Development and application of environmental DNA surveillance for the threatened crucian carp (*Carassius carassius*)

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Abstract

1. The crucian carp (*Carassius carassius*) is one of few fish species associated with small ponds in the UK. These populations contain genetic diversity not found in Europe and are important to conservation efforts for the species, which has declined across its range in Europe. Detection and monitoring of extant crucian carp populations are crucial for conservation success. Environmental DNA (eDNA) analysis could be very useful in this respect as a rapid, cost-efficient monitoring tool.

2. We developed a species-specific quantitative PCR (qPCR) assay for eDNA surveillance of crucian carp to enable non-invasive, large-scale distribution monitoring. We compared fyke netting and eDNA at ponds with (N = 10) and without (N = 10) crucian carp for presence-absence detection. We examined biotic (crucian carp density represented by catch-per-unit-effort estimate - CPUE) and abiotic influences on eDNA detection probability using a hierarchical occupancy model, and eDNA quantification using a mixed-effects model.

3. eDNA analysis achieved 90% detection for crucian carp (N = 10), failing in only one pond where presence was known. CPUE estimate and conductivity had positive and negative influences on eDNA detection probability in qPCR replicates respectively. Similarly, conductivity had a negative effect on DNA copy number, whereas copy number increased with CPUE estimate.

4. Our results demonstrate that eDNA could enable detection of crucian carp populations in ponds and benefit ongoing conservation efforts, but imperfect species detection in relation to biotic and abiotic factors and eDNA workflow requires further investigation. Nonetheless, we have established an eDNA framework for crucian carp as well as sources of imperfect detection which future investigations can build upon.

1. Introduction

The crucian carp (*Carassius carassius*) (Figure 1) is an elusive, benthic fish species popular with anglers (Copp, Warrington & Wesley, 2008b; Sayer et al., 2011). As one of few fish associated with small ponds, this species may have an important ecological role but its relationship with other lentic biodiversity is understudied (Copp & Sayer, 2010; Stefanoudis et al., 2017). Although listed as 'Least Concern' on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, the species has declined throughout its native range of Northwest and Central Europe (Copp et al., 2008b; Sayer et al., 2011), with local extinctions across the UK (Copp & Sayer, 2010). The county of Norfolk in eastern England was believed to hold abundant and widely distributed crucian carp populations, but research indicates heavy (~75%) declines in this region (Sayer et al., 2011). Declines of the crucian carp throughout its range are due to habitat loss (Copp et al., 2008b; Sayer et al., 2011), species displacement by the invasive gibel carp (*Carassius gibelio*) (Copp et al., 2008b; Tarkan et al., 2009; Sayer et al., 2011), and genetic introgression through hybridisation (Hänfling et al., 2005). Indeed, Sayer et al. (2011) observed only 50% of crucian carp ponds to be uninhabited by goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*), or their hybrids with crucian carp.

Prior to the 1970s, crucian carp were thought to have been introduced to the UK alongside common carp and were classed as non-native (Maitland, 1972). Wheeler (1977) deemed the species native to southeast England based on archaeological evidence and a historic distribution that mirrored native cyprinids. Conservation organisations (e.g. English Nature, Environment Agency) later recognised the crucian carp as native and threatened (Smith & Moss, 1994; Environment Agency, 2003), but recent genetic evidence supports anthropogenic introduction of the crucian carp to the UK during the 15th century (Jeffries et

al., 2017). Nonetheless, many introduced species in the UK are now naturalised, and several provide ecological and economical benefits (Manchester & Bullock, 2000). Evidence suggests that the crucian carp is characteristic of small, plant-dominated, high-quality ponds (Copp et al., 2008b; Sayer et al., 2011; Stefanoudis et al., 2017), and English populations contain a substantial proportion of the overall genetic diversity for the species across Europe. English crucian carp populations may buffer species displacement by gibel carp at the European level (Jeffries et al., 2017), but are threatened by hybridisation with goldfish and possible displacement (Hänfling et al., 2005; Tarkan et al., 2009) as well as anthropogenic activity (Copp, Černý & Kováč, 2008a).

In 2010, the crucian carp was designated as a Biodiversity Action Plan (BAP) species in the county of Norfolk (Copp & Sayer, 2010; Sayer et al., 2011). To meet the BAP aims, local conservation efforts have included species reintroduction, pond restoration, and eradication of goldfish (Sayer et al., 2011). However, current distribution records are unreliable as individuals are frequently misidentified as the feral brown variety of goldfish due to high physical similarity (Copp et al., 2008a; Tarkan et al., 2009), and many pond populations are mixtures of true crucian carp and crucian carp x goldfish hybrids (Hänfling et al., 2005). Consequently, distribution maps have been called into question and further monitoring is needed to ensure long-term success of established and reintroduced crucian carp populations (Copp et al., 2008a; Tarkan et al., 2009).

Primarily, crucian carp are surveyed using fyke netting or electrofishing, but these methods can be costly and time-consuming. Environmental DNA (eDNA) analysis offers a potentially rapid and cost-effective approach to fish monitoring (Jerde et al., 2011; Sigsgaard et al., 2015; Wilcox et al., 2016; Hänfling et al., 2016; Hinlo et al., 2017a). Species are identified using DNA deposited in the environment by individuals via secretions, excretions, gametes, blood, or decomposition (Lawson Handley, 2015). eDNA has been applied

worldwide to survey for invasive freshwater fish (Jerde et al., 2011; Keskin, 2014; Robson et al., 2016; Hinlo et al., 2017a), and is now used routinely to monitor Asian carp (*Hypophthalmichthys* spp.) invasion in the Great Lakes, USA (Farrington et al., 2015). A quantitative PCR (qPCR) assay targeting crucian carp was also published in the context of early warning invasion monitoring for fish species that may arrive in Canada (Roy et al., 2017), but was only tested on tissue-derived DNA. Of equal importance to invasion monitoring, eDNA analysis has enhanced surveys for threatened and endangered freshwater fish (Sigsgaard et al., 2015; Schmelzle & Kinziger, 2016; Piggott, 2016; Bylemans et al., 2017).

eDNA analysis has been conducted with conventional PCR (PCR) (Ficetola et al., 2008; Jerde et al., 2011), but qPCR and droplet digital PCR (ddPCR) are suggested to perform better, suffer less from inhibition, and enable abundance or biomass estimation (Nathan et al., 2014). However, these estimates can be inconsistent across habitats and target organisms. In flowing water, Hinlo et al. (2017a) found no relationship between DNA copy number and conventional density estimates of common carp, yet Takahara et al. (2012) observed a positive association between common carp biomass and eDNA concentration in ponds. Environmental variables play a substantial role in abundance/biomass estimation by influencing the ecology of eDNA (Barnes et al., 2014). Variables examined have included temperature, pH, salinity, conductivity, anoxia, sediment type, and UV light (Takahara et al., 2012; Barnes et al., 2014; Pilliod et al., 2014; Keskin, 2014; Strickler, Fremier & Goldberg, 2015; Robson et al., 2016; Buxton et al., 2017b; Buxton, Groombridge & Griffiths, 2017a; Weltz et al., 2017; Stoeckle et al., 2017; Goldberg, Strickler & Fremier, 2018). However, these variables are not always measured and only a handful of studies have assessed their effects in ponds (Takahara et al., 2012; Buxton et al., 2017a, b; Goldberg et al., 2018).

In this study, we developed a species-specific qPCR assay for the threatened crucian

carp. We evaluated presence-absence detection with eDNA compared to fyke netting, and investigated the influence of biotic and abiotic factors on eDNA detection and quantification. We hypothesised that: (1) eDNA and fyke netting would provide comparable presence-absence records for crucian carp, and (2) eDNA detection and quantification would be influenced by crucian carp density, temperature, pH, conductivity, surface dissolved oxygen, macrophyte cover, and tree shading. We provide an eDNA framework for crucian carp monitoring which holds promise for routine survey.

2. Methods

2.1 Study sites

We studied 10 ponds with confirmed crucian carp presence at different densities and 10 fishless ponds in Norfolk (Figure 2). This region is low-lying (<100 m above sea level) and mainly agricultural. All study ponds were selected to be small (<40 m in max. dimension), shallow (<2 m), macrophyte-dominated, with a largely open-canopy and thus minimal shading of the water surface. Ponds were largely surrounded by arable fields, excluding one located in woodland. No specific permits were required for sampling but relevant landowner permissions were obtained.

2.2. Conventional survey

Crucian carp presence-absence was confirmed at each pond by fyke netting between 2010 and 2016. Bar two ponds surveyed in 2013 and 2015, all crucian carp ponds were last surveyed in 2016. Where possible, double-ended fyke nets were set perpendicular to the bank or to beds of aquatic vegetation and exposed overnight (for c. 16 h), with the number of fyke nets set being proportional to pond size. This provided CPUE estimates of relative densities, which are the number of fish captured per fyke net per 16 h exposure. Environmental data were collected between May and August from 2010 to 2017. Conductivity, pH, surface dissolved oxygen, and water temperature were measured with a HACH HQ30d meter (Hach Company, CO, USA), and alkalinity was determined by sulphuric-acid titration using a HACH AL-DT kit (Hach Company, CO, USA). Percentages of macrophyte cover and shading of ponds by trees and scrub were estimated visually.

2.3 eDNA sampling, capture and extraction

Five 2 L surface water samples were collected from the shoreline of each pond using sterile Gosselin™ HDPE plastic bottles (Fisher Scientific UK Ltd, UK) and disposable gloves. Samples were taken at equidistant points around the pond perimeter where access permitted. All ponds without crucian carp were sampled on 22nd August 2016. Water samples were transported on ice in sterile coolboxes to the Centre for Ecology and Hydrology (CEH), Wallingford, stored at 4 °C, and vacuum-filtered within 24 hours of collection. Coolboxes were sterilised using 10% v/v chlorine-based commercial bleach (Elliott Hygiene Ltd, UK) solution and 70% v/v ethanol solution before ponds containing crucian carp were sampled on 25th August 2016. Samples were handled in the same way as those from fishless ponds. For

each pond, a full process blank (1 L molecular grade water) was taken into the field and stored in coolboxes with samples. Blanks were filtered and extracted alongside pond samples to identify contamination.

Where possible, the full 2 L of each sample was vacuum-filtered through sterile 0.45 μm cellulose nitrate membrane filters with pads (47 mm diameter; Whatman, GE Healthcare, UK) using Nalgene filtration units. One hour was allowed for each sample to filter but if filters clogged during this time, a second filter was used. After 2 L had been filtered or one hour had passed, filters were removed from pads using sterile tweezers and placed in sterile 47 mm petri dishes (Fisher Scientific UK Ltd, UK), sealed with parafilm (Sigma-Aldrich®, UK), and stored at -20 °C. The total volume of water filtered and the number of filters used per sample were recorded for downstream analysis (Table S1). After each round of filtration (samples and blanks from two ponds), all equipment was sterilised in 10% v/v chlorine-based commercial bleach (Elliott Hygiene Ltd, UK) solution for 10 minutes, immersed in 5% v/v MicroSol detergent (Anachem, UK), and rinsed with purified water.

All filters were transported on ice in a sterile coolbox to the University of Hull and stored at -20 °C until DNA extraction one week later. DNA was isolated from filters using the PowerWater® DNA Isolation Kit (MO BIO Laboratories, CA, USA) following the manufacturer's protocol in a dedicated eDNA facility at the University of Hull, devoted to pre-PCR processes with separate rooms for filtration, DNA extraction, and PCR preparation of environmental samples. Duplicate filters from the same sample were co-extracted by placing both filters in a single tube for bead milling. Eluted DNA (100 μL) concentration was quantified on a Qubit™ 3.0 fluorometer using a Qubit™ dsDNA HS Assay Kit (Invitrogen, UK). DNA extracts were stored at -20 °C until further analysis.

2.4 Assay design, specificity and sensitivity

We designed a novel qPCR assay to target a 118 bp amplicon (73 bp excluding primers) within the mitochondrial cytochrome *b* (*cytb*) gene, specific to crucian carp. Crucian carp sequences from Jeffries et al. (2016) were aligned using MAFFT in AliView (Larsson, 2014) to sequences downloaded from the NCBI nucleotide (nt) database for 23 closely related species of European freshwater fish (Table S2), and a consensus sequence for each species was identified (Figure 3). Sequences were visually compared to maximise nucleotide mismatches between crucian carp and non-target species, particularly goldfish and common carp, and minimise theoretical risk of non-specific amplification. Mismatches in primer regions were maximised over the probe region to increase specificity (Wilcox et al., 2013). Species-specific primers CruCarp_CytB_984F (5'-AGTTGCAGATATGGCTATCTTAA-3') and CruCarp_CytB_1101R (5'-TGGAAAGAGGACAAGGAATAAT-3'), and corresponding probe CruCarp_CytB_1008Probe (FAM 5'-ATGGATTGGAGGCATACCAGTAGAACACC-3' BHQ1) were selected on this basis.

Primers without probe were tested *in silico* using ecoPCR (Ficetola et al., 2010) against a custom, phylogenetically curated reference database that was constructed for eDNA metabarcoding of lake fish communities in Windermere, Lake District National Park, England, which contains 67 freshwater fish species confirmed or potentially present in the UK (Hänfling et al., 2016). Parameters set allowed a 50-150 bp fragment and maximum of three mismatches between each primer and each sequence in the reference database. Specificity of primers (without probe) was also tested against the full NCBI nucleotide (nt) database using Primer-BLAST (Ye et al., 2012) with default settings.

The primers were tested with PCR, following which primer and probe concentrations, standard curve preparation, and cycling conditions for qPCR were optimised (Supporting

Information: Appendix 1). All subsequent qPCR analyses were performed using the conditions detailed in section 2.5. Primers and probe were validated *in vitro* using tissue DNA (standardised to 1 ng/µL) from fin clips of 10 non-target species (1 UK individual per species) related to crucian carp (Table S3, Figures S1-3). The positive control and No Template Control (NTC) were crucian carp DNA and molecular grade water (Fisher Scientific UK Ltd, UK) respectively. The limits of detection (LOD, the lowest concentration where at least one technical replicate amplified crucian carp DNA) and quantification (LOQ, the concentration at which all technical replicates consistently amplified crucian carp DNA) (Agersnap et al., 2017) were established using the qPCR standards (10^6 to 1 copy/µL) (Figure S4). Five technical replicates were performed for standards, controls, and samples in tests of assay specificity and sensitivity.

2.5 Detection and quantification of crucian carp eDNA

All qPCR reactions were prepared in a UV and bleach (Elliott Hygiene Ltd, UK) sterilised laminar flow hood in the dedicated eDNA facility at the University of Hull. Reactions were performed in a total volume of 20 µL, consisting of 2 µL of template DNA, 1 µL of each primer (Forward 900 nM, Reverse 600 nM), 1 µL of probe (125 nM) (Integrated DNA Technologies, Belgium), 10 µL of TaqMan® Environmental Master Mix 2.0 (Life Technologies, CA, USA), and 5 µL molecular grade water (Fisher Scientific UK Ltd, UK). Once eDNA samples and three NTCs were added to each 96-well plate, the plate was sealed and transported to a separate laboratory on a different floor for addition of the standard curve and three positive controls (crucian carp DNA, 0.01 ng/µL) in a UV and bleach sterilised laminar flow hood.

249 Our standard curve was a synthesised 500 bp gBlocks® Gene Fragment (Integrated
250 DNA Technologies, Belgium) based on GenBank accessions (KT630374 - KT630380) for
251 crucian carp from Norfolk (Jeffries et al., 2016). Copy number for the gBlocks® fragment
252 was estimated by multiplying Avogadro's number by the number of moles. We performed a
253 10-fold serial dilution of the gBlocks® fragment to generate a 6-point standard curve that
254 ranged from 10^6 to 10 copies/µL. eDNA samples were compared to these known
255 concentrations for quantification (Hinlo et al., 2017a). Each standard was replicated five
256 times on each qPCR plate. Similarly, five technical replicates were performed for every
257 sample and full process blank from each pond.
258 After addition of standards and positive controls, plates were again sealed and
259 transported to a separate laboratory on a different floor where qPCR was conducted on a
260 StepOnePlus™ Real-Time PCR system (Life Technologies, CA, USA). Thermocycling
261 conditions consisted of incubation for 5 min at 50 °C, a 10 min denaturation step at 95 °C,
262 followed by 60 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. We
263 used 60 cycles for consistency with optimisation tests, but cycling could be reduced to 45
264 cycles for subsequent applications (see Supporting Information: Appendix 1). A small-scale
265 comparison of eDNA detection and concentration using PCR and qPCR was also conducted
266 (Supporting Information: Appendix 1).
267 Amplifications were considered positive detections if the exponential phase occurred
268 within 45 reaction cycles as the mean C_q value was 40.07 for the LOD (1 copy/µL). A pond
269 was considered positive for crucian carp if two or more of the five technical replicates from a
270 sample returned positive, or more than one sample returned any positive technical replicates
271 (Goldberg et al., 2016). False negatives were obtained for one pond, therefore all samples
272 were tested for inhibition by spiking duplicate qPCR reactions with a known concentration of
273 synthetic crucian carp template (1000 copies/µL) (Jane et al., 2015).

2.6 DNA sequencing

Non-target DNA extracts and full-process blanks that amplified with qPCR were Sanger sequenced alongside a representative eDNA sample from each positive pond (N = 9) to confirm sequence identity. Purification and sequencing was performed by Macrogen Europe (Amsterdam, The Netherlands) in triplicate in the forward direction. Sequences were edited using CodonCode Aligner (CodonCode Corporation, MA, USA) with default settings. Sequences were then manually aligned in AliView (Larsson, 2014) and poor quality sequences were discarded (Figure S5). Primers were removed from remaining sequences, and sequences identified against the full NCBI nucleotide (nt) database using the NCBI BLASTn tool.

2.7 Data analysis

Technical replicates for each qPCR standard that differed by >0.5 C_q from the average of the five technical replicates performed were discarded to minimise bias induced by pipetting error. All technical replicates for eDNA samples were retained, and those which failed to amplify were classed as 0 copies/µL (Goldberg et al., 2016). The C_q values for each set of technical replicates were averaged and quantified to provide a single DNA copy number for each sample. Samples with no positive amplifications were assigned a DNA copy number of zero. DNA copy numbers of samples were then averaged to generate a single DNA copy number for each pond.

All subsequent data analyses were performed in the statistical programming environment R v.3.4.2 (R Core Team, 2017). Effects of water volume filtered, number of filters used, and water sample content on DNA copy number of samples were tested and reported in Supporting Information (see Appendices 1, 2; Figures S6, S7). Results and discussion of the PCR-qPCR comparison are also reported in Supporting Information (Appendices 2-3; Table S4; Figure S8). The R package 'eDNAoccupancy' v0.2.0 (Dorazio & Erickson, 2017) was used to fit a Bayesian, multi-scale occupancy model to estimate eDNA detection probability at sites where crucian carp were confirmed as present by fyke netting. Existing eDNA literature was used to identify biotic and abiotic factors reported to affect eDNA detection, persistence and degradation, and construct hypotheses regarding their effects on eDNA detection probability in water samples (θ), and eDNA detection probability in qPCR replicates (p). No covariates were included at the site level (ψ) as ponds were occupied by crucian carp and eDNA should have been present. At the sample level, more individuals (reflected by CPUE) should increase eDNA concentration and improve detection. Temperature can increase physical, metabolic, or behavioural activity of organisms resulting in more eDNA release, breakdown, and degradation (Takahara et al., 2012; Pilliod et al., 2014; Strickler et al., 2015; Robson et al., 2016; Lacoursière-Roussel, Rosabal & Bernatchez, 2016; Buxton et al., 2017b; Bylemans et al., 2017). Links established between eDNA and pH support greater detectability, concentration, and persistence of eDNA in more alkaline waters (Barnes et al., 2014; Strickler et al., 2015; Goldberg et al., 2018). Conductivity relates to Total Dissolved Solids (TDS) and sediment type, which can impair eDNA detection due to release of inhibitory substances and their capacity to bind DNA (Buxton et al., 2017a; Stoeckle et al., 2017). Vegetated ponds reduce UV exposure thereby preserving eDNA (Barnes et al., 2014), and are susceptible to terrestrialisation which can create anoxic conditions that may slow eDNA degradation (Barnes et al., 2014; Pilliod et al., 2014; Weltz

et al., 2017). At the qPCR replicate level, covariates again included CPUE as higher eDNA concentration should improve amplification success and consistency, whereas conductivity may indicate inhibitory substances that cause amplification failure.

Prior to modeling, all environmental variables were assessed for collinearity using Spearman's correlation coefficient and Variance Inflation Factors (VIFs) calculated using the R package 'car' v2.1-6 (Fox & Weisberg, 2011). Variables were considered collinear and removed if $r > 0.3$ and VIF >3 (Zuur et al., 2009), following which candidate variables (CPUE, conductivity, pH, and percentage of macrophyte cover) were centred and scaled to have a mean of 0 and standard deviation of 1. We constructed 64 models which assumed a constant probability of eDNA occurrence at the site level, and different covariate combinations at the sample and qPCR replicate levels. Models were ranked (Table S5) according to posterior predictive loss criterion (PPLC) under squared-error loss and the widely applicable information criterion (WAIC). The model with the best support was selected for comparison to the null model without covariates at the entire sampling hierarchy.

We examined the influence of biotic and abiotic factors on eDNA quantification using a generalised linear mixed effects model (GLMM) within the R package 'glmmTMB' v0.2.0 (Brooks et al., 2017). Collinearity was assessed as above, leaving CPUE, pH, conductivity, and percentage of macrophyte cover as explanatory variables. Pond was modeled as a random effect to account for spatial autocorrelation in our data set and the influence of other properties inherent to each pond, whereas all other explanatory variables were fixed effects. A Poisson distribution was specified as the nature of the response variable (DNA copy number) was integer count data. Validation checks were performed to ensure all model assumptions were met and absence of overdispersion (Zuur et al., 2009). Model fit was assessed visually and with the Hosmer and Lemeshow Goodness of Fit Test (Hosmer & Lemeshow, 2000) using the R package 'ResourceSelection' v0.3-0 (Lele et al., 2014). Model

predictions were obtained using the predict() function and upper and lower 95% CIs were calculated from the standard error of the predictions. All values were bound in a new data frame and model results plotted for evaluation using the R package 'ggplot2' v2.2.1 (Wickham, 2009). All R scripts and corresponding data have been deposited in a dedicated GitHub repository (https://github.com/lrharper1/crucian_carp_eDNA_surveillance), which has been permanently archived (https://doi.org/10.5281/zenodo.1421602).

3. Results

3.1 Assay specificity and sensitivity

Only crucian carp amplified in ecoPCR, confirming primer specificity. Non-target species returned by primer-BLAST against the full NCBI nucleotide (nt) database were *Barilius bakeri* (a Cyprinid fish restricted to India, 6 mismatches), *Naumovozyma dairensis* (fungi, 8 mismatches), and *Medicago trunculata* (plant, 8 mismatches). Our probe sequence could not be included *in silico* but would likely increase specificity. Tissue extracts from common rudd (*Scardinius erythrophthalmus*) and European chub (*Squalius cephalus*) included in qPCR assay specificity tests were amplified by primers and probe, but possessed low DNA copy number (<10 copies/µL). In a later test, common carp DNA also amplified (<10 copies/µL). However, no amplification was observed for NTCs, fresh tissue extracts obtained from rudd and chub, or eDNA samples from locations where crucian carp were absent and these species were present (data not shown). DNA sequencing confirmed cross-contamination of reference

material, where sequences were either identified as crucian carp or of poor quality (Table 2). Our assay was highly sensitive with a LOD of 1 copy/µL and LOQ of 10 copies/µL.

3.2 qPCR analysis

The qPCR assay had an average amplification efficiency of 93.61% (range 79.61-102.49%) and an average R^2 value of 0.998 (range 0.995-0.999) for the standard curve. Only one plate had a qPCR efficiency lower than 90% but the standard curve quantified as expected, thus qPCR was not repeated. No amplification occurred in NTCs, but the full process blank for one pond (POFA4) amplified (<10 copies/µL). This was the only contaminated blank as the blank for pond POHI filtered alongside POFA4 and POHI samples, and blanks downstream of these samples did not amplify. Partial inhibition (<1000 copies/µL) occurred in a single sample from four different ponds: PYES2 (no crucian carp), RAIL, POHI, and GUES1 (crucian carp present). However, partially inhibited samples all possessed >0 copies/µL when originally tested, and copy number did not differ substantially (higher in one instance) from other samples belonging to the same pond (Table S1). Consequently, partial inhibition did not influence detectability in our study, and problematic samples were not treated for inhibition and qPCRs were not repeated.

3.3 Presence-absence detection

eDNA surveillance detected crucian carp in 90% of the study ponds (N = 10) with confirmed presence. Sanger sequencing of representative samples confirmed species identity as crucian

carp (Table 2). eDNA failed entirely in one pond (CHIP) that contained a sizeable crucian carp population (CPUE = 60.50), but samples from CHIP were not inhibited. Crucian carp DNA was not detected at any sites where the species was absent.

3.4 Factors influencing eDNA detection and quantification

The occupancy model with the best support included CPUE and conductivity as covariates of eDNA detection probability in qPCR replicates (p). The model did not include any covariates of eDNA occurrence probability at sites (ψ) or eDNA detection probability in water samples (θ). Estimates of eDNA detection probability in a qPCR replicate ranged between 0.12 to 1.00 (Table 1), where crucian carp CPUE (parameter estimate = 1.357) and conductivity (parameter estimate = -2.112) played positive and negative roles in eDNA availability respectively (Figures 4a, b). The GLMM identified CPUE (0.020 ± 0.007, $\chi^2_1 = 5.426$, $P = 0.020$) and conductivity (-0.007 ± 0.002, $\chi^2_1 = 8.709$, $P = 0.003$) as significant predictors of DNA copy number, where DNA copy number was greater at higher CPUE (Figure 5a) but decreased as conductivity increased (Figure 5b).

4. Discussion

We developed a novel species-specific qPCR assay to enable large-scale distribution monitoring of the threatened crucian carp using eDNA. Crucian carp were detected at almost all sites with confirmed presence and no false positives were generated. Furthermore, biotic

and abiotic factors that influence eDNA detection and quantification were identified. We discuss areas for improvement in our workflow and provide recommendations for future study.

4.1 Using eDNA for crucian carp conservation

eDNA analysis is often compared to conventional monitoring tools to assess performance, reliability, reproducibility, and prospective applications in conservation programmes. We found strong agreement between eDNA and fyke netting for crucian carp detection, where eDNA detected crucian carp in 90% of ponds with presence confirmed by netting. This high detection and low false negative rate supports the applicability of eDNA analysis to crucian carp presence-absence monitoring, particularly at large spatial scales where fyke netting can be costly and time-consuming, and where ponds are remote with poor access. Abundance estimation is less straightforward as there was uncertainty around the relationship between DNA copy number and crucian carp density. This inconsistency is more likely to result from eDNA than fyke netting due to effects exerted by external factors (section 4.2) and sample processing (section 4.3) on eDNA quality. However, fyke netting also has detection biases that may influence performance comparisons with eDNA. Fyke net surveys are restricted spatially and temporally to pre- and post-spawning as well as spring and autumn when temperatures are low to reduce fish stress in nets. Furthermore, fyke net surveys may fail to capture species that do not have homogenous distribution in their environment, especially where populations contain few individuals (Turner et al., 2012). Netting is also biased towards particular fish size classes that can enter nets through standard European otter (*Lutra lutra*) guards (75 mm in UK), and catchability is further dependent on time of year (Ruane,

Davenport & Igoe, 2012) and even time of day (Hardie, Barmuta & White, 2006). Therefore, effectiveness of standard methods must also be evaluated and eDNA compared to multiple tools before deemed capable or incapable of estimating abundance.

4.2 Factors influencing eDNA detection and quantification

Effects of biotic and abiotic factors on eDNA may vary across target species and ecosystems (Barnes et al., 2014). We found crucian carp density (CPUE) positively influenced eDNA detection probability and DNA copy number. Density is frequently reported to improve detection probability of aquatic species due to more eDNA deposition in the environment (Schmelzle & Kinziger, 2016; Buxton et al., 2017b; Stoeckle et al., 2017), but this relationship is highly variable across study systems and species due to influence of external factors (Strickler et al., 2015; Buxton et al., 2017a; Goldberg et al., 2018). For example, increase in water temperature coincided with breeding activity and heightened DNA release in other fish and amphibian species (Buxton et al., 2017b; Bylemans et al., 2017). In our study, CPUE was collinear with water temperature and thus water temperature was not included in our occupancy model or GLMM. We performed water sample collection in late August, which is outside the reported spawning period for crucian carp (Aho & Holopainen, 2000). However, the association between CPUE and DNA copy number may be linked to increased DNA shedding rates caused by higher metabolic activity in response to warm temperature, as reported for other fish species (Takahara et al., 2012; Robson et al., 2016; Lacoursière-Roussel et al., 2016).

In contrast to CPUE, conductivity had a negative effect on eDNA detection and concentration. Conductivity has been suggested to influence eDNA detection and

quantification, but studies that directly measured this variable have found no discernable effect (Takahara et al., 2012; Keskin, 2014; Goldberg et al., 2018). Conductivity (also measured as TDS) relates to sediment type which influences eDNA detection probability, the rate at which sediment binds eDNA, and release of inhibitory substances (Buxton et al., 2017a; Stoeckle et al., 2017). Notably, the only false negative pond in our study was also the most conductive (760 µs/cm) and possessed dense beds of rigid hornwort (*Ceratophyllum demersum*) that could restrict water movement. Therefore, conductivity may lead to incorrect inferences about species presence and impact conservation management decisions. Further investigation into the effects of conductivity on eDNA detection and quantification is clearly needed.

Our results indicate that samples may have been affected by inhibitory substances despite tests performed to identify inhibition. We spiked qPCR reactions with a known amount of synthetic target DNA; however, an artificial Internal Positive Control gene assay may identify inhibition more effectively (Goldberg et al., 2016). Dilution of eDNA samples (and inhibitory substances present) can release inhibition, but also reduce detection probability (Piggott, 2016) and induce false negatives (Buxton et al., 2017a). We used TaqMan® Environmental Master Mix 2.0 (Life Technologies, CA, USA) in qPCR reactions to counter inhibition (Jane et al., 2015), but it may be advisable to use DNA extraction kits that perform inhibitor removal (Sellers et al., 2018) or include Bovine-serum albumin (BSA) in qPCR reactions (Jane et al., 2015). Alternatively, ddPCR may handle inhibitors better than qPCR and provide more accurate abundance or biomass estimates (Nathan et al., 2014).

Crucially, environmental data were not collected in 2016 for every pond in our study. Our results indicate direction of effects of biotic and abiotic factors on eDNA detection and quantification, but contemporary data are needed for comprehensive interpretation of these relationships. However, it is clear that eDNA practitioners must account for these effects as

well as sample inhibition. The uncertainty around the estimated effects of covariates in our hierarchical occupancy model and GLMM also imply that greater sample volume, sample number, and/or qPCR replication are required to improve the ability and precision of our assay to detect crucian carp eDNA and reduce the potential for false negatives (Schultz & Lance, 2015; Goldberg et al., 2018).

4.3 Optimisation of eDNA workflow

Some non-target DNA extracts used to validate assay specificity were contaminated with crucian carp DNA. Field cross-contamination can occur if reference tissue material is collected from multiple species without sterilising equipment, or eDNA is present on the material collected (Rodgers, 2017). Collection and storage of reference tissue material is an important consideration for eDNA practitioners, particularly those using highly sensitive assays (LOD = 1 copy/μL) (Wilcox et al., 2013, 2016). Dedicated, sterilised equipment should be used when collecting new reference material from different species. From existing reference collections, only non-target samples that were collected on separate and chronologically distinct occasions from target samples should be used (Rodgers, 2017).

Cross-contamination can also arise during water sampling, filtration, DNA extraction and qPCR preparation. Low-level contamination was found in one full process blank but detections from this pond were not omitted as it contained crucian carp and contamination was not observed downstream. All equipment in our study was sterilised by immersion in 10% chlorine-based commercial bleach (Elliott Hygiene Ltd, UK) solution for 10 mins, followed by rinsing in 5% MicroSol detergent (Anachem, UK), and then purified water. However, sterilisation with 50% chlorine-based commercial bleach solution (Goldberg et al., 2016) or

single-use, sterile materials (Wilcox et al., 2016) may further minimise contamination risk.

Many of our eDNA samples were low concentration (<100 copies/µL) which can cause inconsistent qPCR amplification (Goldberg et al., 2016), thus we discuss approaches to maximise eDNA concentration and improve detection probability. The probability of eDNA detection depends heavily on the number of samples and volume of water collected, time of sampling, and sample concentration (Schultz & Lance, 2015; Goldberg et al., 2018). We sampled 5 x 2 L water samples from each pond in autumn 2016, but timing and/or sampling effort may have been inappropriate. A seasonal effect on common carp eDNA detection was observed, where spring sampling generated higher eDNA concentration and detection rates due to greater common carp activity (Turner et al., 2014) and density (Hinlo et al., 2017a). As water sampling did not coincide with fyke netting (spring 2016) in our study, eDNA concentration may not reflect CPUE estimates. Water samples in spring may contain more crucian carp eDNA due to higher activity of individuals, or autumn fyke netting may produce lower CPUE estimates. Parallel seasonal sampling, where water sampling is performed in conjunction with fyke netting at different times of the year, may better align eDNA concentration with CPUE estimates and enable eDNA-based abundance estimates for crucian carp. This is certainly a worthwhile area of research.

Representative sampling is crucial in eDNA surveys. Individuals of a species can be unevenly distributed in the environment, which impacts eDNA detection, distribution, and concentration (Takahara et al., 2012; Eichmiller, Bajer & Sorensen, 2014; Schmelzle & Kinziger, 2016; Goldberg et al., 2018). In lentic ecosystems, eDNA has a patchy horizontal and sometimes vertical distribution, resulting in fine spatial variation (Eichmiller et al., 2014). Studies on common carp revealed eDNA was more concentrated near the shoreline of lentic water bodies (Takahara et al., 2012; Eichmiller et al., 2014), due to aggregations of individuals (Eichmiller et al., 2014). We collected surface water (5 x 2 L) from the shoreline

548 and sampled at equidistant points around the pond perimeter where possible; however, more
549 samples and greater water volumes may be required to improve detection probability (Schultz
550 & Lance, 2015; Goldberg et al., 2018). Fine spatial sampling and occupancy modelling are
551 needed to determine the sample number required to achieve high detection probability and
552 eliminate false negatives (Goldberg et al., 2018). However, the number of samples required
553 will inevitably vary across habitats due to inherently variable physical properties (Schmelzle
554 & Kinziger, 2016).

555 Method of eDNA capture can dictate success of this monitoring tool. Studies of
556 eDNA in ponds (Ficetola et al., 2008; Biggs et al., 2015) have used an ethanol precipitation
557 approach, but this is restricted to small volumes. Filtration allows more water to be processed
558 and minimises capture of non-target DNA, with macro-organism eDNA effectively captured
559 by pore sizes of 1 - 10 μm (Turner et al., 2014). We used a small pore size of 0.45 μm to
560 capture most eDNA particle sizes, although filter clogging prevented the entire sample being
561 processed and may have affected eDNA concentration downstream. Pre-filtering can reduce
562 clogging, but is labour-intensive and increases cost (Takahara et al., 2012). Larger pore sizes
563 have been used in temperate and tropical lentic environments (Takahara et al., 2012; Robson
564 et al., 2016; Goldberg et al., 2018), though independent investigation is needed to determine
565 which pore size maximises target DNA concentration.

566 Comparisons of eDNA yield across filter types and DNA extraction protocols have
567 shown that cellulose nitrate filters stored at -20 °C (this study) often provide best eDNA yield
568 (Piggott, 2016; Spens et al., 2016; Hinlo et al., 2017b). However, different filter types may be
569 optimal for different species, which has consequences for detectability (Spens et al., 2016)
570 and relationships between eDNA concentration and abundance/biomass (Lacoursière-Roussel
571 et al., 2016). Extraction method used, regardless of filter type, will ultimately influence DNA
572 quality and yield. We used the PowerWater® DNA Isolation Kit (MO BIO Laboratories, CA,

USA), but the DNeasy Blood and Tissue kit (Qiagen®, Hilden, Germany) has demonstrated greater yield (Hinlo et al., 2017b). We also combined filters from the same sample for DNA extraction at the bead milling stage, but independent lysis may recover more DNA (Hinlo et al., 2017b). A comparison of DNA extraction protocols is necessary to assess which approach maximises crucian carp eDNA concentration. A new modular extraction method shows promise for eDNA but has yet to be evaluated for targeted qPCR (Sellars et al., 2018).

Finally, detection sensitivity can be enhanced by increasing the number of qPCR technical replicates (Schultz & Lance, 2015; Piggott, 2016). We performed five technical replicates for each of our samples, but other studies have used as many as twelve and only one may amplify (Biggs et al., 2015). More replication may have enabled amplification from the CHIP pond samples, but qPCR cost would inevitably increase. Further replication may also be unnecessary if steps are taken to improve initial concentration of samples instead (Schultz & Lance, 2015).

4.4 Concluding remarks

A primary objective of the Norfolk crucian carp BAP was to obtain a basic understanding of species distribution and population status across Norfolk (Copp & Sayer, 2010). eDNA surveillance for crucian carp will provide a useful, cost-effective alternative to established survey methods where the aim is determining presence-absence. Our assay may detect hybrids where crucian carp were the maternal parent due to use of a mitochondrial marker; however, these detections are also beneficial to the crucian carp conservation effort through the identification of ponds where true crucian carp may still exist, and where contamination with goldfish, common carp and their hybrids has occurred. Alternatively, our assay could be

used as an early warning tool in countries where the crucian carp is considered invasive. The areas we have highlighted require further investigation before eDNA can be used routinely. Nevertheless, eDNA survey could enable large-scale distribution monitoring for crucian carp through rapid screening of existing and new ponds. Fyke netting could then be used to investigate age, sex and size structure of populations, and remove hybrids.

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Conflict of interest

The authors declare no conflict of interest.

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838 **Table 1**. Bayesian estimates of crucian carp eDNA occurrence probability at a pond (ψ), eDNA detection
839 probability in a water sample (θ), and eDNA detection probability in a qPCR replicate (p). Posterior median and
840 95% credible interval (CI) are given for each parameter of the occupancy model. The corresponding catch-per-
841 unit-effort estimate (CPUE) is given for each pond.
842

Pond	Crucian carp (Y/N)	CPUE estimate	ψ		θ		p	
			Posterior median	95% CI	Posterior median	95% CI	Posterior median	95% CI
CAKE	Y	43.00	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.14	0.05 - 0.33
CHIP	Y	60.50	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.12	0.03 - 0.36
GUES1	Y	121.75	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.98	0.86 - 1.00
MYST	Y	6.17	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.93	0.86 - 0.98
OTOM	Y	14.67	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.96	0.91 - 0.99
POFA4	Y	13.67	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.89	0.81 - 0.95
POHI	Y	7.25	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.44	0.28 - 0.59
RAIL	Y	58.17	0.90	0.62 – 1.00	0.83	0.70 - 0.92	1.00	0.99 - 1.00
SKEY1	Y	31.38	0.90	0.62 – 1.00	0.83	0.70 - 0.92	1.00	1.00 - 1.00
WADD3	Y	126.00	0.90	0.62 – 1.00	0.83	0.70 - 0.92	1.00	1.00 - 1.00

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844 **Table 2.** Top NCBI BLASTn hit for Sanger sequences obtained from target DNA (tissue extracts and synthetic
845 gBlocks® Gene Fragment), non-target tissue DNA extracts, full process blanks, and representative eDNA
846 samples that amplified during qPCR. Sample descriptions marked with '!' indicate a poor quality, discarded
847 sequence.
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Sample	Description	Query length	Coverage	E value	Identity	Accession
CrucianCarp-01	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
CrucianCarp-02	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
CrucianCarp-03	!					
Gblock-100copies-01	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
Gblock-100copies-02	!					
Gblock-100copies-03	!					
Rudd-JL-01	*Carassius carassius*	38	100%	9E-10	100%	KR131843.1
Rudd-JL-02	*Carassius carassius*	38	100%	9E-10	100%	KR131843.1
Rudd-JL-03	!					
Rudd-PS-01	!					
Rudd-PS-02	!					
Rudd-PS-03	!					
Chub-PS-01	!					
Chub-PS-02	!					
Chub-PS-03	!					
Chub-JL-01	!					
Chub-JL-02	!					
Chub-JL-03	!					
CommonCarp-01	!					
CommonCarp-02	!					
CommonCarp-03	!					
POFA4-B-01	!					
POFA4-B-02	!					

POFA4-B-03	!					
GUES1-5-01	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
GUES1-5-02	*Carassius carassius*	41	100%	1E-07	95%	KR131843.1
GUES1-5-03	*Carassius carassius*	41	100%	2E-11	100%	KR131843.1
MYST-3-01	*Carassius carassius*	46	100%	4E-14	100%	KR131843.1
MYST-3-02	!					
MYST-3-03	*Carassius carassius*	41	100%	1E-07	95%	KR131843.1
SKEY1-4-01	*Carassius carassius*	35	100%	4E-08	100%	KR131843.1
SKEY1-4-02	!					
SKEY1-4-03	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
OTOM-4-01	!					
OTOM-4-02	!					
OTOM-4-03	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
POHI-2-01	*Carassius carassius*	41	100%	2E-11	100%	KR131843.1
POHI-2-02	!					
POHI-2-03	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
RAIL-4-01	!					
RAIL-4-02	*Carassius carassius*	38	100%	9E-10	100%	KR131843.1
RAIL-4-03	*Carassius carassius*	46	100%	4E-14	100%	KR131843.1
WADD3-4-01	*Carassius carassius*	25	96%	0.034	100%	KR131843.1
WADD3-4-02	!					
WADD3-4-03	*Carassius carassius*	38	100%	9E-10	100%	KR131843.1
CAKE-1-01	!					
CAKE-1-02	!					
CAKE-1-03	!					
POFA4-5-01	!					
POFA4-5-01	!					
POFA4-5-01	!					

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Figure 1. A crucian carp (*Carassius carassius*) individual **(a)** and examples of the study ponds **(b-d)**. Photo **(a)** by John Bailey and photo **(d)** by Sacha Dench.

Figure 2. Map of pond locations in North Norfolk, eastern England, showing the distribution of ponds containing crucian carp (black dots) and ponds where the species is absent (grey dots).

Figure 3. Alignment of consensus sequences for a region of the mitochondrial cytochrome *b* (*cytb*) gene in 24 European freshwater fishes, including the crucian carp. Species-specific primers and probe for the crucian carp are given on the first line. Consensus with primer and probe sequence across species is highlighted in white whereas mismatches are coloured by nucleotide base.

Figure 4. Estimated probability of eDNA detection in qPCR replicates. Points are estimates of posterior medians with 95% credible intervals. Probability of eDNA detection in qPCR replicates increased with higher catch-per-unit-effort (CPUE) estimate **(a)** but decreased as conductivity increased **(b)**.

Figure 5. Relationship between fixed effects and response variable (DNA copy number) in ponds, as predicted by the Poisson GLMM. The 95% CIs, as calculated using the model predictions and standard error for these predictions, are given for each relationship. The observed data (points) are also displayed against the predicted relationships (line). DNA copy number increased with catch-per-unit-effort (CPUE) estimate **(a)**, but decreased as conductivity **(b)** increased.