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Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples

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ABSTRACT

The European weather loach (*Misgurnus fossilis*) represents one of many European freshwater fishes in decline. Efficient monitoring is essential if conservation efforts are to be successful, but due to the species' cryptic biology, traditional monitoring methods currently in use are inefficient, time consuming and likely prone to non-detection error. Here, we investigate the usefulness of environmental DNA (eDNA) monitoring as an alternative or supplementary method for surveying the Danish weather loach population, which is presumed to consist primarily of a single group of no more than 50 individuals. In 2008, the majority of historical Danish localities were surveyed, using traditional fishing techniques. We then applied eDNA methods to a number of these, as well as other potential localities. We successfully detected the weather loach at multiple sites in the single known remaining locality; a result that was later confirmed when local managers caught eight live specimens. Furthermore, the eDNA method indicated presence of the weather loach in another historical locality, where the species has not been observed since 1995. At the remaining localities, weather loach eDNA was not detected, providing further evidence for its absence. Importantly, the eDNA survey required less effort in person-hours and lower costs than the traditional fishing survey. This study confirms that eDNA monitoring is a valid supplement to traditional monitoring methods currently applied to monitor rare freshwater fishes. We propose that by providing reliable distribution data at lower cost and limited effort, the eDNA method can allow for increased management efficiency of endangered freshwater species such as the European weather loach.

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1. Introduction

More than one third of European freshwater fishes are endangered or threatened (Freyhof and Brooks, 2011). A good example is the European weather loach *Misgurnus fossilis*, a species of international conservation concern (Council of the European Union, 1992) that has declined rapidly across its range (Hartvich et al., 2010), and is nearly extinct in Denmark (Møller et al., 2012). Drainage channels are an important habitat of the weather loach in modern-day Europe, where oxbows, backwaters, swamps and periodically flooded pools and meadows – the natural habitats of the weather loach (Lelek, 1987) – are rare. Weeding and dredging of these channels, however, pose a serious threat to the species (Meyer and Hinrichs, 2000). Owing to the cryptic biology of the weather loach, which buries itself in the sediment, monitoring currently relies on cumbersome fishing methods, including electro-

fishing and fishing with traps (Møller et al., 2012; Meyer and Hinrichs, 2000). These methods are associated with a number of methodological difficulties. The former is dependent on heavy equipment and training, while the latter often requires habitats to be accessible by boat. Furthermore, a certain minimum water depth is required when using traps, a problem which can also apply to electrofishing (Copp, 1989). These difficulties may increase the risk of non-detection error, which in the case of rare species can pose a substantial problem (Gardner et al., 1999; Gu and Swihart, 2004). Another important drawback of both methods is their invasiveness. Electrofishing has in many cases been found to have harmful effects on fish, including spinal injuries, bleeding from the gills and vent, and even death (review by Snyder (2003)). In the case of fish traps, our experience suggests that weather loaches caught in traps risk being killed by other fish, such as eel, that are caught as by-catch.

More benign, accurate and faster monitoring methods would greatly benefit conservation efforts aimed at monitoring the weather loach. Monitoring fish species based on environmental DNA (eDNA) obtained directly from water samples is a non-invasive approach, which has been successfully applied to several species in

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various aquatic systems (e.g., Jerde et al., 2011, 2013; Foote et al., 2012; Thomsen et al., 2012a,b; Takahara et al., 2012, 2013; Wilcox et al., 2013, see also Thomsen and Willerslev, 2015), including successful detection of the weather loach in natural freshwater systems (Thomsen et al., 2012a). The decay of eDNA beyond the threshold of detectability in controlled experiments has been shown to occur on a scale of days or weeks (Dejean et al., 2011; Thomsen et al., 2012a,b; Barnes et al., 2014; Pilliod et al., 2014), indicating that the method provides an almost contemporary picture of species presence.

We believe the eDNA method is highly appealing for monitoring the European weather loach due to its cryptic biology, lack of efficient monitoring methods and high conservation priority (listed on the EU Habitat Directive). In this study we test the potential of using eDNA from water samples to monitor the European weather loach in a geographical context where it is very rare; based on monitoring surveys and civilian reports, more than 90% of the Danish weather loach population is presumed to reside at a single locality, and this population consists of at most 50 individuals (Carl et al., 2010).

2. Methods

2.1. Field sites and sample collection

In 2008, ten registered historical Danish localities for the weather loach were surveyed by PRM, HC and others (see Acknowledgments), using traditional methods, including electrofishing, fish traps and landing nets (Fig. 1, Table 1). These localities constitute the majority of natural historical localities in Denmark. Fishing effort was highest in the single known remaining Danish locality of Sølsted Mose, where all accessible water bodies were searched with at least one of the abovementioned methods. On the 2nd and 3rd of May and the 29th of August, 2012, water samples were collected at five of these localities, as well as at two additional historical localities and three unregistered localities (a total of ten localities) (Fig. 1). Within each locality a varying number of sites were sampled (Table 1). At most localities, one or a few sites which were deemed suitable microhabitats for the weather loach

were sampled. Three localities were sampled more intensively, and here sites were chosen in an attempt to obtain a broad spatial coverage of the areas. Most water samples were taken in and around Sølsted Mose, as this is the most recent catch locality. The unregistered localities were also sampled relatively densely, due to their geographic proximity to Sølsted Mose. Sølsted Mose is a degraded raised bog featuring a mosaic of scrub forest, peat hags, ponds, marsh areas and meadows. The wetland is surrounded by farmland and drains into the river Sejersbæk via a number of drainage channels.

Following Ficetola et al. (2008) with slight modifications, water samples of 3×15 mL were collected at each site. Samples were taken at the surface using 50 mL plastic tubes, and immediately after sampling 33.5 mL ethanol 96% and 1.5 mL sodium acetate 3 M was added to each tube. Sampling was performed in triplicates as this has been found to increase the probability of species detection with eDNA, when compared to a single 15 mL water sample (Thomsen et al., 2012a, Supporting Information). In flowing water, samples were taken consecutively in an upstream direction, to avoid re-sampling of water. Disposable gloves were worn at all times during sampling, and were changed between sampling events, and before handling equipment. On both sampling trips, Sølsted Mose was the last locality to be sampled. A total of 54 triplicate samples were taken (Table 1).

2.2. DNA extraction, qPCR and sequencing

Following field work, water samples were stored at -20°C . Prior to extraction, all samples were centrifuged for 30 min at 4700g and RT, supernatants were discarded, and each set of three samples was pooled into a single sample, representing 45 mL of water. Extraction was done using the Qiagen DNeasy Blood & Tissue kit (spin column protocol). Pooled, extracted samples were then analyzed using quantitative PCR with the primers and probe developed by Thomsen et al. (2012a). qPCR reactions for each extracted sample were performed four to eight times on a Stratagene Mx3000P in a 25 μL total volume using 10 μL of TaqMan[®] Environmental Master Mix 2.0 (Life Technologies, Carlsbad, CA, USA), 9 μL of ddH₂O, 3 μL of template DNA, 1 μL of each primer (10 μM) and 1 μL of probe (2.5 μM). Additionally, to check for

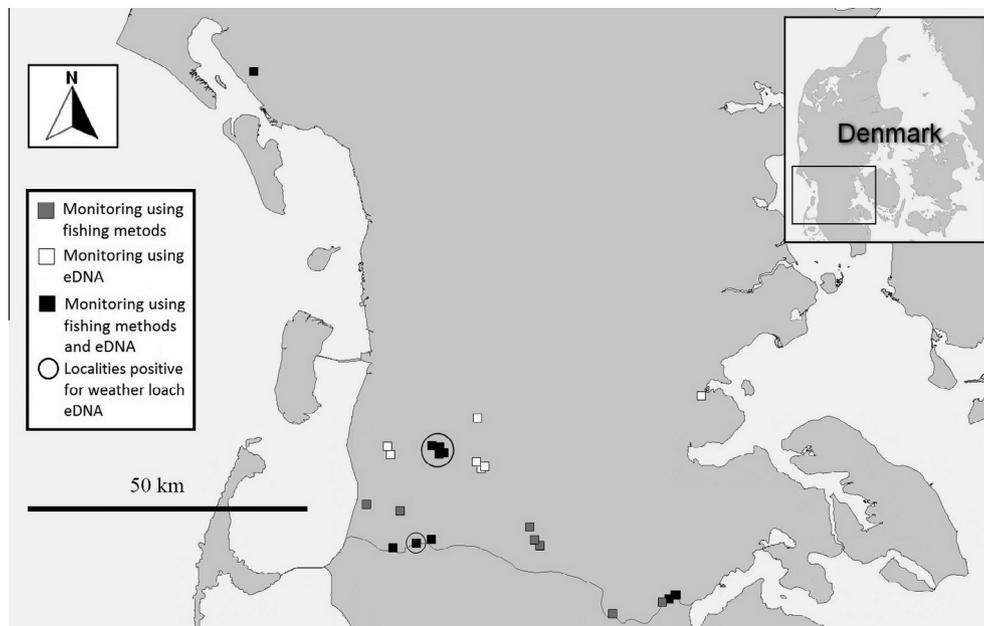


Fig. 1. Overview of the study sites, which are all located in Jutland, Denmark. The monitoring methods used at each locality are indicated by the legend. Localities where the weather loach was detected, i.e. Sølsted Mose and Magisterkogen, are circled.

Table 1

Overview of study localities and monitoring effort for the fishing and eDNA surveys of the present study. For historical localities, the year(s) of recording are indicated as well as the number of specimens caught. An “n” indicates that the number of specimens is not known. Effort for both the fishing and the eDNA survey is measured in person-hours, and includes both field work and lab work in the latter case. The number of sites sampled for eDNA within each locality is indicated, as well as the density of sampling. Where no sampling density is given, the site was a stream or river.

Locality	Historical catch records		This study 2008–2012			
	Year	No. of specimens	Fishing effort (hrs)	eDNA effort (hrs)	eDNA sampling sites	Sampling density (sites/ha)
Rørkær	1921–1922	8	4			
Frøsløv Mose	1936–1937	6	4			
Burkal	<1937	n	4			
Bønderby Sø	1937	n	8			
Løgumgård	<1938	n		3	1	
Bov Enge	<1939	n	4			
Kruså Mølledam	1942	n	6	5	3 ^a	0.26
Rundemølle	1958	1		3	1	
Rudbøl Sø	(1920s–) 1963	n	10	3	2	0.04
Magisterkogen	(1920s–) 1979–1995	11	8	5	1	0.03
Guldager Mølledam	2004	1	4	3	1	0.45
Sølsted Mose and Sejersbæk	(1920s–) 1980–2008	39	300	58	28	5.86
Lægan Harbour (Vidå River)	n/a	n/a		3	1	
Kogsbøl Mose	n/a	n/a		12	4	5.41
Kongens Mose	n/a	n/a		24	12	0.72
Total			352	119	54	

^a One of the 15 mL samples was very turbid and was discarded to avoid inhibition of the pooled sample.

inhibition we used an internal positive control (TaqMan® Exogenous Internal Positive Control), adding 2.5 µL of Exo IPC Mix and 0.5 µL of Exo IPC template DNA to the mixture in at least 2 replicates of each sample (6 µL of ddH₂O was used in these replicates). The PCR incubation program was set to 5 min at 50 °C, 10 min at 95 °C, and then 50 cycles of 95 °C for 30 s and 55 °C for 1 min. Samples that showed signs of inhibition (no initial amplification of the dye Vic) were diluted 1:10 in ddH₂O and re-analyzed through another round of qPCR.

A sample was deemed positive when a sigmoidal amplification curve was detected in at least one qPCR replicate. When more than one qPCR replicate amplified, the average cycle threshold (C_t) value of all replicates showing amplification is reported in the results. The C_t value is a relative measure of the initial concentration of target DNA in a PCR reaction, with lower C_t values indicating higher starting concentrations. Positive qPCR products from field sites were additionally verified on 2% agarose gels stained with Gel-Red™ (Biotium Inc., Hayward, CA, USA). qPCR products were then purified using a Qiagen MinElute PCR purification kit (Qiagen) and cloned using the Topo TA cloning kit from Invitrogen (Life Technologies, Carlsbad, CA, USA). Subsequently, 8 colonies from each plate (corresponding to a field site) were picked, and the inserted PCR fragments were commercially sequenced (Macrogen Europe, Amsterdam, Netherlands). In order to increase cloning success, purified qPCR products were amplified by PCR, using the same primers as in qPCRs, and cloning was then redone, following the procedure described above. The PCR incubation program was set to 5 min at 95 °C, 40 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, a final elongation step of 2 min at 72 °C, and then 10 °C for ∞. A total volume of 25 µL was used, consisting of 18.4 µL ddH₂O, 1 µL of template, 1 µL of each primer (10 µM), 1 µL of dNTPs (2.5 mM) and 0.1 µL AmpliTaq Gold DNA polymerase (Applied Biosystems).

Throughout the study we used separate laboratories for pre- and post-PCR procedures, and employed rigorous controls to monitor contamination including DNA extraction blanks and PCR blanks. The qPCR system for the European weather loach was previously tested for negative amplification on *Cobitis taenia* DNA, the only other cobitid species native to Denmark, as well as on *Anguilla Anguilla*, *Carassius carassius*, *Rutilus rutilus* and *Cyprinus carpio* tissue extracted DNA (Thomsen et al., 2012a). In the present study, the assay was further tested for negative amplification on the stone

loach, *Barbatula barbatula*, by running a qPCR reaction as specified above, but with 1 µL of tissue extracted DNA as the template. These fishes represent a selection of close relatives of the weather loach, which could potentially occur on the investigated localities.

2.3. Monitoring effort

For each monitoring method, the total invested effort, measured in person-hours, was estimated for every locality (Table 1). For the eDNA method this includes sample collection as well as lab work.

2.4. Cost comparison

The cost of materials and reagents for eDNA analysis was estimated, including the cost of extraction, qPCR reagents, cloning, and commercial Sanger sequencing. Availability of a qPCR machine and other lab equipment was assumed, and the cost of various plastics, such as pipette tips and tubes, was not included in calculations. Likewise, the cost of electrofishing gear and other fishing equipment was not included in the cost of fishing. The approximate cost in salaries was determined for fishing and eDNA sampling, based on the estimated effort in person-hours, and the salary of a Ph.D. student.

3. Results

3.1. Results from traditional fishing survey and qPCR

Surveys based on traditional fishing methods in 2008 resulted in detection of the weather loach in a channel in Sølsted Mose, where two individuals were caught in fish traps (Fig. 2). In the same area, in 2012, eDNA from weather loach was detected at five sites; two sites in the River Sejersbæk, two drainage channels, and a pond (Fig. 2). This pond was positive for eDNA in both autumn and spring and showed the highest relative amount of eDNA. The eDNA survey further resulted in detection of the weather loach in the wetland Magisterkogen, where the species has not been detected since 1995 (Table 1). C_t values were 43.35 and 43.93 for the sites in the Sejersbæk (sites 7 and 8 in Fig. 2, respectively), 42.64 and 42.25 for the drainage channels (sites 11 and 23 in Fig. 2, respectively), 42.52 and 41.46 for the pond (site 14 in

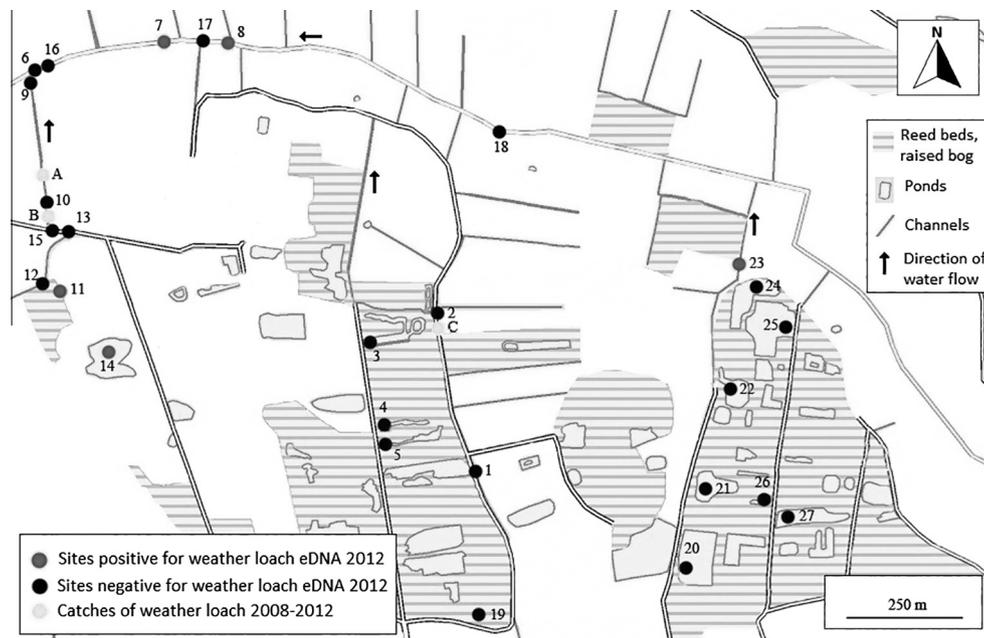


Fig. 2. eDNA sampling sites in the wetland locality of Sølsted Mose, Denmark. Presence or absence of weather loach eDNA at each site and the most recent catch locations are shown. The sampling sites are numbered from 1–27; sites 1–14 were sampled in May 2012 and sites 14–27 were sampled in August 2012. In the 2008 fishing survey of this study two weather loaches were caught at location A. In 2012, local managers caught six specimens at location B and two specimens at location C. The Sejersbæk River can be seen flowing westward, draining the wetland via a number of channels.

Fig. 2. Spring and autumn samples, respectively), and 44.19 for Magisterkogen (Table S1). Most samples showed amplification in just one qPCR replicate, except for site 23, where amplification was detected in three out of four qPCR replicates, and the pond in spring, where all four replicates were positive (Table S1). At the remaining study localities, the weather loach was not detected. Inhibition was detected in a number of samples from Sølsted Mose and Kongens Mose. Dilution of inhibited samples did not yield any additional positive eDNA results. No amplification was detected in extraction blanks and PCR blanks, while included positive PCR controls showed a high level of amplification, and no amplification was detected for the non-target stone loach DNA.

3.2. Sequencing results

Cloning of PCR products and subsequent Sanger sequencing confirmed sequences from two sites in Sølsted Mose and the site in Magisterkogen, which all showed a 100% match to the European weather loach by BLAST against the NCBI Genbank nt database (sequence: TATTCTATCTGGTCTTAATAGT).

3.3. Comparison of monitoring effort

Approximately one person-hour of lab work was spent per water sample. In the field, the effort spent per sample varied with the number of people performing the sampling and the difficulty of accessing the sampling site. The total effort spent surveying a locality was lower with the eDNA method, when compared to the fishing effort expended at the same locality (Table 1). Most importantly, in Sølsted Mose, successful detection of the weather loach required ca. 300 h of fishing vs. ca. 60 h of effort with the eDNA approach.

3.4. Comparison of costs

Approximately 1500 USD was spent on reagents for eDNA analysis. The estimated cost of fishing in salaries was ~8100 USD, while

the cost of field and lab work for the eDNA method was ~2750 USD in salaries.

4. Discussion

In this study, environmental monitoring based on eDNA from water samples, as well as traditional fishing methods, were used to search for the European weather loach in Denmark. Fishing methods resulted in detection of the species in the wetland Sølsted Mose in 2008. At the remaining localities, no loaches were caught. In 2012, the eDNA method led to detection in Sølsted Mose (Fig. 2) and in Magisterkogen. Interestingly, soon after the spring samples had been analyzed in 2012, an intensive survey by local managers resulted in the catch of eight live specimens of the weather loach in Sølsted Mose (Henriksen, 2012), re-confirming the presence of the species in the area. The weather loach has not been detected in Magisterkogen since 1995, but 11 specimens were recorded in the area between 1979 and 1995 (Møller et al., 2012), adding support to the positive eDNA result. At all other localities, no weather loach eDNA was detected, providing further evidence that the species is in fact absent. The weather loach reaches 12 years of age in captivity (Møller et al., 2012) and likely similar ages in the wild, which reduces the bias of comparing 2008 and 2012 results.

4.1. Caveats of eDNA monitoring

False negatives can certainly occur when using the eDNA method (Ficetola et al., 2008; Hyman and Collins, 2012; Thomsen et al., 2012a). This can simply be a result of eDNA molecules being too low in concentration or too patchily distributed to allow detection, or due to PCR inhibition by humic substances (Albers et al., 2013; Jane et al., 2014). Thomsen et al. (2012a) found lower detection rates in running water compared to stagnant waters, probably due to a higher degree of dilution in flowing water. Increasing relative sampling effort in flowing waters would likely alleviate this problem, as the risk of non-detection error is decreased by more intensive sampling (e.g. Gu and Swihart, 2004). In ponds however, Thomsen et al. (2012a) obtained a detection rate of 100% for the

weather loach, and similar detection rates – between 75% and 100% – have been achieved for the species in ditches and ponds in Holland (Herder et al., 2012, 2013; Kranenbarg et al., 2014, all discussed further in Herder et al., 2014). Herder et al. (2013) also confirmed that detection success was higher at sites pointed out in advance as “good” vs. “medium” by species experts, illustrating the importance of ecological knowledge, not only in interpretation of results obtained by eDNA, but also in study design prior to sampling. In the current study, inhibition was detected in a number of samples. Dilution of samples countered inhibition, but most likely decreased the probability of detection. Takahara et al. (2015) found that using smaller volumes of template (2 μ L instead of 5 μ L), led to higher qPCR detection probabilities for the common carp (*Cyprinus carpio*), likely due to inhibition when using large volumes. However, in cases with low concentrations of target DNA, choosing the amount of template will be a compromise between avoiding inhibition and retaining a detectable concentration of target molecules.

False positive results also constitute an issue in eDNA studies, which is of greater concern as it can lead to publication of over-optimistic results (Darling and Mahon, 2011). In the case of eDNA detection of carps in the Great Lakes, many potential sources of false positives have been suggested, including excrements from predators. However, Jerde et al. (2013) concluded that the presence of carp eDNA was most likely to indicate presence of live specimens, as eDNA was only detected where carps had been caught historically, or where later fishing surveys detected the species. Similarly, detections in this study are supported by historical records and/or fishing surveys conducted in the same year. While the fact that five of our samples amplified in just a single qPCR replicate could suggest false positives due to contamination or non-specific amplification, no indications of contamination were found, and sequencing of cloned qPCR products verified the amplifications from Magisterkogen and site 8 in Sølsted Mose as weather loach. Therefore, the low frequency of amplification was likely due to a very low target concentration in the samples.

4.2. Possible effects of environmental variables on detection

While much remains unknown about the importance of environmental variables for the longevity of eDNA molecules, a number of biotic and abiotic factors have been found to have an effect on degradation rates (Barnes et al., 2014). Since a decreased degradation rate may allow eDNA concentrations to build up to a greater degree, differences in environmental conditions between study sites may influence detection probability with the eDNA method. For instance, while UV exposure is generally expected to increase degradation, anoxic conditions and deviations from neutral pH are expected to slow degradation due to decreased activity of microorganisms and extracellular enzymes – although interestingly, higher overall biological activity was found to decrease degradation in a study by Barnes et al. (2014), possibly due to shielding of DNA molecules from UV light by large amounts of algae. Based on current knowledge, we might therefore expect a comparatively high degradation rate in clear running water with a neutral pH, and a slower degradation in stagnant waters with either high or low acidity levels. In our case, it is thus conceivable that degradation rates were more favorable for detection of eDNA in the raised bog sites than the stream sites. It is clear however, that a complex combination of factors determines degradation rates, and much remains to be investigated experimentally.

4.3. Independent sources of eDNA?

Although eDNA can be transported in flowing water over several kilometers (Pilliod et al., 2013; Deiner and Altermatt, 2014),

the relative locations of positive sites in Sølsted Mose and the direction of water flow indicate at least three independent sources of weather loach DNA at the locality (Fig. 2). The pond that was positive for loach DNA is surrounded by reed beds and did not appear to be connected to the positive western drainage channel at the time of sampling, pointing to two separate sources for these sites. The DNA detected in the Sejersbæk however, could originate from the same source as the DNA found at site 23 (Fig. 2). Interestingly, in the Sejersbæk River, positive and negative eDNA results were obtained in between each other across short distances (sites 7, 8 and 17, Fig. 2). A negative result was obtained only 60 m downstream of a positive site, while 140 m further downstream, another positive result was obtained. No strong conclusions can be drawn from these results. However, it could indicate that eDNA was not homogeneously distributed throughout the river, and hence could be closely spatially linked to species presence. Jane et al. (2014) measured eDNA concentrations at distances of 27.5–239.5 m downstream of caged brook trouts in two streams, and found that at high flows, concentrations were similar close to and far from the cages, while at low water flows, concentrations were highest close to the trouts. As the water flow in the Sejersbæk River was low at the time of sampling, it is therefore plausible that the two positive samples represent separate sources of eDNA. This aspect of eDNA and species proximity is very important in a conservation context and in running water in particular, and should be addressed more thoroughly in future studies. However, this should be done using larger water samples than in the current study to avoid false negatives, as 45 mL of water may not be a sufficiently large sample to accurately detect a species in flowing water (Deiner et al., 2015).

4.4. A possible breeding site

The pond that was found to be positive for weather loach eDNA was positive both in spring and autumn. The relative amount of eDNA was also comparatively high in this pond, based on the high proportion of amplifying qPCR replicates in the spring sample. These results may reflect the lower water volume per individual and higher water retention time compared to running water, or a slower degradation rate of eDNA (see Section 4.2). Nevertheless, we speculate that this could also indicate that the pond is a breeding site for the local population, from which individuals can spread to the channel systems when the water level is high. This is supported by the breeding behavior of the weather loach, which prefers warm water rich in vegetation, similar to the conditions in this pond. While weather loach eggs and larvae have never been reported in Denmark (Møller et al., 2012), the breeding season in other European countries is between April and June (Hartvich et al., 2010) and it is therefore reasonable to presume that the eDNA sampling done in May corresponded to the breeding season of the local population. Interestingly, Spear et al. (2015) found that eDNA concentrations for the Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) increased markedly during the breeding season, both in natural river systems and in an aquarium setting with a captive male hellbender. They argue that this result could be highly important for rare aquatic species, as focusing sampling efforts in the breeding season of target organisms may lead to improved detection probabilities, and because monitoring of changes in eDNA concentration may indicate whether endangered populations are reproducing.

4.5. Perspectives of eDNA for conservation

The advantages of eDNA as an applied tool in species management improving detection probability have recently been demonstrated for the invasive American bullfrog (*Lithobates catesbeianus*)

(Dejean et al., 2012), and the bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*) (Jerde et al., 2011, 2013). As such the approach has been predicted a promising future in environmental management (Kelly et al., 2014). The present study emphasizes several advantages of eDNA monitoring of an endangered species. Importantly, the eDNA survey required less effort in person-hours and lower costs than the traditional fishing survey. This comparison is of course most informative in the case of Sølsted Mose, where the weather loach was definitely present during both surveys. A lower required effort of eDNA monitoring compared to conventional methods has previously been demonstrated by Jerde et al. (2011) for carps, and in Holland, eDNA monitoring of the weather loach has been found to be at least one and a half times more cost-effective than electrofishing (Herder et al., 2014), based on observed increases in the detection rate of up to three-fold (Kranenborg et al., 2014). Another advantage of eDNA sampling and analyses is that they are easier to carry out in a standardized manner compared to traditional fishing methods, and are therefore likely less prone to variation due to differences of sampling intensity and training of personnel. Finally, the eDNA method is noninvasive, an important strength in the case of freshwater fishes, where many fishing techniques carry risks of harming the study species (e.g. Snyder, 2003), but also for other freshwater taxa, such as amphibians (Olson et al., 2012). Our results suggest that the European weather loach can be reliably monitored using eDNA, and that this new approach may be very useful for obtaining distribution data for other species. Alternatively, eDNA can be used as an initial, supplementary survey, before traditional studies with traps and electrofishing – thus making the best use of limited time when management decisions are needed quickly.

Conflict of interests

The authors declare no financial or other conflict of interests.

Role of the funding sources

The funding sources had no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocon.2014.11.023>.

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