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RESEARCH

Effects of ivermectin on development of *Calliphora vicina*, Robineau-Desvoidy 1830 (Diptera, Calliphoridae)

Lucía E. Iglesias[1] · Milagros Junco[1] · Juan M. Sallovitz[2] · Adrián L. Lifschitz[2] · Carlos A. Saumell[1]

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Abstract

Ivermectin is one of the most widely used drugs for parasite control. Previous studies have shown a reduction in the abundance and diversity of "non-target" coprophilous organisms due to the presence of ivermectin (IVM) in bovine faecal matter (FM). Due to its breadth of behavioural habits, *Calliphora vicina* is a suitable dipteran species to evaluate the effects of IVM in FM. The aim of this work was to evaluate the effect of five concentrations of IVM in FM (3000, 300, 100, 30, and 3 ng/g) on the development of *C. vicina*. The following endpoints were evaluated: survival (between the first larval stage and emergence of new adults), larval development times to pupation and pupation times to adult, and adult emergence (% sex) and LC_{50}. Sampling was performed from larval hatching at 60 and 120 min and at 3, 4, 5, and 12 h, and every 24 h specimens were weighed until pupae were observed. Data were analysed by ANOVA using a non-parametric Kruskal–Wallis test and as a function of elapsed development time and accumulated degree hours (ADH). Mortality at 3000 and 300 ng/g was 100% and 97%, respectively. There were statistically significant delays in adult emergence time ($p = 0.0216$) and in the ADH ($p = 0.0431$) between the control group (C) and 100 ng/g. The LC_{50} was determined at 5.6 ng/g. These results demonstrate the lethal and sub-lethal effects of IVM on C. *vicina*, while highlighting the usefulness of this species as a bioindicator for ecotoxicological studies.

Keywords Ecotoxicology · Dung pats · Coprofauna · Anthelmintic drug · Blowfly

Introduction

Calliphora vicina, Robineau-Desvoidy 1830 (Diptera: Calliphoridae), is one of the neotropical calyptrate dipteran species with sarcosaprophagous habits, cosmopolitan, and with marked synanthropy (Mulieri et al. 2014). It also has a tendency to endophilia (Faucherre et al. 1999; Mariluis et al. 2008) and high tolerance to low temperatures (Faucherre et al. 1999; Costamagna et al. 2007; Mariluis et al. 2008), which allows its wide distribution and permanence even in cold months in different regions of Argentina (Schnack et al. 1995; Mariluis and Mulieri 2003; Costamagna et al. 2007; Mariluis et al. 2008). Adult specimens, commonly called "blowflies", are characterised by the presence of yellow or orange-yellow basicosta, the reddish gena at least in the anterior half, the lower calyptra with dorsal pilosity (Mariluis 1981; Whitworth 2006; Mulieri et al. 2014), and a metallic blue colouring of the abdomen, the male of this species being holoptic and the female dichoptic. In relation to their feeding habits, they are attracted by plant secretions such as nectar and by flowers with odours similar to those of decomposing organic matter or by exudates released from that source.

The calliphorids are holometabolous insects, so their life cycle includes egg, larval, pupal, and imago or adult stages. Females of *C. vicina* oviposit on different organic substrates on which the hatched larvae feed, performing this function over a wider temperature range than other insects.

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✉ Lucía E. Iglesias
luciaemi@vet.unicen.edu.ar

[1] Área Parasitología y Enfermedades Parasitarias, Dpto. Sanidad Animal y Medicina Preventiva, Centro Investigaciones Sanidad Animal Pública y Ambiental (CISAPA), CIVETAN (UNCPBA-CICPBA-CONICET), Facultad de Ciencias Veterinarias, UNCPBA, Buenos Aires, Argentina

[2] Laboratorio de Farmacología, Dpto. Fisiopatología, CIVETAN (UNCPBA-CICPBA-CONICET), Facultad de Ciencias Veterinarias, UNCPBA, Buenos Aires, Argentina

Descriptive works on preimaginal stages are scarce due to the similar morphology of related species. Among them, Florez and Wolff (2009) elaborated an identification key for immature stages of calliphorids. In the meantime, a comparative study of the three larval stages and a description of the pupal stage of *C. vicina* was contributed by Ubero-Pascal et al. (2012). This study was intended for application in forensic entomology. The detailed work carried out by these authors allows the recognition of structures with significant changes during development, which is of special interest in the determination of postmortem interval (PMI) and highlights aspects of importance in forensic medicine as well as in the field of entomotoxicology.

Like many sarcophagid dipterans, certain calliphorids can facultatively develop myiasis and coprophagous habits, collaborating in the removal of material (Mariluis et al. 2008; Wolff 2010). As such, their specimens can potentially develop the function of bio-indicators, in the first case of PMIs as well as of chemical residues in the faeces of treated animals.

Due to the breadth of behavioural habits, the seasonal distribution in our region, the biotic potential, and the possibility of laboratory rearing on substrates rich in organic matter, *C. vicina* is a suitable dipteran species to assess the effects of IVM present in FM.

Among the various assessment mechanisms, the determination of effects on developmental stages is frequently used. The rate of development and growth of insect larvae can be obtained, among other methods, by calculating the number of accumulated degree days or degree hours (ADD and ADH respectively). Degree-days (°D) represent the accumulation of heat units above a certain temperature, which is the minimum threshold for development, during a period of 1 day. For each day, the difference between the mean daily temperature and the minimum development threshold is calculated. In forensic entomology studies, larval size is considered one of the parameters to determine the minimum PMI, which is related to larval age as a function of time and temperature (Donovan et al. 2006).

However, other environmental factors may have an effect on development that is not negligible with respect to the effect of temperature, such as relative humidity or photoperiod (Marco-Mancebón, 2001).

The objective of this work was to assess the impact of different concentrations of IVM on the growth and development of the dipteran species *Calliphora vicina*, Robineau-Desvoidy. The study focused on several endpoints, including percentage of survival, emergence time of larval, pupal and adult stages (considering sex percentages), and the LC_{50} estimation.

Materials and methods

Location

The different stages of this work were carried out at the Laboratory of Parasitology and Parasitic Diseases, Department of Animal Health and Preventive Medicine (SAMP-CISAPA), Faculty of Veterinary Sciences, National University of the Centre of the Province of Buenos Aires (FCV, UNCPBA), CIVETAN (UNCPBA-CICPBA-CONICET).

Faecal substrate and flies' specimens

The species *Calliphora vicina* (Robineau-Desvoidy), captured in the environment by means of a baited trap, was chosen because its properties allow its cultivation under laboratory conditions. The FM was obtained from Holstein cows (Holando Argentino) of approximately 1 year of age, without previous antiparasitic treatment.

Taxonomic determination of the species, *C. vicina*, was carried out according to the identification keys of Whitworth (2006) and Mulieri et al. (2014). Diet, temperature, humidity, and photoperiod of the adults were as those suggested by the OECD (2016) protocol 228, using a specially designed rearing chamber (Fig. 1).

Chemical reagents

Five concentrations were prepared from a commercial solution of IVM (Bagomectin®, 1% IVM, Series 100). The dilutions in ethanol (99% purity) were prepared as described in previous trials (Iglesias et al. 2018, 2022): high IVM concentrations evaluated in these works (3000 and 300 ng/g) were used to assess survival, while lower IVM concentrations (100, 30, and 3 ng/g) to record sub-lethal effects on the specimens.

Rearing and sampling methodology

The diet of the adults consisted of a mixture of equal parts of sucrose, skimmed milk powder, and dehydrated and pulverised egg yolk. Temperature conditions were adjusted to those relative to the biological cycle based on literature. It was recorded by an electronic sensor (Datalogger Hygrochron iButton®), being the maximum temperature (Tmax) 31.78 °C, the minimum temperature (Tmin) of 22.79 °C resulting in an average temperature of 27.54 °C. The photoperiod was set to12/12 h, regulated by a programmable clock.

To stimulate oviposition, fresh drug-free pig liver was offered. After hatching, larvae of instar I (L1) were

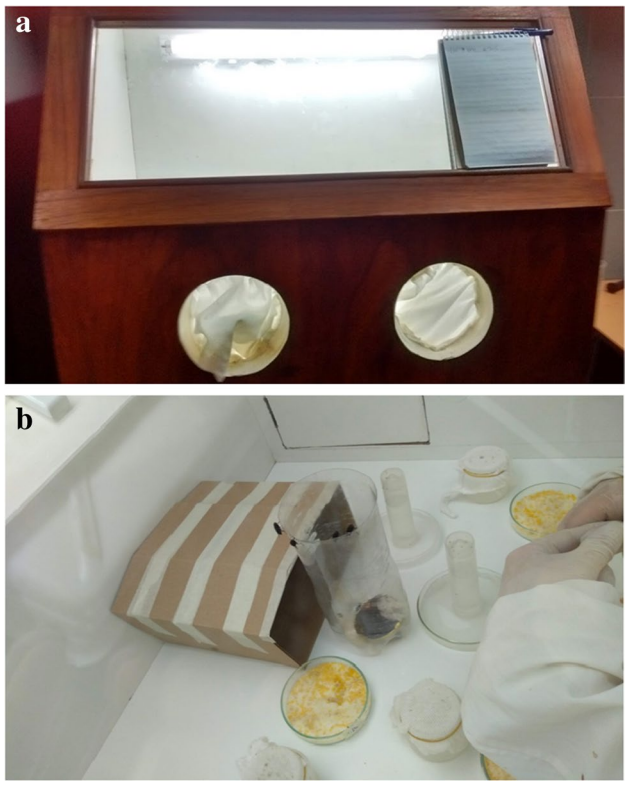

Fig. 1 **a** Rearing chamber used for the development of *C. vicina*; **b** inside of chamber with diet and hydration devices and plates with pig liver for oviposition

transferred to the rearing substrate to complete their development and obtain new generations. The substrate consisted of a mixture of bovine FM, free of anti-parasitic drugs, obtained within 2 h of deposition, kept for at least 3 weeks in freezer (dry matter 28.8%), and bone meal 10% to enrich the protein nutrient content. At completion of the larval stage or instar III (L3) (pre-pupal or also called post-feeding stage), the larvae started to "wander" away from the rearing substrate and contracted into a barrel shape when touched (Donovan et al. 2006).

When this phase of the cycle was recognised, pre-pupae were transferred to a sawdust substrate (resin-free) for pupal development and adult emergence. After obtaining six generations, the toxicity of five concentrations of IVM in FM (3000, 300, 100, 30, and 3 ng/g) was evaluated according to the following endpoints: survival of larval specimens, development times of the different biological stages, mean lethal concentration (LC_{50}), and adult emergence (considering sex percentages).

For each experimental treatment (control without addition of IVM and five different IVM addition levels), five replicates were prepared and each one contained 200 g of FM. Prior to replicate's preparation for each treatment, 30 ml of the corresponding IVM concentration were added to 1 kg of FM; in the case of the control group, a same volume of ethanol was added. Homogenisation of each mixture was performed with an electric mixer for 5 min. The mixtures were left stand for 24 h at room temperature in order to allow the evaporation of the alcohol.

After adjusting the rearing methodology, L1 hatched on pig liver substrate were collected with a brush dipped in distilled water. Then, 50 specimens were placed in each rearing bottle containing 200 g of FM substrate per replicate. This ensured substrate availability of 4 g/larva. In each sampling, the presence and mobility of larvae on substrate, gut contents, and substrate ingestion were observed.

The sampling frequency was determined according to the morphological changes that allow the recognition of the different larval stages, as reported in the literature (Florez and Wolff 2009; Ubero-Pascal et al. 2012).

Specimens were observed at 60 min and 120 min. Observations were then made at 3, 4, 5, and 12 h and then every 24 h until pupae formation was observed. At this time, they were transferred to the sawdust substrate to complete the pupal period. After 24 h, ten specimens randomly sampled from each replicate were weighed on a precision balance (Mettler Toledo ME204) and the data were recorded. To perform this practice, and in order to avoid any artefacts, each specimen was washed with distilled water, cleansed with a fine brush, and excess water was removed with filter paper.

Statistical analysis

Weight data obtained were analysed by ANOVA using a Kruskal–Wallis non-parametric test. For assessing differences in developmental stage, data were analysed as a function of elapsed time in the L1–L3/pre-pupal and pupa-adult stages. Development was also analysed in terms of cumulative degree hours (ADH) (Donovan et al. 2006; Salimi et al. 2018). Percentage of mortality was plotted on a dose–response curve, and LC_{50} was calculated using the same software (GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

At the first observation (60 min), no L1 vitality was recorded in the FM spiked with IVM 3000 ng/g, demonstrating 100% mortality in this group at the start of the trial.

Meanwhile, at the same sampling time, L1 feeding activity was observed on FM spiked with 300 ng/g and C (no IVM) substrates. At 48 h, few specimens were observed on substrate spiked with 300 ng/g under binocular stereoscopic microscope. At 192 h (8 days), pupae were obtained from group C specimens, while no specimens were observed in

replicates 1 to 3 of the rearing flasks containing FM spiked with IVM 300 ng/g. In the remaining flasks, the few L3 specimens developed to pupae or pre-pupae showing a delaying effect of 2 days compared to those in the C group.

Fly emergence in group C began after 19 days (456 h) in all replicates (C1 to C5). This observation coincided with the cycle times recorded in the pre-trial stage of the present work. Meanwhile, there was no emergence in the group treated with IVM 300 ng/g before 22 days (528 h) after the trial beginning, which denotes a delay in both evolution and emergence times, being in accordance with what was observed in larval development.

A survival rate up to adult emergence of 3% was recorded in the treated IVM 300 ng/g replicates, while in the control group (C) 80% of the specimens developed.

Hence, a concentration effect in the treated IVM 300 ng/g group was observed on survival and evolution times, with a 3-day delay in adult emergence. However, it was not possible to determine all the proposed study parameters due to the 100% larval mortality in the treated IVM 3000 ng/g group and the low (3%) survival of individuals in the treated IVM 300 ng/g group.

Based on these results in the treated IVM 300 ng/g group, a second phase of toxicity bioassay was carried out to evaluate the IVM effect at lower concentrations, i.e., 100, 30, and 3 ng/g in bovine FM on the development of *C. vicina*. Table 1 summarises the observations made for all IVM concentrations used. The value sex M/F (%) corresponds to the percentage of adults by sex (male/female) in relation to the total number of L1 placed on substrate (50 specimens).

Considering the survival results at the higher IVM concentrations, no statistical analysis could be performed and only results with lower IVM concentrations were statistically analysed.

Table 1 Parameters used to evaluate the toxicity of five concentrations of IVM on the development of *Calliphora vicina*: survival (percentage), duration of life stages (average), sex ratio at adult emergence, and lethal concentration 50 (LC_{50})

Group	Survival (%)	Duration of instars in days (average)			Sex M/F (%)*
		Cycle	L1-pupa	Pupa-adult	
Control*	80.23	19.5	11.3	10.25	36/35.4
3 ng/g	50.40	23.4	11.4	12	22.4/22
5.6 ng/g LC_{50}					
30 ng/g	35.20	23.4	11	12.4	21.2/10.8
100 ng/g	41.60	24.2	11	13.2	14.4/24.4
300 ng/g	3	23	11	12	–
3000 ng/g	–	–	–	–	–

*Average of both control groups; – without specimens; M/F: male, female; *percentage of adults by sex (M/F) in relation to the total number of L1 placed on substrate

When analysing the results obtained with lower IVM concentrations of 3, 30, and 100 ng/g, a statistically significant difference in pupation to adult times was only observed between the control group and the 100 ng/g-treated group ($p = 0.0431$).

The LC_{50} value (5.6 ng/g IC95, 2.42–14.9 ng /g) was calculated to be close to the lowest concentration used in the bioassay, which resulted in mortality of 49.6% of the tested population (Table 1).

Figure 2 shows, as a dose–response curve, the mortality percentage for the five IVM concentrations.

In the second phase trials, when lower concentrations of IVM were used (3, 30, and 100 ng/g), larval weight was recorded after 24 h as a developmental parameter. The comparative representation of larval weight until pupation is shown in Fig. 3.

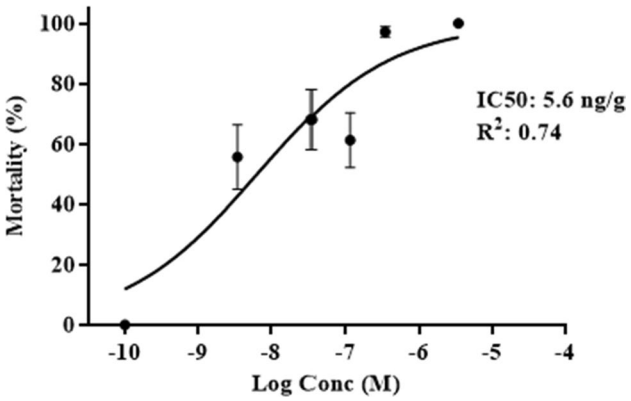

Fig. 2 Percentage of larval mortality of *Calliphora. vicina* in relation to IVM concentration in substrate, expressed as logarithm of IVM molar concentration (M). The points with their standard deviation correspond to the control groups (without addition of IVM), and treated groups (IVM: 3, 30, 100, 300, and 3000 ng/g)

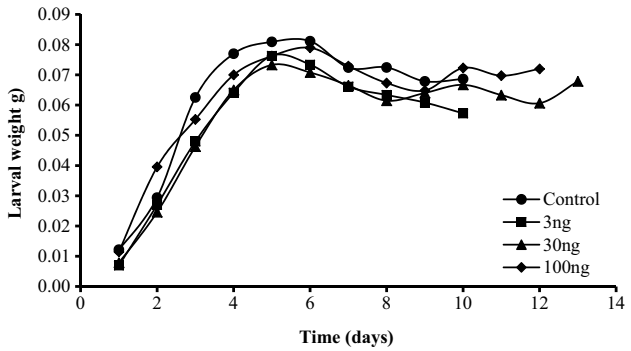

Fig. 3 Variations of larval weight over time during development from L1 to pupa. Each representative line of the experimental groups (control, 3, 30, and 100 ng) is interrupted when the pupal stage is reached. Each experimental group tine is drawn until the time the pupal stage is reached

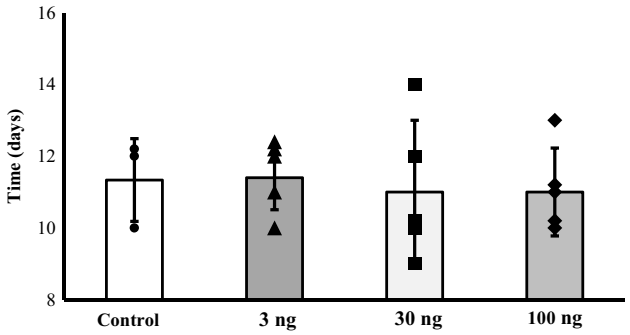

Fig. 4 Development time (days) of *Calliphora vicina* from L1 to pupa in control and IVM-treated experimental groups at concentrations of 3, 30, and 100 ng/g in bovine faeces

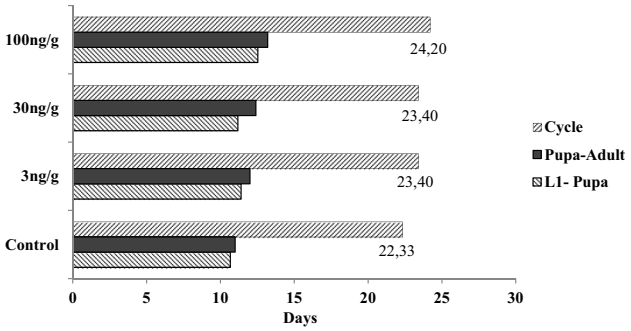

Fig. 6 Average development time for L1-pupa, pupa-adult stages, and cycle length in days

When analysing the larval weights of the different groups (control and IVM-treated) versus time of the evolution rages, i.e., between L1 to L3-pre-pupal and pupal to adult stages, as well as from L1 to L3-pre-pupal as a function of ADH, a statistically significant difference was only observed in the pupa-to-adult evolution times between the control group and the group treated with 100 ng/g in the weight-time analysis ($p = 0.0216$) (Fig. 4), as well as in the ADH ($p = 0.0431$) (Fig. 5). Figure 6 summarises the evolution times between larval, pupal and adult stages, and the duration of the biological cycle for each experimental group.

The LC_{50} determined was 5.6 ng/g. Meanwhile, at a concentration of 300 ng/g, the specimen's mortality reached 97%, which would represent almost 100% lethality, as initially obtained with the IVM concentration of 3000 ng/g (Table 1).

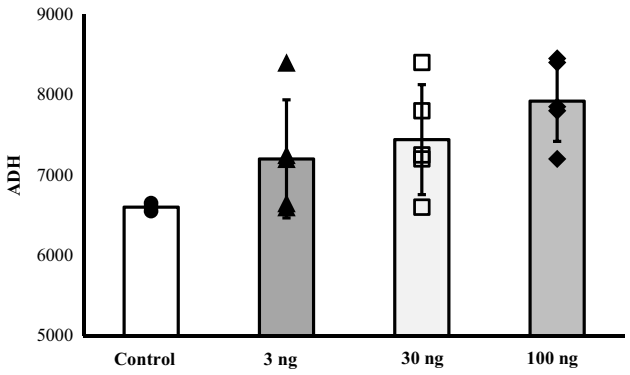

Fig. 5 Average ADH invested by each group (control and IVM-treated: 3, 30, and 100 ng/g) in the period from pupa to adult. The maximum value (7920) corresponded to 100 ng/g and the minimum (6600 ADH) to the control group ($p < 0.05$)

Discussion

Previous studies confirmed the alteration of the coprofauna in faecal masses determined by IVM-treated bovine faeces (Iglesias 1998), highlighting the reduction in both the abundance and diversity of this type of organisms (Iglesias et al. 2006). These reductions were greater on days 1, 3, and 7 post-treatments (dpt), coinciding with the highest IVM concentrations in the faecal masses during an experimental period of 60 days. Among coprophilous arthropods, the Cyclorrapha dipterans (Suborder Brachycera) are more susceptible to IVM compared with other insect orders (Madsen et al. 1990; Floate 1998; Lumaret et al. 2012).

Their feeding and behavioural habits determine early exposure to deworming drug residues in bovine faeces, as these organisms frequent and enter faecal masses in the early stages of colonisation (Desière 1973; Lumaret et al. 2007; Tixier et al. 2015). The immature stages of dipterans were the most affected by the presence of the antiparasitic drug in bovine faeces, an effect that was maintained throughout the experimental time and, with statistical significance, at 3 and 7 dpt.

However, for the selected species, no baseline data were available on the toxicity caused by IVM at concentrations that could be quantified in bovine faeces after treatment. Although the trials were conducted under controlled laboratory conditions and for a limited time, survival data on substrates with different concentrations of IVM showed the effect of this molecule at different elimination times.

Different authors determined the IVM concentration in faeces after subcutaneous or pour-on administration at the therapeutic dose. In the evaluation by Perez Cogollo et al. (2017) were determined 890 ng/g IVM at 3 dpt and 43.9 ng/g at 28 dpt. Moreno Morales et al. (2014) found a concentration of 876 ng/g at 2 dpt and 1.7 ng/g at 20 dpt. Regarding pour on administration, Herd et al. (1996) at 2 dpt found a concentration of 1850 ng/g and 40 ng/g at day 40. In the present research work, minimum and maximum

concentrations were evaluated, which recognise the different concentrations found in the literature.

For example, in previous studies, the maximum IVM concentration in bovine faeces after subcutaneous treatment at the recommended therapeutic dose (0.2 mg/kg) during autumn was 717 ng/g at the onset of colonisation (Iglesias et al. 2006). Even at a concentration close to half of the value reported by Iglesias et al. (2006) (i.e. 300 ng/g), mortality larval stages of *C vicina* was 97%. The lowest concentration used in the toxicity bioassay (3 ng/g) is close to the determined LC_{50} (5.6 ng/g), i.e., the concentration likely to kill 50% of the population of this species. Although the precise elimination time of this concentration was not determined in field tests by these authors, the lowest concentration detected (2.1 ng/g) occurred after 21 days of environmental exposure (Iglesias et al. 2006).

This analysis highlights the lethal effect of the IVM on "non-target" coprophilic organisms and confirms results previously obtained in regional studies.

In the absence of previous data on lethal concentrations of IVM on *C. vicina* species and considering that the methodology described in the OECD protocol for *Scatophaga stercoraria* species was adopted in the toxicity bioassays, some of the values recorded for these latter should be mentioned.

Strong and James (1993) reported toxic effects for 50% of *S. stercoraria* larval stages exposed for 24 h to a concentration of 50 ng/g (dry weight) and to 40 ng/g during a 48-h exposure, while exposure to concentrations of 1 ng/g and 20 ng/g were sufficient to delay adult emergence and pupation by 10 days, respectively.

In reference to sub-lethal effects, the relationship between developmental rate and temperature was found to depend on species and geographic region (Salimi et al. 2018). Thus, the duration of development decreases as temperature increases, with the pupal period occupying a large part of the life cycle. In fact, in the present trial, the pupal period corresponded to half of the life cycle, with the longest time recorded for the substrate added with IVM 100 ng/g (Table 1).

Due to the temperature range of each region, and the temperature tolerance unique to each species during different developmental stages, the ADD/ADH is different for the insect population in each region (Donovan et al. 2006). Hence, for *C. vicina*, these authors estimated the minimum temperature (threshold) for development to be 1 °C and a minimum total of 4700 accumulated degree hours (ADH) in the developmental period between egg and pupa.

In the present work, the sub-lethal effects on the development of *C. vicina* were analysed under two criteria: the weight of the immature stages, as a function of time of evolution for the stages from L1 to L3-pre-pupa and from pupa to adult, and the accumulated degree hours (ADH) as an indicator of development in each experimental group. Despite being delayed in relation to the control group, the analysis of the recorded data showed statistical significance only between the group with the addition of 100 ng/g IVM and the control group in the pupa-to-adult period. In the post-feeding phase, larvae do not ingest food, although they consume energy during the transfer from the food source to a substrate to pupate. As reported by Donovan et al. (2006), larvae shrink in size during this phase, due to physiological and behavioural changes, explaining the slight decrease in larval weight observed in the bioassay prior to the pupal stage (Fig. 3).

Comparing to the results reported by Donovan et al. (2006), the highest cumulative degree hour value of larval stages until pupal development was on average 6840 (±536.66) ADH in the IVM 3 ng/g group. According to previous studies in the region, concentrations similar to the latter were detected in FM of cattle treated subcutaneously with IVM (0.2 mg/kg) after 21 dpt (Iglesias et al. 2006). In relation to the methodology used by Donovan et al. (2006), in this work, we controlled the temperature during the experimental period from L1 to pupation, a condition that allowed us to ensure the fertility of the eggs and to evaluate from hatching to an established number (50) of specimens that came into contact with the IVM-added substrate. Likewise, the specimens under study were subjected to temperatures within the developmental range, without reaching extremes that, as Donovan et al. (2006) reported, could affect survival or prevent development to pupa. Also, these authors highlight the particular biogeographical variation of *C. vicina* in relation to thermal tolerance during development.

Long-term monitoring studies, considering the possibility of bioaccumulation, will be necessary to accurately assess the impact of antiparasitic drugs in the environment without underestimating or overestimating their effects.

Conclusions

Calliphora vicina proved to be an environmental bioindicator due to certain particularities of its life cycle. These facilitated its rearing in the laboratory: the thermal amplitude at which it develops, the duration of the cycle, its biotic potential, and the number of eggs laid on a nutrient-rich substrate such as pig liver, although it can also oviposit on other organic substances. Besides, the size of the larvae allows their manipulation, and the adaptation to the food diet favoured their rearing in bovine faeces until the emergence of the new adults is complete.

However, the evaluated time in one life cycle is not sufficient to confirm the effects of IVM on this species or to predict its impact on the environment in the medium or long term. For this, it will be necessary to consider other assessment models that allow us to observe changes in its interaction with the environment.

In this study, the lethal and sub-lethal effects of different IVM concentrations on *C. vicina* were evidenced by reductions in survival and alterations in evolution times. Hence, the L1 of the FM spiked with IVM 3000 ng/g, did not survive the first hour of exposure, resulting in 100% mortality.

These results demonstrate the ecotoxicity of IVM in FM at concentrations that can be found after IVM administration in actual parasite control programmes in cattle.

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Author contribution All authors contributed to the review and design. Material preparation and data collection and analysis were performed by Lucía Iglesias, Milagros Junco, and Carlos Saumell; sample processing and data analysis were carried out by Lucía Iglesias, Adrián Lifschitz, and Juan Sallovitz. The first draft was written by Lucia Iglesias, and co-authors commented on previous versions of the manuscript. All authors read and proved the final manuscript.

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Data availability The data generated during and/or analysed during the current research are available from the author (Lucía Iglesias) on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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