Methods in Ecology and Evolution

BRITISH ECOLOGICAL SOCIETY

Methods in Ecology and Evolution 2017, **8,** 646–655 doi: 10.1111/2041-210X.12709

An environmental DNA-based method for monitoring spawning activity: a case study, using the endangered Macquarie perch (*Macquaria australasica*)

Jonas Bylemans*[,1,2], Elise M. Furlan[1,2], Christopher M. Hardy[3], Prudence McGuffie[1,4], Mark Lintermans[1] and Dianne M. Gleeson[1,2]

[1]*Institute for Applied Ecology, University of Canberra, Canberra, ACT, Australia;* [2]*Invasive Animals Cooperative Research Centre, University of Canberra, Canberra, ACT 2617, Australia;* [3]*CSIRO Land and Water, GPO Box 1700, Canberra, ACT 2601, Australia; and* [4]*New-South Wales Department of Primary Industries, Batemans Bay, NSW 2536, Australia*

Summary

1. Determining the timing and location of reproductive events is critical for efficient management of species. However, methods currently used for aquatic species are costly, time intensive, biased and often require destructive or injurious sampling. Hence, developing a non-invasive sampling method to accurately determine the timing and location of reproduction for aquatic species would be extremely valuable.

2. We conducted an experimental and field study to determine the influence of spawning, and the mass release of spermatozoa in particular, on environmental DNA (eDNA) concentrations. Using a quantitative PCR approach we monitored changes in nuclear and mitochondrial eDNA concentrations over time.

3. The data from the experimental study and the field survey supported our hypothesis that spawning events are characterized by higher concentrations of nuclear relative to mitochondrial eDNA. Outside of the reproductive period, we find that nuclear and mitochondrial DNA fragments are equally abundant in environmental water samples.

4. We have shown that changes in the relative abundance of nuclear and mitochondrial eDNA can be used to monitor spawning activity of the endangered Macquarie perch. Our method is likely to be transferrable to other aquatic species and can be particularly useful to increase our understanding of the spawning biology of cryptic, rare or threatened species as well as design and evaluate environmental management actions and determine species establishment.

Key-words: aquatic vertebrates, conservation genetics, environmental DNA, reproduction, spermatozoa, wildlife management

Introduction

Monitoring reproduction in aquatic organisms is important for the conservation and management of species and/or populations (Koenig *et al.* 2000; Merz & Setka 2004; King *et al.* 2010; Di Franco *et al.* 2012; Kearns *et al.* 2012). Individual monitoring methods suffer from biases, do not provide direct evidence for reproduction or are unable to distinguish between reproductive failure and high mortality rates of early life-history stages. DNA-based methods provide promising opportunities to overcome these challenges through the monitoring of environmental DNA (eDNA) signals that are correlated with reproductive activity in aquatic organisms.

Many aquatic organisms reproduce sexually through a process called spawning, i.e. the mass release of reproductive cells (oocytes and spermatozoa) into the water column, allowing external fertilization (Harrison *et al.* 1984; Beebee 1996; Coward *et al.* 2002). Determining the timing and location of spawning events is important to: increase our understanding of the species' biology (Harrison *et al.* 1984; Rose 1993; Grant, Chadwick & Halliday 2009); evaluate the reproductive output of populations (Levitan *et al.* 2014); determine population establishment for both invasive and translocated native species (Pearce 2013); and design and evaluate management actions (Koenig *et al.* 2000; King *et al.* 2010; Kearns *et al.* 2012). For aquatic vertebrates relying on external fertilizations (e.g. most fishes and frogs) monitoring reproductive activity can be achieved by destructive, injurious or non-invasive methods (Table 1) (Lefort *et al.* 2015). The extra mortality rate imposed by destructive sampling methods makes them undesirable for monitoring reproduction in rare and threatened species (Tsukamoto 2006; Wei *et al.* 2009; Engstedt, Engkvist & Larsson 2014). On the other hand, injurious methods (i.e. use of acoustic telemetry) are often unable to deliver direct evidence of spawning and non-invasive methods are sensitive to observer biases and taxonomic misidentification (Caswell *et al.* 2004; Miller *et al.* 2012; Ko *et al.* 2013; Koster *et al.* 2013; Diana, Hanchin & Popoff 2015). Overall, all currently available survey

*Correspondence author. E-mail: Jonas.Bylemans@canberra.edu.au

Table 1. Non-exhaustive list of categories for monitoring methods that can be used to monitor reproductive activity in aquatic vertebrates relying on external fertilization. Definitions for the different categories were modified from Lefort *et al.* (2015)

Categories	Definition	Examples
Destructive	Monitoring methods that require sacrificing all or a subset of all organisms (adults, juveniles, larvae or fertilized eggs) collected	Gonad maturation Otolith microchemistry
Injurious	Monitoring methods that require direct contact and may cause physical injury or wounds	Acoustic telemetry
Non-invasive	Monitoring methods that do not affect the physical integrity of the organism, but may affect fitness or behavior	Visual surveys Acoustic surveys

techniques are biased and combining multiple methods to reduce the effects of biases (which is a common practice) increases cost and time requirements. Hence, an efficient non-invasive sampling method that can accurately determine the timing and location of spawning events would be a valuable tool for the management of aquatic biodiversity.

Environmental DNA-based species detection is a relatively new technique which is particularly useful for detecting aquatic species at low densities (Ficetola *et al.* 2008; Thomsen *et al.* 2012a). The sensitivity of this technology can be used to improve presence/absence data for cryptic species (Dejean *et al.* 2012; Thomsen *et al.* 2012b; Sigsgaard *et al.* 2015) and accurately delineate the distribution of species (Jerde *et al.* 2011; Laramie, Pilliod & Goldberg 2014; Bylemans *et al.* 2016a). Furthermore, it has been suggested that the absolute abundance of mitochondrial (mt-) eDNA fragments can be used to determine the reproductive status of populations (Spear *et al.* 2014). Due to the correlation between mt-eDNA abundance and species biomass, an increase in mt-eDNA concentrations during the spawning season might be due to the formation of spawning aggregations (Takahara *et al.* 2012; Lacoursière-Roussel *et al.* 2015; Yamamoto *et al.* 2016). A different approach is thus needed to successfully determine spawning activity from eDNA abundances. As highlighted earlier, spawning is often characterized by the mass release of oocytes and spermatozoa into the water column. While oocytes are relatively large and are often attached to bottom substrate or aquatic vegetation; spermatozoa are small, mobile, more abundant and generally distributed more homogeneously within the waterbody (Cosson *et al.* 2008). As such, spermatozoa are likely to be a major source of eDNA during spawning. Spermatozoa are genetically very different to somatic cells as they contain highly condensed and protected nuclear DNA while the number of mitochondrial genomes is relatively low. It is therefore reasonable to hypothesize that the amount of nuclear (nu-) eDNA fragments will increase relative to mt-eDNA fragments and changes in the ratios between the concentrations of both fragments will be indicative of recent reproductive activity (Coward *et al.* 2002; Islam & Akhter 2012).

Macquarie perch (*Macquaria australasica*) is a medium-bodied freshwater fish endemic to Australia and is currently listed as nationally endangered with only a handful self-sustaining populations remaining (Ingram, Douglas & Lintermans 2000; Lintermans 2007). The abundance and distribution of this species has declined as a result of anthropogenic disturbances and negative interactions with invasive species (Ingram, Douglas & Lintermans 2000; Koehn & MacKenzie 2004; Broadhurst, Ebner & Clear 2009). While multiple recovery actions have been undertaken to ensure the future survival of this species, evaluating their effectiveness is often difficult due to the lack of long-term monitoring surveys and biases associated with individual monitoring methods (Lintermans 2013a, b, 2015). In recent years, efforts have been undertaken to increase our understanding of Macquarie perch spawning biology (Tonkin, Lyon & Pickworth 2010; Broadhurst, Ebner & Clear 2012; Koster *et al.* 2013; Tonkin *et al.* 2015). However, obtaining detailed information about the timing and location of spawning currently requires destructive or injurious sampling methods (Tonkin, Lyon & Pickworth 2010; Tonkin *et al.* 2015). Spawning in Macquarie perch generally occurs when water temperatures reach 14–18 °C and they remain reproductively active for up to 2 months (Ingram, Douglas & Lintermans 2000; Tonkin, Lyon & Pickworth 2010; Tonkin *et al.* 2015). Mature adults undertake spawning migrations and form aggregations at the tail end of pools (Tonkin, Lyon & Pickworth 2010; Koster *et al.* 2013). Spawning occurs both day and night although fish are thought to be more active in the late afternoon and early morning (Tonkin, Lyon & Pickworth 2010; Kearns *et al.* 2012). Gametes are generally released in fast flowing areas of the riffles and the relatively large demersal adhesive eggs will flow downstream and get lodged into the gravel bed until they hatch (Lintermans 2007; Tonkin, Lyon & Pickworth 2010; Kearns *et al.* 2012).

Here, we present a novel eDNA-based method for monitoring reproductive activity in aquatic organisms. Using Macquarie perch as a target species in laboratory and field based studies we show that the relative abundance of mt- and nu-eDNA can be indicative of recent reproductive activity. The presented methodology will be broadly applicable and has the potential to increase our understanding of the reproductive biology of wide variety of species, which could ultimately lead to improved management strategies.

Materials and methods

PRIMER DESIGN AND TESTING

Primers amplifying fragments of c. 150 bp of Macquarie perch mitochondrial (12S) and nuclear (ITS1) DNA were designed, using GENEIOUS v. 7.1.7 (Kearse *et al.* 2012). Target regions were selected on the basis that they are present in multiple copies within a cell and they are highly variable between species (Long & Dawid 1980; Foran 2006; Hardy *et al.* 2011). Both primer pairs were tested *in silico* for undesirable primer interactions and specificity. The best performing primer pairs were tested *in vitro* by running quantitative PCR

(qPCR) reactions on a three point, ten-fold dilution series of genomic DNA from Macquarie perch and closely related co-occurring species. For all positive amplifications PCR products were purified, using the MinElute® PCR purification kit (Qiagen, Hilden, Germany) and Sanger sequenced to confirm the absence of non-specific amplification (for full details on primer development and testing see Appendix S1, Supporting Information).

EXPERIMENTAL PROTOCOL

Prior to the experimental set-up, all equipment was soaked for approximately 20 min in a 10% (v/v) bleach solution and thoroughly rinsed with UV-sterilized tap water to destroy any potential contaminating DNA. Experimental fish, 18 one-year old Macquarie perch, were sourced from the Narrandera Fisheries Centre (NFC) (NSW Department of Primary Industries) and used in two tank configurations. Due to the limited number of individuals, configurations were set-up at different times (October 2014 and December 2014) (Fig. 1). Each set-up consisted of six experimental tanks (three replicates per treatment) and a single Negative Control Tank (NCT), which contained no fish to evaluate potential cross-contamination. The first set-up, containing low (one fish/50 L) and high (five fish/50 L) density Experimental Spawning Tanks (EST), was used to simulate spawning for solitary (i.e., the release of gametes by a single male and female) and broadcast (i.e., the release of gametes by multiple males and females) spawning species, respectively. The second configurations consisted of low and high-density Experimental Control Tanks (ECT) to evaluate the potential impact of the sampling strategy on eDNA concentrations. Water samples (50 mL) were collected prior to stocking tanks with experimental animals to confirm the absence of Macquarie perch eDNA. After introducing experimental animals to the tanks, five samples were collected over a 14-day period for each tank (Fig. 1). After collecting samples at day 14 (336 h), EST were supplemented with a 10 mL mixture of Macquarie perch milt (fish seminal fluid obtained from NFC) and UV-sterilized tap water to replicate a single spawning event. Appropriate milt volumes for low and high density treatments were calculated based on the milt production of single ripe male (10 mL/700 g body weight) (Asmus M., pers. comm.) and the mean body weight of the experimental animals (17·2 ± 7·5 g). An equivalent volume of UV-sterilized tap water was added to all the control tanks (NCT and ECT). Water samples were collected from all tanks for an additional 8 days (Fig. 1). After each sampling event, 50 mL of UV-sterilized tap water was added to each tank to keep water volumes constant.

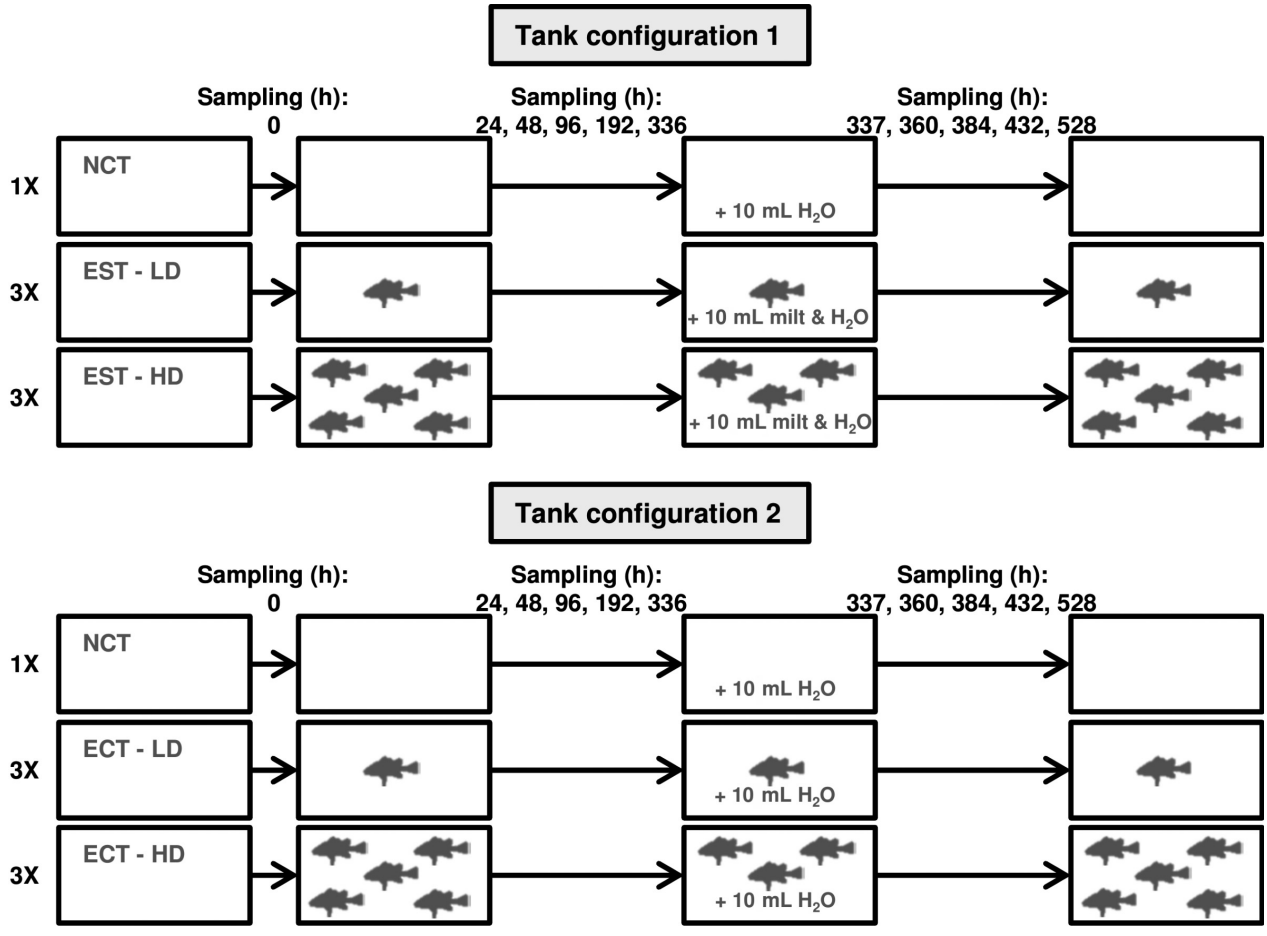

Fig. 1. Schematic representation of the experimental set-up. Each tank configuration consisted of seven tanks containing 50 L of UV-sterilized tap water. The first tank configuration was set-up in October 2014 and consisted of a single Negative Control Tank (NCT) (no fish present) and six Experimental Spawning Tanks with low (one fish/50 L) and high (five fish/50 L) fish densities(EST-LD and EST-HD, respectively). Tank configuration 2 was set-up in December 2014 and contained a single NCT and six Experimental Control Tanks with low (ECT-LD) and high (ECT-HD) fish densities.

FIELD SURVEY

In order to confirm the applicability of our method in the field, a small-scale field survey was conducted targeting known spawning grounds for Macquarie perch within the Upper Murrumbidgee River (UMR) (NSW, Australia). Over a period of 3 years (2012–2015), acoustic telemetry methods were used to track the spawning movements of adult Macquarie perch in the UMR (P. McGuffie, unpubl. data). Six potential spawning riffles, located within a remote section of the UMR, were identified based on this previous research and monitored, using egg collections and eDNA sampling during the spring of 2015 (Fig. 2). Over three sampling events (October 20, 23 and 26), four double-winged fine-meshed (0·5 mm) drift nets were set to capture eggs and confirm spawning at each location. At the top and bottom of each riffle two individual nets were set overnight at a minimum depth of 0·5 m. Nets were retrieved the following days (October 21, 24 and 27) and eggs were collected, transported to a field laboratory and counted. Because of the remote sampling locations, the collection of water samples for eDNA analyses (four 2 L samples from pools downstream of the spawning riffles) was limited to day-time hours. Sampling bottles were sterilized by soaking them for approximately 20 min in a 10% (v/v) bleach solution and thoroughly rinsing with UV-sterilized tap water. Samples were collected before the presumed spawning period (October 8) to obtain baseline information on the relative concentrations of nu- and mt-eDNA. Given that Macquarie perch are reproductively active for 1–2 months (Tonkin, Lyon & Pickworth 2010; Tonkin *et al.* 2015), eDNA sampling was continued after the first records of eggs in the drift nets and was stopped after spawning fish aggregations were observed on the riffles and egg counts showed a clear sign of recent spawning activity (October 24 and 27). A Blank Field Control (BFC) was included at each sampling site and consisted of a 2 L sampling bottle filled with UV-sterilized tap water that was opened on site, closed and submerged in the water. After collection, all samples were stored on ice and transported to the University of Canberra for further processing.

SAMPLE PROCESSING AND ANALYSES

Environmental DNA of all collected samples was captured by filtering water samples immediately (experimental samples) or within 24 h (field samples) through a 1·2 µm glass fiber filter. Before and between filtering samples, all equipment was soaked for 10 min in a 10% (v/v) bleach solution and thoroughly rinsed with UV-sterilized tap water. After sterilization of the filtering equipment, a Negative Equipment Control (NEC) was obtained by filtering 500 mL of UV-sterilized water before loading experimental or field samples. All filters were placed in a 5 mL tube, using sterilized forceps and stored at −20 °C.

Environmental DNA extractions, using the PowerWater DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA, USA), and further analyses were conducted in the trace DNA laboratory at the University of Canberra (Australia). Concentrations of nu- and mt-eDNA in all samples were determined by performing three qPCR replicates per sample for each target fragment, using the SYBR® Select Master Mix (ThermoFisher Scientific, Waltham, MA, USA). All qPCR analyses were performed in a final volume of 20 µL on a 96-well plate, using the Viia7 Real-Time PCR System (ThermoFisher Scientific). For each plate a five point standard curve with three qPCR replicates for each DNA concentration (5×10^6–5×10^2) was used to infer absolute eDNA abundance. Cycling conditions consisted of an initial activation step of 2 min at 95 °C, 55 2-step cycles of 15 s at 95 °C and 1 min at 60°C, and a melting curve step with a continuous increase of 0·05 °C s^{-1} from 60 °C to 95 °C. Amplification curves and melt curves were visually inspected and replicates were omitted if the amplification curve did not show a clear exponential phase or the observed melt curves deviated from those observed in the standard curve samples. Additionally, positive PCR replicates obtained from negative control samples (NCT and BFC) and a random subset (≥10%) of experimental and field samples were purified and sequenced. All obtained sequence reads matched the Macquarie perch target sequence. More detailed information for the sample analysis protocol can be found in the Appendix S2.

From the eDNA copies per reaction we calculated the number of eDNA copies per litre of water collected. A correction for the dilution effect was incorporated for all experimental samples and qPCR replicates showing no amplification were assigned a concentration of zero eDNA copies per litre. The obtained eDNA concentrations were subsequently log transformed, using eqn 1.

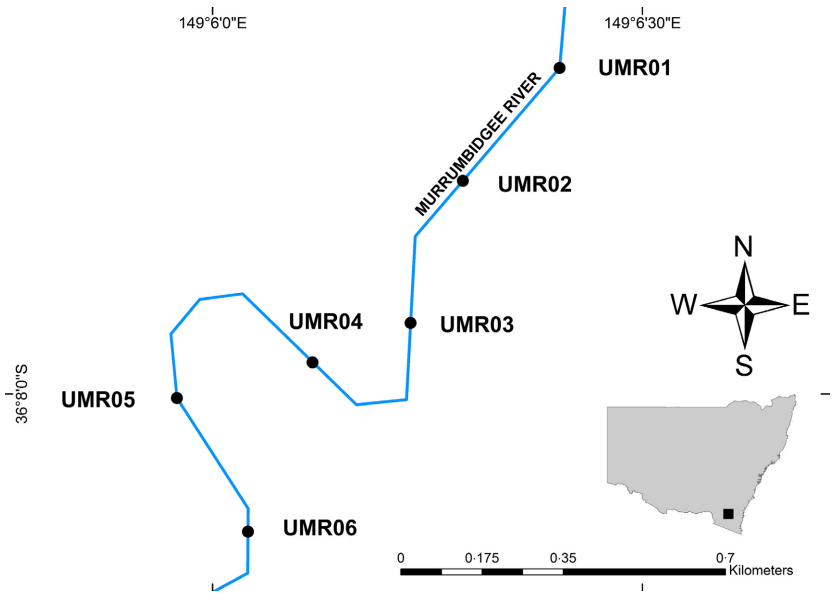

Fig. 2. Map of the sampling locations within the Upper Murrumbidgee River (UMR) (NSW, Australia). Sample locations are numbered from downstream (UMR01) to upstream (UMR06).

$$[\text{eDNA}] = \text{LOG}_{10}((\text{eDNA copies/L}) + 1) \quad \text{eqn 1}$$

The relative abundance of nu- and mt-eDNA fragments was determined on a per sample basis by calculating the ratios of [nu-eDNA] to [mt-eDNA] across all independent qPCR replicates. All graphs used to visualize changes in the log transformed eDNA concentrations and the ratios of [nu-eDNA] to [mt-eDNA] were constructed, using the packages GGPLOT2, GRIDEXTRA and COWPLOT in R v.3.1.3 (R Development Core Team 2010).

Results

PRIMER DESIGN AND TESTING

After *in silico* and *in vitro* testing of the potential primer pairs, the best performing primer combinations were selected for further analyses. Final primer combinations amplified a 148 bp and 157 bp fragment of the 12S and ITS1 region of Macquarie perch, respectively (Table 2). Both primer pairs were considered highly specific as no amplification was observed in closely related co-occurring species (Appendix S1).

EXPERIMENTAL RESULTS

The ECTs showed a rapid increase in nu- and mt-eDNA within the first 24 h after stocking the tanks with Macquarie perch (Fig. 3). After this initial build-up phase, eDNA concentrations reached a plateau at which equilibrium between eDNA production and degradation was achieved. The observed equilibrium concentrations were generally higher in the high-density treatments and this trend was observed for both nu- and mt-eDNA. When evaluating the relative abundance of nu- and mt-eDNA, the results indicate that both target fragments are equally abundant since the calculated ratios do not deviate strongly from one (Fig. 4). Although a small increase in nu-eDNA concentrations can be observed in the low density ECT at 384 h (Fig. 3), a closer inspection of the raw data revealed that this was due to an increase in a single tank. As the ECT were set-up a month after the EST and immature experimental animals were used, this increase in nu-eDNA is unlikely to be caused by the presence of spermatozoa. A more likely explanation for this pattern is the higher natural variation in eDNA concentrations when species densities are low or increased stress levels experienced by individual fish due to the increased temporal sampling between 336 and 384 h.

Within the EST, the nu- and mt-eDNA concentrations generally follow the same trend as in the ECT for the first part of the experiment. After supplementing the tanks with Macquarie perch milt, however, the abundance of both target fragments increases (Fig. 3). Samples collected one hour after milt supplementation had the highest concentrations of both target fragments. While there is no obvious difference in these peak concentrations between density treatments, there is a clear difference in concentrations between target fragments. In both density treatments, milt supplementation resulted in an approximately 100-fold increase in nu-eDNA relative to mt-eDNA. This relative difference in eDNA concentrations remains detectable in the high and low density treatments for approximately 40 to 60 h, respectively (Fig. 4). Additionally, the results indicate that the degradation profiles for eDNA originating from spermatozoa are dependent on the fish density and the target fragment. A comparison between density treatments reveals that spermatozoa eDNA, both nu- and mt-eDNA, degrades faster in the high density tanks. When comparing the nu- and mt-eDNA degradation curves after milt supplementation for both density treatments, the results indicate that the mt-eDNA follows a typical exponential degradation curve while the nu-eDNA degradation curve follows an inverse logistic function (Fig. 3). This trend can also be observed in Fig. 4 where the calculated ratios show a peak 24 h after milt supplementation.

All NEC and the samples from the NCT associated with the ECT showed no signs of contamination. In contrast, contamination was observed in the NCT which was run simultaneously with the EST (Appendix S3). Analyses of all the samples collected from this tank showed low levels of nu-eDNA shortly before and after EST were supplemented with milt while no mt-eDNA was detected. The most likely source of contamination is thus the handling of Macquarie perch milt prior to collecting the 336 h samples which has caused the transfer of few spermatozoa into the associated NCT. Given that only low levels of nu-eDNA were detected in the NCT (i.e., average log transformed nu-eDNA concentration across all positive samples is 2·94 ± 2·32) and no increase in eDNA concentrations was observed in the EST samples collected at 336 h, the observed contamination levels are unlikely to affect the general trends observed.

Table 2. Details of the Macquarie perch specific primers used to target a fragment of the mitochondrial 12S gene and the nuclear ITS1 region

Primer	Sequence (5'-3')	Amplicon length
MP-12S-183F23	CAGCTTACCCTGTG AAGGACTAA	148 bp
MP-12S-330R25	CCTTCAGGATGTA CGTTTCAGTATA	
MP-ITS1-444F21	TAGTTCAATTGC CGTCGTGCA	157 bp
MP-ITS1-600R19	CGACGAGGGA GAGAGAGAC	

FIELD SURVEY

Through the conventional monitoring methods we were able to confirm the absence of spawning migration during the first day of eDNA sampling (October 8, 2015). In addition, egg collections indicated that Macquarie perch were reproductively active at two spawning riffles (UMR04 and UMR06) during October 21st, 24th, and 27th (Table 3). The eDNA analyses clearly show that outside of the reproductive period (October 8, 2015) the ratios between nu- and mt-eDNA concentrations do not deviate from one (Fig. 5). In contrast to the conventional monitoring, eDNA monitoring did not show evidence

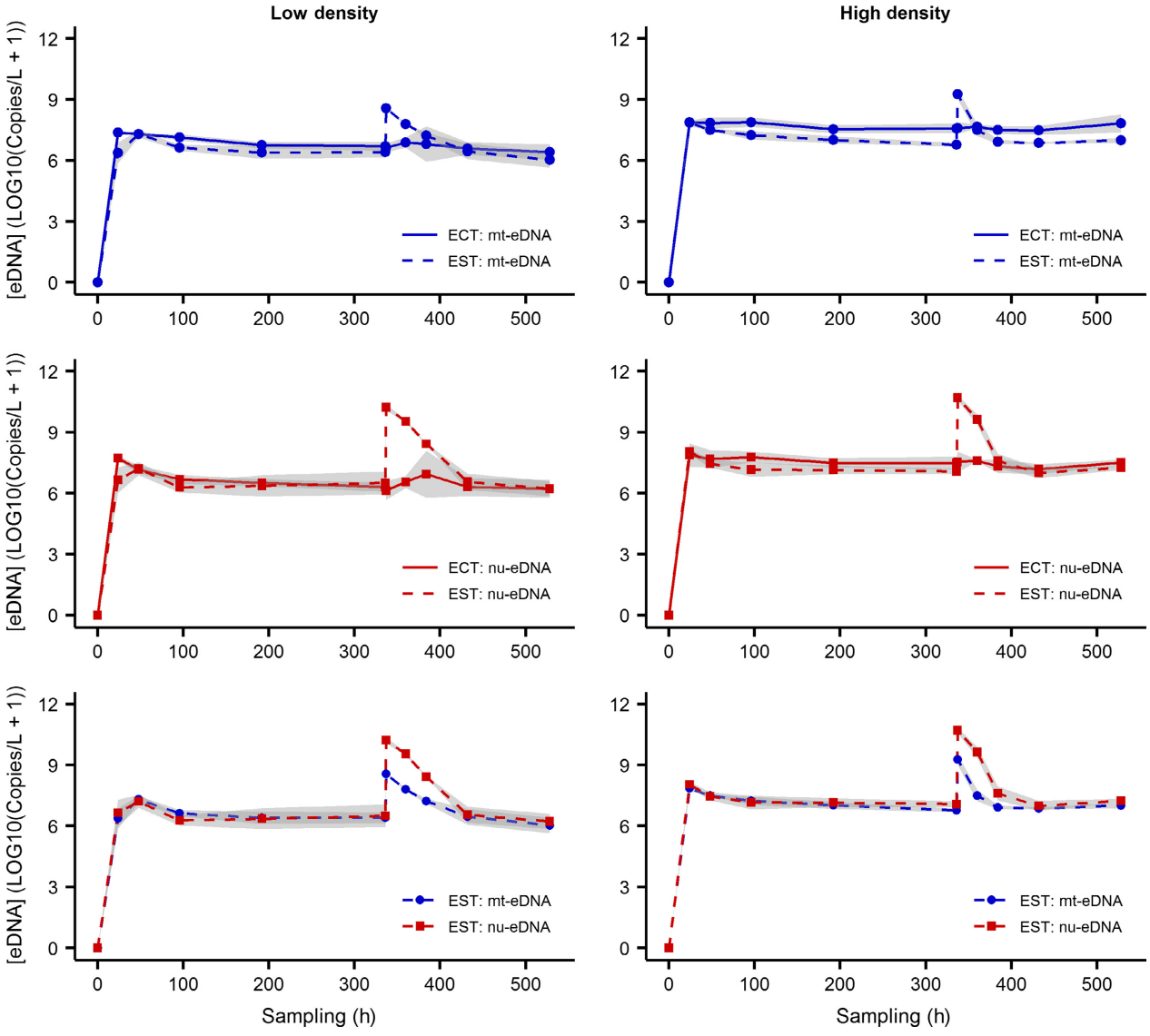

Fig. 3. The log10 transformed Macquarie perch environmental DNA (eDNA) concentrations over time for low and high density treatments. The top and middle graphs give a comparison between the Experimental Control Tanks (ECT) and Experimental Spawning Tanks (EST) for mitochondrial (mt−) and nuclear (nu−) eDNA, respectively. The bottom graphs give a comparison between mt− and nu− eDNA concentrations within the EST. Tanks were supplemented with 10 mL of water (ECT) or a mixture of milt and water (EST) after 336 h. Grey shading represents ±1 SD from the mean.

of spawning activity during October 24th but all samples collected during October 27th showed an increase in ratios between nu- and mt-eDNA (Fig. 5).

The eDNA analyses performed on both BFC's and NEC's yielded one positive amplification for the 12S gene fragment in the BFC associated with the samples collected from UMR04 on October 27th. Given that all other controls tested negative for Macquarie perch DNA and high concentrations were obtained from a single qPCR replicate (1201 DNA copies/reaction), it is highly likely that contamination occurred during the PCR set-up. This was further supported by performed an additional six qPCR replicates for both target fragments for this BFC, which did not produce a positive amplification. As such, contamination due to improper sample handling or DNA extractions can be excluded and the results obtained from all associated samples were not omitted from the analyses.

Discussion

We have shown that changes in the relative abundance of nuclear and mitochondrial eDNA can be used to monitor spawning activity of Macquarie perch. Although we focused on a single species, our methods are likely to be transferrable to other aquatic species relying on external fertilization such as many teleost fish and frog species. While the relatively high numbers of mitochondria found in frog spermatozoa compared to teleost fish might reduce the strength of the eDNA signal (Jamieson 1991; Lee & Jamieson 1992), this may be partially compensated by higher copy numbers of the nuclear

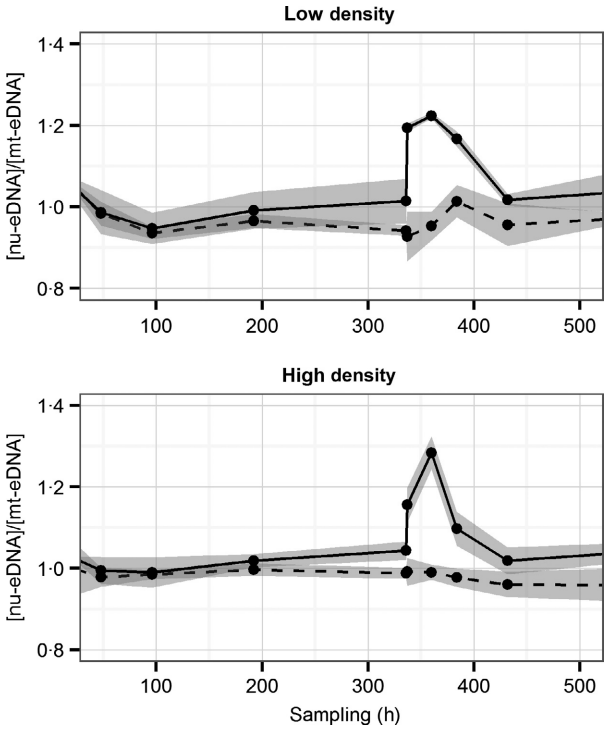

Fig. 4. The ratios between Macquarie perch nuclear and mitochondrial environmental DNA (eDNA) concentrations over time for the low and high density treatments. Solid lines represent the Experimental Spawning Tanks (EST) while dashed lines represent the Experimental Control Tanks (ECT). Ten millilitres of water (ECT) or a mixture of milt and water (EST) were added after 336 h.

Table 3. The number of Macquarie perch eggs collected with drift nets for each sampling site and sampling date

	Drift nets		
Site	21/10	24/10	27/10
UMR01	0	0	0
UMR02	0	0	0
UMR03	0	0	0
UMR04	1	40	399
UMR05	0	0	0
UMR06	4564	485	1585

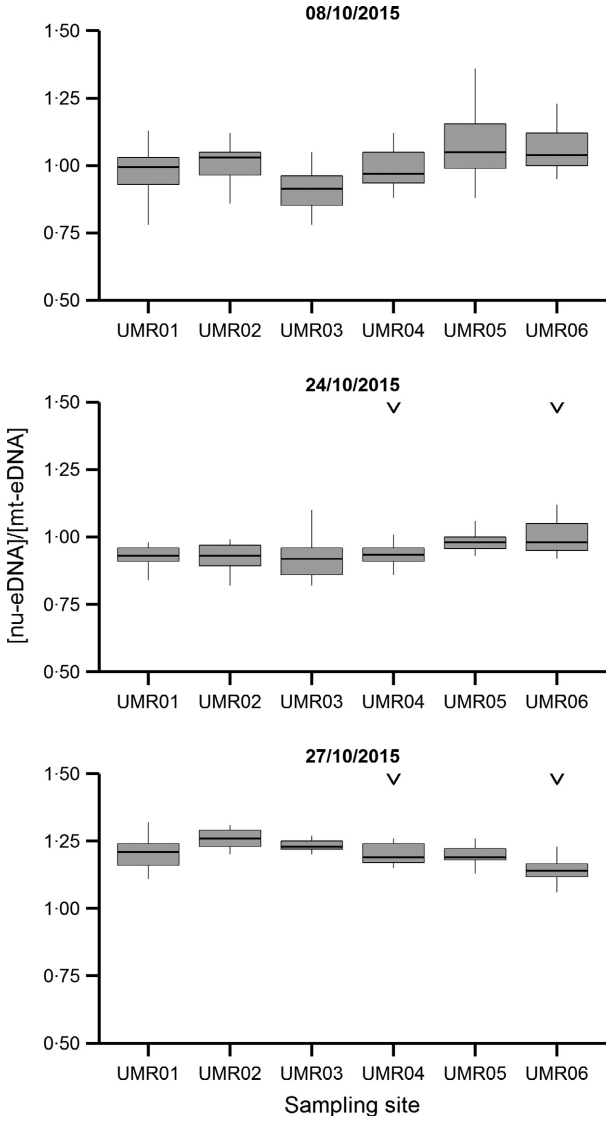

Fig. 5. The ratios between Macquarie perch nuclear and mitochondrial environmental DNA (eDNA) concentrations for all field sites sampled before (October 8, 2015) and during (October 24 and 27, 2015) the presumed spawning period. Black arrow points indicate the sampling dates and sites at which eggs were collected, using drift nets.

ribosomal operon in frog species (Long & Dawid 1980). Besides the direct applications of this method, the results obtained from our study also contradict the popular belief that mitochondrial DNA fragments are more abundant in environmental water samples (Olson, Briggler & Williams 2012). This is consistent with recently published research which has shown that amplicon targets (nuclear vs mitochondrial) do not have a significant effect on eDNA detection rates and the use of nu-eDNA targets might actually increase the sensitivity of eDNA studies (Minamoto *et al.* 2016; Piggott 2016). These findings and the fact that nuclear DNA fragments are thought to degrade faster in environmental samples than mitochondrial DNA (Foran 2006), offers new opportunities to improve the reliability of eDNA-based species detections by targeting both eDNA fragments, using a multiplex PCR.

Although the results obtained from the drift nets and eDNA sampling are inherently biased due to differences in sampling times (i.e., night- and daytime collections, respectively), a comparison between the data collected from both monitoring methods do highlight some of their limitations. Firstly, drift nets were able to collect Macquarie perch eggs during October 24th while eDNA monitoring did not show any sign of recent spawning activity. This observation could indicate that our eDNA method is less sensitive than the use of drift nets to detect low levels of spawning activity. Alternatively, the pattern could be explained if previously deposited eggs were dislodged from the gravel beds and washed into the drift nets. As such, relying on egg counts for the detection of recent spawning activity might suffer from false positive errors. When

comparing eDNA and conventional monitoring results when both showed signs of reproductive activity (October 27, 2015), we find that sites showing the highest ratios between nu- and mt-eDNA do not correspond to the spawning areas as defined by conventional monitoring (Fig. 5). This difference could be explained when taking into consideration water flow and the method used to infer spawning activity. Owing to the limited transport distance of the demersal and slightly adhesive eggs of Macquarie perch, drift nets can determine spawning locations very accurately. However, their labor-intensive nature makes them unsuitable to precisely determine temporal variation in spawning activity. In contrast, our eDNA-based methodology relies on the presence of highly mobile spermatozoa in the water samples to infer spawning. Consequently, the downstream transport of spermatozoa will affect our ability to determine the exact spawning locations. However, eDNA sampling can be highly automated and thus temporal and spatial sampling efforts can be increased to obtain more detailed information on the exact spawning time and location. While this study shows that eDNA-based monitoring can be utilized as a non-invasive method for monitoring reproduction, additional studies comparing conventional methods with our eDNA-based approach are needed to assess the advantages and disadvantages of this method. Future comparative studies in lotic systems will be valuable to better understand the impact of water flow and will benefit from temporal sampling strategies in which sampling time/period is consistent between methods. While the transport of eDNA originating from spermatozoa is likely to be more limited in lentic environments, additional work is needed to determine dispersal rates of spermatozoa in these systems. The application of this method to other species will be beneficial to understand the influence of reproductive behavior (e.g., solitary vs broadcast spawning) on the efficiency of conventional methods and eDNA-based monitoring of reproductive activity. From a management context, determining the magnitude of spawning events is important to monitor changes in the effective population size of species. After milt supplementations, we found that both nuclear and mitochondria eDNA concentrations were higher in the high-density tanks compared to the low-density tanks (i.e., a 3- and 5-fold increase, respectively). Although this indicates that eDNA concentrations might be related to the magnitude of spawning events, more rigorous studies are required to provide conclusive evidence. Finally, the uptake of the presented method will depend strongly on cost and time requirements as these are often limiting factors in monitoring surveys. Surveys using eDNA are generally considered less time-intensive than conventional monitoring and this is particularly true when conventional methods are very time consuming (e.g., collecting and counting eggs) (Jerde *et al*. 2011). Although the methods used in this study are likely to be relatively costly, the use of a multiplex PCR to evaluate the relative abundance of nuclear and mitochondrial eDNA fragments within a single reaction will reduce costs significantly. Finally, it is important to note that within our study contamination was observed in some negative control samples (NCT and BFC). As the presence of contaminating DNA can significantly affect the validity of future studies, we strongly recommend that future research takes into consideration recently published guidelines for eDNA studies (Goldberg *et al*. 2016).

Conclusion

Our eDNA methodology for detecting reproductive activity in aquatic organisms has the potential to increase our knowledge of the reproductive biology of elusive species and help evaluate management actions aimed at increasing the reproductive output of endangered populations. Environmental DNA monitoring provides several advantages in that it is a non-invasive method, highly species-specific and can be highly automated, allowing it to be applied across large temporal and spatial scales. Furthermore, given that this method relies on the detection of the direct products of spawning activity, it could help evaluate whether population declines are caused by spawning failure or high mortality rates of the early life-history stages. While the presented method could have broad applications, future comparative studies are required to better understand the sensitivity and the cost- and time-requirements of this method, which will ultimately determine its potential for species management and conservation.

Authors' contributions

J.B., E.M.F., C.M.H., M.L., and D.M.G. conceived the idea and designed the experimental set-up; J.B. conducted the experimental study and collected the samples; J.B. and P.M. designed and conducted the field survey; J.B. conducted the laboratory work and the data analyses with significant contributions from C.M.H., E.M.F, and D.M.G.; J.B. led the writing of the manuscript. All authors contributed to the writing of the manuscript and gave final approval for publication.

Acknowledgments

We wish to thank the Invasive Animal CRC, NSW Fisheries Scientific Committee and the Australian Wildlife Society for providing funding for this research project. We also acknowledge D. Gilligan and M. Asmus who kindly provided the Macquarie perch used in the experimental study. Further thanks go out to A. Georges, all participants of the Invasive Animal CRC writing workshop (circle D) and two anonymous reviewers for their valuable comments on previous versions of the manuscript. All work was conducted under the University of Canberra Animal Ethic permit CEAE 14-02 and the ACT Government license IF201418.

Data accessibility

Sequences used for the nuclear primer developed have been submitted to GenBank (accession numbers KX342078–KX342086). The qPCR data and the R script used to construct all figures are available in the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.5kq4r). (Bylemans et al. 2016b)

References

Beebee, T.J.C. (1996) *Ecology and Conservation of Amphibians*. Chapman & Hall, London, UK.

Broadhurst, B.T., Ebner, B.C. & Clear, R.C. (2009) Effects of radio-tagging on two-year-old, endangered Macquarie perch (*Macquaria australasica*: Percichthyidae). *Marine and Freshwater Research*, **60**, 341–345.

Broadhurst, B.T., Ebner, B.C. & Clear, R.C. (2012) A rock-ramp fishway expands nursery grounds of the endangered Macquarie perch (*Macquaria australasica*). *Australian Journal of Zoology*, **60**, 91–100.

Bylemans, J., Furlan, E.M., Pearce, L., Daly, T. & Gleeson, D.M. (2016a) Improving the containment of a freshwater invader using environmental DNA (eDNA) based monitoring. *Biological Invasions*, **18**, 3081–3089.

Bylemans, J., Furlan, E.M., Hardy, C.M., McGuffie, P., Lintermans, M.& Gleeson, D.M. (2016b) Data from: An environmental DNA-based method for monitoring spawning activity: a case study, using the endangered l Macquarie perch *(Macquaria australasica)*. *Dryad Digital Repository*. http://dx.doi.org/10.5061/dryad.5kq4r

Caswell, N.M., Peterson, D.L., Manny, B.A. & Kennedy, G.W. (2004) Spawning by lake sturgeon (*Acipenser fulvescens*) in the Detroit River. *Journal of Applied Ichthyology*, **20**, 1–6.

Cosson, J., Groison, A.L., Suquet, M., Fauvel, C., Dreanno, C. & Billard, R. (2008) Marine fish spermatozoa: racing ephemeral swimmers. *Reproduction*, **136**, 277–294.

Coward, K., Bromage, N.R., Hibbitt, O. & Parrington, J. (2002) Gametogenesis, fertilization and egg activation in teleost fish. *Reviews in Fish Biology and Fisheries*, **12**, 33–58.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E. & Miaud, C. (2012) Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. *Journal of Applied Ecology*, **49**, 953–959.

Di Franco, A., Coppini, G., Pujolar, J.M., De Leo, G.A., Gatto, M., Lyubartsev, V., Melià, P., Zane, L. & Guidetti, P. (2012) Assessing dispersal patterns of fish propagules from an effective mediterranean marine protected area. *PLoS ONE*, **7**, e52108.

Diana, J.S., Hanchin, P. & Popoff, N. (2015) Movement patterns and spawning sites of muskellunge *Esox masquinongy* in the Antrim chain of lakes, Michigan. *Environmental Biology of Fishes*, **98**, 833–844.

Engstedt, O., Engkvist, R. & Larsson, P. (2014) Elemental fingerprinting in otoliths reveals natal homing of anadromous Baltic Sea pike (*Esox lucius* L.). *Ecology of Freshwater Fish*, **23**, 313–321.

Ficetola, G.F., Miaud, C., Pompanon, F. & Taberlet, P. (2008) Species detection using environmental DNA from water samples. *Biology Letters*, **4**, 423–425.

Foran, D.R. (2006) Relative degradation of nuclear and mitochondrial DNA: an experimental approach. *Journal of Forensic Sciences*, **51**, 766–770.

Goldberg, C.S., Turner, C.R., Deiner, K. *et al.* (2016) Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, **7**, 1299–1307.

Grant, R.A., Chadwick, E.A. & Halliday, T. (2009) The lunar cycle: a cue for amphibian reproductive phenology? *Animal Behaviour*, **78**, 349–357.

Hardy, C.M., Adams, M., Jerry, D.R., Court, L.N., Morgan, M.J. & Hartley, D.M. (2011) DNA barcoding to support conservation: species identification, genetic structure and biogeography of fishes in the Murray-Darling River Basin, Australia. *Marine and Freshwater Research*, **62**, 887–901.

Harrison, P.L., Babcock, R.C., Bull, G.D., Oliver, J.K., Wallace, C.C. & Willis, B.L. (1984) Mass spawning in tropical reef corals. *Science*, **223**, 1186–1189.

Ingram, B., Douglas, J. & Lintermans, M. (2000) Threatened fishes of the world: *Macquaria australasica* Cuvier, 1830 (Percichthyidae). *Environmental Biology of Fishes*, **59**, 68.

Islam, M.S. & Akhter, T. (2012) Tale of fish sperm and factors affecting sperm motility: a review. *Advances in Life Sciences*, **1**, 11–19.

Jamieson, B.G.M. (1991) *Fish Evolution and Systematics: Evidence from Spermatozoa*. Cambridge University Press, Cambridge, UK.

Jerde, C.L., Mahon, A.R., Chadderton, W.L. & Lodge, D.M. (2011) 'Sight-unseen' detection of rare aquatic species using environmental DNA. *Conservation Letters*, **4**, 150–157.

Kearns, J., Tonkin, Z., O'Mahony, J. & Lyon, J. (2012). *Identification and protection of key spawning habitats for Macquarie Perch in King Parrot Creek: Black Saturday Victoria 2009 - Natural values fire recovery program*. Heidelberg, Victoria. http://www.depi.vic.gov.au/__data/assets/pdf_file/0020/203933/VBRRA-P16-web-rev2.pdf

Kearse, M., Moir, R., Wilson, A. *et al.* (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, **28**, 1647–1649.

King, A.J., Ward, K.A., O'Connor, P., Green, D., Tonkin, Z. & Mahoney, J. (2010) Adaptive management of an environmental watering event to enhance native fish spawning and recruitment. *Freshwater Biology*, **55**, 17–31.

Ko, H.-L., Wang, Y.-T., Chiu, T.-S., Lee, M.-A., Leu, M.-Y., Chang, K.-Z., Chen, W.-Y. & Shao, K.-T. (2013) Evaluating the accuracy of morphological identification of larval fishes by applying DNA barcoding. *PLoS ONE*, **8**, e53451.

Koehn, J.D. & MacKenzie, R.F. (2004) Priority management actions for alien freshwater fish species in Australia. *New Zealand Journal of Marine and Freshwater Research*, **38**, 457–472.

Koenig, C.C., Coleman, F.C., Grimes, C.B., Fitzhugh, G.R., Scanlon, K.M., Gledhill, C.T. & Grace, M. (2000) Protection of fish spawning habitat for the conservation of warm temperate reef fish fisheries of shelf edge reefs of Florida. *Bulletin of Marine Science*, **66**, 593–616.

Koster, W.M., Dawson, D.R., Morrongiello, J.R. & Crook, D.A. (2013) Spawning season movements of Macquarie perch (*Macquaria australasica*) in the Yarra River, Victoria. *Australian Journal of Zoology*, **61**, 386–394.

Lacoursière-Roussel, A., Côté, G., Leclerc, V. & Bernatchez, L. (2015) Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. *Journal of Applied Ecology*, **53**, 1148–1157.

Laramie, M.B., Pilliod, D.S. & Goldberg, C.S. (2014) Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biological Conservation*, **183**, 29–37.

Lee, M.S.Y. & Jamieson, B.G.M. (1992) The ultrastructure of the spermatozoa of 3 species of myobatrachid frogs (Anura, Amphibia) with phylogenetic considerations. *Acta Zoologica*, **73**, 213–222.

Lefort, M.C., Boyer, S., Barun, A., Emami-Khoyi, A., Ridden, J., Smith, V.R., Sprague, R., Waterhouse, B.R. & Cruickshank, R.H. (2015) Blood, sweat and tears: non-invasive vs. non-disruptive DNA sampling for experimental biology. *PeerJ Preprints*, doi:10.7287/peerj.preprints.655v3.

Levitan, D., Boudreau, W., Jara, J. & Knowlton, N. (2014) Long-term reduced spawning in Orbicella coral species due to temperature stress. *Marine Ecology Progress Series*, **515**, 1–10.

Lintermans, M. (2007) *Fishes of the Murray-Darling Basin: An Introductory Guide*. http://www.mdba.gov.au/sites/default/files/pubs/MDBA-Fish-species-book.pdf

Lintermans, M. (2013a) A review of on-ground recovery actions for threatened freshwater fish in Australia. *Marine and Freshwater Research*, **64**, 775–791.

Lintermans, M. (2013b) The rise and fall of a translocated population of the endangered Macquarie perch, *Macquaria australasica*, in south-eastern Australia. *Marine and Freshwater Research*, **64**, 838.

Lintermans, M. (2015) Finding the needle in the haystack: comparing sampling methods for detecting an endangered freshwater fish. *Marine and Freshwater Research*, **67**, 1740–1749.

Long, E.O. & Dawid, I.B. (1980) Repeated genes in eukaryotes. *Annual Review of Biochemistry*, **49**, 727–764.

Merz, J. & Setka, J. (2004) Evaluation of a spawning habitat enhancement site for chinook salmon in a regulated California river. *North American Journal of Fisheries Management*, **24**, 397–407.

Miller, D.A.W., Weir, L.A., McClintock, B.T., Campbell, G.E.H., Bailey, L.L. & Simons, T.R. (2012) Experimental investigation of false positive errors in auditory species occurrence surveys. *Ecological Applications*, **22**, 1665–1674.

Minamoto, T., Uchii, K., Takahara, T., Kitayoshi, T., Satsuki, T., Yamanaka, H. & Doi, H. (2016) Nuclear internal transcribed spacer-1 as a sensitive genetic marker for environmental DNA studies in common carp *Cyprinus carpio*. *Molecular Ecology Resources*, doi:10.1111/1755-0998.12586.

Olson, Z.H., Briggler, J.T. & Williams, R.N. (2012) An eDNA approach to detect eastern hellbenders (*Cryptobranchus a. alleganiensis*) using samples of water. *Wildlife Research*, **39**, 629–636.

Pearce, L. (2013) *Macquarie Perch Refuge Project - Final Report for the Lachlan CMA*. http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0008/551690/macquarie-perch-refuge-project.pdf

Piggott, M.P. (2016) Evaluating the effects of laboratory protocols on eDNA detection probability for an endangered freshwater fish. *Ecology and Evolution*, **6**, 2739–2750.

R Development Core Team (2010) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0.

Rose, G.A. (1993) Cod spawning on a migration highway in the north-west Atlantic. *Nature*, **366**, 458–461.

Sigsgaard, E.E., Carl, H., Møller, P.R. & Thomsen, P.F. (2015) Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation*, **183**, 46–52.

Spear, S.F., Groves, J.D., Williams, L.A. & Waits, L.P. (2014) Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. *Biological Conservation*, **183**, 38–45.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H. & Kawabata, Z. (2012) Estimation of fish biomass using environmental DNA. *PLoS ONE*, **7**, e35868.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Møller, P.R., Rasmussen, M. & Willerslev, E. (2012a) Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS ONE*, **7**, e41732.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L. & Willerslev, E. (2012b) Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, **21**, 2565–2573.

Tonkin, Z., Lyon, J. & Pickworth, A. (2010) Spawning behaviour of the endangered Macquarie Perch *Macquaria australasica* in an upland Australian river. *Ecological Management & Restoration*, **11**, 223–226.

Tonkin, Z., Kearns, J., O'Mahony, J. & Mahoney, J. (2015) Spatio-temporal spawning patterns of two riverine populations of the threatened Macquarie perch (*Macquaria australasica*). *Marine and Freshwater Research*, **67**, 1762–1770.

Tsukamoto, K. (2006) Oceanic biology: spawning of eels near a seamount. *Nature*, **439**, 929.

Wei, Q.W., Kynard, B., Yang, D.G., Chen, X.H., Du, H., Shen, L. & Zhang, H. (2009) Using drift nets to capture early life stages and monitor spawning of the Yangtze river Chinese sturgeon (*Acipenser sinensis*). *Journal of Applied Ichthyology*, **25**, 100–106.

Yamamoto, S., Minami, K., Fukaya, K. *et al.* (2016) Environmental DNA as a 'Snapshot' of fish distribution: a case study of Japanese Jack Mackerel in Maizuru Bay, sea of Japan. *PLoS ONE*, **11**, e0149786.

Received 14 October 2016; accepted 8 November 2016
Handling Editor: M. Gilbert

Supporting Information

Details of electronic Supporting Information are provided below.

Appendix S1. Detailed description of the protocol used to develop and test primers.

Appendix S2. Detailed description of the protocol used for the quantitative PCR analyses.

Appendix S3. Graph showing the log transformed environmental DNA concentrations observed in the Negative Control Tank which was run simultaneously with the Experimental Spawning Tanks.