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Environmental DNA reveals the temporal and spatial extent of spawning migrations of European shad in a highly fragmented river basin

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Abstract

1. Artificial barriers on lowland rivers impede the spawning migrations of anadromous fishes, preventing access to historical spawning areas. In the cryptic European shads *Alosa alosa* and *Alosa fallax* ('shad' hereafter), this has resulted in population declines across their range. Conservation programmes aim to facilitate the passage of migrators over these barriers and so require baseline information on the spatial and temporal extent of current migrations.
2. Here, a shad-specific environmental DNA (eDNA) assay was used to quantify the spatial extent of shad spawning migrations in the River Severn basin, western England. This basin is characterized by the presence of multiple barriers in the lower catchment. In 2017, the eDNA assay was piloted in the River Teme, an important shad spawning tributary, and then applied in 2018 and 2019 across the lower Severn basin.
3. In all years, shad DNA was detected between mid-May and mid-June, with the maximum spatial extent of shad distribution being in early June when shad eDNA was detected upstream of weirs that were generally considered as impassable. In 2018, this included the detection of shad above the most upstream weir on the main River Severn that required individual fish to have passed six weirs.
4. Although barriers inhibit the spawning migrations of shad, this eDNA assay showed that some highly vagile individuals might be able to ascend these barriers and migrate considerable distances upstream. This suggests that efforts to increase the permeability of these barriers could result in relatively high numbers of migrating shad reaching upstream spawning areas. These results demonstrate that this eDNA assay could also be used across their range, to further quantify the spatial extent of their spawning, including in highly fragmented rivers and those where shad are believed to spawn only occasionally and are rarely observed.

KEYWORDS
fish, habitat management, impoundment, migration, restoration, river

1 | INTRODUCTION

The artificial modification of lowland rivers has resulted in profound impacts on biodiversity, with dams and weirs that regulate river flows interrupting longitudinal connectivity. This inhibits the upstream migrations of anadromous fish, affecting the sustainability of their populations (Dudgeon et al., 2006; Rolls et al., 2014). Where these impacts result in population declines and conservation concerns, restoration efforts require information on the temporal and spatial extent of their spawning migrations (Pess et al., 2014).

Mapping the extent of the spawning migrations of anadromous fishes traditionally relies on methods such as visual or telemetry observations of migration or evidence of their spawning, such as the visual identification of redds or sampling of eggs (Antognazza et al., 2019). These methods can, however, require considerable effort and might not be feasible under certain river conditions, such as during high flows (Radinger et al., 2019). In the last decade, the development of environmental DNA (eDNA) detection techniques has provided methods that can be rapidly deployed and provide high spatial resolution of spawning distributions, including in unfavourable conditions (e.g. Deiner et al., 2016; Klymus, Marshall & Stepien, 2017; Maruyama et al., 2018; Tillotson et al., 2018; Wilcox et al., 2018; Itakura et al., 2019).

In aquatic systems, organisms naturally shed DNA into the water (Pilliod et al., 2013), enabling eDNA-based tools to detect species via their DNA fragments (Ficetola et al., 2008). Although these methods can be used simultaneously at different sites with greater repetition (Darling & Mahon, 2011; Baldigo et al., 2017), their ability at detecting species at low abundances can be problematic. This is especially the case in rivers, particularly where a species is present some distance upstream of the sampling location (Jane et al., 2015; Thomsen & Willerslev, 2015; Wilcox et al., 2016), and where DNA settlement on the river bed and its subsequent resuspension can affect the reliability and interpretation of the results (Shogren et al., 2017). In addition, the quantity of DNA shed in the system and its consequent concentration depends not only on the abundance of the target species, but also on the metabolic state, behaviour, and activity of individuals, so a wide range of factors influence the detectability of the species (Goldberg et al., 2011; Barnes et al., 2014; Bracken et al., 2019). In addition, temporal and spatial variation in river flows can strongly influence dilution effects and so affect the subsequent ability to detect DNA within collected water samples (Thalinger et al., 2019).

European shads, *Alosa alosa* (Linnaeus, 1758) and *Alosa fallax* (Lacépède, 1803; 'shad'), are cryptic, anadromous fishes with an incompletely overlapping distribution in many Atlantic river basins (Alexandrino et al., 2006). They are listed in the Bern Convention (Appendix III) and in the Habitats Directive of the European Union (Annexes II and V) (Council of the European Communities, 1992; Aprahamian, Lester & Aprahamian, 1999; Aprahamian et al., 2003). The Habitats Directive ensures the conservation of a wide range of rare, threatened, or endemic animal and plant species, with European Union Member States required to designate 'special areas of conservation' (SACs) for species listed on Annex II. In addition, Annex V lists species whose exploitation may be subject to management measures.

The spawning behaviour of shad involves migrating into rivers in spring, with spawning generally occurring from late April to July in more northern European rivers (Acolas et al., 2004). A notable feature of these shads is their production of reproductively viable hybrids, especially when the species share spawning areas caused by blockages to migration (Jolly et al., 2012). The combination of hybridization and barriers to their spawning migration has been suggested as the reason for their contemporary population declines (Aprahamian et al., 2003). Consequently, shad conservation management requires information on the temporal and spatial extent of their spawning migrations, including how these relate to migration blockages. This information can be challenging to generate, as *A. alosa* can make long upstream migrations (>400 km; Kottelat & Freyhof, 2007) and both species reproduce at night (Aprahamian et al., 2003). Although egg surveys can indicate spawning locations, these are often difficult to perform in deeper waters and require considerable effort to provide information that is, at best, semi-quantitative (Antognazza et al., 2019).

In western Britain, the lower River Severn basin was subject to considerable river engineering in the 19th century, with a series of weirs constructed that enabled navigation further upstream for industrial purposes, but resulted in shad population declines owing to the loss of longitudinal connectivity (Aprahamian, 1988). Its estuary has been designated as a Ramsar site (Ramsar Convention, 1971), for the protection and conservation of wetlands, where migratory fish (such as shad, lamprey, and salmon) are recognized as internationally important species (Joint Nature Conservation Committee, 2008). Under the Habitats Directive, the estuary is also an SAC and thus has conservation protection in order to maintain and restore habitats and species that have been identified as vulnerable (Council of the European Communities, 1992). There are seven diadromous fish species of conservation importance in the Severn Estuary, including the two European shads, where *A. alosa* is considered rare. Both the main River Severn and its major tributary the River Teme, are also Sites of Special Interest under national legislation, with both rivers having some of the only remaining *A. fallax* spawning sites in the UK (Maitland & Lyle, 2005; Noble et al., 2007).

To assist the recovery of shad populations, efforts are now commencing to restore the connectivity of the rivers Severn and Teme by modifying weirs, including the construction of shad-friendly fish passes. A pilot study on the River Teme in 2017 developed and

applied an eDNA assay to quantify the spatial and temporal extent of shad spawning in the basin based only on river water samples (Antognazza et al., 2019). This assay (which cannot differentiate between *A. alosa*, *A. fallax* and their hybrids) was then applied across the basin during the shad migration period in 2018 and 2019. In 2018, water samples were collected weekly across the catchment throughout the spawning period; in 2019, the temporal sampling intensity was reduced but with the spatial extent increased. Despite the reconnection programme, no weirs had been modified in 2018 and only two had been modified in 2019, both on the River Teme (Figure 1). Consequently, the aim of this study was to (i) quantify the shad spawning distribution in the lower Severn basin in 2018 and 2019, with reference to the results from the River Teme survey; (ii) discuss these results in relation to the extent to which the weirs represented shad migration blockages; and (iii) assess the efficacy of using eDNA methods to quantify spawning distributions of anadromous fishes.

2 | MATERIALS AND METHODS

2.1 | Sampling sites

The River Teme is approximately 130 km in length and is impounded in its lower reaches by a weir (Powick Weir; Figures 1 and 2a), with a head of approximately 1.5 m, located 3 km from the confluence with the River Severn, and considered impassable for shad. However, a combination of eDNA-based detection (Antognazza et al., 2019) and shad egg sampling in 2017 had shown that some shad could pass this weir. Thus, in 2018, the River Teme was sampled at three locations (Figure 1; Table S1): (i) downstream of Powick Weir (Powick, site T1); (ii) upstream of Powick and Knightwick weirs (Knightwick, site T2); and (iii) 48 km upstream of Powick Weir at Tenbury (site T3). Knightwick Weir is not considered a barrier to shad movements because of its lower head (Figure 2b). In 2019, the upstream range for eDNA sampling was expanded by adding another sampling location upstream of Tenbury at Dinham (site T4). Two relevant long-term flow datasets were available for the river: the first was close to T3, where the long-term low flow rate (Q95) was 1.5 $m^3 s^{-1}$, median flow rate (Q50) was 8.3 $m^3 s^{-1}$, and high flow rate (Q5) was 52.0 $m^3 s^{-1}$, and the other was close to T2, where Q95 was 2.0 $m^3 s^{-1}$, Q50 was 10.13 and Q5 was 62.8 (Centre for Ecology and Hydrology, 2020). Water samples collected from the river in 2018 were at flows between 3.0 and 12.0 $m^3 s^{-1}$ at T3 and 4.6 to 21.3 $m^3 s^{-1}$ at T2. Flow data were unavailable for 2019, but observations suggested they were conducted at river levels and at flow rates within the range of those encountered in the 2018 sample collection.

The River Severn is approximately 354 km in length and has a series of six weirs in its lower reaches that disrupt its longitudinal connectivity. The primary focus of the study was on the second most downstream weir (Diglis Weir; Figures 1 and 2c), which is located on the non-tidal section of the river. This was because all the weirs further downstream on the Severn (e.g. 'Upper Lode'; Figures 1 and 2d) are known to be passable to shad (Bolland et al., 2019). Sampling sites were downstream (site S1) and upstream (site S2) of Diglis Weir (Figures 1; Table S1). All further sampling sites on the Severn were upstream of Diglis Weir, being upstream of Bevere Weir (site S3; Figure 2e) and upstream of Lincomb Weir (site S4; Figure 2f; Figure 1; Table S1). In 2019, an additional upstream site was added at Ironbridge (site S5; Figure 1; Table S1). Long-term flow data were available for the river in the vicinity of site S1, where Q95 was 15.3 $m^3 s^{-1}$, Q50 was 53.6 $m^3 s^{-1}$ and Q5 was 287.0 $m^3 s^{-1}$. Water

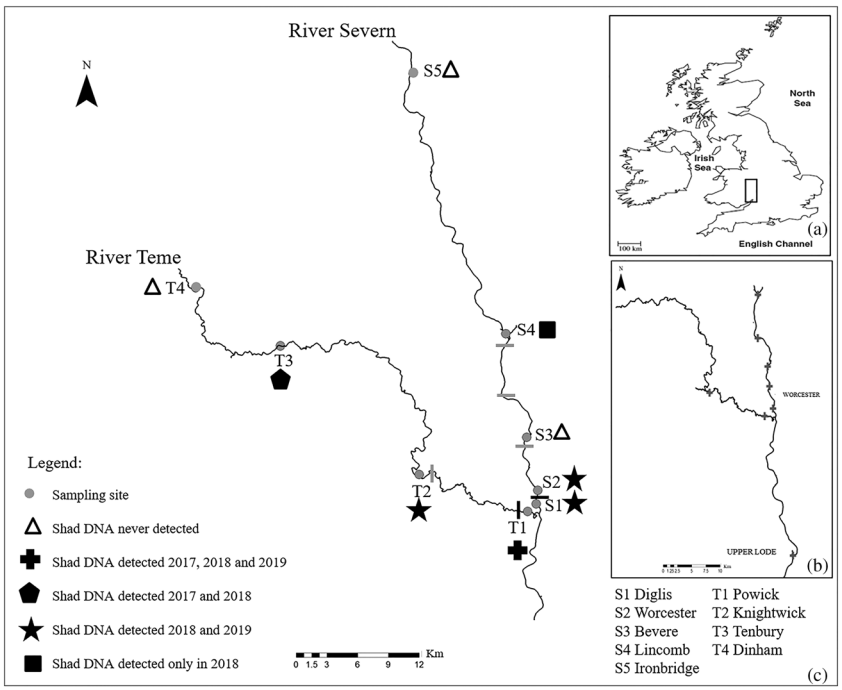

FIGURE 1 (a) Locations of sampling on the River Teme and River Severn, western England, where water samples have been collected during the shad spawning season. (b) Zoom-in of extended River Severn also showing Upper Lode Weir on the lower reach of the river (cf. Figure 2d). (c) Thick black lines refer to the two main impoundments on the Teme and Severn — being Powick and Diglis weirs, respectively (cf. Figure 2a,c). Powick Weir during shad spawning in 2019 was no longer present (cf. Figure 2g). Thick grey lines refer to the other weirs along the two rivers (cf. Figures 2b,d–f and S2). Shad DNA detection is detailed as shown in the key, including positive detection from Antognazza et al. (2019)

FIGURE 2 (a) Powick Weir prior to modifications during sampling in 2017 and 2018, and considered as largely impassable to shad. (b) Knightwick Weir prior to modifications, during sampling in 2017 and 2018, considered an obstacle to migrating shad. (c) Diglis Weir, considered to be impassable to shad. (d) Upper Lode Weir, in the lower reach of the River Severn, considered not to be a major obstacle to fish migration. (e) Bevere Weir, an obstacle to migrating shad on the River Severn. (f) Lincomb Weir, an obstacle to migrating shad on the River Severn. (g) Powick Weir following weir modifications in 2019. (h) Knightwick Weir following modifications in 2019

samples were collected in 2018 at flows of 29.3 to 83.3 $m^3 s^{-1}$ and, as with the River Teme, flow data were unavailable in 2019 but with samples collected under similar river conditions. Sampling included five bi-weekly samples in 2018 (May–July; Table 1) and two samples in 2019 (May and June; Table 2). In the 2019 spawning period, the weirs at Powick (Figure 2g) and Knightwick (Figure 2h) on the Teme had both been modified to facilitate shad passage, but with no modifications yet in place on the River Severn weirs.

2.2 | Sampling methods

Water samples were collected using 1-L sterile plastic bottles by following the two methods developed in the 2017 pilot study and as outlined in Antognazza et al. (2019). In 2018, samples at S3 and S4 were collected via an extendible pole, and at all other locations samples were collected from bridges. These inter-site differences in how the water samples were collected resulted from contrasting site characteristics that meant the methods required to collect water samples in a safe and sterile manner were inconsistent. The validity of using the two sampling methods were tested in the pilot study (Antognazza et al., 2019) and details are not reported here. Five replicate samples and two negatives (collected at the beginning and at the end of sample collection) were collected per site and sampling occasion (in both years). The negative controls consisted of 1-L sterile plastic bottles that were filled with sterile water in the laboratory. These were treated in the same way as sample collection bottles in the field, i.e., the lid was removed and put back on the bottle, and the

TABLE 1 Summary of real-time PCR results after applying cycling threshold cut-off (C_t = 36.166), data collection 2018

Site	Date	*n*	*N* positive samples	*n* qPCR positive	C_t	[eDNA] (ng µl^{-1})	Relative [eDNA] (ng µl^{-1})[a]	SE[b]	RSE (%)[c]	Positive replicates (%)
T3	03-May	5	0	0	>36.166	na	na	na	na	0
Tenbury	14-May	5	0	0	>36.166	na	na	na	na	0
	29-May	5	0	0	>36.166	na	na	na	na	0
	11-Jun	5	2	6	35.030	0.0005	0.0081	0.0033	38%	40%
	25-Jun	5	0	0	>36.166	na	na	na	na	0
	Overall	25	2	6						
T2	03-May	5	0	0	>36.166	na	na	na	na	0
Knightwick	14-May	5	1	2	36.155	0.0070	0.0029	0.0006	19%	13%
	29-May	5	1	2	32.735	0.0010	0.0164	0.0037	29%	13%
	11-Jun	5	0	0	>36.166	na	na	na	na	0
	25-Jun	5	0	0	>36.166	na	na	na	na	0
	Overall	25	2	4						
T1	03-May	5	0	0	>36.166	na	na	na	na	0
Powick	14-May	5	0	0	>36.166	na	na	na	na	0
	29-May	5	1	1	34.765	0.0070	0.0078	na	na	6%
	11-Jun	5	3	9	33.115	0.0456	0.0144	0.0042	26%	60%
	25-Jun	5	0	0	>36.166	na	na	na	na	0
	Overall	25	4	10						
S4	21-May	5	0	0	>36.166	na	na	na	na	0
Lincomb	4-Jun	5	2	2	35.289	0.0135	0.0053	0.0046	4%	13%
	Overall	10	2	2						
S3	21-May	5	0	0	>36.166	na	na	na	na	0
Bevere	4-Jun	5	0	0	>36.166	na	na	na	na	0
	Overall	10	0	0						
S2	03-May	5	0	0	>36.166	na	na	na	na	0
Worcester	21-May	5	0	0	>36.166	na	na	na	na	0
	4-Jun	5	1	3	33.930	0.0143	0.0164	0.0025	18%	20%
	25-Jun	5	0	0	>36.166	na	na	na	na	0
	Overall	20	1	3						
S1	03-May	5	0	0	>36.166	na	na	na	na	0
Diglis	21-May	5	2	5	34.558	0.0060	0.0071	0.0006	95%	33%
	4-Jun	5	0	0	>36.166	na	na	na	na	0
	25-Jun	5	0	0	>36.166	na	na	na	na	0
	Overall	20	2	5						

Note: Collection dates, number of eDNA samples from site (*n*), number of qPCR positive replicates per collection dates (*n* qPCR positive), mean cycling threshold (C_t), detection of shad (environmental DNA (eDNA)), relative concentration of shad eDNA (relative [eDNA]), standard error (SE), relative standard error (RSE as percentage) and percentage of positive qPCR replicates are detailed.

[a]Concentration of shad eDNA standardized across all qPCR runs (cf. Equation 1).

[b]SE = standard error of the mean.

[c]RSE = SE/mean.

closed bottle was then dipped in the water. All samples were immediately stored on ice and then refrigerated overnight. In 2019, all samples were collected from bridges using 1-L sterile plastic bottles by following the method outlined in Antognazza et al., (2019). All samples were immediately stored on ice and then frozen at −80°C (Table S2).

2.3 | Sample filtering and extraction of eDNA

For the 2018 samples, all water samples were filtered through a 0.45-µm cellulose nitrate filter membrane (Whatman™), using a Merck Millipore base glass vacuum filter of 47 mm diameter (Thermo Fisher Scientific), on the day following each sampling event. Filtration

TABLE 2 Summary of real-time PCR results after applying cycling threshold cut-off (C_t = 36.765), data collection 2019

Site	Date	*n*	*N* positive samples	*n* qPCR positive	C_t	[eDNA] (ng μl^{-1})	Relative [eDNA] (ng μl^{-1})[a]	SE[b]	RSE (%)[c]	Positive replicates (%)
T4	21-May	5	0	0	>40.0	na	na	na	Na	0
Dinham	05-June	5	0	0	>40.0	na	na	na	Na	0
	Overall	10	0	0						
T3	21-May	5	0	0	>40.0	na	na	na	na	0
Tenbury	05-June	5	0	0	>40.0	na	na	na	na	0
	Overall	10	0	0						
T2	21-May	5	1	1	36.628	0.0090	0.0134	na	na	6%
Knightwick	05-June	5	3	9	34.695	0.0456	0.0376	0.0040	10%	60%
	Overall	10	4	10						
T1	21-May	5	5	15	34.037	0.0339	0.0530	0.0048	9%	100%
Powick	05-June	5	4	11	33.486	0.0462	0.0717	0.0094	13%	73%
	Overall	10	9	26						
S5	23-May	5	0	0	>40.0	na	na	na	na	0
Ironbridge	10-June	5	0	0	>40.0	na	na	na	na	0
	Overall	10	0	0						
S4	23-May	5	0	0	>40.0	na	na	na	na	0
Lincomb	10-June	5	0	0	>40.0	na	na	na	na	0
	Overall	10	0	0						
S3	23-May	5	0	0	>40.0	na	na	na	na	0
Bevere	10-June	5	0	0	>40.0	na	na	na	na	0
	Overall	10	0	0						
S2	23-May	5	1	1	35.572	0.006	0.0229	na	na	6%
Worcester	10-June	5	0	0	na	na	na	na	na	0
	Overall	10	1	1						
S1	23-May	5	2	2	36.159	0.0115	0.0175	0.0040	23%	0
Diglis	10-June	5	2	5	34.023	0.0362	0.0288	0.0184	27%	0
	Overall	10	4	7						

Note: Collection date (Date, number of eDNA samples from site (*n*), number of qPCR positive replicates per collection dates (*n* qPCR positive), mean cycling threshold (C_t), detection of shad (environmental DNA (eDNA)), relative concentration of shad eDNA (relative [eDNA]), standard error SE), relative standard error (RSE in percentage) and percentage of positive qPCR replicates are detailed.

[a]Concentration of shad eDNA standardized across all qPCR runs (cf. Equation 2).

[b]SE = standard error of the mean.

[c]RSE = SE/mean.

was performed using a three filtration samples PVC manifold (Thermo Fisher Scientific). In 2018, a central vacuum pump system was used, whereas in 2019 filtration was performed using a Merck millipore chemical duty vacuum pressure pump (Thermo Fisher Scientific). Filtration blanks (1 L distilled water) were run before the first filtration and then after every six samples, plus one at the end, to enable testing for contamination at the filtration stage. The filtration was performed in a biological flow cabinet (Nuaire Labguard Class II biological safety cabinet) in a laboratory not dedicated to any DNA processing to minimize contamination risk. Before filtration, all equipment was sterilized under ultraviolet light in a flow cabinet for 20 min. Following each field and control sample filtration, the filter paper was removed using sterile tweezers and placed in an individual power bead tube for DNA extraction, and then stored in a refrigerator. Tweezers were sterilized after each use in 10% Microsol solution (Anachem, Leicester, UK) for at least 10 min and then washed with distilled water. Filtration equipment was sterilized in a 10% commercial bleach solution for 15 min, followed by flushing with tap water and then two washes with distilled water. The day after filtration, DNA was extracted using a DNeasy PowerWater Kit (Qiagen) according to the manufacturer's guidelines and eluted in 100 μl elution buffer. Extraction steps were performed in a biological flow cabinet and all equipment was sterilized under UV light for 20 min before and after extraction. Samples were quantified through Nanodrop and a sub-sample of each was transferred into 96-well plates and stored in the fridge for subsequent use (the following day for qPCR), with the remainder stored at −20°C.

For the 2019 samples, the filtering and extraction processes were as described above, but for logistical reasons the water samples were frozen at −20°C on the day of their collection, with no sample frozen for more than 30 days (Table S2). On the day before processing, the water samples were removed from the freezer, defrosted, and then filtered. Afterwards, filter papers were placed in individual Eppendorf tubes with sterile tweezers and stored at −80°C for up to 25 days before their extraction (Table S2). Then, all samples were randomly extracted (as described for 2018); eluted samples were stored at −80°C until amplification. Amplification reactions were performed between 45 and 62 days after extraction (Table S2).

2.4 | Target DNA amplification

Detection of shad DNA was conducted using the TaqMan® Gene Expression Master Mix UDG assay (Applied Biosystems, Foster City, CA) (Antognazza et al., 2019) that targets shad mitochondrial cytochrome C oxidase subunit I gene segment (70 bp, COI gene; forward primer was 5′-GCGGCTTTGGGAATTGACTAG-3′; reverse primer 5′-GCAAGGAGGAGGAGGAATGAG-3′; assay ID: APMFW3H). The assay specific to *Alosa* spp. was developed and tested in silico by Applied Biosystems (Table S3). In the laboratory, the *Alosa* species-specific COI gene assay was tested for cross-reactivity with pure fish DNA for 16 fish species present in the River Severn catchment (10 ng per fish species): roach *Rutilus rutilus*, minnow *Phoxinus phoxinus*, common bream *Abramis brama*, chub *Squalius cephalus*, perch *Perca fluviatilis*, dace *Leuciscus leuciscus*, bleak *Alburnus alburnus*, grayling *Thymallus thymallus*, brown trout, *Salmo trutta*, Atlantic salmon *Salmo salar*, gudgeon *Gobio gobio*, eel *Anguilla anguilla*, sea lamprey *Petromyzon marinus*, brook lamprey *Lampetra planeri*, carp *Cyprinus carpio*, and European barbel *Barbus barbus*.

The Taqman MGB probe was labelled with the fluorescent reporter dye FAM at the 5′-end and a non-fluorescent quencher MGBNFQ at the 3′-end. The unlabelled primers and Taqman probe were purchased from Applied Biosystems (assay ID: APMFW3H). The TaqMan® Gene Expression Master Mix UDG was used for this assay (Applied Biosystems). qRT-PCR was run in triplicate for each eDNA sample and negative control in 20-μl reactions using 10 μl TaqMan® Gene Expression Master Mix UDG, 1 μl assay mix (primers and probe) and 2 μl of DNA template (undiluted). All reactions were performed in the StepOne real time PCR machine (Applied Biosystems) and analysed by StepOne software v. 2.0 (Antognazza et al., 2019). Thermal cycler conditions were set to a holding stage at 50°C for 2 min to allow UDG enzymatic activity and initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. All negative controls were screened for contamination, and all positive detections from field samples had the corresponding equipment control (filtering and extraction) processed. Samples from 2018 and 2019 were handled separately for all the analyses.

A calibration curve was generated using genomic DNA extracted from shad scales to determine the sensitivity of the assay. A 10-fold serial dilution of shad genomic DNA was prepared in UV-irradiated sterile water to give a template concentration of 10 ng μl^{-1} to 1 fg μl^{-1}. The limit of detection was defined as the lowest concentration of genomic shad DNA detected at least 95% of the time by the qPCR assay. In both 2018 and 2019, the limit of detection of the assay was 1 pg μl^{-1}, with a mean of threshold cycle (C_t-value) of 37. The C_t-values with standard genomic DNA dilutions in the late cycle (>37), which corresponded to 0.1 pg μl^{-1}, were considered unreliable as the probability of detection was <95%. In 2018, when the logarithm of starting material (ng of total genomic shad DNA; *x*-axis) was plotted against the average C_t value (*y*-axis), the resulting line had a slope of −3.291, a *y*-intercept of 27.797, and R^2 of 0.96. The PCR efficiency was calculated as $\left[10^{\left(-\frac{1}{slope}\right)}\right]-1$, yielding an overall efficiency value of 101% (Baldigo et al., 2017). In 2019, when the logarithm of starting material (*x*-axis) was plotted against the average C_t value (*y*-axis), the resulting line had a slope of −4.526, a *y*-intercept of 28.150, and R^2 of 0.97, yielding an overall PCR efficiency value of 66% (Baldigo et al., 2017). For both years independently, PCR efficiency, slope and *y*-intercept were then standardised among all the qPCR amplifications performed. Consequently, the relative concentration of shad DNA in all environmental samples was calculated for each year, as:

$$\text{Water samples in 2018 : Concentration}\left(\text{ng}\,\mu l^{-1}\right) = 10^{\left[\frac{C_t-27.797}{-3.291}\right]}, \quad (1)$$

$$\text{Water samples in 2019 : Concentration}\left(\text{ng}\,\mu l^{-1}\right) = 10^{\left[\frac{C_t-28.150}{-4.526}\right]}. \quad (2)$$

Hereafter, the relative concentration of shad in environmental samples will be referred to as shad DNA or eDNA (Baldigo et al., 2017). Since shad DNA detectability in a fluvial system is affected by multiple factors, the failure to amplify the target in all triplicates suggested that eDNA concentrations were either negligible or below the lower limits of assay quantification; it does not necessarily imply with 100% confidence that no shad were present.

To determine the cut-off of the mean cycling threshold (C_t) values to select the positive qPCR replicates, C_t values of standard genomic DNA dilution were standardized among all the reactions performed. The precision of the within-sample unit replication and the spatial eDNA distribution was evaluated for each site from the relative standard deviation error (RSE); >20% was generally considered as high heterogeneity (either spatially or temporally) or inadequate sample replication (McCune & Grace, 2002). In addition, the percentage of positive qPCR replicates was calculated at each site for each sampling date (Table 1).

The results of the spatial evaluation of eDNA collected in 2018 were plotted in ArcMap 10.3.1 (ESRI Inc.). Given that eDNA in a fluvial system moves from upstream to downstream, then if an upstream site provided a positive shad detection but the downstream one was negative, the assumption was that shad were present in that stretch of the river.

3 | RESULTS

3.1 | Shad eDNA samples from 2018

For the samples amplified in 2018, the C_t value threshold was 36.166; all eDNA samples that resulted positive, but with an average C_t value above this threshold, were considered as unreliable and discarded from further consideration (Table S3). Average C_t values for positive water samples ranged from 32.735 to 36.166, equivalent to relative concentrations of shad DNA from 0.0029 to 0.0164 ng μl^{-1} (Table 1). Positive eDNA detection ranged between one and three samples at a single sampling event per location, and the percentage of positive qPCR replicates ranged between 6% and 60% (Table 1). The results of all extraction controls were negative. The field negative controls displayed no amplification, except two collected at T1 and one at S2 (Figure 1), with samples collected after these negative controls removed from analyses. Only one of the 44 filtration negative controls displayed positive amplification. After checking the order of filtered samples it was assumed that contamination might have occurred from previous filtered samples, as the following control displayed no amplification. Samples filtered between those controls did not show positive amplification. The R^2 values for the qPCR standard curve ranged from 0.86 to 1.00, and the efficiency ranged from 63.53 to 100.

In the River Teme tributary, shad DNA was detected at least once at each sampling location (Table 1). In the River Severn, DNA was detected at least once in all locations except S3 (Table 1). At the beginning of May, the assay only detected shad DNA up to site T2 in the River Teme (Figure S1a), while by the end of May it was detected in the River Severn up to site S1 (Figure S1b). In early June, shad DNA was then detected up to site T3 on the Teme and site S4 on the Severn (Figure S1c). There was no detection of shad DNA in samples collected at the end of June (Figure S1d).

3.2 | Shad eDNA samples from 2019

In 2019, the C_t value threshold was determined as 36.765; as for 2018, samples with higher C_t value were discarded from further consideration (Table S4). Average C_t values for positive water samples per sampling ranged from 33.486 to 36.628, equivalent to relative concentrations of shad DNA from 0.0134 to 0.0717 ng μl^{-1} (Table 2). Positive eDNA detection ranged from one to five samples at a single sampling event, and the percentage of positive qPCR replicates ranged from 6% to 100% (Table 2). All negative controls (field, filtering and extraction) showed no amplifications. The R^2 values for the qPCR standard curve ranged from 0.82 to 0.98, and the efficiency ranged from 54.08 to 77.10.

In the River Severn, DNA was positively detected on each sampling date at site S1, and at site S2 in May, but not at sites S3 to S5 (Table 2; Figure 1). In 2019, the weirs on the lower River Teme had both been modified to facilitate shad passage, and shad DNA was detected on each sampling date at site T1 and site T2, with the proportion of positive samples at site T2 increasing from 13% in 2018 to 60% in 2019 (Tables 1 and 2). In contrast to 2017 and 2018, shad DNA was not detected at site T3 in 2019.

Overall, on the River Teme, shad DNA was detected in all 3 years of sample collection above Powick Weir, with it being detected 48 km upstream in both 2017 and 2018 when the weir had yet to be modified (Antognazza et al., 2019). On the River Severn, shad DNA was always detected in both years of sample collection at site S1, downstream of Diglis Weir, the first weir on the main River Severn that is considered to be generally impassable to migrating shad. However, shad DNA was also detected in both years upstream of Diglis Weir, with it being detected at the most upstream site sampled in 2018 that was located above the most upstream navigation weir on the lower river (site S4).

4 | DISCUSSION

The samples collected in 2018 and 2019 confirmed the ability of the eDNA assay to detect the presence of migrating shad in the rivers, as reported for the shad spawning period in 2017 (Antognazza et al., 2019). The assay has now demonstrated that some shad may have been able to pass the weirs on both the Severn and Teme over a 3-year period, despite the general assumption that these were impassable. In 2017, the eDNA assay was successfully piloted in the River Teme, an important shad spawning tributary, and showed that shad were able to pass the weirs on the lower river, with shad eggs and their DNA detected in upstream areas (Antognazza et al., 2019). When the assay was applied more generally to the lower Severn basin in 2018 and 2019, shad DNA was only detected between mid-May and mid-June, as found in 2017. The maximum spatial extent of shad distribution was in early June, when they were detected upstream of weirs that were generally considered as impassable. In 2018, this included the detection of shad eDNA above the most upstream weir on the main River Severn that required individual fish to have passed six weirs. Nevertheless, with the eDNA assay applied at relatively broad spatial scales, it was unable to identify precisely where shad spawned, only whether they had migrated as far upstream as the samples were collected. The assay only provided traces of recent shad presence, not an estimate of the number of shad actually present.

In 2018, the eDNA assay showed that the highest shad spawning distributions were in early June, coinciding with peaks in shad spawning observed at night by citizen scientists (T. Thorpe, personal communication), as well as in the collection of *Alosa* eggs downstream of Powick Weir (at site T1) (C. Antognazza, unpublished data). These results enabled the sampling effort to be reduced in 2019, with samples only collected in May once fish had been observed as present in the lower river, and in early June to coincide with the previously detected peak shad spawning period. Following the conclusion of the 2018 shad spawning period, Powick Weir (above site T1) and Knightwick Weir (below site T2) on the River Teme were both modified to assist the passage of migrating shad. The 2019 eDNA

samples revealed positive shad detection at site T1 and site T2, with an increased proportion of shad DNA samples being positive. This suggests that a higher number of shad were present at site T2 in 2019 compared with 2018, as might be expected owing to the weir modification.

In general, the choice of sampling methods, extraction, and preservation of eDNA depends on several factors: field accessibility, the target species, and the overall aim of the study, as well as the costs and the available laboratory facilities. In this study, during sampling in 2019, laboratory processing had to be modified for practical reasons, resulting in water samples being preserved in the freezer for several days (Table S2). Even though filtering within 24 hours is still the recommended practice, freezer storage for up to 30 days has no impact on detecting DNA (Hinlo et al., 2017) with detectability being recorded up to 2 months following freezing (Williams, Huyvaert & Piaggio, 2016). However, the freezing of the water samples in 2019 and the lower PCR efficiency could have resulted in low concentrations of shad eDNA not being detected. Lower concentrations might be expected in the upper reaches of the rivers, owing to fewer fish migrating longer distances, and could explain the lack of detection of shad eDNA at Site T3 in 2019 compared with 2018.

There is a rapidly growing number of studies showing a relationship between eDNA concentration per litre and downstream distance, but it is difficult to measure all the necessary parameters in field studies (Laramie, Pilliod & Goldberg, 2015; Tillotson et al., 2018). This is at least partly the result of DNA settlement on the river bed and its subsequent degradation that reduces eDNA concentrations (Goldberg, Strickler & Pilliod, 2015; Wilcox et al., 2016; Shogren et al., 2017). Individual fish behaviour is another factor to be taken into account in relation to the quantity of DNA shed by fish, especially during spawning migrations (Tillotson et al., 2018; Thalinger et al., 2019). Temporal resolution of eDNA detection is important in determining the spawning migrations of anadromous fish, as upstream movements can attenuate DNA shedding, possibly leading to non-detection (Levi et al., 2019). Therefore, an eDNA monitoring system for anadromous fish could be highly effective if samples were collected daily (Levi et al., 2019), although this would generate substantial field and laboratory costs.

The DNA degradation with downstream distance potentially provides an explanation for the positive detection of shad DNA from the most upstream site (e.g. site T3 on the River Teme in 2018) and no detection at the respective downstream sites on the same day (Table 1). Moreover, it was assumed that where shad were able to pass the weirs considered to be largely impassable, the event was limited to a small number of individuals, perhaps taking advantage of elevated water levels facilitating their passage. This assumption would then help to explain the limited spatial distribution of where the eDNA was detected on each sampling occasion.

On the River Severn, Diglis Weir is considered as being largely impassable to shad (Figure 2c). Tracking studies on other species, such as European barbel *Barbus barbus*, have observed no fish movements above this weir, even after individuals accessed the weir pool, and after some tagged individuals were able to pass Powick Weir (Figure 2a), albeit during very high river levels (Gutmann Roberts, Hinder & Britton, 2019). Correspondingly, the positive detections of shad DNA above Diglis Weir might be caused by factors other than shad passing the weir. For example, these detections might reflect the movements and defaecation of piscivorous animals in the river that have recently consumed shad, as some studies suggest that fish-eating birds, especially cormorants (*Phalacrocorax* spp.), can move fish eDNA to areas upstream of barriers (Guilfoyle & Schultz, 2017; Guilfoyle et al., 2017). The movement of fish carcasses, slime, and bird faeces on boats and barges has also shown to be a possible explanation for eDNA detections of fish species in areas where those species have yet to be captured, with DNA persisting for up to 1 month (Merkes et al., 2014). In the River Severn, however, cormorant numbers (*Phalacrocorax carbo carbo* and *Phalacrocorax carbo sinensis*) tend to be greatly reduced in spring, in common with inland waters in England generally, as the birds migrate to coastal areas in March for their breeding season (Britton et al., 2002). Whereas the lower River Severn is navigable, shad DNA was not detected in all sites upstream of Diglis Weir (e.g. it was not recorded in site S3 in 2018, but was recorded further upstream), as might be expected had boat traffic been responsible for its movement. Moreover, even if the River Severn weirs were impassable to shad, these weirs all have locks that maintain connectivity for navigation, potentially providing an alternative route for upstream passage. Powick Weir (Figure 2a) was initially considered impassable to shad until their eggs were detected upstream, with the only reason that egg surveys were not conducted in the River Severn being its deep, impounded nature that inhibits the efficacy of the survey methods used. Thus, the presence of shad DNA in the areas upstream of the weirs was still considered to be largely the result of a potentially small number of live fish bypassing these structures. Indeed, a small number of shad tagged with acoustic transmitters were detected upstream of Diglis Weir during a large flood (flows in the region of Q5) in late June 2019 (P. Davies, unpublished data).

The analysis of the eDNA detection patterns in this study revealed a high spatial heterogeneity of eDNA, particularly in water samples collected in 2018, as suggested from the relative standard error (RSE = SE/mean) of within-site estimates of eDNA concentration (generally >20%). The probability of obtaining at least one positive sample is dependent on the number of repeated field samples collected and on the eDNA concentration present. A meta-analysis of eDNA studies by Willoughby et al. (2016) indicated that field replicates and qPCR replicates influence the probability of eDNA detection. In scenarios where DNA is considered to be rare in the environment (e.g. detection probability fixed at 0.25), by increasing field replicates (n = 10) and having PCR efficiency of 100%, eDNA detection probability still does not exceed 90% (Willoughby et al., 2016). In the present study, the PCR efficiency ranged between 63.5% and 100% in 2018 and between 54.1% and 77.1% in 2019, with five replicate water samples collected per site. With shad DNA likely to be relatively rare in the water, and given this level of

sampling, it can be assumed that the calculated probability of detection would have exceeded 75% in 2018 and 65% in 2019.

Considerable uncertainty remains around the ecology of eDNA in the environment, especially for lotic systems, as many factors (such as transport, dilution, shedding, degradation) and their interaction determines the fate of eDNA in the environment (Shogren et al., 2017; Seymour et al., 2018; Tillotson et al., 2018; Levi et al., 2019). Although the detection rates of eDNA can be relatively high in river water samples (Pilliod et al., 2013), information on the spatial resolution of these detections is often uncertain (Goldberg et al., 2013; Tillotson et al., 2018). Furthermore, the dynamics of its production, persistence, and drift differ according to characteristics of the river and target species (Klymus et al., 2015; Chambert et al., 2018). To increase the knowledge around the ecology of eDNA in the environment, future studies should consider recording multiple abiotic field parameters at each site, such as water temperature, turbidity, river flow, depth, and water chemistry (e.g. pH). Improving knowledge on the behaviour of DNA in fluvial systems is important so that more suitable sampling and laboratory steps can be identified that might be context-dependent according to site-specific characteristics.

This study not only confirmed the ability of an eDNA tool to monitor shad spawning migrations, but also allowed further refinement of field sample collection. For example, restricting sample collection during the period of peak spawning activity (mid-May to beginning of June) enables greater spatial resolution in sample collection, as shown in the 2019 sampling period. For a more accurate quantification of the distribution of anadromous fishes in a river system, daily sampling has been suggested (Levi et al., 2019). Future eDNA sampling in the river could benefit from a further reduction in the duration of the sampling period while increasing the spatial extent of sampling, including sites further upstream in both rivers to identify the full spatial distribution during the peak spawning period. This will be increasingly important as the modifications to weirs (weir removal or fish pass construction) are implemented, which should increase the number of individuals that are able to migrate considerable distances upstream. Indeed, incorporating prior knowledge on the ecology of anadromous species into eDNA survey designs is important, as it allows more informed selection of sampling sites and times, and helps ensure that all appropriate sampling locations are considered (Bracken et al., 2019; Itakura et al., 2019).

In general, only direct observation or capture-based methods are currently considered as official monitoring procedures for migratory fishes of conservation importance, such as shad (Joint Nature Conservation Committee, 2015). Nevertheless, eDNA-based methods are now providing robust approaches that can be integrated into standard monitoring procedures. Although other detection methods, such as telemetry, spawning observations, and egg sampling, provide data at specific sampling points, eDNA methods can contribute a wider understanding of the spawning distributions of migratory fish across time and space. For shad, we argue strongly that to develop a comprehensive understanding of the temporal and spatial patterns in spawning distributions, this eDNA protocol should be considered as a primary monitoring tool. In the River Severn, preliminary data from acoustic telemetry suggested that the upstream extent of the migration of a relatively small number of migrating shad in 2017 to 2019 was less than that demonstrated by eDNA. This suggests that the latter can provide better spatial resolution for when mapping distribution at a broad scale, even if it cannot provide information at finer spatial scales. These results also highlight the potential of eDNA for application to other rivers across the range of the species, including those where shad are relatively rare and others that are also highly fragmented by weirs and dams. With shad considered as imperilled across their entire range (Aprahamian et al., 2003), this eDNA protocol should be an integral part of long-term annual monitoring programmes. This would provide long-term datasets and enable a better understanding of the annual variability of shad spawning distribution by assessing how varying environmental conditions affect the upstream extent of spawning.

This study has demonstrated the value of eDNA protocols, and shown their potential for understanding the spawning migrations of other anadromous fishes, especially non-salmonid species that are often poorly studied (e.g. Bracken et al., 2019). A principal advantage of applying eDNA based methods to adult anadromous fishes in fresh water is that it minimizes disturbance to their spawning migrations, eliminating the need to capture and handle individuals at a sensitive stage of their life cycle (Lucas & Baras, 2001). Nevertheless, for eDNA-based methods to be fully integrated within existing legal monitoring frameworks, there is still the need to optimize the efficacy of these techniques (Tillotson et al., 2018; Belle, Stoeckle & Geist, 2019). This will enable eDNA to be used as a non-invasive alternative monitoring tool, thereby providing even greater support for conservation programmes for threatened species in future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data used in this study will be available at the repository BORDaR and are available from the corresponding author on request.

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SUPPORTING INFORMATION

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

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