Survey of the Wingless Donna Buang Stonefly *Riekoperla darlingtoni* in relation to the proposed Warburton mountain bike trail.

By Eddie Tsyrlin

The juvenile (left) and the adult (right) of *Riekoperla darlingtoni*

Introduction

Mt Donna Buang stonefly, *Riekoperla darlingtoni* (Illies 1968), is one of the two species of wingless stoneflies found in Australia and the only wingless species of stoneflies found in Victoria. *R. darlingtoni* is a cryptic species from family Gripopterygidae, order Plecoptera (stoneflies). Federally, the species is listed as “critically endangered” (Department of the Environment, 2018) and in Victoria, as “threatened” (DELWP, 2018) with recommendation to be listed as “critically endangered” (DSE 2009). Additionally, the species is in the IUCN Red List of Threatened Species (IUCN 2017) due to its extremely limited range of distribution and “a continuing decline in the number of mature individuals, observed from 2005 to 2012” (IUCN, 2017).

Wingless Stoneflies are found within a 1km radius from the summit of Mt Donna Buang. Its suitable habitat includes springs and trickles found down to 900m above sea level within the Yarra Ranges National Park. This species requires high quality of water and habitat. It is likely to be extremely sensitive to any amount of water pollution, sedimentation and any forms of habitat alteration.

Melbourne Water has conducted surveys of the species every August from 2005 to 2016 which showed a significant decline in numbers of (at least 90%) since 2006 (Melbourne Water and DELWP unpublished data). This dataset provided ample evidence and warranted listing this species under the EPBC act.

Apart from the conservation significance stemming from its extremely limited distribution range, the stonefly is an example of an “island species” intricately linked to a narrow range of environmental conditions, such as slow flowing ephemeral springs trickles in forested areas at altitudes above 900 meters. The species has an unusually long life cycle. It takes two to three years for its juvenile aquatic stage (nymph) to develop into the adults. During this time it lives in springs and trickles and digs into moist ground when the springs dry up during the Summer months (Hynes & Hynes 1975). The wingless adults are usually found within 1-2 meters of a stream edge, mostly in rolled pieces of mountain ash bark (Hynes, 1974, personal obs.).

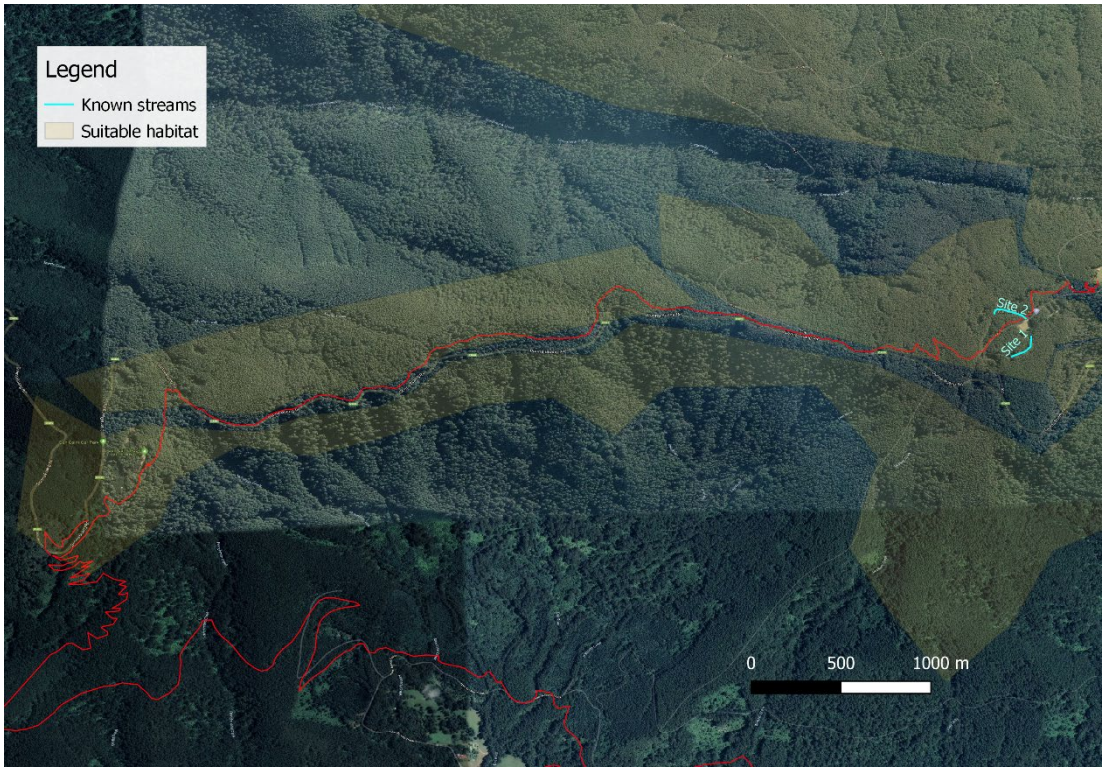
The Yarra Ranges Shire Council sought to investigate the presence / absence of the critically endangered Donna Buang stonefly to address a possible risk posed by the

construction, maintenance and use of a mountain bike trail proposed for the Mt Donna Buang area.

Methods

Study Site

The main study was conducted in the area downstream of the proposed mountain bike trail (Map 1). Ecological Vegetation Community of this area is classified as Montane wet forest dominated by Alpine Ash *Eucalyptus delegatensis* and Shining Gum *E. vnitens,* with cool temperate rainforest patches characterised by Myrtle Beech *Nothofagus cunninghamii* (Ahern, Tsyrlin & Myers 2003).



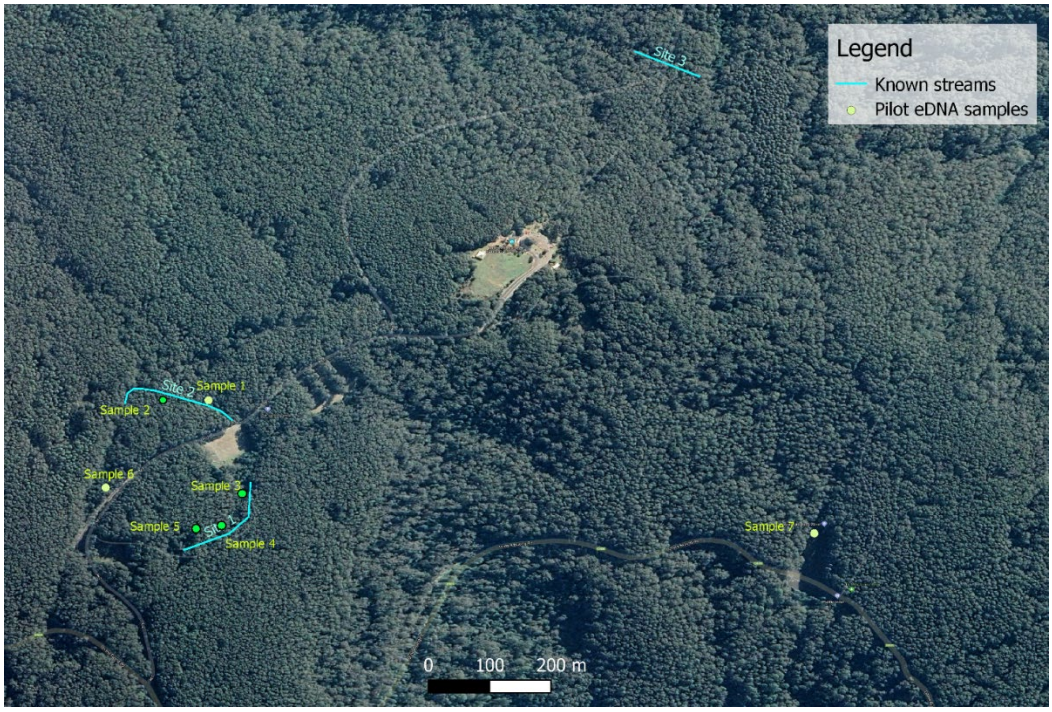
Map 1. The proposed mountain bike trail in relation to the probable stonefly area of distribution of *R. darligntoni.*

eDNA Methodology

Using an environmental DNA (eDNA) approach has been suggested for efficient detection of the stonefly over the brief period of two months in the difficult terrain of Mt Donna Buang. eDNA is an emerging tool for detecting organisms from environmental samples like soil, sediments, marine and fresh waters (Thomsen & Willerslev 2015). This method relies on capturing DNA that an organism sheds into its environment. This DNA is detected using primers and probes unique to the target species. The approach allows detection of cryptic

and rare species without having to physically catch them. It offers an advantage of being sensitive, non-invasive and safe for the operators in the field as they don't need to enter a water body (Griffiths et al. 2016).

Our study had two stages. The first stage was a pilot study (Map 2) to verify the performance of the species-specific primers and probe by sampling habitats where the stonefly is known to occur and habitats where stonefly is known to be absent. Once we were satisfied with the sensitivity and reliability of our methods, we undertook the main survey to locate sites near the proposed trail where the stonefly could be present.



Map 2. Pilot e DNA sites. Bright green circles (Samples 2, 3, 4 and 5) indicate detection of R. darlingtoni DNA. Samples 8 and 9 – Cement creek and Sample 10 – Yarra River are outside of this map. "Known streams" refer to water bodies where larvae have been observed previously. In case of Site 2, the stream continues downstream to point 6 and further but we did not observe larvae beyond the range indicated on the map.

DNA Collection and Extraction

For the pilot study, water samples were collected from 10 different locations (Map 2) on 8 July 2019. For the main study, water samples were collected between 10 and 26 September 2019 on four separate occasions, with additional sampling on the 3rd October 2019 to verify the species present at two sites. The samples were stored for 48 hours at 4C prior to the analysis. To extract DNA from water, we filtered between 400 and 600mL of water using

60mL syringes and Sterivex® 0.22 μm filter unit (Merck, Germany). The filters were stored at -20C until DNA extraction. The Qiagen DNeasy Blood & Tissue Kit was used for DNA extraction with modifications described by Lugg et al. (2017).

Primer Design, And Taqman® qPCR Assays

The marker and probe unique to *R. darlingtoni* were based on the available COI region of mitochondrial DNA sequenced earlier. We used Primer 3 module (version 2.3.7) Geneious program (Geneious Prime® 2019.2.1 n.d.) for the probe and primer design. The uniqueness of the probe and primers was assessed using primer BLAST (Ye et al. 2012). The eDNA primers and probe used for the study were as follows:
Probe: TCACCTCGCCGGAGTCTCCTCGA
Forward primer: CATGCCGGAGCCTCAGTAG
Reverse primer (original sequence): CAAGAGTTATACCGGTGGATCG

eDNA Lab Analysis
"Real-time TaqMan® PCR assays were conducted using a Roche LightCycler 480 system in a 384-well format. 10 μL reactions containing 5 μL of 2 × Qiagen multiplex PCR Master Mix (Qiagen), 0.5 μL 20× TaqMan® Gene Expression Assay, 2.5 μL ddH_2O and 2 μL of DNA were prepared in triplicate. These triplicates are used to minimise pipetting and other technical errors during the PCR essays. After initial testing, we used KAPA Taq enzyme for all PCR essays to overcome the issue of PCR inhibitors present in the water (Wong et al. 2014).

All extractions and qPCR analysis were undertaken in a room that is dedicated to low-quantity DNA sources. Negative controls were included at all stages (DNA extraction, qPCR) so that contamination issues could be identified if present. (Lugg et al. 2017).

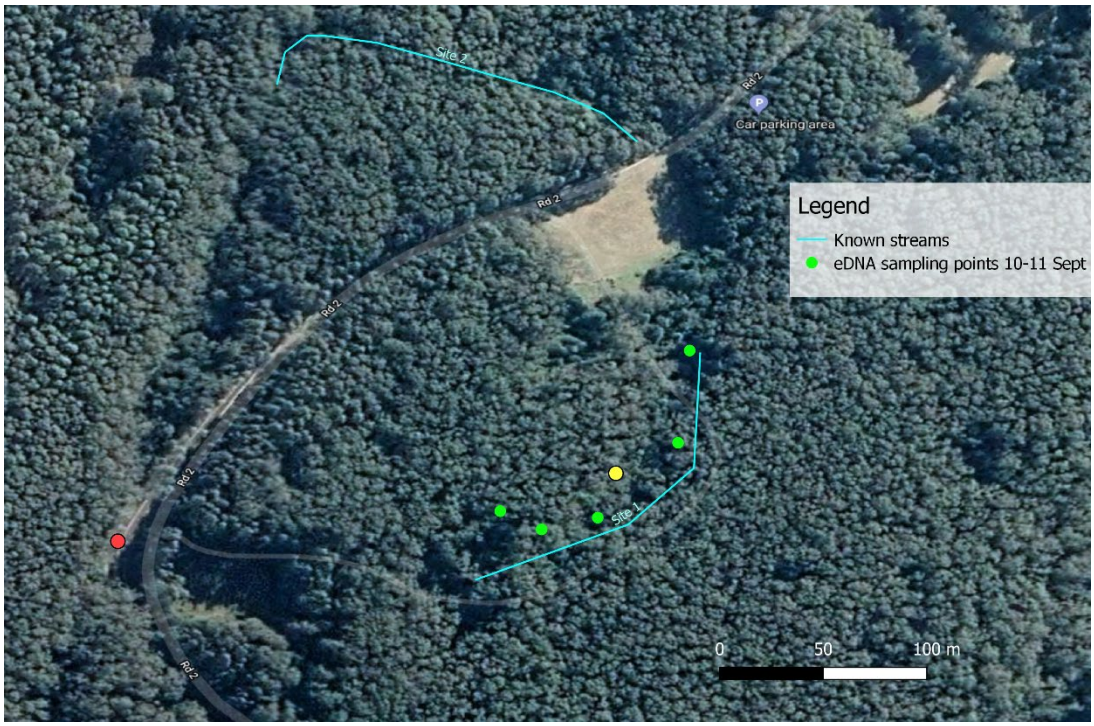
Results

Pilot study

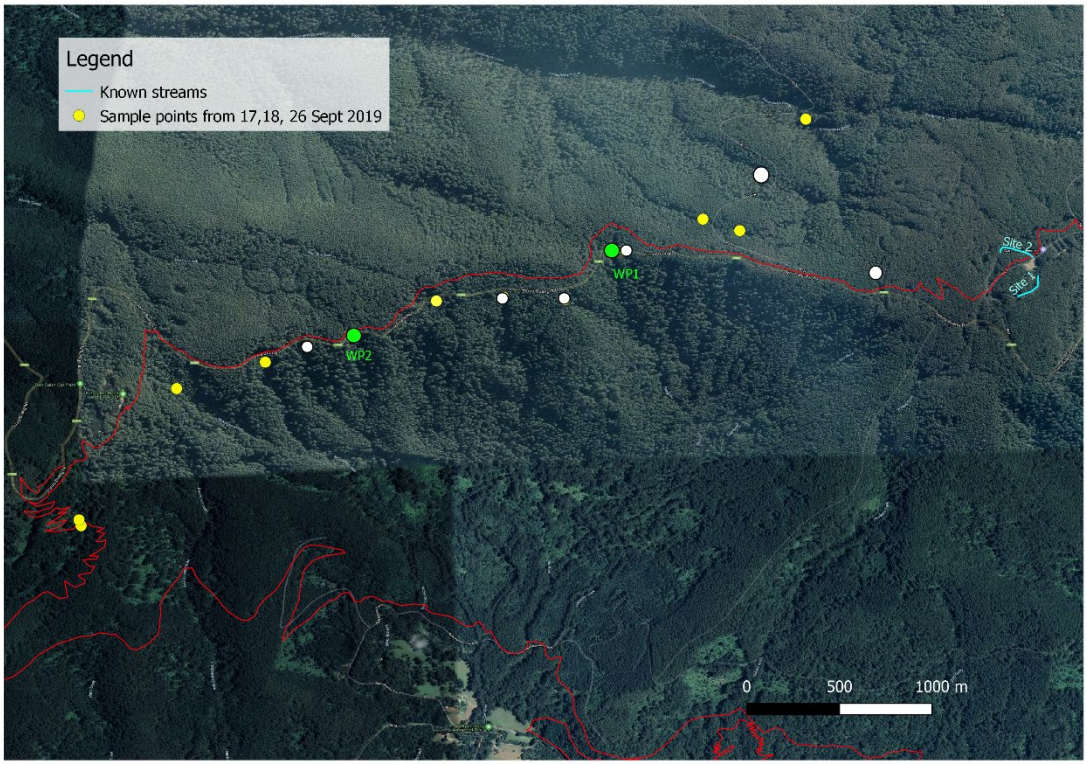
During the pilot study, the target eDNA was successfully detected at Sites 1 and 2 – samples 2, 3, 4, 5 (Map 2) and but not at sites outside of the probable distribution range of the species (Map 1). Results from Samples 6 and 1 were uncertain with one DNA detection out of three replicate vials.

The Main study

We detected stonefly eDNA at all sampling points at Site 1 with one sampling point returning a positive result in two out of three technical replicates (Map 3).



Map 3. eDNA sampling points 10-11 September. Green circles – positive detections, yellow – less certain detection, red – no detection.



Map 4. eDNA sampling points from 17-26 September 2019. White circles indicate absence of water. WP1 and WP2 are points of probable stonefly occupancy.

We also detected *R. darlingtoni* DNA at WP1 and WP2 (Map 4) with one and two out of three technical replicates respectively. An additional sampling round on the 3rd October 2019 produced only one successful DNA amplification out of three technical replicates at WP2. No juveniles or adults of *R. darlingtoni* were observed on 18th September or 3rd October 2019 despite a deliberate search.

Discussion and Recommendations

The results of the study showed that eDNA is a valid and sensitive method for detection of *R. darlingtoni*.
The pilot study results were positive within the known occupancy range at Site 1 and negative in stream where we did not expect them. The fact that we had ambiguous results at sample point 6 is of interest as it shows that larvae can be detected only over a short range of 100m from the point where they were observed (Map 2).

Although, we did not get amplification in all three replicate vials, the results of the main survey suggest that it is highly likely that the species occupies sites WP1 and WP2. However, an observation of the larvae or adults would be desirable to significantly expand the known distribution range of this restricted stonefly with certainty.

Additionally, we have collected another rare *Riekoperla cornuta* at WP1. Because the DNA sequence of *R. conuta* is not yet available, we can not ensure the complete uniqueness of our primers and probe. As such, a small possibility of our DNA marker detecting this species instead of *R. darlingtoni* exists for the time being. Sequencing of *Riekoperla cornuta* is currently in progress.

Recommendations

To the best of available knowledge the proposed trail would not directly cross any waterways where *R. darligtoni* is present or is likely to be present. However, due to high porosity of the soil, all springs in the area are well connected to their catchment. This means that any of the effluent generated during the building and usage of the trail is likely to affect the quality of water and habitat immediately downstream.
Therefore, it is recommended to build and use the trail in the way that:

- Eliminates any pollution that can be soaked into the soil

- Eliminates coarse and fine sediment carried into permanent or ephemeral (occasionally flowing) water bodies
- Does not in any way interrupts the flow rate of the ground waters
- Does not increase sediment from the Donna Buang road flowing into the adjacent springs downstream of the road.

As a way of improvement of the species chance of survival:

- Decrease sediment generated by car park 2 (see Tsyrlin, 2018)
- Decrease the sediment generated from Donna Buang Rd, especially near WP1 and WP2 by installing sediment traps and other appropriate measures. More detailed sediment control actions are similar to those listed in a recent report to Parks Victoria (Tsyrlin, 2018). These actions should involve a road engineer or a similarly qualified specialist working closely with Parks Victoria staff and consultants appointed by the Council.
- Avoid chemical weed control in the vicinity of WP1 and WP2
- Carry out a survey in August 2020 at WP1 and WP2 to confirm the species presence
- Repeat eDNA sampling and analysis at nearby streams in August 2020
- Monitor population size of the species at Site 2 (potentially affected) and Sites 1 and 3 (as a control) to prove that the trail building and use does not result in population size decrease
- Carry out additional eDNA surveys within the species distribution range to identify other potential locations.

Acknowledgements

I would like to thank the Yara Ranges Shire Council for commissioning and facilitating this study. Tarryn Elverd provided the special data on the proposed bike trail and made useful suggestions to improve this report. Huge thanks go to Dr Katie Robertson for helping with the lab work and to Nick Bell, Mengija Liu, and Véronique Paris for helping in the field. I am particularly grateful to Sarah Matthews from Park Victoria for her undying enthusiasm, useful suggestions for this study and courageously braving the icy water for too many hours in the field.

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