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Ivermectin residues disrupt dung beetle diversity, soil properties and ecosystem functioning: An interdisciplinary field study

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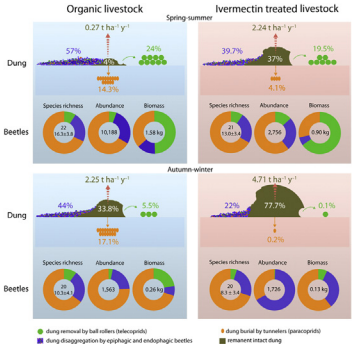
HIGHLIGHTS

- At the short term, ivermectin residues cause a strong decrease in dung relocation and dung spreading by dung beetles.
- Conventional use of ivermectin disrupts diversity by affecting species richness, abundance and biomass of dung beetles.
- Reduction in the functional efficiency of dung degradation resulted in the long-term accumulation of manure.
- Use of ivermectin causes lower quality in soil organic C and the increase of the in-situ mineral N and P production.
- The results of this study highlight that the effects of ivermectin must be investigated from a global perspective.
- The use of this veterinary medical product must be monitored and controlled following a precautionary principle.

GRAPHICAL ABSTRACT



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ABSTRACT

Ivermectin is the most common endectocide used to control parasites affecting livestock. Short-term physiological and behavioural effects of ivermectin on dung beetles may have long-term consequences for beetle populations and ecosystem functioning. Long-term effects of the use of ivermectin can be estimated by comparing dung assemblages and ecosystem functions in areas with conventional ivermectin-treated livestock and environmentally similar areas in which livestock are not treated with veterinary medical products (organic farming). In this study, we investigated both short-term and long-term effects of the administration of ivermectin on the characteristics of dung beetle assemblages and the services they provided in a protected area (Doñana National Park, SW Spain). We examined short-term dung colonization, dwelling, relocation, and disaggregation rates and the associations

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between these processes and the key assemblage parameters of species richness, abundance, biomass and functional diversity. Furthermore, we analysed changes in soil physical-chemical properties and processes. Short-term differences were observed in the total amount of dung relocated by dung beetles at different colonization vs. emigration stages, suggesting that dung beetles in this area were affected by the recent treatments of livestock with ivermectin. Moreover, short-term effects could also be responsible for the significant differences in dung spreading rates between sites. Conventional use of ivermectin disrupted ecosystem functioning by affecting species richness, abundance and biomass. The decrease in diversity parameters was related to a reduction in the functional efficiency, which resulted in the long-term accumulation of dung on the ground and considerable changes in soil functionality.

1. Introduction

Ivermectin is the most common endectocide substance used to control arthropods and nematodes affecting livestock (Campbell et al., 1983). Since the early 1980s, over 5 billion doses have been sold worldwide (Shoop and Soll, 2002). After application, the primary route of excretion is via faeces (Canga et al., 2009), which constitute a food source for very many invertebrate species (Holter, 2016), in addition to an important input of organic matter into soils (Irshad et al., 2013). Dung degradation is a complex process that involves different taxonomic groups, including dung beetles, which may be affected by ivermectin. Previous studies suggest that the massive treatment of entire flocks in a given area can lead to a significant reduction in the number and composition of coprophagous invertebrates, delaying dung degradation (Wall and Strong, 1987; Floate, 1998) and altering soil nutrient cycling (Madsen et al., 1990; Sommer and Bibby, 2002). Short-term effects of ivermectin on dung beetles include increased attractiveness of ivermectin-containing dung (Holter et al., 1993; Lumaret et al., 1993), loss of sensorial and mechanical activity (Verdú et al., 2015), disruption of reproductive physiology (Martínez et al., 2017), and mortality at both adult and larval stages (Wardhaugh and Rodriguez-Menendez, 1988; Krüger and Scholtz, 1997; Iwasa et al., 2007). According to recent evidence, even low doses of ivermectin can significantly decrease the olfactory and locomotor capacity of dung beetles (Verdú et al., 2015). Thus, ivermectin seriously affects basic biological activities of dung beetles such as food detection, intraspecific communication, locomotion and interaction with the environment.

These short-term physiological and behavioural effects of ivermectin on dung beetles may have long-term consequences for beetle populations and ecosystem function. For example, long-term disruptions caused by ivermectin residues could be responsible for increases in local extinctions and changes in guild structure, causing alterations in ecological functioning and ecosystem services (Lobo, 2001; Beynon et al., 2012; Nature-England, 2016). Despite the increasing number of studies investigating the effect of ivermectin on several ecosystem services, no studies have examined long-term effects (several years) of ivermectin residues. Long-term consequences of the use of ivermectin can be anticipated by comparing both dung assemblages and ecosystem functions in areas with conventional ivermectin-treated livestock and environmentally similar areas in which livestock is not treated with antiparasitic substances (organic farming). Unfortunately, these types of comparisons are becoming more difficult because intensive farming has been the dominant livestock system for decades; whereas organic livestock continues to remain scarce (Tilman et al., 2001; IAASTD, 2009). Furthermore, under current regulations, "organic farming" does not guarantee ivermectin-free livestock (Coffey and Baier, 2012). On the contrary, legislation in many countries requires organic livestock farmers to establish preventive health care practices that include the use of synthetic veterinary substances, such as ivermectin (Chander et al., 2011; Agricultural Marketing Service, USDA, 2017; European Union, 2007). In this study, we conducted an experiment in a protected area (Doñana National Park, SW Spain) with a stringent health management program that impeded the use of ivermectin in a core area belonging to a biological reserve (ICTS-DBR) for the last 30 years and a nearby site within the park in which ivermectin has been used to treat livestock. We investigated both short-term and long-term effects of the administration of this medication on the characteristics of dung beetle assemblages and the services they provide. To better understand the long-term effect of ivermectin use, we examined short-term dung colonization, dwelling, relocation, and disaggregation rates and the associations between these processes and the key assemblage parameters of species richness, abundance, biomass and functional diversity. Finally, we analysed changes in soil physical-chemical properties and functions that were potentially derived from the effects of ivermectin on dung beetle assemblages. Specifically, we focused on soil carbon (C), nitrogen (N), and phosphorous (P) pools and availability, in addition to C and N mineralization rates. These variables were all selected because of their importance for maintaining soil productivity and fertility and for the provision of key ecosystem services (Compton et al., 2011).

2. Materials and methods

2.1. Study area and livestock management

The Doñana region is located in SW Spain between the provinces of Huelva and Sevilla. This region hosts a nature reserve that includes both Doñana National Park and Doñana Natural Park. In Doñana National Park, livestock is composed of Andalusian endemic races of feral 'Mostrenca' cows and marsh horses. The climate is Mediterranean with some oceanic influence. Average annual rainfall is approximately 560 mm, and average annual temperature is approximately 17 °C.

The dung beetle assemblages of Doñana National Park have been rigorously studied (Lobo et al., 1997; Cárdenas and Hidalgo, 2006), because the area is one of the most diverse in the Palearctic region for species richness, abundance and guild diversity. We selected two field sites within Doñana National Park that differed in farming history and current use: one located within the Doñana Biological Reserve (DBR-ICTS) and the other, separated by 10 km, outside this reserve, called "Los Sotos." The DBR-ICTS site (traditional organic livestock, hereafter the ECO site) was located in an ivermectin-free area (6794 ha) and was selected because it constitutes a good example of 'organic farming'. The livestock density on the ECO site was 0.33 head of cattle per hectare (PAG-PNDoñana, 2000). In the ECO site, ivermectin (or similar compounds) has never been used. The Los Sotos site (ivermectin-treated livestock, hereafter the IVM site) was also an extensive landscape with similar climate, vegetation (Muñoz-Reinoso and García-Novo, 2005), soil type (Siljeström et al., 1994), livestock density (0.30 head per hectare) and area (6423 ha) to the ECO site but differed in farming history because livestock have been regularly treated with ivermectin and other veterinary medical products since the 1980s. According to a farmer survey conducted in 2014 (Figs. S1–S3 in Supporting Information), ivermectin is the VMP local farmers preferred (92% of VMPs used). Injectable and oral formulations of ivermectin are administrated to livestock in two different periods during the year. In July, horses (with oral gel application) and cows (with subcutaneous injection) are massively treated with ivermectin (generally in combination with praziquantel, for tapeworm control). In September–November, cows, and horses in

a few cases, are treated again. In both periods, 2–4 days after the treatments, all livestock are released into the field, so the complete elimination of the ivermectin residues occurs across the entire grazing area. To verify the use/non-use of ivermectin by farmers in the IVM site and the absence of this VMP in the ECO site during our study, we analysed 15 randomly selected dung pats in each site using a new analytical method (Ortiz et al., 2017). The results provided by these chemical analyses (Table S1 in Supporting Information) strongly supported the ubiquitous occurrence of ivermectin in the dung pats from the IVM site and the absence of this compound in those coming from the ECO site.

2.2. Experimental design

Field experiments were conducted at the selected sites in July 2013 and November 2013 after two antiparasitic treatments. The two different seasons are characterized by the occurrence of dissimilar dung beetle assemblages (Sowig, 1997; Agoglitta et al., 2012). Thus, the experimental design included two factors: 'Management' (sites with and without historical use of ivermectin; IVM and ECO sites, respectively) and 'Season' (spring and autumn). In each one of these four treatments, 40 sampling units were placed on the ground: 20 using fresh cow dung ('Dung' factor) with ivermectin addition (hereafter IVM dung) and 20 without ivermectin addition (hereafter CNT dung). The treated dung bait was spiked with 100 μg kg^{-1} of ivermectin and was prepared following Verdú et al. (2015) protocol. Dung with ivermectin was placed in the ECO site to examine whether ivermectin in dung affected attractiveness to dung beetles. Finally, 10 sampling units of each treatment were examined 12 h after dung placement and the others after 48 h ('Time' factor). During the first 12 h (t_1), the immigration-colonization of beetles to the dung is the primary structuring force of dung assemblages, whereas after emigration of the first colonizers, a second wave of colonization occurs by nocturnal species and dwelling dominates ($t_2 = 48$ h) (Sullivan et al., 2017). The factors 'Season' and 'Time' were included to examine the stability of the results under different ecological scenarios. A total of 160 sampling units were finally placed in the field (2 Managements × 2 Seasons × 2 Dung types × 2 Times × 10 replicates). Animals destroyed three of the sampling units. The sampling units were randomly assigned to a level of each of the factors and placed 25 m apart in each site to minimize potential interference among them in this attractiveness to dung beetles.

Each sampling unit consisted of one adapted plastic washbasin (40 l in volume) filled with soil on which a bait of cow dung was placed in the midpoint (1320 ± 65 g fresh mass, equivalent to 247 ± 23 g dry mass; mean ± SD). To measure dung disaggregation as the rate of the dung pat spreading out on the ground due to dung beetle activity, the size and shape of the dung bait was standardized using cylindrical containers, 14 cm diameter × 7.5 cm height, as mold to prepare baits. To avoid the escape of beetles rolling dung balls, 5 cm of unevenness remained inside the container (Movie S1 in Supporting Information for details). The ivermectin-free dung used as bait was collected from the ECO site during the first 2 h of the morning to avoid dung fauna colonization and to minimize physical-chemical changes in the dung.

These experiments conformed to the Spanish legal requirements related to wildlife conservation and welfare. Additionally, specimen collection and field research in Doñana National Park were conducted with the required permissions.

2.3. Beetle diversity and function measurements

For each of the sampling units, the number of beetles of each species was counted, and the species richness (*S*), total abundance (*N*) and total biomass (*B*, g fresh mass) were measured.

The total amount of dung relocated or manipulated by dung beetles and the specific amount of dung transported by telecoprids (rollers), disaggregated by epiphagic-endophagic beetles (dwellers sensu *lato*) or buried by paracoprids (tunnelers) were estimated in each sampling unit. For rollers, the beetles rolling dung balls were counted, and dung balls were collected, kept in plastic bags and labelled accordingly until determination of dry mass in the laboratory. All the roller dung beetles were removed (and later released ca. 10–20 km from the study sites) to avoid a possible re-colonization of dung by the same individuals, which could overestimate their ecological function. For dwellers, the number of individuals of each species was counted, and only when taxonomic identification was difficult in the field, the beetles were preserved in 70% ethanol for identification in the laboratory. The dung disaggregated by the guild of dwellers was kept in plastic bags and labelled accordingly until dry mass was determined in the laboratory. For tunnelers, the dung relocated in the soil of the washbasin (up to 25 cm in depth) was also kept in plastic bags and dry mass was determined in the laboratory.

The estimates of the fresh mass of dung removed, disaggregated and buried by dung beetles were converted to dung dry mass to avoid biases caused by i) differences in moisture content due to variations in desiccation of dung samples collected in contrasting seasons (summer and autumn), after different times of exposure (12 and 48 h), and differing in the degree of dung fragmentation and ii) sand adhered to dung. Water content and sand-free dry mass of dung were determined in the laboratory by completely desiccating the dung pats at 100 °C for 72 h, which were then weighed using a precision balance (AG104 Analytic Balance; Mettler Toledo, Columbus, OH, USA). Dung mass loss was calculated by subtracting the dry mass of the dung pats from the initial dry mass used as bait (81.30 ± 0.77% water content; mean ± SD).

All measurements were performed simultaneously at both sites for each 'Time' period to avoid possible biases between sites.

2.4. Dung spreading rate

The speed at which disaggregation (i.e., fragmentation and disappearance of dung pats) occurred was measured by calculating the rate of dung spreading, i.e., the area covered for each dung pat per hour (in $cm^2\ h^{-1}$). The slope of the relationship between dung spreading area (cm^2) and time (h) was calculated using a digital image of each dung pat taken at four time periods (t_0 = dung pat placement, and t_6, t_{12} and t_{24} = 6, 12 and 24 h after placement). These images were used to estimate the rate of dung spreading and total disaggregation (after 24 h) and their variation according to the level of the 'Management' and 'Season' factors using a superposition image analysis performed with ImageJ 1.48 free software (http://imagej.nih.gov/ij) to calculate areas. Adobe Photoshop CS5.1 and Adobe Illustrator CS5.1 software (Adobe Systems Inc., San José, California, USA) were used to edit the generated images.

2.5. Soil carbon and nitrogen dynamics

A preliminary assessment of the possible long-term effects of livestock management on soil chemical properties, including extractable nutrients (i.e., available to plants), and soil functions (soil C and N potential mineralization rates) was conducted. In November 2014, 10 independent soil cores (5 cm wide and 15 cm deep) were randomly collected (separated by at least 20 m) in the experimental plots in the ECO and IVM sites. Soil samples were stored at 4 °C in the laboratory until analyses. All measurements were conducted within one month after collection. From each soil core, a 10 g subsample of fresh soil was oven-dried at 60 °C to determine soil water content. Total C and N concentrations were determined by dry combustion with an elemental analyser (LECO TruSpec CN). We measured soil pH with a pH meter using a soil-to-water ratio of 1:2.5 (m/v) and analysed organic matter content by loss-on-ignition (Nelson and Sommers, 1996). To extract soil inorganic N, fresh soil subsamples (10 g) with 40 ml of 2 M KCl were shaken for 1 h at 200 rpm on an orbital shaker and the suspension was filtered through a 0.45 mm Millipore filter. We used these extracts to colorimetrically determine the amounts of NH_4^+–N, NO_3^+–N, and PO_4^{3-}–P (Durán et al., 2008).

The above measurements estimated the in situ concentrations of available inorganic nutrients; therefore, additional analyses with ion exchange membranes (IEMs) were performed to calculate the rate of production of inorganic N (NO_3^- and NH_4^+) and P (PO_4^{3-}) (Durán et al., 2013). IEMs are a useful and reliable alternative to more destructive traditional methods. A total of 10 anionic and cationic exchange membranes were placed at a depth of 10 cm in the soil separated by 20 m in each sampling site. IEMs remained in the field for 30 days, and then the membranes were extracted with 30 ml of 2 M KCl in the laboratory. These extracts were analysed colorimetrically for NO_3^-–N, NH_4^+–N, and PO_4^{3-} contents, as described above.

Potential C and N mineralization rates were determined in a laboratory incubation experiment. Thirty grams of each soil sample ($n = 10$) was incubated in 250 ml jars at optimal moisture (~25%) and temperature (~25 °C) conditions for 2 weeks, after equilibrating at the new temperature and water content conditions for 24 h. Soil water content was adjusted to 25%, and soil samples were weighed and placed in glass jars one day before starting the C mineralization measurements. We kept soil water content constant during the experiment by carefully adding water to compensate for any water loss. We estimated soil carbon mineralization rates by measuring the increase in CO_2 concentration in the sealed glass jars over 90 s on days 0, 1, 2, 3, 6, 9, and 15 with a portable gas analyser (EGM-2; PP-systems, Amesbury, MA, USA). The total amount of carbon mineralized was calculated by interpolation of the soil carbon mineralization rates between measurement dates. Total mineralized carbon was normalized to both dry soil mass and soil initial carbon content. We estimated the potential N mineralization as the net increase in total inorganic N (NH_4^+–N + NO_3^-–N) over the incubation period. Net increases in NH_4^+–N and NO_3^-–N were used to indicate net ammonification and nitrification rates, respectively.

2.6. Statistical analyses

General Linear Models (GLMs) were used to perform a multivariate analysis of variance (MANOVA) with a sigma-restricted parameterization. Species richness (S), log of abundance (log$N$), log of biomass (log$B$, in g), total quantity of dung relocated or managed by dung beetles (in g), and the specific quantities of dung transported by rollers, disaggregated by dwellers or buried by tunnelers were considered dependent variables. The effect of the four formerly described binary categorical factors on these response variables was evaluated in a 2 (Management) × 2 (Season) × 2 (Dung) × 2 (Time) between-groups factorial design. In these analyses, when two-way, three-way or four-way interactions were statistically significant after Bonferroni correction for multiple estimations, the explanation of the additive main effects was obviated.

A one-way ANOVA was used to determine significant differences between the slopes of the relationship between dung spreading area and time and the total area of dung disaggregation after 24 h of exposure. One-way ANOVA tests were also used to estimate the effect of the "Management" factor on soil chemical variables, total C and N mineralized, and N production rates. Variables were log-transformed before analyses when necessary to meet the assumptions of the statistical analyses. All analyses were performed with the statistical software R (R Core Team, 2014).

3. Results

3.1. Species richness, abundance and biomass

A total of 16,316 individuals belonging to 32 species were collected (Table S2 in Supporting Information). 'Management' (Fig. 1a), 'Season' and 'Time' were the statistically significant main factors that explained the variation in species richness (Table 1). Thus, the richest assemblages were in the ECO site, colonizing the dung during the hottest season in the first 12 h. The significant 'Season' * 'Time' interaction indicated

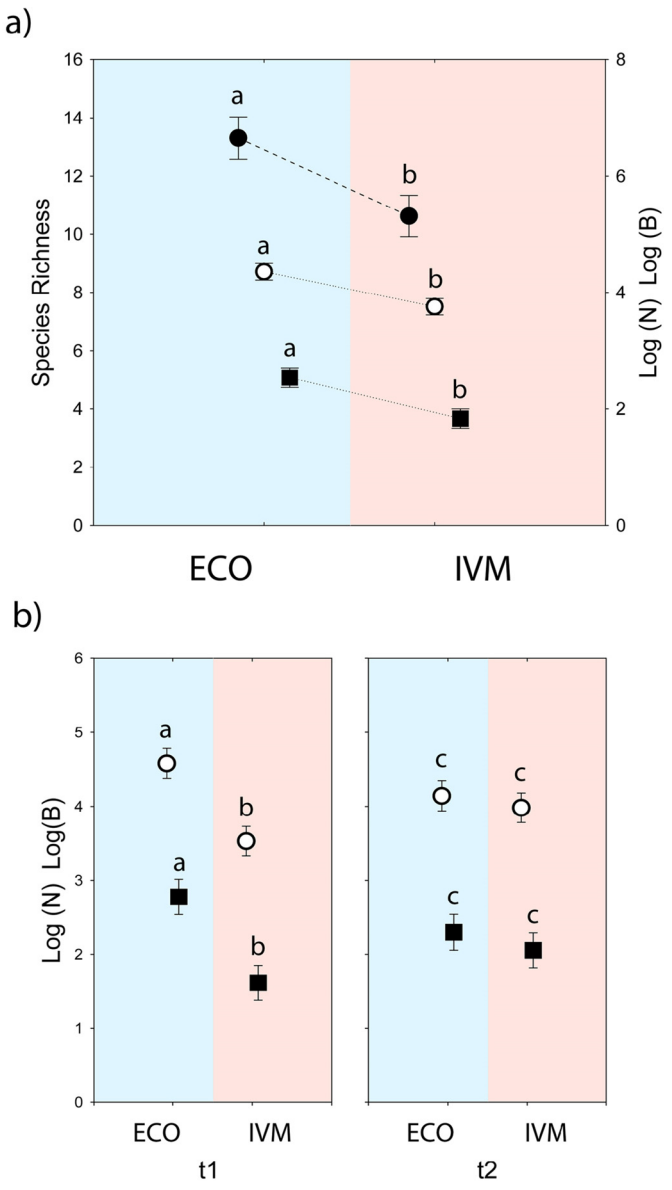

Fig. 1. Dung beetle diversity, population abundance and biomass in conventional and organic livestock systems. a) Species richness (black circles; mean ± s.e.m.), abundance (white circles; log10 transformed data, mean ± s.e.m.) and biomass (black dots; log10 transformed data, mean ± s.e.m.) in conventional (IVM) and organic (ECO) livestock systems; b) abundance and biomass variation between management types and times of colonization (t1, t2). Different letters indicate significantly different means (post-hoc Tukey test $P < 0.05$, after Bonferroni correction; see also Table 1 for general statistical results).

that in the first 12 h (t_1) the richness differed between the two seasons, with a higher species richness at t_1 in the spring-summer season (Table 1; Fig. 1a).

Beetle species abundance was also significantly higher during the hottest season in the ECO site than at the IVM site (Table 1; Fig. 1b). The three two-way interactions and one three-way interaction with the factors 'Management', 'Season' and 'Time' were significant, indicating that the assemblages with the highest abundances inhabited the ECO site during the hottest season in the first 12 h after dung placement. The statistically significant 'Management' * 'Time' interaction indicated that those sampling units with higher abundance were in the ECO site, at t_1 (Fig. 1b). Beetle biomass was also significantly higher during the hottest season in the ECO site (Table 1; Fig. 1b). The significant three two-way interactions revealed that the biomass was particularly high at t_1 (Fig. 1c) in the hottest season and in the ECO site. The 'Season' *

Table 1
Multivariate analysis of variance (MANOVA) derived from General Linear Models to estimate the effects of the four binary factors on the variation of species richness (S), log abundance (log(N)) and log biomass (log(B)). *P*-values after Bonferroni correction: **** ≤ 0.000007; *** ≤ 0.00007; ** ≤ 0.0007; * ≤ 0.003.

	S	Log(N)	Log(B)
	$F_{(1141)}$	$F_{(1141)}$	$F_{(1141)}$
Management	27.05****	35.30****	33.99****
Season	108.99****	103.45****	241.71****
Time	22.14****	0.01	0.03
Dung	0.40	0.50	0.09
Management ∗ season	1.50	52.42****	0.08
Season ∗ time	15.44**	40.69****	15.58**
Management ∗ time	1.04	19.11***	14.54**
Season ∗ dung	6.61	2.84	12.14**
Management ∗ dung	0.85	8.21	0.96
Time ∗ dung	0.27	1.42	0.83
Management ∗ season ∗ time	0.85	18.82***	1.04
Management ∗ season ∗ dung	4.76	8.39	0.50
Season ∗ time ∗ dung	1.95	2.10	0.22
Management ∗ time ∗ dung	1.02	0.04	0.01
Management ∗ season ∗ time ∗ dung	1.69	0.06	0.08

'Dung' interaction was the only one in which the addition of ivermectin to the bait had a significant effect; during the cold season, the beetle biomass in dung pats with ivermectin was reduced (Table 1).

3.2. Total dung relocated

'Management', 'Season' and 'Time' were the statistically significant main factors for the total dung relocated, with the first two factors explaining >50% of total variability (18.2% and 34.3%, respectively; Table 2). Thus, more dung was manipulated and relocated by dung beetles in the ECO site, particularly during the hottest season and at t_1. The statistically significant interaction among these three factors (Table 2 and Fig. 2a) indicated that the timing of maximum dung relocation differed between ECO and IVM sites (with the highest dung relocation at t_2) and that season had an effect, because the highest values of relocated dung occurred during autumn-winter in the ECO site and at t_2. The other significant three-way interaction was 'Management' ∗ 'Time' ∗ 'Dung' (Table 2 and Fig. 2b), which showed that the amount of dung relocated at t_1 (during the first 12 h upon dung placement) was lower in the IVM site and similar in the CNT site to the dung relocated at t_2. However, in the ECO site, the total quantity of relocated dung was higher at t_2 when the dung bait contained ivermectin (IVM dung).

Table 2
Multivariate analysis of variance (MANOVA) derived from General Linear Models to estimate the effects of the four binary factors on the variation of total amount of dung relocated or manipulated by dung beetles (Total), in addition to the specific amount of dung rolled away by telecoprids (Tel), disaggregated by endophagic-epiphagic beetles (End) or buried by paracoprids (Par). *P*-values after Bonferroni correction: **** ≤ 0.000007; *** ≤ 0.00007; ** ≤ 0.0007; * ≤ 0.003.

	Total	Tel	End	Par
	$F_{(1144)}$	$F_{(1144)}$	$F_{(1144)}$	$F_{(1144)}$
Management	83.02****	11.72*	59.26****	36.84****
Season	158.14****	163.80****	38.15****	68.85****
Time	13.12**	1.56	21.61****	4.13
Dung	3.47	1.34	3.35	0.02
Management ∗ season	0.89	0.03	0.06	18.02***
Season ∗ time	0.53	6.05	3.55	1.33
Management ∗ time	5.95	12.94**	0.27	3.13
Season ∗ dung	0.31	0.25	1.12	0.01
Management ∗ dung	0.14	0.01	0.00	0.98
Time ∗ dung	15.40**	12.48**	7.28	1.43
Management ∗ season ∗ time	27.79****	21.56***	10.83*	7.09
Management ∗ season ∗ dung	1.79	3.05	1.19	0.74
Season ∗ time ∗ dung	0.01	2.97	1.42	0.93
Management ∗ time ∗ dung	11.32*	6.68	4.72	4.30
Management ∗ season ∗ time ∗ dung	0.51	0.38	3.61	2.87

3.3. Dung transported by rollers

'Management' and 'Season' were the only two statistically significant main factors for the quantity of dung transported and dispersed by roller dung beetles (Table 2), explaining 3.0% and 42.1% of total variability, respectively. Again, the dung relocated by rollers was higher in the ECO site than in the IVM site during the hottest season. The significant 'Management' ∗ 'Season' ∗ 'Time' interaction indicated that the activity of rollers was reduced during the autumn-winter season and increased at t_1 in the hottest season for the dung deposited in the ECO site (Table 1 and Fig. 2c). Notably, the significant 'Time' ∗ 'Dung' two-way interaction suggested that the activity of roller dung beetles increased when dung contained ivermectin at t_2 (Table 1 and Fig. 2d).

3.4. Dung disaggregation

The fragmentation and disaggregation of dung by endophagic and epiphagic beetles were also explained by the main factors 'Management', 'Season' and 'Time' (Table 2), which explained 19.7%, 12.7% and 7.2% of the total variability, respectively. Thus, the level of disaggregation was also the highest during the hottest season at t_2 and in the ECO site. Again, the significant interaction 'Management' ∗ 'Season' ∗ 'Time' showed that the rates of dung disaggregation at t_1 and t_2 differed strongly between ECO and IVM sites and between seasons, with the lowest values of dung disaggregation corresponding to the IVM site, primarily during the autumn-winter season and at t_2 (Table 2 and Fig. 2e).

3.5. Dung buried by paracoprids

'Management' and 'Season' were the only two statistically significant main factors that explained the dung buried by paracoprid beetles (Table 2), explaining 12.5% and 23.4% of the total variability, respectively. The quantity of buried dung was higher in the ECO site than in the IVM site and during spring-summer than in autumn-winter, although the only statistically significant interaction ('Management' ∗ 'Season') indicated that the seasonal difference was only observed in the ECO site (Table 2 and Fig. 2g).

3.6. Rates of dung spreading

For the two levels of 'Season', after 24 h of exposure, the total dung spreading area and rates of spreading were significantly higher at the ECO site than at the IVM site (Spring-summer: Total area: $F_{1,20} = 27.3$, $P < 0.0001$; Spreading rate: $F_{1,20} = 17.1$, $P < 0.0001$; Autumn-winter: Total area: $F_{1,20} = 37.5$, $P < 0.0001$; Spreading rate: $F_{1,20} = 29.1$, $P < 0.0001$; Fig. 3). In the hottest season, at the ECO site, the spreading rate of dung was 51.7 $cm^2\ h^{-1}$, whereas at the IVM site, the spreading rate decreased to 28.3 $cm^2\ h^{-1}$. During autumn-winter season, a general decrease and a delay were observed in the spreading rate, although the rate in the ECO site was higher than that in the IVM site (Fig. 3).

3.7. Physicochemical soil properties and C and N mineralization rates

Soil organic matter and soil carbon content were very low (Table 3). The soil pH values in the IVM site were significantly higher than those in the soils at the ECO site but values for total C, total N, and C/N ratios were similar. Soils in the IVM site had consistently, but not always significantly, higher extractable PO_4^{-3}–P and lower extractable NH_4^-–N, NO_3^-–N and total inorganic N than soils in the ECO site (Table 3).

Despite the lack of significant differences in the soil nutrient contents between sites, rates of inorganic N and P production over time were markedly higher in the IVM site (Table 3). These differences in the rates of nutrient production were consistent with potential C and N mineralization rates, with both C and N mineralization rates significantly higher in the IVM site than in the ECO site.

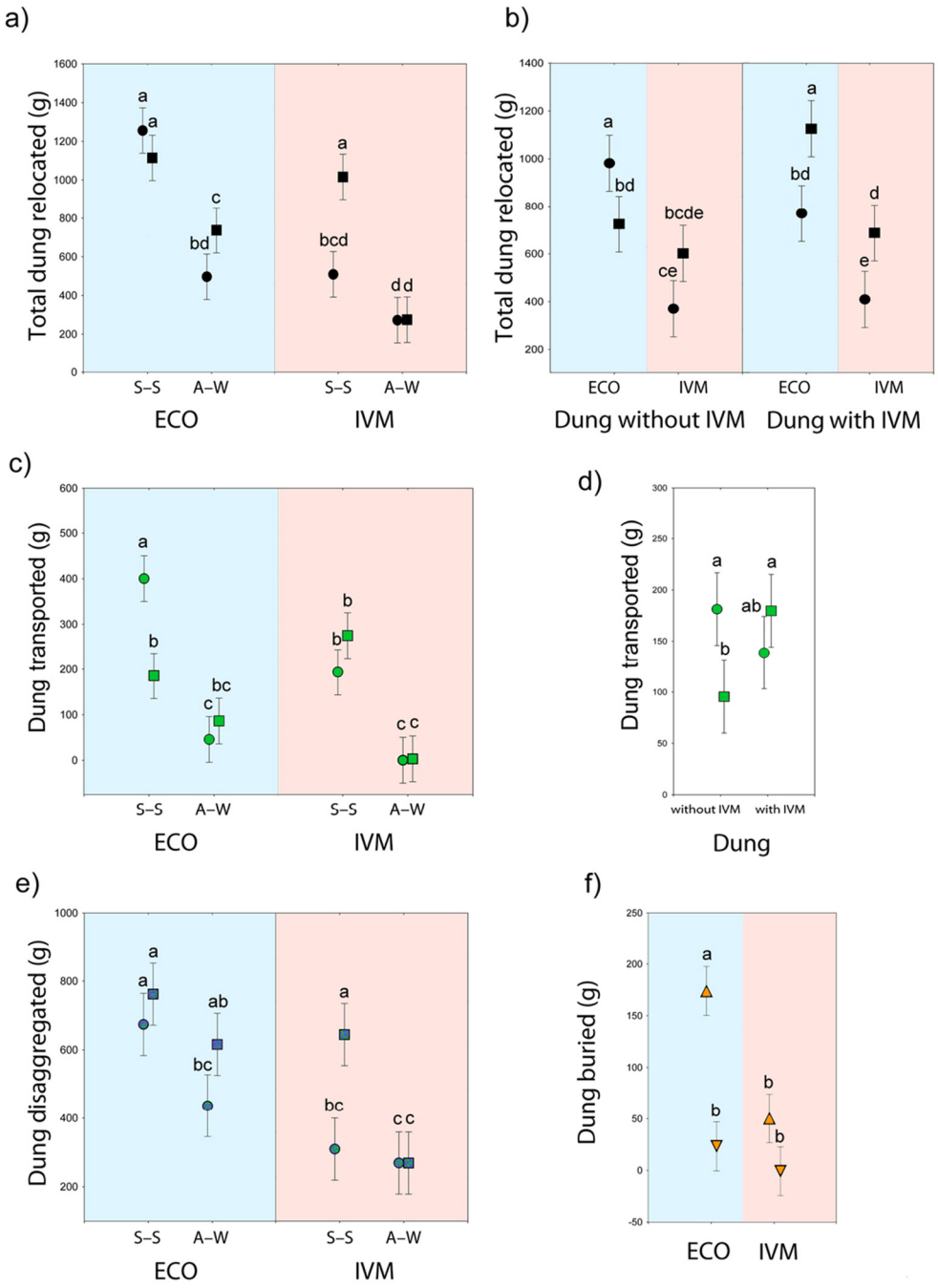

Fig. 2. Dung degradation by dung beetle activity in conventional and organic livestock systems. a) Total dung relocated by dung beetles in conventional (IVM) and organic (ECO) livestock systems in two seasons (S–S: spring–summer; A–W: autumn–winter) and at two different times of dung colonization (circles: t_1; dots: t_2); b) Total dung relocated by dung beetles in IVM and ECO sites for ivermectin-free dung and dung with ivermectin addition; c) Dung transported by roller dung beetles in IVM and ECO sites in each season; d) Dung transported by roller dung beetles, distinguishing ivermectin-free dung and dung with ivermectin addition; e) Dung disaggregated by endophagic (endocoprids and some paracoprids) and epiphagic (some telecoprids) dung beetles in IVM and ECO sites in each season; f) Dung buried by paracoprid dung beetles in IVM and ECO sites. Different letters indicate significantly different means (post-hoc Tukey test $P < 0.05$, after Bonferroni correction; see also Table 1 for general statistical results).

4. Discussion

In contrast to other studies (Lumaret et al., 1993; Floate, 2007; Errouissi and Lumaret, 2010; Römbke et al., 2010), we did not observe increased attractiveness of dung beetles to faeces containing ivermectin. We observed only a significant reduction in dung beetle biomass (but not in number of species or abundance) during the cold season due to ivermectin in dung pads. Furthermore, short-term differences were observed in the total amount of dung relocated by dung beetles at colonization vs. emigration stages (t_1 and t_2). The strong decrease in dung relocation found at the IVM site suggested that dung beetles in this area were affected by the recent treatment of livestock with ivermectin conducted a few days before the bioassay (July 27–31). This veterinary treatment, supported by chemical analyses during the bioassay (Table S1 in Supporting Information), might have contributed to an accumulated ingestion of ivermectin, which could explain the observed delay in feeding behaviour and consequently, reduced functional role of dung beetles in dung decomposition. This observation is consistent with the sublethal effects of ivermectin observed in dung beetles (Verdú et al., 2015) and was corroborated by field observations during this study in which all the dead individuals in the experiment were associated with dung treated with ivermectin placed in the IVM site. Ivermectin was

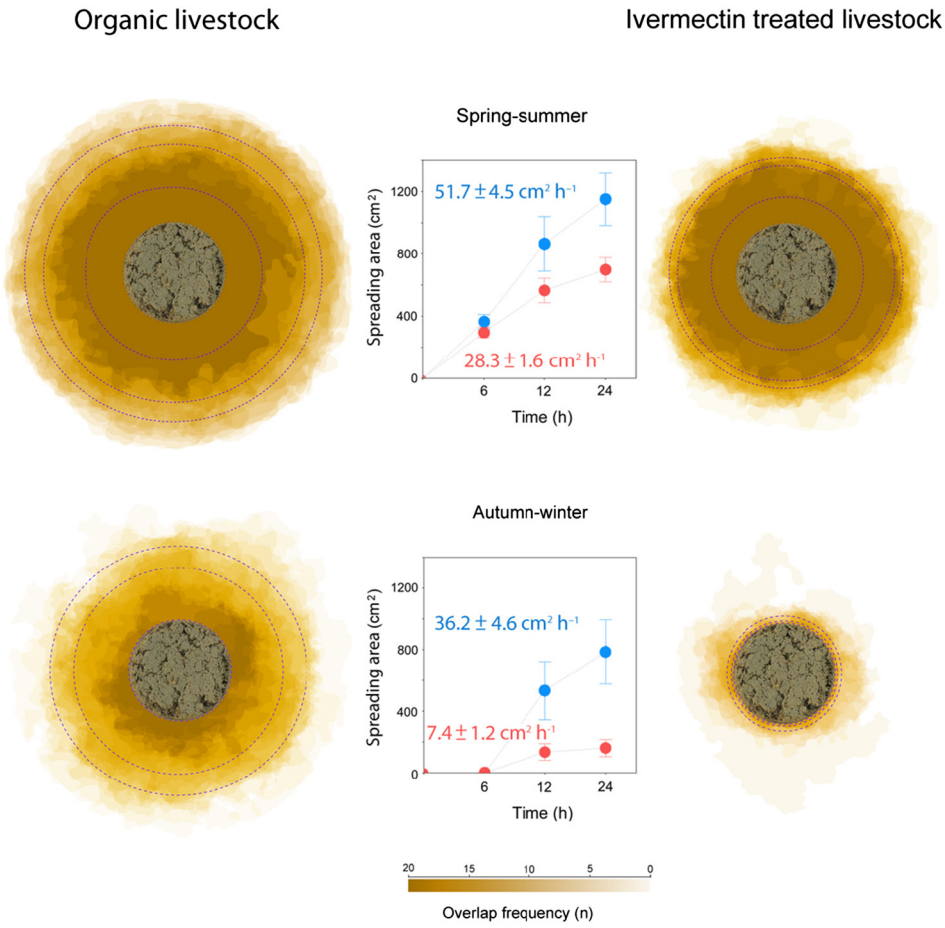

Fig. 3. Rates of dung spreading in conventional and organic livestock systems. Concentric circles (0, 6, 12 and 24 h) in each superposition image. Dots in the graphs (seasons; blue dots = spring-summer; red dots = autumn-winter) represent the average area (mean ± s.e.m.; $n = 20$ for each treatment) of dung spreading (in cm^2) at 6, 12 and 24 h, respectively. Dung spreading rates are expressed in $cm^2\ h^{-1}$ for each livestock management system (ECO and IVM sites) and season. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

also detected in the carcasses of several individuals of *Scarabaeus sacer* ($n = 12$) and *S. cicatricosus* ($n = 8$) found dead inside the washbasins during the present study period (Ortiz et al., 2017). Notably, in the ECO site, no individual died in any of the samples, which suggested that lethal effects require at least several days of drug ingestion (Verdú et al., 2015).

Our results demonstrate that the intensification of livestock management, with an intrinsic increase in the use of veterinary drugs for cattle, is detrimental to dung beetle populations (Lumaret et al., 1993) and might affect dung beetle patterns of diversity (Hutton and Giller, 2003). In this study, the use of VMPs contributed to a long-term negative effect on species richness, abundance and biomass in Doñana National Park.

Both short-term and long-term effects were involved in the differences between IVM and ECO sites in the dung degradation process, as clearly shown by the results of dung spreading. On one hand, differences in dung spreading area could be explained by long-term effects associated with the decrease in dung beetle populations over time, involving in some cases, changes in species composition and a decline in the number of species. On the other hand, short-term effects could also be responsible for the significant differences in dung spreading rates between the sites. Reductions in dung spreading rates at the IVM site compared with those in the ECO site in both seasons could be due to the sublethal effects of ivermectin ingestion, such as stupor and ataxia (Verdú et al., 2015).

Furthermore, conventional use of ivermectin apparently disrupts ecosystem function by affecting species richness, abundance and biomass. The parameters of assemblage diversity were significantly lower in the site with ivermectin-treated livestock than in the other site. The decrease of some dung beetle populations influenced by the use of VMPs (ivermectin primarily) has been reported in other studies as the first indicator of the quality of the dung beetle assemblage

Table 3
Soil chemical variables, extractable nutrients (i.e., available to plants) and nutrient production estimated by ion exchange membranes (IEMs) in the sites with livestock conventionally treated with ivermectin (IVM) and without ivermectin treatment (ECO). Values are the mean ± s.e.m. ($n = 10$), whereas the probabilities are those corresponding to ANOVA analyses.

Variable	IVM	ECO	P
Chemical variables			
Loss on ignition (%)	2.19 ± 0.29	1.53 ± 0.13	0.060
Soil moisture content (%)	9.60 ± 2.04	8.25 ± 0.76	0.064
pH	6.09 ± 0.09	5.36 ± 0.06	<0.001
Total C (%)	1.30 ± 0.28	0.82 ± 0.07	0.235
Total N (%)	0.07 ± 0.01	0.06 ± 0.01	0.433
C/N ratio	20.7 ± 5.60	13.2 ± 0.70	0.290
Extractable nutrients			
NO_3^-–N (mg kg^{-1})	8.36 ± 1.86	10.71 ± 1.78	0.375
NH_4^+–N (mg kg^{-1})	10.41 ± 0.67	13.10 ± 1.45	0.077
Inorganic N (mg kg^{-1})	18.50 ± 1.84	23.80 ± 2.93	0.154
PO_4^{-3}–P (mg kg^{-1})	0.19 ± 0.06	0.11 ± 0.03	0.256
Nutrient production			
NO_3^- (μg cm^2 day^{-1})	279.6 ± 18.21	73.65 ± 17.09	<0.001
NH_4^+ (μg cm^2 day^{-1})	107.1 ± 13.20	60.46 ± 18.61	0.056
Inorganic N (μg cm^2 day^{-1})	386.7 ± 15.90	134.1 ± 33.30	<0.001
PO_4^{3-} (μg cm^2 day^{-1})	4.35 ± 0.85	0.38 ± 0.18	<0.001

(Adler et al., 2016). In Doñana National Park, this phenomenon was observed in both seasons, affecting all dung beetle functional guilds. For example, in the IVM site, the total dung buried by paracoprids was three to seventeen-fold lower than that in the ECO site (4.1% vs. 14.3% in spring-summer and 0.2% vs. 17.1% in autumn-winter, respectively). The negative effect of ivermectin on dung burial activity of some paracoprids such as *Onthophagus taurus*, a common species in Doñana National Park, has been previously reported (Dadour et al., 1999). For rollers and dwellers the amount of dung removed differed up to two orders of magnitude. As a long-term consequence of population decreases, local extinctions would explain the impoverishment observed in the assemblages. In this study, total abundance and biomass declined with species richness and also apparently contributed to the loss of dung beetle function for all guilds. Variations in total and relative abundance and biomass among species can disturb ecosystem function, sometimes in accordance with changes in species richness (Cardinale et al., 2000). For example, in some cases dung beetle species richness, abundance and biomass has been positively correlated with dung burial rates (Larsen et al., 2005). Additionally, it exists a positive effect of species richness on dung decomposition in both ivermectin-treated and untreated dung pads (Beynon et al., 2012). This study supports this relation based on an innovative multifunctional approach that considered the three primary dung beetle functional guilds. As expected, the decrease in diversity parameters was related to a reduction in the functional efficiency, which resulted in the long-term accumulation of dung on the ground (Fig. 4). Indeed, most organic matter input from livestock manure remained on the soil surface at the IVM site, without relocation by dung beetles (approximately 2.24 t ha^{-1} y^{-1} and 4.71 t ha^{-1} y^{-1} remained unaltered in spring and autumn, respectively). Therefore, the efficiency of dung degradation in the IVM site was only 65.5% of the amount of dung processed by beetles at the ECO site during spring-summer. During the autumn-winter period, dung degradation in the IVM site was 33.7% lower than that at the ECO site.

The changes in dung beetle assemblages could have serious consequences for several ecosystem functions and services, including soil permeability, soil bioturbation, plant regeneration, and parasite regulation (Beynon et al., 2012; Mittal, 1993; Vulinec, 2002; Bang et al., 2001; Bang et al., 2005). For example, dung beetles provide ecosystem services to the US cattle industry valued at $380 million/year (Losey and Vaughan, 2006). Moreover, dung beetles contribute to the incorporation of organic matter and nutrients from dung into soils, reducing nutrient losses from leaching and volatilization (Gillard, 1967). For these reasons, we expected that the changes observed in dung beetle activity and diversity affected soil biogeochemical processes after years of livestock management practices at the IVM site. Our results confirmed this hypothesis in part, with considerable changes in soil functionality. The buildup of organic matter in soils is a very slow process (Van-Camp et al., 2004), particularly in Mediterranean oligotrophic soils, and therefore, the absence of significant differences in topsoil nutrient content observed between sites was not unexpected. Other studies have also found that dung beetle activity did not affect soil fertility

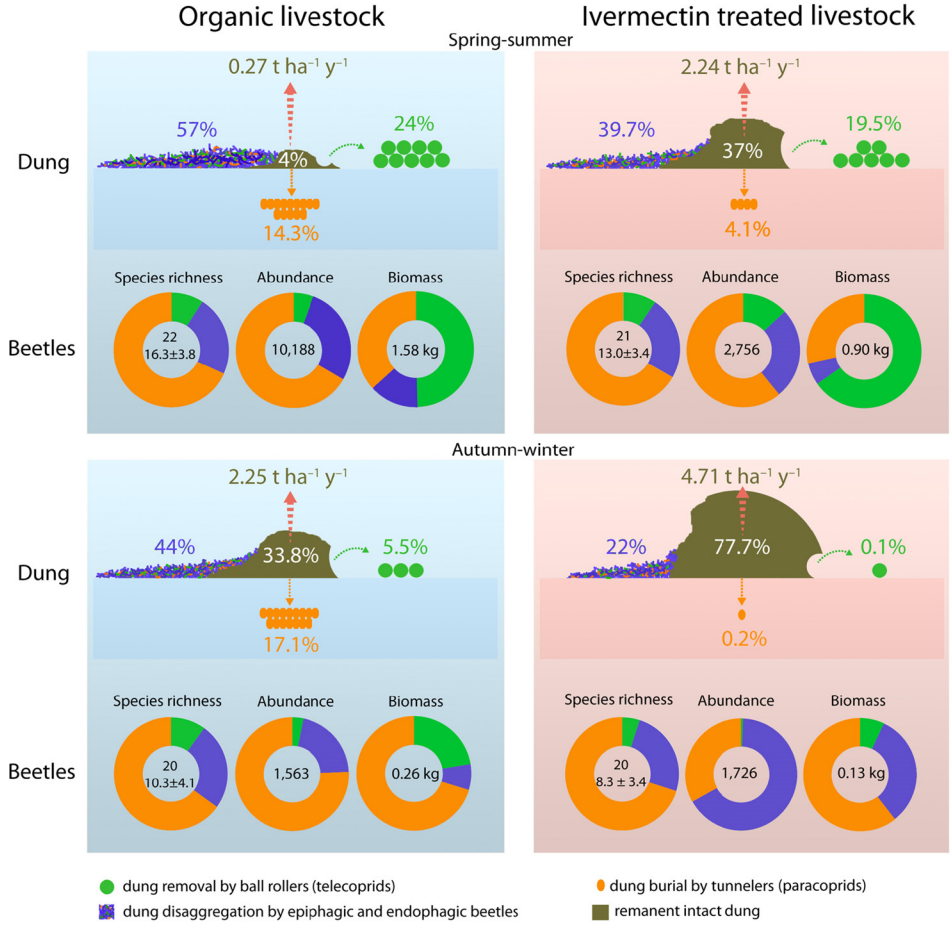

Fig. 4. Schematic summary of differences in dung beetle assemblage structure and ecological function in conventional and organic livestock systems. Assemblage structure is depicted by species richness (cumulative and average number of species: mean ± s.d.), abundance (total number of individuals) and biomass (total fresh dung beetle weight in kg). Average percentages of dung rolled away by telecoprids (in green), disaggregated (in a mixture of blue, green and orange outlines) by *endo*- and epiphagic beetles, and buried by paracoprids (in orange) are also represented. The average percentage of the dung pat not processed by dung beetles (in dark green) and an estimation of the amount of manure accumulated on the soil surface (in t ha^{-1} y^{-1}) are also provided.

(Bertone et al., 2006; Yamada et al., 2007). The lack of significant differences in soil organic matter and nutrients might be due to the 37% and 78% of the dung that remained unaltered at the soil surface in the IVM site in spring and autumn, respectively, whereas higher dung beetle activity might have increased organic matter decomposition and dispersion in the ECO site both at depth (by burrowing paracoprids and telecoprids) and at the soil surface (by endophagic and dwelling beetles) (96% and 66% of total dung relocated in spring and autumn, respectively).

However, significant differences in soil functionality were observed between the IVM and ECO sites. Soils from the IVM site mineralized significantly more carbon and nitrogen than soils from the ECO site. Notably, this pattern reversed when carbon mineralization rates were expressed on an initial carbon basis, which suggested that soils at the IVM site had lower quality soil organic C (as indicated by higher C/N ratios). In addition to higher potential mineralization rates, soils in the IVM site showed consistently significantly higher in-situ mineral N and P production than soils in the ECO site. These higher rates in the IVM site could also be attributed to the accumulation of dung-derived organic matter in the top 10 cm of the soil profile as a result of reduced dung beetle activity. Nevertheless, the increases in nitrification rates observed in ivermectin-treated soils (Konopka et al., 2015) suggest that higher N transformation rates in the IVM site could also be a direct effect of ivermectin on soil properties. Although avermectins apparently have no short-term effects on soil bacteria and fungi (Kollmann et al., 2004), long-term exposure to ivermectin can reduce production and germination of fungal spores in strains of *Fusarium oxysporum* (Kollmann et al., 2004).

To our knowledge, few studies have tested the direct effect of ivermectin on soil microbial populations, although dung beetle activity is known to affect soil microbiota (Slade et al., 2016a, 2016b) and soil biodiversity affects several soil functions (Wagg et al., 2014). Notably, the extractable N (NH_4–N and NO_3–N) and P (PO_4^{3-}–P) values at both sites indicated that the differences in N and P production did not result in significantly larger soil inorganic N and P pools in the IVM than in the ECO site. This apparent contradiction might indicate a decoupling between nutrient production and consumption by plants and microorganisms in the IVM site, particularly for N, leading to a less conservative system and potential increases in nutrient losses (Robertson and Groffman, 2007). This scenario would lead to a gradual decrease in inorganic N pools, compromising the long-term productivity of these already N-limited systems (Diacono and Montemurro, 2010).

5. Conclusions

Our study indicates that ivermectin residues caused profound short-term and long-term ecological effects, disrupting dung beetle assemblages, dung degradation processes, and soil properties and functions. The evidence is increasing that ivermectin residues cause detrimental effects on biodiversity, directly decreasing dung beetle populations. Farm-use intensification of VMPs could result in the intense and continuous accumulation of manure, with cascading effects on functional diversity and ecosystem resiliency and effective functioning. Vegetation diversity (Laliberté et al., 2010), soil physicochemical properties (Konopka et al., 2015), greenhouse gas emission rates (Slade et al., 2016a, 2016b; Penttilä et al., 2013), and carbon sequestration (Wilsey et al., 2002) are all attributes that could also be seriously affected by the indiscriminate use of antiparasitic compounds. The results of this study highlight that the effects of ivermectin must be investigated from a global perspective and that the use must be monitored and controlled following a precautionary principle, as recommended by the CVMP (Committee for Medicinal Products for Veterinary use) of the European Medicines Agency (2017).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.10.331.

Authors' contributions

J.R.V., J.M.L. and F.S.P. conceived and designed the research; J.R.V., C.N., J.M.L., F.S.P., B.G., M.T., J.P.G-T., A.R. and J-P.L. collected the biological samples. V.C., A.J.O., A.R. and J.D. performed the chemical analysis. J.M.L., J.R.V., and F.S.P. applied the statistical tests. J.R.V., J.M.L., F.S.P. J-P.L., A.R. and C.N. wrote the manuscript and the other authors revised the paper.

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Supporting Information

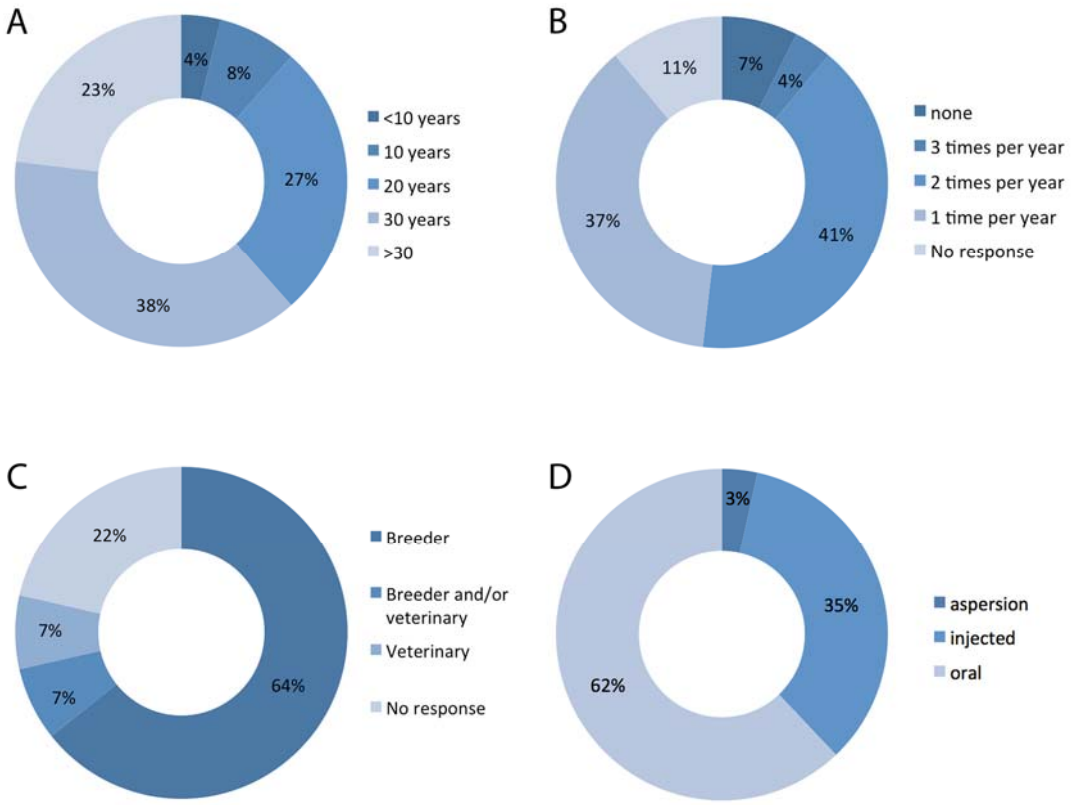

Figure S1. A) Experience of the farmers (in years) with livestock activities in Doñana National Park. The profile of the interviewed farmers shows that the great majority are engaged to this farming activity since very young age. More than the 60% of them had been dedicated to the craft for more than 20 years; **B) Number of antiparasitic applications per year in livestock.** The internal and external parasite treatment scheme consists basically of at least two antiparasitic applications per year. More than 40% apply these substances in June-July and repeat the application another time of the year, at the end of the summer (55%) or at another time **C) People in charge of the administration of antiparasitics to the livestock.** The application of antiparasitics in livestock rests mainly on owners (64%), only 7% of the respondents stated that veterinarians are in charge of this work, and 7% of breeders conduct this activity together with veterinarians; **D) Methods of administration of antiparasitics.** The administration of veterinary medical products (VMP) is mainly given orally (62%, although a significant percentage is administered parenterally). Other substances for non-veterinary use such as bleach are applied by spraying. The preferred method and route of administration of antiparasitics, varied among respondents. For some of them oral drugs are very difficult to administer, while other respondents assert that the application of these drugs via parenteral generates inflammation and infections. The manufacturers agree with this last observation, and they advice against parenteral administration since animals develop infections more easily at the place of application than other kind of livestock. Surveys were conducted at the fairground during the mares gathering season in June 2015 to a random group of 30 farmers.

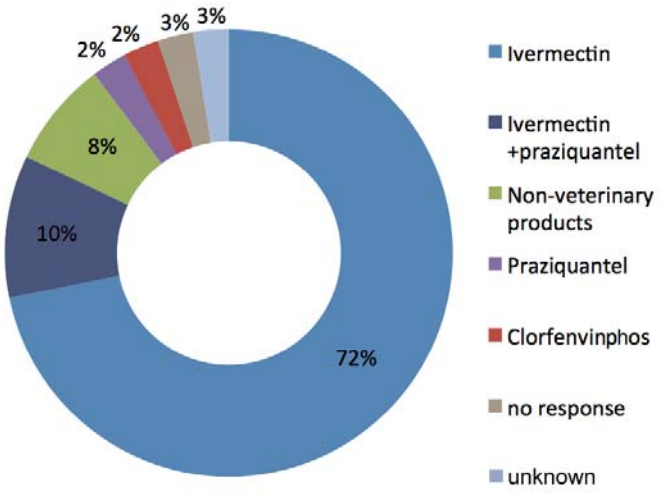

Figure S2. Utilization of substances for parasite control in livestock in Doñana National Park. Regarding the type of substance applied to livestock in the park, Ivermectin is the most used medication for parasite control (82%). At least 8 of the commercial trademarks referred by respondents were Ivermectin (72%) or a mixture of ivermectin and praziquantel (10%). It is important to mention that although in a low percentage and only in the years of high infestation, some owners use specially polluting products such as chlorfenvinphos, a banned substance in the USA and UE, or non-veterinary substances such as bleach, sulfur or other organophosphorus insecticides (dimethoate). Surveys were conducted at the fairground during the mares gathering season in June 2015 to a random group of 30 farmers.

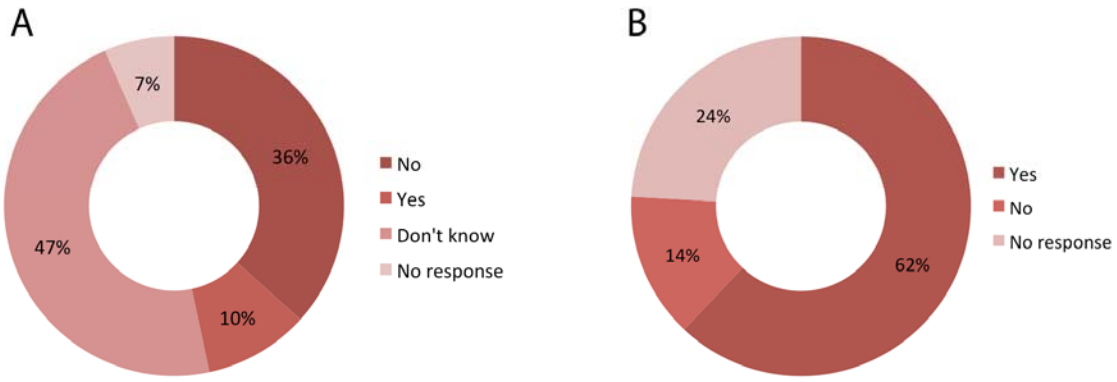

Figure S3. A) Farmers' opinion on whether the use of pesticides can affect the environment and biodiversity. Almost half of the surveyed (47%) did not know that the application of antiparasitics can have effects on the environment and biodiversity. Only 3% of respondents believed that the application of these substances can have an impact on the environment and 36% believed that these substances have no effect on the environment; B) Intention of farmers surveyed to pay a product a bit more expensive but with less effects on the environment and biodiversity. Despite this previous mention, 90% of respondents would be willing to switch to a product less harmful to the environment. A smaller percentage would be willing to buy it even if it cost a little more (62%). Surveys were conducted at the fairground during the mares gathering season in June 2015 to a random group of 30 farmers.

Table S1. Ivermectin determination in sampling sites of Doñana National Park. *Collection of samples*: 15 samples of fresh dung (50 g approx.) in each site were randomly collected and maintained in plastic vials and immediately protected from UV radiation. All samples were maintained in a cooler at 4 ºC until chemical analysis. *Method*: Analytical method based on solvent extraction, followed by continuous solid-phase extraction (SPE) clean-up with polymeric sorbent using liquid chromatography combined with positive electrospray ionization tandem mass spectrometry (LC/ESI$^+$–MS/MS) (1).

Sampling site	Collection samples	Analysis date	IVM (ppb)
IVM	07/10/2014	10/01/2014	0.338
IVM	07/10/2014	10/01/2014	0.568
IVM	07/10/2014	10/01/2014	0.408
IVM	07/10/2014	10/01/2014	0.608
IVM	07/10/2014	11/04/2014	0.355
IVM	07/10/2014	11/04/2014	0.257
IVM	07/10/2014	11/04/2014	0.216
IVM	07/10/2014	11/04/2014	0.829
IVM	07/10/2014	11/04/2014	1.053
IVM	07/10/2014	11/04/2014	0.702
IVM	07/10/2014	11/04/2014	0.323
IVM	07/10/2014	11/04/2014	0.241
IVM	07/10/2014	11/04/2014	0.734
IVM	07/10/2014	11/04/2014	0.623
IVM	07/10/2014	11/04/2014	0.401
ECO	07/10/2014	10/01/2014	Not determined
ECO	07/10/2014	10/01/2014	Not determined
ECO	07/10/2014	10/01/2014	Not determined
ECO	07/10/2014	10/01/2014	Not determined
ECO	07/10/2014	11/04/2014	Not determined
ECO	07/10/2014	11/04/2014	Not determined
ECO	07/10/2014	11/04/2014	Not determined
ECO	07/10/2014	11/04/2014	Not determined
ECO	07/10/2014	11/04/2014	Not determined
ECO	07/10/2014	11/04/2014	Not determined
ECO	07/10/2014	11/04/2014	Not determined
ECO	07/10/2014	11/04/2014	Not determined
ECO	07/10/2014	11/04/2014	Not determined
ECO	07/10/2014	11/04/2014	Not determined
ECO	07/10/2014	11/04/2014	Not determined

IVM: Ivermectin treated livestock: ECO: Organic livestock

Fig S3. Each sampling unit consisted in one adapted plastic washbasin (40 l in volume) filled with soil on which centre a bait (cow dung) was placed (1320 ± 65 g fresh weight, equivalent to 247 ± 23 g of dry weight; mean ± SD). In order to measure dung disaggregation as the rate of spreading out the dung pat on the ground due to dung beetle activity, the size and shape of the dung bait was also standardized by using cylindrical containers 14 cm diameter x 7.5 cm height as moulds for bait preparation. Washbasins were modified by adding sufficiently large drainage holes sealed by a protective nylon mesh (1 mm grid size).

Movie S1. Sampling unit in the field. Each washbasin was buried and filled with the dug soil in order to allow the entrance of running and flying dung beetles but avoiding the escape of roller dung beetles (telecoprids) with their dung balls by leaving 5 cm of unevenness in the interior of the container. Finally, in order to avoid that plastic washbasins would work like pitfall traps for other flightless arthropods, such as spiders and ants, we previously added an innocuous adhesive spray (Axton® Groupe ADEO, France) to stick sand over the exposed parts of the container, which allowed both the freely entrance and escape of these small arthropods.

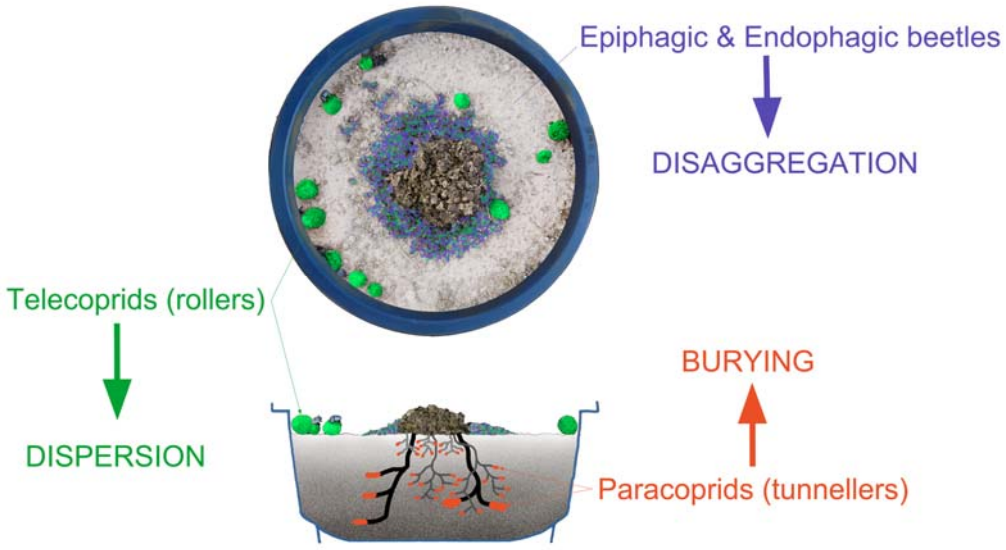

Fig S4. Schematic illustration of different functional groups of dung: rollers (telecoprids), tunnelers (paracoprids), and dwellers (endophagic dung beetles and endocoprids).

Table S2. Dung beetles in sampling sites of Doñana National Park. Different colours correspond to different functional groups: endocoprids (blue), paracoprids (orange) and telecoprids (green).

	Spring-summer		Autumn-winter	
	ECO	**IVM**	**ECO**	**IVM**
Aphodiidae				
Acanthobodilus immundus	66	7	0	0
Aphodius fimetarius	1	0	29	39
Aphodius foetidus	0	0	223	884
Bodiloides ictericus ghardimaouensis	0	0	3	7
Chilothorax lineolatus	0	0	4	22
Colobopterus erraticus	0	0	1	0
Labarrus lividus	1	21	0	0
Nialus varians	0	2	0	0
Otophorus haemorrhoidalis	55	0	0	0
Subrinus sturmi	0	13	0	0
Subrinus vitellinus	153	377	0	1
Geotrupidae				
Ceratophyus hoffmannssegi	1	6	39	29
Geotrupes ibericus	0	0	0	2
Sericotrupes niger	0	0	49	1
Typhaeus momus	0	3	157	63
Scarabaeidae				
Bubas bison	1	0	67	36
Caccobius schreberi	87	53	0	0
Cheironitis hungaricus	24	11	0	0
Copris hispanus	0	1	0	4
Euoniticellus fulvus	805	22	3	2
Euoniticellus pallens	44	15	1	1
Euoniticellus pallipes	72	5	0	2
Onitis belial	1	0	0	0
Onthophagus furcatus	518	430	29	3
Onthophagus maki	2769	851	5	0
Onthophagus marginalis	331	21	7	0
Onthophagus opacicollis	119	8	740	590
Onthophagus punctatus	3	0	111	16
Onthophagus taurus	4402	445	21	11
Onthophagus vacca	2	12	3	1
Scarabaeus cicatricosus	601	250	70	12
Scarabaeus sacer	132	203	1	0
Cumulative Species Richness (*S*)	22	21	20	20
Average Species Richness (S_p)	16.3 ± 3.8	13.0 ± 3.4	10.3 ± 4.1	8.3 ± 3.4
Total Abundance (*N*)	10,210	2,777	1,583	1,746
Total Biomass (*B*, in kg)	1.58	0.90	0.26	0.13

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1. Ortiz AJ, Cortez V, Azzouz A, Verdú JR (2017) Isolation and determination of ivermectin in post-mortem and in vivo tissues of dung beetles using a continuous solid phase extraction method followed by LC-ESI+-MS/MS. *PLoS ONE* 12(2): e0172202. doi:10.1371/journal.pone.0172202