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Searching for a signal: Environmental DNA (eDNA) for the detection of invasive signal crayfish, *Pacifastacus leniusculus* (Dana, 1852)

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Abstract

Environmental DNA (eDNA) is a rapid, non-invasive method for species detection and distribution using DNA deposited in the environment by target organisms. eDNA has become a recognised and powerful tool for detecting invasive species in a broad range of aquatic ecosystems. We examined the use of eDNA as a tool for detecting the invasive American signal crayfish *Pacifastacus leniusculus* in Scotland. Species-specific TaqMan probe and primers were designed for *P. leniusculus* and a robust quantitative PCR (qPCR) assay and DNA extraction protocol were developed. We investigated the detection capability for *P. leniusculus* from water samples in a controlled laboratory experiment and determined whether crayfish density (low = 1 crayfish 5.5 L^{-1} or high = 3 crayfish 5.5 L^{-1}) or length of time in tanks (samples taken at 1, 3 and 7 days) influenced DNA detectability. Additionally, the persistence of DNA was investigated after *P. leniusculus* removal (samples taken at 1, 3 and 7 days post removal). *P. leniusculus* DNA was consistently detected during the entire 7-day period and higher density tanks yielded stronger positive results with lower C_t values. After removal of *P. leniusculus*, there was a rapid and continuous decrease in the detectability of DNA. *P. leniusculus* DNA could only be detected in high density tanks by the end of the 7-day period, while DNA was no longer detectable in low density tanks after 72 hours. Preliminary field experiments sampled water from three sites in winter and five sites in summer. *P. leniusculus* was known to be present at two of these sites. *P. leniusculus* was not detected at any site in winter. However, in summer, positive signals were observed at sites with known *P. leniusculus*, and at sites where *P. leniusculus* was believed to be present anecdotally, but not confirmed. All sites where crayfish were believed to be absent were negative for eDNA. Therefore, eDNA represents a promising technique to detect and monitor invasive *P. leniusculus*, although the presence of detectable amounts of eDNA may be season and location dependent, even where invasive crayfish are known to be present.

Key words: invasive, crustacean, freshwater, qPCR, TaqMan, detection, eDNA

Introduction

Species introduced outside of their native range (alien or non-indigenous species) have the potential to cause irreversible ecological and economic damage (Gherardi et al. 2011), and invasive species are recognised as a significant threat to freshwater biodiversity (Dextrase and Mandrak 2006; Lodge et al. 2000; Sala et al. 2000). Early detection and monitoring of invasive species is key to enabling eradication and preventing spread (Takahara et al. 2013). However, early detection of invasive species after initial introduction is difficult using traditional sampling methods as they inevitably occur at low densities (Keller and Kumschick 2017).

Recently, environmental DNA (eDNA) has emerged as a new tool to detect and monitor invasive species (Jerde et al. 2011; Scriver et al. 2015). eDNA is defined as DNA extracted directly from environmental samples such as soil, sediment or water, without any visual signs of the biological source material from where it came (Thomsen and Willserslev 2015).

Organisms release DNA into the environment through faeces, skin, hair, mucus, urine, gametes, insect exuviae or decomposing individuals (Bohmann et al. 2014; Pedersen et al. 2015; Thomsen and Willserslev 2015). The detection of a target species within aquatic environments using eDNA is rapid and non-invasive (Eichmiller et al. 2014; Goldberg et al. 2015; Sigsgaard et al. 2015). Measurement of eDNA can reduce time and costs associated with traditional sampling methods (Dejean et al. 2012; Evans et al. 2017; Jerde et al. 2011; Sigsgaard et al. 2015; Takahara et al. 2013) and in conjunction with conventional sampling, produce comprehensive biological data for a species (Evans et al. 2017). Furthermore, eDNA allows detection of a target species at any age and of either sex.

Ficetola et al. (2008) were the first to successfully detect the presence of an invasive species, the American bullfrog *Rana catesbeiana* (Shaw, 1802), using eDNA and subsequent studies have successfully applied eDNA to many other taxa (for review see Thomsen and Willersev 2015). The number of targeted single-species studies continues to rise exponentially with focus on rare or invasive macrospecies, which has resulted in large-scale monitoring programmes using eDNA for species such as threatened great crested newt *Triturus cristatus* (Laurenti, 1768) in the UK (Rees et al. 2014; Biggs et al. 2015), and invasive bighead carp *Hypophthalmichthys nobilis* (Richardson, 1845) and silver carp *H. molitrix* (Valenciennes, 1844) in the USA (Farrington et al. 2015).

Detection of aquatic invertebrates may be more difficult than for vertebrates due to the lack of mucous producing structures which are present in fish and amphibians. Instead aquatic arthropods form hard exoskeletons made of chitin, limiting release of extracellular DNA into their environment which may make detection difficult (Cai et al. 2017; Tréguier et al. 2014). However, interest in the use of eDNA for aquatic invertebrates is rapidly increasing, including for several invasive crayfish (Agersnap et al. 2017; Cai et al. 2017; Dougherty et al. 2016; Dunn et al. 2017; Figiel and Bohn 2015; Geerts et al. 2018; Larson et al. 2017; Mauvisseau et al. 2018; Tréguier et al. 2014) and endangered crayfish species (Agersnap et al. 2017; Ikeda et al. 2016).

The American signal crayfish *Pacifastacus leniusculus* (Dana, 1852) is endemic to North America but was introduced to the UK for aquaculture purposes in the 1970's (Holdich et al. 2014), and anecdotal records of introductions to Scotland date back to the 1990's (Freeman et al. 2010), although *P. leniusculus* presence was not officially confirmed until 1995 (Maitland 1996). Within one decade, *P. leniusculus* established populations in eight river catchments and over 58 km of river (Gladman et al. 2009). Thereafter, *P. leniusculus* has spread to more than 20 sites in Scotland, spanning 15 different river catchments (Freeman et al. 2010), and now occupies an estimated 174 km of river (Scottish National Heritage 2015). *P. leniusculus* are known to negatively impact freshwater ecosystems by physically altering habitats (Harvey et al. 2013; Johnson et al. 2011), as well as the community composition (Griffiths et al. 2004; Holdich et al. 2014; Twardochleb et al. 2013). Furthermore, fierce intraspecific competition between *P. leniusculus* and native white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) has led to a rapid decline in native crayfish numbers (Freeman et al. 2010; James et al. 2015). *P. leniusculus* is also a vector for crayfish plague *Aphanomyces astaci* (Schikora, 1906), and *A. pallipes* numbers have been decimated while *P. leniusculus* is unaffected (Alderman et al. 1990; Holdich and Reeve, 1991; Dunn et al. 2009).

New populations of *P. leniusculus* are not only difficult to detect, but can colonise new aquatic environments rapidly. This can make controlling or eradicating *P. leniusculus* populations problematic as well as costly (Gherardi et al. 2011). Traditional methods for detecting and monitoring *P. leniusculus* can be time and labour intensive and are unable to detect populations at low density (Peay 2001), often with large periods of time passing before a population reaches detectable levels after introduction (Hiley 2003). As such, eDNA could prove especially useful for early detection of *P. leniusculus*.

Here, we aim to evaluate the potential of eDNA as a tool for detection of *P. leniusculus*. We developed a species-specific real-time quantitative PCR (qPCR) assay for *P. leniusculus* and investigated persistence of eDNA from this species under controlled laboratory conditions. Preliminary field experiments were also performed at sites with known or anecdotally reported *P. leniusculus* populations and sites where *P. leniusculus* are known to be absent.

Methods

General procedures

All eDNA work was completed in laboratory facilities where crayfish DNA had not been previously handled. DNA extractions were performed in a laboratory dedicated to DNA/RNA extraction, and separated from PCR activities. The workspace was wiped with alcohol wipes before and after use. Reaction mixtures for qPCR assays were prepared in a separate room, which was free of any DNA material. Alcohol wipes

were again used in this room to wipe bench space thoroughly before and after use. Although clean laboratory practices would now include wiping the bench with 50 % bleach solution (Goldberg et al. 2016), we are confident that no contamination occurred using the decontamination procedures outlined. A negative control was extracted alongside every site or tank eDNA sample, and also included in all qPCR plates. No negative control registered a C_t value, confirming no contamination had occurred. qPCR reaction set-up was also prepared in a dedicated space, which had two stations: the first for negative samples and the second for positive. Each station also had separate pipettes and filter pipette tips, as well as being cleaned with DNAZap (Invitrogen) before and after use. Each room had separate equipment including, but not limited to; lab coat, gloves, pipettes, filter pipette tips, sample tubes, sample tube racks and reagents. The qPCR 96 well plates and cover films were kept in the DNA free reaction set-up room.

DNA extraction

For assay development and validation, DNA was extracted from frozen crayfish legs using a standard DNA extraction protocol. Recovered DNA was quantified spectrophotometrically by absorbance at 260 nm.

DNA was extracted from water samples using a Qiagen DNEasy Blood and Tissue kit. Ethanol/sodium acetate treated water samples were centrifuged at 5467 g for 35 minutes at 6 °C (modified from Ficetola et al. 2008), before removing supernatant in one fluid motion taking care not to disturb the pellet. Excess ethanol was blotted away with sterile tissue and 200 µl of ATL Buffer added before vortexing to re-suspend the pellet. This solution was then transferred to a 1.5 ml eppendorf and 20 µl of Proteinase K added and vortex mixed. Samples were incubated at 56 °C for 45 minutes, vortexing occasionally. After incubation, samples were vortexed for a further 15 seconds and 200 µl of Buffer AL added and vortexed to mix. This was followed by the addition of 200 µl of absolute ethanol and vortexing again. This mixture was transferred to a DNeasy column placed in a collection tube and centrifuged at 8000 rpm for 1 minute. The flow through was discarded and the DNeasy column washed again with 500 µl of Buffer AW1 with centrifugation at 8000 rpm for 1 minute. This was repeated with 500 µl of Buffer AW2, but with centrifugation at 14000 rpm for 3 minutes. The DNeasy column was then placed into a fresh, clean and sterile eppendorf and 50 µl of Buffer AE added directly onto the DNeasy membrane. This was incubated at room temperature for 1 minute, before centrifugation at 8000 rpm for 1 minute to collect the purified, concentrated eDNA. Pre-prepared negative controls (15 ml milliQ water) were included and extracted at the same time as each batch of eDNA samples to control for contamination during eDNA preparation. Upon completion of extraction, samples were stored at −20 °C until qPCR could be performed.

Primer design and qPCR

Cytochrome *c* oxidase subunit I (COI) sequences from eight species of crayfish (*A. pallipes*; *Astacus leptodactylus* (Eschscholtz, 1823)*; Astacus astacus* (Linnaeus, 1758)*; Procambarus clarkii* (Girard, 1852)*, Orconectes virilis* (Hagen, 1870)*; Orconectes limosus* (Rafinesque, 1817)*; Procambarus acutus* (Girard, 1852)) with established populations in the wild in the UK (Holdich et al. 2014) and two species of crayfish (*Cherax quadricarinatus* (von Martens, 1868)*; Procambarus fallax* (Hagen, 1870)) not currently known to have populations established in the wild but are present in the aquarium trade in the UK, were identified in GenBank (NCBI). Sequences were aligned using MAFFT (v7.182) and regions were identified where differences existed between species. These regions were used as input to Primer-BLAST (NCBI) with specificity searching for *P. leniusculus* to create the primer pair qPlCOIF (5′-ATAGTTGAA AGAGGAGTGGGTACT-3′) and qPlCOIR (5′-TAA ATCAACAGAAGCCCCTGCA-3′), which amplify an 87 bp fragment of the COI gene. A *P. leniusculus* specific TaqMan probe, SigCrayP1 (FAM-5′-CCTC CTCTAGCAGCGGCTATTGCTCATGC-3′-BHQ1) was designed to bind within this region. These primers and probe were selected from three different probe/primer combinations which met criteria for Taqman design, and which differed by up to 4 bp either side of probe/primer sequences of final target sites. The primer/probe combination which generated the lowest signal after 40 cycles of qPCR of negative controls and the highest positive signal was selected. The best performing primer/probe combination, included a 3 bp overlap of the sense probe and the reverse primer (Supplementary material Table S1). The primer pair and probe were also evaluated for specificity on tissue-derived DNA samples from 10 *P. leniusculus* individuals and 10 of the only other species of crayfish found in Scotland*, A. pallipes* (tissue provided by Moneycarragh Fishfarm, Co Down, Ireland) using conventional PCR.

The limit of detection (LOD) and quantification (LOQ) of the assay were determined under simulated field assay conditions. Serial dilutions of crayfish DNA were added to 15 ml of water from Airthrey

Loch, a site where crayfish are absent. Ethanol precipitation from 15 ml of water was decided upon based on the only other published crayfish eDNA study at the time, Tréguier et al. (2014). These samples were processed in triplicate, and represented eight serial dilutions to give final water sample concentrations of 167 to 0.0000167 pg μl^{-1}. LOQ was defined as the lowest concentration showing a log linear progression from the highest DNA concentration, while LOD was defined as the lowest concentration where all triplicate samples registered a C_t value. All assays were run for 40 cycles and if no C_t value was registered after this time, the sample was considered negative for *P. leniusculus* DNA.

TaqMan qPCR was performed on a Mastercycler ep *realplex* (Ependorf) in a total volume of 10 µl: 5 µl SensiFAST Probe No-ROX (Bioline); 0.4 µM of each primer; 0.1 µM TaqMan probe; 1.1 µl milliQ water; 3 µl DNA template. Cycling parameters included an initial denaturation step at 95 °C for 5 minutes, followed by 40 cycles at 95 °C for 15 seconds, and annealing step of 60 °C for 15 seconds at which point fluorescence was measured. Each qPCR assay had three replicates per water sample, plus three replicates of a positive control containing crayfish DNA and three of the negative control (milliQ water extracted alongside eDNA samples). Three no template controls (NTCs), where 3 µl DNA template was replaced with 3 µl milliQ water in the reaction mixture, were also added to each qPCR well plate. A sample was considered positive where a C_t value was recorded for any replicate. Negative controls did not register C_t values in the TaqMan assays (i.e. no amplification occurred), but a C_t value of 40 was designated as negative in order to facilitate graphical representation of the results.

Laboratory study

Under Scottish National Heritage licence, 12 *P. leniusculus* individuals were collected in November 2014 from Loch Ken (55.043744, -4.1205),located near Castle Douglas in Dumfries and Galloway, Scotland. All 12 *P. leniusculus* individuals (1 female, 11 males) used in this study were similar-sized (mean carapace length = 44.1 ± 1.5 mm; mean weight = 23.4 ± 2.5 g).

Nine Ferplast Geo Medium tanks (30 cm × 20 cm × 20.3 cm) were set-up in a temperature-controlled room of 14 °C under a 12:12 hr photoperiod. Tanks were filled to a volume of 5.5 L using tap water from another location to minimise contamination risk, and an air stone added. Of the nine tanks, three served as a control (0 crayfish), three as "low" density (1-crayfish) and three as "high" density (3-crayfish). The 11 male *P. leniusculus* were randomly allocated to each density category, while the single female *P. leniusculus* was assigned to a low density category. Each density was randomly allocated to one of the nine tanks.

The laboratory eDNA trial lasted a total of 14 days. *P. leniusculus* were kept in the tank a total of seven days. During this time, no shelter was provided and crayfish were not fed. Three 15 ml water samples were taken 1, 3 and 7 days after *P. leniusculus* introduction. On the seventh day, all *P. leniusculus* were removed from the tank and euthanised by freezing. Water samples were then taken 1, 3 and 7 days after *P. leniusculus* removal. Clean gloves and sterile universal centrifuge tubes were used for each tank during each time point in order to minimise possible cross contamination between tanks. Water was taken using individual 15 ml sterile universals from the centre of the water column for each sample within the tank to avoid disturbing the debris on the bottom. Water was then added to pre-prepared universals containing 35 ml absolute ethanol and 1.5 ml 3M sodium acetate solution (pH 5.2). All samples were then stored at −20 °C until DNA extraction could be completed.

Field study

Sampling for *P. leniusculus* eDNA was performed in November 2014 and in July 2016. In November 2014, three sites (Table 1) were selected to be included in a preliminary field eDNA trial. The first site, Airthrey Loch was free of *P. leniusculus*. The second and third sites were selected on the basis of having well established *P. leniusculus* populations. The sites chosen on this basis were Daer Water in the upper reaches of the River Clyde at Elvanfoot and Loch Ken where the *P. leniusculus* population is estimated to be between 1.06 and 9.05 crayfish m^{-2} (Ribbons and Graham 2009). All sites were sampled during late November 2014. In July 2016, five sites (Table 1) were selected for sampling. These were Loch Ken (3 sub-sites; A, B and C,) the River Teith (3 sub-sites; A, B and C), the Row Burn (2 sub-sites; A and B), Airthrey Loch and the Forth & Clyde canal. In addition to Loch Ken, *P. leniusculus* had previously been recorded in the Row Burn, while there are anecdotal reports of *P. leniusuclus* from the River Teith. *P. leniusculus* have not been recorded in Airthrey Loch or in the Forth & Clyde canal.

Tréguier et al. (2014), stated that sediment disturbance could risk leading to the release of "ancient" DNA fragments and lead to false positive results, while Turner et al. (2015) found that the upper 2 cm

Table 1. *P. leniusculus* eDNA detection results of field sampling.

Location	Latitude, N	Longitude, W	Date	+ve field replicates[a]	+ve qPCR replicates[b]	C_t mean, range
Airthrey Loch	56.14717	-3.921895	November 2014	0/3	0/9	–
Loch Ken	**55.043744**	**-4.1205**	**November 2014**	**0/3**	**0/9**	–
Daer Water	**55.433967**	**-3.648208**	**November 2014**	**0/3**	**0/9**	–
River Teith A	56.167294	-4.028683	July 2016	1/6	1/18	34.26, -
RiverTeith B	56.166231	-4.021725	July 2016	2/6	2/18	35.36, 35.05–35.67
River Teith C	56.150886	-3.994386	July 2016	3/6	4/18	34.64, 33.39–36.81
Row BurnA	**56.167431**	**-4.024731**	**July 2016**	**2/6**	**2/18**	**33.60, 33.27–33.92**
Row Burn B	**56.166797**	**-4.022439**	**July 2016**	**2/6**	**2/18**	**34.95, 34.07–35.82**
Airthrey Loch	56.14717	-3.921895	July 2016	0/6	0/18	–
Forth & Clyde Canal	55.934136	-3.478817	July 2016	0/6	0/18	–
Loch Ken A	**55.009**	**-4.056**	**July 2016**	**6/6**	**9/18**	**36.52, 34.73–38.51**
Loch Ken B	**55.007394**	**-4.048908**	**July 2016**	**6/6**	**15/18**	**35.48, 31.19–38.69**
Loch Ken C	**55.010614**	**-4.059175**	**July 2016**	**0/6**	**0/18**	–

a, number of sub-sites where any sample showed a positive signal.
b, total number of positive signals in all qPCR replicates.
Sites where *P. leniusculus* is known to be present are denoted in bold

of sediment contained bigheaded Asian carp *Hypophthalmichthys* spp. eDNA that was 8–1800 times more concentrated than water samples. Therefore, in order to standardise environmental water sampling, a Van Dorn sampler was used at all sites. As *P. leniusculus* is a benthic species, the Van Dorn sampler allowed samples to be taken from the water column as close to the bottom as possible but without disturbing subsurface sediments.

Three (November 2014) or six (July 2016) Van Dorn samples were taken at each site, from which a 15 ml water subsample was taken and added to pre-prepared collection tubes containing 35 ml of ethanol and 1.5 ml 3M sodium acetate solution. Clean gloves and sterile 50 ml polypropylene centrifuge tubes were used at each site to collect the samples, and the Van Dorn sampler was cleaned with a 70 % alcohol solution before and after use to minimise the risk of possible cross contamination between sites. Samples were frozen at −20 °C upon return to the laboratory until DNA extraction could take place.

Statistical analysis

For the laboratory tank experiment, differences among density and replicates were explored separately for the 7-day post *P. leniusculus* introduction and 7-day post *P. leniusculus* removal periods, using a univariate general linear model (GLM). Subsequent post hoc tests were performed using a Bonferroni adjustment to identify significant interactions. Residuals were visually inspected for normality.

All statistical analyses were conducted using SPSS (V 22.0). Values reported are mean ± SE unless otherwise stated. Significance level was defined as $p < 0.05$.

Results

Primer design

The designed qPCR primer pair qPlCO1F /qPlCO1R successfully amplified a 87 bp region of the COI gene for only *P. leniusculus* DNA. As the designed primer pair did not amplify *A. pallipes* DNA, it was concluded that qPlCO1F/qPlCO1R were specific for *P. leniusculus*.

LOD for the TaqMan assay was 0.00167pg μl^{-1} in water samples and LOQ was 0.167pg μl^{-1} (Figure 1).

Laboratory study

P. leniusculus eDNA was detected in both 1-crayfish and 3-crayfish density tanks for the full 7-day period after *P. leniusculus* introduction. Results from the 3-crayfish density tanks yielded a much stronger positive value than those obtained from the 1-crayfish density tanks (Figure 2). During the entire post *P. leniusculus* removal period, eDNA could still be detected in the 3-crayfish density tanks. However, *P. leniusculus* eDNA was only detected in a single 1-crayfish density tank during the 1-day and 3-day post *P. leniusculus* removal sampling periods. *P. leniusculus* eDNA was no longer detectable in 1-crayfish density tanks by the final 7-day post *P. leniusculus* removal sampling period (Figure 2). There were also positive signals from 1 replicate of each 0-crayfish tank in the initial sampling points. As the parallel extracted negative control samples did not produce C_t values, it is concluded these positives are likely a result of contamination during tank set-up or sampling.

For the 7-day post *P. leniusculus* introduction period, density was found to be significant

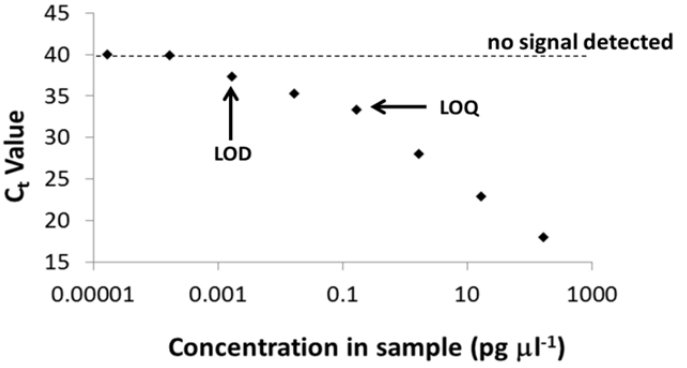

Figure 1. Limit of detection (LOD) and limit of quantification (LOQ) of assay. Serial dilutions of *P. leniusculus* DNA were spiked into loch water and extracted as described in text, before being subjected to TaqMan probe qPCR assay. Points on graph represent means of triplicates. "No signal detected" signifies that the assay did not produce a C_t value after 40 cycles, i.e. there was no amplification of *P. leniusculus* DNA.

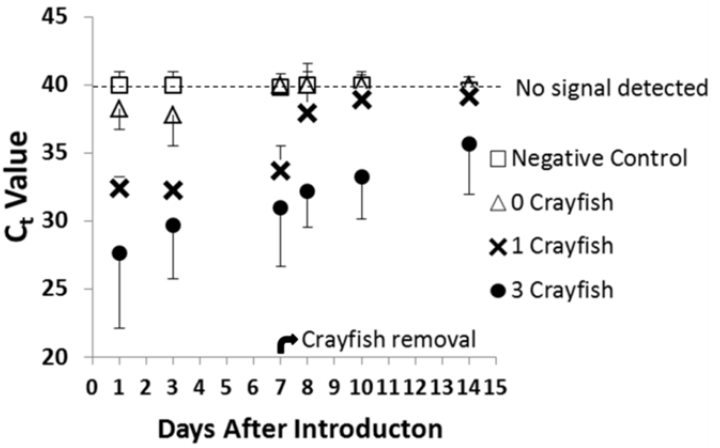

Figure 2. Mean (± SD) C_t value obtained for each tank (0-, 1- or 3-crayfish density) sampled, as well as each negative control processed at each of the three time points within the 7-day post *P. leniusculus* introduction period and the 7-day post *P. leniusculus* removal period. "No signal detected" signifies that the assay did not produce a C_t value after 40 cycles, i.e. there was no amplification of *P. leniusculus* DNA.

($F_{2,4} = 52.043$, $p < 0.005$, $n^2 = .963$) while replicates were not ($F_{2,4} = 1.422$, $p = 0.342$, $n^2 = .416$). Overall, lower mean C_t values were observed in tanks with 3-crayfish density (23.86) than 1-crayfish density (30.16) and 0-crayfish (36.05), indicating a stronger positive signal for tanks with more *P. leniusculus* present. There was no significant interaction between density and replicates. Bonferroni post hoc tests revealed that all three densities were significantly different from one another (all p values < 0.005; 0-crayfish 36.05 ± 0.77, 1-crayfish 30.16 ± 0.77, 3-crayfish 23.86 ± 0.77). For the 7-day post *P. leniusculus* removal period, there was once again a significant difference between densities ($F_{2,4} = 28.054$, $p < 0.005$, $n^2 = .933$) but no significant difference between replicates ($F_{2,4} = 2.352$, $p = 0.211$, $n^2 = .540$). However, there was a significant interaction between *P. leniusculus* density and replicates ($F_{4,18} = 4.258$, $p < 0.005$, $n^2 = .486$). The Bonferroni post hoc test revealed that all densities were significantly different from one another (all p-values < 0.05; 0-crayfish 37.26 ± 0.43, 1-crayfish 35.42 ± 0.43, 3-crayfish 28.34 ± 0.43). For tanks containing 3 crayfish, the mean C_t value for replicates 1 (31.23 ± 0.75) and 3 (26.32 ± 0.75) were significantly different ($p < 0.001$) from one another during the final sampling time point (7-days post *P. leniusculus* removal).

It is important to note that the amount of *P. leniusculus* eDNA detected continually decreased from 1-day after *P. leniusculus* introduction through to 7-days after *P. leniusculus* introduction. This finding was unusual as it was expected that the amount of *P. leniusculus* eDNA present during the 7-day introduction period would increase as time passed, or at the very least level off. This trend continued after *P. leniusculus* were removed, as expected.

Field study

A positive result for *P. leniusculus* DNA presence was defined as a recorded C_t value for any replicate and for any sample from a particular location, conditional on all parallel processed negative controls (distilled water) showing no C_t on the same qPCR assay plate. Not all positive samples, as defined above, exhibited positive signals in all qPCR sample triplicates. These results are summarised in

Table 1. *P. leniusculus* eDNA was not detected at any of the three sites sampled during November, even from Loch Ken where crayfish were known to be present and abundant. In contrast, samples taken in July indicated positive results from Loch Ken, although not at all sub-sites sampled at this location. In addition, positive signals were recorded in the River Teith system where *P. leniusculus* have been anecdotally reported, and in the Row Burn, a tributary of River Teith, where *P. leniusculus* have previously been confirmed. Airthrey Loch and the Forth & Clyde canal, where *P. leniusculus* have never been observed, samples were all negative. Negative controls all failed to produce C_t values, indicating no contamination occurred during the DNA extraction process. Positive controls were also included in each plate and consisted of qPCR reactions spiked with *P. leniusculus* DNA. These samples registered consistent positive C_t values in all assay plates, therefore serving to confirm that the qPCR assay was working as expected.

Discussion

We have developed a reliable and specific assay for *P. leniusculus* eDNA in water samples. It should be noted that the primers used in this study were only validated on *P. leniusculus* obtained from one population in Loch Ken, and sequenced COI PCR products obtained from the global primer pair (HCO 2198/LCO 1490) (Folmer et al. 1994) from three individuals within this population showed some variation in COI gene sequence (Table S1). One individual had a mismatch of a single base pair within both the forward primer (qPlCO1F) and the probe. The base pair mismatches in this *P. leniusculus* individual were located close to the 5′ end on both the primer and the probe. It is possible that this would not impact the specificity of the primers and probe used in this study, although this is unknown. *P. leniusculus* is a highly genetically diverse species, and was recently revealed to be a species complex comprising of multiple cryptic lineages (Larson et al. 2012; Larson et al. 2016). As such, the primers presented in this paper may not work for all cryptic *P. leniusculus* lineages, and therefore before conducting further eDNA studies it would be advisable to test the primers and probe on other available populations of *P. leniusculus* in Scotland and elsewhere. Based on the limited database information in Table S1, there may be evidence of less diversity in introduced European populations. Agersnap et al. (2017) and Dunn et al. (2017) designed and used primers, and in the case of Agersnap et al. (2017) a probe, in similar positions on the COI gene as reported in this study. A recent survey (Petrusek et al. 2017) of crayfish COI sequences from isolates across Europe also showed no variation in this probe region, despite indicating some haplotype diversity elsewhere in the COI gene. This would suggest that modifications of the reported primer pair and probe using the COI sequence alignment of *P. leniusculus* from varying geographical locations (Table S1) would account for issues detecting cryptic lineages using the eDNA assay, although in its current form it would be specific for the vast majority of European populations. The assay was also tested on *A. pallipes* (native crayfish) and *Salmo salar* (Linnaeus, 1758) DNA and no amplification of these non-target species was found, further indicating specificity.

Although development of a sensitive qPCR assay in the laboratory has been demonstrated on numerous occasions, there are still many issues associated with the reliable detection of species using this method. These include defining cut-offs for C_t values being used to define a positive, the number of positive replicates within a plate being used to distinguish a true positive from background noise, the treatment of negative replicates, and the optimum standardised number of replicates to be used within a study – currently anywhere between three and twelve or more (Sigsgaard et al. 2015; Thomsen and Willersev 2015). Additionally, models to estimate occurrence and detection probabilities can be incorporated into the design of an eDNA monitoring program to account for some of these issues (Dorazio and Erickson 2017; Hunter et al. 2015). In this case we opted for a TaqMan probe assay, having found SYBR green based assays to be unsuitable and prone to numerous issues with false priming and positives. The advantage of the TaqMan assay, and no doubt other dual label hydrolysis probe assays, is that it is sensitive and has little or no background interference. In our hands, samples with no *P. leniusculus* DNA generated no signal after 40 cycles of qPCR, whereas samples with target DNA of more than 0.00167pg μl^{-1} reliably generated a signal in laboratory validation.

The persistence of detectable concentrations of eDNA is important in understanding how eDNA analysis can effectively be utilised for a target species, whether that is simply determining presence/absence or estimating abundance/biomass. This study demonstrated that the persistence of *P. leniusculus* eDNA is influenced by time and the number of individuals present. When *P. leniusculus* were present, eDNA was continually detected over the course of the 7-day period for all 3-crayfish density tanks. For the 1-crayfish density tanks, eDNA was detected for the entire 7-day period except for one tank on the seventh day. However, despite *P. leniusculus* presence within the

tanks, eDNA was observed to decrease as time passed. The concentration of eDNA was lower in all instances on the seventh day compared to the first. This observation was unusual as it was expected that the longer *P. leniusculus* were in the tanks, the greater the concentration of eDNA.

Thomsen et al. (2012) conducted a similar experiment with varying densities (0, 1, 2, or 4) of common spadefoot toad *Pelobates fuscus* (Laurenti, 1768) and great crested newt *T. cristatus* larvae. While individuals were present, a significant effect of density and time on eDNA concentration was observed where a greater number of individuals resulted in a greater concentration of eDNA, similar to this study. However in contrast to this study, Thomsen et al. (2012) also observed increasing concentrations of eDNA over the period of time when individuals were present, as well as reporting that *P. fuscus* had consistently higher concentrations of eDNA than *T. cristatus*. The authors suggest this may be due *P. fuscus* larvae being substantially larger and more active than *T. cristatus*. This may explain the decreasing concentrations of eDNA throughout this study as *P. leniusculus* were very inactive during the 7-day period, which may have resulted in eDNA not being released to any great extent. The inactivity of *P. leniusculus* is in direct contrast with the findings of Figiel and Bohn (2015), who investigated the use of eDNA as a method of detection for the crayfish *Procambarus zonangulus* (Hobbs, 1990) under laboratory conditions. The authors observed that *P. zonangulus* were active during the entirety of their 15-day experiment, regardless of the time of day or the presence of conspecifics. Moreover, Cai et al. (2017) report that food availability can influence eDNA excretion rates. In the various eDNA aquarium studies conducted by Figiel and Bohn (2015), Dunn et al. (2017), Cai et al. (2017) crayfish were fed during the experiment. In our study and the one by Mauvisseau et al. (2018) investigating eDNA persistence, crayfish were maintained without food. It is therefore possible that by not providing a source of food, crayfish were not excreting as much DNA as they might have been if feeding behaviours were permitted. Therefore, feeding rate might be a major determinant of DNA excretion in crayfish and further study investigating the relationship between availability of food and the concentration of eDNA released is needed. Furthermore, Dunn et al. (2017) report variation in the concentration of eDNA between tanks, with male *P. leniusculus* releasing less eDNA than ovigerous females suggesting that non-ovigerous mature crayfish are not releasing DNA at a constant rate. In this study, the single female *P. leniusculus* was assigned to a 1-crayfish density tank. This was to avoid any potential mating behaviour which could increase the amount of DNA excreted and skew the results.

After *P. leniusculus* removal, there was a rapid and continuous decrease in eDNA detection. By 7-days post *P. leniusculus* removal, eDNA could only be detected in two of the 3-crayfish density tanks. Our study is one of only two to investigate the persistence of *P. leniusculus* eDNA in a controlled experiment. Mauvisseau et al. (2018) investigated the persistence of *P. clarkii, O. limosus* and *P. leniusculus* eDNA over a period of 34 days after crayfish removal. In contrast to our study, Mauvisseau et al. (2018) did not detect *P. leniusculus* during the period after removal until homogenisation of the tank water before the final water collection. Although it is unclear as to why *P. leniusculus* eDNA was not detected prior to the final day, this suggests that *P. leniusculus* eDNA may persist longer than 1–2 weeks as found in other freshwater eDNA studies (Dejean et al. 2011; Piaggio et al. 2014; Thomsen et al. 2012). Consequently, given the results of our study, and those of Mauvisseau et al. (2018), greater understanding of eDNA degradation rates for *P. leniusculus* is needed before eDNA can be used for large-scale presence/absence monitoring of *P. leniusculus*.

Our own study would indicate that many environmental samples, even from locations with high crayfish abundances, can have low eDNA concentrations. This would indicate that eDNA is better suited to determining contemporary presence/absence of *P. leniusculus* rather than estimating abundance. For example, Tréguier et al. (2014) found eDNA to only detect *P. clarkii* in 59 % of ponds where trapping confirmed presence. Additionally, both Larson et al. (2017) and Dougherty et al. (2016) found eDNA concentrations to not be correlated with crayfish abundance. However, although Agersnap et al. (2017) agree that eDNA is sensitive enough for detection of crayfish but not for quantification, the authors do report that in their study there is a clear relationship between high eDNA concentrations at sites with high densities of *P. leniusculus* and the converse to be true for low density sites. Given the differing results between this study, Larson et al. (2017), Dougherty et al. (2016) and those presented by Agersnap et al. (2017), it is clear further work is required before eDNA concentrations can be correlated to abundance of a crayfish species such as *P. leniusculus*.

Given the lack of signal in all negative control results, we chose to define any replicate, whether field or technical qPCR replicate, which generated a signal (i.e. recorded a C_t value before 40 cycles) as a positive identification of *P. leniusculus* within a water body. It has been suggested that some of the

standard detection thresholds defined in literature may need relaxing for eDNA studies (Thomsen and Willersev 2015). Additionally, Thomsen and Willersev (2015) suggest that if high C_t values are used to define detection, sequencing of the resultant product is crucial for confirmation of detection of target species. This was not done for either the aquaria or field trials in this study. Doing so may have improved the reliability of the positive results obtained in the aquaria trials and given a better indication of eDNA persistence for *P. leniusculus*. However, as the positive and negative controls in this study did not return unusual results, it can be concluded that the laboratory protocols employed were robust and no contamination occurred.

It is also important to consider the amount of template used in a qPCR assay to avoid inhibition, and yet still retain detectable amounts of DNA (Sigsgaard et al. 2015). For example, Takahara et al. (2015) found that reducing the template volume from 5 μl to 2 μl, increased detection probability for the common carp *Cyprinius carpio* (Linnaeus, 1758) when using qPCR. The authors surmised that this could be due to increased inhibition encountered when using larger template volumes. Furthermore, Biggs et al. (2015) demonstrated a detection rate of only one in twelve replicates for some samples, which indicates very low concentrations of target DNA. This study had only three technical replicates per sample and therefore any results, negative or positive, may not be reliable. Our procedure also used a relatively simple sampling protocol consisting of three or six sample replicates per site of 15 ml each. This method has the advantage of ease of performance, simple equipment requirements, and as the samples are added directly into ethanol, minimises post-sampling eDNA breakdown. The reported success rate for detection of eDNA has been high (> 80 %) using only three 15 ml water samples (Dejean et al. 2012; Ficetola et al. 2008; Foote et al. 2012; Thomsen et al. 2012). However, the assay could be made more sensitive by sampling larger volumes and/or more replicates. Existing crayfish eDNA studies use varying eDNA sampling methodologies. Mauvisseau et al. (2018), Dunn et al. (2017), Tréguier et al. (2014) and this study use modified ethanol precipitation methods, while Larson et al. (2017), Dougherty et al. (2016), Agersnap et al. (2017), Ikeda et al. (2016), Cai et al. (2017) and Figiel and Bohn (2015) used filtration methods. Additionally, Geerts et al. (2018) recently investigated different eDNA sampling and extraction methods for *P. clarkii* and found results to vary based on primer choice and DNA extraction method. As a result, it is clear that there is a need for a more standardised protocol for using eDNA to detect crayfish.

Furthermore, as discussed above, the main criticism of the ethanol precipitation method is that the volume of water sampled is relatively low and unless a species is found at high densities, and is consequently releasing high quantities of eDNA into the environment, detection by eDNA is unlikely. Also, if an organism does not have a high level of mobility, the area in which eDNA is present is limited. Therefore, if water samples are not taken in close proximity to an organism, the probability of detection by eDNA is low. Based on this reasoning, more samples should have been taken at each site during November to increase the probability of detecting *P. leniusculus* eDNA. However, if moulting is indeed a mechanism for DNA release in aquatic arthropods (Tréguier et al. 2014), such as *P. leniusculus*, then time of year should also be taken into account in eDNA studies. The moulting season for *P. leniusculus* occurs between July and September inclusive (Reeve 2004) and during November it would be likely that crayfish were releasing relatively low amounts of DNA. Moreover, *P. leniusculus* activity varies with season. During winter months *P. leniusculus* spends the majority of time in torpor (a period of inactivity), often in refuges (Peay 2000). Consequently *P. leniusculus* were not very mobile during the November sampling period in this study and DNA is likely to be restricted to certain locations, which may also have further decreased the probability of detection. This reasoning is supported by the work of de Souza et al. (2016) who found that seasonality does indeed have a strong effect on eDNA detection. The authors found that eDNA detection can be directly correlated with seasons of peak activity and breeding.

Spear et al. (2015) suggest that where temporal differences may influence detection probabilities, researchers should conduct pilot studies over several months to determine the optimal sampling period. Sampling in July from the same site (Loch Ken) as in November generated a large number of positive sample results. We suggest that this is due to greater crayfish activity, moulting, and possibly lower water flow/level. In fact, Dunn et al. (2017) found that egg bearing females significantly increased the amount of eDNA present in aquaria conditions. However, even in July, one site at Loch Ken still failed to generate positives. Although sampling error may account for the differences observed, it is also possible that there may be locations within water bodies at which target eDNA may be absent, possibly due to variations in hydrography or unsuitable habitat for *P. leniusculus*. Given that positive and negative locations within Loch Ken in July were not far apart (approx. 1–2 km), this would indicate that eDNA may break down rapidly, as observed in some laboratory tank

experiments. While this may be problematic in terms of false negatives, particularly seasonally, short persistence means eDNA carried in moving water is less likely to cause "false" positives in non-crayfish bearing areas. Consequently, future research should focus on the mechanisms of DNA release by aquatic arthropods as well as determining how temporal differences affect eDNA detection rates for *P. leniusculus*.

Conclusion

This study has demonstrated the detection of *P. leniusculus* by eDNA in both riverine and lake systems. The assay described here is sensitive, specific and should be applicable to most, if not all, European populations of *P. leniusculus*. Positive results were obtained from water bodies with known high *P. leniusculus* abundance, as well as riverine environments with probable low abundances. Additionally, eDNA detected *P. leniusculus* in water bodies where presence was suspected but trapping has failed to confirm.

However, it was apparent that the assay and procedure used in this study were performing at limits of detection, even in areas of high abundance, and in these areas, not all sub-locations were positive for *P. leniusculus* eDNA. Furthermore, time of year could have a large effect on detection frequency. While some of these sensitivity issues might be addressed by sampling of larger water volumes, a greater understanding of temporal and spatial eDNA shedding and persistence, as well as models to estimate detection and occurrence probabilities, are required before using eDNA as a tool for screening large numbers of aquatic systems in Scotland for invasive *P. leniusculus*.

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Supplementary material

The following supplementary material is available for this article:

Table S1. The *P. leniusculus* COI target region for TaqMan Assay.

This material is available as part of online article from:
http://www.reabic.net/journals/mbi/2018/Supplements/MBI_2018_Harper_etal_Table_S1.pdf