Locating the threatened Glenelg Freshwater Mussel in the lower Glenelg River system

1. Remote sensing and physical sampling

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Front cover photo: Crawford River, lower reaches (main image), Glenelg Freshwater Mussel (inset) (Tarmo A. Raadik).

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Locating the threatened Glenelg Freshwater Mussel in the lower Glenelg River system

Remote sensing and physical sampling

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Context:

Hyridella glenelgensis, the Glenelg Freshwater Mussel (GFW Mussel), is a small, fragile, almond-shaped native bivalve known only from the Glenelg River system in south-western Victoria. Since its description in 1898 the species was largely forgotten until 1990 and 2000, when a few individuals were found in the Crawford River, a tributary of the Glenelg River. In 2005 a survey found GFW Mussel was restricted to a single population in a 10 km section of the Crawford River and had undergone an extensive decline in range and abundance. The species is now listed as threatened in Victoria and critically endangered nationally.

Targeted surveys between 2015 and 2018 found a further decline in abundance and distribution of the species in the Crawford River and located two additional but small and isolated remnant populations. The decline of GFW Mussel is a result of various factors, including a decline in the flow regime (flow permanency) and instream and riparian habitat condition, leading to a loss of resilience (e.g. small range, low abundance, genetic decline) and high risk of extinction from stochastic events (e.g. fire, sedimentation). The threat to the species was recently exacerbated by a large fire in the Crawford River catchment.

Comprehensive knowledge of the distribution and status of GFW Mussel in the largest remaining population (Crawford River) is required to establish a benchmark for on-going monitoring as a foundation for conservation management. Improved survey techniques for such a small, cryptic, species are essential for improving this knowledge.

This study represents the most detailed assessment of GFW Mussels undertaken to date and forms the basis for the development of an on-going monitoring program for the conservation of the species.

Aims:

The aim of this project was to improve our current knowledge of the distribution of GFW Mussel in the Crawford River and identify extant populations in nearby streams. To support these objectives, a further task was to improve detection by developing and trialling a method for the mussel using detection of their DNA from water samples (environmental DNA, or eDNA) to complement physical sampling. As the mussels do not need to be physically located, this is considered a remote sensing method.

Methods:

Based on previous survey data as a guide, 39 sites were selected for assessment in the lower reaches of the Glenelg River system: 26 on the Crawford River for assessment of GFW Mussel, and 13 on other streams to search for new populations or verify the continuing presence of other populations of GFW Mussel. Physical sampling for both live individuals and shells (dead individuals) was undertaken by hand ‘brailing’ (feeling for mussels in the substrate using fingers) and included and assessment of basic water quality parameter, and a visual assessment of coarse instream habitat parameters that are important for GFW Mussel (water presence, level, and flow perenniality, and predominant substrate type). Relative abundance/density of mussels was expressed as the number of individuals per 30 minutes of searching. Replicate water samples were collected before physical sampling from a subset of all sites using a filtering unit for eDNA water sample collection, for later analysis in the laboratory. The results of physical sampling were used to verify the results of the eDNA analysis.

Several potentially useful probes to target a specific region of the mtDNA 16S gene for GFW Mussel DNA, were designed, developed and tested, based on tissue samples from GFW Mussel and closely related mussels and following a qualitative PCR (qPCR) approach, and the most suitable one was selected. On return to the laboratory DNA was extracted from the replicate eDNA samples, each underwent qPCR reactions to test for positive detections of target DNA and tested for PCR inhibition which may lead to false negative results (no detection of target DNA whilst DNA was present).

Results:

1. Physical survey

- GFW Mussel populations were recorded from all streams from which they had been found previously (Crawford River, Moleside Creek, Glenaulin Creek).
- The current distribution of the GFW Mussel in the Crawford River (the largest extant population) has contracted from a 28 km stream length in 2005 to the most downstream 5 km of the system (82% decline). The lower portion of this reach also contained the highest abundance of individuals.
- Based on the presence of shells, the former distribution of the species in the Crawford River extended farther upstream from the Lyons–Hotspur Road to at least the Steep Bank area in the Crawford Regional Park.
- The distribution of the GFW Mussel in the Crawford River has contracted to a section of stream which is perennially flowing, sustained by ground-water outflow: live individuals were absent from intermittent sites upstream, including those which had permanent pools. This supports a dependency of GFW Mussel with flowing water: this was previously unknown.
- The relationship between GFW Mussel and perennial flow is further supported by its continuing presence in similar reaches in Moleside Creek and Glenaulin Creek, and its absence in Scott Creek and Minnie Creek, where it was historically present, which are now intermittent.
- A population of GFW Mussel was discovered for the first time in the Stokes River. This brings to four the number of known populations.

2. eDNA detection

- DNA of GFW Mussel was positively detected (2–3 of 3 replicates) from 22 replicate water samples taken from 10 sites, indicating eDNA detection is possible for this species.
- Many additional ‘possibly positive’ detections were also recorded, where less replicates were positive. These may indicate true positive detections, but low stream DNA.
- There was good concordance between the results from eDNA remote sensing and physical sampling, with both techniques recording the presence of GFW Mussel in Moleside Creek, Glenaulin Creek, and the Crawford River.
- There were some discrepancies between the sampling methods, e.g. mussels physically detected but no, or only a ‘possibly positive’, detection via eDNA, ‘possibly positive’ eDNA detections at sites where no live mussels found, and positive eDNA detections where no mussels have been found.
- Remote sensing of GFW Mussel using eDNA has potential to be a valuable tool to undertake initial, rapid screen of catchments to guide more costly intensive physical sampling. However, to achieve a high level of detection confidence in eDNA surveillance, a greater understanding of the discrepancies (listed above) between results of the two techniques is required. Therefore, further verification of both techniques is needed involving additional sampling at these sites to confirm results: this is necessary and valuable part of eDNA method development.

Conclusions and implications:

This project has demonstrated that GFW Mussel continues to persist in the Crawford River (main population), and in two small populations in Moleside Creek and Glenaulin Creek, however it has declined significantly in the Crawford River and is now restricted to perennially flowing, groundwater sustained reaches in the most downstream portion of the catchment. Overall, the species is therefore still in decline, even though an additional, small, population was discovered in Stokes River. Field surveys have been instrumental in defining the distribution and abundance of the species and can now be used to design a rigorous monitoring program to monitor population status, as well as outcomes of recovery actions. The development of a remote sensing method for rapid detection of GFW Mussel is a valuable complementary tool to physical sampling but will benefit from further development to increase detection probability and confidence in the technique.

The dependency of GFW Mussel with perennially flowing habitats is a significant outcome, important in the context of the conservation management for the species at a landscape and local scale, in addition to management of surface water inflows for overall river health. Further research and management are therefore required on catchment groundwater/surface water interactions (local/regional scales) and specific stream spring outflow areas and research on mussel physiological requirements and tolerance to reduced flows.

1 Introduction

The Glenelg Freshwater Mussel, *Hyridella glenelgensis* (GFW Mussel) is a small (18–51 mm long), fragile, almond-shaped native bivalve, only known from the Glenelg River system in south-west Victoria (Cotton and Gabriel 1932; McMichael and Hiscock 1958; Playford and Walker 2008). The species was first described in 1898 from specimens collected from the Glenelg River near Dartmoor (lower catchment) and Roseneath (mid-catchment) in Victoria (Dennant 1898). After the late 1920s no further specimens were recorded until 1990, and subsequently in 2000, when a few live individuals were found in the Crawford River, a tributary of the Glenelg River (Walker et al. 2001).

An assessment of the status of the species in 2005 indicated that it was restricted to a 10 km section of the Crawford River in its lower reaches, near Dartmoor (Playford 2005; Playford and Walker 2008). This demonstrated that the species had undergone an extensive decline in range and abundance. The species is now considered threatened and is listed as such under the *Flora and Fauna Guarantee Act 1988* (Vic.). It is also listed as critically endangered nationally under the *Environmental Protection and Biodiversity Conservation Act 1999* (Cth) and internationally by the International Union for Conservation of Nature (Walker et al. 2014a).

The decline of the species is likely to be a result of various factors, including changes in flow regime, drought, decreased frequency of flood events, increased sedimentation and bank erosion, and loss of aquatic and riparian vegetation due to land clearing and grazing by stock (Walker et al. 2014a). Considering the decline in range and abundance of GFW Mussel, there is now a high risk that stochastic events such as sedimentation, floods, drought and predation, as well as genetic decline and loss of evolutionary adaptability through inbreeding, could cause the extinction of the species.

Threats to the species were recently exacerbated by a large fire in the Crawford River Regional Park in early 2020, with consequent potential for erosion of soil and ash during high-intensity rainfall from the burnt catchment leading to instream sedimentation and poor water quality.

This project is part of a broader program of the Glenelg Hopkins Catchment Management Authority that aims to improve habitat quality through revegetation in the Crawford River and thus increase the ability of GFW Mussel to recover from adverse effects caused by the 2020 fire and improve its resistance and resilience to threats.

The current sub-project has two objectives:

- Undertake a detailed survey for GFW Mussel in the Crawford River.
- Identify other extant populations in nearby streams.

As GFW Mussels are small and usually buried in sandy substrates in streams, physical detection can be difficult and time-consuming, particularly if the abundance is low. Consequently, a specific task to support the two objectives was to improve detection by developing and trialling a remote-sensing method for GFW Mussels, using detection of their DNA from water samples (environmental DNA, or eDNA) to complement physical sampling.

The results of the project will be important in understanding the species' recovery following the 2020 bushfires, and in understanding its present extent and establishing a benchmark for monitoring the species into the future.

1.1 Relevant recent conservation work

After 2005 no additional work on the conservation of GFW Mussel was undertaken until ARI commenced surveys in 2014, funded by the following Victorian Government programs:

- Victorian Environmental Partnerships Program (VEPP) in 2014–2015.
- Critical Action and Strategic Partnerships Program (CASP – TSPI) in 2015–2016.

- Biodiversity On-ground Actions Program (BOA) in 2017–2018 (Raadik 2019).

A project to investigate potential drought refuge for aquatic fauna in unregulated streams, funded by the Water and Catchment Division of the Department of Environment, Land Water and Planning, involved survey work in 2016, which contributed to the knowledge of the status and distribution of GFW Mussel at that time (Raadik and Nicol 2018).

In these projects a total of 208 sites were visited in the lower to mid reaches of the Glenelg River system in the presumed historical range of GFW Mussel, in an area extending from Moleside Creek north to upstream of Dergholm, and east to upstream of Hamilton. In addition, 39 sites in the adjacent Portland Coast basin were also surveyed. Survey outcomes specifically contributed data on presence/absence and habitat status (abundance, quality, etc.) for GFW Mussel, and were immediately included in ongoing conservation management (Raadik 2019; T. Raadik, unpublished data).

Notable outcomes from this work for GFW Mussel were:

- Confirmation that the species is restricted to a small range in the lower Glenelg River system.
- Confirmation that the largest population persists in the Crawford River system.
- Confirmation of the absence of the species in the Glenelg River.
- Discovery of two additional, small, extant populations in the adjacent Glenaulin Creek and farther south in Moleside Creek.
- All three known populations are geographically isolated from each other.
- Discovery of two locations where the species was historically present, based on the discovery of dead shells: Scott Creek north of Dartmoor and Minnie Creek, in a pine plantation, farther north near Myaring.
- All extant populations appeared to persist in reaches of stream with permanent flow, supported probably by groundwater discharge.

This new information was pivotal for the development of the current project.

1.2 Study location and site selection

Based on the recent survey work cited above, the study location for this project was restricted to an area encompassing all known extant populations of GFW Mussel, including the two previous locations where only shells were found (Figure 1). This encompassed an area extending from the Moleside Creek in the south, north past Dartmoor to near Myaring, and east to Hotspur.

The focus stream was the Crawford River, which was sampled extensively (26 sites) from its junction with the Glenelg River upstream to past the Crawford Regional Park (burnt zone), including the area of 'The Neck' where the Glenelg Hopkins CMA was undertaking riparian revegetation works. Survey sites were also established in nearby streams with the following characteristics:

- Sustain known populations of GFW Mussel: Moleside Creek and Glenaulin Creek.
- Known to have previously sustained populations of GFW Mussel: Scott Creek and Minnie Creek.
- Potentially contain GFW Mussel: Stokes River.
- Permanently flowing in their lower reaches and therefore may support GFW Mussel: Glenelg River tributary (site 5) and Limestone Creek.

Sites were established in permanently flowing and/or intermittent stream reaches.

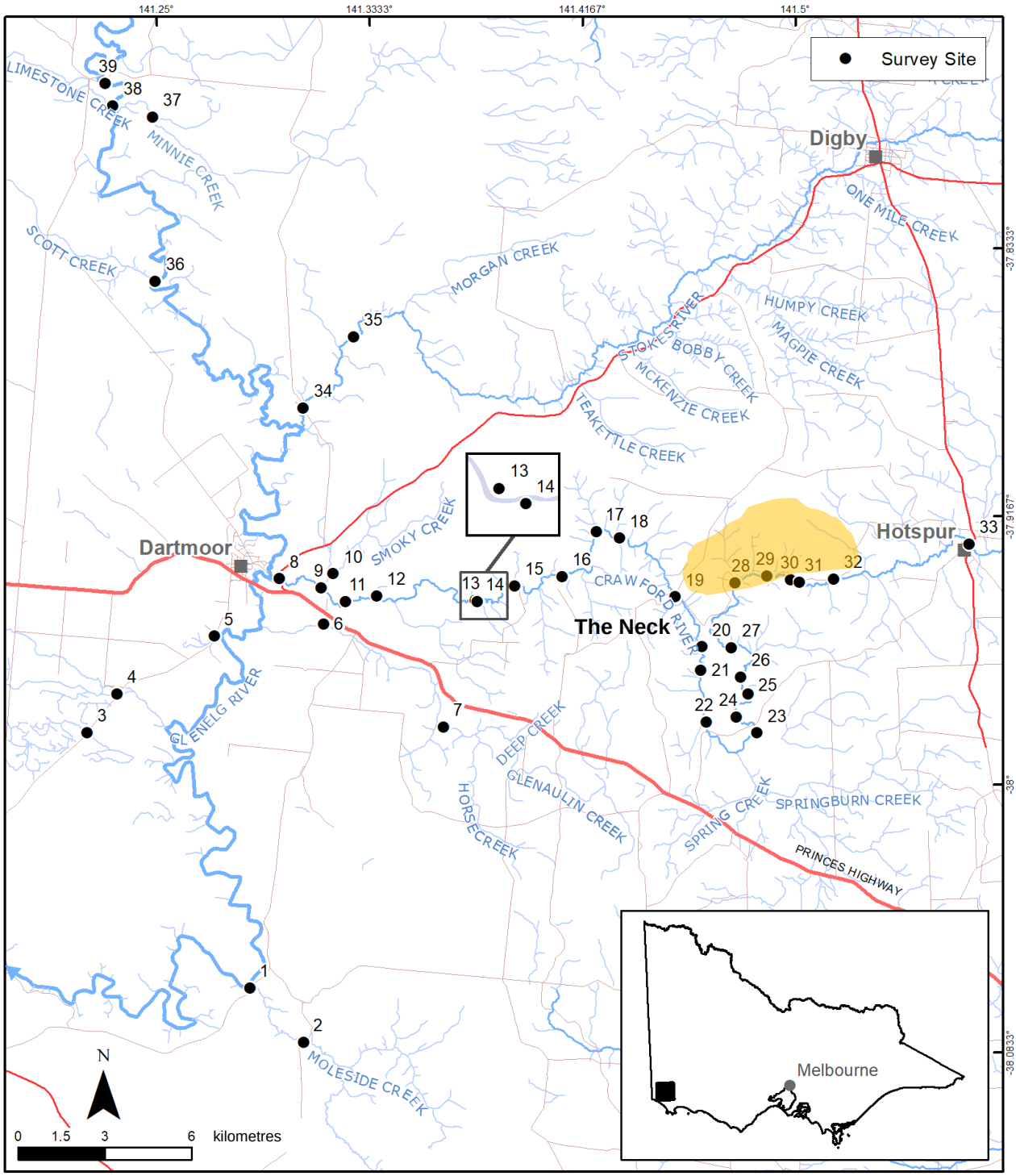

Figure 1. Location of survey sites in the lower Glenelg River system, south-west Victoria, including 'The Neck' riparian vegetation rehabilitation reach and the approximate area of the 2020 fire (orange shaded area).

2 Methods

2.1 Field survey

The field survey consisted of the collection of water quality and habitat data from survey sites, as well as physical sampling for GFW Mussels and the collection of replicate water samples for eDNA analysis.

2.1.1 Water quality and habitat characteristics

The following water quality parameters were recorded in situ at a range of survey sites at a depth of 0.2 m below the water surface, using a calibrated water-quality multimeter: electrical conductivity (µS/cm) (EC, standardised to 25 °C), pH, dissolved oxygen concentration (mg/L), turbidity (NTU) and water temperature (°C).

Site-based, fine-scale habitat characteristics for GFW Mussel have been identified previously (Playford 2005; Playford and Walker 2008) and are not repeated here. However, the following coarse habitat features related to flow and substrate, which we consider important for GFW Mussel survival, were visually noted and recorded at survey sites:

- predominant flow habitat (pool, glide, riffle, run, etc.).
- flow type, related to the amount of stream flows (e.g. no flow, trickling, slow or moderate flow).
- an estimate of the usual flow status of the reach (permanent, intermittent, drying to pools or drying completely), based on a visual assessment of stream morphology (e.g. channel depth and degree of heterogeneity) and previous observations).
- predominant stream substrate (bedrock, rock, pebble, gravel, coarse sand, fine sand, silt, mud, clay, organic material).

2.1.2 Physical sampling

Physical sampling for GFW Mussels was undertaken where water presence and depth allowed (i.e. the site held water and its depth was under 500 mm at times in the reach). Sampling consisted of one or more people gently brailling the substrate using the fingers of both hands to detect buried mussels (Figure 2), and visually searching the bank just above the water level for the presence of shells. Sampling was conducted in an upstream direction for a minimum of 30 minutes, but often longer if large amounts of instream structure was encountered.

Any live mussels, shells or shell fragments detected were removed from the stream and identified to species based on morphological characteristics. Live GFW Mussels were then measured for maximum length (mm), weighed (to the nearest 0.1 g) and, along with any other mussels found, returned to the water at the site of capture.

Figure 2. Brailling for Glenelg Freshwater Mussels (Tarmo A. Raadik)

2.1.3 eDNA water sample collection

Three replicate water samples were collected from each of the sites where eDNA collection was to be undertaken (Table 1A). Samples were collected from the stream in flowing reaches where possible, using an ANDe™ backpack eDNA filtration system (Figure 3). For each replicate, water was filtered through a 1.2 or 5.0 µm filter at a rate of 1 L/min until the filter became clogged or a volume of 7 L was reached. The volume of water filtered per sample was recorded, and each filter was uniquely numbered and placed into a portable refrigerator within 30 minutes of collection for storage.

Figure 3. Collecting water samples for eDNA analysis using ANDe™ backpack eDNA filtration system
Clockwise from top left: using the unit mid-stream; sampling backwater areas; a 1.2 µm self-preserving filter; connecting a filter to the unit; water being sucked from the stream and through the filter. (Tarmo A. Raadik)

Strict sampling procedures were followed to eliminate the risk of cross-contamination between collection sites. In short, samples were collected with the pole on the unit extended to allow the operator to remain downstream and away from the filtering end. To further eliminate the risk of DNA contamination single use gloves and tweezers were used to transfer the eDNA filter at each site.

2.2 Environmental DNA probe development and water sample analysis

Environmental DNA offers a cost effective, non-invasive method for species detection. However, eDNA detection has not been undertaken for GFW Mussel, as a species-specific DNA "probe" or primer was required to be develop before quantitative PCR (qPCR) based analysis could commence.

2.2.1 *In silico* probe development

The design of a species-specific probe was confounded by the presence of other mussel species in the survey area. Given the suggested rarity of GFW Mussel in the region, the probe had to be sufficiently sensitive to detect minute concentrations of DNA in samples.

As mitochondrial DNA (mtDNA) generally provides the strongest signal within eDNA samples, as opposed to nuclear DNA (Bylemans et al 2018), mtDNA gene regions were used to develop the probe. DNA sequences for the mtDNA 16S and CO1 genes were generated for GFW Mussel, several closely related species of *Hyridella* — Narracan Freshwater Mussel (*H. narracanensis*), Austral River Mussel (*H. australis*), Freshwater Mussel (*H. drapeta*), Depressed (or Flattened) River Mussel (*H. depressa*), and an undescribed *Hyridella* species from the Fitzroy River system, close to the Glenelg River system (T. Raadik, unpublished data) — as well as the co-occurring Slow Water Mussel (*Velesunio ambiguus*). The closely related *Lortiella froggatti* from north-western Australia was used as an out-group.

A TaqMan®-style qPCR method was used in detecting eDNA, as probe-based chemistries have been shown to demonstrate higher specificity to the target DNA (Wilcox et al. 2013). However, other methods are possible (e.g. Sybr Green) and offer different advantages, such as using melt-curve analyses to examine cross-species amplification (e.g. Larson et al 2017).

Primer and probe development was then undertaken, attaining specificity by ideally ensuring:

- the PCR product was less than 200 base pairs (bp) long (extended to 250 bp if necessary).
- primers were at least 20 bp with melting point (Tm) > 55 °C (Tm would, however, be reduced if deemed necessary during primer development) with no mismatches within GFW Mussel and > 2 mismatches to other species.
- probes were at least 24 bp, with Tm > 60 °C (Tm would once again be reduced if deemed necessary during probe development), with no mismatches within GFW Mussel, and > 4 mismatches to other species. .

Initial alignments were undertaken in Geneious version 9 (https://www.geneious.com).

The web-based tool ssPRIMER was used to design species specific primers with the above specifications (https://www.mattortonapps.com/shiny/ssPRIMER/; accessed 14/05/2021).

Probe trials

No primer–probe combinations were deemed suitable using the CO1 gene, but the 16S gene provided several suitable primer–probe options. An initial trial of the two best candidate primer–probe sets (see results) was undertaken. PrimeTime qPCR probe assays (Integrated DNA Technologies) with a 2:1 primer:probe ratio and 5' dye / 3' quencher were used to trial probes, with all assays using 6-FAM/ZEN/IBQF dye–quencher combination for high signal to noise qPCR reactions.

Initial trials for amplification success were undertaken with GFW Mussel DNA extracted from mussels collected from the target site. In addition, species specificity was tested against potentially co-occurring mussel species — the Slow Water Mussel and Pea Mussel (*Corbicula australis*) — as well as *Hyridella australis, H. narracanensis* and the Fitzroy River *Hyridella* species.

The qPCR conditions for all initial probe assays were: 5.0 µl PrimeTime Gene Expression Master Mix (IDT), 0.5 µL 20X Primetime qPCR Assay, 2.5 µl H20, and 2.0 µL of 5ng/µL of DNA extraction, and initial trials were undertaken on the Chai Open qPCR (https://www.chaibio.com/) with the following reaction conditions: 95 °C for 3:00 minutes, 50 cycles of 95 °C for 20 seconds, and tests of annealing temperatures ranging from 60 to 65 °C for 30 seconds. For tests of species specificity, multiple DNA extractions were used from all test species/lineages, and qPCR reactions were run in triplicate.

A synthetic double-stranded DNA sequence (gBlocks Gene Fragment, Intergrated DNA Technologies) representing the GFW Mussel 16S sequence was generated, and a dilution series was run to test the efficiency of the qPCR probes at both 60 °C and 65 °C. Sensitivity was indicated as sufficient where PCR efficiency fit within the recommended R2 of > 0.95.

2.2.2 eDNA sensitivity tests and analysis

DNA was extracted from each replicate sample from each site following a modified Qiagen DNeasy protocol (Spens et al. 2017). To do this, each filter was removed from its individually labelled packet and cut in half. One half of the filter was returned to the packet from which it had been removed and placed back into cold storage for future analysis if required. The other half was cut into 3–5 mm strips using scissors that had been sterilised using 50% bleach. Filter pieces were then air-dried to remove ethanol and placed in 1.5 µL tubes, to which was added 720 µL of buffer ATL and 80 µL of Proteinase K. The filters were then digested overnight at 55 °C.

The following day 400 µL of the supernatant was placed into two separate tubes, with equal volumes of Buffer AL and 100% ethanol. Both tubes were then sequentially passed through DNA mini spin columns, until all DNA was bound to the columns. The standard DNeasy protocol was then followed. As DNA extractions occurred over multiple sessions, extraction blanks were made in each extraction session (following the same procedure, but without the addition of filters) as controls to check for contamination.

Quantitative PCR (qPCR) reactions were then undertaken using developed species-specific primers (see section 2.4.1, section 2.4.2). PrimeTime qPCR probe assays (Integrated DNA Technologies) with a primer–probe ratio of 2:1 and 5' dye / 3' quencher were used, with all assays using 6-FAM/ZEN/IBQF dye/quencher combination. The qPCR conditions for all probe assays were as developed: 5.0 µL PrimeTime Gene Expression Master Mix (IDT), 0.5 µL 20X Primetime qPCR Assay, 2.5 µL H_20 and 2.0 µL of DNA extraction. For all samples, including controls, qPCRs were run in triplicate on a BioRad CFX96 Touch thermal cycler under the following conditions: 95 °C for 3:00 minutes, 50 cycles of 95 °C for 20 seconds, and 60 °C annealing for 30 seconds. Only samples where 2 of 3 replicates returned a positive result were counted as successful.

To test for potential false negatives in the results, testing for PCR inhibition was undertaken. PCR inhibition can be introduced by the co-extraction of factors such as plant polyphenols and humic acid. All DNA extractions were spiked with control DNA and amplified with a control qPCR probe. Samples that failed to amplify for the control probe were purified using OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA), and retested using the control probe; this step was repeated if necessary to ensure that PCR reactions were not inhibited.

3 Results

In all, 39 sites were selected and 37 were sampled (Figure 4) to determine the spatial extent of GFW Mussel in the survey area (see Appendix Table A1). Field sampling was undertaken over two one-week periods, in late January 2020 and late March 2020. Combined physical and eDNA water sampling was conducted at 25 sites, physical sampling alone at 10 sites, and eDNA alone at 2 sites; 2 other sites were found to be dry and therefore were not sampled (Figure 4). The method employed at each site (physical and/or eDNA water sampling) was determined in the field in response to the presence and depth of water (Figure 4, Appendix Table A1). Fencing and revegetation works had been undertaken by the Glenelg Hopkins CMA immediately adjacent to site 21 and site 25 (Figure 4).

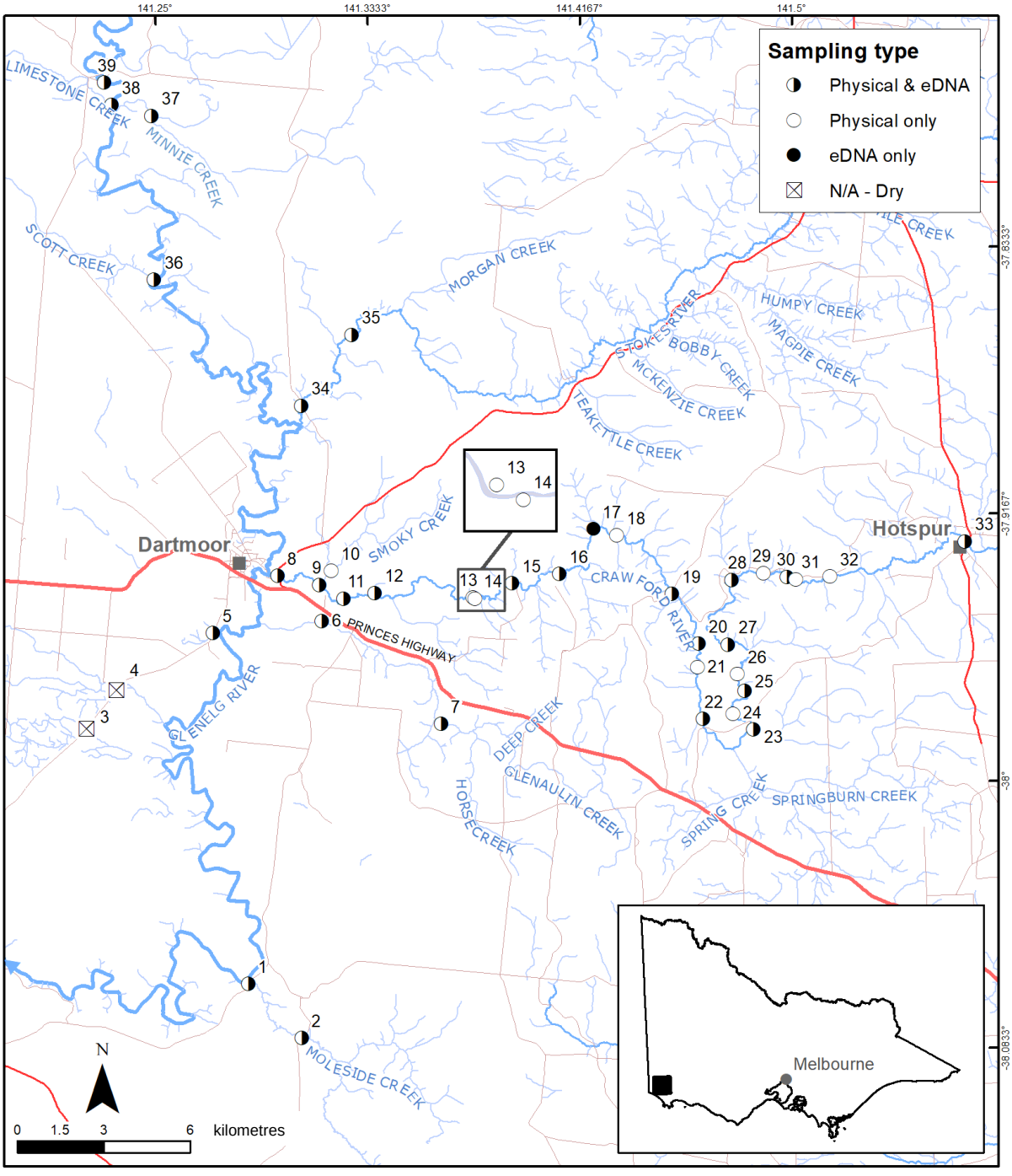

Figure 4. Type of sampling undertaken at each survey site. Sites 3 and 4 were not sampled because they were dry

3.1 Water quality and habitat characteristics

Water quality parameters (Table 2) varied across all sites and survey periods, as follows:

- Electrical conductivity was generally high, ranging from 750 to 3310 µS/cm (mean 1404 µS/cm).
- Water temperature ranged from 16.7 to 23.0 °C.
- Dissolved oxygen levels were reasonably high, ranging from 6.8 to 10.9 mg/L (mean 8.8 mg/L).
- pH was basic, ranging between 7.3 and 7.8 (mean 7.6).
- Turbidity was low, ranging between 0 and 140 NTU (mean 6.8 NTU).
- Water hardness was high, ranging between 180 and 880 ppm $CaCO_3$ (mean 348 ppm $CaCO_3$).

Specifically, water quality parameters at sites where live GFW Mussel were detected (Table 2) were as follows; values are mean and range:

- electrical conductivity 1244 µS/cm (820–1920) µS/cm).
- water temperature 18.7 °C (16.8–21.1 °C).
- dissolved oxygen 7.7 mg/L (6.8–10.9 mg/L).
- pH 7.6 (7.3–7.8).
- turbidity 21 NTU (0–140 NTU).
- water hardness 337 ppm $CaCO_3$ (180–540 ppm $CaCO_3$).

Coarse habitat parameters also varied between sites (Table 2). In general, the flow habitat at sampled locations was predominantly pools or riffles, but the amount of flow varied from moderate flow to no flow, most sites having low flow. Similarly, predicted flow permanency at sites varied from intermittent (either possibly complete drying or remnant pools), to permanent flow, but most sites were considered intermittent.

Sites with permanent water flow were in Moleside Creek (sites 1 and 2), Glenaulin Creek (sites 6 and 7, a tributary of the Glenelg River (site 5), Minnie Creek (site 37), Limestone Creek (site 38) Glenelg River (site 39). On the Crawford River only the lower three or four sites (8, 9, 11 and possibly 12) were considered to have permanent flow, although site 16 in the middle reaches of the system might also have permanent flow (Table 2).

Water and flow characteristics at sites at which live GFW Mussel have been found (historical and current data) can be summarised as consisting predominantly of pool or riffle habitat, usually in permanent stream reaches with low to moderate flow during summer (Table 2). An exception was site 35 on Stokes River, where the reach was considered to have intermittent flow. Sites where only shells of GFW Mussels were detected were intermittent flow reaches, usually in pool, riffle or glide habitat, with no or low flow levels, except sites 23 and 25 on the Crawford River which had low to moderate flows during the sampling period.

Predominant instream substrate in the Glenelg River and tributary streams consisted predominantly of sand; in the Crawford River it consisted of sand in the lower reaches but mud, silt and clay in the mid to upper reaches (Table 2). As described previously (Playford 2005; Playford and Walker 2008), the predominant substrate at sites where live GFW Mussels were detected was sand (Table 2).

Characteristics of selected survey sites are shown in Figures 5 and 6.

Table 1. Water quality parameters, flow and habitat characteristics at survey sites

EC – electrical conductivity @ 25 °C; Temp – water temperature; DO – dissolved oxygen; Turb. – turbidity; Hard. – hardness. Yellow shading – sites where live Glenelg Freshwater Mussels were located; Orange shading – sites where only GFW Mussel shells were found.

Site	Waterbody	EC (µS/cm)	Temp. (°C)	DO (mg/L)	pH	Turb. (NTU)	Hard. (ppm $CaCO_3$)	Flow habitat	Flow amount	Estimated reach flow status	Predominant substrate
1	Moleside Creek	805	16.7	9.3	7.5	0	300	riffle	moderate	permanent	fine sand, silt
2	Moleside Creek	820	16.8	10.4	7.8	0	300	pool	moderate	permanent	coarse sand, limestone bedrock
3	Glenelg River tributary	–	–	–	–	–	–	dry	dry	intermittent / ephemeral	coarse sand
4	Glenelg River tributary	–	–	–	–	–	–	dry	dry	intermittent / ephemeral	coarse sand
5	Glenelg River tributary	750	18.7	9.2	7.5	0	320	riffle	moderate	permanent	fine sand
6	Glenaulin Creek	1460	17.5	9.2	7.8	140	540	glide	moderate	permanent	silt, fine sand
7	Glenaulin Creek	1545	19.2	8.8	7.5	0	380	riffle	moderate	permanent	gravel, coarse sand, silt
8	Crawford River	1305	18.2	8.7	7.7	0	290	riffle	moderate	permanent	fine sand
9	Crawford River	1210	17.6	8.8	7.6	0	340	riffle	moderate	permanent	fine sand, gravel
10	Smokey Creek	1165	19.5		7.8	0	–	pools	very low	intermittent	sand, clay
11	Crawford River	1210	18.7	8.6	7.5	0	300	pool	low	permanent,	coarse sand, silt, organics, bedrock
12	Crawford River	1920	18.7	8.3	7.5	0	410	almost pool	very low	possibly intermittent, or very low flow	gravel, pebble, coarse sand, mud
13	Crawford River	–	–	–	–	–	–	glide/pool	low	intermittent, probably dries	silt, mud
14	Crawford River	–	–	–	–	–	–	dry	dry	intermittent, dries	silt, mud, clay
15	Crawford River	1195	21.1	–	7.8	0	–	isolated pool	no flow	intermittent, dries to pools	mud, silt, clay

Site	Waterbody	EC (µS/cm)	Temp. (°C)	DO (mg/L)	pH	Turb. (NTU)	Hard. (ppm $CaCO_3$)	Flow habitat	Flow amount	Estimated reach flow status	Predominant substrate
16	Crawford River	1230	23.0	7.1	7.5	0	330	riffle	very low	possibly just permanent (seeps), or intermittent	coarse and fine sand, clay
17	Crawford River	1400	17	9.7	7.3	0	300	pool	no flow	intermittent, dries to pools	mud
18	Crawford River	–	–	–	–	–	–	glide/pool	low	intermittent, probably dries	coarse and fine sand, silt, mud
19	Crawford River	1415	19.3	8.9	7.3	0	300	pool	no flow	intermittent, dries to pools	coarse sand, mud, organics
20	Crawford River	–	–	–	–	–	–	glide/ backwater	low to moderate	intermittent, dries to pools	mud, clay, organics
21	Crawford River	–	–	–	–	–	–	glide/pool	low	intermittent	mud, silt, clay, fine sand
22	Crawford River	1285	20.5	10.9	7.8	0	260	glide, pool	very low	intermittent	mud, silt, clay, coarse sand
23	Crawford River	–	–	–	–	–	–	glide/pool	low to moderate	intermittent, dries to pools	mud, silt, clay, fine sand
24	Crawford River	1224	19.7	9.8	7.8	10	200	riffle/glide	low	intermittent	mud, silt, clay, organics
25	Crawford River	1180	19.4	9.7	7.5	0	220	riffle/run/ pool	low to moderate	intermittent	clay, mud, silt, organics
26	Crawford River	–	–	–	–	–	–	riffle/run/ pool	low to moderate	intermittent	clay, organics, fine sand
27	Crawford River	1470	18.5	6.8	7.5	5	300	riffle/glide/ pool	low to moderate	intermittent, dries to pools	mud, silt clay, organics
28	Crawford River	1200	20.1	–	7.5	0	–	riffle/glide, end of pool	low	intermittent	mud, silt, coarse sand, clay, organics

Site	Waterbody	EC (µS/cm)	Temp. (°C)	DO (mg/L)	pH	Turb. (NTU)	Hard. (ppm $CaCO_3$)	Flow habitat	Flow amount	Estimated reach flow status	Predominant substrate
29	Crawford River	–	–	–	–	–	–	pool	low	intermittent	mud, coarse sand, silt, clay
30	Crawford River	1265	17.6	10	7.8	0	270	pool	low	intermittent	mud, clay silt
31	Crawford River	–	–	–	–	–	–	riffle/glide/ pool	low	intermittent	mud, silt, clay, fine and coarse sand, organics
32	Crawford River	1770	22	7.3	7.7	0	470	glide/pool	low	intermittent	mud, silt, clay, fine sand, organics
33	Crawford River	3310	19.8	10.6	7.8	0	880	pool	no flow	intermittent	mud, clay, silt
34	Stokes River	1565	19.9	6.8	7.5	5	340	pool	low	possibly intermittent, drying to pools	coarse sand, silt, rock
35	Stokes River	830	20.8	7	7.5	10	180	pool	low	intermittent, dries to pools	coarse and fine sand, mud, silt, clay
36	Scott Creek	–	–	–	–	–	–	riffle	moderate	intermittent (now)	coarse and fine sand
37	Minnie Creek	1520	19	9	7.7	0	380	pool/riffle	very low	intermittent (now)	coarse sand
38	Limestone Creek	2800	19	8.5	7.0	0	500	glide	moderate	permanent	limestone bedrock, coarse sand
39	Glenelg River	2450	21.2	8.1	7.8	0	400	glide	moderate	permanent	coarse sand

Figure 5. Examples of site characteristics on the Crawford River.
(A) Lower catchment, below Dartmoor–Hamilton Road, (site 8); (B) Balds Plantation, site 13; (C) upstream of East Greenwald Road, site 16; (D) western side of 'The Neck', McEachern Plantation, site 20; (E) eastern side of 'The Neck', McEachern Plantation, site 26; north-eastern side of 'The Neck', McEachern Plantation, site 27. (Tarmo A. Raadik)

Figure 6. Examples of site characteristics on the Glenelg River and tributary streams
(A) Glenaulin Creek, site 6; (B) Scott Creek, just upstream of junction with Glenelg River, site 36; (C) Glenelg River at the old bridge downstream of Myaring–Pieracle Road, site 39; (D) Minnie Creek in pine plantation, site 37. (Tarmo A. Raadik)

3.2 Physical survey

Live GFW Mussels as well as shells were detected during this survey (Figure 7). Two other mussel species (Pea Mussel and Slow Water Mussel) were also found (Figure 8). Of the 35 sites physically sampled, live GFW Mussels were found at seven sites in four streams: Moleside Creek (site 2),Glenaulin Creek (site 6), Crawford River (sites 8, 9, 11 and 12), and Stokes River (site 35) (Figure 4). Only dead shells of GFW Mussels were found at a further seven on three streams: Crawford River (sites 13, 16, 23, 25, 29), Scott Creek (site 36) and Minnie Creek (37) (Figure 4).

In the Crawford River, live mussels were restricted to the lower reaches of the system, from near the junction with the Glenelg River upstream to about Winnap Siding (site 12). This section conforms to the reach of the Crawford River considered to have the most reaches of permanent stream flow (Table 2).

Figure 7. Glenelg Freshwater Mussel (*Hyridella glenelgensis*); (left) individual instream, (right) enlarged image. (Tarmo A. Raadik)

Figure 8. Additional species of freshwater mussels: (left) Pea Mussel (*Corbicula australis*) and (right) Slow Water Mussel (*Velesunio ambiguus*). (Tarmo A. Raadik)

Live GFW Mussels had been collected previously from Moleside Creek, Glenaulin Creek and Crawford River. However, the detection of the species in the Stokes River is new (Figures 9 and 10), though still from within the presumed historical range of the species. Shells have also previously been collected from the mid reaches of the Crawford River up to the eastern crossing of the Lyons–Hotspur Road (site 23), and in Scott Creek and Minnie Creek (sites 36 and 37) (Figure 10). The detection of shells in the Crawford River at sites 25 and 29 is a range extension, indicating that the species was at least historically present farther upstream.

Figure 9. New record of GFW Mussel from the Stokes River (Tarmo A. Raadik)

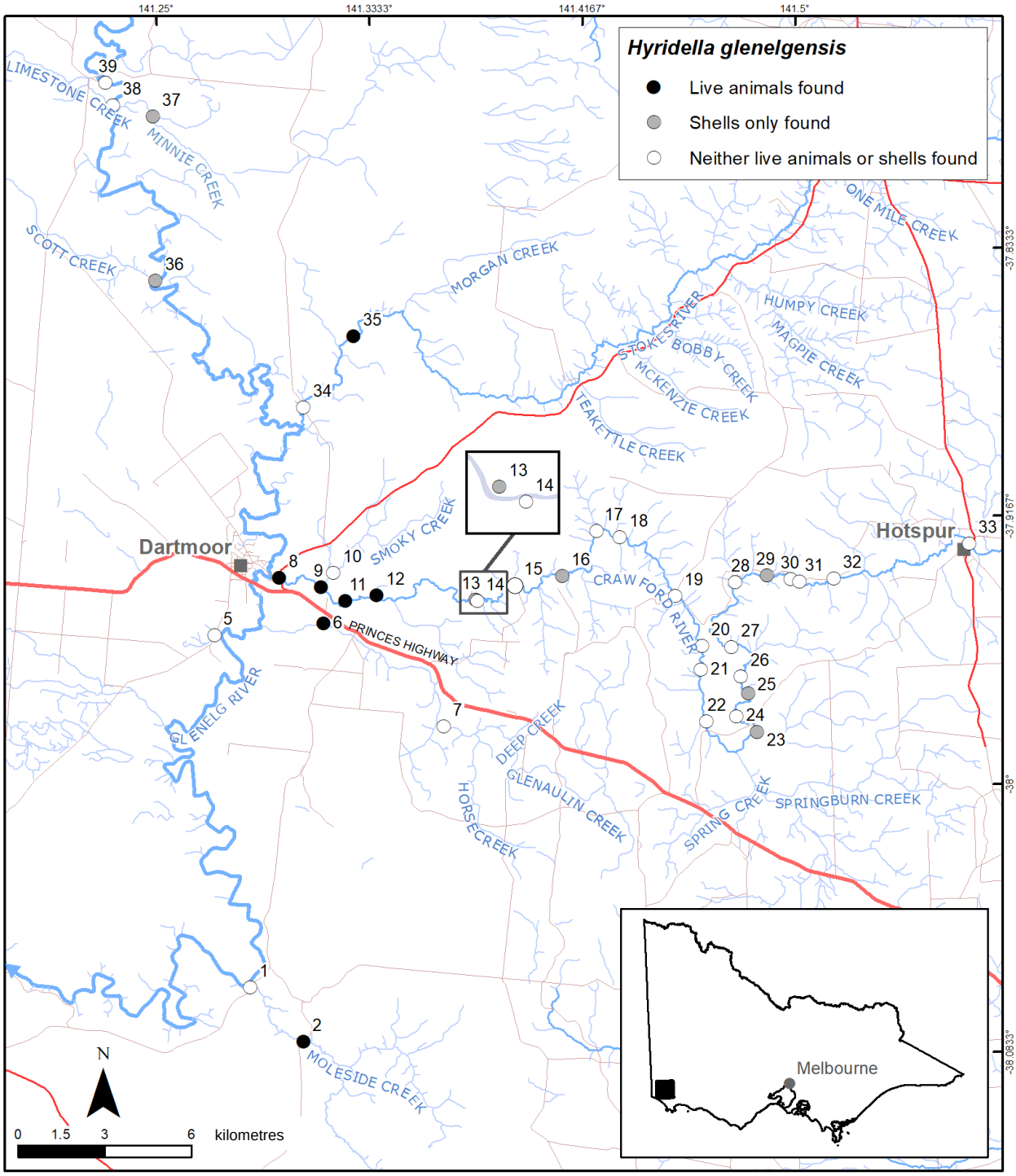

Figure 10. Results of physical sampling: location of sites where live individuals or shells of Glenelg Freshwater Mussel were found.

The distribution of the Slow Water Mussel and Pea Mussel at the survey sites is shown in Figure 11. The Pea Mussel was relatively widespread in the study area, and the Slow Water Mussel was distributed along most of the length of the Crawford River.

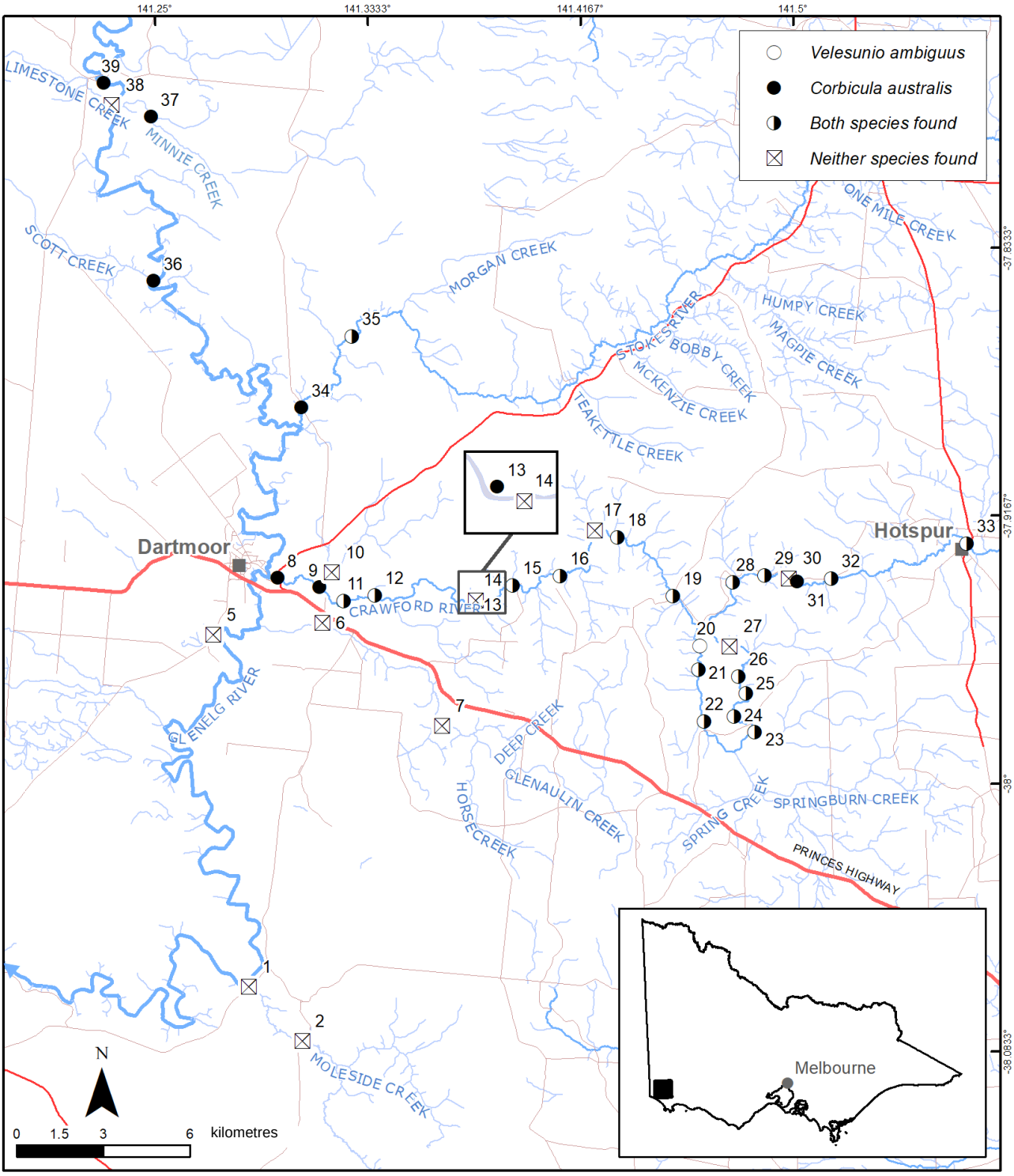

Figure 11. Results of physical sampling: sites where the Slow Water Mussel (*Velesunio ambiguus*) and Pea Mussel (*Corbicula australis*) were found.

The abundance of live individuals or shells of GFW Mussels collected at each survey site, including the total search site, and a measure of effort (number of mussels per 30-minute search) are shown in Table 3.

The highest density of mussels was recorded in the Crawford River, though many of these were dead shells. The highest density of live mussels was in the Crawford River at site 11. Surprisingly, 35 shells were found in the Crawford River at site 16, upstream from the end of East Greenwald Road, where no live mussels were detected (Figure 12).

Table 2. Locations where GFW Mussels were detected (live or shells) by physical sampling

* – data from previous monitoring (T. Raadik, unpublished data); D – shells; **L** – live shells.

Site	Waterbody	Search time (minutes)	Relative abundance	No. per 30-minutes
2*	Moleside Creek	60	5 **L**, 3 D	4.0
6	Glenaulin Creek	35	5 D	4.3
8	Crawford River	120	10 **L**	2.5
9*	Crawford River	60	5 **L**, 3 D	4.0
11	Crawford River	15	12 **L**	24.0
12*	Crawford River	70	1 **L**, 9 D10	8.5
13	Crawford River	70	2 D	0.9
15*	Crawford River	60	2D	1.0
16	Crawford River	90	35 D	11.7
23	Crawford River	80	1 D	0.7
25	Crawford River	135	1 D	0.2
29	Crawford River	30	1 D	1.0
35	Stokes River	45	3 **L**	2.0
36*	Scott Creek	45	2 D	1.3
37	Minnie Creek	90	3 D	1.0

Figure 12. Some of the large number of mussel shells found in the Crawford River upstream of East Greenwald Road. (Tarmo A. Raadik)

3.3 Environmental DNA

3.3.1 *In silico* probe development and trial

Of the two genes tested for primers, only 16S met the minimum standards specified for primer design, and therefore no primer pairs for CO1 were likely to be specific to GFW Mussel and not also amplify co-occurring species. Based on *in silico* tests (Table 3), five primer–probe sets were developed for the 16S gene. However, it was clear from the sequencing data that GFW Mussel shares a very close evolutionary history with *H. narracanensis* and the Fitzroy River *Hyridella* species. Therefore it was not possible to produce a primer–probe set that was specific for GFW Mussel, based on the tissue available. However, as neither of these species (see above) occur in the Glenelg River catchment and the probe does not detect other mussels present in the system (Slow Water Mussel and Pea Mussel), it will only detect GFW Mussel in the catchment.

Table 3. Primers/probes designed *in silico* using GenBank sequences and ssPRIMER

Probe name	Gene	Length (bp)	Forward primer	Reverse primer	Probe sequence
HG_16_1	16S	101	TATACGAGATAATGCCTGCC	ACTGGCCTCAAATTAAAAGG	GATAACCATTCAACGGCCGCGTTAG
HG_16_2	16S	106	GGTAGCGTAATAAACAGCCT	TTGAGCCTTTTCACTCAAAG	AAGGCAACACGAAGAGCAGCTTTTT
HG_16_3	16S	158	AACACGAAGAGCAGCTTTTT	CCAACCAAACCTAGGGTAAT	ATACAAAAAGACGAAAAGACCCCGC
HG_16_4	16S	117	GGAGCTTTACCCTTCAGTTA	TGCTATGATAGAAAGATTTGGAAG	TTATTACCCTAGGTTTGGTTGGGGC
HG_16_5	16S	105	GGAACAATCAATCTTCCAAATC	TTCAAGTTGGATTACGCTGT	AGAATAAAAAGAAGCTACCCCGGGG

Probe Trials

Two probes (HG_16_3, HG 16_4) were initially tested as potentially suitable (Table 3). Initial qPCRs using two GFW Mussel DNA extractions from the Crawford River (target site) demonstrated successful amplification for both primer pairs. HG 16_4 demonstrated slightly higher PCR efficiency and so was preferred for additional tests for GFW Mussel.

Specificity and Sensitivity

As expected from the primer design, both primer–probe sets showed equal efficiency when amplifying GFW Mussel, *H. narracanensis* and the Fitzroy River *Hyridella* species. Using a dilution series of the synthetic GFW Mussel G-Block, we determined that PCR reactions for HG_16_4 primer–probe set were highly efficient (R2 < 0.99) and that this probe set had a detection limit of 19 copies of the 16S GFW Mussel gene. At very high DNA concentrations, HG_16_4 primer/probe set showed minor amplification of non-target species (Slow Water Mussel, Pea Mussel and Austral River Mussel), but dilution series for the non-target species demonstrated that those PCR reactions were highly inefficient ($R^2 < 0.60$) and had detection limits 100 000 times that of GFW Mussel, meaning that the eDNA sample would need to be saturated with the off-target DNA to detect a false positive score.

Analyses were undertaken at higher annealing temperatures (up to 65 °C), which reduced the detectability and efficiency of the non-target amplification even further, but at 65 °C the detection limits for GFW Mussel increased to 850 copies of the 16S GFW Mussel gene. Therefore it was deemed that HG_16_4 was suitable for detection of GFW Mussel DNA from eDNA samples, and that the use of an annealing temperature of 60 °C would enable better detection of low DNA amounts.

3.3.2 eDNA analysis

The results of qPCR tests showed that GFW Mussel DNA was detected in 22 replicate samples from 10 sites (Table 4). This indicates a positive detection of GFW Mussel DNA from Moleside Creek (sites 1 and 2), Glenaulin Creek (sites 6 and 7), a Glenelg River tributary (site 5), Stokes River (site 34), and Crawford River (sites 8, 9, 11 and 28) (Figure 13). All positive samples returned Cq scores ranging from 34.0–39.5.

Sites 8–9 in the Crawford River had the highest detection, with up to 19 000 copies of GFW Mussel DNA detected and at least one order of magnitude higher concentration of DNA detected at this site than at other sites. Several additional samples registered a positive qPCR result in only 1 of 3 replicates ('possible' in Table 4), which may be due to either false positive reactions or very low DNA copy number present; therefore further investigation is warranted at these sites.

Table 4. Site and location where Glenelg Freshwater Mussel DNA was detected

Site	Waterbody	Sample no.	Positive replicates	eDNA presence	DNA copies detected
1	Moleside Creek	1	3	**yes**	393
1	Moleside Creek	2	2	**yes**	2409
1	Moleside Creek	3	3	**yes**	668
2	Moleside Creek	1	3	**yes**	1244
2	Moleside Creek	2	3	**yes**	1293
2	Moleside Creek	3	3	**yes**	946
5	Glenelg River	1	1	possible	
5	Glenelg River	2	2	**yes**	1336
5	Glenelg River	3	1	possible	
6	Glenaulin Creek	1	1	possible	
6	Glenaulin Creek	2	2	**yes**	1233
6	Glenaulin Creek	3	3	**yes**	1082
7	Glenaulin Creek	1	1	possible	
7	Glenaulin Creek	2	3	**yes**	2094
7	Glenaulin Creek	3	3	**yes**	4291
8	Crawford River	1	3	**yes**	15905
8	Crawford River	2	3	**yes**	19322
8	Crawford River	3	3	**yes**	5670
9	Crawford River	1	3	**yes**	12911
9	Crawford River	2	3	**yes**	5897
9	Crawford River	3	3	**yes**	10938
11	Crawford River	1	3	**yes**	4855

Site	Waterbody	Sample no.	Positive replicates	eDNA presence	DNA copies detected
11	Crawford River	2	3	**yes**	2765
11	Crawford River	3	3	**yes**	5693
12	Crawford River	1	1	possible	
15	Crawford River	1	1	possible	
16	Crawford River	1	1	possible	
22	Crawford River	2	1	possible	
25	Crawford River	2	1	possible	
27	Crawford River	1	1	possible	
28	Crawford River	1	2	**yes**	2932
28	Crawford River	2	1	possible	
28	Crawford River	3	1	possible	
30	Crawford River	1	1	possible	
30	Crawford River	2	1	possible	
34	Stokes River	1	1	possible	
34	Stokes River	2	1	possible	
34	Stokes River	3	2	**yes**	332
39	Glenelg River	1	1	possible	

Positive detections include at least 1 of 3 sample replicates per site with a positive detection; possible detections include at least 1 of 3 sub-replicates of a sample replicate with a possible detection.

All negative samples tested positive for the control DNA, therefore GFW Mussel false negatives associated with poorly performed qPCR reactions were not present in these results.

There was concordance between positive eDNA and physical sampling results: GFW Mussel was detected by both in Moleside Creek (site 2), Glenaulin Creek (site 6) and the Crawford River at sites 8, 9 and 11 (Figure 13). An eDNA positive detection also occurred on the site 1 on Moleside Creek, downstream of a known population at site 2, and in the lower site (34) on the Stokes River, which is also downstream of a known population (site 35). A positive detection was also from the Crawford River site 28, well upstream of any known population, and similarly in a small tributary of the Glenelg River (site 5) upstream of where the stream plummets approximately 20 m over a short distance into the Glenelg River, and in Glenaulin Creek (site 7) upstream of a known population of mussels (Figure 13).

Possible positive detections were from the Crawford River at a mix of sites with no physical detections or detections of mussel shells only, all upstream of the known mussel population, and at site 12, where the water samples for analysis were taken 50 mm above where live mussels were subsequently detected. An additional possible positive detection was from site 39 on the Glenelg River, which is the most upstream site in this study and is near Minnie Creek, a former location for the species.

No eDNA was detected in samples from the two former GFW Mussel population sites in Scott Creek (site 36), and Minnie Creek (site 37), or the nearby permanently flowing Limestone Creek from which no live mussels of shells have been detected despite repeated sampling (T. Raadik, unpublished data). Lastly, the detection of a new population of GFW Mussel in the Stokes River (site 35) by physical sampling, was not

corroborated by a positive eDNA detection, even though, like the Crawford River site 12, the water samples were taken 50 mm directly above live mussels.

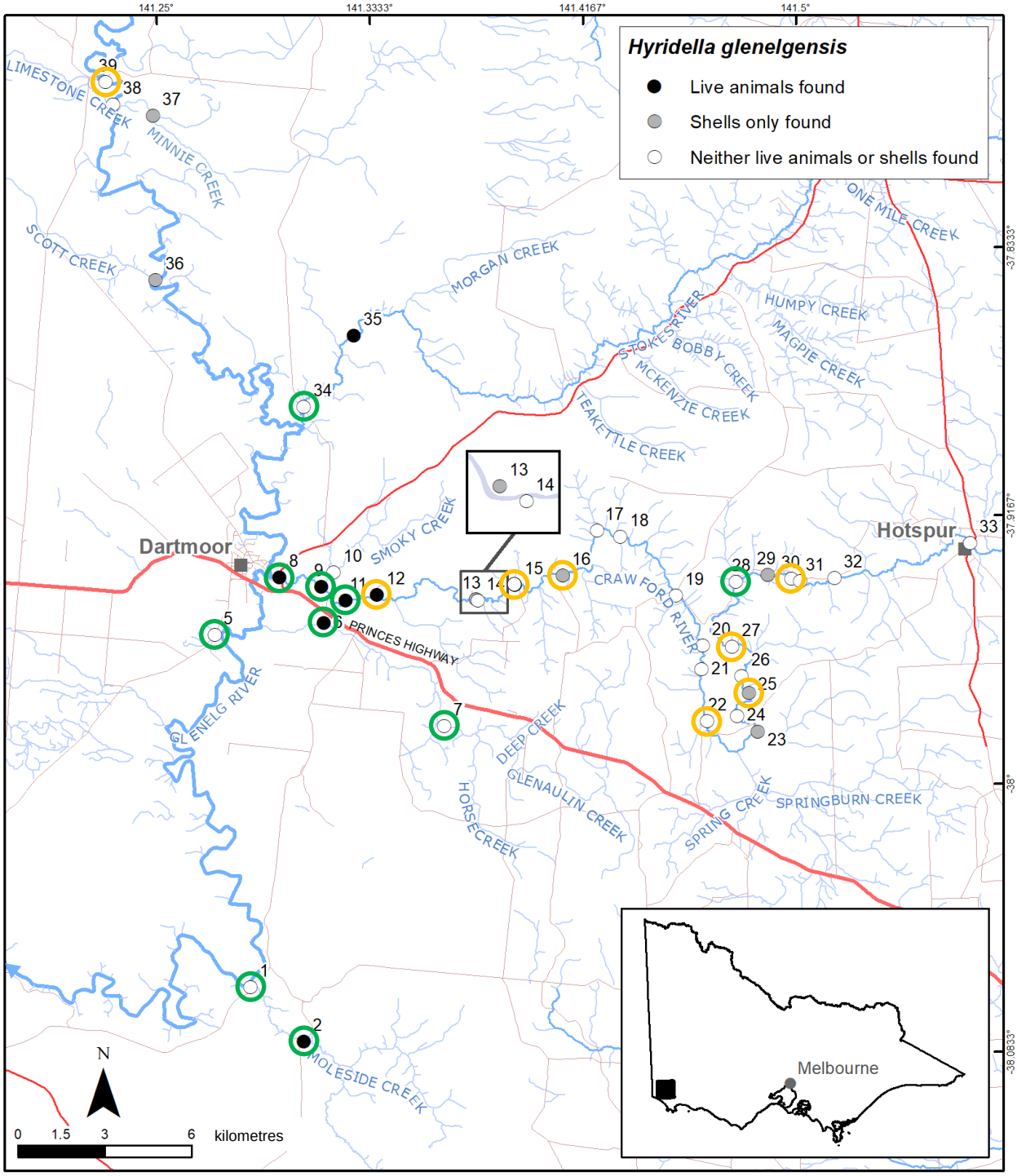

Figure 13. Remote sensing results: location of positive (green circles) and possible positive (orange circles) eDNA detections for Glenelg Freshwater Mussel. No coloured circle indicates no detection of DNA.

4 Discussion

This project has refined the distribution of GFW Mussel in the Crawford River and established a baseline of distribution and mussel abundance which can form the basis of a monitoring program. With respect to the 2020 fire in the mid-portion of the catchment, detailed physical sampling has confirmed that the species still exists in the Crawford River in the lower reaches (Winnap Siding and downstream) and confirmed previous suggestions that the species appears to be absent from the mid- to upper catchment where it was historically present (Walker et al. 2001). In addition, we have also confirmed the ongoing persistence of the species in two small, isolated populations in Moleside Creek and Glenaulin Creek, which were discovered in 2015 (Raadik 2019; T. Raadik, unpublished data).

Additional locations for GFW Mussel have also been found in the Crawford River system, based on new collections of shells. These may reflect extirpated (historical) populations, or the presence of nearby (upstream) remnant populations. Significantly, a new population of the species was discovered in the Stokes River, the next largest tributary of the Glenelg River to the north of the Crawford River. This brings the total number of GFW Mussel populations to four, increasing from the one population known in the Crawford River in 2005 (Playford and Walker 2008). These discoveries have occurred over the last six years when intensive surveys for the species commenced (Raadik 2019), which also located evidence of two historical populations based on the discovery of shells (Scott Creek and Minnie Creek).

Whilst the species was sparsely distributed along a 28 km reach of the Crawford River in 2005, extending from the Lyons/Hotspur Road to the Glenelg River (Playford and Walker 2008), we confirm that its current distribution has reduced to a 5 km reach from the Glenelg River junction upstream to Winnap Siding Road. This section of the Crawford River appears to have the best water, and stream flow, permanency, with flows appearing to be supported by groundwater outflow, particularly during dry periods (T. Raadik, pers. obs.). This observed dependence of GFW Mussel on flowing water is supported by its presence in the lower reaches of the Crawford River, as well as in the lower Moleside Creek, and in Glenaulin Creek, which also have permanent flow. The species was also historically present in Minnie Creek, a tributary of the Glenelg River farther north in the catchment. This system appears to have been a permanent stream but is now surrounded by pine plantation and flows appear intermittent.

The discovery of dead shells from two locations farther up the Crawford River, to the Steep Bank camping area off The Boulevard, confirms the historical distribution of GFW Mussel extended upstream to at least that area: no evidence of GFW Mussel from here was found in the detailed survey in 2005 (Playford 2005; Playford and Walker 2008). Potentially additional sampling upstream from this location may locate additional shells.

As searching for small freshwater mussels is difficult and time consuming, we developed and trialled a remote sensing method to detect mussel DNA from water samples (eDNA), as a complementary tool. A genetic ‘probe’ was first developed to detect a short sequence of mussel DNA, and then used to screen water samples collected from survey sites for mussel DNA, with positive detections of DNA indicating the presence of live mussels in the stream at some point upstream of the water sample location. The eDNA analysis resulted in several positive detections, as well as potentially positive detections where a positive DNA detection was produced by a low number of replicate samples per site.

The combination of remote sensing using eDNA analysis and the physical searches have provided relatively complementary survey results. For most sites where GFW Mussel were physically detected, the eDNA also provided a positive detection. The presence of live mussels at these locations validates the positive eDNA results, confirming that this technique has value in detecting live GFW Mussels.

However, some results differed. A positive eDNA detection was recorded for site 28 (near Kingfisher camping area), upstream in the Crawford catchment, despite no physical detection of mussels within the vicinity of this site, however shells were located farther upstream. A similar result was also present at site 7, the most upstream site in the Glenaulin Creek. Both site 28 and site 7 suggest that GFW Mussel has a greater range than the physical surveys suggest. In addition, the possibly positive eDNA results were also concentrated in the upper part of the Crawford River. Therefore, more detailed physical searches are

required in the upstream reaches of these streams to validate results. Further, a positive detection for GFW Mussel was not substantiated by the location of live mussels during physical sampling at site 5 on a small, permanently flowing tributary of the Glenelg River. Again, more detailed sampling in this system is also needed for validation.

Three positive replicate eDNA samples were also recorded from site 1 on Moleside Creek, despite no physical evidence from searches. However, however, this site is directly downstream from a live population, and one farther upstream at sites 2, and therefore downstream transport of DNA to site 1 is possible. A similar explanation may be derived for the discrepancy between sites 34 and 35 in the Stokes River, with the downstream site showing a positive eDNA signal, whilst the upstream site was negative despite containing a population of mussels. The positive eDNA signal at the downstream site further supports the notion of downstream movement of DNA.

The downstream movement of DNA may also influence the strength of a detection; the two most downstream sites (8 and 9) in the Crawford River, which contains the largest population of the GFW Mussel, clearly had the highest concentration of GFW Mussel DNA of all positive detection sites. The lack of a positive detection for the Stokes River population may be due to the high abundance of the much larger Slow Water Mussel, which was about 33 times as abundant, possibly resulting in a low abundance of GFW Mussel in the Stokes River, which is indicated by only 350 copies of DNA recorded at the downstream site. This needs further investigation.

Consequently, while we have demonstrated a positive benefit of potentially using eDNA detection to initially screen sites for GFW Mussel presence to focus physical survey effort, additional work is required to understand anomalous results from this project to improve the accuracy and detection probability of eDNA sampling.

The persistence of live GFW Mussel populations appears to be linked to groundwater-fed reaches of streams where water flow is maintained, as all known extant populations are present in such reaches. This may therefore strongly influence the distribution of the species, and potentially its abundance, and declining groundwater outflow may be a key driver of GFW Mussel decline. An investigation of change (i.e. decline) in flow permanency along reaches of the mid to upper Crawford River has not been undertaken but is suspected based on the number and location of dead mussels and lack of live populations and may be responsible for the downstream decline in the distribution of GFW Mussel since 2005. This may also be responsible for the loss of the population in Minnie Creek, where the replacement of the natural forest by pine plantations may have reduced groundwater outflow volumes.

Thus a decline in groundwater and surface water volumes, leading to greater intermittency in stream flow and water persistence, may be a greater threat to GFW Mussel then previously understood, particularly as the threat operates at a landscape scale. In this respect, and particularly pertinent to GFW Mussel, is the proliferation of Blue Gum (*Eucalyptus globulus*) and Radiata Pine (*Pinus radiata*) plantations, which have been implicated in lowering water tables, drying springs, and reducing surface water flows (SKM 2008). Consequently, the careful management of refuge locations for GFW Mussel, in areas where the species currently persists, should be a high priority, with the protection and possibly improvement of groundwater outflow volumes of primary importance.

Extraction of groundwater and surface water typically increases in the region during summer, which in turn results in an increase in the salinity and temperature of stream water and lower dissolved oxygen concentrations, all of which may be lethal to the species (Walker et al. 2014a). Water extraction also reduces the quantity and depth of surface water, which in turn exposes sheltered littoral areas occupied by mussels (Walker et al. 2014a), leading to potentially increased predation and death by desiccation. These effects are likely to be exacerbated by climate-change, since the Glenelg region is predicted to experience a more variable climate, increased temperatures and less rainfall (Jones and Durack 2005; Alamgir et al. 2014), resulting in less groundwater and surface water, reduced stream flow, and increased frequency and severity of droughts and bushfires. Small streams like Minnie Creek, Scott Creek, Stokes River, Crawford River and Glenaulin Creek are likely to be especially vulnerable due to their size and the prevalence of anthropogenic threats in their catchments.

The management of leached chemicals such as herbicides and pesticides that are commonly used on agricultural land and eucalypt plantations (Carnegie et al. 2017) also requires management at the landscape

scale. These can impact GFW Mussel populations because mussels are filter feeders and bioaccumulate contaminants in their tissues (Walker et al. 2001).Therefore it is vital to prevent chemicals entering waters at or upstream of GFW Mussel refuge habitat, including tributary streams and dry drainage lines adjacent to populations. This risk is therefore high for all GFW Mussel populations which drain from private or public land, except for the population in Moleside Creek which is mainly in a national park.

Unimpeded stock access has also been implicated in the loss of freshwater mussel populations elsewhere in Australia (Walker et al. 2001; Brainwood et al. 2006).

At a local level, fencing and revegetation work is fundamental in maintaining the health of streams, particularly mussel refuge habitats, as it assists recovery of the riparian zone, is critical in limiting threatening anthropological driven threats, and controlling stock access to streams. This reduces the possibility of GFW Mussels being trampled, and the occurrence of erosion and compaction of sediments, pollution of water, and destruction of vegetation (both in-stream and riparian vegetation; Walker et al. 2014a).

Lastly, an emerging threat to the long-term persistence of GFW Mussel is the increasing presence of the introduced Common Carp (*Cyprinus carpio*) in the Glenelg River system. It was originally introduced into Rocklands Reservoir and subsequently confirmed in the Glenelg River below the storage in 2002 (Brown et al. 2003). Common Carp have now spread to the lower reaches of the Glenelg River, being recorded from the estuary (C. Solum, Glenelg Hopkins CMA, pers. comm.), and no instream barriers are present to prevent it moving into the Crawford River, Stokes River and Glenaulin Creek. This species can be very abundant (Davies et al. 2008) and is responsible for the destruction of wetland and stream habitats and the displacement and decline of numerous native fauna, including several species of fish and invertebrates (Sheldon and Walker 1993; Walker et al. 2014a); and they also eat molluscs. Further, there is evidence that Common Carp are not effective hosts for glochidia, the parasitic larval stage of freshwater mussels (Walker 1981; Walker et al. 2001; 2014b; DPIPWE 2009; Klunzinger et al. 2012).

Like other threatened species persisting at a few sites (e.g. Furlan et al. 2012; Brown et al. 2021), GFW Mussel is at a high risk of extinction. Consequently it would benefit from the establishment of additional populations within catchments in the region as a risk mediation strategy, in addition to protecting and rehabilitating existing habitat. This could be achieved by translocating wild individuals and raising and releasing captive-bred individuals. However, not enough individuals are currently available in the small extant populations to support wild-to-wild translocation, suitable translocation sites have not been found, and captive breeding has not been attempted. These actions fall within a broader framework of species recovery and ecological restoration, driven by maintaining or re-establishing evolutionarily sustainable populations and wild habitats (Collares-Pereira and Cowx 2004; Shute et al. 2005).

5 Conclusion

This field-based project has contributed valuable knowledge for the conservation of the Glenelg Freshwater Mussel, particularly in relation to its persistence following fire, its reliance on flowing stream reaches, and the establishment of a monitoring program. The development and successful trial of a complementary remote-sensing technique (environmental DNA analysis) as a highly efficient detection tool to target physical sampling for the species further improves the ability to potentially detect remnant populations of the species and to monitor the presence of live individuals without disturbance.

The identification of a dependency of GFW Mussel on perennially flowing habitats is a significant outcome and is important in the context of the conservation management for the species at a landscape scale (groundwater aquifer management to maintain outflow) and local scale (refuge habitat protection). This is in addition to management of groundwater and surface water inflows for overall river health. Further research and management are required on catchment groundwater – surface water interactions (at local and regional scales) and specific stream spring outflow areas (location, volume, duration), and research on the mussel's thermal and respiratory requirements and its use of non-flowing remnant pools.

References

Alamgir, M., Pert, P. L. and Turton, S. M. (2014). A review of ecosystem services research in Australia reveals a gap in integrating climate change and impacts on ecosystem services. *International Journal of Biodiversity Science, Ecosystem Services and Management* **10**(2), 112–127.

Brainwood, M., Burgin, S., and Byrne, M. (2006). Is the decline of freshwater mussel populations in a regulated coastal river in south-eastern Australia linked with human modification of habitat? *Aquatic Conservation* **16**(5), 501–516.

Brown, P., Sivakumaran, K.P., Stoessel, D., Giles, A., Green, C. and Walker T. (2003). *Carp Population Biology in Victoria*. Report 56. Marine and Freshwater Resources Institute, Department of Primary Industries, Snobs Creek. Victoria.

Brown, K., Tambyahm T., Fenwick, J., Burke, A., Grant, P., Gegarty-Cremer, S., Muller, J. and Bode, M. (2021). Choosing optimal trigger points for ex situ, in toto conservation of single population threatened species. bioRxiv, doi: https://doi.org/10.1101/2021.01.08.425867 (preprint).

Bylemans, J., Gleeson, D.M., Hardy, C.M. and Furlan, E. (2018). Toward an ecoregion scale evaluation of eDNA metabarcoding primers: A case study for the freshwater fish biodiversity of the Murray–Darling Basin (Australia). *Ecology and Evolutiion* **8**(17), 8697–8712.

Carnegie, A., Lawson, S., Cameron, N., Wardlaw, T. and Venn, T. (2017). *Evaluating the Costs and Benefits of Managing New and Existing Biosecurity Threats to Australia's Plantation Industry*. Forest and Wood Products Australia, Melbourne.

Collares-Pereira, M., and Cowx, I. (2004). The role of catchment scale environmental management in freshwater fish conservation. *Fisheries Management and Ecology* **11**, 303–312.

Cotton, B.C. and Gabriel, C.J. (1932). Australian Unionidae. *Proceedings of the Royal Society of Victoria* **44**, 155–160.

Davies, P.E., Harris, J.H., Hillman, T.J. and Walker, K.F. (2008). SRA Report 1: A report on the ecological health of rivers in the Murray– Darling Basin, 2004–2007. Prepared by the Independent Sustainable Rivers Audit Group for the Murray–Darling Basin Ministerial Council. Murray-Darling Basin Commission, Canberra.

Dennant, J. (1898). Description of a new species of *Unio* from the River Glenelg. *Proceedings of the Royal Society of Victoria* **10**, 112–113.

DPIPWE (2009). Status of fish communities and observations on South Esk Freshwater Mussel (*Velesunio moretonicus*) populations in the Macquarie River catchment upstream of Lake River. Water Assessment Aquatic Ecology Report WA 09/02. Department of Primary Industries, Parks, Water and Environment, Hobart.

Furlan, E., Stokolosa, J., Griffiths, J., Gust, N., Ellis, R., Huggins, R.M. and Weeks, A.R. (2012). Small population size and extremely low levels of genetic diversity in island populations of the platypus, *Ornithorhynchus anatinus*. *Ecology and Evolution* **2**(4), 844–857.

Jones, R. and Durack, P. (2005). *Estimating the Impacts of Climate Change on Victoria's Runoff Using a Hydrological Sensitivity Model.* CSIRO Atmospheric Research, Melbourne.

Klunzinger, M.W., Beatty, S.J., Morgan, D.L., Thomson, G.J. and Lymbery, A.J. (2012). Glochidia ecology in wild fish populations and laboratory determination of competent host fishes for an endemic freshwater mussel of south-western Australia. *Australian Journal of Zoology* **60**, 26–36.

Larson, E. R., Renshaw, M. A., Gantz, C. A., Umek, J., Chandra, S., Lodge, D. M., and Egan, S. P. (2017). Environmental DNA (eDNA) detects the invasive crayfishes *Orconectes rusticus* and *Pacifastacus leniusculus* in large lakes of North America. *Hydrobiologia* **800**(1), 173–185.

McMichael, D.F. and Hiscock, I.D. (1958). A monograph of the freshwater mussels (Mollusca: Pelecypoda) of the Australian region. *Australian Journal of Marine and Freshwater Research* **9**, 372–508.

Playford, T.J. (2005). Conservation status of the endangered Glenelg River mussel *Hyridella glenelgensis* (Dennant, 1898) (Unionoida: Hyriidae). Unpublished BSc Honours Thesis. School of Earth and Environmental Sciences, The University of Adelaide.

Playford, T.J. and Walker, K.F. (2008). Status of the endangered Glenelg River Mussel *Hyridella glenelgensis* (Unionoida: Hyriidae) in Australia*. Aquatic Conservation: Marine and freshwater Ecosystems* **18**(5), 679–691.

Raadik, T.A. (2019). Recovery actions for Glenelg Freshwater Mussel. Biodiversity On-ground Actions Regional Partnerships and Targeted Actions Project 2017–18. Published Fact Sheet. Arthur Rylah Institute for Environmental Research, Department of Environment, Land, Water and Planning, Heidelberg. 2 pp.

Raadik, T.A. and Nicol, M.D. (2018). Assessing condition and value of potential aquatic refuges in small, unregulated Victorian streams: A methodology. Report to Catchments, Waterways, Cities and Towns Division, DELWP. Arthur Rylah Institute for Environmental Research, Department of Environment, Land, Water and Planning, Heidelberg. 26 pp.

Sheldon, F. and Walker, K.F. (1993). Short communication. Pipelines as a refuge for freshwater snails. *Regulated Rivers: Research & Management* **8**(3), 295–299.

Shute, J.R., Rakes, P.L. and Shute, P.W. (2005). Reintroduction of four imperilled fishes in Abrams Creek, Tennessee. *Southeastern Naturalist* **4**, 93–110.

SKM (2008). Water and land use change study: Stage 3 Case Studies. Water and land Use change in the catchments of: Darlots Creek, Eumeralla River, Moyne River, Merri River. Sinclair Knight Merz, Report to Glenelg Hopkins Catchment Management Authority and Water and Land Use Change Steering Committee. Project VW03647.

Spens, J., Evans, A.R. Halfmaetern, D., Knudsen, S.W., Sengupta, M.E., Mak, S.S.T., Sigsgaard, E.E. and Hellström, M. (2017). Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods in Ecology and Evolution* **8**(5), 635–645.

Walker, K.F. (1981). *Ecology of freshwater mussels in the River Murray*. Australian Water Resources Council, Canberra.

Walker, K.F., Byrne, M., Hickey, C.W. and Roper, D.S. (2001). Freshwater mussels (Hyriidae) of Australasia. In: Bauer, G. and Wächtler, K. (Eds.) *Ecology and Evolution of the Freshwater Mussels Unionoida*, pp. 5–31. Springer, New York.

Walker, K.F., Jones, H.A. and Klunzinger, M. (2014a). *Hyridella glenelgensis*. The IUCN Red List of Threatened Species 2014: e.T58609631A58628791. http://dx.doi.org/10.2305/IUCN.UK.2014-3.RLTS.T58609631A58628791.en

Walker, K.F., Jones, H.A. and Klunzinger, M.W. (2014b). Bivalves in a bottleneck: taxonomy, phylogeography and conservation of freshwater mussels (Bivalvia: Unionoida) in Australasia. *Hydrobiologia* **735**(1), 61–79.

Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R., and Schwartz, M. K. (2013). Robust detection of rare species using environmental DNA: the importance of primer specificity. *PLOS One* **8(3)**, e59520.

Appendix

Table A1. Survey site location, date of sampling, and type of sampling undertaken

– not physically sampled but persistence of GFW Mussel known from previous monitoring from 2014–2019 (T. Raadik, unpublished data).

Site	Waterbody	Location	Latitude	Longitude	Date	Physical sampling	Physical Sampling duration (min)	eDNA sampling
1	Moleside Creek	Below falls, downstream Winnap/Nelson Rd	-38.05463	141.27161	23/03/2021	Y	30	Y
2		Kentbruck Road	-38.07214	141.29182		#	60	Y
3	Glenelg River	Tributary, Wanwin Road	-37.96155	141.22403		N	-	N
4		Tributary, Wanwin Road	-37.97309	141.21161		N	-	N
5		Tributary, Wanwin Road	-37.94471	141.26300		Y	30	Y
6	Glenaulin Creek	upstream end of plantation, downstream of Winnap/Nelson Road	-37.94221	141.30631		Y	35	Y
7		off track, upstream of Bacci Creek	-37.97579	141.35162	24/03/2021	Y	30	Y
8	Crawford River	at Dartmoor/Hamilton Road	-37.92764	141.28950	23/01/2021	Y	120	Y
9		Spencer's Road	-37.93092	141.30568	24/03/2021	#	60	Y
10	Smokey Creek	off track, off Spencer's Road	-37.92676	141.31072		Y	30	N
11	Crawford River	upstream of Spencers Road	-37.93550	141.31513		Y	15	Y
12		at Winnap Siding Road	-37.93412	141.32736		#	70	Y
13		off border track, north-west edge of Balds Plantation, off Balds Road	-37.93669	141.36605	21/01/2021	Y	70	N

Site	Waterbody	Location	Latitude	Longitude	Date	Physical sampling	Physical Sampling duration (min)	eDNA sampling
14	Crawford River	off border track, north-west edge of Balds Plantation, off Balds Road	-37.93708	141.36684	24/03/2021	Y	5	N
15		at pool downstream of Johnsons Road	-37.93277	141.38174		#	60	Y
16		upstream of end of East Greenwald Road	-37.93038	141.40048	21/01/2021	Y	105	N
		upstream of end of East Greenwald Road			24/03/2021	Y	30	Y
17		in Crawford Lake	-37.91678	141.41480	25/03/2021	N[1]	-	Y
18		upstream from Crawford Lake	-37.91907	141.42369	21/01/2021	Y	70	N
19		Hiscocks Crossing on Big Hill Road	-37.93800	141.44400	25/03/2021	Y	35	Y
20		off track, NW edge of McEachern Plantation	-37.95380	141.45433	19/01/2021	Y	90	Y
21		off track, west edge of McEachern Plantation, upstream of The Neck	-37.96107	141.45369	20/01/2021	Y	60	N
22		south-west side of McEachern Plantation	-37.97732	141.45495		Y	90	N
		south-west side of McEachern Plantation			25/03/2021	N	-	Y

[1] Too deep to sample

Site	Waterbody	Location	Latitude	Longitude	Date	Physical sampling	Physical Sampling duration (min)	eDNA sampling
23	Crawford River	Lyons/Hotspur Road, east crossing	-37.98124	141.47487	19/01/2021	Y	80	Y
24		south-south-east corner of McEachern Plantation	-37.97614	141.46692	20/01/2021	Y	80	N
25		off track, east-south-east boundary of McEachern Plantation	-37.96902	141.47180	19/01/2021	Y	135	N
		off track, east-south-east boundary of McEachern Plantation			25/03/2021	Y	30	Y
26		off track, eastern boundary of McEachern Plantation	-37.96373	141.46930	19/01/2021	Y	90	N
27		off track, north-east side of McEachern Plantation	-37.95444	141.46605		Y	225	Y
28		just downstream of Kingfisher camping area, off The Boulevard	-37.93429	141.46839	20/01/2021	Y	60	N
		just downstream of Kingfisher camping area, off The Boulevard				N	-	Y
29		upstream of Bronzewing camping area, off The Boulevard	-37.93248	141.48105	25/03/2020	Y	30	N
30		at Steep Bank camping area, off The Boulevard	-37.93393	141.49027		Y	30	Y
31		just upstream of Steep Bank camping area, off The Boulevard	-37.93486	141.49366	20/01/2021	Y	60	N

Site	Waterbody	Location	Latitude	Longitude	Date	Physical sampling	Physical Sampling duration (min)	eDNA sampling
32	Crawford River	east end of Crawford River Regional Park, off The Boulevard	-37.93430	141.50710	20/01/2021	Y	60	N
33		at old bridge, Hotspur	–37.92488	141.56082	25/03/2021	N[2]	-	Y
34	Stokes River	at Casterton/Dartmoor Road	-37.87482	141.30147	23/03/2021	Y	30	Y
35		off end of track off Casterton/Dartmoor Road	-37.85327	141.32226		Y	45	Y
36	Scott Creek	from Glenelg R to 50 m upstream	-37.83367	141.24542	22/01/2021	Y	45	N
		from Glenelg R to 50 m upstream			22/03/2021	N	-	Y
37	Minnie Creek	at end of track in plantation	-37.78246	141.24676	18/01/2021	Y	30	Y
		at end of track in plantation			22/03/2021	Y	30	Y
38	Limestone Creek	at ford on old track	-37.77860	141.23143	18/01/2021	Y	30	Y
		at ford on old track			22/03/2021	N	-	Y
39	Glenelg River	at old bridge on Myaring–Pieracle Road	-37.77151	141.22887	23/03/2021	Y	40	Y

[2] Too deep to sample; sampled previously.

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