The effects of sublethal and lethal doses of ivermectin on the reproductive physiology and larval development of the dung beetle *Euoniticellus intermedius* (Coleoptera: Scarabaeidae)

Imelda Martínez M.,[1] Jean-Pierre Lumaret, Rosario Ortiz Zayas, Nassera Kadiri

Abstract—This study assesses the effects of the veterinary medical product ivermectin (IVM) in a range of concentrations on adult reproductive physiology and larval mortality of the dung beetle *Euoniticellus intermedius* (Reiche) (Coleoptera: Scarabaeidae). The ecotoxicological tests comprised eight treatments, including two controls and six increasing ivermectina concentrations (3.16, 10.0, 31.6, 63.2, 100, and 316 µg IVM/kg fresh dung). After 10 days of exposure, the females were dissected and the brood balls counted (fecundity). The brood balls were opened 15 days later and live larvae were counted to estimate larval mortality. Ivermectin altered the morphology of the ovary and stopped vitellogenesis, causing oocyte resorption and thus decreasing fecundity. The 30% threshold of decline in fecundity was reached at 115.9 µg IVM/kg dung, with no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values of 10.0 and 31.6 µg IVM/kg dung, respectively. Larval sensitivity to ivermectin was higher, with a lethal concentration required to kill 50% of the population of 85.9 µg IVM/kg dung, and NOEC and LOEC of 3.16 and 10.0 µg IVM/kg dung, respectively. After cattle were treated with ivermectin at the recommended dose, the ivermectin concentration in their dung during the two first weeks after administration far exceeded the thresholds determined for *E. intermedius*.

Résumé—Les effets de l'ivermectine (IVM) sur la reproduction des femelles d'*Euoniticellus intermedius* (Reiche) (Coleoptera: Scarabaeidae) ont été évalués. La mortalité des larves a également été mesurée. Huit traitements ont été réalisés, soit deux témoins et six concentrations d'ivermectine (3,16 ; 10,0 ; 31,6 ; 63,2 ; 100 et 316 µg IVM/kg de bouse fraîche). Après 10 jours d'exposition, les femelles ont été disséquées et les pelotes fécales fabriquées ont été décomptées (fécondité). Ces boules ont été ouvertes 15 jours plus tard et les larves vivantes dénombrées (estimation de la mortalité larvaire). L'ivermectine modifie la morphologie de l'ovaire et interrompt la vitellogenèse, provoquant une résorption des ovocytes et une diminution de la fécondité. On observe 30% de baisse de fécondité à 115.9 µg IVM/kg bouse fraîche, aucun effet observable (NOEC) à 10,0 µg IVM et un début d'effet (LOEC) à 31,6 µg IVM/kg. Le seuil de sensibilité des larves est un peu plus élevé, avec une concentration létale de 50% (CL50) de 85,9 µg IVM/kg bouse fraîche et des valeurs de NOEC et LOEC de 3,16 et 10,0 µg IVM/kg, respectivement. Après le traitement du bétail, la concentration d'ivermectine dans les bouses dépasse largement les seuils établis ici pour *E. intermedius*.

Introduction

The use of parasiticides for the control of parasites in domestic animals constitutes a major sector of the global animal health effort. Infections of livestock by parasites can affect the overall health of dairy cattle (*Bos taurus* Linnaeus; Mammalia: Bovidae) by causing weight loss and reducing milk yield, and consequently profitability. The macrocyclic lactone ivermectin (IVM) is the most widely used pharmaceutical worldwide (Lumaret *et al*. 2012).

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Its use in livestock has direct toxic effects on dung-breeding insects, with implications for the functioning of pasture ecosystems. Regardless of the administration route, ivermectin is excreted almost entirely via the faeces. Treated cattle thus excrete residues that are toxic to dung-inhabiting insects, including the dung beetles (Coleoptera: Scarabaeidae) that provide valuable ecosystem services such as biological pest control and soil fertilisation (Nichols *et al*. 2008; Beynon *et al*. 2015). Concerns have been raised that the use of macrocyclic lactones may reduce insect diversity and cause the accumulation of dung on pastures (Floate *et al*. 2005; Lumaret *et al*. 2012). Beynon *et al*. (2012) estimated that, if it is not broken down, dung accumulation could reduce the total area of a permanent pasture by up to 4.8% each year.

The key behavioural trait of tunnelling dung beetles is to excavate tunnels underneath droppings and provide dung for offspring in the form of brood balls. The female lays an egg in a cavity within each individual brood ball that nourishes the larva when it hatches (Halffter and Edmonds 1982). Beetle tunnelling reduces the survival of dung-breeding pest flies and gastrointestinal parasites of livestock, returns nutrients to the soil, and physically moves manure from the fouled forage (Anderson *et al*. 1984). Tunnelling and dung burial also result in increased grass root growth and biological activity in soils under and adjacent to dung pats. Economic and ecological benefits from dung beetles have led to the introduction of several species into pasture habitats around the world and their use for applied studies in biodiversity (Halffter and Favila 1993; Spector 2006). Thus, *Euoniticellus intermedius* (Reiche) (Coleoptera: Scarabaeidae) was first introduced from Africa to Australia between 1971 and 1984 to reduce cattle dung accumulation on pastures resulting from the inability of the native dung beetles to cope with the large moist dung pats produced by the introduced cattle. The second objective of the introduction was to control dung-breeding buffalo flies (*Haematobia exigua* Meijere; Diptera: Muscidae) and bush flies (*Musca vetustissima* Walker; Diptera: Muscidae) (Waterhouse 1974; Edwards 2007). *Euoniticellus intermedius* was introduced from Australia into the southern of the United States of America where it established (Fincher 1986). Since the 1980s, the species has spread to Mexico (Montes de Oca and Halffter 1998) and from there, southward to Costa Rica (Solís and Kohlmann 2012). Today this invasive species is considered one of the most common dung beetles in the grazed pastures of primarily tropical Mexico where its burial activity is substantial (Flota-Bañuelos *et al*. 2012).

Previous studies on the unintended effects of ivermectin (lethal and sublethal effects) on *E. intermedius* mainly focussed on the rate of adult emergence and brood ball production following the treatment of livestock, without measuring the precise concentration of ivermectin in the dung given as food to adults and their larvae (Fincher 1996; Krüger and Scholtz 1997). In these studies, the injection of cattle with ivermectin (200 µg/kg body weight) prevented adult emergence of *E. intermedius* from dung collected two to seven days after treatment. The number of eggs laid by females, which corresponded to the number of brood balls constructed, was similar for both treatment and control groups, except during the peak of ivermectin faecal elimination in the first three days after treatment. Recently, Cruz-Rosales *et al*. (2012) evaluated the effects of three concentrations of ivermectin directly added to the fresh dung (spiked dung; 0.01, 1.0, and 100 ppm) on the reproduction and larval development of *E. intermedius*. At 0.01 ppm (10 µg IVM/kg dung fresh weight) the fecundity of adults was not affected, in contrast to 1.0 ppm (1000 µg/kg dung fresh weight), which drastically reduced fecundity and larval emergence and 100 ppm and killed all parent beetles. In that experiment, the small number and wide range of concentrations did not allow for the calculation of important parameters such as lethal concentration at which 50% individuals die (LC50), no observed effect concentration (NOEC), or lowest observed effect concentration (LOEC), and this made it impossible to come to a definitive conclusion about the effects of ivermectin on female fecundity and larval mortality. Cruz-Rosales *et al*. (2012) suggested that more detailed analyses using concentrations ranging from 0.01 to 1.0 ppm were necessary to establish the harmful and lethal concentrations for *E. intermedius* larvae.

The effects of ivermectin in dung beetles depend on its concentration in the dung, but we are far from completely understanding how this macrocyclic lactone modifies the physiology of

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female ovaries in *E. intermedius*. Furthermore, the way in that ivermectin reduces the fecundity and increases the length of pre-imaginal development has not yet been clarified. Recently, Verdú *et al.* (2015) showed that low ivermectin concentrations paralyses the muscles of adult dung beetles, as it does in nematodes where ivermectin inhibits pharyngeal muscle contraction, leading to paralysis (Turner and Schaeffer 1989; Brownlee *et al.* 1997). These findings have led us to examine the preliminary results of Cruz-Rosales *et al.* (2012) more closely and investigate the possibility that the ingestion of ivermectin by mature *E. intermedius* females induces a decrease in the contraction force of muscles surrounding the ovary, extending the period between the expulsion of successive eggs, and leading to the premature resorption of oocytes and a decrease in fecundity. Similarly, in larvae, an increase in the flaccidness of their muscles may slow down their consumption of the dung reserves buried in the pedotrophic nest and lead to protracted juvenile development.

In the present study, we investigated the effects of exposure to ivermectin, specifically at low concentrations, on *E. intermedius* females and their offspring. The objectives were: (i) to evaluate female fecundity as a function of ivermectin concentration in dung; (ii) to assess the effects of ivermectin on ovarian morphology and physiology; and (iii) to determine the relationship between larval mortality and ivermectin concentration and then estimate the NOEC, LOEC, and LC50 values.

Materials and methods

Study species and laboratory rearing

A laboratory colony of *E. intermedius* was established from beetles collected from Zapoapan Ranch, close to Santiago Tuxtla, Veracruz, Mexico (18°27'N, 95°18'W; 166 m). The cattle had not been treated with ivermectin for six months before beetle collection. The first generation of *E. intermedius* reared in the laboratory was used exclusively for the experiments. Beetles were reared and all experiments were conducted in an insectary at 26–27 °C, 14 hour photoperiod and ~ 70% relative humidity. Beetles for breeding were placed in plastic terraria (57 × 30 × 37 cm) half filled with moist and sterilised sandy soil. For bioassays, pairs of beetles (an individual of each sex together) were placed in a 1-L, gauze-covered plastic container (140 mm in height; 100 mm in diameter). The soil was moistened to a crumbly but compact consistency favourable for nesting. Humidity was maintained constant throughout the reproductive period. Dung used for cultures and all tests was obtained from cattle that had not been treated with any parasiticide for at least three months. The dung was collected fresh from the field and frozen at −18 °C for at least 48 hours before use, then thawed and mixed mechanically for 10 minutes to homogenise moisture (83.6 ± 0.9%), pH (6.40), and nitrogen content. Beetles were supplied with cattle dung *ad libitum*. Beetles used in bioassays were first separated by sex and not fed for 24 hours, and later paired up and placed in the containers (female age at the start of treatment: 7–15 days).

Bioassay

The tests were performed with technical grade ivermectin (CAS-Number: 70288-86-7) with a purity of ⩾ 90% ivermectin B1a and ⩽ 5% ivermectin B1b (Batch number: SLBG8734V, Sigma-18898; Sigma-Aldrich, Toluca, Mexico). As ivermectin is poorly soluble in water, it was first dissolved in acetone (CAS-Number: 1567-89-1; Sigma purity > 99.8%) to obtain the desired concentrations by serial dilution. The acetone/ivermectin solution was then thoroughly mixed into the cattle dung (spiked dung), and kept for at least eight hours at 25 °C to allow the solvent to evaporate (13 mL acetone/2.5 kg dung fresh weight. A protocol derived from standardised bioassays and guidance developed for dung beetles to test the lethal and sublethal toxicity of parasiticide residues in livestock dung was employed (Organisation for Economic Co-operation and Development 2010; Römbke *et al.* 2010). The bioassay comprised eight treatments, including a water control, a solvent (acetone) control, and six increasing ivermectin concentrations). Ivermectin concentrations, treatments C1–C6, were 3.16, 10.0, 31.6, 63.2, 100, and 316 µg IVM/kg dung fresh weight. Seven mating pairs (one male and one female) of *E. intermedius* were used as replicates for each concentration/treatment. Adults were fed three times per week. After 10 days of exposure, the beetles were removed, the females were dissected (reproductive

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system, fat body, gut content) and the brood balls counted. Each brood ball formed from dung stored by adults during days 1–10 contained an egg, which was used to calculate the relationship between female fecundity and ivermectin concentration. The brood balls were opened 15 days later and live larvae were counted to estimate larval mortality. Larvae at different stages of development were killed and fixed in Peterson's KAAD fixative (8 mL toluene, 70 mL 95% ethyl alcohol, 14 mL glacial acetic acid, and 8 mL dioxane), and then stored in 95% ethyl alcohol (Carne 1951).

Morphology and physiology of the ovary

The females of true dung beetles (Coleoptera: Scarabaeidae: Scarabaeinae) have only one ovary (the left one), with a single ovariole (Robertson 1961). The ovary consists of a coiled germarium and a vitellarium containing differentiated oocytes in sequential stages of development. The ovary is connected to an oviduct leading to the vagina, with the spermatheca also joining the vagina (Robertson 1961). The reproductive system is surrounded by muscles whose contractions push the basal oocyte into the ovary during oviposition and move it through the oviduct and vagina during egg laying. In the present study, the reproductive system of each female was removed by dissection in Ringer's solution, then fixed in AFATD (75 mL ethyl alcohol 96%, 10 mL formaldehyde, 5 mL acetic acid, 10 mL dimethylsulphoxide, and 1 g trichloroacetic acid) (Martínez 2002). The ovary was drawn to scale using a stereo-microscope with a camera lucida. The physiological state was defined by using the morpho-physiological techniques described by Martínez (2002) that take into account the size and the number of oocytes per ovary and their colour and morphology. During the dissection of fresh females, body fat quality was evaluated under a stereo-microscope, considering its colour, abundance, and morphological appearance. The quantity of food inside the gut was evaluated by observation through the transparent intestinal wall.

Statistical analysis of fecundity and larval mortality

The statistical analysis of female fecundity, oocyte number per ovary, and larval mortality was run in ToxRat® software version 3.2.1 (Toxrat Solutions 2015). The pair-wise comparison of the two controls (water and solvent) was performed with the Student's *t*-test for homogeneous variances, with $\alpha = 0.05$, two-sided, and with $P(t)$ = probability of sample t for Ho: $\mu 1 = \mu 2$. Female fecundity and larval mortality were corrected with Abbott's formula (Abbott 1925). The comparison of treatments with pooled controls (no significant differences between water and solvent) was also performed with a *t*-test procedure following Williams (1971) (significance $\alpha = 0.05$). The Shapiro–Wilk's test was used to test the distribution of the treatment for normality, in association with a Levene's test on the homogeneity of the variance of the data (Levene 1960; Shapiro and Wilk 1965). The critical effect and threshold concentrations were calculated at the end of the experiment. The LOEC and NOEC values were determined using Fisher's exact test with the Bonferroni–Holm adjustment. The effective concentration (EC) and lethal concentration (LC) values with 95% confidence limits were assessed using a probit analysis with a linear maximum likelihood regression. The valid test for brood ball production (fecundity) required a minimum of 20 offspring in the controls and a coefficient of variation not exceeding 15% (Toxrat Solutions 2015). Similarly, a valid test required that the control mortality of offspring not exceed 10%, and the coefficient of variation not exceed 15%, while for oocyte numbers the minimum detectable difference between the two controls was required to be less than 10%. The fecundity and larval mortality values are reported as mean ± standard deviation.

Results

Female fecundity in relation to ivermectin concentration in dung

Under our experimental conditions, female fecundity (estimated by the number of brood balls, which corresponds to egg production, produced during days 1–10) varied significantly ($F = 6.74$; $P < 0.001$) as a function of ivermectin concentration levels in the diet (Table 1). The largest decrease in brood ball production occurred at treatment C6 (mean 15.1 ± 5.3 brood balls), whereas the number of brood balls was 26.7 ± 3.7 and 25.4 ± 4.6 for the water and solvent controls, respectively. There was no significant difference

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Table 1. Mean number (x) of brood balls (corresponding to the number of eggs deposited) produced by the females of *Euoniticellus intermedius* for a range of ivermectin (IVM) concentrations in fresh dung (µg IVM/kg) and comparison of treatments (C1–C6) with pooled controls.

Treatment	Pooled controls	3.16 (C1)	10.00 (C2)	31.60 (C3)	63.20 (C4)	100.00 (C5)	316.00 (C6)
n	14	7	7	7	7	7	7
x	26.1a	26.7a	23.3ab	21.7ab	19.0b	19.3b	15.1b
s	4.08	2.69	3.15	4.15	3.92	3.25	5.27
s(x)	1.09	1.02	1.19	1.57	1.48	1.23	1.99
Comparison of treatments with pooled controls using the t-test procedure (after Williams 1971)							
% MDD		−11.6	−12.1	−12.3	−12.4	−12.4	−12.5
t		0.36	−1.54	−2.41	−3.84	−3.84	−6.05
t*		−1.68	−1.75	−1.78	−1.79	−1.80	−1.80
Significance		−	−	+	+	+	+

Notes: Means followed by the same letter in row x are not statistically different ($\alpha = 0.05$).

n, number of replicates; s, standard deviation; s(x), standard error; % MDD, minimum detectable difference to pooled controls (in per cent of pooled controls); t, t-test value; t*, critical t for Ho: $\mu 1 = \mu 2 = \ldots = \mu k$; the differences are significant when $|t| > |t^*|$ (the residual variance of an analysis of variance was applied).

between the control and solvent (df = 12; $t = -0.57$; $P(t) = 0.577$; $P(F) = -0.309$), so the two controls were pooled. The results of the Shapiro–Wilk's test (W = 0.984; P(W) = 0.844; number of residues = 40) indicate that the data did not deviate significantly from a normal distribution ($P(W) > 0.05$). Variance homogeneity was confirmed at the 0.05 confidence level (df = 6; $F = 0.725$; $P(F) = 0.632$). The comparison of treatments (C1–C6) with pooled controls using the t-test procedure (after Williams 1971) gave a NOEC value of 10.0 µg IVM/kg dung fresh weight (*i.e.*, concentration C2) and a LOEC of 31.6 µg IVM/kg dung fresh weight for the production of brood balls (Table 1), while the results of the probit analysis gave an EC30 value of 115.9 µg IVM/kg, with lower and upper 95% limits of 78.0 and 183.3 µg IVM/kg dung fresh weight, respectively (Fig. 1). In the probit analysis, the precise EC50 must be obtained when responses are distributed between 25% and 75% (Robertson *et al.* 2007). In this study, the maximum concentration evaluated (316 µg IVM/kg dung fresh weight) produced less than a 50% response. Therefore, we chose LC30 instead of LC50.

Effects of ivermectin on ovary morphology and physiology and on gut content

The number of oocytes per ovary varied significantly ($F = 28.82$; $P < 0.001$) with ivermectin concentration, increasing progressively as ivermectin concentration increased (Table 2). In contrast, the size of the ovary diminished (Fig. 2). The normal distribution and variance homogeneity of data requirements were fulfilled (W = 0.980; P(W) = 0.947), and there was no significant difference between control and solvent ($F = 0.26$; $P = 0.619$), so they were pooled.

In the absence of ivermectin in dung (pooled controls) and at the lowest dose of ivermectin (treatment C1), the number of oocytes in the ovary did not significantly differ ($F = 3.39$; $P = 0.080$); the last (basal) oocyte (white colour) was ready to be expelled, or there was a small white basal oocyte when females had recently oviposited (Fig. 1). In all cases the gut of the females contained food in abundance, their body fat was abundant and white in colour, and their oviduct was distended due to oocyte oviposition. At treatment C2 (corresponding to the NOEC value calculated for fecundity), the early degeneration of the basal oocyte (colour ranging from yellow to orange) was evident in most females, up to three oocytes were under reabsorption per ovary, and there were significantly more oocytes throughout the ovary than at the lowest ivermectin concentration, treatment C1 ($F = 6.42$; $P = 0.024$). The body fat remained abundant but slightly pale yellow and the gut contained much less food than in the controls. At treatment C3, which corresponds to the value, the number of oocytes had increased significantly compared with C2 ($F = 6.81$; $P = 0.021$). The ovary and basal oocyte were smaller than in the controls, the

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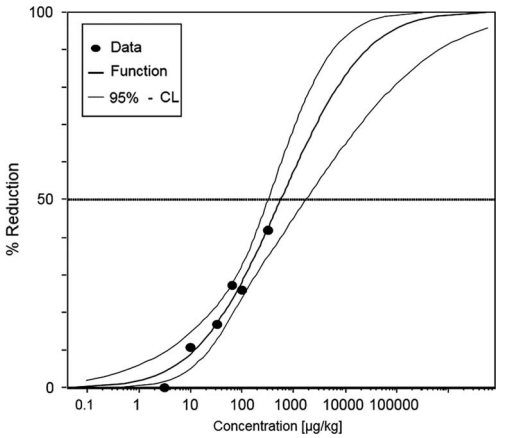
basal oocyte and also the following oocytes (up to three) were being resorbed. The oviduct was not as distended as observed in the controls, treatment C1, and treatment C2. The body fat (yellow in colour) was still abundant but the gut contained little food. No significant difference in oocyte numbers was detected among treatment C3–C6 ($F = 2.01$; $P = 0.182$), though anatomical differences were detected. At concentration treatment C4, up to three oocytes were being resorbed and the body fat was dark yellow, small in quantity, and the gut was almost empty. It was difficult to dissect the females because the tissues were all very weak. At concentration treatment C5, the ovary contained a high number of small yellow or orange coloured oocytes, most of them undeveloped, and up to three oocytes in resorption on average. The body fat (orange in colour) was in short supply and the gut contained little food. At the highest ivermectin concentration (treatment C6), the oocytes were very small and for all the females, the basal oocyte was in resorption, with up to seven oocytes being resorbed simultaneously in the ovary, and the oviduct was never distended. Body fat was almost absent and the gut was empty.

Fig. 1. Effect of increasing ivermectin concentrations (treatments C1–C6) (µg/kg dung fresh weight) on the fecundity of *Euoniticellus intermedius* females. 95% CL, lower and upper 95% confidence limits.



Mortality of larvae in relation to ivermectin concentration in dung

For the water and solvent controls, after 10 days, 26.7 ± 3.7 and 25.0 ± 4.9 live larvae were recovered from the brood balls, respectively. There was no significant difference between the control and solvent (df = 12; $t = -0.74$; $P(t) = 0.475$; $P(F) = -0.262$) so they were pooled. The results of the Shapiro–Wilk's test ($W = 0.979$; $P(W) = 0.634$; number of residues = 42) show that the data did not significantly deviate from a normal distribution ($P(W) > 0.05$). Variance homogeneity was confirmed at the 0.05 confidence level (df = 6; $F = 0.894$; $P(F) = 0.507$).

When the brood balls were opened, it was observed that the movements of the larvae in controls were normal and vigorous. Gradually, as the concentration increased, movement became slower to the point of the complete absence of movement at the highest concentration. Table 3 gives the mean number of live *E. intermedius* offspring, ranging from 25.9 to 2.7 brood balls for the pooled controls and treatment C6, respectively (Table 3). The proportion of variance explained by the dose/response parameters corresponded to a coefficient of determination $r^2 = 0.904$, with $F = 37.74$ and $\chi^2 = 0.101$. Results of the probit analysis gave effective concentrations effective concentration for x% effect of the test items

Table 2. Mean number (x) of oocytes in *Euoniticellus intermedius* female ovary for a range of ivermectin (IVM) concentrations in fresh dung (µg IVM/kg).

Treatment	Pooled controls	3.16 (C1)	10.00 (C2)	31.60 (C3)	63.20 (C4)	100.00 (C5)	316.00 (C6)
n	16	7	9	7	9	7	6
x	9.69a	9.29a	10.33b	11.43c	11.78c	12.43c	12.00c
s	0.479	0.488	1.000	0.535	0.441	0.787	0.894
% Increase		–4.1	6.7	18	21.6	28.3	23.9

Notes: Means followed by the same letter in a column are not statistically different.
n, number of replicates; s, standard deviation; % increase compared with pooled controls.

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Fig. 2. Morpho-physiological changes in female *Euoniticellus intermedius* ovaries when fed for 10 days with dung without ivermectin (water control = WC) and solvent control (SC) and with increasing ivermectin concentrations from treatment C1 to C6 (3.16, 10.0, 31.6, 63.2, 100, and 316 µg IVM/kg dung fresh weight. boa, basal oocyte after egg laying; bof, basal oocyte before egg laying; g, germarium; or, oocytes resorption; ov, oviduct.

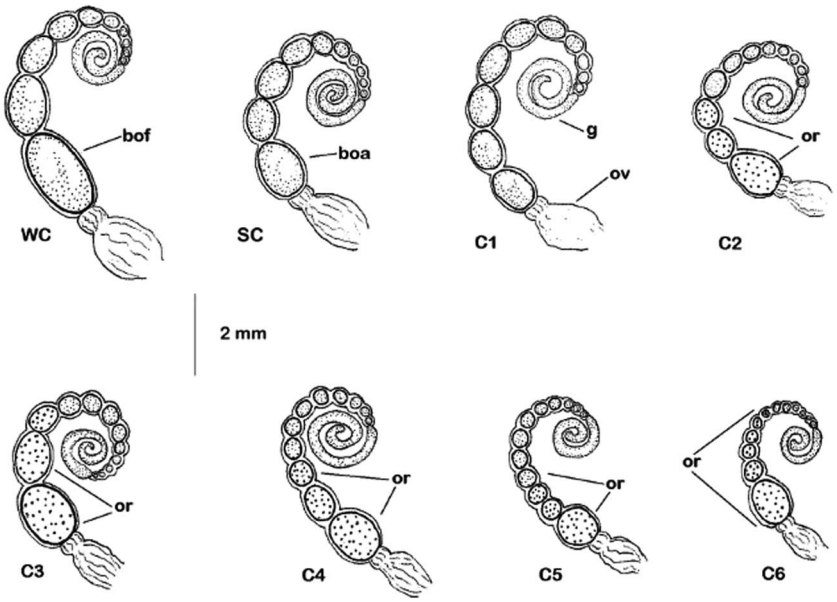

Table 3. Mean number (x) of live offspring of *Euoniticellus intermedius* at day 25 for a range of ivermectin (IVM) concentrations in fresh dung (µg IVM/kg) and comparison of treatments C1–C6 with pooled controls.

Treatment	Pooled controls	3.16 (C1)	10.00 (C2)	31.60 (C3)	63.20 (C4)	100.00 (C5)	316.00 (C6)
n	14	7	7	7	7	7	7
x	25.9a	25.1a	22.1a	19.6ab	15.4b	13.4b	2.70c
s	4.28	4.60	3.67	5.56	3.36	3.51	2.75
s(x)	1.14	1.74	1.39	2.10	1.27	1.32	1.04
Comparison of treatments with pooled controls using the t-test procedure (after Williams 1971)							
% MDD		−12.3	−12.8	−13.0	−13.1	−13.1	−13.2
t		−0.38	−1.96	−3.32	−5.51	−6.57	−12.24
t*		−1.68	−1.75	−1.78	−1.79	−1.80	−1.80
Significance		−	+	+	+	+	+

Notes: Means followed by the same letter in row x are not statistically different ($\alpha = 0.05$).

n, number of replicates; s, standard deviation; s(x), standard error; n, sum of treatment replicates $n(i)$; k, number of treatments; % MDD, minimum detectable difference to pooled controls (in per cent of pooled controls); t, sample t; t*, critical t for Ho: µ1 = µ2 = … = µk; the differences are significant when $|t| > |t^*|$ (the residual variance of an analysis of variance was applied).

and their 95% confidence limits, the computation of variances and confidence limits adjusted to metric data (Christensen and Nyholm 1984) (Fig. 3). The calculated LC50 value was 85.5 µg IVM/kg dung fresh weight, with lower and upper 95% confidence limits of 58.9 and 132.9 µg/kg dung fresh weight, respectively. The Williams multiple sequential t-test procedure gave NOEC and LOEC values of 3.16 and 10.0 µg IVM/kg dung fresh weight, respectively, corresponding to concentrations of treatments C1 and C2 (Table 3).

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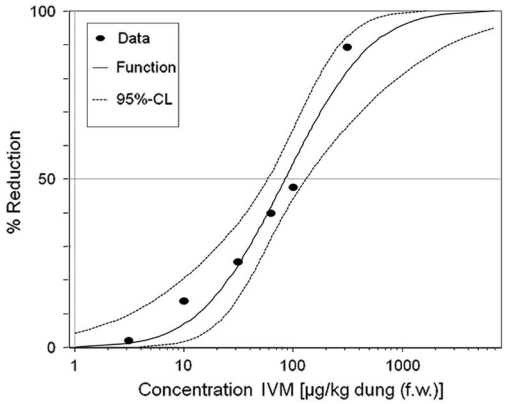
Fig. 3. Effect of increasing ivermectin concentration (treatments C1–C6) (µg IVM/kg dung fresh weight) on larval mortality of *Euoniticellus intermedius* females. 95% CL, lower and upper 95% confidence limits.



Discussion

Effect of ivermectin on female fecundity and ovary morphology

Without ivermectin in dung, the fecundity of *E. intermedius* is high and, under laboratory conditions, each female may produce ~140 eggs during her life (I.M.M., unpublished data). Under our experimental conditions, no significant effect (NOEC) on female fecundity was detected at 10.0 µg IVM/kg dung fresh weight but this was significantly lower than the LOEC value (31.6 µg IVM/kg dung), with a 30% decrease (EC30) in brood ball production (*i.e.*, eggs) at 115.9 µg IVM/kg dung fresh weight. These results can be compared with ivermectin concentrations in the droppings of animals after veterinary treatment. Following a topical treatment of cattle (500 µg IVM/kg body weight), Wohde *et al.* (2016) reported concentrations of 2845–5029; 2480–7675, and 692–341 µg IVM/kg dung fresh weight in dung excreted three, seven, and 14 days after treatment, respectively. These values correspond to 969–1006, 496–1535, and 138–68 µg IVM/kg dung fresh weight for comparison with fresh weight values used in the present study. They far exceed the thresholds determined for *E. intermedius* in our study, which means that the droppings of animals can significantly affect female fecundity for at least two weeks after cattle treatment.

Ovarian morphology and gut content followed the same concentration thresholds. However, the first signs appeared very early, at treatment C2 (*i.e.*, 10.0 µg IVM/kg dung fresh weight), before fecundity was significantly affected. At the LOEC threshold, the ovary was visibly smaller and multiple oocytes had been resorbed.

Usually females start to oviposition five to seven days after their emergence, with two to three eggs laid every 24 hours, which means that yolk synthesis is rapid and that the females need to feed throughout the oviposition period. The changes observed in the gut content indicate that the females gradually stopped feeding, with a consequent reduction in body fat reserves and the cessation of yolk synthesis in the oocytes. During this metabolic phase, the highly lipophilic ivermectin present in the dung can migrate to the fat body and the ovary, to be incorporated into the yolk. In dung beetles, ivermectin may act on the ovary by stopping vitellogenesis and inducing the resorption of oocytes. Our results can be explained in part by the mechanisms that control reproduction in insects. Ovulation and oviposition are both triggered by environmental factors (nutrition, temperature, photoperiod, and chemical products) and by internal factors (neurohormonal and hormonal regulation) (Martínez and Caussanel 1984; Martínez 1995; Martínez and Huerta 1997; Martínez and Cruz 1998).

In the present study, the detailed effects of a low concentration of ivermectin on beetle vitellogenesis were assessed for the first time, revealing the different states of oocyte resorption. We show the progressive cessation of oocyte expulsion and their resorption, specifically the successive blocking of the oocytes that accumulate behind. The action of ivermectin should not be generalised, and further investigations are needed to determine more precisely how this parasiticide affects the neuroendocrine organs and the ovary, and stops vitellogenesis.

It is known that the muscular system of beetles is affected by ivermectin (Verdú *et al.* 2015) so females have trouble moving, preparing brood balls and ovipositing; in the extreme, they cannot move and eventually die (Cruz Rosales *et al.* 2012). Ivermectine was indeed shown to paralyse the muscles of the dung beetle *Scarabaeus cicatricosus* Lucas (Coleoptera: Scarabaeidae) (Verdú *et al.* 2015), and in nematodes where it

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inhibits pharyngeal muscle contractions (Turner and Schaeffer 1989; Brownlee *et al*. 1997). In *S. cicatricosus*, ivermectin decreased the olfactory and locomotor capacity of adults, preventing beetles from performing basic biological activities (Verdú *et al*. 2015). These authors reported significant effects of ivermectin at doses close to the LOEC value identified in our study. Therefore, the increasing difficulty of movement in adults and larvae, and the gradual decrease in egg production in *E. intermedius* females were most likely due to the action of ivermectin. Under natural conditions, once reproductive activity ends, females stop burying dung and preparing brood balls, and their ovary enters a resorption stage (Halffter and Edmonds 1982). The remaining oocytes are broken down, with some having internal clusters of follicular cells that form a resorption body. The ovary is surrounded by contracting muscles that push oocytes through the ovariole, causing the expulsion of the terminal oocyte during oviposition. The oviduct and especially the vagina have thick muscle layers, which operate during copulation, oviposition, and fertilisation. With a decrease in muscular contraction, the females would be unable to produce more eggs due to the slow progress of oocytes. While not analysed in detail, the behaviour of males was also affected (I.M.M., unpublished data). Wardhaugh *et al*. (2001) demonstrated that newly emerged adults of the dung beetle *Onthophagus taurus* (Schreber) (Coleoptera: Scarabaeidae) were also sensitive to ivermectin residues, with signs of delayed sexual maturation in the adults that did not die. Our results are also consistent with those obtained for the dung beetle *Onthophagus landolti* Harold (Coleoptera: Scarabaeidae), for which fecundity at 100 µg IVM/kg dung µg IVM/kg dung fresh weight decreased to only 2% in comparison with the controls, and to zero at higher concentrations (Pérez-Cogollo *et al*. 2015).

Effect of ivermectin on larval mortality

Regarding larval mortality, the NOEC and LOEC ivermectin concentrations in dung were lower than those obtained for female mortality, and the LC50 was much lower (85.5 µg IVM/kg dung fresh weight) than the value of 50% calculated for female fecundity (560 µg). As pointed out earlier for female fecundity, the ivermectin concentrations in the dung excreted by treated animals far exceed the lethal threshold values for larvae (Wohde *et al*. 2016).

In the present experiment, surviving larvae continued to feed on dung containing ivermectin, but an increase in the flaccidness of their muscles may have slowed down their consumption of dung and led either to death, or to protracted juvenile development as reported by Cruz Rosales *et al*. (2012) and Pérez-Cogollo *et al*. (2015). Ivermectin blocks nerve signals by interfering with the glutamate-gated chloride channel receptors and this affects membrane stability: the target species is paralysed and dies as a result of the inhibition of inter-neural and neuromuscular transmission (Martin *et al*. 2002). This was also demonstrated in mosquitoes where ivermectin causes paralysis and mortality (Gardner *et al*. 1993). In a specific case, the larvae of *Culex quinquefasciatus* Say (Diptera: Culicidae) suffered from ataxia (dysfunction of the part of the nervous system which coordinates muscle movements), followed by death when exposed to ivermectin (Nunes Alves *et al*. 2004).

General outcomes and conclusion

Our results are relevant at a time when the Society of Environmental Toxicology and Chemistry Expert Group, Dung Organism Toxicity Testing Standardisation has started working on the development of a new Organisation for Economic Co-operation and Development guideline covering the testing of the tunnelling dung beetle *Onthophagus taurus* (Schreber) to complement the guidance document on the determination of the toxicity of chemicals to dweller dung beetles (Organisation for Economic Co-operation and Development 2010). It is becoming increasingly evident that adult and offspring mortality rates cannot be the main criteria in understanding the role of stressors in the environment. Results from the present study clearly reveal the relevance of considering the sublethal effects on dung organisms. In the international ring test performed in the context of this guideline preparation, it became clear that such an endpoint is more sensitive, and provides a more comprehensive understanding of toxic effects, than mortality does.

Moreover, our results may be useful for modelling the effects of cattle treatments on dung beetle populations. Many authors did not take into

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account the decline in adult fecundity at low doses of parasiticides when larvae exhibited no apparent developmental problems. For instance, in models and experiments using pyrethroids in flies, direct toxicity effects were mostly studied disregarding possible decreases in fecundity, so it was assumed that toxicity would be negligible for adults, delaying the effects on populations of dung-breeding insects by only one or two days (Vale and Grant 2002). However, a model taking into account the effect of deltamethrin on the breeding success of dung beetles in the field suggested that a single treatment may cause a reduction of up to 75% in beetle activity by the end of the season (Wardhaugh *et al*. 1998).

Ivermectin is the most widely used pharmaceutical worldwide. Very little has been reported on the sublethal toxicity of ivermectin residues, such that their adverse effects are being underestimated in current models. More serious concerns are the uncertainties regarding the level of the toxicity decline in successive droppings produced by animals after the peak of elimination of veterinary medical products (Vale and Grant 2002; Beynon *et al*. 2015; Wohde *et al*. 2016). The results of the current study are therefore timely in that they may be used to model the total impact of veterinary medical products on dung beetle populations more accurately. More generally, taking both lethal and sublethal effects into account should lead to a refinement of the existing and future models, and a better overall estimation of the impact of veterinary residues on the natural environment.

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References

Abbott, W.S. 1925. A method of computing the effectiveness of an insecticide. Journal of Economic Entomology, **18**: 265–267.

Anderson, J.R., Merritt, R.W., and Loomis, E.C. 1984. The insect-free cattle dropping and its relationship to increased dung fouling of rangeland pastures. Journal of Economic Entomology, **77**: 133–141.

Beynon, S.A., Peck, M., Mann, D.J., and Lewis, O.T. 2012. Consequences of alternative and conventional endoparasite control in cattle for dung-associated invertebrates and ecosystem functioning. Agriculture, Ecosystems & Environment, **162**: 36–44.

Beynon, S.A., Wainwright, W.A., and Christie, M. 2015. The application of an ecosystem services framework to estimate the economic value of dung beetles to the U.K. cattle industry. Ecological Entomology, **40**(Supplement 1): 124–135.

Brownlee, D.J., Holden-Dye, L., and Walker, R.J. 1997. Actions of the anthelmintic ivermectin on the pharyngeal muscle of the parasitic nematode, *Ascaris suum*. Parasitology, **115**: 553–561.

Carne, P.B. 1951. Preservation techniques for scarabaeid and other insect larvae. Proceedings of the Linnean Society of New South Wales, **76**: 26–30.

Christensen, E.R. and Nyholm, N. 1984. Ecotoxicological assays with algae: Weibull dose response curves. Environmental Science & Technology, **18**: 713–718.

Cruz Rosales, M., Martínez, I.M., López-Collado, J., Vargas-Mendoza, M., González-Hernández, H., and Fajersson, P. 2012. Effect of ivermectin on the survival and fecundity of *Euoniticellus intermedius* (Coleoptera: Scarabaeidae). Revista de Biología Tropical International Journal of Tropical Biology, **60**: 333–345.

Edwards, P. 2007. Introduced dung beetles in Australia 1967–2007. Current status and future directions. Landcare Australia and The Orica Community Foundation,"Dung Beetles for Landcare Farming", Sinnamon Park, Australia.

Fincher, G.T. 1986. Importation, colonization, and release of dung-burying scarabs. *In* Biological control of muscoid flies. Volume 61. *Edited by* R.S. Patterson and D.A. Rutz. Miscellaneous publications. Entomological Society of America, College Park, Maryland, United States of America. Pp. 69–76.

Fincher, G.T. 1996. Ivermectin pour-on for cattle: effects on some dung inhabiting insects. Southwestern Entomologist, **21**: 445–450.

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https://doi.org/10.4039/tce.2017.11 Published online by Cambridge University Press

Floate, K.D., Wardhaugh, K.G., Boxall, A.B.A, and Sherratt, T.N. 2005. Faecal residues of veterinary pharmaceuticals: non-target effects in the pasture environment. Annual Review of Entomology, **50**: 153–179.

Flota-Bañuelos, C., López-Collado, J., Vargas-Mendoza, M., Fajersson, P., González-Hernández, H., and Martínez-M., I. 2012. Efecto de la ivermectina en la dinámica espacio-temporal de escarabajos estercoleros en Veracruz, México. Tropical and Subtropical Agroecosystems, **15**: 227–239.

Gardner, K., Meish, M., Meek, C.L., and Bivin, W.S. 1993. Effects of ivermectin in canine blood on *Anopheles quadrimaculatus*, *Aedes albopictus* and *Culex salinarius*. Journal of the American Mosquito Control Association, **9**: 400–402.

Halffter, G. and Edmonds, W.D. 1982. The nesting behavior of dung beetles (Scarabaeinae). An ecological and evolutive approach. Instituto de Ecología, Mexico City, Mexico.

Halffter, G. and Favila, M. 1993. The Scarabaeinae (Insecta: Coleoptera), an animal group for analysing, inventorying and monitoring biodiversity in tropical rainforest and modified landscapes. Biology International, **27**: 15–21.

Krüger, K. and Scholtz, C.H. 1997. Lethal and sub-lethal effects of ivermectin on the dung-breeding beetles *Euoniticellus intermedius* (Reiche) and *Onitis alexis* Klug (Coleoptera, Scarabaeidae). Agriculture, Ecosystems & Environment, **61**: 123–131.

Levene, H. 1960. Robust tests for equality of variances. *In* Contributions to probability and statistics: essays in honor of Harold Hotelling. *Edited by* I. Olkin, S.G. Ghurye, W. Hoeffding, W.G. Madow, and H.B. Mann. Stanford University Press, Palo Alto, California, United States of America. Pp 278–292.

Lumaret, J.-P., Errouissi, F., Floate, K., Römbke, J., and Wardhaugh, K. 2012. A review on the toxicity and non-target effects of macrocyclic lactones in terrestrial and aquatic environments. Current Pharmaceutical Biotechnology, **13**: 1004–1060.

Martin, R.J., Robertson, A.P., and Wolstenholme, A.J. 2002. Mode of action of the macrocyclic lactones. *In* Macrocyclic lactones in antiparasitic therapy. *Edited by* J. Vercruysse and R.S. Rew. Centre for Agriculture and Biosciences International Publishing, Wallingford, United Kingdom. Pp 125–140.

Martínez, M.I. 1995. Observations on reproductive control in females of two species of *Canthon* Hoffmannsegg (Coleoptera: Scarabaeidae: Scarabaeinae). Bollettino Museo Regionale di Scienze Naturali Torino, **13**: 327–343.

Martínez, M.I. 2002. Técnicas básicas de anatomía microscópica y de morfometría para estudiar los insectos. Boletín de la Sociedad Entomológica Aragonesa, **30**: 187–195.

Martínez, M.I. and Caussanel, C. 1984. Modifications de la pars intercerebralis, corpora allata, gonades et comportement reproducteur chez *Canthon cyanellus* (Coleoptera, Scarabaeinae). Comptes Rendus de l'Académie des Sciences de Paris 299, série III, **14**: 597–602.

Martínez, M.I. and Cruz, R.M. 1998. Effects of nourishment on the gonadal maturation in *Canthon cyanellus cyanellus* LeConte (Coleoptera Scarabeidae: Scarabaeinae). The Coleopterists Bulletin, **52**: 237–243.

Martínez, M.I. and Huerta, C.C. 1997. Coordinated activity of the ovary, pars intercerebralis and corpus allatum during the prenesting and nesting cycles of *Copris incertus* Say (Coleoptera Scarabaeidae: Scarabaeinae). The Coleopterists Bulletin, **51**: 351–363.

Montes de Oca, E. and Halffter, G. 1998. Invasion of Mexico by two dung beetles previously introduced into the United States. Studies on Neotropical Fauna and Environment, **33**: 37–45.

Nichols, E., Spector, S., Louzada, J., Larsen, T., Amezquita, S., and Favila, M.E. 2008. The Scarabaeinae research network. 2008. Ecological functions and ecosystem services provided by Scarabaeinae dung beetles. Biological Conservation, **141**: 1461–1474.

Nunes Alves, S., Serrão, J.E., Mocelin, G., and Lane de Melo, A. 2004. Effect of ivermectin on the life cycle and larval fat body of *Culex quinquefasciatus*. Brazilian Archives of Biology and Technology, **47**: 433–439.

Organisation for Economic Co-operation and Development. 2010. Test No. 122: guidance document on the determination of the toxicity of a test chemical to the dung beetle *Aphodius constans.* Organisation for Economic Co-operation and Development Environment, Health and Safety Publications, Series on Testing and Assessment. ENV/JM/MONO (2010) 13. Organisation for Economic Co-operation and Development Publishing, Paris. France.

Pérez-Cogollo, L.C., Rodríguez-Vivas, R.I., Delfín-González, H., Reyes-Novelo, E., and Ojeda-Chi, M.M. 2015. Lethal and sublethal effects of ivermectin on *Onthophagus landolti* (Coleoptera: Scarabaeidae). Environmental Entomology, **44**: 1634–1640.

Robertson, J.G. 1961. Ovariole number in Coleoptera. Canadian Journal of Zoology, **39**: 245–263.

Robertson, J.L., Russell, R.M., Preisler, H.K., and Savin, E. 2007. Bioassays with arthropods, 2nd edition, CRC Press, Boca Raton, Florida, United States of America.

Römbke, J., Barrett, K., Blanckenhorn, W.U., Hargreaves, T., Kadiri, N., Knäbe, S., *et al.* 2010. Results of an international ring test with the dung fly *Musca autumnalis* in support of a new OECD test guideline. Science of the Total Environment, **408**: 4102–4106.

Shapiro, S.S. and Wilk, M.B. 1965. An analysis of variance test for normality (complete samples). Biometrika, **52**: 591–611.

Solís, A. and Kohlmann, B. 2012. Checklist and distribution atlas of the Scarabaeinae (Coleoptera: Scarabaeidae) of Costa Rica. Zootaxa, **3482**: 1–32.

Spector, S. 2006. Scarabaeine dung beetles (Coleoptera: Scarabaeidae: Scarabaeinae): an invertebrate focal taxon for biodiversity research and conservation. The Coleopterists Bulletin, **60**: 71–83.

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https://doi.org/10.4039/tce.2017.11 Published online by Cambridge University Press

Toxrat Solutions. 2015. ToxRat Professional 3.2.1. Software for the statistical analysis of biotests. Toxrat Solutions, Alsdorf, Germany.

Turner, M.J. and Schaeffer, J.M. 1989. Mode of action of ivermectin. *In* Ivermectin and abamectin. *Edited by* W.C. Campbell. Springer-Verlag, New York, New York, United States of America. Pp 73–78.

Vale, G.A. and Grant, I.F. 2002. Modelled impact of insecticide-contaminated dung on the abundance and distribution of dung fauna. Bulletin of Entomological Research, **92**: 251–263.

Verdú, J.R., Cortez, V., Ortiz, A.J., González-Rodríguez, E., Pinna, J.M., Lumaret, J.-P, *et al*. 2015. Low doses of ivermectin cause sensory and locomotor disorders in dung beetles. Scientific Report, **5**: 1–10. https://doi.org/10.1038/srep13912.

Wardhaugh, K.G., Holter, P., and Longstaff, B. 2001. The development and survival of three species of coprophagous insect after feeding on the faeces of sheep treated with controlled-release formulations of ivermectin or albendazole. Australian Veterinary Journal, **79**: 125–132.

Wardhaugh, K.G., Longstaff, B.C., and Lacey, M.J. 1998. Effects of residues of deltamethrin in cattle faeces on the development and survival of three species of dung-breeding insect. Australian Veterinary Journal, **76**: 273–280.

Waterhouse, D.F. 1974. The biological control of dung. Scientific American, **230**: 100–109.

Williams, D.A. 1971. A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics, **27**: 103–117.

Wohde, M., Blanckenhorn, W.U., Floate, K.D., Lahr, J., Lumaret, J.-P., Römbke, J., *et al*. 2016. Analysis and dissipation of the antiparasitic agent ivermectin in cattle dung under different field conditions. Environmental Toxicology and Chemistry, **35**: 1924–1933. https://doi.org/10.1002/etc.3462.

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