalog numbers: 81.08.01 – 8499; 74.10.02 – 8500; 83.07.04 – 8502; 83.07.05 – 8503; 83.07.06 – 8504; 83.07.07 – 8505; 84.07.08 – 8506; 84.07.09 – 8507; 85.02.10 – 8508; 85.02.11 – 8509; 87.10.12 – 8510)

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Neomys fodiens

American Museum of Natural History (AMNH) Mammal Collection: Mammals, http://data.gbif.org/datasets/resource/8439 (2013-03-06)

Banc de dades de biodiversitat de Catalunya: Banc de dades de biodiversitat de Catalunya-VerteCat, http://data.gbif.org/datasets/resource/2473 (2013-02-22)

Biological Station of Doñana (CSIC): Estacion Biologica Donana - CSIC, Mammal Collection, http://data.gbif.org/datasets/resource/1759 (2013-02-22, 2013-03-06)

Centre d'estudis de la neu i de la muntanya d'Andorra (CENMA), Institut d'Estudis Andorrans: Vertebrates of the Andorra , http://data.gbif.org/datasets/resource/12760 (2013-03-06)

Cornell University Museum of Vertebrates: CUMV Mammal Collection, http://data.gbif.org/datasets/resource/14897 (2013-02-22)

European Distributed Institute of Taxonomy (EDIT) 2007-2010 - All Taxa Biodiversity Inventory + Monitoring (ATBI+M) in "Parc National du Mercantour" (France) and "Parco Naturale delle Alpi Marittime" (Italy) , http://data.gbif.org/datasets/resource/7949 (2013-03-06)

Field Museum: Field Museum of Natural History (Zoology) Mammal Collection, http://data.gbif.org/datasets/resource/14349 (2013-02-22, 2013-03-06)

GBIF-Sweden: Mammals (NRM) http://data.gbif.org/datasets/resource/1041 (2013-03-06)

GBIF-Sweden: Lund Museum of Zoology (MZLU), http://data.gbif.org/datasets/resource/1533 (2013-03-06)

KU Mammal Collection: KUBI Mammalogy Collection, http://data.gbif.org/datasets/resource/14480 (2013- 02-22, 2013-03-06)

Mammal Collection of the Spanish Museo Nacional de Ciencias Naturales, online database (www.gbif.es), http://data.gbif.org/datasets/resource/302 (2013-02-22)

Michigan State University Museum (MSU) Vertebrate Collection, http://data.gbif.org/datasets/resource/541 (2013-02-22)

Museu de Ciències Naturals de Barcelona (MCNB) Chordate GBIF online database: MCNB-Cord, http://data.gbif.org/datasets/resource/9126 (2013-02-22, 2013-03-06)

Museum of Comparative Zoology, Harvard University: Museum of Comparative Zoology, Harvard University, http://data.gbif.org/datasets/resource/14100 (2013-03-06)

Museum of Southwestern Biology: MSB Mammal Collection (Arctos), http://data.gbif.org/datasets/resource/14904 (2013-02-22, 2013-03-06)

MVZ Mammal Catalog, http://data.gbif.org/datasets/resource/8121 (2013-03-06)

National Museum of Nature and Science, Japan: Mammal collection of National Museum of Nature and Science, http://data.gbif.org/datasets/resource/614 (2013-03-06)

Netherlands Biodiversity Information Facility (NLBIF): Zoological Museum Amsterdam, University of Amsterdam (NL) – Mammalia, http://data.gbif.org/datasets/resource/12489 (2013-03-06)

NMNH Vertebrate Zoology Mammals Collections, National Museum of Natural History, Smithsonian Institution, 10th and Constitution Ave. N.W., Washington, DC 20560-0193 (http://www.nmnh.si.edu/), http://data.gbif.org/datasets/resource/1837 (2013-02-22, 2013-03-

06)

Palomi, J. L, Gisbert, J. y Blanco J. C. (eds.) 2007. Atlas y Libro Rojo de los Mamíferos Terrestres de España. Dirección General para la Biodiversidad. SECEM-SECEMU. Madrid, 588 pp., http://data.gbif.org/datasets/resource/3369 (2013-02-22)

Royal Belgian Institute of Natural Sciences: RBINS collections, http://data.gbif.org/datasets/resource/13690 (2013-03-06)

Senckenberg: Collection Mammalia SMF, http://data.gbif.org/datasets/resource/8313 (2013-03-06)

SPN - Service du Patrimoine naturel, Muséum national d'Histoire naturelle, Paris: Inventaire National du Patrimoine Naturel (I002) : Mammifères de France, http://data.gbif.org/datasets/resource/14281 (2013-02-22)

University of California, Davis Wildlife and Fisheries Museum, http://data.gbif.org/datasets/resource/736 (2013-02-22)

ZOOTRON - 5 database at Museum of Zoology, University of Navarra (MZNA – UNAV.ES; A.H. Ariño), http://data.gbif.org/datasets/resource/791 (2013-02-22)

Appendix S2 – Variable selection and model validation – outputs of Maxent modeling procedure

Table S1. Analysis of variable contributions in the final MaxEnt model for *Neomys fodiens.*

Variable	Percent contribution	Permutation importance
bio17	44.9	13.6
bio10	16.2	13.5
bio18	14.8	11.6
bio11	6.7	16.1
bio15	6	17.8
alt	5.4	10.5
bio2	4.7	14.9
bio9	1.3	1.9

Table S2. Analysis of variable contributions in the final MaxEnt model for *Neomys anomalus.*

Variable	Percent contribution	Permutation importance
alt	38.4	32.9
bio9	38	19.7
bio15	13.5	6
bio2	5	4.9
bio18	2.9	11.7
bio11	1.5	7.9
bio14	0.6	16.8

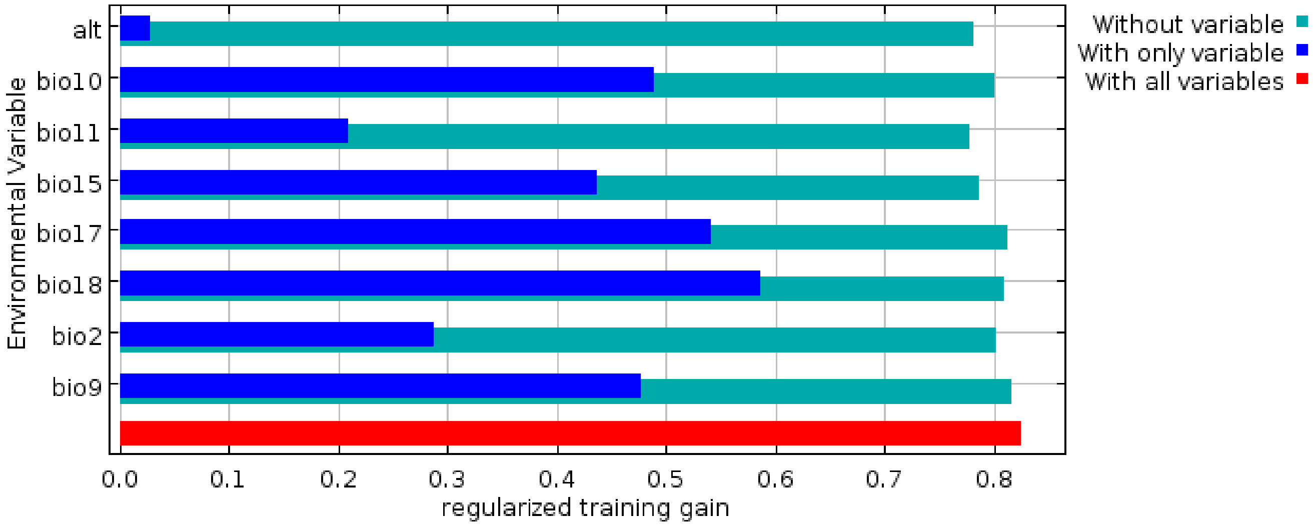

Figure S1. Jackknife of regularized training gain for *Neomys fodiens*.

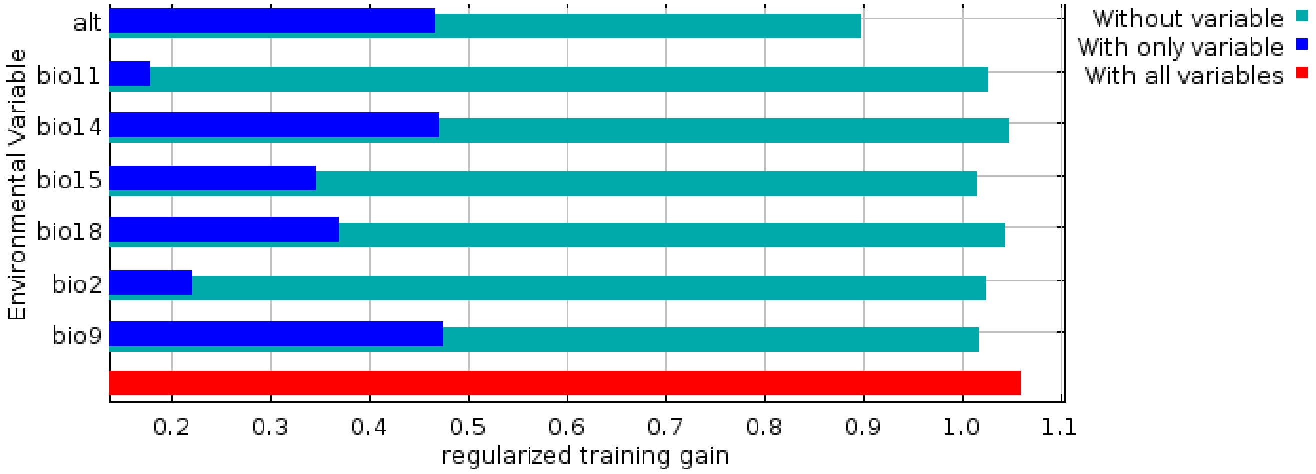

Figure S2. Jackknife of regularized training gain for *Neomys anomalus*.

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

The multidisciplinary and time-scaled approach followed in this thesis allowed to identify two main factors that may affect the distribution of the Mediterranean water shrew: severe climatic alterations, that were extremely important in the past and that are also projected to have a significant impact in the future distribution of the species; and the interspecific competition with a congeneric species, which is one of the main ecological factors regulating the current habitat preferences of the species and consequently its distributional pattern. Such conclusion was reached by i) the examination of the phylogeographic structure of the Mediterranean water shrew across its distribution range; ii) the investigation of the role of interspecific competition regulating habitat preferences and semi-aquatic behaviour; iii) the study of the potential alterations on the southern border of the distribution range under different climate scenarios.

Up to now the phylogeographic structure of the Mediterranean water shrew was poorly known due to the reduced sampling from several areas across its distribution range. The sampling effort carried out during this PhD study allowed to considerably increase the number and geographic range of samples ever used in the molecular analysis of this species. The most striking result obtained from this study was the identification of a highly divergent genetic lineage in the Iberian Peninsula. The difficulty to capture Mediterranean water shrews is also the main reason for the small number of studies concerning basic aspects of its biology and ecology, such as habitat preferences, swimming abilities, underwater foraging or diet. Most of the information given for the species is, in fact, inferred by comparative studies conducted on the more common Eurasian water shrew. As here shown, the ecological (di)similarities between the two species are strongly influenced by competitive interactions. The Mediterranean water shrew tends to adjust its ecological requirements, being more similar to the Eurasian water shrew in areas where this species is absent, confirming the competitive release hypothesis. The demonstration of the role of competition between water shrews is particularly relevant to better forecast the consequences of severe climate changes. In the case of water shrews, a rapid climate warming will not only contract the southern limit of both species but will also change the size of sympatric and allopatric areas, thus altering the proportion of areas where competitive release can occur.

The present thesis was intended to increase significantly the knowledge about one of the least studied small mammal species not only of the Portuguese but also of the whole European fauna. The data here presented provide relevant insights on the factors regulating the distribution of the Mediterranean water shrew, highlighting the need to clarify the taxonomic status of the Iberian populations. The detection of a unique genetic lineage of this species in the Iberian Peninsula makes the reported findings highly relevant from an evolutionary perspective, having important conservation implications.

6.1 Main findings

Chapter 2: Phylogeography of water shrews

The hypothesis of the existence of Mediterranean glacial refugia was only confirmed for the Mediterranean water shrew. A well-defined phylogeographic structure was found for this species, consisting in three mtDNA lineages that were named according to their geographical location. Two of these lineages were composed by haplotypes located on the Mediterranean region: the first constituted by haplotypes found in the Iberian Peninsula (Iberian lineage) and the second including haplotypes mainly from the Italian Peninsula (Italian lineage). The third lineage was composed by mtDNA haplotypes spread over a larger area, including central and eastern Europe, Balkans and the Anatolia region (Eastern lineage). The mean genetic distances calculated between the three lineages were highly variable, being the Iberian lineage highly divergent when compared with the other two lineages. The genetic distance found between this lineage and the other two lineages is higher than the genetic distance calculated in other studies between well-defined shrew species (Fumagalli et al. 1999; Castiglia et al. 2007). The mtDNA differentiation between Iberian populations and the remaining populations of the Mediterranean water shrew is congruent with the sub-specific classification proposed by Corbet (1978): *N. anomalus anomalus* Cabrera, 1907, occurring exclusively in the Iberian Peninsula and *N. anomalus milleri* Mottaz, 1907, occurring in the remaining of the species' distributional range. This result is in agreement with a potential cryptic speciation of the Mediterranean water shrew in the Iberian Peninsula.

No distinct mtDNA lineages were found among populations of the Eurasian water shrew. Instead, a star-like network phylogeny indicated a rapid post-glacial colonisation across the distribution range of the species. Such phylogeographic pattern is consistent with multiple northern and central refugia, as already postulated for other small mammal species, particularly those tolerant to cold temperatures (Deffontaine et al. 2005; Kotlík et al. 2006; Vega et al. 2010). The current southwestern limit of the Eurasian water shrew is the North of the Iberian Peninsula. Corbet (1978) also identified in this region a distinct geographic unit, *N. fodiens milleri*, distinct from the sub-species occupying the remaining part of Europe, *N. fodiens fodiens*. The lack of relationship between mtDNA haplotypes and the location from which they were sampled, including the Iberian *N. fodiens*, provided no evidence for sub-specific differentiation. As such, no evidence was found for the persistence of this species in Mediterranean glacial refugia.

The evolutionary history of the two water shrew species gives important insights on their current distribution. The identification of Mediterranean refugia only for *N. anomalus* indicates that this species is less tolerant to low temperatures than *N. fodiens*. Accordingly, *N. fodiens* is currently distributed farther north than *N. anomalus*. Additionally, the northern expansion of *N. anomalus* could be also constrained by the rapid post-glacial re-colonisation of the dominant *N. fodiens*. Low temperatures and interspecific competition may have contributed to the isolation of the Iberian and the Italian lineages of the Mediterranean water shrew.

Chapter 3: Habitat selection by the Mediterranean water shrew

The results described in this chapter showed that in present allopatric areas in the Iberian Peninsula the Mediterranean water shrew selects habitats similar to the habitats usually occupied by the Eurasian water shrew, thus displaying a habitat shift congruent with the competitive release hypothesis. All monitored specimens in Serra da Estrela were captured at a short distance from a main watercourse, the Mondego River, characterized by a water depth and a water flow usually more appropriate for the occurrence of *N. fodiens* (Rychlik 2000). The trapping success of *N. anomalus* was determined by seasonal climate-induced variations that promoted significant alterations in the morphology of the river, rather than other factors like interspecific competition with other syntopic shrews, such as the Iberian shrew, *Sorex granarius* or the Greater white-toothed shrew, *Crocidura russula*. Thus, water shrews were more abundant during periods when water depth and water flow reached minimum levels (i.e. summer and autumn) and less abundant when the water levels increased (i.e. winter and spring).

Different levels of habitat overlap were found between co-occurring shrews, depending on the habitat preferences of each species. As such, the species showing the highest habitat overlap with *N. anomalus* was *S. granarius* due to its preference for ecotone areas, which in this case corresponded to the transitional area between the river bank and a pine forest. *Crocidura russula* was the most abundant shrew species in the study plot, occurring preferentially in drier habitats. Considering the spatial distribution of species over the study plot it was possible to establish a wet-to-dry gradient of preferences: *N. anomalus*, *S. granarius* and *C. russula*, consistent with spatial segregation. Shrew movements inferred by successive recaptures indicated differences in the habitat use between species, *N. anomalus* being the only species using both river banks simultaneously. The use of both banks indicates that this species may have swimming and diving abilities similar to *N. fodiens* allowing it to forage in deep water. This is one of the first studies highlighting a habitat shift by allopatric *N. anomalus* which, in the case of the populations occurring in the Iberian Peninsula, may have important implications in the conservation planning of a potential distinct taxonomic unit.

Chapter 4: Swimming and diving behaviour of water shrews

The competitive release hypothesis was also supported by the swimming and diving behaviour of allopatric Mediterranean water shrews trapped in Serra da Estrela and tested in experimental conditions. The swimming ability of these animals resembled that of the Eurasian water shrew in several parameters such as stroke frequency of the hind feet while swimming, undulatory movements of the tail and floating capacity. The diving ability of allopatric *N. anomalus* was also distinct from that previously observed in *N. anomalus* populations sympatric with *N. fodiens* (Mendes-Soares and Rychlik

2009). However, in this case it also differed from what had been observed for *N. fodiens*. Diving profiles of allopatric *N. anomalus* were highly variable, not allowing the discrimination between the frequency of shallow dives (typical of a wading foraging mode), and the frequency of deep and long duration dives (characteristic of a diving foraging strategy; Rychlik 1997). The indication that these animals probably use more than one strategy to search for food was given by the experimental observation of their foraging behaviour. Although allopatric *N. anomalus* still relied on obtaining food mainly from terrestrial areas, some animals were able to dive and find food in deep water, a behaviour observed for the first time in this species. Overall, the behavioural observations of *N. anomalus* from Serra da Estrela supported an extension of the ecological niche of the species, consistent with the habitat shift previously reported in chapter 3.

Chapter 5: Geographical distribution models of water shrews

The hypothesis that the effect of interspecific competition between water shrew species can influence their distribution at a regional scale was partially falsified. The distribution models obtained for each water shrew species present in the Iberian Peninsula were mainly influenced by altitude, with both species occurring preferentially in mountain areas. Differences in thermal tolerance were also found, with *N. anomalus* occurring generally in warmer and drier areas than *N. fodiens*. The resulting distribution pattern of these species in the Iberian Peninsula shows that *N. fodiens* is confined to the mountains in the north of the Peninsula while *N. anomalus* is occurring not only in those same areas but also in other mountain ranges, including the Iberian Central System and the Betic System. The effect of interspecific competition in the distribution of water shrews in the Iberian Peninsula was only detected by the significant difference found between the mean altitude of allopatric and sympatric populations of *N. anomalus*. In the northern mountains of the Iberian Peninsula where both water shrew species occur, *N. fodiens* occurs at higher altitudes than *N. anomalus*. However, the mean altitude of *N. anomalus* increases significantly towards the mean altitude of *N. fodiens* in areas where this later species is absent. Altitude shifts in *N. anomalus* support the hypothesis of competitive exclusion in the presence of *N. fodiens* and the hypothesis of competitive release under the absence of the dominant species.

The climate changes projected by the IPCC (2013) for the next 30 to 50 years will induce significant reductions in the climatically suitable areas for *N. anomalus* and *N. fodiens* in the Iberian Peninsula. The distribution of both species is expected to suffer a northward contraction, which will be more evident in *N. fodiens* than in *N. anomalus*. However, the climate changes are estimated to induce stronger ecological and evolutionary consequences for the later species, due the effects of interspecific competition. Firstly, the populations that are now sympatric with *N. fodiens* will probably become allopatric, which may induce a habitat shift. Secondly, the future area of sympatry between *N. anomalus* and *N. fodiens* will probably correspond to the entire range of this later species in the Iberian Peninsula. Thirdly, most populations of *N. anomalus* that now occupy allopatric areas and benefit from competitive release, are expected to disappear. This would clearly compromise the persistence of the distinct Iberian lineage of this species.

6.2 Is the Mediterranean water shrew a model for assessing climate change effects?

The current fragmented pattern of the Mediterranean water shrew is in great extent a consequence of past severe climatic events that promoted the isolation of some populations in southern refugia, such as the Iberian Peninsula or the Italian Peninsula. Contrary to other mammal species that were able to re-colonise vast areas as climatic conditions improved, several populations of the Mediterranean water shrew remained isolated as translated in its phylogeographic structure. One of the main factors that has most likely contributed and still does for the persistence of the fragmented distributional pattern of the Mediterranean water shrew is the interspecific competition with the Eurasian water shrew. As showed for the Iberian Peninsula, this species is apparently more adapted to colder conditions than the Mediterranean water shrew. This fact has probably contributed for a fast re-colonisation of Europe by the Eurasian water shrew as revealed by its lack of phylogeographic structure. As such, the Mediterranean water shrew was prevented to occupy optimal wet habitats due the presence of the Eurasian water shrew. Without the fast re-colonisation of the Eurasian water shrew, the current distribution pattern of the Mediterranean water shrew would have been much less fragmented since its current allopatric populations are significantly more effective in exploring aquatic resources than sympatric populations co-occurring with the Eurasian water shrew.

The findings of the present thesis confirm that water shrews are among the mammal species projected to be more affected by climate changes in Europe (Thuiller *et al.*, 2011). A potential climate warming will affect the distribution of both water shrew species significantly changing the proportion between sympatric and allopatric areas. As such, particularly the Mediterranean water shrew can be considered a very interesting model species for testing several global changes hypothesis, namely the effects of climate warming in animal communities.

6.3 Future directions

One of the main findings of this PhD study is the mtDNA divergence between Iberian *N. anomalus* and the remaining populations occurring outside the Iberian Peninsula. In order to clarify the taxonomic status of the Iberian *N. anomalus* efforts should be carried out not only to obtain more samples from this region but also to continue the molecular analysis using a multilocus approach (mitochondrial, sex-linked and autosomal loci).

It is also critical to investigate the role of the genetic differentiation of the Iberian *N. anomalus* in the ecological and behavioural changes here reported between allopatric and sympatric populations. Morphological studies conducted not only in the Iberian Peninsula but also in central and eastern Europe consistently support the hypothesis that the main factor driving significant differences between allopatric and sympatric populations of *N. anomalus* is competition (or lack of it) with *N. fodiens* (Rácz and Demeter 1998; Kryštufek and Quadracci 2008; Popov and Zidarova 2008). One way to evaluate the influence of the genetic differentiation in the ecology and behaviour of different *N. anomalus* populations is by comparing sympatric and allopatric populations from the same genetic lineage. As such, comparative studies should be conducted using sympatric and allopatric *N. anomalus* from the Iberian Peninsula, as well as, sympatric and allopatric populations occurring in the Italian Peninsula and in central and eastern Europe. Besides the effects of the genetic differentiation of each genetic lineage, it should also be tested if intraspecific differences between sympatric and allopatric populations of *N. anomalus* have a genetic basis or if they are driven by phenotypic plasticity. Such analysis will be important to forecast the preferential habitats that *N. anomalus* can potentially occupy in the case of *N. fodiens* disappearance from some of the current sympatric areas, as estimated in different scenarios of climate change.

The perception that the habitat preferences of *N. anomalus* may vary significantly according to both abiotic and biotic interactions, is crucial to establish an accurate conservation strategy for the species, especially considering future climate changes that may significantly reduce climatically suitable areas for the species. It will be especially important to clarify the consequences of climate change over the entire range of the species because the contraction of southern populations, as described for the Iberian Peninsula, may be followed by a northern expansion, such as that recently uncovered by the occurrence of *N. anomalus* in latitudes so far considered out of its northern range limit (Lithuania, Balčiauskas and Balčiauskienė 2012). The conservation risk of *N. anomalus* in the Iberian Peninsula may be particularly higher than in other regions because these populations will most likely continue to be isolated by the Pyrenees, and unable to expand northwards. Global warming may lead to a higher dependence on main watercourses, which in the Iberian Peninsula could represent an additional risk for the species, as riparian areas are among the most threaten habitats in this region. A higher dependence on watercourses may also increase the competition with other semi-aquatic species such as the endemic Pyrenean desman, *Galemys pyrenaicus*, a species already considered vulnerable due to multiple factors negatively affecting its habitat, such as dam construction and water pollution. Moreover, the genetic divergence here reported indicates that Iberian *N. anomalus* populations may constitute an Evolutionary Significant Unit that should be managed accordingly and probably differently from the remaining conspecific populations (Moritz 1994).

6.4 References

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