Development of ethically acceptable techniques for invertebrate wet-pit trapping: A report to the Department of Parks and Wildlife's Animal Ethics Committee

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Summary

This work was initiated in an endeavour to address animal ethics concerns over vertebrate bycatch deaths when wet pits are used for sampling invertebrates. These concerns arose primarily because vertebrate bycatch is invariably subjected to drowning over prolonged time frames, often in ethylene glycol and formalin which are both noxious chemicals and likely significant irritants. Furthermore vertebrate material inadvertently caught this way has been of limited scientific value due to desiccation and distortion of morphological features and lack of DNA fixation.

Firstly, we attempted to identify alternative chemical solutions that would ensure rapid euthanasia of non-target vertebrates but also provide improved morphological and genetic fixation for all target and non-target species. We were unable to identify any chemical additives that could directly deal with rapid euthanasia issues and concluded that, aside from physical exclusion of vertebrates, which is almost impossible if traps are also to be effective for macroinvertebrates, that effective invertebrate pitfall trapping is best addressed through the use of a low density fluid (< 0.9 kg/l) reducing the ability of any animals to remain buoyant on the fluid surface. This then became a mandatory criteria.

Secondly, ethanol (CH_3CH_2OH) was identified as the most appropriate low density solution (~0.79 kg/l) due to its inherent preservative properties; however its volatility and therefore rapid evaporation are problematic. Consequently, we attempted to mitigate evaporation through the utilisation of an overlay, creating a barrier between the ethanol and the atmosphere. While we trialled several compounds that were effective in reducing evaporation they all invariably either impeded fixation, as much of the material remained trapped at the interface between the overlay and the ethanol, or the overlay compound directly damaged captured material. Experiments were then conducted with a number of surfactants in an attempt to disrupt the surface tension at the chemical interface and allow all material to transition through to the ethanol. All surfactants trialled proved to be ineffectual.

Thirdly, the focus was switched to moderating ethanol evaporation through the addition of a miscible, but less volatile liquid, propylene glycol ($C_3H_8O_2$), which also has some desirable preservation qualities. Estimates of evaporation rates of solutions of 70-80% ethanol, with the remainder propylene glycol, varied markedly between laboratory experiments and field trials with evaporation rates in the field being considerably higher. While the addition of propylene glycol alone was not sufficient to extend the viability of standard two litre wet pit traps beyond a

week or so in the field, modifying pit traps, by extending their height and reducing the aperture to volume ratio, enabled traps with four litres of 80% ethanol to be deployed for up to 35 days (mean daily maximum temperature of 36°C) without the concentration of ethanol dropping below 70% by volume or the density rising above 0.9 kg/l.

Throughout all of the trials, assessments were made of the quality and quantity of DNA that could be extracted from invertebrates and vertebrate bycatch with differing concentrations and ratios of ethanol and propylene glycol. In all cases the higher concentrations of ethanol yielded superior results for quality and quantity of DNA. However we were able to extract and amplify DNA to a level suitable for generalised barcoding from material preserved in concentrations as low as 70% ethanol/30% propylene glycol.

Material preserved well in ethanol/propylene glycol and was clearly superior to that of ethylene glycol/ formalin, or our initial trials of ethanol with an overlay, for subsequent detailed morphological studies.

In conclusion, while we view ethanol as the most desirable preservation fluid, the use of overlay systems to address evaporation negatively affected the preservation of captured material by restricting transferal to the ethanol layer. We found no way to adequately address this issue, even with the use of surfactants to lower surface tension between the overlay and ethanol layers. However a preserving solution with a concentration of at least 80% ethanol/20% propylene glycol can provide effective preservation of morphological characters and adequate DNA preservation for barcoding for both target invertebrates and non-target vertebrates while simultaneously reducing ethanol evaporation. This concentration also maintains a density that should ensure a rapid death to vertebrate bycatch.

The use of modified traps with high aperture to volume ratios, along with the use of shading devices will maximise the length of time traps can be deployed over. In the worst case scenario where all ethanol evaporates, propylene glycol would still remain ensuring captured material did not desiccate, although we have not examined the effect of this on preservation characteristics.

Introduction

Wet pitfall trapping has been used for at least the last 30 years as a method to sample species and assemblages of ground dwelling invertebrates over time frames ranging from days to several months or longer. This sampling method utilises open containers of various dimensions (often one or two litre plastic jars) buried flush with the ground and containing solutions that preserve

invertebrates that fall into the container. Captured animals are killed by drowning and subsequently fixed in the "preservative" solution. When these traps are set over long time frames they require trap designs and/ or preservative solutions that have little or no evaporation.

A number of chemicals have been used in wet pitfall traps and these include methylated spirits, various concentrations of formaldehyde, gault's solution (sodium chloride, chloral hydrate, potassium nitrate and water), ethanol (60-95% ethanol), trisodium phosphate, picric acid, water with detergent, propylene glycol and ethylene glycol (e.g. Gurdebeke and Maelfait 2002, Pekár 2002, Southwood and Henderson 2000, Standen 2000 and, Walker and Crosby 1998). A search of the internet using google scholar (http://scholar.google.com.au/) at the time of this report identifies ethylene glycol and ethanol as the most referenced agents along with the search terms of "invertebrate pit trap", with 2180 and 6240 results respectively. For long term sampling, ethylene glycol has generally been the chemical of choice due to its inherent stability and resilience to evaporation. While ethylene glycol has remained a principal preservative, its toxicity to fauna if consumed and risk to the environment (Sutherland 2006), along with the sometimes high capture rate of vertebrates (bycatch), have raised concerns for its continued use.

One of the least desirable consequences of wet pitfall trapping is the number of vertebrates that can be unintentionally caught and this has been identified as a major ethical concern in recent years. The problem extends beyond just the number of animals caught to the way in which they die. Death invariably occurs through drowning after the animals become fatigued from swimming. Adding further to this problem is the likely level of distress from the irritant properties of most chemicals as the densities of these chemicals generally allow animals to remain buoyant for considerable periods of time, thus prolonging the suffering. Additionally, identification of animals using morphological characters caught in this manner can be difficult, and often, most vertebrates caught in wet pitfalls have little scientific value and are generally discarded. Ethylene glycol in particular leaves vertebrates discoloured, desiccated, distorted and in some instances partially decomposed, as well being a poor DNA preservative (Dillon *et al.,* 1996).

As a method for sampling invertebrates in biogeographic survey, biodiversity monitoring and environmental impact assessments it is highly desirable to continue the use of wet pitfall trapping due to the utility and efficiency of the technique. However, for this to occur the ethical issues around vertebrate bycatch need to be addressed. This of course should have a focus on reduction,

because eliminating all bycatch will be exceedingly difficult, if not impossible, and it also needs to address the level of distress associated with capture and drowning in a wet pit as well as on enhancing the scientific value of bycatch through improving morphological and molecular fixation. The reduction in capture rates is a complex issue and one that requires an entirely different approach to that of more humane euthanasia and improving scientific value, and was beyond the scope of this work.

The focus of this project has been on attempting to reduce distress prior to death in vertebrate bycatch and enhancing their scientific value; at the same time not compromising the effectiveness of traps for capturing and preserving their target organisms, the ground dwelling invertebrates. In exploring these issues we undertook assessments in the laboratory and in the field on a variety of potential alternative chemistries and trap modifications and we report on those here. Some of our avenues of investigation ended after irresolvable issues were encountered either in the laboratory or in the field. This led to the exploration of alternate approaches. These issues generally related to 1) quality of preservation of target invertebrates, 2) the influence on species and number of species of invertebrates caught 3) quality of preservation of morphological and molecular attributes of vertebrate bycatch, 4) control of evaporation of preservative, and/or combinations of all four of the above. While we believe we have been able to present methodologies that address most of these, there still remains an issue in respect to longevity of deployment due to preservative evaporation. To ensure that any future investigations attempting to address this remaining issue don't repeat all of the same unproductive pathways we examined, we have documented these also. Consequently, we present the work undertaken in chronological sequence starting with trials and techniques that were found to be unworkable, for reasons that are outlined, and concluding in methods and trials that we believe represent the best way forward. The following key criteria were used to set the targets for a workable wet pitfall trapping methodology.

- Ensuring rapid euthanasia of non-target species.
- Preservation of specimens to a standard suitable for taxonomic research (maintains visible morphological characters).
- Preservation of material suitable for molecular research
- Stability of solution
- Handling safety and viability for field application

However, it should be noted that the utility and acceptability of what is proposed here still ultimately rests with animal ethics committees to determine whether the potential reduction in distress to vertebrate bycatch and improved scientific value outweighs the ethical costs associated with the use of this type of trap. There is also considerable scope for continued work on how best to reduce vertebrate bycatch while not compromising target species. The merging of techniques that achieve reduction in bycatch, rapid euthanasia of captures, along with improved scientific value of captures presents the best long term solution to the ethical dilemmas.

1.0 Preliminary examination

1.1 Assessments of chemicals additives for rapid euthanasia

In the initial phase of this project we performed a literature search to identify a number of potential compounds that could be used as additives and have a documented use as an anaesthetic and/or euthanasia solution via transdermal absorption in appropriate species (Table 1). For the purposes of euthanasia in pit traps however, transdermal absorption is only likely to offer a functional application in amphibian species, and even then the chemicals used as preservatives in wet-pits are likely to result in death prior to any significant effect by anaesthetic agents. Some absorption may be expected in vertebrates via inhalation or oral ingestion, but data on effect via these routes is lacking and again this would be unlikely to result in a satisfactory reduction in animal distress.

There are also possible negative effects for the chemicals listed in Table 1 and these include direct chemical irritation (oral, ocular, dermal), stability of such solutions in the field and, their compatibility with other liquids, all of which would require investigation.

As many of these chemicals are restricted and hazardous if incorrectly used, this would pose significant usage problems. Also, almost all documented applications of these additives incorporates immersion of the animal in various dilutions with water, which is generally not possible to reproduce within a functional wet-pit trap environment as water does not have preservative qualities and readily evaporates. Subsequently, we determined that none of these compounds were likely to provide an effective, stable and safe solution for euthanasia in wet-pit traps under field conditions. Vertebrates were still likely to succumb to drowning in the same prolonged manner as has generally been the case in the past.

Further investigation into anaesthetic and euthanasia additives was therefore not continued beyond this initial assessment.

Table 1: Compounds and application rates that have a documented use as anaesthetic and/or euthanasia solutions via transdermal absorption in appropriate species

Compound	Application	Institution/Reference
Clove Oil/Eugenol/Aqui-S	10 drops per litre of water (0.65 ml per litre). Dissolving it in a little ethanol improves its solubility.	South Australian Museum 2016
	300- 450mg/l of water	University of Queensland
	15-20mg/ml (Aqui-S)	University of Queensland
	450 mg/L of water	Lafortune *et al.* 2001
Tricaine Methane Sulfonate (MS-222)	250 mg/litre is appropriate, must be neutralised with buffering agents to prevent irritation	South Australian Museum 2016
	0.1% - .3% sol (1-3gm/L buffered to 7-7.4 pH)	University of Queensland
	1 g/L	Kimberlee *et al.* 2010
Benzocaine	Ventral application of a 20% benzocaine over-the-counter oral gel (frogs)	South Australian Museum 2016
	25-100mg/l (fish)	Moon and Stabenau 1996
	200-300 mg/L of solution (frog)	University of Queensland
Chlorbutanol	0.20%	University of Queensland
Ethanol	Immersion of the sedated animal in 20% ethanol will result in death	Conroy *et al.* 2009

1.2 Physical attributes of solutions to increase speed of euthanasia

As it was considered that chemical additives would be ineffectual in providing rapid euthanasia for vertebrate bycatch, trials switched to examining physical aspects of solutions that might reduce animal duress through a reduction in time taken for an individual to drown. For this we looked at preserving/fixing solutions with a low density such that vertebrates, with a density of around 1.079 kg/l (Kessler 1996), would not be able to remain buoyant and swim on the surface for extended periods. A wet-pit trap medium significantly less than 1.079kg/l would result in the most rapid death and minimise the animals suffering.

Table 2 below presents some comparative properties for typical concentrations of ethanol, water and the other conventional wet pit chemicals of ethylene glycol and propylene glycol. At 20°C 100% ethanol (CH_3CH_2OH) has a density of 0.789 kg/l as compared to ethylene glycol ($C_2H_6O_2$) with 1.113 kg/l and propylene glycol ($C_3H_8O_2$) with 1.036 kg/l. Ethanol is particularly viable from a density perspective for the purpose of reducing natural buoyancy in vertebrates because even at 0° C the density of 70% ethanol remains below 0.86 kg/l, and as temperatures and/or concentrations of ethanol increase the density further decreases. Densities of 100% and 70% ethanol at differing temperatures are presented in Figure 1.

As can be seen from Table 2 the options for low density solutions were limited as the only chemicals recognised as having suitable morphological and genetic fixative properties, which also had significantly lower density than that of vertebrates, were ethanol and methylated spirits. For all practical purposes these two chemicals have almost identical properties varying only by methylated spirits being 98% ethanol with the inclusion of approximately 2% methanol as a denaturing agent. The addition of methanol is as a bittering and odour agent as well as being toxic when ingested. Little data is available on the effects of methanol on preservation of specimens and DNA however Simmons (1995) notes that it is a powerful solvent that can oxidise to formaldehyde and is therefore not recommended as a preservative for animal collections. The effects of industrial methylated spirits (IMS) on DNA preservation of invertebrates found that the methanol denaturant could have a significant degrading effect, particularly in respect to time (Carter 2003).

Table 2: Physical properties of common chemicals used in preservation

Preservative	Density (kg/l @ 20^0C)	Density (kg/l @ 0^0C)	Boiling point (^{0}C)	Other properties
Ethylene glycol	1.113	1.096	197.3	Odourless, highly referenced, antifreeze, not known to preserve DNA, considered harmful if ingested
Propylene glycol	1.036	1.017	188	Highly referenced, antifreeze, hygroscopic, unknown effects on DNA quality, non-volatile
Water	0.99	.99	100	Without additives has no preservative property. Moderate evaporation rate. Degrades DNA
Ethanol (70%)	0.85	0.86	84.9	Highly referenced, evaporates readily, flammable, volatile. Water component degrades preservation of DNA
Ethanol (80%)	0.83	0.84	82.8	Highly referenced, evaporates readily, flammable, volatile. Water component degrades preservation of DNA
Ethanol (90%)	0.81	0.82	80.6	Highly referenced, evaporates readily, flammable, volatile. Water component degrades preservation of DNA
Methylated spirits (100%)	0.80	0.81	78	Evaporates readily, flammable, volatile and has the same general properties of 100% ethanol apart from the addition of 2% methanol. DNA preservation less than ethanol. Methanol component can oxidise to formaldehyde.
Ethanol (100%)	0.789	0.80	78.5	Highly referenced, especially on DNA preservation, evaporates readily, flammable, volatile

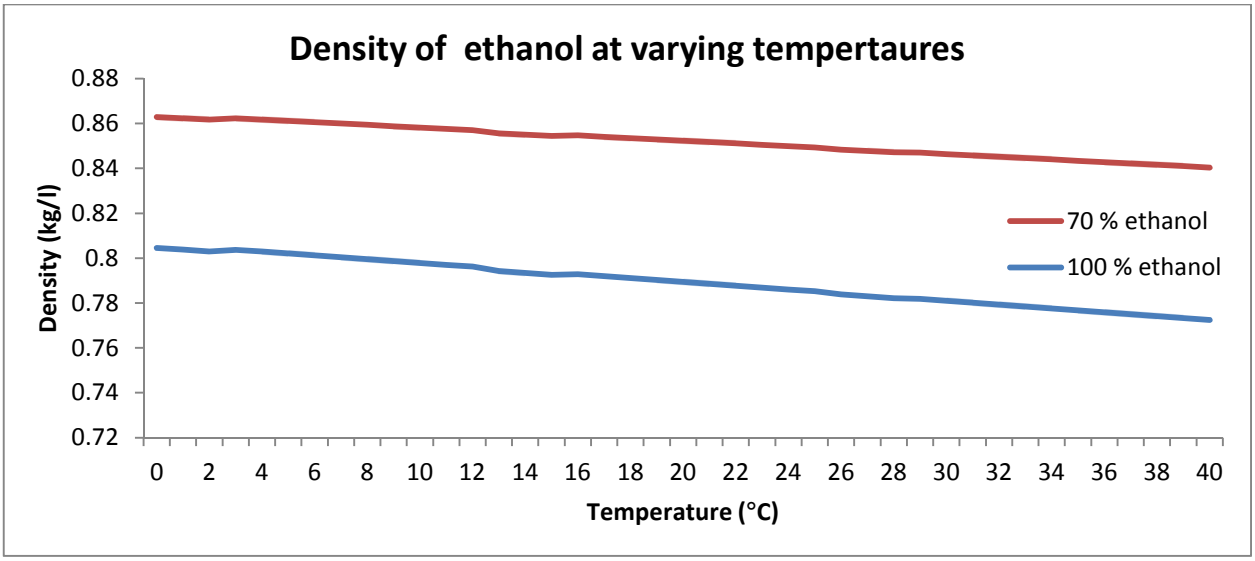

Figure 1: Density relationship of 100 % ethanol and 70% ethanol to temperature

As ethanol is one of the most widely used materials for DNA preservation, as well as invertebrate specimen preservation and storage, we focused work on how it might be used under field conditions for long durations as a wet-pit trap solution. Other advantages of ethanol include no toxic additives and that it is readily available in large quantities for scientific research. However ethanol is not without undesirable properties, volatility in particular with concomitant rapid evaporation, especially with increasing temperature. Consequently, for this to be a viable solution for time frames beyond several days of deployment necessitates finding a method of mitigating evaporation while simultaneously retaining the critical low density properties required for euthanasia.

2.0 Laboratory Trials

2.1 Controlling evaporation of ethanol with overlays

Our initial trials in the laboratory showed that evaporation of 70% ethanol concentration could be as high as 45% over as few as six days when temperature is held at 45°C. This was from 80ml of ethanol in an 80ml beaker. For 100% ethanol this would be even greater due to the absence of any water fraction. One way to moderate evaporation is with the introduction of an overlay on the ethanol to create a barrier to the atmosphere, a technique often used in certain types of laboratory work for moderating evaporation. We therefore trialled a number of different compounds, primarily oils, but also a variety of other commercially available agents, that would float on

various concentrations of ethanol. For any of the overlays to have potential for application in invertebrate wet pit traps all of the following properties are required:

[1] Must be less dense than the concentration of ethanol (>70%) used in the trap
[2] Must be stable for the life of a trapping program
[3] Must be resistant to evaporation
[5] Significantly reduce ethanol evaporation
[6] Safe to humans and to the environment
[7] Should not adversely affect the killing/preserving process of either target or non-target species and,
[8] Should not increase capture rates of non-target species over those already observed through the use of other conventional wet pitfall trapping solutions such as ethylene glycol.

A list of potential overlays that were examined and some of their properties are presented in Table 3.

For all of the mineral oils, apart from light oil and Sigma oil, the densities were too heavy to float on any ethanol concentration at or above 70% @ 20°C so were not considered further. Sigma oil was slightly denser than the light oil and as both were mineral oils with other properties almost identical we chose to undertake trials with the light oil only due to its lower density. While it is possible to find reference to the density of olive oil ranging between 0.8-0.9 kg/l, we were unable to locate any specific brands or sources at the lower end of this density scale (< 0.85 kg/l) and thus no trials were undertaken with this as an overlay. Other chemicals that could create an overlay but were not continued with were Silicon, Paraffin Oil and Kerosene. The former two had densities that would only just form an overlay at the lower end of the acceptable ethanol concentration of 70% while kerosene was not used as it was highly aromatic and its other properties were replicated in the more refined Lamp Oil. This then left only WDLube, Lamp oil, and Light oil to perform evaporation trials. Table 4 shows at which concentrations of ethanol these, and the other compounds identified above, would readily form overlays.

Table 3: List of potential overlays and some of their properties

Overlay trialled	Density (kg/l @ 20^0C)	Boiling Point (^{o}C)	Other properties
Mineral Oil (heavy)	0.862	218	LD50 Oral - Rat - male and female - > 5,000 mg/kg (OECD Test Guideline 401) LC50 Inhalation - Rat - male and female - 4 h - > 5 mg/l (OECD Test Guideline 403) LD50 Dermal - Rabbit - male and female - > 2,000 mg/kg (OECD Test Guideline 402)
Mineral Oil (light)	0.838	218	as above
Mineral Oil (nujol)	0.880	218	as above
Mineral Oil (pure)	0.877	218	as above
Mineral Oil (spectroscopy)	0.851	218	as above
Mineral Oil (Sigma)	0.840	218	as above
Olive Oil	0.80-0.9	300	LD50/LC50: CAS# 8001-25-0: Draize test, rabbit, skin: 100 mg/48H Moderate; Carcinogenicity: CAS# 8001-25-0: Not listed by ACGIH NIOSH, NTP, or OSHA.
Paraffin Solution	0.85	150	Swallowed: Expected to be of low toxicity. Eye: Irritant. Skin: Prolonged contact may cause dermatitis. Inhaled: Inhalation of vapours or mists may cause irritation to the respiratory system. Chronic: Prolonged contact may cause dermatitis.
Octanol	0.82	195	LD50 Oral (mouse)1790 mg/kg Swallowed: Expected to be very hazardous Eye: very hazardous-irritant. Skin: hazardous-irritant Inhaled: Inhalation is considered hazardous
Lamp Oil	0.79	190	Swallowed: Expected to be of low toxicity.

Overlay trialled	Density (kg/l @ 20^0C)	Boiling Point (^{o}C)	Other properties
			Eye: Irritant. Skin: Prolonged contact may cause dermatitis. Inhaled: Inhalation of vapours or mists may cause irritation to the respiratory system. Chronic: Prolonged contact may cause dermatitis.
Kerosene	0.80	150	Expected to be of low toxicity LD50 Oral (rat) > 2000 mg/kg LC50 Inhalation greater than near-saturated vapour concentration (rat, 4h) LD50 Dermal (rabbit) > 2000 mg/kg
Silicon	0.85	250	No significant acute toxicological data identified in literature search
WDlube	0.80	183	Oral rat LD50- >5000 mg/kg; Skin rabbit LD50- >3160 mg/kg.

Table 4: Comparison between different liquids as ethanol overlays

Overlay	Ethanol Concentration			
	70%	80%	90%	100%
Paraffin Oil	+[1]	-	-	-
Silicone	+[1]	-	-	-
Sigma grade Oil	+	-	-	-
Light Oil	+	-	-	-
Octanol	+	+	-	-
Olive Oil (light)[2]	+	+	-	-
Lamp Oil	+	+	+	-
Kerosene	+	+	+	-
WD lube	+	+	+	-

+ Point of equivalence where liquid forms an overlay, [1] marginal equivalence, [2] unable to identify any source.

2.2 Testing of overlays to reduce ethanol evaporation

An initial comparative trial was undertaken with light oil as an overlay on 70% ethanol and non-layered ethanol to measure evaporation at different temperatures. Without an overlay, up to 25% of ethanol (70%) by weight was lost to the environment through evaporation over 30 days in a 37^0C water-bath (Fig. 2C, blue dots). This result differed from our initial ethanol evaporation trial due to the use of a narrower 60ml vessel with a much reduced surface area exposed to the atmosphere, and thus became an important aspect for further work. As these tests were only comparative with and without overlays this variation was not critical. This rate of loss was lower at lower temperatures (Fig. 2A and 2B) but still significant. A light oil overlay on 70% ethanol gave a clear reduction in evaporation at all temperatures trialled (Fig. 2A, 2B and 2C, red dots).

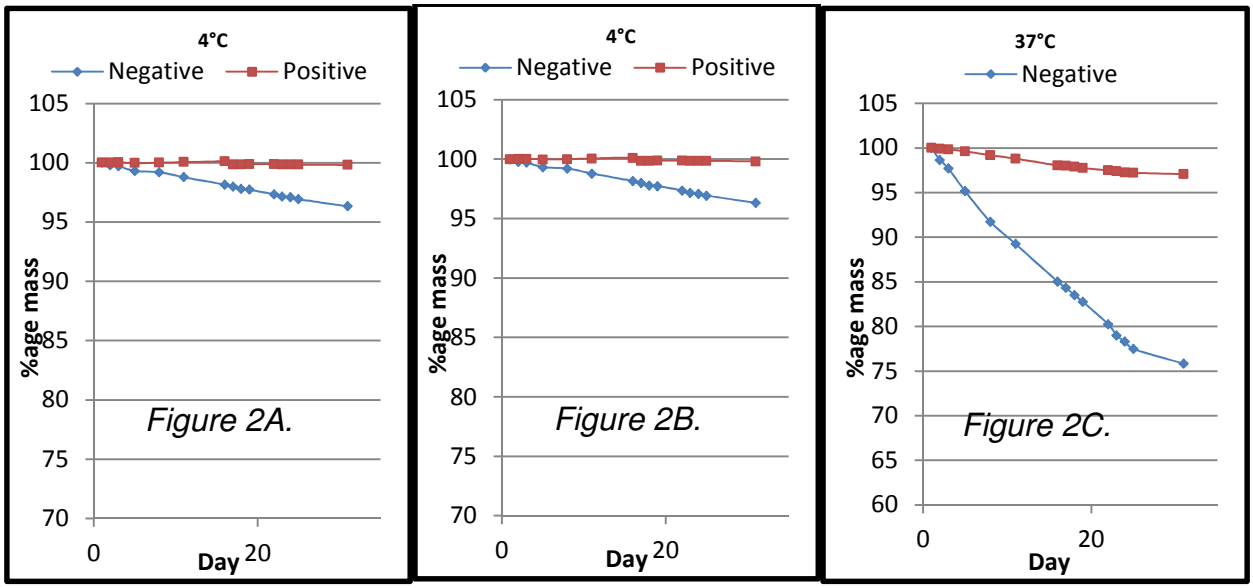

Figure 2: Evaporation of ethanol (70%) at different temperatures with (red) and without (blue) a light mineral oil overlay

While this initial trial was promising from the evaporation perspective, for preservation of DNA the desirable concentration of ethanol is 100% as the water fraction of diluted concentrations is known to degrade DNA over time (Carter 2003), and this concentration more rapidly penetrates cellular membranes and deactivates DNase activity (King and Porter 2004). Carter (2003) also demonstrated that higher molecular weight extractions and DNA quality was achieved from invertebrates through the use of high concentrations of ethanol in the initial preservation of specimens.

Only five of the liquids we identified for trials in reducing evaporation (Table 3) could form an overlay at anything above an 80% concentration of ethanol so this concentration then became the

minimum standard that we used for further investigation of ethanol evaporation reduction in the laboratory.

We investigated whether WDLube and Lamp oil, the two lowest density liquids identified, both of which would form overlays on a 90% concentration of ethanol, would also have moderating effects on ethanol evaporation. This was done through placement of an overlay of each liquid over 90% ethanol, along with a 90% ethanol control, in a 45°C water bath in fume hood with air circulating. The volume of the overlays and the ethanol were 20ml in a narrow 60ml glass jar. Levels were then monitored over a 4-day period. Exposure to a higher temperature than previous trials, along with air circulation, was employed to better replicate the sort of conditions that may be encountered in the field. The WDLube performed well with no loss of the overlay and only a small reduction in ethanol volume. There was some loss of the lamp oil overlay and of ethanol. While this loss of ethanol was greater than that for the WDLube sample, it was significantly less than that of the control. However the evaporation rate of the lamp oil would probably require moderating of its own volatility if it were to be used in the field, possibly through the addition and mixing with a slightly heavier oil such as paraffin. The fact that these two oils are miscible means it is possible to create oil at any density between 0.85 kg/l and 0.79 kg/l. For example a ratio of 1:3 of paraffin and lamp oil would result in an oil of 0.80 kg/l, still light enough to form an overlay on 90% ethanol @ 20°C. This slightly denser oil should have less volatility and a slightly improved reduction of ethanol evaporation.

2.3 Effect of overlays on transferal of biota to preserving fluid

Having identified two potential liquids for moderating evaporation of ethanol under laboratory conditions, further examination was required to see whether these overlay systems would ensure rapid submersion of target (invertebrates) and non-target (vertebrates) species, and subsequent transfer through to the ethanol layer for preservation. Rather than undertake any unnecessary experiments on live vertebrates, and given the body mass of the smallest model invertebrates (juvenile crickets ~ 25mg) is less than that of the smallest vertebrates (*Menetia sp.* ~ 200mg), we considered that submersion of vertebrates and transfer through to ethanol would occur if it did for equivalent or smaller sized invertebrates, particularly as the surface area to mass ratio of invertebrates is generally considerably larger than vertebrates.

The model invertebrate species used in these laboratory trials were crickets, adults and juveniles, as they were readily available from pet supply stores. The initial tests were done by dropping crickets into 80% ethanol as this was the absolute minimum concentration of ethanol (and therefore highest density) we wanted to use. Also, it was presumed that any increased concentrations above 80% should have better results in terms of both euthanasia efficiency and DNA preservation.

Adult and juvenile crickets dropped into an 80% ethanol solution submerged immediately and were rapidly killed. The introduction of an oil overlay did not significantly delay the euthanizing process, however, juvenile crickets (25 mg; mean; n = 20) did not enter the ethanol layer and remained on the interface between the overlay and ethanol (Figure 3). Further investigation indicated that target species with a mass less than 300mg (n = 20) would remain at the overlay/fixative interface and not transition through to the preserving ethanol layer below. The precise mass was indeterminate as it is likely influenced by a number of factors including body size, shape, morphological structures such as hair and, temperature of the fluid (changes in specific gravity). Under operational conditions in the field this size class of invertebrate (<300mg) can form a significant portion of typical wet pit captures. By increasing the ethanol concentration above 80% and utilizing WDLube or a lamp oil overlay, the size class of invertebrates transferring to the ethanol was slightly improved but still not complete.

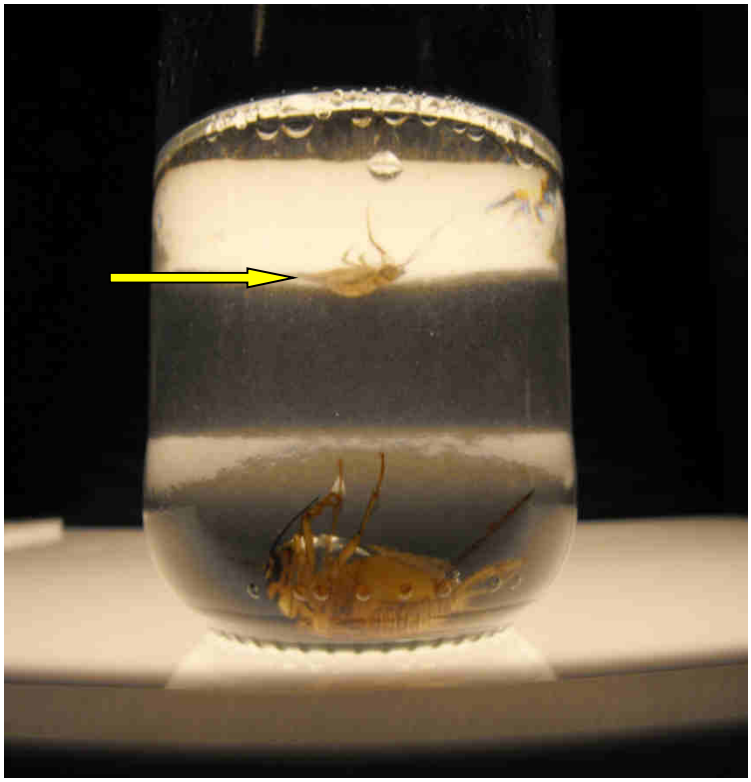

Figure 3: Juvenile cricket (~25mg - yellow arrow) and adult cricket (~350mg) placed in oil overlay on 80% ethanol

2.4 Effect on surfactants in mitigating surface tension between overly and ethanol

Surfactants are known for lowering the surface tension of liquids and, in the context of this project, the interfacial tension between two liquids. In an attempt to lower the surface tension between the overlays and ethanol, the characteristic that we considered to be the limiting factor in transferal of all size classes of invertebrates to the ethanol, we investigated a number of surfactants. The surfactants we investigated are commonly used in laboratory work and these were sodium docecyl sulphate (SDS; anionic), cetyltrimethyl ammonium bromide (CTAB; cationic) and, Triton X-100 (non-ionic/neutral). Ethanol (80%) was prepared with and without 5% of SDS, CTAB or Triton X-100 and then overlayed with the same volume of the overlays. When a healthy adult cricket (350 mg) was dropped into a 'wet pit' trap of overlay/ethanol (non-surfactant control) it struggled briefly within the overlay before swimming through the boundary layer and becoming submerged in the ethanol fixative. It rapidly died/drowned and descended to the bottom of the container. These trials were replicated for each of the surfactants with similar observations. When the trial was repeated with a healthy juvenile cricket (25 mg) with the non-surfactant control they were unable to break through the boundary to the ethanol fixative but instead rapidly died/drowned in the overlay and then descended onto and remained on the

overlay/ethanol interface. This trial was again repeated for each of the surfactants with none of the crickets (25mg) transferring through the interface to the ethanol. It was therefore concluded there was no significant reduction of surface tension between the overlay and ethanol when the ethanol was fortified with the surfactants sodium docecyl sulphate (anionic), Triton X-100 (non-ionic/neutral) or cetyltrimethyl ammonium bromide (cationic).

2.5 Impact of Wet-pit Preservative on Specimen Morphological Integrity

As we demonstrated that in low density solutions, with or without overlays, our larger model invertebrates would rapidly submerge and drown, we were confident that for larger organisms in general, including vertebrate bycatch for which the smallest (*Menetia sp.*) weigh around 500mg, captures would probably transgress the boundary layer into the ethanol, we focused on examination of the preservative capabilities of ethanol.

An understanding of the effect(s) of ethanol under differing temperatures and durations on the structural integrity of specimens is essential for development of a functional wet pit methodology. Therefore we placed adult crickets in a range of ethanol concentrations (60% to 90%) and incubated them for 12 weeks at a range of temperatures (4^0C to 37^0C). Specimens were examined at 2, 4, 8 and 12-week intervals, and their morphological characteristics compared with those of a control (Figure 4). All specimens were easily mounted on card with pins regardless of ethanol concentration, temperature or duration of exposure. However exposure to 90% ethanol at 37^0C, for 8 weeks, resulted in hind limbs becoming less flexible with a tendency to break if not carefully handled. By the 12 weeks in 90% ethanol, all limbs and antenna were somewhat brittle and more prone to breaking accidentally than specimens from other concentrations/durations and there were also some signs of slight shrinkage and disfigurement. However, as long term storage of specimens from wet pits for research usually takes place in ethanol solutions the condition of dry test organisms at the end of the trial was not viewed as a significant issue for morphological preservation. The trial indicated that external features remain in slightly better condition if they are not exposed to high concentrations of ethanol and high temperatures for extended periods.

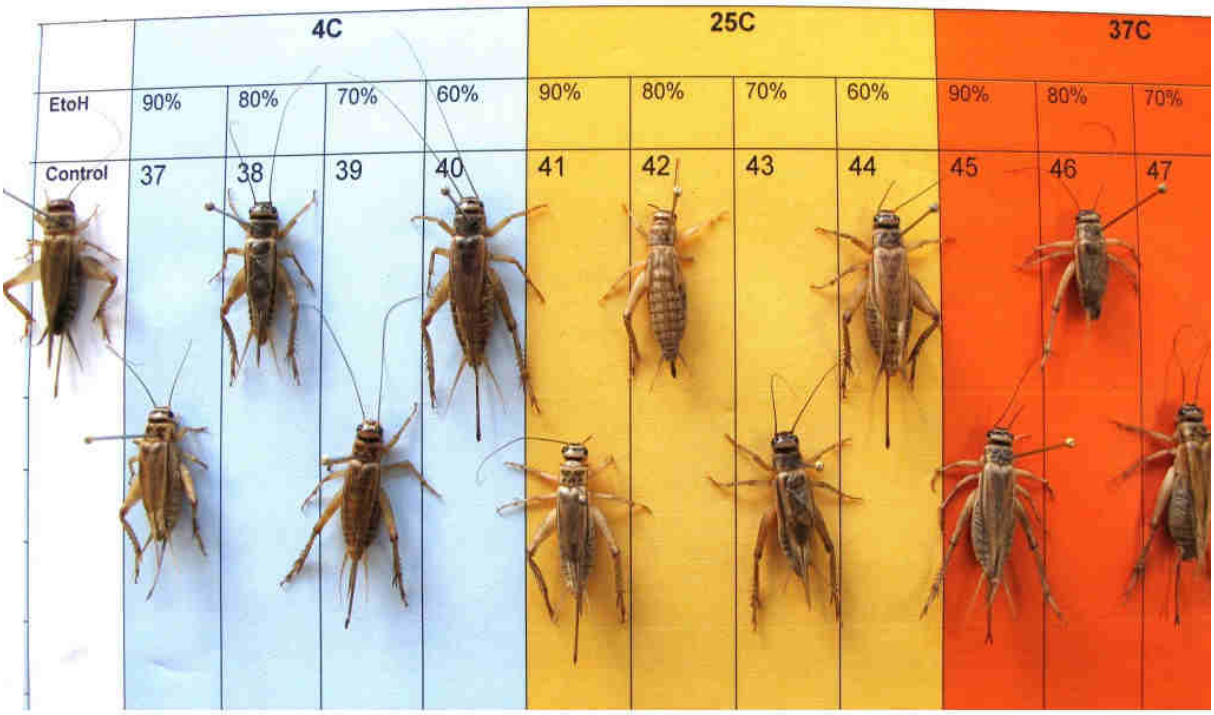

Figure 4: Mounted specimens from different concentrations, durations and temperatures of ethanol

2.6 Impact of Wet-pit Preservative on Molecular Preservation-Laboratory trial

Beyond an assessment of external morphology we also examined samples from the crickets for DNA preservation. The type of molecular examination that can be undertaken is limited by the size (and quality) of the isolated DNA. Molecular applications such as cloning can require large DNA fragments (more than 2 kilobase pairs), whilst detection of single nucleotide polymorphisms using Sanger DNA sequencing is achievable from smaller fragments (less than 200 base pairs). To allow the broadest applicability of molecular research methods, it is preferable that high quality, i.e. high molecular weight DNA, is preserved in scientific specimens. The best preserved DNA from our temperature and duration trials resulted from using 90% ethanol, and this was relatively unaffected by temperature or duration of exposure. However, prolonged exposure (12 weeks, 37^0C) of specimens to 60% ethanol resulted in a higher proportion of smaller DNA fragments. Despite the extent of degradation, DNA isolates contained high molecular weight DNA in excess of 2 kilobase pairs. Such DNA is generally regarded as suitable for molecular examination, for example PCR amplification and DNA sequencing. Indeed, each isolate was positive for the 690 bp invertebrate mitochondrial gene by standard PCR amplification (Table 5). No adverse effects from any ethanol concentration were detected.

Table 5: Detection of 690 base pair Invertebrate Mitochondrial Gene by PCR amplification

		4C				25C				37C			
Ethanol (%)	**C**	**90**	**80**	**70**	**60**	**90**	**80**	**70**	**60**	**90**	**80**	**70**	**60**
Week 2	+	+	+	+	+	+	+	+	+	+	+	+	+
Week 4	+	+	+	+	+	+	+	+	+	+	+	+	+
Week 8	+	+	+	+	+	+	+	+	+	+	+	+	+
Week 12	+	+	+	+	+	+	+	+	+	+	+	+	+

C = Control: Thawed adult cricket and untreated

3.0 Field Trials

3.1 Field trial introduction

While there were some limitations as to the effectiveness within each of the investigations undertaken in the laboratory, we had enough information to further explore our findings and apply them under field conditions. To this end we undertook two trials, one within Kalbarri National Park in May of 2011 and a second in banksia woodland on the northern Swan Coastal Plain in November of 2011 with the aim to assess the following criteria:

1. adequate morphological preservation for target and non-target species (vertebrate bycatch)
2. adequate DNA preservation for target and non-target species
3. stability and longevity for field operation and,
4. no increased rates of capture of non-target species over traditional wet pit trapping methods utilising ethylene glycol

The previous laboratory trials had already demonstrated that captured animals would rapidly become submerged in low density solutions and as it is not be feasible to examine this further in a field setting, beyond observation at to where within a solution captures are positioned, this was not specifically investigated.

Failure in meeting any one or more of the above criteria would result in rejection of that chemistry for long term usage.

3.2 First field trial of overlay methods

For the Kalbarri field trial the overlay and ethanol concentration were based on laboratory trials and aimed at the highest possible concentration of ethanol that would readily maintain the overlays of WDLube or a modified lamp oil. The lamp oil was modified by mixing with paraffin oil so as to reduce the overall evaporation rate of the overlay and further reduce ethanol evaporation. Paraffin oil was used for dilution because of the following characteristics:

1. paraffin oil and lamp oil are miscible with each other
2. it introduces no odour or colour
3. it is more effective at reducing ethanol evaporation than lamp oil
4. paraffin oil is resistant to evaporation and,
5. it is readily available and cheap to buy

However on its own paraffin oil is too dense to form an overlay on concentrations of ethanol much above 70%, which is too low for the best molecular preservation and also has a density (0.85 kg/l @20°C) that would most likely prolong the time in which animals may be able to stay buoyant and swim. In addition, the lower the density of oil overlays the smaller the size class of invertebrate that are likely to migrate through the oil/ethanol interface into the ethanol due to a lower interface boundary tension. The mixture of lamp oil and paraffin oil used was a ratio of 75:25 respectively producing an oil overlay with a density 0.805 kg/l at 20°C. This enabled application with an ethanol concentration of 85% as this has a density of around 0.82 kg/l at 20°C. This concentration of ethanol also remained functional for use with WDLube which has a density of 0.80 at 20°C.

We established three sites at Kalbarri in differing habitats with each having a grid of twenty (4 x 5) two litre plastic jars at five metre spacing buried flush in the ground. Groups of five pits were selected at random at each site and allotted one of three treatments -lamp/paraffin oil (75:25) mix over 85% ethanol, WDLube over 85% ethanol and 100% ethylene glycol. The volumes for the oil/ethanol and the WDLube/ethanol were 500ml/1000ml totalling 1500ml in a container. For the

ethylene glycol we used 1500ml. The remaining five pits were dry control pit. Pits were in operation for 10 days from 13th to 25th May 2016 (closed for two nights/days due to rain) and dry pits were checked each morning and any vertebrate captures immediately released, while invertebrate captures were transferred into jars of ethanol for later identification. Fourteen remote cameras were distributed across the three sites and between the different chemistries for the duration of the sampling to investigate visitation by vertebrates, particularly mammals. Figure 5 shows a typical heath site (a), a wet pit trap with a WDLube overlay (b), remote cameras set up on wet pits (c) and, a wet pit with an oil overlay (d).

Figure 5a: Sand plain habitat

Figure 5b: Wet pit trap with WDLube

Figure 5c: Remote triggered cameras

Figure 5d: Wet pit trap with oil

3.3 Results for field trial at Kalbarri

3.3.1 Comparison of invertebrate captures between different chemistries

During this trial there were over 2054 invertebrate captures representing at least 32 families, 126 genera and more than 325 species.

For the lamp/paraffin oil only there were some 817 captures of around 208 species. Only 18% of these invertebrate captures migrated through into the bottom ethanol layer with a few invertebrates remaining on the surface of the oil but the majority within the oil mixture at the interface with the ethanol. For the WDLube there were 275 captures of some 55 species. While the captures were lower than for the oil, 68% of them transitioned through into the ethanol preservative and almost none remained floating on the surface, which was an improvement. Ethylene glycol caught a similar number and species to that of the oil/ethanol pits with 729 individuals from some 195 species. All captured material was submerged. Finally the dry pits had only 233 individuals from around 95 species. These lower figures for dry pits could result from predation within the dry pits, or that jumping, flying and climbing insects can escape and that with no fluid with any odour there is no attraction for any species to this type of trap. Captures in dry pits are more likely to be entirely random events rather than through any attraction.

3.3.2 Comparison of invertebrate assemblages between different chemistries

Different chemistries and trap types have been shown to capture differing elements of invertebrate communities (e.g. Schmidt et al. 2006, Jud and Schmidt-Entling 2008, Knapp and Ruzicka 2012). While we documented the differences in trap capture rates in terms of species and total individuals captured, we also ran a classification to broadly examine how variable invertebrate assemblage structure could be depending on the trap chemistry used (Figure 8). Association was determined with the Bray-Curtis dissimilarity measure on square root transformed abundance data using the software package Primer-E (Clarke and Gorley 2015). Here the greatest level of similarity was between ethylene glycol and lamp/paraffin oil but that was still only 62 percent. The similarities between the other pairs of chemistries or dry pits were lower and ranged between 34 and 40 percent. This trial categorically indicates that resolved community structure is likely to be dependent on the chemistry used and that each method will give somewhat differing results from the others.

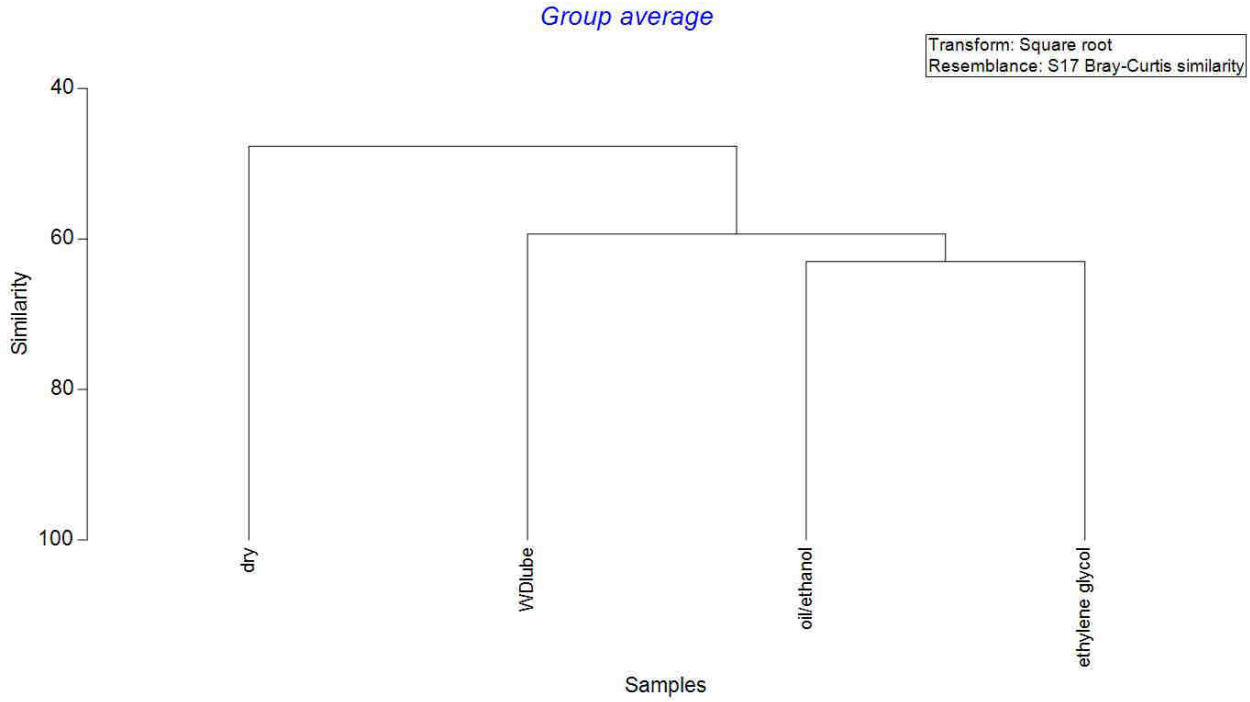

Figure 6: Similarity of samples from all treatments combined at Kalbarri. Measure used was Bray Curtis on square root transformed abundance data.

3.3.3 Vertebrate captures

For vertebrates there were twelve frogs, three rodents and one lizard caught. Three of the frogs and the lizard were caught in dry traps. One hopping mouse was caught in an oil/ethanol trap and two were caught in ethylene glycol traps. Three frogs were caught in oil/ethanol traps, four in WDLube and two in ethylene glycol traps. The cameras detected only three vertebrates (Figures 7a, 7b and 7c) and although each of these either investigated or passed near traps, none were caught in the traps.

Figure 7a: Ethylene glycol pit

Figure 7b: Oil/ethanol pit

Figure 7c: Oil/ethanol pit

Overall captures of vertebrates were quite low and had there not been any rain over two days and one night during the trial we would have expected no captures of frogs as this weather markedly increased their activity. As each trap type had the same effort (150 trap nights) and a total of four vertebrate captures each there was no statistically significant difference in any of the trap types. While this capture rate of 2.7 individuals /100 trap nights is low and would have been much lower without the frogs (0.5 individuals / 100 traps nights) this is almost certainly not indicative of capture rate under hot conditions when most reptiles are increasingly active.

3.3.4 DNA preservation of target and non-target species

Examination for DNA isolation using a QIAGEN Blood and Tissue Kit was undertaken for invertebrate and vertebrate tissue samples which had been preserved in trial pits in the field using the oil overlay on 85% ethanol. Samples had remained in the overlay/85% ethanol fluid at ambient temperatures (cycling from a minimum of 4.4°C to maximum 29.5°C) for 30 days prior to storage in 100% ethanol at room temperature (~25°C). The samples shown in figure 8, lanes 1-14, comprised a tail tip from two mammals (*Notomys alexis,* lanes 1 & 2), toes from four frogs (*Arenophryne xiphorhyncha,* lanes 3-6), a leg from five spiders (families Zoridiae, lanes 7-9 and Idiopidae, lanes 10 & 11) and a leg from three beetles (Carabidae, lanes 12 - 14). Despite some degradation, high molecular weight DNA was present in all samples although less was extracted from the Carabidae than for the other samples as can be seen in the gel scan (Figure 8 lanes12-14).

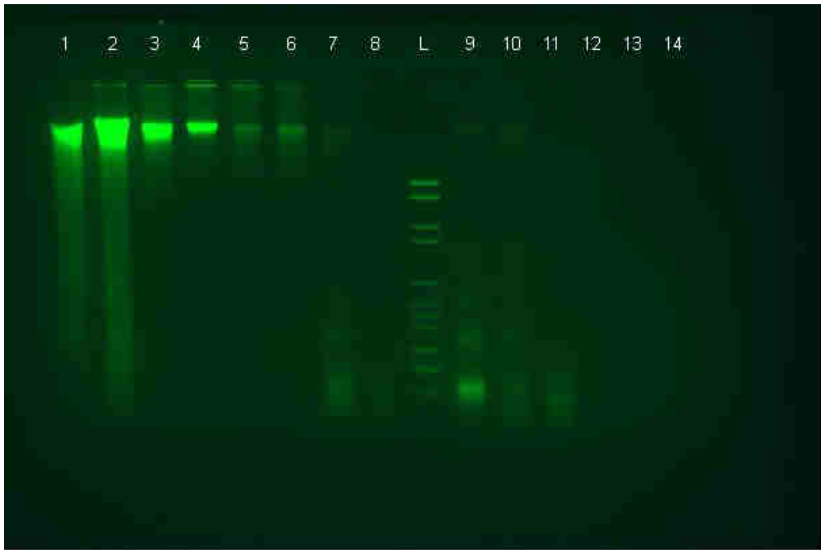

Figure 8: DNA samples in agarose gel for invertebrate and vertebrate samples collected during a field trial of oil overlay on 80% ethanol (Notomys alexis, lanes 1 & 2, Arenophryne xiphorhyncha, lanes 3-6, Zoridiae lanes 7-9, Idiopidae lanes 10 & 11, Carabidae la lanes 12 – 14). The ladder, L, is 2 kilobase pairs.

3.3.5 Morphological preservation

This quality of morphological preservation was variable between the differing chemistries. The size of invertebrates, and whether they were covered in hair like structures or had open wings or not strongly influenced whether they would readily transfer into the ethanol in the oil/ethanol traps. This in turn influenced the quality of preservation with those in the ethanol layer being generally well preserved, although small hard bodied invertebrates in the oil layer did not appear to suffer any visible degradation over the course of the 10 days of the field trial either. However the cool conditions may have assisted in slowing down any decomposition. With the WDLube there were problems with staining due to the dark colour and this also permeated into the ethanol layer. This resulted in significant difficulties in identification of captured material. While more of the captures in this type of trap ended up in the ethanol than for the oil/ethanol traps, for the material caught in WDLube layer they showed significant levels of deterioration and were not considered useful as specimens. In addition the aromatic nature of WD Lube was also a potential hazard when sorting of material was undertaken in an enclosed laboratory environment. This necessitated the use of a fume hood. On the basis of these results WDLube was considered unsuitable as a viable wet pit solution and no further trials were undertaken with it as we could not identify ways

of mitigating any of the identified issues. Ethylene glycol performed as expected, and while in this short term trial adequately preserved morphological features for invertebrates, vertebrate captures had begun to rot and looked somewhat desiccated. However the aim was not to examine the properties of ethylene glycol as it is some of the properties it exhibits that are the reason for identifying alternatives.

3.3.6 Chemical stability under field conditions

While we had already identified under laboratory conditions ethanol with an overlay of oil has some level of evaporation it is important to understand how this may operate under field conditions in a standard trap with an aperture of 80mm, a height of 200mm and a volume of 2,000ml. To quantify this we measured the volumes of fluids at the end of the trial to compare with the initial volumes and ascertain the average loss for each of the fluids. Unfortunately there was some rain over a total of four days during our study and while the amount was variable across the sites, we were unable to quantify how much fell at any individual site. A rain gauge within Kalbarri National Park Recorded almost 40mm over the duration of our survey however on the heaviest nights (~25mm) we had placed lids on traps as a precaution. While we are certain that none of the traps received anywhere near the 15mm recorded while they were open and this did not significantly change the evaporation results, it is likely to have slightly inflated the amount of ethanol measured at the end of the trial. Subsequently the following values for ethanol are indicative only and real losses due to evaporation would be equal to or slightly higher than specified. The volume of water 15mm would contribute to a trap of 80 mm diameter if it fell directly on the trap would be 75ml. In a worst case scenario the actual evaporation of ethanol could then have been as much as 75ml more than we measured. Temperatures recorded in the National Park ranged from a mean maximum of 23.9ºC down to a mean minimum of 11.5ºC.

The average loss for ethanol within the oil/ethanol traps was 433±14.1 ml (or 508 ml when including maximum potential rainfall) while for the oil fraction it was 43±6.3 ml. For the WDLube/ethanol traps the ethanol was 430±18.8 ml (or 505 ml including maximum potential rainfall) and for the WDLube, 50.3±5.5. This differed somewhat from the laboratory results in that the WDLube evaporated less than the lamp oil, and

was significantly better at reducing ethanol evaporation. Ethanol evaporation was now almost equivalent between the two overlays, indicating the modification of lamp oil by the addition of the slightly more stable but heavier paraffin oil had the desired effect on evaporation. There was no loss for the ethylene glycol. It would be expected that as the overlays evaporate the rates of ethanol evaporation would marginally increase and thus it is not a simple linear relationship in terms of calculating duration to zero ethanol. However if we assumed a straight line then the best case scenario under these conditions would be that the ethanol would last for approximately 24 days. This would be very similar for either of the overlays used in this trial. If we accounted for the addition of a maximum of 75ml of additional evaporation due to the increased volume from 15mm of rainfall then the longevity of traps would fall just under 20 days before all ethanol was lost.

3.4 Second field trial of overlay methods

A second field trial was undertaken from 3rd^{h} to the 18th of November 2011 in banksia woodland on the northern Swan Coastal Plain. The purpose here was to examine methods under different climatic conditions (warmer temperatures), in a different habitat type and, for a slightly longer duration than the trial undertaken at Kalbarri. Despite encountering issues in transferal of captures through to the ethanol layer during the Kalbarri trial we continued with the paraffin/lamp oil and ethanol as there was some level of preservation of the material caught in the oil and it was also possible that the surface tension between layers would be reduced and more effective with an increase in temperature. Concentrations of ethanol and the mixture of paraffin and lamp oil remained the same as the previous trial as higher concentrations of ethanol required lower density oil, which results in increased evaporation of both the lamp oil and the ethanol. If we used lower concentrations of ethanol with higher density oils to increase solution longevity under field conditions we were unlikely to meet the overall objectives of morphological and molecular preservation or rapid euthanasia of bycatch. For comparative purposes dry pits and pits with ethylene glycol were again also used. As already mentioned we did not persist with trials of WDLube, primarily due to the staining, low capture rates and the difficulties in handling. Due to the higher temperatures expected in November than for the winter Kalbarri trial, and in an attempt

to negate the rate of evaporation, as well as protect solutions from rain, all traps were set with a small corrugated iron roof (Figure 9).

For this trial four sites were trapped, all of which were a combination of proteaceous heath and banksia woodland. Fifteen traps were set at each site in a three by five array at five metre intervals between each trap. Again traps were identified at random to contain either the oil/ethanol, ethylene glycol or to remain dry. Therefore five traps at each site were allocated to each of the treatments. Traps were opened and run for 14 consecutive nights. Traps were checked early in the morning each day so as to remove captures from the dry traps and to ensure there wasn't a problem with excessive captures in any of the wet pits. As with previous trials any vertebrate captures were identified and released from dry pits while captures of invertebrates were transferred to ethanol for later identification and to provide a comparison with the captures in other pit types.

Data collected from each of the sites was pooled for analytical purposes as it was the overall operation of traps we were interested in in terms of 1) evaporation rates 2) invertebrate capture rates 3) overall differences in species composition 4) level of morphological preservation and 5) any broad bias towards vertebrate captures. We did not undertake molecular analysis from this trial as we believe the analysis undertaken after the Kalbarri work was adequate for an initial assessment of oil over ethanol as a fixative.

Figure 9: Pit trap with weather cover

3.5 Results for field trial on northern Swan Coastal Plain

3.5.1 Comparison of invertebrate captures between different chemistries

For this trial there were a total of 3019 invertebrate captures from 53 families, at least 107 genera and more than 240 species. The oil and ethanol traps were responsible for 1559 captures (150 species), the ethylene glycol 2086 captures (146 species) with only 189 individuals (64 species) in the dry traps. Of the captures in the oil and ethanol traps only 670 individuals or 43% had migrated through to the ethanol with the rest remaining in the oil at the interface with the ethanol. Despite the less than complete transferal to ethanol these captures was a significant numerical improvement from those during the Kalbarri trial.

3.5.2 Comparison of invertebrate assemblages between different chemistries

Utilising the same Bray-Curtis dissimilarity measure with the same square root transformation as the previous trial, the oil/ethanol and ethylene glycol had assemblages

with a similarity of 59% while the dry pits with ethylene glycol and oil/ethanol had similarities of only 29% and 30% respectively (Figure 10). These data are comparatively similar to those of the previous Kalbarri trial with close agreement between the assemblage similarities for each of the trap types. This further re-enforces that resolved invertebrate community structures are influenced by the chemicals used in the traps.

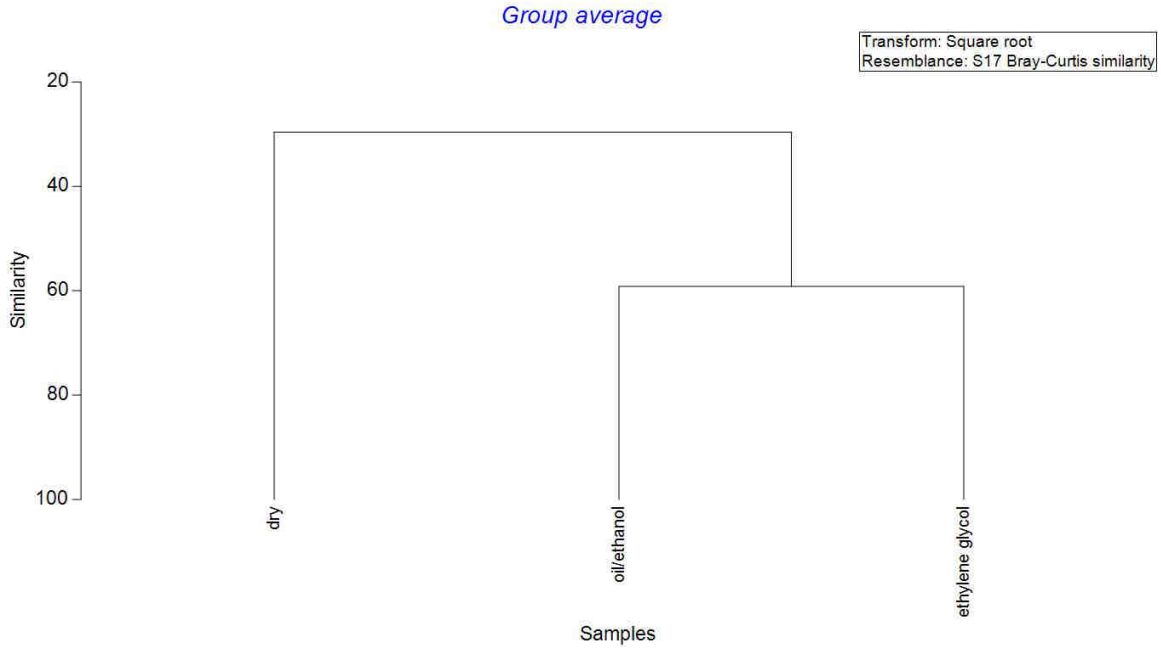

Figure 10: Similarity of samples from all treatments combined in banksia woodland on the Swan Coastal Plain. Measure used was Bray Curtis on square root transformed abundance data.

3.5.3 Vertebrate captures

There were 16 lizards 2 frogs and 1 snake caught in these traps giving a vertebrate capture rate of 2.1 individuals / 100 trap nights, which is less than that of 2.7 for the previous trial at Kalbarri, although the captures here were dominated by lizards rather than frogs. In terms of individual trap type captures oil/ethanol traps were responsible for five lizards (2 individuals / 100 traps nights), ethylene glycol for nine lizards and one frog (3.3 individuals / 100 traps nights) and dry traps had two lizards, one snake and one frog (1.7 individuals / 100 trap nights). While the ethylene glycol had more captures than either of the other trap types it was again not statistically significant.

The 11 cameras that were placed across the trap types (four each on the oil/ethanol and ethylene Glycol traps and three on the dry traps) detected five skinks, three dragons, one

monitor, two echidnas and a fox. The number of detections was split across all trap types and the only animals that briefly stopped to examine the traps were two skinks and the fox (Figure 11a and 11 b), the remainder past traps but appeared to have no interest. None of the animals caught on camera ended up caught in the traps, or at least the traps the cameras were focused on.

Figure 11a: A fox briefly investigating an ethylene glycol trap

Figure 11b: A skink investigating an oil/ethanol trap

3.5.4 Morphological preservation

A morphological examination of specimens caught in the oil traps indicated that for material that transferred through to the ethanol it preserved well with no obvious signs of deterioration or fungal growth. However the results were more variable for material caught in the oil layer with some larger soft bodied animals not preserving particularly well and showing signs of decomposition and deterioration. This was particularly the case for moths and butterflies, although these groups do not generally preserve well in any fluid, including ethylene glycol. Most small invertebrates did not transition through to ethanol and therefore these groups are probably the most vulnerable to degradation in this type of chemistry. Some fungal growth was identified on some individuals caught in the oil layer and the level of growth may be influenced by the length of time in the trap as well as proximity to other captures that also have fungal growth. There was no particular pattern to which species caught in the oil layer developed fungal growth.

3.5.5 Chemical stability under field conditions

Evaporation rates of the oil and ethanol were slightly higher for this trial than they had been for the Kalbarri trial however this was not unexpected as temperatures ranged from a mean maximum of 22.3ºC down to a mean minimum of 11.8ºC . There were four days in which it rained totalling 19mm however as we had installed corrugated roofs over each of the traps this did not contribute to an increase in volume for any of the traps. The average volume of ethanol lost from the traps was more than 465ml.

4.0 Summary of oil based overlays on ethanol

While the initial lab trials for using an overlay over ethanol showed some promise, in actual field application, where we could find solutions to fulfil most of our objectives, there were always one or two of the objectives that couldn't be met. For example oils that had the greatest moderation of ethanol evaporation could only be used with low concentrations of ethanol. This was less than optimal as a molecular fixative and was unlikely to be as efficient for rapid euthanizing of vertebrate bycatch due to higher

densities. The surface tension at the interface between the ethanol and the oil also had significant limitations by restricting the proportion of captures sinking through into the ethanol. Where we were able to increase transferal of captures through to the ethanol by replacing oils with WDLube we encountered issues of low capture rates of invertebrates and the captures were stained brown and covered with an oil film that made them difficult to work with. The collected material was assessed as unsuitable to contribute to curated collections such as those of Museums. The aromatic nature of WDLube was also a problem during handling. The overlay that showed the most promise was a mixture of lamp and paraffin oil (ratio of 75:25 respectively) on an 85% solution of ethanol. This met targets in terms of ensuring rapid submersion of vertebrate bycatch and this concentration of ethanol provides reasonable DNA preservation over the short term. The capture rates of invertebrates did not differ markedly from those of ethylene glycol although species composition was somewhat divergent with approximately 60% similarity in the pooled captures from both field trials. However the primary drawback that remained was the lack of transferal of target organisms into the ethanol with only 43% making the transition. Fortification with surfactants made no difference and while increased temperatures appeared to improve the transferal by lowering specific gravity, and perhaps surface tension at the interface between the oils and ethanol, this was not to a level suitable for survey with subsequent preservation and storage of captures.

A summary of findings from both laboratory trials and field trials are documented in point form below for ease of reference.

- Compounds designed to euthanize through transdermal absorption are not considered an effective method for vertebrate bycatch because animals will almost invariably drown prior to any affect from the chemicals which are too slow to act.
- While drowning is not viewed as an ethical demise for accidental captures of vertebrates in wet pit traps, ensuring that animals do not have a prolonged death is essential. The only way we can see to achieve a quicker death is to reduce the capacity for animals to remain at the surface of a wet pit. Lowering specific gravity of solutions well below that of vertebrate bycatch limits their capacity to remain at the surface and is the most viable solution to ensure rapid euthanasia.

- Quality of preservation of morphological characteristics, for both invertebrate and vertebrate, from within ethanol at concentrations of up to 90% were of a high standard in the short term (less than 8 weeks at 37°C).
- The quality of DNA that could be extracted from target organisms increased with higher concentrations of ethanol although it was still possible to extract high molecular weight DNA from invertebrates preserved in 60% ethanol over a 12 week period in laboratory trials. The 85% concentration used in the field provided high molecular weight DNA from a range of samples derived from invertebrates and vertebrates.
- Overlays of oils can mitigate evaporation of ethanol, although the higher the concentration of ethanol the more volatile the oils are that can float on the surface and the less they mitigate evaporation.
- The boundary layer formed between oil and ethanol has a surface tension that is restrictive to a high percentage of invertebrates transitioning through into the ethanol layer and material caught at the boundary layer is prone to degradation through fungal attack and rotting, particularly for soft bodied organisms.
- All vertebrate captures readily passed through a boundary layer into the ethanol
- Surfactants trialled had no effect in reducing the surface tension and allowing a greater proportion of invertebrates through to the ethanol.
- Ethanol still remains the preferred preservative because of it molecular and morphological fixative properties, as well as its low specific gravity.

5.0 Ethanol and Additive Trial

Despite the lack of success in utilising an overlay with ethanol, ethanol still remained the preferred wet pit solution as it fulfilled more of the criteria we required than any other identified solutions. Mitigation of evaporation, while not interfering or detrimentally affecting preservative properties, continued to pose the primary problem. Thus our focus switched to an examination of whether an additive, along with modifications to trap design, could reduce evaporation to a level that would enable trap deployment over extended time frames of several weeks or longer.

Options for additives were limited to liquids that were miscible with ethanol and would not significantly compromise any of the primary criteria for preservation, density, or safety. Subsequently tests were restricted to the addition of propylene glycol (and water) as other than its density it was the next most viable solution to ethanol with recognised preservation qualities for morphology and DNA (Boase & Waller, 1992) and the additional advantage of being resistant to evaporation under normal climatic conditions.

5.1 Laboratory Tests

To ascertain effects on evaporation a number of trial solutions were prepared and evaporated under controlled laboratory conditions to assess how long it may be possible to deploy the given liquids. Additionally, tests were also conducted with pure (>99%) ethanol, to allow a basis of comparison for the evaporation rates of each of the solutions trialled. These solutions were either evaporated to dryness or until all of the more volatile components (primarily the ethanol) had been evaporated. The test solutions were:

- 70 % Ethanol with 30% propylene glycol by volume (0.86 kg/l)
- 70 % Ethanol with 30% water by volume (0.85 kg/l)
- 60 % Ethanol with 20% propylene glycol and 20% water by volume (0.88 kg/l)
- Ethanol (>99%) (0.789 kg/l)

Initial trials were all undertaken in 1L beakers to ensure that there were no differences in evaporation that maybe attributable to vessel geometry. Each test solution was prepared, weighed and placed in an incubator set to a constant temperature of 50^{o}C. Each solution was then removed and weighed at regular intervals, such that the evaporation rate by mass could be monitored.

The results of these trials are shown in Figure 12, where the cumulative mass loss (evaporation) is shown for each liquid over time. It can clearly be seen from comparison to the linear fit that the presence of another liquid in the ethanol will cause a decrease in evaporation rate. It is also clear that the solutions containing propylene glycol will not

evaporate to dryness, indicating that if used in the field specimens collected would not dry out/decompose due to the complete evaporation of the ethanol.

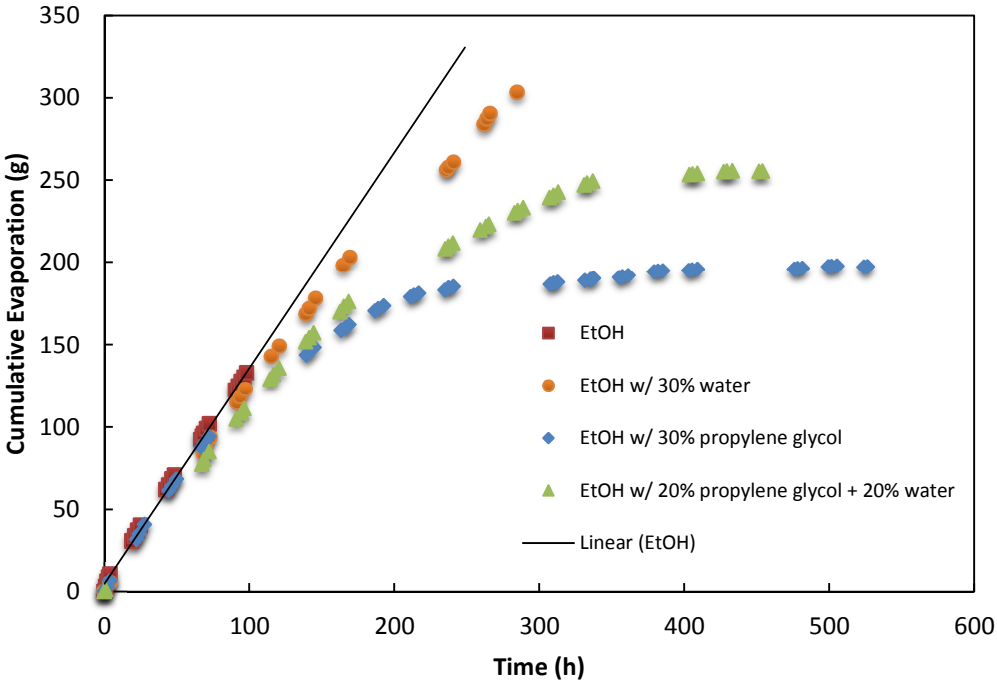

Figure 12: Cumulative mass loss of the trial solutions over time

Given the promising results with the trial solutions the experiment was repeated utilising 1L plastic containers typical of those often used for invertebrate wet pit surveys. The dimensions of these were approximately 160mm in height with a diameter of 80mm. The results are shown in Figure 13.

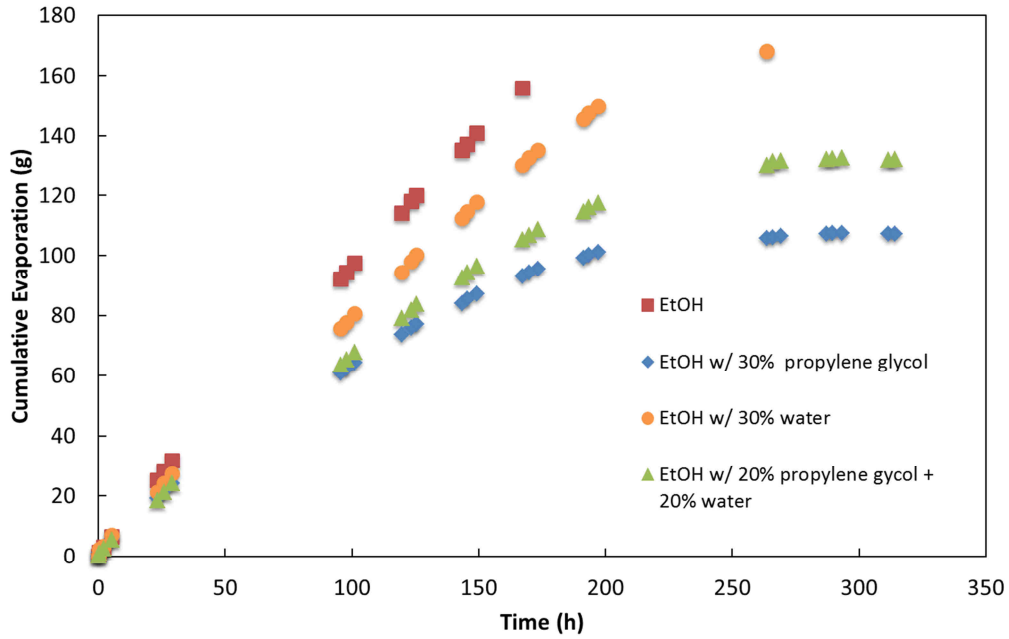

Figure 13: Cumulative mass loss over of the trial solutions in the traps over time

The results in Figure 13 show a similar pattern to those in Figure 12, the differing residual values of propylene glycol/water in the two cases was due to different initial volumes in the traps. The comparison of ethanol evaporation rates in the cases using only ethanol reveals a lower evaporation rate in the traps than in the beakers (~ 0.9 g/h for the traps vs. ~ 1.3 g/h for the beakers) indicating that the trap aperture probably has some effect.

While the two cases utilising the propylene glycol, show a residual volume of solution that does not evaporate and may act to keep trapped specimens preserved, there would be an increase in bulk density of the solution as the more ethanol evaporates. Therefore after a time, the solution would no longer meet the density requirement. Based on the results shown in Figure 12 it was determined that the propylene glycol containing solutions would no longer meet the density requirement after ~ 150 hours (6.25 days). However, it should be possible to predict the amount of time a larger volume of solution would last and it is estimated that solutions containing 70% ethanol would maintain the density requirement until at least 50% of the ethanol has evaporated. The ethanol evaporation rate from each solution in these laboratory trials was assumed constant and was predicted based on a linear fit to the initial part of the dataset. There is therefore a tendency to over-predict the evaporation rate and hence the traps could potentially be used longer than predicted.

An alternative trap design was also trialled, which allowed a higher volume of liquid while having a slightly smaller aperture than that of a typical trap. This was essentially a container with a tube attached to it (see Figure 15), and can be seen to give improved performance. The trials using the alternative trap design are shown in Figure 14.

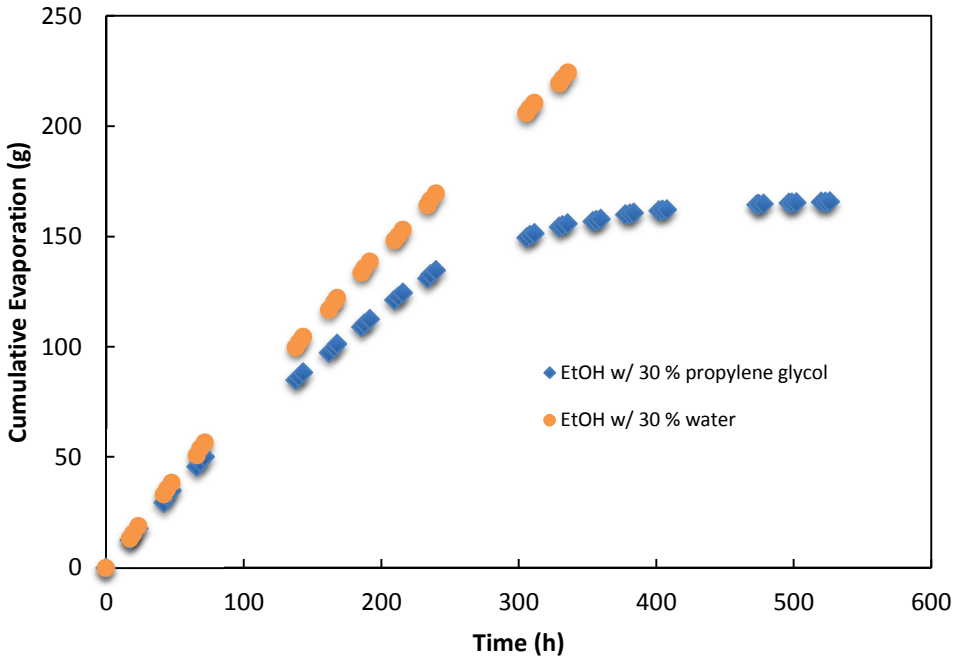

Figure 14: cumulative mass losses over time for the alternate trap design, both 30% propylene glycol and 30% water solutions were trialled.

Figure 15: The alternate trap design

Figure 16 shows a comparison of the performance of the existing and the modified traps, using 500 mL of each solution. The mass loss shown is only the amount of ethanol that would need to be evaporated before the traps no longer met the density requirement, based on the previously stated assumption. Hence all plots are linear. It can be seen that even with a relatively low liquid volume trap performance is improved.

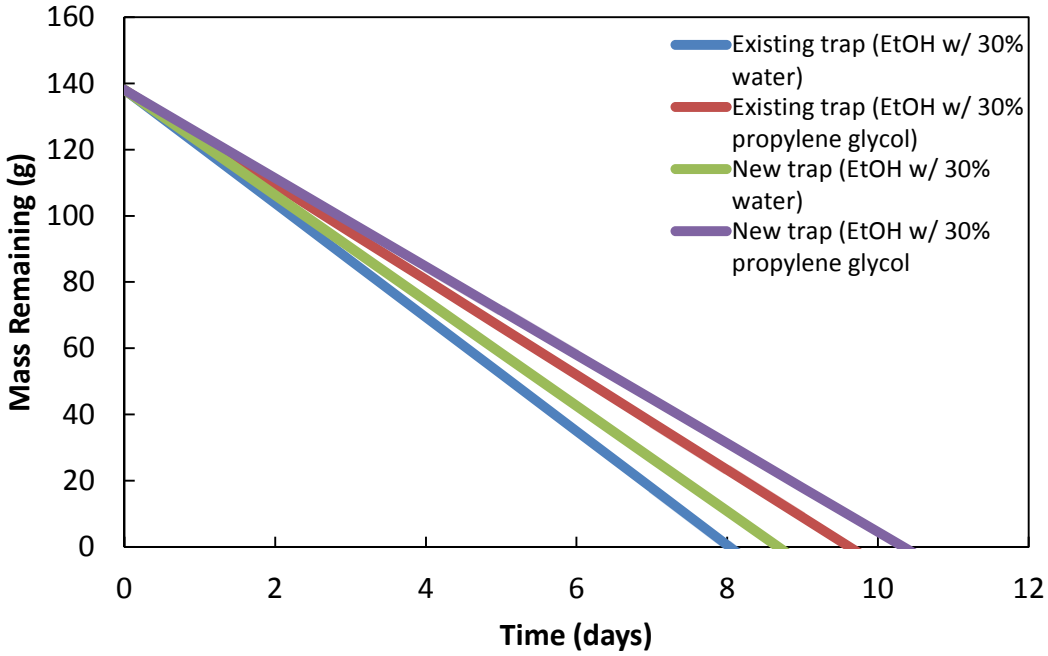

Figure 16: Performance of the exiting trap and the new trap with 2 different solutions

The trials were repeated with temperature fluctuation as this was a limitation in the original test method used as it utilised a fixed temperature. Any traps deployed in the field would be subject to daily temperature fluctuations. In certain regions (e.g. the Pilbara) there is also the possibility of day time temperatures exceeding 50^{o}C, particularly at ground level, as well as night time temperatures that are considerably lower.

To account for this the incubator was attached to a timer, so that it only operated during the day. The incubator was set to 65^{o}C, during the day but would shut off over night, allowing the temperature to decrease and thus more closely resembling normal day/night temperature cycles.

To allow comparison between the alternate trap design and the original traps, both were used. The liquids used were the same as used for the tests shown in Figure 16, though in this test only three traps were used rather than four.

The results of the temperature variation trial are shown in Figure 17. The difference between the ethanol-water mixtures and ethanol-propylene glycol mixture can be clearly seen where the blue markers, defining the remaining mass of the ethanol-propylene glycol mixture, plateau at around 300 hours with around 150g of fluid remaining, whereas at the other ethanol/water traps are already dry. The key advantage of the ethanol-propylene glycol mixture is therefore that the propylene glycol component won't completely evaporate which would prevent any collected specimens from drying out or decomposing.

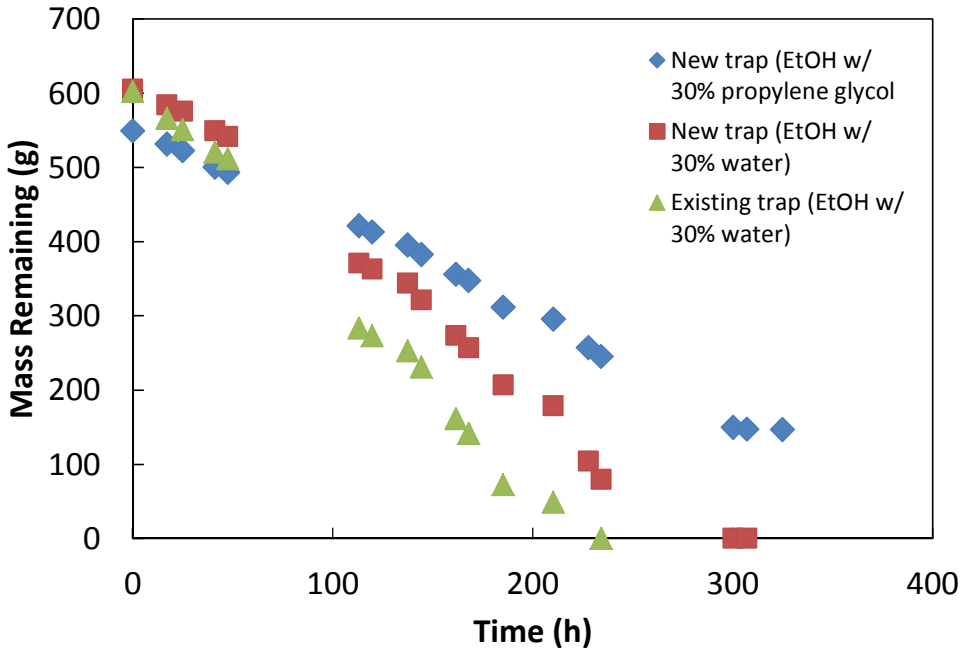

Figure 17: Mass remaining in the traps during the varying temperature trials

5.1.2 DNA preservation and fixation

In order to assess the DNA preservation quality of the tested solutions, cockroach specimens were added to and left in the solution as they evaporated in the incubator. They were introduced at the start of the tests and removed before the trap containing the

ethanol-water evaporated altogether, a total period of 8 days. The specimens were then transferred into 100% ethanol for storage prior to molecular analysis. This analysis indicated that DNA could be extracted from specimens "captured" in the ethanol-propylene glycol solution; however, this was of lower quality than the DNA extracted from specimens in ethanol alone.

Figure 18 shows the bottom of one of the traps (ethanol w/ 30% propylene glycol). The cockroaches were introduced individually to assess the effect of liquid density. Two of the specimens sank immediately to the bottom of the trap upon introduction and stopped moving. The largest cockroach sank about halfway and moved around for a few seconds before becoming stationary. The large cockroach remained floating mid-solution until the specimens were removed 8 days later.

Figure 18: Cockroach "test subjects" were introduced to the trap containing 70% ethanol and 30% propylene glycol

In terms of the quantity of DNA recovered from the cockroach specimens, 100% ethanol gave the best yields followed by 70% ethanol. The samples from 70% ethanol/30% PG had considerably less recoverable DNA (~1/5 that of the ethanol samples) although when run on a gel still appeared to be good quality. The samples preserved in 60% ethanol / 20% H20 / 20% PG however had very low yields and when run on a gel appeared to be of low quality. Each of the samples from the differing preservatives was then subjected to a PCR test using the general invertebrate barcoding primers, and surprisingly all samples amplified to a level where they could be sent for sequencing if required-a desirable objective for much of the DNA work that might be undertaken for survey collected material. However, the barcoding primers are very general and amplify

DNA quite readily whereas the use of more specialised primers could make it more difficult to use the DNA from the PG preserved specimens. In summary, for this trial, and for specimens to be most useful for a range of DNA applications, 70-100% ethanol is preferred, but if propylene glycol is used then 70% ethanol/30% propylene glycol is likely to give better results than using the 60% ethanol /20% propylene glycol/20% water. What remains unclear from this trial is how well the DNA would be preserved over longer durations with greater temperature extremes and variability.

5.2 Field trials

In order to ensure the results from the laboratory tests were applicable in the field, a set of trials were conducted at Dryandra, an open woodland reserve in the Wheatbelt region of Western Australia, in March 2014. These were important as there were two principle limitations to the laboratory tests. The first being the lack of atmospheric conditions (e.g. direct sunlight, wind, humidity) and the second the possibility that the air in the incubator may become saturated with vapour, thereby reducing the amount of liquid lost from the traps. A variation to the laboratory trials was the use of 80% ethanol / 20% propylene glycol rather than the 70% / 30% used in the laboratory trials as higher concentrations of ethanol had at least two desirable aspects. Firstly, from our lab trials it appeared that higher concentrations of ethanol gave better DNA fixation and this would likely be important under the more variable climatic conditions in the field. Secondly, higher concentrations of ethanol keep the fluids overall density as low as possible for the longest duration, an important attribute for rapid euthanasia.

As the traps used in the field trials were installed in the ground it was impractical to remove and weigh the remaining fluid at regular intervals so instead a liquid depth measurement was taken as a measure of evaporation. This could later be expressed as a mass loss, based on trap geometry and liquid density.

As well as a mechanism of validating laboratory results the field trials also provided an opportunity to test two differing trap geometries as well as the use of shading above the traps in an attempt to further limit evaporation. The two tested trap geometries were a standard 2L plastic container with an 80mm aperture and a 5L bucket with an extended narrow 70mm aperture.

The modified trap geometry has a number of advantages over the standard trap type which include greater fluid volume, lower aperture to surface area ratio and, as a direct consequence of the height of the neck and therefore the depth below soil surface that fluid is held, a reduction in temperature and therefore volatility. It is envisaged that all of these aspects would enable deployment over longer durations than those of more conventional unshaded traps.

5.2.1 Evaporation in standard pits under field conditions

For this trial five standard two litre plastic pits were placed in the open and buried with their apertures flush to the ground surface. Each was filled with approximately 1300 g of 80% ethanol and 20 % propylene glycol, equivalent to one and a half litres of fluid. Depth measurements of the fluid were taken at least daily over a nine day period. The values were converted to remaining mass of fluid over time and are presented in Figure 19.

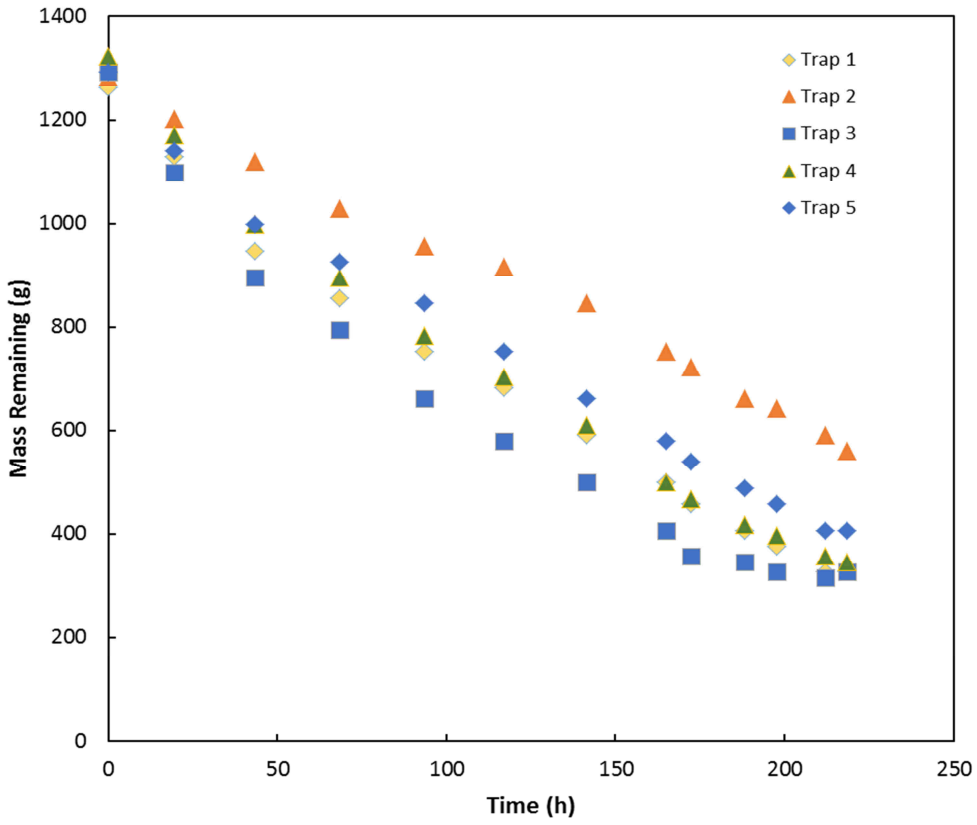

Figure 19: Results for field trials for standard traps

Evaporation rates were markedly higher than those observed during laboratory trials with an average hourly loss of 4.5 ± 0.8 g/h compared with 1.94 ± 0.09 g/h, a 2.3 fold increase! This is likely a direct result of the exposed environment in which heat from direct exposure to the sun along with constant airflow across the trap surface maximise evaporation. What is also evident from Figure 19 is the high degree of variation in evaporation between traps highlighting also the effects of environmental conditions at very fine scales. With evaporation at this average rate and with the initial mass/volume of fluid used in this trial, traps would have lost 50% of the fluid after approximately six days of operation.

5.2.2 Effects of shading traps

The field trials also explored the possibility of using shaded traps as a means to further reduce evaporation. These trials were conducted using a relatively small trap just to compare evaporation rates with and without shade. Six traps were used and all were buried to ground level with their apertures exposed to the environment (Figure 20). Small plywood square was placed approximately 10cm above each of three traps and each was filled with 600g of 80% ethanol and 20 % propylene glycol, equivalent to 750mm of fluid. As with the standard pits, depth of fluid was measured daily and remaining fluid mass calculated. The results from this are shown in Figure 21.

Figure 20: One of the test sites at Dryandra, with 3 shaded and 3 unshaded traps

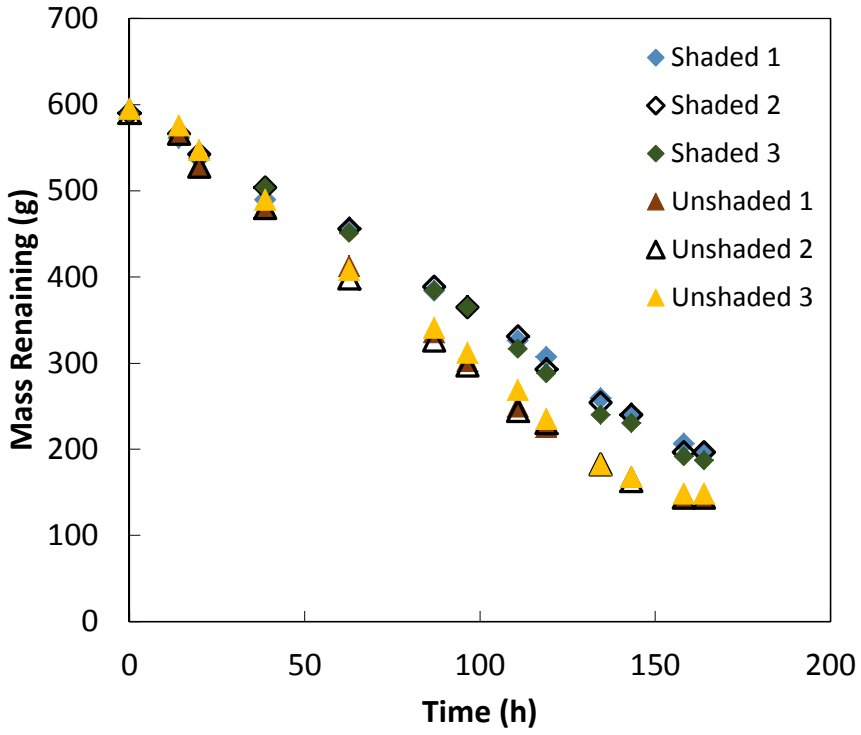

Figure 21: Results for field trials using shaded and unshaded traps

There was a small but clear reduction in evaporation rate for all traps when shade was provided. The average evaporation rates for the shaded and unshaded traps were 2.49 ± 0.06 g/h and 2.92 ± 0.02 g/h, respectively, a difference in this case of approximately 0.43 g/h or just over 10g per day. Measurements taken at the site give an indication of how shading moderates temperature. The bare ground had a mean daily maximum of 62ºC while the shaded sites were 48 ºC. This translated into in to fluid temperature differential of approximately 5ºC with unshaded traps having a mean temperature of 33ºC and those with shade 28 ºC. Subsequently while in this instance the variation was small it may nevertheless be useful, particularly under extremes of high temperature and over extended time frames.

5.2.3 Evaporation rates of a modified pit

While laboratory trials indicated that a modified trap design with a large volume and relatively small aperture improved trap performance it was necessary to test this in the field. This was particularly important as from the field trials of standard traps we had

already identified significant variation between results obtained in the laboratory and the field, with evaporation rates considerably higher in the latter. This trial consisted of the deployment of three modified traps which were five litre plastic buckets (diameter =200mm with a PVC tube of aperture 70mm and length 130mm inserted through the lid extending above the lip of the bucket by approximately 60mm. Differences between the standard and modified trap along with the placement of the PVC aperture are shown in Figures 22a and 22 b.

Figure 22a: (top) showing the difference in dimensions between a standard trap (right) and modified traps (left) and Figure 22b: (bottom) showing the position of the PVC aperture tube in the lid for the modified trap.

Each of the traps was again buried so the aperture was flush with the ground and for these traps they were left in position for a total of 53 days. Each trap was filled with 3350g of 80% ethanol and 20 % propylene glycol, equivalent to four litres of fluid. As

before, depth of fluid was measured at regular intervals for the first 12 days and then intermittently when opportunity arose over the ensuing 41 days. Measurements were also again converted to mass for comparative purposes and a graph of the results is presented in Figure 23.

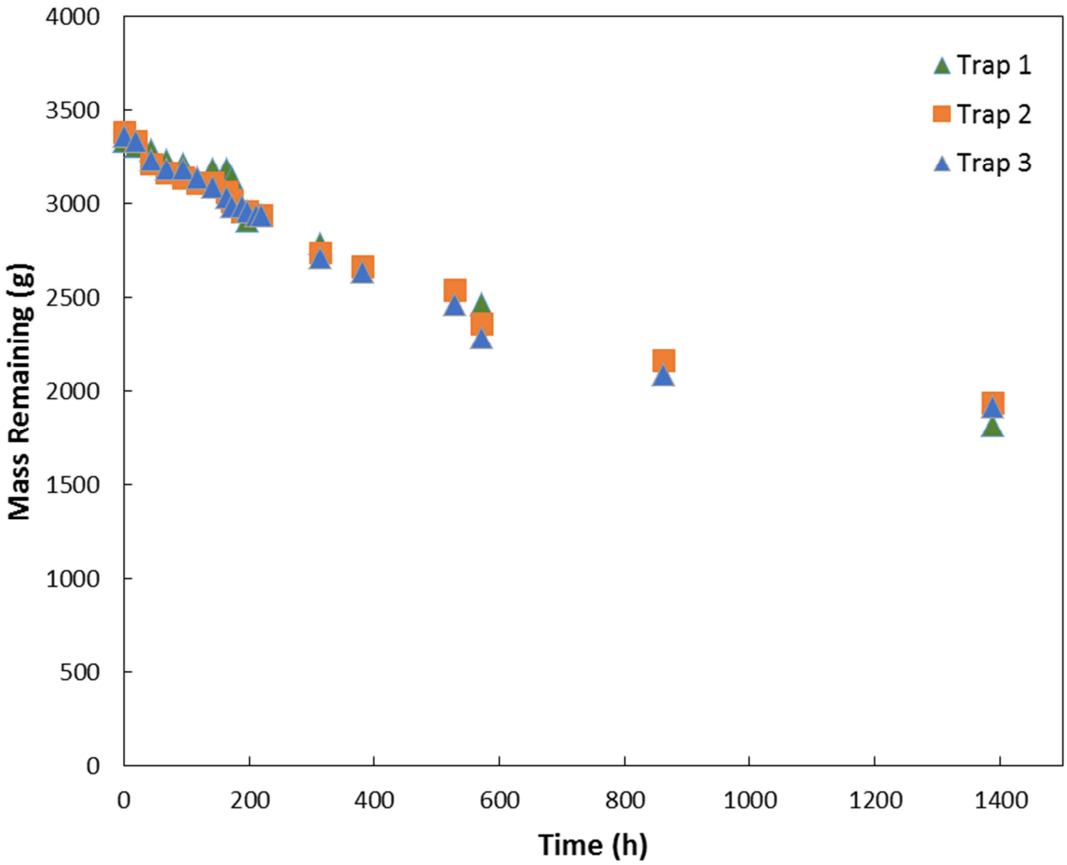

Figure 23: Results of trials with modified traps

The three plots in Figure 23 show that the larger trap with a modified aperture could be deployed for over 50 days (1400 hours) without running out of fluid. The mass loss of fluid over the first 24 days (576 hours), which was almost linear, was 1.72 ± 0.18 g/h. At the end of 24 days the concentration by volume was 70.1% ethanol with a density of 0.86 kg/l. This rate was not only a significant improvement from that observed from the field trials of standard traps, with less than half of the evaporation rate, but was also a slight improvement on anything we had managed in the laboratory trials. Over the following 12 days (288 hours) the rate of evaporation was 0.89 ± 0.35 g/h with the fluid after 36 days now 67% ethanol by volume with a density 0f 0.87 kg/l. At the end of trial after 53 days over 1600ml of fluid remained (40% of the original volume) and this was 50% ethanol by volume with a density of 0.91 kg/l. for the modified traps The

evaporation rate was not an overall reduction but results from the non-volatile and denser propylene glycol making up an increased proportion of the remaining fluid.

To retain a low fluid density and adequate preservation characteristics in an operational context it would be desirable to maintain a mass of not less than 2400g as this would be equivalent to approximately 70% ethanol and 30% propylene glycol and a density of around 0.86 kg/l. Although this concentration is at the lower end of the desirable DNA fixation concentration for material, most material would have initially been subjected to higher concentrations of ethanol. This would still enable a deployment of approximately 25 days under equivalent conditions to those used for this study which were typical of summer in southern Western Australian latitudes.

5.2.4 An assessment of DNA preservation from field caught material

A number of specimens (including reptile, mammal, and arachnid) were collected as part of the invertebrate wet pit trial at Dryandra and assessed for use in molecular barcoding applications for species identification (http://www.barcodeoflife.org/). Preservative types included 100% ethanol (standard preservation technique, used as a control), 100% ethylene glycol, and the trial solution containing 80% ethanol and 20% propylene glycol. Individuals used in this molecular study and their preservation method are listed in Table 6.

Table 6: Specimens and preservative types used in the study.

Number	Specimen	Preservative type
1	*Lerista distinguenda*	100% ethanol (collected November 2014)
2	*Ctenotus buchanani*	100% ethanol (collected November 2014)
3	*Ctenotus schomburgkii*	100% ethylene glycol
4	*Lerista distinguenda*	ethanol 80% propylene glycol 20%
5	*Ctenotus buchanani*	ethanol 80% propylene glycol 20%
6	*Lerista distinguenda*	ethanol 80% propylene glycol 20%
7	Spider	ethanol 80% propylene glycol 20%
8	*Lerista distinguenda*	ethanol 80% propylene glycol 20%
9	Scorpion	ethanol 80% propylene glycol 20%
10	*Mus musculus*	ethanol 80% propylene glycol 20%
11	*Lerista distinguenda*	100% ethylene glycol
12	*Mus musculus*	100% ethylene glycol

DNA was extracted from the tail tip in reptiles, an ear notch in mammals, and a leg for the arachnids by using a standard ‘Salting-out’ technique (http://www.liv.ac.uk/~kempsj /IsolationofDNA.pdf) with some modifications. The concentration of DNA was

recorded using a Qubit 2.0 Fluorometer and DNA product was also checked by using agarose gel electrophoresis with results viewed on an imaging machine (Figure 24). The DNA extracted from specimens was variable in quality and quantity. As with our earlier DNA analysis, and not unexpectedly, the highest quality DNA (high molecular weight) came from material preserved immediately in 100% ethanol as controls (Lanes 1 and 2). DNA samples extracted from the two arachnids had the lowest quality and quantity (Lanes 7 and 9) while the remaining samples all contained DNA and showed no strong differentiation between preservation in ethylene glycol or the mixed solution of 80% ethanol and 20% propylene glycol. For comparative purposes lanes 1, 4, 6, 8 and 11 are all from the reptile *Lerista distinguenda* and span the three treatments with lane 1 100% ethanol, lanes 4, 6 and 8 ethanol 80% propylene glycol 20% and lane 11 100% ethylene glycol.

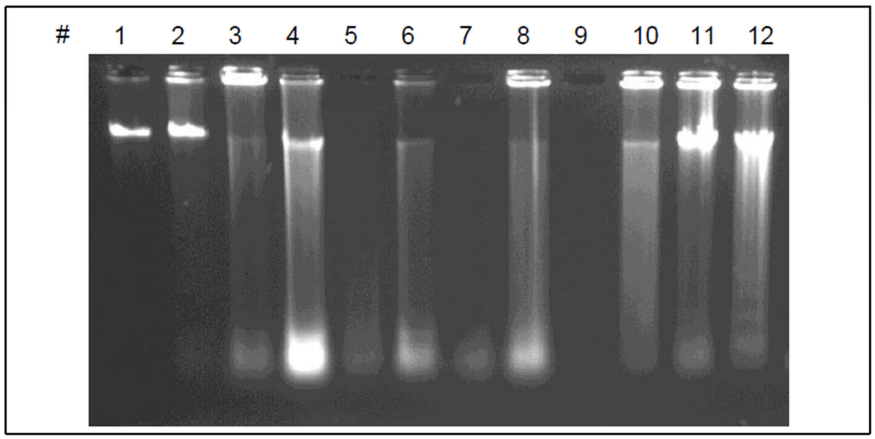

Figure 24: Image of agarose gel plate showing quality of DNA product and where lane numbers correspond to sample number in Table 6.

Polymerase chain reaction (PCR) was used to amplify the standard barcoding gene Cytochrome Oxidase Subunit 1 (CO1) mitochondrial gene using the LCO1490 (forward) and HCO2198 (reverse) primers for all reptiles and arachnids while the COILWW26F (forward) and HCO2198 (reverse) primers were used for the mammals. The PCR product was also checked by using agarose gel electrophoresis and the results viewed on an imaging machine (Figure 25). Despite variation in template quality all samples were successful in the amplification of the CO1 gene.

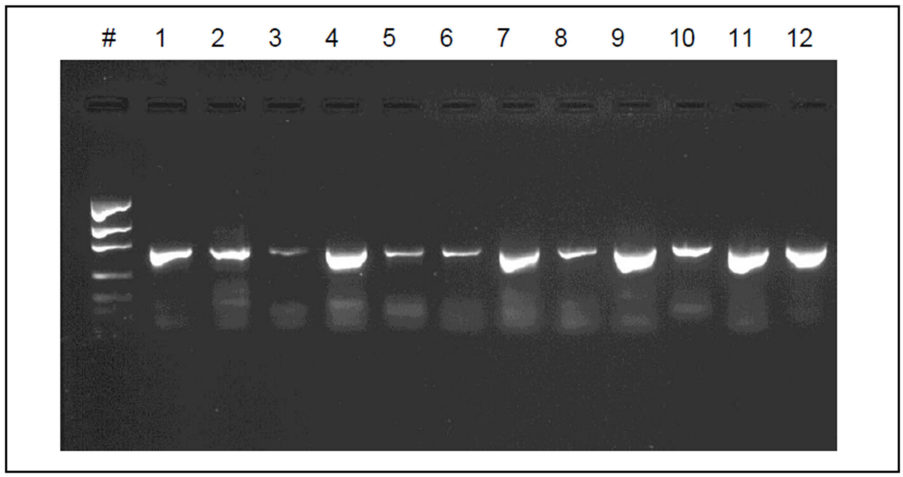

Figure 25: Image of agarose gel plate showing quality of PCR product and where lane numbers correspond to sample number in Table 6.

The PCR products were sent to the Australian Genome Research Facility (AGRF; Nedlands, W.A) for Sanger sequencing (single direction only, using the reverse primer HCO2198). Sequences were edited and trimmed manually and then aligned using the ClustalW function in the program *BioEdit* (http://www.mbio.ncsu.edu/bioedit/bioedit.html). They were then checked for compatibility within all taxonomic groups (Genbank online DNA database). Sequences appeared to match to at least Family level for the reptiles, Species level for the mammals, and at least Order level for the arachnids (lower order identification of specimens used was unknown), confirming correct barcode identification of the specimen. It was not possible to match to species level for the reptiles and arachnids due to an under-representation of Australian native fauna species in the database.

Therefore, for this trial, viable DNA material for use in standard genetic analysis such as DNA barcoding was obtained from specimens collected in each of the preservation types. However, further investigation would likely be necessary if more complex DNA analyses were required.

6.0 Conclusions and Final Recommendations

It was clear from laboratory trials that the addition of propylene glycol to ethanol would have a moderating effect on evaporation and that both temperature and trap geometry also have an influence on this. However, field trials demonstrated that laboratory

estimates significantly underestimated evaporation for standard aperture traps and would therefore overestimate the duration over which a trap might be deployed. This variation likely related to limitations of the laboratory tests in that, there were minimal temperature fluctuations, no direct sunlight, no wind and the possibility that the air in the incubator became saturated with ethanol vapour. The use of the modified trap gave a significant improvement on evaporation rates, and providing enough fluid is present within a trap, could allow deployment of up to 24 days or more. While volume or mass of fluid is fundamental, it is likely that the tube extension played an important role by minimising air flow over the surface of the fluid, allowing the fluid to remain deeper in the substrate and thus reducing overall temperature, and even by possibly maintaining ethanol saturation in the tube thus limiting further evaporation. In principle the primary aim for a trap should be to maintain the lowest aperture to volume/mass ratio while ensuring fluid is held at the greatest soil depth possible by using a tube between the pit and the soil surface. The difference in evaporation rate for the modified traps versus standard traps was more than just initial volume. While there was only 1.5 litres in the standard trap as opposed to 4 litres in the modified trap, multiplying the fluid for the standard trap by 2.6 would result in duration of 18 days before the solution had 66% ethanol volume remaining. For the modified trap it takes ~ 36 days before the concentration has reduced to 67% ethanol so in essence a small reduction in aperture (~10mm) and increased depth has almost halved the evaporation rate.

The longevity of deployment of traps can be further extended, if only marginally, by the use of a shading device that not only reduces temperature but disrupts air flow across the traps aperture. It is likely that the more surface area is shaded the greater the effect will be as the amount of both direct and radiant heat will be reduced from around the aperture.

Other technological modifications that could be explored include mechanisms for the automatic refilling of ethanol via the use of a constant flow cannula where precise evaporation is known. Preferably though, a system using a float valve would enable the regulation of ethanol concentration in a solution without the requirement to know precise evaporation rates. However these steps are only likely to be necessary for long duration deployments in areas that are not easily serviceable on a regular basis.

While all of our trials of invertebrates and vertebrate have supported the understanding that quality and quantity of DNA is superior when preserved in 100% ethanol (Quicke *et al.* 1999, King & Porter 2004), as opposed to the other solutions trialled, we have still been able to extract and amplify DNA to a level suitable for generalised barcoding, and in some cases potentially more sophisticated analysis. This is a significant improvement from the use of previous chemistries such as ethylene glycol and formalin and would appreciably value add to targeted fauna and to vertebrate bycatch. However, this is an area that warrants continued investigation under various operational conditions to ascertain limitations in duration and the effects of more extreme temperature.

The addition of propylene glycol to ethanol improves preservation of morphological characters for long terms storage of Museum specimens (Boase & Waller 1992, Carter 2003) and subsequently we see no negative issues with the addition of propylene glycol for preservation of morphological characters in the field. All specimens caught in the field with the ethanol/ propylene glycol solution were determined to be in better condition than those caught with the other chemistries trialled, or those from long term trapping utilising ethylene glycol/formalin. How well preserved material remains after extended time frames under harsh conditions again remains untested but it is reasonable to conclude that they will remain superior to those in ethylene glycol under the same conditions.

Throughout our field trials there was no significant variation to the numbers of vertebrate bycatch caught with any of the trialled chemistries when compared to that of the more conventional fluid of ethylene glycol. While it is preferable to have no vertebrate bycatch, we believe the chemistry we propose here will minimise distress to inadvertent captures while significantly increasing their scientific value. While the scope of our work was not on exclusion of vertebrate bycatch this is clearly another area that could and should receive further attention. If reductions can be managed without compromising the efficacy for target species or reducing their scientific value then the likelihood of the continued use of invertebrate wet pits for biogeographic survey and monitoring of invertebrate species/ communities is improved.

The main points and recommendations therefore are:

- A minimum starting concentration of 80% ethanol and 20% propylene glycol is recommended, although for short duration sampling this could be increased to upwards of 90% ethanol, although a concomitant increase in evaporation would result.
- Minimum levels for the solution should be ~ 70% ethanol and 30% propylene glycol as quality of DNA fixation diminishes with reduction in ethanol concentration and the density of the solution increases.
- The use of a narrow tube (~70 mm diameter) attached to a high volume container can give extended durations of trap deployment and with an initial four litres of 80% ethanol and 20% propylene glycol up to 35 days is achievable under warm to hot and dry temperatures without going below the minimum recommended fluid concentration.
- While identifying appropriate ways of euthanizing vertebrate bycatch is problematic, we feel that outside physical exclusion, which is extremely difficult in itself, we have the best result attainable through maintaining a solution well below a density of 1 kg/l in which animals should not be able to swim for extended periods. This is further improved by using large volumes of fluid in which most animals would be unable to stand or prop on the bottom of the trap. We would recommend keeping the fluid density below 0.9 kg/l and this would be achieved for ethanol/ propylene glycol solutions where the ethanol concentration remains above 55%.
- If the worst case scenario occurs where there has been complete evaporation of ethanol the original volume of propylene glycol would still remain ensuring that captured material did not dry out or desiccate, and depending on duration, may still have recoverable DNA.
- When material is collected from traps, samples for DNA extraction should be transferred to 100% ethanol and then kept as cold as possible therefore minimizing degradation of the DNA while long term anatomical preservation should be in 70% ethanol (Quicke *et al.* 1999).

7.0 Acknowledgments

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