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ORIGINAL PAPER

Impact of the invasive alien topmouth gudgeon (*Pseudorasbora parva*) and its associated parasite *Sphaerothecum destruens* on native fish species

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Abstract The Asian cyprinid *Pseudorasbora parva* is considered to be a major threat to native fish communities and listed as an invasive alien species of European Union concern. Our study aims to gain evidence-based knowledge on the impact of both *P. parva* and its parasite *Sphaerothecum destruens* on native fish populations by analysing fish assemblages and body condition of individuals of native fish species in floodplain water bodies that were invaded and uninvaded by *P. parva*. We explored the use of environmental DNA (eDNA) techniques to detect *S. destruens*. Prevalence of *S. destruens* in native fish species was assessed. Fish samplings showed significantly negative correlations between the abundance of *P. parva* and the native *Leucaspius delineatus*, and *Pungitius pungitius* and three biodiversity indices of the fish assemblages (Simpson's diversity index, Shannon–Wiener index and evenness). Contrastingly, the abundances of the native *Gasterosteus aculeatus* and *P. parva* were positively related. In nearly all isolated water bodies with *P. parva*, this species is outnumbering native fish species. No effect of *P. parva* presence was found on

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body condition of native fish species. *Sphaerothecum destruens* was demonstrated to occur in both *P. parva* and *G. aculeatus*. *Gasterosteus aculeatus* is suggested to be an asymptomatic carrier that can aid the further spread of *S. destruens.* Analysis of eDNA proved to be a promising method for early detection of *S. destruens*, here showing that *S. destruens* presence coincided with *P. parva* presence. The ongoing invasion of both *P. parva* and *S. destruens* is predicted to pose a significant risk to native fish communities.

Keywords Biodiversity threat · eDNA · *Gasterosteus aculeatus* · *Leucaspius delineatus* · Pathogen · *Pungitius pungitius*

Introduction

Invasive species affect ecosystem functions and services (Vilà et al. 2010) and can have a high economic impact (Cook et al. 2007). Fish species are important invaders, with a high number of species introduced by human activities outside their original biogeographic region (Strayer 2010). Predation, habitat degradation, competition for resources, hybridization and disease transmission are considered key factors in association with invasive alien fish introductions (Gozlan et al. 2010a). Threats caused by alien pathogens, often associated with other invasive species that serve as reservoirs, are poorly understood and deserve more attention in risk assessment and management of biological invasions (Roy et al. 2017). Pathogen pollution caused by spill-over from invasive fish species goes largely undetected in ongoing monitoring schemes, clouding the causes of their detrimental effects on naïve native species (Andreou and Gozlan 2016).

The cyprinid topmouth gudgeon (*Pseudorasbora parva*) and its associated pathogen *Sphaerothecum destruens* are an example of such a cryptic, invasive duo. Their invasion initially started via aquaculture trade (Sana et al. 2017), with *P. parva* serving as an unaffected reservoir. *Pseudorasbora parva* is listed as an invasive alien species of European Union concern (European Commission 2016). There is a growing need for evidence-based knowledge on its impact and effective management measures.

Pseudorasbora parva originates from China and South-East Asia (Bănărescu 1999). This species has rapidly colonised Europe from countries around the Black Sea, where it was introduced unintentionally in the 1960s (Gavriloaie et al. 2014; Simon et al. 2015). The range expansion of *P. parva* is regarded as one of the most compelling examples of fish invasion in the world (Gozlan et al. 2010b). As a ubiquitous, sedentary, hardy, omnivorous fish species (Bănărescu 1999), it occurs in both lentic and lotic waters, but is most abundant in lentic conditions such as well vegetated ponds, small lakes, and side channels of rivers (Gozlan et al. 2010b). It adapts well to new man-made habitats in both its introduced range and its native range (Onikura and Nakajima 2012). This high plasticity in life history traits and broad and opportunistic habitat use explains its success as an invader (Gozlan et al. 2010b). *Pseudorasbora parva* is considered to be a major threat to native fish communities. It was demonstrated that only four years after introduction into a lake, the native fish species composition and community structure had dramatically shifted due to competition (Britton et al. 2007). In addition, Gozlan et al. (2005) found that *P. parva* is a healthy carrier *of S. destruens*, a fish pathogen which has the capacity to threaten native fish populations.

Sphaerothecum destruens, previously named rosette agent and first discovered in 1984, belongs to the Rhinosporidiidae, a family of aquatic pathogens that belong to the Dermocystida, an order of the class Mesomycetozoea (Ichthyosporea) (Arkush et al. 2003).This class is phylogenetically classified on the boundary of animals and fungi (Mendoza et al. 2002). All known Mesomycetozoean species live in symbiotic relationships with animals and most of them are aquatic (Glockling et al. 2013). *Sphaerothecum destruens* lives as intracellular spores within its host that are released into the water via the host's urine, bile or gut epithelium. Here it forms active, uniflagellate zoospores that can infect other hosts (Arkush et al. 2003; Andreou 2010; Al-Shorbaji et al. 2015). *Sphaerothecum destruens* tolerates a wide range of temperatures (Andreou et al. 2009) and can survive up to 26 days as a zoospore at 4 °C.

Sphaerothecum destruens became known for causing mortality in salmonid fishes in aquaculture (Arkush et al. 2003). It was subsequently identified as the cause of mortality and spawning inhibition in populations of *Leucaspius delineatus* (Gozlan et al. 2005; Andreou et al. 2011; Paley et al. 2012). Preliminary examination showed susceptibility of

Table 1 Use of (sub)datasets for each section in the results

Data in Methods	Used for analysis in results section	Collected in year	Number of sites included in analysis
Fish assemblage and habitat characterization	Effects of *Pseudorasbora parva* abundance on fish assemblage	2015	39
Fish assemblage and habitat characterization	Habitat conditions in sampled water bodies	2015	39
Fish assemblage and habitat characterization	Effects of *Pseudorasbora parva* presence on fish condition	2015	54
Prevalence of *Sphaerothecum destruens*	Prevalence of *Sphaerothecum destruens* in native fish and *Pseudorasbora parva*	2014	1
Environmental DNA sampling and analyses	Presence of *Sphaerothecum destruens* in water bodies	2016	39

other cyprinids to the parasite as well (Gozlan et al. 2005). An increasing number of studies showed that *S. destruens* negatively impacts local native fish populations in Europe (Gozlan et al. 2009; Andreou 2010; Peeler et al. 2011; Andreou et al. 2012; Ercan et al. 2015). *Abramis brama*, *Rutilus rutilus* and *L. delineatus*, all species native to Western Europe, are highly susceptible to infection by *S. destruens* (Al-Shorbaji et al. 2015). However, the impact of *P. parva* or *S. destruens* on abundance, body condition and reproduction of native fish in situ are poorly known.

Our study aims to increase the body of knowledge on the impact of *P. parva* and *S. destruens* on native fish assemblages. We are addressing the following research questions: (1) how do fish assemblages differ in water bodies with or without *P. parva* in terms of abundance and reproductive success of native species and community diversity? (2) does *P. parva* presence have an effect on body condition of native fish? and (3) what is the prevalence of *S. destruens* in native fish species? As there is a need for quick and reliable methods to detect non-native pathogens, we also address the following research question: (4) can environmental DNA be used as a molecular method to detect *S. destruens* in situ?

Methods

Data were collected over the period of 2014–2016 for the analyses of effects of *P. parva* abundance on the fish assemblage, effects of habitat conditions in sampled water bodies, effects of *P. parva* presence on fish condition, prevalence of *S. destruens* in native fish and *P. parva* and finally the presence of *S. destruens* in water bodies (Table 1).

Fish assemblage and habitat conditions

We selected 54 water bodies (oxbow lakes, shallow lakes and ponds) in river floodplains of the IJssel, Meuse, Nederrijn and Waal River. These water bodies were selected using the following criteria: (a) Potential presence of *P. parva* according to the Dutch National Database Flora and Fauna (NDFF 2015), (b) No permanent hydrological connection with the main stream or a side channel, (c) Similarity in habitat characteristics (e.g., depth and surface area, for habitat characteristics per sampling site see Online Resource 1), (d) Suitability for sampling with a seine net.

These criteria were set to reduce variance in the fish species composition created by habitat variables, as our aim was to detect effects caused by *P. parva*. The areas of sampled water bodies ranged from 100 to 80,000 m^2. In total 54 sites located in the floodplains were visited (Fig. 1) and sampled using a seine net (21 m long, 2.4 m high, mesh size 4×4 to 10×10 mm). Fifteen sites could not be sampled sufficiently with this gear type due to high vegetation cover and/or water depth (Fig. 1). Hence, 39 sites were included in the analyses of effect on fish assemblages and body condition (Table 1). Sampling of the fish populations was carried out from October to December 2015. The seine net was used while wading and provided adequate data on juvenile and small fishes in shallow habitats. The sampling area ranged from 0.04 to 82.35% of the surface area of water bodies and was used to calculate fish densities (number of fish m^{-2}). All caught fishes were identified, weighed (accuracy

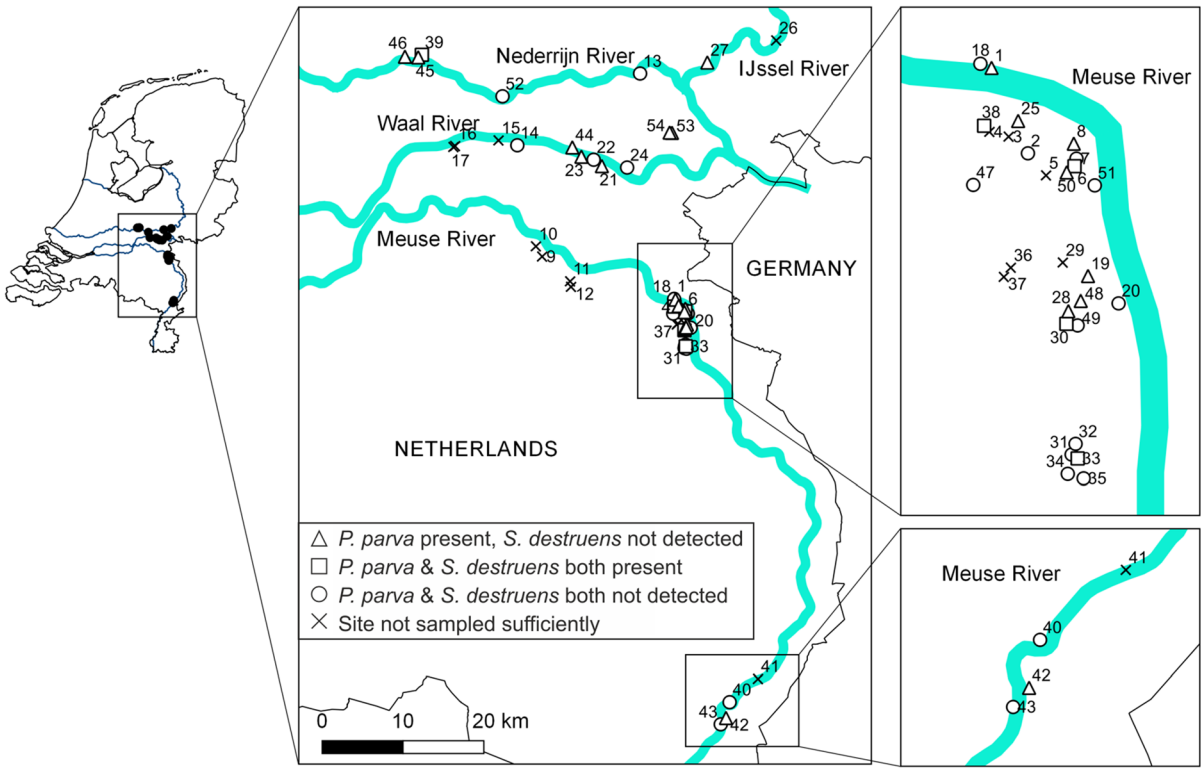

Fig. 1 Sampling sites with *Pseudorasbora parva* and/or *Sphaerothecum destruens* and without these species at the floodplains of the rivers IJssel, Meuse, Nederrijn and Waal in the Netherlands

0.05 g) and their total lengths (TL, from tip of snout to longer caudal fin lobe, accuracy 1 mm) measured in the field. Young of the year (YOY) were distinguished, based on length. Each individual was assigned to being a YOY, based on known YOY thresholds (Kranenbarg et al. 2010). Small fishes (< 35 mm) were pooled for weighting. In case a large number (> 50) of the same species and length were encountered, a representative number was weighed and measured and the remaining individuals were only counted. Subsequently, the fishes were released. Using the obtained data, Simpson index (1-D), Shannon–Wiener index, species richness (total number of fish species) and evenness (a distribution of abundance among species within a community) were calculated (Wilsey and Potvin 2000). Habitat and soil parameters which included coverage percentage of aquatic vegetation, littoral vegetation, and substrate (mud, sand, gravel and rocks), and tree branches in the water were visually estimated. The water transparency (cm) was determined using a Secchi disk (measured vertically). Water temperature (°C), conductivity (µS cm^{-1}) and salinity (PSU) were measured at the site with the use of a Model 30 m (YSI incorporated). A water sample was taken and at the same day pH and alkalinity (eq l^{-1}) were measured in the laboratory. Water samples in polyethylene bottles were stored in the freezer at a maximum storage time of 75 days until analysis. Metal ions were analysed using an ICP analyser (Thermo Electron corporation IRIS Intrepid II XDL). Concentrations of nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{3-}), chloride (Cl^-) and potassium (K^+) were determined using an Auto Analyzer 3 system (Bran and Luebbe, Norderstedt Germany).

Prevalence of *Sphaerothecum destruens*

To determine infection rates of *S. destruens* in *P. parva* and three native fish species, eighty fish were caught in the Teelebeek stream (WGS84 coordinates 51.718, 5.929) in December 2014. In total 20 *P. parva*, 20 *Gasterosteus aculeatus*, 20 *Barbatula barbatula* and 20 *Rutilus rutilus* individuals were caught by means of dip-net fishing and euthanized with

benzocaine (100 mg l^{-1}). The fishes were individually preserved in tubes with ethanol (96%) and stored at − 20 °C. Of each individual fish, kidneys, liver and gonads were dissected out of the body and pooled prior to DNA extraction (Miller et al. 1998). DNA was extracted using the Powersoil DNA kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacture's protocol. Polymerase Chain Reaction (PCR) was performed on the extracted DNA using primers targeting the ribosomal 18S rRNA gene of *S. destruens* (Gozlan et al. 2005): (R1) 5′-GAA-GTC-ACA-GGC-GAT-TCG-G-3′, (F2) 5′-ACA-GGG-CTT-TTT-AAG-TCTTGT-3′ and (R2) 5′-ATG-GAG-TCA-TAG-AAT-TAA-CAT-CC-3′. For the first PCR, the F2 and R1 primer combination were used, yielding a 909 base pair product. All PCR results were examined using 1% agarose gel electrophoresis. To improve specificity of the PCR and exclude false positives, a nested PCR was carried out on the previously obtained PCR product using the F2 and R2 primer combination which yielded a 600 base pair product. Both primer combinations were also used separately (not as nested primer pair) for PCR. As a positive control, a plasmid containing a fragment encoding for the 18S rRNA of *S. destruens* was used (Gozlan et al. 2005, 2009). The PCR for both the first and the nested PCR was done using an initial denaturation of 5 min at 94 °C. Subsequently, 35 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min using a gradient of 50–60 °C and elongation for 1.5 min at 72 °C were performed. Finally, an elongation step of 10 min at 72 °C was performed. The analysis was performed in 2015.

The nested PCR products that yielded a 600 base pair product were purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). PCR products were directly Sanger sequenced by Base-Clear B.V. (Leiden, Netherlands). The sequences were analysed using Chromas Lite version 2.4.4 (Technelysium Pty Ltd, Australia) and a BLASTN analysis (http://www.ncbi.nlm.nih.gov) was performed. Sequence alignment was performed using ClustalW (Larkin et al. 2007). Phylogenetic analysis was performed, to show possible genetic diversity within the amplified 18S fragments of the different samples obtained with above described primers targeting the 18S rRNA gene of *S. destruens*, linked to the location of sampling. The phylogenetic analysis was performed also to validate the outcomes of our eDNA experiment, to record and assess site specificity of populations of *S. destruens*. The phylogenetic analysis was performed with the software package MEGA 7.0.14 (Tamura et al. 2011) using the Neighbour-joining method and the p-distance model with pairwise deletion and 1000 bootstrap replications. The obtained sequences from the examined fishes were deposited in GenBank under the following accession numbers: MF319869 to MF319872.

Environmental DNA sampling and analyses

Each eDNA sample was composed of 40 water samples from a water body taken with a sterile 50 ml tube, up to 50 cm deep and later mixed in a sterile plastic bag. From the two litre homogeneous mixed water sample, a 15 ml sample was conserved in a sterile 50 ml tube, with 3 M 5 ml sodium acetate (pH 5.2) and 30 ml ethanol (100%) and separately stored at − 20° C (Dejean et al. 2011), in a sealed sterile plastic bag to avoid cross contamination between tubes. During the sampling, for each spot, fresh gloves were used and discarded after sampling. Great care was taken to avoid cross-contamination of the samples during eDNA sampling. DNA extraction was performed per sample separately on a decontaminated work spot. For the PCR, preparations were performed in a PCR UV chamber (Plas-Labs model 825) and negative controls were used (sterile Milli-Q).

Environmental DNA, biological material and solid particles were pelleted by 30 min centrifugation of the 50 ml sample tube at 4000 g at 4 °C. DNA was extracted from the pellet using the Powersoil DNA kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's protocol. After DNA isolation, a fragment of the 18S rRNA gene was amplified with PCR using F2 and R1 primers, followed by a nested PCR using F2 and R2 primers and Sanger sequenced as described above. Since the primers target the 18S rRNA gene of *S. destruens*, only a similarity of the amplified product with known *S. destruens* sequences could be established. Obtained PCR products of a similar size as the amplification product of the positive control of *S. destruens* were cloned using the pGEM®-T Easy Cloning Vector (Promega, Madison, USA) and transformed in *E. coli* XL1Blue competent cells. Clones were subtracted to blue/white screening, positive clones were randomly picked, and the plasmids were subsequently isolated using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) and

Sanger sequenced by BaseClear B.V. (Leiden, The Netherlands). The analysis was performed in 2016. The obtained sequences from the eDNA samples were deposited (provided with latitude and longitude coordinates) in GenBank under the accession numbers: MF319861 to MF319868.

Statistical analyses

Effect of Sphaerothecum destruens and Pseudorasbora parva on co-occurring fish species

To test for the expected effect of *P. parva* on native fish populations, generalized linear models (GLM) were used. First, we tested the relation between the abundance of *P. parva* and the abundance of each of the other fish species that were found living together with *P. parva*. Therefore, the abundance of each fish species was separately included as the response variable in the GLMs and the abundance of *P. parva* was included in the model as an explanatory variable. These analyses were performed for six species that were found in ≥ 7 sites. Furthermore, the relation between the abundance of *P. parva* and each diversity index (Simpson index, Shannon–Wiener index, species richness and evenness) was tested. Since the response variables were in many cases integers, we used the Poisson or negative binomial distribution, depending on over-dispersion. When the response variable data were non-integers, GLMs with normal distributions were applied when criteria for these models were met. The models best fit was chosen based on Akaike's Information Criterion (AIC). These analyses were conducted with the use of R version 3.6.0 (R Core Team 2019) using the MASS package (Venables and Ripley 2002).

Multivariate analyses of habitat conditions

Multivariate analyses using Principal Component Analysis (PCA) were performed to provide insight in the physico-chemical characteristics of the sampled sites. One PCA was performed on the obtained chemical habitat data, and one PCA was performed on the physical habitat data. The data were first standardized (Z-score) by subtracting the obtained values by the mean. The resulted value was divided by the standard deviation. Permutational multivariate analysis of variance (PERMANOVA) was performed to determine if the difference between the presence or absence of *P. parva* is explained by ordering the sites along the first principal component (PC1). The PERMANOVA was performed with 999 permutations and based on Euclidean distance. Multivariate analyses were performed with the use of R version 3.6.0 (R Core Team 2019) using the vegan package (Oksanen et al. 2016).

Effect of Pseudorasbora parva on body condition

Body conditions of several species in the presence and absence of *P. parva* were compared, to test whether presence of *P. parva* decreases condition of native species. In this analysis, we used data from 54 sampled sites (Fig. 1). For testing differences in the length–weight relationship (also referred to as condition) of individuals, the isometric/allometric growth was determined (Santos et al. 2002). The length–weight relationship of individuals per species is expressed by:

$$W = a \cdot L^b \tag{1}$$

Here, W is the recorded weight (g), L is the total length (from tip of snout to longer caudal fin lobe, cm), a is a body form coefficient and b is the exponent which indicates relative growth (Froese 2006). The values a and b were obtained by fitting least-squares regression on the natural log length as a function of the natural log weight for each species. Value b is the slope of the regression line in logarithmic form, value a is calculated as the exponent of the intercept. Subsequently, the mean relative weight (W_{rm} which is an indication of the body condition) per individual was calculated by:

$$W_{rm} = 100 \cdot \frac{W}{a_m L^{b_m}} \tag{2}$$

in accordance with Froese (2006). Welch Two Sample t tests were used for comparing the relative weight per fish species between the sites with and the sites without *P. parva* using R version 3.6.0 (R Core Team 2019). However, the sample sizes of these two groups largely differed for most of the fish species and were regarded too unequal (when the sample size of the larger group outnumbered the smaller group by more than half). Therefore, only *Carassius gibelio, Gasterosteus aculeatus, Pungitius pungitius* and *Tinca tinca* could be tested.

eDNA related analyses

It is expected that the occurrence of *S. destruens* coincides with the occurrence of *P. parva*. To test whether *S. destruens* is randomly distributed in sites, a Chi square test was performed. The expected values for this test were determined using the number of sites with *P. parva* (P +), the number of sites without *P. parva* (P−), and the number of sites with *S. destruens,* assuming the *S. destruens* can be found in these sites proportionally to P+ and P−. A Welch Two Sample *t* test on surface area was performed between sites harbouring *S. destruens* and sites where it was not detected to test if *S. destruens* detection was related to surface area. This was tested as eDNA detection limits are affected by *P. parva* densities (Sana 2016), assuming to be related to surface area of water bodies. This test was performed with the use of R version 3.6.0 (R Core Team 2019).

Results

Fish assemblage and habitat conditions

In total, 12,607 individuals representing 21 fish species were captured, measured and weighed at 39 different sites (Table 2). *Gasterosteus aculeatus* was the most abundant species with 7,933 individuals found at 27 sites (Table 2). Densities of *P. parva* ranged from 0.01 to 9.85 individuals m^{-2} (Table 2, Online Resource 2). *Pseudorasbora parva* was found to be present at 21 sites (Fig. 1) and co-occurred with 14 other fish species: *Abrama brama, Carassius auratus, Carassius gibelio, Cobitis taenia, Cyprinus carpio, G. aculeatus, Gobio gobio, L. delineatus, Neogobius melanostomus, Proterorhinus semilunaris, Pungitius pungitius, Rhodeus amarus, Scardinius erythrophthalmus* and *Tinca tinca* (Online Resource 2). *Pseudorasbora parva* was the most abundant species based on densities in 10 out of 21 water bodies, while in 8 out of these 21 water bodies, *G. aculeatus* was the most abundant species. Where present, *P. parva* made up on average 48% (± SE 6.6%) of the total number of individuals (Online Resource 2). Young of the year (YOY) (≤ 35 mm, Gozlan et al. 2010b) of *P. parva* were present in 17 out of 21 (81%) water bodies and were not recorded in water bodies with low densities of *P. parva* of < 0.30 individuals m^{-2}. Principal Component Analysis and PERMANOVA show that the presence or absence of *P. parva* is not explained by chemical (F = 0.40; $p = 0.55$) or physical habitat (F = 0.08; $p = 0.80$) variables (Online Resource 3). Online Resource 1 summarizes all physico-chemical habitat characteristics of sampled sites.

Effects on fish assemblage and body condition

Pseudorasbora parva abundance showed significantly negative relations with *L. delineatus* abundance (Z = − 2.051; $p < 0.05$) and *P. pungitius* (Z = − 2.033; $p < 0.05$) (Fig. 2). Three diversity indices showed significantly negative relations with *P. parva* abundance, when including *P. parva* in the calculation of these indices: Simpson's diversity index of fish assemblages (t = − 3.506; $p < 0.01$), Shannon–Wiener index (t = − 3.2; $p < 0.01$) and evenness (t = − 3.104; $p < 0.01$) (Fig. 2). A positive significant correlation was found with *G. aculeatus* abundance (Z = 2.03; $p < 0.05$) (Fig. 2). No correlation was found between *P. parva* and three other co-occurring species (*P. semilunaris, R. amarus* and *T. tinca*). Also, no correlation was found between *P. parva* abundance and species richness. None of the diversity indices showed a significant relation with *P. parva* abundance, when *P. parva* was excluded from the index calculations.

YOY co-occurring with *P. parva* were found for the following species: *C. gibelio, G. aculeatus, G. gobio, L. delineatus, N. melanostomus, P. pungitius, R. amarus, S. erythrophthalmus* and *T. tinca* (Table 2).

No differences in condition (mean relative body weight W_{rm}) in the presence or absence of *P. parva* of co-occurring fish species could be demonstrated. Only *C. gibelio*, *G. aculeatus*, *P. pungitius* and *T. tinca* could be tested because sample sizes of other fish species were regarded too unequal (sample size threshold: when the sample size of the larger group outnumbered the smaller group by more than half). Due to the limited number of sites where *S. destruens* was detected (n = 5), body condition of individuals of other fish species than *P. parva* by the mean relative weight W_{rm} was only compared in the presence or absence of *P. parva*, and not for the presence or absence of *S. destruens*.

Table 2 Overview of fish data sorted by family

Family	Scientific name	N individuals	N sites total	N sites co-occurring with *P. parva*	N sites YOY with *P. parva* present	N sites YOY with *P. parva* absent	Mean density (N m^{-2})	Density range (N m^{-2})	Weight range (g)	Length range (cm)
Centrarchidae										
	*Lepomis gibbosus**	15	1	0	1	0	0.004	0.14	0.31–0.60	2.6–3.6
Cobitidae										
	Cobitis taenia	7	5	3	2	0	0.002	0.01–0.02	0.50–7.0	5.5–12
Cyprinidae										
	Abramis brama	1	1	1	0	0	0.00034	0.01	1400	52
	*Carassius auratus**	1	1	1	0	0	0.00016	0.01	4.5	7.4
	*Cyprinus carpio**	3	3	2	0	0	0.002	0.01–0.05	57–7000	2.4–67
	Rutilus rutilus	7	5	0	0	0	0.004	0.02–0.06	3.3–35	7.4–15
	Gobio gobio	8	1	1	1	0	0.002	0.07	0.20–0.55	3.4–4.5
	*Aspius aspius**	10	3	0	0	0	0.003	0.01–0.08	3.2–7.3	7.4–10
	Tinca tinca	13	8	2	1	5	0.007	0.004–0.15	0.20–145	2.6–22
	Scardinius erythrophthalmus	21	4	2	2	2	0.012	0.002–0.42	0.05–2.5	3.0–6.8
	Leuciscus idus	34	1	0	0	1	0.026	1.0	0.20–0.60	2.6–4.5
	*Carassius gibelio**	49	3	3	1	0	0.016	0.01–0.52	6.3–14	8.1–14
	Rhodeus amarus	159	13	4	4	3	0.11	0.004–1.6	0.10–4.0	2.6–6.7
	Leucaspius delineatus	1721	7	3	2	4	2.3	0.03–62	0.13–4.6	2.5–8.5
	*Pseudorasbora parva**	1737	21	–	–	–	1.1	0.01–9.8	0.05–8.3	1.5–9.7
Gasterosteidae										
	Pungitius pungitius	755	19	11	7	4	0.94	0.01–20	0.20–2.0	2.3–7.6
	Gasterosteus aculeatus	7933	27	15	11	8	2.7	0.01–21	0.05–4.5	0.3–7.4
Gobiidae										
	*Neogobius melanostomus**	2	2	1	1	0	0.001	0.01–0.03	1.8–7.9	5.5–8.5
	*Neogobius fluviatilis**	27	4	0	0	1	0.017	0.06–0.45	0.30–4.4	3.6–7.5
	*Proterorhinus semilunaris**	71	9	1	0	3	0.029	0.01–0.44	0.30–2.8	3–6.9
Percidae										
	Perca fluviatilis	33	4	0	0	1	0.013	0.04–0.27	0.90–5.5	4.8–8.4

N represents the total number of individuals caught at all sites during sampling. Mean density is the number of individuals/sampled surface area (m^2) of all (39) sites. Density range displays the lowest and highest density (N m^{-2}) of the sites sampled surface. Weight range represents the minimum and maximum weight of the encountered individuals and length range shows their minimum and maximum length. * indicates an alien species; *YOY* Young of the year

Prevalence of *Sphaerothecum destruens* in native fish and *Pseudorasbora parva*

Kidneys, liver and gonads of eighty individuals of fish, comprising four species (each n = 20), were tested for the presence of *S. destruens* to establish infection rates. For 13 out of 20 samples isolated from *G. aculeatus*, PCR resulted in fragments matching the expected size of the PCR product for *S. destruens*, when using the F2 × R1 primer combination

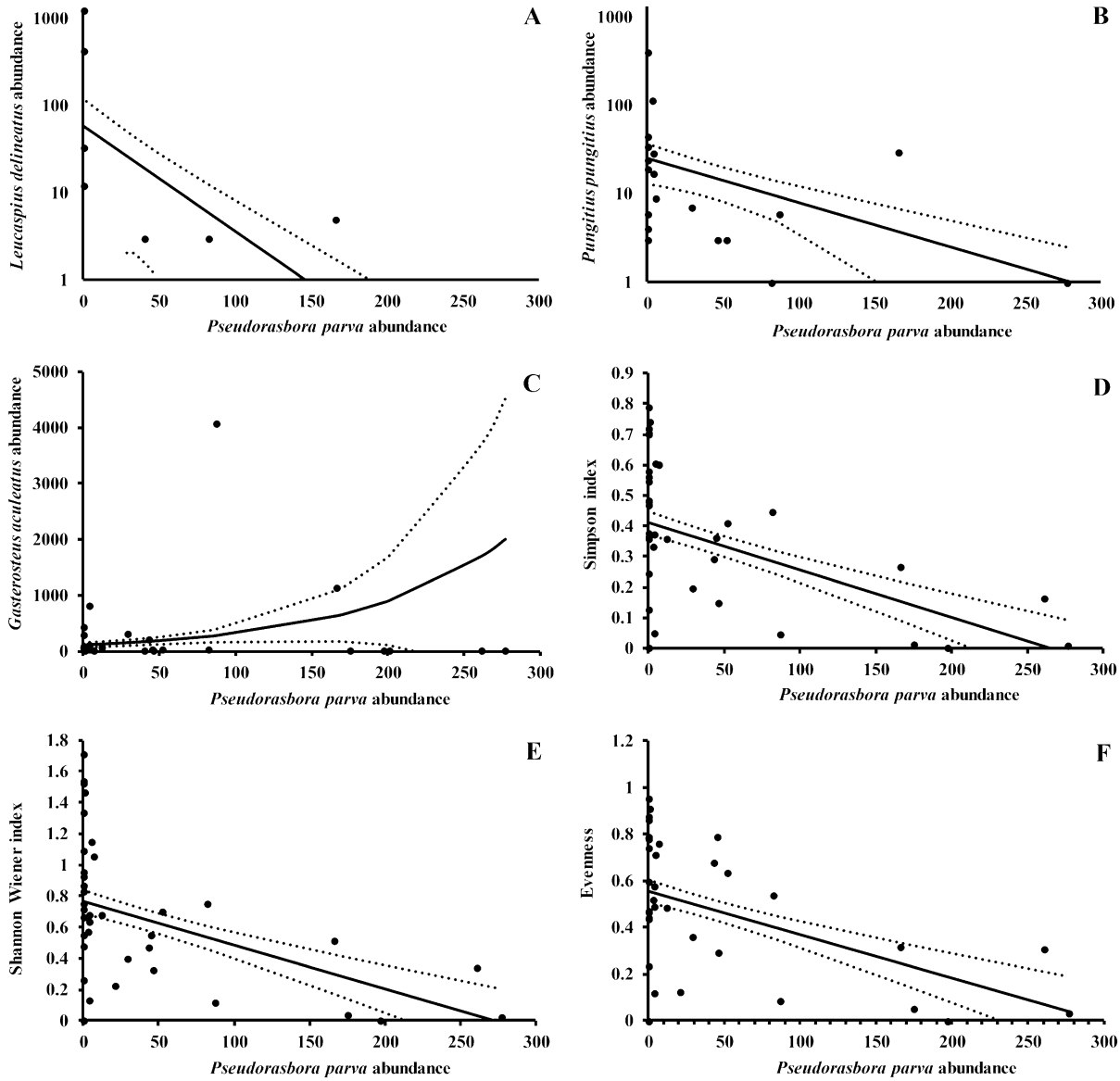

Fig. 2 Obtained values (dots) and fitted values (solid line) with 95% confidence intervals (dotted line) of significant correlations by GLMs between *Pseudorasbora parv*a abundance and **a** *Leucaspius delineatus* abundance (log scale, Z = − 2.051; $p < 0.05$), **b** *Pungitius pungitius* abundance (log scale, Z = − 2.033; $p < 0.05$), **c** *Gasterosteus aculeatus* abundance (Z = 2.03; $p < 0.05$), **d** Simpson diversity index (t = − 3.506; $p < 0.01$), **e** Shannon–Wiener index (t = − 3.2; $p < 0.01$) and **f** evenness (t = − 3.104; $p < 0.01$)

(n = 10), F2 × R2 primer combination (n = 2). One of the fishes showed fragments for both primer combinations (n = 1). From these 13 samples, eight were randomly selected for sequencing. BLAST search analysis using BLASTN gave a positive match with *S. destruens* for five of the *G. aculeatus* individuals. For four of these samples (22R2, 25R1, 52R1 and 53R1), showing 97–99% similarity with the 18S rRNA gene of *S. destruens*, phylogeny could be performed. One sample that showed mixed sequences was excluded from the phylogenetic analysis. Prevalence of *S. destruens* in *G. aculeatus* is at least 25% (5

out of 20 specimens that were analysed), but might be as high as 50%, as only 8 out of 13 PCR products were sequenced. For at least 18 out of 20 sampled specimens (90%) from *P. parva,* PCR resulted in fragments matching the expected size of the PCR product for amplification product for 18S rRNA gene of *S. destruens*, when using either primer combination F2 × R1 or F2 × R2. From these samples, six (showing the highest intensity on agarose gel) were selected for sequencing. Sequence analysis and phylogeny showed that five out of six samples best matched *S. destruens* using BLASTN analysis (similarity 97–99% and 0.0 E-values). Prevalence of *S. destruens* in *P. parva* in this study is at least 25% (5 out of 20), but might be as high as 75%, as only 6 out of 18 PCR products were sequenced. These results were excluded from the phylogenetic analysis as they showed mixed sequences. *Sphaerothecum destruens* was not found in any of the *R. rutilus* and *B. barbatula* individuals.

Presence of *Sphaerothecum destruens* in water bodies

For five eDNA samples out of 39 water bodies, a nested PCR fragment of the expected size could be perceived, positively matching the 18S rRNA gene of *S. destruens*, with high similarity (97–99%) and 0.0 E-values, after BLASTN analysis (Online Resource 4).

In all five sites where *S. destruens* was found, presence of *P. parva* was confirmed by data of the fish surveys (Fig. 1; Online Resource 2). There is no clear pattern in the presence or absence of *P. parva* and *S. destruens* in relation to physico-chemical parameters, as supported by the PERMANOVA (Online Resource 3). Sites harbouring *S. destruens* were significantly smaller ($t = -3.4359$, $df = 33.074$, $p < 0.01$) in surface area ($\bar{x} = 218.1$ m^2; ± SE 82.1 m^2; $n = 5$) than sites where *S. destruens* was not detected ($\bar{x} = 9964.4$ m^2; ± SE 2775.2 m^2; $n = 34$). The results suggest that *S. destruens* is more likely to be detected in smaller water bodies, possibly due to relatively higher DNA concentrations due to lower water volume. Of the 21 sites where *P. parva* is present, presence of *S. destruens* could not be confirmed in 16 sites. In addition, there are 18 sites without *P. parva* or *S. destruens*. To test whether the occurrence of *S. destruens* coincides with *P. parva*, we used a Chi square test with 5 sites with *P. parva* and *S. destruens* and 0 sites without *P. parva* and with *S. destruens* (observed values). Based on the number of sites with *P. parva* (21) and without *P. parva* (18), we assumed a random distribution of *S. destruens* over the sites and hence the expected values for this test were 2.7 sites with *P. parva* and S. *destruens* and 2.3 sites without *P. parva* and with *S. destruens*. The test showed that the distribution of *S. destruens* significantly deviates from the expected values ($p = 0.0384$, Chi square = 4.29, $df = 1$). This test suggests that the occurrence of *S. destruens* coincides with *P. parva*.

Discussion

Effect of *Pseudorasbora parva* on native fish species

Pseudorasbora parva is rapidly colonizing new water bodies since its first record in the Netherlands in 1992 (Spikmans et al. 2013). This species is likely to persist in newly colonised areas and reproduces successful in most water bodies it occupies, making further expansion of its distribution highly likely. The number of individuals of *P. parva* across sites comprised almost half of all fishes that could be caught (Online Resource 2). Dominance in densities of *P. parva* was earlier shown in Dutch floodplain lakes (Pollux and Korosi 2006), an English lake (Britton et al. 2007) and a Polish river (Witkowski 2009). Densities of *P. parva* in floodplain lakes with lentic conditions ranged from 0.01 to 9.85 individuals m^{-2} (Table 2), and are relatively high compared to densities found in lotic conditions in Italy (Carosi et al. 2016). Our study shows a negative relation between the abundance of *P. parva* and that of *L. delineatus* and *P. pungitius,* and the Simpsons diversity index, Shannon–Wiener index and evenness of the fish assemblages in the water bodies studied (Fig. 2). In laboratory conditions, *P. parva* and *S. destruens* have been associated with high mortality of *L. delineatus* (Gozlan et al. 2005; Andreou et al. 2012). Gozlan et al. (2005), Ercan et al. (2015) and Carosi et al. (2016) have recorded severe declines of several freshwater fish species associated with *P. parva* and *S. destruens* in the wild. In contrast, a population of *L. delineatus* in a French lake showed no decline after the introduction of *P. parva* (Carpentier et al. 2007), but information on the

presence of *S. destruens* was not available for this site. *Gasterosteus aculeatus* was the only species with abundances positively related to *P. parva.* Combined with the molecular results, this suggests *G. aculeatus* may be a healthy carrier (see section *Sphaerothecum destruens* prevalence in co-occurring fish species).

The non-random selection of our sampling sites was focussed on temporarily isolated, shallow and stagnant water bodies in floodplains along large rivers because this type of water was known to harbour high densities of *P. parva* (Pollux and Korosi 2006). Our study revealed that this type of water is only favoured by a limited number of limnophilic and eurytopic species, such as *L. delineatus,* and is less favoured by other fish species, such as *G. gobio, L. idus* and *A. brama*. Most co-occurring species were only encountered in a few water bodies and effects of *P. parva* or *S. destruens* on their abundance could therefore not be established. Eleven species were found to reproduce in the presence of *P. parva* (Table 2), including *L. delineatus*. However, each water body was sampled at only one moment in time. Therefore, local extinction of species after *P. parva* colonisation might have taken place unnoticed as data on the historic composition of the fish assemblages are lacking. Factors other than *S. destruens* might also affect diversity in invaded water bodies, such as trophic interactions between *P. parva* and co-occurring fish species (Britton et al. 2010; Didenko and Kruzhylina 2015), habitat competition and hydrological events, such as flooding and droughts.

No effect of the presence of *P. parva* or *S. destruens* on the body condition of four co-occurring species (*C. gibelio*, *G. aculeatus*, *P. pungitius* and *T. tinca*) was found. Carosi et al. (2016) demonstrated a lower body condition of three native fish species where these species co-occurred with *P. parva* and suggested that this was caused by trophic overlap between species. Trophic overlap was indeed shown between *P. parva* and the native *R. rutilus* (Britton et al. 2010). On the other hand, Rola et al. (2019) showed high trophic plasticity for *P. parva* and a shift to a lower trophic level in the presence of competitors, resulting in a lower condition of *P. parva* individuals. Therefore, prediction of the trophic impact of invasive *P. parva* is difficult.

Sphaerothecum destruens prevalence in co-occurring fish species

Sphaerothecum destruens was found present in both *P. parva* and *G. aculeatus* individuals. Prevalence was at least 25% in *G. aculeatus*, possibly higher (up to 50%). For *P. parva,* the prevalence rate is at least 25% but possibly higher (up to 75%), since only 6 out of 18 PCR fragments were sequenced and 90% of the tested fishes resulted in a nested PCR fragment of the correct size. Prevalence in *P. parva* in the same study area was previously assessed at 67 to 74% (Spikmans et al. 2013). The prevalence in *G. aculeatus* might be an underestimation, as we did not perform sequencing on all samples with PCR product of matching size.

Sphaerothecum destruens is a generalist and known to have a broad host species spectrum (Arkush et al. 2003; Gozlan et al. 2009; Andreou et al. 2012; Andreou and Gozlan 2016) and it is being found in an increasing number of fish species in wild populations: *Oncorhynchus tshawytscha, Oncorhynchus kisutch* (Arkush et al. 1998), *P. parva* (Gozlan et al. 2005), *L. delineatus* (Andreou et al. 2011), *Dicentrarchus labrax, Lepomis gibbosus, Oxynoemacheilus* sp., *Petroleuciscus smyrnaeus, Squalius fellowesii* (Ercan et al. 2015), *Leuciscus leuciscus, Rutilus rutilus, Squalius cephalus* and *Salmo trutta* (Sana et al. 2018). In experimental or hatchery conditions, several other fish species have proven to act as hosts: *Oncorhynchus mykiss* (Arkush et al. 1998), *Salmo salar* (Paley et al. 2012), *A. brama* and *C. carpio* (Andreou et al. 2012). Several species mentioned here co-occur with *P. parva* in the Netherlands.

In our study no *S. destruens* infection of *B. barbatula* or *R. rutilus* could be found, although these samples were taken in the presence of *P. parva* and *G. aculeatus* known to be carrying *S. destruens*. However, Andreou et al. (2012) found high mortality rates in *R. rutilus* due to *S. destruens*. *Rutilus rutilus* might either have been immune to initial infection, able to clear early stages of parasitism or developed a latent infection (carrier state) that is more difficult to detect with PCR (Andreou et al. 2012). This is possibly due to co-existence with *P. parva* that has inhabited this area for 20 years. Sana et al. (2018) found that *B. barbatula*, *Cottus gobio* and *G. aculeatus* all tested negative as a host for *S. destruens*, even though *S. destruens* presence in the sampled stream was proven by eDNA detection.

In our study *S. destruens* was demonstrated for the first time in a population of *G. aculeatus*. While *L. delineatus* and *P. pungitius* abundances were lower in the presence of *P. parva*, for *G. aculeatus* we demonstrated an increasing abundance (Fig. 2). *Gasterosteus aculeatus* commonly co-occurred with *P. parva* (71% of water bodies, n = 21) and was the numerically dominant species in 38% of these water bodies. It also frequently co-occurred with *S. destruens* (80% of water bodies, n = 5). Their body condition was unaffected by the presence of *P. parva* and/or *S. destruens*. Presence of YOY indicates *G. aculeatus* reproduces in sites where *S. destruens* was found. Scharsack et al. (2007) studied the habitat-specific immune response of *G. aculeatus* and found that it is able to rapidly adapt its immune system to local conditions to cope with non-trophically transmitted parasites. Species-dependent susceptibility to *S. destruens* was shown in an experiment with *A. brama*, *R. rutilus* and *C. carpio*, viz. 53%, 37% and 8% mortality, respectively (Al-Shorbadji et al. 2015; Andreou et al. 2012). Since the wide host species range of *S. destruens* as described above, *G. aculeatus* might function as an asymptomatic carrier of this parasite. Therefore, *G. aculeatus* may act as a reservoir for *S. destruens* and thereby promoting disease outbreaks. This has direct implications for control programmes aimed at *S. destruens*. Further histopathological screening is needed to confirm the role of *G. aculeatus* as a reservoir.

Sphaerothecum destruens eDNA detection

Using eDNA, DNA fragments showing 97–99% similarity with the 18S rRNA gene of *S. destruens* were found to be present in five out of 21 (24%) of the sites where *P. parva* was found (Online Resource 4). Phylogenetic analysis shows minor genetic differences between more spatially separated sites, site 39 in relation to site 6, 30, 33 and 38 (Fig. 1; Online Resource 4). For example, more samples are required to determine spatial genetic variation of *S. destruens* in more detail. Environmental DNA is a promising, non-invasive, sensitive and specific method to detect *S. destruens* (Sana et al. 2018) as the species has a free-living zoospore stage with a broad temperature tolerance (Andreou et al. 2009) and (nested) PCR-amplification is extremely sensitive in detecting this particular zoospore life stage (Mendonca and Arkush 2004; Bass et al. 2015). Sampling techniques can be of great influence on the outcomes of eDNA studies (Hinlo et al. 2017; Goldberg et al. 2016). In our study the water bodies were sampled in late autumn/early winter at low water temperature. Zoospore concentrations and zoosporulation duration of *S. destruens* are known to increase at temperatures between 4 and 15 °C (Andreou et al. 2009). Detection of *S. destruens* eDNA is limited in turbid conditions, due to absorption of the DNA to soil particles and detection is also more difficult with low *P. parva* densities (Sana et al. 2018). Furthermore, we found that sites where *S. destruens* was detected were significantly smaller in surface area than sites where *S. destruens* was not detected. This indicates that the chance of detection is higher in the case of sites with a small surface area, likely due to relatively smaller volumes and higher DNA concentrations. In our study *S. destruens* was strictly found in the presence of *P. parva*. However, in 16 sites where *P. parva* was present, no *S. destruens* was found. Since we amplified a relatively large DNA fragment for detecting *S. destruens*, due to DNA degradation this might have yielded negatives meaning that our results are conservative. In addition, Sana et al. (2018) used longer primers that were more specific to detect *S. destruens* and real-time PCR, which is therefore recommended for use in future eDNA studies. Optimizing the eDNA sampling and analysis protocol in accordance with Mendonca and Arkush (2004) and Sana et al. (2018) is important as it can be useful to better understand the scale of *S. destruens* distribution, providing information for risk analysis and setting up control programs. Furthermore, eDNA can be used to screen fish hatcheries to help prevent further spread of the parasite.

Risk management

Effects of both *P. parva* and *S. destruens* easily go undetected (Andreou and Gozlan 2016). Ongoing monitoring programmes for ecological status assessments of water systems according to the EU Water Framework Directive, conservation status of protected areas according to the EU Habitat Directive and the Dutch national long-term fish monitoring programme (De Graaf et al. 2016) are unable to detect declines of native fish associated with *P. parva* or *S. destruens.* These programmes do not assess fish populations at a yearly basis and are limited to specific target species

and habitats (lotic and large lentic water bodies). It is recommended to set up a long-term fish population monitoring programme in lentic water bodies, combined with an epidemiological survey using molecular techniques, to better understand the role of both *P. parva* and *S. destruens* in population changes of native fish species.

Since August 2016, *P. parva* is listed as an invasive alien species of European Union concern, making introduction, possession, trade and transport illegal in compliance with the EU Regulation 1143/2014. All EU member states are obliged to eradicate or at least control and contain its populations to prevent further spread and to mitigate harmful effects. Eradication of established populations of *P. parva* is not likely to be successful at large spatial scales in hydrologically connected water bodies. Therefore, cost-effective strategies seem to be limited to control population densities and prevent further spread of *P. parva*. Eradication of *P. parva* as a host, however, is not expected to prevent establishment of *S. destruens* in adjacent fish communities due to spore transmission through water (Al-Shorbaji et al. 2015). Restoring and conserving pristine ecosystems to prevent further loss of biodiversity can decrease transmission of pathogens (Keesing et al. 2010). In the current study *P. parva* was found to be the dominant species in many waters where it occurred, implying that lowering *P. parva* densities can limit the transmission of parasites such as *S. destruens* (Al-Shorbaji et al. 2015). Lemmens et al. (2015) show that the abundance of *P. parva* can be suppressed by *Esox lucius* stocking, enhancing the biotic resistance of fish communities. We also suggest focussing attention on minimizing the human assisted dispersal of *P. parva* via (ornamental) trade and angling associated stockings.

Conclusions

Since its introduction to Europe, *P. parva* has spread rapidly. *Pseudorasbora parva* poses risks to biodiversity due to dietary competition with native species, and hosting and spreading of the parasite *S. destruens.* This study confirms negative effects of *P. parva* on abundances of *L. delineatus, P. pungitius* and diversity of fish assemblages (i.e. Simpson index, Shannon–Wiener index and evenness) in floodplain water bodies along the rivers IJssel, Meuse, Nederrijn and Waal. However, a positive correlation was found between the abundance of *P. parva* and *G. aculeatus*. This relation, combined with the demonstrated infection of *G. aculeatus* with *S. destruens*, suggests *G. aculeatus* to be an asymptomatic carrier, further increasing the risk of *S. destruens* spreading to other water bodies. eDNA is a promising method for early detection of *S. destruens* and facilitates rapid response (e.g. eradication or control of its reservoir host). The ongoing colonisation of *P. parva* and its function as carrier of the parasite *S. destruens* will pose a significant risk to the native fish communities. The wide spread *Gasterosteus aculeatus* might substitute *P. parva* as a viable *S. destruens* host, implying that eradication or population control of *P. parva* does not necessarily lead to minimizing the risk of disease emergence caused by *S. destruens*.

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Authors' contributions Authors Frank Spikmans and Pim Lemmers contributed equally to this paper.

Compliance with ethical standards

Ethical approval The project was approved by the Royal Netherlands Academy of Arts and Sciences ethics committee and was performed under Animal Experimentation Law: WoD Licence No. TRC/VWA/2012/4275.

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