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The effect of antifreeze (ethylene glycol) on the survival and the life cycle of two species of necrophagous blowflies (Diptera: Calliphoridae)

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Abstract

Entomotoxicology involves the analysis of the presence and the effects of toxicological substances in necrophagous insects. Results obtained by entomotoxicological studies may assist in the investigation of both the causes and the time of death of humans and animals. Ethylene glycol (EG) is easy to purchase, sweet and extremely toxic. It may be consumed accidentally or purposefully, in an attempt to cause death for suicidal or homicidal intent. Several cases report fatalities of humans and animals. The present study is the first to examine the effects of EG on the survival, developmental rate and morphology of two blowfly species, (Diptera: Calliphoridae) typically found on corpses and carcasses: *Lucilia sericata* (Meigen) and *L. cuprina* (Wiedemann). Both species were reared on substrates (beef liver) spiked with three different concentrations of EG that could cause death in either a human or cat: $1/2LD_{50}$ (T1), LD_{50} (T2), $2LD_{50}$ (T3), in addition to a control treatment (C) with no EG.

Results of this research show that: a) both species are unable to survive if reared on a food substrate spiked with the highest concentration of EG (T3), while lower and medium concentrations (T1, T2) affect, but not prevent, the survival and the completion of the life cycle of such species; b) adults of *L. sericata* eclose only in C and T1, while adults of *L. cuprina* in both C, T1, T2; however, c) the developmental time of both species reared in T1 and T2 is statistically slower than the control; d) the body length of the immatures of both of the species reared in T1 and T2 is statistically smaller than the control.

Keywords

Ethylene glycol; Entomotoxicology; *Lucilia sericata*; *Lucilia cuprina.*

1. Introduction

Entomological evidence in a legal investigation can provide valuable information and the discipline now known as *forensic entomology* is accepted worldwide in the court of law (1). Beside assisting in the estimation of the minimum time since death (or minPMI, minimum Post Mortem Interval), insects found at the crime scene may be used in the toxicological evaluation of body remains (2). Insects may in fact represent a valuable alternative matrix to detect drugs or other toxic substances in the absence of body tissues or biological fluids (e.g. body in a high deposed stage of decomposition, skeletonized body, mummified body). The interface of entomology and toxicology is known as *entomotoxicology* (3). In recent years, the analysis of blowflies feeding on decomposing bodies of humans and animals has been considered a reliable forensic tool as a means of identifying many different toxicological substances, such as metals, pesticides, medical and recreational drugs that the subject potentially ingested, or was exposed to, before death (4-6). Furthermore, previous studies have also demonstrated that the bioaccumulations of these substances may cause significant effects on the morphology, the developmental time and survival of the blowflies (7-9). If overlooked, the alterations of the insects' anatomy and physiology due to the presence of toxicological substances in the food substrate, may have serious consequences on the potential use of the insects for the estimation of the minPMI (4).

Studies already present in the literature have highlighted the effects of several toxicological substances (5, 6), however, ethylene glycol has not been considered in any previous entomotoxological study.

Ethylene glycol (1,2-ethanediol) (EG), commonly referred to as antifreeze, is soluble chemical solvent widely marketed for commercial use in household products, as well as for coolant purposes in automobiles and machinery (10).

Small doses of EG may lead to severe toxicity and consequentially result in death. In humans, the lethal dose of 95% EG is 1-1.5 mL/kg body weight. A similar lethal dose can be observed in cats, however dogs have a higher lethal dose of 4-6 mL/kg (11, 12). Due to its high solubility, the toxin is quickly absorbed from the gastrointestinal tract and redistributed throughout the rest of the body targeting the central nervous system, cardio-pulmonary system and renal system, where it acts on the organs by producing metabolites with toxic by-products that lead to acute kidney failure, central nervous system depression and tachycardia (13, 14).

Ethylene glycol can be easily found in many products, and it is simple to purchase because of both its availability and its cheap cost. This accessibility combined with its sweet taste makes it a fairly common overdose agent, both in cases of accidental (especially pediatric) ingestions and in suicide (15). Home alcohols for recreational purposes are commonly adulterated with EG to improve their taste and “sting” (16). A wide range of case reports in the scientific literature, as well as in news around the world, describe the ingestion of the toxin either by humans or animals (e.g. (10, 12, 15-18)). In 2002, there were 5816 reported cases of EG poisoning in the United States and in 2003 it was the most common chemical responsible for death in the United States (16). The Royal Society for the Prevention of Cruelty to Animals, recognises EG as one of the most common causes of death to pets in the UK, with the majority of intoxication cases reported being due to inappropriately storing or disposing of the solvent after usage (19).

In the present research, larvae of the common carrion blowflies *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) and *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae)) were reared on substrates spiked with different concentrations of EG, based on the LD_{50} for humans and/or cats. The aim of this research was to determine the effects of the presence of different concentrations of EG on the survival, developmental rate and morphology (length of larvae and pupae) on these blowfly species. Results of this research will aid future investigations where EG is a potential cause of death to both humans and animals.

2. Materials & Methods

2.1. Preparation of foodstuff and rearing of larvae

L. sericata and *L. cuprina* are green metallic blowfly species commonly found throughout the world. While *L. sericata* is present worldwide, *L. cuprina* is mostly present in Australia, North America and Africa (20). Both species are the earliest to arrive and colonise fresh decomposing corpses, and may be pivotal in minPMI estimations (20).

L. sericata and *L. cuprina* fly colonies were reared in the forensic entomology laboratory of the department of Veterinary & Life Sciences at Murdoch University, Western Australia. The design of the experiment was performed following previously described procedures by Magni et al. (7-9). Adult flies were caught from the wild near Murdoch University (-32° 03' 59.47" S; 115° 50' 6.29" E), identified by an entomologist using the keys of Smith (21) and placed in mesh cages (Bugdorm® 32.5 x 32.5 x 32.5 cm). Each cage contained one species only, and the adults were

periodically replenished to prevent inbreeding. Species used in this experiment were harvested from a fourth-generation laboratory culture. Flies were supplied with tap water and sugar *ad libitum*. A small tray containing 150 g of fresh beef liver was provided with the purpose of allowing ovary development of the females. The tray was left in the cage for 48 hours then removed. After five days, the fly species were supplied with another tray of 150 g of fresh beef liver, with the purpose of the oviposition of the females. The tray was left in the mesh cages for 3 hours to allow the deposition of a sufficient number of fly eggs and in order to use eggs of a similar age laid by different females for the purpose of the experiment. Once the tray was removed from the cage, for each species, four egg clusters of approximately 1.2 g (1000 eggs) were removed with a fine paintbrush and singularly placed in trays (17 x 12 x 5 cm) with 150 g of fresh beef liver previously prepared for the experiment. For each treatment the beef liver samples were spiked and homogenized with increasing concentrations of EG that could cause death in either a human or a cat (T1=1/2$LD_{50,}$ 2.1mL/150g; T2= $LD_{50,}$ 4.2mL/150g, T3=2$LD_{50,}$ 8.4mL/150g) (11), in addition to a control sample of beef liver containing no EG solution. It was determined from a preliminary trial that this amount (150g) was sufficient and more beef liver spiked with the same amount of EG solution was added in the treatment that needed as the trial progressed. The homogenization of the experimental beef liver involved using A11 basic Analytical mill (IKA1-Werke GmbH & Co.), followed by the dispersion of the analytical standard by using T18 digital ULTRA-TURRAX (IKA1-Werke GmbH & Co.). The trays with the experimental beef livers and egg clusters were placed in separate large plastic boxes (22 x 40 x 20 cm) containing sand 5cm deep, sealed with mesh covers and elastic bands. The experiment was performed at a laboratory temperature of ±25^{o}C with approximately 20% RH and a photoperiod (h) of 12:12

(L:D). The temperature was recorded by Tinytag data-loggers every 15 min and the average temperature was calculated every hour.

2.2. Sample collection

Collection and preservation of samples were carried out following previously standardised forensic entomology guidelines (22). Samples consisting of 30 individuals were collected from all treatments, sacrificed in hot water (<80'C) and preserved in ethanol 70%. The growth of the larvae as well as the starting of the post-feeding, the pupation and the eclosion of the adults was checked every day at the same time (9 am) during the experiment time. As reported in table s 3 and 4, the sampling of both *L. sericata and L. cuprina* started when the flies were in the second instar of life (L2) and, when possible, it continued for the entire duration of the insect immature life, considering samples in third instar (L3), post-feeding instar (PF) and pupae (P). After one day of preserving the samples, length measurements between the most distal parts of the head and the eighth abdominal segment (23) were carried out using a digital caliper (Mitutoyo Digital Caliper 6"- 500-196-30).

Another sample consisting of 100 larvae of both *L. sericata and L. cuprina* in PF instar from each treatment were collected and placed in separate boxes, in order to record the developmental time from the deposition of the eggs to the pupation and the developmental time from the deposition of the eggs to the eclosion of the adult flies. Additionally, the survival percentage to the instar of pupa and to the instar of adult was recorded. For this purpose, the total number of daily pupated individuals, the time to eclosion and the total number of emerging adults were documented.

2.3. Statistical analysis

The effect of EG on the length of *L. sericata* and *L. cuprina* in different treatments were analysed by one-way analysis of variance (ANOVA) and Tukey test. Pupation and eclosion rate were analysed by one-way ANOVA and Pearson's chi-squared test. Statistical significance was set at $p < 0.05$. Calculations were performed using IBM SPSS Statistics 22 software package.

3. Results

3.1. Developmental time

Developmental time from oviposition to pupation and from oviposition to eclosion for both of the species reared in the controlled treatments was consistent with the tables of growth available in previous research (24, 25) (Tables 1, 2). However, the developmental time of *L. cuprina* and *L. sericata* reared in T1, T2 and T3 was significantly different in the presence of the different concentrations of EG.

In particular, the developmental time to reach both the pupal and adult instar was statistically slower in comparison with the control. This was observed and analysed in *L. cuprina* reared in T2 and T3, and in *L. sericata* reared in T1 where the life cycle was completed. Meanwhile, the developmental time of *L. cuprina* reared in T3 and *L. sericata* reared in T2 and T3 could not be compared to other treatments as the specimens were unable to survive and reach the pupal instar (see 3.2).

A summary of the effects of EG on *L. cuprina* and *L. sericata*'s developmental time is reported in Tables 1 and 2.

3.2. Survival

Egg clusters of *L. cuprina* and *L. sericata* placed on the food substrate spiked with the highest concentration of EG (T3) were unable to hatch, and did not survive (Tables 1, 2).

With regards to *L. sericata*, all the specimens reared in T1 showed 100% survival until the instar of pupa, with only 72% of the pupae able to eclose into adults (Table 2). Specimens reared in T2, instead, were able to survive only until the instar of L3 (Table 2 and 4) and therefore not considered for statistical comparisons with other treatments.

On the other hand, specimens of *L. cuprina* reared in T1 and T2 were able to reach the instar of pupa and adult, but in both cases the survival was statistically less than the control (Table 1).

A summary of the effects of EG on *L. cuprina* and *L. sericata*'s survival rate is reported in Tables 1 and 2.

3.3. Larval & pupal length

As no eggs of either *L. cuprina* and *L. sericata* placed in T3 hatched no data regarding larval and pupal lengths were obtained (Table 3,4, see also 3.2). Overall, both species reared on food substrates spiked with EG showed statistically shorter lengths with respect to the control (Table 3, 4). Obtained data also show that specimens reared in T2 are statistically shorter than specimens reared in T1 (Table 4).

A summary of the effects of EG on the larval and pupal lengths of *L. cuprina* and *L. sericata* is reported in Table 3 and 4.

4. Discussion

The availability of products containing EG are widespread and easily found on the market due to high demands for the solvent's mechanical and household uses. Currently, there are many cases reporting deaths that have involved EG poisoning or suspected poisoning (10-13, 15-19, 26, 27). Obviously, there is some importance of conducting studies documenting the effects of EG on insects utilising human remains. At present this research is the first to address the effects of EG on forensically important blowflies.

Evaluation of the results provides evidence that the presence of EG in the food substrate can induce significant alterations to the morphology, the survival and the development of both the blowfly species investigated.

In particular, when the food substrate is spiked with an EG concentration that can certainly cause death in humans and cats (T3) (11, 12), egg clusters of both *L. cuprina* and *L. sericata* are not able to hatch into larvae following deposition. This of course can be extrapolated to other animals, such as dogs, where the LD_{50} is higher than humans and cats (11, 12). However, the experimental design of this research follows the structure of previous publications that considers the manual placement of egg clusters on the different treatments (7-9). Therefore, it is possible that, an adult female would simply avoid or delay the deposition of the eggs onto a toxic substrate. When conducting this study, it was noted that the food substrate spiked with EG produced a very strong and distinctive odour which may have detered the adult female fly from ovipositing on this substrate. In the light of the fact that EG products

that can be purchased have different odours – from odourless, to sweet or toxic – this aspect of the research requires further investigation.

Overall, *L. cuprina* and *L. sericata* have a similar survival rate when developing on a substrate spiked with EG, but both have a survival rate lower than the control. Both species are able to survive and complete their life cycle if the amount of EG spiked with the food substrate doesn't exceed the $1/2LD_{50}$ for humans or cats (T1 in this research). Instead, when the amount of EG in the food substrate reaches the LD_{50} for humans or cats (T2 in this research), only a few specimens of *L. cuprina* are able to survive (3% to the pupal instar, 2% to the adult instar), while no specimens of *L. sericata* reached the pupal instar and therefore the adult instar.

As well, EG effects the morphology of the immatures of both *L. cuprina* and *L. sericata.* As the concentration of EG present in the food substrate increases, the longer time it required for both species to complete their life cycle and the smaller the size of the insect (larvae and pupae). This must be taken in careful consideration when the minPMI is estimated with either the use of the isomegalen- and isomorphen-diagram (24) or with the use of the tables of growth available in the literature (21, 25).

To conclude, the aim of this research was to determine the effects that EG may have on insects utilising decomposing remains. The data obtained in this research will contribute to a more accurate minPMI in forensic investigations, when a corpse or a carcass has been allegedly poisoned with EG. Future directions of this research will be focused on the development and the validation of a gas chromatography-mass spectrometry (GC-MS) method for the detection of EG in the blowflies. This method

will allow forensic toxicologists to use insects as a non-conventional matrix for the identification of the presence of EG in remains that are under investigation (28).

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1.

Table 1

Time (days ± S.E.) from oviposition to pupation and to eclosion of *L. cuprina* larvae, which were exposed to either liver containing different amount of ethyl glycol, or to the control liver. The table shows also the number of larvae dead prior to pupation, the number of not emerged adults, and the number of survivals. The groups indicated in brackets (i.e., C, T1, T2, T3) are the ones significantly different ($P < 0.05$) from the group indicated in the corresponding column. N/A = not applicable, since larvae belonging to T3 never reached the instar of PF.

Treatment	**Control (C)**	**T1**	**T2**	**T3**
Amount of ethyl glycol spiked with liver	0 ml/150g	2.1 ml/150g	4.2 ml/150g	8.4 mL/150g
Larvae PF N=	100	100	100	N/A
Time (days) from oviposition to pupation	8.56 ± 0.15 (T1, T2)	9.92 ± 0.22 (C, T2)	15.33 ± 0.88 (C, T1)	N/A
Larvae dead prior to pupation	0	48	97	N/A
Pupae	100 (T1, T2)	52 (C, T2)	3 (C, T1)	N/A
Survival % from PF to pupation	100%	48%	3%	N/A
Pupae N=	100	52	3	N/A
Time (days) from oviposition to eclosion	18.71 ± 0.26 (T1, T2)	24.33 ± 1.28 (C, T2)	21.50 ± 0.50 (C, T1)	N/A
Not emerged adults	8	44	1	N/A
Emerged adults (Survival)	92 (T1, T2)	8 (C, T2)	2 (C, T1)	N/A
Survival % from pupation to eclosion	92%	15%	67%	N/A
Survival % from oviposition to eclosion	92%	8%	2%	N/A

Table 2

Time (days ± S.E.) and percentage of survival from oviposition to pupation and to eclosion of *L. sericata* larvae, which were exposed to either liver containing different amount of ethyl glycol, or to the control liver. The table shows also the number of larvae dead prior to pupation, the number of not emerged adults, and the number of survivals. The groups indicated in brackets (i.e., C, T1, T2, T3) are the ones significantly different ($P < 0.05$) from the group indicated in the corresponding column. N/A = not applicable, since larvae belonging to T2 and T3 never reached the instar of PF.

Treatment	Control (C)	T1	T2	T3
Amount of ethyl glycol spiked with liver	0 ml/150g	2.1 ml/150g	4.2 ml/150g	8.4 mL/150g
Larvae PF N=	100	100	N/A	N/A
Time (days) from oviposition to pupation	5.57 ± 0.05 (T1)	8.45 ± 0.07 (C)	N/A	N/A
Larvae dead prior to pupation	0	1	N/A	N/A
Pupae	100	100	N/A	N/A
Survival % from PF to pupation	100%	100%	N/A	N/A
Pupae N=	100	100	N/A	N/A
Time (days) from oviposition to eclosion	12.40 ± 0.64 (T1)	16.07 ± 0.35 (C)	N/A	N/A
Not emerged adults	3	28	N/A	N/A
Emerged adults (Survival)	97 (T1)	72 (C)	N/A	N/A
Survival % from pupation to eclosion	97%	72%	N/A	N/A
Survival % from oviposition to eclosion	97%	72%	N/A	N/A

Table 3

L. cuprina larvae and pupae mean lengths (mm ± S.E.) related to instar of life (L2=second instar, L3=third instar, PF=post-feeding instar, P=pupa instar). The groups indicated in brackets (i.e. C, T1, T2) are the ones significantly different ($P<0.05$) from the group indicated in the corresponding column. For each time of exposure and each treatment N=30. N/A = not applicable, since egg clusters placed in T3 never hatched.

L. cuprina mean length (mm ± S.E.)					
Treatment		Control (C)	T1	T2	T3
Amount of ethyl glycol spiked with liver		0 ml/150g	2.1 ml/150g	4.2 ml/150g	8.4 mL/150g
Life instar Sampling day	L2 Day 2	12.04 ± 0.15 (T1, T2)	9.01 ± 0.28 (C, T2)	4.15 ± 0.08 (C, T1)	N/A
	L3 Day 3	15.44 ± 0.12 (T1, T2)	13.08 ± 0.14 (C, T2)	4.65 ± 0.05 (C, T1)	N/A
	L3 Day 4	13.85 ± 0.18 (T2)	14.27 ± 0.12 (T2)	4.85 ± 0.15 (C, T1)	N/A
	PF Day 5	11.95 ± 0.18 (T1, T2)	11.10 ± 0.28 (C, T2)	5.10 ± 0.14 (C, T1)	N/A
	P (C=Day 8; T1= Day 9; T2=Day 15)	7.23 ± 0.06 (T1, T2)	6.75 ± 0.05 (C, T2)	4.51 ± 0.02 (C, T1)	N/A

Table 4

L. sericata larvae and pupae mean lengths (mm ± S.E.) related to instar of life (L2=second instar, L3=third instar, PF=post-feeding instar, P=pupa instar). The groups indicated in brackets (i.e. C, T1, T2) are the ones significantly different ($P<0.05$) from the group indicated in the corresponding column. For each time of exposure and each treatment N=30. N/A = not applicable, since egg clusters placed in T3 never hatched, while larvae in T2 never reached the PF instar.

L. sericata mean length (mm ± S.E.)					
Treatment		Control (C)	T1	T2	T3
Amount of ethyl glycol spiked with liver		0 ml/150g	2.1 ml/150g	4.2 ml/150g	8.4 mL/150g
Life instar Sampling d	L2 Day 2	11.18 ± 0.62 (T1, T2)	6.12 ± 0.18 (C, T2)	4.24 ± 0.07 (C, T1)	N/A
	L3 Day 3	12.78 ± 0.57 (T1, T2)	10.74 ± 0.13 (C, T2)	4.52 ± 0.07 (C, T1)	N/A
	L3 Day 4	13.30 ± 0.33 (T1, T2)	12.05 ± 0.18 (C, T2)	5.28 ± 0.12 (C, T1)	N/A
	PF Day 5	12.64 ± 0.22 (T1, T2)	11.66 ± 0.35 (C, T2)	N/A	N/A
	P (C=Day 6; T1= Day 8)	7.50 ± 0.06 (T1, T2)	5.79 ± 0.10 (C, T2)	N/A	N/A

Highlights

- Entomotoxicology considers the effects of toxicological substances in insects
- Ethylene glycol is easy to purchase, sweet and extremely toxic
- Several cases report fatalities of humans and animals caused by ethylene glycol
- Ethylene glycol affects the survival and growth rate of carrion blowflies
- Ethylene glycol affects the morphology of carrion blowflies