

Evaluating long-term ivermectin use and the role of dung beetles in reducing short-term CH₄ and CO₂ emissions from livestock faeces: a mesocosm design under Mediterranean conditions

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Abstract. 1. Greenhouse gas (GHG) emissions from livestock contribute significantly to global warming, and a reduction of this source of emissions is crucial in achieving the goal of mitigating global warming.

2. CO₂ and CH₄ emissions from dung pats were analysed by means of a mesocosm experiment in a Mediterranean ecosystem. The experiment consisted of a total of 30 mesocosms distributed across three treatments: a well-preserved, undisturbed dung beetle assemblage associated with organic livestock; a dung beetle assemblage that was impoverished as a result of the long-term use of veterinary medical products; and a control treatment without dung beetles.

3. Corrections related to insect respiration allow researchers to provide more precise measurements of CO₂ emissions from dung, especially in the initial and final phases of dung exposure, when the percentage of CO₂ emitted by dung beetles can become greater than the emissions from the dung pats themselves.

4. The effects of dung beetles on CO₂ and CH₄ emissions are much more accentuated in warm-temperate conditions than in northern temperate areas previously studied. Mediterranean assemblages remove and spread dung faster and more effectively than do northern dung beetle assemblages characterised by a lower functional richness and beetle abundance and biomass.

5. From a livestock management viewpoint, mesocosms representing areas with impoverished dung beetle assemblages, due to the long-term use of veterinary medical products, such as ivermectin, emitted 1.6- and 2.8-fold higher total CO₂ and CH₄, respectively, than mesocosms mimicking sites with untreated livestock.

Key words. Artificial warming, biodiversity and ecosystem function, greenhouse gas emissions, ivermectin, off-axis integrated cavity output spectroscopy.

Introduction

The intensification of agriculture has had a significant impact on the environment, contributing an estimated 10–12% of the total greenhouse gas (GHG) emissions, mostly caused by the livestock sector (Smith *et al.*, 2014). Currently (data from 2010), the contribution to warming directly attributable to

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livestock GHG emissions represents 23% of all human activities. Direct livestock emissions occur in the form of carbon dioxide (CO₂), methane (CH₄), and nitrous oxide (N₂O). CO₂ has been described as the dominant GHG driving climate change, while CH₄ is the second most important GHG, with a radiative forcing 34 times greater than that of CO₂ over 100 years (Myhre *et al.*, 2013). Furthermore, although CH₄ has a shorter lifetime than CO₂ in the atmosphere (*c.* 12.4 years according to Reisinger & Clark, 2018), it has a much higher warming efficacy per molecule and per kg. The contributions of CH₄, N₂O, and

CO_2 have been estimated to be 14%, 5% and 3%, respectively (Reisinger & Clark, 2018). If GHG emissions are not reduced, livestock will directly contribute c. 5% of the predicted total warming by 2100 (Reisinger & Clark, 2018). A reduction in livestock emissions consequently appears to be crucial to support the stringent global mitigation goals (Wollenberg *et al.*, 2016).

Although the literature indicates that the vast majority of GHG livestock emissions originate from enteric fermentation (Reisinger & Clark, 2018), cattle dung deposited on the soil surface is also a non-negligible source of GHG emissions (Holter, 1991; Jarvis *et al.*, 1995; Holter, 1997; Saggar *et al.*, 2004; Bel-larby *et al.*, 2013) because fermentation continues after dung deposition, which in turn may enhance soil methanogenesis (Cai *et al.*, 2017). Some mesocosm studies have reported that the action of dung beetles substantially influences GHG emissions from dung pats (Penttilä *et al.*, 2013; Iwasa *et al.*, 2015; Hammer *et al.*, 2016; Slade *et al.*, 2016a; Piccini *et al.*, 2017, 2018). Dung beetles constitute a highly diversified and specialised group of species that contribute significantly to key ecological processes such as dung decomposition, nutrient recycling, soil bioturbation and aeration, parasite control, plant growth, seed dispersal and alterations to soil microbial communities (Bornemissza & Williams, 1970; Hanski, 1987; Yamada *et al.*, 2007; Nichols *et al.*, 2008; Slade *et al.*, 2016a; Milošić *et al.*, 2017). Depending on the duration of the study, the presence of dung beetles has been shown to significantly increase the total CO_2 emitted from dung pats (Iwasa *et al.*, 2015) and to have a negligible influence on the total CO_2 emissions (Penttilä *et al.*, 2013; Hammer *et al.*, 2016; Piccini *et al.*, 2017). Both the respiratory activity of the beetles themselves and the aerobic microbial respiration caused by the action of dung beetles (Slade *et al.*, 2016b) are likely to be the main sources of CO_2 , which is emitted at a higher proportion during the initial days that dung beetles are present. Methane emissions, however, seem always to decrease when dung beetles are present (Penttilä *et al.*, 2013; Iwasa *et al.*, 2015; Hammer *et al.*, 2016; Slade *et al.*, 2016a; Piccini *et al.*, 2018; but see Piccini *et al.*, 2017), probably as a consequence of the decrease in anaerobic conditions due to the aeration caused by dung beetle activities.

The low number of available studies on the effects of dung beetles on GHG emissions from livestock dung, the contrasting durations of mesocosm experiments (from 7 to 59 days), and the different species compositions in the previously conducted studies may help to explain these partially conflicting results. There is also geographical bias in the research carried out so far, because most of the studies have been conducted in cold or cold-temperate localities at a latitude $> 42^\circ$ (Penttilä *et al.*, 2013; Iwasa *et al.*, 2015; Hammer *et al.*, 2016; Piccini *et al.*, 2018). At such locations, the species richness of dung beetle assemblages is considerably lower than that in the Mediterranean region (Hanski, 1980; Lumaret & Lobo, 1996). For example, approximately two species per dung pat can be collected in Finland (Roslin, 2001), whereas 13 species and 380 individuals can be collected on average in dung pats in southern Spain (Lobo *et al.*, 1997). Furthermore, dung beetle assemblages in central and northern European regions are mainly composed of small dweller species of Aphodiinae,

some small tunnellers belonging to the genus *Onthophagus* (Scarabaeinae), and very few large Geotrupinae tunnellers. By contrast, Mediterranean dung beetle assemblages are usually diverse from a functional point of view, comprising dwellers, large and small tunnellers, and rollers. Moreover, the role of dung beetles in dung degradation is also proportionally lower in northern Europe than in the Mediterranean because of the higher importance of other groups exploiting herbivore faeces, such as Diptera or Lumbricidae (Holter, 1979).

Usually, two different techniques are used to measure GHG soil fluxes: either the samples are collected in the field by syringe for laboratory analysis using gas chromatography (GC), or real-time flux measurements are obtained onsite to avoid the delays and sample transport errors that this entails. In recent years, the implementation of new technologies, such as cavity ring-down spectroscopy (CRDS) with the more accurate off-axis integrated cavity output spectroscopy (OA-ICOS) technique, and Fourier transform infrared spectroscopy have provided a higher level of precision in gas flux measurements, making them more robust and suitable for field applications (Romanini *et al.*, 2014; Butterbach-Bahl *et al.*, 2016). Some studies have shown that the GC technique is severely limited in detecting CH_4 fluxes under both laboratory and field conditions as compared with CRDS (Christiansen *et al.*, 2015). Moreover, in a field comparison, the minimum (analytical) detectable fluxes for GHGs were more than one order of magnitude lower for the CRDS technique than for GC (Brannon *et al.*, 2016). As a consequence, analysers based on OA-ICOS and CRDS technologies are now emerging as more reliable alternatives in GHG studies in different scenarios (Mortazavi *et al.*, 2013; Christiansen *et al.*, 2015; Gelfand *et al.*, 2015; Martin & Moseman-Valtierra, 2015; Brannon *et al.*, 2016).

The OA-ICOS technique has enabled us to obtain not only onsite real-time measurements of GHG emissions but also corrected estimates, by considering the CO_2 emissions generated by the respiration of the target species. Specifically, our primary purpose is to determine the effects of dung beetle assemblages on the dynamics of CO_2 and CH_4 fluxes from dung pats by means of a mesocosm experiment simulating two contrasting Mediterranean farming scenarios. Thus, we compare GHG emissions from dung pats with: (i) an undisturbed dung beetle assemblage that typically colonises dung pats in an organic livestock farming area; and (ii) an impoverished dung beetle assemblage, such as the one occurring in a locality subjected to the long-term use of veterinary medical products (VMPs; see Verdú *et al.*, 2018). Additionally, we sought for the first time to discriminate CO_2 inputs due to insect, dung pat, and soil respiration under Mediterranean conditions.

Materials and methods

Study area and mesocosm experimental design

The experiment was conducted in the Doñana Biological Reserve (DBR-ICTS), an area located within Doñana National Park. We selected this site because it is a Spanish site that has been free of the use of VMPs since its commercial use (Verdú *et al.*, 2015; Verdú *et al.*, 2018) and because it hosts a

Table 1. Results of general linear models (repeated-measures ANOVA design) to estimate the effects of the three considered treatments (treatment factor: dung-control mesocosms, rich mesocosms and poor mesocosms) and the eight different periods analysed after the placement of dung in each mesocosm (time factor) on CO₂, CH₄ and dung spreading rate (DSR).

	CO ₂	CH ₄	DSR
Treatment	$F_{2,27} = 0.21^{\text{NS}}$	$F_{2,27} = 11.89^{***}$	$F_{2,27} = 82.98^{***}$
Time	$F_{7,189} = 82.33^{***}$	$F_{7,189} = 45.62^{***}$	$F_{6,162} = 210.09^{***}$
Treatment × time	$F_{14,189} = 5.01^{***}$	$F_{14,189} = 4.22^{***}$	$F_{12,162} = 47.58^{***}$

*** $P \leq 0.001$.

species-rich and functionally diverse dung beetle assemblage (Lobo *et al.*, 1997). The climate is Mediterranean with some oceanic influence. The average annual rainfall is *c.* 560 mm, and the average annual temperature is *c.* 17 °C. During the fieldwork period (23–30 June 2017) the average temperature in the area was 25.0 °C, ranging from 23.4 to 37.5 °C. No rainfall was recorded in the area during the study period (for more details, see Appendix S1).

To study the effects of dung beetle activity on dung CO₂ and CH₄ emissions, we conducted a mesocosm experiment under field conditions. Mesocosms were constructed using a cylindrical exclusion cage 30 cm in diameter with an external mesh of 1.5 mm (Figs. 1a, 2a). The exclusion cages were buried 10 cm into the ground to prevent the beetles from escaping (see later) or entering from the outside. A total of 30 mesocosms were placed in the field separated by a distance of 5 m. A dung pat (1.32 ± 0.65 kg fresh weight) was placed inside each mesocosm. The mesocosms were assigned to three different treatments (i.e. 10 mesocosms per treatment): (i) dung pats without dung beetles (dung-control mesocosms); (ii) dung pats with an undisturbed dung beetle assemblage (rich mesocosms); and (iii) dung pats with impoverished dung beetle assemblages (poor mesocosms) both in the number of species and in their abundance or biomass. Additionally, 10 GHG emission measurements were carried out on the ground near the mesocosms to estimate soil respiration when neither dung pats nor dung beetles were present (soil control).

Fresh cow dung was collected during the first 2 h after daybreak to avoid dung colonisation by beetles and to minimise physical and chemical changes in the dung. The collected cow dung was homogenised, and the size and shape of the dung pats were standardised using cylindrical containers of diameter 14 cm and height 7.5 cm.

The dung beetle composition for the rich and poor mesocosms was determined taking into account the results of a previous study comparing the dung beetle assemblages at two nearby localities differing in their history of cattle anti-parasite (ivermectin) treatments (Verdú *et al.*, 2018). Thus, one treatment (rich mesocosms) simulates the species composition and abundances of undisturbed dung beetle assemblages occurring in pats located in those areas where the cattle have never been treated with anti-parasitic compounds, and the other treatment (poor mesocosms) represents the impoverished assemblages occurring in the areas with treated cattle, where species abundances are almost eight times lower than in the undisturbed

sites (Fig. 3). The species composition and abundances for each mesocosm were established considering the average number of individuals obtained by direct sampling in 40 dung pats (Verdú *et al.*, 2018). All of the individuals ($n = 2860$) belonging to 10 dung beetle species necessary to conduct the experiment were collected from fresh dung pats in the study area (Fig. 3). Once collected, the beetles were sorted into species and kept in plastic containers with moist paper at 20 °C until they were used in the field experiment.

To analyse the temporal patterns in the emissions of GHGs in the different treatments (treatment factor), the emissions from each dung pat were measured at eight different times after dung placement (time factor): 0 h (at 09.00 hours on 23 June, immediately after dung placement inside the exclusion cages), 2 h (the moment at which the dung beetles were added), and 6, 11, 26, 34, 48, and 168 h. These times were established based on dung colonisation patterns by dung beetles and the spreading rates found in a previous study (Verdú *et al.*, 2018), also taking into account the fact that beyond the maximum period of 168 h, CO₂ and CH₄ emissions can be considered negligible in our surveyed locality. The exclusion cages were opened at 48 h after their placement, allowing the emigration of beetles due to the loss of attractiveness of dry dung pats to them after this time in the study area (Verdú *et al.*, 2018).

Soil chamber design and GHG flux measurement

A temporary/portable chamber was constructed following the recommendations of Parkin and Venterea (2010) to minimise artefacts during GHG flux measurements (Fig. 1b). The chamber body (diameter 40 cm, height 24.5 cm) was made of aluminium (thickness 3 mm), a non-reactive material, to reduce the absorption of sunlight and minimise temperature effects during the measurements. Under-pressurisation or over-pressurisation of the chamber caused by flow restrictions in the air circulation can result in significant errors (Davidson *et al.*, 2002). This problem was avoided by assembling an appropriate chamber vent capable of maintaining pressure equilibrium between the inside and outside of the chamber under different wind conditions following the design described by Xu *et al.* (2006) (Fig. 1c). To ensure the correct performance of the chamber design, the pressure, temperature and RH were measured using a weather mini-station (Windoo 3; JDC Electronic SA, Yverdon-les-Bains, Switzerland) located inside the chamber and connected externally to a smartphone (Fig. 1b). The microenvironmental conditions recorded during data acquisition are summarised in Appendix S1.

The CO₂ and CH₄ emitted from the dung pats, dung beetles and soil were surveyed from inside the chamber through two 4-m-long Tygon® chemical- and UV-resistant tubes (Versilon R-3400; Saint-Gobain Performance Plastics, Akron, Ohio) (outer diameter 6.4 mm, inner diameter 3.2 mm). One end of the inlet tube was attached inside the chamber using standard Luer fittings (Cole-Parmer Instrument Company, Vernon Hills, Illinois), and the other end was attached to an ultraportable gas analyser (LGR-UGGA) based on OA-ICOS technology (Los Gatos Research, Inc., San Jose, California). The tube used for

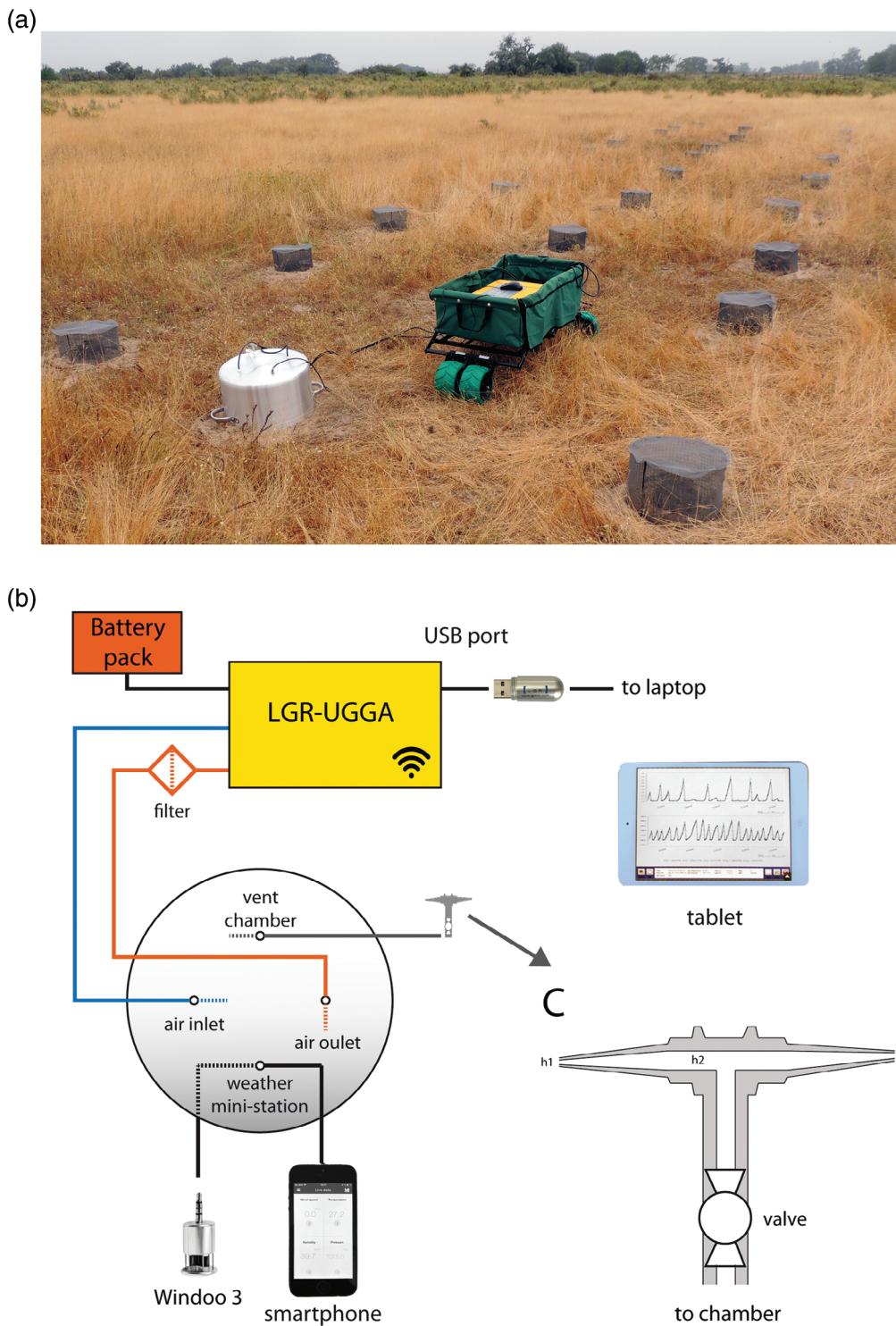


Fig. 1. Experimental setups used in onsite and real-time greenhouse gas flux measurements from dung pats. (a) Field experimental mesocosm design displaying three different treatments [dung pats without dung beetles (dung control), dung pats with a rich dung beetle assemblage (rich mesocosm), and dung pats with impoverished dung beetle assemblages (poor mesocosm)], each one including 10 artificial dung pats separated by distances of 5 m. (b) Design of soil chamber, configuration of gas circulating system and data acquisition equipment. (c) Cross-sectional view of the vent chamber; h_1 and h_2 are the edge and the central distances between the upper and the lower halves of the vent, respectively, after the design proposed by Xu *et al.* (2006). LGR-UGGA, Los Gatos Research ultraportable gas analyser. [Colour figure can be viewed at wileyonlinelibrary.com].

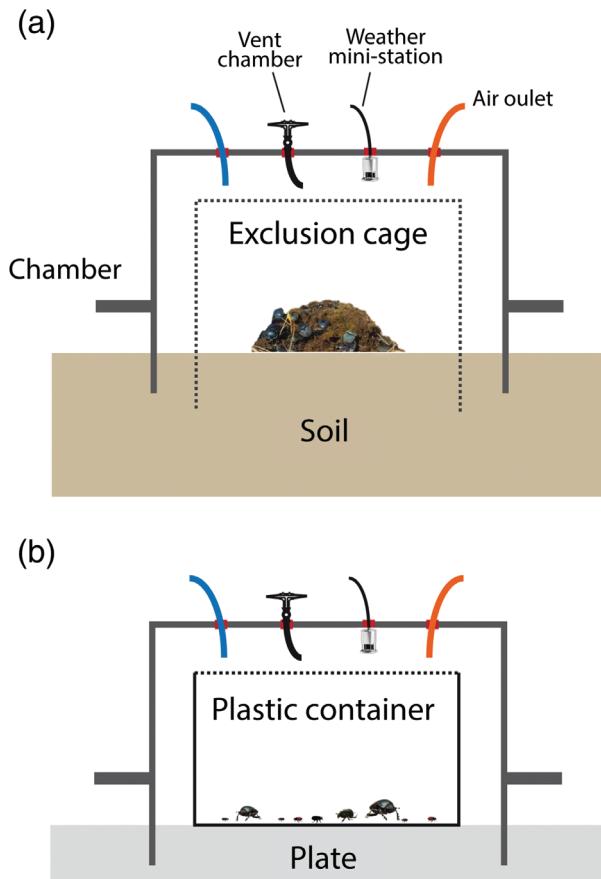


Fig. 2. Cross-section of soil chamber designed to measure: (a) greenhouse gas flux emissions from dung pats in the field; and (b) CO₂ emission from dung beetle respiration in the laboratory. [Colour figure can be viewed at wileyonlinelibrary.com].

air to enter the LGR-UGGA was equipped with a particle filter (1 µm). The other tube was used for outgoing air moving back into the chamber, creating a closed loop between the chamber and the LGR-UGGA (Fig. 1b). Concentrations (in ppm) of CH₄ and CO₂ were logged with the LGR-UGGA every 5 s for 1.5 min for each sample. Between each pair of measurements, the chamber was removed from the site and vented for 30 s until the gas levels decreased to the same level as the surrounding air.

The LGR-UGGA is able to detect ranges of 0.01–100 ppm CH₄ and 1–20 000 ppm CO₂ with precision of <0.9 ppb CH₄ and <140 ppb CO₂ (for 5 s repeatability). Moreover, the LGR-UGGA uses all the measured absorption spectra to accurately correct the water vapour dilution and absorption line broadening effects, reporting CH₄ and CO₂ on a dry mole fraction basis without drying or post-processing.

Greenhouse gas emissions were calculated by estimating the concentration increase during the chamber closures and were linearly fitted considering that the increase in gas concentration inside the chamber is constant (Livingston & Hutchinson, 1995). The slopes of these linear adjustments (in ppm h⁻¹) were calculated using the software EXPEDATA v.1.2.5 (Sable Systems International, North Las Vegas, Nevada). The OA-ICOS technology

permits a great number of point measures to be obtained in a short period of time. In our case, linear regressions were conducted using 15–20 points, obtaining R² coefficients of c. 0.99 in all cases. The GHG flux values (F) in ppm h⁻¹ were transformed to g m⁻² day⁻¹ using the following equation:

$$F = \frac{24 \times P \times V}{10^6 \times R \times A \times (T + 237)} \times M \times b$$

where P is the barometric pressure (mBar) measured inside the chamber, R is the universal gas constant {0.083 145 10 [(bar L K⁻¹] mol⁻¹], V is the volume of the chamber, A is the area of the chamber, T is the temperature in °C inside the chamber, b is the calculated regression slope (ppm h⁻¹), and M is the molecular weight of each gas (CO₂ = 44 and CH₄ = 16).

To evaluate the global warming effect of the studied GHG emissions from dung pats among the different treatments, we converted CH₄ flux emissions to CO₂ equivalents by multiplying the obtained values by 34 (Myhre *et al.*, 2013), the IPCC (2013) global warming potential impact factor for a 100-year time period.

CO₂ flux corrections

The CO₂ detected by the LGR-UGGA in each mesocosm sample is the sum of the dung pat, soil and dung beetle emissions. To correct this possible overestimate of CO₂ fluxes in the mesocosm samples, the emissions produced by the soil and dung beetles were subtracted from the total estimations. To determine the CO₂ emissions due to dung beetle respiration, the same method described earlier was used, although in this case the chamber was closed at the bottom in contact with the soil by means of a polyurethane plate fitted to the shape of the chamber and soft paraffin to ensure the joints were impermeable. The dung beetle specimens included in the rich and poor mesocosms were placed in a plastic container with an external mesh (Fig. 2b). Concentrations (in ppm) of CO₂ were logged with the LGR-UGGA every 5 s for 10 min for each sample. In these cases, linear regressions were conducted using at least 50 points, obtaining R² coefficients of c. 0.99 in all cases. For each treatment, 10 replicates were used to determine the CO₂ emissions due to dung beetle respiration. All measurements were conducted in the DBR-ICTS under the same environmental conditions.

Dung spreading

The process by which dung disaggregation occurred by the action of dung beetles was analysed by calculating the dung spreading area (in cm²) using digital images of each dung pat taken during each experimental time point. All images were edited and analysed using the free software IMAGEJ 1.48 (<http://imagej.nih.gov/ij>).

Statistical treatment of the data

As the GHGs emitted from the dung pats in the three treatments (dung-control mesocosms, rich mesocosms and poor

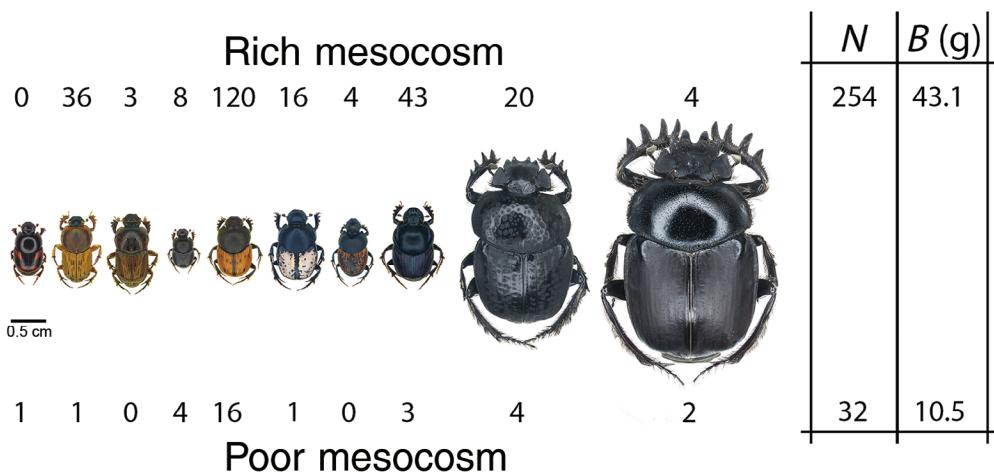


Fig. 3. Scarabaeinae dung beetle species, number of individuals (*N*) and total biomass (*B*; fresh weight) used in each mesocosm treatment. Rich mesocosms were dung pats with a rich dung beetle assemblage simulating the functional composition and biomass of dung pats located in those areas in which the cattle had never been treated with anti-parasitic compounds. Poor mesocosms were dung pats with impoverished dung beetle assemblages such as those occurring in the areas with cattle treated with ivermectin. Species names (from left to right): *Caccobius schreberi* (Linnaeus, 1776), *Euoniticellus fulvus* (Goeze, 1777), *Euoniticellus pallipes* (Fabricius, 1781), *Onthophagus furcatus* (Fabricius, 1781), *Onthophagus maki* (Illiger, 1803), *Onthophagus marginalis* (Gebler, 1817), *Onthophagus opacifollis* (Reitter, 1893), *Onthophagus taurus* (Schreber, 1759), *Scarabaeus cicatricosus* (Lucas, 1846) and *Scarabaeus sacer* (Linnaeus, 1758). [Colour figure can be viewed at wileyonlinelibrary.com].

mesocosms) were estimated repeatedly, general linear models with a repeated-measures ANOVA design were used to analyse the obtained results. Treatment (three levels) was the between-group factor, and time (eight levels) was the repeated-measures factor because it represents the repeated measurement of GHG emissions from the same dung pat at different times. Sigma-restricted parameterisation coding was used as the categorical predictor variable. The statistical significance ($P \leq 0.01$) of the two main effects (time and treatment) and the time \times treatment interaction were examined, but if the interaction was statistically significant, the main effects were ignored in the interpretation of the results. Tukey honestly significant difference (HSD) *post hoc* tests after Bonferroni corrections were used to examine the statistically significant differences in mean emissions among the levels of the time and treatment factors.

For both GHGs and treatments, cumulative fluxes were estimated (in $\text{g m}^{-2} \text{ kg}^{-1}$) as the area under the curve (AUC) using Reimann sums (Marsden *et al.*, 1993) and normalised by the average fresh weight of the dung pats ($1.32 \pm 0.65 \text{ kg}$). One-way ANOVA was used to analyse the obtained results. Tukey HSD *post hoc* tests after Bonferroni corrections were used to examine the statistically significant differences among treatments. All these calculations were performed using STATISTICA v.12 (StatSoft, 2013).

Results

GHG emissions by faeces and dung spreading

Dung beetles clearly reduced CO_2 and CH_4 emissions from fresh dung pats in both rich and poor mesocosms compared with the dung-control treatment (Fig. 4). The cumulative CO_2

and CH_4 emissions for the whole 168-h period significantly ($P < 0.001$) differed among treatments in the case of CO_2 ($F_{2,27} = 31.8$, $R^2 = 0.702$), CH_4 ($F_{2,27} = 43.5$, $R^2 = 0.763$), and CO_2 -equivalent emissions ($F_{2,27} = 42.0$, $R^2 = 0.757$). For both GHGs, the highest total emissions corresponded to the dung-control treatment ($111.3 \pm 11.4 \text{ g m}^{-2} \text{ kg}^{-1}$ for CO_2 and $0.44 \pm 0.05 \text{ g m}^{-2} \text{ kg}^{-1}$ for CH_4 ; mean \pm 95% CI), while the poor mesocosms had lower values ($85.3 \pm 5.1 \text{ g m}^{-2} \text{ kg}^{-1}$ for CO_2 and $0.25 \pm 0.03 \text{ g m}^{-2} \text{ kg}^{-1}$ for CH_4 ; Fig. 4), and the rich mesocosms had the lowest figures ($53.1 \pm 8.7 \text{ g m}^{-2} \text{ kg}^{-1}$ for CO_2 and $0.10 \pm 0.02 \text{ g m}^{-2} \text{ kg}^{-1}$ for CH_4 ; Fig. 4). The *post hoc* Tukey HSD tests indicated that the mean values of the two GHGs differed significantly among the three treatments ($P < 0.01$ in all cases). The conversion of the total CH_4 and CO_2 flux emissions into CO_2 equivalents showed that the total GHG emissions in the dung-control mesocosms ($126.3 \pm 12.6 \text{ g m}^{-2} \text{ kg}^{-1}$) were 1.3- and 2.2-fold higher than those in the poor mesocosms ($93.7 \pm 9.7 \text{ g m}^{-2} \text{ kg}^{-1}$) and rich mesocosms ($56.4 \pm 9.0 \text{ g m}^{-2} \text{ kg}^{-1}$), respectively. In addition, the total GHG emissions, transformed into CO_2 equivalents, were 1.7-fold higher in the poor mesocosms than in the rich mesocosms.

The treatments showed different patterns of emission dynamics. The CO_2 emissions showed a clear flux pattern of rapid increase that progressively declined after 6–11 h (Fig. 5a). The Tukey HSD *post hoc* tests showed that the emissions of CO_2 were higher in the rich mesocosm treatment just 6 h after the start of the placement of the experiment in the morning, the stage at which the maximum flux of CO_2 was obtained (Fig. 5a). However, after a complete daytime period (11 h after experimental placement), this flux began to significantly decrease in the rich mesocosms, so the obtained emissions did not differ significantly among the three treatments (Fig. 5a). With the

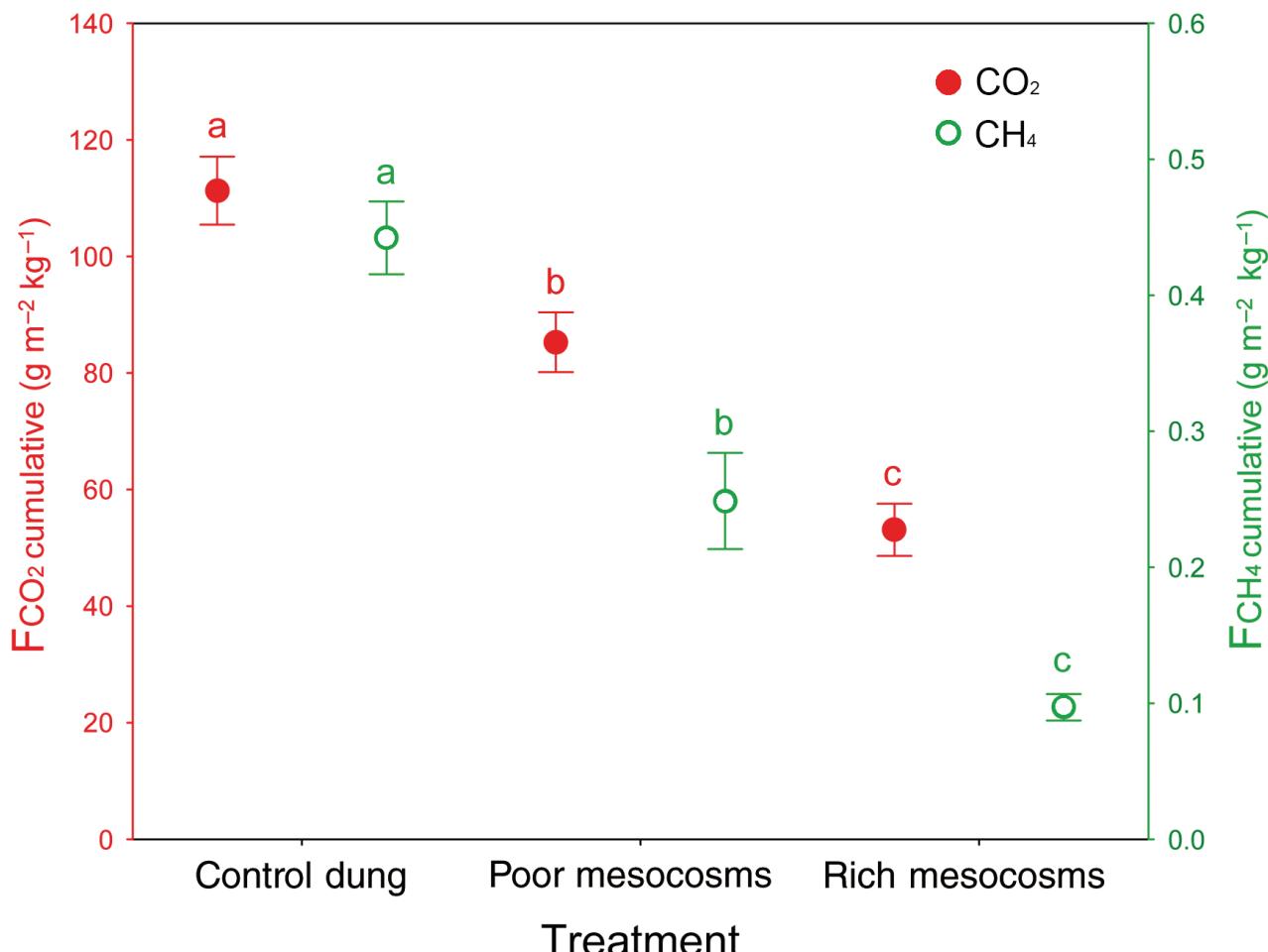


Fig. 4. Cumulative fluxes of CO_2 and CH_4 from dung pats in Mediterranean conditions. Dots show means \pm SEM estimated by a general linear model (for statistical details, see text). Different letters indicate significantly different means between greenhouse (*post hoc* Tukey test, $P < 0.05$, after Bonferroni correction). [Colour figure can be viewed at wileyonlinelibrary.com].

exception of the dung-control mesocosms, the CO_2 emissions continued to decrease significantly at night (26 h). During the second cycle of diurnal activity (34 h) or after the end of the second day (48 h), the CO_2 emissions in the rich mesocosms seemed to be slightly lower than those in the dung-control and poor mesocosms ($P = 0.02$ and $P = 0.05$, respectively). Finally, after 1 week, all CO_2 emissions were similar and very low (Fig. 4a).

The estimates of CH_4 emissions also showed a clear flux pattern of significant increase of methane emissions 2 h after the start of the experiment that remained high until 11 h, subsequently decreasing after a daytime period of solar exposure. Tukey HSD *post hoc* tests show that the methane emissions fell drastically at 11 h in the rich mesocosms ($P < 0.001$) without significant subsequent variation at 26, 34 and 48 h, whereas the CH_4 emissions were significantly higher when the dung beetle assemblage was impoverished. After 1 week, all CH_4 emissions were zero (Fig. 5b).

The area over which dung pats were spread as a result of dung beetle activities increased over time, and this rate was

significantly higher in the rich mesocosms than in the poor mesocosms and also higher in the poor mesocosms than in the dung-control mesocosms (Fig. 5c).

GHG emissions by dung beetles and soil

The mean fluxes of CO_2 due to beetle respiration differed significantly between the rich and poor mesocosms (Student's *t*-test = 6.58, d.f. = 148, $P < 0.0001$). For the rich mesocosms, beetles emitted a flux of $15.29 \pm 1.54 \text{ g CO}_2 \text{ day}^{-1}$, whereas for the poor mesocosms the value was $2.65 \pm 0.36 \text{ g CO}_2 \text{ day}^{-1}$. The proportion of CO_2 exhaled by the dung beetles in the rich mesocosms ranged from 14% to 85% of the total CO_2 emitted during the experiment (dung + dung beetles + soil). In the case of the poor mesocosms, the variation ranged from 4% to 18% with respect to the total CO_2 emitted (Fig. 4d). In the soil, the gas flux measurements showed zero emissions for CH_4 and were relatively low for CO_2 ($2.08 \pm 1.13 \text{ g m}^{-2} \text{ day}^{-1}$).

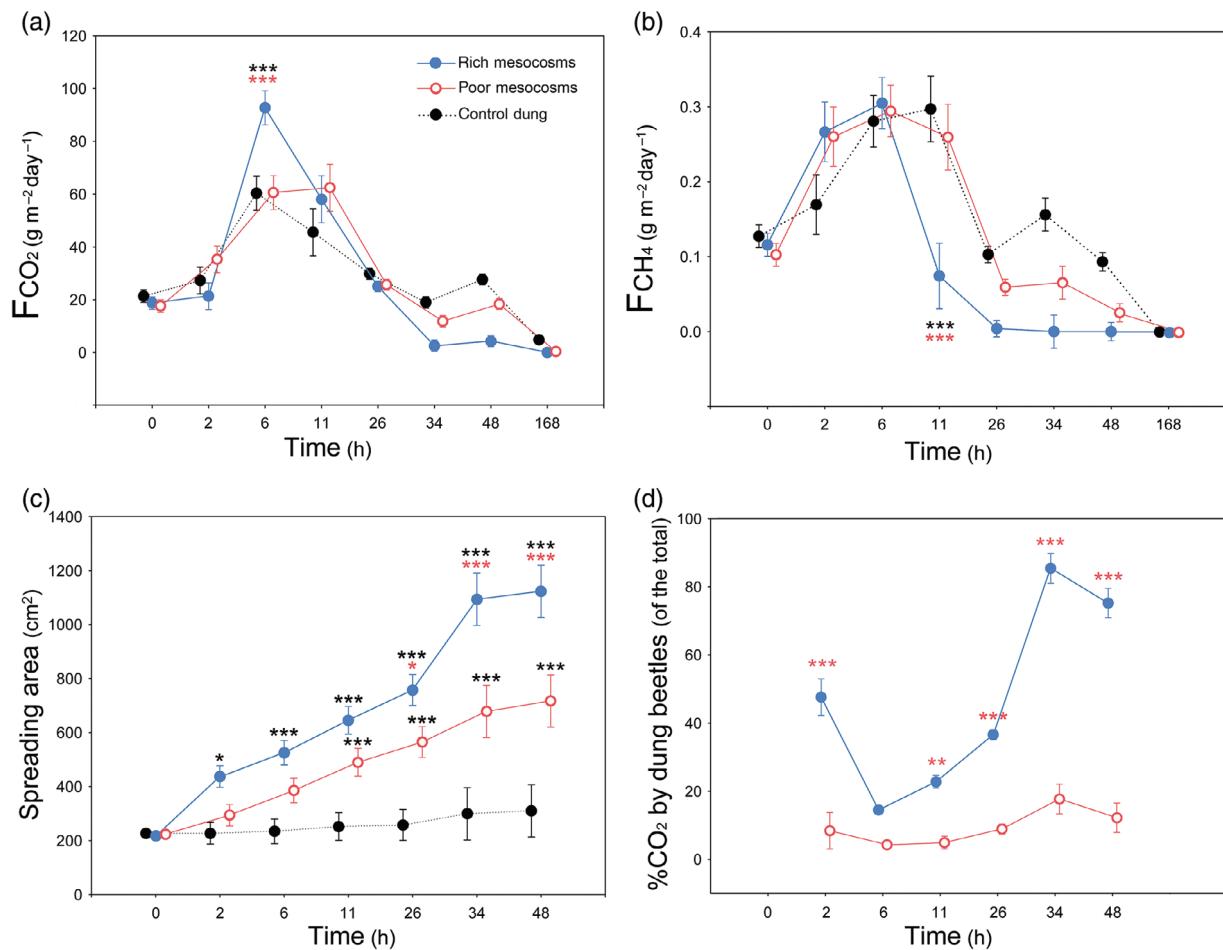


Fig. 5. (a–d) Corrected fluxes of CO₂ (a) and CH₄ (b) from dung pats, and effect of dung beetles on dung spreading (c) and CO₂ emission by respiration (d). Dots show means \pm SEM estimated by a general linear model (for statistical details, see Table 1 and text). To avoid overlap between data points, values were slightly offset on the time axis. To better discern values between treatments during the first time periods (0–11 h) the x-axis does not reflect a continuous timescale (black asterisks indicate significant differences with control dung; grey asterisks indicate significant differences between rich and poor mesocosms; *P < 0.05, **P < 0.01, ***P < 0.001, post hoc Tukey test after Bonferroni correction). [Colour figure can be viewed at wileyonlinelibrary.com].

Discussion

Dung beetle effects on GHG flux dynamics

The GHG emissions from dung pats in this Mediterranean system were seriously affected by the activity of dung beetles, as reported in previous studies carried out in other regions (Jarvis *et al.*, 1995; Holter, 1997; Penttilä *et al.*, 2013; Iwasa *et al.*, 2015; Slade *et al.*, 2016a; Piccini *et al.*, 2017). However, in this study, the effects of dung beetles were not only higher and time-limited (see later) but also dependent on the total abundance and total biomass of the dung beetle assemblages, as evidenced by the experimental assemblages simulating those occurring in areas subjected to different historical anti-parasite treatments (Verdú *et al.*, 2018). Thus, the richest dung beetle assemblage (rich mesocosms) with greater abundance and biomass increased the emissions of both CO₂ and CH₄ in the early moments of the dung degradation process (6 h for CO₂

and 2–6 h for CH₄) and also caused a significant decrease in emissions later when dung degradation increased: after 34 h for CO₂ and after 11 h in the case of CH₄. The consequence of this early increase in CO₂ emissions due to the action of undisturbed dung beetle populations is that these emissions are subsequently reduced after 2 days of daytime solar incidence. The dynamics of CO₂ and CH₄ emissions differ when dung pats are colonised by a healthy or impoverished assemblage of dung beetles, as evidenced by the differences between the rich and poor mesocosms. The peak in CO₂ emissions observed in the rich mesocosms after 6 h did not occur in the poor mesocosms, while the CO₂ emissions were also high after 11 h in both treatments. Additionally, a smaller but significantly higher increase in CO₂ emissions was observed again after 48 h in the poor mesocosms. In the case of CH₄, the emissions did not differ between the rich and poor mesocosms up to 6 h but were drastically reduced at 11 h after the start of the experiment (complete exposure to daytime conditions). These differences in the dynamics of CO₂

and CH₄ emissions must be associated with the increase in dung spreading in the undisturbed assemblages, because a larger amount of dung that had not been disaggregated by the beetles (*c.* 50%) remained in the impoverished assemblages (Fig. 5c,d). The decrease in CH₄ emissions in dung colonised by dung beetles has been related to the aeration promoted by the tunnelling activity of dung beetles inside and beneath the dung pats (Penttilä *et al.*, 2013; Iwasa *et al.*, 2015; Slade *et al.*, 2016a). In our case, however, dung spreading by the activity of rollers and tunnellers (see Fig. 5c), rather than digging beneath the dung pat, appears to be the most important process explaining these reductions in GHG emissions (Verdú *et al.*, 2018). Future experiments should aim to discern the comparative effects of species richness, functional richness, abundance and biomass on GHG emissions under Mediterranean conditions through a full factorial design that takes into account combinations of the different levels of these factors.

Dung beetle and climate effects on cumulative GHG emissions

Our results reveal that the presence of dung beetles significantly reduced total CO₂ and CH₄ emissions from dung pats. Thus the cumulative CO₂ emissions were *c.* 23% and 52% lower and the CH₄ emissions were *c.* 44% and 78% lower in the poor and rich mesocosms, respectively, than in the dung-control mesocosms. The CO₂ equivalents were thus 26% lower in the poor mesocosms and 55% lower in the rich mesocosms. In other field studies using mesocosms, the activity of dung beetles contributed to an increase in the total CO₂ emissions in the system (Penttilä *et al.*, 2013; Iwasa *et al.*, 2015) or a tendency for the CO₂ flux to decrease only when individuals of larger species, such as *Copris lunaris*, are considered (Piccini *et al.*, 2017). Our study thus clearly shows that a ‘healthy’ dung beetle assemblage is able to reduce the GHG emissions from dung pats and that a decrease in the total abundance and total biomass of dung beetles due to the long-term effect of ivermectin use may affect these emissions.

From a geographical viewpoint, the cumulative GHG emissions by dung pats clearly show that in addition to dung beetles, climate also affects GHG emission patterns. The amount of time during which GHG emissions from dung pats occurred in our study was very short in comparison to the times reported in other published studies. Thus, climate appears to be a crucial factor related to the time at which gas emissions stop in dung pats not colonised by beetles (dung-control mesocosms). For example, subtracting pasture soil emissions, a study in northern Europe (southern Finland, 60°N) reported cumulative CO₂ emissions around 1642 g m⁻² kg⁻¹ for dung pats without dung beetles during the first 50 days after dung deposition (Penttilä *et al.*, 2013). However, our study indicates that CO₂ emissions from dung pats without dung beetles completely disappear after 7 days, with cumulative emissions *c.* 111.3 g CO₂ m⁻² kg⁻¹ dung, a value almost 15 times lower. In the case of CH₄ our detected cumulative emissions are 0.44 g m⁻² kg⁻¹ dung, whereas this value would be more than four times higher in southern Finland (a reported mean value of 1.77 g m⁻² which is equivalent to 1.51 g m⁻² kg⁻¹ dung after subtracting pasture soil emissions).

From a global perspective, our study highlights the combined effects of both abiotic conditions (climate) and dung beetle assemblages on GHG emissions. Thus, the available data indicate that the different times over which dung pats emit GHGs in the field and the decreasing pattern of functional dung beetle diversity along the latitudinal gradient from southern to northern Europe (Hanski, 1980; Hortal *et al.*, 2011) should have profound consequences in terms of GHG emissions. In boreal and temperate regions (e.g. Finland), mesocosms were established considering the low number of species and individuals encountered per natural dung pat (median two species per pat, range 1–8 species; *n* = 797 samples) (Slade *et al.*, 2016a from Roslin, 2001), while in warm Mediterranean sites, such as Doñana National Park, the species composition and abundances used in the mesocosms were based on the highly diverse dung beetle assemblages (e.g. rich mesocosms: median 12 species per pat, range 7–13 species, median abundance = 457 individuals per pat, median biomass = 57 g per dung pat; *n* = 20 samples) (Verdú *et al.*, 2018). The different results obtained in this study compared with those reported by other authors (Penttilä *et al.*, 2013) probably exemplify the differences in the effects of dung beetles on GHG emissions in contrasting environments, as suggested by Slade *et al.* (2016a).

Environmental implications of ivermectin treated livestock

A recent study conducted during spring–summer in Doñana National Park showed that the efficiency of dung degradation in areas where livestock is managed using VMPs is only 65.5% of the amount of dung processed by beetles at sites where livestock is free of VMPs. This implies that different proportions of the organic matter input from livestock manure remain on the soil surface in the two areas (*c.* 2.24 and 0.27 tonnes ha⁻¹ year⁻¹ of dung would remain unaltered in poor and rich sites, respectively) (Verdú *et al.*, 2018). According to the results obtained here, it is possible to estimate that in sites with impoverished dung beetle assemblages due to the traditional use of VMPs, *c.* 164.6 kg ha⁻¹ of CO₂ and 0.65 kg ha⁻¹ of CH₄ (corresponding to 186.7 kg ha⁻¹ of CO₂ equivalents) could be emitted into the atmosphere owing to the presence of unaltered and accumulated dung on the soil, considering the spring–summer seasons only. In sites with no use of VMPs and thus characterised by well-established dung beetle assemblages, the GHG values would still be lower: 19.8 kg ha⁻¹ of CO₂ and 0.08 kg ha⁻¹ of CH₄ (corresponding to 22.5 kg ha⁻¹ of CO₂ equivalents) during spring and summer. Thus, changes in dung beetle assemblages could have serious consequences for several ecosystem functions and services, including a decrease in GHG emissions.

Our results also suggest that the joint effect of climate and dung beetles on GHG emissions in Mediterranean conditions may seriously reduce GHG emissions from dung pats deposited on the soil when compared with those originating from enteric fermentation. Methane emissions due to enteric fermentation seem to oscillate from 135 to 390 g day⁻¹ per cow (Crutzen *et al.*, 1986; Niu *et al.*, 2018; Reisinger & Clark, 2018), while the dung deposited on the soil surface by a cow in a day

(24 kg day⁻¹; see Haynes & Williams, 1993) can generate up to 77.76 g CH₄ day⁻¹ per cow along the complete cycle of degradation in temperate or cold-temperate conditions (Saggar *et al.*, 2004). In our case, the CH₄ emissions from dung would be c. 10.56 g CH₄ day⁻¹ in control dung not colonised by dung beetles (i.e. seven times less) and 2.33 g CH₄ day⁻¹ in dung exploited by a dung beetle assemblage not affected by ivermectin use (33 times less). Thus, the existence of a functionally diverse and ivermectin-free dung beetle assemblage under Mediterranean conditions can cause a substantial reduction in GHG emissions from dung left in pastures, which has been estimated as the second most important source of livestock GHG emissions (Tubiello *et al.*, 2013; Slade *et al.*, 2016a). This result may also indicate a comparatively higher importance of enteric fermentation in terms of GHG emissions, highlighting the importance of developing effective strategies to reduce such emissions (Duin *et al.*, 2016).

Methodological improvement of GHG flux measurements

Substantial improvements in the state of the art regarding dung beetle-mediated effects on GHG emissions were obtained in this study. On the one hand, the use of the OA-ICOS technique allows reliable real-time onsite measurements to reduce the time over which samples were measured (c. 1.5 min per sample) and thus the total duration of each sampling event (c. 60 min per sampling event for 40 samples). In the conventional chamber-based trace gas flux measurement protocols (Parkin *et al.*, 2003) used in other related studies, the time required to obtain data similar to those provided here is very long (c. 30 min per sample and c. 4 h per sampling event for a total of 21 samples). This drawback indicates the importance of having appropriate controls and/or corrections of emission rates of GHGs due to temperature variations during a field experiment (Penttilä *et al.*, 2013). Usually, it is accepted that shorter enclosure times reduce the systematic errors that occur as a result of the use of closed chambers (Venterea & Baker, 2008; Christiansen *et al.*, 2015). Furthermore, an additional advantage of the CRDS technique is that the total volume analysed for each sample is near 1 litre, whereas in the conventional approach, a total volume of c. 5–50 ml is usually sampled, and only 0.25–1 ml of this sample is actually analysed by GC (Parkin *et al.*, 2003).

On the other hand, the CDRS technique adapted to measure insect respiration provided corrected GHG measurements by considering the CO₂ emissions generated by the dung beetles. In this study, the use of two treatments with different assemblage structures highlights the importance of considering the respiration of insects to correct estimates of CO₂ fluxes from dung in studies in which the respiration of the organisms must be taken into account. These corrections allow us to provide more precise measurements of CO₂ emissions from the dung, specifically in the initial and final phases of dung exposure, when the percentage of CO₂ emitted by dung beetles can become greater than the actual emissions from the dung pats (Fig. 5d).

Conclusions

In agreement with the comments of Slade *et al.* (2016a), we demonstrate here that the effects of dung beetles on CO₂ and CH₄ emissions are much more accentuated under warm-temperate conditions. Although the high ambient temperatures in southern Spain play an important role in substantially reducing the lifetime of dung pats, as shown in our dung-control mesocosms, the relatively high dung beetle taxonomic and functional diversity in this region could also affect emissions because more diverse and abundant assemblages remove and spread dung faster and more effectively. Despite the crucial roles of dung beetle richness and population abundance in enhancing dung degradation and reducing GHG emissions, our results also highlight the detrimental consequences of the ongoing steep decline in dung beetle assemblages in these warmer regions due to indiscriminate and traditional VMP use. Further studies exploring this topic across broad latitudinal and/or altitudinal gradients are needed to better understand the true role of dung beetles as GHG mediators and their implications in terms of climatic warming worldwide.

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Author contributions

JRV, JML and FSP conceived and designed the research; JRV, FSP, JML and VC collected the biological samples and performed the GHG measurements; JML, JRV, and FSP applied the statistical tests; and JRV, JML, FSP and VC wrote the manuscript.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

File S1. Environmental conditions in the field area and microenvironmental conditions inside the chamber during field measurements.

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