A comparison of anesthesia techniques for entomological experimentation: Longevity of the leaf-mining fly pest *Scaptomyza flava* Fallén (Drosophilidae)

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Historically, handling insects usually involved their being killed as early studies used these organisms for work on genetics, physiology and/or pesticide bioassays. With the advancement of these research topics, the development of behavioral studies and the sensitivity of culturally important, threatened or endangered species, approaches that focus on non-lethal preparation of the insects for experimentation have become important. Anesthesia has been used as one of these non-lethal approaches to provide researchers flexibility when designing experiments. Two common anesthetics used for insect experimentation are carbon dioxide and chilling. These anesthetics have been used frequently in the literature but their sub-lethal effects on insects are poorly studied. Another that has potential for experimental use is triethylamine (TEA). This chemical shows promise because of its ease of use and potency as an insect anesthetic, but evidence, if any, of the sub-lethal effects is almost non-existent in the literature. A series of experiments was carried out to find the optimal exposure times and/or concentrations for each of these three approaches. Once an optimal treatment was found for each approach, these were compared to each other in a subsequent experiment. It was found that TEA is a far superior anesthetic when recording/observing fly longevity.

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Short running title: A comparison of anesthesia techniques for entomological experimentation

Abstract

Historically, handling insects usually involved their being killed as early studies used these organisms for work on genetics, physiology and/or pesticide bioassays. With the advancement of these research topics, the development of behavioral studies and the sensitivity of culturally important, threatened or endangered species, approaches that focus on non-lethal preparation of the insects for experimentation have become important. Anesthesia has been used as one of these non-lethal approaches to provide researchers flexibility when designing experiments. Two common anesthetics used for insect experimentation are carbon dioxide and chilling. These anesthetics have been used frequently in the literature but their sub-lethal effects on insects are poorly studied. Another that has potential for experimental use is triethylamine (TEA). This chemical shows promise because of its ease of use and potency as an insect anesthetic, but evidence, if any, of the sub-lethal effects is almost non-existent in the literature. A series of experiments was carried out to find the optimal exposure times and/or concentrations for each of these three approaches. Once an optimal treatment was found for each approach, these were compared to each other in a subsequent experiment. It was found that TEA is a far superior anesthetic when recording/observing fly longevity.

Key words: Anesthesia, Chilling, CO_2, *Scaptomyza flava*, Triethlyamine

Introduction

Handling of insect specimens for taxonomy or genetics has historically involved preparing/storing them in liquids or in a dry state, such as pinning. However, for behavioral work, these methods are not appropriate, although in the latter case, the organisms may still have to be benignly anesthetized to facilitate aging, sexing and for setting up experiments. Also, the recent acceleration of genetic, physiological, and ecological research on invertebrates has necessitated the use of living organisms. Another important aspect is the research on culturally important species or those that are threatened or endangered. This usually requires live specimens so that they can subsequently be translocated and released. Anesthesia is an approach for all these cases and has been developed over the past few decades. Historically, experiments began in the 1920s to investigate possible techniques to render insects immobile without killing them (Willis, 1925). Many techniques have been developed since then, each with their own advantages and disadvantages (Wedberg & Clarke, 1947; Worthen & Moore, 1991; Ratterman, 2003; Chen & Hillyer, 2013). For instance, ether is an effective anesthetic but is harmful to the user, whereas chilling is benign to the user but has a shorter anesthetic effect (Barron, 2000; Ratterman, 2003). If the intention is to work on the behavior, ecological fitness or other aspects of the insect's biology, it becomes important to know how these approaches will affect the insect. Therefore, alternative techniques that do not harm the researcher or the insect are desired (Champion De Crespigny & Wedell, 2008; Cooper, 2011; Smith *et al.*, 2014). Three types of anesthesia have historically been used with varying success in the literature. These are carbon dioxide (CO_2), chilling and triethylamine (TEA). This paper compares these three approaches, because of the possibly unjustified popularity of both CO_2 and chilling, and the relatively unknown sub-lethal effects of TEA. This compound in particular is used in a commercial product initially developed for anesthesia of *Drosophila* spp. in education (Ratterman, 2003), but little work has been carried out to elucidate physiological mechanisms

of action and lethal and sub-lethal effects on insects (Ratterman, 2003). This study will focus on the sub-lethal effects that these approaches may have, with longevity as the key 'fitness' trait measured.

Carbon dioxide as an insect anesthetic

CO_2 has been used for decades as an anesthetic agent for insects, starting as early as the 1920s (Willis, 1925). At high concentrations, this gas interferes with signals that trigger central nervous system function, and can stimulate some behavior (e.g. foraging for food) at low concentrations (Nicolas & Sillans, 1989; Badre *et al.*, 2005). Due to its ease of use, reproducible results, and safety for humans, CO_2 has often been employed as the primary insect anesthesia technique (Nicolas & Sillans, 1989; Badre *et al.*, 2005; Champion De Crespigny & Wedell, 2008). However, care must be taken when using this gas, as it can also have adverse effects on insect behavior and fertility (Ribbands, 1950; Champion De Crespigny & Wedell, 2008). Another drawback with of this gas is that it has to be stored under pressure, which has safety issues for the user because of the weight of the cylinder in which it is provided, which can lead to injury (Artiss & Hughes, 2007). Depending on duration of anesthesia rental/purchase costs can become costly because of the equipment needed for application and storage.

Chilling as an insect anesthetic

As with CO_2, chilling has been commonly used as an insect anesthetic for many years, because of its ease and safety of use (Wedberg & Clarke, 1947; Nilson *et al.*, 2006; Champion De Crespigny & Wedell, 2008). It allows the researcher to take advantage of triggering insect chill coma, which is a threshold in which the neuromuscular activity comes to a halt at low temperatures (MacMillan & Sinclair, 2011). However, results are variable as they depend on the environment from which the insects were collected or reared and the insect species' response to cold stress. For example, some insects from tropical regions have less tolerance of temperature fluctuations than do species in more temperate climates (David *et al.*, 1998; Barron, 2000; Reynolds & Orchard, 2011). Some experiments have successfully used chilling alone

(Reynolds & Orchard, 2011), while one had to use a CO_2 in conjunction with chilling to increase survival after recovery from anesthesia (Nilson *et al.*, 2006). Others have found additional complications from chilling, because disruption of mating behavior after recovery can be caused by condensation. This damages the wings which are used in mating displays in some insect groups (Artiss & Hughes, 2007).

Triethylamine as an insect anesthetic

Common use of TEA started in the early 1990s for studies mostly involving *Drosophila* spp. (Worthen & Moore, 1991; Kauffmann *et al.*, 1995). Subsequently, this compound was used to facilitate the observation of functioning structures (e.g. viewing the heart and spiracles) inside living dissected insects (Vogler & Ocorr, 2009; Boppana & Hillyer, 2014). The generally accepted disadvantage of this compound is its volatility (Ratterman, 2003; Artiss & Hughes, 2007), which could lead to acute toxicity in humans if it is handled inappropriately. To mitigate this, appropriate handling procedures and personal protective equipment are necessary. This compound can be used in small diluted quantities, as only a drop is needed for anesthetizing an entire 35ml vial of insects (Fresia *et al.*, 2001). This makes TEA cost-effective, easily transportable and reduces risks to researchers; it also retains its effectiveness when used in that way.

Materials and Methods

Scaptomyza flava Fallén (Drosophildae) were kept in colonies in 60x60x60cm BugDorms (http://bugdorm.megaview.com.tw/) and reared on trays of *Brassica juncea* v. Mizuna, which were kept at 22 ± 2°C in controlled temperature rooms at the Bio-Protection Research Centre, Lincoln, New Zealand. These were used because further experiments were planned as part of a larger research project.

CO_2 exposure times

Several series of range-finder treatments were implemented and a complete range was evaluated for optimal exposure time of CO_2. These times were made up of 10 periods (5, 60, 600, 900, 1200, 1500, 1800, 4800, and 6000 seconds) and replicated 3 times. This was done because there is inconsistent information in the literature on how best to anesthetize insects with this compound (Nicolas & Sillans, 1989; Nilson *et al.*, 2006; Colinet & Renault, 2012). 25 flies were chosen from the colonies. CO_2 was applied by inserting a tube into a LabServ P35 35ml vial sealed with a foam plug (Fig. 1). Then flies were sexed under a dissecting microscope with 16x and 63x magnification. Next, 20 flies were chosen as close to a 1:1 ratio of males to females as was possible and the sexes were put into separate vials with meshed caps. Each vial contained an Eppendorf tube filled with water and plugged with cotton (Fig. 1) so the flies could re-hydrate themselves. The Eppendorf tube was fixed to the side of the vial to protect anesthetized flies from being crushed by possible movement of tube, because vials were set on their side to be monitored. These treatments were monitored until all flies recovered.

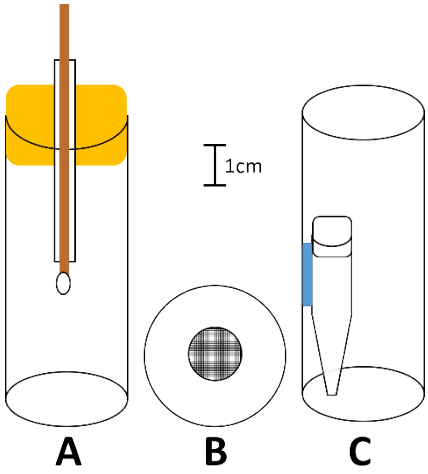


Fig. 1. Foam plugged 35ml vial with straw and swab inserted for application of TEA (A), meshed cap (B) and vial with cotton-filled 2ml

Eppendorf tube for monitoring recovery (C).

Chilling exposure times

A range finder test was carried out for temperatures and exposure periods. 15 temperature/exposure combinations were chosen with 3 replications. The temperatures chosen were 0, 2 and 4°C because of the climate in which these flies overwinter (Seraj, 1994) and using an Australasian study (Reynolds & Orchard, 2011) as guide. The exposure times were 2, 4, 8, 16, and 24 hours chosen from the range finder. This was carried out using vials sealed with foam plugs and floated in a temperature-controlled water bath (± 0.5°C). The flies were sexed directly from the colonies using a loupe and were collected by aspiration because the treatments in this approach did not meet the 15-minute threshold. This period was needed to sex 25 flies after the treatments were applied. After the exposure period the flies were put into vials as described above (Fig. 1) and monitored until all flies recovered.

TEA exposure times and dosages

There were 30 time/concentration combinations evaluated (10, 20, 30, 40, 50, 60 s and 100/0, 75/25, 50/50, 25/75; TEA%/ethanol% respectively) with 3 replications. These treatments used a cotton swab soaked in the chemical and the former was inserted into the vial through a plastic drinking straw, which was then sealed with a foam plug (Fig. 1). A new cotton swab was used for each application of TEA. The swab and straw were removed after exposure and the flies were continually knocked down to the bottom of the container until they were all immobilized. These were observed for 6 h.

Comparing anesthesia treatments

148 The experimental design selected the optimal treatments from each of the three range-finder methods
149 above and included a control. The experimental arrangement was a randomized-block design with 3
150 replications. After the flies were sexed and put into their respective vial, they were monitored for the
151 first 6 h at 5-minute intervals. They were then monitored in 24 h intervals until all flies died. Deaths
152 were recorded at each time interval.

153 *Analysis*

154 For the first set of experiments, Kaplan-Meier survival analysis in R Studio (3.3.1) was used for the
155 recovery time. Knockdown time and deaths were also used, but were run with ANOVA because
156 normality was demonstrated (Shapiro-Wilks <0.0001). The parameters used to evaluate the results for
157 the initial experiments were: amount of time to knockout, recovery time and number of deaths after 24
158 h. Kaplan-Meier survival analysis using R Studio (3.3.1) used the recovery time and deaths after
159 anesthesia to determine optimal treatments.

160 **Results**

161 ***Optimal treatments***

162 *TEA*

163 The 25% TEA was not effective because most flies were not fully anesthetized before they regained full
164 mobility. 100% TEA led to a relatively high mortality rate (3-10%, $\bar{x}$- 6.2 ± 2.3). 50% was not chosen
165 because of possible IP infringement of a product called FlyNap that uses a 50% solution of TEA (Supply,
166 2012; Binkley, 2016). Because of this 75% TEA was evaluated at different exposures to find the optimal
167 treatment. 75% TEA at 60 s had fewer deaths than at 50 s (0, 10 ± 1.7 respectively). Fewer flies were
168 awake after 1h for 50 and 60 s exposure than at 40 s (0 ± 0.9, 8.1 ± 2.6 respectively). 75% TEA at 60s

knocked down the flies significantly faster than all other treatments (p-values < 0.05). From this data, 75% TEA was chosen and applied to the flies for 60 s for the comparison experiment.

CO_2

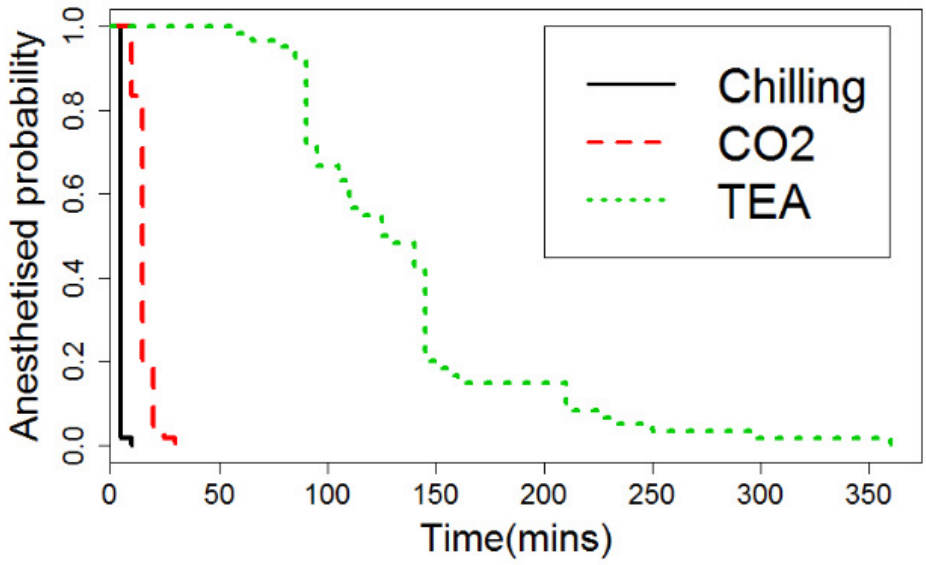
The 5 and 60 s exposures did not meet the 15min exposure threshold. The 1800 s exposure led to a large proportion of deaths (19.33% ± 26.9%). The 600, 900, 1200, and 1500 s exposures were not significantly different, but at 1500 s the longest mean recovery time (1450.67 ± 315.85s) occurred among these four treatments. The 1500 s (25min) exposure for CO_2 was thus chosen because of this long recovery time with no deaths.

Chilling

The exposure times and deaths were not significantly different (p-values = 0.903 and 0.322 respectively) for all temperatures, but at 0°C, recovery time was different when compared to 2°C and 4°C (p-values< 0.0001), where 0°C had the highest recovery time (483.76 ± 209.88). The 8 h exposure treatment had the highest mean recovery time (706 ± 277.32) among the 0°C treatments. The treatment chosen for the comparison experiment was 0°C at an 8 h exposure because of the relatively long recovery time and ease of implementation.

Comparison of treatments

Recovery time from anesthesia was significantly different among all treatments (p-value < 0.0001, Fig. 2), whereas longevity was not significantly different (p-value = 0.1145). TEA had the largest recovery time (median = 128 ± 18), CO_2 was second (median = 15 ± 0) and chilling had the lowest (median = 5 ± 0).



188

189 *Fig. 2*. Proportion of flies still anesthetized as time

190 progresses for the optimal treatments. Control was

191 zero as they were not anesthetized.

192 **Discussion**

193 TEA was the optimal anesthetic among those selected for this study to minimize mortality and provide

194 anesthetized flies to carry out potential experimentation. Comparing these methods has demonstrated

195 that the two most widely used anesthetic methods (CO_2 and chilling) may not be the most effective. TEA

196 is easier to handle, is faster to use and much more readily portable (dependent on health and safety

197 regulations in particular countries). Results here have shown that using CO_2 or chilling can be ineffective

198 when trying to perform observations with anesthetized insects, despite their frequent use (Perron et al.,

199 1972, Smith et al., 2004). This work therefore opens up new possibilities for insect anesthesia, especially

200 in the context of rapidly-advancing molecular, ecological and bio-systematics areas in which killing the

201 insect target is often undesirable (Oi *et al.*, 2013; Price *et al.*, 2015; Cheng & Lin, 2016; Sikulu-Lord *et al.*,

202 2016). Other than the effects on longevity, potential sub-lethal effects of TEA were not investigated in

this study, but could include changes to fecundity, host-searching and flight (Voinovich *et al.*, 2012; Chen *et al.*, 2013). It is also important, however, to reiterate that this compound does have negative health consequences if proper precautions are not followed (Sciencelab, 2013).

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