Northern Australia Environmental Resources Hub

National Environmental Science Programme

Standard operating procedures for collecting and extracting Gouldian Finch eDNA

Report

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CHARLES DARWIN UNIVERSITY

NORTHERN TERRITORY GOVERNMENT

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Cover photographs

Front cover: Adult and juvenile Gouldian Finches (*Erythrura gouldiae*) and three Long-Tailed Finches (*Poephila acuticauda acuticauda*), photo Alexander and Eckhard Garve.

Back cover: Jawoyn Rangers, Ken Duffill (left) and Ryan Barrwei (right) collecting waterhole samples for Gouldian eDNA research, photo Alea Rose.

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1. Standard Operating Procedure 1: Gouldian counting and waterhole monitoring 2018

Photo by Brydie Hill, Species Conservation Unit, NT Department of Environment and Natural Resources.

1.1 Introduction

Waterhole monitoring capitalises on the granivorous diet of Gouldian Finches requiring them to drink water to survive. Monitoring is undertaken in the late dry season when Gouldians and other seed eating birds are drawn to the small number of waterholes that remain in a region to drink. Ideally all sources of water in a region are monitored to get a reasonable estimate of population metrics to compare between years. Local rain just prior or during the monitoring period provides abundant alternative water sources, that can affect the bird count results because birds can drink from raindrops on leaves, reducing the number of birds that will use the waterholes being monitored.

A monitoring program for Gouldian Finches in the Yinberrie Hills based on waterhole counts was commenced by the Conservation Commission of the Northern Territory in 1996 to determine trends in population and health indices to assess if populations were stable or improving at key sites. The program went through a number of reviews and modifications. The most recent analysis concluded that the index produced does not provide an absolute measure of population size and it is acknowledged that high levels of variability in counts and an unbalanced data set resulted in the monitoring program having low statistical power to detect change over time. The waterhole monitoring program was discontinued in 2013 in favour of exploring options to determine breeding attempts in the Yinberrie Hills. This option is still under investigation.

1.2 Training

Before monitoring commences some training is required. All observers undertaking the waterhole counts should:

- view the “Gorgeous Gouldian Finch” DVD and are familiar with how to identify the finches they will see
- be familiar with identification of juvenile Gouldian Finches
- know how to record the data on the datasheet
- know how to identify the other seed-eating birds that come into drink

1.3 Waterhole locations

Table 1.1 provides coordinates for the waterholes around the Mt Todd mine in the Yinberrie Hills for which site coordinates are available. However, waterholes are dynamic and can change over time and they may not all have water every year. It is very important that waterholes are accurately identified. Because of the better wet season in 2016/17 many are likely to be full, although the current dry season has been hot. If waterholes are all full you may want to wait until as late as possible in the dry season to undertake the monitoring as bird presence and numbers at each waterhole are likely to be affected by alternative water sources in the region.

Two weeks before the counts start at each site, there needs to be a reconnaissance trip to all the waterholes to check if they have water. Note this takes some time.

During reconnaissance:

- Flag the path into the waterhole if necessary

- Flag the actual waterhole with the name of the waterhole written on the flagging tape (so there can be no confusion on waterhole names when written on data sheet)

Depending on the previous wet season's rainfall, the waterholes can be in a different spot to the previous GPS location. Waterholes could:

- Not be in exactly the same place as the year before. Generally, if the waterhole is within 200m either side of where the waterhole was last year – then that waterhole becomes the waterhole to count. Please note: this (shifted) waterhole will have the same name as last year's waterhole (just remember to take a GPS location of the waterhole and write that on the datasheet)
- Have no water – there is no point in counting that waterhole but do a scout around just to make sure there isn't a waterhole close by – walk the creekline to make sure
- Be lots of little waterholes – if this is the case it is good to do an early morning reconnaissance a few days before the counts begin and see which holes are being used by Gouldians coming into drink. If it is more than one waterhole that is being used, put observers on all ones where the birds are drinking from and then lump all the counts as if it is one waterhole.

Location and names of waterholes can be seen in Figure 1.1, Figure 1.2, Figure 1.3 and Figure 1.4.

*Table 1.1. Coordinates for waterholes in the Yinberrie Hills). Post 2008, only the 12 sites indicated were monitored * sites have had cameras set up at them by TNRM. +TNRM noted that the water levels are very low in late August and may be dry by late September.*

Name	Latitude	Longitude	post 2008
CP1	-14.106	132.0935	
CP2*	-14.124	132.0613	
CP3	-14.1546	132.0681	
CP4	-14.1607	132.103	
Cut	-14.1289	132.059	
Edith Falls camp	-14.1783	132.1864	
Fg 5	-14.1521	132.0683	
FG5	-14.1527	132.0681	yes
Figtree	-14.1513	132.0691	yes
Froghole	-14.1502	132.0698	yes
JC05*	-14.123	132.0623	yes
JG10*+	-14.1234	132.0594	yes
JG35*+	-14.1195	132.0549	yes
K?	-14.1592	132.0997	
K7	-14.1607	132.0986	yes
NorthWater	-14.1091	132.088	yes
NW01	-14.1088	132.0875	yes
NW05	-14.1076	132.0958	yes
Poachers*	-14.1235	132.0612	yes
Soak +	-14.1235	132.0601	yes
Vista	-14.1422	132.1116	

1.4 Waterhole count methodology

Summary

- Each waterhole needs to be counted three times (three mornings) (this does not have to be on consecutive mornings) but counted within the monitoring session
- Counts start around sunrise (times will vary depending site)
- Counts last for three hours
- Adult Gouldian male, female and juveniles are recorded (including head colour of adults)
- All seed-eating birds using the waterholes are to be counted but priority is to count Gouldian's
- At the end of each morning's session, completed datasheets handed to the coordinator.

Equipment needed by waterhole observers

Make sure each observer has the following equipment:

- Binoculars
- Clipboard and count datasheet (and you understand how to record data)
- 2 x pencils
- Watch or clock
- Waterhole monitoring SOP
- Chair (optional)
- Water (and food)
- Bird book
- Sunscreen and hat
- GPS

Choosing your observation point at the waterhole

Finches will drink surprisingly close to you; at times much less than 10 metres, but the closer you are sitting to the waterhole the more easily the birds can be frightened by even the slightest movement. Flightiness has serious potential to disrupt your counts, and may even frighten the birds to another waterhole on this or the subsequent day. Try to avoid sitting too close to the waterhole or making abrupt movements. We recommend a distance of about 15 metres, more if you're a restless sitter.

Other issues to consider when you are selecting where to put your chair:

- Have the sun behind you so you can see through your binoculars clearly
- Make sure that your shadow is not on or near the water as any movement will flush the birds
- Try to avoid being higher than the finches though this is not always possible
- Have as clear a view of as much of the waterhole as possible
- Try to avoid having to shift position once you have started counting
- When counting at larger waterholes consider where the finches are likely to drink. They prefer gently sloping edges such as beaches, or rocks just above the water surface. If the pool is very long sitting at one end can encourage the birds to drink at the other end and give you a clear line of sight

Species identification

Female Gouldian Finches can be very dull, much more so than bird books suggest. However, like the male they will always have a definite face patch and purple chest. Both red head and black head males and females can be seen.

Juvenile Gouldian Finches lack the distinct coloured face patch and purple chest of the adults. Juveniles are basically a dull olive colour especially on the back, though some may have patches of brighter feathers beginning to moult through as they get older. All juvenile Gouldian finches have a dark bill. If you have any doubts, please view the "Gorgeous Gouldian Finch" DVD.

Please remember that inaccurate identifications are worse than no counts at all!

Counting birds and recording the counts on the datasheet

Each counting session lasts three hours. For ease of recording, the datasheet is split into 15-minute periods. Nonetheless, it is a continuous count. A copy of the datasheet is in Table 1.2.

Counting commences shortly after dawn usually around 0700. Count only birds that come down to drink. Sometimes lots of Gouldian's come into drink at once and you may not be able to count individual birds. In this case, estimate group sizes, e.g. 10 black-headed male Gouldians, 5 red-headed female Gouldians, 25 Juveniles, and 40 Longtails. Put these numbers down as your count. Return to actual individual counts as soon as you can.

If it gets really busy with lots of birds coming down to drink (and this can happen) DON'T PANIC. The priorities are:

- 1st – count Gouldians, adults and juveniles
- 2nd – count Hooded Parrots
- 3rd – count other finch species
- 4th – count other seed-eating birds such as Peaceful and Diamond Doves

The best method of recording numbers is in a tally system. Four vertical strokes and a horizontal stoke through them represents five. At the end of each morning count, tally each 15-minute segment for each species, and write the total, circled, in the lower right corner of the appropriate square.

Other finches/grass eating birds that you could see

Generally there are a number of more common finches that you will encounter – please know what they look like:

- Long-tailed Finch
- Masked Finch
- Double-barred Finch
- Pictorella Mannikin
- Chestnut-breasted Mannikin
- Peaceful Dove
- Diamond Dove
- Bar-shouldered Dove
- Common Bronzewing
- Hooded Parrot

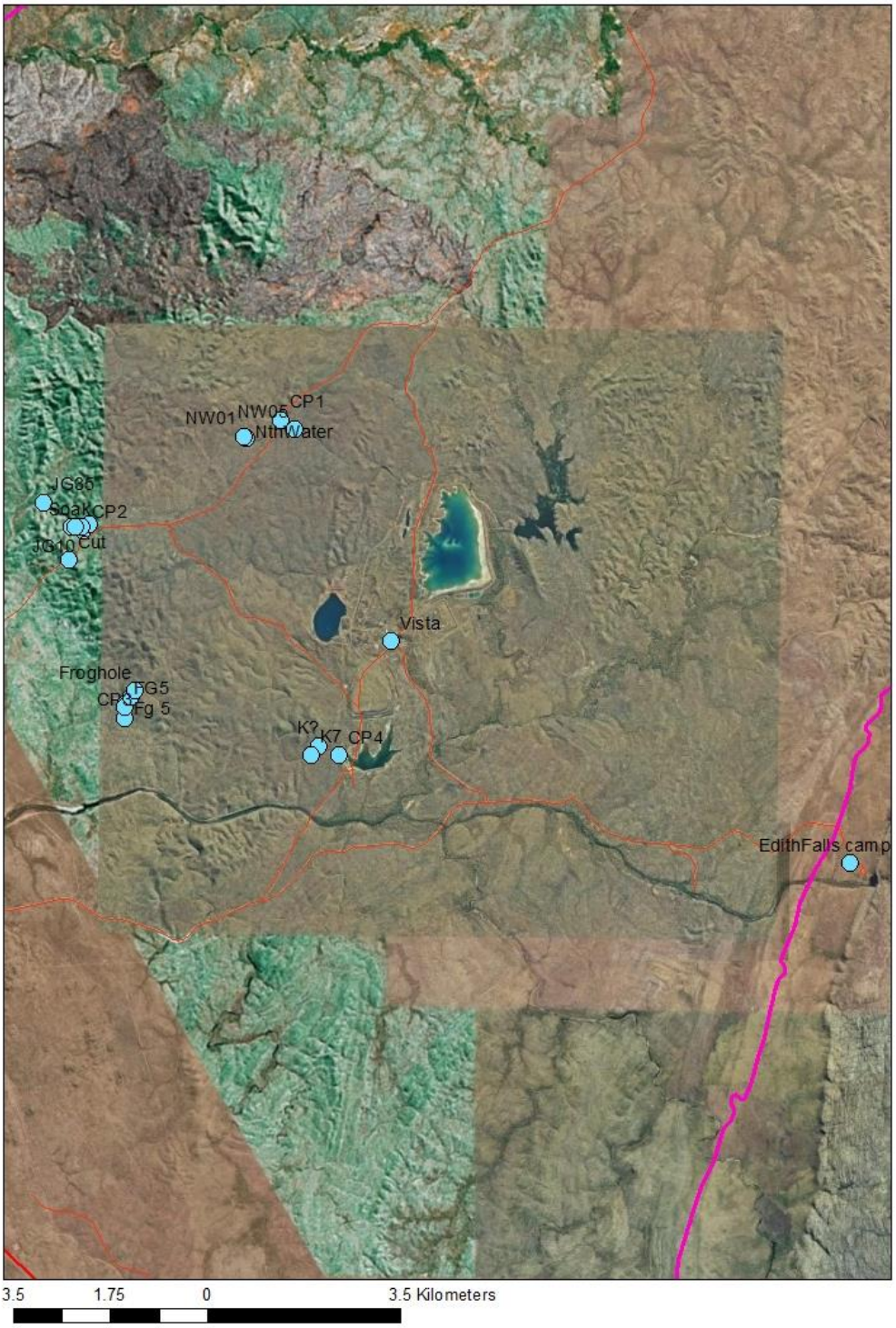


Figure 1.1. Waterhole monitoring locations in the Yinberrie Hills.

Figure 1.2. Locations of the northern waterholes in the Yinberrie Hills.

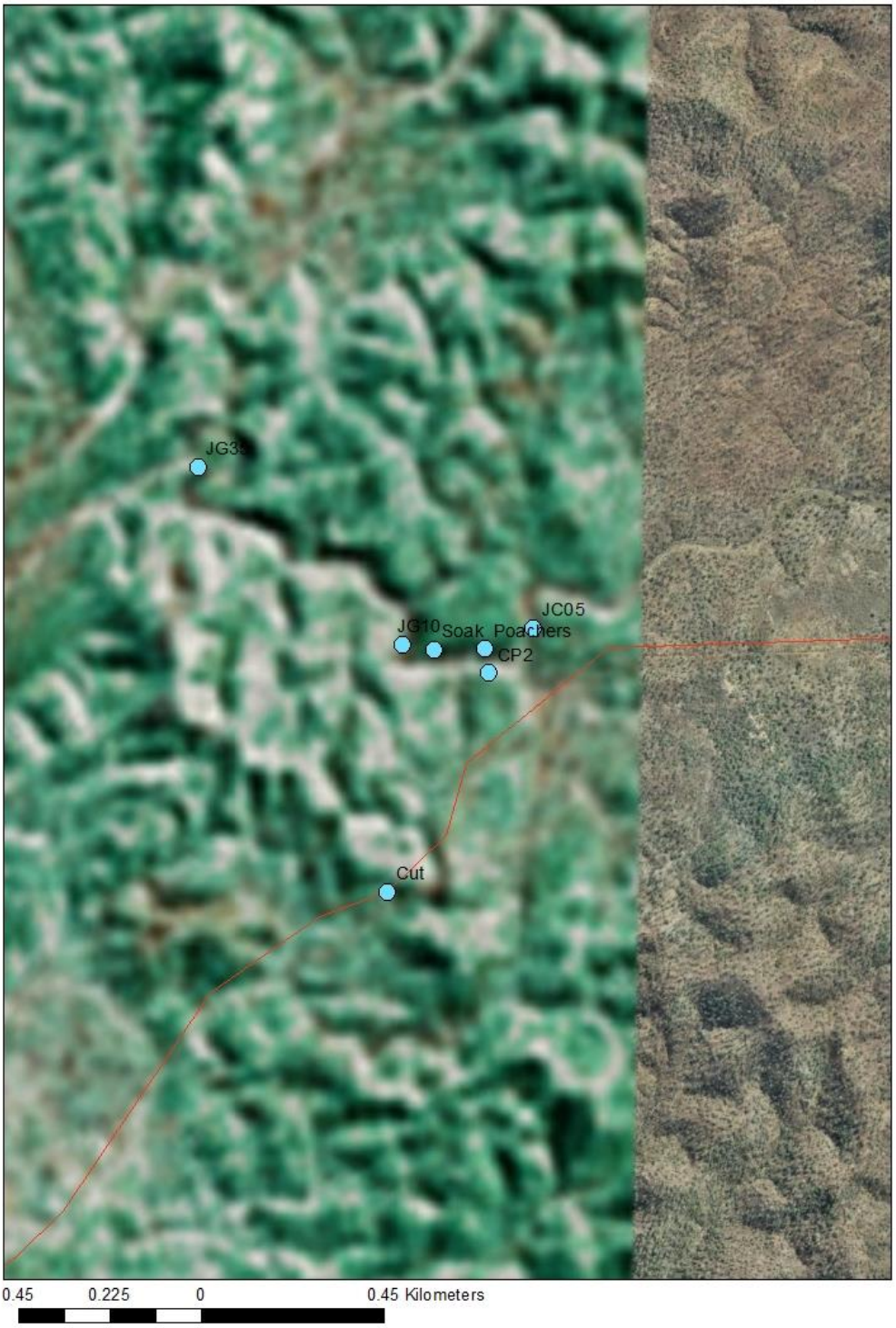


Figure 1.3. Location of western waterholes in the Yinberrie Hills.

Figure 1.4. South-western waterholes in the Yinberrie Hills.

Table 1.2. Gouldian Finch counts datasheet – Yinberrie Hills September 2018.

Waterhole location: Yinberries **Waterhole name:**

GPS:

Time	Gouldian					Masked		Longtail		Double-barred	Pictorella	Other granivores		
	Male		Female		Juvenile	Adult	Juvenile	Adult	Juvenile					
	Red	Black	Red	Black										
0700-0715														
0716-0730														
0731-0745														
0746-0800														
0801-0815														
0816-0830														
0831-0845														
0846-0900														
0901-0915														
0916-0930														
0931-0945														
0946-1000														
Time first sunrays hit the water……					Observer distance from water……					Observer……				
Date……					Weather at 0800	warm	cold	sunny	cloudy	still	breeze	windy		
General notes														

2. Standard Operating Procedure 2: Gouldian eDNA waterhole collection

Final version: 4.0, 06/10/18

Derived from: Gouldian eDNA fieldwork SOP, 03/06/18

2.1 Purpose

- To trial the deployment of the Gouldian eDNA test at waterholes.
- The following protocol was developed for collecting and filtering waterhole samples to trial the eDNA detection of Gouldian finches.

2.2 Overview of waterhole sampling method

- Number of waterholes = 6.
- Number of samples = 96 samples (54 samples + 6 negative controls + 36 storage experiment samples).
- Collect physicochemical properties at each waterhole and site in triplicate
- Waterhole categories:
  - Two waterholes with high Gouldian finch counts (>20 Gouldians)
  - Two waterholes with medium Gouldian finch counts (<10 Gouldians)
  - Two waterholes with 0 Gouldian finch counts (after 3 counts)
- Three sampling sites at each waterhole (see Figure 2.1 for site examples):
  - Site 1: where Gouldian's were drinking (zero distance) called 'At drinking site' (ADS)
  - Site 2: near where Gouldian's were drinking (50cm from zero) called 'Near drinking site (NDS)
  - Site 3: further from drinking site (2m from zero – or closer if waterhole is small – say the centre. The full dimensions of the waterhole should be measured) called 'Far from drinking site (FDS)

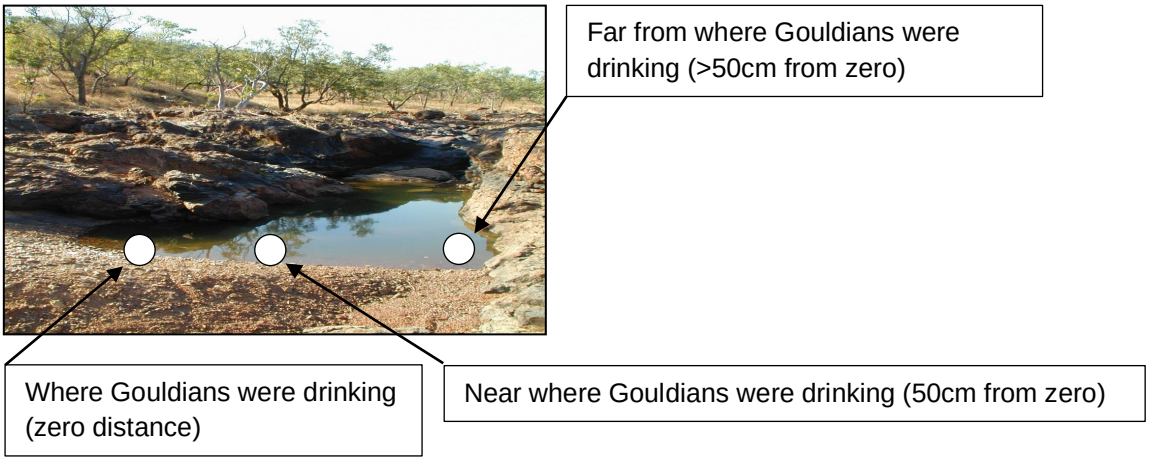


Figure 2.1. Example waterhole with x3 field site locations. Please note, during sampling, avoid 'slimy' areas as DNA degrades quickly in these locations.

2.3 Quality control

Collect in triplicate (x3 samples) for each site (9 samples per waterhole + 1 negative control + 6 storage experiment samples)

- Perform a negative site control at each waterhole (expose a bottle of high pure water to the air for 2 mins)
- For each site, rinse bottles x3 times with water before collecting (to prevent water contamination, pour water rinses into a waste container until finished sampling)
- If waterhole is fully exposed (no shade) or covered (no patches of sunlight) etc. make a note
- Perform bird counts for three days before sampling to get an average of waterhole visitations

2.4 Equipment

- Masterflex Peristaltic Pump with x1 batteries & car charger (if no access to power)
- Sartorius Stedium Microstart Filter unit (if have access to power)
- Nalgene tubing with connector (2 types of tubing: x1 soft and x1 hard)
- HANNA (portable hydrolab with GPS & data logger) and carry bag
- USB for HANNA data logs
- Clipboards
- Bottle extension poles
- Small petrie dishes
- Parafilm
- Scissors
- Pens (permanent markers, black pens)
- Roll of sticky tape
- Sterile 100mL Pall filter cups with 0.45µm filter paper (PALL CORPORATION)
- 500mL glass waste jar
- Waste container (for temporarily holding water)
- 250mL Nalgene bottles, sterilised (plus field negatives filled with HP water)
- Soft esky
- Ice bricks for filter paper storage in field
- Nitrile gloves
- Waste bags
- Measuring tape
- Cable ties (for attaching tubing to extension poles)
- Spare batteries
- Bottle with High Pure water (for flushing tubing)
- Cannon camera (x3 batteries & charger)
- Satellite phone
- Vehicle with fridge
- Backpack for sites to carry samples in esky
- Field sampling plan (included above)
- Field collection SOP (included below)
- Field sample processing SOP (included below)

- Filtration set-up SOP (included below)
- HANNA SOP (included below)
- Bird Count SOP 1
- Copy of bird count table (Table 1.2)
- Copy of field checklist table (Table 2.1)
- Copy of approved field plans, maps, movement requisitions & risk assessments
- Talent release forms for photos

2.5 Procedure

Field collection for Gouldian eDNA at waterholes

Notes before starting	Perform bird counts for 3 days before collecting water samples
	Perform Day 3 bird count before sampling
	Always wear nitrile gloves during sampling and change gloves between samples
	Avoid slimy/algal areas (eDNA degrades quickly in these locations)
	Photograph the waterhole & each site sampled and describe conditions (windy, water appearance, depth etc)
	Perform a negative control before sampling

Day 1 bird counts

- At each waterhole, perform bird count according to Bird Count SOP 1
- Record count details in bird count table (Table 1.2)
- Photograph waterhole and describe conditions

Day 2 bird counts

- At each waterhole, perform bird count according to Bird Count SOP 1
- Record count details in bird count table (Table 1.2)
- Photograph waterhole and describe conditions

Day 3 bird counts and water sampling

- At each waterhole, perform bird count according to Bird Count SOP 1
- Record count details in bird count table (Table 1.2)
- Photograph waterhole and describe conditions
- Perform x1 negative control (open the x1 250mL Nalgene bottle filled with HP water to air for 2 minutes, seal, and store at 4°C)
- At each waterhole site (sites = at drinking site; near drinking site; further from drinking site), take x3 physicochemical measurements & the GPS location using HANNA – mark off on 'field checklist' table
- Prime the 250mL Nalgene bottles before collecting water from each sample site (sites = at drinking site; near drinking site; further from drinking site) by:

    - Rinsing the bottle by partially filling with the site water and swirling. Empty water into a waste container (note – empty waste container back into waterhole once sampling is complete)
    - Repeat bottle rinse 3 times
    - Moving back to the sample location (i.e. where birds were drinking), fill the 250mL Nalgene bottle to the 200mL mark
    - Repeat process until have x3 200mL replicates from 3 sites. Store samples at 4°C
- For the 'at drinking site,' prime and collect x3 replicates for the eDNA small storage experiment (do not filter until after 2 weeks – back in lab) and store at 4°C
- Total number of samples per waterhole = 16 (x9 samples + x1 negative control + x3 storage experiment samples)
- To get an estimation of the waterhole's water volume, use a measuring tape, measure the length, width and water height from multiple waterhole points and record dimensions on a sketched image of the waterhole
- When complete, tick off on field checklist table

Taking physicochemical measurements and recording GPS waypoints with the HANNA (at each waterhole on Day 3)

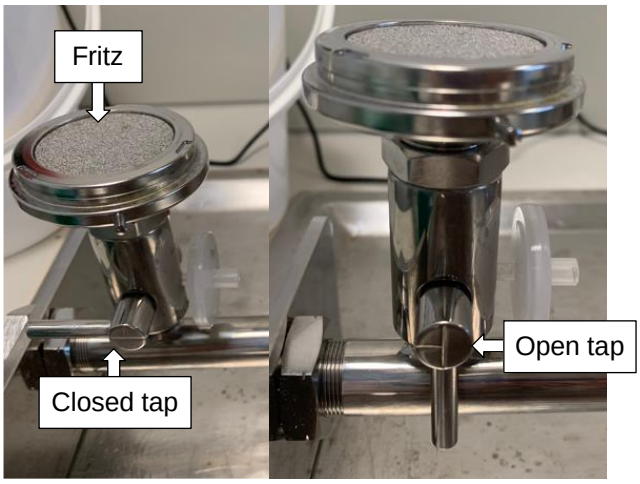
- Rinse probe cup x3 with site water (empty into waste container)
- Fill cup with water and immerse probes
- Allow readings to settle then record GPS location and probe readings using the data logger (see HANNA manual for details)
- Dispose of water into waste container (to go back into waterhole once all sampling is complete) and repeat last step another 2 times (x9 readings in total per waterhole)
- upload data to a computer each day (save a copy to the USB located in Manual bag) – see HANNA manual for details
- When complete, tick off on field checklist table

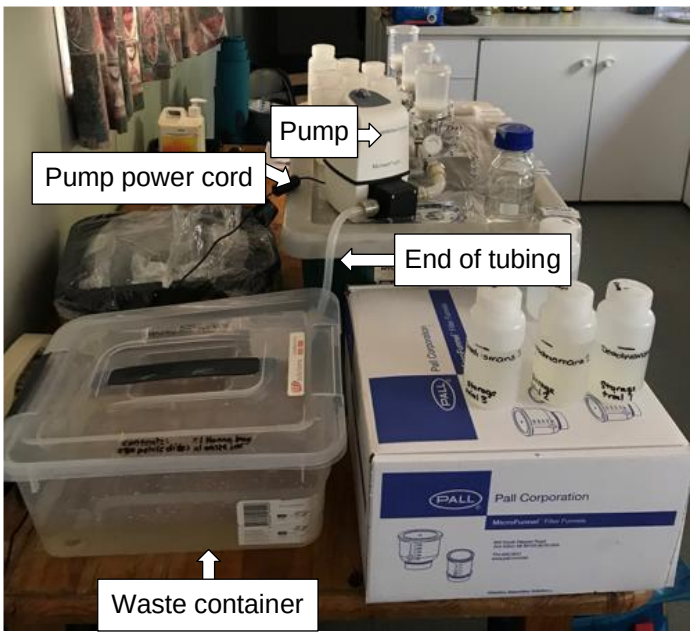
Filtering water samples to capture eDNA

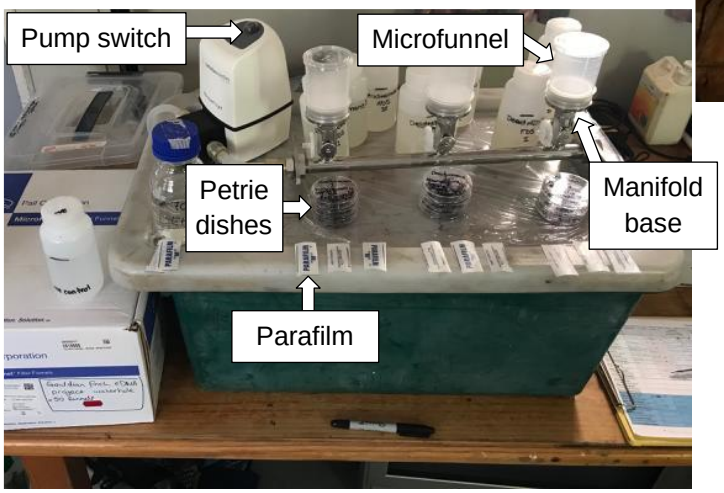
Notes before starting	Process within 12hrs of waterhole sample collection
	Always wear nitrile gloves during sampling and change gloves between samples
	Filter the negative controls first
	Label petrie dishes with site/waterhole name, date and replicate number
	For each sample, filter (see below for filtration method) a 100mL aliquot of the x9 water samples and x1 negative control using a new sterile 0.45µM Filter cup for each sample
	DO NOT filter the x3 storage experiment samples-keep x3 refrigerated only

Filtering water samples at a powered site with the Sartorius filter manifold

- Set-up the pump and manifold filtration unit as described in the images below
- For each sample, filter a 100mL aliquot of the x9 water samples and x1 negative control using a new sterile 0.45µM Filter cup for each sample
- Store the remaining 100mL of each sample at room temperature (to be filtered 2 weeks later as 'abuse' samples)
- Carefully remove the porous metal 'fritz' from the three bases on the manifold and temporarily store in a clear dry area until filtration is complete
- Plug the manifold pump into a power source and insert the discharge end of the manifold tubing into the waste collection container
- Checking the red cap is removed from the filter funnel, place the sterile funnel unit ontop of the manifold base and gently press down to secure
- Once set up, pour 100mL of water sample into assembled filtration unit and turn on suction by turning on the pump
- Open the base tap and allow entire water volume to pass through filter (record volume & time processed)
- Close the base tap, snap off funnel and insert the bottom half of the filter unit into a small labelled petrie dish (label top and bottom side of dish with waterhole name, date, and replicate number)
- Seal with parafilm and store filter at 4°C
- Record the starting time for filtering and volume filtered
- Keep filtered samples cool and out of the sun in esky with ice or ice bricks (refrigerate when possible)



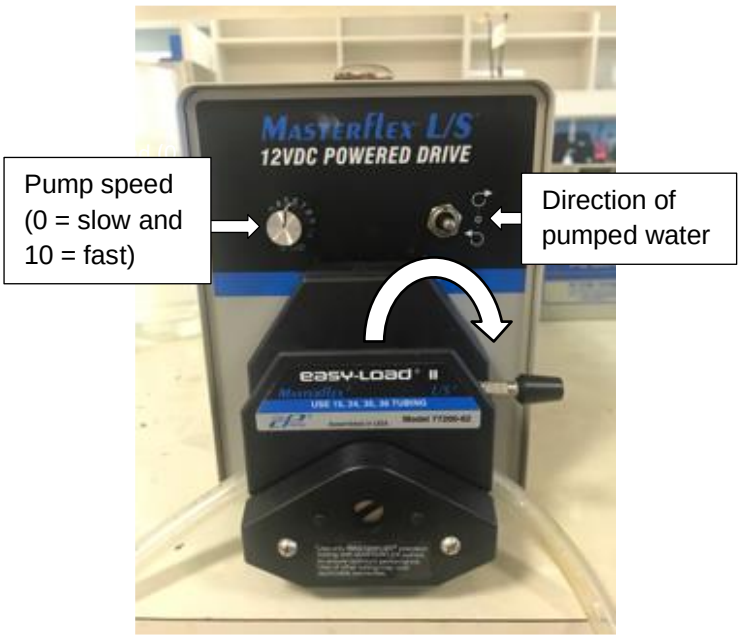




Filtering water samples at an un-powered site with the peristaltic pump and waste jar

- Set-up the pump and waste jar as described in images below
- Connect a battery to the pump unit (see images below for connections)
- Pull lever to the left to open pump
- Insert soft Nalgene tubing into the pump and ensure tube is within aligned within the guide arches – important: do not use rigid tubing in pump
- Once tubing is in position, pull lever to the right to close the pump and secure the tubing. Adjust the pump speed and direction of pumped water using the knob and switch (note – do not exceed a speed of 5 or 6)
- Remove red cap from bottom of the filter unit and place on top of the waste jar
- Attach the end of the soft tubing to spout of the waste collection bottle and insert the end of the rigid tubing into the waste container
- Once set up, pour 100mL of water sample into assembled filtration unit and turn on suction by flicking the water direction switch into the upright position
- For each sample, filter a 100mL aliquot of the x9 water samples and x1 negative control using a new sterile 0.45µM Filter cup for each sample
- Store the remaining 100mL of each sample at room temperature (to be filtered 2 weeks later as 'abuse' samples)
- Avoiding touching the filter paper, snap off funnel and insert the bottom half of the filter unit into a small labelled petrie dish (label top and bottom side of dish with waterhole name, date, site and replicate number)
- Seal with parafilm and store filter at 4°C
- Record the starting time for filtering and volume filtered
- Keep filtered samples cool and out of the sun in esky with ice or ice bricks (refegerate when possible)


12VDC POWERED DRIVE
easy-load II
USE 15, 24, 35, 36 TUBING
Model 77200-62


Pump speed (0 = slow and 10 = fast)
Direction of pumped water
MASTERFLEX L/S
12VDC POWERED DRIVE
easy-load II

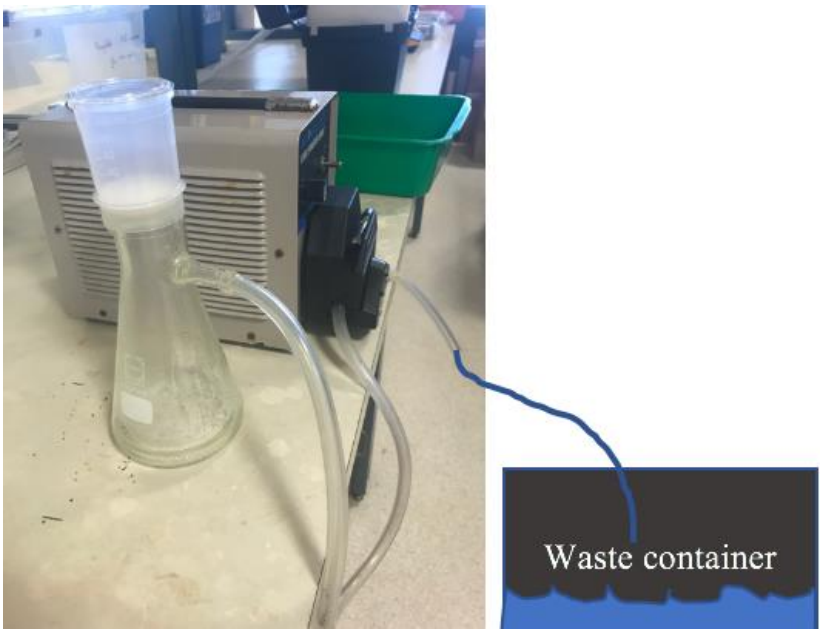

Waste container

Table 2.1. Field checklist.

Field checklist – Record waterhole name, filtered water volume, and tick off each item when completed.

Waterhole & sample site characteristics (High>20; Med<10; Low=0)	Waterhole	Date	Time	Sample ID (Waterhole-Date-Sun/Shade-At/Near)	Sample Depth	Measurements of waterhole (length, width, depth)	X3 days of finch counts ☑	Photos (w'hole & site) ☑	Phys chem & GPS location ☑			Site -ve control (HP water) ☑	200mL rep 1	200mL rep 2	200mL rep 3	Storage trial (x6 100mL at ADS)
High bird counts site 1 **At drinking site**																
High bird counts site 1 **Near drinking site**																
High bird counts site 1 **Far from drinking site**																
High bird counts site 2 **At drinking site**																
High bird counts site 2 **Near drinking site**																
High bird counts site 2 **Far from drinking site**																
Med bird counts site 1 **At drinking site**																
Med bird counts site 1 **Near drinking site**																
Med bird counts site 1 **Far from drinking site**																
Med bird counts site 2 **At drinking site**																
Med bird counts site 2 **Near drinking site**																

Med bird counts site 2 **Far from drinking site**																
0/low bird counts site 1 **Shallow beach**																
0/low bird counts site 1 **Near shallow beach**																
0/low bird counts site 1 **Far from shallow beach**																
0/low bird counts site 2 **Shallow beach**																
0/low bird counts site 2 **Near shallow beach**																
0/low bird counts site 2 **Far from shallow beach**																

3. Standard Operating Procedure 3: eDNA extraction from filtered water

Final version: 1.3, 15/12/17

Derived from: Original QIAGEN DNeasy Blood & Tissue Kit

3.1 Purpose

- To extract total DNA from filtered water samples.
- The following protocol was developed for eDNA detection of Gouldian finches and saltwater crocodiles but can be used generally for total DNA extraction from water.

3.2 Overview of method modifications

- Because the DNeasy kit was not designed to accommodate large filter papers containing trapped eDNA, the original protocol was changed to include additional treatment steps and greater buffer volumes.
- To ensure that DNA was extracted from the entire filter, samples were first snap frozen in liquid N_2 and pulverised to 2–3 mm fragments. The volumes of Buffer ATL, proteinase K, AL and ethanol were also *tripled* to cope with the filter volume.

3.3 Equipment

- MoBio PowerLyser™ (MoBio)
- Additional QIAGEN reagents: Buffer ATL, proteinase K and Buffer AL
- Molecular grade 100% ethanol (SIGMA-ALDRICH)
- Additional DNase/RNase free 2mL screw capped tubes (SSI, lot no. 16126)
- Sterile 2mm glass beads
- Biohazard cabinet
- Nuclease-free water
- Centrifuge
- Vortex
- Pipettes
- BIOLINE Shaker (BIOLINE)
- Nanodrop One (THERMO-SCIENTIFIC)

3.4 Procedure

Sample preparation prior to extraction

- Tube & glass bead preparation in a sterilised biohazard cabinet
  - Before use, soak the 2mm glass beads in virkon for 1hr, rinse in Nuclease-free water three times then UV-sterilise for 10mins
  - Allow beads to dry in the biohazard cabinet
  - Add four of the sterile, 2mm glass beads into a labelled, sterile, 2mL Free Standing Screw Tube (SSI, lot no. 16126)
- Pulverising filters prior to digestion step in DNeasy protocol

- Place filter into the prepared bead-tube with sterile forceps (do not roll too tight or beads cannot move)
- To snap-freeze filters, immerse the 2mL tube in liquid nitrogen
- Disrupt the sample with Mo Bio PowerLyserTM 24 at 3,200 rpm for 30 secs
- Repeat the procedure at least 3 times, i.e., put the 2mL tube in liquid nitrogen and repeat sample disruption until the sample forms small 2–3mm fragments
- Follow modified extraction protocol as outlined below:

eDNA extraction of filters

Notes before starting	Centrifuge samples at room temperature
	Vortex samples by pulse-vortexing for 5–10 secs
	Heat Buffer AL and Buffer ATL to dissolve salts at 56°C for 10–15 min
	Add 100% Ethanol to Buffer AW1 and Buffer AW2 concentrates
	Thaw frozen filters to room temperature
	Preheat BIOLINE Shaker to 56°C

- Add 540µL Buffer ATL and 60µL Proteinase K (step 1a)
- Mix by vortexing
- Incubate at 56°C in BIOLINE Shaker overnight
- Add 600µL Buffer AL and mix thoroughly by vortexing
- Centrifuge at 6000 x g (8000 rpm) 2 min
- Insert a pipette tip into tube and draw up solution (avoiding filter pellet/debris – use technique outlined in DNeasy Soil DNA kit)
- Transfer solution to a new 2mL collection tube (not provided) – expect ~1mL
- Add 600µL ethanol 100%, thoroughly vortex
- Centrifuge at 6000 x g (8000 rpm) 1 min
- Avoiding pellet, transfer 600µL of the solution into a DNeasy Mini spin column inserted into a 2mL collection tube (new tube)
- Centrifuge at 6000 x g (8000 rpm) 1 min and discard flow-through
- Avoiding pellet, transfer 600µL of the solution into the DNeasy Mini spin column (repeat until all solution is loaded onto spin column)
- Centrifuge at 6000 x g (8000 rpm) 1 min and discard flow-through
- Place spin column into a new tube
- Add 500µL Buffer AW1
- Centrifuge at 6000 x g (8000 rpm) 1 min and discard flow-through
- Place spin column into a new tube
- Add 500µL Buffer AW2
- Centrifuge at 20,000 x g (14,000 rpm) 3 min and discard flow-through
- Place spin column into a new tube (either 1.5 or 2mL tube)
- Elute DNA by adding 100µL AE to the centre of the spin column
- Incubate for 1 min at room temperature
- Centrifuge at 6000 x g (8000 rpm) 1 min
- Increase eluted DNA yield by adding another 100µL AE to spin column

- Incubate for 1 min at room temperature
- Centrifuge at 6000 x g (8000 rpm) 1 min
- Nanodrop 1µL of purified DNA samples using AE as a blank
- Store samples in sealed box at -80°C

3.5 Quality control

- To check for DNA contamination during the extraction process, extract x3 ‘blanks’ filters during your extraction. Blanks are filters that have filtered Nuclease-free water (make sure the filtered Nuclease-free water volume is the same as that of the field samples)
- To check for DNA contamination in the field, a ‘field blank’ (filtered Nuclease-free water exposed to the air for 2mins in the field) should be collected during sampling
- Duplicate (or triplicate) samples should be collected in the field for quality control

4. Standard Operating Procedure 4: Extracting DNA from finch blood

Final version: 1.1, 15/12/17

Derived from: Original QIAGEN DNeasy Blood & Tissue SOP (starting from step 1c for nucleated blood)

4.1 Purpose

- To extract total DNA from finch blood.
- The following protocol was developed for extracting DNA from the blood of Gouldian, Masked and Long-tailed finches but can be used generally for total DNA extraction from bird blood.

4.2 Overview of method modifications

- If finch blood was collected and immediately frozen or preserved in 100% ethanol and not treated with anticoagulant, then extend the 56°C incubation time and repeat vortexing multiple times to fully resuspend the blood before adding 100% ethanol.

4.3 Equipment

- Molecular grade 100% ethanol (SIGMA-ALDRICH)
- Centrifuge
- Vortex
- PBS, pH 7.2 (50mM potassium phosphate, 150mM NaCl)
- Pipettes
- BIOLINE Shaker (BIOLINE)
- Nanodrop One (THERMO-SCIENTIFIC)

4.4 Procedure

Finch blood DNA extraction

Notes before starting	Centrifuge at room temperature
	Vortex samples by pulse-vortexing for 5–10 secs
	Heat Buffer AL to dissolve salts at 56°C for 10–15 min
	Add 100% Ethanol to Buffer AW1 and Buffer AW2 concentrates
	Thaw frozen blood samples to room temperature
	Preheat BIOLINE Shaker to 56°C

- Add 20µL Proteinase K (step 1c) into a 1.5 or 2mL centrifuge tube
- Add 10µL of anticoagulant-treated blood (finch blood)
- Adjust volume to 220µL with 210µL of PBS (x1 PBS from lab)

- Vortex for 15 secs directly before next step
- Add 200µL Buffer AL (without added ethanol) and mix thoroughly by vortexing
- Incubate blood samples at 56°C in BIOLINE Shaker for 10 min (NOTE: vortex and repeat 56°C heating step until blood samples have completely suspended)
- Add 200µL ethanol 100%, thoroughly vortex
- Transfer solution into a DNeasy Mini spin column in a 2mL collection tube (*new tube*)
- Centrifuge at *6000 x g* (8000 rpm) 1 min and discard flow-through
- Place spin column into a *new tube*
- Add 500µL Buffer AW1
- Centrifuge at *6000 x g* (8000 rpm) 1 min and discard flow-through
- Place spin column into a *new tube*
- Add 500µL Buffer AW2
- Centrifuge at *20,000 x g* (14,000 rpm) 3 min and discard flow-through
- Place spin column into a *new tube* (either 1.5 or 2mL tube)
- Elute DNA by adding 200µL AE to the centre of the spin column
- Incubate for 1 min at room temperature
- Centrifuge at *6000 x g* (8000 rpm) 1 min
- Nanodrop 1µL of purified DNA samples using AE as a blank
- Store samples in sealed box at -80°C

5. Standard Operating Procedure 5: *E. gouldii* and finch eDNA test

Final version: 3.1, 29/08/18

Derived from: original method

5.1 Purpose

- The following qPCR protocol was developed to test the Gouldian finch probe on DNA extracted from blood or water samples.
- The qPCR test is dual purpose:
  - Uses Quantitect SYBR green to test for three finch species – Gouldian, Masked and Long-Tailed Finches – on the green channel (all double stranded DNA from the primers); and
  - The Gouldian-specific probe (unique Gouldian DNA sequence) on the yellow channel.

5.2 Quality control

- To ensure the qPCR reaction is valid, in each qPCR, always include the following:
  - A positive control (Gouldian DNA) because if the positive control is not detected then the reaction has failed
  - A negative Gouldian probe control (e.g. Long-tailed or Masked Finch DNA)
  - No Template control (NTC) – PCR product with no DNA added. If the NTC is detected, then the reaction is contaminated and should be repeated
- Run all samples and NTCs in duplicate to check for random error
- Intepretation of raw Ct values:
  - 0 = no fluorescence and therefore no Ct value
  - >50 = negative result but the late Ct suggests degraded DNA fragments binding
  - Low Ct values = high concentration of target DNA in sample
  - High Ct values = low concentration of target DNA in sample

5.3 Equipment

- Quantitect SYBR Green qPCR mastermix (QIAGEN)
- FinchCR forward (TCAGGTACCATAYAGCCCAA) and reverse primers (CCTAGGAGGTGGGCGAATTC)
- Gouldian-specific probe (E_gouldii_internal-TET 5'CGTCACCCGTAATGCTAGGGA)
- Bovine serum albumin (QIAGEN)
- DNA extracted from finch blood (Gouldian, Masked and Long-tailed)
- eDNA extracted from Aviary water
- Additional DNase/RNase free 2mL Eppendorf tubes
- DNase/RNase free 0.2mL qPCR tubes and lids
- PCR assembly room
- Nuclease-free water
- Microfuge
- Vortex

- Pipettes
- Pipette filter tips
- LabCleaner (QIAGEN) for decontaminating workbench and PCR assembly from DNA
- Rotor Gene-Q qPCR machine (QIAGEN)
- qPCR cold block (QIAGEN)

5.4 Procedure

Notes before starting	Never bring DNA, gloves or lab coats into the assembly room
	Decontaminate all working surfaces and equipment before use
	Keep qPCR mastermix protected from light and cool
	Only use pipette filter tips
	Change gloves regularly to minimise sample contamination risks
	Do not talk around open tubes
	Thaw DNA samples in fridge before adding to the reaction

Preparing qPCR reaction

Preparing mastermix of primers and probe in PCR assembly room – do not bring DNA into assembly room

- Decontaminate assembly area with LabCleaner
- Protected from light, thaw primers, probes and mastermix components
- Label a sterile 2mL Eppendorf tube
- According to how many samples are being tested (including the positive control and NTC), use the table below to work out how much of each component to add to the tube
- Add all components except the DNA
- Vortex for 10 secs and spin for 3 secs
- Remove mastermix from assembly room and take into lab to aliquot into 0.2mL qPCR tubes and add DNA samples

FinchCR_primers and probe

FinchCR_primers and probe	Working stock (µM)	Final Conc in rx (µM)	1xµL (per reaction)
dH2O			7.7
Forw Primer	20	0.4	0.4
Rev Primer	20	0.4	0.4
Probe: E_gouldii_internal_TET 5'	20	0.4	0.4
BSA	10,000ng/µL	50ng/µL	0.1
Quantitect SYBR MM	2x	1x	10
DNA			1
Final volume			20

Adding DNA samples to tubes

- Decontaminate work area with LabCleaner
- Aliquot 19µL of the mastermix into the 0.2mL qPCR tubes
- Vortex DNA samples for 10 secs and spin for 3 secs
- Add 1µL of DNA to 0.2mL qPCR tubes
- Seal tubes with qPCR lids and label with position number
- Insert tubes into Rotor Gene- Q qPCR machine

Programming the qPCR machine

- Once the tubes are secured, set the qPCR reaction conditions to those outlined in the table below:

qPCR CONDITIONS:		
Hold		
20 min	94 C	
60 cycles of		
10 sec	94 C	
30 sec	**60 C**	annealing temp
30 sec	72 C	**acquire** (Yellow & Green)
Hold		
2 min	72 C	

6. Standard Operating Procedure 6: Aviary experiments and water filtering

Final version: 2.0, 27/08/18

Derived from: original method

6.1 Purpose

- The following protocol was developed for eDNA detection of Gouldian Finches in aviary trials. Trials include:
  - To determine whether the Gouldian eDNA test can detect DNA in water
  - Test what volume of water is needed to detect Gouldian DNA
  - To test how long before Gouldian eDNA was detectable
  - Test how long the Gouldian eDNA persists in the water
- Observe Gouldian drinking behaviour
- To optimise the Gouldian eDNA test for deployment in the field.

6.2 Overview of method

- This protocol is separated into three sections:
  - Aviary set-up and sampling (aviary set-up, aviary camera sampling, and water collection);
  - eDNA trials (optimal sampling volume, first point of detection, and last point of detection); and
  - Sample preservation

6.3 Equipment

- Brinno TLC200 Timelapse camera with SD card
- White, 20L rectangular container (Bunnings)
- Measuring container
- Steel aviary mesh (Bunnings)
- Steel fencing crimps
- Gaffer tape
- Virkon
- KimWipe tissues (low lint)
- Tweezers
- Parafilm
- Petri dishes
- 20mL, 50mL, 100mL and 200mL bottles
- 3-fold water filtration unit (Sartorius)
- Sterile 0.45µm filter papers (PALL CORPORATION)
- 70% ethanol (SIGMA-ALDRICH)
- Biohazard cabinet
- Nuclease-free water
- High pure water

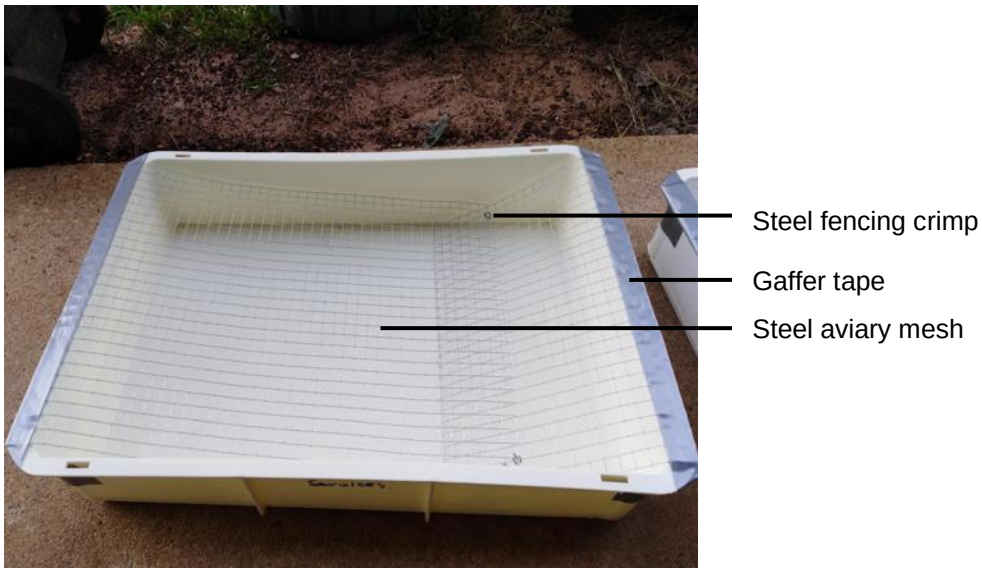
6.4 Procedure

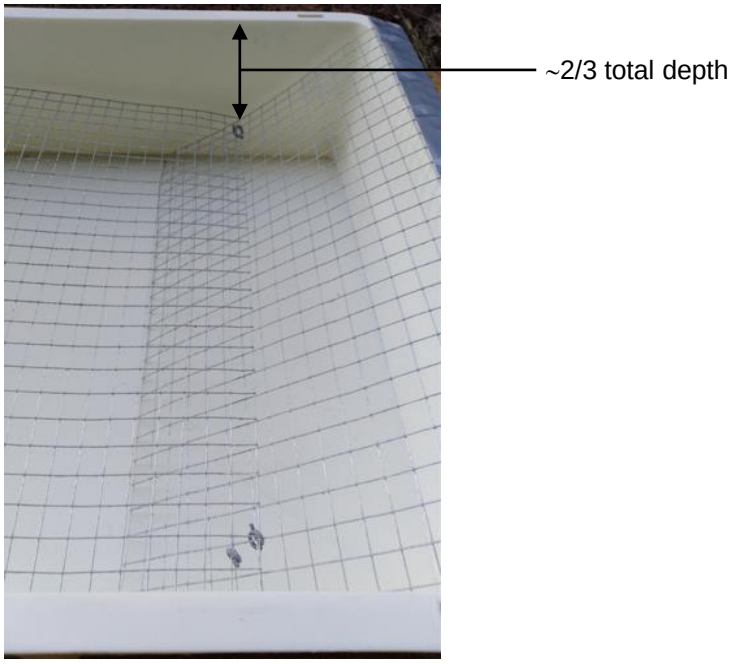
Aviary set-up and sampling

Notes before starting	Sterilise 20L plastic containers by rinsing with Virkon, triple rinsing with high pure water and exposing to UV light for 10mins. Cover with cling film to prevent contamination until use.
	Ensure camera batteries and SD card have sufficient power and storage to last for the full 3-day period or replace batteries and SD card as required
	Note: The Brinno camera would not be suitable for monitoring small birds (e.g. finches) at a distance of >3m and it would be difficult to differentiate small birds of similar appearance/colour at a distance of >2m (particularly in dappled lighting or complex habitat)
	Identical containers should be used for both aviary water and control water
	Wear gloves whilst collecting samples and replace gloves between water source/ sampling location to avoid cross contamination
	Prior to field work, sterilise 250mL bottles by rinsing with Virkon, triple rinsing with high pure water and exposing to a UV light source for 10minutes

Aviary set-up

- Before use, soak the 250mL bottles in virkon for 1hr, rinse in Nuclease-free water three times then UV-sterilize for 10mins
- Set a camera <3m from the container to record all interactions between the finches and the water body
- Fit the container with a ramp constructed from aviary mesh, descending from each end of the tray, lengthwise, these ramps should meet in the middle at a depth of ~ 2/3 the depth of the container, fixed together using fencing clips (images below). (ensure that the water level does not drop below the lowest section of the ramp within the period that the container is in the aviary)
- Remove all other possible sources of drinking water from the aviary.
- Place a plastic tray in the aviary in a position with no (or few as possible) overhanging branches
- Fill the container with 18L of tap water using a bucket with graduated measuring marks
- Leave the water in the aviary for three consecutive days (72hrs)





Camera sampling

- Set the Brinno TLC200 Timelapse camera to the following settings:
  - Capture rate: 2frames/sec
  - Playback rate:10frames/sec
  - Image quality: Better
  - Timer: 07:00–18:30

Water collection

- Draw water from below the surface of the water, using a sterile 60mL syringe, collect a total of 200mL per sample. Avoid collecting from too close to the substrate if possible, to reduce the collection of settled debris
- Place collected samples on ice as soon as possible after collection
- Filter samples according to sample preservation protocol (section 4.3 below) within 24hrs of collection, until further processing

eDNA trials

Notes before starting	For first point of detection trial: sampling times should aim to capture half of the birds' daily activity at each sampling point. (Here the 12:30pm sampling point represents 7am–12:30pm and the 7am sample represents activity from 12:30pm–6pm the previous day, collecting the later sample at 6pm each day is preferred if possible)
	For last point of detection trial: Measure depth, temperature and turbidity at each sampling point
	For last point of detection trial: Remaining volume to be calculated from depth measurements

Optimal water sampling volume

- Collect triplicates of 20mL, 50mL, 100mL and 200mL from the water body immediately after setting up the trial, to act as an initial control
- Place samples on ice
- Process samples according to sample preservation protocol within 24hrs, until further processing
- Collect triplicates of 20mL, 50mL, 100mL and 200mL from the water body at 72hrs from commencement of the trial
- Place samples on ice
- Process samples according to sample preservation protocol within 24hrs, until further processing
- Repeat trial x3

First point of detection

- Collect triplicates of 200mL samples at 07:00pm and 12:30pm
- Place samples on ice
- Process samples according to sample preservation protocol within 24hrs, until further processing
- Repeat sample collection each day up until 72hrs
- Repeat trial x3

Point of last detection

- Remove the water from the aviary at 72hrs by securing a lid on the container (A layer of cling film under the lid may be used to form a seal if the lid itself does not already contain a seal)
- Transport the water to a suitable area where the container can be exposed to direct sunlight
- Fill another sterile, plastic container with tap water to act as a control, to the same volume as the aviary water by filling the container to the same depth, measured using an accurate ruler. Ensure no contamination occurs between containers
- Cover both containers with a rectangle of aviary mesh to prevent any animals from accessing the water, secure with bulldog clips

- Measure pH at commencement of trial
- Collect a triplicate of 200mL samples from aviary water and an additional 200mL sample of the control water at commencement of trial (0hrs)
- Place samples on ice
- Process samples according to sample preservation protocol (below) within 24hrs of collection, until further processing
- Repeat sample collection and processing every three hours for the first 12hrs of the trial (3hrs, 6hrs, 9hrs, 12hrs)
- Repeat sample collection and processing at 24hr intervals each day, starting 24hrs after commencement, up until three 200mL samples can no longer be collected
- Measure pH at time of collecting final samples
- Repeat trial x3

Sample preservation

Notes before starting	Replicate samples may be filtered using the same funnel consecutively
	Sterilise filter funnels with ethanol and high pure water, as described below, between filtering samples from different sources
	Assigning each funnel to a specific source, if possible, reduces contamination risk
	Pour samples carefully to avoid any splashback that may contaminate adjacent funnels

- Sterilise filtering manifold by attaching filtering funnels, cover the inside of each funnel with 70% Ethanol allowing the ethanol to saturate the fritz and wipe over the funnel with a sterile wipe
- Turn on the filtering system, open the valves for each filter and drain any remaining ethanol
- Close valves
- Fill each funnel to the top with high pure water
- Open the valves to drain the water from the funnels
- Once drained, close valves
- Remove filter funnels
- Place a 0.45micron filter papers on each of the fritz, using tweezers sterilised in 70% ethanol
- Replace the filter funnels
- Pour one sample into each filtering funnel
- Open valves to allow filtration of the samples
- Close valves and remove filter funnels
- Place each filter paper in a labelled petri dish using sterilised tweezers and seal the circumference with parafilm
- Repeat filtration process for remaining samples, sterilising the filtering system in between if changing sample source
- Store filtered samples at -20°C

Northern Australia Environmental Resources Hub

National Environmental Science Programme

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