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HYDROLOGICAL ALTERATION, CRITICAL SWIMMING SPEED AND LIFE HISTORY IN INLAND FISH

Carlos Cano-Barbacil

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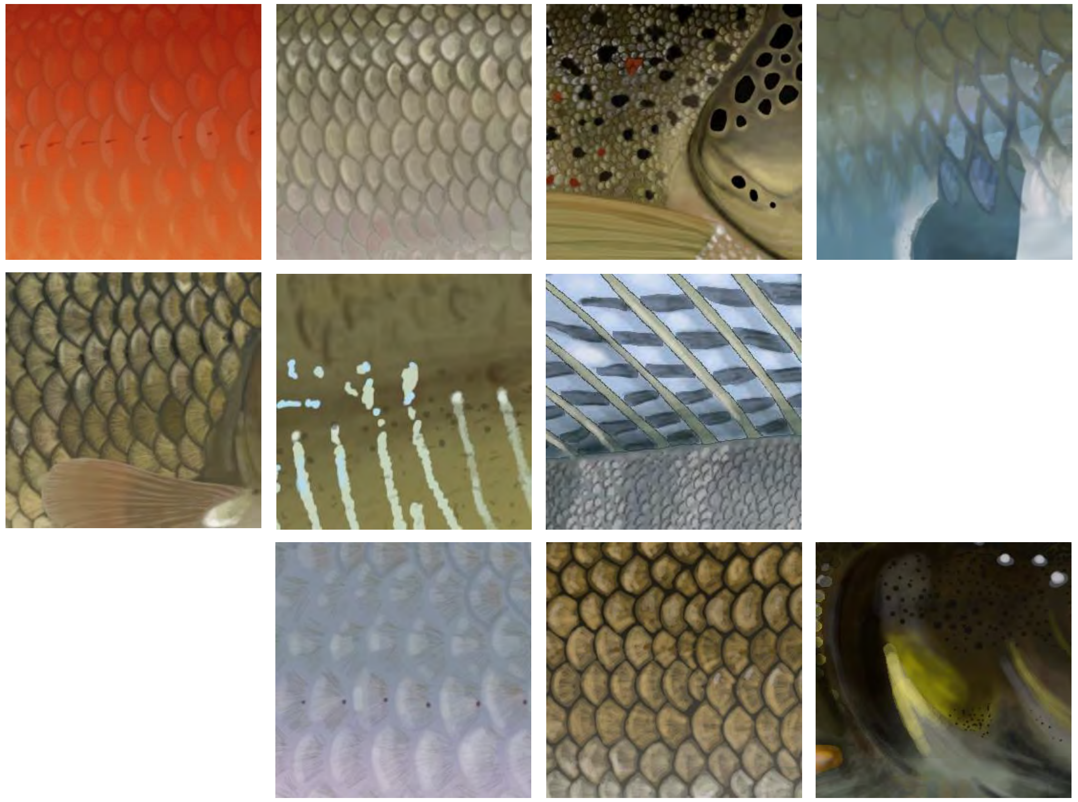

Inland waters are among the most biodiverse but also threatened ecosystems on Earth. This thesis explores through the analysis of fish traits and species' distributions the ecological filtering produced by the break in connectivity caused by damming on Iberian fish species, as well as the mechanisms that favour the proliferation of alien fish in Iberian rivers.

Illustrations: Carlos Cano-Barbacil

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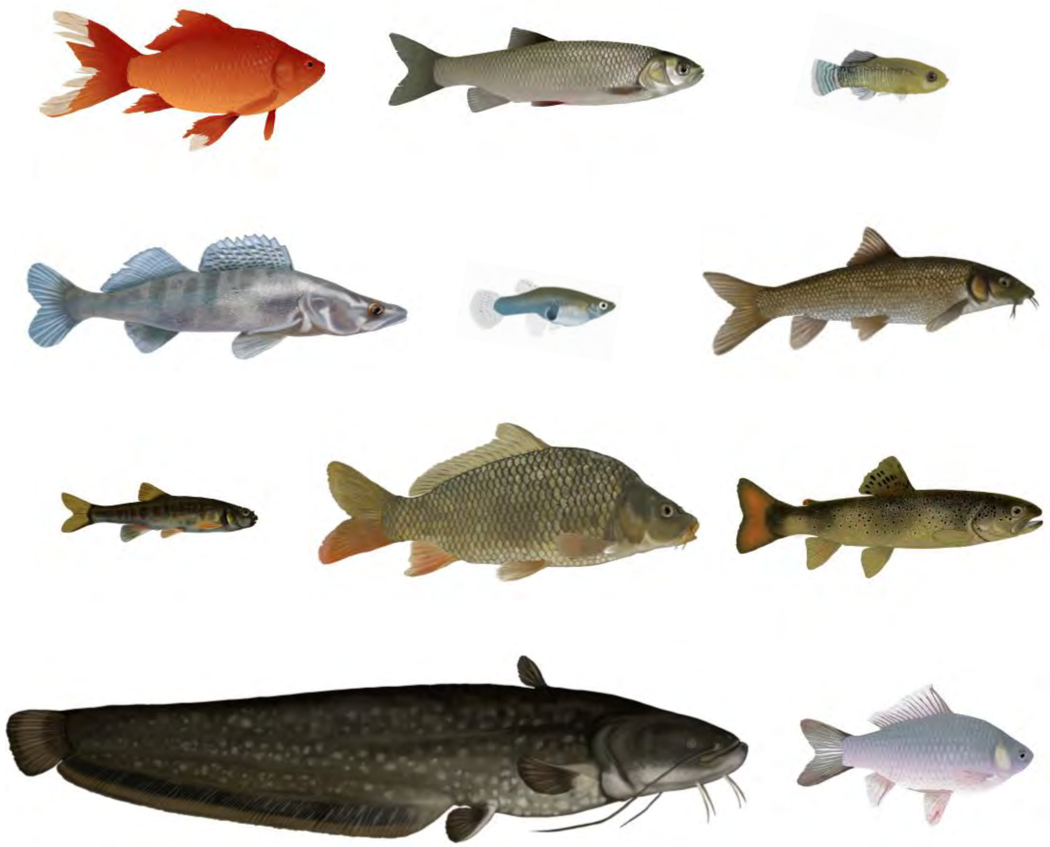
Hydrological alteration, critical swimming speed and life history in inland fish

2022

DOCTORAL THESIS

Hydrological alteration, critical swimming speed and life history in inland fish



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DOCTORAL THESIS

Hydrological alteration, critical swimming speed and life history in inland fish

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Doctoral Program in Water Science and Technology

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Thesis submitted in fulfilment of the requirements to obtain the Degree of Doctor at Universitat de Girona

The following thesis contains five annexes containing the supplementary materials corresponding to each chapter of the work

Hereby, Dr. Emili García-Berthou from Universitat de Girona and Dr. Johannes Radinger from Leibniz-Institute of Freshwater Ecology and Inland Fisheries,

CERTIFY:

That the doctoral thesis entitled '*Hydrological alteration, critical swimming speed and life history in inland fish*' submitted by Carlos Cano-Barbacil to obtain the degree of Doctor at Universitat de Girona has been completed under our supervision and meets the requirements to opt for the International Doctor mention.

In witness whereof and for such purposes as may arise, the following certification is signed:

Dr. Emili García-Berthou Dr. Johannes Radinger

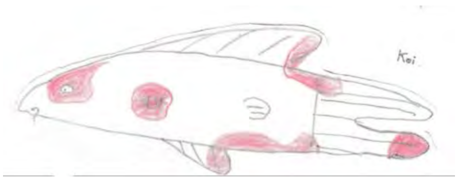
Girona, 01/05/2022

A vosotras.

Valeria, Mari Jose, Natalia y Clàudia.

Thanks

Desde pequeño, cuando buceaba en la piscina de casa de mis abuelos Valeria y Regino entre carpas y carpines, me ha fascinado toda la vida que habita bajo el agua. De hecho, si habláis con mi madre os dirá que de niño dedicaba gran parte de mi tiempo libre a dibujar peces, de todas las formas y colores. Lo que jamás me imaginé (y creo que ella tampoco), es que dedicaría cuatro años de mi vida a hacer una tesis sobre ellos.



Pero el camino que he hecho para llevar a cabo este trabajo no lo he recorrido sólo. Si hoy estoy en disposición de defender esta tesis, ha sido sobre todo gracias a mi familia. Porque, aunque los últimos cuatro años hemos estado a más de 700 km de distancia, ellos se encargaron los veinticuatro años anteriores de darme las herramientas para poder hacer frente a los obstáculos y a los desafíos a los que me he enfrentado durante esta etapa. Gracias a los abuelos, que nos dedicaron todo su tiempo y cariño a cuidarnos, desde bien pequeños hasta que supimos valernos por nosotros solos. Gracias a mi madre, por todo, pero en especial por velar para que nunca me faltase nada y pudiera dedicar el tiempo necesario a estudiar y formarme como persona. Gracias a mi padre por transmitirme el amor por la naturaleza, por las historias de expediciones y lugares remotos, y por llevarme al campo a ver pájaros y bichos en general desde bien pequeño. Y por supuesto, gracias a Natalia, mi ‘hermana favorita’, por darme el momento más feliz de mi vida cuando me enteré de que tendría una hermanita, y por haberme dado la mejor compañía desde entonces.

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Lo cierto es que durante una tesis doctoral hay muchos momentos alegres, momentos de descubrimiento y de diversión; pero también hay algún momento de estrés y amargura. Por eso es fundamental estar bien rodeado, y yo he tenido la inmensa suerte de contar con las buenas gentes del *seminari* y demás compañeros del departamento. Gracias a todos los integrantes del *seminari* 1.0, David, Giulia, Cesc, Marina, Irene T., Laura B., Jordi C. y María A., por integrarme con tanto cariño en el grupo. Gracias a todos los que fueron llegando después, Maria A., Álex, Andrés, Anna C., Judit, Anna D., Irene G., Amina, Joan, Luis y Ada, por mantener siempre el buen rollo en el despacho, por los buenos momentos escalando pedruscos, buscando renacuajos o tomando una cerveza en Can Paco. Gracias al resto de compañeros del pasillo por acompañarme en esta aventura, con mención especial a Jordi B., mi botánico de cabecera, y su inseparable Fiona, a Maria B., a Alba y JP, a Lorena y Laura D., a Raül, Jorge, Marta, Xènia y Mar. Gracias a Anna Vila por sus visitas al *seminari* que siempre nos sacan una sonrisa, y al resto de profesores por estar siempre dispuestos a ayudar. Gracias también a los amigos que dejé en Madrid, Doval, Paquito, Rodri, Toni y Dani, porque uno siempre puede contar con ellos en cualquier momento y para cualquier cosa, por loca que sea.

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All the images and photos contained in this thesis are created and taken by Carlos Cano-Barbacil (except for those figures indicated with a different author name). If you wish to reproduce any of the images, please contact me in advance and I will be glad to share my work:
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List of abbreviations

$a_{3.0}$: Body form factor
AgrPrc: Average agricultural land use with a given sub-catchment
AIC: Akaike Information Criterion
ANCOVA: Analysis of covariance
ANOVA: Analysis of variance
AnnPrec: Average annual precipitation within sub-catchment
AUC: Area under the receiver operating characteristic curve
BIO4: Temperature seasonality
BIO10: Mean temperature of warmest quarter
BIO11: Mean temperature of coldest quarter
BIO15: Precipitation seasonality
BIO16: Precipitation of wettest quarter
BIO17: Precipitation of driest quarter
BL: Body length
BM: Brownian motion
BRT: Boosted regression trees
BW: Brackishwater
CR: Critically endangered
CI: Confidence interval
CT_{max}: Critical thermal maxima
D_o: Observed disagreement between values assigned for a particular trait by different raters across a set of species
D_e: Disagreement expected by chance
***df*:** Degrees of freedom
DisSea: Distance to the sea
Dir: Direct
e.g.: *Exempli gratia* (‘for example’)
Elev: Elevation
EMMs: Estimated marginal means
EN: Endangered
Eur: Euryhaline
FL: Fork length
ForPrc: Average forest land use within a given sub-catchment
FW: Freshwater
GBIF: Global Biodiversity Information Facility
GE: Geographical extent
GLM: Generalised linear models
Grad: Gradual
GS: Grain size
HoD: Homogeneity of dispersion
ID: Identity
i.e.: *Id est* (‘in other words’)
IUCN: International Union for Conservation of Nature
J: Jaccard index
LC: Least concern
LC_{50}: Maximum concentration that killed 50% of the individuals in the experiment
LMM: Linear mixed model
LoA: Limits of agreement
LoResCp: Local reservoir capacity
M: Marine
MeanTem: Annual mean temperature
mtry: Number of variables per level
N: Total number of databases
N_i: Number of databases that describe at least one trait of species i

N_k: Number of databases that consider trait *k*
NA: Not applicable / no data available
NB: Niche breadth
NE: Not evaluated
NM: Niche marginality
NP: Niche position
NT: Near threatened
ntree: Number of trees
OMI: Outlying mean index
ORCDRC: Soil organic carbon across sub-catchment
OU: Ornstein-Uhlenbeck models
PCA: Principal components analysis
PCoA: Principal coordinates analysis
PeA: Peripheral alien species
PeN: Peripheral native species
Per: Peripheral
PERMANOVA: Permutational analysis of variance
PET: Potential evapotranspiration
PGLMM: Phylogenetic generalised linear mixed model
PGLS: Phylogenetic generalised least squares
PHIHOX: Soil pH in H_2O across sub-catchment
PNC: Phylogenetic niche conservatism
PopDen: Population density
ppt: Parts per trillion
PrA: Primary alien species
PrecSeas: Average precipitation seasonality within sub-catchment
Pri: Primary
PrN: Primary native species
PS: Phylogenetic signal
RDA: Redundancy analysis
RF: Random forests
RME: Relative mean elevation
S_j: Number of species considered in database *j*
S_{kj}: Number of species with available information on trait *k* in database *j*
SD: Standard deviation
SDM: Species distribution model
SeA: Secondary alien species
Sec: Secondary
SeN: Secondary native species
SL: Standard length
SolRadiat: Solar radiation
Slo: Slope
sp. / spp.: Species (singular) / species (plural)
SSO / StrOrdSt: Strahler's stream order
Ste: Stenohaline
StrOrdSh: Shreve's stream order
T: Temperature
T_{ij}: Number of traits available for species *i* in database *j*
T_j: Number of traits considered in database *j*
TemAnnRan: Temperature annual range
TL: Total length
TopInd: Topographic index
TS: Time step
TSS: True skill statistic
U_{crit}: Critical swimming speed
UpResCp: Upstream reservoir capacity

UrbPrc: Average urban land use within a given sub-catchment

UTM: Universal Transverse Mercator

VIF: Variance inflation factor

vs.: *Versus* (‘against’)

VU: Vulnerable

List of publications

CHAPTER I: Cano-Barbacil, C.[1]; Radinger, J.[1,2] & García-Berthou, E.[1] 2020. Reliability analysis of fish traits reveals discrepancies among databases. *Freshwater Biology*, 65(5): 863-877. https://doi.org/10.1111/fwb.13469

CHAPTER II: Cano-Barbacil, C.[1]; Radinger, J.[1,2]; Argudo, M.[1]; Rubio-Gracia, F.[1]; Vila-Gispert, A.[1] & García-Berthou, E.[1] 2020. Key factors explaining critical swimming speed in freshwater fish: a review and statistical analysis for Iberian species. *Scientific Reports*, 10: 1-12. https://doi.org/10.1038/s41598-020-75974-x

CHAPTER III: Cano-Barbacil, C. [1]; Radinger, J.[1,2] & García-Berthou, E.[1] (Under review). The importance of seawater tolerance and native status in mediating the distribution of inland fishes.

CHAPTER IV: Cano-Barbacil, C.[1]; Radinger, J.[1,2]; Grenouillet, G.[3] & García-Berthou, E.[1] 2022. Phylogenetic signal and evolutionary relationships among traits of inland fishes along elevational and longitudinal gradients. *Freshwater Biology*, 67(5): 912–925. https://doi.org/10.1111/fwb.13890

CHAPTER V: Cano-Barbacil, C. [1]; Radinger, J.[1,2]; Olden, J.D.[4] & García-Berthou, E.[1] (Under review). Estimates of niche position and breadth vary across spatial scales for native and alien inland fishes.

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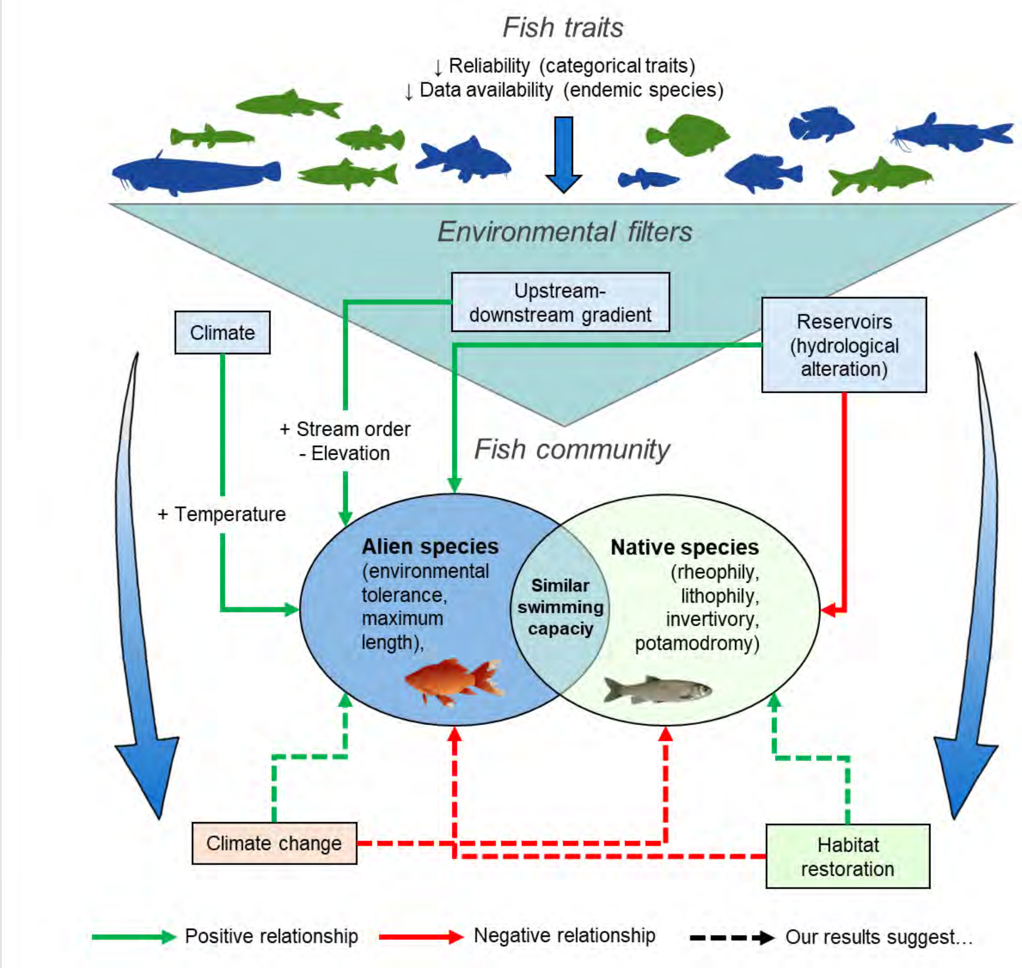
Graphical abstract



Summary

Fresh waters are among the most biodiverse ecosystems globally but, at the same time, also the most threatened. Serious threats to freshwater ecosystems are, for example, habitat alteration, invasive alien species, pollution, water abstraction and climate change. One of the most pervasive indicators of anthropogenic impacts on rivers is hydrological alteration caused by artificial barriers, which is known to reinforce the decline of local biodiversity and ecosystem services, and the species homogenisation of inland fish communities. Previous studies showed that the novel lentic habitats created by dams and habitat modifications occurring downstream of dams favour the establishment of alien fish, as many of them commonly prefer lentic habitats with warmer water temperatures compared with native fish. Thus, jointly studying species distribution patterns and species traits and their evolutionary history provides an essential link to better understand species' responses to environmental change and biological invasions, and to design effective management tools and policies. For instance, swimming performance (often measured in the laboratory as critical swimming speed [U_{crit}]) is a particularly important trait in aquatic organisms, mediating their fitness, habitat selection or survival. However, there are few studies that evaluated its evolutionary relationship with other traits, with fish distribution patterns or with invasion success in freshwater ecosystems.

The Iberian Peninsula is a well-suited region to study the effects of climate, land use change and hydrological alteration on the distribution of native and alien fish species, due to its complex orography and diverse climate and ichthyofauna. Thus, this thesis aimed to evaluate the relationship of distribution patterns and traits of inland fishes, and to understand the ecological filtering with a special focus on hydrological alteration using the Iberian ichthyofauna as a study system. The research performed included: (1) a compilation of Iberian inland fish trait data and corresponding reliability analysis of the different data sources; (2) the analysis of swimming capacity of native and alien Iberian inland fishes using random forests models, generalised linear mixed model and linear models; and (3) studies of the distribution patterns and niche characteristics of Iberian fish using species distribution models and the outlying mean index, as well as the analysis of relationships of these distribution patterns with fish traits using phylogenetic and non-phylogenetic approaches.

The results showed that there is an important lack of data availability regarding critical swimming speed (U_{crit}) and other traits of Iberian inland fishes, especially for endemic species. There exist also significant discrepancies in categorical/binary feature attribution among databases. Thus, a consensus trait database and a complete swimming performance dataset for

Iberian inland fishes is provided, which constitute the first attempt of a comprehensive, regional database summarising trait information with unprecedented coverage.

Historical and climatic predictors were found, in general, more important than land use and anthropogenic factors in shaping the distribution of inland fishes. However, the results showed significant differences in the importance of predictors explaining the distributions of native *vs.* alien species and even larger differences among Darlington’s divisions. Specifically, the distribution of most strictly freshwater native species is mainly determined by the long-term basin boundaries, and are more prevalent in upstream and middle reaches, whereas secondary and peripheral species especially occur in lowland reaches near to the coast. By contrast, alien species tend to occur in the hydrologically altered, warmer and lowermost reaches of the river mainstems. In fact, damming promoted the occurrence of large-bodied alien and tolerant fishes, and impinges native invertivore and rheophilic species. However, despite having different habitat preferences, native and alien species showed similar swimming performance.

The results of this thesis also support that the estimation of species’ niche metrics strongly depends on the geographical extent considered in the analyses, and therefore, the latter should be carefully selected based on the main objective of the study and on the characteristics of the target species. Alien fishes of the Iberian Peninsula had a wider environmental tolerance than native species, although the distribution of some of them is strongly restricted by basin boundaries and therefore, their regional niche is not in equilibrium. As a consequence, they might be able to colonise new areas in the future, and thus, the prevention of new introductions is the most effective measure to prevent their establishment and spread. Finally, a number of management applications emerging from the results and future research prospects are discussed, ultimately aiming at supporting an improved and effective management of invasive and conservation of threatened native species populations.

Resumen

Las aguas continentales se encuentran entre los ecosistemas con mayor biodiversidad a nivel global, pero al mismo tiempo, están gravemente amenazados por la alteración del hábitat, las especies invasoras, la contaminación, la extracción de agua y el cambio climático. En concreto, uno de los mejores indicadores del impacto humano sobre los ríos es la alteración hidrológica causada por las barreras artificiales. Es sabido que la construcción de presas y embalses provoca el declive de la biodiversidad local, la pérdida de servicios ecosistémicos y la homogenización de las comunidades piscícolas. Estudios previos han demostrado que los nuevos hábitats lénticos creados por las presas, así como la serie de modificaciones producidas aguas debajo de la presa, favorecen el establecimiento de especies invasoras de peces mejor adaptadas a las nuevas características del medio que las especies nativas. Así, el estudio conjunto de los patrones de distribución con los rasgos de las especies y su historia evolutiva es esencial para entender mejor las posibles respuestas de las especies a eventuales cambios ambientales e invasiones biológicas, y al mismo tiempo, planificar medidas de gestión eficaces. Por ejemplo, el rendimiento de natación (medido a menudo en el laboratorio como velocidad crítica de natación [U_{crit}]) es un rasgo particularmente interesante de analizar en organismos acuáticos, ya que de él dependen la reproducción, la selección del hábitat o la supervivencia. Sin embargo, hay pocos estudios que evalúen sus relaciones evolutivas con otros rasgos, con los patrones de distribución o con el éxito invasor en ecosistemas acuáticos.

La península ibérica es una región apta para el estudio de los efectos del clima, de los cambios en el uso de suelo y de la alteración hidrológica sobre la distribución de los peces autóctonos e introducidos debido a su compleja orografía y a la diversidad de climas y especies de peces que en ella habitan. Por lo tanto, el objetivo principal de esta tesis es evaluar las relaciones entre los patrones de distribución y los rasgos de las especies, así como estudiar el filtrado ecológico producido por la alteración hidrológica usando la ictiofauna continental ibérica como caso de estudio. Para llevar a cabo este estudio realizamos diferentes tareas específicas: (1) recopilación de información sobre los rasgos de las especies y análisis de fiabilidad de las bases de datos empleadas; (2) análisis de la capacidad de natación de las especies autóctonas e introducidas usando modelos de 'random forests', modelos lineales generalizados mixtos y modelos lineales; y (3) estudio de los patrones de distribución y de las características del nicho usando modelos de distribución de especies y el 'outlying mean index', así como análisis de la relación entre estos patrones de distribución con los rasgos de los peces usando métodos comparativos y métodos no filogenéticos.

Los resultados mostraron que actualmente existe poca disponibilidad de datos sobre la capacidad de natación (U_{crit}) y otros rasgos para peces continentales ibéricos, en especial para las especies endémicas. Se encontraron discrepancias significativas en la asignación de los rasgos binarios entre bases de datos. Además, se generó una base de datos consenso y un set de datos con todos los experimentos realizados para medir la capacidad de natación de las especies ibéricas de aguas continentales. Esta información constituye el primer intento de generar una base de datos regional que resume toda la información de rasgos disponible con una cobertura sin precedentes.

Los modelos de distribución de especies mostraron que los factores históricos y climáticos son, en general, más importantes que los factores antropogénicos a la hora de explicar los patrones de distribución de los peces continentales. Sin embargo, se encontraron diferencias significativas en la importancia de las variables entre especies autóctonas e introducidas, y aún diferencias mayores entre las tres divisiones de Darlington. En concreto, la distribución de la mayoría de especies autóctonas estrictamente de agua dulce (primarias) está principalmente condicionada por las barreras geográficas, y son más prevalentes en los cursos altos y medios de los ríos. Por su parte, las especies secundarias y periféricas ocupan sobre todo los tramos bajos cerca de la costa. Por el contrario, las especies introducidas tienden a ser más prevalentes en medios con mayor alteración hidrológica, mayor temperatura media anual, y en tramos bajos de los cauces principales. De hecho, los resultados mostraron que la construcción de presas facilita la ocurrencia de especies introducidas de gran tamaño con una elevada tolerancia ambiental, mientras que desplaza a especies autóctonas invertívoras que habitan en medios lóticos. Sin embargo, a pesar de que las especies autóctonas e introducidas parecen seleccionar el hábitat de forma distinta, muestran una capacidad de natación similar.

Los resultados de esta tesis apoyan que la caracterización del nicho de las especies depende en gran parte de la selección de la extensión geográfica de los datos considerados en el análisis. Por lo tanto, esta debe ser seleccionada cautelosamente en función del objetivo principal del trabajo y de las características de las especies estudiadas. Se encontró que las especies de peces introducidas en la península ibérica tienen una mayor amplitud de nicho (tolerancia ambiental) que las especies autóctonas, a pesar de que la distribución de muchas de ellas está muy restringida también por las barreras geográficas y por lo tanto su nicho regional no está en equilibrio. Como consecuencia, estas especies podrían colonizar en el futuro nuevas regiones dentro de la península ibérica. Es por tanto fundamental evitar nuevas introducciones con el objeto de evitar futuras invasiones. Finalmente, se discuten una serie de medidas de gestión propuestas en base a los resultados obtenidos en esta tesis, con la finalidad de mejorar la gestión de las poblaciones de especies invasoras y la conservación de las especies autóctonas amenazadas.

Resum

Les aigües continentals es troben dins dels ecosistemes amb major biodiversitat a escala global, però al mateix temps, estan molt amenaçades per l'alteració de l'hàbitat, les espècies invasores, la contaminació, l'extracció d'aigua i el canvi climàtic. En concret, un dels millors indicadors de l'impacte humà sobre els rius és l'alteració hidrològica causada per les barreres artificials. De fet, se sap que la construcció de preses i embassaments provoca la pèrdua de biodiversitat local i de serveis ecosistèmics, així com l'homogeneïtzació de les comunitats de peixos. Estudis previs han demostrat que els nous hàbitats lenítics produïts per les preses, així com la sèrie de modificacions produïdes aigües avall de la presa, afavoreixen l'establiment d'espècies invasores de peixos millor adaptades a les nous característiques del medi que les espècies natives. Per tant, l'estudi conjunt dels patrons de distribució, dels trets i de la seva història evolutiva és fonamental per entendre millor les possibles respostes de les espècies a eventuals canvis ambientals i invasions biològiques, i al mateix temps, per planificar mesures de gestió eficaces. Per eixample, la capacitat de natació (mesurat normalment al laboratori com velocitat crítica de natació [U_{crit}]) és un tret particularment interessant d'analitzar en organismes aquàtics, ja que d'aquest tret depenen la reproducció, la selecció de l'hàbitat o la supervivència. Però, n'hi ha pocs estudis que estudiïn la seva relació evolutiva amb altres trets, amb els patrons de distribució o amb l'èxit invasor en ecosistemes aquàtics.

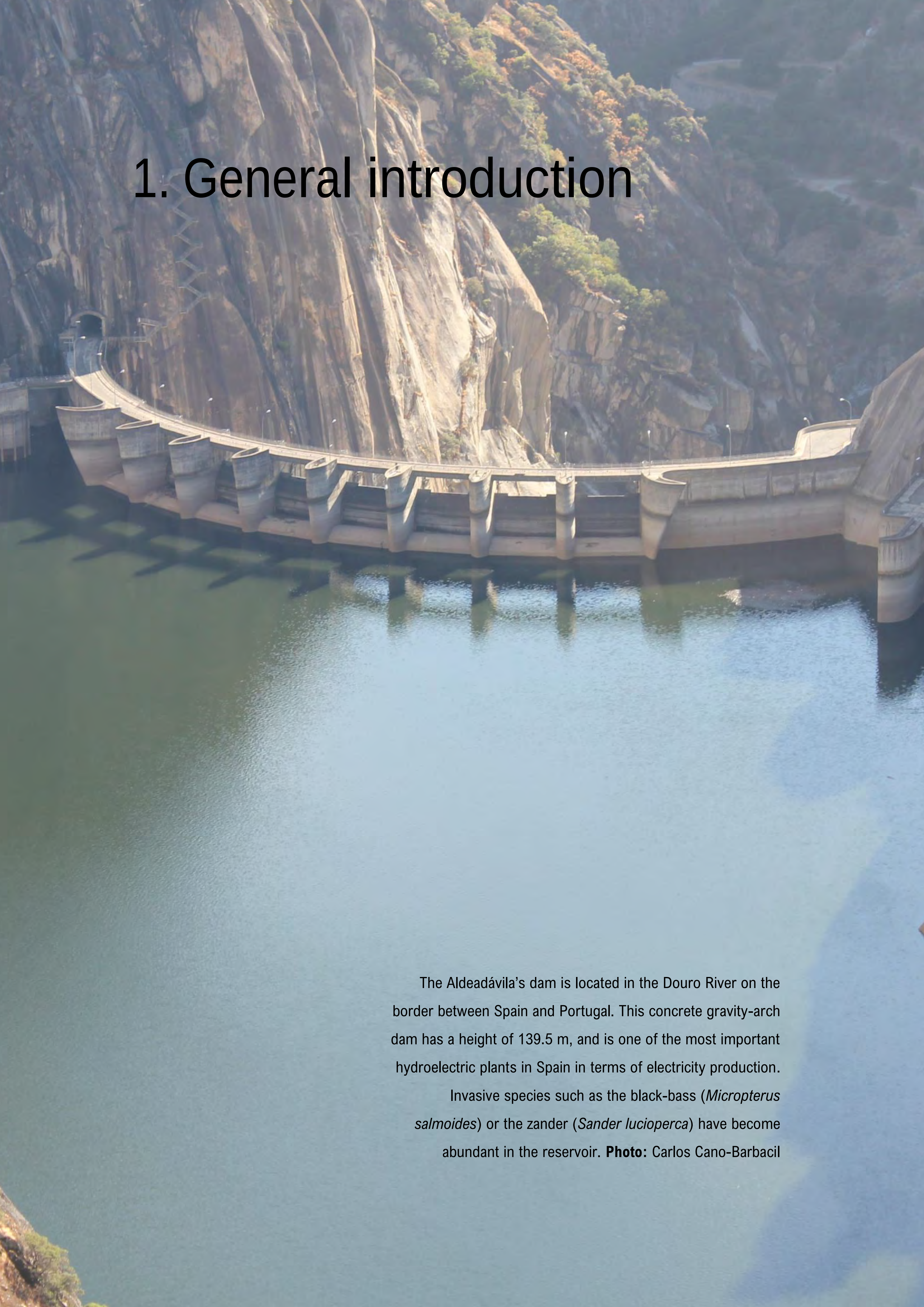
La península Ibèrica és una regió apta per l'estudi dels efectes del clima, dels canvis en l'ús del sol i de l'alteració hidrològica sobre la distribució dels peixos autòctons i introduïts degut a la seva orografia complexa i a la seva diversitat de climes i espècies de peixos. Per tant, l'objectiu principal d'aquesta tesi és avaluar les relacions entre els patrons de distribució i els trets de les espècies, així com estudiar el filtratge ecològic produït per l'alteració hidrològica fent servir els peixos ibèrics com cas d'estudi. Per portar a terme aquest estudi acomplim diferents tasques específiques: (1) recopilació d'informació sobre els trets de les espècies i anàlisis de fiabilitat de les bases de dades empleades; (2) anàlisis de la capacitat de natació de les espècies autòctones i introduïdes fent servir models de 'random forests', models lineals generalitzats mixtos i models lineals; i (3) estudi dels patrons de distribució i de les característiques del nínxol fent servir models de distribució d'espècies i el 'outlying mean index', així com anàlisis de la relació entre aquests patrons de distribució amb els trets dels peixos fent servir mètodes comparatius i mètodes no filogenètics.

Els resultats van indicar que actualment existeix poca disponibilitat de dades sobre la capacitat de natació (U_{crit}) i altres trets per peixos continentals ibèrics, especialment per espècies endèmiques. Es van trobar discrepàncies significatives en l'assignació dels trets binaris entre bases de dades. A més a més, es va generar una base de dades consens i un set de dades amb tots els experiments realitzats per mesurar la capacitat de natació de les espècies ibèriques d'aigües continentals. Aquesta informació constitueix el primer intent de generar una base de dades regional que resumi tota la informació de trets disponible amb una cobertura sense precedents.

Els models de distribució d'espècies van mostrar que els factors històrics i climàtics són, en general, més importants que els factors antropogènics a l'hora d'explicar els patrons de distribució dels peixos continentals. Però, es van trobar diferències significatives en la importància de les variables entre espècies autòctones i introduïdes, i encara diferències majors entre les tres divisions de Darlington. En concret, la distribució de la majoria d'espècies autòctones estrictament d'aigua dolça (primàries) està principalment condicionada per les barreres geogràfiques, i són més abundants en els trams alts i mitjans dels rius. Per altra banda, les espècies secundàries i perifèriques ocupen sobretot els trams baixos a prop de la costa. Al contrari, les espècies introduïdes són més abundants en hàbitats modificats amb major alteració hidrològica, amb una temperatura mitjana anual més elevada, i en trams baixos dels rius principals. De fet, els resultats van mostrar que la construcció de preses facilita l'ocurrència d'espècies introduïdes de gran mida amb elevada tolerància ambiental, mentre que desplaça a espècies autòctones insectívores que viuen en hàbitats lòtics. Però, a pesar que les espècies autòctones i introduïdes semblen seleccionar l'hàbitat de manera diferent, mostren una capacitat de natació semblant.

Els resultats d'aquesta tesi secunden que la caracterització del nínxol de les espècies depèn en gran part de la selecció de l'extensió geogràfica de les dades considerades en l'anàlisi. Per tant, l'extensió geogràfica ha de ser seleccionada cautelosament en funció de l'objectiu principal de l'estudi i de les característiques de l'espècie estudiada. Es va trobar que les espècies de peixos introduïdes en la península Ibèrica tenen una major amplitud de nínxol (tolerància ambiental) que les espècies autòctones, tot i que la seva distribució està molt limitada també per les barreres geogràfiques i, per tant, el seu nínxol no està en equilibri. Com a conseqüència, aquestes espècies introduïdes podrien colonitzar en el futur noves regions dins de la península Ibèrica. En conseqüència, és fonamental evitar noves introduccions amb el propòsit d'evitar futures invasions. Finalment, es discuteixen una sèrie de mesures de gestió propostes d'acord amb els resultats obtinguts en aquesta tesi, amb la finalitat de millorar la gestió de les poblacions d'espècies invasores i la conservació de les espècies autòctones amenaçades.

1. General introduction



The Aldeadávila's dam is located in the Douro River on the border between Spain and Portugal. This concrete gravity-arch dam has a height of 139.5 m, and is one of the most important hydroelectric plants in Spain in terms of electricity production. Invasive species such as the black-bass (*Micropterus salmoides*) or the zander (*Sander lucioperca*) have become abundant in the reservoir. **Photo:** Carlos Cano-Barbacil

1.1. A brief overview of global freshwater fish diversity and its conservation status

Rivers, lakes and wetlands are among the most biodiverse and dynamic ecosystems, despite covering only less than 1% of the Earth surface. In fact, of the 35500 fish species known, 51% inhabit fresh waters. However, 30% of those 18000 inland fish species are considered at risk of extinction due to manifold interacting threats that cause the decline of fish populations (IUCN, 2019; Fricke, Eschmeyer & Van der Laan, 2021). It is well known that the rate of decline of vertebrate populations is higher in fresh waters than in marine or terrestrial environments (He *et al.*, 2019). Especially, in the last 50 years, populations of migratory freshwater fish have declined by 76% (Deinet *et al.*, 2020), populations of freshwater mega-fishes (i.e. fish heavier than 30 kg) have even more drastically decreased by 94% (He *et al.*, 2019), which is due to their complex habitat requirements and particular life-history strategies. The main reasons for these population declines, species extirpations and extinctions are river fragmentation, the introduction of non-native species, pollution, water abstraction, climate change and habitat transformation (see Figure 1.1; Reid *et al.*, 2019). Despite their relevance and the multiple threats they face, inland fishes, and freshwater ecosystems in general, remain an afterthought for policymakers and the public at large (WWF, 2021).

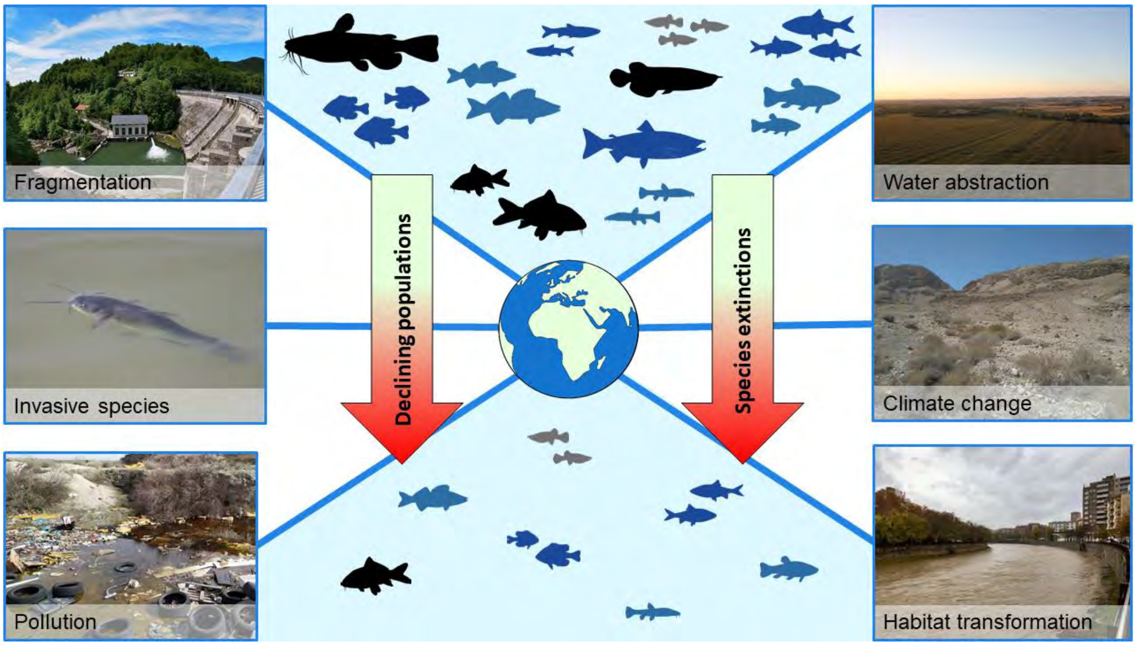


Figure 1.1. Main drivers of inland fish population declines and species extinctions. Photos by Carlos Cano-Barbacil.

1.2. Hydrological alteration and alien species: two main ecosystem transformation drivers

Human history has always been linked to freshwater resources, attracting human settlements and inducing the development of agriculture, industry and urbanisation (Kummu *et al.*, 2011). Today, over 50% of the world's population lives closer than 3 km to a surface freshwater body, and only 10% of the population lives further than 10 km away (Kummu *et al.*, 2011). As consequence, freshwater ecosystems around the world are threatened by human activities that alter environmental conditions by changing land use and climate, by introducing alien species, or by changing river morphology (Stevenson & Sabater, 2010; Carpenter, Stanley & Vander Zanden, 2011). Thus, subsequent effects have occurred on river hydrology, habitat availability, nutrient cycles, sediment transport, or inputs of toxic compounds (Stevenson & Sabater, 2010; Tanentzap *et al.*, 2021). These trends are predicted to exacerbate in future years because the human population is still growing, people is migrating from rural to urban areas, and because of the stresses that global change imposes on water quality and availability (Vitousek *et al.*, 1997; Grimm *et al.*, 2008a b; Gudmundsson *et al.*, 2021).

One of the most pervasive indicators of human impacts on rivers is the interruption of longitudinal connectivity caused by artificial barriers to free flow (Vitousek *et al.*, 1997). Human usage of rivers and their resources has been closely associated with their fragmentation (Carpenter *et al.*, 2011). Recent studies revealed that there are over 2.8 million dams (with reservoir areas >1000 m^2), and over 500,000 km of rivers and canals for navigation and transport around the world (Grill *et al.*, 2019). Only in Europe, there is a density of 0.74 barriers per kilometre (Belletti *et al.*, 2020). The damming of natural flows and diversion of water for irrigated agriculture are substantially altering hydrologic regimes and causing the 'drying up' of rivers, modifications of the temperature regimes and large-scale disruption of sediment transport (Bernstein, 2002; Nilsson *et al.*, 2005; Poff *et al.*, 2007; Olden & Naiman, 2010). This in turn causes the rapid decline of local biodiversity and ecosystem services, and the taxonomic homogenisation of fish communities (Rahel, 2002; Clavero & Hermoso, 2011; Fuller, Doyle & Strayer, 2015; van Puijenbroek *et al.*, 2019). The decrease in species richness and the reorganisation of the fish assemblage caused by damming occurs mainly because the environment has been heavily modified and the majority of the native ichthyofauna is often not well adapted (in terms of e.g. locomotion, behaviour, and feeding traits) to cope with these newly created lentic habitats (Agostinho *et al.*, 1999; Gomes & Miranda, 2001). The effects on migratory fish are exacerbated because of the loss of suitable conditions e.g. for the reproduction, and the fragmentation of essential migratory pathways (Gomes & Miranda, 2001). Moreover, effects of damming on inland fishes might even be underestimated, because of time lags of effects showing up, and the difficulty of generating a single, straight-forward signal of fragmentation that applies to all aquatic species (Fuller *et al.*, 2015; dos Santos *et al.*, 2022). However, damming is not the only cause of hydrological alteration, which is also induced by surface water diversion, groundwater extraction, urbanisation and modification to natural drainage networks for flood protection (Stewardson *et al.*, 2017).

In addition, novel habitats created by the modification of the natural flow regime may induce the establishment of alien fish, as many of them prefer more lentic habitats and warmer water temperatures than the native fish. Often, these alien fishes replace the native species in such hydrologically transformed ecosystems, as they have a broad physiological tolerance, generalist resource requirements and life-history traits that enhance consumption and growth (Vila-Gispert, Alcaraz & García-Berthou, 2005; Olden, Poff & Bestgen, 2006; Orrù, Deiana & Cau, 2010; Gido *et al.*, 2013; Radinger, Alcaraz-Hernández & García-Berthou, 2019). Thus, species invasions constitute a major source of change and decline in global biodiversity, because of competition for trophic resources, behavioural interference, hybridisation, disease transmission and habitat alteration and predation (Leunda, 2010; Bellard, Genovesi & Jeschke, 2016); and entail economic costs of billions of dollars annually worldwide (Oreska & Aldridge, 2011; Simberloff *et al.*, 2013; Fournier *et al.*, 2019; Angulo *et al.*, 2021; Diagne *et al.*, 2021; Haubrock *et al.*, 2021).

1.3. The importance of studying native and alien inland fish distribution patterns

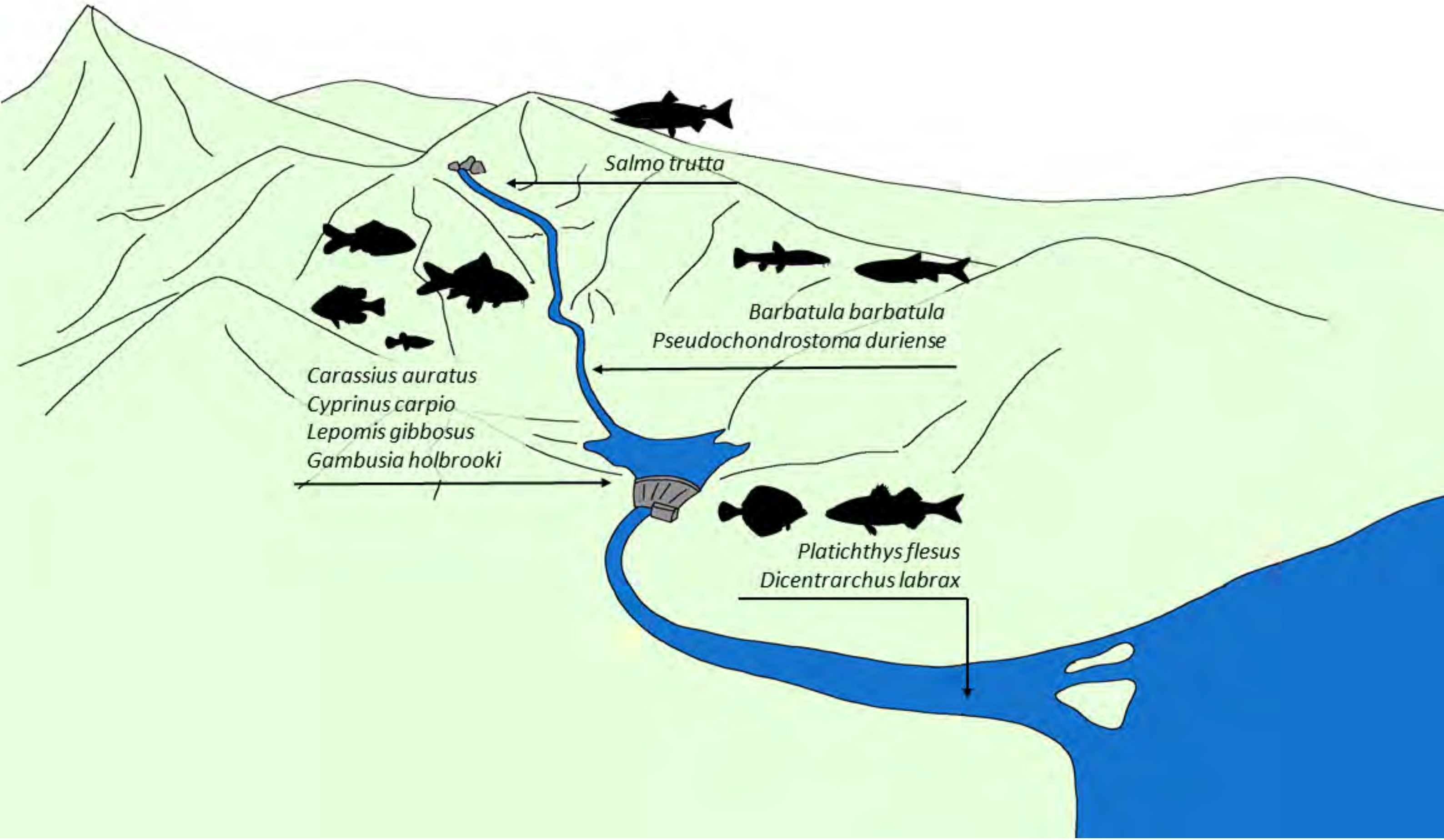
Understanding the main drivers of species distribution patterns is one of the central goals of ecology, biogeography and conservation biology (Filipe *et al.*, 2009; Marcer *et al.*, 2013). This has already started in the 19th century, when Alfred Russel Wallace (1876) began to study the geographical distribution of species. More recently, a number of different techniques to characterise species niches, such as gradient analysis (Austin, 1987; Ter Braak & Prentice, 1988), the outlying mean index (OMI; Dolédec, Chessel & Gimaret-Carpentier, 2000) or species distribution models (SDMs; Akçakaya, 2000; Guisan & Zimmermann, 2000; Guisan & Thuiller, 2005; Peterson, 2006), have become commonly used across terrestrial, freshwater, and marine ecosystems. They are numerical tools that combine species occurrences or abundances with different environmental predictors, and are applied to gain ecological and evolutionary insights, to predict species ranges or to project the potential distribution of alien species (Thuiller, 2003; Elith & Leathwick, 2009; Gallien *et al.*, 2012). Specifically, these techniques assume that species are at quasi-equilibrium with the environment, i.e. in the case of biological invasions they assume that alien species have already reached all suitable sites and are absent from all unsuitable places

(Guisan & Thuiller, 2005; Gallien *et al.*, 2012). However, the realised regional niche of alien species is not usually in quasi-equilibrium with the regional environment, as it is limited by their invasion history, dispersal constrains, environmental conditions and biotic interactions (Wilson *et al.*, 2007). Thus, using a combination of both regional and global occurrence data might provide more informative results when analysing the distribution of alien species (Roura-Pascual *et al.*, 2009; Gallien *et al.*, 2012) than using regional occurrence data from the invaded region only. However, studies may analyse regional distributions (e.g. Dullinger *et al.*, 2009; Barbet-Massin *et al.*, 2018), for example, when their main objective is to account for the specificities of local adventive ranges.

In freshwater ecosystems, modelling species distribution and characterising their ecological niche has frequently been used to understand effects of hydrologic alteration and habitat fragmentation in native and alien species populations (Radinger *et al.*, 2019), and to unveil the main factors that drive successful establishments of invasive alien species (Korsu *et al.*, 2012; Murphy, Grenouillet & García-Berthou, 2015; Bae, Murphy & García-Berthou, 2018). In addition, these techniques have been also applied to understand the environmental constraints and the evolutionary history of species (Filipe *et al.*, 2009), to generate valuable information for conservation management of endangered and rare species (Marcer *et al.*, 2013), or to assess the future environmental suitability under different climate change scenarios and to identify potentially threatened species (Buisson *et al.*, 2008; Markovic *et al.*, 2014).

Specifically, the distribution of inland fish species is well known to be constrained by a plethora of factors. For instance, and in contrast to assemblage organisation in marine or terrestrial ecosystems, previous studies concluded that historical factors and barriers to dispersal (i.e. long-term basin boundaries) exert greater constraints on native inland ichthyofauna than do contemporary climatic conditions (Filipe *et al.*, 2009). These historical factors are especially important for strictly freshwater organisms with very limited dispersal capacity. Thus, the ability to survive and disperse through seawater is also considered an important factor to understand contemporary geographical patterns of inland fishes (Myers, 1938; Darlington, 1948). However, current climatic conditions (e.g. Murphy *et al.*, 2015; Bae *et al.*, 2018), elevational and longitudinal gradients (e.g. Cook *et al.*, 2004; Kirk, Rahel & Laughlin, 2021), as well as anthropogenic factors (e.g. Radinger, Alcaraz-Hernández & García-Berthou, 2018) are also well known to shape and modulate fish distribution and assemblages in river ecosystems at larger spatial scales (see Figure 1.2). In fact, temperature and precipitation-related predictors have been proved as strong correlates of species occurrences, as well as hydrological alteration and human impact indicators in the case of some alien species such as *Gambusia holbrooki* and *Micropterus salmoides* (Filipe *et al.*, 2009; Murphy *et al.*, 2015; Bae *et al.*, 2018). However, although multiple studies have studied the relationships of historical and environmental variables, and the distribution of freshwater fish species (Filipe *et al.*, 2009; Dias *et al.*, 2014; Carvajal-Quintero *et al.*, 2019), both the effect of seawater tolerance on contemporary inland fish distribution (but see Filipe *et al.*, 2009) and the inclusion of these anthropogenic predictors in the analyses have been usually neglected in previous studies despite, as explained above, habitat modifications might facilitate species invasions (Bae *et al.*, 2018).

Figure 1.2. Historical connexions of river basins, current climatic conditions, elevational and longitudinal gradients, and anthropogenic factors shape the contemporary distribution of inland fish species along river ecosystems.



1.4. Trait-based approaches: a useful tool in ecology

The inclusion of species' traits (i.e. any characteristic that reflects a species adaptation to its environment; Menezes, Baird & Soares, 2010) into analyses have been commonly used in applied and theoretical ecology and in evolutionary research (Violle *et al.*, 2007; Dolédec & Statzner, 2010). Trait-based approaches enable to address macroecological questions across zoogeographic regions by potentially reducing species-specific context dependency (Hortal *et al.*, 2015). Thus, linking distribution patterns and niche properties with species traits have promoted a more complete view of the general rules and patterns that shape the spatial structure of species assemblages and a better understanding of responses to environmental change and the invasion process (Poff *et al.*, 2006; Frimpong & Angermeier, 2010; Thuiller *et al.*, 2012).

Specifically, trait-based approaches have been widely used to obtain a more comprehensive mechanistic understanding of the environmental filters selecting species from a regional species pool (Lebrija-Trejos *et al.*, 2010; Madrigal-González *et al.*, 2020). The 'environmental filter' concept has its roots in the study of plant communities but has obtained great attention recently (Kraft *et al.*, 2015), as it has been applied in many studies of community assembly, succession and invasion biology (e.g. Madrigal-González *et al.*, 2020). In fact, three main assembly processes (i.e. filters) are known to determine the success of introduced species in the new territory (Richardson & Pyšek, 2012). First, abiotic conditions of the invaded area filter species based on their traits and ecological niches (Pyšek & Richardson, 2008). Second, competition from the native species selects among the environmentally adapted aliens those that can coexist within local communities. Ultimately, natural or human mediated dispersal regulates which species spread into natural communities across the region (Gallien & Carboni, 2017). However, recent studies have criticised the ability to accurately identify the different mechanisms that shape biological communities as biotic interactions (e.g. competition) can produce similar patterns to those induced by environmental filtering (Kraft *et al.*, 2015; Cadotte & Tucker, 2017).

In fish ecology, trait-based approaches have been used to evaluate the interspecific patterns of fish traits and identify fish contrasting life history strategies. For instance, Winemiller and Rose (1992) classified North American fishes into three type of strategists. Fishes with 'periodic' strategy show late maturity in order to attain a size sufficient for production of a large clutch and adult survival during periods of suboptimal environmental conditions. The 'opportunistic' life-history strategy identifies fishes with early maturation, continuous reproduction over a long spawning season and rapid larval growth. Finally, fishes with 'equilibrium' strategy have a suite of traits often associated with the traditional *K*-strategy as large eggs, parental care and small clutches. Trait-based approaches and the fish strategies defined by Winemiller and Rose

(1992) have also been applied to study changes in fish assemblages along gradients of habitat degradation or hydrological variation (Hoeinghaus, Winemiller & Birnbaum, 2007; Tedesco *et al.*, 2008), to evaluate the effects of river restoration (Dolédec *et al.*, 2015), or to compare the different strategies of native versus alien fish species (Vila-Gispert *et al.*, 2005). In fact, recent studies showed that damming can cause significant effects on fish communities (Mims & Olden, 2013), favouring the proliferation of equilibrium strategists and the decline of more opportunist, rheophilic, benthic species and feeding specialists (Arantes *et al.*, 2019a). However, more studies are needed to better understand mechanisms of biotic responses to anthropogenic changes in river ecosystems.

However, although the development of open global fish databases such as FishBase (Froese & Pauly, 2019) or FISHMORPH (Brosse *et al.*, 2021) has favoured the accessibility to trait information, there is not yet a single database covering all fish species and all relevant traits. For instance, FishBase provides comprehensive ecological information for less than 2000 out of the 17134 described freshwater species (Brosse *et al.*, 2021). Morphological, reproductive and ecological features such as fish maximum length, longevity, fecundity, diet, habitat selection or swimming capacity are well established at the species level for North American ichthyofauna (Frimpong & Angermeier, 2010) and most common European species (Kuczynski *et al.*, 2018), but are still lacking for a large part of the world fish fauna (Brosse *et al.*, 2021). Actually, available datasets show different coverage and gaps regarding some specific traits and regarding less abundant or rare species (Statzner, Bonada & Dolédec, 2007). In fact, current knowledge of inland fish traits is generally based on a haphazard set of studies, with many species, regions, and ecosystems still unexplored (Frimpong & Angermeier, 2010), and is often established using expert judgment with scarce quantitative data and with a lack of standardised measurement protocols (e.g. Noble *et al.*, 2007). In addition, no study has analysed so far, the reliability (i.e. the agreement observed among different datasets) of assigned traits among the most common fish databases currently in use, even though it is key to provide meaningful and robust results and conclusions. Therefore, the development of consensus databases that collect and homogenise fish trait information so that it can be used in analyses of fish responses to global changes is very much needed (Brosse *et al.*, 2021).

1.4.1. Fish swimming performance

Swimming performance is a particularly crucial trait in fish and other aquatic organisms, mediating their fitness and survival (Jones, Kiceniuk & Bamford, 1974; Tudorache *et al.*, 2008), and playing an important role in habitat selection, feeding behaviour and reproduction (Plaut, 2001). Thus, swimming performance has been the subject of study in numerous works (see

Katopodis & Gervais, 2016). Critical swimming speed (U_{crit}), an experimental measurement of prolonged swimming performance, has been typically used as a standard measurement of fish swimming capabilities (Brett, 1964; Plaut, 2001). To estimate U_{crit}, individuals are forced to swim against water flow of increasing velocity until fatigue, i.e. the moment at which the fish can no longer maintain its position in the current (Kolok, 1999; Beecham *et al.*, 2009). This measurement has been used to assess the effects of different environmental and anthropogenic factors on fish fitness and survival (Plaut, 2001; Rajotte & Couture, 2002), or to design fish passes (Clough *et al.*, 2004a).

Several studies have evaluated the effects of experimental factors such as water temperature or fish size on U_{crit} (e.g. Srean *et al.*, 2016). For example, it is well known that U_{crit} increases as temperature rises below the optimum and then decreases as temperature rises above this optimum temperature (Hammer, 1995; Claireaux, Couturier & Groison, 2006). There is also clear evidence that swimming performance increases significantly with fish size (Srean *et al.*, 2016). However, there are still few studies that have analysed interspecific variability in swimming and its evolutionary relationship with other fish traits or with environmental gradients. Moreover, despite some previous studies demonstrated that the demands of fish on locomotion in flowing waters differ from those in stagnant waters (McGuigan *et al.*, 2003), the mechanisms by which many alien fish have competitive advantage over native species in calm waters remain poorly understood.

1.4.2. Analysing fish trait evolution and its relationship with the environment

There is a long history of comparative studies that evaluate the relationship of two traits across different species, or that analyse the link between one trait with an specific environmental variable (Felsenstein, 1985). However, it is important to notice that species are part of a hierarchically structured phylogeny, and thus, they do not represent statistically independent points (Felsenstein, 1985). It implies that closely related species tend to show similar morphological, trophic, reproductive, behavioural and ecological traits, due to common ancestry (Harvey & Pagel, 1991; Kamilar & Cooper, 2013). That is the reason why phylogenetic analyses are useful when researchers are interested in co-evolution of pair of traits or syndromes of traits, and why they have become widespread in the last years (Losos, 2008; de Bello *et al.*, 2015).

It is well known that fish species show adaptations to specific environments and that the functional trait composition of fish assemblages change across the river continuum and along a gradient of hydrologic variability (Wagner *et al.*, 2009; Olden & Kennard, 2010; Pease *et al.*, 2012). For instance, Pease *et al*. (2012) found that high-elevation streams lacking pool habitats harboured fish species with more fusiform bodies. However, few studies have analysed the trait–environment relationships of inland fish species along the elevational and longitudinal gradients of the rivers from an evolutionary perspective (Comte, Murienne & Grenouillet, 2014), or even the evolutionary relationships among traits. Therefore, little is known about how the evolution of traits correlates with species niches or with their distribution patterns. Specifically, the evolutionary relationship between the critical swimming speed (U_{crit}), the Winemiller-Rose typology and environmental conditions has been barely studied.

1.5. The Iberian ichthyofauna as a case study

Mediterranean-climate regions, and specifically, the Iberian Peninsula, are well suited to study the effects of climate, land use change and hydrological alteration on the distribution of native and alien species due to their particular characteristics. First of all, the Iberian Peninsula provides an excellent case study for comparing the contributions of historical and contemporary environmental factors because of the long-term existence of basin boundaries (e.g. Pyrenean mountain range), the high proportion of endemic species and a well-known evolutionary history of Iberian ichthyofauna (Filipe *et al.*, 2009). Secondly, the flow regime of Iberian rivers reflects the precipitation pattern, which is generally scarce during summer, with consequent low flows and high water temperatures; and could be very high during spring and fall, leading to high flows and even flash floods (Sabater & Barceló, 2010; Bonada & Resh, 2013). Damming has great negative ecological effects because reservoirs heavily transform the natural regimes of rivers into artificial lentic environments. Actually, more than 1500 barriers fragment Iberian rivers (MAPAMA, 2020).

Western and southern Europe, including the Iberian Peninsula, is one of the main global invasion hotspots where introduced species represent more than a quarter of the total number of species (Leprieur *et al.*, 2008). In Iberian inland waters, 125 alien naturalised animal species have been identified (Muñoz-Mas & García-Berthou, 2020), constituting one of the most important threats to native fauna, and generating a clear biotic homogenisation among basins (Clavero & García-Berthou, 2006). In fact, some of them, like *Cyprinus carpio* or *G. holbrooki,* are listed as one of the 100 worst invasive alien species (Lowe *et al.*, 2000).

Compared with other regions, Mediterranean fresh waters are low in fish species richness with a large part of its freshwater fauna being endemic and threatened according to IUCN criteria (Smith & Darwall, 2006). Specifically, the Iberian fresh waters are inhabited by 68 native fish species, of which 41 are endemic, belonging to 31 genera and 20 families; and 32 alien species, belonging to 29 genera and 13 families (Figure 1.3 and Figure 1.4). Following the IUCN Red List of Threatened Species (2019), 8 native species are critically endangered (CR), 11 endangered (EN) and 13 vulnerable (VU). Almost 45% of the native species show declining population trends (Mota, Rochard & Antunes, 2016), and some populations of native diadromous fish have decreased by about 90% since the 1950s (Maceda-Veiga, 2013), in agreement with the global trends shown above. Following Darlington's simplification of Myers' classification (Darlington, 1948, 1957), of 100 fish species (68 native and 32 alien) considered in this study,63 are primary (i.e. fish that cannot survive in seawater and are thus strictly confined to fresh water), 7 secondary (i.e. fish that mostly live in fresh water but show a little salt-tolerance and can thus eventually survive in seawater) and 30 are peripheral fish species (i.e. fish that occur in fresh waters but have high salt-tolerance, such as diadromous or species of marine origin; Figure 1.3).

In general, river headwaters and tributaries of the Iberian Peninsula are the most preserved stretches, and are inhabited by brown trout (*Salmo trutta*; see Figure 1.2) and some cyprinids adapted to these cool-water habitats with steep slopes and coarse substrates (e.g. *Barbus meridionalis* and *B. haasi*). Middle and lower reaches are dominated by primary fish species such as *Luciobarbus* and *Squalius* spp., and some peripheral fishes (e.g. *Anguila anguilla*, *Alosa* spp. or *Salaria fluviatilis*). The few secondary species that are native to the Iberian Peninsula (*Aphanius* spp. and *Valencia hispanica*) are mainly distributed in coastal lagoons or river estuaries (Doadrio, 2001).

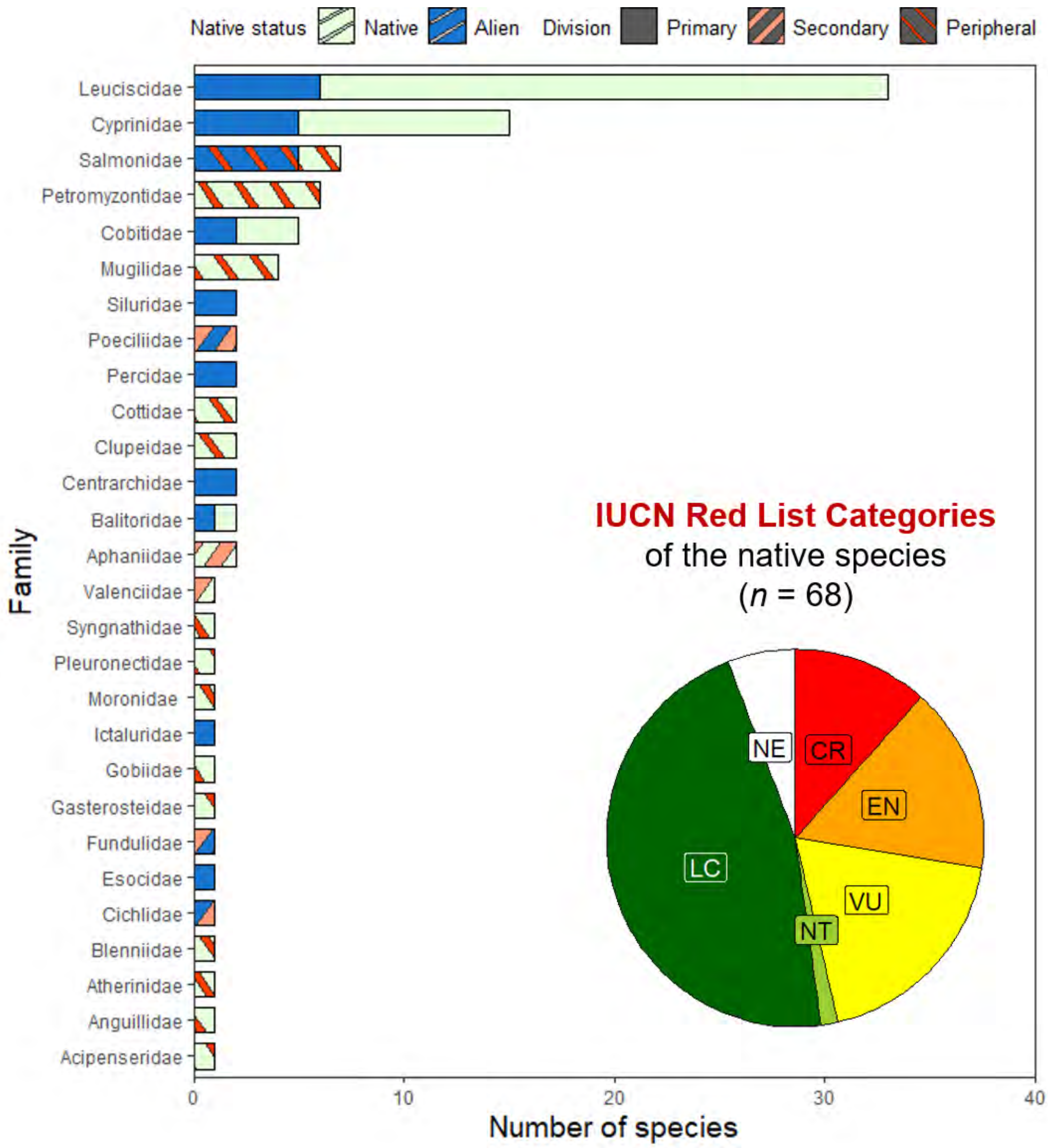


Figure 1.3. Number of inland fish species of each family present in the Iberian Peninsula. Native species are represented by light green bars, while alien species are represented by dark blue bars. Striped patterns show Darlington's divisions (i.e. primary species or fish that cannot survive in seawater and are thus strictly confined to fresh water; secondary species or fish that mostly live in fresh water but show a little salt-tolerance and can thus eventually survive in seawater; and peripheral species or fish that occur in fresh waters but have high salt-tolerance, such as diadromous or species of marine origin). The pie chart at the bottom shows the proportion of endangered native species following the IUCN categories and criteria. LC = least concern; NT = near threatened; VU = vulnerable; EN = endangered; CR = critically endangered; NE = not evaluated.

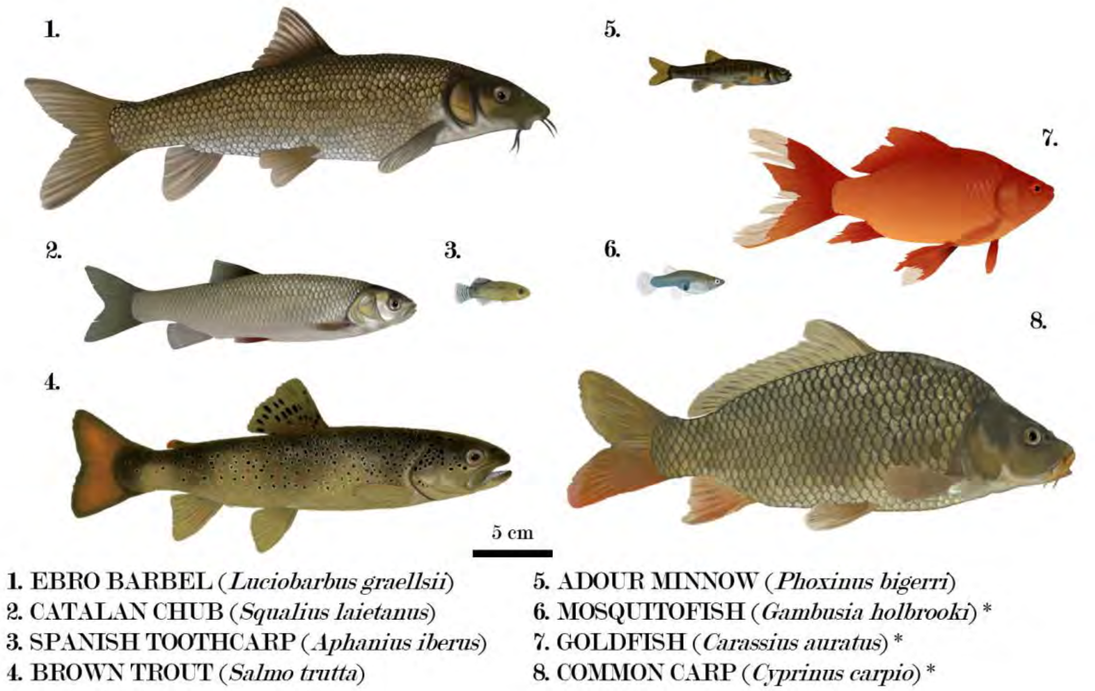
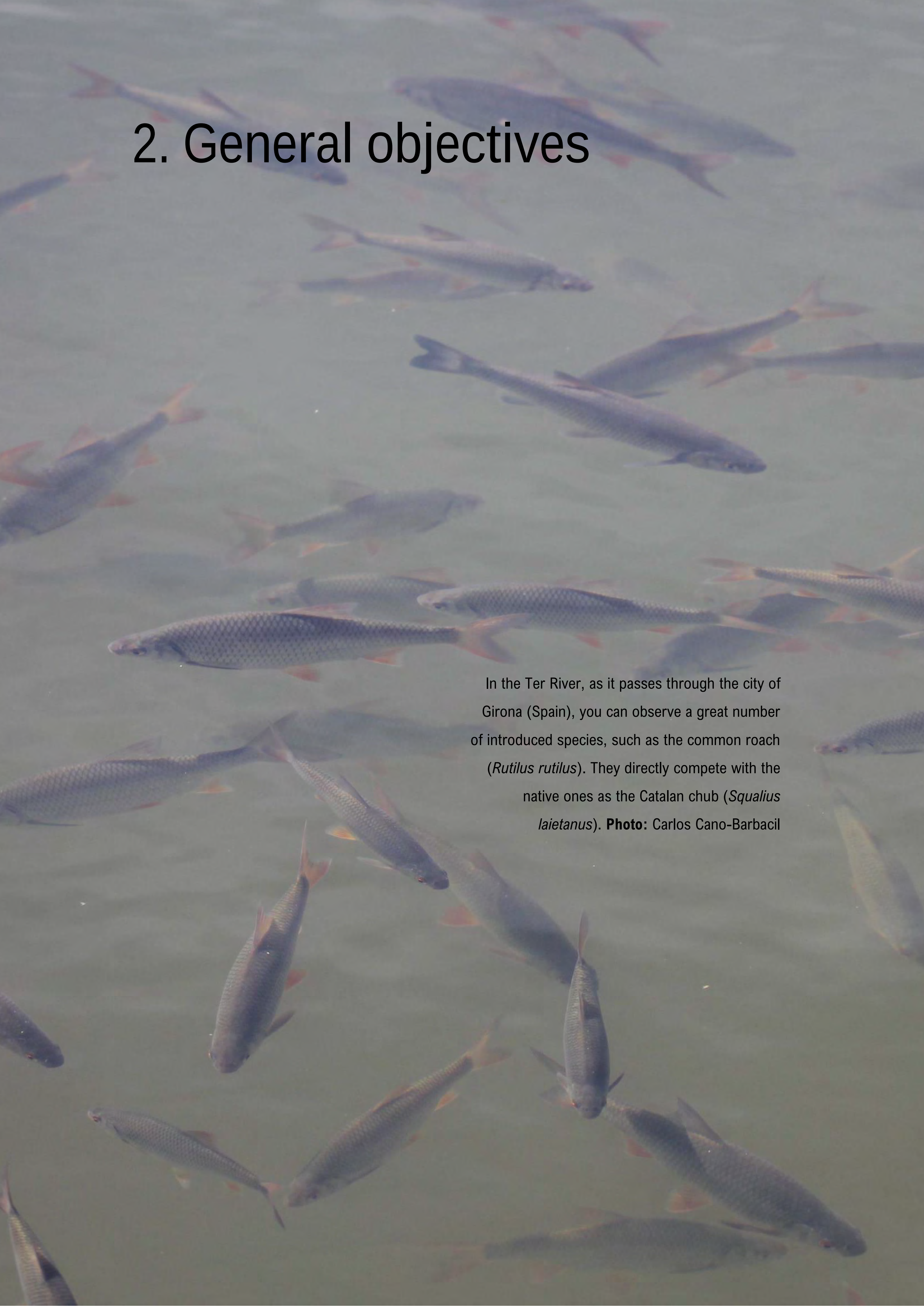


Figure 1.4. Some representative species of the Iberian Peninsula. Alien species are indicated by asterisks. Illustrations by Carlos Cano-Barbacil.

Life history strategies of Iberian fish are relatively well known (see e.g. Vila-Gispert *et al.*, 2005). Native fish populations of the Iberian Peninsula have developed a life history with morphological, physiological and behavioural traits to overcome the irregular flow regime (Gasith & Resh, 1999; Doadrio *et al.*, 2011; Lorenzo-Lacruz *et al.*, 2012). In general, native species are characterised by small size, short longevity, early maturity, low fecundity, multiple spawnings per year, and long reproductive span, which corresponds well to opportunistic life history strategy (Vila-Gispert *et al.*, 2005). By contrast, alien species are characterised by traits of periodic strategists: large size, high longevity, late maturity, high fecundity, partly multiple spawnings per year, and short reproductive span. There is also some evidence that hydrological alteration favours these periodic alien species (Bae *et al.*, 2018). However, the ecological filtering produced by hydrological alteration in Iberian fish has not been demonstrated.



2. General objectives

In the Ter River, as it passes through the city of Girona (Spain), you can observe a great number of introduced species, such as the common roach (*Rutilus rutilus*). They directly compete with the native ones as the Catalan chub (*Squalius laietanus*). **Photo:** Carlos Cano-Barbacil

This thesis is built on five studies focused on the general goal of evaluating the relationship between environmental factors and the distribution patterns of Iberian inland fishes and their traits, and to understand if species traits explain the ecological filtering produced by hydrological alteration. Specifically, the present thesis is structured in five chapters (see Figure 2.1) elaborating on following objectives and hypotheses:

- **Objective 1:** To assess the reliability of species traits and the consistency of trait information among different databases currently in use, identify well-studied versus data-deficient traits and species, and provide an open, updated, consensus trait database for all fish species inhabiting the Iberian Peninsula (*Chapter I*).
    - **Hypothesis 1:** It was hypothesised that reliability (i.e. the agreement observed among databases) would be lower for traits established using expert judgment with scarce quantitative data, and that species with very restricted distributions would show generally lower data coverage.
- **Objective 2:** To compile the most comprehensive empirical dataset of swimming performance (i.e. U_{crit}) for Iberian inland fishes, and to compare the role of species identity, taxonomic affiliation and other experimental variables on U_{crit}, using the machine learning technique ‘random forests’ (*Chapter II*).
    - **Hypothesis 2:** It was expected that larger and streamlined fish would show higher U_{crit} and that temperature would be one of the main factors influencing U_{crit}.
- **Objective 3:** To test for differences in U_{crit} between native and alien species of the Iberian Peninsula (*Chapter II*).
    - **Hypothesis 3:** It was hypothesised that alien species would show lower U_{crit} than native fishes as many successful invaders in the Iberian Peninsula prefer lentic habitats, while native species would be better adapted to flowing waters.
- **Objective 4:** To assess the importance of different environmental variables in shaping the current distribution of Iberian inland native and alien fish, and specifically, to evaluate the role of hydrological alteration (*Chapter III*).

  - **Hypothesis 4:** It was hypothesised that native and alien species in the Iberian Peninsula would show contrasting importance of predictors. We expected that hydrological alteration and temperature would be more important and positively correlated with alien species presence.

- **Objective 5:** To understand the relationship between these distributional drivers and fish traits (*Chapter III*).

  - **Hypothesis 5:** Although similar studies dealing with the ecological filtering that hydrological alteration produces in European inland fish are scarce, it was hypothesised that alien limnophilic and periodic species would be more prevalent at sites with higher temperatures and hydrological alteration. By contrast, the presence of native rheophilic fish was expected to be negatively correlated with these variables.

- **Objective 6:** To measure the phylogenetic signal of several morphological, trophic, habitat use, and reproductive traits in Iberian inland fish, and to test for correlated evolution of these traits with elevational and longitudinal distribution (*Chapter IV*).

  - **Hypothesis 6:** It was expected that the majority of traits would show a phylogenetic signal and that fish body size and other morphological traits are more conserved across the phylogeny than others such as trophic traits. Although studies analysing the trait–environment relationships of inland fish species along the elevational gradient of the rivers from an evolutionary perspective are scarce, an evolutionary correlation between fish traits and elevational and longitudinal distribution was also expected.

- **Objective 7:** To test for differences in ecological niche breadth and position among Iberian native and alien species, and to compare these results at different geographical extents (*Chapter V*).

- **Hypothesis 7:** It was hypothesised that alien species would show higher tolerance (i.e. they should be generalist taxa that occur in a wide range of environmental conditions), while native fishes would show more marginal niche position (i.e. specialists that deviate from general habitat conditions). It was also expected to find contrasting results depending on the geographical extent used in the analyses. Specifically, a greater proportional increase of niche breadth of widely distributed species (e.g. alien fishes) was expected when analysing global data.

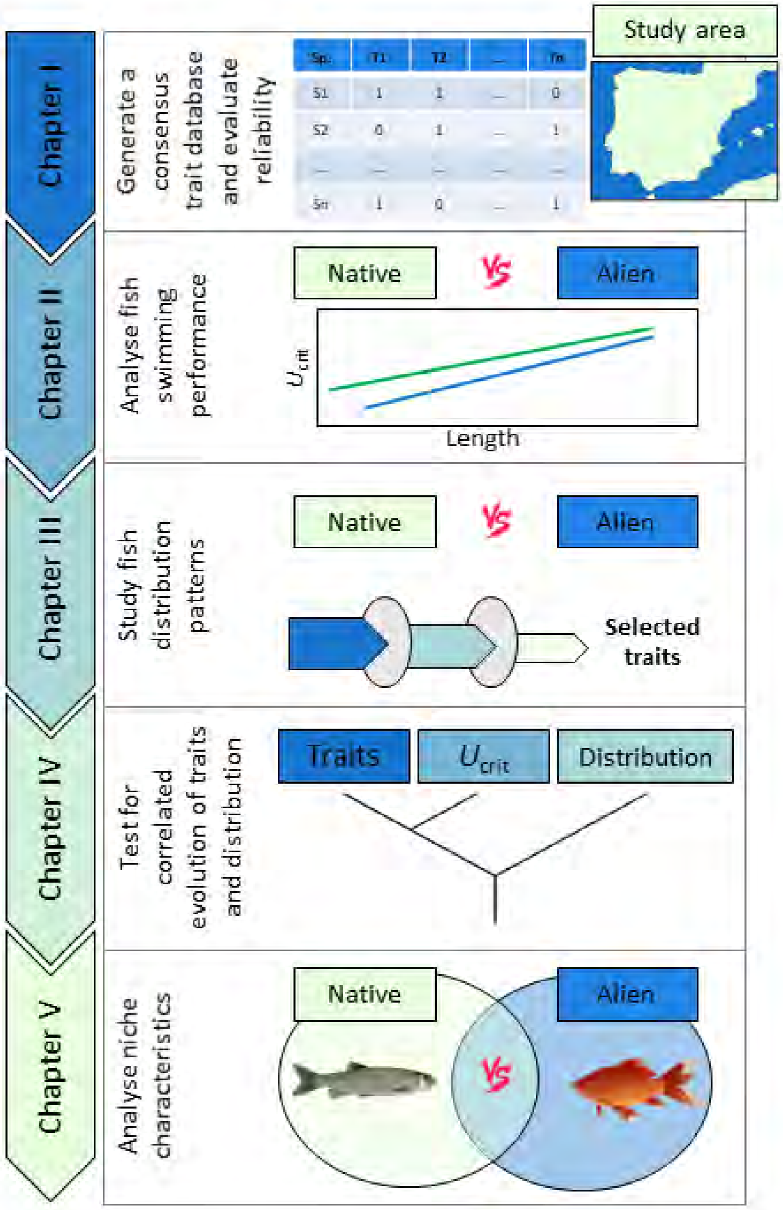
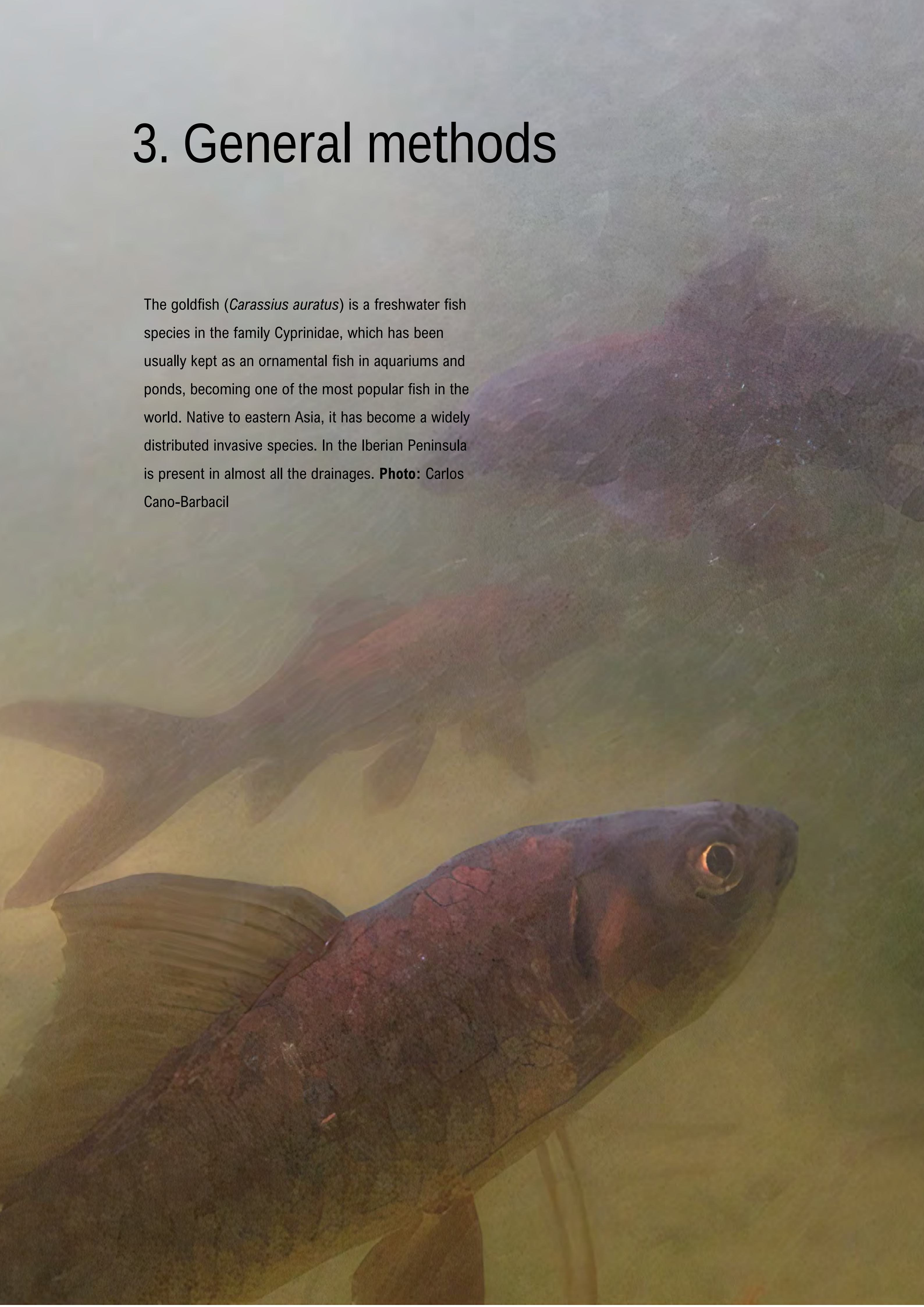


Figure 2.1. Graphical abstract of the main objectives of the thesis.

3. General methods



The goldfish (*Carassius auratus*) is a freshwater fish species in the family Cyprinidae, which has been usually kept as an ornamental fish in aquariums and ponds, becoming one of the most popular fish in the world. Native to eastern Asia, it has become a widely distributed invasive species. In the Iberian Peninsula is present in almost all the drainages. **Photo:** Carlos Cano-Barbacil

This section summarises the main characteristics of the study area, outlines the data sources used to compile trait information and occurrence records of Iberian inland fishes, and resumes all the statistical analyses applied in this thesis. More specific information about the methodological aspects can be found on the *Methods* section of each chapter.

3.1. Study area overview

This thesis is focused on the inland waters and fishes of the Iberian Peninsula, which is located in the southwest corner of the European continent, and has an extension of 583254 km^2. This region encompasses four main climates within a relatively small geographical space. Following the Köppen-Geiger climate classification, the northern half of the Iberian Peninsula is dominated by a Mediterranean oceanic climate with warm summers (Csb; Figure 3.1a), the southern half by a Mediterranean climate with dry and hot summers (Csa; Figure 3.1b), SE Spain by a semiarid climate (Bsk, Bsh, Bwk and Bwh; Figure 3.1c), and mountainous areas by an oceanic climate (Cfb and Cfa; Figure 3.1d) (Kottek *et al.*, 2006; AEMET, 2011). Furthermore, the Iberian Peninsula is a mountainous territory with a complex orography (Figure 3.2a), in which elevations over 1500 m are common and the highest peak rises to 3480 m, causing large variations in climate at local and regional scales (Sabater *et al.*, 2009; Bayón & Vilà, 2019).

In the Iberian Peninsula there are five large rivers longer than 650 km. Four of them drain into the Atlantic Ocean (Douro, Tagus, Guadiana, Guadalquivir) and one into the Mediterranean Sea (Ebro; see Figure 3.2). This drainage asymmetry is consequence of the particular geology of the Iberian Peninsula, as the central plateau is tilted to the west and surrounded by the Iberian System to the east (Sabater *et al.*, 2009). Hence, Atlantic rivers are longer and have lower gradient than the Mediterranean ones, which tend to be torrential with irregular flow regimes due to the high interannual variability in rainfall, alternating prolonged periods of drought with floods of variable intensity (Doadrio, 2001). By contrast, rivers flowing to the Cantabric Sea are short, but mighty because of high rainfall.

Moreover, the Iberian Peninsula is one of the most impacted regions by dams as human influence on freshwater ecosystems has a long history in this territory, resulting in the regulation of many Iberian rivers (Sabater *et al.*, 2009; Grill *et al.*, 2019). Actually, over 1500 large dams fragment Iberian rivers, and some of them are interconnected with artificial channels (e.g. Tagus – Segura transbasin diversion), facilitating inter-basin transfer of biota and threatening sensitive species (Sabater *et al.*, 2009; MAPAMA, 2020).

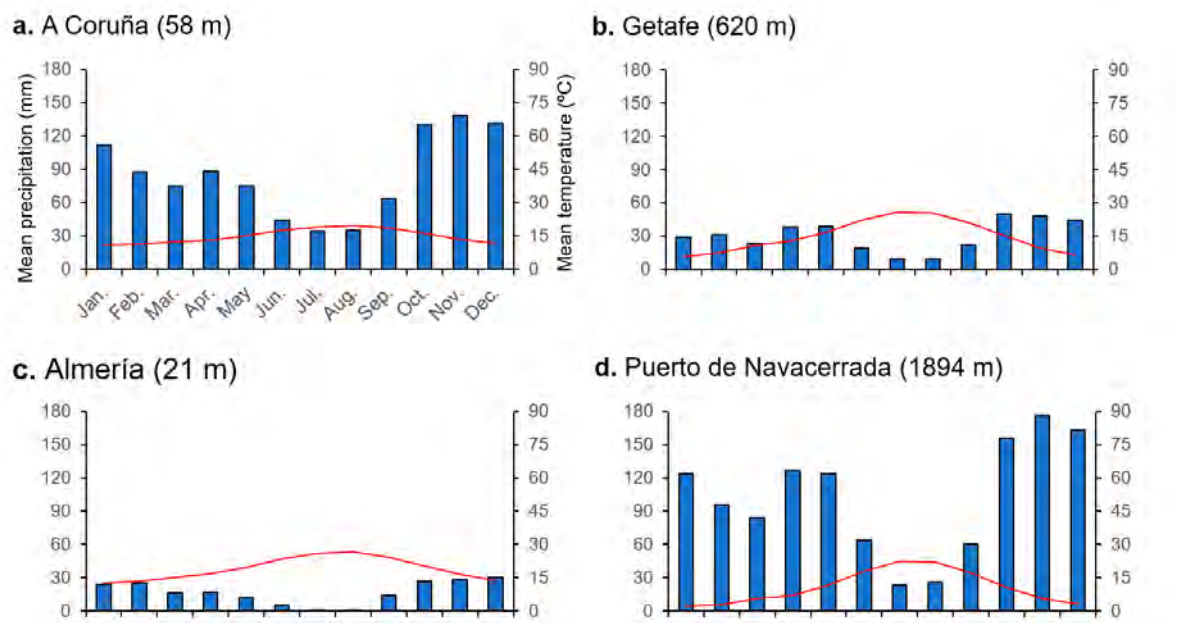


Figure 3.1. Climatic diagrams of four locations in the Iberian Peninsula with contrasting climatic characteristics: (a) A Coruña, (b) Getafe, (c) Almería and (d) Puerto de Navacerrada. Blue bars indicate the mean monthly precipitation and the red line indicates the mean monthly temperature. Source: Agencia Estatal de Meteorología (AEMET).

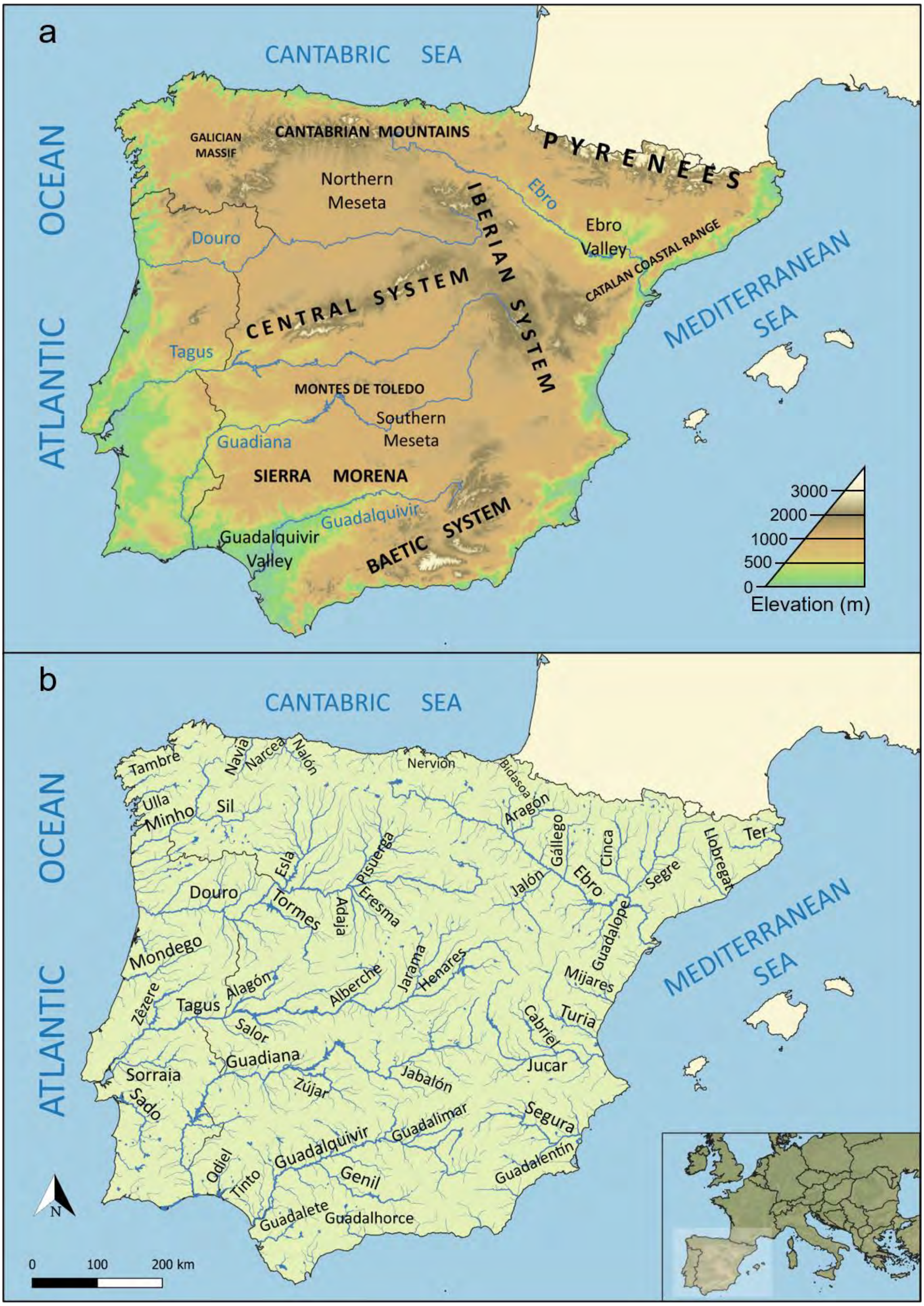


Figure 3.2. (a) Physical map of the Iberian Peninsula. (b) Main rivers of the Iberian Peninsula. Source: Spanish National Center for Geographic Information (http://centrodedescargas.cnig.es/).

3.2. List of the species studied

The list of inland Iberian fishes mainly followed Doadrio *et al.* (2011) and Kottelat & Freyhof (2007), and was completed with the new species described (e.g. the three new species of the genus *Lamprea*; Mateus *et al.*, 2013) and the latest introduced species established in the study area (López *et al.*, 2012; Aparicio *et al.*, 2013; Aparicio, 2015; Ribeiro *et al.*, 2015; Merciai *et al.*, 2018). We classified all species using Darlington's divisions (Darlington, 1948) into three major eco-evolutionary groups (i.e. primary, secondary and peripheral). Darlington's divisions (a simplification of the original Myers' classification) were assigned using taxonomic families following Berra (2001). We also classified the endangered native species following the IUCN categories and criteria (IUCN, 2019), and compiled the introduction date for those alien species from a recently published review (Muñoz-Mas & García-Berthou, 2020).

3.3. Data sources

We compiled fish trait data from 19 complete and recently updated data sources: peer-reviewed papers (n = 10); fish index manuals (n = 6); online databanks (n = 2); and books (n = 1) (see Table S4.1 for specific references). The consensus trait database generated in the *Chapter I*, was subsequently completed for the following analyses using other 15 scientific publications (see Table S6.1 for further details). Similarly, U_{crit} experimental data was collected from 79 literature sources published from 1959 to 2020 (see Table S5.1 for further details). Secondly, we collected occurrence data for all established Iberian freshwater fishes between 2000 and 2020. Regional and global presence information was primarily obtained from GBIF (Global Biodiversity Information Facility; GBIF.org, 2019a), the Freshwater Biodiversity Data Portal (Biofresh, 2021), Spanish atlases (Doadrio, 2001; Doadrio *et al.*, 2011) and the Portuguese 'Carta Piscícola Nacional' (Ribeiro *et al.*, 2007), and was complemented with 19 additional sources (see Table S6.1 for further details). The phylogenetic relationships of the studied Iberian species were obtained from a recent phylogenetic tree of ray-finned fishes (Rabosky *et al.*, 2018), using the function 'fishtree_phylogeny' of the R-package 'Fish Tree' (Chang *et al.*, 2019). We also compiled information regarding the salinity (see Table S6.7.) and thermal tolerance (see Comte & Olden, 2017a; Kärcher *et al.*, 2019) of Iberian fish species. Finally, we compiled several climatic, topographic, land use and anthropogenic variables from online databases (see methods in *Chapter III*, *IV* and *V* for further details) and did subsequent calculations in QGIS 3.4.14 (QGIS Development Team, 2019) in order to provide the adequate format.

3.4. Brief overview of the statistical analysis used

To achieve the proposed objectives, a number of statistical techniques was used, which are summarised in Table 3.1. Further details about statistical analyses are described in each chapter.

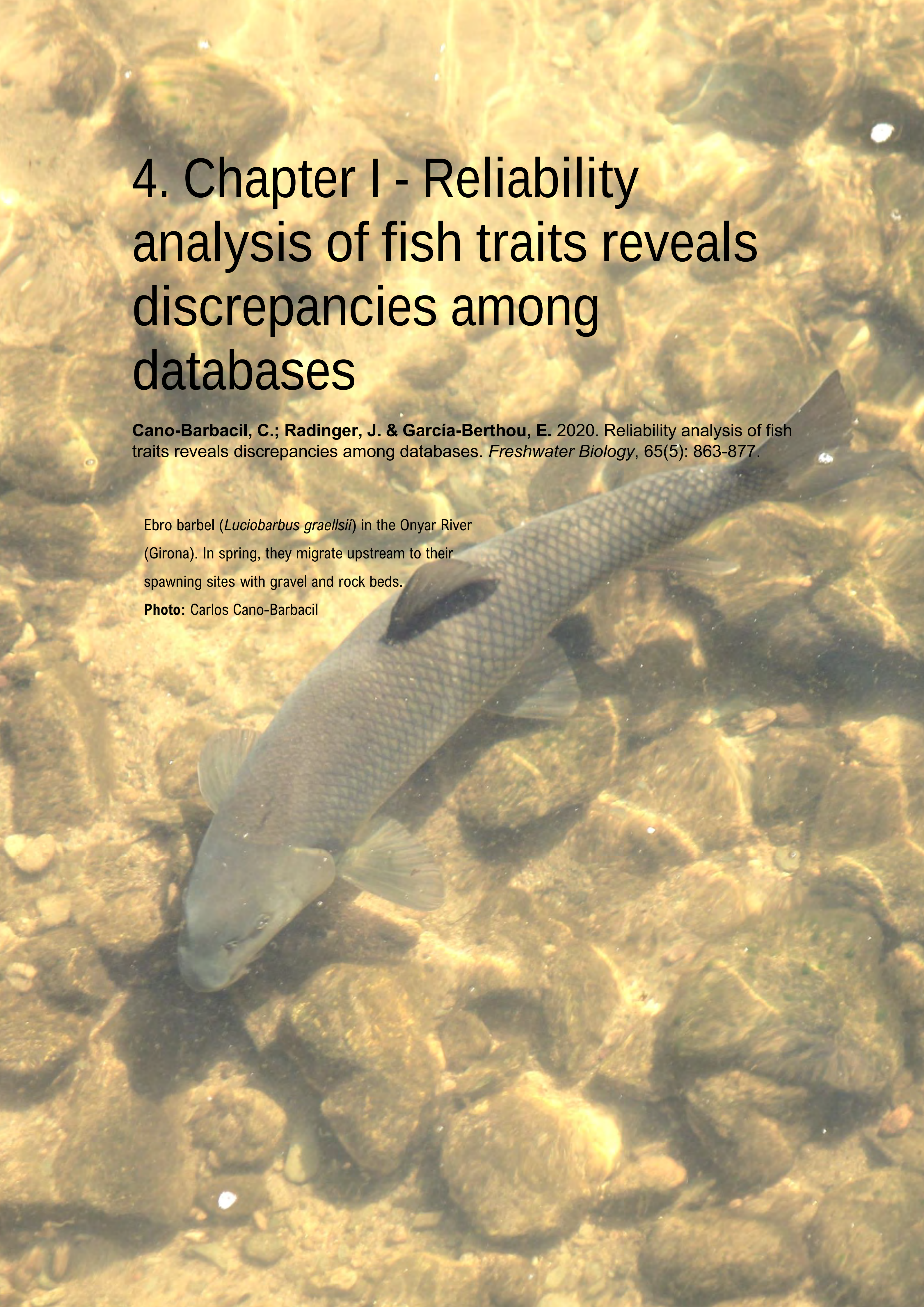
Table 3.1. Statistical techniques used for each chapter of this thesis. See more detailed information in the methods section of each chapter.

Chapter	Statistical technique	Purpose of the analysis
I	Generalised linear models	Test for differences in fish traits among databases while accounting for differences among species.
	Reliability analysis	Measure the agreement among databases.
	Spearman's rank correlations	Analyse the relationship among trait use, trait-specific data availability and trait reliability.
	Linear models	Test for effects of measurement scale (continuous or binary) and trait type (morphological, habitat use, trophic, or reproductive) on trait reliability, trait use, and trait-specific data availability. Test for effects of latitudinal range, species' year of description, migration behaviour, and conservation and native status on species' coverage and species-specific trait data availability.
	Bland-Altman analysis	Compare η^2 of linear models with partial R^2 of generalised linear models.
II	Random forests	Analyse which predictors better explained U_{crit} variation.
	Analysis of covariance (linear model)	Test for differences in U_{crit} between native and alien species, among families, and among body shape categories, after accounting for fish body length.
	Linear mixed models	Quantify the relative roles of species and other predictors on U_{crit} and further test for heterogeneous slopes.
	Bland-Altman analysis	Compare the predicted Ucrit values using estimated marginal means with those obtained using random forests.
III	Species distribution models	Model the current distribution of Iberian inland fish using different algorithms.
	Permutational analysis of variance	Test for differences in the quality of the model's predictions and in the variable importance of predictors among primary, secondary and peripheral native and alien fish species.
	Redundancy analysis	Explore the importance of specific environmental variables in determining the distribution of different traits of freshwater fish.

Chapter	Statistical analysis	Purpose of the analysis
IV	Measurement of phylogenetic signal	Evaluate if traits are more conserved than expected by chance across the phylogeny.
	Analysis of covariance (linear model)	Analyse the effect of trait measurement scale (i.e. continuous or binary) and trait type (i.e. morphological, trophic, reproductive and habitat use) on phylogenetic signal measure.
	Brownian motion and Ornstein-Uhlenbeck models	Evaluate how elevational distribution has evolved across the fish phylogeny.
	Principal coordinates analysis	Identify the main fish life-history strategies and visualise possible correlations among fish traits.
	Generalised additive models	Test for a relationship between the ordination axes and elevation.
	Phylogenetic generalised least squares	Analyse the influence of mean elevation on fish traits and the correlation among traits.
	Generalised linear models	Analyse the influence of mean elevation on fish traits and the correlation among traits.
V	Outlying mean index analysis	Calculate niche position, marginality and breadth of each species considered.
	Pearson's correlation	Evaluate the differences in the niche metrics when using climatic and longitudinal variables.
	Linear models	Analyse the relationship among climatic niche metrics calculated using different geographical extents considering the species native status, the Darlington's division and the interaction among factors. Relate climatic niche metrics calculated using different geographical extents of alien species considering their introduction date.
	Permutational analysis of variance	Test for differences in niche metrics among native and alien, and primary, secondary and peripheral fish species.

4. Chapter I - Reliability analysis of fish traits reveals discrepancies among databases

Cano-Barbacil, C.; Radinger, J. & García-Berthou, E. 2020. Reliability analysis of fish traits reveals discrepancies among databases. *Freshwater Biology*, 65(5): 863-877.



Ebro barbel (*Luciobarbus graellsii*) in the Onyar River (Girona). In spring, they migrate upstream to their spawning sites with gravel and rock beds.

Photo: Carlos Cano-Barbacil

Chapter summary

Trait-based approaches are commonly used in ecology to understand the relationship between biodiversity and ecosystem functioning, environmental filtering or biotic responses to anthropogenic perturbations. However, little is known about the reliability of assigned traits and the consistency of trait information among different databases currently in use.

Using 99 native and alien Iberian inland fish species, we investigated a total of 27 biological and ecological traits for their consistency among 19 different databases and identified less reliable traits, that is, traits with high disagreement among databases. Specifically, we used generalised linear models and inter-rater reliability statistics (Krippendorff's α) to test for differences in trait values among databases. We also identified well-studied versus data-deficient traits and species.

Our results show notable discrepancies and low reliability for several biological and ecological traits such as microhabitat preference, omnivory, invertivory, rheophily, and limnophily. Least reliable traits were mainly categorical (rather than continuous) and established by expert judgment and without a clear definition or a common methodology. Interestingly, categorical traits such as rheophily or limnophily, which showed significantly lower reliability, concurrently showed higher data availability and use than continuously scaled traits. Such uncertainties in trait assignments could affect bioassessment and other ecological analyses. Species with smaller distributional ranges and those that have been described more recently, presented lower coverage and data availability in trait databases. We encourage further standardisation of fish trait measurement protocols to help improve the robust application of bioassessment indices and trait-based approaches.

Keywords: trait-based approaches, habitat requirements, Krippendorff's α, freshwater fish, Iberian Peninsula

4.1. Overview

Species traits are widely used in theoretical and applied ecology (Dolédec & Statzner, 2010; Frimpong & Angermeier, 2010) and evolutionary research (Violle *et al.*, 2007; Pyron *et al.*, 2011), often replacing strictly species-based approaches (Menezes *et al.*, 2010; Moretti *et al.*, 2017; Zakharova, Meyer & Seifan, 2019). Trait-based approaches offer advantages in quantifying and predicting impacts of disturbances on communities (Mouillot *et al.*, 2013) and ecosystem processes and functioning (Naeem, Duffy & Zavaleta, 2012). Trait-based approaches also enable one to address macroecological questions by potentially reducing species-specific context dependency and allowing for generalisation across communities and ecosystems (Statzner *et al.*, 2001; McGill *et al.*, 2006; Suding *et al.*, 2008; Hortal *et al.*, 2015; Kunstler *et al.*, 2015; Moretti *et al.*, 2017). Trait-based approaches are employed in many organism groups such as plants (Guittar *et al.*, 2016; Kergunteuil *et al.*, 2018), animals (Poff *et al.*, 2006; Frimpong & Angermeier, 2010; Luck *et al.*, 2012; Castro, Dolédec & Callisto, 2018), phytoplankton (Litchman & Klausmeier, 2008), and microorganisms (Allison, 2012; Ortiz-Álvarez *et al.*, 2018; Guittar, Shade & Litchman, 2019). However, there is considerable disparity in the methodological advancements among different organism groups. Specifically, trait-based approaches in animal ecology are considered lagging behind the advances made in plant ecology during the last two decades (Luck *et al.*, 2012). These advances include, for example, the development of standardised methods for selecting and measuring plant traits (McIntyre *et al.*, 1999; Cornelissen *et al.*, 2003; Pérez-Harguindeguy *et al.*, 2013). Nevertheless, important progress has also been made in recent years with respect to traits of freshwater animals, particularly macroinvertebrates, such as the development of public online databases that facilitate biodiversity conservation, bioassessment and the development of ecological theory (Statzner *et al.*, 2007; Schmidt-Kloiber & Hering, 2015).

Many different types of traits are used in the ecological literature, causing ambiguity in defining 'what' actually constitutes a trait (Violle *et al.*, 2007; Flatt & Heyland, 2011; Breed & Moore, 2016). Following Menezes *et al.* (2010), traits are any characteristic that reflects a species adaptation to its environment and can be classified into two types of features ('traits' hereafter): (1) biological traits describing life cycle, physiological and/or behavioural characteristics including maximum body size, longevity or feeding and reproductive strategies, and (2) ecological traits or requirements that are linked to habitat preferences, water flow, pollution or temperature tolerances.

Trait approaches constitute a valuable tool that has also frequently been employed in fish ecology. Commonly used traits in fish ecology relate to fish morphology (e.g. body size and form), reproductive features (e.g. age at maturity, egg size), trophic position (e.g. piscivory, omnivory or invertivory), or habitat preference (e.g. rheophily or limnophily) (Frimpong & Angermeier, 2010). These traits can be either reported as continuous variables like most morphological traits, which are often directly measured on individuals, or as categorical variables like most trophic and habitat traits, which are frequently established by expert criteria (e.g. Noble *et al.*, 2007).

Fish traits have, for example, been employed to examine the roles of environmental variables and biotic interactions in determining the structure of stream fish assemblages, with trait-based approaches often performing better than taxonomic-based analyses to infer ecological responses to environmental variation (e.g. Hoeinghaus, Winemiller & Birnbaum, 2007). Moreover, trait-based approaches also allow one to evaluate changes in fish assemblages along gradients of habitat degradation or hydrological variation (Berkman & Rabeni, 1987; Poff & Allan, 1995; Goldstein & Meador, 2005; Hoeinghaus *et al.*, 2007; Tedesco *et al.*, 2008; Dolédec *et al.*, 2015). For example, Olden & Kennard (2010) showed that an increase in hydrologic variability promoted opportunistic species traits (i.e. small fishes with early maturation and short generation time), while dam-induced dampening of flow variability favoured periodic or equilibrium strategists (i.e. species with longer generation time and in general higher fecundity or juvenile survivorship). Similarly, Dolédec *et al.* (2015) demonstrated that river restoration (i.e. increasing lateral connectivity and minimum flow) generally favoured traits of low fecundity, intermediate growth rates, late maturation age, intermediate length at maturity, large size and high mobility. Other studies used fish traits to compare features of successful vs. failed alien fish introduction and to compare features of successful alien vs. native fish species (Erös, 2005; Vila-Gispert *et al.*, 2005; Olden *et al.*, 2006; Ribeiro *et al.*, 2008; Grabowska & Przybylski, 2015), to assess species responses to climate change (Daufresne *et al.*, 2003; Chevalier *et al.*, 2018), and to predict and understand local species extinctions (Angermeier, 1995; Parent & Schriml, 1995; Johnston, 1999; Reynolds, Webb & Hawkins, 2005; Olden, Poff & Bestgen, 2008). Fish traits also constitute a central component of biotic indices that have been widely used to evaluate the integrity of freshwater ecosystems (Oberdorff *et al.*, 2002; Logez & Pont, 2011; García-Berthou *et al.*, 2015). The main advantage of trait-based indices is the large-scale applicability, as organism responses can be anticipated by their traits and thus allow comparisons across geographical regions that do not necessarily share the same species pool (Bonada *et al.*, 2006). Most fish indices use traits such as 'rheophily' as an indicator of river impoundment and channelisation effects (Pont *et al.*, 2006; Holzer, 2008). Other traits such as 'diadromy' are commonly used as indicators of disruption of longitudinal connectivity (EFI+ CONSORTIUM, 2009).

Trait-based approaches are, however, only as good as the trait data that underpin them. For this reason, unambiguous, reliable and comprehensive information on biological and ecological traits for freshwater fish is essential. This requires detecting knowledge gaps regarding trait information and discrepancies among fish-trait databases. Presently, there are many independent databases that concurrently cover the same species pool of a given geographical region, but which might provide inconsistent information for some species. Reliability analysis is widely used in psychology and other social sciences to evaluate the agreement or consistency among measurements or raters (Krippendorff, 2004; Gwet, 2008). More specifically, inter-rater reliability statistics quantify the closeness of scores assigned by a pool of raters (e.g. databases) to the same observation (Gwet, 2008). Although there are websites that compile a large amount of data on fish (e.g. FishBase.org), there is generally no single database covering all species and all relevant traits, even for small geographical areas (Kremer *et al.*, 2017). In addition, these databases often include fishes that are not evenly studied and thus have different coverage and gaps regarding some specific trait information, presumably due to methodological reasons, e.g. required high efforts to collect less abundant or rare species (Statzner *et al.*, 2007). This is particularly the case for the Iberian Peninsula, which is home to a large number of endemic fishes (over 40), many of them described in the last 20 years (Doadrio *et al.*, 2011), but also hosts a large number of non-native fish species (currently about 30 established).

Therefore, the principal objectives of this study are: (1) to test for differences in fish traits among databases while accounting for differences among species; (2) to assess the reliability of assigned traits; and (3) to identify well-studied vs. data-deficient traits and species, using Iberian fish as a case study. Additionally, we provide an up-to-date and comprehensive consensus trait database for native and alien fish species of the Iberian Peninsula. We hypothesised that most of the variation of traits would be due to inherent differences among species. However, we also expected a notable part of the variation for some traits to be explained by a uniform bias in trait assignment of particular databases. We hypothesised that reliability (i.e. the agreement observed among databases) would be higher for continuous compared to categorical traits, because the latter are often established using expert judgment with scarce quantitative data (e.g. Noble *et al.*, 2007). We also hypothesised that more recently described and endemic species would generally have more knowledge gaps regarding their traits due to the difficulty of obtaining reliable empirical data for rare, endemic or endangered species (Kunin & Gaston, 1991; Nakagawa & Freckleton, 2008; Tyler *et al.*, 2012; Tsianou & Kallimanis, 2016; Radinger, Kail & Wolter, 2017).

4.2. Methods

4.2.1. Data compilation

We collected comprehensive trait data for all inland fishes (including diadromous) of the Iberian Peninsula (*n* = 99), considering both native (*n* = 68) and alien (*n* = 31) species, belonging to 56 genera and 28 families, according to a recently proposed classification (Freyhof, Özuluğ & Saç, 2017; Schönhuth *et al.*, 2018). As alien species, we only included those having established self-sustaining populations in the Iberian Peninsula. The list of inland Iberian fishes mainly followed Doadrio *et al*. (2011) and Kottelat & Freyhof (2007), and was completed with more recently described native species (Mateus *et al.*, 2013) and alien species lately recorded (López *et al.*, 2012; Aparicio *et al.*, 2013; Aparicio, 2015; Ribeiro *et al.*, 2015; Merciai *et al.*, 2018). The Iberian Peninsula was selected as the study area because it is a well-defined biogeographical area and its freshwater fauna is relatively well studied (Doadrio *et al.*, 2011).

We identified and compiled information from the most complete and recently updated data sources (‘databases’ hereafter) that included trait data of fish species present in the Iberian Peninsula, thereby aiming to avoid the use of very correlated or duplicated databases. In total, trait data were obtained from 19 different databases (Table S4.1): peer-reviewed papers (*n* = 10); fish index manuals (*n* = 6); online databanks (*n* = 2); and books (*n* = 1). Databases with focal geographical area outside the Iberian Peninsula (but covering Iberian species) were also included, as our purpose was not to reflect regional features but rather to capture as much variability of traits as possible and because regional studies often rely on global rather than local data. A major data source for this study were Doadrio’s atlases (Doadrio, 2001; Doadrio *et al.*, 2011), which constitute the most comprehensive and used fish guides in Spain. We gathered all traits that were available in at least three out of the 19 databases investigated. For the analyses, polytomous categorical traits with *k* different levels were converted to *k* binary/dichotomous trait variables (i.e. dummy variables; Hardy, 1993) by keeping the full information of the original variable but allowing comparisons with binary-expressed variables of other databases. For example, a categorical feeding trait with levels ‘piscivore’, ‘invertivore’ or ‘omnivore’ was converted to three binary variables that coded the presence or absence of three respective traits (piscivory, invertivory and omnivory). In total, we compiled data for 27 fish traits (10 continuous and 17 binary; Table 4.1). We identified synonyms used to refer to the same trait or category to unify trait information and to allow further comparative analyses (Table S4.2). The raw data collected are available at figshare (DOI: 10.6084/m9.figshare.8168267).

Table 4.1. Traits compiled for freshwater fishes of the Iberian Peninsula. Total number of species with data available for a particular trait and number of databases that consider a particular trait. Trait data were collected for a total of 99 species from 19 databases. See the Supplementary Information for the raw data and further information on the databases.

Trait (and TYPE)	**Scale of measurement**	**Number of species with data available**	**Number of databases considering the trait**
MORPHOLOGICAL			
Maximum length (cm)	Continuous	98	7
Maximum weight (g)	Continuous	54	4
Fusiform shape	Binary	94	3
Elongated form	Binary	96	4
Eel-like form	Binary	96	4
TROPHIC			
Invertivory	Binary	91	12
Omnivory	Binary	91	12
Piscivory	Binary	88	10
REPRODUCTIVE			
Maximum longevity (years)	Continuous	68	8
Reproductive span (months)	Continuous	84	7
Mean fecundity (eggs/female)	Continuous	59	8
Maximum fecundity (eggs/female)	Continuous	62	4
Egg size (mm)	Continuous	47	8
Age at maturity (years)	Continuous	70	8
Length at maturity (cm)	Continuous	54	5
Parental care	Binary	93	8
Single spawning	Binary	87	6
HABITAT USE			
Rheophily	Binary	90	11
Limnophily	Binary	90	9
Potamodromy	Binary	98	7
Long migration	Binary	94	9
Benthic	Binary	96	8
Water column	Binary	99	8
Tolerant	Binary	87	7
Intolerant	Binary	92	6
Lithophily	Binary	91	14
Phytophily	Binary	86	12

4.2.2. Statistical analyses

We first used generalised linear models (GLMs), an extension of linear models that allows for non-normal errors and heteroscedasticity (McCullagh & Nelder, 1989), to test for differences in fish traits among databases while accounting for differences among species. GLMs were fitted each with the reported value of a specific fish trait as the response variable and database and species as independent predictor variables. We used the ‘gamma’ family and ‘log’ link function to model continuous traits, and the ‘binomial’ distribution and ‘logit’ function for binary traits. The partial R^2 values of the GLM models were calculated by dividing species and database deviances by the null deviance. Consequently, partial R^2 values allowed estimating the percentage of variation in reported values of a specific trait that can be attributed to either differences among databases, differences among species or residuals (i.e. particular combinations of species and databases). We assessed the robustness of these analyses by calculating η^2 of linear models of the log-transformed continuous traits, fitted with the same predictors (species and database) and comparing both statistics (partial R^2 vs. η^2) through a Bland-Altman analysis (Bland & Altman, 1986; see Supplementary materials S4).

We used inter-rater reliability statistics, which are quality indicators that measure the agreement among independent observers (Hayes & Krippendorff, 2007; Gwet, 2010). Of the many reliability indices that have been proposed, we used Krippendorff’s α (Krippendorff, 2004) because it has been recently proposed as the standard reliability measure (Hayes & Krippendorff, 2007) and has many advantages: it has no restrictions on the number of databases tested or varying sample sizes; it enables one to handle incomplete or missing data (Krippendorff, 2011; Zapf *et al.*, 2016); and, in contrast to many other reliability measures, it can be computed for any type of variable (nominal, ordinal, interval, and ratio) and thus enables one to compare them (Hayes & Krippendorff, 2007). Krippendorff’s α corresponds to $1 - D_o/D_e$ where D_o is the observed disagreement between values assigned for a particular trait by different raters (i.e. databases) across a set of species, and D_e is the disagreement expected by chance. Krippendorff’s α embraces many other measures (such as Spearman’s or Pearson correlations) as special cases and generally ranges from 0 to 1, with values of 1 indicating perfect reliability, 0 indicating absence of reliability, and higher values indicating higher reliability (i.e. agreement among databases in our case) (Krippendorff, 2004, 2011). As a rule of thumb, variables with α values of 0.8 are considered as reliable in content analysis and those with $\alpha < 0.667$ should not be considered; however, higher standards should be expected in experimental sciences (Krippendorff 2004). We applied the function ‘kripp.boot’ of the R package ‘kripp.boot’ (Proutskova & Gruszczynski, 2017; R Core Team, 2018) to obtain the bootstrapped Krippendorff’s α coefficient and its 95% confidence interval, CI (based on 1000 bootstrap samples), applying the ratio method for continuous traits and the nominal method for binary traits.

To determine which traits were more studied, we calculated two variables: trait use and trait-specific data availability in the databases. Trait use was defined as the percentage of databases that considered a particular trait relative to the total number of databases:

$$Use\ of\ trait\ k = \frac{N_k}{N} \times 100$$

where N_k = number of databases that consider trait k; and N = total number of databases (n = 19). Trait-specific data availability was defined as the average across all databases of the percentages of species with assigned data for a particular trait in a database relative to the total number of species studied in that database:

$$Data\ availability\ for\ trait\ k = \frac{1}{N_k}\sum_{j=1}^{N}\frac{S_{kj}}{S_j} \times 100$$

where N_k = number of databases that consider trait k; S_{kj} = number of species with available information on trait k in database j; and S_j = number of species considered in database j. To analyse the relationship among trait use, trait-specific data availability and Krippendorff's α, we used Spearman's rank correlations. To test for effects of measurement scale (continuous or binary) and trait type (morphological, habitat use, trophic or reproductive) on Krippendorff's α, trait use and trait-specific data availability we used linear models with logit transformation of the response variables (Warton & Hui, 2011).

We also calculated species' coverage and species-specific trait data availability, which describe the information status of a particular species regarding its traits. Species' coverage was defined as the proportion of databases that consider a particular species:

$$Coverage\ of\ species\ i = \frac{N_i}{N} \times 100$$

where N_i = number of databases that describe at least one trait of species i; and N = total number of databases (n = 19). Species-specific trait data availability was defined as the average of the percentages of trait data available for a particular species in a database relative to the total number of traits considered in that database:

$$Data\ availability\ for\ species\ i = \frac{1}{N_i}\sum_{j=1}^{N}\frac{T_{ij}}{T_j} \times 100$$

where N_i = number of databases that describe at least one trait of species *i*; T_{ij} = number of traits available for species *i* in database *j*; and T_j = number of traits considered in database *j*. We modelled species' coverage and species-specific data availability using linear models following the same methodology already employed to model trait use and availability. Latitudinal range (Froese & Pauly, 2019), the species' year of description (Froese & Pauly, 2019), migration behaviour (diadromous or non-diadromous; see consensus database available in figshare repository, DOI: 10.6084/m9.figshare.8168267), the conservation status (International Union for Conservation of Nature [IUCN] Red List Categories: from least concern to critically endangered; IUCN, 2019) and native status (endemic, native or alien; Doadrio, 2001; Doadrio *et al.*, 2011; Froese & Pauly, 2019; Clavero, 2019) were used as predictors potentially influencing the indices of species' coverage and species-specific trait data availability. To generate a consensus database for all investigated Iberian fish species, we calculated the mean, the median and the standard deviation for all traits.

4.3. Results

Most of the variation within traits was due to differences among species (average partial R^2 across traits = 0.735) rather than due to differences among databases (average partial R^2 = 0.080), as indicated by the GLMs (Figure 4.1 and Table S4.3). For example, maximum weight was the trait with most explained variability due to differences among species (partial R^2 = 0.977, Figure 4.1). However, for some selected traits such as limnophily (partial R^2 = 0.309), water column microhabitat (partial R^2 = 0.219) or rheophily (partial R^2 = 0.191), a notable part of the variation was explained by differences among databases, that is, particular databases were uniformly biased in trait assignment and tended to have higher (or lower) values for the assessed trait than other databases (Figure 4.1 and Table S4.3). For example, three species (*Carassius auratus*, *Cyprinus carpio* and *Rutilus rutilus*) were considered limnophilic in three databases, but not limnophilic in two other databases. Many of the traits that showed a large variation among databases were binary traits (limnophily, rheophily, elongated body form or water column, Table S4.3). Of the 10 traits with the greatest explained variation in total, seven were continuous. However, reproductive span showed a low total variation explained (R^2 = 0.615) despite being a continuous trait, with a large part of the variance being due to databases (Figure 4.1). The consensus database of 27 traits for the 99 freshwater fishes that inhabit the Iberian Peninsula is available via figshare (DOI: 10.6084/m9.figshare.8168267).

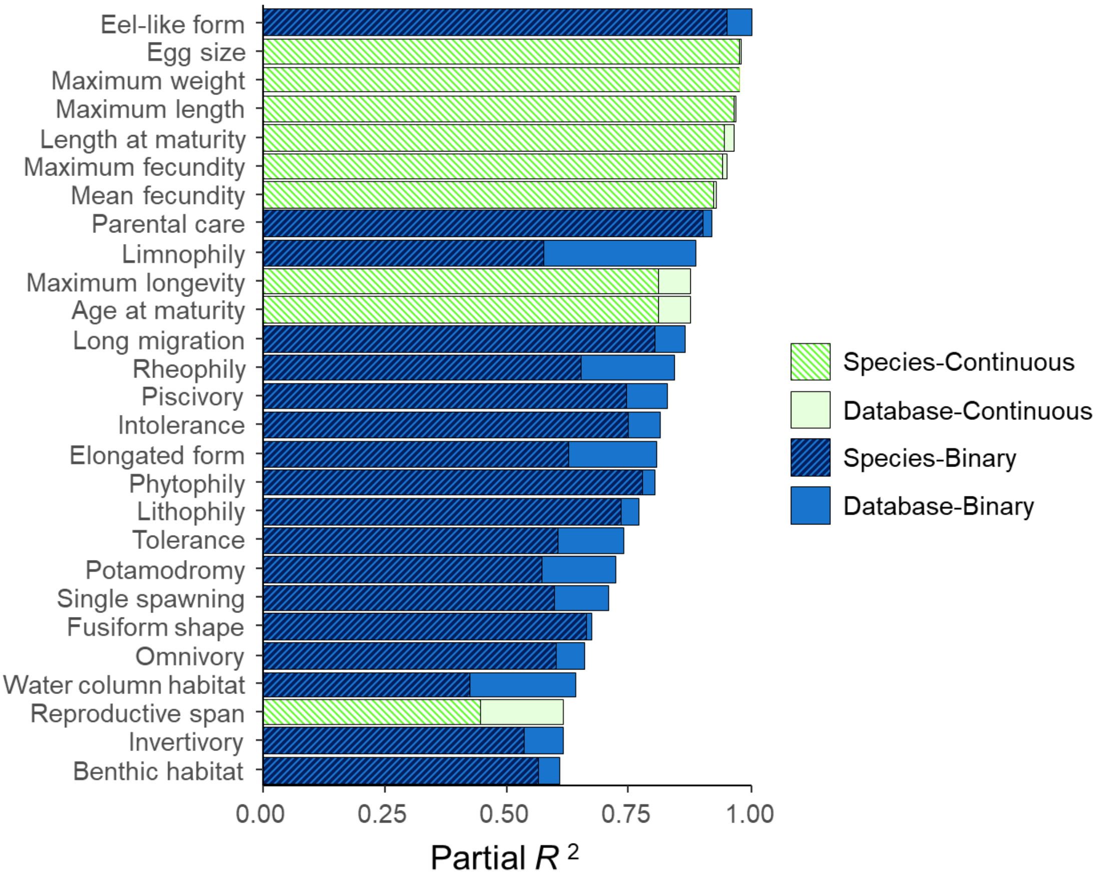


Figure 4.1. Variation in traits explained (partial R^2 obtained with Generalised Linear Models) by species vs. databases for continuous and binary traits analysed. Analyses included 19 trait databases and data of 99 Iberian fish species.

Reliability, that is, the agreement of trait information among databases measured by Krippendorff's *α*, varied with measurement scale (continuous vs. binary), with continuous traits being more reliable than binary ($P = 0.032$, Figure 4.2a and Table 4.2). Accordingly, 12 of the 13 most unreliable traits were binary (Figure 4.3 and Table S4.4). Reproductive span showed a much lower reliability than the other continuous traits (Figure 4.3 and Table S4.4). However, differences due to the trait type were statistically not clear ($P = 0.847$, Figure 4.2a and Table 4.2). The traits that showed the highest reliability were egg size ($\alpha = 0.945$), maximum length ($\alpha = 0.919$), and eel-like form ($\alpha = 0.873$), whereas elongated form ($\alpha = 0.299$), reproductive span ($\alpha = 0.218$) and water column microhabitat use ($\alpha = 0.214$) were the least reliable (Figure 4.3 and Table S4.4). We also observed that Krippendorff's *α*-values of binary traits had a greater uncertainty (i.e. larger confidence interval) compared to those of continuous traits (Figure 4.3 and Table S4.4).

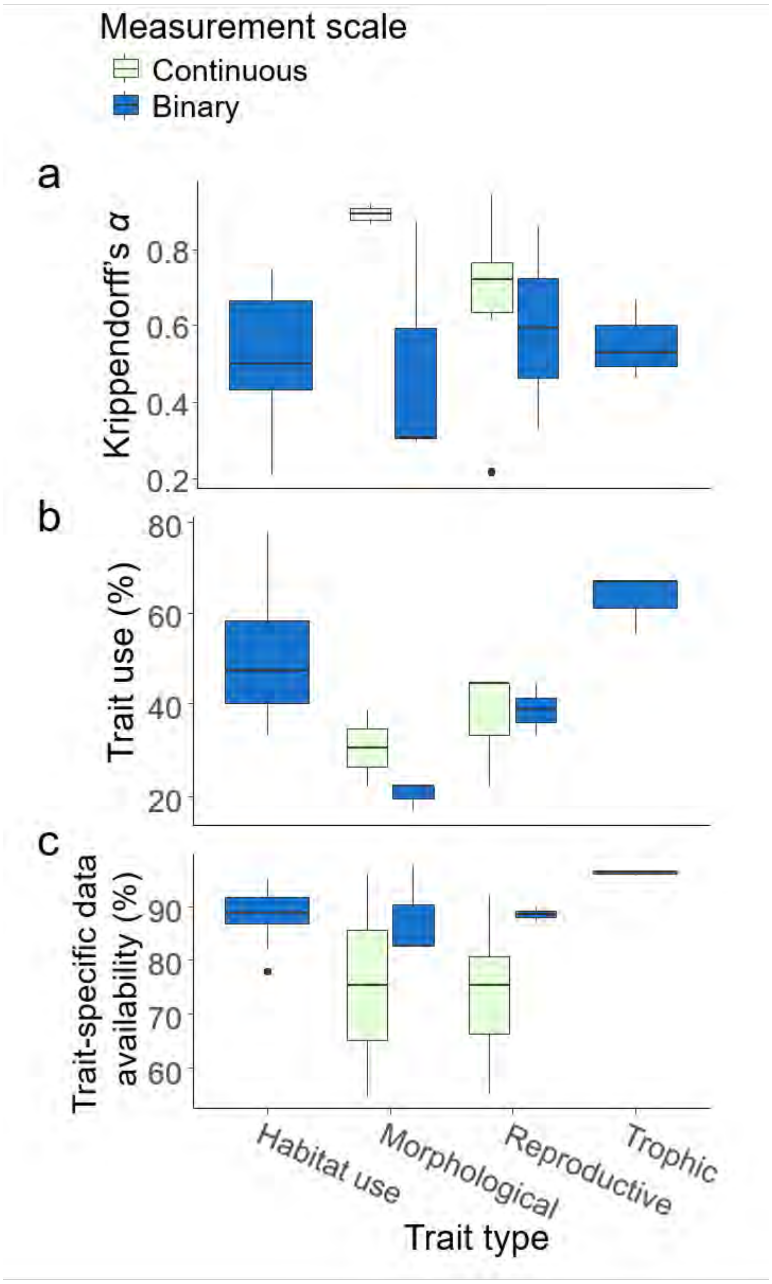


Figure 4.2. Effects of trait type and measurement scale on (a) trait reliability (Krippendorff's *α*), (b) trait use (percentage of databases that included the trait) and (c) trait-specific data availability (percentage of species with trait values reported, averaged across databases) for 99 Iberian fish species as reported in 19 trait databases. Boxes correspond to the 25th and 75th percentiles; lines inside a box show the median; whiskers extend to the last observation within 1.5 times the interquartile range from the quartiles and outliers are indicated by filled circles.

Table 4.2. Linear models of Krippendorff's α, trait use (percentage of databases that included the trait) and trait-specific data availability (percentage of species with trait values reported, averaged across databases) in response to measurement scale and trait type as categorical factors; and linear models of species' coverage (percentage of databases that included the species) and species-specific trait data availability (average of the 'percentages of trait data available for a particular species in a database relative to the total number of traits considered in that database') in response to latitudinal range, description year, migration behavior, native status and IUCN category. R^2_{adj} = adjusted coefficient of determination in parenthesis; *df* = degrees of freedom.

Response variable (R^2_{adj})	Factor	Sum of squares	*df*	*P*
Krippendorff's α (0.103)	Measurement scale	5.680	1	0.032
	Trait type	0.870	3	0.847
	Scale × Type	2.060	1	0.181
	Residual	22.602	21	
Trait use (0.512)	Measurement scale	1.031	1	0.049
	Trait type	6.392	3	0.001
	Scale × Type	0.218	1	0.348
	Residual	4.980	21	
Trait-specific data availability (0.296)	Measurement scale	6.700	1	0.004
	Trait type	3.391	3	0.183
	Scale × Type	0.058	1	0.765
	Residual	13.392	21	
Species' coverage (0.464)	Latitudinal range	37.727	1	<0.001
	Description year	37.326	1	<0.001
	Migration behavior	3.493	1	0.079
	Native status	8.792	2	0.022
	IUCN category	6.385	4	0.226
	Residual	87.001	79	
Species-specific trait data availability (0.691)	Latitudinal range	57.190	1	<0.001
	Description year	32.069	1	<0.001
	Migration behavior	1.971	1	0.042
	Native status	1.793	2	0.150
	IUCN category	1.924	4	0.390
	Residual	36.392	79	

Trait use showed significant differences among trait types ($P = 0.001$), with trophic traits being the most considered in databases, followed by habitat use traits. There were also noticeable differences in trait use related to its measurement scale ($P = 0.049$). Specifically, continuous measures of morphological and reproductive traits were more often included in databases than binary traits in those trait categories (Figure 4.2b). Phytophily, piscivory and lithophily were traits that are often included in databases and concurrently more reliable than many others (Figure 4.4a). By contrast, water column and benthic microhabitats, limnophily, invertivory and potamodromy were also often considered but were found less reliable (Figure 4.4a).

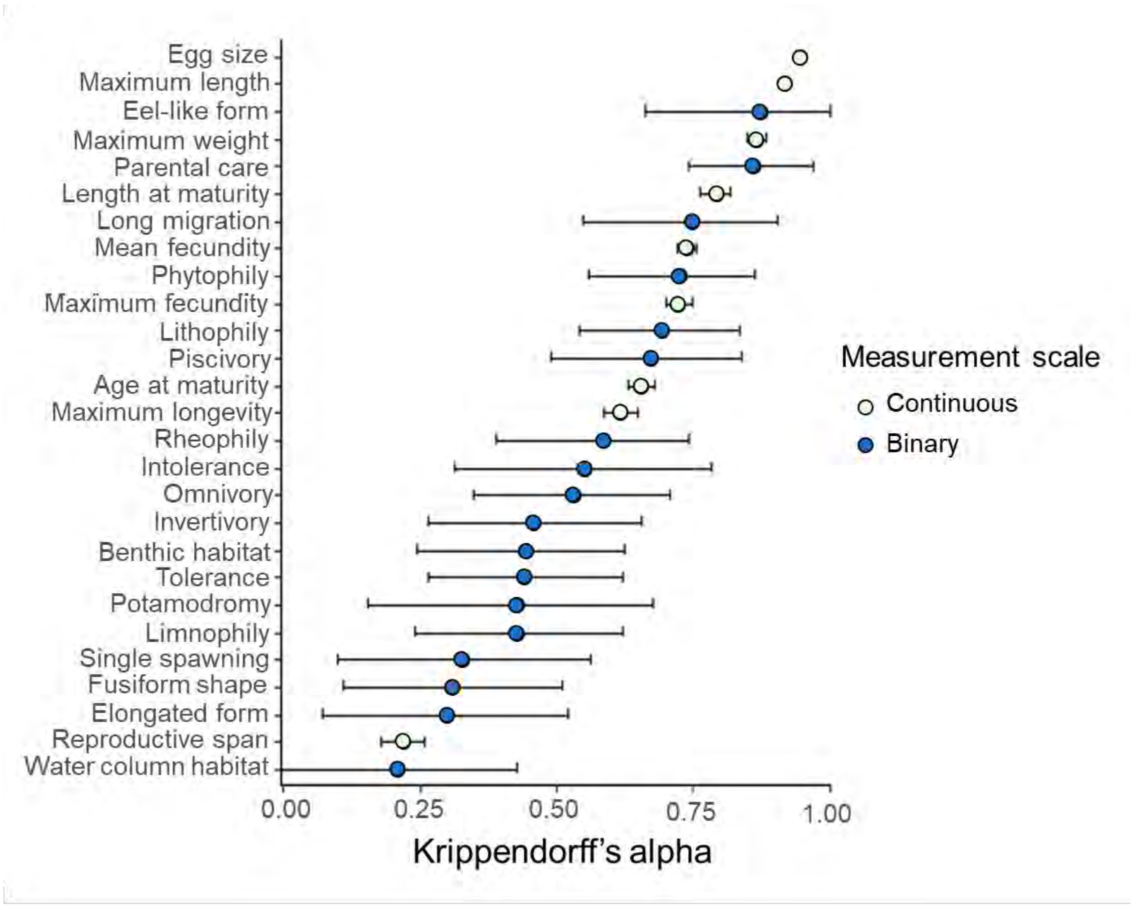


Figure 4.3. Krippendorff's *α* (bootstrapped mean and corresponding 95% confidence interval) for the different traits studied of 99 Iberian fish species as reported in 19 trait databases. Open and filled circles correspond to continuous and binary traits, respectively.

There was greater trait-specific data availability for binary than for continuous traits (P = 0.004) but the differences among trait types were statistically not clear (P = 0.183, Figure 4.2c and Table 4.2). Trait use in databases was only weakly related to trait-specific data availability (r_s = 0.414; n = 27; P = 0.032). For example, egg size is a trait considered in many databases but often only available for a few species, thus representing the trait with second highest percentage of missing values (Figure 4.4b). Conversely, maximum length was only rarely included in databases, but available for many species when considered. Reliability (Krippendorff's *α*) was not clearly related to trait use (r_s = -0.040; n = 27; P = 0.843) or availability (r_s = -0.362; n = 27; P = 0.063).

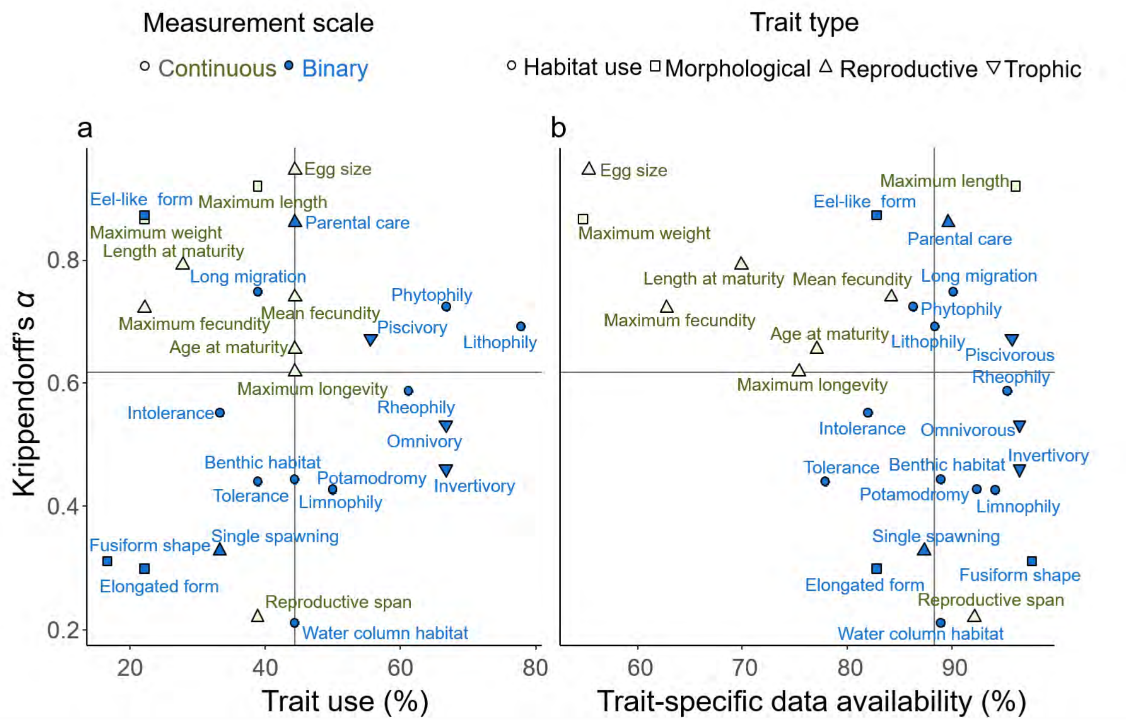


Figure 4.4. Relationship between (a) trait use (percentage of databases that included the trait) and (b) trait-specific data availability (percentage of species with trait values reported, averaged across databases) with trait reliability (Krippendorff's *α*). Vertical lines represent the median value of trait use and trait-specific data availability and horizontal continuous lines represent the median value of Krippendorff's *α*.

Species' coverage (i.e. how often a particular species is considered in trait databases) was mostly related to the latitudinal range of the species ($P < 0.001$), its year of description ($P < 0.001$) and its native status ($P = 0.022$, Table 4.2). Fish species with smaller latitudinal range and those that have been described more recently were less frequently included in databases, whereas alien species showed higher species' coverage than native and, especially, endemic fish. However, migration behaviour ($P = 0.079$) and IUCN category ($P = 0.226$) had no clear statistical effects on a species' coverage (Figures 4.5a and 4.5b and Table 4.2). Species-specific trait data availability was influenced by the latitudinal range ($P < 0.001$), description year ($P < 0.001$) and migration behaviour of a species ($P = 0.043$, Table 4.2). Species described more recently had less available trait data in databases (Table 4.2). For example, *Squalius malacitanus* and *Squalius valentinus*, both described in 2006, had less than 25% of data on their traits available; whereas widespread species such as *Dicentrarchus labrax* (97.4%), *Misgurnus anguillicaudatus* (91.6%), or the diadromous *Anguilla anguilla* (90.5%) had more information available (Figures 4.5c and 4.5d). Species' coverage and species-specific trait data availability were highly positively correlated (r_s = 0.818; n = 89; $P < 0.001$).

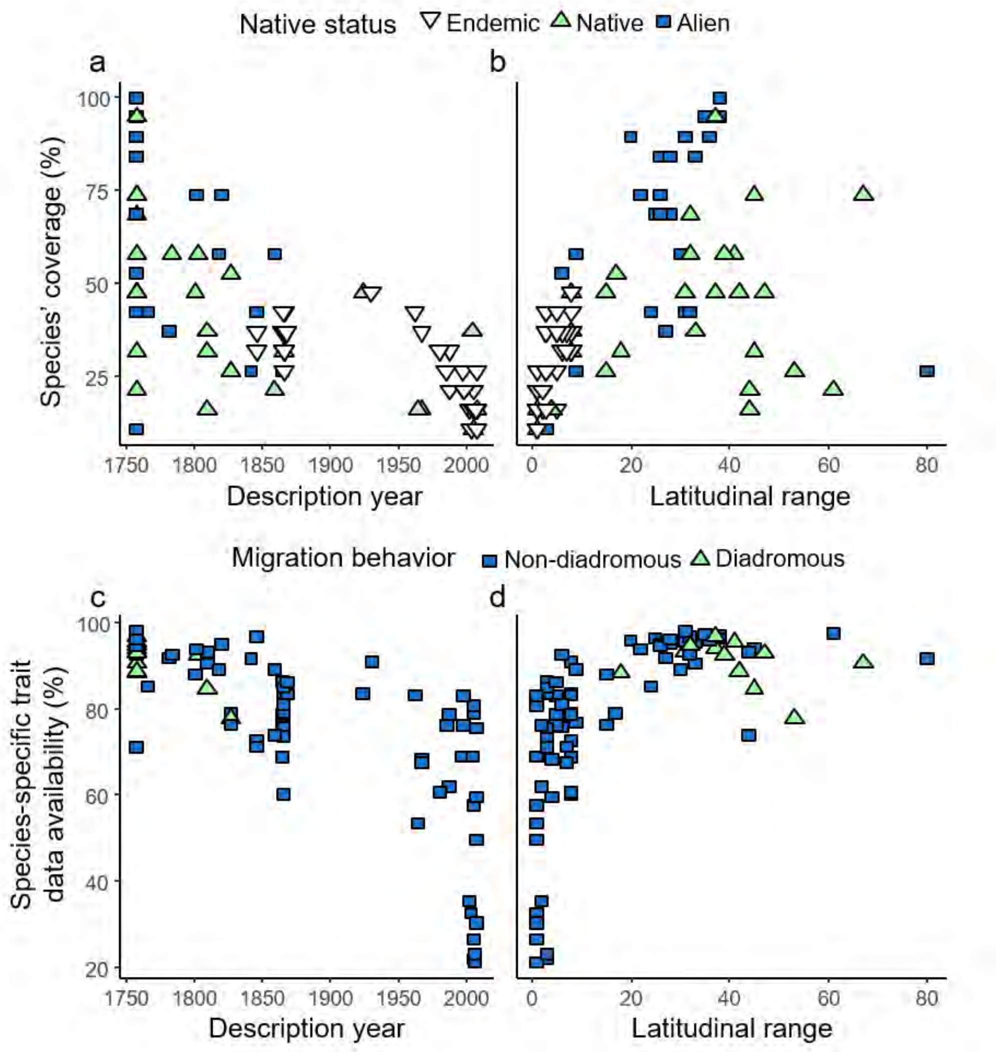


Figure 4.5. Relationship of species coverage (percentage of databases that included the species) with (a) species' description year and (b) species' latitudinal range, and relationship of species-specific trait data availability (average of the percentages of trait data available for a particular species in a database relative to the total number of traits considered in that database) with (c) species' description year and (d) species' latitudinal range, in 19 fish trait databases for 99 species of the Iberian Peninsula.

4.4. Discussion

To our knowledge, this is the first study to assess the statistical reliability of species traits in different databases, and the first with a focus specifically on native and alien Iberian inland fishes. We also assessed the frequency of use and availability of in total 27 different fish traits and tested factors that potentially explain them. Results show numerous discrepancies in Iberian fish species traits for the 19 databases analysed, and reveal knowledge gaps regarding some traits and specifically concerning rare species.

4.4.1. Trait data state of knowledge

As hypothesised, we found a notable part of the variation for some traits explained by the uniform bias in trait assignment of particular databases, specifically for non-continuous (i.e. categorical/binary) traits. This also translates into a generally lower reliability (i.e. the agreement observed among databases) for categorical than for continuous traits, as also observed in the psychological literature (Markon, Chmielewski & Miller, 2011). The lower reliability of categorical traits might be either because they are established mostly by expert judgment, or because there are large discrepancies among trait definitions. In general, there are two sources of uncertainty of a trait which are addressed below: (1) epistemic uncertainty, due to limitations of the measurement, insufficient data, subjective judgment and natural variability; and (2) linguistic uncertainty, due to the use of vague or ambiguous vocabulary (Regan, Colyvan & Burgman, 2002).

Our results indicated rather high observed variability of categorical trait assignments, which might be related e.g. to subjective expert judgment (Regan *et al.*, 2002; Sutherland & Burgman, 2015). Accordingly, previous studies showed that the precision of expert-assigned species traits depends on the familiarity with the species or the system studied, the social context and individual beliefs, values or experiences (Burgman *et al.*, 2011; McBride *et al.*, 2012; Radinger *et al.*, 2017). Importantly, assignments to habitat preference categories like limnophily and rheophily, which are mainly established by expert criteria, showed rather low reliability, that is, low agreement among databases. Similarly, feeding traits are often established by expert judgment based on data from limited geographic regions and might be then transferred to closely related but unstudied species. However, both traits are widely used in fish indices and in trait-based studies (see Frimpong & Angermeier, 2010). As a result, errors in characterising habitat preferences and feeding traits could, for example, affect biotic assessments of river impoundment and/or channelisation effects, or assessments of the trophic structure of a fish community (Noble *et al.*, 2007), respectively. By contrast, the majority of the analysed continuous traits (e.g. body size) are commonly established based on individual measurements. This might result in higher reliability as indicated by our results but might also take higher efforts to obtain such trait information. The challenges associated with obtaining continuous traits might be a reason why our study showed lower trait-specific data availability for these traits compared to categorical traits. Specifically, reproductive traits were among the least covered continuous traits in the analysed databases because features such as egg size, reproductive span, fecundity or age at maturity are generally difficult to measure.

Another important source of low reliability might be related to the intraspecific variability of traits, that is, differences of traits within species for instance due to different environments or geographical variation (Radinger *et al.*, 2017; Bonada & Dolédec, 2018). Intraspecific trait variability is frequently neglected as trait values are summarised as averages per species (Beck *et al.*, 2012), or it is assumed to be negligible compared to interspecific variability (Albert *et al.*, 2011), which might lead to biased results (Albert *et al.*, 2010). For example, a study on plants revealed that the accuracy of mean trait values within species retrieved from databases is specifically lower in plastic traits (Cordlandwehr *et al.*, 2013), that is, those that respond to environmental factors (Lusk *et al.*, 2008). Among the reproductive traits, the least reliable continuous trait investigated in this study was reproductive span (i.e. the length of a species' breeding season). Here, the observed low reliability might be related to the rather large intraspecific variability of this trait (Blanck & Lamouroux, 2007). We also note that some fish show important intraspecific variation in their diet within different ambient conditions (Blanco *et al.*, 2003), depending on age and ontogenetic development (Eggold & Motta, 1992; Sánchez-Hernández *et al.*, 2018), depending on the food availability over the year (Weliange & Amarasinghe, 2003), and under the presence of invasive alien species (Adams, 1991; Feyrer *et al.*, 2003), which complicates assignment of trophic traits.

The reliability of trait information might also be related to linguistic uncertainties and the standardisation of traits (i.e. their use without applying a common and standardised methodology or definition). Non-standardised and even contradictory trait definitions can reduce reliability and, as shown by previous studies, can prevent authors from readily comparing their findings (Costello *et al.*, 2015). This issue might even increase as trait-based studies are adopted more widely and, thus, the number of trait definitions and terms will potentially increase (Degen *et al.*, 2018).

The rather low reliability of trophic traits as revealed in this study, might point to the generally low standardisation in this group of traits, despite several authors trying to introduce a common classification (Gerking, 1994; Goldstein & Simon, 1999). The use in databases compiled in our study ranged from two (invertivorous and omnivorous; Belliard & Roset, 2006; Oliveira, Ferreira & Santos, 2016) to seven categories (parasitic, detritivorous, zoobenthivorous, zooplanktivorous, piscivorous and phytivorous; Aarts & Nienhuis, 2003) for trophic traits. Analogously, the low reliability of habitat preferences might also be related to unclear definitions. For example, true water column fishes can only occur in deeper lowland stretches of rivers or in lakes where the vertical spatial scale allows differentiation (Noble *et al.*, 2007), while in headwater or shallow streams this vertical scale is limited. Nevertheless, it is common to classify also headwater species in one of these two categories. For example, brown trout (*Salmo trutta*), which inhabits headwater stretches of rivers and partly uses the stones as shelter (Heggenes, 1988), is generally considered a water column species. However, species like *S. trutta* are rather distinct from the classical water column species described for lakes (e.g. *Coregonus* spp. occurring in

European lakes outside the Iberian Peninsula). The databases analysed here also showed discrepancies in the classification of potamodromy: while some sources define potamodromous species as those fish that migrate between different river zones (Pont *et al.*, 2006; Holzer, 2008), others specify potamodromous fish as those that migrate over at least 100 km within a river system (Froese & Pauly, 2019). Another example of a trait that is commonly used in fish ecology but lacks a standardised definition is tolerance, especially general tolerance (Noble *et al.*, 2007), which aggregates a species' ecological tolerance (or its opposite concept, which is sensitivity) to specific stressors. Consequently and because of its proneness to subjectivity compared to other more reliable traits such as the lithophily trait, some fish indices even explicitly exclude the number of intolerant species as a contributing criterion (Oberdorff *et al.*, 2002). Conversely, body size (i.e. maximum length), which might also have been affected by issues of different definitions (e.g. total, standard, or fork length) or statistics (maximum, 'typical', estimated from regressions), showed high reliability. It might be assumed that the rather high reliability of maximum length is due to the relatively lower intraspecific variability compared to its interspecific variability, making differences among databases very small relative to differences among species.

4.4.2. Lack of species information

Generally, biodiversity data are often incomplete or suffer from biases, being conspicuous and often focused on economically valuable species from temperate and accessible regions (Hortal *et al.*, 2015). Our results revealed that diadromous species have greater species-specific trait data availability than strict freshwater species. This might be related to the fact that: (1) many diadromous species investigated in this study, such as *A. anguilla,* have a wide distribution range and thus, are better studied; and (2) migration is a focus area in fish ecology and has been extensively studied over the past century (e.g. Schmidt, 1923). Moreover, as hypothesised, species that have been described more recently were characterised by rather low data availability in the analysed databases. Some of these recently described species have been included in only two trait databases such as three species of lampreys described in Portugal in 2013 (Mateus *et al.*, 2013). Our results show that species with a small distribution range as well as endemic species are covered by fewer databases and their knowledge is likely to be especially scarce since most of their traits have not yet been studied. This lack of knowledge in trait information for rare and recently described species constitutes a major limiting factor in many studies, for example, when calculating functional diversity indices (Pakeman, 2014). If bioassessment is the purpose it would be desirable to have at least reliable information for common species and principal traits involved in bioassessment (habitat preferences, migratory behaviour, tolerance and feeding habits).

4.4.3. Future directions and conclusions

Our results indicate that increased efforts to complement categorical, expert-based trait assignments by empirically derived continuous trait information could lead to an overall improvement of trait reliability in freshwater fish. For example, numerous studies already successfully used stable isotopes as indicators of a fish's trophic level (Hesslein *et al.*, 1991; Jennings *et al.*, 2002). Furthermore, form factor, roundness and aspect ratio could be used as continuous descriptors of body shape; and body size might constitute a valuable continuous proxy of movement trait, specifically in potamodromous fish, given the close relationship between fish length and movement distance (Radinger & Wolter, 2014). In cases where the use of continuous variables is not possible and expert judgment is necessary, structured protocols for elicitation could be used for establishing fish traits and to counter subjective judgment biases (McBride *et al.*, 2012). Furthermore fuzzy coding, a procedure to structure the biological and environmental information and to describe the affinity of a species to specific trait categories might constitute a valuable approach (Chevenet, Dolédec & Chessel, 1994; Persat, Olivier & Pont, 1994). Several authors have also proposed solutions to standardise heterogeneous trait data in order to improve the reliability linked to ambiguity of traits (Kattge *et al.*, 2011b; Schneider *et al.*, 2018). Generic database structures facilitate the exchange and analysis of information, and ensure compatibility and comparability among databases. In particular, plant and terrestrial invertebrates ecologists have made substantial progress in defining a common set of useful traits and developing detailed sampling protocols (McIntyre *et al.*, 1999; Cornelissen *et al.*, 2003; Violle *et al.*, 2007; Pérez-Harguindeguy *et al.*, 2013). However, such standardised protocols for recording trait data and corresponding database tools are not yet available or applied in freshwater fish, but are greatly needed. Corresponding efforts might ultimately contribute to a global or continental, and standardised fish trait database, as already available for other taxonomic groups (Kleyer *et al.*, 2008; Kattge *et al.*, 2011a; Storchová & Hořák, 2018).

The consensus trait dataset for Iberian freshwater fishes that comes along with this study constitutes a reference source that provides information for 99 species and 27 traits. Although not being strictly based on standardised trait data, this is the first attempt of a comprehensive, regional database that summarises trait information for Iberian freshwater fishes with unprecedented coverage.

There are some limitations of our study that are partly related to the underlying species trait databases. For example, we acknowledge that some trait assignments build on little available data and that the investigated databases might show some degree of relatedness. More specifically, we observed that some datasets used information from other databases studied and thus even ecologically wrong trait information might have been shared among the different data sources. Moreover, the traits used in regional studies often do not correspond to local data but originate from other regions. For example, traits of invasive alien species included in Vila-Gispert *et al.* (2005) were mainly obtained from studies from outside the Iberian Peninsula. This might have also impinged on the results of our analyses, as they rely on underlying sources.

To conclude, reliable information on species traits is crucial for providing meaningful and robust trait-based analyses (Zapf *et al.*, 2016) and for avoiding biases in trait-based bioassessments. In this study we identified the most unreliable fish traits and improved our understanding of the sources' uncertainty in specific fish traits. With our consensus trait dataset for Iberian freshwater fishes we provide a comprehensive regional reference source for further fish trait-based studies. We strongly encourage efforts towards more standardised and feasible quantification of fish traits, in particular, of commonly understudied, endemic and rare fish species. This will ultimately help to improve the reliability and robust application of fish trait-based approaches and fish indices.

5. Chapter II - Key factors explaining critical swimming speed in freshwater fish: a review and statistical analysis for Iberian species

Cano-Barbacil, C.; Radinger, J.; Argudo, M.; Rubio-Gracia, F.; Vila-Gispert, A. & García-Berthou, E. 2020. Key factors explaining critical swimming speed in freshwater fish: a review and statistical analysis for Iberian species. *Scientific Reports*, 10: 18947.

The pumpkinseed (*Lepomis gibbosus*) is a freshwater fish native to North America. This species was introduced to European waters, and it is currently included in the ‘List of Invasive Alien Species of Union concern’.

Photo: Carlos Cano-Barbacil

Chapter summary

Swimming performance is a key feature that mediates fitness and survival in aquatic animals. Dispersal, habitat selection, predator-prey interactions and reproduction are processes that depend on swimming capabilities. Testing the critical swimming speed (U_{crit}) of fish is the most straightforward method to assess their prolonged swimming performance. We analysed the contribution of several predictor variables (total body length, experimental water temperature, time step interval between velocity increments, species identity, taxonomic affiliation, native status, body shape and form factor) in explaining the variation of U_{crit}, using linear models and random forests. We compiled in total 204 studies testing U_{crit} of 35 inland fishes of the Iberian Peninsula, including 17 alien species that are non-native to that region. We found that body length is largely the most important predictor of U_{crit} out of the eight tested variables, followed by family, time step interval and species identity. By contrast, form factor, temperature, body shape and native status were less important. Results showed a generally positive relationship between U_{crit} and total body length, but regression slopes varied markedly among families and species. By contrast, linear models did not show significant differences between native and alien species. In conclusion, the present study provides a first comprehensive database of U_{crit} in Iberian freshwater fish, which can be thus of considerable interest for habitat management and restoration plans. The resulting data represents a sound foundation to assess fish responses to hydrological alteration (e.g. water flow tolerance and dispersal capacities), or to categorise their habitat preferences.

Keywords: alien species, body length, Iberian Peninsula, swim tunnel, temperature, U_{crit}

5.1. Overview

Swimming performance represents one of the most important features that mediate fitness and survival of fish and other aquatic animals (Jones *et al.*, 1974; Watkins, 1996; Burgess, Booth & Lanyon, 2006; Tudorache *et al.*, 2008). It plays a crucial role in dispersal, migration, habitat selection, predator-prey interactions and reproduction (Taylor & McPhail, 1985; Videler, 1993; Kolok, 1999; Reidy, Kerr & Nelson, 2000; Plaut, 2001; Wolter & Arlinghaus, 2003). Swimming performance in fish is traditionally assessed using swim tunnels and ecohydraulic flumes (Videler, 1993; Wilson & Egginton, 1994; Claireaux *et al.*, 2006; McKenzie & Claireaux, 2010; Katopodis & Gervais, 2016; Katopodis, Cai & Johnson, 2019) and can be classified into three categories: sustained, prolonged and burst swimming (Beamish, 1978). Sustained swimming is aerobically fuelled and can be maintained for long time periods, typically more than 200 min, without muscular fatigue (Beamish, 1966; Brett, 1967; Hoover, Zielinski & Sorensen, 2017). The maximum swimming speed of which fish are capable is burst swimming, which can be maintained only for shorter periods (typically < 20–30 s) and is fuelled anaerobically (Beamish, 1978; Hoover *et al.*, 2017). Prolonged swimming is the transitional mode between sustained and burst swimming and is not barely distinguishable from burst swimming in some species (Hoover *et al.*, 2017). Prolonged swimming is partly fuelled by aerobic and anaerobic metabolism, and can be maintained for intermediate intervals of time (1–200 min) (Beamish, 1978; Hoover *et al.*, 2017).

Since Brett's work (1964), many authors have opted for determining critical swimming speed (U_{crit}), as a measurement of prolonged swimming performance, while measuring oxygen consumption rates at the same time (Hammer, 1995). To measure U_{crit}, individual fish are forced to swim against water flow of increasing velocity until fatigue, i.e. the moment at which the fish can no longer swim and maintain its position in the current (Kolok, 1999; Beecham *et al.*, 2009).

U_{crit} is well known to be positively related to body size, including both body length (Beamish, 1978; Wolter & Arlinghaus, 2003) and body mass (Srean *et al.*, 2016; Rubio-Gracia *et al.*, 2020). Swimming performance also depends on body shape (Webb, 1984b a; Walker, 2000; Boily & Magnan, 2002) and fin form (Webb, 1984b; Nicoletto, 1991; Videler, 1993; Plaut, 2000a). For example, most of the fast-cruising fish have well streamlined bodies that reduce drag forces and recoil energy losses (Sfakiotakis, Lane & Davies, 1999). Muscle function (Webb & Weihs, 1983; Kieffer, 2000), swimming mode (Hertel, 1966; Sfakiotakis *et al.*, 1999; Müller *et al.*, 2001), and fish behaviour (Katopodis & Gervais, 2012) are also important factors that influence fish swimming performance. Thus, U_{crit} is strongly size-dependent (Katopodis & Gervais, 2012) and specific to groups of species displaying similar swimming performances (Wolter & Arlinghaus, 2003; Katopodis & Gervais, 2016). U_{crit} is also known to depend on the experimental setups and, increases with shorter step-time intervals between velocity increments during the experiment (Peterson, 1974).

Previous studies have shown that abiotic factors, such as water temperature affect the U_{crit}. In fact, a bell-shaped relationship between temperature and U_{crit} has repeatedly been reported (Randall & Brauner, 1991; Koumoundouros *et al.*, 2002; Oufiero & Whitlow, 2016). This means that U_{crit} ascends as temperature rises below the optimum temperature and descends as temperature rises above the optimum temperature (Hammer, 1995; Claireaux *et al.*, 2006). Nevertheless, some studies only detected significant decrease in swimming performance with lower water temperatures (Claireaux *et al.*, 2006; Fangue *et al.*, 2008b). Similar bell-shaped relationships have also been observed between swimming speed and pH (Randall & Brauner, 1991) or salinity (Glova & McInerney, 1977; Randall & Brauner, 1991; Nelson, Tang & Boutilier, 1996; Plaut, 2000b; Yetsko & Sancho, 2015). Other studies have noted the negative effects of several pollutants such as metals and nutrients on fish swimming performance (Peterson, 1974; Howard, 1975; Randall & Brauner, 1991; Nikl & Farrell, 1993; Beaumont, Butler & Taylor, 1995b a; Shingles *et al.*, 2001; Brown *et al.*, 2017).

The demands of fish on locomotion in flowing water differ from those in still water as fish need to avoid downstream displacement in lotic environments such as rivers and streams (McGuigan *et al.*, 2003). In general, fish species that inhabit in fast flowing riverine habitats tend to show higher U_{crit} than those that inhabit in slower flowing riverine or lentic habitats (Langerhans, 2008; Leavy & Bonner, 2009). Because of the close relationship between habitat conditions and fish swimming performance, several studies have assessed U_{crit} of species in different environments to understand the ecological consequences of anthropogenic perturbations in rivers such as hydrologic alteration, habitat fragmentation (Toepfer, Fisher & Haubelt, 1999), or navigation (Wolter & Arlinghaus, 2003), and to suggest corresponding mitigation measures. For example, U_{crit} has commonly been used to estimate maximum flow velocities in fish passes that assist species to move up or downstream of barriers or that impede the spread of invasive alien species (Katopodis, 2005; Peake, 2008a; Katopodis & Gervais, 2012, 2016; Katopodis *et al.*, 2019).

The number of studies and the availability of data regarding U_{crit} in fish have consistently grown in the last years (Katopodis & Gervais, 2012, 2016). However, many studies on fish swimming speeds have focused either on salmonids (McKenzie & Claireaux, 2010) because of their commercial and recreational interest (Glova & McInerney, 1977; Booth *et al.*, 1997; Peake, McKinley & Scruton, 1997; Shingles *et al.*, 2001), and on long-distance migratory fish such as potamodromous and diadromous species (Katopodis & Gervais, 2012; Silva *et al.*, 2018). By contrast, studies evaluating U_{crit} for many other species are rather limited (Haro *et al.*, 2004). This is particularly the case for many Mediterranean fish (Alexandre *et al.*, 2016), specifically for rare or local endemic species, which are frequently threatened (IUCN, 2019). Thus, general knowledge on the effects of factors such as body length and temperature on swimming performance in many of these Mediterranean fish species is lacking. Moreover, many regions in the world such as our study area, the Iberian Peninsula, are increasingly invaded by alien species. It has been shown that alien species replace the more flow-adapted native species in hydrologically altered systems (Boix *et al.*, 2010; Bae *et al.*, 2018). However, the mechanisms by which the invasive alien species have competitive advantage over native species in calm, stagnant waters are still poorly understood. Therefore, a thorough understanding of the swimming capacities of both native and alien species may provide insights into the reasons of this replacement, which can be a result of great importance for the management of water bodies (e.g. habitat assessments of alien and native species, and development of efficient fish passages at physical or velocity barriers for native fish).

The objectives of this study are: (1) to compile the most comprehensive empirical dataset of U_{crit} for Iberian freshwater fishes; (2) to compare the role of species identity, taxonomic affiliation, body length, body shape, time step interval between velocity increments and experimental temperature on U_{crit}, using for the first time the machine learning technique 'random forests' (RF); and (3) to test for differences in U_{crit} between native and alien species. We hypothesised that larger fish and more streamlined species would show higher U_{crit} (Kolok, 1999) and that temperature would be one of the main factors that influence U_{crit} (Randall & Brauner, 1991). Particular temperature effects are expected when experimental temperatures are beyond a species' ecological thermal range. We also hypothesised that alien species would show weaker swimming performance than native fishes because many successful freshwater invaders in the Iberian Peninsula are considered limnophilic, i.e. preferring lentic habitats, compared to the more flow-adapted, often rheophilic native species (Boix *et al.*, 2010; Bae *et al.*, 2018).

5.2. Methods

5.2.1. Data compilation

We attempted to compile U_{crit} data for all the current inland fish species inhabiting the Iberian Peninsula, including native and established alien species. The list of species mainly followed Doadrio *et al.* (2011) and Kottelat & Freyhof (2007) and was completed with few more recently described native species (Mateus *et al.*, 2013) and alien species lately recorded (Benejam *et al.*, 2005; López *et al.*, 2012; Aparicio *et al.*, 2013; Aparicio, 2015; Ribeiro *et al.*, 2015; Merciai *et al.*, 2018). Out of the 68 native and 32 alien naturalised inland fishes of the Iberian Peninsula, we found U_{crit} data for 35 species (18 native and 17 alien), from 79 literature sources published from 1959 to 2020 (Table S5.1), which include data for 8 species (3 native and 5 alien) from our previous work (Srean *et al.*, 2016; Rubio-Gracia *et al.*, 2020; Rubio-Gracia *et al.*, 2020). Data extraction occasionally implied digitising figures, using ImageJ2 software (Rueden *et al.*, 2017), to estimate U_{crit} values that were not provided in tables or within the text of the respective literature. We excluded works that investigated gradients or extreme values beyond the salinity or pH natural range of species, or that investigated the effect of pollution on swimming performance. In addition to U_{crit} values, we collated eight additional explanatory variables for further analyses. Besides species identity, family and native status (native vs. alien), these included fish body length, body shape, body form factor, time step interval and water temperature as described for the experiments. We used body length rather than body mass due to better data availability. U_{crit} and fish body length were converted to uniform units: relative U_{crit} (BL s^{-1}) was converted to absolute U_{crit} (cm s^{-1}); fork length (FL) or standard length (SL) were converted to total length (TL) using published length-length relationships (Ramseyer, 1995; Froese & Pauly, 2019). We obtained the species-specific body shapes indicating whether a fish has a fusiform (i.e. spindle-shaped and streamlined body), elongated (i.e. tubular body), short and deep (i.e. almost circular and laterally compressed body), or eel-like form (i.e. long and snake-like body) from FishBase (Froese & Pauly, 2019). Finally, we calculated the species-specific body form factor ($a_{3.0}$) (Froese, 2006) using the parameters *a* and *b* of the weight-length relationship retrieved from FishBase using the following equation:

$$a_{3.0} = 10^{\log a - S(b-3)}$$

where *S* is the slope of the regression of log *a* vs. *b*. For cases of insufficient data on weight–length relationships to estimate *S*, we used the recommended mean value of -1.358 (Froese, 2006). The form factor is an estimate of the coefficient *a* if exponent *b* was 3. This form factor is commonly used to compare body shape differences among populations or species (Verreycken, Van Thuyne & Belpaire, 2011; Neat & Campbell, 2013) and increases from eel-like to elongated, fusiform and short and deep body shapes (Froese, 2006). All the experiments considered were carried out at temperatures within a natural thermal range of each species. Raw data compiled are available at Figshare (DOI: 10.6084/m9.figshare.10260722).

5.2.2. Statistical analyses

We used random forest (RF) (Breiman, 2001), as implemented in the package 'party' (Hothorn, Hornik & Zeileis, 2006) of the R software (R Core Team, 2020), to analyse which of the six predictors best explained U_{crit}. RF is a machine-learning technique that is frequently used because of their advantages, including computational efficiency on large databases with many correlated predictors, the provision of estimates of variable importance, the ability to impute missing data while maintaining accuracy, and the handling of non-linearities and interactions (Breiman, 2001; Tuulaikhuu, Guasch & García-Berthou, 2017). Specifically, RF computed with package ‘party’ has the advantage of providing unbiased variable selection compared to other software packages, because it is more accurate when predictors are correlated and vary in their measurement scale or number of categories (Strobl *et al.*, 2007, 2008). We used species identity, family, native status (native vs. alien) and body shape (eel-like, elongated, fusiform or short and deep) as categorical factors, and TL, time step interval, water temperature and form factor as continuous predictors. In a first step, we searched for the optimal hyperparameters, i.e. number of trees (ntree) and number of variables per level (mtry) using the ‘mlr’ R-package (Bischl *et al.*, 2016). Consequently, we used 550 trees to build the RF as increasing the number of trees did not substantially affect the results of explained variation or variable importance (Liaw & Wiener, 2002), and seven variables were randomly sampled as candidates at each split. We measured the percentage of variation explained (i.e. pseudo-R^2) of the final model obtained. We used the conditional permutation scheme to estimate variable importance, which reflects the true impact of each predictor more reliably than a marginal approach (Strobl *et al.*, 2008). For species, TL and time step interval, we generated partial dependence plots (Friedman, 2001) to graphically illustrate the conditional effect of a predictor while accounting for other predictors.

We used analysis of covariance (ANCOVA) to further investigate the effects and explanatory power of the predictors considered in the RF and to test for specific hypotheses. The general model included fish TL, species identity, and their interaction to test the assumption of homogeneous slopes in the standard ANCOVA (García-Berthou & Moreno-Amich, 1993); time step interval and temperature and its quadratic term as predictors, since bell-shaped relationship is commonly accepted as the typical effect of temperature on U_{crit} (Randall & Brauner, 1991; Koumoundouros *et al.*, 2002). Another model included fish TL, species identity, time step interval, temperature and its quadratic term as predictors without considering interaction terms. Similarly to the RF-approach, we used the ANCOVA model to compute estimated marginal means (EMMs), using the ‘emmeans’ package (Searle, Speed & Milliken, 1980; Lenth, 2018), for the species identity factor and to compare the predicted U_{crit} values with those obtained using RF. For that purpose, we applied the Bland-Altman analysis (Bland & Altman, 1986), an established protocol for assessing agreement between two different measuring methods, using the ‘blandr’ R package (Datta, 2017). Specific hypotheses that we tested using ANCOVA were: whether there is an overall difference in U_{crit} between (1) native and alien species, (2) among families, and (3) among body shape categories, after accounting for fish body length. We did not consider the length × factor interaction when it was clearly non-significant ($P > 0.10$) and thus used a standard ANCOVA in these cases (García-Berthou & Moreno-Amich, 1993). In all models, TL and the response variable (U_{crit}) were $\log_{10}$-transformed to satisfy the assumptions of normality, homoscedasticity and linearity.

Finally, we used a linear mixed model (LMM) accounting for species-specific differences using the R-package 'lme4' (Bates *et al.*, 2015) to quantify the relative roles of species and other predictors and further test for heterogeneous slopes. We used U_{crit} as response variable, TL, time step interval and temperature as fixed-effect covariates and species as random effects in a random slopes model. This approach allows each species to have different slopes, i.e. the covariates have different effects for each species. The random slopes model was selected over the random intercepts model due to lower AIC values (AIC = -147.3 and AIC = -124.6, respectively) and a significant likelihood ratio test ($\chi^2 = 52.7$, $df = 15$, $P < 0.001$). We also used the 'ranova' function of the R-package 'lmerTest' (Kuznetsova, Brockhoff & Christensen, 2017) to test the random-effect terms in the model. Finally, we calculated *p* values and the marginal and conditional R^2 (Nakagawa & Schielzeth, 2013; Johnson, 2014; Nakagawa, Johnson & Schielzeth, 2017; Barton, 2018) with the 'lmerTest' (Kuznetsova *et al.*, 2017) and 'MuMIn' R-packages (Nakagawa & Schielzeth, 2013; Johnson, 2014; Nakagawa *et al.*, 2017; Barton, 2018), respectively. The marginal R^2 describes the variability explained by the fixed effects, while the conditional R^2 describes the variability jointly explained by the fixed and the random effects.

5.3. Results

The eight explanatory variables used in the RF model (i.e. species identity, family, fish total length [TL], body shape, form factor, time step interval, water temperature and native status) explained 72.8% of the variation in U_{crit}. The most important explanatory variable out of the eight tested was TL (54.1% variable importance), followed by family (9.9%), time step interval (5.1%) and species identity (1.7%). Form factor (1.1%), temperature (0.4%), body shape (0.3%) and native status (0.2%) were of low importance (Figure 5.1). Analysis of partial dependence of U_{crit} on TL revealed a steady but nonlinear increase of U_{crit} up to a body size TL ≤ 400 mm (Figure S5.1) where it reached a plateau, since very few fish in the dataset were longer than 400 mm. In contrast, the partial dependence plot on time step intervals showed a decrease of U_{crit} up to a time step interval ≤ 40 min (Figure S5.2) where it stabilised. After accounting for fish body length and all other predictor variables, common roach (*Rutilus rutilus*), European bass (*Dicentrarchus labrax*), perch (*Perca fluviatilis*), zander (*Sander lucioperca*) and brown trout (*Salmo trutta*) displayed the highest U_{crit}. By contrast, largemouth bass (*Micropterus salmoides*), European flounder (*Platichthys flesus*), channel catfish (*Ictalurus punctatus*), Tagus and Douro nase (*Pseudochondrostoma polylepis* and *P. duriense*) and pumpkinseed (*Lepomis gibbosus*) showed the lowest U_{crit} (Figure 5.2).

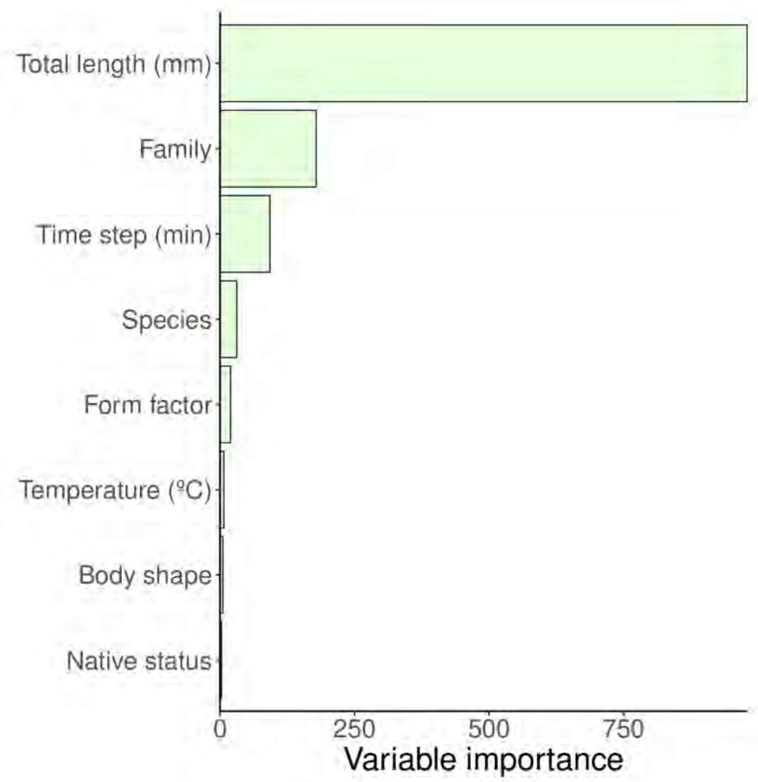


Figure 5.1. Variable importance of predictors of U_{crit} according to the random forest model. Variable importance is the difference in prediction accuracy (i.e. the number of observations classified correctly) before and after permuting a variable, averaged over all trees (Strobl *et al.*, 2008); and represents the effect of a variable in both main effects and interactions. Total percentage of explained variation was 72.8%.

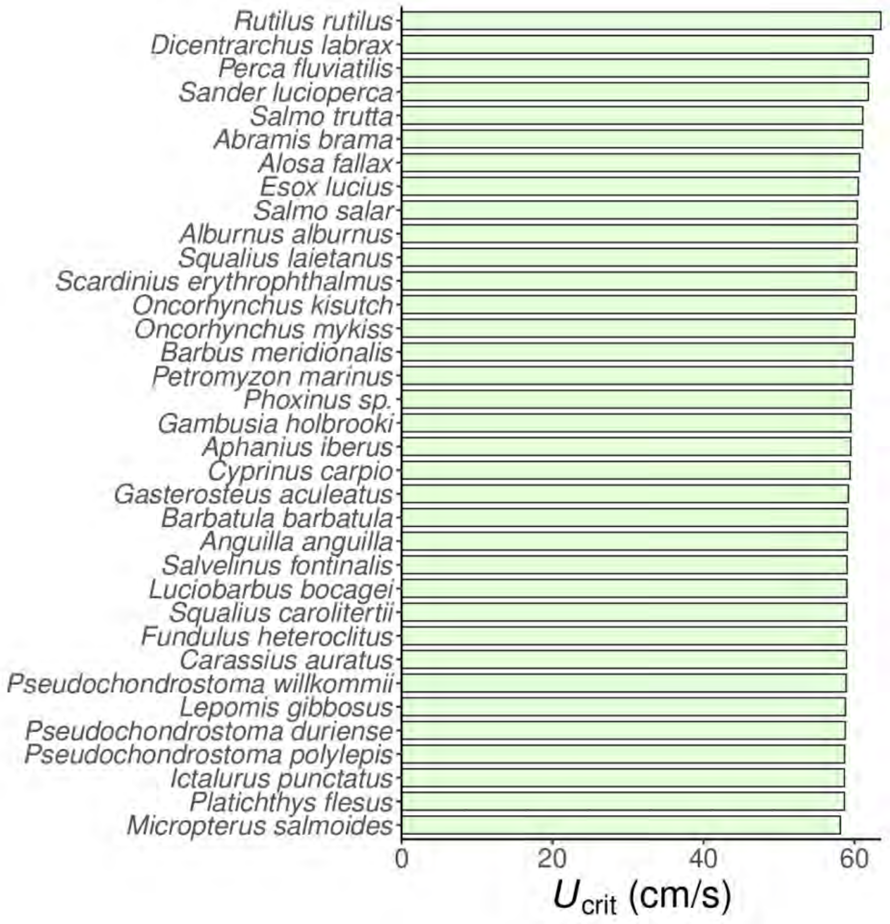


Figure 5.2. Partial dependence of U_{crit} across fish species based on the random forest model.

In the analysis of covariance (ANCOVA) model, 84.6% of the variance was explained by the considered explanatory variables: TL, species identity, and their interaction, temperature and time step interval. The TL × species identity interaction was significant, i.e. the slopes of the U_{crit} - TL relationship varied markedly among species (Figure 5.3 and Table 5.1), but was generally positive (approximately linear on a log-log scale) for species with significant relationships. In cyprinids for example, slope was flatter for common carp (*Cyprinus carpio*) than for roach (Figure 5.3a and Table S5.2). The ANCOVA was in agreement with the RF model, showing that fish body length (i.e. $\log_{10}$ TL) and fish species identity (and its interaction with length) explained most of the variation in U_{crit} (Table 5.1). In agreement also with the RF model, time step interval showed a significant negative effect on U_{crit}. Temperature was much less important but significant in the linear model, whereas its quadratic term was not (Table 5.1). Figure 5.4 shows the relationship of U_{crit} with TL and temperature for two common and well-studied fish species (roach and brown trout). Again, U_{crit} showed an increase with fish body length, reaching its maximum at intermediate temperatures, as observed particularly in roach (Figure 5.4a).

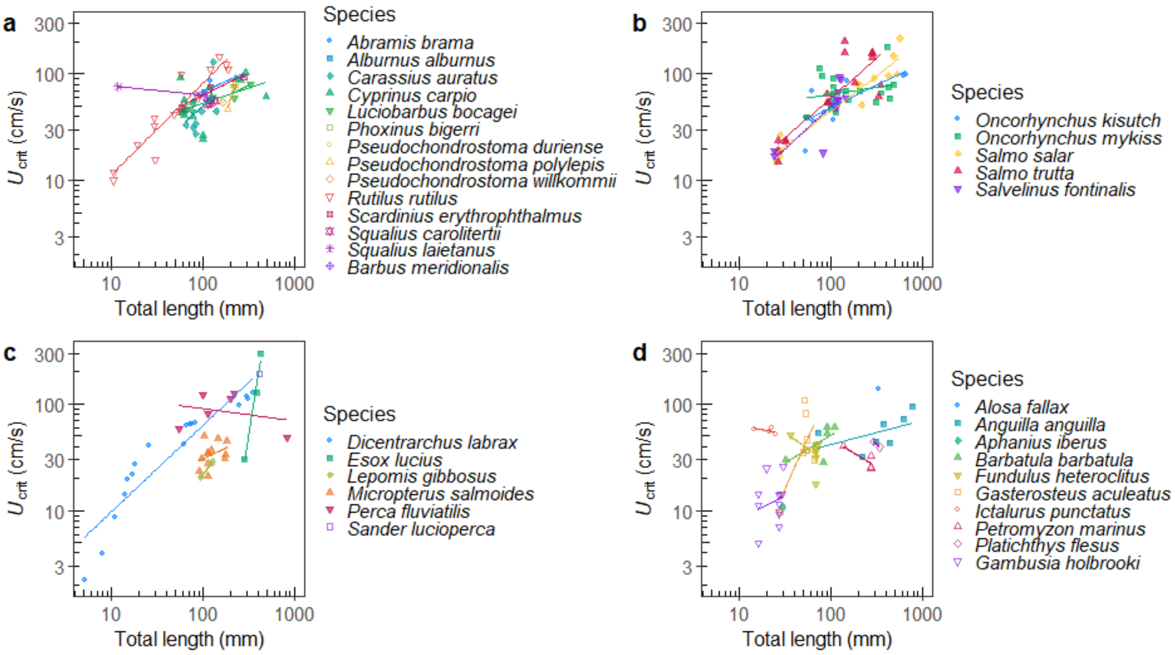


Figure 5.3. Relationship of U_{crit} with fish total length (TL) across species belonging to: (a) Cyprinidae and Leuciscidae; (b) Salmonidae; (c) Percidae, Moronidae, Centrarchidae and Esocidae; and (d) other families. Only lines for significant regressions are shown (see Table S5.2 for statistics).

The relationship between U_{crit} and TL also varied notably among families, both in intercepts and slopes (Figure S5.3 and Tables S5.3 and S5.4). Cyprinids for example, displayed lower swimming performance than other families studied, especially for longer fish lengths. However, the model accounting for family explained about 1.6% less of the total variance compared to the model with species identity, because there was some variability among species within families, e.g. within cyprinids, leuciscids and salmonids (Figure 5.3a and 5.3b).

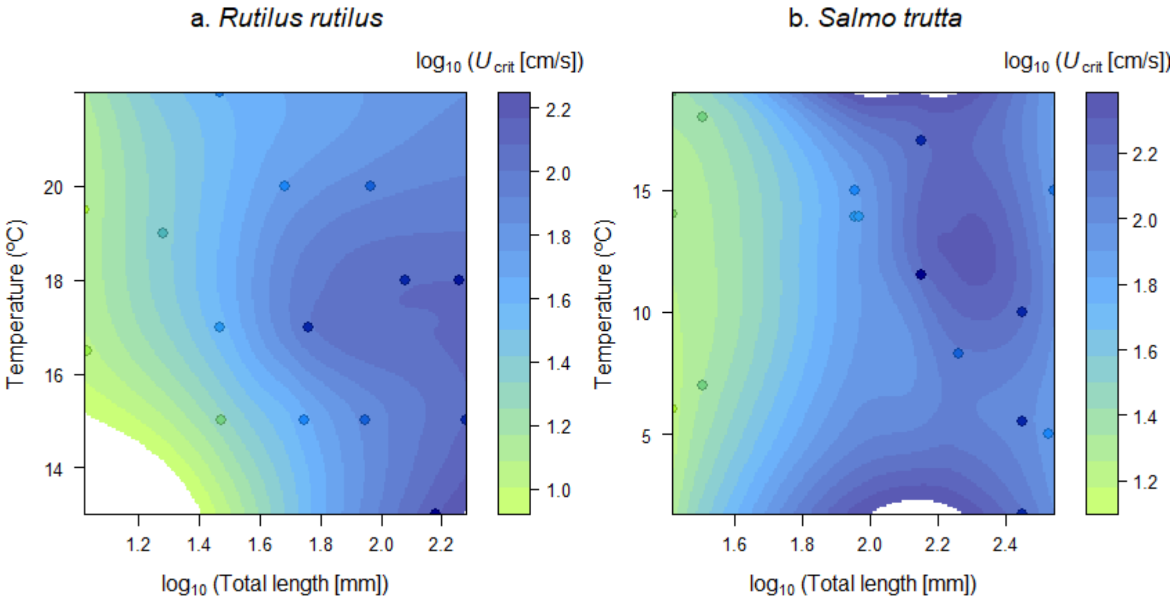


Figure 5.4. Surface plots relating U_{crit} with fish total length (TL) and temperature for two well-studied species: (a) *Rutilus rutilus*, and (b) *Salmo trutta*. Note log_{10}-transformations for U_{crit} and TL variables.

A linear model with only body shape and TL but without species identity explained much less variation, despite being significant (Table S5.4). The slopes were also significantly different among groups of body shape with fusiform and elongated species showing higher swimming performance for a given length than species with eel-like and short and deep forms (Figure S5.4). By contrast, native and alien species did not show significant differences (Figure S5.5 and Table S5.4). The estimated marginal means (EMMs) revealed that (after controlling for length) zander, roach, perch and brown trout were the species with the highest U_{crit}, whereas European flounder, eel, pumpkinseed and Spanish toothcarp (*Aphanius iberus*) showed the lowest U_{crit} (Figure S5.6). Comparing the EMMs and the partial dependence of U_{crit} on species obtained with the RF model, we observed that both results were highly correlated ($r = 0.680$, Figure S5.7) and showed no clear differences (mean difference = -2.19, 95% confidence interval = [-8.74, 4.36], Figure S5.8).

Finally, the results of the linear mixed model (LMM) showed that fixed effects (TL, time step interval and water temperature) explained 50.5% of the variation (marginal R^2), whereas the variation explained with the model also including random effects (species) increased up to 88.2% (conditional R^2). This again highlights the differences in U_{crit} among species and the heterogeneity of slopes of the U_{crit} - length relationship. Overall, the LMM revealed a positive effect of TL (coef. = 0.604, SE = 0.072, $P < 0.001$, Figure S5.9) and a negative effect of time step interval (coef. = -0.003, SE = 0.002, $P = 0.004$) on U_{crit}. By contrast, the effect of temperature was statistically not clear in the LMM (coef. = 0.012, SE = 0.002, $P = 0.987$).

Table 5.1. Linear model of critical swimming speed (U_{crit}) in response to total length, fish species, temperature and time step interval. R^2_{adj} = adjusted coefficient of determination in parentheses; *df* = degrees of freedom; *P* = *P* value.

Response variable (R^2_{adj})	**Variable**	**Sum of squares**	***df***	***P***
$\log_{10}$ (U_{crit} [cm s^{-1}])	$\log_{10}$(Total length [mm])	14.474	1	<0.001
(0.846)	Species	3.015	34	<0.001
	Temperature (°C)	0.594	1	<0.001
	Temperature^2	0.023	1	0.245
	Time step interval (min)	0.228	1	<0.001
	$\log_{10}$(Total length [mm]) × Species	1.863	25	<0.001
	Residual	2.403	140	

5.4. Discussion

This study is the first that comprehensively compiles and investigates a well-established measurement of prolonged swimming performance, i.e. critical swimming speed (U_{crit}), for 35 freshwater fish species currently inhabiting the Iberian Peninsula. Our results reinforce the importance of several factors that influence U_{crit}, with fish body length and taxonomic family being the most important predictors, followed by time step interval, species, the form factor, water temperature, species' body shape and native status.

Analogously to previous studies, our results revealed that fish body length is a key biological factor to understand swimming performance (Beamish, 1978; Plaut, 2001; Katopodis & Gervais, 2012, 2016). It is well known that absolute critical swimming speed (U_{crit} expressed in cm s^{-1}) scales with fish body length (Mateus, Quintella & Almeida, 2008), as already described in earlier studies of sustained and prolonged swimming (Thompson, 1917). Furthermore, for many species U_{crit} generally increases with the square root of fish length (Katopodis & Gervais, 2012, 2016). In our study, U_{crit} scales with fish body length following the typical allometric equation or power function, which is generally estimated through linear regression of log-transformed variables:

$$\log U = a + b \cdot \log L$$

where U is swimming speed and L is fish body length (Beamish, 1978). However, other studies also described the relationship with simple linear regressions without log-transformations (Mateus *et al.*, 2008; Romão *et al.*, 2012). Besides body length, it is important to note that body mass may also be an important predictor of U_{crit}, especially when it comes to comparing swimming abilities among species with different body shapes, swimming and propulsion types (Beamish, 1978; Videler, 1993; Ohlberger, Staaks & Hölker, 2006; Rubio-Gracia *et al.*, 2020). Although not tested in this study, body mass is directly related to body volume and, therefore, to energy expenditure needed to move against the flow (Ohlberger *et al.*, 2005; Srean *et al.*, 2016). Moreover, energy costs of swimming (i.e. the amount of energy necessary to transport one unit of body mass per unit of distance) are negatively associated with body mass because of the lower surface area to volume ratio in larger fish (Schmidt-Nielsen, 1972; Webb, 1975). Thus, the surface in contact with water per unit of volume is larger in small fish, increasing the friction drag and the relative dissipated energy (Sfakiotakis *et al.*, 1999). In addition, there is a direct association between body volume and muscle mass and number of myofilaments, which favours swimming performance (Hammer, 1995). As expected, body shape significantly influenced fish swimming performance. Earlier studies showed that body shape also influences the energetic costs associated with swimming (Ohlberger *et al.*, 2006; Rubio-Gracia *et al.*, 2020; Rubio-Gracia *et al.*, 2020). In general, streamlined fish tend to maximise thrust *while minimising drag and recoil energy losses* (Webb, 1975; Langerhans & Reznick, 2010). Correspondingly, fish evolve body forms that enhance steady swimming (i.e. swimming at constant-speed in a straight line) in open-water habitats, high-flow environments, and areas with relatively high competition for patchily-distributed resources (Langerhans & Reznick, 2010). Steady swimming is generally enhanced with a streamlined body shape, a shallow caudal region and a high aspect ratio of the caudal fin (Weihs, 1973; Froese, 2006). In agreement with this, our results showed that elongated and fusiform body shapes are better adapted to swim steadily. On the other hand, species that present the opposite suite of morphological traits such as eel-like and short and deep bodies tend to optimise unsteady swimming (i.e. more complicated locomotor patterns in which changes in velocity or direction occur, such as fast-starts, rapid turns, braking, and burst-and-coast swimming; Webb, 1984a).

Despite the general U_{crit} – body length relationship, we found large variability among fish species as indicated by the significant interaction of TL and species identity and associated contrasting slopes. For example, we found that eastern mosquitofish has lower U_{crit} than many other species for a given length, as shown in a previous study (Srean *et al.*, 2016). These differences might be due to fish species and populations having evolved over long-term periods, thereby adopting different abilities and strategies towards environmental and ecological conditions (Katopodis & Gervais, 2012). For example, a previous study showed that cyprinids living in fast flowing habitats showed higher U_{crit} values compared to fish species preferring slow flowing waters, independently of phylogenetic relationships (Fu *et al.*, 2014). Other studies found that long-distance migratory fish show higher swimming capabilities than those migrating over shorter distances (Tudorache *et al.*, 2008). Moreover, other species adapted to a specific environment such as bottom-dwelling or flatfish species, usually perform poorly in U_{crit} (Duthie, 1982; Knaepkens, Maerten & Eens, 2007; Tudorache *et al.*, 2008). Consistent with these earlier findings, our results also indicated that benthic and flatfish species like European flounder have relatively lower U_{crit}. In addition, our results revealed taxonomic family as a good predictor of U_{crit} despite marked differences in lifestyle and form among species within the same family (Killen *et al.*, 2016). For example, in salmonids we revealed brown trout as a species with a high estimated U_{crit}, while brook charr showed comparably lower U_{crit} for a given body length. These differences in swimming capacity might be related to differences in their habitat preferences with brook charr (*Salvelinus fontinalis*) being generally found in slow-flowing pools whereas brown trout prefers faster riffle areas (Peake *et al.*, 1997). The relationships of habitat preferences and swimming capacity have also been shown in cyprinids and leuciscids because of their distribution in a wide variety of habitats and their associated morphological diversity (Fu *et al.*, 2014; Killen *et al.*, 2016; Schönhuth *et al.*, 2018). However, we acknowledge that our analyses might have been affected by differences in data availability which might also affect the predictive power of the variable 'species'.

In agreement with previous research, the duration of the step-test interval had an effect on mean critical velocity (Peterson, 1974). U_{crit} increases for short time steps and reaches an asymptote at time step intervals between 30 and 60 minutes (Peterson, 1974), as we observed in our results. Thus, given these differences in swimming performance when using different time intervals, we therefore strongly recommend standardising and carefully choosing the U_{crit} protocol to prevent misleading understanding of fish swimming performances. In addition, we also examined the effects of temperature on U_{crit}, which is also one of the most important abiotic factors influencing fish swimming performance (Fry, 1947, 1971; Brett, 1971; Webb, 1975). Specifically, the relationship between U_{crit} and temperature is commonly described by a bell-shaped curve (Randall & Brauner, 1991). Others demonstrated this bell-shaped relationship for juvenile sea bass, whose swimming speed increased as temperature rose from 15 to 25 °C and then decreased (Koumoundouros *et al.*, 2002). Mechanistically, this can be explained by a general decline of all physiological processes at low temperatures (e.g. a decrease in power generated by the muscle) that also reduces U_{crit} (Randall & Brauner, 1991; McKenzie & Claireaux, 2010). As temperature increases, there is a positive effect on muscle functioning, and its associated power generation contributes to an increase in swimming performance (Johnston & Temple, 2002; Rome, 2007). Nevertheless, when temperature exceeds the optimum range, the oxygen-carrying capacity of the blood decreases and restrains oxygen delivery to the tissues (Randall & Brauner, 1991). In contrast to this bell-shaped relationship of temperature and U_{crit}, we only found a positive relationship with temperature. This lack of observed decline at high temperatures might be due to different reasons. On one hand, the bell-shaped curve is strongly influenced by rates of temperature acclimation previous to the experiment, being most marked in fishes that are exposed to intense temperature change, and increasingly less pronounced with acclimation time (McKenzie & Claireaux, 2010). On the other hand, most experiments were conducted only at temperatures that are within the normal thermal tolerance range of a species, i.e. optimum or colder temperatures, rather than covering a long temperature gradient (Videler & Wardle, 1991). Finally, some studies revealed asymmetric relationships between temperature and U_{crit} showing only significant swimming performance decreases at low temperatures (Claireaux *et al.*, 2006; Fangue *et al.*, 2008b).

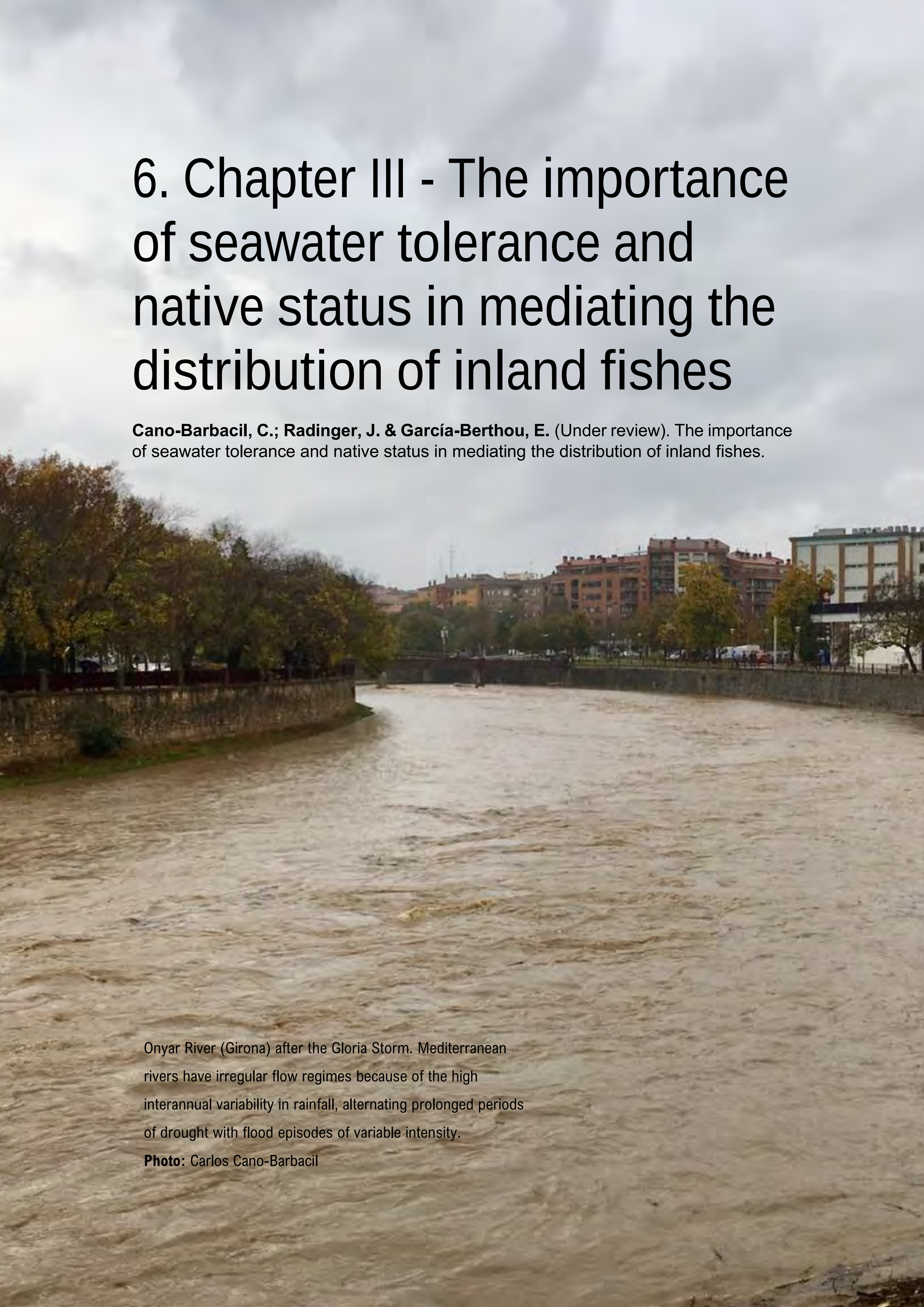
Our results revealed that both native and alien species have similar prolonged swimming performance, after accounting for body size. This finding is fairly surprising according to the apparent differences in habitat preferences between the two class of groups. Several studies showed that alien fish dominate Iberian reservoir habitats with their artificially stable limnological conditions (Rodríguez-Ruiz, 1998; Aparicio *et al.*, 2000; Corbacho & Sánchez, 2001; Clavero, Blanco-Garrido & Prenda, 2004; Carol *et al.*, 2006; Leunda, 2010). By contrast, native species, mostly cypriniforms, are considered more adapted to lotic habitats with naturally more fluctuating flow regimes and, in particular, with frequent occurrence of high-flow events (Propst & Gido, 2004; Gido *et al.*, 2013; Pool & Olden, 2015; Srean *et al.*, 2016). However, several alien species that are often classified as limnophilic (Cano-Barbacil, Radinger & García-Berthou, 2020) showed relatively high swimming capacities (e.g. zander, northern pike [*Esox lucius*], common bleak [*Alburnus alburnus*]). These species have in common that are pelagic and some of them also have high trophic level lifestyles, which has been shown to favour swimming performance and maximum aerobic capacity (Killen *et al.*, 2016). It suggests, therefore, that the classification of fish according to their habitat preferences is not always a good proxy of their prolonged swimming performance, and that U_{crit} may be more related to ecological demands of species (Fu *et al.*, 2014). In addition, not all alien fish of the Iberian Peninsula are inhabiting reservoirs or lentic habitats, as in the case of the non-native rainbow trout (*Oncorhynchus mykiss*) that rather prefer rivers with moderate to rapid flows (Doadrio, 2001). Moreover, the native freshwater fish fauna of the Iberian Peninsula is characterised by a low number of families, but with a considerable degree of diversification of species (Doadrio, 2001). Thus, it may be further hypothesised that the high diversity of species and forms could have counteracted the variation in U_{crit} across species, independently of their origin (native or alien). Ultimately, some invaders can colonize novel environments using other swimming strategies, like fast-start swimming (Tierney *et al.*, 2011), and specific meso- or microhabitats. Thus, U_{crit} might not be necessarily the main character determining ecological success and, therefore, invasiveness.

Our results might be limited by different issues. First, reported measurements of U_{crit} might be skewed by experimental setups, such as the effects of chamber type and length. Indeed, it has been shown that fish can reach higher U_{crit} values in longer flumes (Haro *et al.*, 2004; Peake & Farrell, 2006; Tudorache *et al.*, 2007b; Kern *et al.*, 2018). However, when taking data from different sources, we were not able to control for the effect of the flume characteristics on swimming performance measurements because they are often not reported. Second, small sample sizes and the narrow ranges of investigated fish lengths studied for some species might contribute to the large variability in regression line slopes found in this study. This was considered in our results and only provided significant regression lines (e.g. for species with larger sample sizes). Moreover, issues of data availability also affected the selection of predictors. For example, we were not able to analyse the effect of some variables like fish weight, which is often not provided in swimming performance studies, or habitat preference which is often rather unclear for the endemic species of the Iberian Peninsula (Cano-Barbacil *et al.*, 2020).

To sum up, this study showed that fish body length is the most relevant explanatory variable of U_{crit} out of the eight considered predictor variables. Other important predictors were fish taxonomic affiliation (family and species identity) and the time step interval between velocity increments used during the experiment. Even though we found overall effects of body shape, form and water temperature on U_{crit}, their relative importance as predictors were much lower. In contrast to our expectations, we did not find clear differences in U_{crit} between native and alien fish species, after accounting for size. Therefore, this suggests that prolonged swimming performance might not be always related to the invasiveness of species in recipient ecosystems, although this needs further testing. We conclude that, besides advancing the fundamental understanding of prolonged swimming performance in Iberian freshwater fishes, our findings also provide the foundation to support their management. The compiled dataset comprises the so far most comprehensive information on U_{crit} of the Iberian ichtyofauna. However, we note that swimming speed determined for fishes confined in a respirometer do not necessarily translate directly to free-swimming individuals in the field (Peake & Farrell, 2006) and thus should be used cautiously. However, until additional research is conducted on free-swimming fish, U_{crit} data represent the best information available (Peake, 2008b). Thus, our results may be used as species-specific estimates of U_{crit}: (1) to design fish bypasses estimating maximum allowable water velocities in order to improve river connectivity (Peake, 2008b), (2) to develop barriers for the exclusion of invasive alien species (Katopodis & Gervais, 2016), (3) to assess the effects of damming and hydrologic alteration on river fish, and (4) to categorise fish habitat preferences and restrictions, since a species swimming performance might be a limiting factor of its presence in a given habitat.

6. Chapter III - The importance of seawater tolerance and native status in mediating the distribution of inland fishes

Cano-Barbacil, C.; Radinger, J. & García-Berthou, E. (Under review). The importance of seawater tolerance and native status in mediating the distribution of inland fishes.



Onyar River (Girona) after the Gloria Storm. Mediterranean rivers have irregular flow regimes because of the high interannual variability in rainfall, alternating prolonged periods of drought with flood episodes of variable intensity.

Photo: Carlos Cano-Barbacil

Chapter summary

Unveiling the ecological and historical factors that underlie species distributions has challenged ecologists for a long time. The main objective of this chapter is to understand the role of environmental variables explaining the distribution of three major eco-evolutionary groups of inland fishes (Darlington's divisions: primary, i.e. strict freshwater; secondary, i.e. salt-tolerant; and peripheral, i.e. diadromous and estuarine) as well as its introduced status, and how these variables are related to fish traits.

We modelled distributions of the most common inland fish species across the Iberian Peninsula to compare the importance of different predictors among the three Darlington's divisions and between native and alien species. To explore the importance of specific environmental variables in determining the distribution of different traits of inland fish, variable importances obtained from species distribution models were subjected to a redundancy analysis.

Darlington's divisions differ significantly in salinity tolerance, in distribution overlap, in the importance of distribution predictors, and associated life history traits. Topographic and climatic variables (e.g. basin, temperature) were generally more important than land use and anthropogenic factors (e.g. hydrological alteration) in explaining fish distributions. We found significant differences in the importance of variables explaining the distribution of native vs. alien species and especially among Darlington's divisions. River basin was most important for primary native and many alien species. Increasing mean temperature and damming were positively associated with the presence of alien species but were less important for native fishes. Tolerant, large-bodied, warm-water alien fishes introduced from more hydrologically stable habitats were often associated with damming and environmental degradation.

Despite marked differences in the distribution patterns of native and alien species, evolutionary and introduction histories as well as seawater tolerance are central factors explaining the current distribution of inland fishes. Darlington's divisions proved useful for addressing ecological and biogeographical questions at broader spatial scales.

Keywords: alien species, biological invasions, Darlington's divisions, endemic species, freshwater fish, hydrologic alteration, Iberian Peninsula, Mediterranean rivers, Myer's divisions, species distribution models

6.1. Overview

Unravelling the ecological and historical factors that underlie species distributions and biodiversity patterns has challenged ecologists and biogeographers for a long time. In an increasingly human-dominated world, where global biodiversity is changing at an unprecedented rate (Sala *et al.*, 2000), unveiling the variables that explain the distribution of species is of key importance to understand environmental impacts, species invasions, and the often simultaneous decline of many native species as well as to implement appropriate management measures (Markovic *et al.*, 2014). This is particularly relevant in freshwater ecosystems, which are among the most diverse but, at the same time, most threatened ecosystems globally (Albert *et al.*, 2021). Fresh waters are threatened by manifold interacting factors such as habitat degradation and alteration through land use changes and damming, pollution, invasive alien species, and climate change (Grill *et al.*, 2019). As a consequence, more than a quarter of all freshwater fauna is threatened or has recently become extinct (IUCN, 2019).

In contrast to terrestrial organisms, for which current climatic conditions and topography seem dominant in determining species' distributions, freshwater fish ranges are also markedly maintained by basin boundaries (Filipe *et al.*, 2009). Thus, the historical connection among river basins, as well as the fish tolerance to seawater are important factors to understand contemporary geographical patterns of freshwater fishes (Darlington, 1948; Filipe *et al.*, 2009). Myers (1938, 1949) recognised that the distribution of fish is mediated by their different ability to survive and disperse through seawater, and proposed a classification of inland fish based on their eco-evolutionary history and euryhalinity. Darlington (1948) reviewed and simplified this classification of inland fish into three major eco-evolutionary groups (hereafter, Darlington's divisions): (1) primary fish, whose ancestors entered inland waters much earlier, cannot survive in seawater and are thus strictly confined to fresh water; (2) secondary fish, which mostly live in fresh waters but show some salt-tolerance and can thus may survive in seawater; and (3) peripheral fish, which occur in fresh waters but have high salt-tolerance, such as diadromous or species of marine origin. This classification is based on taxonomic families and in general primary species such as cyprinids, characids and most siluriforms have low salinity tolerance in contrast to secondary species such as cichlids and cyprinodontiforms (McDowall, 2010), the latter comprising certain species with the highest salinity tolerance known among fishes (Schultz & McCormick, 2013). Since its introduction, Darlington's classification has been frequently used to address questions in freshwater zoogeography and may be used as proxy of seawater tolerance (Berra, 2001). Primary fishes are naturally absent from oceanic islands such as New Zealand, Madagascar, the West Indies and most of Australia in contrast to secondary and peripheral families, which were able to reach these areas because of their higher salinity tolerance (Darlington, 1948). For this reason, fish also reflect the faunal boundary between Australia and Southeast Asia (known as Wallace's line) better than other vertebrate groups (Berra, 2001). Similarly, secondary fishes are more prevalent and diversified in Central America because they colonised it before the final uplift of the Isthmus of Panama, and 10 million years before primary fishes (Smith & Bermingham, 2005). Myers' or Darlington's classifications are generally supported and used by many of the most comprehensive, recent fish monographs (Bănărescu, 1990; Berra, 2001; Doadrio, 2001; Kottelat & Freyhof, 2007; McDowall, 2010). Although numerous studies have analyzed the relationships of environmental variables and the distribution of freshwater fish species (Carvajal-Quintero *et al.*, 2019), the effect of seawater tolerance on contemporary inland fish distribution, and therefore, the differences among the three Darlington's divisions, has been barely investigated (Smith & Bermingham, 2005; Filipe *et al.*, 2009).

An analysis of contemporary fish distributions must also consider the native status of a species (i.e. whether a species is native or not to a given region). This is relevant to draw meaningful conclusions about the importance of historical and ecological variables (Sax, Stachowicz & Gaines, 2005), as alien species often have different distribution patterns and drivers than native species. For instance, previous studies showed that temperature and other climate-related variables markedly influence freshwater fish invasion success (e.g. warm temperatures favour the establishment and spread of many alien species) (Bae *et al.*, 2018).

In addition, anthropogenic factors such as land use change have altered the range size distribution of fish species (Radinger *et al.*, 2016). Specifically, dams causing fragmentation of river networks and modifications of the natural flow and sediment regimes have been associated with changes in diversity and taxonomic homogenisation of fish communities, favouring the presence of alien species and hindering native ones (Johnson, Olden & Vander Zanden, 2008). For instance, damming often facilitates the establishment and proliferation alien species with a suite of traits (Cano-Barbacil *et al.*, 2020) that corresponds well to a periodic strategy (Winemiller & Rose, 1992; i.e. limnophilic and phytophilic species that maximises age-specific fecundity at the expense of optimising turn-over time and juvenile survivorship; see Vila-Gispert *et al.*, 2005). In addition, the distribution and abundance of migratory, estuarine, rheophilic and lithophilic species is heavily impacted by dams due to the loss of connectivity and accessibility to essential habitats and the alteration of the flow regime (Lassalle, Crouzet & Rochard, 2009).

Mediterranean-climate regions are well suited to study the mechanisms that explain differences in the distribution patterns of inland fish. They harbor a very particular fauna, rich in endemic but also alien species, and they often show strong anthropogenic perturbation (Leprieur *et al.*, 2008). Specifically, the Iberian inland fish fauna comprises 68 native species, of which 41 are endemic, and 32 alien species. Furthermore, numerous barriers cause rivers of the Iberian Peninsula to be more fragmented and impacted by dams than many other European rivers (Grill *et al.*, 2019). Over the last decades, and concurrent with the proliferation of alien species and the increase in the number of dams, native fish populations of the Iberian Peninsula have considerably declined (Doadrio *et al.*, 2011).

Against this background, the main objectives of this study are: (1) to assess the importance of climatic, topographic and anthropogenic variables in shaping the current distribution of primary, secondary and peripheral native and alien fish of the Iberian Peninsula; (2) to evaluate the role of hydrological alteration in the distribution of these three eco-evolutionary species' groups; and (3) to understand the relationship between the importance of distributional drivers and fish traits. We hypothesised that primary, secondary and peripheral species would show contrasting importance of predictors because of their fundamental differences in evolutionary history and salinity tolerance. We expected that geographic restrictions by the river basin would be of particular importance for primary native species due to their limited dispersal ability. We also hypothesised that temperature and hydrological alteration would be important and positively correlated with alien species presence, because many of them are rather thermophilic and well adapted to stagnant waters. Finally, we hypothesised that limnophilic and tolerant species would be more prevalent at sites with warmer temperatures and higher hydrological alteration, while rheophilic fish presence would be negatively associated with these variables.

6.2. Methods

6.2.1. Study area

The study area comprised the Iberian Peninsula (see Figure 6.1a), which is characterised by its complex orography and high spatial and temporal climate variability. Following the Köppen-Geiger climate classification, the southern half of the Iberian Peninsula is dominated by a Mediterranean climate with dry and hot summers, SE Spain by a semiarid climate, the northern half by a Mediterranean oceanic climate with warm summers and mountainous areas by an oceanic climate (Kottek *et al.*, 2006). Additionally, there are over 1500 large dams (MAPAMA, 2020), mostly for agricultural irrigation and other human uses.

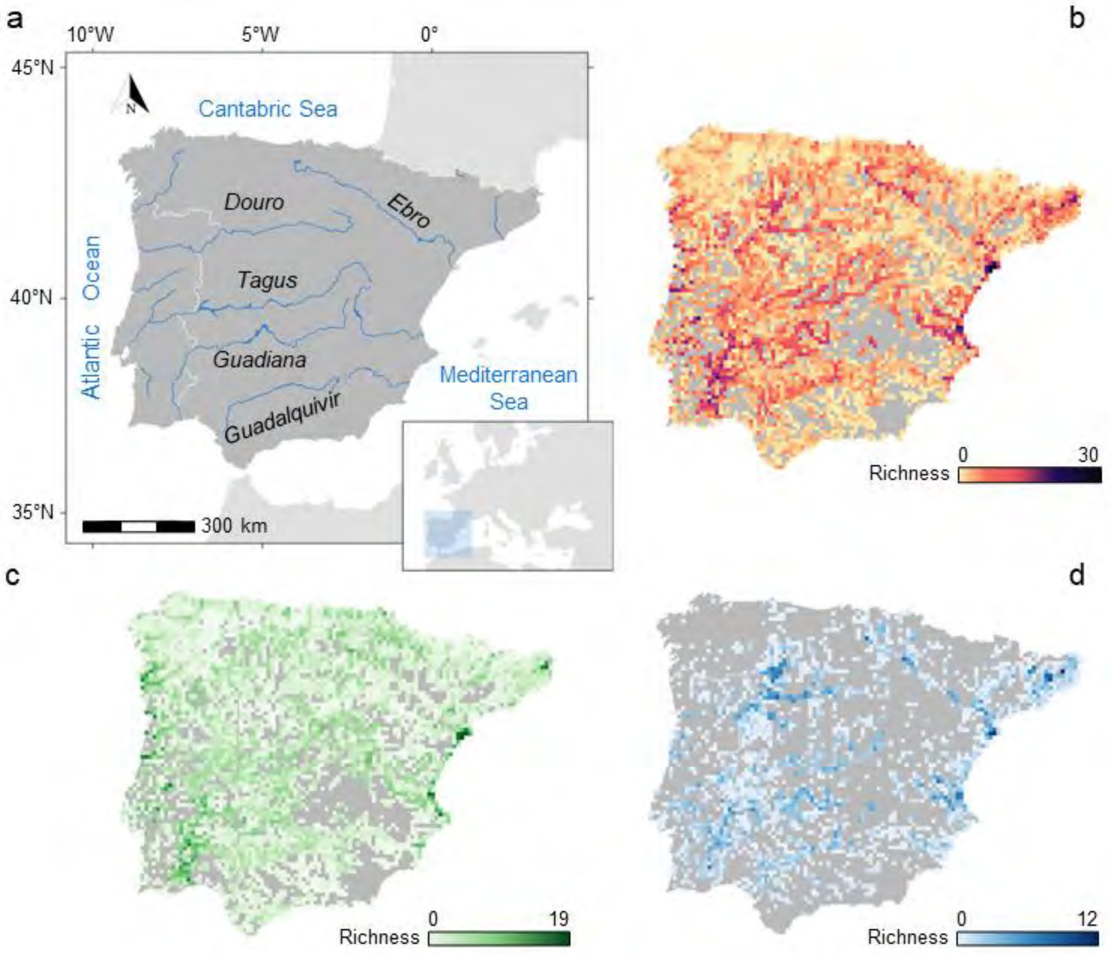


Figure 6.1. (a) Map of the study area with major Iberian rivers. (b) Observed total fish species richness, (c) richness of native fish species, and (d) richness of alien fish species in the Iberian Peninsula. Projection: WGS 84 / Pseudo-Mercator - EPSG:3857.

6.2.2. Fish data

We compiled occurrence data for all established Iberian inland fishes between 2000 and 2020. These comprise 68 native (including diadromous and estuarine) and 32 alien fish species. Presences were mainly obtained from GBIF (Global Biodiversity Information Facility; GBIF.org, 2019a) and the Portuguese 'Carta Piscícola Nacional' (Ribeiro *et al.*, 2007), and complemented with 19 additional published studies (see Table S6.1). GBIF data were mainly based on Doadrio's atlas (2001), which is the most comprehensive fish study of Spain (Figure 6.1). The spatial resolution for subsequent modelling was set to 10 × 10 km which reflects the most widely used resolution in the species' occurrence records (see Doadrio, 2001). Darlington's divisions of the fish species were assigned using taxonomic families following Berra (Berra, 2001). We used this classification because: (1) Darlington's divisions are well associated with salinity tolerance data for the few species that have quantitative data; (2) other classifications (e.g. euryhalinity or use of brackishwater) are similar but more based on expert criteria than Darlington's; and (3) statistical analyses using data on salinity tolerance generally provided similar results (see Appendix S6.1 for more details). Finally, we also compiled data of sixteen morphological, reproductive and habitat use species traits ('traits' hereafter; see Table S6.2) mainly from Cano-Barbacil *et al.* (2020) and complemented with other sources (Table S6.1).

6.2.3. Environmental data

We compiled climatic, topographic, land use and anthropogenic variables (Table S6.3). We obtained environmental data layers from online databases and did subsequent calculations in QGIS 3.4.14 (QGIS Development Team, 2019). Predictor variables were rescaled to a modelling grid with a resolution of 10 × 10 km UTM (Universal Transverse Mercator, i.e. 100 km^2, $n = 6142$ total cells) to agree with the grain of our species data. Following Dormann *et al.* (2013), we removed strongly correlated variables with Pearson correlation coefficients $|r| \geq 0.7$. Using hierarchical cluster analysis based on the correlation matrix (Figure S6.1), we selected only one variable from each group of predictors based on its ecological relevance and previous literature. We then calculated variance inflation factors (VIF), using the R-package 'HH' (Heiberger, 2019), and checked that VIF < 5 (Kock & Lynn, 2012). A total of 13 predictor variables were finally used for SDM development (Table S6.3; maps of all predictors are provided in Appendix S6.2).

As climatic predictors, we used: mean air temperature as indicative of water temperature, which is a crucial driver of inland fish distributions (Murphy *et al.*, 2015; Bae *et al.*, 2018); average precipitation within each sub-catchment as representative of water discharge (Garvey *et al.*, 2000); average precipitation seasonality (i.e. a measure of variation in monthly precipitation over the course of the year) as surrogate of the flow regime, a key environmental factor determining riverine dynamics (Lane *et al.*, 2017); and solar radiation, reported as influential for spawning and growth of fishes (Williamson *et al.*, 1997). As topographic variables we selected: terrain slope; the topographic index, i.e. a function of the catchment area and the slope gradient that is commonly used to quantify topographic control on hydrological processes (Sørensen, Zinko & Seibert, 2006); distance to the sea; and Strahler's stream order as a proxy of stream size and longitudinal position within a river system (Strahler, 1957). As indicators of anthropogenic perturbation, we used: percentage of agricultural and urban land use in the catchment upstream (i.e. percent surface of altered land use in the river basin upstream of a certain UTM), which are, for example, correlated to impairment of water, habitat quality and siltation (Bae *et al.*, 2018); upstream accumulated reservoir capacity (i.e. the accumulated volume of water stored in reservoirs upstream of each modelling grid in the river network), as an indicator of the changes in flood magnitude and mean flow produced by damming (Batalla, Gómez & Kondolf, 2004; Bae *et al.*, 2018); and local reservoir capacity (i.e. the volume of water stored in each 10 × 10 km modelling grid cell), as a measure of the direct influence of reservoirs on fish species occurrence (Rahel, 2002). Finally, we also included the water district (hereafter, 'basin ID') to account for biogeographic units and evolutionary history since our main focus is to understand factors explaining the current distribution of fish species rather than to know their potential distribution. 'Basin ID' consisted of single river basins in the case of large rivers, or sets of small coastal rivers that share similar faunas and environmental characteristics.

6.2.4. Statistical analyses

We first used a multivariate permutational analysis of variance (PERMANOVA) to test for differences in the current distribution of primary, secondary and peripheral native and alien species. We used the 'adonis2' function of the R-package 'vegan' (Oksanen *et al.*, 2017). We used 999 permutations and Jaccard distances. Compared to other methods, PERMANOVA has the advantage of not making distributional assumptions and permitting various distance measures and designs. We also calculated the distribution similarity of the three Darlington's divisions using the Jaccard index (*J*). For that purpose, we used the functions 'vegdist' and 'meandist' of the R-package 'vegan'.

To develop species distribution models (SDMs), we used the BIOMOD computational framework, as implemented in the R-package 'biomod2' (Thuiller *et al.*, 2019). To avoid potential biases, we did not model recently established introduced species and only analyzed distributions of species with occurrence records in at least 20 modelling grid cells. In total, 51 native and 17 alien species were considered in our distribution models. We used four different algorithms that have been frequently applied to a variety of taxa and that showed good accuracy and complementary advantages: generalised linear models (GLM), boosted regression trees (BRT), random forests (RF), and Maxent. GLM are an extension of linear models to allow for heteroscedasticity and non-normal errors (McCullagh & Nelder, 1989). We used GLMs with binomial distribution and a logit link function. BRT combine the strengths of regression trees (i.e. models that relate a response variable to their predictors by recursive binary splits) and boosting (Elith, Leathwick & Hastie, 2008) by proceeding through sequential improvements using a numerical optimisation algorithm that adds a new tree at each step. RF are model-averaging approaches where each tree depends on the values of a randomised subset of predictors and with the same distribution for all trees in the forest (Breiman, 2001). RF showed better prediction accuracy than other SDM techniques with minimal overfitting (Marmion *et al.*, 2009). Maxent algorithm models species distributions using species' presence records and a 'background' sample of environments in the study area, and applies the maximum-entropy principle for fitting the model (Guillera-Arroita, Lahoz-Monfort & Elith, 2014). For additional details of all the modelling options selected, see Table S6.4.

Our datasets did not include reliable absence locations because of inconsistent sampling effort. Therefore, we generated three pseudo-absence datasets (each n = 1000) among background grid cells for each species (Barbet-Massin *et al.*, 2012). We used a random selection of pseudo-absences, a procedure generally yielding reliable SDMs (Barbet-Massin *et al.*, 2012). Random selection of pseudo-absences is the best strategy when using regression techniques (e.g. GLM) and yields good models when using classification and machine-learning techniques (e.g. RF, BRT) (Barbet-Massin *et al.*, 2012). We calibrated the models ten times using randomly selected 70% of the data and validated against the remaining 30% based on cross-validation. We evaluated the predictive accuracy of the different SDM algorithms using four statistics (Table S6.5): the area under the receiver operating characteristic curve (AUC), the true skill statistic (TSS), sensitivity and specificity. We computed an ensemble forecast, built for each species using models with a AUC score greater than 0.7, weighted by their AUC in order to increase prediction accuracy and to overcome prediction uncertainty from individual modelling techniques (Marmion *et al.*, 2009).

We computed variable importances for each species-specific ensemble model to determine the most influential environmental factors, using the internal procedure of 'biomod2'. This methodology applies Pearson correlation between the standard predictions (i.e. fitted values) and predictions where the variable under investigation has been randomly permutated. If the correlation is high (i.e. small difference between both predictions), a variable is considered less important for the model (Thuiller *et al.*, 2009). Variable importance ranges from 0 to 1, with higher values indicating greater importance of a predictor. As the variable 'basin ID' could potentially mask effects of other environmental predictors, we additionally computed all the SDMs without considering 'basin ID' to compare results. Models including 'basin ID' resulted in similar importance of the other variables but generally higher predictive accuracy (see Appendix S6.3). Therefore, we decided to base all subsequent analyses on the set of SDMs including 'basin ID' as predictor variable.

To test for differences in variable importance of predictors and AUC of models among primary, secondary and peripheral native and alien fish species we used PERMANOVA. We used univariate PERMANOVAs to analyze differences of variable importance of predictors and AUC in native status and Darlington's divisions and its interaction, and a multivariate PERMANOVA including all predictor variable importances. We used 999 permutations and Euclidean distances for the PERMANOVA. We also tested for homogeneity of dispersions for the different predictors among groups using the function 'betadisper' of the R-package 'vegan'.

To explore the importance of specific environmental variables in determining the distribution of different traits of inland fish (see e.g. Magadzire *et al.*, 2019), variable importances obtained from our SDMs were subjected to a redundancy analysis (RDA) (Legendre & Legendre, 2012), using the 'rda' function of the R-package 'vegan'. This technique extracts and summarises the variation in a set of response variables (i.e. variable importances) that can be explained by a group of explanatory variables (i.e. fish traits). We used 'arcsin' transformation for response variables to ameliorate linearity and normality. We assessed the significance for each term by using permutation tests (999 permutations). As the trait dataset contained missing data, we imputed the 2.6% of missing values to avoid potential drawbacks of analyses that omit these cases (Nakagawa & Freckleton, 2008), and because it allowed us to increase the overall number of species with complete data by 29.4%. We used the 'imputeFAMD' function of the R-package 'missMDA', which allows the imputation of missing values of mixed datasets comprising of continuous and categorical variables. We used five components to predict the missing entries as estimated using the 'estim_ncpFAMD' function. We then computed two analyses of variance (ANOVA) to analyze how the scores of the two first axis varied among native status and Darlington's divisions and its interaction. As a complementary approach (de Bello *et al.*, 2015), to account for the non-independence of trait data among species due to phylogenetic relatedness (Felsenstein, 1985), we also performed a principal components analysis (PCA) on the variable importance dataset to extract synthetic axes, and then we related the first two axes to the set of species traits using phylogenetic generalised least squares (PGLS), using the 'pgls' function of the 'caper' package (Orme *et al.*, 2018). The maximum likelihood estimate of λ was incorporated as a parameter in the PGLS model, thus controlling for phylogenetic dependence in the data in a manner that is optimal for the data set (Freckleton, Harvey & Pagel, 2002). The phylogenetic tree of the studied species was obtained from a recent ray-finned fishes phylogeny (Rabosky *et al.*, 2018), using the function 'fishtree_phylogeny' of the R-package 'Fish Tree' (Chang *et al.*, 2019). All statistical and modelling tasks were performed with the software R, version 3.5.1 (R Core Team, 2020).

6.3. Results

We found that actual distributions of fish (as mapped in current fish atlases) varied between native and alien species (PERMANOVA, $R^2 = 0.023$; $P = 0.007$), but especially among the three Darlington's divisions ($R^2 = 0.065$; $P = 0.001$). Although the similarity of species distributions was generally very low (different species are often found in different basins; see Appendix S6.4), secondary and peripheral species are more similar in their distributions (mean $J = 0.056$) compared to primary species ($J = 0.030$ with secondary and 0.026 with peripheral).

For all species ensemble models, average cross-validated AUC scores were high (ranging from 0.856 to 0.990 with a mean value of 0.945, Table S6.5) but varied among Darlington's divisions (PERMANOVA, $R^2 = 0.115$; $P = 0.014$), between native and alien species ($R^2 = 0.049$; $P = 0.045$), and with significant interaction between Darlington's divisions and native status ($R^2 = 0.108$; $P = 0.022$). AUC were lower for alien than for native species and particularly higher for native primary fish (Figure S6.2). High values of true skill statistics (TSS) ranging from 0.604 to 0.977 with a mean value of 0.829 also indicated good performance of the models (Table S6.5). High specificity (ranging from 81.4 to 98.9 with a mean value of 91.8) and sensitivity (ranging from 78.9 to 99.1 with a mean value of 91.3) of the models pointed to a great proportion of correctly predicted background points and presences, respectively (Table S6.5). Projected SDM maps for the 68 inland fish in the Iberian Peninsula are provided in Appendix S6.4.

In general, climatic and topographic predictors were more important than land use and anthropogenic predictors (Figure S6.3). However, multivariate PERMANOVA revealed significant differences in the importance of predictors explaining the distributions of native *vs.* alien species and even larger differences among Darlington's divisions (25 and 6.5% of explained variation, respectively), with no clear interactions or differences in dispersions (Figure 6.2 and Table 6.1). Specifically, 'basin ID' was the most important predictor across species (Figure S6.3) but showed marked differences in variable importance among Darlington's divisions. It was particularly important for primary species (mean = 0.429; SD = 0.244), but less important for secondary (mean = 0.292; SD = 0.253) and peripheral species (mean = 0.210; SD = 0.151; Figure 6.2b). 'Basin ID' was the most important variable for many endemic species (Figure 6.3a) and those alien species that are present yet in a few specific basins (Figure S6.4). Overall, 'distance to the sea' was the second most important variable in our models (Figure S6.3). It was the most important variable for several peripheral (mean = 0.452; SD = 0.268) and secondary (mean = 0.401; SD = 0.304) native species present in estuaries or coastal lagoons (Figures 6.2, 6.3b and S6.5) where it was negatively related to their occurrence probability. 'Annual mean temperature' was overall the second most important variable for alien species (mean = 0.174; SD = 0.122; Figure 6.2a). Its importance was significantly greater than for native fish (mean = 0.086; SD = 0.113). With increasing temperatures, the occurrence probability was increasing for 15 out of the 17 alien species studied (Figure S6.6). Conversely, increasing temperatures were associated with decreasing occurrence probability of some native fish, such as *Salmo trutta* (Figure 6.3c) or *Achondrostoma arcasii*. The importance of 'annual mean temperature' was also higher for secondary species than for peripheral and primary fish (Figure 6.2b).

'Upstream reservoir capacity' was overall the sixth most important variable (Figure S6.3). Its variable importance differed among the three Darlington's divisions studied but mainly between native and alien fish. ‘Upstream reservoir capacity’ variable importance was significantly greater for alien and peripheral fish (Figure 6.2). Analysis of homogeneity of dispersions showed also that the dispersion across groups was not homogeneous (Table 6.1). Specifically, alien species showed greater variability in the importance of the ‘upstream reservoir capacity’ (mean = 0.064; SD = 0.053) than native ones (mean = 0.020; SD = 0.033). This variable was especially important and positively related with the occurrence of several alien species like *Silurus glanis*, *Esox lucius* or *Cyprinus carpio* (Figures 6.3d and S6.7). ‘Upstream reservoir capacity’ was negatively but less markedly related with the occurrence of 12 out of the 29 primary native species studied. Moreover, SDMs revealed a positive relationship between 'upstream reservoir capacity' and the occurrence of peripheral native species. By contrast, the mean effect of ‘local reservoir capacity’ on this group of species was negative. Finally, the variables ‘slope’, ‘topographic index’ and ‘solar radiation’, or those related to anthropogenic disturbance, such as ‘agricultural’ or ‘urban percentage land uses’ were, in general, much less important predictors of fish species distribution at the scale of the Iberian Peninsula.

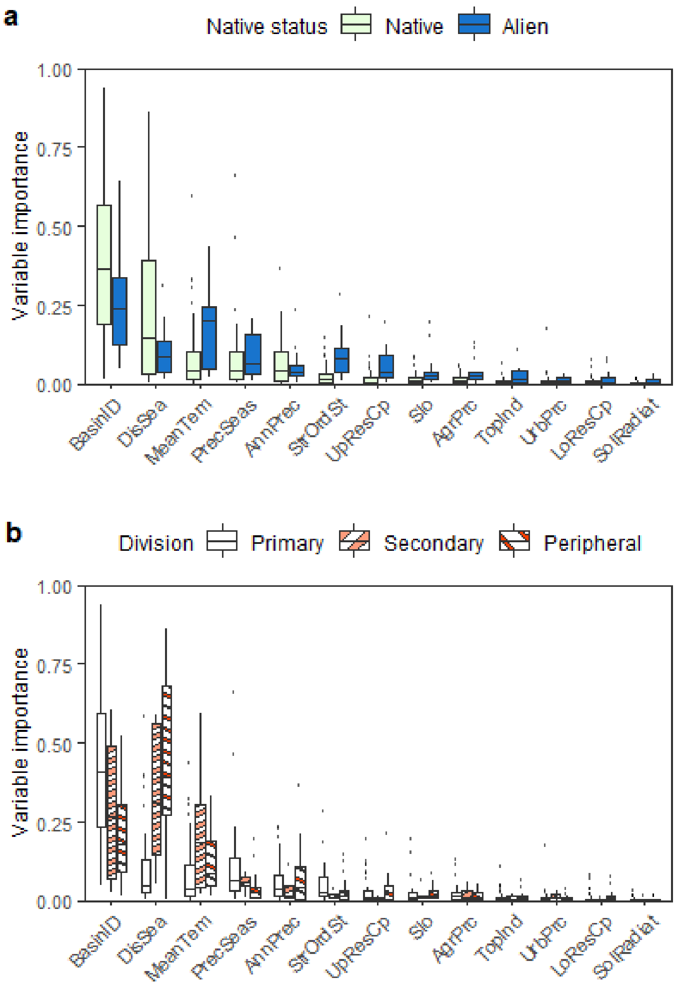


Figure 6.2. Importance of the different predictor variables used in the SDMs (a) across native status (i.e. alien vs. native) and (b) Darlington’s divisions of inland fish (i.e. primary, secondary and peripheral). Boxes correspond to the 25th and 75th percentiles; lines inside a box show the median; whiskers extend to the last observation within 1.5 times the interquartile range from the quartiles and outliers are indicated by empty circles. Predictor variable abbreviations are defined in Table 6.1.

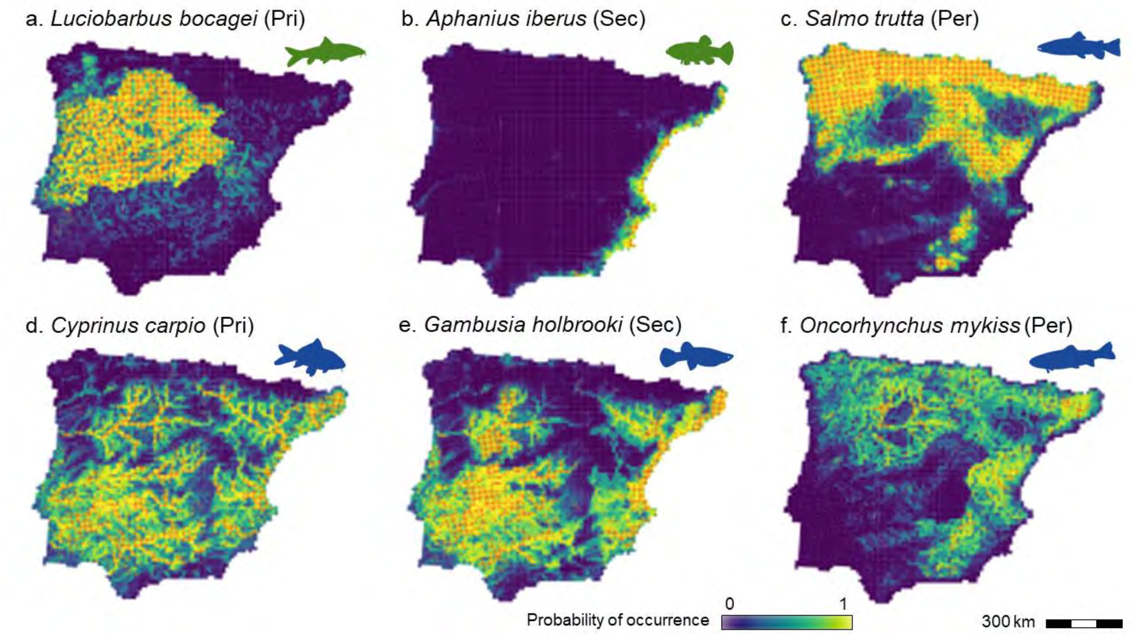


Figure 6.3. Projected species distribution models (SDM) maps for six paradigmatic inland fish species in the Iberian Peninsula. Upper panels with green silhouettes show native species; lower panels with blue silhouettes show alien species. Red points correspond to species occurrences. Pri = primary; Sec = secondary; Per = peripheral. Projection: WGS 84 / Pseudo-Mercator - EPSG:3857. Silhouettes were obtained from http://phylopic.org/.

Table 6.1. Results of the PERMANOVA and homogeneity of dispersions analysis across native status (NS) and Darlington's fish divisions (D). Coefficients of determination (R^2) and positive/negative mean effects for the six groups are shown for PERMANOVA. *P* values for PERMANOVA are expressed with asterisks (*** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05; . ≤ 0.1). *P* values are also shown for homogeneity of dispersions analysis. PrN = primary native species; SeN = secondary native species; PeN = peripheral native species; PrA = primary alien species; SeA = secondary alien species; PeA = peripheral alien species; NA = not applicable. DisSea = distance to the sea; MeanTem = annual mean temperature; PrecSeas = average precipitation seasonality within sub-catchment; AnnPrec = average annual precipitation within sub-catchment; StrOrdSt = Strahler's stream order; UpResCp = upstream reservoir capacity; Slo = slope; AgrPrc = average agricultural land use with a given sub-catchment; TopInd = topographic index; UrbPrc = average urban land use within a given sub-catchment; LoResCp = local reservoir capacity; SolRadiat = solar radiation.

Response variable	PERMANOVA										Homogeneity of dispersions
	NS	D	NS × D	Residuals	Mean effect						*P*
					PrN	SeN	PeN	PrA	SeA	PeA	
All	**0.064** **	**0.242** ***	0.031	0.661	NA	NA	NA	NA	NA	NA	0.324
Basin ID	0.038 .	**0.228** ***	0.028	0.705	NA	NA	NA	NA	NA	NA	0.150
DisSea	**0.082** **	**0.368** ***	0.027	0.523	+	-	-	+	-	+	**0.032**
MeanTem	**0.101** **	**0.116** *	0.062 .	0.721	-	+	+	+	+	-	**0.017**
PrecSeas	0.006	0.075	0.037	0.881	-	+	+	-	+	-	0.183
AnnPrec	0.007	0.014	0.010	0.969	-	-	+	-	-	+	0.383
StrOrdSt	**0.280** ***	0.041	0.018	0.660	+	+	+	+	+	+	0.144
UpResCp	**0.196** ***	**0.081***	0.054	0.669	+	+	+	+	+	-	**0.037**
Slo	**0.114** *	0.020	0.003	0.863	-	-	-	-	-	+	0.211
AgrPrc	**0.159** **	0.001	0.031	0.809	+	+	-	+	+	-	0.139
TopInd	**0.151** **	0.040	0.013	0.796	+	+	+	+	+	+	0.101
UrbPrc	0.003	0.008	0.015	0.975	-	+	+	+	+	-	0.950
LoResCp	**0.084** *	**0.087** *	0.048	0.781	+	-	-	+	+	-	**0.050**
SolRadiat	**0.173** **	0.015	0.010	0.802	+	+	-	+	+	-	0.183

RDA revealed that 35.3% of the variation in variable importance can be explained by differences between fish traits ($F_{16, 51} = 1.737$, $P = 0.001$). Species whose distributions were highly sensitive to 'annual mean temperature', 'stream order', 'upstream reservoir capacity' and 'slope' were mainly characterised by being tolerant and larger-bodied species (Figure 6.4). Moreover, many of these species were alien fish (Figure S6.8). Primary native species, whose distributions were particularly conditioned by 'basin ID', were mainly rheophilic, invertivory and potamodromous (Figure 6.4). RDA indicated that most peripheral species are diadromous and larger-bodied species (Table S6.2). The ANOVA results showed that the scores of the first axis (i.e. RDA1) varied among Darlington's divisions ($F_{2,62} = 15.1$; $P < 0.001$), between native and alien species ($F_{1,62} = 43.3$; $P < 0.001$), and with significant interaction between Darlington's divisions and native status ($F_{2,62} = 3.3$; $P = 0.042$; Figure S6.9). The ANOVA of the second axis did not show clear differences among Darlington's divisions nor between native and alien fish.

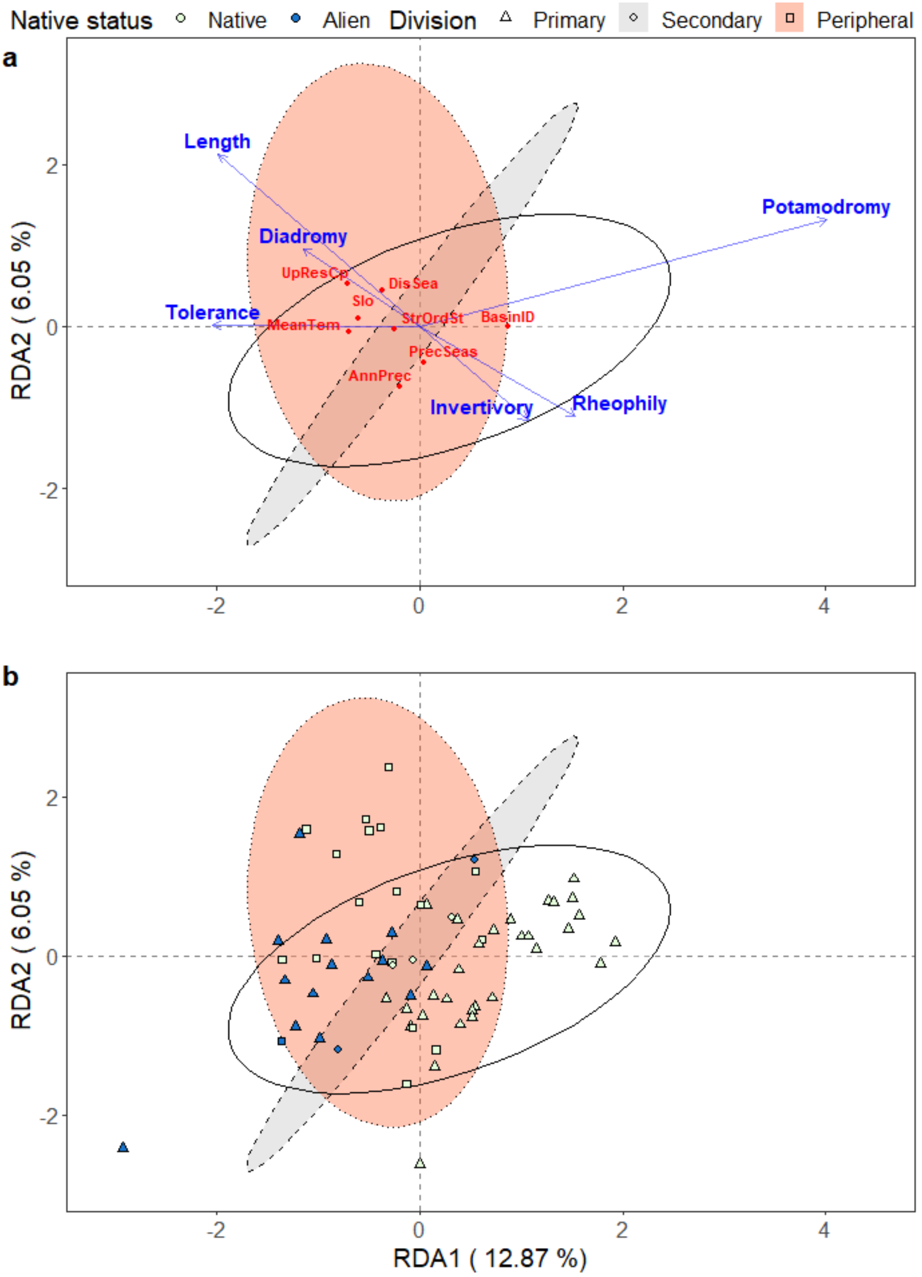


Figure 6.4. (a) Correlation biplot based on redundancy analysis (RDA) of variable importance of the different environmental predictors used in the SDMs. Species traits are shown as blue arrows. For clarity, only significant and marginally significant traits ($P < 0.10$, see Table S6.2 for further information) and 8 most important environmental variables in SDMs are shown. Predictor variable abbreviations are defined in Table 6.1. Note that continuous traits (e.g. fish maximum length) were previously $\log_{10}$-transformed. (b) Individual plot where each dot represents one species. Light green shows native species, while dark blue shows alien species. 95% confidence ellipses are shown for primary (white), secondary (grey) and peripheral (light red) fish.

The PCA explained 40.3% of the variable importance variation with two axes (Figure S6.10). The first PCA axis identified a dominant gradient of variable importances that contrasts species whose distribution is mainly constrained by river basin boundaries with species whose distribution is more influenced by climatic and anthropogenic factors (e.g. precipitation, temperature and human impacts). The second axis contrasts species whose distribution is affected by the continentality (i.e. species that occur in coastal areas *vs*. species that occur in the interior of the Iberian Peninsula). In contrast to the results of the RDA, PGLS only showed a positive relationship between omnivory and the first axis of the PCA (estimate = 2.534, t = 3.240, P = 0.002; Table S6.6).

6.4. Discussion

Our results showed that regardless of differences in the distribution patterns between native and alien species, evolutionary and introduction histories as well as seawater tolerance are central factors explaining the current distribution of Iberian inland fishes. For instance, we found that secondary species have a more similar distribution to peripheral than to primary species. The distribution of *Fundulus heteroclitus* in the Atlantic coast of North America or in southwestern Iberia (where it was introduced and dispersed to different river estuaries) and *Aphanius* spp. along Mediterranean salt marshes exemplifies that despite the likely absence of long-distance movements through salt waters in modern times (Moyle & Cech, 2004), the distribution patterns of such secondary species are very different from primary fishes, which are often confined to a few river basins.

Hence, our hypothesis that primary, secondary and peripheral fish species would generally show contrasting importance of different climatic, topographic and anthropogenic predictors in explaining their distribution was supported by our results. Results revealed marked and clear differences between the eco-evolutionary groups suggested by Darlington (1948), which were even more pronounced than differences between native and alien species. More specifically, our results showed that ‘basin ID’ was the most important variable for primary native species, suggesting that these species are confined to their particular and potentially isolated drainage systems and that any migration to other basins might (naturally) only be enabled by slow geological processes (e.g. river captures or changes in sea level) (Myers, 1938). For example, native *Squalius* and *Luciobarbus* genera as well as other native cyprinids have experienced extensive speciation processes in the Iberian Peninsula after the formation of the different basins and geographical barriers (Doadrio *et al.*, 2011). Correspondingly, a recent study investigating global fish distributions revealed that the historical connection among river basins during Quaternary low-sea level periods constitutes a good predictor explaining range sizes in freshwater fishes (Carvajal-Quintero *et al.*, 2019). Although its effect is not as remarkable as for primary native species, ‘basin ID’ was also the most important variable for several alien species such as: *Australoheros facetus*, mostly present in the Guadiana basin (Hermoso, Blanco-Garrido & Prenda, 2008); and *Scardinius erythrophthalmus*, whose distribution is mainly restricted to the basins of the Ebro and Eastern Pyrenees (Doadrio, 2001). By contrast, ‘basin ID’ was much less important for peripheral fish, as their geographical ranges are not necessarily restricted by the sea given their ability to migrate, or disperse through seawater (McDowall, 2010) and thereby enter and colonise other river catchments.

In accordance with our hypothesis and previous studies (Murphy *et al.*, 2015; Bae *et al.*, 2018), temperature was found to be a key environmental variable for explaining the distribution of alien species. This further emphasises the thermophilic character of many alien species (e.g. *Gambusia holbrooki*), with higher temperatures favouring their reproduction and competitive capacity (Carmona-Catot, Magellan & García-Berthou, 2013). Climate change models for the Iberian Peninsula predict temperature increases, most pronounced during the summer months, and changes in precipitation with increased variability over the year (Álvarez Cobelas, Catalán & García de Jalón, 2005). Warmer temperatures and reduced flows could create novel suitable habitats for introduced species in future, thereby facilitating their establishment and invasion (Carmona-Catot *et al.*, 2013). 'Annual mean temperature' also showed an important effect on several secondary species that are also thermophilic such as the native *Aphanius baeticus* and *Valencia hispanica*. Some native species showed considerable negative effects of temperature on their geographic distributions. This includes, for example, *S. trutta*, a well-known cold-water species that is mainly distributed through the north of the Peninsula and in mountain ranges, and which is sensitive to high temperatures (Elliott & Elliott, 2010). Thus, climate change could produce severe impacts on both taxonomic and functional components of the native ichthyofauna (de Oliveira *et al.*, 2019).

Results indicated that topographical and climatic variables were more influential drivers of the distributions of Iberian inland fishes than anthropogenic factors. In agreement with previous studies (Murphy *et al.*, 2015; Bae *et al.*, 2018), variables such as 'agricultural' and 'urban land uses' had little effect on fish distribution at the spatial scale of the Iberian Peninsula. This contrasts with other previous studies, which found land use an important driver of fish distributions in large river catchments (Radinger *et al.*, 2016, 2019). This disagreement may result from the different spatial scales considered and because climatic predictors are often more important especially at larger spatial scales (Guisan & Thuiller, 2005).

In addition, 'local reservoir capacity', which indicates the presence and size of local reservoirs, was also of less importance for explaining fish distributions in our study. This partly contrasts with previous studies describing local reservoirs as an important predictor of the distribution of some alien species (e.g. *Micropterus salmoides*) that dominate Iberian reservoirs, and whose presence can be explained by altered environmental conditions and increased propagule pressure (Johnson *et al.*, 2008). Surprisingly, the observed importance of dams in explaining native fish distributions was relatively low compared to topographical and climatic variables. We found a negative effect of ‘local reservoir capacity’ on peripheral fish distribution. As migratory and estuarine species cannot pass river barriers, their presence is negatively associated to reservoirs. It is well documented that dams and other river barriers have greatly reduced the range sizes and spawning areas of many anadromous species. In the Iberian Peninsula, anadromous species lost up to 80% of habitats in many river basins (Mateus *et al.*, 2012). Nevertheless, the variable 'upstream reservoir capacity', which describes the degree of modification of the natural flow regime and other ecological features resulting from upstream impoundment, was the most important anthropogenic factor, especially related to the distributions of alien and peripheral species. This might point to the importance of cumulative effects of dams on fish distributions which might differ from local impacts of single reservoirs.

Our results agree with previous studies, which have indicated that dams and their associated alteration of the seasonal and inter-annual flow variability, the creation of impoundments and the modification of sediment transport is likely to favour alien fish (Radinger *et al.*, 2019). The greater importance of ‘upstream reservoir capacity’ than ‘local reservoir capacity’ for alien species might indicate that the presence of these fish is not only associated to the reservoir itself – which is often considered a site of high propagule pressure of alien species – but rather to the regulated river reaches that are severely hydrologically altered. Moreover, the RDA showed that alien species are mainly tolerant fish with rather large flexibility in their requirements regarding water quality and habitats, and are larger-bodied than primary native species. These traits were mainly related to the variable importance of ‘mean annual temperature’ and ‘upstream reservoir capacity’. Our results agree with previous studies of the Iberian Peninsula (Vila-Gispert *et al.*, 2005) showing that alien species correspond well to the periodic life-history strategy defined by Winemiller and Rose (1992): in fact, alien fish represent large-sized species with long longevity, late maturity, high fecundity, few spawning bouts per year, and short reproductive span. However, traits of many alien fishes result from human selection of species with particular desirable features such as large body-size (e.g. game and commercial fishes) or wide ecological tolerance to ensure successful establishment (Alcaraz, Vila-Gispert & García-Berthou, 2005; Grabowska & Przybylski, 2015). In contrast, primary native fish show more opportunistic traits, i.e. they show early maturation, frequent reproduction over an extended spawning season, rapid larval growth, and rapid population growth rates (Winemiller & Rose, 1992). By altering environmental conditions and reducing flow variability with the construction of barriers and dams, the introduction of alien fish from seasonal habitats (central European and southeastern North American streams) that are more hydrologically stable has been favored (Vila-Gispert *et al.*, 2005). The disagreement observed between the results of the RDA and the results after accounting for phylogenetic relatedness among species (i.e. PGLS) could be due to the absence of an evolutionary relationship between the distributional drivers considered in this study and fish traits.

The weaker performance of the SDMs of alien species as indicated by lower AUC values might reflect that they have not yet fully realised their distributions in the Iberian Peninsula. For example, some alien species have only been found in single drainage basins, which does not mean that environmental conditions in other basins are unsuitable for future colonisation. Therefore, their current distributions are also much influenced by their introduction histories and basin boundaries. Nevertheless, SDMs constitute a valuable tool to identify those regions which are particularly vulnerable to the establishment of invasive alien species (Barbet-Massin *et al.*, 2018; Perrin *et al.*, 2021). They are useful instruments to identify important areas for early monitoring and to prioritise eradication efforts if necessary.

Although Darlington's classification has received some criticism (Rosen, 1974; Sparks & Smith, 2005), it is widely applied and well related to experimental seawater tolerance (see Appendix S6.1). Our results showed that it is a useful classification system to address ecological and biogeographical questions as the species of the same division are generally closer in their distribution patterns. The scarcity of quantitative data on seawater tolerance makes this classification a helpful proxy. We also note that there are some limitations that might have affected our modelling results, related to both methodological issues and uncertainties associated with observational data. We minimised multicollinearity among predictor variables and selected methods that are relatively robust to it. However, we acknowledge that in river systems many environmental variables develop along the upstream-downstream gradient. Thus, we cannot exclude that some of our investigated distributional patterns might be rather related to predictors not explicitly considered but which covary with the selected variables. In addition, the variables upstream and local reservoir capacity explained much of the shifts below dams in flood magnitude and mean monthly flow, but not changes in annual runoff or median daily flows (Batalla *et al.*, 2004), as the effect of a reservoir depends not only on its capacity, but also on its operation. Thus, these metrics only represent a portion of the potential hydrologic alterations that rivers suffer. Moreover, we did not consider the temporal dynamics of species distributions. Here, we used fish samplings aggregated over several years because there is barely time series or fish monitoring in the Iberian Peninsula. For that reason, we had to assume similar climate, land use and hydrological alteration over that time. Nevertheless, we acknowledge that alien fish introduction, establishment and spread are complex time-dynamic processes (Dominguez Almela *et al.*, 2020). Therefore, we encourage further studies to analyze time-series datasets to understand the dynamics of species invasion processes and the potential decline of native species distribution ranges. Finally, the dendritic structure of river networks has been frequently argued against use of species atlas grid-based data for modeling freshwater fish distributions. However, SDMs based on grid could also show high predictive performance and are a good alternative in those cases where stream network related data are not available (Markovic, Freyhof & Wolter, 2012).

In summary, our results shed light on a central topic in biogeography and reveal the main variables that shape the distribution patterns of inland fish species of the Iberian Peninsula. Specifically, we found that topographic and climatic predictors are more important than land use and anthropogenic variables in explaining Iberian fish distributions. Native and alien species showed marked differences in the importance of factors explaining their distribution. Thus, our results are an important contribution to the prioritisation in alien species management and to identify areas that might become invaded. Particularly, the marked differences among Darlington's divisions reflect that this eco-evolutionary classification is strongly related to environmental variables driving species distributions, with species of the same division generally showing analogous distribution patterns. This further leads to the conclusion that different tolerances to salinity of the three divisions studied and possibly associated factors largely influence the current distribution of inland fish.

7. Chapter IV - Phylogenetic signal and evolutionary relationships among traits of inland fishes along elevational and longitudinal gradients

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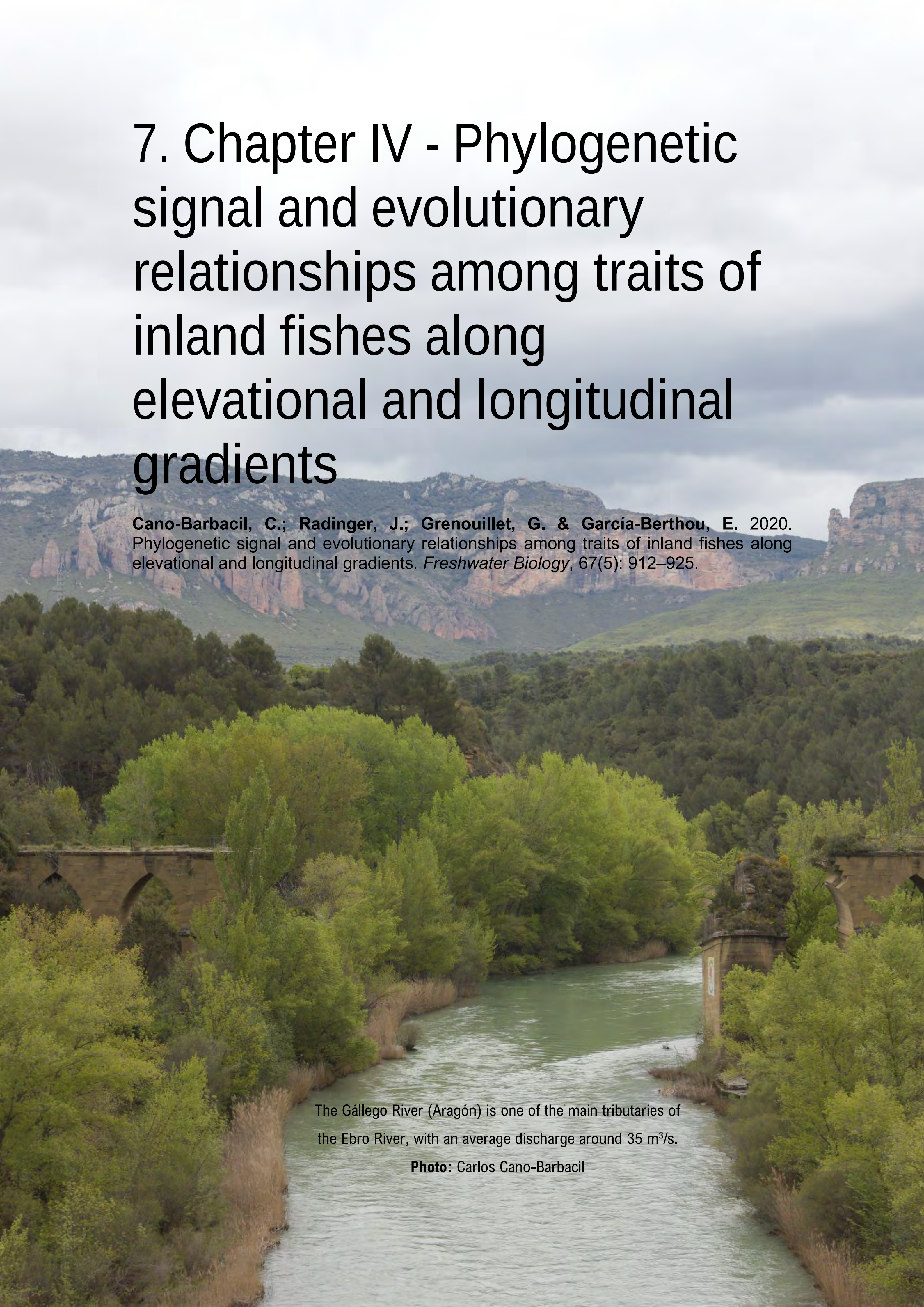


The Gállego River (Aragón) is one of the main tributaries of the Ebro River, with an average discharge around 35 m^3/s.

Photo: Carlos Cano-Barbacil

Chapter summary

Understanding the main drivers of species distributions is one of the main goals of ecology. However, the relationships between traits and elevational and longitudinal distributions in inland fishes, as well as their underlying evolutionary processes, have been less investigated. Thus, we aimed to quantify and assess the relationships among several types of traits resulting from species' evolutionary histories by measuring their phylogenetic signal across inland fishes of the Iberian Peninsula. We also aimed to test for correlated evolution of these traits with elevation and stream size (i.e. stream order), to test whether a species' suite of traits and their elevational and longitudinal niche tend to evolve together.

We compiled data on 23 fish biological and ecological traits for 30 inland fish species present in the Iberian Peninsula. We quantified their phylogenetic signal using four complementary indices (Pagel's λ, Blomberg's K and Abouheif's C_{mean} for continuous and $-D + 1$ for binary traits). We used both phylogenetic and non-phylogenetic methods to evaluate the relationship among traits and their relationship with elevation and stream order.

We found a significant phylogenetic signal for 65% of the studied traits. Phylogenetic signals were quite variable, but we did not detect clear differences between continuous and binary traits or among trait types (i.e. morphological, trophic, reproductive and habitat use). Evolutionary models revealed that elevational and longitudinal distribution showed little evidence for directional trends of evolution. Hence, species elevational and longitudinal niches tend to resemble to those of the common ancestor. Many fish traits were inter-correlated as revealed by phylogenetic methods, indicating correlated evolution of pairs of traits. For example, reproductive traits such as maximum longevity, fecundity and age at maturity tended to evolve together with fish maximum length. Consequently, certain orders of fish showed shared suites of traits. Comparative methods revealed a significant positive relationship of parental care with elevation and stream order. By contrast, non-phylogenetic analyses and multivariate analyses indicated positive relationships between elevation and rheophily and lithophily, and a negative relationship between stream order and invertivory and rheophily.

Overall, our results point to a notable phylogenetic signal in many traits of inland fishes and a strong phylogenetic structure in their functional traits along their elevational and longitudinal gradients. Thereby our results contribute to an improved understanding of species' adaptations to environmental changes with important conceptual and practical implications for minimising further species losses.

Keywords: alien species, body length, Iberian Peninsula, swim tunnel, temperature, U_{crit}

7.1. Overview

Understanding the main drivers of species distribution patterns and the mechanisms of coexistence is the central goal of ecology. Competition for resources and other ecological interactions often lead to the divergence of clades into multiple niches and the appearance of novel traits (Rüber, Verheyen & Meyer, 1999; Gilbert *et al.*, 2018). Over the course of evolution, some taxonomic groups accumulate morphological and ecological variation among their constituent species, others produce more similar species and others can show parallel evolution (Rüber *et al.*, 1999; Sidlauskas, 2008). However, closely related taxa tend to show similarities in many characteristics, including morphological, trophic, reproductive, behavioural or ecological traits, due to common ancestry (Harvey & Pagel, 1991; Kamilar & Cooper, 2013). This phylogenetic relatedness can be measured by the 'phylogenetic signal' (hereafter PS), defined by Blomberg & Garland (Blomberg & Garland, 2002) as the 'tendency for related species to resemble each other more than they resemble species drawn at random from the tree'. Previous studies found that the PS varies substantially across trait types (Freckleton *et al.*, 2002; Blomberg, Garland & Ives, 2003; Kamilar & Cooper, 2013). Most but not all traits display significant PS, which tends to be strongest in morphological traits such as body size, intermediate in life-history and physiological traits, and low in behavioural traits (Blomberg *et al.*, 2003; Kamilar & Cooper, 2013). For instance, in primates, dietary traits and climatic niche were among the variables with lowest PS (Kamilar & Cooper, 2013). Comte *et al.* (2014) found that fish traits related to morphological attributes and trophic position showed stronger phylogenetic clustering than other reproductive and habitat use characteristics.

The retention of niche-related ecological traits over time, causing that closely related species are more ecologically similar than would be expected based on their phylogenetic relationships (Losos, 2008), is termed 'phylogenetic niche conservatism' (hereafter PNC) and strong PS has often been interpreted as evidence of it (Wiens *et al.*, 2010). Some degree of PNC is likely in many species and its understanding is important to inform potential responses to global warming or species introductions in new areas (Wiens & Graham, 2005; Wiens *et al.*, 2010). For instance, species with little tolerance to encompass the new environmental conditions and with strong PNC must either migrate or go extinct, while species with more evolutionarily labile traits could potentially adapt (Holt, 1990; Wiens *et al.*, 2010). Nevertheless, strong PS can result from PNC or from Brownian motion-like evolutionary change (e.g. due to genetic drift or randomly fluctuating natural selection) (Losos, 2008; Wiens *et al.*, 2010). So, PS is seen as a necessary but insufficient condition for PNC (Losos, 2008) and their relationship is complex (Revell, Harmon & Collar, 2008; Wiens *et al.*, 2010). So far, there is no universal test for PNC (Wiens *et al.*, 2010) but a recent, promising approach is to compare the relative fit of different evolutionary models to the data, including the Brownian motion (BM) model and models of stasis or stabilising selection such as Ornstein-Uhlenbeck (OU) models (Losos, 2008; Kozak & Wiens, 2010; Wiens *et al.*, 2010). The BM model assumes that the correlation structure among trait values is proportional to the extent of shared ancestry for pairs of species (Felsenstein, 1973), and works reasonably well as a model of trait evolution (Beaulieu *et al.*, 2012). The OU models incorporate both selection and drift and are more general than pure drift models based on BM (Butler & King, 2004). They have been proved useful in a variety of contexts as they can capture the heterogeneity in the evolutionary process (Beaulieu *et al.*, 2012; Pennell *et al.*, 2015). In fact, several OU models with different degrees of complexity have been proposed, allowing to translate hypotheses regarding evolution in different selective regimes into explicit models (Hansen, 1997; Butler & King, 2004; Beaulieu *et al.*, 2012).

If traits have PS, it is often useful to apply phylogenetic methods (i.e. comparative methods), which have become a standard ecological tool in recent decades (Losos, 2008). When used in combination with trait-based approaches, phylogenetic analysis can strengthen hypothesis testing and generate new insights (de Bello *et al.*, 2015), as these methods account for the non-independence of species in statistical analyses due to shared evolutionary history (Felsenstein, 1985; Revell *et al.*, 2008). For instance, it can prove useful to consider phylogeny when assessing evolutionary mechanisms underlying present trait-environment patterns (de Bello *et al.*, 2015). However, non-phylogenetic analyses answer questions at different evolutionary scales (de Bello *et al.*, 2015) and are also informative, particularly when well-resolved phylogenies are not available for study taxa (Losos, 1999). Comparing the results of comparative and non-phylogenetic analyses can also inform about the existence of PNC and thus, as a rule of thumb, it might be useful to apply both techniques to trait data (de Bello *et al.*, 2015).

Elevational and longitudinal gradients (i.e. stream size or upstream-downstream) are well-studied in river ecosystems. Both spatial gradients covary and display variation in many environmental variables such as water temperature, stream flow, habitat features and productivity (Vannote *et al.*, 1980; Jones, Augspurger & Closs, 2017). They are also well known to shape fish communities, with changes in fish abundance, richness, species composition and traits (Cook *et al.*, 2004). Several studies have already analysed the trait–environment relationships of freshwater fishes along these spatial gradients (Kennedy, Peterson & Fausch, 2003; Pease *et al.*, 2012; Jones *et al.*, 2017). For instance, some studies revealed that species from uppermost reaches have more fusiform bodies, larger egg sizes and longer spawning seasons, but smaller body sizes and smaller clutches than species from lower river reaches (Winemiller & Rose, 1992; Pease *et al.*, 2012; Jones *et al.*, 2017). Similarly, Kennedy et al. (2003) also revealed a significant intraspecific trait variability across the elevation gradient. However, few studies have addressed this issue from a phylogenetic perspective (Comte *et al.*, 2014). Therefore, little is known about which traits or groups of traits (e.g., morphological, trophic, reproductive and habitat use traits) are the most conserved in inland fishes, and how their evolution correlates with their elevational and longitudinal distributions.

The Iberian Peninsula is well suited to study the evolutionary assembly of fish species and traits along spatial gradients because of its complex orography, diverse climate, and particular ichthyofauna. This region is a mountainous territory with a broad range of elevation rising from the sea level, over a large central plateau (Meseta Central) to the peaks of over 3000 m (Sabater *et al.*, 2009; Bayón & Vilà, 2019). Moreover, Iberian freshwaters are inhabited by 68 native fish species, of which 41 are endemic and they have been subjected to very prolonged isolation and speciation processes (Doadrio, 2001), but they are also inhabited by 32 alien species, some of them widespread throughout the planet.

Our main objectives were: (1) to compare the PS of several morphological, trophic, habitat use, and reproductive traits in inland fishes (i.e. species from freshwater ecosystems, including diadromous fishes and a few marine species that enter rivers), and (2) to test for correlated evolution of these traits with elevational and longitudinal distribution (i.e. if traits and species niche tend to evolve together) under three models of niche evolution (i.e. Brownian motion, Ornstein-Uhlenbeck stasis and Ornstein-Uhlenbeck trend models). We hypothesised that the majority of traits would show PS (Johnson & Stinchcombe, 2007) but its magnitude would vary among trait types (i.e. morphological, trophic, reproductive or habitat use). Specifically, we predicted that fish body size and other morphological traits would show higher PS than reproductive or habitat traits as in other taxonomic groups (Blomberg *et al.*, 2003; Kamilar & Cooper, 2013; Comte *et al.*, 2014). Finally, we hypothesised that fish traits would display correlated evolution with elevational and longitudinal gradients, since the functional trait composition of fish assemblages is known to change across the river continuum (Pease *et al.*, 2012).

7.2. Methods

7.2.1. Trait dataset and swimming performance estimation

We obtained 23 fish biological and ecological traits ('traits' hereafter, see Table S7.1) data of inland fishes present in the Iberian Peninsula from two recently published databases (Cano-Barbacil *et al.*, 2020; Cano-Barbacil *et al.*, 2020), which contain trait information for the 100 inland fish species present in this territory. In total, we analysed 30 species (12 native and 18 alien species) that had complete data on traits (Cano-Barbacil *et al.*, 2020). Of all traits considered, seven were described as continuous and 16 as binary variables. Traits considered were related to fish morphology (*n* = 3), trophic position (*n* = 3), reproduction (*n* = 6) and habitat use (*n* = 11) and were selected for their ecological relevance (i.e. features commonly used in trait-based approaches that are known to influence the reproductive success, individual survival and fitness; see Table S1 for further details). Specifically, species-specific critical swimming speed (U_{crit}), a measure of a species' swimming performance, was estimated from a recently compiled dataset of 196 experiments (Cano-Barbacil *et al.*, 2020) for all species considered in this work. U_{crit} is a standard experimental measure of prolonged swimming performance, which mediates fitness, survival and habitat selection of fish (Plaut, 2001; Cano-Barbacil *et al.*, 2020). We used random forests (RF) with the R-package 'party' (Hothorn *et al.*, 2017) to estimate a standard U_{crit} value for each species (see results in Figure S7.1) while accounting for effects of experimental setups (water temperature, time step interval between velocity increments, and individual fish total length). RF were built based on optimal hyperparameters calculated using the R-package 'mlr' (Bischl *et al.*, 2016), involving 550 trees with three variables randomly sampled at each split.

Finally, we calculated the relative mean elevational distribution (i.e. mean elevational distribution of each species divided by the maximum elevation of the Iberian Peninsula) and the mean longitudinal distribution (i.e. using Strahler's stream order; Strahler, 1957) for 28 out of the 30 inland fish species considered in this study (see also Figures 7.1 and S7.2, and Table S7.2). These means were calculated using the presence data available from the Instituto da Conservação da Natureza e das Florestas (Portugal) and the Global Biodiversity Information Facility (GBIF; GBIF.org, 2019a), with a grid resolution of 10 × 10 km UTMs (Universal Transverse Mercator, i.e. 100 km^2, *n* = 6142 total cells). The full ranges of elevation and stream orders studied were sampled fairly uniformly with respect to effort, as the majority of the occurrence data came from standardised samplings on which the fish atlases of Spain and Portugal are based (Doadrio, 2001; Ribeiro *et al.*, 2007; Doadrio *et al.*, 2011). Even though elevation is not a factor that directly determines fish distribution, it is considered a well suited proxy of important variables such as water flow velocity or temperature (see Figure S7.3) and has been previously used to evaluate and summarise inland fish distribution (Comte *et al.*, 2014). For our dataset, elevation was weakly, but significantly correlated with stream order (see Figure S7.4). Specifically, we used the relative mean elevational distribution because using the absolute mean elevational distribution could lead to incorrect conclusions if species occupy different relative niches in different geographic regions (e.g. headwater species of the Iberian Peninsula might be lowland species in other regions).

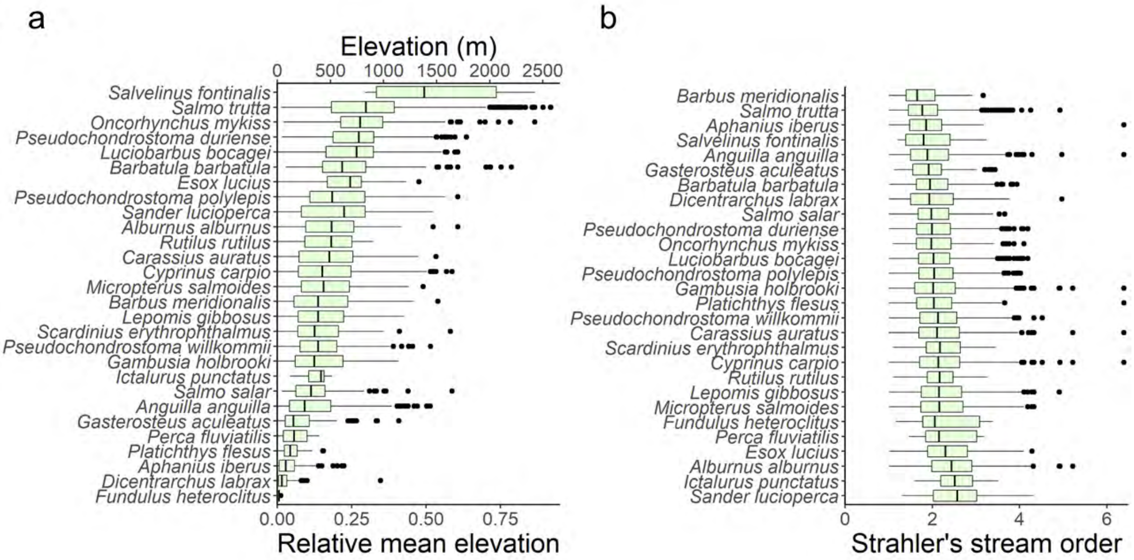


Figure 7.1. (a) Elevation and relative mean elevation, and (b) Strahler's stream order associated with the presences of 28 inland fish species in the Iberian Peninsula (see Table S7.2 for further details). Elevation and Strahler's stream order were calculated with a grid resolution of 10 × 10 km UTMs (Universal Transverse Mercator, i.e. 100 km^2, n = 6142 total cells). Relative mean elevation was calculated as the mean elevational distribution of each species divided by the maximum elevation of the Iberian Peninsula (3479 m).

7.2.2. Phylogenetic signal in species' traits and elevational distribution

For the comparative analyses, we first obtained the phylogenetic tree of the studied Iberian species from a recent phylogeny of ray-finned fishes (Rabosky *et al.*, 2018), using the function 'fishtree_phylogeny' of the R-package 'Fish Tree' (Chang *et al.*, 2019). We then estimated the PS of single traits using different, complementary indices (Münkemüller *et al.*, 2012). For continuous traits and elevational and longitudinal distribution, we used Pagel's λ (Pagel, 1999), Blomberg's K (Blomberg *et al.*, 2003) and Abouheif's C_{mean} (Abouheif, 1999) and their associated tests. Values of λ, K and C_{mean} with larger deviations from zero all indicate stronger relationships between species traits and phylogeny (Münkemüller *et al.*, 2012). Under a Brownian motion (BM) model, Pagel's λ and Blomberg's K are expected to be equal to 1, i.e. where trait evolution follows a random walk along the branches of the phylogeny (Münkemüller *et al.*, 2012) and species inherit their features from ancestors but randomly and slowly vary at a constant rate through time (Comte *et al.*, 2014). For both statistics, values of λ and K equal to 0 indicate that there is no PS in the studied trait, while values between 0 and 1 suggest some level of trait lability (Comte *et al.*, 2014), i.e. change of traits during evolution. The upper limit of Pagel's λ is close to one, while Blomberg's K can take higher values that indicate stronger trait similarity among related species than expected under BM (Münkemüller *et al.*, 2012). By contrast, Abouheif's C_{mean} is an autocorrelation index that is not based on any evolutionary model (Münkemüller *et al.*, 2012), with larger deviations from zero indicating stronger PS. We used these three measures of PS because they are complementary since their performance depends on the underlying evolutionary model, sample size and the possible existence of errors in the topology of the phylogeny (Münkemüller *et al.*, 2012). For proper interpretation, we tested whenever possible if these estimates of PS were significantly different from 0 (P_0) and from 1 (P_1).

To measure the PS of binary traits we calculated the D statistic (Fritz & Purvis, 2010). D ranges within the interval $(-\infty, \infty)$, with values lower than 1 indicating trait conservatism. To allow comparison with Blomberg's K statistic, D was transformed to $-D + 1$ (Goberna & Verdú, 2016), as an indicator of: (a) no significant signal ($-D + 1 \sim 0$); (b) traits more conserved than expected by chance but less than expected under BM ($0 < -D + 1 < 1$); (c) traits conserved as expected under BM ($-D + 1 \sim 1$); or (d) traits more conserved than expected under BM ($-D + 1 > 1$) (Fritz & Purvis, 2010). Pagel's λ and Blomberg's K were calculated with the R-package 'phytools' (Revell, 2012) and the C_{mean} statistic with 'adephylo' (Jombart, Balloux & Dray, 2010). Prior to all statistical analyses, continuous trait data were $\log_{10}$-transformed to comply with the assumptions of parametric tests. D statistic was calculated with the R-package 'caper' (Orme *et al.*, 2018). As results using K and λ were very similar, and K also informs about trait variation that is more similar than expected under BM and is easily comparable to $– D + 1$ when using binary traits, we used K value for further analyses of continuous traits.

To analyse the effects of trait measurement scale (i.e. continuous or binary) and trait type (i.e. morphological, trophic, reproductive and habitat use) on PS measure, we used a two-way ANOVA. In the ANOVA, we also calculated η^2, which is a measure of effect size based on the proportion of the total variance in the dependent variable that is associated with each individual factor or source of variation in the design (Richardson, 2011), and in the case of a single quantitative predictor is identical to the coefficient of determination r^2.

We used the Brownian motion (BM) model (Felsenstein, 1973) and two different Ornstein-Uhlenbeck (OU_{stasis} and OU_{trend}) models to evaluate how elevational and longitudinal distribution have evolved in fish. The OU_{stasis} model fits a random walk with a central tendency equal to the root value (Z_0) with an attraction strength proportional to the parameter α (Butler & King, 2004). The OU_{trend} model also includes an evolutionary trend, i.e. the optimum (Θ) is not the same as the root value (Z_0), with a single optimum for all species (Beaulieu *et al.*, 2012; Blomberg, Rathnayake & Moreau, 2020). We applied the function 'fitContinuous' of the package 'geiger' (Pennell *et al.*, 2014) to fit the BM model, and the 'OUwie' function of the package 'OUwie' for the OU models.

7.2.3. Trait correlation with elevation and tests of correlated evolution

In order to identify the main fish life-history strategies and to visualise possible correlations among fish traits, we performed a principal coordinate analysis (PCoA), using the 'wcmdscale' function of the 'vegan' R-package (Oksanen *et al.*, 2017) and log-transformation of continuous traits. To analyse if these ordination axes were related to elevation and stream order, we fitted two smooth surfaces using the 'ordisurf' function of 'vegan'. To test for PS of the set of traits, we also computed Pagel's λ, Blomberg's K and Abouheif's C_{mean} of these two axes.

To test for the relationship of species traits (as response variables) with mean elevation and stream order (as predictors), we performed phylogenetic generalised least squares (PGLS) using the 'pgls' function for continuous traits, and the 'brunch' function for binary traits, both in the 'caper' package (Orme *et al.*, 2018). The maximum likelihood estimate of λ was incorporated as a parameter in the PGLS model for continuous traits, thus controlling for phylogenetic dependence in the data in a manner that is optimal for the data set (Freckleton *et al.*, 2002). We also used generalised linear models (GLMs) without considering phylogenetic information (de Bello *et al.*, 2015), with gamma and binomial distributions for continuous and binary traits, respectively. In order to analyse and evaluate differences between non-phylogenetically and phylogenetically corrected analyses, we reconstructed the ancestral state of those significant traits using the 'contMap' function in the 'phytools' R-package (Revell, 2012, 2013).

Finally, to test for correlated evolution among fish traits, we performed PGLS using the 'pgls' function for continuous traits and binary PGLMM (phylogenetic generalised linear mixed model) with binomial error structure using the 'binaryPGLMM' function of the 'ape' package (Paradis & Schliep, 2019) for binary traits (Gilbert *et al.*, 2018). For the non-phylogenetic analyses, we also used Pearson and Spearman correlation analyses, for continuous and binary traits, respectively. All statistical analyses and modelling tasks were performed with the software R, version 3.5.1 (R Core Team, 2020).

7.3. Results

7.3.1. Phylogenetic signal in species' traits and elevational and longitudinal distribution

We found statistically significant PS in relative mean elevational distribution using Pagel's λ (λ = 0.904, P_0 = 0.004, P_1 = 0.115), Blomberg's K (K = 0.538, P_0 = 0.035, P_1 = 0.123) and Abouheif's C_{mean} (C_{mean} = 0.343, P_0 = 0.007). Moreover, results of evolutionary models on elevational distribution of inland fishes revealed that the OU_{stasis} model was preferable, with an AIC_c weight of 0.475 (Table 7.1). However, the BM model was just slightly worse, showing an AIC_c weight of 0.405, whereas the OU_{trend} model was less likely. The best model (i.e. OU_{stasis}) suggested that the current elevational distribution of fish species tended to resemble to that of the common ancestor, showing a stabilising selection around a fixed elevation root value (Z_0 = 331.7 m). The attraction strength to this root value, i.e. the strength of niche selection, was proportional to α = 0.007. The rate of divergence, i.e. the rate of stochastic evolution away from the root value, was proportional to σ^2 = 0.003. By contrast, we did not find statistically significant PS in longitudinal distribution using Pagel's λ ($\lambda < 0.001$, $P_0 = 1.000$, $P_1 < 0.001$), Blomberg's K ($K = 0.337$, P_0 = 0.064, P_1 = 0.006) and Abouheif's C_{mean} ($C_{\text{mean}} = 0.071$, $P_0 = 0.266$). In addition, we found that the $\text{OU}_{\text{stasis}}$ evolutionary model was preferable for longitudinal distribution, with an AIC_c weight of 0.786 (Table 7.1). This suggested that the evolutionary pattern of longitudinal distribution was similar compared with that of elevational distribution. It showed stabilising selection around fixed stream order root value (Z_0 = 2.154) with an attraction strength proportional to α = 0.025, and a rate of divergence $\sigma^2 = 7.4 \cdot 10^{-5}$.

Table 7.1. Results of three evolutionary models of niche evolution (Brownian motion, Ornstein-Uhlenbeck stasis and Ornstein-Uhlenbeck trend models) to understand how elevational and longitudinal distributions have evolved in Iberian inland fish. The adjusted Akaike information criterion (AIC_c) and model weight are shown. σ^2 = rate of divergence (i.e. the rate at which taxa diverge from each other through time; it is unit dependent and cannot be compared); Z_0 = root value (i.e. starting value of the common ancestor of the species studied); α = attraction strength (i.e. strength of trait selection); θ = optimum (i.e. evolutionary optimal value); '-' indicate parameters not considered by the model.

Variable	**Model**	**AIC_c**	**AIC_c weight**	**σ^2**	**Z_0**	**α**	**θ**
Relative mean elevation	Brownian motion	23.362	0.405	0.002	353.0	-	-
	$\text{OU}_{\text{stasis}}$	23.044	0.475	0.003	331.7	0.007	-
	OU_{trend}	25.783	0.120	0.003	8.8	0.007	1033.5
Strahler's stream order	Brownian motion	-91.695	0.015	$2.7 \cdot 10^{-5}$	2.146	-	-
	$\text{OU}_{\text{stasis}}$	-99.662	0.786	$7.4 \cdot 10^{-5}$	2.154	0.025	-
	OU_{trend}	-96.922	0.200	$7.4 \cdot 10^{-5}$	1.007	0.025	2.168

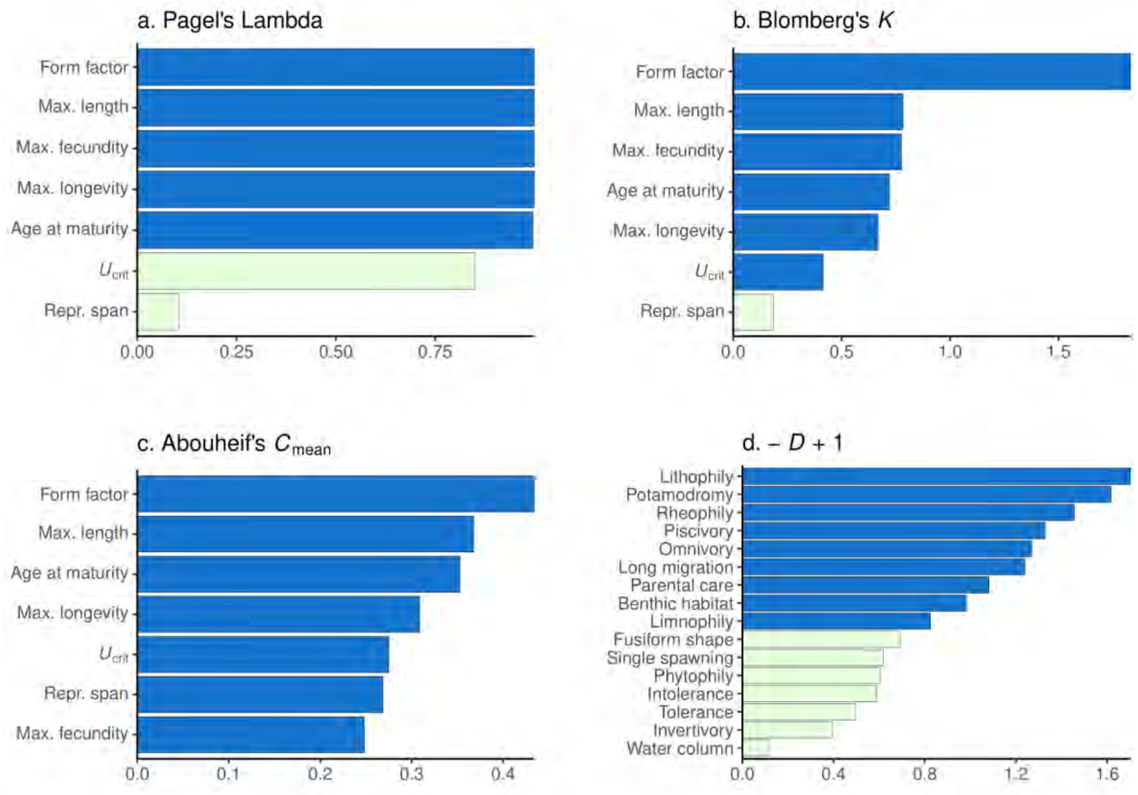


Figure 7.2. Phylogenetic signal in Iberian fish species traits using (a) Pagel's λ, (b) Blomberg's K, and (c) Abouheif's C_{mean} for continuous traits; and (d) $-D + 1$ for binary traits. Darker color indicates traits with significant phylogenetic signal (i.e. $P_0 < 0.05$).

Continuous species traits showed different levels of PS, with the three indices used yielding highly consistent results (Figures 7.2a, b and c). We also found clear PS in 5 and 6 out of 7 continuous traits using Pagel's λ and Blomberg's K, respectively (Figure 2a and b). By contrast, 'reproductive span' was not clearly related to phylogeny, as indicated by both measures ($\lambda = 0.105$, $P_0 = 0.764$; $K = 0.185$, $P_0 = 0.465$). Critical swimming speed (U_{crit}) showed clear PS using Blomberg's K ($K = 0.415$, $P_0 = 0.006$) but not with Pagel's λ ($\lambda = 0.851$, $P_0 = 0.172$). Blomberg's K varied widely across traits, most of them showing values between 0 and 1. Only 'form factor' had $K > 1$, indicating that this morphological trait is more phylogenetically conserved than expected under BM. Using Abouheif's C_{mean}, all seven traits exhibited significant levels of PS (Figure 7.2c). For example, species within salmonids, perciforms (e.g. *Micropterus salmoides*, *Sander lucioperca* or *Dicentrarchus labrax*), and cypriniforms (*Cyprinus carpio* or *Abramis brama*) tended to show larger body sizes (i.e. maximum length), while the cyprinodontiforms (e.g. *Gambusia holbrooki* and *Aphanius iberus*) showed small body sizes (Figure 7.3). A similar pattern can be observed for form factor and fecundity, whereas this is less the case for reproductive span and U_{crit}, which are more labile traits.

Of the 16 considered binary traits, 9 showed significant PS (Figure 7.2d). Seven traits (i.e. lithophily, potadromy, rheophily, piscivory, omnivory, long migration and parental care) even had –*D* + 1 > 1, indicating that these traits are more conserved than expected under BM. Figure 7.4 shows how traits with a high PS have been conserved throughout the phylogeny. For instance, lithophilic spawning has been conserved in all salmonid species, all species of the genus *Pseudochondrostoma* and in two closely-related cyprinid species (*Luciobarbus bocagei* and *Barbus meridionalis*); and potamodromy has been conserved only in some cypriniform species. However, PS did not clearly depend on trait type ($\eta^2 = 0.077$, $F_{3,\ 16} = 0.523$, $P = 0.673$), measurement scale ($\eta^2 = 0.016$, $F_{1,\ 16} = 0.329$, $P = 0.574$), or their interaction ($\eta^2 = 0.106$, $F_{1,\ 16} = 1.075$, $P = 0.365$) (Figure S7.5).

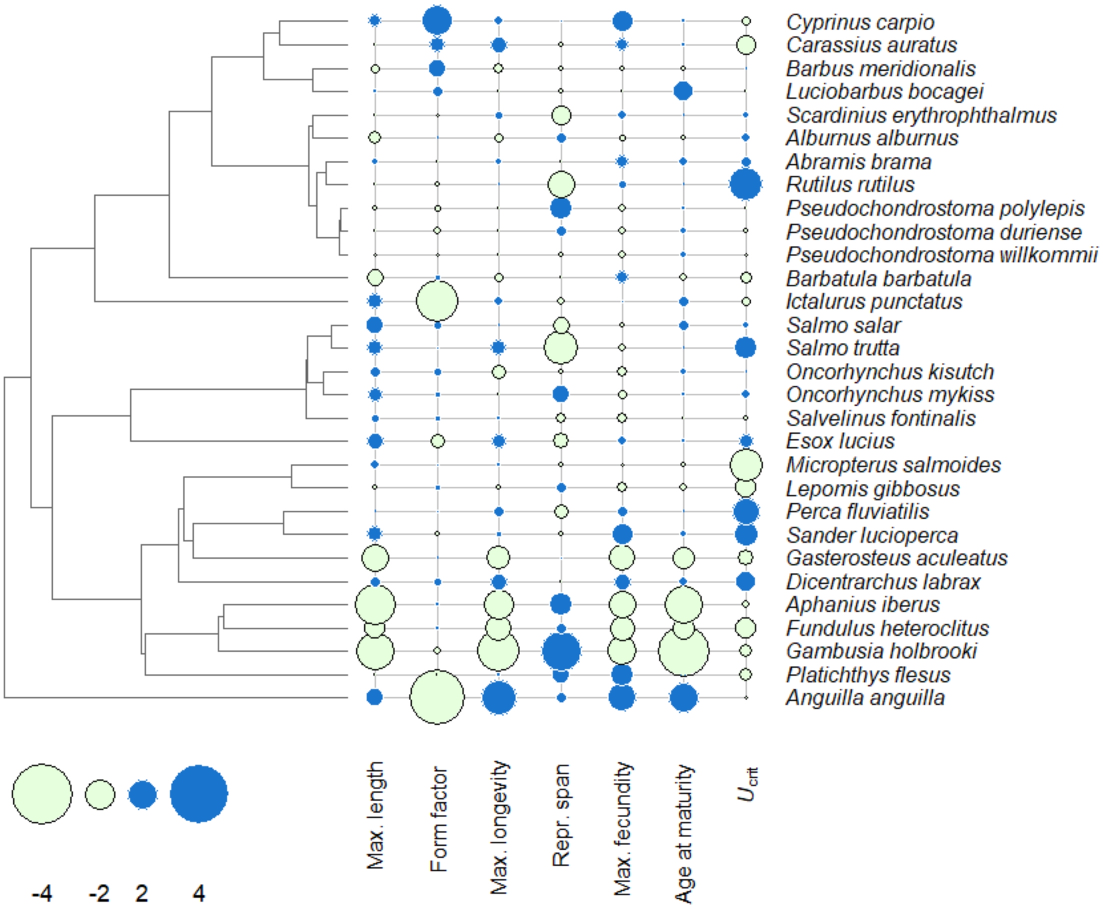


Figure 7.3. Phylogenetic tree annotated with a matrix of continuous traits (circle size represents the standard deviate) associated with each species.

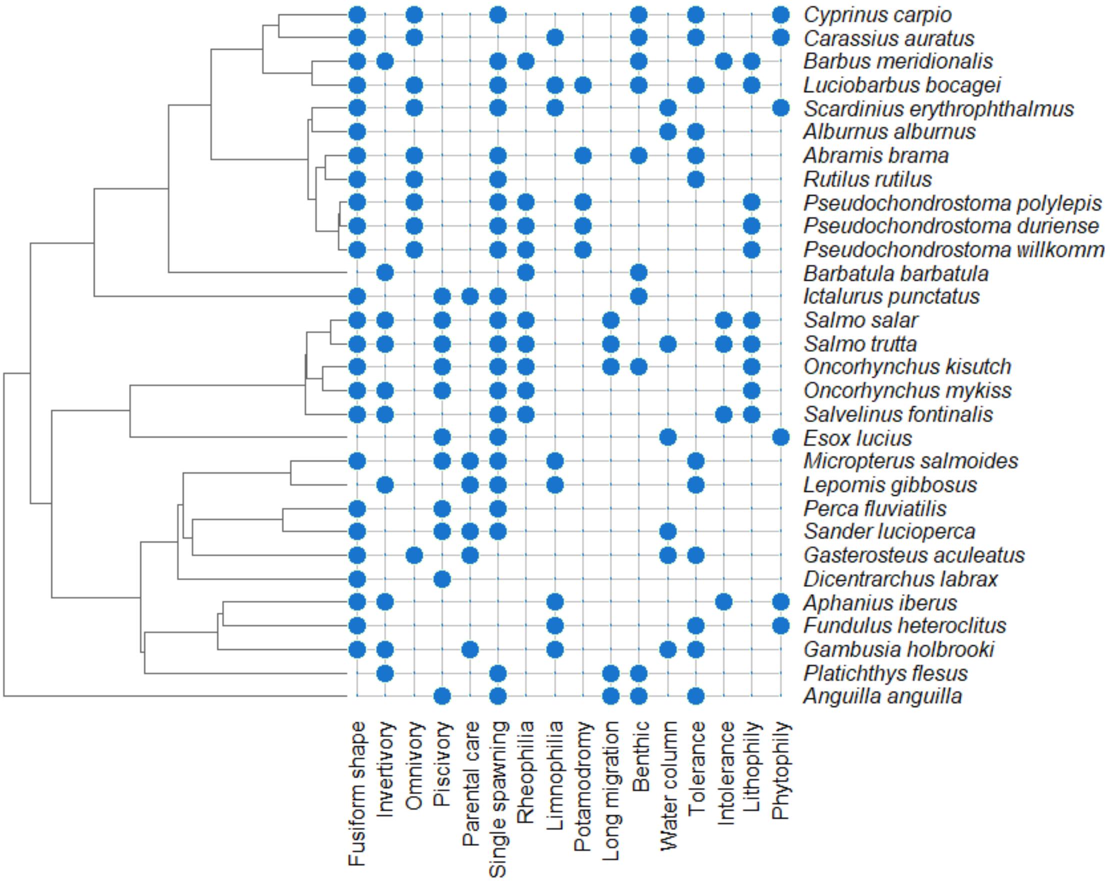


Figure 7.4. Phylogenetic tree annotated with a matrix of binary traits associated with each species.

7.3.2. Trait correlation with elevation and tests of correlated evolution

The PCoA results showed that Iberian inland fishes vary in their trophic, morphological, habitat, and reproductive traits (Figure 7.5). The fitting of the smooth surface showed that changes in the fish traits were related to elevation (Figure 7.5a; $R^2_{adj} = 0.228$, $F_{3.11,\,9} = 0.887$, $P = 0.048$) and stream order (Figure 7.5b; $R^2_{adj} = 0.379$, $F_{3.98,\,9} = 0.887$, $P = 0.005$). The first principal axis summarised approximately 26.0% of overall variation in the trait dataset, and described an ecological and life-history gradient. Species with negative scores on the first PCoA axis were rheophilic, lithophilic, piscivorous and large-sized species often migratory and with high swimming performance (i.e. higher U_{crit} values) and fewer spawning bouts (mostly salmoniforms and other upland species). By contrast, species with positive scores were lowland, limnophilic, phytophilic, tolerant species with large reproductive span and high parental care (Figure 7.5c). The second axis (15.8% of explained variation) distinguished among lowland species: with negative scores, large-sized, omnivorous species with late maturity and high fecundity and longevity (mostly lowland cyprinids); with positive scores, small, short-lived species with the opposite suite of traits (e.g. cyprinodontiforms) (Figure 7.5c). Specifically, cypriniforms showed more diverse life-history strategies than other fish orders such as salmoniforms or cyprinodontiforms. Some cypriniforms are rheophilic, lithophilic and with high swimming performance (e.g. *Barbus meridionalis*), whereas other species have high fecundity and water quality tolerance, and a phytophilic reproduction (e.g. *C. carpio*) (Figures 7.5 and S7.6).

The first ($\lambda = 0.999$, $P_0 < 0.001$; $K = 0.698$, $P_0 = 0.001$; $C_{\mathrm{mean}} = 0.517$, $P_0 = 0.001$) and the second axes ($\lambda = 0.552$, $P_0 = 0.015$; $K = 0.408$, $P_0 = 0.007$; $C_{\mathrm{mean}} = 0.354$, $P_0 = 0.003$) of the PCoA also showed significant PS for the three statistics used, indicating that closely related species tended to exhibit a similar suite of fish life-history traits (Figure S7.7).

Using PGLS, we observed a significant positive relationship of parental care with both elevation and stream order (Table 7.2). However, in other PGLS models, a large amount of the variation of elevational distribution was explained by traits (e.g. potamodromy, benthic and lithophily). Results obtained without considering phylogenetic information (i.e. GLMs) indicated several significant present-day relationships. For instance, we found a negative correlation between stream order and invertivory. Similarly, rheophily was positively related with elevation but negatively with stream order, whereas lithophily was also positively associated with elevation (Table 7.2). Ancestral state reconstruction of these four traits showed that their evolution followed a pattern of multiple independent character gains (and losses). We found five gains of the invertivory trait with one subsequent reversal (Figure S7.8a), three gains of parental with one subsequent reversal (Figure S7.8b), four gains of rheophily (Figure S7.8c) and three gains of lithophily (Figure S7.8d).

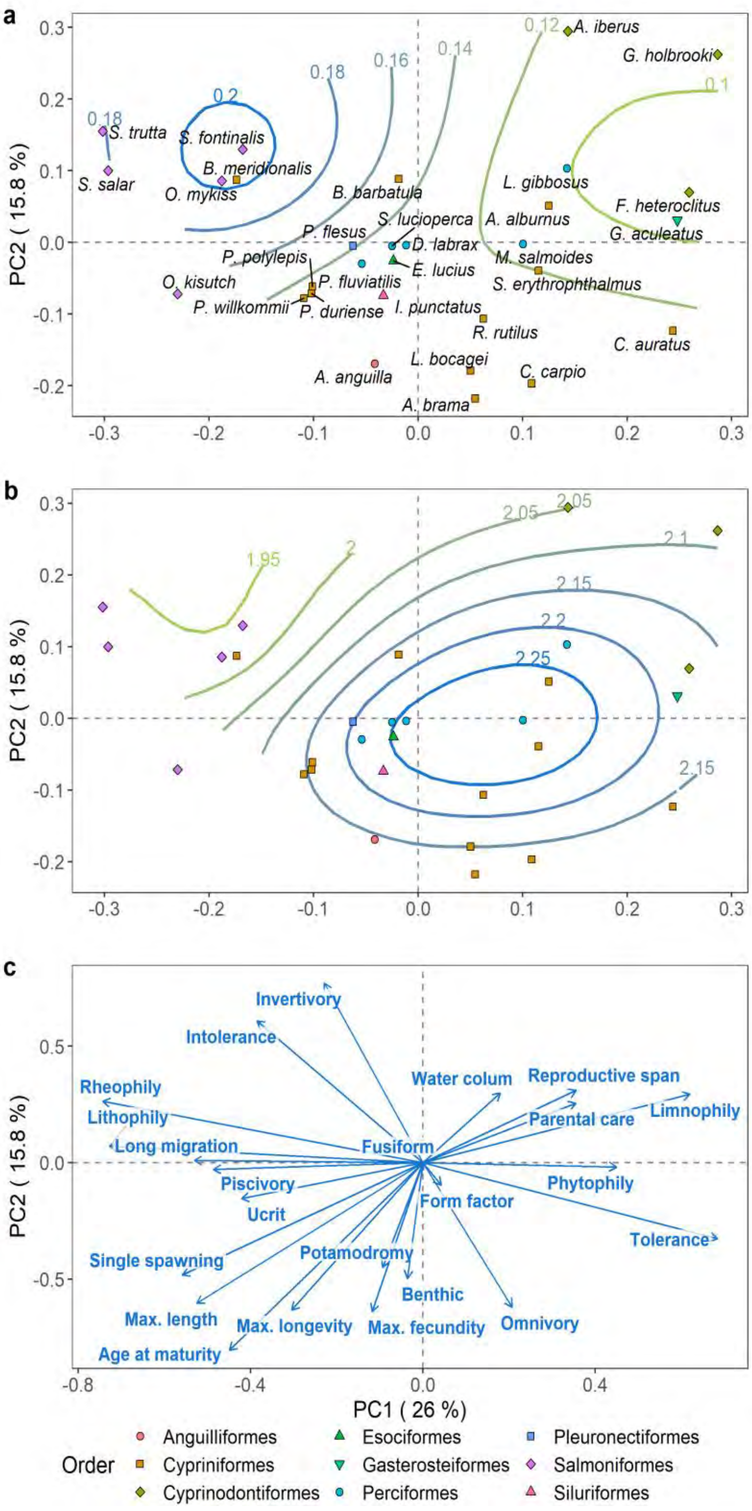


Figure 7.5. Principal coordinate analysis of the 23 biological and ecological traits for the fish species of the Iberian Peninsula. (a) Species scores with smooth response curves of relative mean elevation overlaid. Blue colors of the isopleths represent high elevation while green colors correspond to low elevations. (b) Species scores with smooth response curves of mean Strahler's stream order overlaid. Blue colors of the isopleths represent high stream orders while green colors correspond to low stream orders. (c) Eigenvector plot of the traits. The first axis distinguishes headwater species, with negative scores (i.e. rheophilic, lithophilic, piscivorous and large-sized species with high swimming performance) from lowland species, with positive scores (i.e. limnophilic, phytophilic and tolerant species). The second axis distinguishes among lowland species: with negative scores, large-sized, omnivorous species with late maturity and high fecundity and longevity; with positive scores, small, short-lived species with the opposite suite of traits.

Table 7.2. Relationship of fish traits with relative mean elevation and mean Strahler's stream order. For phylogenetically corrected analyses, we used phylogenetic generalised least squares (PGLS) for continuous traits and the 'brunch' function for binary traits, both from the 'caper' package (Orme *et al.*, 2018). For non-phylogenetically corrected analyses, we used generalised linear models (GLM) with gamma and binomial distributions for continuous and binary traits, respectively. Partial r^2 for each predictor and their *P* values expressed as asterisks (* ≤ 0.05; . ≤ 0.1), and R^2 of the full model are shown. RME = relative mean elevation; SSO = Strahler's stream order. Note that predictors and continuous traits were log-transformed for the PGLS.

Trait (and TYPE)	**Measurement scale**	**Phylogenetic generalised least squares**			**Generalised linear models**		
		r^2_{RME}	r^2_{SSO}	R^2	r^2_{RME}	r^2_{SSO}	R^2
MORPHOLOGICAL							
Max. total length (cm)	Continuous	0.007	0.112 .	0.112	0.057	0.013	0.070
Form factor	Continuous	0.089	0.003	0.089	0.001	0.051	0.052
Fusiform shape	Binary	0.147	0.068	0.147	0.004	$4.5\cdot10^{-5}$	0.004
TROPHIC							
Invertivory	Binary	0.001	0.641 .	0.650	0.010	**0.299** *	0.309
Omnivory	Binary	0.015	0.004	0.021	0.003	$2.8\cdot10^{-3}$	0.003
Piscivory	Binary	0.458	0.301	0.616	$6.7\cdot10^{-6}$	0.029	0.029
REPRODUCTIVE							
Max. longevity (years)	Continuous	0.003	$7.3\cdot10^{-5}$	0.004	0.001	0.003	0.004
Repr. span (months)	Continuous	0.065	0.002	0.069	0.066	0.010	0.076
Max. fecundity (no. eggs)	Continuous	$2.1\cdot10^{-4}$	0.049	0.053	$1.5\cdot10^{-4}$	0.063	0.063
Age at maturity (years)	Continuous	0.006	0.107 .	0.135	0.008	$1.0\cdot10^{-5}$	0.008
Parental care	Binary	**0.974** *	**0.966** *	0.991	0.011	0.130 .	0.141
Single spawning	Binary	0.117	0.106	0.141	0.147 .	0.002	0.149
HABITAT USE							
U_{crit} (cm/s)	Continuous	0.000	0.043	0.005	0.017	0.010	0.027
Rheophily	Binary	0.309	0.842 .	0.849	**0.252** *	**0.280** *	0.532
Limnophily	Binary	0.145	0.060	0.169	0.045	0.003	0.048
Potamodromy	Binary	0.400	0.119	0.541	0.032	0.009	0.041
Long migration	Binary	0.470	0.768	0.786	0.029	0.142 .	0.171
Benthic	Binary	0.624	0.325	0.635	0.005	0.011	0.017
Water column	Binary	0.361	0.019	0.366	0.014	0.046	0.060
Tolerance	Binary	$3.5\cdot10^{-4}$	0.422 .	0.425	0.023	0.023	0.046
Intolerance	Binary	0.131	0.863 .	0.863	0.005	0.678 .	0.683
Lithophily	Binary	0.676	0.226	0.920	**0.263** *	0.253 .	0.516
Phytophily	Binary	0.338	$5.0\cdot10^{-4}$	0.338	0.052	0.021	0.073

Moreover, using PGLS we found that several continuous traits were positively inter-correlated (e.g., maximum total length, maximum longevity, maximum fecundity and age at maturity; Table S7.3). Reproductive span was negatively correlated with maximum longevity, age at maturity and U_{crit} (Table S7.3). For binary traits, we found positive correlations between 'intolerance' and 'invertivory', 'rheophily' and 'lithophily', and 'limnophily' and 'phytophily', and a negative correlation between 'lithophily and 'tolerance' (Table S7.4). Using conventional correlation analyses, we found similar correlation patterns among fish traits (Figure S7.9 and S7.10).

7.4. Discussion

7.4.1. Phylogenetic signal in species' traits and elevational and longitudinal distribution

In our study we measured the PS of several morphological, trophic, habitat use, and reproductive traits in inland fishes and tested for correlated evolution of these traits with elevational distribution. In agreement with our first hypothesis, most of the studied traits showed significant PS. Form factor and maximum length were the continuous traits with the highest PS detected. This is in accordance with previous studies in fish and other species groups that also showed that morphological traits often have strong PS (Blomberg *et al.*, 2003; Kamilar & Cooper, 2013; Sternberg & Kennard, 2014). Although PS was quite variable, we did not find clear differences among types of traits, in contrast to previous works (Blomberg *et al.*, 2003; Kamilar & Cooper, 2013). These potential discrepancies might be due to different reasons. For instance, because of smaller differences in PS among trait types in freshwater fishes compared with other taxa, or because of low statistical power due to high PS variability combined with lesser data availability and lower diversity of traits in freshwater fishes. Regarding trophic traits, we found significant PS in two (omnivory and piscivory) of the three traits considered. Previous phylogenetic findings regarding trophic traits were rather inconsistent. For instance, Comte *et al.* (2014) found that trophic position showed strong phylogenetic clustering in 32 European inland fishes, while Wagner *et al.* (2009) found an opposite pattern for 32 Tanganyikan cichlid species. The latter could be due to the rapid radiation observed in cichlid species accompanied by trophic diversification (Rüber *et al.*, 1999). With respect to other traits, for example, we found that sensitivity to degradation of water quality and habitat (i.e. tolerance and intolerance traits) did not show clear PS. Similarly, a previous study on fish sensitivity to toxicants revealed that the PS was only significant for 24% of the chemicals analysed (Hylton *et al.*, 2018). Finally, some reproductive traits such as parental care appeared to show also strong PS, that is in agreement with previous findings (Sternberg & Kennard, 2014).

Results of our evolutionary analysis of elevational and longitudinal distribution revealed the OU_{stasis} as the best performing model. However, in the case of elevational distribution, the parameter α, which measures the strength of selection in OU models, was very close to zero, suggesting that the deterministic part of the OU model was negligible and that the model might be simplified to the BM model of pure drift (Butler & King, 2004). Thus, we can conclude that elevational and longitudinal distributions showed little evidence for directional trends of evolution and that the ecological niche of fish species tends to resemble to those of ancestors. Similarly, previous studies showed that the evolution of other traits such as trophic position or body size in diadromous and non-diadromous Clupeiformes fit much better to OU models than BM models (Bloom, Burns & Schriever, 2018), as OU models can capture the heterogeneity in the evolutionary process and reflect the variability of the trait value among species (Münkemüller *et al.*, 2015; Pennell *et al.*, 2015).

Evaluating trait lability and the relationship of traits with the environment is essential for managing biodiversity and minimising further species losses. Our results and further work could be helpful to understand the possible responses of inland fishes to environmental change. This study reinforces previous findings suggesting that in a scenario of ongoing climate change, the consequences on the inland ichthyofauna could be detrimental, especially in freshwater fishes with limited dispersal capacity (Markovic *et al.*, 2014). Additionally, our results suggest that the majority of traits studied and the elevational niche could not be able to evolve as fast as the changes of the environment, as revealed by the strong PS. Although there is some evidence of local adaptation to temperature changes in fishes (Jensen *et al.*, 2008), our results suggest that fish species tend to retain their ancestral niche characteristics. Hence, those species inhabiting closer to their thermal limits would be likely to face increased extinction risk (Comte *et al.*, 2014). Accordingly, Markovic *et al.* (2014), for example, suggested that eight European fish species are predicted to experience total range loss under future climate change.

7.4.2. Trait correlation with elevation and tests of correlated evolution

Our results also indicate that several traits show correlated evolution, i.e. pairs of traits tend to evolve together because of processes such as natural selection or mutation. In addition, we found that the variation in some fish traits was correlated to their elevational and longitudinal distributions, especially when using non-phylogenetic methods. This suggests suites of traits that covary along the elevational and longitudinal river gradient (Vannote *et al.*, 1980) and correlated evolution of traits less related to this environmental gradient. Some relationships are rather unsurprising and likely related to well-known co-occurring characteristics of riverine ecosystems (i.e. higher flow velocities are typically found in high elevation gravel-bed streams, while vegetated areas are more often associated with lowland lentic habitats). Thus, species inhabiting upstream stretches are typically rheophilic, lithophilic and intolerant, whereas those from lower reaches are more frequently limnophilic, phytophilic and tolerant (Belliard, Berrebi Dit Thomas & Monnier, 1999). Specifically, GLMs revealed positive relationships between elevation and traits such as rheophily and lithophily, and negative relationships between stream order and invertivory and rheophily. However, the relationship of these traits with elevation and stream order became non-significant after accounting for phylogenetic relatedness. This disagreement observed between phylogenetic and non-phylogenetic methods could result from small sample sizes, which might be insufficient to reject the null hypothesis after correcting for phylogenetic relatedness, as evidenced by the ancestral state reconstructions (i.e. few transitions between character states). However, it might also be due to the absence of an evolutionary relationship between elevation and stream order and these fish traits. In fact, we were not able to distinguish between both causes and, therefore, these results should be taken with due caution.

In agreement with previous studies (see Blanck & Lamouroux, 2007; Barneche *et al.*, 2018), our results also showed evolutionary correlations among several reproductive traits (e.g. maximum longevity and fecundity, and age at maturity) and fish maximum length. For instance, PCoA revealed that salmoniform species showed large size, long longevity and late maturity, which corresponds well to the periodic life-history strategy as defined by Winemiller and Rose (1992), while cyprinodontiforms species have opportunistic traits such as small body size, early sexual maturation or continuous reproduction (Winemiller & Rose, 1992; Vila-Gispert *et al.*, 2005). The periodic strategy seems to be dominant in temperate and tropical lotic ecosystems, while the opportunistic strategy is more common in productive lowland habitats subjected to disturbances such as intermittent streams, ephemeral pools or salt marshes (Winemiller, 2005). Cypriniforms are the most diverse order of the fish species studied, showing different strategies and a considerable morphological, physiological and swimming performance diversity as seen in previous studies (Howes, 1991; Cano-Barbacil *et al.*, 2020).

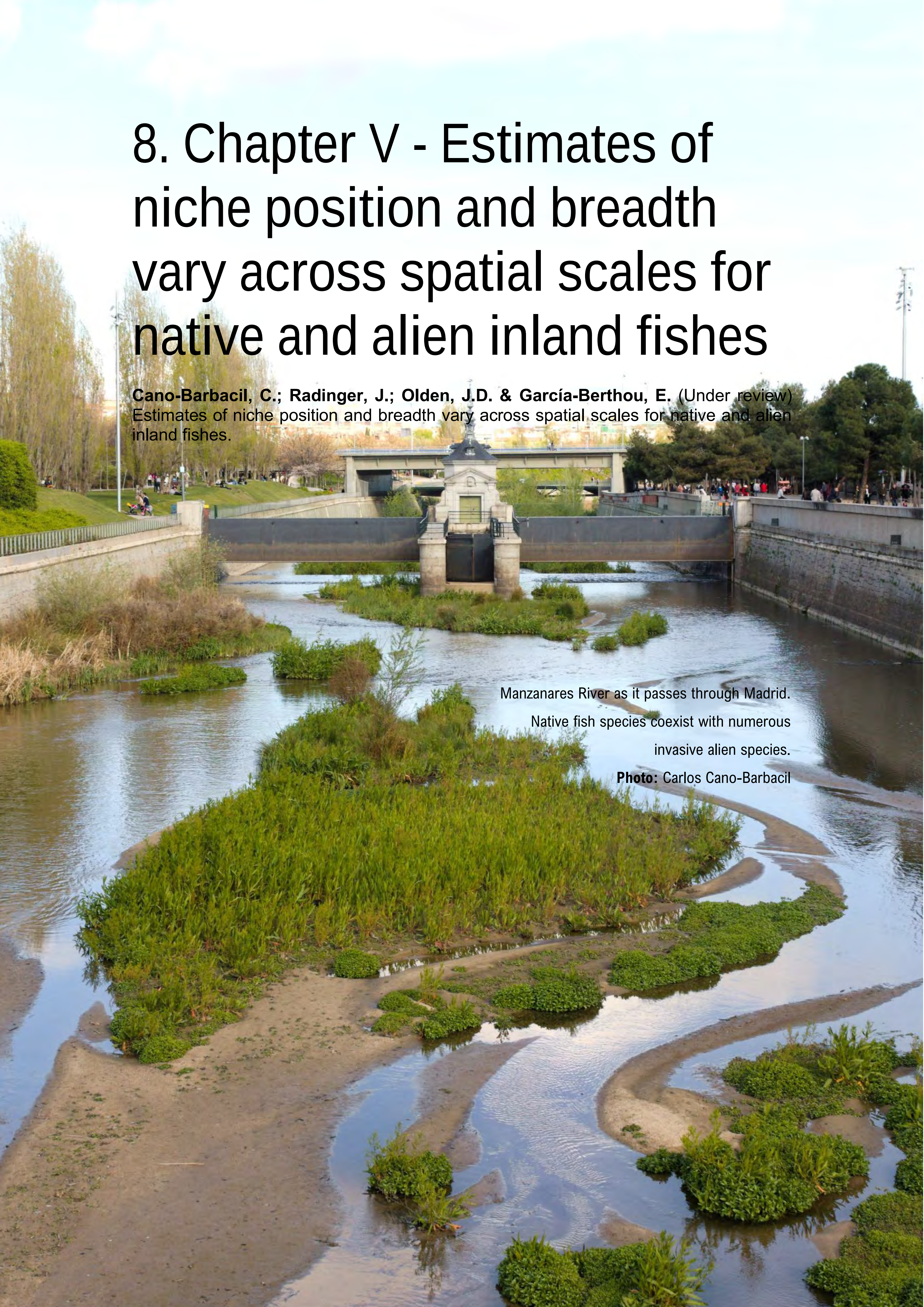
As already explained, we consider the Iberian Peninsula as a good case study to evaluate the evolutionary process of fish traits along spatial gradients, due to strong elevational and climatic gradients and a particular fauna. Our study area is representative of rivers with relatively low fish richness but with many threatened endemisms and invasive species. Our results constitute a solid base on which to develop future studies, showing an overview of the fish trait lability and its evolutionary relationship with elevational and longitudinal distribution. However, we note that there are some limitations that might have affected our results, related to both methodological issues (e.g. small sample size due to low trait data availability of Iberian species) and uncertainties associated with trait data (e.g. low reliability of some categorical traits and lack of information on intraspecific variability; Cano-Barbacil *et al.*, 2020) as already discussed.

7.5. Conclusions

Overall, we found significant phylogenetic signal for 65% of the studied traits but no clear differences between continuous and binary, or among morphological, trophic, reproductive and habitat use traits. Evolutionary models revealed that elevational and longitudinal distributions showed little evidence for directional trends of evolution, and thus that the ecological niche tends to resemble that of the common ancestor. Phylogenetic methods showed that several traits such as maximum length and some reproductive traits are inter-correlated, reflecting that those traits have evolved together. We also found a significant positive relationship of parental care with elevation and stream order, using PGLS. However, we found a positive relationship between elevation and traits like rheophily and lithophily, and a negative relationship between stream order and invertivory and rheophily when using non-phylogenetic methods. In sum, our study suggests that the well-known evolutionary relationship among fish species traits and distribution, and the strong phylogenetic signal observed for some features could have important implications for adaptation to global change, since many species would not be able to evolve and adapt to the new environmental conditions.

8. Chapter V - Estimates of niche position and breadth vary across spatial scales for native and alien inland fishes

Cano-Barbacil, C.; Radinger, J.; Olden, J.D. & García-Berthou, E. (Under review) Estimates of niche position and breadth vary across spatial scales for native and alien inland fishes.



Manzanares River as it passes through Madrid. Native fish species coexist with numerous invasive alien species. **Photo:** Carlos Cano-Barbacil

Chapter summary

The niche concept, and particularly the position and breath of a species' niche are crucial to understand species distributions and their ecological and evolutionary responses to natural and anthropogenic changes to the environment. Macroecological inference is substantially affected by the selection of a certain geographical extent. This is particularly relevant for understanding alien species introduction success and associated species' niche characteristics which can be estimated either from their invaded region, their native region or overall. However, surprisingly little is known how the estimation of species niche breath and position, especially those of non-native species, is affected by the geographical extent under consideration. Here we estimate and compare climatic and longitudinal niche metrics of native and alien Iberian inland fishes using outlying mean index (OMI) for different geographical extents spanning from the regional to the global scale. Furthermore, we investigate how the introduction date of alien species affects the niche characterisation at the regional invaded scale.

Niche metrics largely differed depending on the considered geographical extent as well as between species which are native or alien to the study region, with most alien fishes showing greater environmental tolerance. However, differences in climatic niche position between native and alien species observed at a global scale vanished at a regional scale. The niche breadth of widely distributed alien species was highly underestimated when only considering the invaded region, and further governed by species' introduction date to the invaded area. Although river longitudinal and climatic niche position were correlated, our results suggest the existence of two independent ecological niche axes, reflecting a differential response to contrasting environmental factors. Therefore, estimating niches of freshwater species, especially of alien invaders must build on a carefully selected geographical extent that is tailored to the objective of the study and the ecology of the species involved. We suggest that using also global data, not only those comprising a specific (e.g. invaded) region, will improve the estimation of niche characteristics of widely distributed organisms, particularly regarding the climatic niche, or to evaluate the invasive potential of a species.

Keywords: biological invasions, freshwater fish, Iberian Peninsula, invasive species, niche breadth, niche marginality, niche position, outlying mean index, river ecosystem

8.1. Overview

The ecological niche is considered fundamental in ecology, evolution, and conservation biology. It is used to understand the distribution, abundance and resource use of organisms, and the response of species to natural and anthropogenic environmental change (Elton, 1927; Devictor *et al.*, 2010; Sexton *et al.*, 2017; Carscadden *et al.*, 2020). At geographical scales, the Grinnellian niche (hereafter 'species niche') describes the totality of environmental requirements that allow a species to exist and successfully reproduce (Chase & Leibold, 2003; Anderson *et al.*, 2011). More specifically, the niche can be defined as the *n*-dimensional set of abiotic and biotic conditions under which a species or population can maintain non-negative growth rates without immigration (Carscadden *et al.*, 2020).

Presence-absence or abundance are commonly used to estimate properties or characteristics of species niches (Pearman *et al.*, 2008; Carscadden *et al.*, 2020). Characterisation of the niche is possible using a suite of different metrics, such as the position, optimum, or breadth of environmental conditions that influence where a species occurs. For example, niche position is defined as the mean environmental condition across all areas occupied by a species (Roughgarden, 1974; Carscadden *et al.*, 2020) and can inform about niche marginality by comparing the mean use with the available conditions in a given region. The niche optimum describes the conditions where population growth is maximised, however past research has demonstrated that it is generally difficult to distinguish from the niche position. Niche breadth can be defined as the range of conditions included within the niche and can be more informative than point estimates such as niche position or optimum, since it more holistically represents the needs and tolerances of species (Dolédec *et al.*, 2000; Carscadden *et al.*, 2020). Recent decades have witnessed an array of different techniques to quantify the dimensions of species' niches (Hirzel & Le Lay, 2008), including gradient analysis (Austin, 1987; Ter Braak & Prentice, 1988), species distribution (or ecological niche) models (Guisan & Zimmermann, 2000; Guisan & Thuiller, 2005; Peterson, 2006; Elith & Leathwick, 2009), or the outlying mean index (OMI; Dolédec *et al.*, 2000).

Robust quantification of species niches is important to enhance our understanding, and prediction, of native species responds to environmental change including e.g. climate change and habitat destruction (Wiens & Graham, 2005; Karasiewicz, Dolédec & Lefebvre, 2017), and the potential of species to invade and have impacts in new regions (Larson, Olden & Usio, 2010; Korsu *et al.*, 2012). For example, previous studies found that generalist species with wider niche breadths (i.e. tolerant organisms that can maintain fitness over a broader range of abiotic conditions) are more likely to be invasive (Higgins & Richardson, 2014; Granot, Shenkar & Belmaker, 2017). However, a few studies pointed that some invasive species had a highly marginal habitat niche position compared to native species (Korsu *et al.*, 2012), reflecting more specialised habitat requirements. A recent study revealed also that the successful establishment of alien species is more probable when the introduction sites are inside the native climatic niche (Broennimann *et al.*, 2021).

A longstanding challenge in ecology is that estimating a species' niche fundamentally depends on the environmental variables considered. Previous attempts have focused predominantly on single classes of variables such as climatic or topographic predictors, or combining different types of variables in a single analysis (Siqueira *et al.*, 2009; van de Meutter *et al.*, 2010; Chejanovski & Wiens, 2014; Rocha *et al.*, 2018). Other research has shown that analysing different environmental gradients separately has great potential to identify evolutionary trade-offs (Chejanovski & Wiens, 2014), to better understand the relationship between a species' regional distribution and local abundance (Siqueira *et al.*, 2009; Rocha *et al.*, 2018), to test the climate variability hypothesis in an elevational gradient (Pintanel *et al.*, 2022), and to study how local and climate variables explain variation in the species niche (Alahuhta *et al.*, 2017a).

Estimating niche characteristics can also be strongly affected by the geographical extent (GE), or the total space, over which analyses are made (Rahbek, 2005; Barve *et al.*, 2011; Acevedo *et al.*, 2012; Jarnevich *et al.*, 2017; Sillero *et al.*, 2021). In general, niche models including data from the entire species' range are more accurate compared to approaches that consider only partial distributions of species (Barbet-Massin, Thuiller & Jiguet, 2010; Sánchez-Fernández, Lobo & Hernández-Manrique, 2011; Raes, 2012; Carretero & Sillero, 2016; Sillero *et al.*, 2021). This is because larger GE includes greater variability of the environmental space in which the species occurs, thereby reducing the risk of truncating species-environment response curves. This ultimately leads to an improved and more informed characterisation of the species' niche. Moreover, inadequate coverage of the full distributional range of species increases the risk of underestimating the breadth of the species niche and the importance of contributing environmental factors (e.g. climate). This is especially likely when the regional realised niches do not correspond with the global niche (Barbet-Massin *et al.*, 2010; Sillero *et al.*, 2021), which can result in large biases for those species that are widely distributed or considered non-native (hereafter alien) to a given region (Wilson *et al.*, 2007; Sillero *et al.*, 2021). For example, the realised regional niche of alien species could differ from the regional equilibrium niche depending on invasion history (i.e., time since initial introduction) or the presence of geographic or anthropogenic barriers to movement (Wilson *et al.*, 2007). By contrast, niche models built on GE that is much larger than a species' distributional area might result in findings that have little meaning for understanding a species environmental niche or to inform species management (Acevedo *et al.*, 2012; Mainali *et al.*, 2015). Thus, analysing partial distributions can be useful and informative when the objective is to identify regional distribution constraints, as these might have contrasting patterns in different parts of a species' global range (Castro, Muñoz & Real, 2008; Martínez-Freiría *et al.*, 2008). In addition, especially in modelling invasive alien species that yet have fully established in a region, comparing models built on different GE can create important insights on a species invasiveness (Gallien *et al.*, 2012). Therefore, using a combination of both global and regional occurrence data might be the best option to analyse the niche characteristics of species (Roura-Pascual *et al.*, 2009; Gallien *et al.*, 2012; Granot *et al.*, 2017).

Despite recent inquiry, surprisingly little is known on how GE may affect the characterisation of the native and alien species niches. In this study, we used the outlying mean index (OMI), a widely used technique for niche characterisation (Dolédec *et al.*, 2000; Heino & Soininen, 2006; Korsu *et al.*, 2012; Rocha *et al.*, 2018; Escoriza, 2021), to estimate and compare niche metrics of native and alien inland fishes of the Iberian Peninsula as a case study. Our main objectives were to: (1) compare climate-based niche metrics (niche position, niche marginality and niche breadth) estimated using OMI analyses at three different GE previously used in the literature (from regional to global) and analyse its dependency on species introduction date for alien species; and (2) test for differences in climatic and river position niches between native and alien species of the Iberian Peninsula. We hypothesised that niche metrics are scale-dependent and predicted: (i) that the estimation of niche characteristics is more accurate at larger GE, particularly for alien and other widely distributed species (e.g. diadromous), for which niche breadth is underestimated using regional-scale data; and (ii) that this bias is larger for recently introduced species.

8.2. Methods

8.2.1. Bibliographic review

We systematically reviewed the literature for published studies using OMI analyses to quantify species niche attributes observed in inland water ecosystems (see Table S8.1). The literature search was performed using Google Scholar (consultation date: 1st February 2022) with the keywords: "outlying mean index" & "freshwater". The search returned 198 compatible results; however, we only considered the 106 peer-reviewed studies that specifically used OMI analyses on organisms linked to inland water ecosystems. Next, from each study we collated information about the focal taxa, the GE of the study, the types of environmental variables used, and if applicable, whether single or groups of variables were used in the OMI analysis.

8.2.2. Study area and data compilation

Our study was conducted in the Iberian Peninsula, which is well suited to study the climatic and longitudinal niche of species because of its complex orography, diverse climate, and particular fish fauna (Cano-Barbacil *et al.*, 2022). It is characterised by a broad range of elevation rising from the sea level, over a large central plateau (Meseta Central) to the peaks of over 3000 m (Sabater *et al.*, 2009; Bayón & Vilà, 2019). The Iberian Peninsula is also characterised by a high spatial and temporal climate variability. The northern half is dominated by a Mediterranean oceanic climate with warm summers (Csb), the southern half a Mediterranean climate with dry and hot summers (Csa), southeastern Spain has a semiarid climate (BSk), and mountainous areas and the northernmost Peninsula an oceanic climate (Cfa and Cfb) (Kottek *et al.*, 2006; AEMET, 2011). Iberian inland waters are inhabited by 100 different fish species (including diadromous and estuarine fish), of which 32 are alien, some of them widespread throughout the world (Cano-Barbacil *et al.*, 2020).

We compiled occurrence data across the world for all established Iberian inland fishes between 2000 and 2020. The dataset was mainly sourced from the Global Biodiversity Information Facility (GBIF.org, 2019a; see Table S8.2 for specific references), the Portuguese 'Carta Piscícola Nacional' (Ribeiro *et al.*, 2007), and Doadrio's atlas (2001), which is the most comprehensive fish study of Spain. It was completed with an up-to-date occurrence database of the Iberian Peninsula published by Cano-Barbacil *et al.* (Cano-Barbacil *et al.*, 2022) and with the data available in the Freshwater Biodiversity Data Portal (Biofresh, 2021). The final dataset contains presence/absence data for 51 native and 17 alien species. To avoid potential biases, all species had at least 20 occurrence records in the Iberian Peninsula.

We selected six climatic variables and six predictors describing the longitudinal river position (i.e. upstream-downstream gradient) with known associations with the composition of inland fish communities (Broennimann *et al.*, 2007; Murphy *et al.*, 2015; Bae *et al.*, 2018) (see Table S8.3 for further details). Following previous studies (Comte & Grenouillet, 2013; Conti *et al.*, 2015), we selected climatic variables related to extremes for species survival and seasonality, which have been proved informative to understand large-scale species distributions: temperature seasonality (BIO4) as representative of temperature stability; mean temperature of warmest quarter (BIO10) and mean temperature of coldest quarter (BIO11) as measures of extreme temperature conditions; precipitation seasonality (BIO15) as surrogate of the flow regime; and precipitation of wettest (BIO16) and driest (BIO17) quarter as measures of extreme precipitation and drought conditions. Variables describing river longitudinal position included: elevation; slope; the topographic index (i.e. a function of the catchment area and the slope gradient that is commonly used to quantify topographic control on hydrological processes, Sørensen *et al.*, 2006); distance to the sea; and Shreve's and Strahler's stream orders as indicators of stream size (Strahler, 1957; Shreve, 1966; Cano-Barbacil *et al.*, 2022). Climatic information was globally available, while longitudinal position variables were available only for the Iberian territory. All spatial data were used at a resolution of 5 minutes (~10 × 10 km at the equator).

8.2.3. Outlying mean index analysis

OMI analyses is an ordination technique well suited to calculate different niche metrics and for identifying the most influential environmental factors for community structure and organisation (Dolédec *et al.*, 2000). Niche position (NP) was estimated as the species score of the first ordination axis (Kleyer *et al.*, 2012; Arribas *et al.*, 2014; Alarcón & Cavieres, 2018; Rodrigues *et al.*, 2019). Niche marginality (NM), which is often also referred to as NP (Korsu *et al.*, 2012; Heino & Grönroos, 2014; Carscadden *et al.*, 2020), is defined as the distance between the mean habitat conditions used by species and the mean habitat conditions of the studied area, and can be especially useful for understanding species' range size, abundances or extinction vulnerability (Dolédec *et al.*, 2000; Carscadden *et al.*, 2020). Thus, species with high NM values have more marginal niches (i.e. occur in less common habitats in the sampling area), while species with low NM values have non-marginal niches (i.e. occur in common habitats in the in the sampling area); therefore, species with intermediate and more extreme NP generally have low and high NM, respectively. Niche breadth (NB), also named tolerance in OMI, measures the amplitude of the distribution of each species along the sampled environmental gradients (Dolédec *et al.*, 2000). Thus, species with high NB values are those distributed in a wide range of environmental conditions (i.e. generalist taxa), while species with low values of NB are those occurring across a limited range of environmental conditions (i.e. specialist taxa). Following previous studies (Siqueira *et al.*, 2009; van de Meutter *et al.*, 2010; Chejanovski & Wiens, 2014; Rocha *et al.*, 2018), we analysed separately the climatic and longitudinal niches.

We computed the climatic OMI analyses at three different scales, using the six climatic predictors previously compiled. First, we ran an OMI analysis using the global distribution of species (Wüest *et al.*, 2015; Comte & Olden, 2017b), as most of the native peripheral and alien species present in the Iberian Peninsula are also distributed worldwide (e.g. *Gambusia holbrooki*, *Micropterus salmoides, Cyprinus carpio*). Second, we performed a restricted OMI analysis using a convex polygon as background (Mainali *et al.*, 2015), created around the occurrence set of all the species studied (see Figure S8.1). Finally, we ran a regional OMI analysis using only fish occurrences in the Iberian Peninsula, which is the equivalent to the most frequent approach in the literature (Table S8.1). We $\log_{10}$-transformed the predictors BIO15, BIO16 and BIO17 to correct positive skewness and better approximate linear relationships.

We used three independent estimates of the climatic tolerance of species based on previous data compilations (see Comte & Olden, 2017a; Kärcher *et al.*, 2019): critical thermal maxima (CT_{max}), which is the temperature at which individuals lose critical motor function; thermal range, defined as the difference between the maximum temperature and the minimum temperature of occurrence; and mean water temperature, calculated by averaging the transformed monthly average air temperatures of occurrence, which were calculated considering the entire distribution of the species. We evaluated the relationship among computed climatic niche metrics and the three previous climatic tolerance variables using Pearson correlations. We then analysed the effect of geographical extent on climatic niche characteristics according to the global, restricted and regional metrics while considering the species native status and potential interactions, by using analyses of covariance (ANCOVA). We also compiled the introduction date of the alien species established in the Iberian Peninsula (Muñoz-Mas & García-Berthou, 2020) and performed a multiple regression analysis to relate regional with global and restricted niche metrics considering their introduction date.

In addition, we conducted a regional OMI analysis using river longitudinal position variables, because they reflect certain niche dimensions different from climatic predictors – despite their partial correlation (Chejanovski & Wiens, 2014; Rocha *et al.*, 2018). Prior to analyses, we $\log_{10}$-transformed the predictors slope, Shreve's and Strahler's stream order to address skewness and linearity assumptions. Longitudinal variables were only available for freshwater (and brackish) sites, and thus analyses were restricted to freshwaters only excluding potential marine occurrences of species. The OMI analyses were conducted by using the 'niche' function of the R-package 'ade4' (Dray & Dufour, 2007), which takes as inputs the table with the species' presence/absence data and a principal component analysis (PCA) of the environmental variables. Subsequently, we used Pearson's correlation to evaluate the differences in the niche metrics (i.e. NP, NM and NB) between using climatic and longitudinal variables at the regional scale.

In order to test for differences in NP, NM and NB among native and alien species we used univariate permutational analysis of variance (PERMANOVA), which has the advantage of not assuming a specific probability distribution (e.g. normality). We used the 'adonis2' function of the R-package 'vegan', and used 999 permutations and Euclidean distances. We also tested for homogeneity of dispersions among groups using the function 'betadisper' of the R-package 'vegan', since the PERMANOVA is sensitive to heterogeneous dispersions. All statistical analyses and graphics were performed with R, version 3.5.1 (R Core Team, 2020).

8.3. Results

8.3.1. Geographical extent affects the estimation of climatic niche metrics

The bibliographic review reveals that the vast majority of published studies (~97%) evaluating niche characteristics using OMI analyses were carried out at regional or even local scale, ignoring in many occasions the full range of aquatic species (Table S8.1). However, we found that niche metrics estimation strongly depends on the GE considered. According to our analysis of inland fishes present in the Iberian Peninsula, we found marked differences between the regional and larger scales (i.e. global and restricted). In fact, correlation analyses revealed that NM and NB are more similar at global and restricted scale compared to regional scale (Figure 8.1). We also observed strong correlations among the three different estimates of the climatic tolerance of species compiled (i.e. critical thermal maxima [CT_{max}], thermal range and mean water temperature). Most importantly, we found that these estimates of climatic tolerance are also strongly correlated with the global, but specially with the restricted climatic NP, NM and NB. Across studied niche metrics, NP was generally more correlated with the estimates of the climatic tolerance than NM, especially at regional scale. Species with wide climatic NB showed broad thermal range. Warmwater species (i.e. species with high CT_{max}, and inhabiting areas with high mean water temperature) generally showed narrower thermal range and, therefore, lower values of climatic NB.

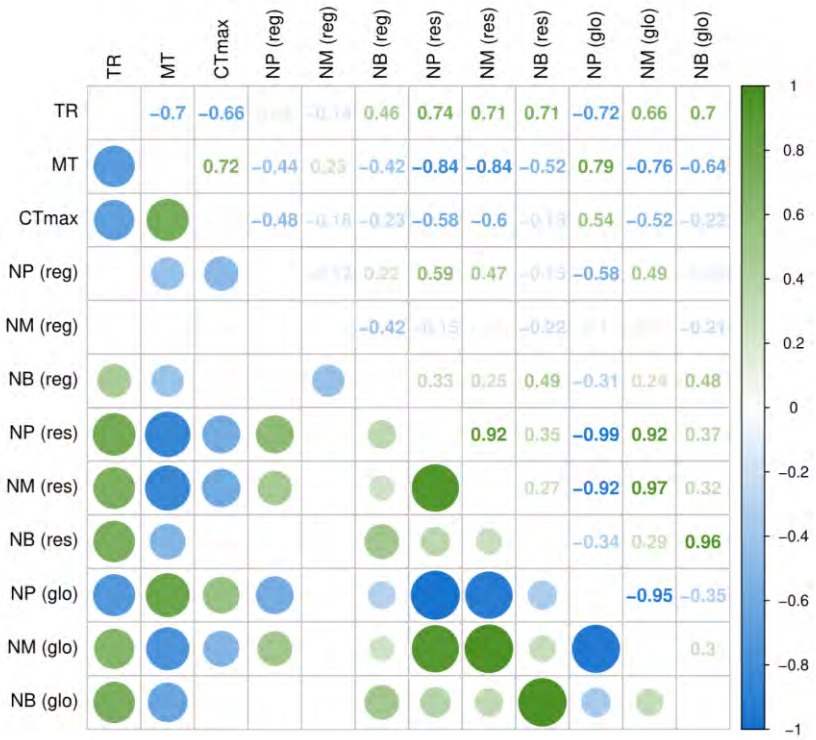


Figure 8.1. Correlation matrix of global (glo), restricted (res) and regional (reg) climatic niche position (NP), niche marginality (NM) and breadth (NB) and thermal range (TR), mean water temperature (MT) and critical thermal maxima (CT_{max}), the latter three expressed in °C. Pearson's correlation coefficients are shown above the diagonal. No circles are given for non-significant correlations (*P*> 0.05).

We found that relationships between climatic global and restricted NM and NB, and between global and regional NP varied with native status (see Figure 8.2 and significant interaction terms in Table S8.4) and were often weaker for alien species. The latter indicates that the mean habitat characteristics of some alien species are different in the introduced area than in their area of origin. The correlations of global and restricted NM with regional NM were not significant (Table S8.4). We also found that the first two axes of the OMI analyses at large scales generally accounted for more explained variation of species niches (98.8% and 97.9% for global and restricted analyses respectively) than the regional analyses (91.2%). For further details on OMI analyses results and most influential environmental variables, see Appendix S8.1.

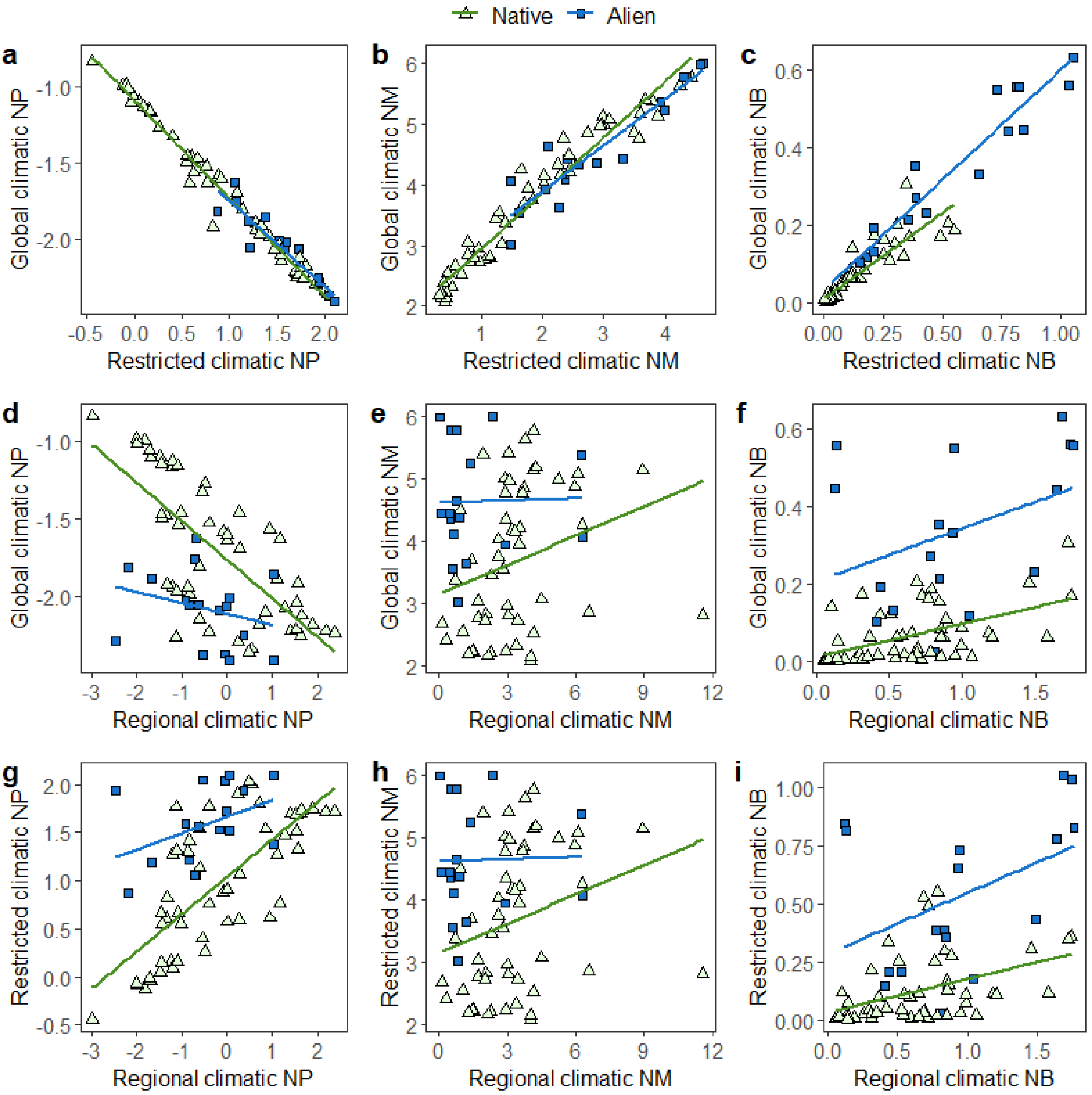


Figure 8.2. Relationships between global, restricted and regional niche metrics for native (green triangles) and alien (blue squares) species. NP = niche position; NM = niche marginality; NB = niche breadth.

The relationship between regional and global NB for alien species depended on the species year of introduction (see significant interaction in Table S8.5). An interaction plot (Figure 8.3) revealed that the slope of the relationship between regional and global NB is practically nil (or even negative) for recently introduced species, while was markedly steeper for species introduced more distantly in the past. Thus, some alien species recently introduced (in the past 40 years) to the Iberian Peninsula that had high environmental tolerance (i.e. wide NB) at the global-scale (e.g. *Australoheros facetus* and *Ictalurus punctatus*) demonstrated narrower NB at regional-scale analysis (Figure 8.3). By contrast, other species that have been introduced at least one century ago (e.g. *Cyprinus carpio*, *Oncorhynchus mykiss*, *Lepomis gibbosus*) showed wide NB at both global and regional scales. However, we did not find clear effects of the introduction date on the relationships regional *vs.* restricted or regional *vs.* global NP or NM (Table S8.5).

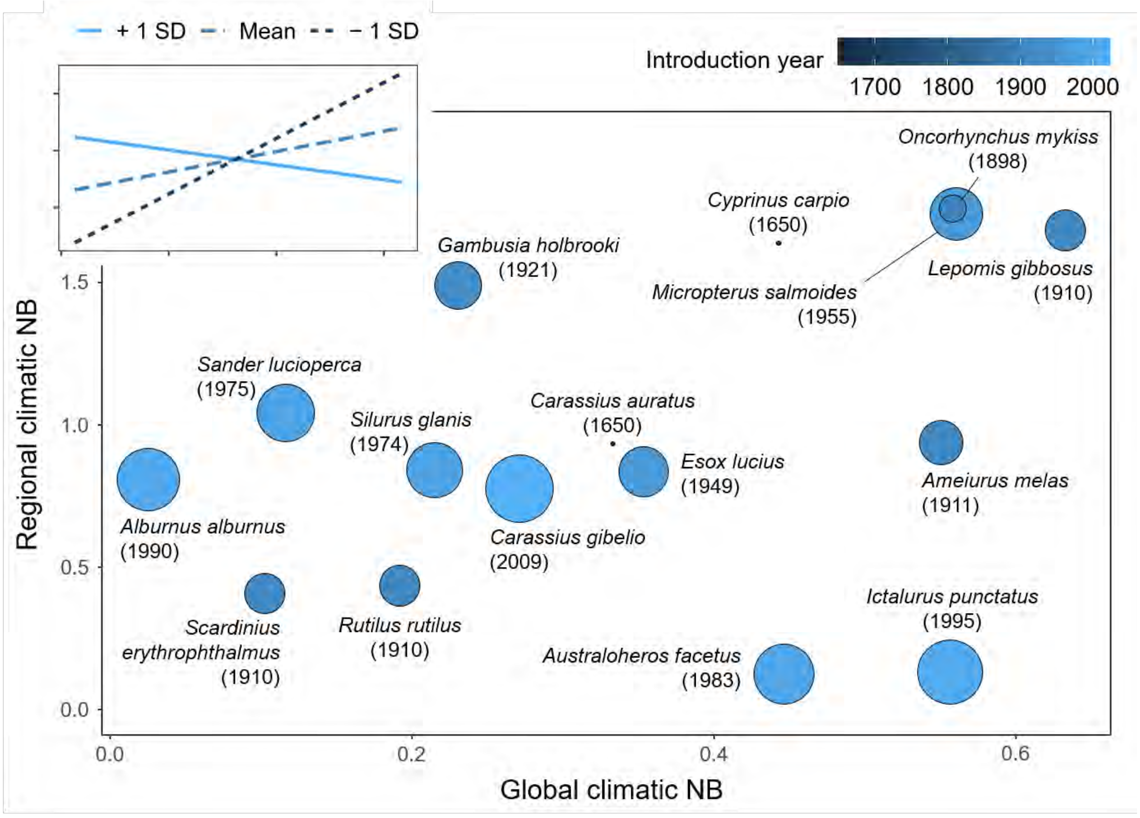


Figure 8.3. Relationship between estimates of regional and global climatic niche breadth (NB). The introduction year in the Iberian Peninsula is shown for each alien species and is proportional to the area (and color) of the circles. The interaction plot (upper left) shows regression lines for three approximately equal-sized groups with the highest (+1 SD), middle (Mean) and lowest (-1 SD) third of the data (by introduction date).

8.3.2. Relationship between climatic and longitudinal niche metrics

In our analysis of Iberian inland fishes, the correlation between regional climatic and longitudinal NM was significant but weak (*r* = 0.363, *P* = 0.002) and stronger between climatic and longitudinal NP (*r* = -0.612, *P* < 0.001; Figure 8.4), while climatic and longitudinal NB were not clearly correlated (*r* = 0.156, *P* = 0.204). Species that inhabit warm regions with high precipitation seasonality were those present in large lowland rivers, whereas those that occur in areas with rainy summers, were present in mountain rivers with steep slopes at high elevations (Figure 8.4). However, some species like the three-spined stickleback (*Gasterosteus aculeatus*) or the Atlantic salmon (*S. salar*) currently only occur in the cooler northern part of the Iberian Peninsula but mostly in small rivers or lowermost reaches. The mosquitofish (*G. holbrooki*) occupies a similar longitudinal position than stickleback but is barely present in northern Spain, because it prefers warmer waters (Figure 8.4). Similarly, both *Platichthys flesus*, *Atherina boyeri* and *Aphanius baeticus* are species only present in the lowermost reaches and coastal lagoons but *P. flesus* is comparably more prevalent in the cooler, northern region, where the two other species are absent. Likewise, although species like *Squalius pyrenaicus* and *Alosa fallax* occupy habitats with similar climatic characteristics, their longitudinal niches are at opposite ends of the gradient. These examples and the weak correlations mentioned above support the existence of two independent ecological niche axes and distinct niche properties related to climatic and longitudinal characteristics. In this light, it was surprising that our literature review indicated that only 5% of previous studies have evaluated the contribution of different types of environmental variables to aquatic species niche characteristics separately (Table S8.1).

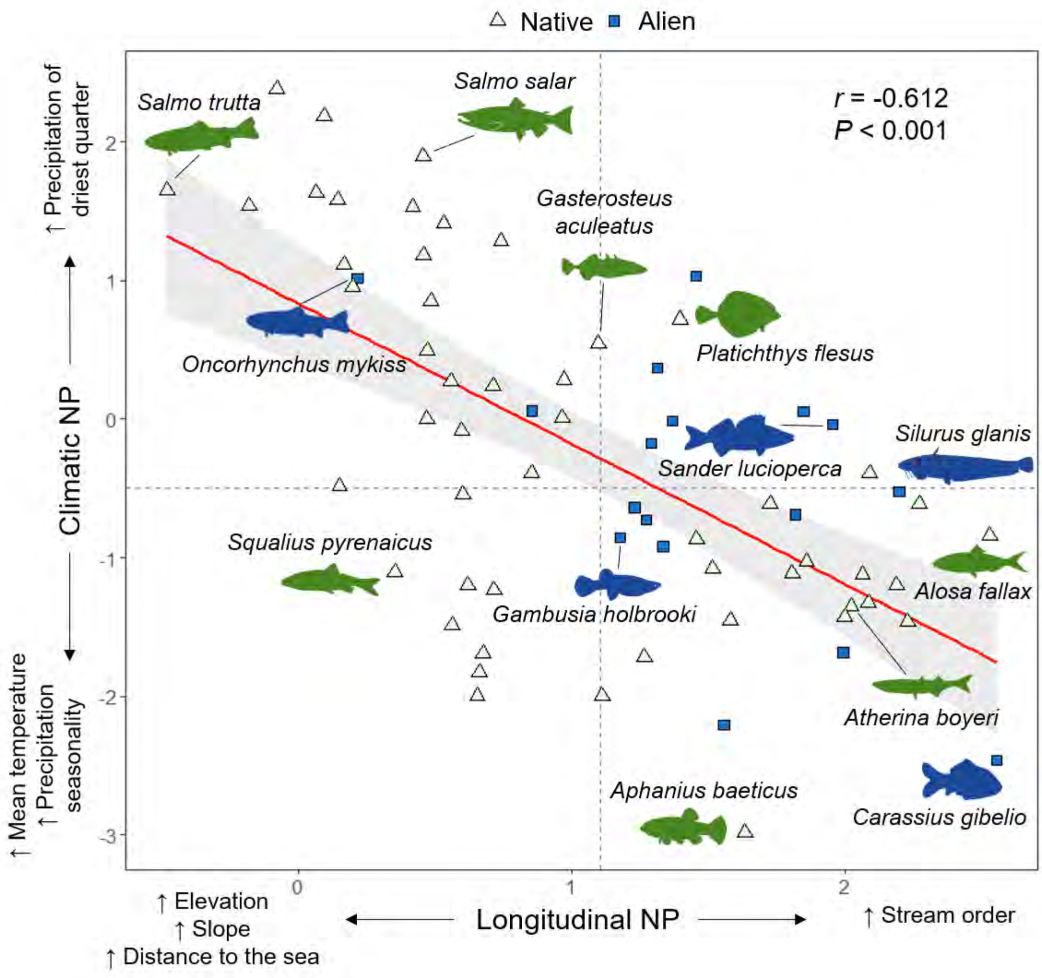


Figure 8.4. Relationship between climatic and longitudinal niche positions (NP) obtained by outlying mean index analyses for native (green triangles) and alien (blue squares) Iberian fishes. Red line shows linear regression across all species, the shaded band represents the corresponding 95% confidence interval. Species silhouettes were obtained from http://phylopic.org/.

8.3.3. Native and alien species showed contrasting niche characteristics

Climatic NP at global and restricted scales varied between native and alien species (Figure 8.5, Table S8.6 and Appendix S8.1). Alien species mostly occur in areas with rainy summers and warm winters, when analysing their global distributions. However, the differences were not significant when using analyses only with regional data (Table S8.6). Climatic NB and NM also varied between native and alien species at the three scales of analysis. Alien species showed higher climatic marginality at global and restricted scales, while at regional scale native species are those with the most marginal niches. Similarly, alien species showed wider climatic NB than native fishes of the Iberian Peninsula, especially at global and restricted scales of analysis. PERMANOVAs of niche parameters at global and restricted scale showed higher explained variation than with regional data only. Longitudinal NP and NB also varied with native status (Figure 8.5 and Table S8.6), whereas the differences were not significant for NM. Native species have narrower NB and have a different NP than alien species, which mainly occur in the lowermost reaches (Figure 8.4).

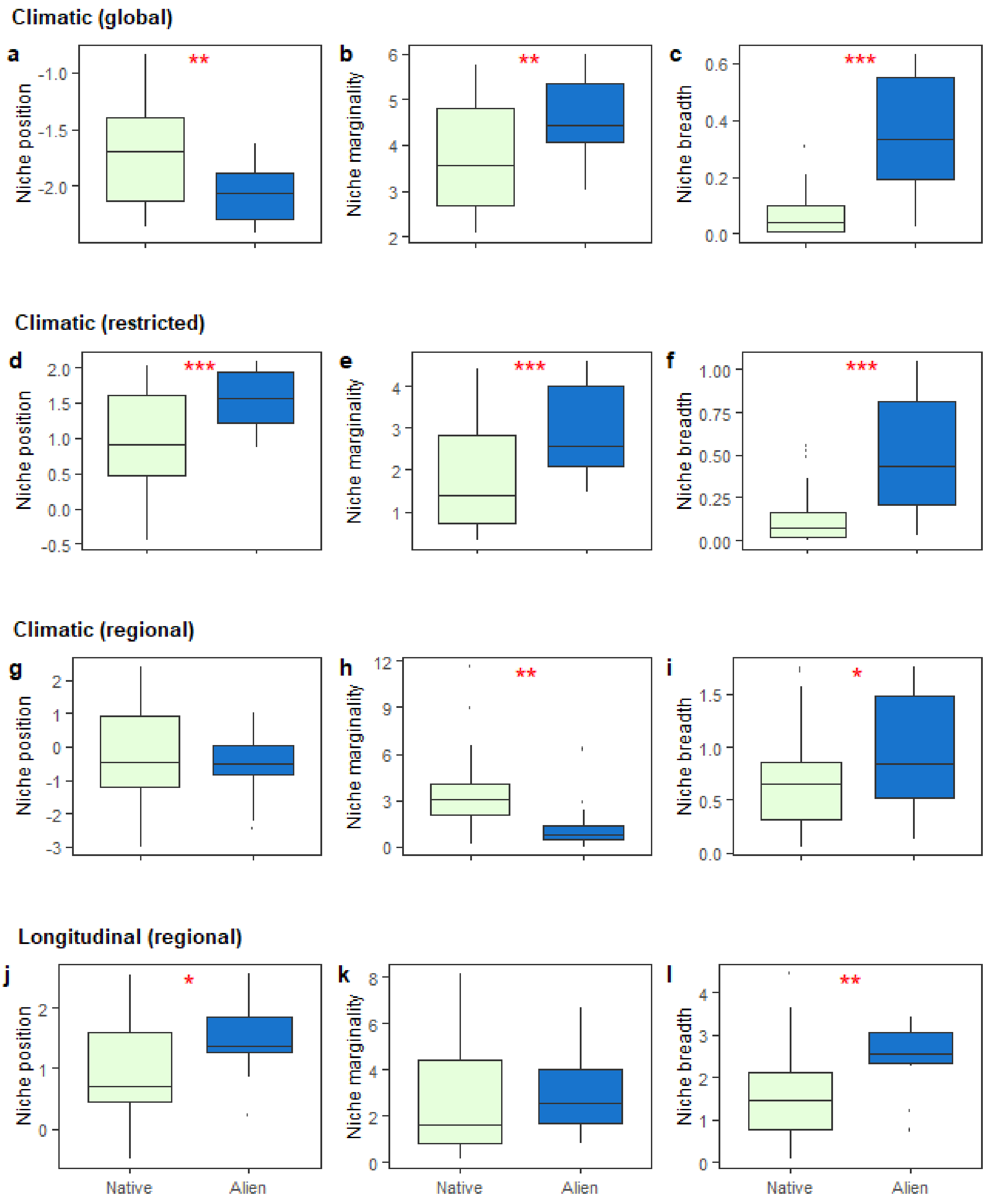


Figure 8.5. Niche position, marginality and breadth of 68 Iberian freshwater fish by native status. Boxes correspond to the 25th and 75th percentiles; lines inside a box show the median; whiskers extend to the last observation within 1.5 times the interquartile range from the quartiles and outliers are indicated by empty circles. *P* values of PERMANOVAs (Table S8.6) are expressed with asterisks (*** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05).

8.4. Discussion

Our study reveals marked differences in species' niche metrics depending on the geographical extent of the investigation. Analyses conducted at larger scales resulted in stronger associations between niche metrics and independent measures from the literature (i.e. thermal range, mean water temperature and critical thermal maxima). This largely agrees with previous studies emphasising the importance of selecting appropriate geographical extent in species distribution modelling (Barve *et al.*, 2011; Sánchez-Fernández *et al.*, 2011; Acevedo *et al.*, 2012; Jarnevich *et al.*, 2017). Using a global or a restricted GE can better reflect the niche characteristics of widely distributed taxa, particularly alien species. This contrasts with the frequent use of OMI analysis that focus solely on regional extents (see Table S8.1 for further references).

The climatic niche breadth of alien and other widely distributed species (e.g. diadromous species) were found to be generally underestimated at regional compared to global geographical extent. Differences in climatic niche breadth across geographical extents suggests climatic disequilibrium in alien species distribution range in the Iberian Peninsula. This was especially evident for species that occupy a small distribution area with a narrow range of climatic conditions and whose distribution is mainly limited by basin boundaries (see Early & Sax, 2014). As a consequence, the invasive potential of alien species in the Iberian Peninsula might far exceed what is reflected in their current regional distributions, thus suggesting that alien species are poised to spread to new areas in the future if the opportunity arises. For instance, non-native species with wide climatic niche breadth such as some salmonids or *Phoxinus* spp. (Tales, Keith & Oberdorff, 2004) could establish in the harsher environments of northernmost Iberia and upper headwaters of river basins, that currently support just a small number of alien species.

Associations between niche metrics at different geographical extents differed according to native status. This result is supported by previous research reporting that some alien species can invade climatically distinct niche spaces following its introduction into new regions (Broennimann *et al.*, 2007; Tingley *et al.*, 2014; Bujan *et al.*, 2021), owning to their wide environmental tolerance and (thermal) plasticity. Our findings emphasise ongoing calls to more carefully consider species' autecology and select appropriate spatial scales of investigation when estimating the range potential of invasive species (Jarnevich *et al.*, 2017). Moving forward, we recommend the application of comparative approaches that combine both regional and global niche estimates. This will support more informative insights about constrains on species distributions across diverse geographies and provide a more nuanced predictions of distributional change in the future (Gallien *et al.*, 2012).

In agreement with our hypothesis and previous studies (Alexander & Edwards, 2010), we found that introduction date was strongly associated with estimations of alien species' climatic niche characteristics. For instance, most recently introduced fish species in the Iberian Peninsula (e.g. *A. facetus* and *I. punctatus*) demonstrated narrower NB when quantified at the regional scale despite having wider NB (i.e. high environmental tolerance) at the global scale. By contrast, other species that have been introduced more than one century ago such as *C. carpio* or *C. auratus* and that have likely experienced multiple introduction events over time (Clavero & Villero, 2014) displayed wider NB at the regional scale. Previous research suggests that niche characteristics in newly invaded areas are related to the date of introduction, in addition to other factors that include the number and location of introduction events, and the environmental suitability of the new range (Alexander & Edwards, 2010). This includes some research showing that time since introduction was unexpectedly negatively related with niche expansion of certain species in the invaded area (Early & Sax, 2014). In our case, we also found that some species, despite being introduced in 1910 (*Rutilus rutilus* and *Scardinius erythrophthalmus*), showed narrow NB at regional extent but they also exhibited a narrow NB at global extent.

Our results also suggest that ecological niches related to climatic conditions versus longitudinal position may be distinct. This agrees with previous studies (Buisson *et al.*, 2008) reporting that the first ordination axis of the OMI analysis distinguished the well-known upstream–downstream gradient of rivers (Vannote *et al.*, 1980), while the second axis reflected a temperature gradient. This further implies that coolwater fishes are not only restricted to the most upstream part of the rivers and there are also some cool water species that specifically inhabit downstream rivers (Buisson *et al.*, 2008). For instance, *P. flesus* and *A. boyeri* are species only present in river estuaries. However, *P. flesus* only occurred in estuaries north of the Tajo estuary (39°N) and is more prevalent in cooler areas at the northern part of the Iberian Peninsula, where *A. boyeri* is absent (Cabral *et al.*, 2007; França, Costa & Cabral, 2011). We note that the upstream-downstream position can be even variable even within species. For example, in the Iberian Peninsula *S. trutta* is mainly found in the headwaters as it prefers clean, cool and well-oxygenated streams. However, in northern basins this species has been found to occupy smaller downstream river reaches close to the sea (Doadrio *et al.*, 2011).

Finally, our results suggest that alien fishes present in the Iberian Peninsula are generalist species, as they usually occur in habitats with average climatic conditions across the study area (i.e. low regional climatic marginality), and they demonstrate wide global climatic niche breadth. This agrees with previous studies reporting that the most important features of alien species successfully invading altered ecosystems are a broad physiological tolerance, generalist resource requirements, traits that enhance consumption and growth, and life history attributes (e.g. long longevity, late maturity, high fecundity, multiple spawnings per year, and short reproductive span) enabling them to survive in habitats where many native species could not (Vila-Gispert *et al.*, 2005; Orrù *et al.*, 2010; Kärcher *et al.*, 2019). Our results indicate that alien species such as *G. holbrooki* and *Micropterus salmoides* tend to occur in the lower main river reaches of which many are hydrologically altered (Clavero & Hermoso, 2011; Bae *et al.*, 2018). In contrast to alien species, native fishes largely displayed narrower climatic and longitudinal niche breadths. This tends to support the idea that Iberian fish distributions are mainly determined by river basin boundaries, pointing to a non-equilibrium state of assemblages with contemporary environmental conditions (Filipe *et al.*, 2009). The implications is that native specialist species of the Iberian Peninsula continue to be vulnerable to the effects of climate change and habitat loss because of synergistic effects of a small range size and narrow niche (Slatyer, Hirst & Sexton, 2013).

In conclusion, this study sheds light on a long-standing methodological challenge regarding how the estimation of species niche breadth and position is affected by the geographical extent of investigation. Specifically, we found that species' niche attributes varied substantially according to the geographical extent considered. We posit that performing a regional OMI analysis is sufficient to gain a basic understanding of regional environmental constraints or to characterise niche attributes of narrowly-distributed species. However, in the case of alien species, time since introduction (and thus opportunities for secondary spread) markedly influence the estimates of regional niche characteristics in the invaded area. Therefore, quantifying niche characteristics of species with broader distributions, may require a larger, potentially global-scale analysis to avoid underestimating their environmental tolerance (i.e. niche breadth). Moreover, despite correlations of niche metrics based on river longitudinal and climatic gradients, our results suggest that these two characteristics produce distinct information regarding species niche properties. Finally, we found significant differences in niche metrics between alien and native species, with Iberian alien fishes showing generally greater longitudinal and climatic niche breadth than their native counterparts. Moving forward, we recommend that non-native species with wide niche breadth should be a specific focus in pre-invasion risk assessments and management, in order to reduce risks of new invasions and prevent associated ecological and economic damages.

9. General discussion

Invasive alien fishes may have also diverse detrimental impacts on native amphibians and other taxonomic groups. In the image, a common midwife toad tadpole (*Alytes obstetricans*).

Photo: Carlos Cano-Barbacil

Freshwater ecosystems around the world are heavily impacted by global change (Grill *et al.*, 2019). In particular, Iberian rivers suffer the effects of high habitat fragmentation and its consequent hydrological alteration, which modifies the natural flow regime and leads to changes in ecosystem functions and community composition (Clavero & Hermoso, 2011; Sabater *et al.*, 2018), and facilitates the establishment of invasive alien species (see e.g. Bae *et al.*, 2018). Unraveling the functional and ecological differences between native and invasive alien species has great scientific and practical interests (Alcaraz *et al.*, 2005). Thus, current lines of research focus on linking distribution patterns and niche properties with species traits and their evolutionary history in order to better understand species' responses to environmental change and the invasion process (Poff *et al.*, 2006; Frimpong & Angermeier, 2010; Thuiller *et al.*, 2012). Although the ecological filtering caused by altered flow regimes has been studied in North American rivers (Mims & Olden, 2013), the biological consequences of hydrological alteration across European, and especially Iberian freshwater ecosystems have been barely investigated (but see Radinger *et al.*, 2019). In an attempt to address these goals, this thesis focused on evaluating the distribution patterns of Iberian fishes, and specifically, unravelling effects produced by hydrological alteration, through the study of fish traits and their ecological niche.

9.1. Compiling Iberian fish trait data, not an easy task

Although the development of global databases in recent years has facilitated access to information on traits of inland fish species (Froese & Pauly, 2019; Brosse *et al.*, 2021), there is still an important knowledge gap for some traits and species (Statzner *et al.*, 2007). This is also the case for experimental data on fish swimming performance. Specifically, the number of studies regarding critical swimming speed (U_{crit}) of fish has considerably grown in the last years (Katopodis & Gervais, 2012, 2016). However, the data availability for many Mediterranean species is still rather limited (Haro *et al.*, 2004; Alexandre *et al.*, 2016).

Our trait data compilation on Iberian fish traits (*Chapter I*) revealed that species with a narrow distribution range (e.g. endemic species) have been less studied than widespread taxa. In fact, diadromous species have greater trait data availability than strictly freshwater species, as many of former have a wide distribution range and have been extensively studied over the past century (e.g. Schmidt, 1923). This is also applicable to the specific case of the study of swimming performance (*Chapter II*). Many studies of fish swimming capacities have focused either on salmonids, especially in North America, because of their commercial and recreational interest (Glova & McInerney, 1977; Booth *et al.*, 1997; Peake *et al.*, 1997; Shingles *et al.*, 2001; McKenzie & Claireaux, 2010), and on migratory species (Katopodis & Gervais, 2012; Silva *et al.*, 2018). Similarly, we found that quantitative data on fish salinity tolerance (supplementary analyses in

Chapter III, Appendix S6.1) was also very scarce for Iberian species (Godinho & Ferreira, 1998). Specifically, we only found data for 19 out of 100 species, of which the great majority were alien species and none of them were endemic.

We also showed that most recently described species were characterised by rather low trait data availability. Taxonomic knowledge on the Iberian ichthyofauna has advanced in recent years thanks to genetic studies, which has caused a significant number of species being described in the last 20 years (Doadrio *et al.*, 2011). Therefore, the functional and ecological features of some of these recently described species, such as three lampreys described in Portugal in 2013 (Mateus *et al.*, 2013), have been barely studied, and there are no experiments to date related to their swimming performance or salinity tolerance.

In addition, reliability analysis revealed a generally low reliability for some traits (*Chapter I*). Especially, we found a lower agreement in trait assignment among databases for categorical than for continuous features, as observed in previous studies (Markon *et al.*, 2011). This lower reliability might be either because categorical traits are mainly established by expert judgment and not supported by quantitative data (i.e. epistemic uncertainty), or because there are large discrepancies among trait definitions (i.e. linguistic uncertainty) (Regan *et al.*, 2002). In fact, we also face this issue when trying to classify Iberian species as stenohaline or euryhaline following the existing records of the literature (*Chapter III*). The majority of studies considered do not provide the criteria or empirical evidence used for such categorisation, and in some cases, they did not share a common definition of each category. Actually, few studies based this classification on quantitative data and considered as freshwater stenohaline species those able to survive in a narrow range of salinity that includes fresh waters (Schultz & McCormick, 2013). However, many other studies based this categorisation on Myers and Darlington's divisions, and considered freshwater stenohaline and primary species as synonyms (Noble, Cowx & Starkie, 2003; Costedoat & Gilles, 2009).

Another important source of variation in trait assignment among databases might be related to intraspecific variability, which is generally neglected in trait datasets (Beck *et al.*, 2012). However, some fish traits are known to have important intraspecific variability, such as reproductive span (Blanck & Lamouroux, 2007), species diet (Blanco *et al.*, 2003; Feyrer *et al.*, 2003; Weliange & Amarasinghe, 2003; Sánchez-Hernández *et al.*, 2018) or even swimming performance. In fact, we found that despite the significant differences among species, the size of the individual is the most important factor determining its swimming performance (*Chapter II*).

Thus, the lack of knowledge in trait information for several Iberian species might constitute an important limiting factor in many trait-based approaches and bioassessment studies. However, the generated datasets on fish traits, critical swimming speed and the seawater tolerance as collated for this thesis might be reference sources for future studies on Iberian ichthyofauna that will ultimately help to improve the reliability and robust application of trait-based approaches and fish indices (see section *9.4. Management implications* for further details). Specifically, the consensus trait database generated in *Chapter I* constitutes the first attempt of a comprehensive, regional database that summarises highly relevant trait information for Iberian inland fishes with unprecedented coverage.

9.2. General differences between native and alien fish distribution patterns

Species distribution and niche models of this thesis revealed that native and alien species of the Iberian Peninsula have contrasting distribution patterns and niche characteristics. The distribution of most native species (especially primary natives) is mainly determined by the long-term basin boundaries. This is in agreement with previous studies indicating that geographical barriers and historical factors often exert greater constraints on native inland fish distributions than climatic or anthropogenic factors (Filipe *et al.*, 2009). Moreover, we observed contrasting patterns of native vs. alien species along the upstream-downstream gradient, as indicated by significant differences found in the longitudinal niche position between groups (Figure 9.1). Specifically, primary native species were found more prevalent in upstream and middle reaches, whereas secondary and peripheral species especially occur in lowland reaches near the coast (*Chapters III*, *IV* and *V*). In accordance to previous research (Radinger *et al.*, 2019), alien species tend to occur in the lowermost reaches of the main river stems.

This longitudinal pattern might be linked to several environmental factors that change along the elevational gradient of streams such as flow velocity, river bed substrate and water temperature (Vannote *et al.*, 1980). In fact, we found that air temperature was positively correlated with alien species oc currences, and therefore, is a key factor to understand their current distribution (*Chapters III* and *V*). This finding emphasises the thermophilic character of many alien species. For instance, suitable spawning temperatures for alien *Cyprinus carpio*, *Alburnus alburnus* and *Gambusia holbrooki* are 16–22, 17–28 and >15–16°C, respectively (Pen & Potter, 1991; Mann, 1996); and it is also well-known that the competitive capacity of some invasive alien species is favoured with higher temperatures (Carmona-Catot *et al.*, 2013). Radinger *et al.* (2019) found that mean annual air temperatures of approximately 15.5°C as a lower threshold for the occurrence of many alien species in the Ebro basin. Similarly, the occurrence of secondary endemic species of the Iberian Peninsula (*Aphanius* spp. and *Valencia hispanica*) is also closely related to warm climates; whereas some other native species showed considerable negative responses in their geographic distributions to warm temperatures. For example, the brown trout (*Salmo trutta*) is rather sensitive to high temperatures (Elliott & Elliott, 2010), and thus, its distribution in the Iberian

Peninsula is restricted to the northernmost latitudes of this territory and to headwater streams (Doadrio, 2001).

Moreover, we found that alien species showed broader climatic and longitudinal niche breadth than native fishes of the Iberian Peninsula (*Chapter V*). Previous studies already found that the most important features of alien species successfully invading altered ecosystems are generalist resource requirements and a broad physiological tolerance. These features enable alien fishes to survive in habitats where many native species cannot. However, we found that the introduction date of invasive alien species to the new territory is a key factor shaping their niche breadths at regional scale, despite it is also known to be influenced by other genetic constraints of niche evolution, the genetic structures of native populations, the number and location of introduction events, and the environmental characteristics of the new range (Alexander & Edwards, 2010). For instance, recently introduced species (e.g. *Australoheros facetus* and *Ictalurus punctatus*) showed narrower niche breadth in the Iberian Peninsula despite having wide environmental tolerance when considering their global distributions. Conversely, other species that have been introduced more than one century ago, such as *C. carpio* or *Carassius auratus*, showed also wide niche breadth when analysing their distributions in the Iberian Peninsula.

Finally, in agreement with the natural flow regime paradigm (Poff *et al.*, 1997), which predicts that native fishes are more likely to benefit from natural flows, whereas hydrologically altered flow regimes would favour alien species, we found that alien fishes are more prevalent in reservoirs and hydrologically altered sections of the lowland main river stems (*Chapters III*, *IV* and *V*). In fact, most of the alien species successfully introduced are native from hydrologically stable habitats from central European and southeastern North American streams, and therefore, they are better adapted to these lentic habitats (Vila-Gispert *et al.*, 2005). Radinger *et al.* (2019) arrived to similar conclusions in the Ebro river, where alien species dominate in reaches with higher habitat fragmentation and land use transformation. Similarly, previous studies that analysed the distribution patterns of *G. holbrooki* and *Micropterus salmoides* in the Iberian Peninsula also found that the number or capacity of upstream reservoirs positively influenced their establishment (Murphy *et al.*, 2015; Bae *et al.*, 2018).

9.3. Fish traits can explain the ecological filtering produced by the hydrological alteration

These homogenised rivers due to damming and flow regulation have significant hydrological and biological consequences for freshwater ecosystems as explained above (Poff *et al.*, 1997), promoting changes in the community functional diversity (Clavero & Hermoso, 2011; Mims & Olden, 2013; Arantes *et al.*, 2019b). These changes in fish assemblages can be explained by differences in life-history strategies (Vila-Gispert *et al.*, 2005) or physiological limitations (Frimpong & Angermeier, 2010). In fact, previous studies found that novel lentic habitats created by dams and their associated reduction of flow variability favour the establishment of equilibrium-strategist species with broad physiological tolerance, generalist resource requirements, trophic plasticity (e.g. omnivores) and life-history traits that enhance consumption and growth (Vila-Gispert *et al.*, 2005; Marr *et al.*, 2013; Mims & Olden, 2013; Agostinho *et al.*, 2016). Our results also agree with previous studies showing that altered ecosystems mainly harbor invasive alien large-bodied and tolerant fishes with rather large flexibility in their requirements regarding water quality (*Chapter III*; see Figure 9.1). Especially, piscivorous game species with long longevity, late maturity, high fecundity, few spawnings per year, and short reproductive span were overrepresented among the established alien fishes in the Iberian reservoirs (Vila-Gispert *et al.*, 2005; Clavero & Hermoso, 2011).

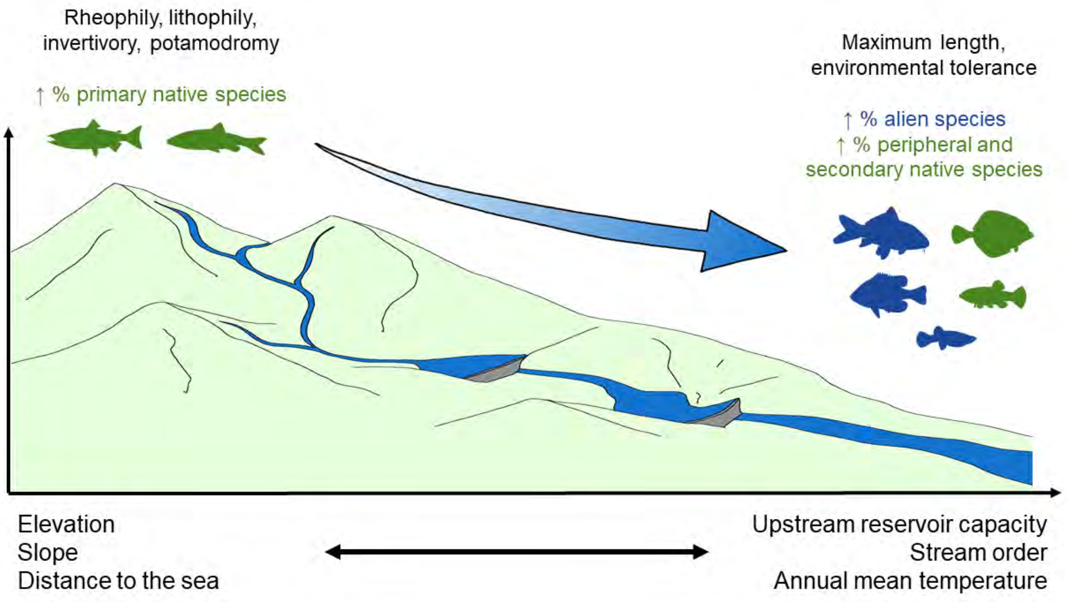


Figure 9.1. Schematic representation summarising the main results of the *Chapters III*, *IV* and *V*.

We found damming acting as ecological filter against rheophilic, litophilic, invertivorous and potamodromous species, which tend to occur in small high-elevation streams (*Chapters III* and *IV*). Many of the species exhibiting these traits are primary native fishes, mostly cypriniforms, and are considered more adapted to lotic habitats with naturally more fluctuating flow regimes with frequent occurrence of high-flow events (Propst & Gido, 2004; Gido *et al.*, 2013; Pool & Olden, 2015; Srean *et al.*, 2016). Previous studies also showed a negative effect of flow regulation on more opportunistic strategists (i.e. small fishes with early maturation, frequent reproduction over an extended spawning season, small eggs and clutches, rapid larval growth, and rapid population turnover rates) such as some Iberian native species (Winemiller & Rose, 1992; Vila-

Gispert *et al.*, 2005; Mims & Olden, 2013). Similarly, large herbivorous and invertivorous species are usually affected after dam constructions (Arantes *et al.*, 2019b), despite some studies found a positive relationship between invertivorous density and reservoir size (dos Santos *et al.*, 2017). The negative effects of river fragmentation on migratory fishes are also well known (dos Santos *et al.*, 2017; Arantes *et al.*, 2019b). Dams disrupt migratory pathways and reduce the availability of feeding and reproductive habitats (Gomes & Miranda, 2001). Specifically, potamodromous have declined even more than diadromous fish populations on average (-83% vs. -73%) (Deinet *et al.*, 2020).

Especially in Mediterranean rivers, native species are often specifically adapted to lotic habitats and to the frequent occurrence of high-flow events and alien fishes replace native species predominately in areas where the hydrological regime is altered (Vila-Gispert *et al.*, 2005; Boix *et al.*, 2010; Bae *et al.*, 2018). Hence, we hypothesised that differences in swimming performance among species might explain these contrasting distribution patterns (Gido *et al.*, 2013; Rubio-Gracia *et al.*, 2020). In fact, we expected to find lower swimming performance in alien species of the Iberian Peninsula. However, our results do not support this hypothesis, reflecting that native and alien species have rather similar swimming performance (*Chapter II*). We even observed that some alien species, commonly classified as limnophilic, displayed high U_{crit} values (*Rutilus rutilus*, *Perca fluviatilis*, *Sander lucioperca*, *Esox lucius* and *Alburnus alburnus*) that are similar to swimming speeds found in native Iberian species (Rubio-Gracia *et al.*, 2020). Moreover, we did not find any relationship between fish swimming performance and the elevational and longitudinal distribution of species (*Chapter IV*). This suggests that swimming performance alone does not explain species' habitat selection, and therefore, it may be more related to other ecological, morphological, physiological or even behavioural features (Ward, Schultz & Matson, 2003). In fact, pelagic and high trophic level lifestyles appear to favour locomotor performance (Killen *et al.*, 2016).

Therefore, and as also concluded by Radinger *et al.* (2019) in their study on the Ebro river, our results might be also viewed in the context of Darwin's naturalisation conundrum, as alien Iberian fishes present some common features with native species, but at the same time they generally have some distinctive traits. Darwin's naturalisation conundrum postulates two contradictory hypotheses regarding factors influencing biological invasions. First, it postulates that alien species closely related to native species and with shared similarity would be more likely to pass environmental filters and successfully establish; but at the same time, it posits the importance of functional distinctiveness of alien species from native species to avoid competitive exclusion and facilitate their establishment (Thuiller *et al.*, 2010; Park *et al.*, 2020).

Overall, our results highlight the difficulty of finding universal traits that favour the introduction and establishment of invasive alien species due to the functional diversity of alien species and the diverse aims of introductions (see also Alcaraz *et al.*, 2005; Olden *et al.*, 2006; Ribeiro *et al.*, 2008). However, the similarity between our results and those of previous studies in the Iberian Peninsula (Vila-Gispert *et al.*, 2005; Ribeiro *et al.*, 2008; Radinger *et al.*, 2019; Rubio-Gracia *et al.*, 2020), and even in other regions (Moyle & Marchetti, 2006; Mims & Olden, 2013; dos Santos *et al.*, 2017; Arantes *et al.*, 2019b) suggests that some generalisations could be made, at least for regions with similar environmental and climatic characteristics (Ribeiro *et al.*, 2008).

9.4. Management implications and future directions

The magnitude of the current biodiversity crisis presses to urgently identify and prioritise effective management actions (Olden *et al.*, 2010; Maceda-Veiga, 2013). However, freshwater research is still poorly linked to conservation ecology, despite inland waters are one of the most threatened ecosystems in the world (Maceda-Veiga, 2013). As already explained in the *Introduction,* the Iberian Peninsula has a long history of biological invasions and anthropogenic disturbances. This has led to the poor conservation status of Iberian inland fishes, with 48% of the species considered in this thesis categorised as vulnerable, endangered or critically endangered, according to IUCN criteria, which might be further exacerbated by climate change. Therefore, effective management actions should be taken and coordinated through Spain and Portugal, and should focus on three main aspects: (1) the restoration of natural riverine habitats, their connectivity and hydrologic regimes; (2) the prevention of new introductions and spread of invasive species; and (3) the prioritisation of areas for fish conservation (Maceda-Veiga *et al.*, 2010; Markovic *et al.*, 2014; Radinger *et al.*, 2019).

This thesis provides relevant information on the main factors that determine the distribution of Iberian inland fishes and open data resources that can be used to design policies that help to achieve these goals and preserve the native ichthyofauna. First, the consensus trait dataset generated in this study constitutes a reference source that provides trait information for all fish species established in Iberian inland waters. This information can be used in the application of trait-based bioassessments and to obtain meaningful and robust results. In fact, fish indices such as IBICAT2b or such as EFI+ (e.g. Almeida *et al.*, 2017), which consider some fish traits (e.g. lithophily, intolerance or piscivory) to estimate the ecological status or river health, are widely used by researchers and public administrations (Figuerola, Maceda-Veiga & de Sostoa, 2012; Maceda-Veiga, Green & De Sostoa, 2014; García-Berthou *et al.*, 2015). Moreover, functional diversity indices are also used to estimate anthropogenic impacts as they are known to be sensitive to both biotic and abiotic degradation, even on species-poor assemblages (Colin *et al.*, 2018).

Therefore, a more accurate application of fish and functional diversity indices can help to better understand the causes of decline in native inland fish populations and to plan effective conservation strategies (Colin *et al.*, 2018).

Secondly, the reported dataset on fish swimming capacity can have several management applications in order to mitigate the effects of habitat fragmentation. Swimming ability is a major determinant of barrier passage success and migration rates (Haro *et al.*, 2004; Tudorache *et al.*, 2008; Jones *et al.*, 2020), which might also depend on other factors such as morphology, sex, phenotypic plasticity, seasonal behaviour changes or environmental conditions (Srean *et al.*, 2016; Silva *et al.*, 2018, 2021; Jones *et al.*, 2020). Specifically, species-specific estimates of U_{crit} and U_{crit} vs. fish length regressions provided in this thesis can be used to estimate maximum allowable water velocities in order to facilitate fish passage over vertical barriers and to improve river connectivity (Peake, 2008b; Katopodis *et al.*, 2019). Similarly, our results can be used to develop barriers that selectively limit the spread of invasive alien species (Katopodis & Gervais, 2016), to design selective fishways that prevent the passage of alien species but allow the passage of native ones (Silva *et al.*, 2018), or to plan deliberate high-flow releases from reservoirs during the breeding season of alien fishes in order to minimise their recruitment and control their populations (Harvey, 1987; Erman, Andrews & Yoder-Williams, 1988). For instance, *G. holbrooki* and *Lepomis gibbosus* are small-bodied fish, respectively inhabiting the water-column and bottom of lentic habitats, with a low swimming capacity compared to other species (Srean *et al.*, 2016). Thus, these characteristics suggest that a particular high water flow level (i.e. water velocities >20 cm s^{-1}) could help to control or eradicate their populations and prevent future invasions (Korman, Kaplinski & Melis, 2011; Gido *et al.*, 2013; Srean, 2015). Despite U_{crit} has been commonly used to estimate fish swimming capacity, it is known that swimming performance derived in the laboratory might underestimate actual abilities of free-swimming individuals in the field (Peake & Farrell, 2006; Silva *et al.*, 2018; Ruiz Legazpi *et al.*, 2019). However, free-swimming experiments are scarce, especially for fish present in the Iberian Peninsula (but see Ruiz Legazpi *et al.*, 2019). We encourage to further study the swimming capacity of native Iberian species and established alien carrying out free-swimming experiments to better preserve native fauna and determine the mechanisms that drive biological invasions in Iberian rivers. However, until additional research is conducted, the results provided in this thesis represent useful information for Iberian fish species management.

Species distribution models (SDM) and niche characterisation implemented in this thesis (*Chapter III* and *V*) can be used to formulate and implement conservation measures. They constitute a well-established tool to predict future invasions, to identify threatened species or to establish priority areas that need to be preserved (Filipe, Cowx & Collares-Pereira, 2002; Guisan *et al.*, 2013). This is particular relevant, since the current protected areas in the Mediterranean basin are small compared with other regions in the world and are mainly established within the terrestrial realm (Hermoso & Clavero, 2011). Moreover, our integrative studies of both species' distributions and traits can be of fundamental importance to better understand the environmental filtering on fish assemblages, and the consequences of habitat degradation. For instance, the relative high importance of anthropogenic hydrological alterations suggests that restoration efforts (e.g. dam removal) might reduce alien species abundance, although some species like *G. holbrooki* are unlikely to be extirpated (Murphy *et al.*, 2015). However, given the relative greater importance of historical and climatic factors rather than anthropogenic perturbation as distributional drivers of the majority of alien species, once they are introduced in a suitable area, there will be few opportunities to limit their establishment and spread (see also Murphy *et al.*, 2015). Therefore, the prevention of new introductions would be the most appropriate, effective and economical management tool in invasion biology and must become a priority (Ricciardi & Rasmussen, 1998; García-Berthou *et al.*, 2005; Gallien *et al.*, 2012; Radinger *et al.*, 2019), especially in those areas where their occurrence has been predicted by the SDMs but not observed (Murphy *et al.*, 2015). Similarly, outlying mean index (OMI) analyses suggest that there is a certain climatic disequilibrium in alien species distributional range in the Iberian Peninsula, especially for those species that occupy a small area limited by basin boundaries (see Early & Sax, 2014). These results are congruent with those obtained using SDMs and support that alien species could be able to colonise further new territories in the future if the opportunity arises. Therefore, we also suggest to include those alien species with wide niche breadth in horizon scanning studies and pre-invasion risk assessments, in order to minimise risks of new basin invasions (*Chapter V*).

According to IUCN criteria, one of the main threats to Iberian ichthyofauna is climate change (Maceda-Veiga, 2013). Future climatic models for the Iberian Peninsula predict temperature increases, most pronounced during the summer months, and modifications in precipitation regimes with increased variability over the year (Álvarez Cobelas *et al.*, 2005). Moreover, water flow intermittency is expected to further exacerbate with climate change because of the consequent growth in human water demands (Hermoso & Clavero, 2011). Although the effects of climate change were not explicitly tested in this thesis, the study of species thermal tolerance (*Chapter V*), and the trait adaptative potential of species in evolutionary terms (*Chapter IV*) might help to better understand species' responses to climate change (Markovic *et al.*, 2014). Under this scenario, our results suggest that the consequences on the Iberian native ichthyofauna could be very harmful, since inland fishes have a limited dispersal capacity (see also Markovic *et al.*, 2014) and since their traits might not evolve sufficiently fast to cope with the changes that occur in the environment (*Chapter IV*). In addition, the majority of native species showed narrow climatic breadth and thermal tolerance, indicating that changes in precipitation and temperature patterns might have a negative effect on their populations (*Chapter V*). Warmer temperatures and reduced flows could also favour the emergence of novel suitable habitats for alien species, facilitating their establishment and invasion (*Chapter III*; Carmona-Catot *et al.*, 2013). For instance, alien fishes, especially those with wide environmental tolerance (Tales *et al.*, 2004), could be able to colonise in the near future the northernmost Iberia and headwater streams, which are currently not yet occupied by (many) alien species (*Chapter V*). Moreover, the distribution range of thermophilic alien species is expected to increase under climate change due to warmer temperatures and reduced flows (*Chapter III*; Murphy *et al.*, 2015). Overall, the results presented in this thesis represent a valuable tool to face the challenge of preserving the native Iberian ichthyofauna. Thus, a close cooperation between researchers, managers and policy makers is essential to promote effective conservation plans and management.

10. General conclusions

The village of Maderuelo (Segovia) is located on the shores of the Linares reservoir (Riaza River). The reservoir floods a limestone canyon carved by the river over millions of years in which the old town of Linares del Arroyo was located.
Photo: Carlos Cano-Barbacil

- A notable part of the variation for some fish traits was explained by a systematic bias in trait assignment by particular databases, specifically for categorical/binary traits. Therefore, increased efforts to complement these categorical traits by empirically-derived continuous data, and the development of standardised protocols for recording trait information could lead to an improvement of trait reliability in inland fish. This will further help to improve the robust application of fish trait-based approaches and fish indices (*Chapter I*).

- Traits were less studied in fish species with small latitudinal range and those that have been described more recently. Alien species showed higher data coverage than native and, especially, endemic fishes (*Chapter I*).

- The consensus trait and the swimming performance databases for Iberian inland fishes that come along with this thesis constitute two reference sources (*Chapters I* and *II*). Specifically, the trait dataset is the first attempt of a comprehensive, regional database that summarises trait information with unprecedented coverage.

- Fish total body length was the most relevant explanatory variable of critical swimming speed (U_{crit}), followed by fish taxonomic affiliation (family and species identity) and the time step interval between velocity increments considered during the experiment. The relative importance of body shape, form and water temperature on U_{crit} were much lower (*Chapter II*).

- Historical and climatic factors are more important than land use and anthropogenic variables in explaining fish distributions of the Iberian Peninsula. However, we found great differences in the importance of factors explaining fish distributions between native and alien species and especially among Darlington’s divisions. This further leads to the conclusion that the eco-evolutionary history and the different seawater tolerances of inland fishes largely mediate their current distribution (*Chapter III*).

- Our results revealed that hydrologically altered ecosystems promote the occurrence of alien large-bodied and tolerant fishes with rather large flexibility in their requirements regarding water quality, and excluding those native invertivore and rheophilic species (*Chapters III* and *V*).

- Although Iberian alien species tend to occur in the lowermost and lentic reaches, native and alien fishes did not show clear differences in U_{crit}, suggesting that prolonged swimming performance might not be always related to the invasiveness of species or to habitat selection (*Chapters II*, *III* and *IV*).

- We found significant phylogenetic signal for 65% of the considered traits but no clear differences among morphological, trophic, reproductive and habitat use features, or between continuous and binary traits. Similarly, evolutionary models revealed that fish elevational and longitudinal distributions showed little evidence for directional trends of evolution, and thus that the ecological niche tends to resemble that of the common ancestor (*Chapter IV*).

- Phylogenetic methods showed that several reproductive traits are intercorrelated with fish maximum length, reflecting that those features have evolved together. We also found a positive relationship between elevational niche and traits like rheophily and lithophily, and a negative relationship between stream order and invertivory and rheophily when using non-phylogenetic methods (*Chapter IV*).

- The estimation of species' niche metrics strongly depends on the geographical extent considered in the analyses. Therefore, the latter should be carefully selected based on the main objective of the study and on the autecology of the target species. For instance, in the case of alien species, the time since introduction markedly influences the estimates of regional niche characteristics in the invaded area, and thus their niche characterisation requires a global-scale analysis to avoid underestimating their niche breadth (*Chapter V*).

- Species distribution models and species niche characterisation showed that alien fishes of the Iberian Peninsula have a wider niche breadth than native species and could be able to colonise further new territories in the future if opportunities arise, especially species recently introduced. Thus, the prevention of new introductions and the inclusion of those species with wide niche breadth in pre-invasion risk assessments might be an effective management tool (*Chapter III* and *V*).

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Supplementary materials S4 - Reliability analysis of fish traits reveals discrepancies among databases

Table S4.1. List of nineteen databases used. Original study area and reference are shown.

No.	Original study area	Reference
1	North America	Winemiller & Rose (1992)
2	France	Oberdorff *et al.* (2002)
3	Europe	Aarts & Nienhuis (2003)
4	Iberian Peninsula	Alcaraz, Vila-Gispert & García-Berthou (2005)
5	Spain (Catalonia)	Vila-Gispert, Alcaraz & García-Berthou (2005)
6	Europe	Pont *et al.* (2006)
7	Europe and North America	Jeschke & Strayer (2006)
8	France	Belliard & Roset (2006)
9	Europe	Blanck & Lamouroux (2007)
10	Iberian Peninsula	Ribeiro *et al.* (2008)
11	Europe	Holzer (2008)
12	Europe	Teletchea *et al.* (2009)
13	Germany	Dußling (2009)
14	Spain	Doadrio *et al.* (2011)
15	Spain (Catalonia)	García-Berthou *et al.* (2015)
16	Portugal	Oliveira, Ferreira & Santos (2016)
17	Austria	Haunschmid *et al.* (2017)
18	Global	Froese & Pauly (2018)
19	Global	Olden (2018)

Table S4.2. List of synonyms and descriptions of the traits used in this work.

Trait (and TYPE)	Scale of measurement	Description	Synonyms
MORPHOLOGICAL			
Maximum length (cm)	Continuous	Maximum total length	
Maximum weight (g)	Continuous	Weight of heaviest individual recorded.	
Fusiform shape	Binary	Spindle-shaped fish, cylindrical or nearly so that tapers toward the ends.	Spindle shaped
Elongated form	Binary	Fish with an elongated shape	Very slender
Eel-like form	Binary	Eel-shaped fish	
TROPHIC			
Invertivory	Binary	Species that feed on invertebrates. They compose the largest and perhaps the most diverse trophic class, including species that feed on the smallest midge, to species that consume large molluscs (Goldstein & Simon, 1999).	
Omnivory	Binary	Species that consume considerable amounts of both plant and animal material.	
Piscivory	Binary	Species that eat mainly other fishes. Fish have a wide mouth aperture with needle-like teeth and a strong jaw with marginal and palatal bones. They are capable of capturing active, mobile prey, inclusive of larger invertebrates (Pont *et al.*, 2006). They pursue a prey by stalking, chasing, ambushing or lying-in-wait approach (Simon & Emery, 1995).	Carnivorous
REPRODUCTIVE			
Maximum longevity (years)	Continuous	Maximum individual age reported.	
Reproductive span (months)	Continuous	Length of breeding season.	
Mean fecundity (eggs/female)	Continuous	Average number of eggs per mature females in a single spawning event. Can be used as a proxy of the potential reproductive capacity of an organism or a population. Normally increases with age and size.	
Maximum fecundity (eggs/female)	Continuous	Maximum number of eggs a mature female can produce in a single spawning event. Can be used as a proxy of the potential reproductive capacity of an organism or a population. Normally increases with age and size.	

Trait (and TYPE)	**Scale of measurement**	**Description**	**Synonyms**
Egg size (mm)	Continuous	Mean diameter of mature oocytes. Egg size effects the size, growth rate, and survival of hatchling larvae, and, consequently, has cascading effects on offspring fitness (Allen & Marshall, 2014).	
Age at maturity (years)	Continuous	Mean age at which fish develop ripe gonads for the first time.	
Length at maturity (cm)	Continuous	Mean length at which fish develop ripe gonads for the first time.	
Parental care	Binary	Any investment by parents in progeny that increases the offsprings' probabilities of surviving and, in hence, reproducing. In fish, parental care can adopt several forms (guarding, nest building, external egg carrying, egg burying, moving eggs or young, ectodermal feeding, oral brooding, internal gestation, brood-pouch egg carrying, etc) (Froese & Pauly, 2019).	Guarders, guard their embryos and/or larvae / Bearers, fish that carry their embryos with them / Eggs guarded / Young guarded, incl. mouth-brooders, species with marsupia, and ovoviviparous species
Single spawning	Binary	Single spawning per season. This trait summarises the seasonality of spawning events (Holzer, 2008).	
HABITAT USE			
Rheophily	Binary	Preferring to live in running water. Species adapted to fluvial habitats.	Lotic / Species preferring fast-flowing and shallow microhabitats within a given reach / Species preferring deep and fast-flowing microhabitats
Limnophily	Binary	Preferring to live in stagnant waters.	Lentic / Species preferring slow and shallow microhabitats / Species preferring slow-flowing and deep microhabitats / Stagnophil
Potamodromy	Binary	Fishes migrate within the river basin having their entire life cycle occurring within fresh waters of a river system. Migrations are cyclical and predictable, showing seasonal return movements to spawning areas.	
Long migration	Binary	Species migrate between freshwater and seawater. This category includes anadromous, catadromous and amphidromous species. Migrations are cyclical and predictable.	Catadromous / Anadromous

Trait (and TYPE)	Scale of measurement	Description	Synonyms
Benthic	Binary	They live, feed and reproduce on the sediment surface. Benthic species are sensitive to siltation and benthic oxygen depletion (Oberdorff & Hughes, 1992).	Demersal
Water column	Binary	Active swimmer species that prefer to live and feed in the water column. Typically feed on drifting and surface invertebrates or other fishes (Oberdorff & Hughes, 1992).	Pelagic / Neustonic / Pelagic-neritic
Tolerant	Binary	Tolerant species have a large water quality and habitat flexibility.	High tolerant
Intolerant	Binary	Intolerant species have a low water quality and habitat flexibility, and are those that first decline with environmental degradation or after a disturbance (Oberdorff & Hughes, 1992).	Low tolerant / Intolerant to degradation / Intolerant to low O2
Lithophily	Binary	Species that deposit eggs on a rock, rubble or gravel bottom where their embryos and larvae develop (Balon, 1975). Lithophilic spawners are particularly sensitive to siltation, requiring clean gravel substrates for reproductive success (Berkman & Rabeni, 1987; Belliard *et al.*, 1999).	
Phytophily	Binary	Species that scatter or deposit eggs with an adhesive membrane to submerged, live or dead, plants. They are usually adapted to habitats with muddy bottoms and low oxygen concentration (Balon, 1975).	

Table S4.3. Generalised Linear Models (GLM) of analysed traits, with species and database as categorical factors. Species, database and null deviance, and *P* values of species and database are shown.

Trait (and TYPE)	**Species deviance**	$P_{species}$	**Database deviance**	$P_{database}$	**Null deviance**
MORPHOLOGICAL					
Maximum length (cm)	372.09	<0.001	1.16	<0.001	385.32
Maximum weight (g)	654.31	<0.001	0.17	0.793	669.79
Fusiform shape	164.46	<0.001	2.18	0.336	248.37
Elongated form	154.41	<0.001	43.87	<0.001	246.33
Eel-like form	127.83	0.014	6.59	0.086	134.42
TROPHIC					
Invertivory	337.19	<0.001	48.90	<0.001	629.16
Omnivory	385.60	<0.001	36.10	<0.001	642.42
Piscivory	316.33	<0.001	36.16	<0.001	425.71
REPRODUCTIVE					
Maximum longevity (years)	116.25	<0.001	9.40	<0.001	143.34
Reproductive span (months)	33.72	<0.001	13.04	<0.001	75.98
Mean fecundity (eggs/female)	921.16	<0.001	8.56	0.027	1000.80
Maximum fecundity (eggs/female)	710.65	<0.001	9.57	<0.001	757.07
Egg size (mm)	36.43	<0.001	0.05	0.710	37.29
Age at maturity (years)	87.00	<0.001	6.76	<0.001	107.23
Length at maturity (cm)	74.12	<0.001	1.67	<0.001	78.63
Parental care	328.57	<0.001	6.40	0.494	364.81
Single spawning	154.64	<0.001	29.04	<0.001	258.70
HABITAT USE					
Rheophily	343.17	<0.001	100.94	<0.001	527.75
Limnophily	252.96	<0.001	135.84	<0.001	439.40
Potamodromy	217.05	<0.001	57.84	<0.001	379.32
Long migration	336.32	<0.001	25.35	0.001	418.19
Benthic	298.24	<0.001	23.54	0.001	530.29
Water column	194.74	<0.001	100.94	<0.001	461.81
Tolerant	197.94	<0.001	43.96	<0.001	328.14
Intolerant	194.64	<0.001	16.95	0.005	259.69
Lithophily	518.29	<0.001	25.85	0.018	707.98
Phytophily	380.91	<0.001	11.03	0.441	489.15

Table S4.4. Krippendorff's *α* for the different traits studied (bootstrapped mean and corresponding 95% confidence interval).

Trait (and TYPE)	Krippendorff's *α*	95 % confidence interval	
		Lower limit	Upper limit
MORPHOLOGICAL			
Maximum length (cm)	0.919	0.913	0.925
Maximum weight (g)	0.866	0.848	0.882
Fusiform shape	0.311	0.110	0.510
Elongated form	0.299	0.071	0.522
Eel-like form	0.873	0.663	1.000
TROPHIC			
Invertivory	0.460	0.265	0.655
Omnivory	0.532	0.348	0.708
Piscivory	0.672	0.490	0.839
REPRODUCTIVE			
Maximum longevity (years)	0.618	0.586	0.649
Reproductive span (months)	0.219	0.177	0.260
Mean fecundity (eggs/female)	0.739	0.722	0.756
Maximum fecundity (eggs/female)	0.722	0.699	0.748
Egg size (mm)	0.945	0.937	0.953
Age at maturity (years)	0.656	0.632	0.679
Length at maturity (cm)	0.792	0.764	0.819
Parental care	0.860	0.742	0.971
Single spawning	0.329	0.098	0.563
HABITAT USE			
Rheophily	0.588	0.391	0.742
Limnophily	0.426	0.242	0.621
Potamodromy	0.428	0.154	0.675
Long migration	0.749	0.550	0.904
Benthic	0.445	0.246	0.623
Water column	0.211	0.000	0.427
Tolerant	0.441	0.264	0.621
Intolerant	0.552	0.312	0.783
Lithophily	0.693	0.543	0.834
Phytophily	0.724	0.558	0.862

Partial R^2 comparation with eta squared (η^2) using Bland-Altman analysis

To assess the robustness of the results on explained deviance (partial R^2) obtained using generalised linear models (GLM), we calculated eta squared (η^2) of linear models for the log-transformed continuous traits, fitted with the same predictors (species and database). Eta squared is simply the Sum of squares (SS) of the factor divided by the total SS, is equivalent to r^2 and widely used as a measure of effect size (Thompson, 2007; Richardson, 2011). We used regression analysis to allow to compute η^2 from the values of partial R^2 reported in Figure 4.1, which can also be computed from Table S4.3. However, to compare the two statistics (partial R^2 vs. η^2) we applied the Bland-Altman analysis (Bland & Altman, 1986) because conventional correlation and regression analyses (e.g. testing if the slope is 1) are misleading. We used the 'blandr' R package (Datta, 2017) to carry out these analyses.

The two methods are highly correlated (r = 0.9998) (Figure S4.1) and show no clear bias (mean difference = -0.0003, 95% confidence interval = -0.0049, 0.0042) (Figure S4.2), because linear models with log-transformations and GLMs with gamma errors often but not always produce similar results (Manning & Mullahy, 2001).

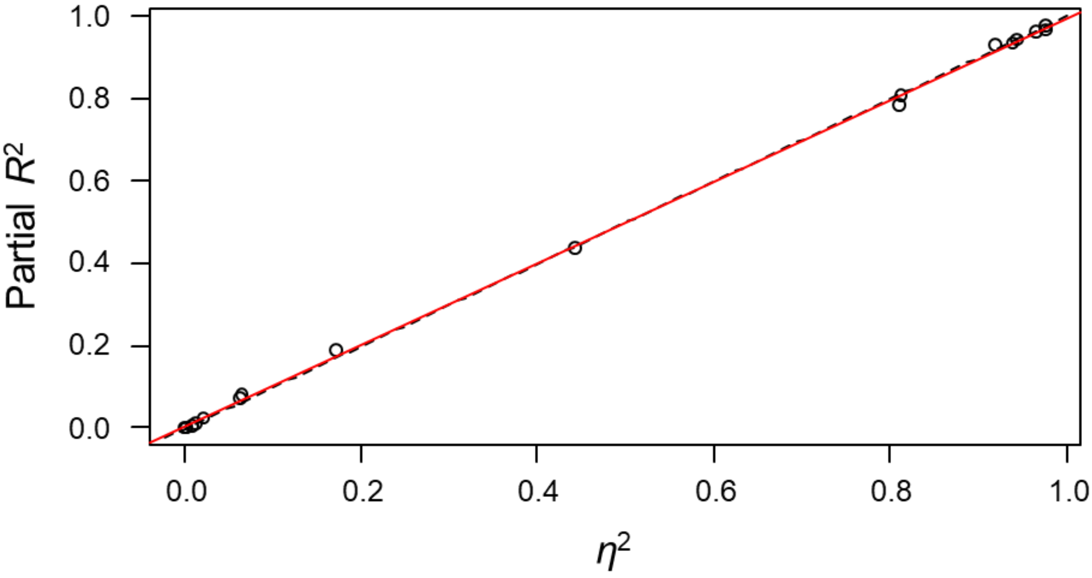


Figure S4.1. Relationship of eta squared (η^2) of linear models (with log-transformation) with partial R^2 of the generalised linear models with databases and species as predictors. The line of equality, that is, the line on which all points would lie if the two methods gave exactly the same reading every time (Bland & Altman, 1986) in dashed black. The linear regression function (η^2 = 0.002941 + 0.992741 partial R^2) in solid red is given to allow to compute η^2 from the partial R^2 given elsewhere in the manuscript.

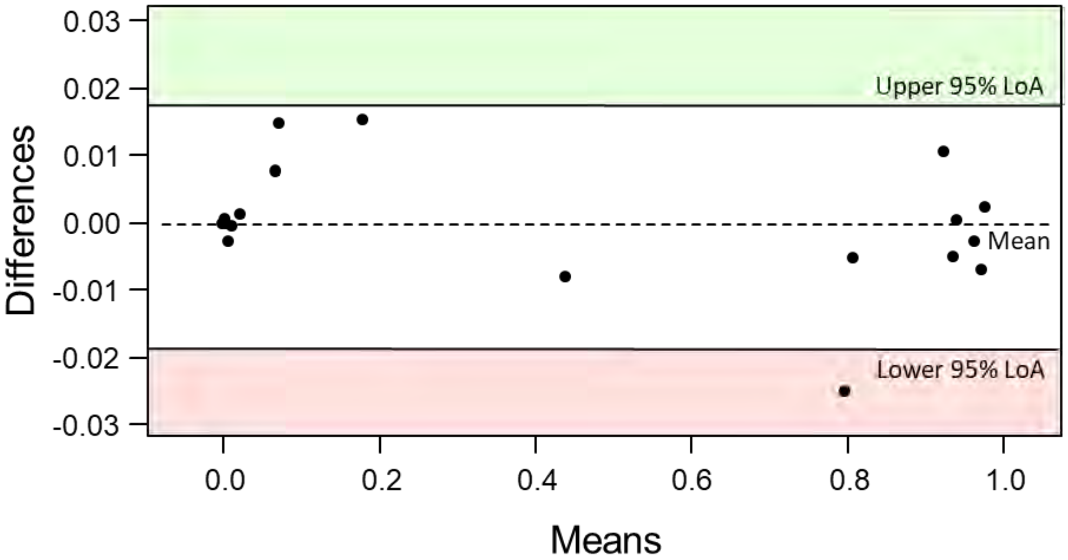


Figure S4.2. Bland-Altman plot for the agreement between eta squared (η^2) of linear models (with log-transformation) with partial R^2 of the generalised linear models with databases and species as predictors. Dotted line represents the average difference between eta squared (η^2) and partial R^2 (i.e. bias), while solid lines represent the upper and lower 95% confidence limits of agreement (LoA).

Supplementary materials S5 - Key factors explaining critical swimming speed in freshwater fish: a review and statistical analysis using Iberian species

Table S5.1. Summary table for all Iberian fish species (including alien species). Mean U_{crit} values and statistics for total length (TL), time step (TS) and temperature (T) and references are given. *n* = number of U_{crit} data used.

Species	U_{crit} (cm/s)	TL_{mean} (mm)	TL_{min} (mm)	TL_{max} (mm)	TS_{mean} (min)	TS_{min} (min)	TS_{max} (min)	T_{mean} (°C)	T_{min} (°C)	T_{max} (°C)	*n*	References
Abramis brama	79.6	159.7	79.0	280.0	20.1	0.3	30.0	16.1	12.3	18.0	3	(Ohlmer & Schwartzkopff, 1959; Clough *et al.*, 2004a)
Alburnus alburnus	68.0	103.0	103.0	103.0	20.0	20.0	20.0	20.0	20.0	20.0	1	(Rubio-Gracia *et al.*, 2020)
Alosa fallax	139.8	336.0	336.0	336.0	0.3	0.3	0.3	18.7	18.7	18.7	1	(Clough *et al.*, 2004b)
Anguilla anguilla	62.0	435.7	72.0	785.4	20.0	0.2	40.0	17.6	12.0	23.0	6	(McCleave, 1980; Mckenzie *et al.*, 2003; Quintella *et al.*, 2010; Tudorache *et al.*, 2015)
Aphanius iberus	10.8	29.6	29.6	29.6	20.0	20.0	20.0	25.0	25.0	25.0	1	(Rubio-Gracia *et al.*, 2020)
Barbatula barbatula	28.2	82.6	82.6	82.6	20.0	20.0	20.0	15.0	15.0	15.0	1	(Tudorache *et al.*, 2008)
Barbus meridionalis	58.2	115.5	110.0	120.9	20.0	20.0	20.0	20.5	20.0	21.0	2	(Rubio-Gracia *et al.*, 2020)
Carassius auratus	39.5	75.9	64.8	97.0	19.4	15.0	20.0	18.4	10.0	25.0	9	(Pang, Cao & Fu, 2011; Yang, Cao & Fu, 2012; Yan *et al.*, 2012; Penghan, Cao & Fu, 2014; Starrs *et al.*, 2015)
Cyprinus carpio	57.7	151.0	58.3	495.0	25.7	20.0	60.0	18.2	10.0	25.0	14	(Heap & Goldspink, 1986; West, Brauner & Hochachka, 1994; Tudorache *et al.*, 2007b, 2008; Pang *et al.*, 2011; Yan *et al.*, 2012)
Dicentrarchus labrax	52.6	99.5	5.1	355.0	7.1	2.0	20.0	20.5	14.0	25.0	17	(Carbonara *et al.*, 2006, 2010; Basaran, Ozbilgin & Ozbilgin, 2007; Basaran *et al.*, 2009; Leis *et al.*, 2012)
Esox lucius	82.3	170.8	21.4	425.0	8.8	3.0	30.0	17.3	16.0	18.0	6	(Ohlmer & Schwartzkopff, 1959; Jones *et al.*, 1974; Peake, 2004)
Fundulus heteroclitus	36.6	64.8	36.5	73.6	11.2	10.0	20.0	20.7	5.2	32.4	8	(Fangue *et al.*, 2008a; Yetsko & Sancho, 2015; Brown *et al.*, 2017)
Gambusia holbrooki	13.4	24.2	16.2	30.0	12.1	5.0	20.0	20.1	10.0	30.0	12	(Grigaltchik, Ward & Seebacher, 2012; Seebacher *et al.*, 2012; Sinclair *et al.*, 2014; Starrs *et al.*, 2015; Srean *et al.*, 2016; Rubio-Gracia *et al.*, 2020)
Gasterosteus aculeatus	30.9	45.1	28.0	54.4	15.0	5.0	20.0	16.0	15.0	18.0	3	(Tudorache, Blust & De Boeck, 2007a; Seebacher *et al.*, 2016)
Ictalurus punctatus	57.5	195.1	147.0	225.2	26.7	20.0	30.0	24.0	20.0	27.0	3	(Hocutt, 1973; Beecham, 2004; Beecham *et al.*, 2014)
Lepomis gibbosus	24.7	111.0	95.1	127.0	40.0	20.0	60.0	20.0	20.0	20.0	2	(Brett & Sutherland, 1965; Rubio-Gracia *et al.*, 2020)
Luciobarbus bocagei	71.7	257.5	219.8	332.5	30.0	30.0	30.0	16.8	16.0	18.5	3	(Mateus *et al.*, 2008; Alexandre *et al.*, 2014)
Micropterus salmoides	34.7	129.2	92.2	182.3	22.1	15.0	30.0	16.8	5.0	25.0	12	(Farlinger & Beamish, 1977; Kolok, 1991, 1992; Cooke, Kassler & Philipp, 2001)
Oncorhynchus kisutch	57.9	187.6	53.3	661.1	45.9	15.0	60.0	12.3	3.0	23.0	11	(Griffiths & Alderdice, 1972; Howard, 1975; Glova & McInerney, 1977; Taylor & McPhail, 1985; Brauner,

Species	U_{crit} (cm/s)	TL_{mean} (mm)	TL_{min} (mm)	TL_{max} (mm)	TS_{mean} (min)	TS_{min} (min)	TS_{max} (min)	T_{mean} (ºC)	T_{min} (ºC)	T_{max} (ºC)	*n*	References
												Shrimpton & Randall, 1992; MacKinnon & Farrell, 1992; Lee *et al.*, 2003)
Oncorhynchus mykiss	75.0	215.3	55.0	491.8	17.8	1.0	40.0	13.1	6.0	18.0	17	(Fry & Cox, 1970; Jones *et al.*, 1974; Duthie & Hughes, 1987; Nikl & Farrell, 1993; Hawkins & Quinn, 1996; Peake *et al.*, 1997; Gregory & Wood, 1998; Shingles *et al.*, 2001; Jain & Farrell, 2003; Ralph *et al.*, 2012; Starrs *et al.*, 2015)
Perca fluviatilis	97.7	163.7	100.0	220.0	23.0	15.0	30.0	16.4	15.0	18.0	5	(Ohlmer & Schwartzkopff, 1959; Tudorache *et al.*, 2008; Starrs *et al.*, 2015)
Petromyzon marinus	86.2	606.0	606.0	606.0	30.0	30.0	30.0	15.0	15.0	15.0	1	(Mesa, Bayer & Seelye, 2003)
Phoxinus sp.	49.0	70.7	70.7	70.7	20.0	20.0	20.0	20.0	20.0	20.0	1	(Rubio-Gracia *et al.*, 2020)
Platichthys flesus	41.7	321.0	295.0	347.0	30.0	30.0	30.0	10.0	5.0	15.0	2	(Duthie, 1982)
Pseudochondrostoma duriense	55.0	164.0	164.0	164.0	30.0	30.0	30.0	15.0	15.0	15.0	1	(Branca, 2015)
Pseudochondrostoma polylepis	70.5	211.1	185.7	221.2	30.0	30.0	30.0	17.4	15.0	19.0	4	(Romão *et al.*, 2012; Branca, 2015; Alexandre *et al.*, 2016)
Pseudochondrostoma willkommii	54.0	166.0	166.0	166.0	30.0	30.0	30.0	15.0	15.0	15.0	1	(Branca, 2015)
Rutilus rutilus	84.1	111.0	10.7	190.1	17.9	0.3	30.0	16.3	13.0	20.0	8	(Ohlmer & Schwartzkopff, 1959; Mann & Bass, 1997; Clough & Turnpenny, 2001; Tudorache *et al.*, 2008; Rubio-Gracia *et al.*, 2020)
Salmo salar	67.8	215.1	25.8	575.0	16.7	5.0	30.0	12.5	6.0	18.0	12	(McCleave & Stred, 1975; Heggenes & Traaen, 1988; Booth *et al.*, 1997; Bui *et al.*, 2016; Remen *et al.*, 2016; Hvas & Oppedal, 2017)
Salmo trutta	82.4	148.9	26.1	350.0	11.4	0.3	20.0	11.5	1.7	19.0	15	(Heggenes & Traaen, 1988; Butler, Day & Namba, 1992; Clough & Turnpenny, 2001; Tudorache *et al.*, 2008; Ralph *et al.*, 2012; Taugbøl *et al.*, 2019)
Salvelinus fontinalis	55.6	100.9	24.2	127.8	24.1	0.2	75.0	13.9	6.0	15.0	14	(Peterson, 1974; Beamish, 1980; Heggenes & Traaen, 1988)
Sander lucioperca	191.0	420.0	420.0	420.0	30.0	30.0	30.0	18.0	18.0	18.0	1	(Ohlmer & Schwartzkopff, 1959)
Scardinius erythrophthalmus	84.5	200.0	120.0	280.0	30.0	30.0	30.0	18.0	18.0	18.0	2	(Ohlmer & Schwartzkopff, 1959; Pavlov *et al.*, 1972)
Squalius carolitertii	54.3	123.6	114.0	136.5	30.0	30.0	30.0	18.9	18.9	19.0	3	(Romão, 2009; Romão *et al.*, 2012)
Squalius laietanus	69.8	110.8	104.1	117.5	20.0	20.0	20.0	20.9	20.0	21.9	2	(Rubio-Gracia *et al.*, 2020)

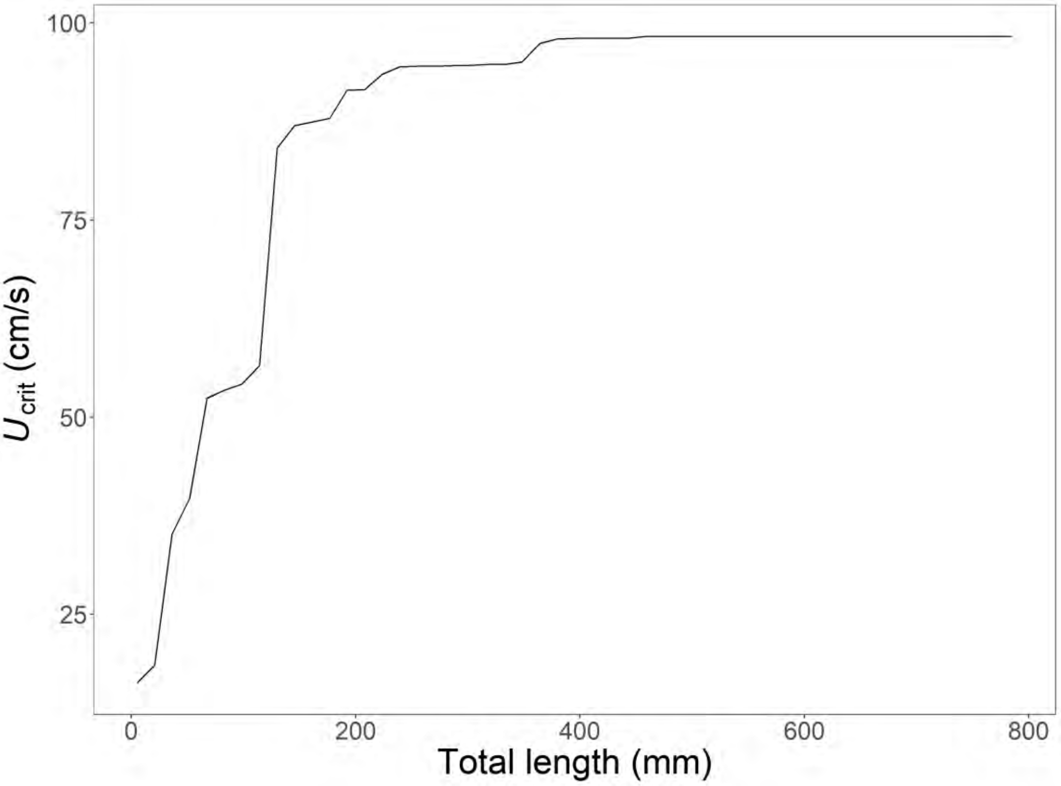


Figure S5.1. Partial dependence of U_{crit} on fish total length based on the random forest analysis of Figure 5.1 (see *Chapter II* for further information).

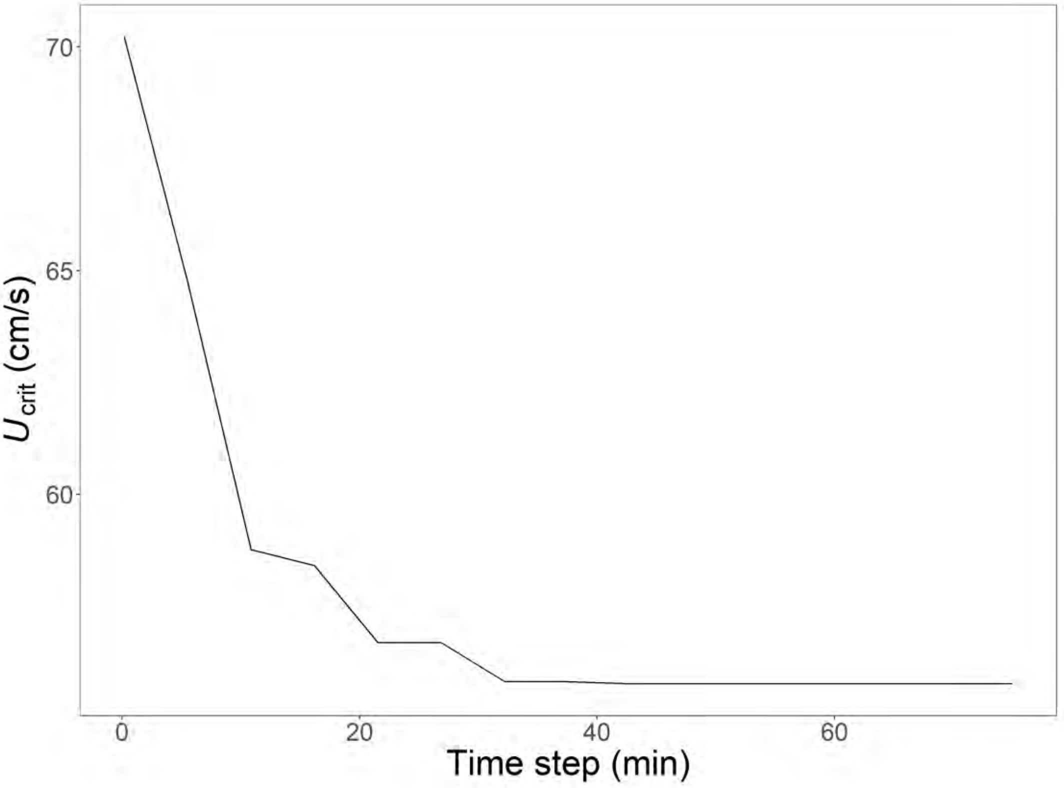


Figure S5.2. Partial dependence of U_{crit} on experimental time step based on the random forest analysis of Figure 5.1 (see *Chapter II* for further information).

Table S5.2. Significant linear regression functions of U_{crit} with fish total length (TL) ($\log_{10} U_{crit} = a + b \log_{10}$ TL) by species (see also Figure 5.3). r^2 = coefficient of determination, P = P value, n = sample size.

Species	***a***	***b***	***r^2***	***P***	***n***
Cyprinus carpio	0.470	0.929	0.348	0.026	14
Dicentrarchus labrax	0.039	1.062	0.860	<0.001	17
Esox lucius	0.576	0.888	0.905	0.003	6
Oncorhynchus kisutch	-0.224	1.339	0.568	0.007	11
Pseudochondrostoma polylepis	1.719	0.329	0.998	0.001	4
Rutilus rutilus	-0.142	1.133	0.956	<0.001	8
Salmo salar	-0.340	1.424	0.926	<0.001	12
Salmo trutta	0.243	0.986	0.733	<0.001	15
Salvelinus fontinalis	0.572	0.821	0.700	<0.001	14

Table S5.3. Significant linear regression functions of U_{crit} with fish total length (TL) ($\log_{10} U_{crit} = a + b \log_{10}$ TL) by family (see also Figure S5.3). r^2 = coefficient of determination, P = P value, n = sample size.

Family	***a***	***b***	***r*²**	***P***	***n***
Cyprinidae	0.262	1.050	0.437	<0.001	28
Esocidae	0.576	0.888	0.905	0.003	6
Leuciscidae	0.104	1.090	0.623	<0.001	26
Moronidae	0.039	1.062	0.860	<0.001	17
Salmonidae	0.156	1.093	0.624	<0.001	69

Table S5.4. Selected linear models of U_{crit} (cm/s) with total length (TL, mm) and different predictors. R^2_{adj} = adjusted coefficient of determination in parentheses, df = degrees of freedom, P = P value, TS = Time step, T = Temperature (°C).

Selected model (R^2_{adj}, AIC)	**Variable**	**Sum of squares**	***df***	***P***
$\log_{10}(U_{crit})$~ $\log_{10}$(TL) × Species + T + T^2 + TS (R^2_{adj} = 0.846, AIC = -197.1)	$\log_{10}$(TL)	14.474	1	<0.001
	Species	3.015	34	<0.001
	Temperature	0.594	1	<0.001
	Temperature^2	0.023	1	0.245
	Time step	0.228	1	<0.001
	$\log_{10}$(TL) × species	1.863	25	<0.001
	Residual	2.403	140	
$\log_{10}(U_{crit})$~ $\log_{10}$(TL) × Species (R^2_{adj} = 0.778, AIC = -124.7)	$\log_{10}$(TL)	14.474	1	<0.001
	Species	3.015	34	<0.001
	$\log_{10}$(TL) × species	1.582	25	<0.001
	Residual	3.530	143	
$\log_{10}(U_{crit})$~ $\log_{10}$(TL) + Species + T + T^2 + TS (R^2_{adj} = 0.768, AIC = -130.0)	$\log_{10}$(TL)	14.474	1	<0.001
	Species	3.015	34	<0.001
	Temperature	0.594	1	<0.001
	Temperature^2	0.023	1	0.397
	Time step	0.228	1	0.003
	Residual	4.266	165	
$\log_{10}(U_{crit})$~ $\log_{10}$(TL) × Family (R^2_{adj} = 0.762, AIC = -131.9)	$\log_{10}$(TL)	14.474	1	<0.001
	Family	2.573	16	<0.001
	$\log_{10}$(TL) × Family	0.937	12	<0.001
	Residual	4.618	174	
$\log_{10}(U_{crit})$~ $\log_{10}$(TL) + Body shape (R^2_{adj} = 0.678, AIC = -96.9)	$\log_{10}$(TL)	14.474	1	<0.001
	Body shape	0.994	3	<0.001
	$\log_{10}$(TL) × Body shape	0.333	3	0.025
	Residual	6.801	196	
$\log_{10}(U_{crit})$~ $\log_{10}$(TL) + Native status (R^2_{adj} = 0.637, AIC = -70.6)	$\log_{10}$(TL)	14.474	1	<0.001
	Native status	0.002	1	0.823
	Residual	8.125	201	

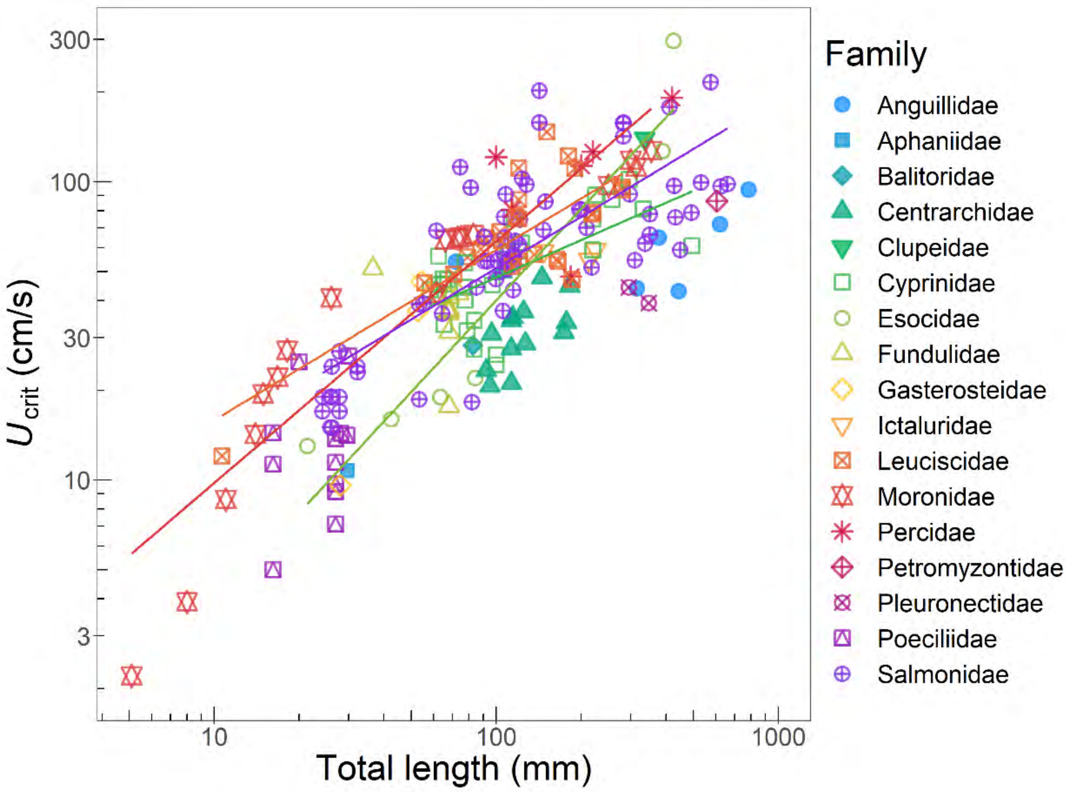


Figure S5.3. Relationship of U_{crit} with fish total length (TL) (note log scales) by taxonomic family.

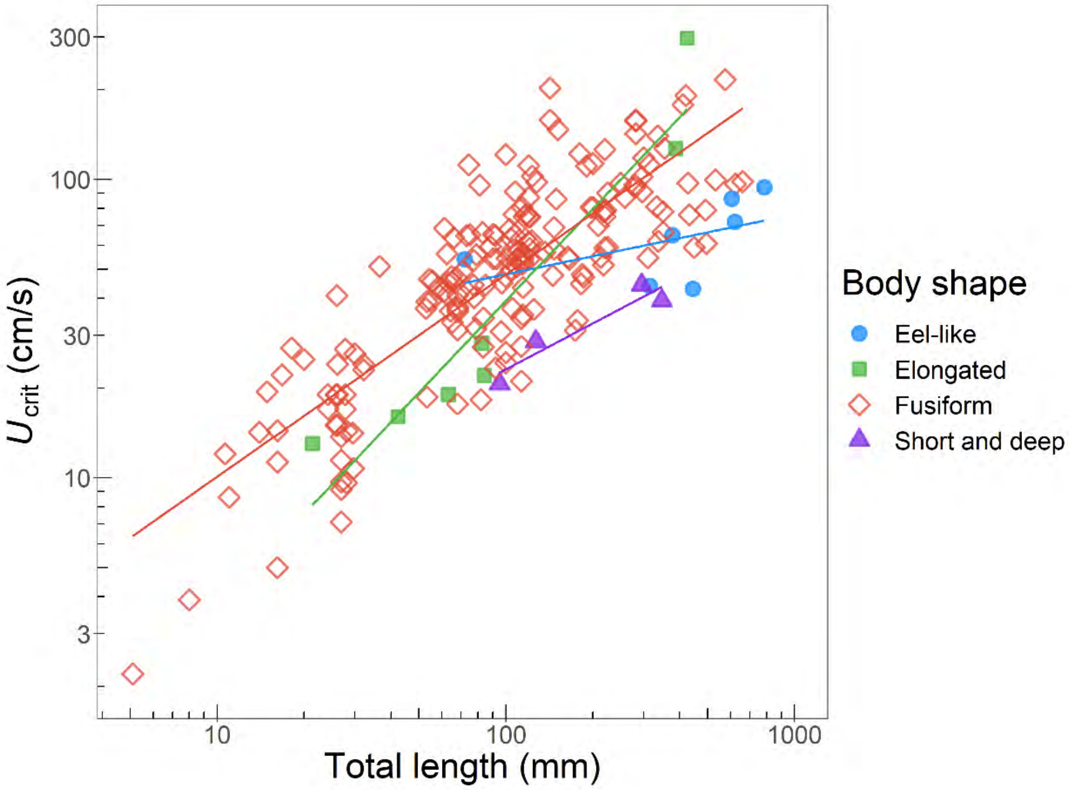


Figure S5.4. Relationship of U_{crit} with fish total length (TL) (note log scales) by body shape.

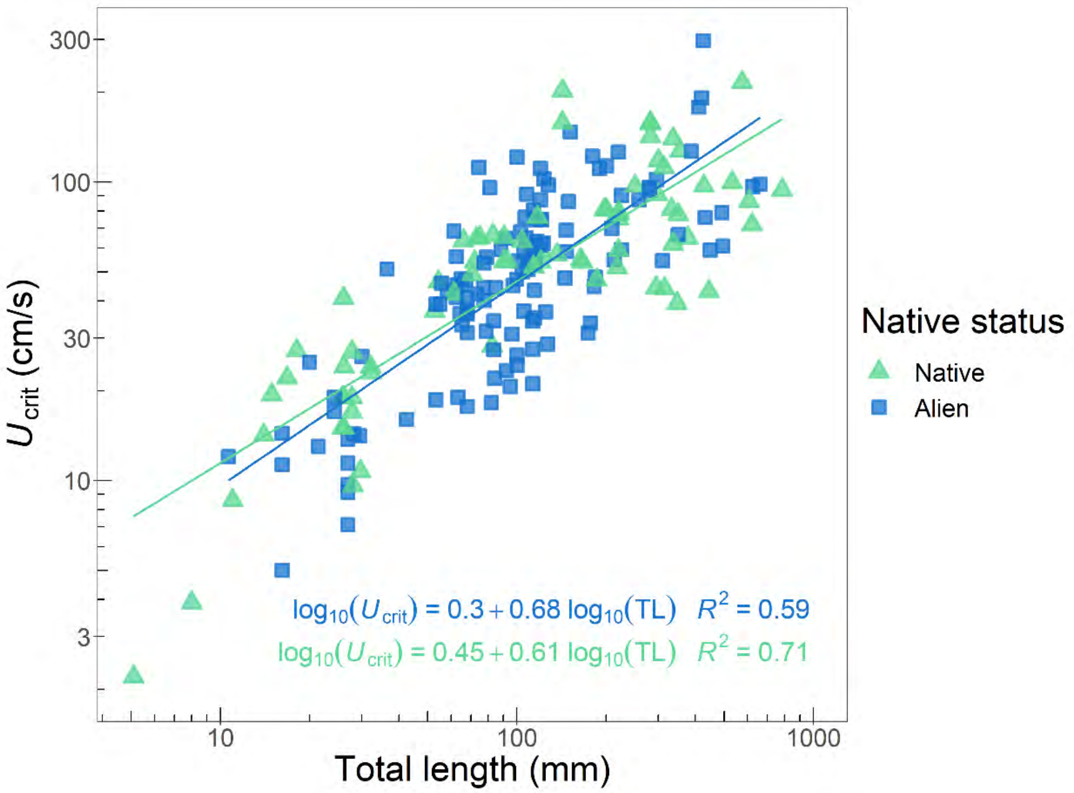


Figure S5.5. Relationship of U_{crit} with fish total length (TL) (note log scales) by native status. Regression linear functions are also shown.

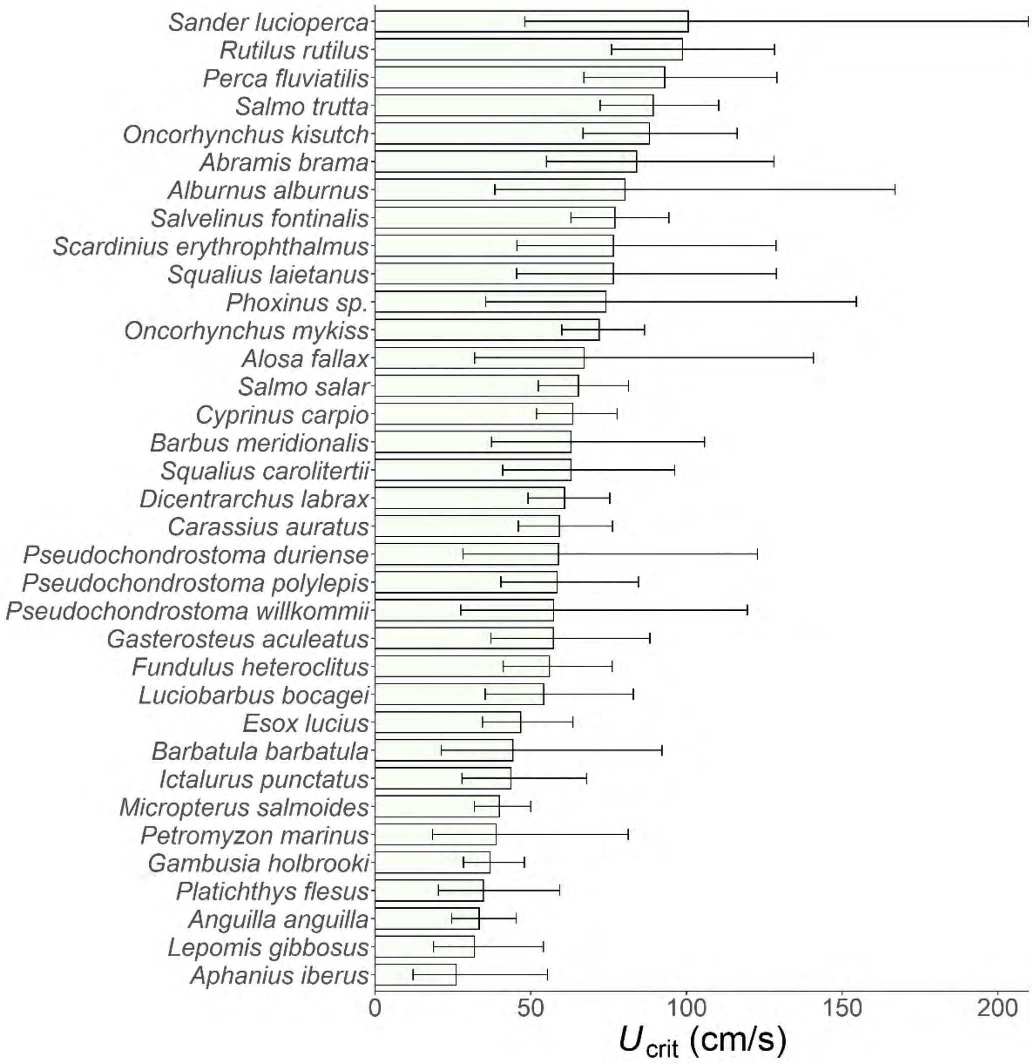


Figure S5.6. Estimated marginal means (EMMs) of the ANCOVA model $\log_{10}(U_{crit})$~ $\log_{10}$(TL) + Species + Time step + Temperature + Temperature^2. Error bars represent the 95% confidence interval.

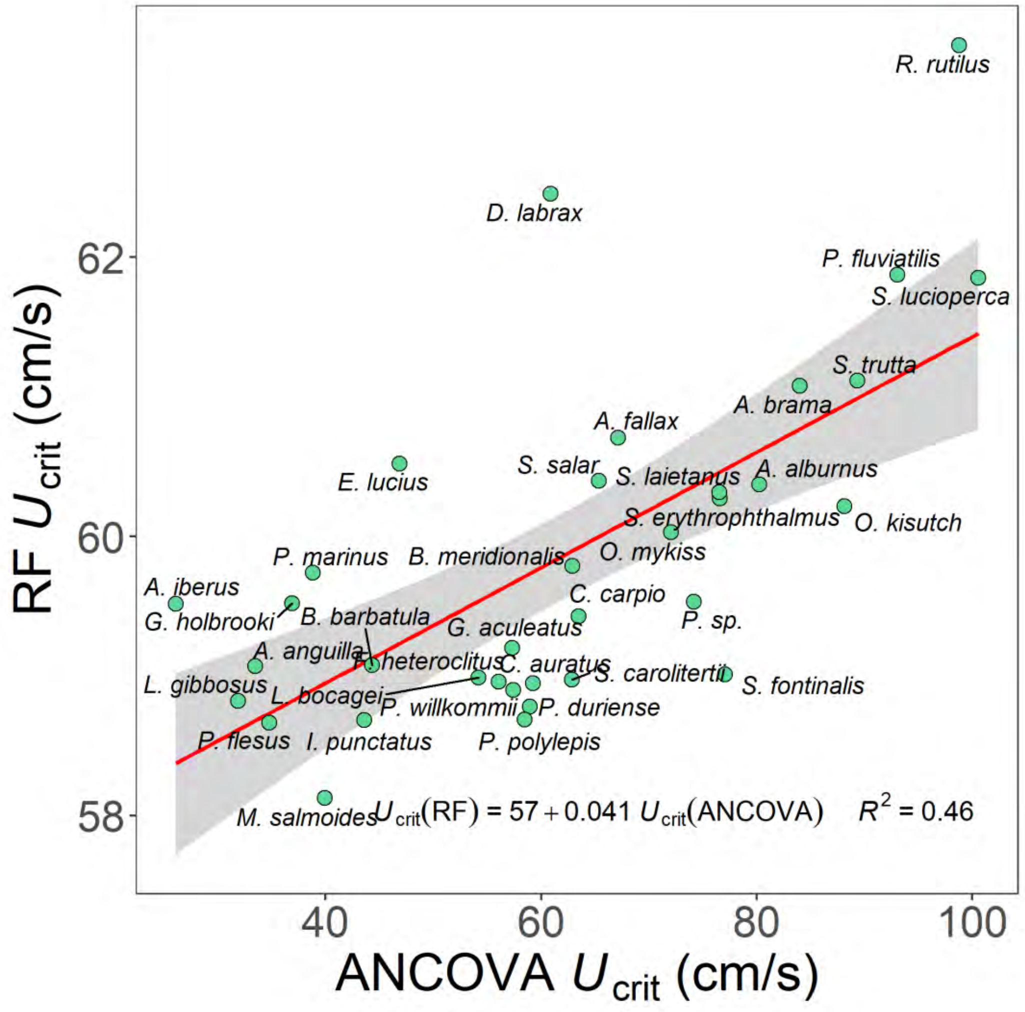


Figure S5.7. Relationship of estimated U_{crit} with random forest (RF, Figure 5.2) with estimated U_{crit} from ANCOVA (Figure S5.5). The red line corresponds to the linear regression function and shaded areas show standard errors. Regression statistics are also given.

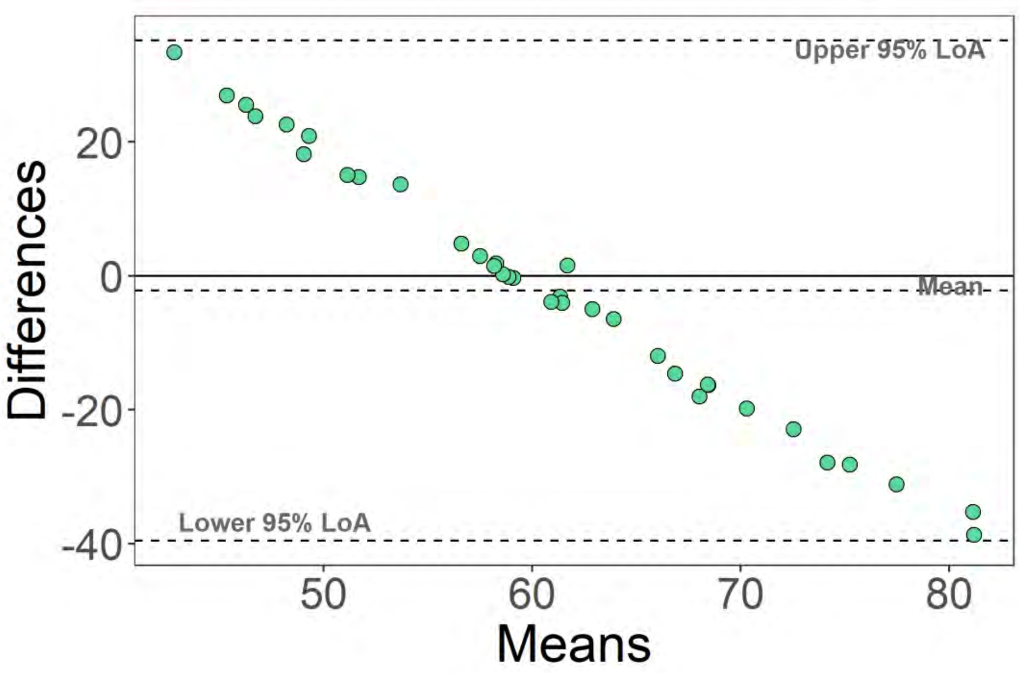


Figure S5.8. Bland-Altman plot for the agreement between estimated U_{crit} with ANCOVA and random forests (RF). The solid line represents the average difference between estimated U_{crit} with the two techniques (i.e. bias), while dotted lines represent the upper and lower 95% confidence limits of agreement (LoA).

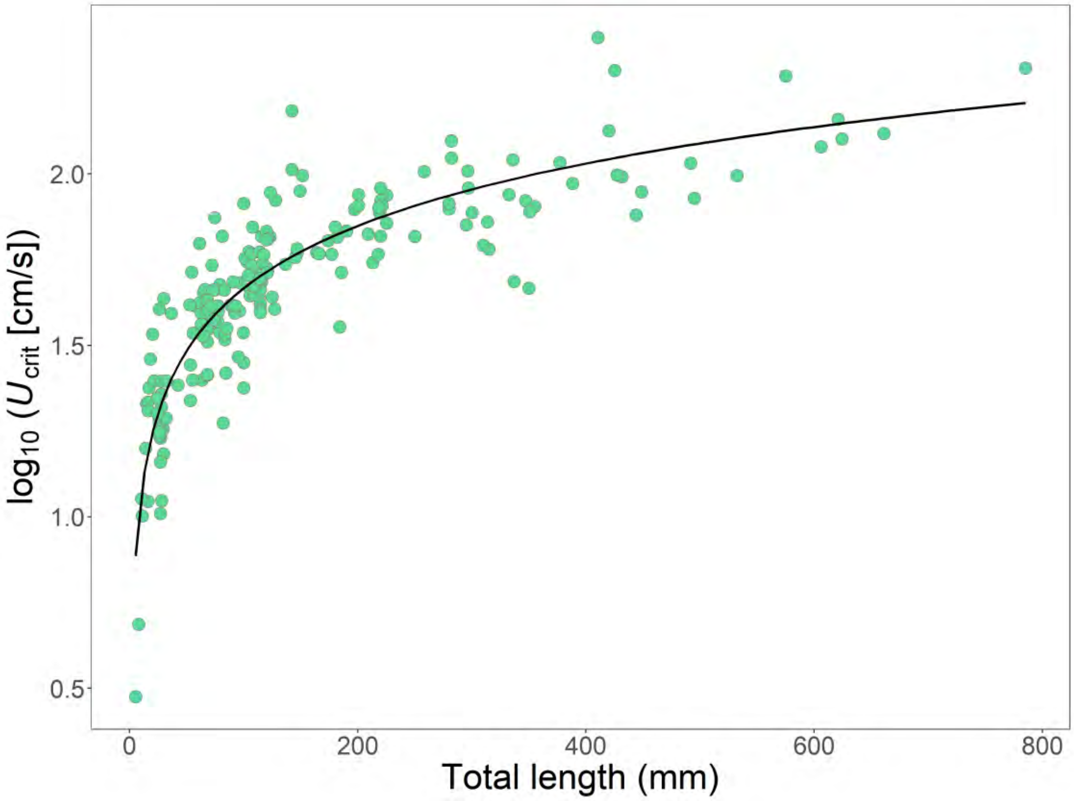


Figure S5.9. Relationship of U_{crit} with fish total length (TL). The black line corresponds to the estimated LMM function. Note that U_{crit} was $\log_{10}$-transformed.

Supplementary materials S6 - The importance of seawater tolerance and native status in mediating the distribution of inland fishes

Table S6.1. References for occurrence records and trait data of the 68 Iberian freshwater fish studied*.*

Species	Occurrence data references	Trait data additional references
Achondrostoma arcasii	(Santos *et al.*, 2006; Ribeiro *et al.*, 2007; GBIF.org, 2019b; Sousa-Santos *et al.*, 2019)	
Achondrostoma oligolepis	(Ribeiro *et al.*, 2007; GBIF.org, 2019c)	
Alburnus alburnus	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; Vinyoles *et al.*, 2007; Hermoso *et al.*, 2008; Fernández-Delgado *et al.*, 2014; Ilhéu *et al.*, 2016; Proyecto LIFE MedWetRivers, 2019; GBIF.org, 2019d)	
Alosa alosa	(Ribeiro *et al.*, 2007; GBIF.org, 2019e)	
Alosa fallax	(Ribeiro *et al.*, 2007; GBIF.org, 2019f)	
Ameiurus melas	(Gante & Santos, 2002; Ribeiro *et al.*, 2007; Hermoso *et al.*, 2008; Fernández-Delgado *et al.*, 2014; Proyecto LIFE MedWetRivers, 2019; GBIF.org, 2019g; Sáez-Gómez & Prenda, 2019)	
Anaecypris hispanica	(Ribeiro *et al.*, 2007; Fernández-Delgado *et al.*, 2014; GBIF.org, 2019h; Sousa-Santos *et al.*, 2019)	(Carrapato & Ribeiro, 2012)
Anguilla anguilla	(Andreu-Soler *et al.*, 2006; Santos *et al.*, 2006; Ribeiro *et al.*, 2007; Fernández-Delgado *et al.*, 2014; GBIF.org, 2019i)	
Aphanius baeticus	(Fernández-Delgado *et al.*, 2014; GBIF.org, 2019j)	
Aphanius iberus	(Andreu-Soler *et al.*, 2006; GBIF.org, 2019k)	
Atherina boyeri	(Ribeiro *et al.*, 2007; GBIF.org, 2019l)	
Australoheros facetus	(Ribeiro *et al.*, 2007; GBIF.org, 2019m)	(Coleman, 1996)
Barbatula quignardi	(GBIF.org, 2019n)	
Barbus haasi	(GBIF.org, 2019o)	
Barbus meridionalis	(GBIF.org, 2019p)	(Benejam *et al.*, 2010)
Carassius auratus	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; Hermoso *et al.*, 2008; Proyecto LIFE MedWetRivers, 2019; GBIF.org, 2019q)	
Carassius gibelio	(Fernández-Delgado *et al.*, 2014; Ribeiro *et al.*, 2015; GBIF.org, 2019r)	(Tarkan *et al.*, 2007)
Chelon labrosus	(Ribeiro *et al.*, 2007; GBIF.org, 2019s)	
Chelon ramada	(Ribeiro *et al.*, 2007; Fernández-Delgado *et al.*, 2014; GBIF.org, 2019t)	(Mousa, 2010)

Species	Occurrence data references	Trait data additional references
Cobitis calderoni	(Santos *et al.*, 2006; Ribeiro *et al.*, 2007; GBIF.org, 2019u)	(Valladolid & Przybylski, 2008; Perdices, 2013)
Cobitis paludica	(Ribeiro *et al.*, 2007; Fernández-Delgado *et al.*, 2014; GBIF.org, 2019v)	
Cobitis vettonica	(GBIF.org, 2019w)	
Cyprinus carpio	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; Hermoso *et al.*, 2008; Fernández-Delgado *et al.*, 2014; Proyecto LIFE MedWetRivers, 2019; GBIF.org, 2019x)	
Dicentrarchus labrax	(Ribeiro *et al.*, 2007; GBIF.org, 2019y)	
Esox lucius	(Ribeiro *et al.*, 2007; Hermoso *et al.*, 2008; Fernández-Delgado *et al.*, 2014; Proyecto LIFE MedWetRivers, 2019; GBIF.org, 2019z)	
Gambusia holbrooki	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; Hermoso *et al.*, 2008; Fernández-Delgado *et al.*, 2014; Murphy *et al.*, 2015; Proyecto LIFE MedWetRivers, 2019; GBIF.org, 2019aa)	
Gasterosteus aculeatus	(Ribeiro *et al.*, 2007; GBIF.org, 2019ab)	
Gobio lozanoi	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; GBIF.org, 2019ac)	
Iberochondrostoma lemmingii	(Ribeiro *et al.*, 2007; GBIF.org, 2019ad; Sousa-Santos *et al.*, 2019)	
Iberochondrostoma lusitanicum	(Ribeiro *et al.*, 2007; GBIF.org, 2019ae; Sousa-Santos *et al.*, 2019)	(Sousa-Santos *et al.*, 2016)
Ictalurus punctatus	(Hermoso *et al.*, 2008; Banha *et al.*, 2017; GBIF.org, 2019af)	
Lampetra planeri	(Ribeiro *et al.*, 2007; GBIF.org, 2019ag)	
Lepomis gibbosus	(Andreu-Soler *et al.*, 2006; Santos *et al.*, 2006; Ribeiro *et al.*, 2007; Hermoso *et al.*, 2008; Fernández-Delgado *et al.*, 2014; Proyecto LIFE MedWetRivers, 2019; GBIF.org, 2019ah)	
Liza aurata	(Ribeiro *et al.*, 2007; GBIF.org, 2019ai)	
Luciobarbus bocagei	(Santos *et al.*, 2006; Ribeiro *et al.*, 2007; GBIF.org, 2019aj; Godinho, 2019)	(Lobón-Cerviá & Fernández-Delgado, 1984)
Luciobarbus comizo	(Ribeiro *et al.*, 2007; GBIF.org, 2019ak)	
Luciobarbus graellsii	(GBIF.org, 2019al)	
Luciobarbus guiraonis	(GBIF.org, 2019am)	
Luciobarbus microcephalus	(Ribeiro *et al.*, 2007; GBIF.org, 2019an)	

Species	Occurrence data references	Trait data additional references
Luciobarbus sclateri	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; Fernández-Delgado *et al.*, 2014; GBIF.org, 2019ao)	
Micropterus salmoides	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; Hermoso *et al.*, 2008; Fernández-Delgado *et al.*, 2014; Bae *et al.*, 2018; Proyecto LIFE MedWetRivers, 2019; GBIF.org, 2019ap)	
Mugil cephalus	(Ribeiro *et al.*, 2007; Fernández-Delgado *et al.*, 2014; GBIF.org, 2019aq)	(Soyinka, 2014)
Oncorhynchus mykiss	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; Fernández-Delgado *et al.*, 2014; Proyecto LIFE MedWetRivers, 2019; GBIF.org, 2019ar)	
Parachondrostoma arrigonis	(GBIF.org, 2019as)	
Parachondrostoma miegii	(GBIF.org, 2019at)	
Parachondrostoma turiense	(GBIF.org, 2019au)	
Petromyzon marinus	(Ribeiro *et al.*, 2007; GBIF.org, 2019av)	(Smith & Marsden, 2009)
Phoxinus bigerri	(GBIF.org, 2019aw)	
Platichthys flesus	(Ribeiro *et al.*, 2007; GBIF.org, 2019ax)	
Pomatoschistus microps	(GBIF.org, 2019ay)	(Miller, 1986; Bouchereau, Joyeux & Quignard, 1989)
Pseudochondrostoma duriense	(Ribeiro *et al.*, 2007; GBIF.org, 2019az; Sousa-Santos *et al.*, 2019)	
Pseudochondrostoma polylepis	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; GBIF.org, 2019ba; Sousa-Santos *et al.*, 2019)	
Pseudochondrostoma willkommii	(Ribeiro *et al.*, 2007; Fernández-Delgado *et al.*, 2014; GBIF.org, 2019bb; Sousa-Santos *et al.*, 2019)	
Rutilus rutilus	(Hermoso *et al.*, 2008; GBIF.org, 2019bc)	
Salaria fluviatilis	(Ribeiro *et al.*, 2007; GBIF.org, 2019bd)	
Salmo salar	(Ribeiro *et al.*, 2007; GBIF.org, 2019be)	
Salmo trutta	(Andreu-Soler *et al.*, 2006; Santos *et al.*, 2006; Ribeiro *et al.*, 2007; Hermoso *et al.*, 2008; Fernández-Delgado *et al.*, 2014; GBIF.org, 2019bf)	
Sander lucioperca	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; Pérez-Bote & Romero, 2009; Proyecto LIFE MedWetRivers, 2019; GBIF.org, 2019bg)	
Scardinius erythrophthalmus	(GBIF.org, 2019bh)	

Species	Occurrence data references	Trait data additional references
Silurus glanis	(Benejam *et al.*, 2007; Moreno-Valcárcel, De Miguel & Fernández-Delgado, 2013; Gago *et al.*, 2016; Proyecto LIFE MedWetRivers, 2019; GBIF.org, 2019bi; Sáez-Gómez & Prenda, 2019)	
Squalius alburnoides	(Santos *et al.*, 2006; Ribeiro *et al.*, 2007; Fernández-Delgado *et al.*, 2014; GBIF.org, 2019bj)	(Ribeiro *et al.*, 2003)
Squalius carolitertii	(Santos *et al.*, 2006; Ribeiro *et al.*, 2007; GBIF.org, 2019bk; Godinho, 2019; Sousa-Santos *et al.*, 2019)	
Squalius laietanus	(GBIF.org, 2019bl)	
Squalius pyrenaicus	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; Fernández-Delgado *et al.*, 2014; GBIF.org, 2019bm; Sousa-Santos *et al.*, 2019)	(Fernández-Delgado & Herrera, 1995)
Squalius valentinus	(GBIF.org, 2019bn)	
Syngnathus abaster	(GBIF.org, 2019bo)	
Tinca tinca	(Andreu-Soler *et al.*, 2006; Ribeiro *et al.*, 2007; Fernández-Delgado *et al.*, 2014; GBIF.org, 2019bp)	
Valencia hispanica	(GBIF.org, 2019bq)	

Table S6.2. Trait description and results of the redundancy analysis (RDA) of variable importances of predictors of Iberian fish distributions ($R^2 = 0.489$) using traits as constraints or explanatory variables. *df* = degrees of freedom, *F* = *F*-statistics, *P* values significant at a level of 0.05 are indicated in bold. B = binary trait; C = continuous trait. Note that continuous traits were previously $\log_{10}$-transformed. *n* = 68.

	Description	***df***	**Variance**	***F***	***P***
Maximum length	Maximum total length expressed in centimetres (C).	1	0.524	3.175	**0.003**
Maximum longevity	Maximum individual age reported expressed in years (C).	1	0.127	0.769	0.638
Rheophily	Species preferring to live in running water and adapted to fluvial habitats (B).	1	0.349	2.118	**0.028**
Limnophily	Species preferring to live in stagnant waters (B).	1	0.105	0.637	0.795
Diadromy	Species that have evolved mechanisms to transition between seawater and freshwater environments (B).	1	0.339	2.056	**0.038**
Potamodromy	Species that migrate within the river basin having their entire life cycle occurring within fresh waters of a river system (B).	1	0.957	5.801	**0.001**
Benthic	Species that live, feed and reproduce on the sediment surface. They are usually sensitive to siltation and benthic oxygen depletion (B).	1	0.157	0.951	0.465
Water column	Active swimmer species that prefer to live and feed in the water column (B).	1	0.132	0.798	0.650
Invertivory	Species that feed on invertebrates (B).	1	0.268	1.622	0.093
Omnivory	Species that consume considerable amounts of both plant and animal material (B).	1	0.223	1.352	0.203
Piscivory	Species that eat mainly other fishes (B).	1	0.161	0.976	0.444
Tolerance	Species with a large water quality and habitat flexibility (B).	1	0.309	1.870	0.054
Intolerance	Species with a low water quality and habitat flexibility. Those that first decline with environmental degradation or after a disturbance (B).	1	0.200	1.213	0.291
Reproductive span	Length of breeding season expressed in months (C).	1	0.260	1.575	0.122
Lithophilic reproduction	Species that deposit eggs on a rock or gravel bottom where their embryos and larvae develop. They are sensitive to siltation, requiring clean gravel substrates for reproductive success (B).	1	0.228	1.381	0.184
Phytophilic reproduction	Species that deposit eggs to submerged, live or dead, plants. They are usually adapted to habitats with muddy bottoms and low oxygen concentration (B).	1	0.248	1.505	0.142
Residuals		51	6.649		

Table S6.3. Predictor variables compiled and their value ranges. Variables used in SDMs of Iberian freshwater fish species are in bold. Data based on 100 km^2 grid cells. Asterisks indicate layers calculated from source data using QGIS (QGIS Development Team, 2019) geoprocessing tools.

Predictor variables (TYPE)	Abbreviation	Range	Source
CLIMATE			
Annual mean temperature (°C)	MeanTem	3.0 – 18.3	1
Temperature annual range (°C)	TemAnnRan	8.7 – 34.2	1
Average annual precipitation within sub-catchment (mm)	AnnPrec	242.0 – 1601.6	2*
Average precipitation seasonality within sub-catchment (%)	PrecSeas	11.1 – 76.5	2*
Solar radiation (W/m^2)	SolRadiat	1633.3 – 2219.7	3
Potential evapotranspiration (mm/day)	PET	1.6 – 7.8	3
TOPOGRAPHY			
Elevation (m)	Elev	0.0 – 2801.6	4
Slope (°)	Slo	0.0 – 27.2	4*
Topographic index	TopInd	-1.3 – 10.3	4*
Distance to the sea (km)	DisSea	0.0 – 928.5	4*
Strahler stream order	StrOrdSt	1.0 – 6.4	4*
Shreve stream order	StrOrdSh	1.0 – 4383.7	4*
Basin ID	BasinID	-	
SOIL			
Soil organic carbon across sub-catchment (g/kg)	ORCDRC	2.5 – 116.6	2*
Soil pH in H_2O across sub-catchment (pH x 10)	PHIHOX	54.4 – 80.2	2*
LAND USE & ANTHROPOGENIC			
Upstream reservoir capacity (km^3)	UpResCp	0.0 – 15098.4	5, 6*
Local reservoir capacity (km^3)	LoResCp	0.0 – 4150.0	5, 6*
Population density (people/km^2)	PopDen	0.0 – 8749.8	7
Average agricultural land use with a given sub-catchment (%)	AgrPrc	0.2 – 99.4	2*
Average forest land use within a given sub-catchment (%)	ForPrc	0.0 – 100.0	2*
Average urban land use within a given sub-catchment (%)	UrbPrc	0.0 – 68.5	2*

Sources: 1 = WorldClim, Global Climate Data (http://worldclim.org/version2); 2 = (Domisch, Amatulli & Jetz, 2015); 3 = Universitat Autònoma de Barcelona, Atlas climático digital de la Península Ibérica (http://www.opengis.uab.es/); 4= Spanish National Center for Geographic Information (http://centrodedescargas.cnig.es/); 5 = (Melo & Gomes, 1992); 6 = Spanish Ministry of Agriculture, Food and Environment (http://sig.magrama.es/); 7 = DIVA-GIS data (http://www.diva-gis.org/datadown).

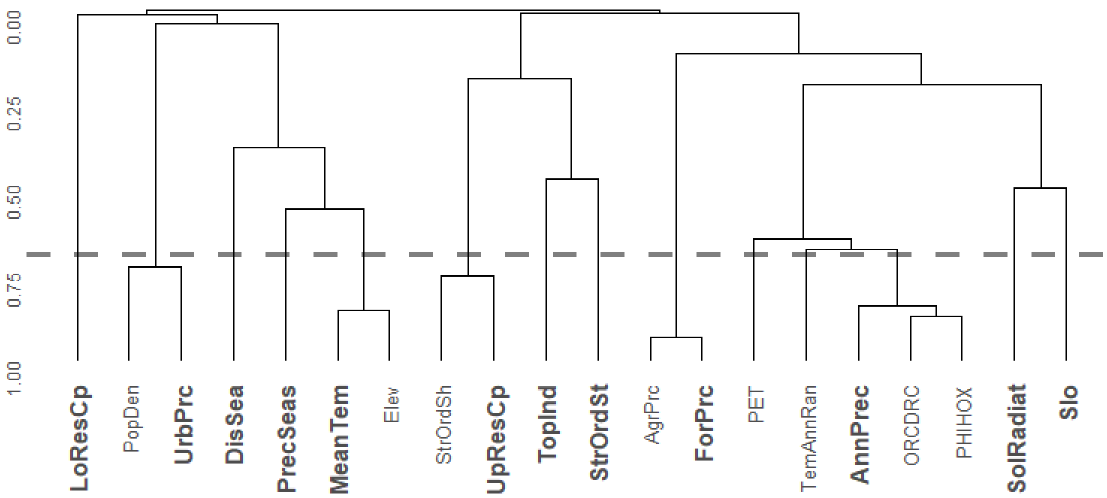


Figure S6.1. Hierarchical cluster analysis based on the correlation matrix to identify correlated (|*r*| ≥ 0.7) and uncorrelated predictor variables. Selected predictors for the SDMs are in bold. See Table S6.3 for abbreviations.

Table S6.4. Modelling options of the four algorithms. We used the default parameters.

Algorithms	**Modelling options**
Generalised linear models (GLM)	type: ‘quadratic’ family: binomial (link = ‘logit’) test: ‘AIC’
Generalised boosted models (boosted regression trees, BRT)	distribution: ‘bernoulli’ n.trees: 2500 interaction.depth: 7 n.minobsinnode: 5 shrinkage: 0.001 bag.fraction: 0.5 train.fraction: 1 cv.folds: 3 keep.data: FALSE verbose: FALSE perf.method: ‘cv’ n.cores: 1
Random forests (RF)	do.classif: TRUE ntree: 500 mtry: 'default' nodesize: 5 maxnodes: NULL
Maxent	background_data_dir: We used the same pseudo absences than for other models as background data. maximumiterations: 200 visible: FALSE linear: TRUE quadratic: TRUE product: TRUE threshold: TRUE hinge: TRUE lq2lqptthreshold: 80 l2lqthreshold: 10 hingethreshold: 15 bea_threshold: -1.0 beta_categorical: -1.0 beta_lqp: -1.0 beta_hinge: -1.0 betamultiplier: 1 defaultprevalence: 0.5

Table S6.5. Number of species occurrences (*n*) used to build distribution models and four associated model evaluation metrics*:* AUC = Area under the receiver operating characteristic curve (ranging from 0 to 1, with values of 0.5 indicating a prediction with no better accuracy than by chance, and values of 1 showing perfect accuracy); TSS = True skill statistic (ranging from -1 to 1, with values of -1 indicating predictive abilities not better than a random model, 0 indicating an indiscriminate model and 1 indicating a perfect model); Specificity (i.e. the proportion of correctly predicted absences) and Sensitivity (i.e. the proportion of correctly predicted presences). SD = Standard deviation.

Species	**Native status**	**Division**	***n***	**AUC ± SD**	**TSS**	**Specificity**	**Sensitivity**
Achondrostoma arcasii	Native	Primary	1681	0.935 ± 0.039	0.769	86.9	90.1
Achondrostoma oligolepis	Native	Primary	135	0.971 ± 0.034	0.920	95.8	96.4
Alburnus alburnus	Introduced	Primary	552	0.921 ± 0.035	0.737	86.9	87.0
Alosa alosa	Native	Peripheral	127	0.950 ± 0.058	0.843	92.5	91.9
Alosa fallax	Native	Peripheral	102	0.935 ± 0.073	0.828	93.6	89.3
Ameiurus melas	Alien	Primary	184	0.941 ± 0.030	0.792	89.0	90.4
Anaecypris hispanica	Native	Primary	158	0.977 ± 0.027	0.929	95.6	97.3
Anguilla anguilla	Native	Peripheral	1214	0.928 ± 0.035	0.750	87.2	87.9
Aphanius baeticus	Native	Secondary	37	0.948 ± 0.079	0.890	98.0	91.1
Aphanius iberus	Native	Secondary	97	0.959 ± 0.051	0.900	96.5	93.6
Atherina boyeri	Native	Peripheral	124	0.935 ± 0.076	0.825	92.8	89.8
Australoheros facetus	Alien	Secondary	76	0.950 ± 0.063	0.875	94.9	92.8
Barbatula quignardi	Native	Primary	108	0.950 ± 0.048	0.856	93.5	92.3
Barbus haasi	Native	Primary	720	0.971 ± 0.027	0.885	92.3	96.3
Barbus meridionalis	Native	Primary	134	0.990 ± 0.014	0.977	98.9	98.8
Carassius auratus	Alien	Primary	877	0.889 ± 0.037	0.650	81.4	83.8
Carassius gibelio	Alien	Primary	40	0.916 ± 0.078	0.775	93.3	84.9
Chelon labrosus	Native	Peripheral	206	0.965 ± 0.046	0.877	92.6	95.3
Chelon ramada	Native	Peripheral	178	0.964 ± 0.029	0.873	92.7	94.8
Cobitis calderoni	Native	Primary	347	0.953 ± 0.033	0.825	89.2	93.4
Cobitis paludica	Native	Primary	1503	0.917 ± 0.040	0.716	86.2	85.5
Cobitis vettonica	Native	Primary	74	0.971 ± 0.038	0.930	97.0	96.0
Cyprinus carpio	Alien	Primary	1602	0.891 ± 0.032	0.652	83.3	82.0

Species	**Native status**	**Division**	***n***	**AUC ± SD**	**TSS**	**Specificity**	**Sensitivity**
Dicentrarchus labrax	Native	Peripheral	134	0.955 ± 0.043	0.882	94.7	93.6
Esox lucius	Alien	Primary	424	0.909 ± 0.042	0.714	86.9	84.7
Gambusia holbrooki	Alien	Secondary	1516	0.919 ± 0.031	0.724	86.7	85.8
Gasterosteus aculeatus	Native	Peripheral	138	0.945 ± 0.054	0.836	92.0	91.7
Gobio lozanoi	Native	Primary	1129	0.899 ± 0.042	0.672	82.8	84.5
Iberochondrostoma lemmingii	Native	Primary	846	0.951 ± 0.033	0.817	88.4	93.4
Iberochondrostoma lusitanicum	Native	Primary	80	0.954 ± 0.052	0.890	96.0	93.1
Ictalurus punctatus	Alien	Primary	36	0.928 ± 0.092	0.821	95.7	86.8
Lampetra planeri	Native	Peripheral	37	0.870 ± 0.079	0.703	89.8	81.6
Lepomis gibbosus	Alien	Primary	1199	0.911 ± 0.036	0.694	84.5	85.1
Liza aurata	Native	Peripheral	123	0.967 ± 0.047	0.896	93.8	95.9
Luciobarbus bocagei	Native	Primary	1568	0.948 ± 0.034	0.799	88.7	91.4
Luciobarbus comizo	Native	Primary	477	0.964 ± 0.036	0.860	91.8	94.4
Luciobarbus graellsii	Native	Primary	802	0.979 ± 0.024	0.919	95.2	96.8
Luciobarbus guiraonis	Native	Primary	294	0.980 ± 0.026	0.941	96.9	97.3
Luciobarbus microcephalus	Native	Primary	295	0.986 ± 0.013	0.939	94.8	99.1
Luciobarbus sclateri	Native	Primary	875	0.972 ± 0.029	0.892	92.8	96.5
Micropterus salmoides	Alien	Primary	1157	0.895 ± 0.037	0.664	83.7	82.8
Mugil cephalus	Native	Peripheral	155	0.963 ± 0.047	0.886	94.6	94.1
Oncorhynchus mykiss	Alien	Peripheral	432	0.912 ± 0.028	0.705	84.6	86.1
Parachondrostoma arrigonis	Native	Primary	65	0.963 ± 0.048	0.915	97.1	94.5
Parachondrostoma miegii	Native	Primary	770	0.973 ± 0.031	0.897	94.4	95.4
Parachondrostoma turiense	Native	Primary	35	0.960 ± 0.054	0.905	97.3	93.2
Petromyzon marinus	Native	Peripheral	206	0.964 ± 0.029	0.868	92.3	94.6
Phoxinus bigerri	Native	Primary	187	0.971 ± 0.024	0.891	92.7	96.5
Platichthys flesus	Native	Peripheral	105	0.960 ± 0.039	0.890	95.4	93.7
Pomatoschistus microps	Native	Peripheral	40	0.911 ± 0.090	0.797	95.6	84.5

Species	**Native status**	**Division**	***n***	**AUC ± SD**	**TSS**	**Specificity**	**Sensitivity**
Pseudochondrostoma duriense	Native	Primary	1073	0.971 ± 0.027	0.885	92.8	95.7
Pseudochondrostoma polylepis	Native	Primary	710	0.963 ± 0.032	0.872	93.2	94.1
Pseudochondrostoma willkommii	Native	Primary	700	0.971 ± 0.030	0.886	93.2	95.5
Rutilus rutilus	Alien	Primary	45	0.917 ± 0.065	0.784	91.6	87.4
Salaria fluviatilis	Native	Peripheral	237	0.944 ± 0.032	0.790	89.1	90.0
Salmo salar	Native	Peripheral	188	0.978 ± 0.024	0.938	96.1	97.7
Salmo trutta	Native	Peripheral	2253	0.951 ± 0.026	0.794	89.8	89.6
Sander lucioperca	Alien	Primary	136	0.924 ± 0.070	0.775	90.6	87.1
Scardinius erythrophthalmus	Alien	Primary	69	0.944 ± 0.047	0.860	94.8	91.3
Silurus glanis	Alien	Primary	92	0.914 ± 0.064	0.758	90.4	85.6
Squalius alburnoides	Native	Primary	1536	0.937 ± 0.036	0.768	85.8	91.1
Squalius carolitertii	Native	Primary	693	0.955 ± 0.031	0.840	90.1	93.9
Squalius laietanus	Native	Primary	126	0.969 ± 0.037	0.923	97.0	95.4
Squalius pyrenaicus	Native	Primary	1308	0.929 ± 0.037	0.750	85.5	89.6
Squalius valentinus	Native	Primary	134	0.976 ± 0.030	0.938	96.9	97.0
Syngnathus abaster	Native	Peripheral	49	0.946 ± 0.049	0.873	97.2	90.2
Tinca tinca	Alien	Primary	242	0.856 ± 0.054	0.604	81.9	78.9
Valencia hispanica	Native	Secondary	39	0.947 ± 0.057	0.865	96.9	89.9

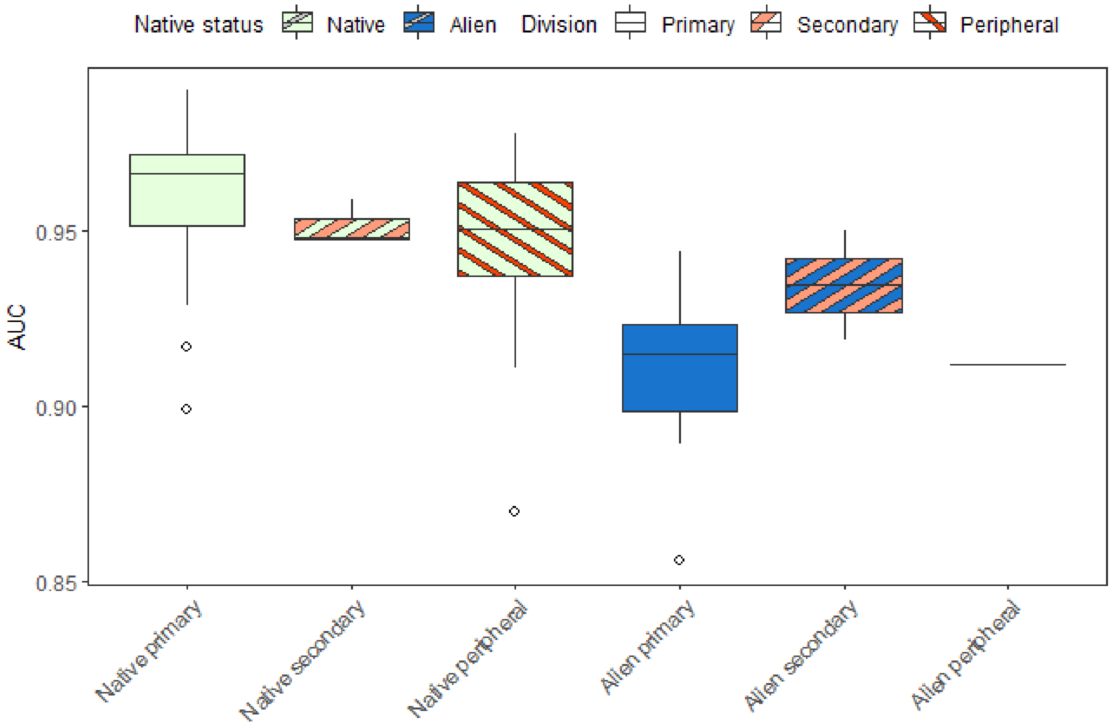


Figure S6.2. Values of the Area Under the receiver operating characteristic Curve (AUC) of SDMs across six groups of Iberian freshwater fish: native primary (*n* = 30), native secondary (*n* = 3), native peripheral (*n* = 18), alien primary (*n* = 14), alien secondary (*n* = 2) and alien peripheral (*n* = 1) species. Boxes correspond to the 25th and 75th percentiles; lines inside a box show the median; whiskers extend to the last observation within 1.5 times the interquartile range from the quartiles and outliers are indicated by empty circles.

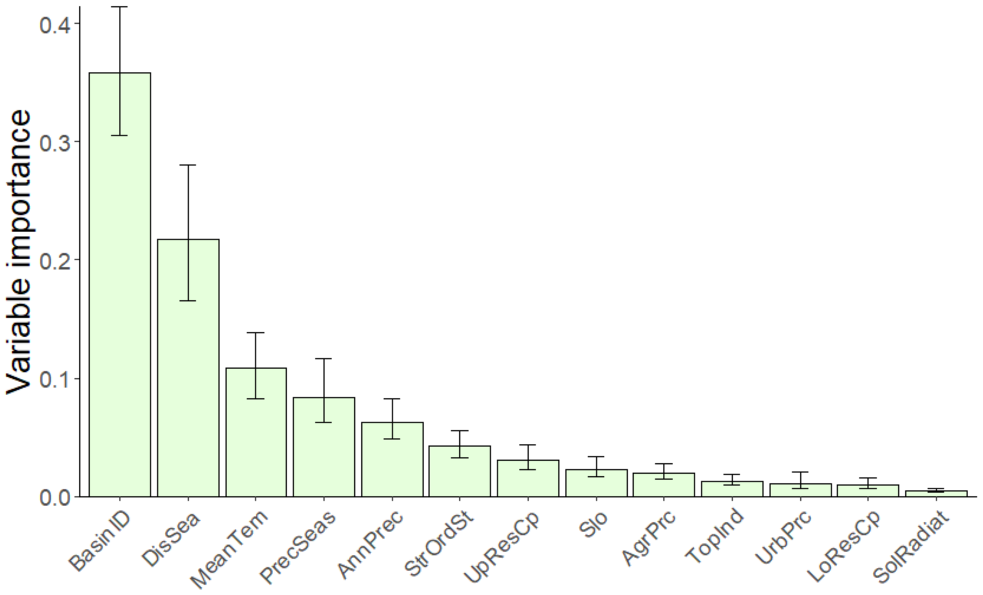


Figure S6.3. Mean variable importance of the different variables used in the species distribution models (SDMs) across the 68 Iberian freshwater species analysed in this study, with its 95% confidence interval based on nonparametric bootstrapping. See Table S6.3 for abbreviations.

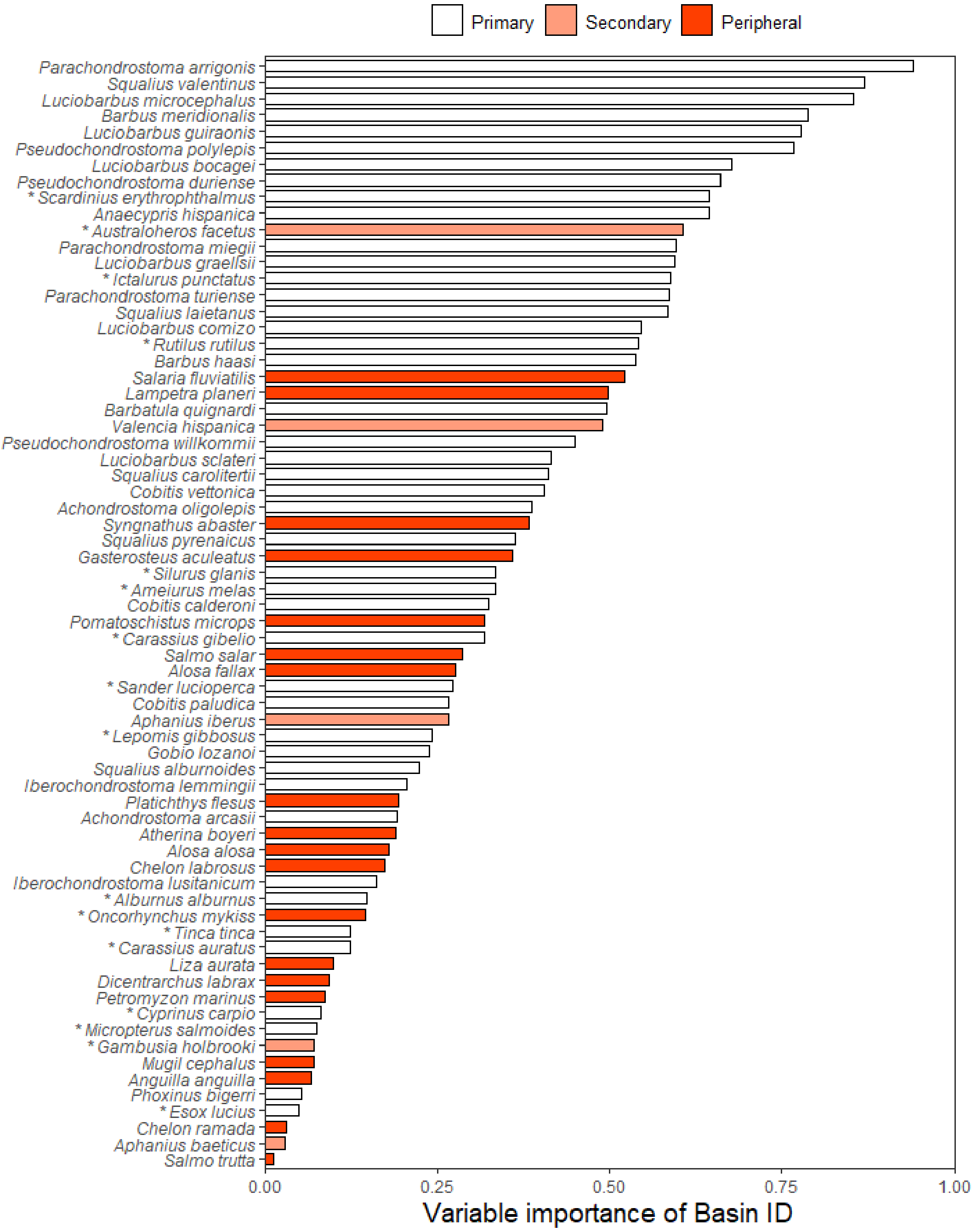


Figure S6.4. Variable importance of ‘Basin ID’ for 68 Iberian freshwater fish species. Alien species are indicated with asterisks (*).

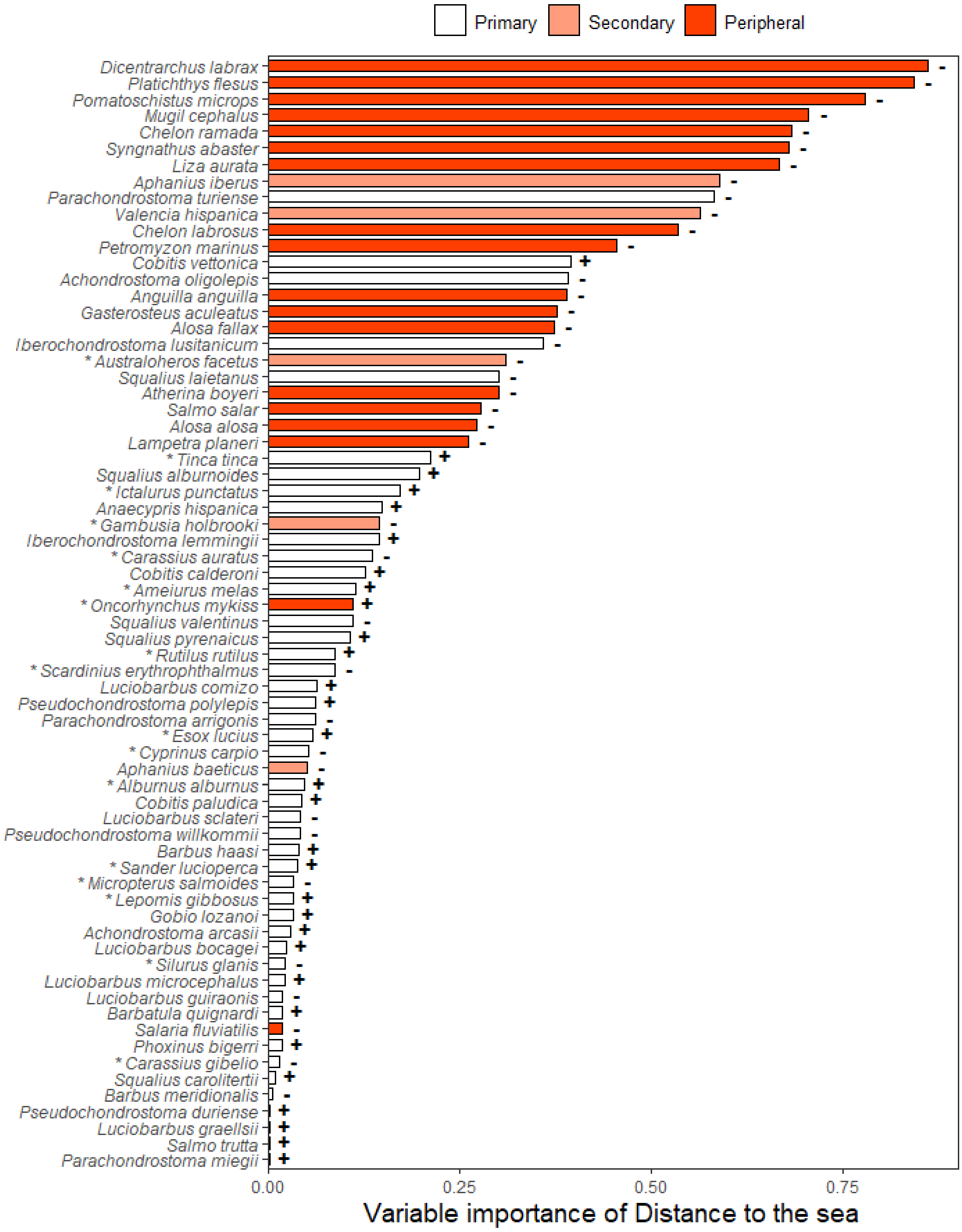


Figure S6.5. Variable importance of 'Distance to the sea' for 68 Iberian freshwater fish species. The signs next to the bars indicate whether the effect of the predictor is positive or negative on the occurrence of a given species. Alien species are indicated with asterisks (*).

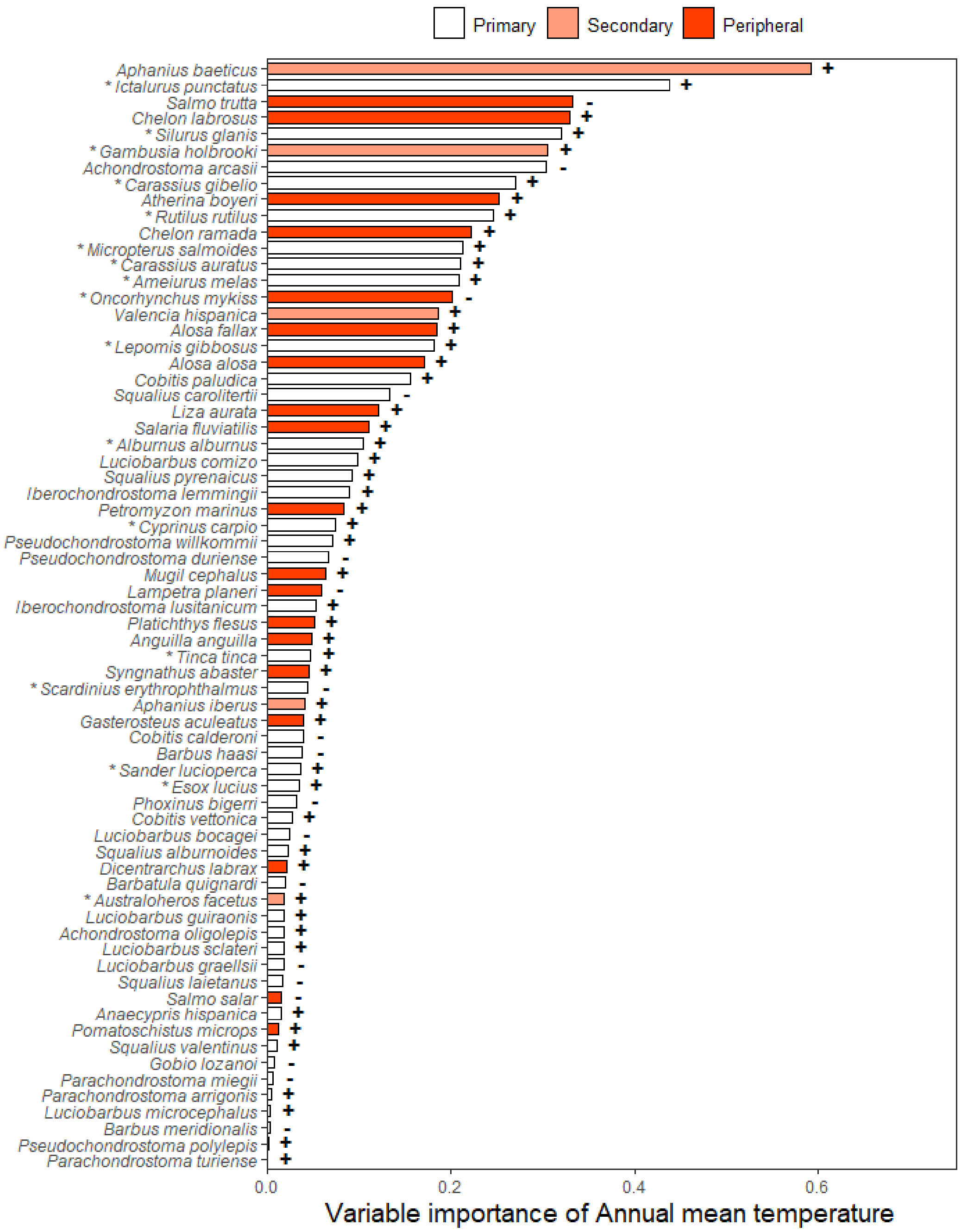


Figure S6.6. Variable importance of ‘Annual mean temperature’ for 68 Iberian freshwater fish species. The signs next to the bars indicate whether the effect of the predictor is positive or negative on the occurrence of a given species. Alien species are indicated with asterisks (*).

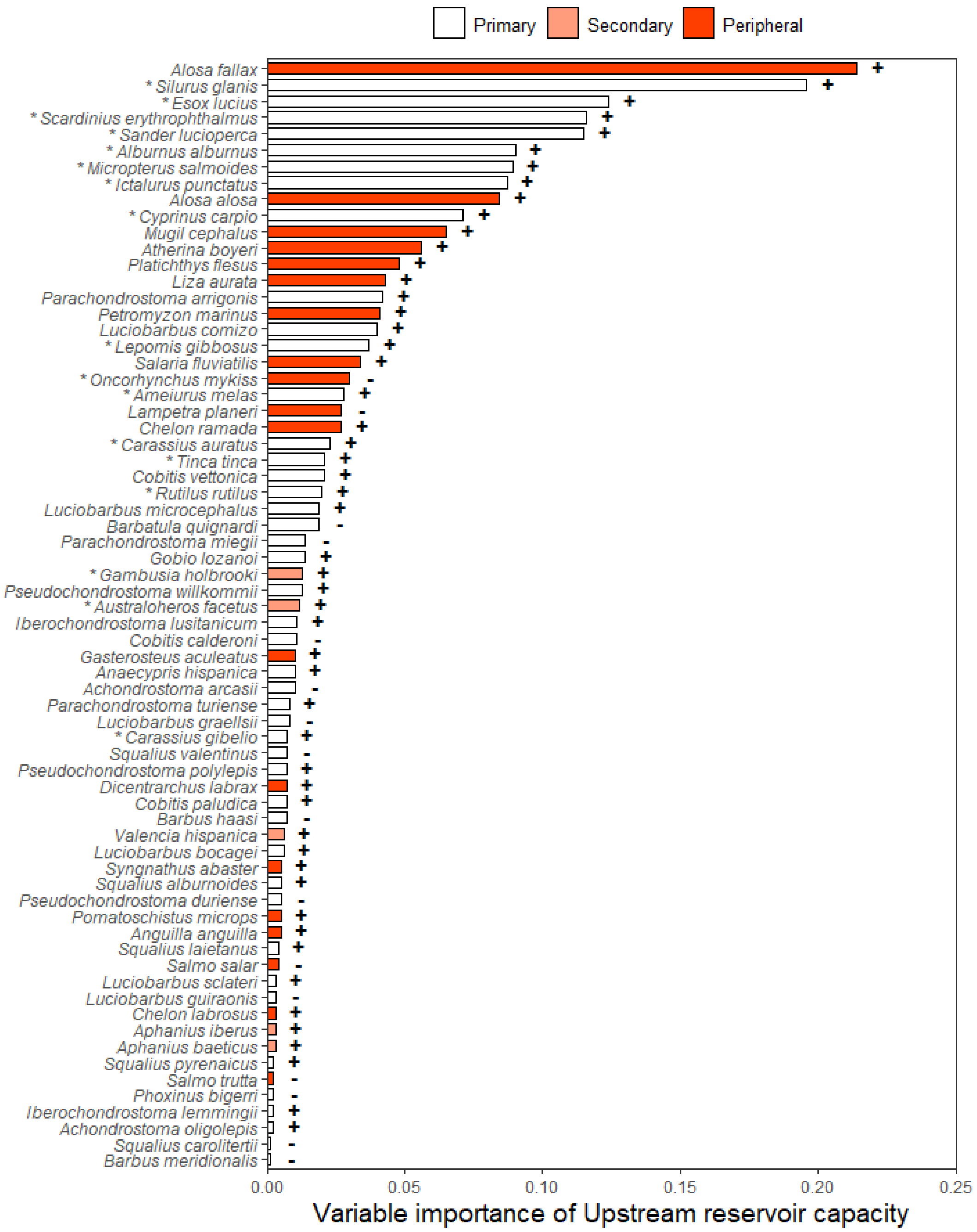


Figure S6.7. Variable importance of ‘Upstream reservoir capacity’ (proxy for hydrological alteration) for 68 Iberian freshwater fish species. The signs next to the bars indicate whether the effect of the predictor is positive or negative on the occurrence of a given species. Alien species are indicated with asterisks (*).

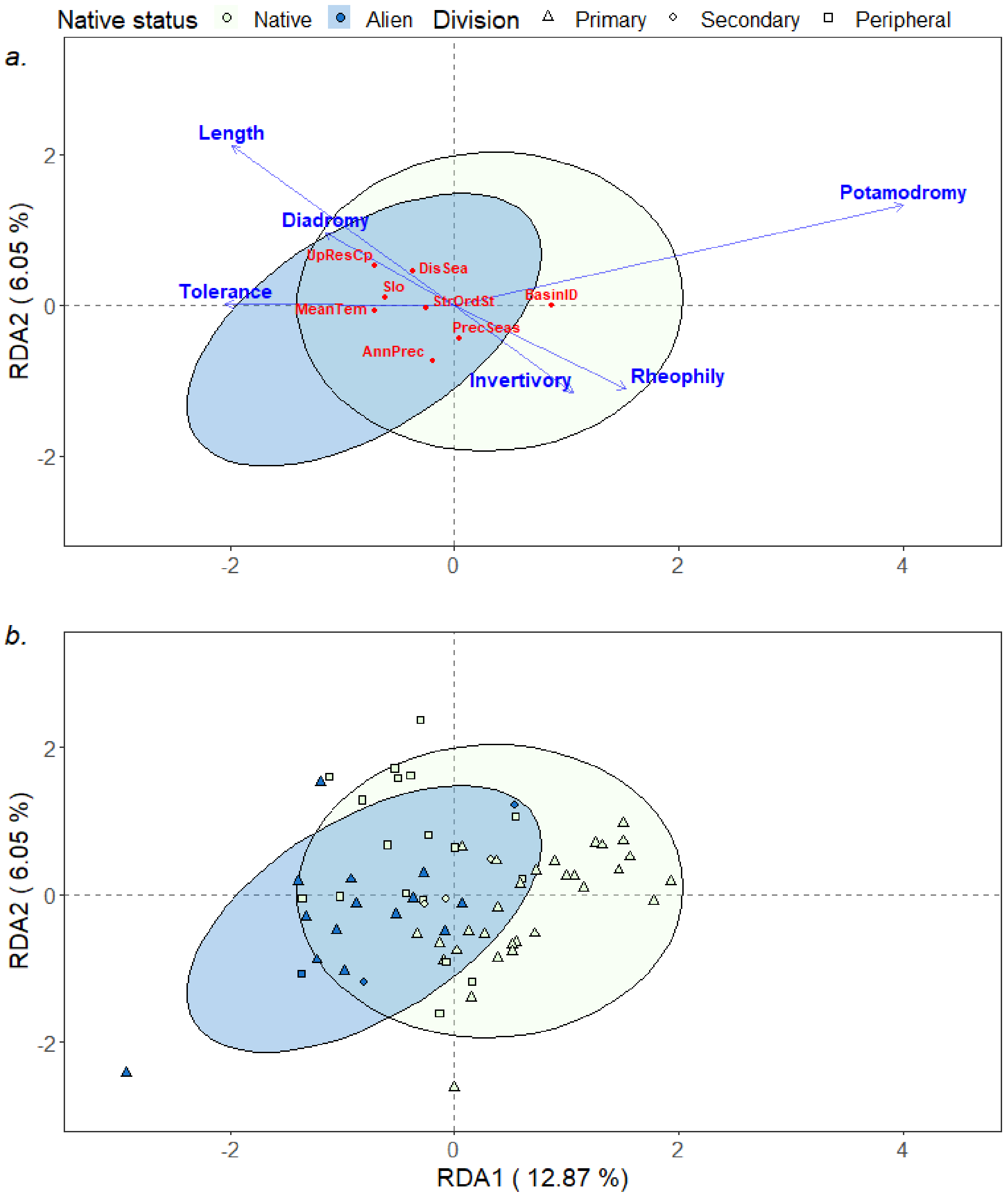


Figure S6.8. (a) Correlation biplot of the redundancy analysis (RDA) of variable importance of the different environmental predictors used in the SDMs. Species traits (constraints in the RDA) are shown as blue arrows. For clarity, only significant and marginally significant traits (*P* < 0.10, see Table S6.3 for further information) and 8 most important environmental variables in SDMs are shown. Predictor variable abbreviations are defined in Table 6.1 and Table S6.3. Note that continuous traits (e.g. fish maximum length) were previously $\log_{10}$-transformed. (b) RDA species scores; triangles, circles and squares represent primary, secondary and peripheral fish, respectively. 95 % confidence ellipses are shown native (light green) and alien fish (blue).

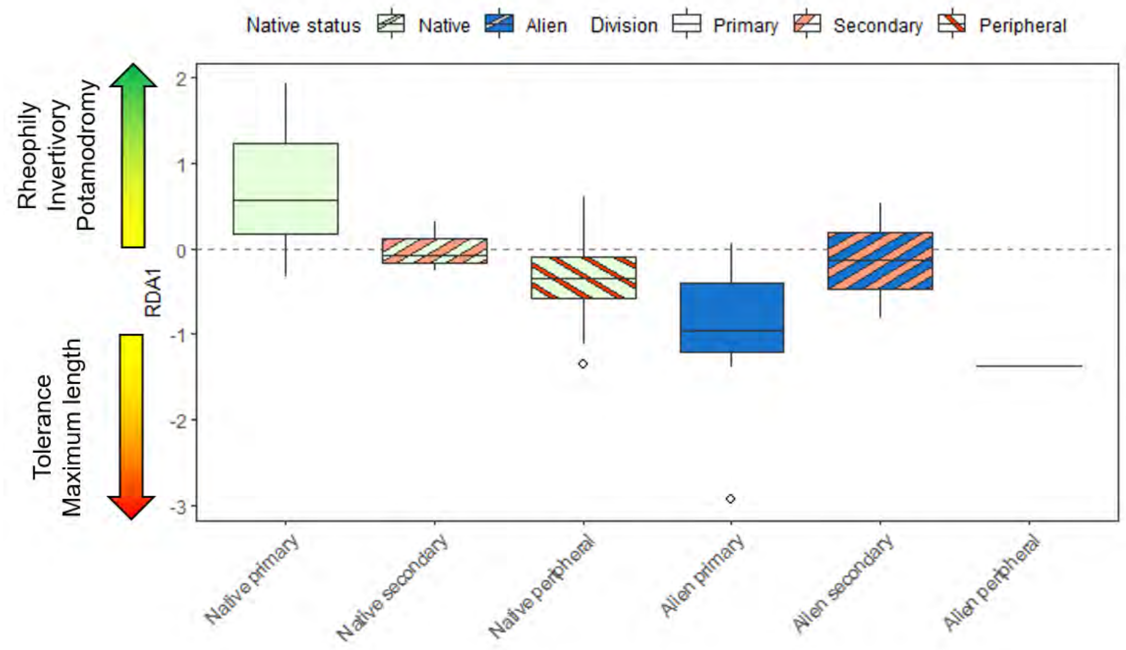


Figure S6.9. First axis scores differences across six groups of Iberian freshwater fish: native primary (*n* = 30), native secondary (*n* = 3), native peripheral (*n* = 18), alien primary (*n* = 14), alien secondary (*n* = 2) and alien peripheral (*n* = 1) species. Boxes correspond to the 25th and 75th percentiles; lines inside a box show the median; whiskers extend to the last observation within 1.5 times the interquartile range from the quartiles and outliers are indicated by circles.

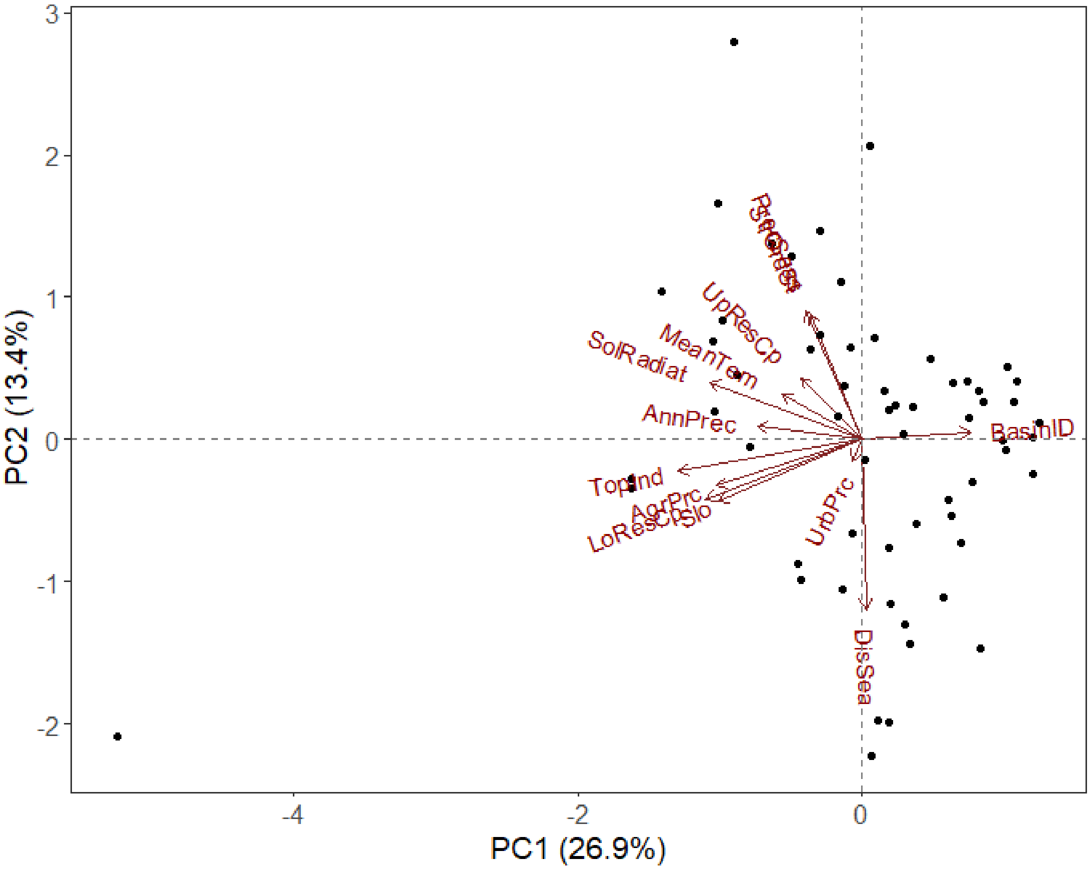


Figure S6.10. Principal component analysis of variable importances of the environmental predictors used in the species distribution models. Arrows represent factor loadings, while points reflect species scores on the first two principal component axes. See Table S6.3 for abbreviations.

Table S6.6. Relationship between traits and the two first axis of the principal components analysis using phylogenetic generalised least squares. Estimates, *t* and *P* values are shown. Note that maximum length, maximum longevity and reproductive span traits were previously $\log_{10}$-transformed.

Trait	**PC1**			**PC2**		
	Estimate	***t***	***P***	**Estimate**	***t***	***P***
Maximum length	0.952	0.741	0.463	0.463	0.492	0.625
Maximum longevity	-0.267	-0.171	0.865	0.921	0.805	0.426
Rheophily	-0.549	-0.658	0.514	-0.730	-1.195	0.239
Limnophily	-0.838	-1.244	0.220	-0.241	-0.489	0.627
Diadromy	0.497	0.407	0.686	-0.723	-0.808	0.424
Potamodromy	-1.113	-1.168	0.249	0.099	0.142	0.888
Benthic	-0.813	-1.262	0.214	-0.451	-0.956	0.344
Water column	-0.264	-0.367	0.718	0.050	0.943	0.925
Invertivory	1.197	1.350	0.184	0.986	1.519	0.136
Omnivory	2.534	3.240	**0.002**	0.534	0.933	0.356
Piscivory	-0.154	-0.158	0.875	0.248	0.349	0.729
Tolerance	-0.476	-0.774	0.443	-0.270	-0.599	0.552
Intolerance	0.379	0.221	0.827	-0.705	-0.561	0.578
Reproductive span	2.319	1.313	0.196	-0.575	-0.445	0.659
Lithophilic reproduction	-0.836	-0.976	0.335	-0.781	-0.289	0.774
Phytophilic reproduction	-0.105	-0.137	0.892	-0.824	-1.471	0.149

Appendix S6.1: The usefulness of Darlington classification

In order to support the usefulness of Darlington classification in our analysis, we compare it here with halotolerance experimental quantitative data, for those species for which they are available, and with other halotolerance classifications. For that purpose, we searched for data on the upper LC_{50} halotolerance limit (i.e. the maximum salt concentration that killed 50% of the individuals in the experiment; Schultz & McCormick, 2013) and/or the halotolerance breadth (range) for inland fishes species of the Iberian Peninsula but only found data for 19 out of 100 species (11 primary, 3 secondary and 5 peripheral) in 13 different studies (see Table S6.7 for references), of which 14 were alien species and 5 were native, but none were Iberian endemics. As Godinho and Ferreira (1998) already highlighted, there is no quantitative information available on the halotolerance of Iberian endemic fish. In order to increase the sample size and robustness of the results we also used data of the upper LC_{50} halotolerance limit for 80 other inland fishes (20 primary, 37 secondary and 23 peripheral species) from Schultz & McCormick (Schultz & McCormick, 2013) that are not present in the study area. We also classified all these species (i.e. the 100 Iberian species and the other 80 inland fish considered) as stenohaline (Schultz & McCormick, 2013) or euryhaline (i.e. 'capable of surviving in a wide range of salinity') following the existing records of the literature (see Table S6.7 for references). Note, however, that many studies do not provide the criteria or empirical evidence used for such classification, in some cases likely rely simply on Myers/Darlington classification and in many cases the classification is not based on empirical data and is thus dubious; for instance, although we did not find empirical data on halotolerance for Iberian endemics, Costedoat & Gilles (2009) suggest that 'the different [European] Cyprinids species are all defined as stenohaline (primary species following Banarescu nomenclature', which we marked as 'stenohaline?' in Table S6.7). Finally, we also compiled the habitat classification of species (freshwater, brackishwater and/or marine) from FishBase (2019), for comparative purposes.

Table S6.7. Seawater tolerance of the 100 Iberian native and alien inland fish*.* Habitat data was obtained from FishBase (2019) and Darlington's division from Berra (2001). The references are for the euryhalinity categorisation and the upper LC_{50} salinity limit, when available. The experimental method to calculate the upper LC_{50} salinity limit is also shown (Dir = Direct; Grad = Gradual). Ste = stenohaline; Eur = euryhaline; FW = freshwater; BW = brackishwater; M = marine; Pri = primary; Sec = secondary; Per = peripheral; ? = doubtful classification based on generalisations (mostly Costedoat & Gilles, 2009) or sometimes contradictory results.

Species	**Upper LC_{50} salinity limit (ppt)**	**Method**	**Euryhalinity**	**Habitat**	**Darlington's divisions**	**References**
Abramis brama			Ste	FW/BW	Pri	(Quigley, 2014)
Achondrostoma arcasii			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Achondrostoma occidentale			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Achondrostoma oligolepis			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Achondrostoma salmantinum			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Acipenser sturio			Eur	FW/BW/M	Per	(Schultz & McCormick, 2013)
Alburnus alburnus			Ste	FW/BW	Pri	(Lindén *et al.*, 1979)
Alosa alosa			Eur	FW/BW/M	Per	(Nachón *et al.*, 2016)
Alosa fallax			Eur	FW/BW/M	Per	(Nachón *et al.*, 2016)
Ameiurus melas	13.8	Dir	Ste	FW/BW	Pri	(Bringolf *et al.*, 2005)
Anaecypris hispanica			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Anguilla anguilla			Eur	FW/BW/M	Per	(Gisbert & López, 2007)
Aphanius baeticus			Eur	FW	Sec	(Elvira, 1995)
Aphanius iberus			Eur	FW/BW	Sec	(Elvira, 1995)
Atherina boyeri			Eur	FW/BW/M	Per	(Gisbert & López, 2007)
Australoheros facetus				FW	Sec	
Barbatula barbatula			Ste	FW	Pri	(Caffrey *et al.*, 2008)
Barbatula quignardi				FW	Pri	
Barbus haasi			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Barbus meridionalis			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Blicca bjoerkna			Ste	FW/BW	Pri	(Wheeler, 1977)
Carassius auratus	16.0	Dir	Ste	FW/BW	Pri	(Schultz & McCormick, 2013)
Carassius gibelio			Ste	FW/BW	Pri	(Rogozin *et al.*, 2011)
Chelon labrosus	53.1	Dir	Eur	FW/BW/M	Per	(Hotos & Vlahos, 1998)
Chelon ramada			Eur	FW/BW/M	Per	(Papa *et al.*, 2003)
Cobitis bilineata				FW	Pri	
Cobitis calderoni				FW	Pri	
Cobitis paludica				FW	Pri	
Cobitis vettonica				FW	Pri	
Cottus aturi			Ste	FW	Per	(Wheeler, 1977)
Cottus hispaniolensis			Ste	FW	Per	(Wheeler, 1977)
Cyprinus carpio	17.0	Dir	Ste	FW/BW	Pri	(Schultz & McCormick, 2013)
Dicentrarchus labrax	90.0	Grad	Eur	FW/BW/M	Per	(Varsamos, 2002)
Esox lucius	14.0	Grad	Ste	FW/BW	Pri	(Schultz & McCormick, 2013)
Fundulus heteroclitus	114.0	Grad	Eur	FW/BW/M	Sec	(Schultz & McCormick, 2013)
Gambusia holbrooki	25.0	Grad	Eur	FW/BW	Sec	(Nordlie & Mirandi, 1996)
Gasterosteus aculeatus			Eur	FW/BW/M	Per	(Münzing, 1963)

Species	Upper LC_{50} salinity limit (ppt)	Method	Euryhalinity	Habitat	Darlington's divisions	References
Gobio lozanoi			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Gobio occitaniae			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Hucho hucho			Eur	FW	Per	(Năstase, Otel & Năvodaru, 2017)
Iberochondrostoma almacai			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Iberochondrostoma lemmingii			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Iberochondrostoma lusitanicum			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Iberochondrostoma olisiponensis			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Iberochondrostoma oretanum			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Ictalurus punctatus	14.0	Dir	Ste	FW/BW	Pri	(Schultz & McCormick, 2013)
Lampetra alavariensis				FW	Per	
Lampetra auremensis				FW	Per	
Lampetra fluviatilis			Eur	FW/BW	Per	(Caffrey *et al.*, 2008)
Lampetra lusitanica				FW	Per	
Lampetra planeri			Eur	FW	Per	(Caffrey *et al.*, 2008)
Lepomis gibbosus	13.6	Dir	Ste	FW/BW	Pri	(Venâncio *et al.*, 2019)
Leuciscus aspius			Ste?	FW/BW	Pri	(Costedoat & Gilles, 2009)
Liza aurata	50.1	Dir	Eur	FW/BW/M	Per	(Shahriari Moghadam *et al.*, 2013)
Luciobarbus bocagei			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Luciobarbus comizo			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Luciobarbus graellsii			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Luciobarbus guiraonis			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Luciobarbus microcephalus			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Luciobarbus sclateri			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Micropterus salmoides			Ste	FW/BW	Pri	(Lowe *et al.*, 2009)
Misgurnus anguillicaudatus			Ste	FW/BW	Pri	(Chang *et al.*, 2002)
Mugil cephalus	50.4	Dir	Eur	FW/BW/M	Per	(Hotos & Vlahos, 1998)
Oncorhynchus kisutch			Eur	FW/BW/M	Per	(Sampaio & Bianchini, 2002)
Oncorhynchus mykiss			Eur	FW/BW/M	Per	(Brijs *et al.*, 2015)
Parachondrostoma arrigonis			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Parachondrostoma miegii			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Parachondrostoma turiense			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Perca fluviatilis	13.0	Dir	Ste	FW/BW	Pri	(Overton *et al.*, 2008)
Petromyzon marinus			Eur	FW/BW/M	Per	(Caffrey *et al.*, 2008)
Phoxinus bigerri			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Platichthys flesus			Eur	FW/BW/M	Per	(Hang & Balment, 2005)
Pomatoschistus microps	51.0	Dir	Eur	FW/BW/M	Per	(Rigal *et al.*, 2008)
Pseudochondrostoma duriense			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Pseudochondrostoma polylepis			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Pseudochondrostoma willkommii			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Pseudorasbora parva	11.5	Dir	Ste	FW/BW	Pri	(Xu *et al.*, 2012)
Rutilus rutilus	14.0	Dir	Ste	FW/BW	Pri	(Schultz & McCormick, 2013)
Salaria fluviatilis			Eur	FW/BW	Per	(Plaut, 1998)
Salmo salar			Eur	FW/BW/M	Per	(Wheeler, 1977)

Species	Upper LC_{50} salinity limit (ppt)	Method	Euryhalinity	Habitat	Darlington's divisions	References
Salmo trutta			Eur	FW/BW/M	Per	(Wheeler, 1977)
Salvelinus fontinalis			Eur	FW/BW/M	Per	(Hiroi & McCormick, 2007)
Salvelinus umbla				FW	Per	
Sander lucioperca			Eur	FW/BW	Pri	(Năstase *et al.*, 2017)
Scardinius erythrophthalmus	12.3	Dir	Ste	FW/BW	Pri	(Solberg, 2012)
Silurus glanis			Ste	FW/BW	Pri	(Sari *et al.*, 1999)
Squalius alburnoides			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Squalius aradensis			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Squalius carolitertii			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Squalius castellanus			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Squalius laietanus			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Squalius malacitanus			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Squalius palaciosi			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Squalius pyrenaicus			Ste?	FW/BW	Pri	(Costedoat & Gilles, 2009)
Squalius torgalensis			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Squalius valentinus			Ste?	FW	Pri	(Costedoat & Gilles, 2009)
Syngnathus abaster			Eur	FW/BW/M	Per	(Năstase *et al.*, 2017)
Tinca tinca	13.8	Grad	Ste	FW/BW	Pri	(Weatherley, 1959)
Valencia hispanica			Eur	FW/BW	Sec	(Doadrio, 2001)
Xiphophorus maculatus	16.5	Dir	Ste?	FW/BW/M	Sec	(Valente *et al.*, 2021)

To analyse the relationship between fish upper LC_{50} salinity limit and other halotolerance classifications (i.e. Darlington, euryhalinity or habitat) we used univariate permutational analysis of variance (PERMANOVA) models. For that pourpose, we also evaluated the complete dataset (i.e. including also the 80 species not present in the Iberian Peninsula) in order to increase the sample size and to make our results more reliable and general. In all models we included the halotolerance experimental method (Schultz & McCormick, 2013) and its interaction with Darlington's divisions, euryhalinity and habitat, respectively, as the method used influences the experimental measurement (Schultz & McCormick, 2013). We used the 'adonis2' function of the R-package 'vegan' (Oksanen *et al.*, 2017), and we tested for homogeneity of dispersions for the different predictors among groups using the function 'betadisper' of the R-package 'vegan', since the PERMANOVA is known to be sensitive to heterogeneous dispersions. We also used a 'pairwise.perm.manova' function of the package 'RVAideMemoire' (Hervé, 2020) to assess differences between Darlington's divisions pairs. We finally studied the relationship between the upper LC_{50} halotolerance limit and the halotolerance breadth using Pearson's correlation.

Results showed that primary fish have much less halotolerance than secondary (*P* = 0.001) and peripheral (*P* = 0.001) species (Figure S6.11a). For instance, the primary species present in the Iberian Peninsula with the largest halotolerance seems to be the common carp (*Cyprinus carpio*), which can tolerate salinities of 17 ppt; while the southern platyfish (*Xiphophorus maculatus*) was the secondary species present in the Iberian Peninsula with the lowest halotolerance, showing an upper LC_{50} halotolerance limit of 16.5 ppt. However, many of the secondary species belonging to the Fundulidae, Cyprinodontidae or Cichlidae families are able to inhabit in hyperhaline waters with more than 50 ppt. In fact, secondary species showed similar halotolerance than peripheral (*P* = 0.104). This model explained 47.4% of the variation in inland fish halotolerance (Table S6.8), reflecting that Darlington's classification could be used as a good proxy of the tolerance to seawater. We also found significant relationship between the upper LC_{50} halotolerance limit and the euryhalinity classification (*P* = 0.001; see Figure S6.11b and Table S6.8). However, results did not show a clear relationship between the upper LC_{50} halotolerance limit and habitat (*P* = 0.064; see Figure S6.11c and Table S6.8), indicating that this classification system does not adequately reflect the fish tolerance to salinity. As previously noted (Schultz & McCormick, 2013), we also found a significant effect of the experimental method on the upper LC_{50} halotolerance limit measurement in all models (Table S8). Additionally, the upper LC_{50} halotolerance limit was highly correlated with the halotolerance breadth (*r* = 0.998), as many of the species considered are tolerant to freshwater, and therefore, the halotolerance breadth is equal in most cases to the upper LC_{50} halotolerance limit.

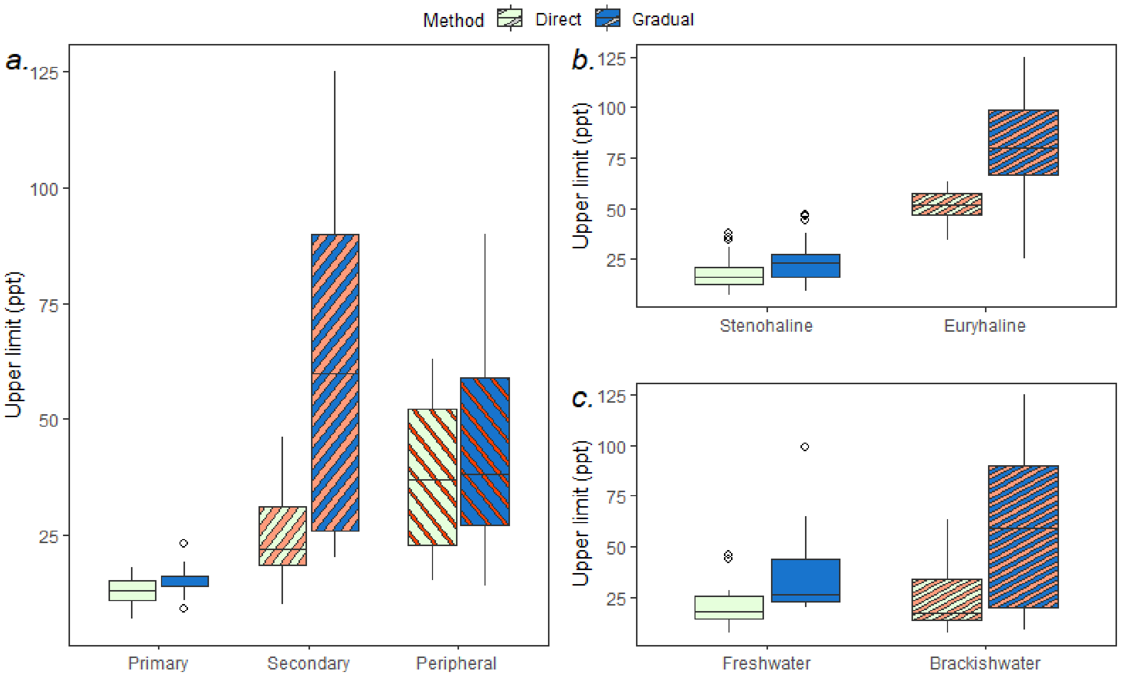


Figure S6.11. Relationship between fish upper LC$_{50}$ salinity limit and different halotolerance classifications: (a) Darlington's divisions (i.e. primary, secondary and peripheral), (b) euryhalinity level (i.e. stenohaline and euryhaline) and (c) habitat (i.e. freshwater and brackishwater). Boxes correspond to the 25th and 75th percentiles; lines inside a box show the median; whiskers extend to the last observation within 1.5 times the interquartile range from the quartiles and outliers are indicated by empty circles.

Table S6.8. Results of the PERMANOVA models analysing the relationship between fish upper LC$_{50}$ salinity limit and different halotolerance classifications (i.e. Darlington, euryhalinity or habitat). Degrees of freedom (*df*), coefficients of determination (R^2), P values for PERMANOVA are shown. P values are also shown for homogeneity of dispersions (HoD) analysis.

Model	**Variables**	***df***	R^2	***P***	***P* (HoD)**
Upper lim. ~ Darlington × Method	Darlington	2	0.302	**0.001**	**<0.001**
	Method	1	0.102	**0.001**	
	Darlington × Method	2	0.070	**0.003**	
	Residuals	123	0.526		
Upper lim. ~ Euryhalinity × Method	Euryhalinity	1	0.711	**0.001**	**<0.001**
	Method	1	0.056	**0.001**	
	Euryhalinity × Method	1	0.037	**0.001**	
	Residuals	125	0.196		
Upper lim. ~ Habitat × Method	Brackishwater	1	0.019	0.064	**0.012**
	Method	1	0.248	**0.001**	
	Habitat × Method	1	0.019	0.066	
	Residuals	125	0.714		

To further analyse the relationship among the different halotolerance classifications (i.e. Darlington, euryhalinity or habitat) using the Iberian species dataset, we constructed contingency tables to summarise the relationship between pairs of variables. We then calculated the corrected contingency coefficient C using the function ‘ContCoef’ of the R-package ‘DescTools’ (Signorell, 2021), and performed a χ^2 test. Results showed that Darlington and euryhalinity classifications were highly associated ($C_{\text{corr}} = 0.948$; $\chi^2 = 73.5$, $df = 2$, $P < 0.001$). For instance, all primary species were considered stenohaline organisms except *Sander lucioperca* (Table S6.9a), which was classified as an euryhaline fish (Năstase *et al.*, 2017). Similarly, all secondary and peripheral fish were considered euryhaline organisms except *X. maculatus*, *Cottus aturi* and *C. hispaniolensis*, which were classified as stenohaline species (Wheeler, 1977; Schultz & McCormick, 2013; Valente *et al.*, 2021). We also found that Darlington and habitat were associated ($C_{\text{corr}} = 0.506$; $\chi^2 = 14.7$, $df = 2$, $P = 0.001$), although, for example, 21 primary species could be found in brackish waters (see Table S6.9b), following the FishBase (2019) classification. Finally, euryhalinity was also associated with habitat ($C_{\text{corr}} = 0.652$; $\chi^2 = 22.1$, $df = 1$, $P < 0.001$), but showed again several inconsistencies when comparing both classification systems (see Table S6.9c).

Table S6.9. Contingency tables that summarise the relationship among the different halotolerance classifications (i.e. Darlington, euryhalinity or habitat). NA = no data available.

a.	**Primary** (*n* = 63)	**Secondary** (*n* = 7)	**Peripheral** (*n* = 30)
Stenohaline (*n* = 60)	57	1 (*Xiphophorus maculatus*)	2 (*Cottus aturi* and *C. hispaniolensis*)
Euryhaline (*n* = 30)	1 (*Sander lucioperca*)	5	24
NA (*n* = 10)	5	1	4

b.	**Primary** (*n* = 63)	**Secondary** (*n* = 7)	**Peripheral** (*n* = 30)
Freshwater (*n* = 52)	42	2	8
Brackishwater (*n* = 48)	21	5	22

c.	**Stenohaline** (*n* = 60)	**Euryhaline** (*n* = 30)	**NA** (*n* = 10)
Freshwater (*n* = 52)	39	3	10
Brackishwater (*n* = 48)	21	27	0

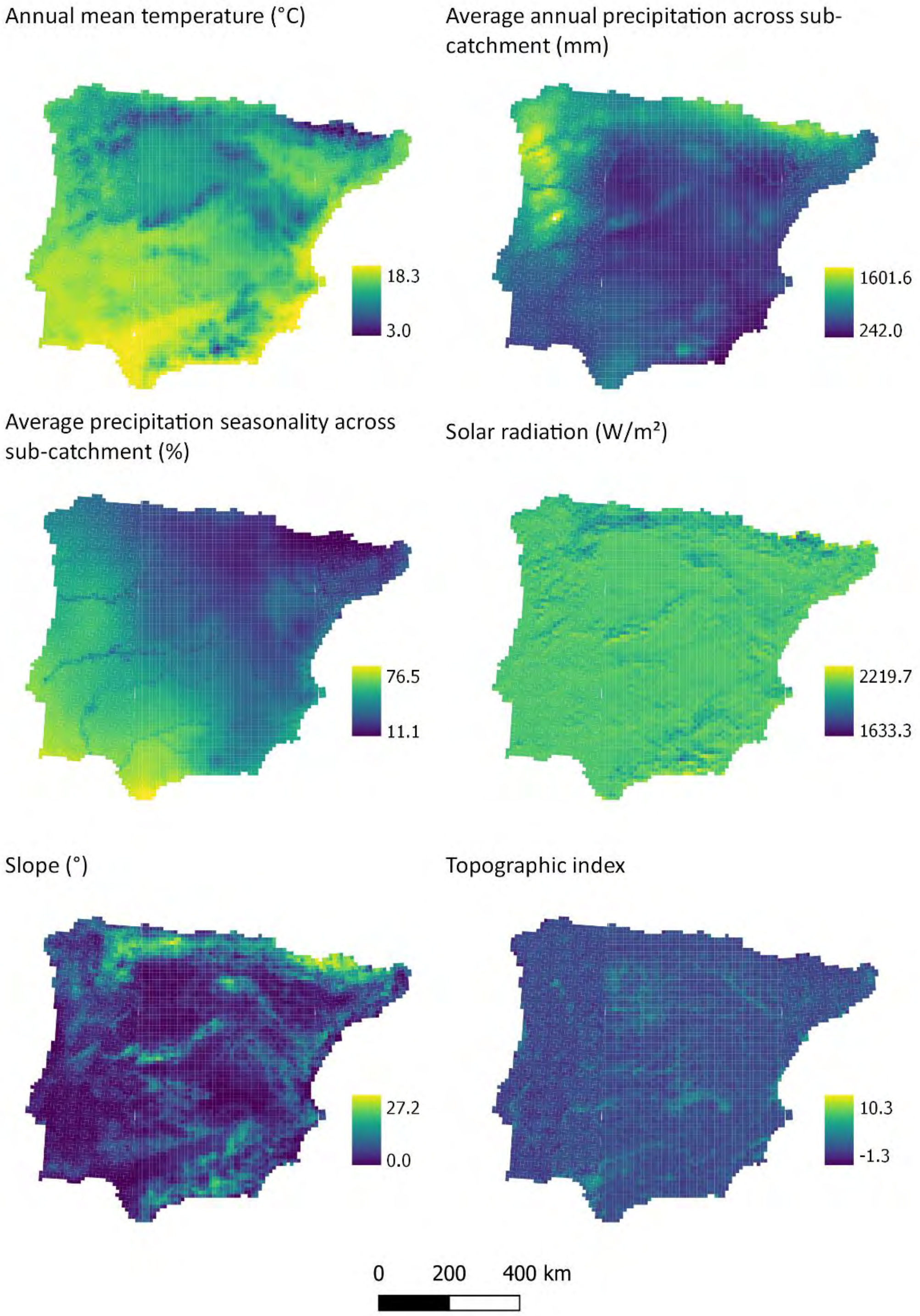
We finally repeated the PERMANOVA analyses for the 63 modeled species with data available on euryhalinity, as this classification system better reflects fish halotolerance than the habitat classification obtained from FishBase. The objective is to test for differences in variable importance of predictors among stenohaline and euryhaline native and alien fish species in order to compare these new results with those obtained using Darlington's divisions. We used again univariate PERMANOVAs to analyse differences of variable importance of predictors in native status and halotolerance and its interaction, and a multivariate PERMANOVA including all predictor variable importances. Multivariate PERMANOVA model using halotolerance levels (i.e. stenohaline/euryhaline) revealed very similar results to that using Darlington's divisions (see Table S6.10 and Table 6.1 of the main text), showing significant differences in the importance of predictors explaining the distributions of native vs. alien species and even larger differences among halotolerance levels (23.3 vs. 6.4 % of explained variation). Univariate PERMANOVAs also showed very similar results, however, they explained less variation for 7 out of the 13 models than those models including Darlington's divisions.

Table S6.10. Results of the PERMANOVA and homogeneity of dispersions analysis across native status (NS) and halotolerance (HT). Coefficients of determination (R^2) are shown for PERMANOVA. *P* values for PERMANOVA are expressed with asterisks (*** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05; . ≤ 0.1). *P* values are also shown for homogeneity of dispersions analysis. NA = not applicable. DisSea = distance to the sea; MeanTem = annual mean temperature; PrecSeas = average precipitation seasonality within sub-catchment; AnnPrec = average annual precipitation within sub-catchment; StrOrdSt = Strahler stream order; UpResCp = Upstream reservoir capacity; Slo = Slope; AgrPrc = average agricultural land use with a given sub-catchment; TopInd = topographic index; UrbPrc = average urban land use within a given sub-catchment; LoResCp = local reservoir capacity; SolRadiat = solar radiation.

Response variable	**PERMANOVA**				**Homogeneity of dispersions**
	NS	**HT**	**NS × HT**	**Residuals**	***P***
All	**0.064** **	**0.233** ***	**0.058** *	0.645	**0.014**
Basin ID	0.038	**0.252** ***	0.054	0.656	**0.007**
DisSea	**0.082** **	**0.323** ***	**0.072** *	0.523	**0.001**
MeanTem	**0.101** *	**0.102** *	0.040	0.757	0.249
PrecSeas	0.007	0.057	0.057	0.880	0.388
AnnPrec	0.007	0.036	0.036	0.921	0.653
StrOrdSt	**0.280** ***	0.008	0.019	0.694	0.380
UpResCp	**0.196** **	0.036	0.047	0.724	0.681
Slo	**0.114** **	0.008	0.011	0.867	0.893
AgrPrc	**0.159** **	0.001	0.066	0.774	0.786
TopInd	**0.151** **	0.016	0.004	0.795	0.969
UrbPrc	0.003	0.000	0.004	0.992	0.974
LoResCp	**0.084** *	0.063	0.042	0.811	0.776
SolRadiat	**0.172** **	0.004	0.032	0.792	0.851

In sum, we can conclude that Darlington's divisions are significantly correlated to the quantitative halotolerance data (i.e. upper LC_{50} salinity limit), and with the FishBase habitat and the euryhalinity classifications. However, the FishBase habitat did not reflect the fish tolerance to salinity, as it is not related with the upper LC_{50} salinity limit. Moreover, the stenohaline/euryhaline classification for Iberian species has been made from very different sources and not from a single and standardised reference list, and most of the information collected is based on expert judgment and not on quantitative data. This can cause the information collected to be not very reliable (Cano-Barbacil *et al.*, 2020) since the different studies may consider different criteria to classify the halotolerance of species. For instance, few studies based this classification on experimental data and considered as freshwater stenohaline species those that can survive in only a narrow range of salinity that includes fresh waters (Schultz & McCormick, 2013); while many other studies based this classification on Myers and Darlington's divisions, and considered stenohaline species and primary species as synonyms (Noble *et al.*, 2003; Costedoat & Gilles, 2009). In fact, some authors such as Costedoat & Gilles (Costedoat & Gilles, 2009) make the same generalisations that Darlington's classification and considered entire families in the same category (e.g. all cyprinids are stenohaline species). Another example of the mistakes that can be made using expert knowledge is the case of *X. maculatus*. It has been typically considered as an euryhaline species (Schreibman & Kallman, 1966), while recent experimental studies revealed that *X. maculatus* shows the typical osmoregulatory capacity of stenohaline fish (Valente *et al.*, 2021). Hence, although we acknowledge that data on quantitative halotolerance limits would be preferable (but is largely unavailable), we suggest that we have shown that using Darlington's divisions is appropriate as a proxy. Moreover, it is a very well established, fully available and standard classification that reflects, not only the halotolerance of fish, but also their eco-evolutionary history (Bănărescu, 1990; Berra, 2001). Similarly, other authors have previously considered that this is the best classification system of inland fish when evaluating their distribution patterns (Bănărescu, 1990). In fact, Myers' and Darlington's classifications are still generally supported and used by many of the most comprehensive fish monographs (Bănărescu, 1990; Matthews, 1998; Berra, 2001; Doadrio, 2001; Kottelat & Freyhof, 2007; McDowall, 2010; e.g. Albert & Reis, 2011).

Appendix S6.2: Maps of predictor variables



Annual mean temperature (°C)

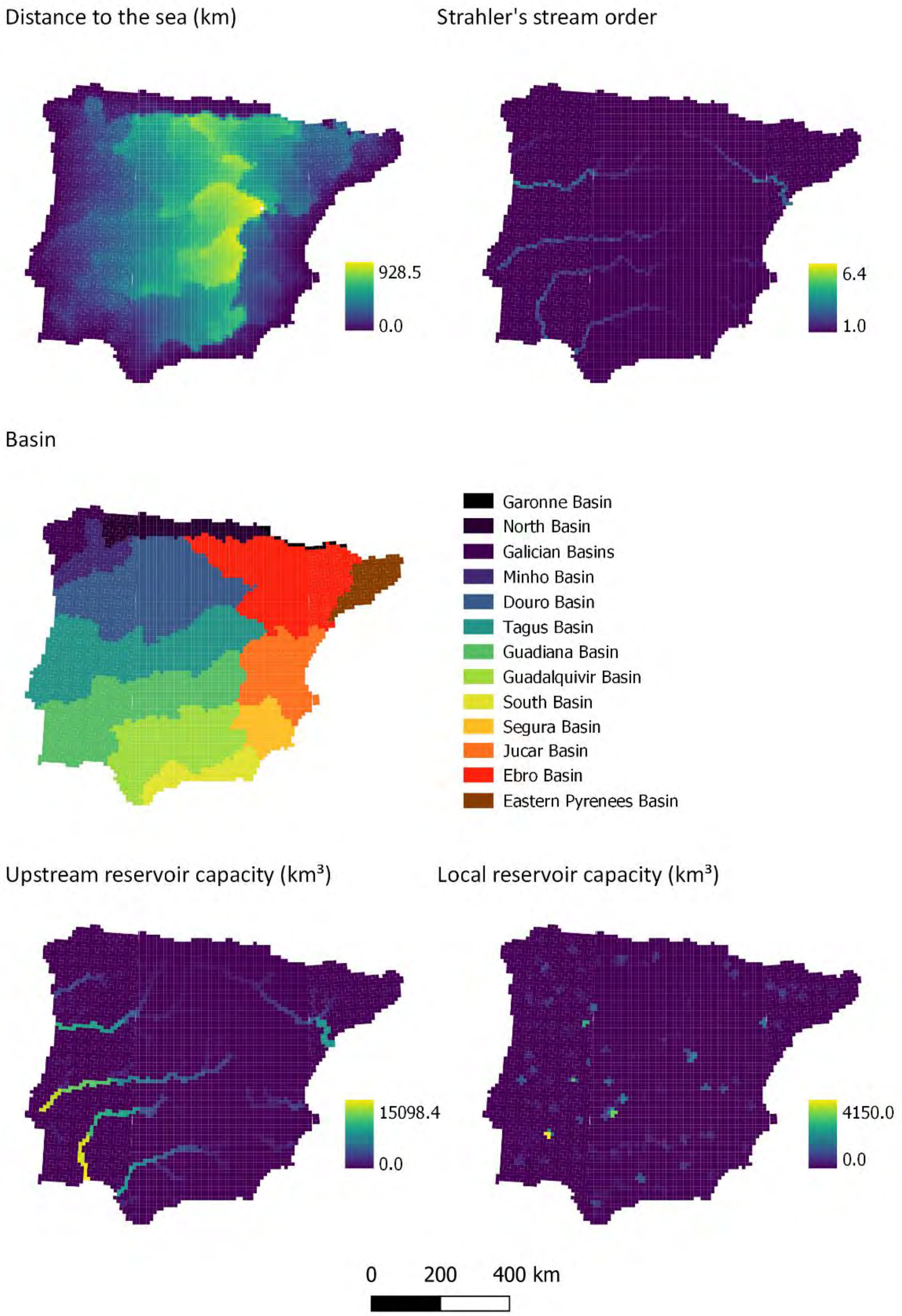
Average annual precipitation across sub-catchment (mm)

Average precipitation seasonality across sub-catchment (%)

Solar radiation (W/m²)

Slope (°)

Topographic index



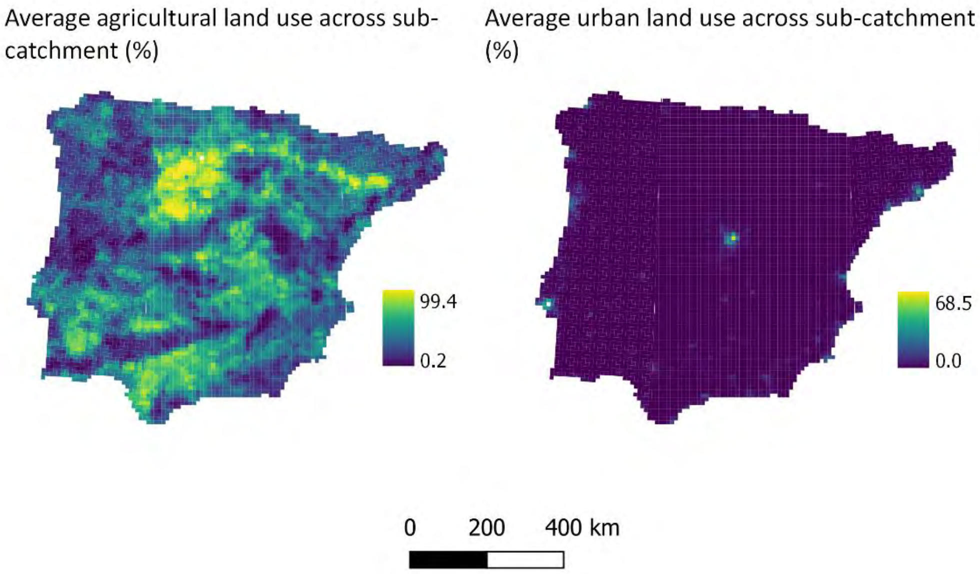
Distance to the sea (km)

Strahler's stream order

Basin

Upstream reservoir capacity (km³)

Local reservoir capacity (km³)



Average agricultural land use across sub-catchment (%)

Average urban land use across sub-catchment (%)

Appendix S6.3: The inclusion of 'basin ID' as predictor variable in the SDMs

As mentioned in the main text, we included the water district (hereafter, 'basin ID') in the SDMs because of three reasons: i) increased predictive accuracy; ii) the goal was to account for biogeographic units and evolutionary history and explain rather the realised distribution more than the potential distribution; iii) the estimates of variable importance were similar than not including basin ID.

First, three out of four of the predictive accuracy statistics used (i.e. AUC, TSS and specificity) showed significantly higher values when including 'basin ID' in the SDMs (see Figure S6.12 and Table S6.11). Tukey test results did not provide any evidence of non-additivity (i.e. no method × species interaction), which is an assumption of the two-way ANOVA without replication. ΔAUC showed a significant interaction between Darlington's divisions and alien status (R_{adj}^2 = 0.156; sum of squares = 0.002, *df* = 2, *P* = 0.021). ΔAUC were positive for most native primary and all alien species, while negative for most native secondary species (Figure S6.13).

Second, not including 'basin ID' resulted in predictions of suitable areas well beyond the current distribution in the Iberian Peninsula (IP). Following Jiménez-Valverde *et al.* (2008), we were more interested in the realised distribution of the Iberian fish species (i.e. the places where species actually live) rather than the potential distribution (i.e. the places where a species could live), which actually would go beyond the IP and is a hypothetical concept that is hard to describe without a high amount of evidence from different sources.

The inclusion of 'basin ID' as a predictor variable in the SDMs could reflect environmental differences among basins, and, therefore, could affect the estimation of the variable importance for the other predictors studied. However, we also computed all the species distribution models excluding 'basin ID' and compared the results of both methods correlating variable importance using Spearman's rank correlation.

Variable importance showed a significant correlation between both methods for all predictors (Table S6.12). Therefore, we used 'basin ID' as a predictor in the SDMs since this seems preferable on conceptual grounds, interesting to compare among Darlington's divisions, and should not change the qualitative conclusions given these observed correlations.

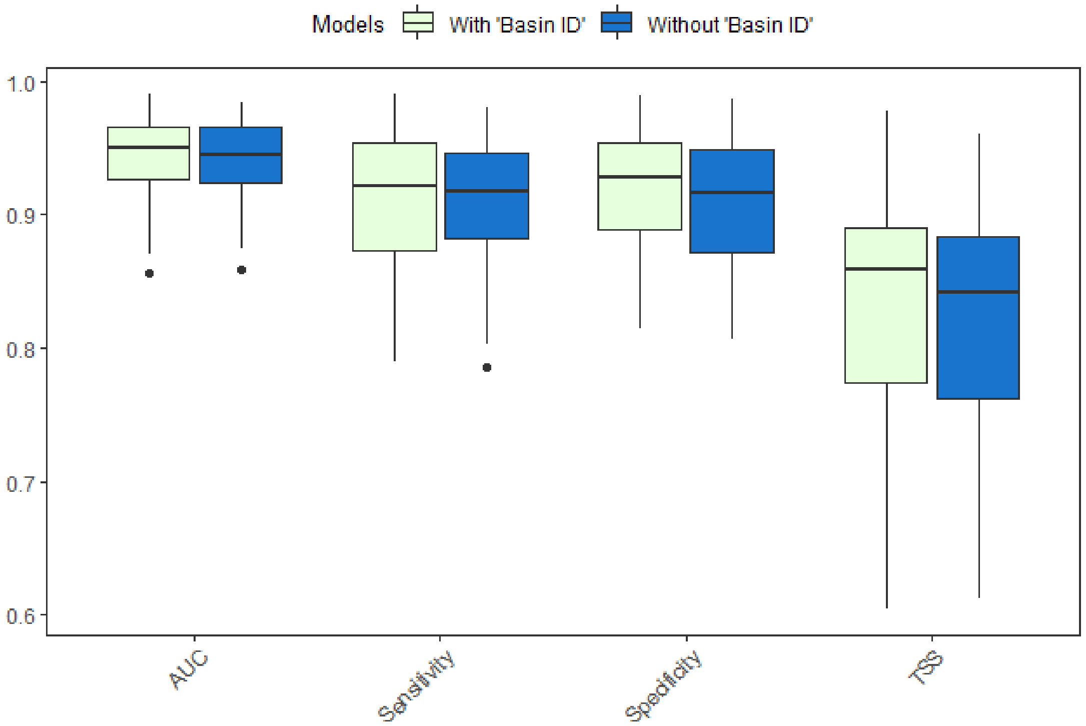


Figure S6.12. Predictive accuracy statistics: area under the receiver operating characteristic curve (AUC), sensitivity, specificity and true skill statistic (TSS). Boxes correspond to the 25th and 75th percentiles; lines inside a box show the median; whiskers extend to the last observation within 1.5 times the interquartile range from the quartiles and outliers are indicated by empty circles.

Table S6.11. Two-way ANOVAs without replication and Tukey additivity tests for each of the predictive accuracy statistics used with method (i.e. with and without basin ID) and species as factors. R_{adj}^2 = adjusted coefficient of determination in parentheses; *df* = degrees of freedom; *P* = *P* value.

Predictive accuracy statistic	Sum of squares	*df*	*P*	Tukey test
AUC (R_{adj}^2 = 0.863)				
Method	0.001	1	**0.028**	Test statistic: 0.021
Species	0.103	67	**< 0.001**	Critical value: 3.986
Residual	0.008	67		The additivity hypothesis cannot be rejected.
TSS (R_{adj}^2 = 0.883)				
Method	0.004	1	**0.045**	Test statistic: 0.348
Species	0.962	67	**< 0.001**	Critical value: 3.986
Residual	0.060	67		The additivity hypothesis cannot be rejected.
Sensitivity (R_{adj}^2 = 0.815)				
Method	0.001	1	0.211	Test statistic: 0.091
Species	0.273	67	**< 0.001**	Critical value: 3.986
Residual	0.028	67		The additivity hypothesis cannot be rejected.
Specificity (R_{adj}^2 = 0.932)				
Method	0.001	1	**0.003**	Test statistic: 1.877
Species	0.275	67	**< 0.001**	Critical value: 3.986
Residual	0.010	67		The additivity hypothesis cannot be rejected.

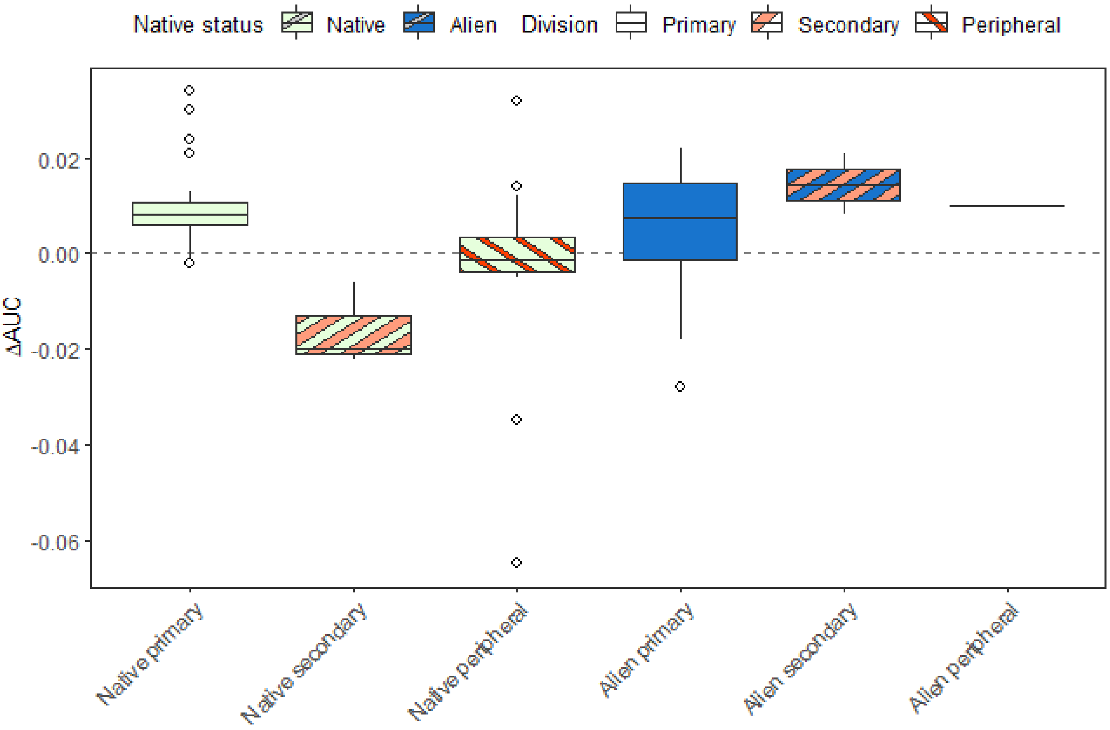


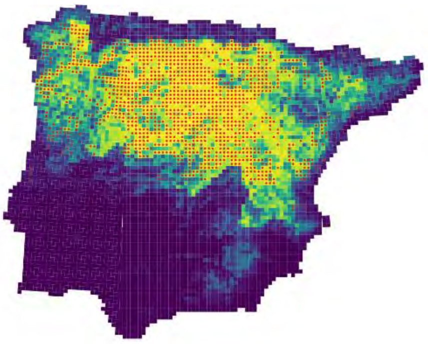
Figure S6.13. AUC differences between two methodologies (i.e. $\text{AUC}_{\text{with 'basin ID'}} - \text{AUC}_{\text{without 'basin ID'}}$) across six groups Iberian freshwater fish: native primary (n = 30), native secondary (n = 3), native peripheral (n = 18), alien primary (n = 14), alien secondary (n = 2) and alien peripheral (n = 1) species. Boxes correspond to the 25th and 75th percentiles; lines inside a box show the median; whiskers extend to the last observation within 1.5 times the interquartile range from the quartiles and outliers are indicated by empty circles.

Table S6.12. Spearman's correlation between variable importances obtained from SDMs with and without considering 'basin ID' as a predictor variable. Spearman's rank correlation coefficient (*ρ*) and *p*-value are given.

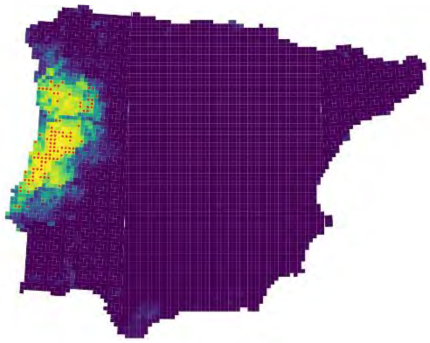
Predictor variables (TYPE)	**Abbreviation**	***ρ***	***p***
CLIMATE			
Annual mean temperature (°C)	MeanTem	0.670	**< 0.001**
Average annual precipitation across sub-catchment (mm)	AnnPrec	0.657	**< 0.001**
Average precipitation seasonality across sub-catchment (%)	PrecSeas	0.538	**< 0.001**
Solar radiation (W/m²)	SolRadiat	0.610	**< 0.001**
TOPOGRAPHY			
Slope (°)	Slo	0.659	**< 0.001**
Topographic index	TopInd	0.587	**< 0.001**
Distance to the sea (km)	DisSea	0.819	**< 0.001**
Stream order Strahler	StrOrdSt	0.933	**< 0.001**
LAND USE & ANTHROPOGENIC			
Upstream reservoir capacity (km^3)	UpResCp	0.886	**< 0.001**
Local reservoir capacity (km^3)	LoResCp	0.837	**< 0.001**
Average agricultural land use across sub-catchment (%)	AgrPrc	0.591	**< 0.001**
Average urban land use across sub-catchment (%)	UrbPrc	0.727	**< 0.001**

Appendix S6.4: Projected distribution maps of Iberian fish species

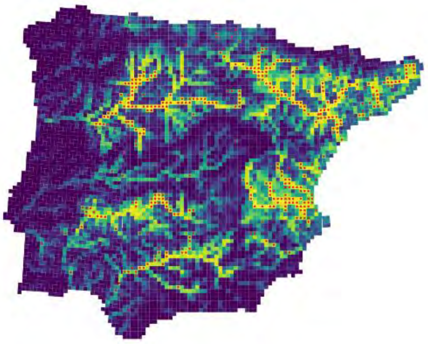
Achondrostoma arcasii



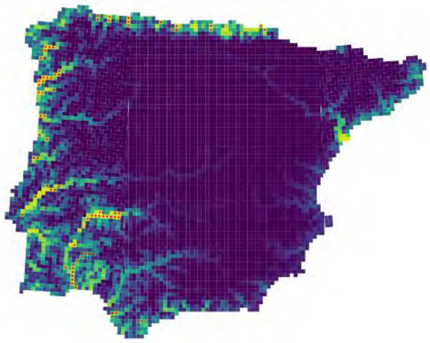
Achondrostoma oligolepis



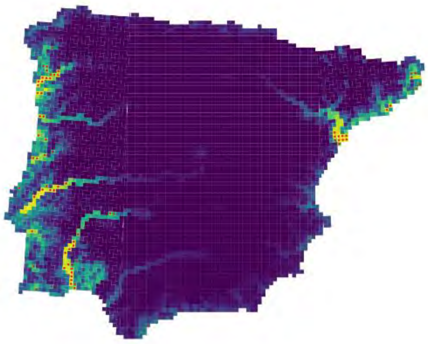
Alburnus alburnus



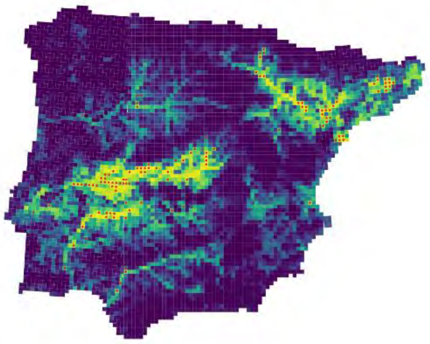
Alosa alosa

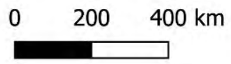


Alosa fallax

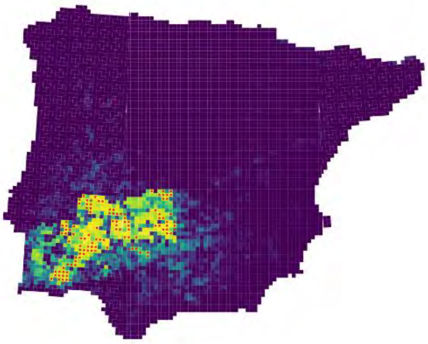


Ameiurus melas

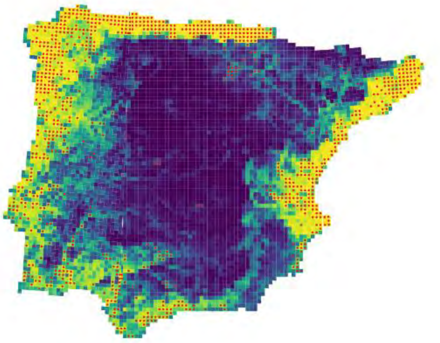




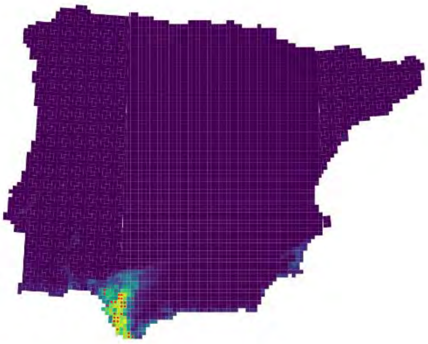
Anaecypris hispanica



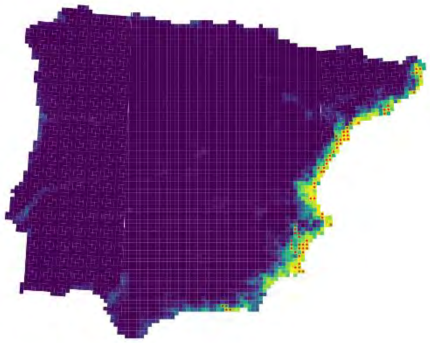
Anguilla anguilla



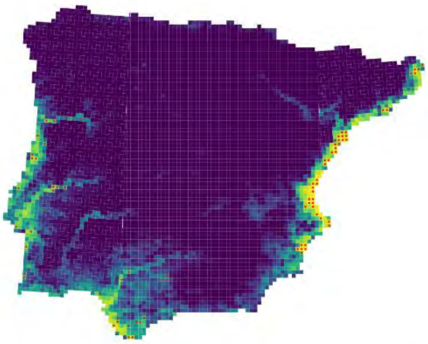
Aphanius baeticus



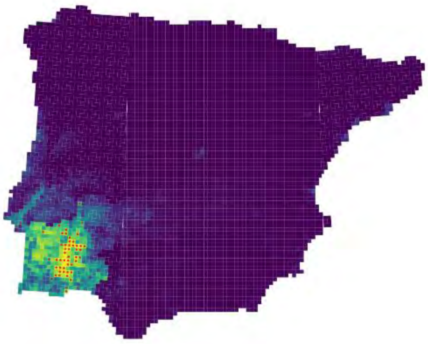
Aphanius iberus



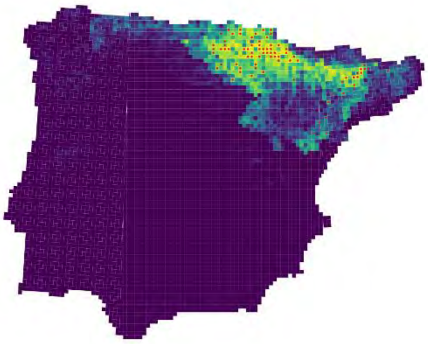
Atherina boyeri



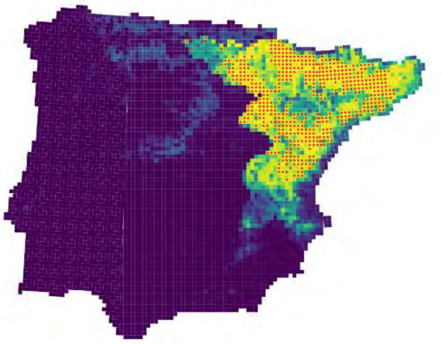
Australoheros facetus



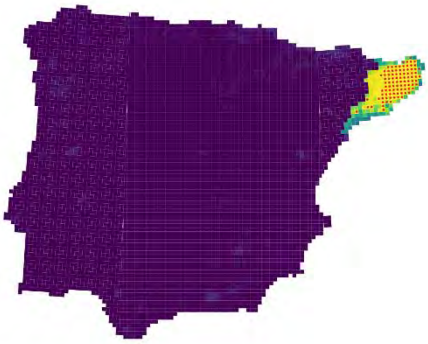
Barbatula quignardi



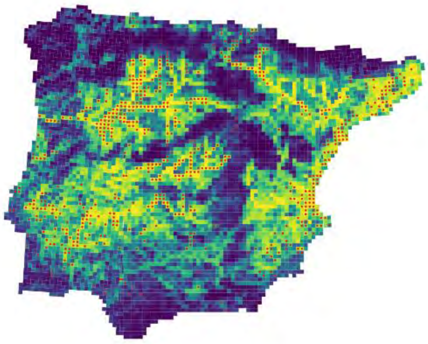
Barbus haasi



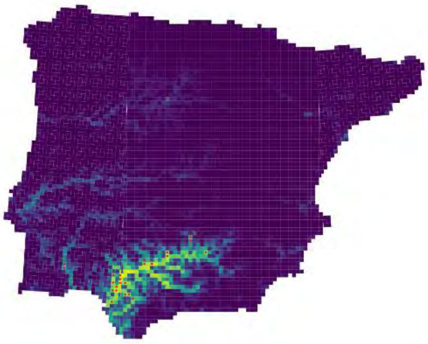
Barbus meridionalis



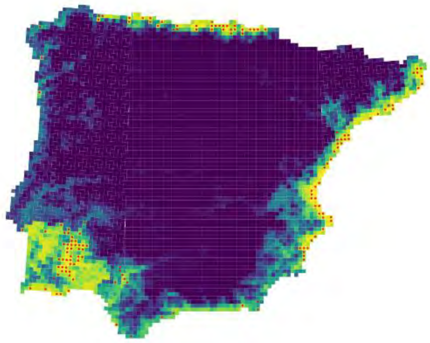
Carassius auratus



Carassius gibelio

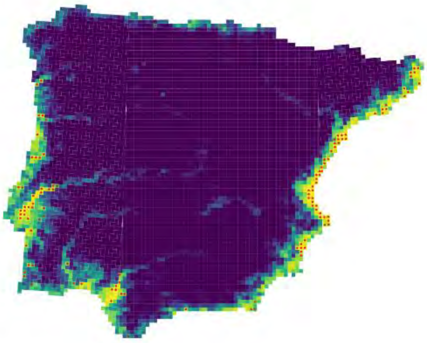


Chelon labrosus

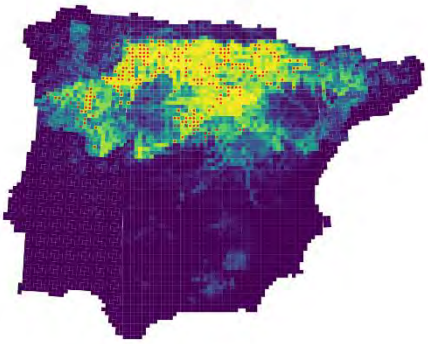


0 200 400 km

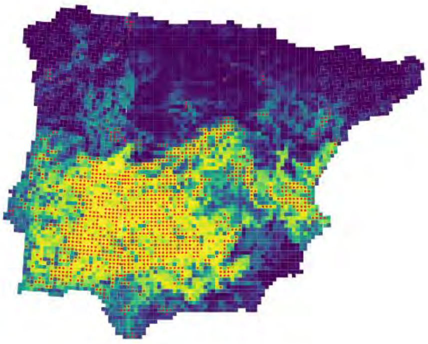
Chelon ramada



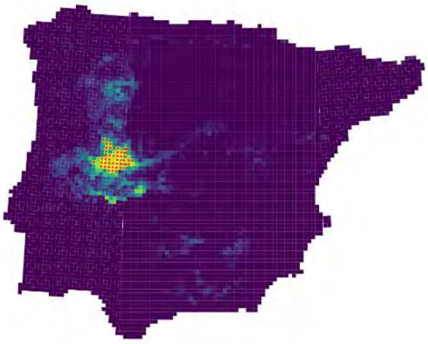
Cobitis calderoni



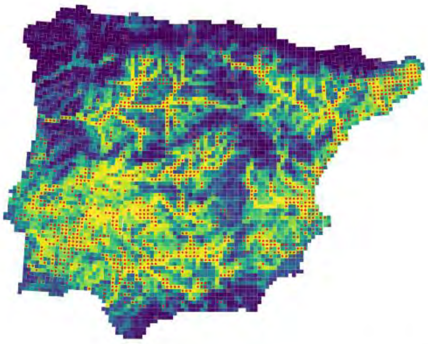
Cobitis paludica



Cobitis vettonica



Cyprinus carpio



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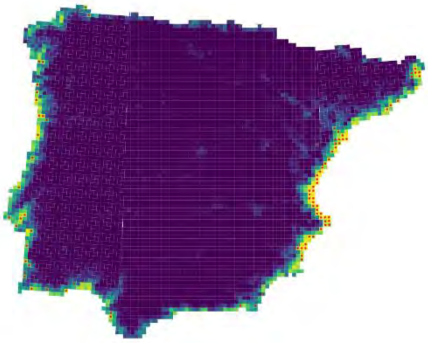
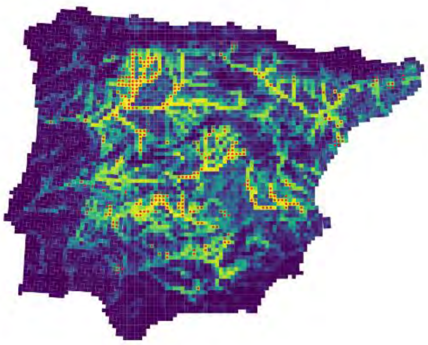
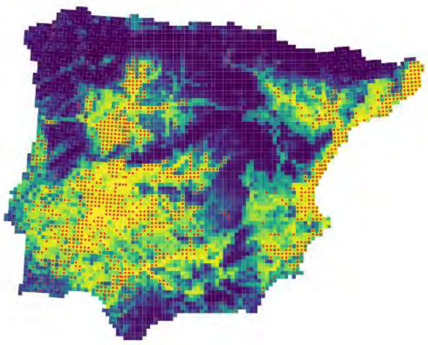


Esox lucius



Gambusia holbrooki



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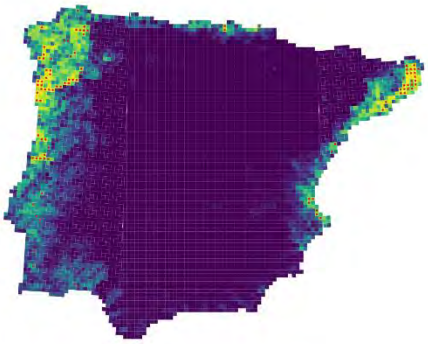
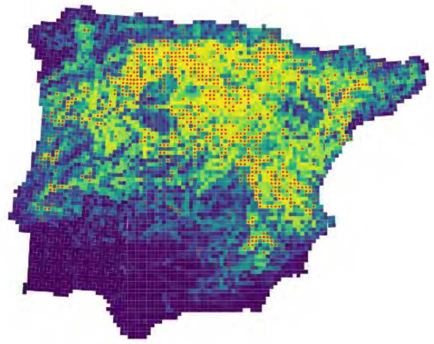
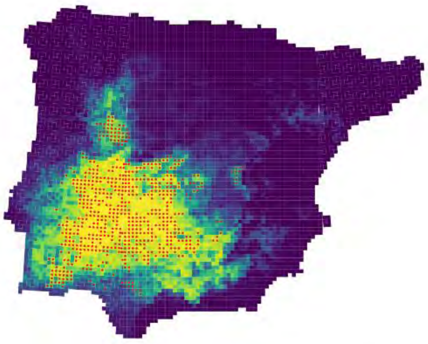


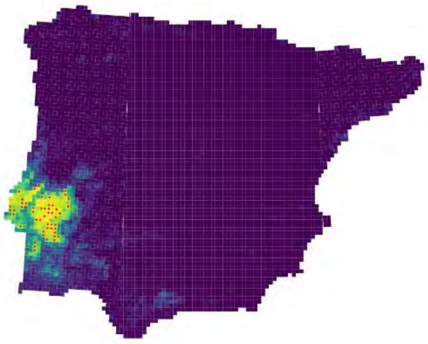
Gobio lozanoi



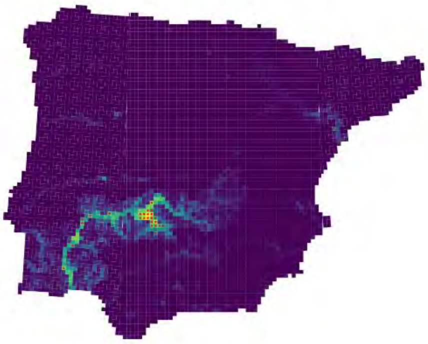
Iberochondrostoma lemmingii



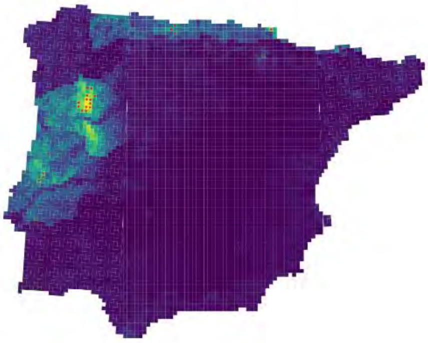
Iberochondrostoma lusitanicum



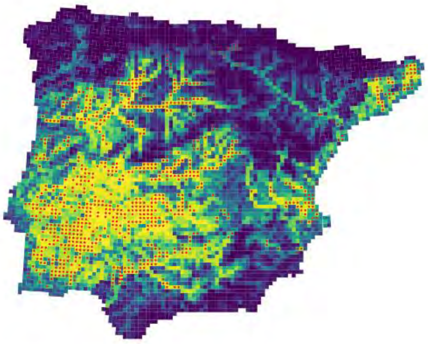
Ictalurus punctatus



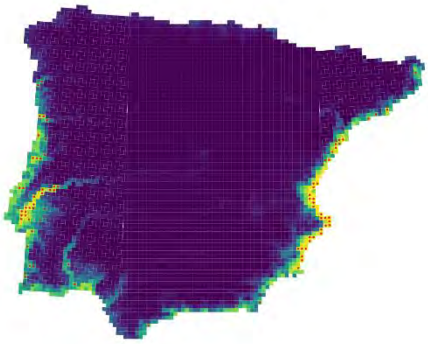
Lampetra planeri



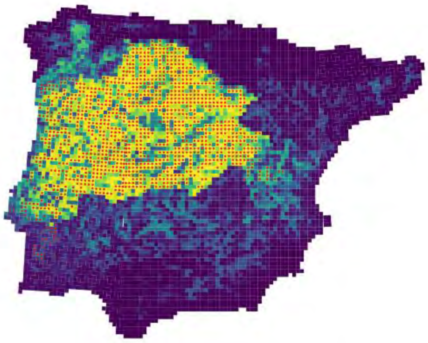
Lepomis gibbosus



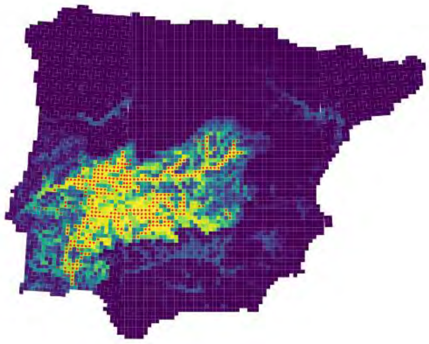
Liza aurata

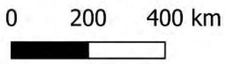


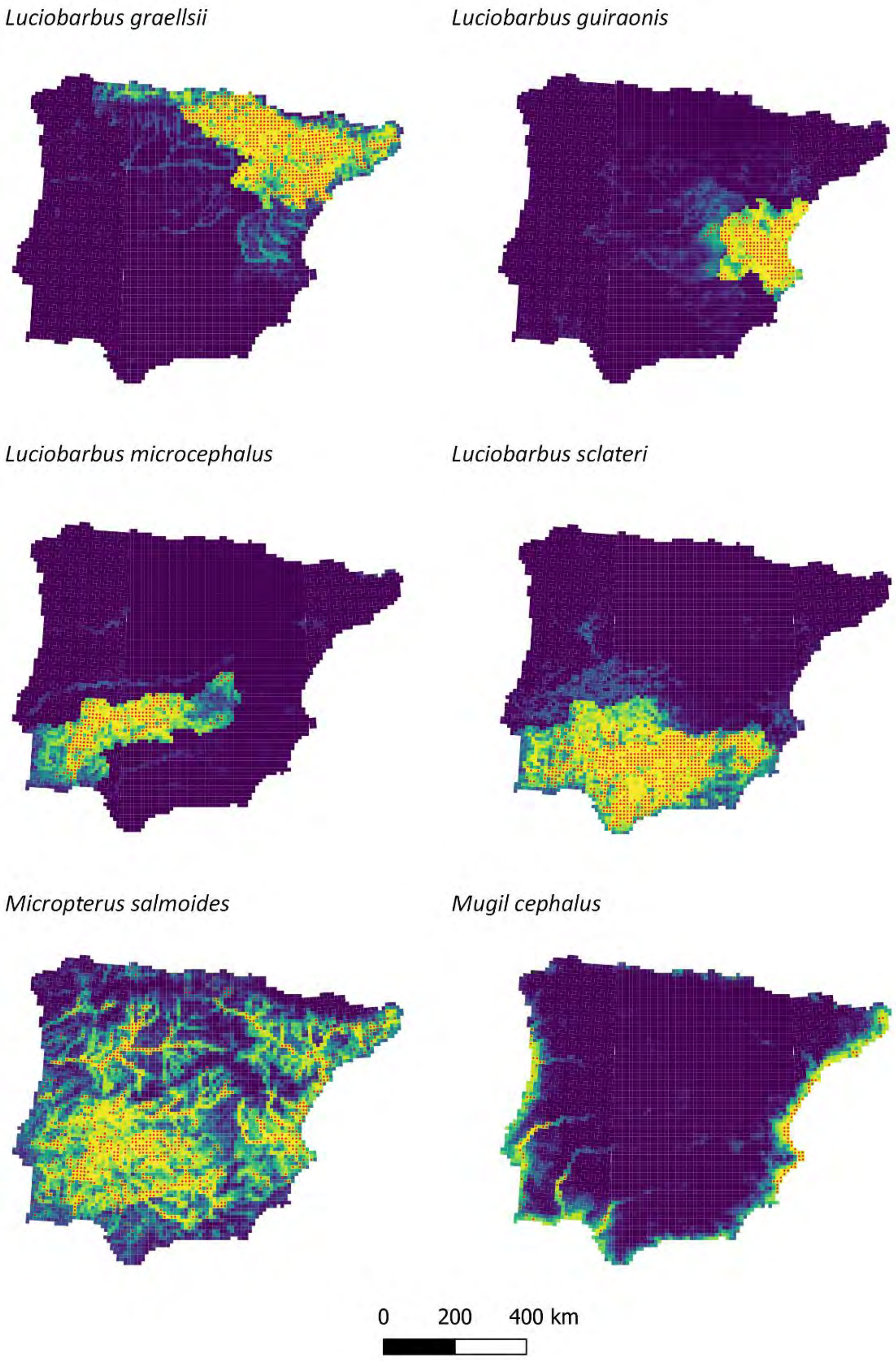
Luciobarbus bocagei



Luciobarbus comizo







Luciobarbus graellsii

Luciobarbus guiraonis

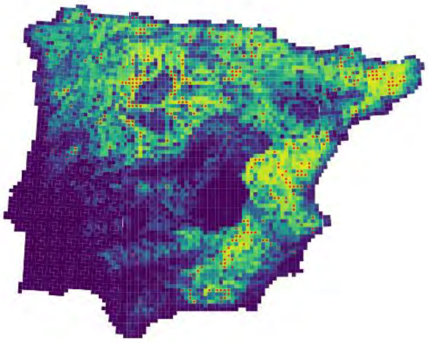
Luciobarbus microcephalus

Luciobarbus sclateri

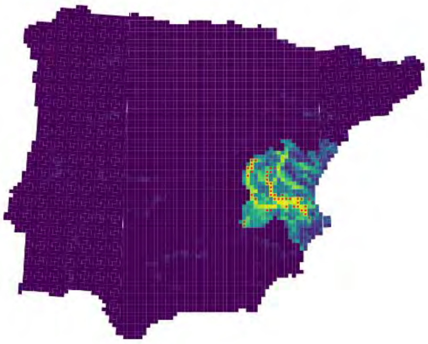
Micropterus salmoides

Mugil cephalus

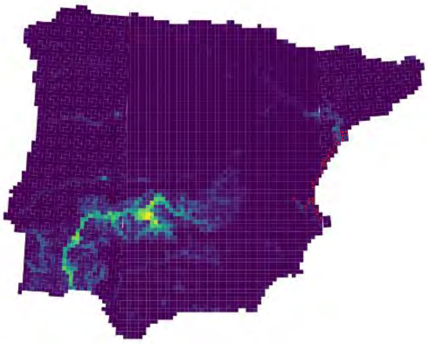
Oncorhynchus mykiss



Parachondrostoma arrigonis



Parachondrostoma miegii



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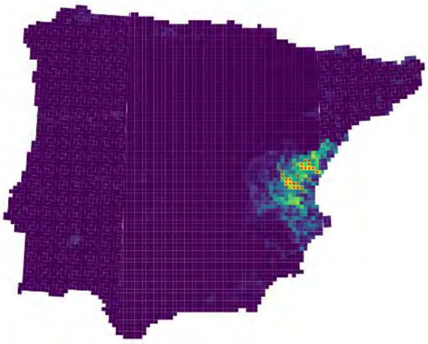

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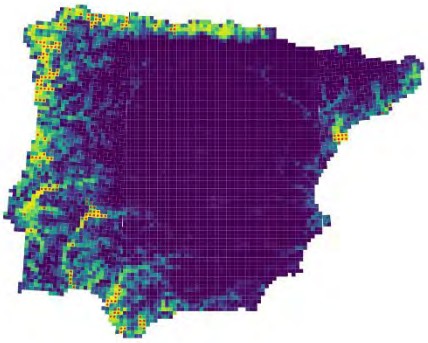

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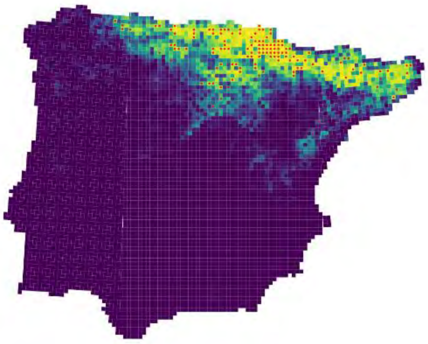
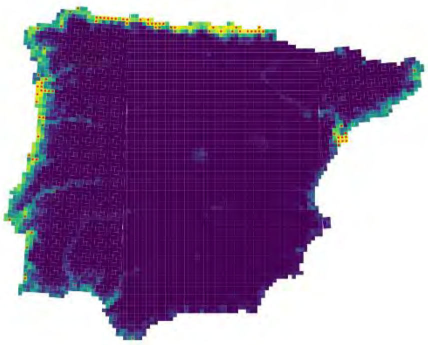
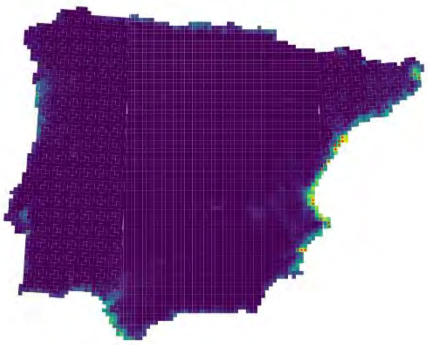


0 200 400 km

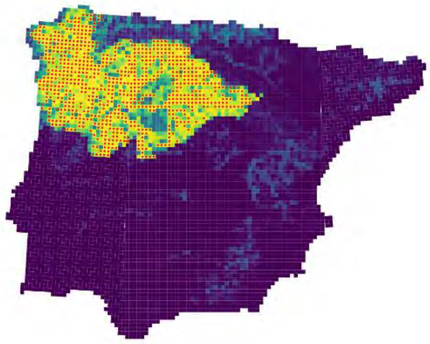
Platichthys flesus



Pomatoschistus microps



Pseudochondrostoma duriense



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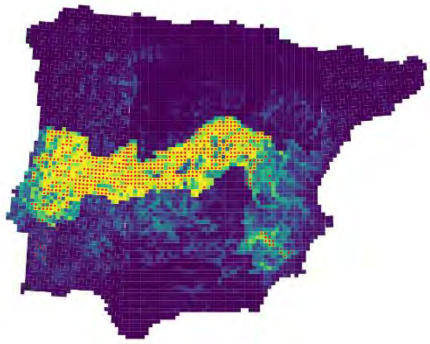
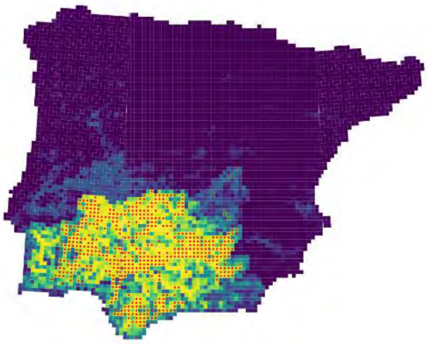
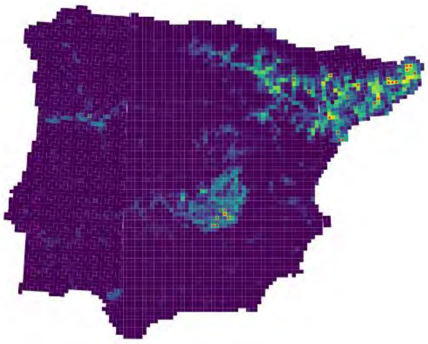


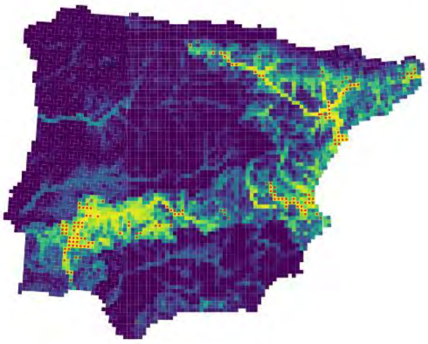
Pseudochondrostoma willkommii



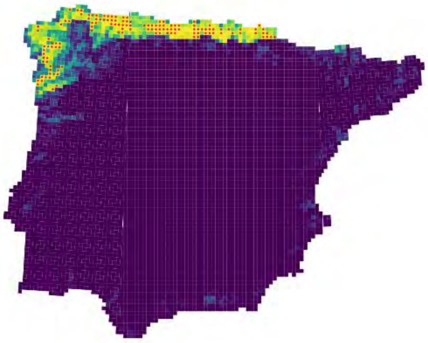
Rutilus rutilus



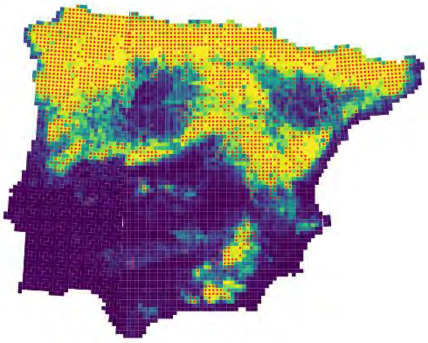
Salaria fluviatilis



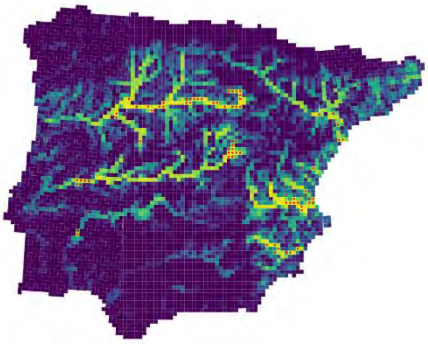
Salmo salar



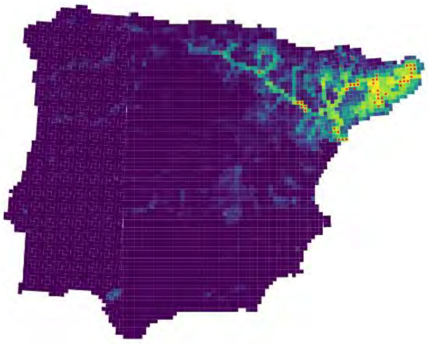
Salmo trutta



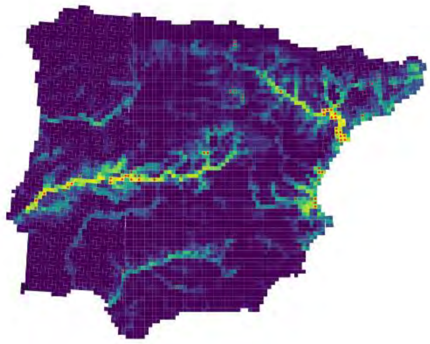
Sander lucioperca



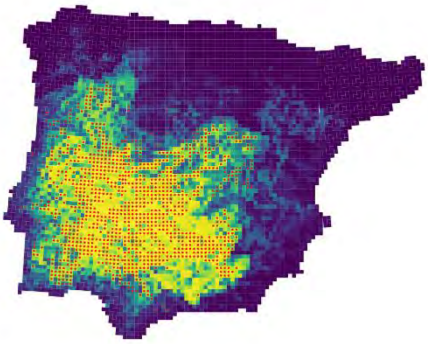
Scardinius erythrophthalmus



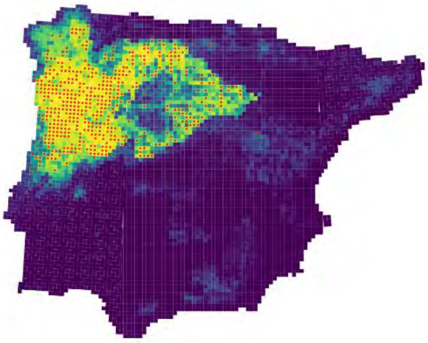
Silurus glanis



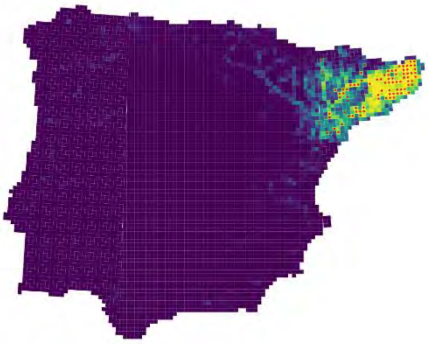
Squalius alburnoides



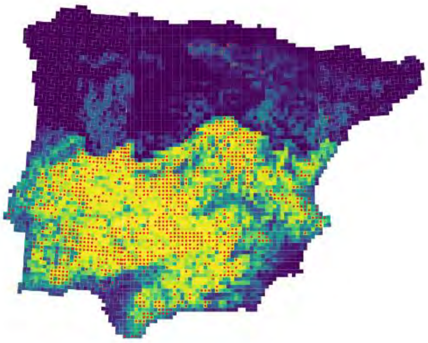
Squalius carolitertii



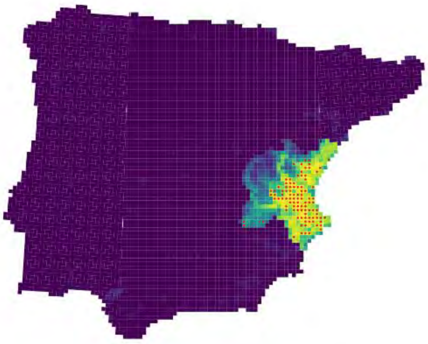
Squalius laietanus



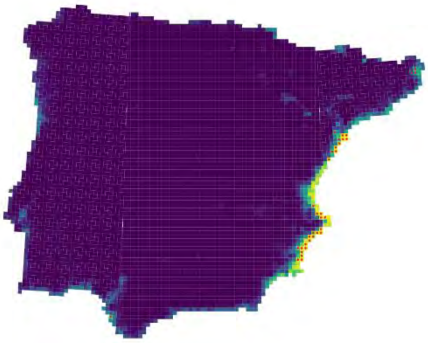
Squalius pyrenaicus

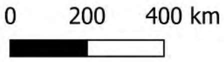


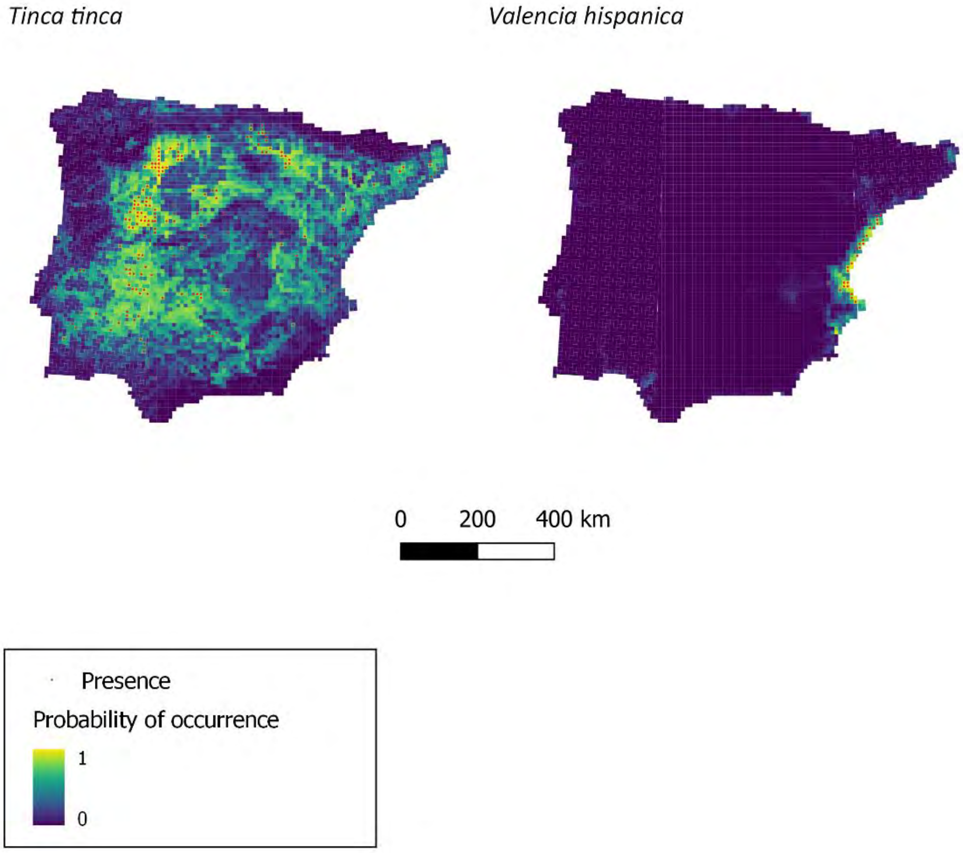
Squalius valentinus



Syngnathus abaster







Tinca tinca

Valencia hispanica

Supplementary materials S7 - Phylogenetic signal and evolutionary relationships among traits of inland fishes along elevational and longitudinal gradients

Table S7.1. List of the 23 traits compiled. Trait type, scale of measurement (i.e. continuous or binary), mean trait value and standard deviation (SD) for continuous traits and proportion for binary traits are shown (n = 30).

Trait (and TYPE)	Scale of measurement	Mean ± SD or Proportion	Description
MORPHOLOGICAL			
Max. total length (cm)	Continuous	70.9 ± 46	Maximum fish total length
Form factor	Continuous	0.008 ± 0.005	Commonly used to compare body shape differences among populations or species. Increases from eel-like to elongated, fusiform and short and deep body shapes (Froese, 2006; Verreycken *et al.*, 2011).
Fusiform shape	Binary	25/30	Spindle-shaped fish, cylindrical or nearly so that tapers toward the ends.
TROPHIC			
Invertivory	Binary	10/30	Species that feed on invertebrates.
Omnivory	Binary	10/30	Species that consume considerable amounts of both plant and animal material.
Piscivory	Binary	11/30	Species that eat mainly other fishes. Fish have a wide mouth aperture with needle-like teeth and a strong jaw with marginal and palatal bones.
REPRODUCTIVE			
Max. longevity (years)	Continuous	15.9 ± 15.4	Maximum individual age reported.
Reproductive span (months)	Continuous	2.5 ± 1.0	Length of breeding season.
Max. fecundity (number of eggs)	Continuous	$5.4 \cdot 10^5 \pm 1.3 \cdot 10^6$	Maximum number of eggs a mature female can produce in a single spawning event.
Age at maturity (years)	Continuous	2.9 ± 1.6	Mean age at which fish develop ripe gonads for the first time.
Parental care	Binary	6/30	Any investment by parents in progeny that increases the offsprings' probabilities of surviving and, in hence, reproducing. In fish, parental care can adopt several forms (guarding, nest building, external egg carrying, egg burying, moving eggs or young, ectodermal feeding, oral brooding, internal gestation, brood-pouch egg carrying, etc) (Froese & Pauly, 2019).
Single spawning	Binary	22/30	Single spawning per season. This trait summarises the seasonality of spawning events.
HABITAT USE			
U_{crit} (cm/s)	Continuous	59.9 ± 7.2	Critical swimming speed (U_{crit}) is a measurement of the prolonged swimming performance of fish (Cano-Barbacil *et al.*, 2020).
Rheophily	Binary	10/30	Preferring to live in running water. Species adapted to fluvial habitats.
Limnophily	Binary	8/30	Preferring to live in stagnant waters.

Trait (and TYPE)	Scale of measurement	Mean ± SD or Proportion	Description
Potamodromy	Binary	5/30	Fishes migrate within the river basin having their entire life cycle occurring within fresh waters of a river system. Migrations are cyclical and predictable, showing seasonal return movements to spawning areas.
Long migration	Binary	5/30	Species migrate between freshwater and seawater. This category includes anadromous, catadromous and amphidromous species. Migrations are cyclical and predictable.
Benthic	Binary	10/30	They live, feed and reproduce on the sediment surface. Benthic species are sensitive to siltation and benthic oxygen depletion (Oberdorff & Hughes, 1992).
Water column	Binary	7/30	Active swimmer species that prefer to live and feed in the water column. Typically feed on drifting and surface invertebrates or other fishes (Oberdorff & Hughes, 1992).
Tolerance	Binary	12/30	Tolerant species have a large water quality and habitat flexibility.
Intolerance	Binary	5/30	Intolerant species have a low water quality and habitat flexibility, and are those that first decline with environmental degradation or after a disturbance (Oberdorff & Hughes, 1992).
Lithophily	Binary	10/30	Species that deposit eggs on a rock, rubble or gravel bottom where their embryos and larvae develop (Balon, 1975). Lithophilic spawners are particularly sensitive to siltation, requiring clean gravel substrates for reproductive success (Berkman & Rabeni, 1987; Belliard *et al.*, 1999).
Phytophily	Binary	6/30	Species that scatter or deposit eggs with an adhesive membrane to submerged, live or dead, plants. They are usually adapted to habitats with muddy bottoms and low oxygen concentration (Balon, 1975).

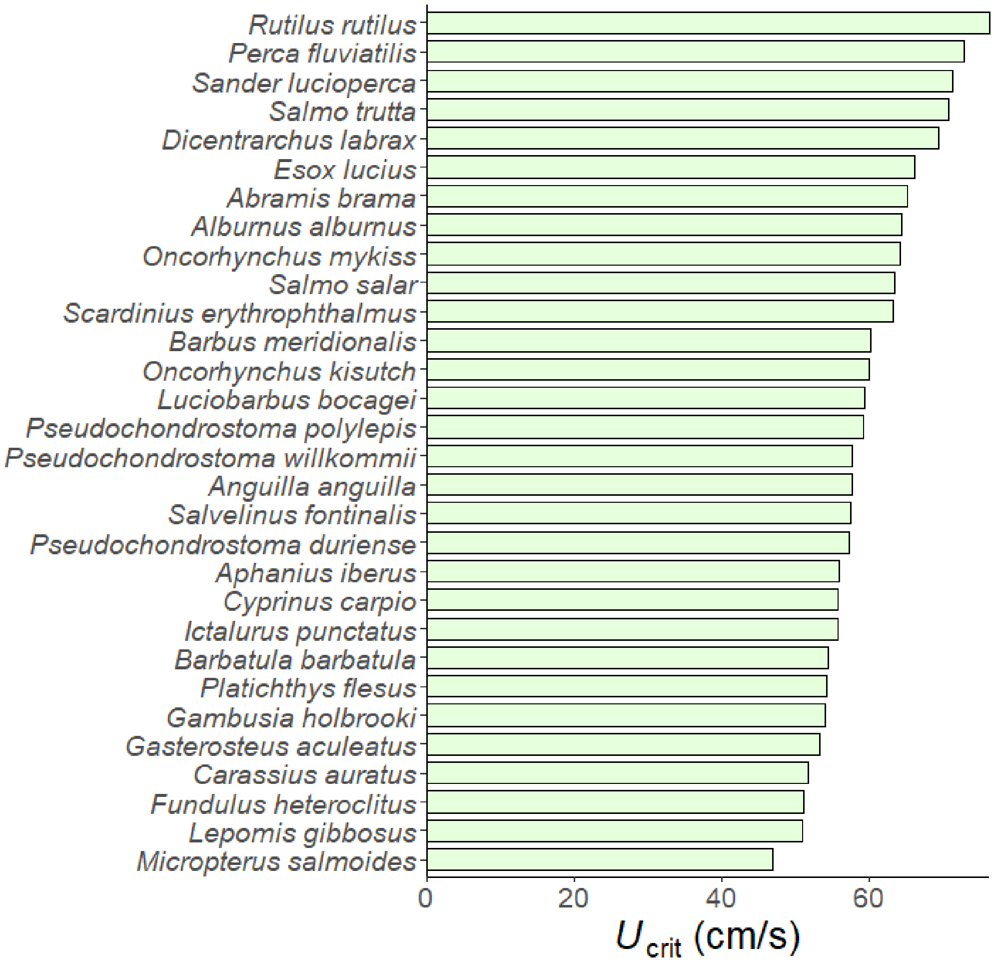


Figure S7.1. Partial dependence of U_{crit} across fish species based on the random forest model. Estimated U_{crit} values for the 30 species studied ranged between 46.8 cm s^{-1} (*Micropterus salmoides*) and 76.2 cm s^{-1} (*Rutilus rutilus*). The four explanatory variables used in the RF model (i.e. species identity, individual fish total length, time step interval between velocity increments and experimental water temperature) explained 73.3% of the variation in U_{crit}.

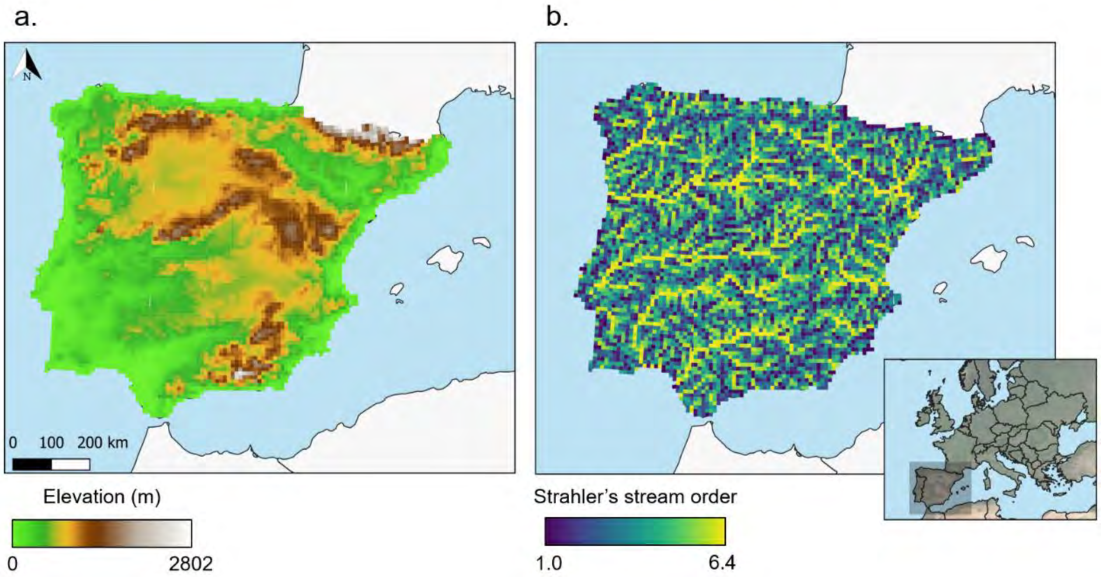


Figure S7.2. (a) Mean elevation and (b) Strahler's stream order maps of the Iberian Peninsula (grid resolution of 10 × 10 km Universal Transverse Mercator, *n* = 6142 total cells). Source: Spanish National Center for Geographic Information (http://centrodedescargas.cnig.es/). Basemap of the general view: Google satellite (obtained through QuickMapServices QGIS plugin), Map data © 2015 Google.

Table S7.2. Mean, maximum, minimum, and range of elevation and Strahler's stream order of inland fish species in the Iberian Peninsula. *n* = sample size.

Species	**Elevation (m)**					**Strahler's stream order**				***n***
	Mean	**Relative mean**	**Maximum**	**Minimum**	**Range**	**Mean**	**Maximum**	**Minimum**	**Range**	
Alburnus alburnus	502.0	0.144	1698.8	0.3	1698.5	2.5	5.2	1.0	4.2	402
Anguilla anguilla	337.1	0.097	1439.0	0.1	1438.9	2.0	6.4	1.0	5.4	898
Aphanius iberus	132.7	0.038	627.5	0.1	627.4	1.9	6.4	1.0	5.4	70
Barbatula barbatula	671.8	0.193	2203.5	82.2	2121.4	2.0	3.9	1.0	2.9	243
Barbus meridionalis	440.1	0.127	1512.8	7.1	1505.7	1.8	3.2	1.0	2.2	94
Carassius auratus	477.0	0.137	1493.6	0.1	1493.5	2.2	6.4	1.0	5.4	639
Cyprinus carpio	457.5	0.132	1643.5	0.1	1643.4	2.2	6.4	1.0	5.4	1166
Dicentrarchus labrax	72.4	0.021	973.1	0.1	973.0	2.0	5.0	1.0	4.0	89
Esox Lucius	629.5	0.181	1334.5	0.3	1334.2	2.4	4.3	1.0	3.3	303
Fundulus heteroclitus	15.5	0.004	33.5	3.1	30.4	2.3	3.4	1.2	2.2	10
Gambusia holbrooki	392.1	0.113	1142.2	0.1	1142.1	2.1	6.4	1.0	5.4	1106
Gasterosteus aculeatus	231.6	0.067	1142.7	0.3	1142.4	2.0	3.4	1.1	2.3	98
Ictalurus punctatus	367.8	0.106	515.2	122.3	392.8	2.5	3.5	1.6	1.9	26
Lepomis gibbosus	413.8	0.119	1193.1	0.3	1192.7	2.2	4.9	1.0	3.9	849
Luciobarbus bocagei	699.4	0.201	1700.1	1.7	1698.4	2.1	4.2	1.0	3.2	1130
Micropterus salmoides	453.5	0.130	1374.1	0.3	1373.7	2.2	4.3	1.0	3.3	841
Oncorhynchus mykiss	815.8	0.235	2422.9	56.7	2366.1	2.1	4.1	1.1	3.0	318
Perca fluviatilis	174.0	0.050	398.4	0.7	397.6	2.3	3.2	1.5	1.7	9
Platichthys flesus	133.2	0.038	434.6	0.1	434.6	2.1	6.4	1.0	5.4	80
Pseudochondrostoma duriense	736.0	0.212	1780.1	44.2	1735.9	2.1	4.2	1.0	3.2	779
Pseudochondrostoma polylepis	578.6	0.166	1700.1	4.8	1695.2	2.1	4.0	1.0	3.0	504
Pseudochondrostoma willkommii	408.5	0.117	1442.1	0.9	1441.2	2.2	4.5	1.0	3.5	521
Rutilus rutilus	488.3	0.140	901.6	0.7	900.9	2.2	3.3	1.1	2.2	33
Salmo salar	360.8	0.104	1646.0	41.1	1604.9	2.0	3.7	1.0	2.7	133
Salmo trutta	842.4	0.242	2574.9	36.2	2538.7	1.8	4.9	1.0	3.9	1665
Salvelinus fontinalis	1465.1	0.421	2422.9	824.2	1598.7	1.9	3.2	1.2	2.0	17
Sander lucioperca	550.3	0.158	1463.4	0.3	1463.1	2.6	4.3	1.3	3.0	94
Scardinius erythrophthalmus	409.5	0.118	1629.7	0.1	1629.6	2.2	3.5	1.1	2.4	49

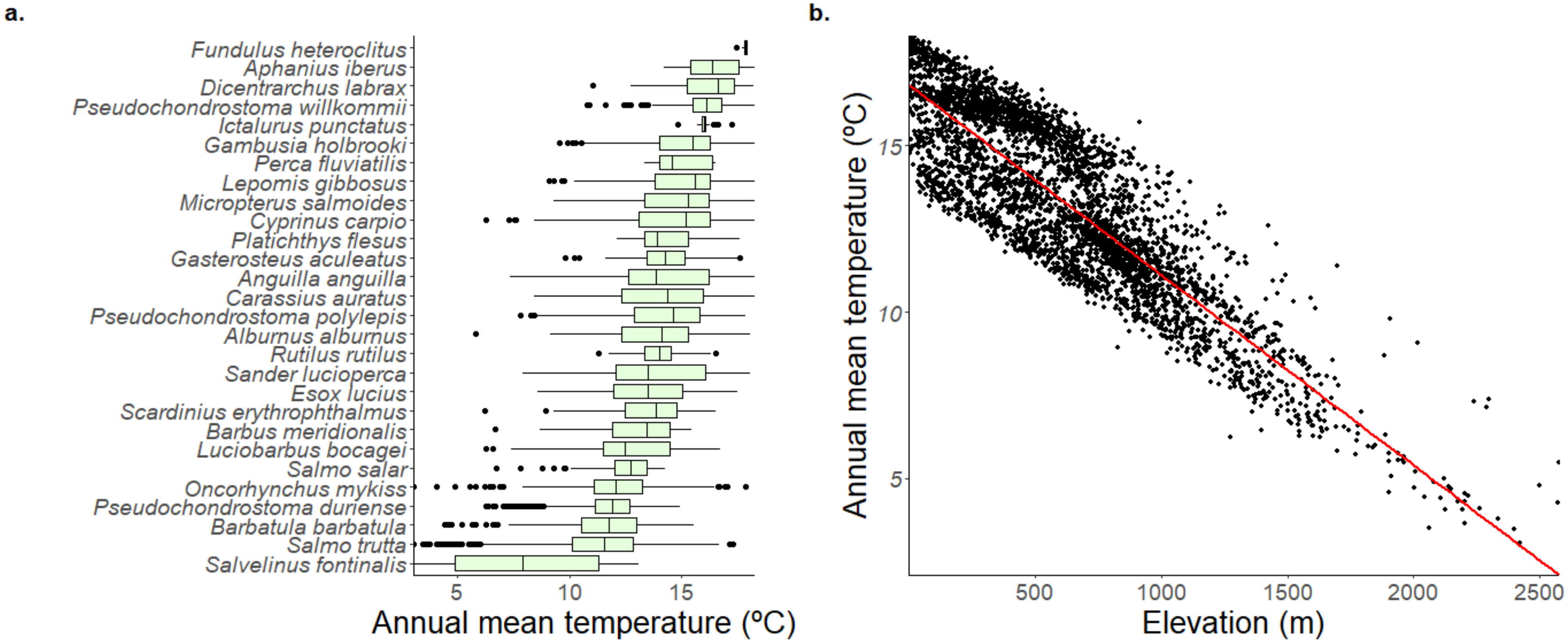


Figure S7.3. (a) Mean air temperature of the presences of 28 freshwater fish species in the Iberian Peninsula. (b) Relationship between mean absolute elevation and mean air temperature (r^2 = 0.728).

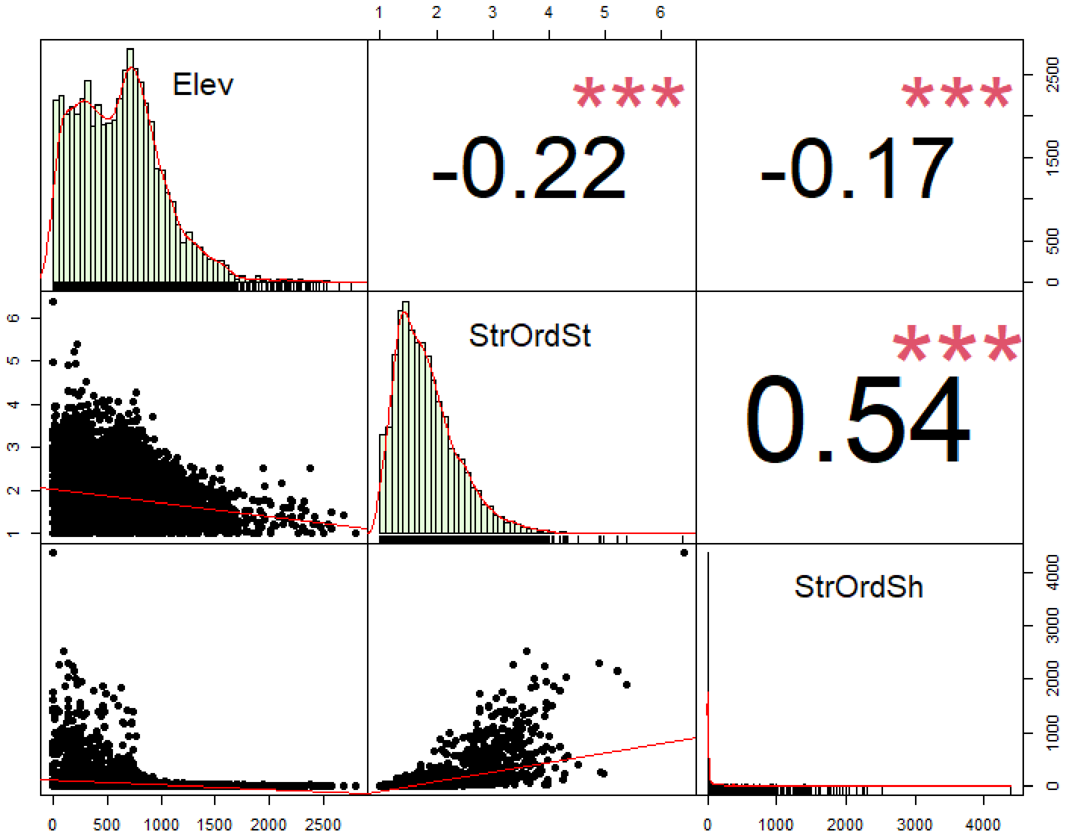


Figure S7.4. Correlation scatterplot matrix of mean elevation (Elev) and two commonly used measures of stream size: Strahler's stream order (StrOrdSt) and Shreve stream order (StrOrdSh). The distribution of each variable is shown on the diagonal; the bivariate scatter plots with a fitted line are shown below the diagonal; Pearson's correlation coefficient and *P* values are shown above the diagonal (***, $P \leq 0.001$). n = 6142.

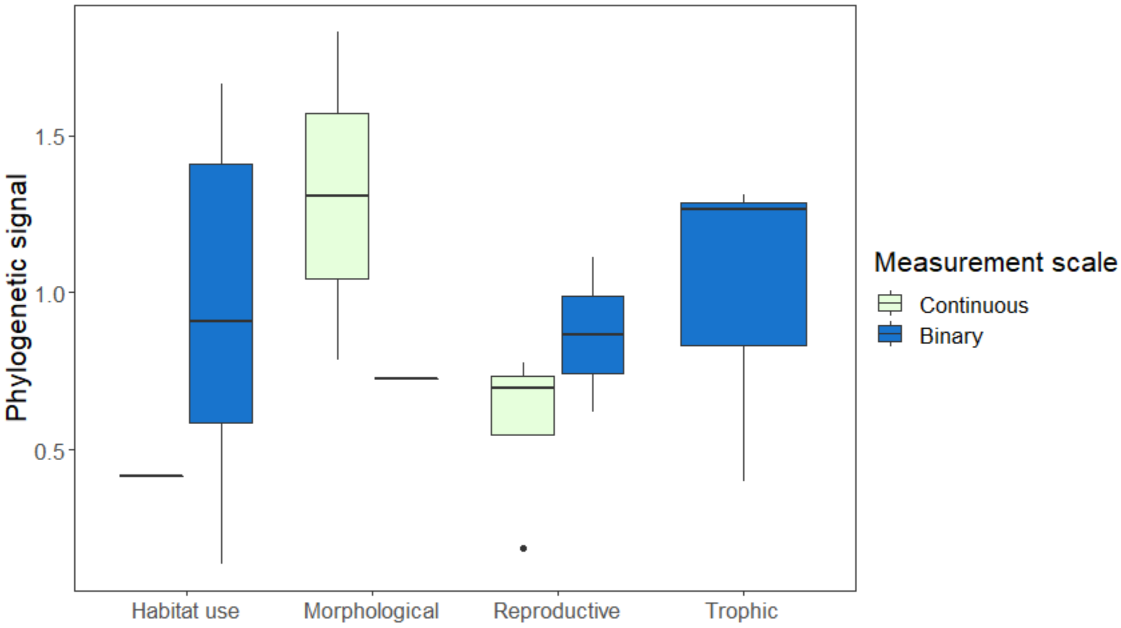


Figure S7.5. Effects of trait type and measurement scale on the phylogenetic signal measurement (–*D* + 1 for binary and Blomberg's *K* for continuous traits). Boxes correspond to the 25th and 75th percentiles; lines inside a box show the median; whiskers extend to the last observation within 1.5 times the interquartile range from the quartiles; and outliers are indicated by filled circles.

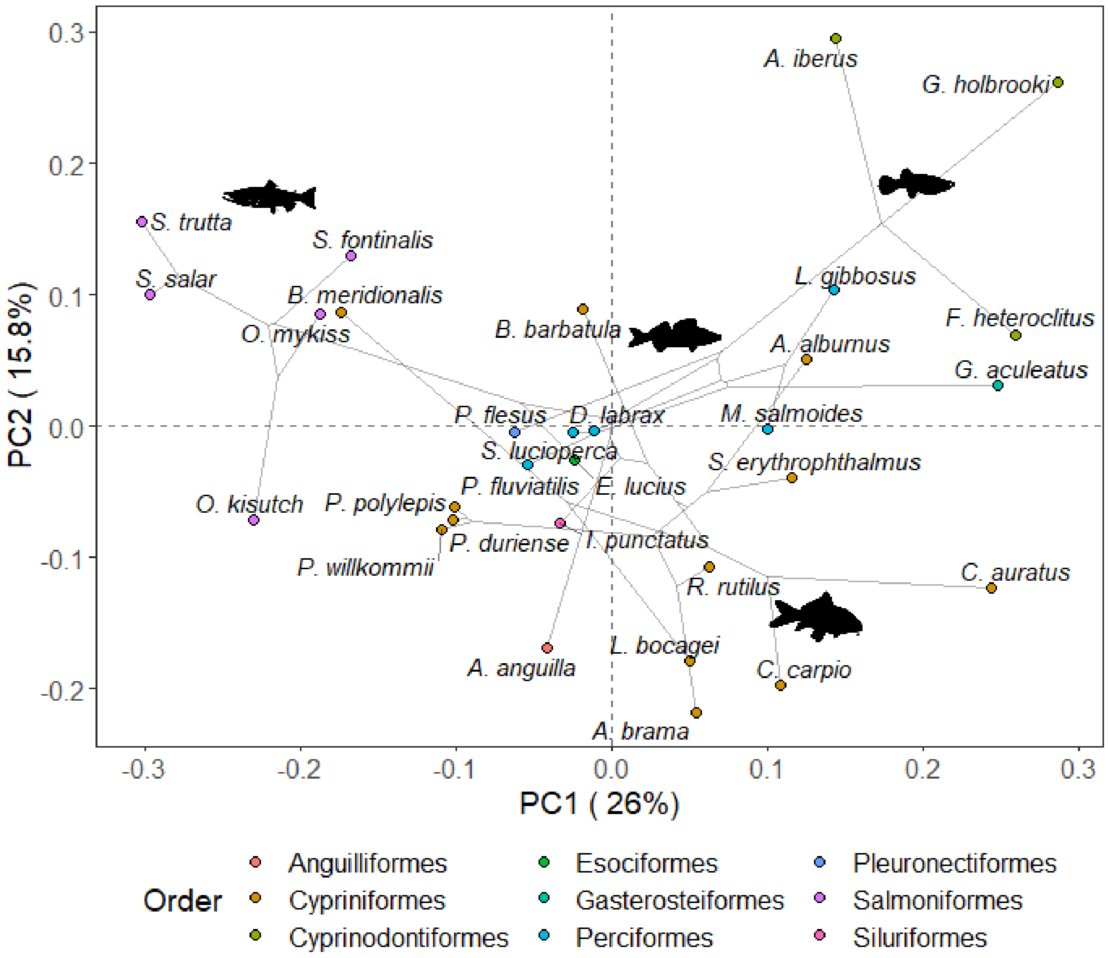


Figure S7.6. Phylomorphospace of the analysis of Figure 7.5 showing species scores; lines connecting taxa represent the branches of the Chang et al. (2019) phylogeny.

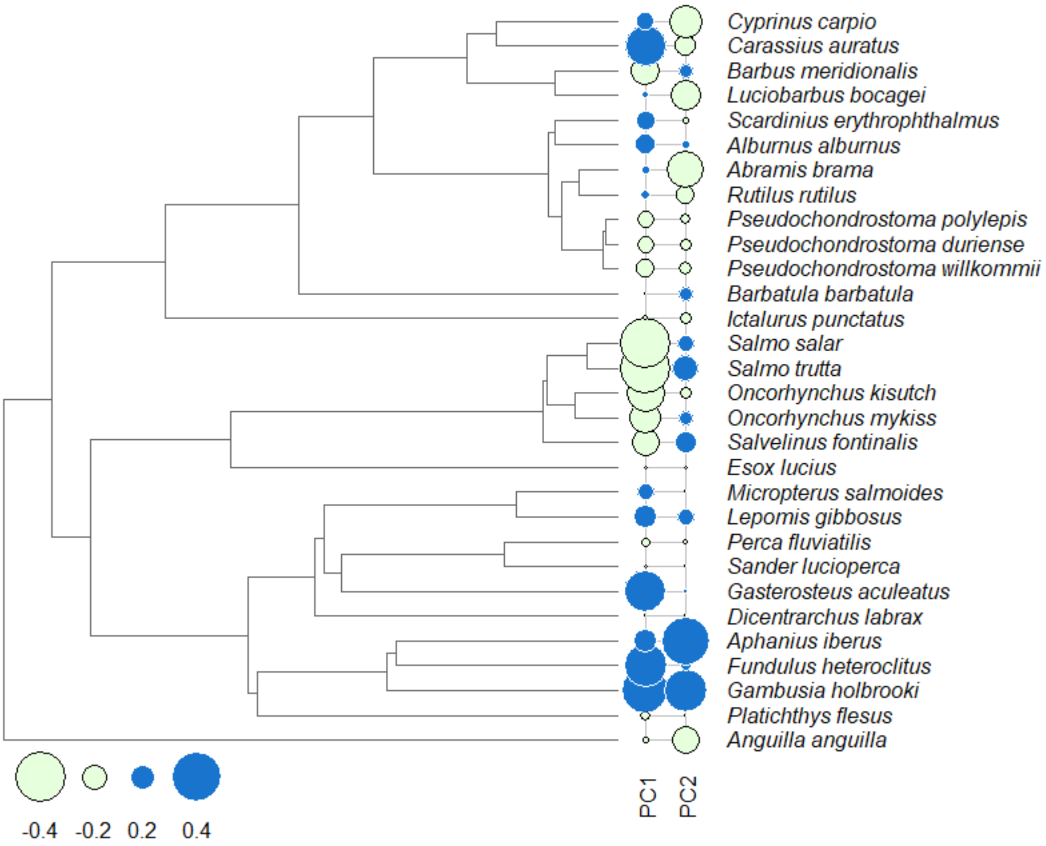


Figure S7.7. Phylogenetic tree annotated with a matrix of the species scores (circle size represents the standard deviate).

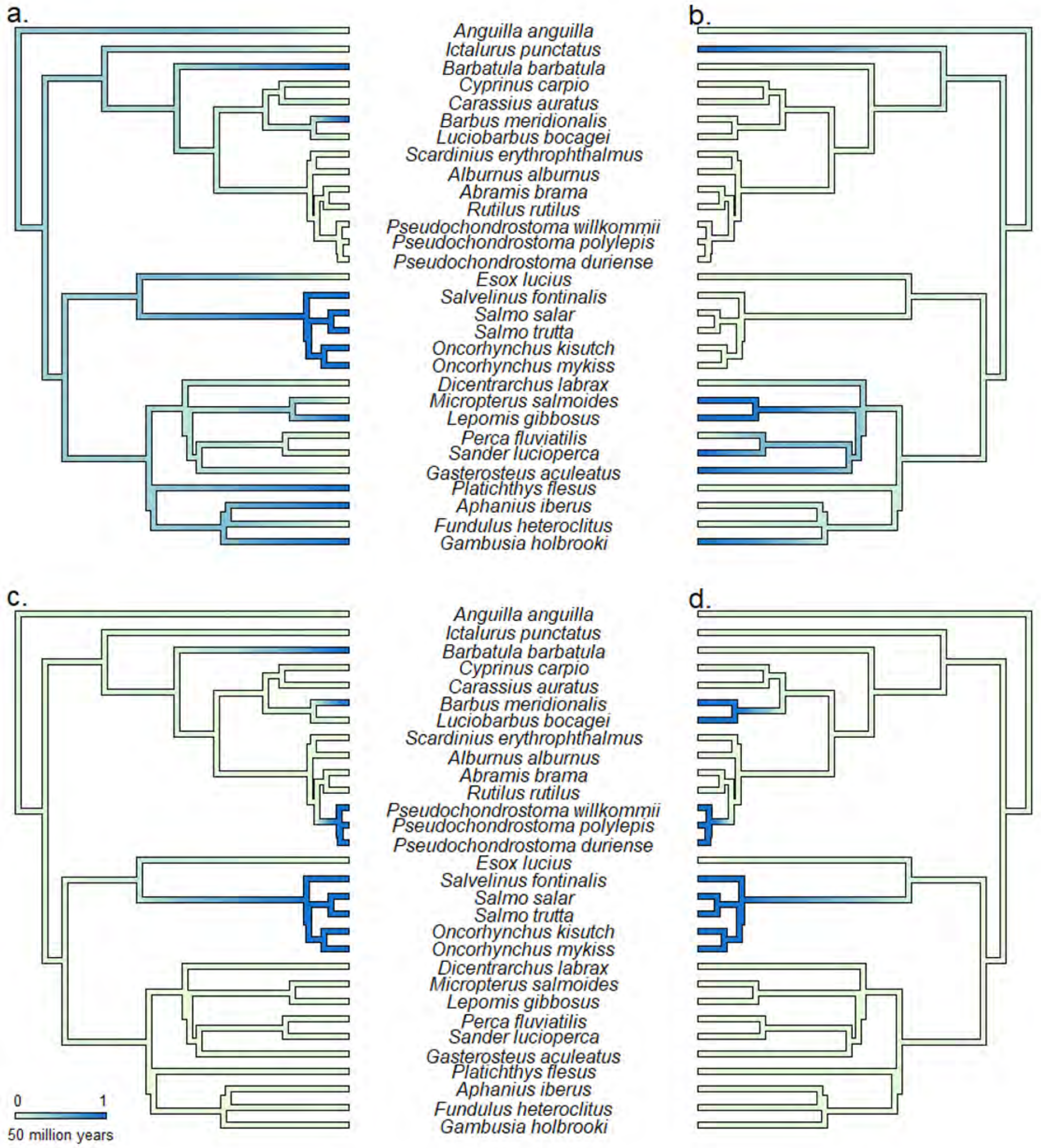


Figure S7.8. Ancestral state reconstruction of (a) invertivory, (b) parental care, (c) rheophily and (d) lithophily traits in Iberian inland fish. Light green color represents the absence of the trait, while the dark blue color represents the presence of the trait. Legend bar length corresponds to a 50 million years period.

Table S7.3. Correlations among continuous traits using phylogenetic generalised least squares. Correlation coefficients and *P* values (in parentheses) are shown. Note that all variables, except for form factor, were previously $\log_{10}$-transformed. $n = 30$.

	Max. total length	**Form factor**	**Max. longevity**	**Reproductive span**	**Max. fecundity**	**Age at maturity**
Form factor	-0.534 (0.388)	—	—	—	—	—
Max. longevity	**0.766** **(< .001)**	-0.064 (0.154)	—	—	—	—
Reproductive span	-0.091 (0.336)	0.004 (0.880)	**-0.300** **(0.025)**	—	—	—
Max. fecundity	**0.678** **(<0.001)**	-0.020 (0.690)	**0.776** **(<0.001)**	-0.337 (0.071)	—	—
Age at maturity	**0.755** **(<0.001)**	-0.047 (0.337)	**0.830** **(<0.001)**	**-0.441** **(0.015)**	**0.527** **(0.003)**	—
U_{crit}	0.099 (0.482)	-0.020 (0.615)	0.240 (0.133)	**-0.497** **(0.005)**	0.243 (0.099)	-0.035 (0.813)

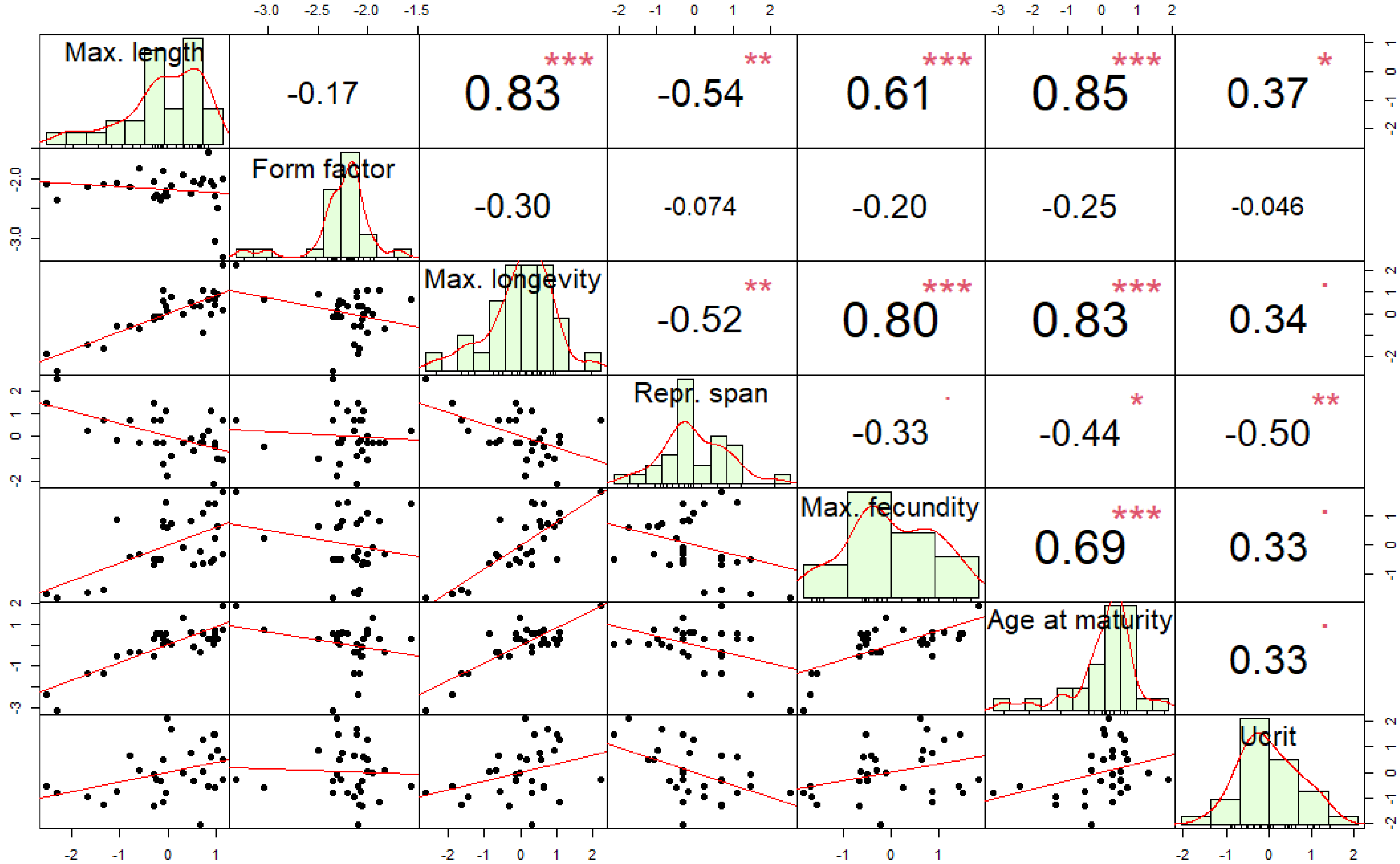


Figure S7.9. Correlation scatterplot matrix of continuous fish traits. The distribution of each variable is shown on the diagonal; the bivariate scatter plots with a fitted line are shown below the diagonal; Pearson's correlation coefficient and *P* values are shown above the diagonal (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ·, $P \leq 0.1$). $n = 30$.

Table S7.4. Correlations among binary traits using phylogenetic generalised linear mixed models for binary data. Correlation estimates and s^2 are shown. The s^2 value is a measure of the phylogenetic signal in the residuals; values further from zero indicate more significant phylogenetic signal in the residuals. Asterisks (*) show significant values ($P < 0.05$). $n = 30$.

	Fusiform	**Invertivory**	**Omnivory**	**Piscivory**	**Par. care**	**Single spaw.**	**Rheophily**	**Limnophily**	**Potamodromy**	**Long migr.**	**Benthic**	**Water column**	**Tolerance**	**Intolerance**	**Lithophily**
Invertivory	-1.71 **s^2: 3.75***	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Omnivory	4.13 **s^2: 0.63***	-6.18 **s^2: 0.13***	-	-	-	-	-	-	-	-	-	-	-	-	-
Piscivory	0.51 s^2: 3.41	-2.21 **s^2: 3.73***	-5.38 **s^2: 2.30***	-	-	-	-	-	-	-	-	-	-	-	-
Par. care	0.43 s^2: 3.08	-0.06 s^2: 1.95	0.33 **s^2: 4.11***	0.33 **s^2: 3.78**	-	-	-	-	-	-	-	-	-	-	-
Single spaw.	-0.55 s^2: 2.91	-0.31 s^2: 1.88*	0.55 **s^2: 3.87***	1.94 **s^2: 3.62**	0.08 **s^2: 3.76**	-	-	-	-	-	-	-	-	-	-
Rheophily	0.47 s^2: 2.64	2.53 **s^2: 2.64***	-0.74 **s^2: 3.88***	-0.32 **s^2: 3.95**	-19.84 **s^2: 1.95***	1.25 s^2: 1.36	-	-	-	-	-	-	-	-	-
Limnophily	0.59 s^2: 2.87	0.26 s^2: 1.86*	0.81 **s^2: 4.11***	-1.91 **s^2: 3.49**	1.05 **s^2: 3.59***	-1.19 s^2: 1.35	-1.20 **s^2: 2.91***	-	-	-	-	-	-	-	-
Potamodromy	2.63 **s^2: 0.66***	-4.37 **s^2: 0.76***	4.56 **s^2: 1.43**	-3.53 **s^2: 2.41**	-2.14 **s^2: 1.85***	3.50 **s^2: 1.26***	1.55 **s^2: 4.06***	-0.21 s^2: 2.17	-	-	-	-	-	-	-
Long migr.	-1.72 s^2: 3.04	0.68 s^2: 1.46	-3.49 **s^2: 2.88**	1.70 **s^2: 3.45**	-1.99 **s^2: 0.69***	4.29 **s^2: 1.42***	0.95 **s^2: 3.99***	-4.21 **s^2: 1.40***	-1.93 **s^2: 1.54***	-	-	-	-	-	-
Benthic	-1.71 s^2: 2.80	-0.06 s^2: 1.93	-0.06 **s^2: 3.98**	0.12 **s^2: 3.76**	-0.61 **s^2: 3.69***	0.52 s^2: 2.49	-0.48 **s^2: 4.52***	-0.57 s^2: 2.65	0.51 **s^2: 4.45***	2.61 **s^2: 4.72***	-	-	-	-	-
Water column	0.32 s^2: 2.69	-0.36 s^2: 1.74	-0.15 **s^2: 3.74**	0.17 **s^2: 3.59**	1.99 **s^2: 4.30***	-1.12 s^2: 2.46	-1.75 **s^2: 4.39***	0.28 s^2: 2.32	-1.72 **s^2: 0.44***	-0.17 **s^2: 3.23***	-4.54 **s^2: 2.20***	-	-	-	-
Tolerance	0.15 s^2: 2.86	-1.30 s^2: 1.86	1.27 **s^2: 3.66***	-1.51 **s^2: 3.50**	1.52 **s^2: 3.71***	-1.21 s^2: 2.15	-25.41 **s^2: 4.23***	1.93 s^2: 0.97	-0.27 **s^2: 3.70***	-0.71 **s^2: 3.34***	0.57 **s^2: 3.36***	0.15 s^2: $1.1 \cdot 10^{-12}$	-	-	-
Intolerance	2.80 **s^2: 0.27***	**5.42*** **s^2: 0.37***	-22.58 **s^2: 3.61***	-1.18 **s^2: 4.16**	-1.88 **s^2: 0.63***	0.30 s^2: 2.12	2.68 **s^2: 4.56***	-0.41 s^2: 2.23	-2.26 **s^2: 1.52***	0.84 **s^2: 3.10***	-1.13 **s^2: 3.60***	-0.23 s^2: $9.1 \cdot 10^{-13}$	-4.92 **s^2: 0.73***	-	-
Lithophily	18.17 **s^2: 1.45***	1.24 s^2: 1.94	0.32 **s^2: 3.85***	-0.21 **s^2: 3.92**	-2.10 **s^2: 0.42***	5.79 **s^2: $4.9 \cdot 10^{-14}$***	**5.14*** s^2: $2.4 \cdot 10^{-7}$	-1.23 s^2: 1.46	3.19 **s^2: 5.85***	1.03 **s^2: 3.76***	-0.61 **s^2: 3.56***	-1.35 s^2: $1.7 \cdot 10^{-14}$	**-2.47*** s^2: 0.96	2.59 s^2: 1.66	-
Phytophily	0.02 s^2: 2.79	-1.22 s^2: 2.05	1.19 **s^2: 4.01**	-1.04 **s^2: 3.50**	-1.98 **s^2: 0.66***	-1.20 s^2: 1.70	-23.70 **s^2: 3.88***	**2.42*** s^2: 2.32	-1.87 **s^2: 0.50**	-8.42 **s^2: 2.96***	-0.40 **s^2: 3.55***	0.64 s^2: $6.3 \cdot 10^{-15}$	0.31 s^2: 1.41	0.20 s^2: 1.78	-23.88 **s^2: 5.01***

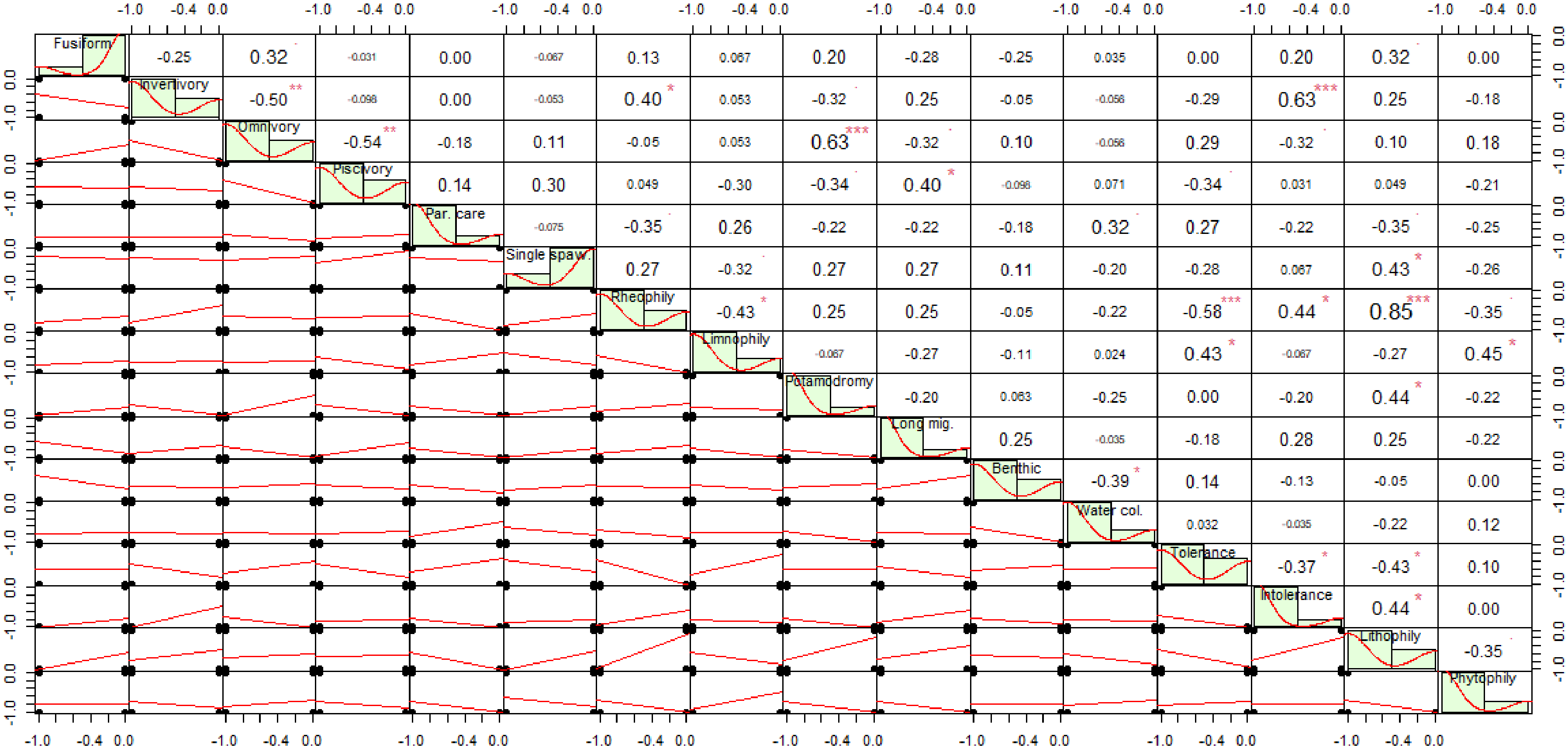


Figure S7.10. Correlation scatterplot matrix of binary fish traits. The distribution of each variable is shown on the diagonal; the bivariate scatter plots with a fitted line are shown below the diagonal; Pearson's correlation coefficient and *P* values are shown above the diagonal (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ·, $P \leq 0.1$). $n = 30$.

Supplementary materials S8 - Estimates of niche position and breadth vary across spatial scales for native and alien inland fishes

Table S8.1. List of studies using outlying mean index (OMI) analyses. The literature search was systematically carried out using Google Scholar (searched on 1st February 2022) with the combination of the keywords: "outlying mean index" & "freshwater". The search returned a total of 198 compatible results. For further analyses we used 106 peer-reviewed studies (displayed here) that actually used OMI analyses to study organisms linked to freshwater, brackish or coastal ecosystems. Literature reference, the studied taxa and the geographic and latitudinal ranges of the study area are provided. The inclusion (+) or exclusion (-) of climatic, topographic or other variables in the OMI analysis is indicated as well as whether studies conducted a single OMI analysis including all variables (-) or several OMI analyses grouping different types of variables (+). See "Notes" for further details.

Reference	**Taxa**	**Geographic range**	**Lat. range**	**Climate**	**Topography**	**Other**	**Separate OMI**	**Notes**
Dolédec et al. 2000	Caddisfly	Lyon (France)	45 °N	+	-	-	-	A single OMI analysis with climatic variables
Dolédec et al. 2000	Fish	Rhône River (France)	43 – 49 °N	-	+	-	-	A single OMI analysis with topographic variables
Fièvet et al. 2001	Fish and shrimp	Guadeloupe (Caribbean Sea)	15 – 18 °N	-	+	+	-	Use topographic, habitat and anthropogenic variables
Choler and Michalet 2002	*Carex* (plants)	French Alps	44 – 46 °N	+	+	+	-	Use climatic, topographic and other habitat variables
Reichard et al. 2002	*Rhodeus sericeus* (fish)	River Morava (Danube basin, Czech Republic)	48 °N	-	-	+	-	A single OMI analysis with hydrological variables
Matejusová et al. 2003	*Pseudodactylogyrus* sp. (Monogenea)	-	-	-	-	+	-	A single OMI analysis with gill variables of the European eel (*Anguilla anguilla*)
Kadlec et al. 2003	*Dactylogyrus* sp. (Monogenea)	-	-	-	-	+	-	A single OMI analysis with gill variables of the European barbel (*Barbus barbus*)
Malard et al. 2003	Invertebrates	Roseg River (Switzerland)	46 – 47 °N	-	-	+	-	A single OMI analysis with hydrological variables
Tales et al. 2004	Fish	France	42 – 51 °N	+	+	+	-	A single OMI analysis with climatic, topographic and hydrological variables
Mérigoux and Dolédec 2004	Invertebrates	Rhône River (France)	43 – 49 °N	-	-	+	-	Use habitat and hydrological variables
Heino 2005	Insects	Koutajoki drainage (Finland)	66 – 67 °N	-	-	+	-	A single OMI analysis with hydrological variables
Mezquita et al. 2005	Ostracods	Iberian Peninsula	36 – 41 °N	-	-	+	-	A single OMI analysis with hydrological variables
Heino and Soininen 2006	Diatoms	Northern Finland	65 – 66 °N	-	+	+	-	Use topographic and other habitat variables
Lappalainen and Soininen 2006	Fish	Southern and central Finland	60 – 67 °N	+	+	+	-	Use climatic, topographic and other habitat variables
Soininen and Heino 2007	Diatoms	Finland	60 – 70 °N	-	-	+	-	A single OMI analysis with hydrological variables
Soininen 2008	Diatoms	Finland	60 – 70 °N	-	-	+	-	A single OMI analysis with hydrological variables
Buisson et al. 2008	Fish	France	44 – 51 °N	+	+	-	-	Use climatic and topographic variables
Dole-Olivier et al. 2009	Stygofauna	Jura massif (France)	47 °N	-	+	+	-	Use topographic and other hydrological variables
Galassi et al. 2009	Stygofauna	Lessinian Massif (Italy)	45 – 46 °N	-	+	+	-	Use topographic and other hydrological variables

Reference	Taxa	Geographic range	Lat. range	Climate	Topography	Other	Separate OMI	Notes
Martin et al. 2009	Stygofauna	Wallonia (Belgium)	50 °N	-	+	+	-	A single OMI analysis with topographic, physicochemical, land use and historical variables
Siqueira et al. 2009	Chironomids	São Paulo State (Brazil)	20 – 25 °S	+	-	+	+	Use local physicochemical predictors and landscape and climatic variables separatedly
van de Meutter et al. 2010	*Trichocorixa verticalis* (Heteroptera: Corixidae)	Marismas del Guadalquivir (Spain)	37 °N	-	-	+	+	Compute two OMI analyses: (1) using salinity-related variables, and (2) including other environmental factors.
Besacier-Monbertrand et al. 2010	Macroinvertebrates	Rhône River (France)	45 – 46 °N	-	-	+	-	A single OMI analysis with hydrological variables
Hof et al. 2010	Amphibians	Global	Global	+	-	-	-	A single OMI analysis with climatic variables
Jones et al. 2012	Fish	Isthmus of Tehuantepec, Mexico	16 – 18 °N	-	+	+	-	A single OMI analysis with topographic and hydrological variables
Gascón et al. 2012	Branchipods	Iberian Peninsula	35 – 44 °N	-	+	+	-	Use topographic and other habitat variables
Korsu et al. 2012	Fish	Northeastern Finland	65 – 70 °N	-	+	+	-	Use topographic and other habitat variables
Carbonell et al. 2012	Hemiptera	Iberian Peninsula	37 – 38 °N	-	-	+	-	A single OMI analysis with hydrological variables
Albouy et al. 2013	Fish	Mediterranean coast	30 – 45 °N	+	-	-	-	A single OMI analysis with climatic variables
Céspedes et al. 2013	Beetles	Iberian Peninsula	35 – 44 °N	-	-	+	-	Use habitat and hydraulic variables
Tornés and Ruhí 2013	Diatoms	Catalonia (Spain)	40 – 43 °N	-	+	+	-	Use topographic and other habitat variables
Kadye and Booth 2013	Fish	Glen Melville Reservoir (South Africa)	33 °S	-	-	+	-	A single OMI analysis with habitat and hydrological variables
Cardo et al. 2013	Insects	Paraná River Delta	34 °S	+	+	+	-	Use climatic, topographic and other hydrological variables
Sutani et al. 2014	Phytoplankton	Japan	37 °N	-	-	+	-	A single OMI analysis with physicochemical variables
Arribas et al. 2014	*Enochrus* sp. (Hydrophilidae)	Europe and northern Africa	27 – 70 °N	+	-	-	-	A single OMI analysis with climatic variables
Chejanovski and Wiens 2014	Amphibians	Eastern North America (from Panama to south USA)	7 – 37 °N	+	-	-	+	Use temperature and precipitation-related variables separatedly
Heino and Grönroos 2014	Fish	Northeastern Finland	65 – 70 °N	-	+	+	-	Use topographic and other habitat variables
Kotta et al. 2014	Aquatic plants	Baltic Sea	57 – 60 °N	-	-	+	-	A single OMI analysis with hydrological variables
Comte et al. 2014	Fish	France	42 – 51 °N	-	+	-	-	A single OMI analysis with topographic variables
Heino 2015	Insects	Finland	65 – 70 °N	-	-	+	-	A single OMI analysis with hydrological variables

Reference	Taxa	Geographic range	Lat. range	Climate	Topography	Other	Separate OMI	Notes
Baastrup-Spohr et al. 2015	Charophytes	Denmark, Sweden, Norway and Finland	55 – 70 °N	-	-	+	-	A single OMI analysis with hydrological variables
Hernández Fariñas et al. 2015	Phytoplankton	France	44 – 51 °N	+	-	+	-	Use climatic and other habitat variables
Rey-Boissezon and Auderset Joye 2015	Charophytes	Alps	46 – 47 °N	-	+	+	-	Use topographic and other habitat variables
Carnicer et al. 2015	*Ostreopsis* cf. *ovata* (dinoflagellate)	Ebro Delta (Spain)	40 – 41 °N	+	-	+	-	Use climatic and other hydrological variables
Falasco et al. 2015	Diatoms	Bossea cave (Italy)	44 °N	-	-	+	-	A single OMI analysis with local variables describing cave characteristics
Abdelahad et al. 2015	Algae	Po plain (Italy)	40 °N	-	-	+	-	A single OMI analysis with hydrological variables
Morandeira and Kandus 2015	Macrophytes	Paraná River floodplain	32 – 33 °S	-	-	+	-	A single OMI analysis with hydrological variables
Geheber and Frenette 2016	Fish	Duck River (US)	36 °N	-	-	+	-	A single OMI analysis with hydrological variables
Cantonati et al. 2016	Diatoms	River Adige (Italy)	45 -46 °N	-	-	+	-	A single OMI analysis with hydrological variables
Mykrä et al. 2016	Fungi	Finland	63 – 66 °N	-	-	+	-	A single OMI analysis with hydrological variables
Carbonell et al. 2016	Insects	Iberian Peninsula	35 – 44 °N	-	-	+	-	A single OMI analysis with hydrological variables
Herkül et al. 2016	Amphipods	Baltic Sea	57 – 59 °N	-	+	+	-	A single OMI analysis with topographic and hydrological variables
Tonkin et al. 2016	Macorinvertebrates	Niger Delta	5 – 7 °N	+	+	+	-	A single OMI analysis with climatic, topographic and hydrological variables
Alahuhta et al. 2017a	Macrophytes	Finland, Sweden and US states of Minnesota and Wisconsin	42 – 70 °N	+	-	+	+/-	Compute OMI analyses in each study area based on three explanatory variable groups: local, climate and combined local–climate variables
Heino and Grönroos 2017	Insects	Finland	65 – 70 °N	-	-	+	-	A single OMI analysis with habitat and hydrological variables
Comte and Olden 2017	Fish	Global	Global	+	-	-	-	A single OMI analysis with climate variables
Piano et al. 2017	Diatoms	Ligurian Alps (Italy)	44 – 45 °N	-	-	+	-	A single OMI analysis with topographic and hydrological variables
Iversen et al. 2017	Insects	Sweden and Denmark	55 – 57 °N	-	-	+	-	A single OMI analysis with vegetation and hydrological variables
Alahuhta et al. 2017b	Macrophytes	Finland, Sweden and US states of Minnesota and Wisconsin	42 – 70 °N	+	-	+	-	A single OMI analysis with climatic and water quality variables

Reference	**Taxa**	**Geographic range**	**Lat. range**	**Climate**	**Topography**	**Other**	**Separate OMI**	**Notes**
Gippet et al. 2017	Ants	Lyon (France)	45 °N	+	+	+	-	Use climatic, topographic and anthropogenic variables
Mykrä and Heino 2017	Invertebrates	Finland	60 – 70 °N	-	+	+	-	Use topographic and other hydrological variables
Escoriza and Hassine 2017	Amphibians	Morocco, Algeria and Tunisia	29 – 38 °N	+	+	+	-	Use climatic, topographic and other hydrological variables
Tonkin et al. 2017	Invertebrates	Hindu-Kush Himalaya (Nepal)	27 – 29 °N	-	-	+	-	A single OMI analysis with hydrological variables
Teittinen et al. 2018	Diatoms	Finland and Norway	68 – 70 °N	-	-	+	-	A single OMI analysis with hydrological variables
Valente-Neto et al. 2018	Insects	São Paulo State (Brazil)	24 °S	-	-	+	-	A single OMI analysis with hydrological variables
Larsen et al. 2018	Invertebrates	Wales	52 °N	-	-	+	-	A single OMI analysis with hydrological variables
Heino and Tolonen 2018	Invertebrates	Finland	66 °N	-	-	+	-	A single OMI analysis with hydrological variables
Karasiewicz et al. 2018	Phytoplankton	Boulogne (France)	50 °N	-	-	+	-	A single OMI analysis with hydrological variables
Rocha et al. 2018	Insects and diatom	Northeastern Finland	70 °N	+	+	+	+	Use topographic and habitat predictors separatedly
Lamb et al. 2018	Pelicans	North America	24 – 45 °N	-	-	+	-	A single OMI analysis with habitat variables
Le Coz et al. 2018	Zooplankton	Scheldt River (France)	49 °N	-	-	+	-	A single OMI analysis with physicochemical variables
Lindholm et al. 2018	Diatoms	Tenojoki drainage (Finland and Norway)	70 °N	-	+	+	-	A single OMI analysis with environmental variables at local and catchment scale and spatial variables
Prat and García-Roger 2018	Chironomids	Vallcebre catchment (Spain)	42 °N	-	-	+	-	A single OMI analysis with hydrological variables
Rodrigues et al. 2019	Turtles	Global	Global	+	-	-	-	A single OMI analysis with climatic variables
Hatvani et al. 2019	Phytoplankton	River Tisza (Eastern Europe)	46 – 49 °N	-	-	+	-	A single OMI analysis with hydrological variables
Alves-de-Souza et al. 2019	*Chaetoceros minimus* and *Hyalodiscus* sp.	Rio de Janeiro	22 °S	-	-	+	-	A single OMI analysis with hydrological variables
Soininen et al. 2019	Diatoms	United States, France, Finland, New Zealand, Antilles and La Réunion	Global	+	-	+	-	Use climatic and local variables
Piano et al. 2019	Ephemeroptera (Heptageniidae)	Piemonte (Italy)	44 – 46 °N	-	-	+	-	A single OMI analysis with hydrological variables
Vilmi et al. 2019	Diatoms and macroinvertebrates	Lake Kitkajärvi (Finland)	64 °N	-	-	+	-	A single OMI analysis with physicochemical variables
Camatti et al. 2019	Zooplankton	Lagoon of Venice (Italy)	45 °N	-	-	+	-	A single OMI analysis with physicochemical variables
Zanon et al. 2019	Phytoplankton	Peixe Angical Reservoir (Brazil)	12 °S	-	-	+	-	A single OMI analysis with hydrological variables

Reference	Taxa	Geographic range	Lat. range	Climate	Topography	Other	Separate OMI	Notes
Lengyel et al. 2020	Diatoms	Hungary and Germany	46 – 54 °N	-	-	+	-	A single OMI analysis with physicochemical variables
Piano et al. 2020	Nemouridae (Plecoptera)	Piemonte (Italy)	44 – 46 °N	-	-	+	-	A single OMI analysis with hydrological variables and considering species competition
Kadye and Booth 2020	Fish	Great Fish River (South Africa)	33 °S	-	-	+	-	A single OMI analysis with hydrological variables
Leboucher et al. 2020	Diatoms	France	42 – 51 °N	-	-	+	-	A single OMI analysis with hydrological variables
Marino et al. 2020	Invertebrates	America	30 °S – 20 °N	-	-	+	-	A single OMI analysis with habitat variables
Lamb et al. 2020	Birds	North America	15 – 75 °N	+	+	+	+	Compute several OMI analyses using different groups of variables and for the different seasons
Saccò et al. 2020	Stygofauna	Western Australia	28 °S	-	-	+	-	A single OMI analysis with hydrological variables
Martins and Ferreira 2020	Elmidae (Coleoptera)	Lapa Grande State Park, Montes Claros, Minas Gerais state (Brazil)	16 °S	-	-	+	-	A single OMI analysis with hydrological variables
David et al. 2020	Plankton	Charente-Maritime marshes (France)	46 °N	-	-	+	-	A single OMI analysis with hydrological variables
Karasiewicz et al. 2020	Algae	France	44 – 51 °N	-	-	+	-	A single OMI analysis with physicochemical variables
Serpa et al. 2020	Invertebrates	Prata River (Brazil)	20 °S	-	-	+	-	A single OMI analysis with hydrological variables
da Silva et al. 2020	Phytoplankton	Cana Brava Reservoir (Brazil)	13 °S	-	-	+	-	A single OMI analysis with hydrological variables
Granzotti et al. 2021	Fish	Paraná River (Brazil)	22 – 23 °S	-	-	+	-	A single OMI analysis with hydrological variables
Guterres et al. 2021	Insects	Brazil	2 – 4 °S	-	-	+	-	A single OMI analysis with hydrological variables
Sánchez-Hernández et al. 2021	Invertebrates and fish	Norway	65 – 67 °N	-	-	+	-	A single OMI analysis with available prey resources variables
Escoriza 2021	Lizards	Mediterranean islands	34 – 45 °N	+	+	+	-	Use climatic, topographic, habitat and biotic variables
Escoriza and Pascual 2021	Reptiles	Morocco, Spain and France	28 – 44 °N	-	+	+	-	A single OMI analysis with topographic and riparian habitat variables
Houliez et al. 2021	Microphytoplankton	Bay of Biscay	43 – 48 °N	-	-	+	-	A single OMI analysis with hydrological variables
Baldrich et al. 2021	Dinoflagellates	Southern Chile	44 – 45 °S	-	-	+	-	A single OMI analysis with hydrological variables
Kolada 2021	Charophytes	Poland	51 – 55 °N	-	-	+	-	A single OMI analysis with hydrological variables
Stewart et al. 2021	Algae	French Alps	44 – 46 °N	-	+	+	-	A single OMI analysis with topographic and hydrological variables
Souza-Silva et al. 2021	Invertebrates	Água Clara cave system (Brazil)	14 °S	-	-	+	-	A single OMI analysis with habitat variables

Reference	Taxa	Geographic range	Lat. range	Climate	Topography	Other	Separate OMI	Notes
López-Peña et al. 2021	Blackflies (Diptera: Simuliidae)	Mediterranean coast of Spain	38 – 40 °N	-	+	+	-	A single OMI analysis with topographic and hydrological variables
Taybi et al. 2021	Hemiptera	Moulouya River (Morocco)	32 – 35 °N	-	+	+	-	A single OMI analysis with topographic and hydrological variables
Tóth et al. 2022	Fish	Hungary	45 – 49 °N	-	-	+	-	A single OMI analysis with hydrological variables

Table S8.2. References for occurrence records of the Iberian inland fish that are present outside the Iberian Peninsula.

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Ictalurus punctatus: GBIF.org (07 May 2021) GBIF Occurrence Download https://doi.org/10.15468/dl.kvpvzh
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Table S8.3. Environmental variables used to compute the outlying mean index (OMI) analysis.

Predictor variables (TYPE)	Abbreviation	Range	Source	Description
CLIMATE				
Temperature seasonality (standard deviation × 100)	BIO4	271.7 – 770.9	1	Temperature seasonality is a measure of the annual temperature stability. Previous studies found a negative correlation of this variable and species richness (Raes *et al.*, 2009).
Mean temperature of warmest quarter (°C)	BIO10	10.6 – 26.3	1	Mean temperature during the warmest three months of the year.
Mean temperature of coldest quarter (°C)	BIO11	-5.0 – 13.1	1	Mean temperature during the coldest three months of the year.
Precipitation seasonality (%)	BIO15	13.9 – 78.3	1	Average precipitation seasonality is a measure of the variation in monthly precipitation totals over the course of the year. It could be used as a surrogate of the flow regime, which is a key environmental factor determining riverine dynamics (Carré & Cheddadi, 2017).
Precipitation of wettest quarter (mm)	BIO16	87.0 – 750.0	1	Accumulated precipitation during the wettest three months of the year.
Precipitation of driest quarter (mm)	BIO17	13.0 – 266.0	1	Accumulated precipitation during the driest three months of the year. It is one of the main predictors in determining fish species richness patterns, as the intensity of the dry periods can condition the survival of freshwater species (Guo *et al.*, 2015).
LONGITUDINAL POSITION				
Elevation (m)	Elev	0.0 – 2801.6	2	Elevation is related to river longitudinal position, water flow velocity or water temperature (Cano-Barbacil *et al.*, 2022).
Slope (°)	Slo	0.0 – 27.2	2	Slope is an indicator of the amount of energy available in the system (Wyrick *et al.*, 2009). Therefore, it could be used as a proxy of water velocity, habitat and substrate type.
Topographic index	TopInd	-1.3 – 10.3	2	The topographic index is a function of the catchment area and the slope gradient commonly used to quantify topographic control on hydrological processes (Sørensen *et al.*, 2006).
Distance to the sea (km)	DisSea	0.0 – 928.5	2	Distance to the sea is a good indicator of the influence of seawater and of the accessibility to upstream waters for peripheral and secondary species.
Strahler's stream order	StrOrdSt	1.0 – 6.4	2	Strahler's stream order state that if two streams of the same order merge, the following downstream segment have that order plus one. When two streams with different order merge, the next downstream segment is assigned the higher number of the two. It

Predictor variables (TYPE)	Abbreviation	Range	Source	Description
				could be used as a proxy of stream size and longitudinal position within a river system (Strahler, 1957).
Shreve’s stream order	StrOrdSh	1.0 – 4383.7	2	Shreve’s stream order assign, at each juncture of two streams, the sum of their orders to the next downstream segment. It could also be used as a proxy of stream size and longitudinal position within a river system (Shreve, 1966).

Sources: 1 = WorldClim, Global Climate Data (http://worldclim.org/version2); 2= Spanish National Center for Geographic Information (http://centrodedescargas.cnig.es/).

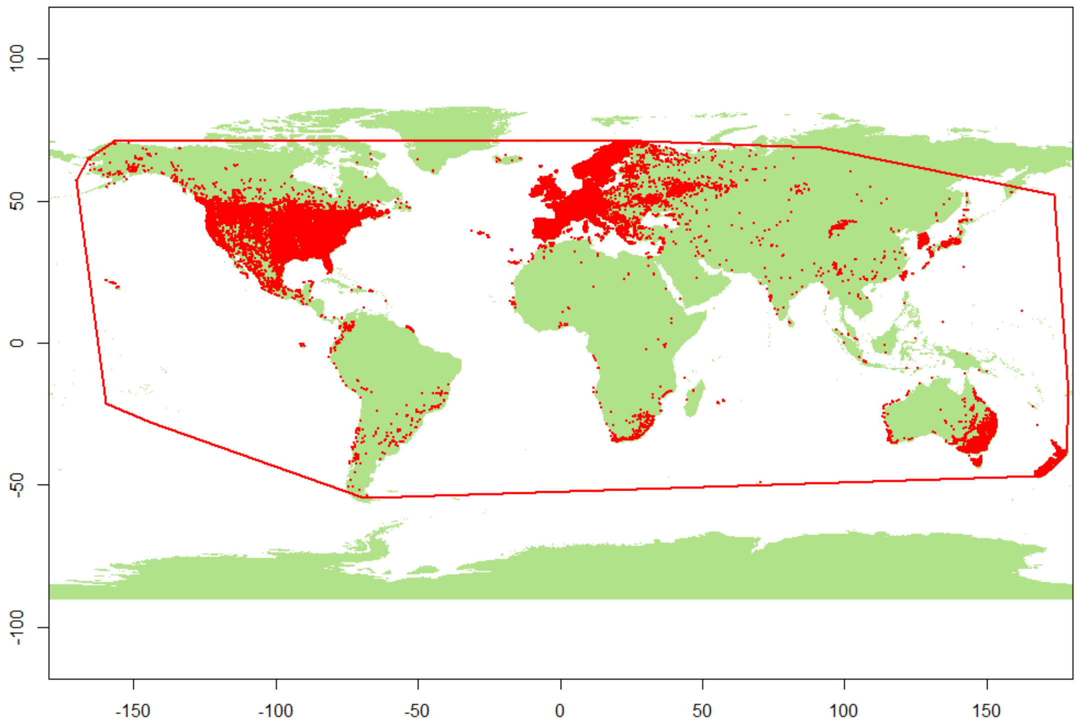

Figure S8.1. Occurrence records of all species considered in this study (red solid circles) and background region (red polygon) used for the restricted outlying mean index (OMI) analysis. Note that marine occurrences were excluded from the analyses.

Table S8.4. Analyses of covariance relating global, restricted and regional climatic niche metrics considering the native status of species (NS) and its interaction with the covariate. Sums of squares (SS) are shown for each predictor and *P* values are expressed with asterisks (*** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05; . ≤ 0.1). GCNP = global climatic niche position; GCNM = global climatic niche marginality; GCNB = global climatic niche breadth; ResCNP = restricted climatic niche position; ResCNM = restricted climatic niche marginality; ResCNB = restricted climatic niche breadth; RegCNP = regional climatic niche position; RegCNM = regional climatic niche marginality; RegCNB = regional climatic niche breadth.

	Niche position			**Niche marginality**			**Niche breadth**		
	Model (R^2_{adj})	**Variable**	**SS (*P*)**	**Model (R^2_{adj})**	**Variable**	**SS (*P*)**	**Model (R^2_{adj})**	**Variable**	**SS (*P*)**
Global vs Restricted	GCNP ~ ResCNP × NS (R^2_{adj} = 0.974)	ResCNP	**12.463** ***	GCNM ~ ResCNM × NS (R^2_{adj} = 0.943)	ResCNM	**86.969** ***	GCNB ~ ResCNB × NS (R^2_{adj} = 0.941)	ResCNB	**1.647** ***
		NS	0.007		NS	0.070		NS	**0.024** ***
		ResCNP × NS	0.016 .		ResCNM × NS	**0.407** *		ResCNB × NS	**0.010** *
		Residuals	0.318		Residuals	5.065		Residuals	0.100
Global vs Regional	GCNP ~ RegCNP × NS (R^2_{adj} = 0.530)	RegCNP	**4.296** ***	GCNM ~ RegCNM × NS (R^2_{adj} = 0.146)	RegCNM	0.488	GCNB ~ RegCNB × NS (R^2_{adj} = 0.592)	RegCNB	**0.412** ***
		NS	**2.355** ***		NS	**15.587** ***		NS	**0.667** ***
		RegCNP × NS	**0.407** *		RegCNM × NS	0.976		RegCNB × NS	0.008
		Residuals	5.744		Residuals	75.460		Residuals	0.694
Restricted vs Regional	ResCNP ~ RegCNP × NS (R^2_{adj} = 0.557)	RegCNP	**10.949** ***	ResCNM ~ RegCNM × NS (R^2_{adj} = 0.181)	RegCNM	0.256	ResCNB ~ RegCNB × NS (R^2_{adj} = 0.523)	RegCNB	**1.154** ***
		NS	**6.527** ***		NS	**22.055** ***		NS	**1.454** ***
		RegCNP × NS	0.599 .		RegCNM × NS	2.428		RegCNB × NS	0.044
		Residuals	13.282		Residuals	88.800		Residuals	2.225

Table S8.5. Multiple regression models relating the regional climatic niche parameters with the global and regional climatic niche estimates, considering the introduction date (ID) of alien species. SE = standard error, *P* values (values < 0.05 are bolded). GCNP = global climatic niche position; GCNM = global climatic niche marginality; GCNB = global climatic niche breadth; ResCNP = restricted climatic niche position; ResCNM = restricted climatic niche marginality; ResCNB = restricted climatic niche breadth; RegCNP = regional climatic niche position; RegCNM = regional climatic niche marginality; RegCNB = regional climatic niche breadth.

Model (R^2_{adj})	**Variable**	**Estimate**	**SE**	***P***
RegCNP ~ GCNP × ID	Intercept	-29.849	126.317	0.817
(R^2_{adj} = 0.000)	GCNP	-16.300	60.748	0.793
	ID	0.014	0.065	0.834
	GCNP × ID	0.008	0.032	0.806
RegCNM ~ GCNM × ID	Intercept	-0.118	0.014	0.999
(R^2_{adj} = 0.000)	GCNM	-2.706	0.310	0.932
	ID	$6.6 \cdot 10^{-4}$	0.071	0.993
	GCNM × ID	0.001	0.016	0.928
RegCNB ~ GCNB × ID	Intercept	-17.486	9.708	0.097
(R^2_{adj} = 0.301)	GCNB	56.494	25.017	**0.043**
	ID	0.009	0.005	0.087
	GCNB × ID	-0.029	0.013	**0.046**
RegCNP ~ ResCNP × ID	Intercept	-27.180	54.919	0.630
(R^2_{adj} = 0.345)	ResCNP	20.065	35.292	0.580
	ID	0.013	0.028	0.657
	ResCNP × ID	-0.010	0.018	0.599
RegCNM ~ ResCNM × ID	Intercept	-18.122	61.015	0.772
(R^2_{adj} = 0.000)	ResCNM	2.174	23.668	0.928
	ID	0.010	0.031	0.744
	ResCNM × ID	-0.001	0.012	0.925
RegCNB ~ ResCNB × ID	Intercept	-18.037	11.582	0.145
(R^2_{adj} = 0.256)	ResCNB	31.397	16.486	0.081
	ID	0.010	0.006	0.134
	ResCNB × ID	-0.016	0.008	0.086

Table S8.6. Proportion of variation in niche metrics (R^2 of PERMANOVA analyses) explained by native status (NS). *P* values are expressed with asterisks (*** ≤ 0.001; ** ≤ 0.01; * ≤ 0.05; . ≤ 0.1). *P* values for tests of homogeneity of dispersions are also shown.

Response variable	**PERMANOVA**		**Homogeneity of dispersions**
	NS	**Residual**	***P***
Climatic niche position (global)	**0.136** **	0.864	**< 0.001**
Climatic niche marginality (global)	**0.128** **	0.872	**0.039**
Climatic niche breadth (global)	**0.523** ***	0.477	**< 0.001**
Climatic niche position (restricted)	**0.156** ***	0.844	**0.002**
Climatic niche marginality (restricted)	**0.156** ***	0.844	0.673
Climatic niche breadth (restricted)	**0.437** ***	0.563	**< 0.001**
Climatic niche position (regional)	0.010	0.990	0.068
Climatic niche marginality (regional)	**0.127** **	0.873	0.525
Climatic niche breadth (regional)	**0.080** *	0.920	**0.002**
Logitudinal niche position (regional)	**0.095** *	0.905	0.099
Longitudinal niche marginality (regional)	0.002	0.998	0.275
Longitudinal niche breadth (regional)	**0.132** **	0.868	0.166

Appendix S8.1

Our global OMI analysis with climatic variables showed that all variables were related to the first axis (97.4% of explained variation) but the most influencing were precipitation seasonality, precipitation of the driest quarter and mean temperature of coldest quarter (Figure S8.2). These variables separated species from areas with wet summers and lower precipitation seasonality (e.g. salmonids such as *Salmo salar or S. trutta*, and *Sander lucioperca*) from species present in regions with the opposite climatic features (e.g. centrarchids such as *M. salmoides*, cypriniforms such as *Luciobarbus* spp. and cyprinondontiforms such as *Aphanius* spp.) (Figure S8.2). The results of the restricted OMI analysis were very similar but with the first axis reversed (Figure S8.3). By contrast, climatic OMI analysis at the regional scale showed that the most important predictors separating the species' climatic niche were precipitation of the driest quarter (BIO17), mean temperature of the warmest quarter (BIO10) and precipitation seasonality (Figure S8.4) and the correlation of some predictors changed of sign, e.g. from negative to positive for BIO10 and BIO17 because warm climates are generally wet in summer at the global scale (e.g. tropics) but dry at the Iberian scale (e.g. Mediterranean climates). These variables separated cold and coolwater species (e.g. salmonids such as *S. salar or S. trutta*, and *Phoxinus bigerri* or *Barbus* spp.) from warmwater species (e.g. centrarchids such as *M. salmoides* and *Lepomis gibbosus* and cyprinondontiforms such as *G. holbrooki* and *Aphanius* spp.) and southern Iberian endemics (e.g. *Luciobarbus* spp.) (Figure S8.4). As the spatial extent was reduced, the explained variation by climatic variables decreased, especially for the first axis.

By contrast, the OMI analysis with river longitudinal variables showed that the most important predictors separating the species' longitudinal niche were Strahler's and Shreve's stream order and elevation (OMI axis 1) and downstream distance to the sea (OMI axis 2) and separated species inhabiting upper reaches from species from the lowermost reaches, mostly native diadromous or endemic cyprinodontiform species and alien primary species (e.g. siluriforms such as *Silurus glanis* or *Ameiurus melas*, and cypriniforms such as *Carassius* spp. or *Alburnus alburnus*) (Figure S8.5).

NM values obtained in the OMI analyses were generally related to the species scores of the first ordination axis (i.e. NP) following a quadratic relationship (Figure S8.6). This implied that sometimes there were species with similar marginality values but that inhabited areas placed in the opposite side of the niche gradient. For instance, *S. trutta* is mainly present in coldwater streams (Elliott & Elliott, 2010; Doadrio *et al.*, 2011), while *A. baeticus* occupy warmwater lagoons, canals and small rivers with high temporal climatic variability (Doadrio *et al.*, 2011), despite both species having a similar NM due to the observed non-monotonic relationship between NP and NM

(Figure S8.6c). Therefore, species scores of the first ordination axis provided an informative indicator of a species' position in the niche gradient. By contrast, focussing on NM informs only about the distance between the mean habitat conditions used by species and the mean habitat conditions of the studied area (Dolédec *et al.*, 2000) which might be misleading.

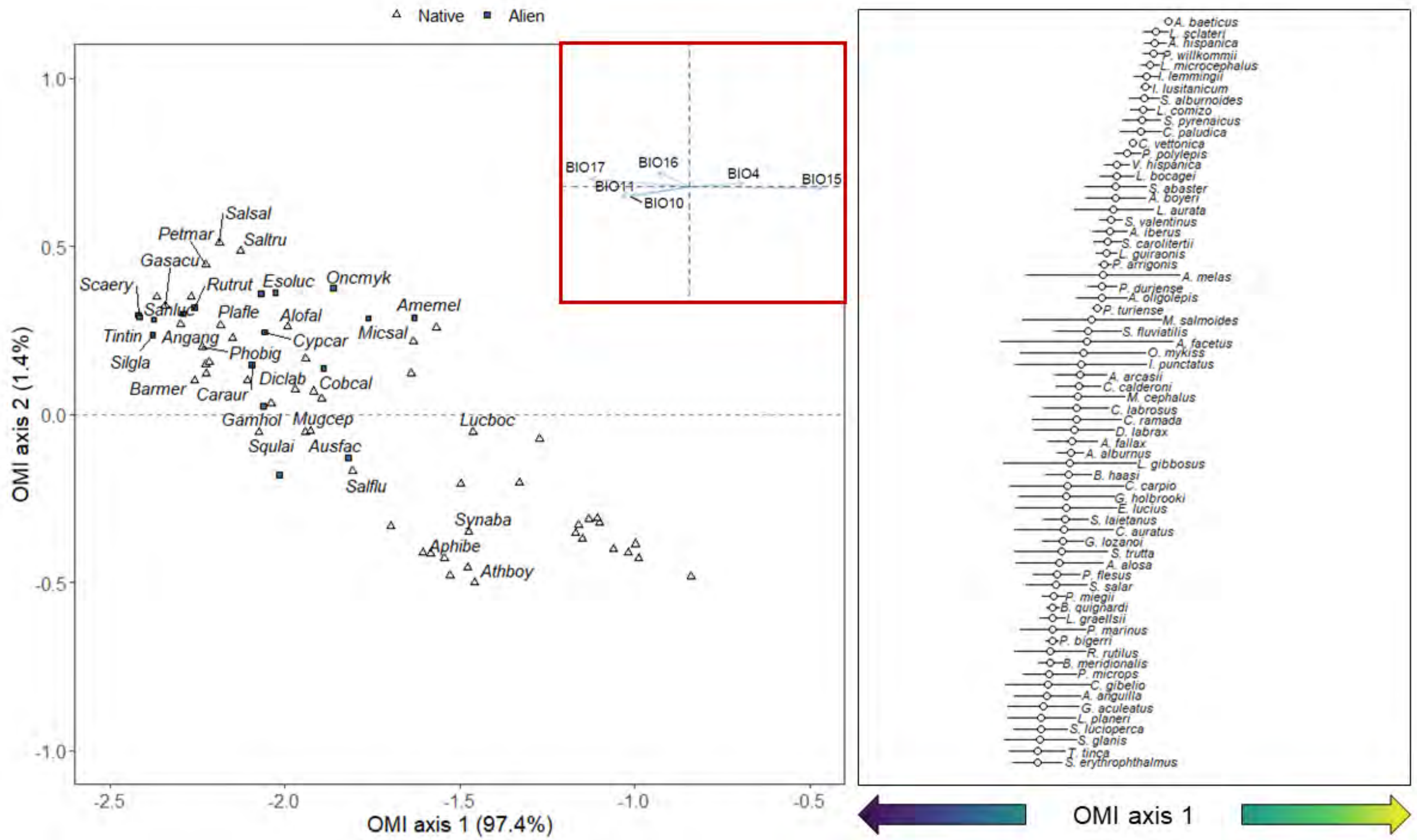

Figure S8.2. On the left, species' positions on the first factorial plane of the Outlying Mean Index (OMI) analyses using climatic variables and the global distributions. See Table S8.3 for abbreviations of the climatic variables. Species with lower marginality are located close to the origin of coordinates. On the right, position of taxa at the average sample-unit scores in the first ordination axis of the OMI analysis. The horizontal lines represent the standard deviation of scores. Species are ordered by their first OMI axis scores, i.e.: species on top for the first axis are those from areas with drier summers and greater precipitation seasonality.

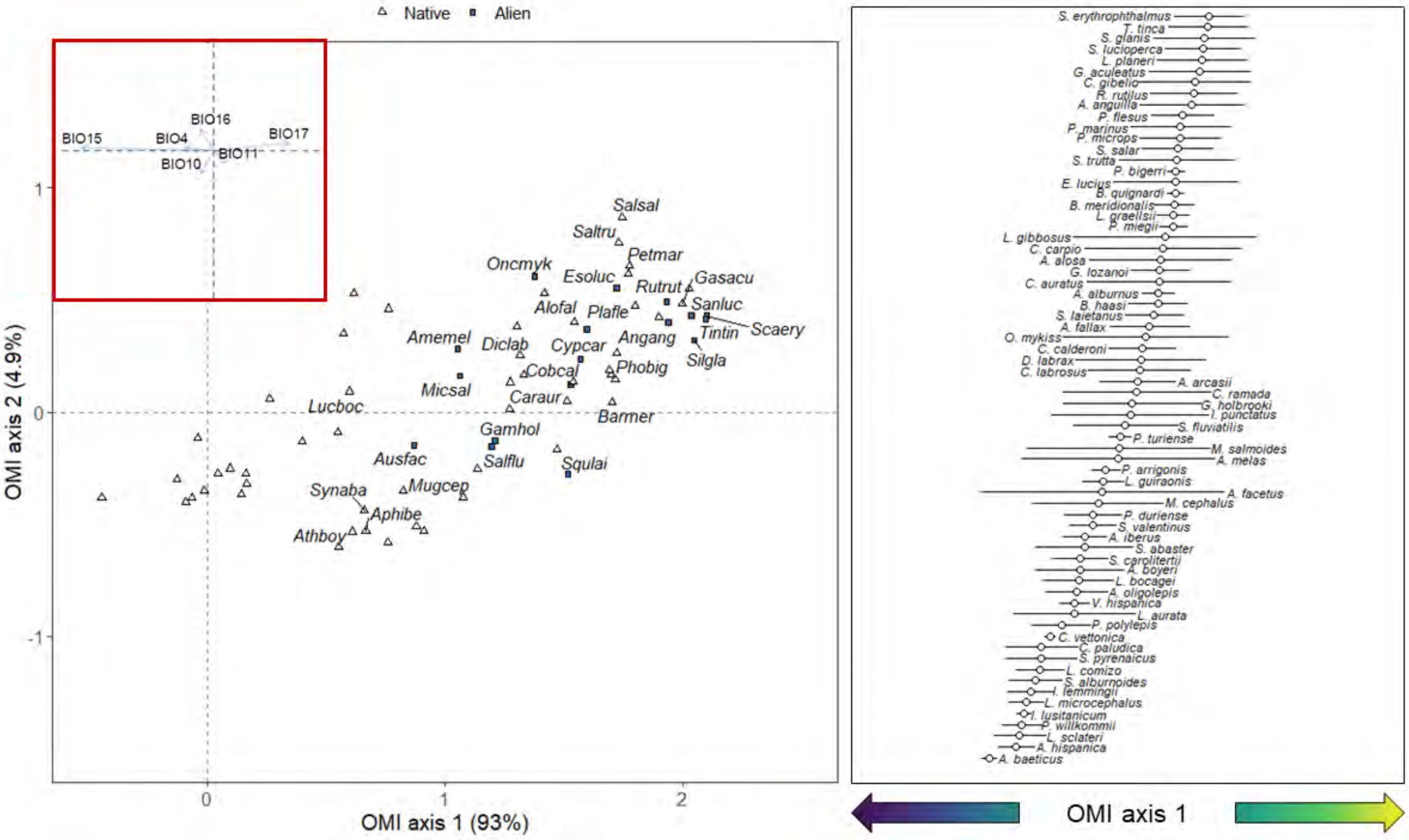

Figure S8.3. On the left, species' positions on the first factorial plane of the Outlying Mean Index (OMI) analyses using climatic variables and the restricted distributions. See Table S8.3 for abbreviations of the climatic variables. Species with lower marginality are located close to the origin of coordinates. On the right, position of taxa at the average sample-unit scores in the first ordination axis of the OMI analysis. The horizontal lines represent the standard deviation of scores. Species are ordered by their first OMI axis scores, i.e.: species on top for the first axis are those from areas with lower temperature and precipitation seasonality.

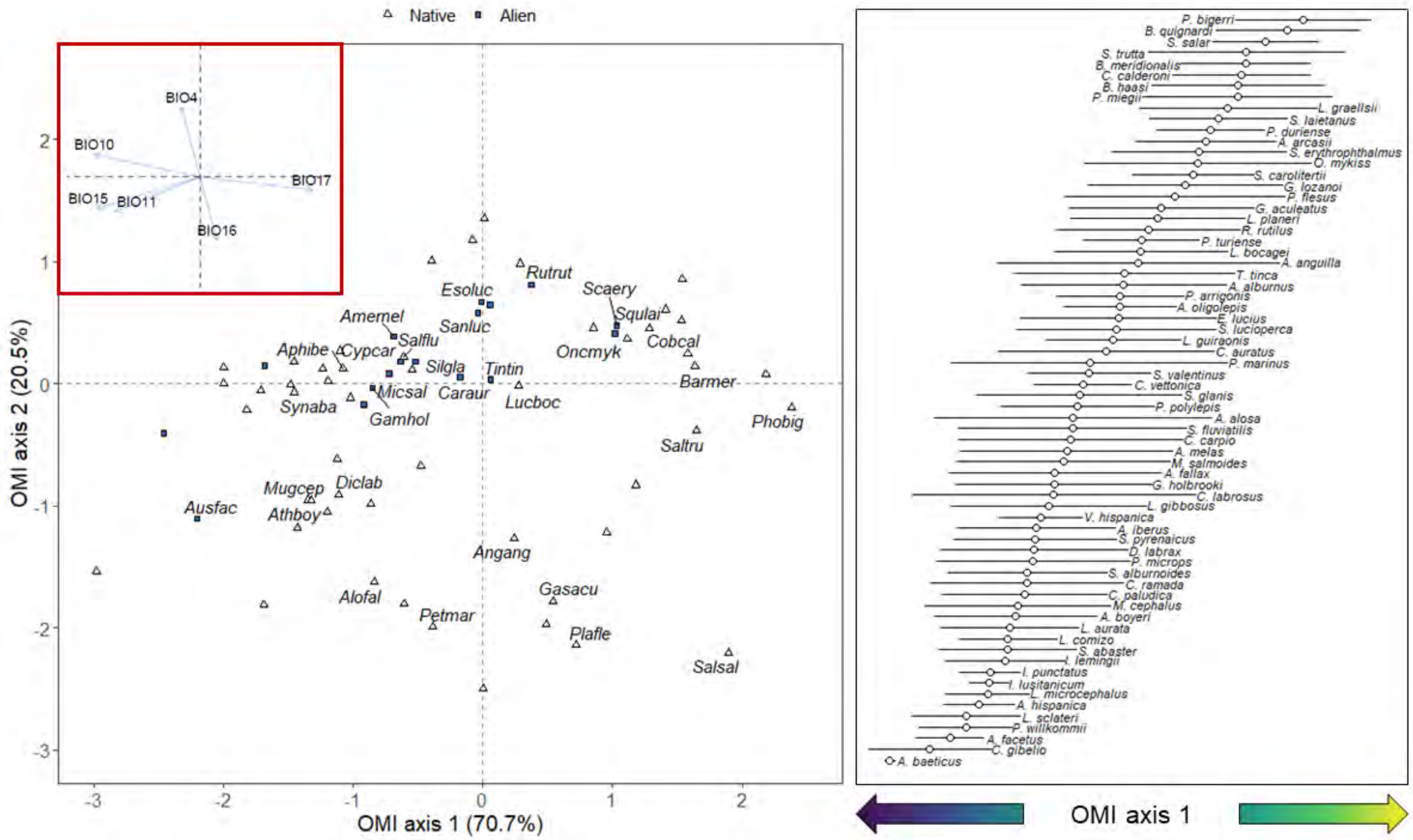

Figure S8.4. On the left, species' positions on the first factorial plane of the Outlying Mean Index (OMI) analyses using climatic variables and the regional distributions. See Table S8.3 for abbreviations of the climatic variables. Species with lower marginality are located close to the origin of coordinates. On the right, position of taxa at the average sample-unit scores in the first ordination axis of the OMI analysis. The horizontal lines represent the standard deviation of scores. Species are ordered by their first OMI axis scores, i.e.: species on top for the first axis are those from areas with lower temperature and precipitation seasonality.

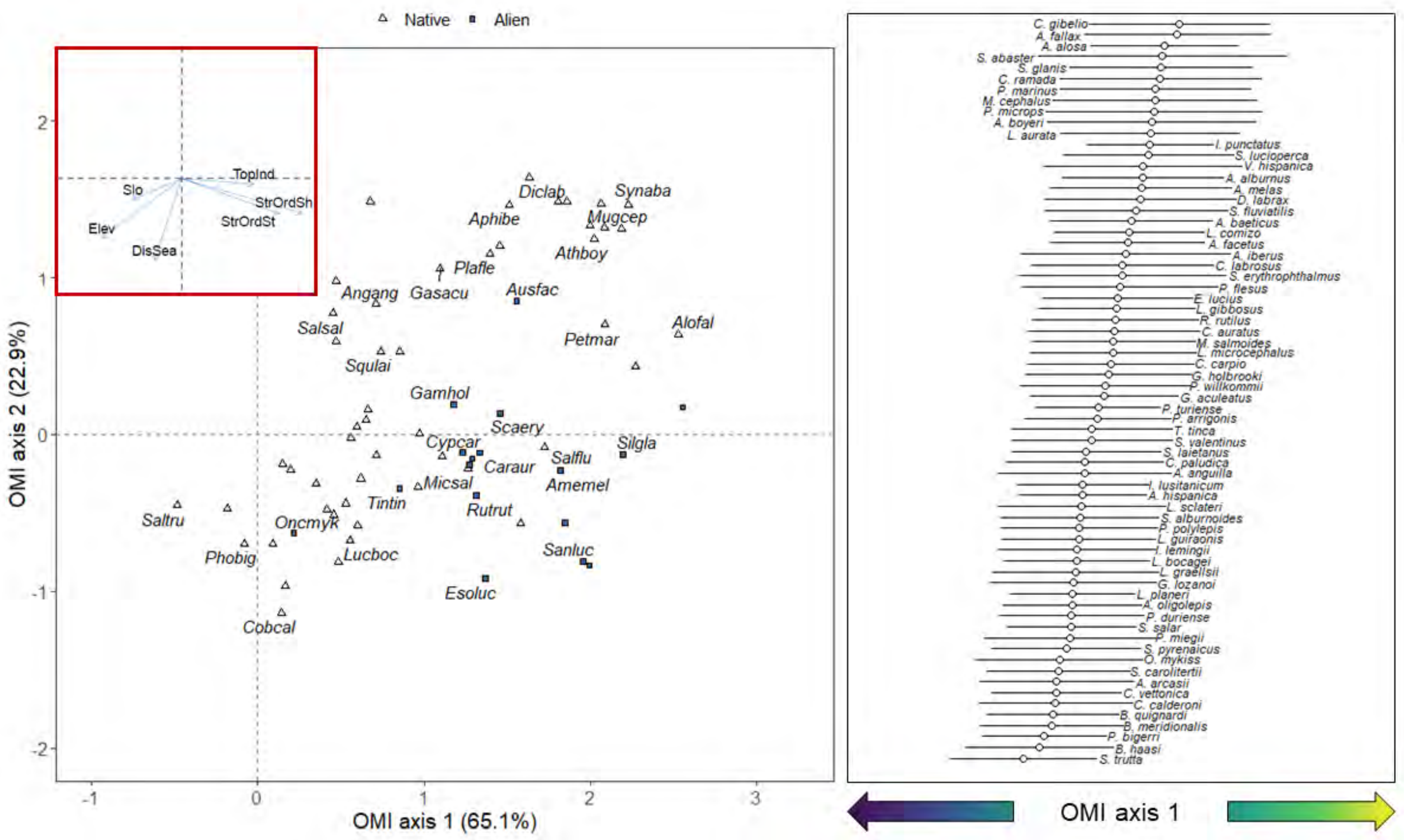

Figure S8.5. On the left, species' positions on the first factorial plane of the Outlying Mean Index (OMI) analyses using river longitudinal variables and the regional distributions. See Table S8.3 for abbreviations of the climatic variables. Species with lower marginality are located close to the origin of coordinates. On the right, position of taxa at the average sample-unit scores in the first ordination axis of the OMI analysis. The horizontal lines represent the standard deviation of scores. Species are ordered by their first OMI axis scores, i.e.: species on top for the first axis are those from lowland areas that inhabit big rivers.

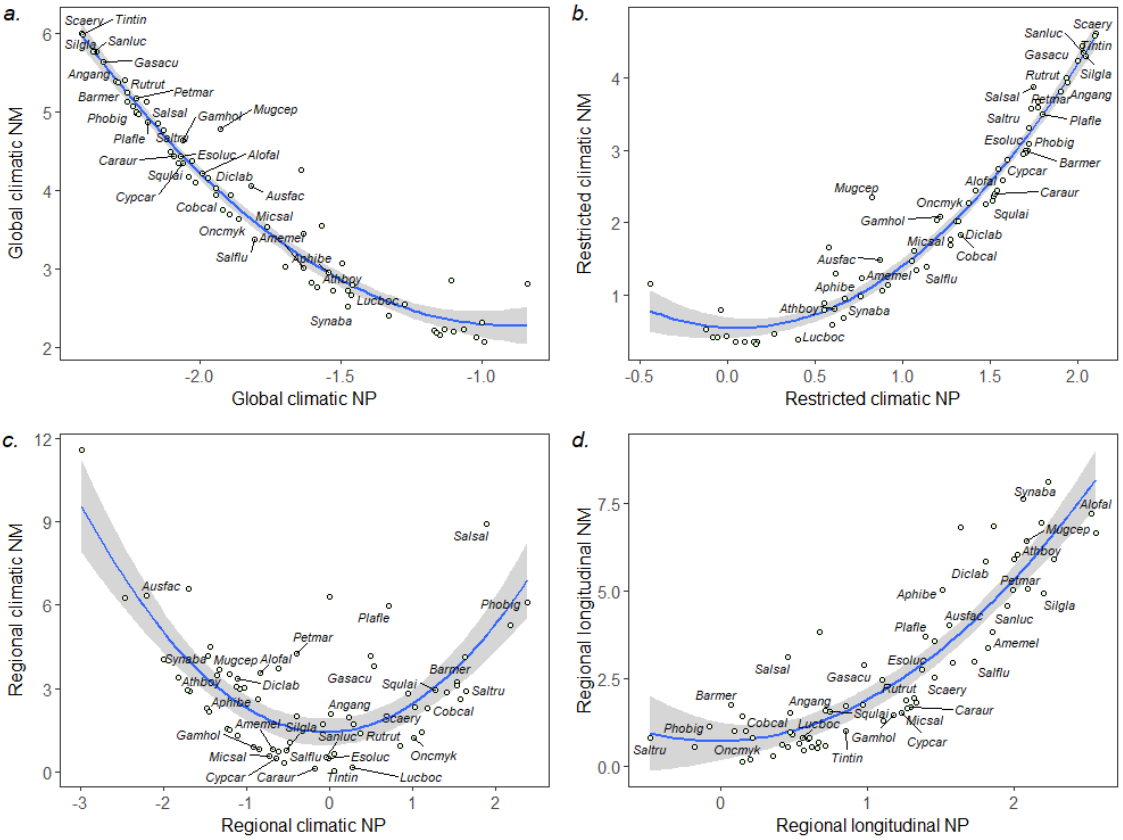

Figure S8.6. Quadratic relationships between species' niche position (NP) and niche marginality (NM) using (a) global climatic (R^2_{adj} = 0.95), (b) restricted climatic (R^2_{adj} = 0.96), (c) regional climatic (R^2_{adj} = 0.53) and (d) regional river longitudinal variables (R^2_{adj} = 0.81). Global analyses used the whole known distribution of the species, whereas the regional analyses were the smallest spatial extent, corresponding to the Iberian Peninsula (see Methods for further explanation). The shaded region represents the standard error.