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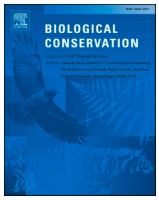

Collapse of native freshwater mussel populations: Prospects of a long-term study

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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) using four native freshwater mussel species (Order Unionida) colonizing two canals of the Ebro River (Spain). Special attention was given to *Pseudunio auricularius* (Spengler, 1793), a critically endangered species. Generalized linear mixed models results showed significant temporal effects on mussel densities, with a continuous decline in all species present, being *Anodonta anatina*, *Potomida littoralis* and *Unio mancus* now considered locally extinct. *Pseudunio auricularius* is still present in the studied canals, but at very low densities (0.01 ind/m^2). Capture-recapture data on *P. auricularius* show a progressive decline in its survival probability, down to 0.15 in 2020 in the Canal Imperial de Aragón, although in the Canal de Tauste 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.

1. 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 and Garcia, 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 and 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 an 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; Rosenberg et al., 2019).

Aquatic ecosystems face an extreme risk of defaunation, and especially freshwater ecosystems, which have long been recognized 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; Dirzo 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 1428 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; Dudgeon, 2019; Eastwood et al., 2022; He et al., 2019; Palombo, 2021; Reid et al., 2019).

Studies on biodiversity decline have been generally assessed thanks to long series of data collected over years of study (Gilmour et al., 2013; Lister and Garcia, 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

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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 and 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 and Garcia, 2018) or other important ecological features. This lack of quantitative data is also problematic for the assessment of ecosystem services provided by freshwater organisms, 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 and 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) (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 (Zieritz et al., 2022; Sousa et al., 2022).

Many studies have tried to unravel the different causes of freshwater mussel mortality but most of these causes remain uncertain (Haag et al., 2019; Haag, 2019; Strayer et al., 2004; Sousa et al., 2022). 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 domestic, industrial and agricultural 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 priéis that most of the effects 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 Red List (Prié, 2021). Currently, this species shows a very restricted distribution that includes Spain, in the Ebro River basin (Altaba, 1990, 1997; Araujo and Ramos, 2000, 2001; Gómez and Araujo, 2008; Nakamura and Guerrero-Campo, 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 and Cochet, 2010; Prié et al., 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 during 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 and Ramos, 1998; Álvarez Halcón, 1998a). Since then, 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 conservation situation of unionid populations 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.

2. 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 function 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 and 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; from there on 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 harbors the highest number of young *P. auricularius* specimens registered so far in Spain (Nakamura et al., 2018b; Guerrero-Campo et al., 2021).

2.1. 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 done 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 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 2 m-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 section affected by

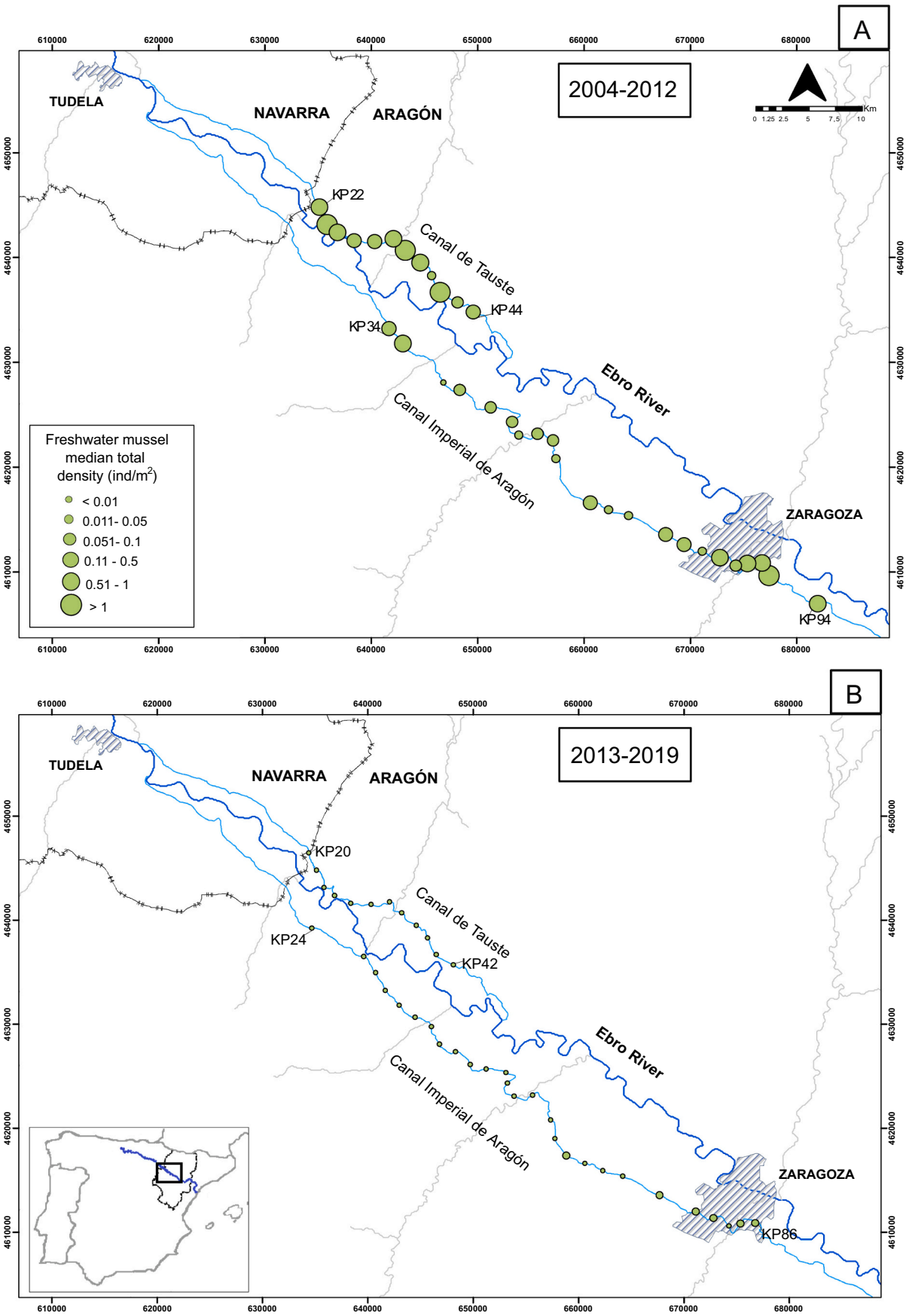

Fig. 1. Study area in the Ebro River Basin (Eastern Iberian Peninsula), with indication of the median density values recorded every 2 km in the Canal Imperial de Aragón and Canal de Tauste in two periods (see the Results section in the text for further explanations): years 2004–2012 (A) and years 2013–2019 (B).

Table 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 maintenance works was, for example, 500 m long, five transversal transects were surveyed. In some sections and due to the presence of *P. auricularius*, transects were set every 50 m. 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 (±0.1 mm). Shell measures were obtained between 2004 and 2019 from both canals. 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. Thanks to the collaboration agreement between the National Science Museum of Natural Sciences and the Aragón Government, information on *P. auricularius* marked and recaptured individuals, starting from 1998 for the CIA and 2002 for CT, could be obtained including date, location and label code.

2.2. Data analysis

Density data ($n = 1088$) of living individuals for each species 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 the number of living individuals (i.e. abundance) of each species as the response variable, and $\log_{10}$ of the sampled area as an offset variable to properly consider abundance as corrected by sampled area (i.e. density). Therefore, we will refer from now to density as the response variable to avoid any confusion. Year was introduced in the models as a fixed factor expected to affect density, whereas KP within each canal, canal itself, and sampling methodology were included as random factors. The sampled location in each canal was approached to the nearest even kilometer in order to reduce the number of categories in the hierarchical random variable KP|Canal to facilitate model convergence. Years 2004–2019 were transformed to a 0–15 scale. Models were built using a negative binomial distribution, as a first option, and a log link function, to account for overdispersion. If the negative binomial model presented convergence problems, we used a Poisson distribution instead. GAMs were carried out with the package *mgvc* (Wood, 2017), and all ZI-GLMM analyses were done 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 found alive or dead. Consequently, we used a joint live and dead encounter (Burham) model parameterization in the MARK software (Cooch and 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. Results

3.1. Changes in mussel density

The overall density of freshwater mussels in both canals drastically decreased over the 16 years of the study (Figs. 1, 2). In the CIA higher densities were mostly observed in its middle and lower stretches before 2013 (2004–2012, Fig. 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, Figs. 1b; 2) when the density fell drastically, registering very low densities (below 0.05 ind/m^2), throughout the entire length of both canals.

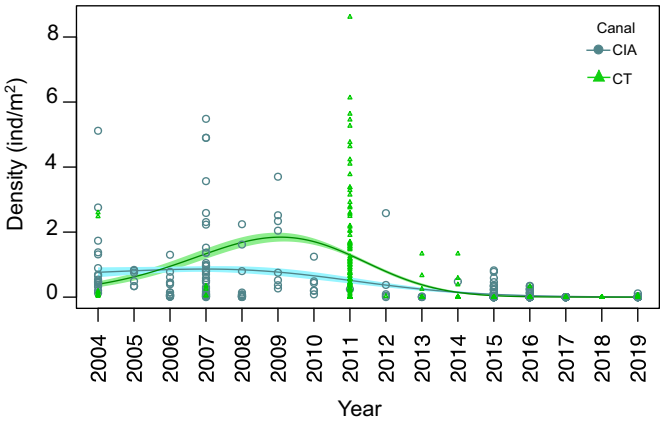

Fig. 2. Total density plots of alive freshwater mussels in CIA and CT between 2004 and 2019, and corresponding GAM models for each canal including predicted values (line) and their standard errors (coloured polygon). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

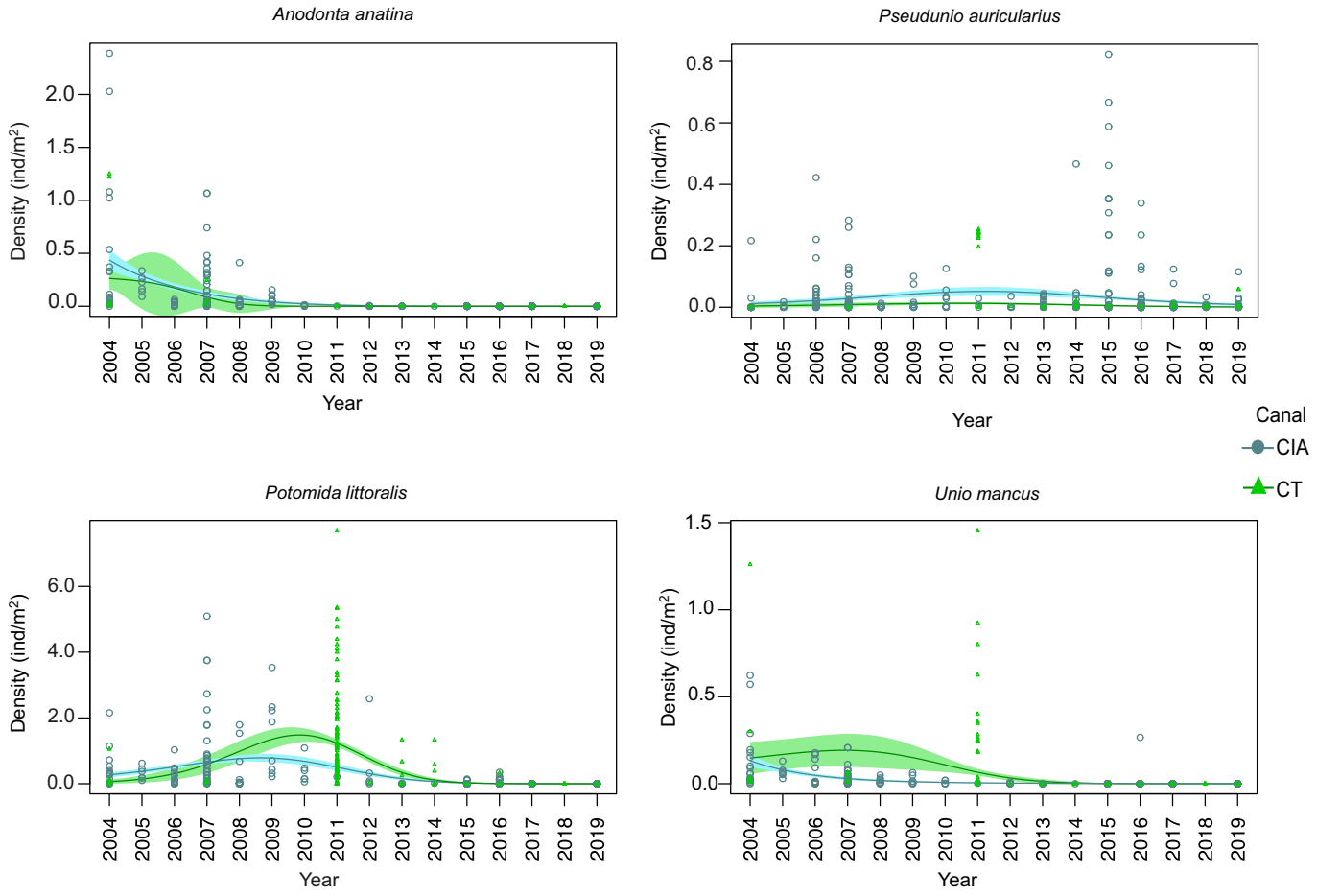

Fig. 3. Density plots of alive freshwater mussels by species in CIA and CT between 2004 and 2019 and corresponding GAM models for each species and canal including predicted values (line) and their standard errors (coloured polygon). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Results of zero-inflated negative binomial (NB) and Poisson (P) GLMMs for the total density 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 = kilometric point within each canal.

		Total Unionids (NB)	*P. auricularius* (NB)	*P. littoralis* (P)	*A. anatina* (NB)	*U. mancus* (NB)
	Observations	467	649	467	467	467
	Deviance	3010.6	2406.2	29,121.2	1174.6	1163.7
	r^2	0.58	0.18	0.73	0.70	0.54
Count part						
Fixed effects	Intercept	2.60 (1.07)*	0.43 (0.23)	0.45 (1.27)	1.67 (0.23)***	0.097 (0.15)
	Year	−0.40 (0.03)***	−0.10 (0.02)***	−0.03 (0.004)***	−0.24 (0.06)***	−0.35 (0.05)***
Random effects	KP:Canal	0.52	0.15	1.49	0.46	0.14
	Canal	0.046	8.55E−09	0.26	3.24E−07	1.39E−09
	Sampling	1.84	7.09E−13	4.08	1.42E−07	0.77
Zero-inflated part						
Fixed effects	Intercept	−43.25 (13.4)**	2.22 (1.12)*	−5.95 (0.53)***	−21.5 (16.5)	−8.19 (2.3)***
	Year	2.31 (0.73)**	−0.77 (0.27)**	0.71 (0.06)***	3.44 (2.54)	1.05 (0.27)***
Random effects	KP:Canal	0.8	4.7	0.63	7.39E−04	4.83E−08
	Canal	226	0.89	9.4E−12	35.5	9.96E−09
	Sampling	1.79E−7	4.99E−08	1.38E−07	2.61E−04	9.45E−11

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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. 2). Mussel density in CT showed estimated values between 1 and 2 ind/m^2 during the first few years of study, with a maximum peak of 8.61 ind/m^2 in one locality in 2011 (the year with the highest sampling effort in this canal, Table 1). Afterwards, density drastically fell down reaching zero in 2015 (Fig. 2). When we analysed the data by species (Fig. 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). Its density was higher in CT than in CIA during 2013–2014, but it fell close to zero thereafter.

The drastic reduction and eventually 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). In CT it had a median density of 0.02 ind/m^2 in 2004 with a 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 (median and maximum density values in 2004 for the CIA = 0.06 and 0.62 ind/m^2 respectively, and 0.02 and 1.26 ind/m^2 for CT) and in both canals it progressively declined year after year (Fig. 3), with the exception of 2011 when a density peak was observed in CT (max value: 1.45 ind/m^2). The species was not found after 2012, except for a unique individual found in 2016, when a greater sampling effort in CIA was performed (Table 1).

The median density of *P. auricularius* in the CIA (Fig. 3) seems to have been maintained low over the years (0.001–0.04 ind/m^2). Exceptional values were registered in 2015 with a maximum value of 0.82 ind/m^2 in one sample; however, the median density in the canal was close to zero. On the other hand, a slight increase in median density was observed in the CT from 2011 to 2019 although the values were always very low (0.001–0.03 ind/m^2).

Most ZI-GLMMs (Table 2) had significant fixed effects of time over both the count and the binomial parts of the model. Considering the model for the overall density of unionids, we found an inverse and

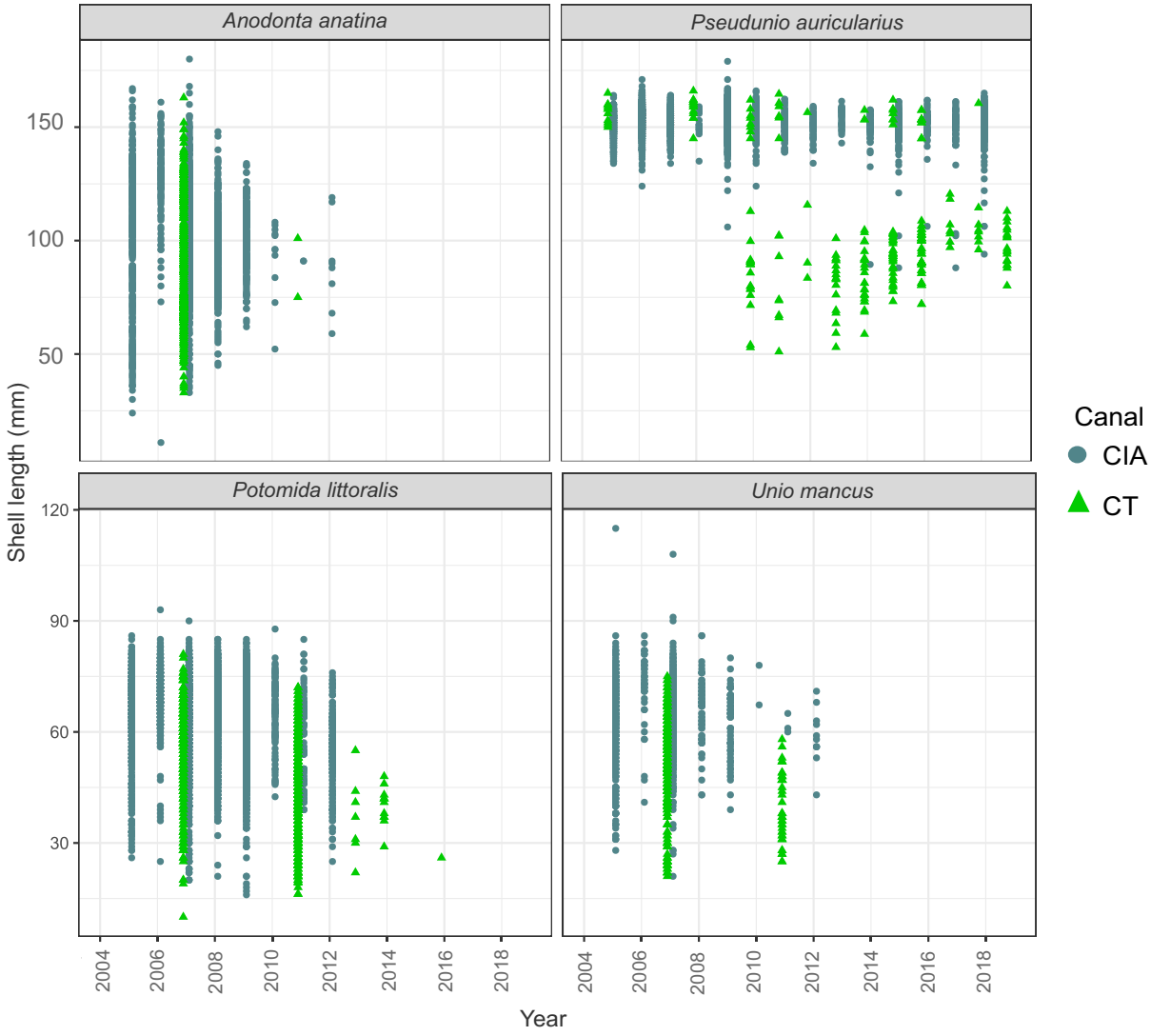

Fig. 4. Freshwater mussel shell lengths by species in CIA and CT, 2004–2019.

significant relationship of time (year) with density (Count part) and a positive relationship with absence probability (ZI part). The sampling methodology and kilometric point (KP) in each canal accounted for much higher variance in density than that explained by the canal itself, but for the ZI part, the canal accounted for higher variance.

Analysing the data by species, the model for *P. auricularius* showed a negative and significant relationship of density with year (−0.10) (Table 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) or the sampling methodology. In the case of *P. littoralis* and *U. mancus*, time (year) was also negatively related to mussel density and positively with its absence probability. In both species, the variance accounted for by sampling methodology in the count part of each model was higher than that by other random factors; the opposite was found for *P. auricularius* and *A. anatina*. The model for *A. anatina* was the only one in which the ZI part was not significant, but the count part also indicated a significant density decline with time. The best fitted models, according to their lower deviance and higher r^2, corresponded to those of *P. littoralis*, *A. anatina* and *U. mancus*, while the model for *P. auricularius* was the one with the weakest relationship between observed and fitted values.

3.2. Population structure

We obtained a total of 19,033 shell measurements of living specimens 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), 10,242 to *P. littoralis* (CIA: 8778, CT: 1464), and 1824 to *U. mancus* (CIA: 1285, CT: 539).

From 2004 to 2007 *A. anatina*, both in the CIA and in the CT, presented young and old specimens (from 11 to 180 mm) (Fig. 4). Starting from 2008, the largest and smallest specimens began to disappear, and only the intermediate-medium sized individuals survived (ca. 75–100 mm long), 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 (mean = 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. In the CIA, *P. littoralis* showed a shell size population structure that included small and large specimens (10–88 mm), with a mean shell size of around 50–60 mm (Fig. 4), which was maintained until the species completely disappeared in 2013. Its population in CT still included adults and juveniles by 2011, but in 2013 and 2014 most of the adult specimens disappeared, and the average shell length decreased to 40 mm. The species was considered locally extinct after 2016 when a single small (26 mm long) specimen was found.

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 long began to be recorded as early as 2010, decreasing the mean shell length in this canal down to 111 mm in 2012. In 2013, the lowest mean value for shell length (80 mm) was recorded, and by 2017 the largest specimens almost completely disappeared from this canal.

3.3. Estimated survival of Pseudunio auricularius

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. 5), but much more pronounced in CIA than in CT. Drops in annual survival

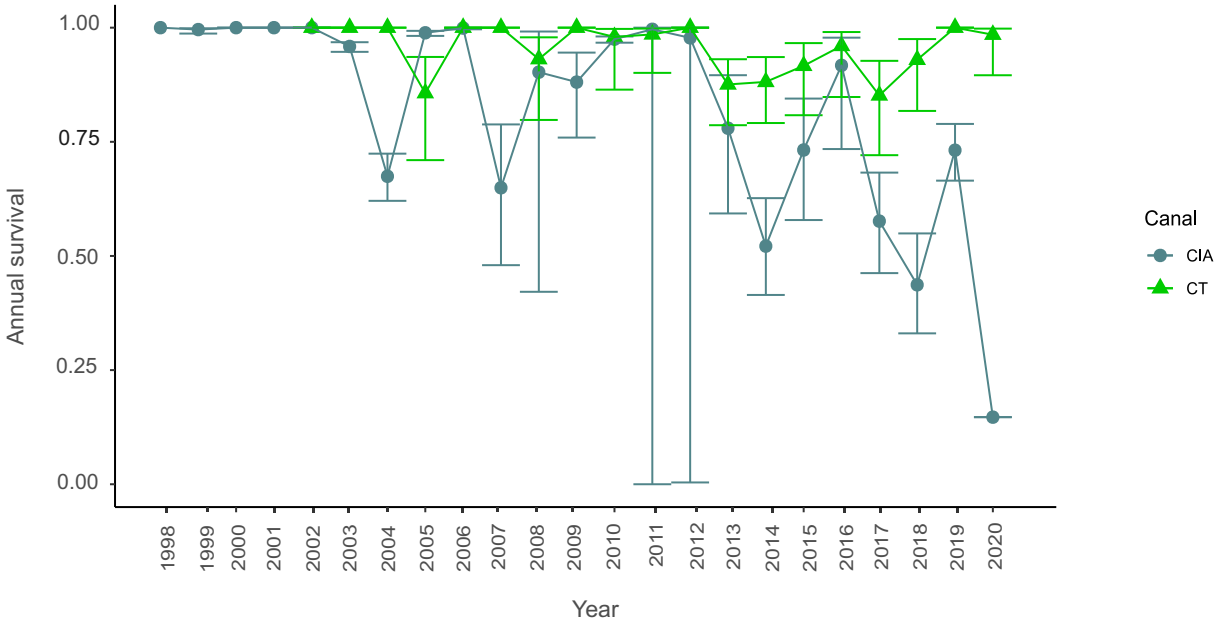

Fig. 5. *Pseudunio auricularius* annual survival rate (symbols: estimate; vertical lines: confidence interval) in CIA (1998–2020) and CT (2002−2020), estimated from capture-recapture data of marked individuals.

were observed in 2004 and 2007 in the CIA, and 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 and 0.9, but several drops were also observed following a similar pattern as in CIA, yet less pronounced.

4. Discussion

4.1. 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, 2019; 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 and Daufresne (2006) or the present survey, are rare. Yet quantitative data allow estimating interannual and spatial variability in a more detailed ecological context and in that way find the best management responses to tackle those changes. The causes for these mussel declines remain mostly unknown (Downing et al., 2010; Haag, 2019), 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) are recent examples of mass mortality episodes that caused abrupt decreases in population densities.

The decline of freshwater mussels in the Ebro River basin in the last decades is well known (Araujo, 2011a,b), especially for *P. auricularius* (Araujo and Álvarez-Cobelas, 2016; Araujo and Ramos, 2000; Guerrero-Campo et al., 2021). Thanks to the work of Haas (1916, 1917) it is known that *P. auricularius* was once very abundant in the middle section of the Ebro River, near Sástago village, where the extraction of thousands of specimens for their use in the artisanal cutlery industry were registered (Álvarez Halcón, 1998b). *Potomida littoralis*, *U. mancus* and *A. anatina*, although colonizing different sections of the Ebro River, were also subjected to recent declines (Araujo et al., 2009; Rubio Millán et al., 2016). However, these declines are not comparable to the ones reported in this study and therefore, the causes of this decline (discussed below) seem to exert greater pressure on the freshwater fauna living in these artificial habitats.

Our data set, despite using different sampling methodologies and sampling efforts along the years (since the low water level in canals depended on whether or not maintenance works were performed), showed a remarkable general decrease in the density of mussels of more than 95 % throughout the studied years. The mussel community composition shifted from four species to only one, *P. auricularius*, which now survive in sympatry with two invasive alien species (Asian clam *Corbicula* spp. and zebra mussel *Dreissena polymorpha* [Pallas, 1771]; Guerrero-Campo 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, such 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 affected fauna may 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 works twice a year (February and November), unlike CT, which only has such works once a year (in February) and usually with a much smaller disturbance; this difference may be acting as a key factor for the contrasting survival of *P. auricularius* between both canals, allowing to maintain its population density in CT, or even increase it during the last years, compared to the CIA.

Gómez and 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 non-native fishes that compete with and prey on native ones, which were the original hosts of freshwater mussels in the area (Nakamura et al., 2022). At present, it is not possible to find any living specimen of *P. littoralis*, *U. mancus* or *A. anatina* in the canals, where they are considered extinct (Guerrero-Campo et al., 2021; Nakamura et al., 2022). Their disappearance was relatively fast, since Gómez and 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 were obtained by us 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 and Araujo (2008) reported mean densities 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 5 times lower (0.01 ind/m^2) mean density in this canal in 2019.

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, 2019), 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 three 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 situation may be one of the reasons why the results of the zero inflated part of the model indicated that time negatively affected the probability of absence, i.e. suggesting an increased probability of finding this species along the study years. This appearance of young individuals slightly increased the density in CT (2019 mean density = 0.02 ind/m^2), filling in the young age classes that were not recorded so far and offering valuable information about this growth stage, previously unknown in the canal. Despite this, our model indicated a progressive decline of density with time for *P. auricularius*, and our analyses of capture-recapture data showed major drops in survival in the CIA, coinciding with the recorded mass mortality event in the central part of this canal (Guerrero-Campo 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 the CIA where the presence of young individuals was, and still is, very rare.

Recently, Aldridge et al. (2022) published a review about threats and opportunities for tackling the global conservation of freshwater mussels in the near future. Knowledge about mussels health, mortality causes, presence of parasites and diseases, and the effects of environmental pollutants are some of the main aspects in which the authors consider it

is necessary to go deeper in future studies. Some of these factors 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 first factor that we considered was habitat degradation. It is sometimes evident but, in many cases, it can be relatively slow and cryptic, and therefore not immediately obvious as a main cause of species decline (Wood and Armitage, 1997). Sousa et al. (2021) reviewed the role of anthropogenic habitats as refuges or ecological traps 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 and Richardson, 2010; Martínez-Abraín and Jiménez, 2016). 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 flow regulation due to the presence of dams, 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 and 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 near the banks, such a replacement was possibly highly detrimental for their survival.

Since massive mortality of *P. auricularius* was detected in the CIA in 2013 (Guerrero-Campo 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 bivalve populations (Guerrero-Campo et al., 2021; Rico Gómez, 2021). Araujo et al. (2021) 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 by cultivated agricultural 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-Campo et al., 2021). The lack of a sound knowledge on the ecotoxicological responses 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, it has been shown that the presence of contaminants can induce oxidative stress in bivalves (Deudero et al., 2015; Khazri et al., 2017).

Haag (2019) 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., 2021; 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 and Jarne Bretones, 2014) and within a decade its density sharply raised up to 15 times the initial values (from 70 ind/m^2 to 1000 ind/m^2; Gimeno 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 and Sousa, 2019; Modesto et al., 2021). 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 ecosystem (Cherry et al., 2005; McDowell et al., 2017; Novais et al., 2017; McDowell and 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 *U. mancus* glochidia (Araujo et al., 2001, 2005, 2009; López and 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 native 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 affect its life-cycle through glochidia filtration 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 Landis et al., 2013; Luck and Ackerman, 2022). High levels of sedimentation can also affect the juvenile mussels by clogging interstitial spaces and causing low oxygen and pH levels in the sediments (Geist and 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 construction of cofferdams during maintenance works, water runoff from the nearby agricultural fields, and landslides from lateral banks due to the total removal of riparian vegetation, could be also contributing to recruitment failure.

4.2. Conservation implications

Recovering freshwater mussel populations can be a key factor for the benefit of the whole aquatic ecosystem due to the ecosystem functions

and services they provide (Aldridge et al., 2022; Eveleens and Febria, 2022; Vaughn, 2018; Zieritz et al., 2022). Habitat restoration actions, such as water and substrate quality improvement (Geist and Hawkins, 2016; Vaughn, 2018), recovery of the native fish community (Galbraith et al., 2018), reduction of siltation to allow the normal development of juveniles (Pandolfi et al., 2022), recovery of the riparian forest that creates fish refuges, reduce diffuse pollution and allow the entry of organic matter from the terrestrial environment (Caskenette et al., 2020), so as improving agricultural practices (Ferreira-Rodríguez et al., 2022), should be considered to facilitate the recovery and conservation of mussel populations in the Ebro basin. 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.

Long-term studies are key to identify temporal trends in long-lived species such as freshwater mussels, allowing 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 highlight important events such as the appearance of young specimens of *P. auricularius* after 15 years since the rediscovery of the species in 1996, the 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 riparian vegetation, or 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, improve communication between stakeholders).

A special attention should be devoted to *P. auricularius*, since this 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 and Ramos, 2000; Guerrero-Campo 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 Europe (Prié et al., 2018), especially in the Iberian Peninsula, making 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 currently being carried out, 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-Campo et al., 2021; Nakamura et al., 2022), suggesting that the cause of mortality might be related to the canal habitat itself.

5. Conclusions

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 negative effects imposed by the spread 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 negative influence of toxic substances. In this long-term work we described an extensive declining trend of freshwater mussels in two canals of the Ebro River, with the 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 young specimens, although its density is still very low. Regular assessments of the health status of freshwater mussel populations, e.g. by using 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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CRediT authorship contribution statement

Conceptualization, Methodology, Field work, Analysis of data: KN, FMJ; Writing - Original draft preparation - Reviewing and Editing: KN, FMJ, RS. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

The datasets generated during and/or analysed during the current study are not publicly available, in order to secure the protection of the target species of the study in their natural habitat, but are available from the first author on reasonable request.

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