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 has usually involved their being killed, but non-lethal approaches are necessary when evaluating the biology of such organisms. Anesthesia has been used as one of these non-lethal approaches. Two common anesthetics used in this way have been carbon dioxide and chilling. These have been used frequently in the literature but have sub-lethal effects on insects that may affect further experimentation. An alternative anesthetic that has potential for experimental use is triethylamine (TEA). This 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. Here, longevity was use as a proxy for fitness as this is a common approach to laboratory work of this type for pests and their natural enemies. A series of experiments were carried out to find the optimal rates for these three selected anesthetics. The organism selected for this work was the fly *Scaptomyza flava* (Fallén), the larvae of which mine the leaves of commercial brassica crops worldwide. It is a 'cosmetic' pest in that damage thresholds for crop rejection are very low and prophylactic use of insecticides is the norm. One way of mitigating the negative environmental aspects of this approach is to enhance biological control. One such way of enhancing biological control is the use of flowering plants, as they can provide alternative resources to natural enemies, which can enhance their ability to control pests. To evaluate these flowering plants, handling of the insects is necessary and anesthesia is used for this purpose. For each anesthetic an optimal rate was found, then these rates were compared to each other in a subsequent experiment. These anesthetics differed markedly in their value in terms of the duration of the anesthesia and other practical considerations. TEA had the longest effect, CO_2 and chilling had the shortest. All three were similar in their effects on longevity (i.e. recovery time and mortality rates).

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Abstract

Historically, handling insects has usually involved their being killed, but non-lethal approaches are necessary when evaluating the biology of such organisms. Anesthesia has been used as one of these non-lethal approaches. Two common anesthetics used in this way have been carbon dioxide and chilling. These have been used frequently in the literature but have sub-lethal effects on insects that may affect further experimentation. An alternative anesthetic that has potential for experimental use is triethylamine (TEA). This 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. Here, longevity was use as a proxy for fitness as this is a common approach to laboratory work of this type for pests and their natural enemies. A series of experiments were carried out to find the optimal rates for these three selected anesthetics. The organism selected for this work was the fly *Scaptomyza flava* (Fallén), the larvae of which mine the leaves of commercial brassica crops worldwide. It is a 'cosmetic' pest in that damage thresholds for crop rejection are very low and prophylactic use of insecticides is the norm. One way of mitigating the negative environmental aspects of this approach is to enhance biological control. One such way of enhancing biological control is the use of flowering plants, as they can provide alternative resources to natural enemies, which can enhance their ability to control pests. To evaluate these flowering plants, handling of the insects is necessary and anesthesia is used for this purpose. For each anesthetic an optimal rate was found, then these rates were compared to each other in a subsequent experiment. These anesthetics differed markedly in their value in terms of the duration of the anesthesia and other practical considerations. TEA

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Key words: Anesthesia, Chilling, CO_2, Triethlyamine, *Scaptomyza flava*, Drosophilidae, Brassicaceae, Leaf-miner

Introduction

A key pest of brassicas worldwide is the leaf-mining fly, *Scaptomyza flava* (Fallén) which causes cosmetic damage that leads to crop rejection by supermarkets/consumers (A. Berrysmith, personal communication). In the latitudes in which leafy salad brassicas are grown, the crop can be harvested all year round, which means that the flies are almost always present (Seraj, 1994). This life history necessitates control throughout the entire harvesting season which usually consists of the prophylactic application of insecticides. One way of ameliorating the negative environmental aspects of this approach is to enhance the effectiveness of biological control by providing alternative food sources for natural enemies. Some flowering plants can provide these, as many parasitoids and other insects can feed on the nectar, which can improve their efficacy (Tylianakis *et al.*, 2004; Gurr *et al.*, 2012). This effect can arise from their increased fecundity and longevity (Gurr *et al.*, 2017). To evaluate different flowering plants and their effects on parasitoids and pests, handling of live specimens is necessary. This is difficult without anesthesia, which allows further experimentation on them.

Historically, documented experiments began in the 1920s to investigate possible techniques to render insects immobile without killing them (Willis, 1925). Many approaches 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. These are carbon dioxide (CO_2), chilling and triethylamine (TEA). Here, we compare 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. for educational purposes, 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 focuses on longevity as the key 'fitness' trait measured because this is the variable that is most usually quantified in laboratory aspects of developing agro-ecological schemes (Berndt *et al.*, 2006; Nafziger & Fadamiro, 2011). TEA is suspected to be a superior anesthetic as it has commercial uses (Supply, 2012; Binkley, 2016).

Carbon dioxide as an insect anesthetic

CO_2 has been used for decades for this purpose, documented 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; Nicolas & Sillans, 1989; Champion De Crespigny & Wedell, 2008). These effects can occur across many insect orders, from longevity and fecundity effects in orthopterans (Chen *et al.*, 2013), to role-switching in social insects such as honey bees (Nicolas & Sillans, 1989). In the Drosophilidae, which includes *S. flava*, CO_2 affects *Drosophila* species' fecundity, survival (Barron, 2000; Fresia *et al.*, 2001; Champion De Crespigny & Wedell, 2008), metabolic processes (Colinet & Renault, 2012) and learning/memory (Margulies *et al.*, 2005). Another drawback with this gas is that it has to be stored under pressure, presenting possible safety issues for the user because of the weight of the cylinders, which can lead to injury (Artiss & Hughes, 2007). Depending on duration of use, rental/purchase costs can become high 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 of use and safety (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 at 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

105 temperature fluctuations than do those in more temperate climates (David *et al.*, 1998; Barron,
106 2000; Reynolds & Orchard, 2011). Some experiments have successfully used chilling alone
107 (Reynolds & Orchard, 2011), while one had to use CO_2 in conjunction with chilling to increase
108 survival after recovery from cold exposure (Nilson *et al.*, 2006). Additional effects reported
109 from chilling have been disruptions to mating behavior and genetic upregulation. Condensation
110 on the wall of the container can damage insect wings, which are used in mating displays in
111 some insect groups (Artiss & Hughes, 2007). Also, copulation latency after recovery has been
112 observed (Barron, 2000). This suggests that exposure to low temperatures may affect
113 reproduction in a range of ways. When observing genetic changes, upregulation of genes in
114 response to cold stress has been observed in *Drosophila melanogaster Meigen* (Zhang *et al.*,
115 2011) especially if it has had repeated exposure. This leads to differences in physiological
116 responses, such as increased cold tolerance, which also occurs during acclimation experiments
117 (Colinet *et al.*, 2012; Marshall & Sinclair, 2012).

118 *Triethylamine as an insect anesthetic*

119 Common use of TEA began in the early 1990s for studies mostly involving *Drosophila* spp.
120 (Worthen & Moore, 1991; Kauffmann *et al.*, 1995). Subsequently, this compound has been used
121 to facilitate the observation of anatomical structures (e.g., viewing the heart and spiracles)
122 inside living dissected insects (Vogler & Ocorr, 2009; Boppana & Hillyer, 2014). The generally
123 accepted disadvantage of this compound is its volatility (Ratterman, 2003; Artiss & Hughes,
124 2007), which could lead to acute toxicity in humans if it is handled inappropriately. To mitigate
125 this, appropriate handling procedures and personal protective equipment are necessary. This
126 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 (because only small quantities are needed) and reduces risks to researchers; it also retains its effectiveness when used in that way.

Materials and Methods

S. flava was kept in colonies in 60x60x60cm BugDorms (http://bugdorm.megaview.com.tw/) and reared on trays of *Brassica juncea* v. m*izuna* seedlings (Figure 1A, Appendix), which were kept at 22°C with a 4°C range in controlled temperature rooms at the Bio-Protection Research Centre, Lincoln University, New Zealand. The mizuna was made available to the flies when it was 3 weeks old. This study comprised three groups of experiments: a preliminary range-finder assessment to find a range of variables to test from each anesthetic, Group 1 – testing multiple rates for each anesthetic to find the optimal treatment and Group 2 – a final comparison to elucidate an overall optimal anesthetic type and rate. For the Group 1 experiment, 25 flies were randomly aspirated from the colonies. The sexes were combined to keep uniformity among the anesthetic treatments because some of the treatment/dose combinations did not meet the 15 min threshold needed to sex the flies. When the optimal rates for each anesthetic application were determined, the Group 2 experiment was carried out. The Group 2 experiment involved aspirating 20 flies that were sexed to get as close to a 1:1 sex ratio as possible. The sexes were then put into separate vials and monitored for recovery (Figure 2A, Appendix). Survival was recorded every 24 h until all flies had died. These methods were derived and adapted from other studies that also evaluated anesthetic methods (Perron *et al.*, 1972; Barron, 2000; Champion De Crespigny & Wedell, 2008; Chen *et al.*, 2013). Recovery of a fly was defined as its ability to fly when disturbed (i.e. in response to the container being gently tapped or in

149 response to researcher movement). For the Group 1 experiment, only three replicates were
150 carried out as a power analysis predicted that too many replicates would have been needed to
151 get ideal resolution in the analyses (Table 1). Each anesthetic used different variables (e.g.,
152 temperature, concentration, etc.) because each had a different method of application. Time to
153 knockdown, which is the time it took for the insect to become immobilized by the anesthetic,
154 was only evaluated in the TEA treatments because it happened more gradually than CO_2 (CO_2
155 was almost instantaneous) and gave more detail in decision making when choosing an optimal
156 rate for TEA. It was also not used for chilling because the vials had to be removed from the
157 water bath to record this variable and thus the treatment would have been disrupted.
158 Otherwise, only recovery time and mortality were compared between rates of the same
159 anesthetic for the Group 1 experiment and between anesthetic treatments for the Group 2
160 experiment.

Table 1 Power analysis for the optimal treatments. Detection for recovery was the variable that determined the number of replicates required as this was the desired resolution for the analysis.

Anesthetic	Pooled Stdev	Detection for Recovery (mins)	T value (95% conf)	Degrees of freedom	Replicates required from desired resolution
CO_2	1418.15	10	2.015	20	163,314
Chilling	339.21	10	2.015	44	9,344
TEA	20.36	10	2.015	185	34

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163 ***Range finders***

164 Each of these three anesthetic methods has been described in the literature, but none has used
165 *S. flava* nor compared all three together. Using several studies as guidelines, a series of

166 preliminary experiments were carried out (range finders) to find the range of the variables in
167 which each anesthetic could be tested. As each anesthetic method has different properties,
168 different variables were measured for each one. CO_2 used only exposure time because
169 concentration could not easily be measured/changed and most published papers do not report
170 concentration used when applying CO_2 (Nicolas & Sillans, 1989; Nilson *et al.*, 2006; Colinet &
171 Renault, 2012). The exposure times were chosen at an exponential rate starting from 5 s.
172 Chilling used exposure time and temperature. The times and temperatures were selected by
173 using several earlier studies as a guide (David *et al.*, 1998; Nilson *et al.*, 2006; MacMillan &
174 Sinclair, 2011; Reynolds & Orchard, 2011). Lastly, TEA exposure times and TEA concentrations
175 were manipulated. The times were measured ad-hoc as all articles found had used a formulated
176 version (Ratterman, 2003; Artiss & Hughes, 2007; Chen & Hillyer, 2013; Boppana & Hillyer,
177 2014) of TEA (FlyNap®, 50% TEA) with which the researcher had previous experience (<3 mins
178 are needed for complete anesthesia). The concentrations used in the range finder were 50%
179 and 100% because the commercial product uses a 50% solution and the compound comes at
180 100%.

181 ***Group 1 - Optimal treatments experiment***

182 *Carbon dioxide*

183 After the range-finder was completed, 10 periods (5, 60, 600, 900, 1200, 1500, 1800, 4800, and
184 6000 s) were selected and replicated 3 times each. The exposure times of 10240 and 20480 s
185 were removed because they were considered too long and expensive to carry out. CO_2 was
186 applied by inserting a tube into a LabServ P35 35ml vial sealed with a foam plug (Fig. 1A). A

187 steady stream of CO_2 that was gentle enough not to blow the flies around in the vial was used.
188 Then flies were sexed under a dissecting microscope with 16x and 63x magnification and moved
189 to new vials. Each new vial contained an Eppendorf tube filled with water and plugged with
190 cotton (Fig. 1C) so the flies could re-hydrate themselves. The Eppendorf tube was fixed to the
191 side of the vial to protect anesthetized flies from being crushed by possible movement of tube,
192 because vials were set on their side to be monitored in the controlled temperature room
193 (Figure 2A, Appendix). These treatments were checked every 5 mins for 8 h or until all flies
194 recovered. 24 h after this period the flies were checked any deaths.

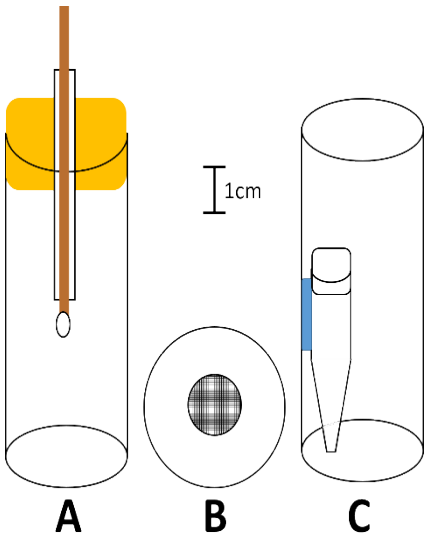

Figure 1 Foam-plugged 35ml vial with straw and swab inserted for application of TEA (A), meshed cap (B) Eppendorf tube filled with water and with a cotton-wool plug. A similar vial setup (no swab and straw) used when applying CO_2 and the vial with a foam plug (A) floated in a water bath for the chilling treatments. All treatments used the vial depicted in C for data collection.

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196

197 *Chilling*

198 15 temperature/exposure combinations (0°C, 2°C, 4°C and 2 h, 4 h, 8 h, 16 h, and 24 h)
199 combinations were chosen with 3 replicates each. Into each vial 25 flies of unknown sex were
200 placed. The vials were then sealed with foam plugs (Fig. 1A) The evaluation was carried out by
201 placing the vials in holes in a Styrofoam float so that they were in the liquid. Which was
202 managed by a temperature-controlled water bath ($\pm$ 0.5°C) using a Low Temperature
203 Circulator LTD-6 (Grant Instruments Ltd, Cambridge, UK). When the targeted exposure had
204 been reached, the vials with the flies were removed and each batch of flies was transferred to a
205 new vial. The new vial contained a water-filled Eppendorf tube and was then monitored in the
206 controlled temperature room as noted above and shown in Figure 1. The flies were monitored
207 every 5 mins for 6 h. 24 h after this period, mortality was recorded.

208 *Triethylamine*

209 There were 30 time/concentration combinations evaluated (10, 20, 30, 40, 50, 60 s and 100/0,
210 75/25, 50/50, 25/75 (TEA %/ethanol %, respectively) with 3 replicates for each combination.
211 These treatments used a cotton swab soaked in the chemical and the former was inserted into
212 the vial through a plastic drinking straw, which was then sealed with a foam plug (Fig. 1A). After
213 exposure time was met, the straw and swab were removed and the flies were gently knocked
214 down to the bottom of the tube until they were fully anesthetised. A new cotton swab was
215 used for each application of TEA. Mortality was recorded as above.

216 ***Group 2 - Final comparison experiment***

The experimental design selected the optimal rates from each of the three anesthetics described above and included a control. The criteria for selecting the optimal treatment from each anesthetic approach was based on meeting the 15 min threshold for sexing, the longest anesthetic period, shortest knockdown and lowest number of deaths after 24 h. The experimental arrangement was a randomized-block design with 3 replicates.

Analysis

As the range finders were run only once to find a range for selecting the experimental variables, no statistical analysis was done. Shapiro-Wilks tests of normality showed that all variables were non-normal (all p-values < 0.001) so non-parametric analyses were needed. For the Group 1 assessments, Kaplan-Meier survival analysis and Cox's Proportional Hazard model with and without Firth's penalized likelihood (coxph and coxphf respectively) was used in R Studio (3.3.1) to analyze the recovery time and knockdown. While generalized linear models (GLM) were used to analyse the death rates. The R packages used for these analyses were coxphf and survival. For the Group 2 experiments, Kaplan-Meier survival analysis was used in conjunction with Cox's Proportional Hazard model with and without Firth's penalized likelihood in R Studio (3.3.1) to analyze both recovery time and longevity. The packages used are the same as noted above.

Results

Range finders

The results from Table 1A (Appendix) were used to select the treatments for each anesthetic type. For CO_2, treatments after 1800 s had a spike in the number of deaths and used up CO_2 supplies rapidly. The canisters were completely exhausted in one or two treatments so were

not used. Next, chilling did not show much variation between treatments so an exponential increase in the exposure was used at each of the three temperatures, starting at 2 h exposures. This led to a final evaluation of 0°,2° and 4°C with exposure times of 2, 4, 8, 16 and 24 h (120, 240, 480, 960 and 1440 mins respectively). Lastly, two TEA treatments were carried out, but all the flies died at or shortly after 60 s exposure. This led to the development of the design that used 25, 50, 75 and 100% TEA where the diluted concentrations used ethanol as the solvent. Each of these concentrations was then paired with 10, 20, 30, 40, 50 and 60 s exposures.

Group 1 - Optimal treatments

Carbon dioxide

The likelihood ratio test and Wald-test (derived from the coxph model) both demonstrated that the treatments were significantly different from controls (all p-values <0.001, Table 2A, Appendix). All the treatments from the survival analysis were also significantly different from the controls (Table 2A medians, Appendix) based on confidence limits (only the lower ones were used as upper limits experienced some quasi-complete separation that is not adjustable even with the Firth's penalized likelihood). The shortest exposures (5 – 600 s) were removed because they did not meet the 15 min (900 s) exposure threshold. The longest three all met the threshold but the 1500sec (25 mins) exposure was chosen because it had a recovery time that overlapped with some of the chosen TEA treatments. If any longevity issues arose they should appear here as this was the treatment immediately before deaths started to occur in the range-finder CO_2 treatments.

Chilling

The chilling exposure times were not significantly different (P = 0.84) and thus were not recorded in Table 2A (Appendix). However, temperatures were significantly different (medians in Table 2 and upper and lower confidence limits) but all three did not meet the 15 min threshold. The longest recovery time was 300 s which occurred in the 0°C treatment and was chosen for use in the final comparison, but the exposure time was one of convenience, at 8 h because of the non-significant differences between exposures.

TEA

The mortality rates were all significantly different from the control (likelihood ratio and Wald-test <0.001, Table 2A, Appendix) and also showed quasi-complete separation when coxphf was used. Using the medians (Table 2A, Appendix), the knockdown times (Table 3A, Appendix) and mean death rates between concentrations, led to the selection of 75% TEA at 60 s exposure. Data for 25% TEA were removed because the flies were not always completely immobilized. The 100% TEA was also removed because there was a spike in mean death rate (Table 4A, Appendix). The decision to choose 75% at 60 s was similar to that for CO_2 because the TEA data showed a spike in deaths and it had shorter knockdown times than the 50% treatment.

Group 2 - Final comparison experiment

The coxph model demonstrated a significant difference between the sexes (P = 0.0281), so all of the results in Table 5A (Appendix) are separated by sex. There were clear differences between all treatments for recovery times (Fig 2), with TEA having the longest recovery (145 and 105 mins) and chilling having the shortest (5 mins). Lastly, all treatments were not significantly

279 different in their effects on the longevity of the flies as seen by the overlap in medians in Table

280 2 and the overlap in survival curves in Figure 3.

Table 2 Median number of days flies in each treatment lived with only water. This was found using Kaplan-Meier survival analysis.

Final comparison longevity

Anesthetic	Conc. TEA	Exposure (sec)	Temp (°C)	Coefficients	Pvalue	Likelihood ratio	Wald-test	Median recovery (days)	Lower CL	Upper CL
Control	N/A	N/A	N/A	N/A	N/A	N/A	N/A	4	3	5
CO_2	N/A	1800	N/A	0.881	0.488	0.0373	0.0294	4	4	5
Chilling	N/A	8	0	0.939	0.73	0.0373	0.0294	4	4	6
TEA	75	60	N/A	1.465	0.0408	0.0373	0.0294	4	3	4

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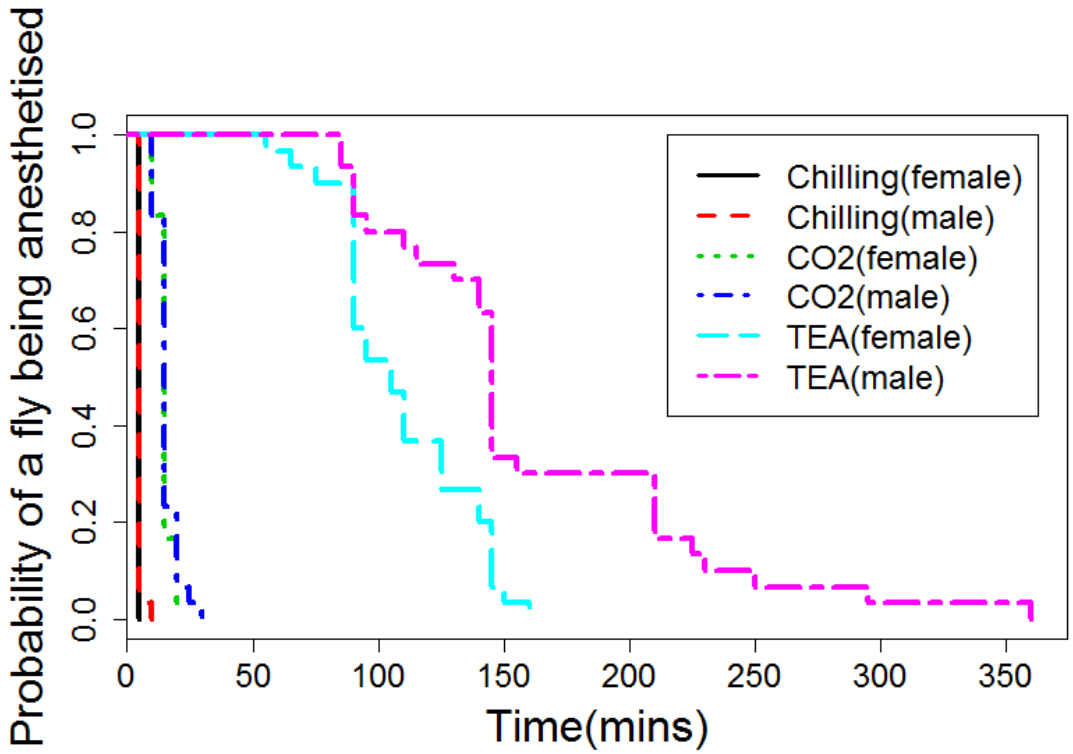

Figure 2 Kaplan-Meier survival analysis curves for recovery times. The curves for the sexes are similar for CO_2 and chilling but are different in TEA. Although the medians with the lower and upper confidence limits show that the curves overlap each other and thus are not treated differently when evaluating each anesthetic. The controls were removed because they were a straight line following the y-axis.

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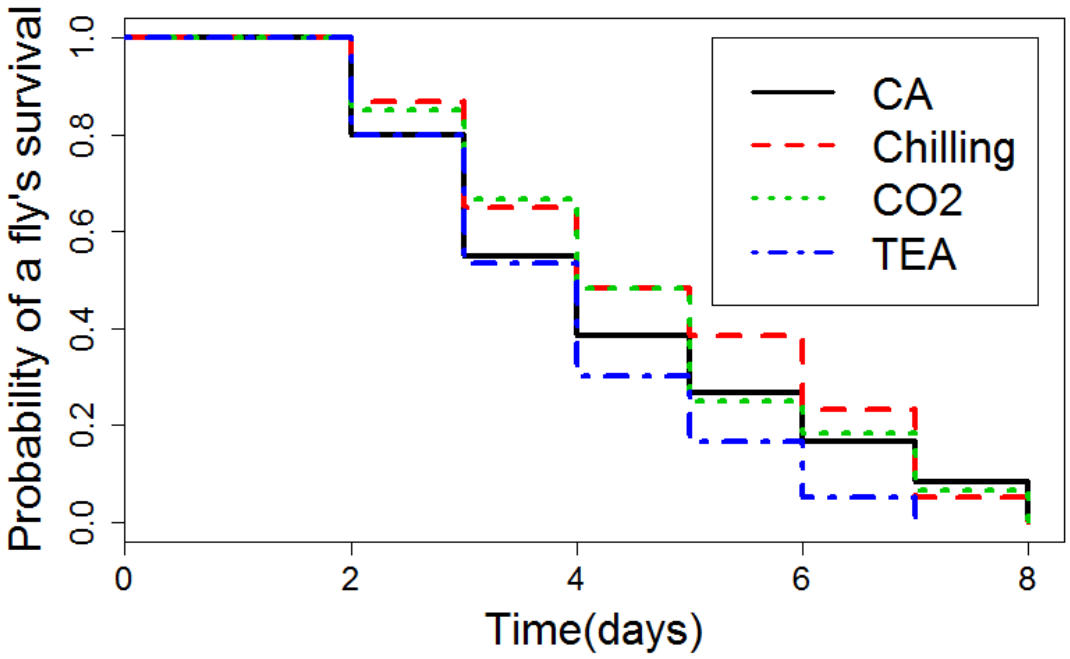

Figure 3 Death rates between the treatments. CA is the control.

299

300 **Discussion**

301 Comparing these methods have shown that each anesthetic affects the flies' recovery
302 differently. This also demonstrates that there may be different uses for each of these
303 anesthetics. For example, when examining the physiology of an insect while it is alive, TEA
304 would be favored an anesthetic because of its long duration of effect after application (Vogler &
305 Ocorr, 2009; Chen & Hillyer, 2013; Boppana & Hillyer, 2014). If movement from one container
306 to the next is the only action desired (Ratterman, 2003; Artiss & Hughes, 2007), then all three

would be equitable. In all cases where a short recovery time is needed, TEA is not suitable, in all other cases, TEA is at least equitable to the other two anesthetics. For this experiment, we chose TEA as the optimal anesthetic compound because of its ease of application, cost effectiveness, duration of anesthetic effect and because duration of recovery time was not an important factor in further experimental designs. Results here have shown that using CO_2 or chilling can be ineffective when trying to perform observations with anesthetized insects within a specific time frame (>15 mins), despite their frequent use (Perron et al., 1972, Smith et al., 2004). This work therefore opens up new possibilities for insect anesthesia, when killing the insect target is often undesirable (Oi *et al.*, 2013; Price *et al.*, 2015; Cheng & Lin, 2016; Sikulu-Lord *et al.*, 2016). Other than the effects on longevity, potential sub-lethal effects of TEA were not investigated in this study, whereas these effects have been thoroughly investigated (and continue to be investigated) with the other two methodologies. The unforeseen effects that TEA could have include, but are not limited to changes in: fecundity, host-searching, memory and flight patterns (Voinovich *et al.*, 2012; Chen *et al.*, 2013). It is also important, however, to reiterate that this compound does have negative human health consequences if proper precautions are not followed (Sciencelab, 2013), but it seems that lower concentration (50%) could be used to lessen the hazards. However, the other two methodologies can also have detrimental effects if handled improperly.

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Appendix

Figure 1A Three-week old *Brassica juncea* 'mizuna' in 'Bugdorms.'

Figure 2A 35 ml vials containing recovering *S. flava*.

Table 1A Preliminary experiments to find a range of variable in which to test each anesthetic method.

Range finder results

Application method	Temperature (°C)	Concentration (% TEA)	Time exposed (sec)	First fly recovered (sec)	Last fly recovered (sec)	Deaths after 24h
CO_2	N/A	N/A	5	60	89	0
CO_2	N/A	N/A	10	38	94	0
CO_2	N/A	N/A	20	14	60	0
CO_2	N/A	N/A	40	46	120	0
CO_2	N/A	N/A	80	68	140	0
CO_2	N/A	N/A	160	93	134	0
CO_2	N/A	N/A	320	118	230	0
CO_2	N/A	N/A	640	363	620	0
CO_2	N/A	N/A	1280	1225	1800	0
CO_2	N/A	N/A	2560	2820	7205	0
CO_2	N/A	N/A	5120	*	N/A	4
CO_2	N/A	N/A	10240	*	N/A	0
CO_2	N/A	N/A	20480	**	N/A	N/A
Chilling	2	N/A	10	6	41	0
Chilling	2	N/A	30	6	176	0
Chilling	2	N/A	60	20	464	0
Chilling	2	N/A	120	6	244	0
Chilling	2	N/A	240	6	270	0
Chilling	2	N/A	1080	6	915	0
Chilling	2	N/A	1440	6	570	0
Chilling	0	N/A	10	6	52	0
Chilling	0	N/A	30	6	128	0
Chilling	0	N/A	60	6	245	0
Chilling	0	N/A	120	6	511	0
Chilling	0	N/A	240	6	786	1
Chilling	0	N/A	1080	6	282	0
Chilling	0	N/A	1440	6	164	0
Chilling	4	N/A	10	6	50	0
Chilling	4	N/A	30	4	154	0
Chilling	4	N/A	60	8	35	0
Chilling	4	N/A	120	5	24	0
Chilling	4	N/A	240	3	48	0
Chilling	4	N/A	1080	4	12	0
Chilling	4	N/A	1440	4	10	0
TEA	N/A	50	80	N/A	N/A	20
TEA	N/A	100	65	N/A	N/A	20

* Left overnight as they didn't recover in an 8hr

** Canister of gas would run out in approximately 5hrs making these not cost effective

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Table 2A Results from the optimal treatment experiments.

Optimal treatments fly recovery

Anesthetic	Conc. TEA (%)	Exposure (sec)	Temp (°C)	coeficients	pvalue**	Likelihood ratio	Wald-test	Median recovery (sec)	Lower CL	Upper CL
CO_2	N/A	5	N/A	-3.348	0.00616	<0.001	0.0000212	45	28.5	N/A
CO_2	N/A	60	N/A	-3.414	0.00527	<0.001	0.0000212	102	83.5	N/A
CO_2	N/A	600	N/A	-3.622	0.00316	<0.001	0.0000212	699	537	N/A
CO_2	N/A	900	N/A	-3.766	0.0022	<0.001	0.0000212	1082	1081.5	N/A
CO_2	N/A	1200	N/A	-4.007	0.00119	<0.001	0.0000212	1161	1161	N/A
CO_2	N/A	1500	N/A	-4.23	0.00069	<0.001	0.0000212	1376	1376.5	N/A
CO_2	N/A	1800	N/A	-4.608	0.000292	<0.001	0.0000212	4485	4383.2	N/A
Chilling	N/A	N/A*	0	-6.461	5.42E-12	<0.001	2.091E-10	14	11	33
Chilling	N/A	N/A*	2	-4.073	0.000000166	<0.001	2.091E-10	50.5	17	177
Chilling	N/A	N/A*	4	-2.71	0.0000619	<0.001	2.091E-10	304	215	648
TEA	25	10s	N/A	-3.225	0.00164	<0.001	0.001	3600	60	N/A
TEA	25	20s	N/A	-3.225	0.00164	<0.001	0.001	3600	N/A	N/A
TEA	25	30s	N/A	-5.785	0.00000391	<0.001	0.001	6492	93	N/A
TEA	25	40s	N/A	-9.258	4.53E-10	<0.001	0.001	5568	72	N/A
TEA	25	50s	N/A	-12.702	4.063E-14	<0.001	0.001	5514	84	N/A
TEA	25	60s	N/A	-15.94	0	<0.001	0.001	5670	64	N/A
TEA	50	10s	N/A	-2.615	0.0115	<0.001	0.000053	6240	87	N/A
TEA	50	20s	N/A	-5.074	0.0000167	<0.001	0.000053	7080	100	N/A
TEA	50	30s	N/A	-8.55	2.35E-09	<0.001	0.000053	6720	95	N/A
TEA	50	40s	N/A	-12.025	2E-13	<0.001	0.000053	7200	93	N/A
TEA	50	50s	N/A	-15.489	0	<0.001	0.000053	7920	93.8	N/A
TEA	50	60s	N/A	-18.702	0	<0.001	0.000053	8760	138	N/A
TEA	75	10s	N/A	-2.562	0.0136	<0.001	0.000146	5490	66	N/A
TEA	75	20s	N/A	-5.992	0.00000636	<0.001	0.000146	7020	60	N/A
TEA	75	30s	N/A	-9.398	1.15E-09	<0.001	0.000146	6570	95	N/A
TEA	75	40s	N/A	-12.589	2E-13	<0.001	0.000146	6840	113	N/A
TEA	75	50s	N/A	-16.064	0	<0.001	0.000146	12240	147	N/A
TEA	75	60s	N/A	-19.542	0	<0.001	0.000146	11052	157	N/A
TEA	100	10s	N/A	-2.562	0.0136	<0.001	0.000109	8520	133	N/A
TEA	100	20s	N/A	-6.037	0.000005	<0.001	0.000109	10680	171	N/A
TEA	100	30s	N/A	-9.511	6.85E-10	<0.001	0.000109	12120	186	N/A
TEA	100	40s	N/A	-12.953	6.79E-14	<0.001	0.000109	11160	159	N/A
TEA	100	50s	N/A	-16.428	0	<0.001	0.000109	12000	156	N/A
TEA	100	60s	N/A	-19.906	0	<0.001	0.000109	14220	195	N/A

* Exposure was not significant (pvalue = 0.84) so they are not reported

** Compared to control

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Table 3A TEA knockdown results using Kaplan-Meier survival analysis and Cox-Proportional Hazard model.

TEA Knockdown

Conc (% TEA)	Exposure (sec)	Likelihood ratio	Wald-test	Median recovery (sec)	Lower CL	Upper CL
25	10	<0.001	4.2E-07	225	210	N/A
25	20	<0.001	4.2E-07	150	110	N/A
25	30	<0.001	4.2E-07	115	86	N/A
25	40	<0.001	4.2E-07	90	86	N/A
25	50	<0.001	4.2E-07	58	45	N/A
25	60	<0.001	4.2E-07	113	55	N/A
50	10	<0.001	4.2E-07	95	78	N/A
50	20	<0.001	4.2E-07	80	70	N/A
50	30	<0.001	4.2E-07	65	40	N/A
50	40	<0.001	4.2E-07	52	25	N/A
50	50	<0.001	4.2E-07	40	18	N/A
50	60	<0.001	4.2E-07	5	5	N/A
75	10	<0.001	4.2E-07	64	26	N/A
75	20	<0.001	4.2E-07	52	45	N/A
75	30	<0.001	4.2E-07	25	23	N/A
75	40	<0.001	4.2E-07	26	12	N/A
75	50	<0.001	4.2E-07	25	5	N/A
75	60	<0.001	4.2E-07	10	0	N/A
100	10	<0.001	4.2E-07	45	44	N/A
100	20	<0.001	4.2E-07	39	35	N/A
100	30	<0.001	4.2E-07	19	18	N/A
100	40	<0.001	4.2E-07	13	5	N/A
100	50	<0.001	4.2E-07	8	6	N/A
100	60	<0.001	4.2E-07	5	0	N/A

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Table 4A Mean death rate from the optimal TEA concentrations. These means did not differ significantly. Exposure times were averaged in each concentration because they were not found to be significantly different. This allowed for better decision making between concentrations.

TEA deaths from optimal treatments

Concentration	Mortality rate
25	3.69
50	0.88
75	3.06
100	6.16

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Table 5A Final anesthetic comparison between sexes and treatments. Cox Proportional Hazard model (columns 6-9) and Kaplan-Meier survival analysis (columns 10-12) were used together to decide on the optimal anesthetic.

Final comparison fly recovery

Anesthetic	Conc. TEA (%)	Exposure (sec)	Temp (°C)	Sex	Coefficients	Pvalue (for sex)	Likelihood ratio	Wald-test	Median recovery (mins)	Lower CL	Upper CL
CO_2	N/A	1800	N/A	Males	-46.38	0.0281	<0.001	0.3057	15	15	15
CO_2	N/A	1800	N/A	Females	-46.38	0.0281	<0.001	0.3057	15	15	15
Chilling	N/A	8	0	Males	-24.61	0.0281	<0.001	0.3057	5	N/A	N/A
Chilling	N/A	8	0	Females	-24.61	0.0281	<0.001	0.3057	5	N/A	N/A
TEA	75	60	N/A	Males	-67.92	0.0281	<0.001	0.3057	145	90	125
TEA	75	60	N/A	Females	-67.92	0.0281	<0.001	0.3057	105	140	210

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