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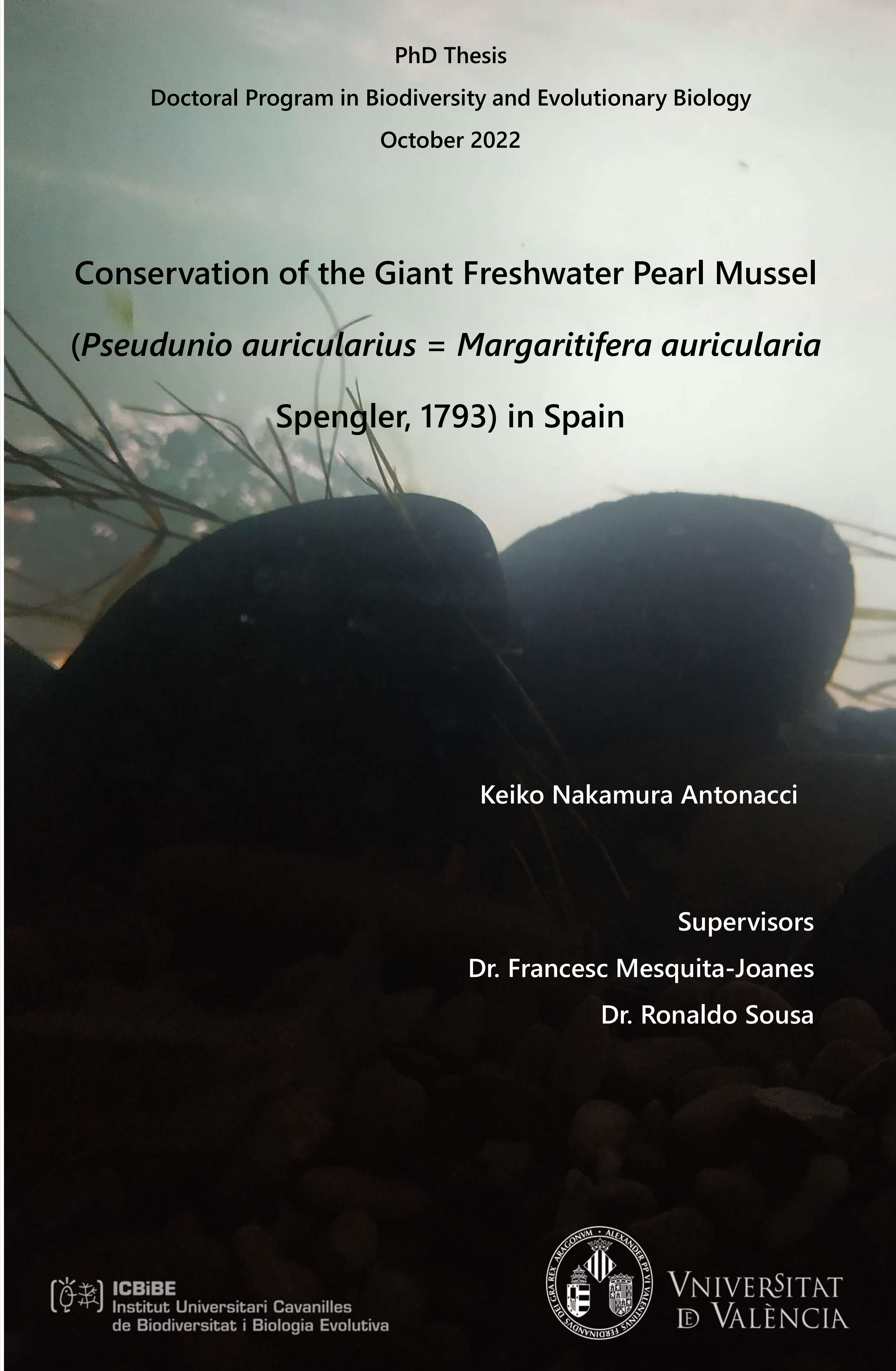
> Silent Spring

PhD Thesis
October 2022
Keiko
Nakamura
Antonacci

Conservation of the Giant Freshwater Pearl Mussel
(*Pseudunio auricularius* = *Margaritifera auricularia* Spengler, 1793) in Spain



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CERTIFY that the student **Keiko Nakamura Antonacci** has carried out under our direction the doctoral thesis entitled "Conservation of the Giant Freshwater Pearl Mussel (*Pseudunio auricularius* = *Margaritifera auricularia* Spengler, 1793) in Spain", to obtain the degree of Doctor.

And for the record, in compliance with current legislation, we issue this certificate in Valencia, on October 24, 2022.

CERTIFICAN que la estudiante **Keiko Nakamura Antonacci** ha realizado bajo nuestra dirección la tesis doctoral titulada: "Conservación de la gran almeja de río (*Pseudunio auricularius* = *Margaritifera auricularia*, Spengler, 1793) en España" para optar al grado de Doctor.

Y para que conste, en cumplimiento con la legislación vigente, se expide el presente certificado en Valencia, el 24 de octubre de 2022.

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Data: 2022.10.21 14:05:47+01'00'
CHAVE MÓVEL

Francesc Mesquita-Joanes **Ronaldo Sousa**

…to my father

Takashi Nakamura

“Man, however much he may like

to pretend the contrary,

is part of nature”

Rachel Carson

Silent Spring

Author’s declaration

I declare that all of the work presented in this thesis is my own and has not been submitted for consideration for a degree at any other university or institution.

The chapters of this thesis that have already been published at the time of its defense are clearly indicated and the quoting is added.

All photographs used in this thesis have been shot by the author.

This thesis is part of the work developed in the framework of the Recovery Plan for *Pseudunio auricularius* (= *Margaritifera auricularia*) in the Aragón region, implemented and funded by the Aragón Government and by the European Agricultural Fund for Rural Development (EAFRD). The actions are developed through the governmental corporation Sociedad Aragonesa de Gestión Agroambiental S.L.U. (SARGA).

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Abstract

Freshwater mussels are one of the most threatened faunal groups in the planet. Among them, the Giant Freshwater Pearl mussel (*Pseudunio auricularius*, Spengler 1793) is the most endangered unionid bivalve in Europe, and the population of the Ebro River basin in Spain is in a critical situation. The aim of this thesis is to increase the scientific knowledge of the current conservation status of the species in Spain in order to apply meaningful management actions to reduce its risk of extinction.

The decline of freshwater mussel (Order Unionida) populations in the Ebro basin is widespread, but the situation in the associated canals is critical, especially since 2013 when an episode of mass mortality of *P. auricularius* in the Canal Imperial de Aragón (CIA) was recorded. In the framework of a long-term study aiming to assess mussel densities in the canals, the obtained results showed a continued and generalized decrease of all native mussel species, ending with the local extinction of *Anodonta anatina* (Linnaeus, 1758), *Potomida littoralis* (Cuvier, 1798) and *Unio mancus* Lamarck, 1819, together with thousands of *P. auricularius*' dead specimens.

One possible explanation for these declines is aging and possible death due to senescence. To find out the relationship between shell length and age in *P. auricularius* in the Ebro basin, its growth pattern was analysed by counting the shell rings of adult and young specimens together with growth data of captive-bred juveniles. The generalized von Bertalanffy and Richards sigmoidal models presented the best fit, showing that the growth rate of *P. auricularius* is relatively slow after 30 years, when maximum growth is reached. The maximum age estimated for a specimen in the Ebro basin was 68 years.

Another possible explanation of the population decline was environmental pollution derived from anthropogenic activities. Considering the lack of previous knowledge on the species' sensitivity, acute toxicity tests against heavy metals and ammonium were carried out for the first time using juveniles from a captive breeding program. In this way, the lethal concentration values (LC_{50} and LC_{10}) were determined for cadmium, copper, nickel, zinc, chromium, lead and ammonium. In addition, non-observed-effect concentration (NOEC) values, lowest-observed-effect concentration (LOEC) values and maximum acceptable toxicant concentrations (MATC) were assessed for each contaminant tested. When comparing the results of *P. auricularius* with available

data from other mussel species, it seems to be more sensitive to cadmium and copper, and less sensitive to nickel, lead, and total ammonia.

As one of the main actions planned for the recovery of the species, laboratory cultures were established to develop a captive breeding protocol for juveniles of *P. auricularius,* by testing the effects of several conditions on survival and growth. The best results corresponded to a treatment in glass containers at a density of 0.2 ind/L, using river water, substrate, detritus and phytoplankton, and without extra aeration. The highest survival and growth rates achieved were 60% at 100 days and 2.56 mm shell length at 30-32 weeks of life. Captive breeding is considered one of the most important management tools to improve the conservation status of *P. auricularius*, and these valuable results allow establishing, for the first time, the best conditions for maximizing juvenile survival under laboratory conditions.

Finally, and with the objective of increasing the survival probabilities of the living specimens left in the CIA, a translocation plan was initiated in 2017. Adult specimens were translocated from the CIA to the Ebro River using plots previously characterized as suitable for the survival of *P. auricularius*. A total of 638 specimens (291 specimens in 2017, 291 in 2018, and 56 in 2019) were translocated. A monitoring survey, one year after, allowed estimating a survival rate between 40 and 70%. On the other hand, the control group left in the CIA had a lower survival rate of just 19.7% after one year. Based on these first results, it was confirmed that the specimens of *P. auricularius* translocated to the main river channel showed higher survival than those that remained in the canal.

Overall, the autecological information collected will provide support for the application of the best restoration measures in order to bend the curve of decline of this iconic riverine species in Spain.

Resumen extendido

Ante la pérdida de biodiversidad a la que nos enfrentamos actualmente, varios autores han sugerido que estamos entrando en la sexta extinción masiva e incluso en una nueva era geológica denominada Antropoceno. Desde el siglo pasado los ecosistemas acuáticos han experimentado enormes impactos a nivel mundial por lo que hoy en día se encuentran entre los más amenazados, y como consecuencia la fauna asociada a ellos también lo está. Las almejas de agua dulce o náyades (Bivalvia, Orden Unionida) son el grupo de invertebrados más amenazado globalmente y cuyas poblaciones han sufrido fuertes declives durante las últimas décadas. La bibliografía especializada atribuye estas disminuciones principalmente a la pérdida y fragmentación del hábitat (p. ej., presas), a la contaminación y el deterioro de la calidad del agua (p. ej., aumento de la deposición de sedimentos finos, aporte excesivo de nutrientes, acumulación de metales pesados, uso de herbicidas y fungicidas en agricultura), a la sobreexplotación (incluidos los peces hospedadores), a la introducción de especies exóticas invasoras y al cambio climático. A pesar de que muchos estudios han tratado de aclarar las diferentes causas de mortalidad de las náyades, la mayoría siguen siendo inciertas y especulativas ya que el gran número de factores potenciales que contribuyen dificulta determinar el tipo de perturbación que ejerce la mayor influencia negativa. El interés por preservar las poblaciones de náyades ha ido en aumento durante las últimas décadas, en parte debido a la desaparición de numerosas especies, y por otra, por los servicios ecosistémicos que brindan y que son importantes para el bienestar humano, como por ejemplo la purificación del agua, el ciclo de nutrientes y la estabilización del hábitat, entre muchos otros.

En España, la cuenca fluvial más grande corresponde a la del río Ebro, la cual alberga algunas de las mejores poblaciones de náyades del país, incluyendo la críticamente amenazada *Pseudunio auricularius* (Spengler, 1793), anteriormente conocida como *Margaritifera auricularia*. Ésta comparte las aguas del Ebro con otras tres especies de náyades: *Anodonta anatina* (Linnaeus, 1758), *Potomida littoralis* (Cuvier, 1798) y *Unio mancus* Lamarck, 1819, estando las últimas dos también catalogadas como especies vulnerables.

Pseudunio auricularius, llamada comúnmente margaritona o almeja perlífera gigante de río*,* es una de las especies más amenazadas del mundo. Se trata de un molusco

bivalvo de agua dulce que tiene su distribución en el Paleártico occidental. Extinguida en gran parte de su área de distribución, en la actualidad limita sus poblaciones a cinco cuencas hidrográficas, la española del río Ebro y cuatro en Francia: Garonnne, Adour, Loire (Vienne-Creuse) y Charente. Los datos del número de ejemplares vivos en Francia, específicamente en el río Charente, arrojan una estimación de 100.000 individuos, por lo que esta se considera hoy en día la mayor población de la especie a nivel mundial. Actualmente, en la Península Ibérica la población más numerosa censada es la que vive en el Canal Imperial de Aragón (CIA), en la provincia de Zaragoza, con alrededor de 6.000 ejemplares censados hasta febrero de 2022. También se encuentran poblaciones en el Canal de Tauste (CT), aunque más exiguas, pero no por eso menos importantes ya que se han censado más de 200 ejemplares en Aragón y 50 en Navarra, de los cuales un gran porcentaje son jóvenes con una longitud de la concha menor o igual a los 10 cm. En el canal natural principal del río Ebro se han censado alrededor de cincuenta individuos adultos vivos y numerosas conchas completas o fragmentos de conchas antiguas. También se han detectado ejemplares, entre ellos algunos de pequeño tamaño (10-11 cm de longitud), en la Acequia de Quinto, un pequeño canal aguas abajo de la ciudad de Zaragoza.

Pseudunio auricularius está clasificada en peligro crítico por la UICN y fue el primer invertebrado en España en ser catalogado como en peligro de extinción. De la misma forma, está incluido en la Directiva Hábitats (Anexo IV: Especies animales y plantas de interés comunitario que necesitan una protección estricta), así como en el Convenio de Berna (Anexo II) sobre la Conservación de la Vida Silvestre y los Hábitats Naturales Europeos, como “fauna que requiere medidas especiales para su protección”.

Como su nombre común "almeja perlífera gigante de río" indica, es una gran náyade que puede llegar a los 18 centímetros de longitud, aunque no produce perlas. Posee unas líneas de crecimiento bien marcadas y un periostraco negruzco-marrón en los adultos y amarillo verdoso en los juveniles. Las valvas son alargadas, ovaladas y bastante comprimidas, siendo el extremo posterior más pronunciado. El borde ventral está normalmente excavado, lo que le da la forma auriculada y de ahí su nombre (en latín “*auricula”* = oreja). Las valvas son gruesas, especialmente en su parte anterior, e internamente son de color blanco nacarado, lo que generó una industria de cuchillos y navajas, que se remonta hasta mediados del siglo XVIII, en los pueblos de la ribera baja

del Ebro, especialmente en Sástago. Sus pobladores usaban el nácar de la concha para decorar los mangos de navajas y cuchillos confeccionados artesanalmente. La valva izquierda presenta dos dientes cardinales gruesos y dos dientes longitudinales posteriores. La valva derecha presenta un diente cardinal grueso, y uno longitudinal posterior que se ajusta entre los dos de la valva izquierda.

En España se creía una especie extinta hasta que en 1995 se encontraron algunos ejemplares en el Delta del Ebro y un año después en el Canal Imperial de Aragón. A partir de ese momento los canales artificiales comenzaron a tener protagonismo en la conservación de las náyades, sobre todo en la comunidad de Aragón, lo que trae como consecuencia que en 2005 se apruebe el primer y único plan de recuperación de la especie en la comunidad (Decreto 187/2005 del Gobierno de Aragón). En dicho plan se plasman las medidas de protección y las acciones de conservación para mejorar su estado de conservación en la región. En este plan también se tiene en cuenta al que se conoce hoy en día como único pez hospedador de la margaritona presente en la cuenca del Ebro y que puede albergar en sus branquias a sus gloquidios (larvas) hasta caer al lecho del rio como juveniles viables. Se trata del pez fraile o blenio de río, *Salaria fluviatilis* (Asso 1801), el cual se encuentra catalogado como "Vulnerable" en el Real Decreto 139/2011 por el que se desarrolla el Listado de Especies Silvestres en Régimen de Protección Especial y el Catálogo Español de Especies Amenazadas.

En las últimas décadas, el descenso de las poblaciones de náyades en la cuenca del Ebro ha sido generalizado. Sin embargo, históricamente en el CIA y en el CT era usual encontrar durante las prospecciones cientos de ejemplares de las cuatro especies de náyades: *P. auricularius*, *A. anatina, P. littoralis* y *U. mancus*, pero la situación se tornó más que crítica, sobre todo desde 2013 cuando se detectó un episodio de mortalidad masiva de *P. auricularius* en el CIA. Ese mismo año ya no se encontraron ejemplares vivos de las otras tres especies de náyades. Durante los últimos 20 años se ha producido un cambio radical en la composición de la fauna bentónica filtrante del fondo de los canales y actualmente sólo se encuentran los últimos ejemplares vivos de *P. auricularius* junto a miles de ejemplares de una especie exótica invasora: la almeja asiática *Corbicula* spp.

Esta tesis tiene como principal objetivo ampliar el conocimiento de la biología y ecología de una de las especies de náyade más amenazada de Europa: *Pseudunio*

auricularius, analizar las posibles hipótesis para explicar las causas de mortalidad en los canales y a la vez proponer acciones de conservación para alejarla de la situación cercana a la extinción en la que se encuentra en España.

Se recopilaron y se analizaron datos de 16 años de monitorización, extraídos del trabajo desarrollado por el Gobierno de Aragón y en el marco del Plan de recuperación de *P. auricularius*. Se estudió la evolución de las densidades de las cuatro especies de náyades que habitaban en los canales (CIA y CT). A lo largo de los años se utilizaron diferentes metodologías de muestreo para evaluar la densidad de las náyades, pero los efectos de la heterogeneidad del muestreo se ajustaron en parte gracias al elevado número de secciones muestreadas y teniendo siempre en cuenta el área muestreada.

Los datos de abundancia de individuos vivos por especie y unidad de área, se clasificaron según año, canal y punto kilométrico (PK) donde se realizó el muestreo, desde 2004 hasta 2019. Teniendo en cuenta el alto número de muestras sin individuos vivos de una o varias especies en el conjunto de datos, se aplicaron modelos lineales generalizados mixtos (GLMM) sobrecargados de ceros (ZI), utilizando el número de individuos vivos de cada especie como variable de respuesta, el año como factor fijo que se espera que afecte su abundancia, el PK dentro de cada canal como factor aleatorio y el logaritmo del área como variable de compensación para considerar adecuadamente la abundancia como dependiente del área muestreada. Los resultados muestran una drástica y generalizada disminución de la densidad de todas las especies a lo largo de los años. En el CIA, antes de 2013 se observó una mayor densidad de individuos en los tramos medio e inferior, a diferencia del CT, donde los ejemplares se distribuyeron uniformemente por todo el canal, alcanzando valores superiores a 1 ind/m^2. La situación cambia radicalmente a partir de 2013 cuando la densidad cae drásticamente, registrándose valores muy bajos, por debajo de 0,05 ind/m^2, en todo su recorrido en ambos canales, finalizando con la extinción de tres de las cuatro especies presentes: *A. anatina, P. littoralis* y *U. mancus*. Los resultados obtenidos de los GLMM-ZI mostraron una relación inversa y significativa del tiempo (año) con la abundancia para todas las especies y una relación positiva con la probabilidad de ausencia (parte ZI) también en todas las especies excepto en *P. auricularius*. El PK en cada canal representó una varianza mucho mayor en la abundancia que la del propio canal, explicando la distribución parcheada de las náyades dentro de los canales.

Adicionalmente se analizaron un total de 19.033 mediciones de longitud de conchas entre 2004 y 2019, analizando para cada especie la fracción de individuos que morían con el pasar de los años. Para *A. anatina, P. littoralis* y *U. mancus* se comprobó la desaparición de los más ejemplares más pequeños y luego los más grandes quedando los de tamaño intermedio hasta su completa extinción. El caso de *P. auricularius* destaca por comenzar a registrarse solo ejemplares adultos en 2005 y a partir de 2010 se registraron los primeros ejemplares jóvenes entre 50 y 100 mm en CT, disminuyendo la longitud promedio en este canal de 150 mm a 111 mm en 2012.

Utilizando el programa MARK, se analizaron datos de captura-recaptura de *P. auricularius* entre 1998 y 2020 para CIA y de 2002 hasta 2020 para CT, para obtener la probabilidad de supervivencia estimada de los individuos marcados en cada canal por separado. En el CIA el resultado fue un descenso de la supervivencia anual de los individuos marcados hasta 0,15 en 2020, a diferencia de en CT que se mantiene cerca de 1. El CIA tiene trabajos de mantenimiento dos veces al año (febrero y noviembre), a diferencia de CT que solo tiene uno (febrero) y con obras menores. Esta diferencia podría estar actuando como un factor clave en la supervivencia diferencial de *P. auricularius* permitiendo mantener la densidad en CT, o incluso aumentarla gracias a los ejemplares jóvenes que se han ido registrando a lo largo de esta última década.

Finalmente, en este primer capítulo de resultados que se acaba de resumir, se discuten las diversas hipótesis planteadas para explicar el declive generalizado de las náyades, entre las que se encuentran: la alteración y fragmentación del hábitat, la posibilidad de la presencia de enfermedades causadas por bacterias, virus o parásitos, la contaminación, la senescencia de la población y la explosión de la población de la especie invasora *Corbicula* ssp.

Por lo que respecta a la alteración del hábitat hay que tener en cuenta que el estudio se lleva a cabo fundamentalmente en canales. Los hábitats artificiales, tales como los canales, pueden en ocasiones proporcionar condiciones adecuadas para el crecimiento y desarrollo de la fauna autóctona debido a que tienen características similares al medio natural. Los canales estudiados ofrecen un hábitat estable para las náyades por la presencia constante de una lámina de agua sin fluctuaciones drásticas, sustrato adecuado, comunidad íctica similar a la del río, entre otros. Sin embargo, los canales son estructuras de riego que cumplen una función de abastecimiento de agua y que necesitan de

mantenimiento y reparaciones constantes, lo que genera problemas a la hora de compatibilizar la supervivencia de la fauna establecida. Las obras de mantenimiento conducen a la destrucción de parte del hábitat, produciendo condiciones de estrés ambiental que, si perduran en el tiempo, pueden llegar a traducirse en mortalidad.

Por otra parte, las mortalidades asociadas a la presencia de parásitos o microrganismos patógenos en las náyades están muy poco estudiadas. Recientemente se han publicado algunos estudios sobre la identificación de virus o bacterias que pueden estar interviniendo en el bienestar de algunas especies de náyades, pero todavía se desconoce si pueden causar la muerte del individuo o si pueden ser la causa directa de mortalidades masivas registradas en localidades tanto en América del Norte como de Europa. En el caso de *P. auricularius,* esta hipótesis ha sido parcialmente evaluada por el Gobierno de Aragón y con la colaboración de varias universidades e institutos de investigación. Sin embargo, los análisis histológicos obtenidos no pudieron demostrar un impacto importante de ningún organismo patógeno sobre los individuos por lo que se debe seguir ahondando en el tema con futuros estudios.

El envejecimiento de la población podría ser otra de las causas de una mortalidad acusada. La edad de los ejemplares de *P. auricularius* en la cuenca del Ebro se desconocía hasta la elaboración de esta tesis, aunque tradicionalmente se consideraba que se trataba de ejemplares de “edad muy avanzada”, por lo que en el momento de registrarse la inusual mortalidad en el CIA surgían dudas de si la muerte podría estar asociada a la senescencia de los individuos. En el capítulo 4 se analizó en detalle la relación entre la edad y la longitud de *P. auricularius* mediante la combinación de tres tipos de datos: el contaje de los anillos de la concha de individuos adultos muertos, el contaje de anillos de especímenes jóvenes vivos en el CT y, finalmente, los datos de crecimiento de juveniles criados en cautividad. Entre los seis modelos de análisis de regresión no lineal que se analizaron para relacionar el tamaño con la edad, los que mejor ajuste mostraron fueron los dos sigmoidales: el modelo generalizado de von Bertalanffy y el de Richards. En este último, la primera parte de la curva sigmoidea mostró un crecimiento exponencial en los estadios juveniles iniciales, hasta un punto de inflexión alrededor de los 7 años. Se discutieron los posibles significados biológicos y ecológicos de ese punto de inflexión como una señal de comienzo de otros procesos biológicos que pudieran adquirir importancia en ese momento, como pueden ser el de la reproducción (madurez sexual), o un cambio en el uso del hábitat, como podría ser que el juvenil saliera a la superficie después de estar viviendo los primeros años totalmente enterrado y comenzara a vivir semienterrado como un adulto.

Se determinó que las conchas de adultos con una longitud, en la mayoría de los casos, entre 144 y 153 mm, tenían una edad estimada de 38-50 años, aunque la edad máxima estimada para un ejemplar en la cuenca del Ebro fue de 68 años. Finalmente, la tasa de crecimiento de *P. auricularius* se ralentizaba notablemente después de los 30 años, a partir de la cual ya se alcanza casi la longitud máxima en los ejemplares adultos, por lo que es difícil estimar la edad de un ejemplar de mayor edad solo con la medida de la longitud.

Diversas son las amenazas de origen antrópico que afectan a las poblaciones de náyades, y la contaminación acuática se considera uno de los principales factores que contribuyen a su desaparición. Las náyades se alimentan por filtración y viven semienterradas en el sustrato y, en consecuencia, la contaminación puede afectar gravemente su fisiología y supervivencia debido a la bioacumulación de metales pesados, pesticidas y otros contaminantes emergentes.

La información ecotoxicológica es esencial para poder desarrollar políticas ambientales acertadas y, por lo tanto, para la conservación biológica y la restauración de las poblaciones de náyades que sustentan su vida en estos ambientes. Hasta ahora, la información de la sensibilidad de organismos acuáticos frente a contaminantes se centraba en organismos no filtradores, tales como vertebrados como el pez cebra *Danio rerio* (Hamilton, 1822) o crustáceos como el langostino o camarón blanco (*Litopenaeus vannamei* [Boone, 1931]), los cuales generalmente muestran una sensibilidad mucho menor que organismos filtradores como las náyades. En general los datos ecotoxicológicos de náyades en Europa son bastante escasos por lo que las referencias para poder comparar son casi inexistentes.

En 2013, después del episodio de mortalidad de *P. auricularius,* así como en años posteriores, se tomaron muestras de agua, sustrato y biota por parte del Gobierno de Aragón para evaluar el papel de la contaminación y la bioacumulación en este suceso, pero al no tener información ecotoxicológica disponible ni tampoco información del umbral de sensibilidad de *P. auricularius*, no se pudo determinar el papel de estos contaminantes detectados en los canales.

En el capítulo 5 se describen los ensayos de toxicidad aguda realizados por primera vez, frente a metales pesados y amonio, utilizando juveniles de *P. auricularius* procedentes de la cría en cautividad y siguiendo la metodología de la guía estandarizada para realizar pruebas de toxicidad aguda de la ASTM (*American Society of Testing and Materials*). Se realizaron ensayos de toxicidad para al cobre (Cu), zinc (Zn), plomo (Pb), cadmio (Cd), cromo (Cr[III]), níquel (Ni) y amonio (TAN), ajustando las concentraciones experimentales para obtener valores de concentración letal (CL_{50}) más precisos. Con el objetivo de hacer comparaciones con estudios anteriores, los valores estimados de CL_{50} se normalizaron a agua de dureza blanda (40–50 mg/L como $CaCO_3$).

Las estimaciones de las concentraciones letales (CL_{50}) en orden decreciente de sensibilidad para *P. auricularius* fueron las siguientes: Cd = 38,85 µg/L, Cu = 58,64 µg/L, Ni = 124,60 µg/L, Zn = 267,40 µg/L, Cr (III) > 1000 µg/L, Pb > 2000 µg/L y nitrógeno amoniacal total (TAN) = 7,53 mg/L. Además, también se estimaron los valores de LC_{10}, NOEC, LOEC y MATC, que son útiles para determinar las condiciones de hábitat más seguras para la especie. Al comparar la sensibilidad de *P. auricularius* con otras especies de náyades americanas éste parece ser más resistente al cobre y al amonio y bastante más sensible al cadmio, zinc y níquel.

La expansión de especies de bivalvos exóticos invasores y sus consecuencias sobre el hábitat es un tema todavía por analizar en profundidad en la cuenca del Ebro. Es una de las hipótesis que se discuten en el capítulo 1 como causa de la mortalidad de *P. auricularius* y otras náyades en los canales, aunque futuros estudios deben estar encaminados a ahondar en los efectos negativos de su presencia en alta densidades.

La almeja asiática, como la mayoría de las especies invasoras, tienen una capacidad reproductiva muy alta y se recupera rápidamente después de experimentar mortalidades, por lo que es fácil pensar que por ello ha terminado reemplazando a las especies nativas en el fondo de los canales. El CIA y el CT fueron colonizados por *Corbicula* spp. en 2006-2007 y en una década aumentó exponencialmente su densidad. Esta invasión ha tenido efectos visibles sobre el hábitat, como por ejemplo modificación de la composición de los fondos de los canales; donde antes había un sustrato de grava bien oxigenado actualmente hay una capa de ejemplares vivos y muertos de *Corbicula* spp. Estos cambios deben haber afectado negativamente a las náyades en puntos tan críticos como por ejemplo la alimentación, por competencia de los recursos dentro del

mismo hábitat, o la reproducción, interfiriendo el ciclo reproductivo de las náyades al ser filtrados los gloquidios por las almejas asiáticas. Los efectos negativos de la almeja asiática sobre las náyades autóctonas también pueden implicar efectos indirectos, tales como los derivados de la muerte y descomposición masiva de la propia especie invasora, provocando aumentos en la concentración de amonio en el agua debido a la descomposición de los tejidos blandos, induciendo mortalidades en el resto de la fauna acuática.

La aplicación de medidas de conservación para mitigar la pérdida de biodiversidad ha aumentado sustancialmente durante los últimos 30 años. Dentro del plan de recuperación de *P. auricularius*, una de las principales acciones de conservación que se establece es el desarrollo de un protocolo de cría en cautividad con el objetivo de contar con generaciones de relevo para reintroducir la especie en el medio natural. La cría en cautividad de náyades en peligro de extinción, como es el caso de *P. auricularius*, puede ser una herramienta eficaz para mantener el potencial evolutivo de sus poblaciones, que de otro modo no resistirían el tiempo suficiente para beneficiarse de la restauración de sus hábitats. De hecho, es una de las soluciones más reconocida para ayudar a preservar estas especies en estado tan crítico y hoy en día se están desarrollando en diversos países y con multitud de especies diferentes de náyades. Sin embargo, no debe considerarse como la única solución para conservar estas especies, ya que son procesos que requieren una importante inversión de tiempo y dinero. Acciones correctivas en el hábitat natural como la restauración de la dinámica fluvial, la mejora de la calidad del agua o la mejora de las poblaciones del pez hospedador, entre otras, deben tomarse en cuenta para tener localidades adecuadas para la reintroducción de los juveniles obtenidos de la cría en cautividad.

En *P. auricularius*, al igual que en el resto de especies de náyades, las larvas denominadas gloquidios necesitan infestar a un hospedador, en este caso las branquias de un pez para completar su desarrollo. El tejido del pez responde envolviendo al parásito para aislarlo y se desarrolla un quiste donde el gloquidio sufre una metamorfosis transformándose en juvenil para luego desprenderse del pez y caer al fondo del río para comenzar su vida libre. El antiguo pez hospedador de *P. auricularius* en el río Ebro era el esturión común *Acipenser sturio* Linnaeus, 1758, el cual habitaba toda la cuenca pero que ya en la década de los 60 se dio por extinto. En la actualidad, la única especie autóctona de la cuenca del Ebro apta para la transformación de los gloquidios de *P. auricularius* en juveniles viables es el blenio de río *Salaria fluviatilis*.

En el capítulo 6 se detallan los pasos de la cría en cautividad de *P. auricularius*, desde la extracción de los adultos grávidos del medio natural, pasando por la recolección de los gloquidios y la infestación de los peces, hasta la recolección de los juveniles recién nacidos. Para el cultivo de los juveniles, desarrollado en dos fases, se ensayaron diferentes combinaciones para la fase I, denominada "detritus boxes": tipo de contenedor (plástico o cristal), origen del agua (río o canal), sustrato y detritus natural (presencia/ausencia), tipos de fitoplancton (marino o dulceacuícola), tasa de alimentación (diaria o semanal), aireación adicional y densidad inicial de juveniles (baja = 0,2 ind/L; alta = 0,4 ind/L). Cada cultivo se cubrió parcialmente con tapa plástica, se mantuvo en oscuridad y a una temperatura controlada de 17-18°C. Cada semana, se comprobaba el estado de los juveniles bajo la lupa binocular y se retiraban los individuos muertos para evitar la proliferación de hongos. Los juveniles que quedaban vivos se contaban y se devolvían al recipiente que previamente había sido limpiado y renovado según las condiciones de cada tratamiento. Una vez los juveniles crecían hasta una longitud de la concha de 1mm se pasaban a la fase II con las mismas condiciones que en la fase I, pero con mayor volumen (20 litros) y con una pequeña bomba que recirculaba el agua.

Se aplicó el análisis de supervivencia de Kaplan-Meier para comparar la supervivencia entre años y entre tratamientos, el análisis de Mann-Whitney para comparar el crecimiento entre años y la regresión de Cox para discernir la mejor opción entre variables del cultivo. Los mejores resultados para la supervivencia y crecimiento de los juveniles se obtuvieron con el tratamiento en recipientes de cristal, a una densidad de 0,2 ind/L, utilizando agua de río, con sustrato, detritus y fitoplancton marino y sin aireación extra. Las mayores tasas de supervivencia y crecimiento alcanzadas, respectivamente fueron del 60% a los 100 días y 2,56 mm de longitud de concha a las 30-32 semanas de vida.

En base a los resultados obtenidos se sugiere que los juveniles de *P. auricularius* a partir de 1 mm de longitud de concha se comienzan a alimentar por filtración, pero aún sin abandonar la alimentación pedal, como sugieren los surcos observados en el sustrato en la fase II. La incorporación de la bomba que circula el agua en los acuarios es un factor muy importante para el rápido crecimiento de los juveniles ya que facilita la filtración del

alimento en suspensión por parte de los juveniles, tal y como lo harían en el medio natural aprovechando las corrientes de agua intersticiales asociadas a la materia orgánica particulada fina, gastando menos energía y facilitando su ingestión al posicionarse orientado hacia el flujo de agua.

Es la primera vez que juveniles de *P. auricularius* se mantienen con vida durante más de un año, lo que abre el camino para pensar en el siguiente paso, que es la futura reintroducción de juveniles en el hábitat, siempre y cuando vaya de la mano de las mencionadas actuaciones de restauración de las condiciones bióticas y abióticas para la colonización exitosa de la especie.

Otra medida, ampliamente utilizada hoy en día para paliar la pérdida acelerada de biodiversidad, es la traslocación de individuos desde una población donante a otra receptora. De acuerdo con la UICN el concepto de traslocación es “el movimiento y liberación intencional de un organismo vivo cuyo objetivo principal es un beneficio de conservación”. Y aunque es considerada una de las herramientas más útiles en conservación, es una herramienta intervencionista que puede acarrear resultados no deseados. La controversia del uso o no de esta herramienta se debe a los riesgos que tiene intrínsecamente una movilización de individuos vivos de una localidad a otra, sobre todo si son de localidades muy diferentes. Entre estos riesgos se encuentran la movilización de patógenos, desestructuración de la población donante o mortalidad de los individuos traslocados por baja adaptabilidad a las nuevas condiciones.

La alta mortalidad de *P. auricularius* detectada en el Canal Imperial desde 2013 precisaba la adopción de medidas urgentes, y entre ellas, desde El Gobierno de Aragón se valoró realizar un traslado de parte de los ejemplares adultos del CIA, por medio de un plan de rescate o plan de traslocación, a áreas donde las condiciones de vida de *P. auricularius* estuvieran libres de las amenazas actuales presentes en el canal, o al menos donde la presión fuera menor. Entre los riesgos evaluados en el plan se encontraba que la mortalidad de los propios ejemplares a traslocar era de partida muy alto, ya que se consideraba que podían estar en un estado débil y/o moribundos por estar inmersos en un proceso de alta mortalidad. Se sumaba a que eran ejemplares adultos adaptados a las condiciones hidrológicas del canal, por lo que su aclimatación a las nuevas localidades en el río, a pesar de ser dentro de la misma cuenca y con el mismo tipo de agua, podía resultar difícil. También se valoró el riesgo de transmisión de posibles enfermedades o

parásitos no descritos, pero se desestimó porque dicho riesgo sería importante en caso de introducción a masas de agua distintas del río Ebro, pero no era el caso ya que el Canal Imperial toma sus aguas del mismo río.

Bajo estas premisas y con el único objetivo de prolongar la supervivencia de estos ejemplares, en 2017 se inició el plan de traslocación, en donde se extrajeron ejemplares adultos del CIA y se transportaron al río Ebro a parcelas previamente caracterizadas como aptas para la supervivencia de *P. auricularius*. Se diseñó un índice para evaluar y escoger las parcelas en el río que comprendía diez variables físicas y químicas, cuatro biológicas y cinco antropogénicas, consideradas como las más influyentes para la supervivencia de la especie, basándose en la bibliografía disponible e incorporando los conocimientos de la cría en cautividad desarrollada y explicada anteriormente. A cada variable se le asignó una puntuación asociada: 1 punto si las condiciones eran poco adecuadas, 3 puntos si eran adecuadas y 5 puntos si eran óptimas para la especie. La suma de los valores de todas las variables dio como resultado una puntuación global para cada localidad, permitiendo elegir aquellas con la puntuación más alta.

El procedimiento de translocación se llevó a cabo de la misma forma en todas las localidades seleccionadas para la posterior comparación entre ellas. Se midieron las variables físico-químicas (pH, conductividad, concentración de oxígeno disuelto, de nitritos y de amonio), tanto en la localidad donante como en la receptora, el mismo día de la translocación; se extrajeron los ejemplares al azar y se verificó que presentaran buenas condiciones externas, se marcaron lo más rápido posible con etiquetas numeradas de plástico además de con un emisor tipo PIT-tag (*Passive Integrated Transponder*). Se tomaron los datos biométricos de cada ejemplar y se transportaron lo más rápido posible, bajo condiciones de alta humedad, utilizando toallas empapadas en agua del CIA, no sin antes devolver al canal los ejemplares del grupo control. En el río Ebro, con la parcela previamente marcada con estacas de madera clavadas al fondo, se colocaron los ejemplares de *P. auricularius* en su posición natural: parcialmente enterrados en el sustrato. El seguimiento de ejemplares se realizó una vez al año, en verano y mediante la visualización directa con la ayuda de un visor (mirafondos) para determinar si los ejemplares estaban vivos o no y luego fueron identificados mediante la lectura a distancia del PIT-tag electrónico evitando así su manipulación.

Se traslocaron un total de 638 ejemplares (291 ejemplares en 2017, 291 en 2018 y 56 en 2019) y se evaluó su probabilidad de supervivencia año tras año hasta el 2020. La supervivencia mínima después de un año para el grupo de 2017 fue del 41,6%; 69 % para el segundo grupo traslocado en 2018 y 49% para el tercer grupo traslocado en 2019. El grupo control dejado en el CIA mostró una supervivencia muy baja, de solo el 19,7%, después de un año. En base a estos primeros resultados se confirma que, hasta el momento, la supervivencia de los ejemplares de *P. auricularius* traslocados al río es mayor que la de los que se quedaron en el propio canal.

Es necesario seguir implementando actuaciones urgentes de conservación tanto en los canales como en el río Ebro para hacer frente a esta alarmante pérdida de biodiversidad de bivalvos uniónidos. Se debe incidir en profundizar en el conocimiento de la biología y ecología de *P. auricularius* y el resto de las especies de náyades presentes en la cuenca del Ebro y de esta forma enfocar la toma de decisiones de gestión basándose en la información específica sobre los requerimientos de cada especie. De la misma forma se debe continuar con las acciones de restauración del hábitat que son fundamentales para permitir la supervivencia de los juveniles en el medio natural y así asegurar la continuidad de las poblaciones. Como apoyo transversal a todas estas acciones, se deben implementar campañas de sensibilización y educación ambiental de forma continua, así como de políticas medioambientales que garanticen la protección estricta de los últimos ejemplares de *P. auricularius* y su hábitat.

Chapter 1

General introduction

1.1 Conservation of freshwater mussels

Freshwater ecosystems are suffering an intense degradation due to several anthropogenic causes (Birk et al., 2020; Reid et al., 2019), and freshwater mussels (Bivalvia, Unionida) are one of the animal groups that have been most negatively affected, triggering an increasing concern among the scientific community (Böhm et al., 2021; Ferreira-Rodríguez et al., 2019; Lopes-Lima et al., 2014; 2017; 2018a, Sousa et al., 2022). This rapid decline in abundance and distribution increased the interest in studying the ecology of freshwater mussels, resulting in a explosion of studies in the last two decades dedicated to better understanding these fascinating animals (Böhm et al., 2021; Ferreira-Rodríguez et al., 2019; Haag, 2019; Lopes-Lima et al., 2014; 2017; 2021a; b; Strayer et al., 2004).

Freshwater mussels play important ecological roles in rivers and lakes, and it is imperative to understand how their disappearance may impact ecosystem functions and services provided by freshwater ecosystems (Dubose et al., 2019; Lopez et al., 2022; Vaughn, 2018; Zieritz et al., 2022). The long list of factors that can contribute to mussel decline makes it challenging to determine which of them has a more significant influence. Anyway, the loss, fragmentation and modification of habitats, water pollution, the introduction of invasive species, climate change, and possible diseases caused by parasites, bacteria and/or viruses appear to be the most influential factors causing local extinctions of freshwater mussels (Lopes-Lima et al., 2017, 2018a, 2021a; Ferreira-Rodríguez et. al., 2019; Sousa et al., 2022).

Some examples of decline of mussel populations in the world are the case of *Margaritifera margaritifera* (Linnaeus, 1758) whose European distribution has been reduced by more than 80% in the last decades (Araujo & Ramos, 2001b; Geist, 2010; Lois et al., 2014; Moorkens et al., 2007; 2017; Sousa et al., 2018; Nogueira et al., 2021; Wengström et al., 2019) and those of *Actinonaias pectorosa* (Conrad, 1834) and *Margaritifera falcata* (Gould, 1850) in North America (Leis et al., 2018). Haag (2019) reported a mussel species richness decline of 72% between 1961 and 2005 in the Conasauga River, Georgia (USA), and a decline in total number of individuals of 97%.

Little is known about the conservation status of freshwater mussel populations in East and Southeast Asia, despite being considered a hotspot for Unionida diversity (Zieritz et al., 2018).

The conservation status of freshwater mussels in Spain is in a critical situation, as several species are included in the Spanish catalog of endangered species (R.D. 139/2011), as well as in the Atlas and Red Book of threatened invertebrates in Spain (Verdú et al., 2011). The IUCN red list classifies the Unionidae species present in Spain as follows: *Anodonta anatina* (Linnaeus, 1758), *A. cygnea* (Linnaeus, 1758) and *Unio pictorum* (Linnaeus, 1758) are listed as "least concern". *Unio mancus* Lamarck, 1819 and *U. delphinus* Rossmässler, 1844 as near-threatened and U. tumidiformis Castro, 1885 as vulnerable. Finally, *Unio ravoisieri* Deshayes, 1847 and *Potomida littoralis* (Cuvier, 1798) are listed as endangered, and *U. gibbus* Spengler 1793 as critically endangered. Regarding the Margaritiferidae family, *Margaritifera margaritifera* (Linnaeus, 1758), is classified as endangered, and *Pseudunio auricularius* (Spengler, 1793) as critically endangered.

1.2 The giant freshwater pearl mussel *Pseudunio auricularius* in Spain

Pseudunio auricularius, formerly known as *Margaritifera auricularia* (Fig. 1.1), is one of the most threatened freshwater bivalves in the world (Prié, 2021). The giant freshwater pearl mussel is the largest native invertebrate in continental Europe with a past distribution in the Western Palearctic colonizing all the major rivers of Western Europe. It was found in Belgium, the Czech Republic, Denmark, Germany, Italy, Luxembourg, the Netherlands, Portugal and the United Kingdom (Altaba, 1990; 1997; Araujo & Ramos, 1998; Araujo & Ramos, 2000a; b; 2001a; b). Currently its known distribution is restricted to two countries: Spain and France.

Pseudunio auricularius was included in the European Habitats Directive (Council Directive 93/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora) in Annex IV (Animal and plant species of community interest in need of strict protection), and it is also listed in the Bern Convention on the Conservation of European Wildlife and Natural Habitats as "fauna requiring special measures to be taken for their protection". It is classified as 'Critically Endangered' by the International Union

for Conservation of Nature (IUCN) (Prié, 2021), and it is classified in the same critical category in the two countries with the remaining living populations (Spain: Royal Decree 439/90, France: Decree of April 23, 2007, article 2). *Pseudunio auricularius* was actually the first invertebrate included in the "Endangered" category in the Spanish national catalogue of threatened species.

Fig 1.1 Two specimens of *Pseudunio auricularius*: adult (top) and young (bottom).

The species was first described by Spengler in 1793 as *Unio auricularius*. In 1819 Lamarck redescribed it using French specimens, as *Unio sinuata*. Haas (1910) later described the genus *Pseudunio* for the Giant Mussel, to differentiate it from the freshwater pearl mussel *Margaritifera margaritifera*. *Pseudunio* was later synonymized with *Margaritana* (=*Margaritifera*) by Ortmann (1911). *Pseudunio* was later used by Haas (1969) in order to separate *M. marocana* (Pallary 1918) from the rest of the species belonging to the genus *Margaritifera*. *Margaritifera auricularia* was recently renamed again as *Pseudunio auricularius* by Lopes-Lima et al. (2018b) based on molecular data and following the identification of a clade distinct from other Margaritiferiidae.

Pseudunio auricularius has several common names in different languages: French: Grande mulette; English: Giant Freshwater Pearl Mussel, Spengler's freshwater mussel; Spanish: margaritona, náyade auriculada, almeja perlífera gigante de río; German: Riesen-Flußperlmuschel (Soler, 2018; Richard & Prié, 2022).

1.2.1 Current distribution

The Ebro River basin is the only Iberian basin where *P. auricularius* is currently present (Altaba 1990, Araujo & Ramos 2000a, Araujo et al., 2009), although it was recorded in the past in the Tagus, Guadiana and Guadalquivir River basins (Araujo & Moreno, 1999). Azpeitia Moros (1933) reported a young specimen in the Tagus River in Toledo, and later a shell was found in Toledo between the years 1990-2000 (Villasante López et al., 2016). Despite this, the species is considered extinct in the Tagus River.

In France, four basins still maintain alive specimens: the Vienne and Creuse Rivers (Loire basin), Charente River (Charente basin), Dronne and Save Rivers (Garonne basin) and the Adour, Luy and Arros (Adour basin) (Cochet, 2001a; b; 2002; Nienhuis, 2003; Prié, et al., 2010; 2018; Richard & Prié, 2022). The largest population reported to date is that of the Charente River, with an estimated population number of 100,000 specimens (Prié et al., 2010).

The best-known populations in Spain are those located in anthropogenic habitats where the flowing water is cut off for annual maintenance works, allowing easy access to specimens, such as the case of three canals in the Ebro River basin: Canal Imperial de Aragón (CIA), Canal de Tauste (CT) and Quinto ditch (Fig. 1.2) (Nakamura et al., 2018b; Nakamura et al., 2022a; Sousa et al., 2021).

The first two localities in Aragón region (Spain) where specimens of *P. auricularius* were found belonged to the Ebro River, one near the town of Escatrón, and another in Zaragoza (Drouët, 1893 and Kobelt, 1903, cited in Álvarez Halcón, 1998b). Later, at the beginning of the 20th century, the German malacologist Fritz Haas studied the Ebro River population (Haas 1916a, b; 1917). By then, the species was thought to have become extinct in most of its former range. Anyway, thanks to these studies it is known that, at that time, the species was very abundant in the Ebro, colonizing the gravel beds at a water depth of 7-8 meters. However, since then and for many decades there were no new records of the presence of live specimens in the Ebro basin, until 1985, when freshly dead specimens appeared during the dredging of an irrigation canal near the Ebro Delta (Altaba, 1990). Later on, alive specimens were also found in the lower Ebro River in 1995 (Altaba, 1997) and almost at the same time (1996) in the Canal Imperial de Aragón (Araujo & Ramos, 1998).

Fig 1.2 Canal Imperial de Aragón (left) and Canal de Tauste (right).

The highest abundance of the species in Spain is found in the Canal Imperial de Aragón (CIA) with almost 6,000 tagged specimens (Guerrero et al., 2021; Nakamura et al., 2019). Since 2013, a high mortality rate was registered; thousands of specimens were found dead and the density in the canal drastically decreased. Currently, in the CIA, and according to the census carried out by the Aragón Government, around 3,000 specimens are left in the canal (Aragón Government, unpublished data). The number of individuals in the Canal de Tauste (CT) is lower. Only 227 specimens have been registered and identified, of which near 170 are still alive. Regardless of its low abundance, it is an important asset for the conservation of the species, as 50% of the specimens are young with a shell length of less than or close to 100 mm (Aragón Government, unpublished data). Furthermore, several dozens of alive specimens have been located in the CT stretch in the region of Navarra, and further 30 specimens are known to remain alive in the Quinto ditch, downstream from Zaragoza city, with the presence of some young specimens (Nakamura et al., 2018a, b).

In the mid-Ebro River main channel (Aragón region), 53 specimens were tagged, out of which 19 have recently been recovered dead. Another alive adult specimen was registered in the main channel of the Ebro River in Navarra and another one in La Rioja region, the latter being the northernmost specimen recorded for the Ebro basin and in Spain.

Fig 1.3 Shell of *Pseudunio auricularius.*

Shells of *P. auricularius* have been found during the last few decades in the Ebro River near Burgos, Álava and La Rioja (Araujo et al., 2007). Recently (2019), a survey promoted by the Government of Castilla y León, reported shells of *P. auricularius* in the Ebro River, near the village of Miranda de Ebro (Burgos), but negative results have been obtained in the area for its presence with the e-DNA technique (Ecohydros, 2019). In the lower Ebro River, the species has practically disappeared and the last two living specimens are maintained in semi-natural conditions (López, pers. com. 2022).

1.2.2 General biology and ecology

Adult individuals of *Pseudunio auricularius* have a shell length up to 18 cm and can weigh between 400 - 500 g (Richard & Prié, 2022). They have a black-brown periostracum in the adult, yellow-brownish in juveniles, a thick and elongated shell, with an auriculate shape and a concave ventral side (hence its name in Latin auricula = ear). The interior is bright white due to the presence of a thick layer of nacre (Fig. 1.3), formerly used in the villages near the Ebro River to decorate the handmade handles of knives (Álvarez Halcón, 1998a, b). The shell hinge shows two posterior lateral teeth in the left valve, and one in the right valve, which is strong and elongated. Robust pseudo-cardinal teeth with a pyramidal shape are present, two on the left valve and one on the right. The imprint of the adductor muscles and the pallial line is well marked (Araujo 2009; 2012).

The umbo and the margins of the shells are usually slightly eroded in adult specimens, but not in younger ones (Nakamura et al., 2018b).

The foot is white, muscular, and very large, which allows the animals to move on and bury themselves in the substrate. As a filter-feeding animal, it has two pairs of gills that are located inside the mantle and above the visceral mass (Araujo 2009, 2012). The gonad, which is usually hermaphrodite in most specimens in Spain (Grande et al., 2001) is embedded in the dorsal part of the foot, inside the visceral mass. No true siphons are present, just a transverse thickening of the posterior ends of the gills which keeps the inhalant and exhalant openings separate. The inhalant opening is lined with sensory papillae similar to small hands (Richard & Prié, 2022).

The age of *P. auricularius* at first reproduction is not known yet, but in Spain, specimens of just 10 cm (corresponding to an age of 10-15 years) produced viable glochidia in captivity conditions (Nakamura et al., 2019). The reproductive cycle begins in winter, between December and February (Araujo et al., 2000). The sperm is released into the water and inhaled by other downstream specimens along with the water they filter. After fertilization, the eggs are incubated in the chamber formed by the four gills, where the embryos develop for one to three months, depending on water temperature, until they become glochidia. In Spain, the glochidia are released from the middle of March to mid-April and this release can be extended until May (Fig. 1.4) (Araujo et al., 2000; K. Nakamura, personal observation in captivity, several years). In French populations it begins a month later (Soler et al., 2018a). The glochidium must find a suitable fish host to attach to their gills and continue their development. The teeth at the margin of the glochidia help them to attach to the fish gills, which responds to the fixation by overgrowing the glochidia forming a cyst in which the mussels developed as ectoparasites. When metamorphosis is complete the glochidium has transformed into a small bivalve, then identified as a juvenile.

An important aspect of the conservation of the species is to know which fish can act as host for the glochidia. The ancient specific host for *P. auricularius* might have been the European sturgeon *Acipenser sturio* (Altaba, 1990; Araujo & Ramos, 2001a), which co-occurs together with *P. auricularius* shells in Pleistocene deposits (Preece, 1988). The sturgeon in the Ebro River became extinct in the 1970's (Elvira et al., 1991; López et al., 2007). Aquarium experiments achieved successful metamorphoses and viable juveniles with the native sturgeon (López et al., 2007), exotic sturgeons: *A. baerii* Brandt, 1869, *A. naccarii* Bonaparte, 1836, and the mosquito fish *Gambusia holbrooki* Girard, 1859 (Altaba & López, 2001; Araujo et al., 2001; 2003); and also, with czech sturgeon *A. ruthenus* Linnaeus, 1758 (Nakamura personal observation 2009-2010). Juveniles were also obtained from the river blenny *Salaria fluviatilis* (Asso, 1801) (Fig. 1.5), the only native fish of the Ebro River basin serving as a suitable host nowadays (Araujo et al., 2001). This fish species is also threatened and legally protected in the Aragón region (Decree 49/1995 of March 28).

Fig 1.4 *Pseudunio auricularius* specimen releasing glochidia conglutinate (black arrow).

Regarding habitat preferences, only that of adult individuals was known until a few years ago (see further description below). Recent studies found that young specimens also occupy the same habitats as adults (Nakamura et al., 2018b). Therefore, it is possible that wherever they detach as juveniles from the host fish, juveniles use and remain in the same habitat until they reach maturity.

Pseudunio auricularius is a species that adapts to a wide range of environmental parameters, especially regarding conductivity and temperature. Among the characteristics of the water where *P. auricularius* usually lives in Spain, we find usually basic pH values (~8), medium-high conductivity (~1000 µS/cm), and well-oxygenated water. In addition, the concentration of calcium ion in the water is remarkably high (in the Ebro River: 150 mg/L, Araujo & Ramos, 2000b), similarly to the sister species *Pseudunio marocanus* that also lives in hard waters (Lopes-Lima et al., 2018b), and clearly different from the species *Margaritifera margaritifera* that lives in soft waters of rivers on acidic soils. However, *P. auricularius* also lives in French Rivers with mean hardness values much lower than in Spain, down to 64.4 mg/L (Richard & Prié, 2022).

Fig 1.5 Host fish *Salaria fluviatilis* next to *P. auricularius* specimen in laboratory conditions.

Pseudunio auricularius mainly occupies gravel and sand bottoms, where it lives semi-buried, although hundreds of specimens living in silty soils have been found in the CIA (Fig. 1.6). They are capable of some active mobility, especially when they are young, and they can bury themselves completely in the sediment. Consequently, their detection is sometimes difficult (Nakamura et al., 2022a). They can dwell in the lateral channels of the river, separated from the main channel by islands, where the water flow is lower and the fish find a habitat to feed, shelter or spawn. They can also be found in areas of rapids and shallow waters, where they live tightly stuck between the stones (Haas 1916a; b; 1917). Freshwater mussels are theoretically more abundant where the host fish spend more time; therefore, the shaded areas of the banks and slopes, where benthic fish like the river blenny spend a large part of their lives, are usually favourable for *P. auricularius*.

Fig. 1.6 *Pseudunio auricularius* specimen semi-buried at the bottom of the CIA.

1.2.3 Main threats

Freshwater mussels (Bivalvia, Unionida) are especially vulnerable to habitat disturbance due to specific biological traits that include a long life-cycle (Bauer, 1983; Lopes-Lima et al., 2017), a sedentary behaviour and low juvenile survival (Sparks & Strayer, 1998; Yeager et al., 1994). They need a stable substrate and appropriate flowing conditions (Strayer et al., 1994) and have a complex reproductive cycle, as they need a fish host to complete it (Galbraith et al., 2018; Modesto et al., 2018). Notwithstanding the specialization of these biological characteristics that may make mussels more prone to particular disturbances related to their ecological preferences and needs, the disappearance of the mussels in the Ebro River and adjacent canals seems to be due to a multitude of human impacts (see below).

The decline of freshwater mussel densities in the canals are much better documented than in the river, due to their better access and easier study. Anyway, the existing populations in the Ebro River seem to have followed a general pattern of regression in all the areas where they are present, as well as throughout Europe, where the current rate of extinction of freshwater mussel populations is considered catastrophic (Lopes-Lima et al., 2018a; 2021b). The historical threats to *P. auricularius* in the Ebro River basin are well known and include the following: the construction of dams along the river, changing the natural flow and the hydrological conditions that prevent the migration of the most suitable host fish; water and sediment pollution; water extraction; hydraulic works (Fig. 1.7); and uncontrolled harvesting of specimens (Altaba, 1990; 2000; Altaba & López, 2001; Alvarez Halcón, 1998; Araujo & Álvarez-Cobelas, 2016; Araujo & Ramos, 2000).

Fig. 1.7 Examples of annual maintenance works in CIA.

The situation of Spanish population of *P. auricularius*, especially in the CIA, is delicate, especially since 2013, when an unusual and very pronounced mortality began to be recorded. In that year a total of 238 dead specimens were registered, 80% of these concentrated in the kilometer 52 of the canal. In 2017, the mussel mortality in that same point reached 100% (Guerrero et al., 2021; Nakamura et al., under revision).

There are several factors that can explain the recent decline of *P. auricularius* and the disappearance of the rest of mussel species in the canals. Each factor can act individually or in synergy, hindering the possible interpretations of their effects on the mussel community. The main hypotheses that were raised (briefly mentioned here since in chapter 1 of this thesis this topic is discussed extensively) are: 1) the absence of recruitment due to either the absence of the host fish (Altaba & López, 2001; Araujo et al., 2001), making it impossible to complete the reproductive cycle, or the increase in siltation, clogging the interstitial space, where newborn juveniles settle (Geist & Auerswald, 2007). In addition, siltation may be interfering with the internal fertilization of freshwater mussels (Gascho Landis et al., 2013) and reduce their filtration activity (Österling et al., 2010); 2) habitat degradation such as the annually maintenance works in the canals and the river (Fig. 1.7); 3) pollution, mainly due to water runoff from the nearby agricultural fields (Nakamura et al., 2021; 2022b); 4) the possible presence of unknown diseases due to bacteria, viruses, or parasites (Brian et al., 2021; Brian & Aldridge, 2022; Richard et al., 2020); 5) the invasion by exotic species such as the Asian clam that today occupies all the canal's bed and various sections of the river, negatively affecting freshwater mussel survival due to competition for resources (Ferreira-Rodríguez et al., 2018; Haag et al., 2021; Kelley et al., 2022; McDowell & Sousa, 2019); and finally, 6) climate change, that is responsible for increasingly stronger and longer droughts (but also stronger floods) that may affect the survival of mussel populations.

1.2.4 Conservation actions

Three LIFE+ projects have been carried out to date dedicated to the conservation of *P. auricularius*. The first in Spain led by the regional government of Catalonia (Generalitat de Catalunya) between 2001 and 2004 (LIFE00 NAT/E/007328). The second carried out by the Aragón Government between 2004 and 2007 (LIFE04 NAT/ES/000033). The Government of Aragón, during this LIFE project approved the recovery plan for the species and from there on it continued to support the conservation program for the species until today. The publication of the Recovery Plan implied holding meetings between the different administrations and the canals managers via the Recovery Plan Monitoring Commission, with the aim of coordinating possible disturbances to the species, avoiding episodes of mortality due to anthropogenic causes. The third LIFE+ project was carried out in France: "LIFE13 BIO/FR/001162: Conservation of the giant pearl mussel in Europe" lead by the Université Francois Rabelais and the Conseil Départemental Charente-Maritimeis. This has been the most complete conservation effort devoted to the species to date in France.

In Spain, and within the framework of the Recovery Plan in the Aragón region (now in the process of updating and publishing a new version), the critical situation of *P. auricularius* has been studied for more than 15 years, including the possible causes that have historically and recently triggered its population decline. The aim of the Recovery Plan involves the commitment to mitigate the impact produced by the canal and river works that can cause habitat modification and disturbances to the species, in order to avoid mortality episodes of freshwater mussels.

A great advance has been achieved since 2014, when a standardized protocol to grow juveniles of the species under captive and semi-natural conditions was established, resulting nowadays in hundreds of juveniles (3-4 cm length) to be reintroduced into their natural habitat (Nakamura et al., 2019).

In the same way, the French government has just published its National Action Plan 2022-2031 for *P. auricularius*, which specifies the adequate conservation actions to recover and conserve the species and its habitat. Furthermore, a PhD thesis on the conservation of *P. auricularius* in France has recently been presented by J. Soler (2018) at the University of Tours. The studies developed in the framework of this thesis allowed discovering new host fish for the species (Soler et al., 2018; 2019), establishing the period of glochidia release in France, and discussing a new threat: the fish *Rhodeus amarus*, which can parasitize the gills of *P. auricularius* by depositing its embryos inside the mussel. This work has increased our knowledge about the species in France and can contribute to improving its conservation in the country as well as in Spain.

1.3 Objectives

The aim of this thesis is to provide updated scientific knowledge to carry out the necessary conservation actions to significantly reduce the risk of extinction of *P. auricularius* in Spain and to reverse or, at least, stop the current process of regression of the species. The specific objectives include the following:

1) To assess the long-term changes (2004-2019) of freshwater mussel densities in two irrigation canals in the Ebro River basin (Canal Imperial de Aragón and Canal de Tauste). To characterise the temporal variation in shell length of freshwater mussels during this period of time, in order to evaluate changes in population structure. To estimate the annual survival of *P. auricularius* from 1999 to 2020 and discuss plausible hypotheses explaining the observed population decline.
2) To analyse, for the first time, the growth pattern of *P. auricularius*, from juveniles just released from their host fish to adulthood.
3) To establish, for the first time, the heavy metal and ammonia tolerance thresholds of *P. auricularius* juveniles originated from captive breeding, by determining the lethal concentration values (LC$_{50}$ and LC$_{10}$) for each contaminant tested.
4) To determine which set of culture conditions could improve the survival and subsequent development of *P. auricularius* juveniles obtained by captive breeding.
5) To assess the survival probability of *P. auricularius* translocated from Canal Imperial de Aragón to selected localities in the Ebro River, with the final goal of increasing its long-term survival.

1.4 Thesis outline

Thanks to the annual monitoring sampling by the Aragón government, we obtained information about the density evolution of the four species of freshwater mussels present in CIA and CT. **Chapter 3** of this thesis, entitled **"Collapse of native freshwater mussel populations: prospects of a long-term study"** rely on these data. In this chapter, the long-term changes (2004-2019) in freshwater mussel densities in the CIA and CT were assessed. Additionally, we studied the temporal variation in shell length of freshwater mussels to have an estimation of the variation of the population age structure. Finally, the annual survival of *P. auricularius* from 1999 to 2020 was also studied using capture-recapture methods, and plausible hypotheses to explain the observed decline were discussed. This chapter has been submitted as a scientific article to **Biological Conservation** for review and has been returned for a major revision (September 2022) following the reviewers' suggestions.

Several mortality episodes of *Pseudunio auricularius* have been recorded in the CIA and CT since 2013. One of the first hypotheses that could explain the registered population declines is the old age of adult specimens (Fig. 1.8). A high proportion of such aged specimens in the CIA could partly justify this phenomenon of high mortality, but it was clear that there had to be other causes that would act as a trigger, given the synchronized dead of many specimens of distinct ages. In addition, the death of many young specimens of other mussel species was also observed (Guerrero et al., 2021). Since the approval of a Recovery Plan in 2005, there was a great speculation about an aging population of adults without apparent recruitment. The length-age relationship was unknown, so there were no data on the population age structure. Given this gap, the aim of **chapter 4, "Modelling growth in the critically endangered freshwater mussel *Margaritifera auricularia* (Spengler, 1793) in the Ebro basin"** was to study the length-age relationship of the species using shells of dead specimens collected in the field during several years, together with young specimens collected alive from Canal de Tauste, and growth data of juveniles reared in the laboratory. All these data were combined in order to create the first growth model of *P. auricularius*. This chapter has been published in the scientific journal **Hydrobiologia** in 2018 (Nakamura et al., 2018b).

Another hypothesis raised to explain mussel decline was the possible negative impact of several contaminants present in the habitat (Fig. 1.8). The Aragón Government

collected a hundred of samples from different sites where high mortality of the species had been detected. In different years, water and sediment samples and tissue from recently dead *P. auricularius* or from the Asian clam *Corbicula* spp. were collected for analysis of toxic substances. The analytical results showed high values of the pesticide terbuthylazine and metolachlor in the CT; ammonium and aluminum concentrations in CIA and organic pesticides in the Ebro River. In the sediments of the CIA, arsenic, nickel and iron were very high in 2017, and copper concentration in sediments from CT reached 531 mg/kg in one sample (Guerrero et al., 2021). The concentration of heavy metals in biological samples was remarkable, especially for lead and cadmium. Based on these results, the aim of the **5th chapter, "Sensitivity of *Pseudunio auricularius* to metals and ammonia: first evaluation"** was to assess the sensitivity of the species to these toxic products using juveniles reared in the laboratory. In this way, we were able to discern if the concentrations found in the habitat might explain the mortality rates reported in the last years. This chapter has been published in the scientific journal **Hydrobiologia** in 2021 (Nakamura et al., 2021).

In **Chapter 6, "Captive breeding of *Margaritifera auricularia* (Spengler, 1793) and its conservation importance"**, the best culture conditions for juveniles of *P. auricularius* in captivity were determined in order to obtain captive breeding specimens large enough to reintroduce them into the natural habitat. **Aquatic Conservation: Marine and Freshwater Ecosystems** was the scientific journal where this chapter was published in 2019 (Nakamura et al., 2019).

Due to the high mortality observed in the CIA, the Aragón Government launched an emergency plan with the aim of increasing the long-term survival of *P. auricularius* specimens, drafting the first translocation plan for the species and distributing it among scientific experts, managers, companies and NGOs that worked with freshwater mussels. From these discussions, the conclusion was that the best option to save the last specimens living in the canal was their translocation from the canal to their natural habitat, the Ebro River. **Chapter 7, "Translocation as an ultimate conservation measure for the long-term survival of a critically endangered freshwater mussel"** analyzes the survival of *P. auricularius* in the first three years after the translocation. This chapter has been published recently in the scientific journal **Hydrobiologia** in 2022 (Nakamura et al., 2022a).

A **general discussion section** has been included to compile all the results obtained from this research and discuss them together in the framework of the recovery plan to preserve *P. auricularius*. Finally, the **conclusions** section highlights the most important results obtained in this thesis.

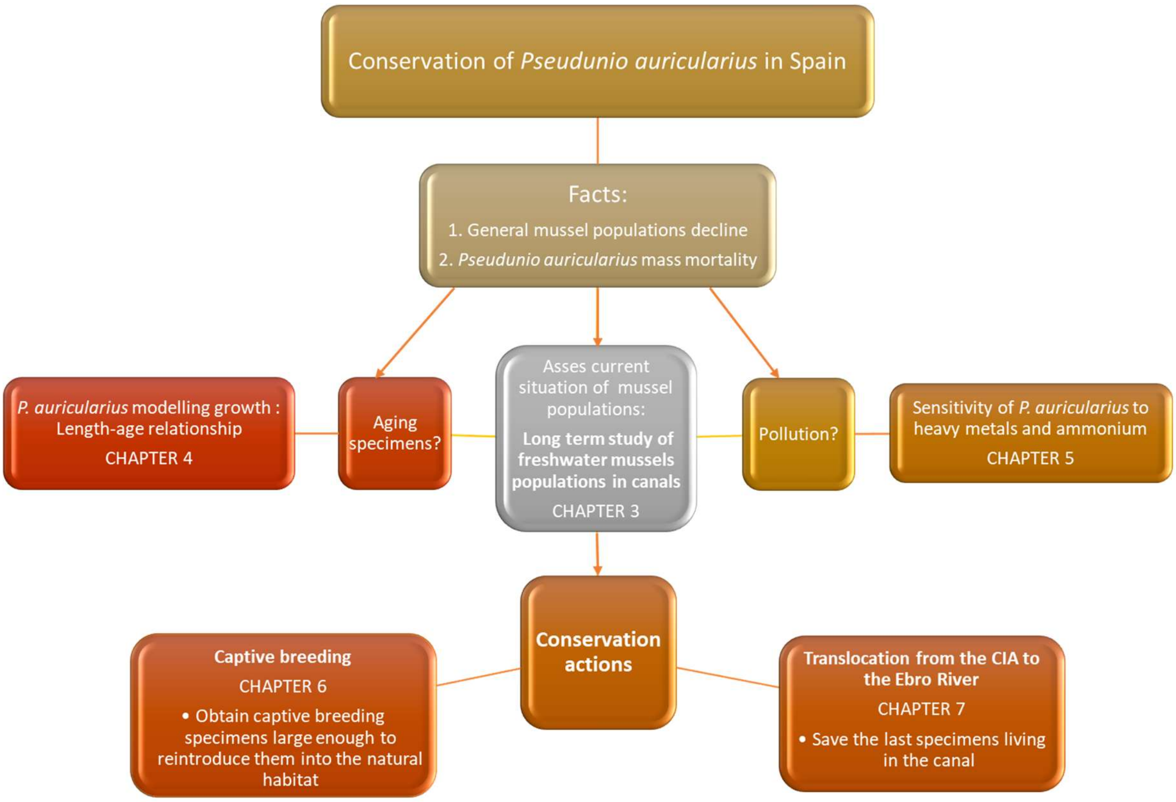

Fig. 1.8 Diagram of the thesis outline.

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Chapter 2

General methods

The work developed in this thesis is part of the *Pseudunio auricularius* (= *Margaritifera auricularia*) Recovery Plan directed and developed by the Government of Aragón through the public company SARGA, since it was approved in 2005.

Each chapter presents its particular methodology section depending on the main objectives, and the techniques used were consequently different in each case.

The study area focuses on the Ebro River basin, one of the largest basins in the Iberian Peninsula. This area is located in northeastern Spain, and 50% of the basin is included in the Aragón region. The Ebro River enters Aragón near the town of Novillas, wherefrom it flows southeast until reaching the Mequinenza reservoir, and then it continues its downstream journey until the Catalan region, where it finally releases its waters into the Mediterranean Sea. The field works in this thesis were carried out in the main channel of the Ebro River and in two adjacent canals: Canal Imperial de Aragón (CIA) and Canal de Tauste (CT). The CIA runs along the Ebro River right bank, is 108 km long and has 30 m^3/s mean water discharge. The CT goes along the left bank, is 44 km long and has a mean discharge of 12.5 m^3/s. Most of both canals' length run through the Aragón region, although they start near the city of Tudela in the Navarra region. Additionally, other small ditches were also visited, such as the Quinto ditch (see chapter 4), downstream from the city of Zaragoza, and other streams, tributaries of the Ebro River, such as the Vero River (see chapter 7).

Sampling in the canals was usually performed when water was cut for maintenance works. These works lasted only two or three weeks, so there was a short window of time for sampling activities. During censusing the species in these canals for monitoring its populations in the field, we also selected specimens for captive breeding, and at the same time collected samples for the toxicological assessmentand and prepared the specimens (tagging, biometry) that later would be translocated from the canal to the river, among others actions. In the case of the Ebro River, the field works were carried out exclusively during the dry season (July-August) when the river has a low level and consequently the accessibility is much easier.

The mussel surveys were usually performed by wading with viewing glasses. Alternatively, professional scuba divers were hired in case of less accessible sites and high water level.

The target species was in all chapters the Giant Freshwater Pearl mussel, *Pseudunio auricularius*, listed as critically endangered by the IUCN as well as in the Spanish catalogue of threatened species. Currently, the unique Spanish population of the species is located in the Ebro River basin, and the greatest abundance so far has been found in the Aragón region, specifically in the CIA.

In Chapter 3, other three mussel species were also taken into account: *Anodonta anatina, Potomida littoralis*, and *Unio mancus*. The last species is listed as vulnerable in Spain. The data used in this chapter have been gathered from sampling campaigns throughout the canals, carried out by the Aragón Government for more than 16 years.

Specimens of *Pseudunio auricularius* handled during the field and laboratory works in this thesis were either adult, young or juvenile individuals. The adult (15-16 cm) and young (8-10 cm) specimens came from the canals wild population, and the juvenile individuals (150-200 µm) were obtained by captive breeding. Juveniles were always handled under a binocular microscope. Captive breeding was carried out in the Aragón Government facilities in Zaragoza, in a location known as La Alfranca, which is described in more detail in chapter 6.

Toxicology and histopathology work was carried out at the University of Zaragoza, Faculty of Veterinary Medicine. The toxicological tests were carried out in the Laboratory of Molecular Toxicology, University of Zaragoza, following the standard methodology for acute tests for freshwater mussels (see chapter 5).

Chapter 3

Collapse of native freshwater mussel populations: prospects of a long-term study

Extant bivalve species present in the Ebro River basin

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3.1 Abstract

Freshwater biodiversity is under threat, but long-term quantitative studies showing major demographic declines in invertebrate species are still scarce. Here we focus on a long-term study (2004 to 2019) of freshwater mussel (Order Unionida) populations in two canals of the Ebro River (Spain): Canal Imperial de Aragón (CIA) and Canal de Tauste (CT). Special attention was given to *Pseudunio auricularius* (Spengler, 1793), a critically endangered species. Data on mussel density show a continuous decline in all four native species present, with *Anodonta anatina, Potomida littoralis* and *Unio mancus* being now considered locally extinct. *Pseudunio auricularius* is still present in the canals, but at very low densities (0.01 ind/m^2). According to capture-recapture data, *P. auricularius* has experienced a progressive decline in survival probability, down to 0.15 in 2020 in the CIA, although in the CT it remains close to 1. Based on these results, we discuss several hypotheses that may explain this rapid collapse of unionid populations. Given the precarious conservation status of freshwater mussels in both canals, effective management measures should be urgently applied, including habitat restoration and captive breeding.

Key words: canals, endangered species, invasive species, mortality, pollution, Unionida

3.2 Introduction

Biodiversity loss at local and global scales is a well-established process (Albert et al., 2021; Damiens et al., 2021; Oliver, 2016; Palombo, 2021), sometimes derived from sudden mass mortality events (Fey et al., 2015; Wernberg et al., 2016) but also due to progressive population declines over time (Ceballos et al., 2017; Lister & García, 2018). Considering this biodiversity crisis, several authors have even suggested that we are entering the sixth mass extinction event and even a new geologic era, the Anthropocene (Ceballos et al., 2015; Ceballos & Ehrlich, 2018; Payne et al., 2016; Palombo, 2021). A decrease in population density is, in many cases, a clear sign that something is negatively affecting the ecosystem, and is interpreted as a warning sign that a population or even a species may disappear in the near future (Ceballos et al., 2015, 2017; Rosemberg et al., 2019). Examples showing these declines are mounting, including an alarming 68% loss

for vertebrate populations monitored between 1970 and 2016 (WWF, 2020; 2022); Rosenberg et al. (2019) reported an abundance decline of 29% in North American avifauna over 48 years of study; Dirzo et al. (2014) found an average 25% decrease in abundance for terrestrial vertebrates and, for invertebrates, a compiled index over the last 40 years showed a mean decline of 45%. Besides density decline per se, the decline of a group of organisms can sometimes cascade along the entire food web. This can cause a general community collapse, as in the Luquillo rainforest in Puerto Rico, where a strong decline in the abundance of arthropods since 1970 caused a synchronous decline from lizards to birds that relayed on the arthropods as their main food source (Lister & García, 2018).

As shown for terrestrial habitats, aquatic ecosystems also face an extreme risk of defaunation, although marine wildlife populations have not yet experienced as severe range contractions as their terrestrial counterparts (Gilmour et al., 2013; McCauley et al., 2015). However, some examples start to pop-up in the literature, including the shift of kelp forests into seaweed turf due to climate change in Australia (Wernberg et al., 2016) or, more recently, the fan mussel (Pinna nobilis Linnaeus, 1758) mass mortality event through the Mediterranean Sea, due to an infection by a haplosporidian parasite (Grau et al., 2022). Conversely, freshwater ecosystems have long been recognised as one of the most threatened habitats, currently declared in crisis (Birk et al., 2020; Collen et al., 2014; Harrison et al., 2018; He et al., 2019; WWF, 2020; 2022). The extinction risk of freshwater species has been consistently higher than that of their terrestrial counterparts (Collen et al., 2014; Lydeard et al., 2004). For example, vertebrate populations decline consistently faster in freshwaters (3.0% annually since 1970) than on land (1.1%) (Dudgeon, 2019; Maasri et al., 2022). Recently, Böhm et al. (2021) assessed the risk of extinction of 1,428 freshwater mollusc species using the IUCN Red List Categories and Criteria and found that almost one-third of them were threatened with extinction. Climate change, pollution, habitat loss and fragmentation, overexploitation and the introduction of pathogens and invasive species are the main causes driving freshwater biodiversity loss (Albert et al., 2021; Damania et al., 2019; Dudgeon et al., 2006, 2019; Eastwood et al., 2022; He et al., 2019; Palombo, 2021; Reid et al., 2019).

The examples of biodiversity decline mentioned above have been mostly assessed thanks to long series of data collected over years of study (Gilmour et al., 2013; Lister & García, 2018; Wernberg et al., 2016). Long-term field studies of animal populations have

indeed been recognized as essential to evaluate biodiversity loss and as an effective tool in conservation biology (Conde et al., 2019; Moussy et al., 2022; Reinke et al., 2019; Rosenberg et al., 2019). These types of surveys allow a broader vision and a more accurate perspective of biological and ecological processes and their temporal variability, being essential for habitat management, especially when endangered species are present. However, most long-term studies are focused on terrestrial habitats, and particularly on vertebrates such as mammals or birds (Conde et al., 2019; Jourdan et al., 2019; Kendrick et al., 2015; Moussy et al., 2022), while few examples exist related to the freshwater environment (Chester & Robson, 2013) and even fewer on freshwater invertebrates (Collen et al., 2014; Inoue et al., 2014; Jourdan et al., 2019; Sanchez Gonzalez et al., 2021). Furthermore, most of the few long-term studies that do exist are mainly based on presence-absence data with a limited number of quantitative studies showing declines in density (Wernberg et al., 2016), biomass (Lister & García, 2018) or other important ecological features. This lack of quantitative data is also problematic for the assessment of ecosystem services provided by biodiversity, because most of these services are highly density dependent (Eastwood et al., 2022).

Freshwater mussels (Bivalvia, Unionida) have biological characteristics that make them especially vulnerable to habitat disturbance: many species are long-lived, do not begin to reproduce until they are 6-12 years old (Bauer, 1983; Lopes-Lima et al., 2017), are sedentary, and have low juvenile survival (Sparks & Strayer, 1998; Yeager et al., 1994). In addition, a stable substrate and appropriate flow conditions are essential for their survival (Strayer et al., 1994). They have a complex reproductive cycle, as they need a fish to act as an intermediary host to develop their larvae (glochidium) (Galbraith et al., 2018; Modesto et al., 2018). Freshwater mussels provide ecosystem services important for human well-being, such as water purification, nutrient cycling and habitat stabilization, among many others (Vaughn, 2018; Zieritz et al., 2022). Nowadays, they are considered the most imperiled group of aquatic organisms, urgently needing protection and conservation measures to revert their decline (Böhm et al., 2021; Ferreira-Rodríguez et al., 2019; Lopes-Lima et al., 2017, 2021a, b). Given this background, and recognizing their significant services, it is important to evaluate their population density, because most of these services rely on healthy and highly dense mussel beds in order to have a significant ecological role.

Many studies have tried to unravel the different causes of freshwater mussel mortality but most of these causes remain uncertain (Haag, 2019 a; b; Strayer et al., 2004). The large number of potential factors contributing to mussel decline makes it difficult to determine which type of disturbance exerts the greatest negative influence. Although the major threats for this faunal group include industrial and agricultural water pollution, ecosystem modification, urban pressure, sedimentation, construction of dams and introduction of non-native invasive species (Lopes-Lima et al., 2017; Ferreira-Rodríguez et. al., 2019), the reality is that most of these threats remain speculative.

The Ebro River basin is the largest in Spain and 50% of its main channel is located in the Aragón region, where despite its moderate water quality (Confederación Hidrográfica del Ebro, 2010-2020), it is currently one of the river sections where populations of freshwater mussels are best preserved (Rubio Millán et al., 2016). Four freshwater mussel species used to live in the Ebro River: *Anodonta anatina* (Linnaeus, 1758), *Potomida littoralis* (Cuvier, 1798), *Unio mancus* Lamarck, 1819, and *Pseudunio auricularius* (= *Margaritifera auricularia* Spengler, 1793). The latter, also known as the Giant Freshwater Pearl Mussel, is one of the most endangered freshwater mussel species in the world and it is classified as Critically Endangered by the IUCN (Prié, 2021). Currently, this species shows a very restricted distribution that includes Spain, in the Ebro River basin (Altaba, 1990; 1997; Araujo & Ramos, 2000; 2001; Gómez & Araujo, 2008; Nakamura & Guerrero, 2008; Nakamura et al., 2018a, b; 2019) and four river basins in France: Charente, Vienne (Loire), Luy (Adour), and Dronne (Garonne) (Cochet, 2001; 2002; Nienhuis, 2003; Prié et al., 2010; 2018). Historically, there was no information on the distribution of the species in Spain since Azpeitia Moros (1933) recorded the presence of young specimens in the Canal Imperial de Aragón (CIA), an ancient canal in the mid Ebro River. No studies were carried out in the second half of the 20th century on this species in the area until 1985, when some specimens were found in the Ebro Delta (Altaba, 1990) and later on, in 1996, in the CIA (Araujo & Ramos, 1998; Álvarez Halcón, 1998). Since then, the canals diverting water from the Ebro River, such as CIA and Canal del Tauste (CT), have been considered as important habitats for freshwater mussels in Spain.

Taking into account the current alarming situation of unionids in the Ebro basin, the aims of this study were to i) assess the long-term changes (2004-2019) of freshwater mussel densities in two irrigation canals (CIA and CT), ii) study the temporal variation

in mean shell length of freshwater mussels from 2004 to 2019 to estimate changes in population structure, iii) evaluate the annual survival of *P. auricularius* from 1999 to 2020 using capture-recapture methods and iv) discuss plausible hypotheses explaining the observed decline.

3.3 Materials and methods

The CIA and CT canals derive their water from the Ebro River, near Tudela city (Navarra region) (Fig. 1). The CIA is 108 km long and has an average water discharge of 30 m^3/s. It was conceived as an irrigation ditch in the 18th century, but was later also used for navigation. Its main objective was to bring water to the city of Zaragoza, and today it continues to fulfill that role, in addition to distributing water for irrigation across the Ebro valley (for more details see Gómez & Araujo 2008; Nakamura et al., 2018b, 2022). The first 30 km of the CIA are located in the Navarra region, where it is fully cemented; therefrom it enters the Aragón region where its bottom is not made of concrete but composed of gravel, sand and silt. The CT is smaller than the CIA, with a length of 44 km and 12.5 m^3/s of mean water discharge. Its bottom is naturalized throughout its length and it harbours the highest number of young *P. auricularius* registered so far in Spain (Nakamura et al., 2018b; Guerrero et al., 2021).

3.3.1 Density, shell length and capture-recapture data

The density data used in this study have been gathered during 16 years (2004-2019) of sampling campaigns throughout both canals, carried out within the framework of a Recovery Plan of *P. auricularius* funded by the Aragón Government. Sampling campaigns were launched whenever water input to a canal was shut for annual maintenance works, i.e. every February and November in the CIA, but only during February in CT. Living and dead freshwater mussels were located by sight using aquascopes when water transparency allowed it, or by palpating the substrate when the water was too turbid to see the bottom. Whenever some work outside these periods was necessary, it was done by hiring professional divers.

Different sampling methodologies were used to assess mussel density (see below and Table I in Supplementary material), partly due to the involvement of various sampling teams through the years, but also depending on the type of maintenance works, the type

of section of the canal, the relative abundance of *P. auricularius* (because of its classification as Critically Endangered), canal spatial heterogeneity or temporal period when the sampling occurred. Nevertheless, the effects of sampling heterogeneity were partly adjusted thanks to the high number of sections being sampled and by taking always into account sampled surface area for any further analysis. In some cases, the selected canal section was surveyed over its full width and all freshwater mussels were collected (FS: full stretch, Table I in Suppl. material). In other cases, three 2m-wide transects parallel to the shore (right, center and left) were inspected along the entire length of the studied section (LONG: longitudinal transect, Table I in Suppl. material). Finally, in other cases, data were collected by covering 0.5-m wide transects perpendicular to the canal (TRANS: transversal transect, Table I in Suppl. material), and repeating these transects every 100 m, so that if the affected section by the maintenance works was, for example, 500 meters long, five transversal transects were surveyed. In some sections and due to the presence of *P. auricularius*, transects were set every 50 meters. Overall, 425 independent samples were completed in the CIA, more frequently obtained during 2007, 2015 and 2016 (52, 50 and 111 samples respectively) (Table 1). In the CT a total of 224 samples were taken, 2011 and 2016 being the years with more samples (101 and 51 samples respectively) (Table 1). In CT no sampling campaigns were carried out in 2005, 2006, 2008, 2009 and 2010 because there were no water cuts, or no maintenance works were performed, and therefore we were not able to collect data (no divers could be hired). Sampling sites were always assigned to the kilometric point (KP) of the canal section being studied, and geographic coordinates (UTM-WGS84) were also obtained.

Freshwater mussels were collected, identified to species level, counted and measured with a manual Vernier calliper (±0.1mm). Shell measures were obtained between 2004 and 2019 from both canals (CIA and CT). Living *P. auricularius* specimens were tagged with a numbered plastic label glued with cyanoacrylate, in order to maintain an updated census of the species. Whenever a high risk of *P. auricularius* mortality was expected due to canal maintenance works, specimens were translocated and, if possible, returned to the same point when the work was finished. Information on *P. auricularius* marked and recaptured individuals, starting in 1998 for the CIA and 2002 for CT, included date, location and label number.

3.3.2 Data analysis

The abundance data (n=1088) of living individuals per species and unit area, were classified according to year, canal (CIA or CT) and kilometric point (KP) where the sampling was carried out, from 2004 to 2019. Samples with area smaller than 0.25 m^2 were discarded for statistical tests, as they were expected to bias the data towards absences, and because they were concentrated in particular years and sections of the canals. This reduced the number of samples being analysed to 649 for *P. auricularius* and 467 for all unionid species (some samples were focused on evaluating only the endangered species *P. auricularius*). We used general additive models (GAMs) to visualize mussel density trends through time, using density as the response variable and year as the predictor variable. Taking into account the high number of samples without living individuals of one or various species in the dataset, we applied zero-inflated (ZI) generalized linear mixed models (GLMMs), using number of living individuals of each species as the response variable, year as a fixed factor expected to affect their abundance, KP as a random factor within each canal, and log10 of the sampled area as an offset variable to properly consider abundance as depending on sampled area. The sampled in each canal was approached to the nearest even kilometer in order to reduce the number of categories in the hierarchical random variable to facilitate model convergence. Years 2004-2019 were transformed to a 0-15 scale. Models were built using a negative binomial distribution and a log link function to account for overdispersion. GAMs were carried out with the package mgvc (Wood, 2017), and all ZI-GLMM analyses with the package glmmTMB (Brooks et al., 2017), both in R version 3.2.2 (R Core Team, 2021). As the estimation of conditional and marginal R^2 is not yet reliable for ZI-GLMM models, we used the correlation between fitted and observed values (r^2) as a simple indicator of model performance, following Byrnes, in Bolker et al. (2022).

In the *P. auricularius* capture - recapture data, we checked which marked individuals had been recaptured each year and if they were alive or dead. Consequently, we used a joint live and dead encounter (Burham) model parameterization in the MARK software (Cooch & White, 2019) to obtain the estimated survival for marked individuals in each canal separately. A series of models were tested to check for effects of year and group of individuals (adults or juveniles, and used or not temporarily in a captive breeding program, see Nakamura et al., 2018b; 2019) on the parameters of probability of survival

(S), recapture (p) and reporting (r), while fidelity (F) was assumed to be fixed. Multimodel selection was done with the Akaike Information Criterion (AIC).

3.4 Results

3.4.1 Changes in mussel abundance

The overall density of freshwater mussels in both canals drastically decreased over the 16 years of the study (Figs. 3.1, 3.2). In the CIA higher densities were observed in its middle and lower stretches before 2013 (2004 - 2012, Fig 3.1a), unlike the CT, where freshwater mussels were more evenly distributed through the entire canal, reaching values above 1 ind/ m^2. The situation changed radically after 2013 (2013 - 2019, Fig. 3.1b; Fig. 3.2) when the density falls drastically, registering very low densities (below 0.05 ind/m^2), throughout the entire length of both canals.

In 2004, the initial median density of all species together was 0.54 ind/m^2 in the CIA and 0.09 ind/m^2 in CT. In the CIA we found maximum density values of 5.11 ind/m^2 in 2004 and 5.48 in 2007. In 2012 we estimated a maximum value of 2.5 ind/m^2, and since then the density decreased down to close to zero and did not recover later on (Fig. 3.2). Mussel density in CT showed a peak in 2011 (8.61 ind/m^2) (also the year with the highest effort in this canal, Table 3.1), but afterwards it drastically fell down below 2 ind/m^2 in 2012-2014 and close to zero later on (Fig. 3.2).

When we analysed the data by species (Fig. 3.3), *P. littoralis* presented in the CIA median densities of 0.30 ind/m^2 in 2004 with maximum values of 2.15 ind/m^2. In 2007 and 2009, high median densities of 0.41 and 1.29 ind/m^2 were registered with maximum values of 5.09 and 3.53 ind/m^2, respectively. In 2013, its density fell down close to zero and it did not recover again over the next years. In CT the median densities of *P. littoralis* were lower than in the CIA and always below 0.03 ind/m^2, with the exception of 2011 when a peak in density was recorded (7.69 ind/m^2), and its density was higher than in CIA during 2013-2014, but close to zero thereafter.

The drastic reduction and even disappearance of *A. anatina* and *U. mancus* from the canals occurred earlier than in *P. littoralis*, both species also registering low densities during the first years of study. In the CIA, *A. anatina* had a median density of 0.11 ind/m^2 in 2004 (maximum value of 2.38 ind/m^2) and by 2010 it had already reached almost zero (0.01 ind/m^2) (Fig. 3.3). In CT it had a median density of 0.02 ind/m^2 in 2004 with maximum value of 1.25 ind/m^2 and by 2011 no live specimens were found. *Unio mancus* had even lower densities at the beginning of the study (2004 median and maximun density values in the CIA = 0.06 and 0.62 ind/m^2, CT = 0.02 and 1.26 ind/m^2, respectively) and in both canals it progressively declined year after year (Fig. 3.3), with the exception in 2011 when reaching a density peak in CT (max value: 1.45 ind/m^2) and then disappearing by 2012, except for a unique value in 2016 higher than 0, when a greater sampling effort in CIA was performed (Table 3.1).

Table 3.1 Number of samples per year in Canal Imperial de Aragón (CIA) and Canal de Tauste (CT) from 2004 to 2019.

Year	CIA	CT
2004	17	10
2005	6	0
2006	24	0
2007	52	4
2008	15	0
2009	10	0
2010	7	0
2011	3	101
2012	6	1
2013	18	13
2014	15	13
2015	50	9
2016	111	51
2017	33	8
2018	23	8
2019	35	6
Total	425	224

The median density of *P. auricularius* in the CIA (Fig. 3.3) seems to have been maintained low over the years (0.001 - 0.04 ind/m^2). Exceptional values were registered in 2015 with unusually high density for the species with a maximum value of 0.82 ind/m^2, however the median density in the canal was close to zero. On the other hand, in the CT a slight increase in median density was observed from 2011 to 2019 although the values were always very low (0.001 - 0.03 ind/m^2).

Most ZI-GLMMs (Table 3.2) had significant fixed effects of time over both the count and the binomial parts of the model. Considering the model for the overall abundance (adjusted by sampling area, i.e. ~ density) of unionids, we found an inverse and significant relationship of time (year) with abundance (Count part) and a positive relationship with absence probability (ZI part). The kilometric point (KP) in each canal accounted for much higher variance in abundance than that explained by the canal itself, but the opposite was true for the ZI part.

Analysing the data by species, the model for *P. auricularius* showed a negative and significant relationship of abundance with year (Table 3.2). However, the ZI part indicated an increase in the probability of finding this species with time (negative estimate for the fixed effects of year on absence probability).

In both parts of the model, the random effects of location (km point) inside each canal seem to account for a higher variability than the effects of the canal (i.e. CIA or CT). In the case of *P. littoralis* and *U. mancus* the ZI part was also significant. In these cases, year was also negatively related to mussel abundance and positively with its absence probability. Intra-canal variance of the probability of finding the species and of its abundance were higher than between-canal variance, according to random effects. The model for *A. anatina* was the only one in which the ZI part was not significant, but the count part also indicated an abundance decline with time. Here, intra-canal variability of abundance seems to be higher than between canals, as well as with other species, but the opposite regarding presence-absence of the species. The best fitted models, according to their lower deviance and higher r^2, correspond to those of *P. littoralis, A. anatina* and *U. mancus*, while the model for *P. auricularius* is the one with the weakest relationship between observed and fitted values.

3.4.2 Shell size population structure

We obtained a total of 19033 shell measurements between 2004 and 2019 for the four mussel species; 2854 corresponded to *A. anatina* (CIA: 2169, CT: 685), 4113 to *P. auricularius* (CIA: 3829, CT: 284), 10242 to *P. littoralis* (CIA: 8778, CT: 1464), and 1824 to *U. mancus* (CIA: 1285, CT: 539).

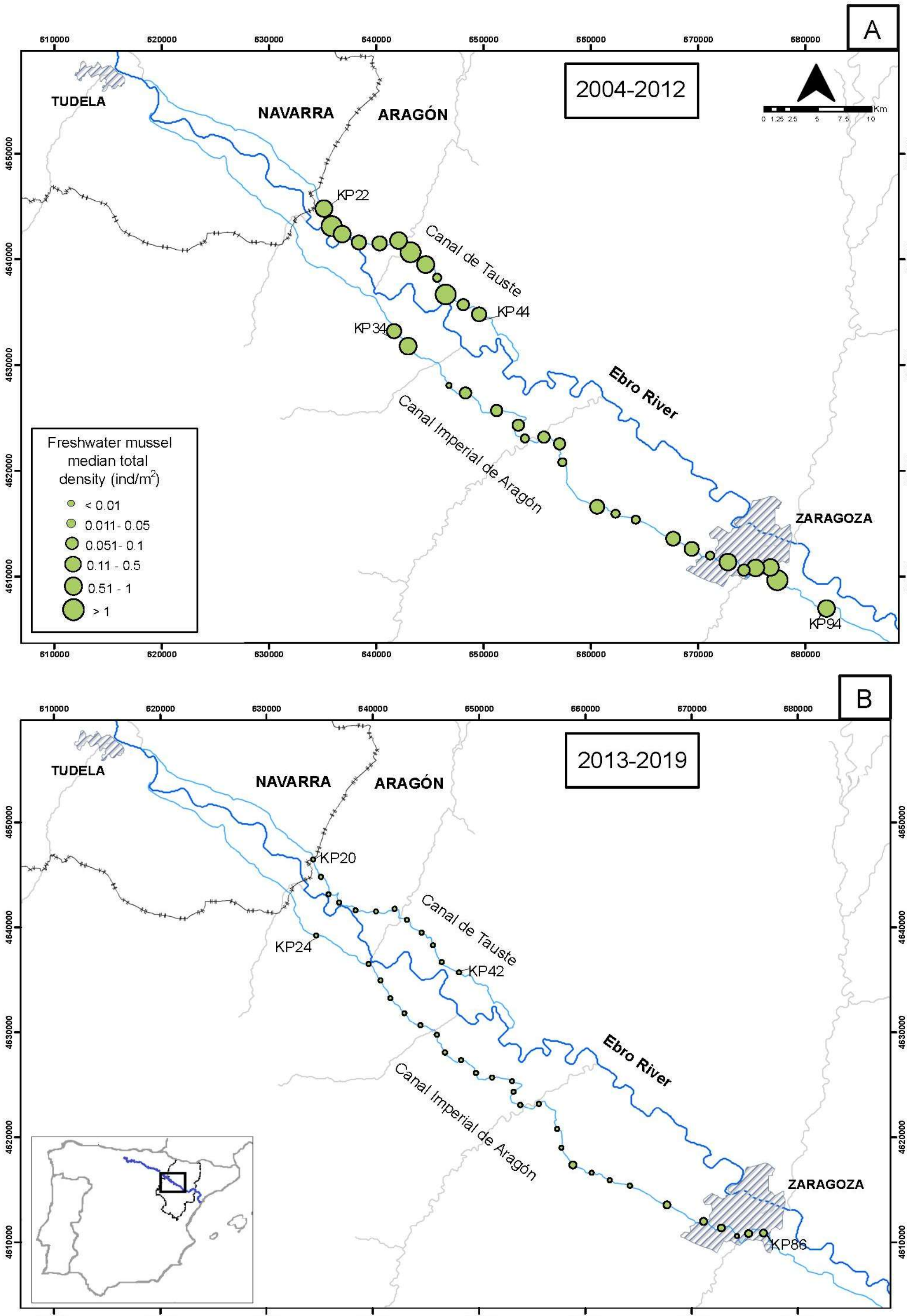

Fig. 3.1 Study area in the Ebro River Basin (Eastern Iberian Peninsula), with indication of the median density values recorded every 2 km in the Canal Imperial de Aragón and Canal de Tauste in two periods (see the Result section in the text for further explanations): years 2004-2012 (A) and years 2013-2019 (B).

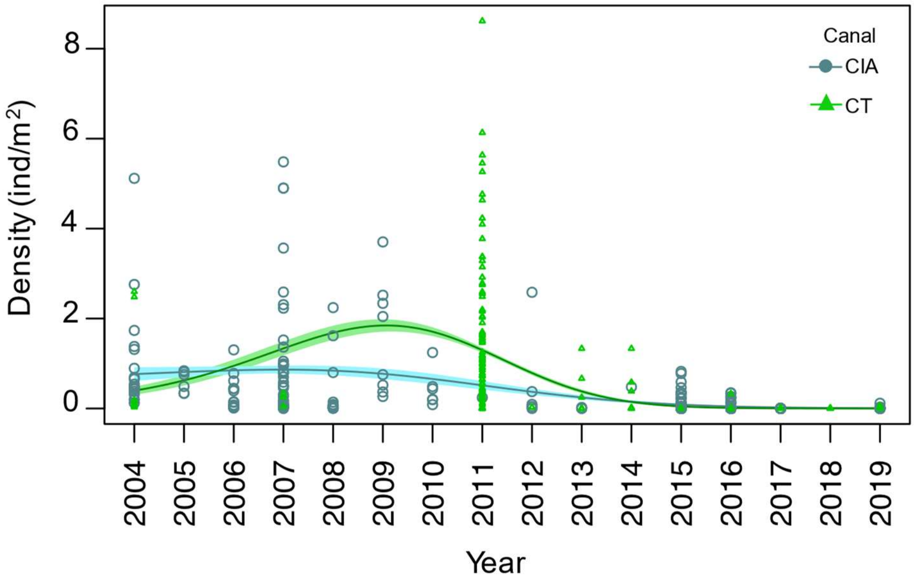

Fig. 3.2 Total density plots of alive freshwater mussels (symbols) in CIA and CT between 2004 -2019, and corresponding GAM models for each canal including predicted values (line) and their standard errors (coloured polygon).

From 2004 to 2007 *A. anatina*, both in the CIA and in the CT, presented small (young) and large (old) specimens (from 11 to 180 mm) (Fig. 3.4). Starting from 2008, the largest and smallest specimens began to disappear, and only the intermediate-medium sized individuals, ca. 75-100 mm long, survived, until the species completely disappeared by 2012. Similarly, *U. mancus* initially maintained a diversity of sizes in the CIA (from 21 to 108 mm), but over the years it was reduced to the presence of only adult specimens (mea n = 60 mm).

In the CT, a reduction of size variability was also observed in this species, the last living specimens being recorded in 2011, with an average shell length of 40 mm. *Potomida littoralis* in CIA showed a shell size population structure including small and large specimens (10-88 mm), with a mean shell size of around 50-60 mm (Fig. 3.4), which was more or less maintained until the species completely disappeared in 2013. The population in CT still had the presence of adults and juveniles by 2011, but in 2013 and 2014 most of the adult specimens disappeared, extinct after 2016 when a single small (26 mm long) specimen was found.

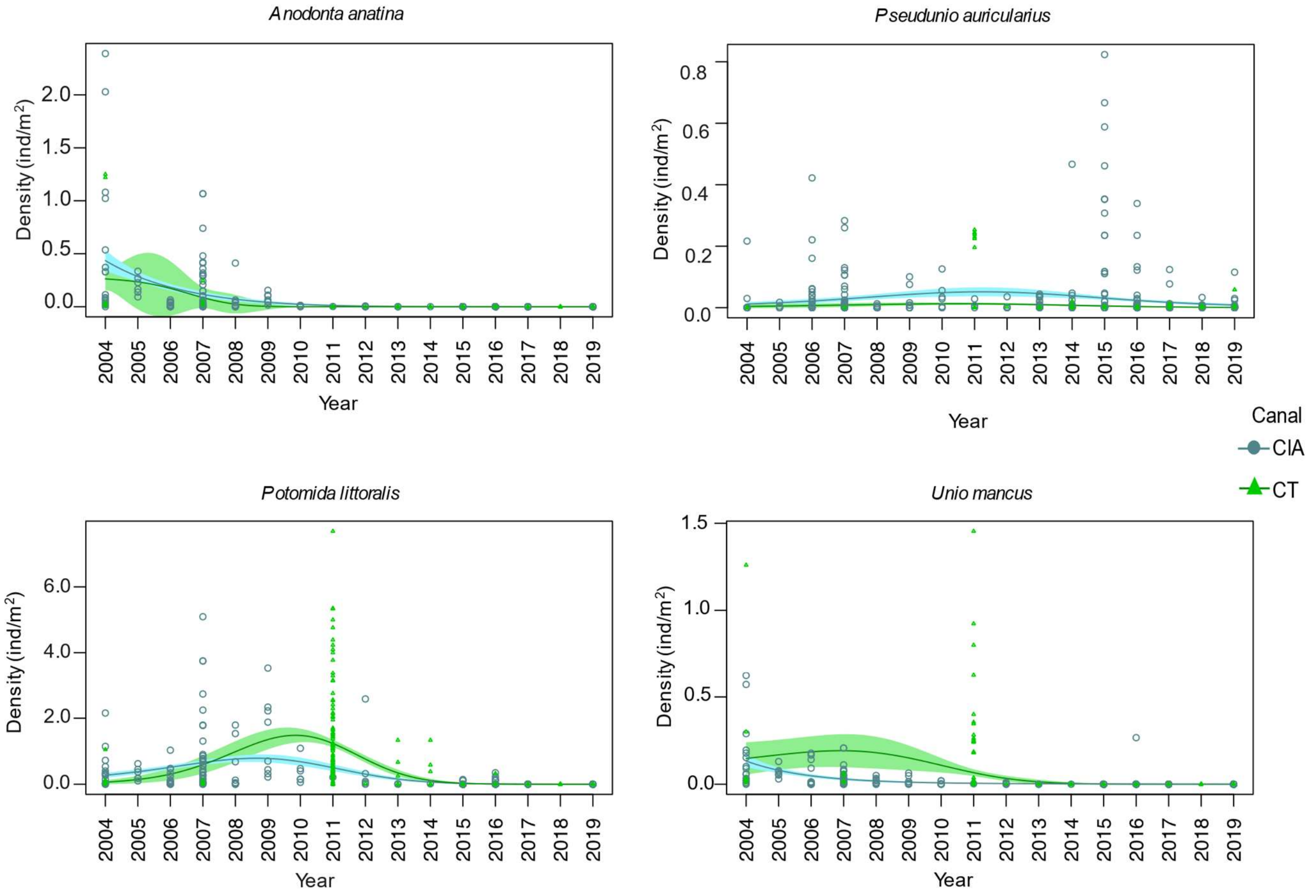

Fig. 3.3 Densities plots of alive freshwater mussels (symbols) by species in CIA and CT between 2004 - 2019 and corresponding GAM models for each species and canal including predicted values (line) and their standard errors (coloured polygon).

Table 3.2 Results of zero-inflated negative binomial GLMMs for the total abundance of unionids and for each species separately. General model performance includes deviance and r^2 (correlation between fitted and observed values). For fixed effects (intercept and year) of count and binary (zero-inflated) parts of the model, estimates (and their standard error between brackets) are shown, so as their significance levels. For random effects, we show their estimated variances. KP:Canal = even kilometric point within each canal.

		Total Unionids	*P. auricularius*	*P. littoralis*	*A. anatina*	*U. mancus*
	Observations	467	649	467	467	467
	Deviance	3010.4	2406.2	2390.1	1174.6	1168.5
	r^2	0.54	0.18	0.62	0.70	0.54
COUNT PART						
Fixed effects	Intercept	3.76 (0.23)***	0.43 (0.23)	2.46 (0.39)***	1.67 (0.23)***	0.98 (0.25)***
	Year	-0.40 (0.03)***	-0.10 (0.02)***	-0.18 (0.06)**	-0.24 (0.06)***	-0.34 (0.05)***
Random effects	KP :Canal	0.54	0.15	0.86	0.46	0.22
	Canal	1.10E-13	7.40E-09	0.007	2.50E-16	1.18E-12
ZERO-INFLATED PART						
Fixed effects	Intercept	-45.97 (15.3)**	2.22 (1.1)*	-13.2 (2.5)***	-24.0 (18.3)	-8.39 (2.5)***
	Year	2.49 (0.87)**	-0.77 (0.27)**	1.37 (0.25)***	4.54 (3.39)	1.07 (0.29)***
Random effects	KP:Canal	2.90E-03	4.7	1.55	9.14E-08	1.07E-07
	Canal	240	0.89	3.62E-13	133	8.06E-12

*p<0.05; **p<0.01; ***p<0

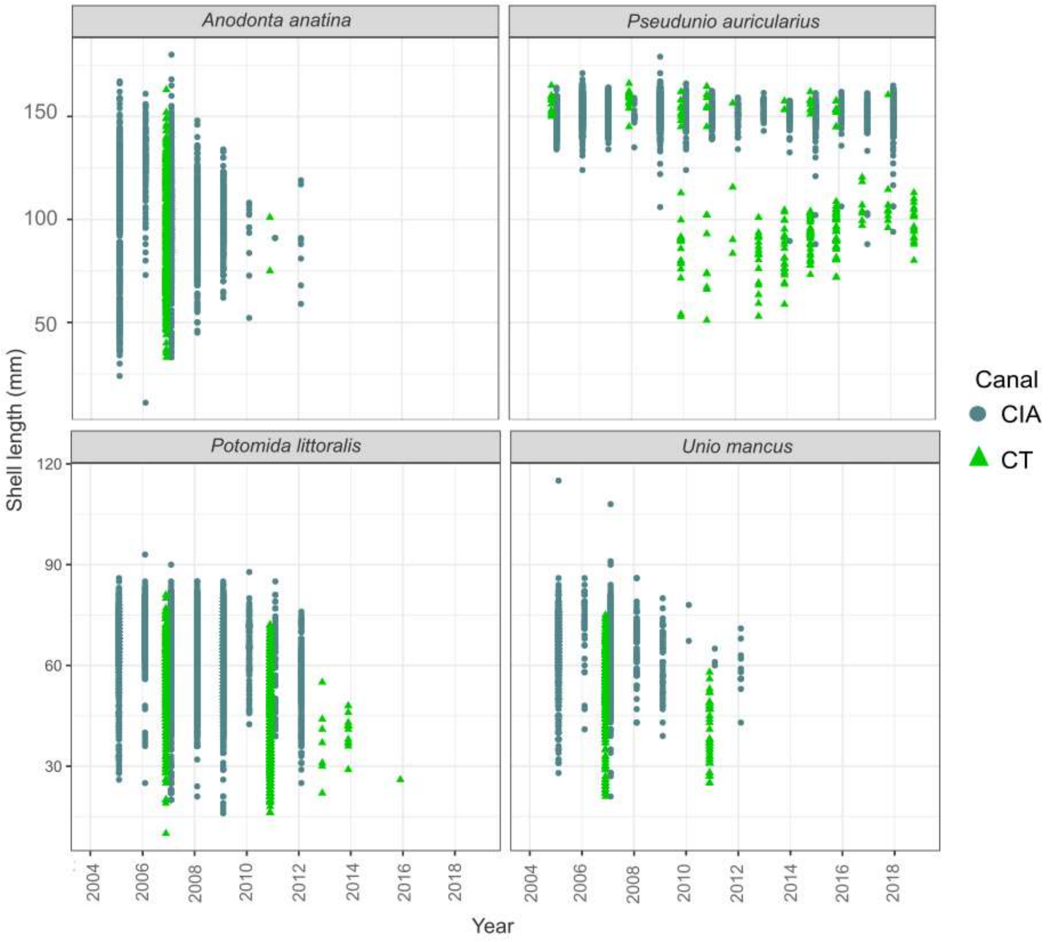

Fig.3.4 Freshwater mussel shell lengths by species in CIA and CT, 2004 – 2019 and the average shell length decreased to 40 mm. The species was considered

In the case of *P. auricularius*, only adult specimens were observed during the first six years of the study (2004-2009) in both canals, with a mean shell length of 152 mm. In 2009, and for the first time in the CIA, a young individual (100 mm long) was registered. However, the average shell size of the population remained around 150 mm and the proportion of young specimens was very low in this canal. More recently (2014-2019), eight specimens between 80 and 100 mm were found in the CIA. In the CT, however, small (young) specimens between 50 and 100 mm began to be recorded as early as 2010, decreasing the average shell length in this canal down to 111 mm in 2012. In 2013, the lowest average value for shell length (80 mm) was recorded, and by 2017 the largest specimens almost completely disappeared from this canal.

3.4.3 Estimated survival of *P. auricularius* from capture-recapture data

We obtained capture-recapture data from 6.133 individuals of *P. auricularius* from both canals: 5901 individuals from the CIA (1998 to 2020) and 232 from the CT (2002 to 2020).

The best survival model obtained for the CIA, according to AIC, included temporal effects (year) on the probabilities of survival, recapture and reporting, but no effects of group of individuals according to size (juvenile or adults) or whether or not they were used temporarily in a captive breeding program. In the CT, the best model also included temporal effects on survival and recapture probabilities, together with effects of group of individuals on reporting probability. These models show a decline in annual survival in both canals through time (Fig. 3.5), although much more pronounced in CIA than in CT. Drops in annual survival were observed in 2004 and 2007 in the CIA, but a year later, in 2005 and 2008, in the CT. Soon after, survival recovered to values close to one in 2010-2012. However, as of 2013, a very pronounced decrease was observed in the CIA, with survival falling down to 0.52 in 2014, 0.43 in 2018 and as low as 0.14 in 2020. On the other hand, survival in CT remained between 0.8-0.9, although several drops were observed following a similar pattern as in CIA, but less pronounced.

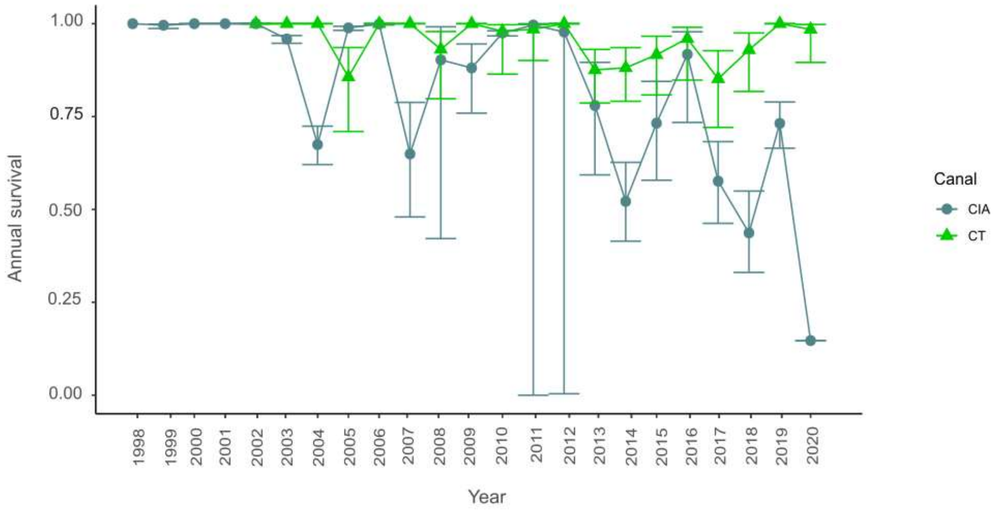

Fig. 3.5 *Pseudunio auricularius* annual survival rate (symbols: estimate; vertical lines: confidence interval) in CIA (1998 - 2020) and CT (2002 - 2020), estimated from capture-recapture data of marked individuals.

3.5. Discussion

3.5.1 Collapse of freshwater mussel populations

Major declines in freshwater mussel populations have been reported worldwide (Böhm et al., 2021; Ferreira-Rodríguez et al., 2019; Haag, 2019b; Lopes-Lima et al., 2014; 2017; 2021a; b). Nevertheless, most of these studies relied on presence/absence data, while quantitative studies based on density declines, such as that of Mouthon & Daufresne (2006) or the present survey, are rare. Yet quantitative data allow estimating interannual and spatial variability in abundance distribution and in that way to put density decline and management responses in a more detailed ecological context. The causes for these mussel declines remain anyway mostly unknown (Downing et al., 2010; Haag, 2019b), and cases such as that of *Margaritifera margaritifera* in Switzerland (Wengström et al., 2019) and Portugal (Sousa et al., 2018; Nogueira et al., 2021), or *Actinonaias pectorosa* in the Clinch River, USA (Leis et al., 2018) show recent examples of mass mortality episodes that caused abrupt decreases in population densities. Our dataset showed an overall mussel density decline of more than 95%, and how the community composition shifted from four species to only one, *P. auricularius*, which now lives in sympatry with two invasive alien species (Asian clam and zebra mussel) (Guerrero et al., 2021, Nakamura et al., 2022).

Mussel densities in the canals varied widely from one section to another despite the general appearance of a homogeneous anthropogenic habitat. In fact, along the canals, there is conspicuous habitat heterogeneity that may partly explain such variation, including different types of substrates or the presence of concrete sections and dam gates modifying water flow and creating lentic areas. Consequently, freshwater mussel distribution is patchy, forming beds where high densities of specimens can be found (Vaughn, 2018). However, in the studied canals that kind of patchy distribution with dense beds was becoming less frequent partly due to translocations induced by the maintenance works, and more recently due to increased mortality events (Nakamura et al., 2022), leaving small groups or solitary specimens remaining across long stretches of the canals. If disturbances are widely spaced in time, the fauna could be able to recover, but if they are continuous and widespread, the probability of survival is drastically reduced and the aquatic biodiversity consequently declines (Moggridge et al., 2014). The CIA presents major maintenance jobs twice a year (February and November), unlike CT, which only has such works once a year (in February) and with smaller works; this

difference may be acting as a key factor in *P. auricularius* differential survival in both canals, allowing it to maintain its density in CT, or even increase it in the last years, compared to CIA.

Gómez & Araujo (2008) considered canals as important refuge ecosystems for freshwater mussels in the Aragón region but, currently, the situation has drastically changed. CIA and CT have become habitats heavily invaded and modified by the Asian clam, together with exotic fish that compete and prey on native fishes, which were the original hosts of freshwater mussels in the area (Nakamura et al., 2022). At present, we are not able to find any living specimen of *P. littoralis*, *U. mancus* and *A. anatina* in the canals, where they are considered extinct (Guerrero et al., 2021; Nakamura et al., 2022). Their disappearance was relatively fast, since Gómez & Araujo (2008) reported overall freshwater mussel densities between 0.02 and 2.38 ind/m^2 in both canals during 2002-2004. These authors reported a mean density around 0.5 ind/m^2 in the CIA for *P. littoralis*, the most abundant species at that time, 0.40 ind/m^2 for *A. anatina* and 0.14 ind/m^2 for *U. mancus*, similar values to our results at the beginning of this study in 2004 (mean densities values: 0.4 ind/m^2, 0.5 ind/m^2 and 0.1 ind/m^2, respectively). In the case of the endangered *P. auricularius*, Gómez & Araujo (2008) reported a mean density of 0.05 and 0.01 ind/m^2 in the CIA and CT respectively for 2002-2004. In our study, although we recorded higher values for *P. auricularius* in 2015 in the CIA (due to the finding of a newly discovered dense patch), we estimated in 2019 a mean density around 5 times lower (0.01 ind/m^2) in this canal.

The mussel species *P. littoralis*, *A. anatina* and *U. mancus* showed a progressive density decline in the canals for a few years prior to their final demise. In these three species, we registered the disappearance of older and younger age groups at the same time, remaining only those of intermediate size, whereas this was not the case for *P. auricularius*. The combined reduction of older and younger individuals may suggest an external cause that triggered that mortality (Haag, 2019b), because in natural population dynamics, and in absence of major disturbances, the death of older specimens is to be expected because of senescence but not so much for younger specimens (Begon et al., 2006).

The population dynamics of *P. auricularius* during the study period differ from those of the other unionids, as it still survives in the canals, although in very low densities. Furthermore, unexpectedly and after more than 14 years of studying *P. auricularius* in

the CT, we found young individuals (5-13 cm long, corresponding to an age of c. 8-25 years; Nakamura et al., 2018b) from 2010 onwards. This finding slightly increased the density in CT (2019 mean density = 0.02 ind/m^2), filling in the juvenile age classes that were not recorded so far and offering some hope for the survival of the population in the mid-term. Despite this, our model found a progressive decline of abundance with time for *P. auricularius*, and our analyses show major drops in survival in the CIA, coinciding with the recorded mass mortality event in the central part of this canal (Guerrero et al., 2021). The appearance of young individuals in CT allowed *P. auricularius* to keep similar density values throughout the years, although many adults were found dead during sampling campaigns, unlike in the CIA where the presence of young individuals was, and still is, very rare.

There are several factors that may explain the significant mussel decline in the canals in recent times, each being able to act individually or in synergy with others, complicating the possible interpretations. The main hypotheses are: habitat degradation, pollution, the presence of unknown diseases, invasion of the Asian clam and/or recruitment failure. Habitat degradation is sometimes evident but, in many cases, it can be relatively slow and cryptic, and therefore not immediately obvious as the main cause of species decline (Wood & Armitage, 1997). Sousa et al. (2021) reviewed the role of anthropogenic habitats as a refuge or ecological trap for freshwater mussels. Some artificial habitats, such as the canals, can provide adequate conditions for the growth and development of native fauna because they have similar characteristics to the natural environment, becoming partial substitutes for the original habitat of the species (Lundholm & Richardson, 2010; Martínez-Abraín & Jiménez, 2015). The studied canals offer stability in terms of the relatively continuous presence of water, unlike the Ebro River, whose flow suffers larger variability and extreme events related to seasonality, and freshwater mussels can be exposed to long droughts or strong winter floods, causing high mortalities (Nakamura et al., 2022). Unfortunately, canals that are actively being used for irrigation or water supply need periodic maintenance. Over the years, the installation of new infrastructures such as lock gates (Gómez & Araujo, 2008) or works such as the replacement of natural earth banks by stone or concrete walls may increase freshwater mussel mortality (Sousa et al., 2021). In fact, the early disappearance of *U. mancus* and *A. anatina* in both canals could be associated with this replacement of earth banks by riprap walls. Since both species were generally found living associated to that

microhabitat, such replacement was possibly highly detrimental for their survival.

Since massive mortality of *P. auricularius* was detected in the CIA in 2013 (Guerrero et al., 2021), numerous sampling campaigns have been carried out by the Aragón Government to determine the possible presence of toxic substances, as well as to investigate the presence of parasites or pathogenic microorganisms in the tissues and shells of recently dead *P. auricularius*, or in living specimens of the Asian clam that shares the same habitat. However, the obtained histological analyses could not demonstrate a major impact of any pathogenic organism on the studied mussel populations (Guerrero et al., 2021; Rico Gómez, 2021). Araujo et al. (2021) indeed reported the presence of viruses, bacteria, and fungi as possible pathogens of *P. auricularius*, and suggested that they may be related to the mass mortality of the species in the CIA, but no conclusive results have been obtained so far.

The two studied canals are surrounded throughout their length by cultivated agriculture fields that make use of their water for irrigation. Runoff from the fields can increase nutrient concentration in the water, as well as drive the incorporation of toxic substances that can cause negative effects on mussels, either by direct toxic effects of these products or indirectly through long-term bioaccumulation (Gillis, 2012). Several toxicants have been detected in samples taken by the regional government, including heavy metals and organic pesticides such as metolachlor or terbuthylazine (Guerrero et al., 2021). The lack of a sound knowledge on the ecotoxicological response of European freshwater mussels does not allow us to conclude that these toxicants might be related to their decline in the canals. In this sense, first results on the sensitivity of *P. auricularius* to heavy metals suggest that it is more sensitive to cadmium and copper, but less sensitive to chromium and lead, compared with other freshwater mussel species (Nakamura et al., 2021). Nevertheless, these tested (or many other non-tested) compounds can produce stress in mussels, influencing their immune systems and making them more easily affected by pathogens. Indeed, the presence of contaminants can induce oxidative stress in bivalves (Deudero et al., 2015; Khazri et al., 2017).

Recently, Haag (2019b) reviewed published information on unionid mortality events reported from 1990 to 2015 in the United States of America (USA), evaluating their common characteristics. This author identified only two factors that could explain these declines: first, some possible disease and, second, the invasion of the Asian clam *Corbicula fluminea*. Regarding diseases in freshwater mussels, they are still poorly studied or unknown. Several works have recently been published on new viruses and parasites that can affect freshwater mussels and on their possible implications in massive died offs (Alfjorden et al., 2021; Araujo et al., 2021, Brian et al., 2021a; b, Chapurina et al., 2020; Goldberg et al., 2019; McElwain, 2019; Richard et al., 2020; Wengström et al., 2019), but their potential detrimental effects for the species found in the Ebro River are mostly unknown. On the other hand, the studied canals were colonized by the Asian clam in 2006-2007 (Guerrero-Campo & Jarne, 2014) and within a decade its density sharply raised up to 15 times the initial values (from 70 ind/m2 to 1000 ind/m2; Gimeno Calvo et al., 2017). This invasion may have negatively affected freshwater mussel recruitment and contributed to the increase of competition with juvenile and adult specimens, affecting their survival (Ferreira-Rodríguez et al., 2018; Haag et al., 2021; Kelley et al., 2022; McDowell & Sousa, 2019). The negative effects of the Asian clam on native mussels may also involve indirect effects such as those derived from the massive death and decomposition of the invasive species. Several authors have reported that sudden mortality events of highly abundant Asian clams can increase the concentration of ammonium in the water due to the decay of soft tissues, affecting the entire aquatic community (Cherry et al., 2005; McDowell et al., 2017; McDowell & Sousa, 2019). The Asian clam has the ability to quickly recover its populations after experiencing massive mortality, since its reproductive capacity is very high (Ilarri et al., 2011; Sousa et al., 2008), this probably being the reason why it has ended up replacing native bivalves in the canals.

Recruitment failure could be another important factor influencing the decline of mussel populations in the canals. Several causes may be involved: 1) Absence of the host fish. The freshwater river blenny (*Salaria fluviatilis* Asso, 1801), a native species identified as the host for *P. auricularius* and *Unio mancus* glochidia (Araujo et al., 2001; 2005; 2009; López & Altaba, 2005), has been rarely detected in the canals during the last decade (Nakamura K., personal observation 2004-2020). On the other hand, non-native species such as wels catfish (*Silurus glanis* Linnaeus, 1758), pike-perch (*Sander lucioperca* Linnaeus, 1758), gambusia (*Gambusia holbrooki* Girard, 1859) and recently black bass (*Micropterus salmoides* Lacépède, 1802) have increased their presence throughout the years (Nakamura et al., 2022; J. Guerrero, personal communication, February 2022) acting negatively on the indigenous fish community. 2) Competition with Asian clam, which can modify and modulate the plankton and microbial communities

(Ilarri et al., 2022; Rong et al., 2021), may reduce mussel reproductive success and even glochidia filtration rates and consequently increase mortality rates (Modesto et al., 2019). 3) Difficulty for successful fertilization due to the scarcity and wide separation of adult individuals (Downing et al., 1993; Mosley, 2012). And, finally 4) the high amount of suspended solids, especially during winter flooding, coincident with the reproductive season of *P. auricularius*, may be interfering with the internal fertilization of freshwater mussels (Gascho et al., 2013). High levels of sedimentation can also affect the benthic juvenile mussels by clogging interstitial spaces and causing low oxygen and pH levels (Geist & Auerswald, 2007) and may also reduce the foraging activity and growth rate of mussels (Österling et al., 2010), negatively affecting their survival. In the canals, internal erosion and sediment deposition may be high, especially after maintenance works. In addition, the constant construction of cofferdams during maintenance works, water runoff from the nearby agricultural fields, and landslides from lateral banks due to the total removal of littoral vegetation, could be also contributing to recruitment failure.

3.5.2 Conservation implications

Long-term studies, necessary to identify temporal trends in long-lived species such as freshwater mussels, allow to better understand their population dynamics and the influence of environment factors, and should therefore be an essential component of sound conservation practice and policy (Moussy et al., 2022). These long-term studies are important to identify seasonal or interannual events that may influence population dynamics, so as to determine the consequences of important threats (Inoue et al., 2014; Sanchez Gonzalez et al., 2021). Our study was key to reveal important events such as the appearance of young specimens of *P. auricularius* after 15 years since the rediscovery of the species in 1996, a sudden mortality event of *P. auricularius* adults in one dense patch in 2013, or the decline and subsequent disappearance of three species of freshwater mussels (*P. littoralis, U. mancus* and *A. anatina*). This information has prompted the modification and adaptation of habitat management by the administrations involved, gradually trying to reconcile mussel survival with the uses of the canals, while trying to alleviate negative impacts (e.g., keeping a minimum water level during maintenance works, ensuring that the stretches do not remain completely dry, careful replacement of substrate in sections with works, reducing removal of riverside trees, and adding water in emergency situations) and reinforce the positive management measures taken so far (e.g.,

notifying maintenance works in advance to, if necessary, translocate the affected mussels, avoid draining all the water in the sections of the canal where it is not necessary, improved communication between stakeholders). Despite this, urgent actions must be taken in the canals, so as in the Ebro River itself, to face this alarming loss of unionid biodiversity. Recovering mussel populations can be effective for the benefit of the whole aquatic ecosystem due to the ecosystem services provided (Eveleens & Febria, 2022; Vaughn, 2018; Zieritz et al., 2022). Habitat restoration actions, such as water and substrate quality improvement (Geist & Hawkins, 2016; Vaughn, 2018), recovery of the fish community (Galbraith et al., 2018), reduction of siltation to permit the juveniles to develop in the interstitial pores of the substrate (Pandolfi et al., 2022), recovery of the riparian forest that creates fish refuges, reducing diffuse pollution and allowing the entry of organic matter from the terrestrial environment (Caskenette et al., 2020), so as improving agricultural practices (Ferreira-Rodríguez et al., 2022), among others, should be considered to allow the recovery of mussel populations. All these actions must be accompanied by environmental policies to ensure that the polluter-pays principle is applied properly, together with effective environmental surveillance (Butchart et al., 2010). Furthermore, consolidating the process of incorporating long-term studies in recovery plans and ensuring continued funding to develop them, would be essential.

Pseudunio auricularius is one of the most threatened freshwater mussels worldwide, and its extinction risk under the current circumstances is very high (Altaba, 1997; Altaba et al., 2000; Araujo & Ramos, 2000; Guerrero et al., 2021; Nakamura et al., 2019; 2022; Prié et al., 2018, Soler et al., 2018; 2019). Its density was and continues to be very low in the Iberian Peninsula, which makes this species highly prone to local extinction. Captive breeding is considered an essential conservation action in the mid and long term to ensure its persistence, so as to reintroduce specimens into the natural environment (Araujo et al., 2003; Nakamura et al., 2019). Other complementary conservation actions are being carried out at present, including the translocation of adult specimens from the CIA to the Ebro River (its natural habitat), resulting in higher survival rates than in the canal (Guerrero et al., 2021; Nakamura et al., 2022), which suggests that the cause of mortality might be related to the canal habitat itself.

3.6 Conclusions

In this work we described a long-term declining trend of freshwater mussels in two canals of the Ebro River, with the eventual disappearance of three out of four species. The only remaining species, the Critically Endangered *P. auricularius* shows also a pattern of increasing mortality rates. Fortunately, one of the two canals, CT, still harbors a population with increasing presence of juveniles, although its density is still very low. Freshwater mussel populations are highly susceptible to a variety of environmental disturbances, making it difficult to attribute their decline or even disappearance to a single cause. Currently, the main threats to the remnant mussel populations in the middle Ebro River and its canals may be acting synergistically, not allowing individual assessment of detrimental factors. Some of the most important are the impact of agricultural activities, the expansion of invasive species, habitat alterations and extreme climatic events such as droughts and floods. The presence of pathogenic agents cannot be ruled out, as well as the influence of toxic substances. Regular assessments of the health status of freshwater mussel populations using e.g., biomarkers, as well as habitat restoration actions, especially those applied at the basin scale, need to be urgently implemented. These actions, in addition to captive breeding and release of juveniles to the natural environment will hopefully increase the chances of recovery of mussel populations in the Ebro River and its connected canals and tributaries.

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Chapter 4

Modelling growth in the critically endangered freshwater mussel *Margaritifera auricularia* (Spengler, 1793) in the Ebro basin

Pseudunio auricularius adult and young specimens

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4.1 Abstract

Here we analyse in detail, for the first time, the growth pattern of the endangered freshwater mussel *Margaritifera auricularia* in the Ebro basin, a life history trait essential for conservation purposes. We combined information on size and age from captive bred juveniles, together with growth annuli from living preadults studied in the field, and empty shells of preadult and adult individuals. We compared the fit of six types of (asymptotic and sigmoid) non-linear growth models and, in terms of residual errors and AIC values, the sigmoid ones were superior, being the Generalized von Bertalanffy and Richards models the best fitted ones. After an initial exponential growth phase in juveniles, growth rate starts to decelerate at an inflection point corresponding to an age of seven years. At an age of about 30 years, the growth rate markedly declines and attains an asymptote at c. 150 mm shell length. Global growth rate is relatively low but comparable to other members of the family, and the maximum age estimated from ring counts was 68 years. We demonstrate that the use of sigmoid models provides more accurate estimation of growth patterns in freshwater mussels, as previously observed for other bivalves.

Keywords Growth models; Mollusca; Unionida; Endangered species

4.2 Introduction

Freshwater mussels (Order Unionida), which include many of the largest freshwater molluscs, present a wide global distribution as a group, but with notably restricted areas, even regional endemics, of their constituent species (Bănărescu, 1990). They may have an important role in freshwater ecosystems in terms of biomass (Vaughn & Hakenkamp, 2001; Araujo & Álvarez-Cobelas, 2016) and function as sentinels of water quality, their absence being usually an indicator of environmental stress (Havlik & Marking, 1987; Mutvei & Westermark, 2001).

During the past century, the impacts experienced worldwide by freshwater ecosystems have triggered a reduction in freshwater mussel populations. As a consequence, unionids are at present one of the most endangered organisms at the global scale (Bogan, 1993; 1998; 2008; Neves et al., 1997; Strayer et al., 2004). Around 70% of freshwater, mussel species in North America and Japan are critically endangered

(William et al., 1993; Vaughn & Taylor, 1999; Negishi & Kayaba, 2010). Similarly, 12 out of 16 species native to Europe are considered endangered or vulnerable (Lopes-Lima et al., 2016).

When evaluating extinction risk and trying to attain the highest efficiency in conservation planning for endangered freshwater mussels, it is necessary to understand their life history traits (San Miguel et al., 2004; Haag & Rypel, 2011; Klunzinger et al., 2014), including information on size, longevity and growth of particular species (Haag, 2009). The study of growth patterns represents one of the key aspects for com- prehending population dynamics processes (Strayer, 2008), and two parameters, i.e. maximum size and longevity, are known to directly relate to reproduction fitness of individuals (Aldridge, 1999).

Growth patterns in freshwater mussels have been relatively well established for many species (Negus, 1966; Neves & Moyer, 1988; Aldridge, 1999; Hastie et al., 2000; Anthony et al., 2001; San Miguel et al., 2004; Outeiro et al., 2008; Akiyama & Iwakuma, 2009; Negishi & Kayaba, 2010; Haag & Rypel, 2011). In this group, so as in other bivalves, the study of size increase with age is based on the presence of concentric rings (annuli) periodically deposited on the external surface of shells through ontogeny. Historically, these rings have been assumed to grow annually, a basic criterion applied to age and growth estimation in bivalves, and validated through different methods in a variety of species (Urban, 2002; Schöne et al., 2004; Helama et al., 2006; Howard & Cuffey, 2006; Haag & Commens-Carson, 2008; Helama & Valovirta, 2008; Rypel et al., 2008; Haag, 2009; Klunzinger et al., 2014). When analysing size–age relationships, different non-linear models have been applied to estimate the growth coefficient (K), the theoretical asymptotic length (L_∞: the predicted mean maximum length for the population) and maximum age (A_{max}) of freshwater mussels and other bivalves (e. g. Urban, 2002; Akiyama & Iwakuma, 2009; Haag & Rypel, 2011). The most widely used model has been based on the von Bertalanffy growth function (VBGF; von Bertalanffy, 1938), even though its use has been criticized by several authors (Colbert et al., 2004; Haag, 2009).

Among members of the family Margaritiferidae, studies on the species *Margaritifera margaritifera* (Linnaeus, 1758) have found a relatively good fit of the asymptotic VBGF curve (Hastie et al., 2000; San Miguel et al., 2004; Outeiro et al., 2008).

However, San Miguel et al. (2004) observed that the growth pattern of *M. margaritifera* was best fitted with a hyperbolic function, for individuals older than 6 years. These authors also found that the L_∞ calculated with the VBGF model underestimated the maximum shell length observed in the field in most populations, contrary to the parameter estimations obtained with the hyperbolic model, which predicted L_∞ values closest to these maximum lengths observed in the field. Other authors have suggested that sigmoidal models, such as the Gompertz function, might also better describe bivalve growth rather than VBGF (Urban, 2002). Indeed, this was the case in the freshwater mussel *M. laevis* (Haas, 1910) from Japan (Akiyama & Iwakuma, 2009). In general, species of the family Margaritiferidae are traditionally considered to have high values of A_{max} and L_∞, with relatively large intraspecific variation among populations. However, they show low K values with reduced variability, as compared to other species of the order Unionida. Consequently, they require long development times (5–13 years) to attain sexual maturity (Haag & Rypel, 2011). The growth of some Iberian populations of *M. margaritifera* shows a fast rate during the first six years, followed by a deceleration, which might indicate deviations in energy allocation from growth to reproduction (San Miguel et al., 2004). According to Bauer (1992), reproductive output in *M. margaritifera* correlates positively to L_∞ and A_{max} and negatively to K.

The growth parameters of freshwater mussels also depend on environmental conditions (Hastie et al., 2000). The observed variability seems to relate to their phenotypic plasticity as a response to habitat stochasticity in freshwater environments (San Miguel et al., 2004). In this framework, European populations of *M. margaritifera* show a positive latitudinal trend in A_{max} and L_∞ (Bauer, 1992; Hastie et al., 2000; San Miguel et al., 2004). Furthermore, variations in growth and size of freshwater mussels have been associated to several factors such as type of substrate (Kesler & Downing, 1997), density of mussels (Bolden & Brown, 2002; Negishi & Kayaba, 2010), availability of food resources (Griffiths & Cyr, 2006), annual water discharge (Rypel et al., 2008) and physical and chemical parameters of the host water (Hinch et al., 1989). Among the last factors, water temperature seems to be one of the most influential on annual growth rates (Parmalee et al., 1980; Bauer, 1992; Schöne et al., 2007; Rypel et al., 2008). In addition, members of the family Margaritiferidae usually present a strong negative relationship between K and A_{max} (Bauer, 1992; Hastie et al., 2000; Haag & Rypel, 2011). This seems

to be a general biological trend, as it has also been observed in a variety of vertebrates, including fish, lizards and mammals (Olsson & Shine, 2002; Metcalfe & Monaghan, 2003; White & Seymour, 2004).

The giant pearl mussel *Margaritifera auricularia* (Spengler, 1793) is one of the most threatened freshwater bivalve species in the world, and it is included in the European Habitats Directive (Appendix IV), so as in the Bern Convention (Appendix II). Furthermore, it is rated by the IUCN Red List as ‘‘critically endangered’’, so as under risk of extinction according to the Spanish National List of Endangered Species. Its abundance and distribution has been reduced during the past decades (Araujo & Moreno, 1999; Araujo & Ramos, 2000), attaining at present a very restricted distribution that includes rivers Adour, Garonne-Dordogne, Charente and Loire in France (Cochet, 2001a, b, 2002; Nienhuis, 2003; Prié et al., 2007; 2008; 2010) and the Ebro River basin in Spain (Altaba, 1990; 1997; Araujo & Ramos, 2000; 2001; Nakamura & Guerrero, 2008; Araujo & Álvarez-Cobelas, 2016). The main factors contributing to its populations decline are habitat destruction, reduction of host fish populations, water pollution, capture for commercial purposes, water abstraction and changes in water flow during reproductive periods (Nakamura & Guerrero, 2008; Araujo & Álvarez-Cobelas, 2016; Lopes-Lima et al., 2016).

Information on growth and development of *M. auricularia* is very scarce (Araujo & Ramos, 1998; Altaba & López, 2001; López et al., 2007). Altaba et al. (2001) estimated a hyperbolic relationship between age and length in *M. auricularia* and suggested that changes in hydrology and water chemistry might drive the growth variability of the species, warning about the high sensitivity of this species to environmental impacts. Nienhuis (2003), working in French rivers, estimated an age of about 43 years for the largest individual collected of *M. auricularia*. Recently, Araujo & Álvarez-Cobelas (2016) estimated an age older than 50 years for shells of about 150 mm long. However, the scarce information available, so as the high extinction risk for this species, claim for a need of deeper knowledge on its growth traits in order to allow more effective conservation strategies. The present survey aims at analysing, for the first time, the complete growth pattern of *M. auricularia*, ranging from juveniles just released from their host fish to adulthood. To this end, we use a combination of information gathered from shell rings and experimental data on juvenile development for evaluating the

statistical fit of asymptotic and sigmoid non-linear models previously proposed for describing bivalve growth.

4.3 Materials and methods

The dataset including size and age of *M. auricularia* used for this study originates from three different sources: (1) empty shells of dead juvenile (preadult) and adult individuals collected in the field; (2) shells of living juvenile (preadult) individuals measured in the field; and (3) the smallest living juvenile individuals reared by captive breeding in the laboratory (Table 1). In this study, we considered juvenile preadult individuals those with a shell length around 100 mm or less; larger individuals were considered adults. This approximate threshold was based on tests in the laboratory, where living individuals larger than 100 mm were observed to be sexually mature and release viable glochidia during the reproductive sea-son. This reproductive output was, however, not observed in individuals 80–90 mm long.

4.3.1 Field data

Most of the field information gathered for this survey come from empty shells of dead individuals found between 2012 and 2015 in the sediment of three sites in the Ebro river basin (Aragón region, Spain) (Fig. 4.1): (1) Canal Imperial de Aragón(CIA) (108 km long and 30 m^3/s mean water discharge); (2) Canal de Tauste (44 km and 12.5 m^3/s), both originating upstream of Zaragoza and (3) Quinto irrigation ditch (21 km; 2 m^3/s), which originates at Pina dam, downstream from the city of Zaragoza (see Gómez & Araujo, 2008).

Sampling campaigns were set up when the water input to these canals, and ditches was closed for maintenance works (February and November for CIA, February in the case of C. Tauste & Quinto), when the water level was very shallow (<50 cm deep), allowing easy access to the bed of the canals, and with the aim of translocating living individuals of freshwater mussels *M. auricularia*, *Potomida littoralis* (Cuvier, 1798), *Anodonta anatina* (Linnaeus, 1758) and *Unio mancus* (Lamarck, 1819) to other areas of the same canal to avoid major damage to their populations by the maintenance works done with

heavy machinery. During these campaigns, the shells of dead individuals of *M. auricularia* were collected for further analyses. Both living and dead individuals of freshwater mussels were located by sight and using a viewing glass when water transparency allowed it, or by palpating the substrate with the hands when the water was too turbid.

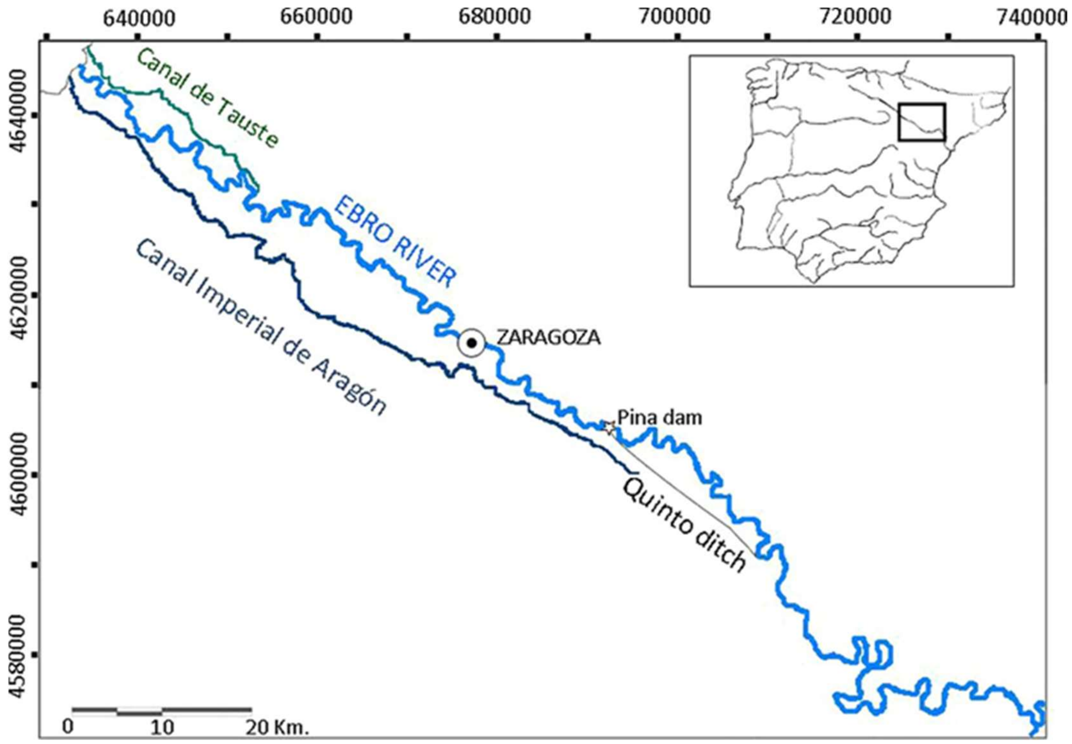

Fig. 4.1 Map of the study area in the Ebro Basin (Eastern Iberian Peninsula), with indication of the three canals where samples of *Margaritifera auricularia* were obtained.

4.3.2 Laboratory experiments

During 2014, 60 Siberian sturgeons (*Acipenser baeri*) were experimentally infested with glochidia of *M. auricularia* following Araujo et al. (2003). The juveniles later released from the host fish were used to establish a captive breeding culture using plastic boxes with detritus, as in Eybe et al. (2013). After several trials with different protocols (to be discussed elsewhere), the best conditions for juvenile growth and survival were established. These consisted of the presence of siliceous substrate, filtered river water, no air bubbling, and added detritus and commercial phytoplankton (Shellfish diet 1800® *Nannochloropsis* sp.). These cultures were checked weekly, and the three largest observed juveniles were measured from each replica available (see Table 1 for number of replicas). Measurements (in µm) were obtained using a digital camera attached to a binocular microscope (Motic SMZ168), by means of the soft- ware Motic Images Plus 2.0.

4.3.3 Counting and measuring shell growth rings

Empty shells of *M. auricularia* collected in the field were processed using the methods suggested by previous authors (Bauer, 1992; San Miguel et al., 2004; Zotin, 2015). First, the external organic layer of the shells (periostracum) was removed using a 5% KOH solution at 60-70°C. Submersion time depended on shell thickness (thicker in *M. auricularia* than in *M. margaritifera*) and size, and varied between 20 min for young stages and 60 min for the largest adult shells. Then, annual rings were identified and counted, being distinguished from non-annual rings following Haag & Commens-Carson (2008) and San Miguel et al. (2004). The fake rings can be distinguished because they are discontinuous and thinner (Neves & Moyer, 1988; Akiyama & Iwakuma, 2009) than annual rings. The total number of annuli per shell was counted by two observers under a stereomicroscope. The maximum length of each ring (i.e. the length of the maximum ring diameter) was measured using a manual calliper (±0.05 mm). The annuli of a set of 11 living juvenile (preadult) individuals were measured and counted in the field using portable lenses. In these small individuals, the shell is very well preserved, so that the umbo and growth rings can be clearly seen. Their first visible ring measured a maximum length of about 5–7 mm, suggesting that it corresponded to their second year, as estimated from size data of younger juveniles reared in the lab. This information was further used to calibrate the corresponding age of the first ring observed in adults with eroded annuli in their umbo.

4.3.4 Data analysis: age calibration

The beginning of the juvenile period was considered as its release from the host fish and established as age 0. The age of the first annulus was estimated from individuals reared in the laboratory experiments (i.e. at approx. 3 mm of length). Considering that juveniles are released approximately in May, the first ring appears during the next winter

(December–February), so that the time interval since detachment to the formation of the first annulus can be established at approximately 245 days (i.e. from May to January). The length of the first visible growth ring of living juveniles (preadults) measured in the field was c. 5–7 mm. According to our data on reared individuals, this annulus corresponds to the second winter growth arrest (age of 245 days plus 1 year). The corresponding ages of the following rings of field individuals were calibrated based on their position relative to this first ring, and assuming that all of them had an annual frequency of appearance. Then, for instance, an annulus at position 4 has an age of 245 days plus 4 years. In adult individuals of *M. auricularia*, as in other freshwater mussels, the umbo area is usually eroded, so that the age of the first observed ring (and consequently all the rings) cannot be directly determined (Bauer, 1992; San Miguel et al., 2004; Akiyama & Iwakuma, 2009). To solve this issue, we used information related to the first annuli of young individuals (4 shells and 11 living juveniles measured in the field), which do not present erosion of the rings at the umbo area. In this way, we could predict the age of the first ring of adults with eroded umbo through a second-order polynomial regression of size versus ring number fitted with the data from juvenile shells.

4.3.5 Data analysis: growth models

In order to find the best fitted pattern of size vs. age in *M. auricularia*, we analysed the relationship between these two variables using the following six models in non-linear regression analysis:

Generalized von Bertalanffy growth function (G-VBGF): $L_t = L_\infty\left(1 - e^{-K(t-t_0)}\right)^D$

Richards (Rich): $L_t = L_\infty\left[1 - De^{[-K(t-t_i)]}\right]^{1/D}$

Gompertz (Gomp): $L_t = L_\infty e^{e^{[-K(t-t_0)]}}$

Logistic (Logis): $L_t = \frac{L_\infty}{1+e^{-K(t-t_0)}}$

Special von Bertalanffy (S-VBGF): $L_t = L_\infty\left(1 - e^{-K(t-t_0)}\right)$

Hyperbolic (Hyper): $L_t = \frac{L_\infty K(t-t_0)}{1+K(t-t_0)}$

where the growth parameters are: L_∞ = the predicted mean maximum length (mm) for the population (asymptotic length); K and $D_{Richards}$ = growth coefficients (y^{-1}); $D_{G\text{-}VBGF}$= surface factor; t =age (years); t_0 = theoretical age at zero length; and t_i = age at the inflection point (years).

It must be noticed that the classical von Bertalanffy growth model, widely used in previous studies with freshwater mussels, is here referred to as the ‘‘Special’’ Bertalanffy growth model, following Urban (2002), as opposed to the ‘‘Generalized’’ von Bertalanffy model, which includes an added parameter D, or surface factor.

The 95% confidence intervals (95% CI) for the parameters of each model were estimated by bootstrapping with 100,000 permutations. The criteria used to compare the tested models included the second- order Akaike information criteria (AICc) and the graphical analysis of Pearson residuals. Finally, the best model was then fitted separately to each population to check for inter-population differences in the parameter estimates, based on the overlap of the 95% CI obtained from bootstrapping (i.e. no overlap between 95% CI indicates significant differences). All these non-linear regression analyses were carried out in R v. 3.2.4 (R Core Team, 2016).

4.4 Results

4.4.1 Summary of mussel shell data

Shell length and estimated age of *M. auricularia* individuals used to build growth models are shown in Table 4.1. In the case of captive brood juveniles, we obtained 5-6 weekly measurements from different cultures, up to a maximum of 78 weeks, depending on available batches. At time 0, juveniles released from their host fish measured 0.199 mm of median length, with little variation among individuals. At an age of 60 weeks, they had

already attained a median length of 3.23 mm, with an interquartile variation of 2.9 - 3.5 mm.

A total of 72 shells from three populations were used to measure and counting all their visible (i.e. not eroded) annuli (Table 4.1): 34 from CIA, 19 from C. Tauste and 19 from Quinto. The median shell length of pre-adults was 93 mm, corresponding to ages from 11 to 14 years. Adult's shells, with an estimated age of 38-50 years measured in most cases between 144 and 153 mm.

Table 4.1 Summary of the shell size and age for the three datasets (i.e. lab juveniles, pre-adults and adults) used to fit the growth models for the freshwater mussel *Margaritifera auricularia*

Stage		***N***	***L* (mm)**			***t* (y)**		
			Median	**Q1**	**Q3**	**Median**	**Q1**	**Q3**
Lab Juv	**Week 0**	34	0.199	0.194	0.204	0	-	-
	Week 12	5	0.470	0.466	0.505	0.231	-	-
	Week 40	6	1.802	1.510	2.472	0.769	-	-
	Week 60	5	3.233	2.850	3.494	1.154	-	-
Pre-ads		15	93.00	84.90	102.00	13.67	12.67	15.17
Adults		57	150.80	144.10	153.60	44.67	39.67	51.67

Lab Juv captive breeding juveniles monitored in cultures under lab conditions, *Pre-ads* shells with young sizes (with no erosion at the umbo), collected from field sampling (both living individuals measured in situ and empty shells collected from the sediment), *Adults* individuals of adult sizes, *N* number of monitored cultures for Lab Juv (see methods section), except for week 0, where N indicates the number of measured individuals; number of sampled individuals for pre-ads and adults, for which annuli size and number were measured, *L* mussel shell length (after KOH treatment in pre-ads and adults), *t* mussel age (according to calibrated number of rings preserved after KOH treatment in pre-ads and adults), *Q1 and Q3* first and third quartiles.

4.4.2 Growth models

The estimated growth coefficient (K) was below 0.3 mm year^{-1} in all the models (Table 4.2) and particularly small in S-VBGF and Hyper (< 0.1 mm year^{-1}). Sigmoidal models, i.e. G-VBGF, Rich, Gomp and Logis, showed a better fit than the asymptotic ones, i.e. S-VBGF and Hyper (Table 4.2; Fig. 4.2). The inflection point in the sigmoidal growth curve, as indicated by Rich, corresponded to an approximate age (t_i) of 7 years. The best fit, in terms of residuals and AICc, corresponded to G-VBGF and Rich, both with an equivalent fitted curve and with a maximum estimated size (L_∞) of 149 mm. Models G-VBGF and Rich (Table 4.2; Fig. 4.2 a-d) did not show any clear pattern in the distribution of residuals. Compared to the best models, Gomp and Logis showed a slight trend to overestimate sizes of juveniles and underestimate those of old individuals, which was more marked in Logis (Fig. 4.2 e-h; $L_\infty = 146$ mm for Gomp and 139 mm for Logis). Finally, an opposite and stronger pattern was observed in S-VBGF and Hyper, underpredicting juvenile sizes and overpredicting old age lengths (Fig. 4.2 i-l; $L\infty = 174$ mm for S-VBGF and 255 mm for Hyper). The anomalous pattern in the residuals was even more pronounced in Hyper than S-VBGF. Both asymptotic models showed the worse fit to the growth data of *M. auricularia*.

The first part of the sigmoid curve in the best two models (G-VBGF and Rich) showed an exponential growth in the initial juvenile stages, up to the inflection point at an age of c. 7 years (according to the Rich model). After this point, growth decelerates steadily until attaining an age of about 30 years, when there is a drastic reduction of growth rate approaching an asymptote, corresponding to a maximum estimated size of nearly 150 mm and no further growth in shell length.

Based on the G-VBGF model and the 95% CI overlap criterion, we found some differences in the growth coefficient (K) among populations (particularly higher values in Quinto; Table 4.3, Fig. 4.3). No further differences among populations were observed in the rest of estimated parameters (i.e. L_∞, t_0 and D), shown by the overlap of the 95% CI between all the locality pairs.

0o**Table 4.2** Summary of the growth models relating shell size with age in the freshwater mussel *Margaritifera auricularia*. The models are sorted from best to worst based on the second-order Akaike Information Criterion (AICc).

Model	**Parameters**												**RSE**	R^2	**AICc**
	L_∞			K			$a / b / t_0 / t_i$			D					
	Est	95% BCI		Est	95% BCI		Est	95% BCI		Est	95% BCI				
		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper			
G-VBGF	148.76	146.41	150.58	0.150	0.144	0.163	-0.338	-2.648	-0.062	3.144	2.884	5.399	9.843	0.957	9878
Rich	148.76	146.43	150.58	0.150	0.144	0.163	7.305	7.150	7.802	0.318	0.185	0.348	9.843	0.957	9878
Gomp	145.71	143.99	147.45	0.179	0.174	0.185	4.474	4.281	4.686	-	-	-	9.933	0.956	9901
Logis	139.16	137.64	140.72	0.284	0.275	0.293	10.285	10.149	10.422	-	-	-	10.846	0.948	10136
S-VBGF	174.40	170.31	178.61	0.064	0.061	0.067	1.582	1.444	1.715	-	-	-	12.826	0.927	10582
Hyper	254.65	245.59	264.10	0.046	0.043	0.050	1.466	1.319	1.606	-	-	-	14.038	0.913	10823

G-VBGF Generalized von Bertalanffy function, *Rich* Richards model, *Gomp* Gompertz model, *Logis* Logistic model, *S-VBGF* Special von Bertalanffy function, *Hyper* Hyperbolic model, L_∞ asymptotic length (mm), *K and* D_{Rich} growth coefficients (y^{-1}), t_0 theoretical age at zero length (G-VBGF, S-VBGF and Hyper models), *a* constant (y) (Gomp model), *b* constant (y) (Logis model), *D* surface factor (G-VBGF model), t_i age at the inflection point (y) (Rich model), *Est* estimate 95%, *BCI* 95% bootstrap confidence interval obtained from 100,000 iterations, *RSE* residual standard error, R^2 coefficient of determination, *AICc* second-order Akaike Information Criterion.

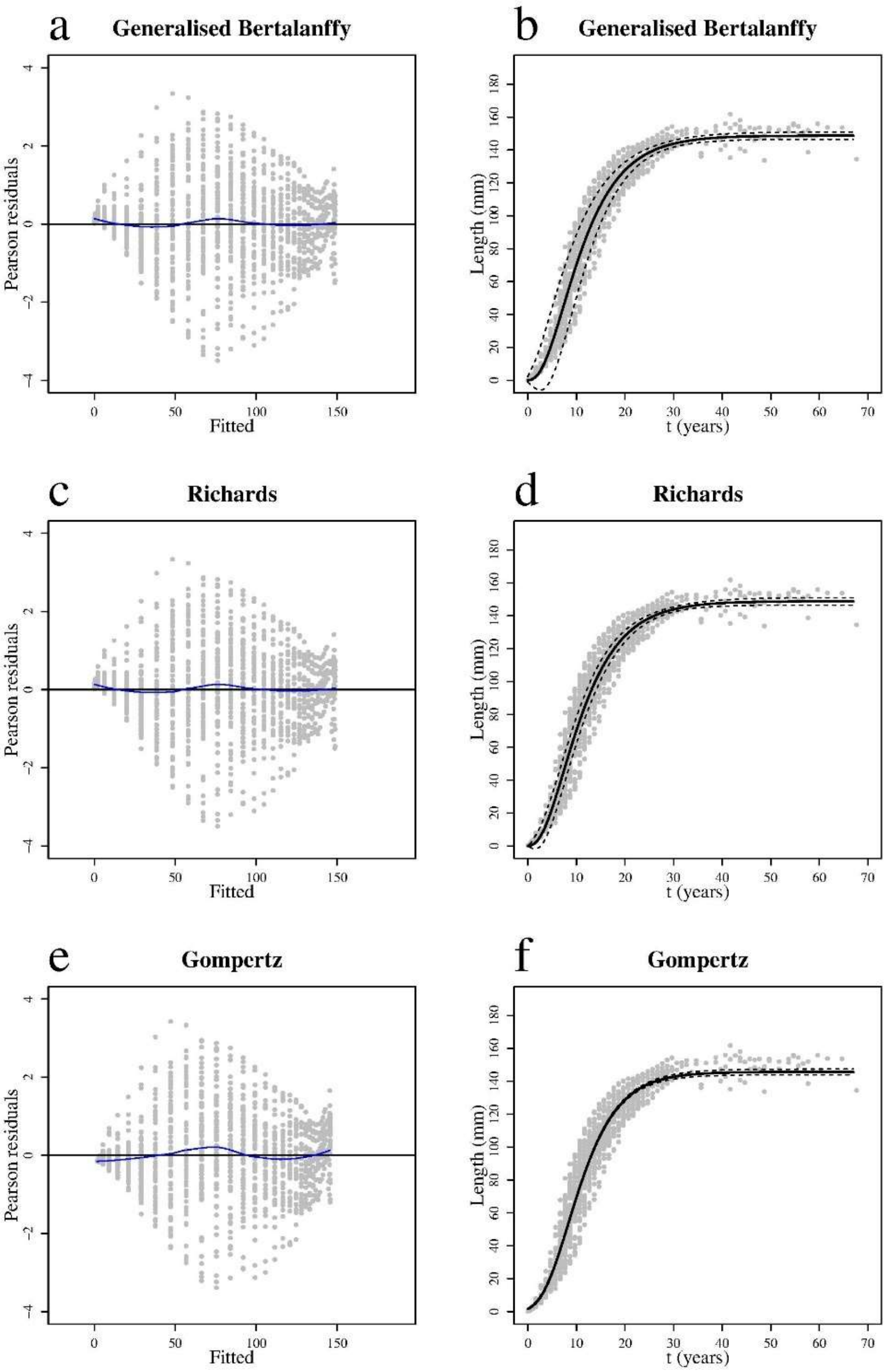

Fig. 4.2a-f Residuals vs. fitted values (a, c, e) and fitted curves (b, d, f) of the growth models for assessing shell growth of the freshwater mussel *Margaritifera auricularia*. The residual trends are represented by a Loess curve (in blue) with a span parameter of 0.75. Black solid lines are fitted curves and dashed lines are 95% confident intervals of fitted curves based on a first-/second-order Taylor expansion approach. The models are sorted from best to worst based on the second-order Akaike Information Criterion.

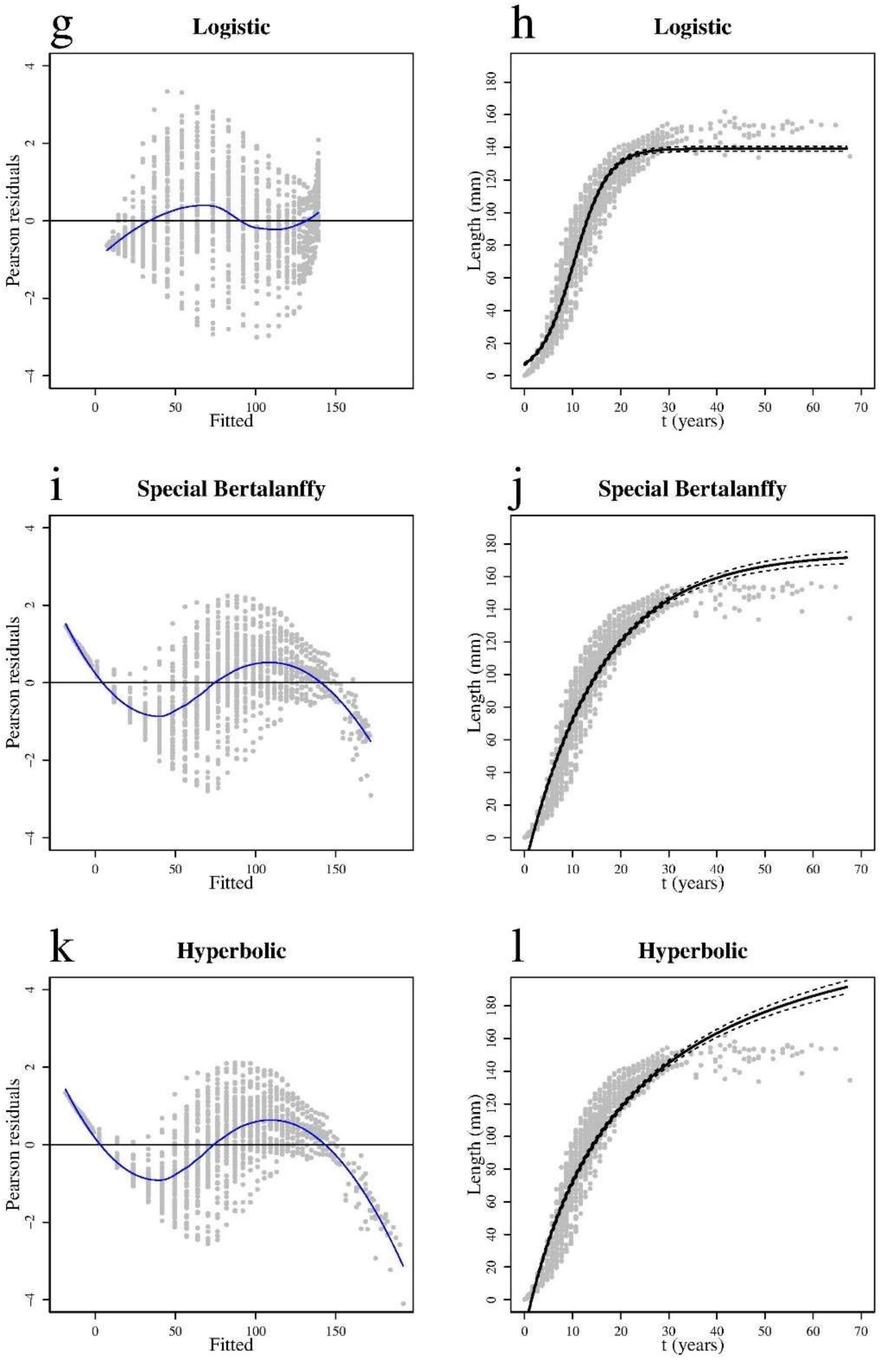

Fig. 4.2 g-l Continued: Residuals vs. fitted values (g, I, k) and fitted curves (h, j, l) of the growth models for assessing shell growth of the freshwater mussel *Margaritifera auricularia*.

Table 4.3 Summary of the growth models for the three studied populations of the freshwater mussel *Margaritifera auricularia* from the Eastern Iberian Peninsula. The assessment is based on model G-VBGF.

	Parameters											
Locality	***L_∞***			***K***			***t_0***			***D***		
	Est	95% BCI		Est	95% BCI		Est	95% BCI		Est	95% BCI	
		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper
C. Imperial	145.81	141.83	149.07	0.136	0.127	0.158	1.192	-1.858	1.571	2.113	1.858	4.391
C. Tauste	153.61	148.78	158.78	0.116	0.101	0.135	0.523	-1.663	1.354	1.983	1.572	3.313
Quinto	148.91	145.82	151.81	0.173	0.158	0.197	0.171	-5.248	1.272	3.405	2.463	12.805

L_∞ asymptotic length (mm), *K* growth coefficient (y^{-1}), *t_0* theoretical age at zero length (y), *D* surface factor, *Est* estimate 95%, *BCI* 95% bootstrap confidence interval obtained from 100,000 iteration

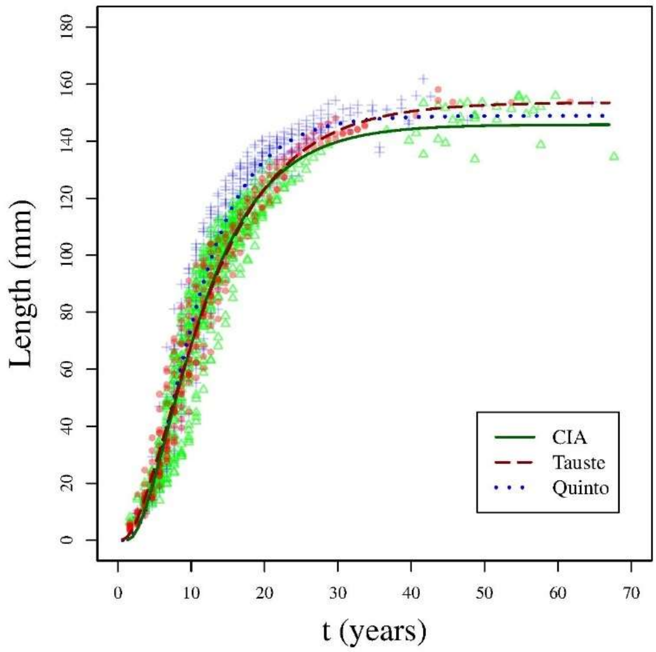

Fig. 4.3 Fitted curves of the Generalized Bertalanffy growth model for the three populations of freshwater mussel *Margaritifera auricularia* from the Eastern Iberian Peninsula. CIA (green line and triangles) = Canal Imperial de Aragón; Quinto (blue dotted line and crosses) = Acequia de Quinto; Tauste (red dashed line and dots) = Canal de Tauste.

4.5 Discussion

4.5.1 Obtaining growth data

The accuracy of growth models notably depends on the range of ages used (Haag, 2009). The present study highlights the importance of having a good representativeness of the whole age range in the study dataset for investigating growth in freshwater mussels. The combination of juvenile experimental data and information from adults collected in the field allows attaining a higher accuracy in growth model fit. In particular, the incorporation of size and age data from juvenile individuals is crucial to soundly establish the most appropriate growth model to understand the life history of bivalve molluscs (Urban, 2002). At present, though, it is very difficult to obtain information of juveniles of *M. auricularia* living in the natural environment, not only because of their small size but also due to the low-field recruitment rates for this endangered species. As we demonstrated, under such circumstances, experimental monitoring of juveniles appears as a successful approach providing this critical information.

On the other hand, the difficulty to obtain solid information on growth of freshwater mussels originates also from methodological issues associated to age estimation of individuals and annuli (Haag & Rypel, 2011). One of the most accepted methods for age estimation of freshwater mussels consists in counting the number of shell growth rings. In this framework, Neves & Moyer, 1988) compare direct counting (i.e. without further shell treatment) of external annuli with the observation of annuli after obtaining fine shell sections, and they found that the first method underestimated the age of species *Pleurobema oviforme* (Conrad, 1834) and *Fusconaia cor* (Conrad, 1834) with an error of 1–5 years. The method used in the present survey, i.e. 5% KOH shell submersion prior to counting its number of annuli, has been previously applied successfully by several authors to species of the family Margaritiferidae, such as *M. margaritifera* (Bauer, 1992; San Miguel et al., 2004; Zotin, 2015), allowing a clear observation and distinction of all type of rings (either true or false), both in juveniles and adults, what is supported in our work with the species *M. auricularia*.

The Margaritiferidae stands among freshwater mussels as one of the families accounting for the oldest maximum ages, attaining in *M. margaritifera* up to 280 years in arctic populations (Mutvei & Westermark, 2001; Lopes-Lima et al., 2016). Hastie et al.

(2000) reports a maximum age interval of 48-123 years for Scottish populations. In the Iberian Peninsula, San Miguel et al. (2004) estimated ages ranging between 35 and 65 years for the same species, and Outeiro et al. (2008) concluded that some individuals could attain more than 60 years. In Japan, *M. laevis* is calculated to attain 21-55 years (Akiyama & Iwakuma, 2009); *M. falcata* lives up to 52 years in British Columbia (Schöne et al., 2007), while *M. laoensis* individuals do not seem to live more than 12 years in Laos (Bolotov et al., 2014). With regard to our focus species *M. auricularia*, Nienhuis (2003) found an average age of 29 years in river Charente, with shells of about 110-120 mm. Comparing those individuals with others in the Ebro river, he concluded that these may be as old as about 60 years, what is in agreement with the maximum age found in the present survey (68 years), and comparable to other species of the family living in similar latitudes (San Miguel et al., 2004). The maximum age of *M. auricularia* estimated by Altaba et al. (2001) using a hyperbolic model threw a value of 159 years for a shell 164 mm long, which is probably an overestimation according to our data. Furthermore, according to our results, the plateau in shell size attained after an age of c. 30 years makes it impossible to calculate any accurate age value for individuals larger than 140-150 mm, as all of them measure approximately the same, independently of their age. It is then necessary to calculate age using an independent method, such as growth ring counts, to estimate an approximate age of large individuals of *M. auricularia*; size is not enough.

Our data about sizes of initial rings of shells in wild populations of *M. auricularia* agree with other surveys focused on this species. Nienhuis (2003) reported that, in *M. auricularia* populations from rivers Loire and Charente, the first growth rings that could be distinguished in the shells measured about 10–14 mm. These values are within the wider range of lengths for initial visible rings of empty shells with eroded umbos from adult individuals in our study (median length of 14.8 mm; interquartile variation of 10.4-19.0 mm). We could see smaller annuli, of c. 5-7 mm, in living juveniles studied in the field. Our data on captive juvenile growth show that the first annulus forms at a size of 3 mm, and the first growth rings detected in wild individuals of young sizes measured in situ corresponded to the second winter growth arrest, so that they seem to lose the ring of the first winter. Therefore, the first annuli reported by Nienhuis (2003) correspond probably to the third to fifth growth ring according to our data. Furthermore, Altaba &

López (2001) found a juvenile shell of *M. auricularia* with a shell size of 5.74 mm and suggested that it might have an age of two years according to the observed rings, in agreement with our findings.

4.5.2 Comparison of growth models

Two main types of mathematical models have been previously proposed to study growth in bivalves: asymptotic and sigmoid. When comparing different possibilities, Urban (2002) found that sigmoid models are most appropriate, and we hereby confirm his findings. The sigmoid curve accurately reflects the development of *M. auricularia* along all the life cycle since detaching from their host fish; after the formation of the first winter annulus at an approximate shell size of 3 mm, they show an exponential growth in size with age, up to an inflection point estimated at an age of 7 years and approximate size of 30 mm. From this age on, growth rate decelerates until a drastic decline in growth at about 140 mm shell length. Then size attains an asymptote close to 150 mm, and no further growth is observed at ages older than about 30 years.

In spite of this pattern, an asymptotic model, i.e. the classic model of von Bertalanffy (S-VBGF), has been recently used commonly to describe growth in various species of freshwater mussels, including members of the family Margaritiferidae, although not always with sound results. Hastie et al. (2000) and San Miguel et al. (2004) state that this model provides an adequate fit to growth data of *M. margaritifera* in Scotland and Spain, respectively, but it must be noticed that their starting ages for the analysis were between 5 and 6 years approximately. Akiyama & Iwakuma (2009) suggest that the S-VBGF does not fit adequately to the growth of *M. laevis*, but the sigmoid type Gomp does. Our results with *M. auricularia* show that the classic S-VBGF does not show a good fit. This asymptotic model does not have an inflection point, so it is perhaps more adequately used to describe growth after the exponential initial growth of younger animals (Ricker 1975, in Akiyama & Iwakuma, 2009) but not appropriate for the whole life cycle (but further problems on largest size estimation and growth rate may arise with this model; see below).

When comparing the growth coefficient (K) of *M. auricularia* with that of other species (using the commonly applied S-VBGF for comparative purposes, $K = 0.064$ mm year^{-1} in our case), we observe that it is similar to other species, but amongst the lowest values reported for the family Margaritiferidae. The species *M. laevis* has a $K = 0.15$ (Akiyama & Iwakuma, 2009); the highest K value observed for *M. margaritifera* in the Iberian Peninsula varies between 0.10 and 0.13 mm year^{-1} (San Miguel et al., 2004), but the K values of Scottish populations are smaller (0.023-0.098; Hastie et al., 2000), in line with our results for *M. auricularia*. Bauer (1992) observed a negative relationship of K with the concentration of calcium in the water. The freshwater mussel *M. auricularia* lives in hard waters in the Ebro basin (Ca concentration of about 145 mg l^{-1}; Araujo, 2006), whereas *M. margaritifera* is mainly found in waters with low ionic concentrations. This might in part explain a reduced K in *M. auricularia* compared to the western Iberian populations of *M. margaritifera*, which live in waters with lower calcium concentration, although the different taxa may respond differently to this factor. Besides these issues, our survey, along the lines of Urban (2002), corroborates that the special von Bertalanffy is not the best model for comparisons of growth parameters, because of lack of adequate sigmoidal fit. First, if we consider the best models fitted for *M. auricularia* (G-VBGF and Rich), their K parameter shows higher values (0.15 mm year^{-1}), twice the value estimated by the S-VBGF, which most probably underestimates general growth rates in these freshwater mussels. Second, the S-VBGF produces an overestimation of 17% for $L\infty$, which increases up to 71% when using the hyperbolic model, as compared with the best fitted models of Rich and G-VBGF. Third, it is probably not adequate to compare growth parameters, even using the same S-VBGF model, among groups of samples when some of them do not include small size juveniles, because the parameters of these types of models can be notably modified depending on such extreme values. For these reasons, we recommend the use of sigmoidal models in future studies on growth of freshwater mussels, such as G-VBGF and Rich. In addition, other potential models might be equally powerful or even superior, as demonstrated in fishes using biphasic models (Minte-Vera et al., 2016), although some previous information on reproductive maturation might be needed, and this is scarce at the moment for *M. auricularia*, as discussed below.

After showing the superiority of sigmoidal models over asymptotic ones for obtaining a more realistic description of growth patterns in freshwater mussels, a new fundamental question arises: Which is the biological meaning of the inflection point at the age represented by the t_i parameter of the Rich model? The change in growth rate trend from increasing to decreasing could be a signal of (1) other biological processes beginning to acquire importance, or (2) a change in habitat use or behaviour occurring at that point. From the point of view of the first hypothesis, growth models provide essential information to understand the life histories of organisms in relation to the ecological strategies for managing energetic resources, such as the trade-off between growth and other processes such as reproduction (Roff, 2002; Minte-Vera et al., 2016). The reproductive yield of freshwater mussels is known to relate positively to maximum shell size (Bauer, 1992), in which *M. auricularia* in the Ebro basin varies between 144 and 153 mm, corresponding to mean ages between 38 and 50 years. Along the life cycle of freshwater mussels, a deceleration of growth is commonly associated to changes in energy allocation, and in the case of *M. margaritifera*, it seems to indicate the beginning of the reproductive period (San Miguel et al., 2004). Haag & Rypel (2011) state that species of the family Margaritiferidae require between 5 and 13 years to attain sexual maturity, a long period compared to other unionid molluscs. Nienhuis (2003) suggests that *M. auricularia* starts to reproduce at an age of about 18-22 years, corresponding according to his estimation to a shell size between 94 and 100 mm. We observed animals in the laboratory reproducing and liberating glochidia at a size of 100 mm (unpublished data) and an estimated age of 13-14 years. Therefore, it remains to be tested whether the threshold age of 7 years related to the inflection point in the sigmoid curve might be related, as suggested for *M. margaritifera*, with the starting of physiological and histological changes to achieve a mature reproductive state and if this is affecting the shift in energy allocation from growth to reproduction. As alternative hypothesis, it is known that in *M. margaritifera*, when juveniles are released from their host, they get buried into the sediment substrate, living there in between interstitial spaces for about 5 years (Hruska, 1999). In the case of *M. auricularia*, Nienhuis (2003) considers it may follow a similar behaviour. If this is correct, the inflection point in growth detected in our models might actually be related to the change in habitat use and behaviour, when after 5 years buried, the juveniles exit to live on the substrate surface, consequently adjusting their life style from an interstitial to a benthic form, with the expected changes in feeding behaviour, response to water flow, oxygen and temperature variations, etc., so as following the developmental trajectory to a mature individual.

4.5.3 Differences in growth parameters between populations

Growth parameters of freshwater mussels are sensitive to environmental pressures, as evidenced, for example, by the positive latitudinal pattern of L_∞ and the negative pattern of K observed in *M. margaritifera*. Regarding the growth parameter L_∞ of our study species, *M. auricularia*, Prié et al. (2008) reported a maximum shell size of 145 mm individuals in the river Charente. The same authors noticed that the northern most populations in the same river were smaller than those from rivers Vienne-Creuse, Oise and Ebro, being the latest the largest ones, in contrast to the expected latitudinal trend in shell size observed for *M. margaritifera*. Probably other factors (natural or perhaps anthropogenic), besides latitude-related ones such as temperature, might be modulating these size patterns in *M. auricularia*. Our results based on the comparison of the best fit models (G-VBGF and Richards) among sites show that the value of L_∞ obtained for each of the three studied populations is very similar (146, 153 and 149 mm), and not significantly different according to bootstrap confidence intervals, corresponding to field data of an average size of adult shells of 150 mm. Therefore, we can consider that adult individuals observed in the field have attained maximum size. Conversely, in other species, largest differences have been found among populations located in different rivers, for instance in *M. margaritifera* (Bauer, 1992; Hastie et al., 2000; Ziuganov et al., 2000; San Miguel et al., 2004) and in *M. laevis* (Akiyama & Iwakuma, 2009).

With regard to growth coefficient, however, we found some differences among populations of *M. auricularia* in their K values, particularly in the Quinto ditch compared to the two other sites. This could be explained by environmental effects on growth. Previous studies have identified temperature, pollutants and food as major controls of shell growth in bivalves (Bauer, 1992; Schöne et al., 2004, 2007, Dunca et al., 2005). Actually, it is known that high water temperature triggers faster growth rates (Bauer, 1992; Hastie et al., 2000; Hochwald, 2001; San Miguel et al., 2004; Negishi & Kayaba, 2010). In populations of *M. margaritifera* from northwest Russia, the individuals with the highest growth rates were those that lived in warmer waters (Semenova et al., 1992). Negus (1966) also observed a positive relationship between intra-annual growth and water temperature in *Anodonta anatina*, and Mutvei & Westermark (2001) found a similar trend between summer air temperature and growth in a population of *M. margaritifera* in southern Sweden. In the present survey, the Quinto ditch has a smaller sized compared to CIA and Canal de Tauste, and even no large differences in temperature must exist among sites in such a small, flat region; we might expect that the water flowing in a small shallow ditch may warm up faster in summer, affecting individual metabolism and possibly speeding up shell growth.

Reis & Araujo (2016) found a positive relationship between *K* and river trophic conditions in *Unio tumidiformis* (Castro, 1885). However, even though a slight eutrophication might favour food availability and increase growth rates up to certain level, further increase of trophic conditions may also negatively alter growth (Strayer, 2014). Furthermore, an excess of suspended solids in the water might reduce filtering efficiency in freshwater mussels, decreasing food uptake and driving slower growth rates (Reis & Araujo, 2016). The Quinto ditch is apparently more eutrophic than the other two sites, although no systematic datasets are available for comparison and there is very scarce knowledge on the environmental factors affecting growth in *M. auricularia*, so future studies should take this into account to try to explain inter-population differences in growth patterns.

Finally, when considering both growth parameters together, L_∞ and *K*, some authors suggested a negative relationship between them in freshwater mussels (e.g. Bauer, 1992; Hastie et al., 2000; Haag & Rypel, 2011; Neves & Moyer, 1988). However, we did not find that relationship comparing between the three populations and using the best model G-VBGF, which is modulated by another further parameter, what makes it more flexible with this regard.

4.6 Conclusions

Our study on growth patterns in *M. auricularia* suggests that the use of juvenile size and age data is instrumental in obtaining a good fit of growth models. Furthermore, we confirm previous findings on the best fit of sigmoid models in comparison to asymptotic ones, despite the common use of the classical growth model of von Bertalanffy. An extended, more general version of the von Bertalanffy function, so as the Richards sigmoid model, which allows an inflection point in growth patterns thanks to an added parameter controlling sigmoicity, is most appropriate. This is particularly true for the extremes of the age range, where sigmoid models are notably superior in establishing an accurate size-age relationship. Finally, we found minor differences among populations with regard to growth rates (but not in final adult size) in *M. auricularia*, probably related to environmental differences among sites that could drive physiological effects on shell growth. Further studies under controlled laboratory conditions are required to establish which are the mechanisms behind these variations in growth rate (e.g. phenotypic plasticity or genetic differentiation).

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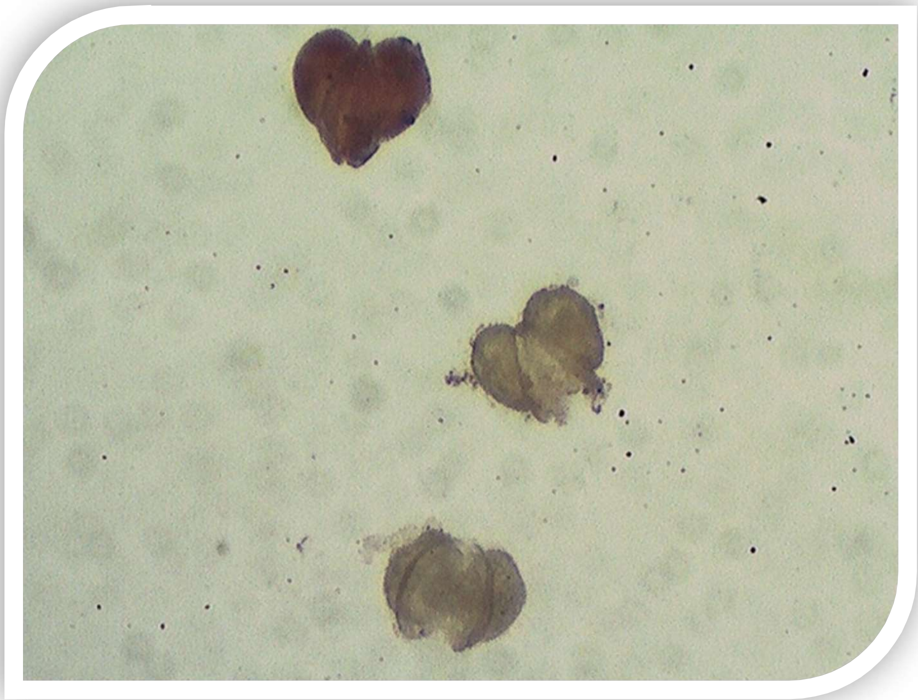
Chapter 5

Sensitivity of *Pseudunio auricularius* to metals and ammonia: first evaluation



Pseudunio auricularius juveniles after 96 h exposed to a toxicant and stained with neutral red (stained red: alive, unstained: dead)

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5.1 Abstract

Populations of the critically endangered freshwater mussel *Pseudunio auricularius* (Spengler, 1793) have been suffering sharp declines, particularly in the Ebro basin (Iberian Peninsula). Among other factors, pollution could be responsible for these declines. We conducted, for the first time, acute toxicological tests (96 h) with heavy metals and ammonia on *P. auricularius* juveniles. The resulting LC_{50} values, in decreasing order of sensitivity, were: Cd = 38.85 μg/L, Cu = 58.64 μg/L, Ni = 124.60 μg/L, Zn = 267.40 μg/L, Cr(III)>1000 μg/L, Pb>2000 μg/L and total ammonia nitrogen (TAN) = 7.53 mg/L. We also report the estimated LC_{10}, NOEC, LOEC and MATC values, which may be used to determine safer habitat conditions. Finally, we compare the obtained LC_{50} values with the concentrations of the toxicants in natural habitats where the species is present. Overall, the results reported here could be used to implement effective conservation actions, such as relocation of specimens to less-polluted sites or reduction of the concentration of pollutants in disturbed habitats. Considering the lack of ecotoxicological studies on freshwater mussels in Europe, this study may also be useful to establish toxicological reference limits for this imperiled faunal group.

Keywords: Acute toxicological test, Ebro River basin, Endangered species, Margaritiferidae, Pollution

5.2 Introduction

Freshwater ecosystems suffer from multiple stressors, including environmental pollution derived from anthropogenic activities, which modify the original characteristics of natural habitats and have a negative impact on their aquatic biota (Mason, 1996; Laws, 2018). Aquatic pollution is considered one of the main factors contributing to the decline of freshwater mussels (Bivalvia, Unionidae) (Lydeard et al., 2004; Faria et al., 2010b; Lopes-Lima et al., 2014; 2017; 2018a; Ferreira-Rodríguez et al., 2019) and their absence in suitable habitats is usually an indicator of environmental disturbance (Havlik & Marking, 1987; Bogan, 1993; Mutvei & Westermark, 2001; Lopes- Lima et al., 2020). Considering the high-conservation importance of this group of molluscs, it is imperative

to understand the potential causes for their decline, including the effects of pollution (Geist, 2015; Ferreira- Rodríguez et al., 2019).

The impacts of aquatic pollution by heavy metals and pesticides have been long and intensely studied through (eco-)toxicological research, being considered an important threat to biodiversity and ecosystem functions, including freshwater supply (Hemelraad et al., 1986a, 1986b; Bringolf et al., 2007b; Moloukhia & Sleem, 2011; Aguilar-Alberola & Mesquita- Joanes, 2012; De Castro-Catalá et al., 2016; Khan et al., 2018). Freshwater mussels are filter feeders and, consequently, pollution may severely affect their physiology and survival because of bioaccumulation of heavy metals such as cadmium, zinc, nickel, copper, lead or mercury, although they may withstand relatively high concentrations in their surrounding environment (Zadory, 1984; Naimo et al., 1992; Naimo, 1995; Byrne & Vesk, 2000; Cope et al., 2008; Byrne, 2016).

Nitrogen compounds, chiefly ammonia, nitrate and nitrite, can also have negative effects on freshwater mussels (Augspurger et al., 2003; Wang et al., 2007a). In addition to the increase in nitrogen compounds directly released to the environment by human activities (e.g. agriculture), the decomposition of organic matter may also increase the amount of ammonia and other nitrogen and phosphorous compounds in fresh- water ecosystems (Laws, 2018). The source of this organic matter is mainly wastewater, but it can also originate from massive die-offs of aquatic organisms. Indeed, the invasion of the Asian clam Corbicula fluminea (O. F. Muller, 1774) in the Iberian Peninsula is increasingly worrisome (Pérez-Quintero, 2008; Sousa et al., 2008a; 2014; Gimeno Calvo et al., 2017) since it can attain very high densities (Sousa et al., 2007; 2008b, c), and high mortalities may eventually occur, for instance in response to extreme climatic events (droughts and floods) (Sousa et al., 2012). The decomposition of organic tissues after high mortalities produce an increase in the concentration of free ammonia and a decline in oxygen concentration, strongly modifying bacterial, fungal and invertebrate communities and ecosystem functioning (Novais et al., 2015; 2016; 2017a, b; McDowell & Sousa, 2019). Such mortality events affecting the whole ecosystem can be very harmful to freshwater mussels, particularly to young individuals (Scheller, 1997; Cherry et al., 2005; McDowell & Sousa, 2019).

Freshwater mussels have a complex reproductive cycle, including a larval stage called glochidium, which is released into the water and must attach to the gills or fins of a suitable fish host (Modesto et al., 2018). The glochidia larvae transform into juvenile mussels, then detach from the fish and drop to the bottom of the water body, where they bury into the sediment and begin a new phase as a free-living juvenile. These juveniles spend most of their lives totally buried into the substrate, where they are most vulnerable to contaminants associated to sediments (Cope et al., 2008), such as heavy metals.

Mussels are, however, largely under-represented in toxicity databases used for the development of Water Quality Criteria (WQC) (Besser et al., 2015; Rai- mondo et al., 2016; Wang et al., 2017). Studies conducted in accordance with the guidelines of the American Society for Testing and Materials (ASTM International, 2013) have shown that mussels are among the most sensitive freshwater taxa to a variety of contaminants, including ammonia, copper, nickel, zinc, chloride, sulfate and potassium. Toxicity values in the U.S. Environmental Protection Agency (U.S. EPA) WQC were developed based on other taxa and, consequently, might not adequately be used to protect native mussels (Augspurger et al., 2007; March et al., 2007; Miao et al., 2010; Gillis, 2012; Wang et al., 2007a; 2008; 2017). This situation is worse in the case of European native freshwater mussels, as there is a lack of information regarding their sensitivity to heavy metals and ammonia. This can be problematic because such kind of ecotoxicological information is essential for environmental regulation and, eventually, biological conservation and restoration of mussel populations, particularly of endangered species.

Pseudunio auricularius (Spengler, 1793) (= *Margaritifera auricularia,* see Lopes-Lima et al., 2018b) is one of the most threatened freshwater mussel species worldwide, classified as Critically Endangered in the IUCN Red List (Araujo & Ramos, 2000; Prié 2010; Lopes-Lima et al., 2014; 2018a). Its former distribution has been reduced by more than 90% in Europe and, nowadays, it is present only in two countries: Spain and France (Nakamura et al., 2018a; Prié, et al., 2018). Since 2013, *P. auricularius* in the Ebro River basin (Spain) has suffered a strong decline, especially in the Canal Imperial de Aragón (Nakamura et al., 2018a, b). Other native freshwater mussels that coexisted with *P. auricularius*, including *Potomida littoralis* (Cuvier, 1798), *Anodonta anatina* (Linnaeus, 1758) and *Unio mancus* Lamarck, 1819, have also experienced similar reductions. These

declines have been probably caused by a combination of factors, including pollution by heavy metals, pesticides, herbicides and fungicides, presence of invasive alien species (IAS) in high densities, or habitat modification, among other possibilities (Nakamura et al., 2018b). In fact, several studies have reported negative effects of pollution on the aquatic fauna of the Ebro River basin (Lavado et al., 2004; 2006; Damásio et al., 2010; Faria et al., 2010a, b; Köck et al., 2010; Navarro et al., 2011; Piña et al., 2011). In particular, Damásio et al. (2010) reported high concentrations of heavy metals in the tissue of the Asian clam in the lower Ebro. Mañosa et al. (2001) reviewed the biotic effects of pollution in the lower Ebro and concluded that it originates from two main sources, i.e., industrial waste and agriculture activities, the later accounting for a large annual input of pesticides and herbicides.

Given this background, the main aim of this study was to establish, for the first time, the heavy metal and ammonia tolerance thresholds of this critically endangered species, by determining the lethal concentration values (LC_{50} and LC_{10}) for each contaminant tested in juvenile individuals, originated from captive breeding by the Aragón Government (Nakamura et al., 2019). We also estimate no-observed-effect concentration (NOEC) values, lowest-observed-effect concentration values (LOEC) and maximum acceptable toxicant concentrations (MATC), which could also be important for future works aimed at comparing the sensitivity of the species to pollutants with their concentrations in the habitat and, in this way, establish WQC fitted to the minimum tolerances of *P. auricularius* and other threatened freshwater mussel species.

5.3 Materials and methods

5.3.1 Toxicological tests

Acute toxicological tests (96 h) were conducted in basic accordance with standard methods (ASTM International, 2013) during 2017 and 2018. Seven different toxicity tests were conducted at least twice: copper (Cu), zinc (Zn), lead (Pb), cadmium (Cd), chromium (Cr[III]), nickel (Ni) and total ammonia nitrogen (TAN), adjusting the experimental concentrations to obtain more precise LC_{50} values.

All toxicity tests were conducted under similar abiotic conditions, without renovation nor aeration and at 20°C. These conditions are similar to the captive breeding program where the juveniles were collected (Nakamura et al., 2019). Polystyrene Petri dishes (50 mL capacity) were used with 25 mL of toxicant solution and 10 juveniles (age 24 h) in each dish. Three replicates were used for each concentration, all of them piled on a tray and covered with plastic film to reduce evaporation. Furthermore, a beaker with hard water was placed inside the tray to establish a water-saturated environment and reduce evaporation from the dishes. The test acceptability criterion was control survival > 90%, and the endpoint of the test was mortality. Test conditions are summarized in Table 5.1.

Three different control waters were initially tested, following ASTM-E729 (ASTM International, 2002): two reconstituted waters (‘‘hard’’ and ‘‘very hard’’) and a ‘‘natural water’’, which is defined as the same water in which the species lives, in this case water from the Ebro River. The minimum requirement for a control water for toxicity testing is that the test organism survive without showing signs of stress, such as discoloration, unusual behavior or death (ASTM International, 2013). In our 96 h tests, survival was > 90% in the three types of water, so all three were considered valid. We finally chose the ‘‘hard’’ water (hardness 160–180 mg/L as $CaCO_3$, alkalinity 110–120 mg/L as $CaCO_3$; ASTM International, 2002) because it fits closer the characteristics of the water where the species lives. In addition, the ‘‘hard’’ water has a lower salt content than the ‘‘very hard’’ water, facilitating the solubility of the toxics for the tests. The use of ‘‘natural water’’ was discarded because its exact composition was unknown and might include reacting substances that could introduce some bias in the analyses of the toxicity tests.

The reconstituted hard water finally used in the experiments was prepared by adding appropriate quantities of selected salts ($NaHCO_3$ = 192 mg/L, $CaSO_4$ = 94.79 mg/L, $MgSO_4 \cdot 7H_2O$ = 244 mg/L and KCl = 8 mg/L) to ultrapure water (18.2 MΩ·cm at 25°C) following the ASTM E729-96 guide (ASTM International, 2002).

Seven compounds were tested: copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), lead chloride ($PbCl_2$), zinc chloride ($ZnCl_2$), nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$), cadmium chloride ($CdCl_2$), chromium (III) chloride hexahydrate ($CrCl_3 \cdot 6H_2O$) and ammonium chloride (NH_4Cl). All chemicals were of analytical grade (Sigma-Aldrich, St Louis, MO, USA).

Table 5.1 Summary of test conditions for conducting acute toxicity tests with *Pseudunio auricularius*, in accordance with ASTM (2013)

Parameters	**Conditions**
Type of test	Static without renewal
Test Duration	96h
Temperature	20°C (heated room)
Type of light	Ambient laboratory light
Photoperiod	14h light 10 h dark
Test chamber	Petri dish (plastic) 50 mL
Volume of test solution	25 mL
Test organism	*Pseudunio auricularius*
Life stage	newly transformed juveniles (24 h)
Number of organisms per chamber	10
Replicates	3 + control
Feeding	No
Aeration	No
Dilution water	ASTM Hard water (hardness 160 to 180 mg/L as $CaCO_3$, alkalinity 110 to 120 mg/L as $CaCO_3$)
Reported toxicity value	LC_{50}, LC_{10}, NOEC, LOEC and MATC
Acceptability test	Survival ≥ 90% in controls

Stock solutions (up to 1 g/L) were prepared in ultrapure water as the first step. The stock solution for Ni had a concentration of 0.1 g/L. The reconstituted ASTM hard water, previously aerated, was used as dilution water in order to prepare the nominal concentrations just before starting each test. For each compound, at least six concentrations plus a control were tested (Table 5.2). Initial test concentrations were chosen based on the ecotoxicological results by Wang et al. (2007a, 2010, 2017) on juvenile mussels of North American species, with special attention to the values obtained for *Margaritifera falcata* (Gould, 1850) as a reference.

Dissolved oxygen (DO) (%), pH, conductivity (µS/cm) and temperature (°C) were measured in all replicates and concentrations, plus the control, at the beginning and the end of the tests, using a multiparametric probe (Thermo Scientific Orion). A water hardness of 170 mg/L as $CaCO_3$ and alkalinity of 115 mg/L as $CaCO_3$ were assumed according to the ASTM guidelines (ASTM International, 2002).

5.3.2 Test organism and mortality assessment

Active juveniles (following the recommendations of ASTM International, 2013) of *P. auricularius* used originated from captive breeding in the facilities of the Aragón Government in La Alfranca (Zaragoza, Spain) during 2017 and 2018 (see Nakamura et al., 2019). For each test, 10 newly transformed juveniles showing mobility were randomly selected. Experimental juveniles were not fed during the 96-h exposure and were previously acclimated to the reconstituted water. The acclimatization was carried out by mixing the water of the captive breeding (natural water) with reconstituted artificial water in a lapse of 24 h (25–75% for 4 h, 50–50% for 4 h, 75–25% for 12 h and 100% of ASTM hard water for 4 h), ensuring that the juveniles had been dwelling in 100% reconstituted water well before starting the ecotoxicological tests.

After 96 h of exposure to toxicants, two methods were used to check juvenile survival in the Petri dishes: vital staining and foot mobility by visual inspection. The last one is recommended by ASTM International (2013). In this guide, survival is checked based on foot mobility with direct observation for, at least, 5 min. The vital staining technique with neutral red dye was also used. This technique has been tested previously in ecotoxicological experiments with other invertebrate species (Crippen & Perrier 1974, Dressel et al., 1972), including juveniles of freshwater mussels (Jacobson et al., 1993a, b). After preliminary staining tests using different combinations of submersion time and dye concentration, the best results were obtained with a concentration of 10 mg/L and a staining time of 20 min, which allowed to easily detect the internally stained organs of the living individuals and did not negatively affect the juvenile performance. When the dye was used in the ecotoxicological tests, those individuals that were neither stained nor showed motility were considered as dead individuals.

Table 5.2 Compounds used to prepare the nominal concentrations for the final ecotoxicological tests (TAN[a]= total ammonia nitrogen, nominal concentrations in mg N/L)

Toxicant	Chemical formula	Purity (%)	Molecular mass (g/mol)	Nominal concentrations (µg/L)
Cd	$CdCl_2$	99.99	183.3	20, 40, 60, 80, 100, 120
Cu	$CuSO_4 \cdot 5H_2O$	99	249.68	50, 60, 70, 80, 90, 100, 120
Ni	$NiCl_2 \cdot 6H_2O$	97	237.71	100, 200, 250, 300, 350, 400, 600
Zn	$ZnCl_2$	98	136.3	200, 400, 500, 600, 700, 800
Cr (III)	$CrCl_3 \cdot 6H_2O$	70	266.5	42, 83, 166, 333, 666, 1000
Pb	$PbCl_2$	99	278.1	800, 1000, 1200, 1400, 1600, 1800, 2000
TAN[a]	NH_4Cl	99.5	53.49	0, 2, 4, 6, 8, 10, 12, 14, 16

5.3.3 Data analysis

Concentrations of metals were log-transformed (*X* values) and mortality values were normalized as percentages (*Y* values), prior to a non-linear (sigmoidal) regression analysis. We obtained dose–response curves that allowed estimating the LC_{10} and LC_{50} values, so as their confidence intervals for each acute test ($a = 0.05$) and Hill coefficients. Data analysis was carried out with GraphPad Prism v. 7 (GraphPad Software, La Jolla, CA, USA). In addition, differences in survival between experimental concentrations were tested by analysis of variance and post hoc Dunnett's tests to estimate NOEC and LOEC values and to obtain the MATC value as a geometric mean of the NOEC and LOEC values.

For comparisons with earlier studies, the estimated LC_{50} values were normalized to a soft hardness water (40–50 mg/L as $CaCO_3$) and both values are reported. Toxicity data for Ni, Cr(III) and Zn were normalized using the equations provided in the U.S. EPA-WQC report (U.S. Environmental Protection Agency, 1996). Cd data were normalized using the criteria from Mebane (2006) based on the U.S. EPA WQC for cadmium (U.S. EPA, 1996). Copper data were normalized using a biotic ligand model (BLM) (U.S. Environmental Protection Agency, 2007). The LC_{50} value estimated for TAN was not modified.

Field water chemistry data (1991–2016) on the concentration of selected toxicants in the Ebro River (at the point of diversion to the Canal Imperial de Aragón, Pignatelli-El Bocal) were obtained from Confederación Hidrográfica del Ebro (CHE; www. chebro.es), to compare field values with the estimated LC_{50} values.

5.4 Results

Mean conductivity, DO, pH and temperature in the acute toxicological tests were similar between control and treatment replicates. Conductivity varied between 549.50 and 669.67 µS/cm, DO between 91.30 and 98.76%, pH between 7.50 and 8.27 and temperature between 18.98 and 20.70°C. Water quality characteristics for each acute toxicity test are summarized in Table 5.3.

Table 5.3 Mean water quality characteristics in acute toxicity tests with *Pseudunio auricularius* (SD = standard deviation, TAN[a] = total ammonia nitrogen, nominal concentrations in mg N/L).

Toxicant	**Nº of samples**	**pH (mean±SD)**		**Conductivity (µS/cm) (mean±SD)**		**Oxygen (%) (mean±SD)**		**Temperature (ºC) (mean±SD)**	
		start	**final**	**start**	**final**	**start**	**final**	**start**	**final**
Cd	7	7.50 (±0.42)	8.03 (±0.05)	550,00 (±7.15)	589.02 (±12.47)	91.30 (±0.67)	91.04 (±0.59)	19.15 (±0.51)	19.97 (±0.14)
Cu	8	8.01 (±0.05)	7.52 (±0.03)	549.50 (±14.71)	530.52 (±8.52)	91.91 (±0.91)	91.44 (±0.96)	19.58 (±1.12)	19.93 (±0.50)
Ni	8	7.72 (±0.06)	7.76 (±0.04)	557.87 (±13.20)	588.44 (±12.99)	93.08 (±0.77)	92.68 (±0.64)	18.98 (±1.10)	20.01 (±0.57)
Zn	7	7.92 (±0.03)	8.13 (±0.02)	564.15 (±4.99)	669.67 (±8.24)	94.10 (±0.93)	91.38 (±0.41)	19.56 (0.40)	20.10 (±0.10)
Cr (III)	7	8.04 (±0.16)	8.27 (±0.02)	570.30 (±6.52)	627.44 (±9.25)	98.66 (±0.86)	98.76 (±0.40)	20.64 (±0.50)	20.40 (±0.39)
Pb	8	7.83 (±0.05)	7.92 (±0.03)	575.64 (±8.30)	635.45 (±10.10)	95.53 (±0.89)	91.20 (±0.61)	19.60 (±0.60)	19.36 (±0.33)
TAN[a]	9	7.33 (±0.03)	7.55 (±0.14)	677.97 (±68.36)	662.28 (±34.03)	95.70 (±1.11)	96.55 (±1.65)	21.60 (±0.50)	20.70 (±0.26)

Control survival was > 90% at the end of all toxicity tests. The LC_{50} values of *P. auricularius* juveniles for the tested compounds, ordered from higher to lower sensitivity based on the concentration (mass) of the toxicant are as follows: Cd > Cu > Ni > Zn >>

Cr(III), Pb >> TAN (Table 5.4). When the lethal concentrations were converted to molarity units, the sensitivity order was the same.

In the toxicity tests in which parameters could be determined, the fit of the model to data always resulted in very stepped curves, with Hill slope coefficients (H.c.) > 2. In the cases of Cu, Ni and Zn the Hill values were around 7, meaning that maximal toxicity is reached within a narrow increase in metal concentration.

Table 5.4 LC_{50}, LC_{10}, NOEC, LOEC and MATC values in hard water for the analysed toxicants tested with *Pseudunio auricularius* (CI: confidence interval, TAN^a = total ammonia nitrogen, units in mg N/L)

Toxicant (µg/L)	**LC_{50} (95% CI)**	**LC_{10} (95% CI)**	**NOEC**	**LOEC**	**MATC**
Cd	38.85 (34.76 – 43.42)	21.45 (16.03 – 28.70)	20	40	28.3
Cu	58.64 (54.27 – 63.36)	43.81 (37.01 – 51.86)	50	60	54.8
Ni	124.60 (109.80 – 141.40)	90.85 (81.38 – 101.40)	0	100	0
Zn	267.40 (237.30 – 301.30)	189.90 (165.30 – 218.10)	200	400	282.8
Cr (III)	> 1000				
Pb	> 2000				
TAN	7.53 (7.031 – 8.056)	5.51 (4.75 – 6.39)	2	4	2.8

After normalizing the results, the sensitivity of *P. auricularius* juveniles generally increased with soft water compared to the original hard water values. For Cd we obtained an LC_{50} of 38.85 µg/L (0.35 µM, H.c. = 3.69) in hard water with a confidence interval (CI $\alpha = 0.05$) between 34.76 and 43.42 µg/L and when normalized to soft water the LC_{50} was 13.95 µg/ L (95% CI 12.48–15.59 µg/L). For Cu, the estimated LC_{50} was 58.64 µg/L with a 95% CI 54.27–63.36 µg/ L (H.c. = 7.53) using hard water. The application of a BLM provided estimated values for LC_{50}= 55 µg/L in hard water and an LC_{50}= 24 µg/L for soft water (Table 5.5). Regarding Ni, we obtained an LC_{50} of 124.60 µg/L (95% CI 109.80–141.40 µg/L, H.c. = 6.95). The estimated value for soft water decreases down to an LC_{50}= 44.24 µg/L (95% CI 38.99–50.21 µg/L) (Table 5.5). In the case of Zn, we obtained an LC_{50}= 267.40 µg/L (95% CI 237.30–301.30 µg/L, H.c. = 6.41) in hard water. The corresponding soft water normalized LC_{50} was 94.81 µg/L (95% CI 84.13–106.83 µg/L). For Cr(III), the tests were valid according to the criterion of acceptability in the controls (< 10% mortality; ASTM International, 2013) but mortality was too low at the highest concentrations (7% at 1000 µg/L) so it was not possible to estimate an LC_{50} value and it is therefore reported here as > 1000 µg/L. The same happened with Pb tests, for which a mortality as low as 10% was obtained at the highest experimental concentration (2000 µg/L).

In the case of TAN, we estimated an LC_{50} = 7.53 mg/L (95% CI 7.03–8.05 mg/L, H.c. = 7.05). The LC_{10}, NOEC, LOEC and MATC values for each metal and TAN are summarized in Table 5.4.

The river water chemistry data, encompassing a period of 25 years (1991–2016) (Table 5.6), showed that the mean field values for the toxicants tested generally remained below the obtained LC_{50} values for *P. auricularius* in hard water. However, there are several episodes, in different years, where values exceeded the LC_{10} value, or even the LC_{50} of the species, as in the case of Cu. The maximum value recorded for Cu was 210 µg/L, 3.6 fold exceeding the LC_{50} of *P. auricularius* (58.64 µg/L) in hard water. Similarly, the maximum value recorded for Zn during this period was of 427 µg/L, i.e. 1.5 fold exceeding the mussel LC_{50} (267.40 µg/L) (Tables 5.4 and 5.6).

Table 5.5 (next page) LC_{50} values in hard water, and normalized LC_{50} in soft water, for *Pseudunio auricularius* and comparison with other freshwater mussels (CI: confidence interval).
[a] = normalized for soft water hardness (50 mg $CaCO_3$/L), [b] = endangered species, [c] = BLM-MODELED LC_{50} (µgCu/L) for soft water hardness (42 mg $CaCO_3$/L), [d] = Cr (VI), TAN[e] = total ammonia nitrogen, units in mg N/L.
Key references = [1]this study, [2]Gibson (2018), [3]Mebane (2006), [4]Mummert et al. (2003), [5]Wang et al. (2007a), [6]Wang et al. (2008), [7]Wang et al. (2010), [8]Wang et al. (2017).

Toxicant	**Species**	**Hardness (mg$CaCO_3$/L)**	**LC$_{50}$ (µg/L) (95% CI)**	**Normalized LC$_{50}$ (µg/L)[a] (95% CI)**
Cd	*Pseudunio auricularius*[b1]	160–180	38.85 (34.76 - 43.42)	13.95 (12.48-15.59)
	Utterbackia imbecillis[3]	-	-	45.7
	Lampsilis siliquoidea[7]	-	-	16 (13-20)
	Lampsilis rafinesqueana[7]	-	-	20(19-22)
Cu	*Pseudunio auricularius*[1]	160–180	58.64 (54.27 - 63.36)	24[c]
	Margaritifera falcata[8]	100	36	16[c]
	Cambarunio iris[4]	160-180	11.4	-
	Epioblasma capsaeformis[b5]	160–180	17 (13-21)	-
	Leptodea leptodon[b5]	160–180	22 (19-27)	-
Ni	*Pseudunio auricularius*[1]	160–180	124.60 (109.80-141.40)	44.24 (38.99-50.21)
	Margaritifera falcata[8]	100	269 (259-280)	150 (144-156)
	Utterbackia imbecillis[8]	100	676 (648-705)	376 (360-392)
	Cambarunio nebulosus[2]	-	-	510
Zn	*Pseudunio auricularius*[1]	160–180	267.40 (237.30-301.30)	94.81 (84.13-106.83)
	Margaritifera falcata[8]	100	447 (397-502)	248 (221-279)
	Utterbackia imbecillis[8]	100	520 (446-606)	289 (248-337)
	Lampsilis siliquoidea[7]	-	-	151
	Lampsilis rafinesqueana[7]	-	-	145
	Cambarunio nebulosus [2]	-	-	436
Cr (III)	*Pseudunio auricularius*[1]	160–180	> 1000	>367.04
	Margaritifera falcata[8]	100	624 (603-647)[d]	-
Pb	*Pseudunio auricularius*[1]	160–180	> 2000	-
	Lampsilis siliquoidea[7]	-	-	142 (124-164)
	Lampsilis rafinesqueana[7]	-	-	188 (161-220)
TAN[e]	*Pseudunio auricularius*[1]	160–180	7.53 (7.03-8.05)	-
	Lampsilis siliquoidea[6]	160–180	5.2 (4.6-5.8)	-
	Epioblasma capsaeformis[b5]	160–180	5.7 (4.5-7.2)	-
	Cambarunio iris [4]	160–180	11.4 (10.1-12.9)	-
	Margaritifera falcata[8]	100	8 (7.7-8.4)	-

Table 5.6 Maximum and mean values with standard deviation (SD) of toxicants analysed in the water of the Ebro River at the point of diversion to the Canal Imperial de Aragón (Pignatelli-El Bocal), period 1991-2016 (Source: CHE, www.chebro.es) (n: number of samples, TAN[a] = total ammonia nitrogen, units in mg N/L).

Toxicant (µg/L)	Maximum	Mean	SD	n
Cd	3	0.35	0.52	196
Cu	210	4.87	13.90	266
Ni	4.6	1.85	0.95	60
Zn	427	33.21	37.83	267
Cr	36	2.39	4.76	202
Pb	50	3.08	5.44	244
TAN [a]	1.03	0.16	0.14	293

5.5 Discussion

For the first time, the sensitivity of *P. auricularius* juveniles to heavy metals and ammonia dissolved in the ambient water has been studied using standard methods. This critically endangered species has been declining during the past decade (Nakamura et al., 2018b), and pollution has been hypothesized to be one of the main potential causes. In this context, the results reported here can be compared to the concentrations of these compounds where the species is present and they can also be used to implement future management measures focused on its conservation and recovery. Furthermore, and considering the scarcity of ecotoxicological studies using European freshwater mussels this study may also be useful for establishing toxicological reference limits in this faunal group.

The continuous input of pollutants into fresh waters and their permanence in the sediments can produce important acute and chronic effects to freshwater mussels, which

have sedentary habits and long-lived life histories (Van Hassel & Farris, 2007; Hartmut & Gerstmann, 2007; Cope et al., 2008; De Castro-Catalá et al., 2016; Baudrimont et al., 2020). The accumulated metals can generate toxic effects such as oxidative stress, changes in immune function, impairment at the whole organism level and eventually death (Gillis, 2012; Gillis et al., 2014; Ufelle &Barchowsky, 2018). Among these metals, Cd, Ni and Pb are considered as priority for water policy (European Water Framework Directive 2008/105/EC), as their toxic properties are well established, so as the fact that they accumulate in many aquatic invertebrates, including for instance *Hyalella azteca* (Saussure, 1858), *Chironomus dilutes* Shobanov, Kiknadze & Butler, 1999 (Besser et al., 2015), the Asian clam *C. fluminea* (Angelo et al., 2007), as well as freshwater mussels (Naimo, 1995; Byrne, 2016; Khan et al., 2018; Baudrimont et al., 2020).

Cd is highly toxic to the aquatic biota (Mebane, 2006) and it can cause serious damages such as impaired growth and enzymatic activity (Naimo, 1995). In freshwater mussels, it may disrupt the calcium metabolism (Hartmut & Gerstmann, 2007) and produce loss of foot mobility, discoloration and even death (Warren, 1996). Cd has been described as an endocrine disruptor in fish (Pierron et al., 2008), and Baudrimont et al. (2020) reported changes in gene expression, gonads tending to be feminized and poorly induced metallothioneins when subjecting *Margaritifera margaritifera* (Linnaeus, 1758) to Cd concentrations of just 2–5 µL for 7 days. Our Cd tests resulted in low LC_{50} values for *P. auricularius* (38.85 µg/L for hard water; 13.95µg/L when adjusted for soft water) and proved this metal to be the most toxic among all compounds tested. The LC_{50} values were similar to those obtained by Wang et al. (2010) in soft water for the American freshwater mussels *Lampsilis siliquoidea* (Barnes, 1823) (LC_{50} = 16 µg/L) and *Lampsilis rafinesquean* Frierson, 1927 (LC_{50} = 20 µg/L) However, Mebane (2006) reported an LC_{50} = 45.7 µg/L for the species *Utterbackia imbecillis* (Say, 1829), about threefold higher than that obtained for *P. auricularius* in soft water (Table 5). Some preliminary analyses of Cd content in the tissue of dead adult specimens of *P. auricularius* confirmed its presence with values around 0.10–0.13 µg/g (Aragón Government, unpublished data). These results might provide further clues on its accumulation and its possible chronic effects up to recent years, when a high mortality has been recorded (Nakamura et al., 2018b). However, this issue remain speculative as other pollutants and/or threats may be involved and so further research is necessary.

The negative effects of Cu on the freshwater fauna are well established (Waller et al., 1993; Borkow & Gabbay, 2009). Copper is known to be highly toxic to invertebrates, including molluscs, and is a common pollutant in riverine ecosystems (Naimo, 1995; Jacobson et al., 1997). Some of its effects on freshwater mussels include inhibition of rhythmic adductor muscle contractions in the glochidium, reduced juvenile feeding, alterations of lipid catabolism, mucous hypersecretion (Jacobson et al., 1997), decrease in body tissue sodium content paralleled by an inhibition of Na^+K^+-ATPase activity, indicating a metal-induced ionoregulatory disturbance, and modification of filtration and oxygen consumption rates (Jorge et al., 2013), among others, including eventually death. Many copper-based products have been intentionally introduced into water bodies, including herbicides, algicides and molluscicides (De Oliveira-Filho et al., 2004). Their use was formerly very common in the Ebro River basin (Mañosa et al., 2001; Damásio et al., 2010). Faria et al. (2010b) report a Cu LC_{50} for glochidia of *Unio mancus* (as *U. elongatulus*, see Araujo et al., 2005) of 86.34 µg/L. This species was selected by Faria et al. (2010b) as a reference model for other molluscs in the Ebro River, including *P. auricularius*. As the authors discussed, previous results indicated that the sensitivity of juveniles to pollutants seems to be similar to that of glochidia (Bringolf et al., 2007a; b; Wang et al., 2007a; b). Therefore, we can compare the LC_{50} obtained for *Unio*'s glochidia with that of *P. auricularius*'s juveniles in this study (58.6 µg/L), and see that the latter are 1.5 fold more sensitive to Cu. Based on this example and to improve the application of safety measures for endangered species, it is recommended, whenever possible, to carry out toxicological tests with glochidia and/or juveniles with species that are generally more sensitive to contaminants. The LC_{50} of Cu for *P. auricularius* was higher than that reported by Mummert et al. (2003) for *Cambarunio iris* (Lea, 1829) (LC_{50} = 11.4 µg/L) and those reported by Wang et al. (2007a) for the endangered species *Epioblasma capsaeformis* (Lea, 1834) (LC_{50} = 17 µg/L) and *Leptodea leptodon* (Rafinesque, 1820) (LC_{50} = 22 µg/L). So, *P. auricularius* appears to be 2–fivefold more tolerant than these species. In addition, when comparing the values of the BLM-modeled LC_{50} (recommended by U.S. EPA, 2007 due to the potential interactions of inorganic and organic copper ligands on its toxicity), *P. auricularius* (LC_{50} = 24 µg/L) is more tolerant

than the phylogenetically closely related species *M. falcata* (LC_{50} = 16 µg/L) (Table 5.5).

Ni and Zn are usually studied together because they are used to form metal alloys, and their effects on freshwater mussels include alterations in growth, behavior and filtration efficiency (Naimo et al., 1995; Angelo et al., 2007). *Pseudunio auricularius* also seems less tolerant to Ni (LC_{50} = 44.24 µg/L in soft water) than other species, as compared to *M. falcata* (LC_{50} = 150 µg/L), *U. imbecillis* (LC_{50} = 376 µg/L) and *Cambarunio nebulosus* (Conrad, 1834) (LC_{50} = 510 µg/L) (Wang et al., 2017; Gibson et al., 2018).

Zinc is commonly used in preparations of alloys, galvanized iron, electroplating, metal spraying, electrical phases, batteries and cable shells. It produces negative effects on fish such as mucus coagulation on the surface of the gills and damage to gill tissues. In addition, its toxicity increases at high temperatures (Hoffman et al., 2002) and eventually resulting in respiratory failure. In a study on Zn toxicity, Keller & Zam (1991) obtained an LC_{50} value for *U. imbecillis* (as *Anodonta imbecillis*) juveniles of438 µg/L in hard water, similar to that reported for C. iris by McCann (1993) (as *Villosa iris*; 418–578 µg/ L), and about twice the value obtained for *P. auricularius* (267.4 µg/L) in hard water in the present study. When we compare the LC_{50} value for Zn corrected for soft water in *P. auricularius* (94.81 µg/L) it is also lower than the values observed by Wang et al. (2010, 2017) and Gibson et al. (2018) for *L. siliquoidea* (151 µg/L), *L. rafinesqueana* (145 µg/L), *M. falcata* (248 µg/L) and *C. nebulosus* (as *Villosa nebulosa*; 436 µg/L). Consequently, *P. auricularius* can be considered the most sensitive of all these mussels to Zn.

U.S. EPA (1996) reports a Cr(III) reference LC_{50}= 291 µg/L for the amphipod *Crangonyx pseudogracilis* (Bousfield, 1958) in soft water (50 mg/L $CaCO_3$). Independently of the oxidation state in which it is found, Cr bioaccumulates in some bivalve species mainly in the digestive gland, but Cr(III) is generally more bioavailable than Cr(VI) (Walsh & O'Halloran, 1997). The estimated Cr(III) LC_{50} for *P. auricularius* is actually quite high (> 367.04 µg/L in soft hardness water), although it is known that Cr(III) is the least toxic form of chrome for the aquatic fauna (Laws, 2018). Future trials should be carried out with Cr(VI) to evaluate its toxicity in *P. auricularius* and compare it to the sensitivity in other freshwater mussels such as *M. falcata,* which has an LC_{50} = 353 µg/L for soft water (Table 5.5).

Lead is the most ubiquitous toxic metal, and is found in almost all habitats (Laws, 2018). In addition to leaded gasolines, which have been gradually replaced in most parts of the world, the main sources of exposure include metal mining and smelting, manufacturing, improper disposal of batteries and historical use of lead pipes and paint (Eisler, 2000). Many studies have shown its adverse effects in various organisms (including freshwater mussels), such as DNA damage, reduction of the filtering activity and bioaccumulation, usually in kidney tissue (Black et al., 1996). *Daphnia (Ctenodaphnia) magna* Straus, 1820 is one of the most frequently tested species for lead toxicological tests. Santos-Medrano & Rico-Martínez (2015) reported an LC_{50} for *D. magna* of 1620 µg/L which, compared to that of *P. auricularius* obtained in this study (>2000 µg/L), suggests that the freshwater mussel could be more resistant. However, bioaccumulation through the life cycle may represent a delayed problem for mussels, as indeed, tissue samples analysed from *P. auricularius* adults collected in the Canal Imperial de Aragón (Zaragoza-Spain) revealed the presence of lead with maximum values of 2.3 mg/ Kg (unpublished data). Therefore, the presence of this metal and its bioavailability in the ecosystem has been confirmed, although this concentration value is not high compared to sites subjected to mining activities (Angelo et al., 2007; Besser et al., 2015). Nevertheless, chronic effects, such as oxidative stress, could be causing significant damage and reducing mussel fitness (Gillis et al., 2014).

Several studies have shown a high sensitivity of freshwater mussels to ammonia and, particularly, juvenile mussels have been found to be more sensitive to TAN than other commonly tested invertebrates (Augspurger et al., 2003; Newton et al., 2003; Wang et al., 2007a, b; 2008). Toxic effects of ammonia include reduced opening of valves for respiration or feeding and reduced ciliary action in bivalves (U.S. EPA 2013), as well as increased mortality (Goudreau et al., 1993). The obtained LC_{50} for TAN in *P. auricularius* (7.5 mg/L) was very similar to that reported by Wang et al. (2017) for *M. falcata* (8 mg/ L), but higher (i.e. more tolerant) than those reported for *L. siliquoidea* (5.2 mg/L; Wang et al., 2008) or *E. capsaeformis* (5.7 mg/L; Wang et al., 2007a, b) (see Table 5). Ammonia concentration peaks produced by massive mortalities of the invasive *C. fluminea* can happen in sites where it coexists with freshwater mussels (Scheller, 1997;

Cherry et al., 2005; McDowell & Sousa, 2019). This phenomenon has been observed in sites inhabited by *P. auricularius*, such as the Canal Imperial de Aragón, where the Asian clam attains average densities higher than 1000 ind/m^2 (Gimeno Calvo et al., 2017). It is, therefore, expected that these *Corbicula* die-offs may contribute to the decline of *P. auricularius* in the Ebro River basin.

At present, the determination of toxicity sources in aquatic ecosystems is challenging, but urgently needed for appropriate risk assessment. To this end, sensitive, but widely distributed organisms must be used to estimate varied tolerance values to protect the entire ecosystem. Wang et al. (2017) and López Gutiérrez et al. (2018) discussed on the need to consider several invertebrate taxa as sentinel species in ecotoxicological assessments. In the Ebro River, freshwater mussels, including *P. auricularius,* may be amongst the most sensitive invertebrates in the ecosystem.

The concentration of toxicants in the field, reported for 1991-2016, are similar to those obtained by Bouza-Deaño et al. (2008) for the period 1981-2004, in the same locality (Pignatelli-El Bocal in the Ebro River), which we used to compare with LC_{50} values in *P. auricularius*. These authors conclude that this locality was one of the most contaminated in the Ebro River, together with a site in the city of Zaragoza. Both sites correspond to areas where *P. auricularius* still thrives. The duration and frequency of these episodes of high concentrations of toxicants in the water are unknown, since the samples were taken once a month, but their presence, even if transient (Milam et al., 2005), could be affecting the overall health of mussels, notably their longevity and reproduction.

With exposures to toxicant concentrations close to the LC_{50} values, it is expected that 50% of individuals could die. Yet, LC_{50} values are just but a starting point; and more parameters must be considered when characterizing the lethal sensitivity of these organisms. For example, it is not very common to report Hill coefficients (which characterize the slope of the sigmoidal relationship) of ecotoxicological curves in this type of studies with aquatic invertebrates. The higher the Hill value, the higher the steepness, and consequently, the higher the increase in mortality with minimal changes of the toxicant concentration if a critical value is reached. Therefore, with toxic concentration only slightly higher than the LC_{50}, 100% mortality is quickly attained and

this can be considered a very significant impact for a critically endangered species; so, it is urgent to develop more stringent measures to control the input of pollutants into the Ebro River and canals. In addition, it should be taken into account that the long- term exposure to low concentrations of these toxicants can have chronic effects in freshwater mussels, with consequences for their survival (Kleinhenz et al., 2019). Several authors report LC_{20} or LC_{10} rather than LC_{50} values (Köprücü & Seker, 2008; Wang et al., 2016; Kleinhenz et al., 2019), which should be safer for the protection of the organisms in the natural environment, especially imperiled species. Other authors, including Moore et al. (1998), suggest the use of NOEC data as the most useful to protect the most sensitive species. In this sense, the obtained NOEC and LOEC values provide an approximation to the maximum toxicant concentrations in the habitat of *P. auricularius* to be considered safe for its survival. However, these values strongly depend on the concentrations tested, and so future works should check survival at narrower ranges of concentrations to confirm (or improve the accuracy of) these values. Actually, the lack of an observed effect at a particular NOEC does not mean that there are no effects at that concentration; indeed, if we compare NOEC with LC_{10} values obtained in our study, we can expect a 10% mortality at NOEC values for Cd, Cu and Zn, as these two indexes show similar estimated concentrations. NOEC and LOEC values strongly depend on the set of concentrations used in the experiments, and a controversy exists on the adequacy of considering these values as the threshold that will be most protective of the whole community in the habitat. Their validity (or lack thereof) has been discussed extensively (see e.g. Laskowski, 1995; Warne & Van Dam, 2008; Jager, 2012; Beasley et al., 2015) and we suggest the use of LC_{10} values as a protective value for the biota in the Ebro River basin.

Overall, the reference values obtained here can be used to implement effective management actions devoted to *P. auricularius* conservation, such as restoring disturbed areas or designing a pollution control program in the areas where the mussels inhabit. These values may also facilitate selecting the most fitted habitats, with low toxicant concentrations, for sake of secure reintroductions of juveniles from captive breeding programs, or to allow safe translocations of adult specimens. These are just some of the actions that could be carried out to improve the status of some populations using the sensitivity values obtained in this study; but further investigations with *P. auricularius*

should also be pursued to test the interaction between toxicants using different mixtures of metals or even other compounds. This work is one of the first steps to try to understand how and why *P. auricularius* is declining so rapidly and to help in its recovery. The ongoing mortality of this critically species in the Ebro River basin calls for urgent protection measures in the few places where it survives, so as for an implementation of additional research on pollution and other threats, including interactions with the invasive Asian clam, the impact of parasites or diseases, or habitat destruction, which are probably also involved in these die-offs and should be carefully evaluated.

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Chapter 6

Captive breeding of *Margaritifera auricularia* (Spengler, 1793) and its conservation importance

Pseudunio auricularius captive-bred juveniles

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6.1 Abstract

Margaritifera auricularia is one of the most endangered freshwater mussels (Bivalvia, Unionida) in the world. Since 2013, the abundance of this species in the Ebro River basin (Spain) has sharply declined, driving the species to the verge of regional extinction. Therefore, any management measures that might facilitate the recovery of this species would be essential for its conservation.

During 2014–2016, captive breeding of *M. auriculari*a allowed the production of >10^6 juveniles, out of which 95% were released into the natural environment, and 5% were grown in the laboratory under controlled conditions. The aim of this experimental work was to establish the best culture conditions for the survival and growth of *M. auricularia* juveniles in the laboratory.

The experiment was divided into two phases: phase I, in which juveniles recently detached from fish gills were cultured in detritus boxes until they reached a shell length of 1 mm; and phase II, in which these specimens were transferred to larger aquaria to grow up to 3–4 mm. The best experimental conditions for juvenile survival and growth corresponded to treatments in glass containers at a density of 0.2 ind. L^{-1}, using river water, with added substrate and detritus, enriched with phytoplankton, and avoiding extra aeration. The highest survival and growth rates attained, respectively, values of c. 60% at 100 days and 2.56 mm in shell length at 30–32 weeks.

This is the first study to report on the long-term survival and growth of juvenile *M. auricularia* in the laboratory, providing essential information in order to implement future conservation measures addressed at reinforcing the natural populations of this highly threatened species in European water bodies.

Keywords: captivity, conservation, endangered species, freshwater mussel, growth, Margaritiferidae, survival.

6.2 Introduction

Freshwater mussels (Bivalvia, Order Unionida) are considered one of the most threatened animal groups in the world, whose populations have suffered sharp declines in recent decades (Bogan, 1993, 2008; Lopes-Lima et al., 2014; 2018; Lydeard et al., 2004; Machordom, Araujo, Erpenbeck, & Ramos, 2003; Neves, Bogan, Williams, Ahlstedt, & Hartfield, 1997; Strayer et al., 2004). These declines are mainly caused by habitat loss and fragmentation (e.g. by dams and other type of physical structures), pollution and deterioration of water quality (e.g. from increased fine sediment deposition, excessive nutrient input, heavy metal accumulation, herbicides and fungicides used in agriculture), overexploitation (including exploitation of fish hosts), introduction of invasive alien species (IAS), and climate change (for a review see Lopes-Lima et al., 2017). Despite their threatened conservation status there is still a noticeable scarcity of information on their com- plex biology (Geist, 2010, 2011; Geist & Auerswald, 2007; Howard & Coffey, 2006; Lopes-Lima et al., 2014), particularly on their reproductive biology and on the fish–mussel relationship (Modesto et al., 2018), which may impair the implementation of effective conservation efforts (Ferreira-Rodríguez et al., 2019).

At present, one recognized way of helping to preserve these species is through captive breeding (Bolland, Bracken, Marin, & Lucas, 2010; Gum, Lange, & Geist, 2011; Moorkens, 2018; Preston, Keys, & Roberts, 2007; Strayer, Geist, Haag, Jackson, & Newbold, 2019). Indeed, Gum et al. (2011) stated that the captive breeding of endangered freshwater mussels can be an efficient tool to maintain the evolutionary potential of their populations, which otherwise would not resist long enough to benefit from the restoration of their habitats. Several projects have previously focused on captive breeding of several species of freshwater mussels, with significant advances during the past two decades (Hastie & Young, 2003; Kovitvadhi, Kovitvadhi, Sawangwong, Thongpan, & Machado, 2006; Lavictoire, Moorkens, Ramsey, Sinclair, & Sweeting, 2016; Preston et al., 2007; Schmidt & Vandré, 2010). Some studies tested different types of diet or substrate, as various culturing systems, to achieve the optimal conditions for the development of juvenile mussels (Barnhart, 2006; Beck & Neves, 2003; Eybe, Thielen, Bohn, & Sures, 2013, 2015; Gatenby, Neves, & Parker, 1996; Gatenby, Parker, & Neves, 1997; Liberty, Ostby, & Neves, 2007). However, only a few projects reached the phase of reintroduction of the bred individuals into the natural environment (Araujo, Feo, Pou, & Campos, 2015;

Kyle, Reid, O'Connor, & Roberts, 2017; Thielen, 2011). In Europe, special attention has been paid to the freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus, 1758) with an important effort devoted to find the best captive breeding techniques (Denic et al., 2015; Eybe et al., 2013, 2015; Geist, 2010; Lavictoire et al., 2016; Moorkens, 2011; Scheder, Lerchegger, Jung, Csar, & Gumpinger, 2014; Sime, 2016; Thomas, Tay- lor, & Garcia de Leaniz, 2010).

More than 200 species of freshwater mussels are included in the IUCN Red List (Lopes-Lima et al., 2018; Lydeard et al., 2004; Prié, 2010). One of these is *Margaritifera auricularia* (Spengler, 1793), where the decline, both in abundance and distribution, has been estimated to be higher than 90%, and is nowadays considered to be close to extinction (Araujo et al., 2009; Prié et al., 2018). It was formerly distributed in all the major rivers in western Europe, either in Mediterranean or Atlantic basins, including Belgium, Czech Republic, Denmark, France, Germany, Italy, Luxembourg, The Netherlands, Portugal, Spain, and the UK (Prié, 2010). The earliest citation of *M. auricularia* in Spain comes from the German malacologist Fritz Haas at the beginning of the 20th century (Haas, 1916, 1917); however, it was later considered to be extinct, until 1985, when a few individuals were found in the lower Ebro River (Altaba, 1990). Later on, another extant population was found in the Canal Imperial de Aragón (CIA) in Zaragoza (Araujo & Ramos, 1998). Today, its known distribution is restricted to the Ebro River basin in Spain (Altaba, 1990, 1997; Araujo & Álvarez-Cobelas, 2016; Araujo & Ramos, 2000a; 2000b; 2001; Gómez & Araujo, 2008; Nakamura et al., 2018; Nakamura & Guerrero, 2008) and four river basins in France: Charente, Vienne (Loire), Luy (Adour), and Dronne (Garonne) (Cochet, 2001; Lopes-Lima et al., 2017; Nienhuis, 2003; Prié et al., 2010, 2018). In Spain, c. 5700 individuals were recorded during the first decade of the 21st century, most of them in CIA, but since 2013 a drastic decline has been observed with a mortality of about 2500 individuals in this canal (Nakamura, Guerrero, Alcántara, Muñoz, & Elbaile, 2018).

In *M. auricularia*, as in other freshwater mussels, the larvae (glochidia) need to infest the gills of a fish to complete their development (Modesto et al., 2018). When ready, the juveniles detach from the fish and fall to the river substrate. Supposedly, the former main fish host of *M. auricularia* in the Ebro River was the common sturgeon *Acipenser sturio* (Linnaeus, 1758), which inhabited the entire basin but is now locally

extinct (Altaba, 1990; Araujo, Bragado, & Ramos, 2001; Elvira, Almodóvar, & Lobón-Cerviá, 1991; López, Altaba, Rouault, & Gisbert, 2007). At present, the unique native species in the Ebro River basin suitable for the transformation of the glochidia of *M. auricularia* into viable juveniles is the river blenny *Salaria fluviatilis* (Asso, 1801) (Altaba & López, 2001; Araujo & Ramos, 2001). Nevertheless, experimental tests with non-native fish species have shown positive results, including the Siberian sturgeon *Acipenser baeri* (Brandt 1869), the Adriatic sturgeon *Acipenser naccarii* (Bonaparte, 1836) (Araujo, Quirós, & Ramos, 2003; López et al., 2007), and the Czech sturgeon *Acipenser ruthenus* (Linnaeus, 1758) (Nakamura, unpublished results). The IAS *Gambusia holbrooki* (Girard, 1859) has also been identified as a viable host for *M. auricularia* in the Ebro River (Araujo et al., 2003; López & Altaba, 2005). Recently, Soler, Boisneau, Wantzen, and Araujo (2018) reported the three-spined stickleback (*Gasterosteus aculeatus* Linnaeus, 1758) as another viable host.

Taking into account that *M. auricularia* is listed as Critically Endangered by the IUCN Red List and also included in Annex II of the Berne Convention and Annex IV of the Habitats Directive (Directive 92/43/ EEC; Council of the European Communities, 1992), a recovery plan was approved in 2005 (Decree 187/2005) for the region of Aragón, with emphasis on habitat protection and reinforcement of the population through a captive breeding programme. The main aim of this study was to determine which set of culture conditions could improve the survival and subsequent development of juveniles obtained by captive breeding. This information is essential for establishing a viable protocol for culturing this species and thereby facilitating the recovery of its populations in Europe.

6.3 Materials and methods

6.3.1 Juvenile production

Eighty-one *M. auricularia* adults were collected from CIA and Canal de Tauste (Zaragoza, Spain) in February 2014 and immediately transported, wrapped between damp towels inside cool boxes, to the aquaculture laboratory set in La Alfranca (Zaragoza, Spain). In February 2015, 71 adults were collected from the CIA and eight from the Quinto ditch (Zaragoza, Spain). In February 2016, 85 adults were collected for

the breeding experiments: 65 from the CIA, 13 from the Canal de Tauste, and seven from the Quinto ditch.

Adult mussels were kept at room temperature (10-12°C) in an Automatic Collection System to collect their glochidia as described by Nakamura, Elbaile, Muñoz, Catalá, and Salinas (2012). Six collecting systems were set, each consisting of a 1000-L tank connected to two containers of approximately 200 L each. Each small tank contained a 15-cm layer of gravel (particle size of 1–3 cm) to allow the mussels to burrow into their natural position in the substrate. Water flowed out through flexible tubes to a 75-µm sieve, which was partially sub- merged in order to maintain the collected glochidia under water. The sieve was checked for the presence of glochidia two or three times each day.

After collection of glochidia, the next step consisted of injecting a concentrated glochidia solution directly onto the gills of Siberian sturgeons. Before infestation, samples of glochidia solution were checked under the microscope to verify glochidia quantity and quality (following Araujo, Cámara, & Ramos, 2002). These samples had a high concentration of glochidia of c. 2 × 106 ind. L^{-1}, similar to values reported for the species in France (Soler, Wantzen, Jugé, & Araujo, 2018).

The number of fish to be infested was calculated depending on their body size. Siberian sturgeons (mean weight of 1.5 kg) were infested by directly injecting 20 ml of the concentrated glochidia solution (~2000 glochidia ml^{-1}) onto their gills with a needleless syringe. Sixty sturgeons were infested in 2014, 100 in 2015 and 50 in 2016. The effectiveness of infestation was checked by direct observation of gills under a magnifying lens. The fish were kept in 3000-L tanks in the external grounds of the laboratory at temperatures ranging between 12 and 15°C, until 2 weeks before the juveniles were expected to start detaching from their gills. At that time, fish were transferred to conical tanks inside the laboratory, at a density of 8-9 fish/m^3. These fish were kept unfed in order to avoid the mixing of detached juveniles with fish faeces. During the following 15 days, the temperature in the conical tanks increased to 20°C (May), when the first juveniles began to detach (amounting to c. 700 total degree-days, Araujo et al., 2003). Juveniles detached from fish were collected daily using a 120 µm sieve, and the number of individuals was estimated from subsamples. Juveniles were subsequently used in the different experiments (described below) or reintroduced into the natural environment.

6.3.2 Juvenile mussel culturing

6.3.2.1 Phase I: Detritus boxes

The culture of juveniles of *M. auricularia* was based on the ‘detritus boxes’ method developed by Eybe et al. (2013) for *M. margaritifera*. The water for these boxes was collected from the Ebro River main channel upstream of the city of Zaragoza and filtered through a 7-µm sieve in 2014 and 2015 and an 18-µm sieve in 2016. Rectangular plastic containers (1-L capacity) were tested in 2014 and 2016, and glass containers in 2015 and 2016. Juveniles (mean shell size ± standard deviation = 192 ± 13.5 µm) were counted under a binocular microscope and allocated to experimental treatments in less than 24 h. A density of 0.4 ind. L^{-1} (i.e. 200 juveniles in 500 ml) was set in 2014 and 2016. In 2015, two densities were tested: 0.2 ind. L^{-1} (100 juveniles in 500 ml) and 0.4 ind. L^{-1}. Silica sand with a particle size of 400–800 µm was used as substrate (following Liberty et al., 2007 and preliminary tests). This sand was previously washed and dried in a furnace at 150°C for 24 h. Detritus used in the boxes was collected weekly by trampling a flooded margin of the Ebro River containing abundant aquatic and riparian vegetation upstream of Zaragoza (Eybe et al., 2013). The collected sample was filtered through an 18-µm sieve, and 25 ml of the resulting water was added to each box. The composition of the detritus was assumed to be a mixture of organic matter, phytoplankton, zooplankton, fine sediments, and a large amount of bacteria and fungi in different proportions (Eybe et al., 2013; Gatenby et al., 1996; Hruska, 1999). Commercial phytoplankton (Reed Mariculture Inc. Campbell, California, USA) was used as additional food. As suggested by Eybe et al. (2013), 200 µl (four drops) of Nanno 3600® (68×10^9 cells ml^{-1}) and 120 µl of Shellfish diet 1800® (SFD; 2×10^9 cells ml^{-1}) were diluted in 10 L of filtered river water. Nanno 3600® is a monospecific concentrate of *Nannochloropsis* sp. (1–2 µm), and SFD is a mixture of six different algae ranging between 4 and 20 µm in diameter (*Isochrysis* sp., *Pavlova* sp., *Thalassiosira weissflogii*, *Thalassiosira pseudonana, Chaetoceros calcitrans*, and *Tetraselmis* sp.) Every 4 weeks the amount of food was increased in the same proportion (+200 µl of Nanno and + 120 of SFD).

Boxes were maintained at a controlled temperature of 17–18°C and partly covered to restrict the light. Boxes were cleaned once per week and their river water was replaced by water enriched in a mixture of food and detritus (according to treatment conditions).

Every week, the state of juveniles was checked under a binocular micro- scope. Those that were alive were counted and returned to the box, which had been previously cleaned and renewed. Dead individuals were removed to avoid proliferation of fungi (Eybe et al., 2013). During experiments carried out in 2014 and 2015, the three largest specimens from each treatment were photographed and measured weekly, using the Motic Image Plus© software. In 2016, specimens were measured monthly using the same method.

Physicochemical parameters pH, conductivity, dissolved oxygen, and temperature were monitored weekly using a multiparametric Thermoscientific ORION portable meter. Nitrite and ammonium concentrations were monitored using colorimetric kits (Visocolor® ECO). After 3 months, the periodicity for these analyses was adjusted to once per month (but always analysing in advance the new water to be used in the boxes).

During 2014, the boxes were checked for juveniles by filtering the substrate. Initially, 120-µm sieves were used, but mesh size was later increased to 250 µm and further to 400 µm, following juvenile growth. To reduce shell damage observed during the filtering process, an alternative elutriation method (i.e. separation of particles according to their density, as described by Lavictoire et al., 2016) was used in 2015. This process consisted of using circular movements to separate juvenile mussels from the heavier substrate. The juveniles suspended in the water were then poured into the sieve, thus avoiding breakage by friction with sand particles.

A multifactorial experiment was carried out in 2014, with six different treatments and four replicates (detritus boxes) per treatment (Table 6.1). Each treatment conditions depended on the combination of adding substrate, detritus, phytoplankton, or extra aeration to filtered river water. The control treatment (Table 6.1, treatment 1), comprising only filtered river water, was used to verify that the juveniles did not survive in the long term without additional food. In 2015, the number of treatments was reduced to three (selecting those that showed the best results in the previous year, with modifications in the amount of food added; see Table 6.1 for details), and the number of replicates varied from three to 15. In 2016 the number of treatments was increased to 12 with the number of replicates ranging from three to nine (Table 6.1).

Table 6.1 Treatments applied to juvenile mussel growth in detritus boxes throughout the study period (2014–2016). √: present, x: absent

Treatment	No. replicates	Year	Density	Container	Water	Phytoplankton	Detritus	Substrate	Feed Rate	Aeration
1	4	2014	High	Plastic	River	x	x	x	x	x
2	4	2014	High	Plastic	River	x	√	√	x	√
3	4	2014	High	Plastic	River	Marine	√	x	Weekly	√
4	4	2014	High	Plastic	River	Marine	√	√	Weekly	x
5	4	2014	High	Plastic	River	Marine	x	√	Weekly	√
6	4	2014	High	Plastic	River	Marine	√	√	Weekly	√
7	12	2015	Low	Glass	River	Marine	x	√	Weekly	x
8	3	2015	High	Glass	River	Marine	x	√	Weekly	x
9	15	2015	Low	Glass	River	Marine	√	√	Weekly	x
10	9	2016	High	Glass	River	Marine	√	√	Weekly	x
11	3	2016	High	Glass	River	Marine	√	√	Daily	x
12	5	2016	High	Glass	Canal	Marine	√	√	Weekly	x
13	3	2016	High	Glass	Canal	Marine	√	√	Daily	x
14	3	2016	High	Glass	River	Freshwater	√	√	Weekly	x
15	3	2016	High	Glass	Canal	Freshwater	√	√	Weekly	x
16	9	2016	High	Plastic	River	Marine	√	√	Weekly	x
17	3	2016	High	Plastic	River	Marine	√	√	Daily	x
18	5	2016	High	Plastic	Canal	Marine	√	√	Weekly	x
19	3	2016	High	Plastic	Canal	Marine	√	√	Daily	x
20	3	2016	High	Plastic	River	Freshwater	√	√	Weekly	x
21	3	2016	High	Plastic	Canal	Freshwater	√	√	Weekly	x

Based on the results obtained in 2014 some culturing conditions were modified in 2015, including the type of container (from plastic to glass), the initial concentration of food (doubled), and the initial density of juveniles per box (reduced from 0.4 to 0.2 ind. L^{-1}; Table 6.1, low density). The role of detritus was also tested by including three different treatments: with or without detritus, plus a high- density treatment without detritus (Table 6.1).

In 2016, when juveniles were c. 100 days old (between June and September), they were transferred to phase II (aquaria) at a shell length of approximately 1 mm. However, in the experiments carried out in 2014–2015, the cultured juveniles remained in phase I until December 2015 (see below).

Plastic containers were used again in 2016 to compare their performance with glass containers (Table 6.1). In addition, river water was compared with that from the CIA, even though the canal water originates from the same river, and another type of food was tested: Algamass (Microalgae Solutions S.L.; ~60 × 10^9 cells ml^{-1}), composed of a mixture of freshwater microalgae: *Chlorella vulgaris* (Beijerinck, 1890) (40%), *Scenedesmus quadricauda* (Brebisson, 1835) (40%), *Neochloris oleobundans* (Chantanachat & Bold, 1962) (12%), and *Pinnularia viridens* (Ehrenberg, 1843) (8%). The initial diet was adjusted using 600 μl of concentrated Algamass, diluted in 10 L of river water, which was increased monthly. A new feeding treatment was also tested, in which a concentrated solution was added daily rather than weekly (only checked with the marine phytoplankton).

6.3.2.2 Phase II: Aquaria

By the end of 2015 and the beginning of 2016, phase II began, using 20-L aquaria (38.5 × 21 × 25 cm) with the same river water and substrate (1 cm thick), and adding a flow of 100 L h^{-1}. Two drops of Shell- fish diet 1800® plus one of Nanno 3600®, previously diluted in water from the same aquarium, were added daily. One month later, the amount of food was increased to three and two drops, respectively. The aquaria were kept at room temperature (12–23°C) and in semi- darkness. In December 2015, the surviving juveniles raised in detritus boxes during 2014 (18 individuals) and 2015 (55 individuals) were transferred to phase II.

In 2016, those juveniles from the detritus boxes (phase I) that were fed with marine phytoplankton and attained 1 mm in shell length were transferred to aquaria

(200 juveniles per aquarium). These juveniles had detached from fish gills in May, and by August the first ones had reached 1 mm. Nine aquaria were set up, all with the same experimental conditions, the only difference among them being the age of the individuals upon entering phase II (i.e. the first aquaria were set with those juveniles that had attained earlier the largest size).

Aquarium 1 was filled during the last week of August, and aquaria 2 and 3 were filled during the first and second week of September, respectively. Aquaria 4–9 were filled during the second week of October. All the remaining juveniles from phase I fed with marine phytoplankton (about 450 juveniles), which were still alive but smaller than 1 mm, were transferred to the last aquaria (8 and 9). Juveniles present in these two aquaria were later combined owing to the high mortality. The results from both these aquaria were analysed together (as 8–9).

Two additional aquaria (10 and 11) were added to the experiment the last week of October with the latest juveniles coming from treatments fed with freshwater phytoplankton. These juveniles were then fed with marine phytoplankton. The density of these aquaria was set to 450 juveniles per aquarium instead of 200.

The size of the three largest individuals was measured once per month. The physicochemical parameters were checked weekly in order to control the potential rise of nitrite and ammonium in the water (following the same procedure as described above), partly renewing it if necessary. During the first months, the aquaria were cleaned every 2 weeks, extracting the juveniles first and then replacing the water and rinsing the substrate. After week 28, cleaning took place every 3 weeks.

6.3.3 Statistical analysis

Kaplan–Meier survival analysis (with the log-Rank test) was used to compare survival between years (up to day 100 before the start of phase II), between treatments during phase I for each year, and between treatments during phase II for year 2016.

Cox's regression with direct selection (also using data up to day 100) was used to test for the effects of different factors within each treatment, separately for each year.

The Shapiro–Wilk test was applied to check for normality in growth data. Mann–Whitney analysis was used to compare growth between years. Only those treatments with the highest growth each year were compared, i.e. one for 2014 and one for 2015. In 2016, comparisons were performed between the two treatments with highest growth rate, fed with marine phytoplankton, and between the two with added freshwater phytoplankton.

A Spearman's correlation coefficient was calculated with data from aquaria 1 to 8–9 (fed with marine phytoplankton) to check for a relationship between survival in phase II and the day juveniles entered this phase.

All statistics were performed using IBM SPSS 19.0 for Windows (IBM, Chicago, IL, USA) and the significance level was set at 0.05.

6.4 Results

6.4.1 Juvenile production

During the breeding experiments in 2014–2016, >10^6 juveniles were collected in the laboratory, with a maximum number of 670,000 juveniles achieved in 2016 (Table 2), of which c. 5% were used in the experiments and 95% were released into the natural environment.

6.4.2 Survival and growth

Survival rate at the end of the first 100 days during phase I was different among the 3 years of experiments ($P < 0.001$), being highest in 2016 (58%), followed by 2014 (41%) and 2015 (37%; Figure 6.1).

Table 6.2 Juvenile production per year and amount assigned to captive breeding

Year	Estimated juvenile production	Number of juveniles breeding in captivity
2014	114,600	112,400 (98.0%)
2015	462,084	7,169 (1.5%)
2016	670,000	30,000 (4.5%)

In 2014, treatment 4, corresponding to a high density of juveniles with river water, added marine phytoplankton, detritus, and substrate, fed weekly, and with no extra aeration, produced the best results in terms of survival and growth rate (Figures 6.2a and 6.3). When comparing treatments 2 and 6 (Table 6.3), which had the same experimental conditions except for the supplementary food in treatment 6, treatment 2 showed a lower mean survival time (65 days) compared with treatment 6 (79 days). Comparing survival time with or without substrate (treatments 3 vs. 6), its presence resulted in an increased mean survival time by c. 19 days ($P < 0.001$). Comparisons between treatments 4 and 6 suggest a negative influence of aeration, which decreased the mean survival time from 86 to 79 days ($P < 0.001$). The effect of detritus (treatments 5 vs. 6) was not significant ($P = 0.567$). The results of Cox's regression for 2014 confirm that the presence of substrate and phytoplankton significantly reduce mortality in about 50% compared with those treatments without (hazard ratio c. 0.5; Table 6.4) and the added aeration increased the probability of mortality by 2.3 times compared with treatments with no aeration.

In 2014, juveniles were maintained in phase I until December 2015, so growth was relatively slow. Shell lengths of 1 and 2 mm, respectively were reached during weeks 28 and 48–49 (Figure 6.3). At that point, an increased growth rate was observed, but it also coincided with the mortality of the largest juveniles, driving a decrease in mean length a few weeks later (week 52). Exponential growth did not occur in this cohort until the next spring (May 2016), around week 95–100 (Figure 6.3). Juveniles raised in 2014 reached 1 cm in length at week 115.

In 2015, the highest survival and growth rates were obtained in treatment 9 (Figures 6.2b and 6.3), with similar conditions as those described for 2014 but in a glass container and at a lower density. Adding detritus resulted in improved mean survival time (81 days instead of 64 days when comparing treatments 7 and 9; $P < 0.001$; Table 6.3). Treatment 7, with low density, produced a higher survival time than treatment 8 (64 vs. 57 days, $P = 0.002$). When testing the effects of different variables with Cox's regression (Table 6.4), the addition of detritus and reduced juvenile density increased survival by 2 and 1.2 times, respectively.

During 2015, shell lengths of 1 and 2 mm were reached during weeks 30 and 54, respectively (Figure 6.3). For juveniles bred in 2015, phase II started at week 32, with an initial exponential increase in length during week 54 and a continuation of growth until they reached about the same size as individuals from the 2014 cohort. Indeed, the juveniles raised in 2015 reached 1 cm in length at week 70, almost 12 months earlier than those raised in 2014.

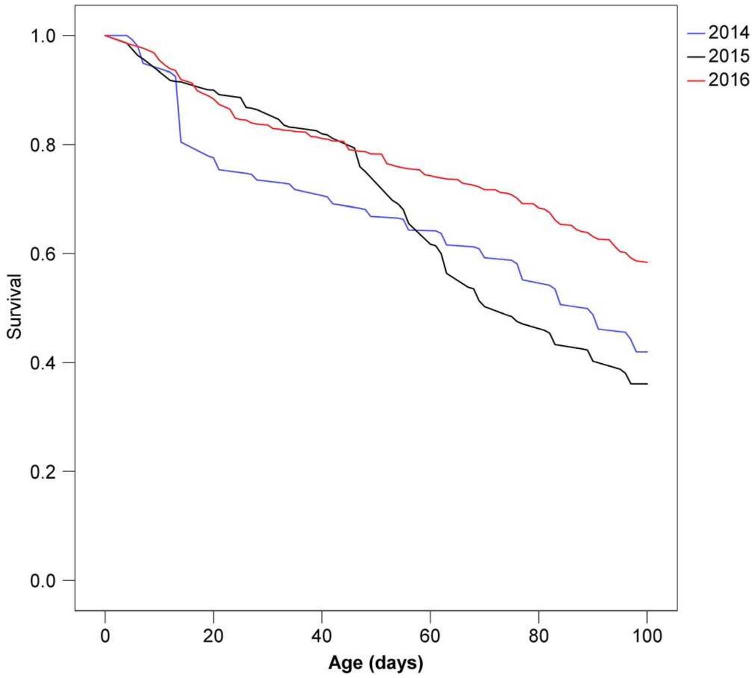

Figure 6.1 Global survival of *Margaritifera auricularia* juveniles in phase I up to day 100, for each year of the experiment across treatments (See Table 6.1 and text for further explanation)

In 2016, treatments 20, 11, and 21 showed the highest survival rates (Figure 6.2c); however, the growth recorded in treatment 20, and generally in treatments with freshwater phytoplankton, was lower when compared with juveniles fed with marine phytoplankton (Figure 6.3). Growth rate in treatments 10 and 16 were the highest recorded values. Therefore, in the balance between survival and growth, treatment 11 was selected as the one with the best results in 2016, despite the fact that the greatest survival was obtained with freshwater phytoplankton. When testing survival time by pairs of treatments for each variable, only four out of 20 comparisons were not significant (Table 6.3) and four categorical variables were significant according to Cox's regression in 2016 (Table 6.4). Both analyses suggest that the probability of survival is higher at daily rather than weekly feeding, and juveniles fed with freshwater phytoplankton showed higher survival (although reduced growth) than those with marine phytoplankton. Regarding water type, the highest survival was obtained with water from the Ebro River. The Kaplan–Meier analysis showed no clear patterns regarding survival time comparing treatments with glass or plastic containers; however, the glass container increased survival by 1.2 times when compared with the plastic container, according to the Cox's regression.

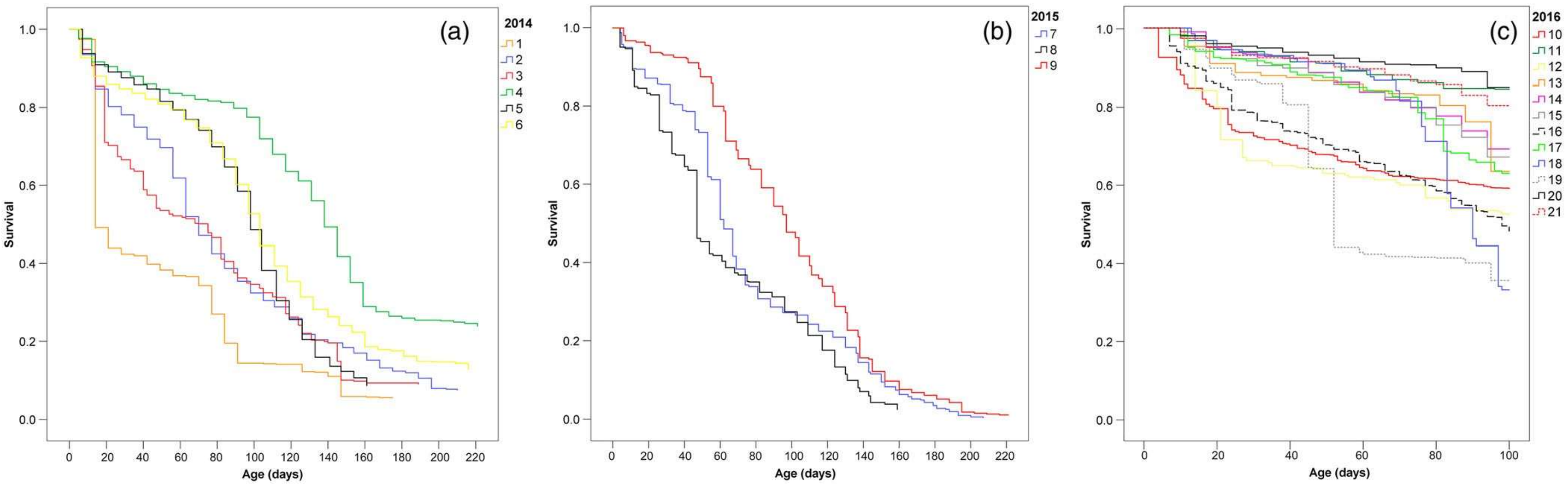

Figure 6.2 Proportion of *Margaritifera auricularia* juveniles surviving in each treatment in phase I, years 2014 (a), 2015 (b), and 2016 (c). Treatment codes as in Table 6.1

The survival of juveniles in phase II was very variable in 2016. The first three aquaria set up in August–September had a final survival rate above 80% after 120 days (220 total days since detached from fish) (Figure 6.4a). The remaining aquaria (4 to 8–9) presented lower survival, the lowest being in aquaria 8–9 with a mortality of almost 100%.

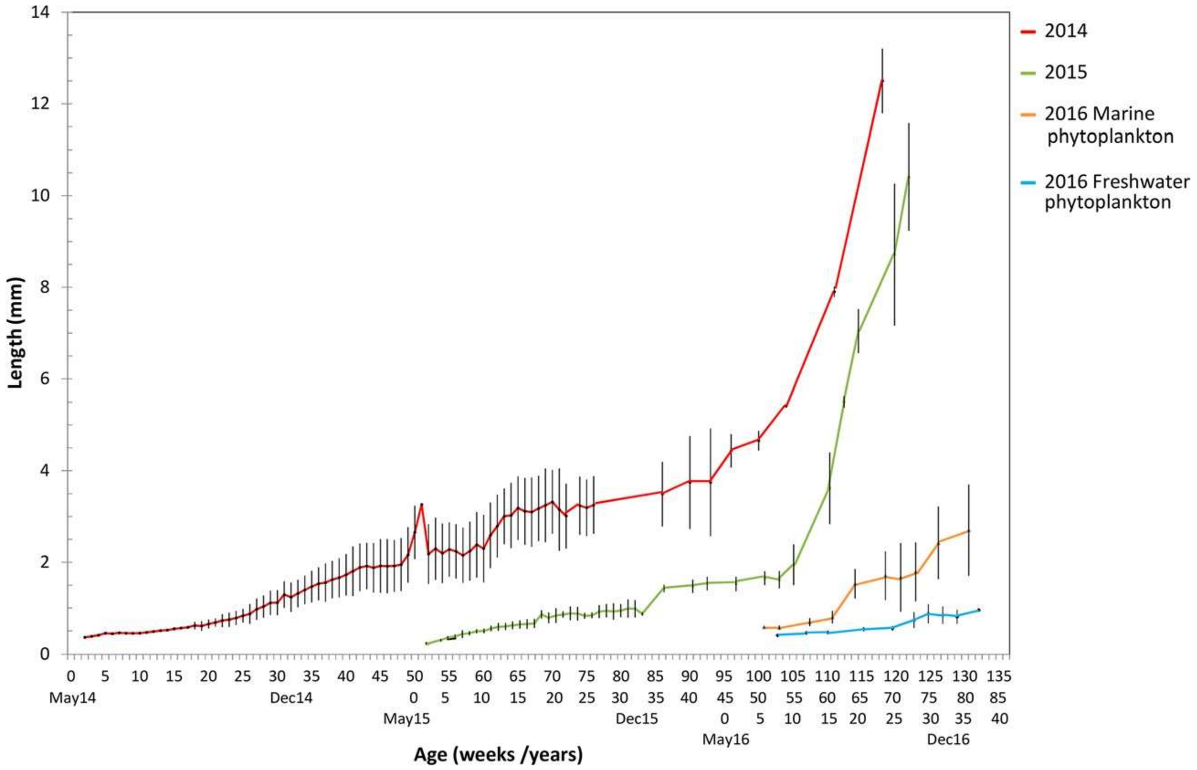

Figure 6.3 Growth of *Margaritifera auricularia* juveniles throughout the study period. Red line: year 2014 (treatment 4). Green line: year 2015 (treatment 9). Orange line: year 2016, marine phytoplankton; week 1 to 17: phase I (treatment 10 + 16); week 17 to 34: phase II (aquaria 1 to 8–9). Blue line: year 2016, freshwater phytoplankton; week 1 to 23: phase I (treatment 14 + 20); week 23 to 34: phase II (aquaria 10–11)

Table 6.3 Pairwise comparison of survival between experimental treatments according to variable tested, and selected conditions with the highest survival rates. Years 2014–2015 and 2016. See Table 1 for treatment explanation. (NS: not significant)

Year	**Variable**	**Treatment**	**Mean survival time (days)**	***P*-value**	**Selected conditions**
2014	Phytoplankton	2 vs. 6	65 vs 79	<0.001	With phytoplankton
	Substrate	3 vs. 6	60 vs. 79	<0.001	With substrate
	Aeration	4 vs. 6	86 vs. 79	<0.001	Without aeration
	Detritus	5 vs. 6	81 vs. 79	0.567	NS
2015	Density	7 vs. 8	64 vs. 57	0.002	Low density
	Detritus	7 vs. 9	64 vs. 81	<0.001	With detritus
2016	Feeding rate	12 vs. 13	68 vs. 86	<0.001	Daily
		10 vs. 11	71 vs. 91	<0.001	Daily
		16 vs. 17	72 vs. 85	<0.001	Daily
		18 vs. 19	83 vs. 65	<0.001	Weekly
	Phytoplankton	12 vs. 15	68 vs. 86	<0.001	Freshwater
		10 vs. 14	71 vs. 87	<0.001	Freshwater
		16 vs. 20	72 vs. 93	<0.001	Freshwater
		18 vs. 21	83 vs. 91	<0.001	Freshwater
	Water	10 vs. 12	70 vs. 68	0.009	River
		11 vs. 13	91 vs. 86	<0.001	River
		14 vs. 15	87 vs. 86	0.445	NS
		16 vs. 18	72 vs. 83	0.003	Canal
		17 vs. 19	85 vs. 65	<0.001	River
		20 vs. 21	93 vs. 91	0.026	River
	Container	12 vs. 18	68 vs. 83	0.078	NS
		11 vs. 17	91 vs. 85	<0.001	Glass
		10 vs. 16	71 vs. 72	0.087	NS
		13 vs. 19	86 vs. 65	<0.001	Glass
		15 vs. 21	86 vs. 91	<0.001	Plastic
		14 vs. 20	87 vs. 93	<0.001	Plastic

The correlation between survival at the end of the year (December 2016) and the day of entry to phase II was negative and significant (Spearman's Rho: -0.745; $P < 0.001$); that is, the later the juveniles enter phase II, the lower their survival rate, as happened in aquarium 7, and especially in aquaria 8–9 (Figure 6.4a). Aquaria 10 and 11, where juveniles were fed with freshwater phytoplankton during phase I, maintained a 70–90% survival rate at the end of the year (Figure 6.4b).

During 2016, growth in phase I was higher for juveniles fed with marine phytoplankton compared with previous years (Figure 6.3) and for those fed with freshwater phytoplankton in the same year. At week 17, juveniles fed with marine phytoplankton already exceeded 1 mm in length and, at the end of 2016 (week 32) they doubled the length reached in previous years at that age with a mean ± SD of 2560 ± 998 µm (compared with 1061 ± 185 µm in 2015 and 1236 ± 323 µm in 2014; Figure 6.3).

Juveniles fed with freshwater phytoplankton grew much slower and remained below 1 mm in length during the entire phase I (Figure 6.3). Their growth rate was still very low in phase II, even though the type of food was changed to marine phytoplankton, and by week 32–33 they had not yet attained 1 mm in shell length (896 ± 19 µm).

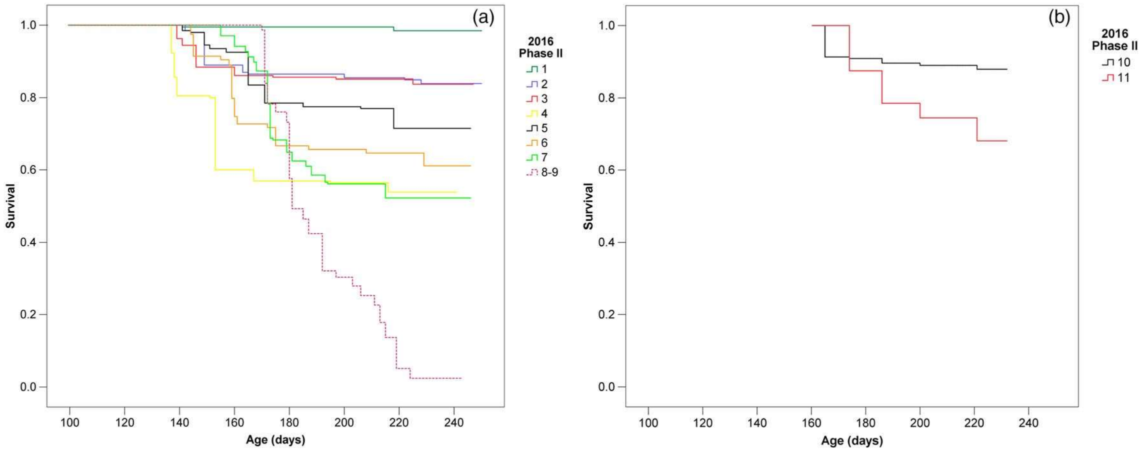

Figure 6.4 Proportion of *Margaritifera auricularia* juveniles surviving in phase II, year 2016. (a) Juveniles from cultures fed marine phytoplankton (starting day aquaria: 1 = 105 days; 2 = 110 days; 3 = 117; 4 = 127; 5 = 128; 6 = 131; 7 = 134; 8–9 = 152). (b) Juveniles from cultures fed freshwater phytoplankton (starting day both aquaria = 162 days)

Table 6.4 Influence of the treatment variables on the survival rate of juveniles in the cultures, as tested with Cox's regression for each year (symbol † and **bold type** indicate the variable conditions that favour survival)

Year	**Variable**	**Hazard ratio (95% confidence interval)**	***P*-value**
2014	Substrate: **presence†** vs. absence	0.542 (0.487–0.602)	<0.001
	Phytoplankton: **presence†** vs. absence	0.559 (0.502–0.622)	<0.001
	Aeration: presence vs. **absence†**	2.327 (1.983–2.730)	<0.001
2015	Detritus: **presence†** vs. absence	0.510 (0.465–0.558)	<0.001
	Density: high vs. **low†**	1.160 (1.045–1.287)	0.005
2016	Type of phytoplankton: **freshwater†** vs. marine	0.380 (0.348–0.414)	<0.001
	Type of water: **river†** vs. canal	0.751 (0.709–0.796)	<0.001
	Container: plastic vs. **glass†**	1.235 (1.165–1.308)	<0.001
	Feed rate: weekly vs. **daily†**	1.499 (1.394–1.612)	<0.001

6.5 Discussion

The results of this study show that *M. auricularia* can be cultured in the laboratory under a set of particular conditions that allow large numbers of viable juveniles to be produced in a few months. This has considerable implications for conservation, as juveniles of *M. auricularia*, a critically endangered species, have been successfully bred in relatively large numbers for the first time to an age older than 2 years and shell size >1 cm, thereby providing support for the recovery of its declining populations through captive breeding.

6.5.1 Phase I

Hruska (1999, 2001) established for the first time a successful method for the breeding of juveniles of *M. margaritifera* using small containers and feeding them with detritus. Eybe et al. (2013) improved the method by using detritus boxes based on plastic containers and feeding juveniles with commercial phytoplankton. The application of this method to *M. auricularia* has produced three cohorts of juveniles (2014, 2015, and 2016); however, a high mortality was observed during the first year of study. Lavictoire et al. (2016), working on *M. margaritifera*, found that this method yields a relatively low number of individuals and requires intensive management, as has been the case also for *M.*

auricularia. Young and Williams (1983) suggested that the early juvenile stage seems to be the most vulnerable to disturbances, so the stability that this method provides seems fundamental.

The first recorded peak of high mortality occurred around day 100, suggesting this is the time period for moving from phase I (detritus boxes) to phase II. In the 0.5-L culture boxes, the resources are probably too rapidly consumed by juveniles, which attain a shell size up to 1 mm, and then need further resources to avoid food shortage (Eybe et al., 2015). In 2016, growth and survival improved substantially in the treatment with a daily feeding rate, most probably also in relation to the earlier transfer to phase II. At this stage, the pumped water cur- rent might have facilitated the capture of suspended food. Feeding juveniles with freshwater phytoplankton should assist their adaptation to natural habitats when released. In fact, several authors have man- aged to breed other freshwater mussel species with this type of food (Kovitvadhi et al., 2006; O'Beirn, Neves, & Steg, 1998). In the case of *M. auricularia*, the large size of the freshwater algae (*S. quadricauda* and *P. viridis*) might have resulted in a conglomerate, which was difficult for the juveniles to feed on (Nakamura, personal observation). Freshwater phytoplankton apparently does not have adverse effects on juvenile survival, as may occur with the presence of salts in the marine food, which can generate metabolic stress (Hart et al., 1991), especially as the amount of food needs to be increased after the first few months. However, in captive breeding, the balance between survival and growth must also be considered, as a faster growth in early juvenile instars may strongly reduce mortality later in colder periods (Schartum, Mortensen, Pittman, & Jakobsen, 2016). In this study, juveniles fed with freshwater algae showed lower growth rates, well below average, so perhaps this food was less nutritious or less edible because of its larger size, than the selected marine algae. Consequently, fresh- water phytoplankton might be disadvantageous as a food resource for the sake of growth, even though a higher proportion of juveniles survived (c. 0.7 survival in 100 days compared with 0.5-0.6 in the mussels fed with marine algae). Future studies should evaluate this issue, using mixtures of species close to those found in the natural environments where *M. auricularia* persists, and testing a combination of marine and freshwater algae, or higher daily feeding rates also using freshwater phytoplankton.

The presence of substrate and detritus, phytoplankton food and the use of river water increased the survival of *M. auricularia* juveniles. Therefore, future treatments aimed at their production must take these variables into account. With regard to water type, the best results were obtained with water from the main channel of the Ebro River, not from the CIA. These results were unexpected because the highest abundance of *M. auricularia* in the Ebro basin has been found in the CIA, rather than in the main river. However, a high mortality has been reported since 2013 in this canal, where pollution and the presence of IAS, among other factors, have been considered potential causes of the mussel population decline (Nakamura, Guerrero, et al., 2018). It is possible, therefore, that water obtained from the Ebro River for the experiments had a better quality and enhanced the survival and growth of *M. auricularia* juveniles. As in *M. margaritifera* (Lavictoire et al., 2016), when detached from their fish host, juveniles of *M. auricularia* get buried in the substrate, thus achieving maximum stability of physical conditions (water flow) in their early stages. The lower survival in the treatment with additional aeration in 2014 might have produced increased disturbance, causing stress and reducing juvenile survival, even though bubbling was very gentle and near the water surface.

A diet with a mixture of several algal species is commonly recommended in captive breeding (Gatenby et al., 1997; 2003). In the present study, the mixture of seven different algae (Shellfish diet 1800®: six species + Nannochloropsis 3600®) provides a wide variety of chemical compounds, including fatty acids, proteins and lipids, as well as a diversity of food size items. Mair (2013) recommended the use of living algae, or alternatively a Shellfish diet®. However, this product is poor in polyunsaturated fatty acids and, consequently, adding living algae such as *N. oleabundans* is highly recommended (Mair, 2013). In the present study, *Nannochloropsis* sp. was used because it is known to have a high lipid content, especially polyunsaturated fatty acids, which are essential as nutritional components for freshwater organ- isms (Krienitz & Wirth, 2006), and juvenile freshwater mussels in particular (Gatenby et al., 1997).

Juvenile survival was higher in treatments with substrate in the 2014 cohort when compared with those without. These results agree with those obtained by O'Beirn et al. (1998) for *Villosa iris*. Gatenby et al. (1996) suggested that the presence of fine sediments helps the digestive activity of juveniles by facilitating crushing of particles, and it may also facilitate pedal feeding. Jones, Mair, and Neves (2005) suggested that the presence of substrate might protect juveniles against predators, allowing them also to bury and adopt a position favouring filter feeding. Some controversy exists about the role of bacteria on the survival and growth of juvenile mussels: for example, Gatenby et al. (1996) considered that bacteria might not be very important whereas Nichols and Garling

(2000, 2002) stated that they could be an important food resource. Vaughn and Hakenkamp (2001) indicated that some freshwater bivalve species supplement suspension feeding in the water column by feeding on organic detritus and bacteria in the sediments, especially in environments with high turbidity where the phytoplankton does not reach high densities. In addition, freshwater mussels in rivers filter more bacteria than pond species (Vaughn & Hakenkamp, 2001). Eybe et al. (2013) also highlighted the role that detritus plays as a biological filter, owing to the presence of nitrifying bacteria that decrease metabolites, up to 50% in the case of ammonium, thus reducing the probability of high mortality by this toxic compound. In the present study, the concentration of nitrite and ammonium in the detritus boxes was always close to zero. So, in *M. auricularia* boxes, the possible major role as a biological filter was attributed to the substrate and not to detritus. The presence of substrate not only allows bacterial proliferation that may reduce the presence of metabolites, but juveniles can probably also use those bacteria as an additional food resource (Nakamura et al., 2015).

Other variables tested, i.e. density, feeding rate, and type of container, may be modified with no major effects on juvenile survival or growth, although some combinations have generated better results than others. Density plays an important role in phase I, because juveniles are very sensitive to interactions such as resource competition, which affects their survival and growth (Eybe et al., 2013; 2015). In this study, low densities (0.2 ind. L^{-1}) minimize these interactions, as those individuals that grow faster and consume a larger amount of resources may influence the survival of the smaller individuals in the same container, with less impact at lower densities. Eybe et al. (2015) performed tests with lower densities (100 juveniles per container) and justified this procedure in order to avoid competition for food. Thus, when lower juvenile densities are used, survival rates may increase.

Daily feeding produced better results than a weekly feeding, prob- ably because it allowed a more efficient use of food resources. Juveniles can filter food particles from the water column, but also use pedal feeding by collecting organic matter available on the substrate. With weekly feeding, most food is soon deposited at the bottom (Nakamura, personal observation) and this is only available for pedal feeding, which is probably less efficient. The combination of both types of feeding leads to higher growth rates of juveniles (Vaughn & Hakenkamp, 2001), as observed during phase II using marine phytoplankton in *M. auricularia*.

In summary, this study has shown that the ideal initial conditions for juveniles of *M. auricularia* must include filtered river water, substrate, detritus, and phytoplankton added daily, and the water must be renewed weekly. The type of container seems to have no effect on the survival of the juveniles but, for practical reasons (i.e. easier cleaning), glass containers are recommended.

6.5.2 Phase II

According to Hastie and Young (2003), the pedal-to-filter feeding transition represents a critical period for the survival of juveniles in captivity, yet the age for this transition is still unknown for *M. auricularia*. Araujo et al. (2018) found that for *M. margaritifera* and *Unio mancus* (Lamarck, 1819) the second metamorphosis occurs at an age of 150–200 and 70 days, respectively. Lavictoire et al. (2016) reported that at age 12 months, *M. margaritifera* continue to feed with the foot and it is only at an age of around 25 months that it becomes a filter feeder, although gills are not completely formed until the mussels are 3 years old. Schartum et al. (2016) indicated that the shift in feeding behavior is a critical transition for the survival of juveniles of *M. margaritifera* and that double feeding – both pedal and filtering – can decrease mortality, especially during winter. In the case of *M. auricularia*, the observations under a binocular microscope through the transparent shell, showed the primordia of gills in juveniles of *M. auricularia* as small as 500–600 μm shell length, suggesting a possible start of mixed feeding at this size. From a shell length of 1 mm onwards, the mussels feed by filtering but without abandoning pedal feeding, as suggested by the grooves observed in the substrate in phase II. The incorporation of circulating water in the aquaria is a very important factor for the rapid growth of juveniles by facilitating filtration. This matches what happens in the natural environment where juvenile mussels take advantage of the interstitial water flow associated with fine particulate organic matter (Yeager, Cherry, & Neves, 1994), spending less energy and facilitating ingestion by orienting towards the current (Englund & Heino, 1996; Vaughn & Hakenkamp, 2001).

High mortalities in juvenile freshwater mussels may have many causes, including hypoxia, metabolite accumulation (Eybe et al., 2013), and the transition process during the second metamorphosis in the laboratory, which many juveniles cannot overcome (Araujo et al., 2018). In the future, more studies are needed to assess the importance of this transition phase to decrease such high mortality rates in captivity.

In a previous study on captive breeding of *M. auricularia*, Araujo et al. (2003) obtained viable juveniles and kept them alive for up to 4–6 weeks; these eventually reached a shell length of 325 µm at the end of that period. Nakamura et al. (2012) grew some juveniles up to 300 µm in 6 weeks and up to 1 mm in 140 days. In the present study, the mean length at week 6 was approximately 440 µm. This larger size probably results from supplementary feeding, based on phytoplankton and detritus, which allows juveniles to grow faster. These growth rates may be compared with those described for *M. margaritifera* raised in captivity, although freshwater pearl mussels attain shorter lengths than *M. auricularia* (Outeiro, Ondina, Fernández, Amaro, & San Miguel, 2007). Hruska (1999) and Eybe et al. (2013) reported a growth of c. 1 mm in about 16–20 weeks for *M. margaritifera,* but lower rates were observed by Schmidt and Vandré (2010) (maximum lengths of 800 µm for juveniles kept for 4 months in the laboratory). In the present study, variable growth was recorded for *M. auricularia* at week 16: juveniles reached 556–609 µm in 2014, 726–818 µm in 2015 and 1400–1500 µm in 2016. Differences between years may be related to the implementation of phase II, facilitating the intake of daily food and therefore increasing growth rate. Such rise in the growth rate of juveniles is important to achieve before the first winter, as it increases survival probability when facing harsher conditions (Schartum et al., 2016). With respect to survival, Eybe et al. (2013) reported a rate of 80% at 110 days for *M. margaritifera* using detritus boxes. In this study, at 100 days the highest recorded survival was almost 60%. Lavictoire et al. (2016), comparing survival rates of different freshwater mussels, showed that mortality can be variable with survival rates from 10 to 80% after 100–200 days in captivity. In fact, the high mortality experienced by juveniles during the first year remains an important obstacle to the successful development of captive breeding of freshwater mussels. Further work and exchange of experiences and methodologies are needed to reduce the high mortality rates during the first year of growth.

Once the juveniles reach a larger size, two further steps are planned towards their successful transfer to the natural environment. Phase III will consist in increasing the volume and therefore the amount of food where the juveniles are kept, maintaining the same type of substrate, water, and daily feeding. Once a shell size of 2-3 cm is reached, phase IV will begin, which will test adaptation to the natural environment using an open system with natural river water and without extra artificial food. These procedures will need a periodic control of survival to assess whether successful reintroduction and population recovery of *M. auricularia* can be achieved in the near future.

6.5.3 Conservation importance

The captive breeding of freshwater mussels should not be regarded as the only solution to conserve these endangered organisms (Strayer et al., 2019). The methods used both for the detritus boxes (phase I) and phase II, require a significant investment of time, logistics (including space to keep the cultures at controlled temperatures) and personnel. The lack of recruitment in natural habitats indicates that these are probably not suitable for all *M. auricularia* life stages, and therefore captive breeding must be complemented by a series of actions aimed at the potential habitats to achieve successful reintroduction of laboratory-reared juveniles. Such actions include the restoration of river dynamics and hydromorphology, an increase of floodplain habitats, improving water quality, and assisting the recovery of microhabitats by ensuring well-oxygenated substrates and a decrease of suspended solids (Dudgeon et al., 2006; Strayer et al., 2019). Remedial actions should remove the likely cause(s) for species decline (Bolland et al., 2010), so the management of fish host populations should also be included as a key management action in order to restore the reproductive potential of freshwater mussels in their natural habitats (Ferreira-Rodríguez et al., 2019).

In Europe, populations of *M. auricularia* are in decline (Prié et al., 2018), and the situation in the Ebro basin has recently become critical. Since 2013, high mortality rates of adults have been recorded and, every year, a large number of dead individuals have been found for this species and for other freshwater mussels such as *Potomida littoralis* (Cuvier 1798), *Anodonta anatina* (Linnaeus, 1758) and *Unio mancus* (Nakamura, Guerrero, et al., 2018). The possible causes of these mortalities are still unknown, but they may include pollution, cli- mate change, diseases, or the impact of the Asian clam *Corbicula* spp., among other possibilities.

The results reported here may become a key management tool to improve the conservation status of *M. auricularia* in the Ebro River basin. Similar methods can be applied to other *M. auricularia* populations or other endangered freshwater mussels (e.g. *M. marocana*) with similar life-cycle traits (Sousa et al., 2016; 2018). These captive programmes and the later release of reared juveniles into natural habitats should only be implemented after the restoration of abiotic and biotic conditions, to allow the successful colonization of *M. auricularia* and the recovery of their populations in the natural habitat.

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Chapter 7

Translocation as an ultimate conservation measure for the long-term survival of a critically endangered freshwater mussel

Tagged specimens of *Pseudunio auricularius*

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7.1 Abstract

Pseudunio auricularius (Spengler, 1793) is one of the most threatened unionid species world- wide. Translocation is considered one of the ultimate actions that can save this species from extinction in the Iberian Peninsula. Since 2013, massive mortalities have been recorded in the Canal Imperial de Aragón (CIA), an anthropogenic habitat where the highest density of *P. auricularius* had been recorded in Spain. An adequacy habitat index was calculated assigning scores to different environmental variables to select the most suitable river stretches receiving the translocated specimens. A total of 638 specimens have been translocated: 291 in 2017, 291 in 2018, and 56 in 2019. The first-year survival in the group of individuals translocated in 2017 was 41.6%. The next year, 95% of these specimens were found alive, suggesting a successful initial establishment. Specimens translocated in 2018 and 2019 showed a survival of c. 69% and 49%, respectively. In contrast, the control group left in CIA in 2017 showed a much lower survival rate of 19.7% after one year, which remained equally low during the next two years. Currently, the conditions in the Ebro River seem to allow a higher survival rate for *P. auricularius* than those in the CIA; nevertheless, future monitoring should confirm their long-term success.

Keywords: Canal Imperial de Aragón, Ebro River basin, Extinction risk, Margaritiferidae, *Pseudunio auricularius*.

7.2 Introduction

Humans have exerted increasing pressure on natural resources, which has a direct negative impact on wildlife (Hunter, 2007; IPBES, 2019). Freshwater ecosystems are not an exception, suffering intense degradation due to pollution, habitat destruction and fragmentation, overexploitation, climate change, and introduction of invasive species (Reid et al., 2019; Birk et al., 2020), resulting in a high rate of biodiversity loss (Dudgeon 2019; Tickner et al., 2020). Freshwater mussels (Bivalvia, Unionida) are among the animal groups that have been most negatively affected by humans, their current situation being of major concern (Haag & Williams, 2014; Lopes Lima et al., 2014, 2017, 2018; Ferreira-Rodríguez et al., 2019; Böhm et al., 2021).

At present, the application of conservation measures to mitigate the loss of biodiversity due to anthropogenic impacts is common practice. One of these measures is the translocation of specimens, an increasingly applied practice during the last 30 years (Armstrong & Seddon, 2008; McMurray & Roe, 2017; Jourdan et al., 2019). The translocation of individuals has been used not only in order to save specimens at risk but also as a methodology to reintroduce species in locations where they had become extinct or to reinforce dwindling populations (Haag & Williams, 2014). According to the IUCN guidelines (IUCN/SSC 2013), the translocation of individuals for conservation purposes is “the intentional movement and release of a living organism where the primary objective is a conservation benefit.” As a concept, translocation seems a relatively straightforward task to carry out but, in fact, it needs a rigorous planning of all the steps involved in the process, including appropriate collection, handling and transport methods, assessment of habitat stability and suitable environmental and biological conditions in the recipient site, among others (Cope et al., 2003; Dunn et al., 2000; Luzier & Miller 2009; Moorkens 2017; Pires et al., 2020). In addition, any translocation plan should include a careful evaluation of the tradeoffs between conservation benefits and the costs and risks for the target species, so as for other species present in the recipient community (Cope & Waller, 1995; IUCN/SSC, 2013; Tsakiris et al., 2017; Brian et al., 2021). Finally, it is also essential to establish a long- term monitoring plan to assess the success or failure of the action (Luzier & Miller, 2009; Germano et al., 2015; Jourdan et al., 2019).

A recent review dealing with reintroduction attempts of aquatic macroinvertebrates concluded that these types of interventions are not as common as they are in terrestrial organisms, and that their viability as a conservation strategy has not been fully evaluated (Jourdan et al., 2019). These authors show that one important factor that may contribute to the failure of translocations is the complex life cycle of the target aquatic organisms. Freshwater mussels have a partly parasitic life cycle. Fertilized mussels release the larvae (glochidia) into the water and these attach to the gills or other body parts of specific host fish species. Once attached to the fish, they undergo a metamorphosis to become small juvenile mussels (see Modesto et al., 2018 for a revision). Therefore, for the specific case of freshwater mussels, any planned translocation always needs to take into account the presence of suitable host fish species to be able to succeed.

The translocation success of freshwater mussels is difficult to assess, and, in some cases, the methodology has been criticized as ineffective (Cope & Waller, 1995;

Haag & Williams, 2014; Germano et al., 2015; Jourdan et al., 2019). For example, Dunn et al. (2000) reported an increase in the mortality of freshwater mussels when translocated at low temperatures. Stodola et al. (2017) reported variable survival depending on species and site. In other cases, high mortalities have been related to predation by raccoons or to specimens being washed away by large floods (Sousa et al., 2012; Stodola et al., 2017; Zając et al., 2019; Hart et al., 2021). Besides survival, sub-lethal effects have also been reported, including losing body condition, which may indicate poor adaptation to the new habitat (Hart et al., 2021).

Other authors obtained positive results when translocating freshwater mussels. For example, Tsakiris et al. (2017) obtained high survival rates for *Quadrula houstonensis* (Lea, 1859) and *Amblema plicata* (Say, 1817) (85 and 99%, respectively), emphasizing that site selection was a key element for success. The work of Valovirta et al. (1998) reports substantial differences in the survival of *Margaritifera margaritifera* (Linnaeus, 1758) when comparing translocations within the same river (survival above 90%) or between different rivers (50% or lower), which rein- forces the importance of environmental and biological characteristics of donor and recipient localities (Jourdan et al., 2019).

The Giant Freshwater Pearl Mussel, previously known as *Margaritifera auricularia* (Spengler, 1793) and recently renamed as *Pseudunio auricularius* (Lopes Lima et al., 2018), is one of the most threatened freshwater mussel species worldwide (Prié et al., 2018; Soler et al., 2018), classified as Critically Endangered in the IUCN Red List (Prié, 2021). Extinct in a large part of its ancient distribution area (Altaba, 1990; Araujo & Moreno, 1999; Araujo & Ramos, 2000), this species is nowadays present in five hydrographic basins: Ebro River (Spain) and Garonne, Vienne-Creuse, Adour, and Charente Rivers (France) (Prié et al., 2018; Soler et al., 2018).

In Spain, *P. auricularius* has a restricted distribution, being only present in the Ebro River basin (Altaba, 1990; Altaba et al., 2001; Araujo & Ramos, 2000; Altaba & López, 2001). The Canal Imperial de Aragón (CIA, Ebro River basin, Spain, for more details see Gómez & Araujo, 2008) was known to harbor around 6000 tagged specimens. Since 2013, the CIA population has suffered a severe and rapid decline estimated as approximately 80% of individuals lost in 2019 (Nakamura et al., 2018a; Guerrero et al., 2021). The causes of this mortality are still under study and may encompass (1) absence of host fish due to the extinction of the original host, the common sturgeon (*Acipenser*

sturio Linnaeus, 1758) in the Ebro Basin (Araujo et al., 2001, 2003; López & Altaba, 2005) and decline of the river blenny [*Salaria fluviatilis* (Asso, 1801)], the only recognized native host fish still present in the Ebro basin for *P. auricularius* (Araujo et al., 2001, 2003), reaching the point of being cataloged as endangered species in the Aragón region (Abad Ibañez & Ginés Llorens, 2020); (2) artificial regulation of river flow due to the construction of dams for hydroelectric power plants and water withdrawals (Araujo & Álvarez-Cobelas, 2016), causing high mortalities; (3) pollution causing lethal or sub-lethal effects in freshwater mussels (Nakamura et al., 2021); (4) possible diseases or parasites (Guerrero et al., 2021); and (5) invasion by non-native species competing for the same resources than native mussels (Gimeno Calvo et al., 2017), including the zebra mussel [*Dreissena polymorpha* (Pallas 1771)] and the Asian clam *Corbicula fluminea* (Müller, 1774), and by fish such as *Silurus glanis* Linnaeus, 1758, *Ameiurus melas* (Rafinesque, 1820), and *Gambusia holbrooki* Girard, 1859 that can compete with or prey on the river blenny (Araujo et al., 2003; López & Altaba, 2005; Araujo & Álvarez-Cobelas, 2016).

Taking into account the high risk of extinction of this important population, the Regional Government of Aragón implemented an emergency action plan for the conservation of *P. auricularius*, discussing its pros and cons with scientific experts, managers, companies, and NGOs working with freshwater mussels. The results of these discussions determined that the best option to preserve the last alive specimens in the CIA was the translocation of adult specimens from this anthropogenic habitat to the natural environment of the Ebro River. Although this river was in the past the main habitat for the species and the source of specimens colonizing the CIA (Haas, 1916a, b, 1917; Azpeitia, 1933), only a few dozen specimens were known to occur currently in the Ebro River (Regional Government of Aragón, unpublished data). Nowadays, environmental conditions in the Ebro River, unlike in the CIA, allow the presence of freshwater mussels [*Potomida littoralis* (Cuvier, 1798), *Anodonta anatina* (Linnaeus, 1758), and *Unio mancus*, Lamarck, 1819], including juvenile specimens, indicating recent recruitment (Nakamura and Guerrero pers. observ.). Although some stretches of the Ebro River are heavily disturbed, it is still possible to find localities that maintain good conditions for freshwater mussels and therefore for *P. auricularius*.

Considering this adverse conservation scenario and the unique opportunity provided by the implementation of an emergency action plan developed by the Regional

Government of Aragón, this study assessed the survival probability of *P. auricularius* translocated from CIA to selected localities in the Ebro River, with the final goal of increasing its long-term survival.

7.3 Materials and methods

7.3.1 Selection of donor and recipient localities

During 2016 a first evaluation was carried out at the CIA to choose the localities from which the specimens would be translocated. We reviewed the census information on *P. auricularius* along the entire canal and choose a section from kilometer 32 to 86, where the density was higher. Then we selected the specific donor localities based on the abundance of alive *P. auricularius* and easy access to the canal. Basic autecological data, including number of specimens and biometric measurements (length, width, height and weight), plus water depth and physical and chemical variables, including pH (± 0.01), conductivity (± 0.01 µS/cm), dissolved oxygen (± 0.1%DO), and temperature (± 0.1 °C), were measured using a multiparametric Thermo Scientific™ ORION portable meter. Nitrite (± 0.05 mg/L), ammonium (± 0.1 mg/L), and phosphate (± 0.1 mg/L) concentrations were estimated using colorimetric kits (Visocolor® ECO). These variables were measured when the specimens were extracted from the canal and again when they were introduced to the new localities in the river.

In the Ebro River, a preselection of possible recipient localities was started during 2016 and continued in the summer of each following year, until 2019. Twelve localities were initially selected based on 1) aerial photographs taken during previous years (1996–2016, www.ign.es/web/comparador_ pnoa/index.html) to assess the stability of the river stretches and avoid localities subjected to strong sedimentation or erosion and 2) technical reports and previous studies (Gómez & Araujo, 2008; Araujo et al., 2009; Araujo & Álvarez-Cobelas, 2016) where the presence of freshwater mussels (Unionids) such as P. littoralis, U. mancus, or P. auricularius had been confirmed. Only one evaluated locality was outside the main channel of the Ebro River: its tributary, the Vero River, near Castillazuelo village (not shown on the map, Fig. 1). It is a Pyrenean stream located to the North of the main study area, 90 km away from the main course of the Ebro River, with the presence of high densities of *P. littoralis* (Regional Government of Aragón, unpublished data), and which has no major pollution problems as is the case in the Ebro River main channel and its close connected canals.

Table 7.1 Limiting environmental values of *P. auricularius* used for the selection of recipient localities in the Ebro River. Based on Altaba (1990, 2001), Araujo & Ramos (2000), Araujo et al. (2001, 2003); Araujo & Álvarez-Cobelas (2016), Nakamura et al. (2018b, 2019), Soler et al. (2018), Wantzen & Araujo (2018).

Variable	Variable code	Less appropriate (Score=1)	Appropriate (Score=3)	Optimum (Score=5)
Physical and chemical variables				
pH	pH	< 7.7 or > 8.5	7.7 – 7.9 or 8.2 – 8.5	7.9 – 8.2
Conductivity (µs/cm)	EC	< 400 or > 2200	400 – 1000 or 1400 – 2200	1000 – 1400
Dissolved oxygen (%)	DO	< 70	70 – 90	>90
Nitrate (mg/L)	NO3	>10	5 – 10	<5
Nitrite (mg/L)	NO2	> 0.5	0.05 – 0.5	< 0.05
Ammonium (mg/L)	NH4	> 0.5	0.3 – 0.5	<0.3
Phosphates (mg/L)	PO4	> 0.5	0.3 – 0.5	<0.3
Depth in summer (cm)	DIS	< 40	40 – 60	> 60
Gravel substrate cover (%)	GSC	< 40	40 – 60	> 60
Dark sediment (%)	DS	> 50	< 50	0
Biological variables				
Freshwater mussels density (ind/m^2)	FWMd	< 1	1 – 2	> 2
Macrophyte cover (%)	MC	50 - 100	10 – 50	< 10
Alive Asian clam density (ind/m^2)	ACd	> 500	200 – 500	< 200
Previous *P. auricularius* density (ind/m^2)	*Pa*_d	0-0.1	0.2 – 0.5	> 0.5
Anthropogenic variables				
Distance to nearby agricultural activity (km)	DAA	< 0.5	0.5 – 1	> 1
Distance to nearby urban treatment plant outlets (km)	DURB	< 0.5	0.5 – 1	> 1
Distance to nearby villages (km)	DV	< 0.5	0.5 – 1	> 1
Fishermen presence (nº encounters)	FP	> 3	1 – 3	0
Accessibility to the point	AP	Difficult	Medium	Easy

An adequacy index was designed to evaluate the preselected localities. The aim was to integrate as many variables as possible that could affect the survival of *P. auricularius*. Localities with previous presence of the species or at least the presence of other living mussel species were especially taken into consideration. The range of values for each variable included in the index was based on available literature about the optimum habitat conditions for the species (Altaba, 1990, 2001; Araujo & Ramos, 2000; Araujo & Álvarez-Cobelas, 2016; Soler et al., 2018) and on 20 years of experience gathered in several projects aiming at conserving the species, both in the natural environment and in captivity (Wantzen & Araujo, 2018; Nakamura et al., 2018b; 2019; 2021). In each recipient locality, a plot (surface area 8-20 m^2) was chosen to assess ten physical and chemical variables, plus four biological and five anthropogenic variables (Table 7.1). First, the presence of mussels and especially *P. auricularius* was confirmed in the plot. The variable "Gravel substrate cover (%)" was visually estimated. We placed a 30 × 30-cm square (replicated 3 times) onto the bottom of the river and registered the substrate percentage cover inside each square looking through an aquascope and before inspecting the substrate to sample the mussels. We used an adapted sediment classification described in Gibson et al. (1998): boulders (64-256 mm), gravel (2-64 mm), sand (0.06-2 mm), and silt (<0.06 mm). The percentage of macrophyte cover and dark sediments (as a proxy for anoxia) on the substrate surface was also visually assessed for each plot. A 0.5-m-wide transect, in the middle of the plot and across its length, was used to assess the density of native unionid mussels. All specimens, alive or dead, were extracted from the transect by palpating the entire surface and, once extracted, they were identified to species level and their density was estimated taking into account the transect area (length of the plot × 0.5 m wide). Living specimens were left back in the transect, naturally buried in the substrate. In addition, three samples were extracted (initial, middle, and end of the transect; variable length in each plot) using 30 × 30-cm squares to assess the density of *C. fluminea*. Once the samples were taken, living and dead specimens were separated to estimate their mean density along the transect. Anthropogenic variables were also taken into account and included the following: nearby human activities such as agriculture and outlets from treatment plants and nearby villages, measuring in both cases the distance in kilometers from the plot to the agricultural field or to the village/city on an orthophoto. Fishermen presence was considered as a threat to the translocation process, first because the location of the new mussels could be noticed and consequently the mussels could suffer acts of vandalism and second because fishermen are expected to enter to the plot to fish and inadvertently disturb the specimens. So, the higher the presence of fishermen, the lower the score that was given to a locality. Furthermore, the variable ‟accessibility to the point” was assessed, providing a positive value for the general evaluation score, i.e., the easier the access, the higher the score. Estimating the access to a sampling site as “easy” did not necessarily mean direct access to the shore through a path (which could be used by fishermen), but rather implied that there were no obstacles (trunks, trees, large stones) or very deep areas, concluding that the site was easily wadable for translocation works. Values of each selected variable were classified into three ranges with an associated score: less appropriate (1 point), appropriate (3 points), and optimum (5 points) conditions (Table 1). The sum of values for all variables resulted in a global score for each locality, which allowed us to choose those with higher scores. Although all the variables were evaluated in the same way to avoid biases, a single variable could be decisive in discarding a locality if it was totally out of range. For example, the presence of sediments with signs of anoxia or the absence of alive mussels were decisive factors to discard a locality.

The preselected localities (Fig. 7.1) were evaluated during three different years: L1–L9 in 2016, L10 in 2017, and L11–L12 in 2018. They were usually evaluated one year before proceeding with the translocation of specimens.

7.3.2 Translocation procedure

Translocations were carried out in autumn, between October and December each year, to avoid extreme air or water temperatures, as well as the most sensitive reproductive period of the species, which in *P. auricularius* occurs in February–March in Spain (Grande et al., 2001). For the procedure we followed these steps: (1) Physical and chemical variables were measured in both, the donor and recipient localities the same day of the translocation, in order to confirm that all the variables were within the optimal range for the species. (2) In the CIA, we chose preferentially those localities that harbored a large number of specimens, in order to leave a control group in that locality. Specimens to be translocated were mostly extracted on foot by direct searching of the bottom of the canal and using an aquascope, while on other occasions they were collected by divers due to high water levels. Taking into account the low number of remaining specimens and the

high mortality in the CIA, the option of leaving a control group for each year of extraction was not considered, so that a single control group was set in the first year of the translocation (2017). (3) Specimens were selected randomly and verifying that they presented good external conditions (without bumps or breaks in the shell and being well-buried in the substrate). Each group of specimens that was extracted from the canal was distributed in more than one locality to avoid losing the survival information of the entire group in case of any local stochastic event. (4) Collected specimens were marked as quickly as possible using two identical numbered tags, one on each side of the shell. The labels were glued to the flattest part of the shell below the umbo with cyanoacrylate glue.

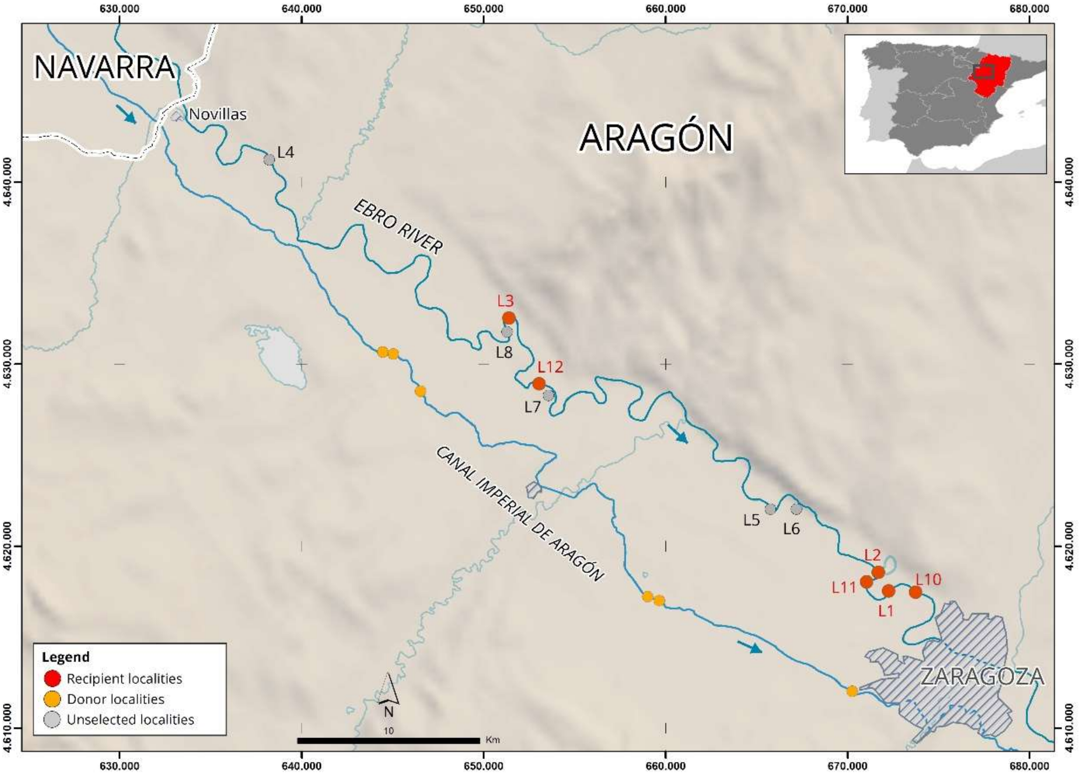

Fig. 7.1 Ebro River and Canal Imperial sections where recipient and donor localities were selected respectively (Aragón region, Spain).

This procedure was done for both groups of specimens: the translocated ones and those that remained as control group. In addition, we fixed an external PIT-tag (Passive Integrated Transponder, FDX-B 12 × 2.12 mm, Loligo Systems®) on the translocated specimens. This PIT-tag was glued onto the posterior end of the shell to facilitate its reading (due to the position adopted by the adult mussel, half of the shell buried in the substrate and leaving the posterior area out of the sediment). We used cyanoacrylate glue during the first-year campaign, but the following years we used a two-component epoxy adhesive (Supertite®) instead (see Results). The PIT TAG READER Agrident GmbH APR500 was used to record the PIT-tag number and associate it to the plastic tag number that had been previously assigned to each specimen. During the first year we used an antenna that was only 0.6 m long, but later on we changed to using a 1.5-m antenna, allowing to sweep a larger and deeper area during field surveys, although the detection sensitivity was the same (15 cm). In addition, before translocation, a small permanent mark was made to each specimen with a Dremel® directly on the shell and using a different symbol for each CIA donor locality, which would allow us in future to assess the survival in the river in relation to a particular donor locality. Biometric data from each specimen were collected using a manual caliper (± 0.05 mm), including shell length, height, and width, so as wet weight with a field scale (Nahita Blue 5171, ± 0.1 g). (5) The collected specimens were kept moist using towels soaked in CIA water and ice to cool the box in which they were transported, but avoiding direct contact with it. The number of specimens in the boxes were kept low to avoid shell dam- age. Transportation was done as quickly as possible. (6) In the recipient localities, the exact final location of the translocated specimens was previously marked with wooden stakes forming a square plot (8–20 m^2). (7) The specimens were placed in their natural position partially buried in the substrate with the help of a garden shovel to open a hole in the substrate. Once the process had finished, location information (UTM coordinates plus identification of landmarks) was taken.

7.3.3 Post-translocation actions

Previous studies recommended permanent monitoring of translocated specimens (Dunn et al., 2000; Jourdan et al., 2019; Luzier & Miller, 2009; IUCN/ SSC, 2013). In large rivers, such as the Ebro River (Spain), this is a difficult task due to the harsh environmental conditions (high depth, poor visibility, and winter floods), an issue that can be even more complicated for species that live buried, such as freshwater mussels (Prié et al., 2018; Hernández et al., 2021). In our case, it was not possible to monitor the specimens during high water levels. Therefore, monitoring was carried out once a year during the river's driest period (July to September). At the same time, annual assessment of control localities (from CIA) were undertaken during October-

November, taking advantage of maintenance works and consequent lower water levels in the CIA. Physical and chemical variables (as described above) were measured at every visit (at least twice a year). Mussel monitoring was carried out by direct observation of the specimens with the help of an aquascope to determine if they were alive or not. For this reason, we waited for the time of the year with the best conditions: low flow, no wind, and no rain that could increase water turbidity through sediment input. Each detected specimen was marked with a small red flag placed next to it to indicate its position and facilitate its identification by means of distance reading its electronic PIT-tag, so avoiding manipulation of specimens. In some plots with a high percentage of silty substrate that was easily resuspended, we used two ropes placed perpendicularly in the center of the plot to divide it into four squares. In this way, we were able to search each square carefully to prevent any visible specimen from going unnoticed. Once the PIT-tag was read, the flag was removed, thus avoiding potentially reading the same specimen several times. If the specimen was dead (with an open shell) it was removed from the plot and recorded. The number of not detected specimens was calculated by summing the numbers of living and dead specimens found and subtracting this from the total number of initially translocated specimens.

All recipient localities were surveyed every year, accounting for a maximum period of three years for those translocated in 2017 and a minimum of one year for those translocated in 2019. The exception was locality L3 that could not be surveyed in 2020, due to high water level. So, we excluded this locality from the calculation of survival for that year. Survival (and mortality) percentages were estimated as the sum of all living (or dead) specimens found that corresponded to a particular year of translocation, divided by the total number of individuals translocated that year and multiplied by 100. The percentages of not detected (ND) individuals were correspondingly calculated as the number of missing individuals (i.e., total minus the sum of dead and living individuals found) that had been translocated a particular year, divided by the total number of individuals translocated that year and multiplied by 100. Chi-square (χ^2) tests were used to check for differences in the frequency between live and dead specimens translocated to the river in 2017 versus the control specimens that remained in the canal the same year, by means of the software SPSS Statistics v.23.

Given the peculiar life cycle of *P. auricularius*, in which the glochidium larvae need to parasitise a suitable fish host (Araujo et al., 2001; López & Altaba 2005; Modesto et al., 2018), particular attention was devoted to this factor. In order to increase future recruitment of *P. auricularius*, we also translocated its known fish host in the Ebro River, the freshwater river blenny (*Salaria fluviatilis*) (Araujo et al., 2001) to four recipient localities, with individuals coming from the Canal de Monegros. Maintenance works are performed annually in this canal located in Huesca province, north of the study area. In this canal, a high density of the river blenny had been reported (Abad Ibañez & Ginés Llorens, 2020) and in October 2019 specimens were collected using hand nets in some shallow stretches of the canal. Specimens were collected in the morning, keeping them in several tanks with water from the same canal and aeration and in the afternoon, they were transported directly to the recipient localities in the Ebro River, where the specimens of *P. auricularius* had already been translocated. The fish were released after a process of acclimatization, for at least two hours, in water from the recipient locality.

7.4 Results

7.4.1 Selection of donor and recipient localities

The best localities for freshwater mussel translocation, according to our index score, were located in the middle Ebro River, upstream from the city of Zaragoza up to Novillas (Fig. 7.1). The selected six localities were L1, L2, L3, L10, L11, and L12 according to their higher scores (Table 7.2).

Out of the six selected localities, those that obtained the best results were L2, L1, and L10 (71, 69, and 69 points, respectively) (Table 7.2). We chose five of the six localities with the highest scores, with the exception of the selected site L3, which had a lower score (55 points). We decided to include L3, despite having 90% macrophyte cover and a moderate-high density of Asian clam (837 ind/m^2), because it was the locality in which the highest number of young *P. auricularius* had been found (this information not being known previously). We found three new specimens in 2017 when we evaluated the plot and two more in 2018, with shell lengths ranging between 10 and 13 cm. On the other hand, L9 was the only locality discarded with a high score (75 points), due to its location in a tributary and not in the main channel of the Ebro River and also because, historically, it was not within the range of the natural distribution of

the species. Despite this situation, we evaluated this locality since it might serve as a backup locality in the case the Ebro River would not work during the first year and mortality in the CIA increased to the point of having to save the last living specimens. Finally, we discarded all other localities because either they suffered desiccation in summer (L8), had 100% macrophyte cover (L4 and L5), sediments presented signs of anoxia (L7), or had very high densities of the invasive *C. fluminea* (L6, L7, and L8) (Table 7.2).

Results of physical and chemical variables were similar between donor and recipient localities, with the exception of phosphates in L12 (0.7 mg/l) and a relatively high value of conductivity in L3 (2676 µs/cm). The substrate of the selected recipient localities was dominated by gravel and sand, and only in L1, L3 and L10 small patches of finer sediment (silt and clay) were observed, especially near the margins where tree logs were present.

Freshwater mussel densities in the selected localities varied between 0.8 and 7.0 ind/m^2, with maximum values in L11 (7.0 ind/m^2) and L1 (4.9 ind/m^2), with the presence of *P. littoralis* and *U. mancus*, and L2 (4.5 ind/m^2) with only *P. littoralis*. Before the translocation, *P. auricularius* specimens were detected in L1, L3, and L10 with densities of 0.15, 0.25, and 0.07 ind/m^2, respectively. Live Asian clam densities were also highly variable, reaching maximum values of 1215 ind/m^2 in L1 and minimum values of 241 ind/m^2 in L12 (Table 7.2).

All localities were affected by nearby agricultural activities. L3 and L12 were also subjected to nearby sewage discharge, but always downstream from the selected translocation plot. The proximity to villages or to the city of Zaragoza was also evaluated and only localities L3 and L12 were less than 1 km away. The presence of fishermen was common along the river, especially in sites L2, L3, and L11.

7.4.2 Translocation results

A total of 638 adult specimens (mean shell length ± SD for 2017 = 153.0 ± 7.3 mm, 2018 = 152.7 ± 7.1 mm, 2019 = 151.6 ± 9.1 mm) were randomly translocated during the three years to the 6 selected localities: 291 specimens in 2017; 291 in 2018; and 56 specimens

Table 7.2 Mean values and scores of the characterization values of limiting variables and other characteristics in CIA control localities and in the Ebro River recipient localities (Score results between brackets, selected localities in **bold**, NA: not available. Variable codes as in Table 7.1).

Variables	CIA Controls	**L1**	**L2**	**L3**	L4	L5	L6	L7	L8	L9	**L10**	**L11**	**L12**
Physical and chemical													
pH	8.20 (5)	8.03(5)	8.06 (5)	7.84 (3)	7.99 (5)	8.12 (5)	8.26 (3)	7.95 (5)	7.99 (5)	8.16(5)	7.72 (3)	7.77 (3)	7.93 (5)
EC	1829 (3)	1948 (3)	2060.5 (3)	2676 (1)	1719(3)	2135(3)	1681(3)	1657(3)	1510(3)	605.2 (3)	1977.5 (3)	2177.5 (3)	1930.5 (3)
DO	117 (5)	99.8 (5)	105 (5)	108 (5)	107 (5)	109(5)	134.8 (5)	105 (5)	94.8(5)	90(5)	103.1 (5)	111.5 (5)	100.2 (5)
NO3	0 (5)	10 (3)	0 (5)	5 (3)	NA	10(3)	5 (3)	5 (3)	5 (3)	1 (5)	0 (5)	5 (3)	0 (5)
NO2	0.03 (5)	0.02 (5)	0.04 (5)	0.05 (3)	0.10 (3)	0.03 (3)	0.05 (3)	0.03 (5)	0 (5)	0.01 (5)	0.03 (5)	0.06 (3)	0.02 (5)
NH4	0 (5)	0 (5)	0 (5)	0 (5)	0 (5)	0 (5)	0.2 (5)	0.2 (5)	0 (5)	0.1 (5)	0.15 (5)	0.10 (5)	0.08 (5)
PO4	0.10 (5)	0.10 (5)	0 (5)	0 (5)	0 (5)	0 (5)	0 (5)	0.7 (1)	0 (5)	0.2 (5)	0 (5)	0 (5)	0.7 (1)
DIS	300–400 (5)	44 (3)	51 (3)	47 (3)	39 (1)	50 (3)	58 (3)	30 (1)	37 (1)	30 (1)	62 (5)	45 (3)	62 (5)
GSC	60 (3)	40 (3)	40 (3)	45 (3)	70 (5)	35 (1)	50 (3)	40 (3)	30 (1)	15 (1)	26 (1)	20 (1)	75 (5)
DS	0 (5)	<50 (3)	0 (5)	<50 (3)	<50 (3)	<50 (3)	<50 (3)	>50 (1)	<50 (3)	<50 (3)	<50 (3)	0 (5)	0 (5)
Subtotal	46	**40**	**44**	**34**	35	36	36	32	36	38	**40**	**36**	**44**

Continued next page…

Biological													
FWMd	0.02 (1)	4.9 (5)	4.5 (5)	1.4 (3)	2 (3)	2 (3)	3.6 (5)	4.9 (5)	1.4 (3)	5 (5)	0.8 (1)	7 (5)	0.9 (1)
MC	10 (5)	80 (1)	90 (1)	90 (1)	100 (1)	100 (1)	70 (1)	40 (1)	30 (3)	**0 (5)**	10 (5)	30 (3)	60 (1)
ACd	1017 (1)	1214.8 (1)	585.2 (1)	837 (1)	74 (5)	20.7(5)	2450 (1)	2722.2 (1)	2855.5 (1)	0 (5)	766.8 (1)	359.3 (3)	240.7 (3)
*Pa*_d	0.08 (1)	0.15 (3)	0 (1)	0.25 (3)	0 (1)	0 (1)	0 (1)	0 (1)	0.04 (1)	0 (1)	0.07 (1)	0 (1)	0 (1)
Subtotal	8	**10**	**8**	**8**	10	10	8	8	8	16	**8**	**12**	**6**
Anthropogenic													
DAA	< 0.5 (1)	0.06 (1)	0.09 (1)	0.28 (1)	0.1 (1)	0.1 (1)	0.32 (1)	0.09 (1)	0.02 (1)	0.03 (1)	0.05 (1)	0.1 (1)	0.05 (1)
DURB	> 2 (5)	> 1 (5)	> 1 (5)	0.1 (1)	>1 (5)	1.5 (5)	0.34 (1)	0.05 (1)	0.88 (3)	> 1 (5)	> 1 (5)	> 1 (5)	0.1 (1)
DV	> 2 (5)	3 (5)	1.8 (5)	1.3 (5)	4 (5)	1.4 (5)	3.4 (5)	0.4 (1)	1.1 (5)	1.2 (5)	1.6 (5)	1.8 (5)	0.3 (1)
FP	>3 (1)	1 (3)	2 (3)	2 (3)	0 (5)	1 (3)	1(3)	1(3)	1 (3)	0(5)	0 (5)	2 (3)	0 (5)
AP	Easy (5)	Easy (5)	Easy (5)	Medium (3)	Difficult (1)	Medium (3)	Easy (5)	Easy (5)	Easy (5)	Easy (5)	Easy (5)	Easy (5)	Easy (5)
Subtotal	17	**19**	**19**	**13**	17	17	15	11	17	21	**21**	**19**	**13**
Total scores	71	**69**	**71**	**55**	62	63	59	51	61	75	**69**	**67**	**63**

in 2019. In the CIA, six control groups (203 specimens) were established in 2017 and their survival assessed during the following three years.

In the group translocated in 2017 we found a significant loss of PIT-tags (approximately 50%) in two of the three selected localities. This situation was possibly related to either the extraordinary winter flood occurred in April 2018 (2037 m^3/s) (Fig. 7.2) or to the initial method of PIT-tag attachment, as we stuck them with cyanoacrylate glue. However, in 2018 and 2019 we used epoxy glue instead for the transponders, obtaining almost zero loss during the next floodings. No loss of both plastic labels at the same time was detected.

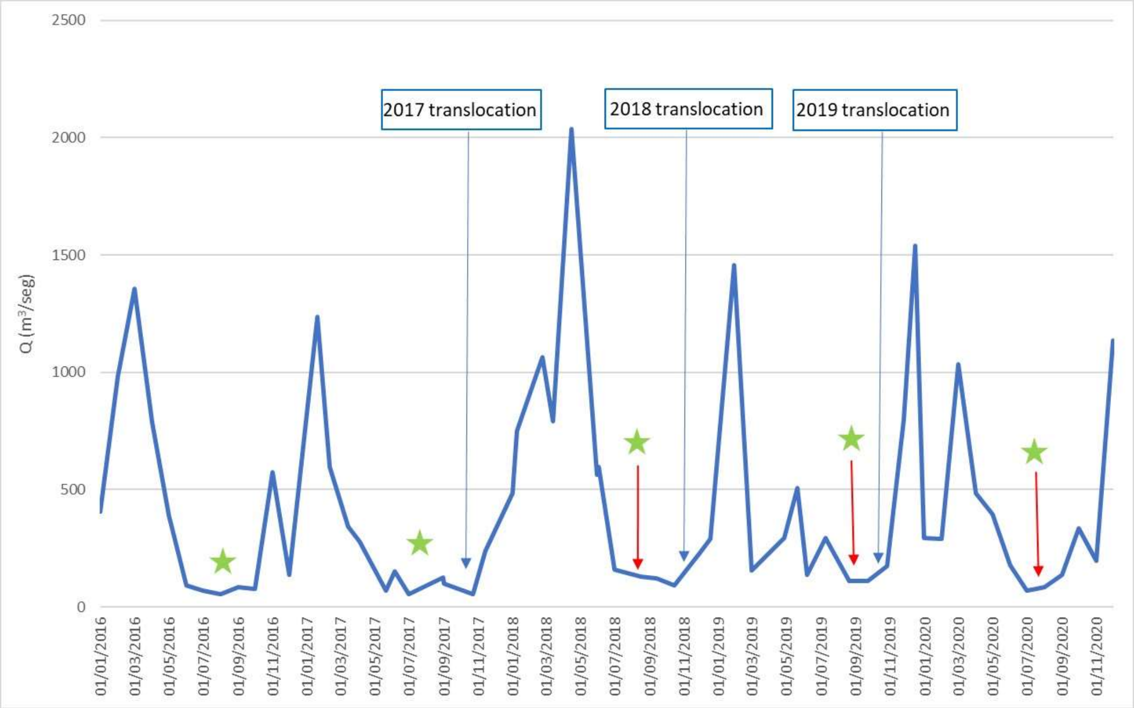

Fig. 7.2 Ebro River flow (m3/seg) during the study years (2016–2020) (blue arrows: translocation actions; red arrows: field survey of the previous year(s) translocation(s); stars: evaluation and selection of the plots)

The mean recovery rate of translocated specimens after the first year was 64.5%, but variable between years of translocation; 48% for specimens translocated in 2017 (and the same for control groups in the canal), 91% for those translocated in 2018, and 54% for individuals translocated in 2019. On the other hand, the mean percentage of specimens not being recovered within the three years and after one-year post-translocation was 35.5%, but with high differences between translocation years (52% for 2017, 8.66% for 2018, and 46% for 2019.

For the specimens translocated in 2017, the overall minimal survival ("minimal" because not all the translocated specimens were found, so we may still expect some undetected specimens to be alive) after one year was 41.6% considering all individuals from different localities together. Separately for each locality, it was 60.2% (n = 118) for L1, 27.5% (n = 109) for L2, and 31.3% for L3 (n = 64). For the specimens translocated in 2018, overall survival was 68.7%, with local survival values of 61% for L1 (n = 218), 96.4% in L2 (n = 28), and 88.9% in L10 (n = 45). After the second year, 95% and 87% of those specimens surviving the first year were again found alive for individuals translocated in 2017 and 2018, respectively. The survival of specimens translocated in 2019, after one year, was 48.7% in L10 (*n* = 37) (Fig. 7.3), excluding data from locality L3 (*n* = 19, not accessed due to the high water level; see above). Accumulated survival during the second and third years for specimens trans- located in 2017, slightly decreased (39.9% in 2019 and 37.1% in 2020). The percentage of specimens that were not detected (ND) was higher than 50% during the three years for this group of specimens and their (accumulated) mortality values reached 11% during the third year. For the specimens translocated in 2018, survival decreased from 68.7% the first year to 60.4% the second year. The percentage of not detected specimens rose from 8.6% the first year to 15.6% the second year. Mortality slightly increased (first year = 22.7%, second year = 24.0%), compared to the initial mortality value. Specimens translocated in 2019 (for which only one locality could be evaluated) showed a survival rate very similar to the proportion of not detected specimens (alive = 48.7%, ND = 46.0%) and only 5.4% of the specimens were found dead. If we only consider the recovered individuals, the estimated average mortality rate was 18% during the first year of translocation.

The control groups presented a very low survival (Fig. 7.3). A survival of only 19.7% was recorded after the first year, with a mortality rate of 27.6% and more than 50% of specimens not detected (ND = 52.7%). The second year, survival slightly increased to 23%, but there was also an increase in mortality, rising up to 40%. In the third year, survival remained at 19% and mortality increased to almost 50% and 34% of specimens were not detected. In the group translocated in 2017, survival was significantly higher in the three studied years when compared with the control specimens left in the canal the same year ($n_{2017} = 494$; first year: $\chi^2 = 26.049$, $P < 0.001$, second year: $\chi^2 = 6.140$, $P < 0.001$, third year: $\chi^2 = 8.138$, $P < 0.001$).

A total of 500 specimens of river blenny were captured in Canal de Monegros and released in four localities: L1 (196 ind), L2 (112 ind), L10 (80 ind), and L11 (112 ind).

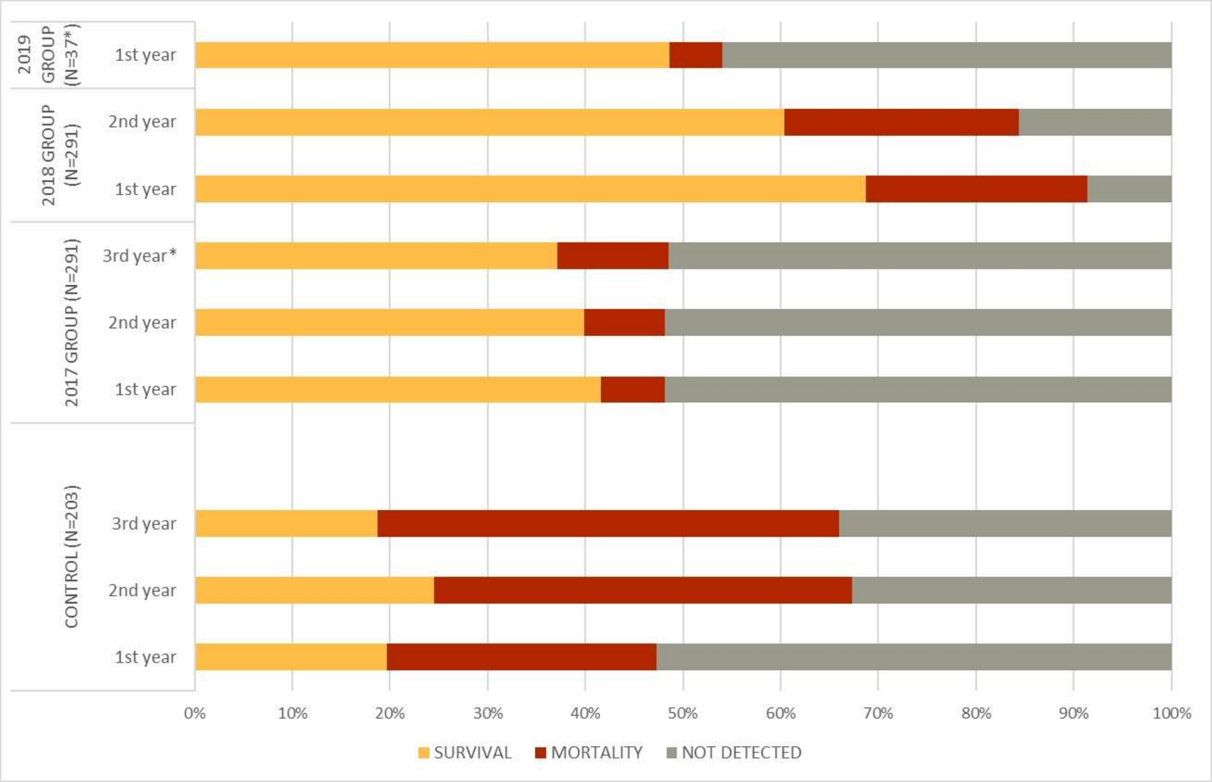

Fig. 7.3 Global survival, detection, and mortality results of *P. auricularius* translocation experiment for the control group and the groups of specimens translocated in 2017, 2018, and 2019 (*one locality could not be checked due to high river water level)

7.5 Discussion

Mitigation translocation is defined by Bradley et al. (2022) as a type of conservation translocation which has the immediate objective of relocating specimens threatened with death. Armstrong and Seddon (2008) reported a substantial increase in publications related to reintroductions and analysis of their effectiveness through subsequent monitoring using different types of organisms. Also, Bradley et al. (2022) reviewed 59 examples of studies that had been carried out to assess the best management options to improve these techniques in future. Clearly, there is a growing scientific interest in translocations, despite its controversy. One of the reasons for such controversy is the scarcity of information on translocation results, due to the lack of long-term monitoring for most of the completed translocation actions (Cosgrove & Hastie, 2001; Haag & Williams, 2014; Germano et al., 2015; Tsakiris et al., 2017; Jourdan et al., 2019).

Translocation of *P. auricularius* was assumed by the Aragón governmental authorities as an emergency task in order to save the last living specimens found in the CIA. This, in addition to captive breeding (Nakamura et al., 2019), is probably one of the few remaining options to avoid the extinction of the species in Spain. In the CIA, the protection of *P. auricularius* and other mussel species is a conservation issue with conflicting interests. On one hand, there is the utilitarian purpose of the canal used to provide water mainly for agriculture and to small villages and the city of Zaragoza. On the other hand, this canal supports a high biodiversity of organisms and their survival is not fully compatible with regular maintenance works. Reaching a balance between the two sides is complex and, at present, the CIA has reached a point of being unsustainable for mussel survival (Guerrero et al., 2021).

Even with a high mortality rate, *P. auricularius* has become the only species of freshwater mussel that still inhabits the CIA, since *P. littoralis*, *A. ana- tina,* and *U. mancus* have all disappeared (Guerrero et al., 2021). All the described disturbances may have turned the canal into an ecological trap for freshwater mussel species (Sousa et al., 2021). The CIA is now- adays an habitat invaded and modified by the Asian clam and the zebra mussel, as well as by a non-native fish directly affecting the mussels reproductive cycle. Wels catfish (*Silurus glanis*) and pike-perch [*Sander lucioperca* (Linnaeus, 1758)] are some of the non- native fish present in the canal that compete and prey on native hosts of the freshwater mussels (Soler et al., 2019), such as the river blenny and barbels (*Barbus graellsii* Steindachner, 1866*, B. haasi* Mertens, 1925). We obtained better survival results than expected for the translocated specimens, considering the under- going process of rapid mortality of *P. auricularius* in the canal. The ecophysiological condition of the translocated specimens, although not assessed, was assumed to be weak (Fig. 4). In addition, the results of the control groups that were left in the canal con- firmed that mortality had not stopped there, showing an increasing mortality reaching almost 50% after three years. Cope & Waller (1995) did an extensive review of translocations with freshwater mussels carried out in the USA

in the 1980s and early 1990s, reporting an average rate of recovered specimens of 43% and an average mortality rate (estimated considering only the recovered individuals) of 49%. In comparison, our results showed a higher recovery rate (65%) and a much lower mortality rate (18%) in the translocated habitats and, consequently, we may conclude that the decision to translocate the specimens probably was the best conservation option at this time.

Fig. 7.4 Photographs of **a**: a group of dead *P. auricularius* specimens from CIA, **b**: selected locality L1 with the transect marked, **c**: selected locality L2, and **d**: selected locality L3

Translocation should be, in all cases, one of the last options to consider for the protection of species that suffer a critical situation. However, where local population declines have the potential to put in dan- ger the persistence of the species, as is the case in *P. auricularius* in the CIA, translocation should be planned and performed (Fig. 7.4; Hart et al., 2021).

7.5.1 Management implications and risk analysis

The success of a translocation action depends on many factors and one of the hardest is the selection of the recipient localities. The adequacy habitat index implemented herein, using what we considered important variables to maximize the establishment and survival of *P. auricularius* and based on previous information and expert knowledge on its habitat preferences, seems to have worked adequately. Nevertheless, more tests using this tool would be needed to confirm its applicability in other contexts or for other species. Furthermore, there are more variables that could be added for future evaluations, e.g., hydrodynamics and hydromorphology (Geist, 2010; Holmgren, 2022), or the previous presence of the host fish in the area (Araujo et al., 2001; López & Altaba, 2005). In addition, the riparian cover should also be evaluated, as it may affect the growth of macrophytes and filamentous algae that negatively impact on mussel survival (Wilson et al., 2011). Finally, the presence of predators such as the red swamp crayfish [*Procambarus clarkii* (Girard 1852)] (Meira et al., 2019), beavers (*Castor fiber* Linnaeus 1758) (Rudzīte, 2005), or fish, such as carp (*Cyprinus carpio* Linnaeus 1758) (Feo et al., 2017), can provide extra information related to mussel mortality.

The percentage of not detected specimens is usually high in translocation monitoring (Cope & Waller, 1995; Fischer & Lindenmayer, 2000; Tsakiris et al., 2017; Zając et al., 2019) and our study was no exception. The high percentage of not detected specimens may be masking part of the survival-mortality results, even though the specimens were marked with PIT- tags in order to facilitate its subsequent location in the river (Kurt et al., 2007). Prié et al. (2018) report for *P. auricularius* a detection of 75% in the Charente River using diving. Our study reported slightly lower values (62%), but using a different methodology (i.e., aquascope surveys). Besides the possible influence of the method used, we have identified two other possible causes of this higher percentage of non-detection: some of the specimens may have been outside the plot (washed away during floods) and/or they were totally buried at the time of monitoring. Hernández et al. (2021), in their study on detectability, state that the variables that most affect the movement behavior of mussels are temperature and the type of substrate. In the Ebro River we had access to the plots only in summer, when water temperature was higher and the mussels were more active; therefore, it is quite possible that many specimens were outside the plot going unnoticed. Consequently, only long-term

monitoring including also areas outside the plots will allow a more accurate evaluation of the recover rate.

When selecting localities for translocation, it is important to take into account the effects of large flood events (Fischer & Lindenmayer, 2000; Jourdan et al., 2019; Hart et al., 2021). The extraordinary flood that occurred in the Ebro River in spring (April 2018, Fig. 7.2) just following the initial translocation a few months earlier caused that 72% and 58% of the specimens were lost in localities L2 and L3, respectively, without the possibility of finding them the following summer, even when a great effort was made to search them some kilometers downstream from the translocation point. It was even noted that the plot was mostly free of Asian clams, which surely were also affected by the flood. Zając et al. (2019) studied the dispersal and mortality of translocated thick-shelled river mussels *Unio crassus* Philipsson, 1788 after a flood, and reported low mortality (< 20%). Furthermore, among living specimens, 15% were buried, 20% were not visible, and 17% were not found. Therefore, it is possible that specimens of *P. auricularius* could be found downstream from the plots for years to come.

There are a multitude of risks when doing a trans- location and they are well summarized in the IUCN translocation guide (IUCN/SSC, 2013; Miller & Payne, 2006). For example, there is a risk of co-dispersal of bacteria, viruses, fungi, or other pathogens with the translocated specimens. Brian et al. (2021) mention that parasites and diseases in freshwater mussels are highly prevalent and may contribute to some of the massive mortalities that have been reported in recent years. Since 2013, when the high mortality of *P. auricularius* was detected in the CIA, the Aragón Government began to investigate the possible causes. From analysis of water samples, sediment, and tis- sue, in search of heavy metals, herbicides, fungicides, and pesticides (Nakamura et al., 2021), and histological sections of dead specimens of *P. auricularius* in search of parasites, bacteria, and fungi, overall results were negative or inconclusive. As Jourdan et al. (2019) stated, selecting a donor population within the same catchment as the recipient site is a simple rule to reduce the risk of transferring allochthonous pathogens or parasites. We followed this recommendation and our translocation actions were planned to take place within the same river basin, and the two systems involved - canal and river - share the same water origin.

In the same way, the inclusion of the host fish *Salaria fluviatilis* in the translocation process may be an important measure to increase the probability of future recruitment and survival of *P. auricularius* in the Ebro River. It is a fish species with a marked territorial behavior which uses shelters for the female to place the eggs (Vinyoles et al., 2002; Vinyoles & Sostoa, 2007). Taking this into account, bricks and flat stones were introduced in the plots near the mussels before the fish were released. In future, it would be interesting to check if there has been an interaction between fish and mussels, either by checking the infestation of fish in the area (checking their gills looking for glochidium larvae) or by assessing the presence of juvenile freshwater mussels in plots with- out previous presence of *P. auricularius*.

7.6 Conclusion

Pseudunio auricularius is facing a serious risk of extinction worldwide (Prié et al., 2018) and the situation in Spain is especially dramatic. The low number of recorded specimens and the disappearance and decline of the two native host fishes in the Ebro basin (sturgeon and river blenny) may impair their recruitment and future survival. Although the environmental characteristics of the river are quite different from those of the canal (higher current velocity, greater annual flow changes, higher macrophyte and algal cover, among others), the results reported here indicate that the translocated specimens have a higher survival in the Ebro River than in CIA. In addition, translocating specimens to various localities in different years can decrease the risk of mortality due to catastrophic events and maximize the probability of survival and establishment of new sub-populations. However, improvements in the methodology and in the index score used to evaluate potential recipient localities should be pursued and future monitoring should assess not only the survival of *P. auricularius* but also its reproduction and recruitment.

7.7 Acknowledgements

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Chapter 8

General discussion

The work developed in this thesis is part of the recovery plan of *P. auricularius* in Aragón, which started in 2005. Results made it clear that the critical situation of this species in the Ebro River basin has not improved, reflecting how hard and difficult it is to improve the status of an endangered species. Actually, the conservation status of *P. auricularius* has deteriorated over the years, especially aggravated by a mortality episode in 2013 in the CIA (Guerrero et al., 2021; Nakamura et al., 2018a), an anthropogenic habitat area where the highest abundance of specimens in Spain had been recorded (Araujo & Ramos, 1998; 2000). This situation was not restricted to *P. auricularius*, since a general decline of *Potomida littoralis, Unio mancus*, and *Anodonta anatina* was also registered. Araujo et al. (2000), at the end of the 1990s, reported an abundance of more than 900 specimens of the four species (including *P. auricularius*) in a single 150 m-stretch in the CIA. A similar abundance was reported in 2004-2005 in the Ebro River and in the canals (Nakamura et al., under review). Nowadays, however, *P. littoralis, U. mancus* and *A. anatina* are considered extinct in the canals. In the river, although their decline seems to be less pronounced, sites with similar abundances to those reported 20 years ago are impossible to find (Guerrero et al., 2021; Nakamura et al., 2022).

Since its classification as critically endangered in Spain, *P. auricularius* has acted as an umbrella species, so that the conservation actions carried out to benefit this species are thought to benefit the entire mussel community, and even the entire aquatic ecosystem. For 20 years, the conservation actions devoted to the conservation of *P. auricularius* have gained significant influence in Spanish administration agencies, promoting the coordination of the different actions carried out in the Ebro basin, and increasing the visibility to this group of fauna (Order: Unionida). For example, during repairing actions of affected areas after floods, the annual maintenance works within the river and canals are nowadays performed taken into account the survival of this species and minimizing its mortality risks. Furthermore, since captive breeding has been successful in Aragón (Nakamura et al., 2019), two important actions have been implemented: the first one consists on the reintroduction of the largest captive-bred juveniles into the Ebro River (2014 to 2019 cohorts) (Fig. 8.1); and the second relates to

the collaboration with other Spanish regions (Catalonia, Navarra, and the Basque Country so far) to test the survival of the juveniles using water from tributaries of the Ebro River rather than from the main channel, so as to evaluate the possibility of survival in other areas. In addition, an increased sampling effort allowed finding young specimens since 2010 in CT and since 2014 in CIA. Most of the specimens found have a total shell length between 8 and 10 cm, corresponding to specimens of approximately 12 -15 years old (Nakamura et al., 2018b). The discovery of those young specimens suggests that natural recruitment is possible and that the recovery of the species in the current habitat conditions may be feasible.

Fig. 8.1 Captive bred juvenile of *Pseudunio auricularius* ready for their reintroduction into the Ebro River.

Unfortunately, and despite all this progress, the conservation situation of the species is increasingly critical, reaching the point of having to try to save the last living adult specimens from the CIA by translocating them to the Ebro River, where they need to tolerate different environmental (e.g. hydrology) conditions.

8.1 *Pseudunio auricularius* mortality: an unsolved issue

The causes of mortality of unionids at a global scale still remain, in most cases, unknown (Haag, 2019). Although recent work is beginning to use different biomarkers to know how to distinguish healthy from unhealthy mussels (Fritts et al., 2015), in most cases the evidence for mortality events is still weak or the true causes of death are still to be discovered (Downing et al., 2010). Pollution, agricultural run-off and eutrophication, droughts and floods, habitat modification and siltation and clogging of stream beds are common causes mentioned during freshwater mussel die-offs (Lopes-Lima et al., 2014; 2017). Examples of uncertainty include the case of *M. margaritifera* in Sweden (Wengström et al., 2019), where specimens with lesions in the digestive gland were reported, but with no apparent direct cause; *M. falcata* in the Upper Bear Creek River, Washington, where individuals with tissue pathologies were found but no specific cause of mortality was determined (Waller & Cope, 2019); and mussel die-off observed in the Big Darby Creek River in Ohio, where all species were affected, and *Actinonaias pectorosa* in Clinch River where a mass mortality was reported without an apparent cause (Leis et al., 2018). These unsolved die-offs contrast with other cases, where the etiological agent is readily known or at least related to some physical or chemical variables, e.g., the case of freshwater mussels in Hawkesbury-Nepean basin (Australia), where the high modification of riparian vegetation associated with anthropogenic activities (urban development and agriculture) were strongly related to the decrease in density and even the extinction of freshwater mussels (Brainwood et al., 2006), or in the marine environment, where the rapid decline in the fan mussel (*Pinna nobilis* L., 1758) detected in Spain in 2016 was related to a new haplosporidium parasite (Grau et al., 2022).

The causes driving the mass mortality of freshwater mussels are often hypothesized but hardly verifiable for different reasons: a direct causal agent is not found, be it a contaminant, a disease, competition, predation, or alterations of the habitat. The overall causes usually include socioeconomic activities (e.g., agriculture, population growth, urbanization) or climate change that determine the frequency and intensity of other anthropogenic pressures (e.g., increased diffuse pollution during each rainy season), and which modify the physical, chemical, and biological characteristics of the ecosystem (Perujo et al., 2021; Sousa et al., 2022).

With regard to *P. auricularius* in Spain, in addition to the reduced population density since its rediscovery in 1996, we must add the high mortality rates with an unknown cause since 2013. The situation in the 1990s was already extremely risky: a small and highly fragmented population (Altaba, 1990; Araujo & Ramos, 1998). However, as the species started to be studied, the population data increased thanks to the annual monitoring sampling program, and while in Catalonia region the species disappeared, in Aragón, the population censuses established that from the initial 2,000

known specimens in 2000 there were actually almost 6,000 in 2013. In 2013, a mass mortality episode was detected accounting for more than half the cumulative mortality in the previous 7 years together (2005-2012: 448 dead specimens found; February 2013: 238 specimens, Fig. 8.2) (Nakamura et al., 2018c). This high and initially localised mortality was the triggering factor for this thesis trying to rise and test several hypotheses explaining these declines.

Fig. 8.2 Dead specimens found in different sections of the CIA.

Climate change is undoubtedly another threat impairing mussel survival in the Ebro River basin. The rise in temperature implicitly involves an alteration of all the components of the hydrological cycle, directly causing a decrease in available water resources as well as a higher level of direct evaporation and evapotranspiration. The main effects of climate change are causing a reduction in river flow and an increase in its variability, which implies that episodes of extreme droughts, combined with strong floods, will probably increase in number and intensity (Karl & Trenberth, 2003; Lespinas et al., 2014), potentially resulting in mussel die-offs (Dubose et al., 2019; Nogueira et al., 2021). This situation is even exacerbated by the continuous extraction of water for use in agricultural activities (Nakamura et al., 2021).

A decreasing river flow may cause situations such as the one experienced in 2017 in a lateral channel of the Ebro River where the water entrance was completely closed. A management work was then carried out with heavy machinery to lower the level of the substrate at the entrance to facilitate the input of water. This was the site where the last group of living specimens of *P. auricularius* had been registered in the Ebro River in Aragón, and the monitoring results of that year confirmed the survival of at least 30 specimens out of 40 previously registered. During the same year, a high mortality of Asian clam *Corbicula* spp. was registered in the area, with hundreds of dead bodies floating on the surface of the water. Two years later, 14 specimens of *P. auricularius* were found dead and only one alive. The question was then which factor might have triggered this mortality to a greater extent: the massive mortality of Asian clams producing high ammonium concentrations due to tissue decay, or the lack of flow triggering high temperatures and decreased oxygen, or a combination of both factors.

The mortality of *P. auricularius* and other freshwater mussels is by no means a new fact, nor has it begun in the Aragón region. Already in 2005, in the Lower Ebro River in Catalonia, mortality episodes were recorded beginning with *P. littoralis, A. anatina*, and *U. mancus*, and finally, *P. auricularius* specimens also died. According to the mussel specialists at that time, the main causes attributed to those mortalities were the biological and hydrological changes produced during those years in the river: the colonization and population explosion of the zebra mussel in the reservoirs upstream, a sharp increase in the water transparency and subsequent exponential increase of macrophytes density. All these changes would have contributed to the reduction of phytoplankton density (necessary as a key food resource to mussels) and increased the sedimentation of fine particles (largely due to the effect of macrophytes) creating unfavorable anoxic conditions for mussels (LIFE 00 NAT/E/007328; López pers. com.). This regime shift, from phytoplankton to a macrophyte-dominated system (Ibáñez et al., 2012a, b; 2020) has been moving upstream and is now also affecting the middle section of the Ebro River, a section considered to have the best environmental conditions for mussels (Rubio et al., 2016). In very dry years like 2012 and 2017, the increase in macrophyte density and water transparency have been evident and could be responsible for ecological changes, from individuals to ecosystems. For example, the reduction of water flow, with the consequence of macrophyte cover increase and accumulation of decomposing organic matter, has lead in the Ebro River to the proliferation of the black fly *Simulium*

erythrocephalum (De Geer, 1776), even causing health problems to humans (Ruiz-Arrondo, 2018). Consequently, the administrations in charge of water and river management respond by deeply cleaning the riverbeds (by dredging) to reduce the macrophyte cover and in this way reduce spawning sites for Simuliidae. This situation is financially costly and is also responsible for the destruction of mussel habitats.

On the other hand, in wet years with extraordinary floods such as those occurred in 2015 and 2018 in the Ebro River, the macrophyte (and Asian clam) density notably decrease, but many mussels also are drifted downstream and sometimes deposited in the banks, which may also impair their survival (Nakamura et al., 2022).

Fig. 8.3 Bottom of the CIA invaded by the Asian clam *Corbicula* spp.

The Asian clam has massively colonized habitats previously occupied by or suitable for *P. auricularius* in the Ebro River basin and currently several authors have demonstrated the negative effects of this invasive species on mussels. This non-native species can negatively affect the recruitment of freshwater mussels and compete with juvenile and adult mussels, affecting their survival (Ferreira-Rodríguez et al., 2018; 2022; Haag et al., 2021; Kelley et al., 2022; McDowell & Sousa, 2019). In the CIA, Gimeno Calvo et al. (2017) reported an average density of alive Asian clams of 1010 ind/m^2, compared to 0.03 ind/m^2 of freshwater mussels (Fig. 8.3). These high densities predictably have multiple effects on the ecosystem, including a sharp drop in primary production (Pigneur et al., 2014) given the filtering capacity of these small bivalves, which can affect the quantity of food available for mussels. At the same time, it can reduce or modify the habitat available for juveniles and even capture sperm, free glochidia and small unionid juveniles due to filtration (Modesto et al., 2018; 2019).

Fig 8.4 Zebra mussels attached to a *Pseudunio auricularius* specimen from the CIA.

The negative effect of the zebra mussel (*Dreissena polymorpha*) on freshwater mussels has been also demonstrated as they can attach to the mussel shells and directly compete for food and affect their biological processes such as respiration or reproduction, and even their ability to burrow in the sediments (Sousa et al., 2011). Its presence has been described in the CIA, with up to 100 dreissenid mussels on a specimen of *P. auricularius* (Fig. 8.4). However, this invasive species is present only in a specific canal section (from kilometers 34 to 39) being very rare in the rest of the canal for unknown reasons.

Introduced fish species can also be an important threat to freshwater mussels by contributing to the disappearance of native compatible fish, or through dilution of glochidia larvae, which may attach to their gills but not develop further and be finally lost in case of attaching to incompatible exotic fish (Douda et al., 2013). Wels catfish (*Silurus glanis* Linnaeus, 1758), pike-perch (*Sander lucioperca* Linnaeus, 1758), and recently black bass (*Micropterus salmoides* Lacépède, 1802) compete and prey on native fish that are becoming increasingly rare in the habitats where mussels live. The European bitterling *Rhodeus amarus* (Bloch, 1782) parasitizes mussels by laying their eggs on the gills, interfering with their basic filter-feeding processes. This fish is expanding to the East and South of France putting the populations of *P. auricularius* in the Creuse River (France) at risk (Soler et al., 2019). Although this fish species is still not present in the Ebro River basin, future monitoring programs should take in account a possible introduction and consequent negative impacts on the native biodiversity, including on *P. auricularius*.

8.2 Significance of the estimated length-age relationship

The beginning of free life in juvenile mussels, according to the knowledge of other Margaritiferidae species, starts by living totally buried in the substrate since they fall from the gills of the host fish. The transition to adulthood occurs when the juveniles emerge from the substrate, which takes about 5 years in *M. margaritifera* (Hruska, 1999). Then, these early adults are usually half-buried and adapt their feeding behaviour in response to water flow, oxygen, and temperature variations (Haag & Rypel, 2011; Hastie et al., 2000; San Miguel et al., 2004). In *P. auricularius,* these basic ecological aspects were mostly unknown until recently. In chapter 4 of this thesis, we suggested that adulthood may be reached at the age of 7 years for *P. auricularius*, coinciding with the inflection point in the growth sigmoidal model. However, under captive conditions, the behavior of juvenile *P. auricularius* seems to be more influenced by temperature and light conditions, as they do not live totally buried as in their natural habitat (Nakamura K., personal, obs.). We have observed that 2 - 3 years-old specimens live on the substrate surface during the entire summer season without burrowing. On the contrary, during winter they are completely buried (Nakamura K., personal, obs.), confirming that the growth and behavior of freshwater mussels strongly depend on environmental conditions (Hastie et al., 2000). Therefore, more autecological studies are necessary to elucidate some of these varied responses to environmental factors.

The aforementioned inflection in the sigmoidal growth curve can also be attributed to another aspect related to age, such as the start of the reproductive stage, which is still unknown in *P. auricularius*. The energy diversion needed to start the reproductive stage substantially slows growth (Roff, 2002; Minte-Vera et al., 2016), so that the timing can be attributed to that inflection point in body growth. It has been proposed in this thesis that for *P. auricularius* the beginning of the reproductive stage could be at an age of 7 years, corresponding to a shell length between 40 - 50 mm and coinciding with the size of the smallest specimens recorded in substrate surface in the wild (53 mm, Canal de Tauste). Haag & Ripel (2011) proposed an age between 5 and 13

years to attain sexual maturity in *M. margaritifera*, but in other unionid mussels it can vary greatly, e.g. 1-2 years for *Lampsilis ornata* (Conrad, 1835), or up to 3–9 years for *Quadrula asperata* (I. Lea, 1861) (Haag & Leann Staton, 2003). In chapter 4, we also reported that laboratory specimens with a shell length of 100-mm (~15 years) can release glochidia. In recent observations we detected that specimens with a shell length of 80 mm (~12 years) could also release glochidia, but some doubts still exist on the age at first reproduction in the reproduction cycle of this species.

Haag & Leann Staton (2003) found out that fecundity increased exponentially with size in 6 of 8 mussel species studied. In contrast, in four species, fecundity decreased in the older animals even though they continued to produce large numbers of glochidia. These basic ecological issues are currently unknown for *P. auricularius* and therefore further studies should be done in order to acquire this important biological information. In theory, this information will be vital to increase juvenile and adult survival probabilities and the reproductive success of mature animals in laboratory and field conditions.

8.3 The toxicology of European freshwater mussels

Freshwater mussels, as filter feeders, are directly exposed to contaminants found in their habitat due to several anthropogenic activities. These contaminants can seriously affect their physiology and therefore their ultimate survival. In some cases, mussels are used as early warning systems for a multitude of human disturbances, including pollution, e.g., several cities in Poland are using freshwater mussels to monitor the quality of the water consumed by 10 million people (https://www.zmescience.com/science/poznan-mussel-water-plants-892524/).

In Europe, toxicological data on native unionid species are scarce. The works developed at the University of Bordeaux in France began with invasive species such as the zebra mussel and especially the Asian clam, studying the metallothionein response to metal exposure, and cadmium and zinc bioaccumulation kinetics (Baudrimont et al., 1997a; b; 1999). More recently, studies have been using *M. margaritifera* adults and juveniles from the Dronne River to assess the response and sensitivity of this mussel to contaminants such as heavy metals, as well as the assessment of transcriptomic and

epigenetic responses, opening the possibility of using this basic information as a protection measure for the species (Baudrimont et al., 2020; Belamy et al., 2020; 2022; Bertucci et al., 2017). In chapter 5 of this thesis, the first sensitivity values of *P. auricularius* against a wide range of metals and ammonia were reported. This is the first step to trying to understand how to improve the conservation status of *P. auricularius* and understand the factors (e.g. pollution) that may be triggering its population decline. Designing a pollution control program as part of a restoring plan for disturbed areas is one of the management actions that could be carried out taking into account the reference values obtained here. This information could also be used for human health purposes, by testing water toxicology with glochidia and/or juvenile mussels, since the CIA water is a resource used by the city of Zaragoza.

Further steps should be aimed at continuing to investigate *P. auricularius* sensitivity to compounds commonly used in agriculture, which are present throughout the Ebro River basin (Nakamura et al., 2022b). For this purpose, juveniles obtained in captive breeding are an excellent resource to develop toxicological tests without sacrificing wild animals, obtaining sensitivity results directly from the target species without having to use surrogate species that do not always respond in the same way. In addition, the use of biomarkers (ABC transporters activity, acetylcholinesterase activity in hemolymph, glycogen content, etc.) in wild adult specimens can be used to obtain information on the health conditions of the species in its own habitat, being potentially useful to predict mortality events.

8.4 Black boxes in *Pseudunio auricularius* biology

This thesis can be considered an important contribution to the knowledge of the biology and conservation of *P. auricularius*. However, some basic aspects of its biology remain unknown, and we discuss some of these gaps in the following paragraphs, suggesting new avenues of future research.

Grande et al. (2001) stated that *P. auricularius* is a hermaphrodite species, based on the study of 13 specimens from the CIA deposited in the Natural Museum of Sciences in Madrid. Out of these 13 specimens, 10 were determined as hermaphrodites and 3 were considered females. The conservation status of the species, when it was rediscovered in

1996 and when the animals for this study were collected, was already critical due to the low population size. Therefore, hermaphroditism could be a consequence of a bottleneck process. However, there are no old references on the diodicity and sex ratio of the species and consequently this issue on the hermaphroditism of the species is still open. In this sense, a histological comparison with French populations that have larger densities could be interesting to pursue.

Each year, gravid adult specimens are collected for captive breeding, so that fertilization process is already fulfilled in the natural habitat, without much information being recovered on reproductive patterns. Such unknown information becomes especially relevant when it comes to studying the causes of reproduction failure and when trying to solve them. For instance, we need to know how the male and female sexual functions are distributed among the specimens in the field, how long the sperm require to find an egg before dying, or what is the number of gametes that a specimen can produce. In 2011, it was impossible for our team to obtain glochidia from the specimens collected in the field (Aragón Government, unpublished data). Several questions arose around this exceptional issue: Was it a problem of distance between adults, meaning that the ones that played separated roles of male and female were too far apart? Was it caused by the absence of male specimens? May it be a similar case to the feminization in carp fish reported in the lower Ebro (Lavado et al., 2004)? Or simply a decrease in the reproductive capacity of very old specimens? Studies with young specimens can shed light on this issue, although at present it is unthinkable and unethical to sacrifice specimens due to the critical situation of the species. Non-lethal techniques such as foot puncture for the extraction of hemolymph could be used, although these techniques have also some caveats (Martínez-Pita et al., 2016; Tsakiris, 2016).

Another aspect about the reproductive cycle of *P. auricularius* that is unknown is the mechanism of attraction between host fish and adult mussels. Questions such as whether the river blenny can see glochidia conglutinates when it passes near the adult specimens and approach them to eat? Or otherwise, the approach is totally random (especially considering that the visibility conditions in the Ebro River are almost null due to high turbidity). These questions remain to be answered. In the same way, the glochidium of *P. auricularius* has a very short life cycle, between 24-48 h (Nakamura K., unpublished data) and if they do not find quickly a suitable host, they die (Fig. 8.5). Controlled mesocosm experiments could give some insights concerning this issue.

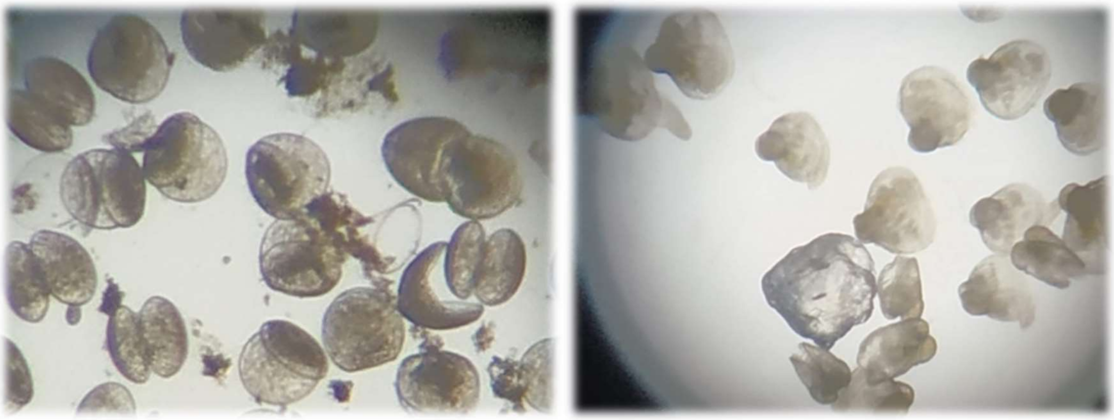

Fig 8.5 Glochidium (left) and juveniles (right) of *Pseudunio auricularius* observed through a binocular microscope.

Knowing juvenile gill development can improve understanding of feeding mechanisms and allow sounder decisions to be made during the captive breeding process. In *M. margaritifera,* it is known that gills are not fully developed until almost 3 years of age (34 months) (Lavictoire et al., 2018). Araujo et al. (2018) reported the transition from pedal feeding to filter-feeding for *M. margaritifera* and *Unio mancus* occurring around 150-200 days and 70 days post-emergence, respectively. In *P. auricularius* it is not known when this change occurs, but based on experience during captive breeding, it seems that it happens upon reaching a millimeter in length, which occurs between 70-90 days (Fig. 8.5), when mortality decreases substantially, which may be related to a change in the feeding strategy (Nakamura et al., 2019).

8.5 Conservation implications

Freshwater bivalves (Unionida) are one of the most endangered invertebrate groups in the world (Böhm et al., 2021; Lopes-Lima et al., 2018a) and are disproportionately imperiled compared to other taxonomic groups (Collen et al., 2014; Dudgeon, 2019; Maasri et al., 2022). They perform key ecological roles and provide valuable environmental services that ultimately can affect human well-being (Dubose et al., 2019; Lopez et al., 2022; Vaugh, 2018; Zieritz et al., 2022).

Considering the high mortality rate of *P. auricularius* during the last decade and the extinction of the other three species of mussels in the canals, together with the hopeful results of the translocations of specimens from the CIA to the Ebro since 2017, it seems clear that the future of the species is in its natural habitat: the river. However, the environmental conditions for unionid mussels in rivers of the Iberian Peninsula are increasingly detiorating due to the multitude of threats discussed throughout this thesis, especially regarding the un-natural river flow that makes it increasingly difficult for the mussels to survive in the rivers, leaving them restricted to small areas that barely conserve the minimum biological and ecological requesites for their survival.

We have suggested that captive breeding and translocations are the main actions that can currently contribute the most to *P. auricularius* survival in Spain. However, if the conditions of the river do not improve in the near future, these efforts will not be effective, since there will be no suitable sites to reintroduce the specimens. Habitat restoration actions, such as the recovery of the fish community, recovery of the riparian forest that creates fish refuges and reducing water pollution, among others, should be considered to allow a successful recolonization of the Ebro River basin by *P. auricularius*.

Habitat restoration is a difficult task and requires, in addition to political will, a lot of time and money due to the complexity of the system, the size of the basin to be managed, and the increasing number of actors that are involved. Consequently, several administrations in charge of controlling different uses of the aquatic system (water, soil, biodiversity, among others), plus farmers and ranchers, fishermen, and recreational and tourism companies need to cooperate in order to apply the most adequate restoration measures. In the same way, the creation of protected areas for *P. auricularius*, especially in areas where there is a confirmed presence of the species or those with recent or historical records, would contribute to its long-term survival. Such areas must be properly identified so that the basin management administration may establish adequate management measures to guarantee the conservation of *P. auricularius* populations and mitigate their habitat alterations.

The EU Biodiversity Strategy for 2030 proposes, among other targets, at least 25,000 km to be restored into free-flowing rivers (Bastino et al., 2021). Theoretically, this will have positive consequences for the habitats of several riverine freshwater mussel species and their host fish. Therefore, new opportunities of funding will be soon available and the application of restoration measures for the conservation of *P. auricularius* should be a priority in order to save this species from extinction. The autecological information collected in this thesis will provide support for the application of the best restoration measures in order to bend the curve of decline of this iconic riverine species in Spain.

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Conclusions

1. A trend of sharp density decline of the freshwater mussel populations in the Ebro River basin during the past two decades was observed, eventually reaching local extinction of three out of the four species previously found in the studied canals (Canal Imperial de Aragón - CIA and Canal de Tauste - CT): *Anodonta anatina, Potomida littoralis* and *Unio mancus.* Nowadays, *Pseudunio auricularius* is the only native freshwater mussel with living specimens remaining in the canals.

2. The study of more than 6000 marked individuals of *P. auricularius* by means of capture-recapture analysis showed a pattern of increasing mortality rates, especially since 2013, when a mass mortality episode was registered in CIA. Annual survival probability decreased repeatedly, attaining final values below 0.25 in 2020 in the CIA. However, the survival of *P. auricularius* remained close to 1 during the same year in CT, whose population structure showed a higher proportion of young individuals than in the CIA.

3. The growth pattern in *P. auricularius* is best fitted by sigmoid rather than asymptotic models, which allows estimating an inflection point during early growth and establishing a more accurate size-age relationship. The inflection point, corresponding to an age of seven years, may be related to the beginning of the reproductive period or the emergence of the juvenile from inside the substrate to its surface.

4. The growth of *Pseudunio auricularius* shows, after the inflection point at an age of seven years, a decelerating rate until an age of about 30 years, when growth attains an asymptote at c. 150 mm shell length. Consequently, shell size is not an accurate enough measure to estimate the age of large individuals of *P. auricularius.*

5. The sensitivity of *P. auricularius* to heavy metals and ammonia was assessed for the first time. These estimations are very valuable when it comes to evaluating potential causes of mortality and to implement effective management actions devoted to the conservation of *P. auricularius* (and other freshwater mussel species). The sensitivity of *P. auricularius* was found to be greater to cadmium and copper, and lower to lead

and nickel. Furthermore, juveniles appear to be quite resistant to relatively high concentrations of total ammonia nitrogen.

6. The first successful protocol for captive breeding of juveniles of *P. auricularius* was here established, making it possible to keep juveniles alive for more than one year, and attaining a shell size larger than one millimeter, after which mortality significantly decreases in captive individuals.

7. The best experimental conditions for *P. auricularius* juvenile survival and growth corresponded to a culture treatment in glass containers at a low density of 0.2 ind. L^{-1}, using river water, with added substrate and detritus, enriched with phytoplankton, and avoiding extra aeration in order to maintain stability within the culture. When the juveniles of *P. auricularius* reach a shell length between 500 µm and 1 mm, they probably begin filter-feeding, without stopping pedal feeding.

8. The translocation to the Ebro River of part of the *P. auricularius* specimens that were still found alive in the CIA was considered a success. A total of 638 specimens were translocated during three consecutive years in which a first-year survival of between 40 and almost 70% was recorded. One year later, ~90% of these specimens were found alive again, suggesting a successful initial establishment. In contrast, the control group left in the CIA in 2017 showed a much lower survival rate of less than 20% after one year, which remained equally low during the next two years.

9. Currently, the conditions in the Ebro River seem to allow a higher survival rate for *P. auricularius* than those in the CIA. However, long-term monitoring will be necessary to check if there is a complete acclimatization of the translocated specimens to the hydrological, physicochemical and biological conditions of the natural habitat.

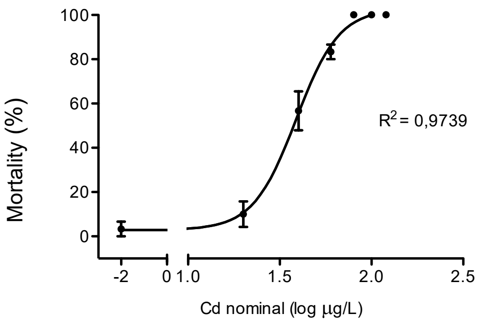
10. Given the *P. auricularius* current conservation situation, and according to our results, captive breeding and translocations would be the actions that could contribute the most to its immediate survival in Spain. However, other priority actions such as habitat restoration and recovery of the native host fish populations should also be considered for a successful recolonization of the Ebro River basin by *P. auricularius*.

Anexes

Chapter 5. Supplementary Material

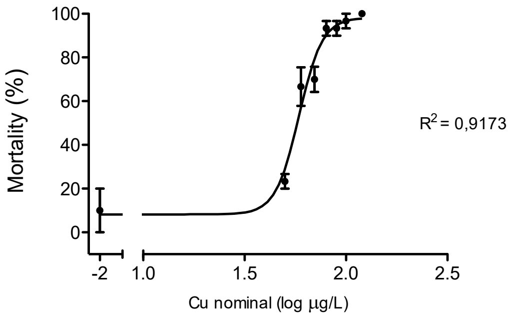
Fig. S1: Mortality response curves for the analysed toxicants tested with *Pseudunio auricularius*.

Cadmium



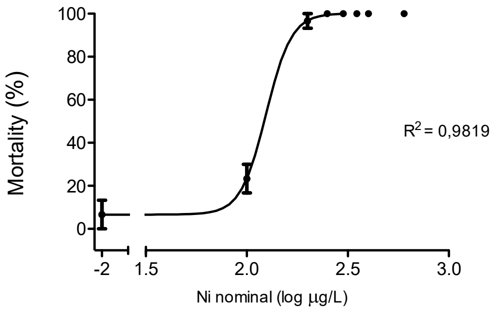
EC50 = 38,85 µg/L

Copper



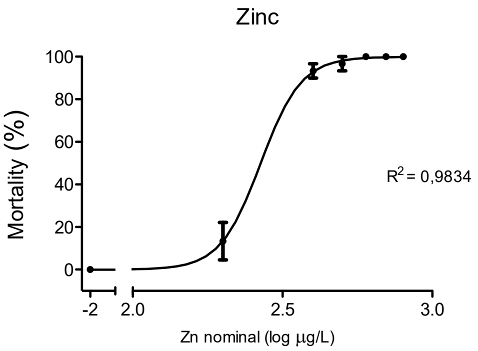
EC50 = 58,64 µg/L

Nickel



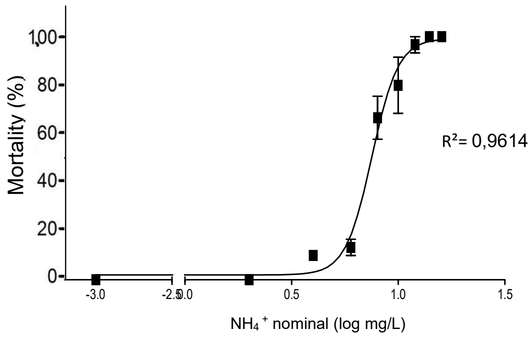
EC50 = 124,6 µg/L

Zinc



EC50 = 267,4 µg/L

TAN (Total ammonia nitrogen)



EC50 = 7,53 mg/L

Results diffusion

Publications related to this thesis (in alphabetic order)

Nakamura, K., Cañete, J., Vijuesca, D., Guillén, N., Sosa, C., Mesquita-Joanes, F., Sousa, R., Ginés, E. & Sorribas, V. (2021). Sensitivity of *Pseudunio auricularius* to metals and ammonia: first evaluation. Hydrobiologia, 848, 2977-2992.

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Communications in conferences

(The underlined author presented the communication)

Nakamura K., Elbaile E., Guillén N., Sosa C. & Sorribas V. (2022). Acute toxicity of two herbicides (prosulfocarb and metolachlor) to the Giant Freshwater Pearl mussel (*Pseudunio auricularius*, Spengler 1793) in the Ebro River basin. XXIV Congreso Español de Toxicología y VIII Iberoamericano. Córdoba, 9-11 noviembre. Póster.

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Nakamura K., Guerrero-Campo J., Ginés E., Sousa R., Mesquita-Joanes F., Alcántara M. (2021). Last chance for *Pseudunio auricularius* in Spain: results from translocation and captive breeding programs. 87th Annual Meeting American Malacological Society. Cape Breton, Nova Scotia, June 14-18. Oral presentation (virtual).

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Nakamura K., Cañete J., Vijuesca D., Sorribas V., Guillen N., Sosa C., Armengol X., Ginés E., Guerrero J., Sousa R., Mesquita-Joanes F. (2018). Sensitivity of *Margaritifera auricularia* (Spengler, 1793) to heavy metals and ammonium: first approximation. V Jornada científica ICBiBE, Valencia October 26th. Oral presentation.

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