An Effective Method for Terrestrial Arthropod Euthanasia

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Summary

As scientific understanding of invertebrate life increases so does the concern for how to end that life in a way that not only minimises (potential) suffering and is non-recoverable, but is also safe for those carrying out the procedure. There is increasing debate on the most appropriate euthanasia methods for invertebrates as their use in experimental research and zoological institutions grows. Their popularity as pet species has also lead to an increase in the need for greater veterinary understanding. Through the use of a local injection of potassium chloride (KCl) initially developed for use in American lobsters, this manuscript describes a safe and effective method for euthanasia in terrestrial invertebrates. Initial work focused on empirically determining the dose for cockroaches then extrapolating to other arthropod species. For this method of euthanasia, we propose the term *'targeted hyperkalosis'* to describe death through terminal depolarisation of the thoracic ganglia as a result of high potassium concentration.

Key Words

Arthropod, anaesthesia, euthanasia, potassium chloride (KCl), *'targeted hyperkalosis'*.

Introduction

A good death is an important part of a good life for all animals in captivity – and a lack of effective euthanasia methods for invertebrates has meant that the practice is not in tune with the theory. This gap in knowledge has gained significant attention recently (Cooper, 2011 and Murray, 2012), as moral concern grows over just what constitutes suffering in these animals. Suitable euthanasia methods need to be not only effective, irreversible and minimise pain, suffering and distress, they must be relatively simple to perform, acceptable to the person conducting the procedure (American Veterinary Medical Association (AVMA) guidelines, 2007), as well as compatible with any research involved.

Battison *et al.* (2000) published a new method of euthanasia for the American lobster (*Homarus americanus*) using injection of a saturated solution of potassium chloride (KCl). This method caused immediate immobilisation and death through circulatory arrest in around one minute and therefore fits the criteria for a euthanasia procedure. Currently anaesthesia followed by immersion in fixative such as 70% ethanol has been cited as a preferred method (Pizzi, 2012) but it is inappropriate for microbiology (Cooper, 2011) or RNA extraction. Freezing is often suggested by private and professional keepers, but this compromises histological examination and is increasingly regarded to be inhumane (Pizzi, 2012) without prior anaesthesia to a depth at which recovery is impossible before death from freezing.

This study extrapolates the work performed by Battison *et al.* (2000) to a range of terrestrial arthropod phyla. Our aim is to produce a universal method of arthropod euthanasia that is not only more effective than current methods, but is quicker, more economical and - importantly for transcriptomic and proteomic studies - preserves tissue quality.

Animal nervous systems contain ion channels permeable to potassium and sodium, which respectively control the flow of these ions across the cell membrane and subsequently the electrical potential. In the field of electrophysiology it is well know that neurones at a resting potential maintain a high intracellular potassium concentration and low intracellular sodium concentration relative to the extracellular environment. Addition of extracellular potassium ions (K^+) in the form of KCl immediately depolarises the cell through neutralisation of the potassium concentration gradient across the cell membrane. This resulting change in potential (voltage) across the cell membrane causes opening of voltage-gated sodium channels. Thus, excess extracellular KCl causes exaggerated sodium influx that depolarises the cell and becomes toxic to it. The excess K^+ remaining in the extracellular environment also prevents repolarisation (Takahashi *et al,* 1999). Therefore application of excess K^+ around the neurones of the thoracic ganglia in the form of KCl abolishes the neural input and results in circulatory collapse and then death.

Evolutionarily conserved across a wide range of arthropod orders, the arthropod ventral nerve cord – with some exceptions - is positioned on the ventral midline and exhibits a chain of serial ganglia. The anterior ganglia are the largest and have been referred to as the 'invertebrate brain' (Gullan and Cranston, 1998) but their main role is processing the large amount of sensory input from the head. Therefore destruction of the brain in invertebrates principally affects the input of sensory information from the eyes and antenna but not from the rest of the body. Consequently a method of euthanasia targeting the ganglia that control the vital functions would be a more appropriate way to successfully terminate arthropod life.

Humans are not the first species to use K^+ ions as pharmacological tools; scorpions have evolved to utilise K^+ ions. Parabuthus species possess potent venom peptides

that act on many ion channels (including K^+ channels). Yet they have also evolved a prevenom with a high concentration of K^+ ions of around 80 mM (Inceoglu *et al*. 2002). Inceoglu *et al*. (2002) studied the effect of this prevenom on two insect species (*Trichoplusia ni* and *Sarcophagia bullata*) and concluded it is an energy efficient way of prey capture through paralysis. We demonstrate that if K^+ is delivered directly to the thoracic ganglia, its effects are rapid and avoid generating potential nociceptive action potentials.

We hereby propose the term *'targeted hyperkalosis'* to describe the euthanasia of invertebrates through local injection of K^+ to deliberately depolarise the thoracic ganglia and bring about rapid death. The direct derivation of *'targeted hyperkalosis'* is local, high potassium state which accurately describes the method.

Materials and Methods

All animals used in this study were euthanised to provide tissue for other research projects and, as such, the development of a suitable method of euthanasia was required. Although the species involved are not currently legally protected, they were maintained under the ethos of the UK Animals Scientific Procedures Act 1986, (A(SP)A).

All animals were acclimatised for two weeks to confirm health and nutritional status, and were weighed during total anaesthesia.

Assessment of Proposed Injection Volume

Prior to any dosing studies, six giant cockroach nymphs (*Blaberus giganteus* (Linnaeus, 1758)) were anaesthetised in the manner described below and allowed to recover in fresh air to record a baseline recovery time.

Ten nymphs, average weight 3.25 g (+/- 1.03 g), were anaesthetised individually in a 670 ml anaesthesia chamber (Reed *et al.* 2011) which delivered carbon dioxide (CO_2) at 0.5 litres per minute through a low range flow meter (Harvard Apparatus, UK). Once anaesthetised, nymphs were abdominally injected with sterile Ringers solution at 100 ul/g body weight, allowed to recover, and then monitored for signs of unusual behaviour and / or illness. The time taken for full induction (i.e. abolishment of the righting reflex) and any movement were recorded along with that of the recovery time. The time to full recovery was defined as the return of the righting reflex. This was important as it enabled us to establish when recovery was expected.

Development of Euthanasia Method

Rather than performing a full dose response curve, three dose groups were selected to assess the initial efficacy of KCl as a method of euthanasia; this was to reduce the number of animals receiving a toxic but non fatal dose. As a suitable euthanasia protocol needs to be effective for every animal every time, standard toxicological parameters such as LD_{50} (lethal dose in 50% of population) and LD_{90} (lethal dose in 90% of population) were not deemed necessary for this study. KCl was dissolved at 300 mg/ml in sterile water, which avoids the need for an incubation step as conducted by Battison *et al.* (2000) and eliminates the risk of precipitation if the solution is kept at cooler temperatures. The solution was sterile filtered through a 0.2 µm filter (Merck Millipore, Watford, Hertfordshire, UK) and aliquoted for storage in 1.5 ml centrifuge tubes (Fisher Scientific, Loughborough, Leicestershire, UK).

B. giganteus were anaethetised as previously described and injected with 300 mg/ml KCl into the first leg sinus via the arthrodial membrane **(Fig. 1)** using a 25G needle and 1 ml syringe (Fisher Scientific, Loughborough, Leicestershire, UK). Doses

assessed were 10 µl/g (n=5 animals 2.72 g +/- 1.26 g), 50 µl/g (n=5 animals 2.92 g +/- 0.71 g) and 100 µl/g (n=10 animals 2.49 g +/- 0.42 g). Double the number of animals was used in the higher dose group to confirm 100% fatality rate.

Extrapolation of Euthanasia Method in Other Species

In order to determine the effectiveness of KCl euthanasia in different terrestrial invertebrates, we tested the method in eight other invertebrate species from eight orders **(table 1)**.

Injections for *G. bimaculatus*, and *L.migratoria* were administered in the first leg sinus via the arthrodial membrane. In *H. membranacea*, the injection was via the arthrodial membrane but in the second leg sinus.

Myriapoda species *S. polymorpha* and *N. americanus* were injected into the joint between the second and third segment along the ventral midline **(fig. 2)**. Phasmids were injected into the ventral midline at the junction between the first leg plate and adjacent ventral plate **(Fig. 3)**.

A. cordubensis and *H. troglodytes* were selected based on the requirement for their venom glands. *A. cordubensis* were dosed centrally via the sternum **(fig. 4)** while *H. troglodytes* were injected into the junction between the second leg coxa and the sternum, rostrally to the genital operculum **(fig. 5)**.

Results

A rising concentration of CO_2 was effective at reversibly anaesthetising, and therefore immobilising, all species used during this study. Individuals from several insect species (particularly *Blattodea* and *Phasmidae*) were observed to vomit during

anaesthesia and occasional exaggerated limb movements were noted. Little other evidence of distress was observed.

Assessment of Proposed Injection Volume

Initial baseline data for *B. giganteus* anaesthesia induction was 1min28s (+/- 6 seconds) for full anaesthesia with recovery taking 1min41s (+/- 13 seconds). After injection of 10% v/w of body weight of sterile Ringers solution whilst under anaesthesia, animals recovered in 1min46s (+/- 9 seconds). No unusual behaviour was noted in the 48 hours' observation time post injection and, subsequently, no further observations were taken. The *E. calcarata* nymph given 100 µl/g sterile Ringers solution into the ventral thoracic cavity recovered after six minutes and showed no detectable behavioural differences up to and beyond the 48 hours' observation time. This demonstrates that the large volume alone (10% v/w of body weight) does not cause any noticeable effect on survival or behaviour.

Battison *et al.* (2000) used ultrasonography to confirm circulatory arrest in *H. americanus*; in this study the authors relied upon irreversible cessation of movement and sensation which was defined as no movement or recovery during a 24 hour period. Doses of 1% and 5% v/w KCl in *B. giganteus* were non fatal; 5 % v/w caused marked local paralysis of forelimbs and antennae which persisted for over one minute; 10% v/w of KCl caused instant paralysis and inward contraction of the limbs as well as total abolishment of all nociceptive responses and non recovery within 24hrs. Therefore 10% v/w was identified as the 100% effective dose for follow on studies.

Results of Extrapolation of Euthanasia Method to Other Species

In *G. bimaculatus* 10% v/w was immediately effective in all but one individual. However, this was a suspected inaccurate dosing as for such small species a 33G needle is required to limit leakage and maintain accuracy. For *L. migratoria*, *H. membranacea* and *E. calcarata* 10% v/w KCl euthanasia was effective in all animals. The only unusual observation made was the forceful autotomisation of a rear leg by one *L. migratoria*.

The *Myriapoda* unique body plan presented a challenge as nerve ganglia and heart tissue are duplicated throughout its length; this most likely brought about the results observed. The *N. americanus* responded to 10% v/w KCl with a wave of paralysis moving anterior to posterior with a maximum latency to death of 13 seconds. Over half of the study group (four animals) were deemed dead before removal of the needle. The passage of paralysis anterior to posterior was slower in *S. polymorpha*, but after a single dose of 10% v/w KCl none of the study group had died within 10 minutes. Immediate paralysis of the anterior segments was evident in all animals while the posterior three segments remained active in all animals. A second dose of 10% v/w KCl was required and resulted in immediate cessation of all movement. As such, a 20% v/w KCl final dose was effective in all the *S. polymorpha* tested and was therefore proposed as the protocol for the *Chilopoda* class of *Arthropoda*.

The 10% v/w KCl dose in the *H. troglodytes* caused immediate paralysis and death in all animals signified by contraction of the limbs and ablation of responses to noxious stimuli (limb crush).

Initial studies on the *Theraphosidae* revealed that injection of 10% v/w into the central nerve ganglion was not possible due to volume limitations and a substantial back pressure was observed in the syringe. Although it was not possible at this time to record haemolymph pressure in the *Theraphosidae* prosoma - and the authors are

unaware of any articles on the subject - reducing the dose to 0.5% v/w and leaving the syringe in place for 10 seconds resolved the issue. Thus 0.5% v/w can be injected into the central sternum as this is technically easier than reaching the ganglia through an arthroidial membrane.

Injection of 0.5 % v/w administered centrally via the sternum was effective in ablating the nervous system and caused death in all animals. 0.5 % v/w injected into the prosoma ganglia is terminal and non recoverable in *Theraphosidae*; haemolymph can be collected via cardiac puncture but only for approximately one minute as this appears to be when the circulation stops. During development of this protocol the authors discovered that direct intra-cardiac delivery of 1% v/w KCl is also an effective euthanasia method for *Theraphosidae* spiders but it does not appear to work for araneomorpha spiders (data not shown). Thus the proposed spider protocol is 0.5% v/w delivered directly to the prosoma ganglia.

Other Species Tested

Representatives of other insect orders can also be euthanised with 10% v/w KCl administered directly via injection to the thoracic ganglia though the sternal membrane between the forelegs in the ventral midline. Adult species tested were the lesser wax moth, (*Achroia grisella* (Fabricius, 1794)*;* Lepidoptera), hover fly (unknown sp.; Diptera) and several beetles (*Pachnoda marginata* (Drury, 1773)), *Smaragdesthes africana oertzeni* (Kolbe, 1895) and *Dicronorrhina derbyana conradsi* (Kolbe, 1909)*;* Coleoptera). For euthanasia of the small insects, a 10 MICROLITER™ glass syringe (Hamilton Company, Reno, Nevada, USA) was used with a 25G needle.

The data presented here is summarised in **Table 2** as a list of proposed euthanasia protocols listed against order. For heavily armoured arthropods such as the crustaceans, the thoracic ganglia can be reached through the arthroidial membrane sinus (Battison *et al*. 2000). For terrestrial arthropods the ventral midline should be used where possible.

Discussion

The data presented here demonstrates that KCl is a rapid and effective method of causing irreversible death in the arthropod species tested. With training this method is easy to perform and causes immediate death that allows rapid tissue collection for experimental transcriptomic and proteomic studies. An understanding of the species' specific neural anatomy is critical to performing this technique. As such, it is important that this is understood prior to working with such animals.

Before obtaining vertebrates for scientific study, it is vital that staff can identify potential suffering and are proficient in carrying out euthanasia. This is detailed in the A(SP)A and we feel this approach should be adopted for invertebrates too. KCl is an ideal agent for euthanasia as it is cheap, effective, doesn't require any special storage, has a very long shelf life and is safe to use. KCl is compatible with a wider range of pathological and research investigations than any of the current protocols (such as transcriptomics and proteomics) with the exception of primary neuronal culture due to the nature of action. However, there are still situations where KCl is impractical such as for very small species (e.g. *Drosophila sp.*) or when culling large numbers of invertebrates, not required for tissue. In these instances anaesthesia followed by a confirmatory procedure such as immersion in fixative or rapid freezing should be

performed, but only as long as the anaesthesia is deep enough so death occurs before recovery is possible.

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Symbols / Abbreviations

A(SP)A: Animals Scientific Procedures Act 1986

AVMA: American Veterinary Medical Association,

CO_2: carbon dioxide

KCl: potassium chloride

LD_{50}: lethal dose in 50% of population

LD_{90}: lethal dose in 90% of population

v/w: volume by weight

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Appendix I: Figure Titles and legends

FIG. 1 BLATTODEA INJECTION SITE

This diagram demonstrates the location of the first arthodial membrane used for euthanasia by targeted hyperkalosis in cockroaches (*Blaberous giganteus,* order *Blattodea*).

FIG. 2 *CHILOPODA* INJECTION SITE

The location of the ventral midline injection site between segments two and three for centipedes (*Solopendra polymorpha*, order *Chilopoda*).

FIG. 3 *PHASMIDAE* INJECTION SITE

Ventral midline (*Eurycantha calcarata*, order *Phasmidae*) illustrated by the red arrow.

FIG. 4 *THERAPHOSIDAE* INJECTION SITE

Central sternum injection site in *Acanthoscurria cordubensis*, order Theraphosidae.

FIG. 5 SCORPION INJECTION SITE

Dosing site for *Hadogenes troglodytes*, order Scorpiones

Appendix II Table Titles and Footnotes

TABLE 1: KCl EUTHANASIA TEST SPECIES' DOSING TABLE

Species	Life stage	n=	(g)*	(g) s.d.**	Dosing
African field cricket (*Gryllus bimaculatus*, (De Geer, 1773))	Adult	6	0.97	+/-0.22	10 %v/w***
Migratory locust (*Locusta migratoria* (Linnaeus, 1758))	Adult	6	1.62	+/-0.29	10 %v/w
Giant Asian mantid (*Hierodula membranacea*, (Burmeister, 1838))	Nymph	5	0.39	+/-0.06	10 %v/w
Sonoran desert centipede (*Scolopendra polymorpha*, (Wood, 1861))	Juvenile	5	0.32	+/-0.2	20 %v/w
Florida millipede (*Narceus americanus*, (Palisot de Beauvois, 1817))	Juvenile	7	2.87	+/-0.81	10 %v/w
Giant spiny stick insect (*Eurycantha calcarata*, (Lucas, 1869))	Nymph	8	2.00	+/-0.89	10 %v/w
Acanthoscurria cordubensis (Thorell, 1894)	Sub-adult	10	18.68	+/-3.21	0.5 %v/w
Flat rock scorpion *(Hadogenes troglodytes* (Peters, 1861))	Juvenile	9	5.08	+/-2.59	10 %v/w

* average weight in grams

**standard deviation of average weight in grams

***volume by weight

TABLE 2 SUMMARY OF EUTHANASIA DOSE PROTOCOLS

Group	Common name	Injection site	Dose
Blattodea	Cockroaches	Between first pair of legs	10% v/w
Phasmidae	Stick insects	Between first pair of legs	10% v/w
Diplopoda	Millipedes	Between second and third segments	10% v/w
Chilopoda	Centipedes	Between second and third segments	20% v/w
Orthoptera	Grasshoppers	Between first pair of legs	10% v/w
Aranae	Spiders	Mid-sternum	0.5% v/w
Scorpiones	Scorpions	Between second pair of legs	10% v/w
Mantodea	Mantids	Between first pair of legs	10% v/w
Coleoptera	Beetles	Between first pair of legs	10% v/w
Diptera	Flies	Between first pair of legs	10% v/w