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ORIGINAL ARTICLE

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The last hideout: Abundance patterns of the not-quite-yet extinct mayfly *Prosopistoma pennigerum* in the Albanian Vjosa River network

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Abstract

1. The mayfly *Prosopistoma pennigerum* (Müller, 1785) (Insecta: Ephemeroptera) once occurred in many European river networks. However, observations decreased in the last decades and the species can be considered largely extinct throughout Europe due to river alterations.
2. Only three extant populations are known from Cabriel (southern Spain), Volga (Russia) and Vjosa (Albania) rivers.
3. We recorded the species along a 150 km stretch in the Vjosa River in three sampling seasons (spring 2018, fall 2018 and fall 2019), counting up to 302 *P. pennigerum* per m^2, the highest recorded abundance for the species to date. Moreover, we detected traces of environmental DNA in a newly designed targeted eDNA assay.
4. In our modelling approach we define the species' niche in a theoretically available niche space given by the Vjosa River network and predict a high probability of presence (θ) in downstream located sections of this river. Expected abundances (λ) could

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be related to a set of environmental variables, importantly to higher discharge and increased sediment dynamics.
5. Simultaneous occurrence of larvae of different sizes at individual sites suggests an asynchronous life cycle, which may be advantageous to cope with the highly dynamic river hydrology.
6. The *P. pennigerum* population in the Vjosa is of key importance for the species' global survival.

KEYWORDS
endangered, flagship species, free flowing, hydromorphology, refugia, river conservation

INTRODUCTION

Increasing demand for energy, transportation, flood protection and other water use has impacted most of European riverscapes (Dynesius & Nilsson, 1994; Grill et al., 2019). This tendency of anthropogenic modification and homogenisation of riverine systems is a global phenomenon and affects both large rivers and increasingly also smaller streams (Belletti et al., 2020; Vitousek et al., 1997). The Balkans harbour a particularly large number of minimally disturbed riverscapes, that are, however, among the most threatened on the continent: ever-increasing demand for economic growth coupled with incentives for hydropower development drives land-use change and river fragmentation that imperil aquatic habitats at large scales, including the Vjosa River network (Schwarz, 2020; Zarfl et al., 2014).

The Vjosa is one of Europe's last large rivers having a mostly undisturbed watercourse with natural hydromorphology and hydrological dynamics (Schiemer et al., 2020). This river sustains a unique fluvial community—including various endemic species or relics that were once widely distributed across Europe such as the critically endangered European eel (*Anguilla anguilla* Linnaeus, 1758) (Meulenbroek et al., 2020; Pike et al., 2020) and the mayfly *Prosopistoma pennigerum* (Müller, 1785) (Graf et al., 2018). The river's biodiversity likely benefits from the maintenance of habitats and habitat heterogeneity in the free-flowing sections of the Vjosa (Hauer et al., 2021) as well as the associated intact metapopulation processes that control and maintain biodiversity over time (Wang & Altermatt, 2019).

As it still provides habitat for the once widespread *P. pennigerum*, the Vjosa River is a prime target to study river ecosystem properties and processes that maintain such species and thus contribute to sought-after reference data at the European scale. Specifically, the charismatic mayfly *P. pennigerum* can serve as a model in minimally disturbed rivers to elucidate its ecological needs and of the species sharing its habitat, both at regional and continental scales (Schletterer et al., 2016).

The mayfly genus *Prosopistoma* Latreille, 1833 is readily identified by its unique larval anatomy: a carapace covers the body, giving the animals the appearance of a subcircular, convex head-carapace-abdomen monocoque. Like all 30 known species of *Prosopistoma*, *P. pennigerum* appears to be associated with minimally or undisturbed conditions. Yet, ecology and phenology of *P. pennigerum* remain enigmatic (Barber-James, 2009; Schletterer & Füreder, 2009). Moreover, the species is currently at the brink of extinction, likely due to large-scale habitat modification and land-use change in European riverscapes (Schletterer & Füreder, 2009). Until the 1960s, *P. pennigerum* was distributed across the entire European subcontinent as a constant inhabitant of the major European river networks, but observations have decreased steadily during the second half of the 20th century (Schletterer et al., 2021; Schletterer & Füreder, 2009). At present, only three remnant populations persist: in the Cabriel (Rueda et al., 2005), Volga (Schletterer & Füreder, 2009) and Vjosa (Graf et al., 2018) rivers. Among those, the southern Balkan Vjosa River merits particular attention because of its largely intact fluvial dynamics and longitudinal connectivity (Schiemer et al., 2020).

Here, we present the first comprehensive characterisation of *P. pennigerum*'s distribution in the Vjosa River from a network-wide survey. In addition, we aim to relate abundance and occurrence patterns of this mayfly to environmental gradients statistically and using a Bayesian finite mixture modelling approach. Moreover, we aim to explore potential adaptations of this species to the fluvial dynamics observed in the Vjosa River, by measuring larval body size distribution along the Vjosa River to learn about life cycle strategy and by discussing dispersal capacities and possible morphological adaptations to highly dynamic flow regimes. We further aim to validate results of a novel targeted environmental DNA-based detection protocol for this mayfly by comparing eDNA detection with observed abundance patterns.

METHODS

Sampling sites, benthic invertebrate fauna sampling and eDNA sampling

A total of 41 sampling sites across the 6704 km^2 Vjosa catchment were selected to reflect the dendritic network structure (Altermatt, 2013) including the Vjosa (Aoos) mainstem and the major tributaries Bence, Dishnica, Drinos, Langarica, Luftina, Sarantaporos, Shushica and Voidomatis. The Vjosa River network spans from the Greek Pindos Mountains to the Adriatic Sea in Southwestern Albania (Supporting information I).

At all sites, physical benthic invertebrate fauna samples were collected three times: in 2018 (April/May; October) and 2019 (September/October). Furthermore, we collected environmental DNA (eDNA) samples (Deiner et al., 2017; Pawlowski et al., 2020). Physical benthic invertebrate fauna samples were taken following the

multi-habitat sampling (MHS) procedure with a 25 × 25 cm 500 μm-mesh square net (AQEM Consortium, 2002). In brief, 20 subsamples were collected along a 50 m stretch according to the prevalent micro-habitat distribution defined by substrate, depth and flow velocities at each sampling site. After pooling, the samples were fixed in 3%–4% formaldehyde in 2018 and in 96% ethanol in 2019 as no benefit of formaldehyde fixation was observed in preliminary comparison (i.e. specimen destruction due to long transport occurred in similar frequency and magnitude in either fixative). In the laboratory, a complete MHS sample was distributed evenly on a rectangular frame net (500 μm) with a 5 × 6 delineated grid (5 × 5 cm). From this grid, 5 out of 30 squares were randomly taken, and all specimens within the subsample were counted and identified to the lowest possible taxonomic level; for most specimens, this was the genus. Finally, specimens were conserved in 96% ethanol and deposited in the Biological Station (WasserCluster Lunz, Lunz am See, Lower Austria) in the collection of Simon Vitecek. Environmental DNA samples were taken upstream of each MHS sampling site in duplicates using sterile equipment. At each site, a volume of 500 ml of stream water was filtered through each of two 0.45 μm Sterivex filter units and stored at −20°C immediately in mobile freezers until further processing (eDNA sampling protocol details followed Mächler et al., 2019) (Supporting information II). In addition, two blanks each at three different sites and dates, that is, in total six field blanks per sampling season, were taken by filtering 500 ml of commercially available deionised water with the same filter and subsequently treated equivalently to the true samples in further laboratory analyses.

eDNA: TaqMan qPCR design and testing

We designed forward and reverse primers as well as corresponding hydrolysis probes for a TaqMan qPCR protocol with Primer3Plus (Untergasser et al., 2012), targeting the mitochondrial Cytochrome c oxidase I gene and amplicon sizes between 75 and 115 bp. Seven candidate primer and probe sets were obtained, of which three were selected for further testing based on amplicon size, melting temperatures and potential primer-dimer and hairpin formations (assessed via MULTIPLEPRIMERANALYZER [Thermo Fisher Scientific]). Further in silico testing involved a Primer-BLAST (Ye et al., 2012) of the selected set against (i) the GenBank library as of 07.ii.2020, (ii) a custom library obtained via the BOLD API containing >35 k barcode sequences (as of 06.ii.2020) of Eurasian benthic invertebrates (Supporting Information III). In silico testing did not recover any non-target matches of any primer or probe with less than 3 bp mismatches.

The three selected candidate primer + probe sets were further tested in vitro by means of standard PCR and a primer + probe set was selected based on amplification success in mock communities with and without *Prosopistoma pennigerum*. These communities were prepared by mixing DNA extracts of single specimens, representing different combinations of taxa abundant in the Vjosa River, including *Caenis* spp. Stephens, 1835, *Ecdyonurus* spp. Eaton, 1868, *Baetis* spp. Leach, 1815, *Rhyacophila* spp. Pictet, 1834, *Leuctra* spp. Stephens, 1836 and *Hydrospyche* spp. Pictet, 1834 (collected in the Vjosa) and a set of *Habroleptoides* spp. Schoenemund, 1929, *Ecdyonurus* spp., *Baetis* spp., *Rhithrogena* spp. Eaton, 1881 and *Epeorus* spp. Eaton, 1881 collected at the Oberer Seebach in Lunz am See. Taxonomic richness of these mock communities varied from 1 to 25. The TaqMan qPCR assay to specifically detect *P. pennigerum* in eDNA samples was optimised by varying primer and probe concentrations in 200 nM/100 nM increments between 200–800 nM and 100–400 nM, respectively (forward primer Proso07fwd: 5′–GCAGGCGTCTCATCTATTTTGG–3′, reverse primer Proso07rev: 5′–TAGAGTTATGCCGGGTGTGC–3′, Probe Proso07pr_rc_MGB: 5′–JOE-TTGTGGTGGTGATAAAATTAACTGCGC–MGB–Q530–3′). Standard curves obtained from an amplicon dilution series (10^1–10^9 amplicons/μl) yielded a reaction efficiency of 88.0% and an R^2-value of 0.995, with a calculated limit of quantification (LOQ) of 1801 copies per 1 μl of template and a limit of detection (LOD) of 59 copies per 1 μl of template according to a curve fitting modelling approach (Klymus & Carl, 2012).

Final PCR reactions contained 10 μl 2× TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific), 600 nM of forward primer (Proso07fwd), 400 nM of reverse primer (Proso07rev), 400 nM of hydrolysis probe (Proso07pr_rc_MGB), 2 μl eDNA extract and ddH_2O up to a final volume of 20 μl. Cycling conditions consisted of an initial denaturation of 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min, performed on a Corbett Rotor-Gene RG-3000 (Qiagen) in standard speed mode.

Detection of *Prosopistoma pennigerum* in eDNA samples via TaqMan qPCRs

A total of 279 eDNA samples across all 41 sampling sites and all three sampling seasons were analysed using the newly developed protocol. Extraction of eDNA was performed using the DNeasy® PowerWater® Sterivex™ Kit (Qiagen GmbH; cat: 14600-50-NF) following the experienced user protocol. Blank extractions were spread across the extractions of the true samples.

Each eDNA sample was tested in five qPCR replicates. With each batch of PCR reactions (encompassing 13 eDNA samples), a total of four PCR negative controls (using ddH_2O instead of eDNA extract), as well as two positive controls were run (1 μl of the 105 copies/μl dilution of the amplified target fragment produced for standard curve generation). All field extraction blanks were run in quintuplicates along with the eDNA samples. To check for presence of potential PCR inhibitors in our samples, we spiked an additional positive control with 1 μl of a randomly chosen sample of the respective batch per PCR run ($N = 22$). A PCR reaction was deemed positive when a sigmoid amplification curve passing the amplification threshold was observed in at least one replicate. Every positive PCR reaction was run on a 2% agarose gel to check for correct amplicon size.

Biometry

To estimate generation numbers and life strategy of *P. pennigerum*, 253 randomly selected specimens collected at 11 sites during the

autumn 2019 campaign were used for measurements of morphological traits by means of a Leica M 125 Stereomicroscope: carapace length (CL), carapace width (CW), head capsule length (HL), head capsule width (HW) and outer-eye-margin to outer-eye-margin distance (EE) (Supporting Information IV). CL measurements correlated strongly with EE (Spearman's $\rho = 0.986$, $S = 37{,}602$, $p < 0.001$) and CW (Spearman's $\rho = 0.985$, $S = 39{,}365$, $p < 0.001$) and were thus used as sole indicator for nymph development (Figure 4b).

Habitat properties and occupied niche space

We computed descriptive statistics of key environmental variables to describe niche space potentially available to *P. pennigerum* throughout the stream network and compared environmental conditions between sites differing in *P. pennigerum* occurrence.

Microhabitat and sediment characteristics

Discharge was calculated based on depth and mean flow velocity measured across transects at a subset of sites spanning the entire river network. These discharge estimates were related to drainage area (Burgers et al., 2014) for each seasonal campaign separately based on a 25 m resolution digital elevation model provided by Copernicus in the scope of the European Union's Earth Observation Programme (EU-digital elevation model v1.1). Through these drainage, area-discharge models discharge was predicted for all sites in each season. In a second step, we computed expected mean flow velocity (V_μ), mean depth, and mean width via published hydraulic scaling relationships (Raymond et al., 2012). These computations were done with the 'WatershedTools' R-package version 1.5.3 (Talluto, 2020).

At each sampling site, microhabitat coverage was estimated along a 50 m stretch as part of the multi-habitat sampling procedure based on sediment grain size classes (AQEM Consortium, 2002). Estimates of microhabitat distribution of sediment grain size classes were then used to compute the median grain size (GS_{q50}). Critical erosion velocity (V_{crit} in m s^{-1}) for the median grain size was defined following Tippner (1972) as: $V_{crit} = 4.6 * z^{(1/6)} * GS_{q50}{}^{(1/3)}$ with z as the depth (m). When $V_\mu > V_{crit}$, then GS_{q50} is expected to be mobilised, and in turn, benthic microhabitats regarded as disturbed (Singer et al., 2005). We further used the ratio of V_{crit}/V_μ as an indicator for the local stability of bed substrates.

Temperature and chemical characteristics

Water temperature was measured as a multi-day average through MiniDOT loggers (PME, Vista, USA) exposed for 2 to 28 days (mean = 8 days). Conductivity was measured at each site in the field with a handheld metre (WTW probe, Xylem, Weilheim, Germany). We filtered water on site through a sterile 0.2 μm Acrodisc GHP filter (GE, Boulder, USA) and stored samples at 4°C until further processing within 4 weeks. To determine ion concentrations, we used ion chromatography (Dionex ICS-200; Thermo Scientific Fisher, Waltham, Germany) for $SO_4{}^{2-}$, Cl^-, and $NO_3{}^-$, and optical emission spectrometry (ICP-OES, Thermo iCAP600 [Thermo Scientific Fisher]) following acidification with 32% HCL to <0.2 pH for cations Ca^{2+}, Mg^{2+}, K^+, and Na^+. Low concentration and minor ions such as dissolved Si and S, as well as reactive P were not included in further analysis.

Statistical analysis

Data were analysed and plotted in the statistical environment R 4.1.1 (R Core Team, 2021). *Prosopistoma pennigerum* size distribution was plotted with the *vioplot* package (Adler & Kelly, 2020). Principal component analysis (PCA) was performed to reduce dimensionality of the water chemistry dataset using the *rda* function of the *vegan* package (Oksanen et al., 2017). Subsequently, a PCA with relevant environmental variables was conducted to assess occupied niche space. To assess the probability of presence and expected abundance of *P. pennigerum* across the Vjosa sampling sites from available environmental information, across seasons and for the respective sampling year, we used a finite mixture model. This model uses a binomial process (governing presence/absence) and a Poisson process (governing abundance if present). Initial modelling of abundances indicated that there were more zero-abundance sites than would be expected under a Poisson distribution. Hence, the chosen mixture model approach accounts for the extra zeros and can be interpreted as consisting of two sub-models: the binomial sub-model informs about the possibility of presence; a predicted zero under this model indicates that *P. pennigerum* should not occur at the site (e.g. because the habitat is inappropriate). A non-zero prediction from the binomial model indicates that *P. pennigerum* might occur, at which point the Poisson sub-model is used to predict abundance. Thus, zero abundances can originate either from the binomial or Poisson sub-models, while non-zero abundances must originate from the Poisson process.

For both sub-models, we described the expected outcome (i.e. probability of presence for binomial, mean count for Poisson) using a linear model. For the binomial model, we used a logistic link to compute the expected probability of presence (θ) from covariates (X_B) as follows:

$$\theta = \text{logit}^{-1}(\alpha_B + X_B\beta_B)$$

where α is the intercept, β is the slope, the subscript B indicates the binomial sub-model, and $\text{logit}^{-1}(y) = e^y/(1 + e^y)$. Similarly, we computed the expected abundance λ (conditional on a non-zero outcome from the binomial process) using a log link:

$$\lambda = e^{\alpha_P + X_P\beta_P}$$

where the P subscript indicates covariates and parameters for the Poisson sub-model. It follows that, for any site, the expected value of abundance is simply $\theta \bullet \lambda$, and the likelihood of an observed abundance N is:

$$\mathrm{pr}(N|\theta, \lambda, X_B, X_P) = \begin{cases} \lambda^N e^{(1-\theta)+\theta e^{-\lambda}} & N=0 \\ \theta \dfrac{\lambda^N e^{-\lambda}}{N!} & N>0 \end{cases}$$

Here, there are two ways to generate a zero abundance: a 'binomial' absence with probability $1-\theta$, and a 'Poisson' absence with probability $\theta \bullet e^{-\lambda}$, which is the probability of presence under the binomial multiplied by the probability of zero abundance under the Poisson. There is only one way to generate non-zeros, which is to have a binomial presence (with probability θ) multiplied with the Poisson likelihood. These models were fitted in a Bayesian context with STAN using the R interface Rstan (Stan Development Team, 2020).

To evaluate the performance of various covariate combinations, we first constructed a set of candidate models by reducing environmental variables to a two-dimensional niche space using PCA for each season. From these, we extracted the PCA axes scores, which we implemented in our mixture model as predictor variables. We built models of increasing complexity, starting with the first model using only the first principal component as predictor. Further candidate models were created by implementing up to six principal components. We compared the candidate models using an approximate leave-one-out cross-validation scheme with the loo R-package (Vehtari et al., 2020). We then selected the most parsimonious model with the minimum leave-one-out information criterion (LOO-IC). LOO-IC is similar in interpretation and scaling to the commonly used Akaike information criterion (AIC); model performance is evaluated using the pointwise density across all posterior simulations, and the model is then penalised for complexity. To avoid overfitting, we further compared model outputs. These analyses suggested that a model including at least a three-dimensional environmental space (i.e. scores of the first, second, and third principal components) predicts presence and abundance of *P. pennigerum* fairly well (LOO-IC score: 'best' 1015.96, 'simplest' 2147.06, and most 'complex' 2052.42).

We then implemented a Bayesian MCMC approach for the selected model with three principal components and drew samples from the posterior distribution with four chains. Each of the four sampling chains ran for 4000 iterations (including 1000 iterations burn-in), and we calculated ratios of effective sample size to total sample size (N_{eff}/N) for θ and λ (mean 0.75, sd = 0.24) to assess independence of posterior estimates. In addition, R-hat values (min = 0.9997, max = 1.0011) indicated good mixing of Markov chains.

RESULTS

Prosopistoma pennigerum presence, abundance, and eDNA detection

Throughout all sampling campaigns, *Prosopistoma pennigerum* was found and detected at least once at a total of 16 sites along a 152 km stretch across the Vjosa River network in both physical samples and the targeted eDNA assay (Figure 1a). In fact, based on physical observations, *P. pennigerum* occupies the Vjosa main stem (12 sites) with a high site fidelity across the 2 years. Further, we found the species sporadically at three sites in tributaries in autumn 2018 (Drinos, Dishnica) and one in autumn 2019 (Langarica) (Supporting Information V). Based on environmental DNA analysis, we detected *P. pennigerum* only at Vjosa mainstem sites. Congruence of *P. pennigerum* presence in physical samples and as indicated by eDNA analysis was low: eDNA detected *P. pennigerum* at 4 out of 10 sites in spring 2018, at 1 out of 14 sites in autumn 2018 and at 7 out of 13 sites in autumn 2019. We never detected eDNA traces of *P. pennigerum* at sites where it was not physically present in the samples. Also, no amplification was observed in any of the field, extraction or PCR negative controls (Figure 1b). *Prosopistoma pennigerum* was not found or detected in the Shushica, Bence, Sarantaporos, Voidomatis or Aoos (i.e. the upper Vjosa mainstem) rivers.

With regard to abundance (Figure 1c), in spring 2018, we found only 14 *P. pennigerum* specimens in a total of 41 multihabitat samples and estimated a median of 1 individual m^{-2} (IQR = 1–1.75) with a maximum of 3 individuals m^{-2}. In autumn 2018, we found a total of 395 specimens across all sites in the entire river network and estimated a median abundance of 12 individuals m^{-2} (IQR = 5–53), ranging from 2 to 82 individuals m^{-2}. In autumn 2019, we collected 1252 *P. pennigerum* specimens and estimated a median abundance of 45.5 individuals m^{-2} (IQR = 17.75–191), ranging from 4 to 302 individuals m^{-2}.

Microhabitat distribution across the river

Physical microhabitat (after Moog et al., 1999) across all sampling sites along the Vjosa network consisted of Megalithal (6% estimated coverage), Macrolithal (14%), Mesolithal (70%), Microlithal (5%), Akal (3%) and minor proportions of Psammal, Pelal and Xylal (2%). Median GS_{q50}, estimated from these microhabitat proportions dominated by Mesolithal, was 14 cm across all sampling sites of the network, which was very similar to the 13 cm estimated for sites with *P. pennigerum* presence. We note a longitudinal decrease of GS_{q50} from up- to downstream located sites.

Flow velocity and discharge

Across all sites and seasons, discharge ranged from 0.36 to 74.89 with a median of 5.15 $m^3\ s^{-1}$, increasing towards downstream and generally higher in spring 2018 than in the two fall campaigns. Sites with *P. pennigerum* occurrence were on average slightly larger rivers with a median discharge of 22.5 $m^3\ s^{-1}$, even though it was also found in some rather small tributaries draining into the Vjosa mainstem. Similarly, we estimated a median velocity of 0.31 $m\ s^{-1}$ across all sites, but 0.47 $m\ s^{-1}$ for sites with *P. pennigerum* occurrence. The ratio V_{crit}/V_{μ}, indicating greater stability of the streambed sediments when >1, was on median 0.77 across all sites and seasons, while it was slightly lower with 0.72 when *P. pennigerum* occurred.

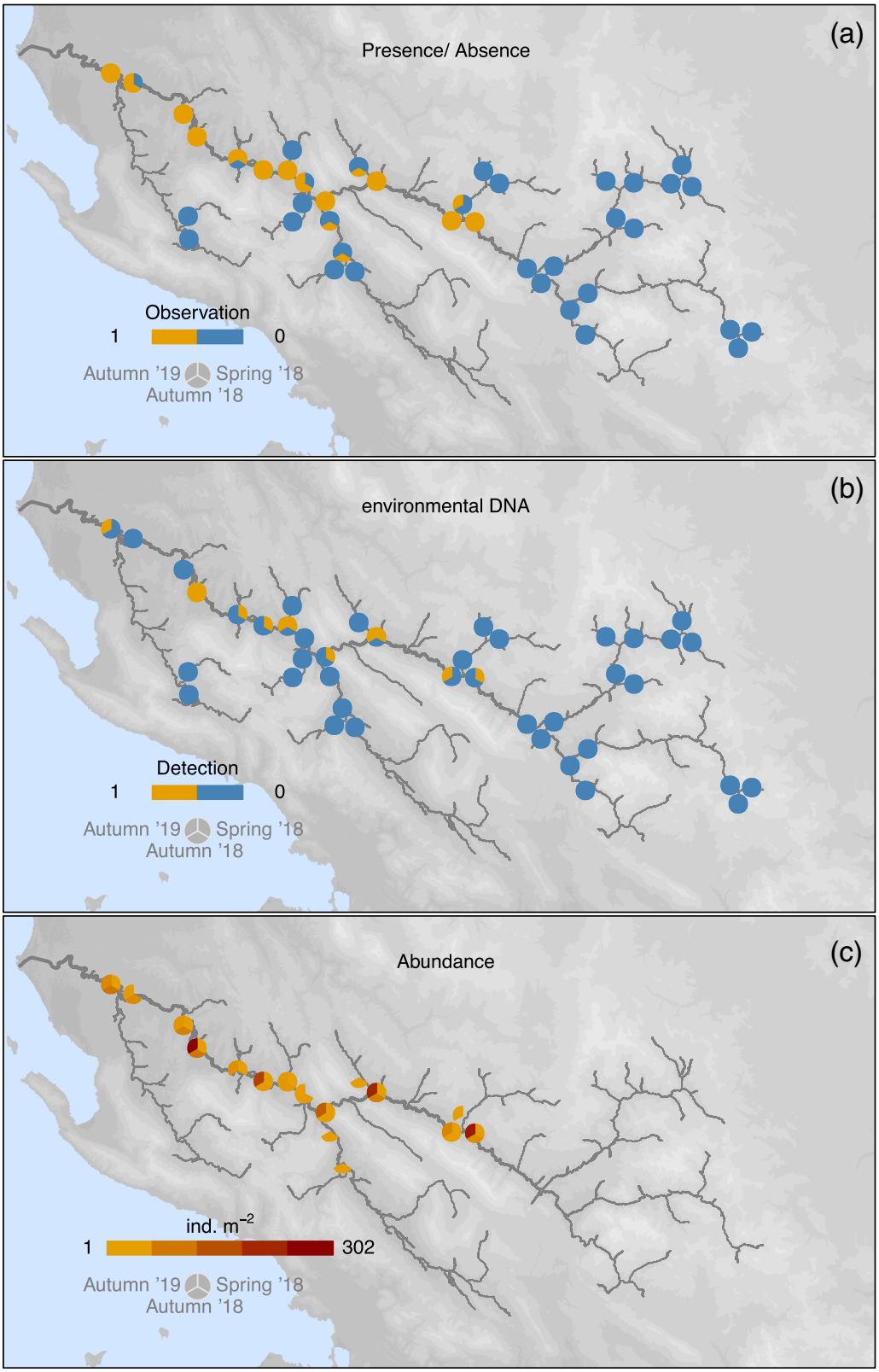

FIGURE 1 Detection of *Prosopistoma pennigerum* in the Vjosa River network. (a) Presence (yellow) and absence (blue) of *Prosopistoma pennigerum* in the Vjosa River network across the different sampling seasons/campaigns. (b) Environmental DNA detections (yellow) of *P. pennigerum* in the Vjosa. (c) Abundance of *P. pennigerum* in the Vjosa. Pie charts represent sampling seasons in a clockwise manner: spring 2018 (12:00–4:00), autumn 2018 (4:00–8:00) and autumn 2019 (8:00–4:00).

Temperature

Daily median water temperature during sampling days was 15.9°C across all sites and seasons with generally higher temperatures in autumn. Temperature increased downstream and was slightly higher when averaged across sites with *P. pennigerum* occurrence.

Chemistry

Descriptive statistics of major ionic constituents computed across all sites and across sites with *P. pennigerum* occurrence showed a slight preference of *P. pennigerum* for sites with higher concentrations (SO_4^{2-}, Cl^-, NO_3^-, Ca^{2+}, Mg^{2+}, K^+, Na^+). Except for NO_3^-, all constituents had lower concentrations in spring when discharge was higher. Most measured ions increased from sites upstream to downstream. Finally, median electric conductivity was 431 µS cm^{-1} (IQR = 329–545) across all sites, but 537 µS cm^{-1} (IQR = 420–571.3) when averaged for sites where *P. pennigerum* occurred.

Overall productivity

As an overall productivity indicator, we estimated median benthic invertebrate abundances of 1360 individuals m^{-2} (IQR = 429–2911), ranging from 4 to 31,522 individuals m^{-2} across all sampling sites and seasons with generally lower abundance in spring than in autumn. Sites with *P. pennigerum* had a higher median invertebrate abundance of 1786 individuals m^{-2}, thus *P. pennigerum* was preferentially found at more productive sites. In these local communities, the median relative abundance of *P. pennigerum* is 0.85% (IQR = 0.2–2.1), ranging from 0.04% to 6.73% (Supporting Information VI).

Principal component analysis

Water chemistry variables and conductivity were summarised in a CA. The first axis explained 60.36% and the second axis 20.74% of variance in the data, respectively. The first axis mostly represents a downstream-directed concentration gradient as ions accumulate with water flowing from sources to sea. The second axis indicates natural

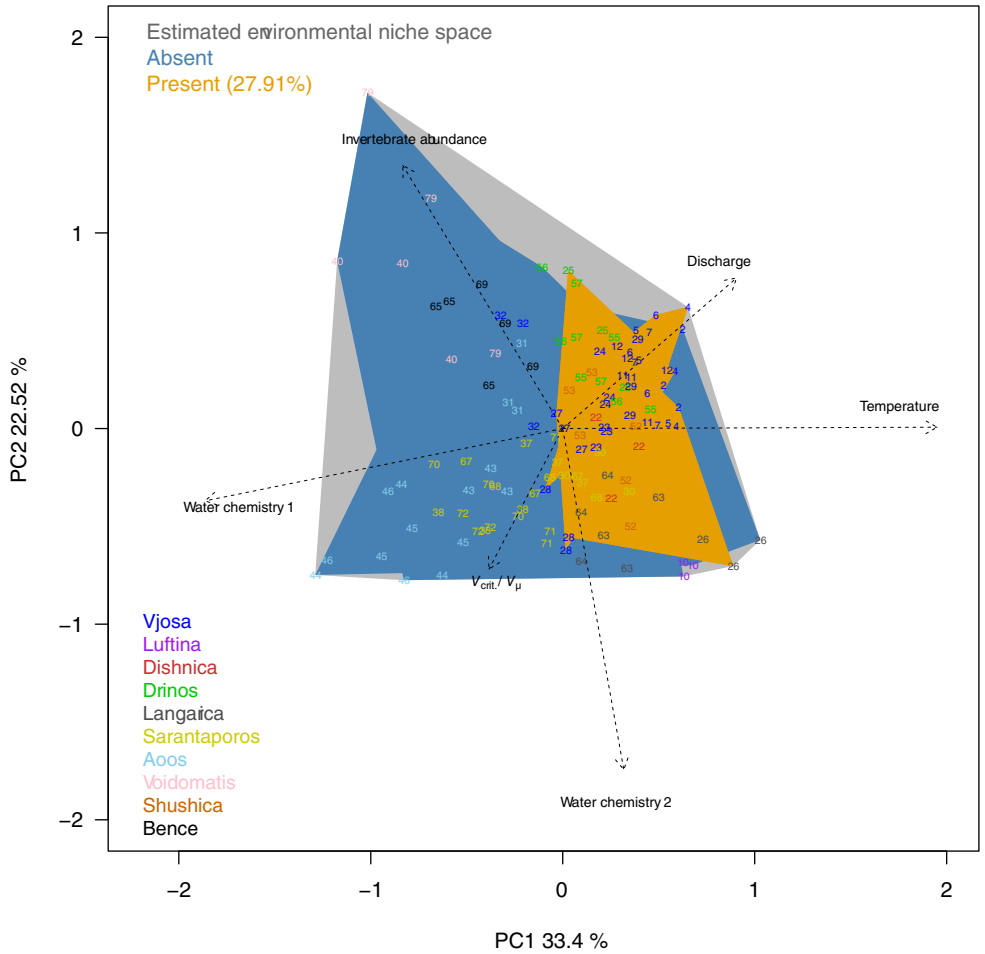

FIGURE 2 Biplot PCA using environmental variables to describe the total and the occupied niche space. PCA axes 1 and 2 explain 33.4% and 22.52% of total variance. The polygons encompass sites with (yellow) and without (blue) *Prosopistoma pennigerum*, respectively; 29.82% of the total available niche space (grey) appears as occupied. Black arrows indicate environmental variables correlation coefficients.

occurrence of dissolved minerals depending on the bedrock geology upstream of the respective sampling site (Supporting Information VII). We described the multivariate niche space available to *P. pennigerum* and the niche actually occupied by *P. pennigerum* with a second PCA. To this aim, we used the first two principal components of the water chemistry data, discharge, V_{crit}/V_{μ} ratio, total abundance of invertebrates and temperature. First and second principal components explained 33.4% and 22.52% of the total variation, respectively. We further set three polygons in this niche space: the first reflects the total estimated environmental niche space (grey), while the second and third polygons connect the sites with the presence of the species (yellow) and sites without the mayfly (blue) for each season, respectively (Figure 2). *Prosopistoma pennigerum* occupied about 27.91% of the total estimated niche space, agreeing with the fact that sites at which *P. pennigerum* occurred are in close vicinity to one another in the PCA space. Separation of occupied versus total niche space or non-occupied niche space was clearer along the first principal component than along the second. We observed a dominant environmental gradient reflecting changes in water chemistry (Water chemistry 1) and temperature along the first principal component. A secondary environmental gradient was defined by water chemistry (Water chemistry 2) and invertebrate abundance along the second principal component. Discharge and V_{crit}/V_{μ} ratio were negatively correlated and angled at 45° to the first and second principal components, thus contributing to both environmental gradients.

Finite mixture model

At sites where *P. pennigerum* was present, we modelled a mean probability of presence (θ) of 0.95 ± 0.08 in spring 2018, $\theta = 0.78 \pm 0.33$ in autumn 2018, and $\theta = 0.81 \pm 0.17$ in autumn 2019, respectively. In sites without occurrences, we modelled a mean $\theta = 0.17 \pm 0.26$ for spring 2018, $\theta = 0.13 \pm 0.21$ for autumn 2018, and $\theta = 0.08 \pm 0.17$ for autumn 2019, respectively (Figure 3a).

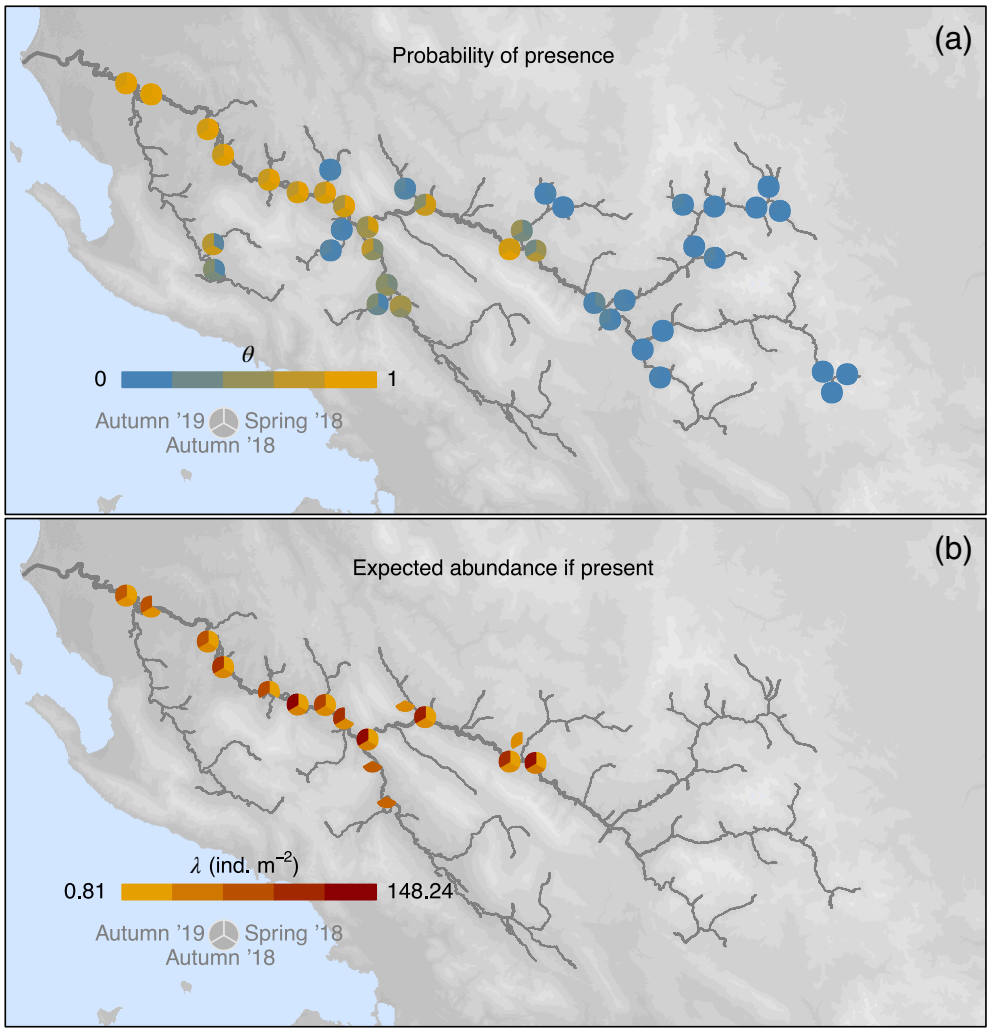

FIGURE 3 Results of finite mixture model predicting presence and abundance of *Prosopistoma pennigerum*. (a) *Prosopistoma pennigerum* probability of presence (θ) in the Vjosa River network: From 0 (most unlikely) to 1 (highly probable). (b) Expected abundance (λ) when *P. pennigerum* is present across the river. Pie charts represent sampling seasons in a clockwise manner: spring 2018 (12:00–4:00), autumn 2018 (4:00–8:00) and autumn 2019 (8:00–4:00).

Expected abundances (λ) are conditional on the presence of *P. pennigerum*. At sites where the species was present our model suggested a mean $\lambda = 1.15 \pm 0.65$ individuals m^{-2} for spring 2018, mean $\lambda = 28.19 \pm 17.7$ individuals m^{-2} in autumn 2018 and mean $\lambda = 104.33 \pm 52.37$ individuals m^{-2} in autumn 2019 (Figure 3b; Supporting Information V).

Biometry

In autumn 2019, *P. pennigerum* larvae ranged in length (CL + HL) from about 0.93 to 4.13 mm, comprising both mature and immature nymphs. Including an estimate of approximately 1 mm for the variable length of the abdomen, this translates to a maximum body length of approximately 5.1 mm. We define mature nymphs, although no clear wing pad development was visible, based on a set of specimens clearly set apart with larger body size, these had a mean EE of 1.43 ± 0.03 SD mm, mean CW of 3.28 ± 0.12 SD mm and mean CL of 3.06 ± 0.18 SD mm (Figure 4a). Along the Vjosa main stem, average body size (as EE) of *P. pennigerum* specimens increased towards downstream sections. However, mature nymphs occurred throughout the river wherever the species was found (Figure 4c).

DISCUSSION

Riverscape dynamics of the Vjosa create braided and anastomosing sections, where habitat turnover is high due to recurrent

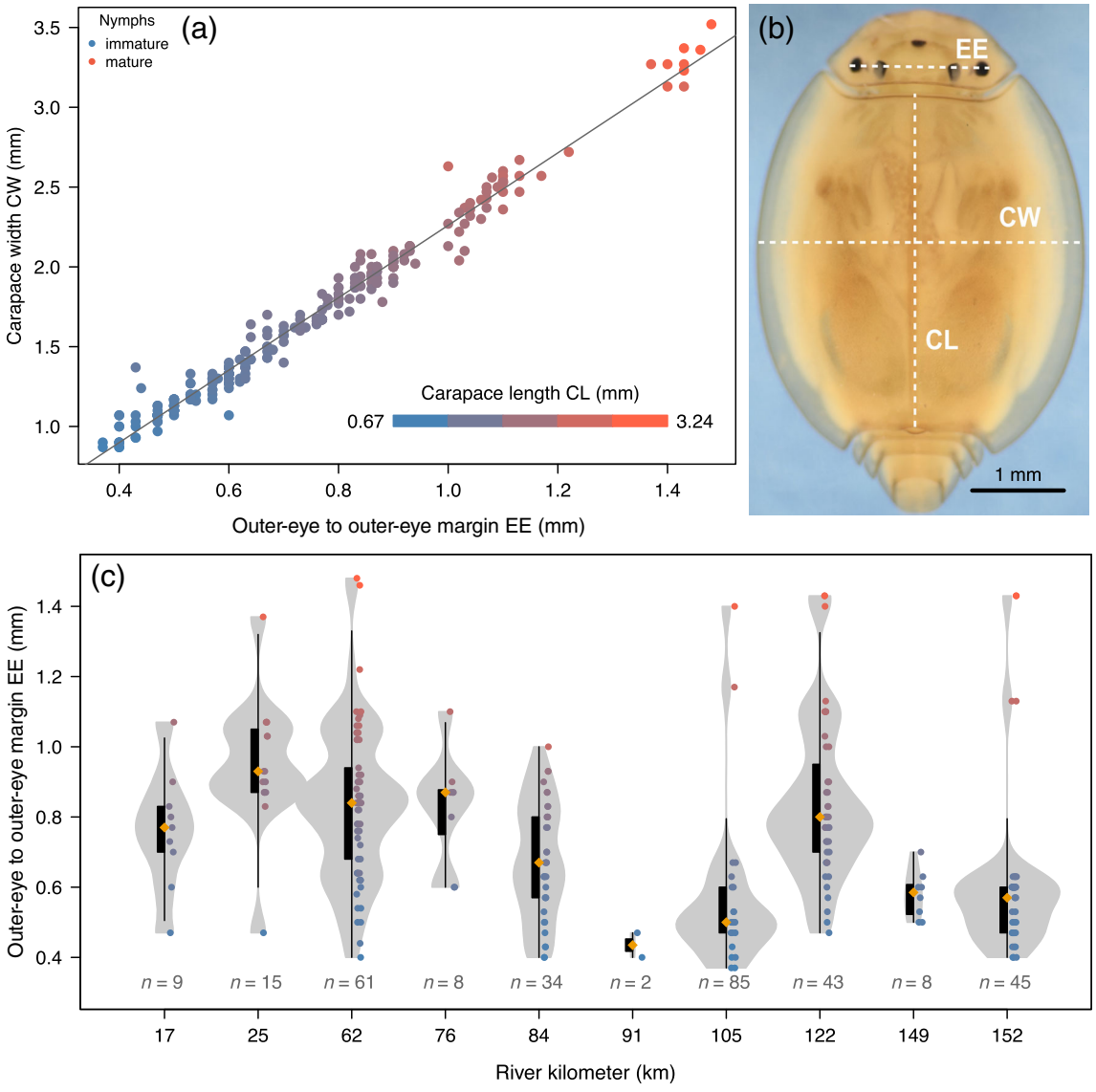

FIGURE 4 *Prosopistoma pennigerum* body size. (a) Size of immature (blue) and mature (red) nymphs of *Prosopistoma pennigerum* specimens of the Vjosa River network: Outer-eye to outer-eye margin (EE) on the x axis, carapace width (CW) on the y axis and carapace length (CL) as colour gradient. Mature nymphs are defined as EE $\mu = 1.43 \pm 0.03$ mm, CW $\mu = 3.28 \pm 0.12$ mm and CL $\mu = 3.06 \pm 0.17$. (b) Measurement scheme on a *P. pennigerum*. (c) Size distribution of *P. pennigerum* along the Vjosa, on the x axis river kilometre measured from the mouth of the river. Black boxplots indicate the median (orange dot), quartiles (boxes) and the 1.5 interquartile range (lines) of the distribution. Violin backgrounds represent the density function of the measured specimens.

re-organisation of the fluvial channel network (Hauer et al., 2021; Schiemer et al., 2020). This is because the Vjosa riverscape has not been a target for hydropower development until recently, and because of the relatively low human population density in the region. Intense hydromorphological modifications were made to curb the original hydrological dynamics elsewhere in European rivers (Hohensinner et al., 2008; Rinaldi et al., 2013; Tockner et al., 2022), likely contributing to the almost complete and widespread extinction of *P. pennigerum* (Schletterer et al., 2021; Schletterer & Füreder, 2009).

Our results indicate that *P. pennigerum* most likely prefers the environmental conditions of the Vjosa main stem: higher discharge and lower bed sediment stability (V_{crit}/V_{μ}) as well as higher water temperatures and lower scores of the first water chemistry gradient (Figure 2). In contrast, we consider environmental conditions in the Bence, Voidomatis, Sarantaporos and upper Aoos tributaries, respectively, as not suitable. In line with our observations, expected probability of presence (θ) increased downstream and was low in the mentioned tributaries. However, high probabilities of presence were also modelled for the Drinos, Shushica and Langarica tributaries, where no or only a few specimens in a single season were collected. These sites also prevented the clear-cut identification of a niche in the PCA: *P. pennigerum* was present in Langarica, Dishnica and Drinos despite rather unsuitable conditions, yet absent from the environmentally suitable Shushica. When *P. pennigerum* was present, modelled abundances (λ) were high and above observed abundances in several cases. Specifically, we observed drops in abundances or null-abundances that were not recovered by our modelling approach in at least one season at sites located directly downstream of the towns of Petran, Tepelena and Poçem, or at sites influenced by increased land-use pressures (Supporting Information VIII). Also, our model returned abundance estimates higher than observed from river km 106 onwards—suggestive for an effect of diffuse habitat degradation in downstream sections. Greater impact from higher human population density and land-use intensity could be expected through artificial groynes stabilising the riverbed downstream of river kilometre 17 and through unmeasured contaminants.

Environmental DNA detection was successful only at sites in which the physical presence of *P. pennigerum* had been established through kicknet sampling. Hence, eDNA did not detect the species at sites without recorded occurrence, even if predicted presence (θ) was high. However, not every observation of *P. pennigerum* was reflected in a positive eDNA signal, resulting in false negatives. We assume that our eDNA sampling effort was not sufficient to match the kicknet sampling, which detects *P. pennigerum* rather reliably. This is in agreement with other studies (e.g. see meta-analysis by Keck et al., 2022) that report poor congruence of specimen and eDNA sampling of benthic macroinvertebrates. The mismatch between observations and eDNA detection may also be due to the low (1 L) water sample volume, which may not be sufficient to detect the entire community or a rare, small-bodied target species (Schabacker et al., 2020; Sepulveda et al., 2019; Troth et al., 2021). Furthermore, the relatively low reaction efficiency (88%) potentially led to the high LOD and LOQ and further to a compromised detection probability of the eDNA assay, resulting in non-detections by eDNA despite physical presence of the target. Given the current state of knowledge, we thus cannot yet recommend to replace benthic kicknet sampling by eDNA sampling, but rather view it as a complementary method.

Our results did not provide conclusive evidence for a particular phenology or life cycle of *P. pennigerum* in the study region: we observed nymphs of all sizes throughout the network in all campaigns, including co-occurring of mature and many immature nymph stages. We interpret this as an indication for an asynchronous univoltine life cycle with multiple overlapping cohorts. Water temperature could be an important control of *P. pennigerum* phenology by affecting development speed and size distribution, but also staged egg development may result in this pattern (Humpesch & Elliott, 2003).

The observed size distribution can be expected to result in the emergence of flying adults over an extended period of time and thus support dispersal of *P. pennigerum*. We consider dispersal potential of *P. pennigerum* as low: data on Prosopistomatidae point at a short adult life and limited flight capacity (Barber-James & de Moor, 2016), while small body size and facultative parthenogenesis reduce overall fecundity (Liegeois et al., 2021). Flying adults must still be expected to be stronger dispersers than the nymphs. Although they deftly swim when disturbed, we expect nymphs to spend most of their time on or in the bed sediments that provide both shelter and food. This behaviour is in line with reports of upstream dispersal distances of at most 200 m without flying for various benthic invertebrates (Arce et al., 2021; Graham et al., 2017). We exclude colonisation by drift dispersal from upstream sections in the tributaries, Aoos or Sarantaporos, as no specimens were found there (including additional qualitative sampling in autumn 2021 and spring and autumn 2022; Martini, Schwingshackl unpub. data).

Based on our observed occurrences along the main stem and three tributaries, we argue that the species must be rather resilient to recurrent natural disturbances that dynamically modify riverscapes. Rapid recovery of population densities as accidentally observed in our study demonstrates high capacity to overcome population bottlenecks. Obligatory or facultative parthenogenesis may be an important trait increasing recovery potential of the species—this is present in some of its congeners but unknown for *P. pennigerum* (Campbell & Hubbard, 1998; Liegeois et al., 2021). Resilience at the population level may benefit from resistance traits at the level of individuals: to survive high discharge events—the most important and frequent disturbance of stream ecosystems—*P. pennigerum* must be adapted to withstand high currents to avoid catastrophic drift. The morphology of *P. pennigerum* (and other Prosopistomatidae) nymphs lends itself to reduced drift vulnerability: the streamlined shape likely acts to reduce drag. In fact, preliminary experimental data indicate that the species is indeed highly drift-resistant when exposed to high water velocities (up to at least 0.6 m s^{-1}; Martini unpub. data). Additionally, nymphs were observed to swim with a speed of about 5 cm s^{-1} and rapidly re-attach to the substrate (Martini unpub. data). Taking together swimming behaviour and habitat requirements, abundance patterns and phenology in the Vjosa, *P. pennigerum* is an inhabitant of highly dynamic fluvial systems with low pollution impact.

CONCLUSION

The mayfly *Prosopistoma pennigerum* still occurs in high abundances in the Vjosa mainstem and occasionally in tributaries. This makes the Vjosa River network one of the last hideouts of this species globally. *Prosopistoma pennigerum* was once widely distributed across all of Europe, yet it is now critically endangered and at risk of global extinction, due to trammels of the natural dynamics of fluvial networks that demolish rivers with a braided or anastomosing morphology across Europe. *Prosopistoma pennigerum* must be considered as well adapted to such strong dynamics, and as a species with a high dispersal and recolonisation potential, at least within a single river network. As such, it could be a primary target for restoration efforts: especially recolonisation of renatured river habitats by *P. pennigerum* could be a significant benchmark for restoration success. Moreover, we argue that the species can be used as a flagship species to promote the conservation of the Vjosa's still intact riverscape because this river is obviously critical habitat for *P. pennigerum*. While calling attention to *P. pennigerum*, we underline the importance of conserving the Vjosa riverscape and its biodiversity. Moreover, this ecosystem can serve as a blueprint for river conservation and restoration. Prudent development of the region must be achieved—ideally by establishing a transboundary protected area.

AUTHOR CONTRIBUTIONS

Jan Martini: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (lead); methodology (equal); software (equal); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Franziska Walther:** Conceptualization (equal); data curation (supporting); investigation (equal); methodology (supporting); software (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Tamara Schenekar:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); writing – original draft (equal); writing – review and editing (supporting). **Emil Birnstiel:** Funding acquisition (equal); investigation (equal); writing – original draft (supporting). **Remo Wüthrich:** Funding acquisition (equal); investigation (equal). **Rebecca Oester:** Investigation (equal); writing – original draft (supporting); writing – review and editing (supporting). **Bernadette Schindelegger:** Data curation (supporting); investigation (supporting); methodology (supporting). **Thea Schwingshackl:** Investigation (equal); validation (equal); writing – original draft (supporting); writing – review and editing (supporting). **Olivia Wilfling:** Investigation (equal); writing – original draft (supporting). **Florian Altermatt:** Funding acquisition (equal); investigation (equal); methodology (equal); writing – original draft (equal); writing – review and editing (supporting). **Lauren Talluto:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); software (equal); visualization (equal); writing – original draft (equal). **Gabriel Singer:** Conceptualization (lead); formal analysis (equal); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); resources (lead); software (supporting); supervision (lead); visualization (supporting); writing – original draft (equal); writing – review and editing (supporting). **Simon Vitecek:** Conceptualization (lead); data curation (equal); formal analysis (equal); investigation (lead); methodology (lead); resources (equal); software (equal); supervision (lead); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (supporting).

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CONFLICT OF INTEREST

There are no conflicts of interest to be declared among the authors of the manuscript.

DATA AVAILABILITY STATEMENT

This work is part of larger project focussing on the Vjosa riverscape. At present, data are available upon request. All data associated with the project will be made publicly available as data publication in the near future, when the project goals will have been accomplished.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Supporting Information I: Map of the Greek-Albanian Vjosa catchment with major tributaries. Red dots indicate sampling sites.

Supporting Information II: Characteristic habitats of *Prosopistoma pennigerum*. (A) Canyon like sampling site 23 (J. Martini 10.2018); (B) fast-flowing sampling site 27 (J. Martini 10.2018); (C) Braided river sampling site 6 (G. Singer 09.2021); (D) Kalivac dam construction site (G. Singer 09.2021).

Supporting Information III: Download link of the custom library obtained via the BOLD API containing >35 k barcode sequences (as of 06.ii.2020) of Eurasian benthic invertebrates as .fas file.

Supporting Information IV: Co-occurring *Prosopistoma pennigerum* specimens of various sizes.

Supporting Information V: Abundance of *Prosopistoma pennigerum*, probability of occurrence (θ) and expected abundances if present (λ) in the Vjosa River network in spring 2018, autumn 2018 and autumn 2019. In yellow sites with P. pennigerum.

Supporting Information VI: Comparison of environmental variables, across all sampling seasons for all sites (grey), sites with *Prosopistoma pennigerum* (yellow) and sites without the species. Boxplots represent the median, IQR, 1.5× range and outliers as dots.

Supporting Information VII: First (A), second (B) and third (C) principal components, based on environmental variables, were used to describe the potential and the occupied niche space along the Vjosa River. We further implemented these scores in the finite mixture model. First (D) and second (E) principal components were implemented in the niche space PCA as water chemistry gradients. Pie charts represent sampling seasons in a clockwise manner: spring 2018 (12:00–4:00), autumn 2018 (4:00–8:00) and autumn 2019 (8:00–4:00).

Supporting Information VIII: Human population density along the Vjosa riverscape and surrounding regions in the Southern Balkans. Human population density along the Vjosa riverscape increases in the downstream sections towards the delta and is generally low in the headwaters and upstream sections of the catchment. Data source: NASA SEDAC, Population Density v4.11, 2020 (CIESIN, 2018).
Reference for this map: Center for International Earth Science Information Network (CIESIN), Columbia University. 2018. Documentation for the Gridded Population of the World, Version 4 (GPWv4), Revision 11 Data Sets. Palisades NY: NASA Socioeconomic Data and Applications Center (SEDAC). https://doi.org/10.7927/H45Q4T5F Accessed 29.v.2022.

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