Received: 5 May 2020 | Revised: 29 September 2020 | Accepted: 30 September 2020

DOI: 10.1002/edn3.151

ORIGINAL ARTICLE

Environmental DNA Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences

WILEY

A new high-resolution melt curve eDNA assay to monitor the simultaneous presence of invasive brown trout (*Salmo trutta*) and endangered galaxiids

Jessica F. Minett[1] | **Carlos Garcia de Leaniz**[1] | **Paul Brickle**[2] | **Sofia Consuegra**[1]

[1]Swansea University, Wales, UK

[2]South Atlantic Environmental Research Institute (SAERI), Stanley, Falkland Islands

Correspondence
Sofia Consuegra, Swansea University, Singleton Park, Swansea, SA2 8PP, Wales, UK.
Email: s.consuegra@swansea.ac.uk

Funding information
This work has been funded by a scholarship from Swansea University and Fortuna Ltd to JFM.

Abstract

Brown trout are highly invasive in the Southern Hemisphere where they support important sport fisheries and aquaculture activities, which may impact endangered native galaxiid fishes and cause conflicts. To protect native galaxiids it is essential to monitor changes in species distributions, but this can be difficult when species are rare or difficult to sample. We developed and validated, both in the laboratory and in the field, a new assay using a high-resolution melt curve (HRM)-eDNA approach to simultaneously detect the presence of two threatened native galaxiids (*Aplochiton zebra* and *Aplochiton taeniatus*) and the invasive brown trout (*Salmo trutta*). Using this method, we found brown trout in 30% of the sampled waterbodies and *Aplochiton* sp. in 15% of them. Galaxiids were solely identified as being present in rivers that lacked brown trout, with both native species coexisting in two of the three rivers where they were detected, despite their different niche preferences. These assays can be used to monitor threatened zebra trout as well as invasive brown trout populations, allowing conservation managers to target areas for intervention.

KEYWORDS

Aplochiton taeniatus, *Aplochiton zebra*, endemic fishes, high-resolution melt curve, invasive species, qPCR-HRM

1 | INTRODUCTION

Understanding species' niche characteristics is essential to predict the consequences of biological invasions (Korsu et al., 2007), but requires being able to accurately identify particular species and their distributions (Darling & Blum, 2007). Species identification can be difficult if they are threatened, at low densities (Jerde et al., 2011) and/or morphologically cryptic (Bickford et al., 2006). This is important because the establishment and dispersal of non-native species often impact native fauna through increased predation, competition for resources, and disease transmission (Ellender & Weyl, 2014; Gozlan et al., 2010). Competition for resources and/or predation can result in the displacement of native species and introgression/hybridization with introduced species, potentially leading to their decline, extirpation or extinction (Huxel, 1999). These negative impacts can be particularly severe for endemic species, especially those found in low abundance and having limited geographic range (Burlakova et al., 2011; Hobbs et al., 2011), and particularly in freshwater ecosystems where invasive species are one of the main drivers of biodiversity loss (Dudgeon et al., 2006; Reid et al., 2019).

The introduction and spread of non-native fishes in freshwater ecosystems have often been attributed to aquaculture and recreational fishing, particularly in the case of salmonid fishes (Garcia de Leaniz et al., 2010), one of the most widespread groups of introduced fishes (Rahel, 2007). Although, few species are known to have become extinct due to the effects of introduced salmonids, declines in abundance and distribution of native and endemic fishes are evident in many countries (Habit et al., 2010; Kadye et al., 2013; McIntosh et al., 2010; Woodford & Impson, 2004; Young et al., 2010). In New Zealand for example, the extinction of the native grayling *Prototroctes oxyrhynchus* has been attributed in part to the introduction of brown trout *Salmo trutta* (McDowall, 2006). Galaxiid fishes, endemic of the Southern Hemisphere, constitute one of the freshwater fish families most seriously threatened by salmonid expansions (Garcia de Leaniz et al., 2010; Habit et al., 2010). Invasive salmonids exert strong selection pressure upon native galaxiids across their ranges, including New Zealand (McIntosh et al., 2010), Chile (Habit et al., 2010), and Australia (Hardie et al., 2006), mainly through predation and competition (Arismendi et al., 2009; Macchi et al., 2007; Penaluna et al., 2009; Soto et al., 2006).

In Chile and the Falkland Islands, the distribution of galaxiids (*Aplochiton* spp.) is determined by historical colonization but also shows strong population structuring, isolation, and reduced genetic diversity in areas affected by salmonids (Vanhaecke et al., 2015). In particular, brown trout have caused widespread ecological damage to areas they have been introduced, and as a result, they have been classified as one of the "100 of the world's worst invasive species" (Lowe et al., 2000). In the Falkland Islands, since its introduction in 1947–1962, brown trout has spread around East and West Falkland (Arrowsmith & Pentelow, 1965; Stewart, 1973), resulting in the once-common native galaxiid, zebra trout (*Aplochiton zebra*) to be classed as threatened, and limited to refuges uninvaded by brown trout south of the islands (McDowall et al., 2001; Ross, 2009). Conservation of *Aplochiton* spp. is complicated because the two known species (*A. zebra* and *A. taeniatus*) are ecologically and morphologically similar and include resident and migratory ecotypes that may confound identification (McDowall, 2006). In fact, until recently both species had been misidentified as *A. zebra* in the Falklands (Vanhaecke et al., 2012). The small sizes of *A. zebra* and *A. taeniatus* juveniles make them particularly susceptible to salmonid predation and displacement (Arismendi et al., 2009; Macchi et al., 2007), which also potentially increases inbreeding and hybridization as a result of population reductions and limited suitable habitat uninvaded by brown trout (Vanhaecke et al., 2012; Wolf et al., 2001). In contrast, the abundance of salmonids seems to be related to propagule pressure (Consuegra et al., 2011) and habitat connectivity (Habit et al., 2012). Previous studies conducted 10 and 20 years ago to assess the distribution of brown trout and native galaxiids in the Falklands (Fowler, 2013; McDowall et al., 2001; Ross, 2009) showed marked reduction in the abundance and distribution of zebra trout since the introduction of brown trout. However, traditional monitoring exercises based on electrofishing are limited by their cost and by the protected and rare nature of *Aplochiton* spp. Electrofishing of rare species often requires increased effort, possibly reducing the number of reaches that can be sampled (Reynolds et al., 2003) and increasing the cost of sampling each reach (Evans et al., 2017). In addition, electrofishing can reduce survival in embryos (Bohl et al., 2009) as well as cause stress, injury and mortality (Miranda & Kidwell, 2010; Panek & Densmore, 2011), which could impact rare and threatened populations.

Environmental DNA (eDNA) released from organisms through blood, urine, skin, mucus, and feces increasingly is used to detect aquatic species that are difficult to locate, identify, and/or are in low abundance, and is particularly useful for conservation programs (Biggs et al., 2015; Robinson, Garcia de Leaniz & Consuegra, 2019). While eDNA metabarcoding is used to target multiple species and often to assess the biodiversity of a system (Deiner et al., 2015; Lacoursière-Roussel et al., 2018), quantitative PCR (qPCR) targets single species and constitutes a reliable method for detecting endangered and invasive species when combined with in vitro controls and amplicon sequencing (Carlsson et al., 2017; Díaz-Ferguson et al., 2014). qPCR in combination with high-resolution melt (HRM) curve analysis allows single-base variations in DNA sequences to be detected based on the DNA product melt temperature in water samples (Ramón-Laca et al., 2014; Robinson et al., 2018; Wittwer, 2009) and has been used with environmental DNA as a sensitive method to detect individual or multiple species, including fishes (Behrens-Chapuis et al., 2018; Robinson, Garcia de Leaniz, Rolla et al., 2019), invertebrates (Robinson, Garcia de Leaniz & Consuegra, 2019; Robinson et al., 2018), and sea turtles (Harper et al., 2020) and plants (Emenyeonu et al., 2018). Here, we developed eDNA-HRM curve analysis assays to map the current distribution of brown trout and both *Aplochiton* species in the Falkland Islands in a non-destructive way, to identify refuges for zebra trout, which then can be prioritized for conservation.

2 | MATERIAL AND METHODS

2.1 | qPCR primer design and optimization

Aplochiton zebra and *A. taeniatus* qPCR primers (AzebAtaeCytbF: 5'-ATGAAATTTTGGCTCTCT-3' and AzebAtaeCytbR: 5'-GAAATATCGGAGGTGTAG-3') were designed to amplify an 89 bp fragment of the cytochrome b region of the mitochondrial (mt) genome (product melt temperature 77.8°C and 79.2°C for *A. zebra* and *A. taeniatus*, respectively). Species-specific qPCR primers (StruttaCytbF: 5'-TATCCTCCATACCTCTAA-3' and StruttaCytbR: 5'-GACCGATGATAATGAATG-3') were designed for *Salmo trutta* to amplify a 139 bp fragment of the mitochondrial cytochrome b region. Both sets of primers were designed using OligoArchitect Primer and Probe Design online software and checked in silico for cross-amplification using NCBI Primer-BLAST (Ye et al., 2012). Both AzebAtaeCytb- and StruttaCytb-qPCR primers were tested in vitro for non-specific amplification against all freshwater fishes present in the Falklands (*A. zebra*, *A. taeniatus*, *Galaxias maculatus*, and *S. trutta*, except *Geotria australis* that may occur intermittently) (McDowall et al., 2001; Vanhaecke et al., 2012).

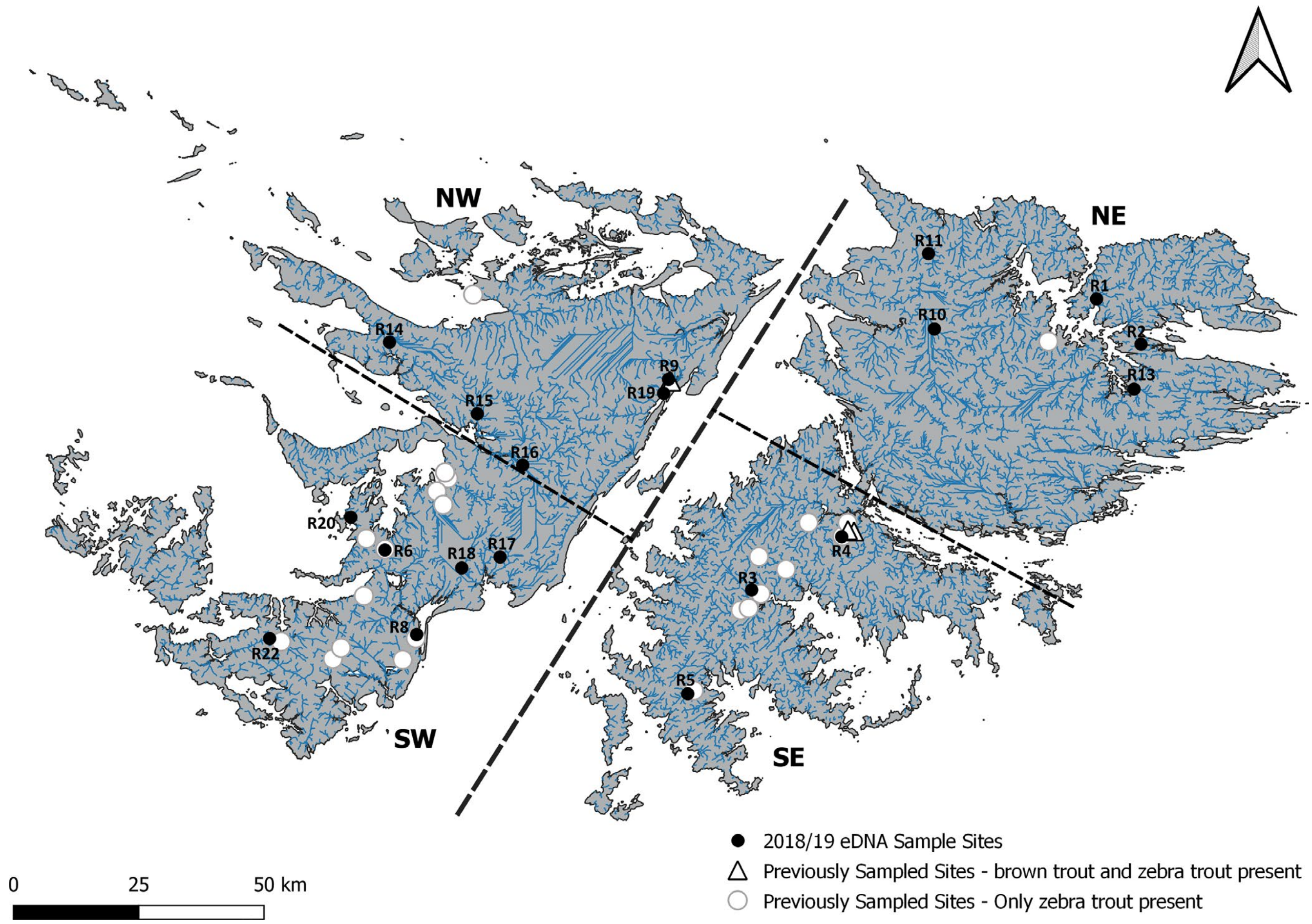

FIGURE 1 eDNA sampling locations in the Falkland Islands. Current eDNA sampling locations (black circles), previously sampled sites where only zebra trout were present (white circles) and previously sampled sites with zebra trout and brown trout present (white triangle), previous sampled data obtained from McDowall et al. (2001), Ross (2009) and Fowler (2013)

Primers were assessed using positive tissue controls (fin clips and muscle tissue) from 12 different *A. zebra* and *A. taeniatus* individuals. DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen, UK). A 10-fold dilution series using pools of DNA from each species (consisting of DNA from six *A. zebra* and six *A. taeniatus*) ranging from 19.7 ng/μl to 1.97×10^{-4} ng/μl and 14.8 ng/μl to 1.48×10^{-4} ng/μl, respectively was conducted in order to determine the limit of detection (LOD) and the limit of quantification (LOQ) as in Robinson et al., (2018). Amplification efficiency, also estimated from the dilution curve, was 79.5% for *A. zebra* and 84.6% for *A. taeniatus* (Bio-Rad, 2013). The annealing temperature for AzebAtaeCytb primers was optimized at 61.5°C. The AzebAtaeCytb-qPCR protocol began with a two min denaturation step at 95°C, followed by 45 cycles of 95°C for 10 s and 61.5°C for 30 s. A HRM step was applied at the end of the real-time PCR reaction, ranging from 65°C to 95°C in 0.1°C increments to test the consistency of amplicon melt temperatures (tm) for each species. To account for any potential intraspecific variation in qPCR product tm, six individuals from five *A. zebra* populations and six from three *A. taeniatus* populations were used for HRM analysis. To assess the ability to detect *A. zebra* and *A. taeniatus* in the same reaction, equal volumes of both species' DNA were pooled from six different individuals of both species at various concentration ratios ranging from 10:90 to 50:50 (e.g., 30:70 dilutions represented in Figure S1).

StruttaCytb-qPCR primers were assessed in vitro using positive tissue controls (fin clips) from nine individual brown trout from a range of populations. DNA was extracted using the Qiagen Blood and Tissue Kit (Qiagen, UK), and amplified in real-time PCR-HRM analysis using the following StruttaCytb protocol: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s, a HRM step was applied to the end of the real-time PCR reaction, ranging from 65°C to 95°C in 0.1°C increments. The annealing temperature for the StruttaCytb primers was optimized at 60°C resulting in an efficiency of 89.4%. A 10-fold dilution series was also carried out ranging from 35.4 ng/μl to 3.54×10^{-4}ng/μl to determine the LOD and LOQ.

AzebAtaeCytb and StruttaCytb primers also were tested using positive eDNA controls (sites where species had been seen during the sampling period) to ensure that the primers would amplify environmental DNA (Figure 2). eDNA samples (nine samples from three different sites × three technical PCR replicates) were spiked with

TABLE 1 Locations and environmental data for eDNA sampling sites, including latitude and longitude, temperature (°C), shade cover (0–3), river width, pH, total dissolved solids (TDS), electrical conductivity (EC), and the total volume filtered

Sampling Date	Site No.	Waterbody	Previously Sampled	Fish Status	Latitude	Longitude	Temp (°)	Shade Cover	Width (m)	pH	TDS (ppm)	EC (S/m)	Total volume Filtered
04/04/18	R1a	Johns Brook	NA	Unknown	−51.48339	−58.29203	5.6	0	1.5	4.5	92	46	872
04/04/18	R1b		NA		−51.48137	−58.29257	6.0	0	5	4.9	102	51	823
04/04/18	R2a	Monty Deans Creek	1999		−51.56585	−58.16645	5.0	0	2	6.1	240	120	850
04/04/18	R2b		1999		−51.56715	−58.15749	5.0	0	2	6.7	240	120	650
09/04/18	R3a	Spots Arroyo	2009	Zebra trout	−51.9902	−59.30946	5.0	0	3	6.9	364	182	900
09/04/18	R3b		2009		−51.9896	−59.28561	6.8	0	3	7.3	370	185	1,200
18/04/18	R4a	Findley Creek Stream	2011	Brown trout & zebra trout	−51.89972	−59.04361	6.8	0	<1	7.3	240	120	1,200
18/04/18	R4b		2011		−51.93139	−59.06011	7.4	0	<1	7.5	288	144	1,200
19/04/18	R5a	North West Arm House Stream	2012	Zebra trout	−52.17283	−59.50553	9.4	0	2	6.8	482	234	1,200
19/04/18	R5b		2012		−52.16641	−59.49236	11.6	0	3	7.1	479	239	1,108
01/05/18	R6a	Fish Creek (2)	2012	Zebra trout	−51.89306	−60.36861	4.0	1	1	5.5	508	254	1,200
01/05/18	R6b		2012		−51.89306	−60.36861	4.0	0	3	6.7	382	191	1,200
02/05/18	R8a	Fish Creek (1)	2012	Zebra trout	−52.05583	−60.29111	4.2	1	2	4.5	240	120	635
02/05/18	R8b		2012		−52.04722	−60.28778	4.2	0	5	4.6	242	121	650
03/05/18	R9a	House Creek	1999	Brown trout & zebra trout	−51.6075	−59.52972	4.2	0	3	4.8	56	28	1,100
03/05/18	R9b		1999		−51.61111	−59.52333	4.2	0	3	4.9	58	29	950
22/09/18	R10a	San Carlos	1999	Brown trout	−51.5095	−58.822	1.6	0	20	3.9	70	35	1,200
30/09/18	R10b		1999		−51.531111	−58.760278	NA	0	15	NA	NA	NA	1,200
03/10/18	R11a	Elephant Beach Pond Stream	1999	Brown trout	−51.395556	−58.771944	2.6	0	5	4.5	92	46	1,200
03/10/18	R11b		1999		−51.434444	−58.773611	5.8	1	2	4.7	94	47	1,200
03/10/18	R13a	Estancia Creek	2008	Brown trout	−51.6475	−58.195833	5.4	2	<1	5.3	92	46	1,200
03/10/18	R13b		2008		−51.646389	−58.188611	5.4	0	15	5.6	704	4,352	1,200
08/10/18	R14a	Herbert Stream	1999	Brown trout	−51.5208333	−60.3277778	5.8	0	10	NA	288	148	900
08/10/18	R14b		1999		−51.5308333	−60.2427778	6.2	1	5	NA	226	110	1,200
08/10/18	R15a	Teal House River	NA	Unknown	−51.6194444	−60.1102778	5.2	1	3	NA	72	36	1,200
08/10/18	R15b		NA		−51.6561111	−60.0841667	6.8	2	4	NA	90	45	1,200
08/10/18	R16a	Chartres River	1999	Brown trout	−51.7516667	−59.9594444	7.8	1	25	NA	92	46	1,192
09/10/18	R16b		1999		−51.8366667	−59.9611111	4.6	1	3	NA	304	152	600
09/10/18	R17a	Doctors Creek	2012	Brown trout	−51.9411111	−60.0522222	4.0	1	3	NA	364	182	1,200
09/10/18	R17b		2012		−51.9147222	−60.0358333	3.6	2	<1	NA	246	123	1,200

(Continues)

TABLE 1 (Continued)

Sampling Date	Site No.	Waterbody	Previously Sampled	Fish Status	Latitude	Longitude	Temp (°)	Shade Cover	Width (m)	pH	TDS (ppm)	EC (S/m)	Total volume Filtered
09/10/18	R18a	Malo Arroyo	NA	Unknown	−51.9313889	−60.1483333	4.0	0	4	NA	364	182	614
11/10/18	R18b		NA		−51.9597222	−60.1569444	1.4	0	7	NA	328	164	600
29/10/18	R19a	Neil Clark Nature Reserve	NA	Unknown	−51.632444	−59.54519	NA	NA	NA	NA	NA	NA	1,200
09/10/18	R19b		NA		−51.9411111	−60.0522222	3.0	0	50	NA	1,660	830	1,200
14/12/19	R19c		NA		−51.632222	−59.545556	NA	2	<1	NA	NA	NA	1,150
06/05/19	R20a	Spring Point	NA	Unknown	−51.8314	−60.4628	NA	2	1	NA	NA	NA	900
06/05/19	R20b		NA		−51.823	−60.4454	NA	2	2	NA	NA	NA	1,000
06/05/19	R22a	Whiskey Creek	2009	Zebra trout	−52.0542	−60.7891	NA	2	2	NA	NA	NA	900
06/05/19	R22b		2009		−52.0416	−60.7155	NA	2	3	NA	NA	NA	900

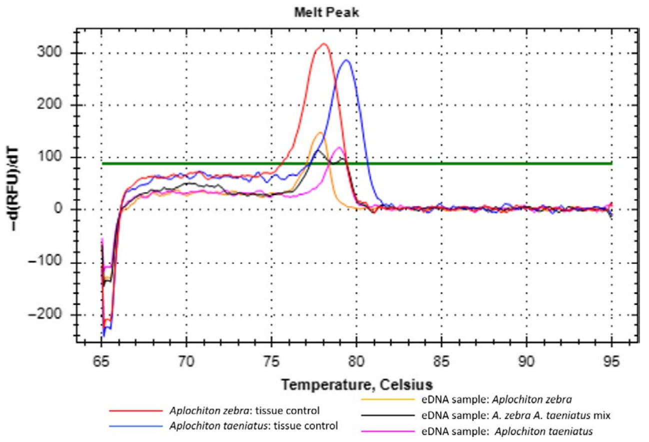

FIGURE 2 qPCR product melt curve profile for positive tissue controls for *Aplochiton zebra* and *Aplochiton taeniatus* and eDNA sample amplifications. Red and blue peaks correspond to positive *A. zebra* and *A. taeniatus* tissue samples respectively, the black peak is from an eDNA sample amplifying both *A. zebra* and *A. taeniatus* simultaneously, and orange and pink peaks correspond to eDNA samples amplifying *A. zebra* and *A. taeniatus*, respectively

positive control DNA (1 µl of *A. zebra* DNA from six individuals, 9.85 ng/µl) to test for possible inhibition in separate reactions.

2.2 | Study populations and eDNA sample collection

We sampled 19 rivers and ponds across the Falkland Islands (Figure 1), eight on East Falkland (five in the North and three in the South), and 11 on West Falkland (five in the North and six in the South). Locations were chosen based on information from monitoring studies conducted 10 and 20 years ago (Fowler, 2013; McDowall et al., 2001; Ross, 2009). Zebra trout had previously been detected at seven of the 19 locations, co-occurring with brown trout at only two locations. Six locations solely supported brown trout populations. The remaining seven rivers had not been surveyed previously ($N = 5$) or were rivers that had been surveyed but where zebra trout or brown trout had not been recorded.

We sampled two sites per river/pond except for R19 Neil Clark Nature Reserve where we sampled three sites; at each site, two water samples were collected from the surface of the water in areas of low flow near the bank of the river, taking precautions to avoid contamination following Robinson, Garcia de Leaniz, Rolla et al. (2019). Three water replicates of 100–200 ml (the final volume depending on the level of particulate organic matter present in the waterbody) were filtered at each site (Table 1). Water was pushed through a syringe filter containing a polyethersulfone (PES) filter membrane with a 0.45 µm pore size using a sterile 50 ml disposable syringe. Filters were then dried by pushing through air before being preserved in 95% ethanol and stored at −20°C until further analyses. To prevent contamination, water sampling bags, syringes, and gloves were disposed of between sites. Negative controls consisting of autoclaved or ultrapure water were filtered instead of river/pond water before sampling at each site. River width, temperature, shade cover, pH, total dissolved solids, and electrical conductivity were measured at each sampling site where possible (Table 1). Due to time and weather constraints, sampling was conducted over two field seasons April-May (Autumn) and September-October (Spring) in 2018, three additional waterbodies were sampled by local citizens, two in May (Autumn) 2019, and a final site sampled in December 2019 (Table 1).

2.3 | eDNA extraction and amplification

eDNA was extracted from 273 field samples (19 waterbodies, 39 sites × two water samples × three replicates and one blank per site, Table 1) using the Qiagen DNeasy PowerSoil Kit (Qiagen, UK), following the manufacturer's instructions. DNA extractions took place in a dedicated eDNA area within an extraction cabinet equipped with a flow-through air system and UV light to minimize the risk of contamination. Extracted DNA was quantified with a Qubit 3.0 fluorometer. Six technical PCR replicates of each sample were amplified in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, UK), in 10µl reaction consisting of 5µl of iTaq Universal SYBR Green Supermix (Bio-Rad, UK), 0.25µl (10µM) of each AzebAtaeCytbF and AzebAtaeCytbR, 2.5µl of ultrapure water, and 2µl of extracted DNA. Amplifications were carried out using the standard AzebAtaeCytb-qPCR protocol as described above, only samples which consistently amplified in at least two technical PCR replicates per site at the target DNA product tm (either 77.8°C ± 0.2 or 79.2°C ± 0.2) were considered to be a positive result (Table S2). Reactions of 10µl also were carried out using the StruttaCytb primers consisting of 5 µl of iTaq Universal SYBR Green Supermix (Bio-Rad, UK), 0.25 µl (10 µM) of each forward and reverse primer, 1.5µl of ultrapure water, and 3µl of DNA. Amplification was carried out using the standard StruttaCytb-qPCR protocol (described above) and only samples that amplified consistently in at least two technical PCR replicates per site at the target DNA product tm (78.7°C ± 0.1) were considered a positive result (Table S2). qPCR reactions were carried out in a dedicated eDNA area; reaction mix was loaded in a DNA-free PCR hood with a flow-through air system and UV light before being transferred to a separate PCR hood to load DNA. Once all eDNA samples had been loaded and sealed two positive controls (one for each species) and a negative control consisting of brown trout or *Galaxias maculatus* DNA also was loaded to control for false positives. Negative filter and extraction controls were run throughout the process. Three additional negative amplification controls consisting of ultrapure water were also added to test for contamination during the entire process (both with eDNA and positive control samples). To confirm primer specificity, a subset of eDNA samples ($N = 4$ brown trout and $N = 9$ *Aplochiton* spp.) was amplified with the qPCR primers using end-point PCR and cloned into a pCR 4-TOPO plasmid cloning vector (TOPO TA Cloning Kit for Sequencing, Invitrogen). In total, 10–25 clones were sequenced per sample using T3 and T7 primers. All samples were cleaned using a sodium acetate/EtOH solution, resuspended in 10 µl HiDi Formamide (Applied Biosystems) and analyzed using Sanger Sequencing on an ABI 3,730 DNA Analyser (Applied Biosystems). Resulting sequences were aligned in BioEdit (v 7.2.5) (Hall, 1999), and input to BLAST (Ye et al., 2006) to confirm species identity.

To determine whether sampling conditions (volume filtered, season, temperature, shade, and total dissolved solids, Table 1) affected amplifications, a generalized linear model using binomial error family was performed in R3.5.3. Using the *drop1* function, individual predictors were dropped from the model until the optimal model based on AIC was obtained.

3 | RESULTS

AzebAtaeCytb and StruttaCytb assays were tested in silico for cross-amplification using NCBI Primer-BLAST (Ye et al., 2012) (Table S1), and we found no cross amplifications with any species present in the Falkland Islands. Primers were also tested in vitro against *S. trutta* and *G. maculatus*, and both species of zebra trout and *G. maculatus*, respectively; no cross amplifications were detected. A 10-fold dilution series of positive control *A. taeniatus* and *A. zebra* DNA (from six individuals respectively) revealed that for *A. taeniatus*, the limit of detection (LOD) was 1.97×10^{-4} ng/µl and for *A. zebra* the LOD was 1.48×10^{-4} ng/µl. The detection threshold for both species of zebra trout at the lowest LOD was 42 cycles and the product melting temperatures (tm) were consistent throughout the dilution series. qPCR product tm showed no overlap between the two species of zebra trout (77.8°C and 79.2°C ± 0.2 for *A. zebra* and *A. taeniatus* respectively; these might vary in zebra trout from different regions, if there were polymorphisms in the amplified region). Using the diagnostic melt curve produced, it was possible to detect the presence of both species when combining varying ratios of pooled DNA (Figure 2). Results from a 10-fold dilution series revealed that the LOD for brown trout was 3.54×10^{-4} ng/µl for the *S. trutta* qPCR assay with a detection threshold of 37 cycles. The nine eDNA samples spiked with positive control *A. zebra* DNA amplified with qPCR product tm at 77.8°C, indicating no signs of inhibition.

We extracted 273 eDNA samples from 19 rivers and ponds in the Falklands retrieving DNA concentrations between 0 and 15 ng/µl across all sites (57 samples had no detectable DNA). Zebra trout DNA was successfully detected in three of the 19 rivers sampled (Table 2), *Aplochiton zebra* in two rivers and *Aplochiton taeniatus* in three, whereas brown trout DNA was detected in six out of 19 rivers (Table 2), three of being the first time. Previously, brown trout and zebra trout had been found together in two of the rivers, R4 (Findley Creek Stream) and R9 (House Creek); however, we found no indication of either species in those. Brown trout and zebra trout DNA were detected at sites where they had been previously found ($N = 3$ in each case) and also at sites where there was visual confirmation eDNA collection (Table 2), supporting the effectiveness of these assays in the field. All negative controls (sampling blanks, extraction blanks, and PCR blanks) failed to amplify for both zebra trout species and brown trout.

Cloning of four brown trout samples resulted in 58 successfully transformed clones whose sequences matched 97.89%–100% *S. trutta* sequences in BLAST (Ye et al., 2006). *Aplochiton* spp. cloning resulted in the successful transformation of 84 clones from nine eDNA samples (N = 2 *A. zebra*, N = 3 *A. taeniatus*, and N = 2 mixed samples), 78 matching 89.66%–100% *A. zebra*, and six matching 91.67%–100% *A. taeniatus* in BLAST, confirming the species identity of the peaks at each of the melting temperatures. Only *A. zebra* sequences were identified in the mixed samples, and non-specific amplification was observed in the remaining clones.

In the final model of the GLM, analyzing potential factors affecting amplification success total water volume sampled was the sole significant predictor (estimate = 0.005, SE = 0.002, t = 2.293, p = .022, AIC = 49.586), indicating that larger volumes of water were more likely to yield successful amplifications (see Table S3 for intermediate model outputs and AICs).

4 | DISCUSSION

The application of our novel AzebAtaeCytb assay allowed us to detect the presence of two threatened galaxiids, which coexisted in some of the sampling locations, and confirmed their presence at three rivers where they had previously been detected with conventional sampling. In addition, using our StruttaCytb assay, we detected brown trout DNA in six rivers, including three where they had not previously been sampled. The assays were validated by sequencing and visual identification.

We failed to detect zebra trout in three rivers where they had previously been identified, including two where the species previously were found to coexist with brown trout. This failure to detect coexistence could be due to brown trout outcompeting native zebra trout, as seen in other streams throughout the Falklands and other counties (Garcia de Leaniz et al., 2010; Valiente et al., 2010). It is possible that the trout caught in Findley Creek Stream and House Creek were new invaders into these areas during the first sampling and, therefore, coexistence

TABLE 2 Previous and current presence/absence data for the three study species at all sampling sites based on previous sampling using electrofishing and on current sampling using eDNA

				Zebra trout current presence			
Waterbody	**Site. No**	**Previously sampled**	**Zebra trout previously present**	***Aplochiton zebra***	***Aplochiton taeniatus***	***Salmo trutta* previously present**	***Salmo trutta* current presence**
Johns Brook	R1	NA	NA	N	N	NA	Y
Monty Deans Creek	R2	1999	N	N	N	N	N
Spots Arroyo	R3	2009	Y	N	Y	N	N
Findley Creek Stream	R4	2011	Y	N	N	Y	N
North West Arm House Stream[a]	R5	2012	Y	Y	Y	N	N
Fish Creek (2)	R6	2012	Y	Y	Y	N	N
Fish Creek (1)	R8	2012	Y	N	N	N	N
House Creek	R9	1999	Y	N	N	Y	N
San Carlos[b]	R10	1999	N	N	N	Y	Y
Elephant Beach Pond Stream	R11	1999	N	N	N	Y	Y
Estancia Creek	R13	2008	N	N	N	Y	N
Herbert Stream[b]	R14	1999	N	N	N	Y	N
Teal House River	R15	NA	NA	N	N	NA	Y
Chartres River	R16	1999	N	N	N	Y	N
Doctors Creek[b]	R17	2012	N	N	N	Y	Y
Malo Arroyo[b]	R18	NA	NA	N	N	NA	N
Neil Clark Nature Reserve	R19	NA	NA	N	N	NA	Y
Spring Point	R20	NA	NA	N	N	NA	N
Whiskey Creek Stream	R22	2009	Y	N	N	N	N

Abbreviations: N, Species not present/detected; Y, species present.

[a]Zebra trout seen during eDNA sampling.

[b]Brown trout caught/seen during eDNA sampling period.

between these species may have been short-lived. However, failure to detect brown trout and zebra trout at rivers where they had previously been found using traditional methods also could be due to low filtration volume, as filtering larger volumes of water increases eDNA capture (Deiner et al., 2015; Muha et al., 2019) and may facilitate detection of rare species and populations (Turner et al., 2014). Although we were able to detect all target species using relatively small volumes of water (100–200 ml per replicate), which were previously shown to be sensitive enough to detect rare species (Robinson, Garcia de Leaniz, Rolla et al., 2019), our analysis indicated that amplifications were affected by the total volume filtered, with detections being more likely with higher volumes (Egeter et al., 2018; Schultz & Lance, 2015; Turner et al., 2014). Therefore, we suggest filtering larger water volumes, at least 1 L per replicate, to maximize detection of rarer target species (Capo et al., 2019; Mächler et al., 2016).

Weather conditions might also have played a role in the detection rates, as sampling was carried out across two field seasons, the first April-May 2018 (Autumn) and the second September-October 2018 (Spring), coinciding with high volume of rain and snowmelt, resulting in more water and faster flowing rivers than in the first sampling season. These high/fast flowing conditions could have led to DNA being flushed out/downstream more quickly, potentially reducing the probability of detecting target species' DNA (Laramie et al., 2015; Pilliod et al., 2014). In addition, seasonal changes in eDNA concentration can occur with breeding, whereby DNA is released into the environment with gametes (Buxton et al., 2017; Doi et al., 2017). Environmental factors such as temperature also can have seasonal impacts, with temperature not only influencing the release of DNA through increased activity, but also impacting its degradation rates (Buxton et al., 2017; Lacoursière-Roussel, Rosabal et al., 2016). However, statistical analyses indicated that season had no effect on amplification, so sampling in two different seasons did not seem to have affected the detection probability in this case. In addition, the spatial distribution and densities of individuals in a river could affect the detection of target DNA, if animals congregated in a specific area and water movement resulted in the clumping of DNA (Furlan et al., 2016). Finally, it is possible that we were not able to detect the presence of brown trout and zebra trout in some streams because they no longer inhabited those areas.

Our analyses distinguished between the morphologically similar *A. zebra* and *A. taeniatus*, enabling the determination of species assemblages when either or both species are present, highlighting the sensitivity of qPCR-based methods over traditional approaches (Evans et al., 2017; Wilcox et al., 2013). Previously, morphological identification was mainly based on stomach size and length, and dorsal spots; however, individuals can lack color patterns especially when small and this colouration should be interpreted with caution (Alò et al., 2013). In addition, identifying species through stomach size and length (Mcdowall & Nakaya, 1988) requires destructive sampling, which is not ideal when working with a threatened species (Barnett et al., 2010; Jardine et al., 2011). Although it is possible to identify *Aplochiton* spp. though DNA barcoding of tissue samples (e.g., fin clips and muscle), this type of sampling could increase mortality as it requires capturing and handling individuals (Vanhaecke et al., 2012), it is more time consuming than collecting water, particularly for rare species such as zebra trout (Reynolds et al., 2003), and is not appropriate endangered species (Falkland Islands Government, 1999;Sanderson et al., 2009).

The introduction of brown trout to the Falkland Islands has posed many risks to the native galaxiids, and the impacts can be seen in all three native species (*Galaxias maculatus* and both *Aplochiton* species) (McDowall et al., 2001; Ross, 2009). Since the introduction of brown trout, zebra trout abundance and distribution have shown a marked decline that resulted in the species being considered threatened in the Falklands (Falkland Islands Government, 1999; McDowall et al., 2001; Ross, 2009). Although we did not detect any coexistence of brown trout and zebra trout in our study, their co-occurrence had been previously observed in the Falkland Islands (McDowall et al., 2001) and in Patagonia, where brown trout has caused dietary changes and decreased body condition in both species of zebra trout (Elgueta et al., 2013).

We also found eDNA from both *Aplochiton* species in two locations where their coexistence had not been previously observed (Vanhaecke et al., 2012). Such species mixing could lead to increased hybridization, known to occur at very low frequencies (Vanhaecke et al., 2012), potentially resulting in outbreeding depression, demographic swamping, and/or genetic assimilation (Esa et al., 2000; Wolf et al., 2001). Hybridization effects of invasions have been observed in pupfish (*Cyprinodon bovinus*) in Texas and Mozambique tilapia (*Oreochromis mossambicus*) in southern Africa where native and invasive species are hybridizing (Echelle & Echelle, 1997; Firmat et al., 2013), and also in New Zealand where introgression between two species of native galaxiid (*Galaxias depressiceps* and *Galaxias sp D*) has been human induced (Esa et al., 2000). It is unknown whether hybrids between *A. zebra* and *A. taeniatus* would be viable, but further research on the potential risks is needed.

To protect the native galaxiids in the Southern Hemisphere, it is important to determine their current distribution and that of invasive salmonids, for which eDNA provides an efficient and cost effective non-invasive tool, as in many recent conservation and monitoring programs (Jerde et al., 2011; Rees et al., 2014). This is particularly valuable in remote/inaccessible areas (Lacoursière-Roussel et al., 2018), such as the Falklands, where it can be very difficult and costly to access and sample using traditional methods due to the limited road network. Information on remaining refugia for galaxiids can be used to prioritize sites for conservation (McGeoch et al., 2016), for example in designating nature reserves and/or Ramsar sites, implementing semi-permeable fish barriers that allow movement of only small native fishes or physically removing brown trout from galaxiid refuges (Chadderton, 2001).

In summary, using newly developed non-destructive eDNA assays, we identified brown trout in locations where it had previously been undetected, suggesting potential expansion of the species in the Falklands, and also detected the coexistence of both *Aplochiton* species. With further optimization, such as using synthetic genes at known concentrations (Wilcox et al., 2013), it may be possible to gain relative estimates of species abundance using qPCR (Lacoursière-Roussel, Côté et al., 2016; Lodge et al., 2012), although our results indicate that water volume is critical for the detection sensitivity. These tools can be used to monitor both threatened galaxiids and invasive brown trout and have the potential to inform conservation managers on their range expansion or contraction to better target areas for intervention (Rees et al., 2014).

ACKNOWLEDGEMENTS

We thank SAERI and their staff for hosting the researcher and providing support with logistics and all the landowners in the Falklands that allowed us to sample on their land. We are grateful for those who assisted with sampling; Amy Guest, Connor McLeod, Megan Boldenow, Peter Nightingale, Nickolas Bonner, Mike Evans, Katherine Ross, Emma Phillips, and Fraser Gould. We also thank Chloe Robinson for assisting with sampling design and Tamsyn Uren Webster for providing support with primer design and testing.

CONFLICT OF INTEREST

Authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

SC, GCL, and PB conceived the idea. JFM carried out the sampling, the laboratory work and the analyses with help from SC. CGL and PB secured the funding. JFM and SC led the writing of the manuscript and all authors contributed critically to the drafts and final version.

ETHICAL APPROVAL

Sampling has been conducted following Home Office regulations and approved by Swansea University Ethics Committees under approval No. 160118/463, 160118/307 and 160118/299.

DATA AVAILABILITY STATEMENT

All raw data are included both in the manuscript and Appendix S1. Sequences produced to confirm eDNA products have been submitted to Genbank Accession numbers: MT858958 - MT859015; MT859016 - MT859095; MT873599 - MT873604.

ORCID

Jessica F. Minett https://orcid.org/0000-0001-6449-0801
Carlos Garcia de Leaniz https://orcid.org/0000-0003-1650-2729
Paul Brickle https://orcid.org/0000-0002-9870-3518
Sofia Consuegra https://orcid.org/0000-0003-4403-2509

REFERENCES

Alò, D., Correa, C., Arias, C., & Cárdenas, L. (2013). Diversity of Aplochiton fishes (Galaxiidea) and the taxonomic resurrection of *A. marinus*. *PLoS One*, *8*(8), e71577. https://doi.org/10.1371/journal.pone.0071577

Arismendi, I., Soto, D., Penaluna, B., Jara, C., Leal, C., & León-Muñoz, J. (2009). Aquaculture, non-native salmonid invasions and associated declines of native fishes in Northern Patagonian lakes. *Freshwater Biology*, *54*(5), 1135–1147. https://doi.org/10.1111/j.1365-2427.2008.02157.x

Arrowsmith, E., & Pentelow, F. T. K. (1965). The introduction of trout and salmon to the Falkland Islands. *Salmon and Trout Magazine*, 119–129.

Barnett, A., Redd, K. S., Frusher, S. D., Stevens, J. D., & Semmens, J. M. (2010). Non-lethal method to obtain stomach samples from a large marine predator and the use of DNA analysis to improve dietary information. *Journal of Experimental Marine Biology and Ecology*, *393*, 188–192. https://doi.org/10.1016/j.jembe.2010.07.022

Behrens-Chapuis, S., Malewski, T., Suchecka, E., Geiger, M. F., Herder, F., & Bogdanowicz, W. (2018). Discriminating European cyprinid specimens by barcode high-resolution melting analysis (Bar-HRM)—A cost efficient and faster way for specimen assignment? *Fisheries Research*, *204*, 61–73. https://doi.org/10.1016/j.fishres.2018.02.007

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K. L., Meier, R., Winker, K., Ingram, K. K., & Das, I. (2006). Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution*, *22*(3), 148–155. https://doi.org/10.1016/j.tree.2006.11.004

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R. A., Foster, J., Wilkinson, J. W., Arnell, A., Brotherton, P., Williams, P., & Dunn, F. (2015). Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). *Biological Conservation*, *183*, 19–28. https://doi.org/10.1016/j.biocon.2014.11.029

Bio-Rad. (2013). *CFX96 TouchTM, CFX96 Touch Deep WellTM, CFX ConnectTM, and CFX384 TouchTM Real-Time PCR Detection Systems - Instruction Manual*. Bio-Rad Laboratories, Inc., 1–178. http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10021337.pdf

Bohl, R. J., Henry, T. B., Strange, R. J., & Rakes, P. L. (2009). Effects of electroshock on cyprinid embryos: Implications for threatened and endangered fishes. *Transactions of the American Fisheries Society*, *138*, 768–776. https://doi.org/10.1577/t08-149.1

Burlakova, L. E., Karatayev, A. Y., Karatayev, V. A., May, M. E., Bennett, D. L., & Cook, M. J. (2011). Endemic species: Contribution to community uniqueness, effect of habitat alteration, and conservation priorities. *Biological Conservation*, *144*(1), 155–165. https://doi.org/10.1016/j.biocon.2010.08.010

Buxton, A. S., Groombridge, J. J., Zakaria, N. B., & Griffiths, R. A. (2017). Seasonal variation in environmental DNA in relation to population size and environmental factors. *Scientific Reports*, *7*, 1–9. https://doi.org/10.1038/srep46294

Capo, E., Spong, G., Königsson, H., & Byström, P. (2019). Effects of filtration methods and water volume on the quantification of brown trout (*Salmo trutta*) and Arctic char (*Salvelinus alpinus*) eDNA concentrations via droplet digital PCR. *Environmental DNA*, *2*, 152–160. https://doi.org/10.1002/edn3.52

Carlsson, J. E. L., Egan, D., Collins, P. C., Farrell, E. D., Igoe, F., & Carlsson, J. (2017). A qPCR MGB probe based eDNA assay for European freshwater pearl mussel (*Margaritifera margaritifera* L.). *Aquatic Conservation: Marine and Freshwater Ecosystems*, *27*(6), 1341–1344. https://doi.org/10.1002/aqc.2788

Chadderton, W. L. (2001). Management of invasive freshwater fish: striking the right balance! Managing Invasive Freshwater Fish in New Zealand. *Proceedings of a Workshop Hosted by Department of Conservation*, *10-12*, 71–83.

Consuegra, S., Phillips, N., Gajardo, G., & Garcia de Leaniz, C. (2011). Winning the invasion roulette : Escapes from fish farms increase admixture and facilitate establishment of non-native rainbow trout. *Evolutionary Applications*, *4*, 660–671. https://doi.org/10.1111/j.1752-4571.2011.00189.x

Darling, J. A., & Blum, M. J. (2007). DNA-based methods for monitoring invasive species: A review and prospectus. *Biological Invasions*, *9*, 751–765. https://doi.org/10.1007/s10530-006-9079-4

Deiner, K., Walser, J. C., Mächler, E., & Altermatt, F. (2015). Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biological Conservation*, *183*, 53–63. https://doi.org/10.1016/j.biocon.2014.11.018

Díaz-Ferguson, E., Herod, J., Galvez, J., & Moyer, G. (2014). Development of molecular markers for eDNA detection of the invasive African jewelfish (*Hemichromis letourneuxi*): A new tool for monitoring aquatic invasive species in National Wildlife Refuges. *Management of Biological Invasions*, *5*(2), 121–131. https://doi.org/10.3391/mbi.2014.5.2.05

Doi, H., Inui, R., Akamatsu, Y., Kanno, K., Yamanaka, H., Takahara, T., & Minamoto, T. (2017). Environmental DNA analysis for estimating the abundance and biomass of stream fish. *Freshwater Biology*, *62*, 30–39. https://doi.org/10.1111/fwb.12846

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z.-I., Knowler, D. J., Lévêque, C., Naiman, R. J., Prieur-Richard, A.-H., Soto, D., Stiassny, M. L. J., & Sullivan, C. A. (2006). Freshwater biodiversity: Importance, threats, status and conservation challenges. *Biological Reviews of the Cambridge Philosophical Society*, *81*, 163–182. https://doi.org/10.1017/S1464793105006950

Echelle, A. A., & Echelle, A. F. (1997). Genetic introgression of endemic taxa by non-natives: A case study with Leon Springs pupfish and sheepshead minnow. *Conservation Biology*, *11*(1), 153–161. https://doi.org/10.1046/j.1523-1739.1997.95427.x

Egeter, B., Peixoto, S., Brito, J. C., Jarman, S., Puppo, P., & Velo-Antón, G. (2018). Challenges for assessing vertebrate diversity in turbid Saharan water-bodies using environmental DNA. *Genome*, *61*, 807–814. https://doi.org/10.1139/gen-2018-0071

Elgueta, A., González, J., Ruzzante, D. E., Walde, S. J., & Habit, E. (2013). Trophic interference by *Salmo trutta* on *Aplochiton zebra* and *Aplochiton taeniatus* in southern Patagonian lakes. *Journal of Fish Biology*, *82*, 430–443. https://doi.org/10.1111/j.1095-8649.2012.03489.x

Ellender, B. R., & Weyl, O. L. F. (2014). A review of current knowledge, risk and ecological impacts associated with non-native freshwater fish introductions in South Africa. *Aquatic Invasions*, *9*(2), 117–132. https://doi.org/10.3391/ai.2014.9.2.01

Emenyeonu, L. C., Croxford, A. E., & Wilkinson, M. J. (2018). The potential of aerosol eDNA sampling for the characterisation of commercial seed lots. *PLoS One*, *13*(8), 1–18. https://doi.org/10.1371/journal.pone.0201617

Esa, Y. B., Waters, J. M., & Wallis, G. P. (2000). Introgressive hybridization between *Galaxias depressiceps* and *Galaxias sp* D (Teleostei: Galaxiidae) in Otago, New Zealand: Secondary contact mediated by water races. *Conservation Genetics*, *1*, 329–339. https://doi.org/10.1023/A:1011511418644

Evans, N. T., Shirey, P. D., Wieringa, J. G., Mahon, A. R., & Lamberti, G. A. (2017). Comparative cost and effort of fish distribution detection via environmental DNA analysis and electrofishing. *Fisheries*, *42*(2), 90–99. https://doi.org/10.1080/03632415.2017.1276329

Falkland Islands Government (1999). Conservation of wildlife and nature ordiance. *The Falkland Islands Gazette Supplement*, *10*, 2–18.

Firmat, C., Alibert, P., Losseau, M., Baroiller, J. F., & Schliewen, U. K. (2013). Successive invasion-mediated interspecific hybridizations and population structure in the endangered cichlid *Oreochromis mossambicus*. *PLoS One*, *8*(5), e63880. https://doi.org/10.1371/journal.pone.0063880

Fowler, D. M. (2013). *Brown trout in the Falklands: Origin, Life history, and public attitudes to an invasive species*. MRes Thesis. Swansea University.

Furlan, E. M., Gleeson, D., Hardy, C. M., & Duncan, R. P. (2016). A framework for estimating the sensitivity of eDNA surveys. *Molecular Ecology Resources*, *16*, 641–654. https://doi.org/10.1111/1755-0998.12483

Garcia de Leaniz, C., Gajardo, G., & Consuegra, S. (2010). From best to pest: Changing perspectives on the impact of exotic salmonids in the southern hemisphere. *Systematics and Biodiversity*, *8*(4), 447–459. https://doi.org/10.1080/14772000.2010.537706

Gozlan, R. E., Britton, J. R., Cowx, I., & Copp, G. H. (2010). Current knowledge on non-native freshwater fish introductions. *Journal of Fish Biology*, *76*(4), 751–786. https://doi.org/10.1111/j.1095-8649.2010.02566.x

Habit, E., Gonzalez, J., Ruzzante, D. E., & Walde, S. J. (2012). Native and introduced fish species richness in Chilean Patagonian lakes: Inferences on invasion mechanisms using salmonid-free lakes. *Diversity and Distributions*, *18*, 1153–1165. https://doi.org/10.1111/j.1472-4642.2012.00906.x

Habit, E., Piedra, P., Ruzzante, D. E., Walde, S. J., Belk, M. C., Cussac, V. E., Gonzalez, J., & Colin, N. (2010). Changes in the distribution of native fishes in response to introduced species and other anthropogenic effects. *Global Ecology and Biogeography*, *19*, 697–710. https://doi.org/10.1111/j.1466-8238.2010.00541.x

Hall, T. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*(41), 95–98.

Hardie, S. A., Jackson, J. E., Barmuta, L. A., & White, R. W. G. (2006). Status of galaxiid fishes in Tasmania, Australia: Conservation listings, threats and management issues. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *16*, 235–250. https://doi.org/10.1002/aqc.722

Harper, K. J., Goodwin, K. D., Harper, L. R., LaCasella, E. L., Frey, A., & Dutton, P. H. (2020). Finding Crush: Environmental DNA Analysis as a Tool for Tracking the Green Sea Turtle *Chelonia mydas* in a Marine Estuary. *Frontiers in Marine Science*, *6*, 1–13. https://doi.org/10.3389/fmars.2019.00810

Hobbs, J. P. A., Jones, G. P., & Munday, P. L. (2011). Extinction risk in endemic marine fishes. *Conservation Biology*, *25*(5), 1053–1055. https://doi.org/10.1111/j.1523-1739.2011.01698.x

Huxel, G. R. (1999). Rapid displacement of native species by invasive species: Eeffects of hybridization. *Biological Conservation*, *89*(2), 143–152. https://doi.org/10.1016/S0006-3207(98)00153-0

Jardine, T. D., Hunt, R. J., Pusey, B. J., & Bunn, S. E. (2011). A non-lethal sampling method for stable carbon and nitrogen isotope studies of tropical fishes. *Marine and Freshwater Research*, *62*(1), 83–90. https://doi.org/10.1071/MF10211

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). “Sight-unseen” detection of rare aquatic species using environmental DNA. *Conservation Letters*, *4*(2), 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Kadye, W. T., Chakona, A., Marufu, L. T., & Samukange, T. (2013). The impact of non-native rainbow trout within Afro-montane streams in eastern Zimbabwe. *Hydrobiologia*, *720*(1), 75–88. https://doi.org/10.1007/s10750-013-1624-4

Korsu, K., Huusko, A., & Muotka, T. (2007). Niche characteristics explain the reciprocal invasion success of stream salmonids in different continents. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(23), 9725–9729. https://doi.org/10.1073/pnas.0610719104

Lacoursière-Roussel, A., Côté, G., Leclerc, V., & Bernatchez, L. (2016). Quantifying relative fish abundance with eDNA: A promising tool for fisheries management. *Journal of Applied Ecology*, *53*(4), 1148–1157. https://doi.org/10.1111/1365-2664.12598

Lacoursière-Roussel, A., Howland, K., Normandeau, E., Grey, E. K., Archambault, P., Deiner, K., & Bernatchez, L. (2018). eDNA metabarcoding as a new surveillance approach for coastal Arctic biodiversity. *Ecology and Evolution*, *8*, 7763–7777. https://doi.org/10.1002/ece3.4213

Lacoursière-Roussel, A., Rosabal, M., & Bernatchez, L. (2016). Estimating fish abundance and biomass from eDNA concentrations: Variability among capture methods and environmental conditions. *Molecular Ecology Resources*, *16*, 1401–1414. https://doi.org/10.1111/1755-0998.12522

Laramie, M. B., Pilliod, D. S., & Goldberg, C. S. (2015). Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biological Conservation*, *183*, 29–37. https://doi.org/10.1016/j.biocon.2014.11.025

Lodge, D. M., Turner, C. R., Jerde, C. L., Barnes, M. A., Chadderton, L., Egan, S. P., Feder, J. L., Mahon, A. R., & Pfrender, M. E. (2012).

Conservation in a cup of water: Estimating biodiversity and population abundance from environmental DNA. *Molecular Ecology*, *21*, 2555–2558. https://doi.org/10.1111/j.1365-294X.2012.05600.x

Lowe, S., Browne, M., Boudjelas, S., & De Poorter, M. (2000). *100 of the worlds worst invasive alien species: A selection from the global invasive species database*, Vol. *12*. Invasive Species Specialist Group.

Macchi, P. J., Pascual, M. A., & Vigliano, P. H. (2007). Differential piscivory of the native Percichthys trucha and exotic salmonids upon the native forage fish *Galaxias maculatus* in Patagonian Andean lakes. *Limnologica*, *37*(1), 76–87. https://doi.org/10.1016/j.limno.2006.09.004

Mächler, E., Deiner, K., Spahn, F., & Altermatt, F. (2016). Fishing in the water: Effect of sampled water volume on environmental DNA-based detection of macroinvertebrates. *Environmental Science and Technology*, *50*, 305–312. https://doi.org/10.1021/acs.est.5b04188

Mcdowall, A. R. M., & Nakaya, K. (1988). Morphological divergence in the two species of *Aplochiton* Jenyns (Salmoniformes : Aplochitonidae): A generalist and a specialist. *American Society of Ichthyologists and Herpetologists*, *1988*(1), 233–236.

McDowall, R. M. (2006). Crying wolf, crying foul, or crying shame: Alien salmonids and a biodiversity crisis in the southern cool-temperate galaxioid fishes? *Reviews in Fish Biology and Fisheries*, *16*, 233–422. https://doi.org/10.1007/s11160-006-9017-7

McDowall, R. M., Allibone, R. M., & Chadderton, W. L. (2001). Issues for the conservation and management of Falkland Islands fresh water fishes. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *11*(6), 473–486. https://doi.org/10.1002/aqc.499

McGeoch, M. A., Genovesi, P., Bellingham, P. J., Costello, M. J., McGrannachan, C., & Sheppard, A. (2016). Prioritizing species, pathways, and sites to achieve conservation targets for biological invasion. *Biological Invasions*, *18*(2), 299–314. https://doi.org/10.1007/s10530-015-1013-1

McIntosh, A. R., McHugh, P. A., Dunn, N. R., Goodman, J. M., Howard, S. W., Jellyman, P. G., & Woodford, D. J. (2010). The impact of trout on galaxiid fishes in New Zealand. *New Zealand Journal of Ecology*, *34*(1), 195–206.

Miranda, L. E., & Kidwell, R. H. (2010). Unintended effects of electrofishing on nongame fishes. *Transactions of the American Fisheries Society*, *139*(5), 1315–1321. https://doi.org/10.1577/t09-225.1

Muha, T. P., Robinson, C. V., Garcia de Leaniz, C., & Consuegra, S. (2019). An optimised eDNA protocol for detecting fish in lentic and lotic freshwaters using a small water volume. *PLoS One*, *14*(7), 1–20. https://doi.org/10.1371/journal.pone.0219218

Panek, F. M., & Densmore, C. L. (2011). *Electrofishing and the effects of depletion sampling on fish health: A review and recommendations for additional study* (pp. 299–308). Khaled Bin Sultan Living Oceans Foundation.

Penaluna, B. E., Arismendi, I., & Soto, D. (2009). Evidence of interactive segregation between introduced trout and native fishes in northern Patagonian rivers, Chile. *Transactions of the American Fisheries Society*, *138*, 839–845. https://doi.org/10.1577/t08-134.1

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2014). Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources*, *14*(1), 109–116. https://doi.org/10.1111/1755-0998.12159

Rahel, F. J. (2007). Biogeographic barriers, connectivity and homogenization of freshwater faunas: It's a small world after all. *Freshwater Biology*, *52*, 696–710. https://doi.org/10.1111/j.1365-2427.2006.01708.x

Ramón-Laca, A., Gleeson, D., Yockney, I., Perry, M., Nugent, G., & Forsyth, D. M. (2014). Reliable discrimination of 10 ungulate species using high resolution melting analysis of faecal DNA. *PLoS One*, *9*(3), 1–8. https://doi.org/10.1371/journal.pone.0092043

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., & Gough, K. C. (2014). The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, *51*, 1450–1459. https://doi.org/10.1111/1365-2664.12306

Reid, A. J., Carlson, A. K., Creed, I. F., Eliason, E. J., Gell, P. A., Johnson, P. T. J., Kidd, K. A., MacCormack, T. J., Olden, J. D., Ormerod, S. J., Smol, J. P., Taylor, W. W., Tockner, K., Vermaire, J. C., Dudgeon, D., & Cooke, S. J. (2019). Emerging threats and persistent conservation challenges for freshwater biodiversity. *Biological Reviews*, *94*, 849–873. https://doi.org/10.1111/brv.12480

Reynolds, L., Herlihy, A. T., Kaufmann, P. R., Gregory, S. V., & Hughes, R. M. (2003). Electrofishing effort requirements for assessing species richness and biotic integrity in western Oregon streams. *North American Journal of Fisheries Management*, *23*(2), 450–461. https://doi.org/10.1577/1548-8675(2003)023<0450:eerfas>2.0.co;2

Robinson, C. V., Garcia de Leaniz, C., & Consuegra, S. (2019). Effect of artificial barriers on the distribution of the invasive signal crayfish and Chinese mitten crab. *Scientific Reports*, *9*(1), 1–11. https://doi.org/10.1038/s41598-019-43570-3

Robinson, C. V., Garcia de Leaniz, C., Rolla, M., & Consuegra, S. (2019). Monitoring the eradication of the highly invasive topmouth gudgeon (*Pseudorasbora parva*) using a novel eDNA assay. *Environmental DNA*, *1*(1), 74–85. https://doi.org/10.1002/edn3.12

Robinson, C. V., Uren Webster, T. M., Cable, J., James, J., & Consuegra, S. (2018). Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA. *Biological Conservation*, *222*, 241–252. https://doi.org/10.1016/j.biocon.2018.04.009

Ross, K. (2009). Freshwater fish in the Falklands: Conservation of native zebra trout. In A report for the Falkland Islands Government and Falklands Conservation. 36 pp.

Sanderson, B. L., Tran, C. D., Coe, H. J., Pelekis, V., Steel, E. A., & Reichert, W. L. (2009). Nonlethal sampling of fish caudal fins yields valuable stable isotope data for threatened and endangered fishes. *Transactions of the American Fisheries Society*, *138*, 1166–1177. https://doi.org/10.1577/T08-086.1

Schultz, M. T., & Lance, R. F. (2015). Modeling the sensitivity of field surveys for detection of environmental DNA (eDNA). *PLoS One*, *10*(10), 1–16. https://doi.org/10.1371/journal.pone.0141503

Soto, D., Arismendi, I., González, J., Sanzana, J., Jara, F., Jara, C., Guzman, E., & Lara, A. (2006). Southern Chile, trout and salmon country: Invasion patterns and threats for native species. *Revista Chilena De Historia Natural*, *79*(1), 97–117. https://doi.org/10.4067/S0716-078X2006000100009

Stewart, 1973Stewart, L. (1973). *Fisheries of the Falkland Islands*. Overseas Development Administration.

Turner, C. R., Barnes, M. A., Xu, C. C. Y., Jones, S. E., Jerde, C. L., & Lodge, D. M. (2014). Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and Evolution*, *5*(7), 676–684. https://doi.org/10.1111/2041-210X.12206

Valiente, A. G., Ayllon, F., Nuñez, P., Juanes, F., & Garcia-Vazquez, E. (2010). Not all lineages are equally invasive: Genetic origin and life-history in Atlantic salmon and brown trout acclimated to the Southern Hemisphere. *Biological Invasions*, *12*(10), 3485–3495. https://doi.org/10.1007/s10530-010-9746-3

Vanhaecke, D., Garcia de Leaniz, C., Gajardo, G., Dunham, J., Giannico, G., & Consuegra, S. (2015). Genetic signatures of historical dispersal of fish threatened by biological invasions: The case of galaxiids in South America. *Journal of Biogeography*, *42*(10), 1942–1952. https://doi.org/10.1111/jbi.12568

Vanhaecke, D., Garcia de Leaniz, C., Gajardo, G., Young, K., Sanzana, J., Orellana, G., Fowler, D., Howes, P., Monzon-Arguello, C., & Consuegra, S. (2012). DNA barcoding and microsatellites help species delimitation and hybrid identification in endangered galaxiid fishes. *PLoS One*, *7*(3), e32939. https://doi.org/10.1371/journal.pone.0032939

Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R., & Schwartz, M. K. (2013). Robust detection of rare species using environmental DNA: The importance of primer specificity. *PLoS One*, *8*(3), e59520. https://doi.org/10.1371/journal.pone.0059520

Wittwer, C. T. (2009). High-resolution DNA melting analysis: Advancements and limitations. *Human Mutation*, *30*(6), 857–859. https://doi.org/10.1002/humu.20951

Wolf, D. E., Takebayashi, N., & Rieseberg, L. H. (2001). Predicting the risk of extinction through hybridization. *Conservation Biology*, *15*(4), 1039–1053. https://doi.org/10.1046/j.1523-1739.2001.0150041039.x

Woodford, D. J., & Impson, N. D. (2004). A preliminary assessment of the impact of alien rainbow trout (*Oncorhynchus mykiss*) on indigenous fishes of the upper Berg River, Western Cape Province, South Africa. *African Journal of Aquatic Science*, *29*(1), 107–111. https://doi.org/10.2989/16085910409503799

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, *13*, 1–11. https://doi.org/10.1186/1471-2105-13-134

Ye, J., McGinnis, S., & Madden, T. L. (2006). BLAST: improvements for better sequence analysis. *Nucleic Acids Research*, *34*(Web Server), W6–W9. https://doi.org/10.1093/nar/gkl164

Young, K. A., Dunham, J. B., Stephenson, J. F., Terreau, A., Thailly, A. F., Gajardo, G., & Garcia de Leaniz, C. (2010). A trial of two trouts: Comparing the impacts of rainbow and brown trout on a native galaxiid. *Animal Conservation*, *13*(4), 399–410. https://doi.org/10.1111/j.1469-1795.2010.00354.x

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Minett JF, de Leaniz CG, Brickle P, Consuegra S. A new high-resolution melt curve eDNA assay to monitor the simultaneous presence of invasive brown trout (*Salmo trutta*) and endangered galaxiids. *Environmental DNA*. 2020;00:1–12. https://doi.org/10.1002/edn3.151