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Effect of pregabalin on the development of *Lucilia sericata* (Meigen, 1826; Diptera: Calliphoridae) in veterinary forensics

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Summary

Pregabalin is the active ingredient of drugs used to treat neurosis, anxiety disorders, neuropathic pain. Due to severe stress, caused by genetic factors but mainly lifestyle, many patients currently suffer from anxiety disorders, and increasingly they are turning to this type of medication. Pregabalin taken in an uncontrolled manner can cause adverse side effects, and indirectly even lead to death. Pregabalin is also used in cats to alleviate anxiety and fear associated with veterinary visits and transport. Administered as an oral solution about 1.5 hours before a scheduled trip, it significantly reduces this anxiety. It is also used to reduce pain in dogs undergoing surgery and for the treatment of neuropathic pain. The purpose of this study was to investigate the effect of pregabalin on the length of the life cycle of *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae). A culture was carried out on a meat medium in the form of a dead laboratory mouse. Observations were carried out using different doses of the active substance (administered orally to the mice): 100 mg/kg (dose 1), 200 mg/kg (dose 2) and 300 mg/kg (dose 3). A control sample (without the drug) was used for comparison. The results showed that pregabalin prolonged the life cycle of the fly relative to the control without the addition of the drug. In addition, it caused a decrease in the body weight of the larvae and pupae and increased their mortality relative to the control sample. By contributing to an understanding of the mechanism of bioaccumulation, this type of study provides valuable information for more accurate estimation of the time of death.

Keywords: forensic entomology, forensic veterinary medicine, drugs, *Lucilia sericata*, pregabalin, post mortem interval

By examining the insects present on a body, the forensic entomologist is able to determine the post-mortem interval and provide helpful information during preliminary proceedings, i.e. an investigation or inquiry (15, 19). For medical-forensic entomology, it is crucial to know the life cycles of the main representatives of necrophagous insects (especially flies) (37). The insects most commonly colonizing dead tissues include necrophagous flies of the family Calliphoridae, such as *Lucilia sericata* (11, 15).

Lucilia sericata is a fly with a characteristic green metallic shine, known all over the world and of great importance in forensic entomology, because its presence on a body can be used to estimate the time of death (12, 15, 17). Eggs may be present on the body just a few hours after death, and larvae are present a few hours later.

The succession of insects and their life cycle are important elements in determining the post-mortem interval (15). However, there are factors that may disturb the development of insects in various ways, accelerating or delaying it. Atmospheric factors have the greatest impact, but various foreign substances may play a role as well. Toxic substances in or on the body are ingested by insects and accumulate in them (2, 9, 11, 16, 18, 36, 41). Various toxicological analyses make it possible to detect the cause of death and can be used as evidence in court, especially in the case of poisoning or overdose of medicines, illegal drugs, or other intoxicants (39).

Pregabalin is a pharmacological agent belonging to the group of GABA neurotransmitters, a derivative of γ-aminobutyric acid (1). The general cellular mechanism of action of pregabalin involves reducing the release of several types of neurotransmitters, including glutamate and noradrenalin. Pregabalin has very broad applications in the treatment of anxiety disorders and in preventing epileptic seizures, including in dogs, but also alleviates neuropathic pain, fibromyalgia, restless leg syndrome, and generalized anxiety (1, 30, 31, 34, 43). Pregabalin has similar effects in the treatment of generalized anxiety disorder to those of antidepressants containing serotonin, an important neurotransmitter in the central nervous system (40). Unfortunately, side effects of pregabalin have been observed in many patients, most often involving disorders of the central nervous system (14, 42). There have also been isolated reports of pregabalin abuse or dependence and even fatalities, mainly involving the simultaneous use of pregabalin and other medicines or alcohol (7, 22, 27). Auditory hallucinations and suicidal thoughts resulting from pregabalin abuse have been observed.

Pregabalin is also used in cats to alleviate anxiety and fear accompanying veterinary visits and transport (23). To alleviate this fear, pregabalin can be given in the form of an oral solution about 1.5 hours before a journey. At a dose of just 5 mg/kg pregabalin statistically significantly reduces anxiety associated with travel ($p < 0.01$) and veterinary visits ($p < 0.01$) compared with a placebo. Pregabalin alleviates pain in dogs undergoing surgery and is used to treat neuropathic pain (33, 35).

The main purpose of the study was to observe the length of the life cycle of *Lucilia sericata* (Calliphoridae) under the influence of the drug pregabalin at three different doses. An attempt was made to determine the duration of the fly's individual development stages, the condition and viability of the larvae, and assessment of body weight of particular development stages (larvae, pupae and adults). The results can provide valuable information for investigations using *Lucilia sericata* in veterinary forensics and forensic entomology in cases when pregabalin overdose is a potential cause of death.

Material and methods

Laboratory trial. The study was carried out in laboratory conditions at ~25°C and relative humidity(RH) ~65%, using a common species of fly of the family Calliphoridae, *Lucilia sericata* (Meigen, 1826), frequently used in forensic entomology.

The biological material used for the experiment consisted of already dead mice. The dead mice were taken from Department of Animal Physiology and Pharmacology in the Institute of Biological Sciences, Faculty of Biology and Biotechnology, Maria Curie-Skłodowska University in Lublin. All procedures related to the killing of animals were conducted in accordance with the European Union Directive (2010/63/EU) and Polish legislation acts. The detailed procedure of killing the mice can be found in the following papers: 25, 26, 38.

Pregabalin was administered to live mice at various doses: 100 mg (dose 1), 200 mg (dose 2) and 300 mg (dose 3). The dead mice were exposed outdoors as bait where *Lucilia sericata* had a free access to them to lay eggs. A control sample without pregabalin was used for comparison of the results. When numerous egg masses had appeared on the material, the samples were placed in plastic containers, covered with air-permeable material, and then shut with a lid. When second-stage larvae appeared, on day 3 of the experiment, they were weighed for the first time. In total they were weighed six times. As the entire life cycle lasted three weeks, weighing was performed twice a week, using an OHAUS PIONEER analytical balance. A total of 120 *L. sericata* larvae were used in the analysis, i.e. 30 larvae in all treatments including the control. The viability and condition of the larvae were monitored every day during the experiment. Pupae were transferred to separate containers and monitored until adult individuals appeared.

Statistical analyses. Descriptive statistics (sums, means, range, medians, and standard deviation) were calculated using PAST ver. 4.09 (13). The normality of the data distribution was checked by the Shapiro-Wilk test. The non-parametric Kruskal-Wallis test for multiple comparisons (H) was used because some of the data had a normal distribution and some did not. The non-parametric Mann-Whitney U test (Z) was used as a post-hoc test. The Shapiro-Wilk, Kruskal-Wallis and Mann-Whitney U tests as well as the graphs were performed using Statistica 13.1. The level of significance was $p = 0.05$.

Because only larvae were present at the time of the first and second weighing, statistical analyses were performed for larvae (Kruskal-Wallis test for the experimental treatments). As pupae were present at the third and fourth weighing, full statistical analyses were carried out for pupae (Kruskal-Wallis test for the experimental treatments). During the fifth and sixth weighing, the number of pupae was in complete due to transformation into imagines, so the data for pupal weight were used only for analyses for the entire duration of the experiment, without analysing differences in the weight of pupae on individual weighing days. Due to the unequal numbers of adult specimens in different experimental treatments on individual weighing days, a single analysis was performed for adult specimens (without separate analyses for weighing days 5 and 6, when imagines were present).

Results and discussion

Larval body weight. Differences in the weight of larvae between treatments were not statistically significant ($H (3, N = 240) = 7.276461$, $p = 0.0636$). The highest values in larval body mass were recorded in the treatment with dose 1. These were: the highest larval weight (0.06737 g), the highest average larval weight (0.03177 g ± 0.02336) and the highest median (0.02690 g) – Figure 1. The lowest values in larval body mass were recorded in the treatment with dose 2. These were: the lowest larval weight (0.00359 g), the lowest average larval weight (0.02729 g ± 0.01881) and the lowest median (0.02212 g) – Figure 1.

Pupal body weight. Differences in the weight of pupae between experimental treatments were statistically significant (H (3, N = 303) = 16.97493, p = 0.0007). The post hoc tests showed statistically significant differences between dose 1 and dose 2 (Z = 3.68, p = 0.0014) and between dose 1 and dose 3 (Z = 3.32, p = 0.0054). The highest (0.05113 g) and lowest (0.00881 g) pupal weights were noted at dose 1 (Fig. 2). The highest average pupal weight (0.04022 g ± 0.00757) as well as the highest median (0.04052 g) were recorded also in the treatment with dose 1. The lowest average pupal weight (0.03628 g ± 0.00958) was noted at dose 3 while the lowest median (0.03714 g) was recorded at dose 2 (Fig. 2).

Imago body weight. Differences in the weight of imagines between experimental treatments were statistically significant (H (3, N = 95) = 19.23185, p = 0.0002). The post hoc tests showed statistically significant differences between the control sample and dose 1 (Z = 4.34, p < 0.0001) and between dose 1 and dose 2 (Z = 2.64, p = 0.0490). The highest (0.03765 g) imago body weight was noted at dose 1 and the lowest (0.00422 g) at dose 2 (Fig. 3). The highest average imago body weight (0.02810 g ± 0.00743) and the highest median (0.02906 g) were recorded in the control sample and lowest average imago body weight (0.01618 g ± 0.00962) and median (0.01116 g) values were noted at dose 1 (Fig. 3).

Growth rate of *Lucilia sericata*. The study showed that the foreign substance affected the growth rate of *Lucilia sericata* at various doses. For all treatments the minimum development time was 13 days. However, the maximum development time increased by two days relative to the control at a dose of 100 mg, lasting 16 days, and by three days at doses of 200 mg and 300 mg, lasting 17 days (Tab. 1).

The shortest time noted for the development of the third larval stage was the same for all treatments, at three days, while the longest time was extended by two or three days in the treatments with the drug relative to the control. The minimum time recorded for pupal development was also the same in all treatments, at

Tab. 1. Length (in days) of *Lucilia sericata* life cycle in the experimental treatments

Stage of development	Experimental treatment			
	Control	100 mg	200 mg	300 mg
Egg	1	1	1	1
1st stage larva	1	1	1	1
2nd stage larva	1	1	1	1
3rd stage larva	3	3-6	3-6	3-5
Pupa	7-8	7-8	7-9	7-10
Length of cycle	13-14	13-16	13-17	13-17

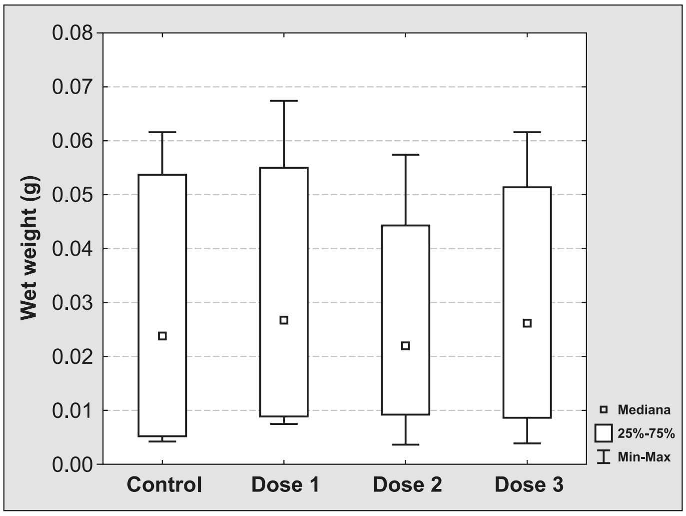

Fig. 1. Difference in the body weight of *Lucilia sericata* larvae during the experiment

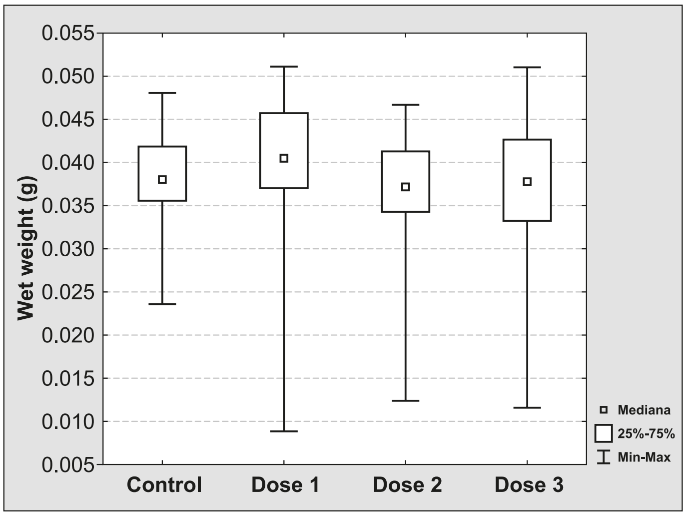

Fig. 2. Differences in the body weight of *Lucilia sericata* pupae during the experiment

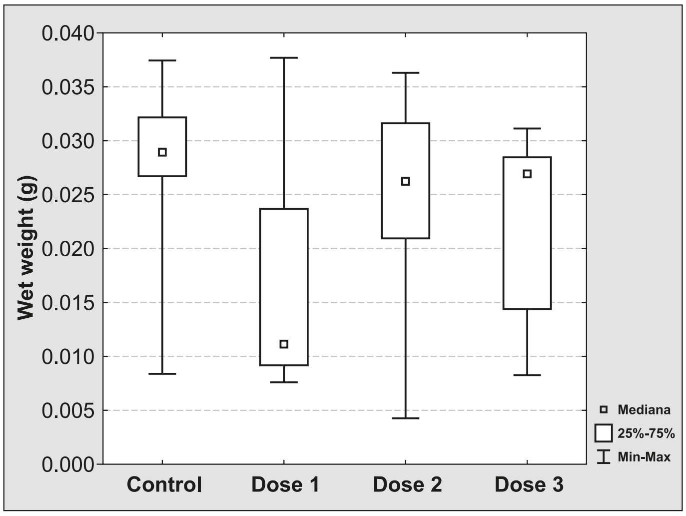

Fig. 3. Differences in the body weight of *Lucilia sericata* imagines during the experiment

seven days. The maximum time was eight days in both the control and at the lowest concentration of the drug (100 mg), and increased by one day with each successive dose – to nine days at 200 mg and 10 days at 300 mg.

The larval stage was present only on the first and second weighing days. The first weighing day was the third day of the life cycle of the insects, and the second weighing day was the sixth day of their life cycle. In all treatments, including the control, only pupae were present on day 12 of the life cycle, i.e. the fourth weighing day. On the fifth weighing day, i.e. the 15th day of the life cycle, there were only four pupae in the control sample, while there were 23 in the sample with dose 1, eight in the sample with dose 2, and 26 in the sample with dose 3, including 10 that had dried up. On day 18 of the experiment, i.e. the sixth weighing day, there were no pupae in the control sample or at dose 2 of the drug, while there were three pupae at dose 1 and 12 at dose 3, including three that were dried up (Fig. 4). On day 15 of the life cycle, before the fifth weighing day, adult individuals were present in all treatments. The most imagines were found in the control sample (26 specimens) and at dose 2 (22 specimens), fewer at dose 1 (7 specimens), and the fewest at dose 3 (4 specimens).

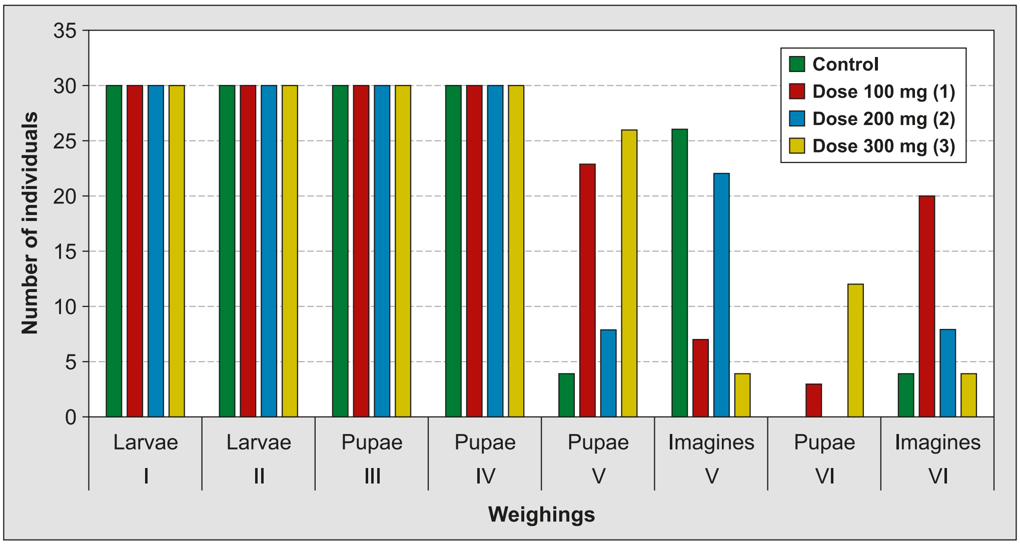

Fig. 4. Numbers of *Lucilia sericata* larvae and pupae during the experiment

Mortality of *Lucilia sericata* larvae and pupae. The study showed high mortality among pupae. The highest mortality was recorded in the samples with the highest dose of the drug (300 mg/kg). All 30 larvae entered the pupal stage, but only 17 developed into adults.

Necrophagous insects, especially their larvae, are of great value to the forensic entomologist and investigators, because when they feed on a body they provide important information that can be crucial in resolving certain doubts. When death is caused by poisoning with various toxic substances or heavy metals, and the dead body is in an advanced stage of decomposition precluding the necessary analyses on internal organs, insect larvae in which foreign substances are accumulated may be helpful (18). The detection of chemical substances which may have caused a person's death in the body of necrophagous insects is important because these substances can influence decomposition rate of the body and thus the insects' rate of development (15, 36, 41).

Experiments by Gosselin et al. (9) using methadone in *L. sericata* showed that sometimes both the foreign substance and its metabolites are detected, while in other cases it is not even possible to detect the foreign substance, because the larvae eliminate the metabolites very quickly.

Our research showed that pregabalin prolonged the life cycle of *Lucilia sericata* by 2-3 days relative to the control. For the control group, the maximum time for full development was two weeks. This time was extended by two days at the dose of 100 mg/kg of the drug and by three days at 200 mg/kg and 300 mg/kg. Development was prolonged at the third larval stage and then in the pupal stage.

Some substances, such as cocaine, are known to shorten the life cycle of insects, while others prolong it, such as petrol, hydrocortisone, malathion, arsenic, carbon monoxide, orhyoscine butylbromide (5, 10, 15). Heroin and morphine prolonged the life cycle of *Sarcophaga peregrine* and *Lucilia sericata* (3, 8). In *Chrysomya albiceps*, tramadol hydrochloride, an opioid, slowed down the life cycle by two days relative to controls (6). In *Chrysomya albiceps* and *Chrysomya putoria*, when diazepam (a psychotropic drug) was added to the medium, larval growth increased rapidly compared to the control without the drug, thus shortening the life cycle of the flies (4).

Some substances may have visible effects only for a short time, such as paracetamol, which in *Calliphora vicina* larvae accelerates development between the second and fourth days of life, leading to a 12-hour difference in estimates of the post-mortem interval (28). Various studies have shown that the same foreign substance may cause different reactions in different species of insects. An example is ketamine, an anaesthetic and analgesic, which has been shown to accelerate the life cycle at the larval stage in *Lucilia sericata* but to prolong it in *Calliphora vomitoria* (44).

Our research showed that pregabalin caused high mortality in the pupae from the biological material with the highest dose of 300 mg/kg, which dried up and shrunk. Some substances reduce the survival rate of flies at the larval and pupal stages. Experimental studies have shown that this effect may be induced by increasing concentrations of elements such as cadmium (Cd), zinc (Zn), copper (Cu), antimony (Sb), barium (Ba) or lead (Pb) in the food of *L. sericata* larvae or by chemical substances such as thiopental, which caused high mortality in *Calliphora vicina* larvae, or ketamine, which had the same effect in *Calliphora vomitoria* (20, 21, 32, 44).

A foreign substance can also affect the body weight of larvae and pupae. Oliveira et al. (29) showed that the analgesic andante spasmodic drug Buscopan slowed down the life cycle of *Chysomya megacephala* and caused a decrease in its body weight. Another study using ketaminein a medium for *Calliphora vomitoria* showed an increase in the body weight of the larvae (44). Studies using metals such as cadmium (Cd) and thallium (Tl) in the food substrate showed the highest average cadmium content in the larvae, while in the case of thallium the highest bioaccumulation factor was noted in the pupae (24). The presence of thallium had a negative effect on larval growth, resulting in pupae of lower average weight and smaller size. As the cadmium concentration in the medium increased, the average weight of the larvae and pupae decreased. In the present study using pregabalin, all doses applied decreased the body weight of *Lucilia sericata* larvae, pupae and imagines. Although the differences in larval weight were not statistically significant, the result was close to significance ($p = 0.0636$), while in the case of pupae and imagines, the differences in body weight were statistically significant.

The examples of analyses conducted by the authors cited above are unfortunately costly, and to minimize error there is a need for many such experiments, which will allow us to understand the basic physiological processes of necrophagous insects (metabolism, bioaccumulation, and excretion of foreign substances).

The study using pregabalin showed that simple observations of biological development are sometimes sufficient to show the effect of a foreign substance on life cycle length, numbers of individuals, and body weight.

Conclusions:

1. Pregabalin had the following effects on the development of *Lucilia sericata*:

a. Prolongation of the life cycle of *L. sericata*. The minimum duration of the complete cycle was 13 days in all experimental treatments, while the maximum development time increased with the concentration of the drug, by 2-3 days relative to the control.

b. A reduction in body weight at each stage of development (larva, pupa and imago).

c. High pupal mortality in the samples with the highest dose of the drug (300 mg/kg).

2. The present study showed that simple observations of development parameters, instead of expensive and complicated bioaccumulation and ecotoxicology analyses, are useful to show the effect of pregabalin concentrations in corpses on life cycle length, numbers of individuals, and body weight of *Lucilia sericata* – the fly used in veterinary forensic and forensic entomology.

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