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ORIGINAL ARTICLE

Environmental DNA detection of the giant freshwater crayfish (*Astacopsis gouldi*)

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Abstract

The giant freshwater crayfish, *Astacopsis gouldi* Clark, 1936, is an endangered endemic freshwater species native to Northern Tasmania. Habitat loss, fishing pressure, and climate change have been identified as threatening processes. The Australian government approved a recovery plan for *A. gouldi* in 2017 that requires routine population surveys to assess the impact of ongoing threats and recovery actions. We developed a novel species-specific probe-based assay targeting a 219 bp fragment in the Cytochrome Oxidase 1 gene region to detect traces of *A. gouldi* DNA in environmental samples as a cost-effective, sensitive, and non-invasive surveillance method to assess the presence of this endangered species. We tested assay specificity against ten crayfish species commonly found in Tasmania within the *Astacopsis*, *Cherax*, *Geocharax*, *Engaeus*, and *Ombrastacoides* genera and determined assay sensitivity using tissue-derived genomic DNA and synthetic oligo standards designed for *A. gouldi*. We then tested water samples collected from aquaria and natural freshwater streams in Northern Tasmania with known occurrence of *A. gouldi*, as well as one site with no known *A. gouldi* occurrence. The probe-based assay designed in this study successfully detected *A. gouldi* DNA and eDNA with a 10 copies/µl limit of detection and showed no amplification of non-targeted co-existing crayfish species. We successfully detected the presence of *A. gouldi* eDNA in water samples from six sites with known occurrences of the species. There was no detection from the negative site. This study validates the use of eDNA-based detection of *A. gouldi* by real-time PCR as a non-invasive monitoring tool to assist field monitoring, assessment, and complement ongoing recovery actions to protect habitable ecosystems of *A. gouldi*.

KEYWORDS
conservation, ecology, eDNA, endangered species, fresh water, Parastacidae

1 | INTRODUCTION

Recent advances in molecular diagnostics include the use of environmental DNA-based technology to detect and monitor single species or communities (Bohmann et al., 2014; Thomsen & Willerslev, 2015). Environmental DNA (eDNA) describes genetic material extracted from environmental samples (water, sediments, ice, and air) (Pilliod et al., 2013). The technique relies on the identification of an organism's DNA in the samples to indicate presence or absence (Pedersen et al., 2015). Several researchers have demonstrated the benefit of eDNA monitoring over conventional methods, including its sensitivity, non-invasiveness, and cost-efficiency (Hinlo et al., 2017; Shaw et al., 2016; Smart et al., 2016). Environmental DNA-based methods are particularly advantageous when dealing with species that are difficult to sample or identify, such as rare or cryptic species, species complexes as well as larval or juvenile stages (Deiner et al., 2017; Ruppert et al., 2019). Because it is well suited for low-density detection (Hayes et al., 2005; Nathan et al., 2014), eDNA-based monitoring is particularly applicable for monitoring animal populations in decline, such as threatened or vulnerable species (Hunter et al., 2018; Jerde et al., 2011).

Australia hosts diverse crayfish fauna, many species of which are threatened. Among them is the giant freshwater crayfish *Astacopsis gouldi* (Clark, 1936), which is considered the largest freshwater crayfish in the world (Walsh & Walsh, 2012). Native to Tasmania's northern river drainages, *A. gouldi* is currently listed as Endangered in the IUCN Red List (IUCN, 2020; Sinclair et al., 2011). Like other crayfish species undergoing population decline globally (Richman et al., 2015), habitat disturbance or modification, fishing pressure, and climate-mediated threats such as droughts and floods have been identified as current ongoing threats to the remaining populations of *A. gouldi* (Commonwealth of Australia, 2017). In 2017, the recovery plan for *A. gouldi* recommended population monitoring to understand movement and genetic connectivity to assess population trends and responses to threatening processes and recovery actions (Commonwealth of Australia, 2017). A sensitive, rapid, and cost-effective method to monitor *A. gouldi* across its distribution is essential for efficient monitoring and timely management interventions.

Environmental DNA-based detection has already been applied to several crayfish species including the signal crayfish *Pacifastacus leniusculus* (Dana, 1852), Japanese crayfish *Cambaroides japonicus* (De Haan, 1841), red swamp crayfish *Procambarus clarkii* (Girard, 1852), spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817), Shasta crayfish *Pacifastacus fortis* (Faxon, 1914), and the rusty crayfish *Faxonius rusticus* (Girard, 1852) (Cowart et al., 2018; Harper et al., 2018; Ikeda et al., 2016; Mauvisseau et al., 2018; Tréguier et al., 2014). Despite concerns that eDNA detection of invertebrates may be challenging due to presence of exoskeletons which may hamper eDNA shedding (Curtis & Larson, 2020; Dunn et al., 2017; Tréguier et al., 2014), as well as poor assay performance when detecting other rare freshwater crayfish species (i.e., *Procambarus clarkia* (Girard, 1852); Tréguier et al., 2014, and *Faxonius eupunctus* Williams, 1952; Rice et al., 2018), studies demonstrate that eDNA-based tools can be effective for crayfish surveillance, even at low densities (Dougherty et al., 2016; Harper et al., 2018). In this study, we developed and validated a sensitive and specific qPCR probe-based assay to enable detection of *A. gouldi* from eDNA water samples. We designed primers and probes specific to *A. gouldi* and tested their specificity and sensitivity in the laboratory. We then applied the method to water samples collected from aquaria and natural freshwater streams in Tasmania where *A. gouldi* is known to occur. In addition, we sampled from a field site with no known *A. gouldi* presence to serve as a negative control. We demonstrate that eDNA-based detection can enhance field monitoring of *A. gouldi*, potentially leading to a more cost-effective survey method and better management for this threatened species.

2 | METHODS

2.1 | Primer development/Assay design

Accessioned tissue samples of *A. gouldi* from across their geographic range, including samples of co-occurring congeneric (*Astacopsis franklinii* and *A. tricornis*) and confamilial species were obtained from the Tasmanian Museum and Art Gallery (TMAG), and the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Todd Walsh Collection. Wild *Astacopsis* and *Engaeus* spp. specimens were also collected by representatives of the Tasmanian Government Inland Fisheries Services (IFS collection permit no. 2019–41) with approval to collect threatened fauna for scientific purposes from the Department of Primary Industries, Parks, Water and Environment (Permit No. TFA 19084). A total of 37 samples representing 13 different species were then sent to the University of Canberra for extraction (Table 1). DNA from each sample was extracted using Qiagen's DNeasy Blood and Tissue Kit following the manufacturer's protocols for tissue samples. Next, a 710-bp region of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene was amplified using the invertebrate universal primers, LCO1490 and HCO2198 (Folmer et al., 1994) in 25 µl PCR reactions with 2.5 µl of DNA, 12.5 µl of MyTaq HS RedMix, 1 µl each of the forward and reverse primers (10 µM), and 8 µl of DEPC-treated water. Polymerase Chain Reaction (PCR) cycling conditions were set at 95°C for 3 min, followed by 35 cycles of 95°C (1 min), 52°C (1 min), and 72°C (1.30 min), followed by a final extension step at 72°C for 10 min on an Eppendorf Mastercycler Pro S. Amplifications were confirmed by gel electrophoresis, using 2% agarose gel stained with SYBR™ Safe DNA gel stain (Invitrogen). Amplified samples were sent to the Australian National University's Biomolecular Resources Facility (ANU-BRF) for Sanger sequencing and accessioned to the National Center for Biotechnology Information (NCBI; accessions W220040- MW220066).

Sequenced amplicons from this study were imported into Geneious 9.1.8 (https://www.geneious.com) and aligned with other CO1 sequences for *A. gouldi* and other *Astacopsis* species downloaded from Genbank. Regions where

TABLE 1 Samples of crayfish species sequenced by this study

Species	Sampling location	Sampling year	Source	University of Canberra voucher code	NCBI accession
Astacopsis gouldi	Frankland River NW Tas	2016	CSIRO (Todd Walsh collection)	AG001	MW220040
Astacopsis gouldi	Frankland River NW Tas	2016	CSIRO (Todd Walsh collection)	AG003	MW220041
Astacopsis gouldi	Frankland River NW Tas	2016	CSIRO (Todd Walsh collection)	AG010	MW220042
Astacopsis gouldi	Great Forester River NE Tas	2016	CSIRO (Todd Walsh collection)	AG016	MW220043
Astacopsis gouldi	Flowerdale River NW Tas	2016	CSIRO (Todd Walsh collection)	AG017	MW220044
Astacopsis gouldi	Flowerdale River NW Tas	2016	CSIRO (Todd Walsh collection)	AG019	MW220045
Astacopsis franklinii	Macquarie River, E Tas	2006	Utas - Anne Watson	AF001	[a]
Astacopsis franklinii	Simmonds Creek SE Tas	2019	Utas - Leon Barmuta	AF002	MW220046
Astacopsis franklinii	Simmonds Creek SE Tas	2019	Utas - Leon Barmuta	AF003	MW220047
Astacopsis franklinii	Simmonds Creek SE Tas	2019	Utas - Leon Barmuta	AF004	MW220048
Astacopsis franklinii	Simmonds Creek SE Tas	2019	Utas - Leon Barmuta	AF005	MW220049
Astacopsis franklinii	Simmonds Creek SE Tas	2019	Utas - Leon Barmuta	AF006	MW220050
Astacopsis franklinii	Simmonds Creek SE Tas	2019	Utas - Leon Barmuta	AF007	[a]
Astacopsis tricornis	Shannon River, central Tas	2012	TMAG anon	AT001	MW220051
Astacopsis tricornis	Lake St Clair, central Tas	2001	TMAG M Driessen	AT002	[a]
Astacopsis tricornis	Lake St Clair, central Tas	2001	TMAG M Driessen	AT003	MW220052
Astacopsis tricornis	Lake St Clair, central Tas	2001	TMAG M Driessen	AT004	[a]
Astacopsis tricornis	Lake St Clair, central Tas	2001	TMAG M Driessen	AT005	MW220053
Engaeus mairener	Lisle, NE Tas	2003	TMAG - FPA	EM001	MW220054
Engaeus mairener	Lisle, NE Tas	2003	TMAG - FPA	EM002	MW220055
Engaeus mairener	South Weld, SW Tas	2011	TMAG - FPA	EM003	MW220056
Engaeus mairener	Underwood, NE Tas	2008	TMAG - FPA	EM004	[a]
Engaeus granulatus	Shearwater, N Tas	2006	TMAG - FPA	EG001	[a]
Engaeus fossor	Gog Range, N Tas	2004	TMAG - FPA	EF001	MW220057
Engaeus fossor	Horton R, NW Tas	2015	TMAG - FPA	EF002	MW220058
Engaeus fossor	Takone, NW Tas	2008	TMAG - FPA	EF003	MW220059
Engaeus fossor	Tewkesbury, NW Tas	2011	TMAG - FPA	EF004	[a]
Engaeus yabbimunna	Somerset, N Tas	2006	TMAG - FPA	EY001	MW220060
Engaeus cunicularius	Tarkine, NW Tas	2015	TMAG - Tarkine Bioblitz	EC001	MW220061
Engaeus lengana	Arthur/Pieman, NW Tas	2015	TMAG - Tarkine Bioblitz	EL001	MW220062
Geocharax tasmanicus	17 Mile Plain, NW Tas	2010	TMAG - AMMR - BushBioblitz	GT001	MW220063
Ombrastacoides huonensis	North Weld Rd, SW Tas	2007	TMAG - FPA	OH001	MW220064
Ombrastacoides sp.	Mt Wedge, SW Tas	2008	TMAG - FPA	OS001	[a]
Cherax destructor	Not recorded	1999	Utas collection - AMMR	CD001	[a]
Cherax destructor	Not recorded	1999	Utas collection - AMMR	CD002	MW220065
Engaeus nulloporius	Little Supply R, Glengarry N Tas	2019	Jo Lyall, NEST	EN001	MW220066

Note: Samples were provided by the Tasmanian Museum and Art Gallery (TMAG), CSIRO's Todd Walsh Collection, the University of Tasmania and local experts.

[a]DNA from these samples was heavily degraded and did not provide high-quality sequences for accession.

differences existed between species were identified and considered during the design of the forward and reverse primers and probe using the Geneious' primer design tool. Primer pair 269F (5′-CCCTTTAGCCGCCTCTATCG-3′) and 489R (5′-GCAAGGACTGGTAGCGAGAG-3′) which amplifies a 219 bp sequence of the CO1 gene were created. A Taqman probe specific for *A. gouldi* (292P, 5′-ATGCAGGAGCCTCAGTTGAC-3′) was also designed to bind within this region, labeled with a FAM reporter at the 5′ and with a Minor Groove Binder (MGB) quencher in the 3′ (Table 2). This primer pair and probe combination was selected

out of two combinations tested because it provided greater sensitivity, higher PCR efficiency, and high specificity during the primer testing.

2.2 | Specificity and sensitivity testing

Specificity of the primers was evaluated in silico by using the BLAST search function on the NCBI website, as well as in vitro using tissue-derived genomic DNA. We tested the assay on six *A. gouldi* tissue samples and 12 other crayfish species known to occur in Tasmania and south eastern Australia (Table 1). The Taqman qPCR was performed in 30 µl reactions consisting of 8 µl PCR water, 15 µl Taqman Environmental Master Mix (Applied Biosystems), 1 µl of each of forward and reverse primers (10 µM), 1 µl probe (10 µM), and 4 µl DNA template. Cycling conditions were as follows: 95°C (10 min), followed by 55 cycles of 95°C (15 s) and 60°C (3 min), followed by a 60°C (30 s) hold stage and a 10°C (infinite hold).

The qPCR efficiency and sensitivity of the Taqman assay were assessed by obtaining the limit of quantification (LOQ) and limit of detection (LOD) using tissue-derived DNA and synthetic standards (Appendix S1). For the synthetic standards, standard curves were established using a dilution series of known concentrations ranging from 10^7 copies/µl and decreasing tenfold down to 1 copy/µl. A similar standard curve was made by analyzing a 1:10 dilution series of DNA extracted from *A. gouldi* tissue (from starting concentration of 10.6 ng/µl down to 10^{-6} ng/µl). Six PCR replicates were used in each dilution step to assess LOD and LOQ. LOD was defined as the last dilution of the standard curve wherein the targeted DNA amplified with a cycle threshold (C_T) below 45 (Bustin et al., 2009; Mauvisseau et al., 2019). The LOQ was defined as the last dilution of the standard curve wherein the target amplified in at least 90% of the qPCR replicates with a C_T below 45 (Mauvisseau et al., 2019). During the specificity and sensitivity testing, triplicate positive and negative control samples were included in each run. Quantitative PCR reactions assays were conducted using the Viia™ 7 Real-Time PCR System (Applied Biosystems).

2.3 | eDNA sampling and field validation

The giant freshwater crayfish is the largest freshwater invertebrate in the world, thought to reach weights up to 6 kg and an outstretched length of more than one meter (Walsh & Haller, 2013) (Figure 1). Typically, they are found in well-shaded streams of all sizes that contain decaying logs and undercut banks in Tasmania. Juveniles are more likely to occur in flowing sections of stream where they use cobbles and boulders to shelter from predation, and they can be found in small headwater streams as well as larger streams. Adults more typically inhabit slower-flowing sections of stream and deep pools where they dig burrows in stream banks (Walsh & Haller, 2013). What they feed on changes to some extent over time, but typically comprises of decaying wood, leaf litter, rotting flesh, and small fish (Walsh & Haller, 2013; Walsh & Walsh, 2012).

Environmental DNA samples were obtained in November 2019 from eight sites in northern Tasmania, Australia (Appendix S2). Two sites were facilities which housed *A. gouldi* in either aquarium tanks or troughs (Lobster Ponds Heaven and Huon Aquaculture Springfield Hatchery, respectively; Appendix S2), five sites were streams known to have populations of *A. gouldi* (Garden of Eden Creek, Great Forester River, Lowries Creek, McKenzie Creek and Minnow River; Appendix S2), and one site was a stream with no known *A. gouldi* populations (West Arm Creek; Appendix S2).

Eight 1 L samples and one 1 L field control were collected from each stream site. Samples were taken approximately 50 cm from opposing riverbanks and along a 100 m gradient when possible. Samples were taken using sterile 1 L bottles in an upstream direction to minimize cross contamination. Field controls for each stream consisted of sterile 1 L bottles filled with UV-filtered distilled water. Each bottle was opened above the water surface for 30 s, closed and submerged for another 30 s. All samples were kept on ice until they were needed for filtration.

Lobster Ponds Heaven had four aquaria housing individual adult *A. gouldi* specimens (approximate length = 140 cm). Each tank had an individual recirculation and aeration system, and water had not been changed for three weeks prior to collection. Three of the tanks held 77.76 liters of water each (Bazza, Danny, Max; Appendix S2), and the fourth tank contained 140.76 liters (Agro; Appendix S2). A single 1 L water sample was collected from the surface of each tank followed by individual field controls. The Huon Aquaculture Springfield had a single trough containing approximately 30 juvenile *A. gouldi* individuals (ca. = 3–4 cm long). This trough had an open flow-through system from the Martial Grove Stream which branched out from the Great Forester River, and at any one time, the trough held 300 L of water. Juvenile crayfish had been kept in the trough for 9 months prior to sample collection. Three 1 L water samples were collected from the trough, one from each end, and one from the middle. A single 1 L field control sample was collected from Huon Aqua farm. All samples and field controls were kept in ice until needed for filtration.

Water samples were filtered within 24 h of sampling using 42 mm, 1.2 µm pore size cellulose nitrate filter papers (Sterlitech, Inc.) and a peristaltic pump (Geopump Series II; Geotech Environmental

TABLE 2 *Astacopsis gouldi-specific* primers targeting the cytochrome oxidase subunit I gene (CO1)

Primer	5′-Sequence-3′	No. of base pairs	Targeted amplicon bp
269F	CCCTTTAGCCGCCTCTATCG	20	219
489R	GCAAGGACTGGTAGCGAGAG	20	
292P	FAM-ATGCAGGAGCCTCAGTTGAC-MGB	20	

FIGURE 1 Representative *Astacopsis gouldi* juvenile (a) and adult (b) specimens

Equipment Inc.). Prior to filtering each sample, 500 ml of UV-sterilized water was passed through the filter equipment as an equipment blank and stored in 100% ethanol. Water samples were filtered in groups of 3 for each site. All filtering equipment was bleached for a minimum of 10 min and rinsed with UV-sterilized water between samples. All filter papers were stored in 100% ethanol immediately after filtration and shipped to the University of Canberra for further analysis.

2.4 | DNA extraction and qPCR of eDNA field samples from Tasmania

DNA extraction and PCR set-up were done in separate rooms inside a dedicated laboratory for trace samples at the University of Canberra, ACT, Australia. DNA from the filter papers was extracted using a modified Qiagen DNeasy© Blood & Tissue extraction protocol. Firstly, the filter papers were transferred to new plastic weigh boats and allowed to air dry inside a biological safety cabinet for a minimum of 20 min before transferring to 15-ml tubes for DNA extraction. The filter papers were then submerged in a lysis solution consisting of 360 µl of buffer ATL and 40 µl of Proteinase K and incubated at 65°C for 1 h. After incubation and vortexing, 400 µl of ethanol and 400 µl Buffer AL were added. Two centrifugation iterations were done to load the contents into the mini-spin column. The manufacturer's protocol was subsequently followed, and eDNA was eluted using 100 µl of Qiagen's buffer AE. DNA extracts were also diluted 1:10. A negative extraction control was included in each batch of DNA extraction to monitor potential contamination during the DNA extraction process.

All neat (undiluted) and diluted samples were initially amplified in duplicate to check for the presence of PCR inhibitors using qPCR assay conditions as described earlier. Samples were considered inhibited when a lower cycle threshold (C_T) value was obtained from the 1:10 dilution compared to the undiluted sample, or if there was a detection seen on the 1:10 sample compared to no detection in the neat sample. If neither the neat or diluted samples amplified to indicate the presence of inhibitors, then neat samples were spiked with a genomic DNA positive control (*Astacopsis gouldi* DNA; MW220042) and amplified to detect inhibition of the genomic control (Supplementary). Samples were considered inhibited if the spiked sample amplified at later cycles compared to the positive control (Supplementary). Diluted samples that showed amplification at earlier stages compared to their corresponding neat sample were used for further testing. If no amplification was observed in neat or diluted samples, then neat samples were used for further testing. All samples were run in triplicate (including negative extraction blanks, field, and negative controls) using the *A. gouldi* Taqman assay. We considered samples as positive if amplification crossed a common threshold determined individually within each qPCR run and if detections had C_T values ≤55 in any of the replicates. If all technical replicates from a sample showed no amplification, then samples were amplified again. If no positive amplification was observed in the re-run qPCR, then a final qPCR was prepared with neat and diluted eDNA samples. If no amplification was observed in any of the qPCR tests, then samples were deemed negative. All positive replicates were purified and sequenced on an AB 3730xl DNA Analyzer at the Genome Discovery Unit—ACRF Biomolecular Resource Facility (John Curtin School of Medical Research, Australian National University) following the manufacturer's protocol for confirmation.

3 | RESULTS

3.1 | Specificity, LOD, LOQ, and amplification efficiency

The qPCR primer pair and probe (269F, 489R, and 292P) developed in this study successfully amplified a 219 bp region of the COI gene in all five *A. gouldi* tissue samples (Table 1). There was no amplification with the other crayfish species tested in this study (Table 1). Using synthetic standards, the LOD was estimated to be 1 copy/µl (C_T = 41) and LOQ at 10 copies/µl (mean C_T ± SD = 39.7 ± 0.87) (Figure 2). LOD and LOQ using *A. gouldi* tissue DNA were estimated at 1×10^{-4} ng/µl (mean C_T ± SD = 39.98 ± 0.97; Figure 2). Amplification efficiencies ranged from 89% to 93% for plates assayed in this study. Positive controls amplified in all plates, and no amplification occurred in the negative template controls.

3.2 | Environmental DNA detection of *A. gouldi*

Astacopsis gouldi eDNA was successfully detected in all sites where the presence of the species had been previously confirmed with cycle thresholds ranging between 38.2 and 45 (Figure 3). Minimal detection was observed in one of four tanks with live *A. gouldi* specimens, with only 2/24 positive technical qPCR replicates (Figure 3; Lobster ponds). These two positive detections came from lobster pond Agro, which was the largest tank with approximately 144 L of water and a 140 cm long specimen (Appendix S2). Similarly, 4/24 and 3/24 technical qPCR replicates of Lowries Creek and Minnow

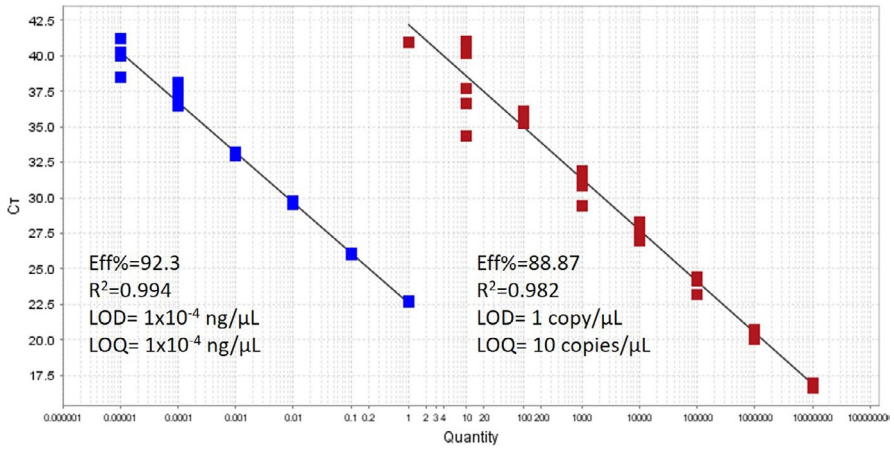

FIGURE 2 Limit of detection and amplification efficiency achieved with the *Astacopsis gouldi* probe-based assay designed in this study. Blue = genomic DNA and red = synthetic oligo

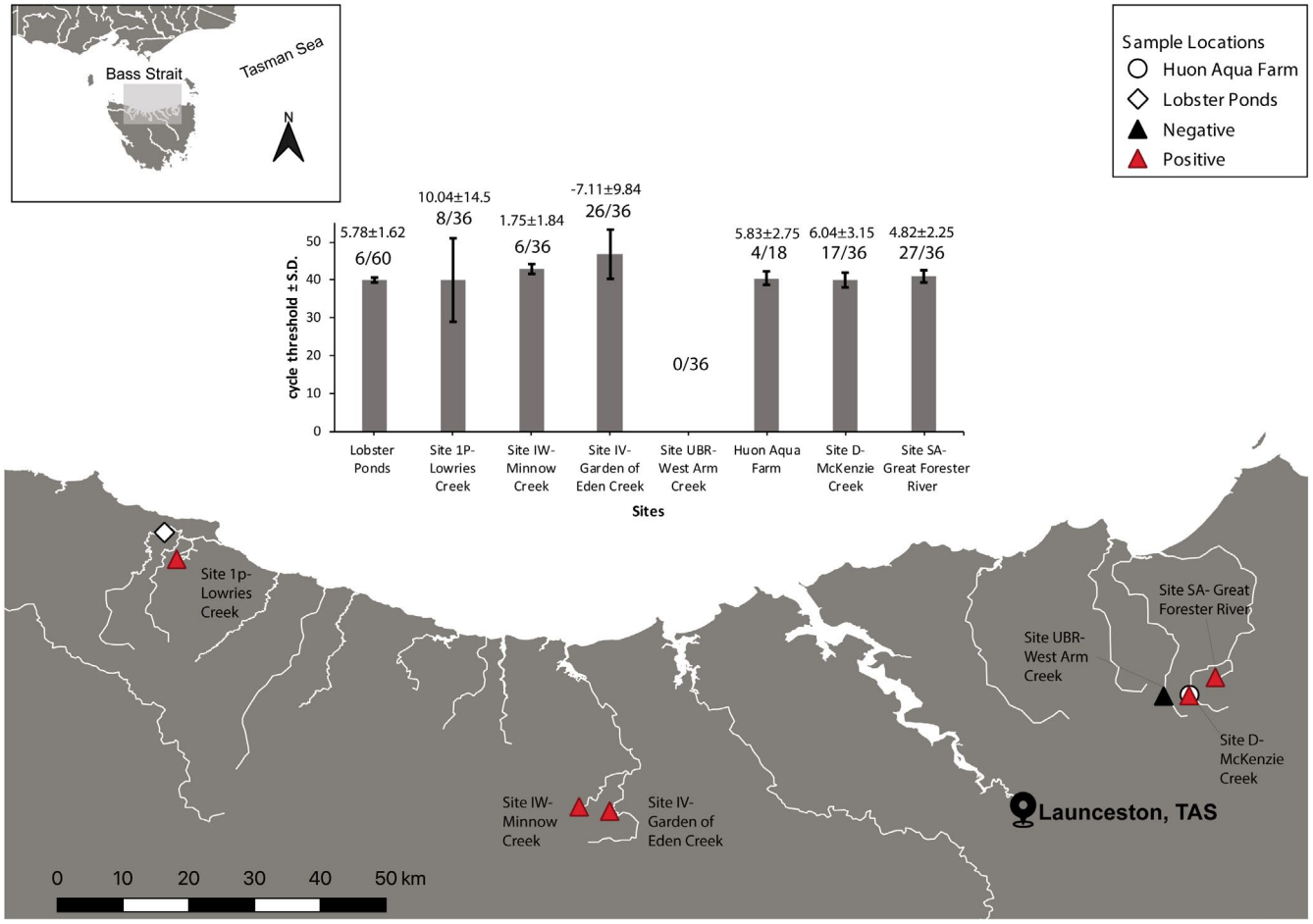

FIGURE 3 *Astacopsis gouldi* environmental DNA detection from sites in Northern Tasmania. The figure shows the mean cycle threshold detections achieved at each site, mean copy number ± Standard deviation, and the proportion of positive/negative qPCR replicates above each site. Negative values in the mean copy number in Site IV-Garden of Eden Creek are a result of positive detections falling out of the standard curve detectable range shown in Figure 2

Creek were positive for *A. gouldi* eDNA, respectively (Figure 3). The only site with no detection was the negative control site (Site UBR-West arm creek; Figure 3). Inhibition was detected in all samples collected during this study (Supplementary). All positive eDNA detections were confirmed by Sanger sequencing and found to have 99%–100% similarity with *Astacopsis gouldi* sequences from this study and NCBI.

4 | DISCUSSION

The giant freshwater crayfish is one of six threatened crayfish species in northern Tasmania (DPIPWE, 2020). Protecting this species in the wild requires reliable monitoring to inform management actions; however, surveying *A. gouldi* populations using traditional methods can be challenging and time-consuming. This study successfully used

the eDNA method to detect *A. gouldi* in captive and natural environments in Tasmania and validates the use of eDNA-based detection as a non-invasive and sensitive tool to detect *A. gouldi* occupancy in wild environments.

This study complements the monitoring requirements in the *A. gouldi* recovery plan (Commonwealth of Australia, 2017). Monitoring population trends to assess the current status of the species and evaluate the effectiveness of recovery actions is a key component for the recovery of *A. gouldi* populations in Tasmania. Environmental DNA-based studies have repeatedly shown the value of using non-invasive molecular methods to monitor freshwater crayfish species (Harper et al., 2018; Ikeda et al., 2016; Mauvisseau et al., 2018; Tréguier et al., 2014). Consistent with previous studies suggesting that small crayfish populations can be detected by targeting eDNA (Cowart et al., 2018; Dougherty et al., 2016; Harper et al., 2018), this study detected *A. gouldi* eDNA in late cycles of real-time PCR reactions, suggesting that minute traces of eDNA were present in water samples from all positive sites in this study. Testing water samples using real-time PCR could inform future monitoring efforts on the presence of *A. gouldi* in low densities, thus making formal occupancy modeling (MacKenzie et al., 2018) feasible for this species across landscapes with varying practices in catchment management.

Our results showed positive detection in only one of four tanks with live *A. gouldi* specimens in Lobster Ponds Haven, indicating a high incidence of false negative errors in these enclosed systems. However, our initial quality control of eDNA samples found that the amplification of diluted samples and spiked DNA standard controls revealed inhibition in water samples collected from all tanks. The presence of inhibitors in environmental samples is a known limiting factor of eDNA-based qPCR amplification that can compromise detection probability (Hunter et al., 2019). Partial or full inhibition of eDNA amplification decreases assay sensitivity and increases the potential for false negatives (McKee et al., 2015). Given that each tank held one large *A. gouldi* adult and that water had not been changed from each tank in three weeks prior to sampling, it is likely that each tank had a high concentration of inhibitors. As such, positive detection in the largest tank at the lobster ponds site (Agro [144 L]: 2/6 positive qPCR replicates) would have likely occurred given that the tank had approximately double the amount of water compared to the others and would in theory have diluted the impact of inhibition (McKee et al., 2015; Minegishi et al., 2019). This imperfect detection is acknowledged in many eDNA surveys, and methods/models have been developed to estimate the sensitivity of eDNA surveys to inform sampling regimens needed to detect the target with high probability (Furlan et al., 2015; Song et al., 2020). This highlights the importance of collecting multiple samples to increase detection probability (Hunter et al., 2019). Similarly, future studies would benefit by utilizing sample storage and extraction methodologies that minimize PCR inhibition and improve amplification, especially in the case of rare species such as *A. gouldi* (see Renshaw et al., 2015; Schrader et al., 2012).

Similarly, we found marginal detections from streams previously reported as having *A. gouldi* populations. Low concentrations of eDNA can result when target animals are small or have limited abundance, or when species shed or excrete eDNA at low rates (Furlan et al., 2015; Hunter et al., 2019). Samples with low eDNA concentrations can result in low positive detection proportions, as was observed with two stream sites in this study (Lowries Creek = 4/24 and Minnow Creek = 3/24 positive qPCR replicates). Similarly, detections from the Garden of Eden creek site showed that the estimated low copy numbers fell out of the out of the standard curve detectable range (copy number/µl ± SD = −7.11 ± 9.84; Figure 3), indicating that the amount of available DNA for detection can be lower than the limit of detection of the TaqMan assay. Within the context of eDNA-based monitoring, these results suggest that these three creeks could potentially have *A. gouldi* populations in low abundance (Cowart et al., 2018); however, there is little understanding on *A. gouldi* shedding and DNA degradation rates to accurately understand how low eDNA copy numbers reflect abundance or population size. Future research should investigate how different DNA extraction, preservation, and sampling methods can maximize DNA recovery for *A. gouldi*, as well as analyzing how DNA shedding rates are affected by environmental conditions to better understand population size and abundance. Nevertheless, eDNA monitoring techniques could help the recovery of *A. gouldi* populations by facilitating the identification of key locations that may warrant protection (Commonwealth of Australia, 2017), monitoring potential home range shifts, constrictions or expansions, and providing an indication on how effective recovery efforts have been.

In conclusion, this study validates the use of a newly developed probe-based qPCR assay used to detect eDNA from endangered *A. gouldi* in Tasmania. Tasmania has a wide range of unique ecosystems with a highly intricate riverine network that is too laborious to successfully monitor by conventional methods. Our findings show the assay successfully detected eDNA from confirmed positive sites, and that eDNA could be amplified by real-time PCR as a non-invasive and sensitive monitoring technique with potential implications for future management measures. Further testing could be done in other ecosystems across the home range of *A. gouldi* to better understand which environmental conditions impact detection probability of the species. Similarly, determining an appropriate eDNA sampling regime in different streams to improve detection probability (Furlan & Gleeson, 2016) would improve implementation of eDNA-based monitoring for management purposes. Research is also needed on seasonality and reproductive activity of the species to identify potentially important seasons to survey *A. gouldi* using eDNA-based methods.

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AUTHOR CONTRIBUTIONS

DG, AW, and PT conceptualized and designed the study. SG, AW, LB, AK, and PT collected the sample. ATG, SG, and RH involved in assay development, testing, and optimization. ATG and RH analyzed and interpreted the data. ATG, RH, and SG wrote the manuscript. All authors revised the manuscript. Funding support: ARC DP 190102837 to LB.

DATA AVAILABILITY STATEMENT

Inhibition tests and Amplification data are freely available at Zenodo in the European Council for Nuclear Research data center (https://zenodo.org/record/4292756#.X8BNz80zZPY; https://doi.org/10.5281/zenodo.4292756). Sequenced data from accessioned tissue samples are available in the National Centre for Biotechnology Information (accession W220040- MW220066).

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SUPPORTING INFORMATION

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

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